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ScienceDirect
Tetrahedron
Tetrahedron 63 (2007) 4725–4729

Terpenoid metabolites of the nudibranch *Hexabranchus sanguineus* from the South China Sea

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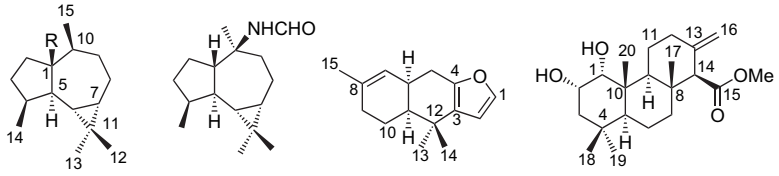
Received 18 December 2006; revised 22 February 2007; accepted 15 March 2007
Available online 18 March 2007

Abstract—Chemical analysis of the secondary metabolite pattern of the nudibranch *Hexabranchus sanguineus* collected from the South China Sea revealed the presence of both sesquiterpenoids and diterpenoids, exhibiting very different structural features. Two new molecules, compounds **1** and **4**, were isolated together with known compounds **2** and **3**, and chemically characterized. This is the first report of terpenoids from *H. sanguineus*. The remarkable diversity of metabolites possessed by *H. sanguineus* supports some recent phylogenetic studies that place the mollusc near the steam of the dorid nudibranch tree.

1. Introduction

The Spanish dancer nudibranch *Hexabranchus sanguineus* (Rüppell and Leuckart, 1828) is a large, brilliantly coloured shell-less sea slug. The metabolite pattern of *H. sanguineus* and its egg masses is remarkable for a suite of unusual oxazole-containing macrolides (ulapualides, kabiramides and halichondramides),[1–3] which play a defensive role and are also responsible for their cytotoxic and antimicrobial activities.[3,4] Similar metabolites were also isolated from the sponge *Halichondria* sp.,[3] which the nudibranch eats. Some of the compounds were even synthesized by different groups.[5–7]

In the course of our studies on the chemical ecology of nudibranch molluscs, some years ago we examined a specimen of *H. sanguineus* collected at the Red Sea, and isolated, in addition to oxazole-containing macrolides, the pigment hurghadin, for which an alternative defensive role was suggested.[8] Our ongoing chemical investigation on the same species has now resulted in the finding, in a single individual collected at the South China Sea, of a series of terpenoid constituents, compounds **1**–**4**, two of which, sesquiterpene **1** and diterpene **4**, are novel molecules. The structural elucidation of these new metabolites is described here. In addition, these chemical data are compared with those reported in the literature for the same mollusc and discussed in terms



1 R = NHCHO
*5 R = CN
*6 R = SCN

2

3

4

*Absolute stereochemistry not implied

Keywords: Nudibranch; *Hexabranchus sanguineus*; Sesquiterpenes; Diterpenes.
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0040–4020/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tet.2007.03.082

of evolutionary trends in feeding and defence for *H. sanguineus* within dorids.

2. Results and discussion

Frozen *H. sanguineus* (one individual) was carefully dissected into mantle and internal organs, which were separately extracted by acetone. A comparative chromatographic analysis of the diethyl ether fractions of the acetone extracts revealed different metabolite patterns for the two parts. A series of red-coloured spots at R_f 0.55–0.25 (diethyl ether) were detected in both fractions by spraying with $Ce(SO_4)_2$, while an Ehrlich-positive compound at R_f 0.45 (light petroleum ether/diethyl ether, 99:1) was observed only in the extract of internal glands of the animal. Both crude extracts (128 mg from mantle and 160 mg from internal organs) were fractionated by silica gel column chromatography followed by either Sephadex LH-20 or reverse-phase HPLC purifications, yielding the main metabolite **1** (18.0 mg) and the minor related compound **2** (1.6 mg) from both anatomical parts, whereas the other major sesquiterpenoid **3** (14.0 mg) and diterpenoid **4** (3.2 mg) were isolated only from the internal organs.

