Supporting Information

Targeting Tumor Associated Carbonic Anhydrase IX and XII: Highly Isozyme Selective Coumarin and Psoralen Inhibitors

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Chemistry

General methods

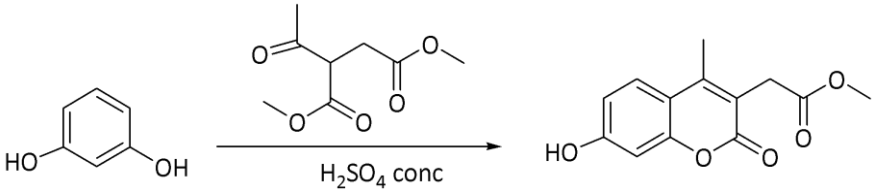
Reagents and solvents were obtained from commercial suppliers and were used without further purification. All melting points were determined by the capillary method on a Stuart SMP30 Digital Advanced apparatus and are uncorrected.

Mass spectra were registered on a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Germany). Compounds were initially dissolved in dimethylsulfoxide (DMSO) at 1 mg/mL concentration. Stock solutions were then diluted 100-fold in ethanol:water 4:1 containing 0.1% of formic acid. Mass spectra were acquired on a Q-Exactive mass spectrometer (Thermo Fisher Scientific) *via* a nano-electrospray interface operating in positive ion mode. Ion transfer tube temperature was 250 °C, whereas S-lens value was 50 units. Full MS spectra were acquired at resolution of 140,000, in the *m/z* range 200-800, using an in-source CID of 20 eV in order to minimize the presence of adducts with DMSO. Mass spectra are reported in Figures S2-S13.

^{1}H-NMR chemical shifts of compounds are reported, and spectra are depicted in Figures S14-S25. All samples were measured in DMSO-*d6* at 278.1 K temperature on a Varian UNITY INOVA 500 MHz or on a Bruker AVANCE III 400 spectrometer. Chemical shifts are reported in ppm. Coupling constants *J* are expressed in hertz (Hz).
TLC chromatography was performed using silica gel plates (Merck F 254), spots were visualized by UV light.

Synthesis and characterization

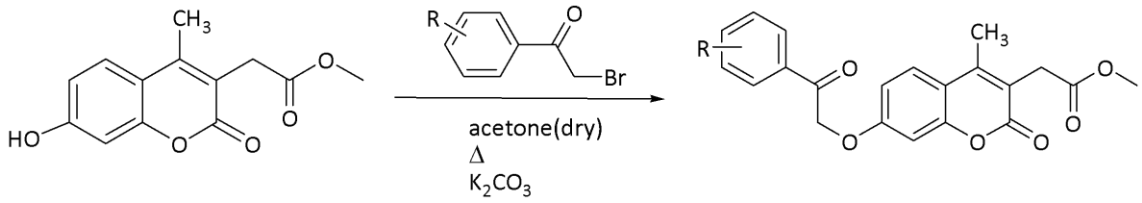
Synthesis of methyl 2-(7-hydroxy-4-methyl-2-oxo-2*H*-chromen-3-yl)acetate (EMAC10150)



A mixture of resorcinol (1 eq), dimethylacetylsuccinate (1 eq) and sulfuric acid 98% (2.8 eq) was vigorously stirred at room temperature. The progression of the reaction was monitored by TLC, using ethyl acetate/n-hexane 2:1. After 30 minutes a homogeneous sticky solid was obtained which was dissolved in methanol and poured into ice water. The mixture was stirred until ice melting and then filtered off to obtain a light yellow solid. The crude product was washed with ethyl ether giving a white powder which was crystallised from ethanol/water.

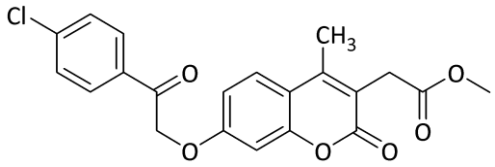
White solid; MW 248.23 g/mol; yield: 68%; Mp 180-182°C
^{1}H NMR (500 MHz, DMSO-*d6*, δ): 2.35 (s, 3H, -CH_3); 3.62 (s, 3H, -OCH_3), 3.64 (s, 2H, -CH_2), 6.71 (d, 1H, -CH aromatic, J_m=2.5), 6.83 (dd, 1H, -CH aromatic, J_o=9; J_m=2,5), 7.65 (d, 1H, -CH aromatic, J_o=8.5;), 10.47 (bs, 1H, -OH phenol).

General procedure for the synthesis of derivatives EMAC10151 A-D



A hot solution of methyl 2-(7-hydroxy-4-methyl-2-oxo-2H-chromen-3-yl)acetate (1 eq) in dry acetone was treated with K_2CO_3(2.5 eq), stirred vigorously and treated with the appropriate α -haloketone (1.1eq). The reaction mixture was heated to reflux and stirred for 1-5 h (course of reaction monitored by TLC using ethyl acetate/n-hexane 2:1) and poured into H_2SO_4 solution (100 mL, 1 N). The resulting precipitate was filtered off and crystallised from ethanol/water.

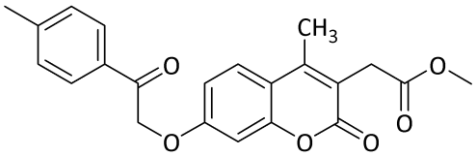
Methyl 2-{7-[2-(4-chlorophenyl)-2-oxoethyl]-4-methyl-2-oxo-2*H*chromen-3-yl}acetate (EMAC10151 A)



White solid; MW 400.81 g/mol; yield 84%; mp 167-168°C

^{1}H NMR (400 MHz, DMSO-*d*6, δ): 2.39 (s, 3H, -CH_3); 3.62 (s, 3H, $COOCH_3$); 3.68 (s, 2H, -CH_2); 5.74 (s, 2H, -CH_2); 7.06 (dd, 1H, -CH aromatic, J_o=7.2; J_m= 2); 7.12 (d, 1H, -CH aromatic; J_m= 2); 7.67 (d, 2H, -CH aromatic, J_o=6.8); 7.77 (d, 1H, -CH aromatic, J_o=7.2); 8.05 (d, 2H, -CH aromatic, J_o=6.8).

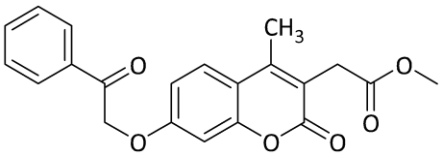
Methyl 2-{7-[2-(4-methylphenyl)-2-oxoethyl]-4-methyl-2-oxo-2*H*chromen-3-yl}acetate (EMAC10151 B)



White crystals; MW 380.39 g/mol; yield 84%; mp 174-176°C

^{1}H NMR (400 MHz, DMSO-*d6*, δ): δ 2.39 (s, 3H, -CH_3); 2.41 (s, 3H, -CH_3); 3.62 (s, 3H, $COOCH_3$); 3.67 (s, 2H, -CH_2); 5.70 (s, 2H, -CH_2); 7.04 (dd, 1H, -CH aromatic, J_o=7.2; J_m=2); 7.08 (d, 1H, -CH aromatic; J_m=2); 7.39 (d, 2H, -CH aromatic, J_o=6.8); 7.76 (d, 1H, -CH aromatic, J_o=7.2); 7.94 (d, 2H,-CH aromatic, J_o=6.8).

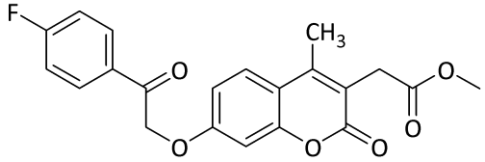
Methyl-2-[4-methyl-7-(2-oxo-2-phenylethoxy)-2-oxo-2*H*-3-chromenyl]acetate (EMAC10151 C)



Off-white crystals; MW 366.36 g/mol; yield 90%, mp 144-145°C

^{1}H-NMR (400 MHz, DMSO-*d6*, δ): 2.39 (s, 3H, -CH_3); 3.62 (s, 2H, -CH_2); 3.67 (3H, s, $COOCH_3$); 5.75 (s, 2H, -CH_2); 7.00 (dd, 1H, -CH aromatic, J_o= 6.8, J_m=2,); 7.04 (d, 1H, -CH aromatic, J_m=2); 7.55 (t, 2H, -CH aromatic, J_o=6); 7.73 (m 2H, CH aromatic)); 8.03 (d, 2H, -CH aromatic, J_o= 6).

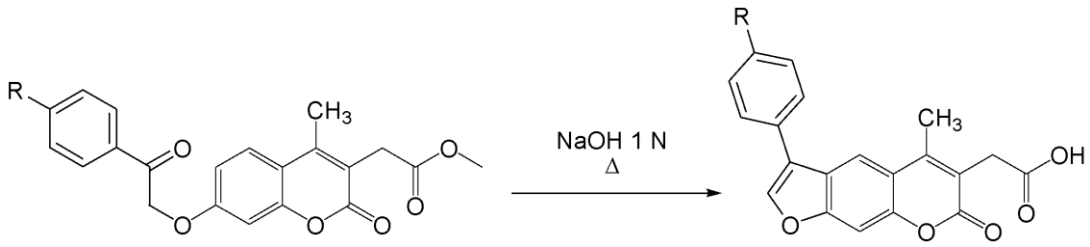
Methyl 2-{7-[2-(4-fluorophenyl)-2-oxoethyl]-4-methyl-2-oxo-2*H*chromen-3-yl}acetate (EMAC10151 D)



Off-white crystals; MW 384.35 g/mol; yield 88%, mp 153-154°C

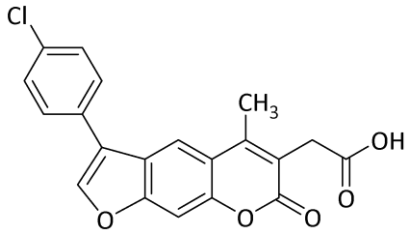
^{1}H NMR (400 MHz, DMSO- *d6*, δ): 2.38 (s, 3H, -CH_3); 3.61 (s, 3H, $COOCH_3$); 3.66 (s, 2H, -CH_2); 5.68 (s, 2H, -CH_2); 7.04 (dd, 1H, -CH aromatic, J_o=7.2, J_m=2); 7.08 (d, 1H, -CH aromatic; J_m=2); 7. 41 (t, 2H, -CH aromatic, J_o=7.2); 7.75 (d, 1H, -CH aromatic, J_o=7.2); 8.11 (t, 2H, -CH aromatic, J_o=7.2).

General procedure for the synthesis of furocoumarins (EMAC10152 A-D)



A solution or suspension of coumarin (1 eq) in propan-2-ol was treated with NaOH solution (4 eq, 1 N). The reaction mixture was heated for 3-4 hours, obtaining a dark solution. The solution was cooled at room temperature and poured into ice water. The pH of this solution was acidified to 4-5 by the addition of concentrated HCl, obtaining a suspension that was filtered off and re-crystallised from propan-2-ol.

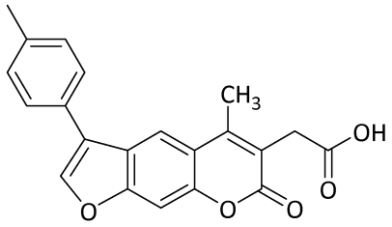
2-(3-(4-chlorophenyl)-5-methyl-7-oxo-7H-furo[3,2-g]chromen-6yl)acetic acid (EMAC10152 A)



White crystal; MW 368.77 g/mol; yield 82%; mp 235-237°C

^{1}H NMR (400 MHz, DMSO- *d6*, δ*)*: 2.54 (s, 3H, -CH_3); 3.65 (s, 2H, -CH_2); 7.59 (d, 2H, -CH aromatic, J_o=6.8); 7.80 (s, 1H, aromatic); 7.86 (d, 2H, -CH aromatic, J_o=6.8); 8.18 (s, 1H, -CH aromatic); 8.51 (s, 1H, -CH aromatic); 12.46 (s, 1H, -COOH).

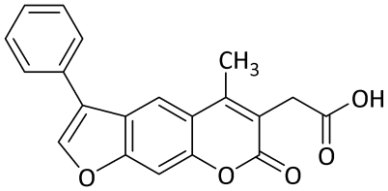
2-[5-methyl-3-(4-methylphenyl)-7-oxo-7H-furo[3,2-g]chromen-6-yl]acetic acid (EMAC10152 B)



White solid; MW 348.35 g/mol; yield 83%; mp 203-205°C

^{1}H NMR (400 MHz, DMSO- *d6*, δ*)*: 2.38 (s, 3H, CH_3, 4-CH_3 phenyl); 2.53 (s, 3H, -CH_3); 3.66 (s, 2H, -CH_2); 7.36 (d, 2H, -CH aromatic, J_o=6.4); 7.71 (d, 2H, -CH aromatic, J_o=6.4); 7.79 (1H, s, -CH aromatic); 8.18 (s, 1H, -CH aromatic); 8.42 (s, 1H, -CH aromatic); COOH not detected.

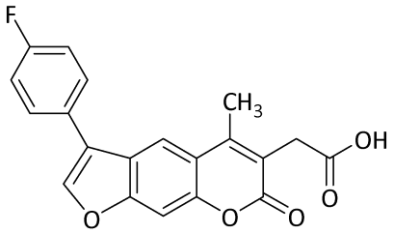
2-{5-methyl-7-oxo-3-phenyl-7H-furo[3,2-g]chromen-6-yl}yacetic acid (EMAC10152 C)



Off-white solid; MW 334.32 g/mol; yield 92%, mp 209-211°C.

