Partial characterization of the pigments produced by the marine-derived fungus Talaromyces albobiverticillius 30548. Towards a new fungal red colorant for the food industry

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A B S T R A C T .

The interest about red color in the food industry has been growing because of its wide application in variety of foods and beverages and also due to the carcinogenic and teratogenic effects of some synthetic colorants. Many ascomycetous fungi naturally synthesize and secrete pigments and thus provide readily available additional and/or alternative sources of natural colorants that are independent of agro-climatic conditions. Some species of *Talaromyces* produce large amounts of *Monascus*-like azaphilone red pigments without any toxins. In this study, *Talaromyces albobiverticillius* 30548 was isolated from the outer slope of the coral reef of the Reunion Island, Indian Ocean. The biosynthesized intracellular and extracellular pigments were extracted by successive cold extractions or by single solvent extraction methods The pigments were then analyzed by HPLC-PDA-ESI/MS system in positive and negative ionization modes. Twelve different compounds were detected and four were tentatively identified as *Monascus*-type pigments, based on the results obtained and the available literature. In particular, N-threonine-monascorubramine, N-glutaryl-rubropunctamine and PP-O were tentatively identified; further, this work reports for the first time on the PDA, MS and NMR characterization of the here named as N-GABA-PP-V (6-[(*Z*)-2-Carboxyvinyl]-N-GABA-PP-V) pigment bearing a *cis* configuration at the C10-C11 double bond, in *Talaromyces albobiverticillius* 30548.

1. Introduction

There is a growing interest for the use of natural colors mainly from the consumers due to the harmful concerns associated with synthetic dyes and pigments. Natural pigments are derived from various sources, mainly from plants and microalgae and have applications in many foods and beverages. However, they have several drawbacks like instability, seasonal availability and high cost when considering the industrial application (Dufossé et al., 2005; Gunasekaran and Poorniammal, 2008; Jiang et al., 2005). Aside from these sources, microorganisms provide an alternative to synthetic pigments as they are able to grow in different culture systems (Campoy et al., 2003; Yan et al., 2005), are independent of climatic conditions and supply of agricultural raw materials (Mapari et al., 2006). Also, some of the pigments produced by microbes possess a high stability towards light, heat and pH. (Joshi et al., 2003; Malik et al., 2012). With these advantages, special attention has been focused on filamentous fungi which are the potential producers of numerous shades of pigments ranging from yellow, red, reddish brown, bronze and maroon (Caro et al., 2012). In fungi, these pigments have been thought to serve different ecological functions, for example, melanins protect them against environmental stress, carotenoids against lethal photo-oxidations, and flavins act as cofactors in enzyme catalysis (Firn and Jones, 2003; Spiteller, 2015).

Fungal colorants can be chemically classified as carotenoids, melanins, polyketides, etc. in which the polyketides constitute the most representative class of pigments. Current industrial fungal productions

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Table 1
Overall compounds detected by HPLC-PDA-ESI/MS in IC and EC extracts[a] of *Talaromyces albobiverticillius* 30548, with reference to the chromatogram shown in Fig. 2.

Compound N.	R.t.	PDA λ nm	MS/ESI	Tentative identification
1	4.89	207, 362	255 $[M + H]^+$	n.i.
2	7.35	223, 390	269 $[M + H]^+$ 267 $[M-H]^-$	n.i.
3	10.04	222,273,422, 511	484 $[M + H]^+$ 482 $[M-H]^-$	N-threonine-monascorubramine
4	10.32	221,273,425, 522	496 $[M + H]^+$; 456 $[M + H-42]^+$ 498 $[M-H]^-$; 454$[M-H-42]^-$	**N-GABA-PP-V (see NMR)**
5	10.92	223, 430, 499	484 $[M + H]^+$ 482 $[M-H]^-$	N-glutaryl-rubropunctamine
6	11.57	225, 409	375 $[M + H]^+$	n.i
7	12.6	222, 280, 461	413 $[M + H]^+$ 411 $[M - H]^-$	n.i.
8	12.8	223,286,458,470	413 $[M + H]^+$ 411 $[M-H]^-$	PP-O
9	13.4	224, 287, 458	459 $[M + H]^+$ 457 $[M-H]^-$	n.i.
10	14.4	225, 421	503 $[M + H]^+$ 501 $[M-H]^-$	n.i.
11	15.2	224, 446	445 $[M + H]^+$ 443 $[M-H]^-$	n.i.
12	15.6	225, 458	459 $[M + H]^+$	n.i.

n.i. = not identified
[a] the analysed samples were extracted with different solvent systems. In Table 1 are reported the compounds detected in all the different samples and, in particular, the EtOAc extract was the most representative as shown in Fig. 3. Only compounds 1, 2 and 6 were detected mainly in the $CHCl_3$ extract.

are running at multi metric tons level with yellow-orange-red food-colorants β-carotene and lycopene, biosynthesized by *Blakeslea trispora* (Finkelstein et al., 1995; López-Nieto et al., 2004; Xu et al., 2007). Polyketide based pigments are structurally complex and involve pathways catalyzed by the enzymes polyketide synthases. The main classes of polyketide pigments include anthraquinones, hydroxyanthraquinones, naphthoquinones, and azaphilone structures, each of which exhibits an array of color hues (Mapari et al., 2010). Since ancient times, azaphilone pigments produced by *Monascus* sp have widely been used in the oriental countries (particularly Japan and China) to color rice wine, koji, soyabean, cheese and meat. However, the use of *Monascus* pigments as food colorants is still forbidden in European countries owing to the time-to-time production of the mycotoxin citrinin (Liu et al., 2005) and also the production of the unwanted cholesterol-lowering drug mevinolin when added to foods (Patakova, 2013). Some species of *Aspergillus* sp *(A. glaucus, A. cristatus,* and *A. repens)* produce hydroxyanthraquinoid (HAQN) pigments like emodin (yellow), physcion (yellow), questin (yellow to orange-brown), erythroglaucin (red), catenarin (red), and rubrocristin (red) along with several mycotoxins such as secalonic acid, oxaline, citrinin, tanzawaic acid A, cyclochlorotine, islanditoxin, luteoskyrin, erythroskyrin, rugulosin or aspergiolide A. Many of these mycotoxins are pigmented and show substitution on both aromatic rings which arise biosynthetically by the polyketide pathway (Caro et al., 2012; Goyal et al., 2016).

In the search to identify potential non-toxic pigment producers for industrial application, several species of fungi have been evaluated and identified belonging to the genus *Paecilomyces, Cordyceps, Penicillium, Aspergillus, Epicoccum, Fusarium* (Cho et al., 2002; Pradeep et al., 2013; Suhr et al., 2002; Unagul et al., 2005). On the other side, several non-pathogenic to humans *Talaromyces* sp producing azaphilone series of yellow and red pigments without the production of mycotoxin seem to be an alternative to *Monascus* red pigments (Frisvad et al., 2013). Azaphilones are interesting set of fungal secondary metabolites namely pigments with pyrone – quinone structures containing a highly oxygenated bicyclic core and a chiral quaternary center (Osmanova et al., 2010). Studies have shown that some *Talaromyces* sp such as *Talaromyces aculeatus, T. pinophilus, T. purpurogenus, T. funiculosus, T. amestolkiae, T. ruber* and *T. stolii* naturally produce polyketide azaphilone *Monascus* red pigments and their amino acid derivatives (Mapari et al., 2008; Mapari et al., 2009). But, the later three species do not diffuse pigments into the culture medium and also *T. purpurogenus* produces mycotoxins such as rubratoxins A and B, rugulovasins and luteoskyrin which limits the biotechnological production of pigments by using this species (Yilmaz et al., 2012). Such compounds, for example, rubratoxin was produced in a high concentration in a rhubarb-wine contaminated with *T. purpurogenus* and induced an immediate liver transplant when consumed by a teenager (Richer et al., 1997; Sigler et al., 1996).

Some other species, specifically *T. atroroseus, T. albobiverticillius, T. minioluteus,* and *T. marneffei* produce diffusing strong red pigments and some yellow pigments. One potential pigment producer among them, namely *T. albobiverticillius* collected from different sources produces several purple-red-orange azaphilone pigments such as monascorubramine (red), monascorubrin (orange), rubropunctatin (orange), PP-R (purple-red) (Mapari et al., 2005; Ogihara et al., 2001; Ogihara et al., 2000) and a series of yellow-orange pigments such as monascin (yellow), mitorubrin (orange-yellow), mitorubrinic acid (yellow) or mitorubrinol (yellow) (Frisvad et al., 2013). (see structures in Table 3)

The current study describes the pigment production from the marine derived fungus *Talaromyces albobiverticillius* strain 30548 isolated from the outer slope of the Réunion island coral reef (Indian Ocean) and the characterization of those pigments by high-performance liquid chromatography-diode array detection-electrospray ionization mass spectrometry (HPLC-PDA-ESI/MS), followed by the isolation of major compound(s) and the structure elucidation of a novel red azaphilone using NMR analysis.

2. Material and methods

2.1. Isolation of fungal strain and identification

The fungus used in this study was sampled from the outer slope of the Réunion island coral-reef (Indian Ocean). After sampling, 5 g of sediment was crushed and cultured on Potato Dextrose Agar (PDA) using serial dilution method. During the period of incubation, several isolates producing colored metabolites were observed visually. The red pigment producing strain was isolated, purified by monospore culture technique and stored at −80 °C for long term preservation. To study its pigment production ability, the fungus was grown on PDA (Samson et al., 2010) (Fig. 1).

The fungal strain was genetically identified as *Talaromyces albobiverticillius* using gene sequencing at molecular level (30548 indicates the Université de La Réunion collection reference number of the newly isolated strain) (Domsch, 1980; Foster et al., 2011).

2.2. Submerged fermentation of fungal strain

For submerged fermentation, Potato Dextrose Broth (PDB) was used as a culture medium and prepared using sterile distilled water. The pH of the culture medium was adjusted to 5.5 ± 0.2 using 0.1 M HCl prior to sterilization at 121 °C for 15 min. Pre culture was prepared by taking a loop of fungus from 7-day old culture grown on PDA Petri plates and transferred into 60 mL sterilized culture medium. The flasks were incubated at 24 °C for 72 h. Cultivations were then carried out in 250 mL Erlenmeyer flasks containing volume of 100 mL sterilized culture

Table 2
^{1}H and ^{13}C NMR spectroscopic data in CD_3OD for compound n. 4 reported in Fig. 4.

Position	δ_C (ppm)[a]	type	δ_H (ppm)[b]	H Mult.[cm] (J (Hz))	COSY[d]	ROESY[e]	HMBC[f]
C2	173.8	C					
C3	102.7	C					
C3a	174.2	C					H-Me9a
C4	99.0	CH	6.66	s		H5	H5
C4a	153.2	C					H4, H8
C5	121.4	CH	6.94	s		H4	H4, H10
C6	151.2	C					H1′, H5, H8
C8	143.5	CH	8.32	s		H1′	H1′
C8a	120.0	C					H4, H5
C9	196.2	C					HMeC9a, H8
C9a	87.1	C					HMeC9a, H4
C-Me9a	30.5	CH_3	1.66	s			
C10	126.7	CH	6.71	d (11.7)	H11	H1′, H11	H5
C11	137.3	CH	6.47	br d (11.7)	H10	H10	
C12	171.1	C					H11
C13	198.7	C					H14
C14	41.3	CH_2	2.80	br t (6.6)	H15	H15	
C15	26.4	CH_2	1.58	m (6.1)	H16, H14	H14	H14
C16	30.3	CH_2	1.32	om	H14		
C17	30.6	CH_2	1.28	om			H15, H14
C18	33.0	CH_2	1.28	om			H20, H16
C19	23.8	CH_2	1.30	om	H20		H20
C20	14.5	CH_3	0.89	br t (7.1)	H19		
C1′	56.0	CH_2	4.14	t (6.5)	H2′	H2′, H3′, H10, H8	H3′, H8
C2′	26.5	CH_2	2.08	p (6.8)	H3′, H1′	H1′	H3′
C3′	31.4	CH_2	2.40	br t (6.6)	H3′, H1′	H1′	
C4′	176.4	C					H3′

a) Chemical shift of the given C. b) Chemical shift of the attached Hs for the given C position c) proton signal multiplicity with standard labeling: br = broad, o = overlapped, m = undefined multiplet, d, t, q, p = doublet, triplet, quadruplet and quintet respectively. d) H labels of the detected H-H COSY connection toward the given H resonance; e) H labels of the detected H-H ROESY connection toward the given H resonance; f) H labels for the detected C-H long-range connections respect to the given C resonance.

medium. The flasks were inoculated with 1% (w/v) 72-h-old pre culture and incubated at 24 °C for 8 days with the agitation of 150 rpm using rotary agitator (Infors Multitron HT).

2.3. Separation and extraction of fungal pigments

After 8 days of fermentation, the culture broth and fungal biomass were separated by centrifugation at 8000 rpm for 6 min (Centrifuge Sigma 3 K 3OH and 19776-H rotor). Both samples were immediately frozen (–80 °C) and then lyophilized into a fine powder (Cryotec cosmos, France). The pigments from both the biomass and the culture filtrate were extracted at room temperature successively with solvents of increasing polarity: *n*- hexane, chloroform, ethyl acetate and ethanol. The crude extracts were dried using rotary evaporator (Büchi, Germany) at 30 °C under reduced pressure yielding red colored dried residues. The residues were then dissolved in 1 mL of methanol (1:1 v/v), filtered through Minisart® syringe filter of 0.20 µm pore size housing with PTFE membrane (Sartorius). The crude filtrates were stored at 4 °C in an amber vial prior to HPLC analysis.

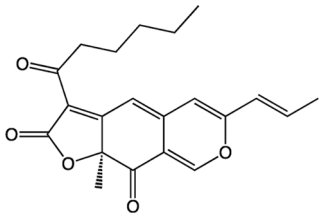
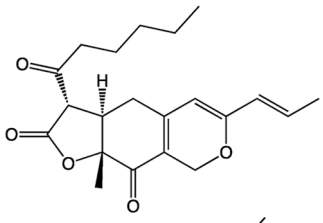
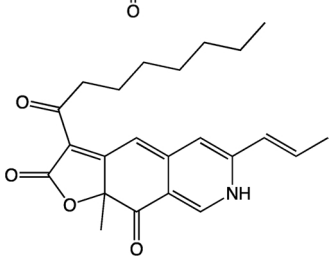
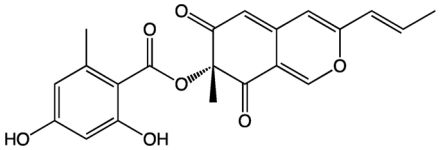
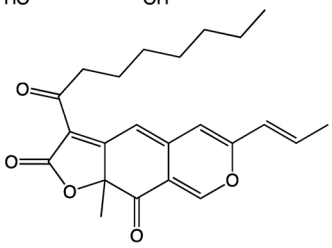
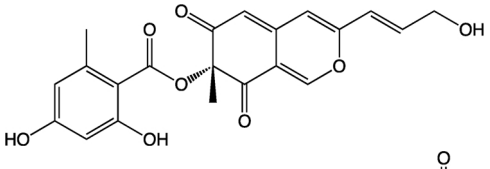
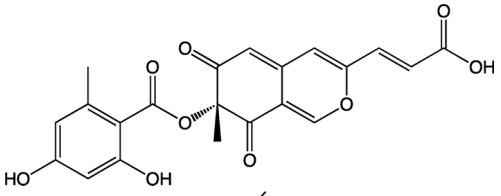
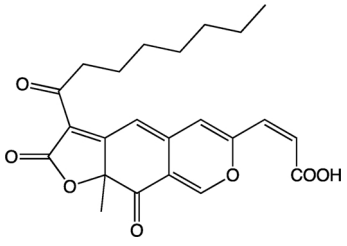
2.4. HPLC-DAD-ESI–MS analysis

The analyses were carried out on a Shimadzu Prominence LC-20A system (Shimadzu, Milan, Italy) equipped with a CBM-20A controller, two LC-20AD pumps, a DGU-$20A_3$ degaser, a SIL-20AC autosampler and a SPD-M20A photo diode array detector. The data were processed with the software Shimadzu Labsolution ver. 5.53. For MS analyses a mass spectrometer was used (LCMS-2020, Shimadzu), equipped with an ESI interface, both in positive and negative ionization modes. HPLC separations were performed on a C18 Kinetex (Phenomenex) column (100 × 2.1 mm-1.7 µm particle size); the mobile phases consisted of water (0.1% formic acid; eluent A) and acetonitrile, (0.1% formic acid; eluent B), using a gradient program as follows: 0 min, 5% B; 15 min, 95% B; 17 min, 95% B; 18 min, 5% B. The flow rate was 0.2 mL/min and the injection volume was 1 µL. The column oven temperature was 30 °C. The UV–vis spectra were acquired in the range of 200–600 nm, while the chromatograms were extracted at 470 nm and 360 nm (sampling frequency: 1,5625 Hz; time constant: 0.64 s). The MS was set as follows: Scan, both ESI positive (+) and negative (−); nebulizing gas flow (N_2): 1.5 L/min; Event Time: 0.3 s; Detector Voltage: 4.5 kV; *m/z* range: 60–600; Interface Voltage: ± 3.5 kV; Interface Temperature: 350 °C; DL Temperature: 250 °C; Heat Block: 400 °C.

2.5. Nuclear magnetic resonance (NMR) spectroscopy

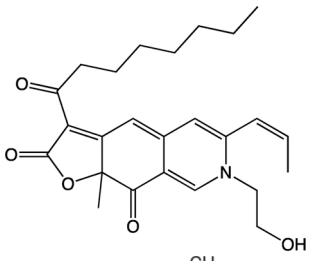
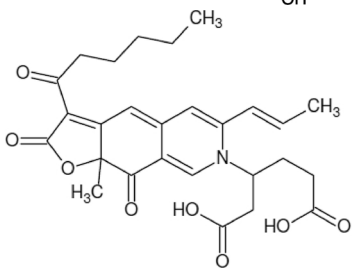
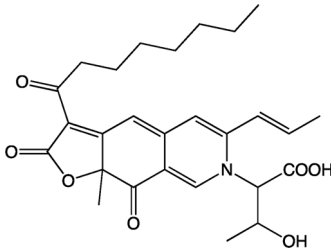
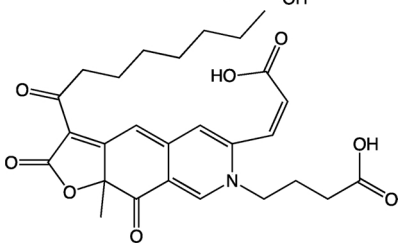
Compound n. 4 in Fig. 2, was collected after LC separation in an almost pure form. The eluent was evaporated and the red residue (around 0.6 mg) was dissolved in 500 µL of CD_3OD and analysed by NMR; afterward the solution was freeze dried and dissolved again in 500 µL of CD_3COCD_3. This strategy was applied in order to rule out possible solvent effects and/or artefacts. ^{1}H and $^{13}C\{^{1}H\}$ NMR spectra of compound n. 4 were recorded on an Agilent Propulse 500 MHz spectrometer equipped with a OneNMR probe and operating at 499.74 and 125.73 MHz respectively. The sample in a 5 mm test-tube was analysed after locking on the deuterated lock signal, search for a good field homogeneity (shimming) and frequency modulation (tuning). The saturation 90° pulse was calculated to be 8us at 59 dB of power level and the protonic spectrum was obtained with 2 s of acquisition time, 2 s of scan delay and 16 scans; all the other techniques were designed starting from this simple experiment. The complete and unambiguous assignment (Table 2 with the numbering scheme in Fig. 4), was confirmed by homo nuclear 2D-COSY, TOCSY and ROESY (Derome, 2013), and heteronuclear (Willker et al., 1993) $^{13}C\{^{1}H\}$-HSQC and ^{13}C–HMBC experiments. Calibration was attained using as internal standard residual proton signal of the solvent (CD_2HOD quintet: δ = 3.31 ppm; CD_3COCD_2H quintet δ = 2.05 ppm and the ^{13}C solvent septuplets at δ = 49.0 ppm and δ = 29.84 respectively) (Gottlieb et al., 1997) and data were processed by vNMRj software and by the PC software package ACD/Lab, which was also exploited to validate the goodness of the structure elucidation.

