Chemical Synthesis of Culmorin Metabolites and their biologic role in Culmorin and Acetyl-Culmorin Treated Wheat Cells

Julia Weber,[a] Marta Vaclavikova,[b] Gerlinde Wiesenberger,[c] Maximilian Haider,[a] Christian Hametner,[a] Johannes Fröhlich,[a] Franz Berthiller,[b] Gerhard Adam,[c] Hannes Mikula,[a] and Philipp Fruhmann[a,d*]

[a]Institute of Applied Synthetic Chemistry, Vienna University of Technology (TU Wien), Austria, E-mail: philipp.fruhmann@tuwien.ac.at
[b]Department of Agrobiotechnology (IFA-Tulln), Center for Analytical Chemistry, University of Natural Resources and Life Sciences, Vienna (BOKU), Austria
[c]Department of Applied Genetics and Cell Biology, University of Natural Resources and Life Sciences, Vienna (BOKU), Tulln, Austria
[d]Center for Electrochemical Surface Technology (CEST), Wiener Neustadt, Austria

Electronic Supplementary Information (ESI)

Content

1) Experimental

a) General remarks

All reactions were performed under argon atmosphere. The progress of the reactions was monitored by thin layer chromatography (TLC) over silica gel 60 F_{254}. All chromatograms were visualized either by UV irradiation (254 or 366 nm) or by heat staining with ceric ammonium molybdate (Hanessian's stain) in ethanol/sulfuric acid.[1] All samples were measured via LC-ESI-MS/MS in negative or positive ionization mode. These measurements were performed on an HCT ion trap mass spectrometer (Bruker, Germany). A TLC-MS interface (Camag, Germany) was used for ESI-MS analysis after TLC. Preparative column chromatography was performed on silica gel 60 (Merck, 40-63 µm) using a Büchi Sepacore™ Flash System or a Grace Reveleris Prep Purification System. Preparative HPLC separation was done on a Grace Reveleris Prep system using a Luna Prep C18(2), 10 µm, 250x10 mm column (Phenomenex). NMR spectra were recorded on a Bruker Avance IIIHD 600Mhz spectrometer equipped with a Prodigy BBO cryo probe or on a Bruker Avance DRX-400 MHz spectrometer at 20°C. Data were recorded and evaluated using TOPSPIN 3.5 (Bruker Biospin). All chemical shifts are given in ppm relative to tetramethylsilane. The calibration was done using residual solvent signals.[2] Multiplicities are abbreviated as s (singlet), d (doublet), t (triplet), q (quartet), b (broad signal). If not stated otherwise, all chemicals were purchased from Sigma Aldrich (Austria/Germany) or ABCR (Germany). Dry solvents were obtained from an in-house PURESOLV facility of it-innovative technology (USA). Molecular sieve 3 Å was activated under vacuum at 200°C before use. HR-MS analysis was carried out from acetonitrile/water solutions (concentration: 10 ppm) by using an HTC PAL system auto-sampler (CTC Analytics AG, Zwingen, Switzerland), an Agilent 1100/1200 HPLC with binary pumps, degasser and column thermostat (Agilent Technologies, Waldbronn, Germany) and Agilent 6230 AJS ESI–TOF mass spectrometer (Agilent Technologies, Palo Alto, United States). In addition to this, also a Thermo Ulitmate 3000 HPLC and Thermo Q Exactive Focus mass spectrometer equipped with the same auto-sampler was used for four samples.

b) Production and Purification of Culmorin

In order to obtain culmorin, two different ways of production, on autoclaved rice and in liquid still cultures were investigated. Two *tri1* knockout strains (IAPT24 (*Fusarium graminearum* PH-1 *tri1*Δ::*hph*) and IAWT2 (*Fusarium graminearum* WG-9 *tri1*Δ::*hph)*[3] selected for purification of the trichothecene calonectrin produced considerable amounts of culmorin and allowed simultaneous purification of culmorin as a by-product.

***Rice cultures using strain* IAPT24**

For the rice culture medium 10 g of long-grained rice and 10 mL Millipore water were filled into 200-mL baby jars. After incubation for one hour at room temperature the jars were autoclaved for 60 minutes at 121 °C. Strain IAPT24 was sporulated in mung bean medium (10 g mung beans were added to 450 mL boiling water and cooked for 20 min. After removal of the mung beans, the extract was filtrated through a folded filter, filled up to 1 L and autoclaved for 20 min at 121°C. Conidia were separated

from mycelia by filtration through a glass-wool filter and sedimented overnight at 4 °C. After removal of the medium spores were re-suspended in water and counted in a Fuchs-Rosenthal chamber.).

36 rice cultures were inoculated with 10^5 spores of IAPT24/jar and incubated at 20°C in the dark for one, two or three weeks, respectively. At each time point 12 glasses were transferred to -20°C and were kept frozen until further use. For extraction all cultures were thawed at room temperature for about one hour, 40 mL ethylacetate (EtOAc) were added to each tube, the rice cakes were broken up with a spatula and subsequently homogenized using an Ultra Turrax at maximum speed. After addition of 60 mL EtOAc the suspensions were shaken at 20°C with 180 rpm for 1 hour. After removal of small samples for analysis all samples were pooled and worked up together as follows: they were centrifuged in 550-g portions with 4000 g at room temperature. The supernatants were collected and pooled. Each rice pellet was extracted once more with 150 mL EtOAc and treated as described above. Finally, all supernatants were pooled and concentrated to a yellow viscous solution of about 500 mL. This remaining crude extract was used for the purification and crystallization step.

***Liquid still cultures using strain* IAWT2**

Strain IAWT2 was sporulated as described above. 100 Magenta boxes containing modified 2-stage medium (2-SM: 3 g/L KH_2PO_4, 0,2 g/L $MgSO_4 \cdot 7\ H_2O$, 5 g/L NaCl, 1 g/L $(NH_4)_2HPO_4$, 40 g/L sucrose, 10 g/L glycerol; 10 mg/L citric acid, 10 mg/L $ZnSO_4 \cdot 6\ H_2O$, 2 mg/L $Fe(NH_4)_2(SO_4)_2 \cdot 6\ H_2O$, 0.5 mg/L $CuSO_4 \cdot 5\ H_2O$, 0.1 mg/L $MnSO_4$, 0.1 mg/L H_3BO_4, 0.1 mg/L $Na_2MoO_4 \cdot 2\ H_2O$; 50 mL/container) were inoculated with 10^5 conidia/jar and incubated as described for the rice cultures. 12 cultures per time point were frozen after one, two or three weeks, respectively. Small aliquots of three samples/time point were used for analysis, the rest and the remaining samples were pooled and the media was concentrated *in vacuo* to a yellow viscous solution of approx. 500 mL which was used for the purification and crystallization step.

Purification and crystallization of culmorin

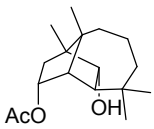
The crude culmorin extract was used for silica gel filtration in order to obtain a pre-concentrated suspension. For this reason, a large and porous suction filter was equipped with filter paper and about 350 g of silica gel 60 (0.015 – 0.040 mm). The crude extract was diluted with a small amount of EtOAc to lower the viscosity and placed on the silica gel layer. EtOAc was subsequently flushed through the material and fractions of around 500 mL were collected. Thin layer chromatography (TLC) in either pure EtOAc or dichloromethane:methanol (95:5) was used to localize CUL in the fractions. The confirmation of the occurrence of CUL was done via the molecular weight using a Camag TLC-MS machine. All CUL enriched fractions were pooled together and concentrated again in vacuo to app. 500 mL of a yellow viscous liquid. This step was repeated two times in order to remove as much by-products as possible and to finally obtain 500 mL of a slightly yellow and still viscous solution. This solution was treated with a small amount of active charcoal followed by column chromatography (DCM:MeOH = 95:5; 400 g silica gel 60) in order to obtain a nearly colourless solution with only minor by-products (according to TLC and TLC-MS). This step was again repeated two times in order to get a colourless solution of nearly pure CUL. This fraction was concentrated in vacuo to a minimal amount

of sticky resin followed by the addition of small amounts of EtOAc (~ 5 mL per portion). This addition was continued till everything of the residue was dissolved and once again a solution was obtained. During this step, colourless crystals precipitated and even more were formed upon standing over night in the fridge. These crystals were filtered, dried under vacuum and confirmed as culmorin via NMR spectroscopy and the available reference literature.[4] Based on this purification procedure, ~120 mg of CUL were obtained from the rice culture with strain IAPT24 and 885 mg of CUL were obtained with the liquid cultures from strain IAWT2. In addition to culmorin, small amounts of 11-AcCul were also present in the media and 42 mg were isolated from a side fraction using several times straight phase chromatography (DCM:MeOH = 98:2, R_f value of 11-AcCul = 0.125). The NMR data of the isolated compound is identical to the synthesized substance.

Culmorin (**1**): ^{1}H NMR (600 MHz, $CDCl_3$): δ = 4.35 (ddd, J = 7.4, 6.5, 4.5 Hz, 1H), 3.82 (d, J = 5.3 Hz, 1H), 2.17 (bs, 2H), 1.90 (d, J = 4.6 Hz, 1H), 1.74 (d, J = 5.1 Hz, 1H), 1.65 (d, J = 6.4 Hz, 2H), 1.48 – 1.26 (m, 6H), 1.00 (s, 3H), 0.91 (s, 3H), 0.86 (s, 3H), 0.81 (s, 3H); ^{13}C NMR (150 MHz, MeOD): δ = 79.7 (d, 1C), 71.2 (d, 1C), 52.5 (s, 1C), 52.1 (d, 1C), 51.3 (s, 1C), 49.7 (d, 1C), 41.4 (t, 1C), 36.3 (t, 1C), 36.0 (t, 1C), 33.0 (s, 1C), 29.4 (q, 1C), 28.9 (q, 1C), 22.7 (t, 1C), 22.2 (q, 1C), 13.3 (q, 1C)

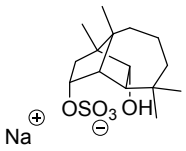
c) Synthetic procedures for culmorin sulfates

11-Acetylculmorin (2)



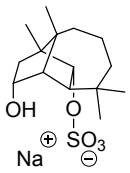
To a solution of culmorin (20 mg, 84 µmol, 1 eq.) in pyridine (2 mL) was added acetyl chloride (6.5 µl, 92 µmol, 1.1 eq.) at -5 °C. After stirring the reaction mixture for 16 h at -5 °C, the reaction was concentrated under reduced pressure and the residue was purified by column chromatography (hexanes: EtOAc 9-17 %) yielding the target compound (21 mg, 89 %); ^{1}H NMR (600 MHz, $CDCl_3$): δ = 5.06 (dt, J = 10.1, 4.3 Hz, 1H), 3.86 (dd, J = 5.2, 1.9 Hz, 1H), 2.18 (d, J = 4.2 Hz, 1H), 2.05 (s, 3H), 1.85 (dd, J = 14.1, 4.4 Hz, 1H), 1.69 (ddd, J = 13.9, 10.2, 2.1 Hz, 1H), 1.58 (d, J = 5.3 Hz, 1H), 1.47 – 1.38 (m, 3H), 1.38 – 1.29 (m, 3H), 0.98 (s, 3H), 0.92 (s, 3H), 0.85 (s, 3H), 0.83 (s, 3H); ^{13}C NMR (150 MHz, MeOD): δ = 171.3 (s, 1C), 79.2 (d, 1C), 74.8 (d, 1C), 53.2 (d, 1C), 52.1 (s, 1C), 51.0 (s, 1C), 47.5 (d, 1C), 41.1 (t, 1C), 35.8 (t, 1C), 33.2 (t, 1C), 32.9 (s, 1C), 29.3 (q, 1C), 29.0 (q, 1C), 22.7 (t, 1C), 22.3 (q, 1C), 21.5 (q, 1C), 13.1 (q, 1C); HRMS calcd for $C_{17}H_{28}NaO_3^+$ $[M+Na]^+$ 303.1931, found 303.1930.

