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Article

Chemical Constituents of the Underground Stem Bark of *Duguetia furfuracea* (Annonaceae)

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No presente trabalho foi realizado um estudo fitoquímico com os diferentes extratos obtidos a partir de órgãos subterrâneos de *Duguetia furfuracea* (Annonaceae), o qual incluiu o teste de toxicidade para *Artemia salina*. O extrato alcaloídico, obtido das cascas do caule subterrâneo, conduziu ao isolamento de (-)-duguetina β-*N*-óxido, de (-)-duguetina, dicentrinona, (-)-*N*-metiltetraidropalmatina e (+)-*N*-metilglaucina. Do extrato etanólico do cerne do caule subterrâneo obteve-se alantoína por precipitação. O óleo volátil e o extrato apolar também foram extraídos das cascas do caule subterrâneo. As substâncias 2,4,5-trimetoxiestireno, α-gurjuneno, aromadendreno, biciclogermacreno, (*E*)-metil-isoeugenol e α-asarona foram isoladas a partir do óleo volátil, e as substâncias policarpol, óxido de β-cariofileno, 2,4,5-trimetóxi-estireno, α-asarona e asaraldeído foram obtidas do extrato em éter de petróleo. Este estudo descreve pela primeira vez o alcalóide β-*N*-óxido de (-)-duguetina, e a ocorrência das substâncias (-)-*N*-metiltetraidropalmatina e (+)-*N*-metilglaucina na família Annonaceae. Todos os extratos se mostraram tóxicos nos testes com *Artemia salina*.

In the present investigation the underground parts of *Duguetia furfuracea* (Annonaceae) were used to conduct a phytochemical study that included the brine shrimp (*Artemia salina*) lethality bioassay. The substances (-)-duguetine β-*N*-oxide, (-)-duguetine, dicentrinone, (-)-*N*-methyltetrahydropalmatine, and (+)-*N*-methylglaucine were isolated from the alkaloid extract of the bark of the underground stem, and the ureide allantoin was also isolated by precipitation from the ethanol extract of the wood of the underground stem. A fresh volatile oil and a nonpolar extract were also obtained from the underground stem bark. The substances 2,4,5-trimethoxystyrene, α-gurjunene, aromadendrene, bicyclogermacrene, (*E*)-methylisoeugenol, and α-asarone were isolated from the fresh volatile oil and polycarpol, β-caryophyllene oxide, 2,4,5-trimethoxystyrene, α-asarone, and asaraldehyde were obtained from the petroleum ether extract. The present study describes for the first time the alkaloid (-)-duguetine β-*N*-oxide and the occurrence of (-)-*N*-methyltetrahydropalmatine and (+)-*N*-methylglaucine in the family Annonaceae. All extracts were active in the brine shrimp lethality bioassay.

Keywords: alkaloids, Annonaceae, aporphines, duguetine β-*N*-oxide*, Duguetia furfuracea*, sesquiterpenoids

Introduction

Nearly 80 species are known in the genus *Duguetia*, which is one of the 128 genera included in the family Annonaceae.[1]

This paper reports the results of our continued phytochemical investigations of the family Annonaceae. We analyzed various extracts obtained from the underground stem bark of *Duguetia furfuracea* (A. St.-Hil.) Benth. & Hook f., which is a shrub distributed throughout the Brazilian state of Mato Grosso do Sul. Two species of the genus *Duguetia* have been found and worked up to date, and little data on their use in folk medicine can be found in the literature.

D. furfuracea is known as "araticum-seco".[2] In folk medicine, the seed powder is mixed with water for use in the treatment of pediculosis,[3] while an infusion of the leaves and twigs is used to treat rheumatism, and a

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medicine derived from this plant is claimed to be useful in the treatment of renal colic.[4] On the other hand, this species is a major invader of pastures and therefore is harmful to the regional economy, which is based predominantly on agribusiness. Thus, the chemical knowledge of the species could contribute to control its weedy behaviour.

The chemical constituents of the aerial parts (leaves and twigs) of *D. furfuracea* are described in three previous papers.[5-7] The first paper describes the isolation of sesquiterpenoids;[5] the other two papers report the isolation of a flavonoid and various alkaloids from an isoquinoline-derived skeleton,[6] and the trypanocidal activity of some of these compounds. One of these papers[7] describes two aporphine alkaloids containing *N*-nitroso functionality.

The present investigation, which adds to the knowledge of this species, led to the isolation of alkaloids, sesquiterpenoids and other constituents from different extracts obtained from the underground stem bark of *D. furfuracea*.

An alkaloid extract was obtained from the dried and finely ground stem bark, from which (-)-duguetine β-*N*-oxide (**1**), (-)-duguetine (**2**), dicentrinone (**3**), (-)-*N*-methyltetrahydropalmatine (**4**), and (+)-*N*-methylglaucine (**5**) were isolated. Also, the ureide allantoin (**6**) was isolated from the ethanol extract of the underground stem (Figure 1).

The substances 2,4,5-trimethoxystyrene (**7**), α-gurjunene (**8**), aromadendrene (**9**), bicyclogermacrene (**10**), (*E*)-methylisoeugenol (**11**), and α-asarone (**12**) were isolated from the fresh volatile oil. The substances polycarpol (**13**), β-caryophyllene oxide (**14**), asaraldehyde (**15**), **7** and **12** were obtained from the petroleum ether extract (Figure 1).

The ^{1}H and ^{13}C NMR data of **1**, including 2D NMR data (NOESY, HMQC and HMBC) and (+) HR-ESIMS characterized this compound as an aporphine alkaloid. To the best of our knowledge, this compound has not been previously reported in the literature. For the first time, substances **6** and **12** are described in the genus *Duguetia* and substances **4** and **5,** in the family Annonaceae.

Results and Discussion

The alkaloid extract was fractionated by column chromatography (CC), yielding a novel alkaloid identified as (-)-duguetine β-*N*-oxide (**1**), and four other known alkaloids (**2**, **3**, **4**, **5**).

Compound **1** was obtained as a brown amorphous solid and exhibited $[\alpha]_D^{20}$ –33.3 (MeOH, *c* 0.001). Its molecular formula ($C_{20}H_{21}NO_6$) was determined using HR-ESIMS (*m/z* 372.1413, $[M+H]^+$). Its FTIR spectrum revealed the presence of a hydroxyl group (3418 cm^{-1}) and an aliphatic system (2959-2846 cm^{-1}). The ^{1}H NMR data showed three hydrogen signals in the aromatic region at δ 7.53 (s), 7.30 (s), and 6.47 (s) and an N-linked methyl group at δ 3.24 (s).

The presence of 1,2-methylenedioxy and 9,10-dimethoxy groups was indicated in the NMR spectrum

Table 1. NMR spectroscopic data of compound **1**

			HMBC		NOESY
C	δ_C	δ_H	$^{2}J_{CH}$	$^{3}J_{CH}$	
1	142.7	-	H-3	O-CH2-O	
1a	115.9	-		H-11	
1b	118.8	-	H-6a	H-3	
2	148.6	-	H-3	O-CH2-O	
3	106.3	6.47 (s)			2.91(H-4)
3a	122.9	-		H-5	
4	27.0	2.91 (m)		H-3	3.59(H-5)
5	66.8	3.59 (m)		$N\text{-}CH_3$	
6a	76.4	4.34 (d, 12.1)	H-7	$N\text{-}CH_3$	3.59(H-5)
7	68.3	5.08 (d, 12.1)	H-6a	H-8	
7a	130.4	-	H-8, H-7	H-11	
8	107.3	7.30 (s)			3.90(OCH_3)
9	147.9	-	H-8	H-11, OCH_3	
10	149.3	-	H-11	H-8, OCH_3	
11	109.8	7.53 (s)			3.84(OCH_3)
11a	119.5	-	H-11	H-8, H-7	
$N\text{-}CH_3$	48.8	3.24 (s)			5.08(H-7)
$O\text{-}CH_2\text{-}O$	101.3	5.93 (d, 1.1) 6.10 (d, 1.1)		H-3	
OCH_3 (9)	55.9	3.84 (s)			
OCH_3 (10)	55.8	3.90 (s)			

^{1}H and ^{13}C NMR spectra were acquired in $CDCl_3$ at 300 and 75 MHz respectively. TMS was used as internal standard, chemical shifts are shown in the δ scale with *J* values (Hz) in parentheses.

by two singlet signals at δ 3.84 (s) and 3.90 (s), and the typical pair of doublets of methylenedioxy protons was observed at δ 6.10 and 5.93 (d, 1.1 Hz). Also, were observed aliphatic protons signals at δ 3.59 (m) and 2.91 (m). These ^{1}H NMR data (Table 1) are in agreement with an aporphine alkaloid having 1,2-methylenedioxy and 7-hydroxy groups with *trans* configuration at 6a,7, exhibiting a large coupling constant for the two doublets at δ 5.08 (d, 12.1 Hz) and 4.34 (d, 12.1 Hz).

The ^{13}C NMR data were also in agreement with the observations (Table 1), but the chemical shift of carbon atoms in the neighborhood of the N-heterocycle suggests the presence of an N-O group, as the N-heterocycle region showed a more pronounced deshielding effect than in duguetine (**2**) (Figure 2).

This effect is in accordance with the molecular formula established by HR-MS, indicating the presence of an additional oxygen atom.[8] By comparing the ^{13}C NMR data of **1** with those of **2**, using N-O models available in the literature, namely oliveroline β-*N*-oxide (6a,7α-H, β-*N*-oxide)[9] and dasymaschaline α-*N*-oxide (6a,7α-H, α-*N*-oxide),[8] and confirming with NOESY the stereochemistry of the *N*-oxide moiety of **1**, it was possible to propose that **1** is a (-)-duguetine β-N-oxide (Table 1).