Table 3
Reported structures of pigmented extrolites produced by different collection strains of *Talaromyces albobiverticillius*, mentioned in (Frisvad et al., 2013) and in this study.

Compound	Chemical structure	Color	Formula	Monoisotopic mass	Average Mass	References
Rubropunctatin		Orange	$C_{21}H_{22}O_5$	354.1467	354.39	Frisvad et al. (2013)
Monascin		Yellow	$C_{21}H_{26}O_5$	358.1780	358.43	Frisvad et al. (2013)
Monascorubramine		Red	$C_{23}H_{27}NO_4$	381.1940	381.46	Frisvad et al. (2013)
Mitorubrin		Orange – yellow	$C_{21}H_{18}O_7$	382.1053	382.36	Frisvad et al. (2013)
Monascorubrin		Orange	$C_{23}H_{26}O_5$	382.1780	382.45	Frisvad et al. (2013)
Mitorubrinol		Yellow	$C_{21}H_{18}O_8$	398.1002	398.36	Frisvad et al. (2013)
Mitorubrinic acid		Yellow	$C_{21}H_{16}O_9$	412.0794	412.35	Frisvad et al. (2013)
PP-O		Red orange	$C_{23}H_{24}O_7$	412.1522	412.43	This study

(*continued on next page*)

Table 3 (*continued*)

Compound	Chemical structure	Color	Formula	Monoisotopic mass	Average Mass	References
PP-R [(10*Z*)-7-(2-hydroxyethyl)-monascorubramine		Purple red	$C_{25}H_{31}NO_5$	425.2202	425.52	Frisvad et al. (2013)
N-glutaryl rubropunctamine		Red	$C_{26}H_{29}NO_8$	483.1893	483.51	This study
N-threonine monascorubramine		Purple red	$C_{27}H_{33}NO_7$	483.2265	483.55	This study
6-[(Z)-2-Carboxyvinyl]-N-GABA-PP-V		Red	$C_{27}H_{31}NO_8$	497.2050	497.53694	This study

3. Results

3.1. Behavior of fungal pigments during extraction(s)

An ideal solvent for fungal pigment extraction must have low toxicity, and must be able to solubilize a range of target pigment molecules (Robinson et al., 2014). Initial extraction trials were conducted with the commonly used solvents from low to high polarity such as *n*-hexane, chloroform, ethyl acetate and ethanol successively which yielded differences in amounts of extracted pigments for both biomass and culture filtrate. Simultaneously, extraction was carried out using ethyl acetate and ethanol as single solvent extraction. On the basis of liquid chromatography-diode array detector (LC-DAD) chromatogram, among the used solvents, ethyl acetate as single solvent was found to be the best solvent for extraction of major pigmented compounds followed by ethanol. Indeed, *Monascus*-like polyketide pigments are hydrophilic in nature, slightly polar and so they are easily handled with polar solvents (Padmavathi and Prabhudessai, 2013). In non-polar solvents like *n*- hexane and chloroform, the extraction and recovery of pigments was very low and chloroform yielded two compounds (peaks 1 and 2) which were unpigmented. The yield from these solvents was very poor compared to ethyl acetate which yielded 12 different compounds and among them 10 compounds were pigmented (Fig. 2).

3.2. Characterization of fungal pigments using HPLC-DAD-ESI–MS

Fig. 2 shows a typical representative chromatogram of the detected pigments (compounds n.s 3–12) in *Talaromyces albobiverticillius* 30548 obtained from the EtOAc pigment extract and detected at the wavelength of 470 nm; in the same Fig. 2, it is also shown an insert representing the better detection for compound n.1 and n.2 obtained from the $CHCl_3$ extract and recorded at the wavelength of 360 nm. Table 1 presents all of the detected compounds, their corresponding retention times, PDA and MS data, with a relative tentative identification based on the obtained spectroscopic data and the comparison with literature data. Together with PDA, an on line MS detector operating in both ESI positive and ESI negative ionization mode was used in order to have a double confirmation of the mass values.

Twelve different compounds were detected and four were tentatively identified as *Monascus*-type pigments (Table 1). The identified compounds 3, 5 and 8 are similar to the already known N-threonine-monascorubramine, N-glutaryl-rubropunctamine and PP-O respectively. Further, one compound was also characterized by NMR analysis and a new structure for this molecule, here named as 6-[(*Z*)-2-Carboxyvinyl]-N-GABA-PP-V, (or as N-GABA-PP-V), is provided for the first time based on PDA, MS and NMR data (see Sections 2.5 and 3.3 of this paper).

Compound 3: Under the assumption that the α-amino acid

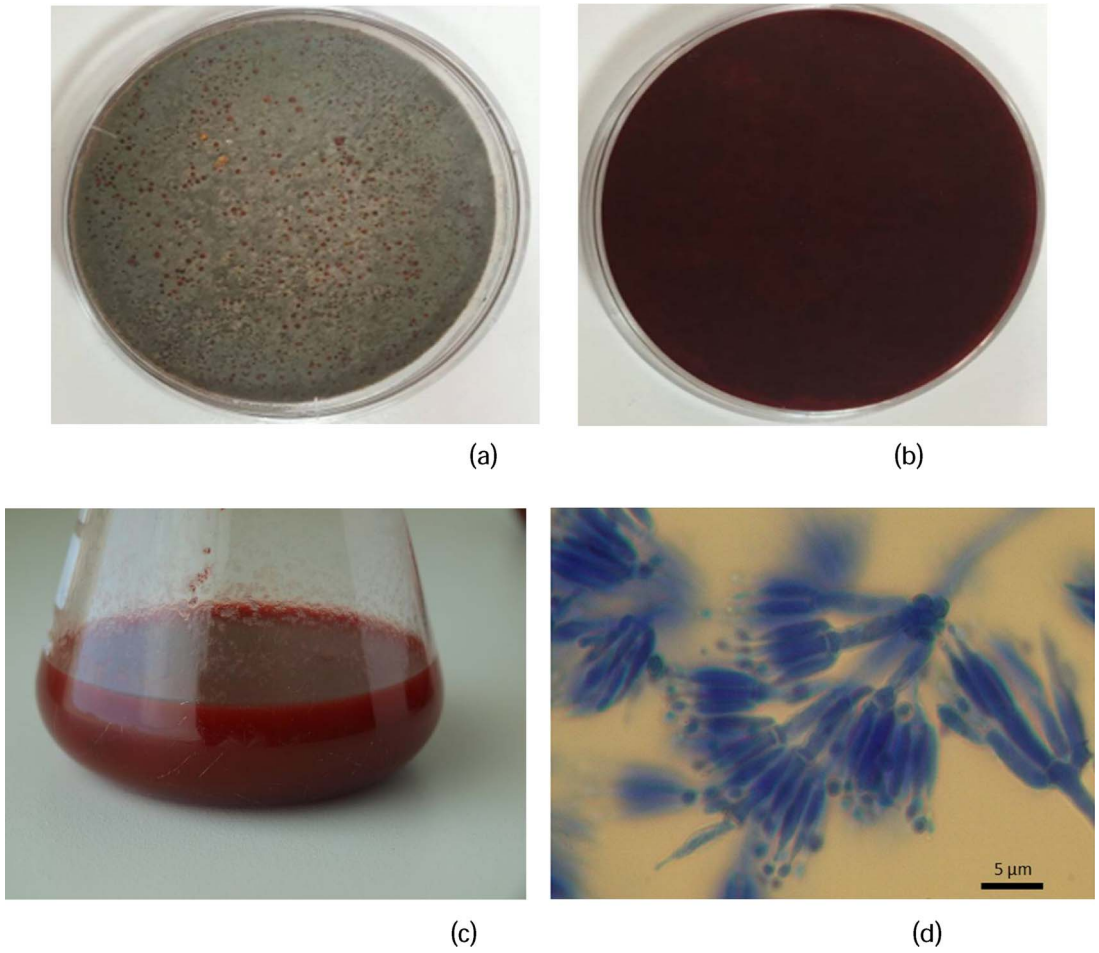


Fig. 1. Morphological features of *Talaromyces albobiverticillius* 30548: (a) Obverse face of fungus grown on Potato Dextrose Agar (PDA) media, (b) Reverse face, (c) Red pigment production in Potato Dextrose Broth (PDB) medium incubated for 7 days at 24 °C, (d) Conidiophores produced on PDA, stained with lactophenol blue (scale bar 5 µm) (for color view, please refer to the online article).

threonine was incorporated into pigment, compound n. 3 was tentatively identified as N-threonine-monascorubramine; the corresponding $[M + H]^+$ *m/z* 484, and $[M - H]^-$ *m/z* 482, pseudomolecular ions mass values were consistent with the values reported by Jung et al., 2003 (Jung et al., 2003) and the corresponding PDA data were also consistent with the reported values for *Monascus* type pigments (Mapari et al., 2008).

Compound 4: Interestingly compound n.4 was here identified as a never previously reported compound. Under the assumption that the γ-amino acid, γ-aminobutyric acid was incorporated into pigment, the name of 6-[(Z)-2-Carboxyvinyl]-N-GABA-PP-V or simply as N −GABA-PP-V was proposed for compound n.4; the structure was determined on the basis of the obtained PDA and MS data and of a detailed NMR investigation (see Sections 2.5 and 3.3 of this paper). The PDA data are in agreement with the reported values for *Monascus* type pigments (Mapari et al., 2008) and the compound showed corresponding $[M + H]^+$ *m/z* 498, and $[M - H]^-$ *m/z* 496, pseudomolecular ions (see Fig. 3) which are consistent with the proposed structure (Fig. 4) for a compound having a molecular formula of $C_{27}H_{31}NO_8$, with a mass value of 497 amu.

The PubChem database (PubChem CID: 44715338) reports the existence of a compound named as *4-{6-[(E)-2-Carboxyvinyl]-9a-methyl-3-octanoyl-2,9-dioxo-9,9a-dihydrofuro[3,2-g]isoquinolin-7(2H)-yl}butanoic* acid with a *trans* configuration at the C10-C11 double bond, but no information was available/reported on the source or in any literatures for this compound. Therefore, this work reports for the first time on the

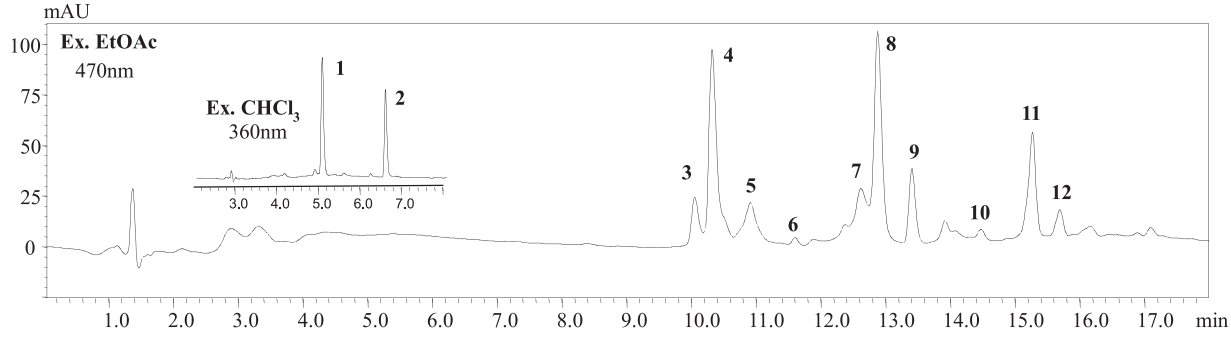


Fig. 2. Chromatogram showing the overall compounds detected by HPLC-PDA- ESI/MS in intracellular (IC) and extracellular (EC) extracts of *Talaromyces albobiverticillius*.

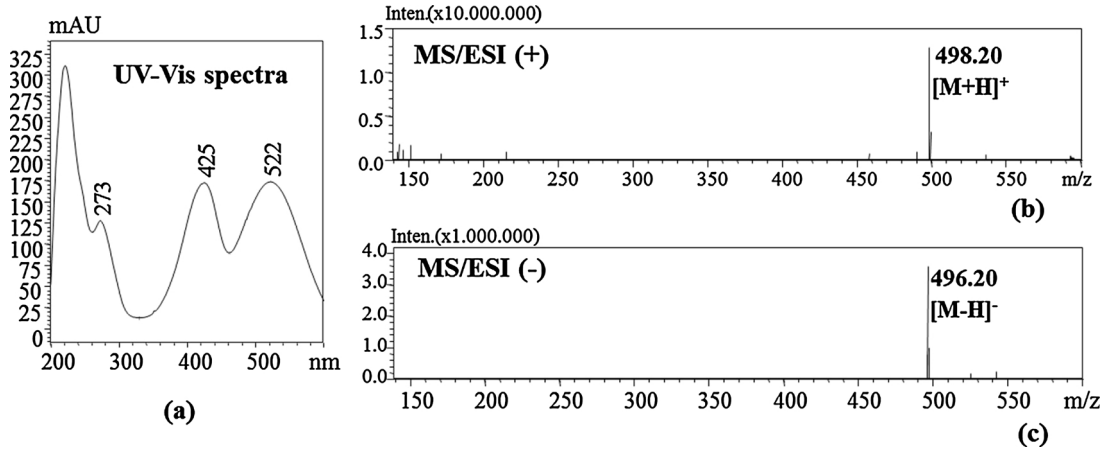


Fig. 3. Structural analysis of red pigments: (a) UV- Vis absorption spectrum of ethyl acetate extract, (b) positive ESI–MS *m/z* spectrum of compound n. 4, (C) negative ESI–MS spectrum.

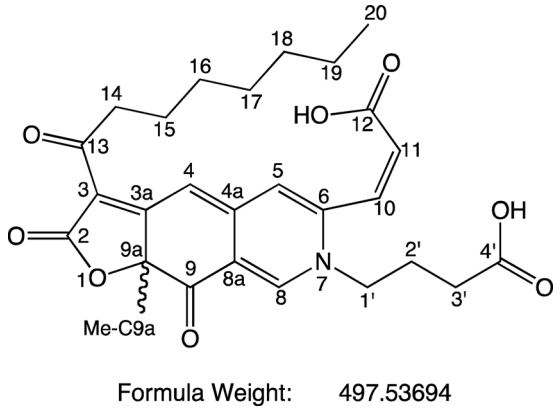


Fig. 4. Molecular structure and carbon atom numbering of compound n. 4. Proposed Name: N-GABA-PP-V, 4-{6-[(E)-2-Carboxyvinyl]-9a-methyl-3-octanoyl-2,9-dioxo-9,9a-dihydrofuro[3,2-g]isoquinolin-7(2H)-yl}butanoic acid.

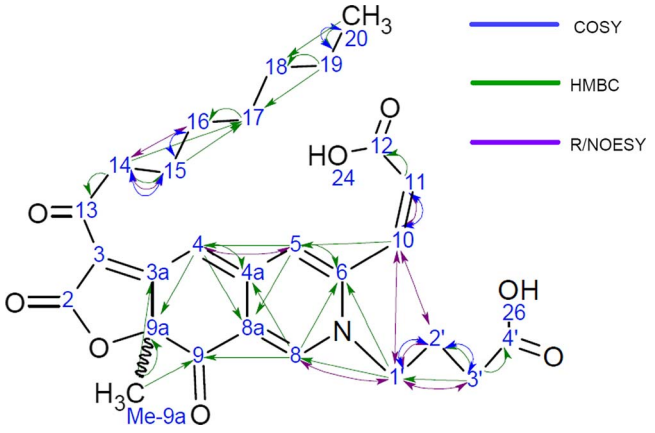


Fig. 5. Selected COSY (doubled headed blue arrows), ROESY (doubled headed red arrows) and HMBC (green arrows) for compound n. 4 reported in Fig. 4 (for color view, please refer to the online article). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

characterization of the pigment N-GABA-PP-V derivative bearing a *cis* configuration at the C10-C11 double bond, in the investigated *Talaromyces albobiverticillius* species.

Compound 5: Compound n.5 was tentatively identified as N-glutaryl-rubropunctamine; it showed the $[M + H]^+$ *m/z* 484 and $[M - H]^-$ *m/z* 482 pseudomolecular ions, and UV–vis absorbance values in agreement with the literature reported values (Mapari et al., 2009).

Compound 8: Compound n.8 was tentatively identified as PP-O; it showed the corresponding $[M + H]^+$ *m/z* 413 and $[M - H]^-$ *m/z* 411 pseudomolecular ions, and UV–vis absorbance values in agreement with the literature reported values (Mapari et al., 2008; Ogihara and Oishi, 2002).

3.3. Characterization of fungal pigments using NMR

As explained in the experimental part, HOMO and HETERO nuclear 2D techniques allows chemical shift (δ) assignments of the ^1H and ^{13}C resonances leading to the structure elucidation (Rotondo et al., 2014; Rotondo et al., 2015). Specifically, HSQC-DEPT experiment defines the direct connection of ^1H resonances to their ^{13}C parent atom resonances. HMBC spectrum is showing ^2J, ^3J and few ^4J ^1H–^{13}C scalar couplings (Table 2), basically consistent with the reported molecular structure and allowed a reasonable assignment of all of the quaternary ^{13}C resonances (Table 2; Fig. 5).

Homo nuclear 2D-TOCSY, which is grouping resonances belonging to the same spin-system (chemical group closely connected through the bonds), clearly distinguished the nature of the azaphilone body and the following side chains: a) the propionyl moiety on the N7 endocyclic atom; b) the acyl seven-membered chain bound to the C3; c) two connected unsaturated CH whose HMBC connection (C10-H5) fixes on the C6endocyclic carbon atom. Terminal CH_2-3 and CH-11 over the N7 and C6 side chains respectively, were connected to carboxylic acid groups because the HMBC spectrum showed correlations between their proton resonances and those of quaternary carbon atoms at 176.4 ppm (C4') and 171.4 ppm (C12) respectively. Chemical nature of these termini was also confirmed by the mass molecular peak of a compound having a molecular formula of $C_{27}H_{13}NO_8$ (see Sections 2.4 and 3.2 of this paper). As the aromatic molecular system is very sensitive to the charge density, some calculated ^{13}C chemical shifts (ACD/Lab calculator) deviate significantly from the experimental value; specifically this is the case of C10, C11 and C4. On the other hand, we are confident about the correct assignment in Table 2 because the well detected HMBC spots reasonably supported by the “through the space” (ROESY) connections (Fig. 5).

These same ROESY connections, joined to the $^3J_{H10\text{-}H11}$ coupling constant (around 11.7 Hz), allowed to definitely assess the Z configuration at the unsaturated C10-C11 bond. After all some other similar molecules, called monascorubramines, were thoroughly analysed (Ogihara and Oishi, 2002), and specifically the compound called PP-R with the same Z configuration and framework has shown compatible NMR constants (Ogihara et al., 2001) thus providing a very sound support to our overall discussion.

