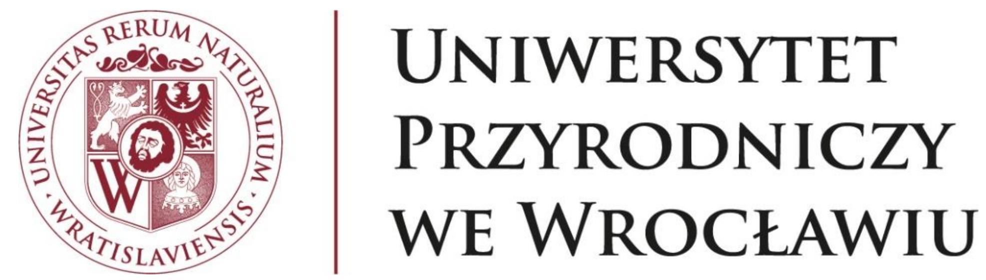

WYDZIAŁ BIOTECHNOLOGII I NAUK O ŻYWNOŚCI

KATEDRA CHEMII

MATEUSZ ŁUŻNY

SYNTEZA I BIOTRANSFORMACJE WYBRANYCH ZWIĄZKÓW FLAWONOIDOWYCH

SYNTHESIS AND BIOTRANSFORMATIONS OF SELECTED FLAVONOID COMPOUNDS

Praca doktorska wykonana pod kierunkiem
Promotora dr. hab. Tomasza Janeczko, prof. UPWr,
Promotora pomocniczego dr inż. Ewy Kozłowskiej

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PUBLIKACJE STANOWIĄCE ROZPRAWĘ DOKTORSKĄ

PUBLIKACJA 1 (P1)

Łużny, M.*; Krzywda, M.; Kozłowska, E.; Kostrzewa-Susłow, E.; Janeczko, T.*
Effective hydrogenation of 3-(2″-furyl)- and 3-(2″-thienyl)-1-(2′-hydroxyphenyl)-prop-2-en-1-one in selected yeast cultures. *Molecule*s, 2019, 24, 3185.

Udział procentowy: 75%

(IF = 3,060; 100 pkt MEiN)

PUBLIKACJA 2 (P2)

Łużny, M.*; Kozłowska, E.; Kostrzewa-Susłow, E.; Janeczko, T.*
Highly effective, regiospecific hydrogenation of methoxychalcone by *Yarrowia lipolytica* enables production of food sweeteners. *Catalysts*, 2020, 10, 1135.

Udział procentowy: 80%

(IF = 3,520; 100 pkt MEiN)

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Łużny, M.*; Tronina, T.; Kozłowska, E.; Dymarska, M.; Popłoński, J.; Łyczko, J.; Kostrzewa-Susłow, E.; Janeczko, T.*
Biotransformation of methoxyflavones by selected entomopathogenic filamentous fungi. *International Journal of Molecular Sciences*, 2020, 21, 6121.

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(IF = 4,556; 140 pkt MEiN)

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STRESZCZENIE W JĘZYKU POLSKIM

Flawonoidy, jako metabolity wtórne roślin, wykazują szereg właściwości biologicznych, m.in. pełnią rolę w ich wzroście i rozwoju, mają właściwości antyoksydacyjne oraz przeciwdrobnoustrojowe. Wykazują również pozytywne działanie wobec organizmu ludzkiego, tj. posiadają m.in. właściwości p/zapalne, p/nowotworowe, kardio- i neuroprotekcyjne, stąd spożywanie produktów roślinnych bogatych w te związki czy dodatkowa suplementacja powinny być brane pod uwagę przy planowaniu codziennych posiłków. Związki flawonoidowe występują w roślinach zawsze w mieszaninie kilku/kilkudziesięciu związków, głównie w formie glikozydów, których biologiczna aktywność może być wzajemnie wzmacniana bądź tłumiona.

Synteza chemiczna *de novo* flawonów, zarówno glukopiranozyloflawonów jak i ich aglikonów, jest zazwyczaj droga i wymaga często zastosowania kosztownych katalizatorów oraz utrzymania drastycznych warunków reakcji. Natomiast w przypadku ekstrakcji poszczególnych związków z roślin, proces ten jest zazwyczaj nieopłacalny ekonomicznie, głównie ze względu na niskie zawartości tych polifenoli w roślinie, w przeliczeniu na gram suchej masy, oraz wysoki koszt rozdziału mieszanin złożonych często z kilkudziesięciu związków.

Celem pracy była synteza nowych, oraz występujących w niewielkich ilościach w roślinach leczniczych, związków flawonoidowych – głównie zawierających podstawnik metoksylowy oraz określenie zdolności katalitycznych wybranych mikroorganizmów – niekonwencjonalnych szczepów drożdży oraz entomopatogennych grzybów strzępkowych do biotransformacji tych związków.

Większość organizmów żywych jest zdolna do uwodornienia wiązania podwójnego. Drożdże posiadają specyficzne enzymy, umożliwiające uwodornienie wiązania podwójnego pomiędzy węglem C2 a C3 chalkonów. W biotransformacjach chalkonów zastosowałem osiem mikroorganizmów (*Yarrowia lipolytica* KCh 71, *Rhodotorula rubra* KCh 4, *R. marina* KCh 77, *R. rubra* KCh 82, *R. glutinis* KCh 242, *Saccharomyces cerevisiae* KCh 464, *Candida viswanathii* KCh 120 oraz *C. parapsilosis* KCh 909). Wszystkie szczepy były zdolne do redukcji wiązania podwójnego w wybranych chalkonach i przekształcały je w oczekiwane dihydrochalkony, jednak wydajność opisanego procesu różniła się znacząco pomiędzy szczepami oraz ze względu na miejsce i ilość podstawników metoksylowych w strukturze zastosowanego substratu.

Jednym z ośmiu szczepów niekonwencjonalnych drożdży wykorzystanych w badaniach był mikroorganizm z gatunku *Yarrowia lipolytica*, który ze względu na status GRAS (Generally Recognized As Safe) może być również stosowany jako suplement/uzupełnienie diety. Zastosowanie drożdży miało na celu uwodornienie wiązania podwójnego pomiędzy węglem C2 a C3 szkieletu chalkonu i wytworzenie związków, o potencjalnie słodkim smaku posiadających właściwości biologiczne zbliżone do chalkonów. Zarówno chalkony jak i uzyskane w wyniku biotransformacji dihydrochalkony zostały przekazane na badania biologiczne w celu wykazania ich aktywności.

Dodatkowo, na podstawie wcześniejszych badań w kulturach entomopatogennych grzybów strzępkowych, w których wykazano zdolność do unikatowej 4-*O*-metyloglikozylacji hydroksyflawonów wybrałem 9 entomopatogennych szczepów grzybów strzępkowych, należących do 4 gatunków (*Beauveria bassiana, B. caledonica, Isaria farinosa* oraz *I. fumosorosea*). Szczepy te zostały przetestowane pod kątem ich katalitycznych zdolności do przekształcenia metoksyflawonów, w celu uzyskania ich lepiej rozpuszczalnych/przyswajalnych pochodnych. Zastosowanie tych katalizatorów pozwoliło na uzyskanie hydroksy- oraz glukopiranozyloflawonów.

W niniejszej pracy doktorskiej przedstawiłem biotransformacje 18 uzyskanych w wyniku syntezy chemicznej zawiązków flawonoidowych. Dziesięć chalkonów poddałem biotransformacji w kulturach niekonwencjonalnych szczepów drożdży otrzymując finalnie 7 dihydrochalkonów (związki **1a-7a**). Osiem metoksyflawonów poddałem biotransformacji w kulturach entomopatogennych szczepów grzybów strzępkowych, uzyskując łącznie 21 produktów. Osiem posiadających w swojej strukturze grupą hydroksylową i trzynaście 4-*O*-metyloglukopiranozylo pochodnych. Jednym z założeń pracy było otrzymanie pochodnych o interesujących właściwościach biologicznych oraz charakteryzujących się lepszą przyswajalnością i rozpuszczalnością w stosunku do zastosowanych substratów, co jest przypisywane glikozydowym pochodnym flawonoidów.

STRESZCZENIE W JĘZYKU ANGIELSKIM

As secondary metabolites of plants, flavonoids exhibit a series of biological properties, e.g. they play a role in plant growth and development, have antioxidant and antimicrobial properties. They also show positive effects on the human organism due to their, e.g. anti-inflammatory, anticancer, cardio- and neuroprotective properties; thus, consumption of plant products containing these compounds or additional supplementation should be considered when planning daily meals. Flavonoid compounds are always present in plants in a complex of several compounds, mainly in the form of glycosides, whose biological activity can be additionally enhanced or suppressed.

De novo chemical synthesis of flavones, both glucopyranosylflavones and their aglycones are generally expensive and commonly require expensive catalysts and the maintenance of harsh reaction conditions. In contrast, when extracting individual compounds from plants, the process is usually not economically viable, mainly due to the low concentrations of these polyphenols in the plant, per gram of dry mass, and the high cost of separating mixtures often composed of dozens of compounds.

The aim of this work was to synthesize new flavonoid compounds and those which occur in small amounts in medicinal plants, mainly containing methoxy substituent, and to determine the catalytic ability of selected microorganisms, unconventional yeast strains and entomopathogenic filamentous fungi for biotransformation of these compounds.

Most living organisms are able to hydrogenate the double bond. Yeasts have specific enzymes that enable hydrogenation of the double bond between the C2 and C3 carbon atom of chalcones. I used eight microorganisms in chalcone biotransformations: *Yarrowia lipolytica* KCh 71, *Rhodotorula rubra* KCh 4, *R. marina* KCh 77, *R. rubra* KCh 82, *R. glutinis* KCh 242, *Saccharomyces cerevisiae* KCh 464, *Candida viswanathii* KCh 120, and *C. parapsilosis* KCh 909. Each exhibited the ability to reduce the double bond in selected chalcones and convert them into the expected products, but the efficiency of the described process differed significantly between the strains.

One of eight strains of unconventional yeast used in the study was a microorganism from the species *Yarrowia lipolytica*, which can also be used as a dietary supplement/supplement due to its GRAS (Generally Recognized As Safe) status. The use of yeast was to hydrogenate the double bond between the C2 and C3 carbon of the chalcone backbone and produce compounds with a potentially sweet taste having biological properties similar to chalcones. Both the chalcones and the biotransformed dihydrochalcones were submitted for biological tests to determine their activity.

Additionally, based on previous studies in entomopathogenic filamentous fungal cultures in which the ability to unique 4-*O*-methylglycosylation of hydroxyflavones was demonstrated, I selected nine entomopathogenic filamentous fungal strains.

They belonged to 4 species (*Beauveria bassiana, B. caledonica, Isaria farinosa*, and *I. fumosorosea*) and were tested for their catalytic ability to convert methoxyflavones to obtain their more soluble/absorbable derivatives. The use of these catalysts yielded hydroxy- and glucopyranosylflavones.

In this dissertation, I presented the biotransformations of 18 substrates. Ten chalcones were biotransformed in cultures of unconventional yeast strains to obtain seven dihydrochalcones (compounds **1a-7a**). Eight methoxyflavones were biotransformed in cultures of entomopathogenic filamentous fungi strains to obtain a total of 21 methoxyflavone derivatives - eight with an attached hydroxyl group and thirteen 4-*O*-methylglucopyranosyl derivatives. The aim of the work was to obtain derivatives with interesting biological properties, better bioavailability and solubility in relation to the substrates used, which is attributed to glycosidic derivatives of flavonoids.

WSTĘP

Flawonoidy stanowią liczną grupę związków występujących w tkankach roślin i wywierają istotny wpływ na ich wzrost i rozwój, nadają barwę kwiatom i owocom oraz są odpowiedzialne za ochronę roślin przed promieniowaniem UV. Związki flawonoidowe są dostarczane do naszego organizmu wraz z produktami roślinnymi, przez co stanowią codzienny składnik naszej diety.[1,2] W większych ilościach obecne są one między innymi w często spożywanych cytrusach, jabłkach, pomidorach, ziemniakach, kiełkach fasoli.[3] Wykazują m.in. działanie przeciwzapalne, p/utleniające oraz p/nowotworowe.[4–6] Związki te ograniczają rozwój patogennych mikroorganizmów, w tym gram-dodatnich i gram-ujemnych bakterii, grzybów[7], a także pierwotniaków.[8] Co więcej, floretyna (dihydrochalkon) jest aktywnym inhibitorem tyrozynazy grzybowej[6,9], co ma wpływ m.in. na proces melanogenezy. Dzięki swoim wysokim i zróżnicowanym aktywnościom biologicznym wiele związków flawonoidowych posiada potencjał do wykorzystania w przemyśle farmaceutycznym. Dihydrochalkony są również wykorzystywane w syntezie chemicznej do uzyskiwania aktywnych biologicznie związków. 2’-Hydroksydihydrochalkon jest wykorzystywany jako blok budulcowy w syntezie propafenonu – substancji czynnej leków przeciwarytmicznych.[10–12]

Na polskim rynku suplementów diety dostępnych jest kilka preparatów zawierających flawonoidy w swoim składzie, jednak oferta firm farmaceutycznych jest wciąż uboga i nie wykorzystuje potencjału prozdrowotnego tej grupy związków. Skupiając się na właściwościach suplementów diety oraz leków, których proces wchłaniania rozpoczyna się już w jamie ustnej, nie można nie wspomnieć o właściwościach smakowych dihydrochalkonów. Związki te w zależności od stężenia, charakteryzują się różnymi smakami (m.in. słodki, słony, mięsny czy umami). Odczucie słodkości jest zbliżone do sacharozy. Zostało to już wykorzystane w przemyśle spożywczym- dihydrochalkon neohesperedyny jest obecnie stosowany jako słodzik i wcześniej określany był numerem E-959. Zastosowanie większej liczby innych dihydrochalkonów jako słodzików poszerzyłoby obecną, „zdrowszą” alternatywę dla cukru i stosowanych słodzików, ze względu na brak działań niepożądanych przypisywanych sacharozie i jednocześnie posiadane przez nie właściwości prozdrowotne.

Rysunek 1. Szkielet 2’-hydroksychalkonu oraz 2’-hydroksydihydrochalkonu

W ostatnich latach przemysł spożywczy koncentruje się m.in. na poszukiwaniu nowych substancji słodzących. W tej grupie dodatków do żywności coraz większą uwagę zyskują dihydrochalkony (Rys. 1.). Zainteresowanie to jest potęgowane faktem, iż naturalne dihydrochalkony są obecne w roślinach i stanowią codzienny składnik naszej diety [1,13], a smak słodki odgrywa dominującą rolę w preferencjach żywieniowych człowieka.[14] Jest on najważniejszą cechą sensoryczną produktów spożywczych. Jest nie tylko źródłem przyjemności, ale również podstawowym bodźcem energetycznym dla organizmu. Badania prospektywne dowiodły korelacji pomiędzy nadmiernym spożyciem cukru i produktów bogatych w cukier a zwiększonym ryzykiem zachorowania na raka trzustki. Biorąc pod uwagę rolę hiperglikemii i hiperinsulinemii w rozwoju tego nowotworu, ustalono, że bezpośrednią przyczyną śmierci tkanek tego narządu jest indukowana pokarmem hiperglikemia oraz wzrost zapotrzebowania i zmniejszenie wrażliwości na insulinę.[15] Coraz więcej dowodów wskazuje również na rolę zwiększonego spożycia cukru w rozwoju nadciśnienia tętniczego, stanów zapalnych i choroby wieńcowej.[16–18] Z tego powodu poszukuje się niskoenergetycznych substytutów, wykazujących właściwości sensoryczne porównywalne z sacharozą, ale zapewniających dodatkowe korzyści zdrowotne. Wzrost zainteresowania zdrowym stylem życia oraz zwiększona zachorowalność na choroby spowodowane zaburzonym metabolizmem związków cukrowych zwiększają popularność produktów zawierających substancje słodzące o obniżonej kaloryczności.[19]

Substancje słodzące definiuje się jako dodatki do żywności, które naśladują uczucie słodkiego smaku, podobnego do sacharozy.[20] Idealny słodzik, oprócz niskiej kaloryczności i wysokiej intensywności słodyczy, zbliżonej do sacharozy, powinien być bezpieczny dla zdrowia konsumenta, a także stabilny w różnych warunkach przetwarzania.[21,22] Mnogość ograniczeń i wad dostępnych na rynku substancji słodzących stworzyła potrzebę poszukiwania nowych związków, które byłyby korzystniejsze pod względem wpływu na zdrowie człowieka i bardziej atrakcyjne dla przemysłu. Znaczne zainteresowanie w tym zakresie budzą dihydrochalkony [23–25] oddziałujące z receptorami smaku słodkiego T1R2.[26] Otrzymywane są one w wyniku uwodornienia chalkonów i wykazują duży potencjał jako innowacyjne substancje słodzące.[27,28] Najlepiej przebadanym związkiem opisanym jako substancja intensywnie słodka jest wspomniany wcześniej dihydrochalkon neohesperydyny.[29]

Rysunek 2. Wzory strukturalne dihydrochalkonów A) floretyny, B) florydzyny oraz C) aspalatyny

Większość roślinnych dihydrochalkonów wykazuje prozdrowotne działanie. Floretyna i florydzyna (występujące w jabłkach) znacząco zmniejszają ryzyko rozwoju chorób sercowo-naczyniowych i cukrzycy[30], natomiast aspalatyna (Rys. 2.) występująca w znaczących ilościach (6-13%) w liściach *Aspalathus linearis* wpływa na redukcję stresu oksydacyjnego i może spowalniać proces starzenia się organizmu.[31] Metoksyflawony (MF) mają dobrze opisane właściwości przeciwnowotworowe i często są bardziej aktywne niż flawony pozbawione grup funkcyjnych.[32] Ze względu na obecność grup metoksylowych związki te wykazują wyższą lipofilowość niż analogi z grupami hydroksylowymi, co bezpośrednio wpływa na ich biodostępność.[33] Najlepszym opisanym do tej pory naturalnym źródłem metoksyflawonów jest pierwiosnek lekarski (*Primula veris* L.), który zawiera te związki w kłączach, korzeniach, kwiatach i liściach, i z którego przygotowuje się głównie leki wykrztuśne.[34]

Potencjał terapeutyczny flawonoidów jest często ograniczony przez ich niską rozpuszczalność i biodostępność. Aglikony charakteryzują się bardzo niską rozpuszczalnością w wodzie.[35] Przyłączenie polarnych cząsteczek, takich jak cząsteczki cukru, może zwiększyć ich aktywność[36], biodostępność[36] i stabilność.[37] Przykładowo, rozpuszczalność w wodzie α-glukozyloizokwercytyny wzrasta ponad 80 000-krotnie, w porównaniu do jej związku macierzystego, kwercetyny.[38,39] Glikozyd - izokwercetyna wykazuje 18 razy wyższą biodostępność niż kwercetyna po podaniu doustnym szczurom[40], a także znacznie wyższą biodostępność w porównaniu do kwercetyny i izokwercetyny w badaniach na ludziach.[41] Wyniki te wskazują, że glikozydowe pochodne flawonoidów wykazują lepsze działanie prozdrowotne niż ich aglikony ze względu na wyższą biodostępność.[42] Dodatkowo, wiadomo, że metoksyflawony posiadają mniejszą aktywność antyoksydacyjną niż hydroksyflawony, jednakże, jednocześnie posiadają zdolność do ochrony komórek przed śmiercią indukowaną stresem retikulum endoplazmatycznego, podczas gdy pozostałe flawonoidy nie wykazują takich właściwości.[43]

Chryzyna (5,7-dihydroksyflawon) ma działanie hamujące aktywność tyrozynazy, umiarkowane działanie hamujące aktywność aromatazy, poprawia rozwój funkcji poznawczych, zmniejsza uszkodzenia mózgu, ma działanie przeciwlękowe i antyestrogenne.[44] Chryzynę można pozyskiwać m.in. z *Passiflora caerulea* L. (męczennica błękitna) lub z miodu pszczelego,[45] natomiast do celów laboratoryjnych i przemysłowych syntetyzuje się ją ze związków pośrednich[46], a obecnie coraz częściej z innych flawonoidów.[44]

Metoksypochodną chryzyny jest 5,7-dimetoksyflawon (5,7-DMF) (**16**), naturalny związek występujący m.in. w kłączu *Boesenbergia pandurata* (Roxb.), tj. rośliny od dawna stosowanej w tradycyjnej medycynie tajskiej,[47] *Piper caninum*[48] czy w *Kaempferia parviflora.*[49] 5,7-DMF wykazywał bardzo małą toksyczność u szczurów, praktycznie bez skutków ubocznych, nawet w dawkach do 3 g/kg masy ciała.[47] Autorzy porównali właściwości przeciwzapalne 5,7-DMF z aspiryną i stwierdzili, że 5,7-DMF jednocześnie hamuje produkcję prostaglandyn (działanie przeciwzapalne) i obniża temperaturę u szczurów. Ponadto, wiadomo również, że 5,7-DMF działa jako inhibitor sarkopenii i jednocześnie

powoduje rozwój masy i objętości mięśni w modelu mysim[50] (aktywność 5,7-DMF jest bardzo podobna do chryzyny).

Wykazano, że transport wewnątrzkomórkowy 5,7-DMF jest około 10-krotnie wyższy niż chryzyny.[51] Ponadto chryzyna była szybko metabolizowana przez ludzką wątrobę, i już po 20-minutowej inkubacji nie obserwowano w komórkach tego związku. W przeciwieństwie do hydroksylowego analoga, 5,7-DMF był stabilny metabolicznie podczas całego 60-minutowego testu.[52,53] Co ciekawe, stwierdzono, że stężenie 5,7-DMF po podaniu doustnym było znacznie wyższe w tkankach niż w osoczu, gdzie jego okres półtrwania wynosił 3,4±2,8 godziny, a całkowite usunięcie z organizmu trwało około 17 godzin.[54] Ponadto wiadomo również, że metabolizm 5,7-DMF w porównaniu z chryzyną jest znacznie ograniczony, co oznacza, że dłużej utrzymuje się on w organizmie, co czyni go obiecującą substancją chemoprewencyjną.[55]

Bazując na różnicach w aktywności biologicznej chryzyny i 5,7-DMF, przeprowadziłem syntezę i biotransformacje szeregu związków flawonoidowych, zawierających podstawniki metoksylowe zarówno w pierścieniu A (w pozycjach 5 i 7) jak i B (przy węglach 3′, 4′ oraz 5′), które zostały opisane w Publikacji **P4.**

Rysunek 3. Podstawowy szkielet flawonu

Flawonoidy (Rys. 3.) nie są jedyną grupą związków, w której udowodniono pozytywny wpływ obecności cząsteczki cukrowej na aktywność związku. Najlepszym przykładem są antybiotyki, takie jak erytromycyna czy wankomycyna, w których obecność grupy cukrowej jest kluczowa dla ich wysokiej aktywności.[37] Obecność i liczba podstawników glikozydowych w cząsteczce flawonoidu, oraz miejsce ich przyłączenia, silnie wpływa na właściwości biologiczne. Naturalnie glikozylowane pochodne flawonoidów występują powszechnie w świecie roślin, jednak ich stężenie w komórkach często jest stosunkowo niskie[56], co utrudnia ich pozyskiwanie z tego źródła. Nowe metody otrzymywania związków glikozylowanych na większą skalę są wciąż potrzebne i poszukiwane.

Wcześniejsze badania wykazały zdolność do unikatowej 4-*O*-metyloglikozylacji hydroksyflawonów obserwowanej w kulturach entomopatogennych grzybów strzępkowych.[36,57] Szczep *Beauveria bassiana* AM 278 katalizował przyłączanie metyloglukozy do grupy hydroksylowej cząsteczki flawonoidu w pozycjach C-7 i C-3′.[58–60] Analogiczną preferencję do selektywnej 4-*O*-metyloglikozylacji grupy hydroksylowej zlokalizowanej przy węglu C-7 zaobserwowano podczas biotransformacji

unikatowych prenylowanych flawonoidów wyizolowanych z chmielu zwyczajnego (*Humulus lupulus*) w kulturach *Beauveria bassiana* AM 446 i AM 278.[61–64] Wykazano efektywną 4-*O*-metyloglikozylację 3-hydroksyflawonu, 6-hydroksyflawonu, 7-hydroksyflawonu, bajkaleiny, kwercetyny, naryngeniny, luteoliny, diosmetyny i daidzeiny w kulturach entomopatogennych grzybów strzępkowych z rodzaju *Isaria*.[35,37,56] Szczep *Beauveria bassiana* ATCC 13144 jest zdolny do jednoczesnej 4-*O*-metyloglikozylacji grupy hydroksylowej zlokalizowanej przy węglu C-3 i hydroksylacji węgla C-4'.[65] Również inne szczepy entomopatogenne charakteryzują się zdolnością do jednoczesnej hydroksylacji i glikozylacji flawonoidów. Funkcjonalizacja ta jest obserwowana głównie przy węglu C-4′ związku flawonoidowego. Tworzenie odpowiednich glikozydów w kulturach *Isaria fumosorosea* KCh J2, *I. farinosa* KCh J1.4, *I. farinosa* KCh J1.6 i *I. farinosa* KCh KW1.2 poprzedzone było właśnie hydroksylacją węgla C-4'. Następujące po sobie hydroksylację węgla C-4' i 4-*O*-metyloglikozylację obserwowano podczas inkubacji flawonu, 3-metoksyflawonu, 5-hydroksyflawonu, 6-metoksyflawonu, 6-metoksyflawanonu i 6-metyloflawonu w kulturach rodzaju *Isaria*.[35,56,66] W oparciu o to zjawisko, w niniejszej pracy poddano biotransformacji flawony z grupami metoksylowymi zlokalizowanymi zarówno w pierścieniu A jak i B, które zostały otrzymane na drodze syntezy chemicznej. Jako biokatalizatory dla tych związków zastosowano szczepy grzybów entomopatogennych o potwierdzonej zdolności do jednoczesnej hydroksylacji/demetylacji i glikozylacji związków flawonoidowych. W niniejszej pracy do przeprowadzenia biotransformacji flawonów zostało zastosowane dziewięć szczepów entomopatogennych grzybów strzępkowych, należących do 4 gatunków: *Beauveria bassiana* KCh J1.5, KCh J2.1, KCh J1, KCh J3.2 i KCh BBT, *B. caledonica* KCh J3.3 i KCh J3.4, *Isaria farinosa* KCh KW 1.1 oraz *I. fumosorosea* KCh J2. Wszystkie mikroorganizmy są dostępne w kolekcji Katedry Chemii UPWr (publikacje **P3** i **P4**).

Wszystkie substraty do syntez chemicznych zostały zakupione w firmie Sigma-Aldrich. Chalkony zostały otrzymane w reakcji kondensacji Claisena-Schmidta, hydroksyacetofenonu (A) z odpowiednim benzaldehydem (B), wg schematu:

Rysunek 4. Synteza chalkonów w reakcji Claisena-Schmidta

	Chalkony	R1	R2	R3	R4	R5	R6
1	2′-metoksychalkon	-OCH3	-H	-H	-H	-H	-H
2	3′-metoksychalkon	-H	-OCH3	-H	-H	-H	-H
3	4′-metoksychalkon	-H	-H	-OCH3	-H	-H	-H
4	2′5′dimetoksychalkon	-OCH3	-H	-H	-OCH3	-H	-H
5	4,6,4′trimetoksychalkon	-H	-H	-OCH3	-H	-OCH3	-OCH3
6	3′4′5′trimetoksychalkon	-H	-OCH3	-OCH3	-OCH3	-H	-H
7	4,6,3′4′5′-pentametoksychalkon	-H	-OCH3	-OCH3	-OCH3	-OCH3	-OCH3
8	4,6-dimetoksychalkon	-H	-H	-H	-H	-OCH3	-OCH3

*Dane dotyczące syntezy chalkonów, zawarte w publikacji **P2**

Wszystkie uzyskane w wyniku syntezy Claisena-Schmidta metoksychalkony zostały poddane reakcji cyklizacji z jodem w DMSO w celu uzyskania metoksyflawonów, wg schematu:

Rysunek 5. Reakcja cyklizacji chalkonów z jodem

*Dane dotyczące syntezy flawonów, zawarte w publikacji **P3**

CEL

Celem pracy była synteza nowych oraz występujących w niewielkich ilościach w leczniczych roślinach związków flawonoidowych oraz określenie zdolności katalitycznych badanych mikroorganizmów – niekonwencjonalnych szczepów drożdży oraz entomopatogennych grzybów strzępkowych do biotransformacji otrzymanych związków.

W celu uzyskania puli dihydrochalkonów, które mogą znaleźć zastosowanie w przemyśle spożywczym do produkcji słodzików, zostały zastosowane drożdże wykazujące zdolność do efektywnego uwodornienia wiązania podwójnego obecnego w chalkonach. Natomiast uzyskanie hydroksypochodnych i glikozydów flawonów o zwiększonej aktywności biologicznej względem substratów zawierających w swojej strukturze podstawniki metoksylowe było możliwe dzięki zastosowaniu szczepów entomopatogennych grzybów strzępkowych o unikatowej zdolności do 4-*O*-metyloglikozylacji.

METODYKA

Biotransformacje prowadzone były w sterylnym podłożu płynnym, zawierającym 1% aminobaku (peptonu bakteriologicznego) i 3% glukozy, rozpuszczonych w wodzie destylowanej. Planując doświadczenia w swojej pracy doktorskiej postanowiłem zastosować wypracowaną w naszej jednostce metodykę.[24,56,59,67,68] Taki zabieg umożliwia porównanie uzyskanych przeze mnie wyników z wcześniej opisanymi w literaturze. Badania przesiewowe oraz biotransformacje preparatywne, prowadzone były w kolbach Erlenmayera odpowiednio o pojemnościach 300 mL (zawierających 100 mL podłoża) oraz 2 L (zawierających 500 mL podłoża). Testy ilościowe zostały wykonane na płytkach 24 dołkowych o pojemności 5 mL, zawierających 3 mL podłoża. Substraty rozpuszczone były w rozpuszczalniku organicznym – DMSO (dimetylosulfotlenek) oraz były dodawane w warunkach sterylnych do podłoża z mikroorganizmami w końcowej fazie ich logarytmicznego wzrostu. Po ustalonym czasie biotransformacji produkty były ekstrahowane octanem etylu i następnie analizowane za pomocą technik chromatograficznych; odpowiednio: TLC oraz GC dla pochodnych chalkonów oraz TLC, HPLC i LC-MS dla pochodnych flawonów. Wszystkie produkty biotransformacji zostały oznaczone metodami spektroskopowymi (^{1}H NMR, ^{13}C NMR oraz widm korelacyjnych). Masę związków potwierdzono za pomocą LC-MS.

Szczegółowo opisane warunki przeprowadzonych biotransformacji, analizę struktury uzyskanych produktów syntez i biotransformacji oraz użyty sprzęt (wraz z parametrami) zostały opisane dokładniej w odpowiednich publikacjach dołączonych do dysertacji.

WYNIKI I DYSKUSJA

Rozprawę doktorską stanowią 4 publikacje (**P1-P4**), w których opisałem biotransformacje związków flawonoidowych: chalkonów (**P1** i **P2**) oraz flawonów (**P3** i **P4**). Biokatalizatorami w pracach **P1** i **P2** były szczepy drożdży z gatunków *Rhodotorula rubra, R. glutinis, Saccharomyces cerevisiae, Candida viswanathii, C. parapsilosis* i *Yarrowia lipolytica*. W pracach **P3** i **P4** opisałem zdolności katalityczne entomopatogennych grzybów strzępkowych z gatunków *Beauveria bassiana, B. caledonica, Isaria farinosa* oraz *I. fumosorosea*.

Zarówno wyżej wymienione gatunki drożdży jak i grzybów były oznaczone metodami biologii molekularnej. Ich zdolność do biotransformacji była opisana we wcześniejszych publikacjach pracowników Katedry Chemii Uniwersytetu Przyrodniczego we Wrocławiu. Wymienione szczepy nie były jednak katalizatorami w transformacjach metoksychalkonów oraz metoksyflawonów.

Wybrane szczepy drożdży, jak i grzybów wykazują w większości zdolność do biotransformacji uzyskanych na drodze chemicznej związków flawonoidowych. Wyniki przeprowadzonych biotransformacji należy podzielić na 4 części, dotyczące odpowiednio:

A. chalkonów (**P1** i **P2**):
   1* zawierających heteroatom w pierścieniu B,
   2* zawierających od 1 do 5 podstawników metoksylowych w swojej strukturze.
B. flawonów (**P3** i **P4**):
   3* zawierających podstawniki metoksylowe tylko w pierścieniu B,
   4* zawierających podstawniki metoksylowe w pierścieniu A lub w A i B.

Do przeprowadzenia biotransformacji zastosowałem 18 substratów uzyskanych w wyniku syntezy chemicznej (10 chalkonów oraz 8 flawonów). W wyniku przeprowadzenia reakcji z zastosowaniem wspomnianych biokatalizatorów otrzymałem łącznie 28 produktów, w tym 7 dihydrochalkonów oraz 21 pochodnych metoksyflawonów - osiem z przyłączoną grupą hydroksylową i trzynaście 4-*O*-metyloglukopiranozylo pochodnych. Trzynaście z nich nie było wcześniej opisanych w literaturze.

STRUKTURY ZWIĄZKÓW UZYSKANYCH W WYNIKU SYNTEZY CHEMICZNEJ I BIOTRANSFORMACJI OPISANE W DYSERTACJI

Chalkony

O OH 1
O S OH 2
O O OH 3
O O OH 4
O OH O 5
O O OH O O 6
O O OH O 7
O O O OH 8
O O O OH O 9
O O O OH O O O 10

Dihydrochalkony

O O OH 1a
O S OH 2a
O O OH 3a
O O OH 4a
O OH O 5a
O O OH O O 6a
O O OH O 7a

Metoksyflawony

11

12

13

14

15

16

17

18

Hydroksyflawony

11a

12a

13a

14d

14e

16a

16b (17a)

16c (17b)

18a

4-*O*-metyloglukopiranozydy flawonów

11b 11c 11d (14a)

11e 12b 13b

14b 14c 15a

15b 15c 15d

18b

W publikacji **P1** opisałem uwodornienie podwójnego wiązania w chalkonach zawierających heteroatom w pierścieniu B (3-(2''-furylo)- (**1**) i 3-(2''-tiofeno-1-(2'-hydroksyfenylo)-prop-2-en-1-onu (**2**)) do dihydrochalkonów w procesie biotransformacji wykorzystując jako katalizatory niekonwencjonalne szczepy drożdży. Najwyższą skuteczność uwodornienia wiązania podwójnego substratu **1** zaobserwowałem w kulturach szczepów *Saccharomyces cerevisiae* KCh 464 i *Yarrowia lipolytica* KCh 71.

O O OH 1 mikroorganizm O O OH 1a

O S OH 2 mikroorganizm O S OH 2a

Schemat 1. Biotransformacja 3-(2″-furylo)-1-(2′-hydroksyfenylo)-prop-2-en-1-onu (**1**) oraz 3-(2″-tiofeno)-1-(2′-hydroksyfenylo)-prop-2-en-1-onu (**2**) przez wybrane szczepy niekonwencjonalnych drożdży

Substrat **1** w kulturach tych szczepów (*Saccharomyces cerevisiae* KCh 464 i *Yarrowia lipolytica* KCh 71) był przekształcony w produkt **1a** z konwersją > 99% (na podstawie analizy GC) już w sześć godzin po rozpoczęciu biotransformacji. Natomiast związek zawierający w swojej strukturze atom siarki (**2**) najskuteczniej przekształcony był w kulturze szczepu drożdży z gatunku *Yarrowia lipolytica* KCh 71 (konwersja > 99%, uzyskana po trzech godzinach inkubacji substratu) (Schemat 1). Zaobserwowałem, że różne szczepy badanych drożdży są w stanie przeprowadzić bioredukcję zastosowanego substratu z różną wydajnością. Wykazałem również, że badane szczepy przeprowadzają uwodornienie z różną prędkością, w zależności od obecności w ich komórkach indukowanych i/lub konstytutywnych ene-reduktaz. Produkty uzyskane w tych reakcjach obserwowałem już po godzinie inkubacji, przy czym konwersja substratu **1** do produktu **1a** wynosiła nawet ponad 50% podczas biotransformacji szczepem *Yarrowia lipolytica* KCh 71 (Publikacja **P1** Tabela 2.).

Największą zaletą tego procesu jest wydajna produkcja jednego produktu, bez powstawania produktów ubocznych.

Na podstawie przeprowadzonych badań opracowałem osiem zgłoszeń patentowych w których opisałem metody otrzymywania dihydrochalkonów. Pięć z nich już uzyskało ochronę patentową (P.426756, P.426757, P.426758, P.426767 oraz P.426768).

Uzyskane wyniki w publikacji **P1** były podstawą do przeprowadzenia biotransformacji chalkonów zawierających w swojej strukturze podstawniki metoksylowe (Tab. 1.). W tych badaniach sprawdziłem nie tylko wydajność biotransformacji w zależności od położenia podstawnika w obrębie pierścienia B (pozycje -orto, -meta, -para), ale również wpływ liczby podstawników metoksylowych oraz porównałem ogólną wydajności procesu biotransformacji pomiędzy szczepami (Publikacja **P2**).

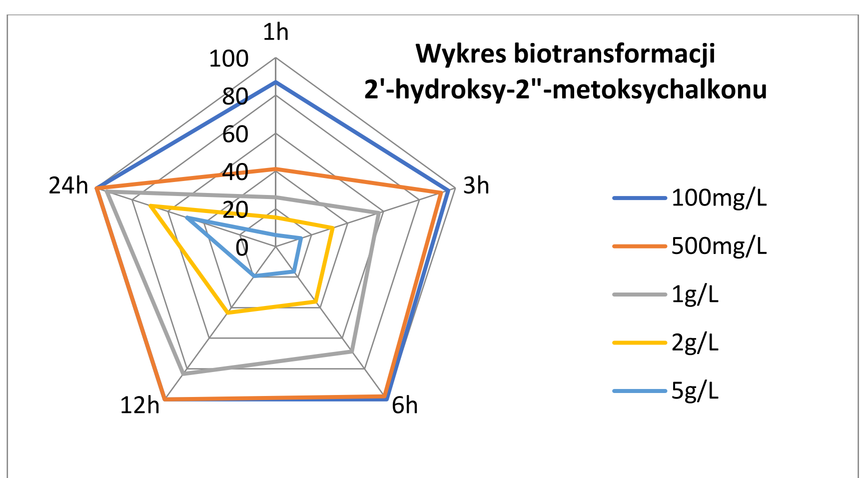
Tabela 1. Zsyntezowane chalkony oraz otrzymane w wyniku biotransformacji dihydrochalkony

Chalkony	R1	R2	R3	R4	R5	R6	Dihydrochalkony
(**3**)	$-OCH_3$	-H	-H	-H	-H	-H	(**3a**)
(**4**)	-H	$-OCH_3$	-H	-H	-H	-H	(**4a**)
(**5**)	-H	-H	$-OCH_3$	-H	-H	-H	(**5a**)
(**6**)	$-OCH_3$	-H	-H	$-OCH_3$	-H	-H	(**6a**)
(**7**)	-H	$-OCH_3$	$-OCH_3$	$-OCH_3$	-H	-H	(**7a**)
(**8**)	-H	-H	-H	-H	$-OCH_3$	$-OCH_3$	-
(**9**)	-H	-H	$-OCH_3$	-H	$-OCH_3$	$-OCH_3$	-
(**10**)	-H	$-OCH_3$	$-OCH_3$	$-OCH_3$	$-OCH_3$	$-OCH_3$	-

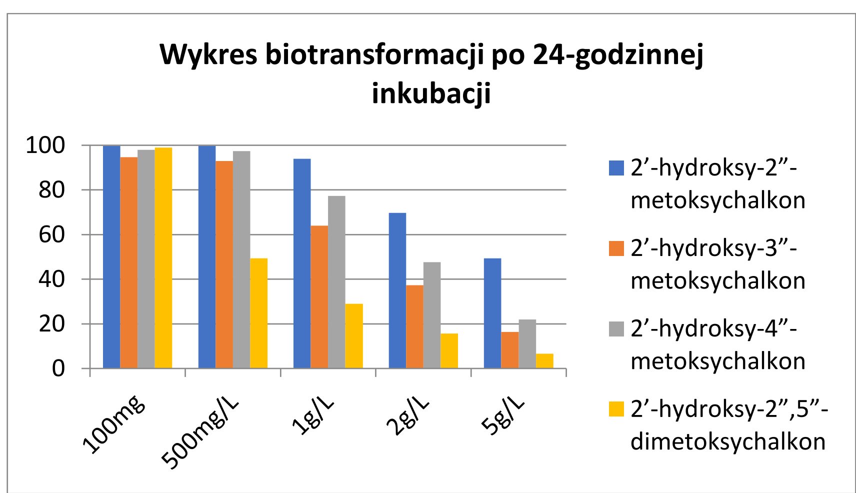
Również w przypadku metoksychalkonów, mikroorganizmem najefektywniej przekształcającym badane związki był szczep *Yarrowia lipolytica* KCh 71, dla którego sprawdziłem również wpływ stężenia substratu na wydajność przemian badanych związków. W hodowli tego szczepu monometoksychalkony (2′-hydroksy-2''-, 3''- i 4''-metoksychalkony) zostały efektywnie uwodornione do odpowiednich dihydrochalkonów przy zastosowaniu substratu w stężeniu 0,5g/1L podłoża już po godzinie inkubacji. Konwersję powyżej 40% po trzech godzinach transformacji obserwowałem również dla stężeń wynoszących 1g/1L pożywki (Schemat 2.). Wraz ze wzrostem liczby podstawników metoksylowych w substracie zaobserwowałem spadek szybkości i wydajności konwersji substratu.

Jedynym wyjątkiem jest 2′-hydroksy-2'',5''-dimetoksychalkon (**6**), który był przekształcany w dihydrochalkon przez szczep *Yarrowia lipolytica* KCh 71 z wydajnością porównywalną z chalkonem zawierającym pojedynczą grupę metoksylową.

Najwydajniej przekształcanymi związkami były chalkony zawierające pojedyncze grupy metoksylowe w pierścieniu B. Co ciekawe, 2′-hydroksy-2″-metoksychalkon (**3**) nawet w stężeniu zwiększonym do 5 g substratu na 1 L pożywki był przekształcany z wydajnością do 50% po 24 godzinach (Schemat 3.). Jednocześnie bardzo wysoka konwersja, od 66 do 91% w zależności od zastosowanego substratu, po jednej godzinie inkubacji wskazuje na bardzo wysoką aktywność enzymów obecnych w komórkach biokatalizatora.



Schemat 2. Konwersja 2′-hydroksy-2″-metoksychalkonu (**3**) przez szczep *Yarrowia lipolytica* KCh 71 przy różnych stężeniach substratu



Schemat 3. Porównanie konwersji substratów we wzrastających stężeniach przez szczep *Yarrowia lipolytica* KCh 71, po 24 godzinach

Redukcja wiązania podwójnego pomiędzy węglami C2 a C3 w szkielecie chalkonu pozwala z wysokim prawdopodobieństwem przypisać te właściwości enzymom OYE (*Old Yellow Enzymes*), które są znane w literaturze naukowej z wydajnej redukcji wiązania podwójnego różnorodnych grup związków, w tym chalkonów. [69–71]

Wraz ze wzrostem liczby grup metoksylowych wydajność procesu malała. W przypadku związków zawierających podstawniki metoksylowe również w pierścieniu A (**8, 9** i **10**) nie obserwowano produktów biotransformacji.

Zastosowanie drożdży z gatunku *Yarrowia lipolytica* nie jest przypadkowe. Jest to mikroorganizm, którego właściwości są szeroko wykorzystywane w przemyśle spożywczym - od produkcji kwasu cytrynowego[72] czy kwasu eikozapentaenowego[73], poprzez zapobieganie gnicia zbiorów[74], aż po produkcję wysokobiałkowej paszy dla zwierząt[75] i niekonwencjonalnej żywności dla ludzi[76]. Wykorzystuje się je również w produkcji różnego rodzaju substancji słodzących[77].

Drożdże tego gatunku zostały dopuszczone do obrotu jako nowa żywność w 2019 roku (Rozporządzenie UE 2019/760).[76] Co istotne, status GRAS (*Generally Recognised As Safe*) definiuje szczepy gatunku *Y. lipolytica* jako atrakcyjne i przyjazne środowisku narzędzie mikrobiologiczne do produkcji nutraceutyków, żywności fermentowanej i suplementów diety.[77] Ponadto, dzięki badaniom przeprowadzonym podczas realizacji pracy doktorskiej, wykazałem, że drożdże tego gatunku mogą być z powodzeniem wykorzystywane do produkcji znacznych ilości różnego rodzaju dihydrochalkonów. Co warte podkreślenia - biotransformacje z zastosowaniem tych mikroorganizmów pozwalają na otrzymanie z dużą wydajnością jednego produktu, co zwiększa szanse na przemysłowe wdrożenie tych procesów. Zastosowane mikroorganizmy nie były zdolne do produkcji innych pochodnych flawonoidów (flawonów, flawanonów czy flawonoli), które występują podczas transformacji chalkonów w kulturach grzybów strzępkowych, bakterii czy glonów.[23,24,67] Modyfikacja budowy chemicznej 2'-hydroksychalkonu prowadzi do powstania *in vitro* pochodnych o zróżnicowanym działaniu przeciwnowotworowym. Taka modyfikacja polegająca na stworzeniu analogów zawierających grupę metoksylową zamiast grupy hydroksylowej spowodowała zmianę siły działania przeciwnowotworowego, w zależności od liczby wprowadzonych grup i ich położenia w badanym związku. Jednak podstawowy mechanizm działania nie uległ zmianie. Działanie cytotoksyczne związku macierzystego i pochodnych było silniejsze w stosunku do linii komórek nowotworowych niż normalnych. Wszystkie przetestowane w tym badaniu związki są otrzymane przeze mnie i wykazują w różnym stopniu aktywność antyproliferacyjną i proapoptyczną oraz zdolność wywoływania uszkodzeń DNA. [78]

Sposoby wytwarzania 10 związków opisanych w obrębie publikacji **P2** zostały objęte ochroną patentową (patenty nr: P.426755, P.426760, P.426761, P.426762, P.426763, P.426764, P.426765, P.426766, P.426769, P.426770).

Chalkony, których syntezę opisałem w publikacji **P2,** następnie przekształciłem w odpowiednie flawony w reakcji cyklizacji z jodem w dimetylosulfotlenku według schematu:

I_2, DMSO
126*C, 2 h

Schemat 4. Reakcja cyklizacji chalkonów z jodem

Biotransformacje metoksyflawonów opisałem w dwóch kolejnych publikacjach (**P3** i **P4**). Katalizatorami zastosowanymi do przekształceń tych związków były entomopatogenne grzyby strzępkowe, które charakteryzują się znacznie bogatszą biblioteką enzymów hydroksylujących i glikozylujących polifenole, w stosunku do drożdży. Na podstawie wcześniejszych badań prowadzonych w naszym zakładzie[56,66,67,79], do przeprowadzenia biotransformacji metoksyflawonów wybrałem 9 szczepów należących do czterech gatunków grzybów: *Beauveria bassiana* KCh J1.5, KCh J2.1, KCh J1, KCh J3.2 i KCh BBT, *B. caledonica* KCh J3.3 i KCh J3.4, *Isaria farinosa* KCh KW 1.1 oraz *I. fumosorosea* KCh J2.

Pierwszą grupę zsyntezowanych metoksyflawonów opisałem w publikacji **P3** (związki o nr **11-15**). Są to związki posiadające grupy metoksylowe tylko w obrębie pierścienia B (Schemat 5):

	R_1	R_2	R_3	R_4	
(**11**)	$-OCH_3$	-H	-H	-H	2′-metoksyflawon
(12)	-H	$-OCH_3$	-H	-H	3′-metoksyflawon
(13)	-H	-H	$-OCH_3$	-H	4′-metoksyflawon
(14)	$-OCH_3$	-H	-H	$-OCH_3$	2′,5′-dimetoksyflawon
(15)	-H	$-OCH_3$	$-OCH_3$	$-OCH_3$	3′,4′,5′-trimetoksyflawon

Schemat 5. Otrzymane metoksyflawony, wykorzystane do przeprowadzenia procesu biotransformacji w publikacji **P3**

Podczas biotransformacji metoksyflawonów zostały zastosowane szczepy entomopatogennych grzybów o udokumentowanej już aktywności (szczepy z gatunków *B. bassiana, I. farinosa* oraz *I. fumosorosea*), jednakże, po raz pierwszy w biotransformacji flawonoidów zostały wykorzystane szczepy z gatunku *Beauveria caledonica* (KCh J3.3 i J3.4).

W odróżnieniu od biotransformacji chalkonów przez niekonwencjonalne szczepy drożdży, podczas biotransformacji flawonów przez entomopatogenne grzyby strzępkowe rzadziej obserwowałem tylko jeden produkt reakcji (zazwyczaj podczas biotransformacji szczepami *B. bassiana* KCh J1 oraz *I. farinosa* KCh KW1.1.). Liczba powstających produktów jest zależna od katalizatora, jednakże należy tutaj zwrócić uwagę na dwa szczepy: *B. bassiana* KCh J1.5, oraz *I. fumosorosea* KCh J2, jako te, posiadające najszerszy wachlarz enzymatyczny, dzięki któremu wyizolowano najwięcej produktów.

Na podstawie struktury wyizolowanych produktów można wnioskować, że glikozylowane pochodne flawonoidów powstają w wyniku przemian kaskadowych. W trakcie inkubacji zachodzą procesy demetylacji i/lub hydroksylacji oraz 4-*O*-metyloglukozylacji, w które zaangażowanych jest kilka enzymów i koenzymów biokatalizatora.

A) Biotransformacje flawonów zawierających grupy metoksylowe w pierścieniu B – Publikacja **P3**

1) Biotransformacje 2'-metoksyflawonu (**11**)

Beauveria bassiana
KCh J1
11
11a

Schemat 6. Biotransformacje 2′-metoksyflawonu (**11**) w kulturze szczepu *B. bassiana* KCh J1

Isaria fumosorosea
KCh J2
11
11b
11c
11d
11e

Schemat 7. Biotransformacje 2′-metoksyflawonu (**11**) w kulturze szczepu *I. fumosorosea* KCh J2

Wyizolowane produkty:

11a) 2′-hydroksyflawon
11b) 2′-*O*-β-D-(4″-*O*-metyloglukopiranozylo)-flawon
11c) 8-*O*-β-D-(4″-*O*-metyloglukopiranozylo)-2′-metoksyflawon
11d) 5′-*O*-β-D-(4″-*O*-metyloglukopiranozylo)-flawon
11e) 3-*O*-β-D-(4″-*O*-metyloglukopiranozylo)-2′-metoksyflawon

Podczas biotransformacji 2′-metoksyflawonu (**11**) zaobserwowałem powstanie łącznie 5 produktów (**11a-11e**) (Schematy 6 i 7). W wyniku biotransformacji w kulturach większości badanych szczepów otrzymałem jeden główny produkt 2′-*O*-β-D-(4″-*O*-metyloglukopiranozylo)-flawon (**11b**). Związku tego nie zaobserwowałem podczas biotransformacji w kulturze szczepu *Beauveria bassiana* KCh J1, który jako jedyny z użytych biokatalizatorów nie posiadał zdolności do 4-*O*-metyloglikozylacji. Związek **11b** powstawał w wyniku następującej po sobie demetylacji i 4-*O*-metyloglikozylacji (Schemat 7.).

Jako jeden z produktów został również scharakteryzowany 2′-hydroksyflawon (**11a**) (Schemat 6.), który powstał w wyniku demetylacji substratu **11**, a po zwiększeniu skali do półpreparatywnej, został on wyizolowany z największą wydajnością z hodowli szczepu *B. bassiana* KCh J1 (z wydajnością 43%).

Pozostałymi produktami transformacji substratu **11**, były glukopiranozyloflawony, posiadające przyłączoną cząsteczkę 4-*O*-glukopiranozydu wraz z zachowaną grupą metoksylową w pozycji 2′ (produkty **11c-e**). Związki te powstały w wyniku następujących po sobie procesów hydroksylacji oraz 4-*O*-metyloglikozylacji. (Schemat 7.).

2) Biotransformacje 3'-metoksyflawonu (**12**)

Schemat 8. Biotransformacje 3′-metoksyflawonu (**12**) w kulturze szczepu *B. bassiana* KCh J1

Schemat 9. Biotransformacje 3′-metoksyflawonu (**12**) w kulturze szczepu *I. farinosa* KCh KW 1.1

Wyizolowane produkty:

12a) 3′-hydroksyflawon
12b) 3′-*O*-β-D-(4″-*O*-metyloglukopiranozylo)-flawon

Kolejny substrat 3′-metoksyflawon (**12**) był najszybciej przekształcanym związkiem przez wszystkie biokatalizatory. Konwersja **12** w hodowlach czterech z dziewięciu biokatalizatorów (*I. fumosorosea* KCh J2, *I. farinosa* KCh KW1.1, *B. bassiana* KCh J1.5 i KCh J3.2) była bliska 100% po trzech dniach inkubacji (Publikacja P3, Tabela 3). Najprawdopodobniej wiąże się to z łatwiejszym dostępem enzymów odpowiedzialnych za proces demetylacji do grupy metoksylowej umiejscowionej w pozycji *meta* w stosunku do podstawnika chromenowego. Jednakże w wyniku tej biotransformacji zaobserwowałem znacznie mniej produktów. Pierwszy produkt **12a** powstał w wyniku demetylacji i został scharakteryzowany jako 3′-hydroksyflawon. (Schemat 8.) Natomiast głównym produktem przemiany 3′-metoksyflawonu u większości badanych szczepów był produkt demetylacji i 4-*O*-metyloglikozylacji, czyli 3′-*O*-β-D-(4″-*O*-metyloglukopiranozylo)-flawon (**12b**). (Schemat 9.)

3) Biotransformacje 4′-metoksyflawonu (**13**)

Schemat 10. Biotransformacje 4′- metoksyflawonu (**13**) w kulturze szczepu *B. bassiana* KCh J1

Schemat 11. Biotransformacje 4′- metoksyflawonu (**13**) w kulturze szczepu *B. bassiana* KCh J1.5

Wyizolowane produkty:

13a) 4′-hydroksyflawon
13b) 4′-*O*-β-D-(4″-*O*-metyloglukopiranozylo)-flawon

Podczas biotransformacji 4′-metoksyflawonu (**13**), konwersje w hodowlach badanych szczepów były porównywalne do tych uzyskanych dla 3′-metoksyflawonu (**12**) w siedmiodniowym okresie biotransformacji. Blisko 100% konwersję zaobserwowałem w hodowli *Isaria farinosa* KCh KW 1.1 oraz czterech szczepów *Beauveria bassiana* (KCh J1.5, J2.1, J3.2, BBT) (Publikacja P3, Tabela 4.). Również w przypadku tego substratu,

ze wszystkich hodowli szczepów entomopatogennych grzybów strzępkowych (za wyjątkiem *Beauveria bassiana* KCh J1) jako główny produkt biotransformacji wyizolowałem 4′-*O*-β-D-(4″-*O*-metyloglukopiranozylo)-flawon (**13b**) (Schemat 11.). Podobnie jak w przypadku dwóch wyżej opisanych związków (**11** i **12**), również dla tego substratu szczep *Beauveria bassiana* KCh J1 był zdolny tylko do demetylacji substratu, w wyniku czego został wyizolowany 4′-hydroksyflawon (**13a**). (Schemat 10.)

Metody i sposoby otrzymywania hydroksyflawonów zostały zastrzeżone w formie patentów o numerach: P.427404, P.427405 i P.427409.

4) Biotransformacje 2',5'-dimetoksyflawonu (**14**)

Schemat 12. Biotransformacje 2′5′-dimetoksyflawonu w kulturach szczepów *I. farinosa* KCh KW1.1 oraz *B. bassiana* KCh J1.5

Schemat 13. Biotransformacje 2′5′-dimetoksyflawonu (**14**) w kulturze szczepu *Beauveria bassiana* KCh J1

Wyizolowane produkty:

14a(11d) 5′-*O*-β-D-(4″-*O*-metyloglukopiranozylo)- 2′-metoksyflawon
14b) 2′-*O*-β-D-(4″-*O*-metyloglukopiranozylo)- 5′- metoksyflawon
14c) 4′-*O*-β-D-(4″-*O*-metyloglukopiranozylo)- 2′,5′-dimetoksyflawon
14d) 5′-hydroksy-2′-metoksyflawon
14e) 4′- hydroksy -2′,5′-dimetoksyflawon

Podczas biotransformacji 2′,5′-dimetoksyflawonu (**14**), głównym produktem był związek powstający w wyniku 4-*O*-metyloglikozylacji poprzedzonej regioselektywną demetylacją grupy CH_3 usytuowanej przy węglu C-5′. W wyniku tych przemian uzyskano

5′-*O*-β-D-(4″-*O*-metyloglukopiranozylo)-2′-metoksyflawon (**14a/11d**). Związek ten zaobserwowałem również podczas biotransformacji 2′-metoksyflawonu (**11**). Drugim produktem powstającym podczas biotransformacji 2′,5′-dimetoksyflawonu (**14**), w kulturze szczepu *B. bassiana* KCh J1.5, który powstawał z niemal dwukrotnie niższą wydajnością niż **14a** (Publikacja P3, Tabela 5.), również był produkt demetylacji oraz 4-*O*-metyloglukozylacji. Strukturę tego produktu ustalono w oparciu o wykonaną analizę strukturalną jako 2′-*O*-β-D-(4″-*O*-metyloglukopiranozylo)-5′-metoksyflawon (**14b**) (Schemat 12). Trzeci produkt powstający z najniższą wydajnością w hodowli *B. bassiana* KCh J1.5 został również zidentyfikowany jako 4-*O*-metyloglukopiranozyd. Jednakże w tym przypadku nastąpiła najpierw hydroksylacja węgla C-4′, a dopiero później 4-*O*-metyloglukozylacja. Otrzymanym produktem był 4′-*O*-β-D-(4″-*O*-metyloglukopiranozylo)- 2′,5′-dimetoksyflawon (**14c**).

W wyniku wykonania biotransformacji preparatywnej w kulturze szczepu *B. bassiana* KCh J1 wyizolowałem również hydroksypochodne. Pierwsza z nich, 5′-hydroksy-2′-metoksyflawon (**14d**), powstała w wyniku demetylacji grupy metylowej usytuowanej przy węglu C-5′, natomiast druga, 4′-hydroksy-2′,5′-dimetoksyflawon (**14e**), – w wyniku hydroksylacji węgla C-4′ (Schemat 13.) .

5) Biotransformacje 3′,4′,5′-trimetoksyflawonu (**15**)

Schemat 14. Biotransformacje 3′,4′,5′-trimetoksyflawonu (**15**) w kulturze szczepu *B. bassiana* KCh J1.5

Wyizolowane produkty:

15a) 3′-*O*-β-D-(4″-*O*-metyloglukopiranozylo)-4′,5′-dimetoksyflawon
15b) 4′-*O*-β-D-(4″-*O*- metyloglukopiranozylo)-3′,5′-dimetoksyflawon
15c) 3′-*O*-β-D-(4″-*O*- metyloglukopiranozylo)-6-hydroksy-4′,5′-dimetoksyflawon
15d) 6-*O*-β-D-(4″-*O*- metyloglukopiranozylo)-3′,4′,5′-trimetoksyflawon

Najwyższą konwersję (ponad 90% po siódmym dniu biotransformacji) 3′,4′,5′-trimetoksyflawonu zaobserwowałem w hodowli *B. bassiana* KCh J1.5 oraz dwóch szczepów *B. caledonica* (KCh J3.3 i KCh J3.4) (Publikacja P3, Tabela 6). W wyniku biotransformacji w zwiększonej skali w kulturze *B. bassiana* KCh J1.5, wyizolowałem cztery produkty (**15a-15d**) (Schemat 14.).

Główny produkt (powstający w większości badanych szczepów) jest efektem demetylacji i 4-*O*-metyloglukozylacji i został scharakteryzowany jako 3′-*O*-β-D-(4″-*O*-metylogluko-piranozylo)-4′,5′-dimetoksyflawon (**15a**).

Drugi produkt przeprowadzonej biotransformacji również powstał w wyniku demetylacji i 4-*O*-metyloglukozylacji, jednakże w tym przypadku metyloglikozyd był dołączany przy węglu C-4′, w efekcie czego powstawał 4′-*O*-β-D-(4″-*O*-metyloglukopiranozylo)-3′,5′-dimetoksyflawon (**15b**). W hodowli *B. bassiana* KCh BBT związki **15a** i **15b** powstawały w stosunku 3:1, w hodowli *I. farinosa* KCh KW 1.1 w stosunku 10:1, a w hodowli *I. fumosorosea* KCh J2 w stosunku 48:1 (Publikacja P3, Tabela 6).

Kolejny produkt, powstający najwydajniej w kulturze szczepu *B. bassiana* KCh J1.5 (ok. 20% po 7 dobie inkubacji) został określony na podstawie widm korelacyjnych, na podstawie których stwierdziłem, że jednostka cukru jest związana z węglem C-6, a związek scharakteryzowałem jako 6-*O*-β-D-(4″-*O*-metyloglukopiranozylo)-3′,4′,5′-trimetoksyflawon (**15d**). Związek ten jest efektem następujących po sobie hydroksylacji i glikozylacji. Ostatnim wyizolowanym produktem biotransformacji był 3′-*O*-β-D-(4″-*O*-metyloglukopiranozylo)-6-hydroksy-4′,5′-dimetoksyflawon (**15c**), który pojawiał się w śladowych ilościach, maksymalnie 6%, po 10 dniach inkubacji w kulturze szczepu *B.bassiana* KCh BBT.

Na podstawie przeprowadzonych doświadczeń powstało 20 zgłoszeń patentowych, dotyczących sposobu wytwarzania glukopiranozyloflawonów.

B) Biotransformacje flawonów zawierających grupy metoksylowe w pierścieniu A oraz w pierścieniu A i B (Schemat 15) – Publikacja **P4**

	R_1	R_2	R_3	
(**16**)	-H	-H	-H	5,7-dimetoksyflawon
(**17**)	-H	$-OCH_3$	-H	5,7,4′-trimetoksyflawon
(**18**)	$-OCH_3$	$-OCH_3$	$-OCH_3$	5,7,3′,4′,5′-pentametoksyflawon

Schemat 15. Otrzymane metoksyflawony, wykorzystane do przeprowadzenia procesu biotransformacji w publikacji **P4**

1) Biotransformacje 5,7-dimetoksyflawonu (**16**)

Schemat 16. Biotransformacje 5,7-dimetoksyflawonu (**16**) w kulturze szczepu *Isaria farinosa* KCh KW 1.1

Wyizolowane produkty:

16a) 3′-hydroksy-5,7-dimetoksyflawon
16b) 4′- hydroksy-5,7-dimetoksyflawon
16c) 3′,4′-dihydroksy-5,7-dimetoksyflawon

2) Biotransformacje 5,7,4′-trimetoksyflawonu (**17**)

Schemat 17. Biotransformacje 5,7,4′-trimetoksyflawonu (**17**) w kulturze szczepu *Isaria farinosa* KCh KW 1.1

Wyizolowane produkty:

17a(16b) 4′-hydroksy-5,7-dimetoksyflawon
17b(16c) 3′,4′-dihydroksy-5,7-dimetoksyflawon

3) Biotransformacje 5,7,3′,4′,5′-pentametoksyflawonu (**18**)

Schemat 18. Biotransformacje 5,7,3′,4′,5′-pentametoksyflawonu (**18**) w kulturach szczepów *B. bassiana* KCh J1.5 and *B. bassiana* KCh J1

Wyizolowane produkty:

18a) 3′-hydroksy-5,7,4′,5′-tetrametoksyflawon
18b) 3′-*O*-β-D-(4″-*O*-metyloglukopiranozylo)-5,7,4′,5′-tetrametoksyflawon

W przypadku flawonów zawierających podstawniki metoksylowe w pozycji 5,7 – pierścienia A (substraty **16-18**), przyłączanie jednostki glikozydowej nie następowało tak wydajnie jak w przypadku substratów zawierających podstawniki tylko w pierścieniu B (**11-15**).

W przeprowadzonych badaniach żaden z badanych mikroorganizmów nie był w stanie przeprowadzić *O*-demetylacji grup metoksylowych obecnych w pierścieniu A. Demetylację obserwowałem jedynie w pierścieniu B badanych substratów. Co zaskakujące, w świetle opisanej wcześniej efektywnej 4-*O*-metyloglukozylacji hydroksyflawonów w kulturach grzybów entomopatogennych,[35,37,80,81] podczas biotransformacji 5,7-dimetoksyflawonów wyizolowałem tylko jeden glikopiranozyd: 3′-*O*-β-D-(4″-*O*-metyloglukopiranozylo)-5,7,4′,5′-tetrametoksyflawon **18b**).

Głównym produktem trzydniowej biotransformacji 5,7-dimetoksyflawonu (**16**) (Schemat 16.) w kulturze szczepu *B. bassiana* KCh J1.5 był 3′-hydroksy-5,7-dimetoksyflawon (**16a**), który został wyizolowany z wydajnością 40%. Analiza NMR i MS wykazała, że związek ten jest produktem hydroksylacji. Stężenie związku **16a** było wyraźnie wyższe niż 5,7-dimetoksy-4′-hydroksyflawonu (**16b**) (**Publikacja 4**, Tabela 1 i Tabela S1 - Materiały uzupełniające). Różnica w ilościach otrzymanych związków **16a** i **16b** świadczy o preferencji monooksygenaz wytwarzanych przez badane szczepy entomopatogenne do umieszczania grupy hydroksylowej w 5,7-dimetoksyflawonie (**16**) przy węglu C-3′. Według wcześniejszych badań głównym miejscem hydroksylacji była pozycja C-4′ pierścienia B. 6-metoksyflawanon w hodowli *I. fumosorosea* KCh J2 był hydroksylowany do pochodnych 4′-hydroksy- i 3′,4′-dihydroksylowych, natomiast 6-metoksyflawon był hydroksylowany do pochodnych 3′- i 4′-hydroksy oraz 3′,4′-dihydroksylowych.[66]

Hydroksylacja węgla C-3′ i 4′-hydroksflawonoidów jest często obserwowana i dobrze scharakteryzowana w komórkach roślinnych.[82,83] Proces hydroksylacji pierścienia B flawonoidów jest katalizowany przez 3′-hydroksylazę flawonoidową (F3′H). Natomiast wyniki badań drzewa filogenetycznego wykazały, że F3′H należy do CYP75B. [82,84]

W przypadku substratu **18** – 5,7,3′,4′,5′-pentametoksyflawonu głównym produktem powstającym w kulturach badanych szczepów był 3′-*O*-β-D-(4″-*O*-metylo)-glukopiranozylo-5,7,4′,5′-tetrametoksyflawon (**18b**) (Schemat 18.). Produkt następujących po sobie regioselektywnej demetylacji oraz 4-*O*-metyloglukozylacji.

Tylko jeden z wykorzystanych szczepów entomopatogennych nie był zdolny do przeprowadzenia tej reakcji - szczep *Beauveria bassiana* KCh J1. Natomiast w hodowli tego szczepu zaobserwowałem najwyższe stężenie produktu regioselektywnej *O*-demetylacji grupy metoksylowej związanej z węglem C-3′. Produktem tego procesu był 3′-hydroksy-5,7,4′,5′-tetrametoksyflawon (**18a**), który był produktem pośrednim, podczas biotransformacji w kulturach pozostałych szczepów, niezbędnym do otrzymania związku **18b**.

PODSUMOWANIE

- Do przeprowadzenia opisanych biotransformacji wykorzystałem 17 szczepów mikroorganizmów, w tym 8 niekonwencjonalnych szczepów drożdży (z gatunków *Rhodotorula rubra, R. glutinis, Saccharomyces cerevisiae, Candida viswanathii, C. parapsilosis* oraz *Yarrowia lipolytica*) – (**P1** i **P2**) , oraz 9 entomopatogennych szczepów grzybów strzępkowych (z gatunków *Beauveria bassiana, B. caledonica, Isaria farinosa* oraz *I. fumosorosea*) – (**P3** i **P4**).
- Jako substratów użyłem 18 związków wytworzonych na drodze syntezy chemicznej (10 chalkonów oraz 8 flawonów), z których otrzymałem łącznie 28 produktów, w tym 7 dihydrochalkonów oraz 21 pochodnych metoksyflawonów - osiem z przyłączoną grupą hydroksylową i trzynaście 4-*O*-metyloglukopiranozylo pochodnych. Trzynaście z nich nie było wcześniej opisanych w literaturze.
- Zastosowane szczepy niekonwencjonalnych drożdży z wysoką regioselektywnością redukowały wiązanie podwójne w chalkonach pomiędzy węglem C2 a C3, czego produktem były odpowiednie dihydrochalkony. Testowane drożdże nie były zdolne do redukcji chalkonów posiadających grupy metoksylowe w pierścieniu A, w pozycjach 4, 6.
- Szczep *Yarrowia lipolytica* KCh 71 przeprowadzał uwodornienie badanych chalkonów najefektywniej.
- Najwięcej produktów biotransformacji flawonów zaobserwowałem, gdy przynajmniej jeden z podstawników metoksylowych znajdował się w pozycji 2′ pierścienia B.
- Zastosowane szczepy entomopatogennych grzybów strzępkowych są zdolne do wprowadzania cząsteczki 4-*O*-metyloglukopiranozy do struktury flawonów posiadających podstawniki metoksylowe w pierścieniu B, po wcześniejszej ich demetylacji.
- Metoksyflawony posiadające podstawniki metoksylowe w pierścieniu A w pozycji 5,7- były najczęściej przekształcane do pochodnych hydroksylowych. Jedynym wyjątkiem był 5,7,3′,4′,5′-pentametoksyflawon, który w wyniku biotransformacji w kulturach szczepów *Beauveria bassiana* KCh J1.5 został przekształcony do 3′-*O*-β-D-(4″-*O*-metyloglukopiranozylo)-5,7,4′,5′-tetrametoksyflawonu (**18b**).
- 4-*O*-metyloglukopiranozydy badanych flawonów powstają w procesie dwuetapowym: metoksyflawony ulegają najpierw demetylacji, a później dochodzi do przyłączenia cząsteczki glikozydu; pozostałe flawony ulegają najpierw hydroksylacji i późniejszemu przyłączeniu 4-O-metyloglukopiranozy.
- Struktury wszystkich otrzymanych produktów określiłem na podstawie analizy ^{1}H NMR, ^{13}C NMR oraz widm korelacyjnych.
- Produkty syntez i biotransformacji flawonów dodatkowo zanalizowałem na podstawie stosunku ich masy do ładunku m/z na podstawie analizy spektroskopowej LC-MS.
- W wyniku przeprowadzonych badań, opisanych w publikacjach **P1-P4** powstało 19 patentów oraz 30 zgłoszeń patentowych dotyczących sposobu wytwarzania przedstawionych w dysertacji związków.

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Oświadczenie

Dotyczące szacunkowego udziału procentowego współautorów publikacji:

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Dane personalne współautora i udział procentowy:

Lp.	Tytuł (stopień), imię i nazwisko, nazwa zakładu pracy.	Udział %	Podpis Autora
1	mgr inż. Mateusz Łużny, Katedra Chemii, Uniwersytet Przyrodniczy we Wrocławiu	75	Łużny
2	Martyna Krzywda, Katedra Chemii, Uniwersytet Przyrodniczy we Wrocławiu	5	M Krzywda
3	mgr inż. Ewa Kozłowska, Katedra Chemii, Uniwersytet Przyrodniczy we Wrocławiu	5	Kozłowska Ewa
4	dr hab. Edyta Kostrzewa-Susłow, Katedra Chemii, Uniwersytet Przyrodniczy we Wrocławiu	5	E Kostrzewa-Susłow
5	dr hab. Tomasz Janeczko, Katedra Chemii, Uniwersytet Przyrodniczy we Wrocławiu	10	[illegible]

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Dotyczące szacunkowego udziału procentowego współautorów publikacji:

Łużny, M.; Kozłowska, E.; Kostrzewa-Susłow, E.; Janeczko, T. Highly effective, regiospecific hydrogenation of methoxychalcone by *Yarrowia lipolytica* enables production of food sweeteners, Catalysts, 2020, 10, 1135.

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Dane personalne współautora i udział procentowy:

Lp.	Tytuł (stopień), imię i nazwisko, nazwa zakładu pracy.	Udział %	Podpis Autora
1	mgr inż. Mateusz Łużny, Katedra Chemii, Uniwersytet Przyrodniczy we Wrocławiu	80	Mateusz Łużny
2	dr inż. Ewa Kozłowska, Katedra Chemii, Uniwersytet Przyrodniczy we Wrocławiu	5	Kozłowska Ewa
3	Prof. dr hab. Edyta Kostrzewa-Susłow, Katedra Chemii, Uniwersytet Przyrodniczy we Wrocławiu	5	E. Kostrzewa-Susłow
4	dr hab. Tomasz Janeczko prof. nadzw. UPWr, Katedra Chemii, Uniwersytet Przyrodniczy we Wrocławiu	10	[illegible]

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Dotyczące szacunkowego udziału procentowego współautorów publikacji:

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Dane personalne współautora i udział procentowy:

Lp.	Tytuł (stopień), imię i nazwisko, nazwa zakładu pracy.	Udział %	Podpis Autora
1	mgr inż. Mateusz Łużny, Katedra Chemii, Uniwersytet Przyrodniczy we Wrocławiu	70	Mateusz Łużny
2	dr inż. Tomasz Tronina, Katedra Chemii, Uniwersytet Przyrodniczy we Wrocławiu	5	Tomasz Tronina
3	dr inż. Ewa Kozłowska, Katedra Chemii, Uniwersytet Przyrodniczy we Wrocławiu	3	Kozłowska Ewa
4	dr inż. Monika Dymarska, Katedra Chemii, Uniwersytet Przyrodniczy we Wrocławiu	3	Dymarska
5	dr inż. Jarosław Popłoński, Katedra Chemii, Uniwersytet Przyrodniczy we Wrocławiu	2	Popłoński
6	mgr inż. Jacek Łyczko, Katedra Chemii, Uniwersytet Przyrodniczy we Wrocławiu	2	Łyczko
7	Prof. dr hab. Edyta Kostrzewa-Susłow, Katedra Chemii, Uniwersytet Przyrodniczy we Wrocławiu	5	E. Kostrzewa-Susłow
8	dr hab. Tomasz Janeczko prof. nadzw. UPWr, Katedra Chemii, Uniwersytet Przyrodniczy we Wrocławiu	10	[illegible]

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Lp.	Tytuł (stopień), imię i nazwisko, nazwa zakładu pracy.	Udział %	Podpis Autora
1.	mgr inż. Mateusz Łuzny, Katedra Chemii, Uniwersytet Przyrodniczy we Wrocławiu	75	Łużny
2.	dr inż. Tomasz Tronina, Katedra Chemii, Uniwersytet Przyrodniczy we Wrocławiu	5	Tomasz Tronina
3.	dr inż. Ewa Kozłowska, Katedra Chemii, Uniwersytet Przyrodniczy we Wrocławiu	5	Kozłowska Ewa
4.	Prof. dr hab. Edyta Kostrzewa-Susłow, Katedra Chemii, Uniwersytet Przyrodniczy we Wrocławiu	5	[signature]
5.	dr hab. Tomasz Janeczko, Katedra Chemii, Uniwersytet Przyrodniczy we Wrocławiu	10	[signature]

PUBLIKACJE

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Effective Hydrogenation of 3-(2″-furyl)- and 3-(2″-thienyl)-1-(2′-hydroxyphenyl)-prop-2-en-1-one in Selected Yeast Cultures. *Molecules,* 2019, 24, 3185.

Article

Effective Hydrogenation of 3-(2″-furyl)- and 3-(2″-thienyl)-1-(2′-hydroxyphenyl)-prop-2-en-1-one in Selected Yeast Cultures

Mateusz Łużny *, Martyna Krzywda, Ewa Kozłowska, Edyta Kostrzewa-Susłow and Tomasz Janeczko *

Department of Chemistry, Wrocław University of Environmental and Life Sciences, Norwida 25, 50-375 Wrocław, Poland
* Correspondence: mat.luzny@gmail.com(M.Ł.); janeczko13@interia.pl (T.J.); Tel.: +48-713-205-195 (T.J.)

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Abstract: Biotransformations were performed on eight selected yeast strains, all of which were able to selectively hydrogenate the chalcone derivatives 3-(2″-furyl)- (**1**) and 3-(2″-thienyl)-1-(2′-hydroxyphenyl)-prop-2-en-1-one (**3**) into 3-(2″-furyl)- (**2**) and 3-(2″-thienyl)-1-(2′-hydroxyphenyl)-propan-1-one (**4**) respectively. The highest efficiency of hydrogenation of the double bond in the substrate **1** was observed in the cultures of *Saccharomyces cerevisiae* KCh 464 and *Yarrowia lipolytica* KCh 71 strains. The substrate was converted into the product with > 99% conversion just in six hours after biotransformation started. The compound containing the sulfur atom in its structure was most effectively transformed by the *Yarrowia lipolytica* KCh 71 culture strain (conversion > 99%, obtained after three hours of substrate incubation). Also, we observed that, different strains of tested yeasts are able to carry out the bioreduction of the used substrate with different yields, depending on the presence of induced and constitutive ene reductases in their cells. The biggest advantage of this process is the efficient production of one product, practically without the formation of side products.

Keywords: biotransformation; chalcone; dihydrochalcone; yeast; sweeteners

1. Introduction

In recent years, there has been growing interest from the food industry in sweeteners. Within this group of substances, dihydrochalcones are increasingly gaining attention. The increase in interest is due to the fact that dihydrochalcones are synthesized by plant cells, and they are a daily part of our diet [1,2]. They are present in citrus, apples, tomatoes, potatoes, bean sprouts, and other plants [3]. Dihydrochalcones have a wide spectrum of activity, such as antiviral (the ability to inhibit dengue virus proteases or herpes simplex virus), and anti-inflammatory and antioxidant activity [4–7]. They are also known for their activity against pathogenic microorganisms, including gram-positive and gram-negative bacteria, as well as fungi [8] and antimalarial or anti-tuberculosis activity [9]. Dihydrochalcone (phlorizin) is an active inhibitor of fungal tyrosinase [6,10]. Dihydrochalcones are also used in chemical synthesis to obtain biologically active compounds. 2′-Hydroxydihydrochalcon is used as a building block in the synthesis of propafenone—the active substance of anti-arrhythmic drugs [11–13].

Schallenberger's hypothesis explains the sensation of sweet taste, according to which the flavor substance and the receptor create contact by hydrogen bonding [14]. This is due to the proton donor groups (AH) and an electron donor (B), which are also present in taste receptors and the structure of sweet substance. The sweetness of dihydrochalcones is related to their structure. The most important element of the structure of these compounds is the substitution of the B-ring in the meta or para position

with at least one -OH group. The compounds that have three adjacent substituents on the B-ring do not have a sweet taste [15]. It is suspected that the structure of the B-ring also affects the perception of non-sweet aftertaste in dihydrochalcones, which do not always correspond to human preferences [16]. Commonly used in the food industry, neohesperidin dihydrochalcone is characterized by a licorice flavor and a cool feeling on the tongue [17]. There is also a group of dihydrochalcone analogs, that have a heteroatom in the B-ring and having non-sweet flavors [2]. This group also describes 3-(2″-furyl)-1-(2′-hydroxyphenyl)-propan-1-one (2), which, depending on the concentration, may have different taste sensations: A concentration of 0.01 ppm does not show any flavor properties; 1 ppm, soapy; umami, bitter; 10 ppm, licorice, soapy, slightly sweet, bitter, vegetable, terpenic; a particularly interesting impression is noted in higher concentrations – 100 ppm, bitter, licorice, light-sweet, celery, umami, and broth [2].

A method for obtaining 3-(2″-furyl)-1-(2′-hydroxyphenyl)-propan-1-one (2) with a conversion of 75%, as a result of the chemical synthesis of 2′-hydroxyacetophenone and furfuryl alcohol with NaOH, in the presence of an iridium catalyst, has been identified in [18]. The direct chemical hydrogenation of chalcones to dihydrochalcones requires the use of metal salts (complexes) of iridium, palladium, ruthenium, or nickel, and the entire reaction (due to its high flammability) must be conducted under strictly controlled conditions [19–22]. There are no reports in the literature regarding the use of biotechnological methods to obtain both, 3-(2″-furyl)- (2) as well 3-(2″-thienyl)-1-(2′-hydroxyphenyl)-propan-1-one (4).

The majority of living organisms are able to hydrogenate the double bond [23,24]. Yeasts possess their specific enzymes, capable of hydrogenating chalcones [9,25,26]. Such activity is also described in relation to other microorganisms, including bacteria, such as *Gordonia* sp. and *Rhodococcus* sp. [27], the entomopathogenic fungus, *Aspergillus flavus* [6], and the cyanobacterium, *Spirulina platensis* [28]. The reduction of activated alkenes by ente reductases [EC 1.3.1.31], which are flavoproteins from the old yellow enzyme (OYE) family, has been investigated in great detail [29–31]. The substrates in these reactions are predominantly hydroxy and methoxy derivatives of chalcones. In this study, we aimed to assess whether the heteroatom-containing substrate in the B-ring would also be accepted by the double-binding dehydrogenase, present in the tested biocatalysts.

The main aim of the study was to assess the capability of yeast strains for the biotransformation of a compound, containing a furan (**1**) and thiophene (**3**) substituent. Yeast strains, used in this experiment, are the subject of many years of research conducted in the Department of Chemistry of Wrocław University of Environmental and Life Sciences, regarding their catalytic capabilities [26,32,33], and have been selected as having a high ability in reduceing the double bond in the chalcone and its derivatives [26–28]. An additional goal was to optimize the biotechnological production of dihydrochalcone (**2**, **4**), which would enable the development of the method of obtaining the product on an increased scale.

2. Results and Discussion

Each of the eight tested microorganisms (*Rhodotorula rubra* KCh 4, *Yarrowia lipolytica* KCh 71, *Rhodotorula marina* KCh 77, *Rhodotorula rubra* KCh 82, *Candida viswanathii* KCh 120, *Rhodotorula glutinis* KCh 242, *Saccharomyces cerevisiae* KCh 464, *Candida parapsilosis* KCh 909) was able to transform both substrates into the expected products, but the efficiency of the described process differs significantly between the strains (Table 1 and Figure 1). High regioselectivity of the biocatalysts' ability to reduce the double bond was observed, which was described before on the example of other compounds, e.g., chalcone containing no substituents and 2′-hydroxychalcone [32,34,35].

Table 1. Relationship between transformation of substrates **1** and **3** [%] and time in the cultures of tested strains.

Time [Days]	Strain Number															
	KCh 4		KCh 71		KCh 77		KCh 82		KCh 120		KCh 242		KCh 464		KCh 909	
	1	3	1	3	1	3	1	3	1	3	1	3	1	3	1	3
1	91	23	98	>99	92	38	98	59	49	84	0	52	98	98	2	9
3	99	33	>99	>99	>99	60	>99	84	>99	99	0	67	>99	99	14	12
7	>99	49	>99	>99	>99	63	>99	97	>99	99	17	87	>99	98	26	16

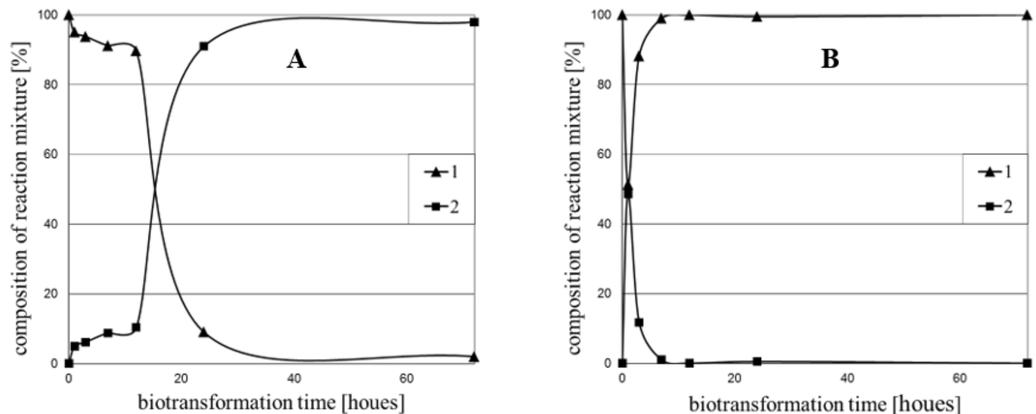

Figure 1. Time dependence of the transformation of chalcone (**1**) in the culture of: (**A**) *Rhodotorula rubra* KCh 4; (**B**) *Yarrowia lipolytica* KCh 71.

In the experiment, 3-(2″-furyl)- (**1**) and 3-(2″-thienyl)-1-(2′-hydroxyphenyl)-prop-2-en-1-one (**3**) were obtained by chemical synthesis, and then converted to 3-(2″-furyl)- (**2**) and 3-(2″-thienyl)-1-(2′-hydroxyphenyl)-propan-1-one (**4**) using biotransformation methods on a semi-preparative scale (Figures 2 and 3). The obtained products **1–4** were purified (isolated yield > 70%) and their structures were determined on the basis of NMR analysis (^{1}H-NMR, ^{13}C-NMR and correlation spectra – HMBC; HMQC, COSY) as well as by gas chromatography (GC) and thin-layer chromatography (TLC) analysis (Supplementary Materials).

Figure 2. Biotransformation of 3-(2″-furyl)-1-(2′-hydroxyphenyl)-prop-2-en-1-one (**1**) by selected yeast cultures. The ring designations and the numbering of carbon atoms have been placed on compound **1**.

Figure 3. Biotransformation of 3-(2″-thienyl)-1-(2′-hydroxyphenyl)-prop-2-en-1-one (**3**) by selected yeast cultures.

The reduction of the double bond was the most efficient in the *Saccharomyces cerevisiae* KCh 464 strain, where over 99% of the product **2** was observed in the reaction medium after six-hour substrate incubation (Table 2). On the first day of the reaction, the four tested microorganisms exceeded the threshold of 90% of the substrate **1** conversion: *R. rubra* KCh 4, *R. rubra* KCh 82, *Y. lipolytica* KCh 71, *R. marina* KCh 77. In the culture of *C. viswanathii* KCh 120, the 90% limit was exceeded after three days of the biotransformation process. A surprisingly low conversion was observed for the strains *R. glutinis* KCh 242 and *C. parapsilosis* KCh 909 (Table 1). High hydrogenation of the double bond in the cultures of these strains was observed for chalcone and its methoxy derivatives [32,36].

Table 2. Transformation of the substrate 1 by selected strains.

Time [h]	Strain Number			
	KCh4	**KCh71**	**KCh120**	**KCh464**
1	5 ± 1.1	52 ± 4.5	2 ± 0.5	40 ± 2.1
3	6 ± 0.1	88 ± 1.6	2 ± 0.4	84 ± 2.9
6	9 ± 0.5	>99 ±0.0	4 ± 0.9	98 ± 0.5
12	10 ± 0.9	>99 ± 0.0	9 ± 0.5	99 ± 0.6

Substrate **3** was most effectively transformed by the *Y. lipolytica* KCh 71 strain. The conversion above 99% was recorded after the third hour of biotransformation. A high efficiency of reduction of the double bond of chalcone derivatives was also confirmed for *Saccharomyces cerevisiae* KCh 464 strain (conversion > 98% after three hours of transformation). For most of the tested strains, a lower conversion of the substrate, containing thiophene (**3**) in its, compared to the furan-containing substrate (**1**), was observed. Interestingly, the strain *Rhodotorula rubra* KCh 4 after twelve hours showed only 10% of substrate **1** conversion, and after 24h it was already 91% (Figure 1), which may indicate that ene reductases are not present in the cell, but their production begins as a response to the stimulus (substrate induction) from the environment. An analogous induction of dehydrogenases (ene reductases) was observed for the strain *C. viswanathii* KCh 120. In the culture of this strain, substrate **3** was converted faster. However, conversion above 98% was achieved for both substrates at the same time in three days. Based on the obtained results, it can be concluded that the constitutive enzyme is present in *Y. lipolytica* KCh 71 and *S. cerevisiae* KCh 464 strains (observations for both substrates **1** and **3**). Biotransformations with these microorganisms show the fastest conversion progress, compared to other used microorganisms for both substrate **1** and **3** (Tables 1–3). Similar observations were obtained during the biotransformation of chalcone and described earlier [32].

Table 3. Transformation of the substrate 3 by selected strains.

Time [h]	Strain Number			
	KCh71	**KCh120**	**KCh242**	**KCh464**
1	67 ± 3.9	0 ± 0.0	1 ± 0.6	35 ± 3.6
3	>99 ± 0.0	10 ± 1.1	5 ± 0.5	64 ± 0.9
6	>99 ± 0.0	14 ± 1.2	9 ± 0.6	83 ± 0.6
12	>99 ± 0.0	25 ± 3.1	12 ± 0.9	98 ± 0.3

The strains of the species *Saccharomyces cerevisiae* are widely described as biocatalysts, effectively reducing the double bond in various compounds – derivatives of chalcones [9], containing methyl, methoxy, hydroxy substituents [37], and even bromine or chlorine [33,38], in both A and B-rings. On the basis of the results obtained in this study, it can be concluded that a chalcone derivative, containing a heteroatom in the B-ring, is effectively transformed by this strain. In previous studies

during biotransformation of chalcones, in addition to the hydrogenation product, a carbonyl reduction product was also observed [28,32,37,39]. In this study, additional products were not observed in any of the reactions carried out for the test compounds.

Biotransformation

The obtained product 3-(2″-furyl)-1-(2′-hydroxyphenyl)-propan-1-one (**2**) was characterized by the following NMR spectrum: ^{1}H-NMR (600 MHz) ($CDCl_3$) δ (ppm): 3.07-3.12 (m, 2H, H-3), 3.34-3.41 (m, 2H, H-2), 6.06 (dq, 1H, *J* = 3.2, 0.8 Hz, H-3″), 6.29 (dd, 1H, *J* = 3.1, 1.9 Hz, H-4″), 6.90 (ddd, 1H, *J* = 8.2, 7.1, 1.2 Hz, H-5′), 7.02 (dd, 1H, *J* = 8.4, 0.9 Hz, H-3′), 7.32 (dd, 1H, *J* = 1.8, 0.8 Hz, H-5″), 7.49 (ddd, 1H, *J* = 8.5, 7.2, 1.6 Hz, H-4′), 7.92 (dd, 1H, *J* = 8.0, 1.6 Hz, H-6′), 12.23 (s, 1H, -O*H*).

^{13}C-NMR (151 MHz, $CDCl_3$) δ = 22.50 (C-3), 36.73 (C-2), 105.69 (C-3″), 110.44 (C-4″), 118.72 (C-3′), 119.12 (C-5′), 119.40 (C-1′), 129.94 (C-6′), 136.56 (C-4′), 141.41 (C-5″), 154.33 (C-2″), 162.56 (C-2′), 204.90 (C-1).

The obtained product 3-(2″-thienyl)-1-(2′-hydroxyphenyl)-propane-1-one (**4**) was characterized by the following NMR spectrum: ^{1}H-NMR (600 MHz) ($CDCl_3$) δ (ppm): 3.27-3.33 (m, 2H, H-3), 3.37-3.43 (m, 2H, H-2), 6.87 (dq, 1H, *J* = 3.4, 1.0 Hz, H-3″), 6.90 (ddd, 1H, *J* = 7.4, 7.1, 1.0 Hz, H-5′), 6.29 (dd, 1H, *J* = 5.1, 3.4 Hz, H-4″), 6.99 (ddd, 1H, *J* = 8.4, 1.1, 0.4 Hz, H-3′), 7.14 (dd, 1H, *J* = 5.1, 1.2 Hz, H-5″), 7.47 (ddd, 1H, *J* = 8.4, 7.3, 1.5 Hz, H-4′), 7.76 (dd, 1H, *J* = 8.1, 1.6 Hz, H-6′), 12.24 (s, 1H, -O*H*).

^{13}C-NMR (151 MHz, $CDCl_3$) δ = 24.16 (C-3), 40.26 (C-2), 118.73 (C-3′), 119.13 (C-5′), 119.38 (C-1′), 123.72 (C-5″), 124.99 (C-3″), 127.06 (C-4″), 129.90 (C-6′), 136.60 (C-4′), 143.40 (C-2″), 162.56 (C-2′), 204.76 (C-1).

3. Materials and Methods

3.1. Substrate

The substrates, used for biotransformation, were obtained by Claisen-Shmidt condensation reaction of 2-hydroxyacetophenone (**5**) with furfural (**6**) or aldehyde **7** [purchased from Sigma-Aldrich (St. Louis, MO, USA)] dissolved in methanol in an alkaline environment at high temperature (Figures 4 and 5) according to the procedure described previously [32,40]. The resulting 3-(2″-furyl)-1-(2′-hydroxyphenyl)-prop-2-en-1-one (**1**) and 3-(2″-thienyl)-1-(2′-hydroxyphenyl)-prop-2-en-1-one (**3**) were used as a substrates for the biotransformation.

Figure 4. Synthesis of 3-(2″-furyl)-1-(2′-hydroxyphenyl)-prop-2-en-1-one (**1**).

Figure 5. Synthesis of 3-(2″-thienyl)-1-(2′-hydroxyphenyl)-prop-2-en-1-one (**3**).

The resulting compound (**1**) was characterized by the following NMR spectral data:

^{1}H-NMR (600 MHz) ($CDCl_3$) δ (ppm): 6.54 (dd, 1H, *J* = 3.4, 1.8 Hz, H-3″), 6.77 (d, 1H, *J* = 3.4 Hz, H-4″), 6.93 (ddd, 1H, *J* = 8.2, 7.2, 1.1 Hz, H-5′), 7.02 (dd, 1H, *J* = 8.4, 0.9 Hz, H-3′), 7.49 (ddd, 1H, *J* = 8.6, 7.2, 1.6 Hz, H-4′), 7.55 (d, 1H, *J* = 15.1 Hz, H-2), 7.56 (d, 1H, *J* = 1.4 Hz, H-5″), 7.68 (d, 1H, *J* = 15.2 Hz, H-3), 7.92 (dd, 1H, *J* = 8.1, 1.6 Hz, H-6′), 12.89 (s, 1H, -O*H*).

^{13}C-NMR (151 MHz, $CDCl_3$) δ = 113.03 (C-4″), 117.29 (C-3″), 117.73 (C-2), 118.66 (C-3′), 118.98 (C-5′), 120.17 (C-1′), 129.77 (C-6′), 131.26 (C-3), 136.44 (C-4′), 145.55 (C-5″), 151.65 (C-2″), 163.66 (C-2′), 193.46 (C-1).

The resulting compound (**3**) was characterized by the following NMR spectral data:

^{1}H-NMR (600 MHz) ($CDCl_3$) δ (ppm): 6.95 (ddd, 1H, *J* = 8.1, 7.1, 1.1 Hz, H-5′), 7.02 (dd, 1H, *J* = 8.4, 1.2 Hz, H-3′), 7.12 (ddd, 1H, *J* = *J* = 5.0, 3.7, 0,3 Hz, H-4″), 7.41 (d, 1H, *J* = 3.6, Hz, H-3″), 7.44 (d, 1H, *J* = 15.2 Hz, H-2), 7.47 (d, 1H, *J* = 4.9 Hz, H-5″), 7.49 (ddd, 1H, *J* = 8.4, 7.2, 1.5 Hz, H-4′), 7.89 (dd, 1H, *J* = 8.1, 1.6 Hz, H-6′), 8.05 (dd, 1H, *J* = 15.2, 0,5 Hz, H-3), 12.85 (s, 1H, -O*H*).

^{13}C-NMR (151 MHz, $CDCl_3$) δ (ppm) = 118.74 (C-3′), 118.95 (C-2), 118.99 (C-5′), 120.06 (C-1′), 128.66 (C-4″), 129.65 (C-6′), 129.68 (C-5″), 132.89 (C-3″), 136.49 (C-4′), 138.00 (C-3), 140.30 (C-2″), 163.69 (C-2′), 193.27 (C-1).

3.2. Microorganisms

The studies were carried out on eight strains of yeasts of the species *Rhodotorula rubra* (KCh 4 and KCh 82), *Rhodotorula marina* (KCh 77), *Rhodotorula glutinis* (KCh 242), *Yarrowia lipolytica* (KCh 71), *Candida viswanathii* (KCh 120), *Saccharomyces cerevisiae* (KCh 464), and *Candida parapsilosis* (KCh 909), which have already been described [26,33], and come from the collection of the Department of Chemistry of Wrocław University of Environmental and Life Sciences, Poland. All the strains were cultivated on a Sabouraud agar consisting of aminobac (5 g), glucose (40 g) and agar (15 g) dissolved in 1 L of distilled water and pH 5.5 and stored in a fridge at 4 °C.

3.3. Analysis

Initial tests were carried out using TLC plates (SiO_2, DC Alufolien Kieselgel 60 F_{254} (0.2 mm thick), Merck, Darmstadt, Germany). The product separation on a plate in cyclohexane is Ethyl acetate eluent (9:1 *v*/*v*). The product was observed (without additional visualization) under the UV lamp for the wavelength of 254 nm.

3.4. Gas Chromatography (GC)

GC analysis were performed using an Agilent 7890A gas chromatograph, equipped with a flame ionization detector (FID) (Agilent, Santa Clara, CA, USA). The capillary column DB-5HT (30 m × 0.25 mm × 0.10 µm) was used to determine the composition of the product mixtures. A temperature program was applied as follows: 80–300 °C, the temperature on the detector: 300 °C, injection 1 µl, flow 1 mL/min, flow H_2: 35 mL/min, air flow; 300 mL/min, time of analysis; 18.67 min. The retention time of the substrate **1**—9,6 min, product **2** retention time—8,5 min. The retention times for compounds having the thiophene substituent **3** and **4** were recorded as 10,8 min, and 9,7 min, respectively (Supplementary Materials).

3.5. NMR Analysis

NMR analysis was performed using a DRX 600 MHz Bruker spectrometer (Bruker, Billerica, MA, USA). The prepared samples were dissolved in deuterated chloroform $CDCL_3$. The analyses performed, included ^{1}H-NMR, ^{13}C-NMR, HMBC (two-dimensional analysis) HMQC (heteronuclear correlation) and COSY (correlation spectroscopy) (Supplementary Materials).

3.6. Screening

Erlenmeyer flasks with a capacity of 300 mL were used for biotransformation; each contained 100 ml of the culture medium – Sabouraud (3% glucose, 1% peptone). The transplanted microorganisms were incubated for three days at 24 °C on a rotary shaker (144 rpm). After this time, the substrates **1** and **3** were added in an amount of 10 mg, dissolved in 1 mL of DMSO (dimethyl sulfoxide). Samples were collected after 1, 3, 6, 12 hours and 1, 3, and 7 day of incubation. After this time, samples were extracted with ethyl acetate, dried with anhydrous magnesium sulfate ($MgSO_4$), and analyzed by TLC and GC.

3.7. Semi-Preparative Scale

Semi-preparative biotransformations were performed in 2 L Erlenmeyer flasks, each containing 500 mL of culture medium (3% glucose, 1% peptone). The transferred microorganisms were incubated for three days at 24 °C on a rotary shaker. After this time, the substrate was added in 100 mg, dissolved in 2 mL of DMSO. After ten days, the product in the mixture was isolated by triple extraction with ethyl acetate (3 extractions of 300 mL). After drying with anhydrous magnesium sulfate, and concentrating the sample by using a rotary evaporator, the obtained product was analyzed by GC and NMR.

The biotransformation product was separated on preparative TLC plates (1000 µm, silica gel plates (Anatech, Gehrden, Germany) with cyclohexane: Ethyl acetate eluent (9:1 *v*/*v*). After separation, the product was scraped from the plate, extracted twice from the gel with ethyl acetate, dried with magnesium sulfate and concentrated by a rotary vacuum evaporator. A one-day transformation of 3-(2″-furyl)-1-(2′-hydroxyphenyl)-prop-2-en-1-one (**1**) (100mg) in *Y. lipolytica* KCh 71 gave 72 mg (colorless oil) of 3-(2″-furyl)-1-(2′-hydroxyphenyl)-propan-1-one (**2**). One-day transformation of 3-(2″-furyl)-1-(2′-hydroxyphenyl)-prop-2-en-1-one (**3**) (100mg) in the same strain culture yielded 76 mg (pale yellow oil) of 3-(2″-thienyl)-1-(2′-hydroxyphenyl)-propan-1-one (**4**). The resulting products were then analyzed by NMR spectroscopy.

4. Conclusions

There is known catalytic activity of ene reductases present in many yeast species, consisting in the hydrogenation of chalcone to dihydrochalcone. In this study, 3-(2″-furyl)- (1) and 3-(2″-thienyl)-1-(2′-hydroxyphenyl)-prop-2-en-1-one (3) were biotransformed using eight yeast strains. The purpose was to test the microorganisms' ability to selectively hydrogenate the double bond in chalcone containing heteroatom, in one of the rings, and select the most efficiently transforming strain. A significant factor in this type of reaction is not only its duration and the efficiency with which the substrate is transformed, but also the lack of by-products. Out of the available microorganisms, the fastest conversions of substrate 1 to the expected product were observed in *Saccharomyces cerevisiae* KCh 464 and *Y. lipolytica* KCh 71 strains. Product 2 was observed following the first hour of biotransformation (constitutive enzyme), and the conversion efficiency >99% was recorded as early as six hours after the start of the biotransformation. High conversion of substrate 1, higher than 90% after 24 hours of biotransformation, was also observed in the *R. rubra* KCh 4 culture. However, when analyzing the composition of the reaction mixture in twelve hours, the observed substrate conversion did not exceed 10%. This biotransformation pattern indicates that it is probably the result of substrate-induced ene reductases. Comparing these data to the conversion of 2-hydroxychalcone to dihydrochalcone, as previously described in [32], where the above-mentioned strains were also tested, we observed that the presence of the heteroatom in the B-ring practically does not affect the transformation efficiency—after 72 hours in the case of KCh 71 and KCh 464 strains; however, it is much more efficient in the case of the strain KCh 4. Substrate 3, which is composed of a sulfur atom in ring B, was analogously converted by the tested biocatalysts in the same way as substrate 1. Substrate 3 was most effectively transformed by *Y. lipolytica* KCh 71 strain culture.

During this biotransformation, 3-(2″-thienyl)-1-(2′-hydroxyphenyl)-propan-1-one (4) was obtained in 99% yield after the third hour of transformation.

Supplementary Materials: Supplementary materials are available online.

Author Contributions: Formal analysis, M.L., M.K. and T.J.; investigation, M.L. and M.K.; methodology, M.L., T.J. and E.K.-S.; project administration, M.L. and T.J.; resources, T.J.; supervision, T.J. and E.K.; visualization, M.L.; writing-original draft, M.L.; writing-review and editing, M.L. and T.J.

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Sample Availability: Samples of the compounds are available from the authors.

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Supplementary Data

Effective Hydrogenation of 3-(2″-furyl)- and 3-(2″-thienyl)-1-(2′-hydroxyphenyl)-prop-2-en-1-one in Selected Yeast Cultures

Mateusz Łużny *, Martyna Krzywda, Ewa Kozłowska, Edyta Kostrzewa-Susłow and Tomasz Janeczko *

Department of Chemistry, Wrocław University of Environmental and Life Sciences, Norwida 25, 50-375 Wrocław, Poland

* Correspondence: mat.luzny@gmail.com(M.Ł.) janeczko13@interia.pl (T.J.); Tel.: +48-713-205-195 (T.J.)

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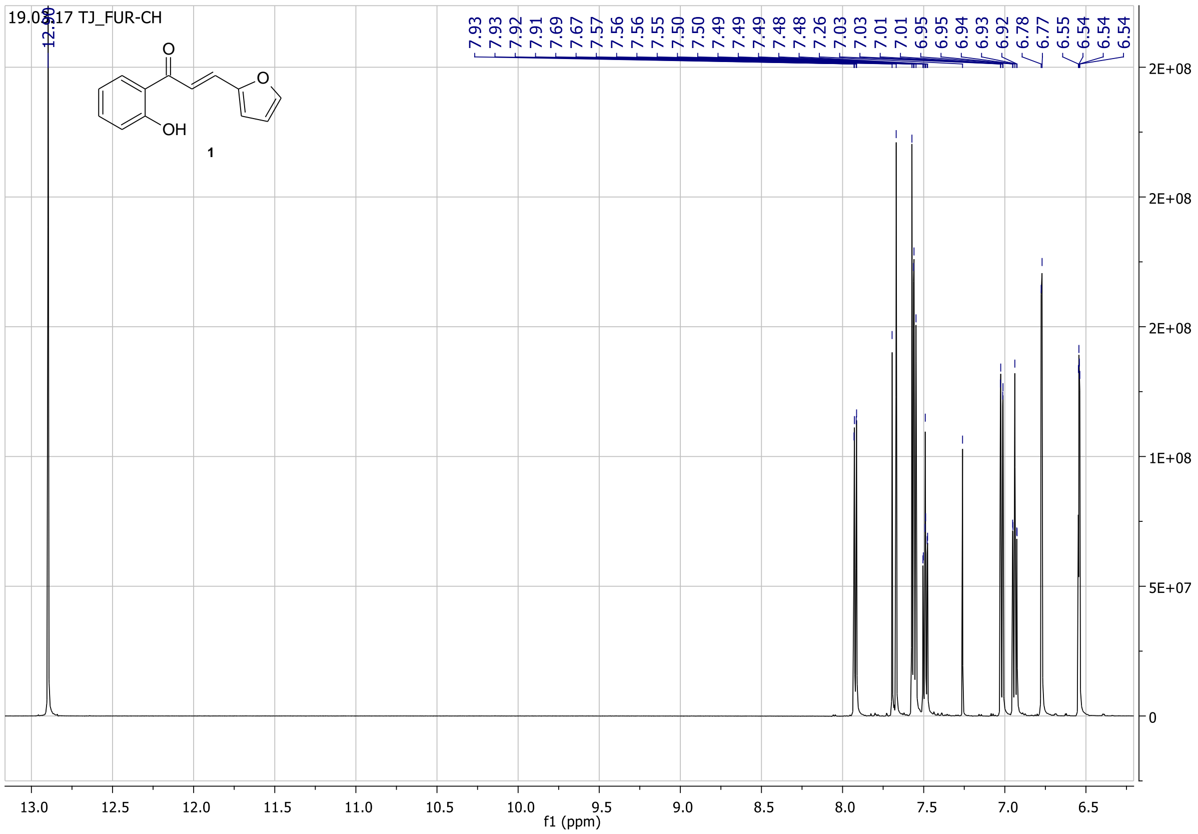
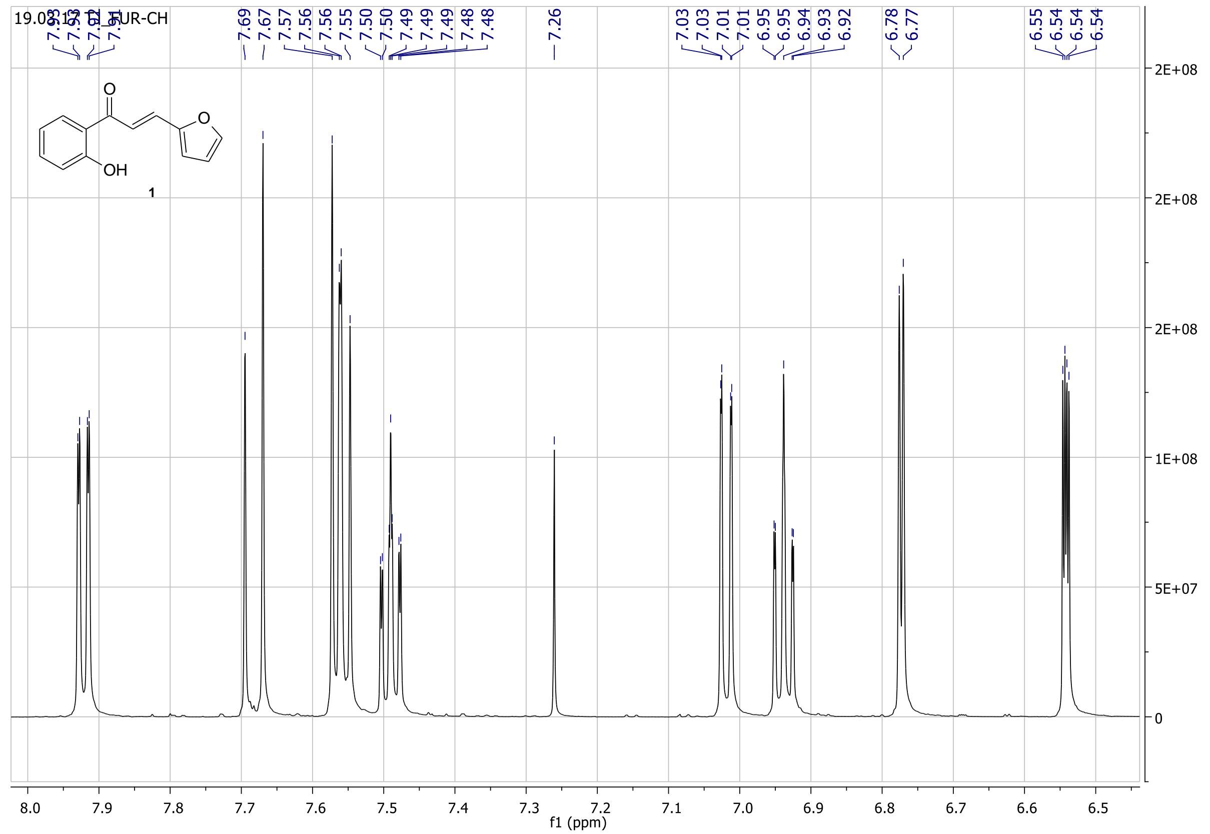
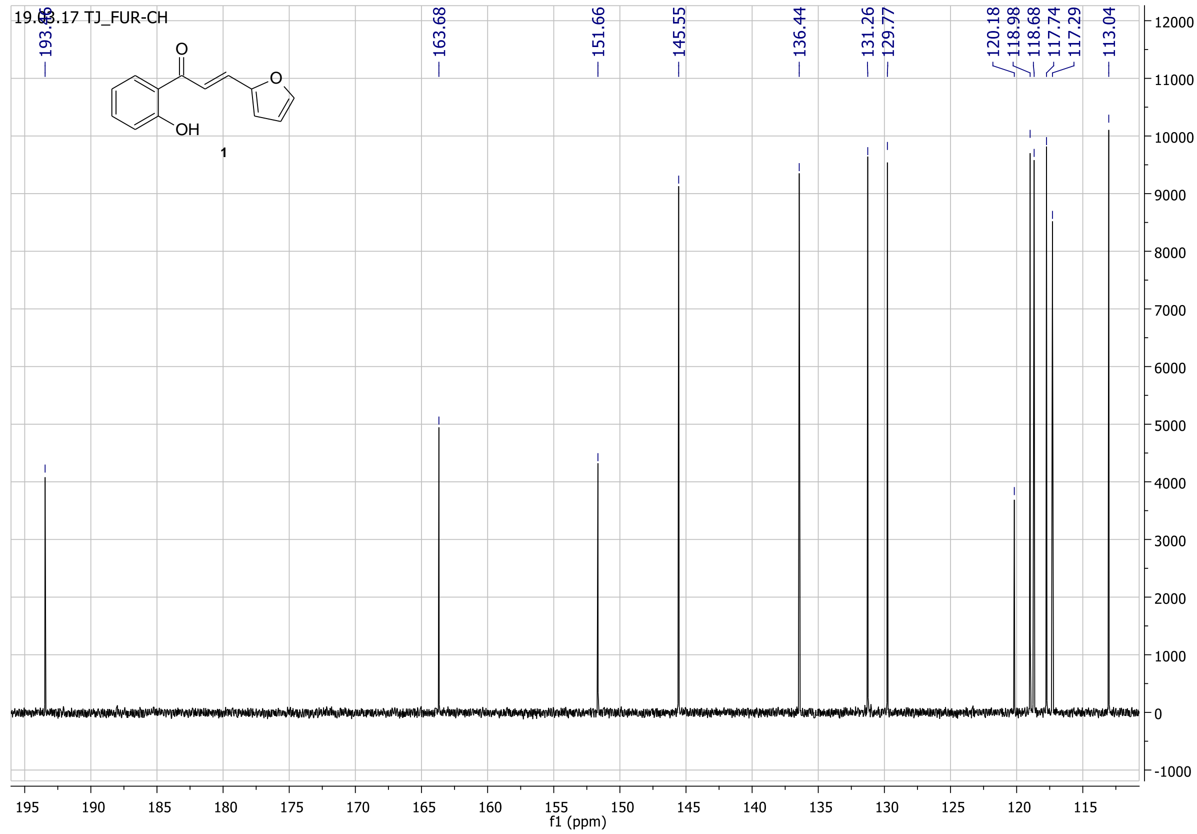
FigureS1. ^{1}H-NMR spectral of 3-(2”-furyl)-1-(2’-hydroxyphenyl)-prop-2-en-1-one (**1**) ($CDCl_3$, 600 MHz)



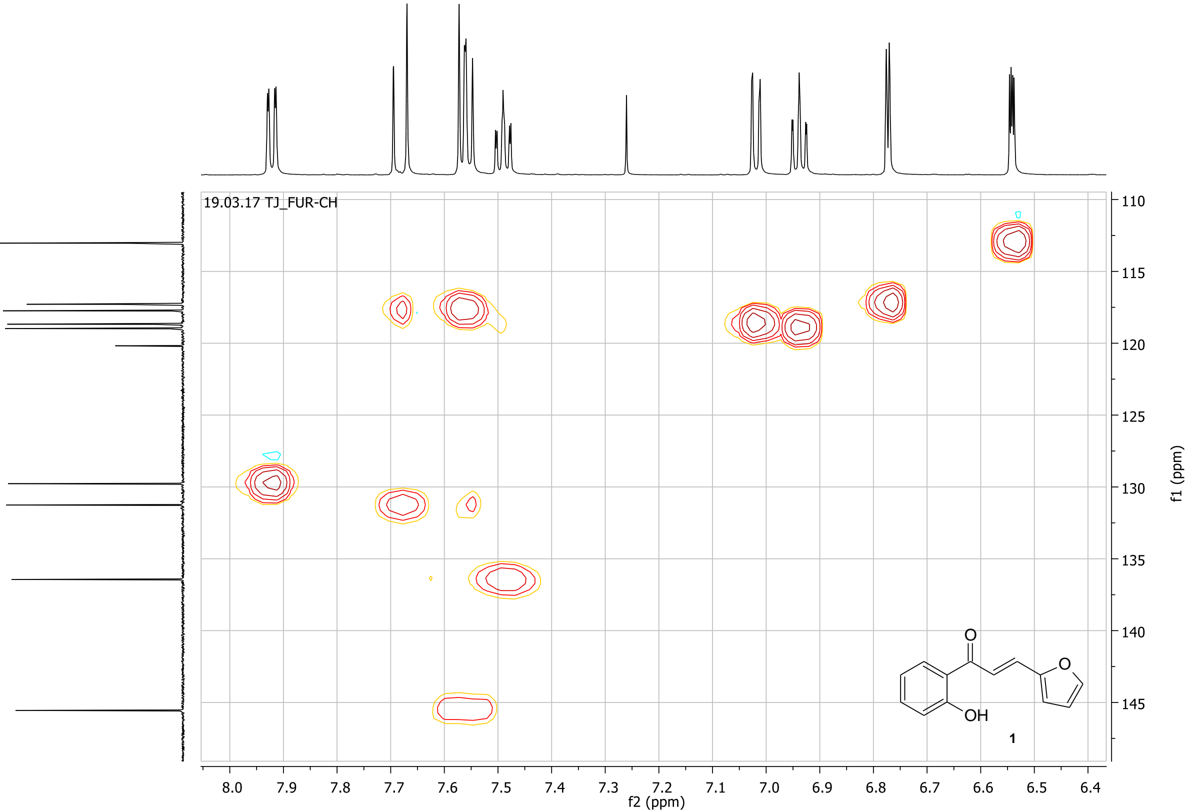
FigureS2. Part of the ^{1}H-NMR spectral of 3-(2”-furyl)-1-(2’-hydroxyphenyl)-prop-2-en-1-one (**1**) ($CDCl_3$, 600 MHz)



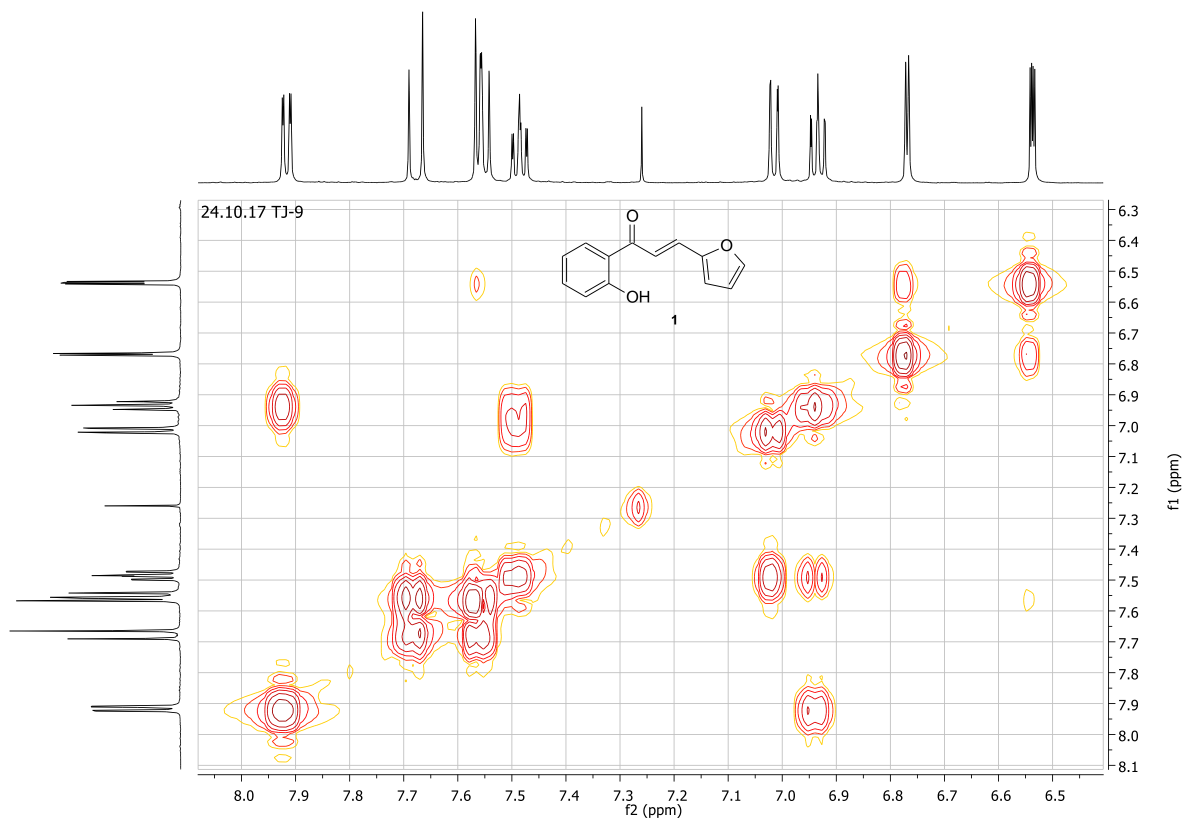
FigureS3. ^{13}C-NMR spectral of 3-(2”-furyl)-1-(2’-hydroxyphenyl)-prop-2-en-1-one (**1**) ($CDCl_3$, 151 MHz)



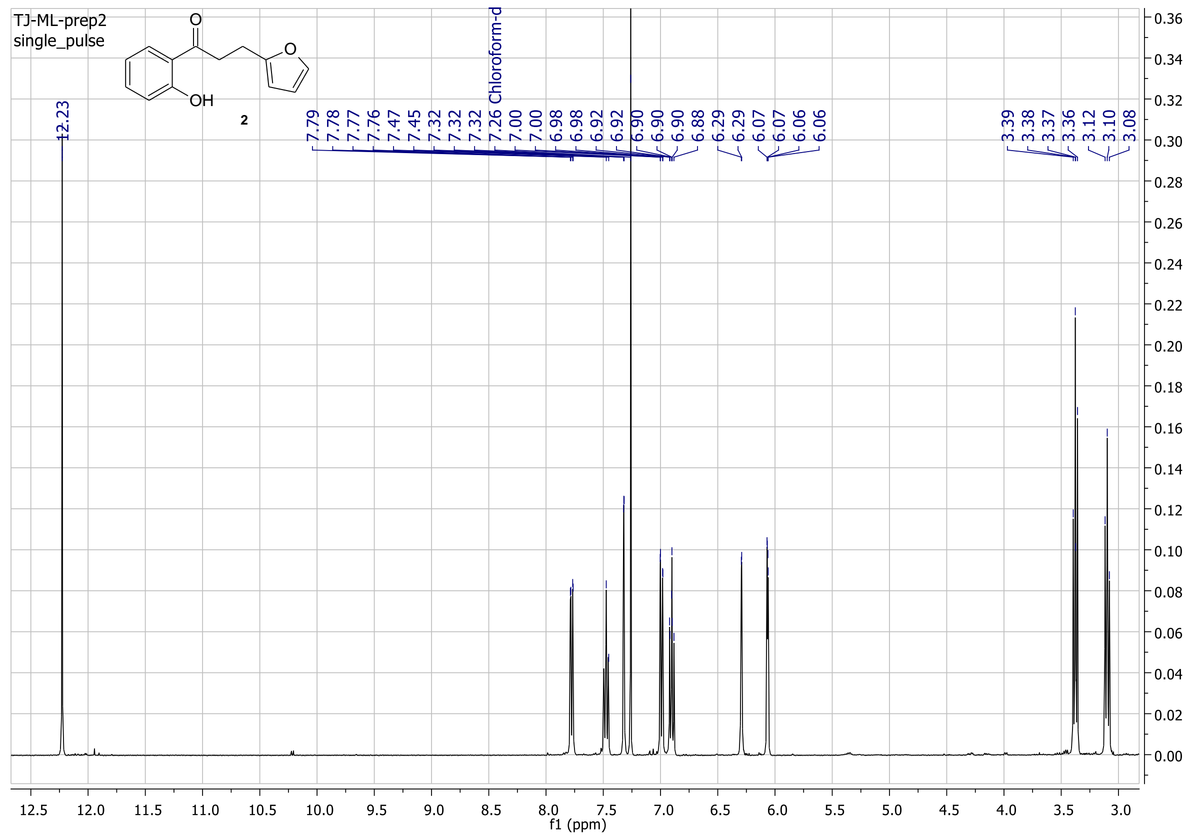
FigureS4. HSQC spectral of 3-(2”-furyl)-1-(2’-hydroxyphenyl)-prop-2-en-1-one (**1**) ($CDCl_3$, 151 MHz)



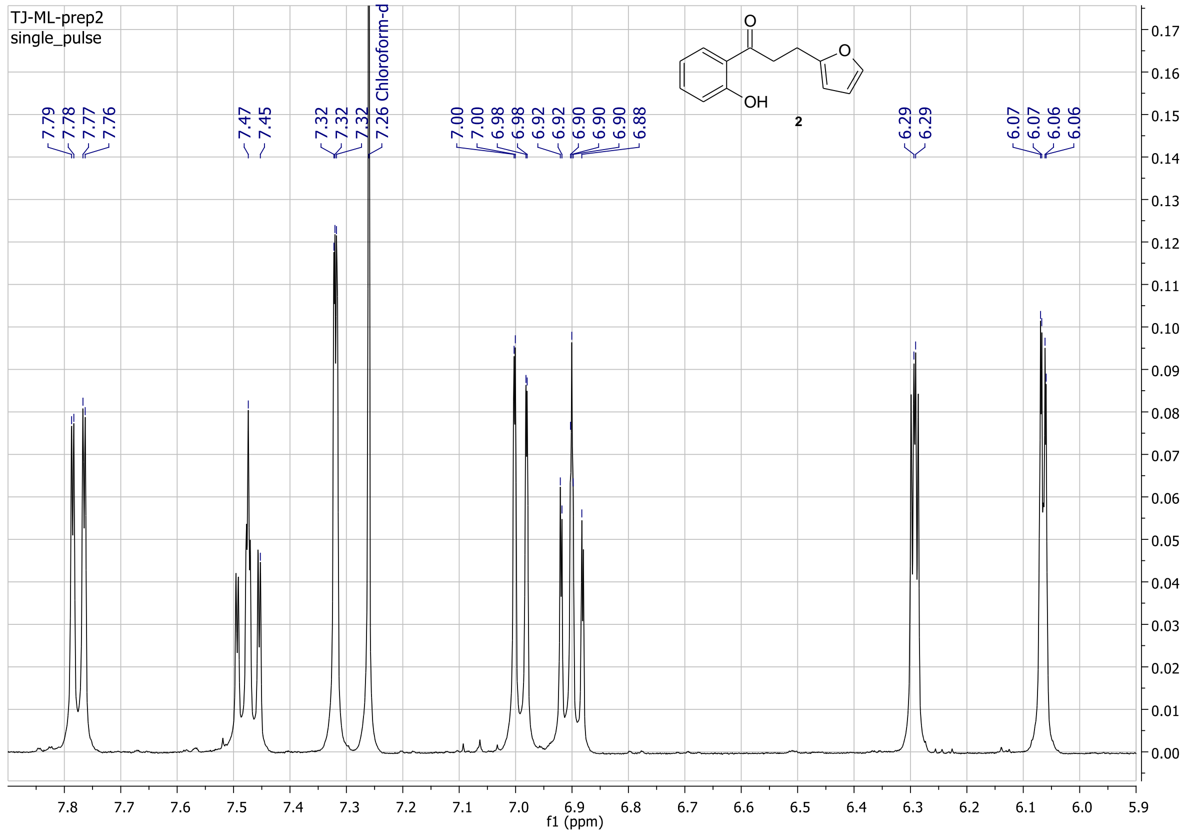
FigureS5. COSY spectral of 3-(2”-furyl)-1-(2’-hydroxyphenyl)-prop-2-en-1-one (**1**) ($CDCl_3$, 600 MHz)



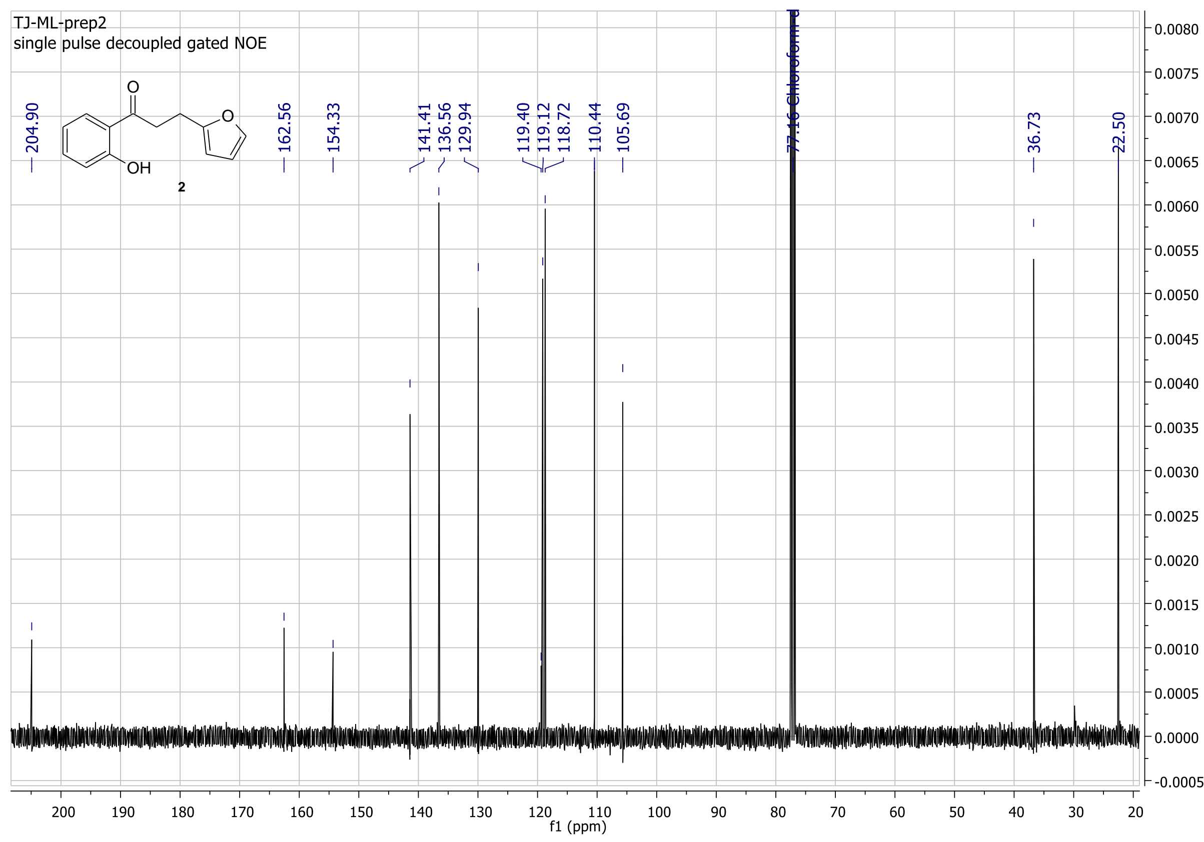
FigureS6. ^{1}H-NMR spectral of 3-(2”-furyl)-1-(2’-hydroxyphenyl)-propan-1-one (**2**) ($CDCl_3$, 600 MHz)



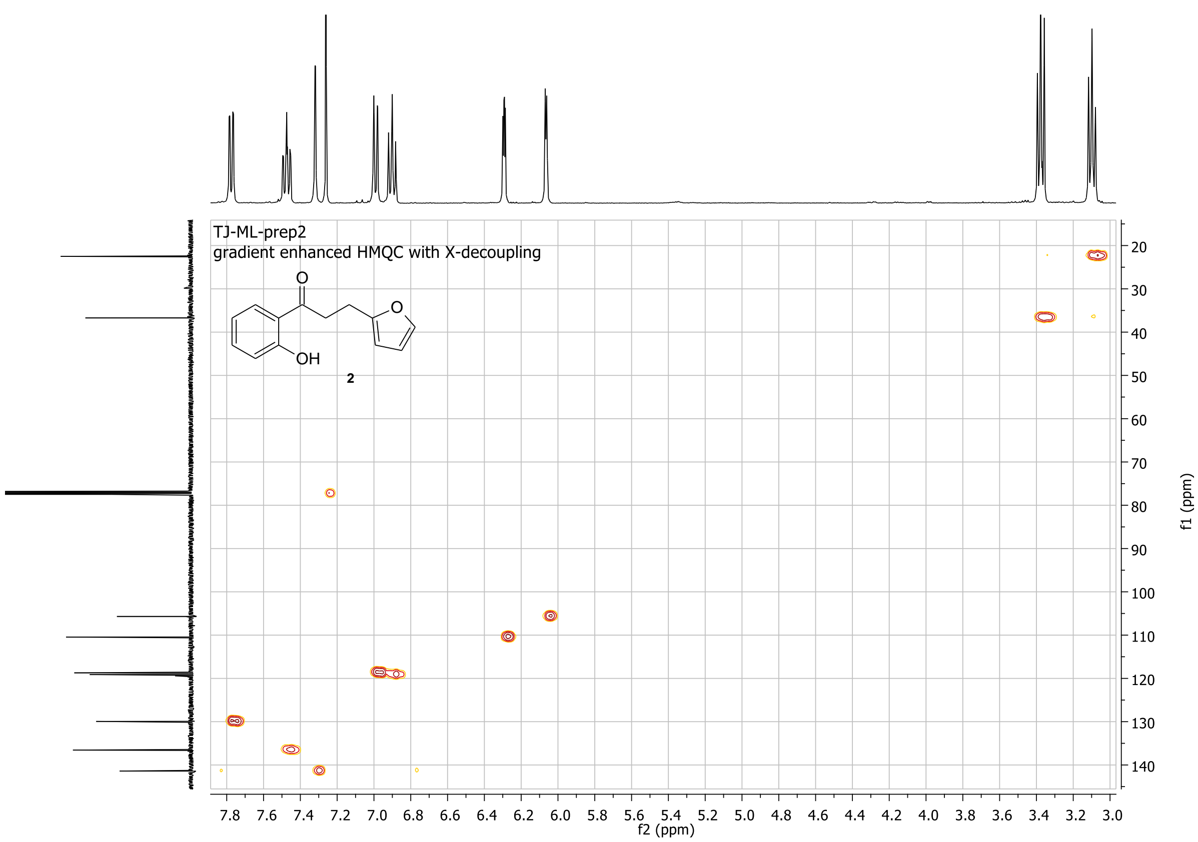
FigureS7. Part of the ^{1}H-NMR spectral of 3-(2”-furyl)-1-(2’-hydroxyphenyl)- propan-1-one (**2**) ($CDCl_3$, 600 MHz)



