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C25 steroids from the marine mussel-derived fungus *Penicillium ubiquetum* MMS330

Thi Phuong Thuy Hoang[a], Catherine Roullier[b], Grégory Genta-Jouve[c,d], Marie-Claude Boumard[b], Thibaut Robiou du Pont[b], Hassan Nazih[b], Yves-François Pouchus[b], Olivier Grovel*[b]

[a] *Institut de Chimie des Substances Naturelles, CNRS UPR 2301, Avenue de la Terrasse, 91198, Gif-sur-Yvette, France*
[b] *EA 2160 - Mer Molécules Santé, Université de Nantes, 44035 Nantes Cedex 1, France*
[c] *Université Paris Descartes, UMR CNRS 8038 CiTCoM, 75006, Paris, France*
[d] *Muséum National d'Histoire Naturelle, Unité Molécules de Communication et Adaptation des Microorganismes, UMR 7245, CP 54, 57 rue Cuvier, 75005 Paris, France*
**Corresponding author at: EA 2160 - Mer Molécules Santé, Université de Nantes, 9 rue Bias, 44035 Nantes Cedex 1, France*
E-mail adress: olivier.grovel@univ-nantes.fr

Abstract

Two new unusual C25 steroid epimers, 24-*O*-methyl-24-*epi*-cyclocitrinol (**1**) and 24-*O*-methylcyclocitrinol (**2**), together with the known oxylipin α-dimorphecolic acid (**3**), were isolated from the extract of cultures of a *Penicillium ubiquetum* MMS330 strain sampled from the blue mussel *Mytilus edulis*. The structures of the new compounds were established based on 1D and 2D NMR spectral analyses and CP3 conformation analysis. LC-MS dereplication of other C25 steroids in culture extracts was also performed on the basis of their HRMS/MS fragmentation patterns, allowing to detect other analogues. Biological activities of compounds **1** and **2** were evaluated as a mixture on the two human cancer cell lines KB and MCF-7.

Keywords

marine-derived fungus; *Penicillium ubiquetum*; C25 steroids; HRMS/MS dereplication

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1. Introduction

C25 steroids with bicyclo[4.4.1]A/B ring have recently been described as an extremely rare family from various terrestrial and marine fungal strains. They share the same tetracyclic skeleton containing two fused seven-membered rings and differ by the decorations of the side chain at position 17, and by the presence or not of a hydroxyl on the octahydro-1*H*-indene moiety. Interestingly, most of them have been found to be produced as mixtures of 24-epimers or 23,24-diastereoisomers. Biosynthesis of these compounds has been proposed to originate from ergosterol (Du et al., 2008; Wang et al., 2019) but the enzymatic sequence and the related gene cluster(s) have still to be discovered. To date twenty eight compounds have been reported (Amagata et al., 2003; Du et al., 2008; Lin et al., 2015; Xia et al., 2014; Ying et al., 2014; Yu et al., 2017), mainly from fungal species belonging to the subgenus *Aspergilloides* of the genus *Penicillium*: *P. citrinum*, *P. decumbens* and *P. janthinellum*. The closely related species *P. chrysogenum*, belonging to the subgenus *Penicillium*, and *Talaromyces purpurogenus* (ex-*P. purpurogenum*) (Samson et al., 2011) have also been described to produce C25 steroids. These compounds could therefore be considered as exclusively produced by *Penicillium* spp. or closely related species (Ying et al., 2014). However the recent discovery of neocyclocitrinol E-G in an endophytic *Colletotrichum* sp. strain means that the capability of building the unusual bicyclo[4.4.1]A/B ring from ergosterol must be more largely distributed in the fungal kingdom than initially thought. Some of the C25 steroids have been evaluated for biological activities. They presented a weak activity either on several human cancer cell lines (Xia et al., 2014; Ying et al., 2014; Yu et al., 2017), on antibacterial activity tests against *Staphylococcus epidermidis* and *Enterococcus durans* (Amagata et al., 2003) or on an acetylcholinesterase inhibitory assay (Yu et al., 2017). Cyclocitrinol, 24-*epi*-cyclocitrinol, neocyclocitrinol B, neocyclocitrinol C and *threo*-23-*O*-methylneocyclocitrinol were shown to induce the production of cyclic AMP in GPR12-transfected CHO cells (Du et al., 2008).

As part of our ongoing research for new natural products from marine-derived fungi (Kerzaon et al., 2009; Roullier et al., 2016b; Vansteelandt et al., 2013), a chemical investigation of a *P. ubiquetum* MMS330 fungal strain isolated from a blue mussel *Mytilus edulis* sampled in the Loire estuary of France (Hoang et al., 2018; Sallenave et al., 1999) was here undertaken. From the culture extract of *P. ubiquetum* MMS330, two new C25 steroids, 24-*O*-methyl-24-*epi*-cyclocitrinol (**1**) and 24-*O*-methylcyclocitrinol (**2**) (Figure 1) were obtained together with the known oxylipin α-dimorphecolic acid (**3**) for which this is the first description as a marine fungal metabolite.

Herein, we report the isolation and structure elucidation of compounds **1**-**3**, the LC-HRMS/MS identification of other C25 steroids produced by *P. ubiquetum* MMS330, and the biological evaluation of compounds **1** and **2** on the two human cancer cell lines KB and MCF-7.

Figure 1. Structures of compounds **1**-**3** isolated from *P. ubiquetum* MMS330.

2. Results and Discussion

2.1. Isolation and Structure Elucidation

Compounds **1** and **2** were first obtained as a 1:1 mixture as observed by ^{1}H NMR spectroscopy. UHPLC-HRESI-MS/MS revealed that they corresponded to isomers with two peaks at *m/z* 415.2835 and 415.2841 (retention time 11.12 min and 11.17 min, respectively) exhibiting the same fragmentation patterns. They were assigned the same molecular formula $C_{26}H_{38}O_4$ with eight degrees of unsaturation. Observation of similar UV spectra (λ_{max} 196 and 246 nm) further confirmed that these two compounds were isomers. 1D and 2D NMR data obtained for the 1:1 mixture revealed that compounds **1** and **2** were nearly identical except for the side chain signals. The mixture was then submitted to further HPLC purification, leading to purified compounds **1** and **2** (0.1 mg each, white amorphous solids). Unambiguous assignment for compounds **1** and **2** was then performed by the combined analysis of the ^{1}H spectra of purified compounds and the 1D and 2D NMR data of the 1:1 mixture.

The ^{1}H NMR spectra and HSQC correlations of both compounds **1** and **2** (Table 1, Figures S1 and S6) displayed four methyl groups including a methoxy (δ_H 0.80, 1.23, 1.37, 3.26 for **1** and 0.82, 1.23, 1.36, 3.26 for **2**), seven methylenes identical for **1** and **2** (δ_H 1.51/2.21, 1.53/1.62, 1.61/1.84, 1.68/2.89, 1.70-1.80, 2.25/2.49 and 2.56), six methines (δ_H 2.11, 2.75, 2.78, 3.50 for both compounds and 1.79, 3.73 for **1**, 1.77, 3.74 for **2**), and four olefinic protons (δ_H 5.49, 5.57 (m, 2H), 5.79 for **1** and 5.50, 5.57 (m, 2H), 5.77 for **2**). Analysis of ^{13}C-NMR spectra (Table 1, Figure S5) revealed the presence of 26 carbons in accordance with the molecular formula, consisting of three methyls (δ_C 14.8, 21.6, 30.0 for **1** and 14.7, 21.4, 29.6 for **2**), one oxygen-bearing sp^3 carbon (δ_C 56.3), seven methylenes (δ_C 22.7, 22.9, 27.7, 27.8, 35.8, 39.6, 41.8), six sp^3 methines (δ_C 48.7, 54.1, 56.1, 60.2, 64.7, 77.9 for **1** and 48.7, 54.1, 56.1, 60.0, 64.7, 77.8 for **2**), four sp^2 methines (δ_C 122.1, 125.3, 128.5, 139.3 for **1** and 122.1, 125.3 128.6, 139.4 for **2**), two sp^3 non protonated carbons (δ_C 46.4, 75.1 for **1** and 46.4, 75.2 for **2**) and three sp^2 non protonated carbons including one carbonyl group (δ_C 146.0, 157.4, 205.3). These NMR data accounted for four out of the eight degrees of unsaturation indicating the presence of a four-ring system.

Table 1. ^{1}H- and ^{13}C- NMR data (500 and 125 MHz, respectively) for **1** and **2** in $CDCl_3$.

Analysis of HMBC and COSY spectra (Figures 2, S7 and S8) revealed a bicyclo[4.4.1] undecadienone A/B ring system in accordance with other previously reported C25 steroids such as isocyclocitrinol A and the revised cyclocitrinol (Amagata et al., 2003; Du et al., 2008; Lin et al., 2015; Xia et al., 2014; Ying et al., 2014; Yu et al., 2017). However, the side chain showed differences either in terms of chemical shifts or HMBC correlations. Both methyl groups H_3-19 (δ_H 0.80 for **1** and 0.82 for **2**) and H_3-21 (δ_H 1.37 for **1** and 1.36 for **2**) showed correlations to the carbon connecting the side chain C-17 (δ_C 60.2 for **1** and 60.0 for **2**). Additionally, the HMBC correlations from H-17 (δ_H 1.79 for **1** and 1.77 for **2**) and H-22 (δ_H 5.79 for **1** and 5.77 for **2**) to C-21 (δ_C 30.0 for **1** and 29.6 for **2**) together with correlations from H-17, H-22 and H-23 (δ_H 5.49 for **1** and 5.50 for **2**) to C-20 (δ_C 75.1 for **1** and 75.2 for **2)** allowed to position the side chain on the four-ring system. The COSY correlations placed the rest of the chain from C-22 to C-25. Finally, the key HMBC correlations from H-24 (δ_H 3.73 for **1**, δ_H 3.74 for **2**) to a methoxy group (δ_C 56.3 identical for **1** and **2**) and from protons of this group (δ_H 3.26 identical for **1** and **2**) to C-24 (δ_C 77.9 for **1** and 77.8 for **2**) assigned this methoxy group at position 24. Based on these data, the planar structure of both compounds **1** and **2** was deduced.

Figure 2. Key COSY, HMBC and NOESY correlations of compounds **1** and **2**.

The study of NOESY spectra (Figure S9) allowed to assign the relative stereochemistry for C-3, C-5, C-9, C-13, C-14, C-17 and C-20 as already described in previous compounds with a similar skeleton (Xia et al., 2014; Ying et al., 2014). The ^{1}H-^{1}H coupling constant between H-22 and H-23 of 15.6 Hz for **1** and 15.8 Hz for **2** confirmed the *trans* double bond at this position for both molecules. However, the ^{1}H-spectrum obtained on the two compounds after HPLC separation and purification exhibited a clear distinction between these two isomers, mainly in the zone between 5.40-5.85 ppm (Figure S2). A close study of chemical shifts and coupling constants, in comparison to described C25 steroids, combined to CP3 calculations allowed to conclude that the two compounds **1** and **2** were epimers at C-24 and to determine their relative configuration on this position. First, the chemical shift of H_3-21 is usually employed as a diagnostic feature for differentiating C-24 epimers in steroids (Goad and Akihisa, 1997). In this way, H_3-21 was 0.01 ppm deshielded in **1** compared to **2** such as it has been described for 20-*O*-methyl-24-*epi*-cyclocitrinol (24*S*, δ_H 1.28) in comparison with 20-*O*-methyl-cyclocitrinol (24*R*, δ_H 1.27) (Xia et al., 2014). Additionally, for **1**, $J^{22,23}$ (15.6 Hz) and $J^{24,25}$ (6.1 Hz) were lower than for compound **2** (15.8 and 6.7 Hz respectively), whereas the coupling constant $J^{23,24}$ was higher for compound **1** (7.6 Hz) than for **2** (7.3 Hz). The same observations were reported for $J^{22,23}$, $J^{23,24}$ and $J^{24,25}$ coupling constants of C-24 epimers of cyclocitrinol and 20-*O*-methyl-cyclocitrinol with lower $J^{22,23}$ and $J^{24,25}$ and a higher $J^{23,24}$ for the 24*S* epimers (Du et al., 2008). According to these data, compound **1** was then proposed as the 24*S* epimer 24-*O*-methyl-24-*epi*-cyclocitrinol and compound **2** as the 24*R* epimer 24-*O*-methylcyclocitrinol. This assignment was further confirmed by means of the CP3 probability calculation (Beniddir et al., 2018; Smith and Goodman, 2009). After GIAO NMR chemical shift calculations, the distinction between both stereoisomers was secured with 73% based on comparing differences in calculated shifts with differences in experimental shifts (Figure 3). From a biosynthetic perspective, the two compounds were assumed to have the same absolute configuration of the [4.4.1]A/B ring as their congeners (Lin et al., 2015; Xia et al., 2014).