^{1}H-NMR (400 MHz, DMSO- *d6*, δ*)*: 2.55 (s, 3H, -CH_3); 3.66 (s, 2H, -CH_2); 7.44 (t, 1H, -CH aromatic, *J* =6), 7.55 (t, 2H, -CH aromatic, *J* =6), 7.82 (m, 3H, -CH aromatic); 8.21 (s, 1H, -CH aromatic); 8.48 (s, 1H, -CH aromatic), 12.46 (s, 1H, -COOH).

2-[3-(4-fluorophenyl)-5-methyl-7-oxo-7H-furo[3,2-g]chromen-6-yl]acetic acid (EMAC10152 D)

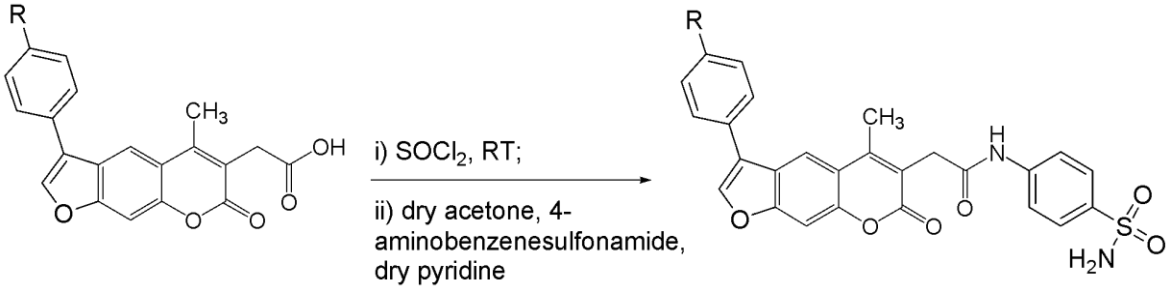


White solid; MW 352.31 g/mol; Yield 85%, mp 245-246°C.

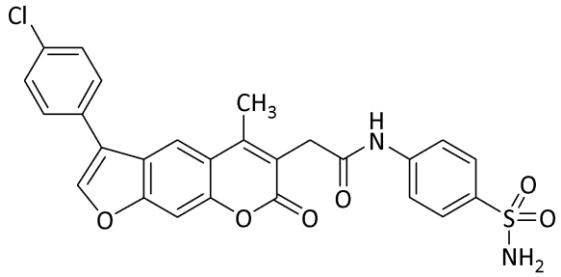
^{1}H NMR (400 MHz, DMSO- *d6*, δ*)*: 2.54 (s, 3H, -CH_3); 3.65 (s, 2H, -CH_2); 7.39 (m, 2H, -CH aromatic); 7.81 (s, 1H, -CH aromatic); 7.87 (m, 2H, -CH aromatic); 8.18 (s, 1H, -CH aromatic), 8.47 (s, 1H, -CH aromatic); COOH not detected).

General procedure for the synthesis of amides (EMAC10153 A-D)

A solution of the **EMAC10153 A-D** acid (1.0 eq) in thionyl chloride (25 mL) was stirred at room temperature until a clear solution was formed. The solvent was evaporated under reduced pressure, and the residue was dissolved in 20 mL of dry acetone. The obtained solution was added dropwise to a stirred solution of 4-aminobenzenesulfonamide (1.2 eq) and dry pyridine (1.1 eq) in 50 mL of dry acetone. After addition, the reaction mixture was stirred overnight at room temperature and monitored by TLC (ethyl acetate/n-hexane 2:1). The precipitate was filtered and washed by a 10% water solution of $NaHCO_3$. Then, the solid was washed with water and filter to afford the desired product.



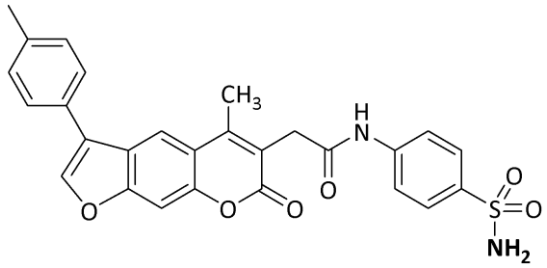
2-[3-(4-chlorophenyl)-5-methyl-7-oxo-7H-furo[3,2-g]chromen-6-yl]-N-(4-sulfamoylphenyl)acetamide (EMAC10153 A)



White solid; MW 522.96 g/mol; yield 55%, mp 311°C and decomposed.

^{1}H NMR (400 MHz, DMSO- *d6, δ)*: 2.59 (s, 3H, -CH_3); 3.85 (s, 2H, -CH_2); 7.21 (bs, 2H, SO_2NH_2); 7.60 (d, 2H, -CH aromatic, J_o=8.8); 7.75 (s, 4H, -CH aromatic, 4-SO_2NH_2 phenyl); 7.84 (s, 1H, -CH aromatic); 7.87 (d, 2H, -CH aromatic, J_o=8.4); 8.22 (s, 1H, -CH aromatic); 8.54 (s, 1H, -CH aromatic); 10.55 (bs, 1H, NH amide).

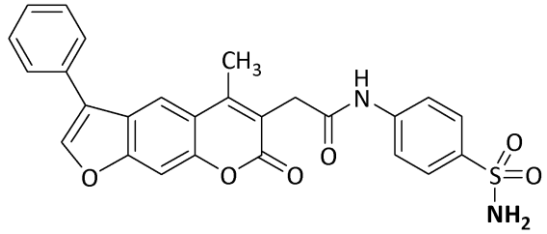
2-[5-methyl-3-(4-methylphenyl)-7-oxo-7H-furo[3,2-g]chromen-6-yl]-N-(4-sulfamoylphenyl)acetamide (EMAC10153 B)



White solid; MW 502.54 g/mol; yield 58%, mp 310°C and decomposed.

^{1}H NMR (400 MHz, DMSO- *d6, δ)*: 2.38 (s, 3H, CH_3, 4-CH_3 phenyl); 2.58 (s, 3H, -CH_3); 3.85 (s, 2H, -CH_2); 7.25 (s, 2H, SO_2NH_2); 7.36 (d, 2H, -CH aromatic, J_o=8.0); 7.71 (d, 2H, -CH aromatic, J_o=8.0); 7.76 (s, 4H, -CH aromatic, 4-SO_2NH_2 phenyl); 7.81 (s, 1H, -CH aromatic); 8.20 (s, 1H, -CH aromatic); 8.43 (s, 1H, -CH aromatic); 10.53 (s, 1H, NH amide).

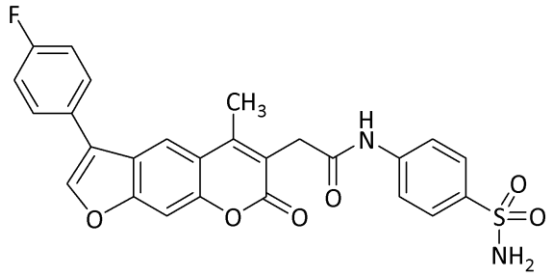
2-{5-methyl-7-oxo-3-phenyl-7H-furo[3,2-g]chromen-6-yl}-N-(4-sulfamoylphenyl)acetamide (EMAC10153 C)



White solid; MW 488.51 g/mol; yield 45%, mp 293-294°C and decomposed.

^{1}H NMR (400 MHz, DMSO- *d6, δ)*: 2.59 (s, 3H, -CH_3); 3.85 (s, 2H, -CH_2); 7.24 (s, 2H, SO_2NH_2); 7.42 (t,1H, -CH aromatic, *J*=8); 7.56 (t, 2H, -CH aromatic, *J*=8); 7.76 (s, 4H, CH, 4-SO_2NH_2 phenyl); 7.83-7.85 (m, 3H, -CH aromatic); 8.22 (s, 1H, -CH aromatic); 8.48 (s, 1H, -CH aromatic); 10.54 (s, 1H, NH amide).

2-[3-(4-fluorophenyl)-5-methyl-7-oxo-7H-furo[3,2-g]chromen-6-yl]-N-(4-sulfamoylphenyl)acetamide (EMAC10153 D)



Grey solid; MW 506.5 g/mol; yield 56%, mp 307°C and decomposed.

^{1}H NMR (400 MHz, DMSO- *d6, δ)*: 2.59 (s, 3H, -CH_3); 3.85 (s, 2H, -CH_2); 7.24 (s, 2H, SO_2NH_2); 7.36-7.40 (t, 2H, -CH aromatic, *J* = 8.0); 7.75 (s, 4H, CH, 4-SO_2NH_2 phenyl); 7.82 (s, 1H, -CH aromatic); 7.88 (t, 2H, -CH aromatic); 8.20 (s, 1H, -CH aromatic), 8.48(s, 1H, -CH aromatic); 10.54 (s, 1H, NH amide).

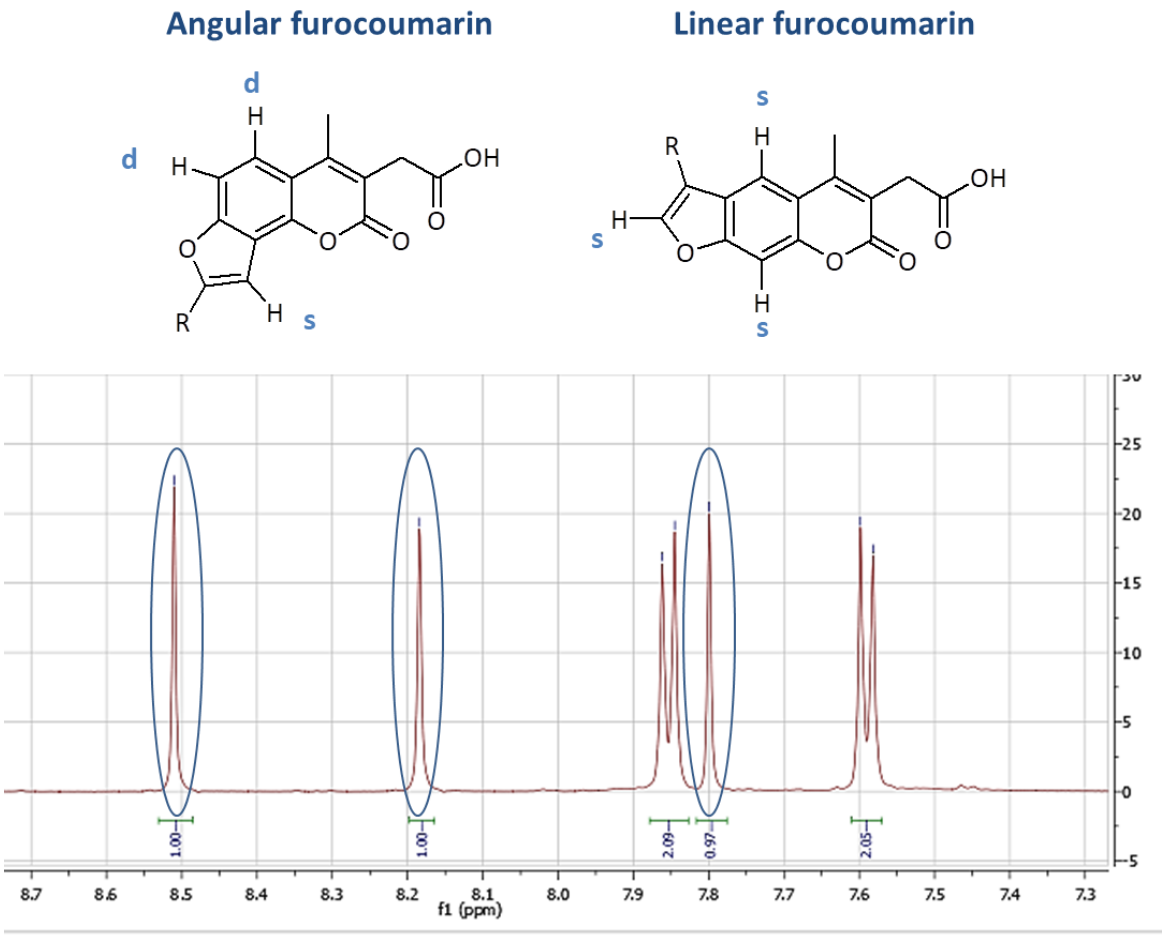


Figure S1. Portion of ^{1}H-NMR spectrum: characteristic signal pattern of synthesized linear furocoumarins.