Monascorubamine (4): ^1H NMR (CD_3OD, 499.7 MHz) δ 8.32 (1H, s, H-8), 6.94 (1H, s, H-6), 6.71 (1H, d, J = 11.7 Hz, H-10), 6.66 (1H, s, H-4), 6.47 (1H, br d, J = 11.7 Hz, H-11), 4.14 (2H, t, J = 6.5 Hz, H-1′),

2.80 (2H, m, J = 6.6 Hz, H-14), 2.40 (2H, br t, J = 6.6 Hz, 2H), 2.08 (2H, p, J = 6.8 Hz H-2′), 1.66 (3H, s, H-Me-9a), 1.58 (2H, m, J = 6.1 Hz,H-15), 1.25 − 1.30 (8H, m, H-16, H-17, H-18, H-19), 0.89 (3H, br t, J = 7.08 Hz, H 20)

^{13}C NMR (CD_3OD, 125.7 MHz) δ 198.7 (C, *C*OOH-12), 196.2 (C, > *C*=O 9), 176.4 (C, C-4′), 174.2 (C, C-3a), 173.8 (C, C-2), 171.1 (C, C-12), 153.2 (C, C-4a), 151.2 (C, C-6), 143.5 (CH, C-8), 137.3 (CH, C-11), 126.7 (CH, C-10), 121.4 (CH, C-5), 120.0 (C, C-8a), 102.7 (C, C-3), 99.0 (CH, C-4), 87.1 (C, C-9a), 56.0 (CH2, C-1′), 41.3 (CH2, C-14), 33.0 (CH2, C-18), 31.4 (CH2, C-3′), 30.6 (CH2, C-17), 30.5 (CH3, *C*H_3-C-9a), 30.3 (CH2, C-16), 26.5 (CH2, C-2′), 26.4 (CH2, C-15), 23.8 (CH2, C-19) 14.5 (CH3, C-20);

EIMS *m/z* 498 [M] + 308 (28)….fragmentations?…….; HREIMS *m/z* 497 (calcd for C27H31NO8).

4. Discussion

Talaromyces species, the teleomorph (sexual reproductive) stages of the well-known *Penicillium* fungi, have a long common history with foods and beverages consumed by human beings. As examples, in Europe, *Penicillium camemberti* or *Penicillium roqueforti* are used in cheese production, and many other *Talaromyces/Penicillium* strains are of great importance in Asia, for soy products.

From a more global point of view, ingredients derived from microbial fermentation are steadily gaining ground in the food industries. Thickening or gelling agents (e.g. polysaccharides such as xanthan, curdlan, gellan), flavour enhancers (yeast hydrolysate, monosodium glutamate), polyunsaturated fatty acids (PUFAs), flavour compounds (gamma-decalactone, diacetyl, methyl-ketones), vitamins, essential amino acids, and acidulants (lactic acid, citric acid) are illustrating this trend. Efforts have been made and continue to be done in order to reduce the production costs of pigments produced by microbial fermentation, since synthetic pigments or those extracted from natural plant sources can often be produced more economically (Dufossé, 2008). The successful marketing of natural pigments such as β-carotene, lutein, and astaxanthin derived from algae (i.e. non-conventional sources; Salvo et al., 2017) or extracted from plants (conventional sources), both as food colorants and nutritional supplements, reflects the presence and importance of niche markets in which consumers are willing to pay a premium for 'natural healthy ingredients'. Among other non-conventional sources, filamentous fungi are known to produce an extraordinary range of pigments that include several chemical classes such as carotenoids, melanins, azaphilones, anthraquinones, flavins, phenazines, quinones, and more specifically, violacein and indigo (Caro et al., 2016; Dufossé, 2008; Fouillaud et al., 2016). The success of any class of pigment produced by fermentation depends on its acceptance by the consumers, regulatory approval, and the capital investment required in bringing the product onto the market. Twenty years ago, influential representatives from food industry expressed doubts about the successful commercialization of algae-derived and fermented food grade pigments due to the high investment required for open ponds, photobioreactors and fermentation facilities, and the extensive and lengthy toxicity studies required by the regulatory authorities. Poor public perception of fungal-derived products for food use had also to be taken into account. Nowadays, some fungal food grade pigments obtained by fermentation already exist on the market worldwide. Among them, fungal *Monascus* pigments, Arpink red™ (now Natural Red™) produced by *Penicillium oxalicum*, riboflavin from the mold fungus *Ashbya gossypii*, lycopene and β-carotene from the tropical mold *Blakeslea trispora*. As an example, the production yield of β-carotene may be as high as 17 g/L of the *Blakeslea trispora* culture medium (Dufossé, 2016).

In the Western World (the Occident), pioneering work about large scale production of fungal colorants was done on carotenoids. Academics knew for a long time that fungi belonging to the order Mucorales are able to produce β-carotene. First papers dealing with *Blakeslea trispora* carotenoid production were published in the late fifties (Ciegler et al., 1959). It took four decades to move to industrial production, waiting for consumer interest about natural colorants, developing biotechnological techniques, and gaining regulatory approval. For this last aspect, Vitatene, a Spanish company, filled a novel foods and novel food ingredients application in 2003, to place lycopene from *Blakeslea trispora* on the European market (under Regulation EC N° 258/97). The positive answer was published on 23 October 2006 (European Commission decision N° 721/2006).

Red azaphilone pigments are similarly known for a long-time by scientists from Asia and, as explained in the introduction, researchers are trying to find new strains, non-mycotoxigenic, to use as an alternative to the citrinin-producing *Monascus*.

Pioneering work started at Denmark Technical University (DTU) during the PhD thesis of Sameer Mapari, with co-workers such as Ulf Thrane, Anne S. Meyer, Jens C. Frisvad and co-funding from the world-leading natural colors Chr. Hansen A/S company, represented by Annette Salskov-Iversen. Many papers were published between 2005 and 2009 (Mapari et al., 2005; Mapari et al., 2006; Mapari et al., 2008, Mapari et al., 2009), setting a general framework in the development of fungal reds. International patents were issued such as EP2262862 (=WO2009026923, priority date August 28, 2007) or EP2011/064152 (=WO2012022765, priority date August 19, 2010). Despite the very nice results obtained in these works, no industrial application of red azaphilone (polyketide) *Monascus*-like pigments appears on the market up to now. A few years later, in 2013, Jens C. Frisvad and co-workers from DTU, the CBS–KNAW Fungal Biodiversity Centre (Utrecht, The Netherlands) and the Department of Biology, Utrecht University (Utrecht, The Netherlands), described a new strain of *Talaromyces*, they named *Talaromyces atroroseus* sp. nov., and recommended as an effective producer of the azaphilone biosynthetic families mitorubrins and *Monascus*-like-pigments without any production of mycotoxins.

In our work, we isolated in the tropical marine environment of Réunion island, Indian Ocean, a different red pigment producing strain belonging to *Talaromyces albobiverticillius* and this paper brings new information about the pigments produced. The literature (Frisvad et al., 2013) lists ten extrolites (excreted metabolites) in seven collection strains (Table 3): mitorubrin $C_{21}H_{18}O_7$ formula weight 382.36 (6 occurrences among 7 strains, *i.e.* 6/7), mitorubrinic acid $C_{21}H_{16}O_9$ formula weight 412.35 (6/7), monascorubramine $C_{23}H_{27}NO_4$ formula weight 381.46 (5/7), rubropunctatin $C_{21}H_{22}O_5$ formula weight 354.39 (4/7), monascorubrin $C_{23}H_{26}O_5$ formula weight 382.45 (2/7), a purpactin (2/7), vermicellin (2/7), PP-R = [(10*Z*)-7-(2-hydroxyethyl)-monascorubramine] $C_{25}H_{31}NO_5$ formula weight 425.52 (1/7), mitorubrinol $C_{21}H_{18}O_8$ formula weight 398.36 (1/7) and monascin $C_{21}H_{26}O_5$ formula weight 358.43 (1/7).

During the research presented here four out of twelve compounds were identified in the investigated pigmented extract from *Talaromyces albobiverticillius* 30548 using HPLC-PDA-ESI/MS and NMR: N-threonine monascorubramine ($C_{27}H_{33}NO_7$ formula weight 483.55), N-glutaryl rubropunctamine ($C_{26}H_{29}NO_8$ formula weight 483.51), PP-O = ((10*Z*)-12-carboxylmonascorubrin) ($C_{23}H_{24}O_7$ formula weight 412.43) and a new compound, a N-GABA-PP-V (6-[(*Z*)-2-Carboxyvinyl]-N-GABA-PP-V) ($C_{27}H_{31}NO_8$ formula weight 497.53), pigment bearing a *cis* configuration at the C10-C11 double bond.

This new compound will enlarge the list of 63 *Monascus* and *Monascus*-like pigments (24 O-containing compounds and 39 N-containing compounds) recently summarized by (Gao et al., 2013). Azaphilones are a large group of pyrano-quinones structures with a high electron acceptor tension determining sensitivity of oxygen in the primary ring. This yields γ- pyridones, exhibiting chromophore properties in which colors depend on their chemical structure. Their name comes from their ability to react with ammonia. Thus in the media, they readily interact with compounds containing amino groups such as proteins, amino acids, or nucleic acids resulting in water soluble colored products. In microorganisms such as fungi, L-glutamate is the main precursor of 4- amino-butyrate (GABA). The production of GABA

is considered as a shunt of the tricarboxylic acid (TCA) cycle which can lead to the production of succinic semi-aldehyde (GABA bypass)(Kumar and Punekar, 1997). The reaction of GABA with O-containing rubropunctatin precursor is mentioned here in *Talaromyces albobiverticillius*, in *Talaromyces* genus, in fungi, for the first time. The specific role of this product in the fungal metabolism has to be clarified. Indeed, the azaphilone skeleton is essential for certain biological activities of these metabolites. The differences observed in their activities can however be ascribed to differences in their reactivity with amines.

The work will continue with large scale cultivation of *Talaromyces albobiverticillius* 30548 in fermenter, analysis of pigmented extracts with liquid chromatography-mass spectrometry ion trap time-of-flight (LCMS-IT-TOF) mass spectrometer (MS) through an atmospheric-pressure chemical ionization (APCI) source, operating in both positive and negative mode, and finally isolation of still unknown compounds for additional NMR.

5. Conclusion

Research efforts on fungal reds will continue in the next years or decades. It is now proven that some *Talaromyces/Penicillium* species are able to produce pigments with no associated mycotoxin(s) (e.g. *Talaromyces atroroseus*; *T. albobiverticillius* − up to now we were unable to detect any mycotoxin(s) in all our extracts, prepared with various solvents, from biomasses produced in many different media). Feeding rats in order to test toxicity on living animals is one of the next steps, as it was done previously with other pigmented extracts or molecules (Jonker et al., 2003; Sanjay et al., 2007).

These *Talaromyces atroroseus*, *T. albobiverticillius* fungal reds will also be challenged by new generations of *Monascus* pigments, biosynthesized by new strains unable to produce the mycotoxin citrinin. The very popular and rapid-evolving technique CRISPR/Cas9 allowing fine targeted genome editing sure opens a new era in molecular biology applied to fungal pigments. More data are soon expected about deletion (s) of polyketide synthase(s) involved in mycotoxin(s) biosynthesis, deletion(s) that should maintain pigment(s) production, such providing safe fungi for the production of food colorants.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jfca.2017.12.036.

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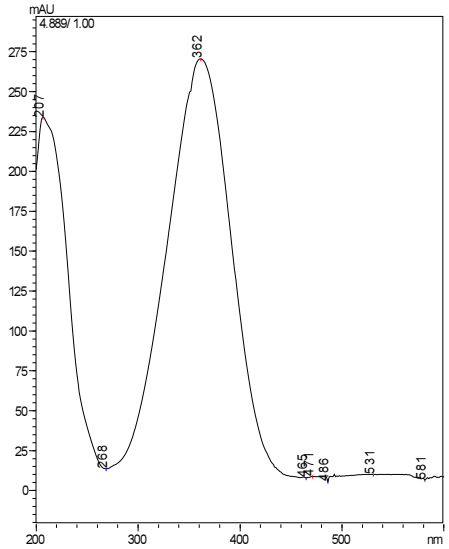
Figure S1. PDA and ESI-MS spectra of azaphilones from *Talaromyces albobiverticillius*

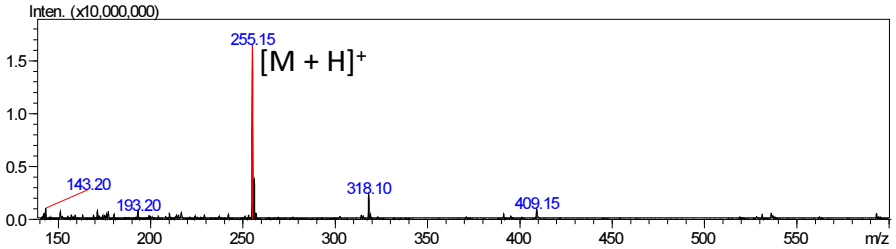
HPLC-PDA-ESI/MS
Sample: EtoAc Single Solvent IC/EC

- Column C18 Phenomenex, Kinetex (100x2.1 mm; 1.7 μm).
- Solvent A: Water (0.1% Formic acid).
- Solvent B: Acetonitrile (0.1% Formic acid).
- PDA: 200-600 nm.
- MS: ESI positive and ESI negative 60-600 m/z.
- Gradient: 0 min, 5% B; 15 min, 95% B; 17 min, 95% B; 18 min, 5% B.

Flow rate: 0.2 ml/min.

PDA Data and ESI (+) /MS Data for Compound N. 1





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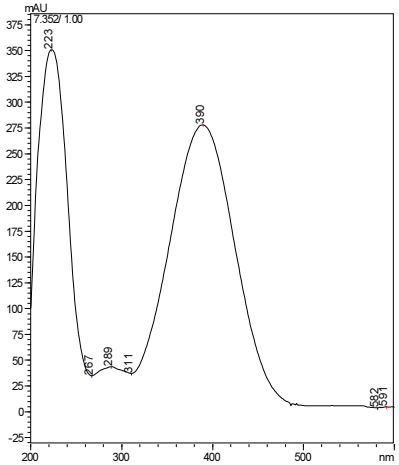
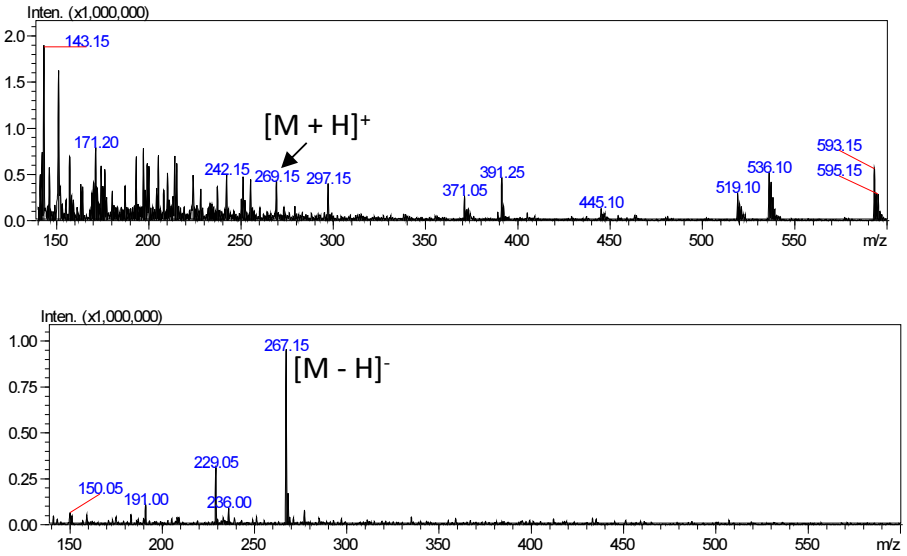
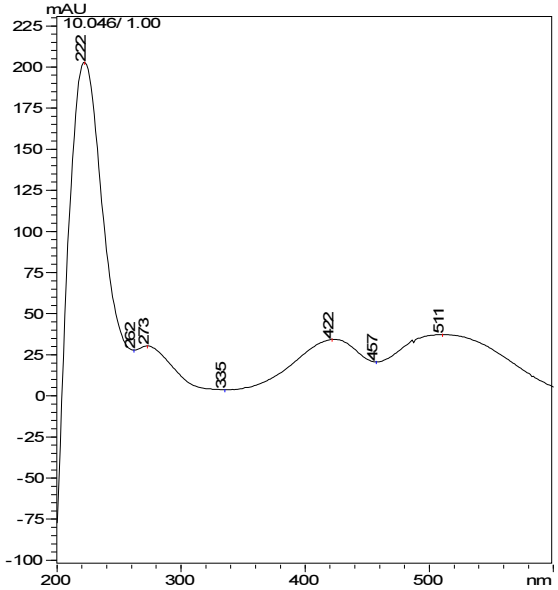
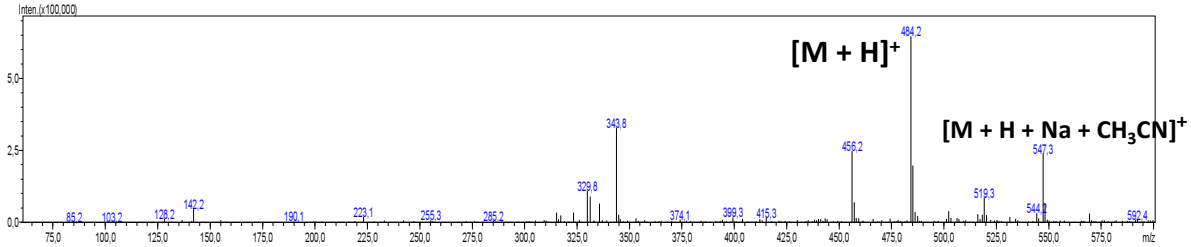
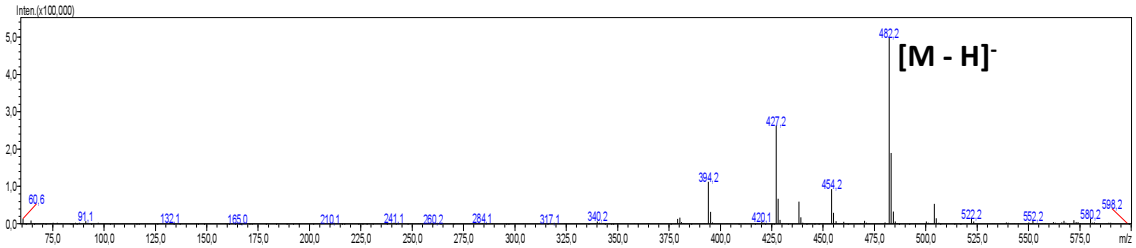




PDA Data and ESI (+) and (-) MS Data for Compound N. 3







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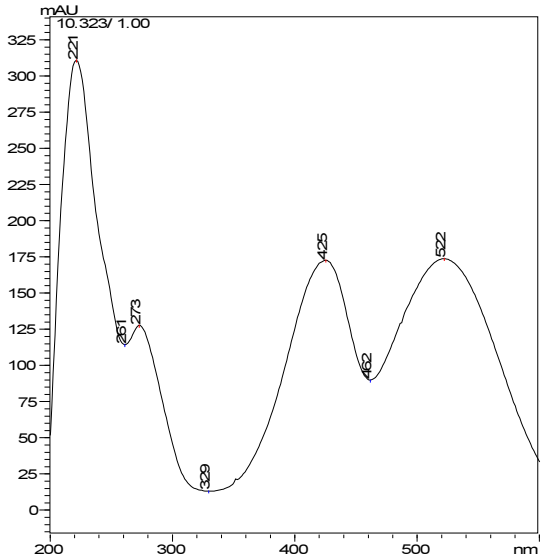