Figure 3. CP3 analysis: comparison of the differences in calculated shifts with differences in experimental shifts for **1** and **2**.

The question that *O*-methylated C25 steroids are true natural products or extraction artifacts has been asked in a previous study (Du et al., 2008). They showed that 20-*O*-methyl-cyclocitrinol epimers could result from the methylation of the corresponding 20-OH natural products, cyclocitrinol and 24-*epi*-cyclocitrinol, by alcoholysis when stirred 48h in MeOH and acetic acid. However, since this study these compounds have been isolated twice from *Penicillium decumbens* (Lin et al., 2015) and *Talaromyces purpurogenus* (Xia et al., 2014). Four other *O*-methylated C25 steroids have also been described from fungal cultures, i.e. *erythro*-23-*O*-methylneocyclocitrinol, *threo*-23-*O*-methylneocyclocitrinol, 23-*O*-methylantineocyclocitrinol A and 23-*O*-methylantineocyclocitrinol B (Xia et al., 2014). It has to be noticed that in our study no MeOH has been used during the extraction process, a priori precluding the formation of methylated products. Nevertheless, to determine whether compounds **1** and **2** were natural

products or not, their presence in the CH_2Cl_2/EtOAc extract was monitored by UHPLC-HRMS/MS profiling. Extracted ion chromatogram (XIC) of *m/z* 415.284 revealed two peaks at 11.12 and 11.17 min corresponding to the elution time of pure compounds **1** and **2** (Figure 4-A). The HRMS/MS spectrum of these two compounds detected in the extract (Figure 4-B) were the same as the two isolated compounds (Figure 4-C), which exhibited fragments ions at *m/z* 397.2758 ($C_{26}H_{37}O_3$, $[M+H-H_2O]^+$, calcd. 397.2742, Δ +3.9 ppm), 383.2591 ($C_{25}H_{35}O_3$ $[M+H-CH_3OH]^+$, calcd. 383.2586, Δ +1.3 ppm), 365.2473 ($C_{25}H_{33}O_2$ $[M+H-CH_3OH-H_2O]^+$, calcd. 365.2480, Δ -2.0 ppm), 313.1814 ($C_{20}H_{25}O_3$ $[M+H-CH_3OH-C_5H_{10}]^+$, calcd. 313.1803, Δ +3.3 ppm), 285.1863 ($C_{19}H_{25}O_2$ $[M+H-CH_3OH-C_5H_{10}-CO]^+$, calcd. 285.1854, Δ +3.0 ppm) and 267.1748 ($C_{19}H_{23}O$ $[M+H-CH_3OH-C_5H_{10}-CO-H_2O]^+$, calcd. 267.1749, Δ -0.3 ppm), this last fragment putatively corresponding to the $C_{19}H_{22}O$ non-decorated tetracyclic backbone shared by all C25 steroids with bicyclo[4.4.1]A/B ring. The presence of compounds **1** and **2** in the extracts established by these observations confirmed that they were not chemical artifacts.

Figure 4. A) UHPLC-HRESIMS/MS profile of extract of *P. ubiquetum* MMS330 and XIC of *m/z* 415.2847 showing the presence of compounds **1** and **2** at t_R 11.1 min; B) HRMS/MS fragmentation spectrum of *m/z* 415.2847 ion detected in *P. ubiquetum* MMS330 extract at t_R = 11.12 min; C) HRMS/MS fragmentation spectrum of pure compound **1**.

In addition to these two new compounds, the known α-dimorphecolic acid, also named 9-hydroxy-10(*E*), 12(*Z*)-octadecadienoic acid or 9-HODE, was isolated and identified from the culture extract of *P. ubiquetum* MMS330. Its structure was elucidated based on the comparison of its ^{1}H-NMR (Figure S11) and HRMS/MS fragmentation data (Figure S12) with those reported in the literature (Garscha et al., 2007; Martin-Arjol et al., 2010; Martini et al., 1996).

2.2. Dereplication of other C25 Steroids

In previous studies focusing on the isolation of C25 steroids from fungi, numerous analogues have been simultaneously isolated, except for the initial publication of cyclocitrinol from a *P. citrinum* strain (Kozlovsky et al., 2000). In order to investigate such possibility in *P. ubiquetum* MMS330, other analogues belonging to this chemical class were searched by dereplication of either the extract or VLC subfractions. For that purpose, the exact mass of $[M+H]^+$ adducts for all the 28 described compounds in this chemical series were searched in LC-HRMS profiles (Figure 5). HRMS/MS spectra of the peaks exhibiting one of these masses were then compared with those of compounds **1** and **2**, and to MS/MS fragmentations described in the literature (Marinho et al., 2005; Xia et al., 2014). In particular, it was noteworthy that the fragmentation pattern of $\Delta^{20\text{-}22}$ compounds is characterized by three successive losses of water, or by one loss of a methoxy group and two losses of water for the 23-*O*-methyl compounds, whereas only two water losses are observed for the $\Delta^{22\text{-}23}$ and $\Delta^{23\text{-}24}$ products. These fragmentations have been described both in APCI and ESI mass spectrometry, leading to suggest their consistency to predict the position of the double bond on the lateral chain.

Thorough investigations of the UHPLC-HRESIMS/MS profiles, searching for these 28 possible $[M+H]^+$ adducts and for their characteristic simultaneous mass losses, led to the detection of six other putative C25 steroids analogues in two VLC fractions. Two pairs of peaks at *m/z* 401.2673 were observed at t_R

8.43/8.56 min and 8.87/9.09 min (Figure 5-B). These four compounds were $C_{25}H_{36}O_4$ isomers (calcd 401.2691, Δ -3.24 ppm). For the two first peaks, the same fragment ions were observed at *m/z* 383.2581 and 365.2475 corresponding to two losses of H_2O. They were deduced to be C25 steroids belonging to the $\Delta^{22\text{-}23}$ or $\Delta^{23\text{-}24}$ subclasses as only two water losses were observed. These four compounds were then tentatively assigned as the known epimer pairs cyclocitrinol and 24-*epi*-cyclocitrinol (major peaks), 22*S*-isocyclocitrinol A and 22*R*-isocyclocitrinol B, i.e. the four known $C_{25}H_{36}O_4$ C25 steroids including a $\Delta^{22\text{-}23}$ or $\Delta^{23\text{-}24}$ double bond. This proposition was reinforced by the supposed biosynthesis of compounds **1** and **2**, which should respectively result from the *O*-methylation of cyclocitrinol and 24-*epi*-cyclocitrinol by a SAM. These two last metabolites have also been systematically found in *Penicillium* strains producing C25 steroids and especially in strains belonging to the section *Citrina* of the subgenus *Aspergilloides*, a section to which belongs the *P. ubiquetum* MMS330 strain (Amagata et al., 2003; Du et al., 2008; Kozlovsky et al., 2000; Lin et al., 2015). In the same way, the 20-*O*-methyl analogues have also been isolated together with cyclocitrinol and 24-*epi*-cyclocitrinol from which they also derive (Xia et al., 2014).

Two isobaric compounds exhibiting the same exact mass than compounds **1** and **2** at *m/z* 415.2847 were also observed as a shouldered peak at t_R 13.63 min (Figure 5-D). Although formula prediction (from $[M+H]^+$, Δ 0.3 ppm) allowed to conclude to $C_{26}H_{38}O_4$ C25 steroid analogues, no fragmentation data could be obtained for these trace compounds, avoiding to give a putative annotation. It could however be envisaged from a biosynthetic point of view that they were 20-*O*-methyl-cyclocitrinol and 20-*O*-methyl-24-*epi*-cyclocitrinol, also resulting from the methylation of cyclocitrinol and 24-*epi*-cyclocitrinol.

Figure 5. A) UHPLC-HRESIMS/MS profile of *P. ubiquetum* MMS330 VLC fraction Fr-10; B) Extracted Ion Chromatograms (XIC) of compounds at *m/z* 401.2673 and characteristic daughter ions searched at *m/z* 383.2581, 365.2475 and 347.2369 by data-dependent scan MS/MS; C) UHPLC-HRESIMS/MS profile of *P. ubiquetum* MMS330 VLC fraction Fr-11; D) XIC of compounds at *m/z* 415.2847 and characteristic daughter ions searched at *m/z* 397.2758, 383.2581, 365.2475 and 347.2369 by data-dependent scan MS/MS (the peaks of compounds **1**, **2** are designated).

2.3. Biological Activity

The 1:1 mixture of compounds **1** and **2** was evaluated for antiproliferative activity against two human cancer cell lines, the epithelial cancer cell line KB and the breast cancer cell line MCF-7. It did not exhibit significant cytotoxicity with 17% and 35% of cell growth inhibition at 30 µM on KB and MCF-7 cell lines respectively.

3. Conclusions

Investigation of the culture extract of *P. ubiquetum* led to the isolation of two further compounds in the rare C-25 steroids series, 24-*O*-methyl-cyclocitrinol and 24-*O*-methyl-24-*epi*-cyclocitrinol, which were demonstrated to be true natural products, probably arising from the *O*-methylation of cyclocitrinol and 24-*epi*-cyclocitrinol. These two last compounds were indeed detected by UHPLC-HRMS/MS together with four other analogues. These results confirm that *Penicillium* strains belonging to the *Aspergilloides*

subgenus are prolific producers of C-25 steroids. Compounds **1** and **2** did not exhibit strong cytotoxic activity. These results were in accordance with previous studies on other human cancer cell lines which have also shown that this class of compounds exhibited only weak inhibition of cancer cell growth (Xia et al., 2014; Ying et al., 2014).

In this study we also report the isolation of α-dimorphecolic acid (9-HODE), a common oxylipin found in plants (Cahoon and Kinney, 2004; McRae et al., 2008; Smith et al., 1960), green algae (Liu et al., 2013), bacteria (Swain et al., 2017) and once detected in the macroscopic fungus *Cordyceps militaris* (Yoon et al., 2005). In fungi, the two main monohydroxylated representatives of oxylipins are 8(*R*)-HODE and 10(*R*)-HODE, which correspond to oxidation products of linoleic acid formed by lipoxygenases such as linoleate diol synthases (Garscha et al., 2007). In *Aspergillus fumigatus* and *A. nidulans* these compounds and other oxylipins are known to play a role in induction of sexual sporulation, in mediation of the balance of asexual to sexual spore ratio, in mycotoxin production and in virulence of pathogenic species (Tsitsigiannis and Keller, 2006; Tsitsigiannis et al., 2004). This study is the first report of α-dimorphecolic acid from an extract of a fungal strain culture.

4. Experimental Section

4.1. General experimental procedures

NMR spectra were recorded in $CDCl_3$ using TMS as internal standard on a Bruker Avance I 500 MHz spectrometer equipped with a cryoprobe. Chemical shifts were referenced using residual solvent $CDCl_3$ as reference (δ_H 7.26 and δ_C 77.16 ± 0.06). HPLC fractionations were carried out on an Agilent 1200 series apparatus (Agilent, USA) with UV detection at 204, 254, 272 and 308 nm. UHPLC-HRESI-MS/MS analyses were performed on a UFLC-MS (IT-TOF) Shimadzu instrument (combining Ion trap and Time of Flight analyzers), using a Kinetex C_{18} column (100 x 2.1 mm) and following previously described conditions (Roullier et al., 2016a). Samples (5 µL) at a concentration of 0.25 mg/mL in MeOH were injected. Silica (60 Å, 35-70 mesh particle size) and C_{18}-bonded silica (50-60 Å) used for Vacuum Liquid Chromatography (VLC) were purchased from Carlo Erba (Val de Reuil, France) and Macherey-Nagel (Düren, Germany) respectively.

4.2. Fungal material fermentation and extraction

The procedures of isolation and identification of the fungal strain, fermentation and extraction used in this experiment were performed as recently reported (Hoang et al., 2018).