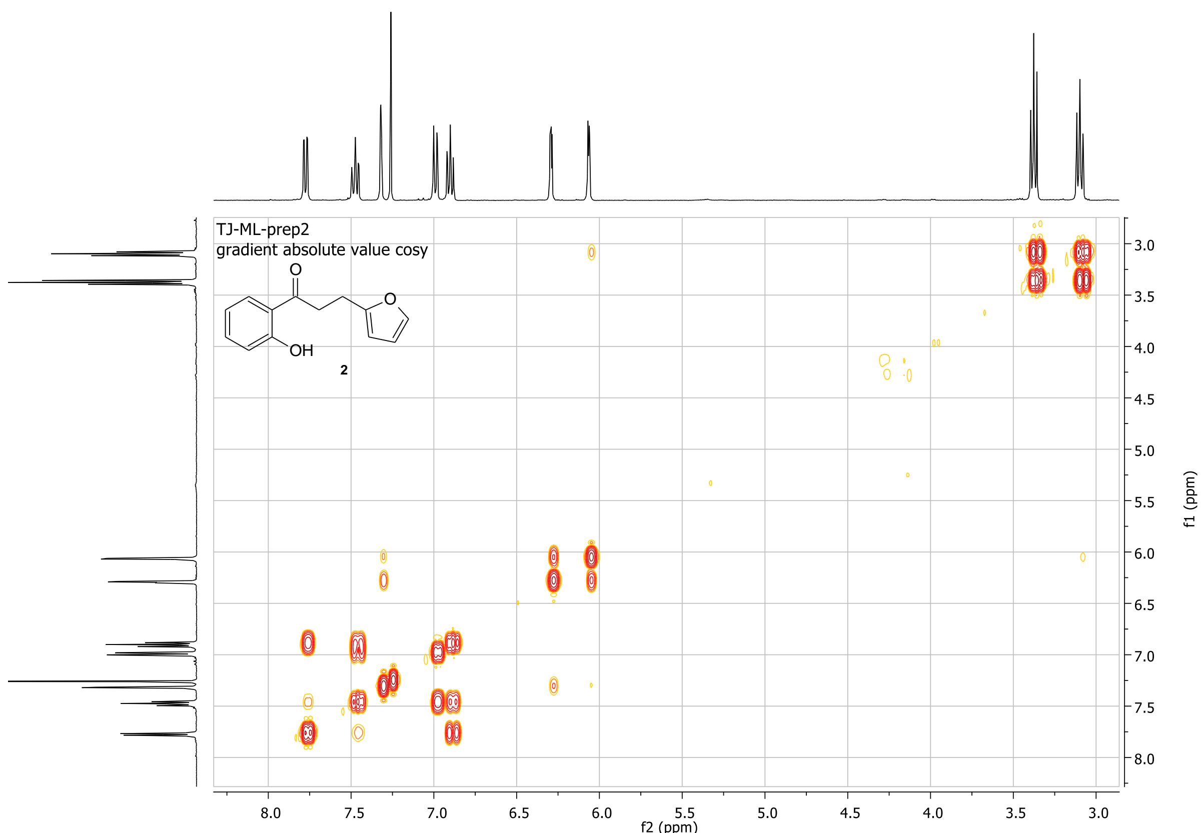
FigureS8. ^{13}C-NMR spectral of 3-(2”-furyl)-1-(2’-hydroxyphenyl)- propan-1-one (**2**) ($CDCl_3$, 151 MHz)



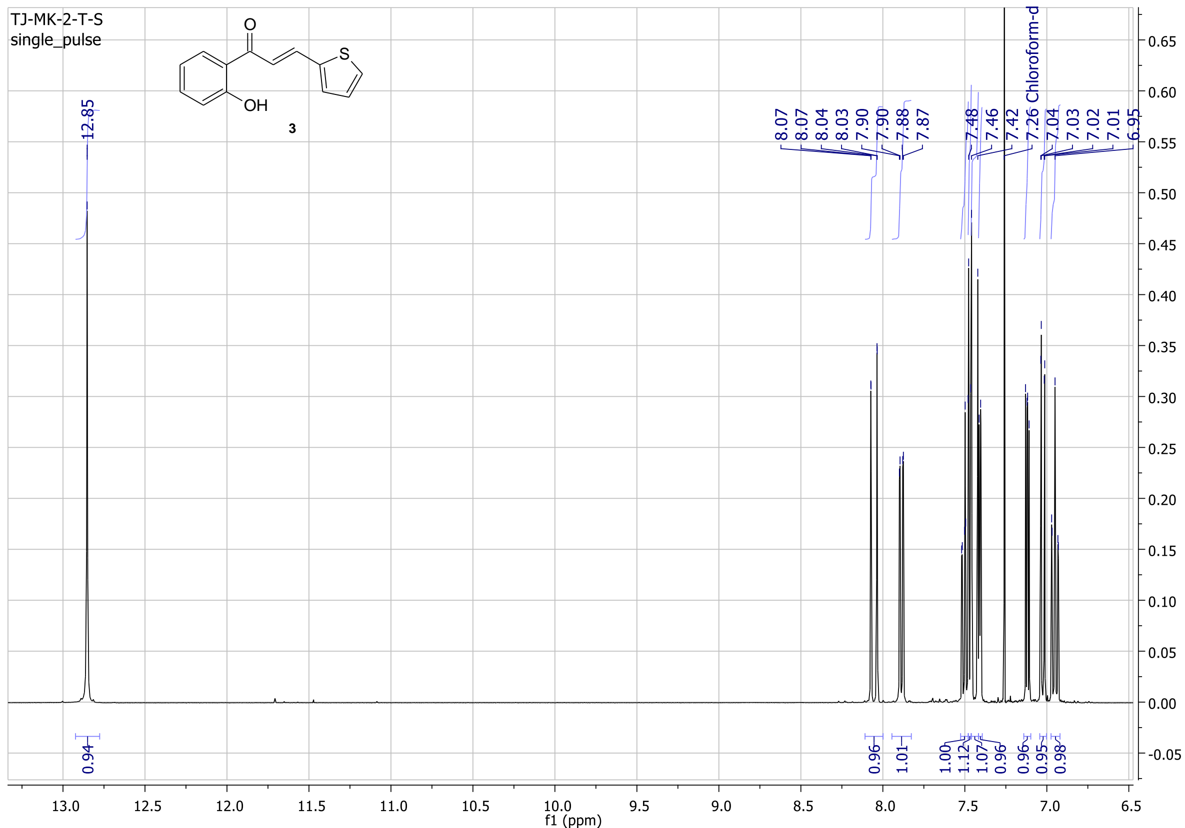
FigureS9. HSQC spectral of 3-(2”-furyl)-1-(2’-hydroxyphenyl)- propan-1-one (**2**) ($CDCl_3$, 151 MHz)



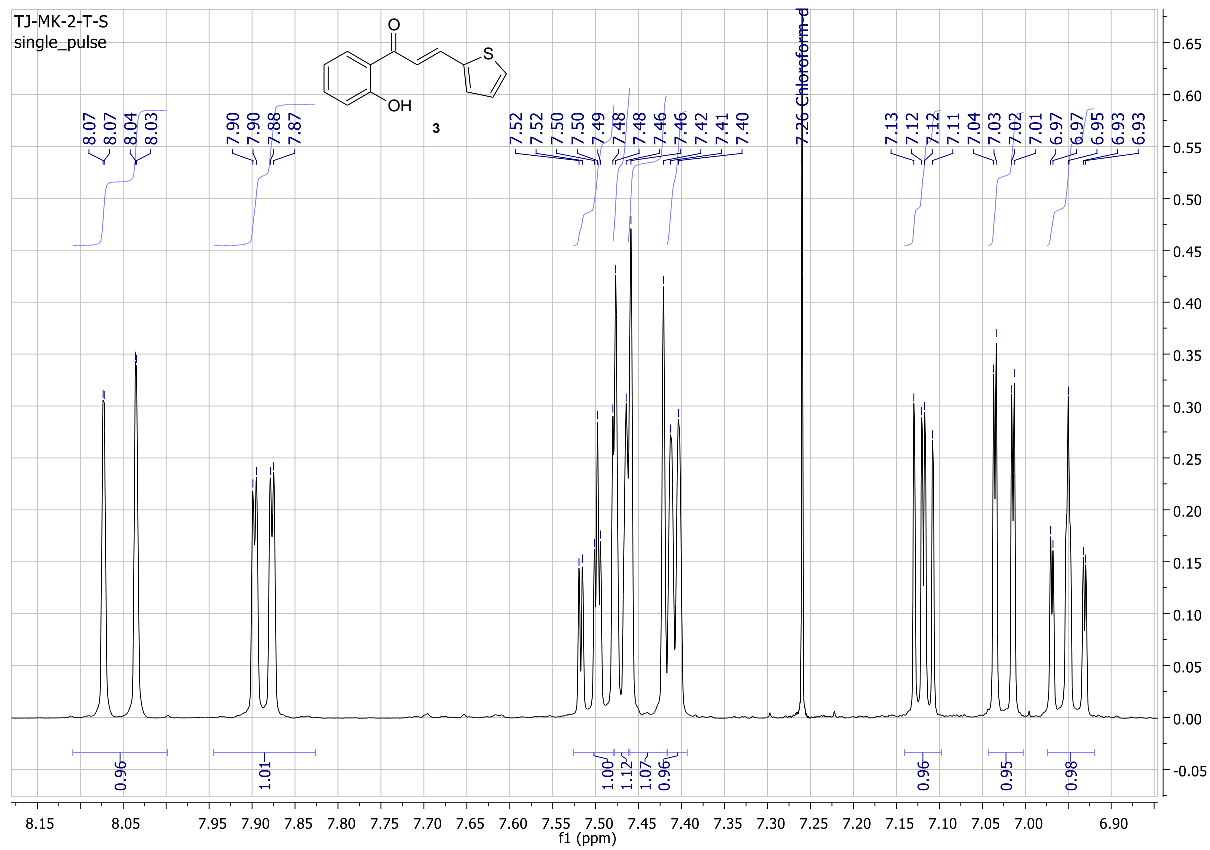
FigureS10. COSY spectral of 3-(2”-furyl)-1-(2’-hydroxyphenyl)- propan-1-one (**2**) ($CDCl_3$, 600 MHz)



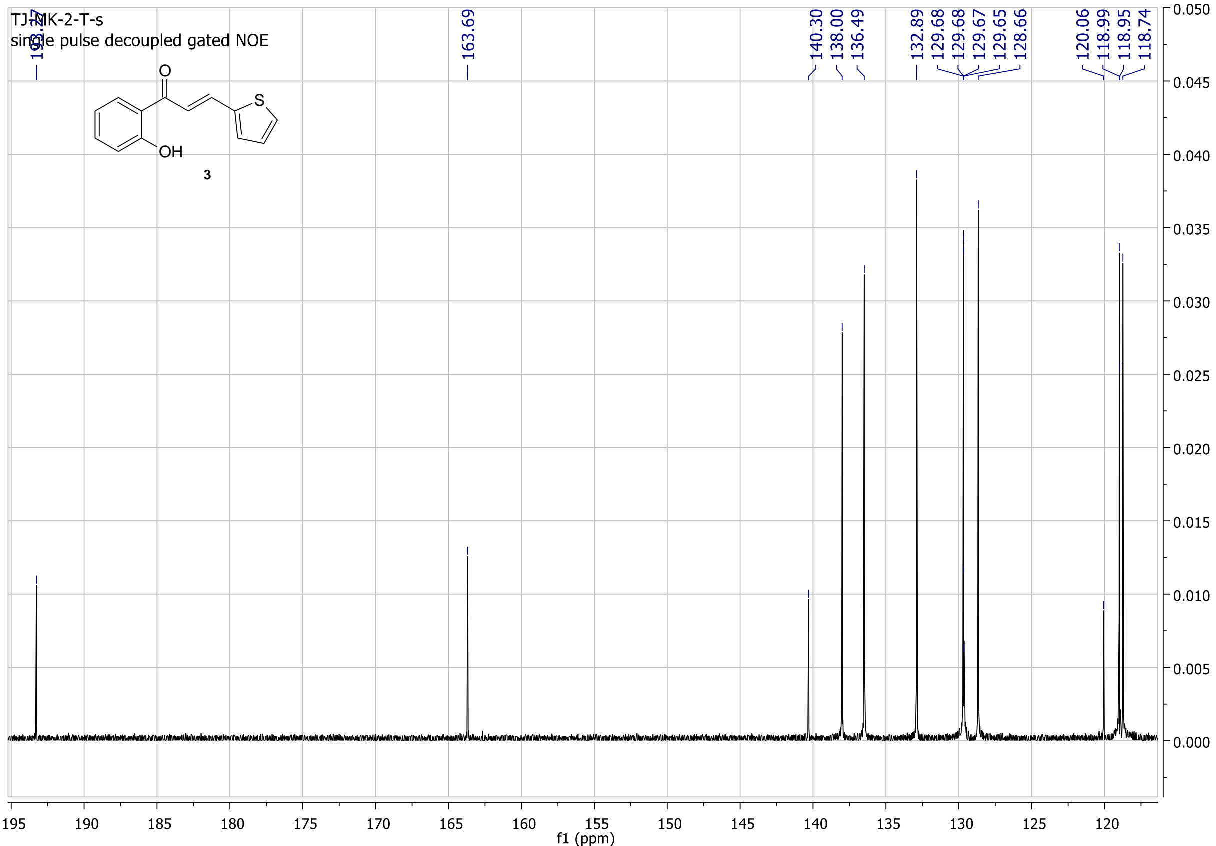
FigureS11. ^{1}H-NMR spectral of 3-(2”-thienyl)-1-(2’-hydroxyphenyl)-prop-2-en-1-one (**3**) ($CDCl_3$, 600 MHz)



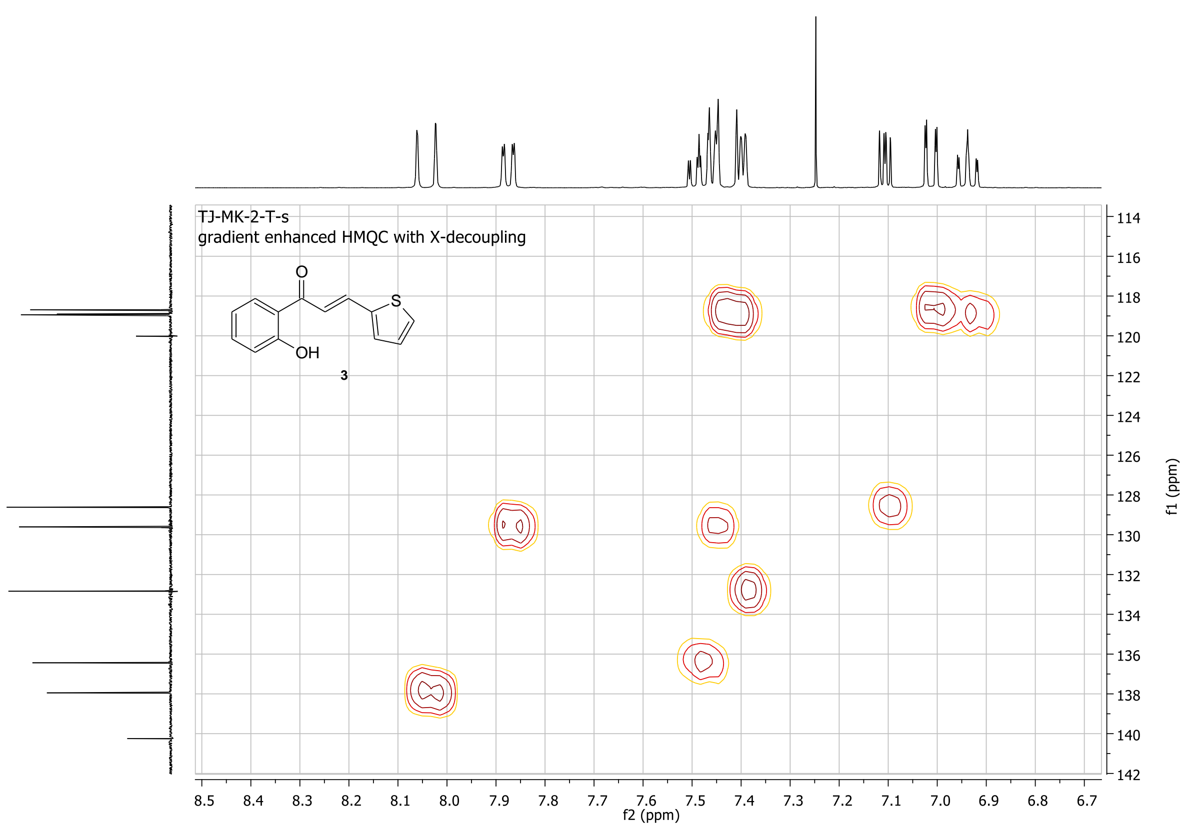
FigureS12. Part of the ^{1}H-NMR spectral of 3-(2”-thienyl)-1-(2’-hydroxyphenyl)-prop-2-en-1-one (**3**) ($CDCl_3$, 600 MHz)



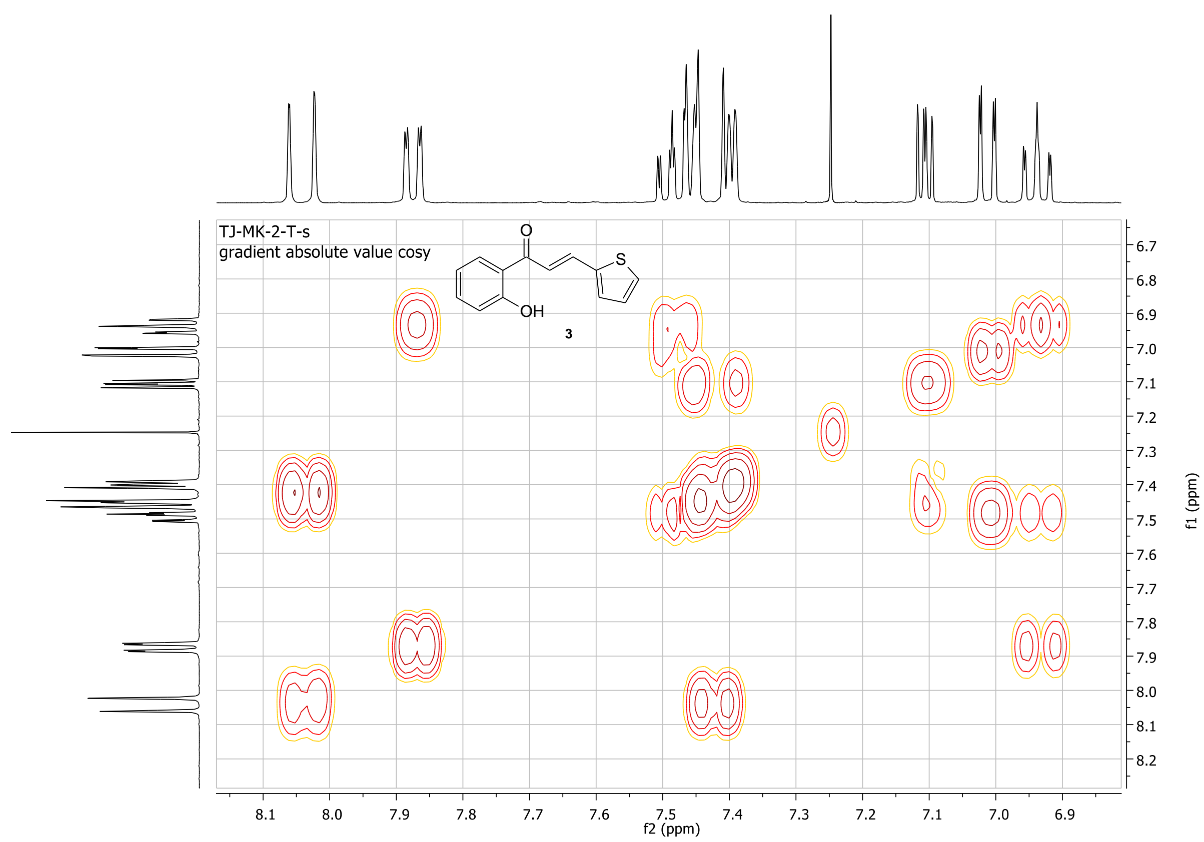
FigureS13. ^{13}C-NMR spectral of 3-(2”-thienyl)-1-(2’-hydroxyphenyl)-prop-2-en-1-one (**3**) ($CDCl_3$, 151 MHz)



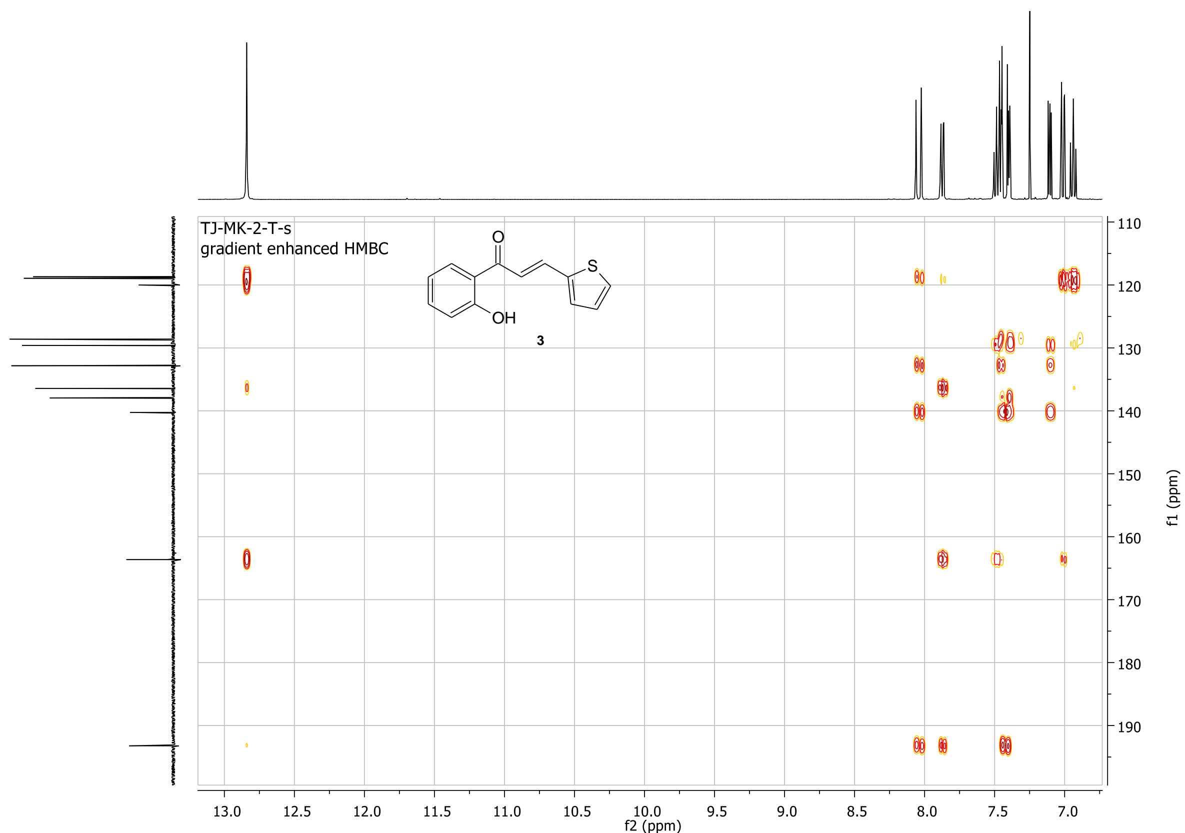
FigureS14. HSQC spectral of 3-(2”-thienyl)-1-(2’-hydroxyphenyl)-prop-2-en-1-one (**3**) ($CDCl_3$, 151 MHz)



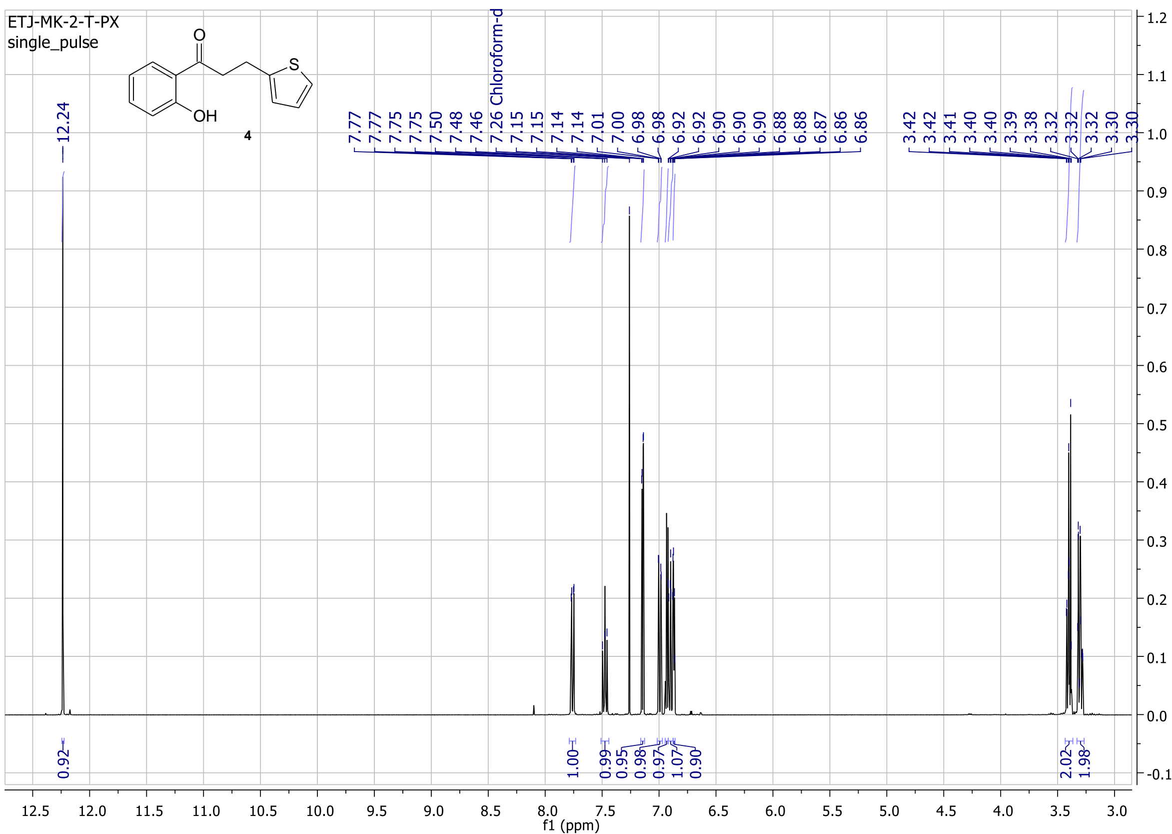
FigureS15. COSY spectral of 3-(2''-thienyl)-1-(2'-hydroxyphenyl)-prop-2-en-1-one (**3**) ($CDCl_3$, 600 MHz)



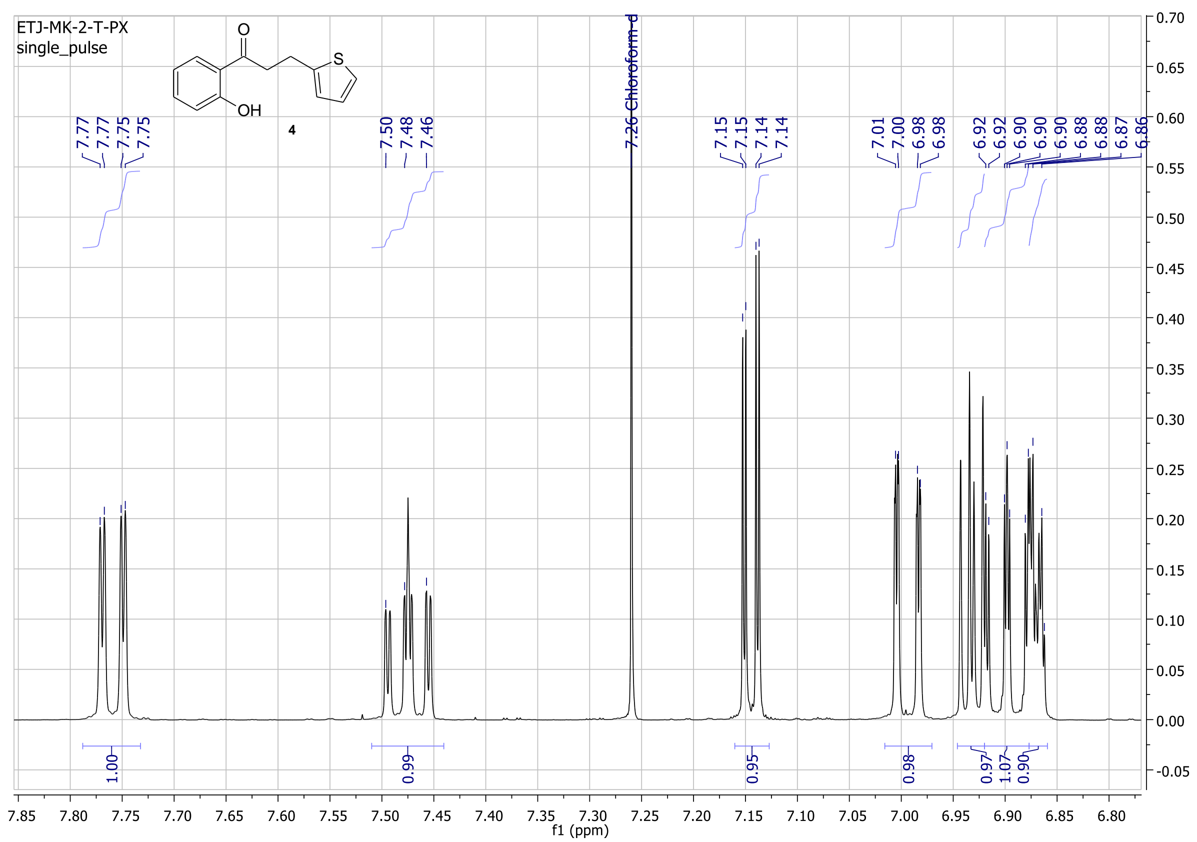
FigureS16. HMBC spectral of 3-(2''-thienyl)-1-(2'-hydroxyphenyl)-prop-2-en-1-one (**3**) ($CDCl_3$, 151 MHz)



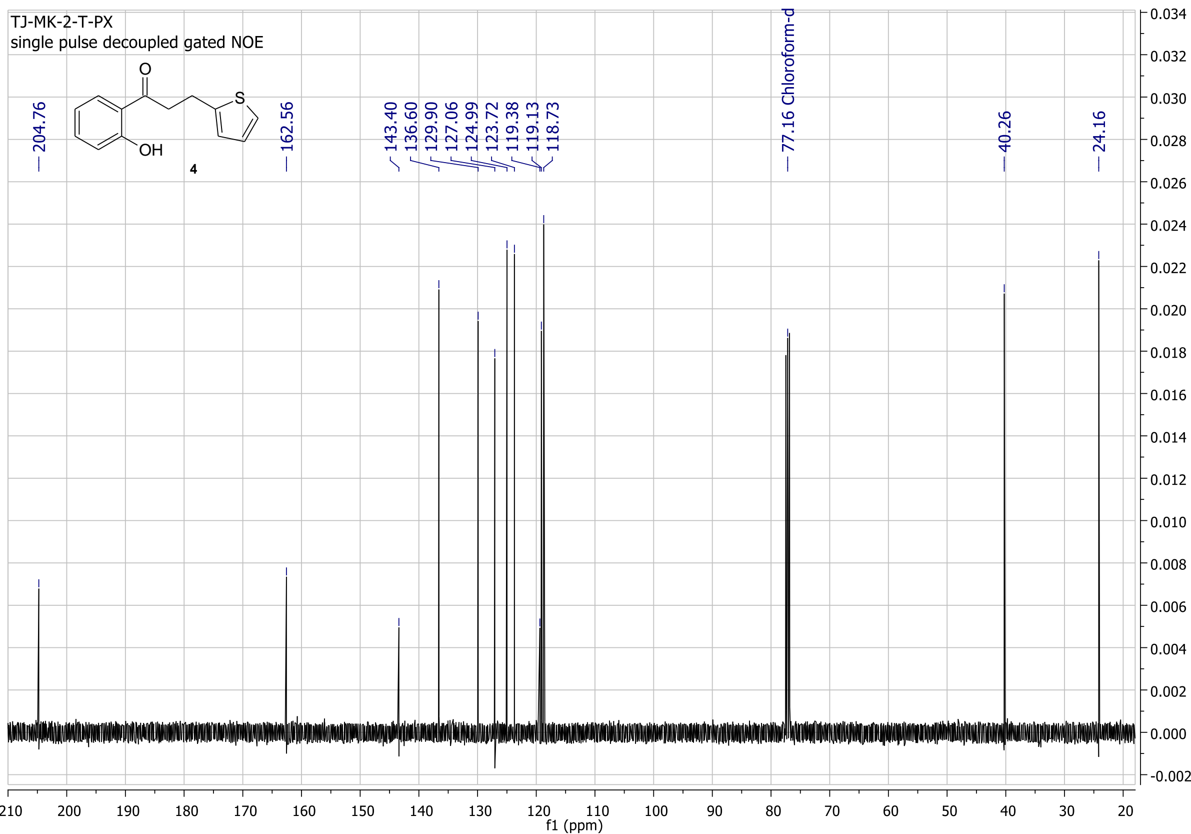
FigureS17. ^{1}H-NMR spectral of 3-(2”-thienyl)-1-(2’-hydroxyphenyl)-propan-1-one (**4**) ($CDCl_3$, 600 MHz)



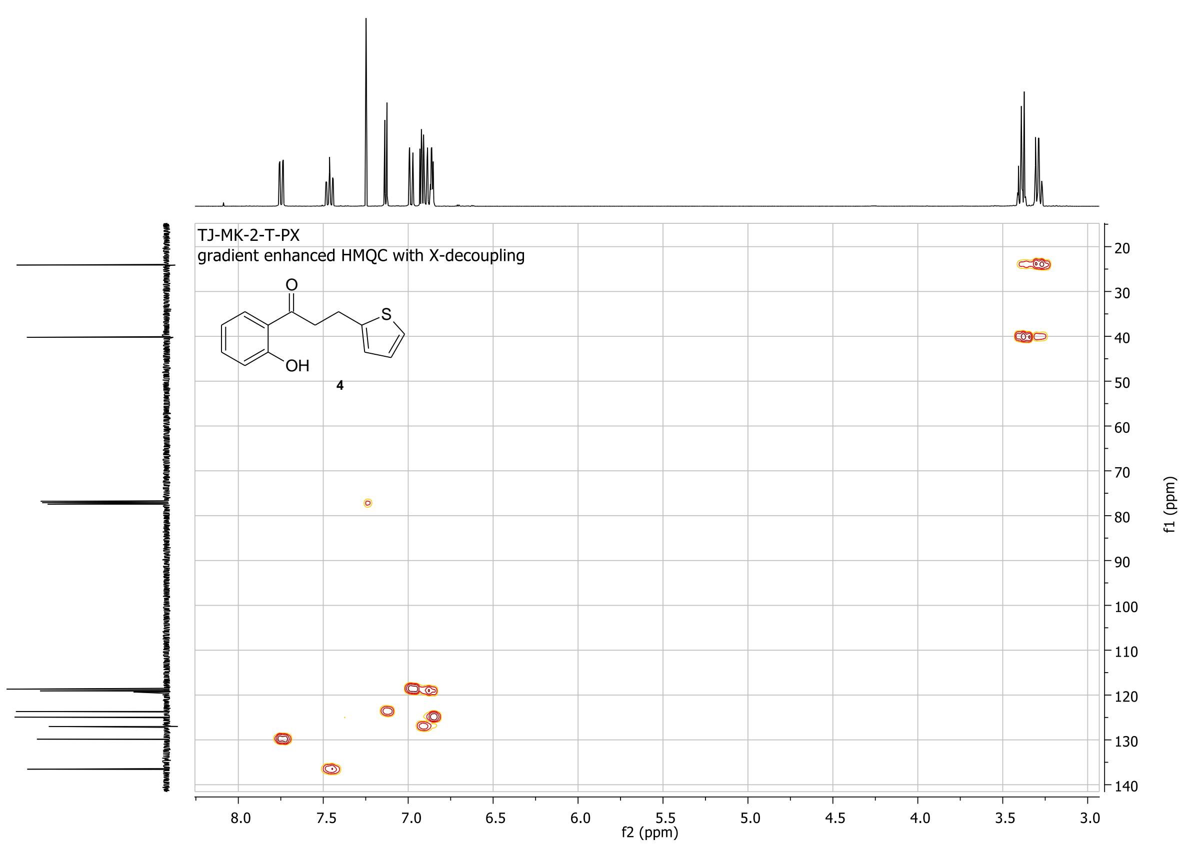
FigureS18. Part of the ^{1}H-NMR spectral of 3-(2”-thienyl)-1-(2’-hydroxyphenyl)- propan-1-one (**4**) ($CDCl_3$, 600 MHz)



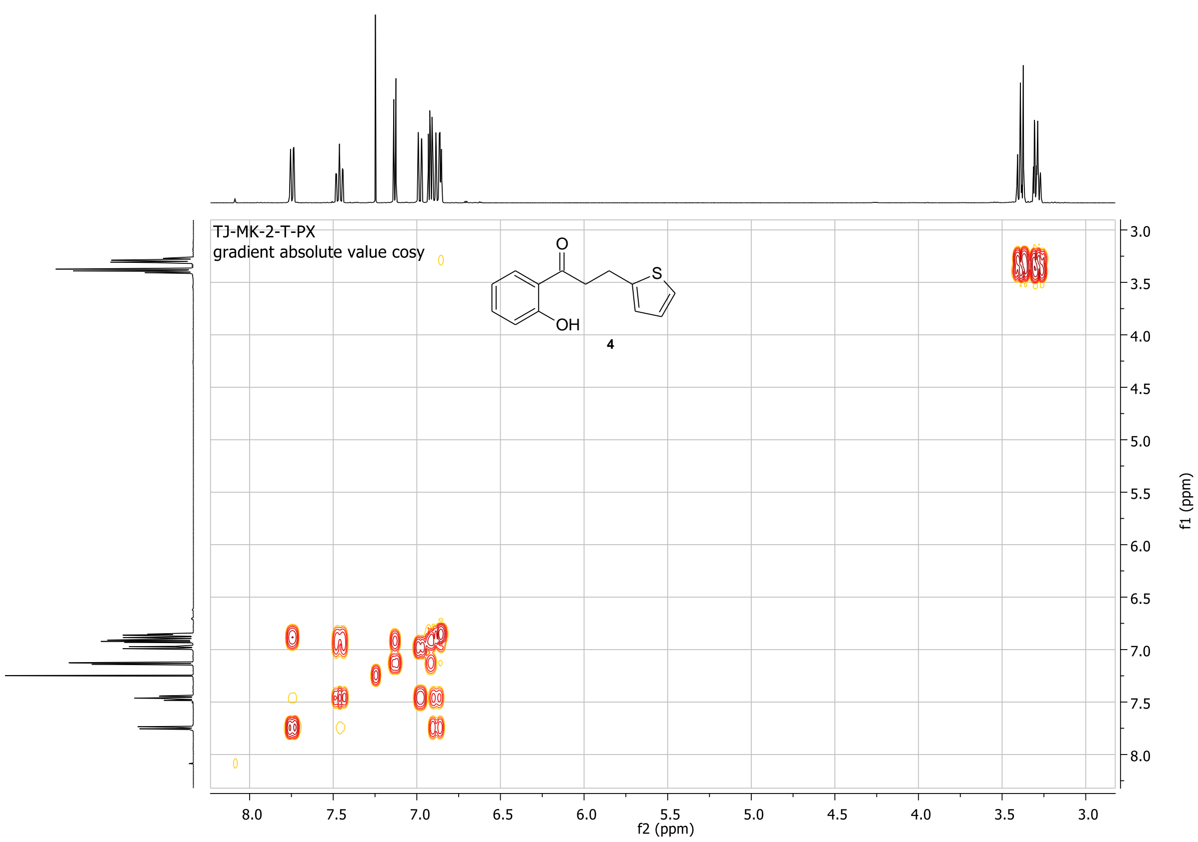
FigureS19. ^{13}C-NMR spectral of 3-(2”-thienyl)-1-(2’-hydroxyphenyl)- propan-1-one (**4**) ($CDCl_3$, 151 MHz)



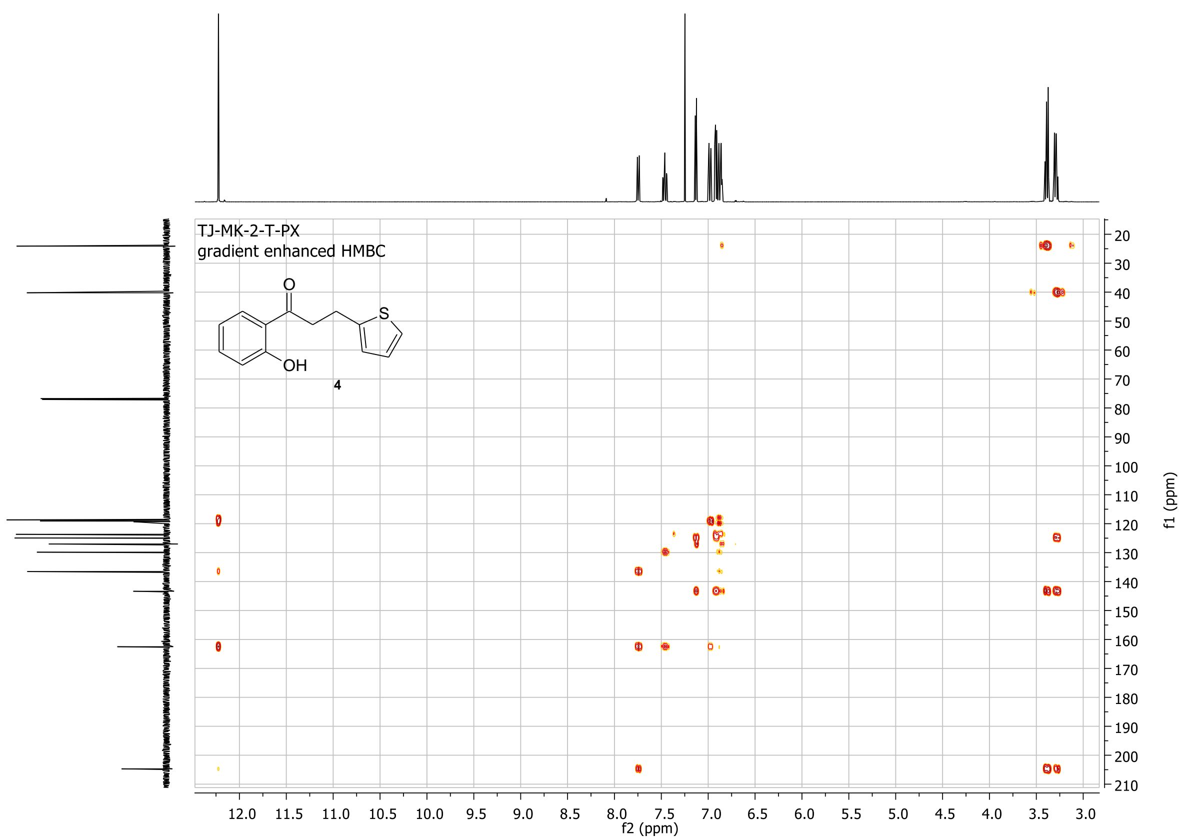
FigureS20. HSQC spectral of 3-(2”-thienyl)-1-(2’-hydroxyphenyl)- propan-1-one (**4**) ($CDCl_3$, 151 MHz)



FigureS21. COSY spectral of 3-(2”-thienyl)-1-(2’-hydroxyphenyl)- propan-1-one (**4**) ($CDCl_3$, 600 MHz)



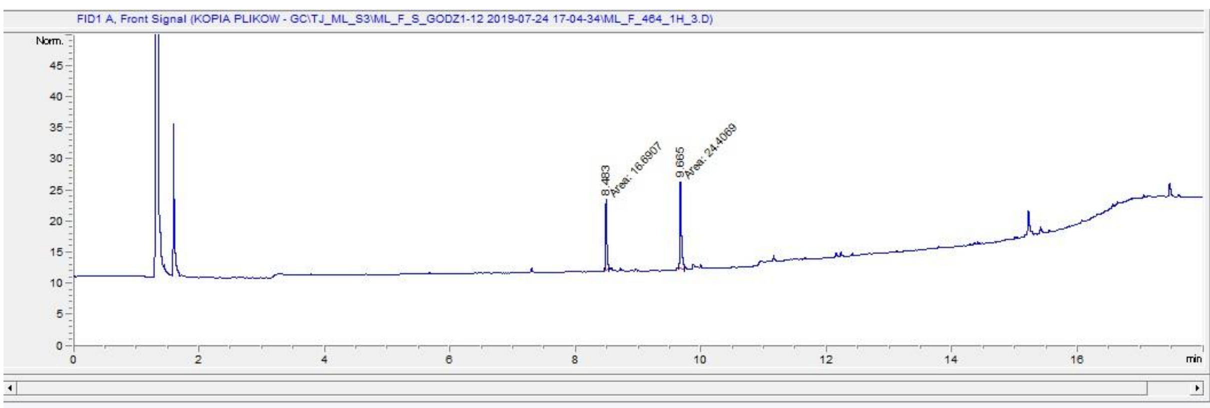
FigureS22. HMBC spectral of 3-(2”-thienyl)-1-(2’-hydroxyphenyl)- propan-1-one (**4**) ($CDCl_3$, 151 MHz)



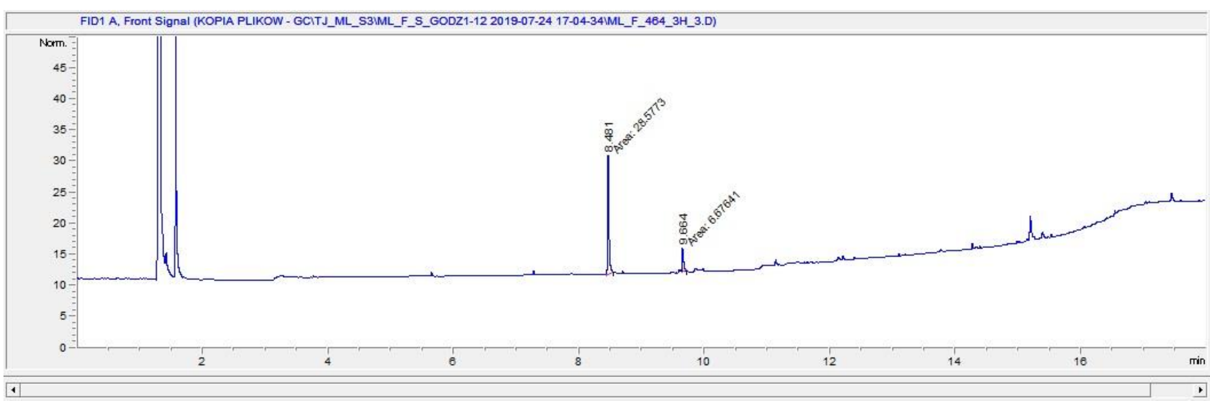
Selected chromatograms of biotransformation course:

FigureS23. Chromatogram presenting the composition of reaction mixture after 1 hour incubation of **1** in the culture of the *Saccharomyces cerevisiae* KCh 464 strain.

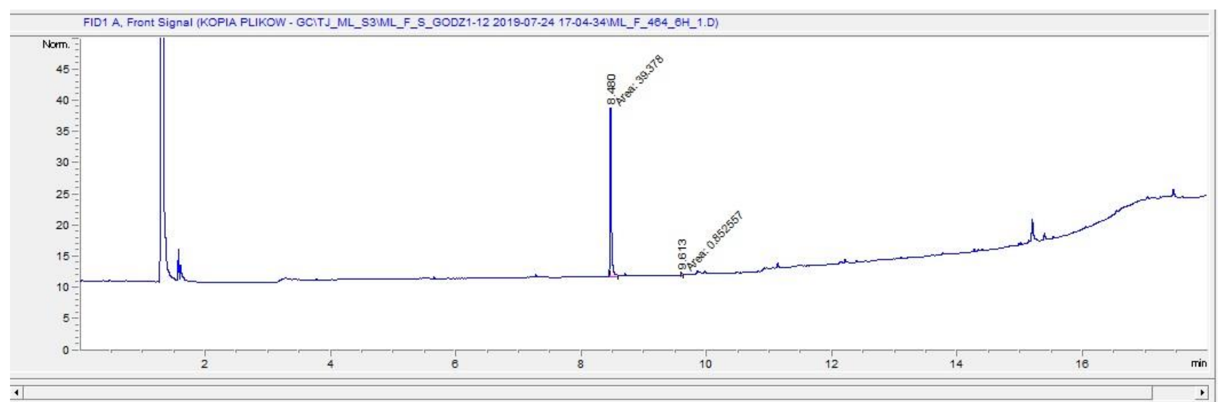
Retention time of the substrate (3-(2”-furyl)-1-(2’-hydroxyphenyl)-prop-2-en-1-one (**1**)) – 9.6min and product (3-(2”-furyl)-1-(2’-hydroxyphenyl)- propan-1-one (**2**)) - 8.5min.



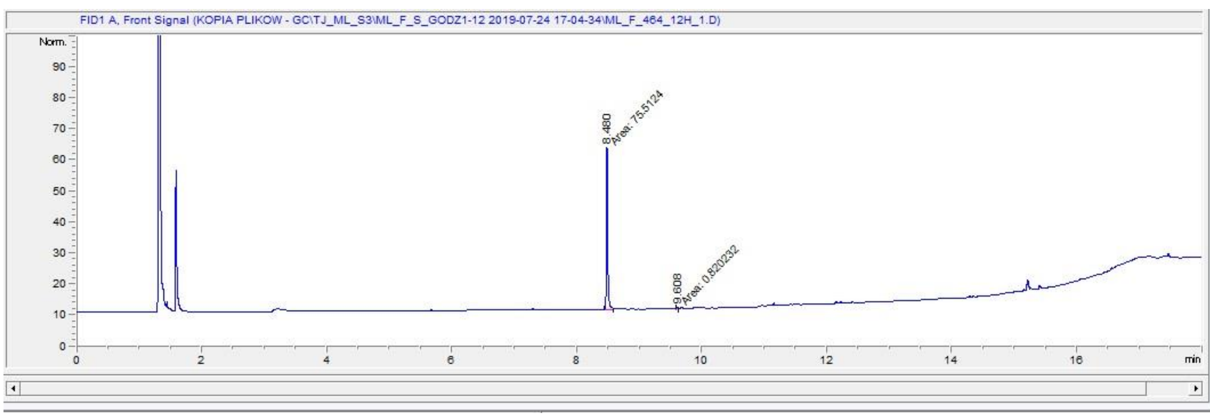
FigureS24. Chromatogram presenting the composition of reaction mixture after 3 hours incubation of **1** in the culture of the *Saccharomyces cerevisiae* KCh 464 strain.



FigureS25. Chromatogram presenting the composition of reaction mixture after 6 hours incubation of **1** in the culture of the *Saccharomyces cerevisiae* KCh 464 strain.

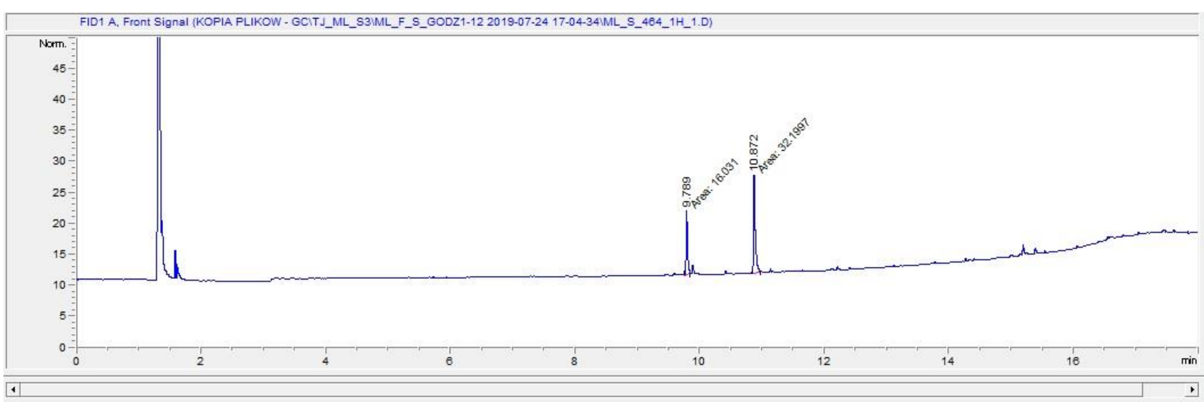


FigureS26. Chromatogram presenting the composition of reaction mixture after 12 hours incubation of **1** in the culture of the *Saccharomyces cerevisiae* KCh 464 strain.

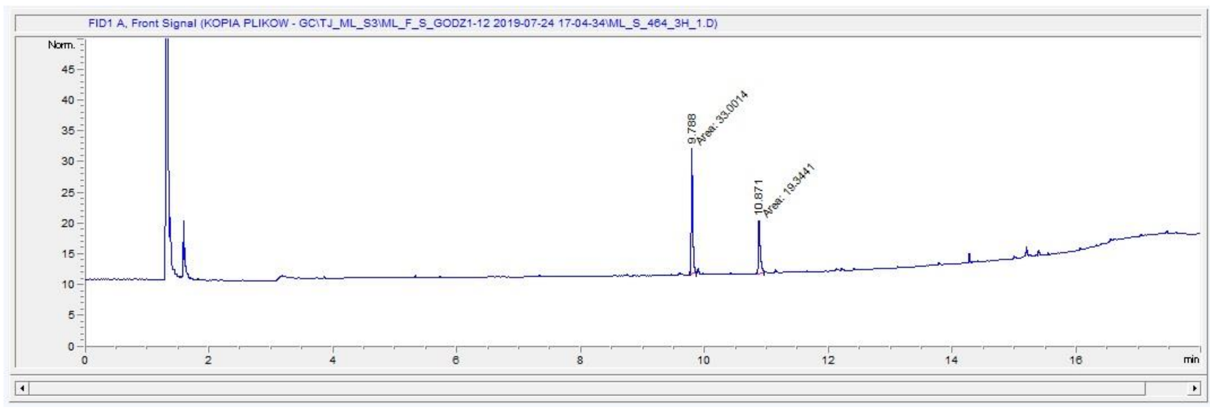


FigureS27. Chromatogram presenting the composition of reaction mixture after 1 hour incubation of **3** in the culture of the *Saccharomyces cerevisiae* KCh 464 strain.

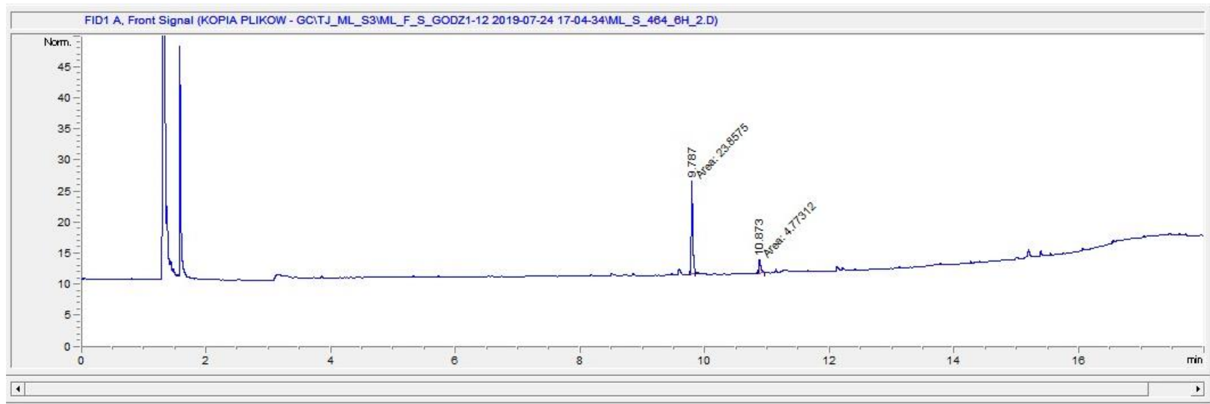
Retention time of the substrate (3-(2”-thienyl)-1-(2’-hydroxyphenyl)-prop-2-en-1-one (**3**)) – 10.8min and product (3-(2”-thienyl)-1-(2’-hydroxyphenyl)- propan-1-one (4)) - 9.7min.



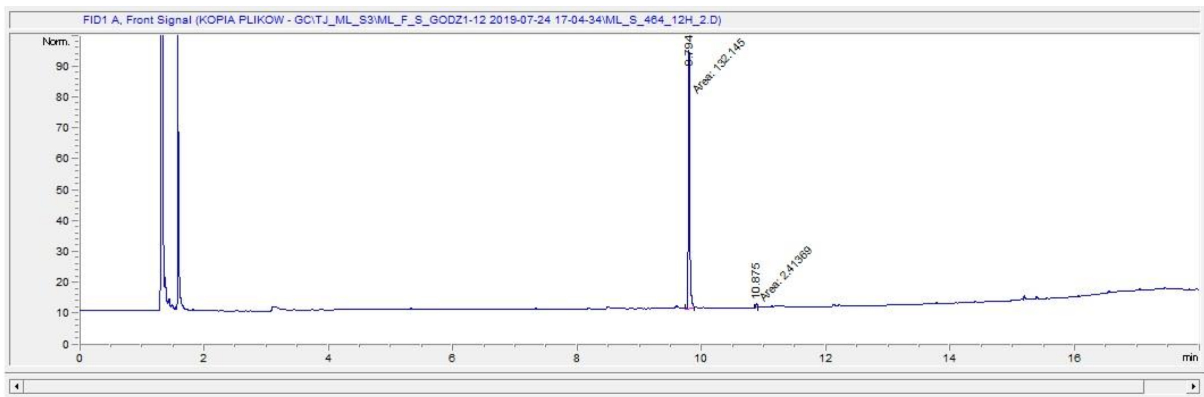
FigureS28. Chromatogram presenting the composition of reaction mixture after 3 hours incubation of **3** in the culture of the *Saccharomyces cerevisiae* KCh 464 strain.



FigureS29. Chromatogram presenting the composition of reaction mixture after 6 hours incubation of **3** in the culture of the *Saccharomyces cerevisiae* KCh 464 strain.



FigureS30. Chromatogram presenting the composition of reaction mixture after 12 hours incubation of **3** in the culture of the *Saccharomyces cerevisiae* KCh 464 strain.



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Highly effective, regiospecific hydrogenation of methoxychalcone by *Yarrowia lipolytica* enables production of food sweeteners. *Catalysts*, 2020, 10, 1135.

Article

Highly Effective, Regiospecific Hydrogenation of Methoxychalcone by *Yarrowia lipolytica* Enables Production of Food Sweeteners

Mateusz Łużny *, Ewa Kozłowska, Edyta Kostrzewa-Susłow and Tomasz Janeczko *

Department of Chemistry, Wrocław University of Environmental and Life Sciences, Norwida 25, 50-375 Wrocław, Poland; e.a.kozlowska@gmail.com (E.K.); ekostrzew@gmail.com (E.K.-S.)

* Correspondence: mateusz.luzny@upwr.edu.pl (M.Ł.); janeczko13@interia.pl (T.J.); Tel.: +48-713-205-195 (T.J.)

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Abstract: We describe the impact of the number and location of methoxy groups in the structure of chalcones on the speed and efficiency of their transformation by unconventional yeast strains. The effect of substrate concentration on the conversion efficiency in the culture of the *Yarrowia lipolytica* KCh 71 strain was tested. In the culture of this strain, monomethoxychalcones (2′-hydroxy-2′′-, 3′′- and 4′′-methoxychalcone) were effectively hydrogenated at over 40% to the specific dihydrochalcones at a concentration of 0.5 g/L of medium after just 1 h of incubation. A conversion rate of over 40% was also observed for concentrations of these compounds of 1 g/L of medium after three hours of transformation. As the number of methoxy substituents increases in the chalcone substrate, the rate and efficiency of transformation to dihydrochalcones decreased. The only exception was 2′-hydroxy-2′′,5′′-dimethoxychalcone, which was transformed into dihydrochalcone by strain KCh71 with a yield comparable to that of chalcone containing a single methoxy group.

Keywords: biotransformations; sweeteners; methoxychalcones; dihydrochalcones; yeast

1. Introduction

Sweet taste plays a dominant role in human food preferences [1]. It is the most important sensory feature of food products. It is not only a source of pleasure but also a basic energy stimulus for the body. Prospective studies have provided information on the correlation between excessive consumption of sugar and sugar-rich products, and an increased risk of pancreatic cancer. Given the role of hyperglycaemia and hyperinsulinaemia in the development of this cancer, it has been established that the direct cause of organ tissue death is the induction of frequent food hyperglycaemia and the increase in demand and reduction of insulin sensitivity [2]. There is also growing evidence of the role of increased sugar consumption in the development of hypertension, inflammation, and coronary artery disease [3–5].

For this reason, low-energy substitutes are being sought, exhibiting physical and chemical properties comparable to sucrose, but providing additional health benefits [6]. The increased interest in a healthy lifestyle and increased incidence of diseases caused by impaired metabolism of sugar compounds enhance the popularity of products containing sweeteners with reduced caloric value [7]. Sweeteners are defined as food additives that mimic the feeling of sweet taste, similar to saccharose [8]. Dihydrochalcones interacting with the receptors of sweet taste, T1R2, are included in this group [9]. An ideal sweetener, apart from low caloric value and sweetness intensity, similar to sucrose, should be safe for the health of the consumer and also stable in various processing conditions [6,10]. The multiplicity of restrictions and disadvantages of sweetener substances available on the market has created the need to search for new compounds that are more beneficial in terms of their impact

on human health and more attractive to industry [11]. Dihydrochalcones raise significant interest in this area [12–14]. They are obtained as a result of hydrogenation of chalcones, and show great potential as synthetic sweeteners [15,16]. The best-studied compound reported as an intensely sweet substance is neohesperidin dihydrochalcone [17]. Desirable biological properties characterise most of the dihydrochalcones found in plants. Phloretin and phlorizin (found in apples) significantly reduce the risk of developing cardiovascular disease and diabetes [18]. Aspalathin is found mainly in larger quantities in the leaves of *Aspalathus linearis* (6–13%), and in the stems it is much less abundant (0.16–0.78%). It affects the reduction of oxidative stress and may slow down the ageing process [19].

The main aim of our research was to obtain, as a result of biotransformation, a bank of dihydrochalcones characterised by a sweet taste. Additionally, we characterised the ability of selected yeast strains to hydrogenate both natural and unidentified in plants chalcones. Unconventional yeasts were used as biocatalysts because of specific enzymes capable of hydrogenation of chalcones [20–23]. Eight microorganisms were chosen to perform biotransformations; however, in this work, we pay special attention to the study of the catalytic capacity of a strain of the species *Yarrowia lipolytica*, which is currently known for its use in the production of many substances essential for the food industry: organic acids [24–26], polyols [27–30], fragrance substances [31,32], hydrolytic enzymes [33–35], and biomass [36]. One of the unique features of *Y. lipolytica* is the ability to use many unconventional carbon sources for its culture, such as alkanes, glycerol, or fatty acids [36–39]. The extraordinary interest in *Y. lipolytica* yeast is due to its strong tolerance to changes in pH, salt concentration, and a wide range of carbon sources that simplify the optimisation of bioprocesses and facilitate the use of non-glucose based raw materials [40,41]. More importantly, the GRAS status (Generally Recognised As Safe) defines strains of the *Y. lipolytica* species as an attractive and environmentally friendly microbiological tool for the production of nutraceuticals, fermented foods, and dietary supplements [41–43]. Yeast of this species received marketing authorisation as a novel food in 2019 (EU Regulation 2019/760). The maximum dose used is 3 g per day for children aged 3 to 10 years. Above the age of 10, the dose is 6 g a day [44]. The ability of strains of this species to effectively hydrogenate chalcones observed in our previous studies [22,23] may, in the future, result in the development of preparations combining the nutritional values of this species' strain, and the health and sensory properties of dihydrochalcones. The combination of chemical and biological methods allows for obtaining new compounds, and the development of unique processes, will enable the development of new ways to obtain sweeteners.

2. Results and Discussion

The main purpose of the study was to assess the capacity of various yeast strains for selective reduction of the double bond in a series of methoxychalcones obtained as a result of chemical synthesis. Additionally, the influence of the number and the position of methoxy substituents on the speed of biotransformation was checked. The selected substrates had one (**1, 2, 3**), two (**4, 6**), three (**5, 7**) or five (**8**) methoxy groups, and also the hydroxyl group at the 2′ position of the A ring (crucial for natural chalcones) (Scheme 1).

Scheme 1. Synthesis of chalcones and dihydrochalcones obtained as a result of transformation by unconventional yeast strains.

Eight microorganisms (*Rhodotorula rubra* KCh 4, *Yarrowia lipolytica* KCh 71, *Rhodotorula marina* KCh 77, *Rhodotorula rubra* KCh 82, *Candida viswanathii* KCh 120, *Rhodotorula glutinis* KCh 242, *Saccharomyces cerevisiae* KCh 464, and *Candida parapsilosis* KCh 909) [13,23] were chosen based on their previously observed high regioselectivity during biotransformation, among others 3-(2″-furyl)-

and 3-(2″-thienyl)-1-(2′-hydroxyphenyl)-prop-2-en-1-one [23]. The strains have a high ability to hydrogenate the double bond between C2–C3 in compounds lacking additional functional groups [13].

Eight substrates (**1–8**) were obtained as a result of chemical synthesis, according to the Claisen–Schmidt reaction (Table 1). Chemically obtained chalcones (**1–8**) and dihydrochalcones (**9–13**) resulting from biotransformation were purified and then characterised by NMR (^{1}H NMR, ^{13}C NMR, correlation spectra and long-range heteronuclear correlation—HMBC (Heteronuclear Multiple Bond Correlation)) and gas chromatography analysis (GC), and as well thin layer liquid chromatography (TLC) analysis.

Table 1. Chalcones obtained by chemical synthesis and dihydrochalcones identify as a results of biotransformations.

Chalcones	Dihydrochalcones	R1	R2	R3	R4	R5	R6
1	**9**	$-OCH_3$	-H	-H	-H	-H	-H
2	**10**	-H	$-OCH_3$	-H	-H	-H	-H
3	**11**	-H	-H	$-OCH_3$	-H	-H	-H
4	**12**	$-OCH_3$	-H	-H	$-OCH_3$	-H	-H
5	**13**	-H	$-OCH_3$	$-OCH_3$	$-OCH_3$	-H	-H
6	-	-H	-H	-H	-H	$-OCH_3$	$-OCH_3$
7	-	-H	-H	$-OCH_3$	-H	$-OCH_3$	$-OCH_3$
8	-	-H	$-OCH_3$	$-OCH_3$	$-OCH_3$	$-OCH_3$	$-OCH_3$

Five out of eight substrates (compounds **1–5**), all having methoxy substituents only in the B ring, were transformed to dihydrochalcones (2′-hydroxy-2″-methoxy-α,β-dihydrochalcone (**9**), 2′-hydroxy-3″-methoxy-α,β-dihydrochalcone (**10**), 2′-hydroxy-4″-methoxy-α,β- dihydrochalcone (**11**), 2′-hydroxy-2″,5″-dimethoxy-α,β-dihydrochalcone (**12**), and 2′-hydroxy- 3″,4″,5″-trimethoxy-α,β-dihydrochalcone (**13**)). In contrast, no conversion of substrates having methoxyl substituents also at the 4′,6′ positions of ring A (substrates **6**, **7,** and **8**), to the expected product was observed, regardless of the used strain. The highest substrate conversion was observed in the culture of the *Yarrowia lipolytica* KCh 71 strain (Table 2). We observed very high conversions (88–99%) of the four used substrates—2′-hydroxy-2″-methoxychalcone (**1**), 2′-hydroxy-3″-methoxychalcone (**2**), 2′-hydroxy-4″-methoxychalcone (**3**), and 2′-hydroxy-2″,5″-dimethoxychalcone (**4**)—in the culture of this strain after just one day. At the same time, as the number of methoxyl groups in the B ring increased, the conversion efficiency to dihydrochalcones decreased. For a substrate containing three methoxyl groups in the 3″, 4″, and 5″ positions (**5**), after reaching a certain level, the biotransformation was stopped and, with the expiration of the time, the substrate was no longer converted.

The results of each reaction were analysed in two aspects: (a) strains performing effective hydrogenation of as many substrates as possible, regardless of their structure. Considering this criterion, the best biocatalyst turned out to be the *Yarrowia lipolytica* KCh 71 strain (Table 2). Similar efficiencies of this strain have also been described during biotransformation of chalcone having heteroatom in the B ring [23]; (b) most efficiently converted substrate. In this case, with the increasing number of methoxyl groups in the B ring, the conversion efficiency of the substrate decreased, while in the presence of methoxyl substituents in the A ring, the product did not occur at all. These results were noted for most of the tested biocatalysts (Table 2).

Table 2. Conversion [%] of the substrates in time.

Substrate	2′-hydroxy-2′′-methoxychalcone (1)			2′-hydroxy-3″-methoxychalcone (2)			2′-hydroxy-4″-methoxychalcone (3)			2′-hydroxy-2″,5″-dimethoxychalcone (4)			2′-hydroxy-3″,4″,5″-trimethoxychalcone (5)		
Incubation Time [days]	**1**	**3**	**7**	**1**	**3**	**7**	**1**	**3**	**7**	**1**	**3**	**7**	**1**	**3**	**7**
Rhodotorula rubra KCh 4	2	3	4	7	8	10	5	5	8	4	5	7	0	0	2
Yarrowia lipolytica KCh 71	98	99	99	96	97	98	88	95	96	99	99	99	7	14	20
Rhodotorula marina KCh 77	3	3	4	8	8	10	10	14	17	2	5	8	0	3	5
Rhodotorula rubra KCh 82	3	3	3	25	30	43	6	8	13	2	4	6	0	4	6
Candida viswanathii KCh 120	98	99	99	70	95	95	98	98	98	0	39	58	6	10	19
Rhodotorula glutinis KCh 242	2	3	14	95	95	95	43	75	94	3	6	6	4	4	6
Saccharomyces cerevisiae KCh 464	10	14	34	6	12	24	74	86	97	3	4	5	3	4	9
Candida parapsilosis KCh 909	44	68	83	33	54	73	29	75	84	12	17	48	4	6	7

Also, the methoxyl substitution position affected the rate and efficiency of the hydrogenation process. These differences are seen the best in the *R. glutinis* KCh 242 strain, in a culture whose conversion of compounds containing one methoxyl group in various positions (in the same ring) is diametrically different. Substrate **1** having a methoxyl group at the C-2″ carbon was 14% converted after seven days of incubation, whereas for substrates **2** and **3** (methoxyl group located at C-3″ and C-4″, respectively) conversion rates exceeded 90% after seven days (Figure 1A). Another preference, selectivity due to the structure of the substrate, was observed for the strain *Saccharomyces cerevisiae* KCh 464, for which a significantly higher conversion was recorded for compound **3**. Compounds with the methoxy group located at the C-2″and C-3″ carbon demonstrate a much slower conversion in the culture of this strain (Figure 1 B). This observation was surprising because strains of this species in many papers are described as effective and universal biocatalysts for the hydrogenation of double bonds, both in chalcones [13,20] and other compounds containing the enone moiety [45,46].

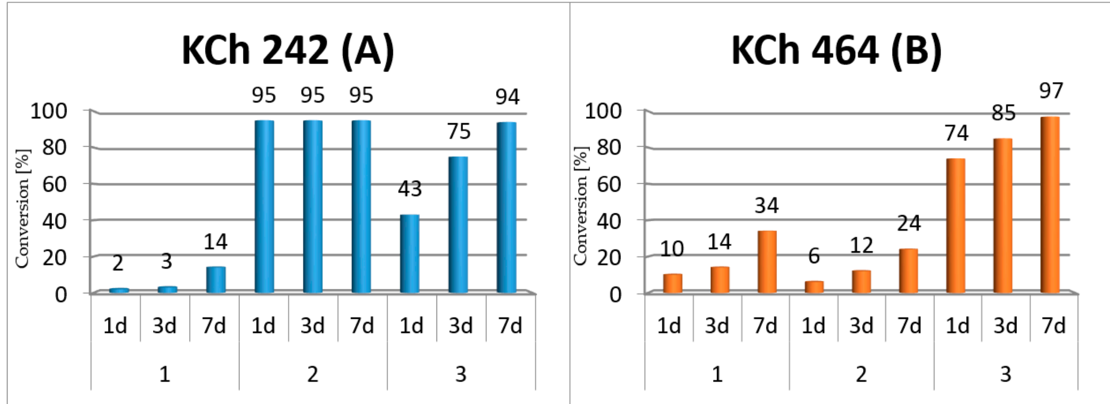

Figure 1. (**A**) Substrate conversionby *R. glutinis* KCh 242 strain after 1 and 7 days of incubation. (**1**) 2′-hydroxy-2″-methoxychalcone, (**2**) 2′-hydroxy-3″-methoxychalcone and (**3**) 2′-hydroxy-4″-methoxychalcone; (**B**) Substrate conversionby *S. Cerevisiae* KCh 464 strain after 1 and 7 days of incubation. (**1**) 2′-hydroxy-2″-methoxychalcone, (**2**) 2′-hydroxy-3″-methoxychalcone and (**3**) 2′-hydroxy-4″-methoxychalcone.

Based on screening tests, we decided to choose *Yarrowia lipolytica* KCh 71 strain for further experiments. This strain showed the highest conversion and the largest number of substrates tested after just one day of biotransformation. In addition, yeast from this species was authorised in 2019 as a novel food (EU Regulation 2019/760). The use of a strain with such properties, as a biocatalyst, minimises the possibility of extraction of any toxic metabolites with the obtained product [44]. For these reasons, we decided to check the progress of biotransformation during the first day of substrate incubation, and the ability of this strain to hydrogenate higher concentrations of tested compounds. At this stage, the four most efficiently convertible compounds were used as substrates (**1–4**). The standard concentration of substrate for screening tests was 100 mg/L. However, we tested and compared the efficiency of this reaction by increasing the scale of the process so that the medium contained a maximum of 5 g of substrate per 1 L of a medium, which is shown in the table (Table 3).

Table 3. Conversion of selected substrates depending on their concentration and biotransformation time, performed in triplicate.

Substrate	Concentration [g/L]	Biotransformation Time				
		1 h	3 h	6 h	12 h	24 h
1	0.1	87 ± 1.2	96 ± 3.5	100 ± 0.0	100 ± 0.0	100 ± 0.6
	0.5	41 ± 7.1	92 ± 3.0	98 ± 0.0	100 ± 0.0	100 ± 0.6
	1	26 ± 4.9	57 ± 6.7	69 ± 7.6	83 ± 7.2	94 ± 0.0
	2	15 ± 1.5	32 ± 2.1	36 ± 1.7	43 ± 4.2	70 ± 4.5
	5	6 ± 1.0	14 ± 2.0	16 ± 2.3	19 ± 2.5	49 ± 0.6
2	0.1	66 ± 8.1	91 ± 5.0	91 ± 8.1	93 ± 1.0	95 ± 1.5
	0.5	46 ± 5.8	77 ± 4.4	85 ± 3.2	90 ± 1.0	93 ± 1.0
	1	27 ± 2.0	41 ± 4.7	49 ± 4.7	60 ± 4.9	64 ± 3.5
	2	14 ± 1.5	25 ± 1.2	30 ± 2.6	31 ± 1.4	37 ± 3.2
	5	5 ± 0.6	8 ± 0.0	9 ± 0.6	10 ± 0.6	16 ± 1.2
3	0.1	74 ± 1.5	95 ± 1.5	98 ± 0.6	98 ± 0.6	98 ± 0.0
	0.5	51 ± 5.3	75 ± 2.6	88 ± 1.7	96 ± 0.6	97 ± 0.6
	1	23 ± 2.5	44 ± 1.5	51 ± 1.0	66 ± 7.0	77 ± 6.4
	2	11 ± 0.6	22 ± 1.0	26 ± 1.2	33 ± 3.2	48 ± 4.0
	5	4 ± 0.6	6 ± 1.2	8 ± 1.7	12 ± 3.0	22 ± 5.0
4	0.1	91 ± 3.5	96 ± 0.6	99 ± 0.0	99 ± 0.0	99 ± 0.0
	0.5	40 ± 3.6	50 ± 3.6	43 ± 2.6	44 ± 0.6	49 ± 1.2
	1	15 ± 1.5	26 ± 3.2	25 ± 3.5	25 ± 2.6	29 ± 2.0
	2	9 ± 1.2	13 ± 2.1	13 ± 1.5	14 ± 1.7	16 ± 2.5
	5	3 ± 0.6	4 ± 0.6	5 ± 0.6	6 ± 1.5	7 ± 1.5

In this part of the experiment, the scale of the process was changed and Riplate square wells were used instead of Erlenmeyer flasks. This test was performed in triplicate. The substrate addition was as follows: 0.3, 1.5, 3, 6, and 15 mg per well, which corresponds to concentrations: 100, 500, 1, 2, and 5 g per 1 L of culture medium, respectively. At the lower concentrations, there is no significant difference between the conversion of various substrates. As the concentration increases, 2′-hydroxy-2″-methoxychalcone (**1**) is transformed the most efficiently, and the conversion is shown in Figure 2B. The lowest conversion rate recorded for this substrate after 24 h was 50%, at the maximum concentration of 5 g/L.

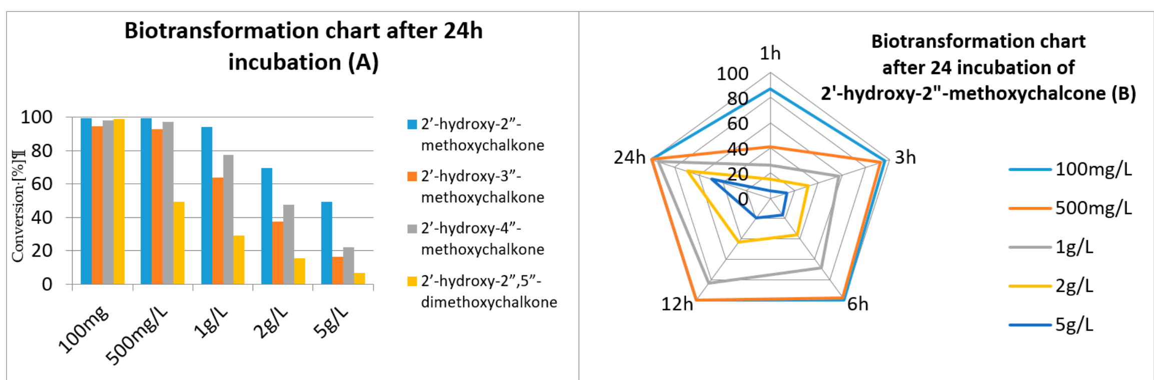

Figure 2. Comparison of conversions of increased substrate concentrations: after 24 h (**A**); conversion of 2′-hydroxy-2″-methoxychalcone (**1**) by *Yarrowia lipolytica* KCh 71 at increasing concentrations of substrate (**B**).

As the concentration increased, significant differences between the conversion of individual compounds started to be visible (Figure 3). The expected product (dihydrochalcone) was also observed during the transformation of the highest substrate concentration but with a much lower yield compared to the screening concentration (100 mg/L). After 24 h of biotransformation, at the highest substrate

concentration (5 g/L), 2′-hydroxy-2″-methoxychalcone was converted the most efficiently—where about 50% conversion was observed (Figure 3A). Under the above conditions, the *Yarrowia lipolytica* KCh 71 strain was also able to transform the other substrates, although with much lower yield (7%–22%) (Table 3). In addition, such a high level of conversion for the tested methoxychalcones, at a concentration of 100 mg/L, is comparable to the previously described studies on the unsubstituted [13] or containing hydroxyl or methyl groups [47] chalcone derivatives.

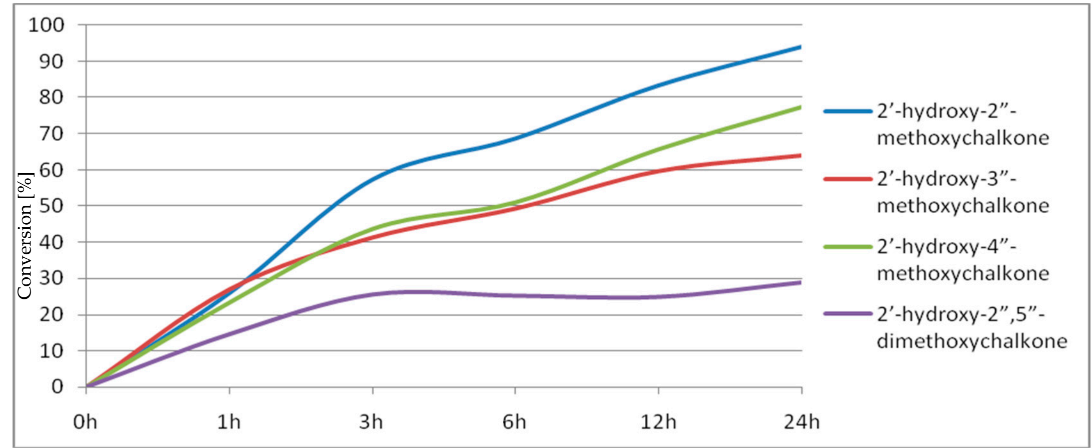

Figure 3. Comparison of conversion of selected substrates to products during 24 h incubation, for a substrate concentration of 1 g/L.

Moreover, the use of yeast from the *Yarrowia lipolytica* species is not accidental. This is a microorganism whose properties are widely used in the food industry—from the production of citric acid [25] or eicosapentaenoic acid [48] by preventing rotting of the harvest [49] and cleaning the soil from petroleum hydrocarbons [50], to the production of high-protein animal feed [51] and unconventional food for humans [44]. It is also used in the production of various types of sweeteners [41]. Moreover, yeast of this species can be successfully used to produce significant amounts of various types of dihydrochalcones. In addition, biotransformations using these microorganisms allow one to obtain with high efficiency a single compound with high purity. The only reaction products were dihydrochalcones (also observed in our previous studies) [23]. The microorganisms used were not able to produce other flavonoid derivatives (flavones, flavanones), which are found during the transformation of chalcones in cultures of filamentous fungi, bacteria, or algae [13,47,52,53].

3. Materials and Methods

3.1. Substrates

The substrates, used for biotransformation, were obtained by Claisen–Schmidt condensation reaction of 2-hydroxyacetophenone or 2′-hydroxy-4′,6′-dimethoxyacetophenone (A*) with benzaldehyde containing methoxylated group(s) in appropriate positions (B*) (purchased from Sigma-Aldrich (St. Louis, MO, USA)) dissolved in methanol in an alkaline environment (Table 1) at a high temperature, according to the procedure described previously [13,23]. The resulting compounds (**1**–**8**) were used as substrates for the biotransformation and their NMR spectral data are identical to those previously published [52,54–56].

3.2. Microorganisms

The research was carried out on eight strains of yeast from the species: *Rhodotorula rubra* (KCh 4 and KCh 82), *Rhodotorula marina* KCh 77, *Rhodotorula glutinis* KCh 242, *Yarrowia lipolytica* KCh 71, *Candida viswanathii* KCh 120, *Saccharomyces cerevisiae* KCh 464, and *Candida parapsilosis* KCh 909 were obtained from the collection of the Department of Chemistry, Wrocław University of Environmental

and Life Sciences (Wrocław, Poland). Whose storage and biocatalytic capacity have been previously described [22,23,57].

3.3. Screening

Erlenmeyer flasks of 300 mL were used for biotransformation on an analytical scale, each containing 100 mL of Sabouraud culture medium (3% glucose, 1% aminobac). Used microorganisms were incubated for three days at 24°C on a rotary shaker (144 rpm)(Eppendorf AG, Hamburg, Germany). After this time, 10 mg of the substrate was dissolved in DMSO (dimethyl sulfoxide) and added. Samples were collected after 1, 3, and 7 days. Portions of 10 mL of the transformation mixture were taken out and extracted with ethyl acetate. The extracts were dried over $MgSO_4$, concentrated *in vacuo*, and analysed by gas chromatography (GC) and thin-layer chromatography (TLC) (SiO2, DC Alufolien Kieselgel 60 F254 (0.2 mm thick), Merck, Darmstadt, Germany).

3.4. Gas Chromatography

GC analysis was performed using an Agilent 7890A gas chromatograph, equipped with a flame ionisation detector (FID) (Agilent, Santa Clara, CA, USA). The capillary column DB-5HT (30 m × 0.25 mm × 0.10 µm) was used to determine the composition of the product mixtures. The temperature programme was applied as follows: 80–300 °C, the temperature on the detector: 300 °C, injection 1 µL, flow 1 mL/min, flow H2: 35 mL/min, airflow; 300 mL/min, time of analysis: 18.67 min. The retention times of the substrates and products are described in Table 4.

Table 4. Compounds mass after extraction (preparative scale) [mg]—in relation to 100 mg of substrate used and retention times based on GC.

Substrate	Product	Isolated Yield [%]	Substrate Retention Time [min]	Product Retention Time [min]
2′-hydroxy-2″-methoxychalcone (**1**)	**9**	47	11.93	10.58
2′-hydroxy-3″-methoxychalcone (**2**)	**10**	33	11.90	10.87
2′-hydroxy-4″-methoxychalcone (**3**)	**11**	40	12.24	11.03
2′-hydroxy-2″,5″-dimethoxychalcone (**4**)	**12**	54	13.36	12.54
2′-hydroxy-3″,4″,5″-trimethoxychalcone (**5**)	**13**	5	14.17	13.46
2′-hydroxy-4′,6′-dimethoxychalcone (**6**)	-	-	13.83	-
2′-hydroxy-4′,6′,4″-trimethoxychalcone (**7**)	-	-	14.69	-
2′-hydroxy-4′,6′,3″,4″,5″-penthamethoxychalcone (**8**)	-	-	16.01	-

3.5. Preparative Scale

Preparative biotransformations were performed in 2 L Erlenmeyer flasks, each containing 500 mL of culture medium (3% glucose, 1% peptone). The transferred microorganisms were incubated for three days at 24°C on a rotary shaker. After this time, 100 mg of the substrate dissolved in 2 mL of DMSO was added. After three days, the product was isolated by triple extraction with ethyl acetate (3 extractions with 300 mL), dried with anhydrous magnesium sulfate, and concentrated *in vacuo*. The transformation products were separated by preparative TLC and analysed (TLC, GC, NMR).

3.6. TLC and NMR Analysis

The course of biotransformation was monitored using TLC plates (SiO_2, DC Alufolien Kiesel gel 60 F254 (0.2 mm thick), Merck, Darmstadt, Germany). Products were separated using preparative TLC plates (Silica Gel GF, 20 × 20 cm, 500 µm, Analtech, Newark, DE, USA) and a cyclohexane: ethyl acetate mixture (9:1, *v*/*v*) as an eluent, according to the method described previously [58]. The product was observed (without additional visualisation) under the UV lamp at the wavelength of 254 nm.

NMR analysis was performed using a DRX 600 MHz Bruker spectrometer (Bruker, Billerica, MA, USA). The prepared samples were dissolved in deuterated chloroform $CDCl_3$. The performed analyses include ^{1}H NMR, ^{13}C NMR, HMBC (two-dimensional analysis) HMQC (heteronuclear correlation) and COSY (correlation spectroscopy) (Supplementary Materials).

3.7. Increasing the Concentrations of Tested Substrates

Transfer (scaling) of the biotransformation scale was carried out in Riplate square wells, 24 wells, to which 3 mL of culture medium with an inoculum of *Yarrowia lipolytica* KCh 71 was added. Microorganisms were incubated for three days at 24 °C on an oscillating shaker (190 rpm)(ELMI, Riga, Latvia). Screening was performed for five substrate concentrations—100 mg/L, 500 mg/L, 1 g/L, 2 g/L, and 5 g/L. The substrate was dissolved in DMSO. A total of 100 µL of the prepared substrate solution was added to each well. The experiment was carried out in triplicate. Samples (500 µL each) were collected after 1, 3, 6, 12, and 24 h of substrate incubation, then extracted with ethyl acetate, centrifuged, and analysed with GC.

4. Conclusions

On account of the constantly growing requirement for sweeteners, new solutions for their production are still being sought, preferably as cheaply as possible, biotechnological and ones that simultaneously fulfil the assumptions of the "green source" theory. The use of microorganisms, which is approved as safe and even allowed for consumption, seems to be an interesting solution. *Y. lipolytica* KCh 71 adapted for biotransformation turned out to be the best of the tested microorganisms to transform methoxychalcones. The most efficiently transformed compounds were chalcones containing single methoxyl groups in the B ring. Interestingly, the substrate with 2′-hydroxy-2″-methoxychalcone even at a scale increased to 5 g substrate per 1 L of medium was convertible with up to 50% yield after 24 h. At the same time, a very high conversion, from 66 to 91% depending on the substrate, after one hour of incubation (Table 2) indicates that the ene-reductase catalysing this process is a constitutive enzyme. However, identification of which group of enzymes catalyzes this reaction requires the use of molecular biology methods.

With the increase in the number of methoxyl groups, the efficiency of the process decreased. For compounds that contain methoxyl substituents also in the A ring, the transformation did not occur at all, including commonly described flavokavain B (2′-hydroxy-4′,6′-dimethoxychalcone).

The challenge facing this type of research is to limit the number of solvents used when extracting products—or to eliminate extraction. This solution would increase the efficiency of the process and, at the same time, would allow the production of a supplement containing the pro-health microorganism as well as a sweet flavonoid product with a significantly different spectrum of activity. Moreover, the use of created dihydrochalcones as sweeteners could have a positive effect on the human body, while enriching our diet and reducing the need to use sucrose and preventing various types of civilisation diseases.

Sweeteners play a significant role in the human diet. Through their palatability, they improve the taste of sweets, drinks, or snacks. Replacing some of the popular sweeteners (e.g., acesulfame) with other, less caloric compounds lacking side-effects is an increasingly common food market practice. The placement of dihydrochalcone, following the dihydrochalcone neohesperidin, in food products could have a more positive effect on the reduction of civilisation diseases than the use of sweeteners without health-promoting properties.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/2073-4344/10/10/1135/s1. Figure S1: ^{1}H NMR spectral of 1-(2′-hydroxyphenyl)-3-(2″-methoxyphenyl)-2-propan-1-one (**9**) ($CDCl_3$, 600 MHz), Figure S2: Part of the ^{13}C NMR spectral of 1-(2′-hydroxyphenyl)-3-(2″-methoxyphenyl)-2-propan-1-one (**9**) ($CDCl_3$, 600 MHz), Figure S3: ^{13}C NMR spectral of 1-(2′-hydroxyphenyl)-3-(2″-methoxyphenyl)-2-propan-1-one (**9**) ($CDCl_3$, 151 MHz), Figure S4: Part of the ^{13}C NMR spectral of 1-(2′-hydroxyphenyl)-3-(2″-methoxyphenyl)-2-propan-1-one (**9**) ($CDCl_3$, 151 MHz), Figure S5: COSY spectral of 1-(2′-hydroxyphenyl)-3-(2″-methoxyphenyl)-2-propan-1-one (**9**) ($CDCl_3$, 151 MHz), Figure S6: Part of the COSY spectral of 1-(2′-hydroxyphenyl)-3-(2″-methoxyphenyl)-2-propan-1-one (**9**) ($CDCl_3$, 151 MHz), Figure S7: HSQC spectral of 1-(2′-hydroxyphenyl)-3-(2″-methoxyphenyl)-2-propan-1-one (**9**) ($CDCl_3$, 151 MHz), Figure S8: HMBC spectral of 1-(2′-hydroxyphenyl)-3-(2″-methoxyphenyl)-2-propan-1-one (**9**) ($CDCl_3$, 151 MHz), Figure S9: ^{1}H NMR spectral of 1-(2′-hydroxyphenyl)-3-(3″-methoxyphenyl)-2-propan-1-one (**10**) ($CDCl_3$, 600 MHz), Figure S10: Part of the ^{13}C NMR spectral of 1-(2′-hydroxyphenyl)-3-(3″-methoxyphenyl)-2-propan-1-one (**10**) ($CDCl_3$,

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Author Contributions: M.Ł. performed the synthesis and biotransformations, interpreted the results, analyzed the spectral data, visualization, data curation, writing, original draft preparation; E.K. methodology, validation, reviewing and editing; E.K.-S. methodology, supervision, interpreted the results, analyzed the spectral data; T.J. conceptualization, validation, interpreted the results, analyzed the spectral data; Writing, reviewing and editing. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

MDPI	Multidisciplinary Digital Publishing Institute
DOAJ	Directory of open access journals
TLA	Three letter acronym
LD	linear dichroism

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Highly Effective, Regiospecific Hydrogenation of Methoxychalcone by *Yarrowia lipolytica* Enables Production of Food Sweeteners

Mateusz Łużny *, Ewa Kozłowska, Edyta Kostrzewa-Susłow and Tomasz Janeczko *

Department of Chemistry, Wrocław University of Environmental and Life Sciences, Norwida 25, 50-375 Wrocław, Poland; e.a.kozlowska@gmail.com (E.K.); ekostrzew@gmail.com (E.K-S.)

* Correspondence: mateusz.luzny@upwr.edu.pl (M.Ł.); janeczko13@interia.pl (T.J.); Tel.: +48-713-205-195 (T.J.)

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Contents (Spectral data of isolated metabolites)

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11. Fig.S11. ^{13}C NMR spectral of 1-(2′-hydroxyphenyl)-3-(3″-methoxyphenyl)-2-propan-1-one (**10**) ($CDCl_3$, 151 MHz)
12. Fig.S12. Part of the ^{13}C NMR spectral of 1-(2′-hydroxyphenyl)-3-(3″-methoxyphenyl)-2-propan-1-one (**10**) ($CDCl_3$, 151 MHz)
13. Fig.S13. COSY spectral of 1-(2′-hydroxyphenyl)-3-(3″-methoxyphenyl)-2-propan-1-one (**10**) ($CDCl_3$, 151 MHz)
14. Fig.S14. Part of the COSY spectral of 1-(2′-hydroxyphenyl)-3-(3″-methoxyphenyl)-2-propan-1-one (**10**) ($CDCl_3$, 151 MHz)
15. Fig.S15. HSQC spectral of 1-(2′-hydroxyphenyl)-3-(3″-methoxyphenyl)-2-propan-1-one (**10**) ($CDCl_3$, 151 MHz)
16. Fig.S16. HMBC spectral of 1-(2′-hydroxyphenyl)-3-(3″-methoxyphenyl)-2-propan-1-one (**10**) ($CDCl_3$, 151 MHz)
17. Fig.S17. ^{1}H NMR spectral of 1-(2′-hydroxyphenyl)-3-(4″-methoxyphenyl)-2-propan-1-one (**11**) ($CDCl_3$, 600 MHz)
18. Fig.S18. Part of the ^{13}C NMR spectral of 1-(2′-hydroxyphenyl)-3-(4″-methoxyphenyl)-2-propan-1-one (**11**) ($CDCl_3$, 600 MHz)

19. Fig.S19. ^{13}C NMR spectral of 1-(2′-hydroxyphenyl)-3-(4″-methoxyphenyl)-2-propan-1-one (**11**) ($CDCl_3$, 151 MHz)
20. Fig.S20. Part of the ^{13}C NMR spectral of 1-(2′-hydroxyphenyl)-3-(4″-methoxyphenyl)-2-propan-1-one (**11**) ($CDCl_3$, 151 MHz)
21. Fig.S21. COSY spectral of 1-(2′-hydroxyphenyl)-3-(4″-methoxyphenyl)-2-propan-1-one (**10**) ($CDCl_3$, 151 MHz)
22. Fig.S22. Part of the COSY spectral of 1-(2′-hydroxyphenyl)-3-(4″-methoxyphenyl)-2-propan-1-one (**11**) ($CDCl_3$, 151 MHz)
23. Fig.S23. HSQC spectral of 1-(2′-hydroxyphenyl)-3-(4″-methoxyphenyl)-2-propan-1-one (**11**) ($CDCl_3$, 151 MHz)
24. Fig.S24. HMBC spectral of 1-(2′-hydroxyphenyl)-3-(4″-methoxyphenyl)-2-propan-1-one (**11**) ($CDCl_3$, 151 MHz)
25. Fig.S25. ^{1}H NMR spectral of 1-(2′-hydroxyphenyl)-3-(2″,5″-dimethoxyphenyl)-2-propan-1-one (**12**) ($CDCl_3$, 600 MHz)
26. Fig.S26. Part of the ^{13}C NMR spectral of 1-(2′-hydroxyphenyl)-3-(2″,5″-dimethoxyphenyl)-2-propan-1-one (**12**) ($CDCl_3$, 600 MHz)
27. Fig.S27. ^{13}C NMR spectral of 1-(2′-hydroxyphenyl)-3-(2″,5″-dimethoxyphenyl)-2-propan-1-one (**12**) ($CDCl_3$, 151 MHz)
28. Fig.S28. Part of the ^{13}C NMR spectral of 1-(2′-hydroxyphenyl)-3-(2″,5″-dimethoxyphenyl)-2-propan-1-one (**12**) ($CDCl_3$, 151 MHz)
29. Fig.S29. COSY spectral of 1-(2′-hydroxyphenyl)-3-(2″,5″-dimethoxyphenyl)-2-propan-1-one (**12**) ($CDCl_3$, 151 MHz)
30. Fig.S30. Part of the COSY spectral of 1-(2′-hydroxyphenyl)-3-(2″,5″-dimethoxyphenyl)-2-propan-1-one (**12**) ($CDCl_3$, 151 MHz)
31. Fig.S31. HSQC spectral of 1-(2′-hydroxyphenyl)-3-(2″,5″-dimethoxyphenyl)-2-propan-1-one (**12**) ($CDCl_3$, 151 MHz)
32. Fig.S32. HMBC spectral of 1-(2′-hydroxyphenyl)-3-(2″,5″-dimethoxyphenyl)-2-propan-1-one (**12**) ($CDCl_3$, 151 MHz)
33. Fig.S33. ^{1}H NMR spectral of 1-(2′-hydroxyphenyl)-3-(3″,4″,5″-trimethoxyphenyl)-2-propan-1-one (**13**) ($CDCl_3$, 600 MHz)
34. Fig.S34. Part of the ^{1}H NMR spectral of 1-(2′-hydroxyphenyl)-3-(3″,4″,5″-trimethoxyphenyl)-2-propan-1-one (**13**) ($CDCl_3$, 600 MHz)
35. Fig.S35. Part of the ^{1}H NMR spectral of 1-(2′-hydroxyphenyl)-3-(3″,4″,5″-trimethoxyphenyl)-2-propan-1-one (**13**) ($CDCl_3$, 600 MHz)
36. Fig.S36. ^{13}C NMR spectral of 1-(2′-hydroxyphenyl)-3-(3″,4″,5″-trimethoxyphenyl)-2-propan-1-one (**13**) ($CDCl_3$, 151 MHz)
37. Fig.S37. Part of the ^{13}C NMR spectral of 1-(2′-hydroxyphenyl)-3-(3″,4″,5″-trimethoxyphenyl)-2-propan-1-one (**13**) ($CDCl_3$, 600 MHz)
38. Fig.S38. HMBC spectral of 1-(2′-hydroxyphenyl)-3-(3″,4″,5″-trimethoxyphenyl)-2-propan-1-one (**13**) ($CDCl_3$, 151 MHz)

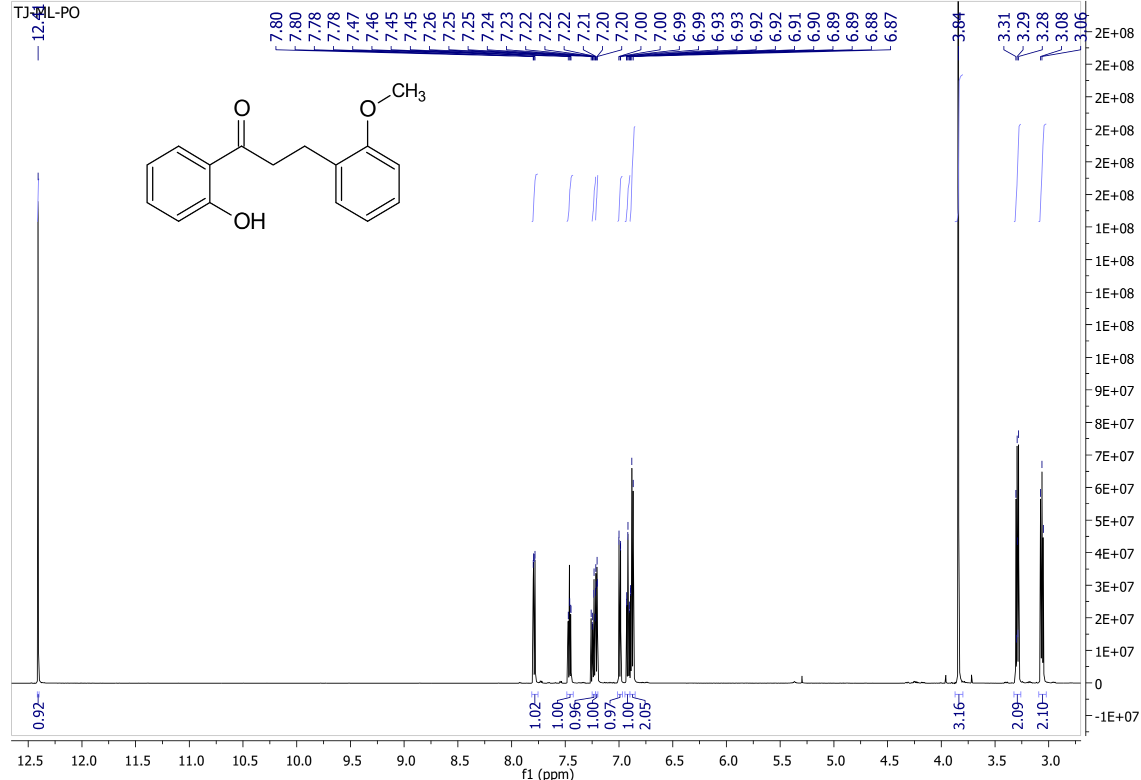

Figure S1. ^{1}H NMR spectral of 1-(2′-hydroxyphenyl)-3-(2″-methoxyphenyl)-2-propan-1-one (**9**) ($CDCl_3$, 600 MHz).

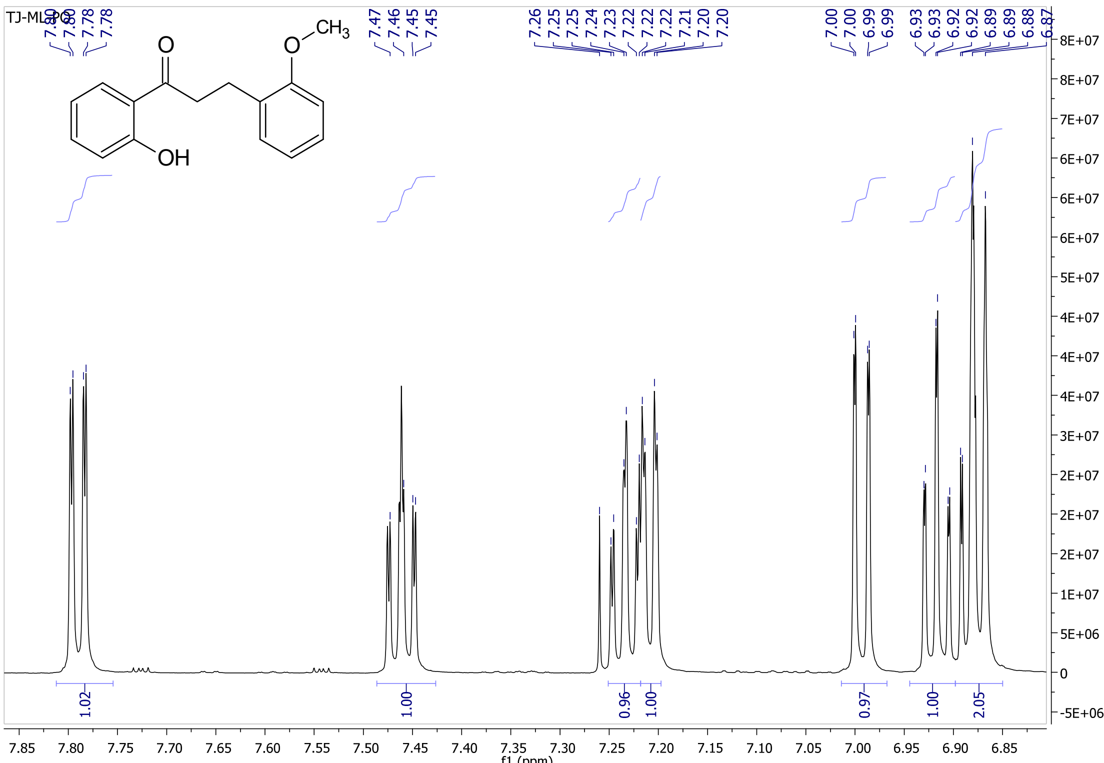

Figure S2. Part of the ^{13}C NMR spectral of 1-(2′-hydroxyphenyl)-3-(2″-methoxyphenyl)-2-propan-1-one (**9**) ($CDCl_3$, 600 MHz).

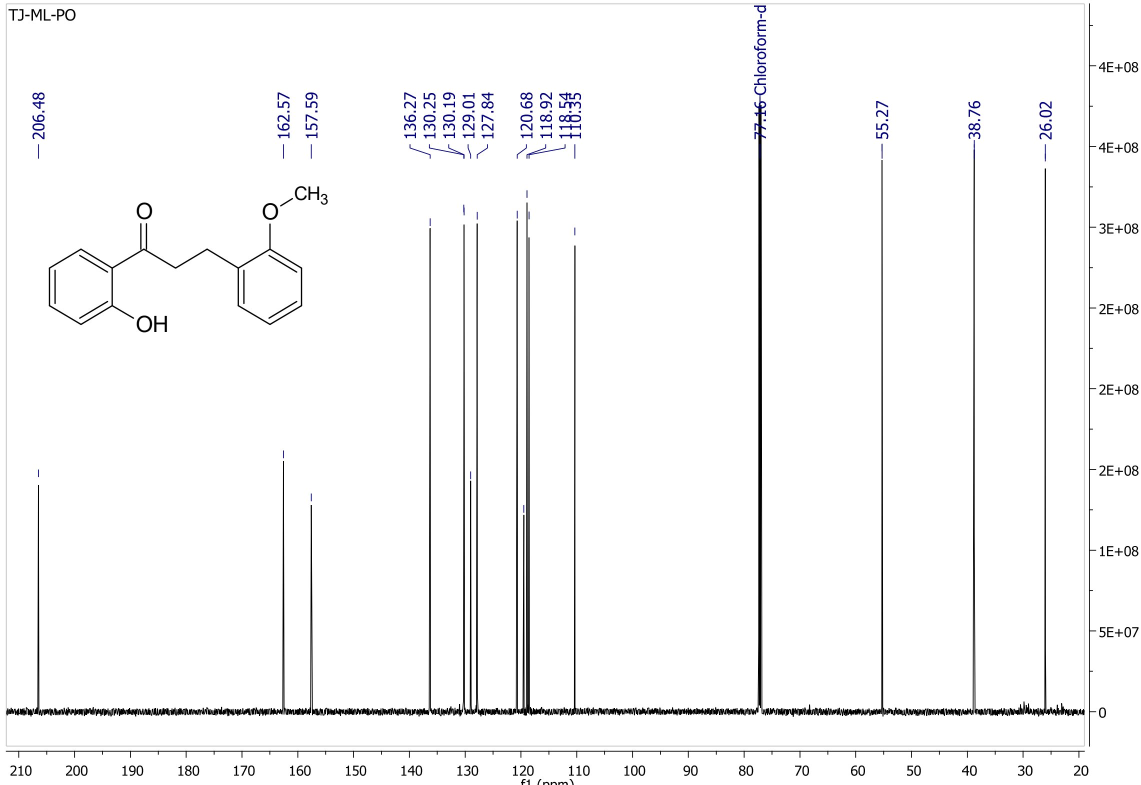

Figure S3. ^{13}C NMR spectral of 1-(2′-hydroxyphenyl)-3-(2″-methoxyphenyl)-2-propan-1-one (**9**) ($CDCl_3$, 151 MHz).

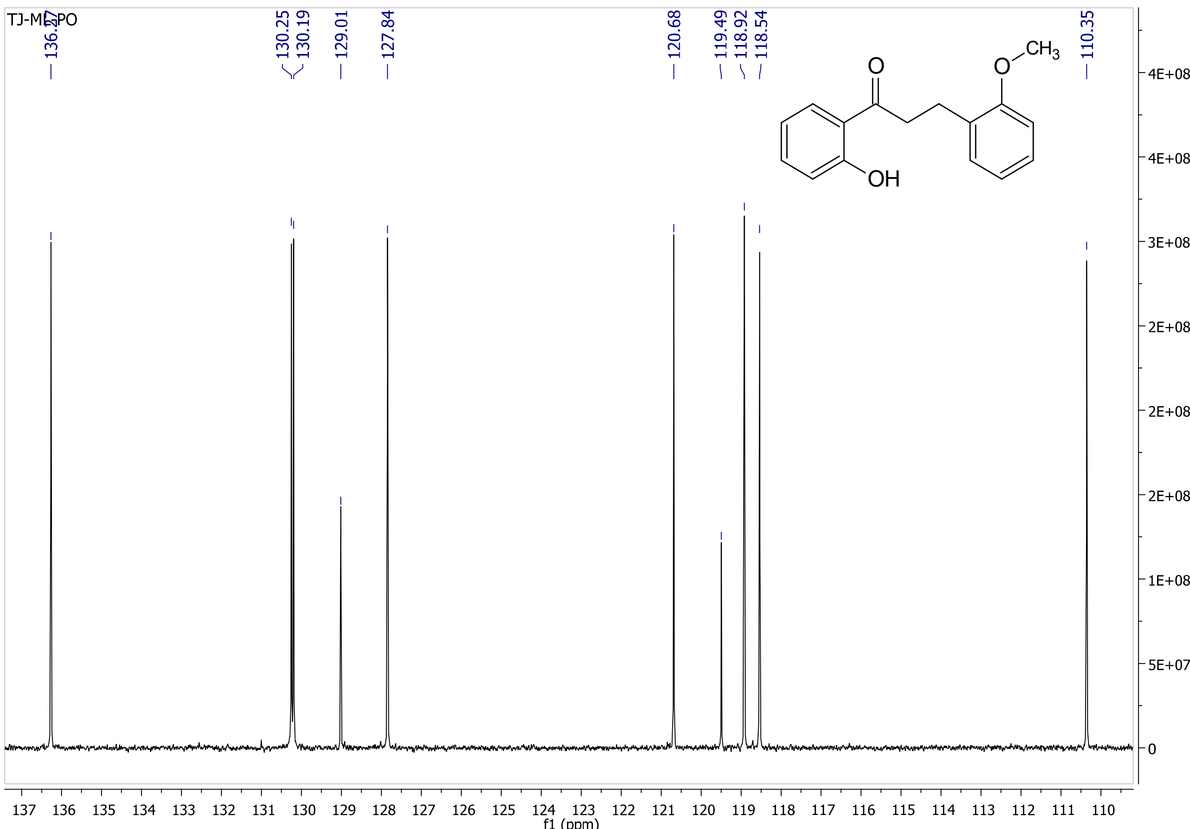

Figure S4. Part of the ^{13}C NMR spectral of 1-(2′-hydroxyphenyl)-3-(2″-methoxyphenyl)-2-propan-1-one (**9**) ($CDCl_3$, 151 MHz).

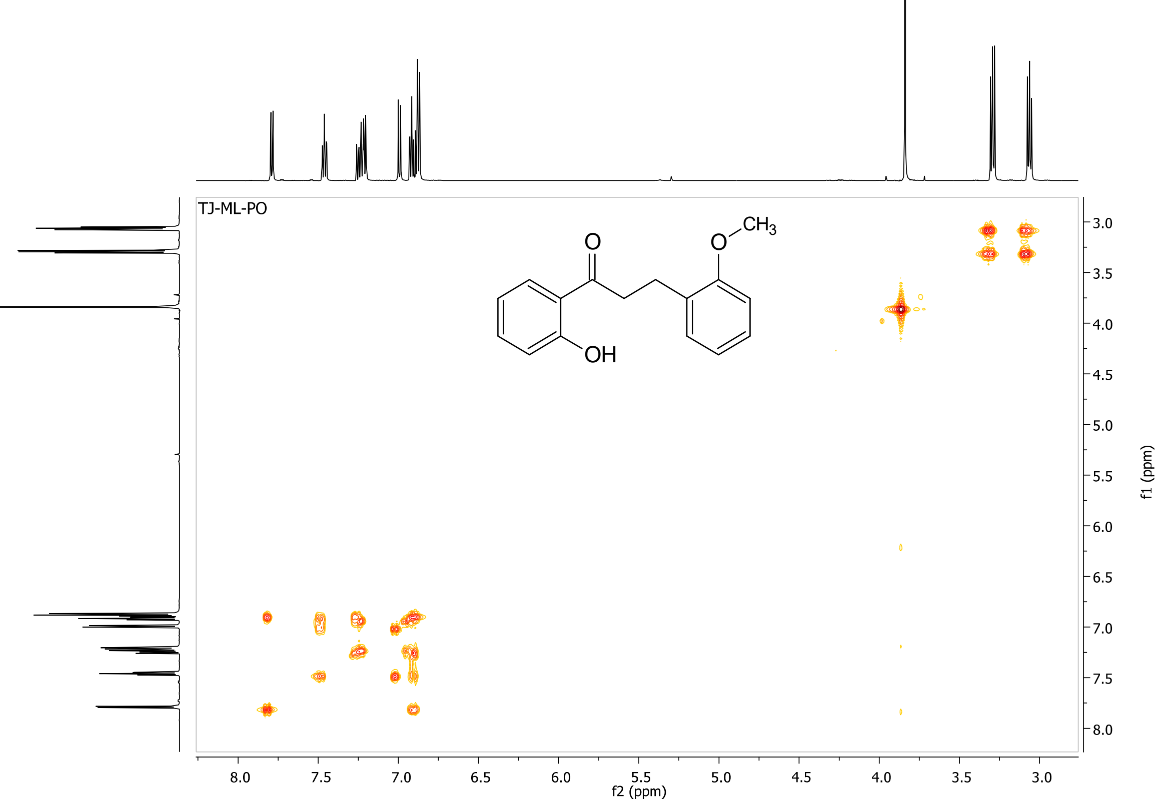

Figure S5. COSY spectral of 1-(2′-hydroxyphenyl)-3-(2″-methoxyphenyl)-2-propan-1-one (**9**) ($CDCl_3$, 151 MHz).

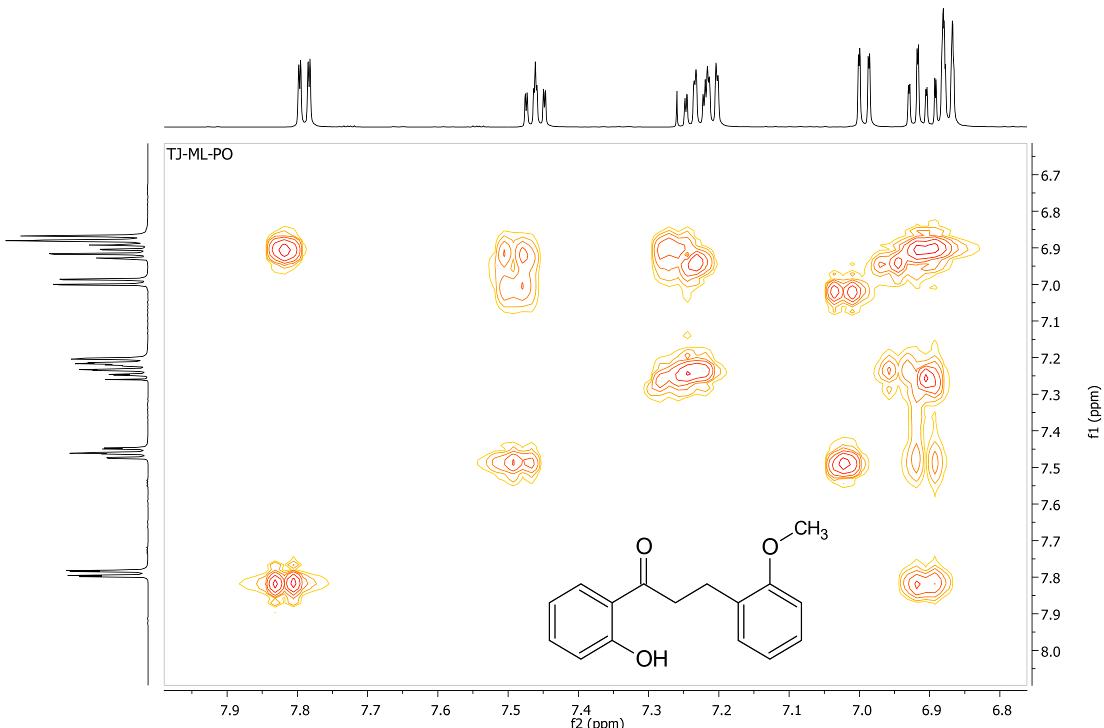

Figure S6. Part of the COSY spectral of 1-(2′-hydroxyphenyl)-3-(2″-methoxyphenyl)-2-propan-1-one (**9**) ($CDCl_3$, 151 MHz).

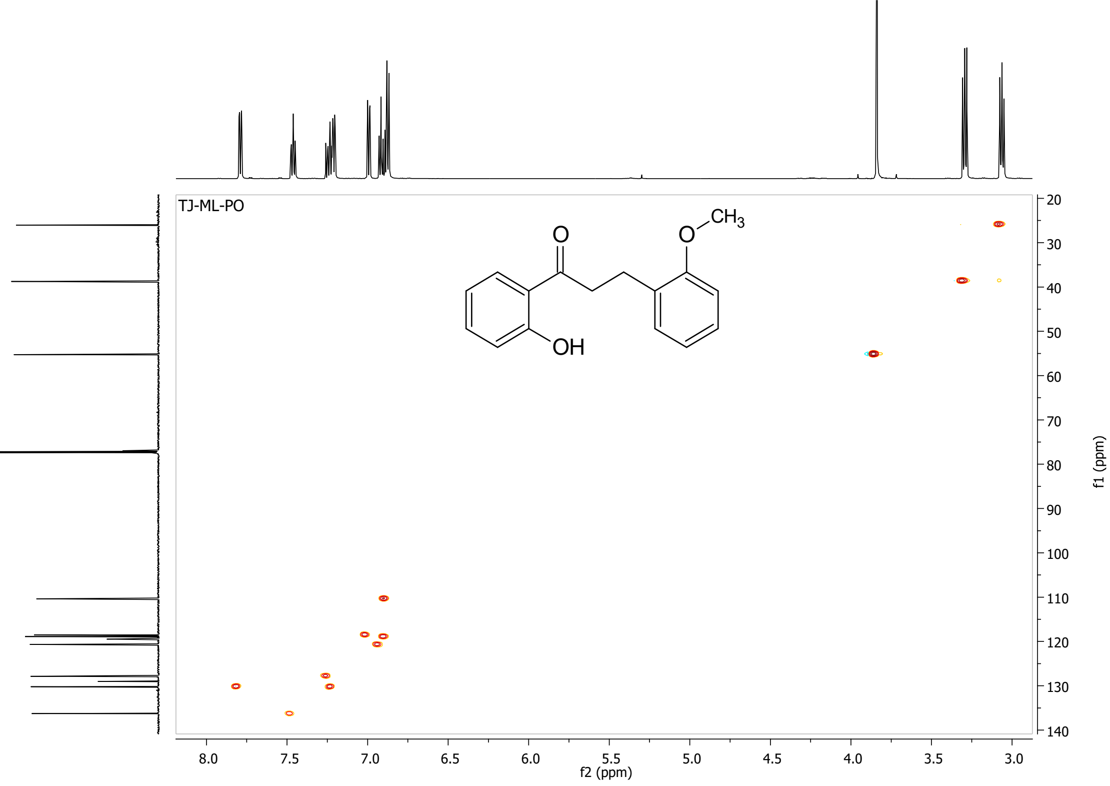

Figure S7. HSQC spectral of 1-(2′-hydroxyphenyl)-3-(2″-methoxyphenyl)-2-propan-1-one (**9**) ($CDCl_3$, 151 MHz).

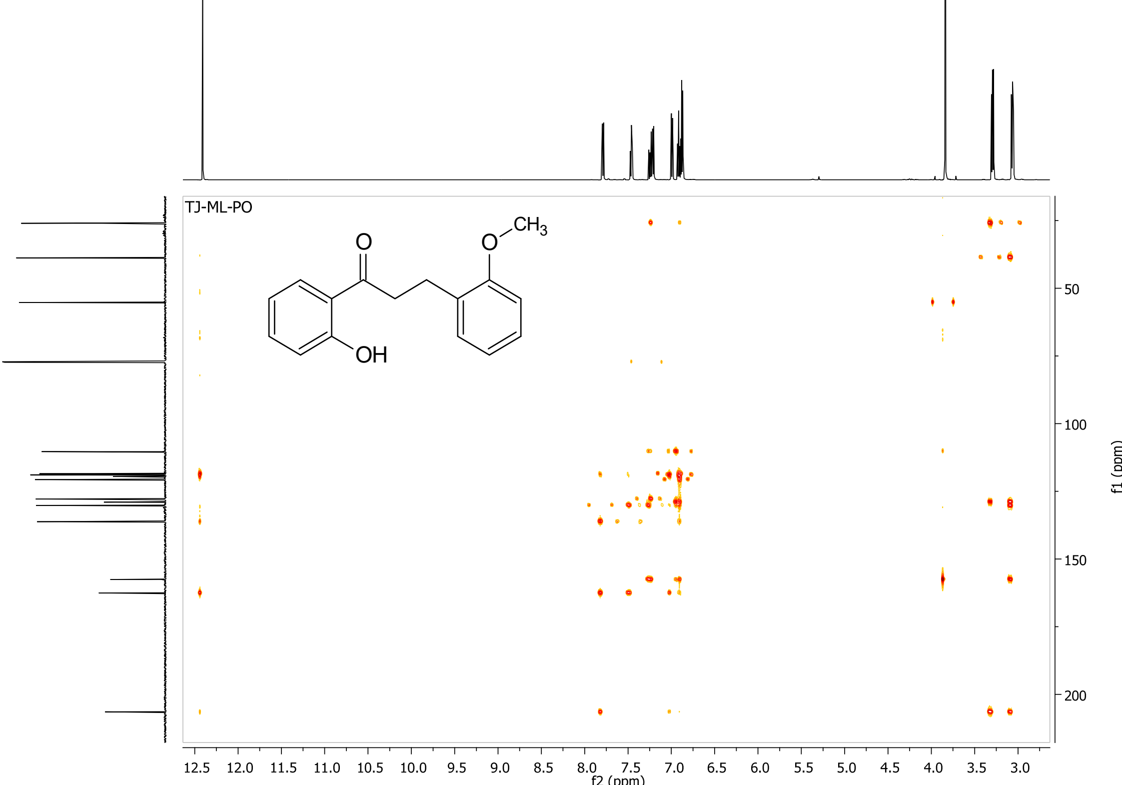

Figure S8. HMBC spectral of 1-(2′-hydroxyphenyl)-3-(2″-methoxyphenyl)-2-propan-1-one (**9**) ($CDCl_3$, 151 MHz).

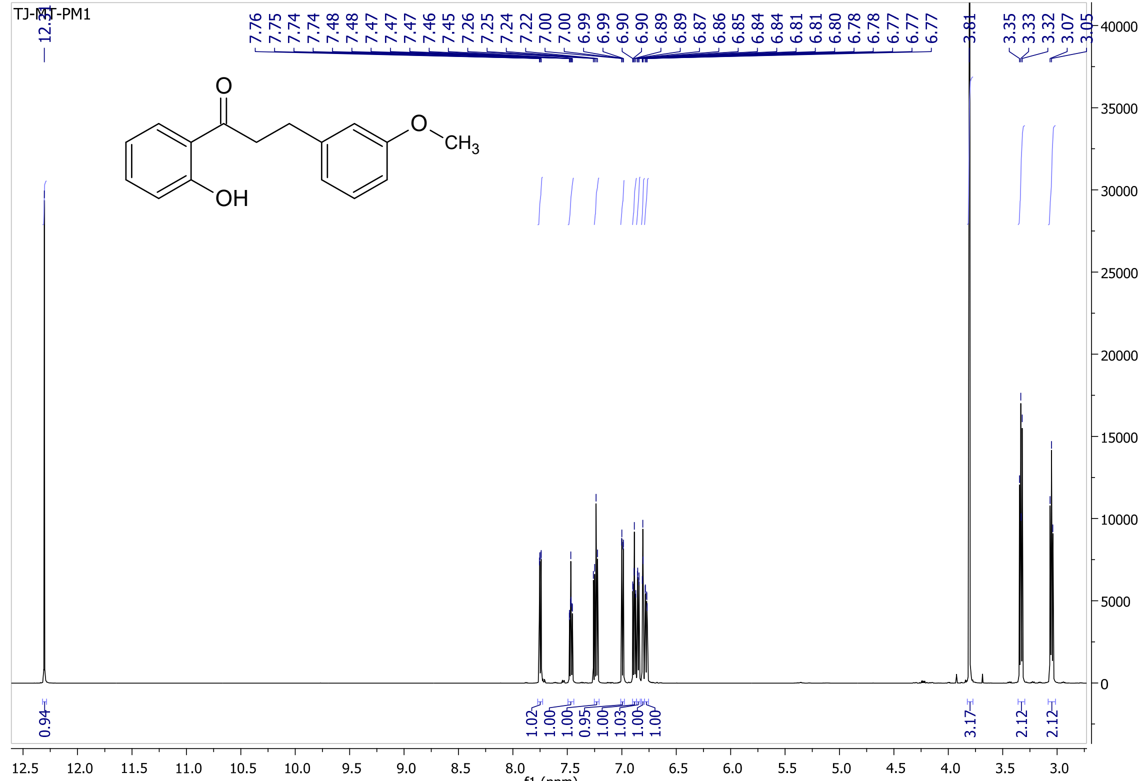

Figure S9. ^1H NMR spectral of 1-(2′-hydroxyphenyl)-3-(3″-methoxyphenyl)-2-propan-1-one (**10**) ($CDCl_3$, 600 MHz).

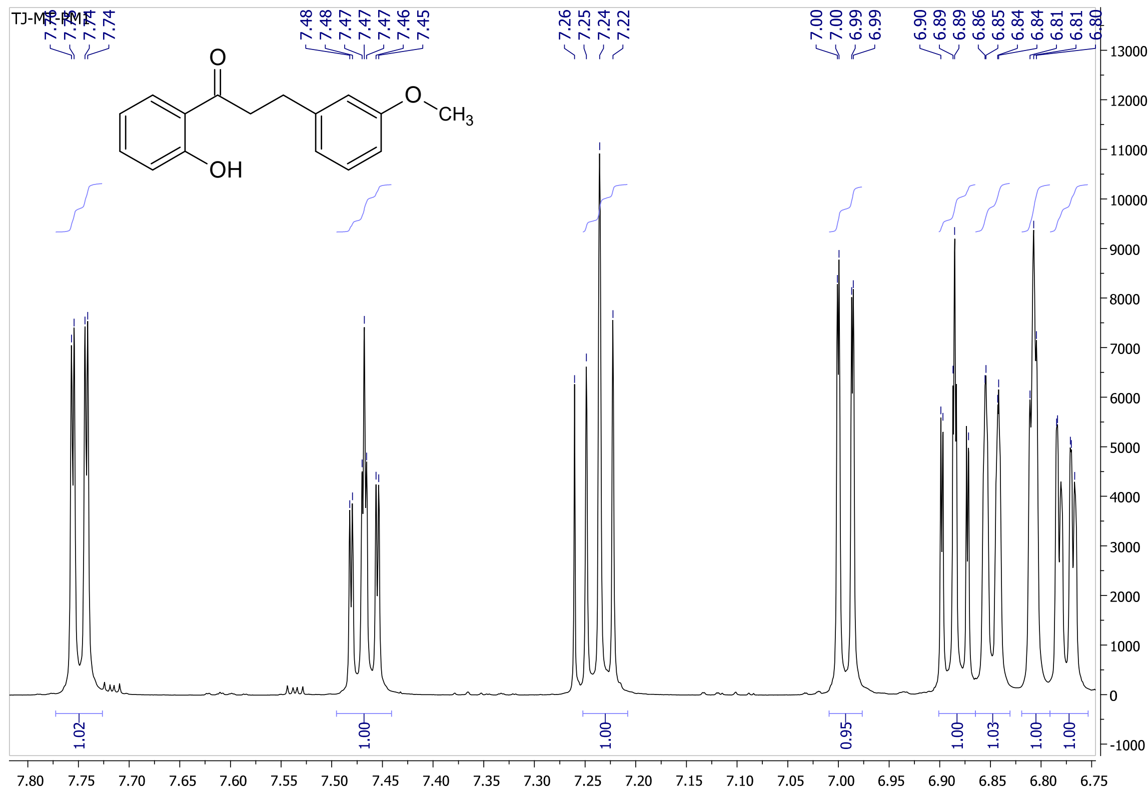

Figure S10. Part of the ^{13}C NMR spectral of 1-(2′-hydroxyphenyl)-3-(3″-methoxyphenyl)-2-propan-1-one (**10**) ($CDCl_3$, 600 MHz).

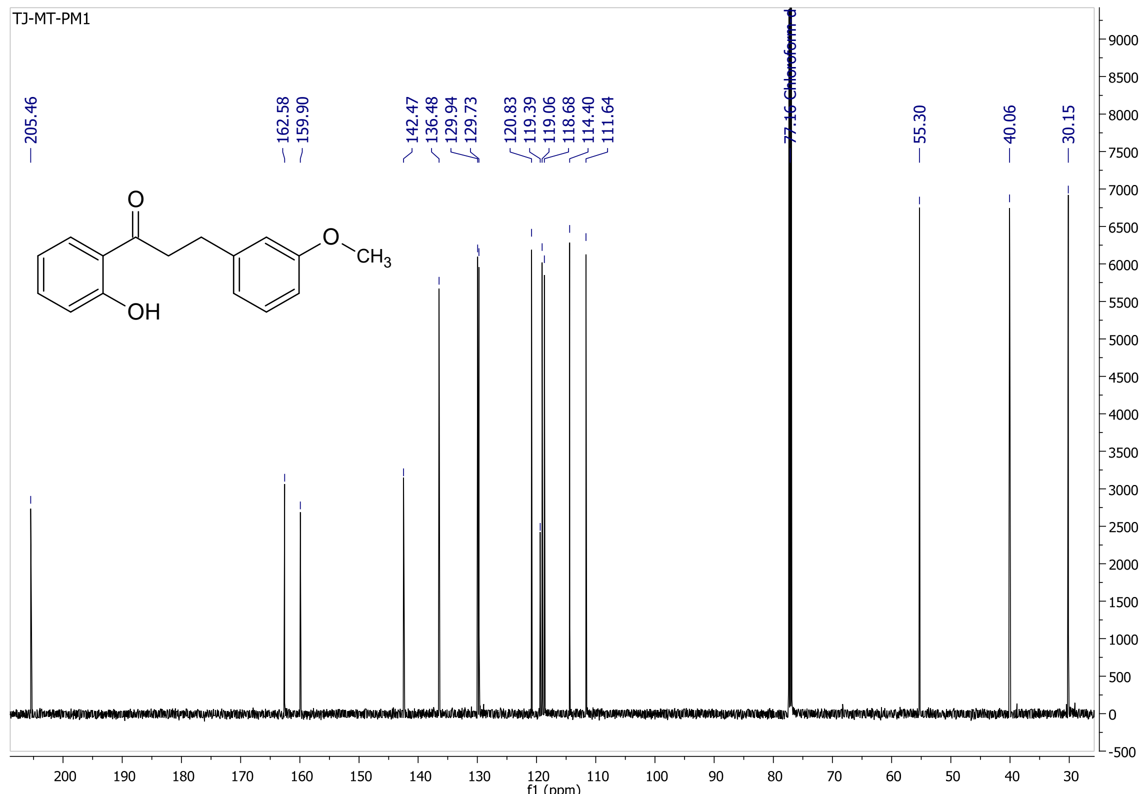

Figure S11. ^{13}C NMR spectral of 1-(2′-hydroxyphenyl)-3-(3″-methoxyphenyl)-2-propan-1-one (**10**) ($CDCl_3$, 151 MHz).

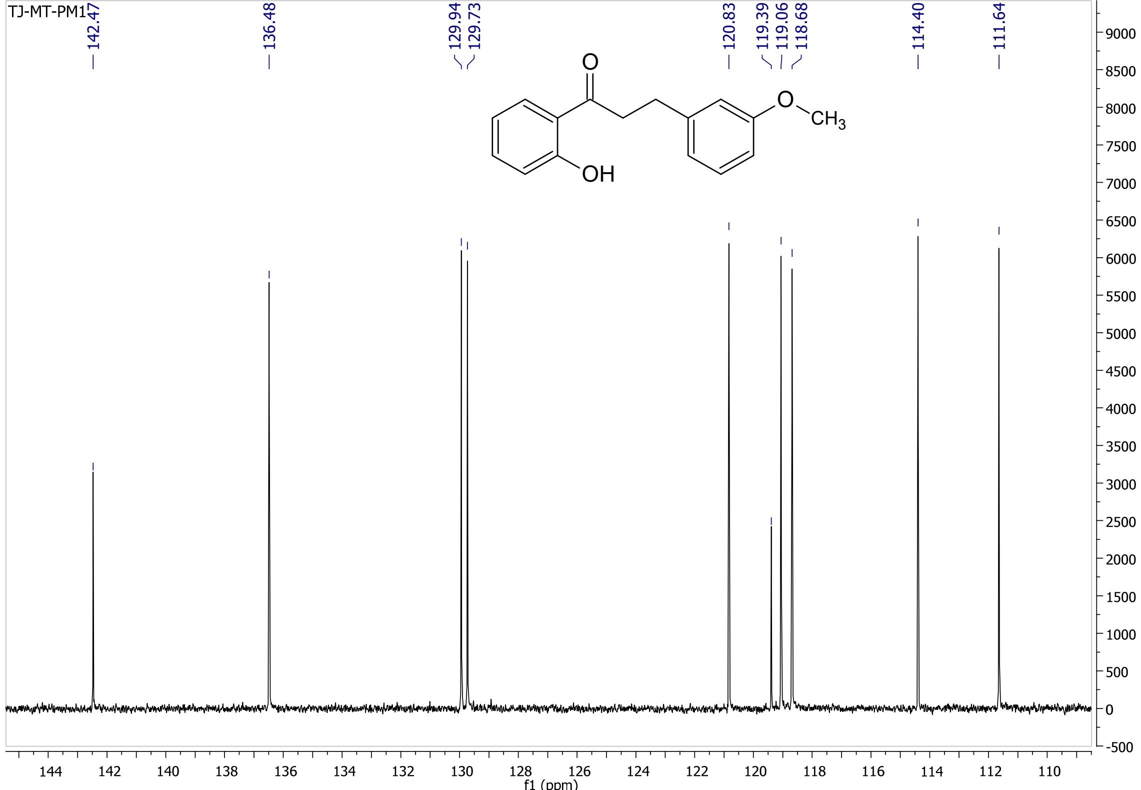

Figure S12. Part of the ^{13}C NMR spectral of 1-(2′-hydroxyphenyl)-3-(3″-methoxyphenyl)-2-propan-1-one (**10**) ($CDCl_3$, 151 MHz).