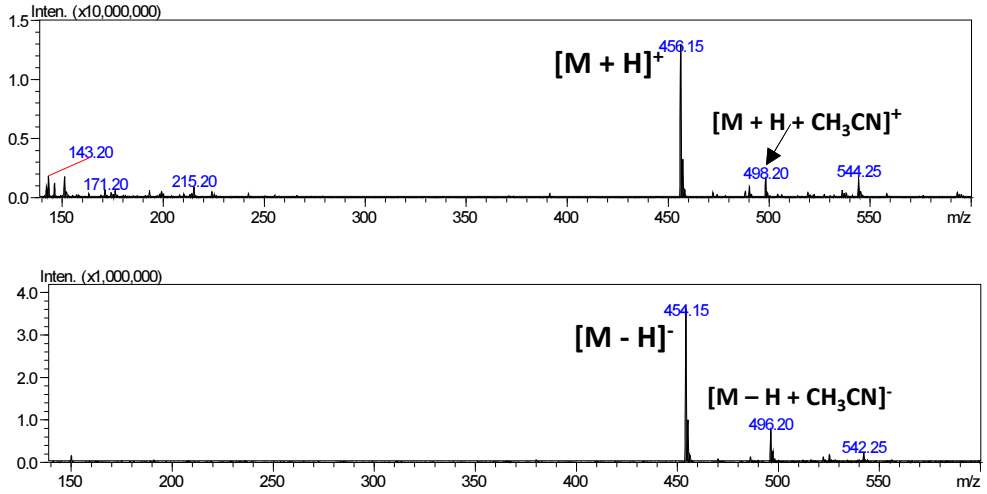

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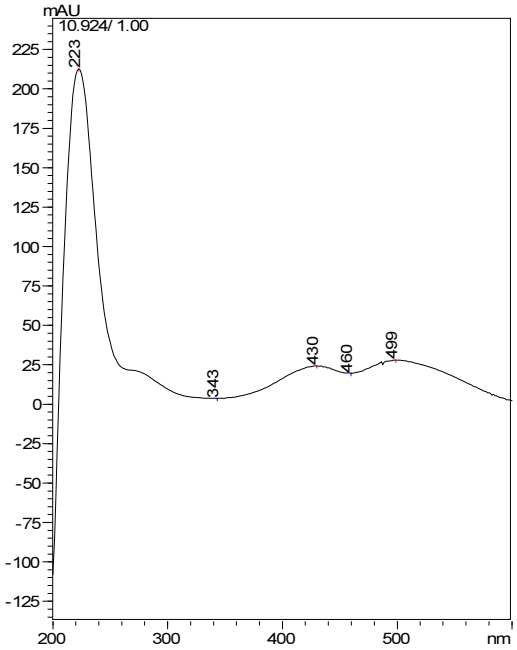

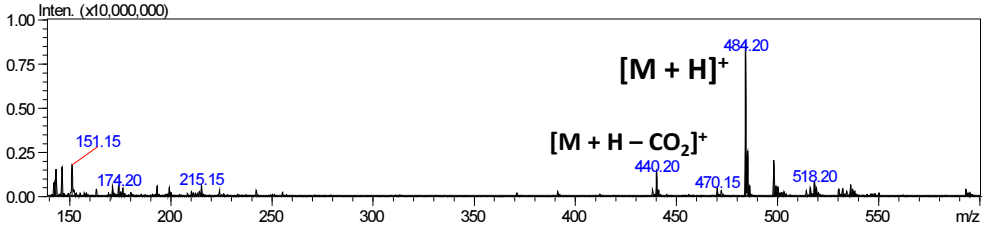

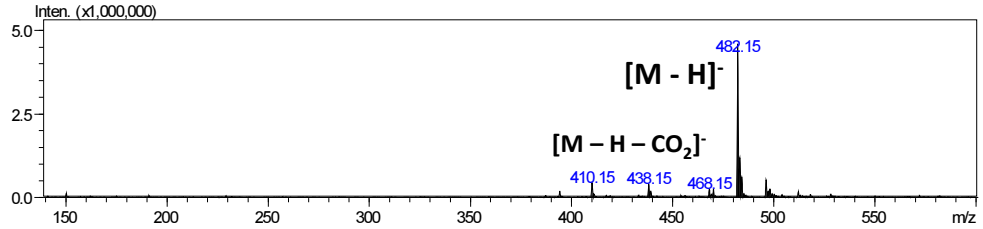

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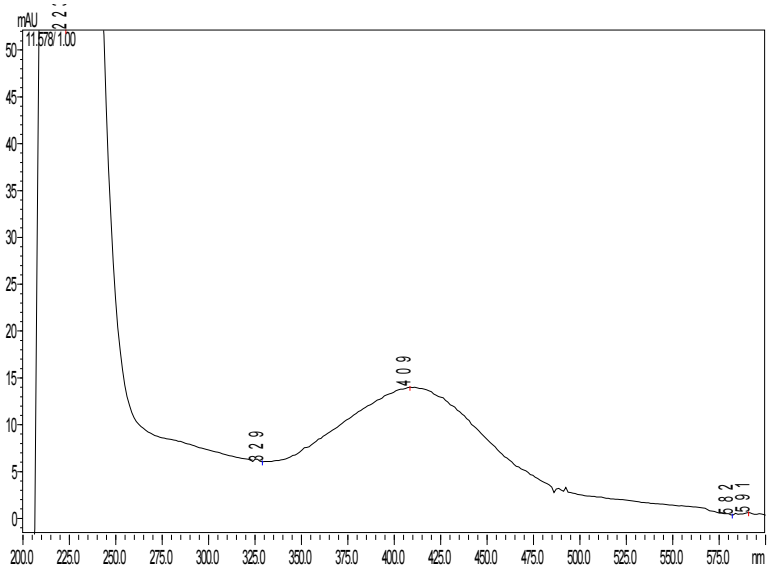

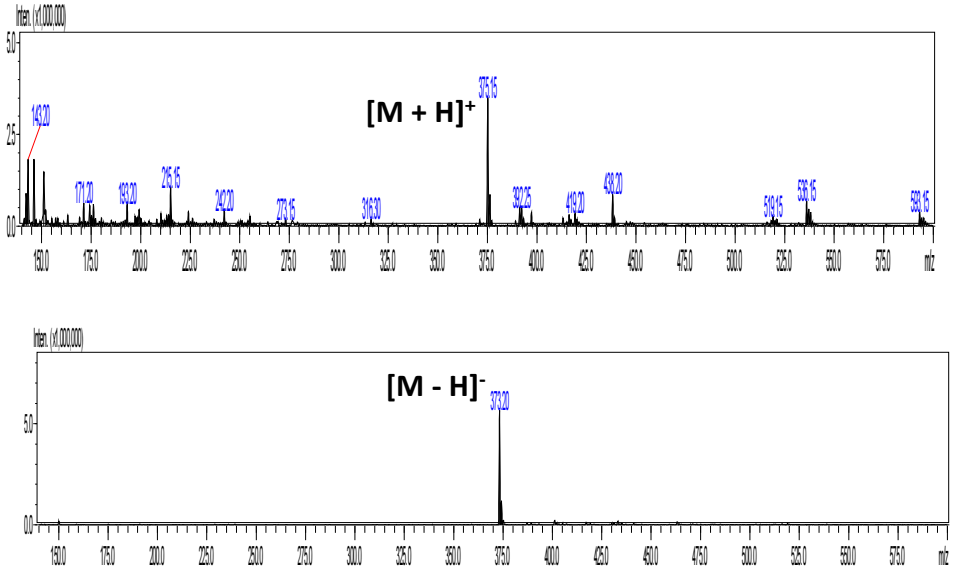

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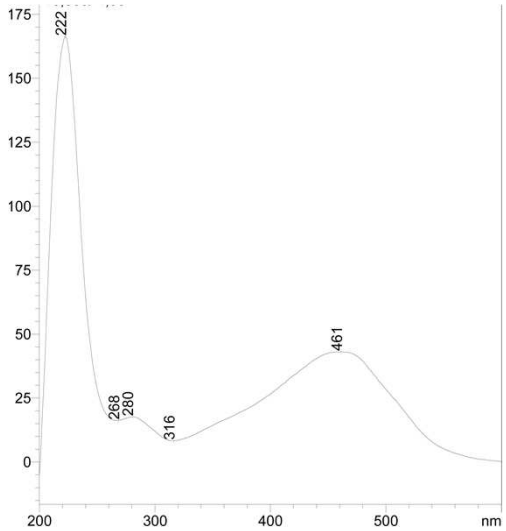

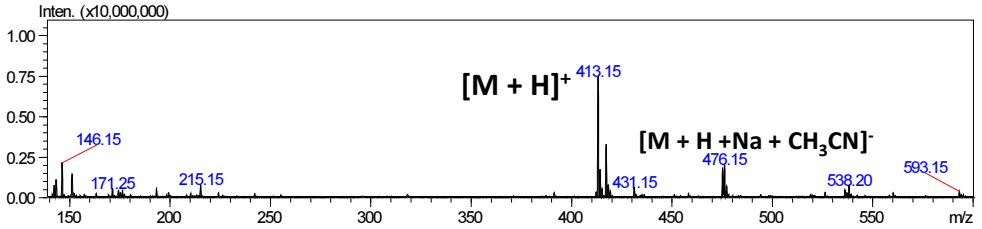

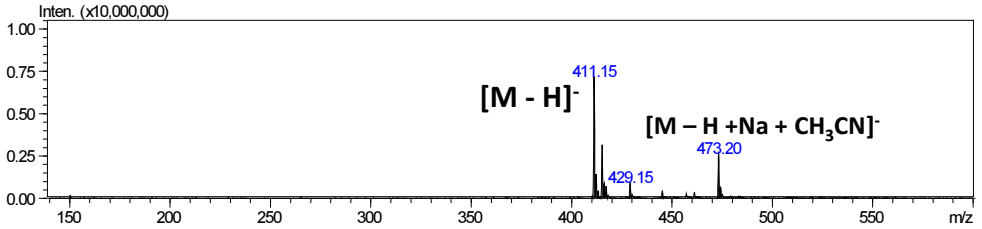

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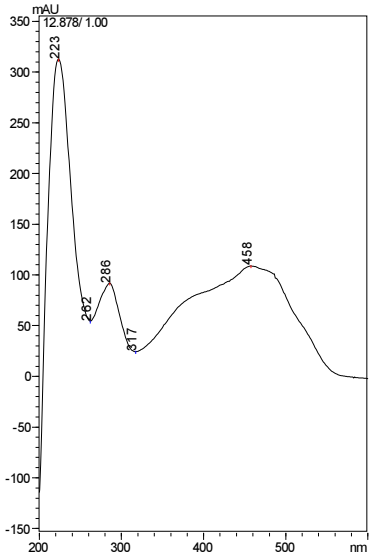
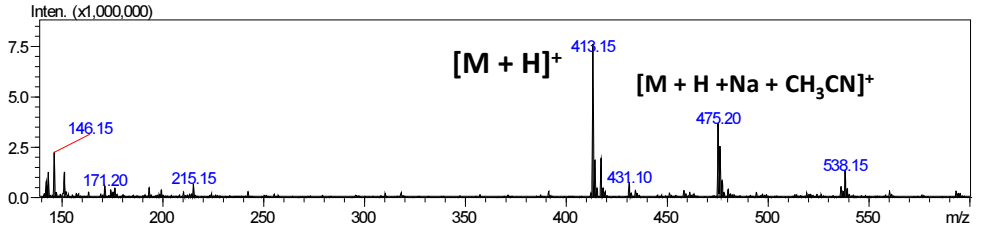
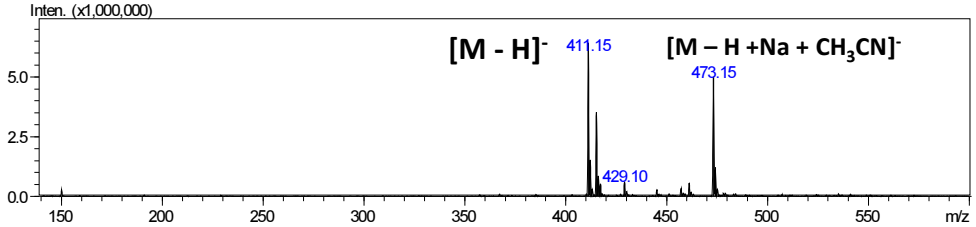
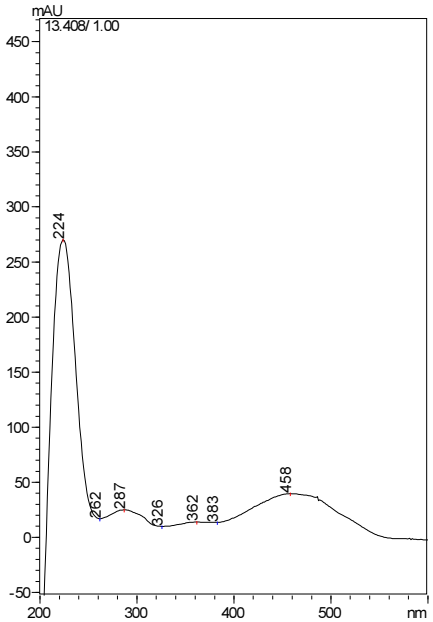
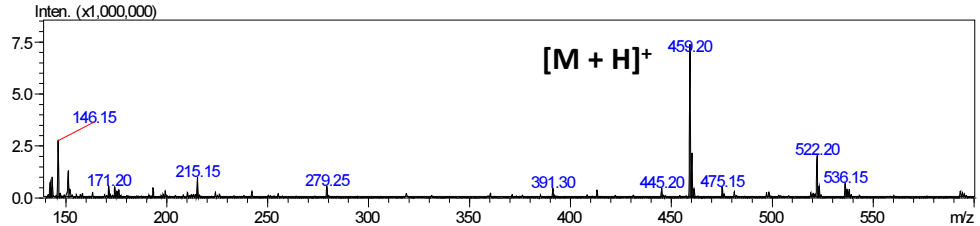


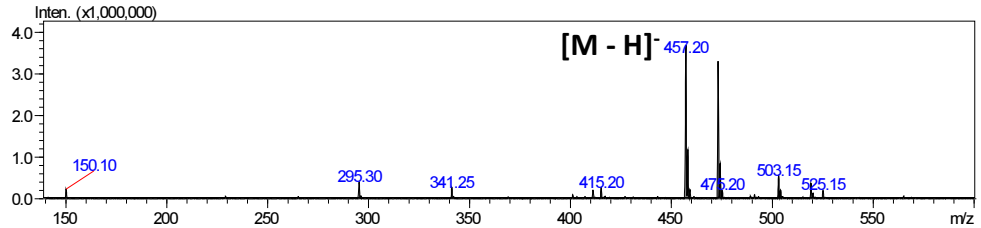




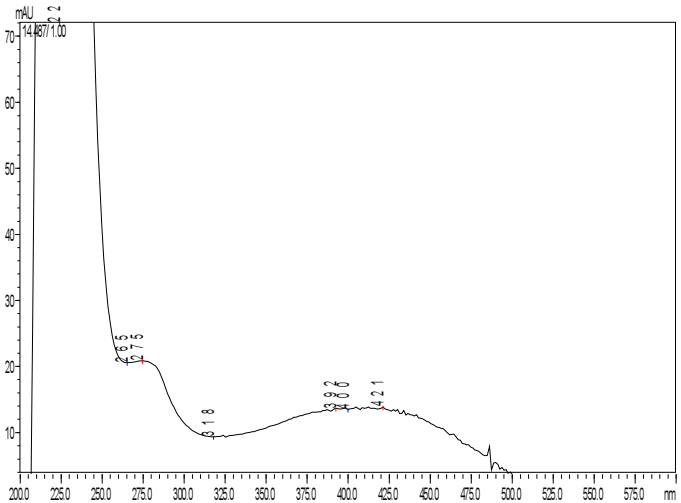
PDA Data and ESI (+) and (-) MS Data for Compound N. 9

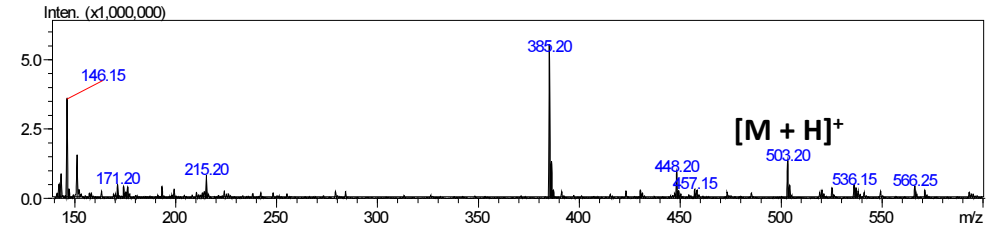


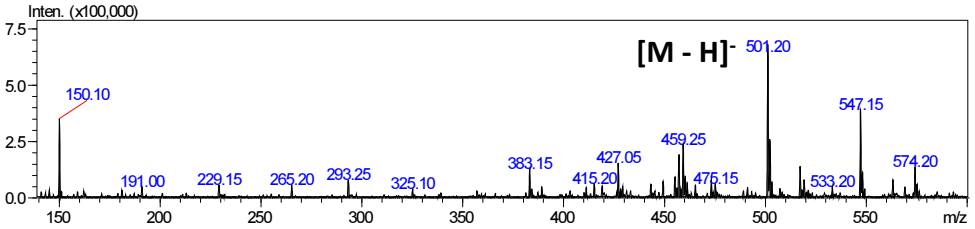




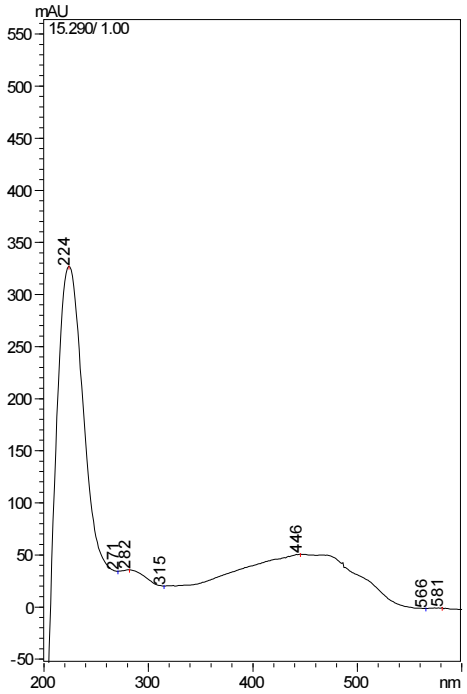
PDA Data and ESI (+) and (-) MS Data for Compound N. 10

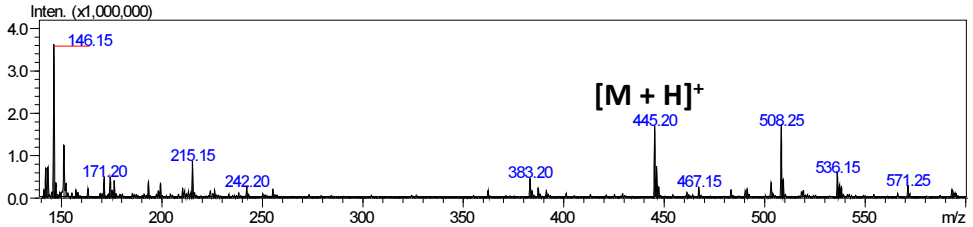


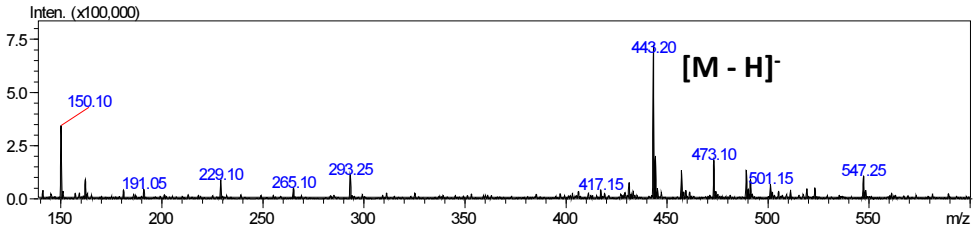




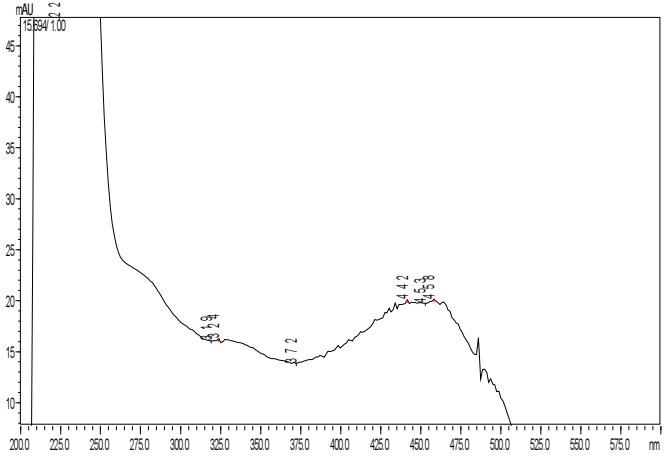
PDA Data and ESI (+) and (-) MS Data for Compound N. 11