Culmorin-11-sulfate, sodium salt (3)



Culmorin (30 mg, 0.13 mmol, 1 eq.) and SO_3•Me_3N (21 mg, 0.15 mmol, 1.2 eq.) was stirred in DMF (1 mL) at 55 °C for 16 h. The solvent was evaporated under high vacuum and the residue was purified by preparative HPLC (water-MeCN 5 to 30 %). To obtain the sodium salt, the product was dissolved in water and passed through a small column packed with sodium ion exchange resin (Amberlite IRC7481 Chelating Resin) to give **3** as a white solid (31 mg, 72 %). ^{1}H NMR (600 MHz, MeOD): δ = 4.85 (dt, J = 10.2, 4.3 Hz, 1H), 3.78 (dd, J = 5.3, 1.8 Hz, 1H), 2.23 (d, J = 4.4 Hz, 1H), 2.02 (dd, J = 14.1, 4.1 Hz, 1H), 1.85 (d, J = 5.6 Hz, 1H), 1.66 (ddd, J = 14.1, 10.3, 2.1 Hz, 1H), 1.55 – 1.44 (m, 3H), 1.42 – 1.35 (m, 2H), 1.35 – 1.29 (m, 1H), 0.98 (s, 3H), 0.95 (s, 3H), 0.93 (s, 3H), 0.81 (s, 3H); ^{13}C NMR (150 MHz, MeOD): δ = 79.8 (d, 1C), 79.5 (d, 1C), 53.1 (s, 1C), 52.7 (d, 1C), 51.4 (s, 1C), 49.4 (d, 1C), 42.5 (t, 1C), 36.9 (t, 1C), 34.9 (t, 1C), 33.9 (s, 1C), 29.8 (q, 1C), 29.1 (q, 1C), 23.7 (t, 1C), 22.7 (q, 1C), 13.2 (q, 1C); HRMS calcd for $C_{15}H_{26}O_5S^-$ $[M-H]^-$ 317.1428, found 317.1426.

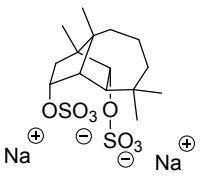
Culmorin-8-sulfate, sodium salt (4)



11-Acetylculmorin (21 mg, 0.08 mmol, 1 eq.) and SO_3•Me_3N (12.5 mg, 0.09 mmol, 1.2 eq.) was stirred in DMF (1.5 mL) at 55 °C for 16 h. The solvent was evaporated under high vacuum. For basic hydrolysis, the crude product was dissolved in MeOH (2 mL), followed by the addition of NaOMe in MeOH (45 µl, 15 wt%, 2 eq.) at 0 °C. The reaction mixture was slowly warmed to room temperature. After stirring for 4 h, the reaction was concentrated under reduced pressure, the residue was dissolved in water and purified via preparative HPLC (water-MeCN 5 to 30 %) to obtain compound **4** (15 mg, 59 %); ^{1}H NMR (600

MHz, MeOD): δ = 4.75 (dd, J = 4.8, 2.2 Hz, 1H), 4.29 (dt, J = 10.1, 4.3 Hz, 1H), 2.13 (d, J = 4.4 Hz, 1H), 1.91 (d, J = 1.9 Hz, 1H), 1.86 (dd, J = 13.8, 4.1 Hz, 1H), 1.60 (ddd, J = 13.5, 9.9, 2.1 Hz, 1H), 1.57 – 1.44 (m, 4H), 1.41 – 1.30 (m, 2H), 1.10 (s, 3H), 0.99 (s, 3H), 0.92 (s, 3H), 0.88 (s, 3H); ^{13}C NMR (150 MHz, MeOD): δ = 89.3 (d, 1C), 71.6 (d, 1C), 53.7 (s, 1C), 52.3 (s, 1C), 50.7 (d, 1C), 50.4 (d, 1C), 42.4 (t, 1C), 37.3 (t, 1C), 37.2 (t, 1C), 33.9 (s, 1C), 29.8 (q, 1C), 29.6 (q, 1C), 23.7 (t, 1C), 22.6 (q, 1C), 14.2 (q, 1C); HRMS calcd for $C_{15}H_{26}O_5S^-$ $[M-H]^-$ 317.1428, found 317.1429.

Culmorin-8,11-disulfate, sodium salt (5)



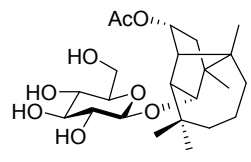
Culmorin (20 mg, 0.084 mmol, 1 eq.) and SO_3•Me_3N (585 mg, 4.2 mmol, 50 eq.) was stirred in DMF (700 µL) at 55 °C for 16 h. The solvent was evaporated under high vacuum, the residue was redissolved in MeOH and filtered to remove the excess of the sulfate complex. The filtrate was concentrated under vacuum and purified by preparative HPLC (water-MeCN 5 to 30 %). To obtain the sodium salt, the product was dissolved in water and passed through a small column packed with sodium ion exchange resin (Amberlite IRC7481 Chelating Resin) to obtain compound **5** as a white solid (32 mg, 87 %); ^{1}H NMR (600 MHz, MeOD): δ = 4.86 (dt, J = 10.2, 4.3 Hz, 1H), 4.75 (dd, J = 5.0, 2.0 Hz, 1H), 2.26 (d, J = 4.7 Hz, 1H), 2.16 (dd, J = 13.9, 4.3 Hz, 1H), 2.11 (d, J = 5.0 Hz, 1H), 1.71 (ddd, J = 14.1, 10.2, 2.2 Hz, 1H), 1.61 – 1.43 (m, 4H), 1.41 – 1.33 (m, 2H), 1.09 (s, 3H), 1.01 (s, 3H), 0.95 (s, 3H), 0.92 (s, 3H); ^{13}C NMR (150 MHz, MeOD): δ = 88.8 (d, 1C), 79.3 (d, 1C), 53.3 (s, 1C), 51.9 (s, 1C), 51.3 (d, 1C), 49.1 (d, 1C), 42.4 (t, 1C), 37.1 (t, 1C), 35.6 (t, 1C), 34.1 (s, 1C), 29.8 (q, 1C), 29.5 (q, 1C), 23.6 (t, 1C), 22.6 (q, 1C), 14.1 (q, 1C); HRMS calcd for $C_{15}H_{24}O_8S_{22}^-$ $[M/_2]^-$ 198.0454, found 198.0452

Preparative HPLC procedures for all culmorin sulfates

Preparative HPLC separation was done on a Grace Reveleris Prep system using a Luna Prep C18(2), 10 µm, 250x10 mm column (Phenomenex). As eluents were used water (A) and MeCN (B) and the flow rate was 10 mL/min. After an initial hold time at 5 % B for 4 min, the proportion of B was linearly increased to 40 % B within the next 10 min, it was hold at 40 % B for 1 min and increased to 90 % within another 1 min.

d) Synthetic procedures for culmorin glucosides

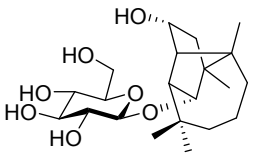
11-Acetylculmorin-8-glucoside (8)



To a solution of culmorin (100 mg, 420 µmol, 1 eq.) and 2,3,4,6-tetra-O-acetyl-α,D-glucopyranosyl-1-(N-phenyl)-2,2,2-trifluoroacetimidate (327 mg, 629 µmol, 1.5 eq.) in dry CH_2Cl_2 (3 mL), molecular sieve (3 Å, 300 mg) was added and the reaction mixture was stirred at room temperature for 30 min.

After cooling the reaction mixture to 0 °C, TMSOTf (7.6 µL, 420 µmol, 1 eq.) was added. At 0 °C the reaction mixture was stirred for 1 h, and quenched by the addition of Et_3N (0.15 eq.). The reaction mixture was filtered through celite and concentrated under reduced pressure. The residue was dissolved in CH_2Cl_2 and purified by flash chromatography (gradient elution hexane:EtOAc) to obtain 11-acetylculmorin-8-tetra-O-acetyl-glucoside with traces of impurities (80 mg, 31 %). This crude fraction (80 mg, 0.13 mmol, 1 eq.) was dissolved in dry MeOH and potassium cyanide (4.3 mg, 0.06 mmol, 0.5 eq.). After stirring for 4 h at room temperature, the reaction mixture was reduced to a volume of 1 mL and purified by preparative HPLC yielding compound **8** (38 mg, 66 %). Preparative HPLC separation was done on a Grace Reveleris Prep system using a Kinetex XB-C18, 5 µm, 100x30 mm column (Phenomenex). As eluents were used water (A) and MeCN (B) and the flow rate was 50 mL/min. After an initial hold time at 10 % B for 2 min, the proportion of B was linearly increased to 68 % B within the next 11 min, and within another 1 min to 90 % B; ^{1}H NMR (600 MHz, MeOD): δ = 5.03 (dt, J = 10.2, 4.3 Hz, 1H), 4.32 (d, J = 7.6 Hz, 1H), 3.94 (dd, J = 4.5, 1.9 Hz, 1H), 3.84 (dd, J = 11.8, 2.5 Hz, 1H), 3.69 (dd, J = 11.7, 5.3 Hz, 1H), 3.36-3.32 (m, 2H), 3.25-3.21 (m, 1H), 3.21- 3.17 (m, 1H), 2.19 (d, J = 4.4 Hz, 1H), 2.08 (dd, J = 14.2, 4.1 Hz, 1H), 2.03 (s, 3H), 1.91 (d, J = 5.0 Hz, 1H), 1.65 (ddd, J = 13.9, 10.2, 2.0 Hz, 1H), 1.59 – 1.45 (m, 3H), 1.41 – 1.31 (m, 3H), 1.03 (s, 3H), 0.98 (s, 3H), 0.92 (s, 3H), 0.87 (s, 3H); ^{13}C NMR (150 MHz, MeOD): δ = 173.3 (s, 1C), 104.7 (d, 1C), 100.0 (d, 1C), 78.3 (d, 1C), 77.5 (d, 1C), 76.8 (d, 1C), 75.4 (d, 1C), 71.6 (d, 1C), 62.7 (t, 1C), 53.4 (s, 1C), 52.8 (d, 1C), 52.4 (s, 1C), 48.6 (d, 1C), 42.2 (t, 1C), 37.0 (t, 1C), 34.9 (t, 1C), 33.7 (s, 1C), 30.3 (q, 1C), 30.0 (q, 1C), 23.6 (t, 1C), 22.7 (q, 1C), 21.2 (q, 1C), 14.1 (q, 1C); HRMS calcd for $C_{23}H_{38}O_8^+$ $[M+Na]^+$ 465.2459, found 465.2469.

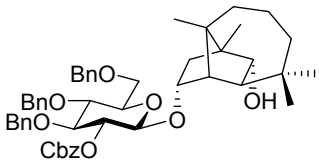
Culmorin-8-glucoside (6)



To a solution of 11-acetylculmorin-8-glucoside (18 mg, 40 µmol, 1 eq.) in MeOH (10 mL) was added potassium cyanide (10 mg, 160 µmol, 4 eq.). The reaction mixture was stirred at room temperature till LC-MS measurements showed full conversion, concentrated under reduced pressure to a volume of 1 mL and purified by preparative HPLC to obtain compound **6** as a white solid (13 mg, 80 %). The separation was done on a Grace Reveleris Prep system using a Luna Prep C18(2), 10 µm, 250x21.2 mm column (Phenomenex). As eluents were used water (A) and MeCN (B) and the flow rate was 20 mL/min. After an initial hold time at 10 % B for 2 min, the proportion of B was linearly increased to 63 % B within the next 11 min, it was hold at 63 % B for 2 min and increased to 90 % within another 5 min; ^{1}H NMR (600 MHz, MeOD): δ = 4.38 (d, J = 7.6 Hz, 1H), 4.31 (dt, J = 10.1, 4.2 Hz, 1H), 3.92 (dd, J = 4.4, 1.5 Hz, 1H), 3.85 (dd, J = 11.7, 2.4 Hz, 1H), 3.68 (dd, J = 11.7, 5.6 Hz, 1H), 3.38-3.28 (m, 2H), 3.27-3.22 (m, 1H), 3.17 (dd, J = 9.3, 7.8 Hz, 1H), 2.04 (d, J = 4.1 Hz, 1H), 1.91 (d, J = 4.7 Hz, 1H), 1.85 (dd, J = 13.7, 4.0, 1H), 1.56 (ddd, J = 13.7, 10.4, 1.8 Hz, 1H), 1.53 – 1.43 (m, 3H), 1.41 – 1.28 (m, 3H), 1.03 (s, 3H), 0.93 (s, 3H), 0.90 (s, 3H), 0.88 (s, 3H); ^{13}C NMR (150 MHz, MeOD): δ = 103.6 (d, 1C), 89.5 (d, 1C), 77.7 (d, 1C), 77.6 (d, 1C), 75.4 (d, 1C), 71.9 (d, 1C), 71.8 (d, 1C), 62.9 (t, 1C), 53.7 (s, 1C), 52.5 (s, 1C), 52.3 (d, 1C), 51.3 (d, 1C), 42.5 (t, 1C), 37.6 (t, 1C), 37.5 (t, 1C), 33.5 (s,

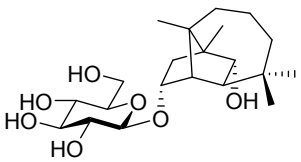
1C), 30.9 (q, 1C), 30.1 (q, 1C), 23.6 (t, 1C), 22.5 (q, 1C), 14.1 (q, 1C); HRMS calcd for $C_{21}H_{36}O_7^+$ $[M+Na]^+$ 423.2354, found 423.2359.

