CHAPTER 3

RESULTS AND DISCUSSION

In the investigation of the chemical constituents from Thai marine organisms, the methanolic extract from the sponge *Ciocalapata* sp. showed the potent antimalarial activity (IC_{50} 0.80 μg/mL). The further investigation led to the isolation of four isonitrile diterpenes and two sterol peroxides, among which one diterpene and one sterol peroxide were new compounds. All the isonitrile diterpenes and sterol peroxides exhibited the antimalarial activity against *Plasmodium falciparum* K1 strain with IC_{50}'s in a range of 0.09-7.13 μM.

3.1 Isolation of the antimalarial compounds from the sponge *Ciocalapata* sp.

The sponge *Ciocalapata* sp. was collected from Kho-Tao, Surat Thani Province, Thailand, in April, 2002. The freeze-dried sponge (279 g) was extracted consecutively using hexane, CH_2Cl_2 and CH_3OH to yield the crude extract from each solvent weighed 7, 6, and 63 g, respectively. The hexane-extract, which showed the most potent antimalarial activity (IC_{50} 0.05 μg/mL), was chosen for the further chromatographic separation including vacuum chromatography over a SiO_2 column (step-gradient, hexane to 50% CH_3OH in CH_2Cl_2). Compound **4** (1.68%) was obtained after repetitive crystallization, and was identified as 8,15-diisocyano-11(20)-amphilectene. Two additional fractions from the fractional pools were also selected for the further isolation. The first one was subjected to the chromatographies with SiO_2 (2% EtOAc in hexane) then with HPLC RP-C18 (10% aq CH_3CN) to yield compound **1** (8-isocyanoamphilecta-11(20),15-diene; 0.10%) as a new compound, along with compounds **2** (7-isocyanoamphilecta-11(20),15-diene; 0.67%), and **3** (8-isocyanoamphilecta-11(20),14-diene; 0.12%). Another fraction was separated with Sephadex LH-20 (50% EtOAc in hexane), SiO_2 (50% EtOAc in hexane; and THF:EtOAc:hexane 9:18:73) and HPLC RP-C18 (5% aq CH_3CN). A new compound, compound **5** (5,9-*epi*-dioxyergostan-6,22-dien-3,8,14-triol; 0.05%), was obtained with compound **6** (5,8-*epi*-dioxyergostan-6,22-dien-3-ol; 0.07%).

3.2 The structure elucidation of the isolated compounds

The discussion on the structure elucidation in this thesis will be divided into two major sections according to the classes of the isolated compounds, i.e. the isonitrile diterpenes and the sterol peroxides. The new compound from each class, which is the focal point in this thesis, will be first elaborated followed by the known ones.

3.2.1 The structure elucidation of the isonitrile diterpenes

3.2.1.1 The structure elucidation of compound 1

Compound **1** was obtained as white amorphous solid (6 mg) by means of chromatographic techniques including a SiO_2 column (2% EtOAc in hexane) and HPLC on a RP-C18 column (Phenomenex®, 10 μm, 250×10 mm; 10% aq CH_3CN, flow rate 4.5 mL/min, t_R 24.0 min).

The molecular formula of compound **1** was established as $C_{21}H_{31}N$ according to the pseudomolecular ion peak $[M+Na]^+$ at *m/z* 320.2338 in HRESIMS (calcd for $C_{21}H_{31}NNa$, 320.2348). The proposed molecular formula required the unsaturation degree of seven. The ^{13}C NMR spectrum (125 MHz, C_6D_6, Figure 3) indicated the presence of one isonitrile functional group (δ 159.9, t, *J* = 4.2 Hz, C-21; and 66.6, t, *J* = 4.2 Hz, C-8) and four olefinic carbons (δ 150.1, C-11; 144.2, C-15; 111.5, C-16; and 105.8, C-20); therefore three ring systems were required. The infrared absorption confirmed the existence of the isonitrile group at ν_{max} 2150 cm^{-1}. The UV spectrum showed the maximal absorption at λ_{max} 244 (log ε 2.38).

The ^1H NMR spectrum of compound **1** (500 MHz, C_6D_6, Figure 4) exhibited three methyls (δ 1.66, s, 3H, H-17; 0.75, br s, 3H, H-18; and 0.82, br s, 3H, H-19) and four doublet olefinic protons (δ 4.66, *J* = 1.1 Hz, H-20b; 4.77, br s, H-20a; 4.77, br s, H-16a; and 4.85, br s, H-16b), along with overlapping signals of six methylenes and six methines (δ 0.50-2.66). Interpretation of ^1H-^1H correlations from the COSY spectrum led to three partial structures. These include fragment **A** at δ 1.67 (m, H-1), 1.78 (m, H-2a), 0.58 (ddd, *J* = 13.2, 11.7, 11.5 Hz, H-2b), 1.93 (m, H-12), 0.65 (ddt, *J* = 10.9, 9.8, 3.2 Hz, H-13), 2.66 (br d, *J* = 14.7 Hz, H-14a), and 1.43 (m, H-14b); fragment **B** at δ 0.79 (m, H-3), 1.13 (br dd, *J* = 10.5, 8.2 Hz, H-4), 1.78 (m, H-5a), 0.48 (dddd, *J* = 11.9, 11.4, 9.4, 3.8 Hz, H-5b), 1.23 (br d, *J* = 13.4 Hz, H-6a), 1.46 (m, H-6b), 0.83 (m, H-7), 0.75 (br s, 3H, H-18), and 0.82 (br s, 3H, H-19); fragment **C** at δ 1.90 (m,

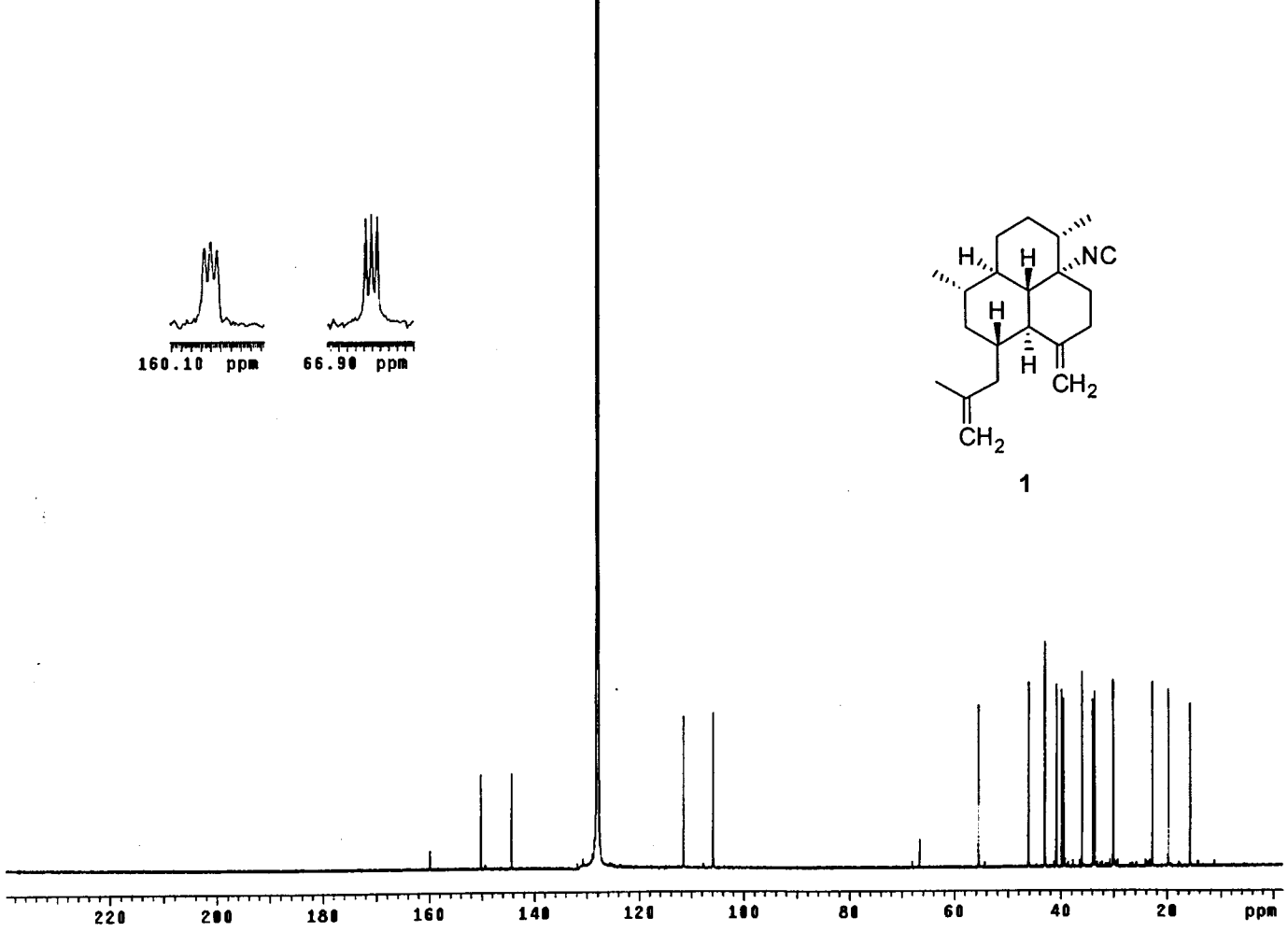


Figure 3 ^{13}C NMR spectrum of **1** (125 MHz, C_6D_6)

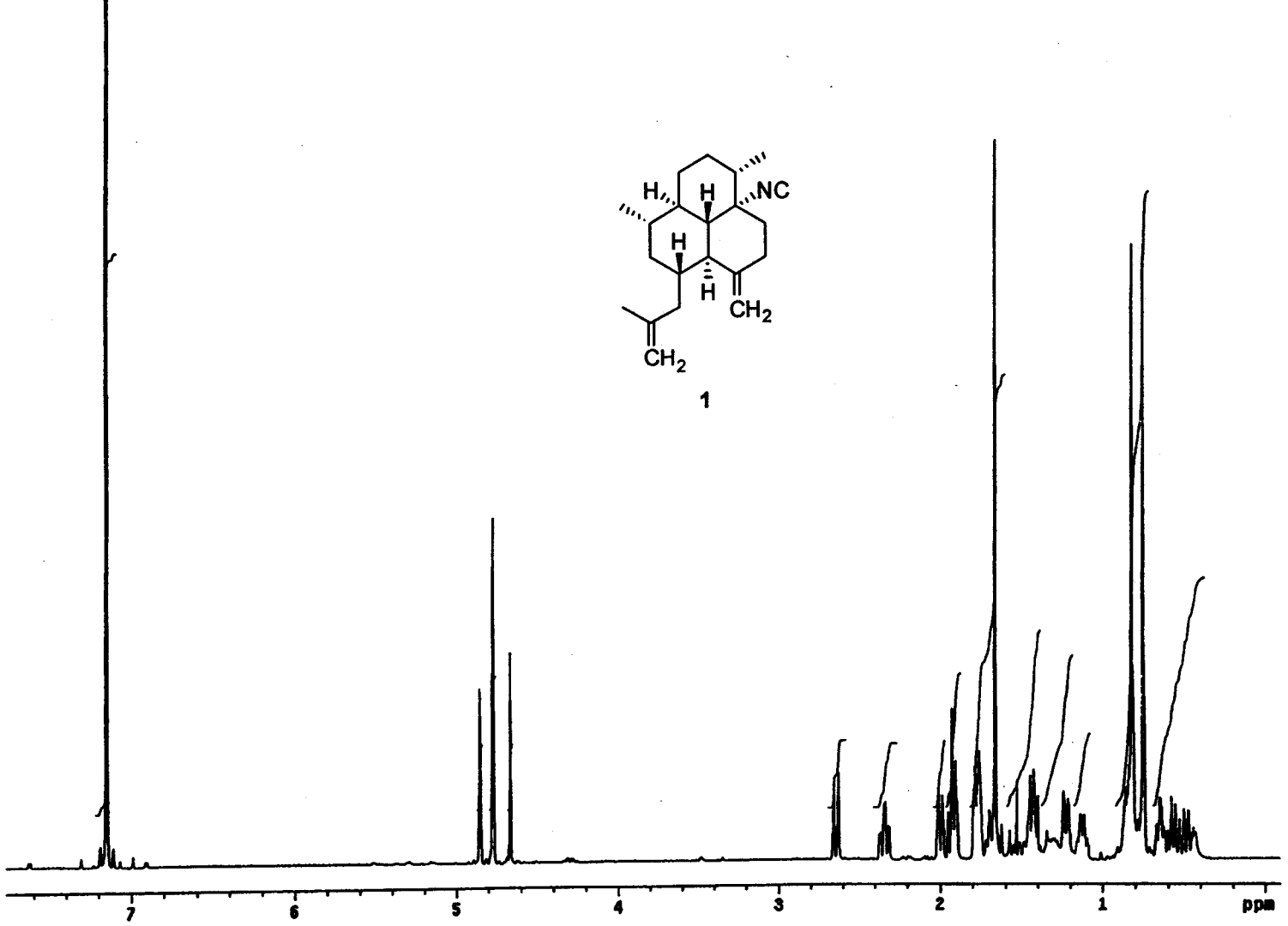
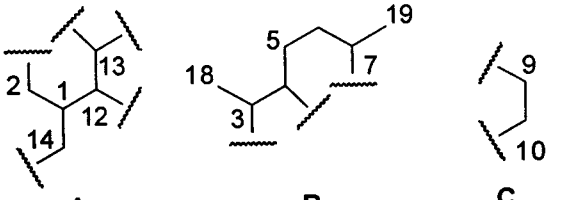