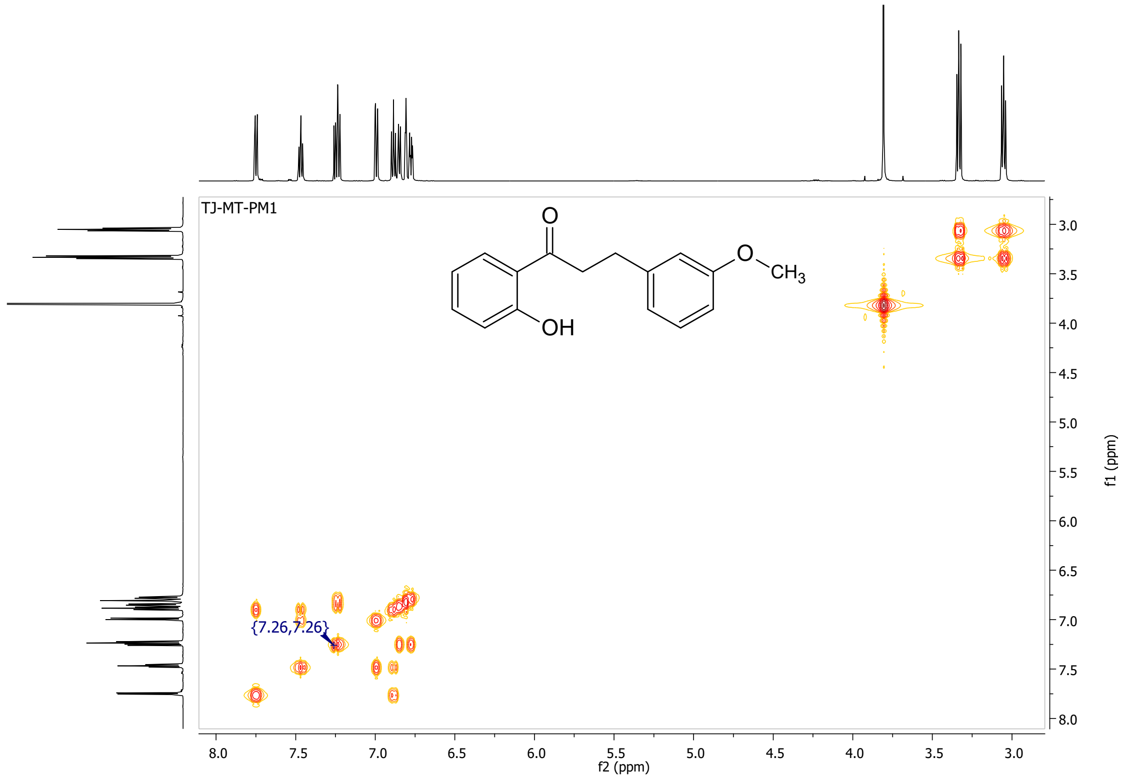

Figure S13. COSY spectral of 1-(2′-hydroxyphenyl)-3-(3″-methoxyphenyl)-2-propan-1-one (**10**) ($CDCl_3$, 151 MHz).

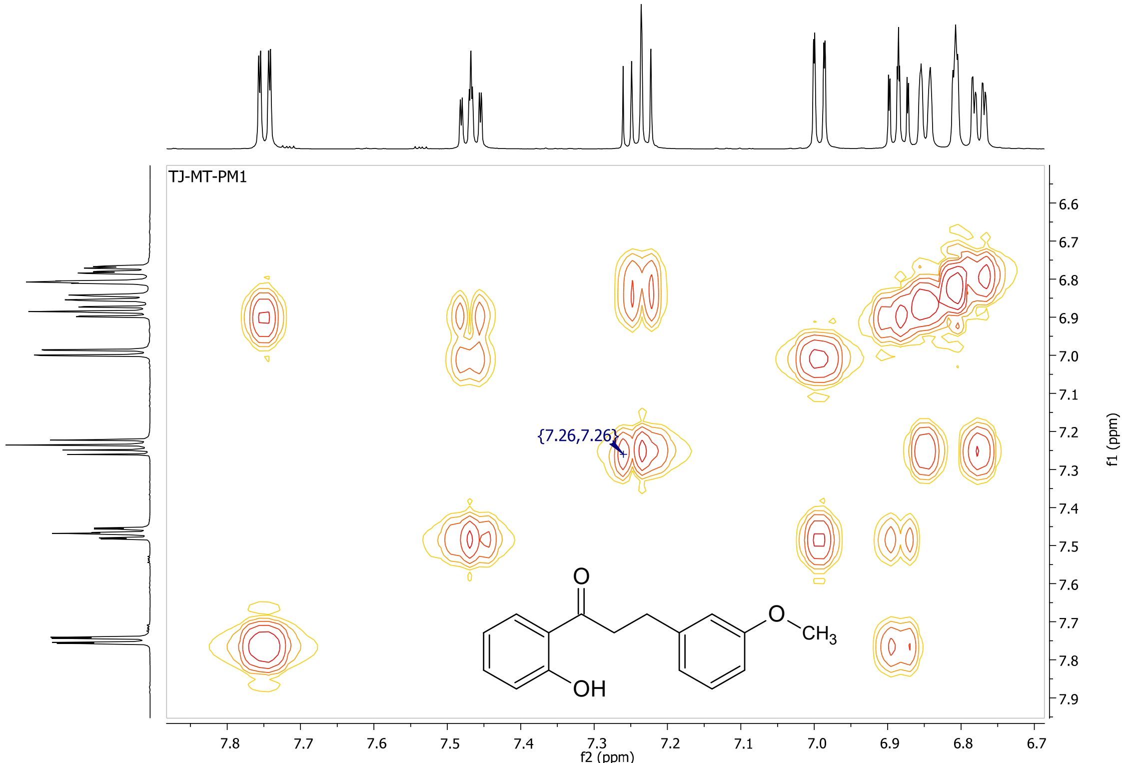

Figure S14. Part of the COSY spectral of 1-(2′-hydroxyphenyl)-3-(3″-methoxyphenyl)-2-propan-1-one (**10**) ($CDCl_3$, 151 MHz).

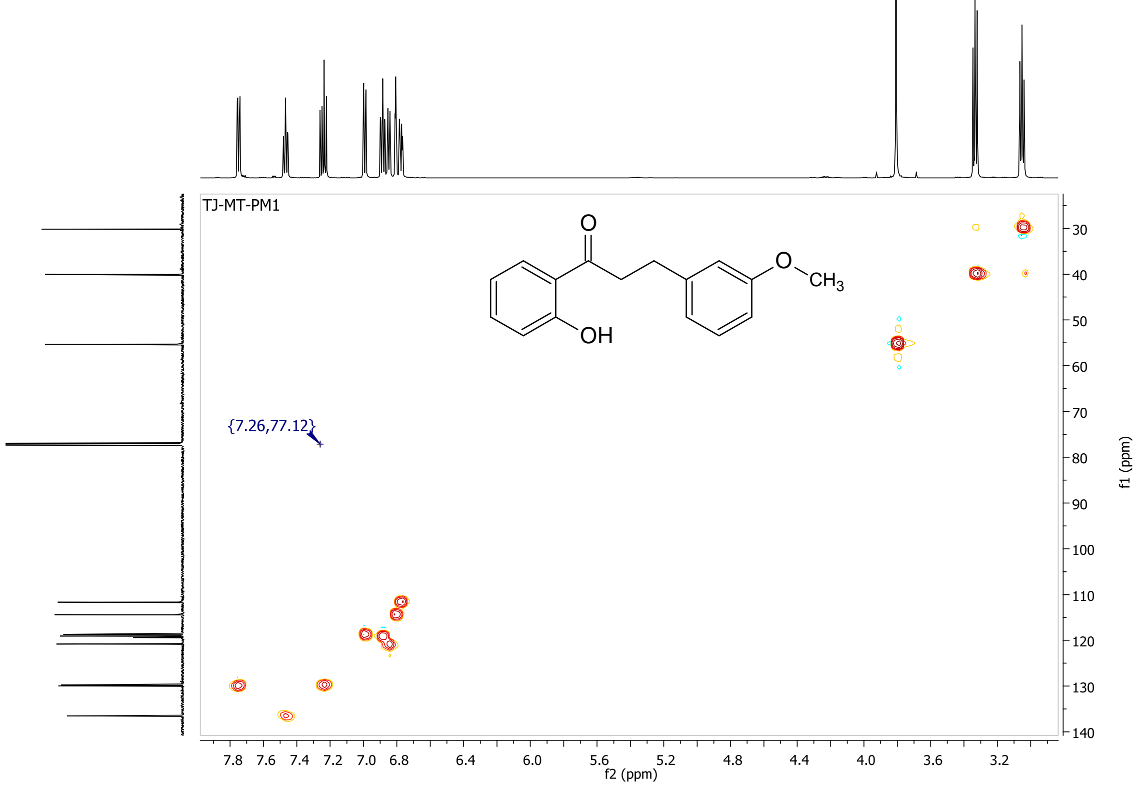

Figure S15. HSQC spectral of 1-(2′-hydroxyphenyl)-3-(3″-methoxyphenyl)-2-propan-1-one (**10**) ($CDCl_3$, 151 MHz).

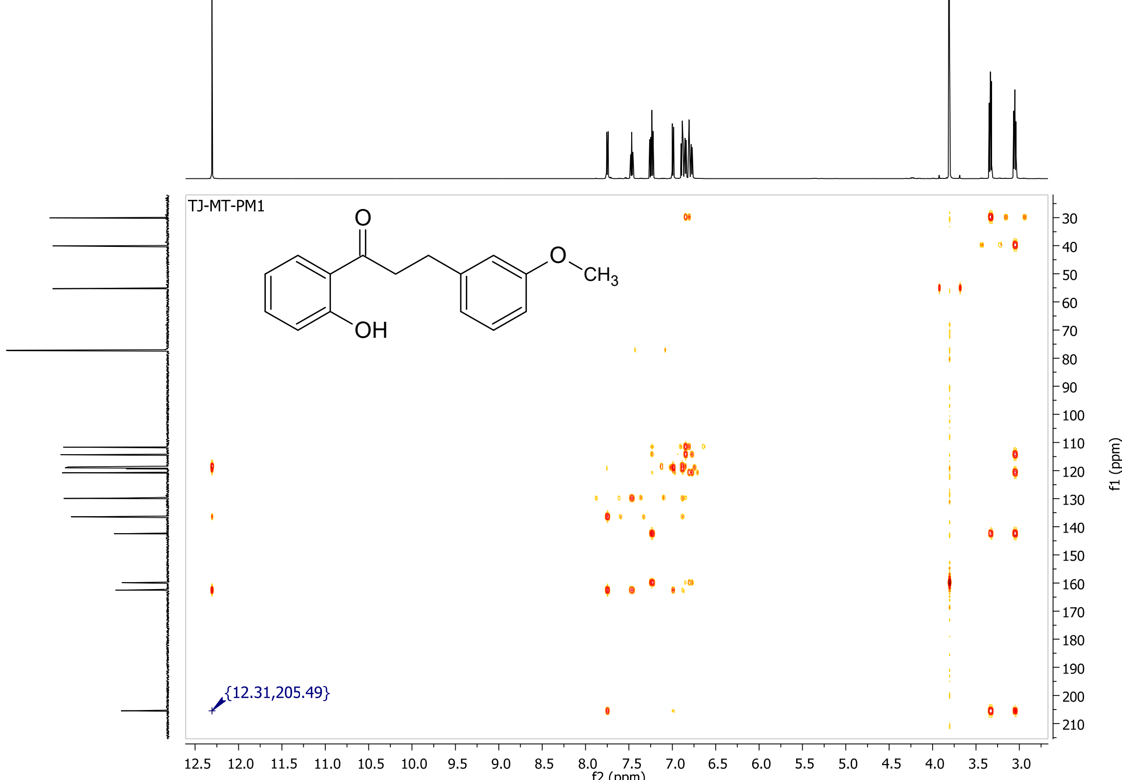

Figure S16. HMBC spectral of 1-(2′-hydroxyphenyl)-3-(3″-methoxyphenyl)-2-propan-1-one (**10**) ($CDCl_3$, 151 MHz).

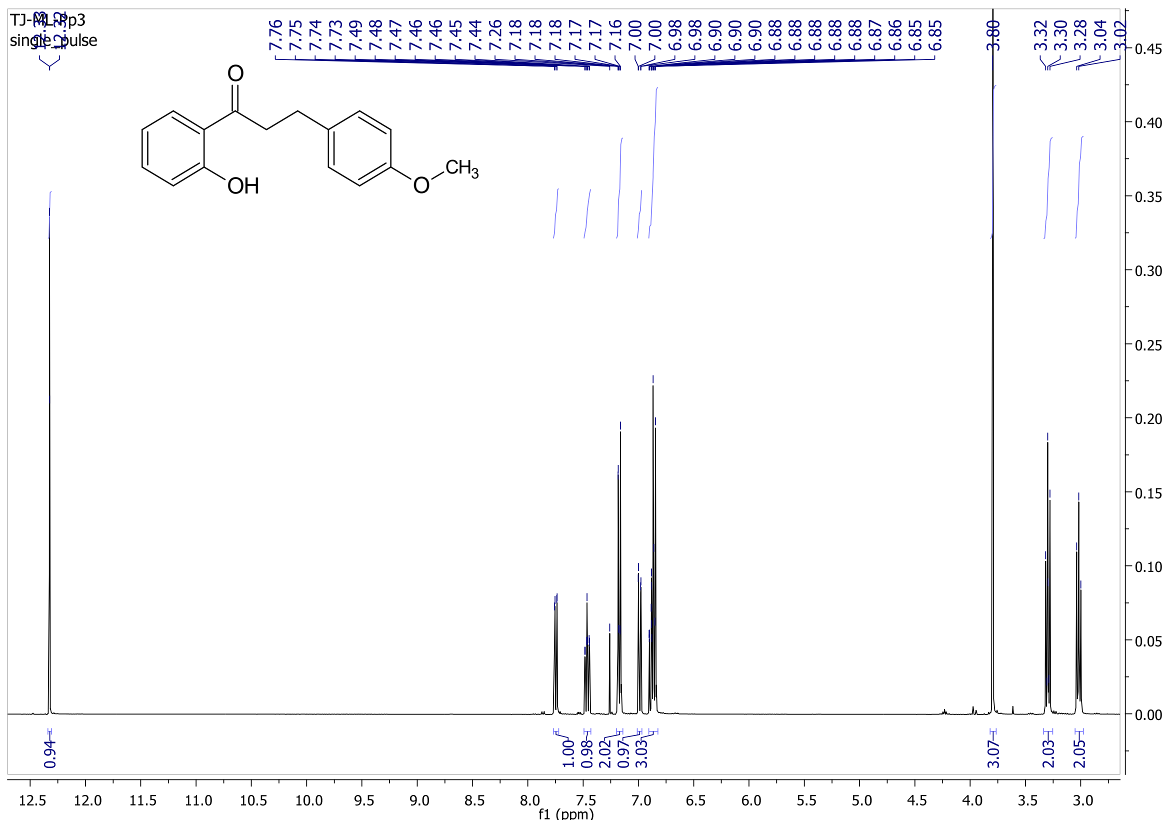

Figure S17. ^{1}H NMR spectral of 1-(2′-hydroxyphenyl)-3-(4″-methoxyphenyl)-2-propan-1-one (**11**) ($CDCl_3$, 600 MHz).

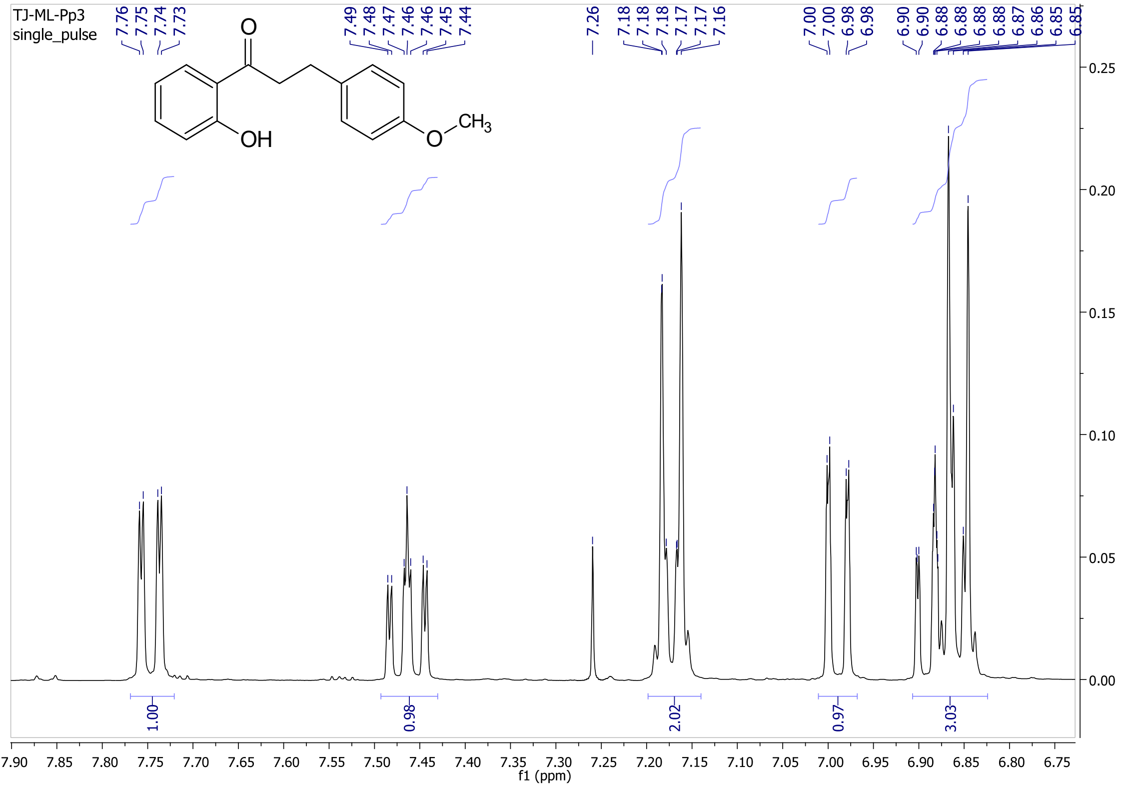

Figure S18. Part of the ^{13}C NMR spectral of 1-(2′-hydroxyphenyl)-3-(4″-methoxyphenyl)-2-propan-1-one (**11**) ($CDCl_3$, 600 MHz).

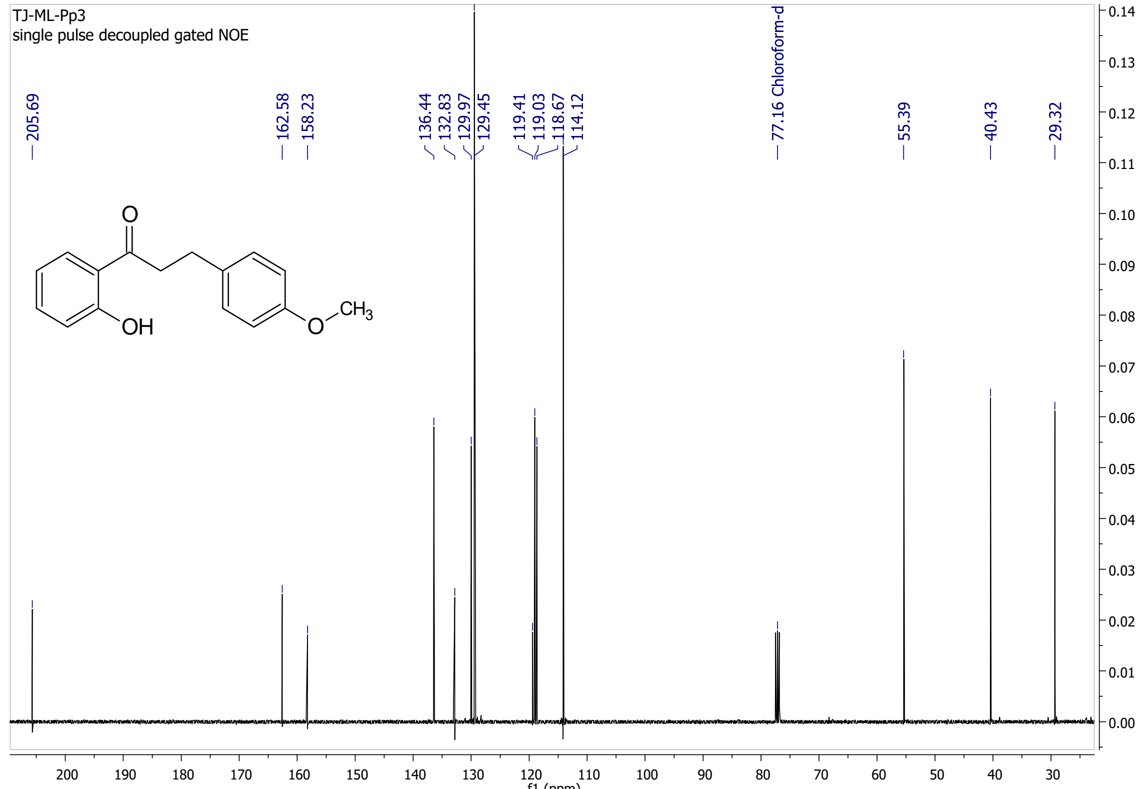

Figure S19. ^{13}C NMR spectral of 1-(2′-hydroxyphenyl)-3-(4″-methoxyphenyl)-2-propan-1-one (**11**) ($CDCl_3$, 151 MHz).

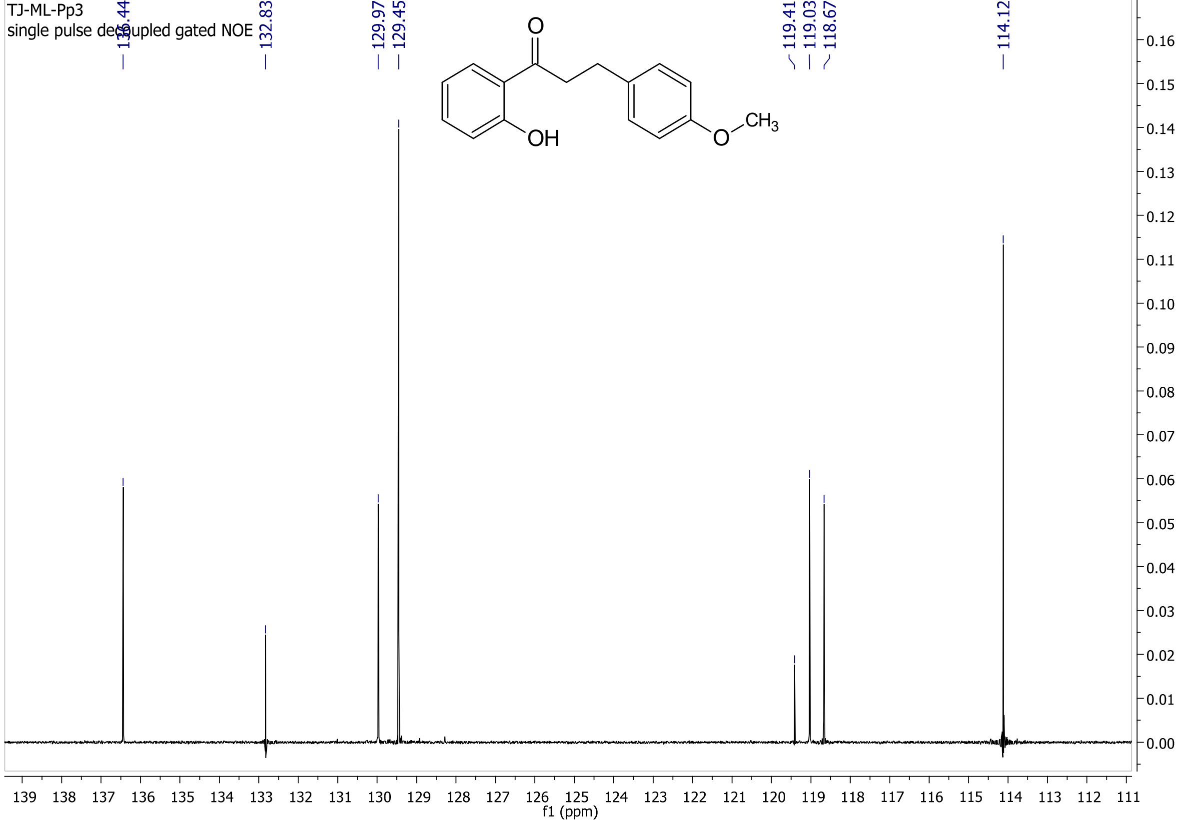

Figure S20. Part of the ^{13}C NMR spectral of 1-(2′-hydroxyphenyl)-3-(4″-methoxyphenyl)-2-propan-1-one (**11**) ($CDCl_3$, 151 MHz).

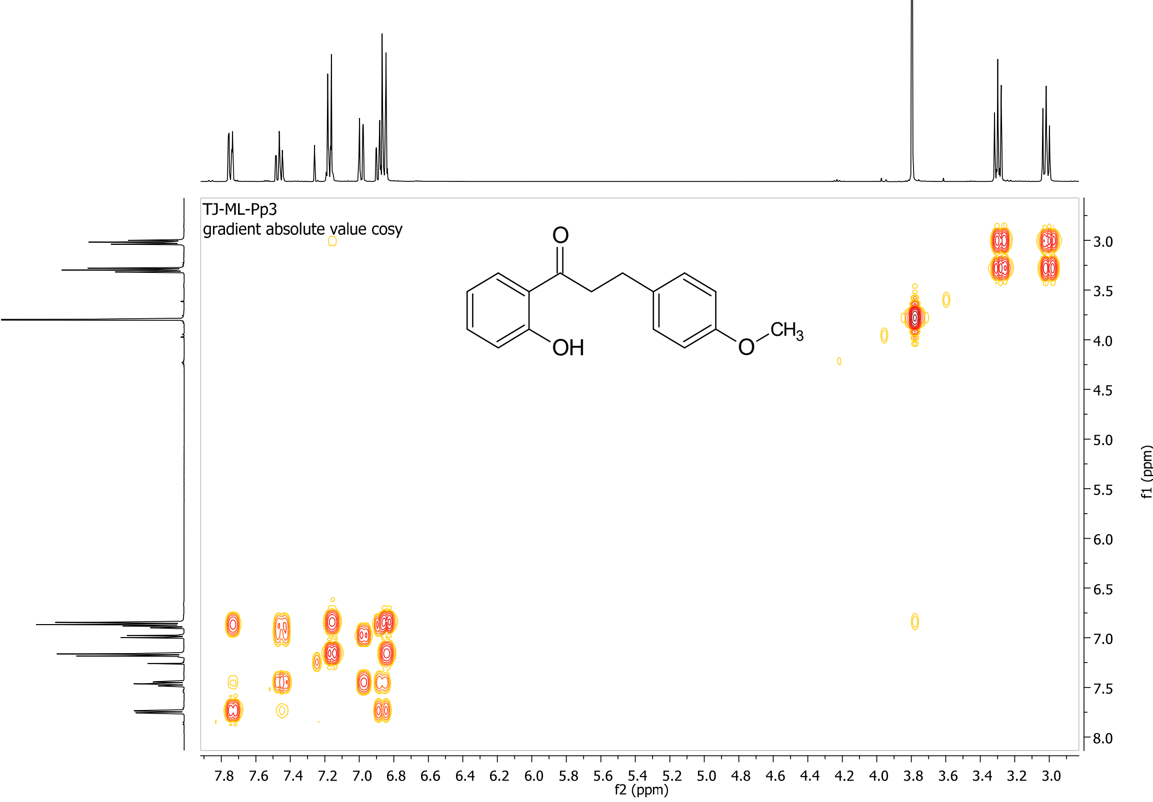

Figure S21. COSY spectral of 1-(2′-hydroxyphenyl)-3-(4″-methoxyphenyl)-2-propan-1-one (**11**) ($CDCl_3$, 151 MHz).

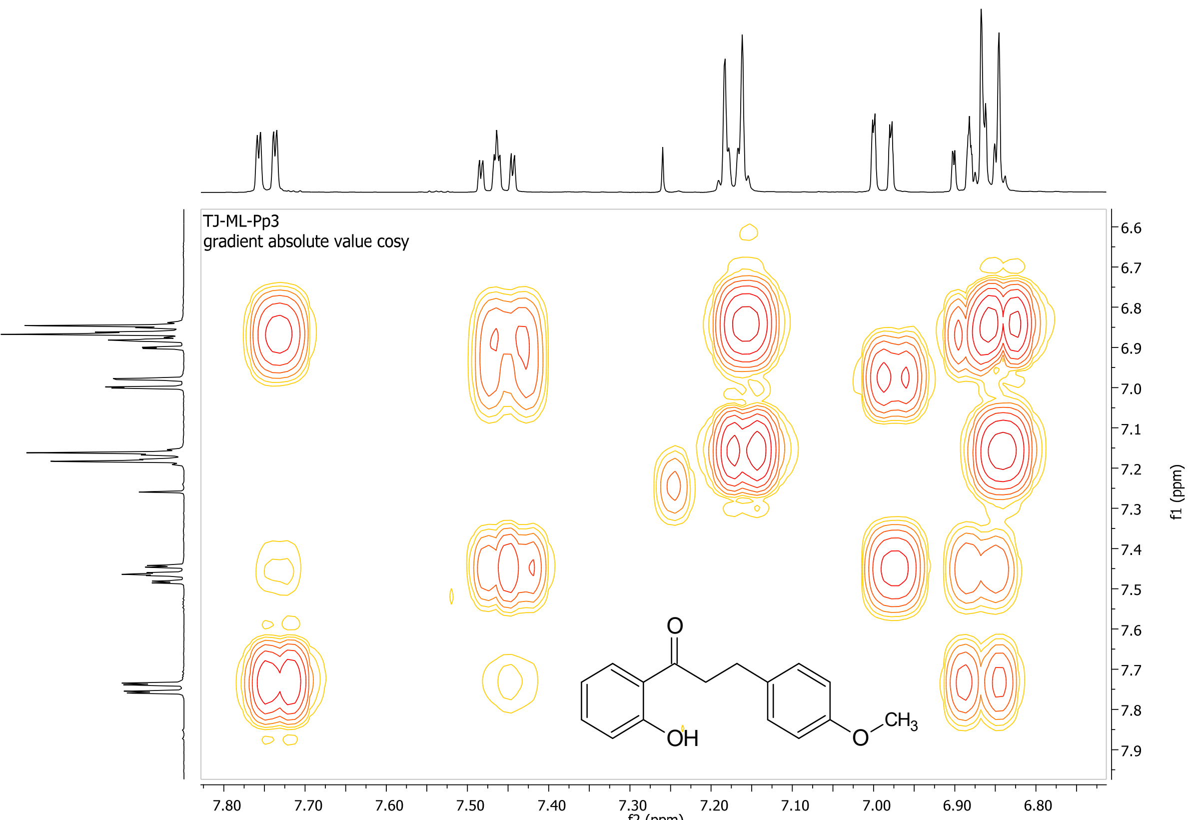

Figure S22. Part of the COSY spectral of 1-(2′-hydroxyphenyl)-3-(4″-methoxyphenyl)-2-propan-1-one (**11**) ($CDCl_3$, 151 MHz).

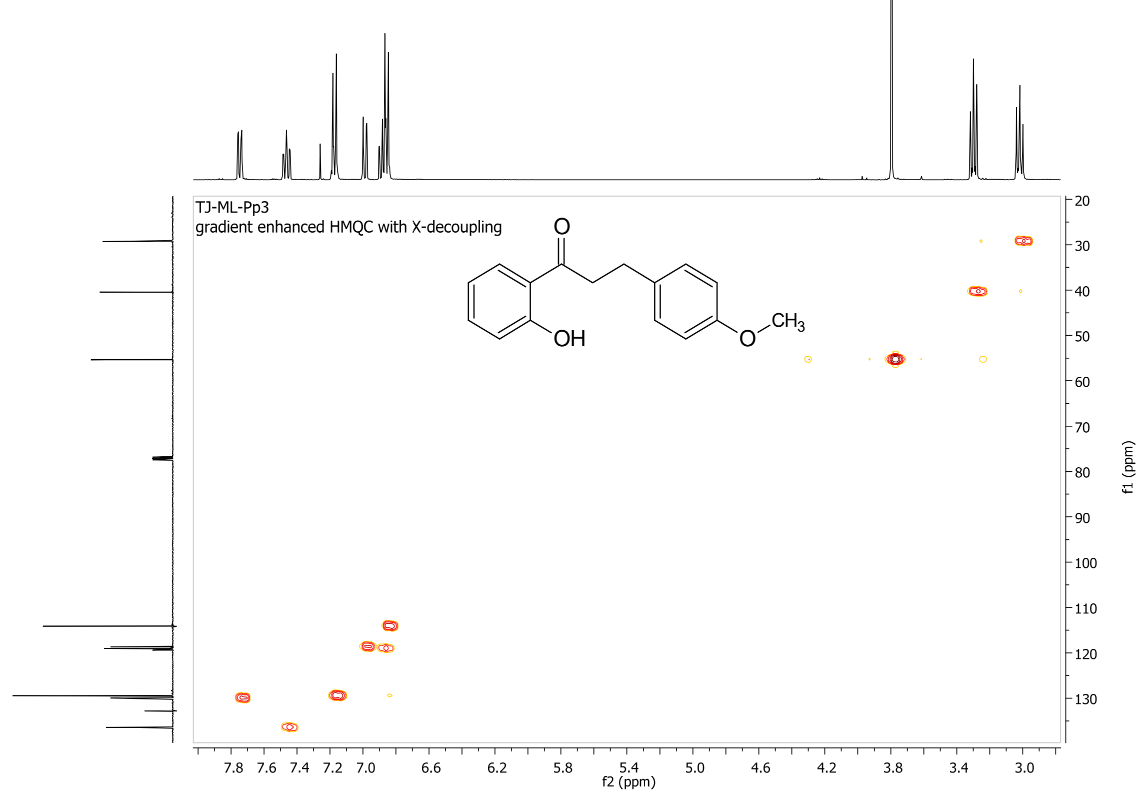

Figure S23. HSQC spectral of 1-(2′-hydroxyphenyl)-3-(4″-methoxyphenyl)-2-propan-1-one (**11**) ($CDCl_3$, 151 MHz).

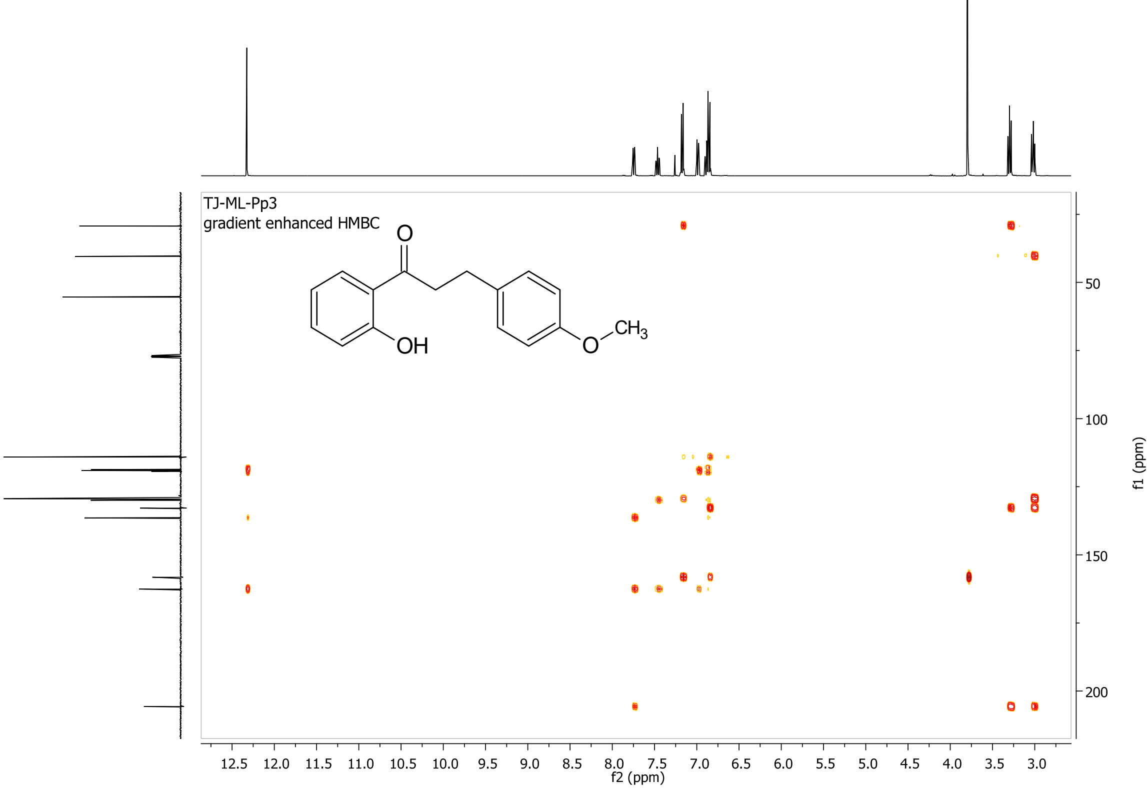

Figure S24. HMBC spectral of 1-(2′-hydroxyphenyl)-3-(4″-methoxyphenyl)-2-propan-1-one (**11**) ($CDCl_3$, 151 MHz).

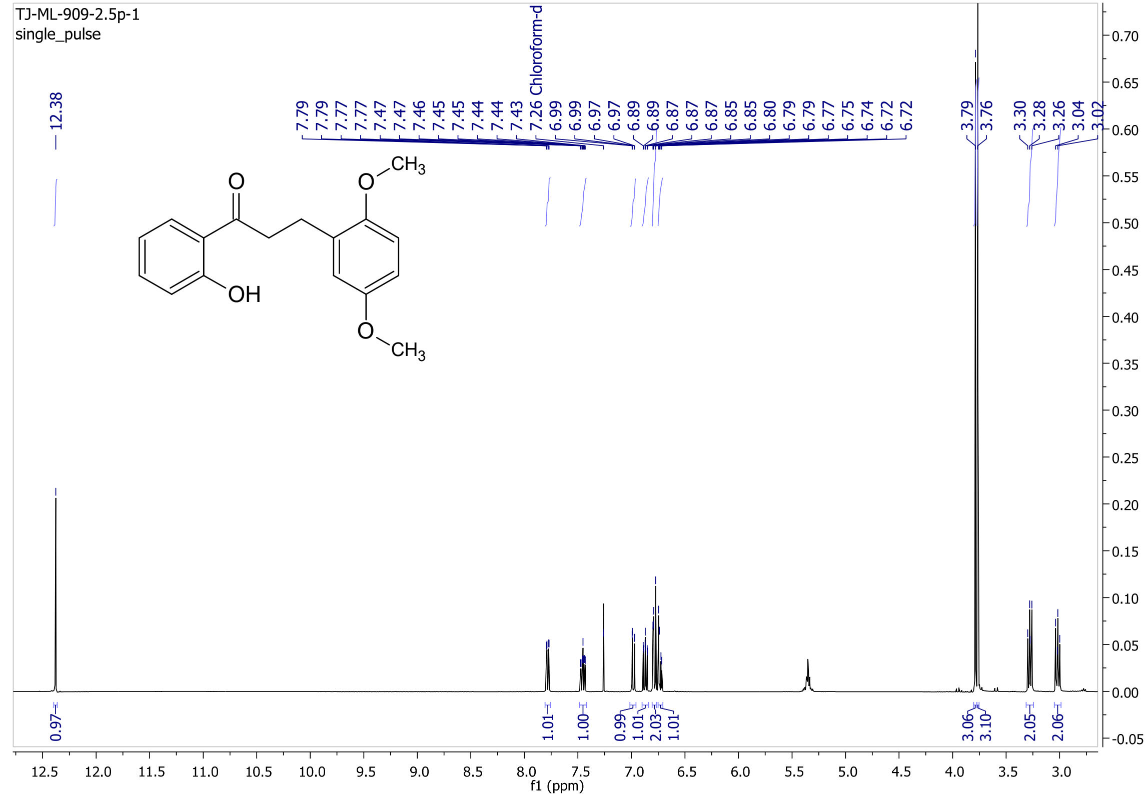

Figure S25. ^{1}H NMR spectral of 1-(2′-hydroxyphenyl)-3-(2″,5″-dimethoxyphenyl)-2-propan-1-one (**12**) ($CDCl_3$, 600 MHz).

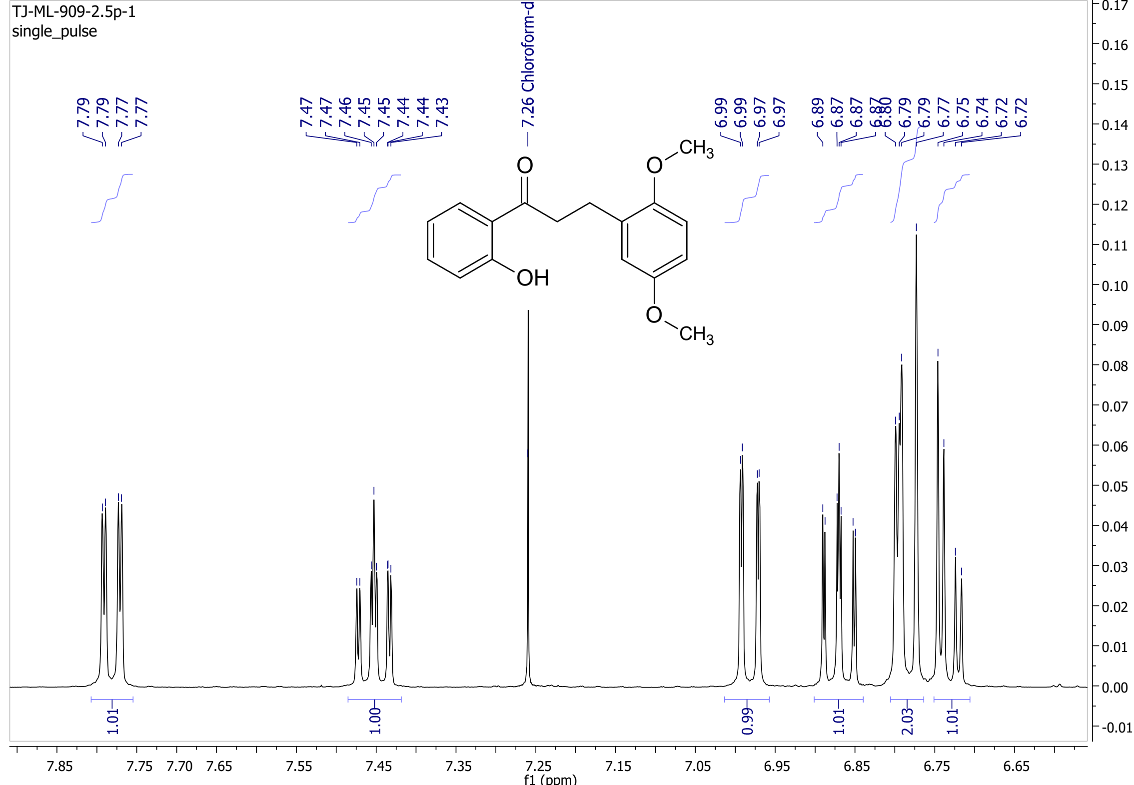

Figure S26. Part of the ^{13}C NMR spectral of 1-(2′-hydroxyphenyl)-3-(2″,5″-dimethoxyphenyl)-2-propan-1-one (**12**) ($CDCl_3$, 600 MHz).

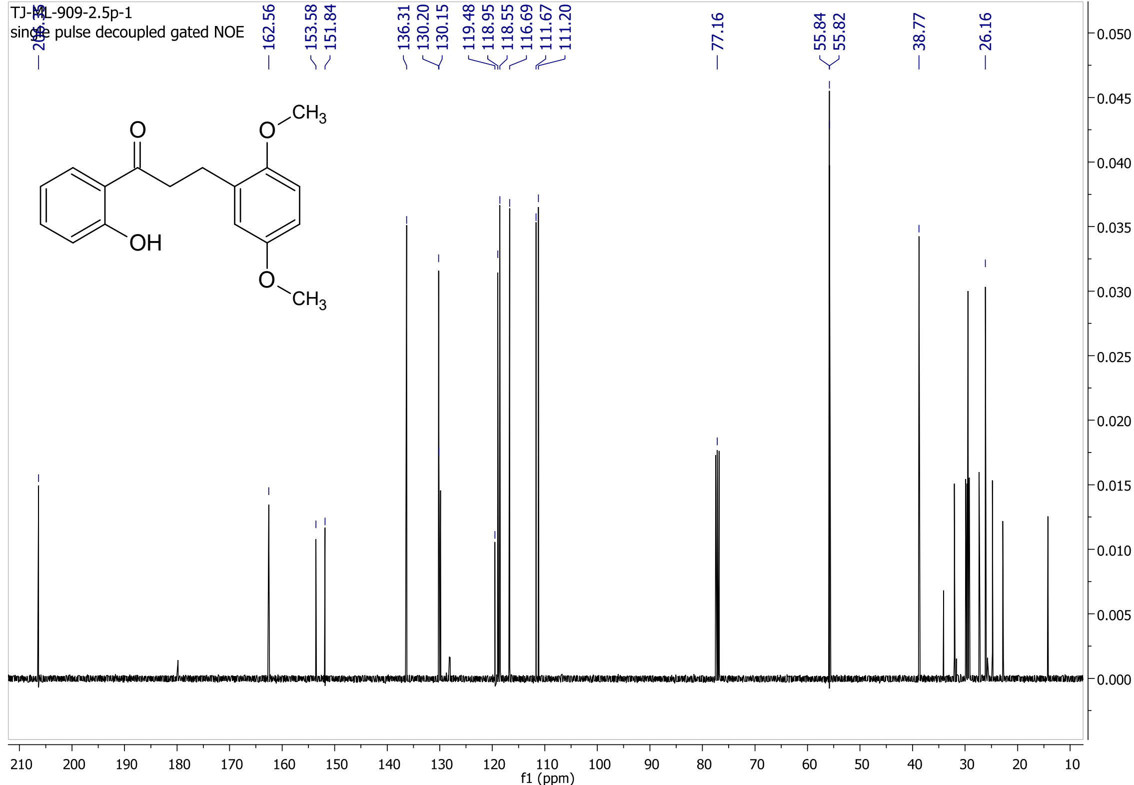

Figure S27. ^{13}C NMR spectral of 1-(2′-hydroxyphenyl)-3-(2″,5″-dimethoxyphenyl)-2-propan-1-one (**12**) ($CDCl_3$, 151 MHz).

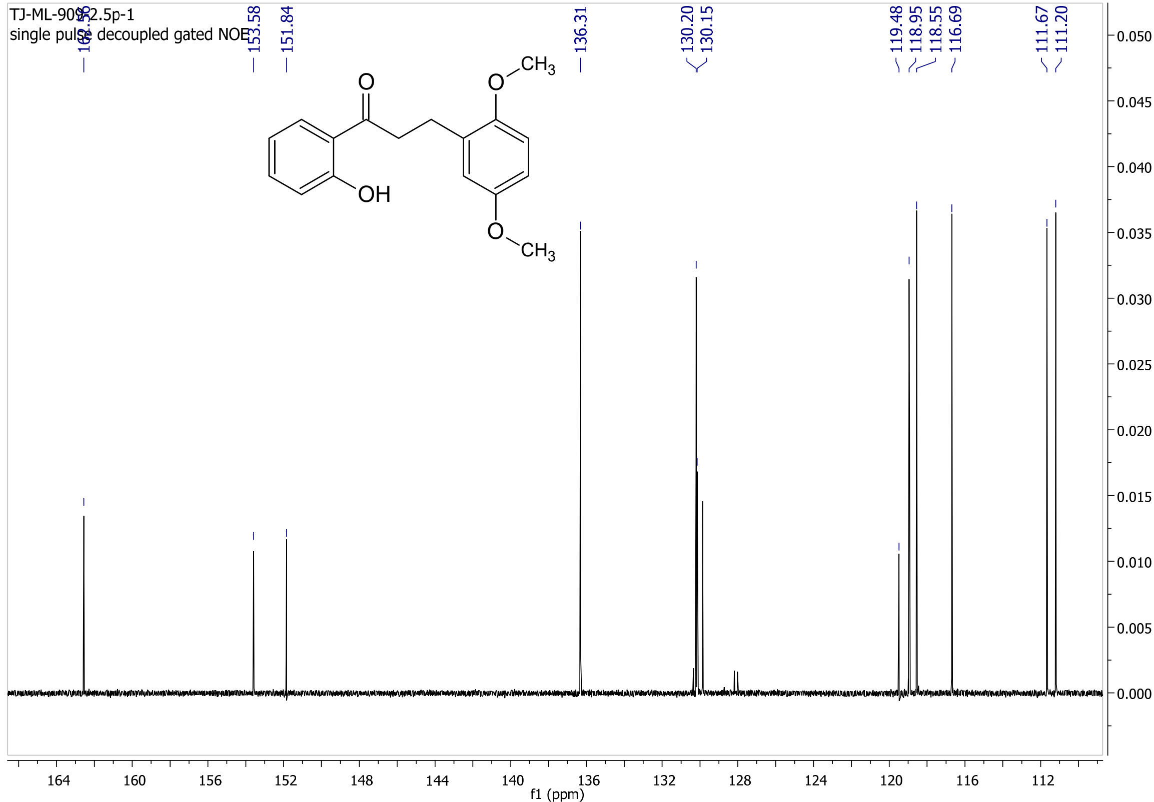

Figure S28. Part of the ^{13}C NMR spectral of 1-(2′-hydroxyphenyl)-3-(2″,5″-dimethoxyphenyl)-2-propan-1-one (**12**) ($CDCl_3$, 151 MHz).

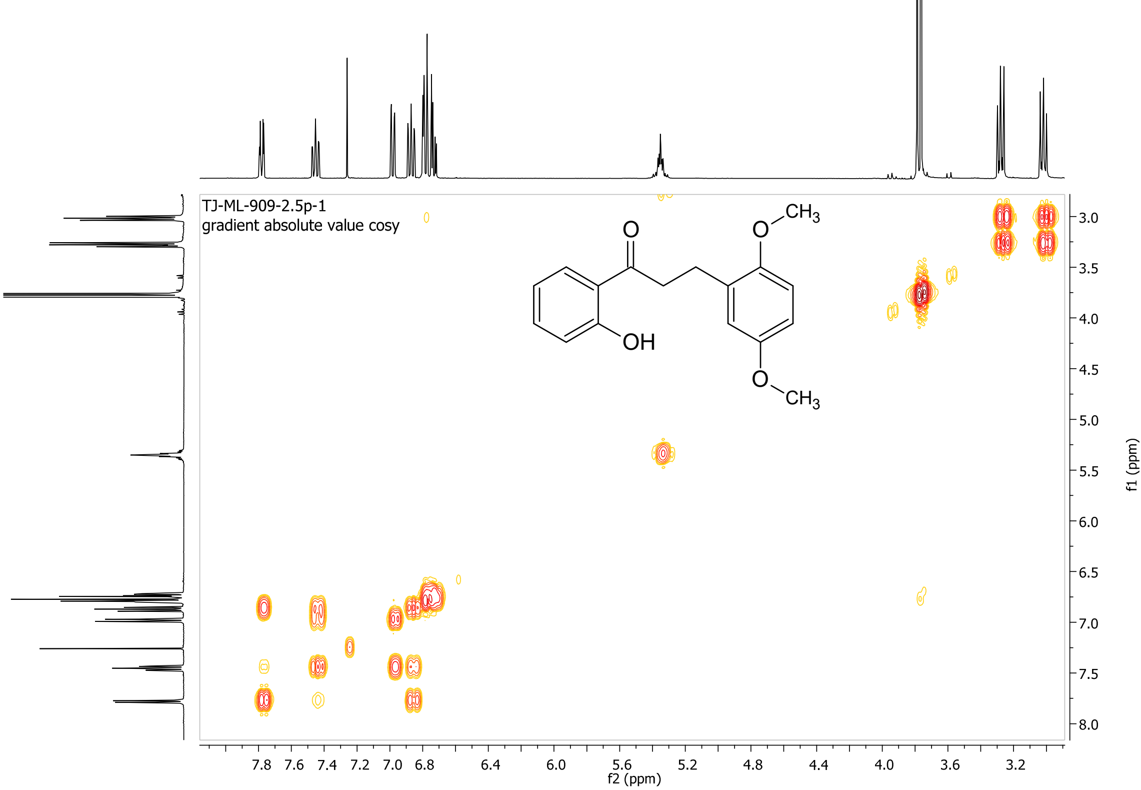

Figure S29. COSY spectral of 1-(2′-hydroxyphenyl)-3-(2″,5″-dimethoxyphenyl)-2-propan-1-one (**12**) ($CDCl_3$, 151 MHz).

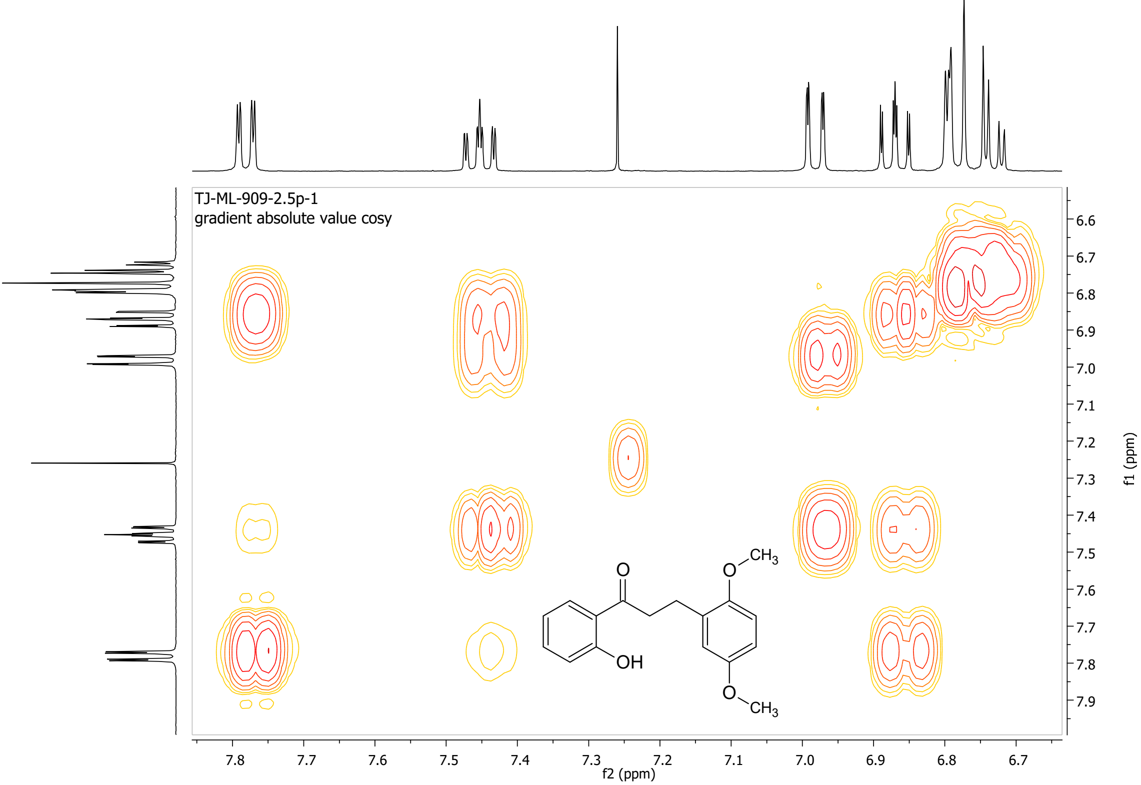

Figure S30. Part of the COSY spectral of 1-(2′-hydroxyphenyl)-3-(2″,5″-dimethoxyphenyl)-2-propan-1-one (**12**) ($CDCl_3$, 151 MHz).

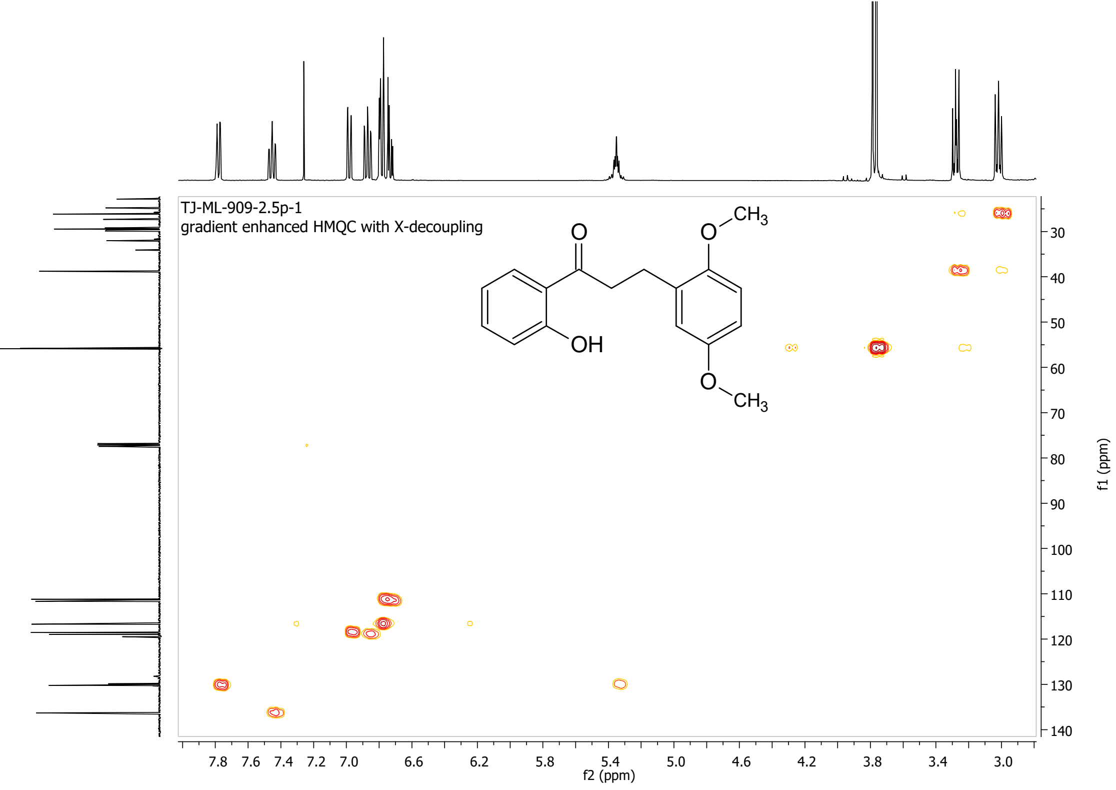

Figure S31. HSQC spectral of 1-(2′-hydroxyphenyl)-3-(2″,5″-dimethoxyphenyl)-2-propan-1-one (**12**) ($CDCl_3$, 151 MHz).

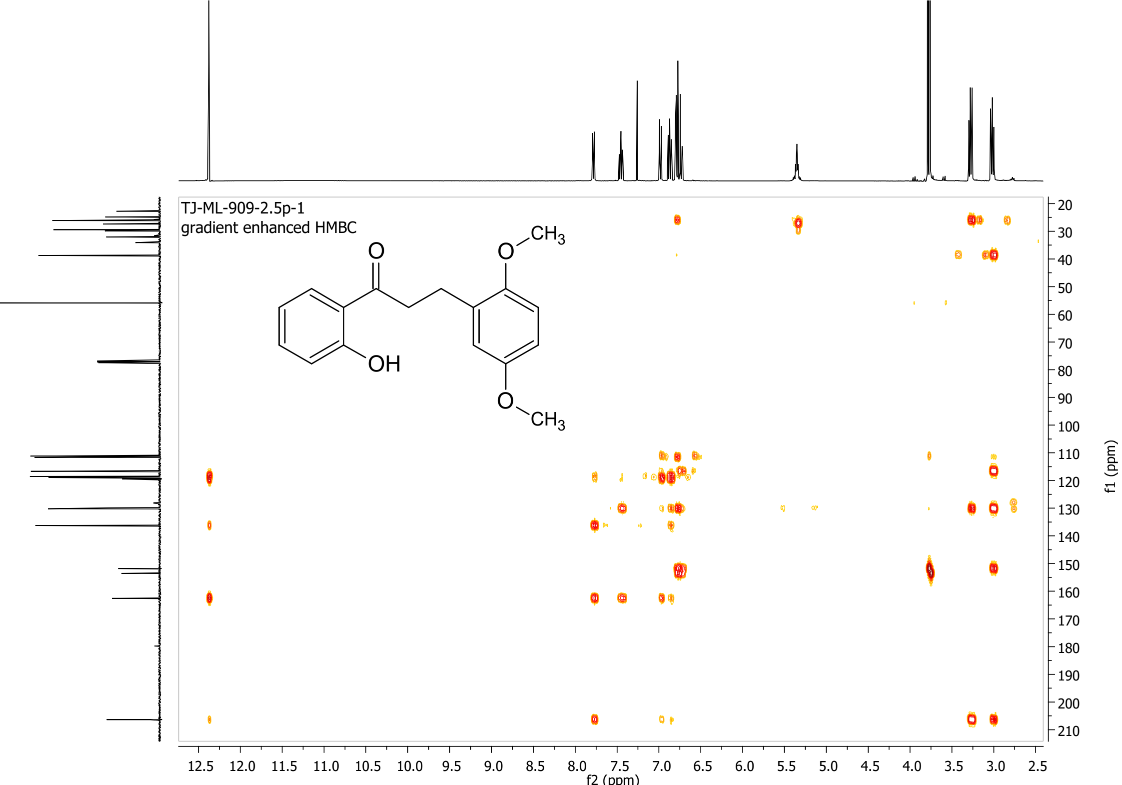

Figure S32. HMBC spectral of 1-(2′-hydroxyphenyl)-3-(2″,5″-dimethoxyphenyl)-2-propan-1-one (**12**) ($CDCl_3$, 151 MHz).

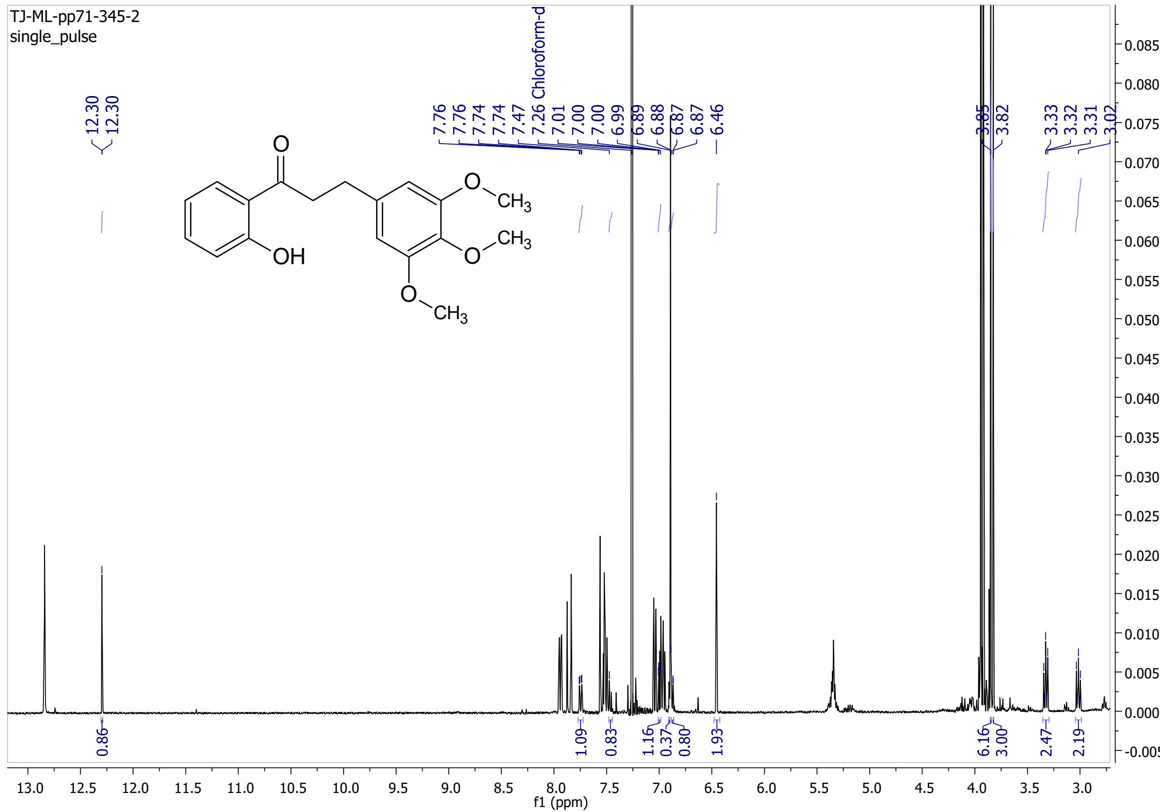

Figure S33. ^{1}H NMR spectral of 1-(2′-hydroxyphenyl)-3-(3″,4″,5″-trimethoxyphenyl)-2-propan-1-one (**13**) ($CDCl_3$, 600 MHz).

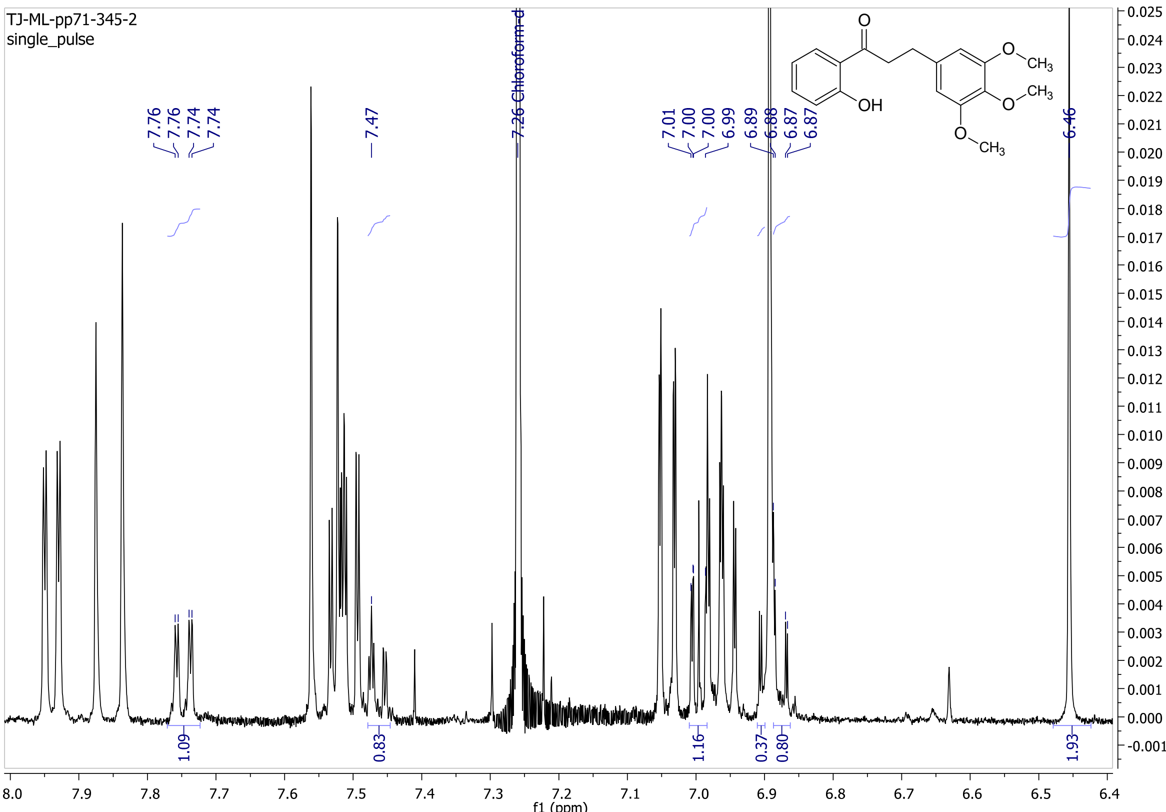

Figure S34. Part of the ^{1}H NMR spectral of 1-(2′-hydroxyphenyl)-3-(3″,4″,5″-trimethoxyphenyl)-2-propan-1-one (**13**) ($CDCl_3$, 600 MHz).

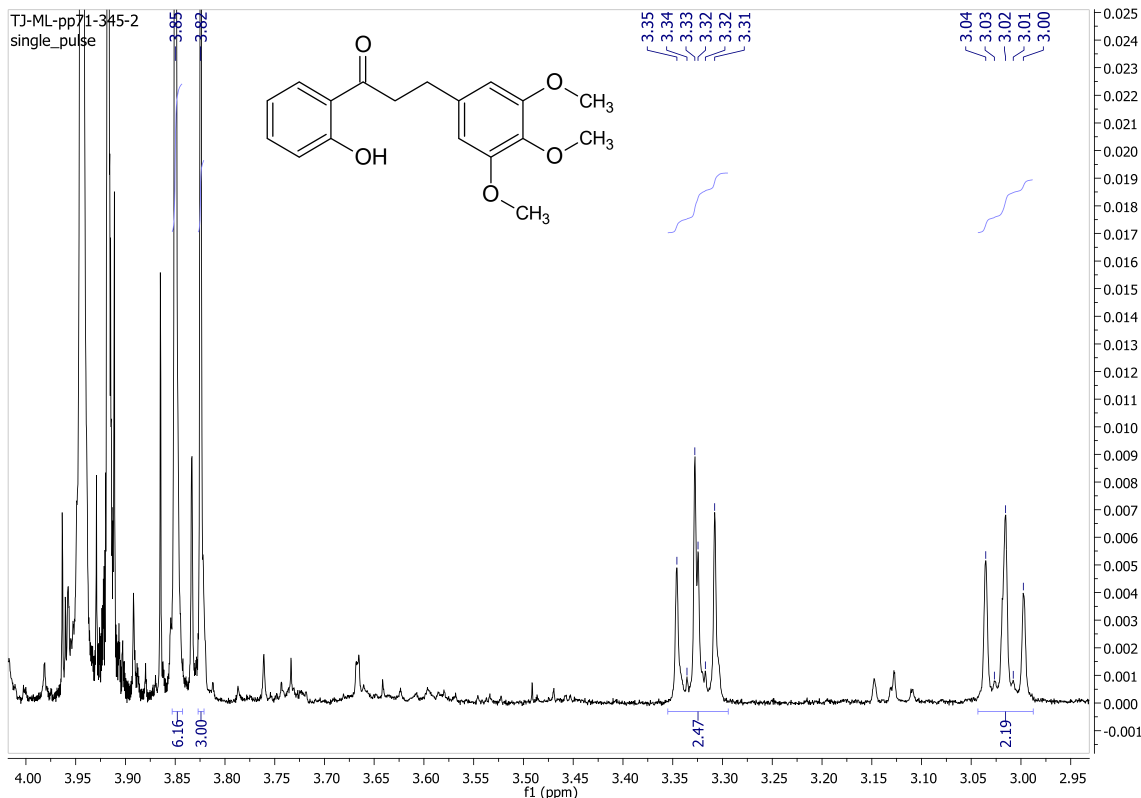

Figure S35. Part of the ^{1}H NMR spectral of 1-(2′-hydroxyphenyl)-3-(3″,4″,5″-trimethoxyphenyl)-2-propan-1-one (**13**) ($CDCl_3$, 600 MHz).

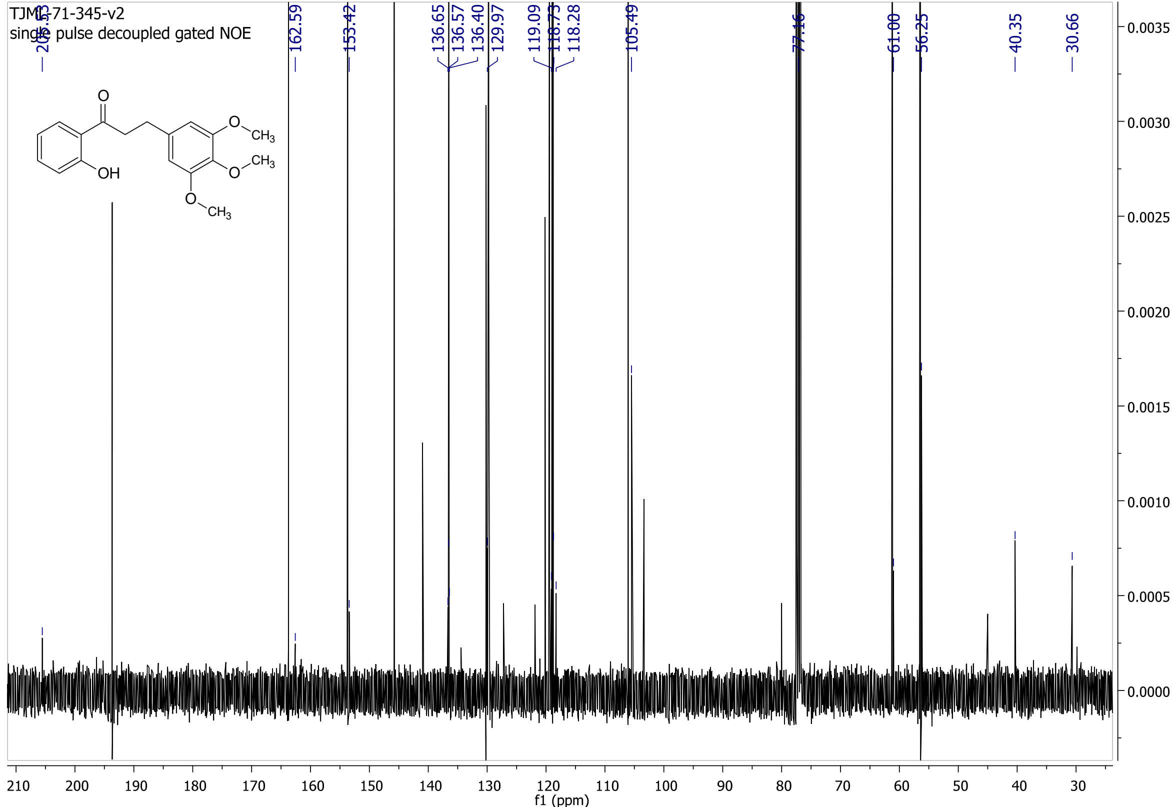

Figure S36. ^{13}C NMR spectral of 1-(2′-hydroxyphenyl)-3-(3″,4″,5″-trimethoxyphenyl)-2-propan-1-one (**13**) ($CDCl_3$, 151 MHz).

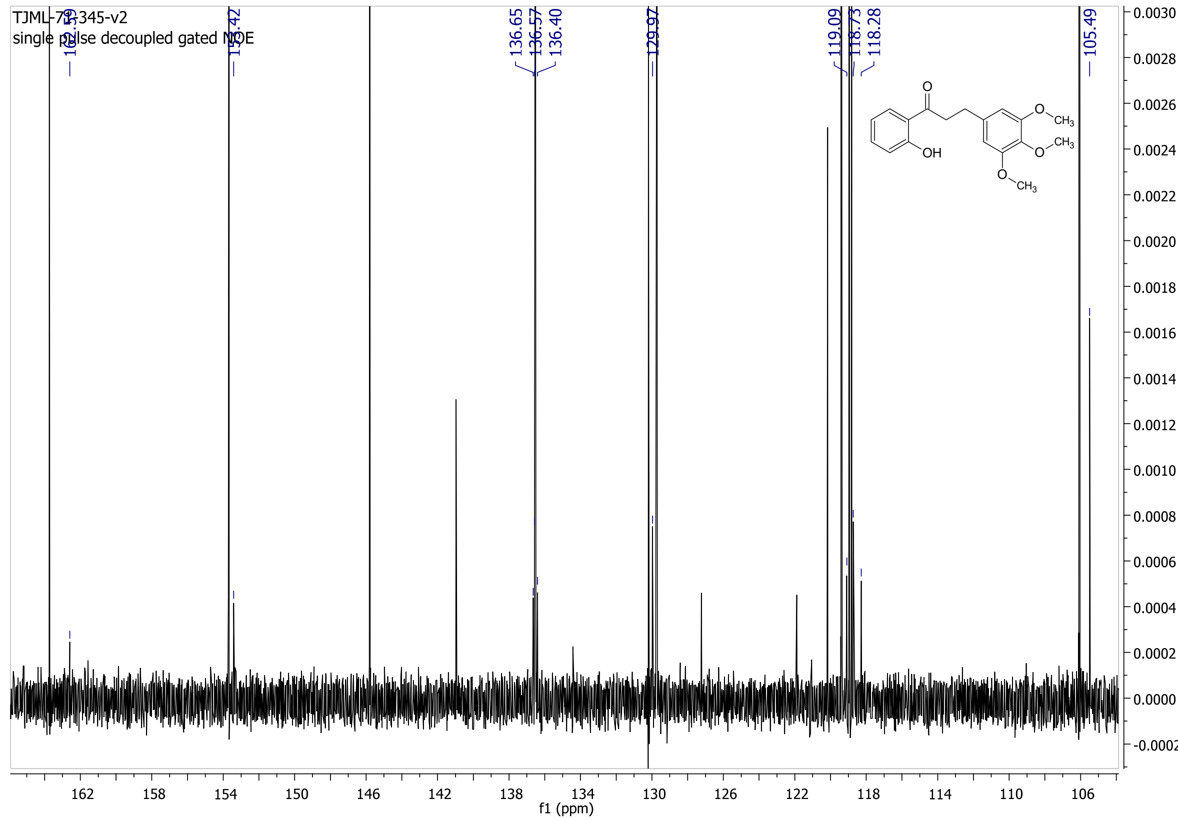

Figure S37. Part of the ^{13}C NMR spectral of 1-(2′-hydroxyphenyl)-3-(3″,4″,5″-trimethoxyphenyl)-2-propan-1-one (**13**) ($CDCl_3$, 600 MHz).

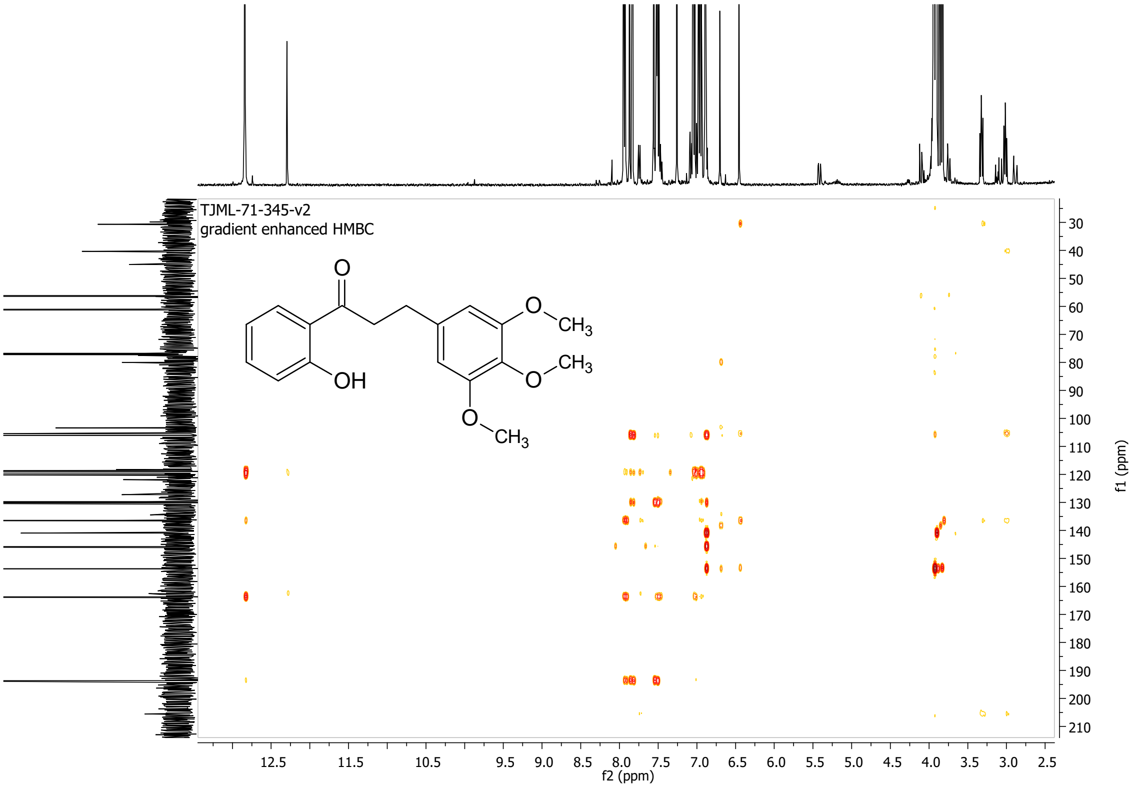

Figure S38. HMBC spectral of 1-(2′-hydroxyphenyl)-3-(3″,4″,5″-trimethoxyphenyl)-2-propan-1-one (**13**) ($CDCl_3$, 151 MHz).

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Łużny, M.*; Tronina, T.; Kozłowska, E.; Dymarska, M.; Popłoński, J.; Łyczko, J.; Kostrzewa-Susłow, E.; Janeczko, T.*

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Article

Biotransformation of Methoxyflavones by Selected Entomopathogenic Filamentous Fungi

Mateusz Łużny *, Tomasz Tronina, Ewa Kozłowska, Monika Dymarska, Jarosław Popłoński, Jacek Łyczko, Edyta Kostrzewa-Susłow and Tomasz Janeczko *

Department of Chemistry, Wrocław University of Environmental and Life Sciences, Norwida 25, 50-375 Wrocław, Poland; tomasz.tronina@upwr.edu.pl (T.T.); e.a.kozlowska@gmail.com (E.K.); monika.dymarska@gmail.com (M.D.); jaroslaw.poplonski@upwr.edu.pl (J.P.); jacek.lyczko@upwr.edu.pl (J.Ł.); ekostrzew@gmail.com (E.K.-S.)

* Correspondence: mat.luzny@gmail.com (M.Ł.); janeczko13@interia.pl (T.J.); Tel.: +48-713-205-195 (T.J.)

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Abstract: The synthesis and biotransformation of five flavones containing methoxy substituents in the B ring: 2′-, 3′-, 4′-methoxyflavones, 2′,5′-dimethoxyflavone and 3′,4′,5′-trimethoxyflavone are described. Strains of entomopathogenic filamentous fungi were used as biocatalysts. Five strains of the species *Beauveria bassiana* (KCh J1.5, J2.1, J3.2, J1, BBT), two of the species *Beauveria caledonica* (KCh J3.3, J3.4), one of *Isaria fumosorosea* (KCh J2) and one of *Isaria farinosa* (KCh KW 1.1) were investigated. Both the number and the place of attachment of the methoxy groups in the flavonoid structure influenced the biotransformation rate and the amount of nascent products. Based on the structures of products and semi-products, it can be concluded that their formation is the result of a cascading process. As a result of enzymes produced in the cells of the tested strains, the test compounds undergo progressive demethylation and/or hydroxylation and 4-*O*-methylglucosylation. Thirteen novel flavonoid 4-*O*-methylglucosides and five hydroxy flavones were isolated and identified.

Keywords: biotransformation; entomopathogenic fungi; methoxyflavones; 4-*O*-methyloglycosylation

1. Introduction

Flavonoids are common in the world of plants as their secondary metabolites, mainly in citrus fruits, olive oil, seeds [1] and vegetables [2]. They function as pigments in coloration of plants [3,4], root growth regulators, and plant defense reaction agents, and are involved in the transport of auxins between plant cells [5]. Flavonoids are present in our diet and have desired pro-health activities, including antioxidant, anti-inflammatory, anti-cancer [3,6,7], antibacterial, antifungal and antiviral activities [6,8]. Some methoxyflavones (e.g., 3′-methoxyflavone) have the ability to prevent parthanatos [9]. It is estimated that we consume a maximum of about 1 g of flavonoids a day, depending on the type and amount of meals consumed [10].

Methoxyflavones have well-described anti-cancer properties and are often more active than flavones without any functional groups [11]. Due to the presence of methoxyl groups, these compounds show stronger lipophilic properties than flavonoids with hydroxyl groups, which directly affects their bioavailability [12]. The best natural source of methoxyflavones described so far is cowslip (*Primula veris* L.), which contains these compounds in the rhizomes, roots, flowers and leaves, and from which mainly expectorants are prepared [13]. The therapeutic potential of flavonoids is often limited by their low solubility and bioavailability.

Aglycons are characterized by very low solubility in water [14]; however, addition of a polar moiety such as sugars may increase their activity, bioavailability [15] and stability [10]. For example, the water solubility of α-glucosyl-isoquercitrin increases more than 80,000-fold compared to its parent

compound, quercetin [16,17]. Isoquercitrin glycoside shows 18 times higher bioavailability than quercetin after oral administration to rats [18], and much higher bioavailability compared to quercetin and isoquercitrin in human studies [19]. These results indicate that glycosyl flavones exhibit better health-promoting effects than their aglycones due to their higher bioavailability [20].

Flavonoids are not the only group of compounds in which the positive effect of the presence of a sugar moiety on the activity of the compound has been proven. The best examples are antibiotics, such as erythromycin or vancomycin, in which the presence of a sugar group is crucial for their high activity [10]. The presence and number of glycosyl substituents in the flavonoid molecule, depending on their position, should exhibit different, although not necessarily positive, properties. Naturally glycosylated derivatives of flavonoids occur widely in the world of plants, but their concentration in cells very often is relatively low [21], which makes their extraction difficult. New methods of obtaining glycosylated compounds on a larger scale are still necessary.

Carrying out the biotransformation of flavonoids to their glycosylated derivatives is less complicated and more effective than their *de novo* chemical synthesis [14], which makes biotransformation a better and cheaper alternative.

The biotransformation of five methoxyflavones with substituents on the B ring is described: 2′-(**1**), 3′-(**2**), 4′-(**3**)-methoxyflavones, 2′,5′-dimethoxyflavone(**4**) and 3′,4′,5′-trimethoxyflavone(**5**). Listed compounds were biotransformed by the strains of entomopathogenic filamentous fungi belonging to the species *Beauveria bassiana* KCh J1.5, J2.1, J3.2, J1, BBT, *Beauveria caledonica* KCh J3.3, J3.4 [21], *Isaria fumosorosea* KCh J2 [22] and *Isaria farinosa* KCh KW 1.1 [21]. Fungi of these species are among the most used for biotransformation, along with *Aspergillus niger*.

Previous studies have shown the capacity for unique 4′′-*O*-methylglycosylation of hydroxyflavones observed in entomopathogenic filamentous fungal culture [15,23]. The strain *Beauveria bassiana* AM 278 catalyzed the methylglucose attachment to the hydroxyl group of a flavonoid molecule at the C-7 and C-3′ positions. Similarly, the application of *Absidia coerulea* as the biocatalyst resulted in the formation of glucosides with a glucose present at the C-7 and C-3′ positions of the same flavonoid compounds [24–26]. An analogous preference to the selective 4-*O*-methylglycosylation of the hydroxyl group located at the C-7 carbon was observed during the biotransformation of unique prenylated flavonoids obtained from the hop plant (*Humulus lupulus*) in cultures of *Beauveria bassiana* AM 446 and AM 278 [27–30]. *Beauveria bassiana* ATCC 13144 is capable of simultaneous 4-*O*-methylglycosylation of the hydroxyl group located at the C-3 and hydroxylation of the C-4′ carbon [31]. Furthermore, other entomopathogenic strains are capable of simultaneous hydroxylation and glycosylation of flavonoids. This functionalization takes place mainly at the C-4-OH position of the sugar moiety. Effective 4-*O*-methylglycosylation of 3-hydroxyflavone, 6-hydroxyflavone, 7-hydroxyflavone, baicalein, quercetin, naringenin, luteolin, diosmetin, and daidzein in entomopathogenic filamentous fungal culture of the genus *Isaria* has been reported [10,14,22]. Moreover, the formation of appropriate glycosides in the cultures of *Isaria fumosorosea* KCh J2, *I. farinosa* KCh J1.4, *I. farinosa* KCh J1.6 and *I. farinosa* KCh KW1.2 cultures was preceded by hydroxylation of the C-4′ carbon. Subsequent C-4′ hydroxylation and 4-*O*-methylglycosylation was observed during the incubation of flavone, 3-methoxyflavone, 5-hydroxyflavone, 6-methoxyflavone, 6-methoxyflavanone and 6-methylflavone in the cultures of the genus *Isaria* [14,22,32]. The capacity of the *I. fumosorosea* KCh J2 strain for simultaneous *O*-demethylation and glycosylation of 2′-, 3′- and 4′-methoxyflavanone was also described [32]. Based on this phenomenon, in this study, we biotransformed flavones with methoxyl groups (in different positions) located in the B ring obtained by chemical synthesis. Entomopathogenic fungal strains with confirmed ability of simultaneous hydroxylation/demethylation and glycosylation of flavonoid compounds were used as biocatalysts. The aim of this research was to investigate the microbial modification of the flavones with methoxyl-blocked carbons preferred for subsequent hydroxylation and glycosylation by the studied strains and to compare it to transformations of substrates without such an obstacle.

2. Results and Discussion

Based on the previously described results concerning the biotransformation of flavonoids in the cultures of entomopathogenic strains, we decided to use five *Beauveria bassiana* strains (KCh J1.5, J2.1, J3.2, J1, BBT), two *Beauveria caledonica* strains (KCh J3.3 and J3.4), and two *Isaria* strains (*I. fumosorosea* KCh J2 and *I. farinosa* KCh KW 1.1) as biocatalysts. *B. bassiana* species are the most common among the entomopathogenic filamentous fungi used in biotransformations, including in the case of flavonoid compounds [25–27]. In our previous studies concerning the biotransformation of steroids we have observed significant differences in the modification of dehydroepiandrosterone (DHEA) in the cultures of five different strains of this species [21]. Therefore, in this study, we decided to determine whether flavonoid compounds will also undergo diverse changes in the cultures of different strains of *B. bassiana*. For the first time in flavonoid biotransformations, we also tested two strains of *Beauveria caledonica* species. The *Isaria fumosorosea* KCh J2 strain has already been described in our previous study as an effective biocatalyst of flavanones and flavones as substrates [14,22,32,33]. Comparing those previously described results for *I. fumosorosea* KCh J2 to the results obtained from the transformations by the *B. bassiana* strains, we noted the similarity in the biotransformations of flavonoid compounds. In both cases, the main products are 4′-*O*-β-D-(4′′-*O*-methyl)-glucopyranosides. We have also observed this ability for strains of *I. farinosa* [14,33]. In this study, we used the *I. farinosa* KCh KW 1.1 strain as a biocatalyst as well.

All used substrates, containing in their structure from one to three methoxyl groups in the B ring, were obtained by a two-step chemical synthesis. In the first stage, five chalcones were synthesized from 2′-hydroxyacetophenone and the appropriate methoxybenzaldehyde in a basic medium. Then, they were transformed into the appropriate methoxyflavones, by reaction with J_2 in DMSO. As a result of these reactions 2′-, 3′-, 4′-methoxyflavone, 2′,5′-dimethoxyflavone and 3′,4′,5′-trimethoxyflavone were obtained.

2.1. 2′-Methoxyflavone (**1**) *Biotransformation*

Biotransformation of 2′-methoxyflavone (**1**) in the cultures of most of the tested strains resulted in one major product (**6**), with a retention time of 11.4 min according to HPLC (Table 1). Furthermore, TLC analysis confirmed that most of the biocatalysts converted 2′-methoxyflavone (**1**) to the same product. This product was identified as 2′-*O*-β-D-(4′′-*O*-methylglucopyranosyl)-flavone (**6**), obtained in all tested strains except *Beauveria bassiana* KCh J1. This compound was formed as a result of subsequent demethylation and 4-*O*-methylglycosylation. However, only in the culture of the *B. bassiana* KCh J1 strain, 2′-hydroxyflavone, was an intermediate product in the biosynthesis of 2′-*O*-β-D-(4′′-*O*-methylglucopyranosyl)-flavone observed. This may be evidence that the substrate demethylation process is crucial for the rate of 2′-*O*-β-D-(4′′-*O*-methylglucopyranosyl)-flavone formation. Among the strains used, only the *B. bassiana* KCh J1 strain does not have the ability of 4-*O*-methylglycosylation; thus only the 2′-hydroxy flavone (**10**) was formed during the biotransformation. The highest concentrations of 2′-*O*-β-D-(4′′-*O*-methylglucopyranosyl)-flavone were identified in the cultures of *Beauveria bassiana* strains: KCh J3.2 (70%); KCh J2.1 (76%); KCh BBT (87%) after 10 days of biotransformation.

The exceptions during the biotransformation of 2′-methoxyflavone (**1**) turned out to be the *I. fumosorosea* KCh J2 strain, where two additional products were also observed (it was not possible to separate compounds **7** and **8** with the selected HPLC program, so in this case UHPLC was performed) (Table 2) and the *B. bassiana* KCh J1 strain, where only one product (**10**) with a retention time of 14.8 min was observed. For this reason, a scale-up biotransformation of these two strains was performed to isolate and characterize the structure of the resulting products. As a result of the scale-up biotransformation of the 2′-methoxyflavone in the culture of the *I. fumosorosea* KCh J2, a total of four products were isolated (Figure 1). Based on NMR analyses, 2′-*O*-β-D-(4′′-*O*-methylglucopyranosyl)-flavone is the major product (**6**). The formation of this compound is possible by successive demethylation and 4-*O*-methylglycosylation. The ^{1}H NMR spectrum shows signals confirming that the structure of the

flavone skeleton has not been changed. The presence, chemical shifts and multiplicities of the signals indicate that the only substituent is on the C-2′ carbon (as in a substrate). The structure of the flavone is confirmed by the COSY, HMQC and HMBC correlation spectra. However, instead of the signal from the protons of the CH_3 group (visible in the ^{1}H NMR substrate spectrum), signals from the sugar unit are visible. The multiplicities and positions of these signals in both the ^{1}H and ^{13}C NMR spectra indicate that a glucose molecule was introduced in place of the CH_3 group. The HMBC spectrum shows the coupling of the signal from the H-1′′ sugar unit proton with the signal from the C-2′ carbon of the flavone skeleton. This coupling indicates the exact place of attachment of the sugar substituent. Additionally, the ^{1}H NMR spectrum shows a singlet derived from three protons (3.46 ppm). In the HMBC spectrum, this signal is coupled to the C-4′′ carbon (sugar substituent), which indicates that the substituent is 4′′-*O*-methylglucopyranoside.

Table 1. Microbial transformation of 2′-methoxyflavone, HPLC conversion.

Strain No.	Substance	Retention Time [min]	Conversion after 1, 3, 7 and 10 Days [%]			
			1	3	7	10
KCh J1.5	**6**	11.4	1	5	33	52
	1	18.2	99	95	67	48
KCh J2.1	**6**	11.4	0	3	43	76
	1	18.2	100	97	57	24
KCh KW 1.1.	**6**	11.4	0	41	99	100
	1	18.2	100	59	1	0
KCh J1	**10**	14.8	0	5	4	8
	1	18.2	100	95	97	92
KCh J2	**7, 8**	10.7	0	5	24	13
	6	11.4	4	19	45	63
	1	18.2	96	76	31	24
KCh J3.4	**6**	11.4	0	0	2	4
	1	18.2	100	100	98	93
KCh J3.2	**6**	11.4	0	13	28	70
	1	18.2	100	87	72	30
KCh J3.3	**6**	11.4	0	1	47	48
	1	18.2	100	99	53	52
KCh BBT	**6**	11.4	0	4	36	87
	1	18.2	100	96	64	13

Figure 1. Biotransformation of 2′-methoxyflavone (**1**) by *I. fumosorosea* KCh J2.

In the ^{1}H NMR spectrum of the next product (**8**) (isolated with a yield of 20%), obtained by scale-up biotransformation of 2′-methoxyflavone in the culture of *I. fumosorosea* KCh J2, there are signals indicating that the product is 4-*O*-methylglycoside. However, the presence of a singlet derived from three protons at 3.90 ppm and its coupling to the C-2′ carbon of the flavone skeleton, visible in the HMBC spectrum, prove that the methoxy group has been preserved. Based on the position and multiplicities of the signals visible in the ^{1}H NMR spectrum and the couplings visible

in the correlation spectra, it can be concluded that an additional substituent was attached at the C-5′ carbon. Additionally, in the HMBC spectrum, the signal from this carbon is coupled with a doublet from the H-1′′ proton of the sugar unit. Based on these data, this compound was identified as 5′-*O*-β-D-(4′′-*O*-methylglucopyranosyl)-2′-methoxyflavone (**8**).

Table 2. *Isaria fumosorosea* KCh J2 transformation of 2′-methoxyflavone (glycosylated derivatives), UHPLC conversion.

Substance	Retention Time [min]	Conversion after 1, 3, 7 and 10 Days [%]			
		1D	3D	7D	10D
6	13.6	1.5	14.5	40.9	22.7
7	11.9	0.7	3.5	12.2	7.4
8	12.2	0.1	3.2	13.6	8.7
1	16.9	97.7	76	26.9	7.8

Based on NMR data, another product was also identified as a glycoside derivative of 2′-methoxyflavone. Based on the chemical shifts of the signals visible in the ^{1}H NMR spectrum of this compound and the couplings visible in the correlation spectra, it can be concluded that the substituent is 4′′-O-methylglycoside, bound to carbon C-8. This compound was isolated in a yield of 4% and identified as 8-*O*-β-D-(4′′-*O*-methylglucopyranosyl)-2′-methoxyflavone (**7**). Another product, identified as 3-*O*-β-D-(4′′-*O*-methylglucopyranosyl)-2′-methoxyflavone (**9**), was isolated, but with a very low yield (<1%). From the ^{1}H NMR spectrum it was found that the structure of 2′-methoxyflavone was retained. At the same time, as in the case of previously described products, signals from 4′′-O-methylglycoside are visible. The lack of a signal of the H-3 proton indicates that the sugar substituent was introduced at the C-3 carbon by *O*-glycosylation reaction. Due to the low concentration of this compound, it was not possible to perform ^{13}C NMR analysis and correlation spectra confirming its structure. Compounds **6**, **7, 8** and **9** are the result of successive hydroxylation and 4-*O*-methylglycosylation.

As a result of up-scale biotransformation of the 2′-methoxyflavone in the culture of the *Beauveria bassiana* KCh J1 strain, one product was isolated with 43.5% yield. On the basis of NMR analyses it was identified as 2′-hydroxyflavone (**10**) (Figure 2). This compound was formed as a result of the *O*-demethylation of 2′-methoxyflavone. The ^{1}H NMR spectrum shows signals confirming that the structure of the flavone skeleton has remained unchanged. The presence and chemical shifts of the signals indicate that the hydroxyl group is on the C-2′ carbon. The structure of the flavone is confirmed by the COSY, HMQC and HMBC correlation spectra. However, instead of the signal from the three protons of the CH_3 group (visible in the ^{1}H NMR substrate spectrum) at 10.03 ppm, the signal from the proton of the hydroxyl group located at C-2′ is visible.

Figure 2. Biotransformation of 2′-methoxyflavone (**1**) by *B. bassiana* KCh J1.

2.2. 3′-Methoxyflavone (2) Biotransformation

The next substrate, 3′-methoxyflavone (**2**), was converted much faster by all tested biocatalysts. It is most likely related to easier access of the enzymes responsible for the demethylation process to the methoxy group situated meta in relation to the chromene substituent (1-benzopyran or chromene). Conversion of 3′-methoxyflavone in the cultures of four out of nine biocatalysts (*I. fumosorosea* KCh J2, *I. farinosa* KCh KW1.1, *B. bassiana* KCh J1.5 and KCh J3.2) was close to 100% after three days of incubation (Table 3).

Table 3. Microbial transformation of 3′-methoxyflavone, HPLC conversion.

Strain No.	Substance	Retention Time [min]	Conversion after 1, 3, 7 and 10 Days [%]			
			1	3	7	10
KCh J1.5	**12**	10.8	58	99	100	100
	2	18.5	42	1	0	0
KCh J2.1	**12**	10.8	14	8	92	100
	2	18.5	86	92	8	0
KCh KW 1.1.	**12**	10.8	38	99	100	100
	2	18.5	62	1	0	0
KCh J1	**11**	13.9	12	79	98	100
	2	18.5	88	21	2	0
KCh J2	**12**	10.8	18	100	100	100
	2	18.5	82	0	0	0
KCh J3.4	**12**	10.8	0	96	100	100
	2	18.5	82	4	0	
KCh J3.2	**12**	10.8	9	100	100	100
	2	18.5	91	0	0	0
KCh J3.3	**12**	10.8	7	11	78	99
	2	18.5	93	89	22	1
KCh BBT	**12**	10.8	6	37	100	100
	11	13.9	0	3	0	0
	2	18.5	94	60	0	0

Similar to the biotransformation of 2′-methoxyflavone (**1**), during the transformation of 3′-methoxyflavone (**2**) in the culture of the *B. bassiana* KCh J1 strain, one of the main products was an effect of demethylation and was identified as 3′-hydroxyflavone (**11**) (Figure 3). All NMR data (Supplementary materials, Figures S35–S39) confirm the structure of this product. This compound was also identified in trace amounts in the culture of the *B. bassiana* KCh BBT strain.

Figure 3. Biotransformation of 3′-methoxyflavone (**2**) by *B. bassiana* KCh J1.

The major product of the transformation of 3′-methoxyflavone in most of the studied strains was the product of progressive demethylation and 4-*O*-methylglycosylation, that is 3′-*O*-β-D-(4′′-*O*-methylglucopyranosyl)-flavone (**12**) (Figure 4). The chemical shifts and multiplicities of the signals visible in the ^{1}H NMR spectrum confirm that the structure of the flavone skeleton has been preserved; the only substituent is on the C-3′ carbon (as in the substrate). The structure of the flavone is also confirmed by the ^{13}C NMR spectra and the correlation with COSY, HMQC and HMBC spectra. The HMBC spectrum shows the coupling of the signal from the H-1′′ proton of the sugar unit with the signal from the C-3′ carbon of the flavone skeleton. The ^{1}H NMR spectrum shows a singlet from the three protons (3.47 ppm). In the HMBC spectrum, this signal is coupled to the C-4′′ carbon (sugar substituent), which proves that the substituent is 4′′-O-methylglucopyranoside.

Figure 4. Biotransformation of 3′-methoxyflavone (**2**) by *I. farinosa* KCh KW 1.1.

2.3. 4′-methoxyflavone (**3**) *Biotransformation*

High conversions of 4′-methoxyflavone (**3**) in the cultures of the tested strains were comparable to those obtained for 3′-methoxyflavone (**2**) at the seven days of biotransformation. Nearly 100% conversion was observed in the culture of *Isaria farinosa* KCh KW 1.1 and four strains of *Beauveria bassiana* (KCh J1.5, J2.1, J3.2, BBT) (Table 4.). Similar to the results of the two abovedescribed compounds (**1** and **2**), the exception is the *Beauveria bassiana* KCh J1 strain, from the culture of which only the demethylation product 4′-hydroxyflavone was isolated (**13**) (Figure 5). Spectral data for this product are provided in supplementary materials (Figures S47–S52). In the culture of the *Beauveria bassiana* KCh J1 strain, the formation of several other products was observed after the seventh day of transformation, but they were formed concentrations that were too low to determine their structure.

Table 4. Microbial transformation of 4′-methoxyflavone (**3**), HPLC conversion.

Strain No.	Substance	Retention Time [min]	Conversion after 1, 3, 7 and 10 Days [%]			
			1	**3**	**7**	**10**
KCh J1.5	**14**	10.6	26	100	100	100
	3	17.8	75	0	0	0
KCh J2.1	**14**	10.6	9	12	97	97
	3	17.8	91	88	3	3
KCh KW 1.1.	**14**	10.6	8	17	100	100
	3	17.8	92	83	0	0
KCh J1	**13**	13.3	1	8	23	83
	3	17.8	99	92	73	17
KCh J2	**14**	10.6	1	8	19	94
	3	17.8	99	92	81	6
KCh J3.4	**14**	10.6	0	9	29	59
	3	17.8	82	91	71	41
KCh J3.2	**14**	10.6	10	98	100	100
	3	17.8	90	2	0	0
KCh J3.3	**14**	10.6	0	2	27	47
	3	17.8	100	98	73	53
KCh BBT	**14**	10.6	6	16	100	100
	3	17.8	94	84	0	0

Figure 5. Biotransformation of 4′-methoxyflavone (**3**) by *B. bassiana* KCh J1.

As a result of scale-up biotransformation with 100 mg of 4′-methoxyflavone (**3**) in the culture of the *Beauveria bassiana* KCh J1.5 strain, 36.5 mg of 4′-O-β-D- (4′′-O-methylglucopyranosyl)-flavone (**14**) was isolated (Figure 6). Its structure was confirmed based on spectroscopic data (Supplementary materials Figures S53–S60).

Figure 6. Biotransformation of 4′-methoxyflavone (**3**) by *B. bassiana* KCh J1.5.

Comparing the biotransformation rate of substrates having a single-methoxy group, it is seen that 3′-methoxyflavone undergoes the fastest demethylation. In the literature to date, in the processes of hydroxylation and 4′′-*O*-methylglucosylation observed in cultures of entomopathogenic strains,

in most cases, functionalization took place at the C-4′ carbon of the flavonoid skeleton [14,22,32,33]. Based on the experiments carried out here, the demethylation and 4′′-O-methylglucosylation processes are the fastest when the methoxy group is located on the C-3′ carbon (Scheme 1)

Scheme 1. Substrate preferences of methoxyflavones affecting the high rate of *O*-methylglucosylation by tested entomopathogenic strains.

2.4. 2′,5′-Dimethoxyflavone (**4**) *Biotransformation*

In the cultures of most of the tested strains the major product of biotransformation of 2′,5′-dimethoxyflavone (**4**) was the 4-*O*-methylglucosylated derivative (the same trend as was observed in case monomethoxy flavone biotransformations). As a result of up-scaled biotransformation of this substrate in the culture of *B. bassiana* KCh J1.5, three products were isolated: **8**, **15** and **16**. These compounds were also observed during biotransformation in other cultures (*B. bassiana* KCh J2.1 and BBT, *B. caledonica* KCh J3.2, and *I. fumosorosea* KCh J2). Compound **8**, with a retention time of 10.7 min (HPLC), was isolated in the highest yield (Table 5).

The ^{1}H NMR spectrum shows signals from the 4-*O*-methylglucopyranosyl substituent. The HMBC spectrum shows the coupling of the signal from the H-1′′ proton of the sugar unit with the signal from the C-5′ carbon of the flavone skeleton. The chemical shifts as well as the coupling of signals observed in the correlation spectra confirm that this product is 5′-*O*-β-D-(4′′-*O*-methylglucopyranosyl)-2′-methoxyflavone (**8**). The next product is 2′-*O*-β-D-(4′′-*O*-methylglucopyranosyl)-5′-methoxyflavone (**15**), the signal of which is observed on the HPLC chromatogram with a retention time of 11.6 min. NMR spectra indicate that it is also a product of demethylation and 4-*O*-methylglucosylation. The HMBC spectrum shows the coupling of the signal from the H-1′′ proton of the sugar unit with the signal from the C-2′ carbon of the flavone skeleton, which indicates that this product is 2′-*O*-β-D-(4′′-*O*-methylglucopyranosyl)-5′-methoxyflavone (**15**). The third product with the lowest yield in the culture of *B. bassiana* KCh J1.5 has also been identified as 4-*O*-methylglucopyranoside. The ^{1}H NMR spectrum shows three singlets from the protons of the -OCH_3 groups. One methoxy group is bonded to the C-4′′ carbon of the sugar unit, the other two to the C-2′ and C-5′ carbons of the flavone skeleton. The B-ring protons of this product give only two singlets at 7.00 and 7.56 ppm. Based on the chemical shifts, multiplicities and couplings observed in the correlation spectra, they were assigned to the protons H-3′ and H-6′, respectively. The NMR spectra (Supplementary materials Figures S70–S76) made for this product indicate that it is 4′-*O*-β-D-(4′′-*O*-methylglucopyranosyl)-2′,5′-dimethoxyflavone (**16**) (Figure 7).

Based on the NMR analyses of the obtained products, it can be seen that demethylation or hydroxylation and 4-*O*-methylglucosylation of 2′,5′-dimethoxyflavone (**4**) take place in the cultures of the strains tested. The highest concentration (over 70%) of 5′-*O*-β-D-(4′′-*O*-methylglucopyranosyl)-2′-methoxyflavone (**8**) in the reaction mixture was observed in the culture of *I. fumosorosea* KCh J2. In the cultures of *B. bassiana* KCh J1.5, KCh J2.1, KCh J3.2 and *I. farinosa* KCh KW1.1 the concentration of 5′-*O*-β-D-(4′′-*O*-methylglucopyranosyl)-2′-methoxyflavone (**8**) is almost twice as high as 2′-*O*-β-D-(4′′-*O*-methylglucopyranosyl)-5′-methoxyflavone (**15**). However, in the cultures of *B. bassiana* KCh BBT and *B. caledonica* KCh J3.3 strains, the concentration of these products was comparable. In the culture of the *B. caledonica* KCh J3.4 strain, several products were observed, but they were formed at low concentrations that prevented their isolation.

Table 5. Microbial transformation of 2′5′-dimethoxyflavone (**4**), HPLC conversion.

Strain No.	Substance	Retention Time [min]	Conversion after 1, 3, 7 and 10 Days [%]			
			1	3	7	10
KCh J1.5	**8**	10.7	47	49	57	52
	16	10.9	4	6	4	9
	15	11.6	24	28	26	31
	4	18.3	25	17	12	8
KCh J2.1	**8**	10.7	20	55	54	67
	16	10.9	0	0	1	0
	15	11.6	8	20	26	25
	4	18.3	73	25	19	8
KCh KW 1.1	**8**	10.7	13	47	49	49
	15	11.6	7	24	30	30
	4	18.3	80	29	21	21
KCh J1	**17**	13.4	25	68	31	20
	18	13.9	5	8	6	3
	4	18.3	69	25	23	41
KCh J2	**8**	10.7	9	62	66	74
	16	10.9	0	0	3	2
	15	11.6	0	12	13	13
	4	18.3	91	26	17	10
KCh J3.4	**15**	11.6	10	11	8	8
	17	13.4	46	51	54	54
	4	18.3	54	49	46	46
KCh J3.2	**8**	10.7	43	50	53	59
	16	10.9	4	5	9	5
	15	11.6	20	24	27	28
	4	18.3	32	21	11	9
KCh J3.3	**8**	10.7	5	36	38	39
	15	11.6	8	46	49	51
	4	18.3	87	18	13	10
KCh BBT	**8**	10.7	37	42	43	43
	16	10.9	1	4	5	9
	15	11.6	38	42	44	44
	4	18.3	23	12	4	4

Figure 7. Biotransformation of 2′5′-dimethoxyflavone (**4**) by *I. farinosa* KCh KW1.1 and *B. bassiana* KCh J1.5 cultures.

Two 2′,5′-dimethoxyflavone (**4**) products were observed in the culture of the *B. bassiana* KCh J1 strain. The main product was identified as 5′-hydroxy-2′-methoxyflavone (**17**). Compared to the spectrum of the substrate, instead of two, only one singlet from the three protons (3.84 ppm) is visible in the ^{1}H NMR spectrum of this product. At the same time, at 9.41 ppm, a singlet derived from the hydroxyl group is observed. The HMBC spectrum shows connections of this signal with signals from carbons C-4′, C-5′ and C-6′, which proves that the methoxyl group situated at the C-5′ carbon was demethylated.

The second product (**18**) is produced in a much smaller amount in the culture of the *B. bassiana* KCh J1 strain. The ^{1}H NMR spectrum shows two signals from protons of -CH_3 groups in positions 3.84 and 3.85 ppm, respectively, and a singlet derived from one proton, in the field characteristic for signals from Ar-OH protons. These data prove the preservation of both methoxy groups and the introduction

of an additional hydroxyl group to the substrate molecule. The chemical shifts and multiplicities of the signals from the protons in the A ring are almost identical to those observed in the ^{1}H NMR of the substrate spectrum. However, the B-ring protons of this product only give two singlets at 6.70 and 7.53 ppm. Based on the shapes and couplings observed in the correlation spectra, H-3′ and H-6′ were assigned, respectively. Additionally, in the HMBC spectrum, the signal from the proton of the hydroxyl group is coupled with the signals from carbons C-3′, C-4′ and C-5′. This information indicates that this compound is 4′-hydroxy-2′,5′-dimethoxyflavone (**18**) (Figure 8). In the culture of this strain, other products of hydroxylation were also formed in the seven-day biotransformation. However, it was not possible to isolate them and determine their structures.

O O O O 4 *Beauveria bassiana* KChJ1 17 OH + 18 OH

Figure 8. Biotransformation of 2′,5′-dimethoxyflavone (**4**) in *Beauveria bassiana* KCh J1 culture.

2.5. *3′,4′,5′-Trimethoxyflavone* (**5**) *Biotransformation*

The highest conversion (over 90% after the seventh day of the biotransformation process) of 3′,4′,5′-trimethoxyflavone was observed in the culture of *B. bassiana* KCh J1.5 and two strains of *B. caledonica* (KCh J3.3 and KCh J3.4). As a result of the scaled-up biotransformation in the culture of *B. bassiana* KCh J1.5, selected based on the results from HPLC (Table 6) and TLC, four products were isolated (**19–22**) (Figure 9). The main product is the effect of progressive demethylation and 4′′-*O*-methylglucosylation. The ^{1}H NMR spectrum shows all signals from the A-ring protons of the flavonoid skeleton with multiplicity and positions similar to the substrate spectrum (3′,4′,5′-trimethoxyflavone (**5**)). However, instead of two singlets, one for six protons, the other for three protons from the protons of the methoxy groups, three singlets from the protons of the -OCH_3 groups are visible (two groups bound to the B ring of the flavone, one to the C-4′′ carbon of the sugar unit). In addition, the signals from the H-2′and H-6′ protons in the substrate spectrum generated a singlet at 7.15 ppm due to the same chemical environment. The spectrum of this product shows two doublets at 7.41 ppm from H-6′ and 7.49 ppm from H-2′. Such differentiation of these signals proves that the substitution took place within the B ring. Based on the correlation spectra, it can be stated that the 4-*O*-methyl glucose substituent is bound to the C-3′ carbon (visible in the HMBC spectrum coupling of the signal from the H-1′′ proton with the signal from carbon C-3′). The most common product of biotransformation of 3′,4′,5′-trimethoxyflavone (**5**) by *B. bassiana* KCh J1 was identified as 3′-*O*-β-D-(4′′-*O*-methylglucopyranosyl)-4′,5′-dimethoxyflavone (**19**).

Furthermore, the second product is a 4-*O*-methylglucoside (**20**). However, the ^{1}H NMR spectrum of **19** shows all signals from the flavonoid skeleton protons with multiplicity and positions similar to the 3′,4′,5′-trimethoxyflavone (**5**) spectrum. A singlet at 3.90 ppm derived from the six protons of methoxy groups (C-3′-OCH_3 and C-5′-OCH_3) is visible. The HMBC spectrum shows the coupling of the signal from the H-1′′ proton with the signal from the C-4′ carbon, which proves that this product is 4′-*O*-β-D-(4′′-*O*-methylglucopyranosyl)-3′,5′-dimethoxyflavone (**20**), which was identified in the cultures of most biocatalysts used. The main compound (**19**) was yielded in 80% of the reaction mixture in the culture of the *B. bassiana* KCh J1.5 strain and two *B. caledonica* strains (KCh J3.3 and KCh J3.4). In the cultures of these strains and the *B. bassiana* KCh J3.2 strain, the concentration of **19** was about five times higher than 4′-*O*-β-D-(4′′-*O*-methylglucopyranosyl)-3′,5′-dimethoxyflavone (**20**). In the culture of *B. bassiana* KCh BBT, compounds **19** and **20** were formed in the ratio of 3:1, in the culture of *I. farinosa* KCh KW 1.1 in the ratio 10:1, and in the culture of *I. fumosorosea* KCh J2 in the ratio of 48:1.

In the culture of the *B. bassiana* KCh J1 strain, even after ten days of 3′,4′,5′-trimethoxyflavone (**5**) incubation, nearly 60% unreacted substrate was observed. The resulting products were formed in very low concentrations, which made them impossible to isolate. Instead, scaled-up biotransformation in the *B. bassiana* KCh J1.5 culture gave two additional products which are formed at very low concentrations in the cultures of most of the tested biocatalysts. The compound with a retention time of 10.3 min was identified as 6-*O*-β-D-(4′′-*O*-methylglucopyranosyl)-3′,4′,5′-trimethoxyflavone (**21**).

Based on the correlation spectra recorded for compound **21**, it was found that the sugar unit is bound to carbon C-6. This compound is an effect of hydroxylation and glycosylation. Table 7 shows the positions of the signals visible in the ^{13}C NMR spectrum of 6-*O*-β-D-(4′′-*O*-methylglucopyranosyl)-flavone (**21**) and the flavone previously described, which were used as a calculation standard [14]. Additionally, the calculated chemical shifts caused by the introduction of the *O*-β-D-(4-*O*-methyl)-glucopyranosyl substituent on the C-6 carbon with respect to the position of analogous signals visible on the flavan spectrum are given. Table 7 shows the signal positions that are visible on the ^{13}C NMR spectrum made for 3′,4′,5′-trimethoxyflavone (**5**) and the calculated signal positions for 6-*O*-β-D-(4′′-*O*-methylglucopyranosyl)-3′,4′,5′-trimethoxyflavone (**21**). The ^{13}C NMR data obtained for compound **21** are compatible with the calculated data (Table 7). Additionally, the structure of this product was confirmed by MS analysis (Supplementary materials Figure S96).

Analogous calculations were made for product **22**. The chemical shifts of the carbon signals caused by the introduction of the hydroxyl group at C-6 were calculated. In this case, the positions of the signals on the ^{13}C NMR spectrum of 6-hydroxy-flavone and flavone without any substituents, previously described [14], were compared and then the shift values were added to the values of the positions of the carbon signals from the 3′-*O*-β-D-(4′′-*O*-methylglucopyranosyl)-4′,5′-dimethoxyflavone. Very high compatibility of the calculated and measured values of the signal positions in the ^{13}C NMR spectrum for 3′-O-β-D-(4′′-O-methylglucopyranosyl)-6-hydroxy-4′,5′-dimethoxyflavone (22) was obtained. The NMR and MS spectra confirm the correct determination of the product structure (Supplementary materials Figures S103–S109).

Figure 9. Biotransformation of 3′,4′,5′-trimethoxyflavone (**5**) by *B. bassiana* KCh J1.5 strain.

Table 6. Microbial transformation of 3′,4′,5′-trimethoxyflavone (**5**), HPLC conversion.

Strain No.	Substance	Retention Time [min]	Conversion after 1, 3, 7 and 10 Days [%]			
			1	**3**	**7**	**10**
KCh J1.5	**21**	10.3	0	1	4	3
	20	10.6	1	6	16	17
	19	10.9	4	32	79	80
	5	16.8	95	61	1	0
KCh J2.1	**21**	10.3	0	1	1	2
	20	10.6	1	2	7	10
	19	10.9	8	19	53	67
	5	16.8	92	79	38	21
KCh KW 1.1	**21**	10.3	0	0	0	0.2
	20	10.6	0	0	1	2
	19	10.9	1	4	11	16
	5	16.8	99	95	88	82
KCh J1	**21**	10.3	7	16	20	20
	22	13.7	0	0	1	2
	5	16.8	91	84	68	59
KCh J2	**20**	10.6	0	0	1	1
	19	10.9	2	13	44	48
	5	16.8	98	86	54	50
KCh J3.4	**20**	10.6	0	5	19	17
	19	10.9	5	45	77	81
	5	16.8	95	50	3	2
KCh J3.2	**21**	10.3	0	1	4	4
	20	10.6	1	3	11	10
	19	10.9	4	17	46	50
	22	13.7	0	1	3	3
	5	16.8	95	77	36	33
KCh J3.3	**20**	10.6	0	4	14	16
	19	10.9	2	22	73	83
	5	16.8	98	74	13	1
KCh BBT	**21**	10.3	0	0	1	0
	20	10.6	0	2	11	11
	19	10.9	0	4	29	38
	22	13.7	0	1	3	6
	5	16.8	100	94	56	45

Flavonoid glycosylation in recent times has been increasingly described, which does not necessarily mean that we fully understood all the processes and enzymes responsible for these reactions. Nine strains of entomopathogenic filamentous fungi—*B. bassiana* KCh J1.5, J2.1, J3.2, J1, BBT, *B. caledonica* KCh J3.3, J3.4, *I. fumosorosea* KCh J2 and *I. farinosa* KCh KW 1.1—used in these experiments were capable to transforming substrates containing a methoxy group(s) on ring B. Often the process of adding a sugar moiety to flavonoids is described as a one-step reaction, in which the end product is formed without the presence of intermediates [24–30]. Based on the structure of the products isolated here, it can be concluded that glycosylated derivatives of flavonoids are the result of cascade changes. During incubation in the studied strains, there takes place the processes of demethylation and/or hydroxylation and 4-*O*-methylglucosylation, in which several enzymes and coenzymes of the biocatalyst are involved. In addition, the position of the substituent within the B ring had a significant impact on the speed and number of products formed. The demethylation and 4-*O*-methylglucosylation processes are fastest when the methoxy group is placed on the C-3′ carbon and slowest when on the C-2′ carbon. However, the largest number of products were formed when the methoxy substituent was in the 2′—position, where the 4-*O*-methylglycose molecule was attached not only within the ring where it was present, but also outside it. The same was true in the case of the flavonoid containing 3 methoxy groups, where attachment of 4-*O*-methylglycose within another ring was also observed.

Table 7. Calculated and measured positions of signals visible in the ^{13}C NMR spectrum.

	F [a]	6GF [b]	Δ Chemical Shifts (6GF-F) [c]	5	Calculated Positions of the Signals 21 (5 + (6GF-F)) [d]	21	6OHF [e]	Δ Chemical Shifts (6OHF-F) [f]	19	Calculated Positions of the Signals 22 (19 + (6OHF-F)) [g]	22
2	163.9	163.8	−0.1	162.44	162.34	162.29	163.6	−0.3	162.32	162.02	161.88
3	108.0	107.2	-0.8	106.87	106.07	106.12	107.0	−1.0	106.78	105.78	105.76
4	178.0	177.7	−0.3	177.17	176.87	176.88	177.9	−0.1	177.22	177.12	177.07
4a	124.8	125.5	0.7	123.29	123.99	124.00	125.7	0.9	123.29	124.19	124.18
5	126.0	111.1	−14.9	124.74	109.84	109.22	108.9	−17.1	124.74	107.64	107.42
6	126.2	156.0	29.8	125.52	155.32	154.59	155.9	29.7	125.53	155.23	154.88
7	134.9	124.9	−10.0	134.20	124.20	124.00	123.7	−11.2	134.25	123.05	122.99
8	119.3	120.6	1.3	118.70	120.00	120.18	120.6	1.3	118.73	120.03	120.02
8a	157.1	152.5	−4.6	155.68	151.08	150.99	151.1	−6.0	155.70	149.70	149.36
1′	132.8	132.8	0.0	126.46	126.46	126.50	133.0	0.2	126.27	126.47	126.51
2′	127.2	127.2	0.0	104.14	104.14	104.09	127.1	−0.1	107.69	107.59	107.52
3′	130.0	130.0	0.0	153.29	153.29	153.29	130.0	0.0	151.25	151.25	151.21
4′	132.5	132.5	0.0	140.63	140.63	140.56	132.3	−0.2	141.31	141.11	141.10
5′	130.0	130.0	0.0	153.29	153.29	153.29	130.0	0.0	153.42	153.42	153.37
6′	127.2	127.2	0.0	104.14	104.14	104.09	127.1	−0.1	104.81	104.71	104.66
1′′						101.90			100.73		100.62
2′′						74.40			73.59		73.55
3′′						76.42			76.55		76.50
4′′						79.18			79.37		79.32
5′′						76.08			75.94		75.88
6′′						60.34			60.50		60.49
C-3′-OCH_3						56.76					
C-4′-OCH_3									60.58		60.53
C-5′-OCH_3						56.76			56.35		56.30
C-4′′-OCH_3						59.66			59.75		59.71

[a] Flavone (**F**); [b] 6-*O*-β-D-(4′′-*O*-methylglucopyranosyl)-3′,4′,5′-trimethoxyflavone (**6GF**); [c] shifts in signals coming from flavone carbons due to the entry of the *O*-β-D-(4-*O*-methyl)-glucopyranosyl substituent; [d] the calculated positions of the signals from compound **21** (chemical shifts induced by *O*-β-D-(4-*O*-methyl)-glucopyranosyl substituent at carbon C-6 were added to the positions of the signals from the 3′,4′,5′-trimethoxyflavone carbons (**5**)); [e] 6-hydroxyflavone (**6OHF**); [f] shifts of carbon signals caused by insertion of the hydroxyl group on C-6 flavone carbon; [g] calculated positions of the signals from compound **22** (chemical shifts caused by the hydroxyl group at carbon C-6 were added to the positions of signals from the 3′-*O*-β-D-(4′′-*O*-methylglucopyranosyl)-4′,5′-dimethoxyflavone carbons (**19**)).

3. Materials and Methods

3.1. Substrates

The substrates 2-hydroxyacetophenone, 2-methoxybenzaldehyde, 3-methoxybenzaldehyde, 4-methoxybenzaldehyde, 2,5-dimethoxybenzaldehyde and 3,4,5-trimethoxybenzaldehyde were purchased from Sigma-Aldrich (St. Louis, MO, USA). Flavones used in biotransformations were synthesized from those substrates (the reactions are described below). The resulting chalcones were used as substrates for the flavone synthesis and their NMR spectral data are identical to those previously published [34–36].

3.2. Synthesis

All used substrates were synthesized in the laboratory by carrying out two kinds of reactions. Firstly five different methoxychalcones were synthesized in the Claisen-Schmidt reaction of 2-hydroxyacetophenone with suitable methoxybenzaldehyde in reaction, described earlier and shown in Scheme 2 [34–38]. The resulting flavones (**1**–**5**) were used as substrates for the biotransformation and their NMR spectral data are identical to those previously published [32,39].

Scheme 2. Chalcone synthesis by Claisen-Schmidt condensation reaction.

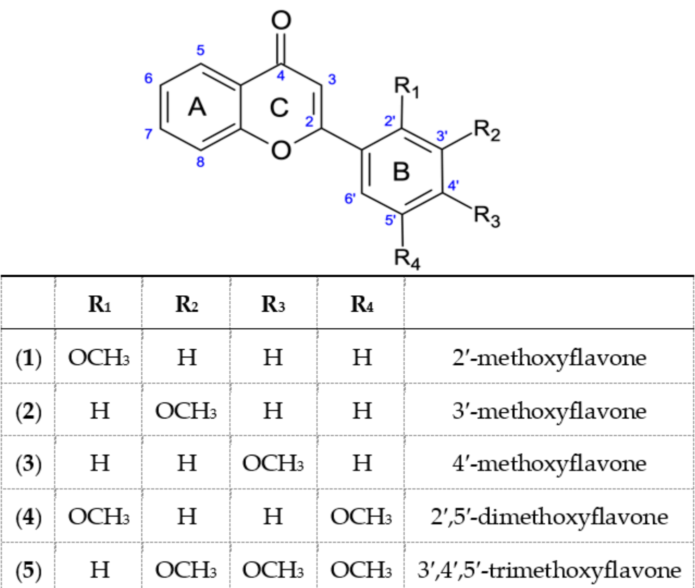
After 2 h of reflux, the product of the Claisen-Schmidt reaction was transferred into an acid environment and filtered using a Buchner funnel. The obtained product (appropriate methoxychalcone) was confirmed by NMR analysis. All other methoxychalcones were synthesized analogously. Methoxyflavones were synthesized from methoxychalcones by reaction with iodine in DMSO with 2–3 h incubation (until the substrate has reacted completely) at 130 °C [40], as presented in the example below (Schemes 3 and 4).

All substrates for biotransformations were synthesized in the same way. The obtained compounds were confirmed by NMR (^{1}H NMR, ^{13}C NMR, COSY, HMBC and HSQC) analysis.

3.3. Microorganisms

The microorganisms *Beauveria bassiana* KCh J1.5, KCh J2.1, KCh J1, KCh J3.2 and KCh BBT, *B. caledonica* KCh J3.3 and KCh J3.4, *Isaria farinosa* KCh KW 1.1 and *I. fumosorosea* KCh J2. were obtained from the collection of the Department of Chemistry, Wrocław University of Environmental and Life Sciences (Wrocław, Poland). Isolation and identification procedures were described in our previous paper [21,22].

Scheme 3. Flavone synthesis. 2′-hydroxy-2-methoxychalcone is converted into 2′-methoxyflavone (**1**).



	R_1	R_2	R_3	R_4	
(1)	OCH_3	H	H	H	2′-methoxyflavone
(2)	H	OCH_3	H	H	3′-methoxyflavone
(3)	H	H	OCH_3	H	4′-methoxyflavone
(4)	OCH_3	H	H	OCH_3	2′,5′-dimethoxyflavone
(5)	H	OCH_3	OCH_3	OCH_3	3′,4′,5′-trimethoxyflavone

Scheme 4. Methoxyflavones obtained and used in the experiment (**1–5**).

3.4. Screening Procedure

Erlenmeyer flasks (300 mL), each containing 100 mL of the sterile cultivation medium (3% glucose, 1% aminobac), were inoculated with a suspension of each entomopathogenic strain and then incubated for 3 days at 24 °C on a rotary shaker. After this time, 10 mg of a substrate was dissolved in 1 mL of dimethyl sulfoxide (DMSO) and added to the interior. Samples were collected on the 1st, 3rd, 7th and 10th day of the process. Then, all products were extracted using ethyl acetate, and extracts were dried using $MgSO_4$, concentrated in vacuo and analyzed using TLC and HPLC methods.

Scale-up Biotransformation

For the scale-up process we used Erlenmeyer flasks (2000 mL), each containing 500 mL of the same cultivation medium (3% glucose, 1% aminobac), which were inoculated in the same way as described above. Three days after inoculation, 100 mg of a substrate was dissolved in 2 mL of DMSO and added to the interior. Samples were collected on the 14th day of the process. Products were extracted three times using ethyl acetate and then analyzed using TLC, HPLC and NMR spectroscopy (^{1}H NMR, ^{13}C NMR, COSY, HMBC and HSQC) analysis.

3.5. Analysis

Initial tests were carried out using TLC plates (SiO_2, DC Alufolien Kieselgel 60 F_{254} (0.2 mm thick), Merck, Darmstadt, Germany). The mobile phase contained a mixture of chloroform and methanol in 9:1 (*v*/*v*) relation. The plates were observed using a UV lamp (254 and 365 nm).

The scale-up biotransformation products were separated using 1000 µm preparative TLC silica gel plates (Anatech, Gehrden, Germany). The mobile phase contained a mixture of chloroform and methanol in a 9:1 (*v*/*v*) ratio. Separation products were scraped out and extracted twice using ethyl acetate.

3.5.1. HPLC

A Waters 2690 instrument equipped with a Waters 996 photodiode array detector, using an ODS 2 column (4.6 × 250 mm, Waters, Milford, MA, USA) and a Guard-Pak Inserts µBondapak C18 pre-column was used to perform HPLC analyses. The mobile phase consisted of eluent A (80% acetonitrile in 4.5% acetic acid solution) and eluent B (4.5% acetic acid) with gradient elution: 0–7 min, 10% A/90% B; 7–10 min, 50% A/50% B; 10–13 min. 60% A/40% B; 15–20 min 80% A/20% B;

20–30 min, 90% A/10% B; 30–40 min, 100% A. The flow rate was 1.0 mL/min, injection volume was 10 μL, detection wavelength 280 nm.

UHPLC

A Thermo Scientific Dionex Ultimate 3000 UHPLC+ instrument (Thermo Scientific, Waltham, MA, USA) with a photodiode array detector (detection in wavelength: 210–450 nm) with a C-18 analytical column ZORBAX Eclipse XDB (5 μm, 4.6 × 250 mm, Agilent, Santa Clara, CA, USA) was used to performed UHPLC analyses. Chromatographic separation was achieved using an isocratic elution of 50% A (0.05% formic acid water solution) and 50% B (methanol containing 0.05% of formic acid) for 2 min, then a linear gradient of B from 50% to 95% for 10 min and isocratic elution of 95% B for 5 min. The flow rate was 1.0 mL/min.

3.5.2. NMR Spectroscopy

The NMR analysis was performed with a DRX 600 MHz Bruker spectrometer (Bruker, Billerica, MA, USA) with an UltraShield Plus magnet and measured in DMSO-d_6 or Acetone-d_6.