Mass spectra of compounds EMAC10151-10153 A-D

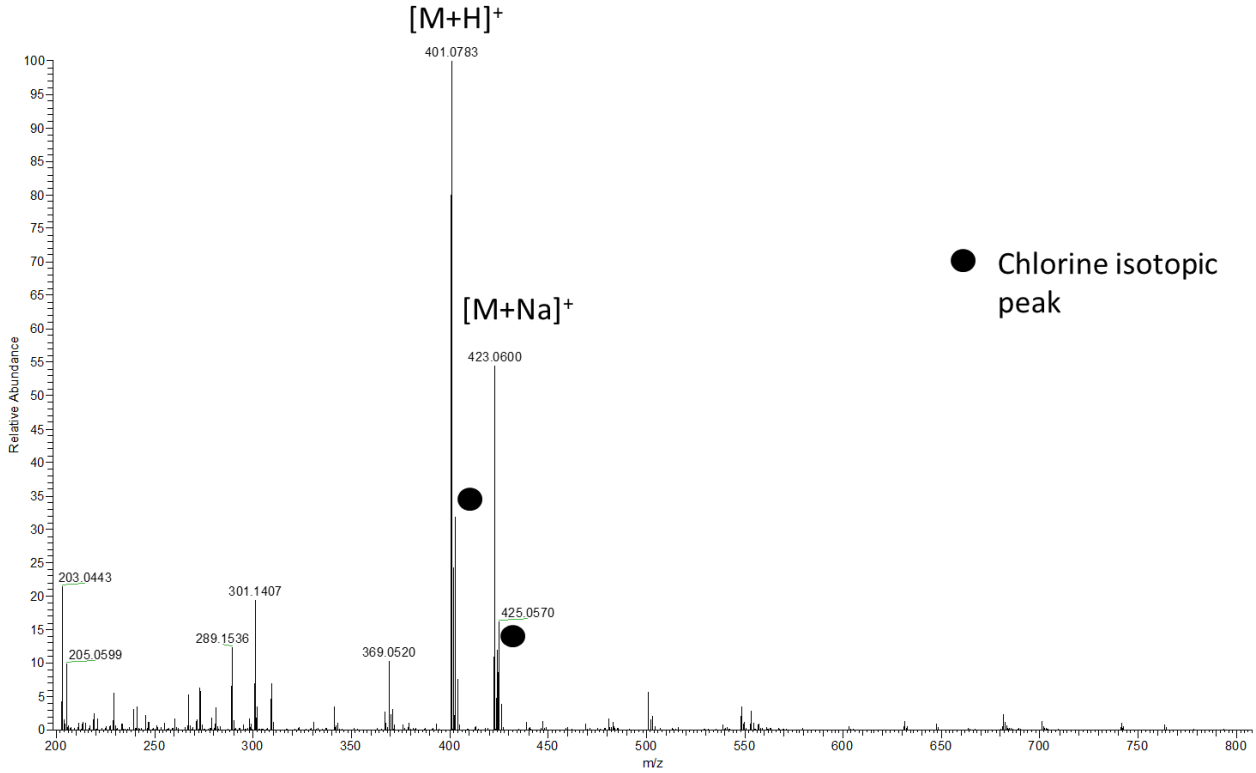


Figure S2. Full Mass Spectrum of compound **EMAC10151 A**. Molecular formula $C_{21}H_{17}ClO_6$, Exact Mass: 400.0714, Theoretical *m/z*: 401.0786, $[M+H]^+$, Found *m/z*: 401.0783, Delta mass: -0.7 ppm.

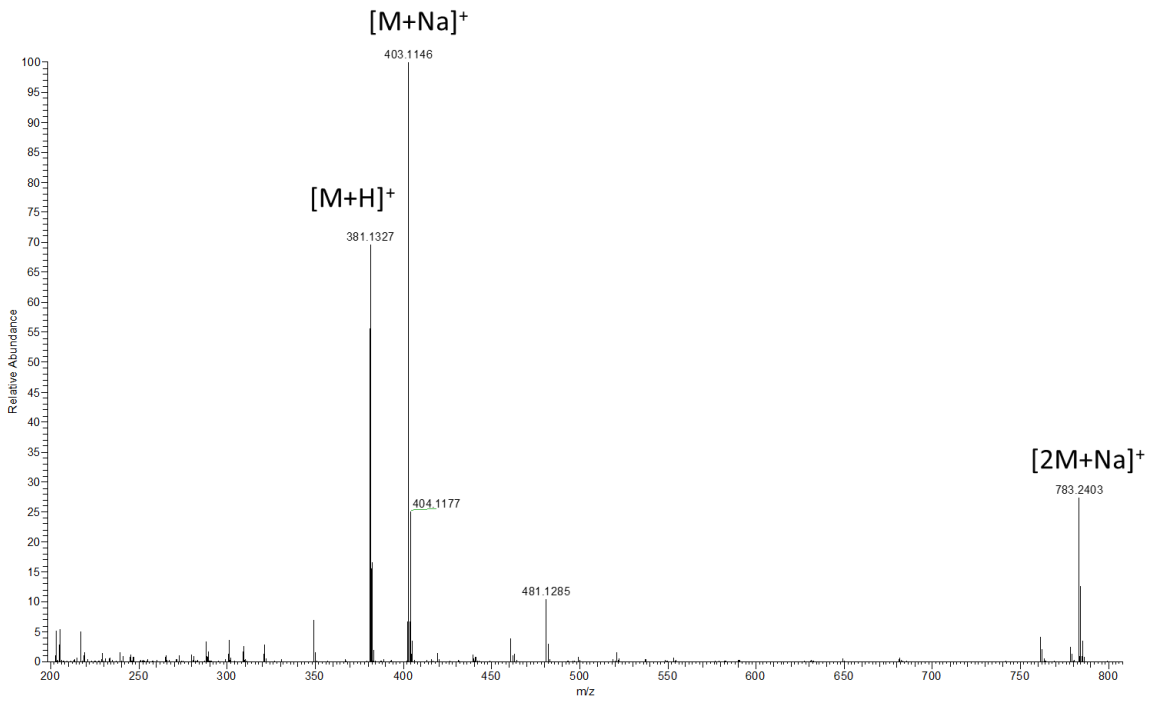


Figure S3. Full Mass Spectrum of compound **EMAC10151 B**. Molecular formula $C_{22}H_{20}O_6$, Exact Mass: 380.1260, Theoretical *m/z*: 381.1333, $[M+H]^+$, Found *m/z*: 381.1327, Delta mass: -1.6 ppm.

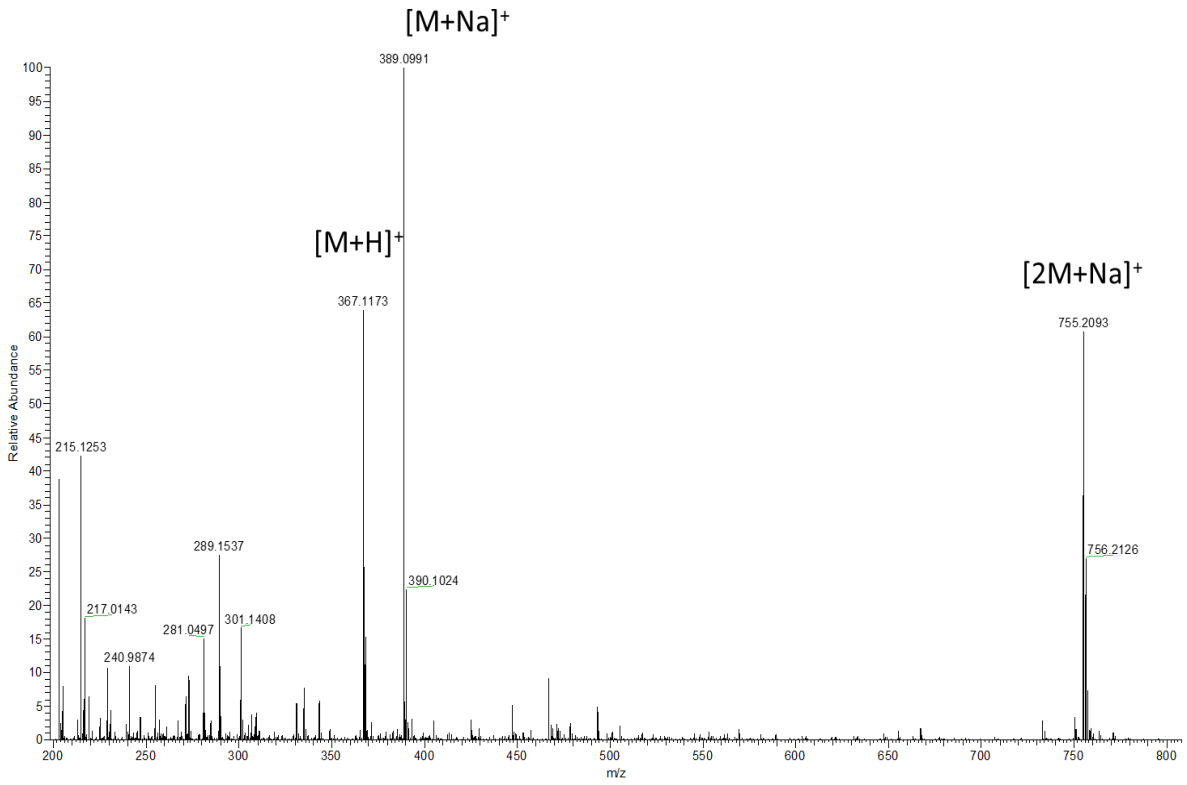


Figure S4. Full Mass Spectrum of compound **EMAC10151 C**. Molecular formula $C_{21}H_{18}O_6$, Exact Mass: 366.1103, Theoretical *m/z*: 367.1176, $[M+H]^+$, Found *m/z*: 367.1173, Delta mass: -0.8 ppm.

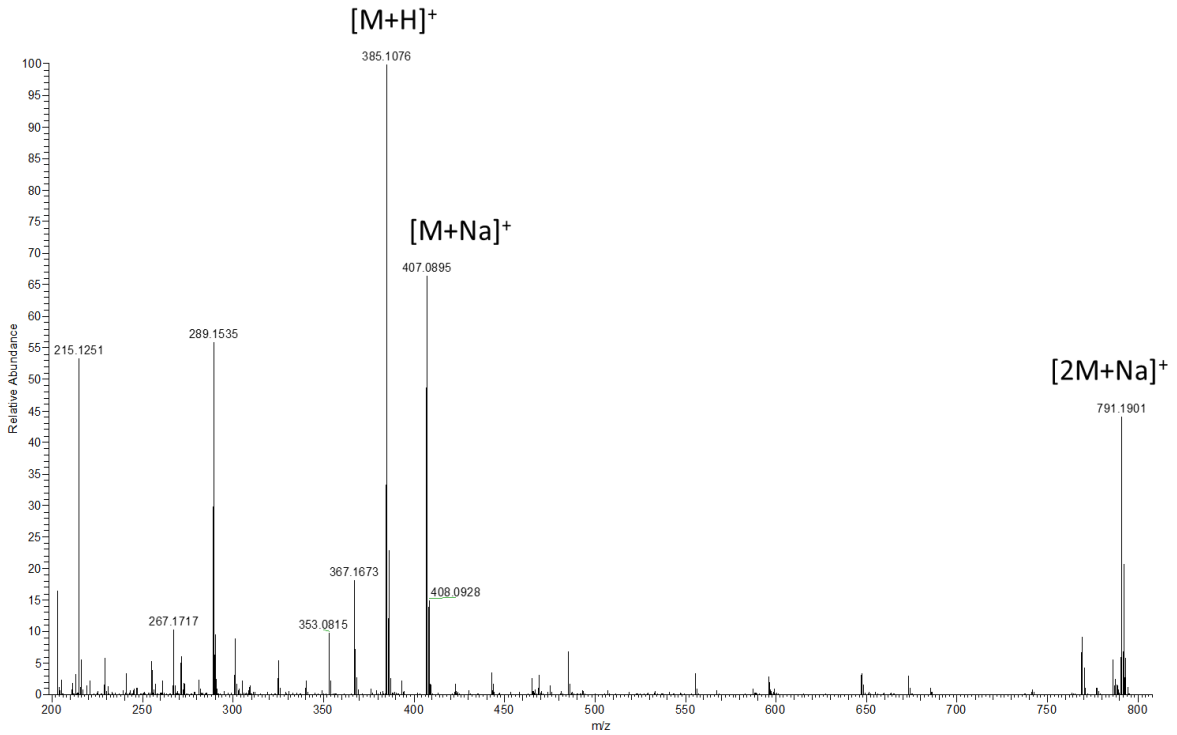


Figure S5. Full Mass Spectrum of compound **EMAC10151 D**. Molecular formula $C_{21}H_{17}FO_6$, Exact Mass: 384.1009, Theoretical *m/z*: 385.1082, $[M+H]^+$, Found *m/z*: 385.1076, Delta mass: -1.6 ppm.

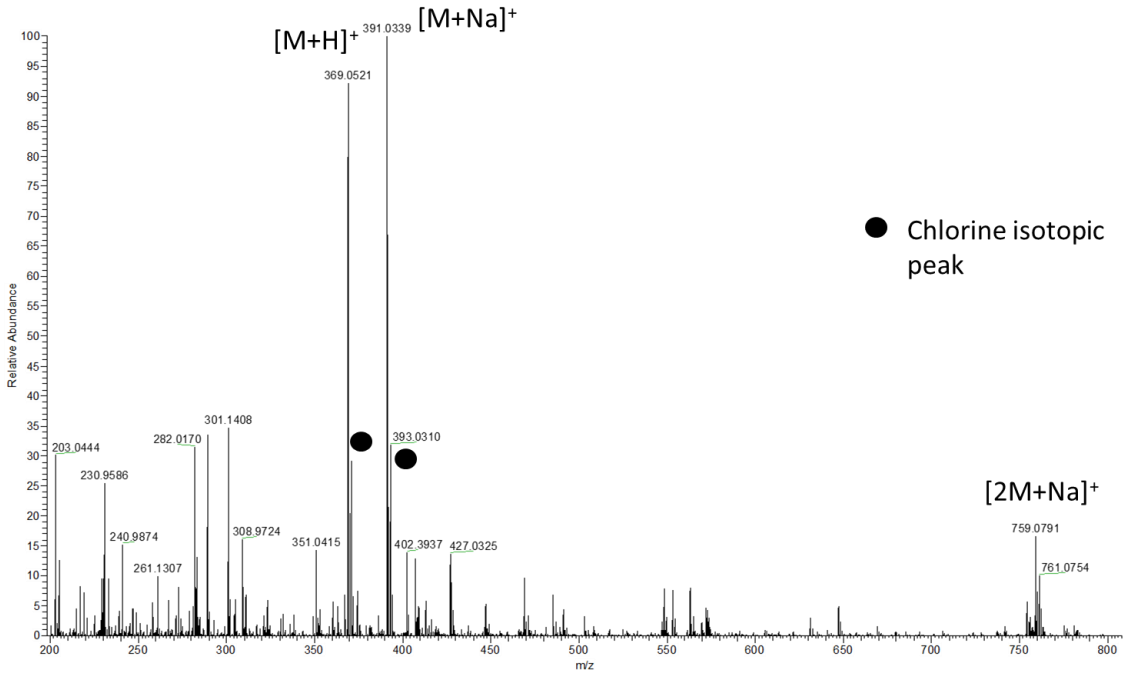


Figure S6. Full Mass Spectrum of compound **EMAC10152 A**. Molecular formula $C_{20}H_{13}ClO_5$, Exact Mass: 368.0452, Theoretical *m/z*: 369.0524, $[M+H]^+$, Found *m/z*: 369.0521, Delta mass: -0.8 ppm.