Allantoin (**6**) was isolated from the ethanol extract by precipitation with acetone and its spectral data were agree with those in the literature.[10] This metabolite

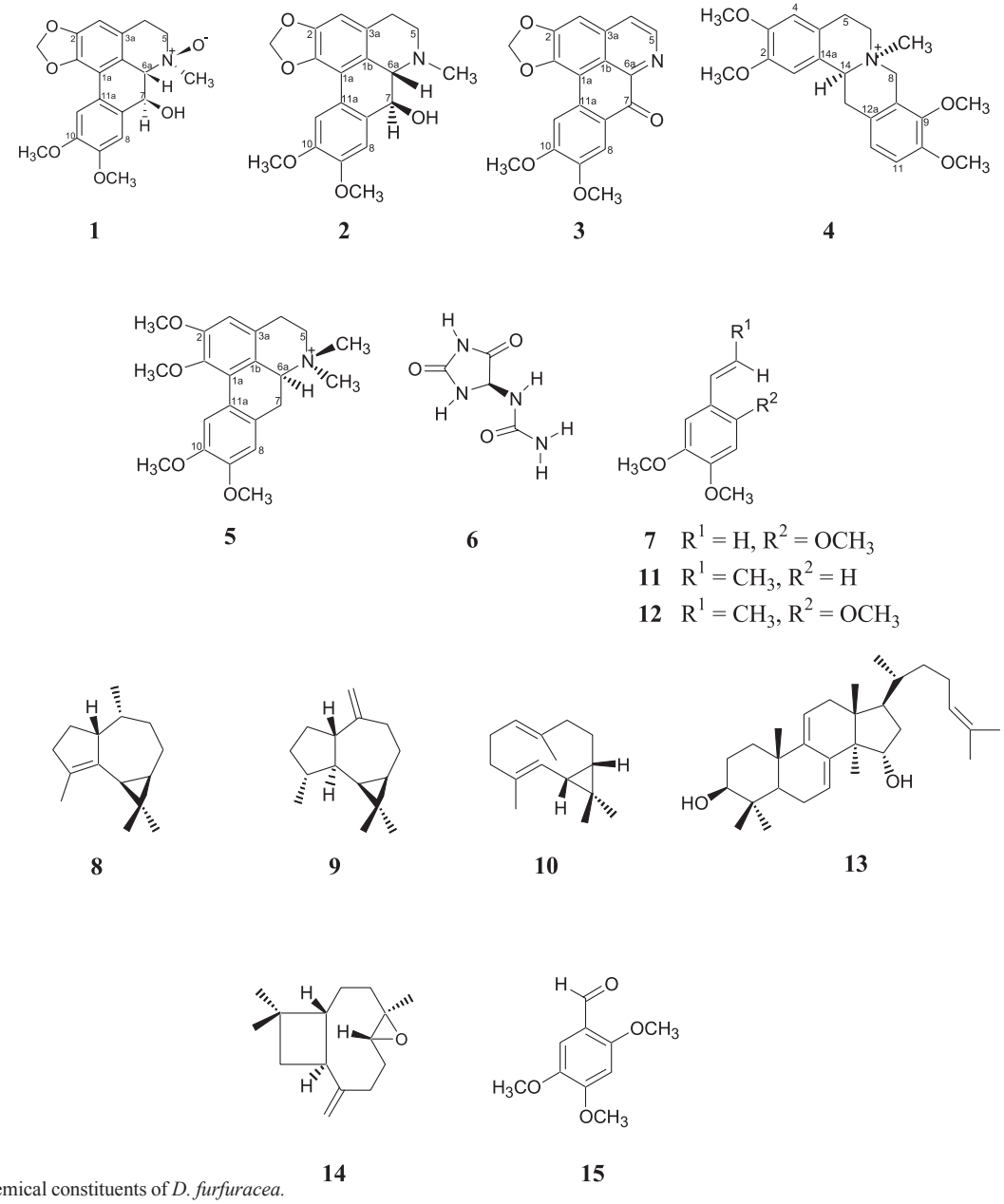


Figure 1. Chemical constituents of *D. furfuracea*.

belongs to the ureide class and plays an important role in the transport and assimilation in some nitrogen-fixing species.[11] Allantoin, which is also used in cosmetics,[12] exhibits anti-inflammatory activity among other properties. This compound has already been obtained in large amounts from the underground parts of other pasture-invading species.[10]

The fresh volatile oil was fractionated by CC on silica gel, yielding several fractions. The fractions, composed of a mixture of two compounds, were subjected to an additional preparative thin layer chromatography (TLC) followed by preparative argentation TLC separation, and six compounds (**7-12**) were isolated and identified by comparison of their spectral data with literature values (see Experimental section). In order to contribute to the chemical knowledge of nonpolar compounds from the fresh stem bark, the fresh volatile oil was subjected to GC/MS analysis. The compounds identified by this method are listed in Table 2.

Table 2. Chemical constitution of the fresh volatile oil of the underground stem bark of *Duguetia furfuracea*

Compounds	Rt	%
α-gurjunene*	31.1	2.1
trans-caryophyllene	31.4	3.2
aromadendrene*	31.9	2.2
α-humulene	33.4	0.8
7,9(11)-drimadiene	34.1	1.5
γ-gurjunene	34.3	0.9
bicyclogermacrene*	35.3	8.6
γ-cadinene	36.3	2.8
2,4,5-trimethoxystyrene*,**	37.7	29.2
palustrol	38.0	1.1
spathulenol	38.8	4.7
caryophyllene oxide**	39.0	2.2
viridiflorol	39.4	1.4
epi-globulol	39.9	6.4
1-*epi*-cubenol	40.7	0.7
epi-α-cadinol	41.2	2.7
valerianol	41.7	1.0
α-asarone*,**	42.4	23.8
asaraldehyde**	43.8	1.6
Not identified	47.3	3.1

Some of the compounds in Table 2 were also identified by conventional phytochemical work up of the fresh volatile oil (*) or petroleum ether extract (**) or both.

In a further chemical investigation of the fresh stem bark, the petroleum ether extract yielded, by the usual phytochemical work up, **7**, **12**, **13**, **14** and **15** (Figure 1).

Volatile and nonpolar compounds have been described in Annonaceae.[13] Essential oils, for instance, are responsible for the fragrance of several species and their chemical composition generally includes well-known monoterpenes, sesquiterpenes, or aromatic compounds.[14]

A noteworthy feature is the fact that the isolation of aromatic compounds such as propenylbenzenes (or vinylbenzenes) has been described predominantly in the underground parts.[14,15] Phenylpropanoid-derived compounds are widespread among vascular plants and play an important role in chemical defense.[16]

With regard to polycarpol (**13**), it has been described as a chemical marker of the family Annonaceae, however it has recently been found in another plant family.[17]

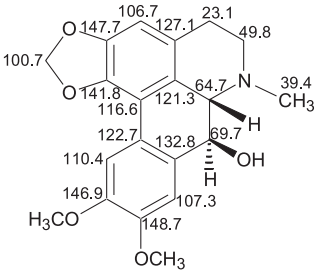
The fresh volatile oil and the petroleum ether and alkaloid extracts were active in the brine shrimp lethality bioassay, with LD_{50} values of 2.6, 6.1, and 36.9 μg mL^{-1}, respectively.

The toxicity exhibited by the extracts and fresh volatile oil might be explained by the presence of active substances previously tested in the brine shrimp lethality bioassay, namely 2,4,5-trimethoxystyrene (**7**) (LD_{50} = 8 μg mL^{-1}),[18] found in both the fresh volatile oil and petroleum ether extract, polycarpol (**13**) (LD_{50} = 254 μg mL^{-1}),[19] in the petroleum ether extract, and several oxoaporphine alkaloids.[20]

Experimental

General experimental procedures

Optical rotations were measured on a Perkin-Elmer 341 polarimeter in MeOH. The FTIR spectra of either KBr pellets or $CHCl_3$ films were obtained on a Perkin Elmer 783 spectrophotometer. The MS system (HR-ESIMS) used



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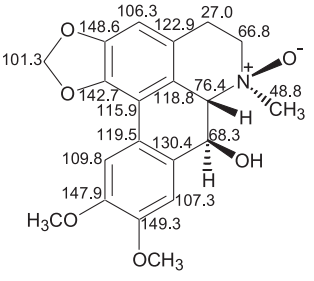

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Figure 2. Comparison between ^{13}C (75 MHz, $CDCl_3$) spectral data of **1** and **2.**

was a quadrupole time-of-flight instrument (UltrOTOF-Q, Bruker Daltonics, Billerica, MA), equipped with an ESI source; the analyses were performed with the mass spectrometer in the positive ion mode. The GC/MS data were obtained on a CG17A/QP5000 Shimadzu GC/MS instrument equipped with a DB-5 capillary column using He as the carrier gas. The ^{1}H and ^{13}C 1D and 2D NMR spectra were recorded at 300 MHz (^{1}H) and 75 MHz (^{13}C) on a Bruker DPX-300 spectrometer.

Plant material

The underground parts (stem bark and wood) of *Duguetia furfuracea* (A. St.-Hil.) Benth. & Hook f. were collected in March 2004, on the UFMS campus in Campo Grande, MS, Brazil, and identified by Prof. R. Mello-Silva. A voucher specimen (No. 023) was deposited in the CGMS Herbarium (UFMS, Campo Grande, MS).

Extraction and isolation of compounds

Ammonium hydroxide was added to 1300 g of air-dried and finely ground bark of the underground stem until pH 9.0 was reached and then subjected to exhaustive extraction with $CHCl_3$. The resulting chloroform layer was concentrated under reduced pressure and a brown residue (68 g) was obtained.

The residue was solubilized in $CHCl_3$ and extracted with 5% HCl; the resulting acid fraction was adjusted to pH 9.0 (NH_4OH) and again exhaustively extracted with $CHCl_3$.

A brown precipitate was obtained (9.2 g). This extract (7.2 g) was fractionated by CC on alumina (70-230 mesh) and eluted with $CHCl_3$:MeOH:H_2O gradient system to yield thirteen fractions (I-XIII).

Fractions IV, X and XI from this column provided **2** (812.1 mg), **4** (69.3 mg) and **5** (11.2 mg). Fraction V (800 mg) was fractionated by CC on silica gel (70-230 mesh) in $CHCl_3$:MeOH:H_2O. Fractions 60-75 yielded **3** (4.6 mg). Fraction VII (378.9 mg) yielded **1** after CC on silica gel (70-230 mesh) eluted with $CHCl_3$:EtOAc:MeOH:H_2O gradient system.

From the ethanol extract of the underground stem, the ureide allantoin (**6**) was isolated by precipitation with acetone.

The alkaloids **2**, **3**, **4** and **5**, as well as the compounds **6-12** were identified by ^{1}H and ^{13}C NMR spectroscopy and compared with literature values.[10,18-31]

*(-)-Duguetine β-N-oxide, (**1**)*

Brown amorphous solid; Rf 0.51 (CHCl3:MeOH, 85.15); $[\alpha]_D^{20}$: –33.3° (MeOH; *c* 0.001,); FTIR (KBr) ν_{max}/cm^{-1}: 3418, 2959, 2929, 2846, 1607, 1515, 1463, 1393, 1340, 1244, 1226, 1217, 1118, 1046, 993, 945, 870; (+) HR-ESIMS *m/z*: 372.1413 $[M+H]^+$ (Calc. for $C_{20}H_{22}NO_6$ requires 372.1436). ^{1}H NMR (300 MHz, $CDCl_3$, δ) and ^{13}C NMR (75 MHz, $CDCl_3$, δ): see Table 1.

*(-)-N-Methyltetrahydropalmatine, (**4**)*

Brown amorphous solid; $[\alpha]_D^{20}$: –104.1° (MeOH; *c* 0.0012). FTIR, ^{1}H and ^{13}C NMR data are in agreement with those reported in the literature.[30]

*(+)-N-Methylglaucine, (**5**)*

Brown amorphous solid; $[\alpha]_D^{20}$: +34.9° (MeOH; *c* 0.0012). FTIR, ^{1}H and ^{13}C NMR data are in agreement with those reported in the literature.[31] A 4-hour hydrodistillation of 300 g of fresh bark of underground stem, using a Clevenger-type apparatus, yielded 1.2% fresh volatile oil.

The oil was fractionated by CC on silica gel (70-230 mesh), and eluted with petroleum ether:$CHCl_3$:MeOH gradient system to yield thirteen combined fractions (I-XIII). Compound **7** (117.1 mg) was identified as the major component of fraction XII, and compound **12** (160.2 mg), of fraction XIII. Fractions II and XI from this column provided **8** (19.5 mg) and **11** (10.1 mg), respectively. Fractions III and VI were purified by preparative/argentation TLC yielding **9** (9.1 mg) and **10** (17.2 mg), respectively.