PDA Data and ESI (+) for compound 12



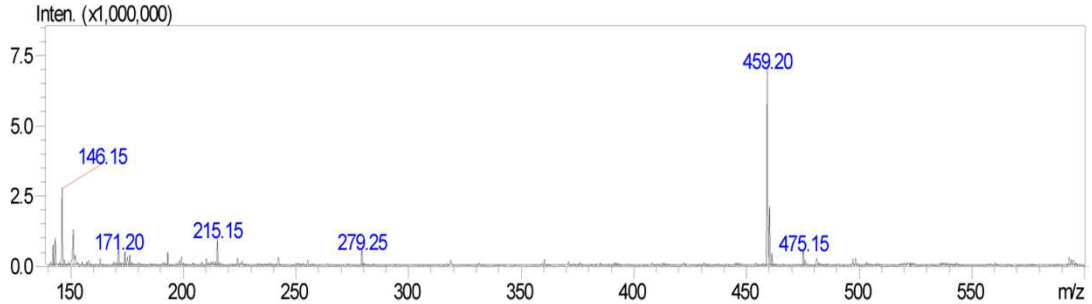
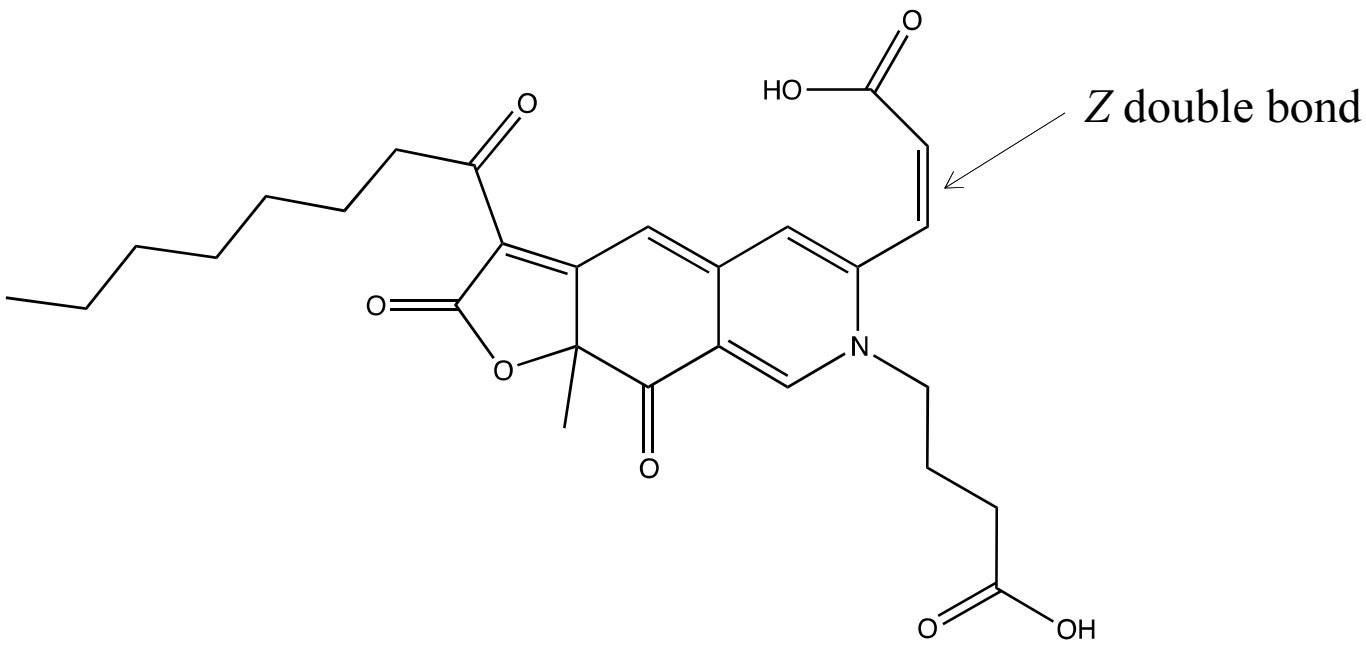


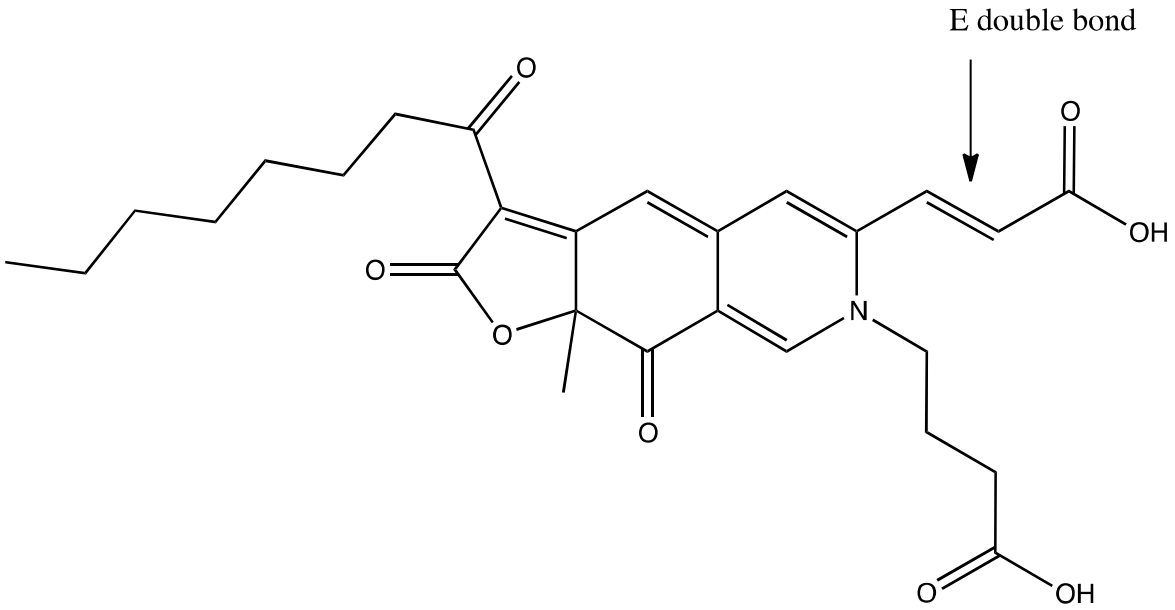
Figure S2. NMR data for compound 4

Proposed Name: N-GABA-PP-V



New proposed structure for compound n. 4 of chromatogram shown in Figure 3

Already reported structure in Chem Spider database with *E* double bond, although not reported in *Talaromyces* species and no source or reference provided.



4-{6-[(E)-2-Carboxyvinyl]-9a-methyl-3-octanoyl-2,9-dioxo-9,9a-dihydrofuro[3,2-g]isoquinolin-7(2H)-yl}butanoic acid
(PubChem CID:44715338). No information available on source and literature.

Supplementary NMR data

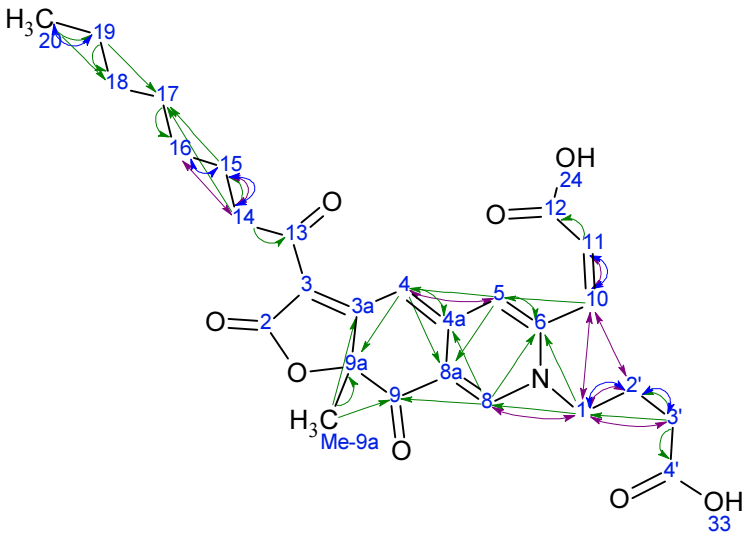


Figure 1S. Molecular scheme of 4 with connections indicated by different colours, specifically green, magenta and blue for HMBC, NOESY and COSY respectively

Table 1S. Total board of the ^{1}H and ^{13}C resonances for compound 4 in methanol.

#	Atom#	C Label	C Shift	XHn	H Label	H Shift	C Calc Shift	H Calc Shift	H Multiplicity	COSY	NOESY	H HMBC	C HMBC
1	20	C20	14.479	CH3	H20	0.887	14.428	0.903	br t (7.08, 7.08)	19			19, 18
2	19	C19	23.755	CH2	H19	1.297	22.763	1.275	m	20		20	17, 18
3	15	C15	26.444	CH2	H15	1.584	24.354	1.444	br quin (6.07)	17, 16, 14	14	14	17
4	2'	C2'	26.513	CH2	H2'	2.081	24.980	2.060	quin	3', 1'	1', 10	3'	
5	17, 16	C16	30.348	CH2	H16	1.324	28.319	1.243, 1.204	br s	15	14		
6	Me-9a	Me-9a	30.542	CH3	H-9a	1.656	21.820	1.695	s				9a, 3a, 9
7	16, 17	C17	30.655	CH2	H17	1.285	28.875	1.204, 1.243	m			19, 15, 14	19
8	3'	C3'	31.456	CH2	H3'	2.400	31.969	2.390	br t (6.65, 6.65)	2'	1'		2', 1', 4'
9	18	C18	33.000	CH2	H18	1.283	31.803	1.235	m			20, 19	
10	14	C14	41.339	CH2	H 14	2.798	39.708	2.984	t	15	17, 16, 15		15, 17, 13
11	1'	C1'	56.020	CH2	H 1'	4.139	52.054	4.394	m	2'	2', 3', 10, 8	3', 8	8, 6
12	9a	C9a	87.125	C			84.863					Me-9a, 4	
13	4	C4	99.015	CH	H 4	6.663	111.376	8.155	s		5	5	9a, 8a, 4a
14	3	C3	102.748	C			112.334						
15	8a	C8a	120.047	C			109.433					4, 5	
16	5	C5	121.402	CH	H 5	6.939	115.535	6.330	s		4	10	4, 8a, 10, 6
17	10	C10	126.671	CH	H 10	6.712	136.095	6.266	d (11.85)	11	2', 1', 11	5	5
18	11	C11	137.320	CH	H 11	6.475	121.932	6.178	br d (12.43)	10	10		12
19	8	C8	143.504	CH	H 8	8.324	140.335	8.958	s		1'	1'	1', 6, 4a, 9
20	6	C6	151.179	C			140.584					1', 5, 8	

#	Atom#	C Label	C Shift	XHn	H Label	H Shift	C Calc Shift	H Calc Shift	H Multiplicity	COSY	NOESY	H HMBC	C HMBC
1	20	C20	14.479	CH3	H20	0.887	14.428	0.903	br t (7.08, 7.08)	19			19, 18
2	19	C19	23.755	CH2	H19	1.297	22.763	1.275	m	20		20	17, 18
3	15	C15	26.444	CH2	H15	1.584	24.354	1.444	br quin (6.07)	17, 16, 14	14	14	17
4	2'	C2'	26.513	CH2	H2'	2.081	24.980	2.060	quin	3', 1'	1', 10	3'	
5	17, 16	C16	30.348	CH2	H16	1.324	28.319	1.243, 1.204	br s	15	14		
6	Me-9a	Me-9a	30.542	CH3	H-9a	1.656	21.820	1.695	s				9a, 3a, 9
7	16, 17	C17	30.655	CH2	H17	1.285	28.875	1.204, 1.243	m			19, 15, 14	19

^{1}H NMR (500 MHz, METHANOL-d_4) δ ppm 0.89 (br t, *J*=7.08 Hz, 4 H) 1.26 - 1.30 (m, 3 H) 1.28 - 1.29 (m, 2 H) 1.28 - 1.32 (m, 4 H) 1.32 (br s, 6 H) 1.58 (br d, *J*=6.07 Hz, 3 H) 1.66 (s, 3 H) 2.05 - 2.13 (m, 2 H) 2.40 (br t, *J*=6.65 Hz, 2 H) 2.78 - 2.82 (m, 2 H) 4.11 - 4.16 (m, 2 H) 6.47 (br d, *J*=12.43 Hz, 1 H) 6.66 (s, 1 H) 6.71 (d, *J*=11.85 Hz, 1 H) 6.94 (s, 1 H) 8.32 (s, 1 H)

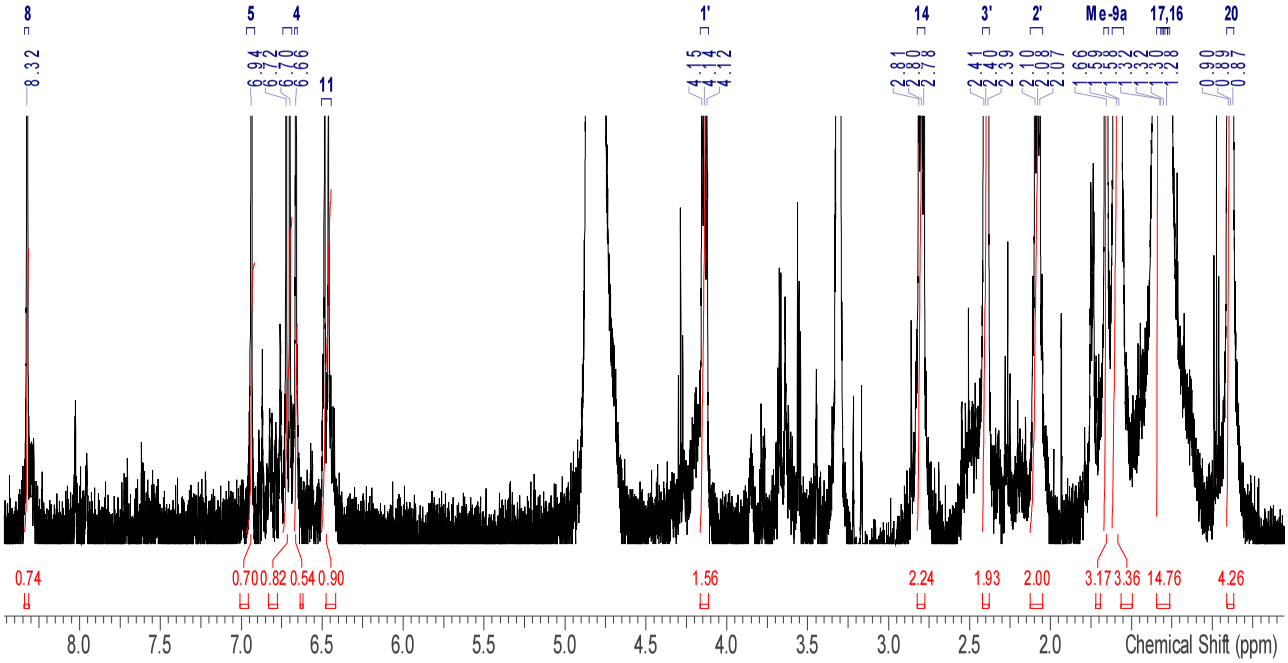


Figure2S. Protonic Spectrum of 4 in Methanol-d4

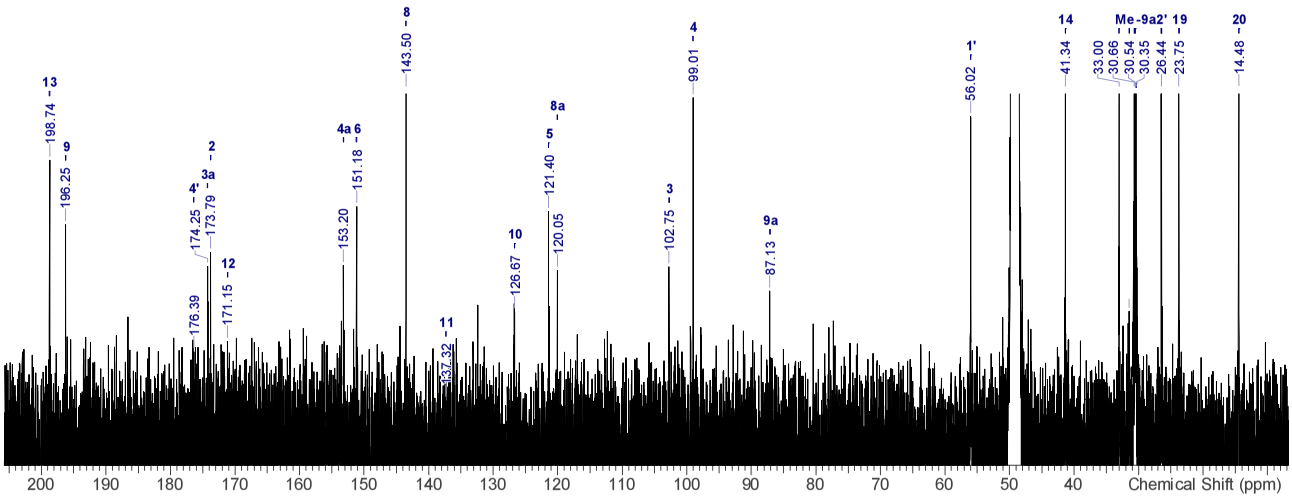


Figure 3S. Carbon spectrum of compound 4 in Methanol-d4

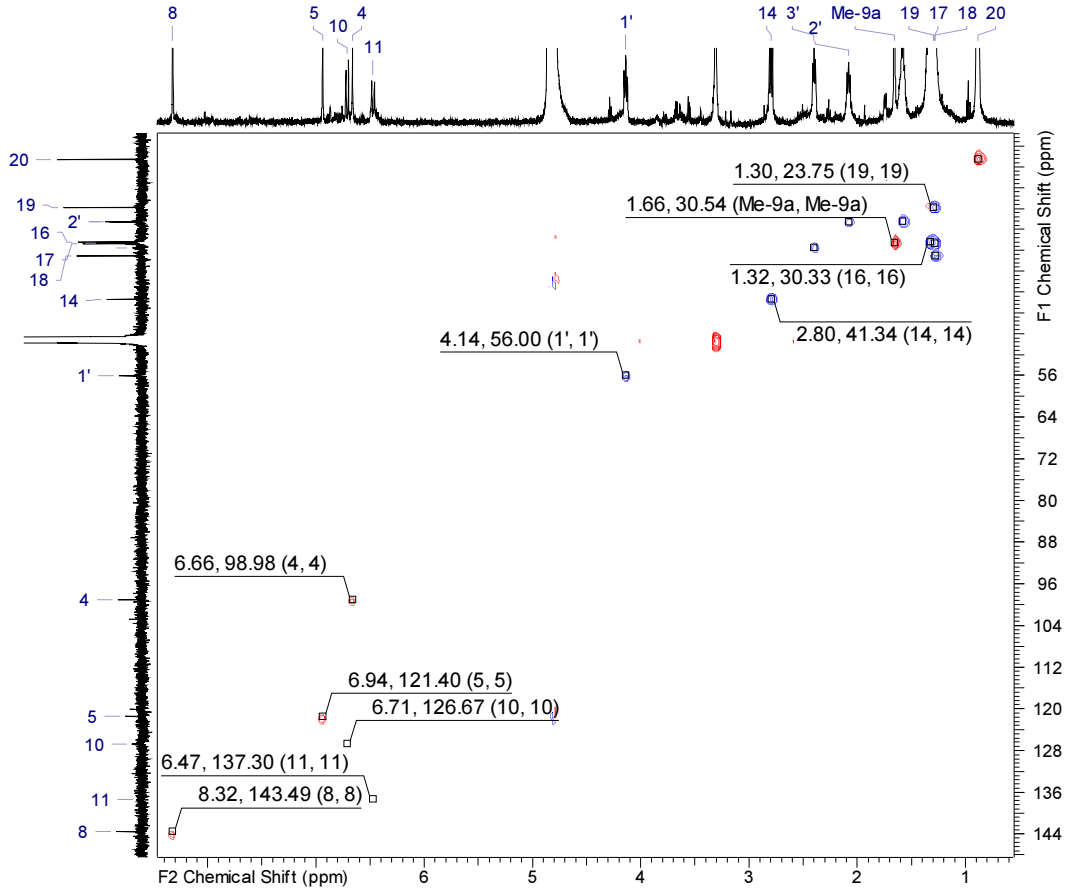


Figure 4S. ^{1}H-^{13}C-HSQC plot for the molecule 4 in methanol-d4

Table 2S. Scheme of the HSQC connections.