4.3. Isolation, purification and structure determination

The CH_2Cl_2/EtOAc 1:1 (v/v) extract (1.06 g) was fractionated by vacuum liquid chromatography (VLC) on a silica gel column with a hexane/EtOAc (100:0 to 60:40 v/v) and CH_2Cl_2/MeOH (100:0 to 0:100 v/v) with a non-linear gradient yielding 13 fractions. Fraction Fr-7 (23 mg) was subjected to VLC on a reverse phase C_{18} column with a step-gradient of H_2O/MeOH from 100:0 to 0:100 (v/v) giving nine fractions. Fraction Fr-7-4 (6.8 mg) was further purified by normal phase silica gel HPLC using a silica gel column (250 x 4.6 mm, CH_2Cl_2/MeOH 98:2 v/v, 1 mL/min) to afford α-dimorphecolic acid **3** (1 mg, t_R = 9 min). Fraction Fr-10 (249.8 mg) was subjected to silica gel column chromatography using a step-gradient of CH_2Cl_2/EtOH from 100:0 to 0:100 (v/v). Among the 12 subfractions obtained, fractions Fr-10-F (11.3

mg) and Fr-10-G (11.2 mg) were fractionated separately by VLC on silica gel with an elution by hexane/acetone mixtures from 95:5 to 30:70 (v/v) to yield 8 subfractions each. Fractions Fr 10-F-6 (2.6 mg), Fr 10-G-5 (2.0 mg) and Fr 10-G-6 (3.0 mg) displayed the same TLC profile and were then combined. They were further fractionated by reverse phase C_{18} column chromatography with H_2O/CH_3CN 1:1 to 0:1 v/v to yield 7 subfractions. Fraction Fr-10-G-F-4 (0.7 mg) contained the mixture of compounds **1** and **2**, which were separated by reversed-phase HPLC using a C_{18} column (250 x 10 mm, H_2O/CH_3CN 30:70 v/v, 2.0 mL/min), affording the individually purified compound **1** (0.1 mg, t_R = 66.7 min) and compound **2** (0.1 mg, t_R = 68.6 min).

4.3.1. 24-O-methyl-24-epi-cyclocitrinol (= (9R,13S,17S,14R,5S,3S)-3-hydroxy-17-[(20S,22E,24S)-20-hydroxy-24-methoxyhex-22-en-20-yl]-13-methyltetracyclo[11.4.1.0^{8,9}.0^{13,14}]octadeca-1(10),8-dien-6-one; 1)

White solid. $[\alpha]_D^{20}$ = +74.9 (1:1 mixture of **1** and **2**, *c* 0.09, $CHCl_3$). UV (MeOH) λ_{max} 196 and 246 nm. ^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) see Table 1. (+)HR-ESIMS *m/z* 415.2835 $[M+H]^+$ (calcd for $C_{26}H_{39}O_4$ 415.2848, Δ -3.2 ppm).

4.3.2. 24-O-methylcyclocitrinol (= (9R,13S,17S,14R,5S,3S)-3-hydroxy-17-[(20S,22E,24R)-20-hydroxy-24-methoxyhex-22-en-20-yl]-13-methyltetracyclo[11.4.1.0^{8,9}.0^{13,14}]octadeca-1(10),8-dien-6-one; 2)

White solid. UV (MeOH) λ_{max} 196 and 246 nm. ^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) see Table 1. (+)HR-ESIMS *m/z* 415.2841 $[M+H]^+$ (calcd for $C_{26}H_{39}O_4$ 415.2848, Δ -1.7 ppm).

4.3.3. CP3 analysis computational details

All DFT calculations have been performed using Gaussian 16 (Frisch et al., 2016). A conformation analysis was conducted using the GMMX plugin and followed by a geometry optimization at the B3LYP/6-31g(d) level. A frequency check was performed at the same level of theory. GIAO NMR properties were calculated at the mpw1pw91/6-311+g(2d,p) level. CP3 probabilities were calculated using our own implementation the algorithm published by Goodman (Smith and Goodman, 2009).

4.4. Cytotoxicity assays

KB and MCF-7 cell lines were purchased from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK). All materials utilized for the test were purchased from Sigma Aldrich (Lyon, France). Cytotoxicity assays were carried for 72 h using the MTT assay (Mosmann, 1983) and following the procedure previously described (Luu et al., 2018; Vansteelandt et al., 2013). Stock solutions at concentration of 5 mM were prepared in EtOH and the final concentration tested in the well was 50 µM. The absorbance was read at 570 nm and 630 nm using a SpectraMax 190 Absorbance Microplate Reader (Molecular Devices, USA). Culture medium in 1% EtOH was used as negative control and docetaxel was used as positive control (IC_{50} of 38 nM for KB cell line and 10.3 nM for MCF-7 cell line).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:

Acknowledgements

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Table 1. ^{1}H- and ^{13}C- NMR data (500 and 125 MHz, respectively) for **1** and **2** in $CDCl_3$.

Position	1			2		
	δ_C	Type	δ_H, multi, *J* (Hz)	δ_C	Type	δ_H, multi, *J* (Hz)
1	122.1	CH	5.57 (m)	122.1	CH	5.57 (m)
2	35.8	CH_2	α : 2.25 (m)	35.8	CH_2	α : 2.25 (m)
			β : 2.49 (ddd, *J* = 13.4, 11.6, 6.1)			β : 2.49 (ddd, *J* = 13.4, 11.6, 6.1)
3	64.7	CH	3.50 (m)	64.7	CH	3.50 (m)
4	41.8	CH_2	α: 2.89 (brd, *J* = 12.8)	41.8	CH_2	α: 2.89 (brd, *J* = 12.8)
			β : 1.68 (m)			β : 1.68 (m)
5	48.7	CH	2.75 (m)	48.7	CH	2.75 (m)
6	205.3	C		205.3	C	
7	125.3	CH	5.57 (s)	125.3	CH	5.57 (s)
8	157.4	C		157.4	C	
9	54.1	CH	2.78 (dd, *J* = 12.2, 6.1)	54.1	CH	2.78 (dd, *J* = 12.2, 6.1)
10	146.0	C		146.0	C	
11	27.8	CH_2	α : 1.61 (d, *J* = 15.0)	27.8	CH_2	α : 1.61 (d, *J* = 15.0)
			β : 1.84 (m)			β : 1.84 (m)
12	39.6	CH_2	α : 1.51 (dt, *J* = 14.3, 4.2)	39.6	CH_2	α : 1.51 (dt, *J* = 14.3, 4.2)
			β : 2.21 (m)			β : 2.21 (m)
13	46.4	C		46.4	C	
14	56.1	CH	2.11 (dd, *J* = 12.5, 6.4)	56.1	CH	2.11 (dd, *J* = 12.5, 6.4)
15	22.7	CH_2	α: 1.62 (m)	22.7	CH_2	α: 1.62 (m)
			β: 1.53 (m)			β: 1.53 (m)
16	22.9	CH_2	1.7 - 1.8 (m)	22.9	CH_2	1.7 - 1.8 (m)
17	60.2	CH	1.79 (t, *J* = 9.9)	60.0	CH	1.77 (brt, *J* = 9.9)
18	27.7	CH_2	2.56 (d, *J* = 6.7)	27.7	CH_2	2.56 (d, *J* = 6.7)
19	14.8	CH_3	0.80 (s)	14.7	CH_3	0.82 (s)
20*	75.2	C		75.1	C	
21	30.0	CH_3	1.37 (s)	29.6	CH_3	1.36 (s)
22*	139.4	CH	5.79 (d, *J* = 15.6)	139.3	CH	5.77 (d, *J* = 15.9)
23*	128.6	CH	5.49 (dd, *J* = 15.6, 7.6)	128.5	CH	5.50 (dd, *J* = 15.9, 7.3)
24*	77.9	CH	3.73 (m)	77.8	CH	3.74 (m)
25*	21.6	CH_3	1.23 (d, *J* = 6.1)	21.4	CH_3	1.23 (d, *J* = 6.7)
OMe	56.3	CH_3	3.26 (s)	56.3	CH_3	3.26 (s)

* assignments for ^{13}C may be interchanged between compounds **1** and **2**

Figure captions

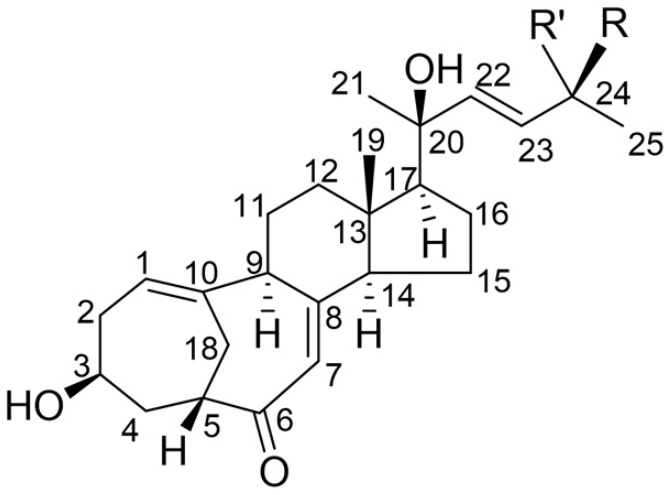
Figure 1. Structures of compounds **1**-**3** isolated from *P. ubiquetum* MMS330.

Figure 2. Key COSY, HMBC and NOESY correlations of compounds **1** and **2**.

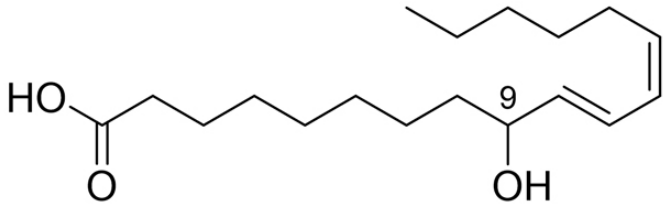
Figure 3. CP3 analysis: comparison of the differences in calculated shifts with differences in experimental shifts for **1** and **2**.

Figure 4. A) UHPLC-HRESIMS/MS profile of extract of *P. ubiquetum* MMS330 and XIC of *m/z* 415.2847 showing the presence of compounds **1** and **2** at t_R 11.1 min; B) HRMS/MS fragmentation spectrum of *m/z* 415.2847 ion detected in *P. ubiquetum* MMS330 extract at t_R = 11.12 min; C) HRMS/MS fragmentation spectrum of pure compound **1**.

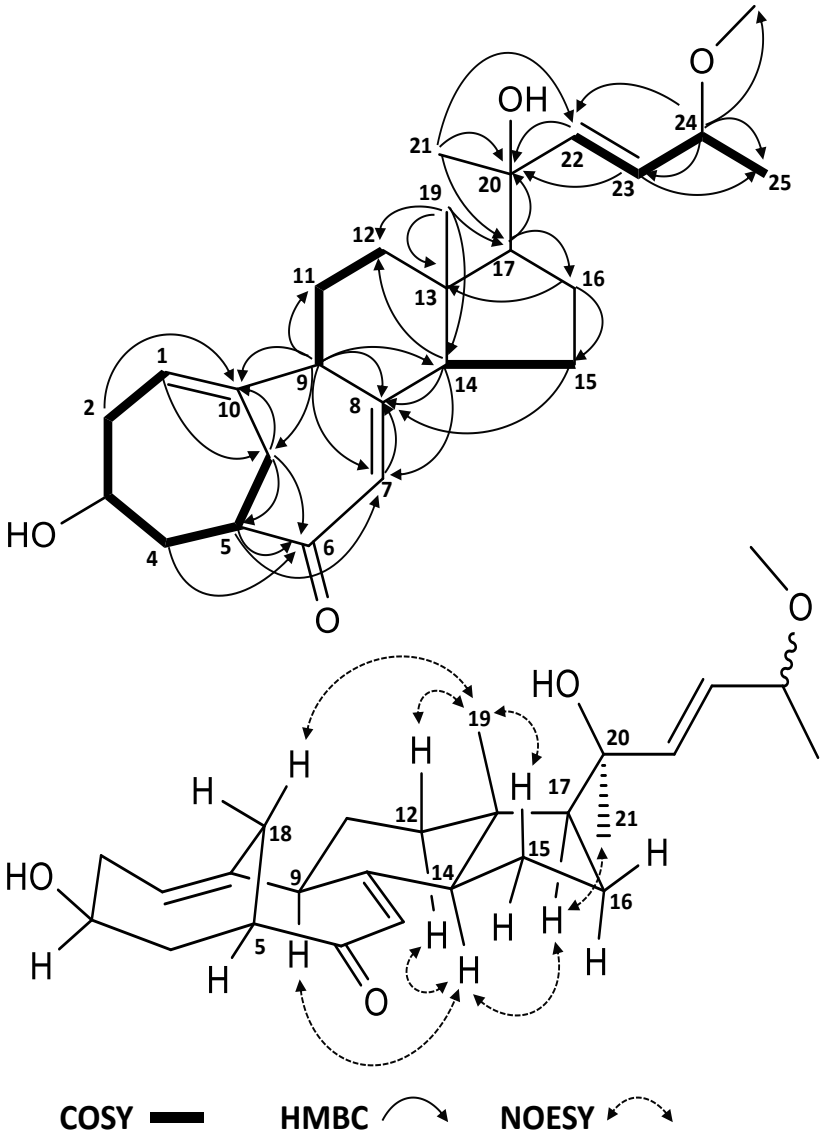
Figure 5. A) UHPLC-HRESIMS/MS profile of *P. ubiquetum* MMS330 VLC fraction Fr-10; B) Extracted Ion Chromatograms (XIC) of compounds at *m/z* 401.2673 and characteristic daughter ions searched at *m/z* 383.2581, 365.2475 and 347.2369 by data-dependent scan MS/MS; C) UHPLC-HRESIMS/MS profile of *P. ubiquetum* MMS330 VLC fraction Fr-11; D) XIC of compounds at *m/z* 415.2847 and characteristic daughter ions searched at *m/z* 397.2758, 383.2581, 365.2475 and 347.2369 by data-dependent scan MS/MS (the peaks of compounds **1**, **2** are designated).