Culmorin-11-(3,4,6-tri-O-benzyl-2-O-benzyloxycarbonyl)-β,D-glucoside (9)



To a solution of glucosyl donor **11** (44 mg, 70 µmol, 1 eq.) and CUL (25 mg, 105 µmol, 1.5 eq.) in dry CH_2Cl_2 (1.5 mL) was added molecular sieve (3 Å, 75 mg) and the reaction was stirred for 2 h at room temperature. After cooling to -10 °C, N-iodosuccinimide (32 mg, 140 µmol, 2 eq.) and TfOH (2 mg, 14 µmol, 0.2 eq.) was added and stirring was continued at -10 °C for 3 h. The reaction was quenched by addition of an aqueous saturated $NaHCO_3$ and Na_2SO_3 solution (1:1), the reaction solution was diluted with CH_2Cl_2 and filtrated over celite. The filtrate was washed with water and brine, dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography (hexanes/EtOAc gradient elution) to obtain the desired product **9** (45 mg, 80 %) in good yields; ^{1}H NMR (400 MHz, CD_2Cl_2): δ = 7.47-7.13 (m, 20H), 5.20 (d, J = 12.1 Hz, 1H), 5.12 (d, J = 12.1 Hz, 1H),4.79 (t, J = 10.5 Hz, 1H), 4.75-4.69 (m, 2H), 4.66 (d, J = 11.3 Hz, 1H), 4.60-4.46 (m, 3H), 4.43 (d, J = 7.8 Hz, 1H), 4.28 (dt, J = 9.4, 4.3 Hz, 1H), 3.78-3.62 (m, 5H), 3.48-3.41 (m, 1H), 2.08 (d, J = 4.7 Hz, 1H), 1.75 (d, J = 5.0 Hz, 1H), 1.56-1.48 (m, 2H), 1.46 – 1.41 (m, 3H), 1.38 – 1.32 (m, 3H), 0.98 (s, 3H), 0.93 (s, 3H), 0.86 (s, 3H), 0.81 (s, 3H); ^{13}C NMR (100 MHz, CD_2Cl_2): δ = 154.8 (s, 1C), 138.72 (s, 1C), 138.67 (s, 1C), 138.6 (s, 1C), 135.9 (s, 1C), 128.9 (d, 2C), 128.8 (d, 1C), 128.72 (d, 2C), 128.70 (d, 2C), 128.69 (d, 2C), 128.6 (d, 2C), 128.4 (d, 2C), 128.21 (d, 2C), 128.18 (d, 2C), 128.1 (d, 1C), 127.9 (d, 2C), 100.3 (d, 1C), 83.0 (d, 1C), 79.5 (d, 1C), 78.5 (d, 1C), 78.3 (d, 1C), 78.2 (d, 1C), 75.4 (t, 1C), 75.31 (t, 1C), 75.27 (d, 1C), 73.7 (t, 1C), 70.3 (t, 1C), 69.3 (t, 1C), 53.0 (d, 1C), 52.3 (s, 1C), 51.1 (s, 1C), 49.0 (d, 1C), 41.7 (t, 1C), 36.1 (t, 1C), 33.6 (t, 1C), 33.3 (s, 1C), 29.7 (q, 1C), 28.7 (q, 1C), 22.9 (t, 1C), 22.4 (q, 1C), 13.3 (q, 1C) ; HRMS calcd for $C_{50}H_{60}NaO_9^+$ $[M+Na]^+$ 827.4130, found 827.4122.

Culmorin-11-β,D-glucoside (7)



To a suspension of the protected Culmorin-11-glucoside **9** (42 mg, 52 µmol, 1 eq.) in dry ethanol (2 mL) was added two small tips of a spatula of Pd/C under argon atmosphere. The argon balloon was changed for a H_2-balloon and the reaction mixture was stirred for 4 h at rt. The reaction mixture was filtered through a syringe filter and the filtrate was concentrated. The residue was dissolved in a mixture of acetonitrile/water (1:2) and purified via preparative-HPLC to yield **7** as a white solid (13 mg, 65 %). The separation was done on a Grace Reveleris Prep system using a Luna Prep C18(2), 10 µm, 250x21.2 mm column (Phenomenex). As eluents were used water (A) and MeCN (B) and the flow rate was 18 mL/min. After an initial hold time at 15 % B for 2 min, the proportion of B was linearly increased to 60 % B within the next 12 min, it was hold at 60 % B for 3 min and increased to 95 % within another 1 min; ^{1}H NMR (600 MHz, MeOD): δ = 4.39 (dt, J = 9.5, 4.2 Hz, 1H), 4.26 (d, J = 7.6 Hz, 1H), 3.87 (dd, J = 11.9, 2.5 Hz, 1H), 3.80 (dd, J = 5.3, 1.7 Hz, 1H), 3.66 (dd, J = 11.9, 5.4 Hz,

1H), 3.35 (t, J = 9.1 Hz, 2H), 3.28 (dd, J = 9.6, 8.6 Hz, 1H), 3.24 (ddd, J = 9.6, 5.6, 2.2 Hz, 1H), 3.19 (dd, J = 9.2, 7.8 Hz, 1H), 2.07 (d, J = 4.4 Hz, 1H), 1.93 (d, J = 5.3 Hz, 1H), 1.79 (dd, J = 13.4, 3.9, 1H), 1.59 (ddd, J = 13.4, 9.6, 1.9 Hz, 1H), 1.53 – 1.43 (m, 3H), 1.40 – 1.36 (m, 2H), 1.34 – 1.26 (m, 1H), 0.98 (s, 3H), 0.92 (s, 3H), 0.88 (s, 3H), 0.81 (s, 3H); ^{13}C NMR (150 MHz, MeOD): δ = 103.5 (d, 1C), 80.0 (d, 1C), 78.7 (d, 1C), 77.90 (d, 1C), 77.87 (d, 1C), 75.2 (d, 1C), 71.7 (d, 1C), 62.8 (t, 1C), 53.1 (s, 1C), 52.0 (d, 1C), 51.8 (s, 1C), 49.9 (d, 1C), 42.6 (t, 1C), 37.0 (t, 1C), 34.8 (t, 1C), 34.0 (s, 1C), 29.8 (q, 1C), 29.2 (q, 1C), 23.6 (t, 1C), 22.7 (q, 1C), 13.5 (q, 1C); HRMS calcd for $C_{21}H_{36}NaO_7^+$ $[M+Na]^+$ 423.2353, found 423.2353.

e) Plant cell experiments

The wheat suspension culture (PC-998, DSMZ, Braunschweig Germany) was maintained in B5 medium and rediluted 1:1 every week as previously described.[5] For the experiment 14 mL round bottom vented tubes (allowing gas exchange) were used (Greiner Bio-One, Kremsmünster, Austria; #191161). The tara of the empty tubes was determined, then 30 µL of a 5,000 mg/L CUL or 11-AcCUL stock solution in DMSO was added and combined with 1.47 mL of the suspension culture (1 day after passage to new medium). The resulting final concentration of the toxin was therefore 100 mg/L (2% final solvent concentration) and the wet weight of the cells per tube was about 250 mg. The tubes were placed upright on a rotary shaker (100 rpm, 20°C) for the duration of the experiment. At the end point the content of the tube was split into three parts (supernatant, wash solution, and pellet extract), which each was brought up to the doubled volume of the initial culture (3 mL) and having a final concentration of 50% methanol. Experiments with CUL were performed in triplicates and with AcCUL in duplicates.

Supernatant: The culture medium was removed by pushing a 1000 µL pipette tip down to the bottom of the 14 mL tube, to avoid aspiration of cells. The medium was completely removed in two steps and transferred to an Eppendorf tube, which was then centrifuged (2 min 5000 rpm) to pellet the small amounts of aspirated fine suspension cells. The cleared supernatant was mixed 1:1 with methanol and transferred into the glass vials for LC-MS culmorin measurement, or further diluted 10-times up to 1000-times with mixture of methanol:water (1:1), depending on analytes concentration.

Washing solution: To remove residual culture medium from the cells and to remove unspecifically adsorbed toxin 750 µL 10% MeOH was added and after gently mixing by hand (to avoid lysing cells) the supernatant was removed by again pushing the tip to the bottom of the tube (beneath the cells). The solution was transferred to an Eppendorf tube. Then another 750 µL aliquot of 10% methanol was added to the cells and the supernatant again removed and combined with the first aliquot. After a short mild centrifugation step (2 min 5000 rpm) 500 µL of the supernatant were combined 1:1 with 90% methanol to give the wash sample.

Pellet extract: the weight of the tube with the remaining cell pellet was determined and the wet weight calculated. To break the cells the tubes were frozen at -80 and thawed by adding 2x 750 µL 100% methanol prewarmed to 37 °C. The suspension was then sonified with a Branson Digital Sonifier (W-250-D, VWR International, Vienna) using a standard resonator with ½'' diameter and the settings: 2x 15 sec, 20% Amplitude. (The resonator was extensively rinsed with methanol between samples.) The resulting suspension was cleared by centrifugation (5 min, 11,000 rpm) and diluted 1:1 with distilled water to also yield a 50% methanol solution.

f) HPLC-MS/MS analysis of culmorin and its metabolites

Semiquantitative analysis of culmorin and its metabolites was performed by high performance liquid chromatography hyphenated to tandem mass spectrometry (HPLC-MS/MS). Since an appropriate separation of all metabolites is rather complicated due to the identical molecular mass and similar structure (e.g. CUL-8-sulfate and CUL-11-sulfate), two different methods were used for analysis of all compounds of interest. Analyses were performed using Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) coupled to 4000 QTRAP mass spectrometer (Applied Biosystems, Foster City, CA) equipped with electrospray ionization (ESI).

In the first method, mass spectrometer was operated in positive ionization mode (ESI+) and covered the analysis of CUL and 11-AcCUL. Chromatographic separation of analytes was carried out using Gemini C18 (Phenomenex) HPLC column (150 x 4.6 mm; 5 µm) operated at 25°C, mobile phase consisted of water:methanol (80:20, v/v) (eluent A) and methanol:water (97:3, v/v) (eluent B), both containing 5 mM ammonium acetate. Time of analysis was 10 min with constant flow rate of 800 µL/min and injection volume of 10 µL. Gradient elution started at 80% of B, was held for 1 min and afterwards increased to 100% B within 5 min and held for another 2 min. Column was equilibrated at initial conditions for 2 min.

Second method involved analysis of CUL-8-sulfate, CUL-11-sulfate, CUL-8,11-disulfate, 11-Ac-CUL-8-glucoside and CUL-8-glucoside. The application of Kinetex Biphenyl UHPLC column (150 x 4.6 mm; 2.6 µm) enabled partial separation of both culmorin sulfates. Column operated at 25°C and the run time of analysis was also 10 min. Mobile phase was running at 300 µL/min and consisted of H_2O with 0.1% acetic acid (eluent A) and methanol with 0.1% acetic acid (eluent B). Gradient started at 3% B, was held for 1 min, then increased up to 90% B within 2 min, and consequently up to 100% B within another 3 min. At 100% B the gradient was held for 2 min and finally decreased down to initial conditions of mobile phase, at which stayed for 2 min.

The following source settings were used: temperature, 550°C; ion spray voltage, 4 kV (positive mode) and 4 kV (negative mode); curtain gas, 30 lb/in^2; source gas one and two, both 50 lb/in^2; and collision gas (nitrogen) set to high. For quantitation, two selected reaction-monitoring transitions per compound

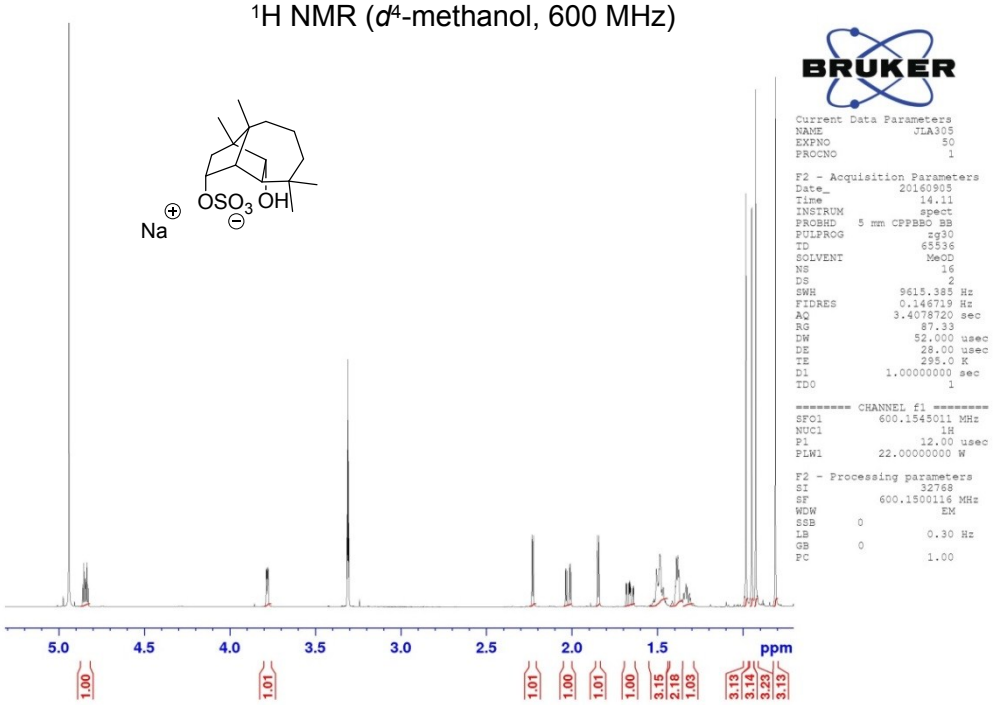
were acquired with a dwell time of 25 ms. Retention times and MS/MS parameters of all analytes are provided in the following table.