2′-*O*-*β*-D-(4′′-*O*-Methylglucopyranosyl)-Flavone (**6**)

^{1}H NMR (600 MHz) (DMSO) δ (ppm): 3.07 (dd, 1H, *J* = 9.5, 9.1 Hz, H-4′′), 3.29–3.35 (m, 1H, H-2′′), 3.41–3.54 (m, 3H, H-3′′, H-5″and one of H-6′′), 3.46 (s, 3H, C-4′′-OCH_3), 3.64 (ddd, 1H, *J* = 11.6, 4.9, 1.6 Hz, one of H-6′′), 4.72 (dd, 1H, *J* = 6.2, 5.0 Hz, C-6′′-O*H*), 5.16 (d, 1H, *J* = 7.8 Hz, H-1′′), 5.27 (d, 1H, *J* = 5.8 Hz, 3′′-O*H*), 5.39 (d, 1H, *J* = 5.5 Hz, C-2′′-O*H*), 7.08 (s, 1H, H-3), 7.22 (ddd, 1H, *J* = 7.3, 6.9, 1.0 Hz, H-5′), 7.35 (dd, 1H, *J* = 8.6, 0.8 Hz, H-3′), 7.50 (ddd, 1H, *J* = 8.1, 7.1, 1.1 Hz, H-6), 7.50 (ddd, 1H, *J* = 8.1, 7.1, 1.1 Hz, H-4′), 7.74 (dd, 1H, *J* = 8.5, 0.7 Hz, H-8), 7.83 (ddd, 1H, *J* = 8.7, 7.1, 1.7 Hz, H-7), 7.93 (dd, 1H, *J* = 7.9, 1.7 Hz, H-6′), 8.06 (ddd, 1H, *J* = 7.9, 1.6, 0.4 Hz, H-5). ^{13}C NMR (151 MHz, DMSO) δ = 59.74 (C-4′′-OCH_3), 60.15 (C-6′′), 73.50 (C-2′′), 75.70 (C-5″), 76.55 (C-3′′), 78.89 (C-4′′), 99.73 (C-1′′), 112.17 (C-3), 115.37 (C-3′), 118.58 (C-8), 120.72 (C-1′), 122.00 (C-5′), 123.20 (C-4a), 124.75 (C-5), 125.37 (C-6), 129.26 (C-6′), 132.70 (C-4′), 134.22 (C-7), 155.30 (C-2′), 156.01 (C-8a), 160.37 (C-2), 177.27 (C-4).

8-*O*-*β*-D-(4′′-*O*-Methylglucopyranosyl)-2′-Methoxyflavone (**7**)

^{1}H NMR (600 MHz) (Acetone-d_6) δ (ppm): 3.27 (dd, 1H, *J* = 9.7, 8.9 Hz, H-4′′), 3.53 (ddd, 1H, *J* = 9.7, 4.7, 2.1 Hz, H-5″), 3.61 (dd, 1H, *J* = 12.5, 4.4 Hz, H-2′′), 3.64–3.74 (m, 2H, H-3′′ and one of H-6′′), 3.85 (ddd, 1H, *J* = 10.9, 4.8, 2.0 Hz, one of H-6′′), 4.03 (s, 3H, C-4′′-OCH_3), 5.18 (d, 1H, *J* = 7.5 Hz, H-1′′), 7.10 (s, 1H, H-3), 7.16 (ddd, 1H, *J* = 7.9, 7.4, 1.1 Hz, H-5′), 7.25 (dd, 1H, *J* = 8.4, 0.9 Hz, H-3′), 7.35 (t, 1H, *J* = 8.0 Hz, H-6), 7.56 (ddd, 1H, *J* = 8.4, 7.4, 1.8 Hz, H-4′), 7.61 (dd, 1H, *J* = 8.1, 1.5 Hz, H-7), 7.74 (dd, 1H, *J* = 8.0, 1.5 Hz, H-5), 8.22 (dd, 1H, *J* = 7.9, 1.7 Hz, H-6′). ^{13}C NMR (151 MHz, Acetone-d6) δ = 56.36 (C-4′′-OCH_3), 60.58 (C-4′′-OCH_3), 61.99 (C-6′′), 75.02 (C-2′′), 77.21 (C-5″), 78.13 (C-3′′), 80.01 (C-4′′), 102.24 (C-1′′), 112.68 (C-3), 113.06 (C-3′), 118.59 (C-5), 120.84 (C-7), 121.26 (C-1′), 121.83 (C-5′), 125.43 (C-6), 125.76 (C-4a), 130.59 (C-6′), 133.51 (C-4′), 147.70 (C-8a), 148.02 (C-8), 159.23 (C-2′), 160.88 (C-2), 178.08 (C-4).

5′-*O*-*β*-D-(4′′-*O*-Methylglucopyranosyl)-2′-Methoxyflavone (**8**)

^{1}H NMR (600 MHz) (DMSO) δ (ppm): 3.02 (t, 1H, *J* = 9.4 Hz, H-4′′), 3.26 (td, 1H, *J* = 9.0, 4.0 Hz, H-2′′), 3.40–3.45 (m, 2H, H-3′′, H-5″), 3.46 (s, 3H, C-4′′-OCH_3), 3.53 (ddd, 1H, *J* = 11.6, 6.2, 5.8 Hz, one of H-6′′), 3.67 (ddd, 1H, *J* = 11.4, 4.7, 1.4 Hz, one of H-6′′), 3.90 (s, 3H, C-2′′-OCH_3), 4.77 (t, 1H, *J* = 5.7 Hz, C-6′′-O*H*), 4.87 (d, 1H, *J* = 7.8 Hz, H-1′′), 5.29 (d, 1H, *J* = 5.4 Hz, C-3′′-O*H*), 5.43 (d, 1H, *J* = 4.5 Hz, C-2′′-O*H*), 6.99 (s, 1H, H-3), 7.20 (d, 1H, *J* = 9.2 Hz, H-3′), 7.27 (dd, 1H, *J* = 9.1, 3.1 Hz, H-4′), 7.50 (ddd, 1H, *J* = 8.0, 7.0, 1.0 Hz, H-6), 7.63 (dd, 1H, *J* = 3.0, Hz, H-6′), 7.76 (dd, 1H, *J* = 7.9, 0.6 Hz, H-8), 7.83 (ddd, 1H, *J* = 8.6, 7.0, 1.6 Hz, H-7), 8.05 (dd, 1H, *J* = 7.9, 1.6 Hz, H-5). ^{13}C NMR (151 MHz, DMSO) δ = 56.39 (C-2′-OCH_3), 59.71 (C-4′′-OCH_3), 60.39 (C-6′′), 73.48 (C-2′′), 75.73 (C-5″), 76.28

(C-3′′), 79.20 (C-4′′), 101.11 (C-1′′), 111.71 (C-3), 113.68 (C-3′), 116.93 (C-6′), 118.61 (C-8), 120.14 (C-1′), 120.88 (C-4′), 123.10 (C-4a), 124.71 (C-5), 125.45 (C-6), 134.33 (C-7), 151.17 (C-5′), 152.88 (C-2′), 155.91 (C-8a), 160.05 (C-2), 177.18 (C-4).

3-*O*-*β*-D-(4′′-*O*-Methylglucopyranosyl)-2′-Methoxyflavone (**9**)

^{1}H NMR (600 MHz) (DMSO) δ (ppm): 3.07 (t, 1H, *J* = 9.3 Hz, H-4′′), 3.27–3.37 (m, 2H, H-2′′ and H-5″), 3.40 (s, 3H, C-4′′-OCH_3), 3.41-3.55 (m, 2H, H-6′′), 3.79 (s, 3H, C-3-OCH_3), 4.45 (t, 1H, *J* = 5.3 Hz, C-6′′-O*H*), 5.14 (d, 1H, *J* = 5.5 Hz, 3′′-O*H*), 5.27 (d, 1H, *J* = 7.8 Hz, H-1′′), 5.31 (d, 1H, *J* = 4.7 Hz, C-2′′-O*H*), 7.06 (td, 1H, *J* = 7.4, 0.8 Hz, H-5′), 7.16 (d, 1H, *J* = 8.3 Hz, H-3′), 7.50-7.55 (m, 2H, H-4′ and H-6), 7.66 (dd, 1H, *J* = 8.4, 0.4 Hz, H-8), 7.73 (dd, 1H, *J* = 7.6, 1.7 Hz, H-6′), 7.82 (ddd, 1H, *J* = 8.6, 7.1, 1.7 Hz, H-7), 8.14 (dd, 1H, *J* = 8.0, 1.4 Hz, H-5).

2′-Hydroxyflavone (**10**)

^{1}H NMR (600 MHz) (Acetone-d$_6$) δ (ppm): 7.07 (ddd, 1H, *J* = 8.0, 7.3, 1.1 Hz, H-5′), 7.13 (dd, 1H, *J* = 8.2, 1.0 Hz, H-3′), 7.19 (s, 1H, H-3), 7.42 (ddd, 1H, *J* = 8.2, 7.3, 1.7 Hz, H-4′), 7.48 (ddd, 1H, *J* = 8.1, 7.1, 1.1 Hz, H-6), 7.70 (ddd, 1H, *J* = 8.4, 1.1, 0.5 Hz, H-8), 7.80 (dd, 1H, *J* = 8.8, 6.9, 1.6 Hz, H-7), 7.99 (dd, 1H, *J* = 7.9, 1.7 Hz, H-5), 10.03 (s, C-2′-O*H*). ^{13}C NMR (151 MHz, Acetone-d$_6$) δ = 112.54 (C-3), 117.93 (C-3′), 119.18 (C-8), 119.58 (C-1′), 120.91 (C-5′), 124.71 (C-4a), 125.85 (C-6), 125.89 (C-5), 129.73 (C-6′), 133.25 (C-4′), 134.62 (C-7), 157.16 (C-2′), 157.32 (C-8a), 161.76 (C-2), 178.25 (C-4).

3′-Hydroxyflavone (**11**)

^{1}H NMR (600 MHz) (DMSO) δ (ppm): 6.94 (s, 1H, H-3), 7.01 (ddd, 1H, *J* = 8.1, 2.5, 0.9 Hz, H-4′), 7.38 (t, 1H, *J* = 7.9 Hz, H-5′), 7.44 (t, 1H, *J* = 1.9 Hz, H-2′), 7.51 (ddd, 1H, *J* = 8.0, 6.9, 1.1 Hz, H-6), 7.53 (ddd, 1H, *J* = 7.8, 1.8, 1.0 Hz, H-6′), 7.77 (ddd, 1H, *J* = 8.4, 1.2, 0.5 Hz, H-8), 7.86 (ddd, 1H, *J* = 8.5, 6.9, 1.6 Hz, H-7), 8.06 (ddd, 1H, *J* = 7.9, 1.7, 0.5 Hz, H-5), 9.90 (s, C-3′-O*H*). ^{13}C NMR (151 MHz, DMSO) δ = 106.93 (C-3), 112.85 (C-2′), 117.23 (C-6′), 118.53 (C-8), 118.88 (C-4′), 123.36 (C-4a), 124.82 (C-5), 125.57 (C-6), 130.29 (C-5′), 132.45 (C-1′), 134.38 (C-7), 155.68 (C-8a), 157.92 (C-3′) 162.73 (C-2), 177.10 (C-4).

3′-*O*-*β*-D-(4′′-*O*-Methylglucopyranosyl)-Flavone (**12**)

^{1}H NMR (600 MHz) (DMSO) δ (ppm): 3.05 (t, 1H, *J* = 9.2 Hz, H-4′′), 3.25–3.34 (m, 1H, H-2′′), 3.42–3.57 (m, 3H, H-3′′, H-5″and one of H-6′′), 3.47 (s, 3H, C-4′′-OCH_3), 3.68 (dd, 1H, *J* = 10.0, 4.9 Hz, one of H-6′′), 4.80 (t, 1H, *J* = 5.2 Hz, C-6′′-O*H*), 5.07 (d, 1H, *J* = 7.8 Hz, H-1′′), 5.31 (d, 1H, *J* = 5.5 Hz, C-3′′-O*H*), 5.46 (d, 1H, *J* = 5.2 Hz, C-2′′-O*H*), 7.07 (s, 1H, H-3), 7.26 (dd, 1H, *J* = 8.2, 2.4 Hz, H-4′), 7.47-7.55 (m, 2H, H-5′ and H-6), 7.73 (d, 1H, *J* = 2.0 Hz, H-2′), 7.76 (d, 1H, *J* = 7.8 Hz, H-8), 7.79-7.87 (m, 2H, H-6′ and H-7), 8.06 (d, 1H, *J* = 7.9 Hz, H-5). ^{13}C NMR (151 MHz, DMSO) δ = 59.76 (C-4′′-OCH$_3$), 60.35 (C-6′′), 73.48 (C-2′′), 75.75 (C-5″), 76.34 (C-3′′), 79.19 (C-4′′), 100.02 (C-1′′), 107.24 (C-3), 113.84 (C-2′), 118.69 (C-6′), 119.79 (C-4′), 120.00 (C-8), 123.36 (C-4a), 124.81 (C-5), 125.63 (C-6), 130.31 (C-5′), 132.52 (C-1′), 134.43 (C-7), 155.72 (C-8a), 157.81 (C-3′), 162.25 (C-2), 177.23 (C-4).

4′-Hydroxyflavone (**13**)

^{1}H NMR (600 MHz) (DMSO) δ (ppm): 6.88 (s, 1H, H-3), 6.92-6.96 (m, 2H, H-3′, H-5′), 7.48 (ddd, 1H, *J* = 8.0, 7.0, 1.2 Hz, 1H, H-6), 7.75 (ddd, 1H, *J* = 8.4, 1.2, 0.4 Hz, H-8), 7.81 (ddd, 1H, *J* = 8.5, 6.9, 1.7 Hz, H-7), 7.95-7.99 (m, 2H, H-2′, H-6′), 8.03 (dd, 1H, *J* = 7.9, 1.7, 0.4 Hz, H-5), 10.32 (s, C-4′-O*H*). ^{13}C NMR (151 MHz, DMSO) δ = 104.83 (C-3), 115.97 (C-3′ and C-5′), 118.39 (C-8), 121.60 (C-1′), 123.34 (C-4a), 124.75 (C-5), 125.35 (C-6), 128.39 (C-2′ and C-6′), 134.06 (C-7), 155.61 (C-8a), 161.00 (C-4′), 163.09 (C-2), 176.91 (C-4).

4′-*O*-*β*-D- (4′′-*O*-Methylglucopyranosyl)-Flavone (**14**)

^{1}H NMR (600 MHz) (DMSO) δ (ppm): 3.06 (t, 1H, *J* = 9.3 Hz, H-4′′), 3.29 (ddd, 1H, *J* = 9.1, 7.8, 5.2 Hz, H-2′′), 3.42-3.49 (m, 2H, H-3′′, H-5″), 3.47 (s, 3H, C-4′′-OCH_3), 3.52 (ddd, 1H, *J* = 11.5, 6.4, 5.1 Hz, one of H-6′′), 3.65 (ddd, *J* = 11.4, 5.0, 1.5 Hz, one of H-6′′), 4.74 (dd, 1H, *J* = 6.2, 5.1 Hz, C-6′′-O*H*), 5.07 (d, 1H, *J* = 7.8 Hz, H-1′′), 5.30 (d, 1H, *J* = 5.5 Hz, C-3′′-O*H*), 5.48 (d, 1H, *J* = 5.2 Hz, C-2′′-O*H*), 6.99 (s, 1H, H-3), 7.18-7.22 (m, 2H, H-3′, H-5′), 7.50 (ddd, 1H, *J* = 8.1, 6.8, 1.3 Hz, H-6), 7.78 (ddd, 1H, *J* = 8.5, 1.3, 0.4 Hz, H-8), 7.83 (ddd, 1H, *J* = 8.5, 6.8, 1.7 Hz, H-7), 8.04 (ddd, 1H, *J* = 7.9, 1.6, 0.5 Hz, H-5), 8.06-8.10 (m, 2H, H-2′, H-6′). ^{13}C NMR (151 MHz, DMSO) δ = 59.74 (C-4′′-OCH_3), 60.18 (C-6′′), 73.37 (C-2′′), 75.69 (C-5″), 76.25 (C-3′′), 78.94 (C-4′′), 99.50 (C-1′′), 105.76 (C-3), 116.54 (C-3′ and C-5′), 118.50 (C-8), 123.33 (C-4a), 124.41 (C-5), 124.78 (C-6), 125.46 (C-1′), 128.11 (C-2′ and C-6′), 134.20 (C-7), 155.65 (C-8a), 160.05 (C-4′), 162.46 (C-2), 177.02 (C-4).

2′-*O*-*β*-D-(4′′-*O*-Methylglucopyranosyl)-5′-Methoxyflavone (**15**)

^{1}H NMR (600 MHz) (DMSO) δ (ppm): 3.05 (t, 1H, *J* = 9.4 Hz, H-4′′), 3.25-3.29 (m, 1H, H-2′′), 3.40-3.45 (m, 2H, H-3′′, H-5″), 3.45 (s, 3H, C-4′′-OCH_3), 3.51-3.55 (m, 1H, one of H-6′′), 3.63 (ddd, 1H, *J* = 12.1, 4.8, 1.7 Hz, one of H-6′′), 3.81 (s, 3H, C-6′′-OCH_3), 4.69 (d, 1H, *J* = 5.5 Hz, C-6′′-O*H*), 5.03 (d, 1H, *J* = 7.8 Hz, H-1′′), 5.24 (d, 1H, *J* = 5.8 Hz, C-3′′-O*H*), 5.34 (d, *J* = 5.6 Hz, C-2′′-O*H*), 7.13 (dd, 1H, *J* = 9.2, 3.2 Hz, H-4′), 7.30 (d, 1H, *J* = 9.2 Hz, H-3′), 7.44 (d, 1H, *J* = 3.1 Hz, H-6′), 7.50 (ddd, 1H, *J* = 8.1, 7.0, 1.1 Hz, H-6), 7.77 (dd, 1H, *J* = 8.3, 0.7 Hz, H-8), 7.83 (ddd, 1H, *J* = 8.6, 7.1, 1.8 Hz, H-7), 8.05 (dd, 1H, *J* = 7.9, 1.8 Hz, H-5). ^{13}C NMR (151 MHz, DMSO) δ = 55.73 (C-2′-OCH_3), 59.72 (C-4′′-OCH_3), 60.21 (C-6′′), 73.55 (C-2′′), 75.70 (C-5″), 76.56 (C-3′′), 78.96 (C-4′′), 100.56 (C-1′′), 112.36 (C-3), 113.87 (C-6′), 117.13 (C-3′), 117.99 (C-4′), 118.71 (C-8), 121.58 (C-1′), 123.20 (C-4a), 124.71 (C-5), 125.37 (C-6), 134.18 (C-7), 149.34 (C-5′), 153.94 (C-2′), 155.99 (C-8a), 160.14 (C-2), 177.27 (C-4).

4′-*O*-*β*-D-(4′′-*O*-Methylglucopyranosyl)-2′,5′-Dimethoxyflavone (**16**)

^{1}H NMR (600 MHz) (DMSO) δ (ppm): 3.00 (t, 1H, *J* = 9.2 Hz, H-4′′), 3.32–3.37 (m, 1H, H-2′′), 3.39–3.43 (m, 1H, H-5″), 3.46 (s, 3H, C-4′′-OCH_3), 3.47-3.54 (m, 2H, H-3′′ and one of H-6′′), 3.64-3.69 (m, 1H, one of H-6′′), 3.84 (s, 3H, C-5″-OCH_3), 3.90 (s, 3H, C-2′′-OCH_3), 4.79 (t, 1H, *J* = 5.1 Hz, C-6′′-O*H*), 5.14 (d, 1H, *J* = 7.9 Hz, H-1′′), 5.32 (d, 1H, *J* = 5.6 Hz, C-3′′-O*H*), 5.49 (d, 1H, *J* = 5.6 Hz, C-2′′-O*H*), 6.98 (s, 1H, H-3), 7.00 (s, 1H, H-3′), 7.48 (ddd, 1H, *J* = 8.0, 7.1, 1.3 Hz, H-6), 7.56 (s, 1H, H-6′), 7.79 (dd, 1H, *J* = 8.4, 1.3 Hz, H-8), 7.81 (ddd, 1H, *J* = 8.5, 7.1, 1.5 Hz, H-7), 8.03 (dd, 1H, *J* = 7.9, 1.5 Hz, H-5). ^{13}C NMR (151 MHz, DMSO) δ = 56.41 (C-2′-OCH_3), 56.69 (C-6′-OCH_3), 59.75 (C-4′′-OCH_3), 60.39 (C-6′′), 73.27 (C-2′′), 75.96 (C-5″), 76.67 (C-3′′), 79.36 (C-4′′), 99.48 (C-1′′), 101.40 (C-3′), 110.56 (C-3), 111.82 (C-1′), 113.06 (C-6′), 119.60 (C-8), 123.11 (C-4a), 124.66 (C-5), 125.29 (C-6), 134.33 (C-7), 142.81 (C-4′), 150.23 (C-5′), 153.23 (C-2′), 155.78 (C-8a), 160.31 (C-2), 177.12 (C-4).

5′-Hydroxy-2′-Methoxyflavone (**17**)

^{1}H NMR (600 MHz) (DMSO) δ (ppm): 3.84 (s, 3H, C-2′-OCH_3), 6.94 (s, 1H, H-3), 6.97 (dd, 1H, *J* = 8.9, 3.0 Hz, H-4′), 7.09 (d, 1H, *J* = 9.0 Hz, H-3′), 7.32 (d, 1H, *J* = 3.0 Hz, H-6′), 7.50 (ddd, *J* = 8.0, 7.1, 0.8 Hz, H-6), 7.71 (d, 1H, *J* = 8.2 Hz, H-8), 7.83 (ddd, 1H, *J* = 8.6, 7.2, 1.7 Hz, H-7), 8.05 (dd, 1H, *J* = 7.9, 1.4 Hz, H-5), 9.41 (s, C-5′-O*H*). ^{13}C NMR (151 MHz, DMSO) δ = 56.33 (C-2′-OCH_3), 111.57 (C-3), 114.07 (C-3′), 115.01 (C-6′), 118.43 (C-8), 119.36 (C-4′), 120.29 (C-1′), 123.13 (C-4a), 124.77 (C-5), 125.42 (C-6), 134.33 (C-7), 150.80 (C-2′), 151.12 (C-5′), 155.89 (C-8a), 160.53 (C-2), 177.16 (C-4).

4′-Hydroxy-2′,5′-Dimethoxyflavone (**18**)

^{1}H NMR (600 MHz) (DMSO) δ (ppm): 3.84 (s, 3H, C-5′-OCH_3), 3.85 (s, 3H, C-2′-OCH_3), 6.70 (s, 1H, H-3′), 6.95 (s, 1H, H-3), 7.47 (ddd, 1H, *J* = 8.1, 6.4, 1.9 Hz, H-6), 7.53 (s, 1H, H-6′), 7.78 (ddd, 1H, *J* = 8.6, 7.2, 1.7 Hz, H-7), 7.81 (d, 1H, *J* = 8.2 Hz, H-8), 8.02 (dd, 1H, *J* = 7.9, 1.5 Hz, H-5), 10.07 (s, C-4′-O*H*). ^{13}C NMR (151 MHz, DMSO) δ = 56.17 (C-5′-OCH_3), 56.64 (C-2′-OCH_3), 101.11 (C-3′), 109.36

(C-1′), 109.90 (C-3), 112.87 (C-6′), 118.51 (C-8), 123.11 (C-4a), 124.63 (C-5), 125.16 (C-6), 133.89 (C-7), 141.87 (C-4′), 151.41 (C-5′), 153.86 (C-2′), 155.74 (C-8a), 160.67 (C-2), 177.08 (C-4).

3′-*O*-*β*-D-(4′′-*O*-Methylglucopyranosyl)-4′,5′-Dimethoxyflavone (**19**)

3.04 (t, 1H, *J* = 9.1 Hz, H-4′′), 3.31–3.36 (m, 1H, H-2′′), 3.48 (s, 3H, C-4′′-OCH_3), 3.46–3.58 (m, 3H, H-3′′, H-5″ and one of H-6′′), 3.70 (dd, 1H, *J* = 9.9, 5.0 Hz, one of H-6′′), 3.81 (s, 3H, C-4′-OCH_3), 3.91 (s, 3H, C-5′-OCH_3), 4.84 (t, 1H, *J* = 5.5 Hz, C-6′′-O*H*), 5.07 (d, 1H, *J* = 7.8 Hz, H-1′′), 5.32 (d, 1H, *J* = 5.6 Hz, C-3′′-O*H*), 5.46 (d, 1H, *J* = 5.7 Hz, C-2′′-O*H*), 7.11 (s, 1H, H-3), 7.41 (d, 1H, *J* = 2.0 Hz, H-6′), 7.49 (ddd, 1H, *J* = 8.1, 5.3, 2.9 Hz, H-6), 7.54 (d, 1H, *J* = 2.0 Hz, H-2′), 7.81-7.84 (m, 2H, H-7 and H-8), 8.04 (ddd, 1H, *J* = 8.0, 1.5, 0.6 Hz, H-5). ^{13}C NMR (151 MHz, DMSO) is presented in Table 7.

4′-*O*-*β*-D-(4′′-*O*-Methylglucopyranosyl)-3′,5′-Dimethoxyflavone (**20**)

3.03 (t, 1H, *J* = 9.1 Hz, H-4′′), 3.31–3.36 (m, 1H, H-2′′), 3.44 (s, 3H, C-4′′-OCH_3), 3.46–3.58 (m, 3H, H-3′′, H-5″ and one of H-6′′), 3.69 (dd, 1H, *J* = 9.9, 5.0 Hz, one of H-6′′), 3.90 (s, 6H, C-3′-OCH_3 and C-5′-OCH_3), 4.52 (dd, 1H, *J* = 5.9, 4.9 Hz, C-6′′-O*H*), 5.14 (d, 1H, *J* = 7.6 Hz, H-1′′), 5.19 (d, 1H, *J* = 5.5 Hz, C-3′′-O*H*), 5.23 (d, 1H, *J* = 4.9 Hz, C-2′′-O*H*), 7.14 (s, 1H, H-3), 7.39 (s, 2H, H-2′ and H-6′), 7.49 (m, 1H, H-6), 7.81-7.84 (m, 2H, H-7 and H-8), 8.04 (ddd, 1H, *J* = 8.0, 1.5, 0.6 Hz, H-5). ^{13}C NMR (151 MHz, DMSO) δ = 56.76 (C-3′-OCH_3 and C-5′-OCH_3), 59.66 (C-4′′-OCH_3), 60.34 (C-6′′), 74.40 (C-2′′), 76.08 (C-5″), 76.42 (C-3′′), 79.18 (C-4′′), 101.90 (C-1′′), 104.89 (C-2′, and C-6′), 106.78 (C-3), 118.73 (C-8), 123.33 (C-4a), 124.77 (C-5), 125.53 (C-6), 126.27 (C-1′), 134.21 (C-7), 137.35 (C-4′), 152.93 (C-3′ and C-5′), 155.71 (C-8a), 162.44 (C-2), 177.22 (C-4).

6-*O*-*β*-D-(4′′-*O*-Methylglucopyranosyl)-3′,4′,5′-Trimethoxyflavone (**21**)

^{1}H NMR (600 MHz) (DMSO) δ (ppm): 3.08 (t, 1H, *J* = 9.3 Hz, H-4′′), 3.28 (ddd, 1H, *J* = 9.1, 7.3, 4.7 Hz, H-2′′), 3.36–3.39 (m, 1H, H-5″), 3.41–3.46 (m, 2H, H-3′′ and one of H-6′′), 3.47 (s, 3H, C-4′′-OCH_3), 3.62–3.67 (m, 1H, one of H-6′′), 3.75 (s, 3H, C-4′-OCH_3), 3.91 (s, 6H, C-3′-OCH_3 and C-5′-OCH_3), 4.74 (dd, 1H, *J* = 6.4, 5.0 Hz, C-6′′-O*H*), 5.01 (d, 1H, *J* = 7.8 Hz, H-1′′), 5.28 (d, 1H, *J* = 5.5 Hz, C-3′′-O*H*), 5.46 (d, 1H, *J* = 5.2 Hz, C-2′′-O*H*), 7.14 (s, 1H, H-3), 7.39 (s, 2H, H-2′ and H-6′), 7.51 (dd, 1H, *J* = 9.1, 3.1 Hz, H-7), 7.58 (d, 1H, *J* = 3.1 Hz, H-5), 7.83 (d, 1H, *J* = 9.2 Hz, H-8). ^{13}C NMR (151 MHz, DMSO) is presented in Table 7.

3′-*O*-*β*-D-(4′′-*O*-Methylglucopyranosyl)-6-Hydroxy-4′,5′-Dimethoxyflavone (**22**)

^{1}H NMR (600 MHz) (DMSO) δ (ppm): 3.03 (t, 1H, *J* = 9.1 Hz, H-4′′), 3.30–3.35 (m, 1H, H-2′′), 3.47 (s, 3H, C-4′′-OCH_3), 3.43–3.57 (m, 3H, H-3′′, H-5″ and one of H-6′′), 3.68-372 (m, 1H, one of H-6′′), 3.80 (s, 3H, C-4′-OCH_3), 3.90 (s, 3H, C-5′-OCH_3), 4.81 (t, 1H, *J* = 5.4 Hz, C-6′′-O*H*), 5.05 (d, 1H, *J* = 7.9 Hz, H-1′′), 5.29 (d, 1H, *J* = 5.7 Hz, C-3′′-O*H*), 5.43 (d, 1H, *J* = 5.7 Hz, C-2′′-O*H*), 7.02 (s, 1H, H-3), 7.25 (dd, 1H, *J* = 9.0, 3.0 Hz, H-6), 7.31 (d, 1H, *J* = 3.0 Hz, H-8), 7.38 (d, 1H, *J* = 2.0 Hz, H-6′), 7.50 (d, 1H, *J* = 2.0 Hz, H-2′), 7.68 (d, 1H, *J* = 9.0 Hz, H-5), 10.02 (s, 1H, -O*H*). ^{13}C NMR (151 MHz, DMSO) is presented in Table 7.

3.5.3. LC-MS

Molecular formulas of products were confirmed by LC-MS 8045 SHIMADZU analysis. The mobile phase was a mixture of 0.1% aqueous formic acid v/v (A) and acetonitrile (B). The program was as follows: 80% B and 20% A in 5 min. The flow rate was 0.3 mL/min and the injection volume was 2 µl. The column (Kinetex 2.6 µm C18 100 Å, 100 mm x 3 mm, Phenomenex, Torrance, CA, USA) was operated at 30 °C. The major operating parameters were as follows: nebulizing gas flow: 3 L/min, heating gas flow: 10 L/min, interface temperature: 300°C, drying gas flow: 10 L/min, data acquisition range m/z 100–1000 Da; ionization mode—positive. Data were collected with LabSolutions (Shimadzu, Kyoto, Japan) software.

4. Conclusions

To sum up, obtaining 18 novel flavonoids via whole-cell fungal biotransformations was described, 13 of them being 4-*O*-methylglucosides and 5 hydroxy flavones. To the best of our knowledge, these flavone glucosides have never been reported before.

Our previous studies have shown that the ability of 4-*O*-methylglycosyl attachment to the hydroxyl group in the flavonoid molecule is unique for entomopathogenic filamentous fungi. In this publication, we have demonstrated the capacity of these strains to produce 4-*O*-methylglucoside flavonoids, the formation of which is preceded by the effective *O*-demethylation and/or hydroxylation of the methoxyflavones obtained by chemical synthesis.

The performed biotransformations resulted in products that may indicate that glycosylation is not a one-step process, but is likely the result of an enzyme cascade as intermediates also appear. Obtaining these products is, therefore, possible due to the use of whole cells cultures of biocatalysts containing many interacting enzymes.

Some of the entomopathogenic fungi used (e.g., *B. bassiana* KCh J1.5 or *I. fumosorosea* KCh J2) were able to introduce a sugar group into the flavone ring by hydroxylation and then 4-*O*-methylglycosylation. This sequence resulted in four different glycosylated products in a one-pot reaction. The demethylation and 4-*O*-methylglucosylation processes are fastest when the methoxy group is on the C-3′ carbon and slowest when the methoxy substituent is on the C-2′ carbon. However, placing the substituent at the C-2′ position results in the highest number of end products. We have obtained 13 new glycosylated flavones (4-*O*-methylglucopyranosides) and five hydroxy derivatives. Each of the obtained products was determined by means of HPLC, LC-MS and NMR (Supplementary materials Figures S1–S109).

Additionally, research on the properties of flavonoids, including their glycoside derivatives, is still ongoing, and efficient methods for obtaining such compounds are sought continuously. The methods presented in this publication allow significant amounts of glycoside derivatives to be obtained in an efficient and relatively cheap way while following the principles of "green chemistry".

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/21/17/6121/s1.

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Abbreviations

MDPI	Multidisciplinary Digital Publishing Institute
DOAJ	Directory of Open Access Journals
TLA	Three letter acronym
LD	linear dichroism

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Supplementary Data

Biotransformation of methoxyflavone by selected entomopathogenic filamentous fungi

Mateusz Łużny*, Tomasz Tronina, Ewa Kozłowska, Monika Dymarska, Jarosław Popłoński, Jacek Łyczko, Edyta Kostrzewa-Susłow and Tomasz Janeczko*

Department of Chemistry, Wrocław University of Environmental and Life Sciences, Norwida 25, 50-375 Wrocław, Poland; e.a.kozlowska@gmail.com (E.K.), ekostrzew@gmail.com (E.K-S.)

* Correspondence:mat.luzny@gmail.com(M.Ł.) janeczko13@interia.pl (T.J.); Tel.: +48-713-205-195

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Fig.S29. ^{1}H NMR spectral of 2'-hydroxyflavone (**10**) (Acetone-d_6, 600 MHz)
Fig.S30. ^{13}C NMR spectral of 2'-hydroxyflavone (**10**) (Acetone-d_6, 151 MHz)
Fig.S31. COSY spectral of 2'-hydroxyflavone (**10**) (Acetone-d_6, 151 MHz)
Fig.S32. HMQC spectral of 2'-hydroxyflavone (**10**) (Acetone-d_6, 151 MHz)
Fig.S33. HMBC spectral of 2'-hydroxyflavone (**10**) (Acetone-d_6, 151 MHz)
Fig.S34. MS analysis 3'-hydroxyflavone (**11**)
Fig.S35. ^{1}H NMR spectral of 3'-hydroxyflavone (**11**) (DMSO-d_6, 600 MHz)
Fig.S36. ^{13}C NMR spectral of 3'-hydroxyflavone (**11**) (DMSO-d_6, 151 MHz)
Fig.S37. COSY spectral of 3'-hydroxyflavone (**11**) (DMSO-d_6, 151 MHz)
Fig.S38. HMQC spectral of 3'-hydroxyflavone (**11**) (DMSO-d_6, 151 MHz)
Fig.S39. HMBC spectral of 3'-hydroxyflavone (**11**) (DMSO-d_6, 151 MHz)
Fig.S40. MS analysis flavone 3'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**12**)
Fig.S41. ^{1}H NMR spectral of flavone 3'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**12**) (DMSO-d_6, 600 MHz)
Fig.S42. Flavone part of the ^{1}H NMR spectral flavone 3'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**12**) (DMSO-d_6, 600 MHz)
Fig.S43. Glucopyranoside part of the ^{1}H NMR spectral flavone 3'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**12**) (DMSO-d_6, 600 MHz)
Fig.S44. ^{13}C NMR spectral of flavone 3'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**12**) (DMSO-d_6, 151 MHz)
Fig.S45. HMQC spectral of flavone 3'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**12**) (DMSO-d_6, 151 MHz)
Fig.S46. HMBC spectral of flavone 3'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**12**) (DMSO-d_6, 151 MHz)
Fig.S47. MS analysis 4'-hydroxyflavone (**13**)
Fig.S48. ^{1}H NMR spectral of 4'-hydroxyflavone (**13**) (DMSO-d_6, 600 MHz)
Fig.S49. ^{13}C NMR spectral of 4'-hydroxyflavone (**13**) (DMSO-d_6, 151 MHz)
Fig.S50. COSY spectral of 4'-hydroxyflavone (**13**) (DMSO-d_6, 151 MHz)
Fig.S51. HMQC spectral of 4'-hydroxyflavone (**13**) (DMSO-d_6, 151 MHz)
Fig.S52. HMBC spectral of 4'-hydroxyflavone (**13**) (DMSO-d_6, 151 MHz)
Fig.S53. MS analysis flavone 4'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**14**)
Fig.S54. ^{1}H NMR spectral of flavone 4'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**14**) (DMSO-d_6, 600 MHz)
Fig.S55. Flavone part of the ^{1}H NMR spectral flavone 4'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**14**) (DMSO-d_6, 600 MHz)
Fig.S56. Glucopyranoside part of the ^{1}H NMR spectral flavone 4'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**14**) (DMSO-d_6, 600 MHz)
Fig.S57. ^{13}C NMR spectral of flavone 4'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**14**) (DMSO-d_6, 151 MHz)
Fig.S58. COSY spectral of flavone 4'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**14**) (DMSO-d_6, 151 MHz)
Fig.S59. HMQC spectral of flavone 4'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**14**) (DMSO-d_6, 151 MHz)
Fig.S60. HMBC spectral of flavone 4'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**14**) (DMSO-d_6, 151 MHz)
Fig.S61. MS analysis 5'-methoxyflavone 2'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**15**)
Fig.S62. ^{1}H NMR spectral of 5'-methoxyflavone 2'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**15**) (DMSO-d_6, 600 MHz)
Fig.S63. Flavone part of the ^{1}H NMR spectral 5'-methoxyflavone 2'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**15**) (DMSO-d_6, 600 MHz)

Fig.S64. Glucopyranoside part of the ^{1}H NMR spectral 5'-methoxyflavone 2'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**15**) (DMSO-d_6, 600 MHz)
Fig.S65. ^{13}C NMR spectral of 5'-methoxyflavone 2'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**15**) (DMSO-d_6, 151 MHz)
Fig.S66. COSY spectral of 5'-methoxyflavone 2'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**15**) (DMSO-d_6, 151 MHz)
Fig.S67. HMQC spectral of 5'-methoxyflavone 2'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**15**) (DMSO-d_6, 151 MHz)
Fig.S68. HMBC spectral of 5'-methoxyflavone 2'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**15**) (DMSO-d_6, 151 MHz)
Fig.S69. MS analysis 2',5'-dimethoxyflavone 4'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**16**)
Fig.S70. ^{1}H NMR spectral of 2',5'-dimethoxyflavone 4'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**16**) (DMSO-d_6, 600 MHz)
Fig.S71. Flavone part of the ^{1}H NMR spectral 2',5'-dimethoxyflavone 4'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**16**) (DMSO-d_6, 600 MHz)
Fig.S72. Glucopyranoside part of the ^{1}H NMR spectral 2',5'-dimethoxyflavone 4'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**16**) (DMSO-d_6, 600 MHz)
Fig.S73. ^{13}C NMR spectral of 2',5'-dimethoxyflavone 4'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**16**) (DMSO-d_6, 151 MHz)
Fig.S74. COSY spectral of 2',5'-dimethoxyflavone 4'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**16**) (DMSO-d_6, 151 MHz)
Fig.S75. HMQC spectral of 2',5'-dimethoxyflavone 4'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**16**) (DMSO-d_6, 151 MHz)
Fig.S76. HMBC spectral of 2',5'-dimethoxyflavone 4'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**16**) (DMSO-d_6, 151 MHz)
Fig.S77. MS analysis 5'-hydroxy-2'-methoxyflavone (**17**)
Fig.S78. ^{1}H NMR spectral of 5'-hydroxy-2'-methoxyflavone (**17**) (DMSO-d_6, 600 MHz)
Fig.S79. ^{13}C NMR spectral of 5'-hydroxy-2'-methoxyflavone (**17**) (DMSO-d_6, 151 MHz)
Fig.S80. COSY spectral of 5'-hydroxy-2'-methoxyflavone (**17**) (DMSO-d_6, 151 MHz)
Fig.S81. HMQC spectral of 5'-hydroxy-2'-methoxyflavone (**17**) (DMSO-d_6, 151 MHz)
Fig.S82. HMBC spectral of 5'-hydroxy-2'-methoxyflavone (**17**) (DMSO-d_6, 151 MHz)
Fig.S83. MS analysis 4'-hydroxy-2',5'-dimethoxyflavone
Fig.S84. ^{1}H NMR spectral of 5'-hydroxy-2'-methoxyflavone (**17**) and 4'-hydroxy-2',5'-dimethoxyflavone (**18**) (DMSO-d_6, 600 MHz)
Fig.S85. Part of the ^{1}H NMR spectral 5'-hydroxy-2'-methoxyflavone (**17**) and 4'-hydroxy-2',5'-dimethoxyflavone (**18**) (DMSO-d_6, 600 MHz)
Fig.S86. ^{13}C NMR spectral of 5'-hydroxy-2'-methoxyflavone (**17**) and 4'-hydroxy-2',5'-dimethoxyflavone (**18**) (DMSO-d_6, 151 MHz)
Fig.S87. HMQC spectral of 5'-hydroxy-2'-methoxyflavone (**17**) and 4'-hydroxy-2',5'-dimethoxyflavone (**18**) (DMSO-d_6, 151 MHz)
Fig.S88. HMBC spectral of 5'-hydroxy-2'-methoxyflavone (**17**) and 4'-hydroxy-2',5'-dimethoxyflavone (**18**) (DMSO-d_6, 151 MHz)
Fig.S89. MS analysis 4',5'-dimethoxyflavone 5'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**19**) and 3',5'-dimethoxyflavone 4'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**20**)
Fig.S90. ^{1}H NMR spectral of 4',5'-dimethoxyflavone 5'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**19**) and 3',5'-dimethoxyflavone 4'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**20**) (DMSO-d_6, 600 MHz)
Fig.S91. Flavone part of the ^{1}H NMR spectral 4',5'-dimethoxyflavone 5'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**19**) and 3',5'-dimethoxyflavone 4'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**20**) (DMSO-d_6, 600 MHz)
Fig.S92. Glucopyranoside part of the ^{1}H NMR spectral 4',5'-dimethoxyflavone 5'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**19**) and 3',5'-dimethoxyflavone 4'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**20**) (DMSO-d_6, 600 MHz)

Fig.S93. ^{13}C NMR spectral of 4’,5’-dimethoxyflavone 5’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**19**) and 3’,5’-dimethoxyflavone 4’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**20**) (DMSO-d_6, 151 MHz)

Fig.S94. HMQC spectral of 4’,5’-dimethoxyflavone 5’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**19**) and 3’,5’-dimethoxyflavone 4’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**20**) (DMSO-d_6, 151 MHz)

Fig.S95. HMBC spectral of 4’,5’-dimethoxyflavone 5’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**19**) and 3’,5’-dimethoxyflavone 4’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**20**) (DMSO-d_6, 151 MHz)

Fig.S96. MS analysis 3’,4’,5’-trimethoxyflavone 6-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**21**)

Fig.S97. ^{1}H NMR spectral of 3’,4’,5’-trimethoxyflavone 6-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**21**) (DMSO-d_6, 600 MHz)

Fig.S98. Flavone part of the ^{1}H NMR spectral 3’,4’,5’-trimethoxyflavone 6-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**21**) (DMSO-d_6, 600 MHz)

Fig.S99. Glucopyranoside part of the ^{1}H NMR spectral 3’,4’,5’-trimethoxyflavone 6-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**21**) (DMSO-d_6, 600 MHz)

Fig.S100. ^{13}C NMR spectral of 3’,4’,5’-trimethoxyflavone 6-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**21**) (DMSO-d_6, 151 MHz)

Fig.S101. HMQC spectral of 3’,4’,5’-trimethoxyflavone 6-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**21**) (DMSO-d_6, 151 MHz)

Fig.S102. HMBC spectral of 3’,4’,5’-trimethoxyflavone 6-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**21**) (DMSO-d_6, 151 MHz)

Fig.S103. MS analysis 6-hydroxy-4’,5’-dimethoxyflavone 3’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**22**)

Fig.S104. ^{1}H NMR spectral of 6-hydroxy-4’,5’-dimethoxyflavone 3’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**22**) (DMSO-d_6, 600 MHz)

Fig.S105. Flavone part of the ^{1}H NMR spectral 6-hydroxy-4’,5’-dimethoxyflavone 3’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**22**) (DMSO-d_6, 600 MHz)

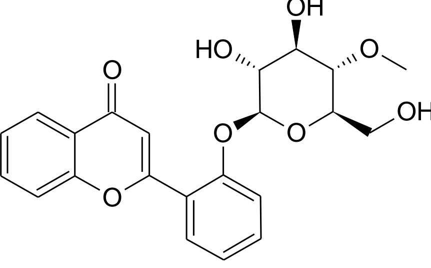
Fig.S106. Glucopyranoside part of the ^{1}H NMR spectral 6-hydroxy-4’,5’-dimethoxyflavone 3’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**22**) (DMSO-d_6, 600 MHz)

Fig.S107. ^{13}C NMR spectral of 6-hydroxy-4’,5’-dimethoxyflavone 3’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**22**) (DMSO-d_6, 151 MHz)

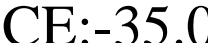
Fig.S108. HMQC spectral of 6-hydroxy-4’,5’-dimethoxyflavone 3’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**22**) (DMSO-d_6, 151 MHz)

Fig.S109. HMBC spectral of 6-hydroxy-4’,5’-dimethoxyflavone 3’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**22**) (DMSO-d_6, 151 MHz)

Fig.S1. MS analysis flavone 2'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**6**)



Molecular Formula = $C_{22}H_{22}O_8$
Formula Weight = 414.40528
Precursor: = 415.4000
CE (collision energy): -15.0


CE:-35.0

CE:-45.0

Fig.S2. ^{1}H NMR spectral of flavone 2’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**6**) (DMSO-d_6, 600 MHz)

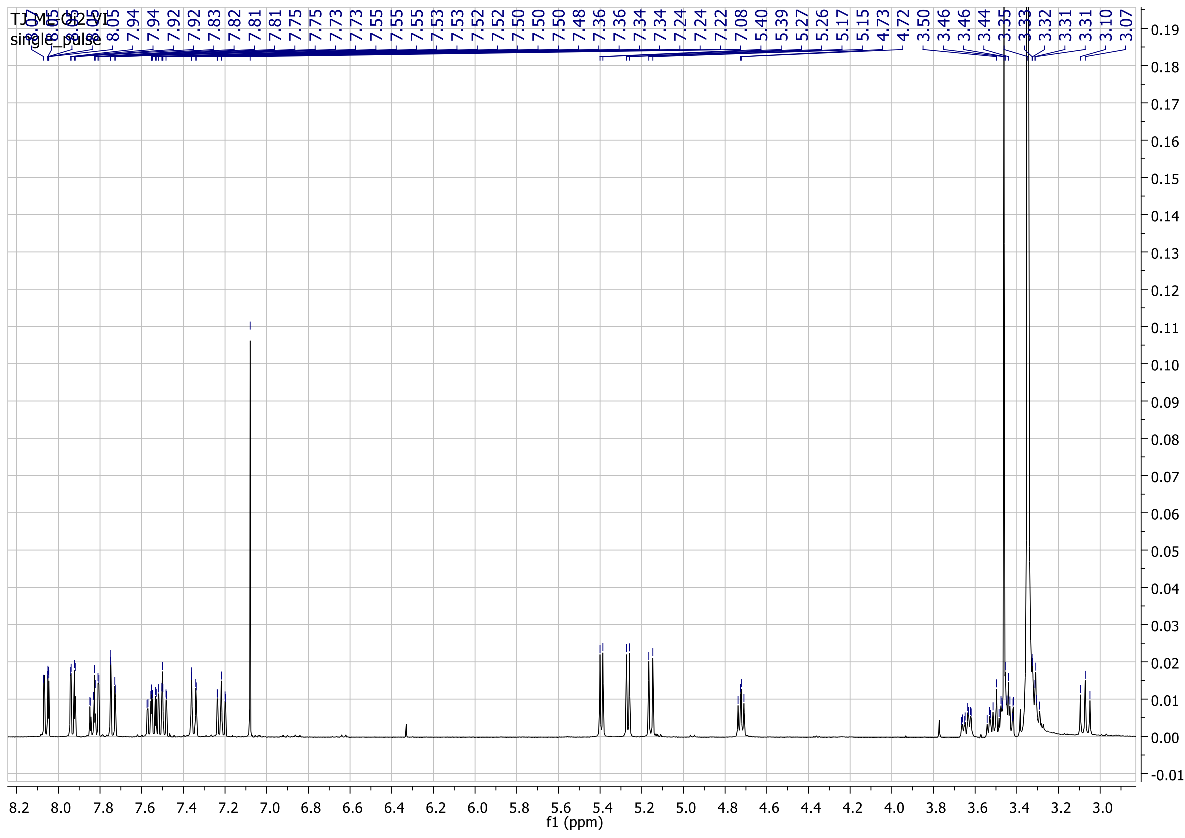


Fig.S3. Flavone part of the ^{1}H NMR spectral flavone 2’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**6**) (DMSO-d_6, 600 MHz)

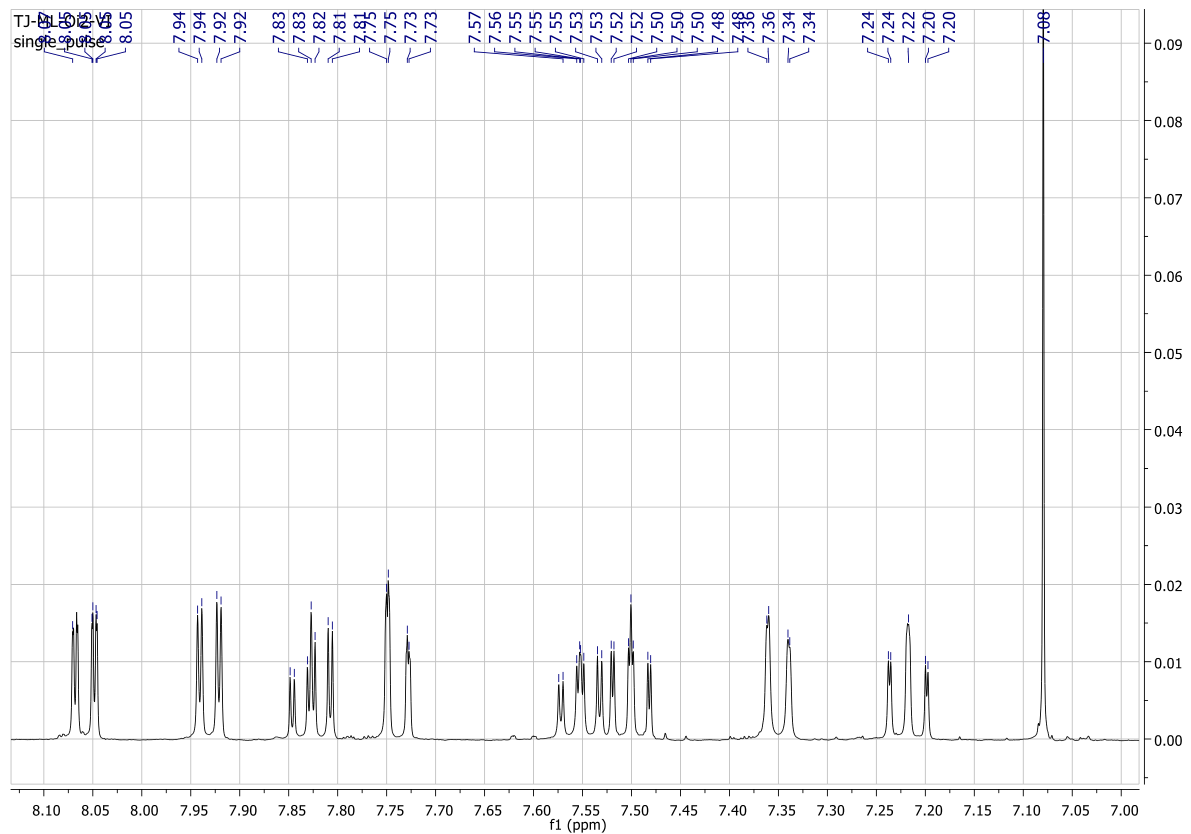


Fig.S4. Glucopyranoside part of the ^{1}H NMR spectral flavone 2’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**6**) (DMSO-d_6, 600 MHz)

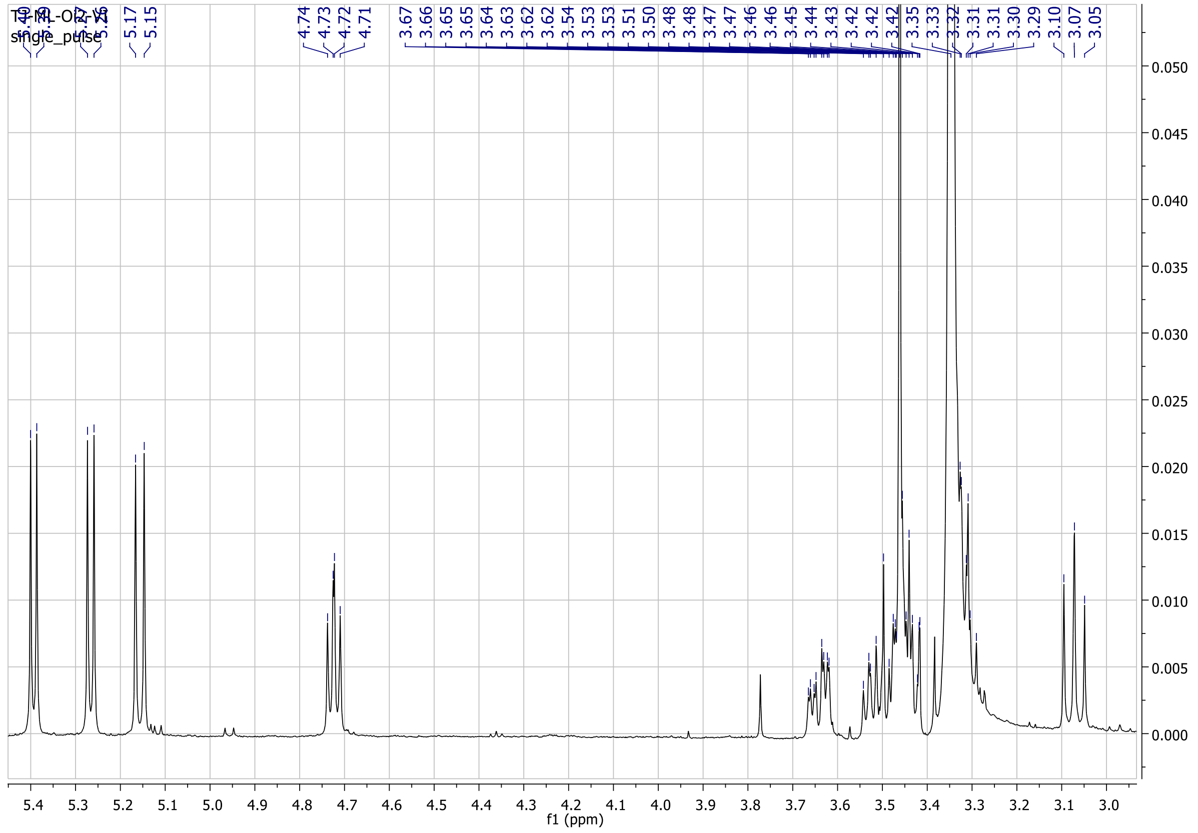


Fig.S5. ^{13}C NMR spectral of flavone 2’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**6**) (DMSO-d_6, 151 MHz)

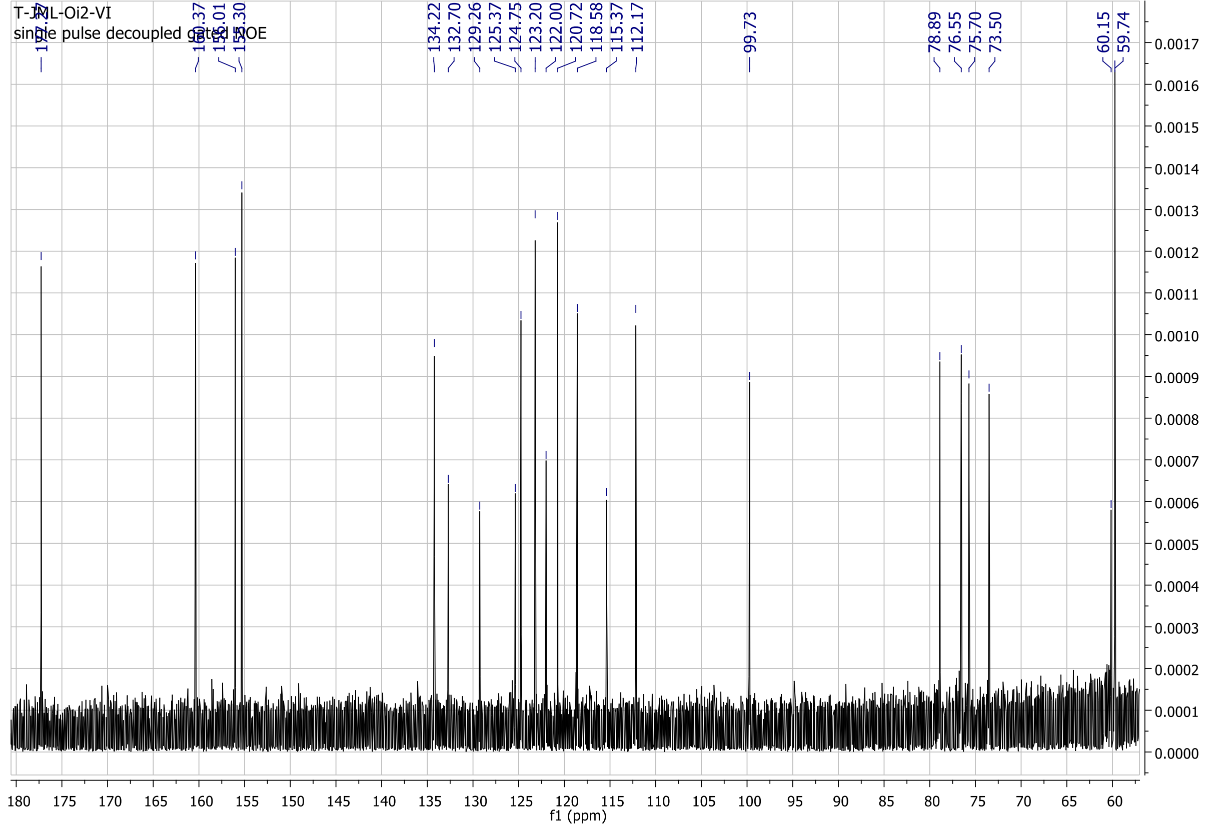


Fig.S6. COSY spectral of flavone 2'-*O*-β-D-(4''-*O*-methyl)-glucopyranoside (**6**) (DMSO-d_6, 151 MHz)

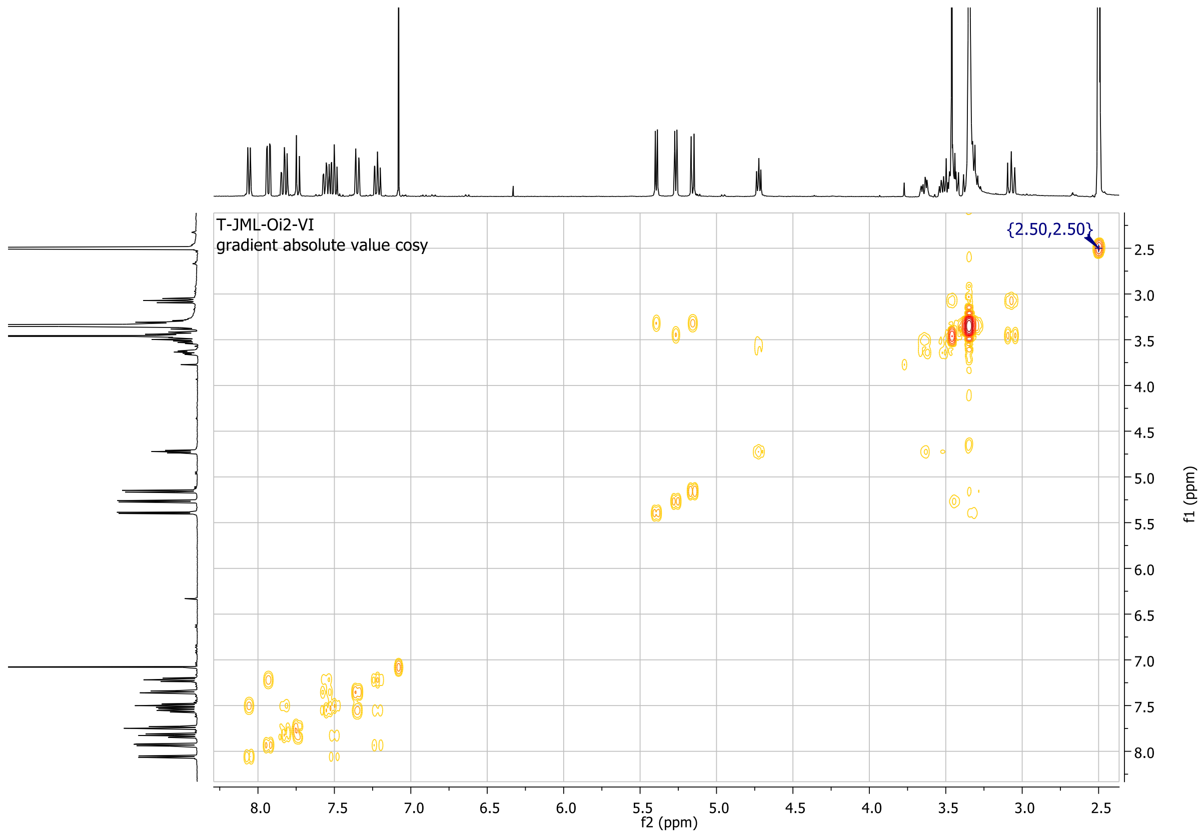


Fig.S7. HMQC spectral of flavone 2'-*O*-β-D-(4''-*O*-methyl)-glucopyranoside (**6**) (DMSO-d_6, 151 MHz)

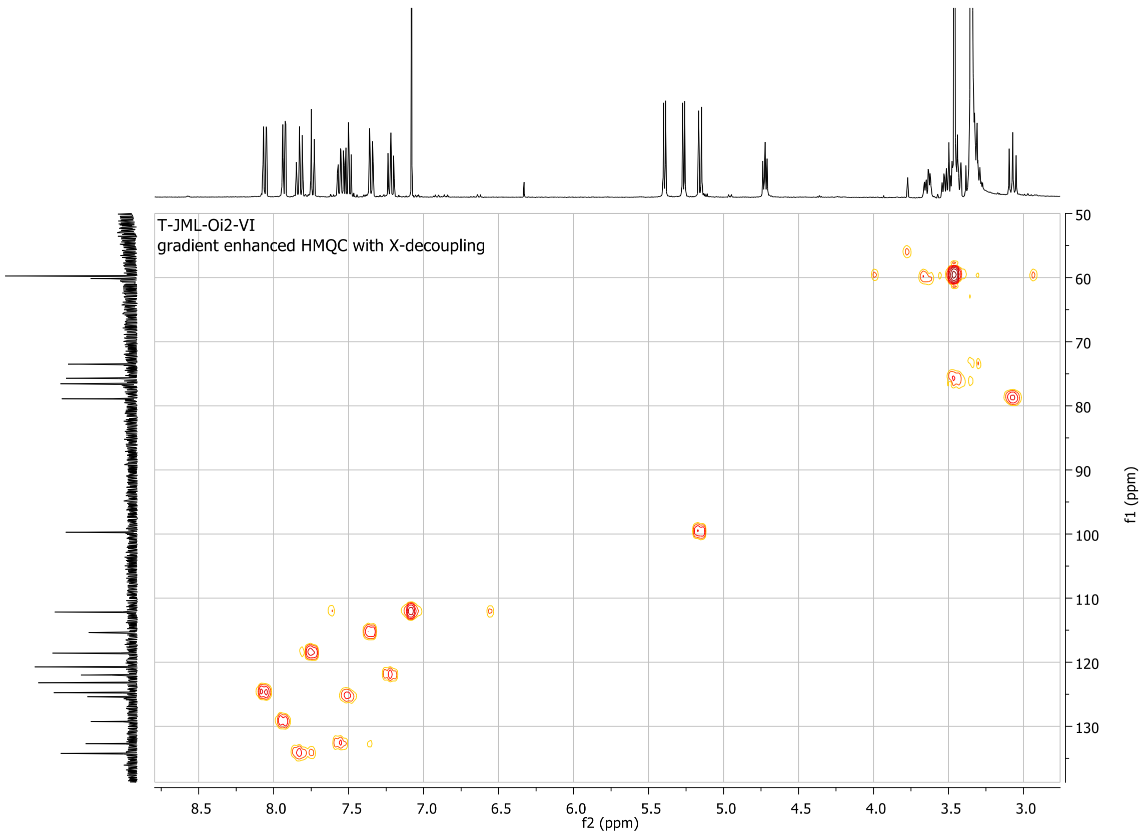


Fig.S8. HMBC spectral of flavone 2’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**6**) (DMSO-d_6, 151 MHz)

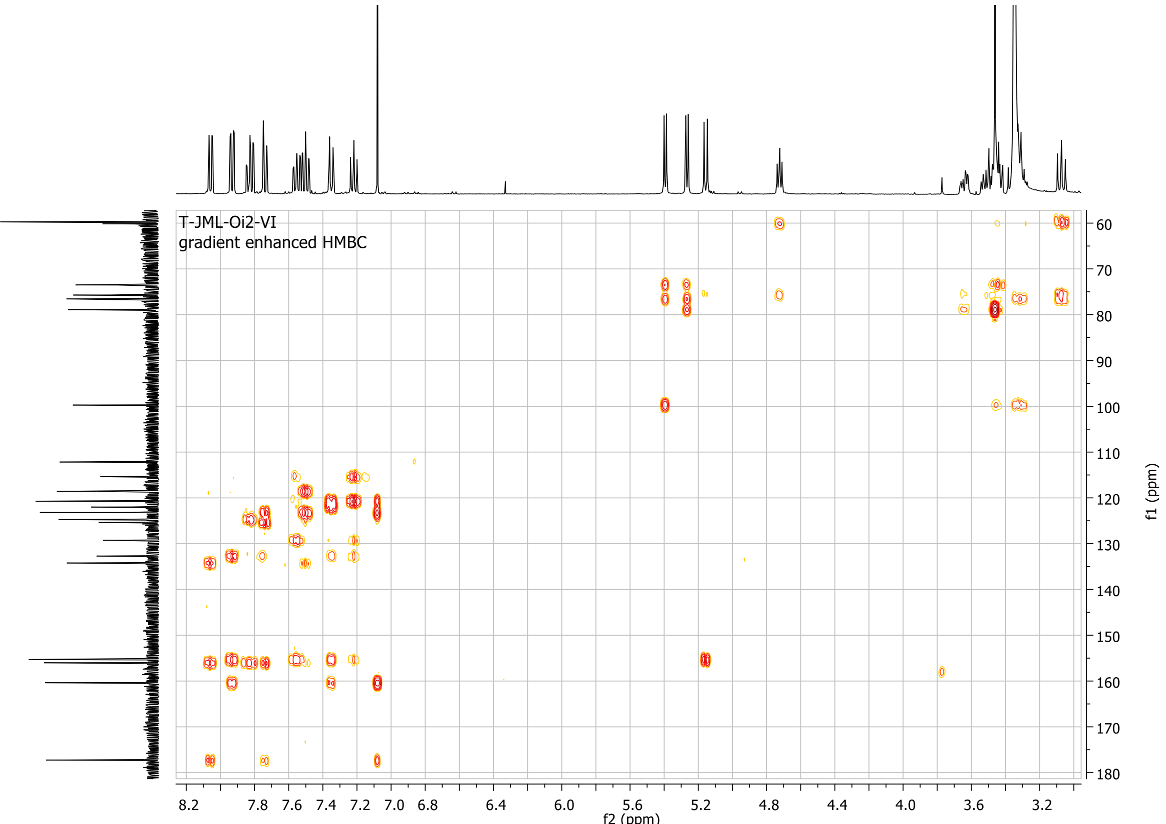
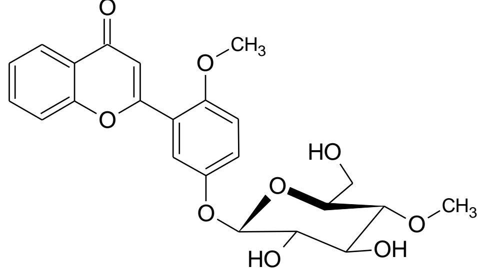


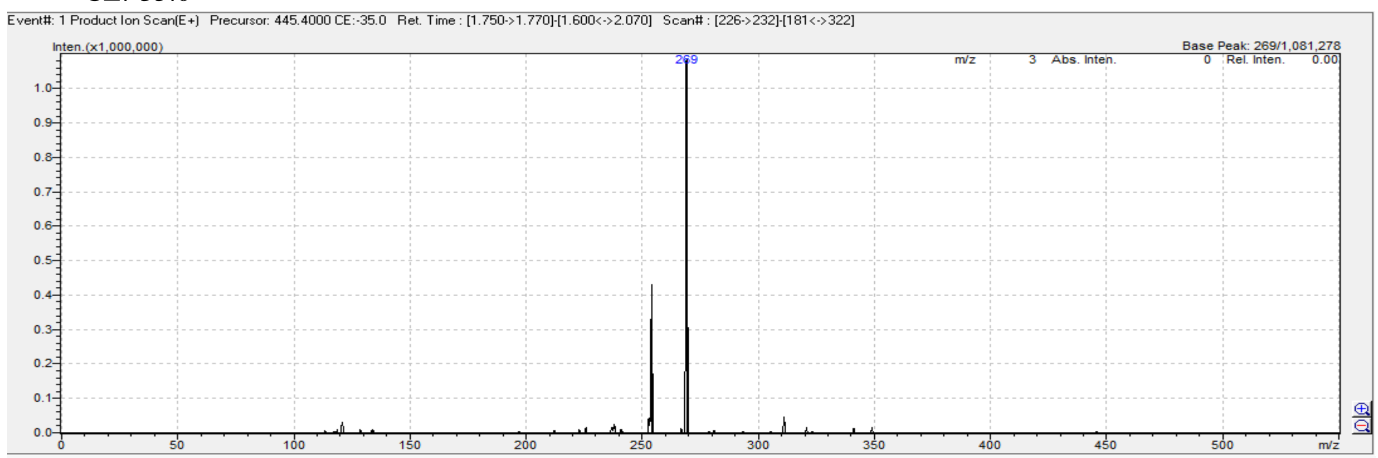
Fig.S9. MS analysis 2'-methoxyflavone 5'-*O*-β-D-(4''-*O*-methyl)-glucopyranoside (**8**)



Molecular Formula = $C_{23}H_{24}O_9$
Formula Weight = 444.43126
Precursor =445.4000

CE: -15.0

CE:-35.0



CE:-45.0

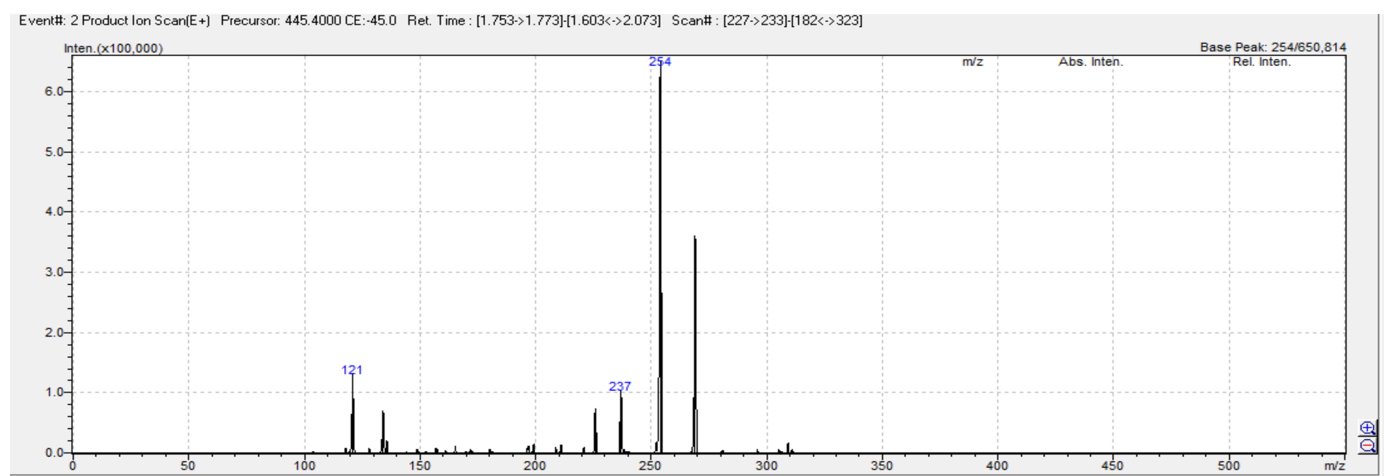


Fig.S10. ^{1}H NMR spectral of 2’-methoxyflavone 5’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**8**) (Acetone-d_6, 600 MHz)

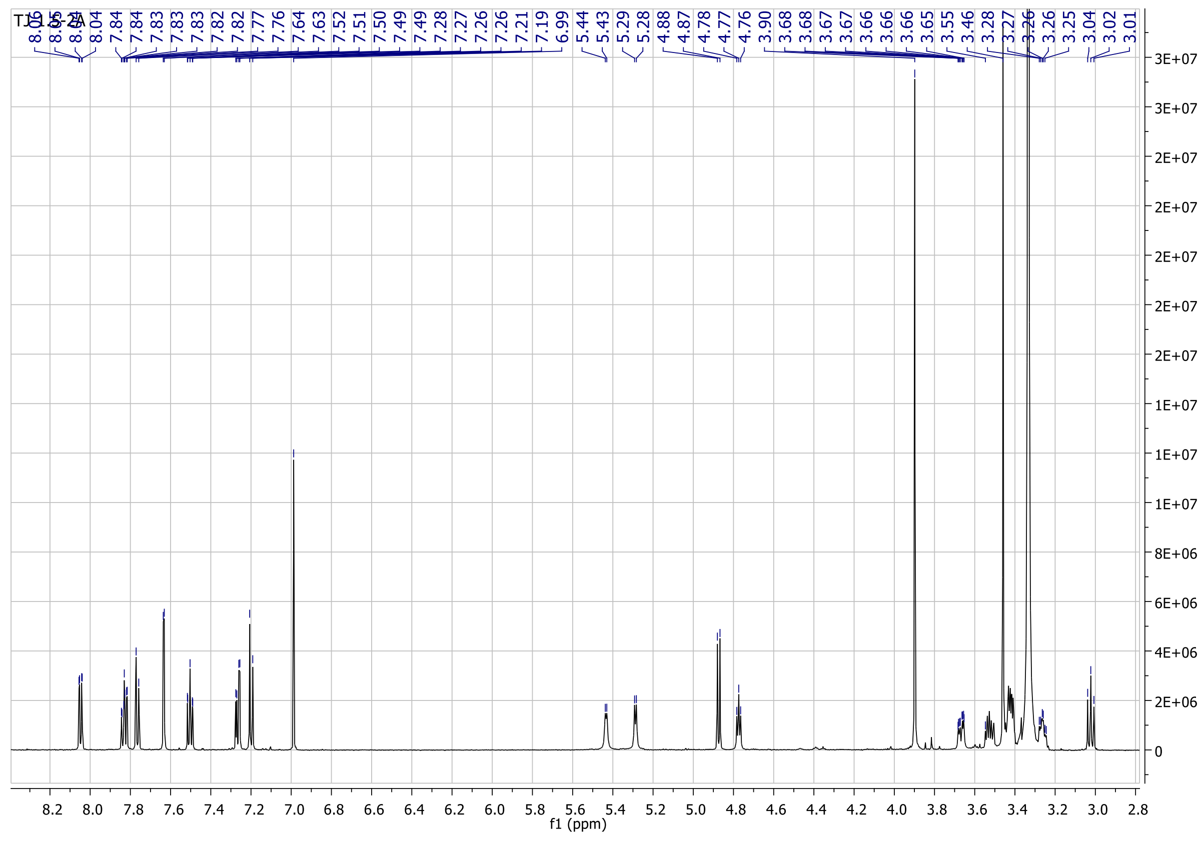


Fig.S11. Flavone part of the ^{1}H NMR spectral 2’-methoxyflavone 5’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**8**) (Acetone-d_6, 600 MHz)

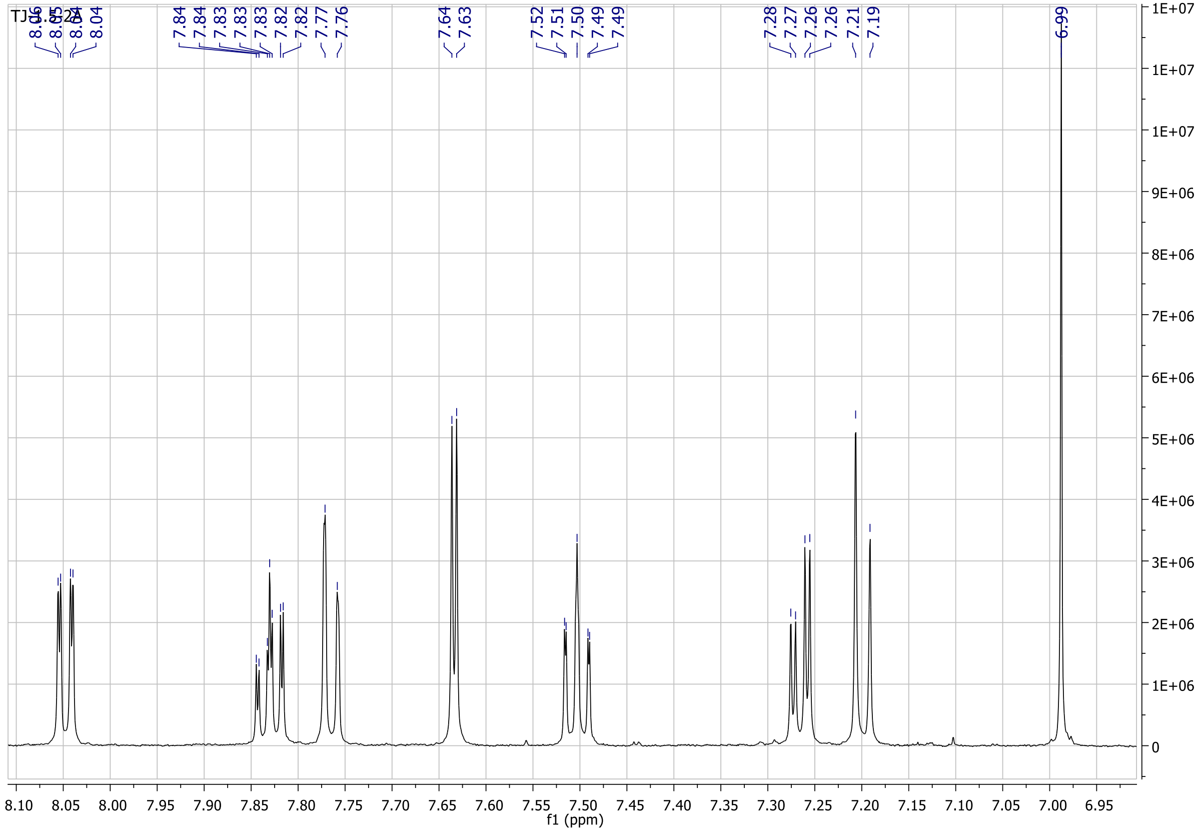


Fig.S12. Glucopyranoside part of the ^{1}H NMR spectral 2'-methoxyflavone 5'-*O*-β-D-(4''-*O*-methyl)-glucopyranoside (**8**) (Acetone-d_6, 600 MHz)

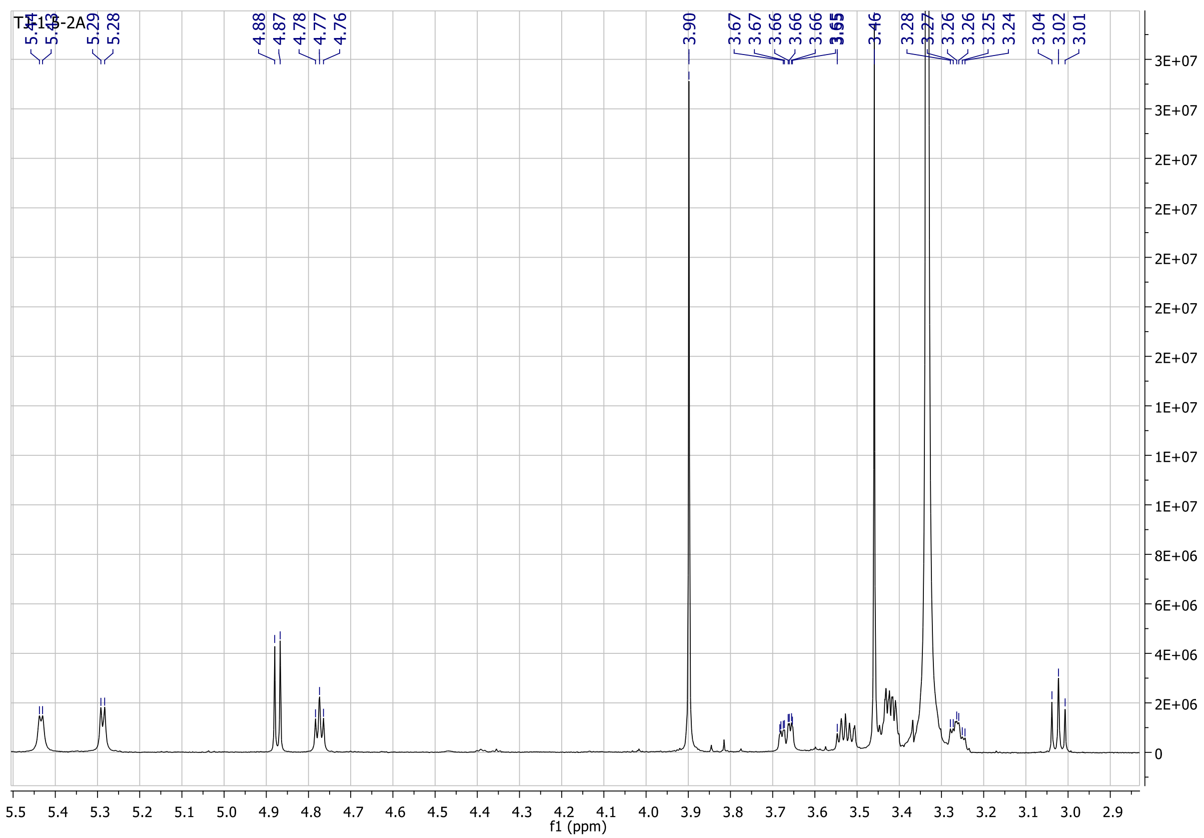


Fig.S13. ^{13}C NMR spectral of 2'-methoxyflavone 5'-*O*-β-D-(4''-*O*-methyl)-glucopyranoside (**8**) (Acetone-d_6, 151 MHz)

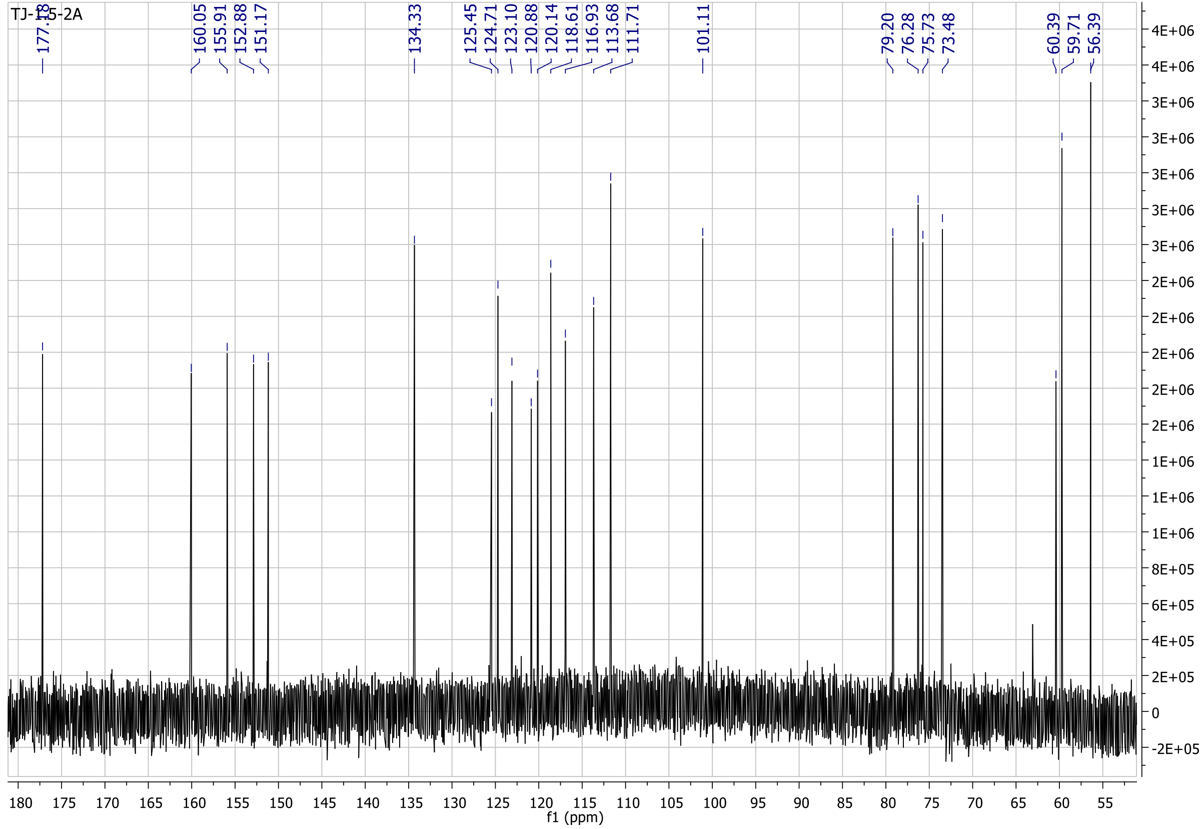


Fig.S14. COSY spectral of 2'-methoxyflavone 5'-*O*-β-D-(4''-*O*-methyl)-glucopyranoside (**8**) (Acetone-d_6, 151 MHz)

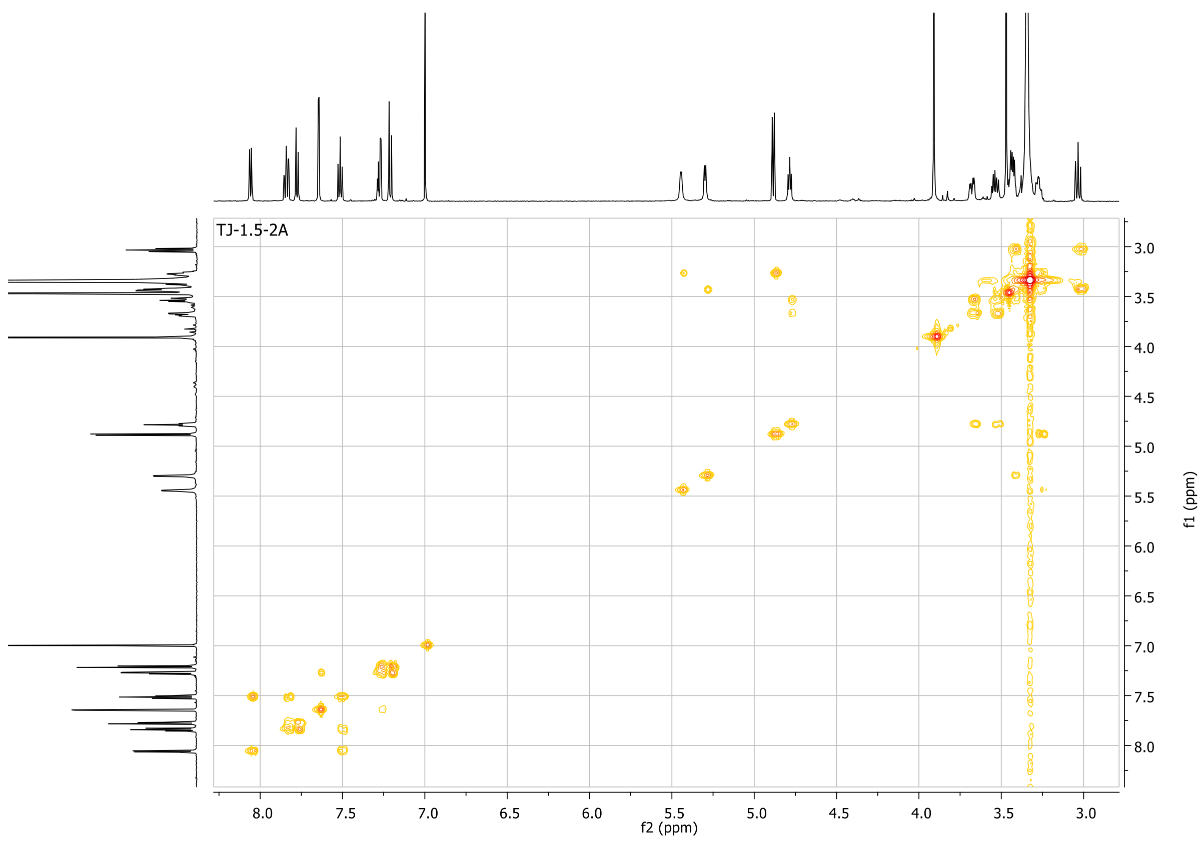


Fig.S15. HMQC spectral of 2'-methoxyflavone 5'-*O*-β-D-(4''-*O*-methyl)-glucopyranoside (**8**) (Acetone-d_6, 151 MHz)

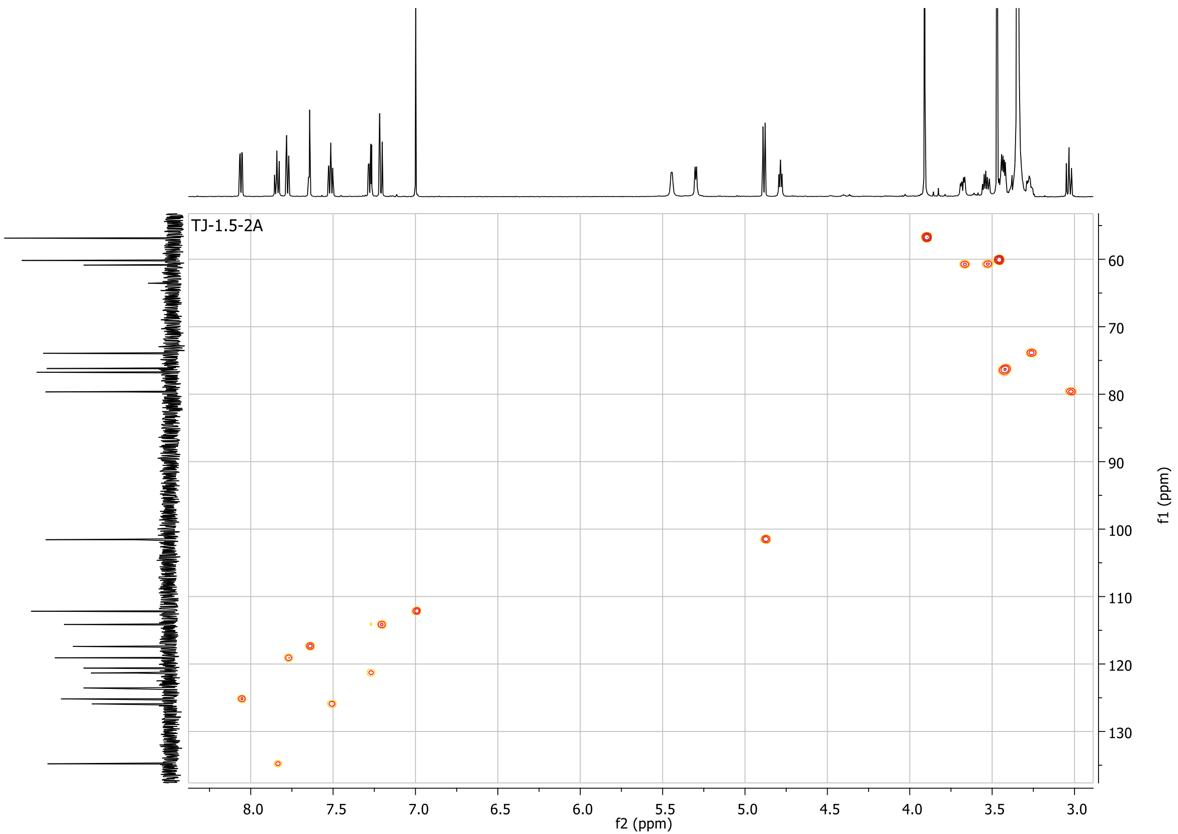


Fig.S16. HMBC spectral of 2'-methoxyflavone 5'-*O*-β-D-(4''-*O*-methyl)-glucopyranoside flavone (**8**) (Acetone-d_6, 151 MHz)

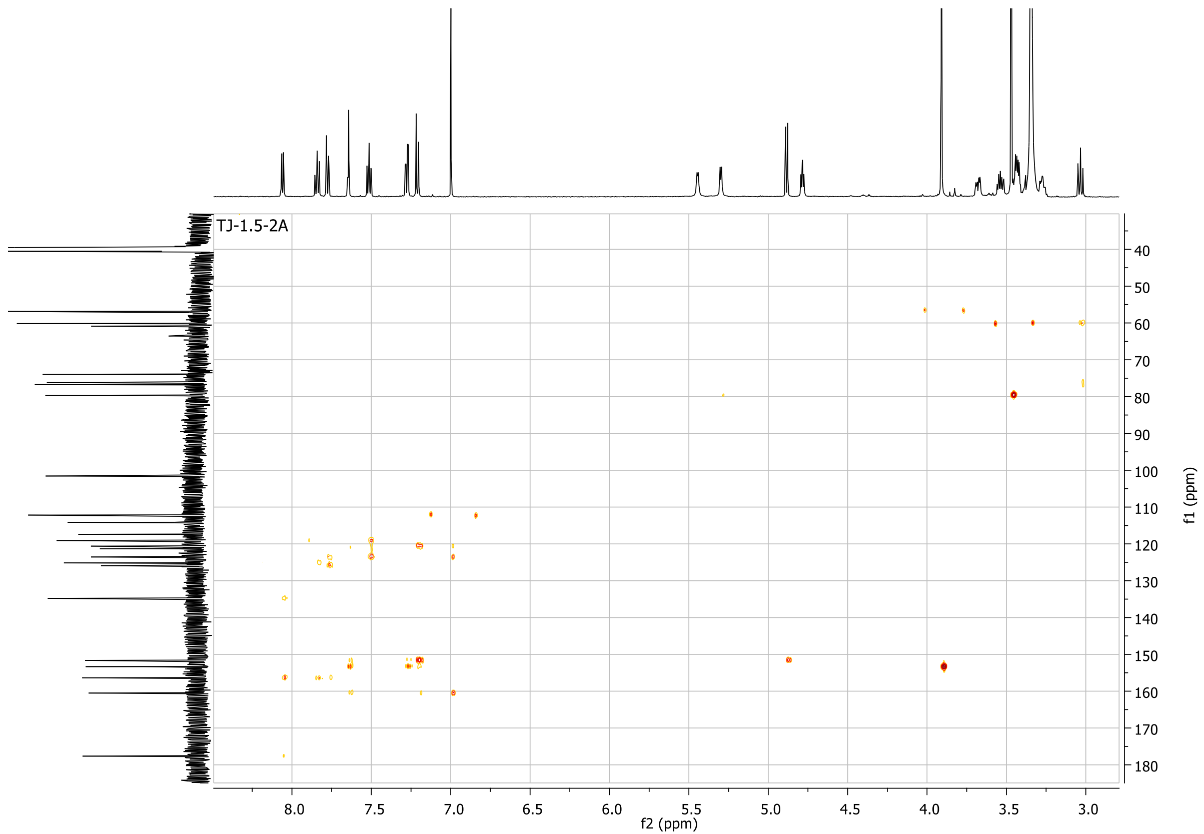
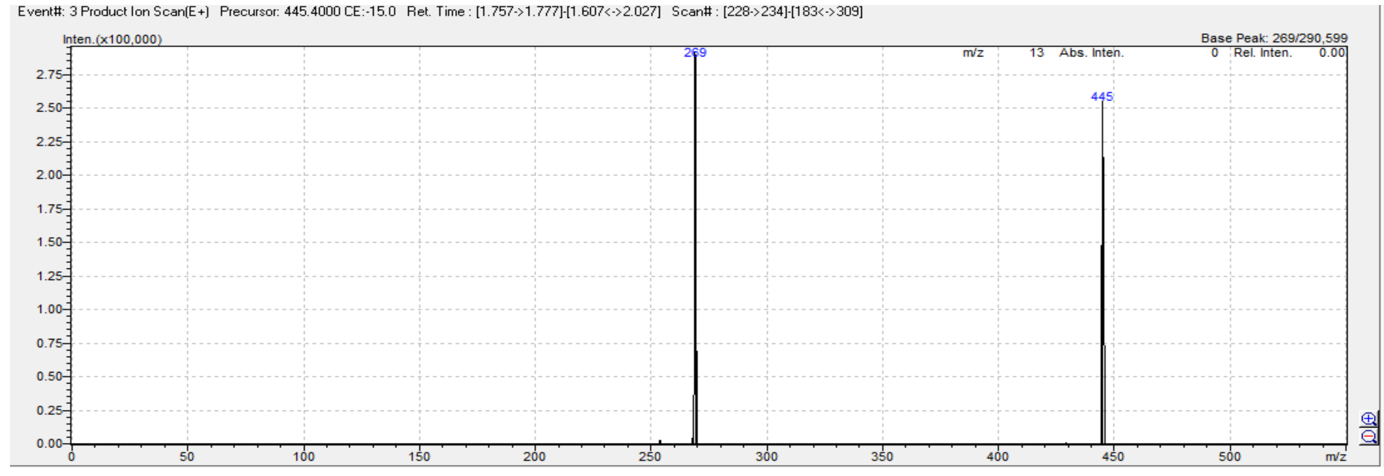


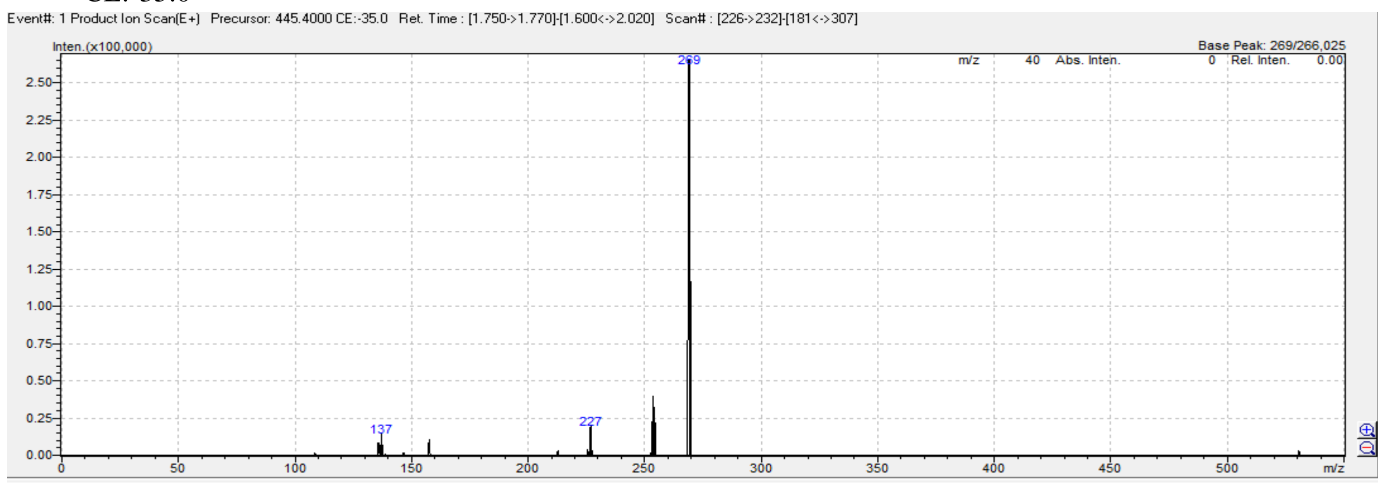
Fig.S17. MS analysis 2’-methoxyflavone 8-*O*-β-D-(4”-*O*-metyloglukopiranozylo)-2’-metoksyflawon (**7**)

Molecular Formula = $C_{23}H_{24}O_9$
Formula Weight = 444.43126
Precursor =445.4000

CE: -15.0



CE:-35.0



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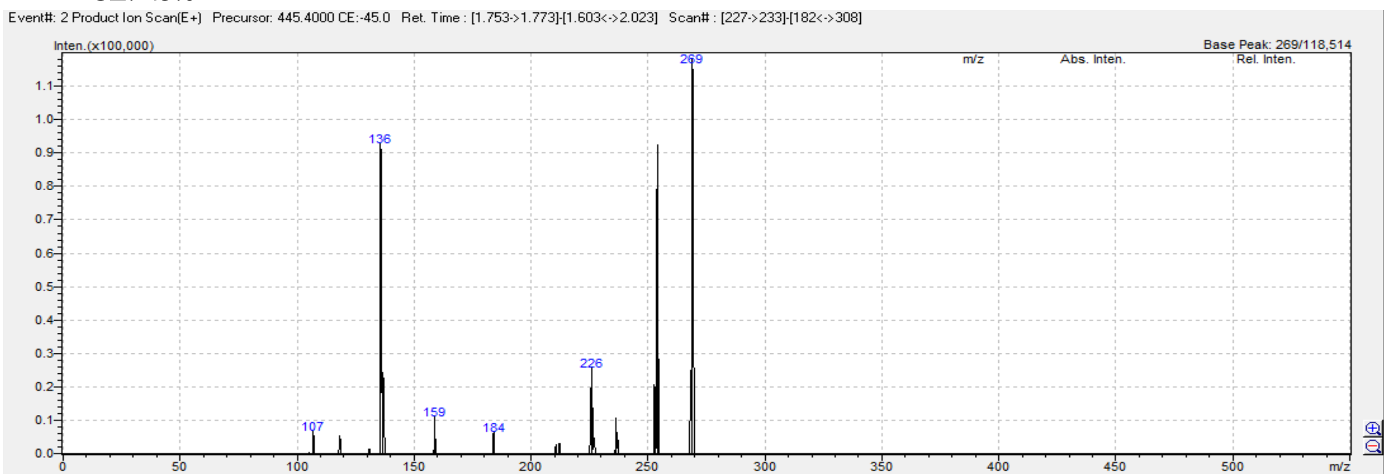


Fig.S18. ^{1}H NMR spectral of 2'-methoxyflavone 8-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**7**) (Acetone-d_6, 600 MHz)

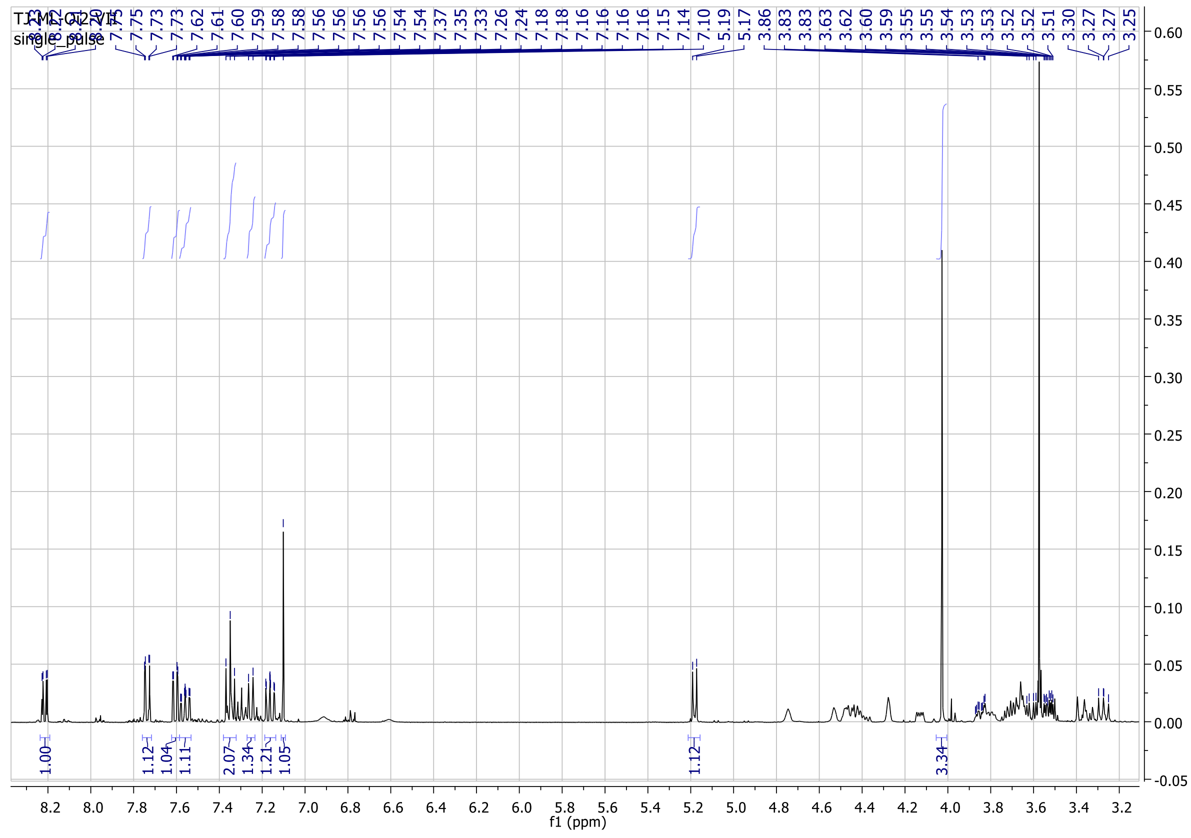


Fig.S19. Flavone part of the ^{1}H NMR spectral 2'-methoxyflavone 8-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**7**) (Acetone-d_6, 600 MHz)

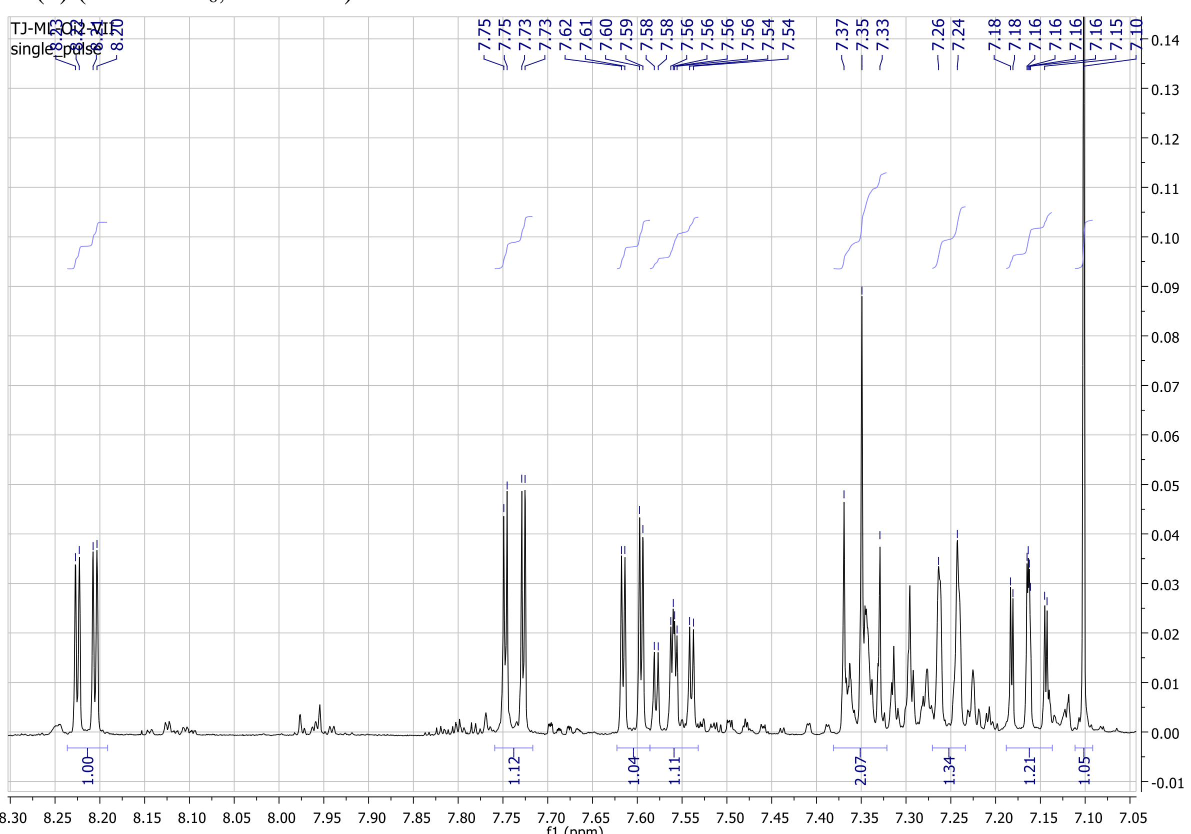


Fig.S20. ^{13}C NMR spectral of 2'-methoxyflavone 8-*O*-β-D-(4''-*O*-methyl)-glucopyranoside (**7**) (Acetone-d_6, 151 MHz)

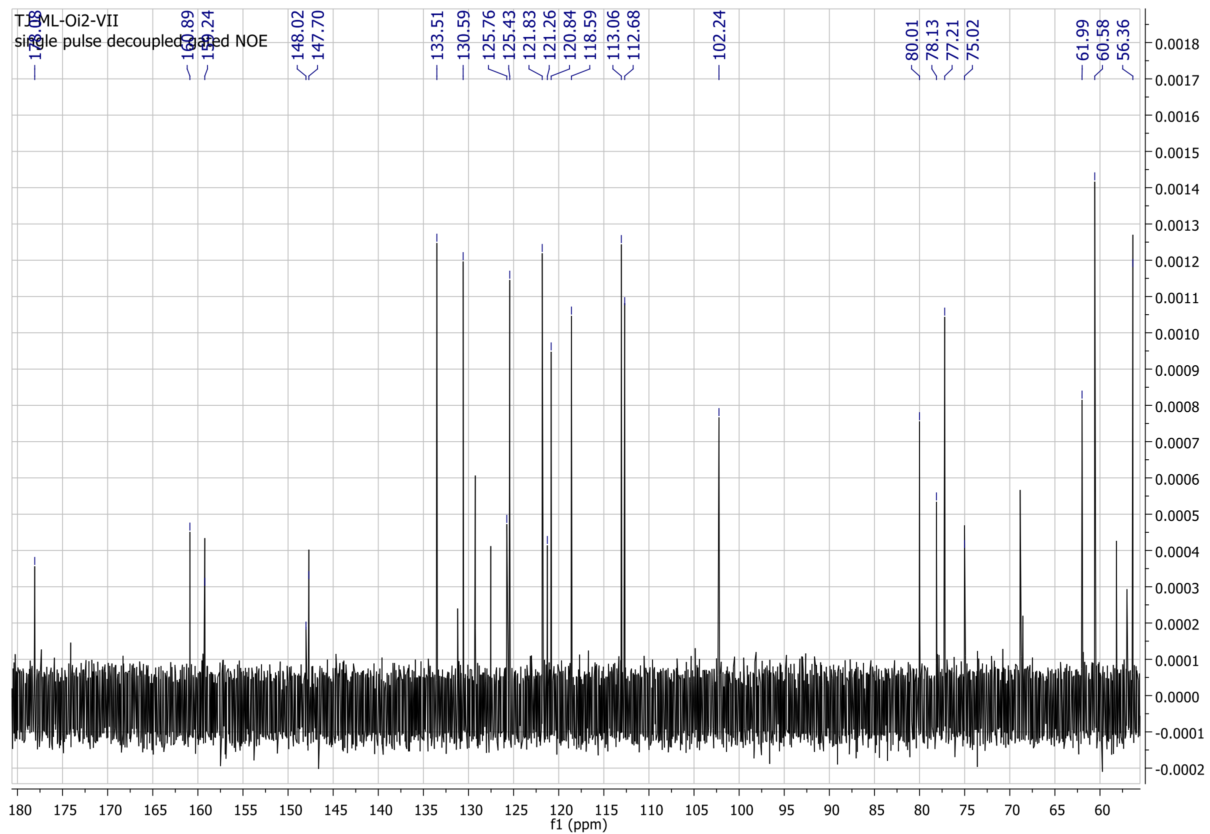


Fig.S21. COSY spectral of 2'-methoxyflavone 8-*O*-β-D-(4''-*O*-methyl)-glucopyranoside (**1**) (Acetone-d_6, 151 MHz)

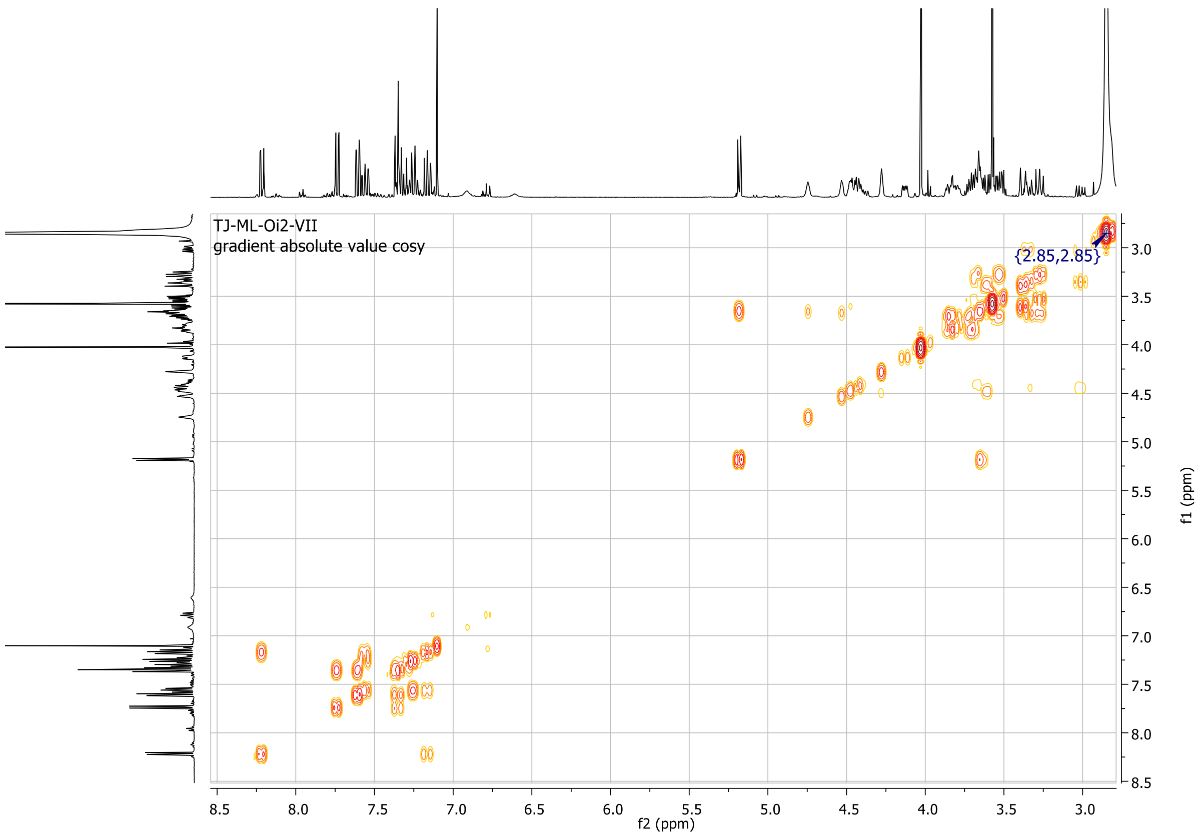


Fig.S22. HMQC spectral of 2'-methoxyflavone 8-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**7**) (Acetone-d_6, 151 MHz)

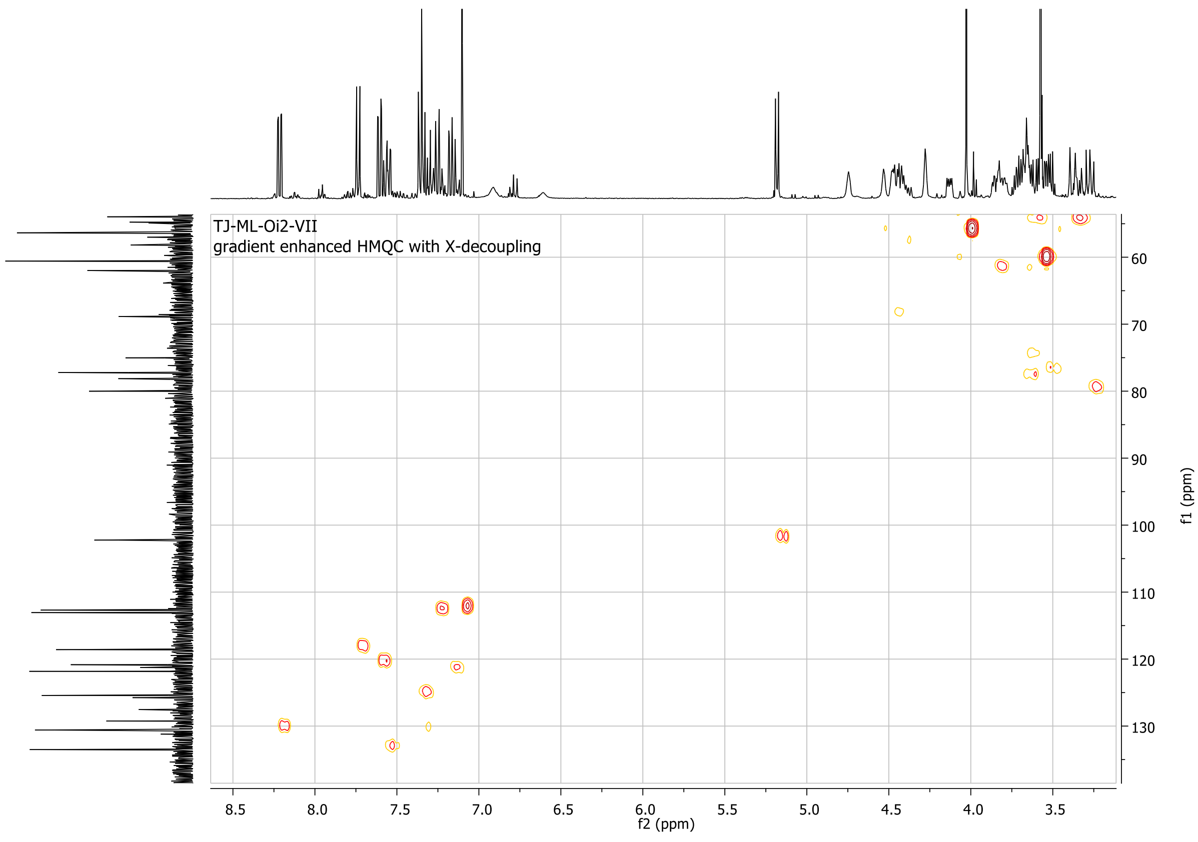


Fig.S23. HMBC spectral of 2'-methoxyflavone 8-*O*-β-D-(4"-*O*-methyl)-glucopyranoside flavone (**7**) (Acetone-d_6, 151 MHz)

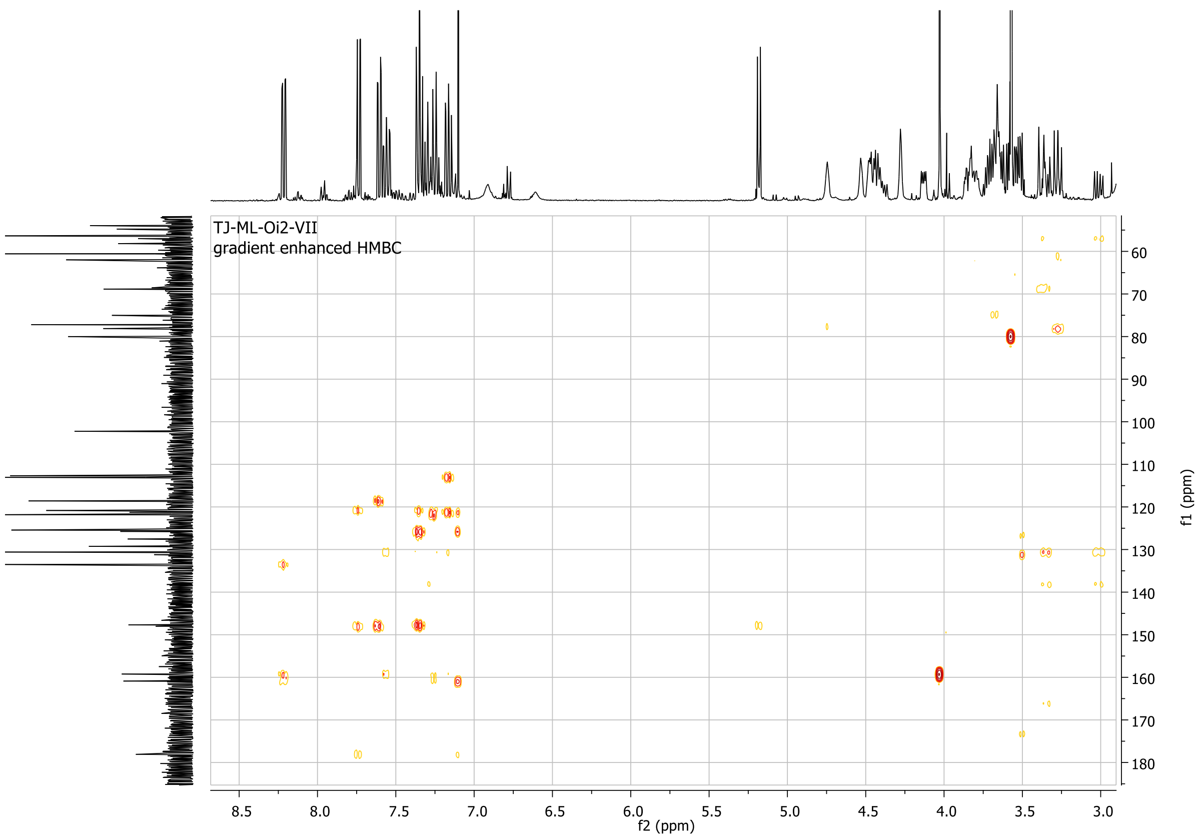
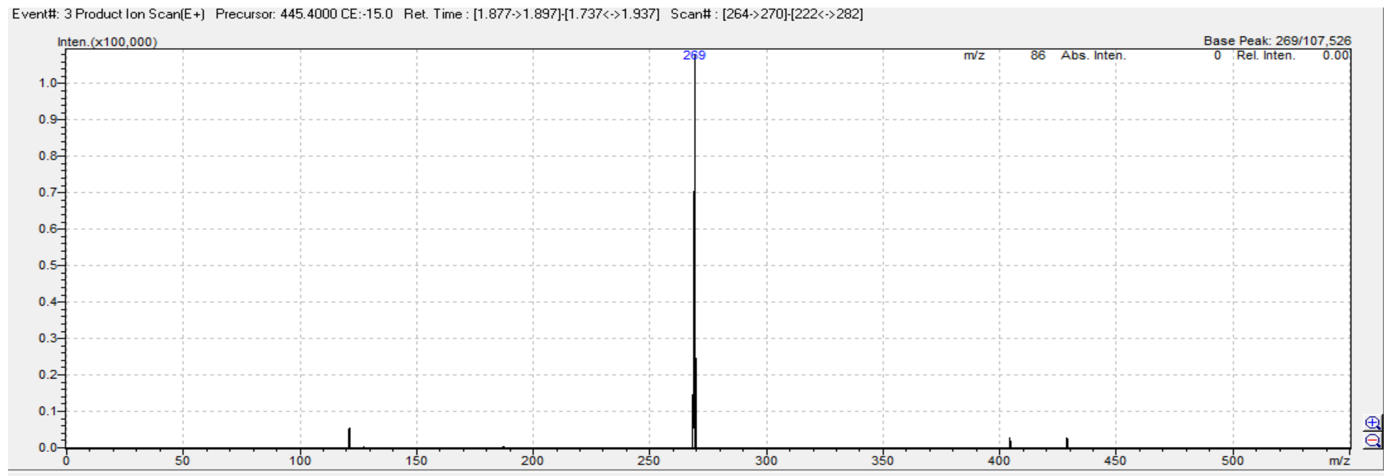


Fig.S24. MS analysis 3-*O*-β-D-(4”-*O*-metyloglukopiranozylo)-2’-metoksyflawon (**9**)

Molecular Formula = $C_{23}H_{24}O_9$
Formula Weight = 444.43126
Precursor =445.4000

CE: -15.0



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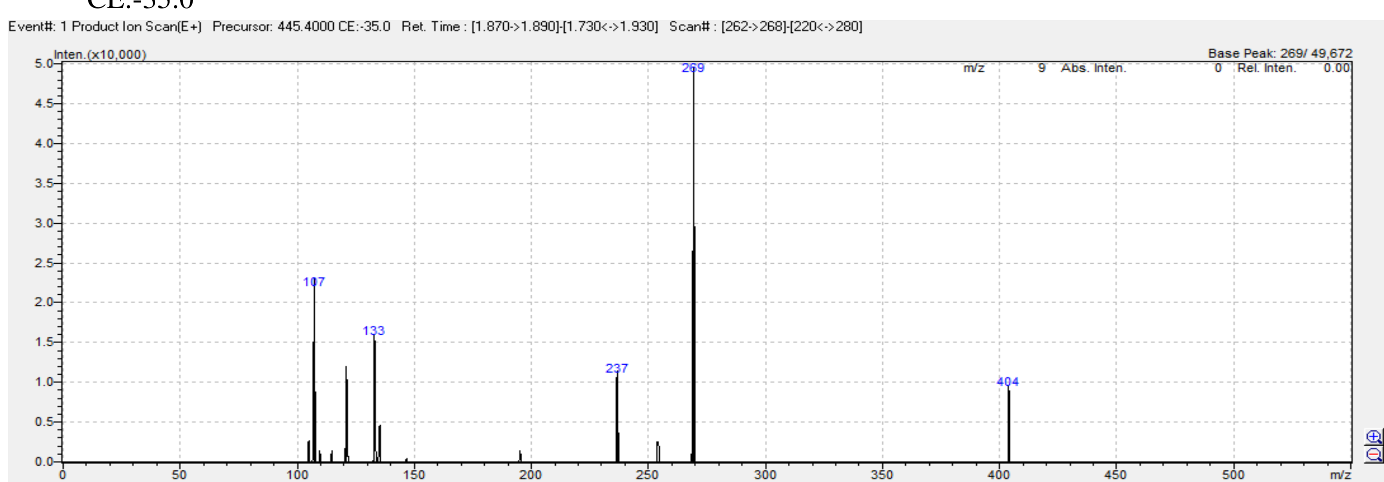


CE:-45.0

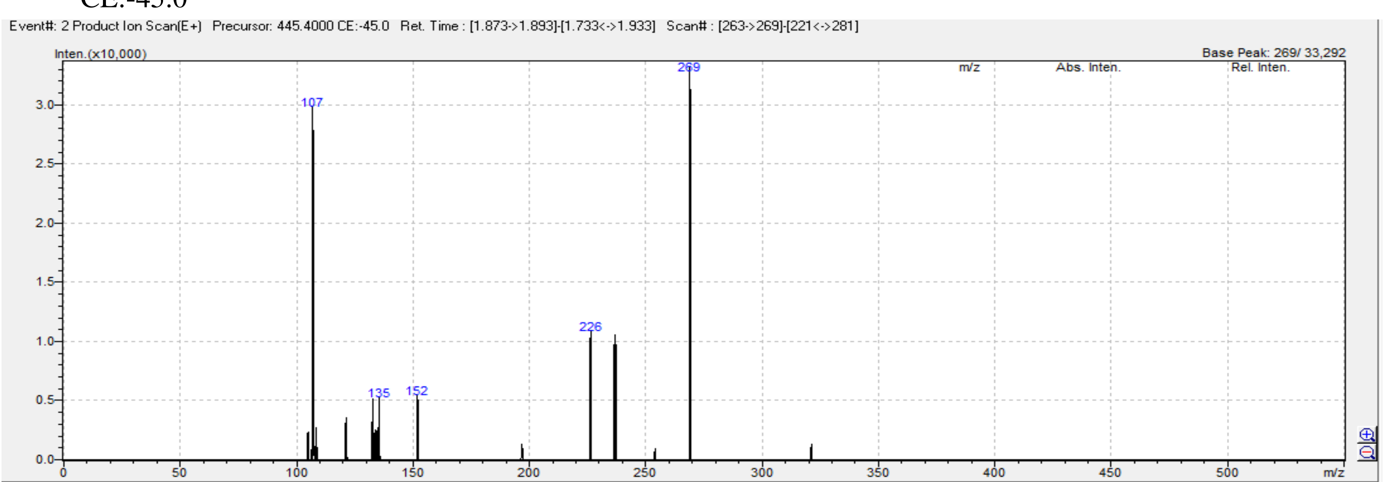


Fig.S25. ^{1}H NMR spectral of 2'-methoxyflavone 3-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**9**) (DMSO-d_6, 600 MHz)

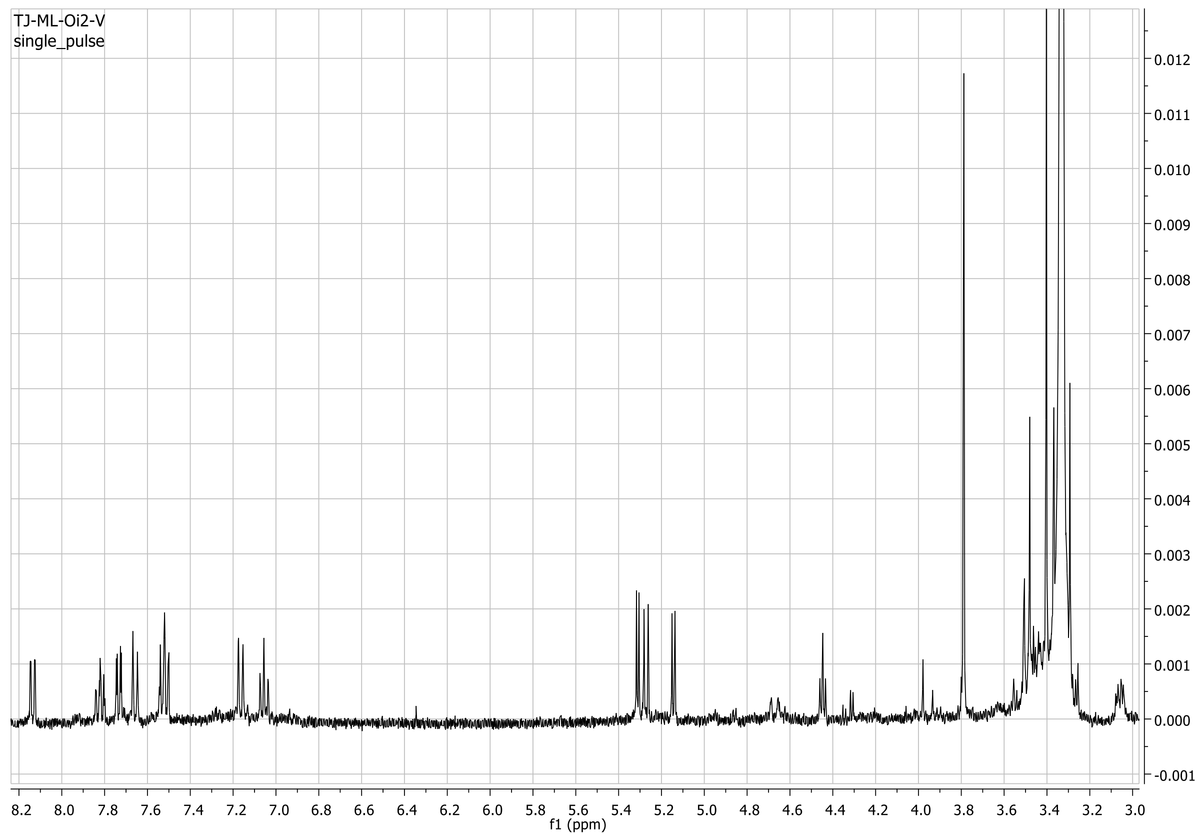


Fig.S26. Flavone part of the ^{1}H NMR spectral 2'-methoxyflavone 3-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**9**) (DMSO-d_6, 600 MHz)