Figure 4 ^{1}H NMR spectrum of **1** (500 MHz, C_6D_6)

H-9a), 0.86 (ddd, J = 13.1, 11.7, 2.3 Hz, H-9b), 2.34 (ddd, J = 13.0, 13.0, 4.4 Hz, H-10a), and 2.00 (ddd, J = 13.0, 2.3, 2.2 Hz, H-10b) as shown.



The three fragments were connected by means of the HMBC spectral analysis (Table 4). The connection of fragment **A** to fragment **B** was based on the correlations from C-2 (δ 39.8) to H-3, H-4, and H-18, and from C-3 (δ 35.9) to H-13. The fragment **C** was connected to fragments **A** and **B** according to the correlations from C-8 (δ 66.6) to H-9, H-10, H-12, H-13, and H-19. The olefinic functionalities were assigned as C-11 (δ 150.1) and C-20 (δ_C 105.8; δ_H 4.77, br s, H-20a; 4.66, d, J = 1.1 Hz, H-20b), and as C-15 (δ 144.2) and C-16 (δ_C 111.5; δ_H 4.77, br s, H-16a; 4.85, br s, H-16b). This was based on the HMBC correlation from C-11 to H-9, H-10, H-12, H-13, and H-20, and from C-15 to H-1, and H-16, and from C-16 to H-14, and H-17 (Figure 5). The characteristic triplet signal of C-8 (δ 66.0, t, J = 4.2 Hz) also indicated the substitution of an isonitrile (Iwashima et al, 2002). The chemical shift of the isonitrile carbon C-21 was found at δ 159.9. The structure of **1** was therefore purposed to be a new isonitrile diterpene derivative, designated as 8-isocyanoamphilecta-11(20),15-diene. The ^{13}C and ^{1}H NMR spectral data of **1** are summarized in Table 4.

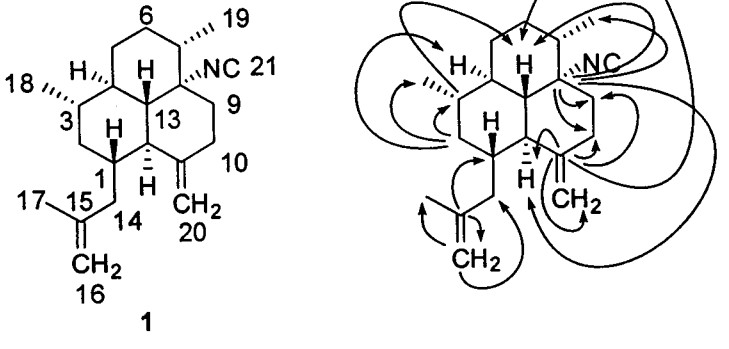


Figure 5 Structure of **1** with crucial HMBC correlation (C⟶H)

Table 4 NMR data of **1** (500 MHz for ^{1}H and 125 MHz for ^{13}C; C_6D_6)

Position	^{13}C (mult)	^{1}H (mult; *J* in Hz)	HMBC correlation (C→H)
1	33.9 (CH)	1.67 (m)	H-2, H-3, H-14
2 a	39.8 (CH_2)	1.78 (m)	H-3, H-4, H-12, H-18
b		0.58 (ddd; 13.2, 11.7, 11.5)	
3	35.9 (CH)	0.79 (m)	H-4, H-5, H-13, H-18
4	43.0 (CH)	1.13 (br dd; 10.5, 8.2)	H-3, H-6, H-13, H-18
5 a	30.0 (CH_2)	1.78 (m)	H-3, H-6, H-13
b		0.48 (dddd; 11.9, 11.4, 9.4, 3.8)	
6 a	30.1 (CH_2)	1.23 (br d; 13.4)	H-5, H-7
b		1.46 (m)	
7	40.8 (CH)	0.83 (m)	H-6, H-19
8	66.6 (C)[a]	-	H-9, H-10, H-12, H-13, H-19
9 a	39.4 (CH_2)	1.90 (m)	H-10
b		0.86 (ddd; 13.1, 11.7, 2.3)	
10 a	33.6 (CH_2)	2.34 (ddd; 13.0, 13.0, 4.4)	H-9, H-20
b		2.00 (ddd; 13.0, 2.3, 2.2)	
11	150.1 (C)	-	H-9, H-10, H-12, H-13, H-20
12	46.1 (CH)	1.93 (m)	H-1, H-13, H-14, H-20
13	55.5 (CH)	0.64 (ddt; 10.9, 9.8, 3.2)	H-4, H-6, H-12
14 a	43.0 (CH_2)	2.66 (br d; 14.7)	H-1, H-2, H-16
b		1.43 (m)	
15	144.2 (C)	-	H-1, H-16
16 a	111.5 (CH_2)	4.77 (br s)	H-14, H-17
b		4.85 (br s)	
17	22.7 (CH_3)	1.66 (s, 3H)	H-16
18	19.7 (CH_3)	0.75 (br s, 3H)	H-2
19	15.7 (CH_3)	0.82 (br s, 3H)	-

Table 4 (cont.)

Position	^{13}C (mult)	^{1}H (mult; *J* in Hz)	HMBC correlation (C→H)
20 a	105.8 (CH_2)	4.77 (br s)	H-10, H-12
b		4.66 (d; 1.1)	
21	159.9 (C)[a]	-	-

Note; [a] t, J = 4.2 Hz.

It is worth mentioning here that, during the course of the structure elucidation of **1**, the resonances of the two methyl groups on C-18 and C-19 were mischievously misleading. When in C_6D_6, the two methyls resonated as broad singlet, despite strong coupling evidently observable as cross resonances on the ^{1}H-^{1}H COSY spectrum (H-18 to H-3; H-19 to H-7). The theoretical doublet multiplicity (J = 6.1 Hz, both) of each methyl was observed only when $CDCl_3$ was employed (Figure 6). Whereas it is widely known that solvent effects cause the deviation in chemical shifts, especially when aromatic solvents such as C_6D_6 are involved, such effects on multiplicity and coupling constants have never been fully documented. To date, this is the first observation of the coupling constant deviation of the methyl on decalin system due to the change in operating NMR solvents.

The relative configuration of **1** was determined based on the nOe-ds experiments. Dipolar couplings among H-1, H-3, H-7, and H-13; and between H-4 and H-12 indicated that H-1, H-3, H-7, and H-13 resided on the same plane of the structure, whereas H-4 and H-12 did on the opposite (Figure 7). The absolute configuration of **1** was proposed as 1*S*, 3*S*, 4*R*, 7*S*, 8*S*, 12*S*, and 13*S*, relative to that of 8,15-diisocyano-11(20)-amphilectene (**4**), which is the prototype of the compounds in its class and also the major isonitrile diterpene isolated in this investigation (Ciavatta et al, 2005).

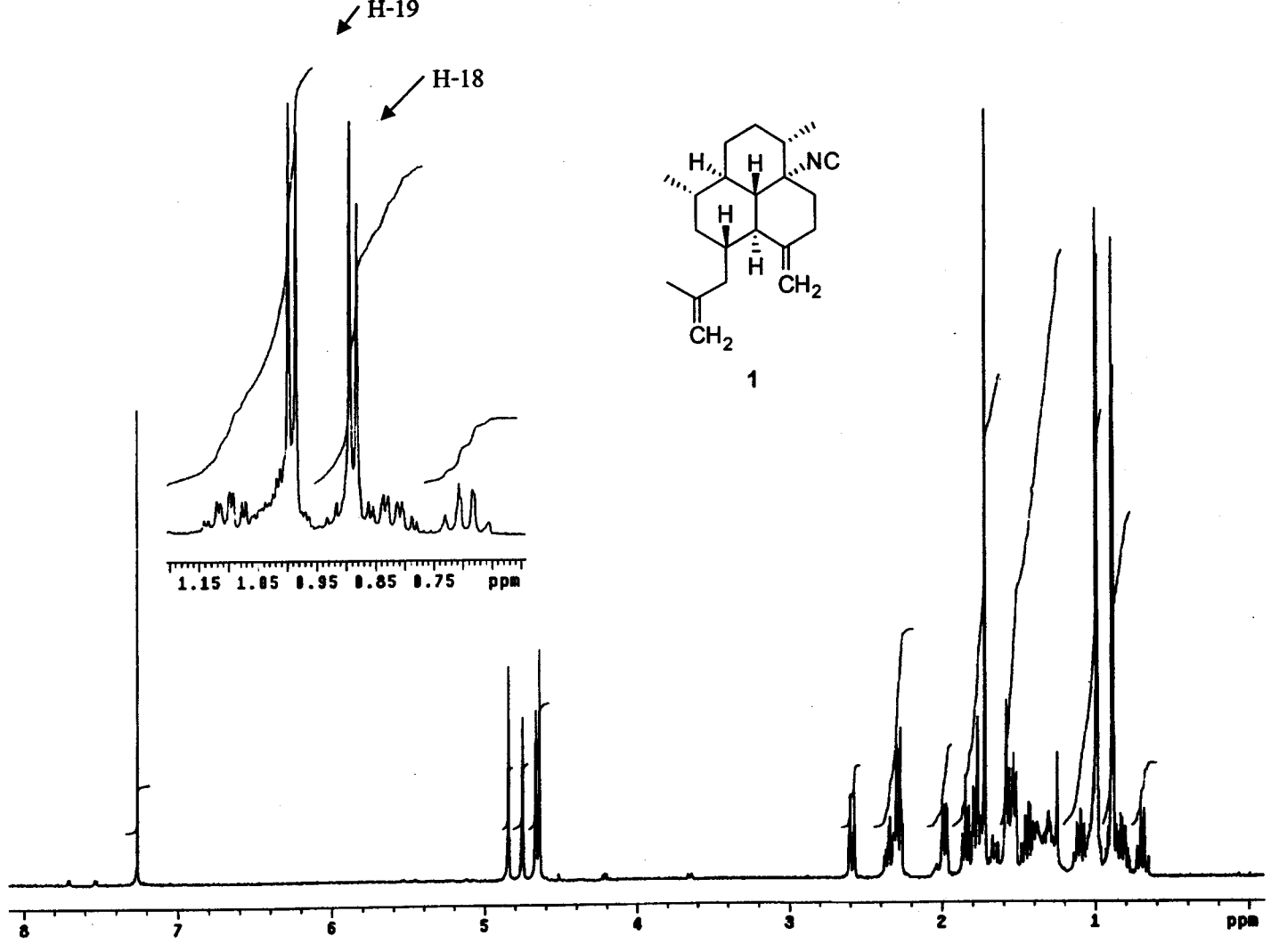


Figure 6 ^{1}H NMR spectrum of **1** (500 MHz, $CDCl_3$)