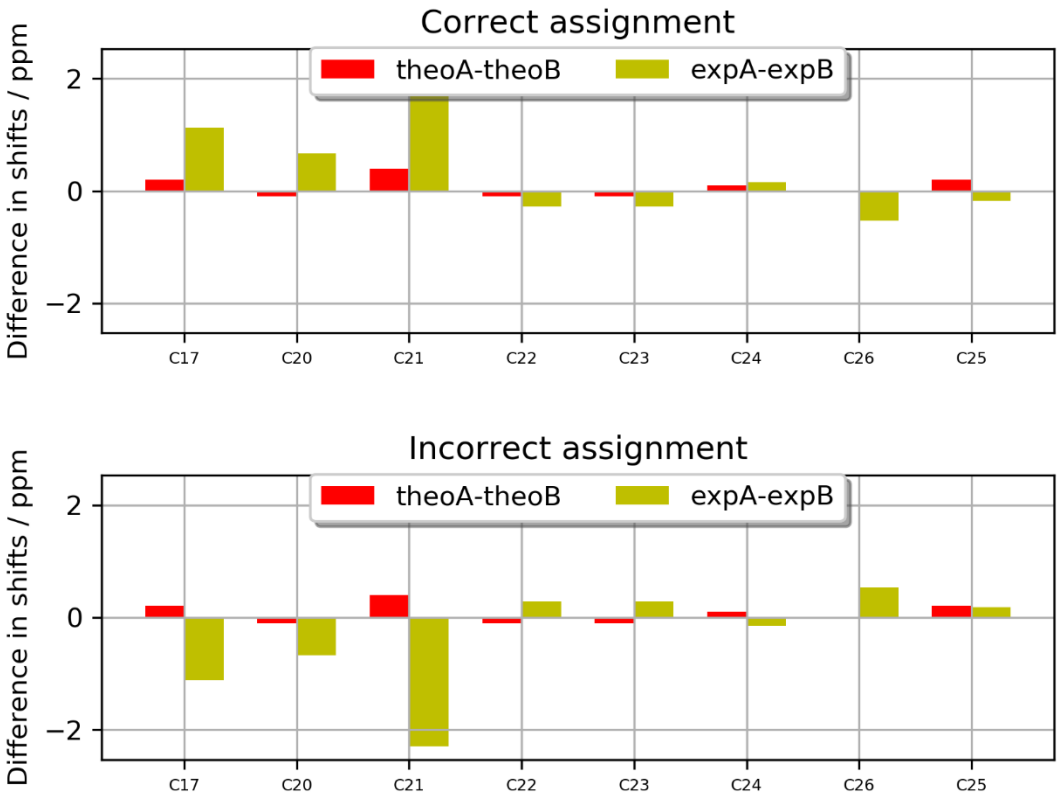


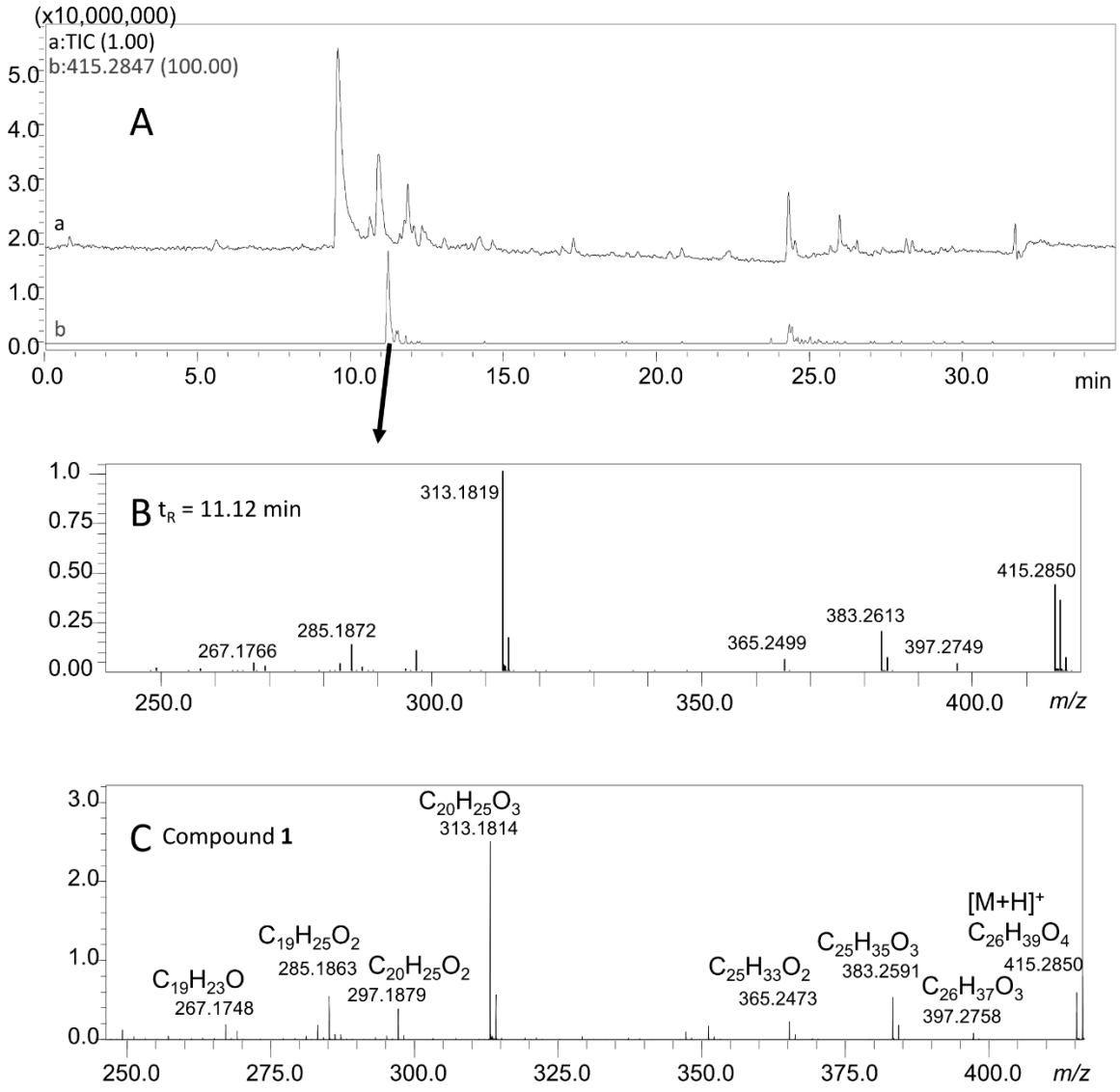
	R	R'
1	-OMe	-H
2	-H	-OMe

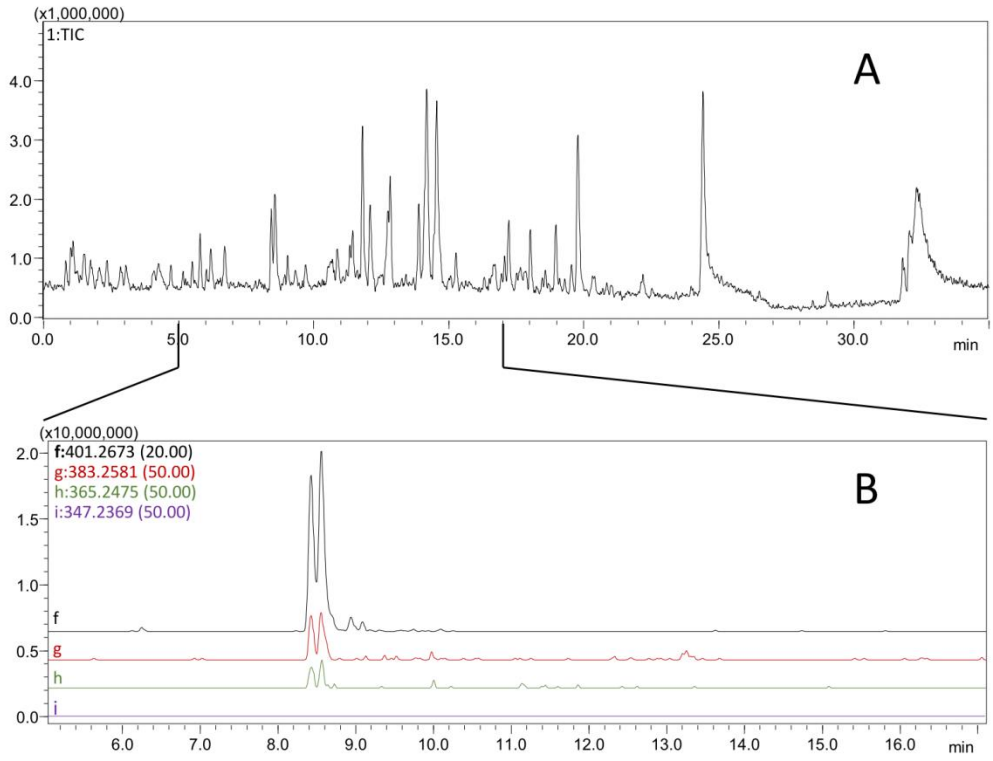


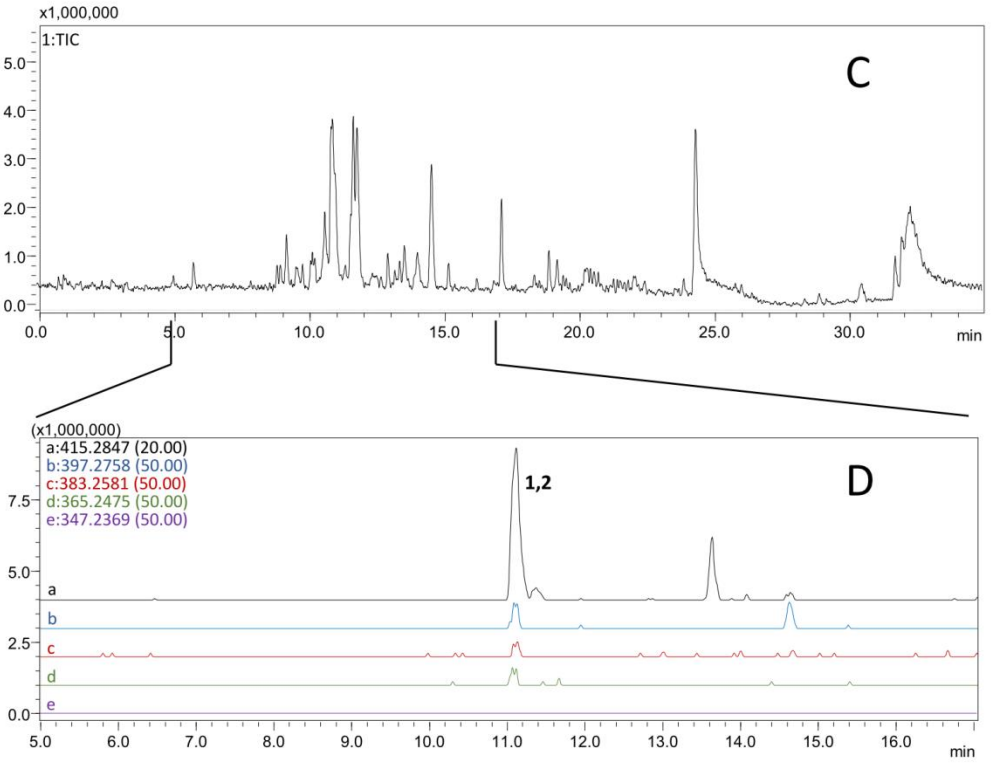
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COSY
HMBC
NOESY


Correct assignment
theoA-theoB
expA-expB
Difference in shifts / ppm
2
0
−2
C17
C20
C21
C22
C23
C24
C26
C25
Incorrect assignment
theoA-theoB
expA-expB
Difference in shifts / ppm
2
0
−2
C17
C20
C21
C22
C23
C24
C26
C25