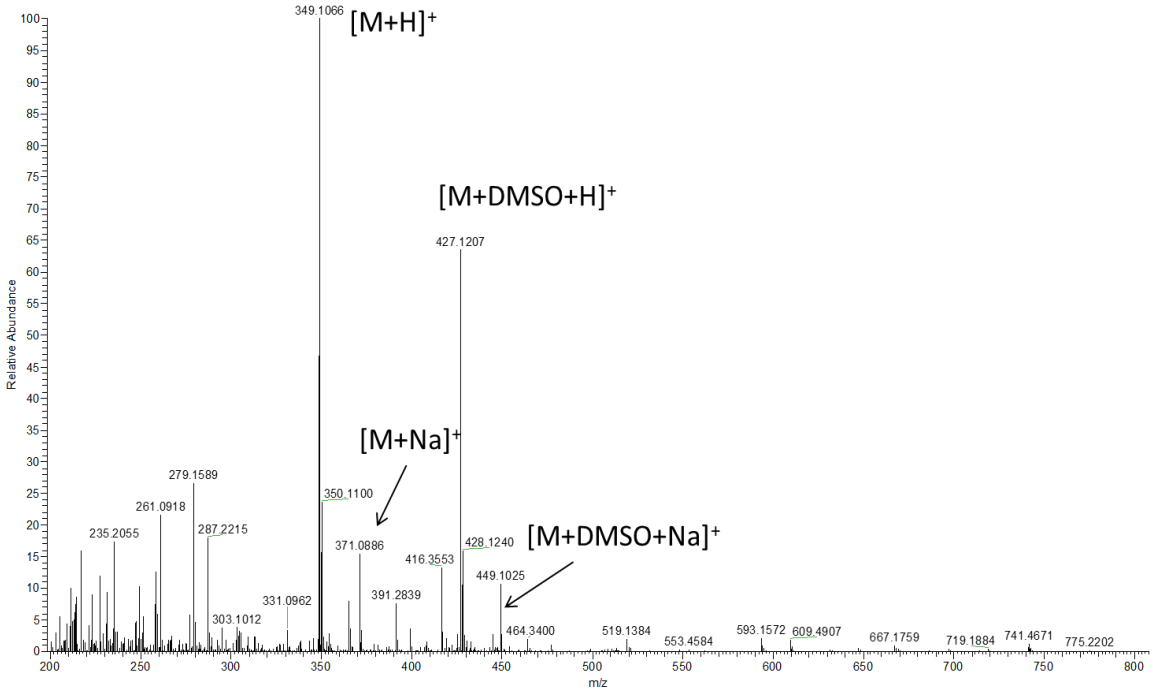


Figure S7. Full Mass Spectrum of compound **EMAC10152 B**. Molecular formula $C_{21}H_{16}O_5$, Exact Mass: 348.0998, Theoretical *m/z*: 349.1071, $[M+H]^+$, Found *m/z*: 349.1066, Delta mass: -1.4 ppm.

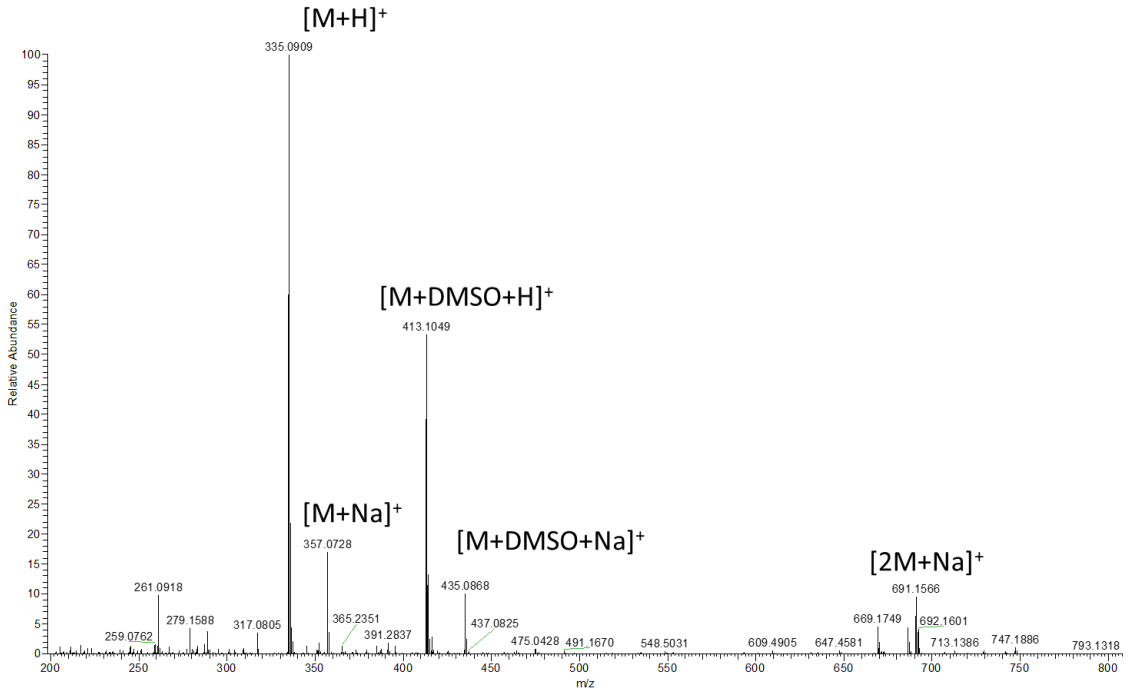


Figure S8. Full Mass Spectrum of compound **EMAC10152 C**. Molecular formula $C_{20}H_{14}O_5$, Exact Mass: 334.0841 Theoretical *m/z*: 335.0914, $[M+H]^+$, Found *m/z*: 335.0909, Delta mass: -1.5 ppm.

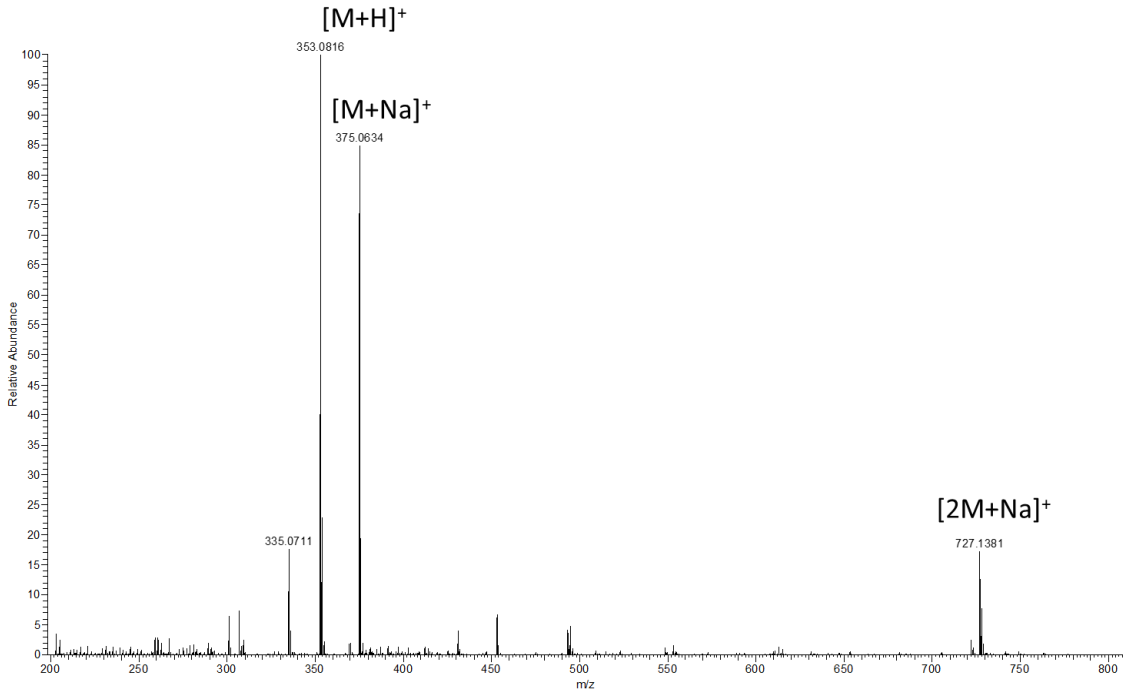


Figure S9. Full Mass Spectrum of compound **EMAC10152 D**. Molecular formula $C_{20}H_{13}FO_5$, Exact Mass: 352.0747, Theoretical *m/z*: 353.0820, $[M+H]^+$, Found *m/z*: 353.0816, Delta mass: -1.1 ppm.

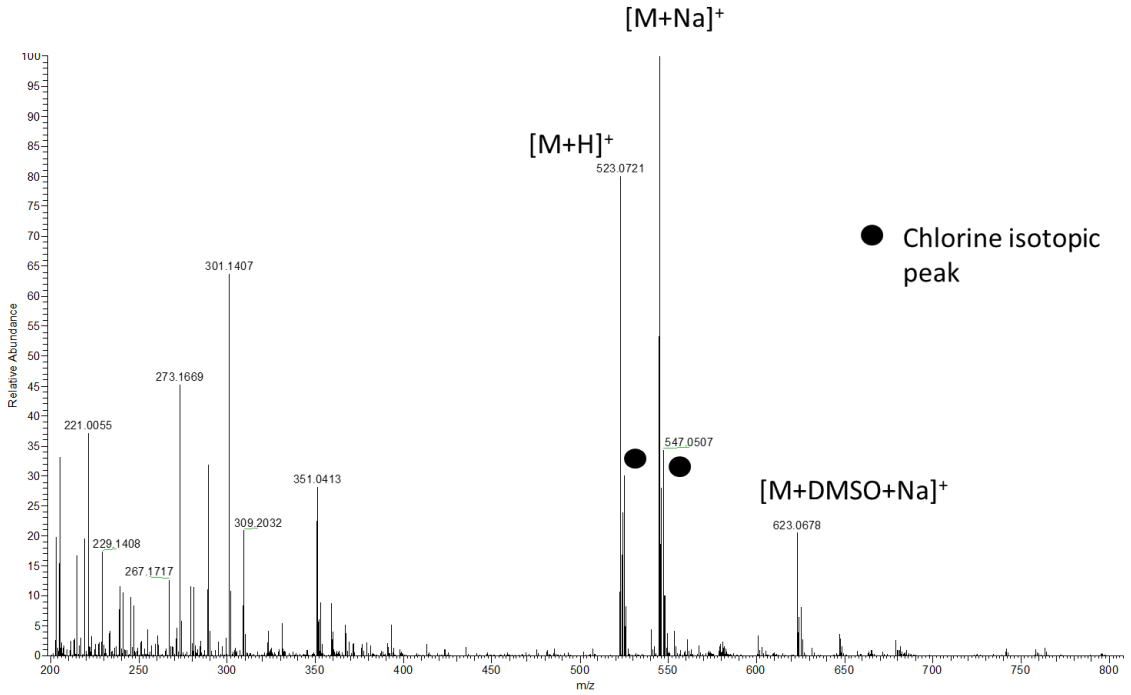


Figure S10. Full Mass Spectrum of compound **EMAC10153 A**. Molecular formula $C_{26}H_{19}ClN_2O_6S$, Exact Mass: 522.0652, Theoretical *m/z*: 523.0725, $[M+H]^+$, Found *m/z*: 523.0721, Delta mass: -0.8 ppm.