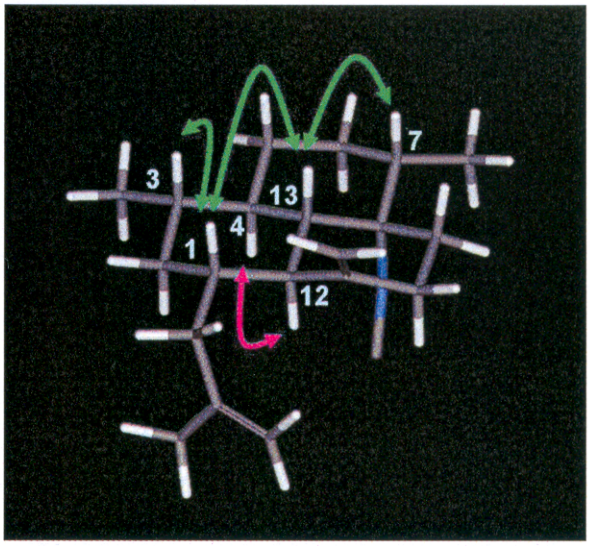


Figure 7 nOe Correlations of **1**

3.2.1.2 The structure elucidation of compound 2

Compound **2** was obtained from the same chromatographic conditions as those for **1**, with t_R of 20.8 min. The molecular formula of compound **2** was established to be $C_{21}H_{31}N$ according to the pseudomolecular ion peak $[M+Na]^+$ at *m/z* 320 in the ESIMS spectrum. The proposed molecular formula required the unsaturation degree of seven. Similar to **1**, the unsaturation degree was analyzed to belong to an isonitrile, two olefins, and three rings. The close identity between the molecular formulae of **1** and **2**, as well as that of other spectral data, including the ^{13}C and ^{1}H NMR (Figures 8 and 9), was evident, and indicated the isomerism between the two compounds. Compound **2** differed from **1** primarily in that the resonances of the triplet carbon changed from that of C-8 (δ 66.6 for **1**) to C-7 (δ 59.3 for **2**), indicating that the isonitrile substitution shifted accordingly. The structure of **2** was therefore proposed to be 7-isocyanoamphilecta-11(20),15-diene, and the spectral data were confirmed based on those previously reported (Ciavatta et al, 1999; see Table 5 for summarized NMR data). The absolute configuration as shown was referred to the configuration of **4** (see 3.2.1.4), and was also confirmed by the specific rotation ($[\alpha]_D$ = +63; *c* 0.25, $CHCl_3$, as reported in Ciavatta et al, 1999; $[\alpha]_D$ = +65, *c* 1.20, $CHCl_3$). Also similar to **1**, the solvent effect from C_6D_6 on the methyl multiplicity was observed with the resonance of H-18 (δ 0.77, br s). The coupling constant between H-18 and H-3 as observed when the ^{1}H NMR experiment was performed in $CDCl_3$ was 6.4 Hz.

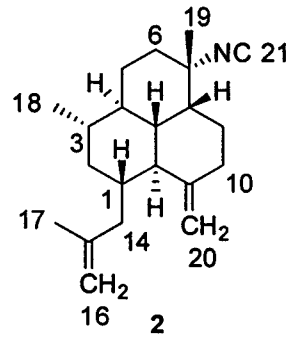


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Table 5 NMR data of **2** (500 MHz for ^{1}H and 125 MHz for ^{13}C; C_6D_6)

Position	^{13}C (mult)	^{1}H (mult; *J* in Hz)	Position	^{13}C (mult)	^{1}H (mult; *J* in Hz)
1	34.9 (CH)	1.58 (m)	10 a	33.9 (CH_2)	2.24 (ddd; 14.2, 5.9, 1.6)
2 a	41.3 (CH_2)	1.84 (ddd; 13.9, 3.4,	b		2.02 (ddd; 14.2, 9.6, 9.4)
		3.4)	11	148.8 (C)	-
b		0.50 (br d, 11.4)	12	49.2 (CH)	1.35 (dd; 10.8, 10.6)
3	39.9 (CH)	1.08 (m)	13	45.5 (CH)	0.87 (br dd; 10.8, 10.6)
4	39.9 (CH)	1.42 (m)	14 a	42.3 (CH_2)	2.72 (br d; 13.7)
5 a	24.7 (CH_2)	1.74 (br ddd; 13.0, 7.8,	b		1.44 (m)
		7.1)	15	144.3 (C)	-
b		0.66 (dddd; 13.0, 6.4,	16 a	111.7 (CH_2)	4.86 (br s)
		3.8, 3.2)	b		4.80 (br s)
6 a	33.6 (CH_2)	1.43 (m)	17	22.0 (CH_3)	1.68 (br s, 3H)
b		1.06 (m)	18	19.4 (CH_3)	0.77 (br s, 3H)
7	59.3 (C)[a]	-	19	28.7 (CH_3)	0.98 (t; 1.8, 3H)
8	42.3 (CH)	1.19 (m)	20 a	106.6 (CH_2)	4.86 (br s)
9 a	21.8 (CH_2)	1.64 (m)	b		4.67 (br s)
b		1.66 (m)	21	158.5 (C)[a]	-

Note; [a] t, $J = 5.0$ Hz.

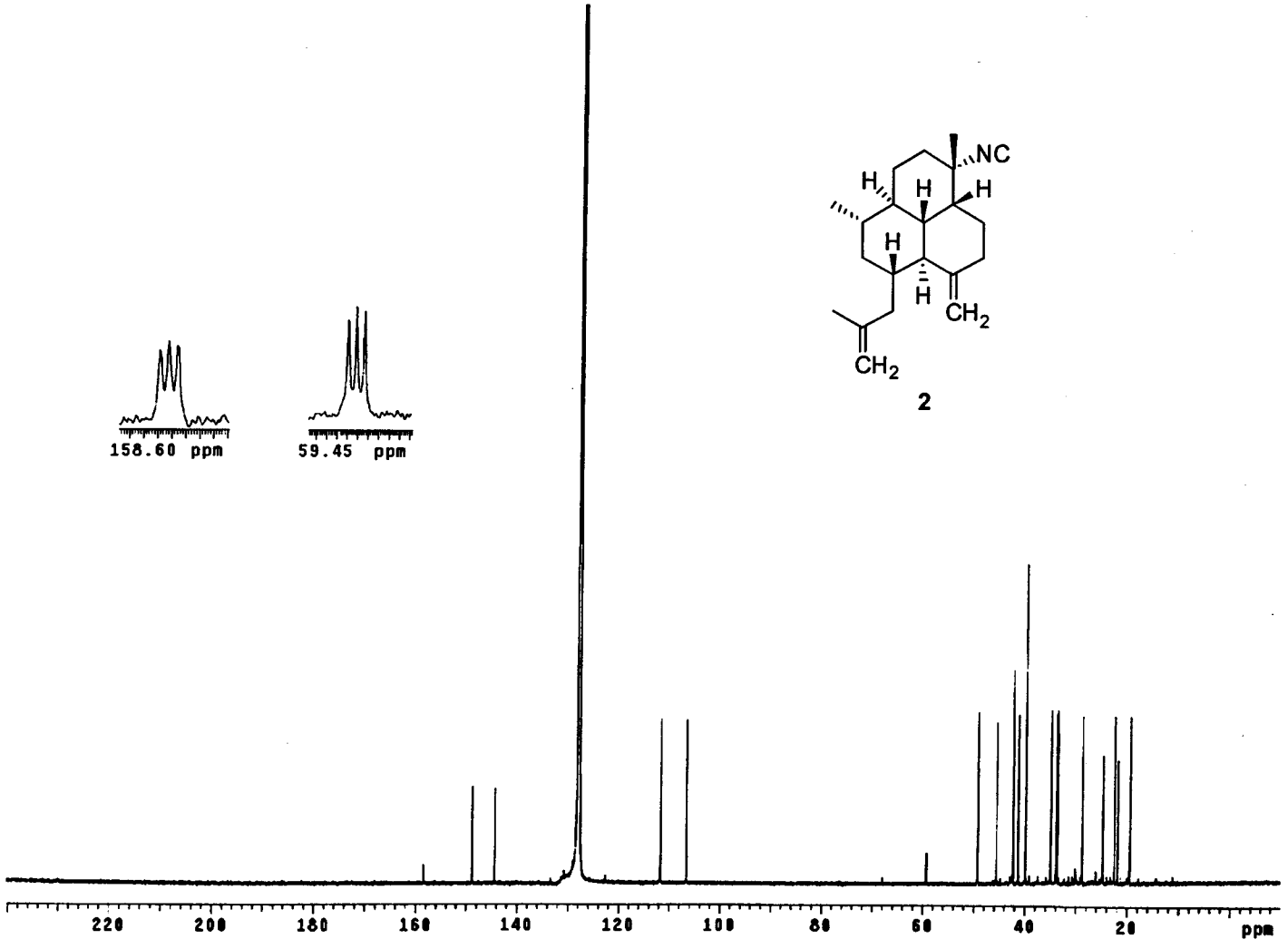

Figure 8 ^{13}C NMR spectrum of **2** (125 MHz, C_6D_6)

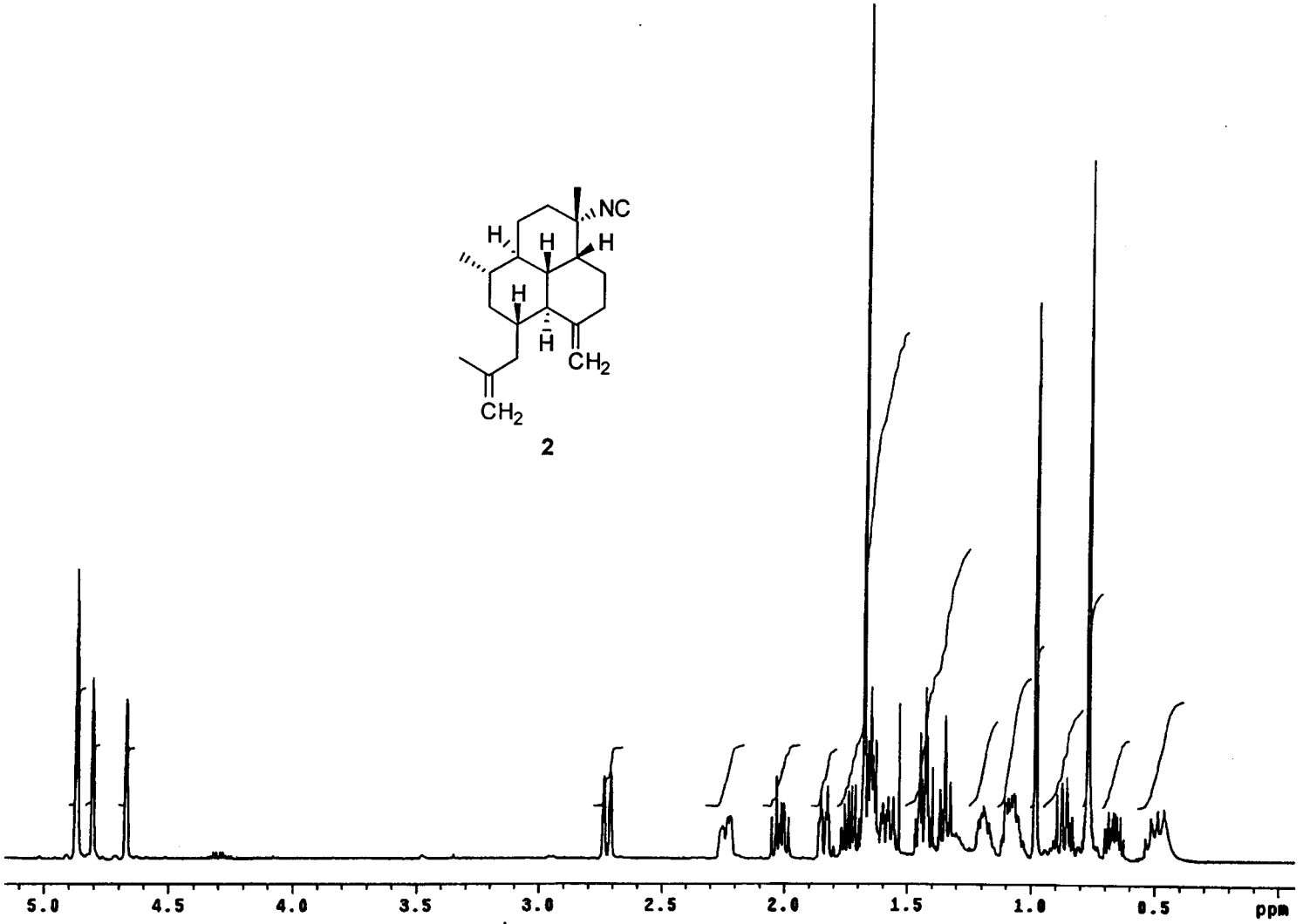

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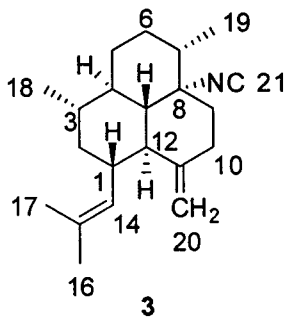
Figure 9 ^{1}H NMR spectrum of **2** (500 MHz, C_6D_6)