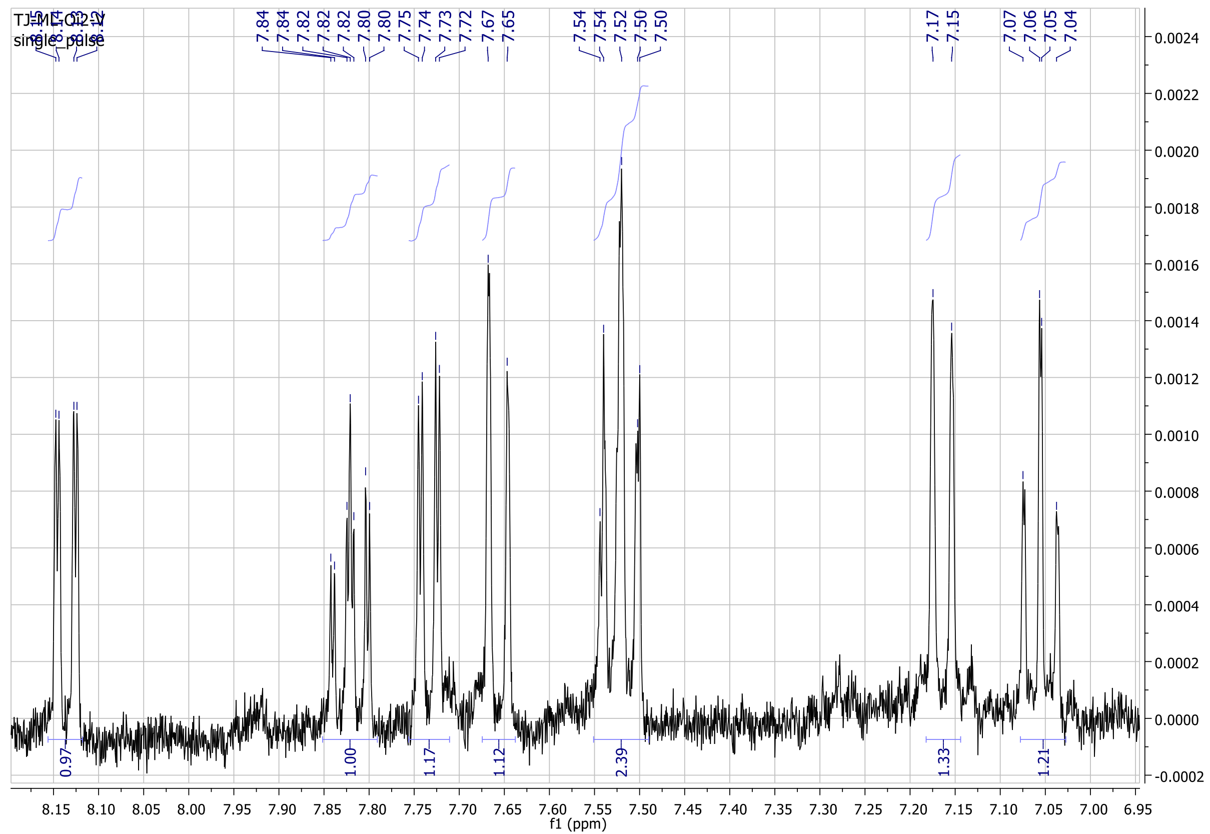


Fig.S27. Glucopyranoside part of the ^{1}H NMR spectral 2'-methoxyflavone 3-*O*-β-D-(4''-*O*-methyl)-glucopyranoside (**9**) (DMSO-d_6, 600 MHz)

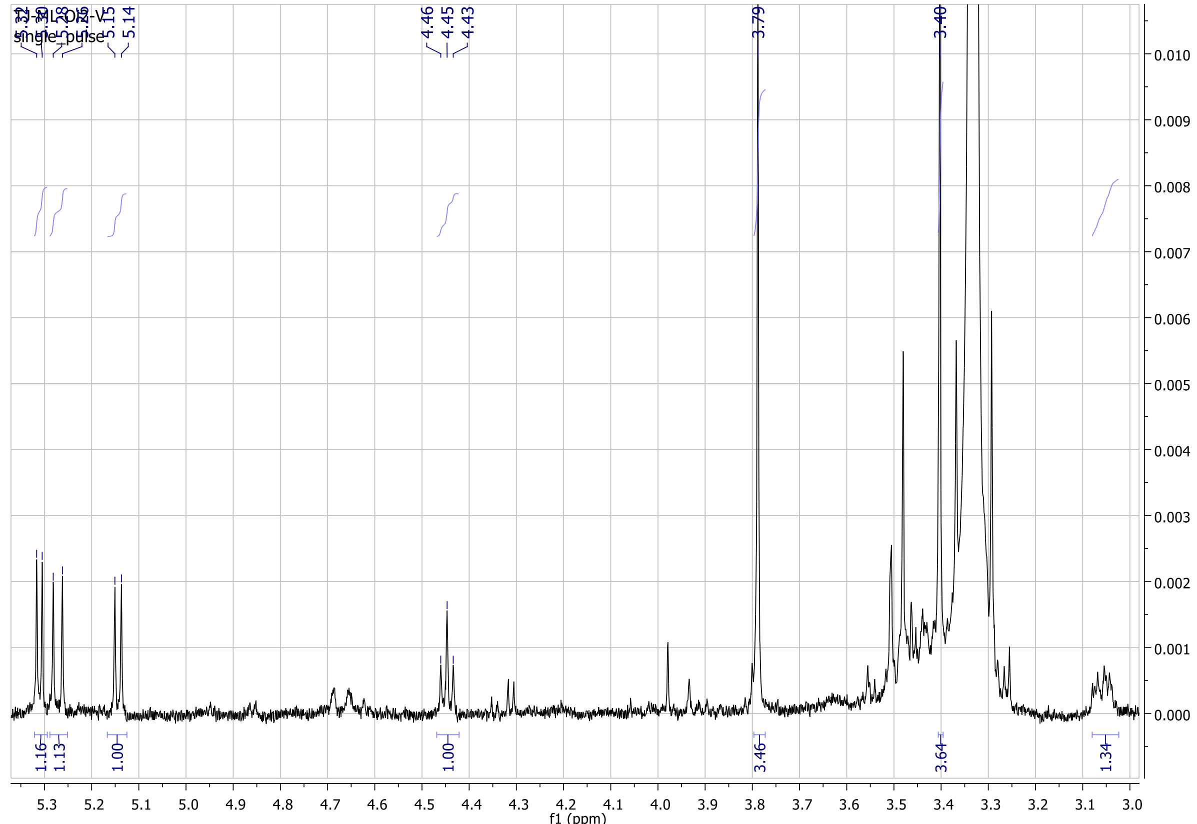
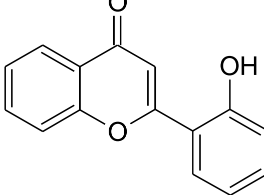
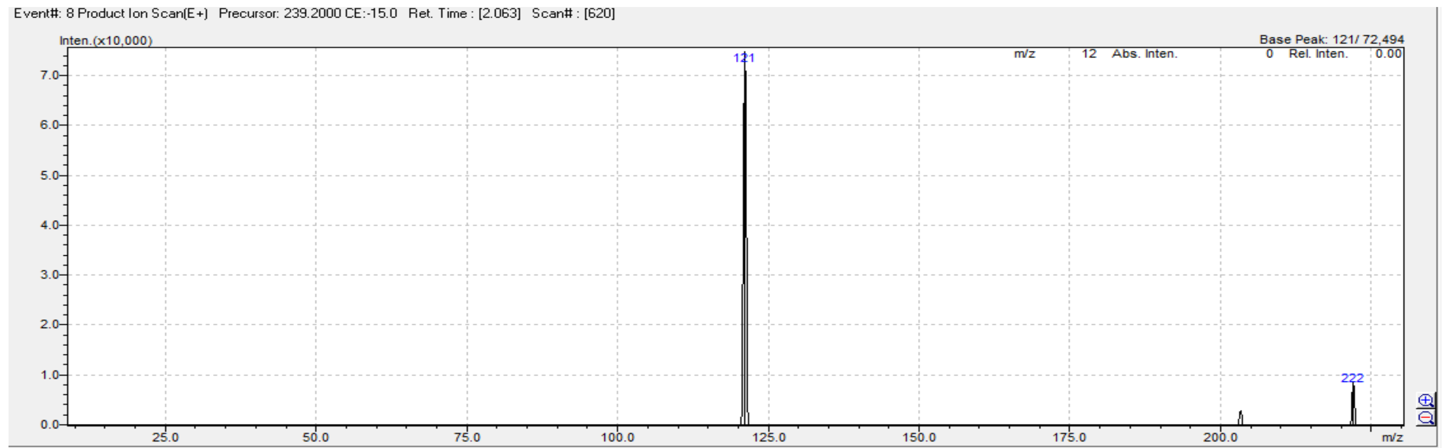


Fig.S28. MS analysis 2'-hydroxyflavone (**10**)

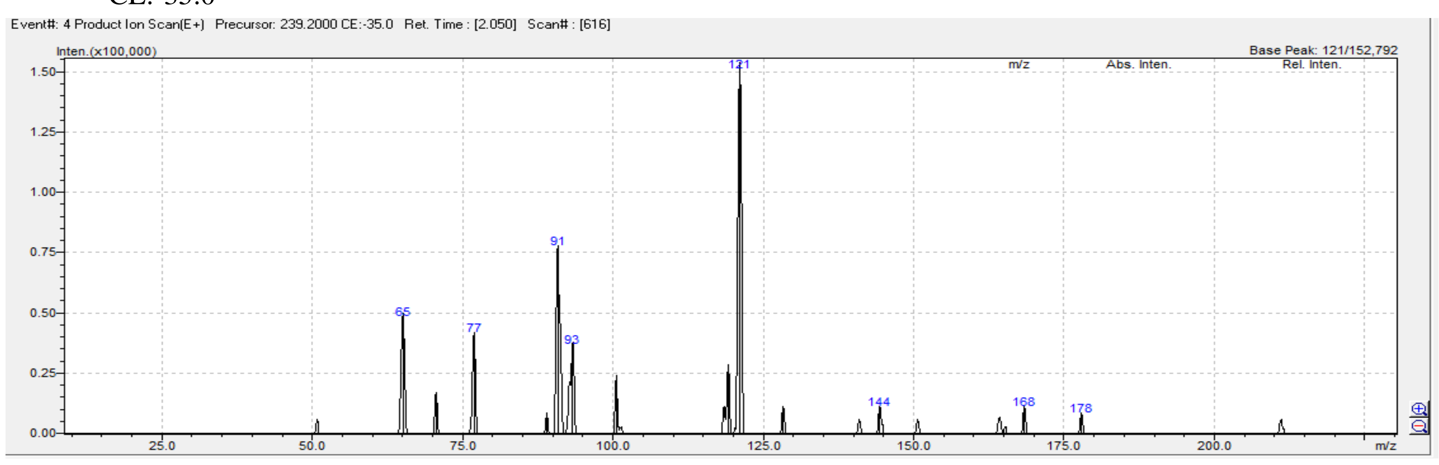


Molecular Formula: $C_{15}H_{10}O_3$
Formula Weight: 238.2381
Precursor: 239.2000

CE: -15.0



CE:-35.0



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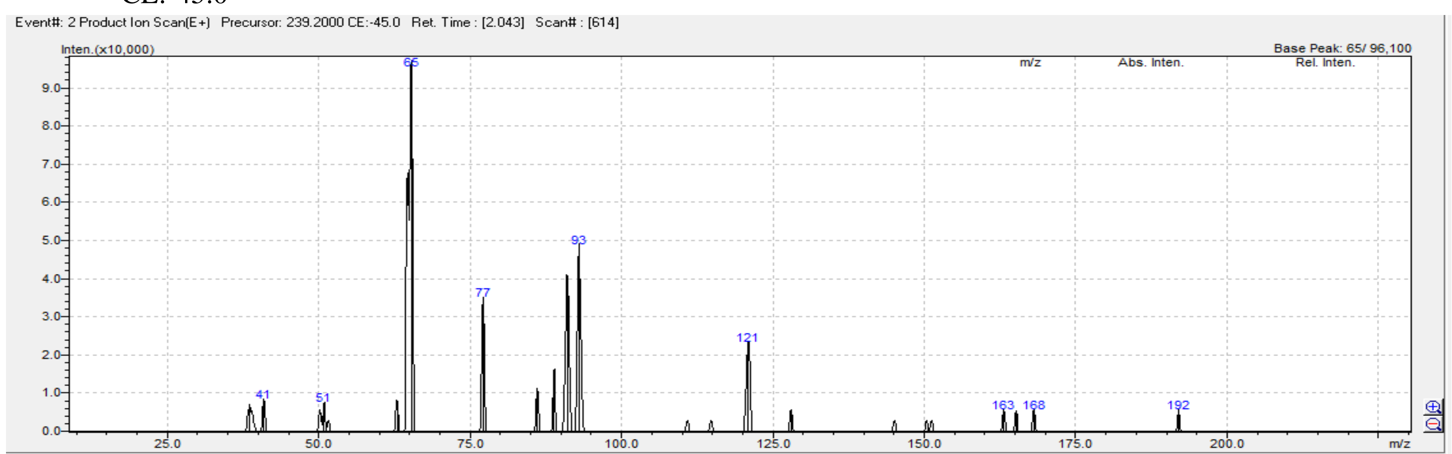


Fig.S29. ^{1}H NMR spectral of 2'-hydroxyflavone (**10**) (Acetone-d_6, 600 MHz)

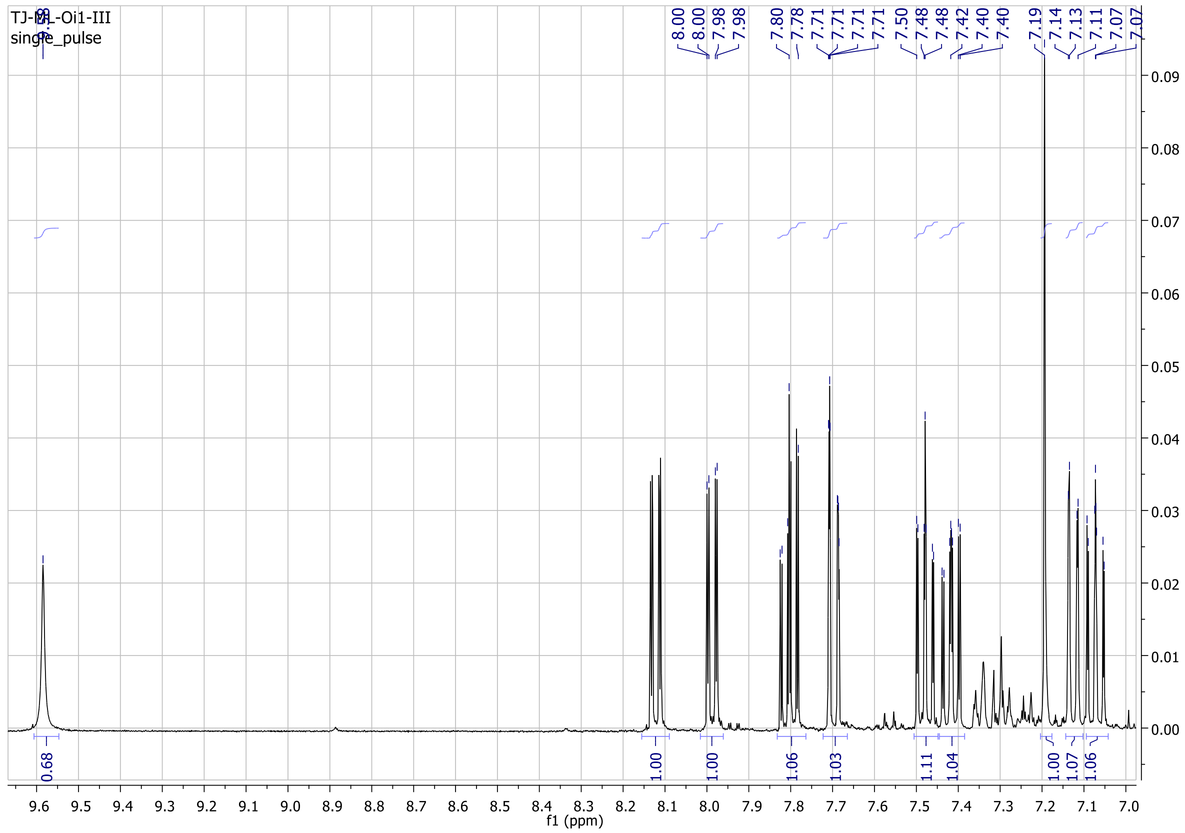


Fig.S30. ^{13}C NMR spectral of 2'-hydroxyflavone (**10**) (Acetone-d_6, 151 MHz)

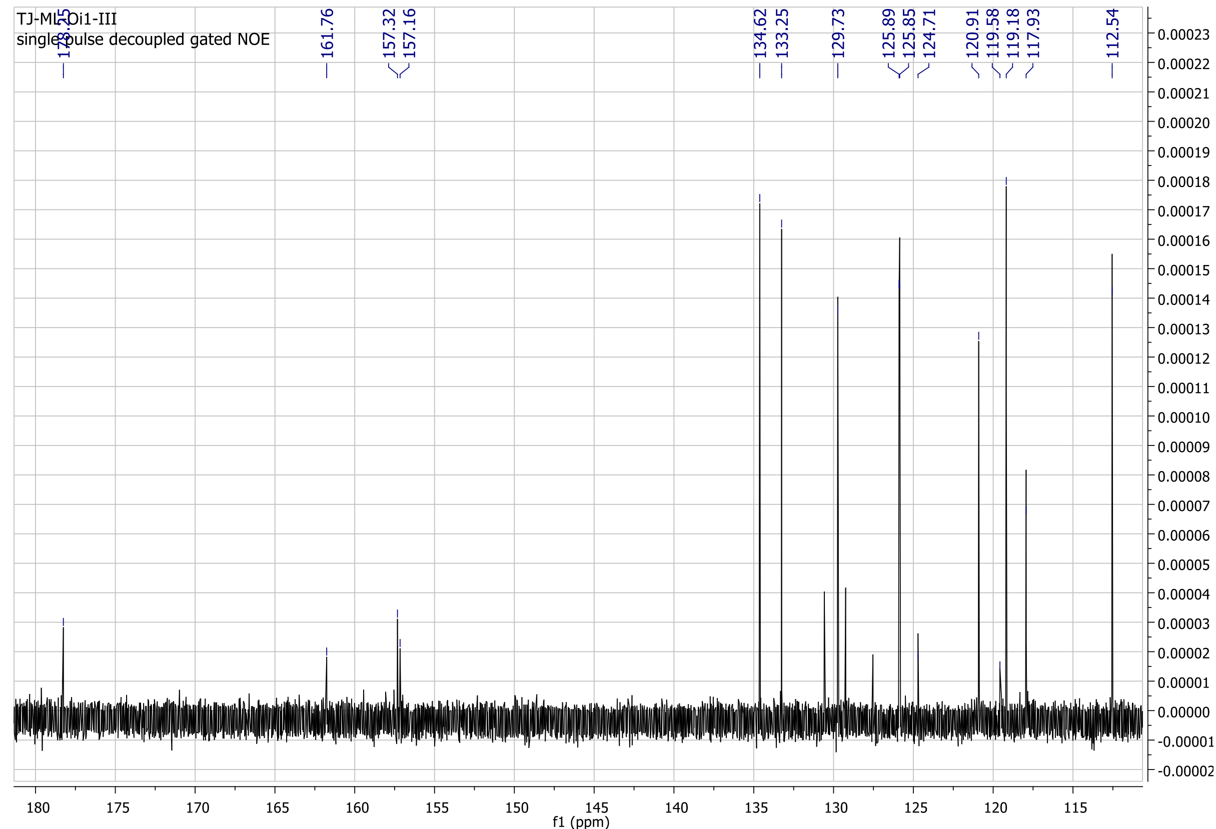


Fig.S31. COSY spectral of 2'-hydroxyflavone (**10**) (Acetone-d_6, 151 MHz)

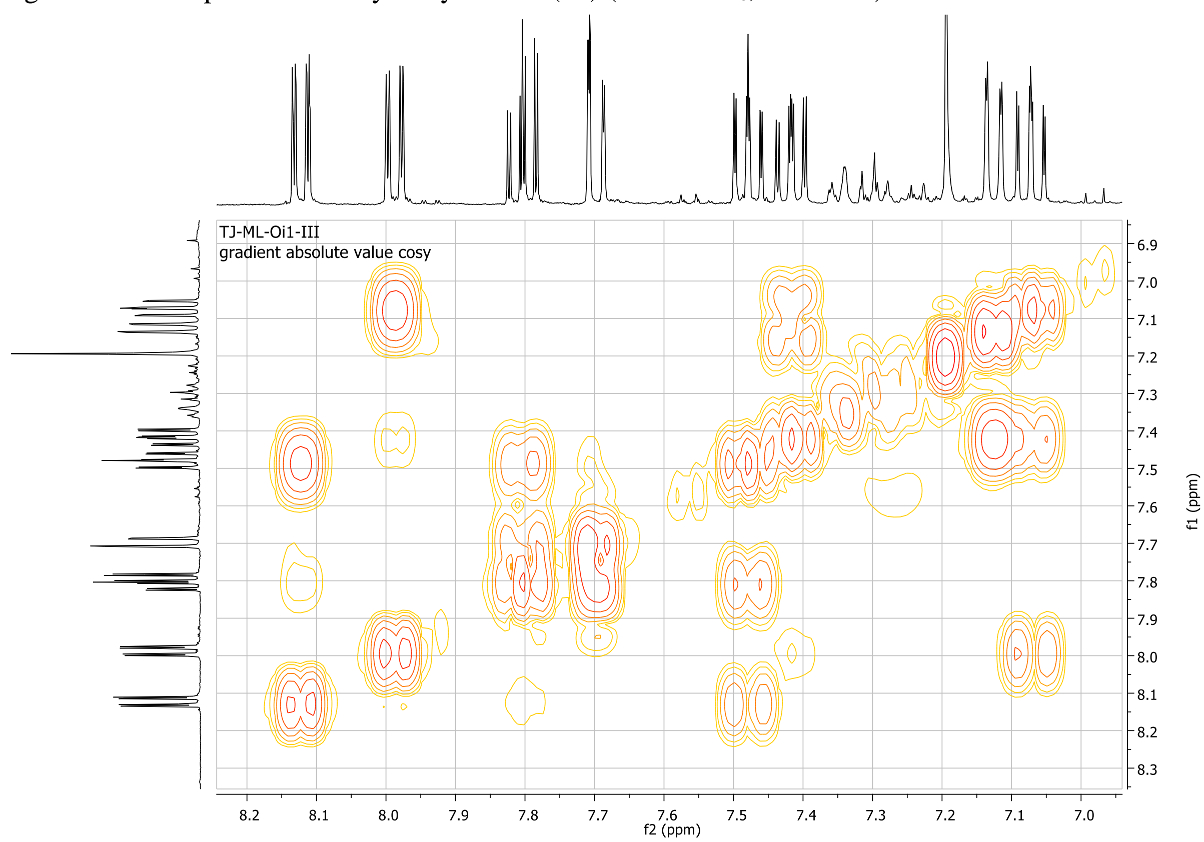


Fig.S32. HMQC spectral of 2'-hydroxyflavone (**10**) (Acetone-d_6, 151 MHz)

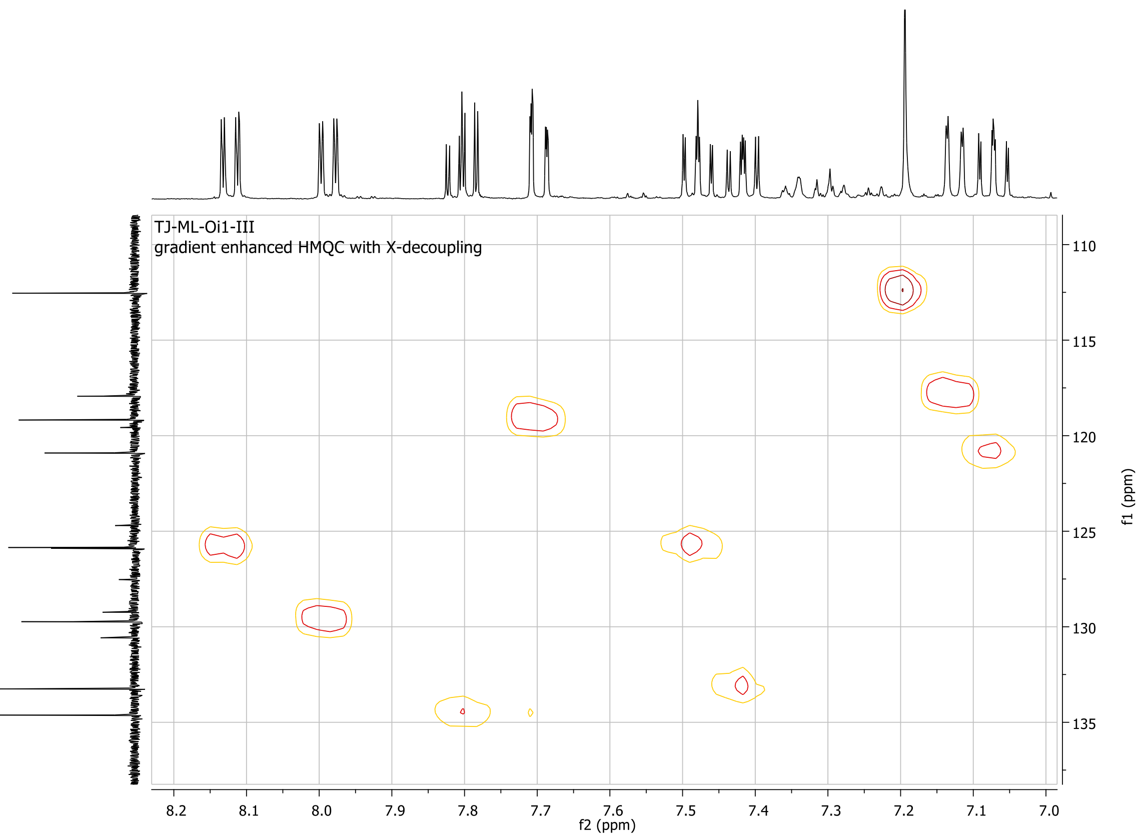


Fig.S33. HMBC spectral of 2’-hydroxyflavone (**10**) (Acetone-d_6, 151 MHz)

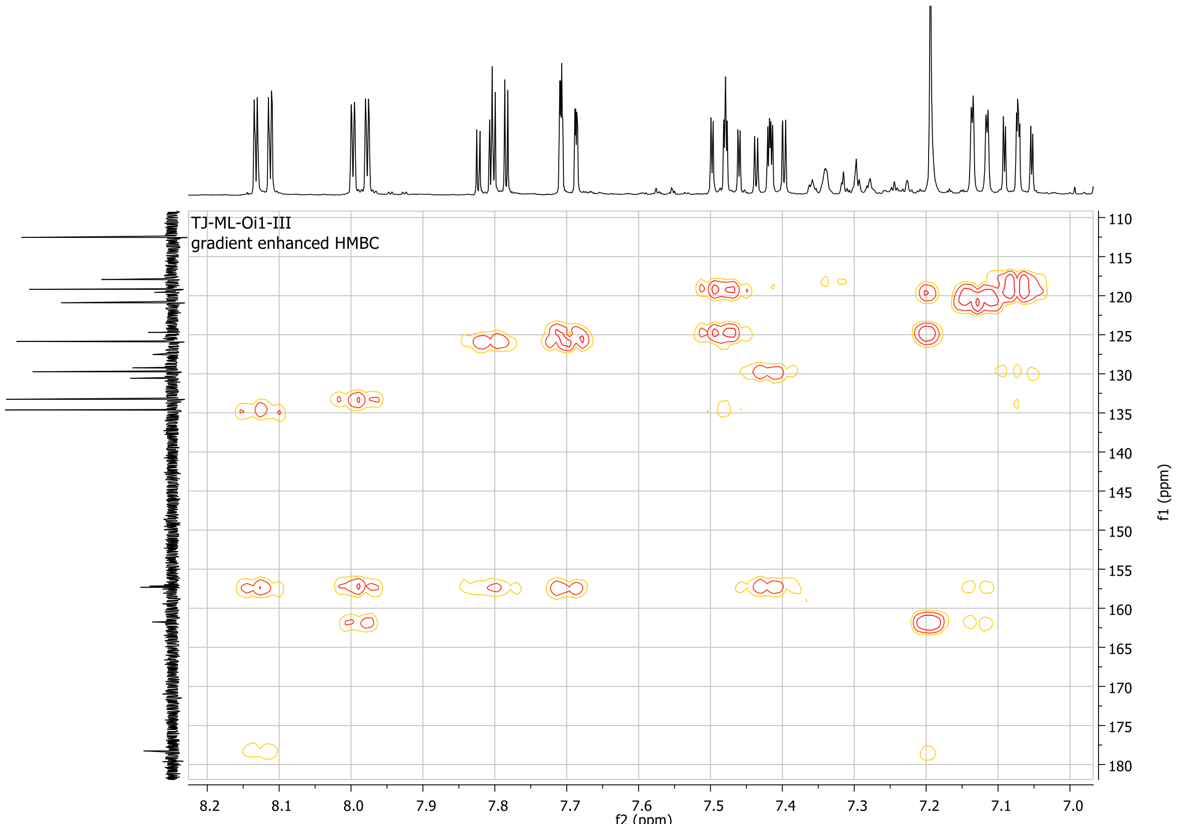
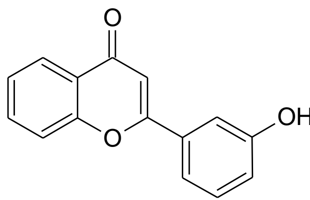
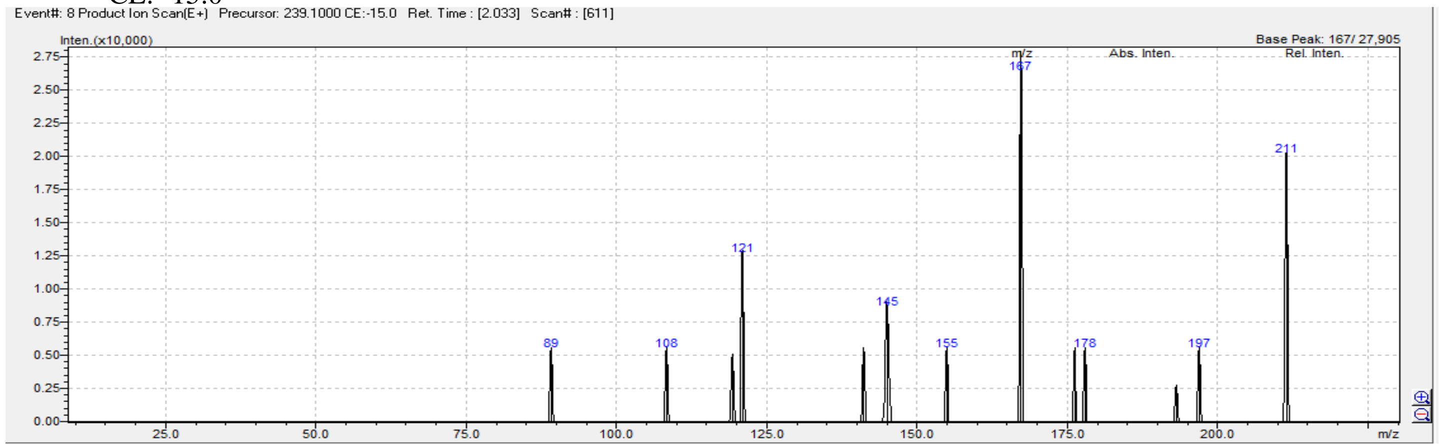


Fig.S34. MS analysis 3’-hydroxyflavone (**11**)

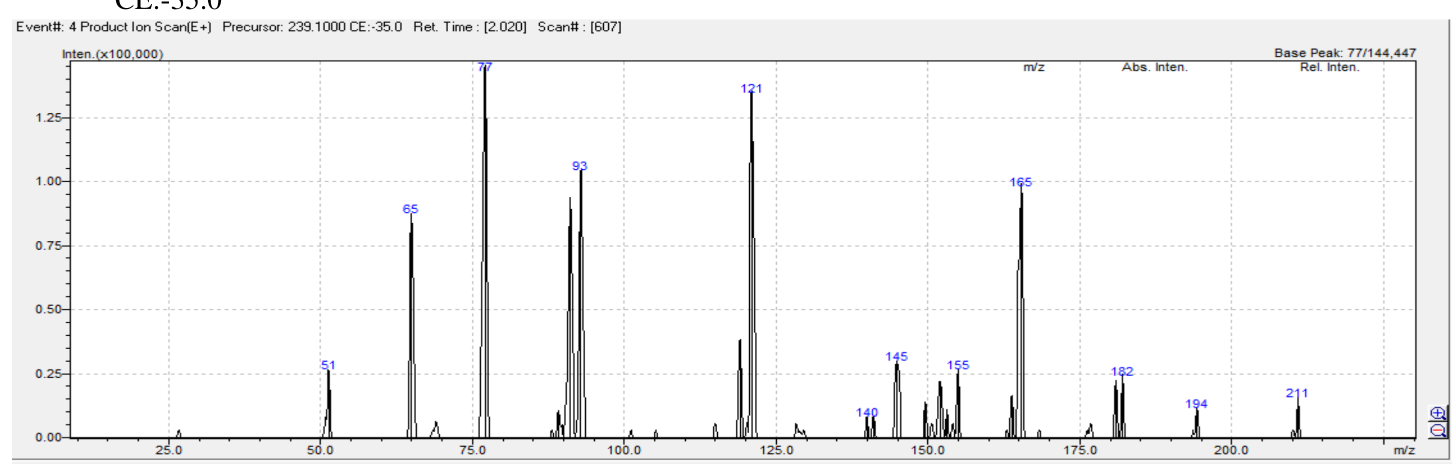


Molecular Formula: $C_{15}H_{10}O_3$
Formula Weight: 238.2381
Precursor: 239.2000

CE: -15.0



CE:-35.0



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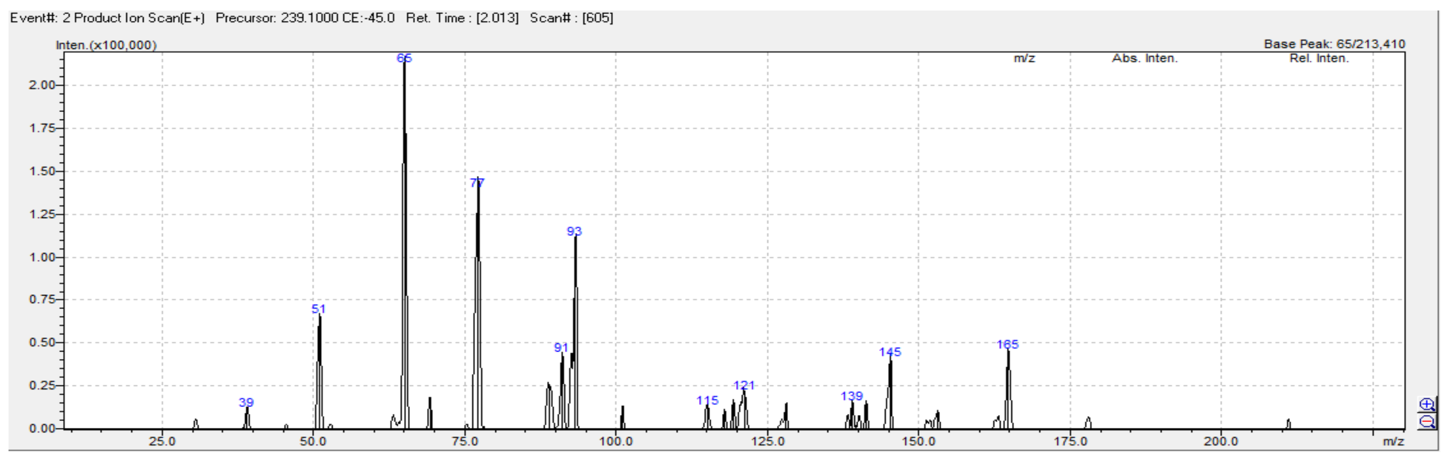


Fig.S35. ^{1}H NMR spectral of 3'-hydroxyflavone (**11**) (DMSO-d_6, 600 MHz)

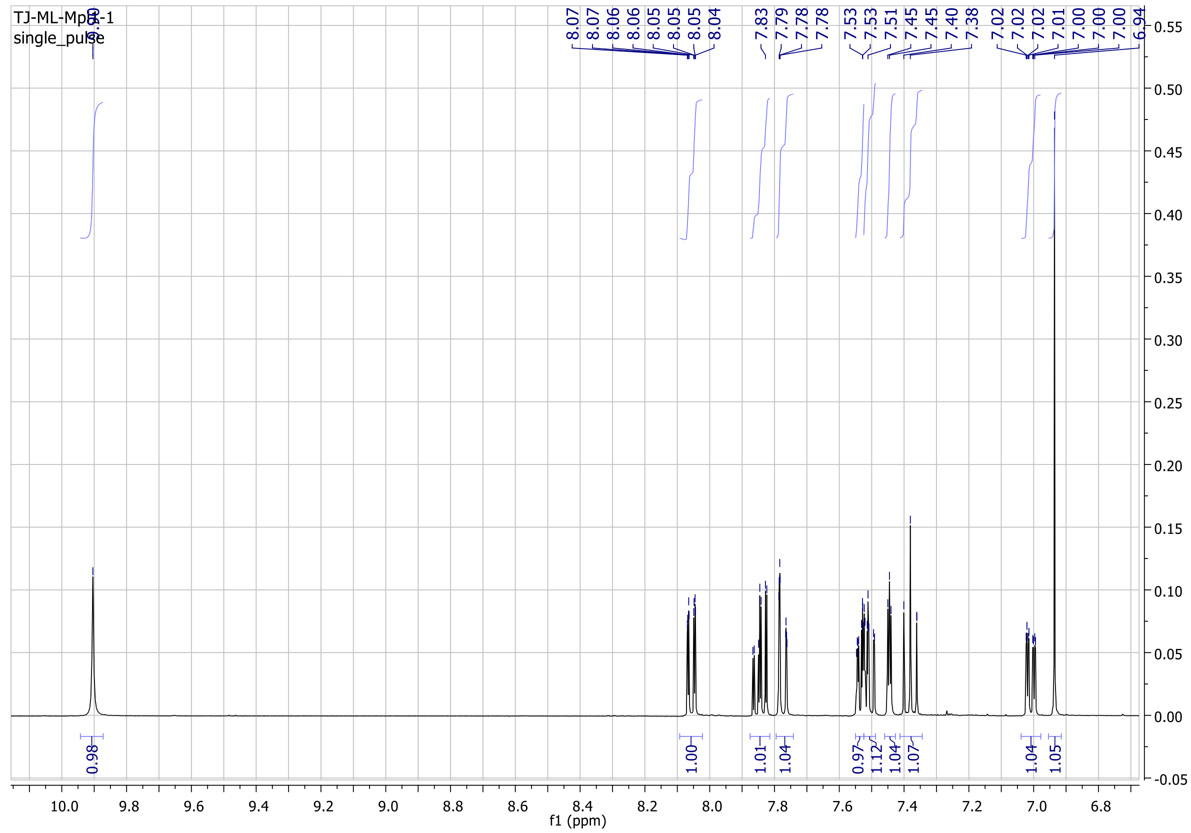


Fig.S36. ^{13}C NMR spectral of 3'-hydroxyflavone (**11**) (DMSO-d_6, 151 MHz)

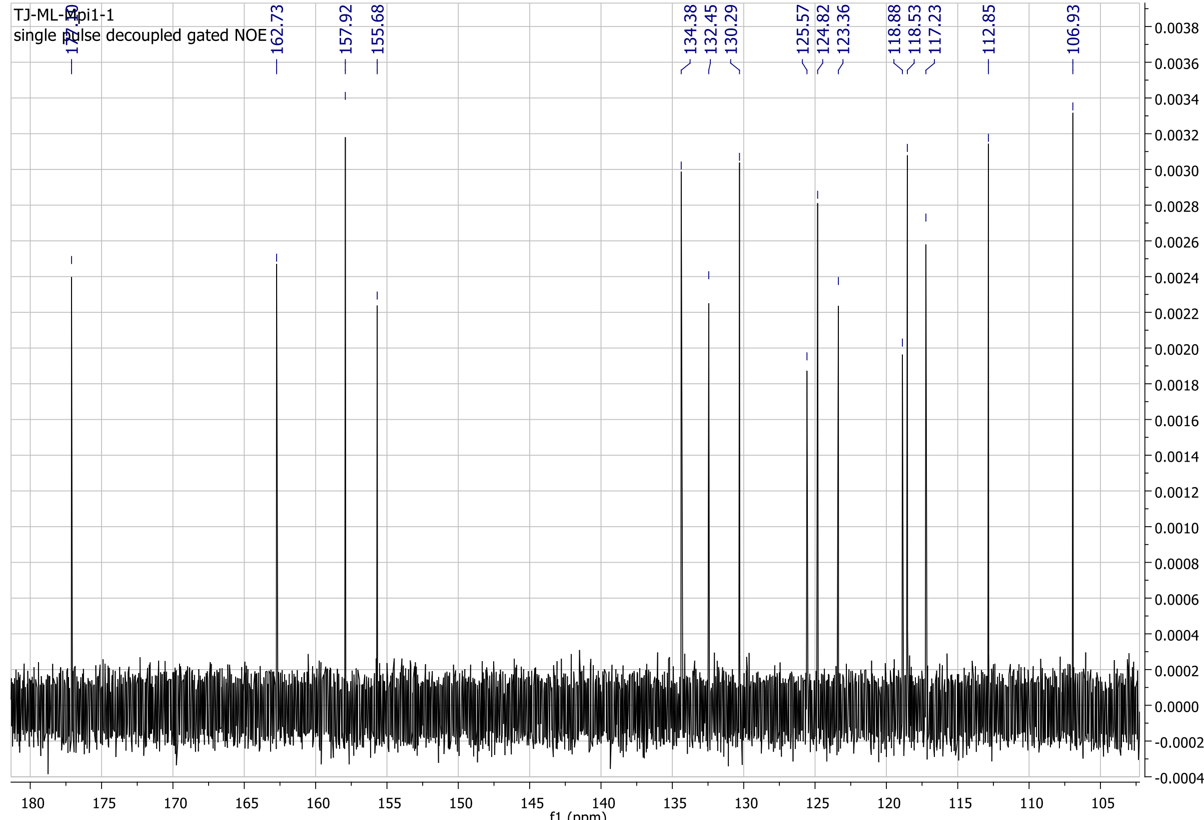


Fig.S37. COSY spectral of 3'-hydroxyflavone (**11**) (DMSO-d_6, 151 MHz)

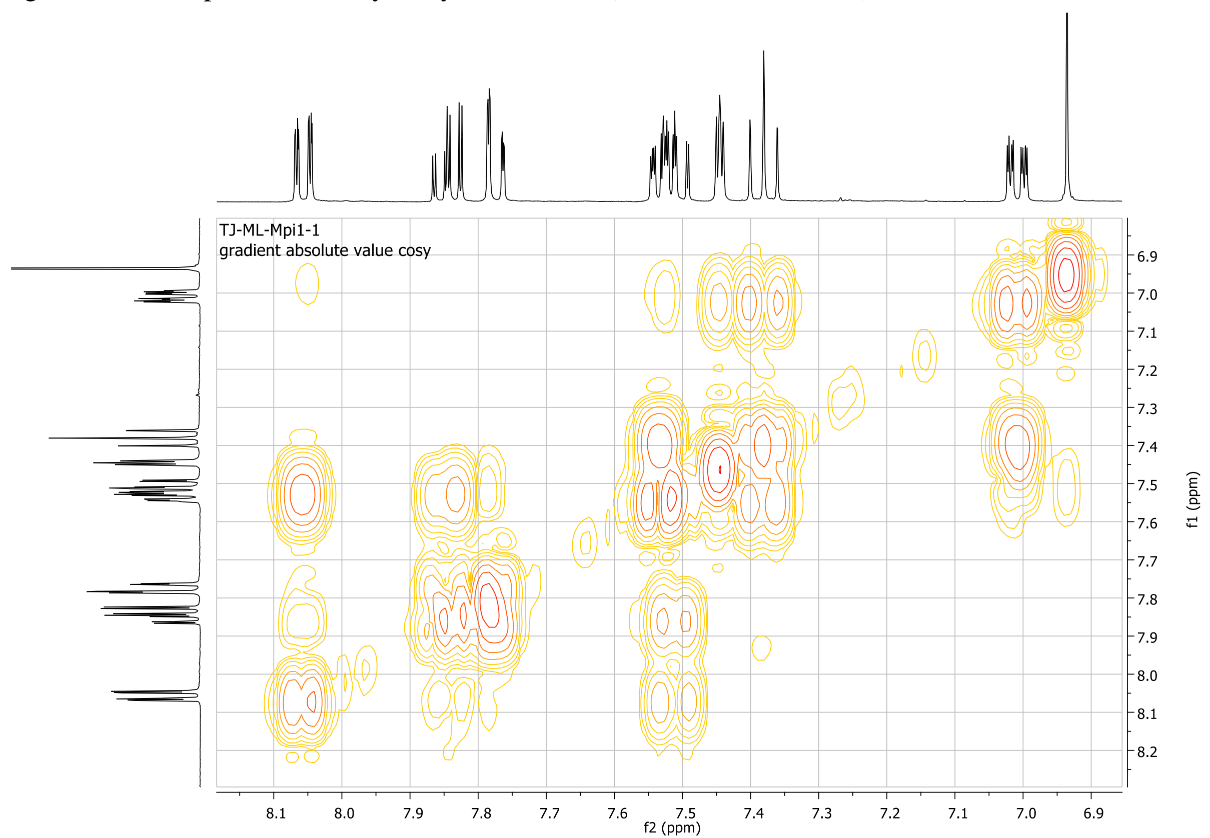


Fig.S38. HMQC spectral of 3'-hydroxyflavone (**11**) (DMSO-d_6, 151 MHz)

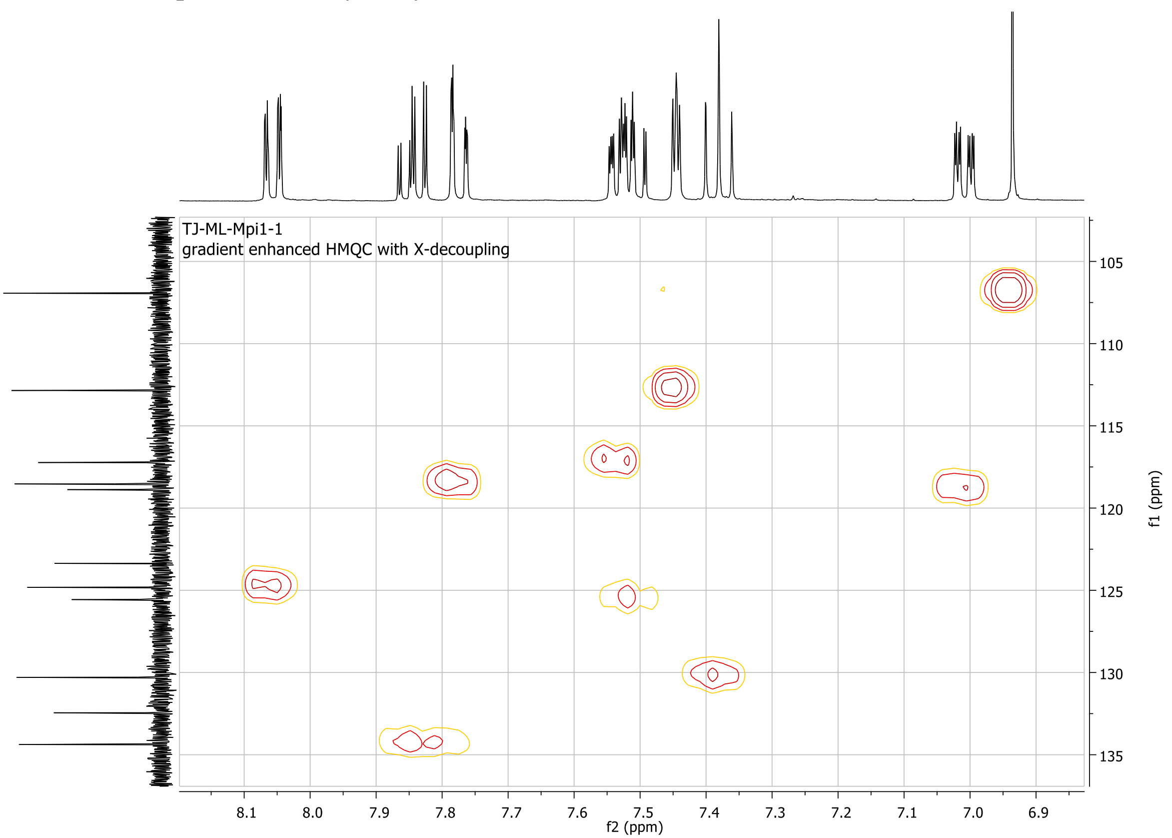


Fig.S39. HMBC spectral of 3'-hydroxyflavone (**11**) (DMSO-d_6, 151 MHz)

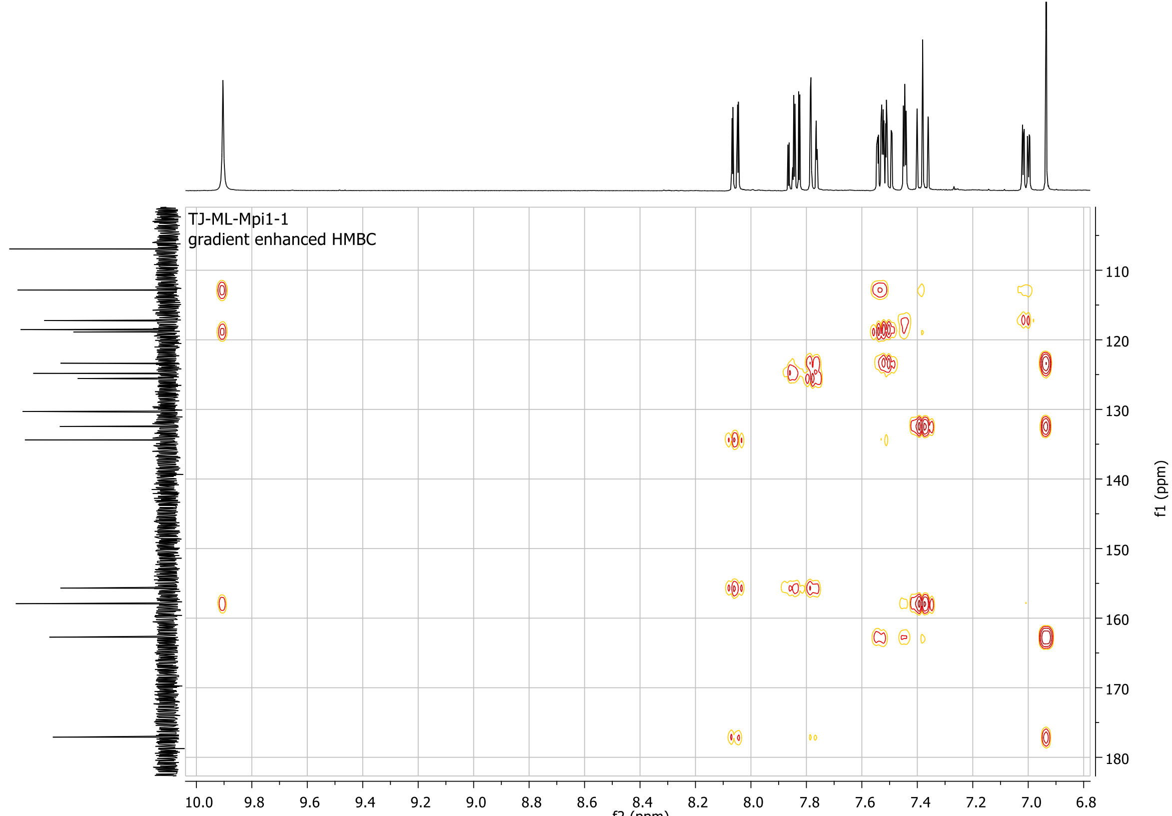
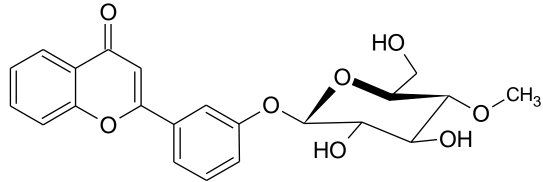
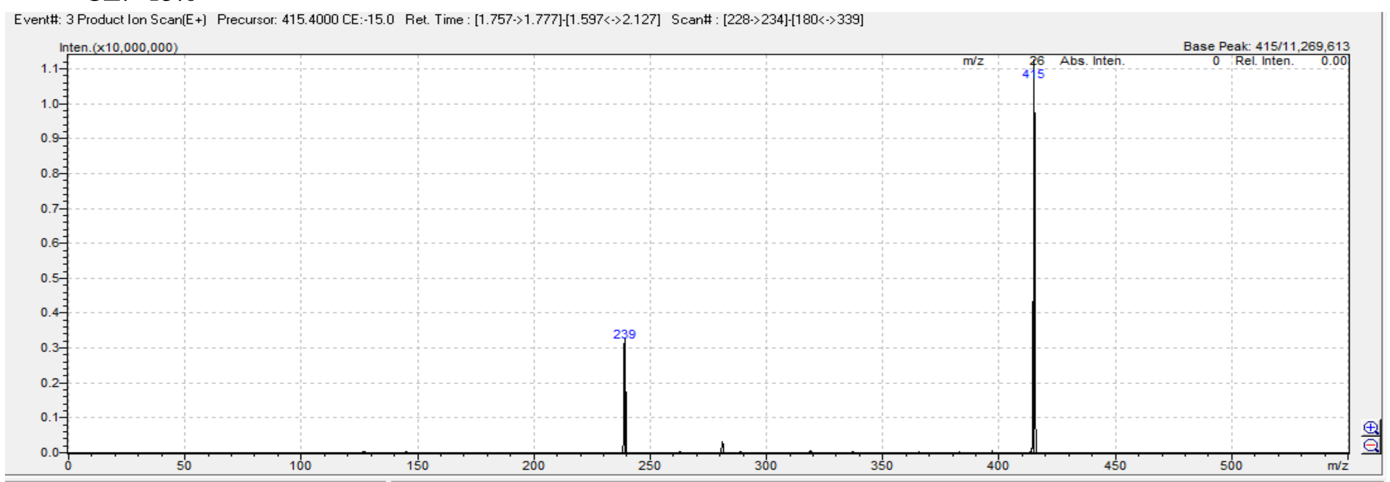


Fig.S40. MS analysis flavone 3'-*O*-β-D-(4''-*O*-methyl)-glucopyranoside (**12**)



Molecular Formula = $C_{22}H_{22}O_8$
Formula Weight = 414.40528
Precursor =415.4000

CE: -15.0



CE:-35.0

CE:-45.0

Fig.S41. ^{1}H NMR spectral of flavone 3'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**12**) (DMSO-d_6, 600 MHz)

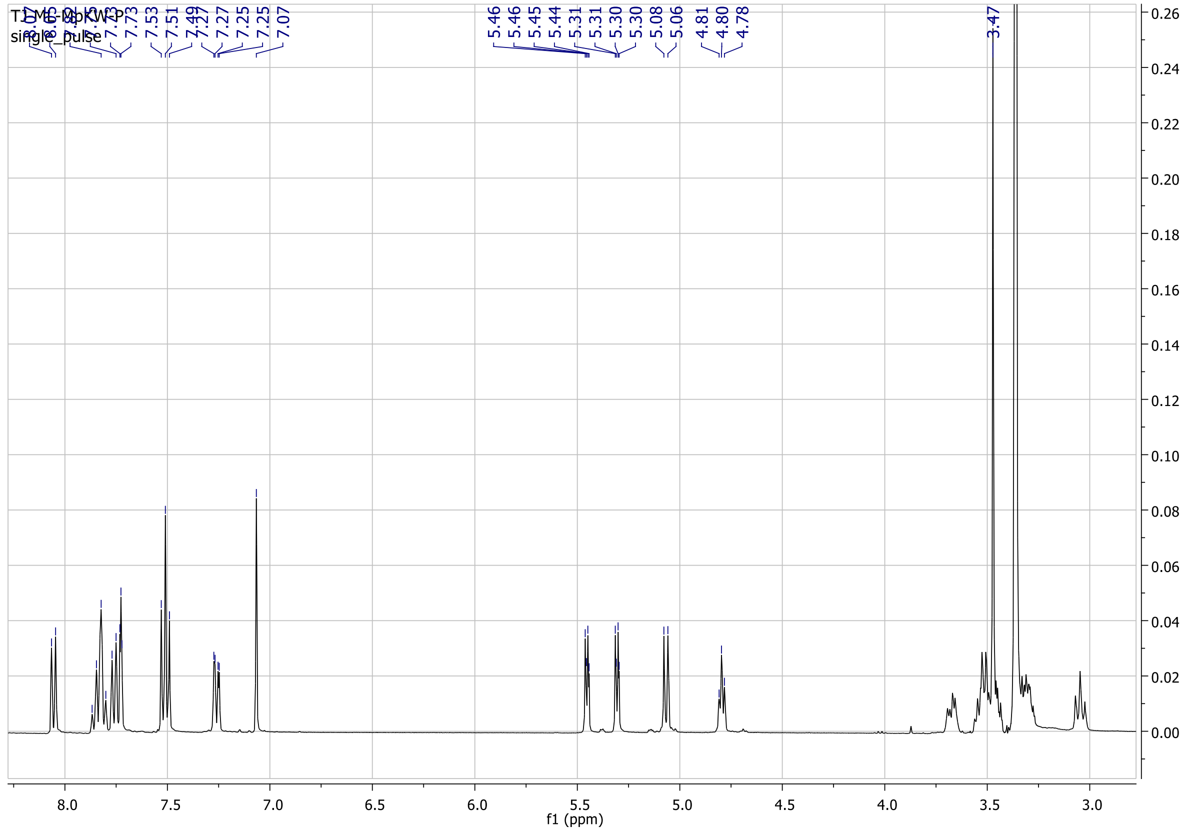


Fig.S42. Flavone part of the ^{1}H NMR spectral flavone 3'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**12**) (DMSO-d_6, 600 MHz)

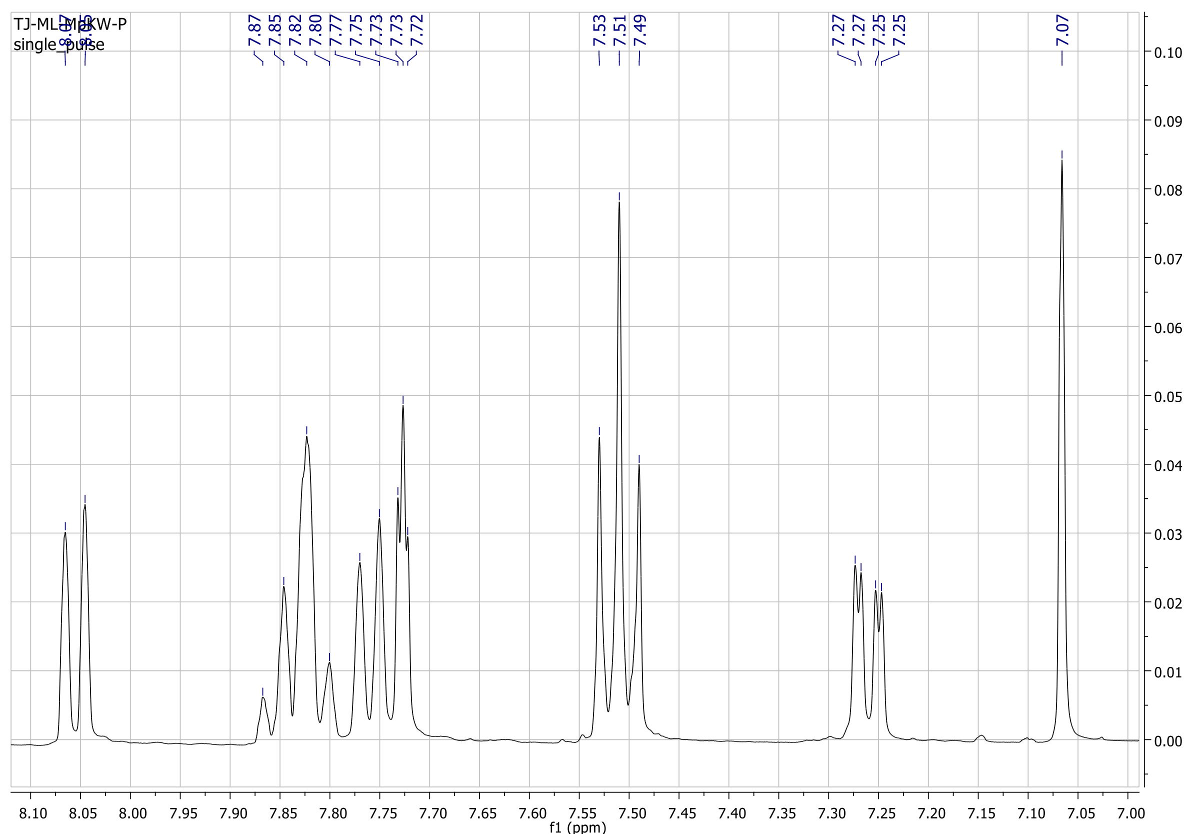


Fig.S43. Glucopyranoside part of the ^{1}H NMR spectral flavone 3’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**12**) (DMSO-d_6, 600 MHz)

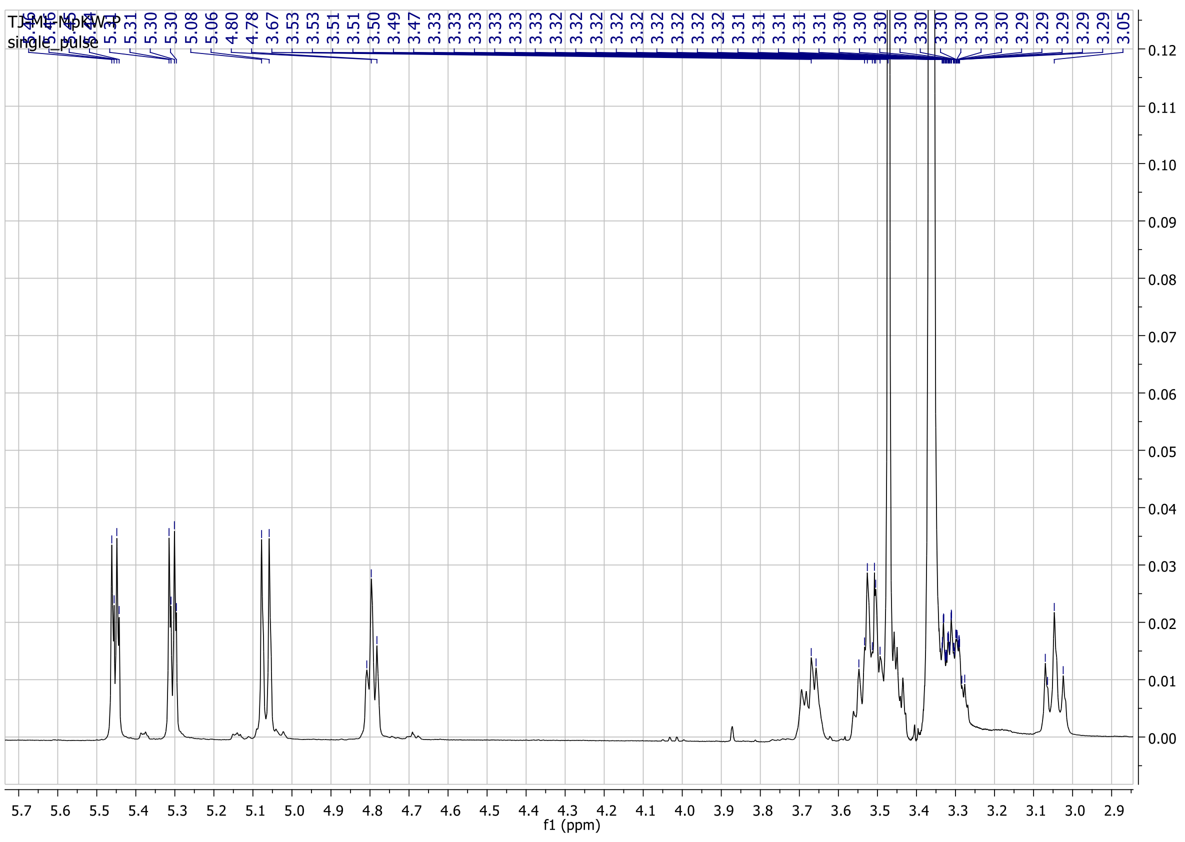


Fig.S44. ^{13}C NMR spectral of flavone 3’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**12**) (DMSO-d_6, 151 MHz)

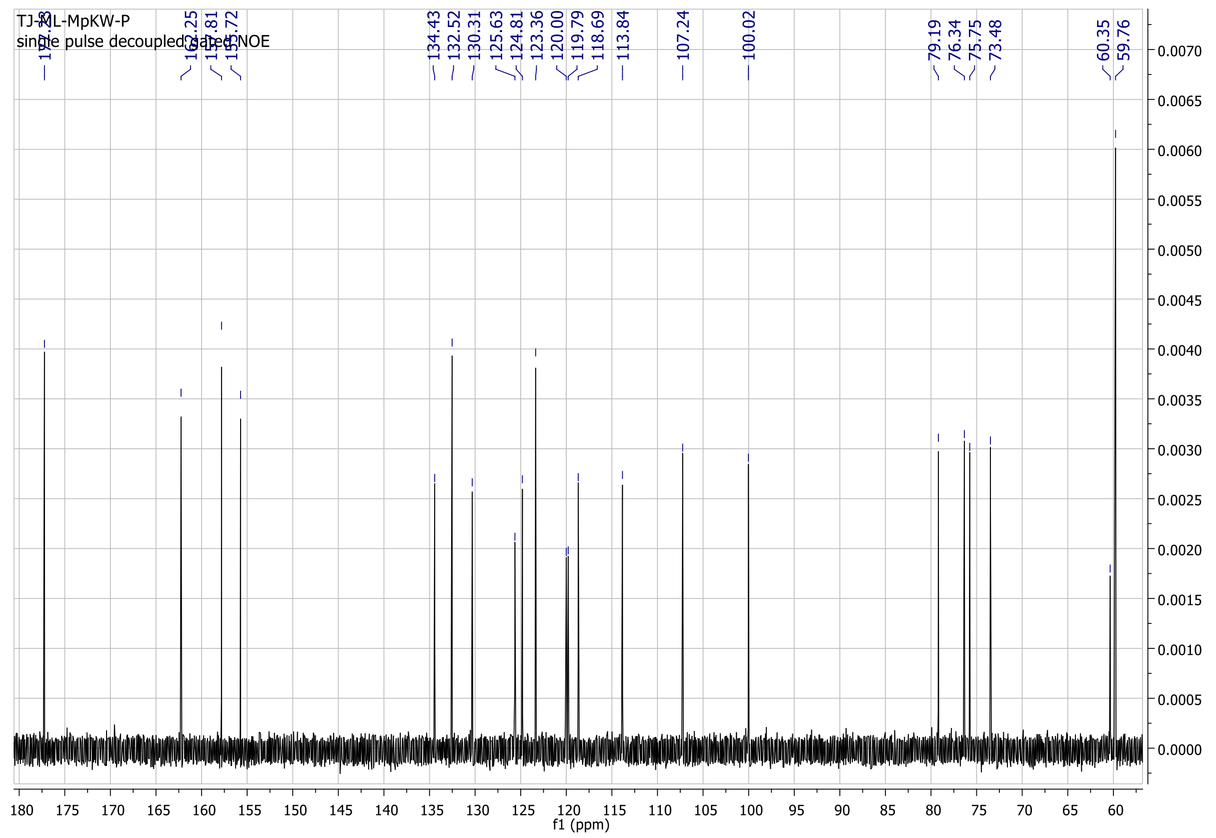


Fig.S45. HMQC spectral of flavone 3’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**12**) (DMSO-d_6, 151 MHz)

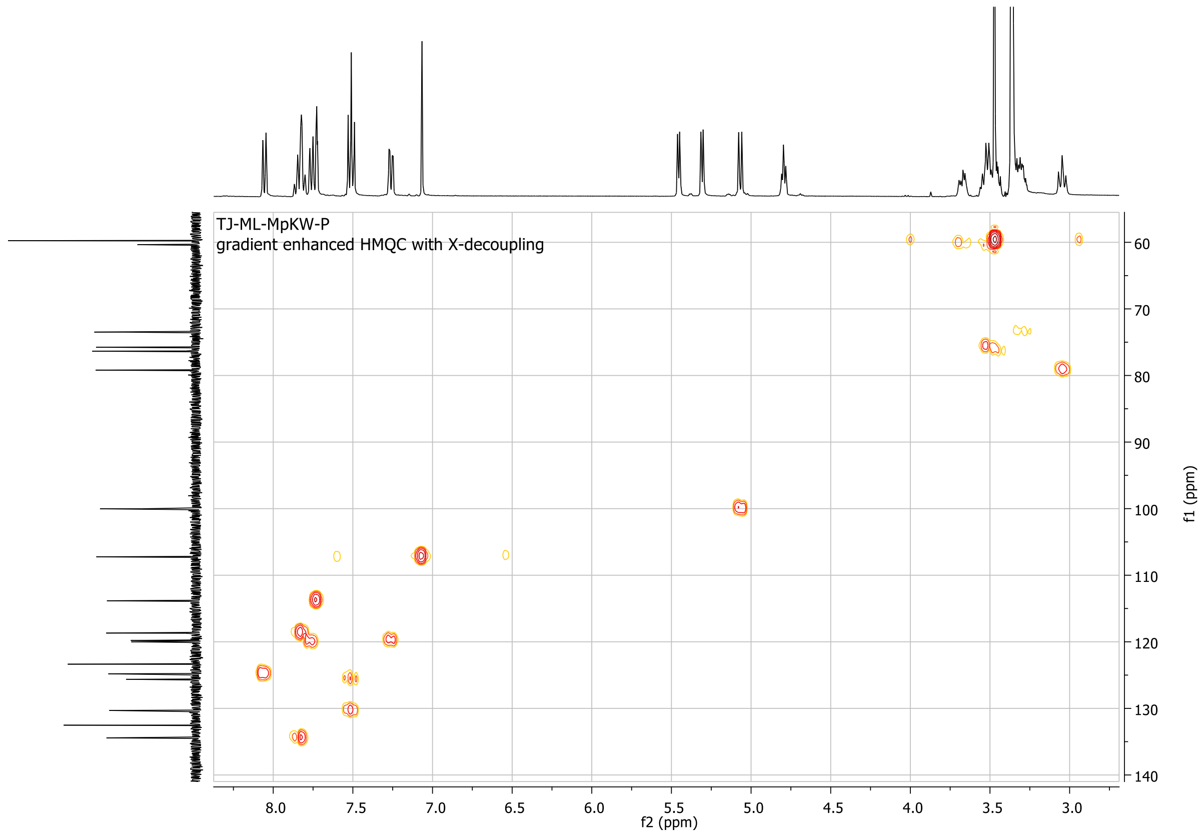


Fig.S46. HMBC spectral of flavone 3’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**12**) (DMSO-d_6, 151 MHz)

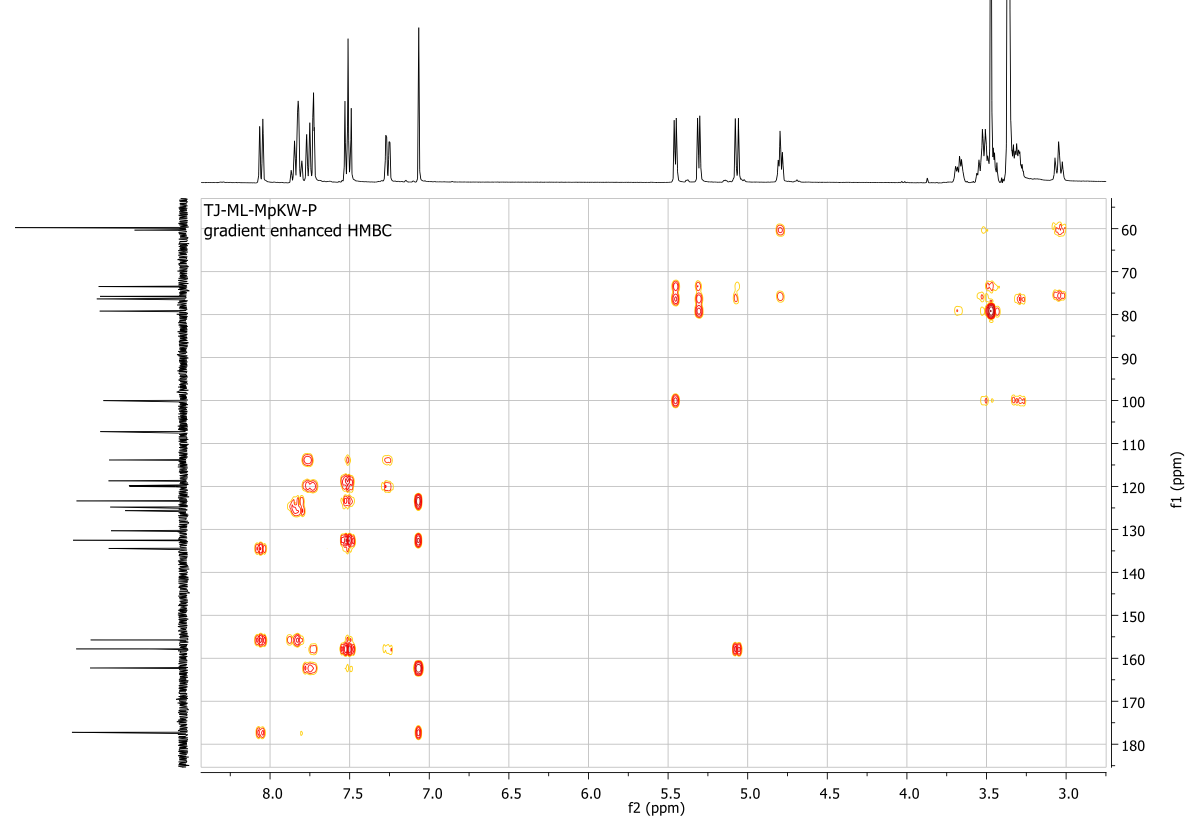
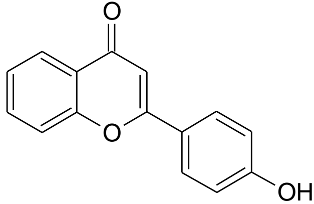
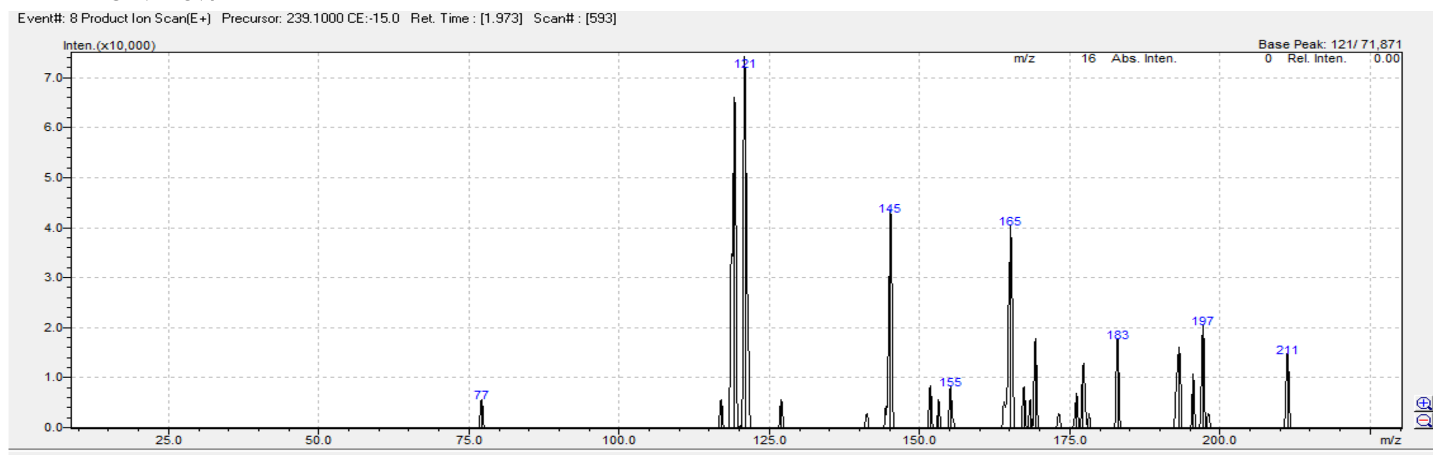


Fig.S47. MS analysis 4'-hydroxyflavone (**13**)

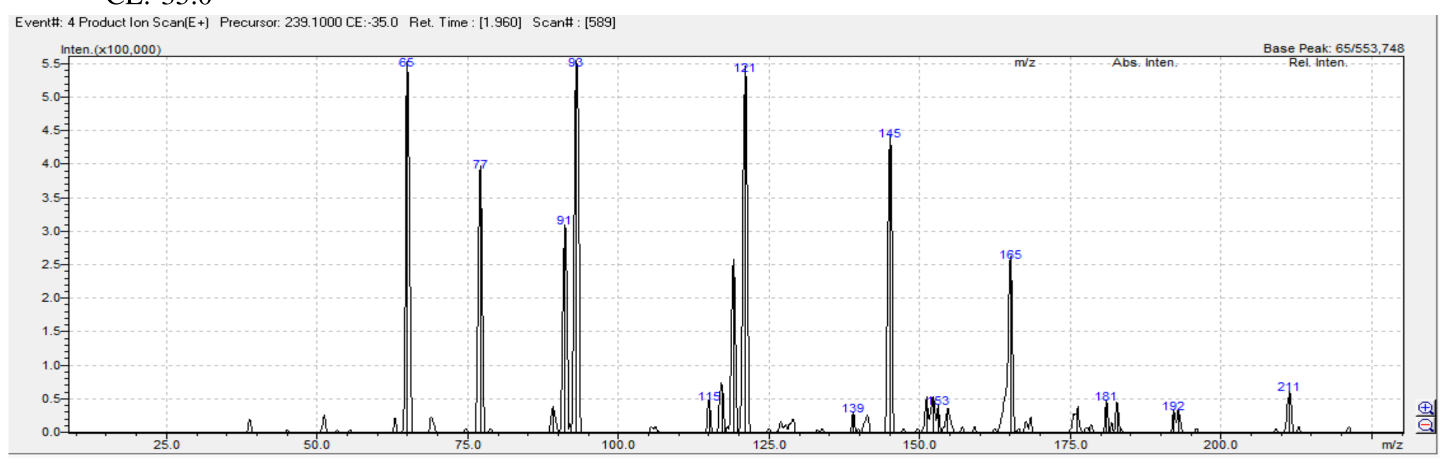


Molecular Formula: $C_{15}H_{10}O_3$
Formula Weight: 238.2381
Precursor: 239.2000

CE: -15.0



CE:-35.0



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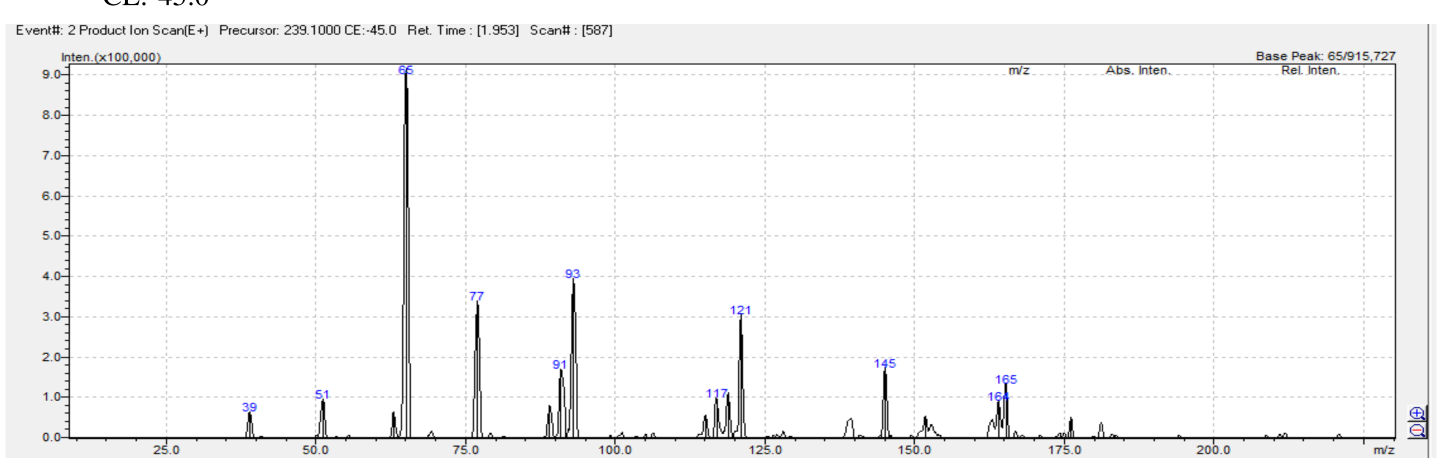


Fig.S48. ^{1}H NMR spectral of 4'-hydroxyflavone (**13**) (DMSO-d_6, 600 MHz)

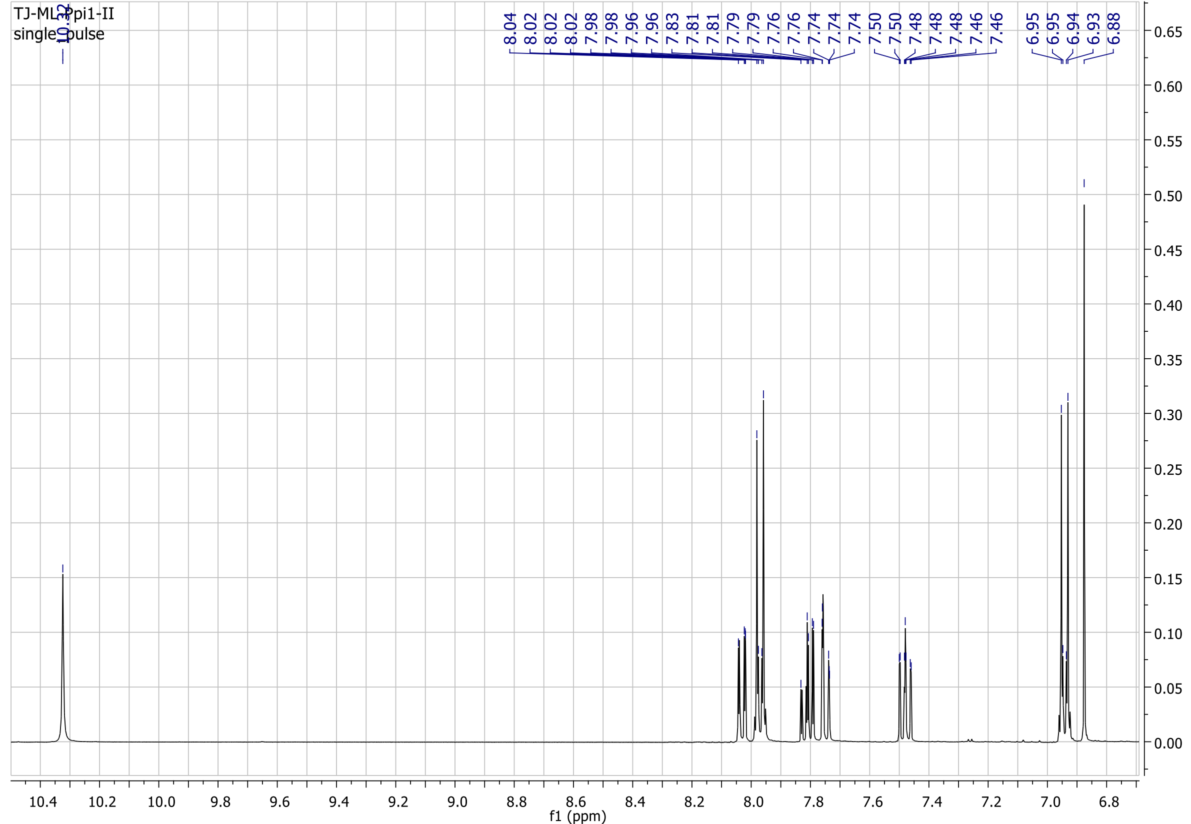


Fig.S49. ^{13}C NMR spectral of 4'-hydroxyflavone (**13**) (DMSO-d_6, 151 MHz)

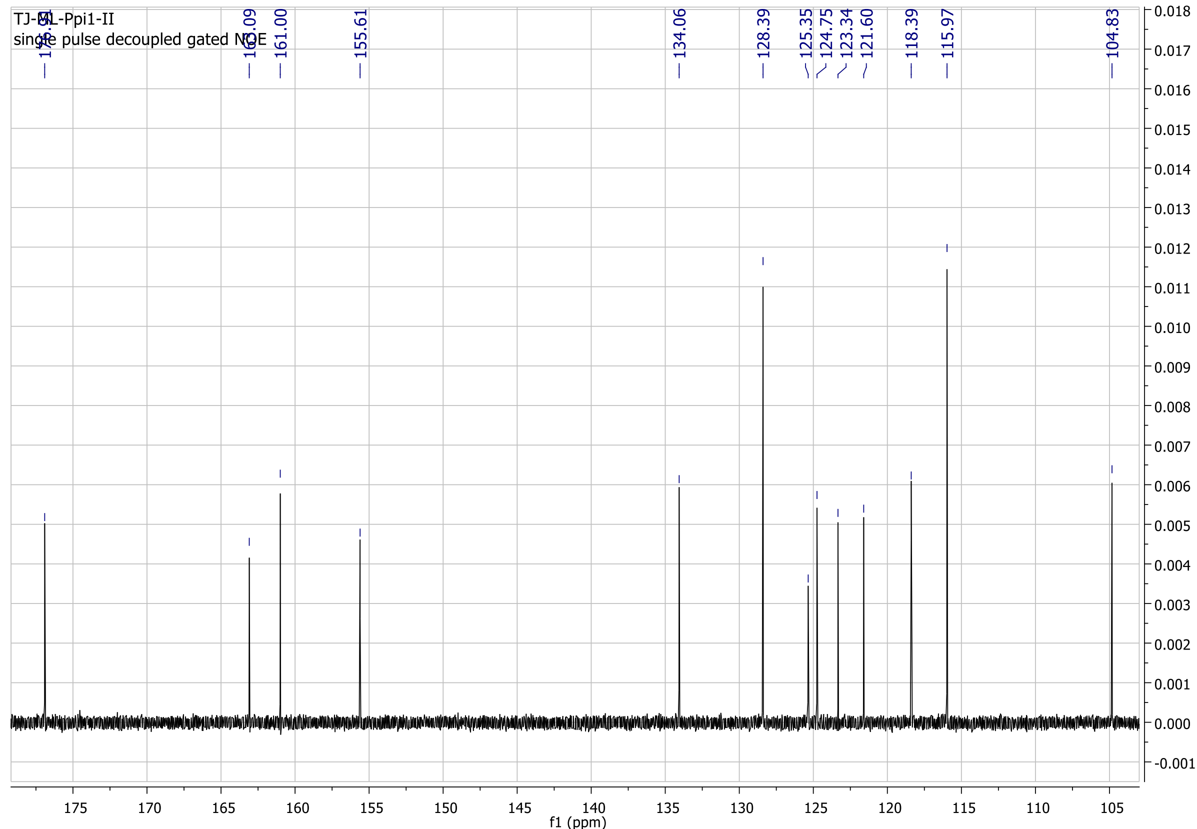


Fig.S50. COSY spectral of 4'-hydroxyflavone (**13**) (DMSO-d_6, 151 MHz)

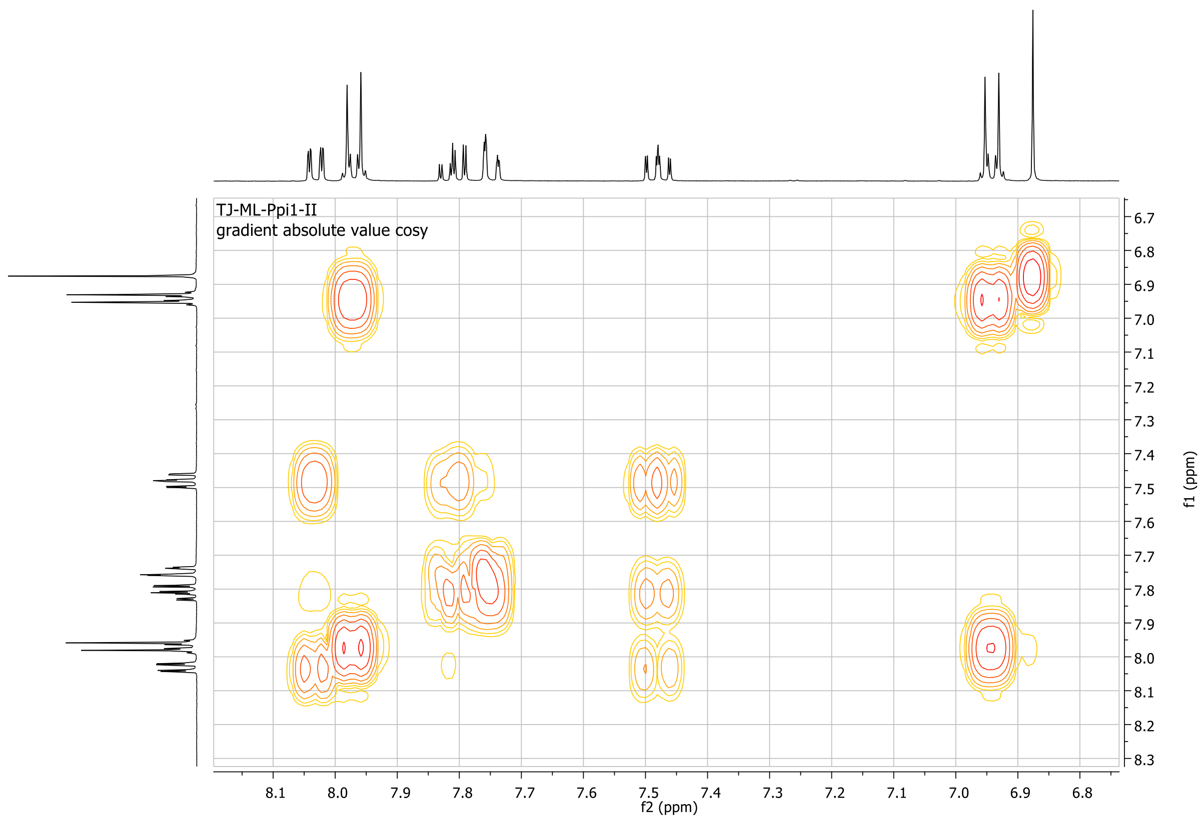


Fig.S51. HMQC spectral of 4'-hydroxyflavone (**13**) (DMSO-d_6, 151 MHz)

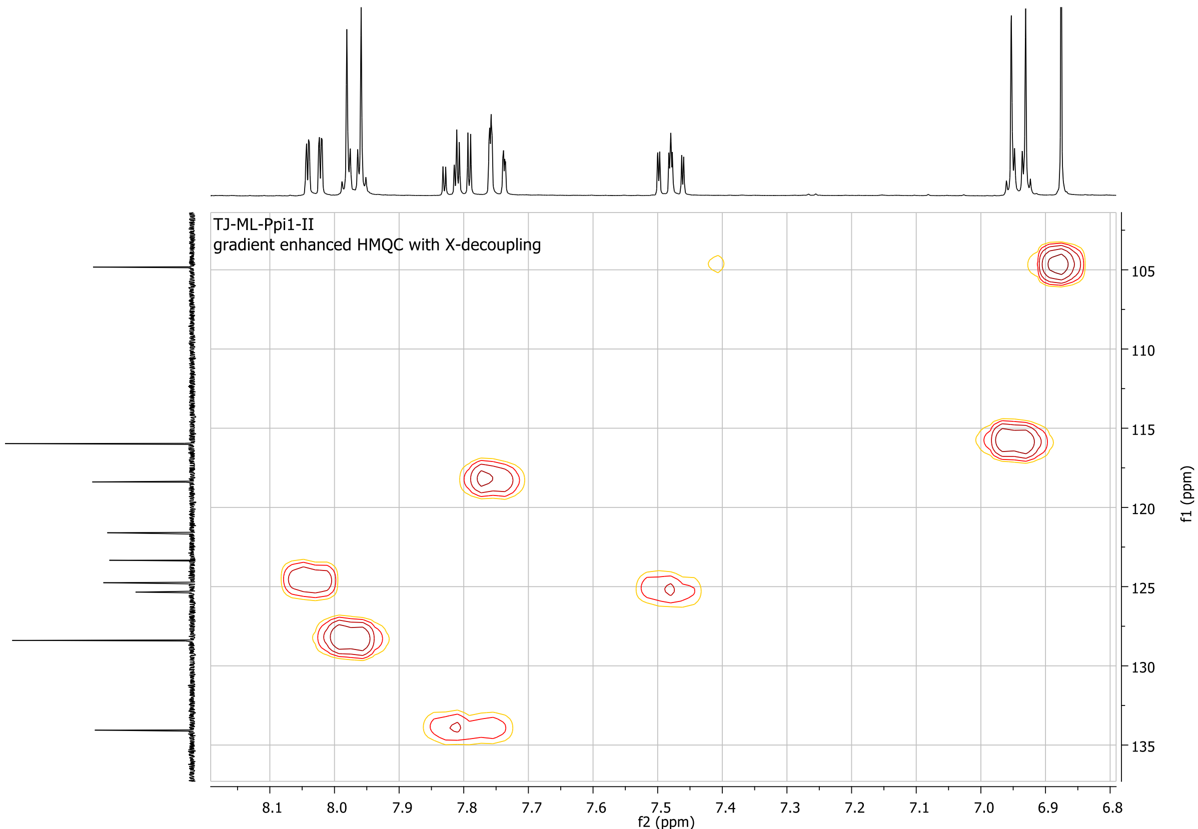


Fig.S52. HMBC spectral of 4’-hydroxyflavone (**13**) (DMSO-d_6, 151 MHz)

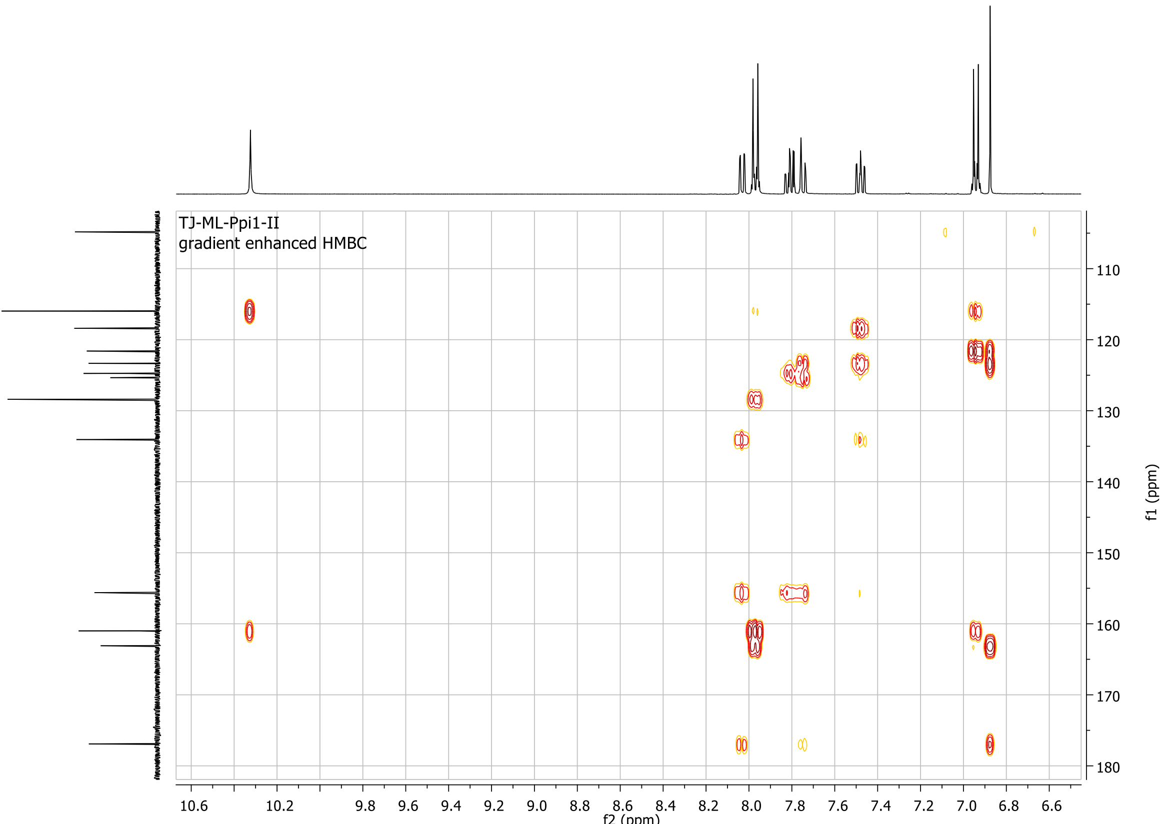
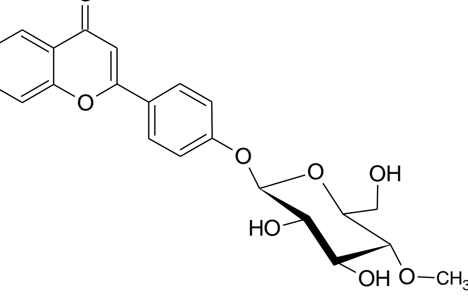


Fig.S53. MS analysis flavone 4'-*O*-β-D-(4''-*O*-methyl)-glucopyranoside (**14**)



Molecular Formula = $C_{22}H_{22}O_8$
Formula Weight = 414.40528
Precursor =415.4000

CE: -15.0

CE:-35.0

CE:-45.0

Fig.S54. ^{1}H NMR spectral of flavone 4’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**14**) (DMSO-d_6, 600 MHz)

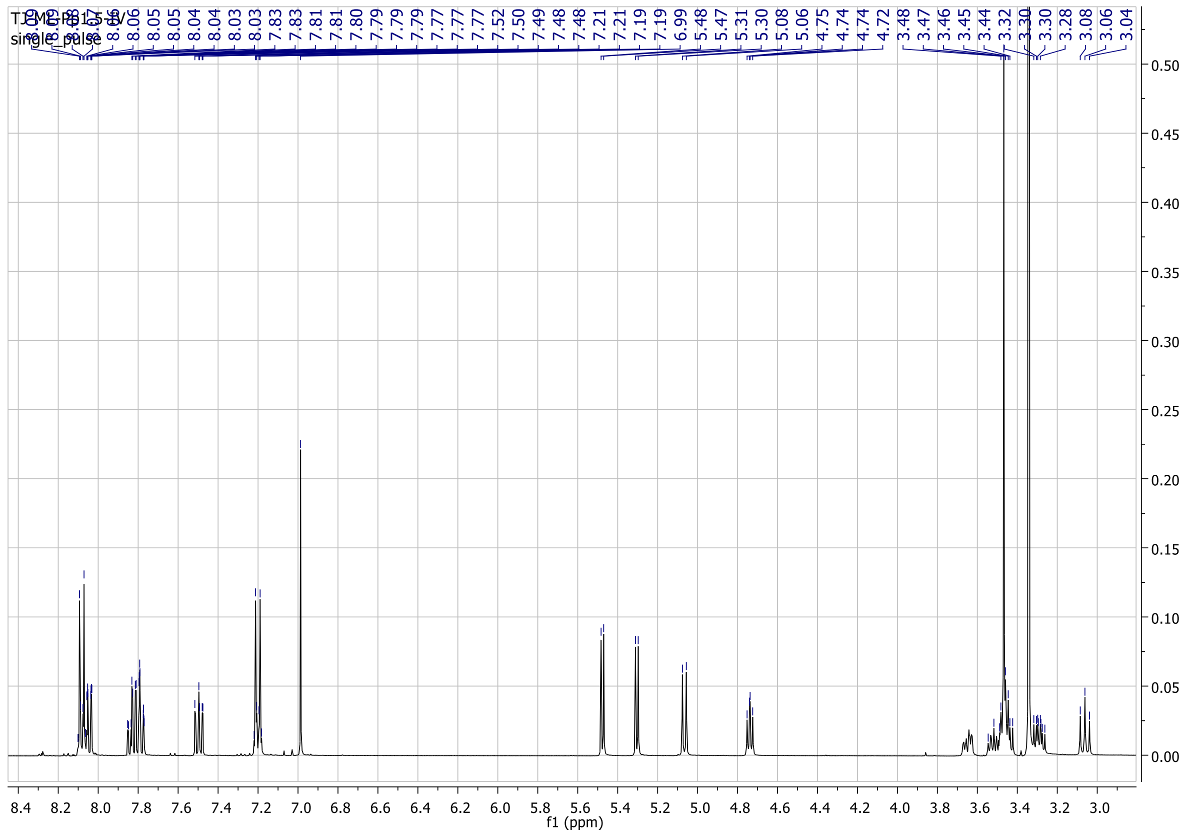


Fig.S55. Flavone part of the ^{1}H NMR spectral flavone 4’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**14**) (DMSO-d_6, 600 MHz)

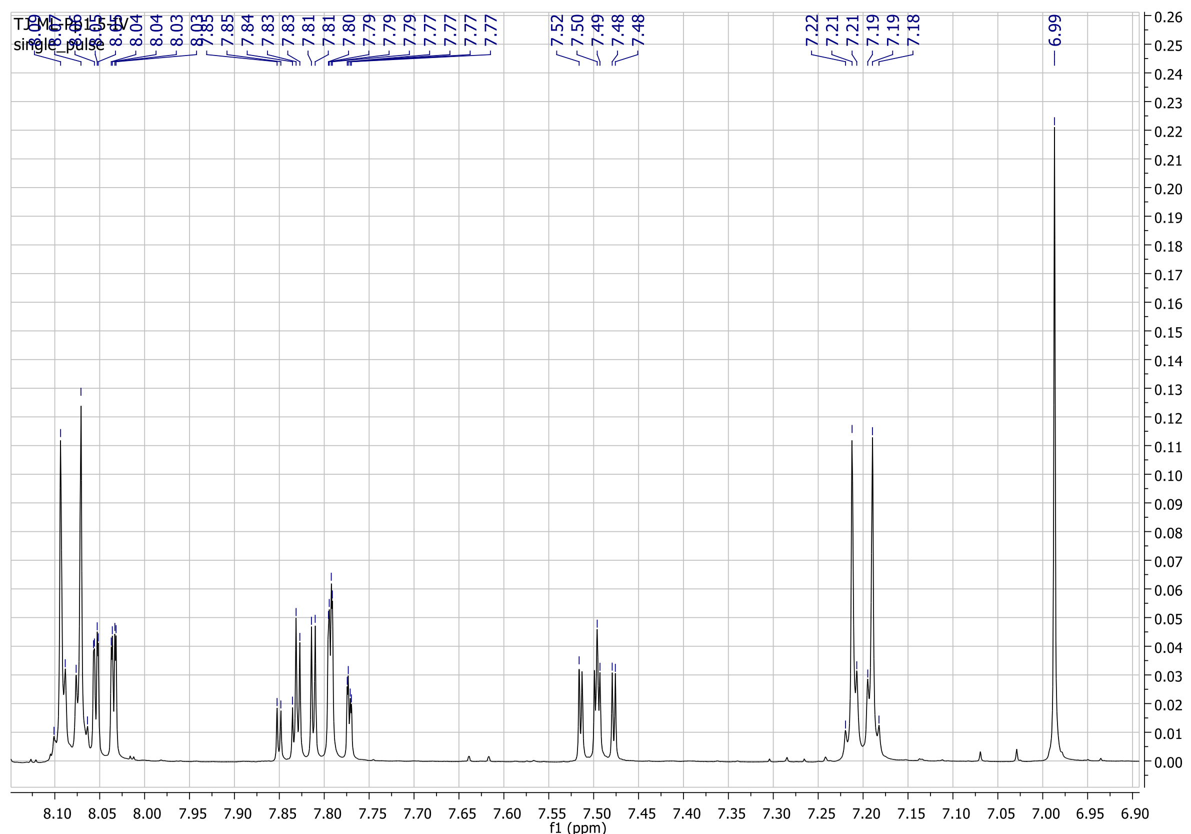


Fig.S56. Glucopyranoside part of the ^{1}H NMR spectral flavone 4'-*O*-β-D-(4''-*O*-methyl)-glucopyranoside (**14**) (DMSO-d_6, 600 MHz)

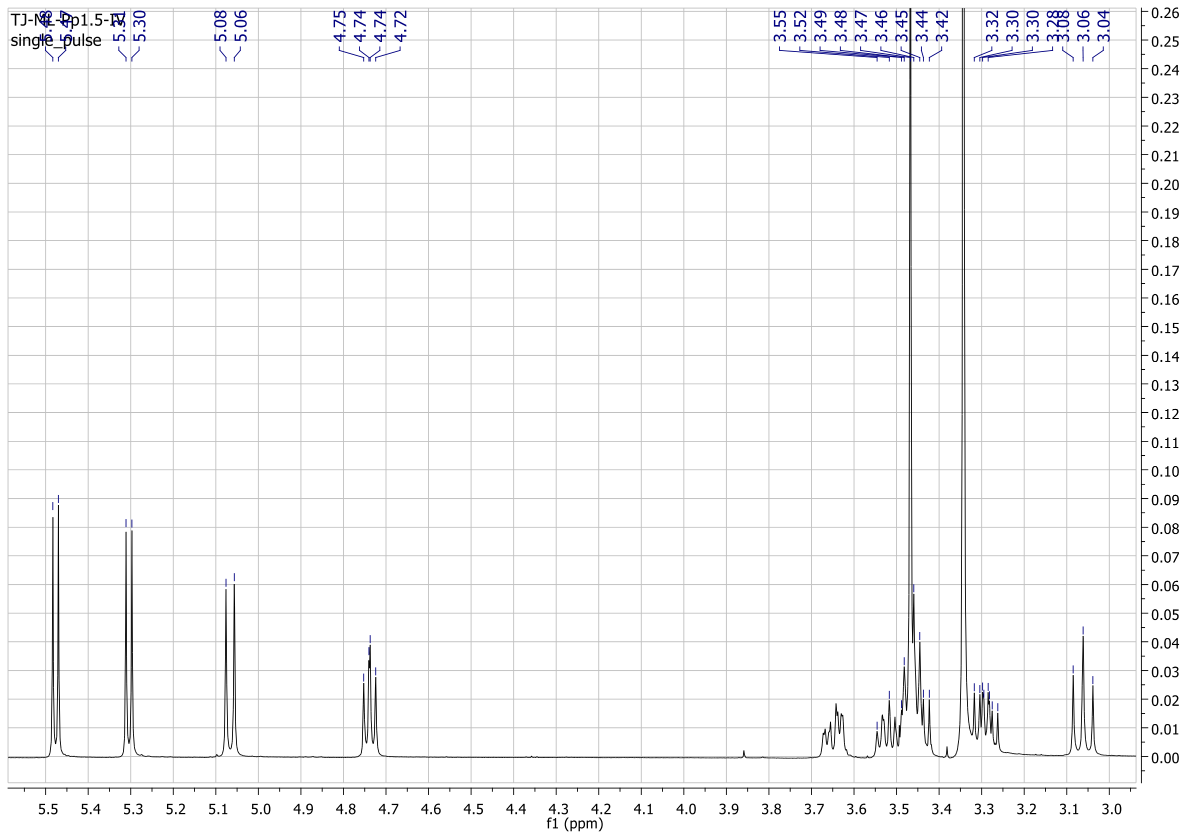


Fig.S57. ^{13}C NMR spectral of flavone 4'-*O*-β-D-(4''-*O*-methyl)-glucopyranoside (**14**) (DMSO-d_6, 151 MHz)

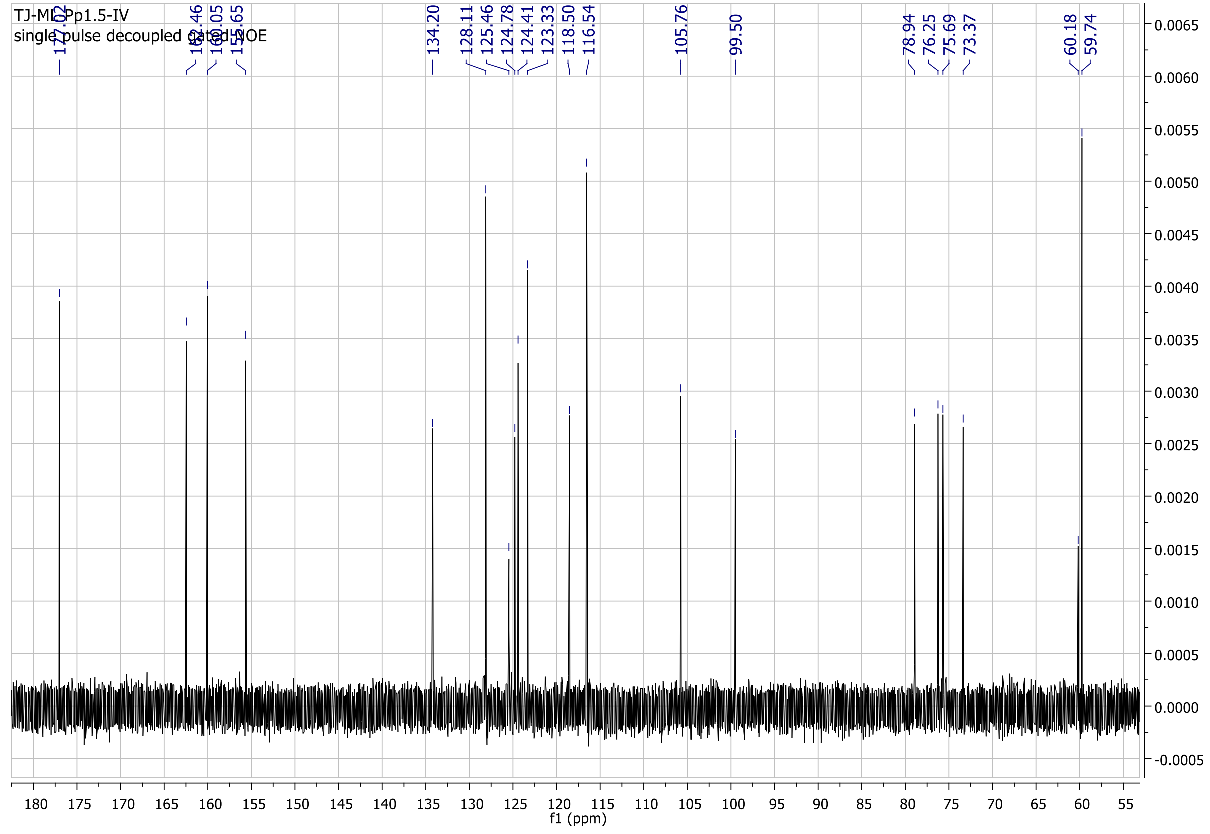


Fig.S58. COSY spectral of flavone 4’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**14**) (DMSO-d_6, 151 MHz)

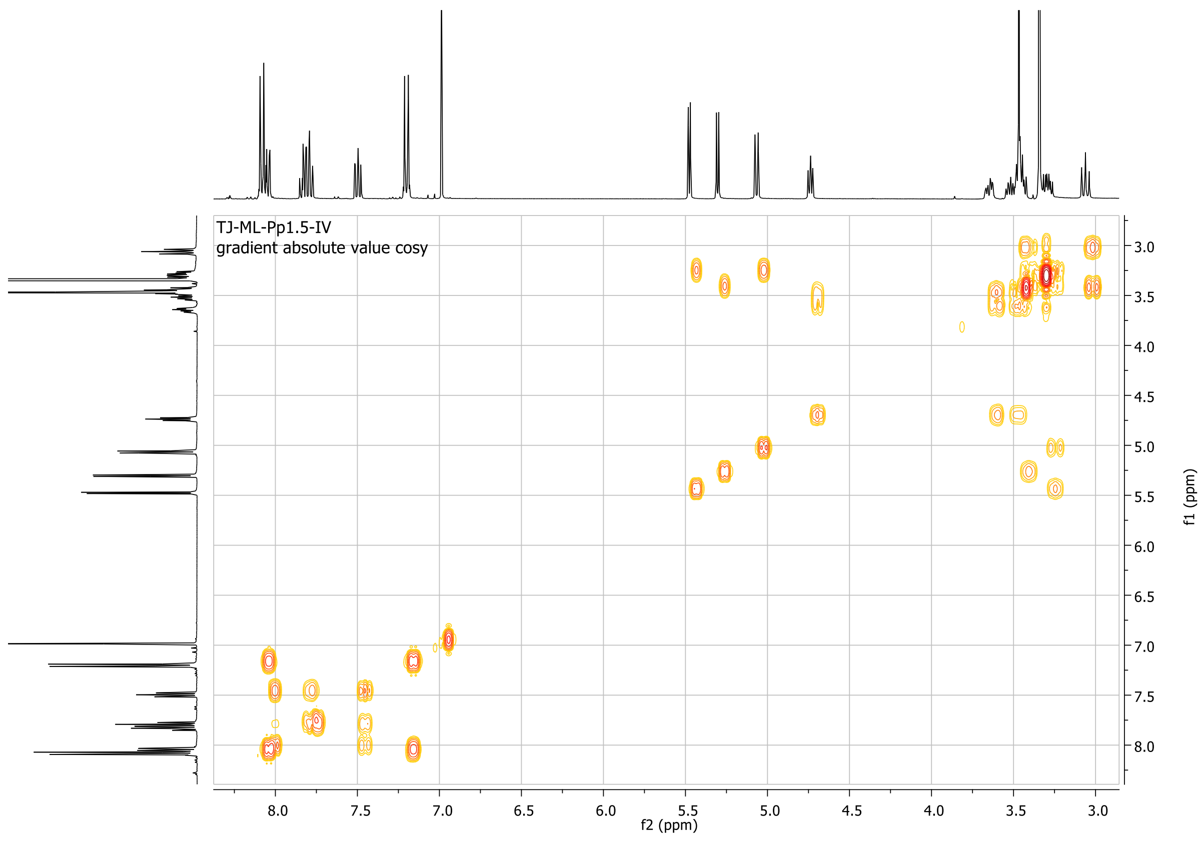


Fig.S59. HMQC spectral of flavone 4’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**14**) (DMSO-d_6, 151 MHz)

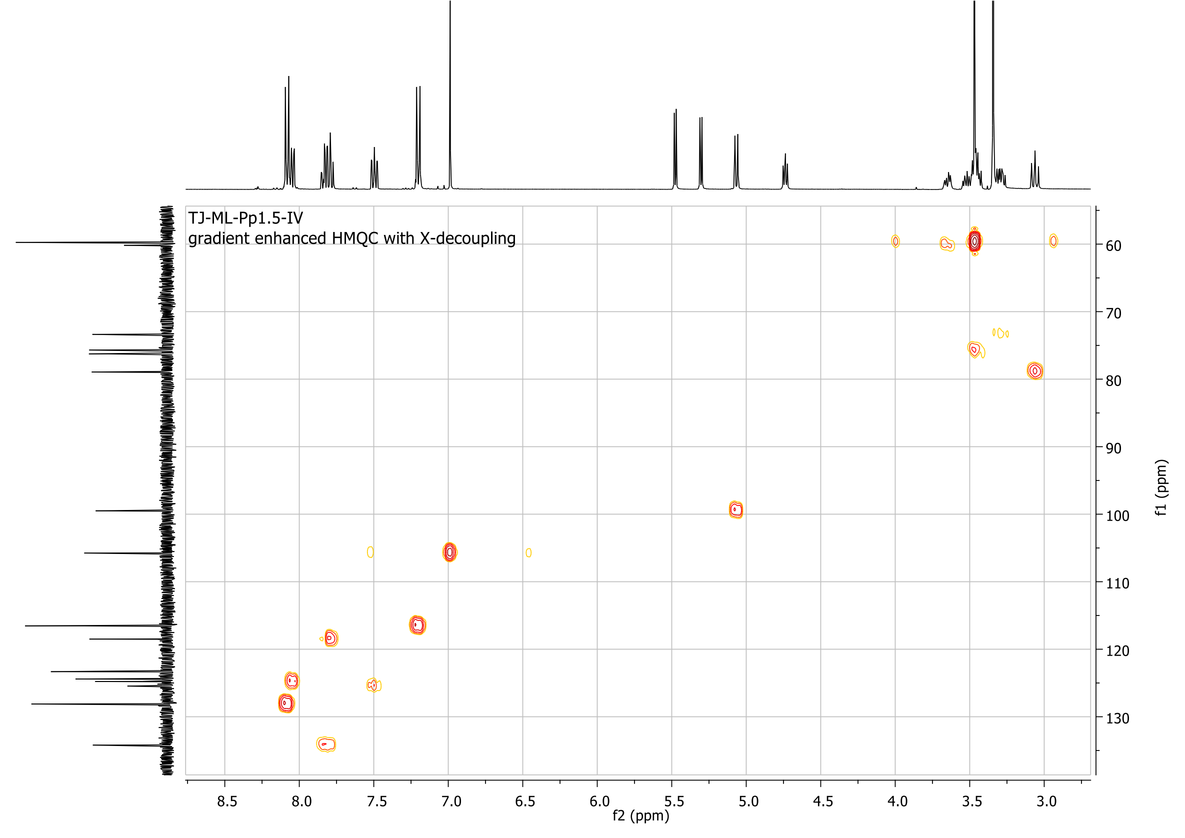


Fig.S60. HMBC spectral of flavone 4'-*O*-β-D-(4''-*O*-methyl)-glucopyranoside (**14**) (DMSO-d_6, 151 MHz)

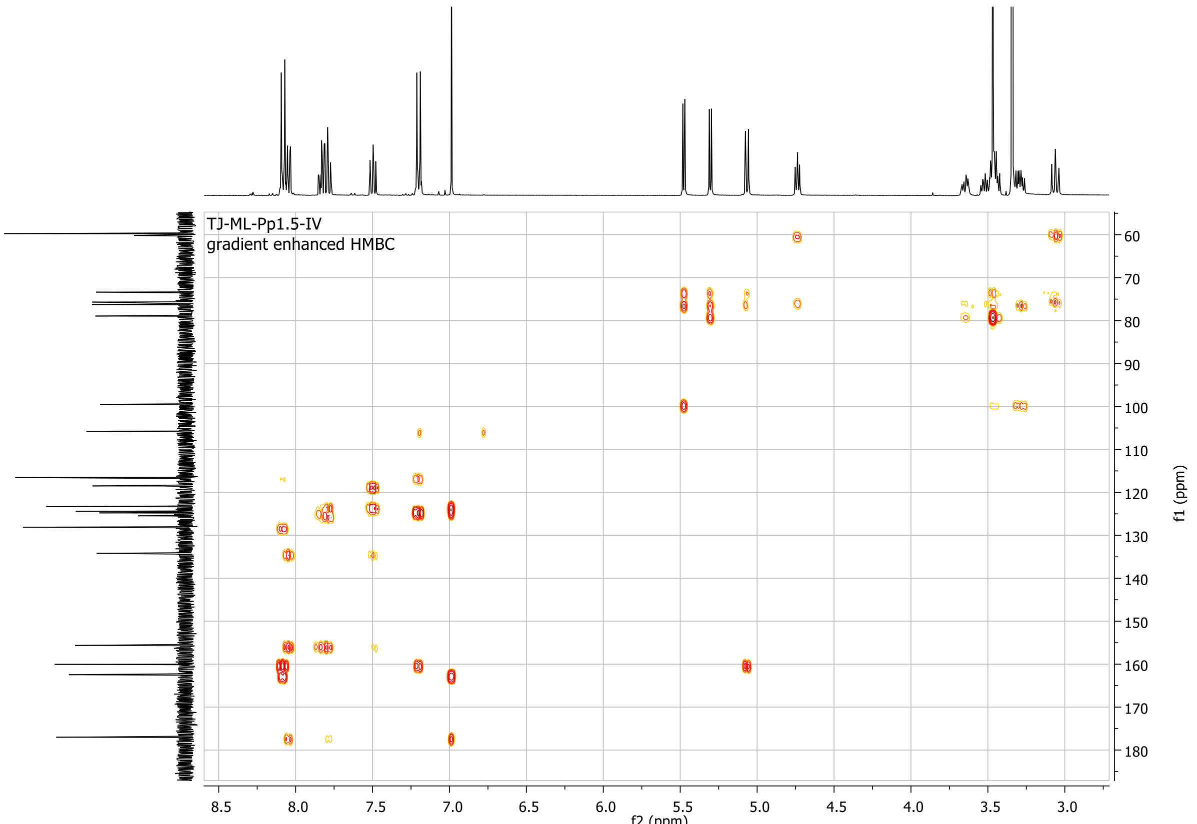
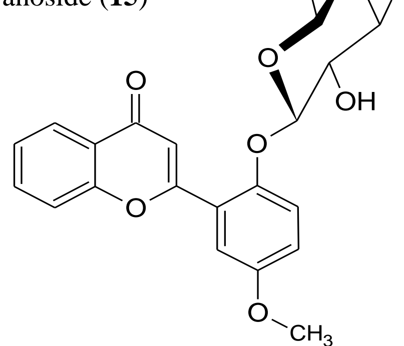


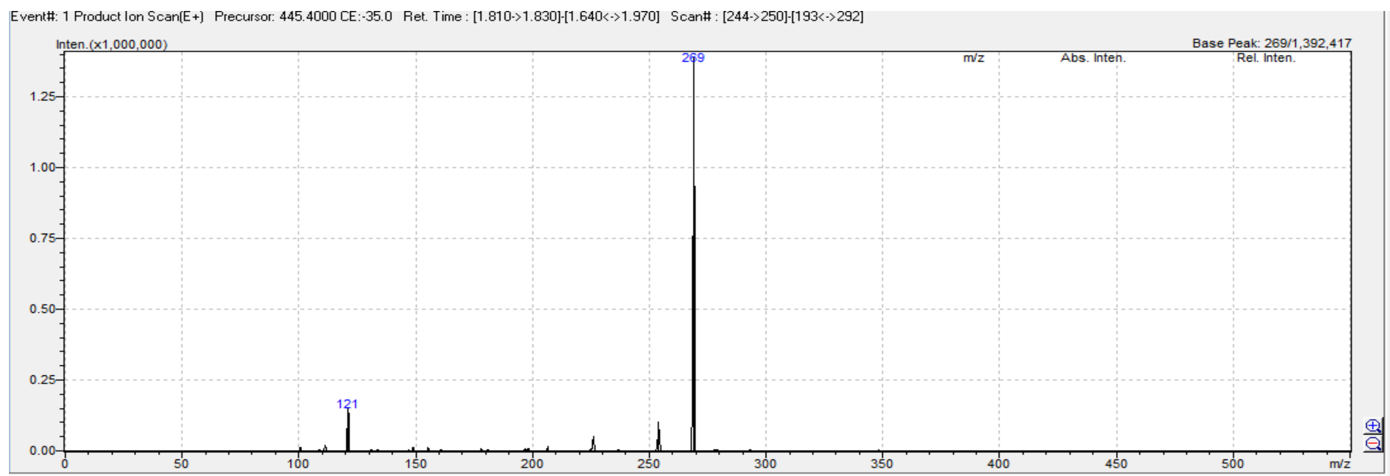
Fig.S61. MS analysis 5'-methoxyflavone 2'-*O*-β-D-(4''-*O*-methyl)-glucopyranoside (**15**)



Molecular Formula = $C_{23}H_{24}O_9$
Formula Weight = 444.43126
Precursor =445.4000

CE: -15.0

CE:-35.0



CE:-45.0

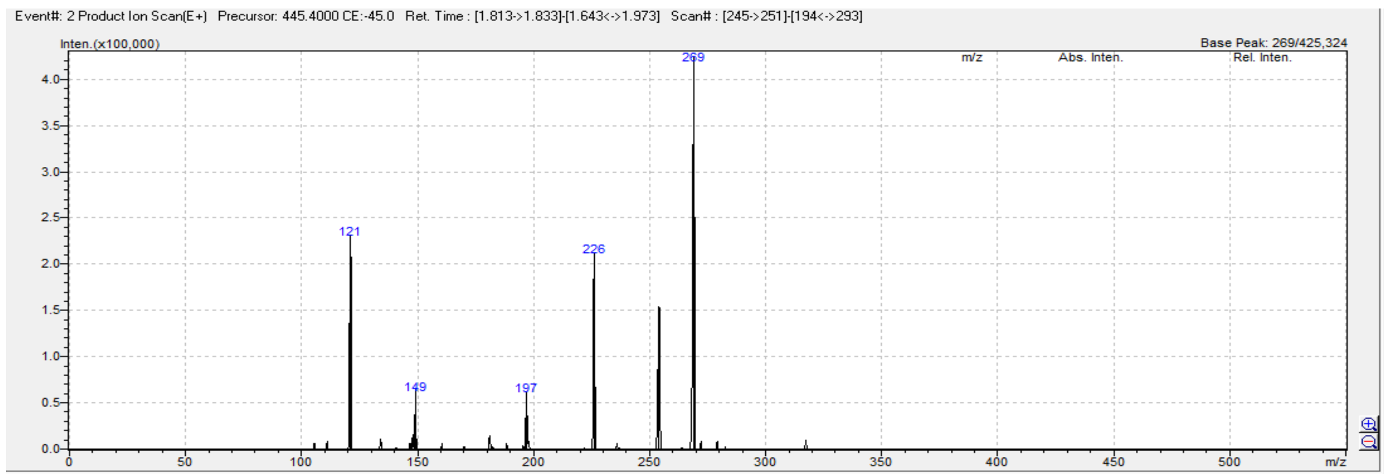


Fig.S62. ^{1}H NMR spectral of 5'-methoxyflavone 2'-*O*-β-D-(4''-*O*-methyl)-glucopyranoside (**15**) (DMSO-d_6, 600 MHz)

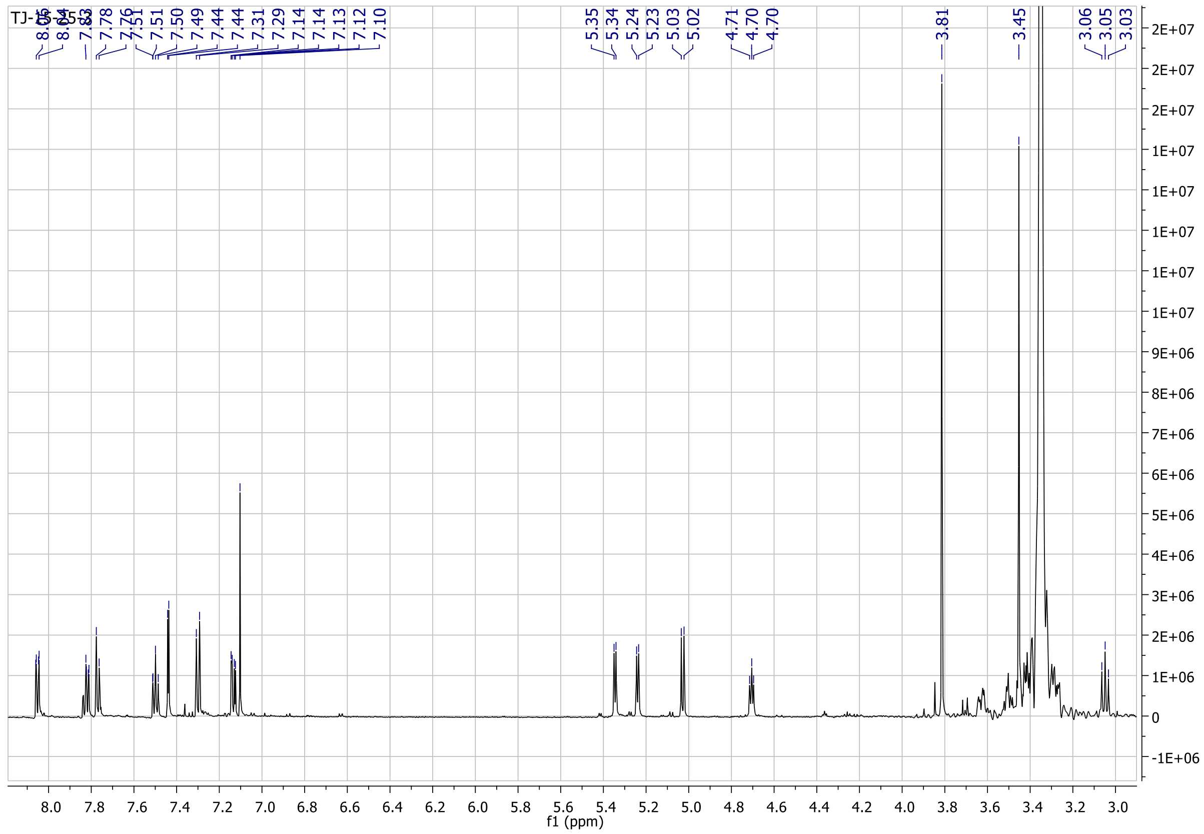


Fig.S63. Flavone part of the ^{1}H NMR spectral 5'-methoxyflavone 2'-*O*-β-D-(4''-*O*-methyl)-glucopyranoside (**15**) (DMSO-d_6, 600 MHz)

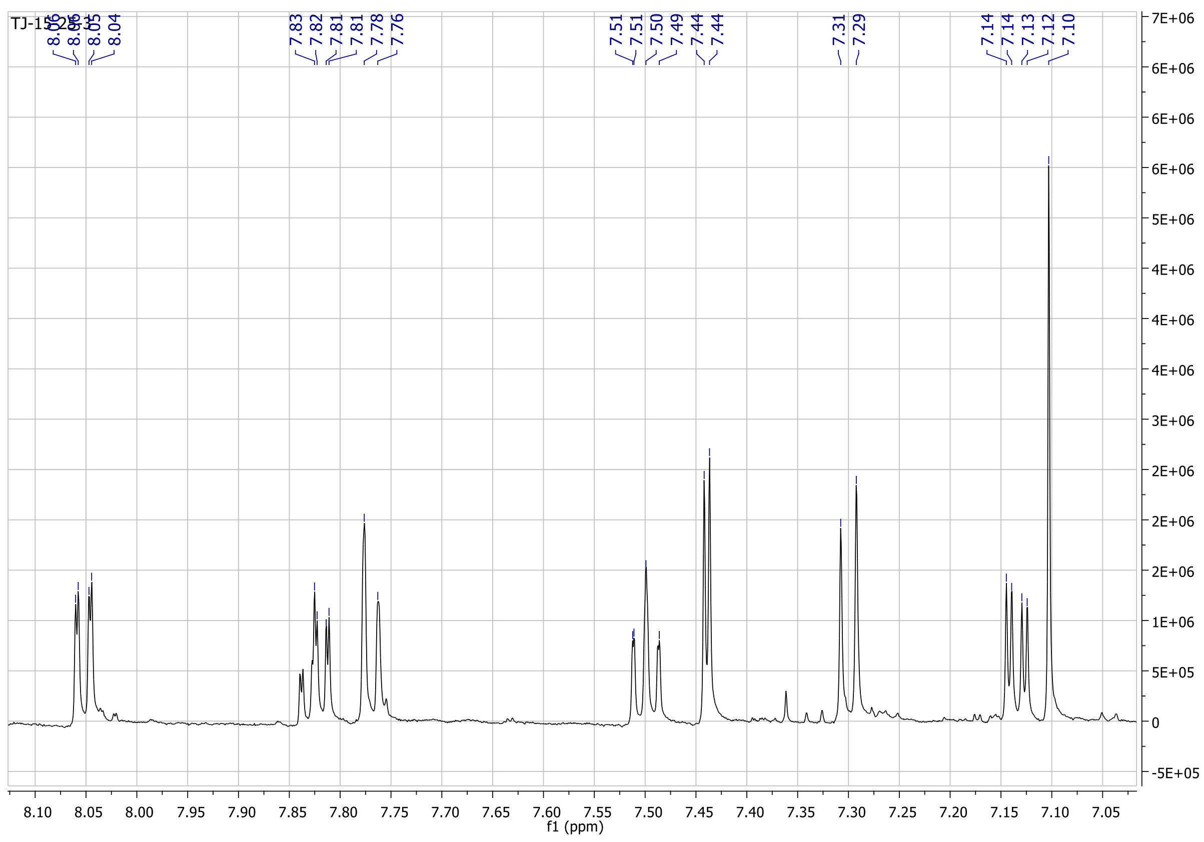


Fig.S64. Glucopyranoside part of the ^{1}H NMR spectral 5’-methoxyflavone 2’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**15**) (DMSO-d_6, 600 MHz)

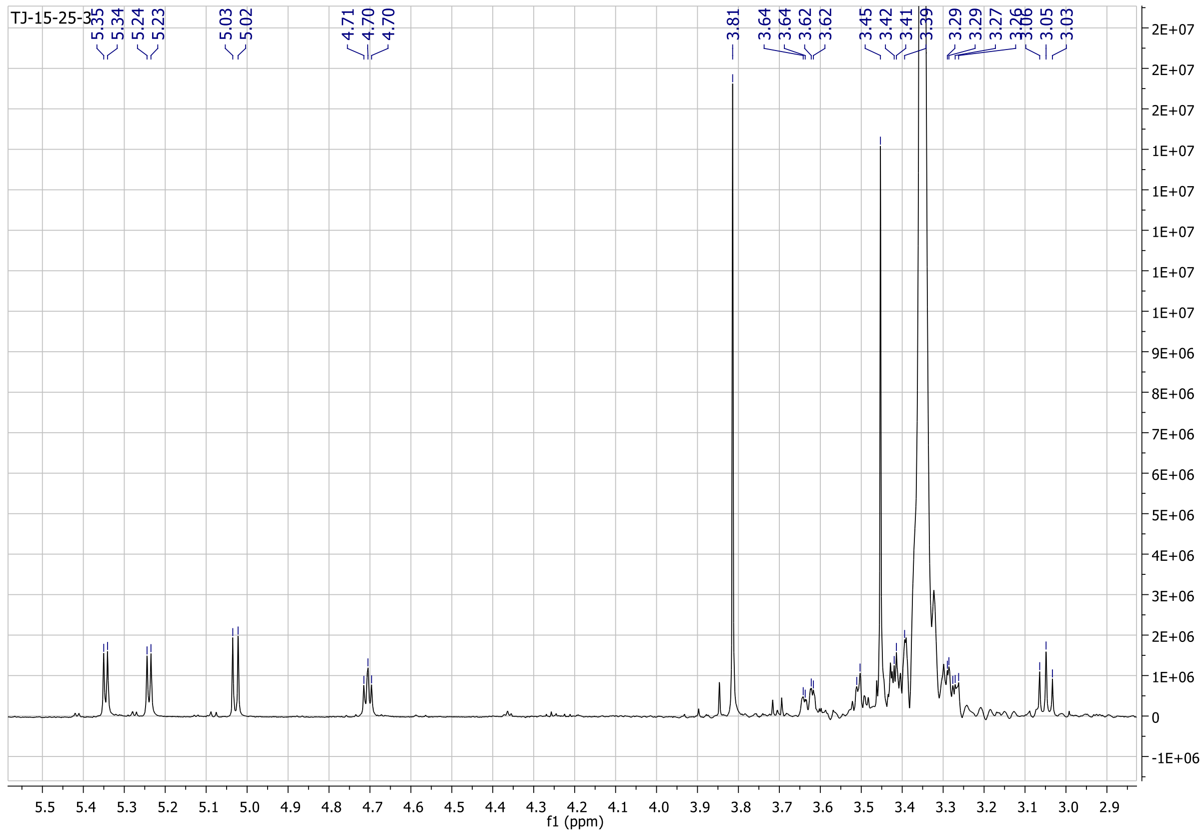


Fig.S65. ^{13}C NMR spectral of 5’-methoxyflavone 2’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**15**) (DMSO-d_6, 151 MHz)