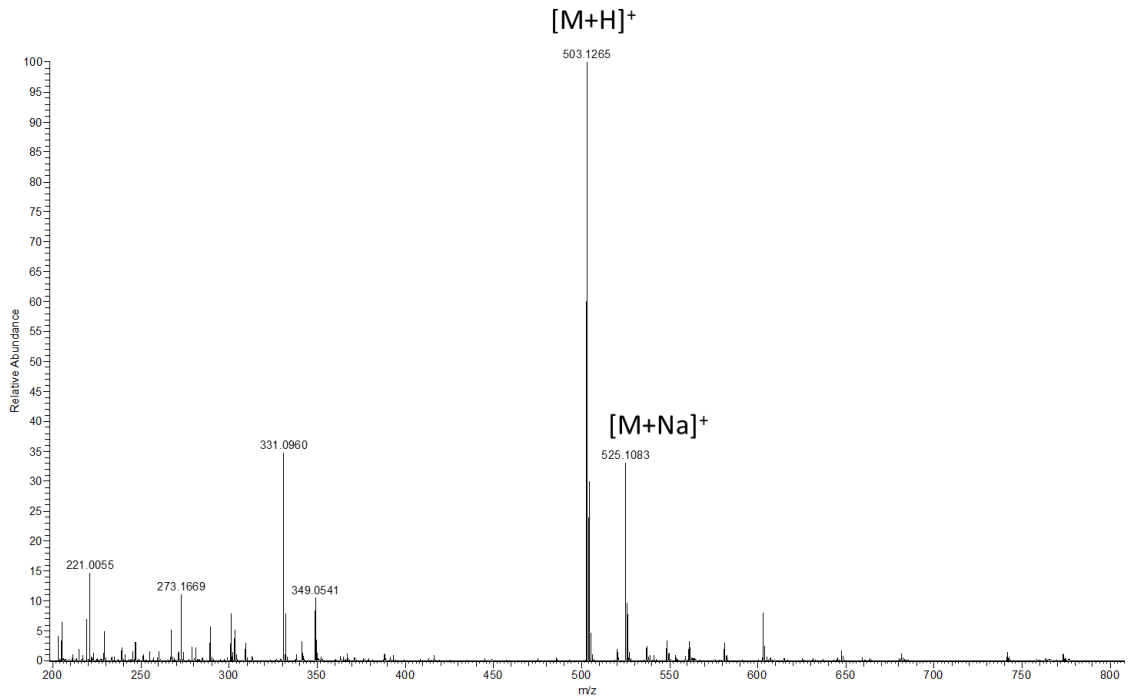


Figure S11. Full Mass Spectrum of compound **EMAC10153 B**. Molecular formula $C_{27}H_{22}N_2O_6S$, Exact Mass: 502.1199, Theoretical *m/z*: 503.1271, $[M+H]^+$, Found *m/z*: 503.1265, Delta mass: -1.2 ppm.

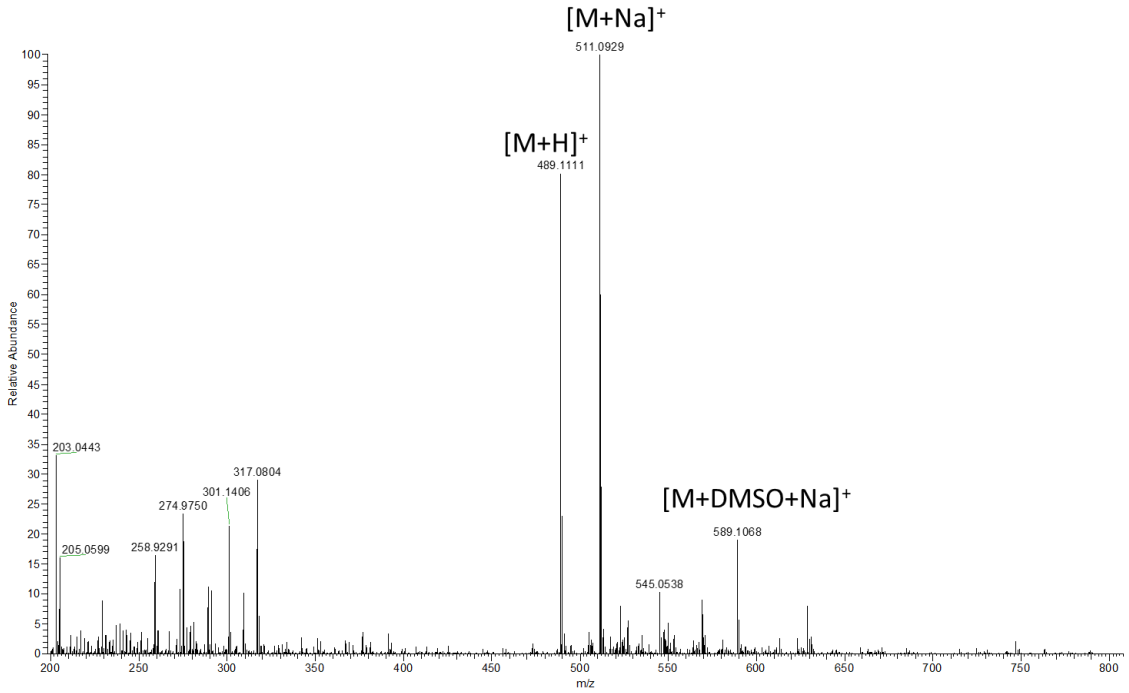


Figure S12. Full Mass Spectrum of compound **EMAC10153 C**. Molecular formula $C_{26}H_{20}N_2O_6S$, Exact Mass: 488.1042, Theoretical *m/z*: 489.1115, $[M+H]^+$, Found *m/z*: 489.1111, Delta mass: -0.8 ppm.

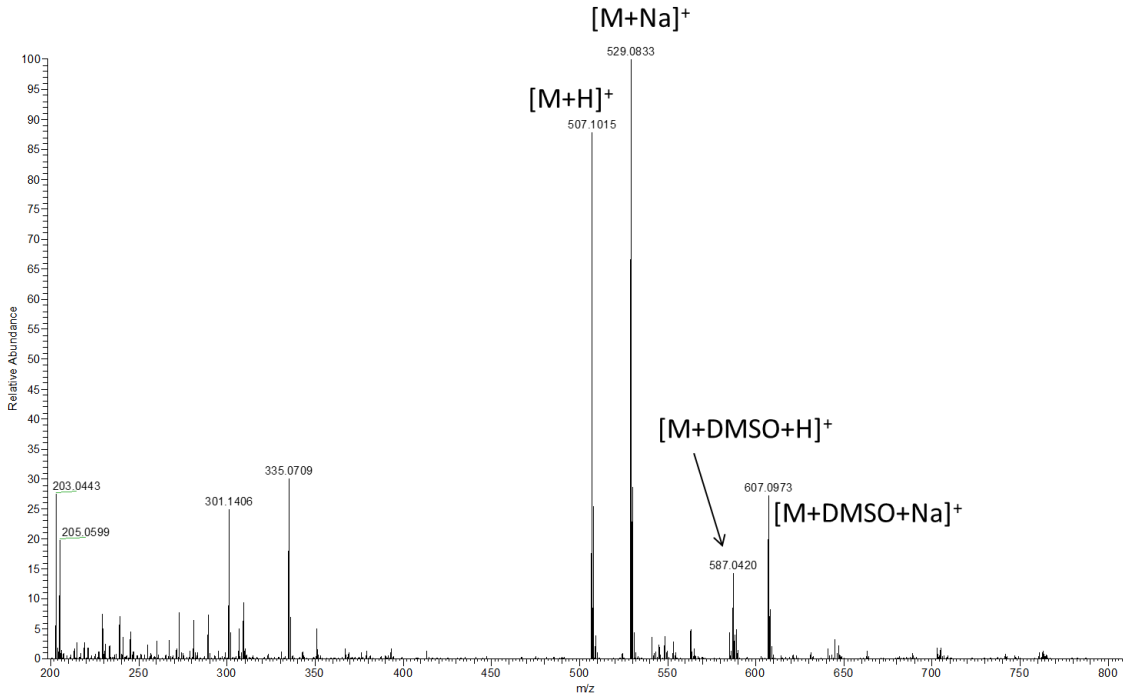


Figure S13. Full Mass Spectrum of compound **EMAC10153 D**. Molecular formula $C_{26}H_{19}FN_2O_6S$, Exact Mass: 506.0948, Theoretical *m/z*: 507.1021, $[M+H]^+$, Found *m/z*: 507.1015, Delta mass: -1.2 ppm.

[1]HNMR of compounds EMAC10151-10153 A-D

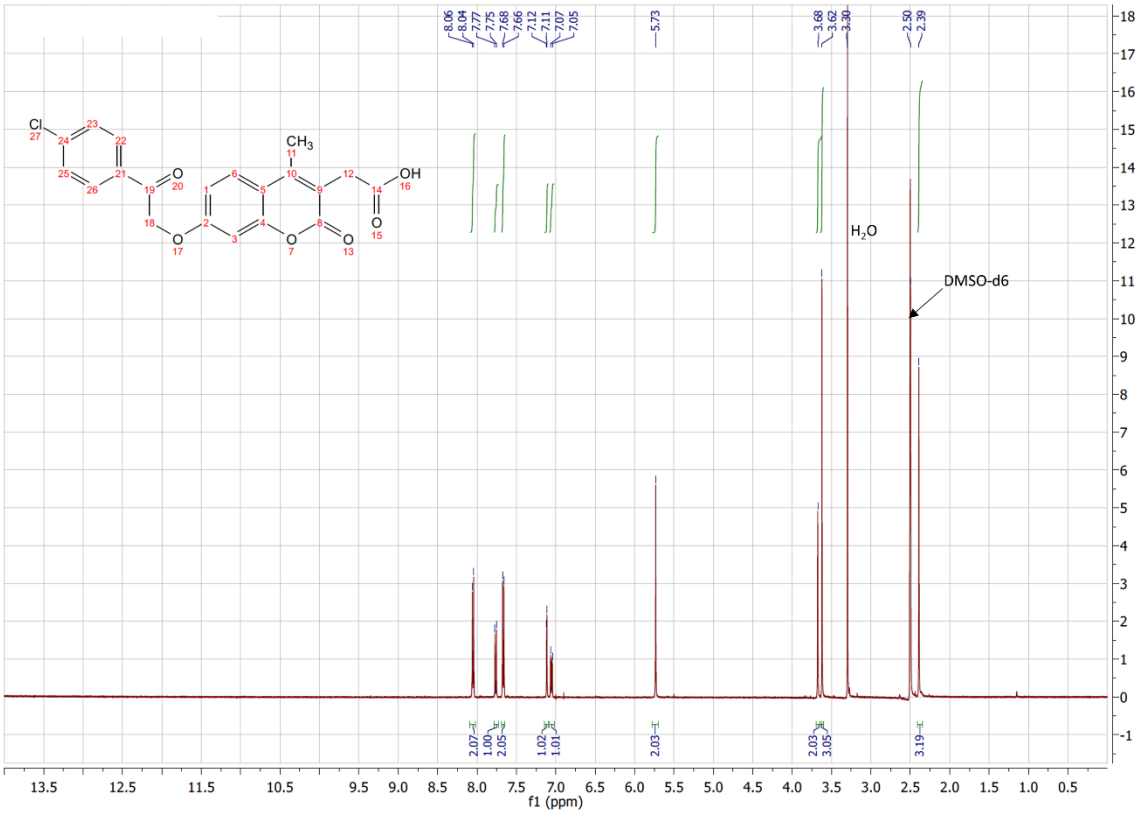


Figure S14. [1]H NMR spectrum (400 MHz, DMSO-*d6*) of **EMAC10151 A**.

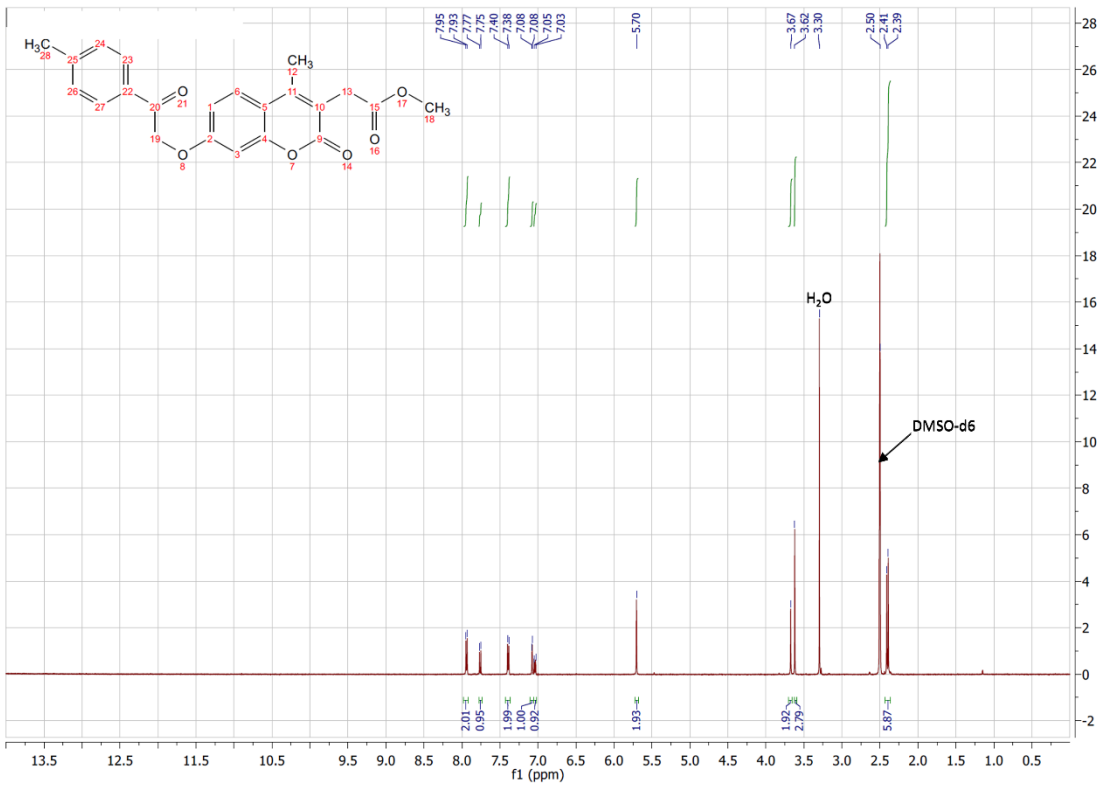


Figure S15. [1]H NMR spectrum (400 MHz, DMSO- *d6*) of **EMAC10151 B**.