A separate amount of air-dried and finely ground bark of the underground stem (155 g) was subjected to exhaustive petroleum ether extraction in a Soxhlet apparatus; the resulting extract was concentrated *in vacuo* to dryness (13 g). The precipitate from this extract was purified by recrystallization from hexane and $CHCl_3$ providing **13** (176.4 mg). The resulting extract (5 g) was then fractionated by CC on silica gel (70-230 mesh) using hexane:$CHCl_3$:MeOH as elution gradient. The fractions showing similar spots on TLC were combined into twelve fractions (I-XII). Fractions I and IX provided **14** (11.8 mg) and **15** (12.3 mg), respectively. Fraction X (235.2 mg) yielded a mixture of **7** and **12**.

Brine shrimp lethality test

The brine shrimp (*A. salina* Leach) toxicity tests were conducted using second instar larvae according to the method of McLaughlin. Stock solutions of samples were prepared by dissolving 15 mg of the test material in 5 mL of sea water containing 1% (v/v) DMSO. The assays were carried out in triplicate on samples at a concentration of 500, 50, 5.0 and 0.5 μg mL^{-1}. Positive (Quinidine Sulfate) and negative (sea water containing 0.1% (v/v) DMSO)

controls were included in each bioassay in order to verify the susceptibility of the brine shrimps. LD_{50} values were determined from 24 h counts, by Probit Analysis.[32]

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Chemical Constituents of the Underground Stem Bark of *Duguetia furfuracea* (Annonaceae)

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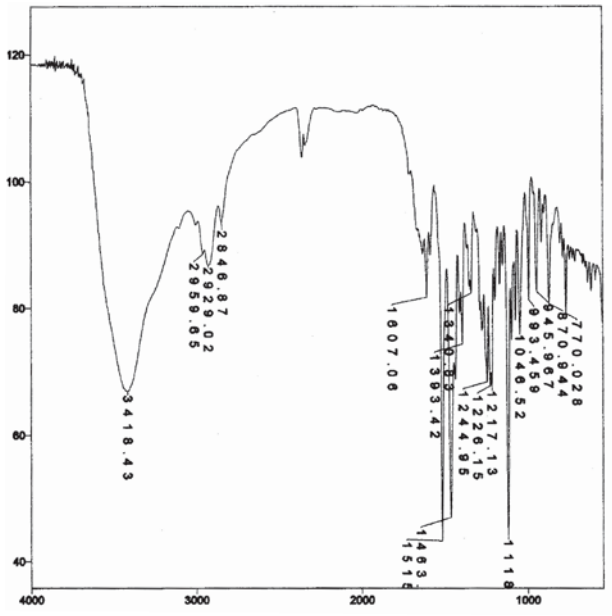

Figure S1. FTIR spectrum of compound **1.**

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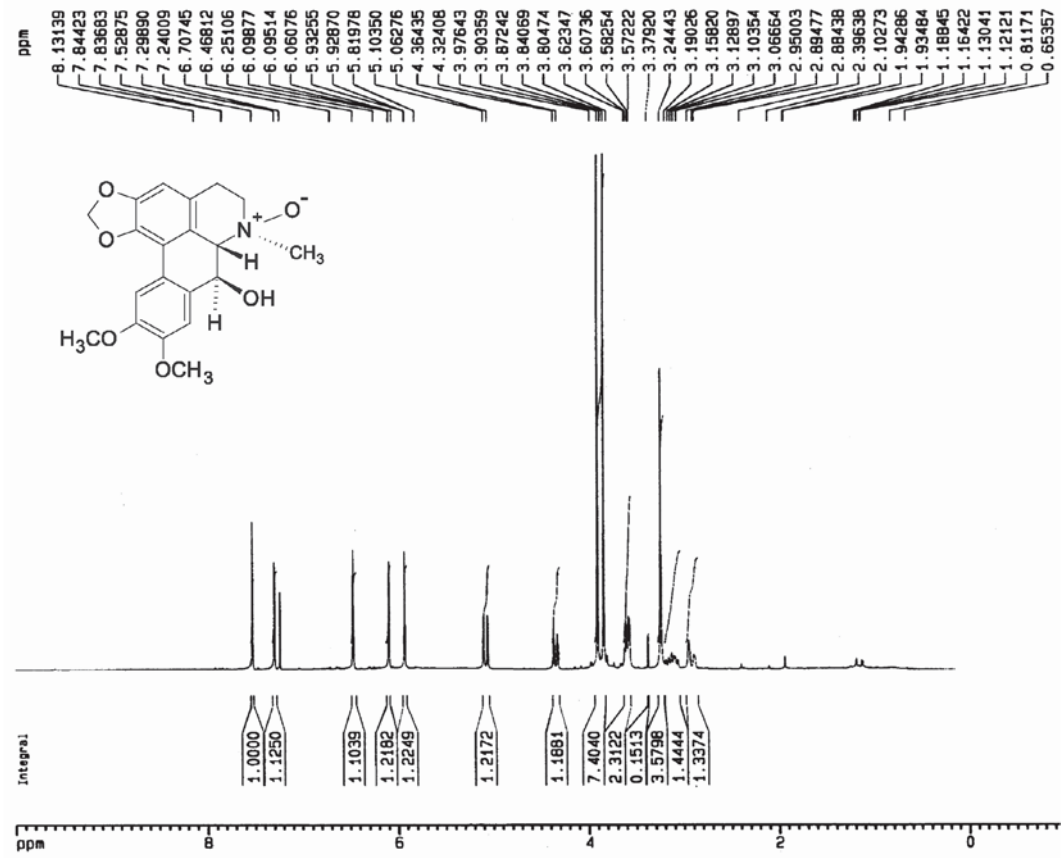

Figure S2. ^{1}H NMR spectrum of compound **1** (300 MHz, $CDCl_3$).

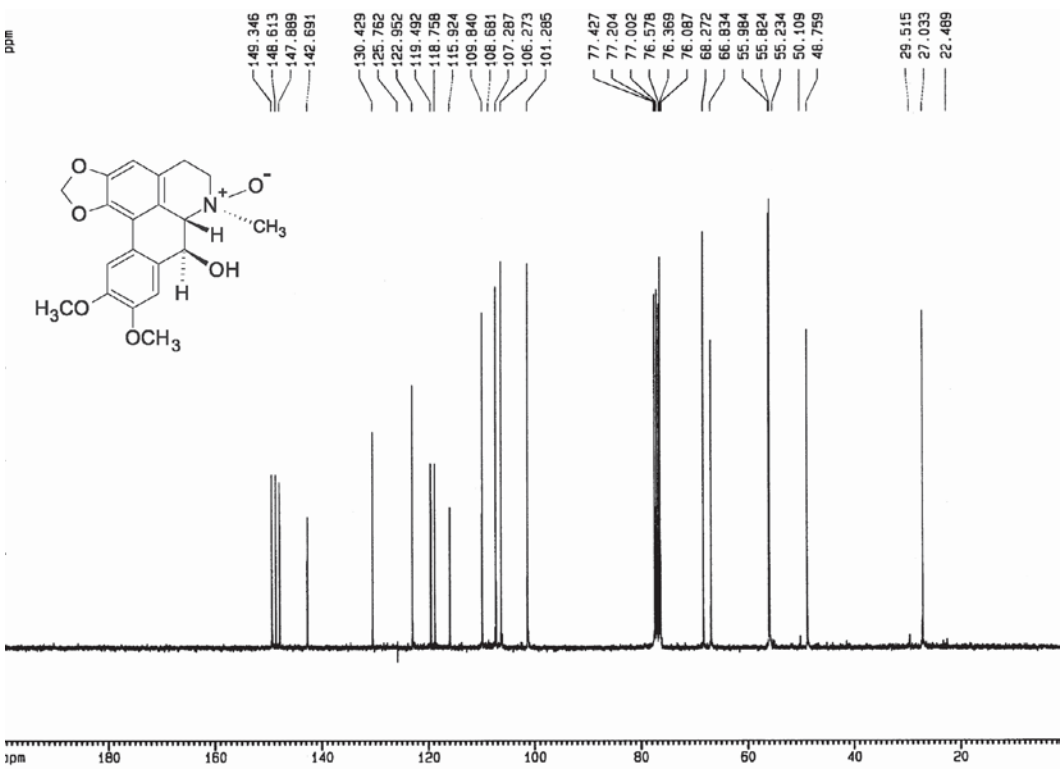

Figure S3. ^{13}C NMR spectrum of compound **1** (75 MHz, $CDCl_3$).

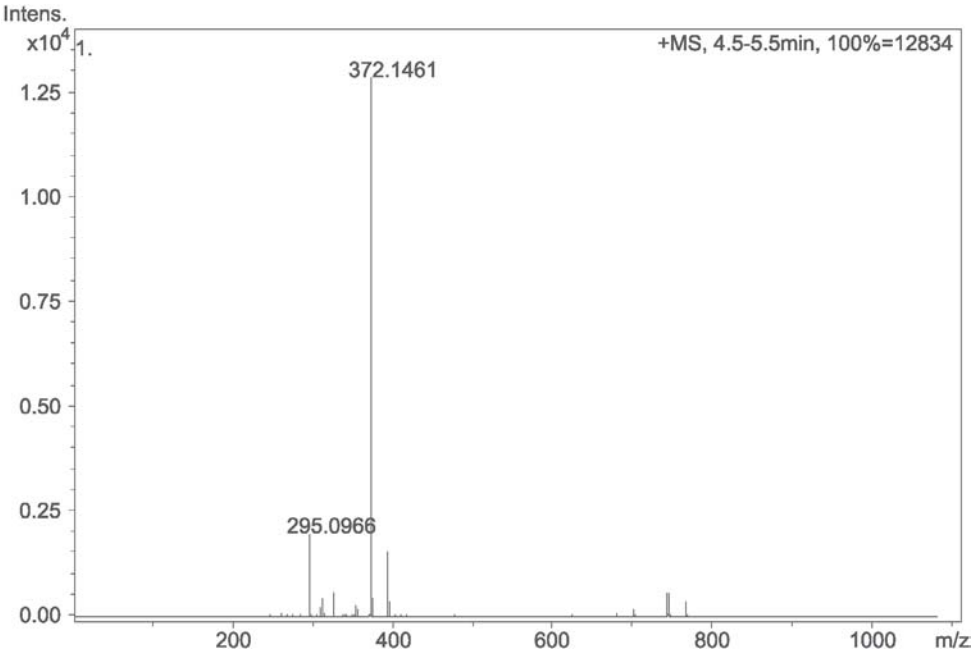

Figure S4. HR-ESIMS spectrum of compound **1** (positive mode).