No.	F2 Atom	F1 Atom	F2 (ppm)	F1 (ppm)
1	Me-9a	Me-9a	1.66	30.54
2	1'	1'	4.14	56.00
3	2'	2'	2.08	26.51
4	3'	3'	2.40	31.48
5	4	4	6.66	98.98
6	5	5	6.94	121.40
7	8	8	8.32	143.49
8	10	10	6.71	126.67
9	11	11	6.47	137.30
10	14	14	2.80	41.34
11	15	15	1.58	26.44
12	16	16	1.32	30.33
13	17	17	1.29	30.65
14	18	18	1.28	32.99
15	19	19	1.30	23.75
16	20	20	0.89	14.47

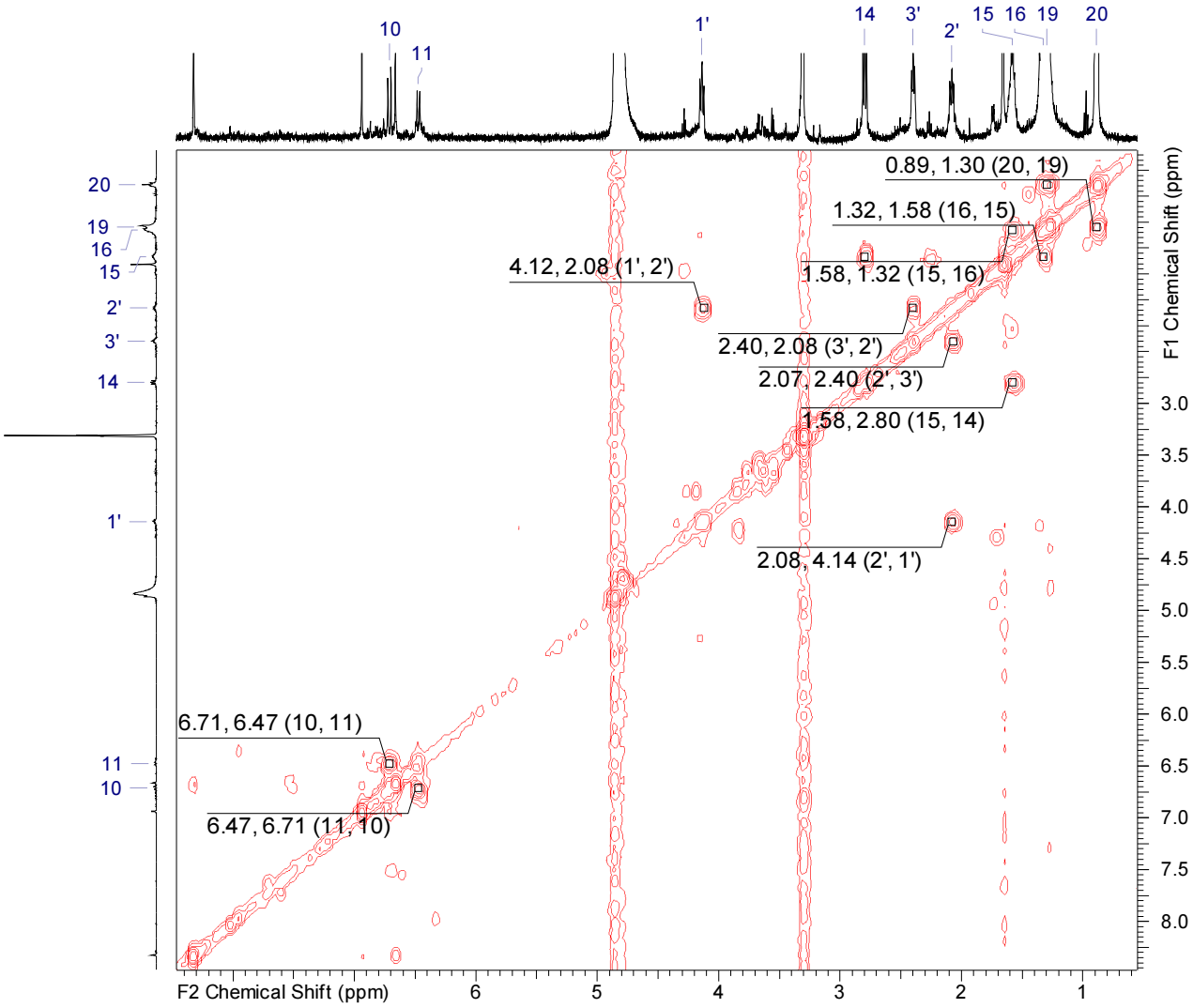


Figure 5S. ^{1}H-^{1}H-COSY plot for the molecule 4 in methanol-d4

Table 3S. Scheme of the COSY connections.

No.	F2 Atom	F1 Atom	F2 (ppm)	F1 (ppm)
1	2'	1'	2.08	4.14
2	1'	2'	4.12	2.08
3	3'	2'	2.40	2.08
4	2'	3'	2.07	2.40
5	11	10	6.47	6.71
6	10	11	6.71	6.47
7	15	14	1.58	2.80
8	14	15	2.80	1.58
9	16	15	1.32	1.58
10	15	16	1.58	1.32
11	20	19	0.89	1.30
12	19	20	1.30	0.89

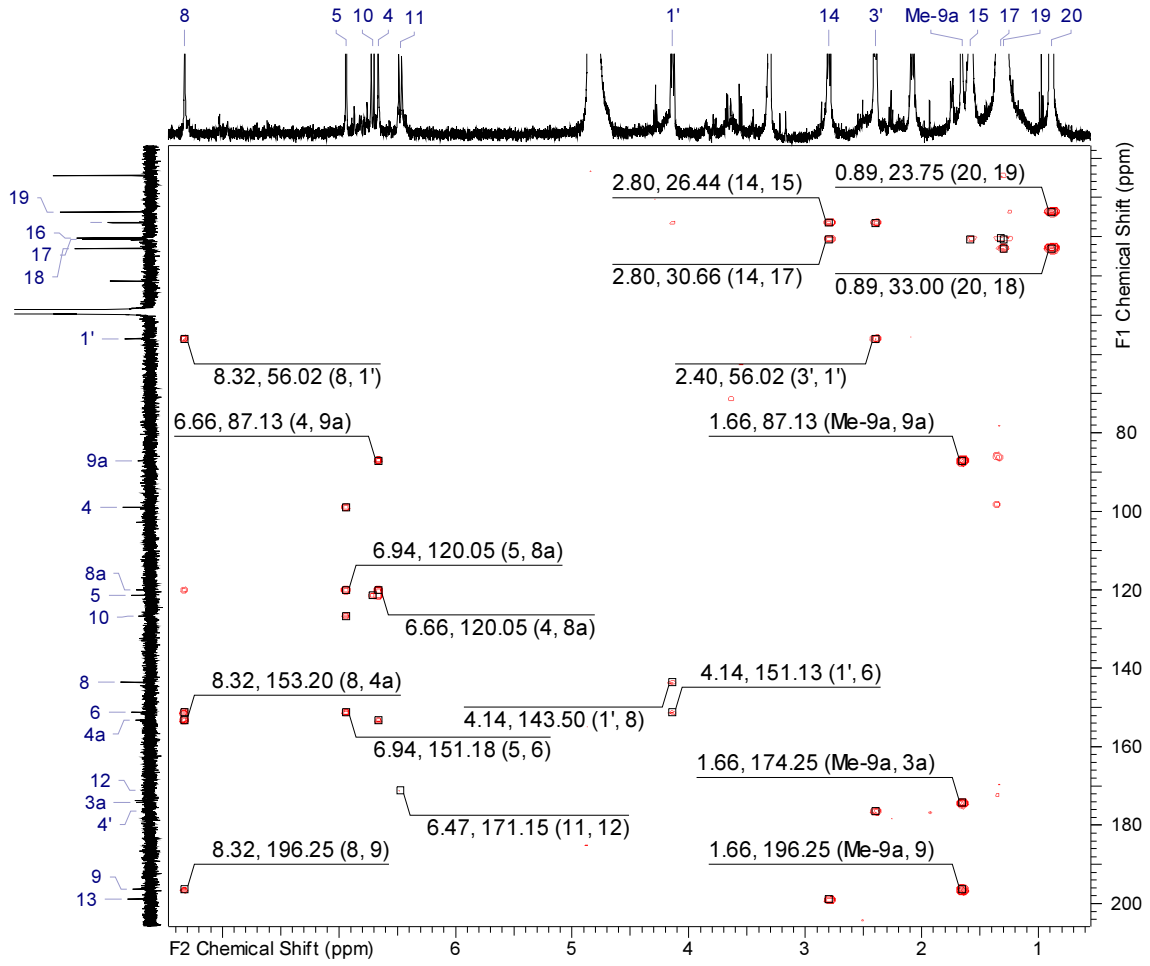


Figure 6S. ^{1}H-^{13}C-HMBC plot for the molecule 4 in methanol-d4

Table 4S. Scheme of the HMBC connections.

No.	F2 Atom	F1 Atom	F2 (ppm)	F1 (ppm)
1	3'	1'	2.40	56.02
2	8	1'	8.32	56.02
3	3'	2'	2.40	26.51
4	Me-9a	3a	1.66	174.25
5	5	4	6.94	99.01
6	3'	4'	2.40	176.39
7	4	4a	6.66	153.20
8	8	4a	8.32	153.20
9	10	5	6.71	121.40
10	1'	6	4.14	151.13
11	5	6	6.94	151.18
12	8	6	8.32	151.18
13	1'	8	4.14	143.50
14	4	8a	6.66	120.05
15	5	8a	6.94	120.05
16	Me-9a	9	1.66	196.25
17	8	9	8.32	196.25
18	Me-9a	9a	1.66	87.13
19	4	9a	6.66	87.13
20	5	10	6.94	126.67
21	11	12	6.47	171.15
22	14	13	2.80	198.74
23	14	15	2.80	26.44
24	17	16	1.32	30.35
25	14	17	2.80	30.66
26	15	17	1.58	30.66
27	19	17	1.30	30.66
28	19	18	1.30	33.00
29	20	18	0.89	33.00
30	20	19	0.89	23.75

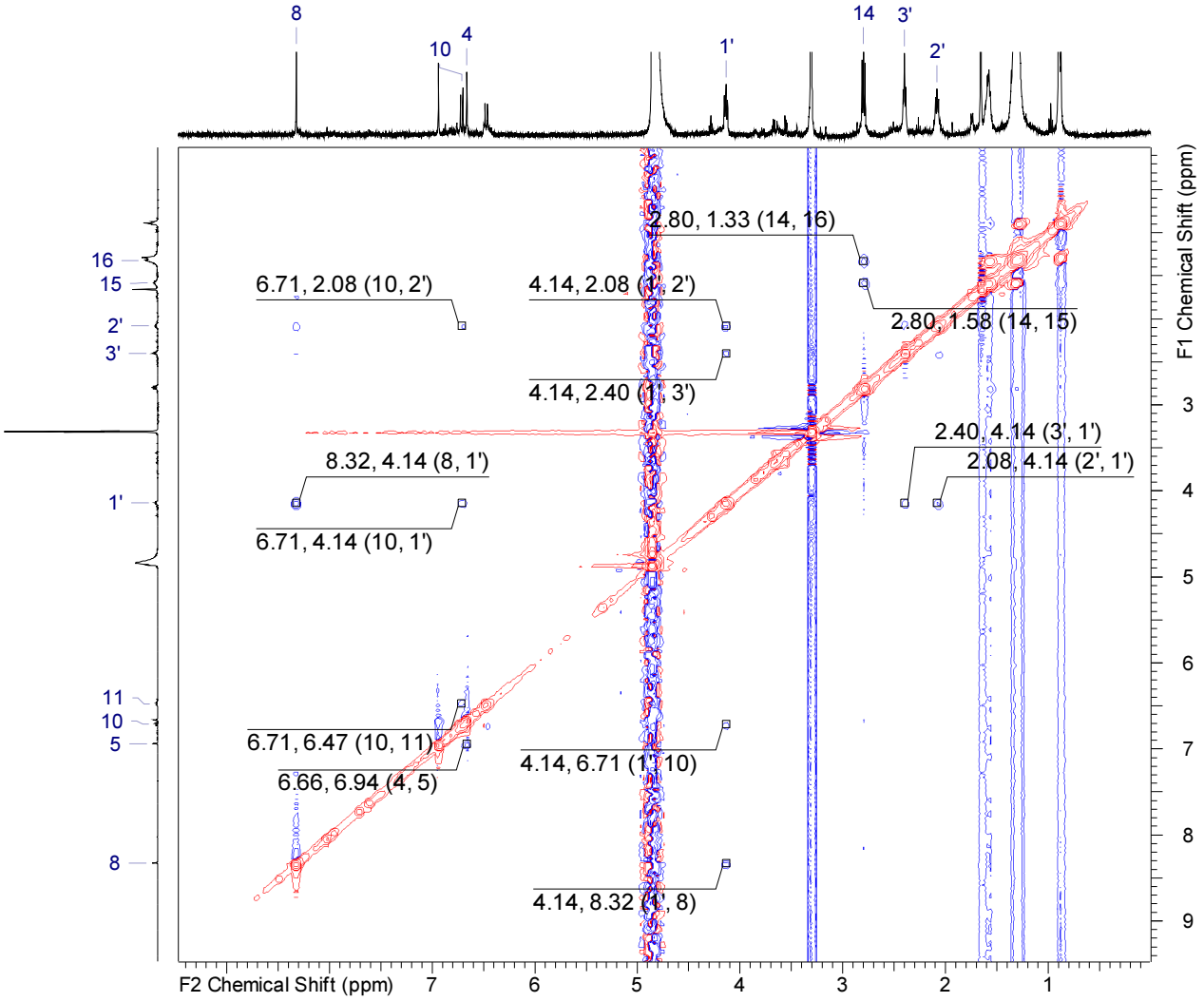
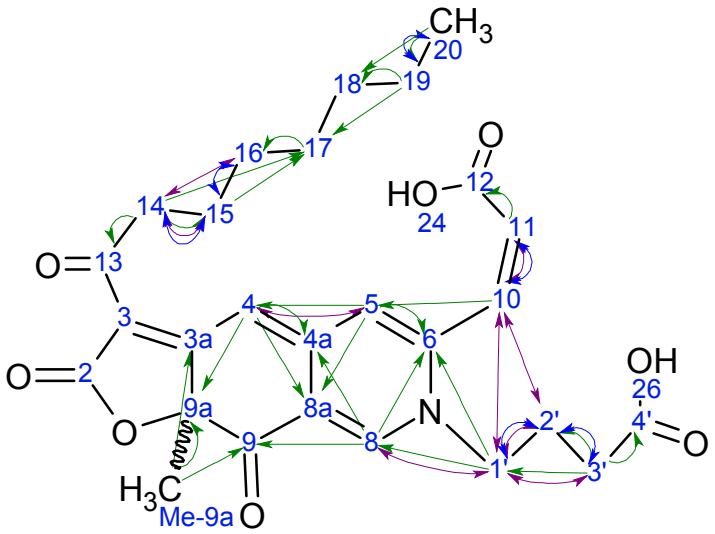
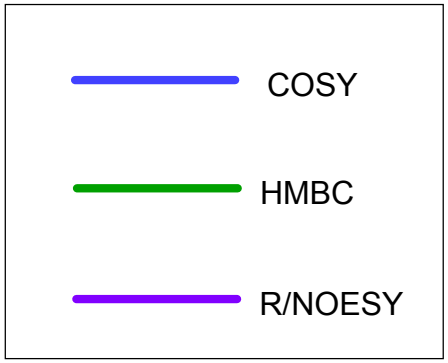


Figure 7S. Noesy spectrum in methanol-d4

Table 5S. Scheme of the NOESY connections.

No.	F2 Atom	F1 Atom	F2 (ppm)	F1 (ppm)
1	2'	1'	2.08	4.14
2	3'	1'	2.40	4.14
3	8	1'	8.32	4.14
4	10	1'	6.71	4.14
5	1'	2'	4.14	2.08
6	10	2'	6.71	2.08
7	1'	3'	4.14	2.40
8	4	5	6.66	6.94
9	1'	8	4.14	8.32
10	1'	10	4.14	6.71
11	10	11	6.71	6.47
12	14	15	2.80	1.58
13	14	16	2.80	1.33





#	Atom#	C Label	C Shift	XHn	H Label	H Shift	C Calc Shift	H Calc Shift	H Multiplicity	COSY	NOESY	H HMBC	C HMBC
1	20	C20	14.479	CH3	H20	0.887	14.428	0.903	br t (7.08, 7.08)	19			19, 18
2	19	C19	23.755	CH2	H19	1.297	22.763	1.275	m	20		20	17, 18
3	15	C15	26.444	CH2	H15	1.584	24.354	1.444	br quin (6.07)	17, 16, 14	14	14	17
4	2'	C2'	26.513	CH2	H2'	2.081	24.980	2.060	quin	3', 1'	1', 10	3'	
5	17, 16	C16	30.348	CH2	H16	1.324	28.319	1.243, 1.204	br s	15	14		
6	Me-9a	Me-9a	30.542	CH3	H-9a	1.656	21.820	1.695	s				9a, 3a, 9
7	16, 17	C17	30.655	CH2	H17	1.285	28.875	1.204, 1.243	m			19, 15, 14	19
8	3'	C3'	31.456	CH2	H3'	2.400	31.969	2.390	br t (6.65, 6.65)	2'	1'		2', 1', 4'
9	18	C18	33.000	CH2	H18	1.283	31.803	1.235	m			20, 19	
10	14	C14	41.339	CH2	H 14	2.798	39.708	2.984	t	15	17, 16, 15		15, 17, 13
11	1'	C1'	56.020	CH2	H 1'	4.139	52.054	4.394	m	2'	2', 3', 10, 8	3', 8	8, 6
12	9a	C9a	87.125	C			84.863					Me-9a, 4	
13	4	C4	99.015	CH	H 4	6.663	111.376	8.155	s		5	5	9a, 8a, 4a
14	3	C3	102.748	C			112.334						
15	8a	C8a	120.047	C			109.433					4, 5	
16	5	C5	121.402	CH	H 5	6.939	115.535	6.330	s		4	10	4, 8a, 10, 6
17	10	C10	126.671	CH	H 10	6.712	136.095	6.266	d (11.85)	11	2', 1', 11	5	5
18	11	C11	137.320	CH	H 11	6.475	121.932	6.178	br d (12.43)	10	10		12
19	8	C8	143.504	CH	H 8	8.324	140.335	8.958	s		1'	1'	1', 6, 4a, 9
20	6	C6	151.179	C			140.584					1', 5, 8	

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#	Atom#	C Label	C Shift	XHn	H Label	H Shift	C Calc Shift	H Calc Shift	H Multiplicity	COSY	NOESY	H HMBC	C HMBC
21	4a	C4a	153.201	C			143.636					4, 8	
22	12	C12	171.145	C			169.827					11	
23	2	C2	173.790	C			169.147						
24	3a	C3a	174.252	C			171.778					Me-9a	
25	4'	C4'	176.392	C			177.759					3'	
26	9	C9	196.252	C			189.149					Me-9a, 8	
27	13	C12	198.745	C			199.835					14	