Analyte	Tr (min)	Q1	Q3	Declustering potential (V)	Collision energy (V)
CUL	4.25	256.2	221.3	41	17
		256.2	203.2	41	21
11-AcCUL	4.85	298.3	203.1	41	17
		298.3	161.1	41	33
CUL-8-glucoside	5.74	459.3	399.1	-70	-24
		459.3	58.9	-70	-48
11-AcCUL-8-glucoside	6.09	501.3	441.0	-75	-24
		501.3	399.1	-75	-34
CUL-8-sulfate	5.50	317.1	96.8	-145	-56
		317.1	79.9	-145	-116
CUL-11-sulfate	5.62	317.1	96.9	-140	-60
		317.1	79.9	-140	-116
CUL-disulfate	5.11	198.1	79.9	-70	-44
		198.1	96.9	-70	-36

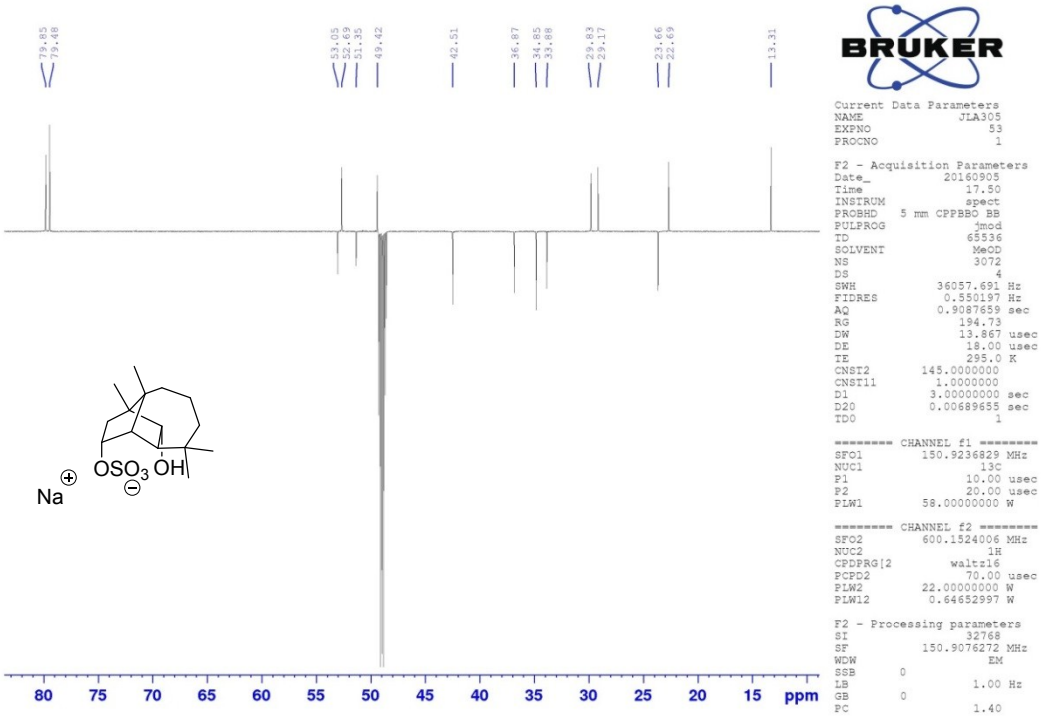
2) Copies of NMR spectra

Culmorin-11-sulfate, sodium salt (3)

^{1}H NMR (*d*^{4}-methanol, 600 MHz)

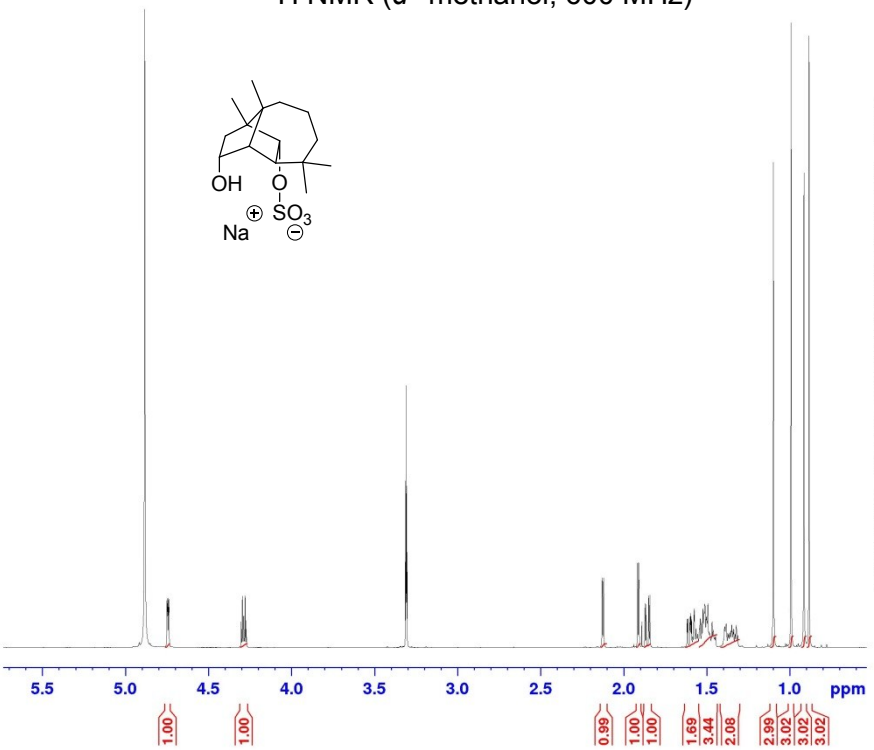


^{13}C NMR (*d*^{4}-methanol, 150 MHz)



Culmorin-8-sulfate, sodium salt (4)

^{1}H NMR (*d*^{4}-methanol, 600 MHz)

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FIDRES            0.146719 Hz
AQ                3.4078720 sec
RG                    77.94
DW                   52.000 usec
DE                   28.00 usec
TE                   295.0 K
D1                1.00000000 sec
TD0                        1

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NUC1                     1H
P1                   12.00 usec
PLW1          22.00000000 W

F2 - Processing parameters
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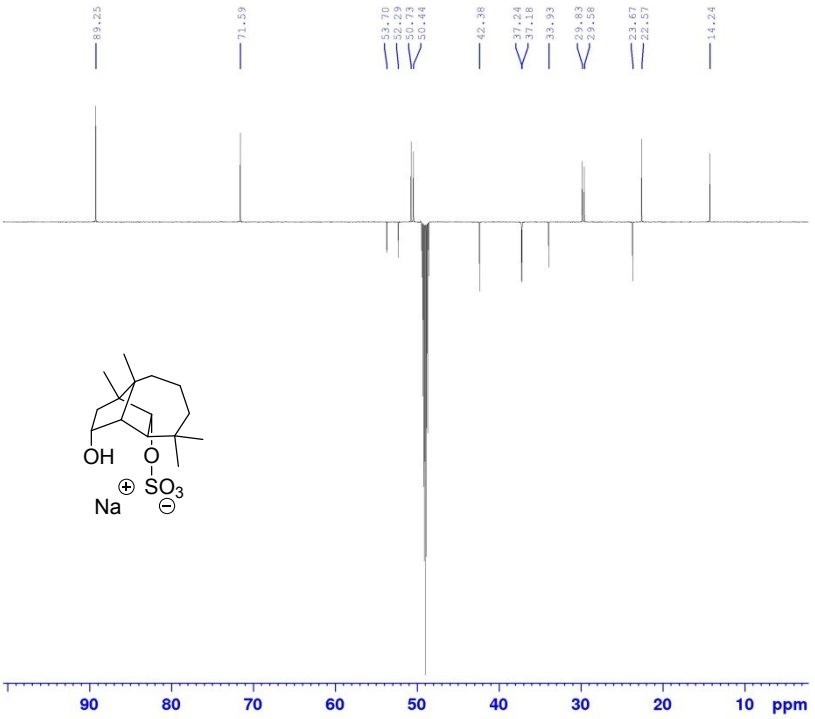
^{13}C NMR (*d*^{4}-methanol, 150 MHz)

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FIDRES            0.550197 Hz
AQ                0.9087659 sec
RG                    194.73
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TE                   295.0 K
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TD0                        1

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NUC1                     13C
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F2 - Processing parameters
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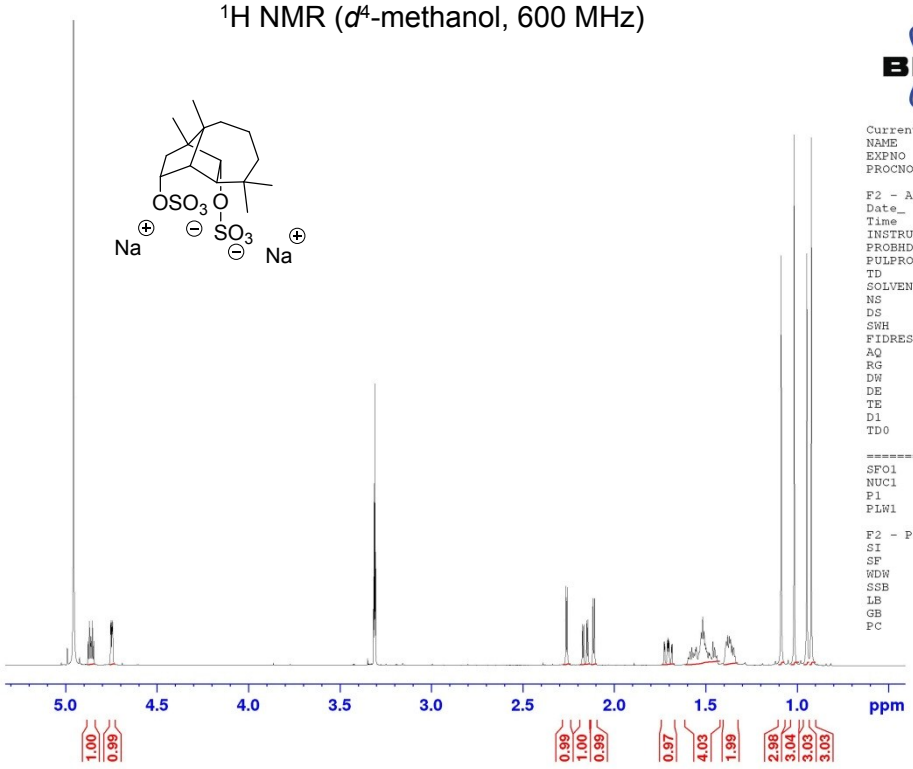
Culmorin-8,11-disulfate, sodium salt (5)

^{1}H NMR (d^{4}-methanol, 600 MHz)



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SOLVENT                MeOD
NS                       16
DS                        2
SWH                9615.385 Hz
FIDRES             0.146719 Hz
AQ                 3.4078720 sec
RG                    77.94
DW                   52.000 usec
DE                   28.00 usec
TE                   295.0 K
D1                1.00000000 sec
TD0                        1

======== CHANNEL f1 ========
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NUC1                       1H
P1                      12.00 usec
PLW1            22.00000000 W