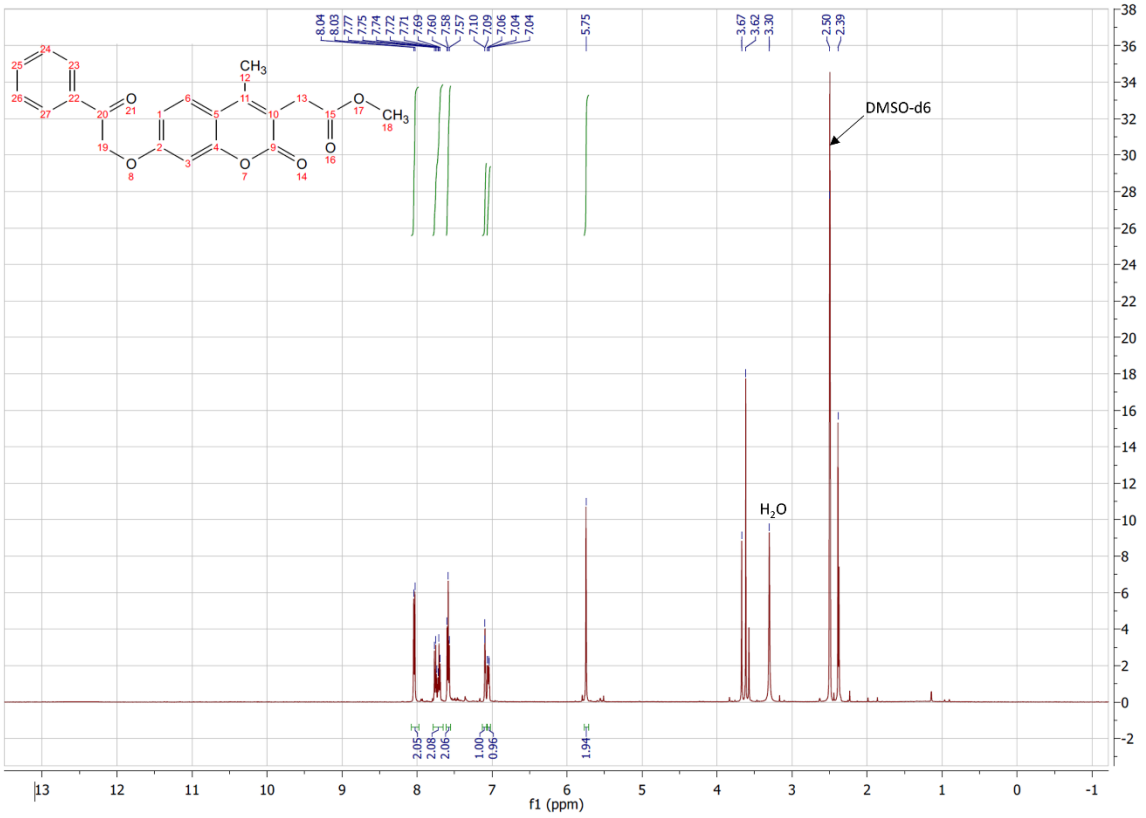


Figure S16. ^{1}H NMR spectrum (400 MHz, DMSO- *d6*) of **EMAC10151 C**.

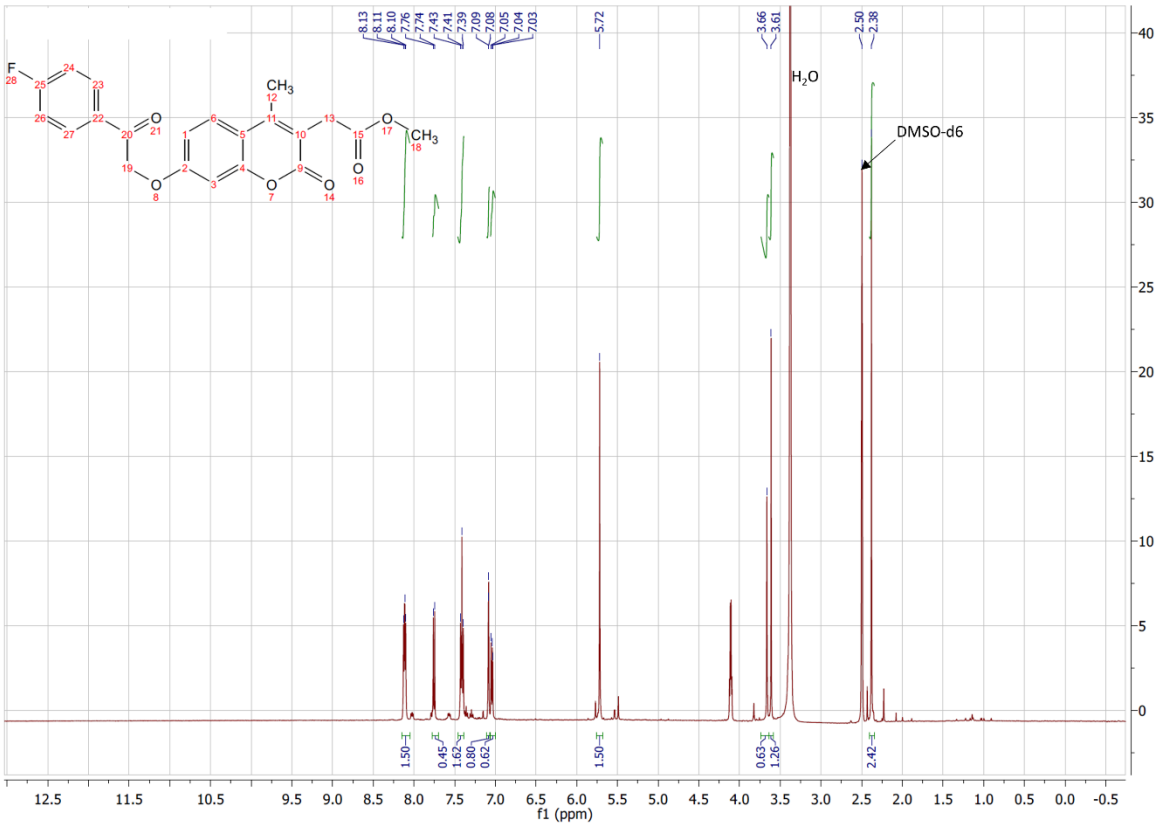


Figure S17. ^{1}H NMR spectrum (400 MHz, DMSO- *d6*) of **EMAC10151 D**.

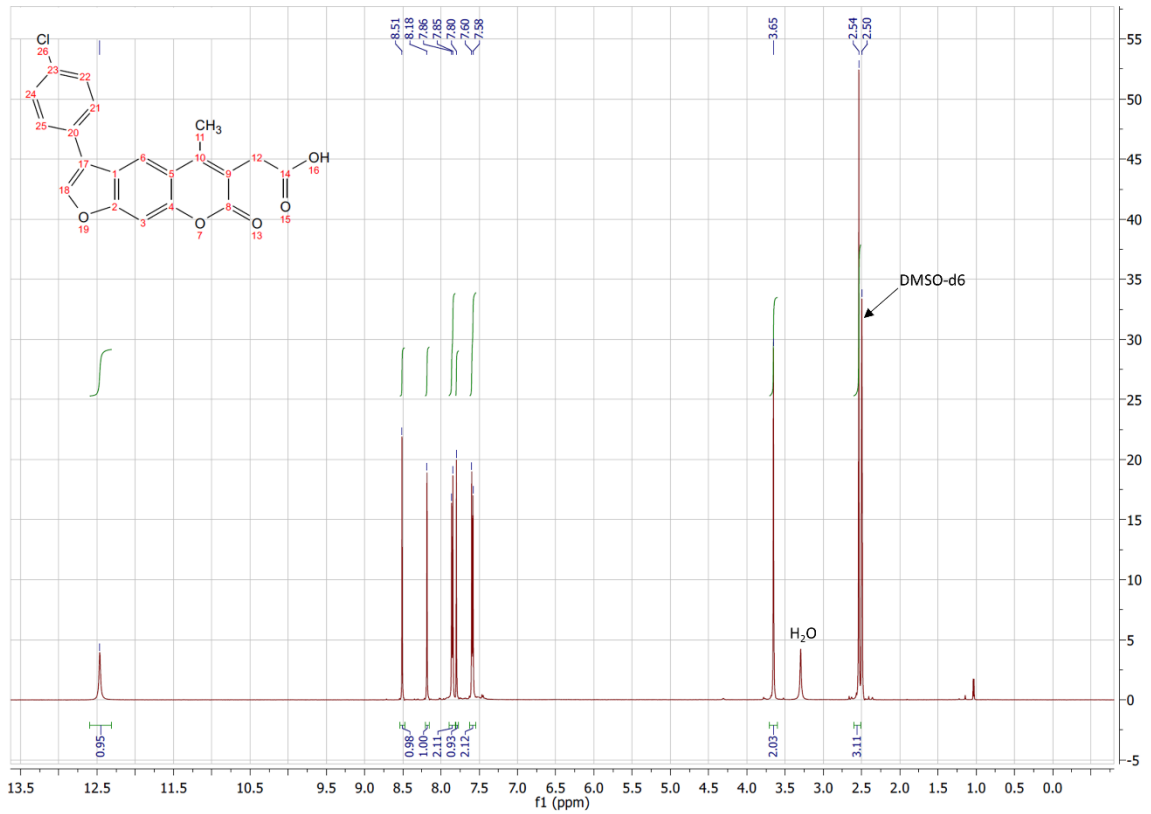


Figure S18. ^{1}H NMR spectrum (400 MHz, DMSO- *d6*) of **EMAC10152 A**.

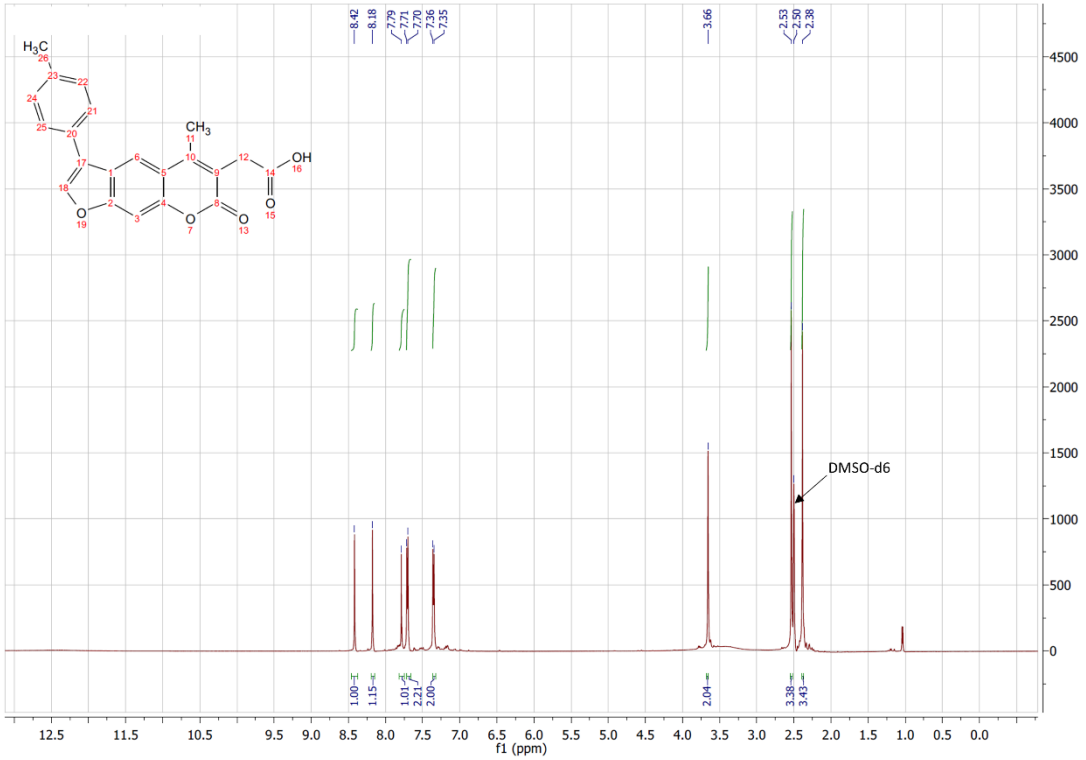


Figure S19. ^{1}H NMR spectrum (400 MHz, DMSO- *d6*) of **EMAC10152 B**.