3.2.1.3 The structure elucidation of compound 3

Along with **1** and **2**, compound **3** was obtained as white amorphous solid (4 mg), and was eluted from the HPLC step as mentioned in 3.1 at t_R of 22.6 min. The molecular formula of compound **3** was proposed as $C_{21}H_{31}N$ based on the pseudomolecular ion peak $[M+Na]^+$ at ***m/z*** 320 in ESIMS. The similarity among the molecular formulae and spectral data of compounds **1**, **2**, and **3** was again apparent, and indicated that **3** was an isomer of **1** and **2**. The isonitrile functionality of **3** was placed on C-8 (δ 66.5) due to the ^{13}C-^{14}N coupling (t, J = 3.7 Hz) as observed previously in **1** (Figures 10). The major difference, on the other hand, was at the olefinic functionalities. Whereas compound **1** and **2** possessed two terminal vinyl methylenes, one of the vinyl methylenes was absent in the spectra of **3** and instead was found as a trisubstituted olefin, resonating at δ_H 4.88 (br d, J = 8.5 Hz, H-14) and at δ_C 131.8 (C-14) and 128.5 (C-15) (Figures 10 and 11; Table 6). Compound **3** was therefore proposed as 8-isocyanoamphilecta-11(20),14-diene (Mitome et al, 2004). The absolute configuration was proposed based on the same argument as those discussed for **1** and **2**, with a closely related $[\alpha]_D$ to that previously reported (observed $[\alpha]_D$ = -27; *c* 0.38, $CHCl_3$; reported $[\alpha]_D$ = -34; *c* 0.40, $CHCl_3$; Mitome et al, 2004). The two methyls of H-18 and H-19 resonated with the coupling constants of 5.5 and 6.4 Hz, respectively, when $CDCl_3$ was used for the NMR experiment.



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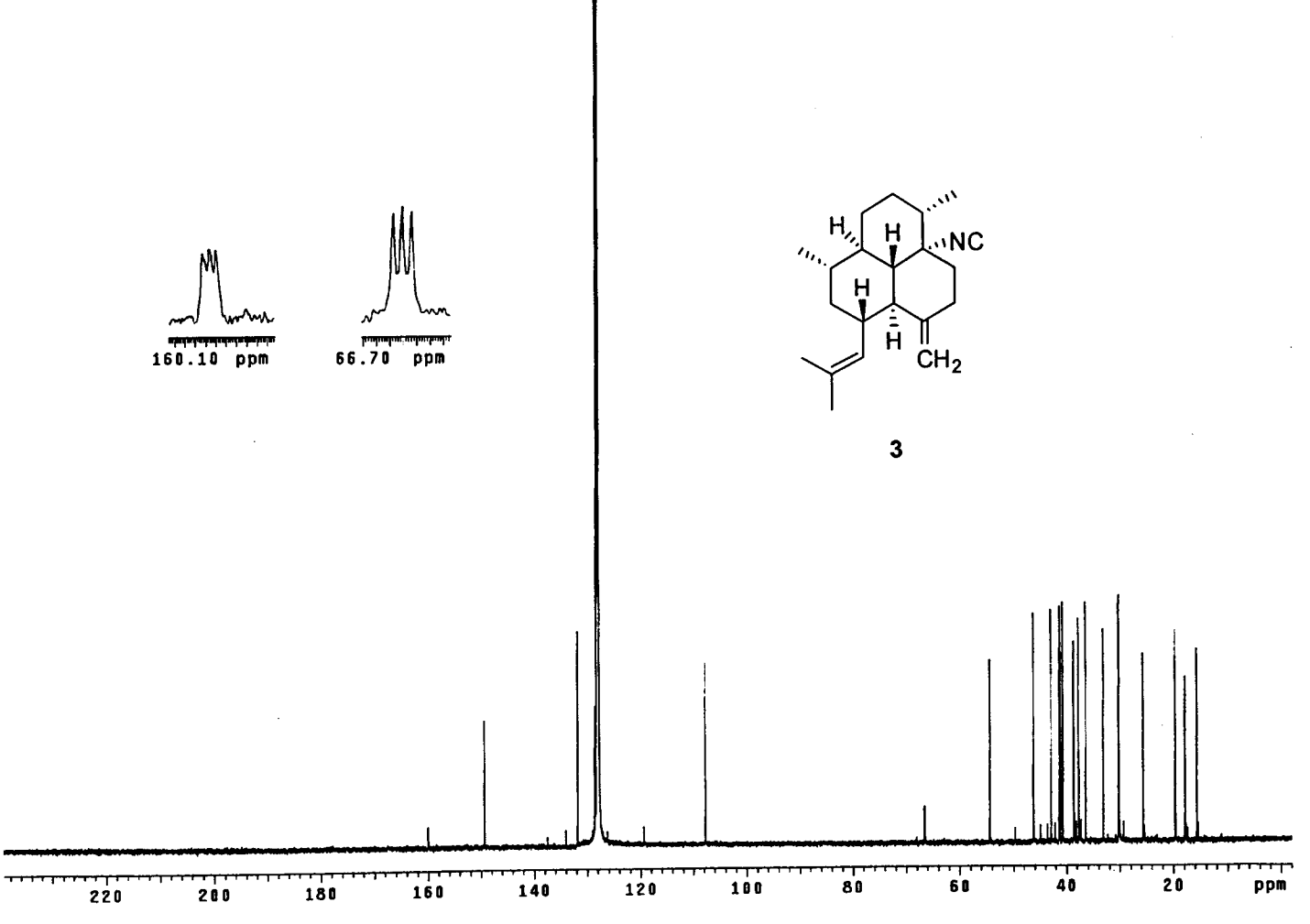

Figure 10 ^{13}C NMR spectrum of **3** (125 MHz, C_6D_6)

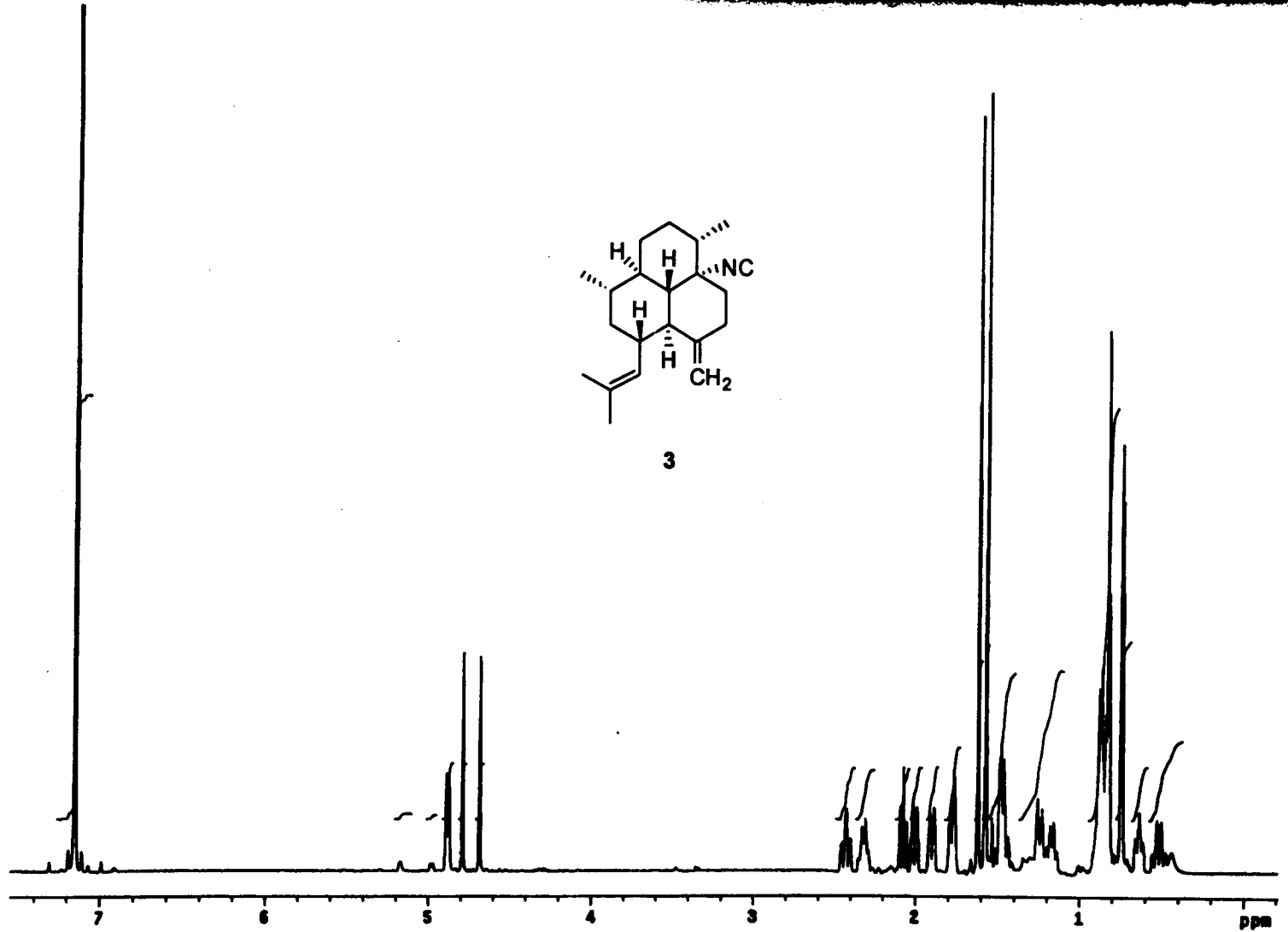

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Figure 11 ^{1}H NMR spectrum of 3 (500 MHz, C_6D_6)

Table 6 NMR data of **3** (500 MHz for ^{1}H and 125 MHz for ^{13}C; C_6D_6)

Position	^{13}C (mult)	^{1}H (mult; *J* in Hz)	Position	^{13}C (mult)	^{1}H (mult; *J* in Hz)
1	37.7 (CH)	2.32 (br ddd; 10.8, 10.0, 4.3)	10 a	33.1 (CH_2)	2.43 (ddd; 13.3, 13.0, 2.0)
			b		2.01 (ddd; 13.3, 2.1, 2.0)
2 a	41.2 (CH_2)	1.47 (m)	11	149.3 (C)	-
b		0.87 (m)	12	46.2 (CH)	2.07 (dd; 10.8, 10.7)
3	36.3 (CH)	0.88 (m)	13	54.3 (CH)	0.64 (ddt; 11.0, 10.7, 3.6)
4	42.9 (CH)	1.16 (br dd; 11.0, 10.6)	14	131.8 (CH)	4.88 (br d; 8.5)
5 a	30.1 (CH_2)	1.78 (br d; 10.6)	15	128.5 (C)	-
b		0.52 (dddd; 13.5, 13.2, 13.1, 4.1)	16	17.8 (CH_3)	1.57 (br s, 3H)
			17	25.6 (CH_3)	1.62 (br s, 3H)
6 a	30.1 (CH_2)	1.47 (m)	18	19.6 (CH_3)	0.75 (dd; 6.0, 2.1, 3H)
b		1.24 (br d; 14.7)	19	15.7 (CH_3)	0.83 (br d; 6.4, 3H)
7	40.7 (CH)	0.83 (m)	20 a	107.7 (CH_2)	4.79 (br s)
8	66.5 (C)[a]	-	b		4.68 (d; 1.4)
9 a	38.6 (CH_2)	1.90 (br dd; 13.0, 2.0)	21	159.9 (C)[a]	-
b		0.85 (m)			

Note; [a] t, $J = 3.7$ Hz.