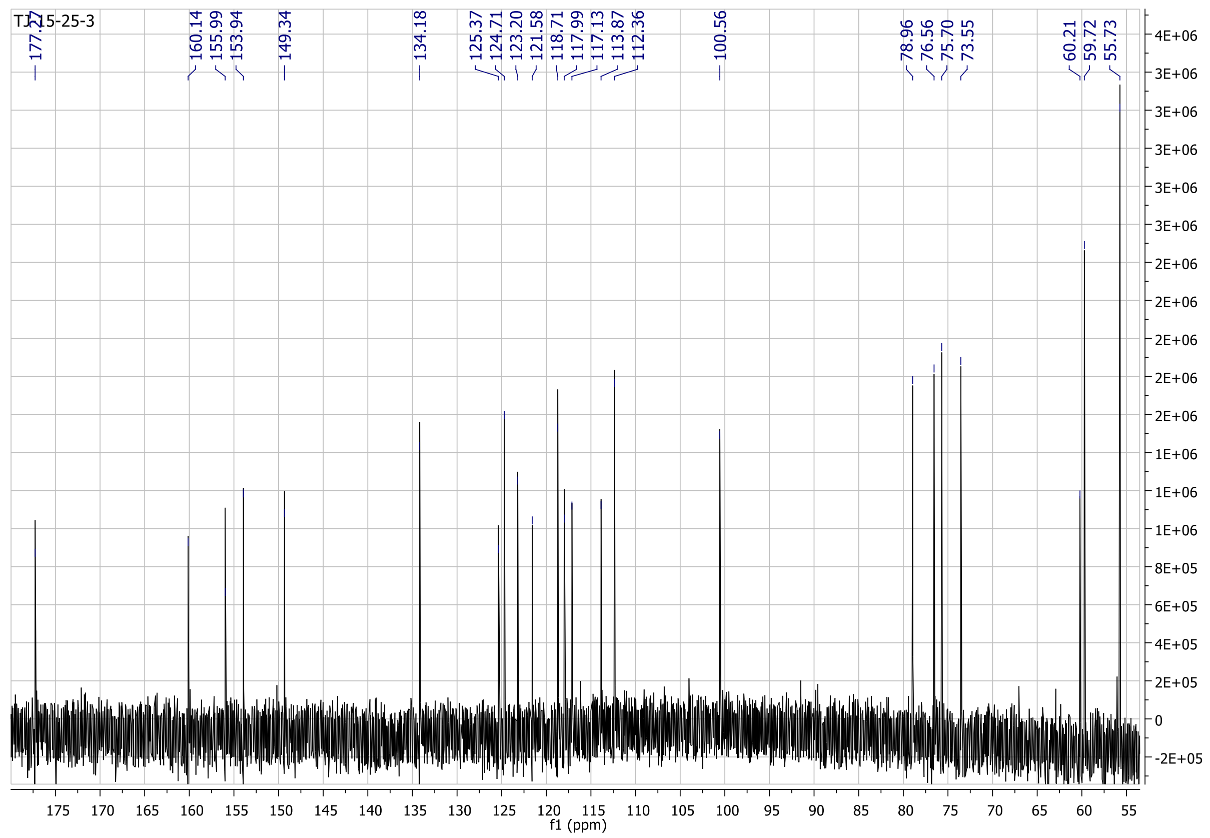


Fig.S66. COSY spectral of 5'-methoxyflavone 2'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**15**) (DMSO-d_6, 151 MHz)

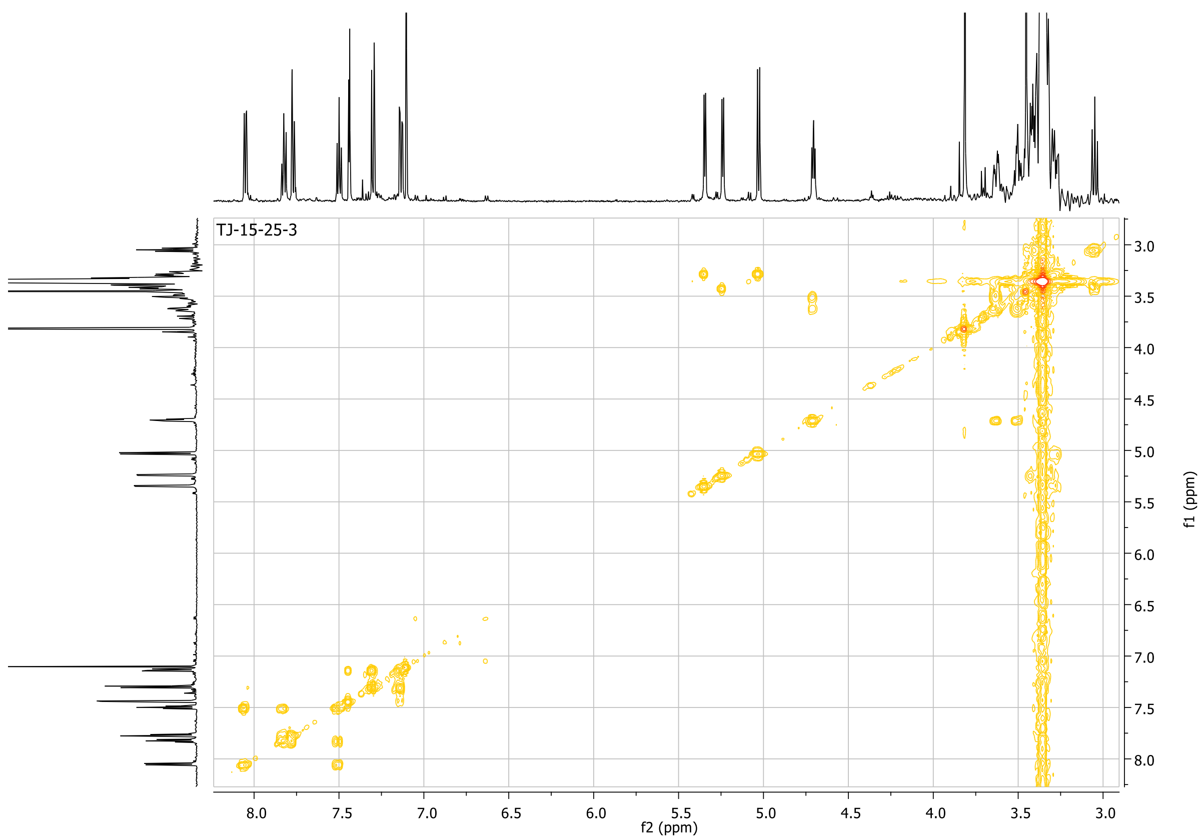


Fig.S67. HMQC spectral of 5'-methoxyflavone 2'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**15**) (DMSO-d_6, 151 MHz)

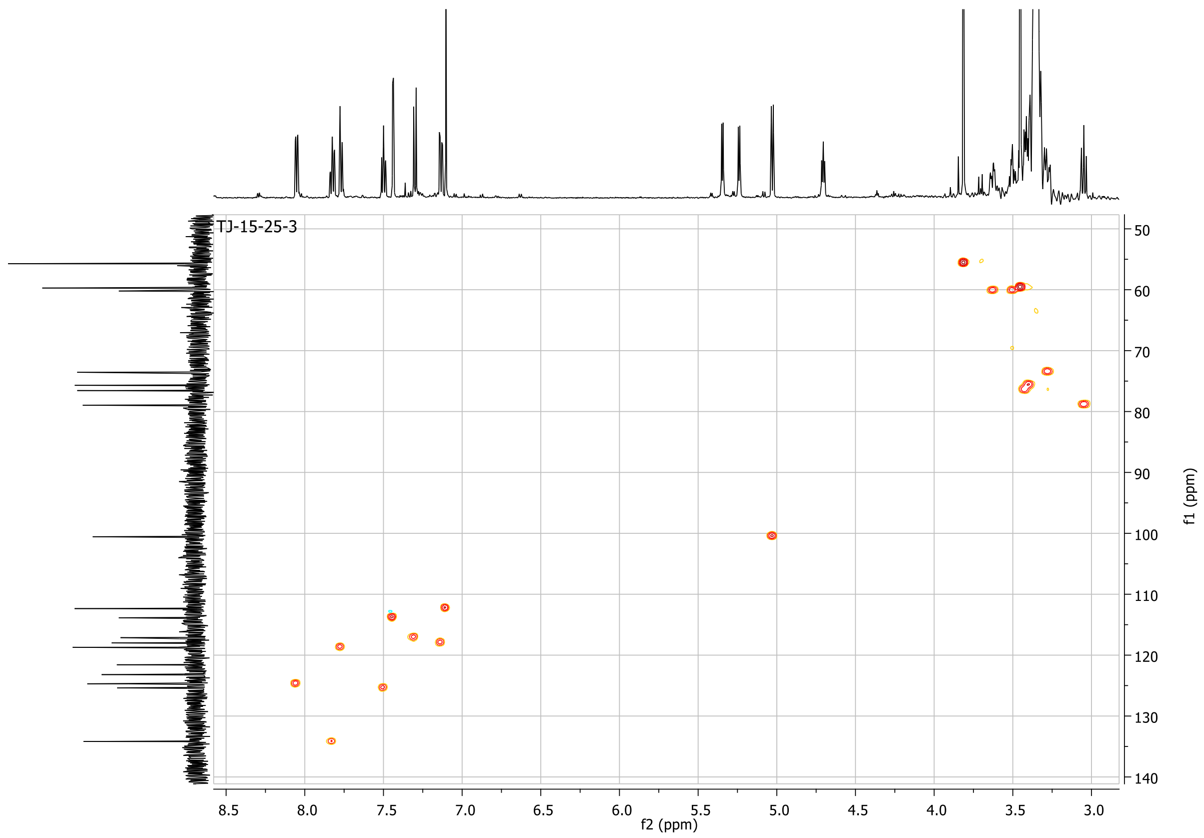


Fig.S68. HMBC spectral of 5’-methoxyflavone 2’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**15**) (DMSO-d_6, 151 MHz)

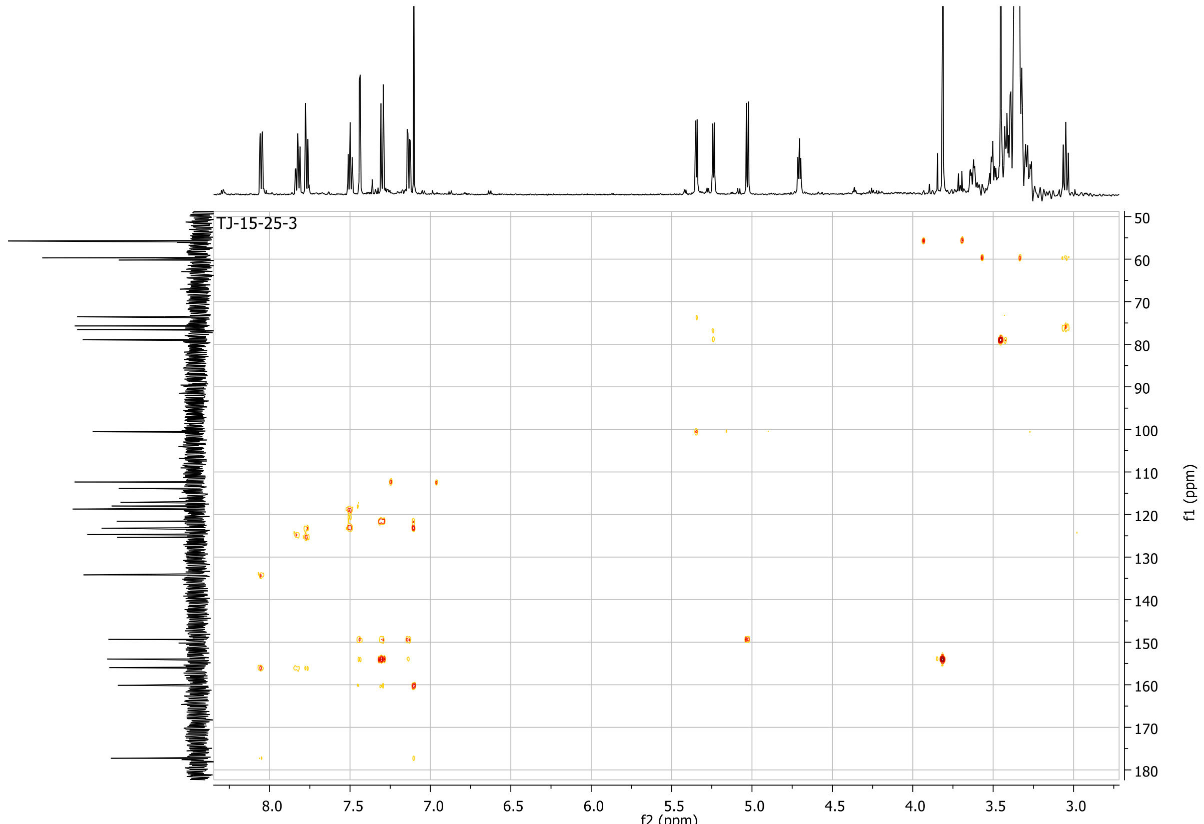
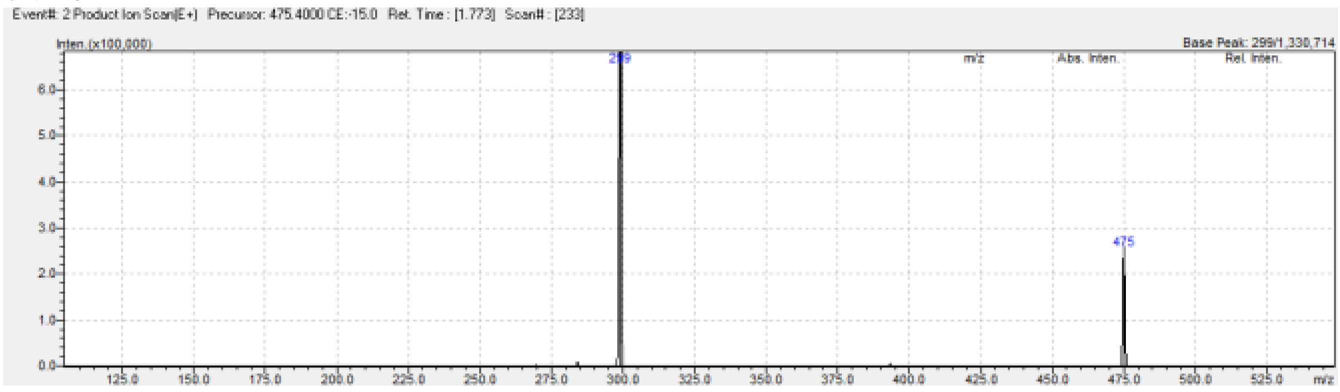


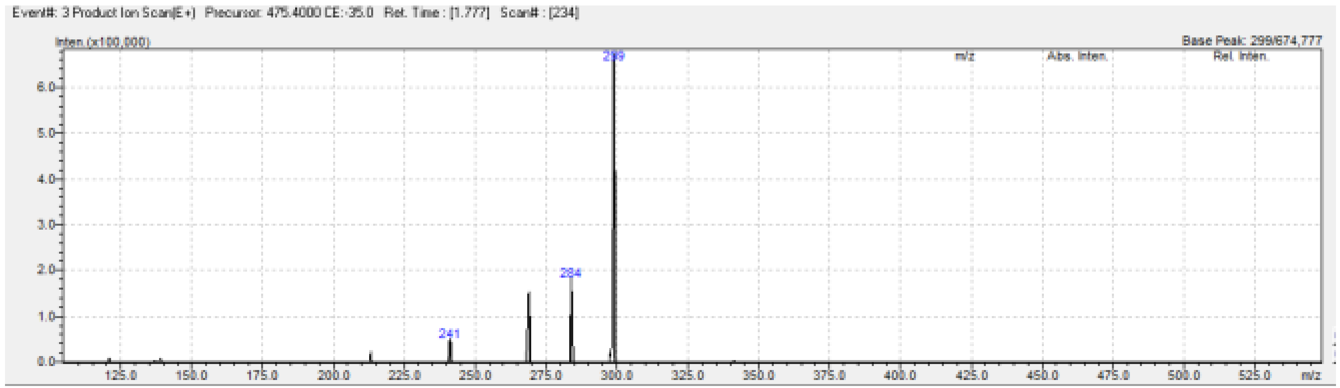
Fig.S69. MS analysis 2’,5’-dimethoxyflavone 4’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**16**)

Molecular Formula = $C_{24}H_{26}O_{10}$
Formula Weight = 474.45724
Precursor: =475.4000

CE: -15



CE:-35



CE:-45

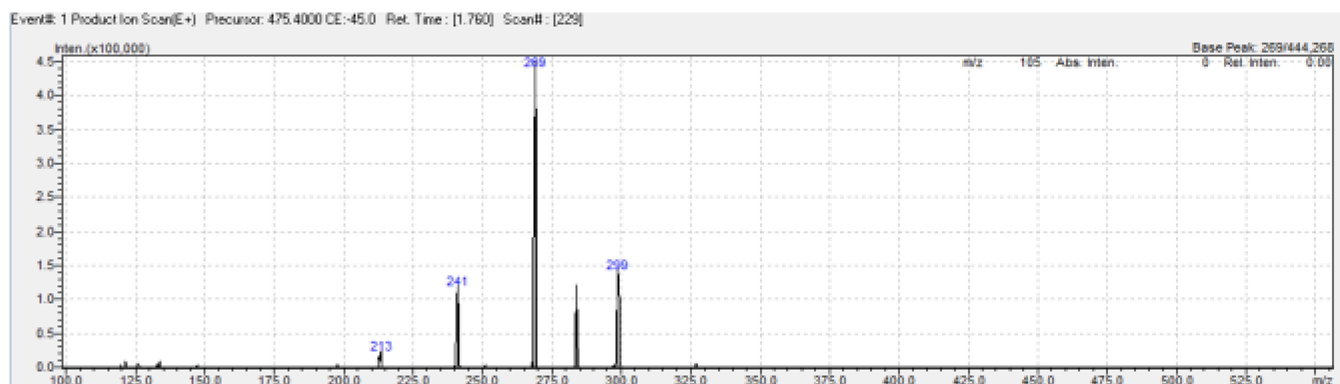


Fig.S70. ^{1}H NMR spectral of 2’,5’-dimethoxyflavone 4’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**16**) (DMSO-d_6, 600 MHz)

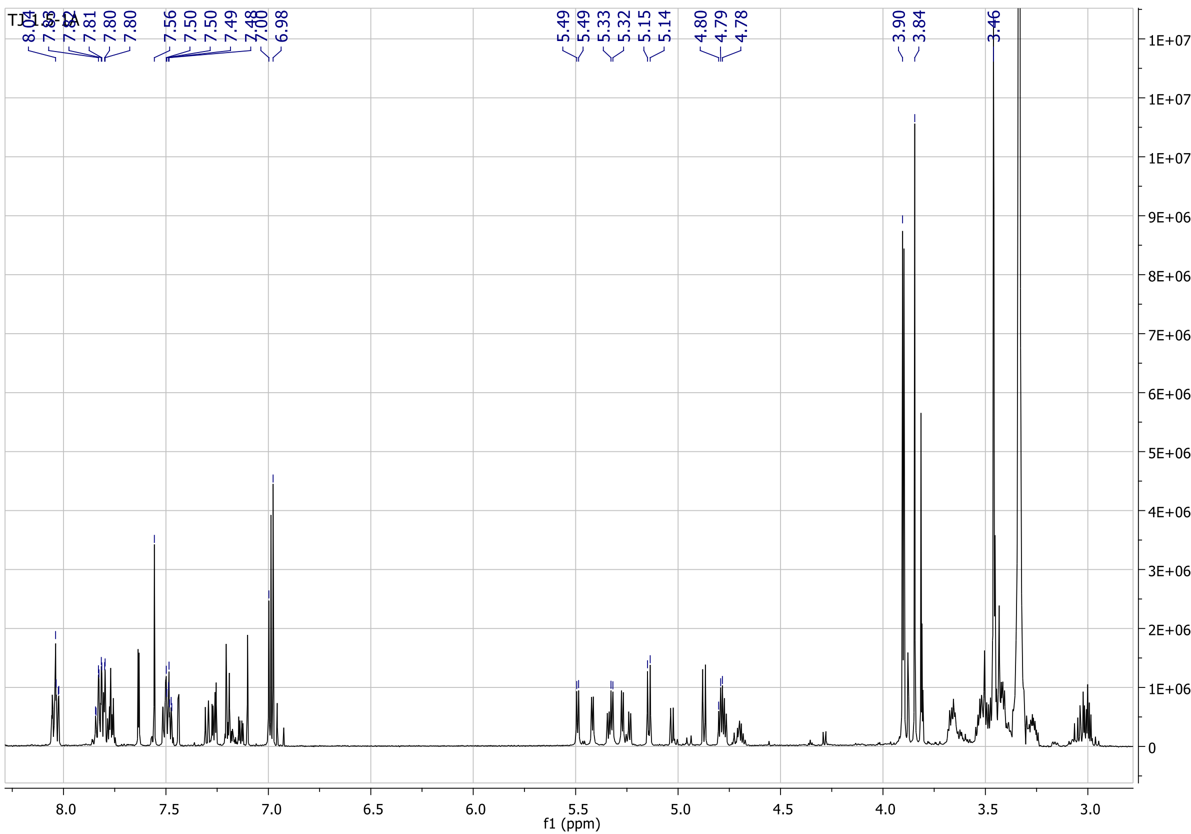


Fig.S71. Flavone part of the ^{1}H NMR spectral 2’,5’-dimethoxyflavone 4’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**16**) (DMSO-d_6, 600 MHz)

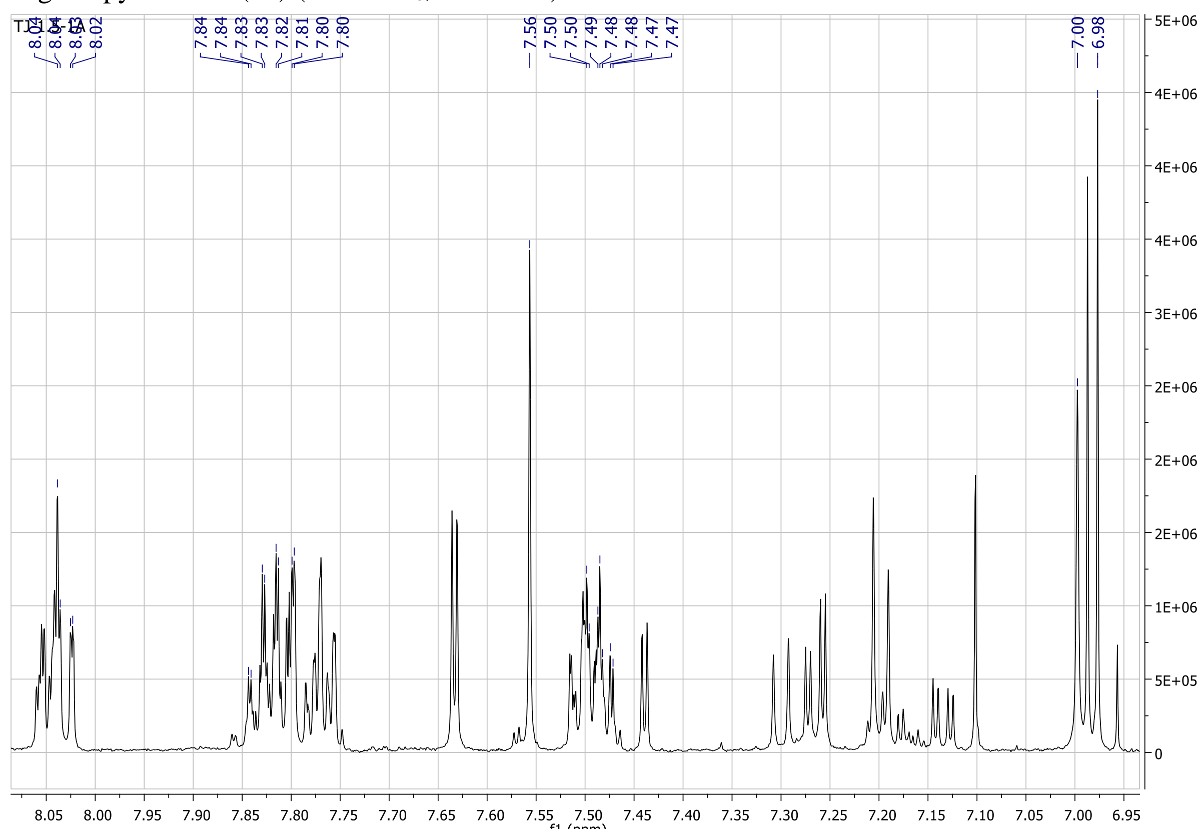


Fig.S72. Glucopyranoside part of the ^{1}H NMR spectral 2',5'-dimethoxyflavone 4'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**16**) (DMSO-d_6, 600 MHz)

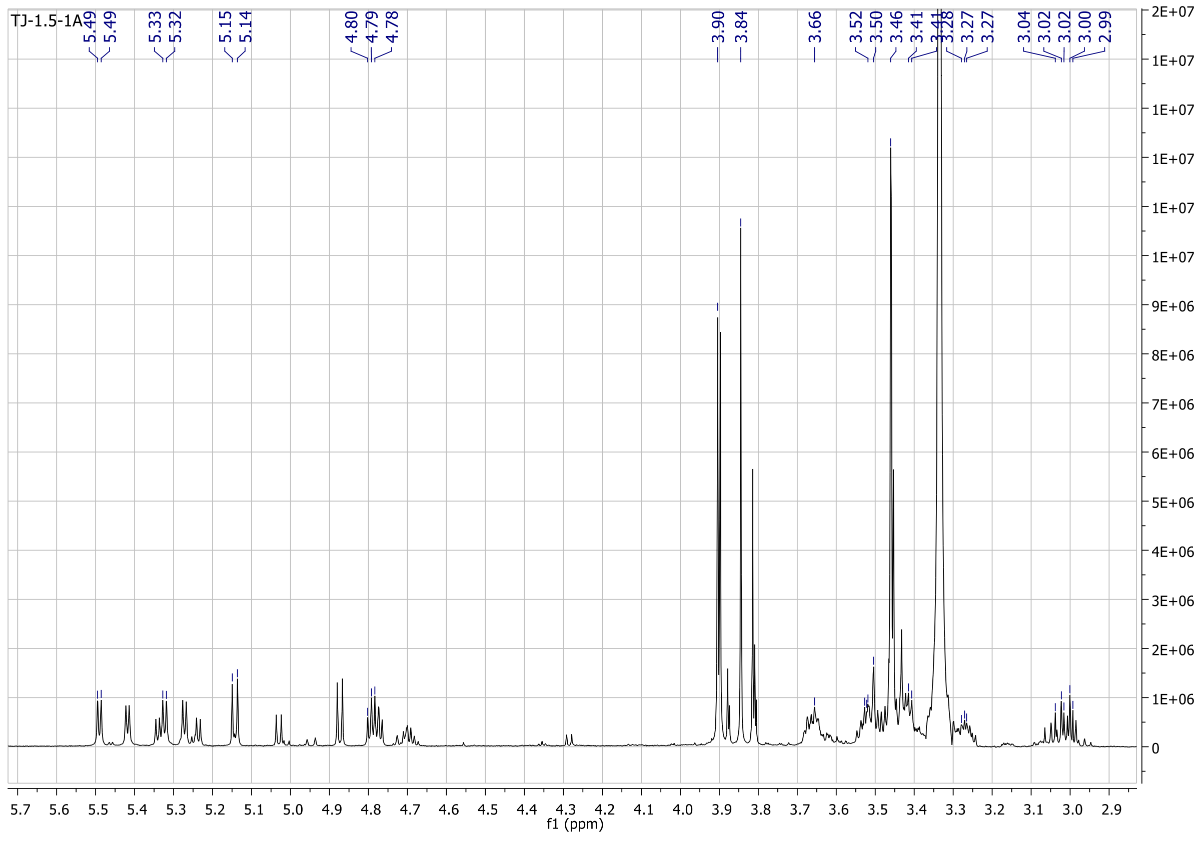


Fig.S73. ^{13}C NMR spectral of 2',5'-dimethoxyflavone 4'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**16**) (DMSO-d_6, 151 MHz)

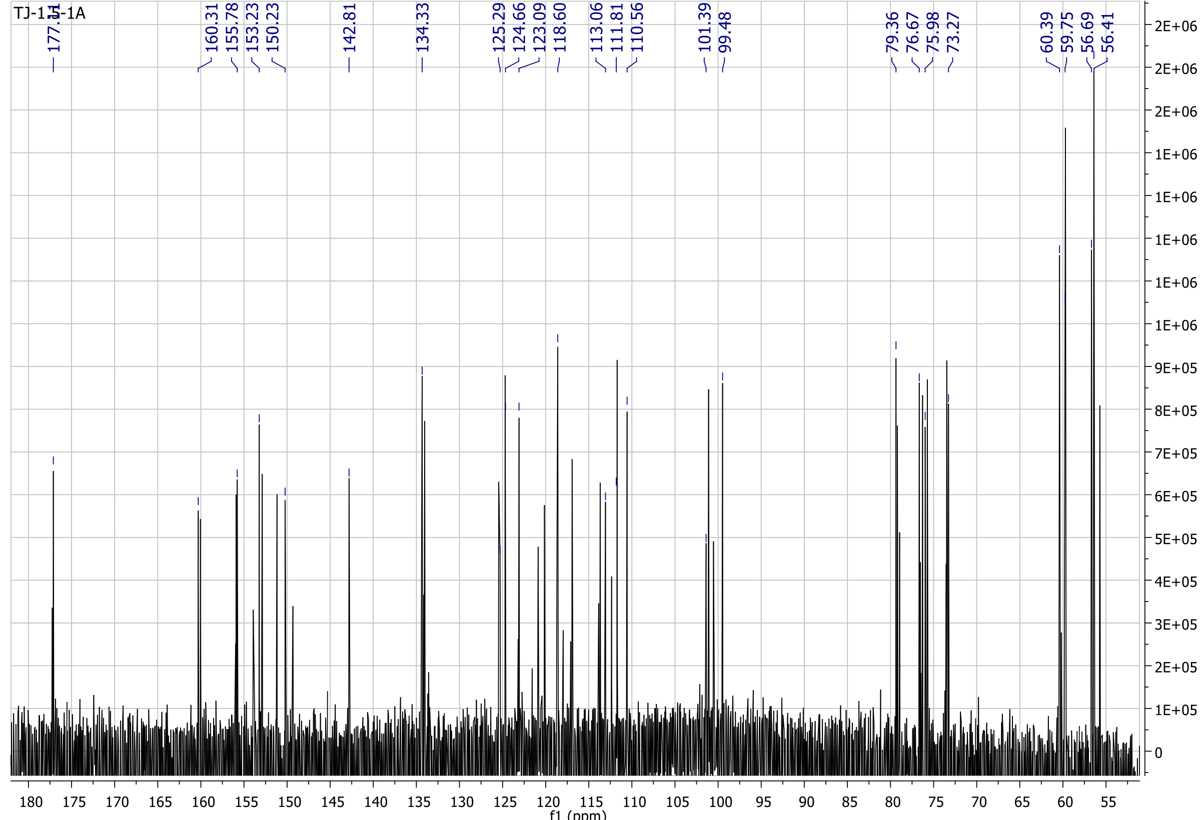


Fig.S74. COSY spectral of 2’,5’-dimethoxyflavone 4’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**16**) (DMSO-d_6, 151 MHz)

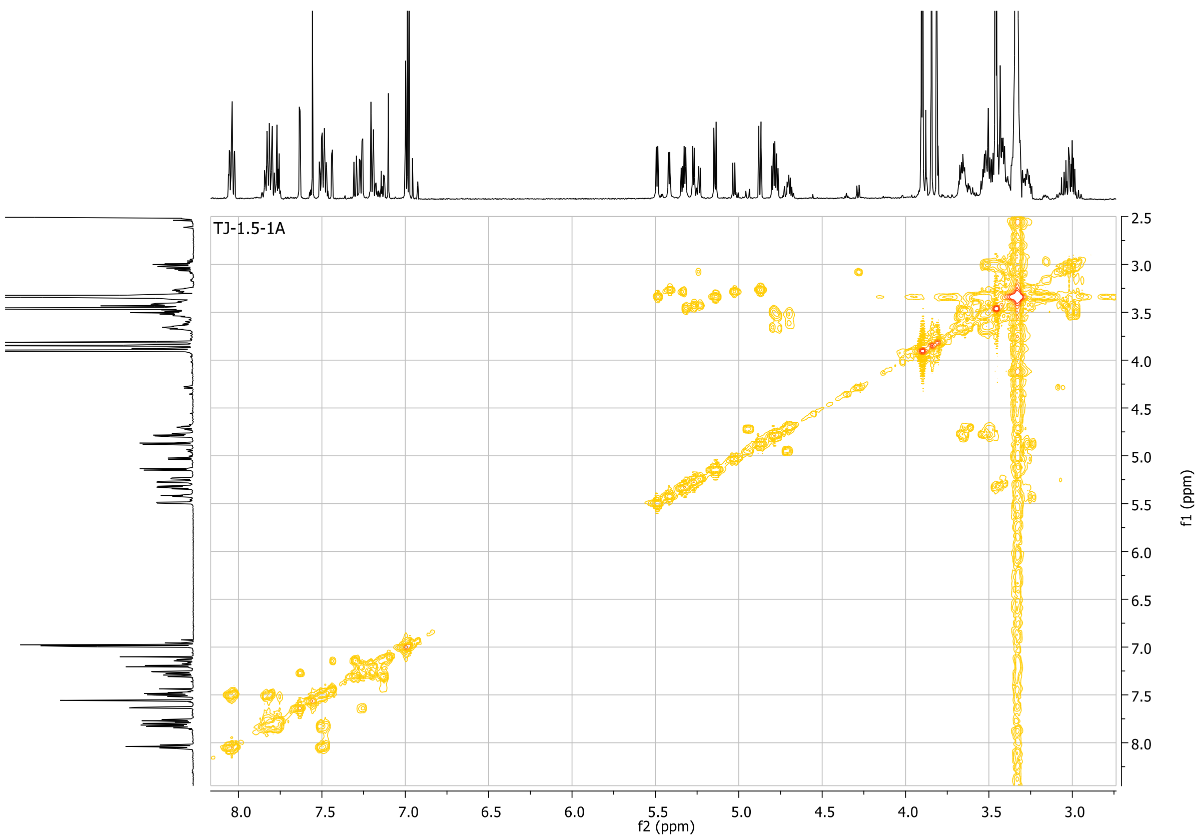


Fig.S75. HMQC spectral of 2’,5’-dimethoxyflavone 4’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**16**) (DMSO-d_6, 151 MHz)

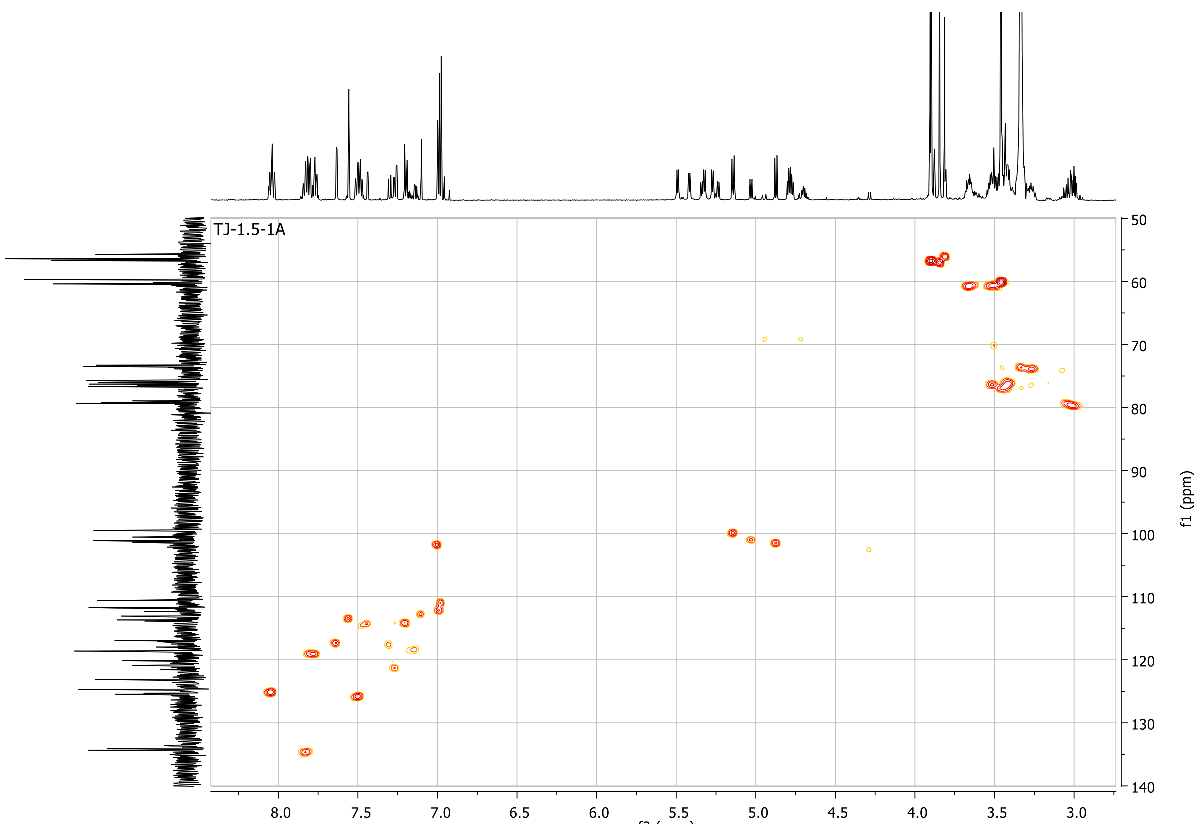


Fig.S76. HMBC spectral of 2',5'-dimethoxyflavone 4'-*O*-β-D-(4''-*O*-methyl)-glucopyranoside (**16**) (DMSO-d_6, 151 MHz)

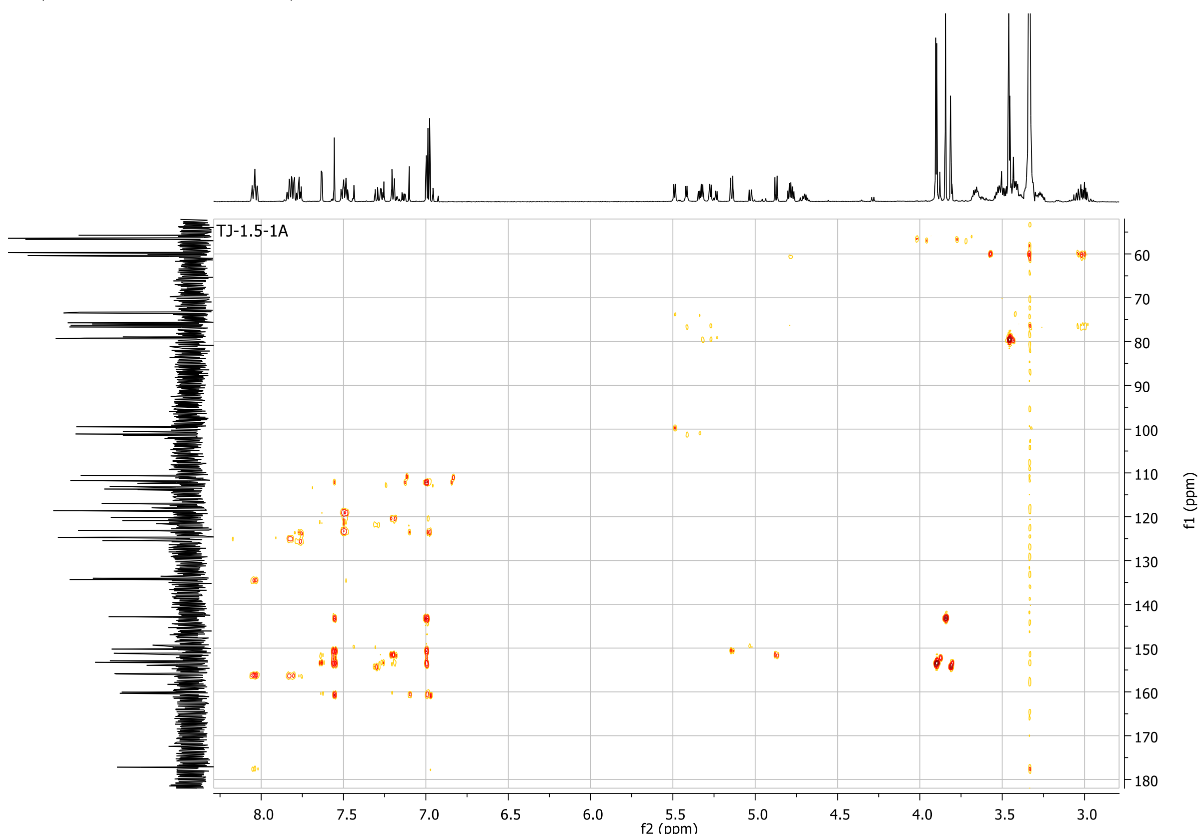
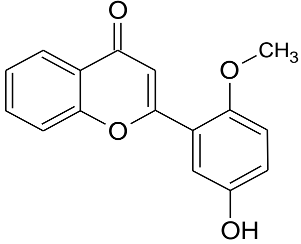
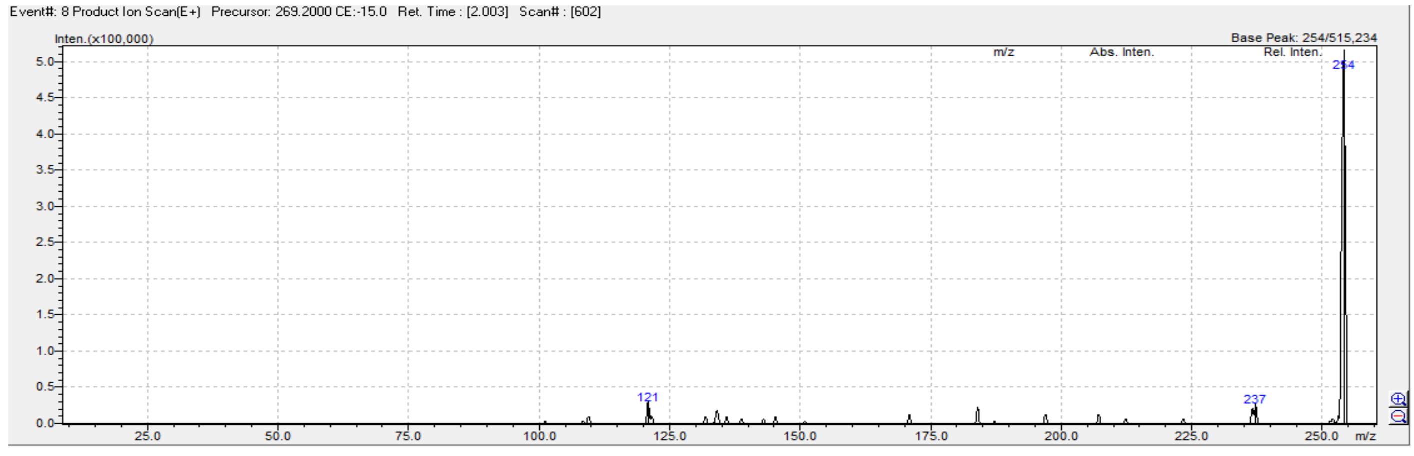


Fig.S77. MS analysis 5'-hydroxy-2'-methoxyflavone (**17**)

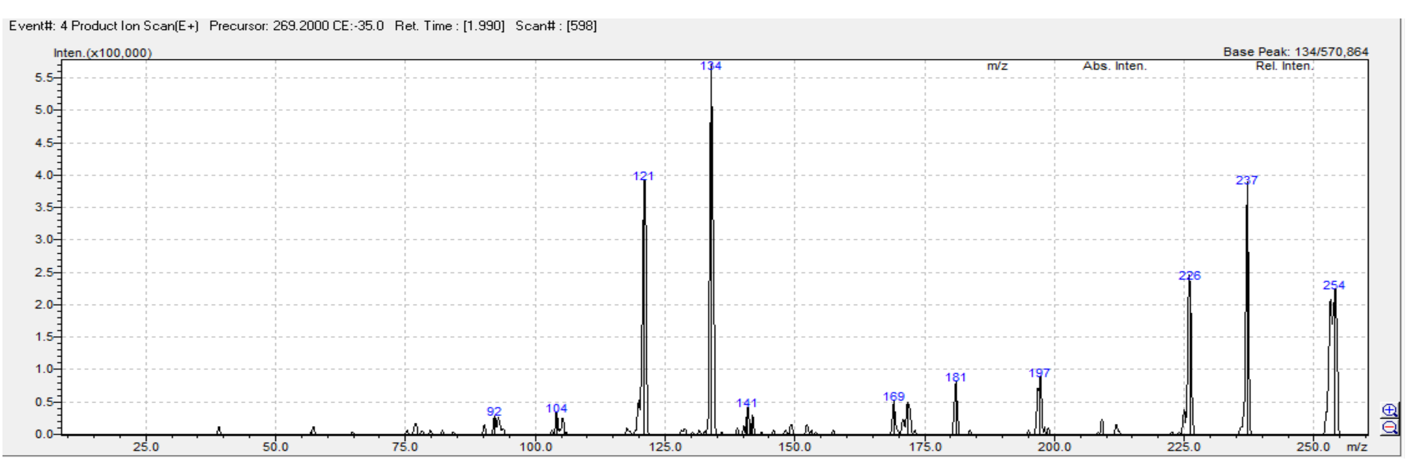


Molecular Formula: $C_{16}H_{12}O_4$
Formula Weight: 268.26408
Precursor: 269.2000

CE: -15.0



CE:-35.0



CE:-45.0

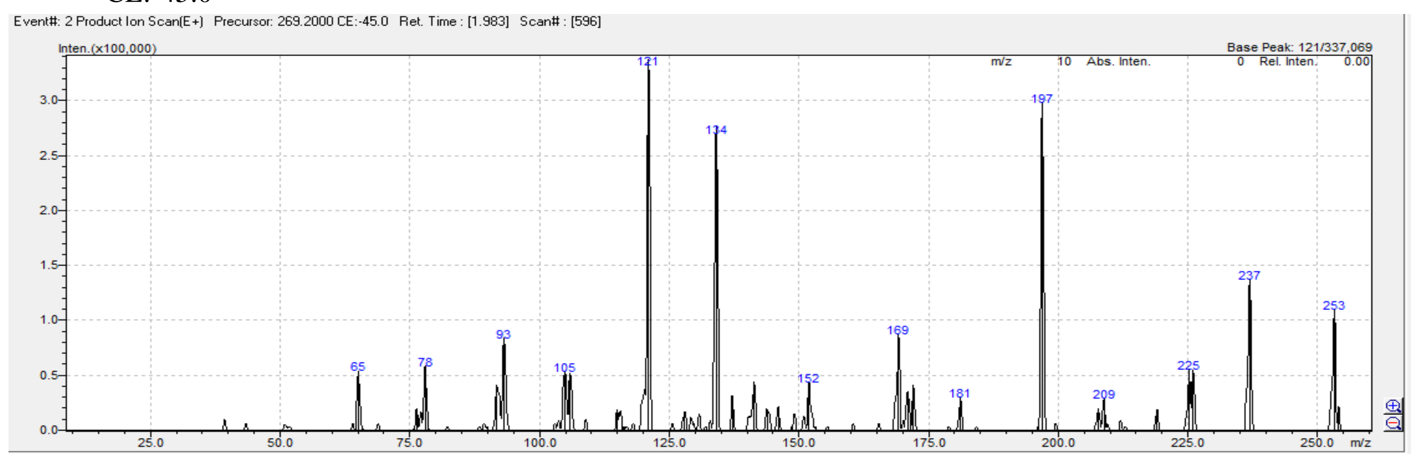


Fig.S78. ^{1}H NMR spectral of 5'-hydroxy-2'-methoxyflavone (**17**) (DMSO-d_6, 600 MHz)

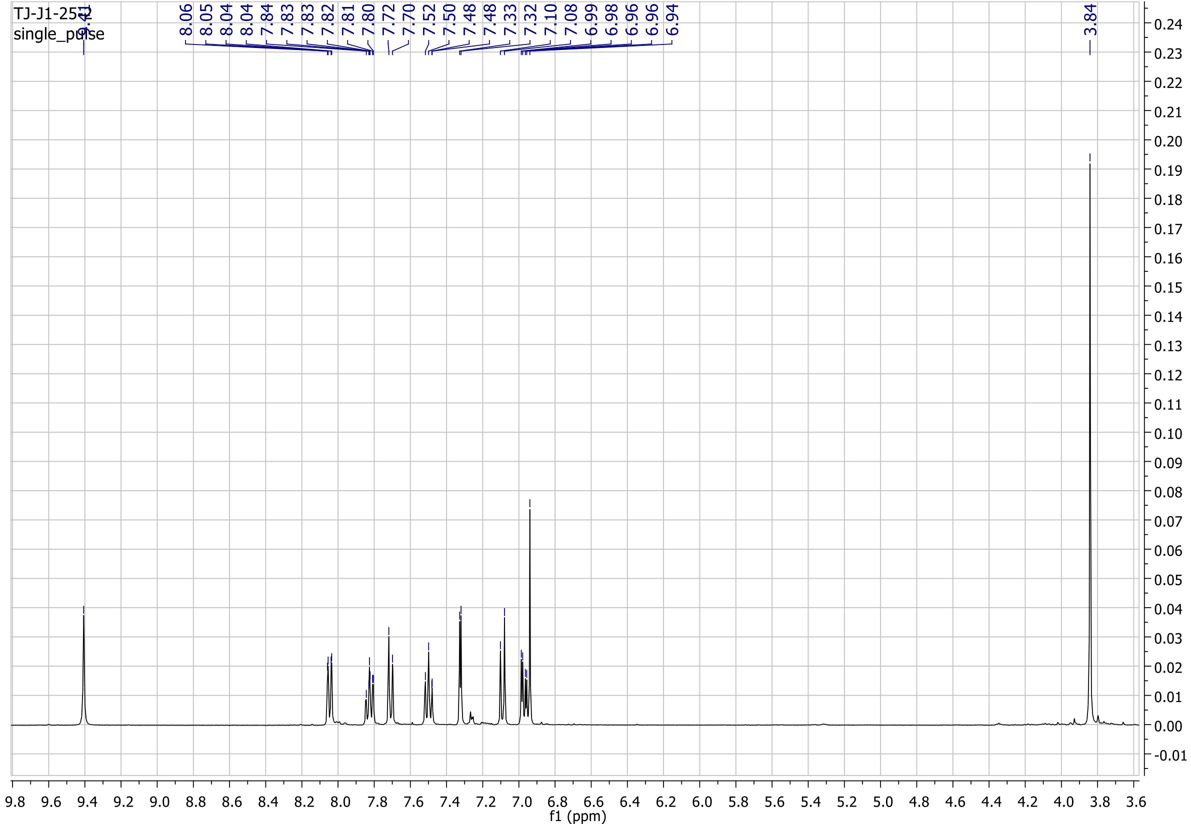


Fig.S79. ^{13}C NMR spectral of 5'-hydroxy-2'-methoxyflavone (**17**) (DMSO-d_6, 151 MHz)

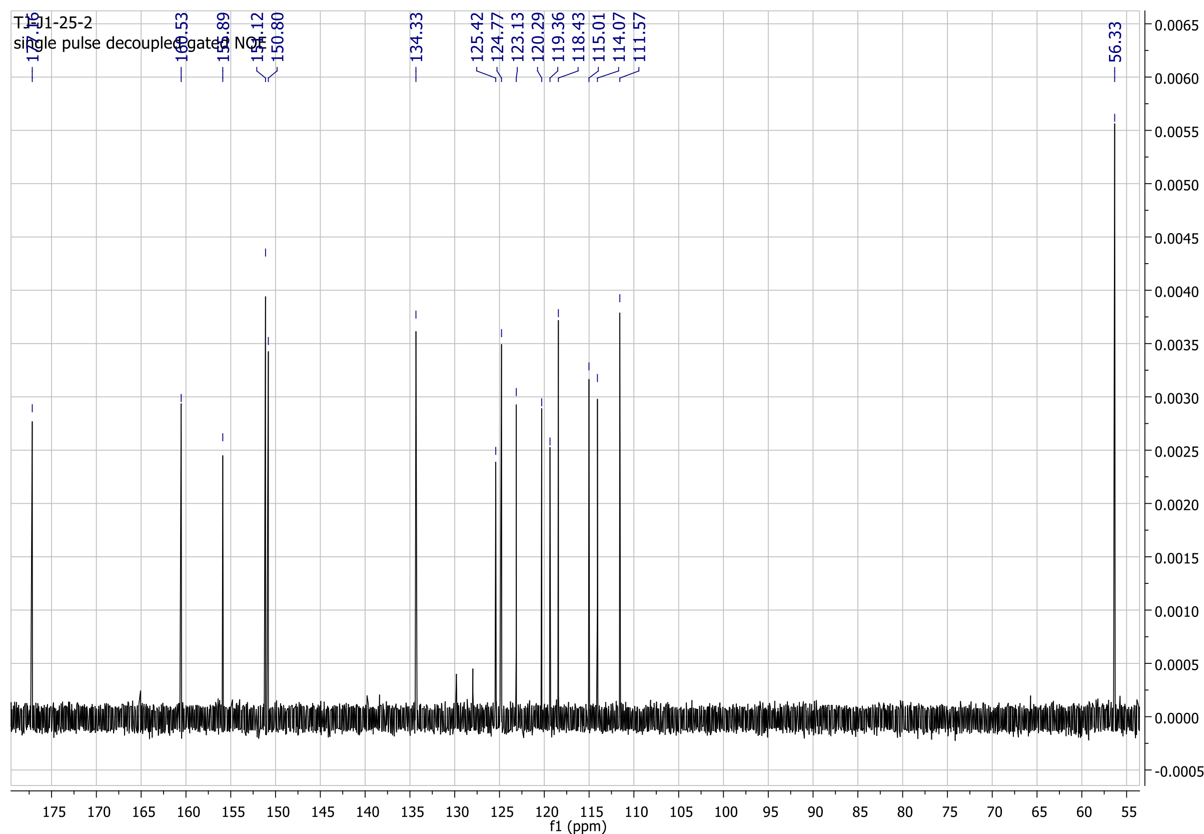


Fig.S80. COSY spectral of 5’-hydroxy-2’-methoxyflavone (**17**) (DMSO-d_6, 151 MHz)

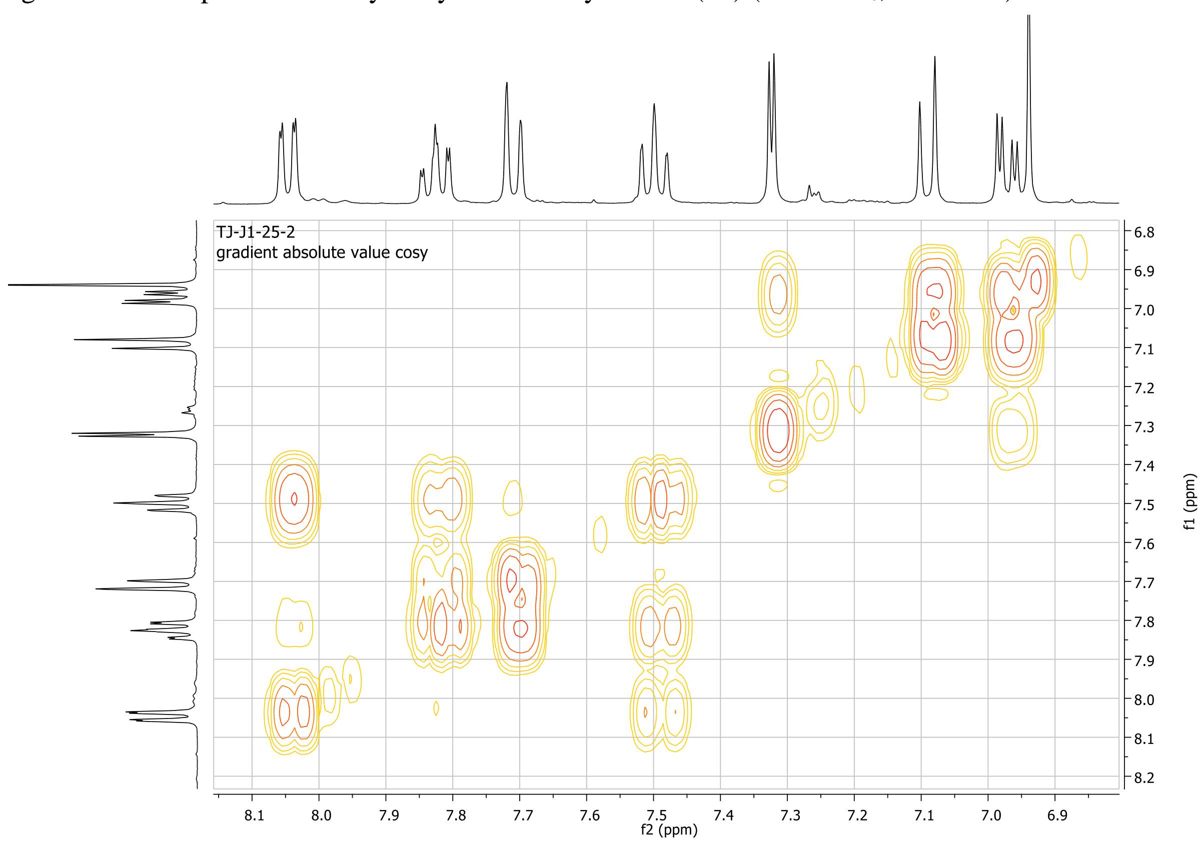


Fig.S81. HMQC spectral of 5’-hydroxy-2’-methoxyflavone (**17**) (DMSO-d_6, 151 MHz)

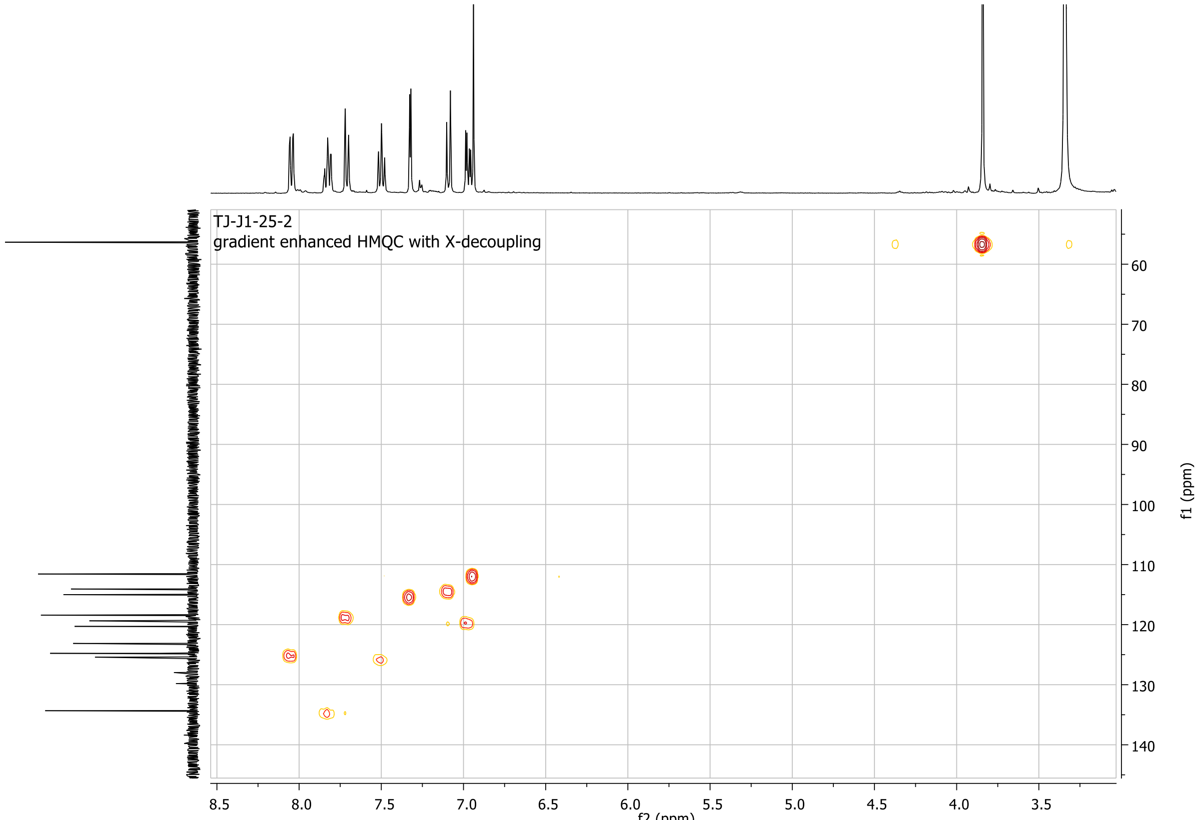


Fig.S82. HMBC spectral of 5’-hydroxy-2’-methoxyflavone (**17**) (DMSO-d_6, 151 MHz)

TJ-J1-25-2
gradient enhanced HMBC

f1 (ppm)

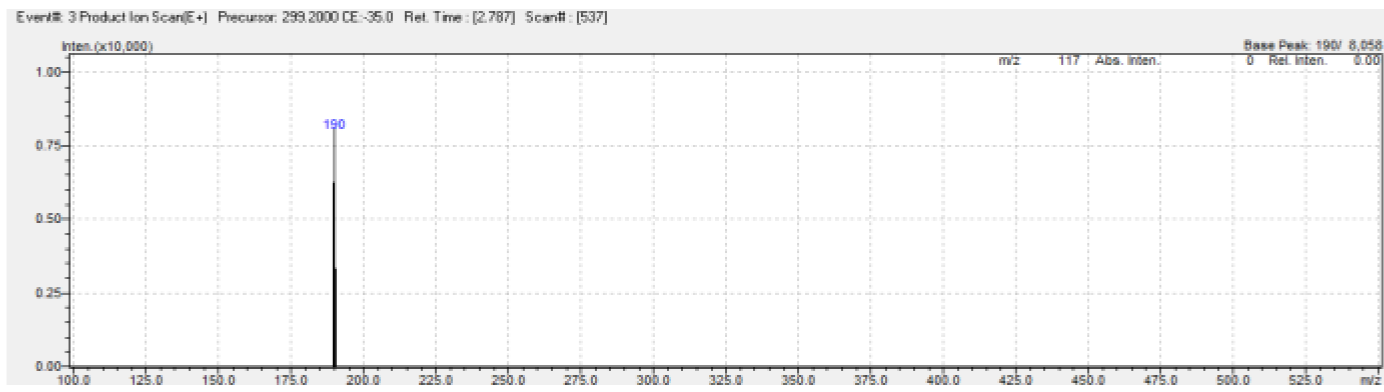
f2 (ppm)

Fig.S83. MS analysis 4’-hydroxy-2’,5’-dimethoxyflavone (**18**)

Molecular Formula = $C_{17}H_{14}O_5$
Formula Weight = 298.29006
Precursor: = 299.2000

CE: -15

CE:-35



CE:-45

Fig.S84. ^{1}H NMR spectral of 5’-hydroxy-2’-methoxyflavone (**17**) and 4’-hydroxy-2’,5’-dimethoxyflavone (**18**) (DMSO-d_6, 600 MHz)

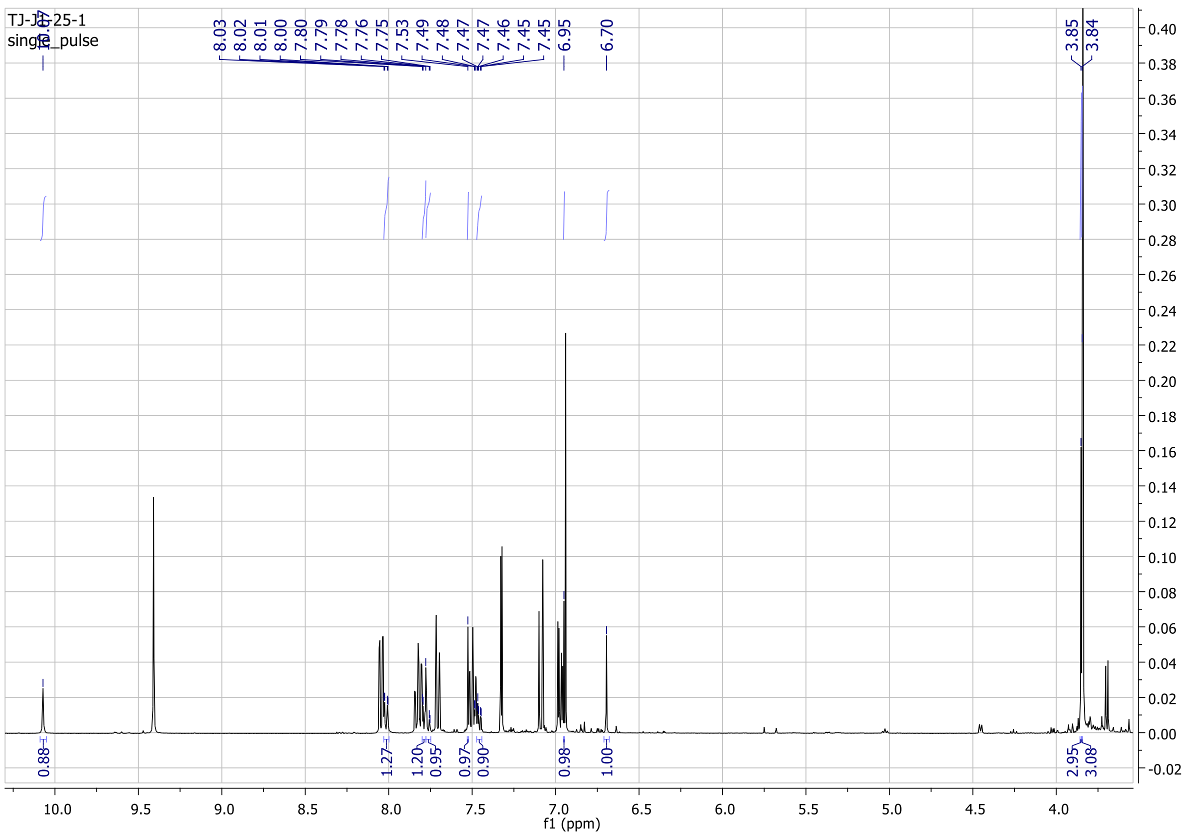


Fig.S85. Part of the ^{1}H NMR spectral 5’-hydroxy-2’-methoxyflavone (**17**) and 4’-hydroxy-2’,5’-dimethoxyflavone (**18**) (DMSO-d_6, 600 MHz)

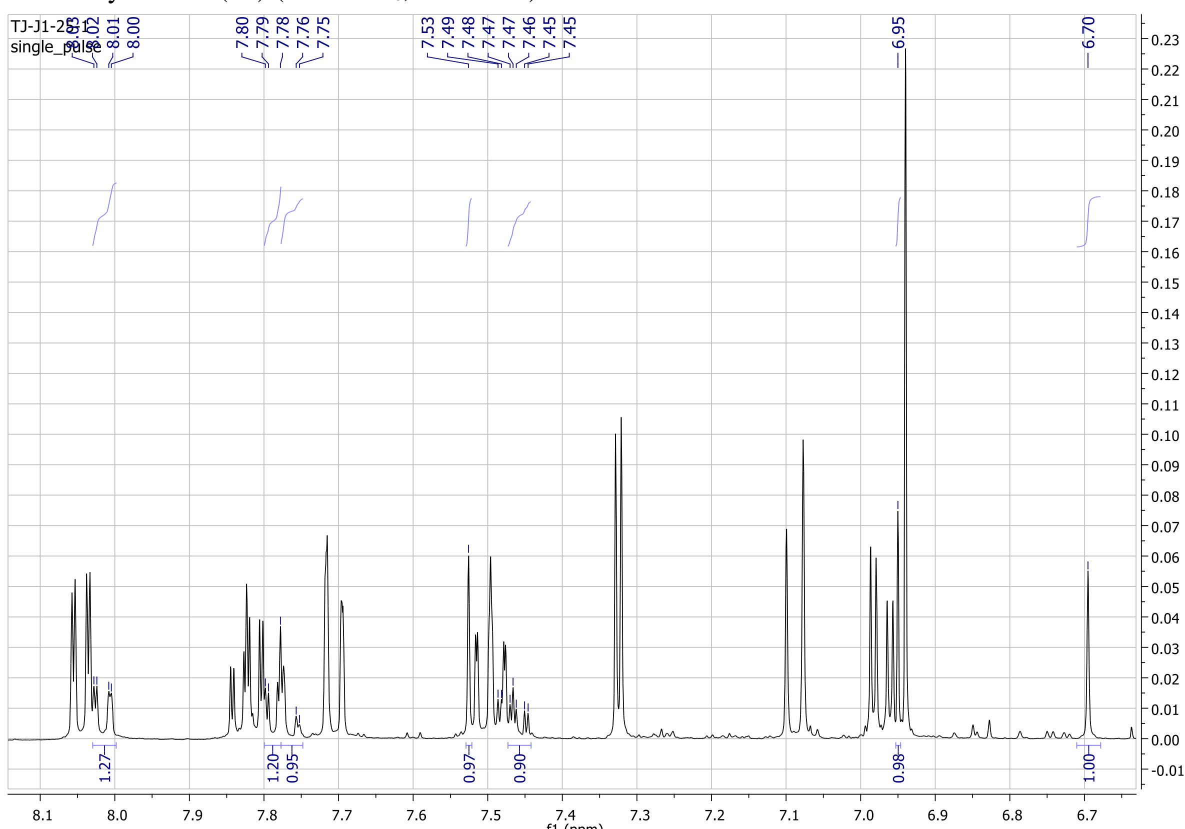


Fig.S86. ^{13}C NMR spectral of 5’-hydroxy-2’-methoxyflavone (**18**) and 4’-hydroxy-2’,5’-dimethoxyflavone (**18**) (DMSO-d_6, 151 MHz)

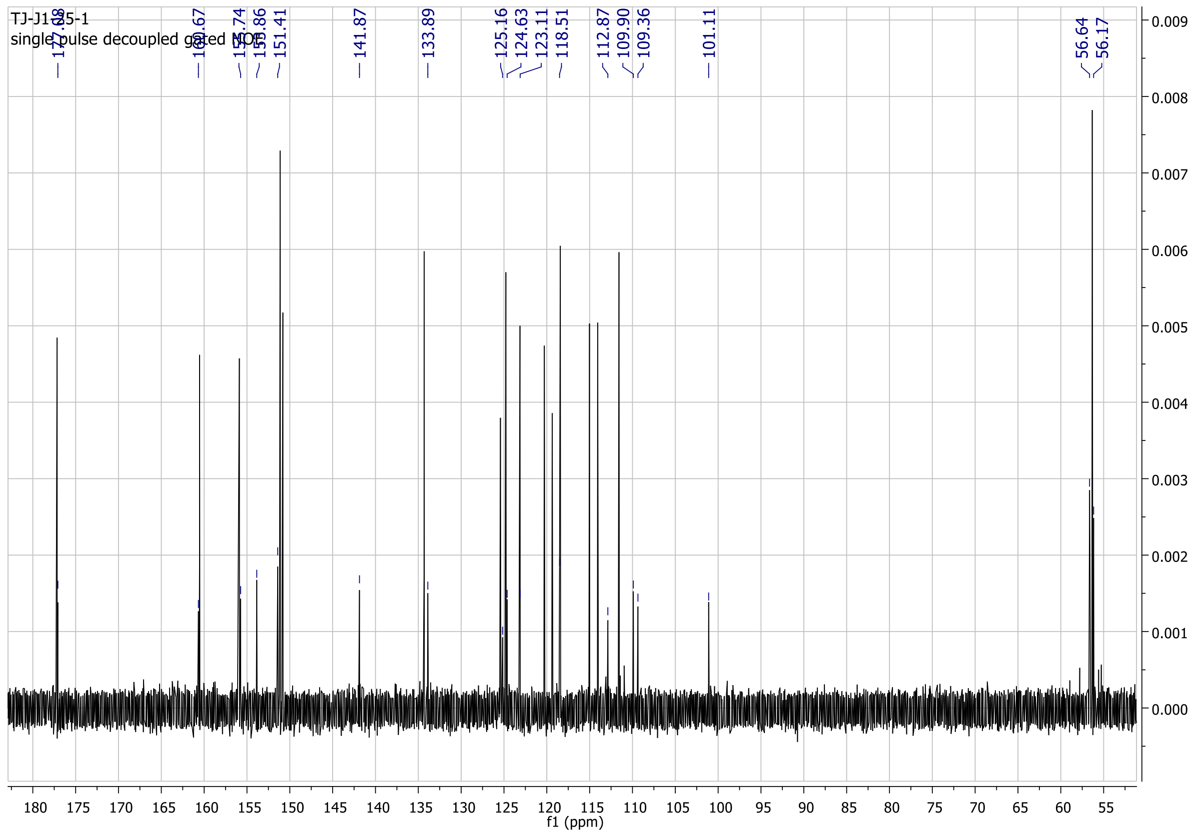


Fig.S87. HMQC spectral of 5’-hydroxy-2’-methoxyflavone (**17**) and 4’-hydroxy-2’,5’-dimethoxyflavone (**18**) (DMSO-d_6, 151 MHz)

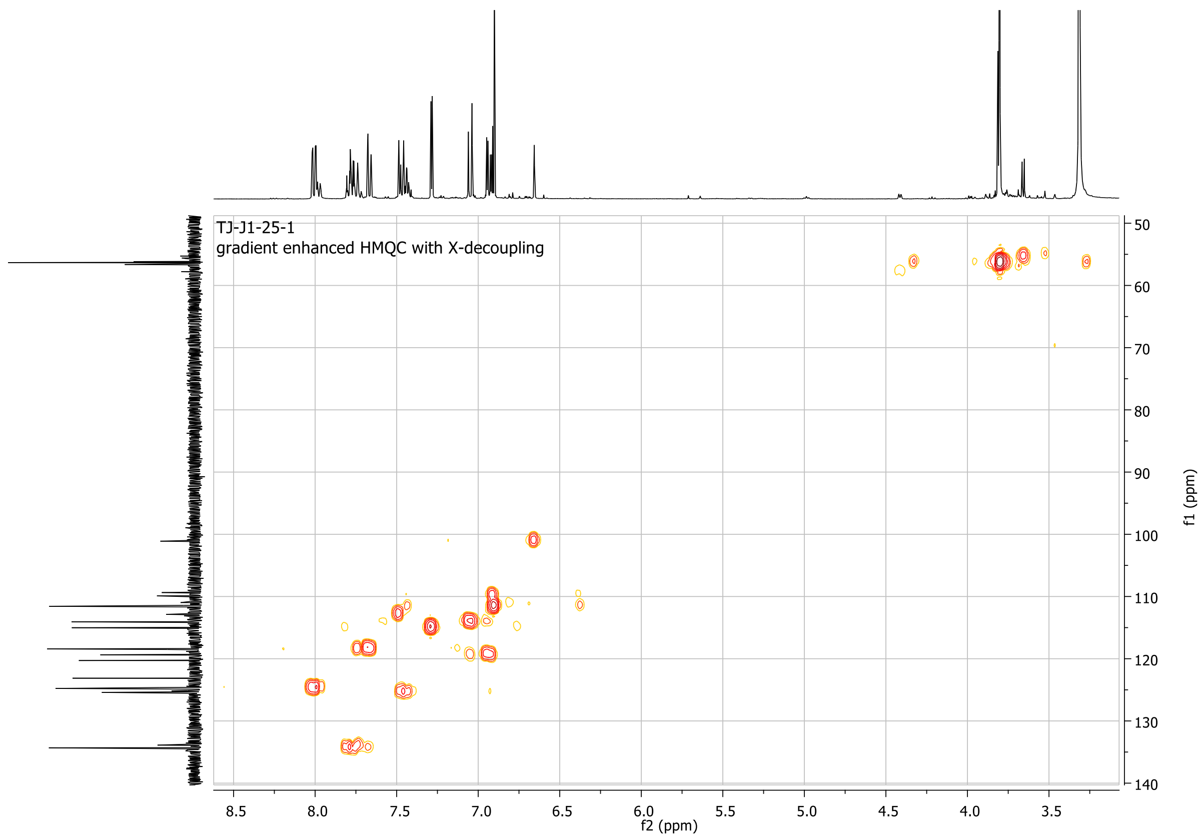


Fig.S88. HMBC spectral of 5'-hydroxy-2'-methoxyflavone (**17**) and 4'-hydroxy-2',5'-dimethoxyflavone (**18**) (DMSO-d_6, 151 MHz)

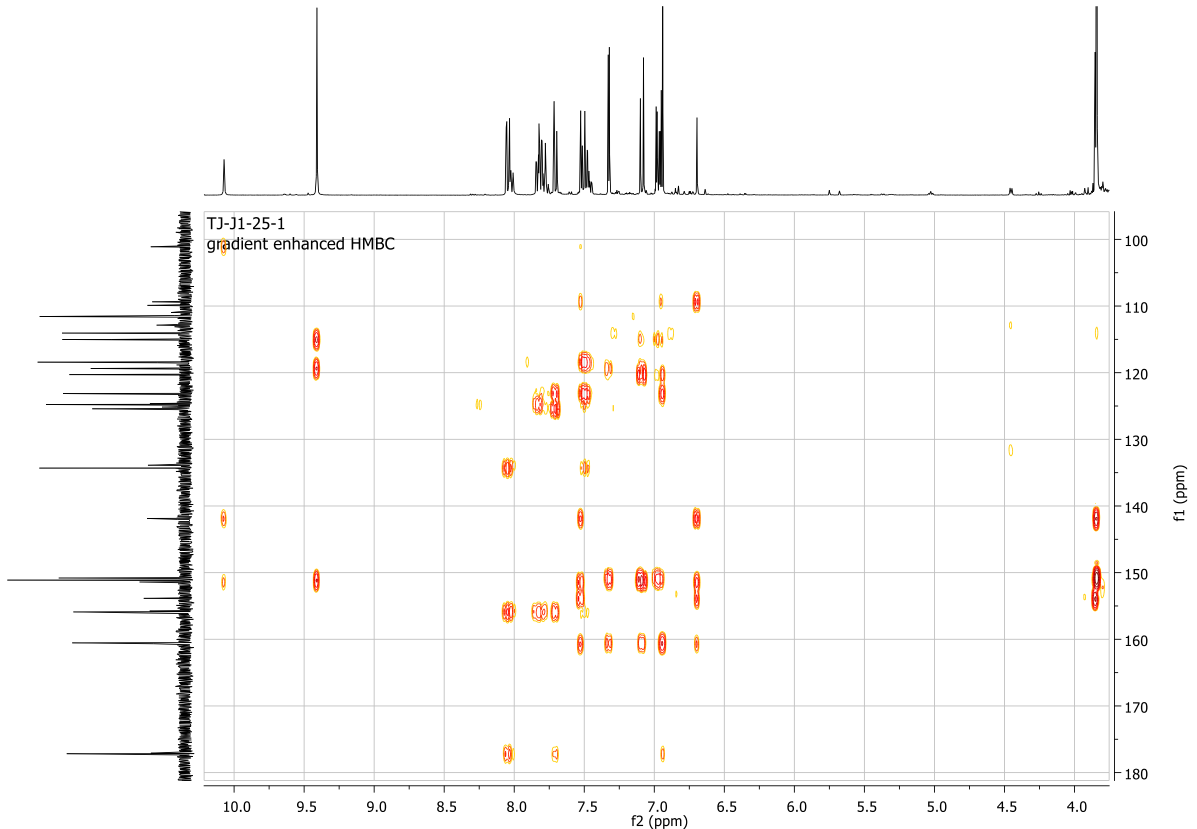
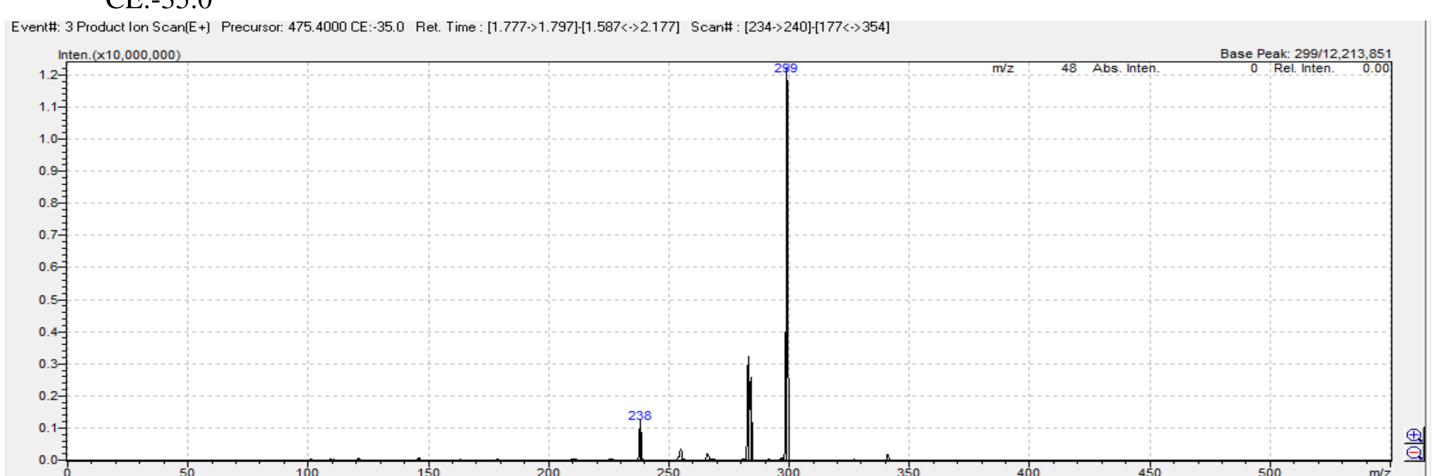


Fig.S89. MS analysis 4’,5’-dimethoxyflavone 5’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**19**) and 3’,5’-dimethoxyflavone 4’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**20**)

Molecular Formula = $C_{24}H_{26}O_{10}$
Formula Weight = 474.45724
Precursor =475.4000

CE: -15.0

CE:-35.0



CE:-45.0

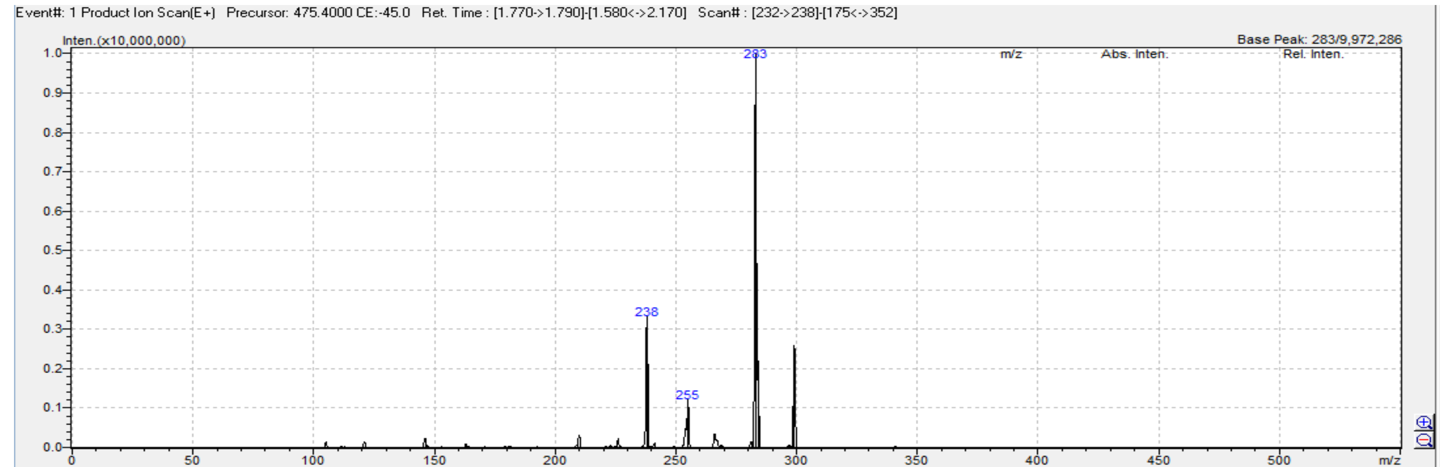


Fig.S90. ^{1}H NMR spectral of 4’,5’-dimethoxyflavone 5’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**19**) and 3’,5’-dimethoxyflavone 4’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**20**) (DMSO-d_6, 600 MHz)

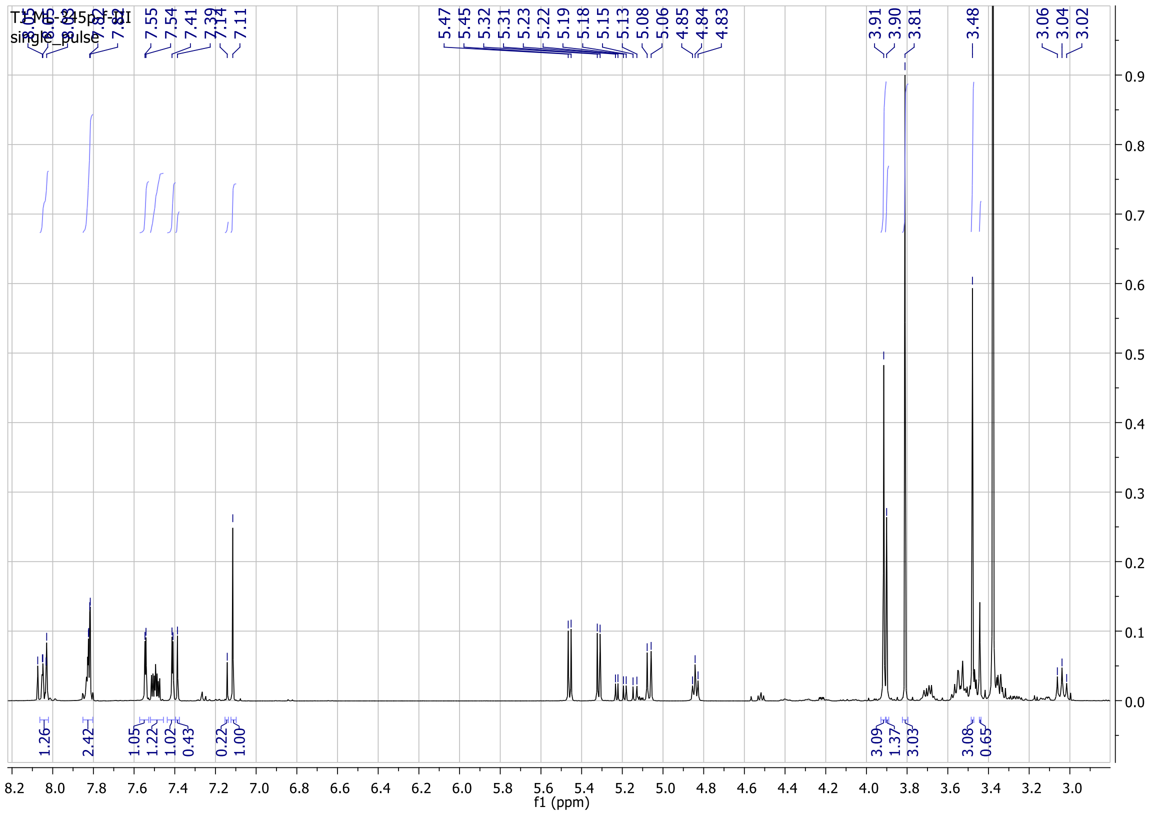


Fig.S91. Flavone part of the ^{1}H NMR spectral 4’,5’-dimethoxyflavone 5’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**19**) and 3’,5’-dimethoxyflavone 4’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**20**) (DMSO-d_6, 600 MHz)

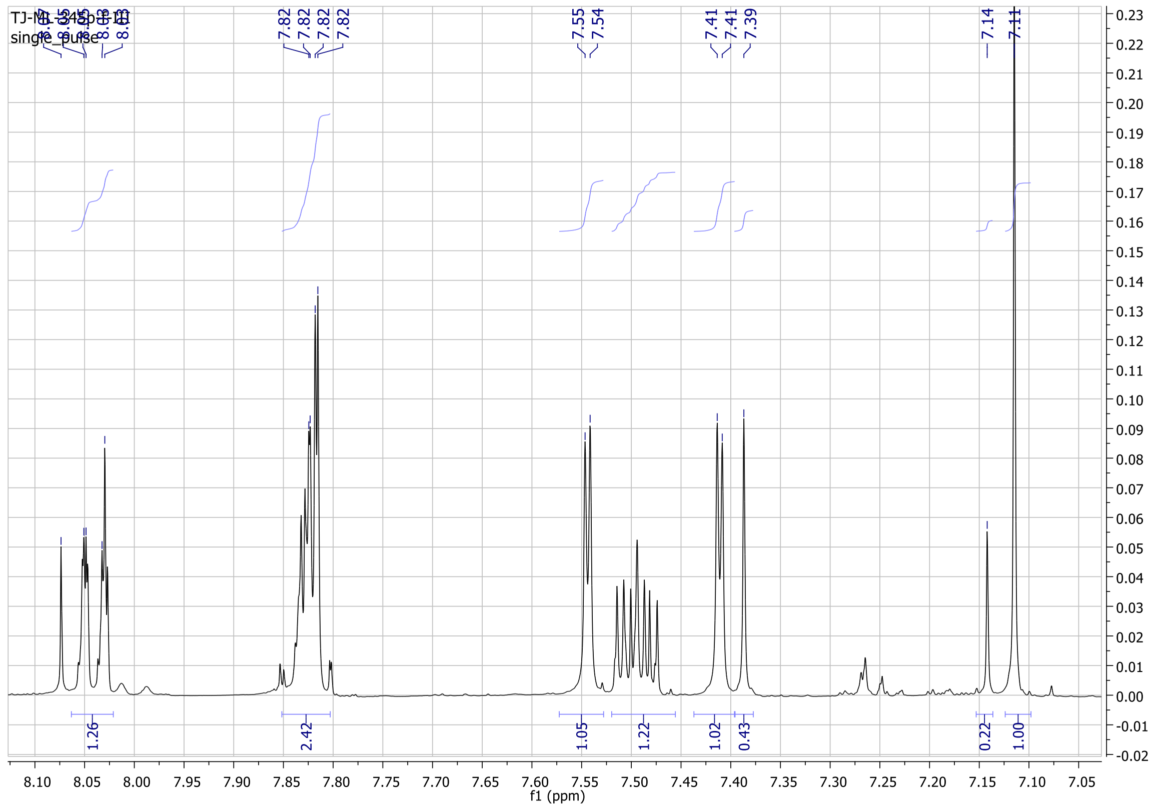


Fig.S92. Glycoside part of the ^{1}H NMR spectral 4',5'-dimethoxyflavone 5'-*O*-β-D-(4''-*O*-methyl)-glucopyranoside (**19**) and 3',5'-dimethoxyflavone 4'-*O*-β-D-(4''-*O*-methyl)-glucopyranoside (**20**) (DMSO-d_6, 600 MHz)

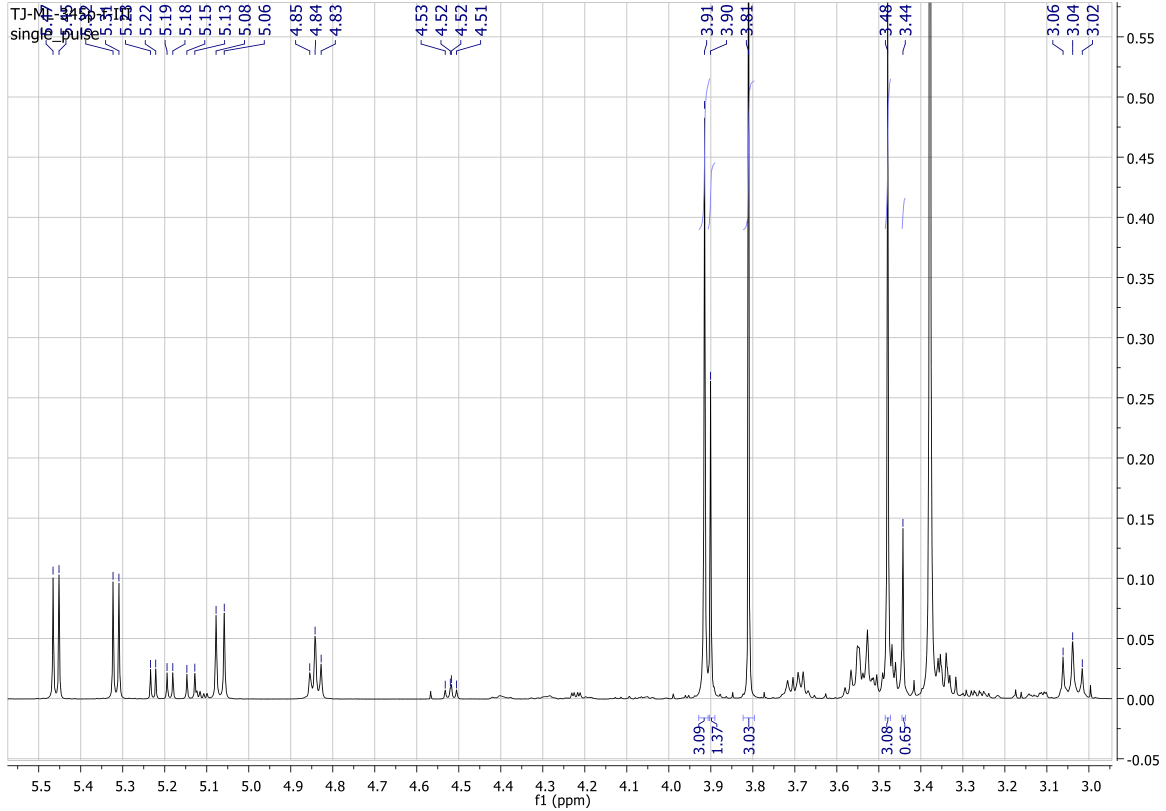


Fig.S93. ^{13}C NMR spectral of 4',5'-dimethoxyflavone 5'-*O*-β-D-(4''-*O*-methyl)-glucopyranoside (**19**) and 3',5'-dimethoxyflavone 4'-*O*-β-D-(4''-*O*-methyl)-glucopyranoside (**20**) (DMSO-d_6, 151 MHz)

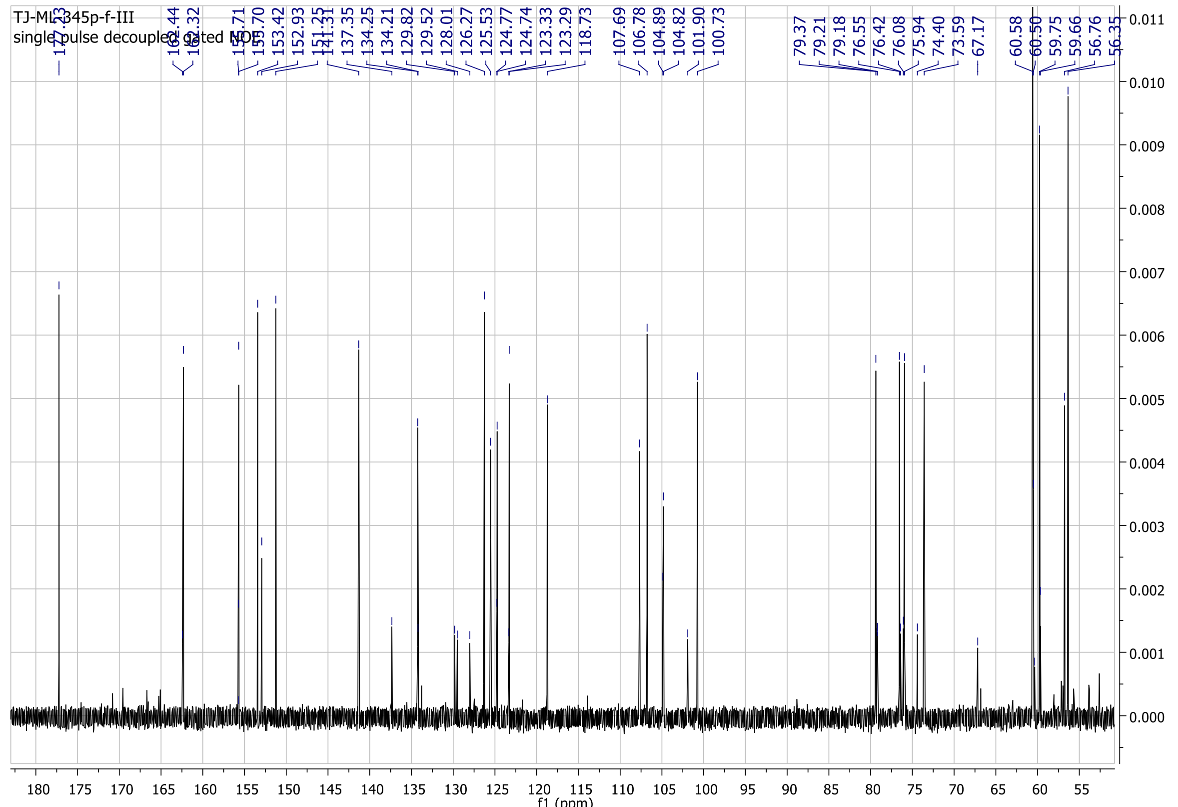


Fig.S94. HMQC spectral of 4',5'-dimethoxyflavone 5'-*O*-β-D-(4''-*O*-methyl)-glucopyranoside (**19**) and 3',5'-dimethoxyflavone 4'-*O*-β-D-(4''-*O*-methyl)-glucopyranoside (**20**) (DMSO-d_6, 151 MHz)

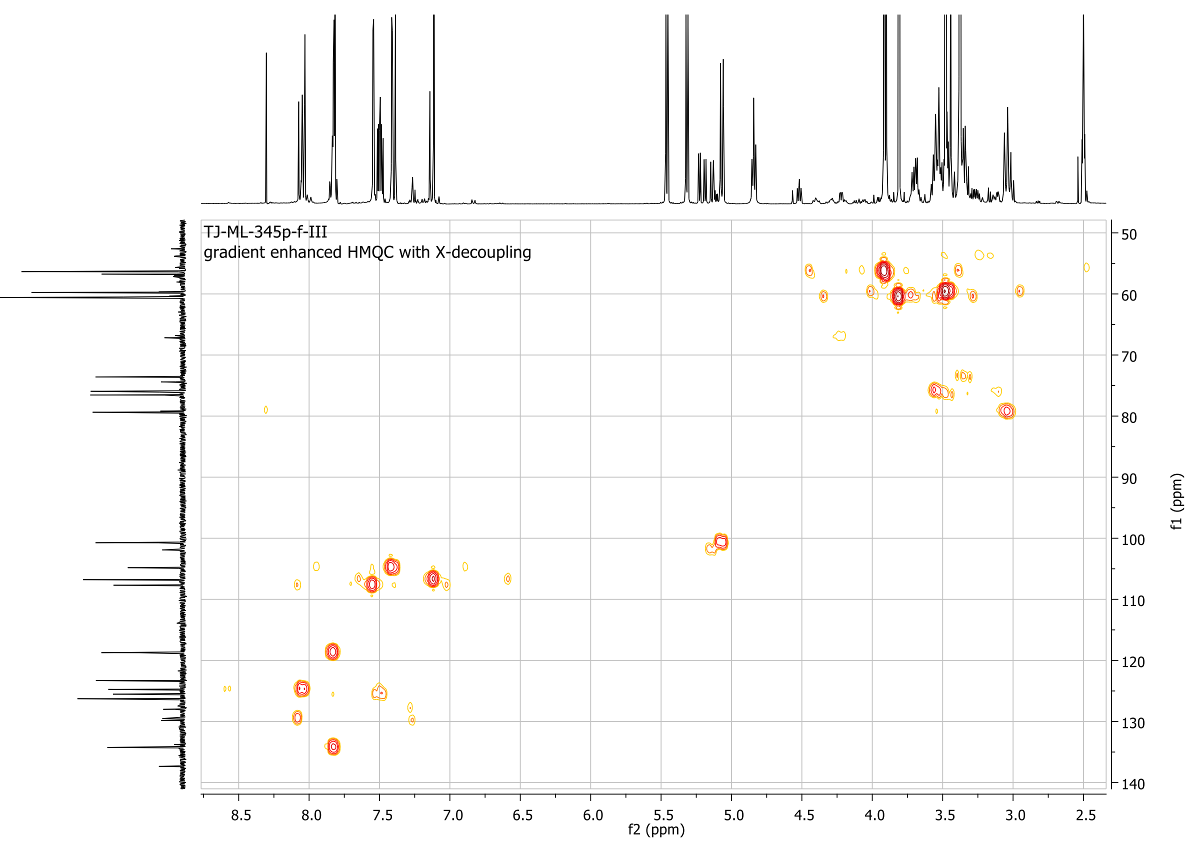


Fig.S95. HMBC spectral of 4',5'-dimethoxyflavone 5'-*O*-β-D-(4''-*O*-methyl)-glucopyranoside (**19**) and 3',5'-dimethoxyflavone 4'-*O*-β-D-(4''-*O*-methyl)-glucopyranoside (**20**) (DMSO-d_6, 151 MHz)

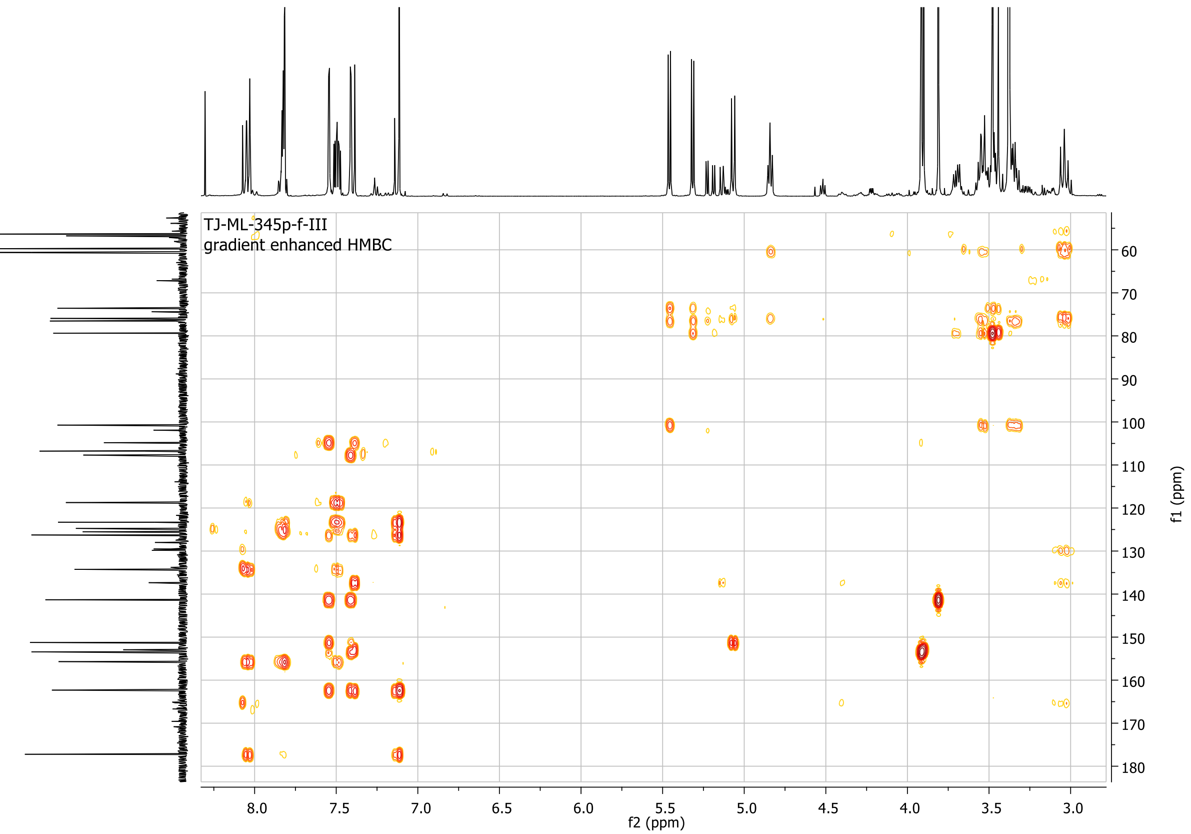
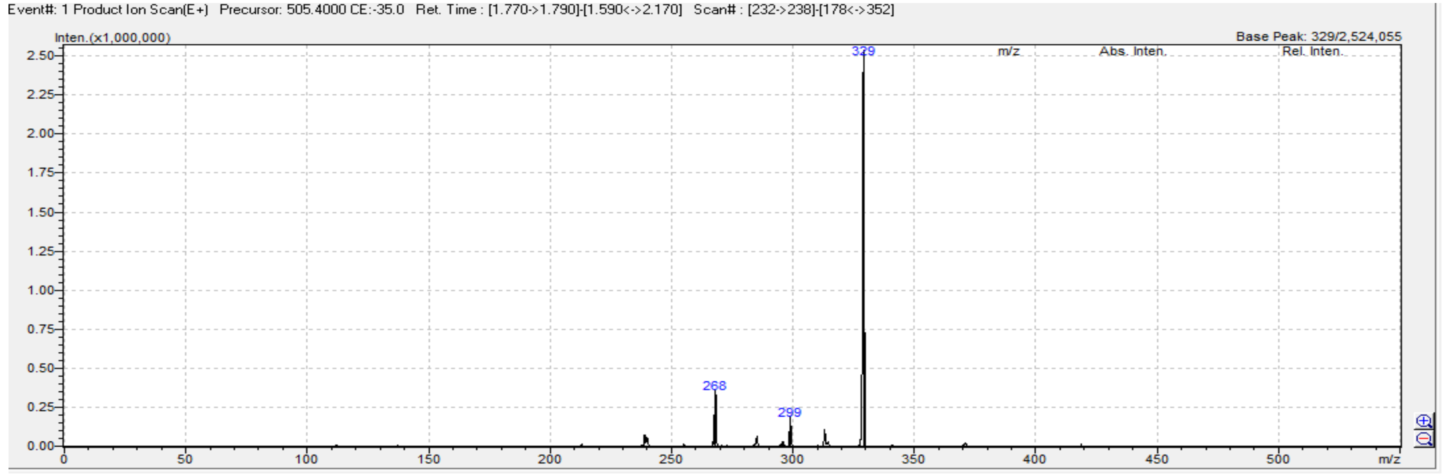


Fig.S96. MS analysis 3’,4’,5’-trimethoxyflavone 6-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**21**)

Molecular Formula = $C_{25}H_{28}O_{11}$
Formula Weight = 504.48322
Precursor: =505.4000

CE: -15.0

CE:-35.0



CE:-45.0

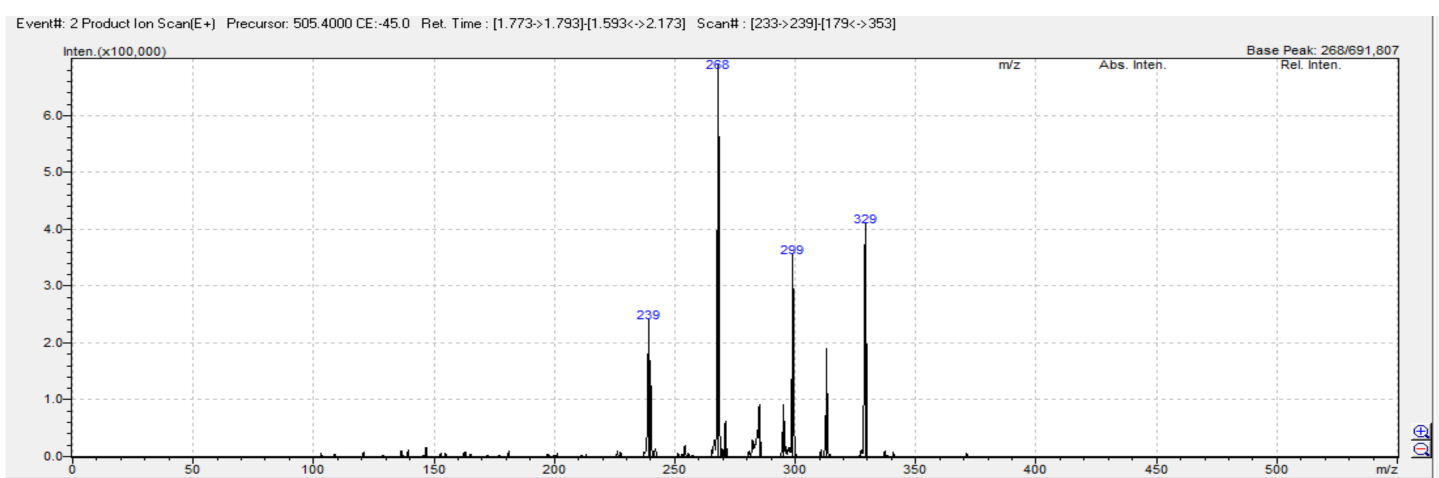


Fig.S97. ^{1}H NMR spectral of 3’,4’,5’-trimethoxyflavone 6-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**21**) (DMSO-d_6, 600 MHz)

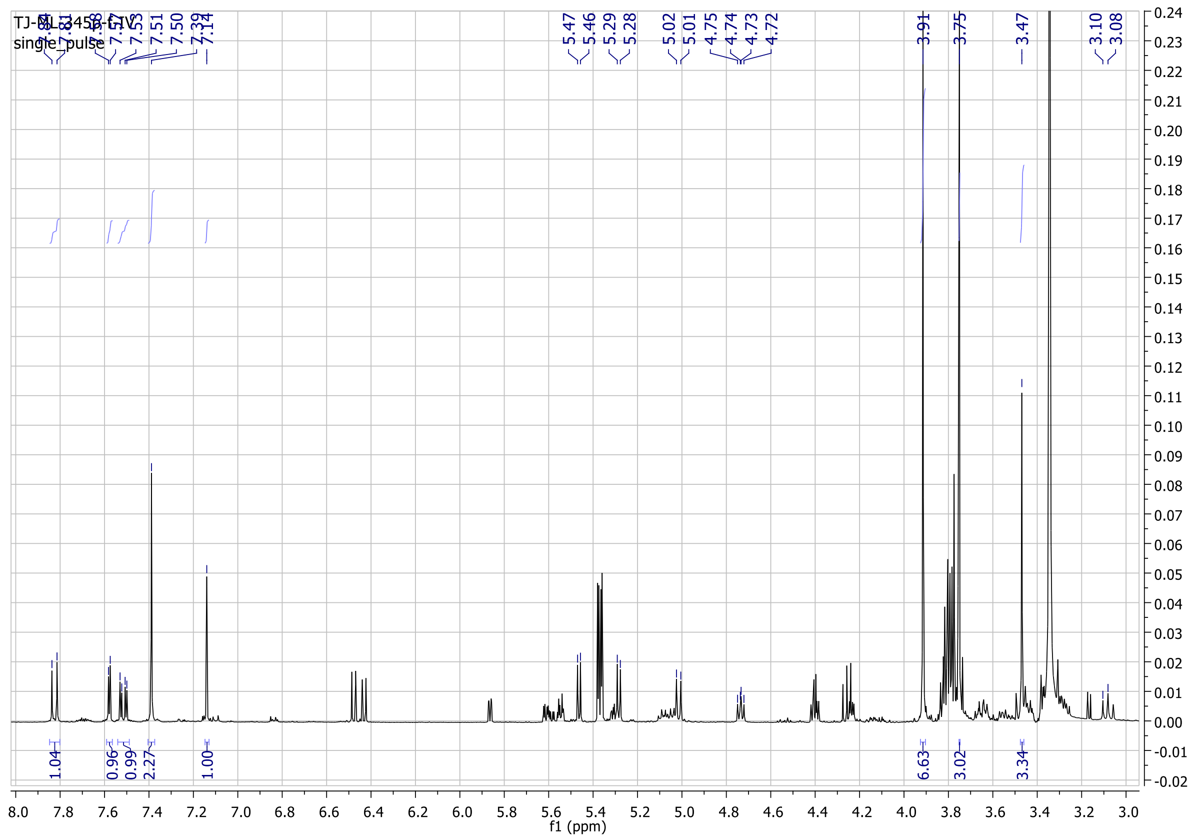


Fig.S98. Flavone part of the ^{1}H NMR spectral 3’,4’,5’-trimethoxyflavone 6-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**21**) (DMSO-d_6, 600 MHz)

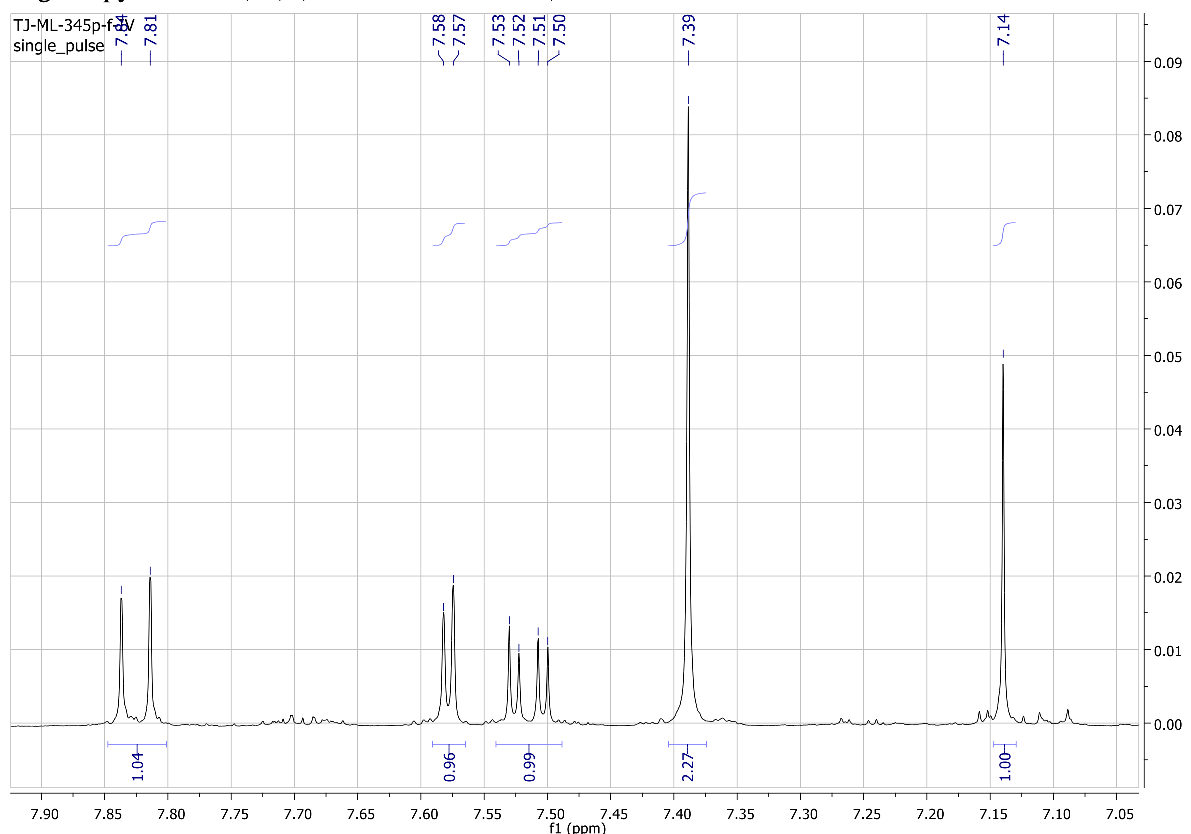


Fig.S99. Glucopyranoside part of the ^{1}H NMR spectral 3’,4’,5’-trimethoxyflavone 6-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**21**) (DMSO-d_6, 600 MHz)

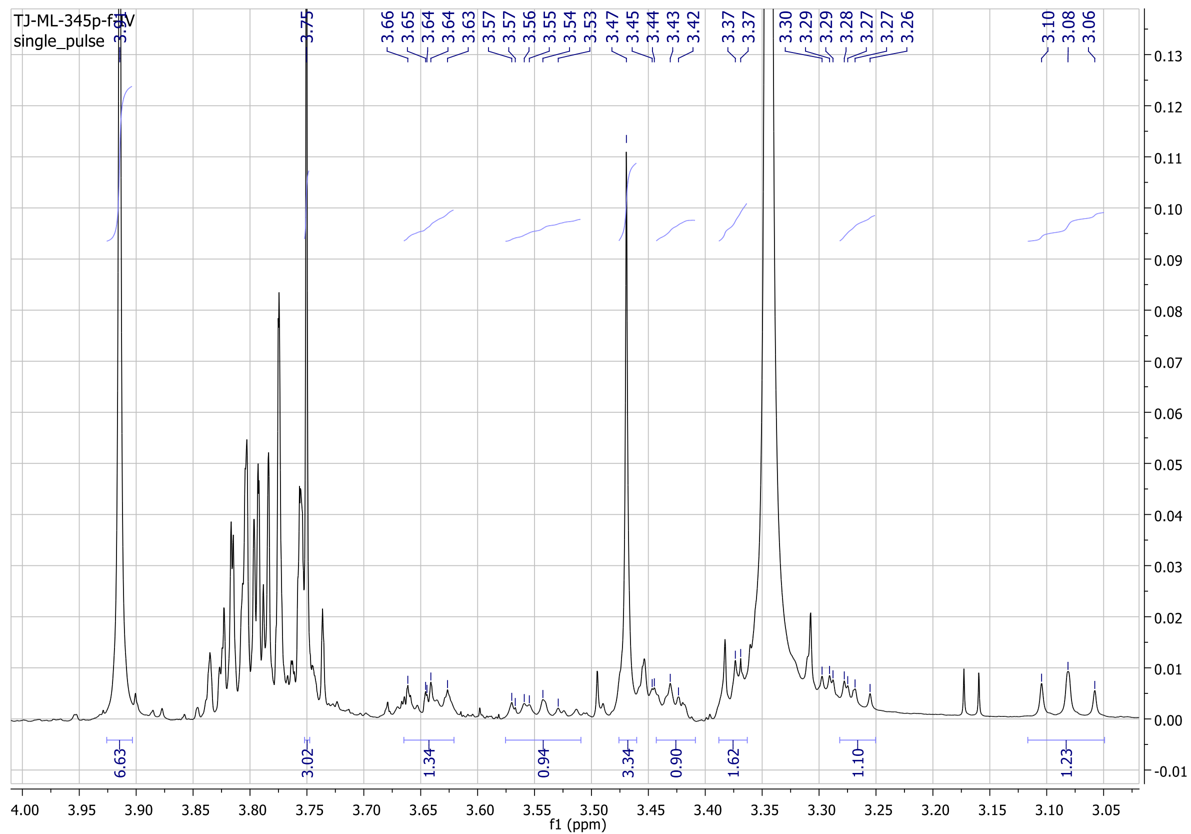


Fig.S100. ^{13}C NMR spectral of 3’,4’,5’-trimethoxyflavone 6-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**21**) (DMSO-d_6, 151 MHz)

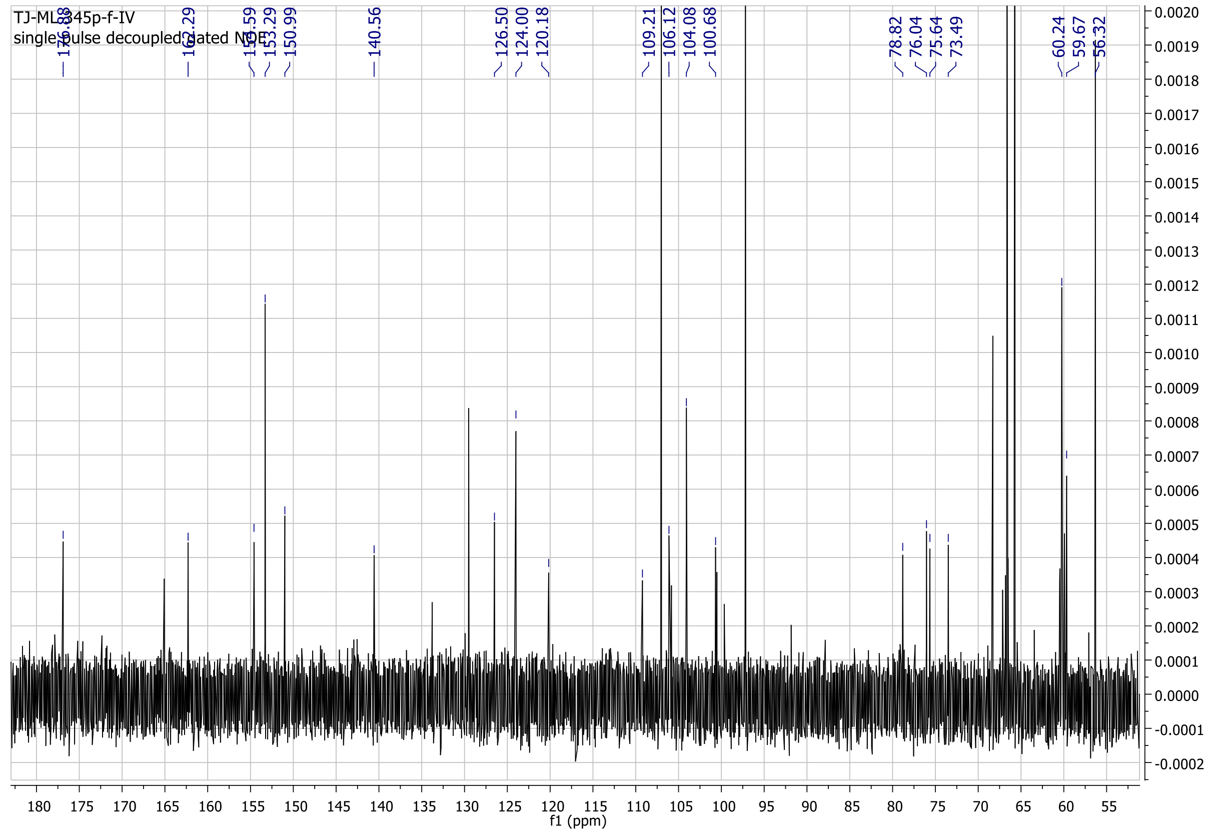


Fig.S101. HMQC spectral of 3',4',5'-trimethoxyflavone 6-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**21**) (DMSO-d_6, 151 MHz)

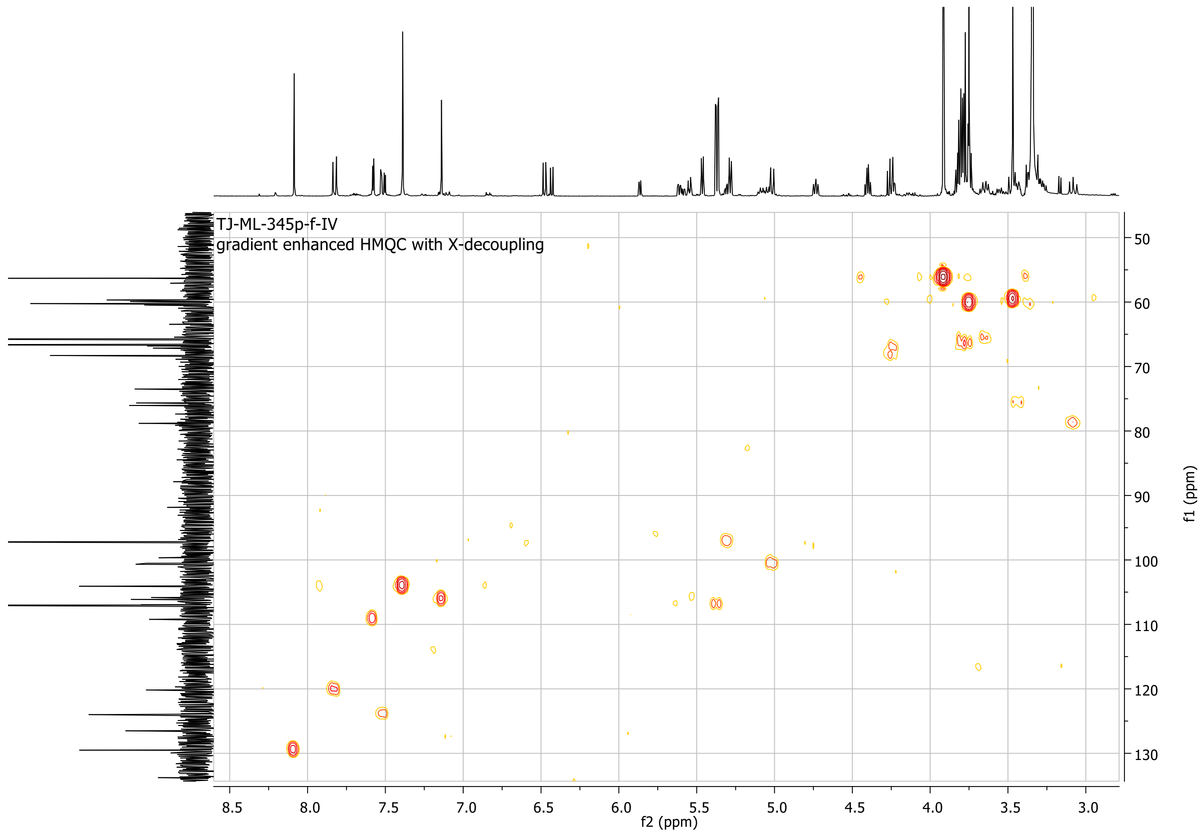


Fig.S102. HMBC spectral of 3',4',5'-trimethoxyflavone 6-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**21**) (DMSO-d_6, 151 MHz)

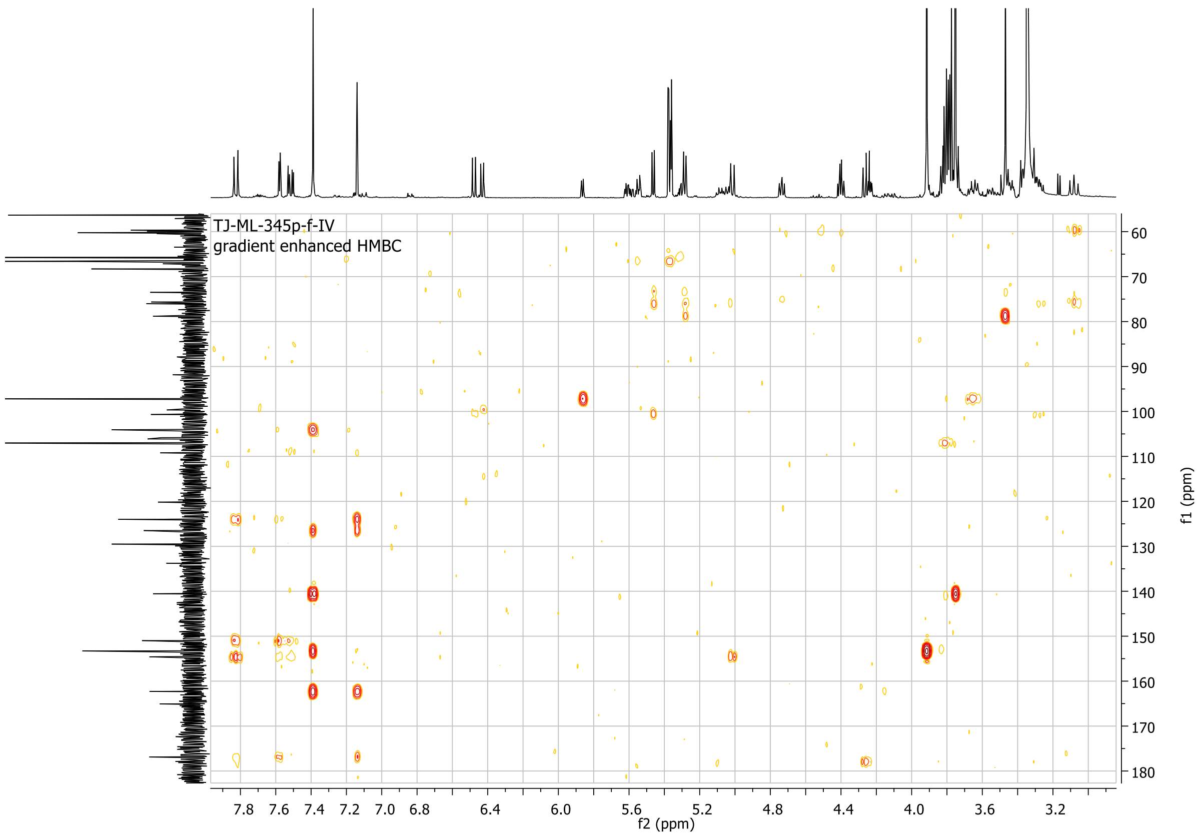
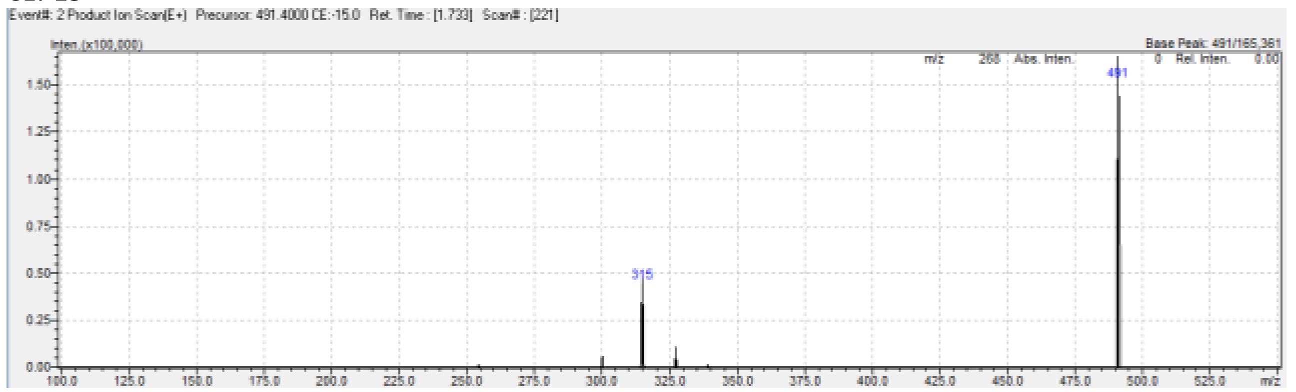


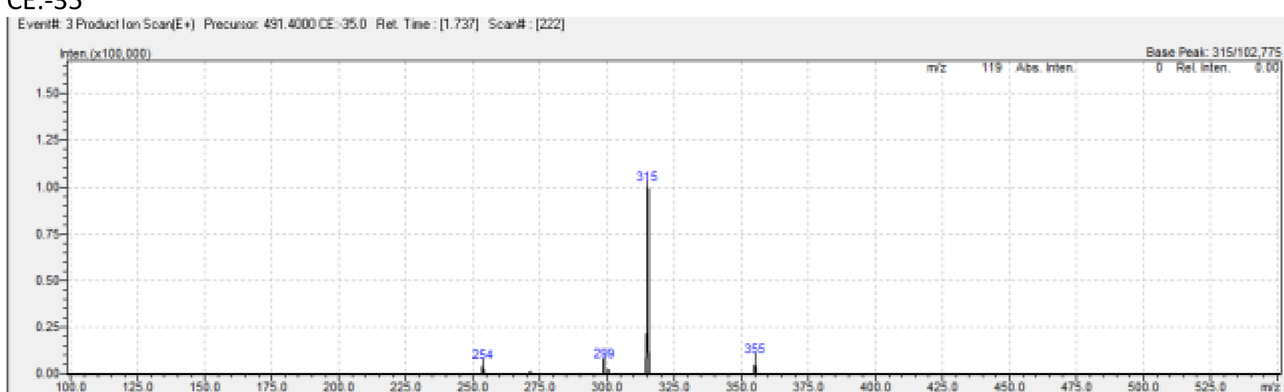
Fig.S103. MS analysis 6-hydroxy-4’,5’-dimethoxyflavone 3’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**22**)

Molecular Formula = $C_{24}H_{26}O_{11}$
Formula Weight = 490.45664
Precursor: = 491.4000