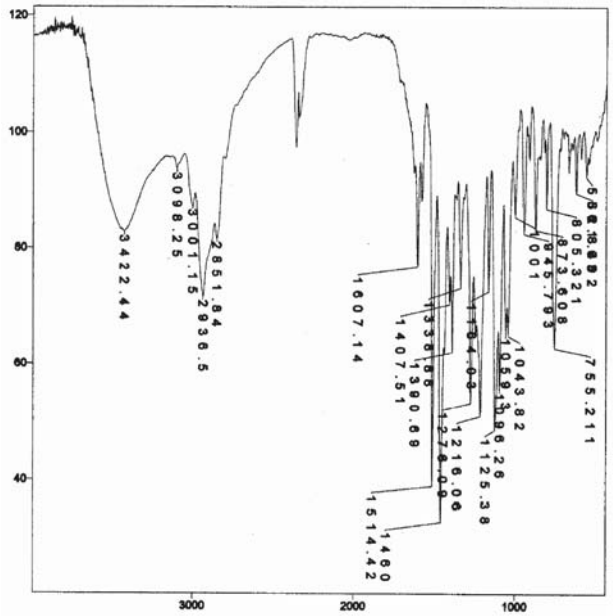

Figure S5. FTIR spectrum of compound **2.**

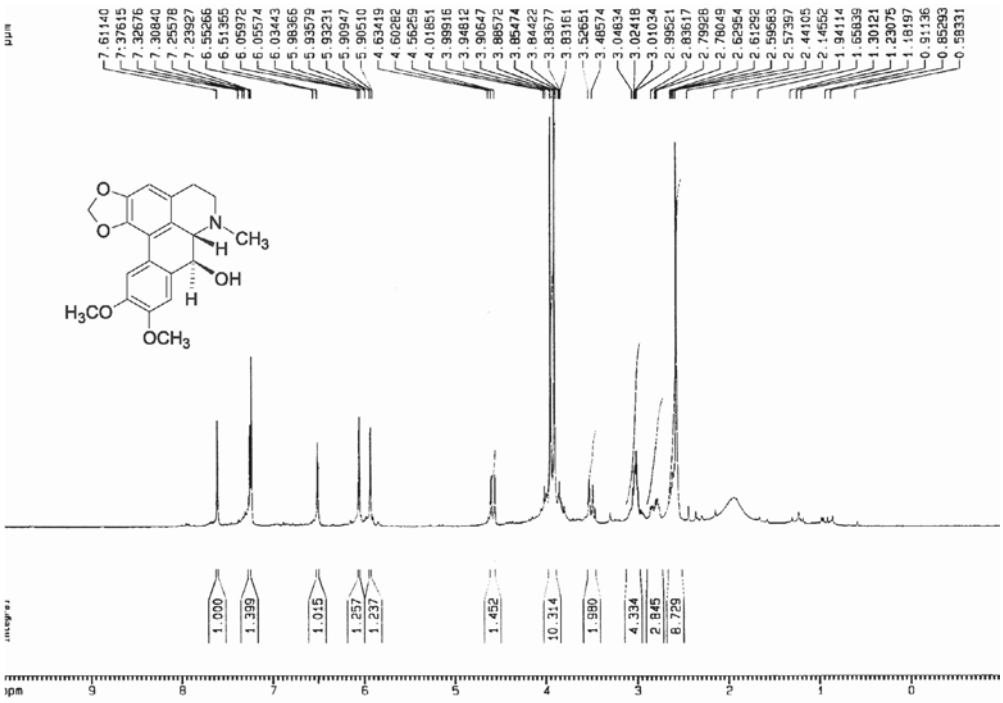

Figure S6. ^{1}H NMR spectrum of compound **2** (300 MHz, $CDCl_3$).

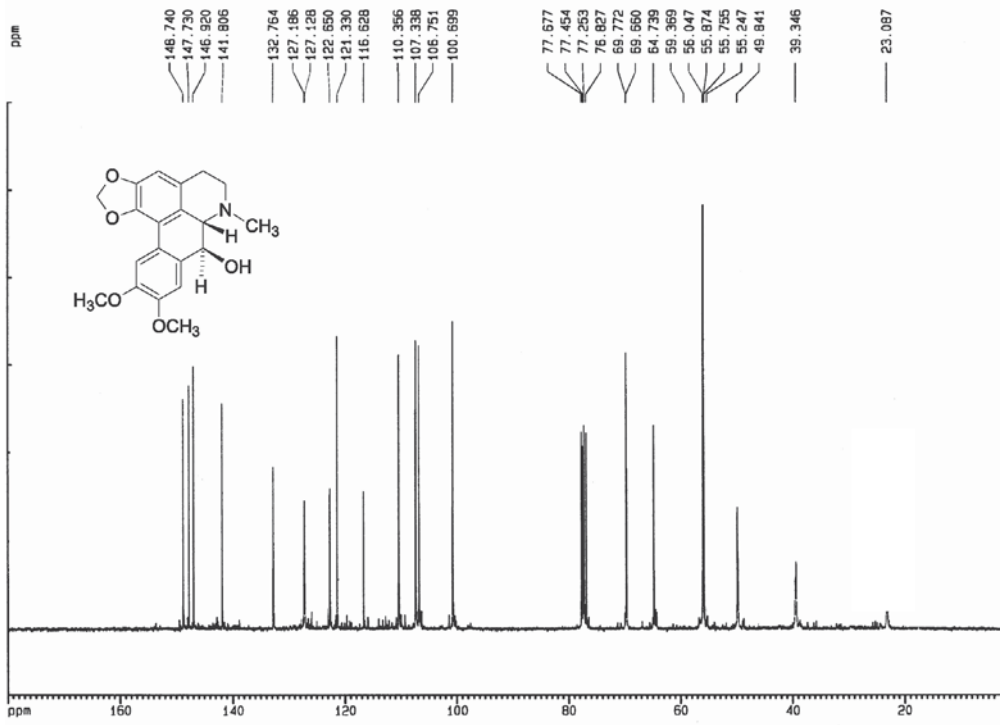

Figure S7. ^{13}C NMR spectrum of compound **2** (75 MHz, $CDCl_3$).

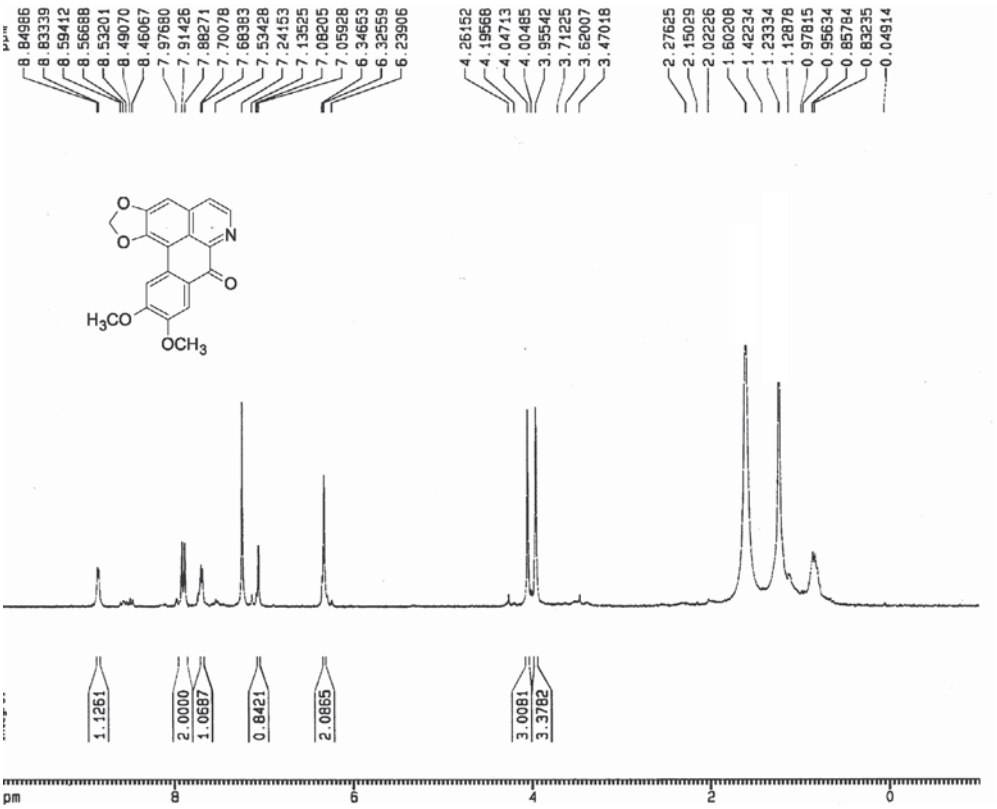

Figure S8. ^{1}H NMR spectrum of compound **3** (300 MHz, $CDCl_3$).

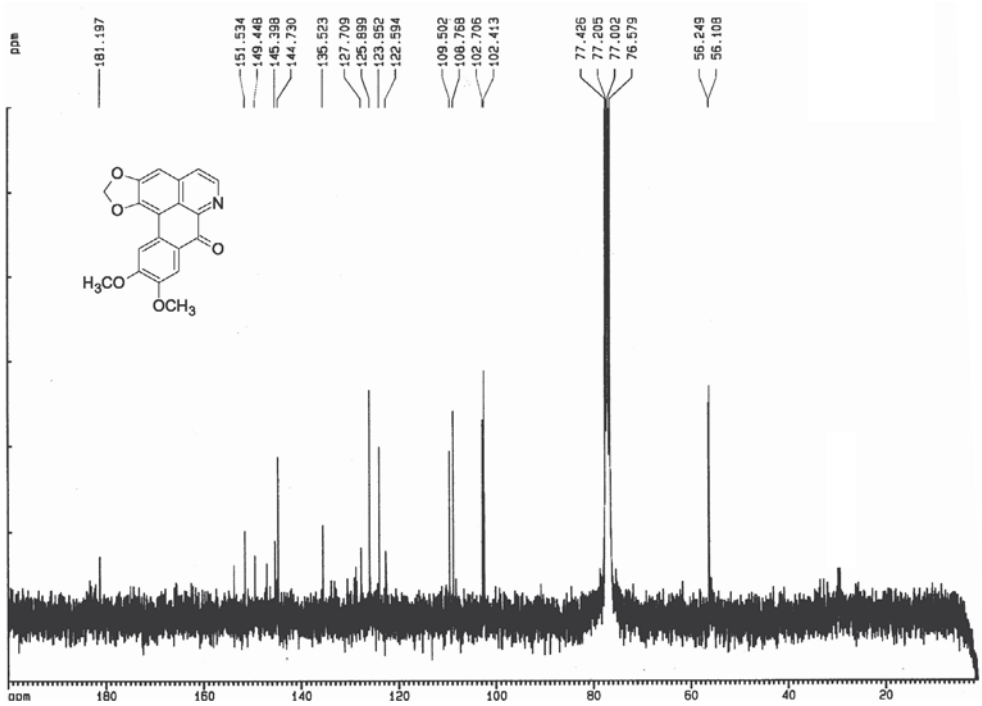

Figure S9. ^{13}C NMR spectrum of compound **3** (75 MHz, $CDCl_3$).