3.2.1.4 The structure elucidation of compound 4

Compound **4** was the major component, obtained as white needles from hexane (101 mg), after the chromatographic separation on a SiO_2 column (step-gradient, hexane to 50% CH_3OH in CH_2Cl_2). The molecular formula of compound **4** was established as $C_{22}H_{32}N_2$ according to the pseudomolecular ion peak $[M+Na]^+$ at *m/z* 347 in ESIMS. The unsaturation degree of eight included two isonitriles, one olefin, and three rings. The infrared absorption confirmed the existence of the isonitrile groups at ν_{max} 2150 cm^{-1}. The UV spectrum showed the maximal absorption at λ_{max} 244 ($\log \varepsilon$ 2.41).

The NMR spectra of **4** (Figures 13 and 14) were almost identical to those of **1**, **2**, and **3**, with nevertheless two isonitriles and one olefin instead of one isonitrile and two vinyls as seen in the previous three. The two isonitrile functionalities were proposed to reside on C-8 (δ 66.7) and C-15 (δ 56.3), based on the ^{13}C-^{14}N coupling (t, J = 3.7 and 5.0 Hz, respectively; Figure 13), whereas the only vinyl group remaining here was on C-11 (δ 149.8) and C-20 (δ_C 106.5; δ_H 4.77, br s, H-20a; and 4.71, br s, H-20b). Confirmed by the X-ray crystallographic analysis (Figure 12), the structure of **4** was proposed as 8,15-diisocyano-11(20)-amphilectene (Ciavatta et al, 1999). The absolute configuration as shown was based on that reported by Ciavatta et al (2005), with the comparable $[\alpha]_D$'s (observed $[\alpha]_D$ = -56; *c* 0.28, $CHCl_3$; reported $[\alpha]_D$ = -56; *c* 1.50, $CHCl_3$). Please also note that the configuration as shown in Figure 12 is arbitrarily enantiomeric to the actual proposed herein. The doublet methyls of H-18 and H-19 were found coupled to H-3 and H-7 with the coupling constants of 6.0 and 6.3 Hz, respectively, in $CDCl_3$.

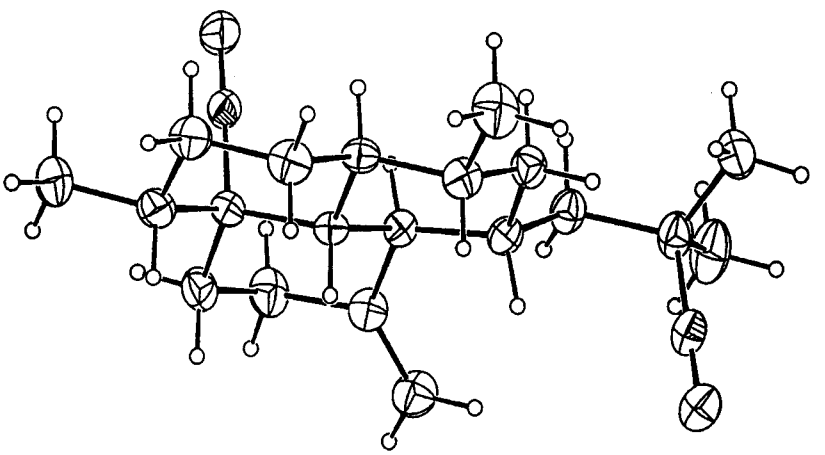


Figure 12 Computer-generated perspective drawing of **4**.

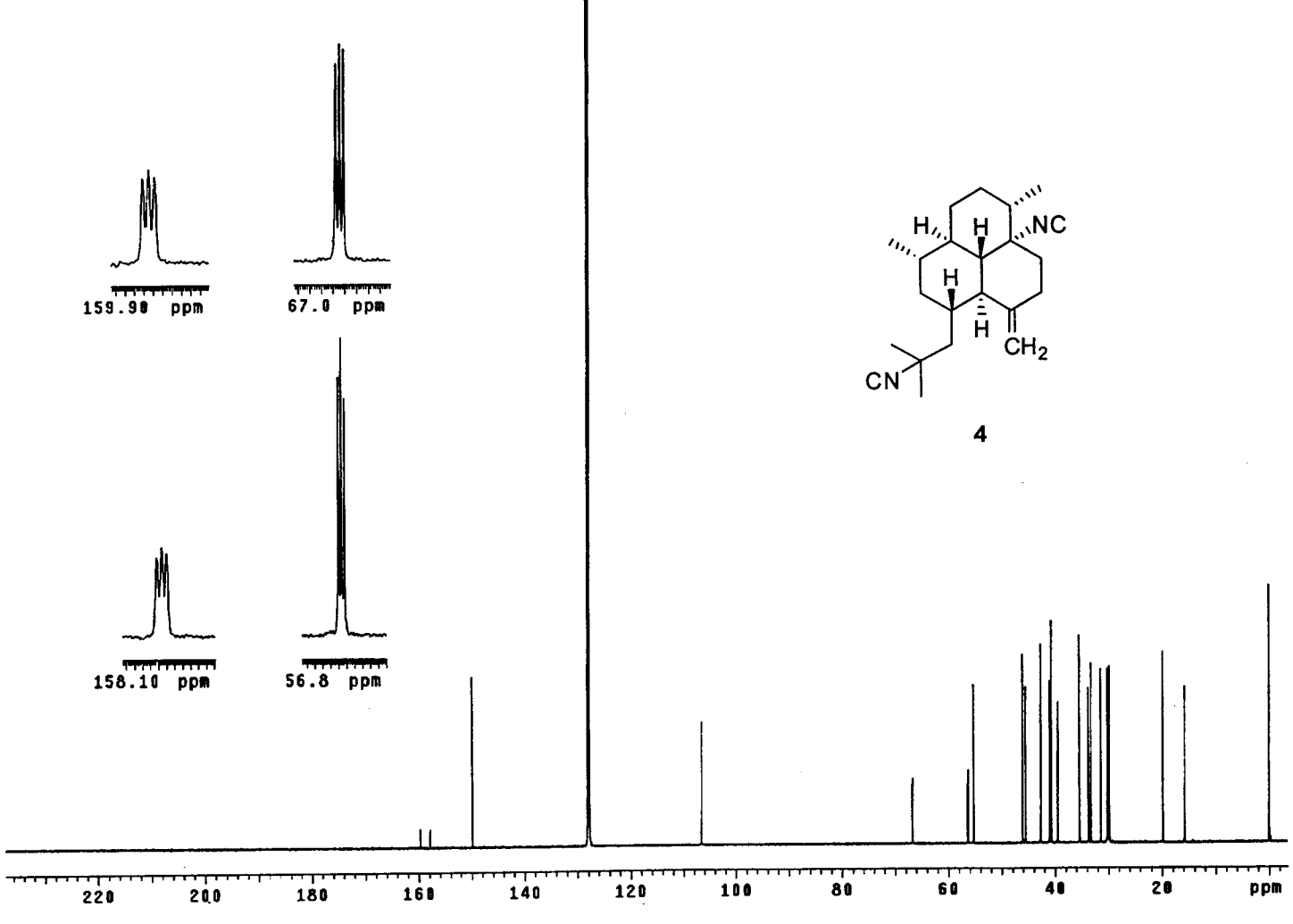


Figure 13 ^{13}C NMR spectrum of **4** (125 MHz, C_6D_6)

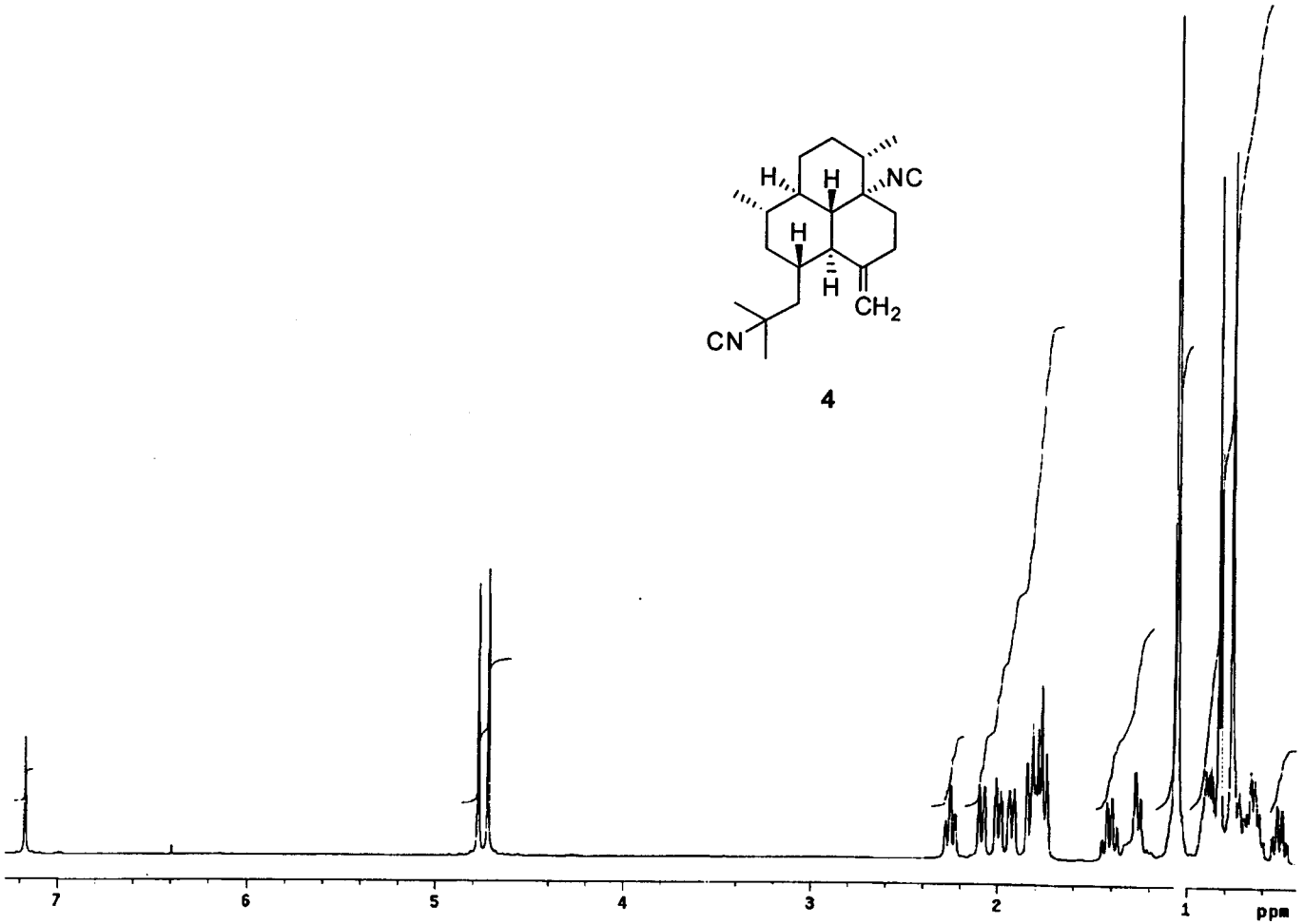
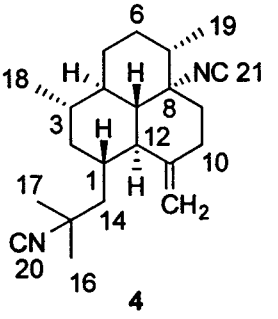


Figure 14 ^{1}H NMR spectrum of **4** (500 MHz, C_6D_6)



4

Table 7 NMR data of 4 (500 MHz for ^{1}H and 125 MHz for ^{13}C; C_6D_6)