Known sponge metabolites, compounds **2** and **3**, were identified by comparison of their spectral data (NMR, MS, $[\alpha]_D$) with the literature as axamide-2[9,10] and *ent*-furodysinin,[11–15] respectively. The new compounds **1** and **4** were subjected to a careful spectroscopic analysis in order to determine their structures. In addition, the assignment of ^{13}C NMR values of **2** (*cis*/*trans*, 1:1), not described in the previous papers, has been made in the present work (see Table 1).

The molecular formula $C_{16}H_{27}NO$ of compound **1**, which was deduced by HRESIMS on the sodiated-molecular ion at *m*/*z* 272, was the same as axamide-2 (**2**). It also showed spectral data very similar to those of **2**, in particular displaying signals due to the same alloaromadendrane skeleton and formamide functional group (Table 1). In fact, in the ^1H NMR spectrum were present typical signals due to *cis*-cyclopropyl protons at δ 0.48 (dd, J=9 and 11 Hz, H-6) and 0.72 (ddd, J=7, 9 and 11 Hz, H-7) and to a formamide group (*trans*-isomer) at δ 8.17 (d, J=12 Hz, –CHO) and 5.86 (d, J=12 Hz, –NHCO–). Four methyls were detected at δ 1.06 (s, H_3-12), 1.03 (s, H_3-13), 0.95 (d, J=7 Hz, H_3-14) and 0.87 (d, J=6 Hz, H_3-15), this latter indicating that in compound **1** the formamide group was located in a different position compared with **2**. Mainly the *trans*-isomer of the two possible rotational isomers of formamide **1** was detected in the $CDCl_3$ solution. Analysis of ^1H–^1H COSY spectrum of **1** led to the determination of all proton sequence from C-2 to C-10, whereas HMBC experiments allowed the assignment of the formamide group at C-1 as reported in formula. In fact, a diagnostic long-range correlation was observed between C-1 (δ 69.2) and the formamide proton at δ 8.17. The relative stereochemistry of **1** was deduced by analysis of ^1H–^1H coupling constants, NOE difference and NOESY experiments as well as by comparison of ^{13}C NMR data with literature values of model compounds. First of all, the large coupling constant (J=11 Hz) between H-5 and H-6 indicated a *trans*-diaxial stereochemistry for these two protons. Irradiation of H-6 signal caused a significant enhancement of –NH proton (8.5%) implying a *trans*-junction between rings A and B. Steric interactions were also detected between –NH proton and the two secondary methyl groups at C-4 and C-10 indicating that H_3-14 and H_3-15 were orientated in the same side as formamide moiety, as reported in formula **1**. The proposed stereochemistry was supported by comparison of carbon values of **1** with those of isonitrile **5** and isothiocyanate **6**, previously isolated from *Acanthella* sponges,[16,17] the relative stereochemistry of

Table 1. NMR data[a] for **1**[b] (*trans*) and axamide-2 (**2**)[b,c]

No.	**1**			**2** (*trans*)	**2** (*cis*)
	δ_H, m, J in Hz	δ_C, m[d]	HMBC (H–C)	δ_C, m[d]	δ_C, m[d]
1		69.2, s		56.7, d	53.6, d
2α	1.50, m	36.0, t	1, 5	26.8, t	26.8, t
2β	2.30, dd, 6.4, 14.0		1, 4, 5		
3α	1.32, m	31.8, t	1, 5	34.2, t	34.4, t
3β	1.99, m		1, 4, 5		
4	2.25, m	34.2, d	1, 5, 14	36.5, d	36.5, d
5	1.66, t, 11.3	48.0, d	1, 4, 6, 10, CHO	38.9, d	38.7, d
6	0.48, dd, 8.9, 11.3	22.2, d	4, 8, 11, 12, 13	26.8, d	26.8, d
7	0.72, ddd, 6.6, 8.9, 11.1	27.2, d	5, 6, 12	28.6, d	28.6, d
8α	1.04, m	24.6, t		20.0, t	20.0, t
8β	1.90, ddd, 6.6, 7.1, 14.7		6, 7, 9, 10		
9α	1.44, dd, 6.1, 14.3	32.3, t	1, 8, 10, 15	44.8, t	39.8, t
9β	1.31, m		1, 8, 10		
10	1.33, m	47.3, d		57.9, s	59.6, s
11		20.5, s		20.2, s	20.2, s
12	1.06, s	28.9, q	6, 7, 13	28.6, q	28.6, q
13	1.03, s	15.3, q	5, 6, 7, 12	15.8,[e] q	15.8,[f] q
14	0.95, d, 7.3	19.2, q	3, 4, 5	16.1,[e] q	16.1,[f] q
15	0.87, d, 6.4	17.9, q	1, 9, 10	18.6, q	18.0, q
CHO	8.17, d, 12.4	163.2, d	1	162.6, d	160.0, d
NH	5.86, d, 12.4				