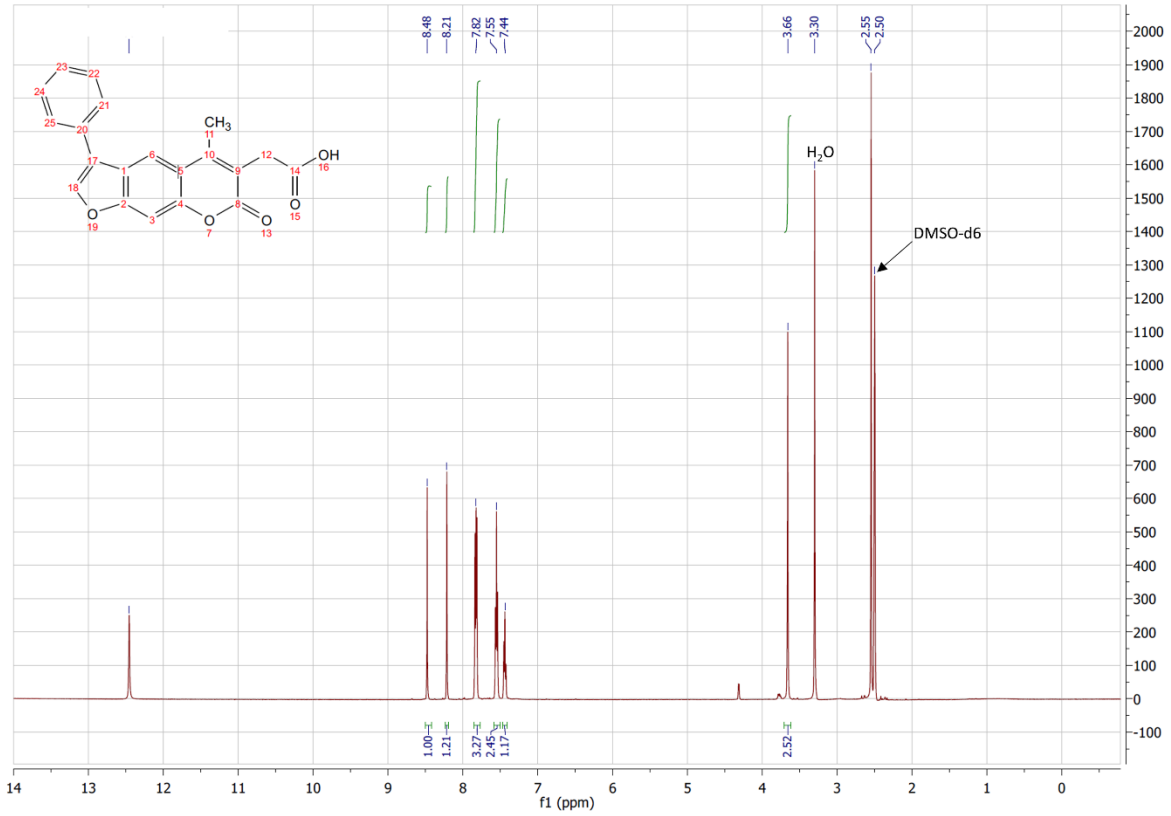


Figure S20. [1]H NMR spectrum (400 MHz, DMSO- *d6*) of **EMAC10152 C**.

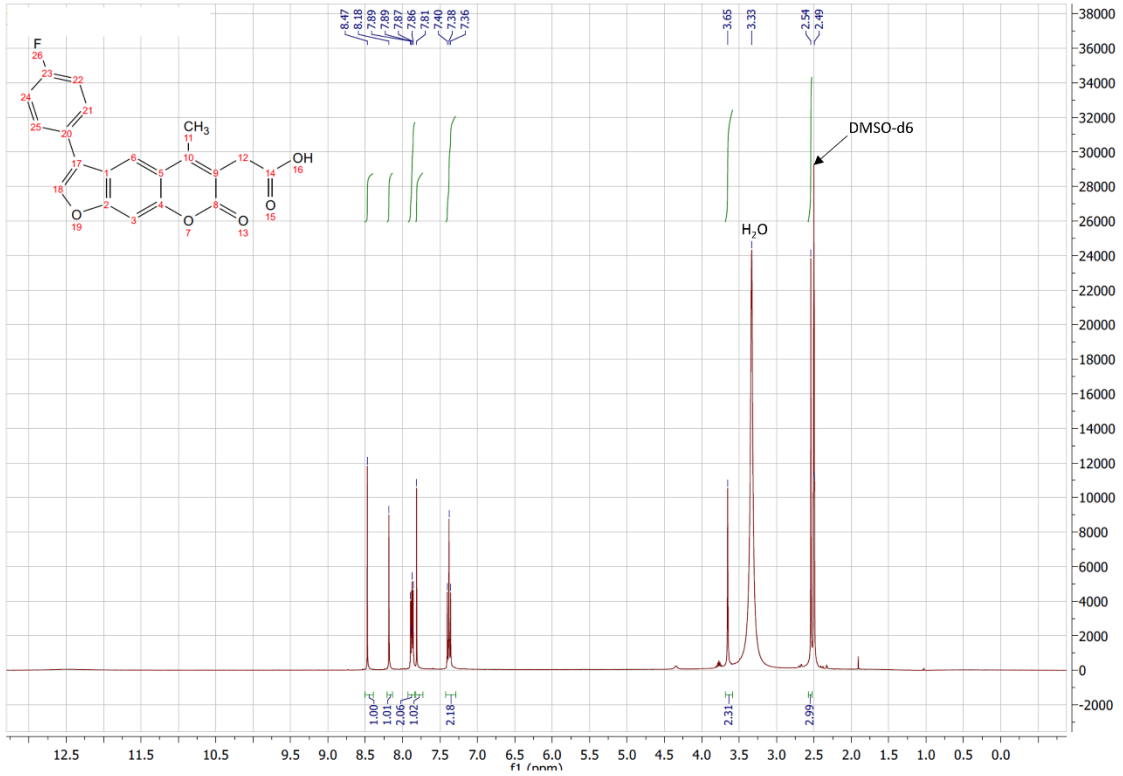


Figure S21. [1]H NMR spectrum (400 MHz, DMSO- *d6*) of **EMAC10152 D**.

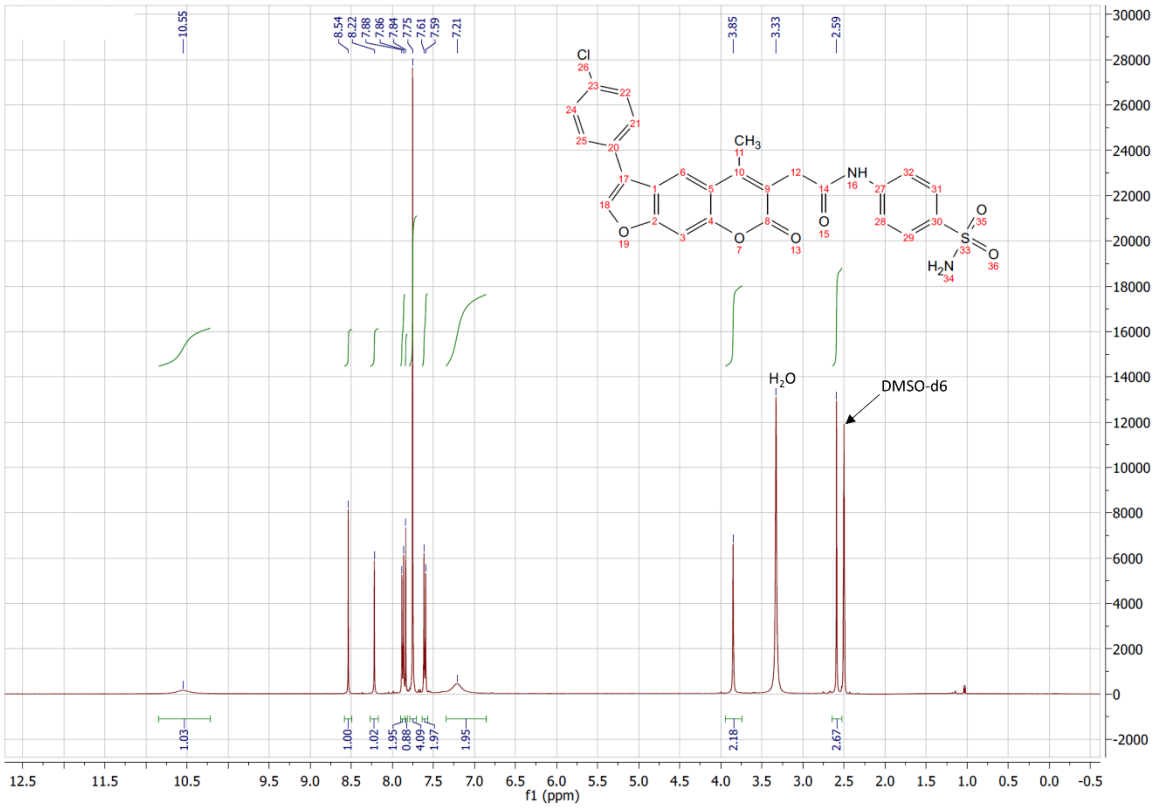


Figure S22. ^{1}H NMR spectrum (400 MHz, DMSO- *d6*) of **EMAC10153 A**.

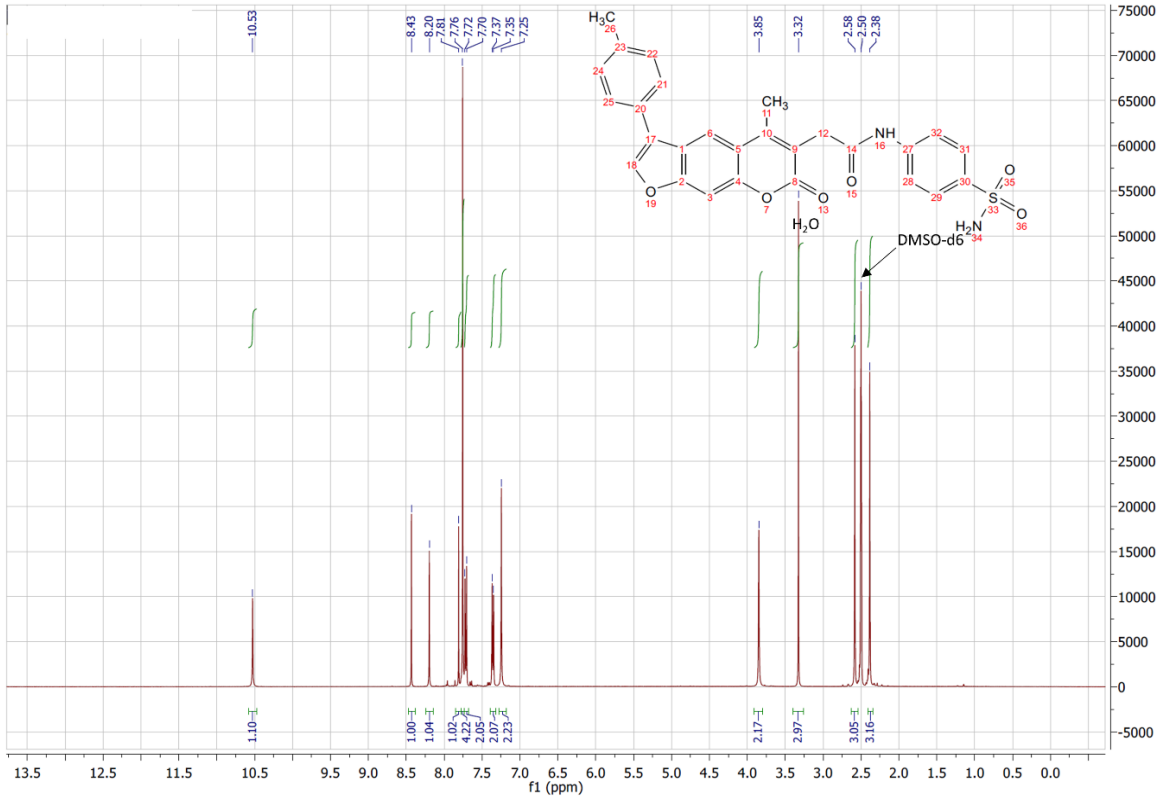


Figure S23. ^{1}H NMR spectrum (400 MHz, DMSO- *d6*) of **EMAC10153 B**.

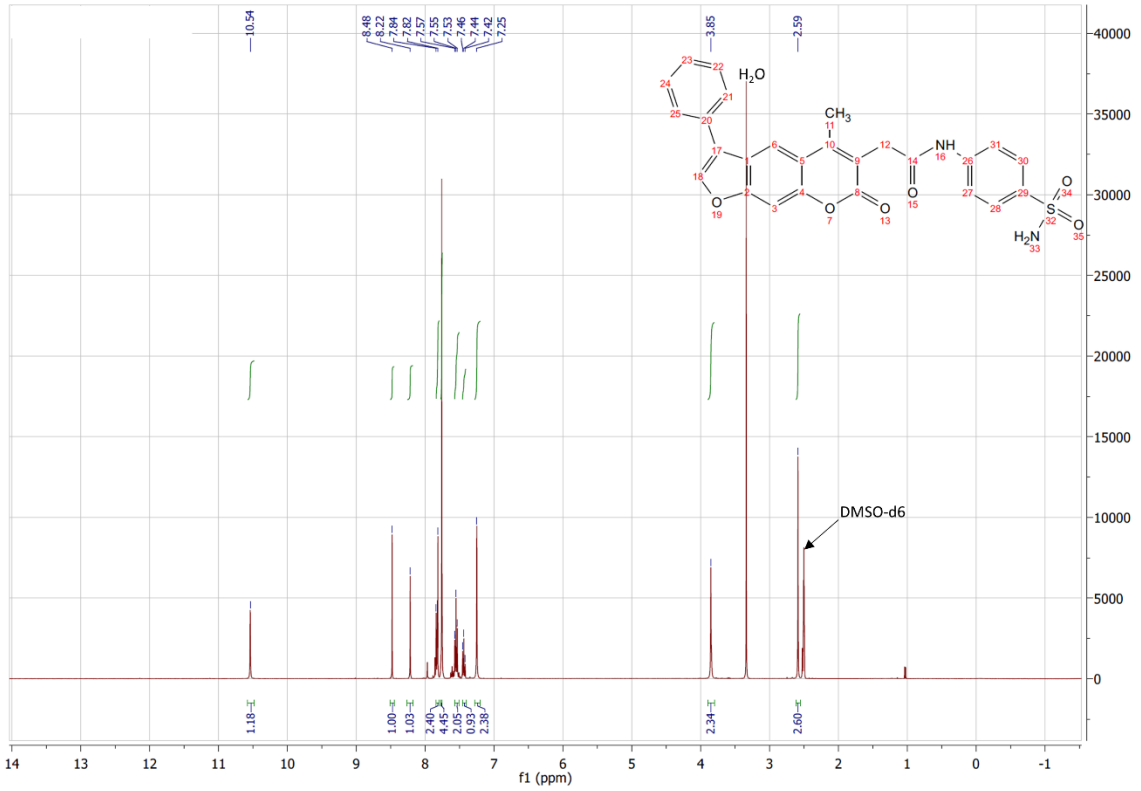


Figure S24. [1]H NMR spectrum (400 MHz, DMSO- *d6*) of **EMAC10153 C**.