(x10,000,000)
a:TIC (1.00)
b:415.2847 (100.00)
A
a
b
5.0
4.0
3.0
2.0
1.0
0.0
0.0
5.0
10.0
15.0
20.0
25.0
30.0
min
B t_R = 11.12 min
313.1819
415.2850
383.2613
285.1872
267.1766
365.2499
397.2749
1.0
0.75
0.50
0.25
0.00
250.0
300.0
350.0
400.0
m/z
C Compound 1
$C_{20}H_{25}O_3$
313.1814
$[M+H]^+$
$C_{26}H_{39}O_4$
415.2850
$C_{19}H_{25}O_2$
285.1863
$C_{20}H_{25}O_2$
297.1879
$C_{19}H_{23}O$
267.1748
$C_{25}H_{33}O_2$
365.2473
$C_{25}H_{35}O_3$
383.2591
$C_{26}H_{37}O_3$
397.2758
3.0
2.0
1.0
0.0
250.0
275.0
300.0
325.0
350.0
375.0
400.0
m/z


(x1,000,000)
1:TIC
A
4.0
3.0
2.0
1.0
0.0
0.0
5.0
10.0
15.0
20.0
25.0
30.0
min
(x10,000,000)
f:401.2673 (20.00)
g:383.2581 (50.00)
h:365.2475 (50.00)
i:347.2369 (50.00)
B
2.0
1.5
1.0
0.5
0.0
f
g
h
i
6.0
7.0
8.0
9.0
10.0
11.0
12.0
13.0
14.0
15.0
16.0
min


x1,000,000
1:TIC
C
5.0
4.0
3.0
2.0
1.0
0.0
0.0
5.0
10.0
15.0
20.0
25.0
30.0
min
(x1,000,000)
a:415.2847 (20.00)
b:397.2758 (50.00)
c:383.2581 (50.00)
d:365.2475 (50.00)
e:347.2369 (50.00)
1,2
D
7.5
5.0
2.5
0.0
a
b
c
d
e
5.0
6.0
7.0
8.0
9.0
10.0
11.0
12.0
13.0
14.0
15.0
16.0
min

Supplementary data

C25 steroids from the marine mussel-derived fungus *Penicillium ubiquetum* MMS330

Thi Phuong Thuy Hoang[a], Catherine Roullier[b], Grégory Genta-Jouve[c,d], Marie-Claude Boumard[b], Thibaut Robiou du Pont[b], Hassan Nazih[b], Yves-François Pouchus[b], Olivier Grovel*[b]

[a] *Institut de Chimie des Substances Naturelles, CNRS UPR 2301, Avenue de la Terrasse, 91198, Gif-sur-Yvette, France*

[b] *EA 2160 - Mer Molécules Santé, Université de Nantes, 44035 Nantes Cedex 1, France*

[c] *Université Paris Descartes, UMR CNRS 8038 CiTCoM, 75006, Paris, France*

[d] *Muséum National d'Histoire Naturelle, Unité Molécules de Communication et Adaptation des Microorganismes, UMR 7245, CP 54, 57 rue Cuvier, 75005 Paris, France*

**Corresponding author at: EA 2160 - Mer Molécules Santé, Université de Nantes, 9 rue Bias, 44035 Nantes Cedex 1, France*

E-mail adress: olivier.grovel@univ-nantes.fr

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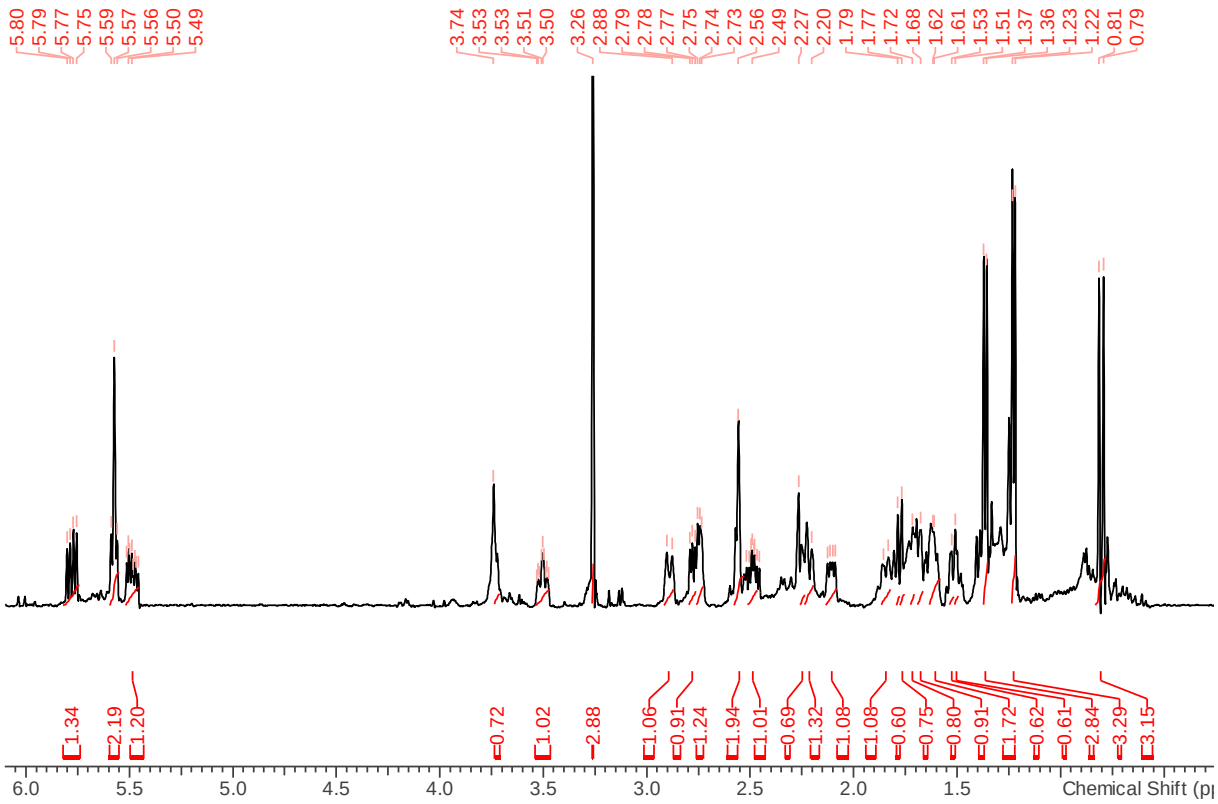


Figure S1. ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) of compounds 1 and 2 (mixture).

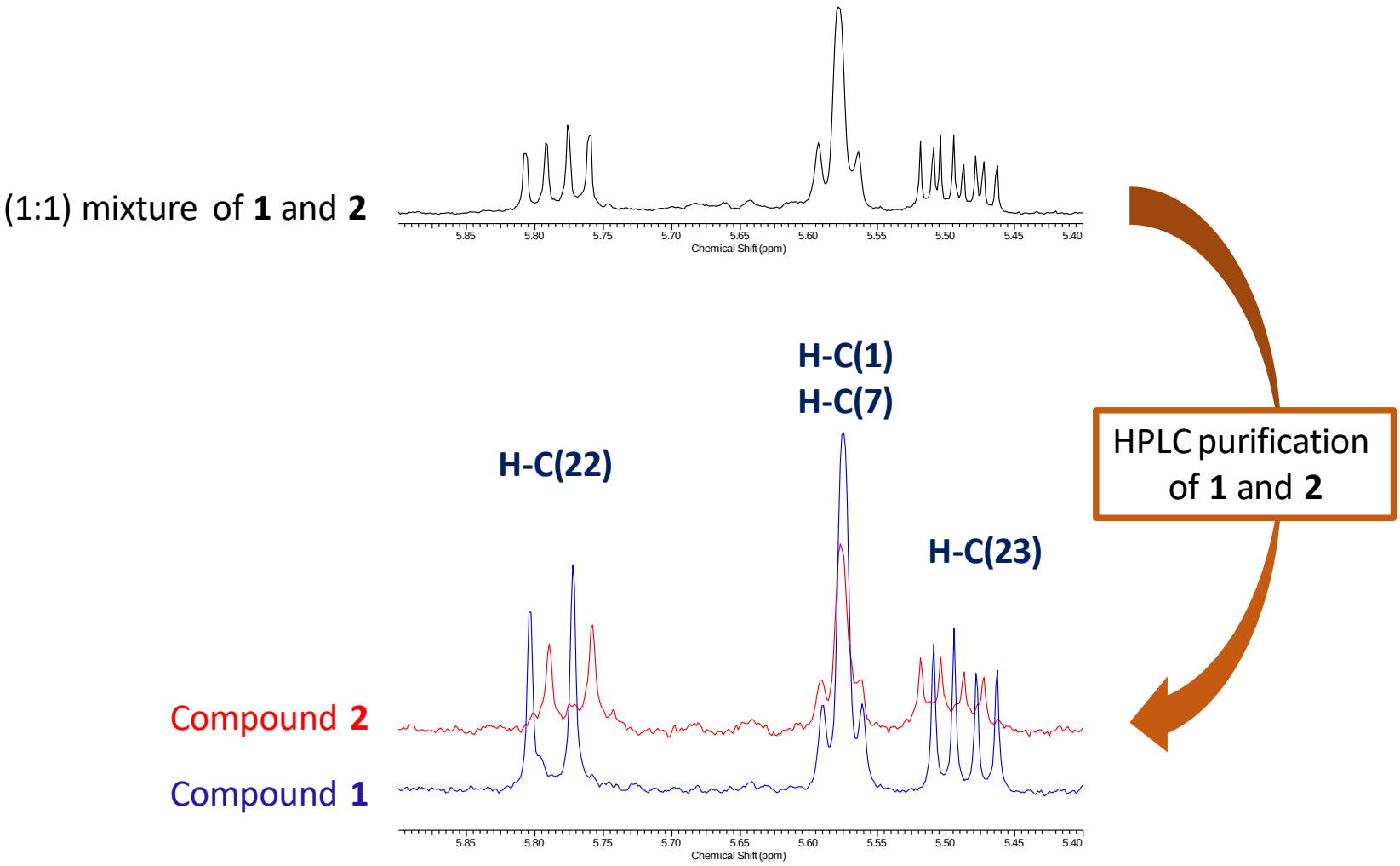


Figure S2. ^{1}H-NMR spectrum of the 1:1 mixture and of the HPLC-purified compounds 1 and 2 (δ_H 5.40-5.90 ppm, 500 MHz, $CDCl_3$).

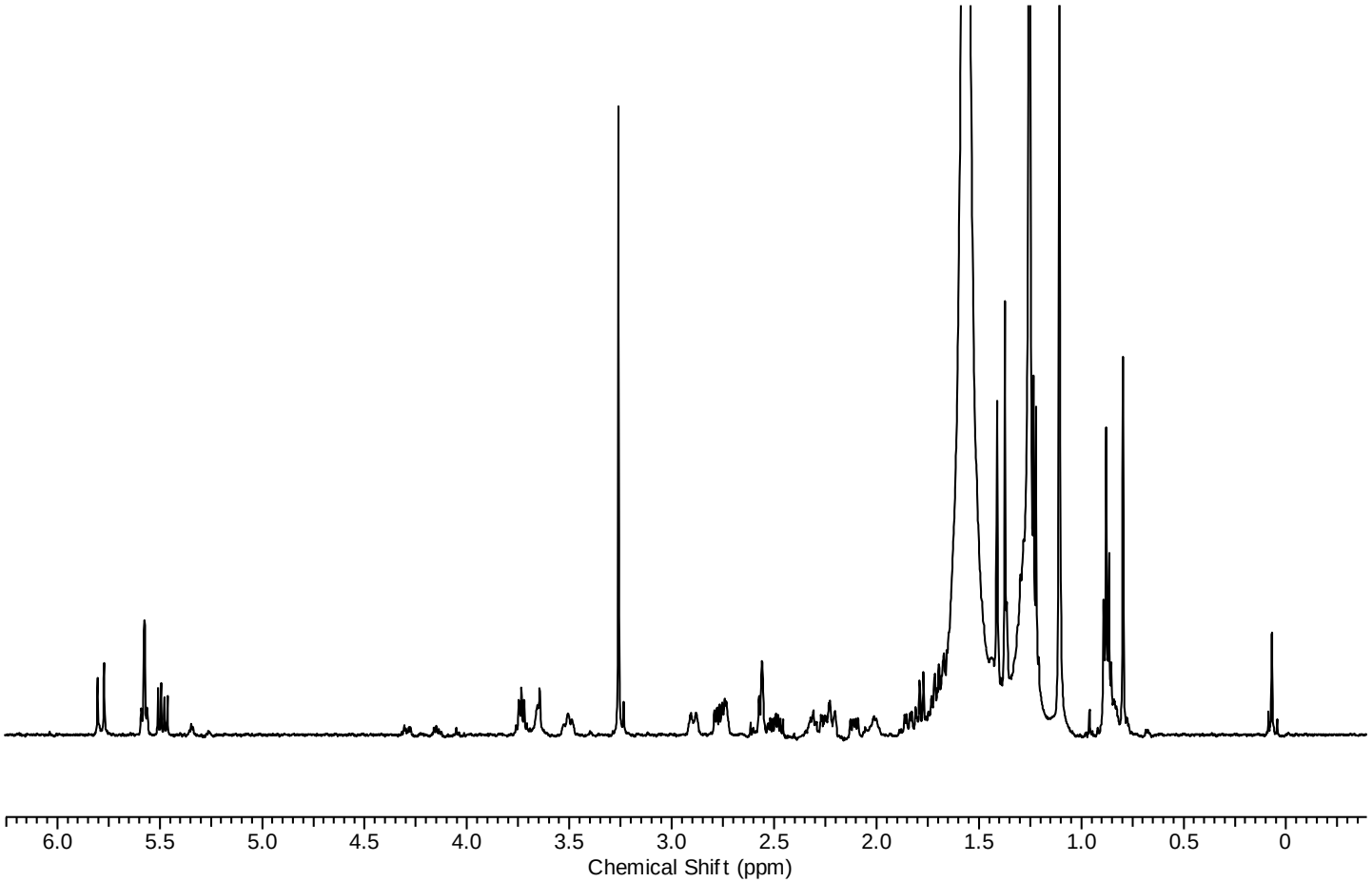


Figure S3. ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) of compound 1.