[a] Bruker-DRX-400 spectrometer, $CDCl_3$, chemical shifts (ppm) referred to $CHCl_3$ (δ_H 7.26) and to $CDCl_3$ (δ_C 77.0).
[b] Assignments made by ^1H–^1H COSY, HMQC, HMBC and NOESY.
[c] ^{13}C NMR values were not reported in the previous papers (Refs. 9 and 10).
[d] By DEPT sequence.
[e,f] Assignments may be reversed.

which has been determined by different methods. In fact, a substantial similarity of δ ^{13}C was observed, with the expected β- or γ-*gauche* effects on the resonances of C-2 (δ 36.6 in **1**, δ 39.7 in **6**), C-6 (δ 22.2 in **1**, δ 24.3 in **6**) and C-10 (δ 47.3 in **1**, δ 49.1 in **6**) due to the formamide substituent at C-1 in the place of –NCS group. All carbon and proton resonances of **1** were assigned by 2D NMR experiments as reported in Table 1. Due to the positive value of $[\alpha]_D$, the absolute stereochemistry of **1** should be proposed to be the same as that of (+)-palustrol, as reported by Braekman et al.[17] In that same paper, a formamide derivative was obtained by chemical conversion of compounds **5** and **6**, but it was not characterized.

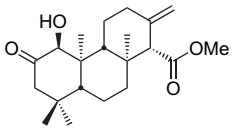
Compound **4** had the molecular formula $C_{21}H_{34}O_4$, deduced by HRESIMS on the sodiated-molecular ion at *m/z* 373. Analysis of ^1H and ^{13}C NMR spectra immediately revealed the diterpenoid nature of the carbon skeleton of **4** exhibiting a methyl ester function [δ_C 172.7 (s) and 50.9 (q), δ_H 3.64 (s)], an exocyclic double bond [δ_C 141.6 (s) and 108.2 (t); δ_H 4.68 (br s) and 4.83 (br s)] and two secondary hydroxyl groups [δ_C 73.6 (d) and 66.3 (d); δ_H 3.59 (m) and 4.09 (ddd, J=2.9, 4.4 and 12.1 Hz). The ^1H NMR spectrum showed signals due to four tertiary methyls [singlets at δ 0.86 (6H, H_3-18 and H_3-20), 0.93 (3H, H_3-19), 1.08 (3H, H_3-17)] that suggested a diterpene tricyclic framework, according to the five unsaturation degrees required by the molecular formula. An isolated proton resonating at δ 2.89 (s, H-14) was located in the α position to the carboxyl methyl ester group. ^1H–^1H COSY experiments established three distinct spin systems that were assigned to the three rings as reported in Table 2. In particular, the carbinol proton at δ 3.59 was correlated to the other alcoholic methine at δ 4.09, which was further coupled with a methylene [δ 1.55 (m) and 1.43 (m), H_2-3], supporting the location of both secondary hydroxyl groups in the same ring. These data were consistent with the structure of an isocopalane acid methyl ester displaying a vicinal diol function in ring A. HMBC experiments corroborated this structural hypothesis (see Table 2), in particular significant long-range connectivities were observed between H-14 and C-15, between H-1 and both C-5 and C-20 and between H_3-19 and C-2 confirming the position of the carboxyl function and of the two secondary hydroxyl groups as reported in formula **4**.