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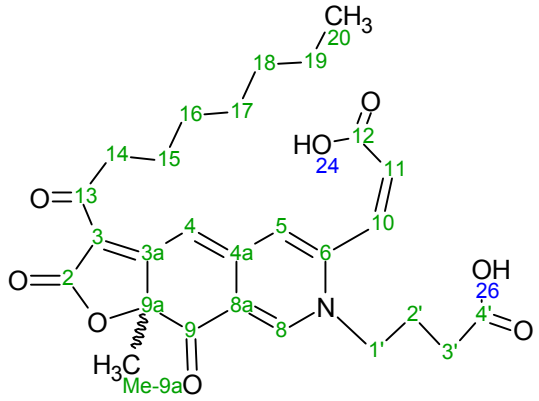
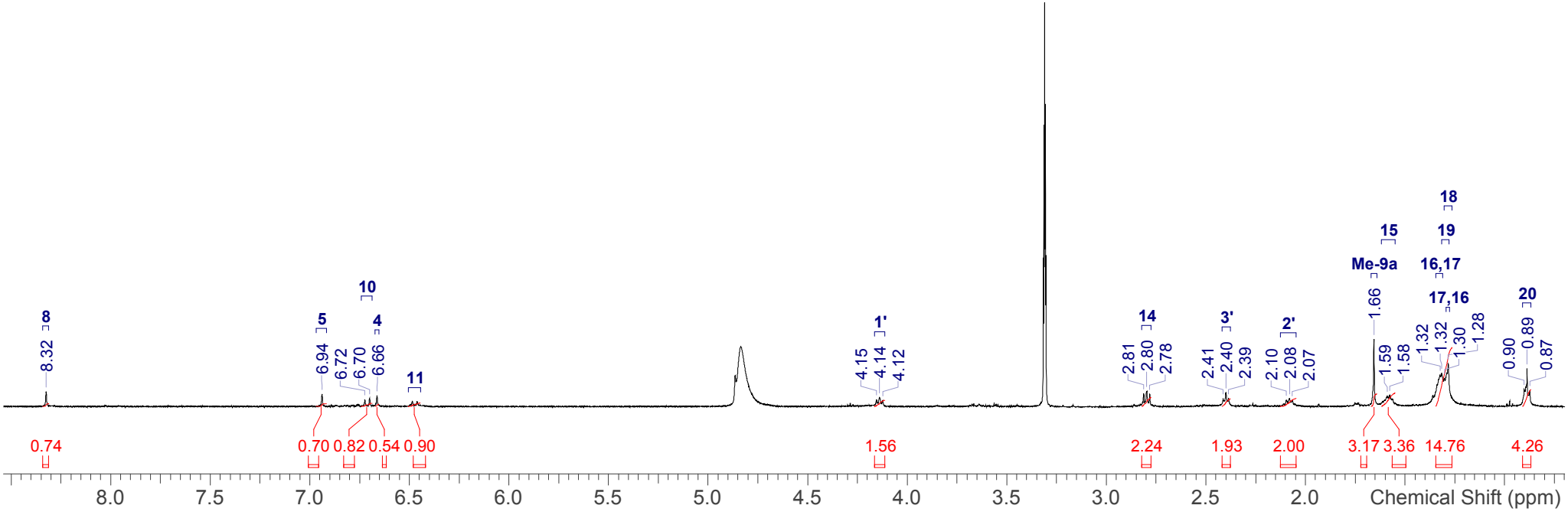
Spectrum Offset (Hz)	2369.4717	***Spectrum Type***	standard
Sweep Width (Hz)	4734.56	***Temperature (degree C)***	25.000



^{1}H NMR (500 MHz, METHANOL-d_4) d ppm 0.89 (br t, *J*=7.08 Hz, 4 H) 1.26 - 1.30 (m, 3 H) 1.28 - 1.29 (m, 2 H) 1.28 - 1.32 (m, 4 H) 1.32 (br s, 6 H) 1.58 (br d, *J*=6.07 Hz, 3 H) 1.66 (s, 3 H) 2.05 - 2.13 (m, 2 H) 2.40 (br t, *J*=6.65 Hz, 2 H) 2.78 - 2.82 (m, 2 H) 4.11 - 4.16 (m, 2 H) 6.47 (br d, *J*=12.43 Hz, 1 H) 6.66 (s, 1 H) 6.71 (d, *J*=11.85 Hz, 1 H) 6.94 (s, 1 H) 8.32 (s, 1 H)



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Spectrum Type	standard	***Sweep Width (Hz)***	31249.52
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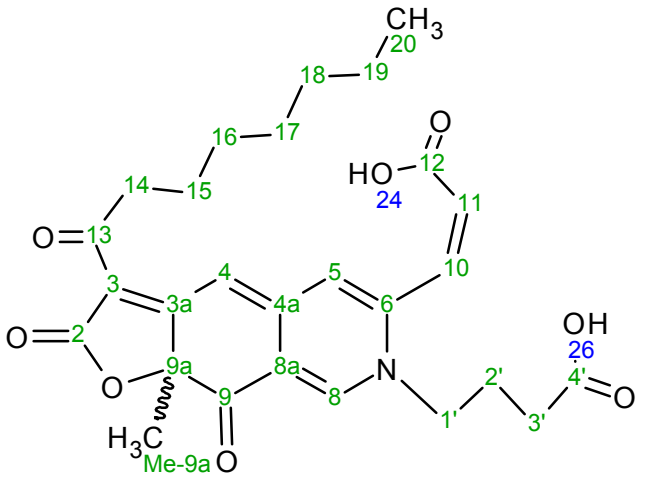

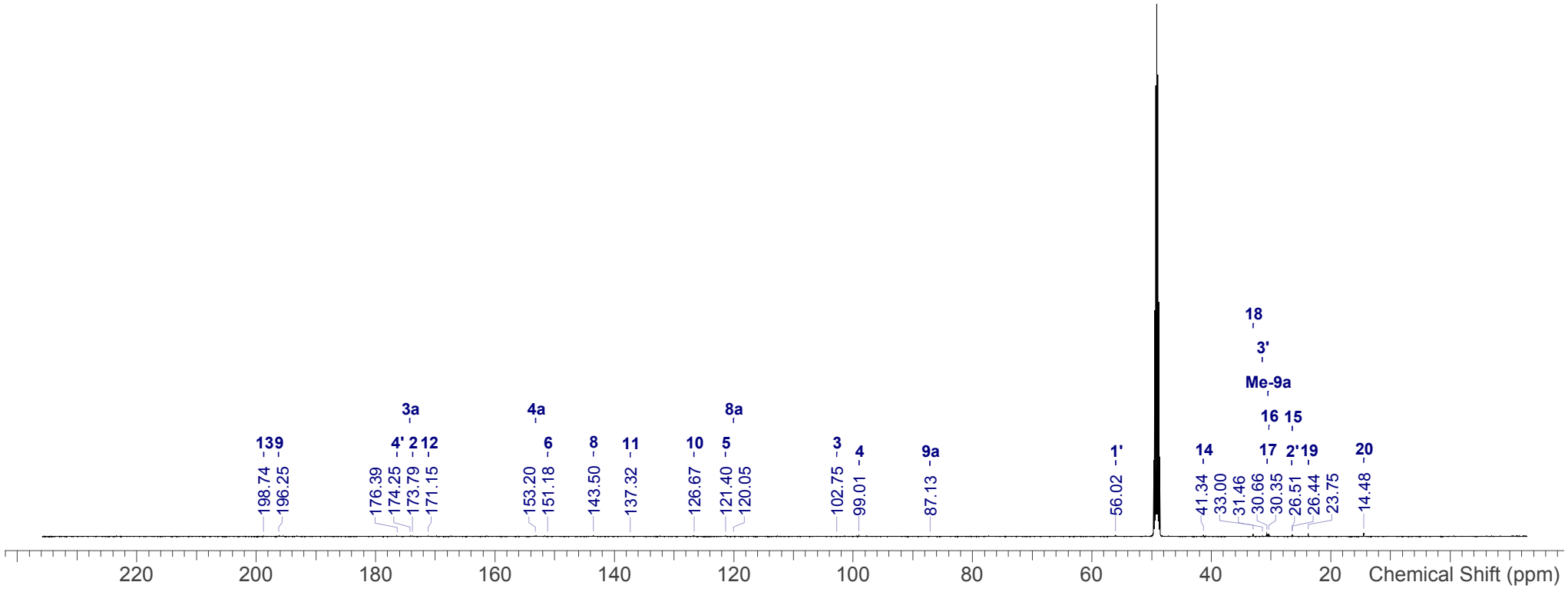

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Points Count	(2048, 1024)	***Pulse Sequence***	gHSQCAD
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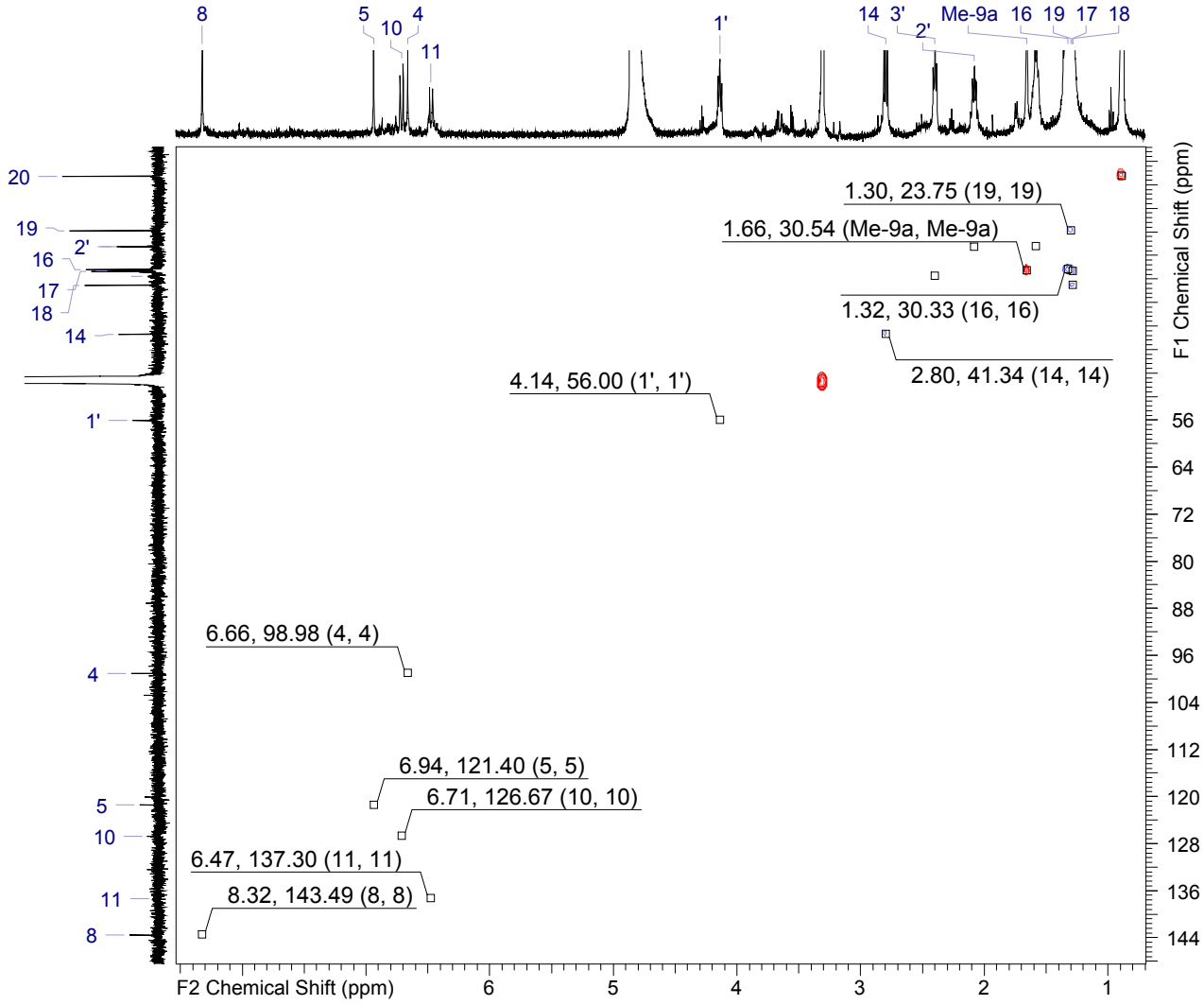
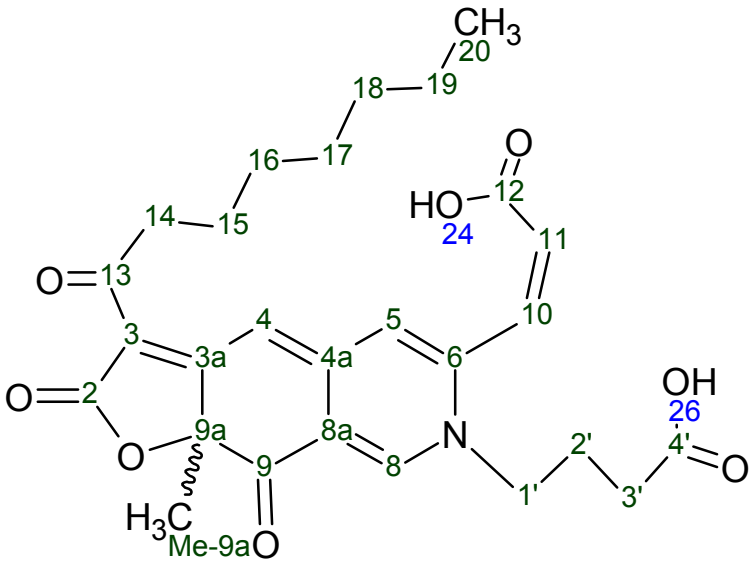
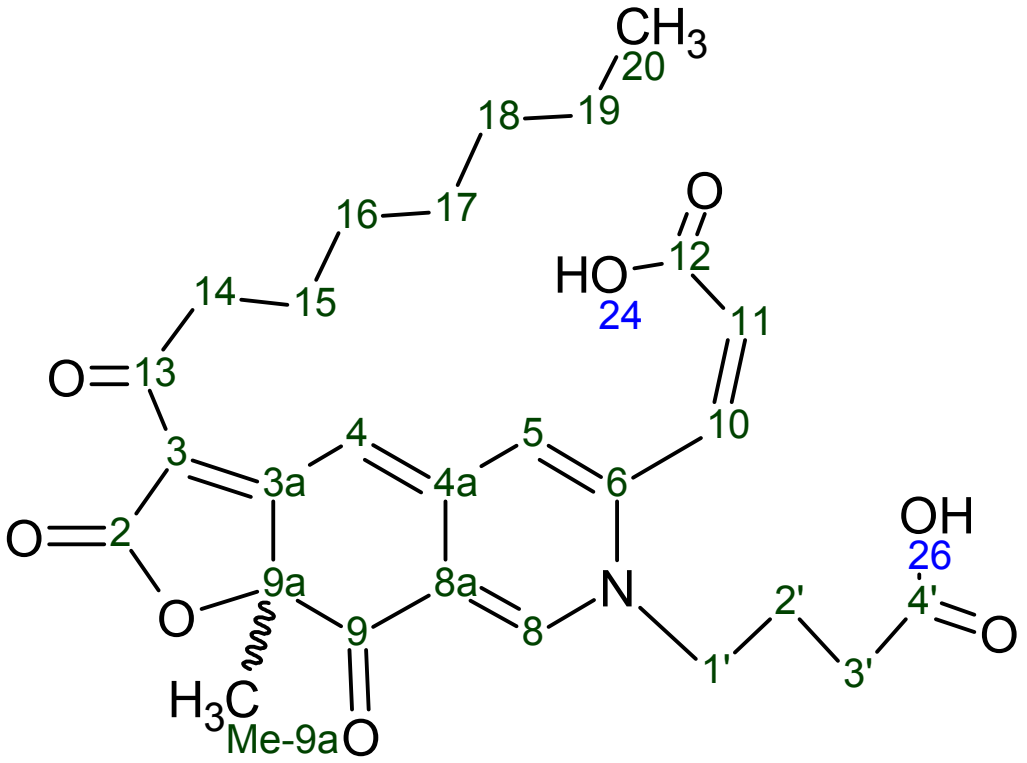
Sweep Width (Hz)	(4732.54, 17450.19)		





No.	F2 Atom	F1 Atom	F2 (ppm)	F1 (ppm)
1	Me-9a	Me-9a	1.66	30.54
2	1'	1'	4.14	56.00
3	2'	2'	2.08	26.51
4	3'	3'	2.40	31.48
5	4	4	6.66	98.98
6	5	5	6.94	121.40
7	8	8	8.32	143.49
8	10	10	6.71	126.67
9	11	11	6.47	137.30
10	14	14	2.80	41.34
11	15	15	1.58	26.44
12	16	16	1.32	30.33
13	17	17	1.29	30.65
14	18	18	1.28	32.99
15	19	19	1.30	23.75
16	20	20	0.89	14.47