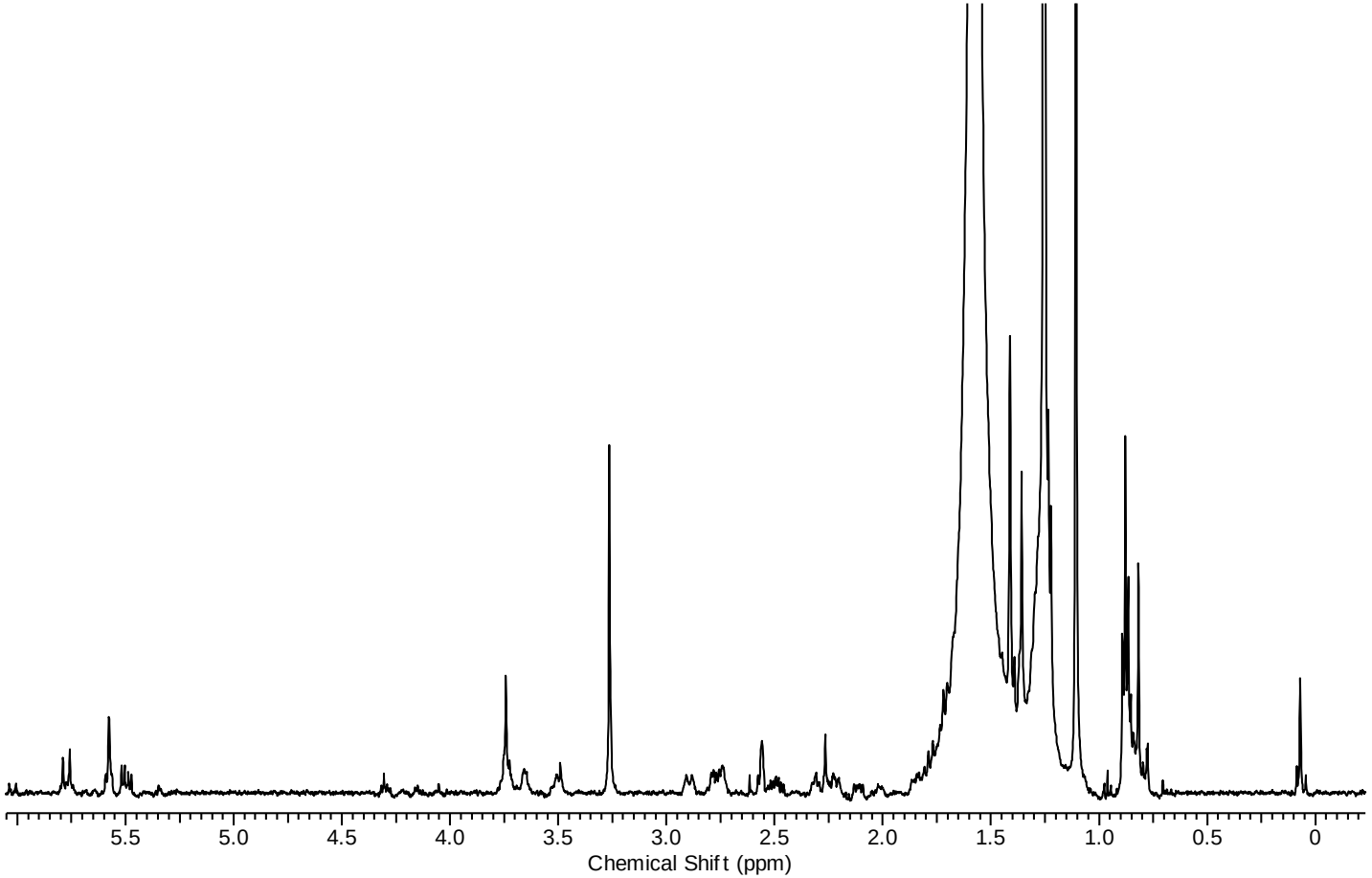


Figure S4. ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) of compound 2.

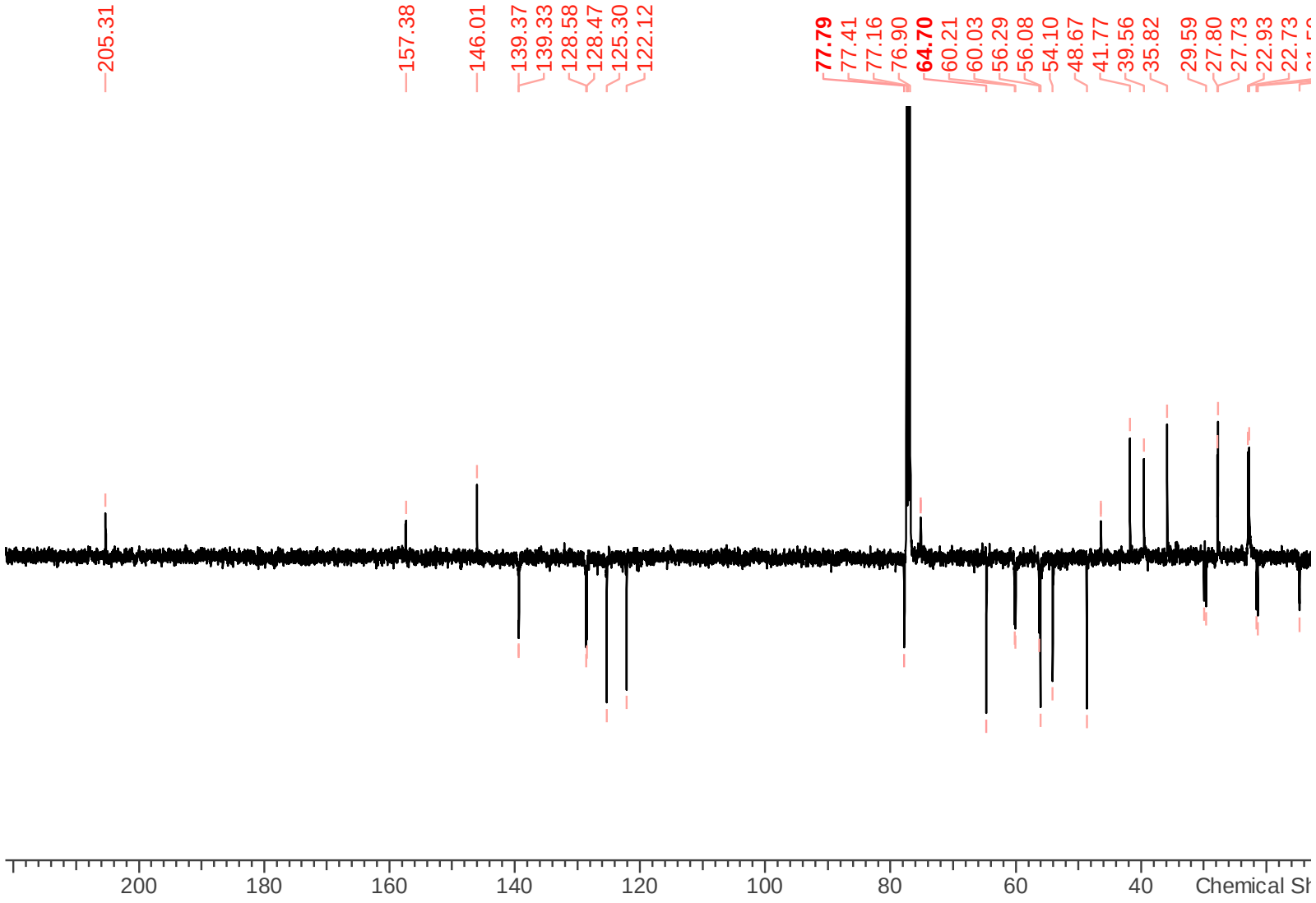


Figure S5. ^{13}C-NMR spectrum (125 MHz, $CDCl_3$) of compounds 1 and 2 (mixture).

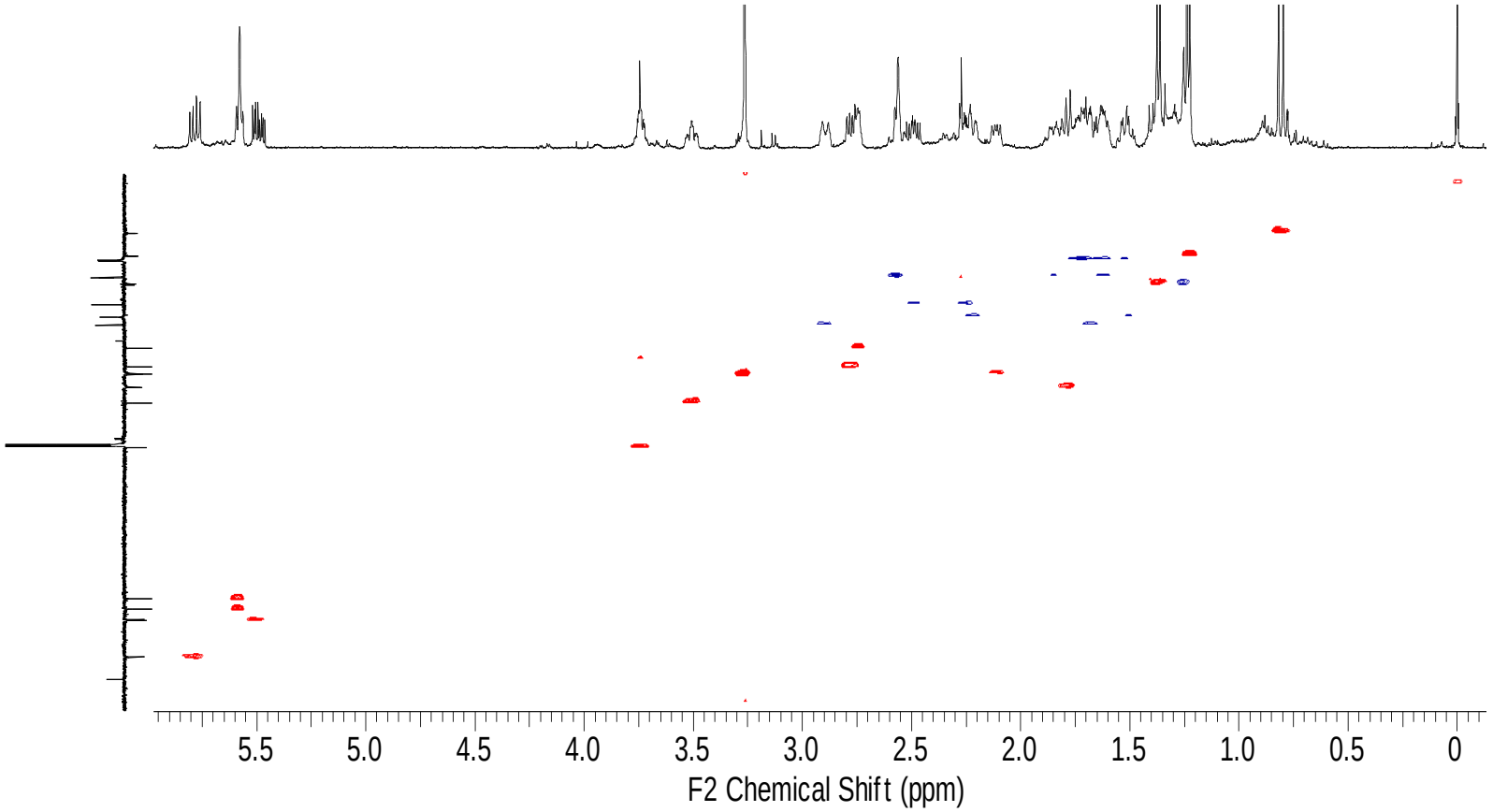


Figure S6. HSQC spectrum (500 MHz, $CDCl_3$) of compounds 1 and 2 (mixture).