Table 2. NMR data for **4**[a,b]

No.	δ_H, m, J in Hz	δ_C, m[c]	HMBC (H–C)
1	3.59, m	73.6, d	2, 3, 5, 20
2	4.09, ddd, 2.9, 4.4, 12.1	66.3, d	
3α	1.55, m	43.3, t	1, 2, 4, 18, 19
3β	1.43, m		1, 2, 5, 19
4		34.3, s	
5	1.32, dd, 2.5, 12.5	47.9, d	18, 19, 20
6α	1.38, m	18.1, t	5, 7, 8
6β	1.56, m		
7α	1.32, m	39.7, t	8
7β	1.58, m		5, 8, 9, 17
8		42.2, s	
9	1.78, dd, 2.6, 12.4	50.2, d	7, 8, 10, 11, 12, 14, 20
10		39.7, s	
11α	1.70, dt, 2.6, 12.9	22.0, t	9, 10, 12, 13, 20
11β	1.48, m		
12α	2.13, dt, 5.4, 13.2	35.8, t	11, 13, 16
12β	2.42, ddd, 2.0, 4.4, 13.2		9, 11, 13, 14, 16
13		143.5, s	
14	2.89, s	63.1, d	7, 8, 12, 13, 15, 16, 17
15		172.0, s	
16a	4.68, br s	108.2, t	12, 13, 14
16b	4.83, br s		12, 14
17	1.08, s	15.2, q	7, 9, 14
18	0.86, s	22.3, q	3, 5, 19
19	0.93, s	33.1, q	2, 3, 4, 5, 18
20	0.86, s	16.4, q	1, 9
OMe	3.64, s	50.9, q	15
1-OH	1.65, m		1, 2, 3, 9
2-OH	2.08, m		1, 3

[a] Bruker-DRX-400 spectrometer, $CDCl_3$, chemical shifts (ppm) referred to $CHCl_3$ (δ_H 7.26) and to $CDCl_3$ (δ_C 77.0).
[b] Assignments made by ^1H–^1H COSY, HMQC, HMBC and NOESY.
[c] By DEPT sequence.

The relative stereochemistry of **4** was deduced from coupling constants as well as by NOESY experiments. Analysis of J_{H-H} values in the ^1H NMR spectrum suggested an equatorial orientation for H-1 resonating as a sharp multiplet whereas the vicinal proton H-2 has to be axial due to the large $J_{2,3}$ value (12 Hz) with H-3ax. Diagnostic NOE effects were detected between H_3-20 and both H-1 and H-2, and between H-9 and both H-5 and H-14, in agreement with the proposed stereochemistry. The *ent*-isocopalane absolute configuration of **4** was determined by comparing the CD profile with those of a series of isocopalane and *ent*-isocopalane diterpenoids,[18–23] the absolute stereochemistry of which has been secured by synthesis. Isocopalane and *ent*-isocopalane diterpenoids exhibiting oxidized ring A are quite rare in nature even though they have been already reported from sponges (i.e., **7**)[24] and nudibranchs (i.e., **8**).[23]



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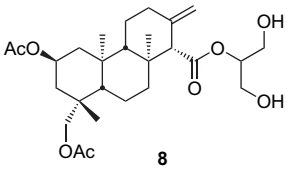

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3. Conclusion

The secondary metabolite pattern of the nudibranch *H. sanguineus* from the South China Sea was significantly different from that observed in previous chemical studies on the same mollusc. In fact, it was characterized by terpenoids with very different structural features, most likely deriving from distinct sponges, whereas the typical macrolides were absent. The main metabolite **1** and the related axamide-2 (**2**), based on alloaromadendrane framework, are members of an interesting group of nitrogen-containing sesquiterpenoids that are typical metabolites of marine sponges of the genera *Axinella* and *Acanthella*,[25] also isolated from some phyllidiid nudibranchs feeding on these sponges. Axamide-2 (**2**) has only been reported as a minor metabolite of Mediterranean *Axinella cannabina*.[9,10] *ent*-Furodysinin (**3**), the absolute stereochemistry of which has been determined by synthesis,[12] is a typical furanosesquiterpene metabolite of sponges belonging to genus *Dysidea*[11] and has been also reported from *Hypselodoris* nudibranchs[13,14] as well as from *Ceratosoma* nudibranchs.[15] The occurrence of *ent*-furodysinin in *Hypselodoris* and *Ceratosoma* nudibranchs has been ascribed to their probable dietary relationship with *Dysidea* sponges. In particular, the transfer of compound **3** from