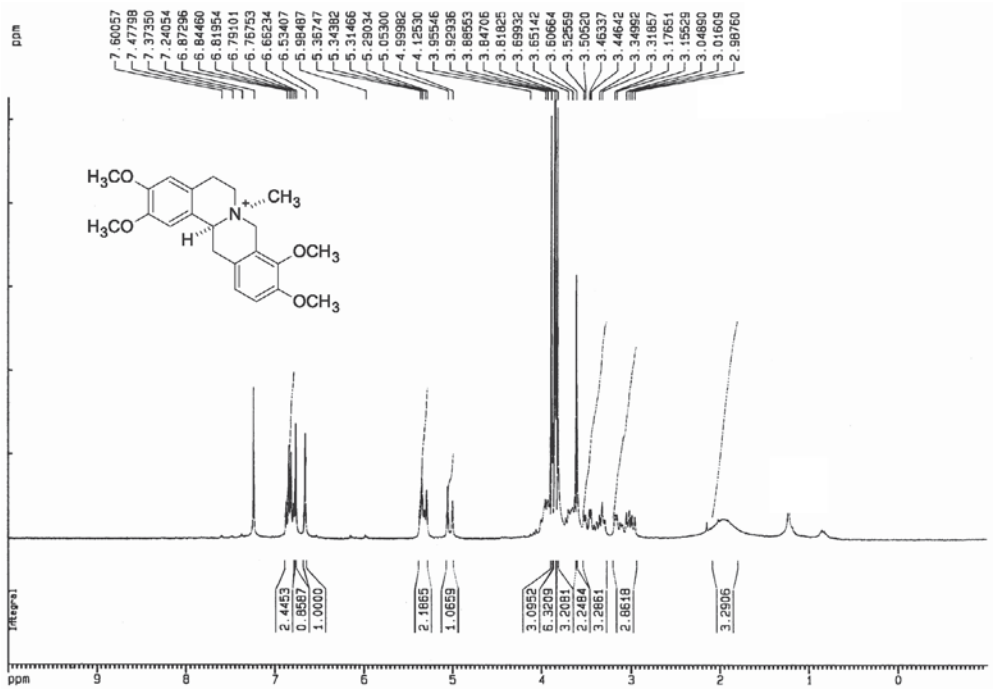

Figure S10. ^{1}H NMR spectrum of compound **4** (300 MHz, $CDCl_3$).

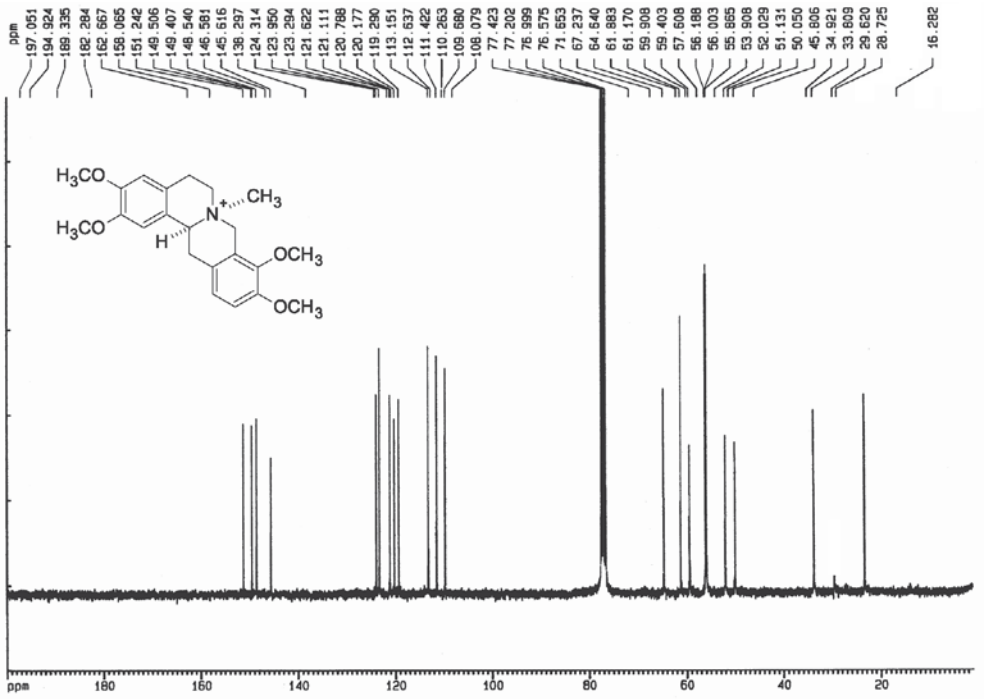

Figure S11. ^{13}C NMR spectrum of compound **4** (75 MHz, $CDCl_3$).

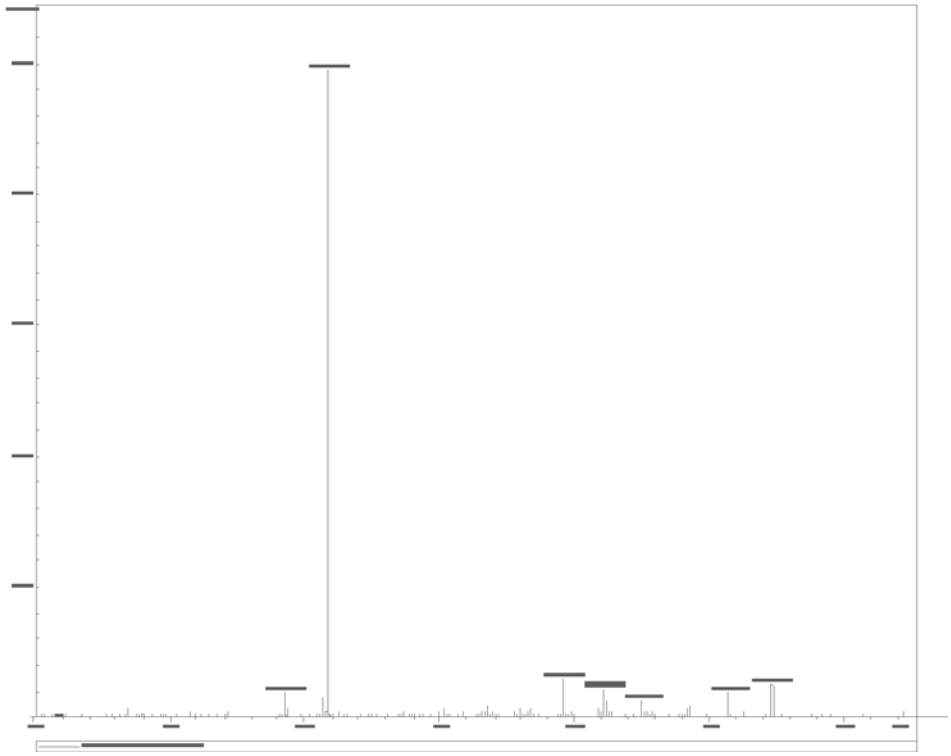

Figure S12. HR-ESIMS/MS spectrum of compound **4** (positive mode).

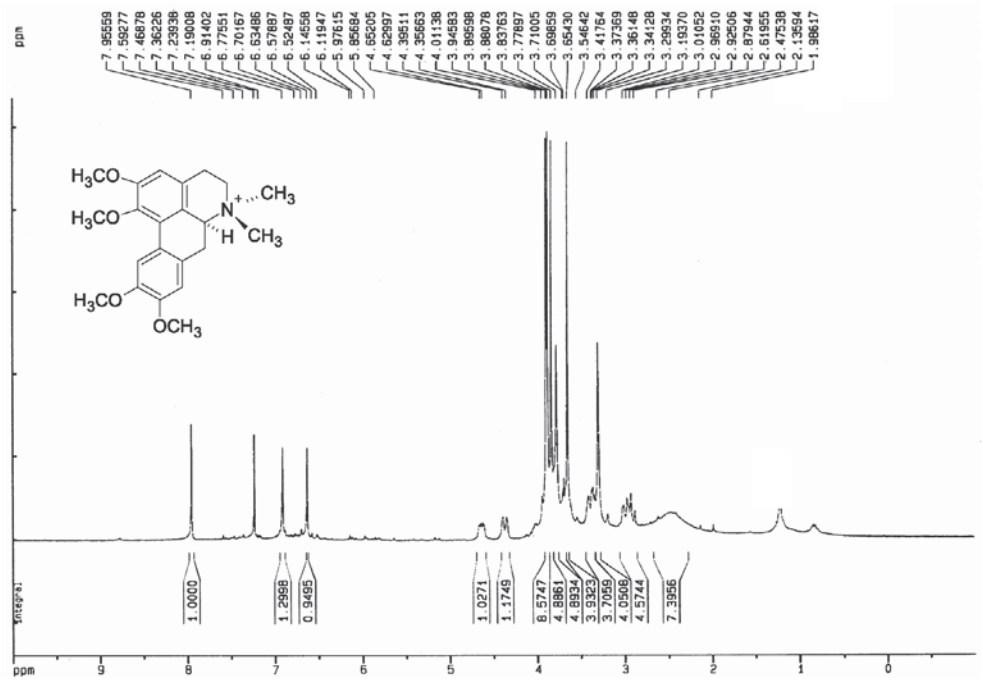

Figure S13. ^{1}H NMR spectrum of compound **5** (300 MHz, $CDCl_3$).

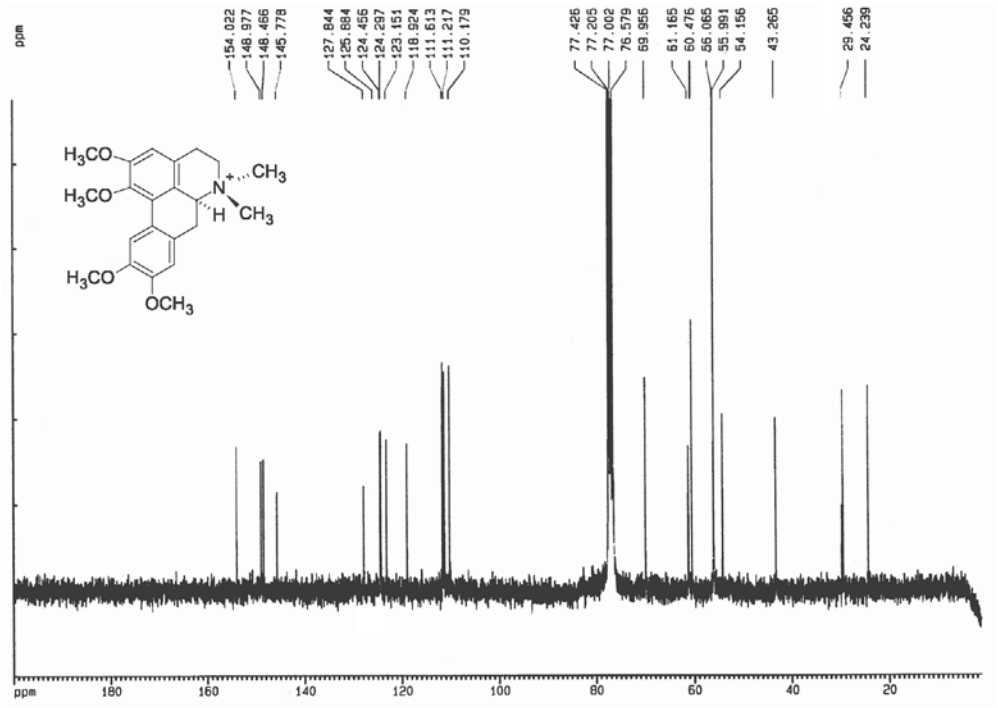

Figure S14. ^{13}C NMR spectrum of compound **5** (75 MHz, $CDCl_3$).