CE:-15



CE:-35



CE:-45

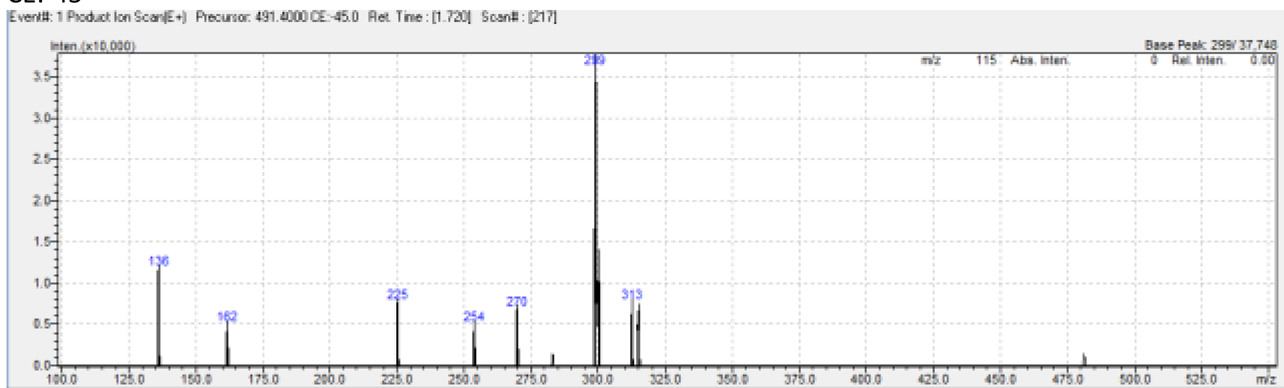


Fig.S104. ^{1}H NMR spectral of 6-hydroxy-4',5'-dimethoxyflavone 3'-*O*-β-D-(4''-*O*-methyl)-glucopyranoside (**22**) (DMSO-d_6, 600 MHz)

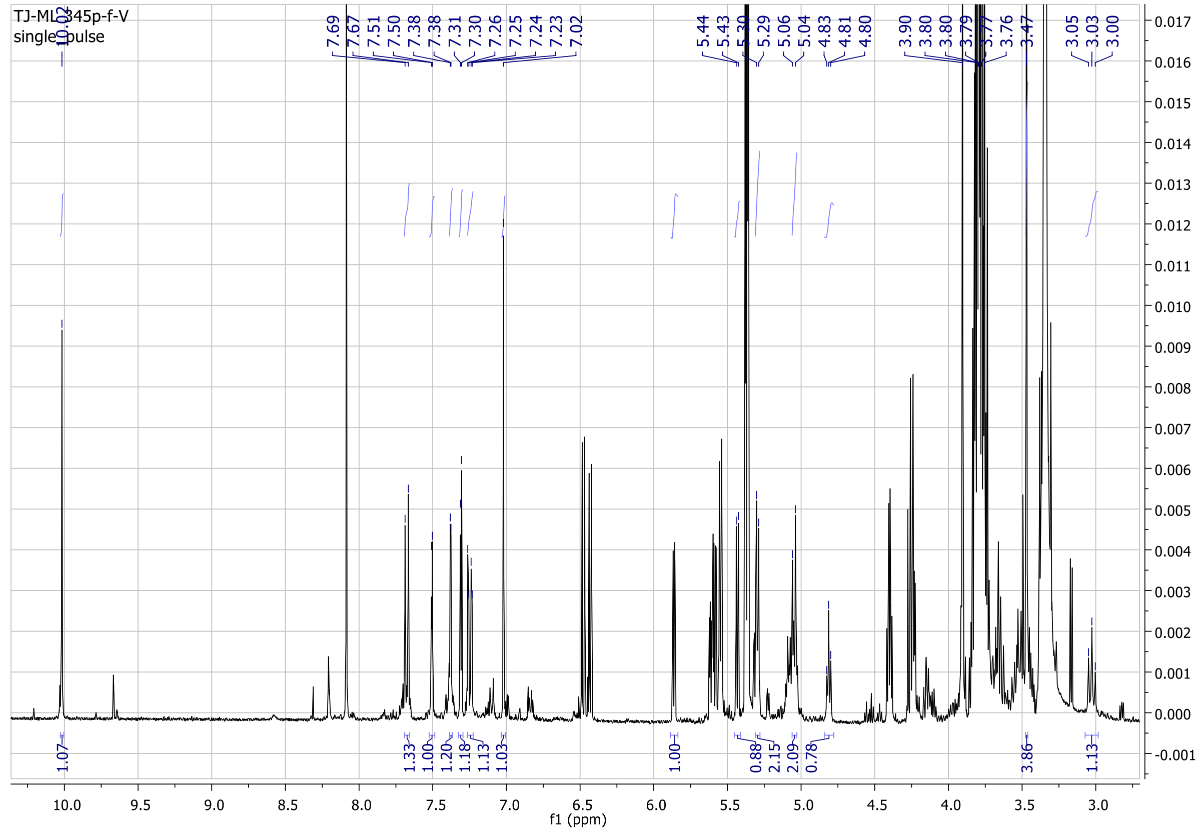


Fig.S105. Flavone part of the ^{1}H NMR spectral 6-hydroxy-4',5'-dimethoxyflavone 3'-*O*-β-D-(4''-*O*-methyl)-glucopyranoside (**22**) (DMSO-d_6, 600 MHz)

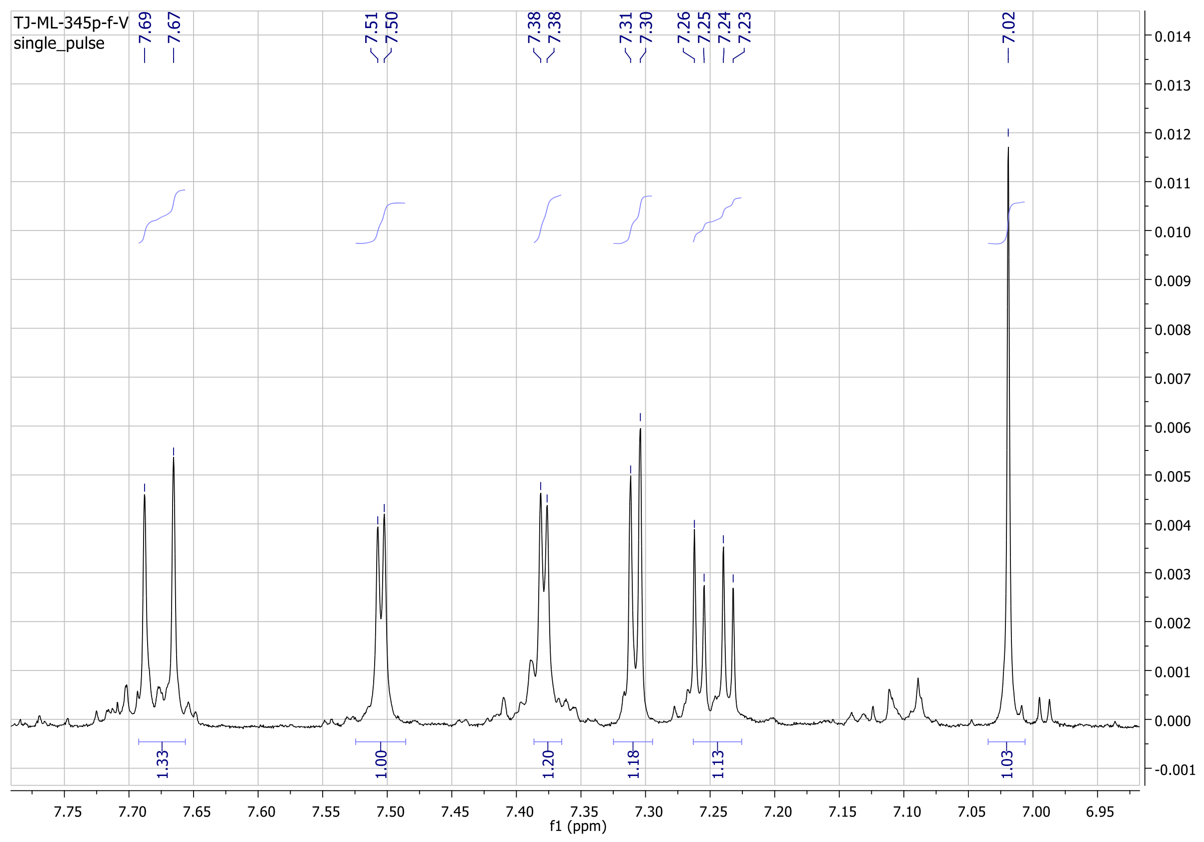


Fig.S106. Glucopyranoside part of the ^{1}H NMR spectral 6-hydroxy-4',5'-dimethoxyflavone 3'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**22**) (DMSO-d_6, 600 MHz)

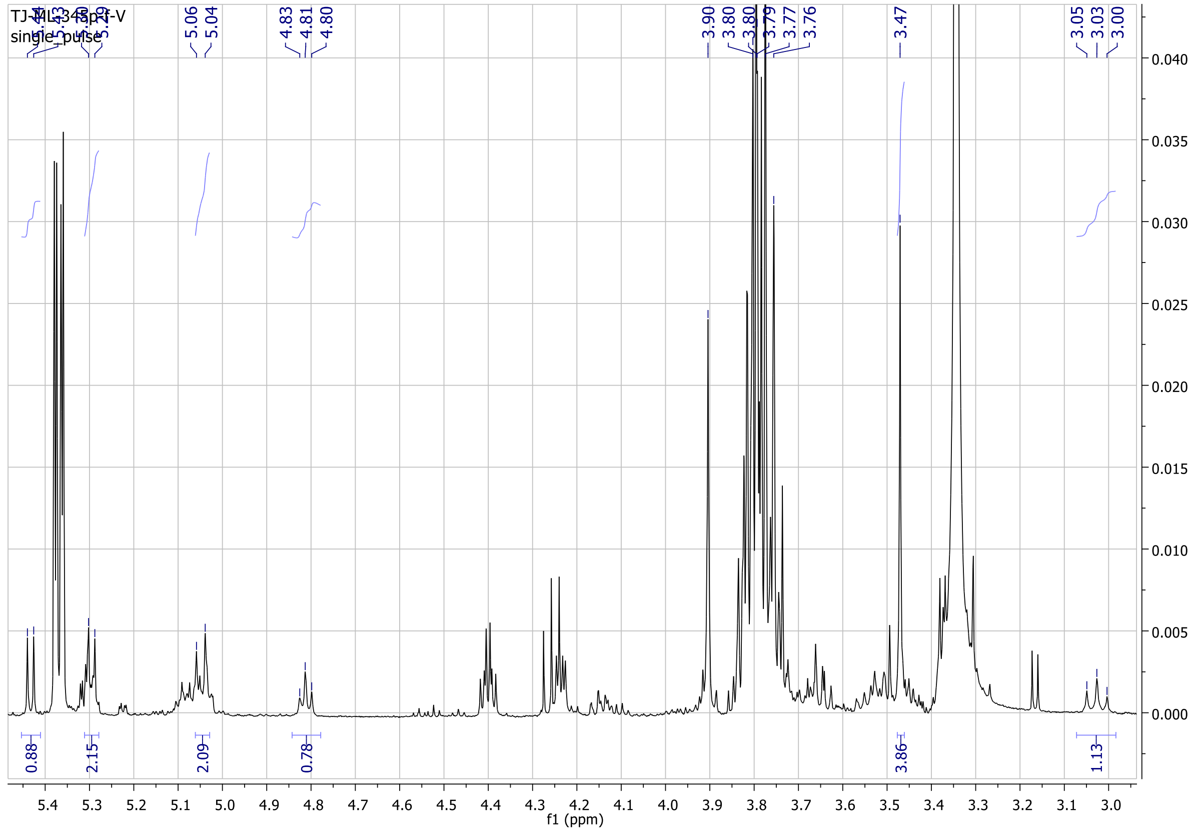


Fig.S107. ^{13}C NMR spectral of 6-hydroxy-4',5'-dimethoxyflavone 3'-*O*-β-D-(4"-*O*-methyl)-glucopyranoside (**22**) (DMSO-d_6, 151 MHz)

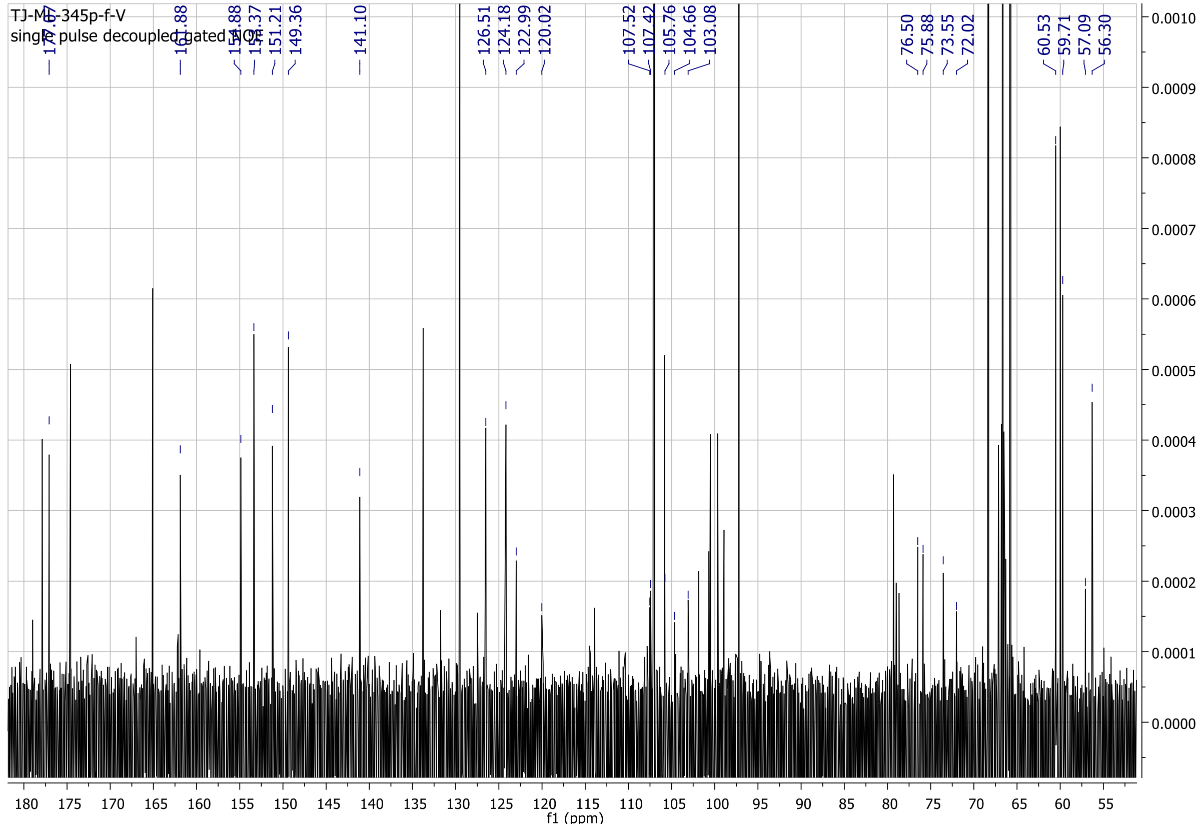


Fig.S108. HMQC spectral of 6-hydroxy-4’,5’-dimethoxyflavone 3’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**22**) (DMSO-d_6, 151 MHz)

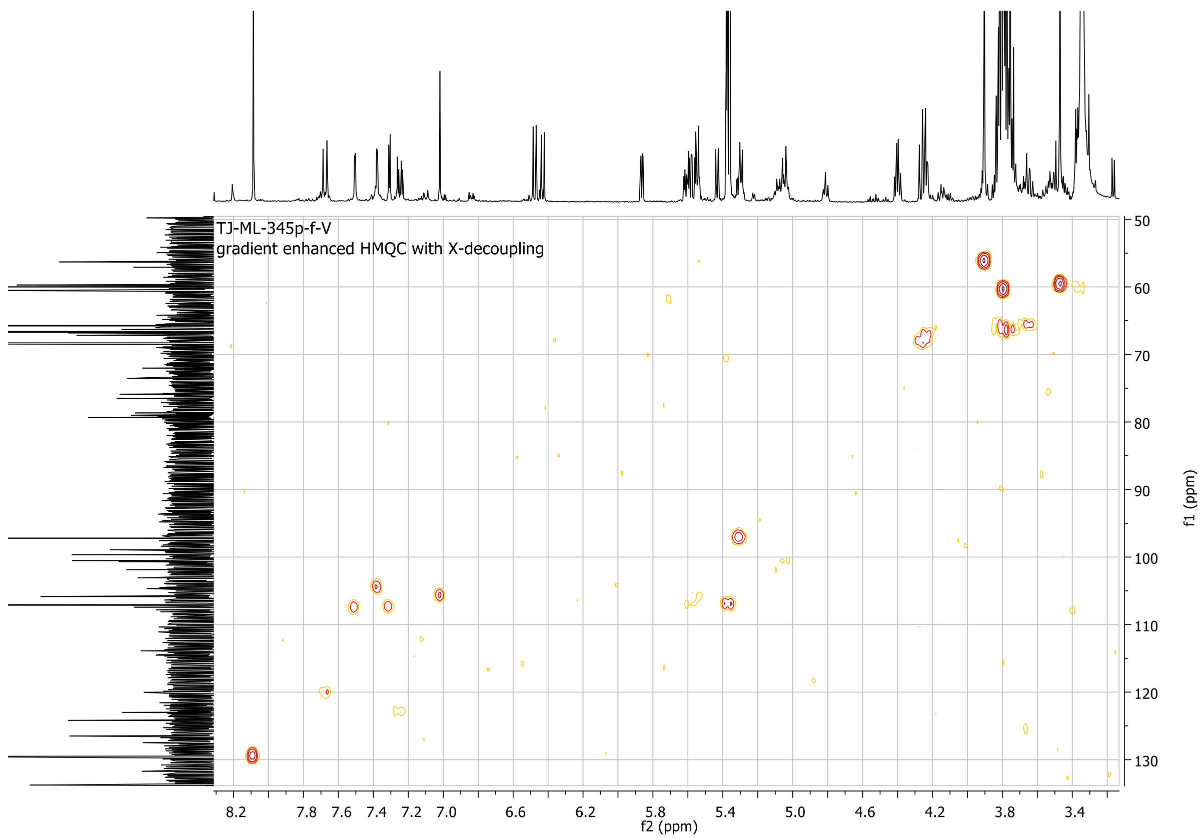
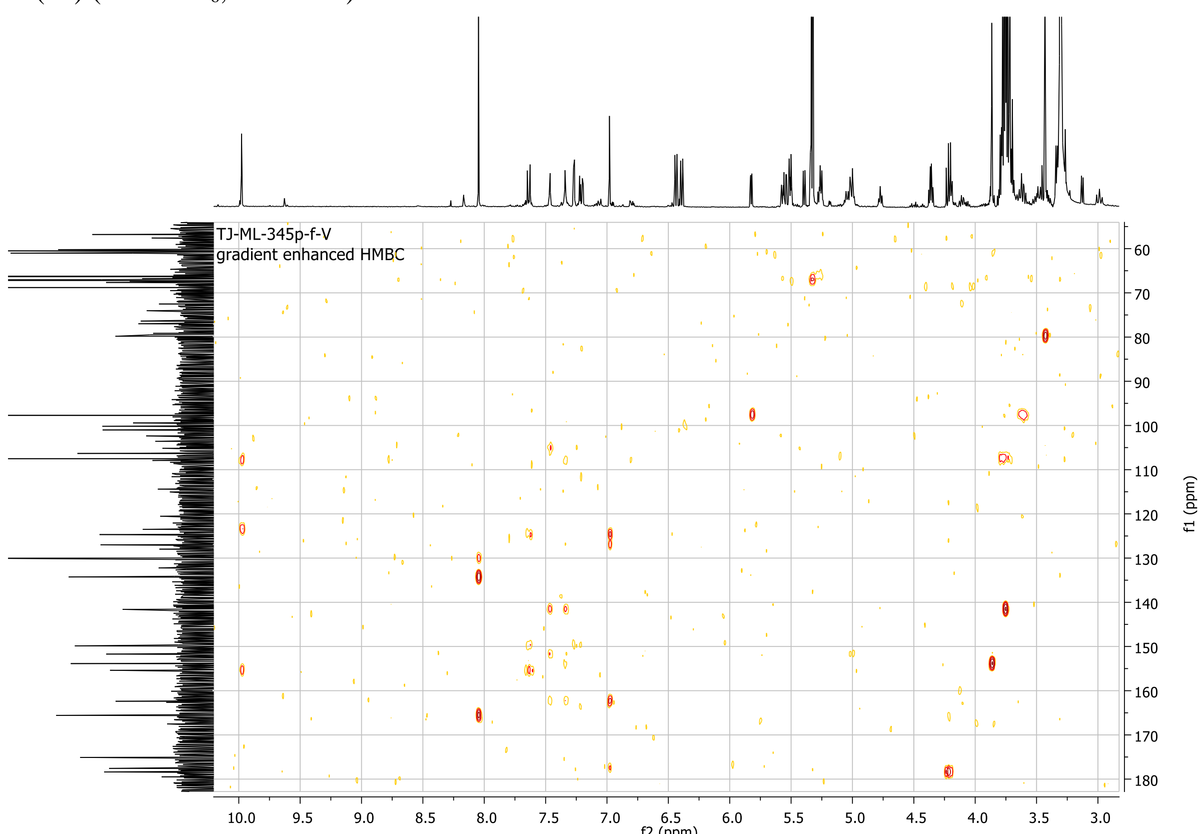


Fig.S109. HMBC spectral of 6-hydroxy-4’,5’-dimethoxyflavone 3’-*O*-β-D-(4”-*O*-methyl)-glucopyranoside (**22**) (DMSO-d_6, 151 MHz)



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Biotransformation of 5,7-Methoxyflavones by Selected Entomopathogenic Filamentous Fungi

Mateusz Łużny,* Tomasz Tronina, Ewa Kozłowska, Edyta Kostrzewa-Susłow, and Tomasz Janeczko*

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ABSTRACT: 5,7-Dimethoxyflavone, a chrysin derivative, occurs in many plants and shows very low toxicity, even at high doses. On the basis of this phenomenon, we biotransformed a series of methoxy-derivatives of chrysin, apigenin, and tricetin obtained by chemical synthesis. We used entomopathogenic fungal strains with the confirmed ability of simultaneous hydroxylation/demethylation and glycosylation of flavonoid compounds. Both the amount and the place of attachment of the methoxy group influenced the biotransformation rate and the product's amount nascent. Based on product and semi-product structures, it can be concluded that they are the result of cascading transformations. Only in the case of 5,7,3′,4′,5′-pentamethoxyflavone, the strains were able to attach a sugar molecule in place of the methoxy substituent to give 3′-*O*-*β*-D-(4″-*O*-methylglucopyranosyl)-5,7,4′,5′-tetramethoxyflavone. However, we observed the tested strains' ability to selectively demethylate/hydroxylate the carbon C-3′ and C-4′ of ring B of the substrates used. The structures of four hydroxyl-derivatives were determined: 4′-hydroxy-5,7-dimethoxyflavone, 3′-hydroxy-5,7-dimethoxyflavone, 3′-hydroxy-5,7,4′,5′-tetramethoxyflavone, and 5,7-dimethoxy-3′,4′-dihydroxyflavone (5,7-dimethoxy-luteolin).

KEYWORDS: *biotransformation, Beauveria sp., Isaria sp., methoxyflavones, 5,7-dimethoxyflavone, 4-O-methyloglycosylation*

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1. INTRODUCTION

Nowadays, it is estimated that there are over 9000 flavonoid compounds present in the plant world.[1] These secondary metabolites fulfill a number of functions, including regulating the overall development of plants, pigmentation, or protecting against ultraviolet (UV) radiation.[2] In addition, new biological properties of flavonoids, such as a positive effect on model organisms (including the human body) after their consumption, are constantly investigated. Studies show that flavonoid compounds can have a wide range of applications from frequently described antioxidant, anticancer, anti-inflammatory,[2,3] antifungal, antibacterial, or antiviral ones[3,4] up to the use in the treatment of Alzheimer's disease[5] or reduction of blood glucose levels (tested on rat models).[6] It is highly likely that we do not know all their properties, and discovering new possibilities of using these substances will take a long time.

Flavonoids show positive effects when tested on cell lines, but usually at a concentration that, when scaled up to the human body, may exceed the maximum daily dose of the drug.[7] It is mainly related to the poor bioavailability and low absorption of these compounds due to their low water solubility.[8] So far, the most frequently described flavonoids have been naturally occurring derivatives containing the hydroxy substituent(s) located at carbon C-5 and C-7. A model example of such a compound (besides quercetin, apigenin, luteolin, and diosmetin) is chrysin (5,7-dihydroxyflavone). It has been determined that chrysin (apart from its antibacterial, anti-inflammatory, antiallergic, anticancer, and antioxidative properties) has an antityrosinase-inhibiting effect and a moderate aromatase-inhibiting effect, improves the development of cognitive functions, reduces brain damage, and has antianxiety and antiestrogenic activities.[9] Chrysin can be extracted, among other sources, from *Passiflora caerulea* L. (blue passion flower) or bee honey,[10] while for laboratory and industrial use, it is synthesized from intermediate compounds[11] and now more and more often from other flavonoids.[9]

Flavonoids with hydroxyl groups are usually characterized by much higher biological activities than counterparts with other substituents (*e.g.*, methoxy).[12] However, the substitution of the hydroxy group, for example, with a methyl or methoxy group, can change their biological activity while increasing the compound's absorbability due to their better lipophilic properties.[13] What is more, introducing a sugar moiety into the flavonoid structure increases its water solubility and, thus, often its biological bioavailability.[8] The lower biological response of such compounds may be compensated by their ability to penetrate biological membranes, including the blood−brain barrier, and a higher level of accumulation in the body, compared to the starting substances.[14]

The methoxy derivative of chrysin is 5,7-dimethoxyflavone (5,7-DMF), a natural compound that can be found, among other sources, in the rhizome of *Boesenbergia pandurata* (Roxb.), that is, a plant which has been used for a long time in traditional Thai medicine,[15] *Piper caninum*,[16] or in

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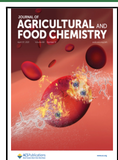

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Scheme 1. Example of Chalcone Synthesis by Claisen−Schmidt Condensation Reaction and Flavone Synthesis

NaOH, MeOH reflux for 2h; I_2, DMSO 130°C, 2h

Scheme 2. Glycoside Hydrolysis with Sulfuric Acid

H_2SO_4, H_2O rt, 0.5h (7 → 8)

Kaempferia parviflora.[17] 5,7-DMF showed very low toxicity, with practically no side effects, even at doses up to 3 g/kg body weight, tested in rats.[15] The authors compared the anti-inflammatory properties of 5,7-DMF with aspirin and found that 5,7-DMF simultaneously inhibits prostaglandin production (anti-inflammatory effect) and lowers the rat's temperature. It was also reported that 5,7-DMF acts as an inhibitor of sarcopenia and, at the same time, causes the development of muscle mass and volume in a mouse model,[18] whose properties are very similar to chrysin.

It was demonstrated that the intracellular transport of 5,7-dimethoxyflavone was approximately 10-fold higher than that of chrysin (5,7-dihydroxyflavone).[19] Moreover, chrysin was rapidly metabolized by the human liver (S9 fraction), with no parent compound remaining after a 20 min incubation. In contrast, 5,7-DMF was metabolically stable over the whole 60 min time-course studied.[20] Interestingly, it was found that the concentration of 5,7-DMF after oral administration was significantly higher in the tissues of the animal than in the plasma, where its half-life was 3.4 ± 2.8 h, and its complete removal from the body took about 17 h.[5] In addition, it has also been shown that the metabolism of 5,7-DMF in comparison to chrysin is significantly reduced, which means that it lasts longer in the body, making it a promising chemopreventive substance.[14]

For this reason, we decided to perform biotransformations of three methoxyflavones obtained by chemical synthesis, containing methoxy substituents at major positions, that is, at the C-5 and C-7 carbon: 5,7-dimethoxyflavone (dimethylchrysin), 5,7,4′-trimethoxyflavone (trimethylapigenin), and 5,7,3′,4′,5′-pentamethoxyflavone (pentamethyltricetin). These compounds were previously identified in medicinal plants: 5,7-DMF, for example, in *K. parviflora*; 5,7,4′-trimethoxyflavone, for example, in *P. caninum;*[16] and 5,7,3′,4′,5′-pentamethoxyflavone in *Murraya paniculata*,[21] whereas the biocatalysts used include strains of entomopathogenic filamentous fungi belonging to the species *Beauveria bassiana* (KCh J1.5, KCh J2.1, KCh J3.2, KCh J1, and KCh BBT), *B. caledonica* (KCh J3.3, KCh J3.4),[22] *Isaria fumosorosea* KCh J2,[8] and *I. farinosa* KCh KW 1.1.[22] Strains from these species have a unique capacity for 4-*O*-methylglycosylation of flavonoids. This reaction is most often described for flavonoid compounds containing a free hydroxyl group in their structure.[3,23−28] The ability of entomopathogenic strains to hydroxylate and 4-*O*-methylglycosylate flavonoid compounds was also described.[8,29,31] In our previous work, we also observed that they perform demethylation and then 4-*O*-methylglycosylation of methoxyflavones.[30,31]

The present research aimed to evaluate the catalytic possibilities of entomopathogenic fungi toward flavones containing methoxy groups located at carbon C-5 and C-7.

2. MATERIALS AND METHODS

2.1. Substrates. The substrates 2-hydroxy-4,6-dimethoxyacetophenone, benzaldehyde, 4-methoxybenzaldehyde, and 3,4,5-trimethoxybenzaldehyde were purchased from Sigma-Aldrich (St. Louis, MO, USA). Flavones used in biotransformations were synthesized from those substrates, and the reactions are described below. The resulting chalcones were used as substrates for the flavone synthesis, and their NMR spectral data are identical to those previously published.[32]

2.2. Synthesis. All used substrates were synthesized in the laboratory by carrying out two kinds of reactions. First, five different methoxychalcones were synthesized in the Claisen−Schmidt reaction of 2-hydroxy-4,6-dimethoxyacetophenone with suitable benzaldehyde. The reaction was described previously[32,33] and is shown in Scheme 1. The resulting flavones (**1**−**3**) were used as substrates for the biotransformation. Their NMR spectral data are identical to those previously published.[32]

After 2 h of reflux, the product of the Claisen−Schmidt reaction was transferred into an acid environment and filtered using a Buchner funnel. The obtained product (appropriate methoxychalcone) was confirmed by NMR analysis. All other methoxychalcones were synthesized analogously. Methoxyflavones were synthesized from methoxychalcones by reaction with iodine in DMSO with 2−3 h incubation (until the substrate had reacted completely) at 130 °C,[34] as presented in the example above (Scheme 1). All substrates for biotransformations were synthesized in the same way, as described in our recent publication.[31] Detailed information on the synthesis is included in the Supporting Information. The course of biotransformation was monitored using thin-layer chromatography (TLC). The composition of product mixtures was established by HPLC. The obtained compounds were confirmed by NMR [^{1}H NMR, ^{13}C NMR, correlation spectroscopy (COSY), HMBC and HSQC] analysis and their mass was checked by liquid chromatography (LC)−mass spectrometry (MS) analysis (Supporting Information).

2.2.1. Hydrolysis of 3′-O-β-D-(4″O-Methylglucopyranosyl)-5,7,4′,5′-tetramethoxyflavone (7). The reaction for glycoside

Figure 1. Biotransformation of 5,7-dimethoxyflavone (**1**) by *I. farinosa* KCh KW 1.1.

hydrolysis with sulfuric acid was carried out on the basis of the study by Zhang *et al.*[35] with solvent modification. In this case, the sample was dissolved in dimethyl sulfoxide (DMSO). The reaction was carried out in a round bottom flask on a magnetic stirrer. 15 mg of compound (7) was dissolved in 800 μL of DMSO. 500 μL of concentrated sulfuric acid (96% v/v) was added dropwise to the sample; then 500 μL of water was added very carefully (Scheme 2). The progress of the reaction was controlled by TLC plates every 30 min. After two hours of reaction, when the starting substrate was no longer observed, 5 mL of H_2O was added to the reaction. Then, the reaction mixture was extracted three times with ethyl acetate. Organic fractions were collected together, and the remains of water were removed with anhydrous $MgSO_4$. Then, the sample was evaporated using a vacuum evaporator. The sample was then analyzed by NMR and LC−MS spectroscopy to confirm the structure of the product obtained.

2.3. Microorganisms. The microorganisms *B. bassiana* KCh J1.5, KCh J2.1, KCh J1, KCh J3.2, and KCh BBT; *B. caledonica* KCh J3.3 and KCh J3.4; *I. farinosa* KCh KW 1.1; and *I. fumosorosea* KCh J2 were obtained from the public culture collection of the Department of Chemistry, Wrocław University of Environmental and Life Sciences (Wrocław, Poland). Isolation and identification procedures of all strains were described in our previous papers.[8,22]

2.4. Screening Procedure. Erlenmeyer flasks (300 mL), each containing 100 mL of the sterile cultivation medium (3% glucose, 1% aminobac-bacteriological peptone—enzymatic hydrolysate of selected animal tissue high in free amino acids and low molecular mass peptides), were inoculated with a suspension of each entomopathogenic strain and then incubated for 3 days at 24 °C on a rotary shaker. After this time, 10 mg of a substrate dissolved in 1 mL of dimethyl sulfoxide (DMSO) was added. Samples were collected on the 1st, 3rd, 7th, and 10th day of the process. Then, all products were extracted using ethyl acetate, and extracts were dried using $MgSO_4$, concentrated *in vacuo*, and analyzed using TLC and HPLC methods.

2.4.1. Scale-up Biotransformation. For the scale-up process, Erlenmeyer flasks (2000 mL) were used, each containing 500 mL of the same cultivation medium (3% glucose, 1% aminobac), which was inoculated in the same way as described above. Three days after inoculation, 100 mg of a substrate dissolved in 2 mL of DMSO was added. Processes of substrate conversion were performed individually depending on substrates and previously obtained HPLC results. Products were extracted three times using ethyl acetate and then analyzed using TLC, HPLC, and NMR spectroscopy (^{1}H NMR, ^{13}C NMR, COSY, HMBC, and HSQC) analysis.

2.5. Analysis. Basic analyses were carried out using TLC plates (SiO_2, DC Alufolien Kieselgel 60 F_{254} (0.2 mm thick), Merck, Darmstadt, Germany). The mobile phase contained a mixture of chloroform and methanol in a 9:1 (v/v) ratio. The plates were observed using a UV lamp (254 and 365 nm). The scale-up biotransformation products were separated using 1000 μm preparative TLC silica gel plates (Anatech, Gehrden, Germany). The mobile phase contained a mixture of chloroform and methanol in a 9:1 (v/v) ratio. The products were isolated by scraping out successive bands and extracted twice with ethyl acetate.

2.5.1. HPLC. A Waters 2690 instrument equipped with a Waters 996 photodiode array detector, using an ODS 2 column (4.6 × 250 mm, Waters, Milford, MA, USA) and a Guard-Pak Inserts μBondapak C18 pre-column, was used to perform HPLC analyses. The mobile phase consisted of eluent A (80% acetonitrile in 4.5% acetic acid solution) and eluent B (4.5% acetic acid) with gradient elution: 0−7 min, 10% A/90% B; 7−10 min, 50% A/50% B; 10−13 min. 60% A/40% B; 15−20 min 80% A/20% B; 20−30 min, 90% A/10% B; 30−40 min, 100% A. The flow rate was 1.0 mL/min, injection volume was 10 μL, and detection wavelength was 323 nm.

2.5.2. NMR Spectroscopy. The NMR analysis was performed with a DRX 600 MHz Bruker spectrometer (Bruker, Billerica, MA, USA) with an UltraShield Plus magnet and measured in DMSO-d_6 or $CDCl_3$. All NMR data is available in the Supporting Information.

2.5.3. LC−MS. Molecular formulas of products were confirmed by LC−MS 8045 SHIMADZU analysis. The mobile phase was a mixture of 0.1% aqueous formic acid v/v (A) and acetonitrile (B). The program was as follows: 80% B and 20% A in 5 min. The flow rate was 0.3 mL/min, and the injection volume was 2 μL. The column (Kinetex 2.6 μm C18 100 Å, 100 mm × 3 mm, Phenomenex, Torrance, CA, USA) was operated at 30 °C. The major operating parameters were as follows: nebulizing gas flow: 3 L/min, heating gas flow: 10 L/min, interface temperature: 300 °C, drying gas flow: 10 L/min, data acquisition range m/z 100−1000 Da; and ionization mode-positive. Data were collected with LabSolutions (Shimadzu, Kyoto, Japan) software.

3. RESULTS AND DISCUSSION

The 5,7-methoxyflavones obtained by chemical synthesis were biotransformed in the cultures of nine entomopathogenic filamentous fungal strains. The cultures of five *B. bassiana* strains (KCh J1.5, KCh J2.1, KCh J3.2, KCh J1, and KCh BBT), two *B. caledonica* strains (KCh J3.3 and KCh J3.4), and two Isaria strains (*I. fumosorosea* KCh J2 and *I. farinosa* KCh KW 1.1.) were used as biocatalysts. All mentioned strains were used in our recent work,[31] in which we described the catalytic abilities of these fungi toward flavones containing a methoxy group/groups within the B ring.

All the used substrates, containing one to three methoxy groups in the structure, were obtained by a two-step chemical synthesis. Three chalcones were synthesized from 2′-hydroxy-4,6-dimethoxyacetophenone and the appropriate benzaldehyde in a basic medium in the first stage. Then, they were transformed into the appropriate methoxyflavones by reaction with I_2 in DMSO. As a result of these reactions, 5,7-

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dimethoxyflavone, 5,7,4′-trimethoxyflavone, and 5,7,3′,4′,5′-pentamethoxyflavone were obtained.

3.1. Biotransformation of 5,7-Dimethoxyflavone (1). Biotransformation of 5,7-dimethoxyflavone (**1**) in the cultures of most of the tested strains resulted in three products (**4−6**) (Figure 1). None of the isolated products contained a sugar molecule in its structure. Such a result of the biotransformation of this substrate is surprising because, in our previous research, we mainly observed 4-*O*-methylglycosylation products preceded by hydroxylation/demethylation.[30,31] After 7-day incubation of 5,7-dimethoxyflavone (**1**) in the culture of the *B. bassiana* KCh J1.5 strain, the main product constituted almost 80% of the reaction mixture, with a retention time of 11.4 min according to HPLC (Table S1—Supporting Information.), and was identified as 5,7-dimethoxy-3′,4′-dihydroxyflavone (**4**).

The ^{1}H NMR spectrum of this product shows signals confirming that the structure of the flavone A and C rings has been preserved. The presence and multiplicity of the signals indicate that the modification has been made in the B ring of the flavone skeleton. The presence and chemical shifts of signals in both the ^{1}H and ^{13}C NMR spectra and the correlation spectra (COSY, HMQC, and HMBC) indicate that 5,7-dimethoxyflavone (**1**) underwent hydroxylation at the carbon C-3′ and C-4′ (shifted signals visible in the ^{13}C NMR spectrum from these carbons are in the field of 145.70 and 148.96 ppm, respectively).

The NMR spectra characterized the other two products (**5** and **6**) (Figure 1.) as 5,7-dimethoxy-4′-hydroxyflavone (**5**) and 5,7-dimethoxy-3′-hydroxyflavone (**6**) (Supporting Information). The molecular mass of these compounds was confirmed by LC−MS analysis. As a result of three-day preparative biotransformation of 5,7-dimethoxyflavone (**1**) in the culture of the *B. bassiana* KCh J1.5 strain, 5,7-dimethoxy-4′-hydroxyflavone (**5**) was isolated with 10% yield. The multiplicities and positions of the signals visible in both the ^{1}H and ^{13}C NMR spectra and the correlation spectra (COSY, HMQC, and HMBC) indicate that this product has one hydroxyl group in its structure. The ^{1}H NMR spectrum shows two multiplets (each derived from two protons) characteristic for a *para*-substituted aromatic ring. In the HMBC spectrum, the signal from the hydrogen of the hydroxyl group (δ = 10.22 ppm) is coupled with the signal from the C-3′ and C-5′ carbon, which confirms the structure of the isolated product **5**.

The main product of this three-day biotransformation by *B. bassiana* KCh J1.5 was 5,7-dimethoxy-3′-hydroxyflavone (**6**), which was isolated with a yield of 40%. The NMR and MS analysis indicated that this compound is a product of hydroxylation. The shape and positions of the signals present in both the ^{1}H and ^{13}C NMR spectra and the correlation spectra (COSY, HMQC, and HMBC) indicate that the 5,7-dimethoxyflavone skeleton was preserved, and the B ring was hydroxylated. In the HMBC spectrum, the signal from the hydroxyl group (δ = 9.87 ppm) is coupled with the signals from the C-2′, C-3′, and C-4′ carbon, which confirms the structure of 5,7-dimethoxy-3′-hydroxyflavone (**6**). This compound was the main product (observed between 1 and 3 days of the substrate incubation process) in the tested strains' cultures. The concentration of compound **6** was significantly higher than that of 5,7-dimethoxy-4′-hydroxyflavone (**5**) (Tables 1 and S1—Supporting Information). The difference in the amounts of obtained compounds **5** and **6** proves the preference of enzymes in the cells of the tested entomopathogenic strains to put the hydroxyl group in 5,7-dimethoxyflavone (**1**) at carbon C-3′. Previous studies described the entomopathogenic strains' preference for the hydroxylation of flavonoid substrates into the C-4′ position. This tendency was observed during the biotransformation of flavone, 5-hydroxyflavone, 6-methylflavone, and 7-aminoflavone in *I. fumosorosea* KCh J2 culture.[8,24] 6-Methoxyflavanone in the culture of *I. fumosorosea* KCh J2 was hydroxylated to 4′-hydroxy and 3′,4′-dihydroxy derivatives, while 6-methoxyflavone was hydroxylated to 3′-hydroxy, 4′-hydroxy, and 3′,4′-dihydroxy derivatives.[30] All products isolated in the mentioned studies were corresponding β-D-(4-*O*-methyl)-glucopyranosides.[8,24,30]

Table 1. Product Yields at 7th Day of Biotransformation of 5,7-Dimethoxyflavone (1) and 5,7,4′-Trimethoxyflavone (2), According to HPLC

strain no.	substrate	composition of products [%] (6)	(5)	(4)
B. bassiana KCh J1.5	1	17.0	2.5	79.5
	2	0	64.5	0.4
B. bassiana KCh J2.1	1	1.5	0	1.7
	2	0	62.6	0.3
I. farinosa KCh KW 1.1.	1	1	0	1.2
	2	0	17.2	0.4
B. bassiana KCh J1	1	21.4	1.7	9.8
	2	0	46.1	2.4
I. fumosorosea KCh J2	1	44.4	0.4	54.8
	2	0	8.6	0.4
B. caledonica KCh J34	1	18.8	1.7	6.4
	2	0	7.7	0
B. bassiana KCh J3.2	1	30.7	3	4.5
	2	0	34.8	1.9
B. caledonica KCh J3.3	1	10.2	0.7	4
	2	0	6.8	0
B. bassiana KCh BBT	1	30.9	3.9	7.5
	2	0	60.2	3.6

3.2. Biotransformation of 5,7,4′-Trimethoxyflavone (2). Two products were isolated (**4** and **5**) (Figure 2) after incubation of 5,7,4′-trimethoxyflavone (**2**) in the cultures of the tested entomopathogenic strains. These compounds were also observed in the biotransformation of 5,7-dimethoxyflavone (**1**). In this case, the main product was 5,7-dimethoxy-4′-hydroxyflavone (**5**), which is the result of the *O*-demethylation of the substrate used. An analogous effective regioselective demethylation of 5,7,4′-trimethoxyisoflavone and sinensetin was previously described using a strain of the species *Aspergillus niger*.[36,37]

Product **5** was produced by the enzymes of all the microorganisms used in the studies. In the cultures of three of them (*B. bassiana* KCh J1.5, KCh J2.1, and KCh BBT), this product was observed with over 60% conversion (after seven days of substrate incubation according to HPLC (Table 1). Extending the biotransformation process to 10 days resulted in increased production of 5,7-dimethoxy-4′-hydroxyflavone (**5**) up to 60 and 75% for two other strains, *I. fumosorosea* KCh J2 and *I. farinosa* KCh KW 1.1., respectively (Scheme 3). Of equal importance, the concentration of 5,7-dimethoxy-3′,4′-dihydroxyflavone (**4**) in the reaction mixture increased after a prolonged time. In the cultures of *B. bassiana* KCh J2.1 and KCh BBT and *I. farinosa* KCh KW 1.1. strains, a concentration exceeding 10% was reached, while in the *B. bassiana* KCh J1 strain culture, the concentration of this product was recorded

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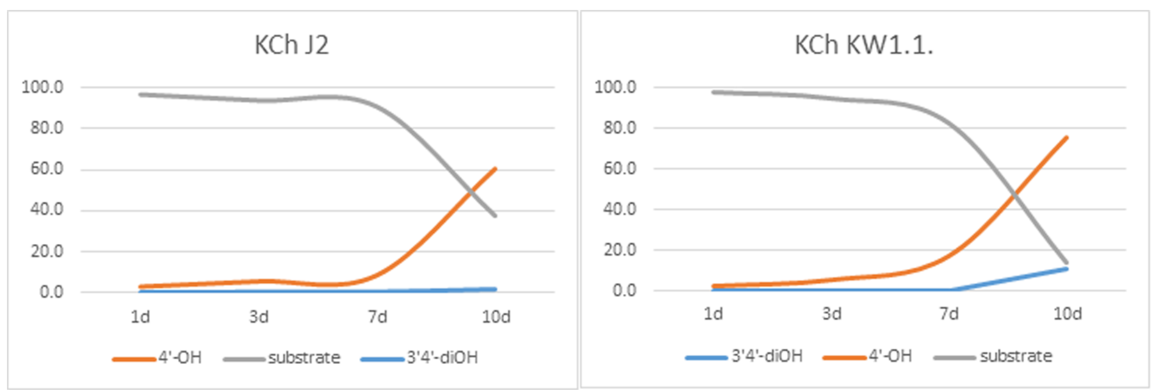
Figure 2. Biotransformation of 5,7,4′-trimethoxyflavone (**2**) by *I. farinosa* KCh KW 1.1.

Scheme 3. Microbial Transformation of 5,7,4′-Trimethoxyflavone (2) in *I. fumosorosea* KCh J2 and *I. farinosa* KCh KW 1.1 in Time



at the level of 26% after ten days of transformation (Tables 1 and S2—Supporting Information). Hydroxylation of C-3′ carbon and 4′-hydroxyl flavonoids is often observed and well-characterized in plant cells.[38,39] The hydroxylation pattern of the B ring of flavonoids is determined by the flavonoid 3′-hydroxylase (F3′H). The phylogenetic tree results showed that F3′H belongs to CYP75B.[38,40]

The application of the *Bacillus megaterium* cytochrome P450 BM3 (CYP450 BM3) and its mutants for the hydroxylation of naringenin to eriodictyol was described by Chu *et al.*[41] As a result of conducting *in vitro* experiments with human liver microsomes and recombinant enzymes, aromatic hydroxylation was also observed at position 3′ of the B ring of naringenin and sakuranetin.[42] The dominant enzyme responsible for this hydroxylation is CYP1A2, whereas other human liver cytochromes P450 are CYP2C19, CYP2D6, CYP2E1, and CYP3A4.[42]

3.3. Biotransformation of 5,7,3′,4′,5′-Pentamethoxyflavone (PMF) (3). The main transformation product of 5,7,3′,4′,5′-pentamethoxyflavone (**3**) in the culture of the *B. bassiana* KCh J1.5 strain nascent with a conversion of >99% after just three days was 3′-*O*-*β*-D-(4″-*O*-methylglucopyranosyl)-5,7,4′,5′-tetramethoxyflavone (**7**). This product was also observed in the other tested strains' cultures but with much lower efficiency (max 20–24% conversion after 7 days) (Table 2). Moreover, the *B. bassiana* KCh J1 strain was the only one of the tested strains unable to attach the sugar molecule into the flavonoid. In this culture, the highest concentration of the regioselective *O*-demethylation product of the methoxy group bound to the C-3′ carbon was observed. The product of this process was 5,7,4′,5′-tetramethoxy-3′-hydroxyflavone (**8**) (Figure 3). Such regioselective demethylation is consistent with the observations previously presented by Nielsen *et al.*[43] *In vitro* biotransformation of tangeretin and nobiletin (the typical polymethoxy flavonoids in plants[44,45]) by rat liver microsomes indicated that the demethylation only occurs at the C-3′ and C-4′ of the B ring.[43] On the other hand, successive *O*-demethylation and 4-*O*-methylglucosylation were previously described during the biotransformation of methoxyflavones and flavanones in cultures of entomopathogenic filamentous fungi of the genera *Beauveria* and *Isaria*.[30,31]

Structure (**8**) was characterized on the basis of data obtained by NMR analyses, and its molecular mass was confirmed by LC–MS analysis. Demethylation of only one group is confirmed by the presence on the ^{1}H NMR spectrum in the position 3.79–3.90 ppm, where only 3 singlets originate from the protons of four methoxyl groups. Additionally, different chemical shifts of signals coming from H-2′ and H-6′ protons (7.48 and 7.34 ppm, respectively) inform that the symmetrical structure of the substrate B ring has changed. On the ^{1}H NMR spectrum, signals coming from the sugar substituent are visible. On the basis of the analysis of multiplicity and chemical shifts of signals coming from this substituent, it was identified as glucose and was visible in the HMBC spectrum of the coupling between the signal coming from the protons of the group $-OCH_3$ visible in the position 3.47 ppm with C-4 carbon of the sugar unit clearly indicating that this substituent is 4-*O*-glucopyranoside. In comparison, on the HMBC spectrum of compound **7**, there is also visible coupling of the doublet coming from the hemiacetal proton H-1″ with the signal from C-3′ carbon which unambiguously confirms the structure of 3′-*O*-*β*-D-(4″-*O*-methylglucopyranosyl)-5,7,4′,5′-tetramthoxyflavone (**7**).

The second product observed during the biotransformation of compound **3** in the studied strains' cultures was a result of regioselective demethylation—3′-hydroxy-5,7,4′,5′-tetramethoxyflavone (**8**), an intermediate product, essential to obtaining compound **7**. The structure of 3′-hydroxy-5,7,4′,5′-tetramethoxyflavone (**8**) was confirmed by comparing its experimental data with the standard obtained as a result of acid deglucosylation of compound **7**. The molecular mass of product **8** was confirmed by LC–MS analysis, and the structure was confirmed by NMR analysis. The shape and position of the signals visible in both the ^{1}H and ^{13}C NMR

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Table 2. Microbial Transformation of 5,7,3′,4′,5′-Pentamethoxyflavone (3), According to HPLC

strain no.	number of compound	retention time [min]	composition of products [%] after 1, 3, and 7 days		
			1	3	7
B. bassiana KCh J1.5	7	10.7	27.9	99.5	99.9
	8	13.0	11.9	0.5	0.1
	3	15.1	60.2	0.0	0.0
B. bassiana KCh J2.1	7	10.7	0.0	0.6	5.3
	8	13.0	1.8	6.2	12.0
	3	15.1	98.2	93.2	82.8
I. farinosa KCh KW 1.1.	7	10.7	0.7	4.1	15.4
	8	13.0	8.5	22.7	29.0
	3	15.1	90.8	73.2	55.6
B. bassiana KCh J1	7	10.7	0.0	0.0	0.0
	8	13.0	2.7	38.7	56.9
	3	15.1	97.4	61.3	43.1
I. fumosorosea KCh J2	7	10.7	0.0	3.0	3.9
	8	13.0	0.0	0.0	0.0
	3	15.1	100.0	97.0	96.1
B. caledonica KCh J34	7	10.7	1.2	18.6	19.8
	8	13.0	0.4	1.9	3.5
	3	15.1	98.4	79.6	76.7
B. bassiana KCH J3.2	7	10.7	0.0	3.1	24.0
	8	13.0	0.3	3.9	16.7
	3	15.1	99.7	93.0	59.2
B. caledonica KCh J3.3	7	10.7	0.9	4.8	7.0
	8	13.0	1.4	0.2	0.4
	3	15.1	97.7	95.1	92.6
B. bassiana KCh BBT	7	10.7	1.0	2.1	5.5
	8	13.0	2.7	7.3	17.2
	3	15.1	96.3	90.6	77.3

spectra and correlation spectra (COSY, HMQC, HMBC) indicate that this product has one hydroxyl group in its structure (singlet in position 9.55 ppm). The ^{1}H NMR spectrum of this product shows four singlets derived from the protons of the methoxy groups. In the HMBC spectrum, the signal from the hydroxyl group (δ = 9.55 ppm) is coupled with the signals from the C-2′, C-3′, and C-4′ carbon, which confirms the isolated product's structure.

The hydroxyl analogues of the 5,7-dimethoxyflavone substrates described in this work (dihydroxyflavone with substituents in the 5 and 7 positions) have already been transformed by the filamentous fungi (including strains of the *B. bassiana* species) to appropriate glycosidic derivatives, where the sugar molecule was mainly attached to carbon C-7′ and C-3′.[28]

In our study, none of the tested microorganisms were able to *O*-demethylate the methoxy groups present in the A ring. Demethylation was observed only in the B ring of the tested substrates. Surprisingly, in the light of the previously described effective 4-*O*-methylglucosylation of hydroxy flavonoids in the cultures of entomopathogenic fungi,[3,23,24,46] only one glucopyranoside was isolated during the biotransformation of 5,7-dimethoxyflavones—3′-*O*-β-D-(4″-*O*-methylglucopyranosyl)-5,7,4′,5′-tetramethoxyflavone (**7**). Two other substrates described in this work underwent ortho-hydroxylation leading to 5,7-dimethoxy-3′,4′-dihydroxyflavone (**4**) (dimethoxy-luteolin). Flavonoid compounds containing hydroxyl groups in the 3′ and 4′ positions in their structure show the desired positive effect on the human body.[47] Luteolin is highly pharmacologically effective in inflammatory and neurodegenerative diseases.[48] In addition, luteolin has been reported as a potential anticancer agent that could inhibit a wide range of human cancers.[47,49,50]

The entomopathogenic fungal strains used in the study showed the capacity for selective demethylation/hydroxylation in the B ring of the substrates used at C-3′ and C-4′ carbon. Three monohydroxy products were obtained: 4′-hydroxy-5,7-dimethoxyflavone (**5**), 3′-hydroxy-5,7-dimethoxyflavone (**6**), and 3′-hydroxy-5,7,4′,5′-tetramethoxyflavone (**8**). It was also demonstrated that the tested strains have produced enzymes capable of orthohydroxylation, resulting in 5,7-dimethoxy-3′,4′-dihydroxyflavone (**4**) (dimethoxy-luteolin). In our previous study, 4-*O*-methylglucosides were the major biotransformation products of methoxyflavones. In this case, where the A ring is substituted in two positions (5,7), the tested microorganisms mainly performed the demethylation and hydroxylation reaction. The location of the methoxyflavones in the A ring—characteristic of many natural flavones—makes them modify only in the B ring.[31]

In summary, the manuscript describes a biotransformation study of 5,7-methoxyflavones by selected entomopathogenic filamentous fungi. Moreover, efficient methods of obtaining bioactive flavonoids are sought continuously. So far, the best and most frequently described flavonoids are naturally occurring derivatives containing the hydroxy or methoxy substituents located at carbon C-5 and C-7. A model example of such a compound (besides quercetin, apigenin, luteolin, and diosmetin) is chrysin (5,7-dihydroxyflavone). The methoxy

Figure 3. Biotransformation of 5,7,3′,4′,5′-pentamethoxyflavone (**3**) by *B. bassiana* KCh J1.5 and *B. bassiana* KCh J1.

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derivative of chrysin is 5,7-dimethoxyflavone, which also occurs in many plants and shows very low toxicity, with practically no side effects, even at high doses (even up to 3 g/kg body weight, tested in rats).[15] Based on this phenomenon, in this study, we biotransformed a series of methoxy derivatives of chrysin, apigenin, and tricetin obtained by chemical synthesis. We used entomopathogenic fungal strains with the confirmed ability of simultaneous hydroxylation/demethylation and glycosylation of flavonoid compounds, like *B. bassiana*, *B. caledonica*, *I. fumosorosea*, and *I. farinosa*, as biocatalysts. Both the amount and the place of attachment of the methoxy group influenced the biotransformation rate and the product's amount nascent. All obtained products were characterized by spectroscopic methods. On the basis of products and semi-products structures, it can be concluded that they are the result of cascading transformations. Five compounds were obtained; among them, four are hydroxyl derivatives of substrates and one 4-*O*-methylglucoside. Each of the obtained products was determined by HPLC, LC−MS, and NMR analysis (Supporting Information). The only glycosylation product was identified as 3′-*O*-*β*-D-(4″-*O*-methylglucopyranosyl)-5,7,4′,5′-tetramethoxyflavone (**7**), which was obtained during the 5,7,3′,4′,5′-pentamethoxyflavone biotransformation. These results clearly indicate that entomopathogenic filamentous fungi produce numerous active enzymes capable of effectively converting flavonoid compounds of varying structures. High yield and short time of conversion of substrates are crucial for increasing the described process into an industrial scale. Moreover, the structure of the obtained products promotes their usage in the food and pharmaceutical industries as active substances in health-promoting preparations.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.1c00136.

NMR data of all compounds described in the publication; MS analysis, ^{1}H NMR, ^{13}C NMR, COSY, HMQC, and HMBC of all substrate and biotransformation products; efficiencies of chalcone synthesis during the Claisen−Schmidt reaction; and flavone synthesis efficiencies by cyclization reactions (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Mateusz Łużny − *Department of Chemistry, Wrocław University of Environmental and Life Sciences, 50-375 Wrocław, Poland;* orcid.org/0000-0002-0646-2295; Email: mat.luzny@gmail.com

Tomasz Janeczko − *Department of Chemistry, Wrocław University of Environmental and Life Sciences, 50-375 Wrocław, Poland;* orcid.org/0000-0002-9757-6684; Phone: +48-713-205-195; Email: janeczko13@interia.pl

Authors

Tomasz Tronina − *Department of Chemistry, Wrocław University of Environmental and Life Sciences, 50-375 Wrocław, Poland;* orcid.org/0000-0002-8317-8976

Ewa Kozłowska − *Department of Chemistry, Wrocław University of Environmental and Life Sciences, 50-375 Wrocław, Poland;* orcid.org/0000-0001-7366-8608

Edyta Kostrzewa-Susłow − *Department of Chemistry, Wrocław University of Environmental and Life Sciences, 50-375 Wrocław, Poland;* orcid.org/0000-0002-9259-0161

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jafc.1c00136

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Notes

The authors declare no competing financial interest.

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SUPPORTING INFORMATION

Biotransformation of 5,7-methoxyflavones by selected entomopathogenic filamentous fungi

Mateusz Łużny*, Tomasz Tronina, Ewa Kozłowska, Edyta Kostrzewa-Susłow and Tomasz Janeczko*

Department of Chemistry, Wrocław University of Environmental and Life Sciences, Norwida 25, 50-375 Wrocław, Poland; e.a.kozlowska@gmail.com (E.K.), ekostrzew@gmail.com (E.K-S.)

* Correspondence: mat.luzny@gmail.com (M.Ł.) and janeczko13@interia.pl (T.J.); Tel.: +48-713-205-195

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NMR data of all compounds described in the publication:

5,7-dimethoxyflavone (**1**)
^{1}H NMR (600 MHz) (DMSO-d_6) δ (ppm): 3.83 (s, 3H, C-5-OCH_3), 3.90 (s, 3H, C-7-OCH_3), 6.51 (d, 1H, J = 2.3 Hz, H-6), 6.77 (s, 1H, H-3), 6.86 (d, 1H, J = 2.3 Hz, H-8), 7.52-7.58 (m, 3H, H-3', H-4', H-5'), 8.02-8.06 (m, 2H, H-2', H-6').
^{13}C NMR (151 MHz, DMSO-d_6) δ = 56.01 (C-7-OCH$_3$), 56.12 (C-5-OCH$_3$), 93.40 (C-8), 96.33 (C-6), 108.40 (C-3), 108.39 (C-4a), 125.94 (C-2', C-6'), 129.07 (C-3', C-5'), 130.90 (C-4'), 131.44 (C-1'), 159.24 (C-8a), 159.59 (C-2), 160.31 (C-5), 163.83 (C-7), 175.72 (C=O).

5,7,4'-trimethoxyflavone (**2**)
^{1}H NMR (600 MHz) ($CDCl_3$) δ (ppm): 3.87 (s, 3H, C-4'-OCH_3), 3.90 (s, 3H, C-7-OCH_3), 3.94 (s, 3H, C-5-OCH_3), 6.36 (d, 1H, J = 2.3 Hz, H-6), 6.55 (d, 1H, J = 2.3 Hz, H-8), 6.61 (s, 1H, H-3), 6.97-7.00 (m, 2H, H-3', H-5'), 7.80-7.83 (m, 2H, H-2', H-6').
^{13}C NMR (151 MHz, DMSO-d_6) δ = 55.60 (C-4'-OCH$_3$), 55.88 (C-7-OCH$_3$), 56.55 (C-5-OCH$_3$), 92.93 (C-8), 96.23 (C-6), 107.64 (C-3), 109.20 (C-4a), 114.48 (C-3', C-5'), 123.87 (C-1'), 127.76 (C-2', C-6'), 159.96 (C-8a), 160.95 (C-2), 160.99 (C-5), 162.21 (C-4'), 164.10 (C-7), 177.80 (C=O).

5,7,3',4',5'-pentamethoxyflavone (**3**)
^{1}H NMR (600 MHz) ($CDCl_3$) δ (ppm): ^{1}H NMR (600 MHz) (DMSO) δ (ppm): 3.92 (s, 3H, C-4'-OCH_3), 3.93 (s, 3H, C-7-OCH_3), 3.95 (s, 6H, C-3'-OCH_3 and C-5'-OCH_3), 3.96 (s, 3H, C-5-OCH_3), 6.39 (d, 1H, J = 2.2 Hz, H-8), 6.57 (d, 1H, J = 2.2 Hz, H-6), 6.69 (s, 1H, H-3), 7.08 (s, 2H, H-2', H-6').
^{13}C NMR (151 MHz, DMSO-d_6) δ = 55.99 (C-7-OCH$_3$), 56.51 (C-3'-OCH$_3$ and C-5'-OCH$_3$), 56.61 (C-5-OCH$_3$), 61.17 (C-4'-OCH$_3$), 93.04 (C-6), 96.42 (C-8), 103.56 (C-2', C-6'), 108.75 (C-3), 109.16 (C-4a), 126.79 (C-1'), 141.05 (C-4'), 153.66 (C-3', C-5'), 160.02 (C-8a), 160.87 (C-2), 161.06 (C-5), 164.36 (C-7), 177.74 (C=O).

3',4'-dihydroxy-5,7-dimethoxyflavone (**4**)
^{1}H NMR (600 MHz) (DMSO-d_6) δ (ppm): 3.82 (s, 3H, C-5-OCH_3), 3.89 (s, 3H, C-7-OCH_3), 6.46 (s, 1H, H-3), 6.49 (d, 1H, J = 2.3 Hz, H-6), 6.88 (d, 1H, J = 2.3 Hz, H-8), 6.87 (d, 1H, J = 8.9 Hz, H-5'), 7.34-7.37 (m, 2H, H-2', H-6').
^{13}C NMR (151 MHz, DMSO-d_6) δ = 55.94 (C-7-OCH$_3$), 56.08 (C-5-OCH$_3$), 93.21 (C-8), 96.17 (C-6), 106.18 (C-3), 108.26 (C-4a), 113.05 (C-2'), 115.92 (C-5'), 118.22 (C-6'), 121.74 (C-1'), 145.70 (C-3'), 148.96 (C-4'), 159.11 (C-8a), 160.26 (C-2 and C-5), 163.58 (C-7), 175.60 (C-4).

4'-hydroxy-5,7-dimethoxyflavone (**5**)
^{1}H NMR (600 MHz) (DMSO-d_6) δ (ppm): 3.82 (s, 3H, C-5-OCH_3), 3.89 (s, 3H, C-7-OCH_3), 6.49 (d, 1H, J = 2.3 Hz, H-6), 6.59 (s, 1H, H-3), 6.83 (d, 1H, J = 2.3 Hz, H-8), 6.89-6.92 (m, 2H, H-3', H-5'), 7.87-7.90 (m, 2H, H-2', H-6'), 10.22 (s, 1H, C-4'-OH).
^{13}C NMR (151 MHz, DMSO-d_6) δ = 55.94 (C-7-OCH$_3$), 56.06 (C-5-OCH$_3$), 93.32 (C-8), 96.16 (C-6), 106.13 (C-3), 108.25 (C-4a), 115.83 (C-3', C-5'), 121.37 (C-1'), 127.84 (C-2', C-6'), 159.12 (C-8a), 160.06 (C-2), 160.24 (C-5), 160.51 (C-4'), 163.58 (C-7), 175.65 (C-4).

3'-hydroxy-5,7-dimethoxyflavone (**6**)
^{1}H NMR (600 MHz) (DMSO-d_6) δ (ppm): 3.83 (s, 3H, C-5-OCH_3), 3.90 (s, 3H, C-7-OCH_3), 6.51 (d, 1H, J = 2.3 Hz, H-6), 6.64 (s, 1H, H-3), 6.82 (d, 1H, J = 2.3 Hz, H-8), 6.96 (ddd, 1H,

J = 8.1, 2.4, 0.5 Hz, H-4'), 7.33 (t, 1H, *J* = 7.9 Hz, H-5'), 7.37 (t, 1H, *J* = 2.2 Hz, H-2'), 7.45 (brd, 1H, *J* = 7.8 Hz, H-6'), 9.87 (s, C-3'-O*H*).
^{13}C NMR (151 MHz, DMSO-d_6) δ = 56.06 (C-7-O*C*H$_3$), 56.17 (C-5-O*C*H$_3$), 93.36 (C-8), 96.38 (C-6), 108.25 (C-3), 108.42 (C-4a), 112.51 (C-2'), 116.83 (C-6'), 118.49 (C-4'), 130.26 (C-5'), 132.23 (C-1'), 157.91 (C-3'), 159.26 (C-8a), 159.79 (C-2), 160.35 (C-5), 160.35 (C-7), 175.77 (C-4).

3'-*O*-β-D-(4''-*O*-methyloglucopiranozylo)- 5,7,4',5'-tetramethoxyflavone (**7**)
^{1}H NMR (600 MHz) (DMSO-d_6) δ (ppm): 3.00 (t, 1H, *J* = 9.3 Hz, H-4''), 3.30-3.34 (m, 1H, H-2''), 3.45 (dd, 1H, *J* = 9.0, 5.6 Hz, H-3''), 3.47 (s, 3H, C-4''-OC*H*$_3$), 3.48-3.56 (m, 2H, H-5'' and one of H-6''), 3.69 (dd, 1H, *J* = 10.3, 5.1 Hz, one of H-6''), 3.79 (s, 3H, C-4'-OC*H*$_3$), 3.83 (s, 3H, C-5-OC*H*$_3$), 3.90 (s, 6H, C-5'-OC*H*$_3$ and C-7-OC*H*$_3$), 4.84 (t, 1H, *J* = 5.5 Hz, C-6''-O*H*), 5.03 (d, 1H, *J* = 7.9 Hz, H-1''), 5.29 (d, 1H, *J* = 5.6 Hz, C-3''-O*H*), 5.42 (d, 1H, *J* = 5.7 Hz, C-2''-O*H*), 6.51 (d, 1H, *J* = 2.3 Hz, H-6), 6.84 (s, 1H, H-3), 6.91 (d, 1H, *J* = 2.3 Hz, H-8), 7.33 (d, 1H, *J* = 2.0 Hz, H-6'), 7.48 (d, 1H, *J* = 2.0 Hz, H-2').
^{13}C NMR (151 MHz, DMSO-d_6) δ = 55.84 (C-7-O*C*H$_3$), 55.97 (C-5'-O*C*H$_3$), 56.08 (C-5-O*C*H$_3$), 59.71 (C-4''-O*C*H$_3$), 60.51 (C-4'-O*C*H$_3$), 60.56 (C-6''), 73.58 (C-2''), 75.90 (C-5''), 76.49 (C-3''), 79.40 (C-4''), 93.52 (C-8), 96.26 (C-6), 100.88 (C-1''), 104.37 (C-6'), 107.33 (C-2'), 108.04 (C-3), 108.29 (C-4a), 126.03 (C-1'), 140.93 (C-4'), 151.26 (C-3'), 153.35 (C-5'), 159.17 (C-2), 159.26 (C-8a), 160.23 (C-5), 166.64 (C-7), 175.78 (C-4).

3'-hydroxy-5,7,4',5'-tetramethoxyflavone (**8**)
^{1}H NMR (600 MHz) (DMSO-d_6) δ (ppm): 3.74 (s, 3H, C-4'-OC*H*$_3$), 3.83 (s, 3H, C-5-OC*H*$_3$), 3.88 (s, 3H, C-5'-OC*H*$_3$), 3.91 (s, 3H, C-7-OC*H*$_3$), 6.51 (d, 1H, *J* = 2.3 Hz, H-6), 6.70 (s, 1H, H-3), 6.82 (d, 1H, *J* = 2.3 Hz, H-8), 7.12 (d, 1H, *J* = 2.1 Hz, H-6'), 7.14 (d, 1H, *J* = 2.1 Hz, H-2'); 12.84 (s, 1H, -O*H*).
^{13}C NMR (151 MHz, DMSO-d_6) δ = 55.98 (C-7-O*C*H$_3$), 56.09 (C-5'-O*C*H$_3$), 56.14 (C-5-O*C*H$_3$), 60.09 (C-4'-O*C*H$_3$), 93.29 (C-8), 96.28 (C-6), 101.63 (C-6'), 107.27 (C-2'), 107.84 (C-3), 108.29 (C-4a), 126.09 (C-1'), 139.08 (C-4'), 150.53 (C-3'), 153.53 (C-5'), 159.15 (C-8a), 159.60 (C-2), 160.27 (C-5), 163.72 (C-7), 175.66 (C-4).

Table S1. Efficiencies of chalcone synthesis during the Claisen-Schmidt reaction (**1a**, **2a** and **3a**)

Ketone [g]	Aldehyde [g]	NaOH [g]	MeOH [mL]	Purified chalcone [g]	Chalcone number
1.1	1.3	2.5	50	1.4	**1a**
5.0	3.5	10.0	50	8.2	**2a**
5.0	5.0	10.0	100	9.1	**3a**

1a **2a** **3a**

Table S2. Flavone synthesis efficiencies by cyclization reactions

Chalcone [g]	Iodine [g]	DMSO [mL]	Purified flavone [g]	Flavone number
1.4	0.14	50	0.62	**1**
3.5	0.35	100	2.43	**2**
4.0	0.40	100	2.98	**3**

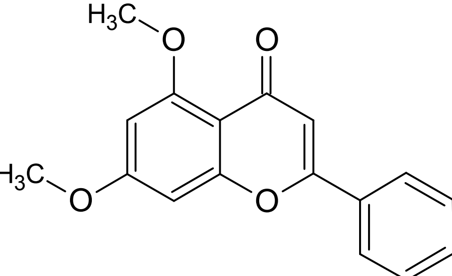
Table S3. Microbial transformation of 5,7-dimethoxyflavone (**1**), HPLC conversion.

Strain No.	**Substance**	**Retention Time [min]**	**Conversion after 1, 3, 7 and 10 Days [%]**			
			1	**3**	**7**	**10**
Beauveria bassiana KCh J1.5	(**4**)	11.4	1.9	14.6	79.5	79.3
	(**5**)	12.4	0.7	3.8	2.5	2.7
	(**6**)	12.9	10.7	52.3	17.0	16.7
	(**1**)	16.1	86.8	29.3	1.0	1.3
Beauveria bassiana KCh J2.1	(**4**)	11.4	0.2	1.0	1.7	1.7
	(**6**)	12.9	0.6	1.2	1.5	1.5
	(**1**)	16.1	99.2	97.8	96.8	96.8
Isaria farinosa KCh KW 1.1.	(**4**)	11.4	0.3	0.6	1.2	1.6
	(**6**)	12.9	0.9	0.8	1.0	1.4
	(**1**)	16.1	98.8	98.6	97.8	97.0
Beauveria bassiana KCh J1	(**4**)	11.4	0.8	5.1	9.8	9.9
	(**5**)	12.4	0.0	0.6	1.7	2.0
	(**6**)	12.9	2.5	12.4	21.4	22.4
	(**1**)	16.1	96.8	81.9	67.1	65.6
Isaria fumosorosea KCh J2	(**4**)	11.4	4.0	23.7	54.8	67.8
	(**5**)	12.4	0.5	2.1	0.4	0.3
	(**6**)	12.9	16.6	71.0	44.4	31.5
	(**1**)	16.1	78.9	3.3	0.3	0.4
Beauveria caledonica KCh J34	(**4**)	11.4	0.0	1.7	6.4	6.9
	(**5**)	12.4	0.0	1.2	1.7	1.8
	(**6**)	12.9	0.1	6.0	18.8	17.9
	(**1**)	16.1	99.9	91.2	73.1	73.5
Beauveria bassiana KCH J3.2	(**4**)	11.4	0.4	1.4	4.5	7.0
	(**5**)	12.4	0.1	0.9	3.0	3.9
	(**6**)	12.9	2.9	10.4	30.7	42.8
	(**1**)	16.1	96.6	87.3	61.8	46.4
Beauveria caledonica KCh J3.3	(**4**)	11.4	0.0	1.1	4.0	5.5
	(**5**)	12.4	0.0	0.7	0.7	0.9
	(**6**)	12.9	0.0	3.6	10.2	13.7
	(**1**)	16.1	100.0	94.7	85.2	80.0
Beauveria bassiana KCh BBT	(**4**)	11.4	0.8	3.3	7.5	9.7
	(**5**)	12.4	1.5	2.4	3.9	4.1
	(**6**)	12.9	3.2	12.4	30.9	33.5
	(**1**)	16.1	94.5	81.9	57.7	52.8

Table S4. Microbial transformation of 5,7,4′-trimethoxyflavone (**2**), HPLC conversion.

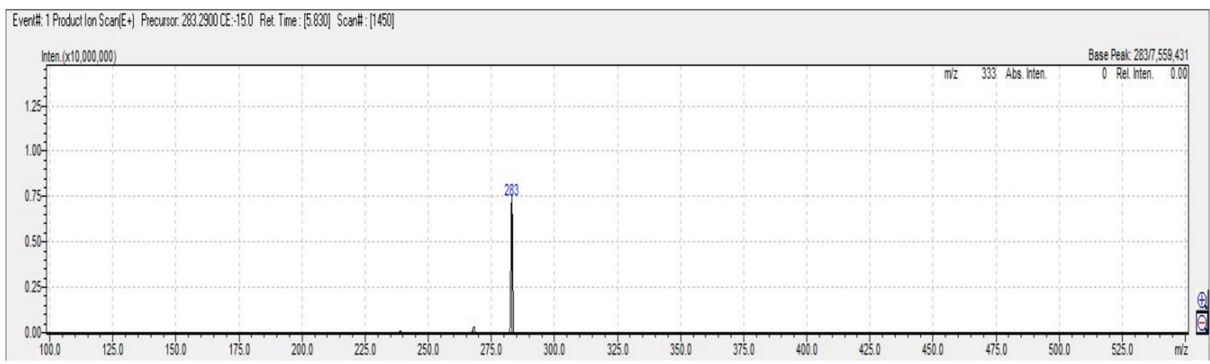
Strain No.	Substance	Retention Time [min]	Conversion after 1, 3, 7 and 10 Days [%]			
			1	3	7	10
Beauveria bassiana KCh J1.5	(**4**)	11.4	0.7	3.9	0.4	0.3
	(**5**)	12.4	31.7	43.3	64.5	83.9
	(**2**)	16.2	67.5	52.8	35.2	15.8
Beauveria bassiana KCh J2.1	(**4**)	11.4	0.2	2.5	0.3	10.1
	(**5**)	12.4	10.7	31.6	62.6	71.1
	(**2**)	16.2	89.1	66.0	37.1	18.8
Isaria farinosa KCh KW 1.1.	(**4**)	11.4	0.0	0.0	0.4	10.9
	(**5**)	12.4	2.3	5.3	17.2	75.4
	(**2**)	16.2	97.7	94.7	82.5	13.7
Beauveria bassiana KCh J1	(**4**)	11.4	0.0	1.1	2.4	2.2
	(**5**)	12.4	2.4	13.9	46.1	60.2
	(**2**)	16.2	97.6	84.9	51.5	37.7
Isaria fumosorosea KCh J2	(**4**)	11.4	0.0	0.3	0.4	2.2
	(**5**)	12.4	3.0	5.6	8.6	60.2
	(**2**)	16.2	97.0	94.1	91.0	37.7
Beauveria caledonica KCh J34	(**4**)	11.4	0.0	0.0	0.0	1.2
	(**5**)	12.4	0.5	2.9	7.7	13.3
	(**2**)	16.2	99.5	97.1	92.3	85.6
Beauveria bassiana KCH J3.2	(**4**)	11.4	0.0	0.0	1.9	3.1
	(**5**)	12.4	2.4	8.7	34.8	47.3
	(**2**)	16.2	97.6	91.3	63.4	49.6
Beauveria caledonica KCh J3.3	(**4**)	11.4	0.0	0.0	0.0	1.5
	(**5**)	12.4	0.6	4.1	6.8	7.9
	(**2**)	16.2	99.4	95.9	93.2	90.6
Beauveria bassiana KCh BBT	(**4**)	11.4	0.0	1.2	3.6	10.7
	(**5**)	12.4	7.7	34.6	60.2	58.6
	(**2**)	16.2	92.3	64.2	36.2	30.8

Fig.S1. MS analysis flavone 5,7-dimethoxyflavone (**1**)

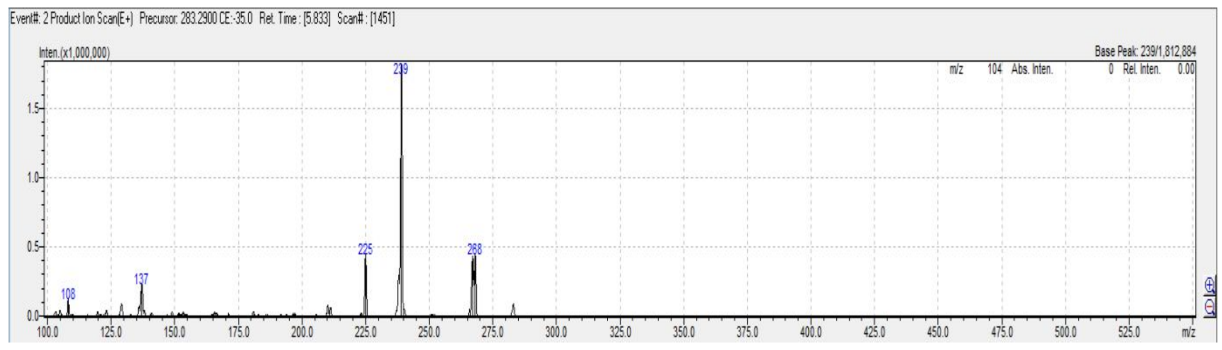


Molecular Formula	= $C_{17}H_{14}O_4$
Formula Weight	= 282.29066
Precursor:	= 283.2900

CE (collision energy): -15.0



CE: -35.0



CE: -45.0

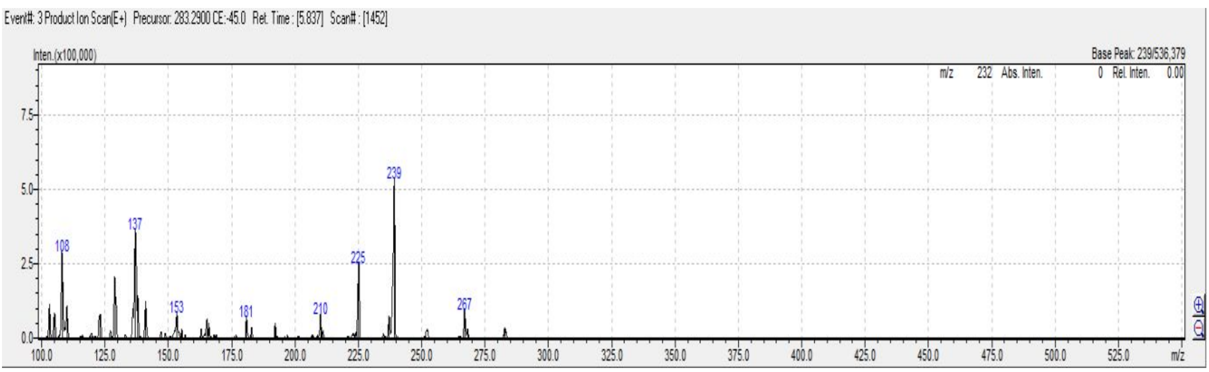


Fig.S2. ^{1}H NMR spectrum of 5,7-dimethoxyflavone (**1**) ($CDCl_3$, 600 MHz)

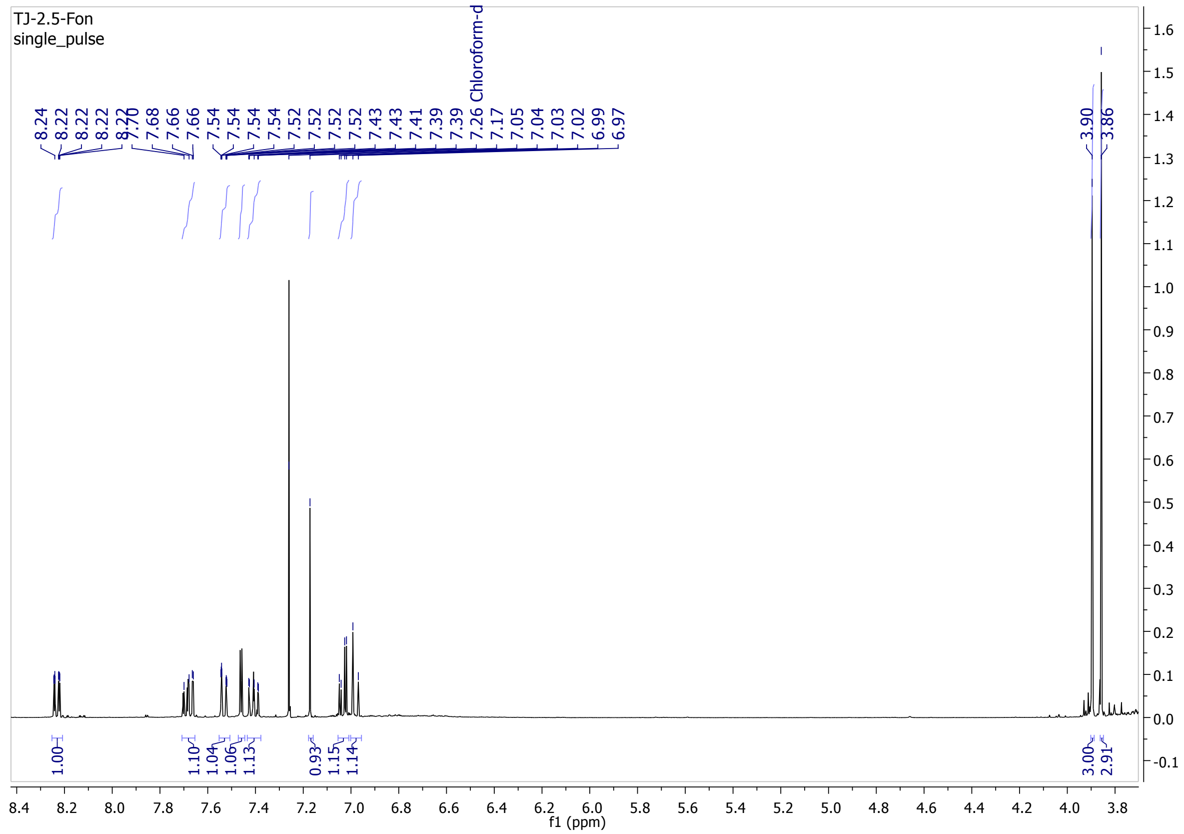


Fig.S3. Flavone part of the ^{1}H NMR spectrum 5,7-dimethoxyflavone (**1**) ($CDCl_3$, 600 MHz)

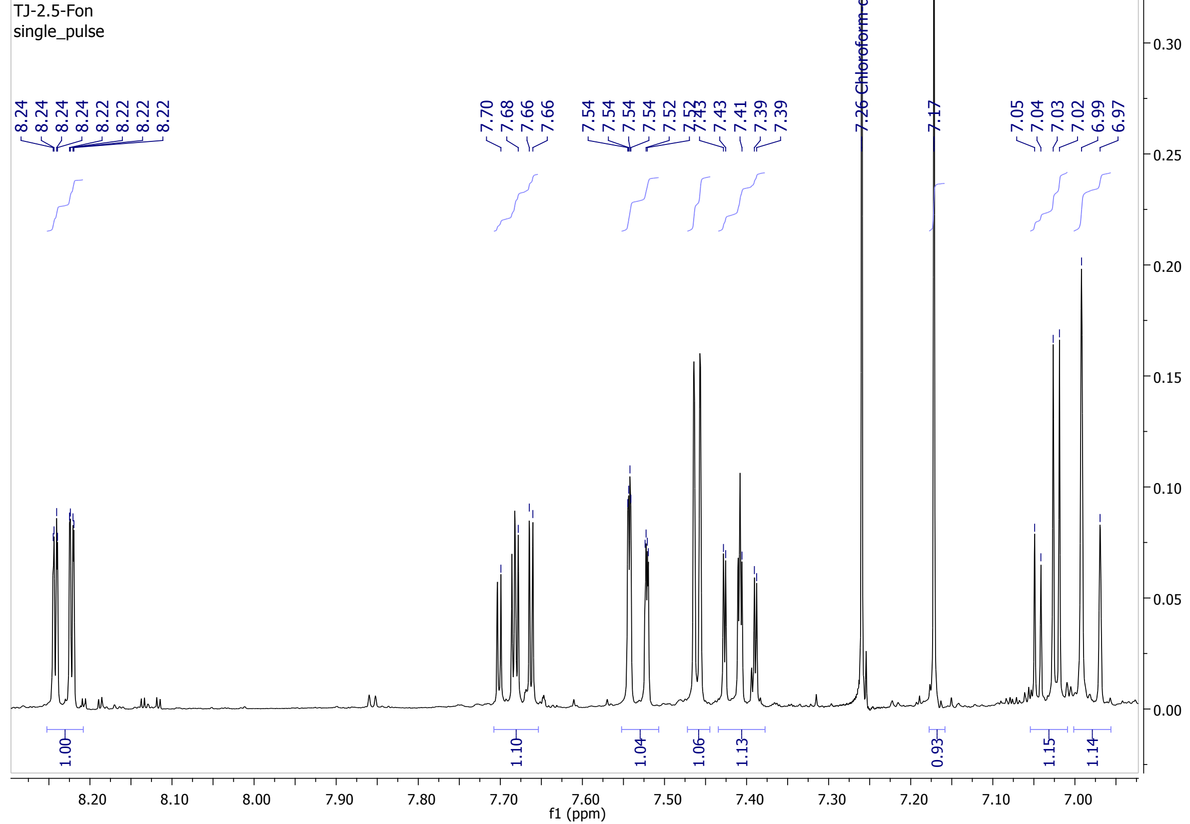


Fig.S4. ^{13}C NMR spectrum of 5,7-dimethoxyflavone (**1**) ($CDCl_3$, 151 MHz)

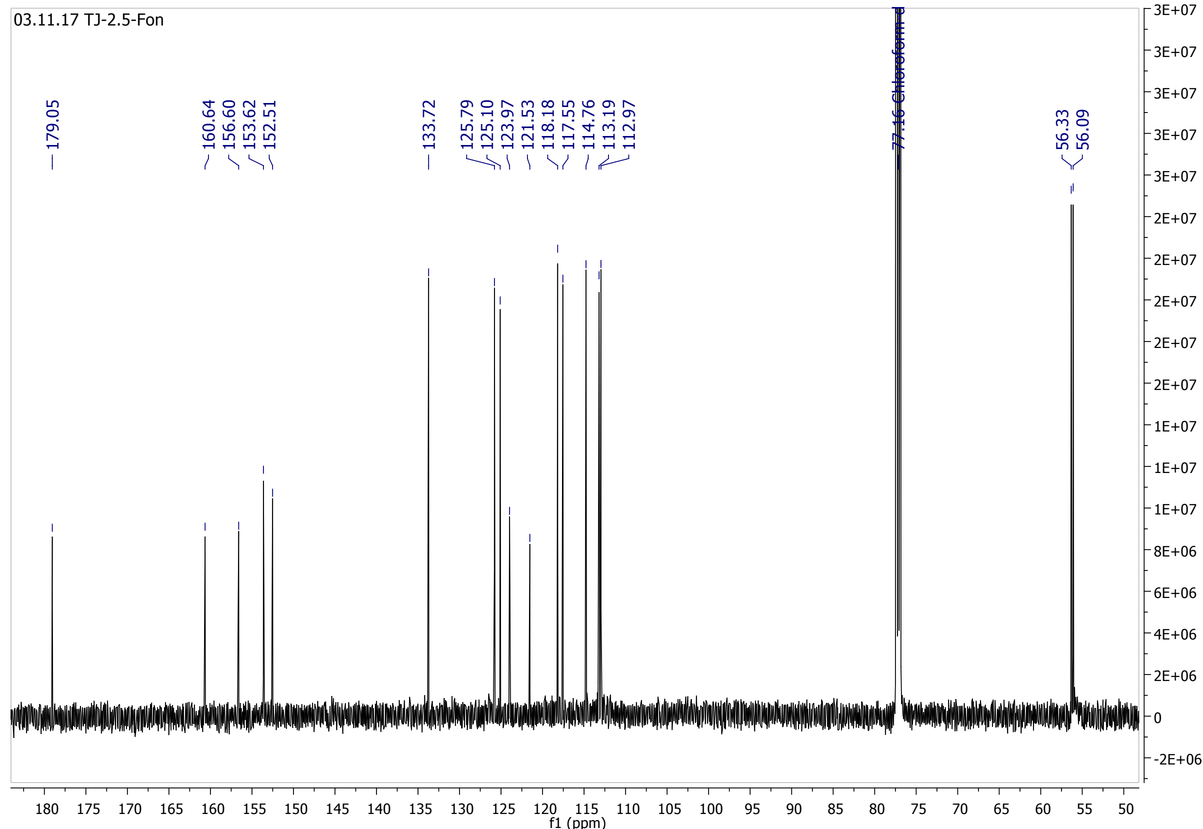


Fig.S5. COSY spectrum of 5,7-dimethoxyflavone (**1**) ($CDCl_3$, 151 MHz)

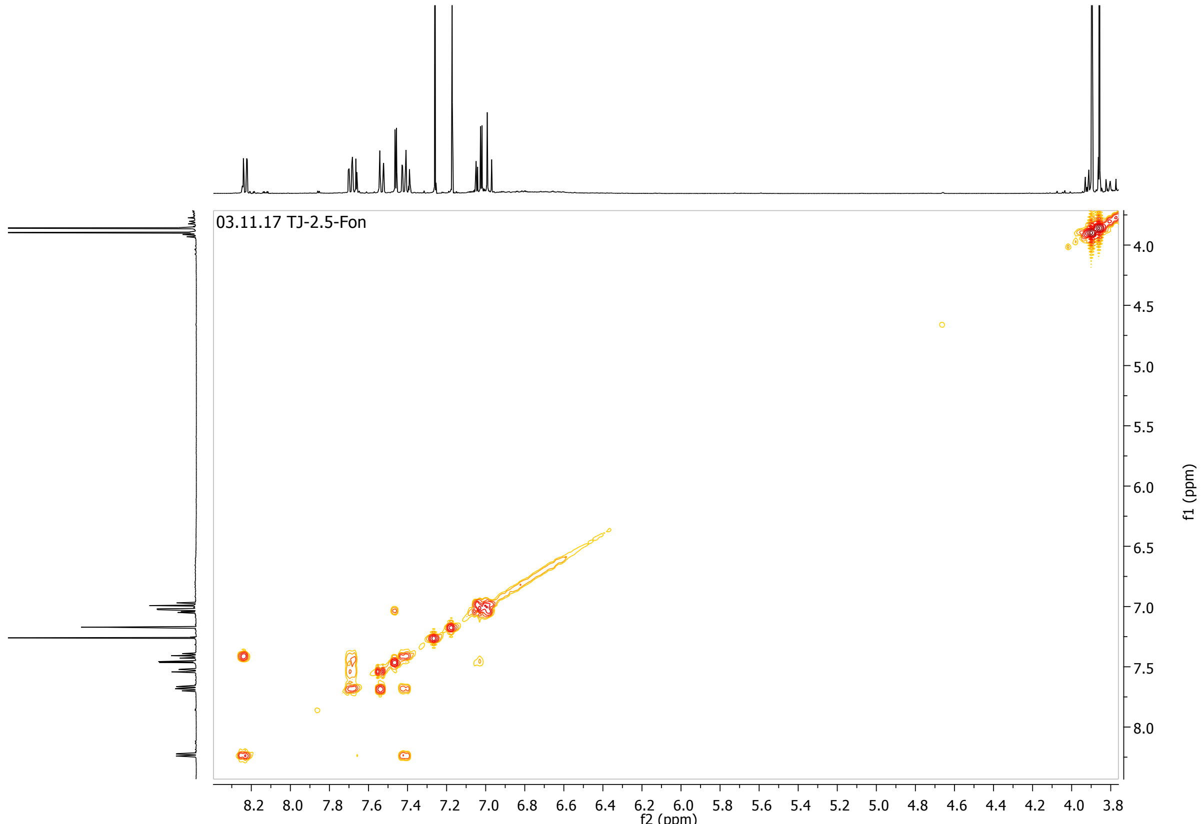


Fig.S6. HMQC spectrum of 5,7-dimethoxyflavone (**1**) ($CDCl_3$, 151 MHz)

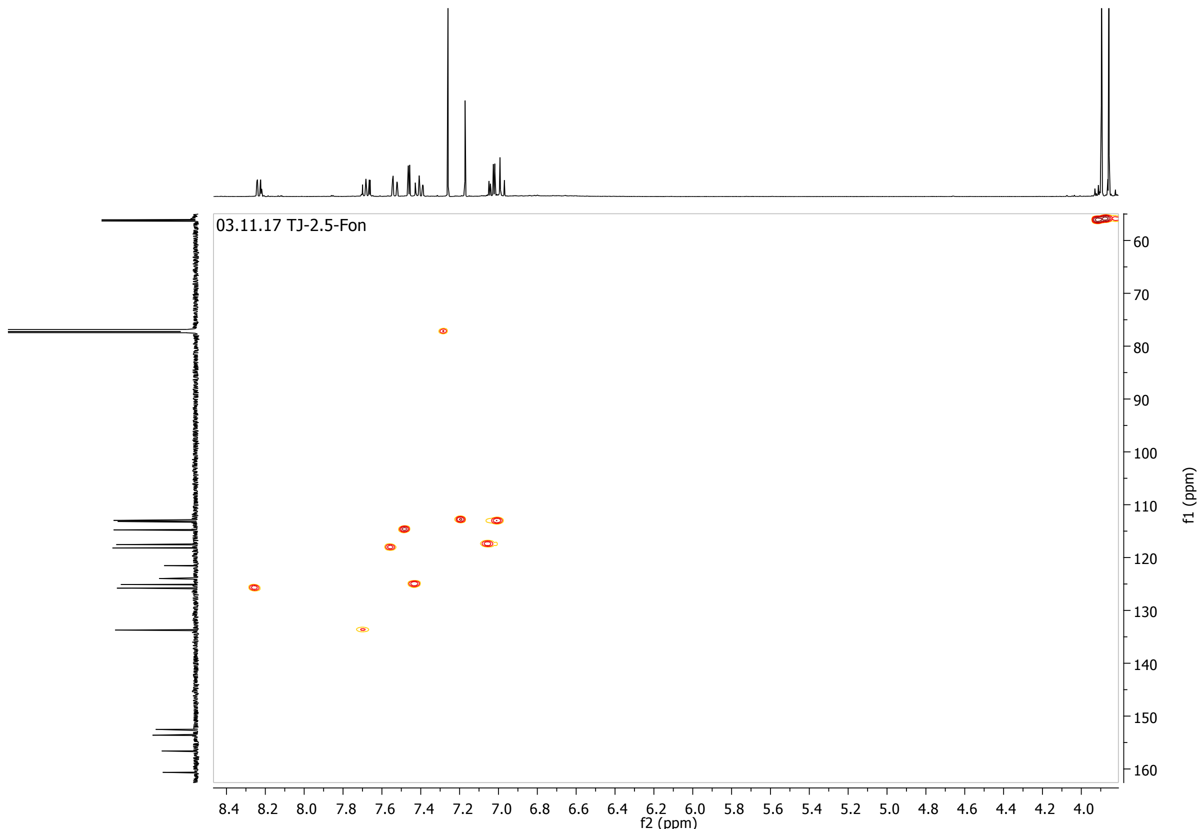
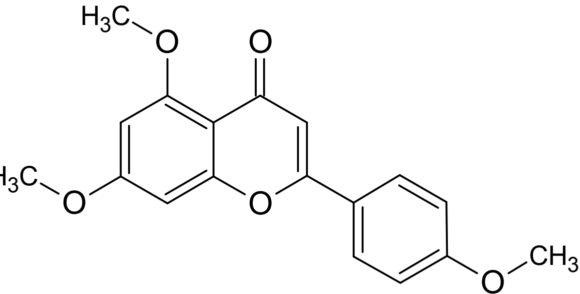
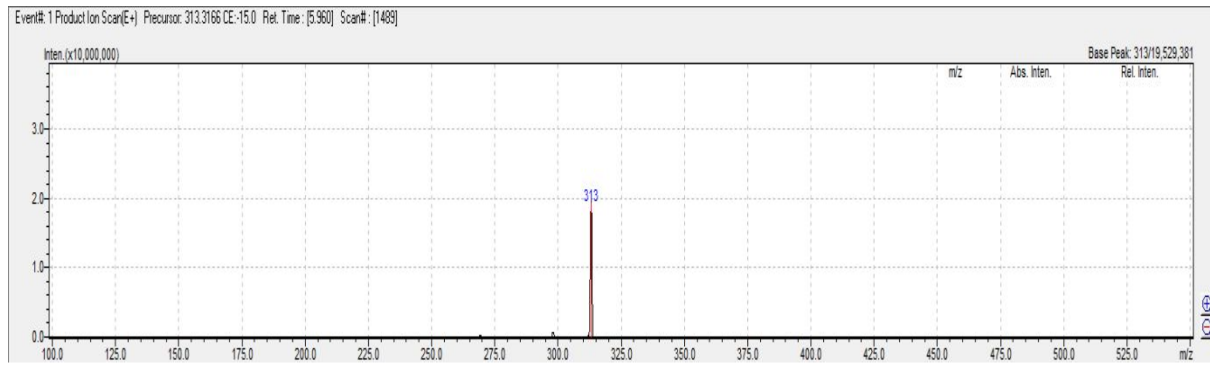


Fig.S7. MS analysis 5,7,4'-trimethoxyflavone (**2**)

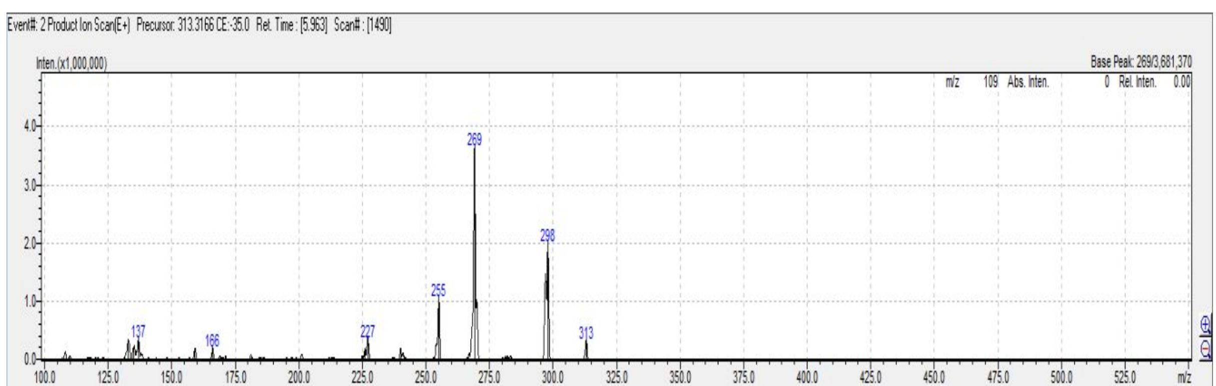


Molecular Formula = $C_{18}H_{16}O_5$
Formula Weight = 312.31664
Precursor = 313.3166

CE: -15.0



CE: -35.0



CE: -45.0

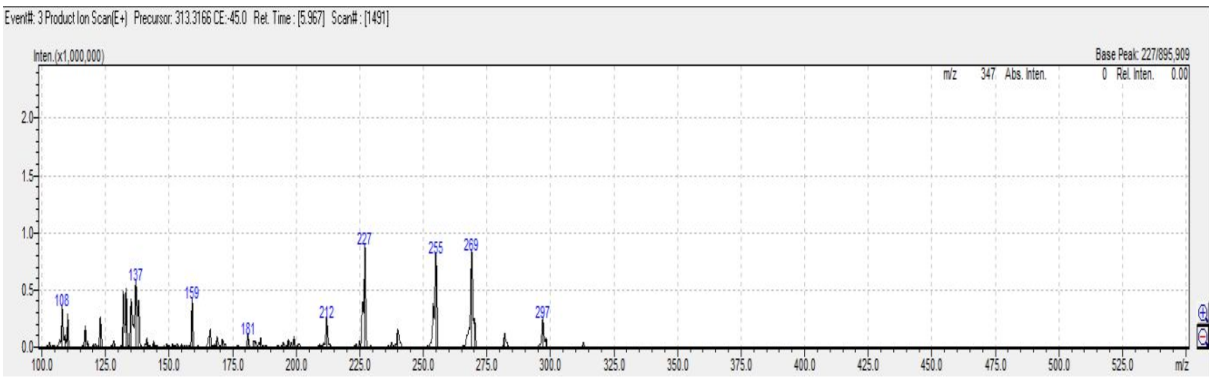


Fig.S8. ^{1}H NMR spectrum of 5,7,4'-trimethoxyflavone (**2**) ($CDCl_3$, 600 MHz)

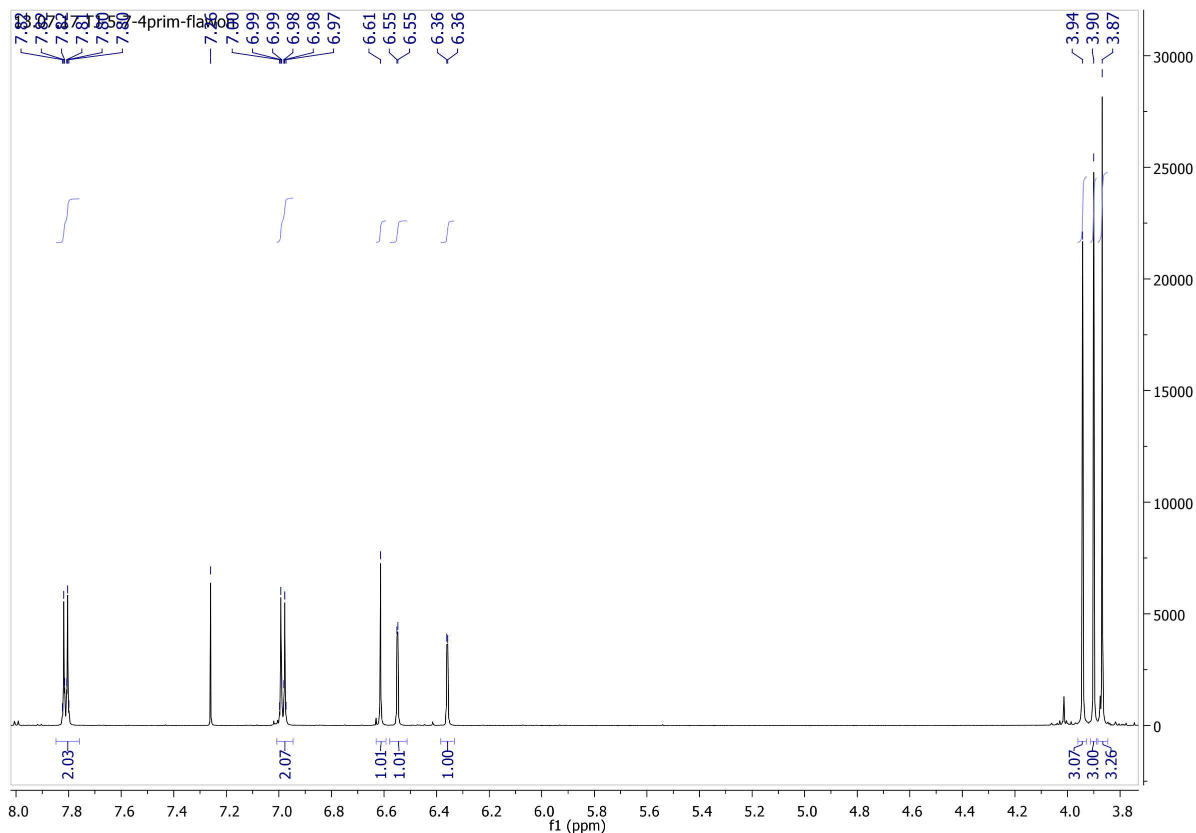


Fig.S9. Flavone part of the ^{1}H NMR spectrum 5,7,4'-trimethoxyflavone (**2**) ($CDCl_3$, 600 MHz)

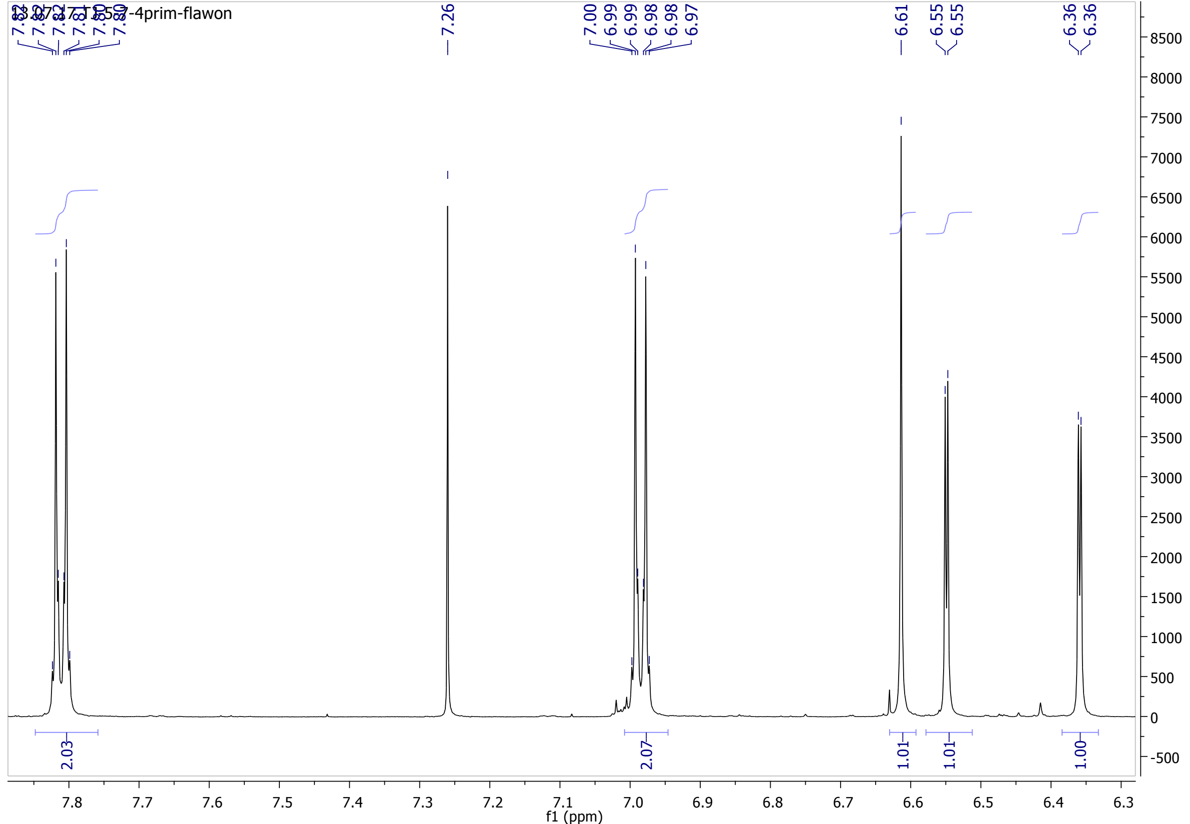


Fig.S10. ^{13}C NMR spectrum of 5,7,4'-trimethoxyflavone (**2**) ($CDCl_3$, 151 MHz)

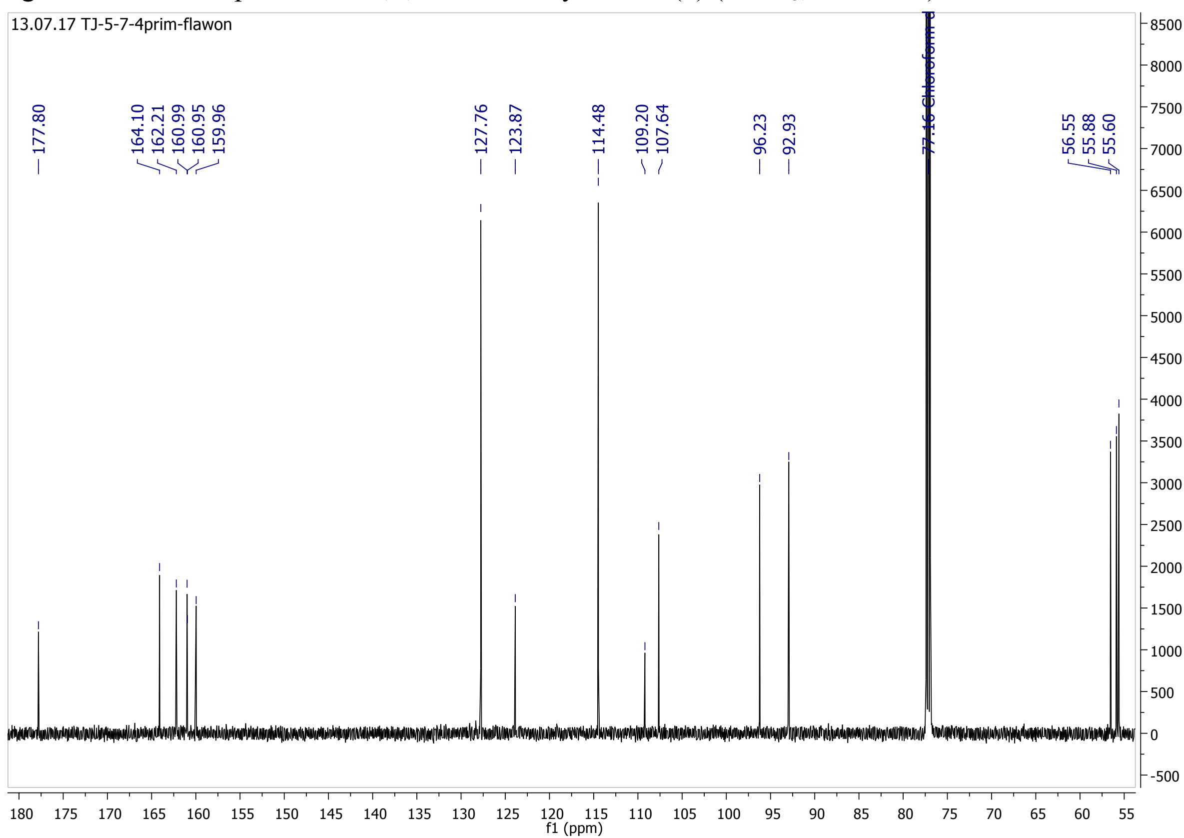


Fig.S11. COSY spectrum of 5,7,4'-trimethoxyflavone (**2**) ($CDCl_3$, 151 MHz)

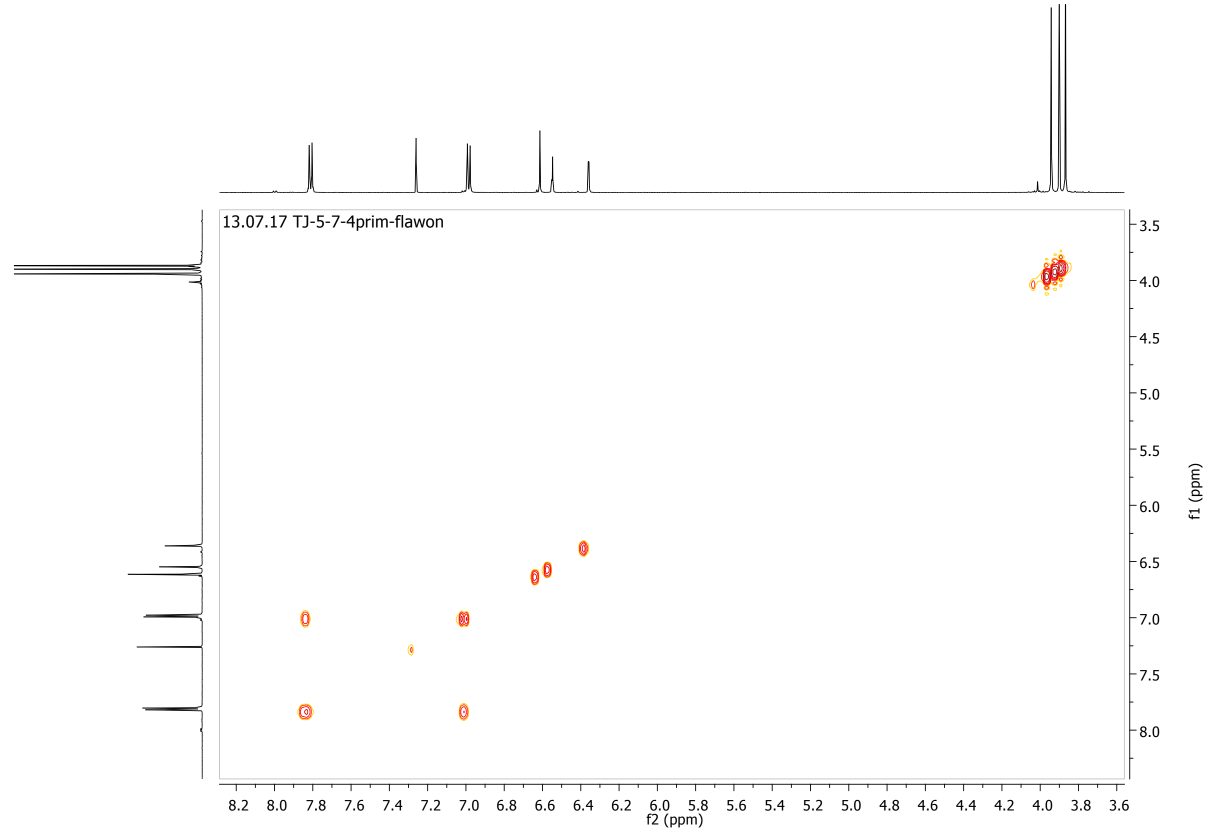


Fig.S12. HMQC spectrum of 5,7,4’-trimethoxyflavone (**2**) ($CDCl_3$, 151 MHz)

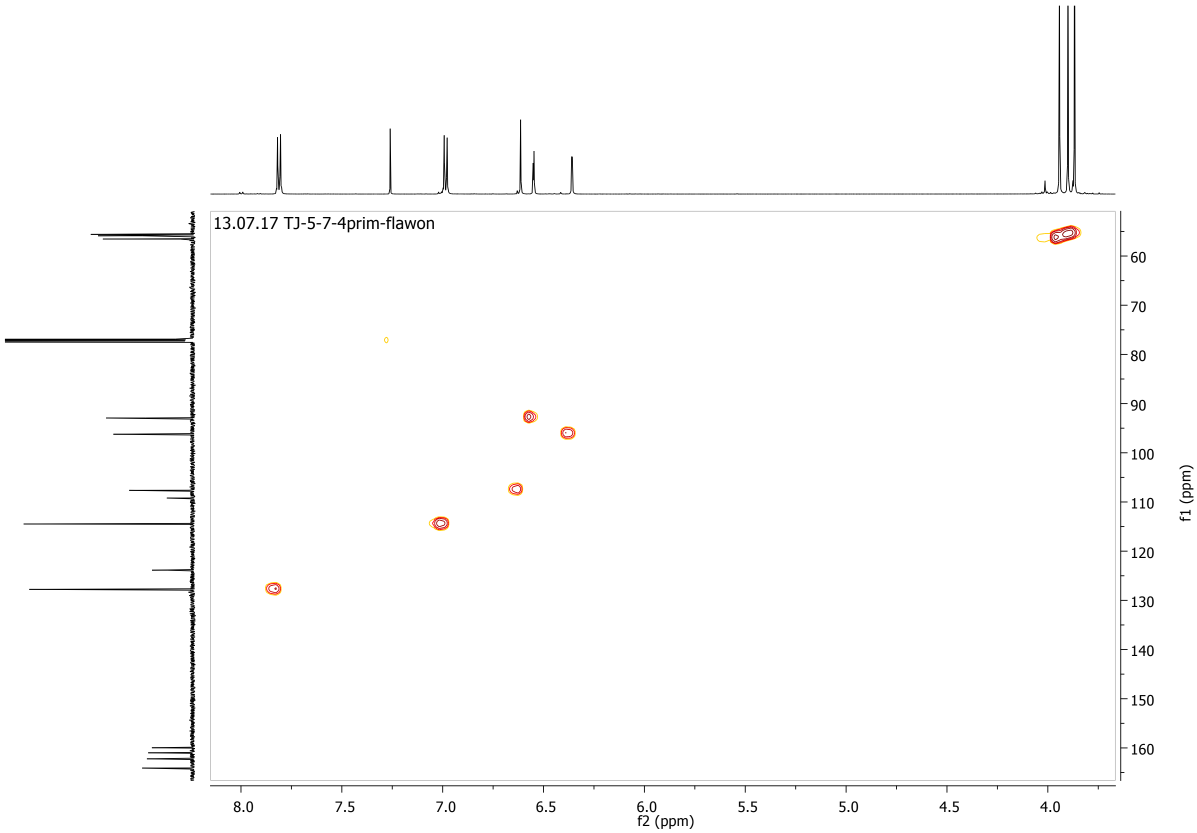


Fig.S13. HMBC spectrum of 5,7,4’-trimethoxyflavone (**2**) ($CDCl_3$, 151 MHz)

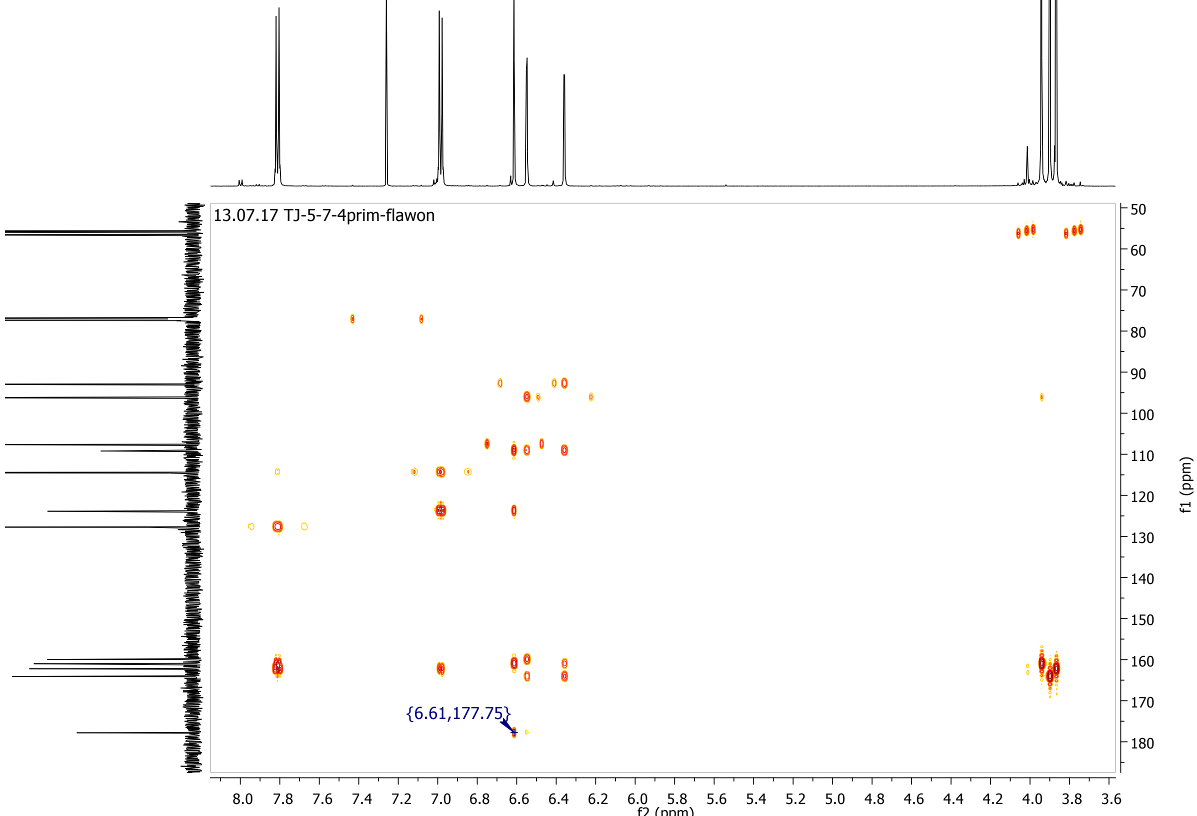
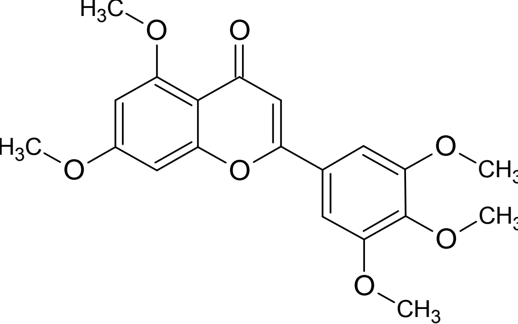
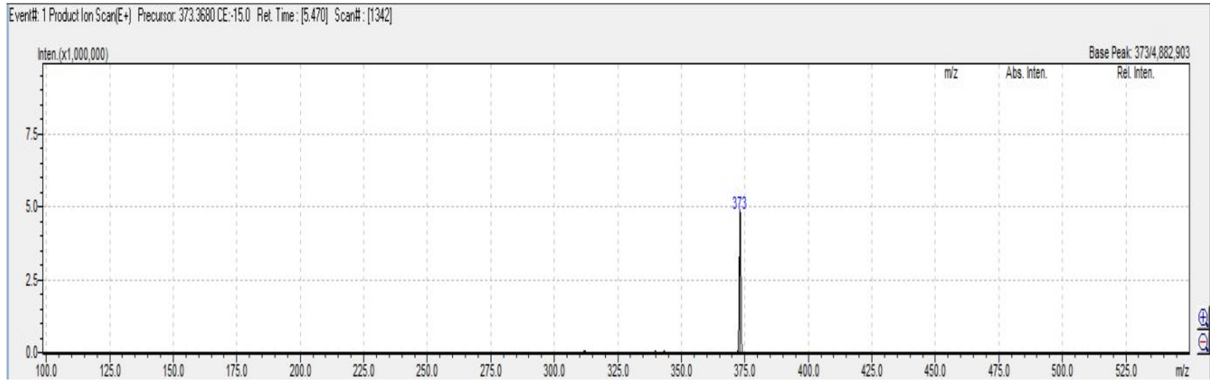


Fig.S14. MS analysis 5,7,3',4',5'-pentamethoxyflavone (**3**)

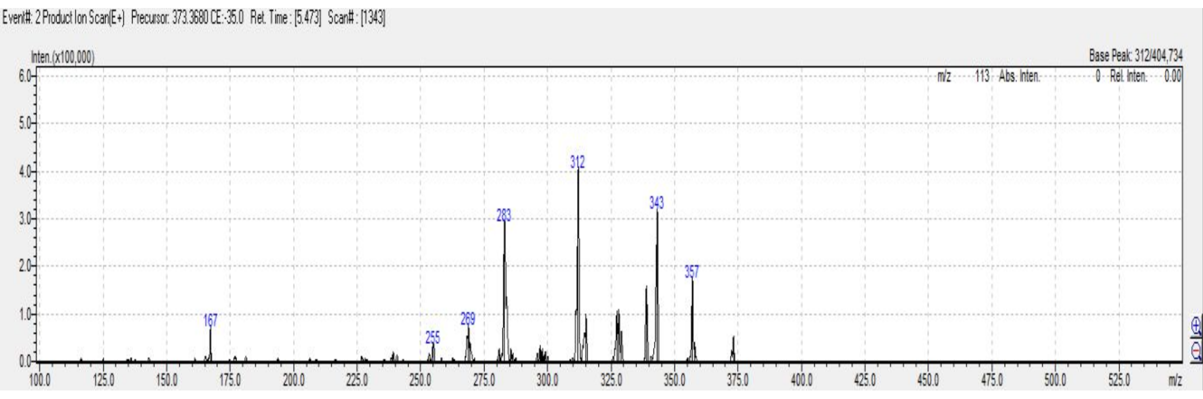


Molecular Formula = $C_{20}H_{20}O_7$
Formula Weight = 372.3686
Precursor = 373.3680

CE: -15.0



CE: -35.0



CE: -45.0

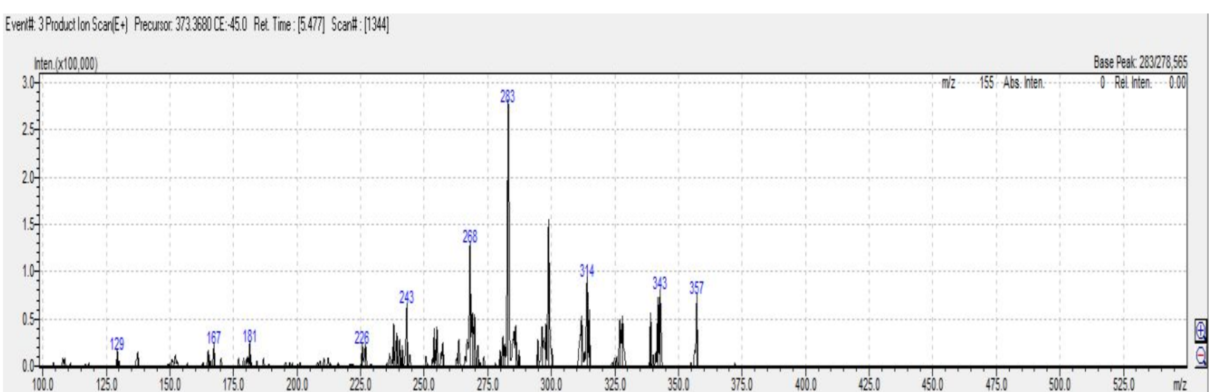


Fig.S15. ^{1}H NMR spectrum of 5,7,3',4',5'-pentamethoxyflavone (**3**) ($CDCl_3$, 600 MHz)

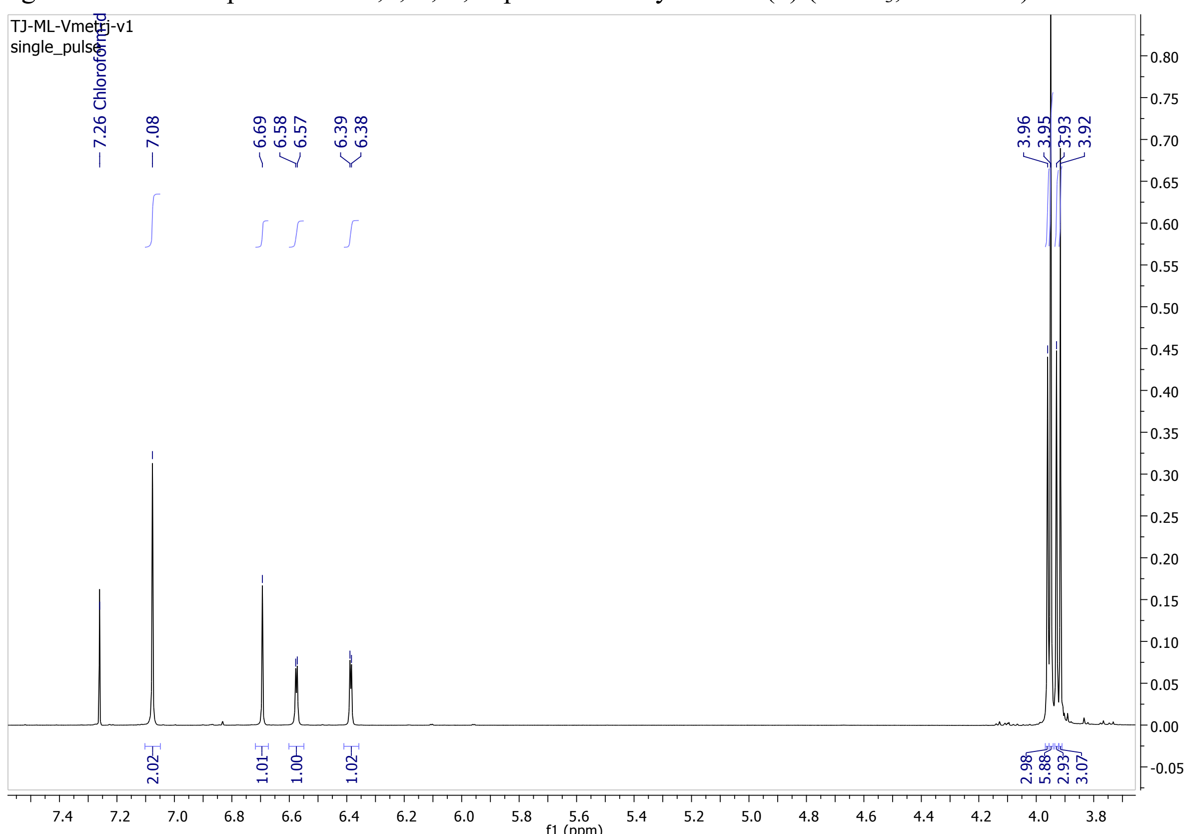


Fig.S16. Flavone part of the ^{1}H NMR spectrum 5,7,3',4',5'-pentamethoxyflavone (**3**) ($CDCl_3$, 600 MHz)

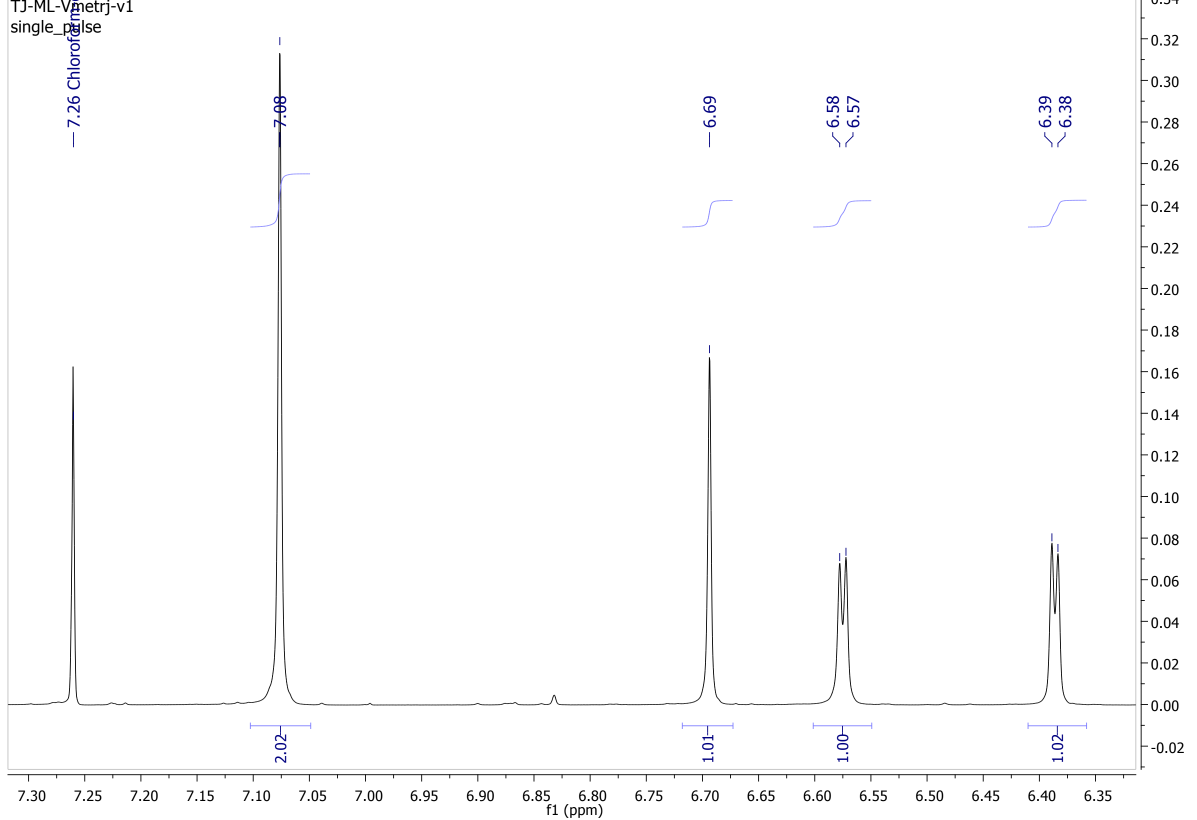


Fig.S17. ^{13}C NMR spectrum of 5,7,3',4',5'-pentamethoxyflavone (**3**) ($CDCl_3$, 600 MHz)