sponge to mollusc has been rigorously demonstrated for *Hypselodoris webbi* feeding on *Dysidea fragilis*.[14] Finally, compound **4** displays the *ent*-isocopalane skeleton that is widely present in metabolites of sponges of the genus *Spongia*[26,27] and also of doridiidae nudibranchs[18,28] in which a de novo biosynthetic origin of isocopalane diterpenoids has been demonstrated.[28]

In contrast, previous chemical studies on different populations of *H. sanguineus* resulted in the isolation of oxazole-containing macrolides[1–4] that are also present in the sponge[3] on which the nudibranch was feeding. Our results show that the mollusc is able to feed on distinct sponges and to sequester secondary metabolites with different chemical features.

These results allow us to elaborate somewhat on evolutionary trends in feeding and defence. The latest phylogenetic studies report for the genus *Hexabranchus* some primitive characteristics and place it near the stem of the dorid nudibranch tree.[29] It is uncertain whether the ancestral dorids were generalist or specialist sponge feeders. However, within the dorids, there does seem to be a general trend in the direction of increasing specialization in the use of secondary metabolites. For example, within the Chromodorididae the anatomically primitive genus *Cadlina* contains a broad range of metabolites, whereas the more derived genera are more specialized: *Glossodoris* mainly on sesterterpenoids, *Hypselodoris* on sesquiterpenoids, *Chromodoris* on diterpenoids and *Phyllidia* on isocyanides. *Hexabranchus* would appear to be unspecialized with respect to the range of metabolites that it utilizes. It contains typical sponge metabolites as would be expected if sponges were the food of the common ancestor of the dorids.[30] These metabolites are characteristic of several groups that are more specialized. In its isocyanides it resembles the Phyllidiidae. In its terpenoids it resembles the Chromodoridiidae.

In conclusion, remembering the previous studies on *H. sanguineus*, this chemical study confirms that *Hexabranchus* species are generalist sponge feeders and offer further support to the phylogenetic tree suggested by Valdés.[29]

4. Experimental

4.1. General

Si-gel chromatography was performed using precoated Merck F_{254} plates and Merck Kieselgel 60 powder. HPLC purification was carried out on a Waters liquid chromatography equipped with a UV detector. Optical rotations were measured on a Jasco DIP 370 digital polarimeter, and CD curves were recorded in EtOH solution at a concentration of 0.0005 M on a JASCO 710 spectropolarimeter. The IR spectra were taken on a Bio-Rad FTS 7 spectrophotometer. 1D and 2D NMR spectra were acquired in $CDCl_3$ (reported δ values are referred to C*H*Cl_3 at 7.26 ppm) on a Bruker Avance-400 operating at 400 MHz, using an inverse probe fitted with a gradient along the *Z*-axis. ^{13}C NMR spectra were recorded on a Bruker DPX-300 operating at 75.5 MHz (δ values are reported to *C*DCl_3, 77.0 ppm) using a dual probe. ^{1}H and ^{13}C NMR assignments were supported by ^{1}H–^{1}H COSY, HMQC, HMBC and NOESY experiments. EIMS spectrum was recorded on a Thermo Focus GC Polaris Q spectrometer (electron impact 70 eV) equipped with an ion trap. Both ESIMS and HRESIMS spectra were recorded on a Q-TOF Micro LC–MS–MS mass spectrometer.

4.2. Biological material

A single individual of *H. sanguineus* was collected along the coast of Xiaodong Hai, Hainan Province, China, in December 2001, at a depth of 20 m. The specimen was identified by one of us (E.M.) and immediately frozen at −20 °C. Subsequently, it was transferred to Italy, at ICB for the chemical work.