Position	^{13}C (mult)	^{1}H (mult; *J* in Hz)	Position	^{13}C (mult)	^{1}H (mult; *J* in Hz)
	33.3 (CH)	1.80 (m)	10 a	33.7 (CH_2)	2.25 (ddd; 13.3, 12.8, 4.1)
a	41.0 (CH_2)	2.08 (br d; 13.3)	b		2.00 (ddd; 13.3, 4.1, 2.7)
b		0.62 (m)	11	149.8 (C)	-
	35.4 (CH)	1.04 (m)	12	46.1 (CH)	1.78 (m)
	42.7 (CH)	0.76 (m)	13	55.2 (CH)	0.67 (m)
a	29.8 (CH_2)	1.25 (br d; 13.8)	14 a	45.5 (CH_2)	1.81 (m)
b		0.50 (dddd; 13.4, 13.2,	b		0.71 (m)
		11.9, 4.1)	15	56.3 (C)[b]	-
a	30.0 (CH_2)	1.74 (m)	16	30.2 (CH_3)	1.05 (br s, 3H)
b		1.41 (dddd; 13.7, 13.2,	17	31.4 (CH_3)	1.05 (br s, 3H)
		12.4, 3.7)	18	19.8 (CH_3)	0.76 (br s, 3H)
	40.7 (CH)	0.84 (m)	19	15.7 (CH_3)	0.82 (d; 6.4, 3H)
	66.7 (C)[a]	-	20 a	106.5 (CH_2)	4.77 (br s)
a	39.5 (CH_2)	1.93 (br d; 13.3)	b		4.71 (br s)
b		0.88 (m)	21	157.8 (C)[b]	-
			22	159.7 (C)[a]	-

Note; [a] t, $J = 3.7$ Hz

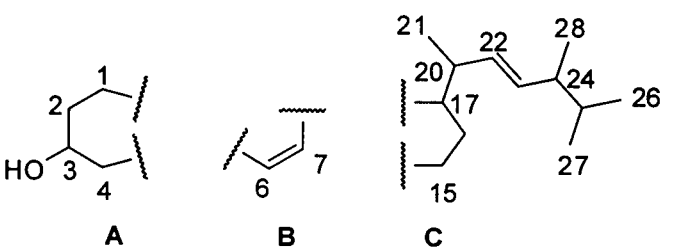
[b] t, $J = 5.0$ Hz.

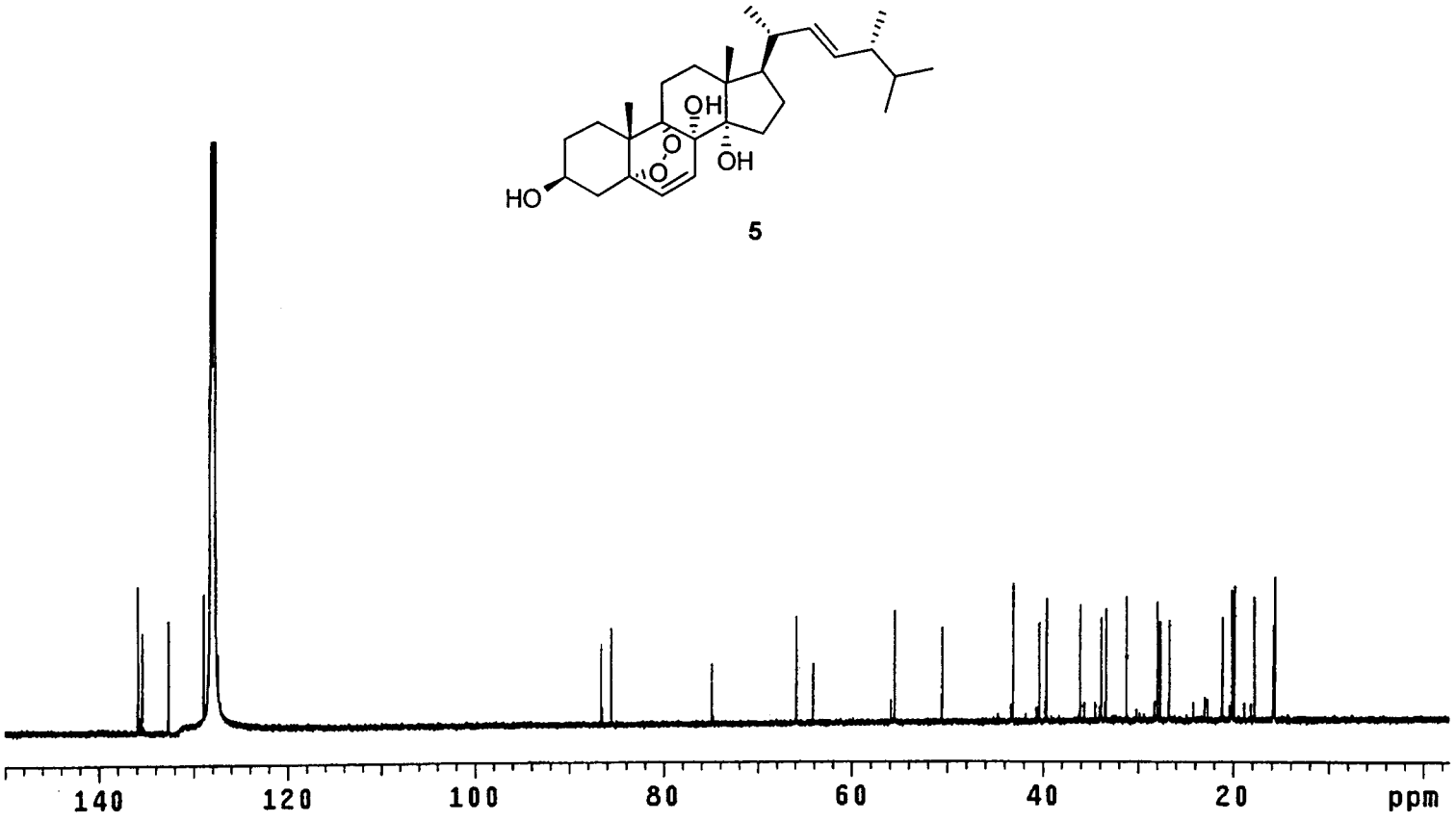
3.2.2 The structure elucidation of the sterol peroxides

3.2.2.1 The structure elucidation of compound 5

Compound **5** was obtained as white solid (3 mg) by means of a series of chromatographic techniques as followed; SiO_2 (step-gradient hexane to 50% CH_3OH in CH_2Cl_2), Sephadex LH-20 (50% EtOAc in hexane), SiO_2 (50% EtOAc in hexane; then THF:EtOAc:hexane 9:18:73), and HPLC RP-C18, (5% aq CH_3CN, t_R 21.1 min). The molecular formula of **5** was $C_{28}H_{44}O_5$, based on the numbers of carbons and protons suggested by the HMQC spectrum. The EI mass spectrum, although did not show the molecular peak as expected, showed an $[M-H_2O]^+$ signal at *m/z* 442. This was confirmed by the HR-EI mass spectrum, which also exhibited an pseudomolecular ion peak $[M-H_2O]^+$ at *m/z* 442.3085 (calcd for $C_{28}H_{42}O_4$, 442.3083), therefore concurring with the proposed molecular formula. This molecular formula indicated the unsaturation degree of seven, belonging to two olefinic double bonds and five rings.

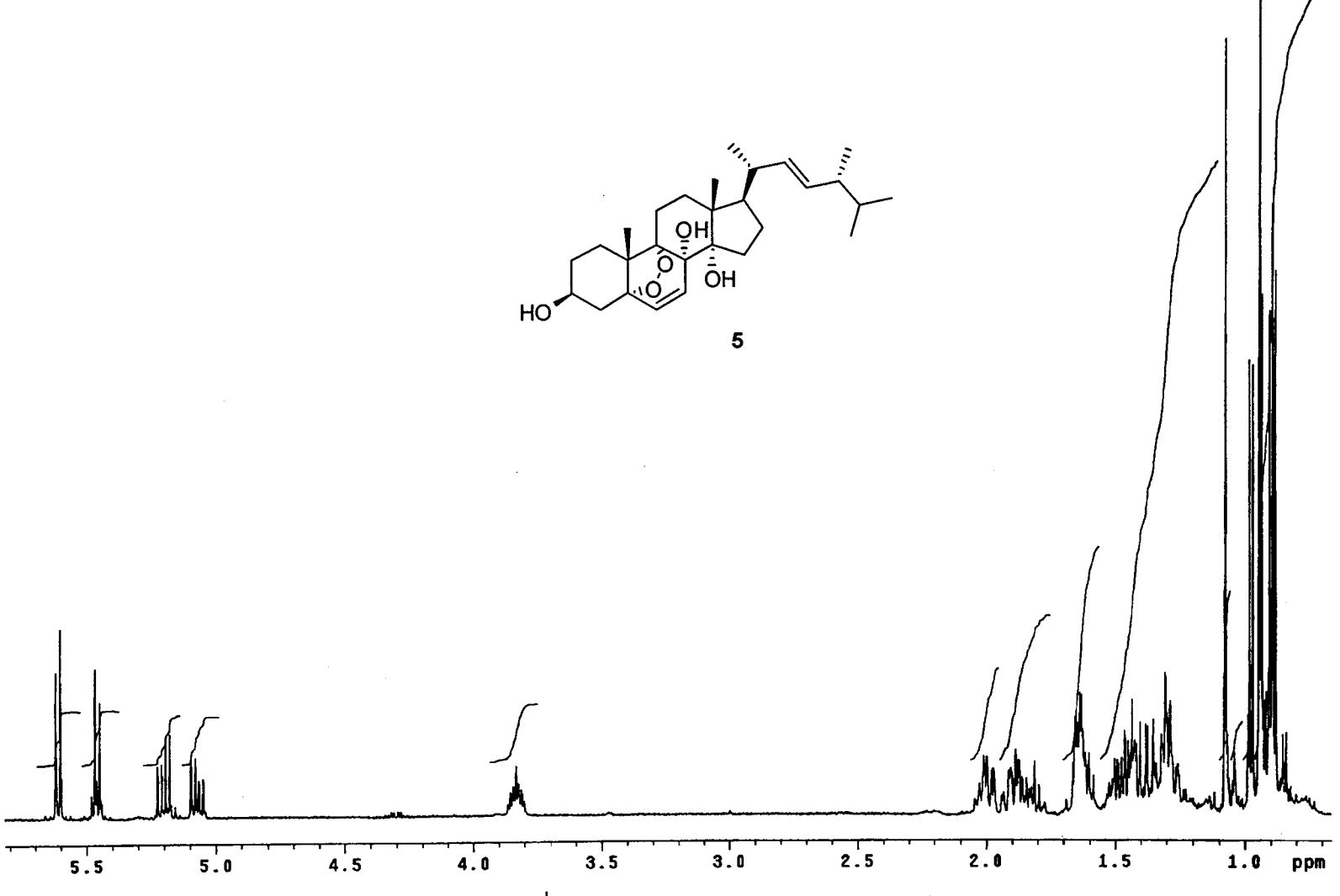
The ^{13}C and ^1H NMR spectra (Figures 15 and 16) showed characteristic resonances of steroid core skeleton, i.e., overlapping high-fielded methylene and methine signals with characteristic methyls of C-18 and C-19. All the high-fielded resonances were connected based on ^1H-^1H couplings observed from the COSY spectrum to yield three major fragments. These included fragments **A** at δ 1.92 (br d, J = 13.3 Hz; H-1_{ax}), 1.05 (ddd, J = 13.3, 3.3, 3.2 Hz; H-1_{eq}), 1.26 (m; H-2_{ax}), 1.61 (m; H-2_{eq}), 3.82 (dddd, J = 11.0, 9.6, 5.0, 4.5 Hz; H-3), 1.34 (m; H-4_{ax}) and 1.98 (dd, J = 14.2, 5.0 Hz; H-4_{eq}); fragment **B** at δ 5.61 (d, J = 9.6 Hz; H-6) and 5.45 (d, J = 9.6 Hz; H-7); fragment **C** at δ 1.80 (m; H-15a), 1.28 (m; H-15b), 1.58 (m; H-16a), 1.55 (m; H-16b), 1.32 (m; H-17), 2.02 (br d, J = 8.2 Hz; H-20), 0.93 (d, J = 6.9 Hz, 3H; H-21), 5.07 (dd, J = 15.1, 8.2 Hz; H-22), 5.20 (dd, J = 15.1, 7.8 Hz; H-23), 1.86 (m; H-24), 1.48 (m; H-25), 0.89 (d, J = 6.8 Hz, 3H; H-26), 0.88 (d, J = 6.9 Hz, 3H; H-27), and 0.97 (d, J = 6.9 Hz, 3H; H-28).