F2 - Processing parameters
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PC                      1.00
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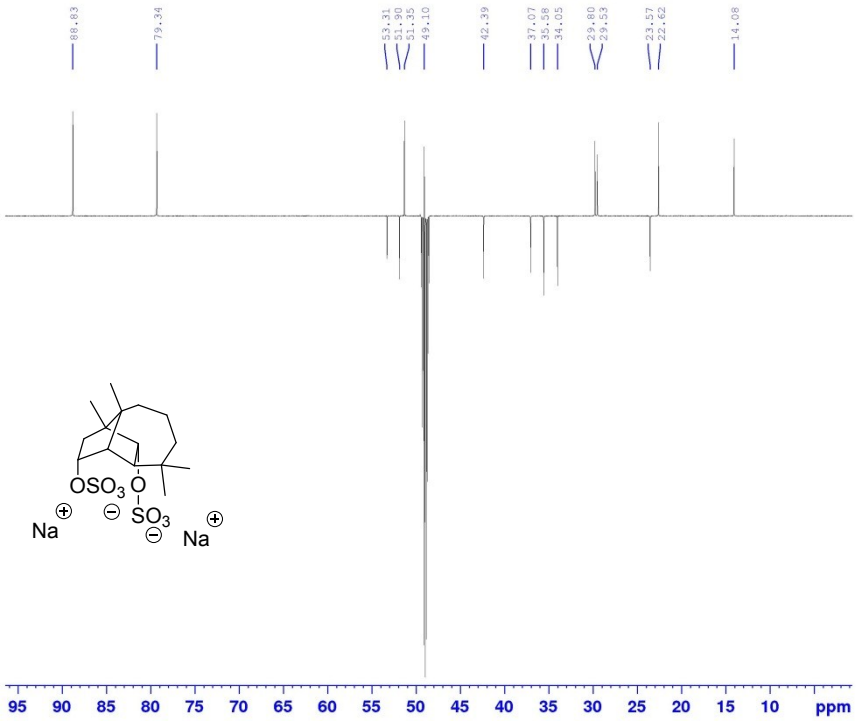
^{13}C NMR (d^{4}-methanol, 150 MHz)



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FIDRES             0.550197 Hz
AQ                 0.9087659 sec
RG                   194.73
DW                   13.867 usec
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TE                   295.0 K
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P1                      10.00 usec
P2                      20.00 usec
PLW1            58.00000000 W

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NUC2                       1H
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PCPD2                   70.00 usec
PLW2            22.00000000 W
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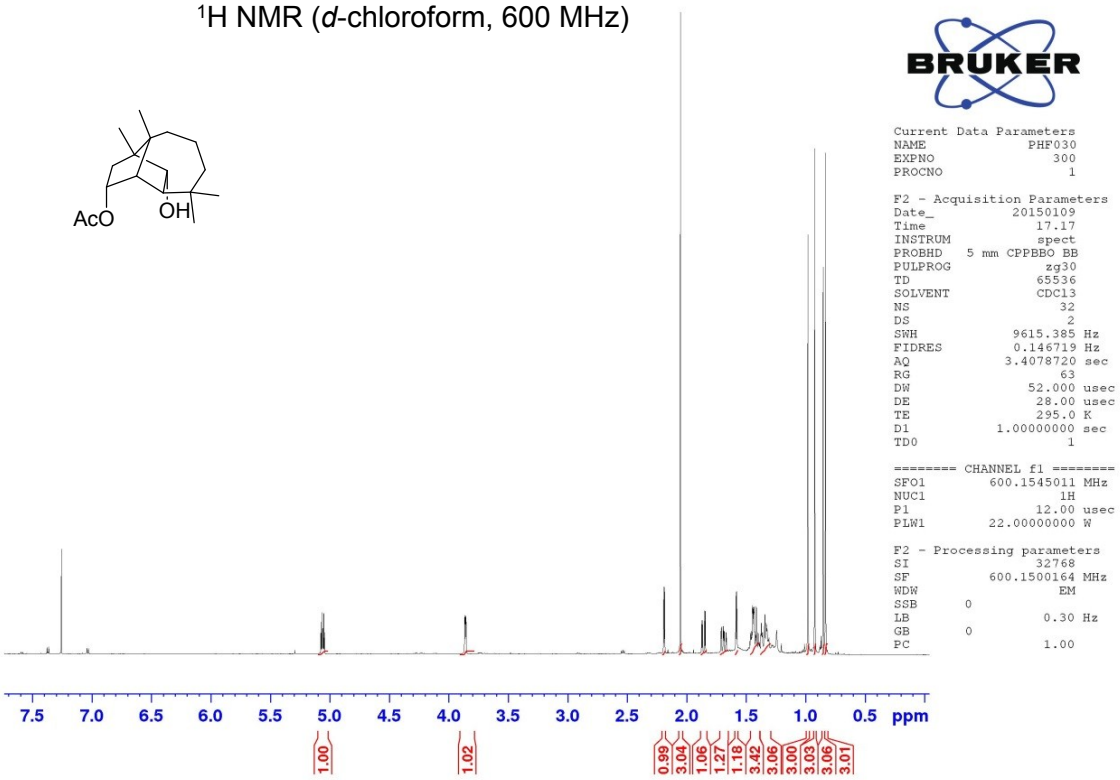
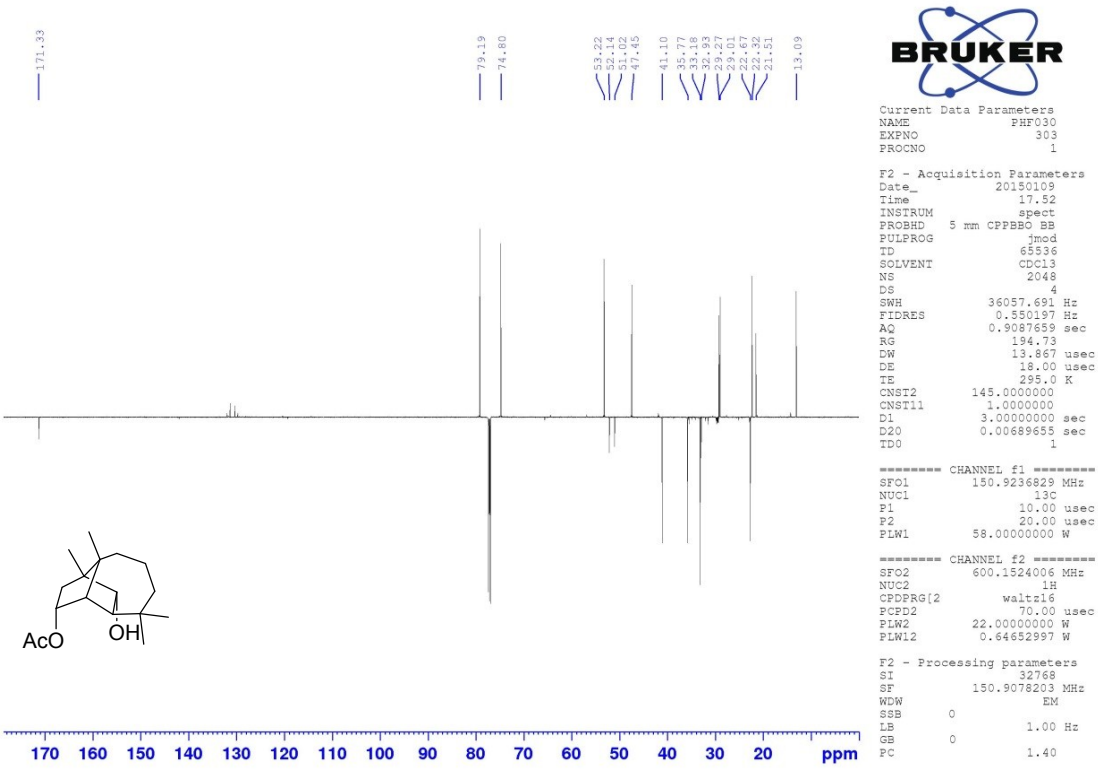
11-Acetylculmorin (2)

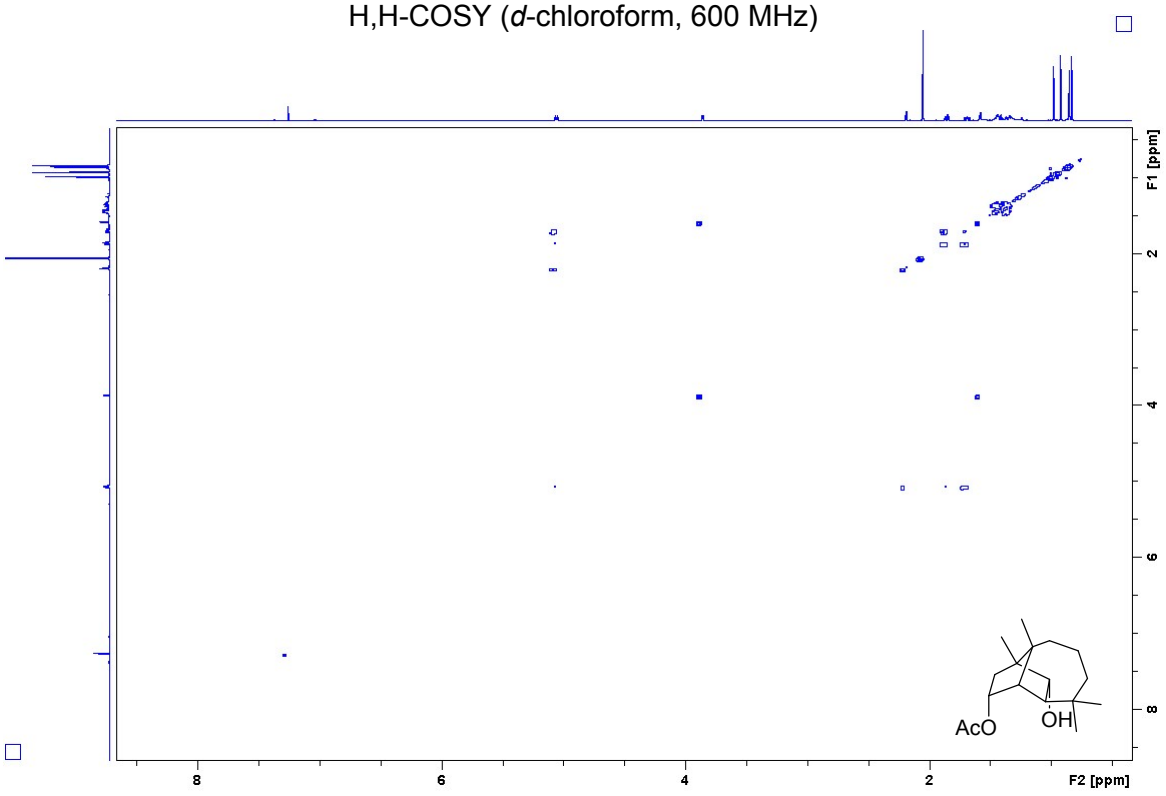
^{1}H NMR (*d*-chloroform, 600 MHz)



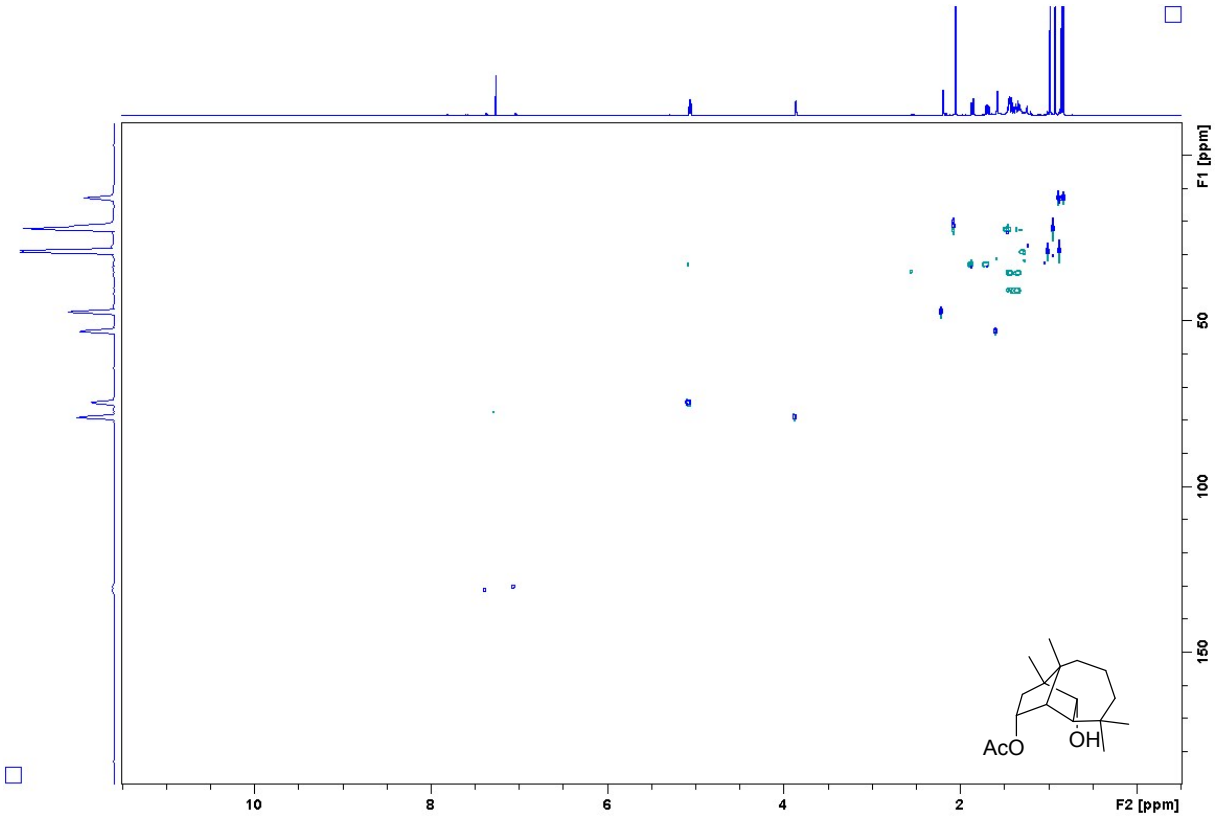
^{13}C NMR (*d*-chloroform, 150 MHz)