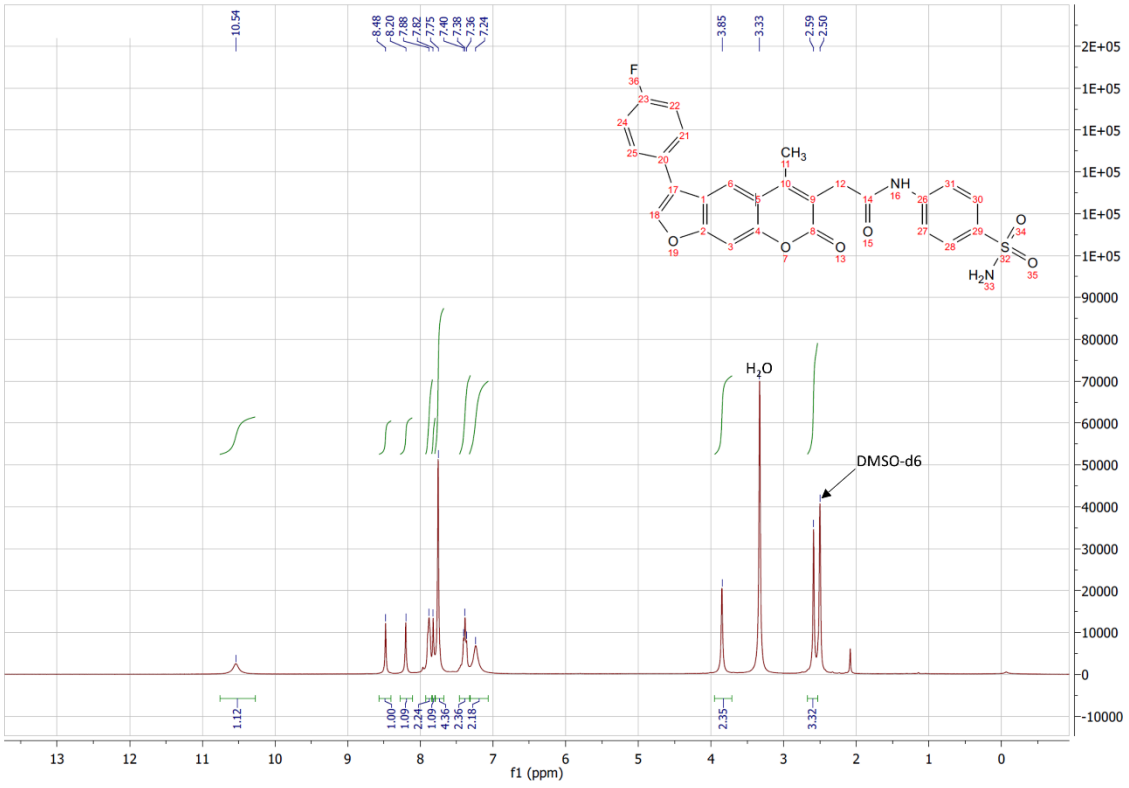


Figure S25. [1]H NMR spectrum (400 MHz, DMSO- *d6*) of **EMAC10153 D**.

Molecular modeling

Docking experiments were carried out in order to predict the putative binding mode of compounds **EMAC10152 D** and **EMAC10153 B.** The ligands were built with Maestro GUI[1] considering the possibility that coumarin ring can be hydrolyzed, if able to reach the catalytic site,[2] and not, if coumarin moiety stay far from the catalytic site, as in the case of benzenesulfonamide hybrids **EMAC10153**. Therefore, two possible structures were considered for psoralen **EMAC10152 D,** closed and hydrolized, while three possible features were taken into account for the **EMAC10153 B** hybrid: closed, closed ionized and hydrolized. In fact, sulfonamide group was considered both ionized and not, since the ionization of this moiety depends on its ability to occupy the basic catalytic site of the enzyme (Figure S26). Psoralens and their corresponding hydrolyzed Z isomers were ionized at pH 7.4 by means of LigPrep.[3] .

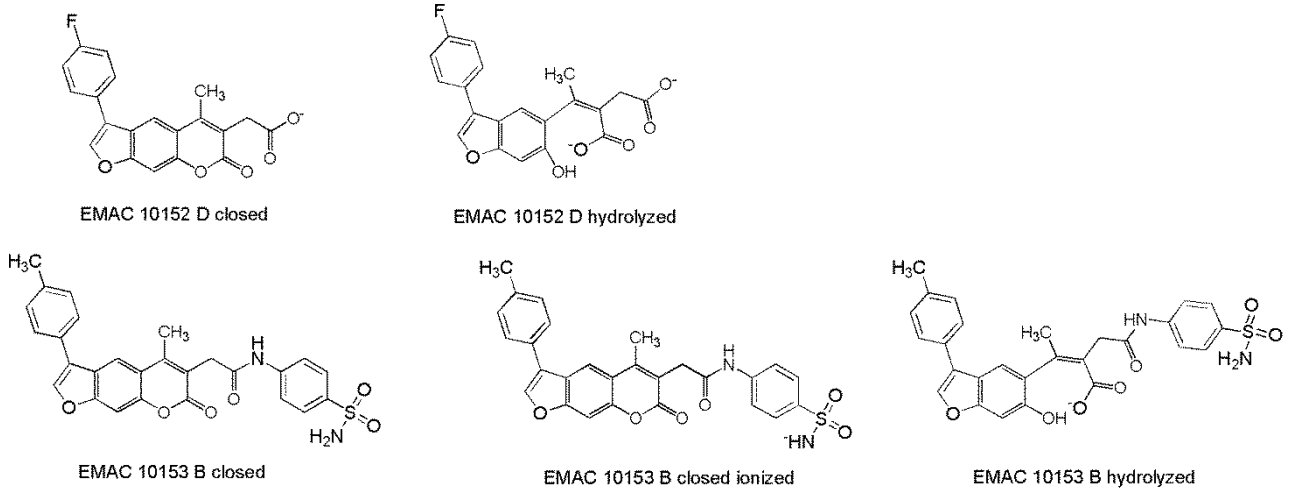


Figure S26. Docked compounds ionized at pH 7.4 with hydrolyzed and closed coumarin moiety.

The compounds were subjected to a conformational search protocol with MacroModel version 9.2,[4] considering Merck molecular force fields (MMFFs)[5] as force field and the implicit solvation model Generalized Born/Surface Area (GB/SA) water.[6] All conformational search parameters were left as default. The compounds were energy minimized using Polak-Ribier Conjugate Gradient (PRCG) method, 5000 iterations and a convergence criterion of 0.05 kcal/(mol Å).

Protein preparation was performed with Protein Preparation module in Maestro GUI[1] starting from protein structure PDB models (3f8e), (5fl4), and (4ww8) for CA II, IX, and XII respectively.[2, 7]All the water molecules were removed.

Docking experiments were performed by means of previously validated protocol[8] by applying Glide Quantum-Mechanical Polarized Docking (QMPL).[9] The Grid box was centered on the co-crystallized ligand and all parameters were set up as default.

The docking results suggested a different mechanism for the two compounds: the acid derivative is able to reach the catalytic pocket and be hydrolyzed, while the sulfonamide derivative do not, since the coumarin moiety is forced outside the pocket. Therefore, best pose complexes were subjected to post-docking procedure based on energy minimization. 10.000 steps of the Polak-Ribier conjugate gradient (PRCG) minimization method were conducted on the top ranked theoretical complexes using OPLS_2005 force field. The optimization process was performed up to the derivative convergence criterion equal to 0.1 kJ/(mol*Å)$^{-1}$. Depictions were taken with Maestro GUI[1] and Pymol.[10]

Table S1. Docking G-scores (kcal/mol) of **EMAC10152 D** and **EMAC10153 B**-hCA complexes. The results related to the proposed mechanisms are reported in bold character.

Compound	hCAII	hCAIX	hCAXII
EMAC10152 D-closed	-4.729	-9.037	-9.463
EMAC10152 D-hydrolyzed	**-4.267**	**-8.626**	**-8.733**
EMAC10153 B-closed	-5.053	-7.327	-5.813
EMAC10153 B closed ionized	**-4.813**	**-9.662**	**-7.915**
EMAC10153 B-hydrolyzed	-5.646	-6.960	-6.192

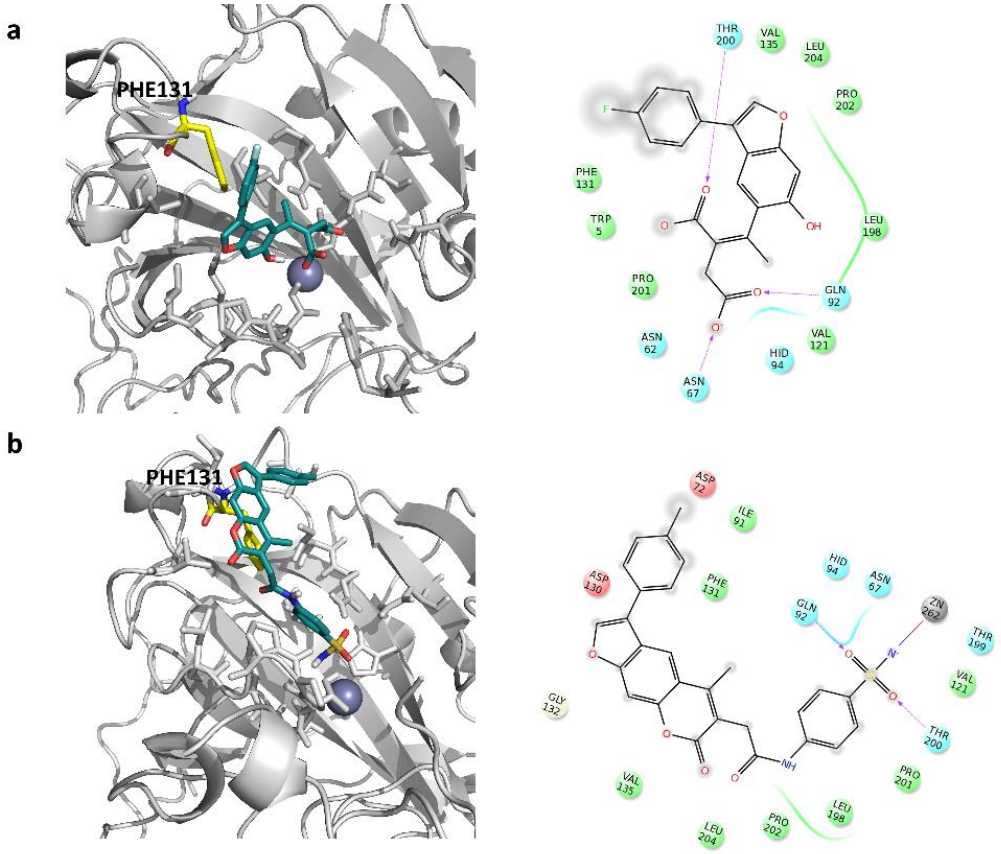


Figure S27. Three-dimensional representation of the putative binding mode obtained by docking experiments of (a) **EMAC10152 D** and (b) **EMAC10153 B** into hCA II (gray) with their relative 2D representation of the complexes stabilizing interactions with the residues of the binding site.

Biological evaluation

Carbonic anhydrase inhibition assay

The purification of cytosolic CA isoenzymes (CA I and CA II) were previously described with a simple one-step method by a Sepharose-4B-L tyrosine-sulphanilamide affinity chromatography[11]. The protein quantity in the column effluents was determined spectrophotometrically at 280nm. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was applied with a Bio-Rad Mini Gel System Mini-PROTEINV R system (Hercules, CA), BioRad Laboratories, Inc., China after purification of both CA isoenzymes. Briefly, it was performed in acrylamide for the running (10%) and the stacking gel (3%) contained SDS (0.1%), respectively. Activities of CA isoenzymes were determined according to a method by Verporte

et al[12]. The increase in absorbance of reaction medium was spectrophotometrically recorded at 348nm. Also, the quantity of protein was determined at 595nm according to the Bradford method[13]. Bovine serum albumin was used as standard protein. The IC50 values were obtained from activity (%) versus compounds plots[14]. For calculation of KI values, three different concentrations were used. The Lineweaver–Burk curves were drawn and calculations were realised[14].

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