5

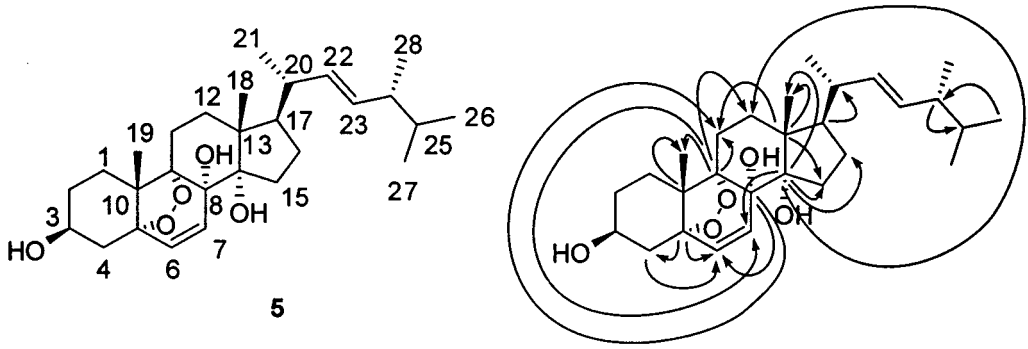
Figure 15 ^{13}C NMR spectrum of **5** (125 MHz, C_6D_6)



5

Figure 16 ^{1}H NMR spectrum of **5** (500 MHz, C_6D_6)

In order to connect all the three fragments, long-range C-H correlations from HMBC spectrum were analyzed. Fragment **A** was connected to **B** based on correlations from C-4 (δ 36.1) to H-6 and from C-5 (δ 85.5) to H-4 and H-6. Further correlation from C-14 (δ 74.8) to H-7 connected fragments **B** and **C**. For fragment **C** alone, a series of correlations from C-17 (δ 55.5) to H-20, from C-24 (δ 43.1) to H-25, and from C-26 (δ 19.9) to H-24 confirmed the proposed connectivity. The quaternary carbons at δ 40.4, 50.5, 64.1, 74.8, 85.5, and 86.6, were assigned to C-13, C-10, C-8, C-14, C-5, and C-9, respectively, by means of the HMBC spectral analysis. The correlations involved here included those from C-5 to H-4, and H-6; from C-8 to H-6, and H-11; from C-9 to H-7, H-11, H-12, and H-19; from C-10 to H-19; from C-13 to H-11, H-15, and H-18; and from C-14 to H-7, H-12, H-15, H-16, and H-18.



5

Figure 17 Structure of **5** with crucial HMBC correlations (C⟶H)

For the substituting groups on C-10 and C-13, two methyl groups of C-19 and C-18 were respectively assigned according to the correlations discussed earlier, therefore constructing a typical tetracyclic dimethyl steroid core skeleton. The low-fielded chemical shifts of C-5 (δ 85.5) and C-9 (δ 86.6), however, were typical to the peroxy-bearing carbon chemical shifts (Barrero et al, 2005; Christian et al, 2001; Monaco et al, 1987). A dioxy bridge, thus, was proposed to link between the two carbons complementing the additional unsaturation degree as indicated. The remaining oxygenated functional groups, coherent to that suggested in the molecular formula, were proposed here as all three hydroxyl groups, substituting on C-3, C-8, and C-14, based on the chemical shifts at δ 65.9, 64.1, and 74.8, respectively. The structure of **5** was proposed as 5,9-*epi*-dioxyergostan-3,8,14-triol, a new member of cyclic-peroxy sterol. The ^{13}C and ^{1}H NMR spectral data of **5** were summarized in Table 8.

Table 8 NMR data of **5** (500 MHz for ^{1}H and 125 MHz for ^{13}C; C_6D_6)

Position	^{13}C (mult)	^{1}H (mult; *J* in Hz)	HMBC correlation (C→H)
1 ax	27.9 (CH_2)	1.92 (br d; 13.3)	H-19
eq		1.05 (ddd; 13.3, 3.3, 3.2)	
2 ax	31.2 (CH_2)	1.26 (m)	H-4
eq		1.61 (m)	
3 ax	65.9 (CH)	3.82 (dddd; 11.0, 9.6, 5.0, 4.5)	H-4
4 ax	36.1 (CH_2)	1.34 (m)	H-6
eq		1.98 (dd; 14.2, 5.0)	
5	85.5 (C)	-	H-4, H-6
6	135.9 (CH)	5.61 (d; 9.6)	H-7
7	128.9 (CH)	5.45 (d; 9.6)	H-6
8	64.1 (C)	-	H-6, H-11
9	86.6 (C)	-	H-7, H-11, H-12, H-19
10	50.5 (C)	-	H-19
11 ax	20.1 (CH_2)	1.44 (m)	-
eq		1.64 (m)	
12 ax	33.8 (CH_2)	1.37 (m)	H-11, H-18
eq		1.67 (m)	
13	40.4 (C)	-	H-11, H-15, H-18
14	74.8 (C)	-	H-7, H-12, H-15, H-16, H-18
15 a	27.6 (CH_2)	1.80 (m)	H-16
b		1.28 (m)	
16 a	26.6 (CH_2)	1.58 (m)	-
b		1.55 (m)	
17	55.5 (CH)	1.32 (m)	H-16, H-18, H-20, H-21
18	15.8 (CH_3)	0.93 (s, 3H)	-
19	15.6 (CH_3)	1.06 (s, 3H)	-
20	39.6 (CH)	2.02 (br d; 8.2)	H-22, H-23

Table 8 (cont.)

Position	^{13}C (mult)	^{1}H (mult; *J* in Hz)	HMBC correlation (C→H)
21	21.1 (CH_3)	0.93 (d; 6.9, 3H)	-
22	135.4 (CH)	5.07 (dd; 15.1, 8.2)	H-21, H-24
23	132.6 (CH)	5.20 (dd; 15.1, 7.8)	H-20, H-24
24	43.1 (CH)	1.86 (m)	H-22, H-23, H-26, H-27, H-28
25	33.3 (CH)	1.48 (m)	H-27
26	19.9 (CH_3)	0.89 (d; 6.8, 3H)	H-25
27	20.1 (CH_3)	0.88 (d; 6.9, 3H)	H-24
28	17.8 (CH_3)	0.97 (d; 6.9, 3H)	H-23, H-24

The relative configuration of compound **5** was determined on the basis of the coupling constant analysis and nOe-ds experiments. The large coupling constants of H-3 (J = 11.0 and 9.6 Hz) indicated the axial orientation. The C-3 hydroxyl group was therefore assigned as an equatorial β orientation. Upon the irradiation of H-7, the enhancements of H-18 and H-19 were observed (Figure 18). As the two methyls resided on the β plane as axial methyls due to their chemical shifts (δ 15.8, C-18; 15.6, C-19), the olefinic part of the bicyclic moiety therefore resided on the same plane, i.e., β orientation. The dioxy bridge, on the other hand, was assigned as α. For the side chain on C-17, the configuration shown here was proposed based on the similarity in the chemical shifts to most ergostane derivatives (Wang et al, 2008). The configurations of all chiral centers of **5** were therefore proposed as 3*S*, 5*S*, 8*S*, 9*S*, 10*S*, 13*R*, 14*S*, 17*R*, 20*S*, and 24*R*.

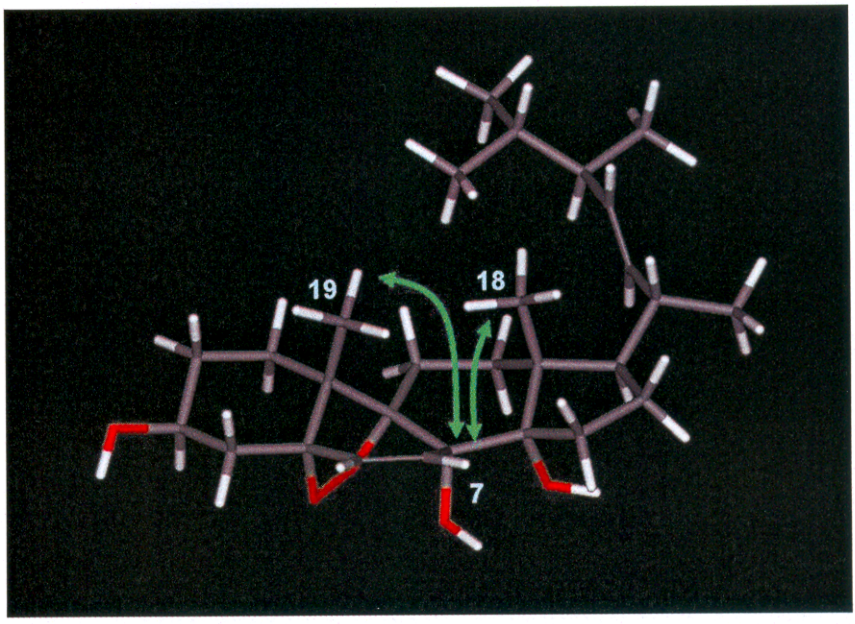


Figure 18 nOe Correlations of **5**

3.2.2.2 The structure elucidation of compound 6

Compound **6** was obtained as white solid (4 mg) from the same isolation steps as those of **5**. The retention time of **6** from the final HPLC separation step was 33.8 min.

The molecular formula of **6** was established as $C_{28}H_{44}O_3$ based on the molecular ion peak $[M]^+$ at *m/z* 428 in EIMS spectrum. Almost identical to those of compound **5**, the spectral data of **6**, including the ^{13}C and ^{1}H NMR spectra (Figures 19 and 20), indicated that the two compounds were closely related, bearing the similar ergostane-type steroid skeleton. The major differences among **5** and **6** were observable in the ^{13}C NMR spectrum, in which two carbons formerly assigned to C-9 and C-14 for compound **5**, shifted towards the up-fielded region, respectively resonating at δ 51.7 and 52.0 in the spectrum of **6**. This corresponded well with the molecular formula of **6** with two oxygen atoms less than that of **5**, suggesting the absence of two hydroxyl groups formerly seen in **5**. Also, the dioxy-bearing carbons, characterized by the low-fielded chemical shifts, were now assigned to C-5 (δ 81.6) and C-8 (δ 78.8). Therefore, the dioxy bridge of **6** migrated from a 5,9 dioxy into a 5,8 one. The structure of **6** was proposed to be 5,8-*epi*-dioxyergostan-3-ol (Kobori et al, 2006; Nam et al, 2001), with the relative configuration referred to those reported in Nam et al (2001). The NMR chemical shifts of **6** are summarized in Table 9.