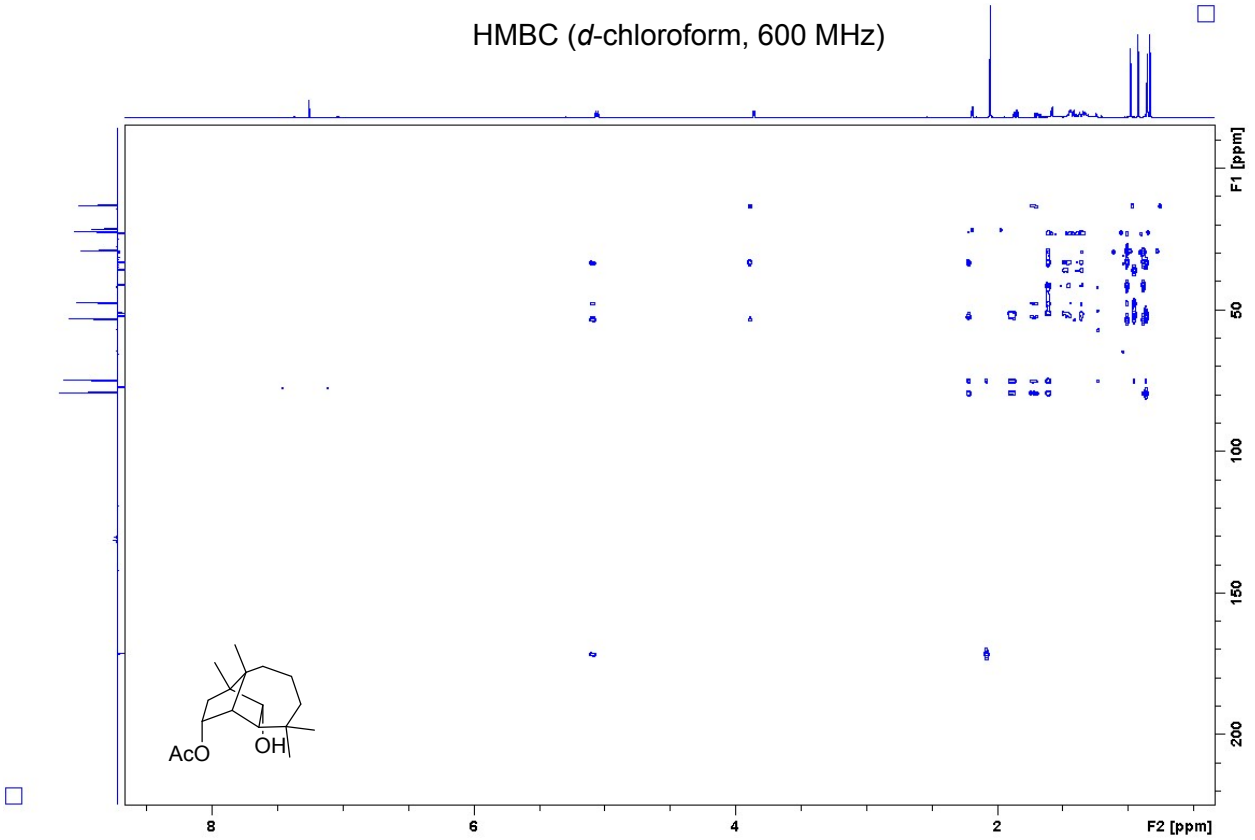
H,H-COSY (*d*-chloroform, 600 MHz)



HSQC (*d*-chloroform, 600 MHz)

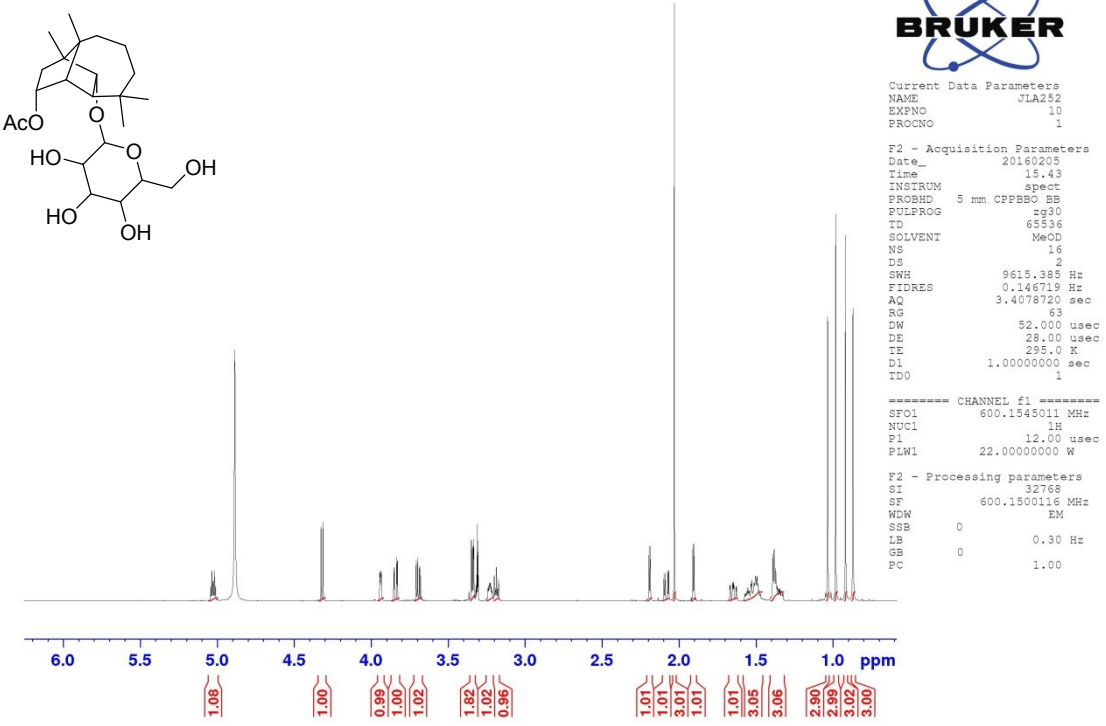


HMBC (*d*-chloroform, 600 MHz)

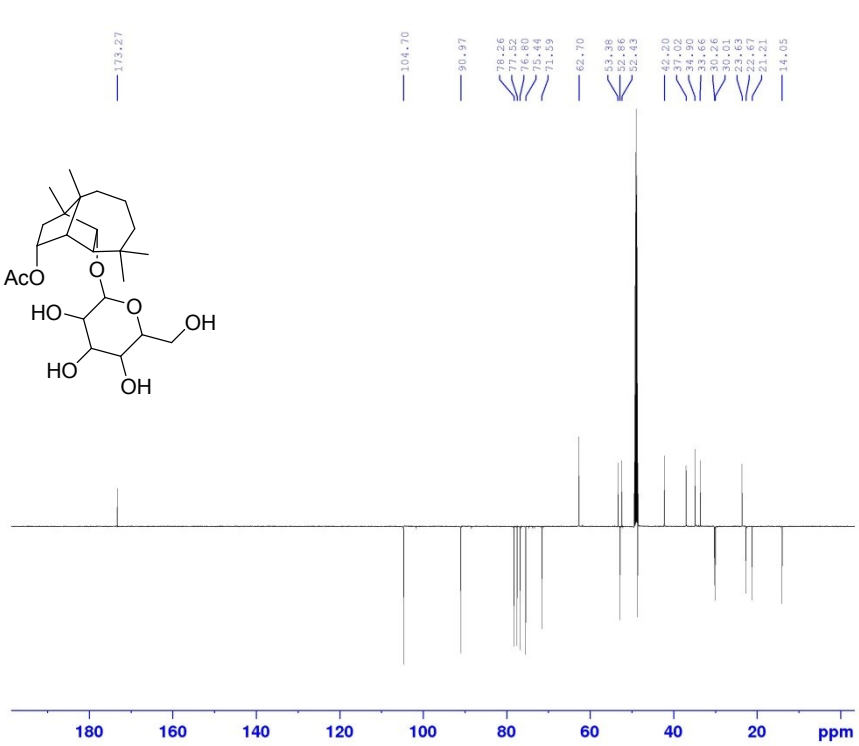


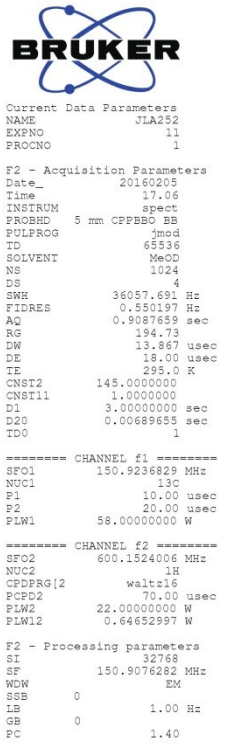
11-Acetylculmorin-8-β,D-glucoside (8)

^{1}H NMR (*d*^{4}-methanol, 600 MHz)

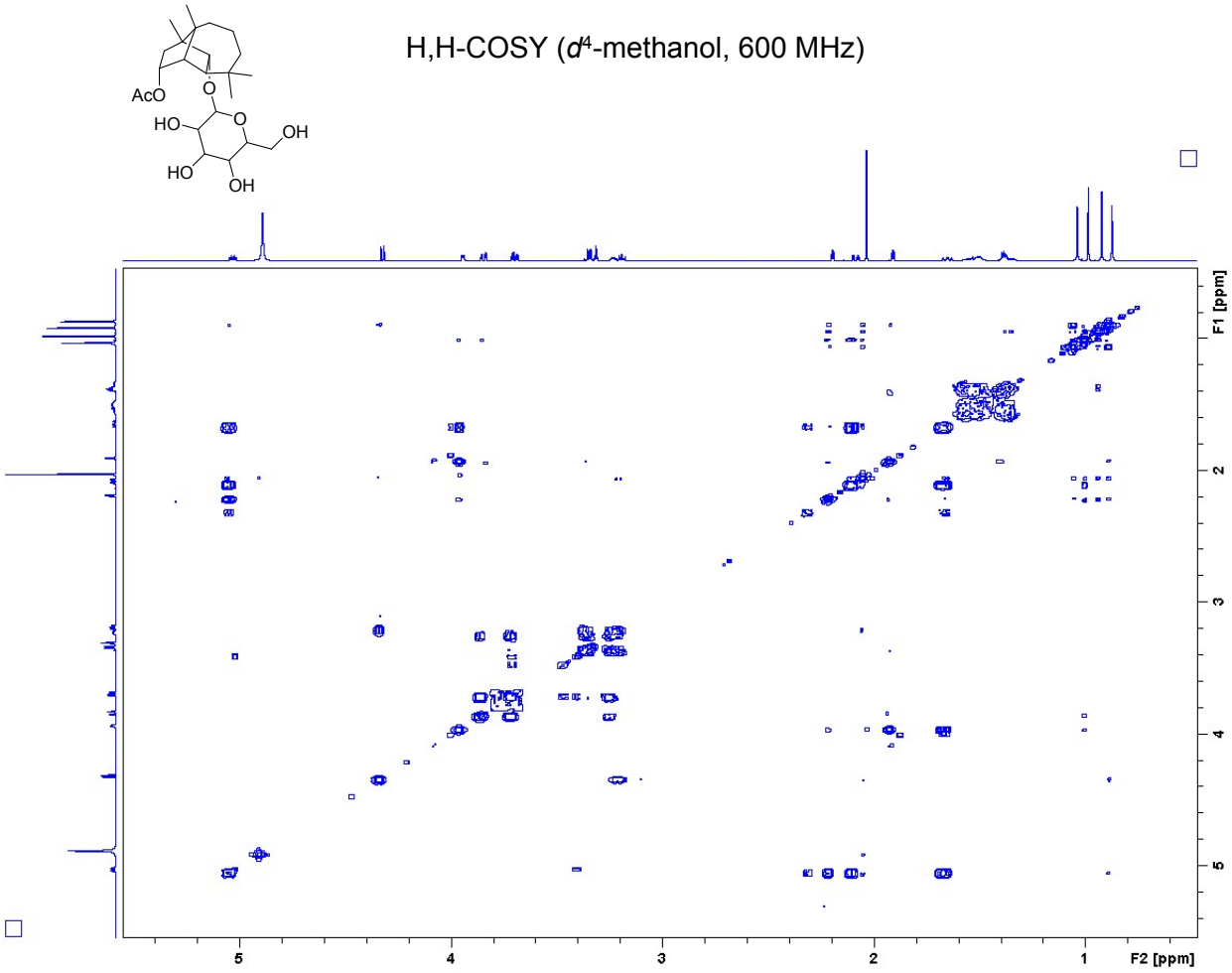


^{13}C NMR (d^4-methanol, 150 MHz)