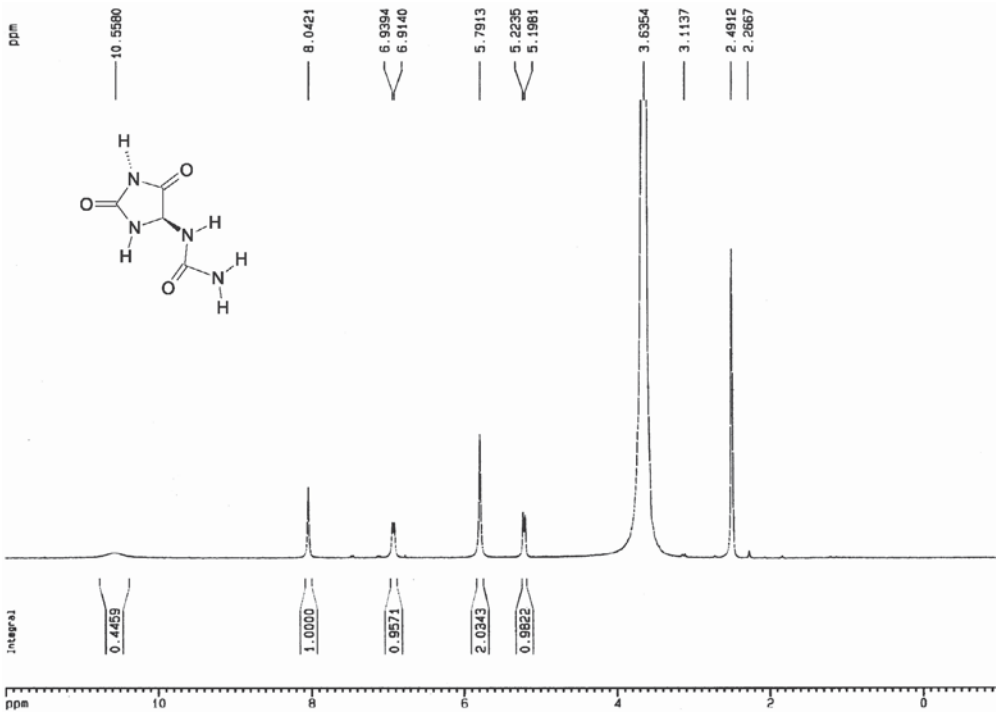

Figure S15. ^{1}H NMR spectrum of compound **6** (300 MHz, DMSO-d_6).

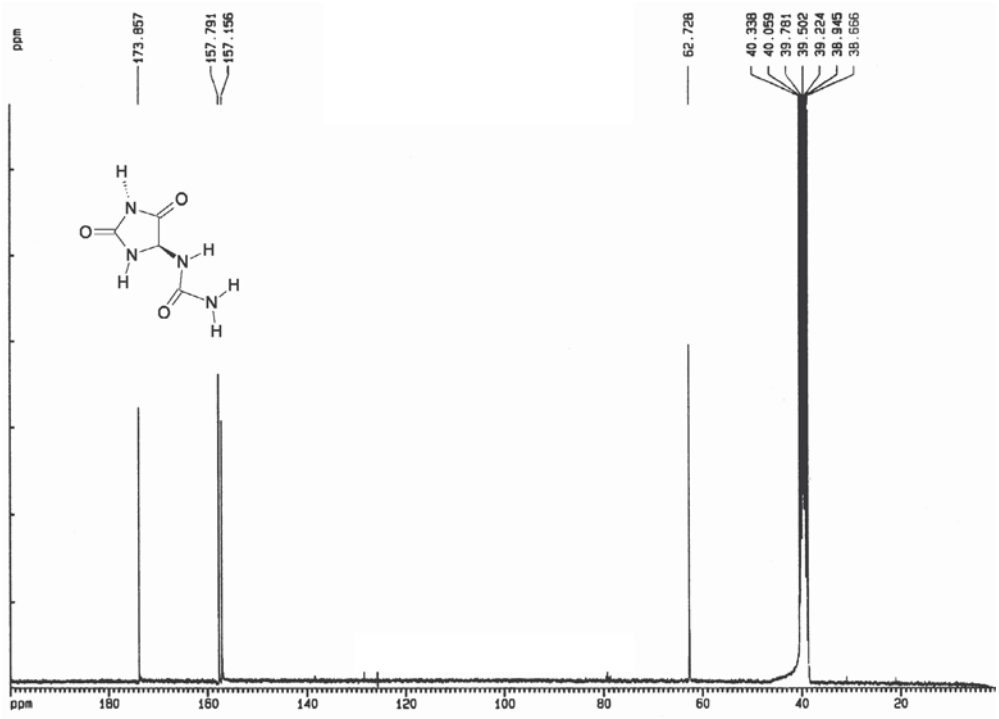

Figure S16. ^{13}C NMR spectrum of compound **6** (75 MHz, DMSO-d_6).

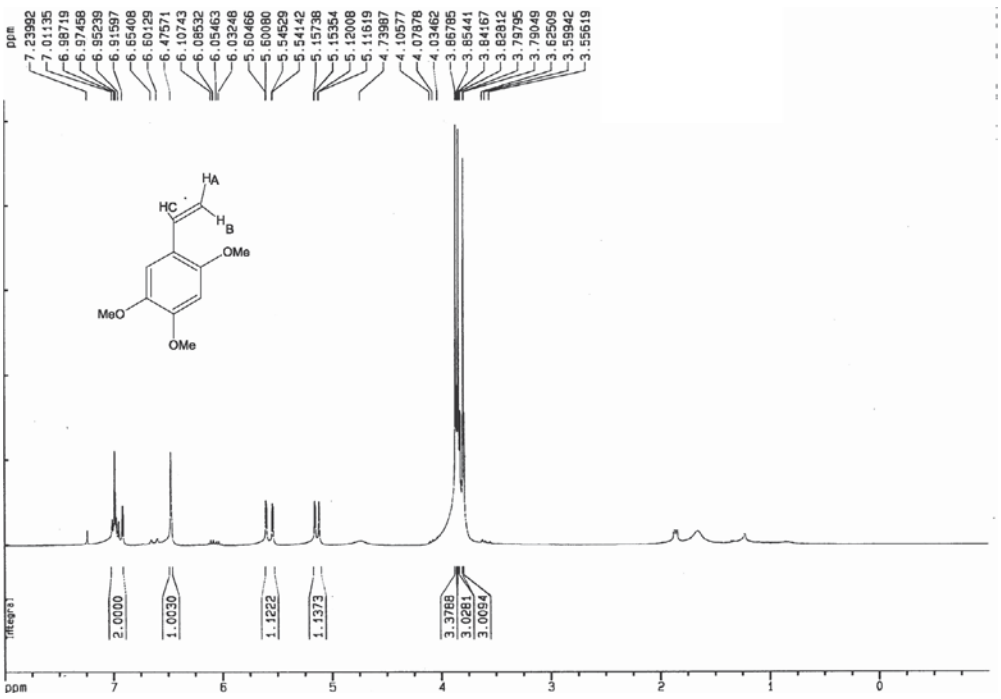

Figure S17. ^{1}H NMR spectrum of compound **7** (300 MHz, $CDCl_3$).

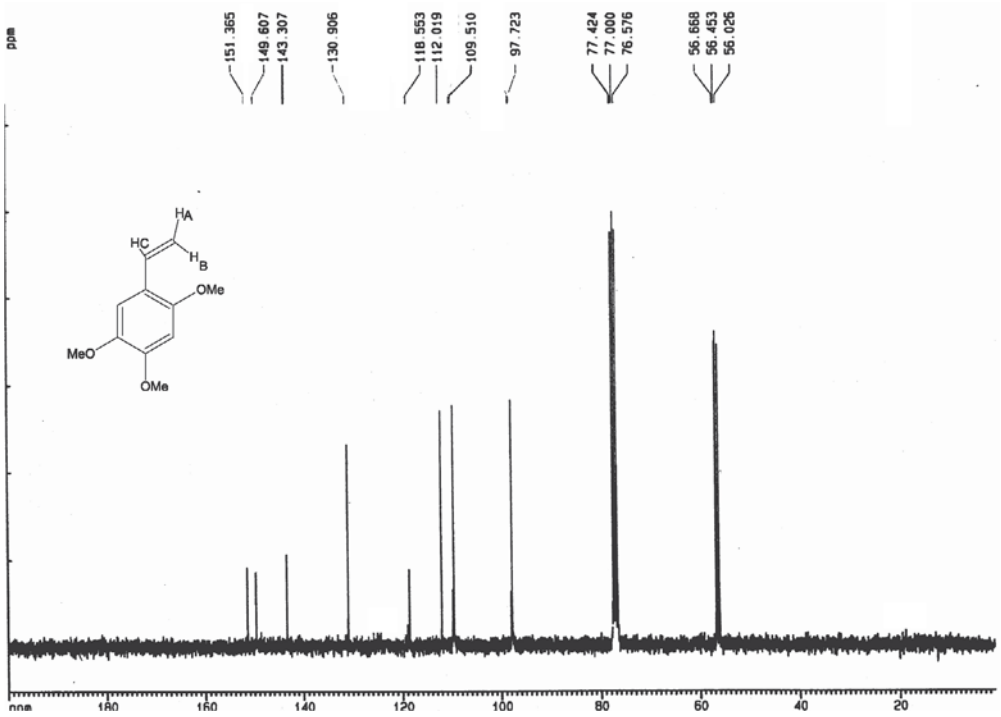

Figure S18. ^{13}C NMR spectrum of compound **7** (75 MHz, $CDCl_3$).

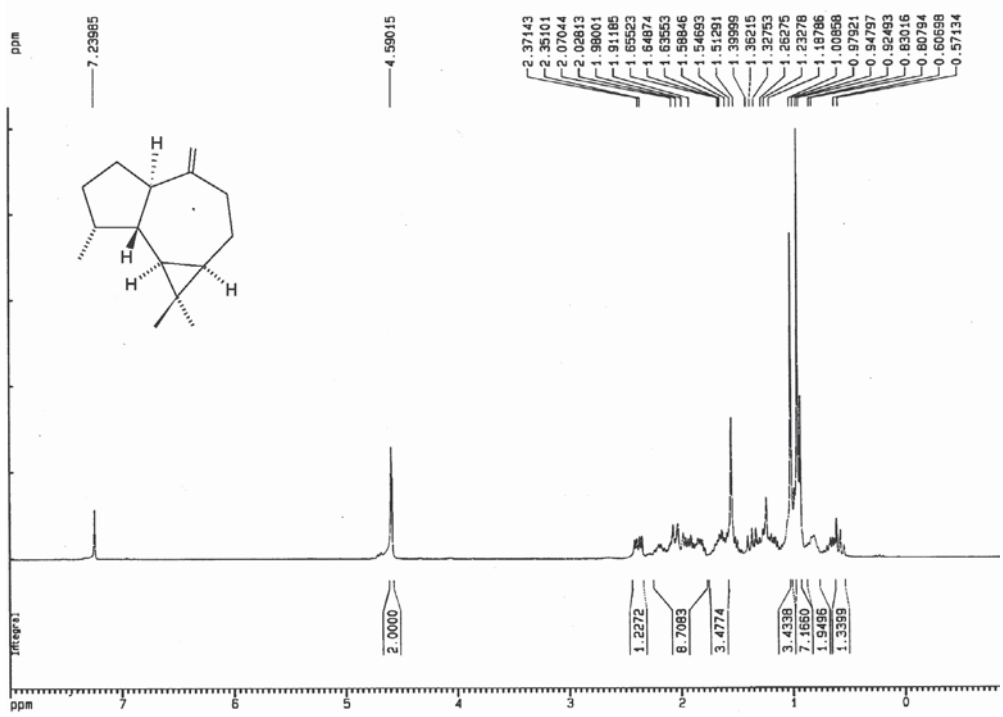

Figure S19. ^{1}H NMR spectrum of compound **9** (300 MHz, $CDCl_3$).