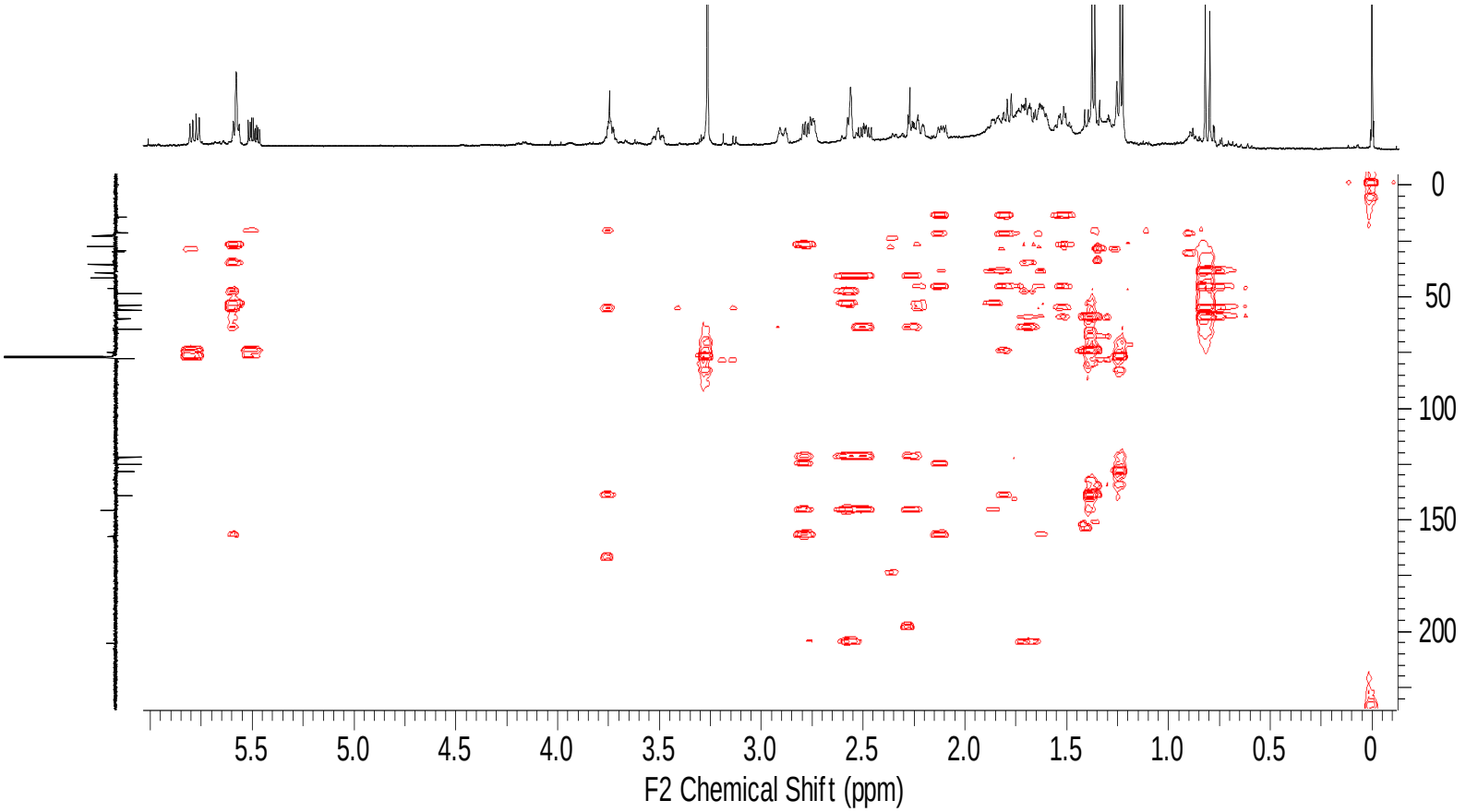


Figure S7. HMBC spectrum (500 MHz, $CDCl_3$) of compounds 1 and 2 (mixture).

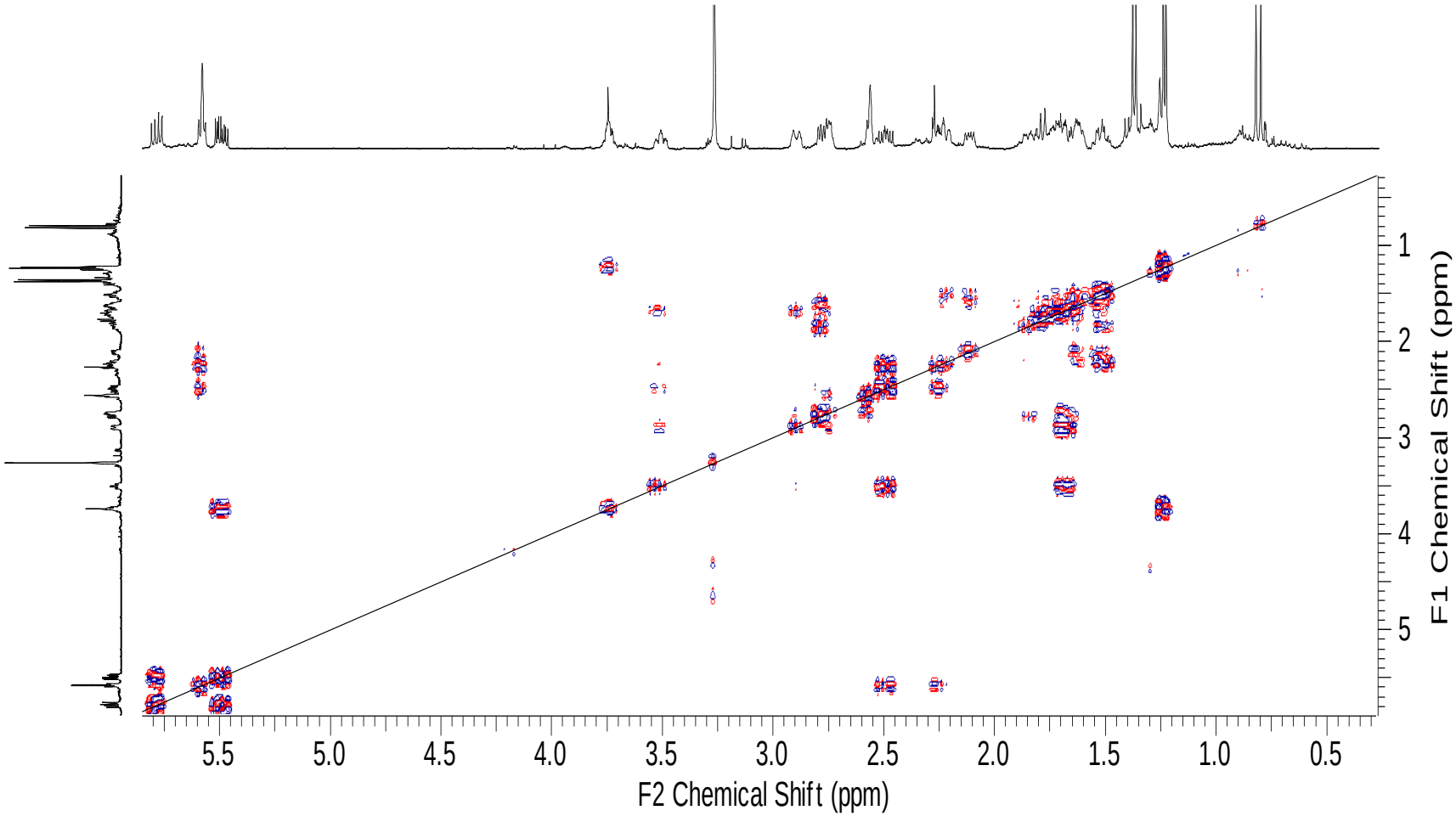


Figure S8. COSY spectrum (500 MHz, $CDCl_3$) of compounds 1 and 2 (mixture).

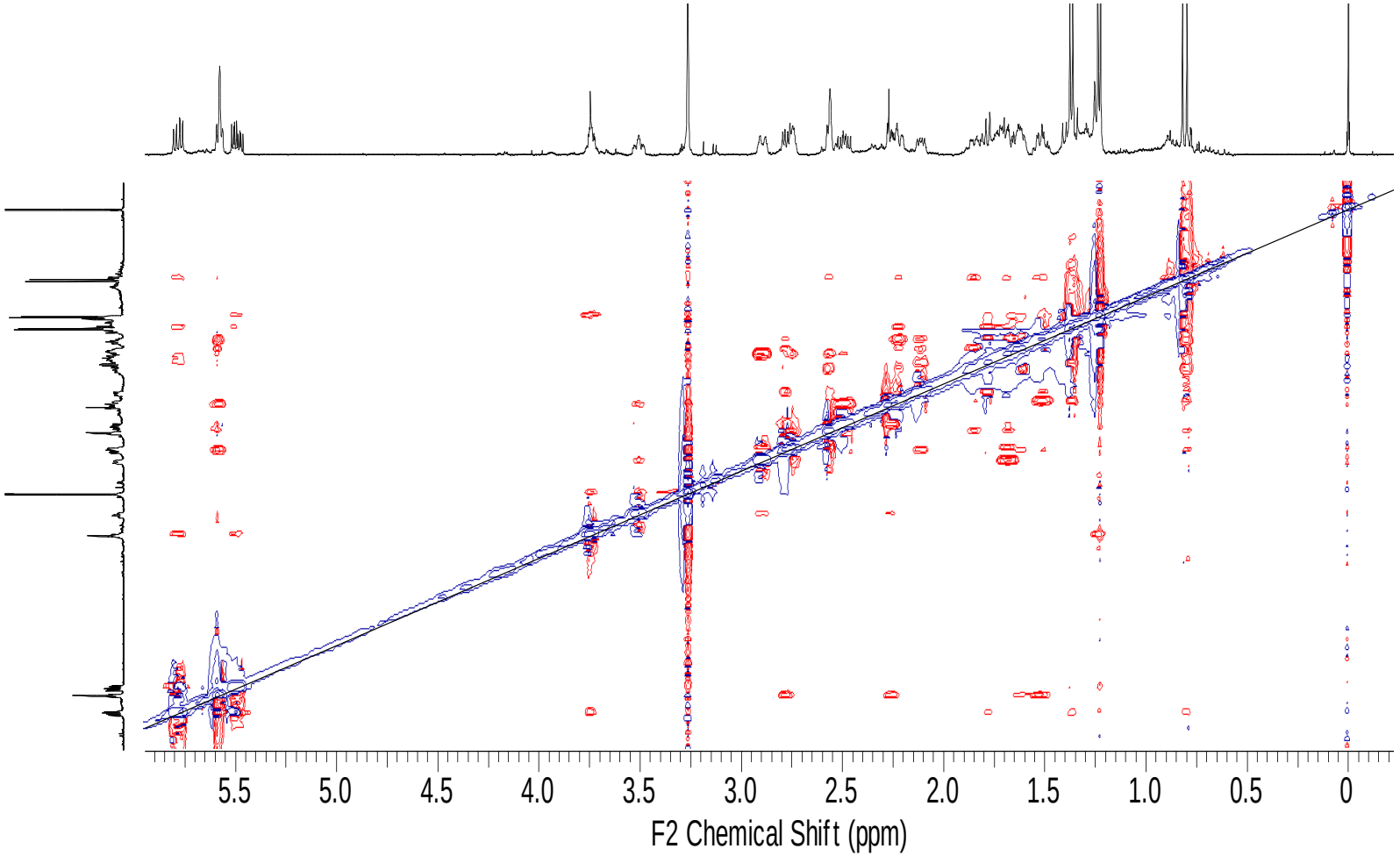


Figure S9. NOESY spectrum (500 MHz, $CDCl_3$) of compounds 1 and 2 (mixture).