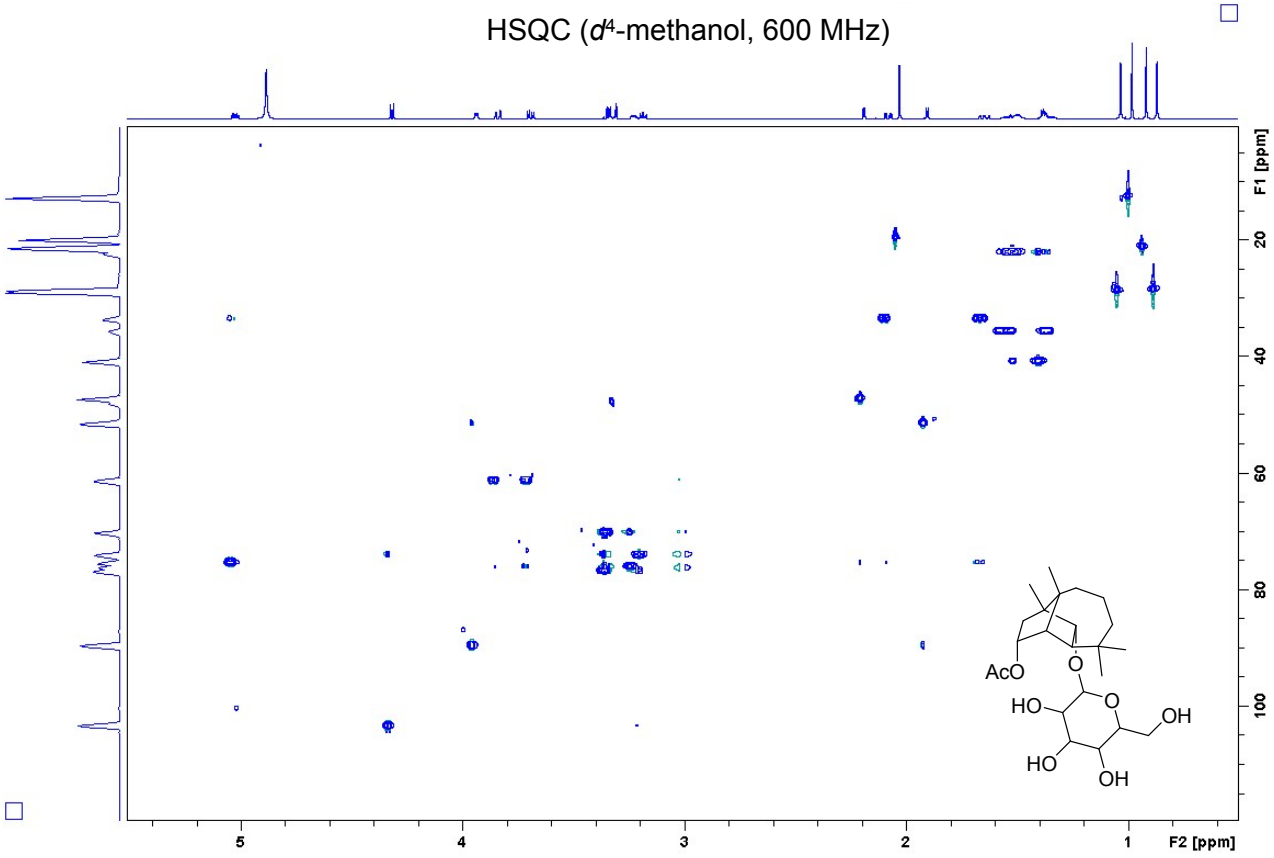




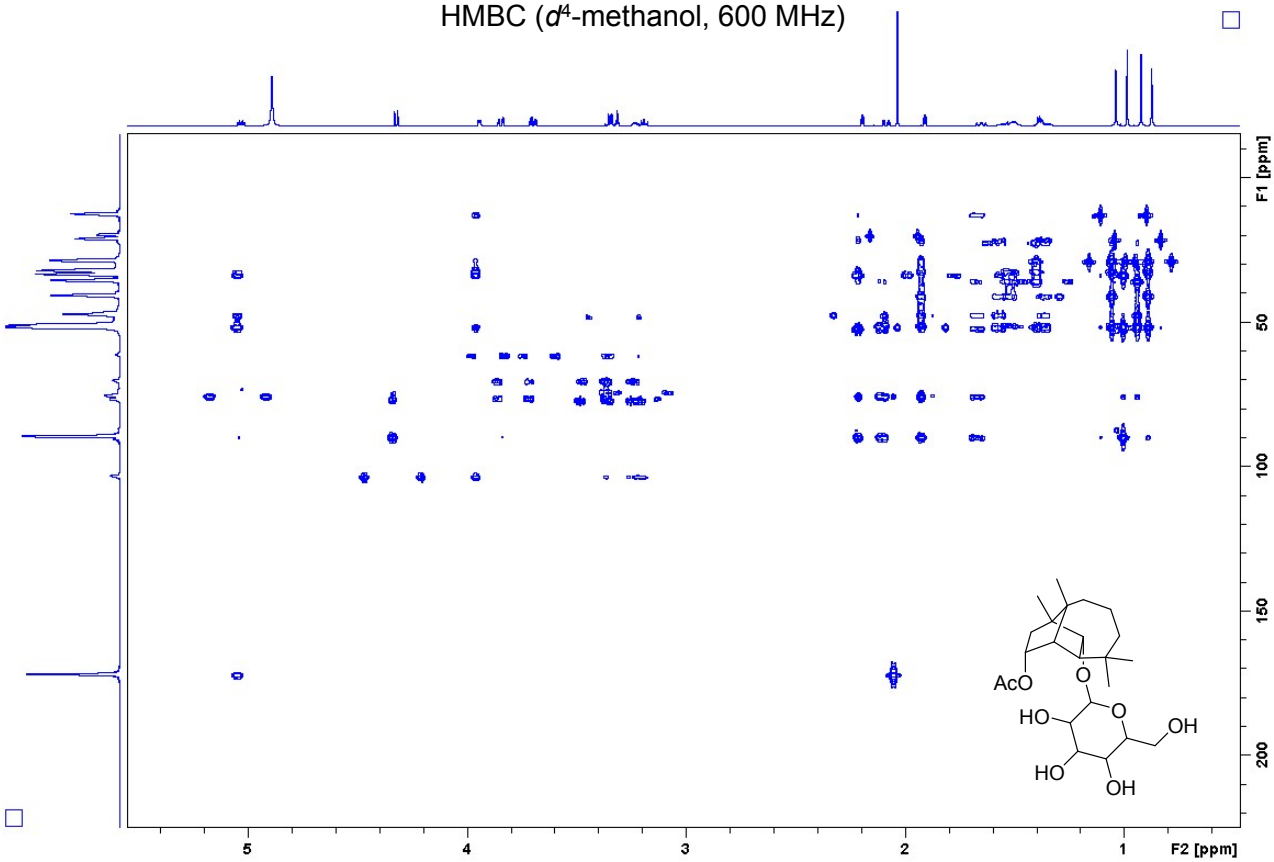
H,H-COSY (d^4-methanol, 600 MHz)



HSQC (d^4-methanol, 600 MHz)

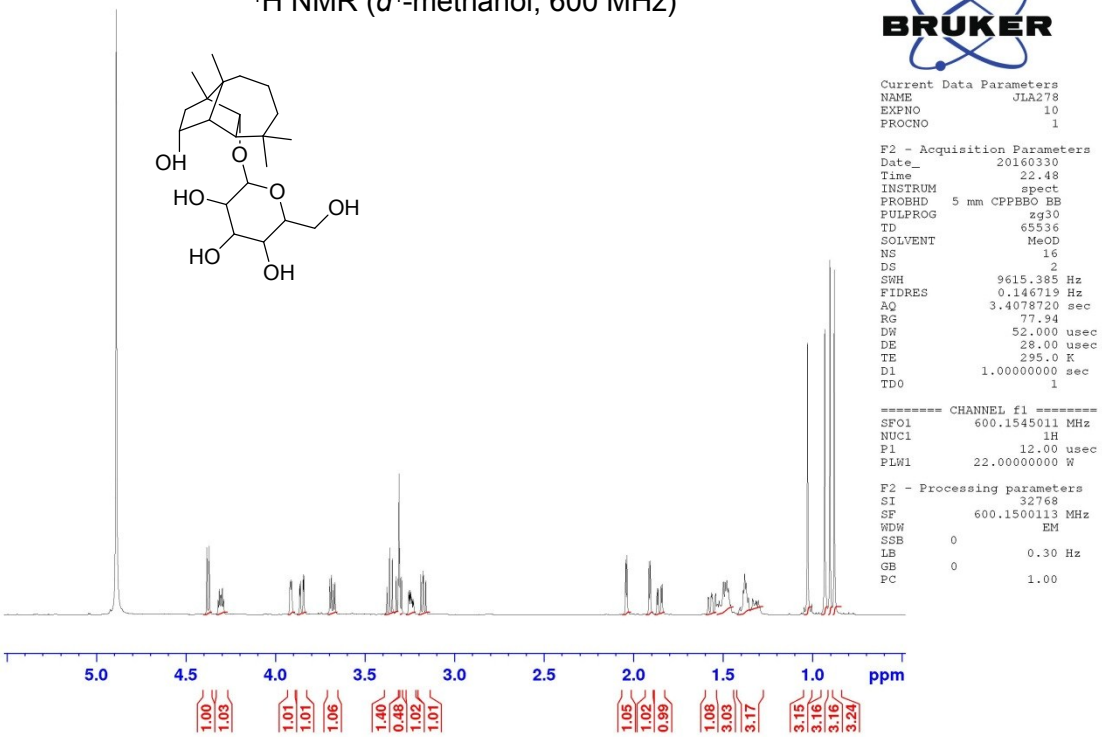


HMBC (d^4-methanol, 600 MHz)



Culmorin-8-β,D-glucoside (6)

^{1}H NMR (*d*^{4}-methanol, 600 MHz)



BRUKER

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FIDRES             0.146719 Hz
AQ                 3.4078720 sec
RG                    77.94
DW                   52.000 usec
DE                   28.00 usec
TE                   295.0 K
D1                1.00000000 sec
TD0                        1

======== CHANNEL f1 ========
SFO1             600.1545011 MHz
NUC1                       1H
P1                      12.00 usec
PLW1            22.00000000 W

F2 - Processing parameters
SI                        32768
SF               600.1500113 MHz
WDW                        EM
SSB          0
LB                      0.30 Hz
GB           0
PC                      1.00
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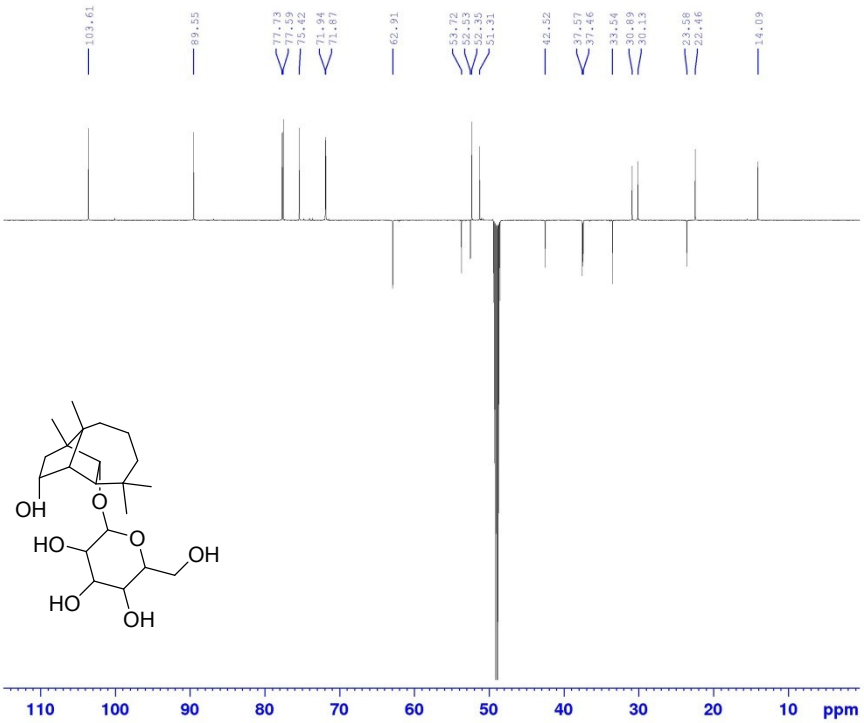
^{13}C NMR (*d*^{4}-methanol, 150 MHz)