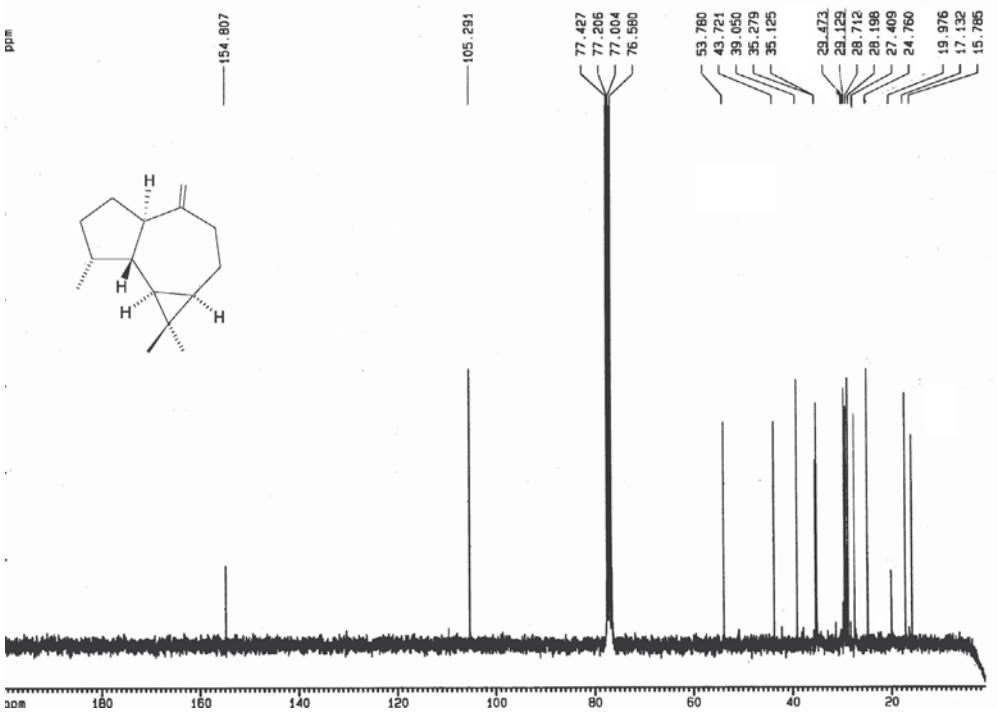

Figure S20. ^{13}C NMR spectrum of compound **9** (75 MHz, $CDCl_3$).

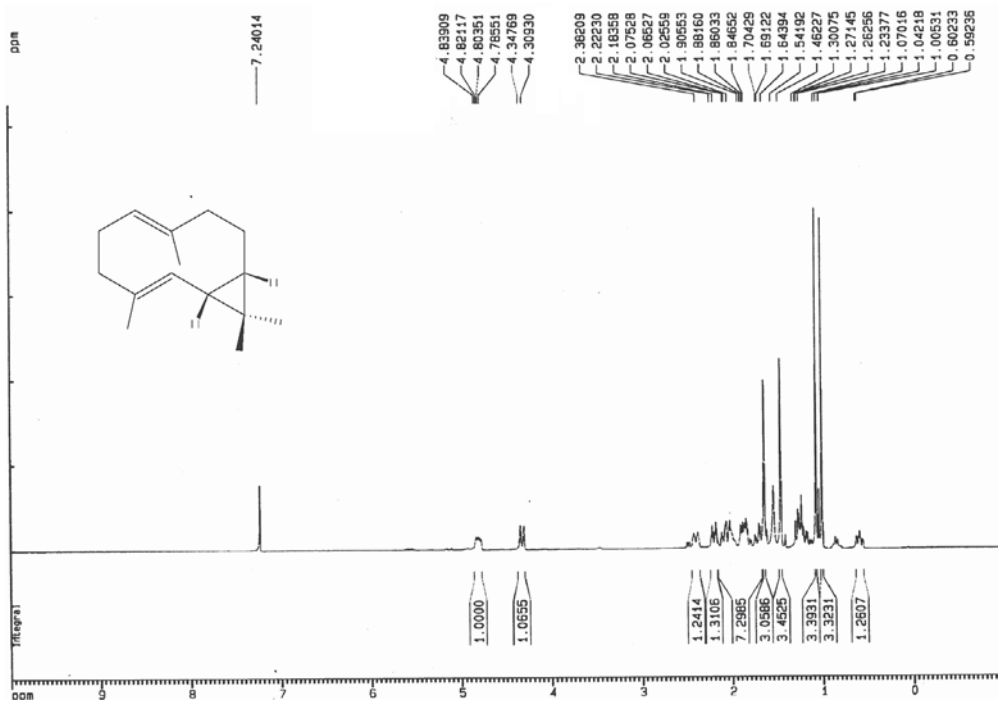

Figure S21. ^{1}H NMR spectrum of compound **10** (300 MHz, $CDCl_3$).

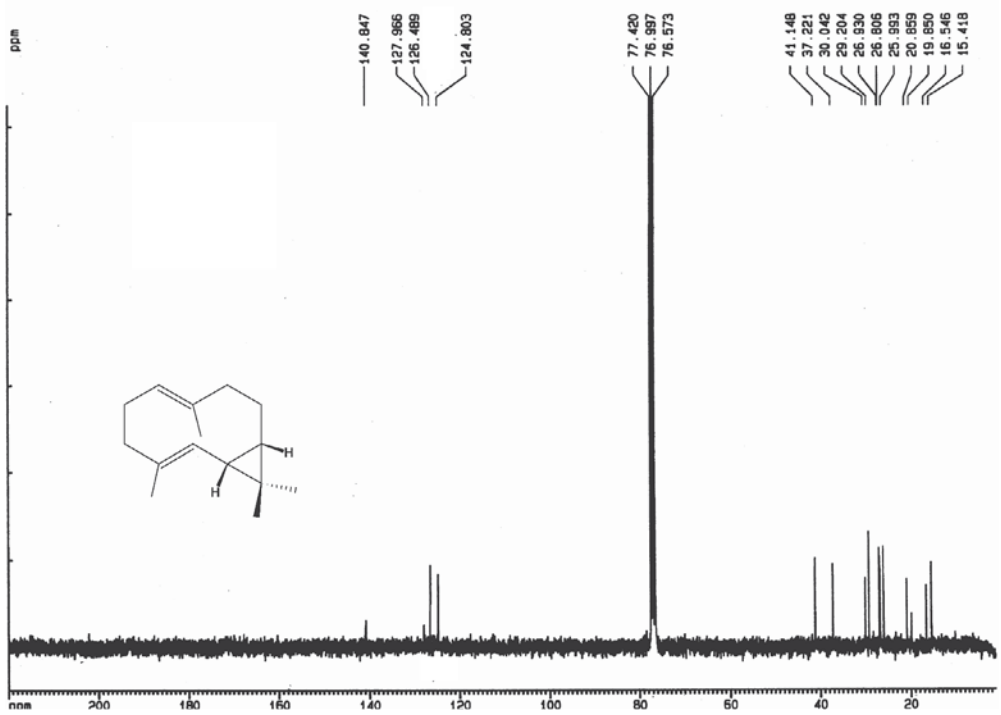

Figure S22. ^{13}C NMR spectrum of compound **10** (75 MHz, $CDCl_3$).

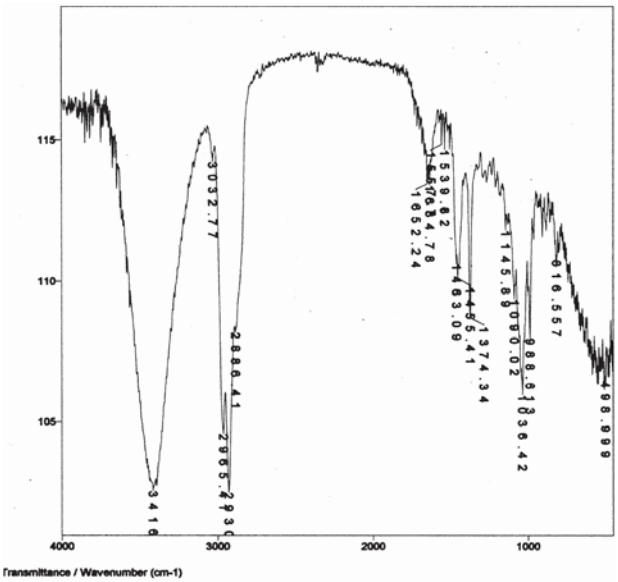

Figure S23. FTIR spectrum of compound **13.**

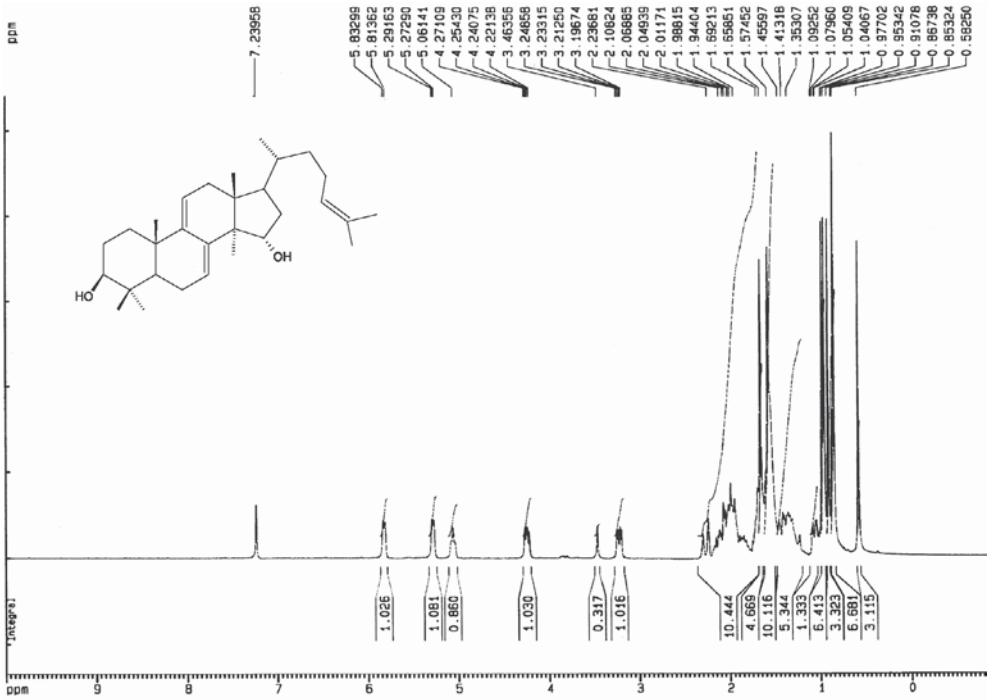

Figure S24. ^{1}H NMR spectrum of compound **13** (300 MHz, $CDCl_3$).