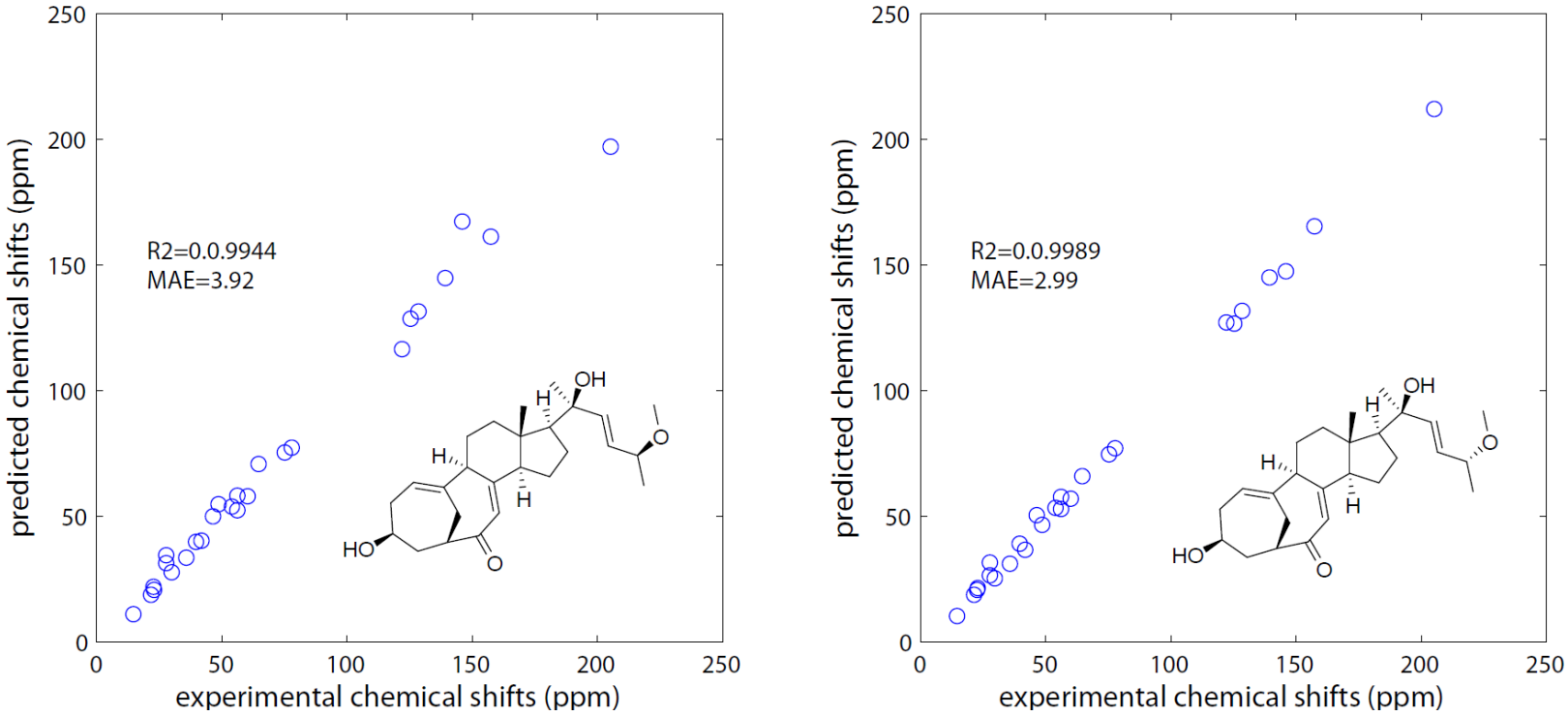


Figure S10. Comparison between experimental and DFT calculated chemical shifts for compounds 1 and 2.

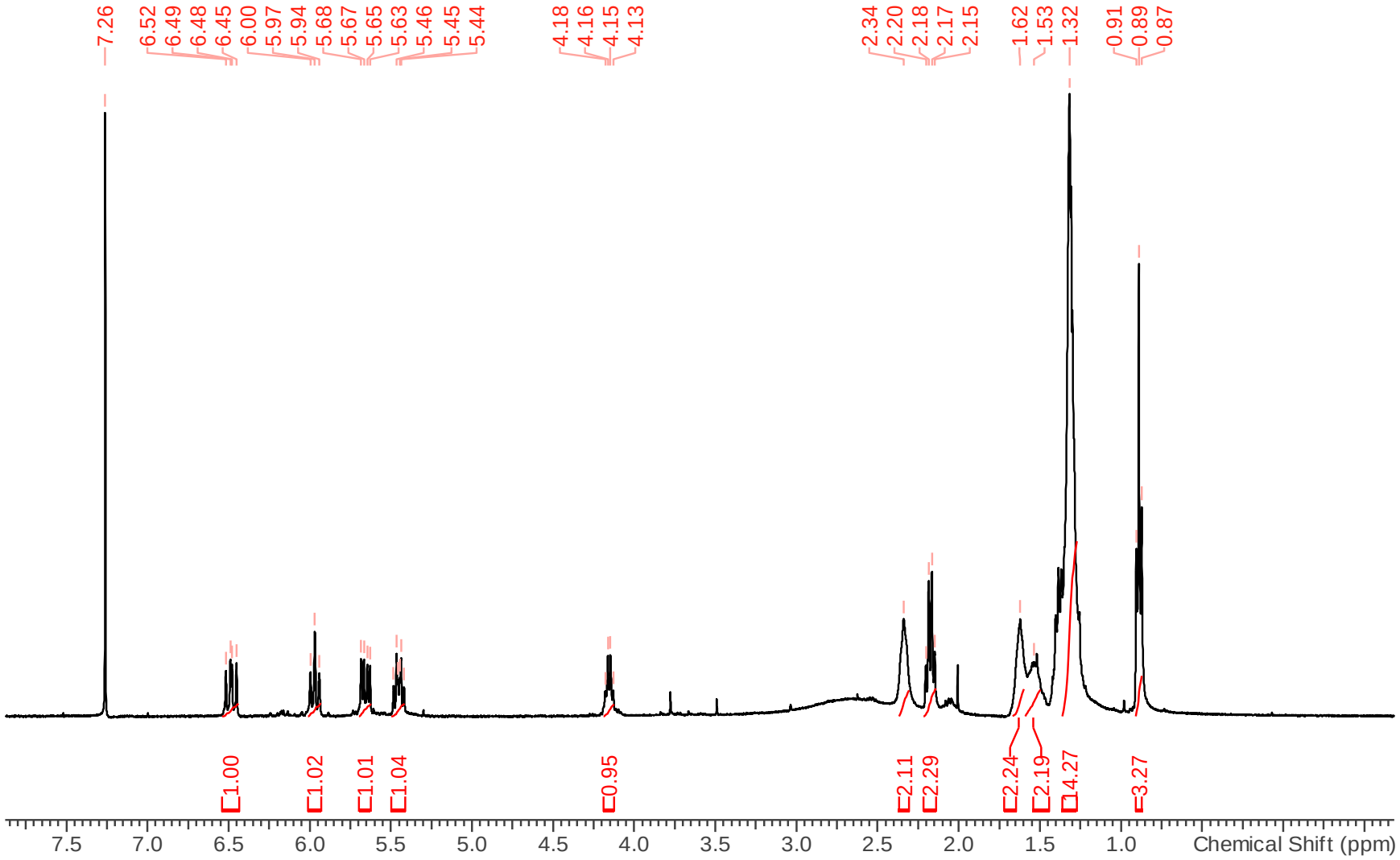


Figure S11. ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) of α-dimorphecolic acid

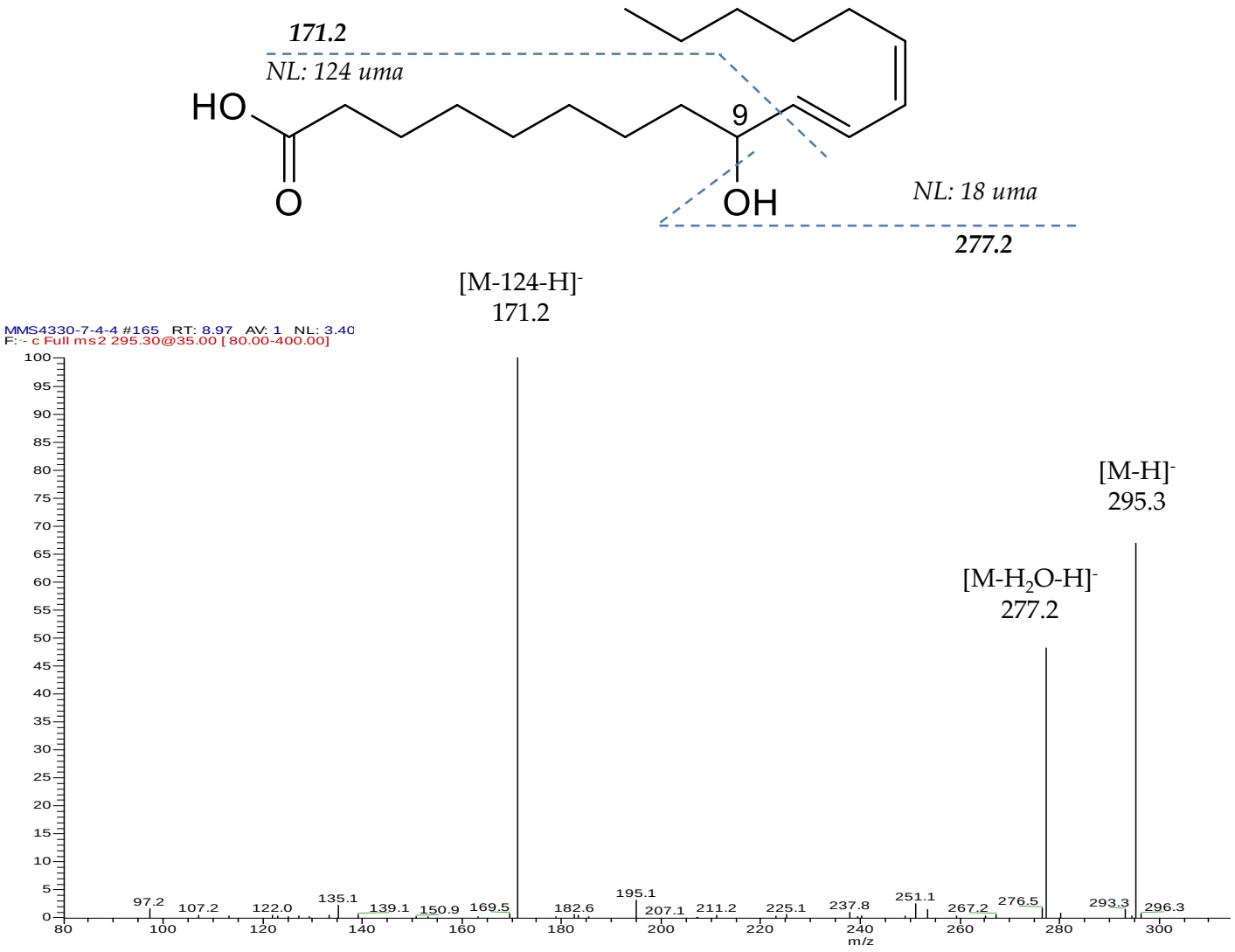
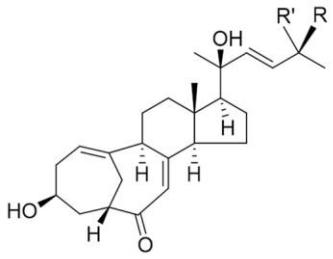
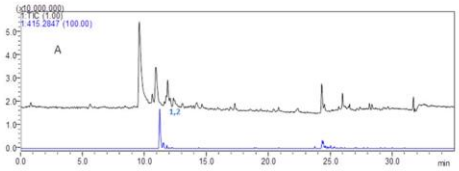


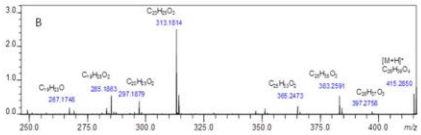
Figure S12. MS/MS spectrum of α-dimorphecolic acid in negative ionization mode




R'
R
OH
H
H
H
HO
H
O

1, 2


TIC (1.00)
A
1,2
min


B
313.1614
285.1563
297.1679
365.2473
383.2591
415.2650
397.2756
m/z