BRUKER

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Current Data Parameters
NAME                  JLA278
EXPNO                     13
PROCNO                     1

F2 - Acquisition Parameters
Date_              20160331
Time                   4.27
INSTRUM                spect
PROBHD   5 mm CPPBBO BB
PULPROG                jmod
TD                    65536
SOLVENT                MeOD
NS                      5120
DS                         4
SWH               36057.691 Hz
FIDRES             0.550197 Hz
AQ                 0.9087659 sec
RG                    194.73
DW                   13.867 usec
DE                   18.00 usec
TE                   295.0 K
CNST2            145.0000000
CNST11             1.0000000
D1                 3.00000000 sec
D20                0.00689655 sec
TD0                        1

======== CHANNEL f1 ========
SFO1             150.9236829 MHz
NUC1                      13C
P1                      10.00 usec
P2                      20.00 usec
PLW1            58.00000000 W

======== CHANNEL f2 ========
SFO2             600.1524006 MHz
NUC2                       1H
CPDPRG[2              waltz16
PCPD2                   70.00 usec
PLW2            22.00000000 W
PLW12            0.64652997 W

F2 - Processing parameters
SI                        32768
SF               150.9076273 MHz
WDW                        EM
SSB          0
LB                      1.00 Hz
GB           0
PC                      1.40
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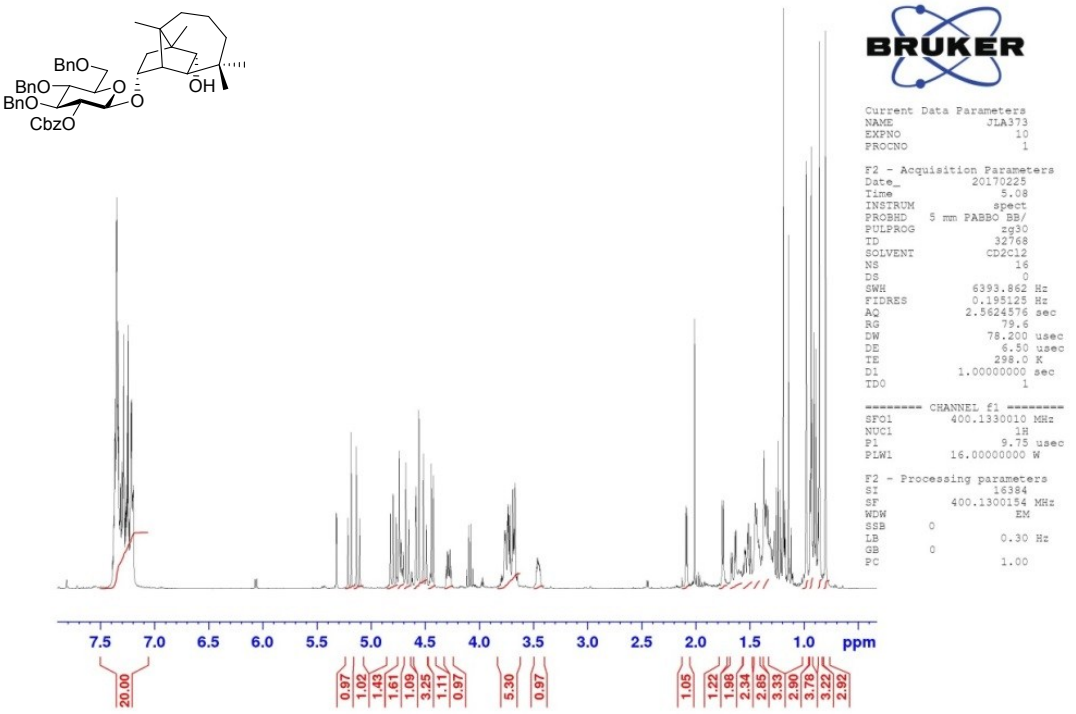
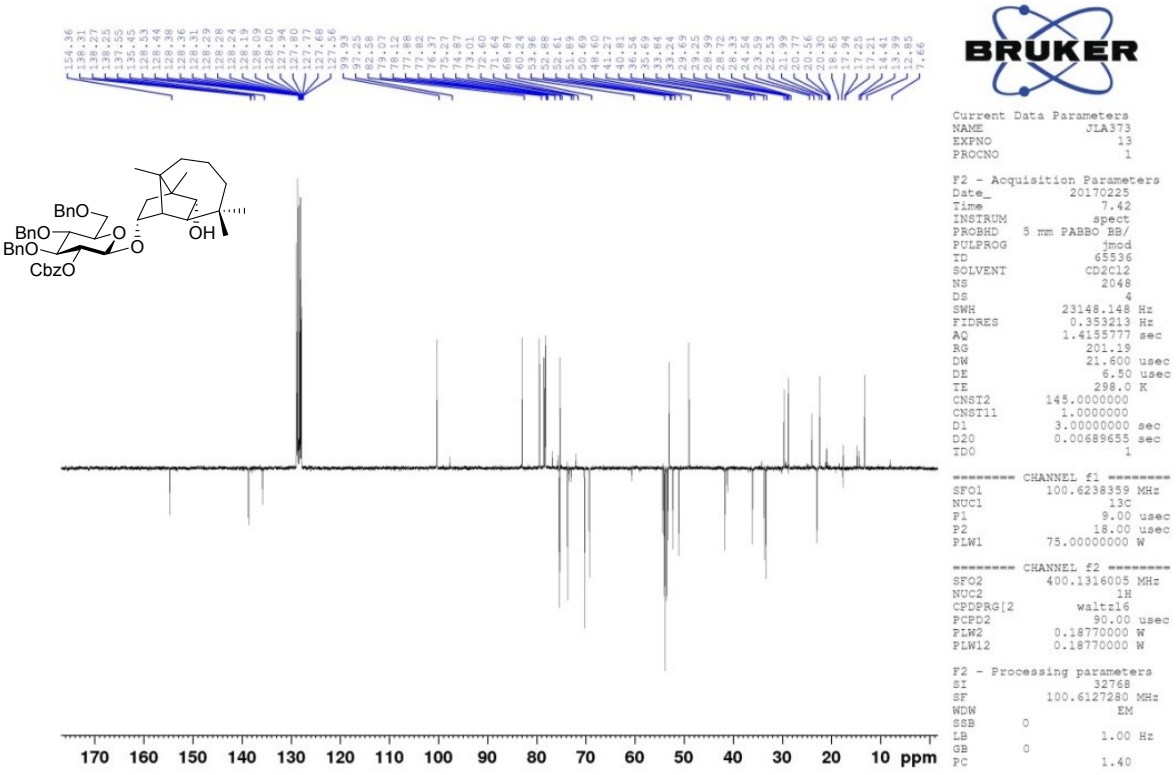
Culmorin-11-(3,4,6-tri-O-benzyl-2-O-benzyloxycarbonyl)-β,D-glucoside (9)

^{1}H NMR (d^{2}-methylene chloride, 400 MHz)

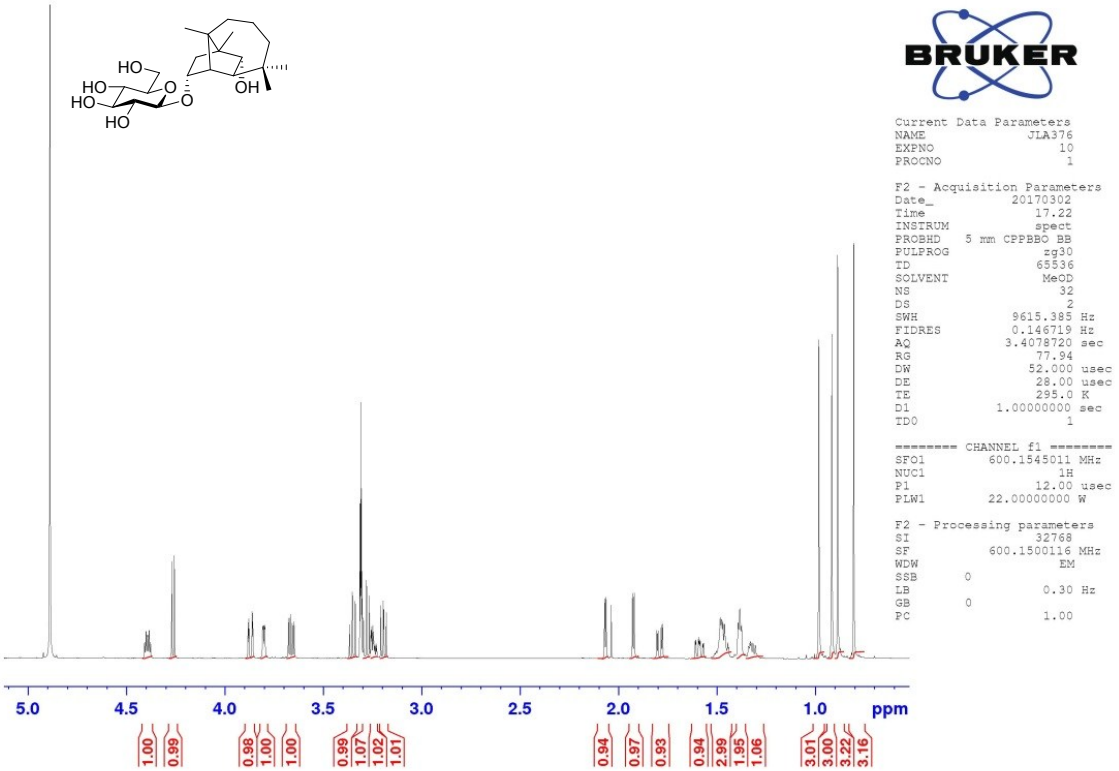


^{13}C NMR (d^{2}-methylene chloride, 100 MHz)

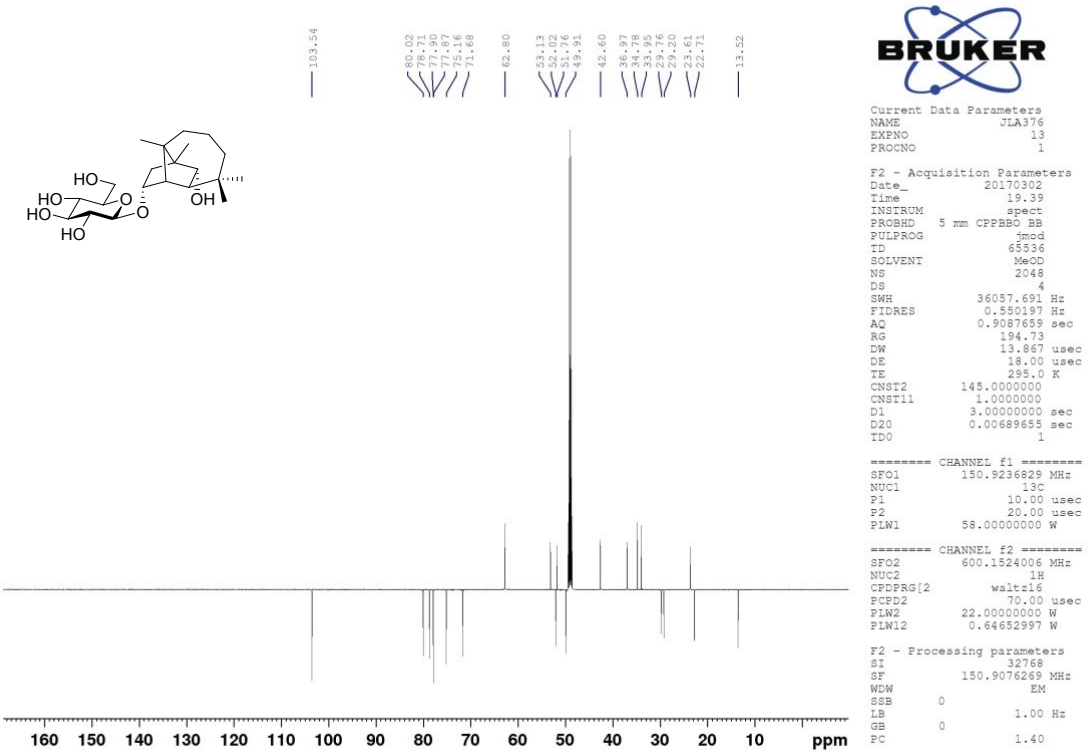


Culmorin-11-β,D-glucoside (7)

^{1}H NMR (*d*^{4}-methanol, 600 MHz)



^{13}C NMR (*d*^{4}-methanol, 150 MHz)



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