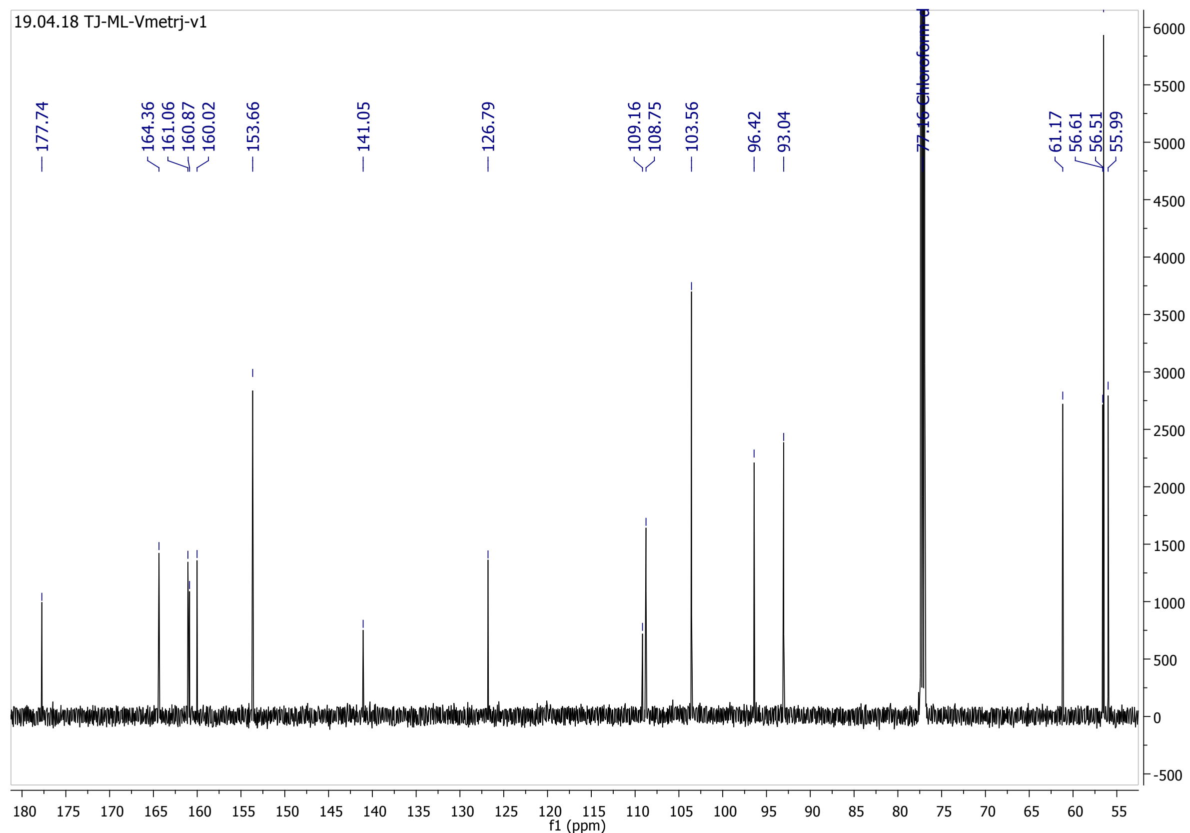


Fig.S18. COSY spectrum of 5,7,3',4',5'-pentamethoxyflavone (**3**) ($CDCl_3$, 600 MHz)

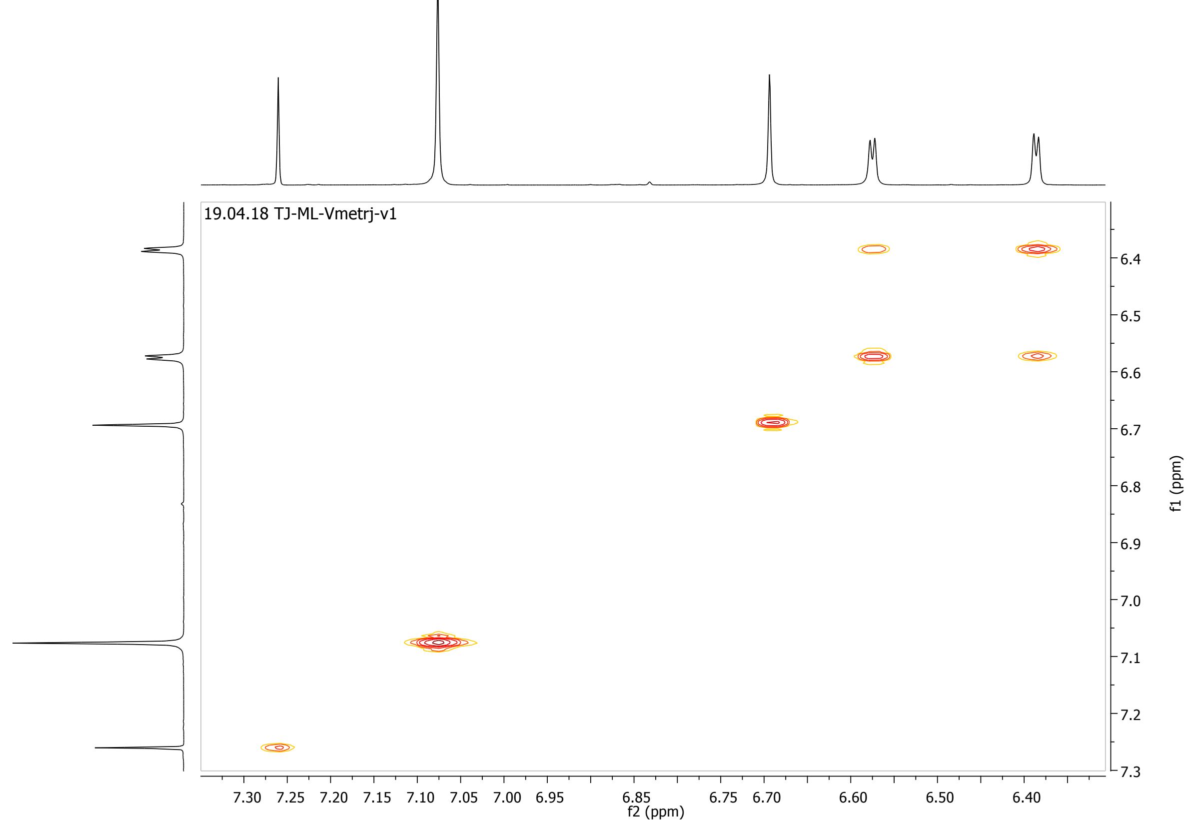


Fig.S19. HMQC spectrum of 5,7,3',4',5'-pentamethoxyflavone (**3**) ($CDCl_3$, 600 MHz)

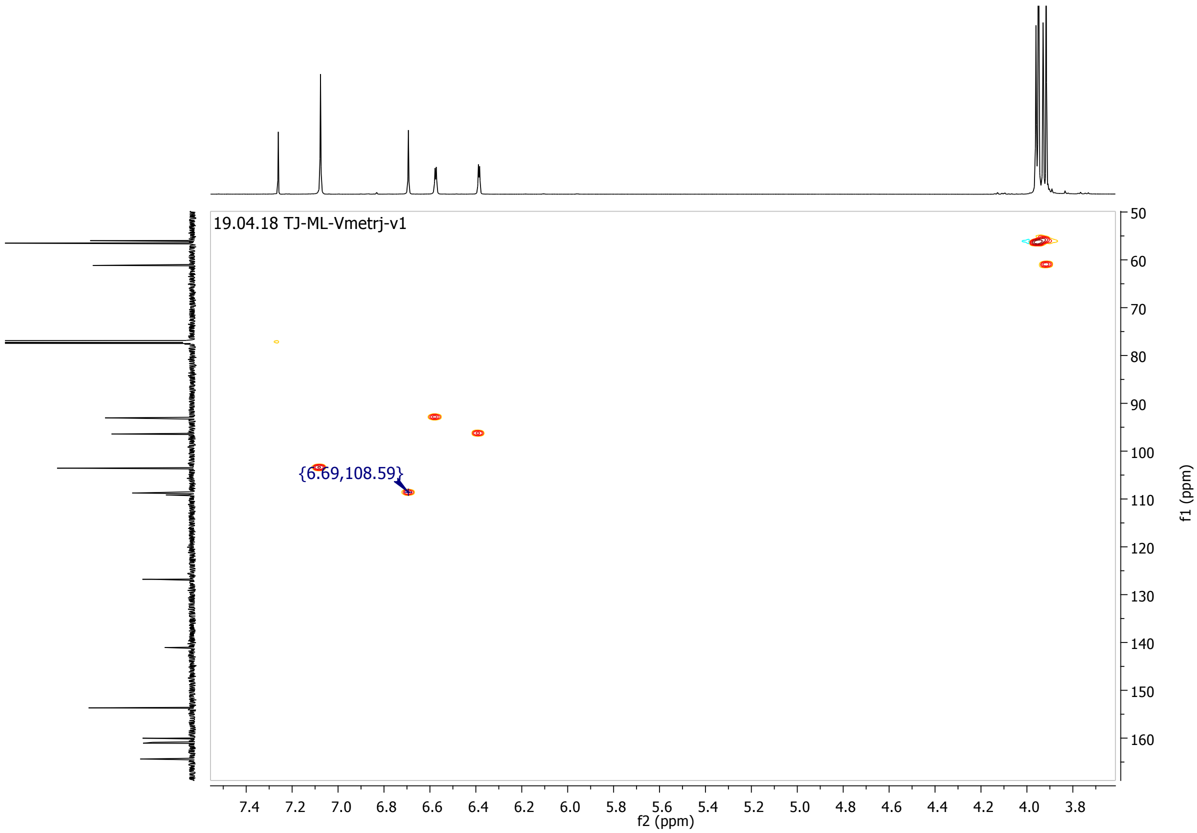


Fig.S20. HMBC spectrum of 5,7,3',4',5'-pentamethoxyflavone (**3**) ($CDCl_3$, 600 MHz)

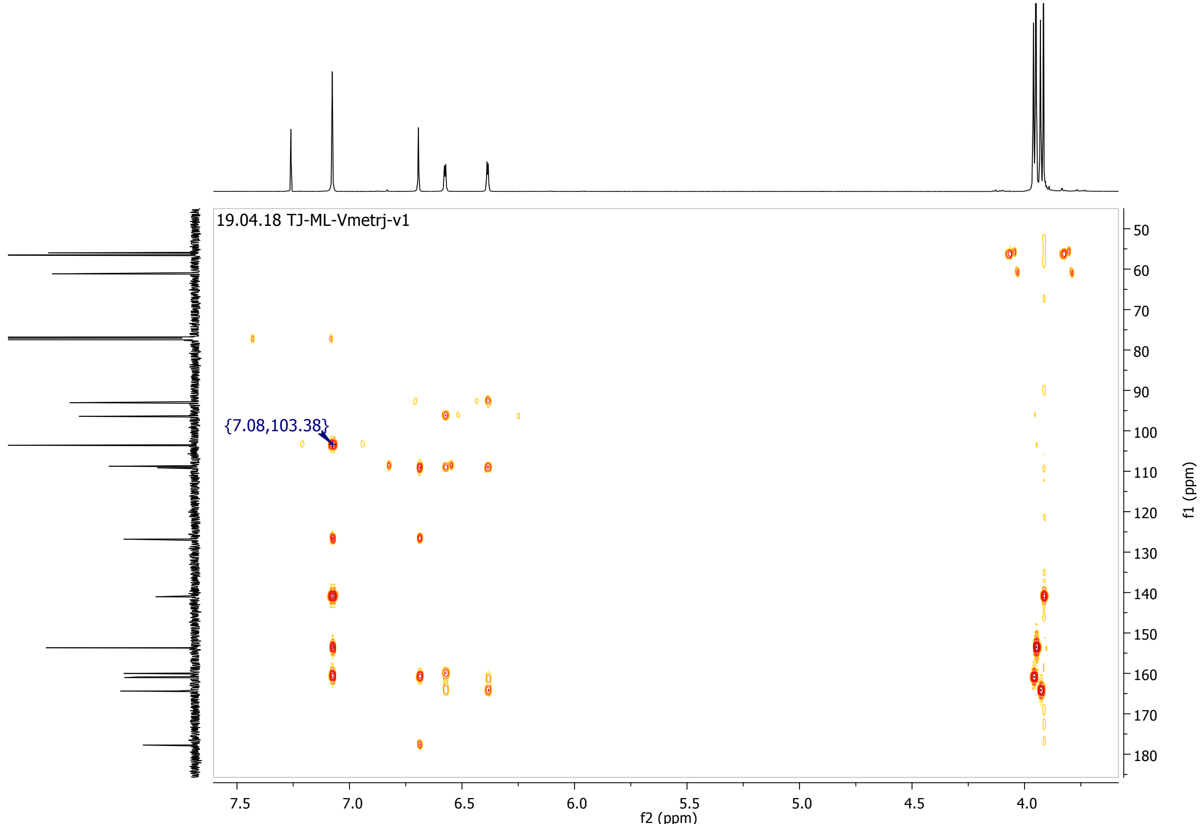
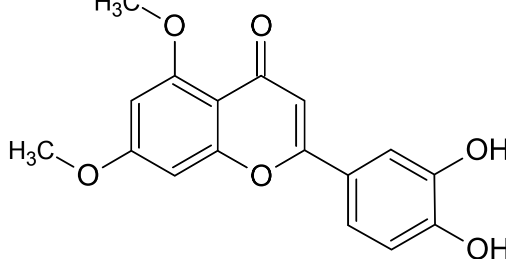
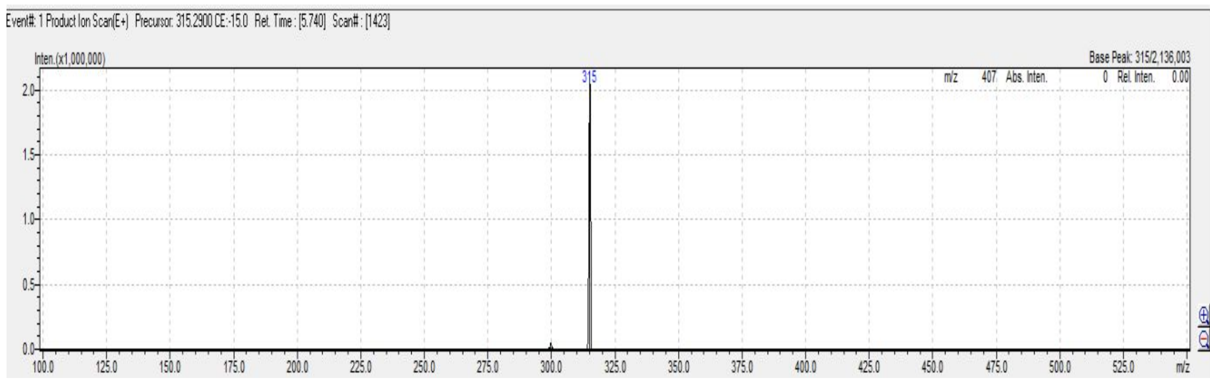


Fig.S21. MS analysis 3’,4’-dihydroxy-5,7-dimethoxyflavone (**4**)

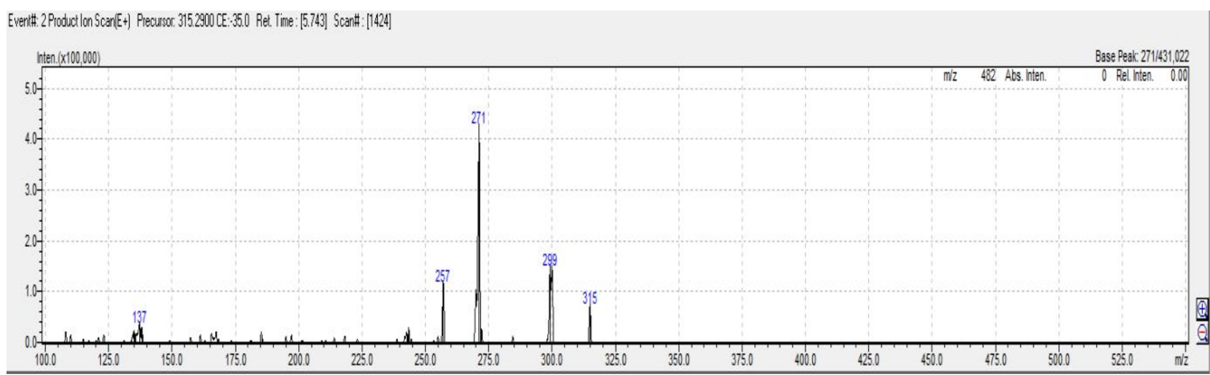
Molecular Formula: $C_{17}H_{14}O_6$
Formula Weight: 314.2894
Precursor: 315.2900



CE: -15.0



CE: -35.0



CE: -45.0

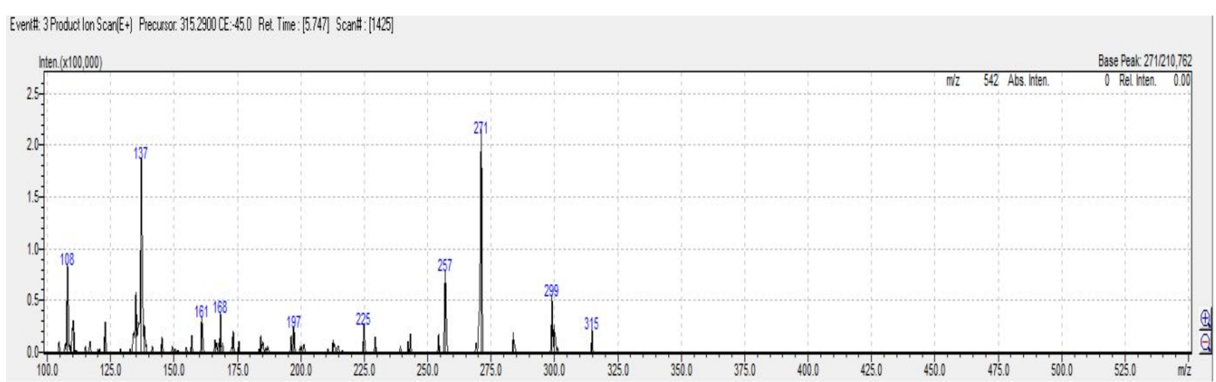


Fig.S22. ^{1}H NMR spectrum of 3',4'-dihydroxy-5,7-dimethoxyflavone (**4**) (DMSO-d_6, 600 MHz)

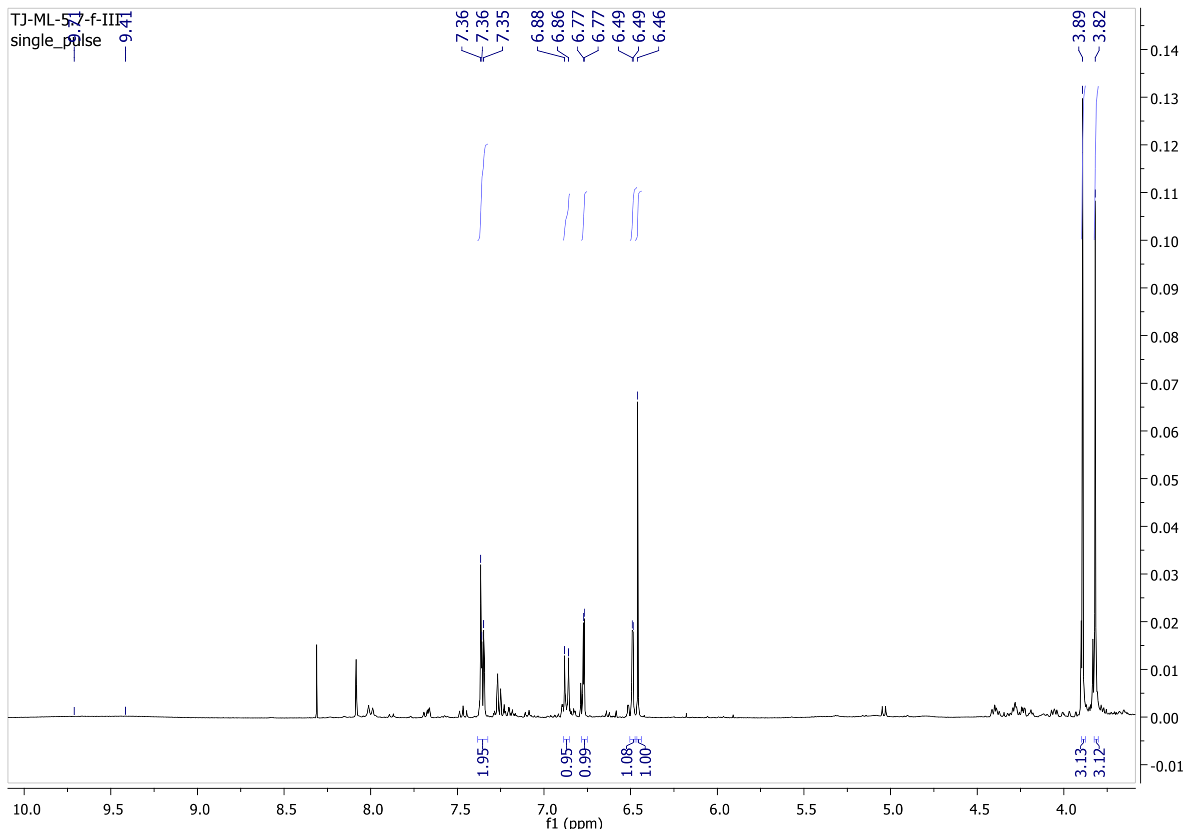


Fig.S23. Flavone part of the ^{1}H NMR spectrum 3',4'-dihydroxy-5,7-dimethoxyflavone (**4**) (DMSO-d_6, 600 MHz)

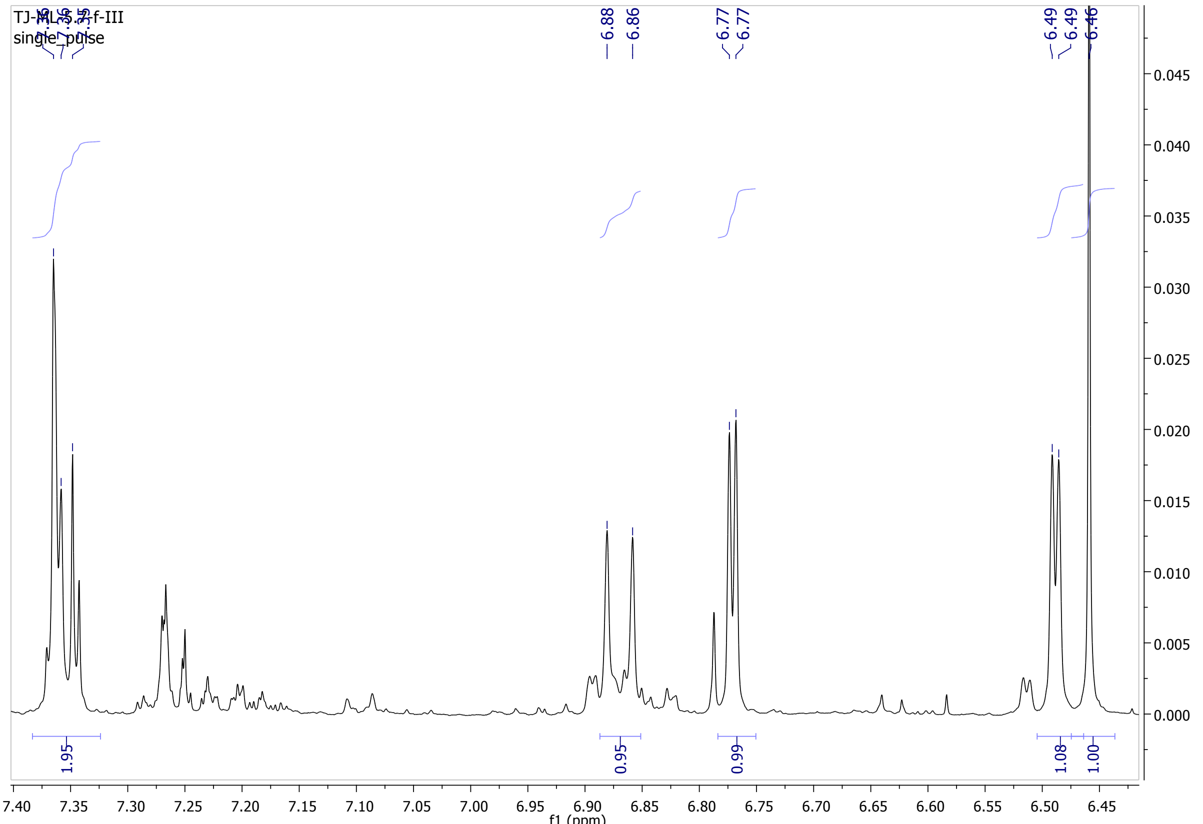


Fig.S24. ^{13}C NMR spectrum of 3',4'-dihydroxy-5,7-dimethoxyflavone (**4**) (DMSO-d_6, 151 MHz)

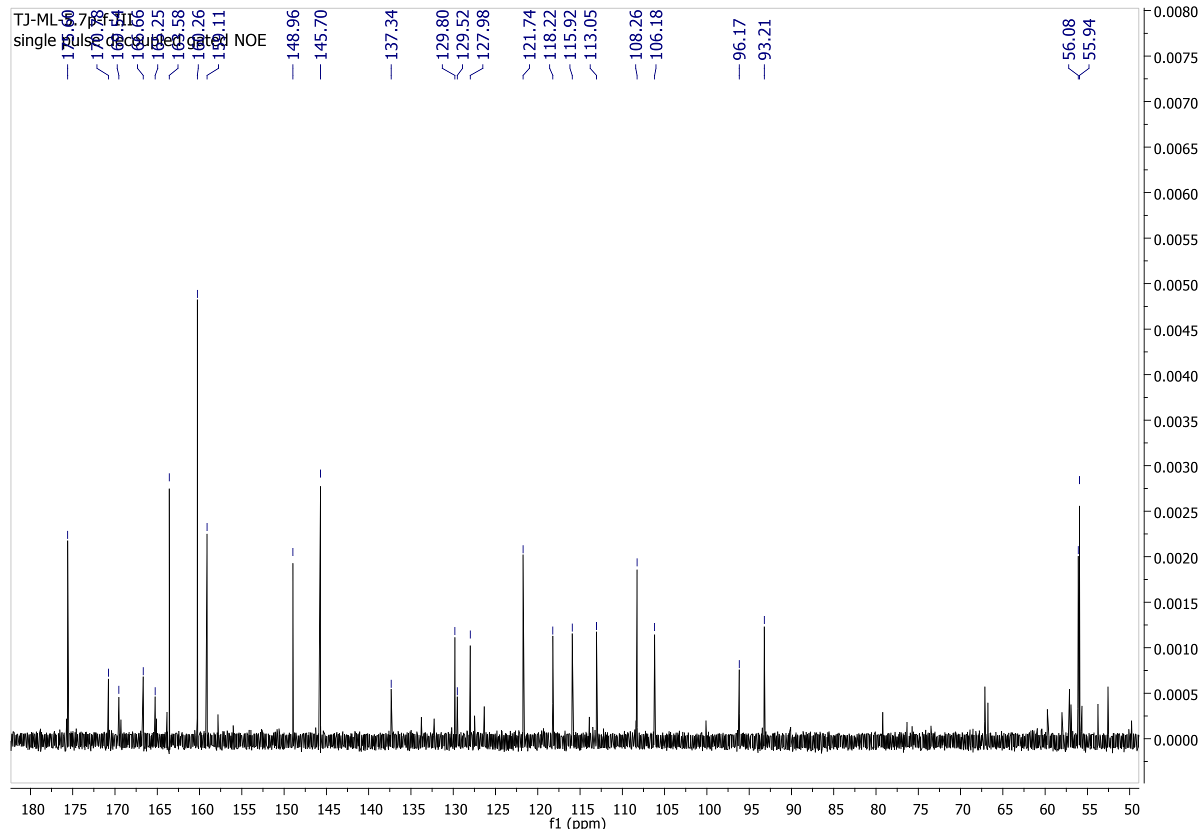


Fig.S25. COSY spectrum of 3',4'-dihydroxy-5,7-dimethoxyflavone (**4**) (DMSO-d_6, 151 MHz)

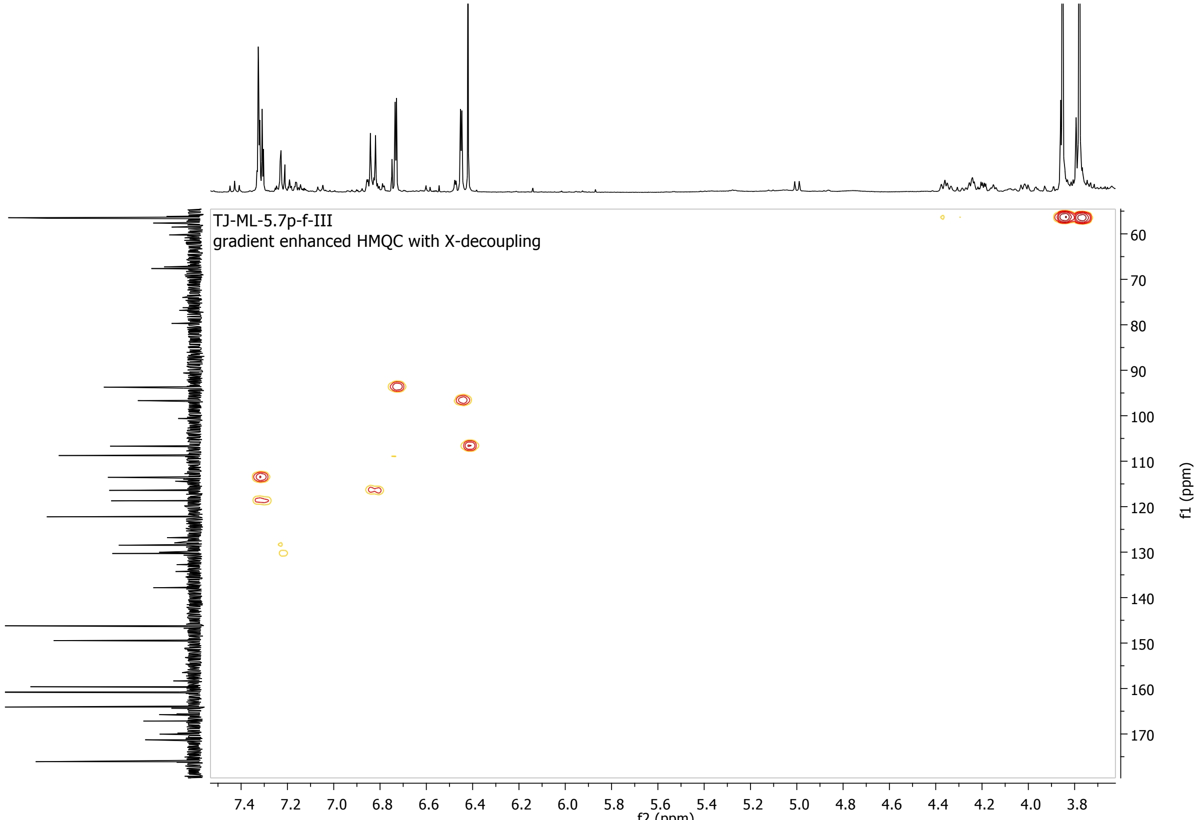


Fig.S26. HMBC spectrum of 3',4'-dihydroxy-5,7-dimethoxyflavone (**4**) (DMSO-d_6, 151 MHz)

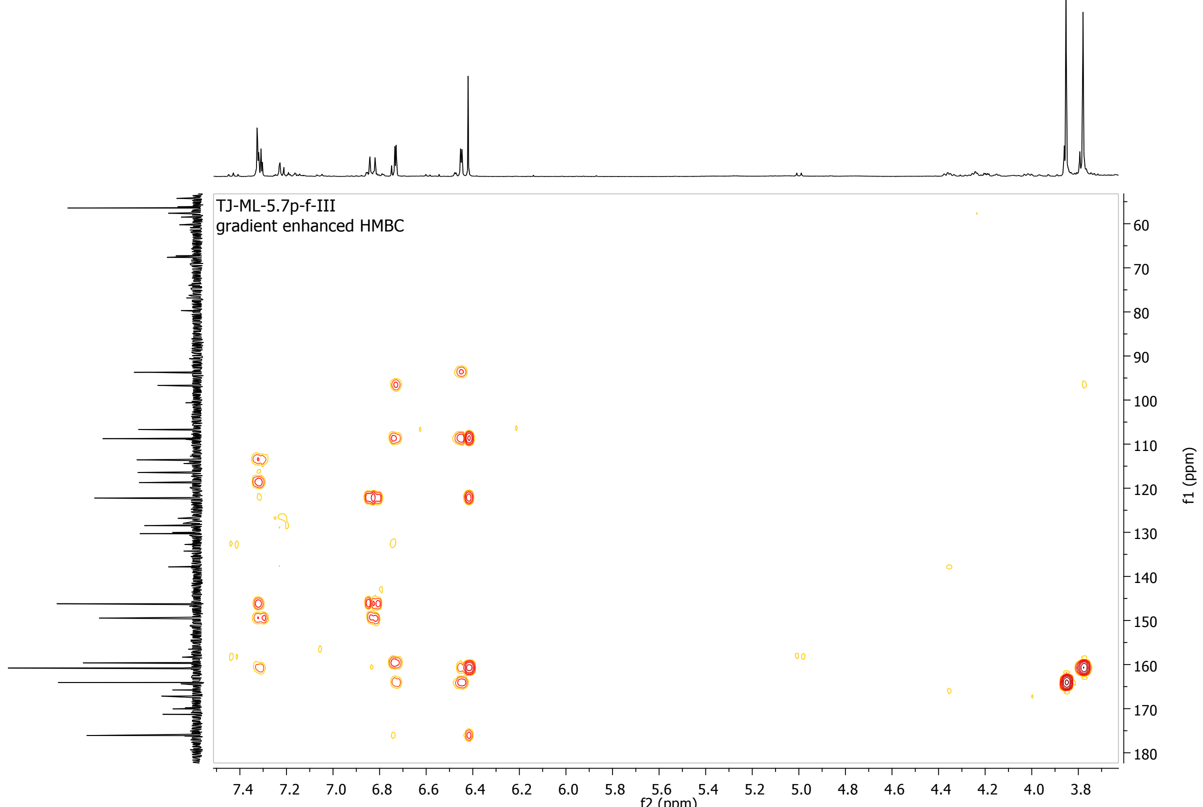
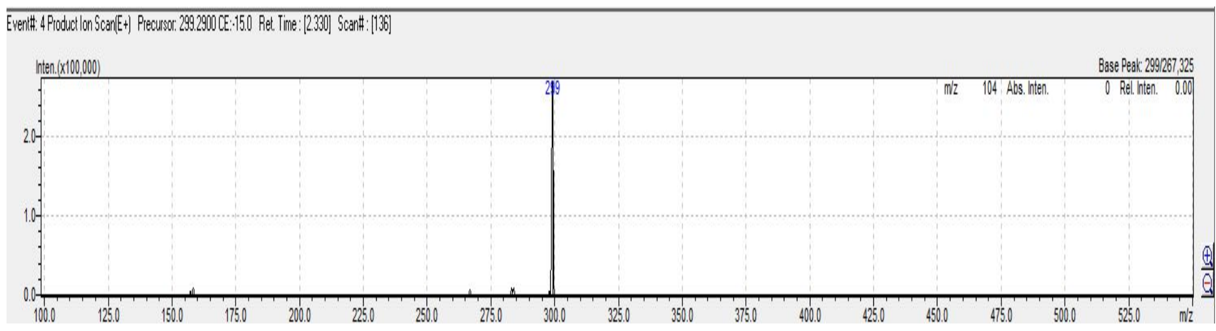


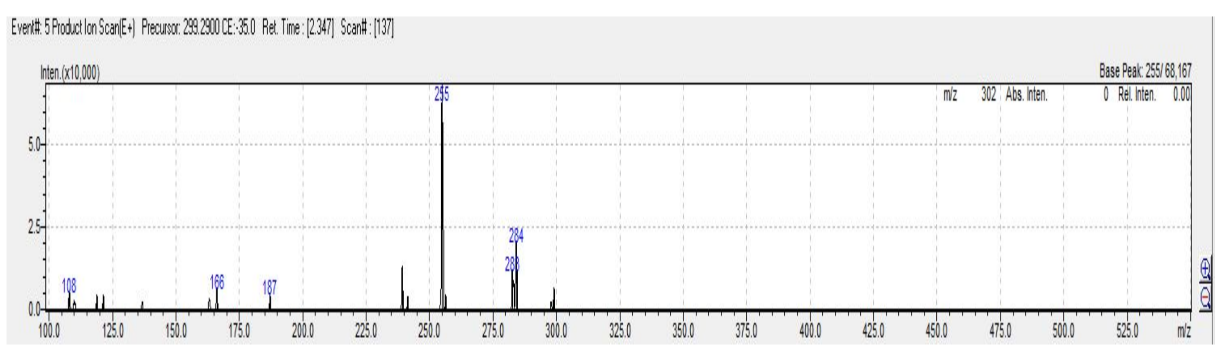
Fig.S27. MS analysis flavone 4'-hydroxy-5,7-dimethoxyflavone (**5**)

Molecular Formula = $C_{17}H_{14}O_5$
Formula Weight = 298.29006
Precursor = 299.2900

CE: -15.0



CE: -35.0



CE: -45.0

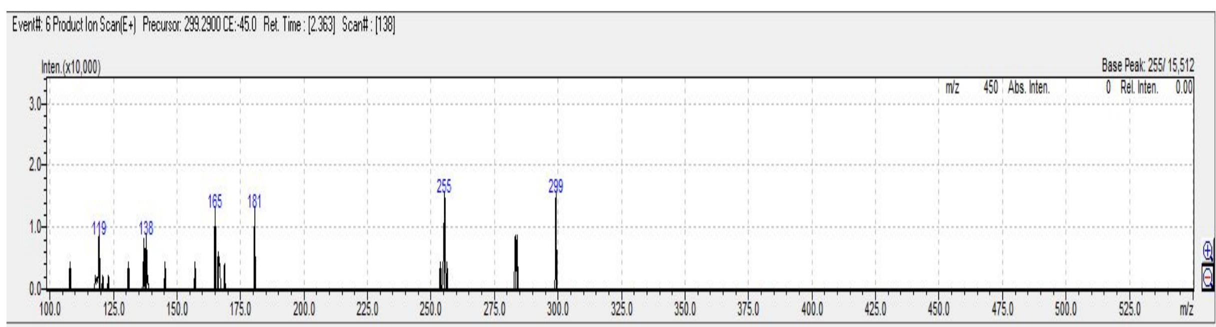


Fig.S28. ^{1}H NMR spectrum of 4'-hydroxy-5,7-dimethoxyflavone (**5**) (DMSO-d_6, 600 MHz)

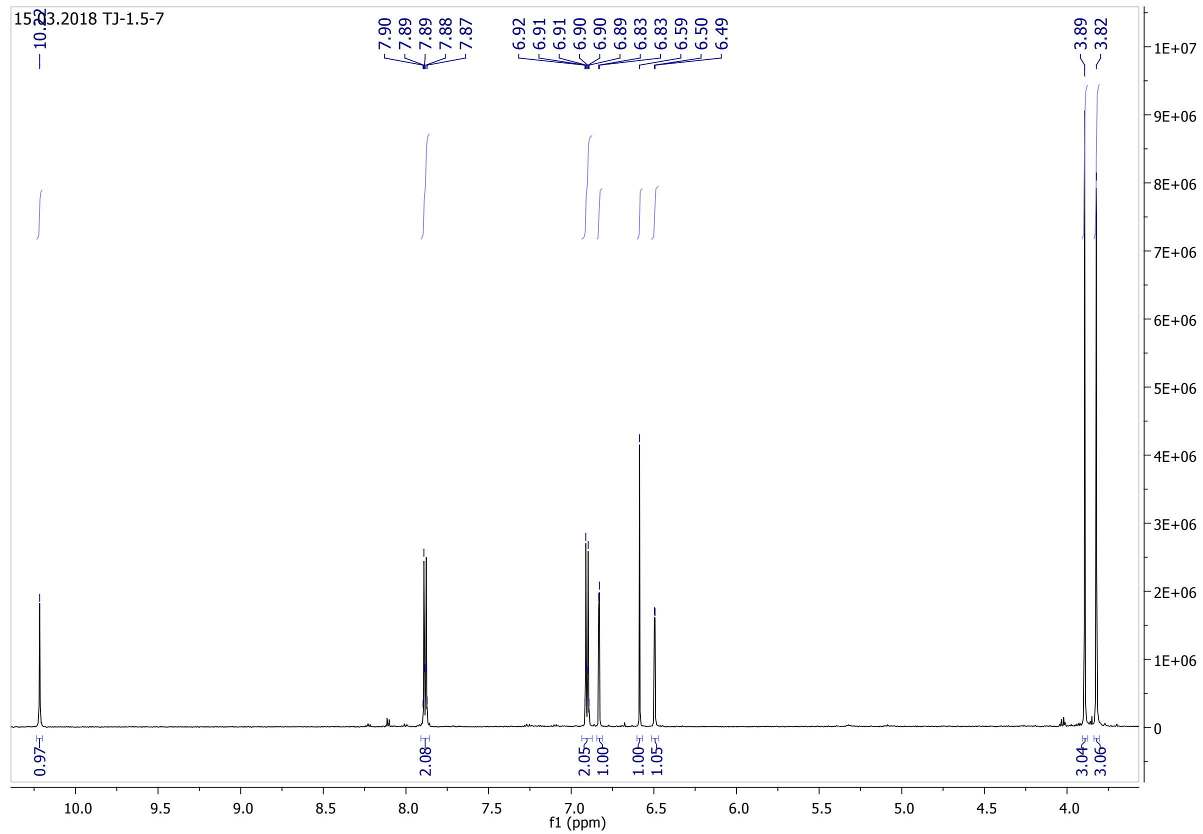


Fig.S29. Flavone part of the ^{1}H NMR spectrum 4'-hydroxy-5,7-dimethoxyflavone (**5**) (DMSO-d_6, 600 MHz)

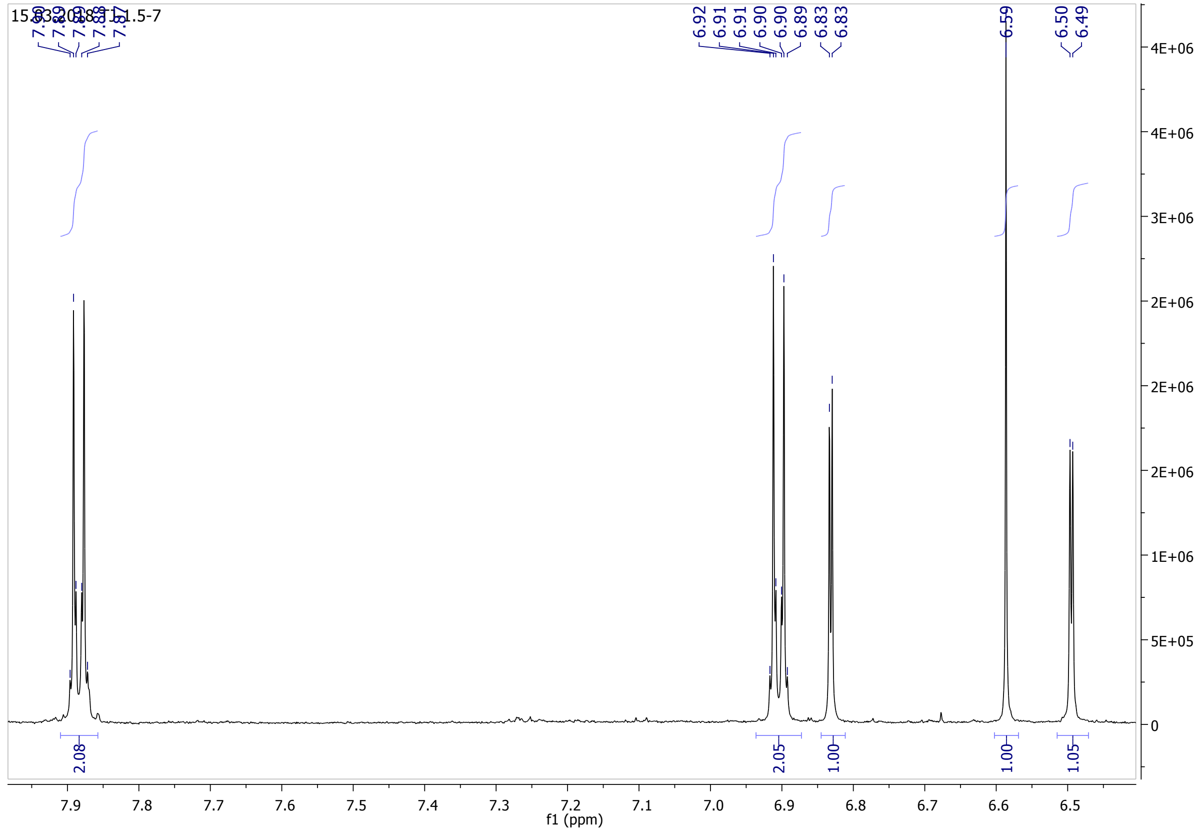


Fig.S30. ^{13}C NMR spectrum of 4'-hydroxy-5,7-dimethoxyflavone (**5**) (DMSO-d_6, 151 MHz)

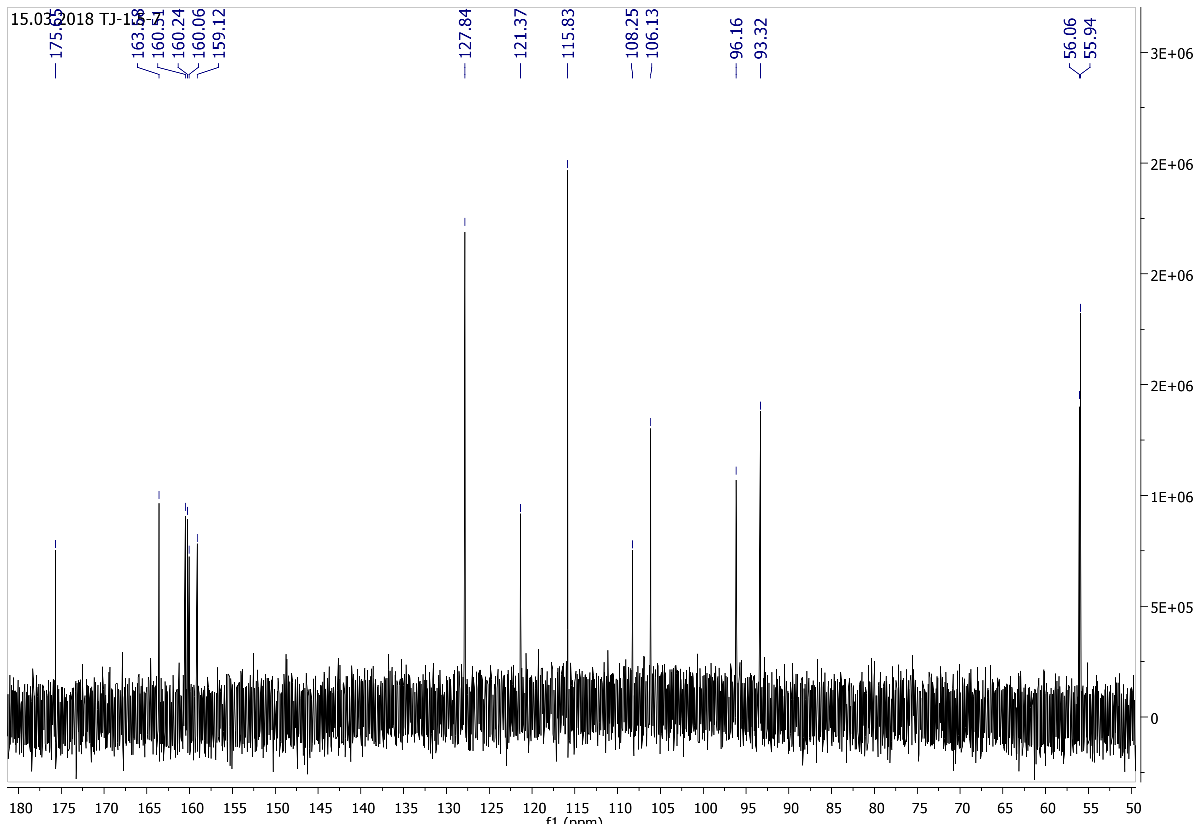


Fig.S31. COSY spectrum of 4’-hydroxy-5,7-dimethoxyflavone (**5**) (DMSO-d_6, 600 MHz)

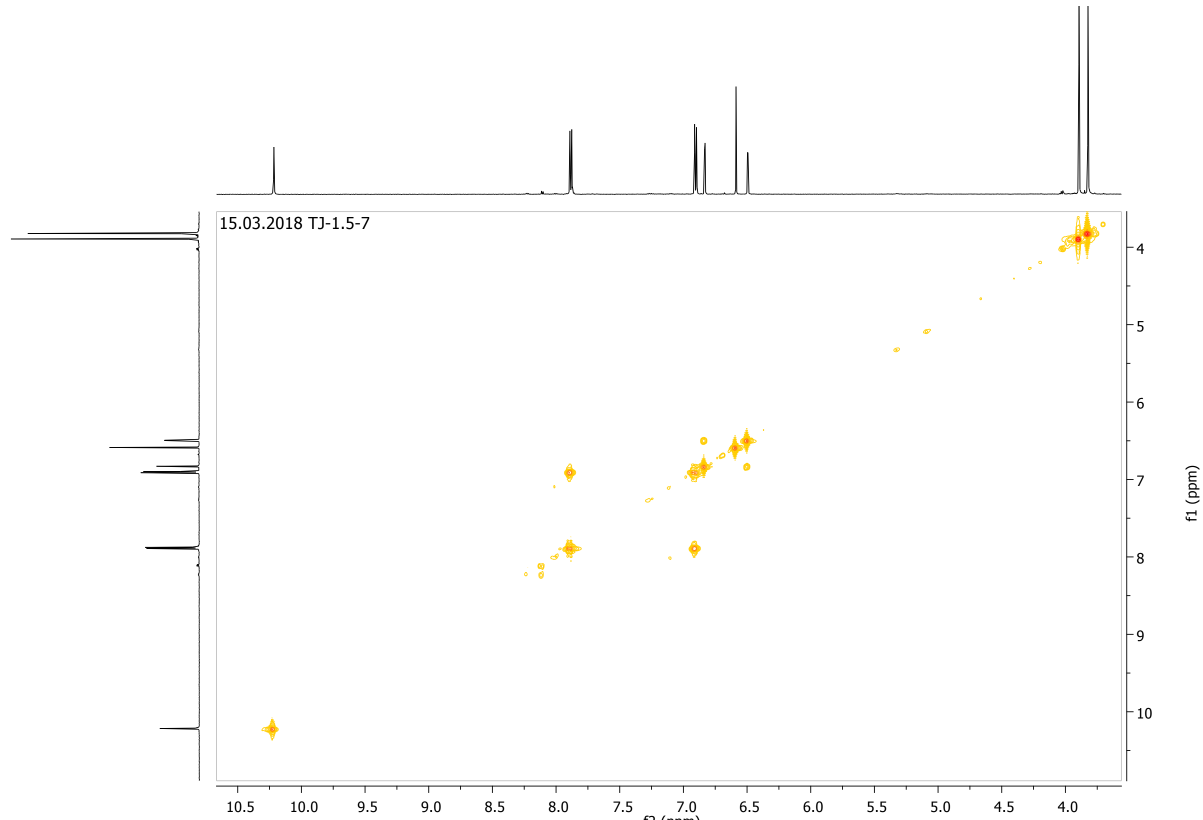


Fig.S32. HMQC spectrum of 4’-hydroxy-5,7-dimethoxyflavone (**5**) (DMSO-d_6, 151 MHz)

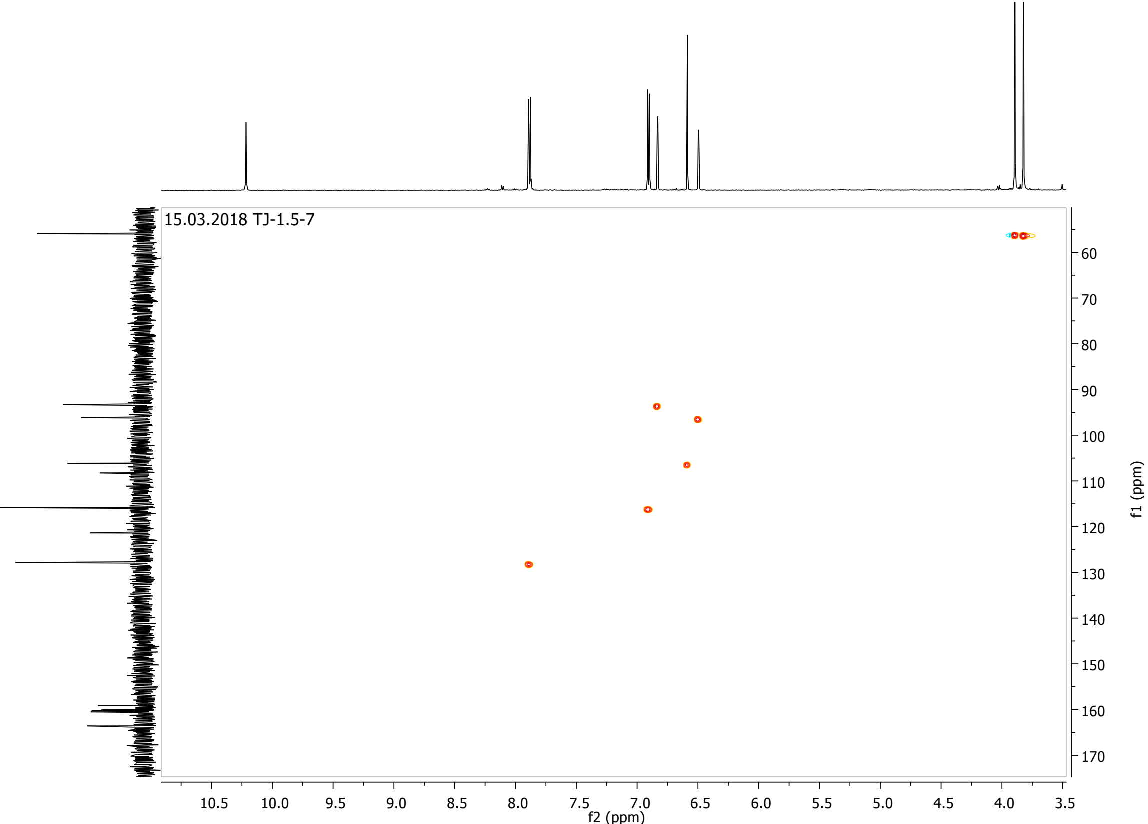


Fig.S33. HMBC spectrum of 4’-hydroxy-5,7-dimethoxyflavone (**5**) (DMSO-d_6, 151 MHz)

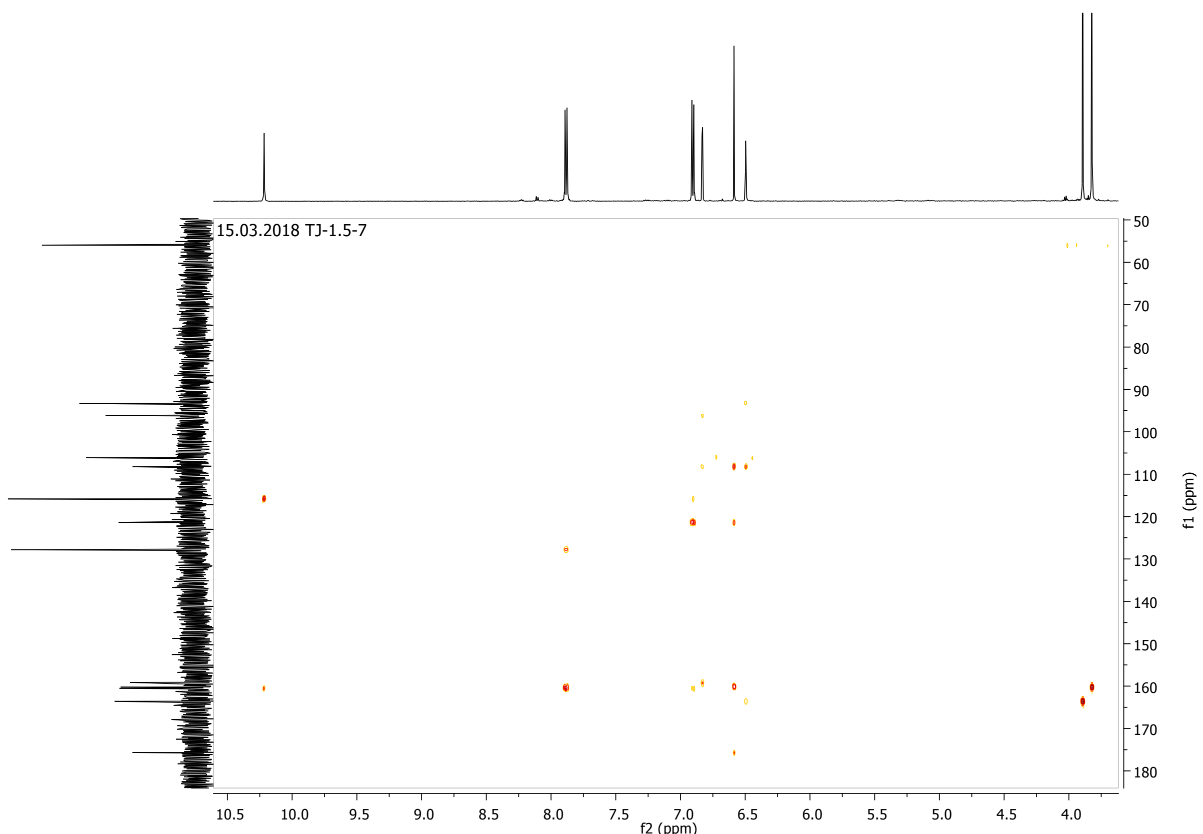
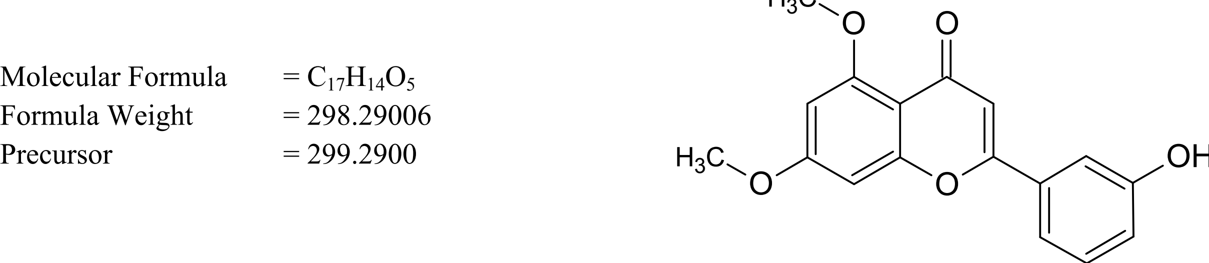
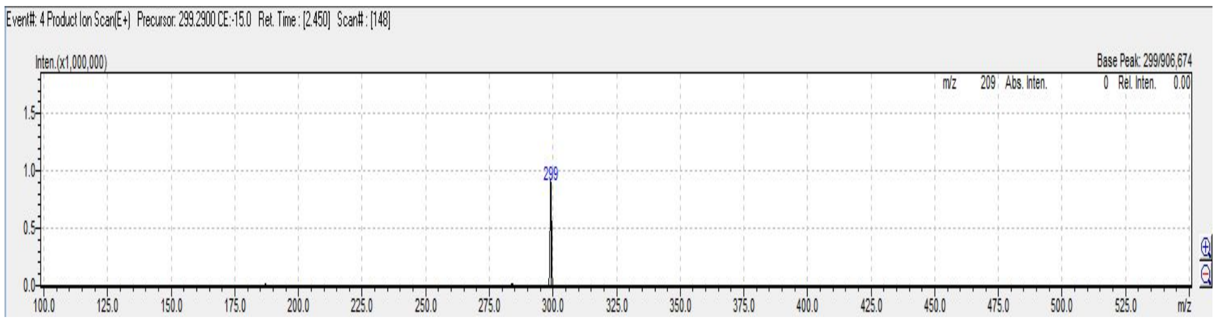


Fig.S34. MS analysis 3’-hydroxy-5,7-dimethoxyflavone (**6**)

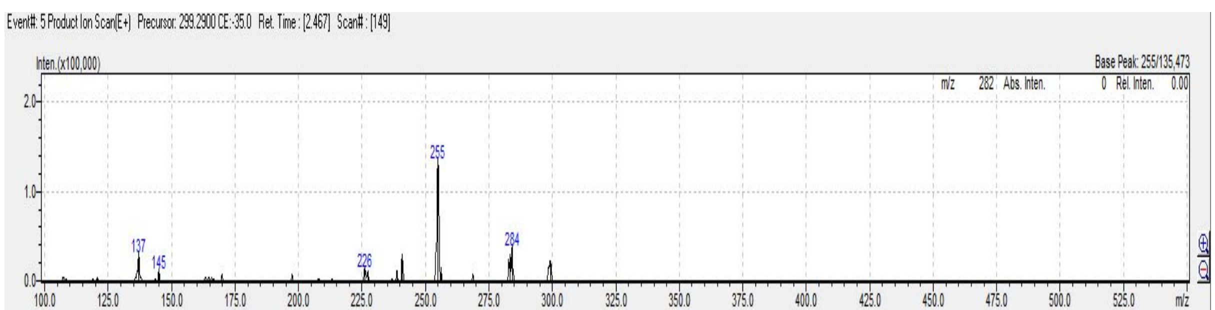
Molecular Formula = $C_{17}H_{14}O_5$
Formula Weight = 298.29006
Precursor = 299.2900



CE: -15.0



CE: -35.0



CE: -45.0

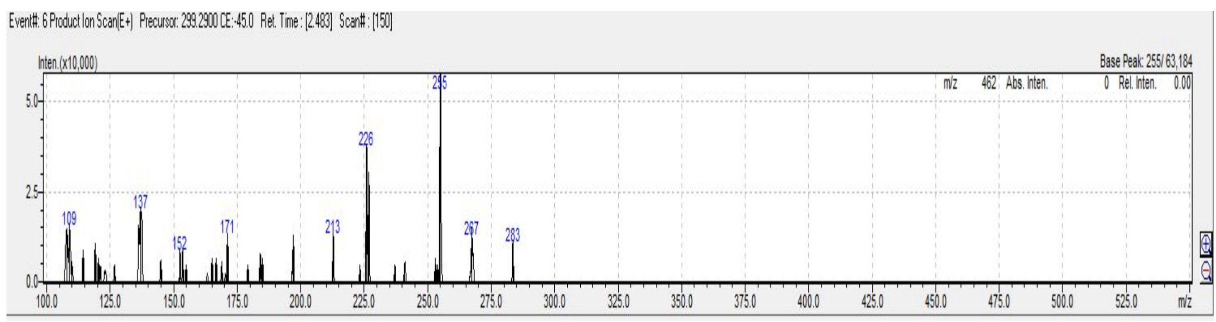


Fig.S35. ^{1}H NMR spectrum of 3'-hydroxy-5,7-dimethoxyflavone (**6**) (DMSO-d_6, 600 MHz)

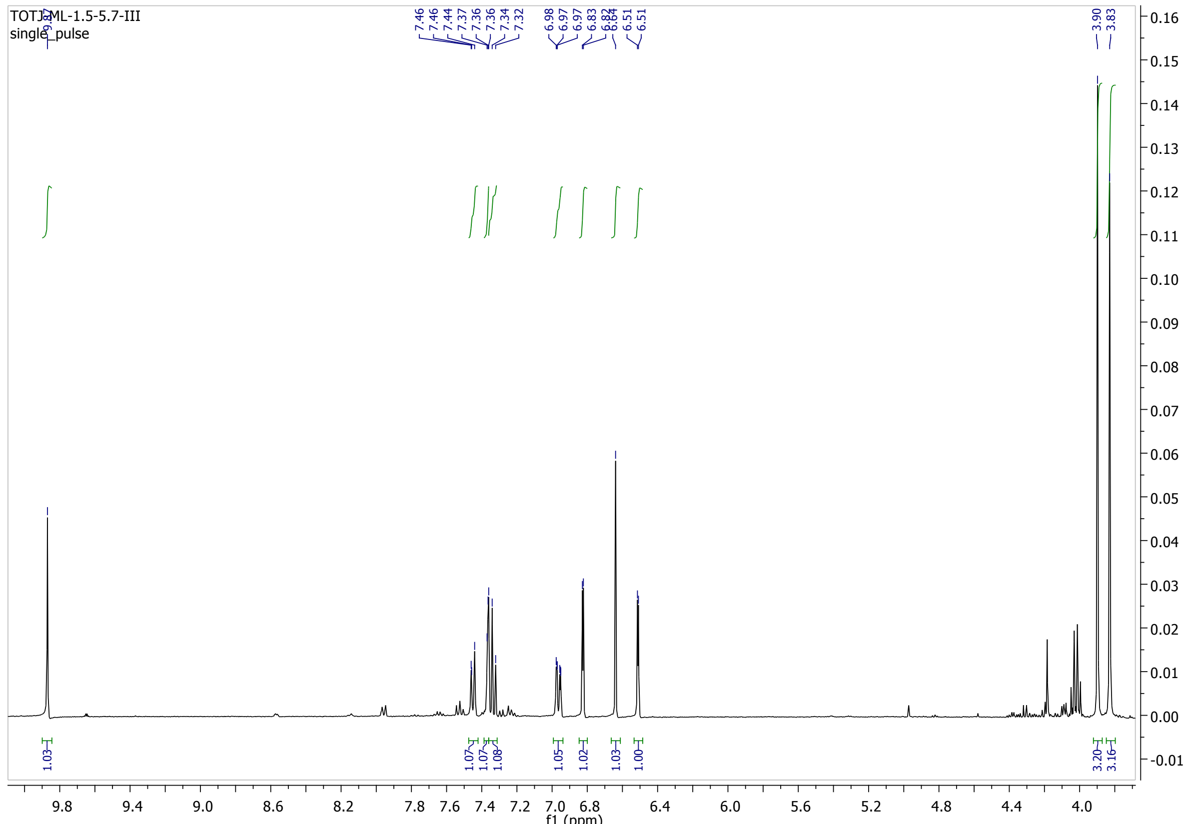


Fig.S36. Flavone part of the ^{1}H NMR spectrum 4'-hydroxy-5,7-dimethoxyflavone (**5**) (DMSO-d_6, 600 MHz)

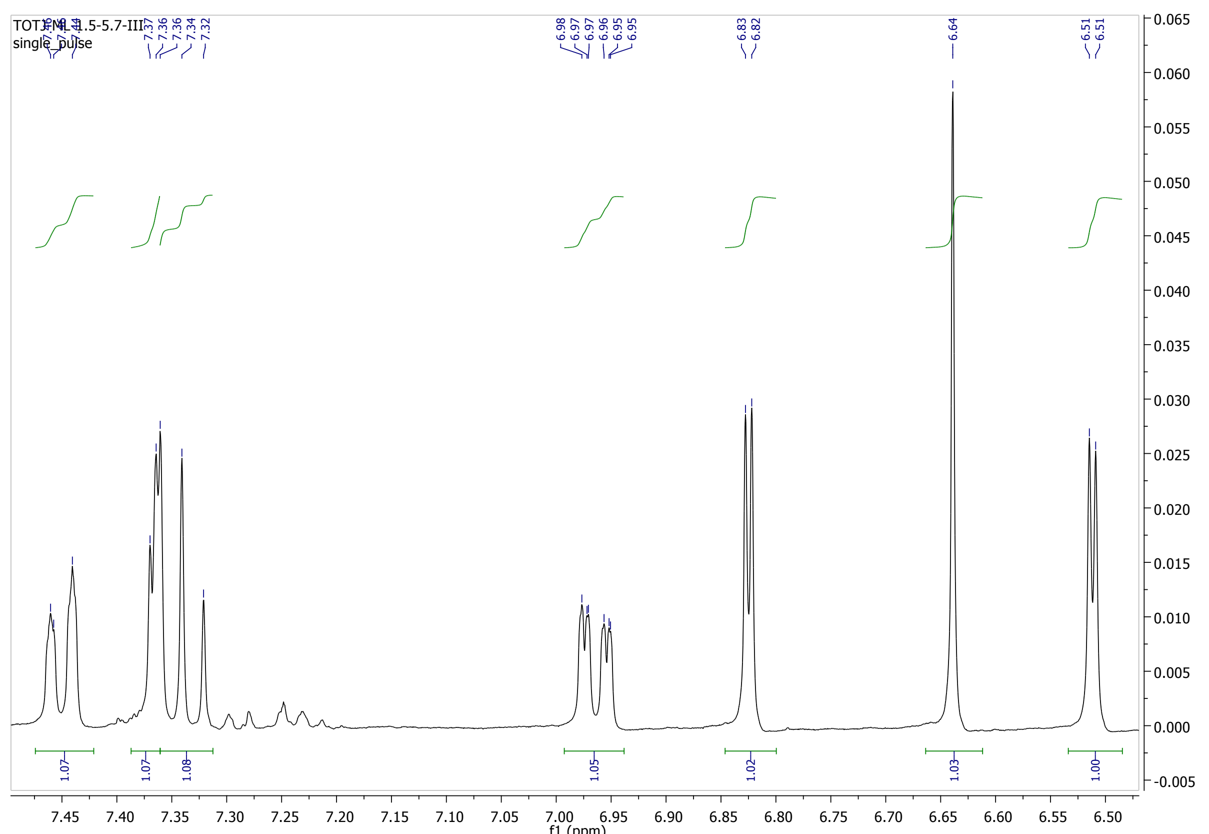


Fig.S37. ^{13}C NMR spectrum of 3’-hydroxy-5,7-dimethoxyflavone (**6**) (DMSO-d_6, 151 MHz)

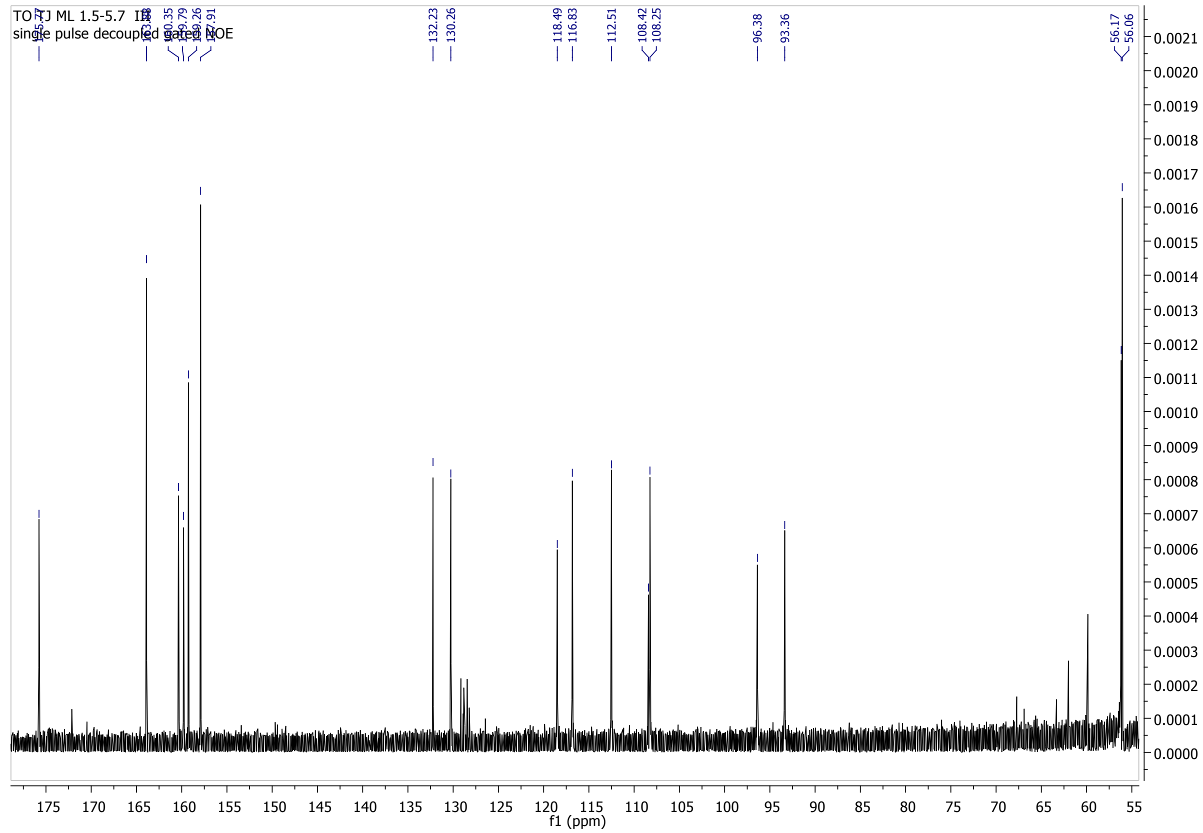


Fig.S38. COSY spectrum of 3’-hydroxy-5,7-dimethoxyflavone (**6**) (DMSO-d_6, 151 MHz)

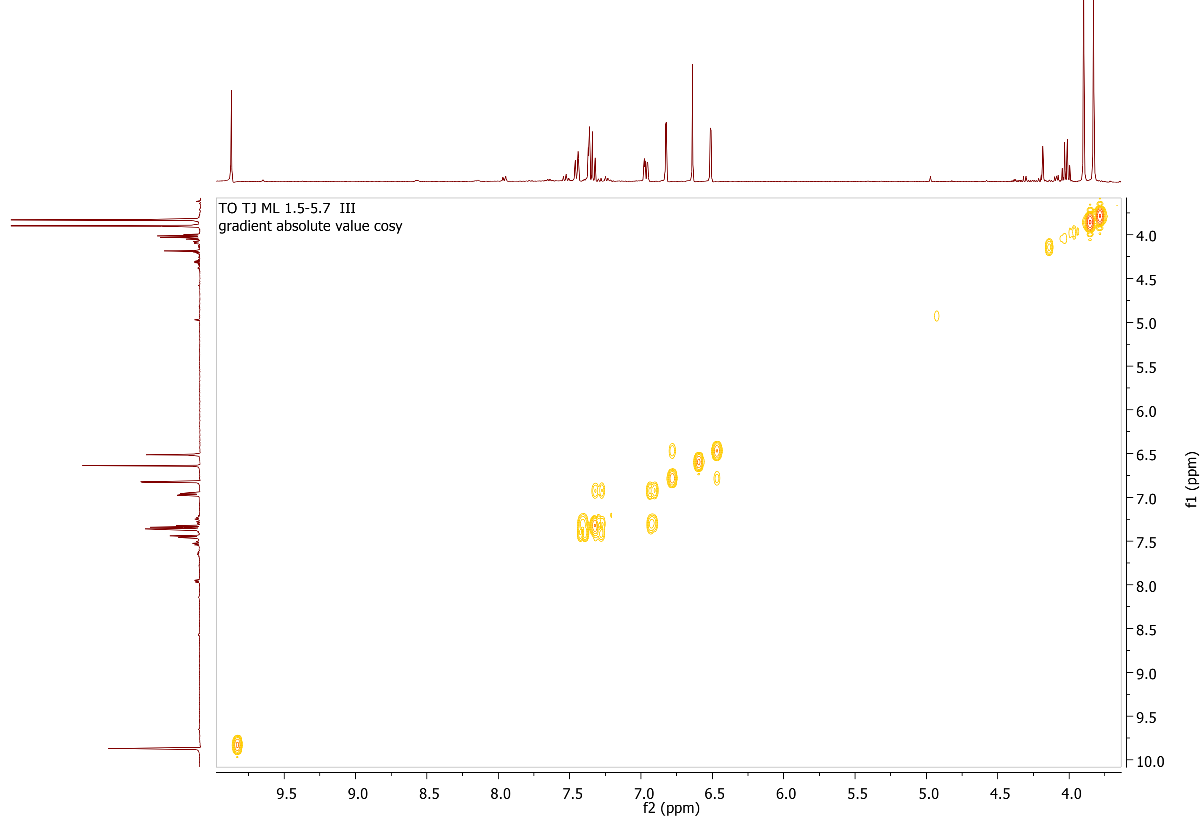


Fig.S39. HMQC spectrum of 3’-hydroxy-5,7-dimethoxyflavone (**6**) (DMSO-d_6, 151 MHz)

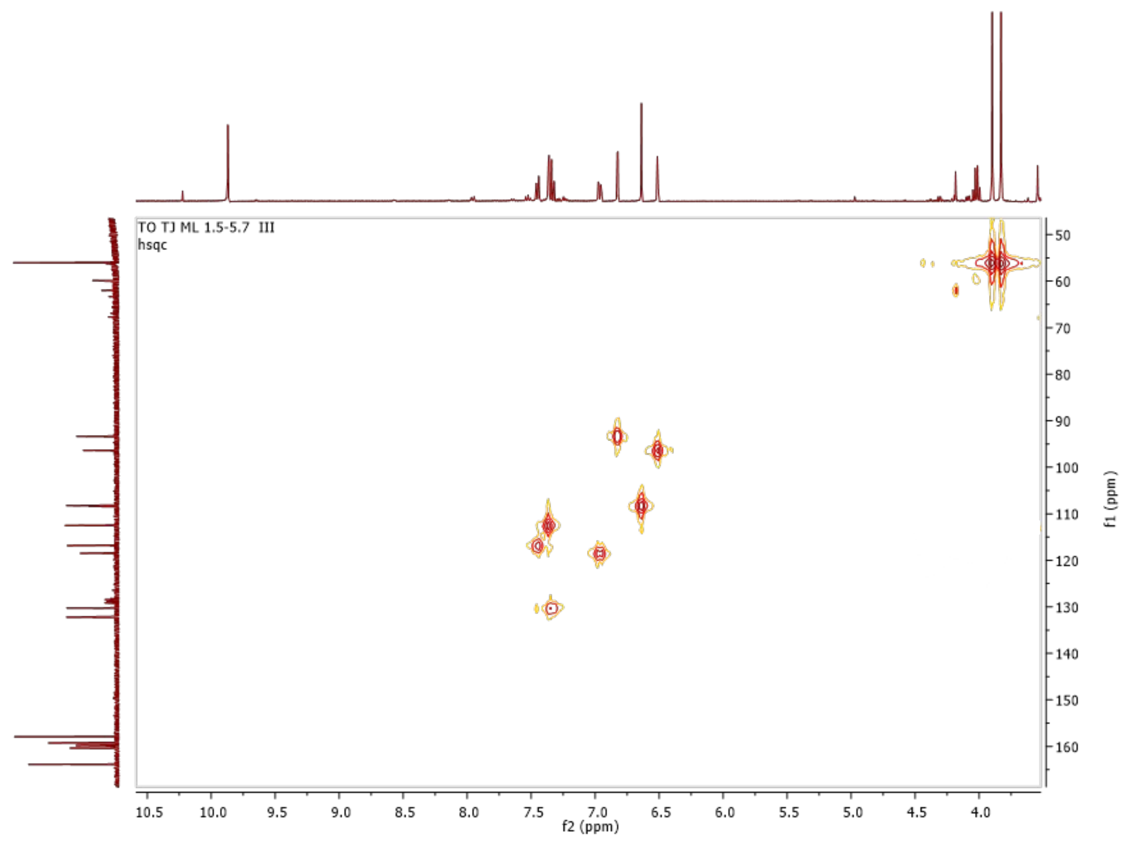


Fig.S40. HMBC spectrum of 3’-hydroxy-5,7-dimethoxyflavone (**6**) (DMSO-d_6, 151 MHz)

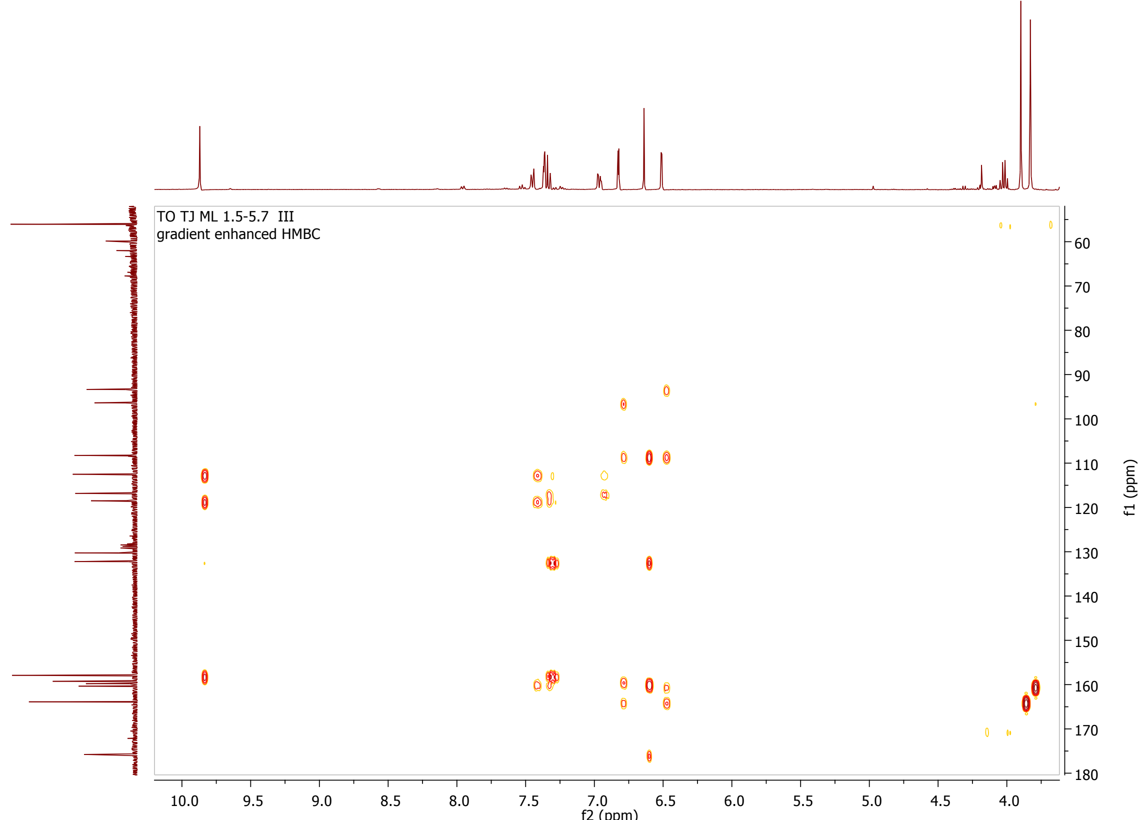
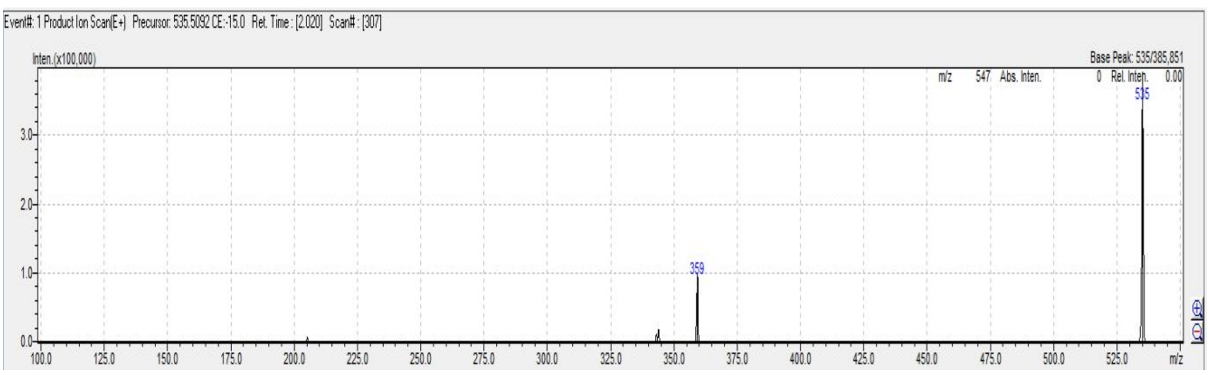


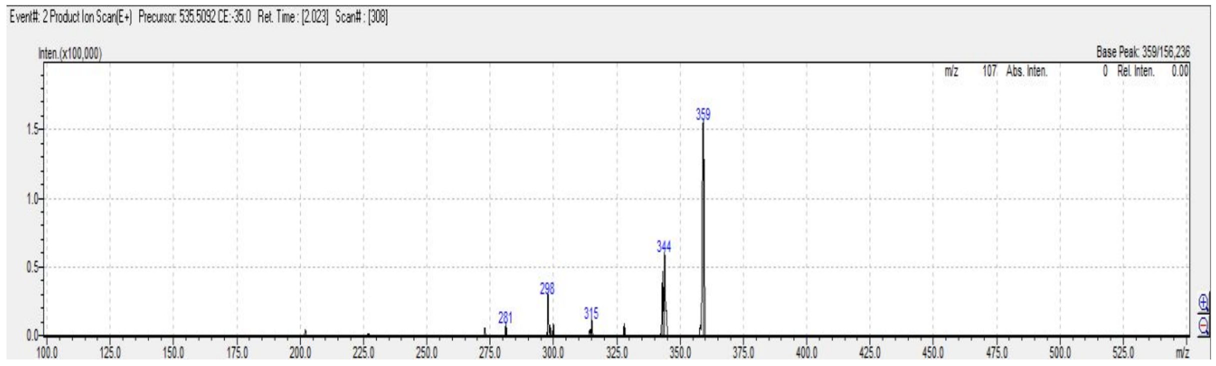
Fig.S41. MS analysis flavone 3’-*O*-β-D-(4′′-*O*-Methylglucopyranosyl)-5,7,4’,5’-tetramethoxyflavone (**7**)

Molecular Formula = $C_{26}H_{30}O_{12}$
Formula Weight = 534.5092
Precursor = 535.5092

CE: -15.0



CE: -35.0



CE: -45.0

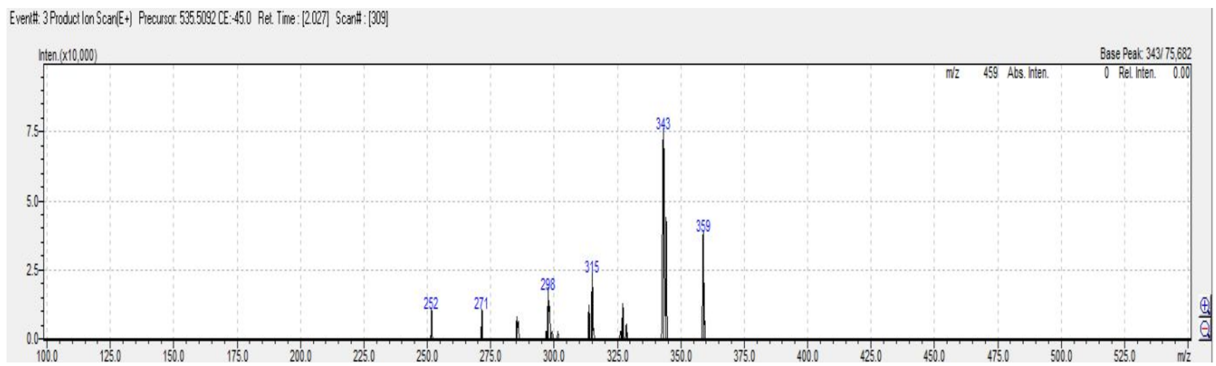


Fig.S42. ^{1}H NMR spectrum of flavone 3'-*O*-β-D-(4″-*O*-Methylglucopyranosyl)-5,7,4',5'-tetramethoxyflavone (**7**) (DMSO-d_6, 600 MHz)

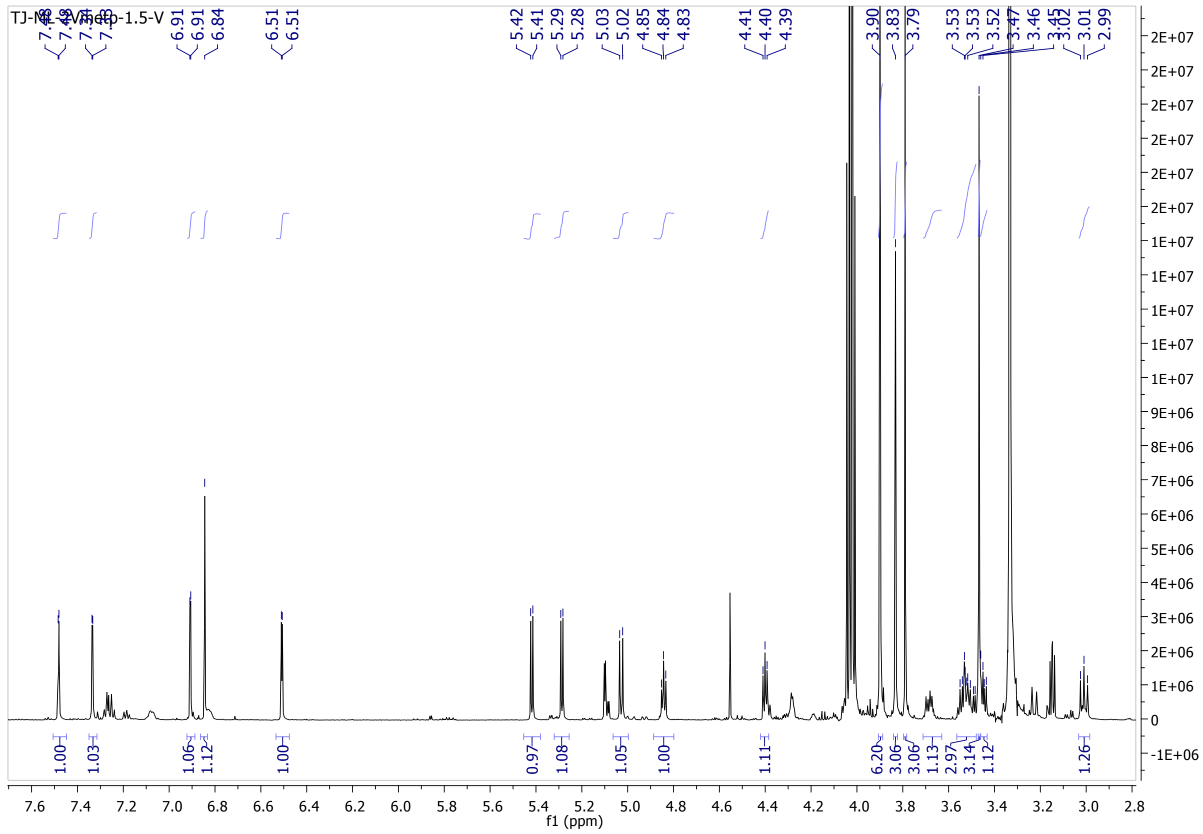


Fig.S43. Flavone part of the ^{1}H NMR spectrum flavone 3'-*O*-β-D-(4″-*O*-Methylglucopyranosyl)-5,7,4',5'-tetramethoxyflavone (**7**) (DMSO-d_6, 600 MHz)

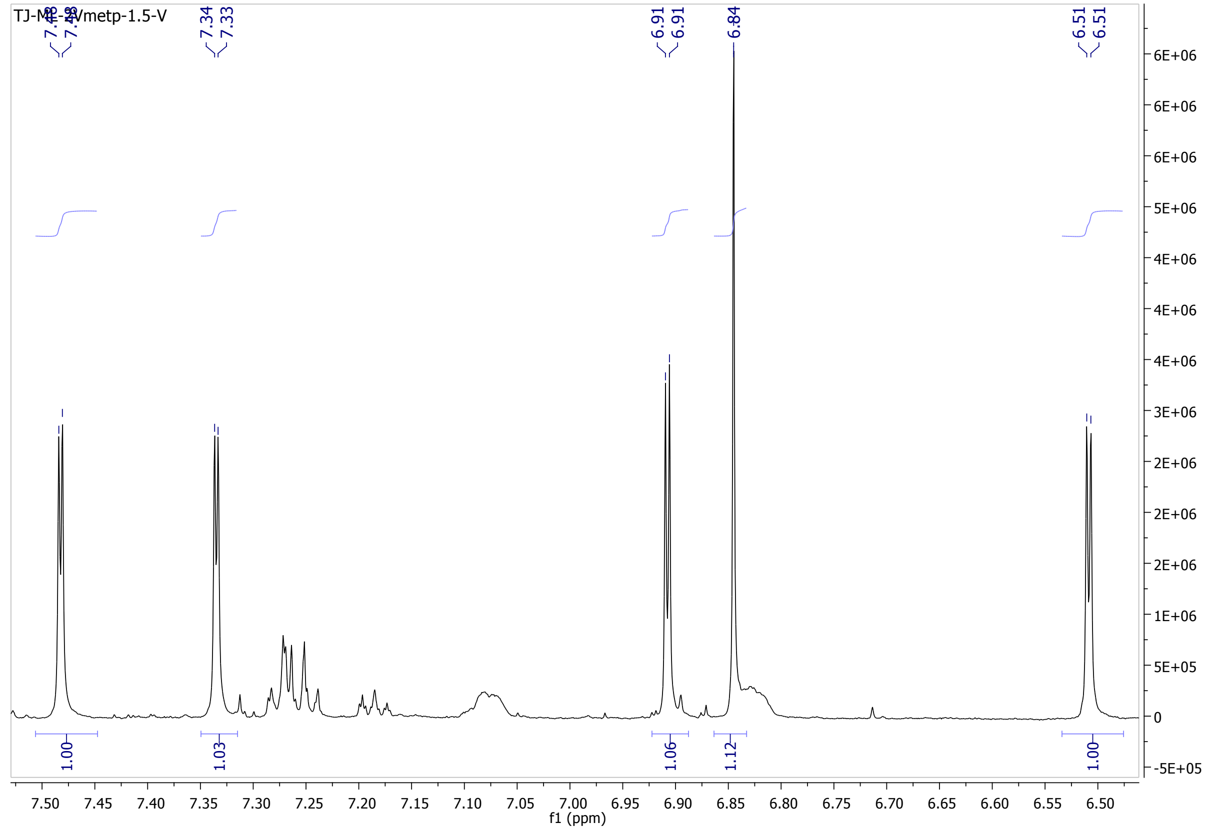


Fig.S44. Glucopyranoside part of the ^{1}H NMR spectrum flavone 3'-*O*-β-D-(4″-*O*-Methylglucopyranosyl)-5,7,4',5'-tetramethoxyflavone (**7**) (DMSO-d_6, 600 MHz)

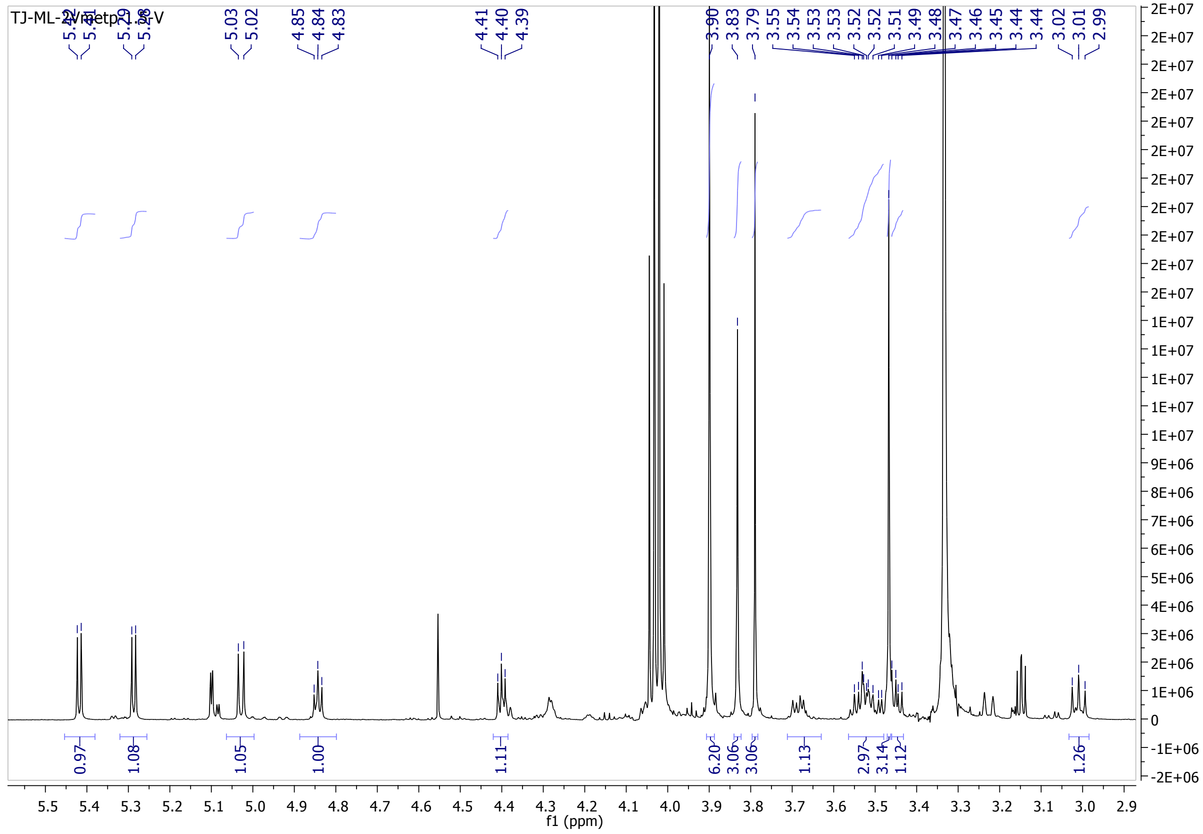


Fig.S45. ^{13}C NMR spectrum of flavone 3'-*O*-β-D-(4″-*O*-Methylglucopyranosyl)-5,7,4',5'-tetramethoxyflavone (**7**) (DMSO-d_6, 151 MHz)

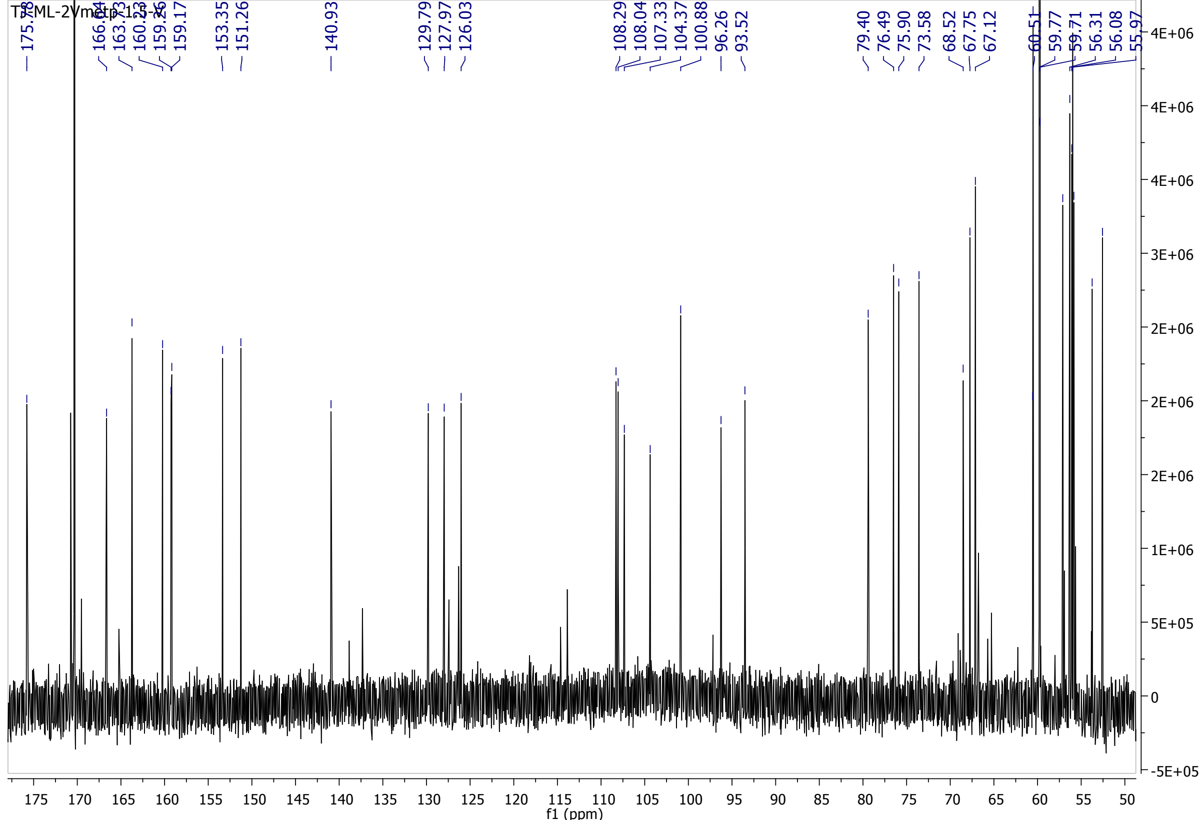


Fig.S46. HMQC spectrum of flavone 3'-*O*-β-D-(4″-*O*-Methylglucopyranosyl)-5,7,4',5'-tetramethoxyflavone (**7**) (DMSO-d_6, 151 MHz)

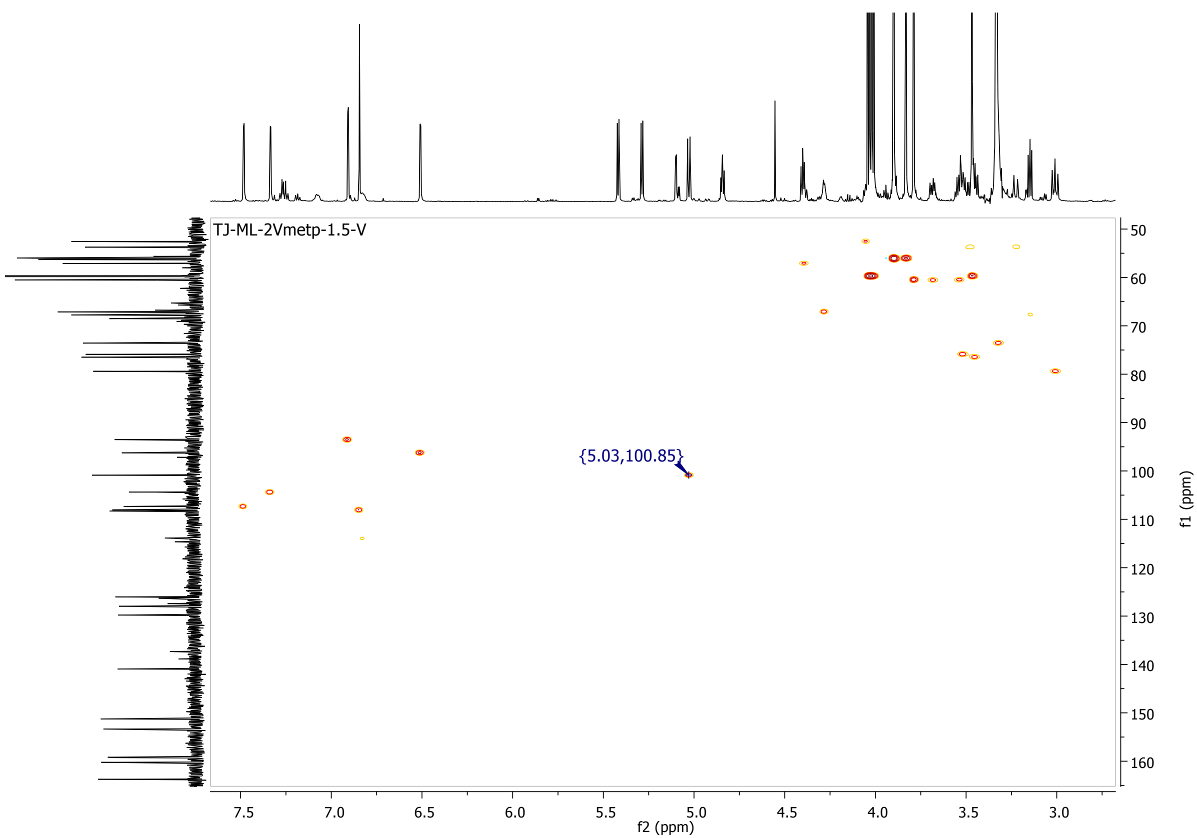


Fig.S47. HMBC spectrum of flavone 3'-*O*-β-D-(4″-*O*-Methylglucopyranosyl)-5,7,4',5'-tetramethoxyflavone (**7**) (DMSO-d_6, 151 MHz)

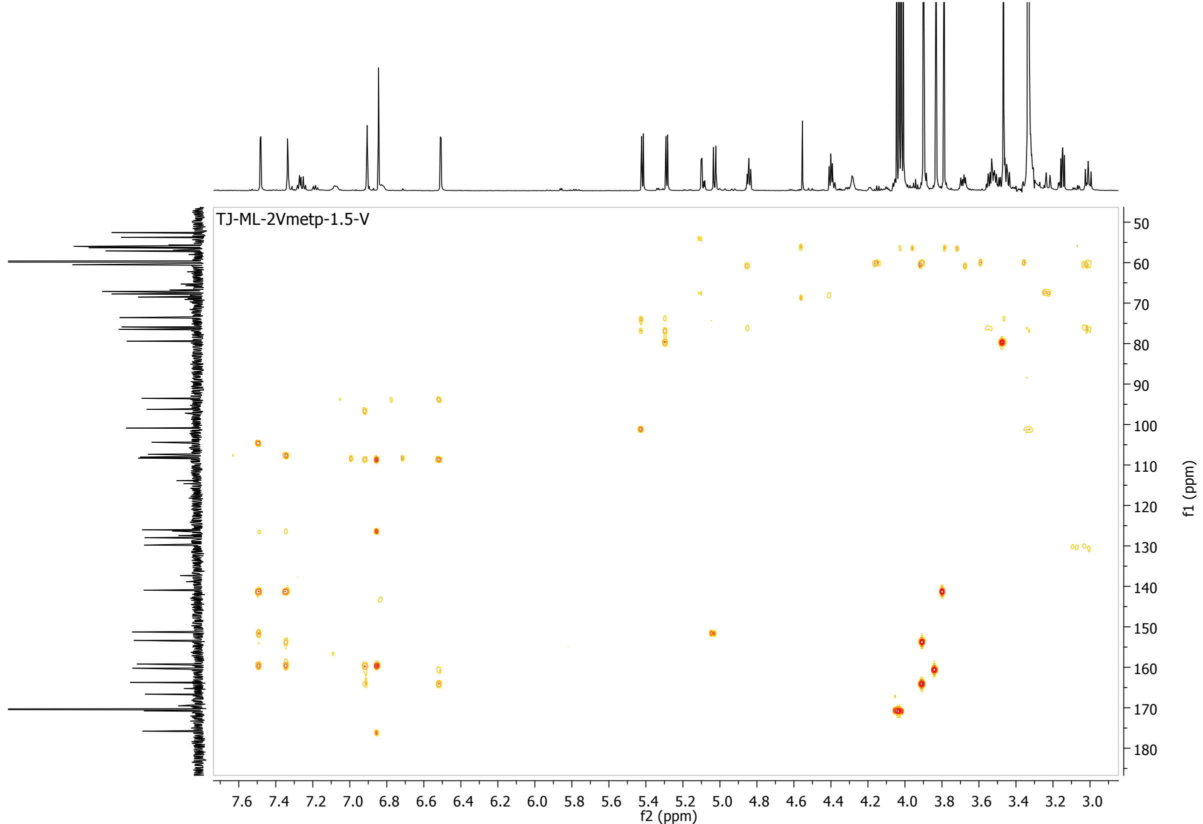
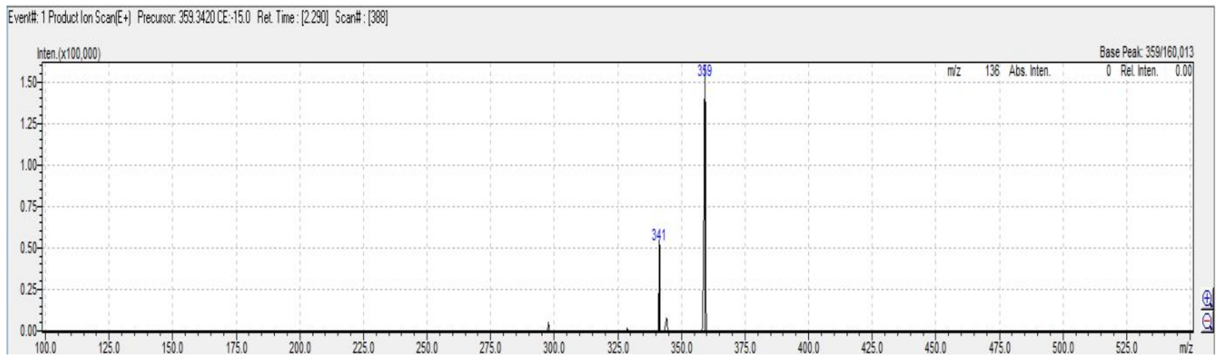


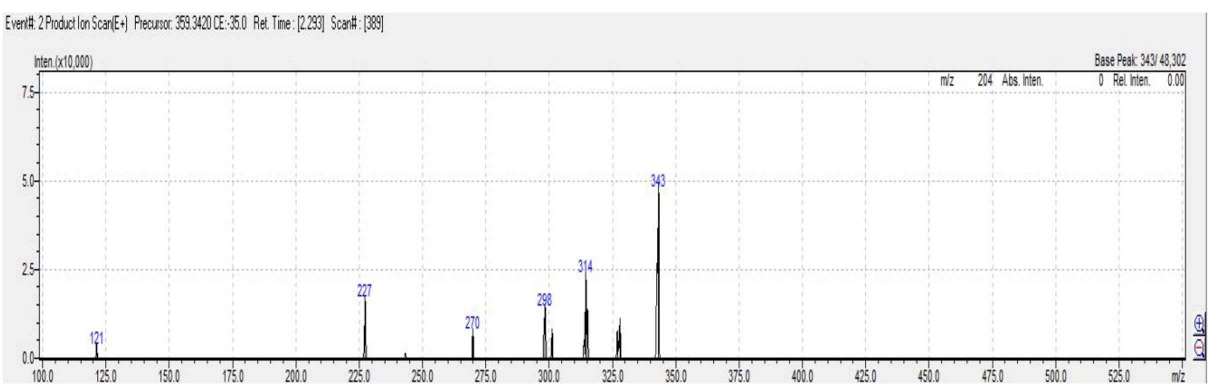
Fig.S48. MS analysis 3'-hydroxy-5,7,4',5'-tetramethoxyflavone (**8**)

Molecular Formula = $C_{19}H_{18}O_7$
Formula Weight = 358.34202
Precursor = 359.3420

CE: -15.0



CE: -35.0



CE: -45.0

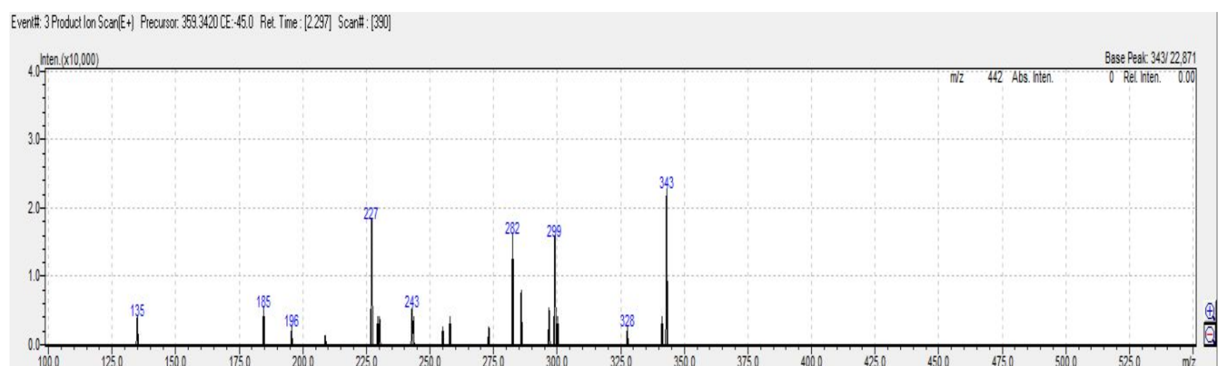


Fig.S49. ^{1}H NMR spectrum of 3’-hydroxy-5,7,4’,5’-tetramethoxyflavone (**8**) (DMSO-d_6, 600 MHz)

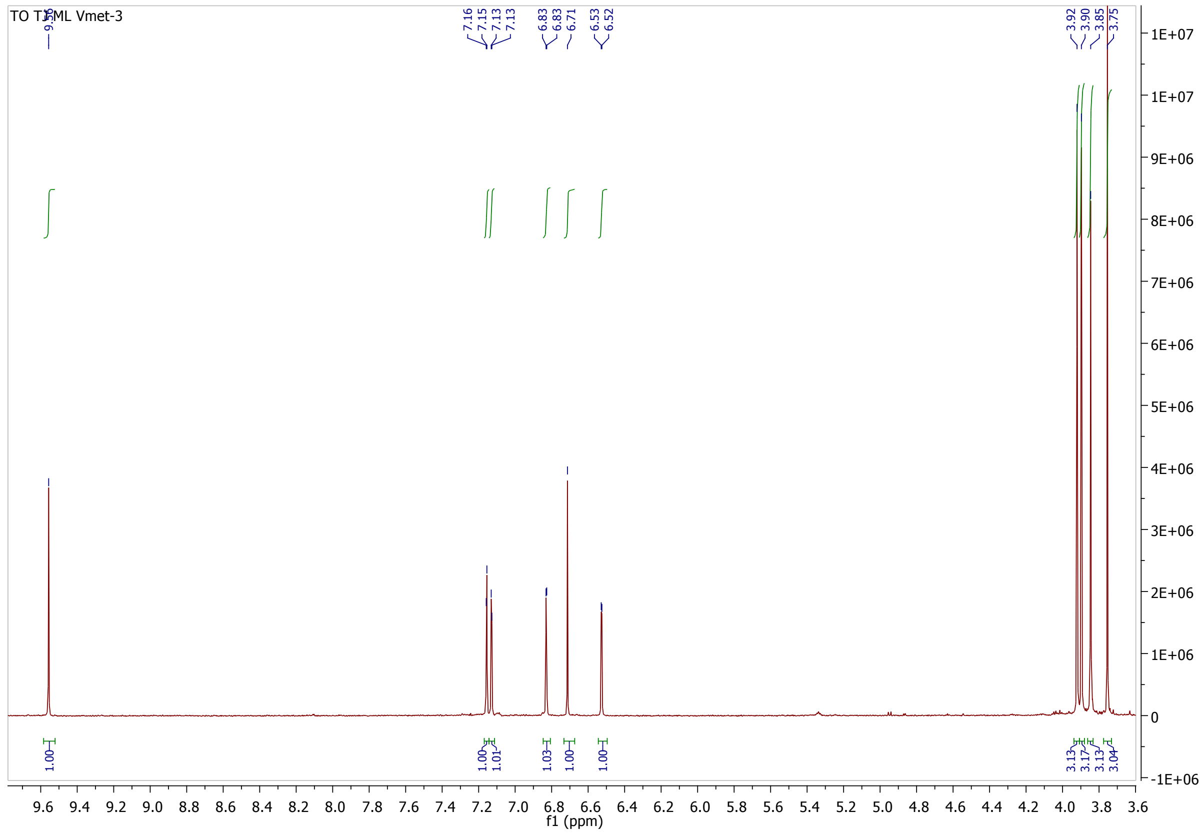


Fig.S50. Flavone part of the ^{1}H NMR spectrum 3’-hydroxy-5,7,4’,5’-tetramethoxyflavone (**8**) (DMSO-d_6, 600 MHz)

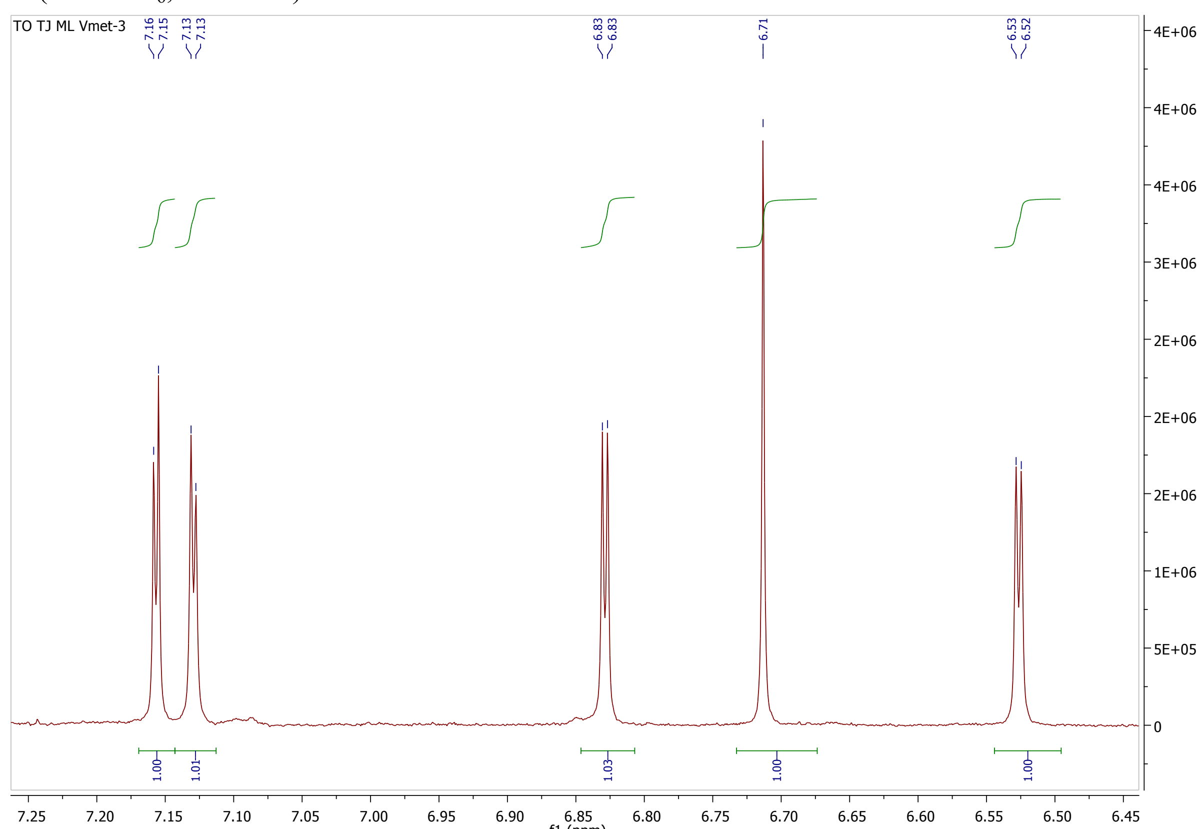


Fig.S51. ^{13}C NMR spectrum of 3'-hydroxy-5,7,4',5'-tetramethoxyflavone (**8**) (DMSO-d_6, 600 MHz)

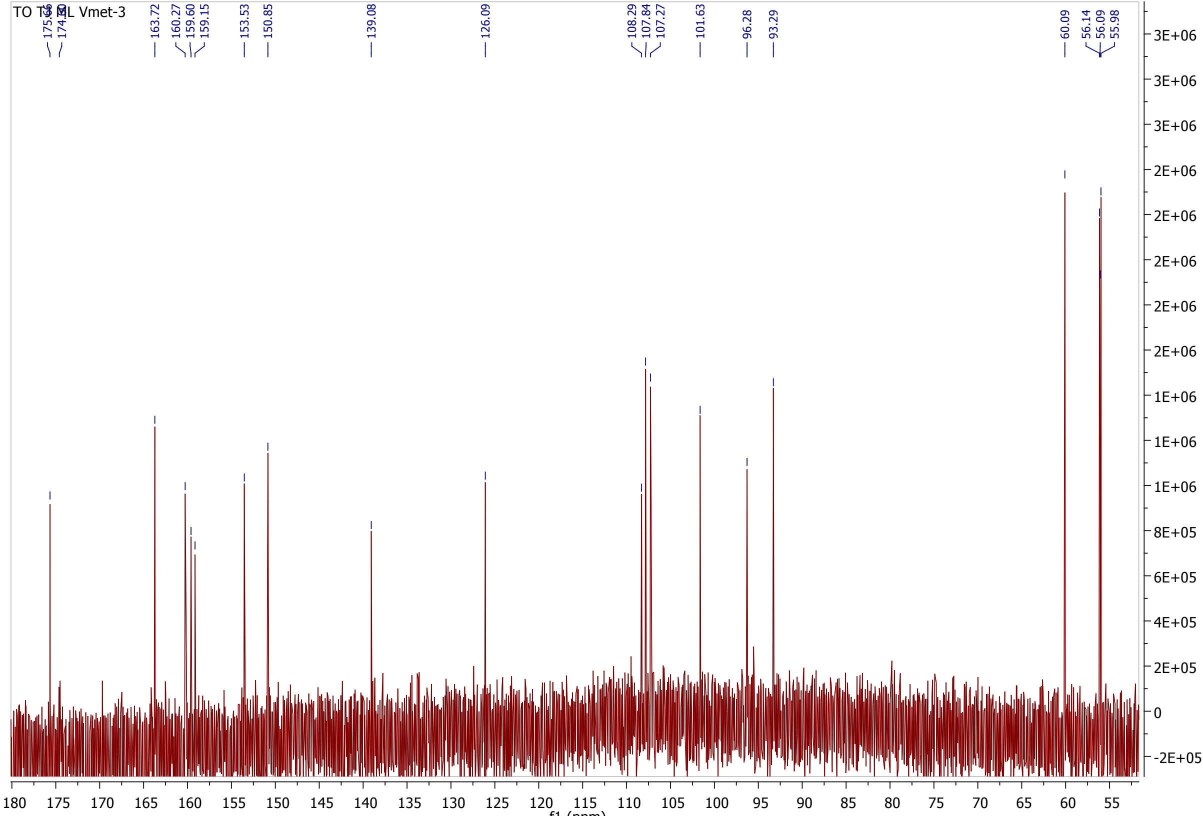


Fig.S52. COSY spectrum of 3'-hydroxy-5,7,4',5'-tetramethoxyflavone (**8**) (DMSO-d_6, 600 MHz)

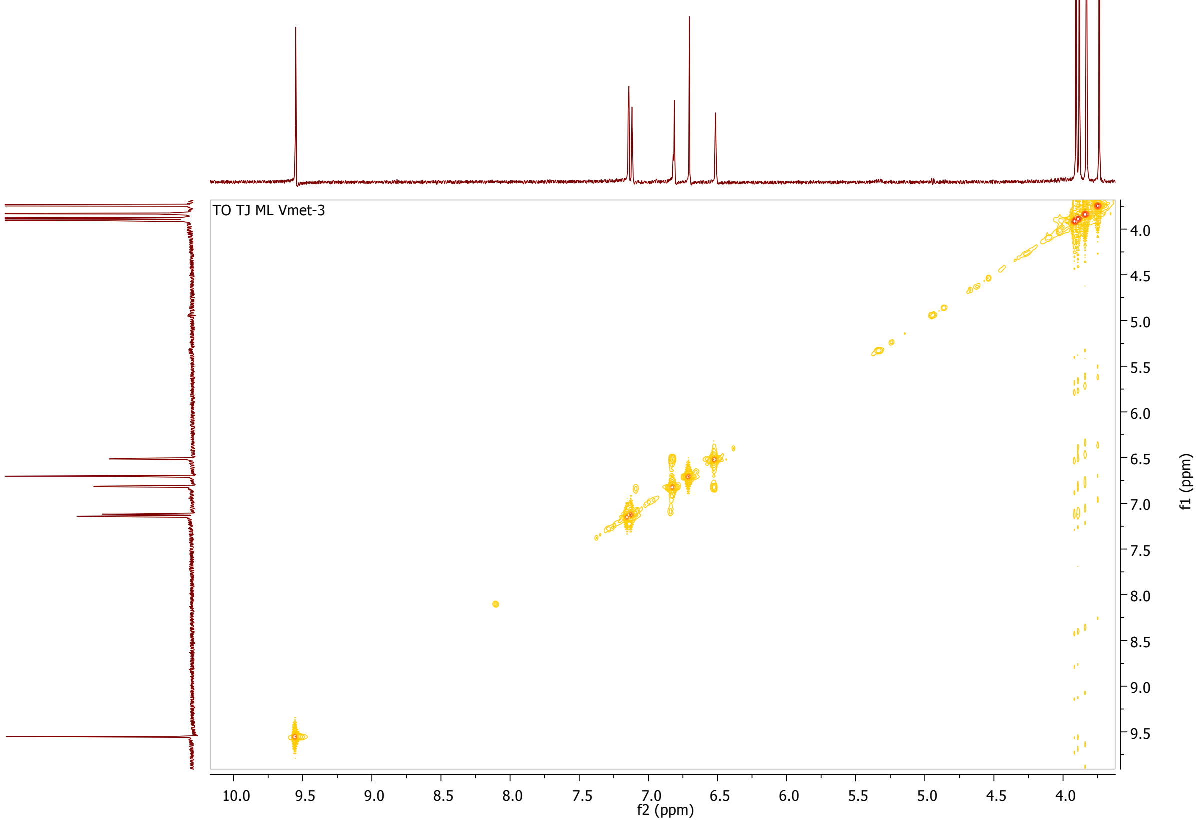


Fig.S53. HMQC spectrum of 3'-hydroxy-5,7,4',5'-tetramethoxyflavone (**8**) (DMSO-d_6, 600 MHz)

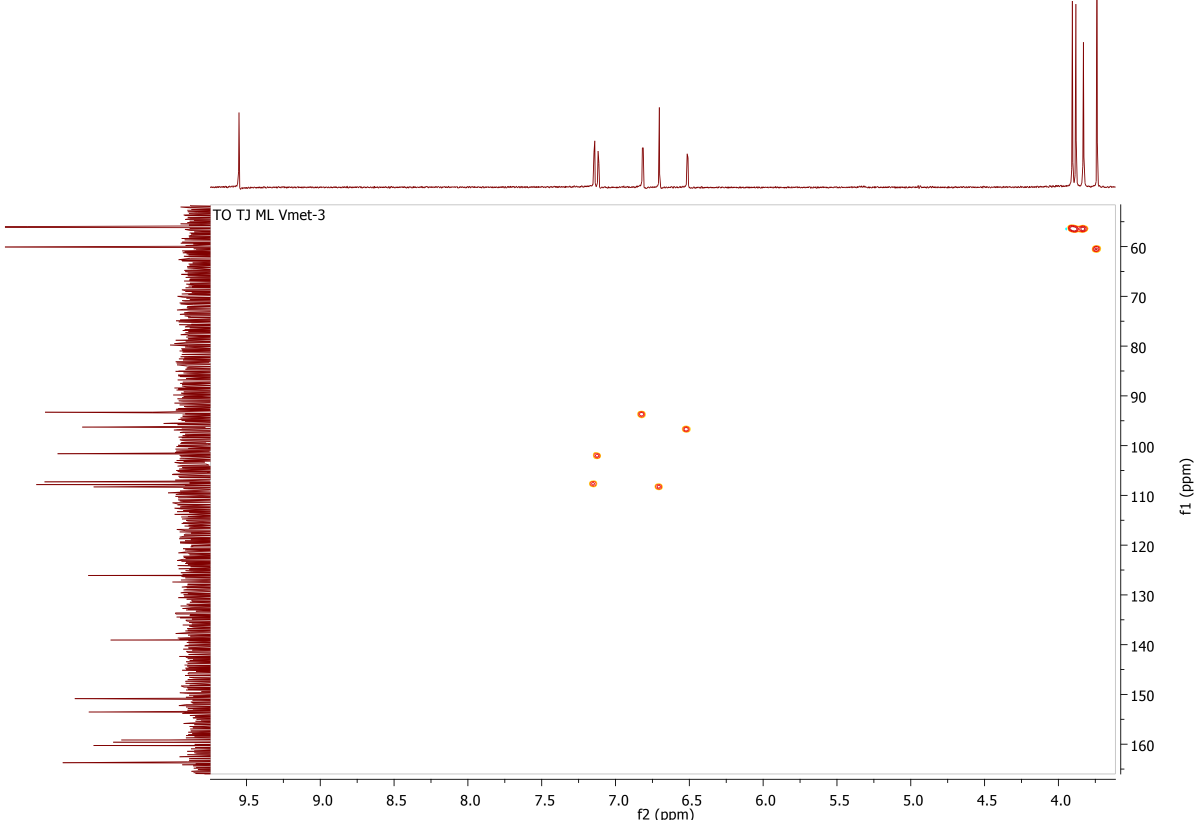
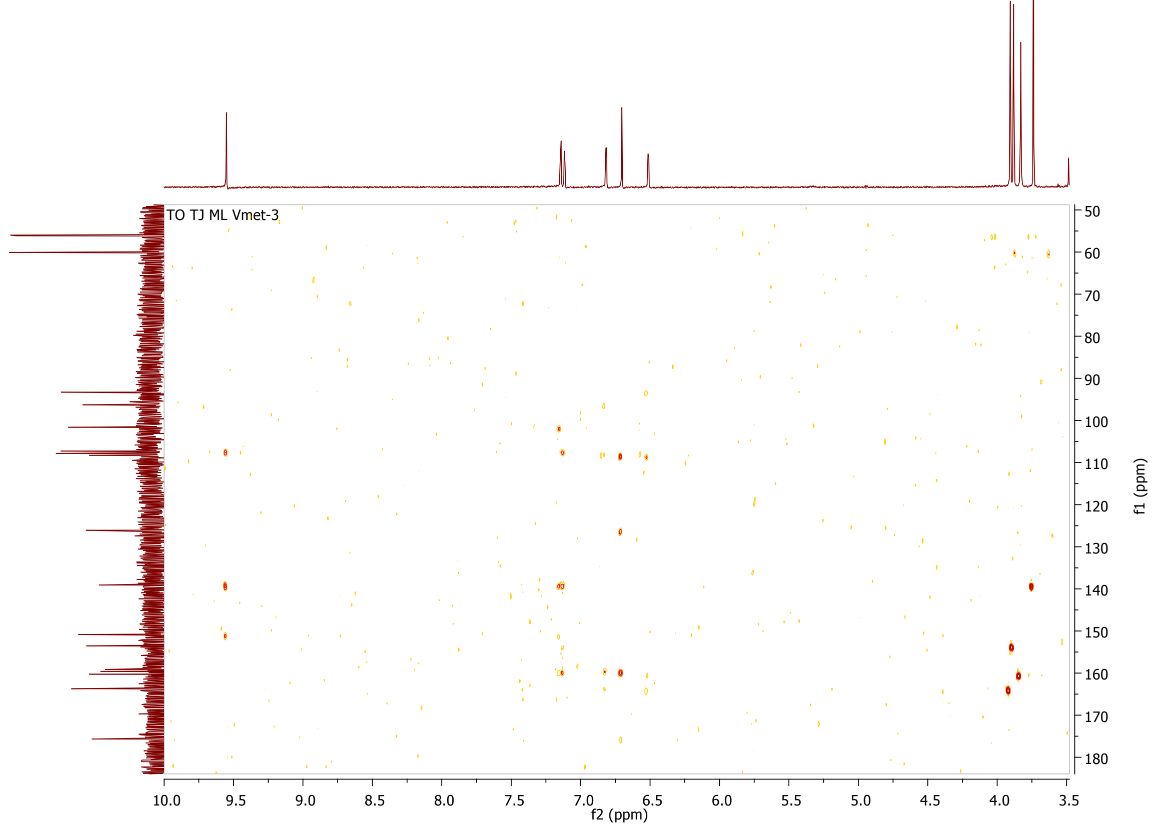


Fig.S54. HMBC spectrum of 3'-hydroxy-5,7,4',5'-tetramethoxyflavone (**8**) (DMSO-d_6, 600 MHz)



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    P.426765_78_18 (23.08.2018) /23.11.2020

15. Łużny M., Kozłowska E, Dymarska M., Kostrzewa-Susłow E., Janeczko T.:
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18. Łużny M., Kozłowska E, Dymarska M., Kostrzewa-Susłow E., Janeczko T.:
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   Sposób wytwarzania 4'-hydroksy-5,7-dimetoksyflawonu
   Zgłoszenie oznaczono numerem: P.428752 (31.01.2019)
2. Łużny M., Kozłowska E., Kostrzewa-Susłow E., Janeczko T.:
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7. Łużny M., Kozłowska E., Tronina T., Kostrzewa-Susłow E., Janeczko T.:
   4′-O-β-D-(4′′-O-Metyloglukopiranozylo)-2′,5′-dimetoksyflawon i sposób wytwarzania 4′-O-β-D-(4′′-O-metyloglukopiranozylo)-2′,5′-dimetoksyflawonu
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9. Łużny M., Kozłowska E., Tronina T., Kostrzewa-Susłow E., Janeczko T.:
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10. Łużny M., Kozłowska E., Tronina T., Kostrzewa-Susłow E., Janeczko T.:
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D-(4″-O-metyloglukopiranozylo)-5′-metoksyflawonu
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17. Łużny M., Kozłowska E., Tronina T., Kostrzewa-Susłow E., Janeczko T.:
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Projekty badawcze

1. „Otrzymywanie naturalnych flawonoidów o zwiększonej biodostępności metodami biotechnologicznymi" – grant NCN SONATA., nr 2015/17/D/NZ9/02060 (wysokość finansowania 599 920 zł). Czas realizacji 09.03.2016-08.03.2019 – udział w projekcie.
2. Zadanie badawcze: Dotacja Statutowa „Synteza i biotransformacje wybranych związków flawonoidowych", nr B030/0005/18 (wysokość finansowania 40 176 zł). Czas realizacji 30.05.2018-31.12.2018 - kierownik zadania badawczego
3. Zadanie badawcze: Innowacyjny Doktorat - „Chemoenzymatyczna synteza dihydrochalkonów – potencjalnych słodzików", nr N070/0012/2020 (wysokość finansowania 30 000 zł). Czas realizacji 30.05.2020-31.12.2020 – kierownik zadania badawczego