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Points Count	(2048, 1024)	***Pulse Sequence***	gCOSY
Solvent	METHANOL-d4	***Spectrum Type***	COSY
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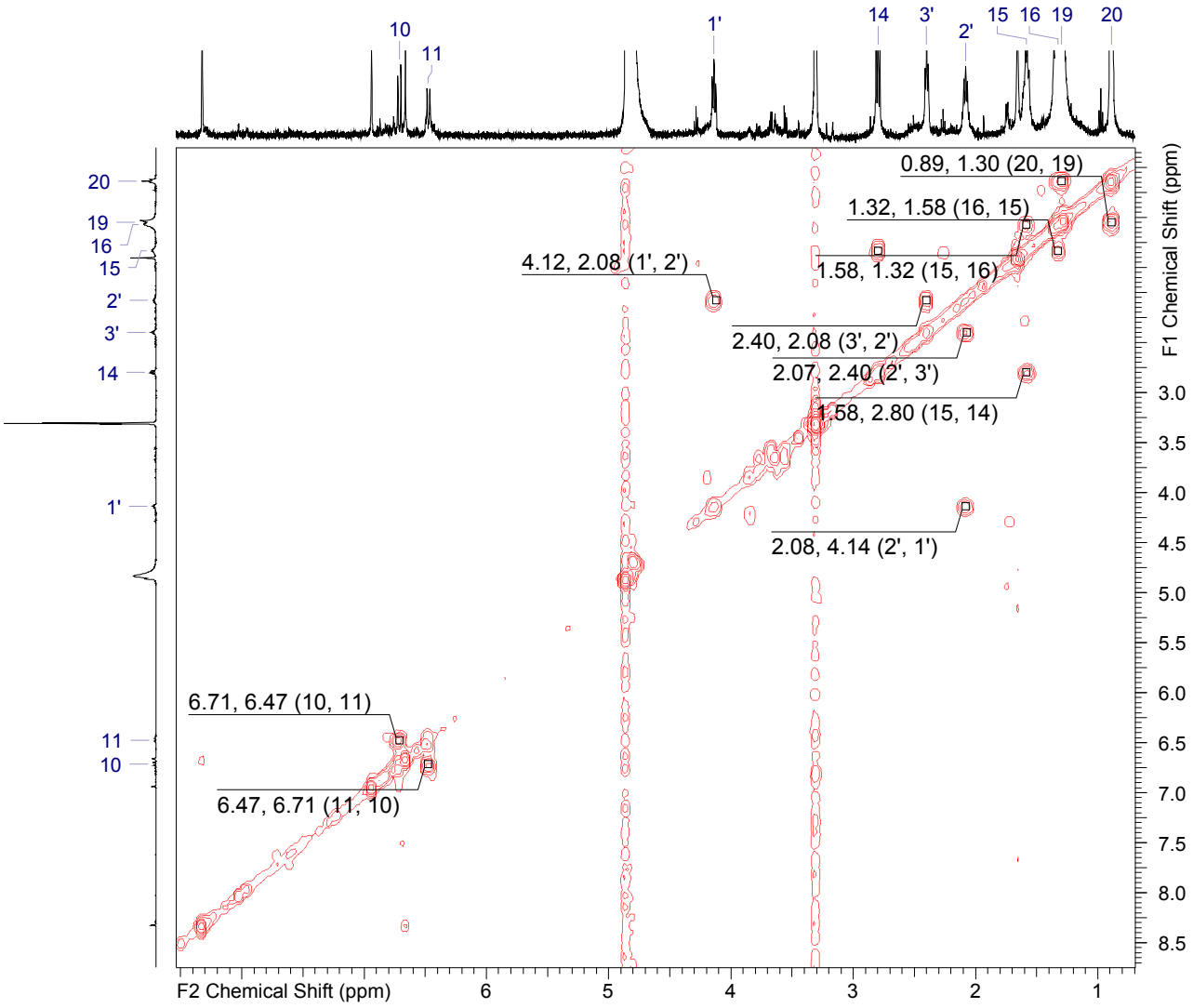
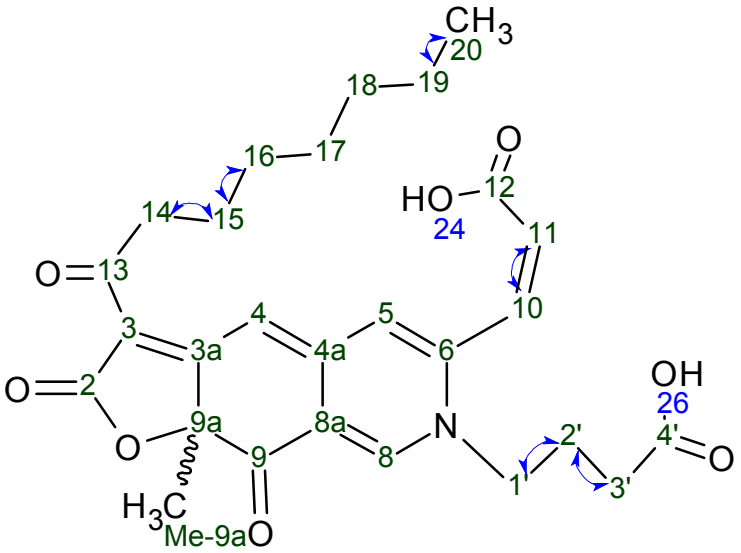
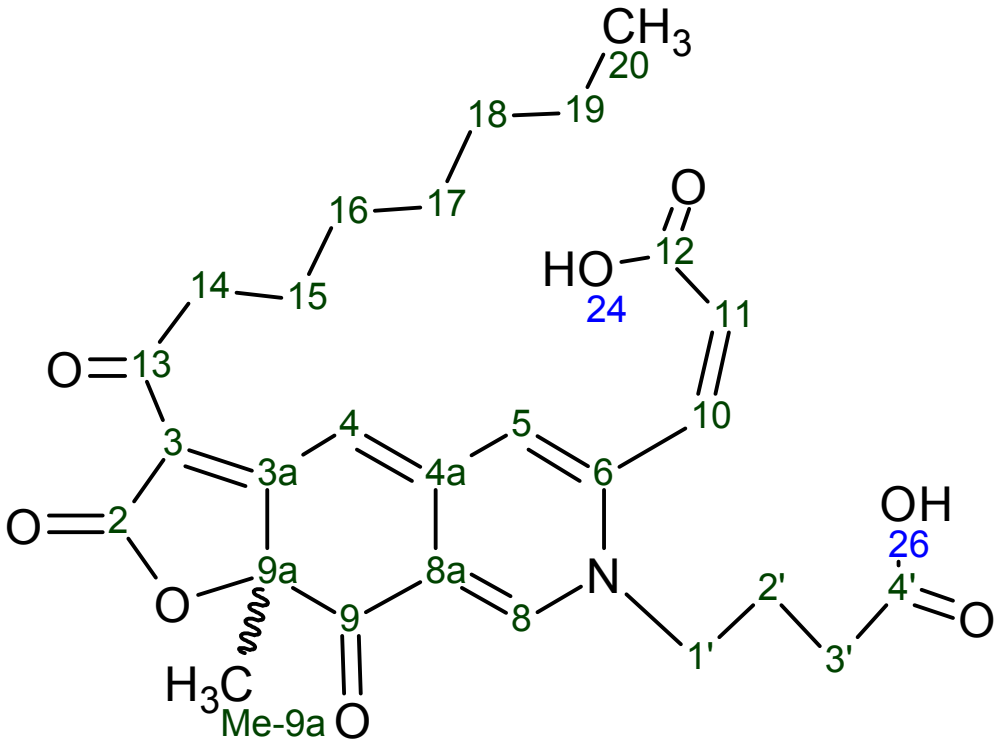




No.	F2 Atom	F1 Atom	F2 (ppm)	F1 (ppm)
1	2'	1'	2.08	4.14
2	1'	2'	4.12	2.08
3	3'	2'	2.40	2.08
4	2'	3'	2.07	2.40
5	11	10	6.47	6.71
6	10	11	6.71	6.47
7	15	14	1.58	2.80
8	14	15	2.80	1.58
9	16	15	1.32	1.58
10	15	16	1.58	1.32
11	20	19	0.89	1.30
12	19	20	1.30	0.89



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Points Count	(2048, 1024)	***Pulse Sequence***	gHMBCAD
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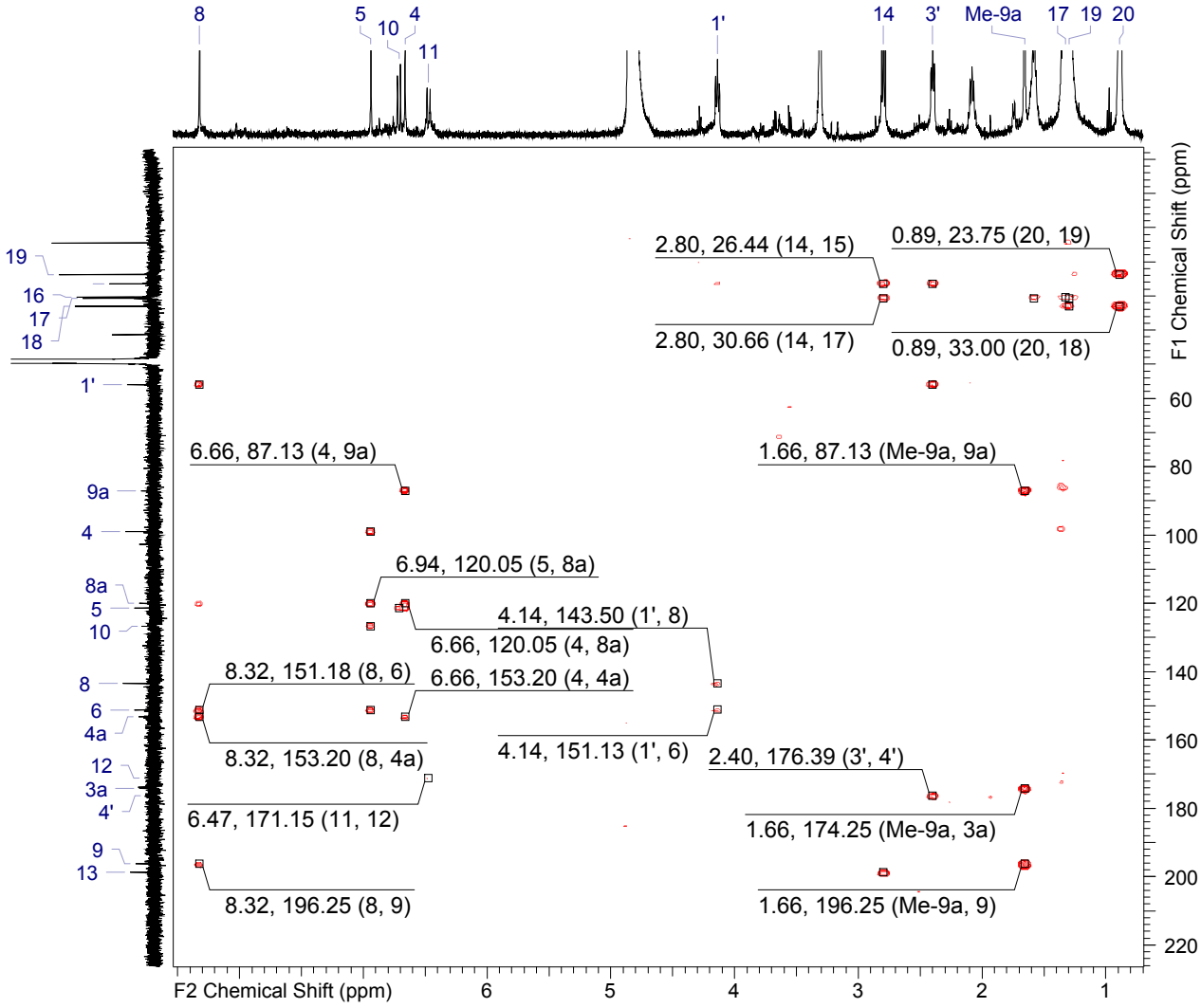
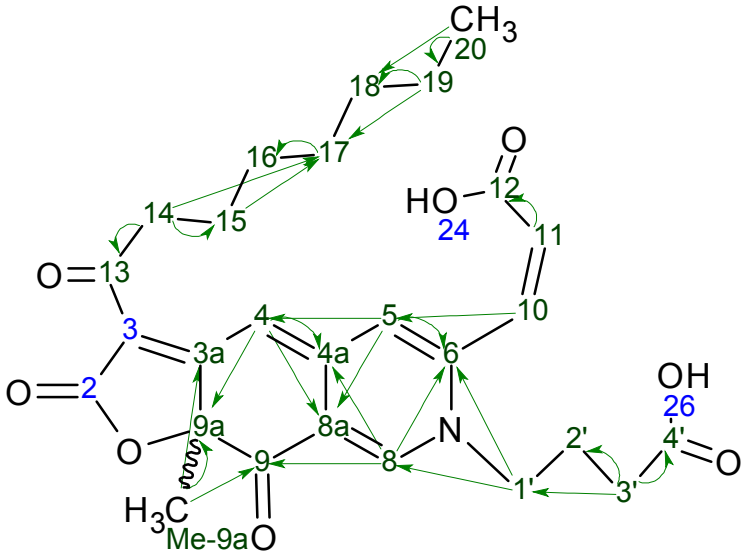
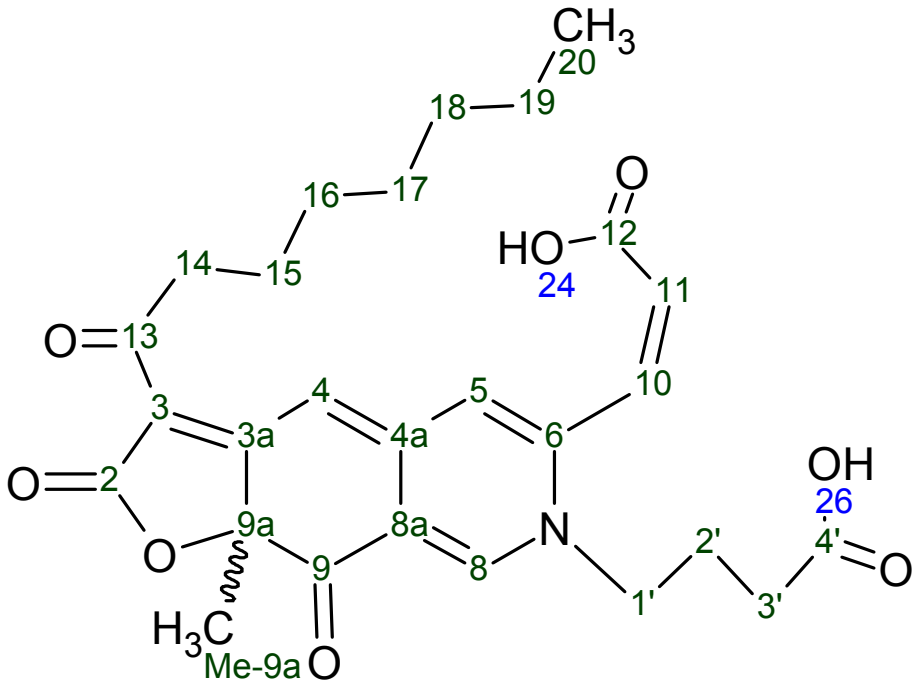
Sweep Width (Hz)	(4732.54, 30136.45)		

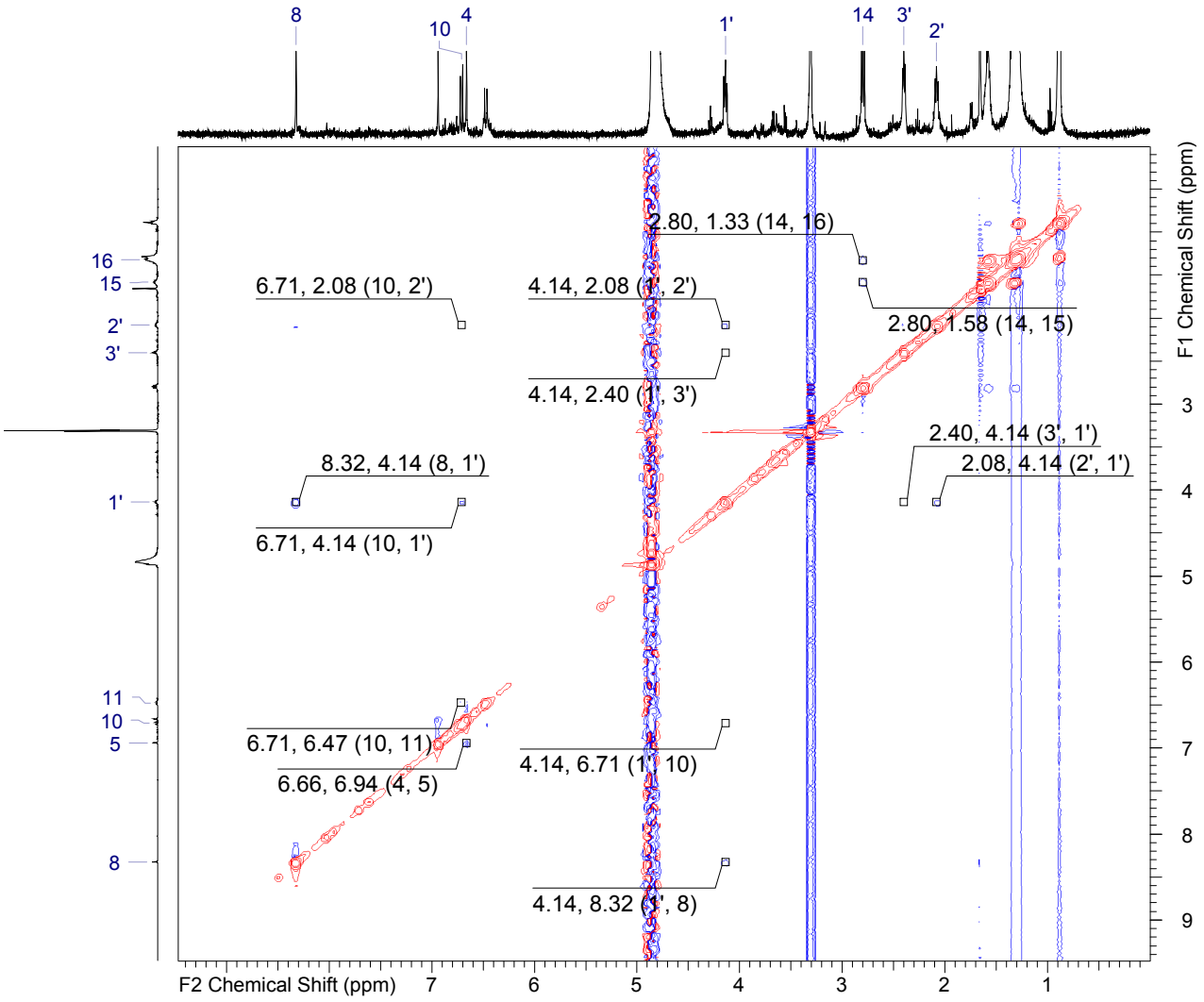


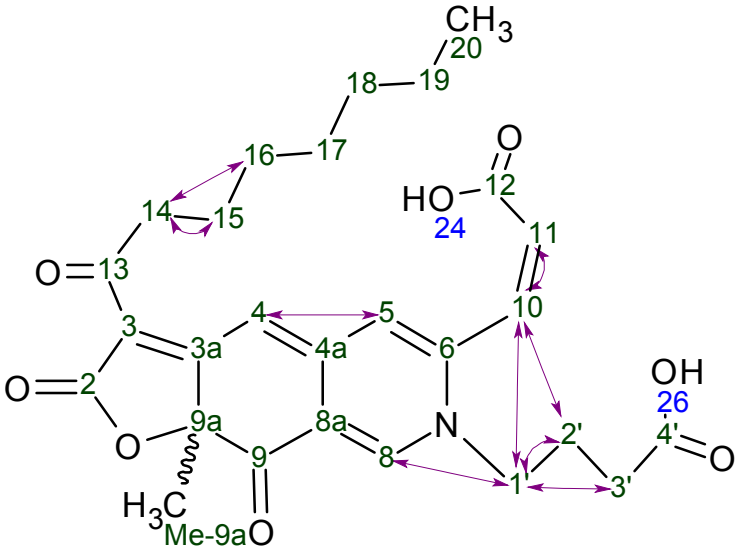


No.	F2 Atom	F1 Atom	F2 (ppm)	F1 (ppm)
1	3'	1'	2.40	56.02
2	8	1'	8.32	56.02
3	3'	2'	2.40	26.51
4	Me-9a	3a	1.66	174.25
5	5	4	6.94	99.01
6	3'	4'	2.40	176.39
7	4	4a	6.66	153.20
8	8	4a	8.32	153.20
9	10	5	6.71	121.40
10	1'	6	4.14	151.13
11	5	6	6.94	151.18
12	8	6	8.32	151.18
13	1'	8	4.14	143.50
14	4	8a	6.66	120.05
15	5	8a	6.94	120.05
16	Me-9a	9	1.66	196.25
17	8	9	8.32	196.25
18	Me-9a	9a	1.66	87.13
19	4	9a	6.66	87.13
20	5	10	6.94	126.67
21	11	12	6.47	171.15
22	14	13	2.80	198.74
23	14	15	2.80	26.44
24	17	16	1.32	30.35
25	14	17	2.80	30.66
26	15	17	1.58	30.66
27	19	17	1.30	30.66
28	19	18	1.30	33.00
29	20	18	0.89	33.00
30	20	19	0.89	23.75



Acquisition Time (sec)	(0.2999, 0.0338)		
Comment	Calibration for OneNMR on 08Jun2016 Sample : F19 S/N test sample		
Date	11 Jun 2016 11:12:54	***Date Stamp***	Jun 10 2016
File Name	C:\DATI\lavori\ALIM\DanieleGiuffrida\azaphil3_ACD\azaphilone3CD3OHnew_1Hroesy300ms.fid\fid		
Frequency (MHz)	(499.8072, 499.8072)	***Nucleus***	(1H, 1H)
Number of Transients	32	***Original Points Count***	(1420, 160)
Points Count	(2048, 1024)	***Pulse Sequence***	ROESYAD
Solvent	METHANOL-d4	***Spectrum Type***	ROESY
Sweep Width (Hz)	(4732.54, 4730.22)		





No.	F2 Atom	F1 Atom	F2 (ppm)	F1 (ppm)
1	2'	1'	2.08	4.14
2	3'	1'	2.40	4.14
3	8	1'	8.32	4.14
4	10	1'	6.71	4.14
5	1'	2'	4.14	2.08
6	10	2'	6.71	2.08
7	1'	3'	4.14	2.40
8	4	5	6.66	6.94
9	1'	8	4.14	8.32
10	1'	10	4.14	6.71
11	10	11	6.71	6.47
12	14	15	2.80	1.58
13	14	16	2.80	1.33