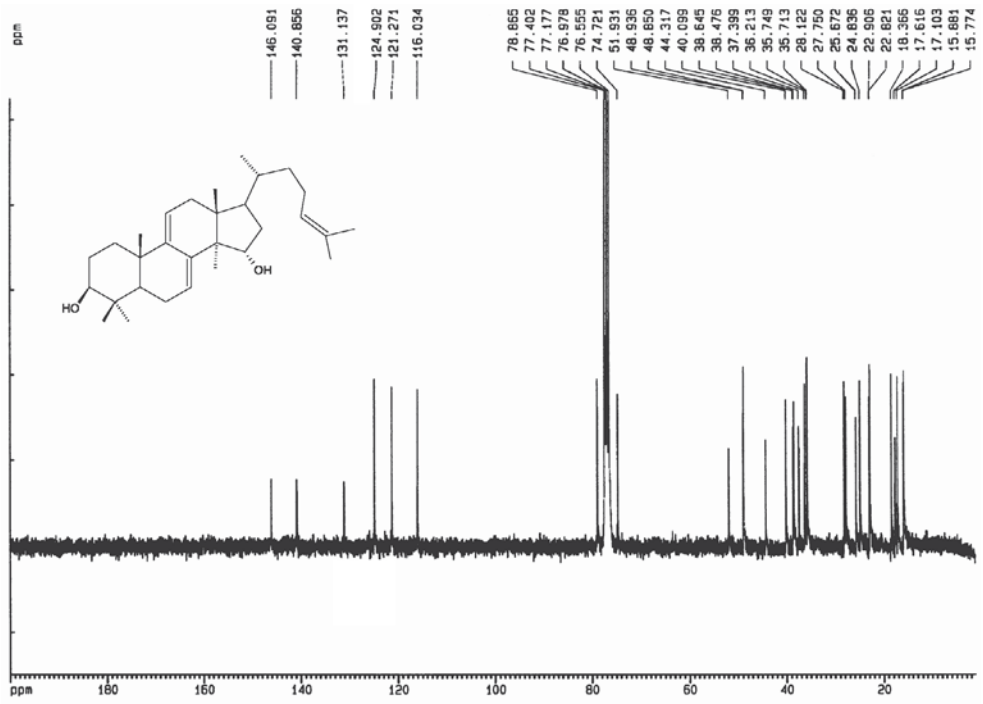

Figure S25. ^{13}C NMR spectrum of compound **13** (75 MHz, $CDCl_3$).

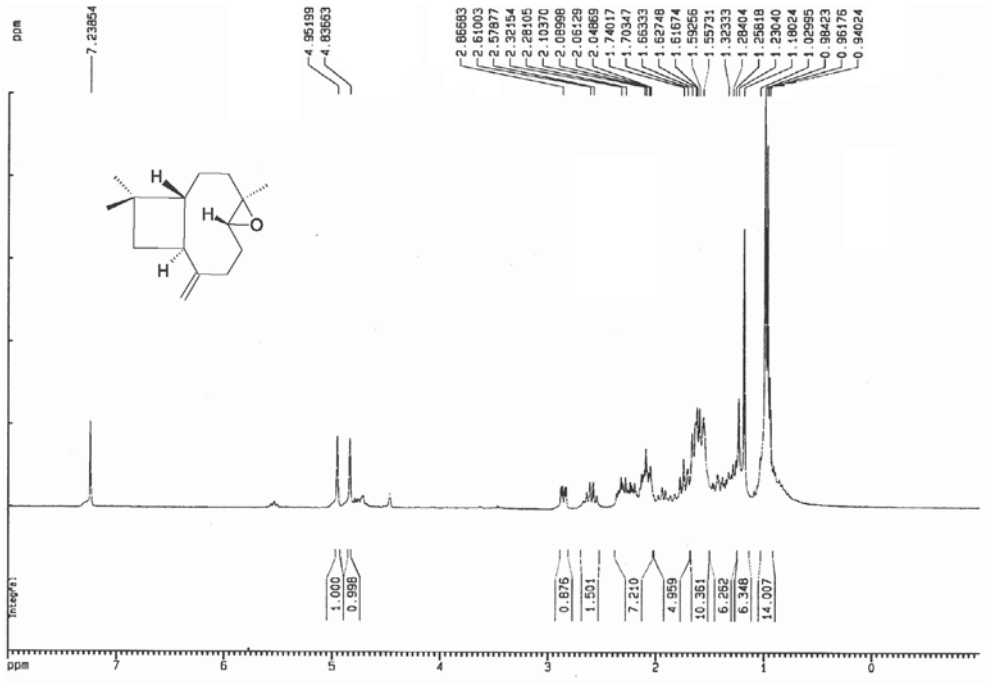

Figure S26. ^{1}H NMR spectrum of compound **14** (300 MHz, $CDCl_3$).

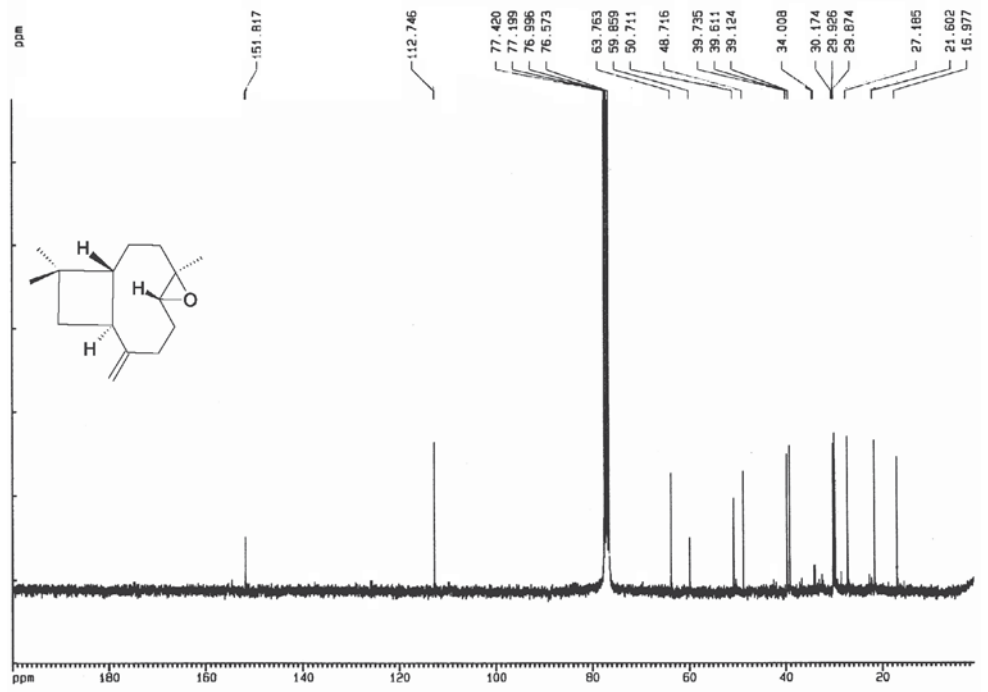

Figure S27. ^{13}C NMR spectrum of compound **14** (75 MHz, $CDCl_3$).

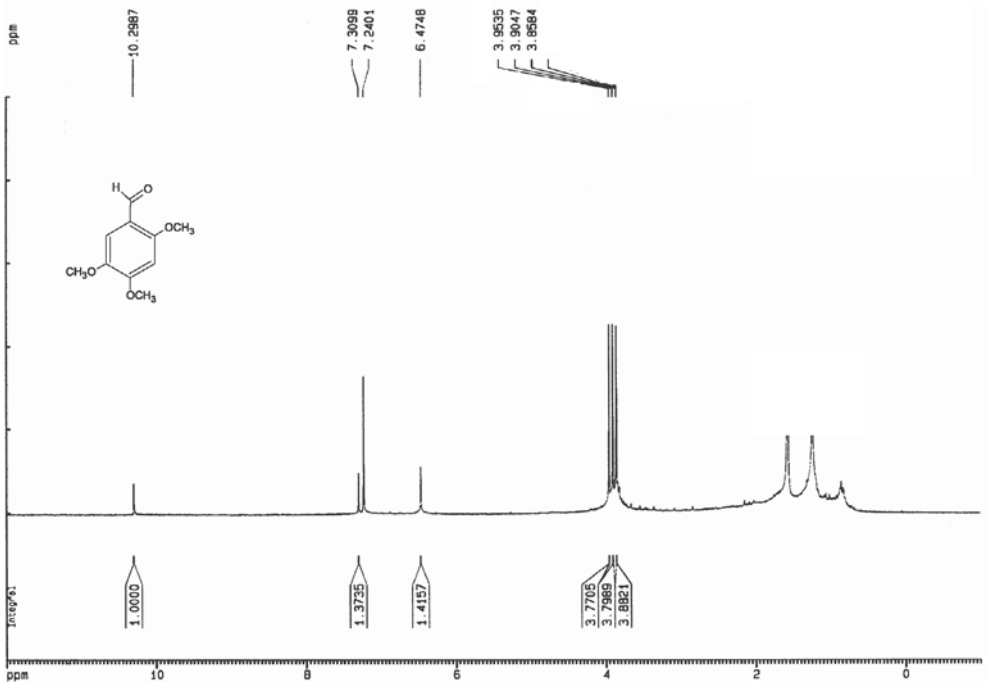

Figure S28. ^{1}H NMR spectrum of compound **15** (300 MHz, $CDCl_3$).

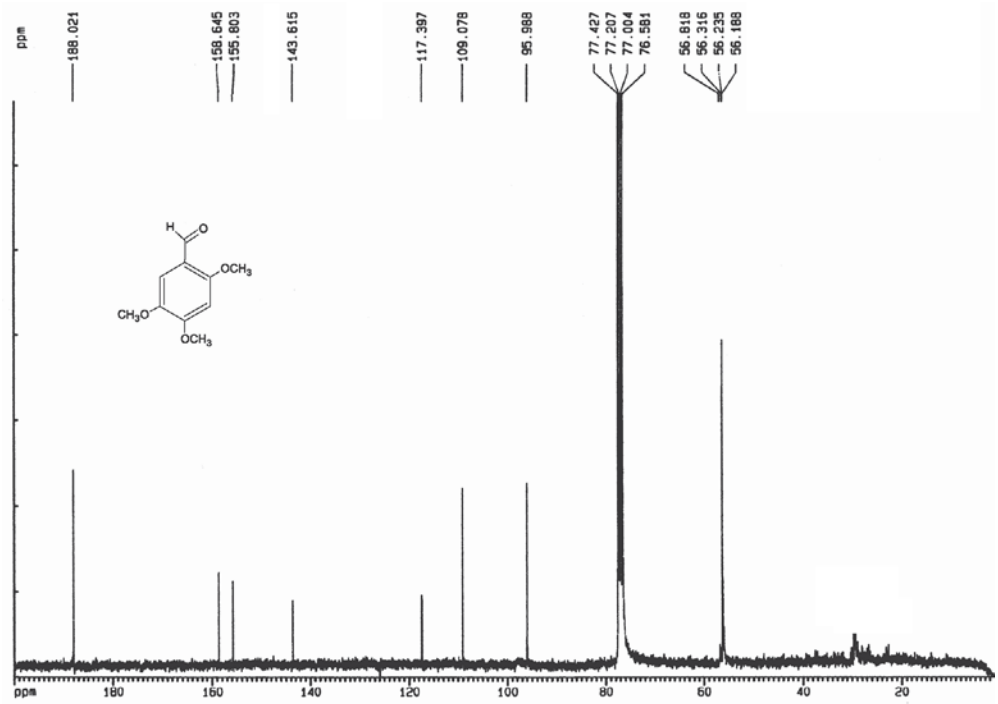

Figure S29. ^{13}C NMR spectrum of compound **15** (75 MHz, $CDCl_3$).