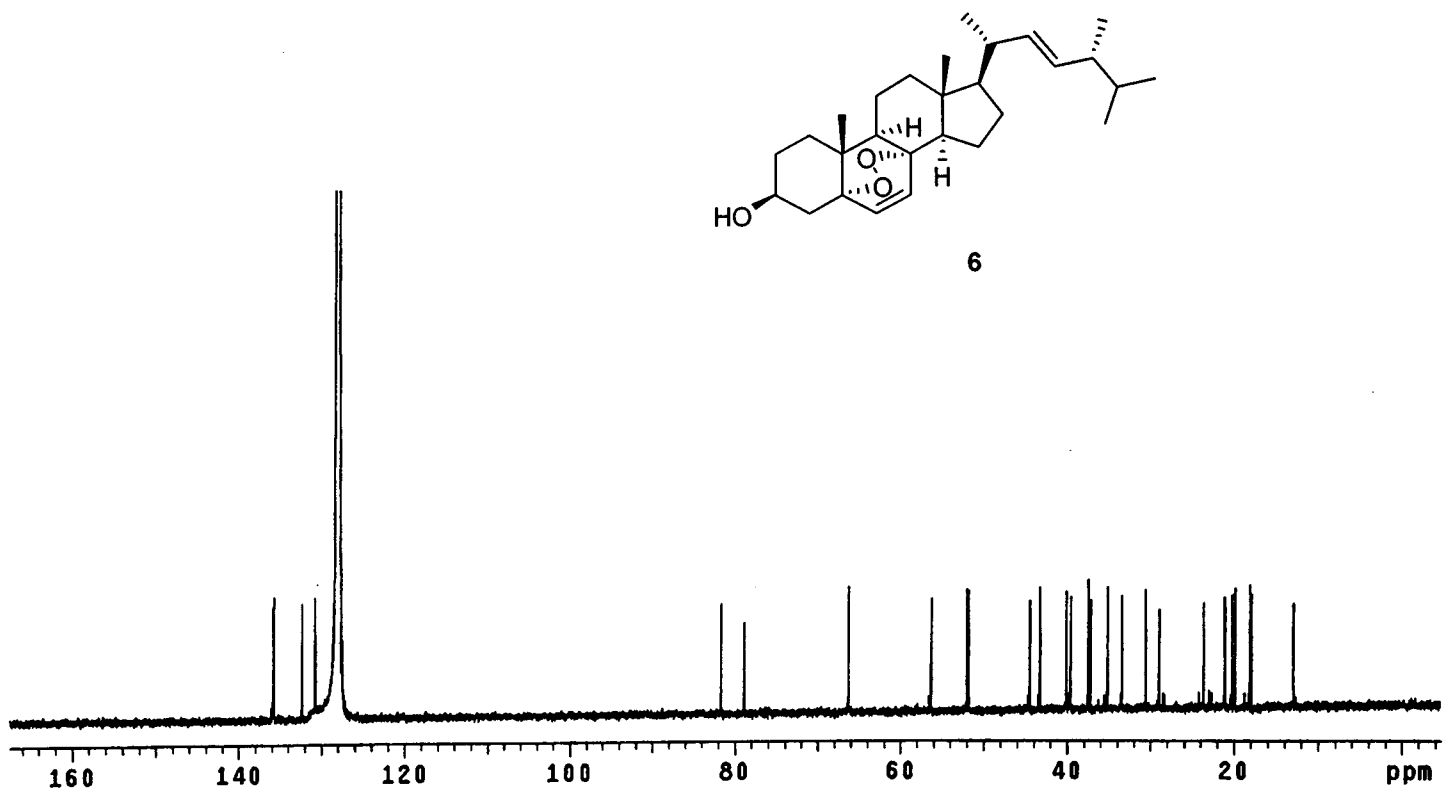


Figure 19 ^{13}C NMR spectrum of **6** (125 MHz, C_6D_6)

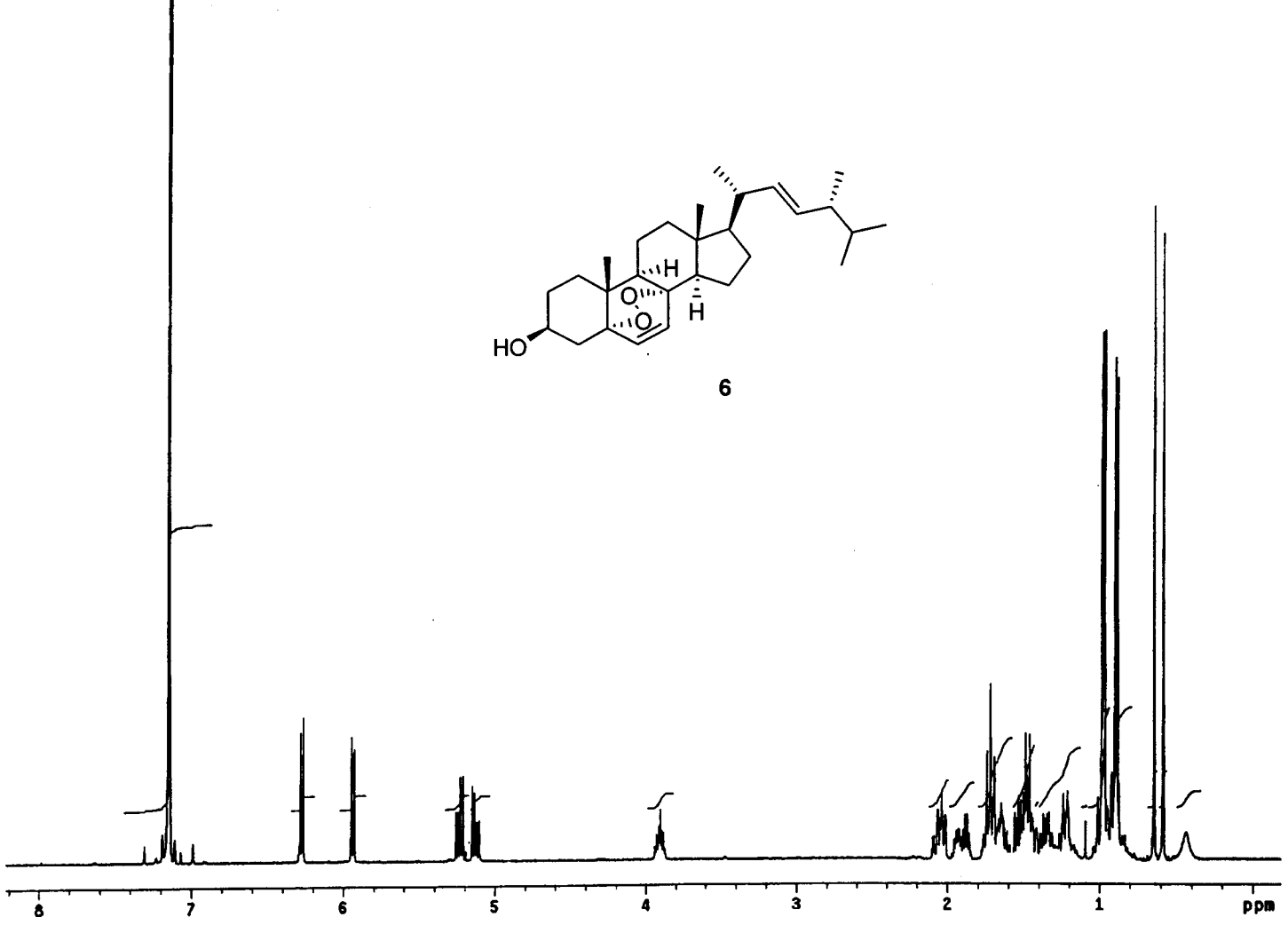
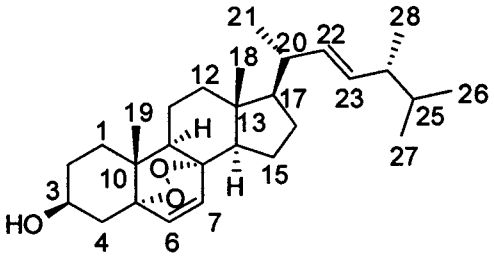


Figure 20 ^{1}H NMR spectrum of **6** (500 MHz, C_6D_6)



6

Table 9 NMR data of **6** (500 MHz for ^{1}H and 125 MHz for ^{13}C; C_6D_6)

Position	^{13}C (mult)	^{1}H (mult; *J* in Hz)	Position	^{13}C (mult)	^{1}H (mult; *J* in Hz)
1 ax	35.1 (CH_2)	2.08 (br dd; 13.8, 3.2)	13	44.4 (C)	-
eq		1.48 (m)	14	52.0 (CH)	1.54 (br d; 7.4)
2 ax	30.5 (CH_2)	1.43 (m)	15 a	21.6 (CH_2)	1.75 (m)
eq		1.66 (m)	b		1.33 (m)
3 ax	66.2 (CH)	3.90 (dddd; 11.0, 10.6,	16 a	28.9 (CH_2)	1.38 (m)
		5.9, 5.0)	b		1.23 (m)
4 ax	37.4 (CH_2)	1.70 (m)	17	56.2 (CH)	0.98 (br d; 6.9)
eq		2.03 (br dd; 4.0, 1.9)	18	12.9 (CH_3)	0.59 (s, 3H)
5	81.6 (C)	-	19	18.2 (CH_3)	0.66 (s, 3H)
6	135.7 (CH)	5.94 (d; 8.4)	20	40.1 (CH)	1.93 (qdd; 6.9, 9.2)
7	130.7 (CH)	6.27 (d; 8.4)	21	21.1 (CH_3)	0.99 (d; 6.9, 3H)
8	78.8 (C)	-	22	135.8 (CH)	5.12 (dd; 15.1, 8.6)
9	51.7 (CH)	1.51 (m)	23	132.3 (CH)	5.24 (dd; 15.1, 7.7)
10	37.2 (C)	-	24	43.8 (CH)	1.87 (br qd; 6.9, 13.7)
11 ax	23.6 (CH_2)	1.62 (m)	25	33.4 (CH)	1.47 (m)
eq		1.21 (m)	26	20.2 (CH_3)	0.89 (d; 6.9, 3H)
12 ax	39.6 (CH_2)	1.73 (m)	27	19.8 (CH_3)	0.88 (d; 6.8, 3H)
eq		0.92 (br dd; 6.4, 2.7)	28	17.6 (CH_3)	0.97 (d; 6.9, 3H)

3.3 Biological activities

All the four isonitrile diterpenes and two sterol peroxides were subjected to the biological activity determinations, namely the antimalarial activity against *P. falciparum* K1 strain, and cytotoxicity against MCF-7 cell line. The activities of all the tested compounds are shown in Table 10.

For the isonitriles, the IC_{50}'s for the antimalarial activity was found in the range of 10^{-2}-10^{0} μM. This was in a comparable range to that of the standard dihydroartemisinin (IC_{50} = 4.4 nM). Also, the reported potencies as shown were all in good agreement to those previously reported (IC_{50}'s in 10^{-2}-10^{0} μM range) (Wright and König, 1996). In particular, compound **4**, which was the most active compound reported here, showed the potency in the closest magnitude to that of the standard. The sterol peroxides, although less active with IC_{50}'s in the magnitude of 10^{0} μM, were however considered among the strong antimalarial agents that have been reported from natural products.

Table 10 The antimalarial activity and cytotoxicity of isolated compounds

Compounds	Antimalarial activity (IC_{50} in μM)	Cytotoxicity (IC_{50} ± SD in μM)
8-isocyano-amphilecta-11(20),15-diene (**1**)	0.98	inactive[a]
7-isocyanoamphilecta-11(20),15-diene (**2**)	1.07	inactive[a]
8-isocyano-amphilecta-11(20),14-diene (**3**)	0.44	NA[b]
8,15-diisocyano-11(20)-amphilectene (**4**)	0.09	9.02 ± 0.1
5,9-epidioxyergostan-6,22-dien-3,8,14-triol (**5**)	7.13	0.0032 ± 0.0002
5,8-epidioxyergostan-6,22-dien-3-ol (**6**)	6.28	0.0254 ± 0.0016
artemisinin	0.01	12.14
dihydroartemisinin	0.004	-
camptothecin	-	0.0016 ± 0.0001

Note: [a] Tested samples showed lower than 10% inhibition against the targeted cell line at the highest concentration of 5 μg/mL.

[b] The IC_{50} was unable to be calculated due to statistically unagreeable results.

The cytotoxicity of all four isonitriles is in a range of 10^0-10^2 μM, and also agreed well with the reported data (Wright and König, 1996). On the other hand, the potent cytotoxicity in the nM scale of the steroidal peroxides, which is in a comparable magnitude to that of standard camptothecin, is more interesting and suggests a promising potential for the further application. The unusual functional groups associated with both classes of compounds focused in the report are the aspects of interest. Both isonitrile and peroxy functionalities have been regarded among the bioactive functional groups, and were responsible for several biological activities (Angerhofer and Pezzuto, 1992; Dembitsky, 2007). Bearing strong redox potentials, the isonitrile and peroxy groups were proposed to target on the oxidation of various biological metal-protein complexes, therefore either causing a direct damage on the metals or the complexes themselves, or generating free radicals and/or reactive species that were harmful to biological systems of invading cells; i.e., the parasites and cancerous tissues (Murakami et al, 2004; Singh et al, 2002; Wright et al, 2001).