4.3. Extraction and isolation procedures

Frozen *H. sanguineus* (one individual) was carefully dissected into mantle and internal organs, which were separately extracted by acetone. After removing the organic solvent, the aqueous residue was partitioned with Et_2O (3×20 mL). Soluble Et_2O fractions were concentrated at reduced pressure to yield 128 mg of Et_2O extract from the mantle, and 160 mg from the internal organs that were separately chromatographed on silica gel column using a petroleum ether/Et_2O gradient as eluent. The mantle extract yielded two terpene-containing fractions at R_f 0.54 and 0.27 (diethyl ether), fractions A (6.1 mg) and B (18.8 mg), along with usual lipid and steroid components. Fraction A was subjected to Sephadex LH-20 ($CHCl_3$) chromatography to afford pure compound **1** (2.9 mg), whereas fraction B was purified by reverse HPLC (Kromasil RP-18 (5 μm, 250× 10 mm), MeOH/H_2O (7:3), 3.0 mL/min, UV 200 nm) to give compound **2** (0.3 mg). The extract of internal organs yielded, in order of elution time, pure compound **3** [14.0 mg, R_f 0.45 (light petroleum ether/diethyl ether, 99:1)] and, analogously with mantle extract, two terpene-containing fractions at R_f 0.54 and 0.27 (diethyl ether), fractions 1 (22.2 mg) and 2 (8.2 mg), along with usual lipid and steroid components. Fraction 1 was subjected to Sephadex LH-20 ($CHCl_3$) chromatography to afford, in order of elution time, pure compound **1** (15.0 mg) and a mixture, which was further purified by *n*-HPLC (Kromasil Si (5 μm, 250×4.6 mm), *n*-hexane/*iso*-propanol (98:2), 1.0 mL/min, UV 200 nm, 17.0 min) to give compound **4** (3.2 mg). Fraction 2 was subjected to reverse HPLC in the same conditions as fraction B to yield compound **2** (1.4 mg).

4.4. Spectral data of compounds 1–4

4.4.1. Compound 1. $C_{16}H_{27}NO$, colourless oil, $[\alpha]_D^{20}$ +16.7 (*c* 1.0, $CHCl_3$). IR (liquid film) ν_{max} 3560 and 1683 cm^{-1}. ^{1}H and ^{13}C NMR spectral values are given in Table 1. ESIMS *m*/*z* 521.11 ([2M+Na]$^+$), 272.06 ([M+Na]$^+$). HRESIMS *m*/*z* 272.2005 (calcd for $C_{16}H_{27}NONa$, 272.1990).

4.4.2. Axamide-2 (2). $C_{16}H_{27}NO$, colourless oil, $[\alpha]_D^{20}$ +79.0 (*c* 0.14, $CHCl_3$); $[\alpha]_D^{20}$ lit.[9] +37.5 (*c* 0.9, $CHCl_3$). Selected ^{1}H NMR ($CDCl_3$, 300 MHz): δ (*trans*) 8.28 (1H, d, *J*=12.3 Hz, CHO), 5.63 (1H, d, *J*=12.3 Hz, NH), 1.95 (1H, m, H-1), 1.21 (3H, s, H_3-15), 1.01 (3H, s, H_3-12), 1.00 (3H, d, *J*=6.9 Hz, H_3-14), 1.00 (3H, s, H_3-13), 0.63 (1H, ov, H-7), 0.58 (1H, ov, H-6); δ (*cis*) 8.01 (1H, d, *J*=2.1 Hz, CHO), 5.16 (1H,

d, J=2.1 Hz, NH), 2.44 (1H, m, H-1), 1.25 (3H, s, H_3-15), 1.00 (3H, s, H_3-12), 0.99 (3H, s, H_3-13), 0.92 (3H, d, J= 6.9 Hz, H_3-14), 0.63 (1H, ov, H-7), 0.58 (1H, ov, H-6). ^{13}C NMR spectral values are given in Table 1. ESIMS m/z 521.10 ([2M+Na]$^+$), 272.04 ([M+Na]).

4.4.3. *ent*-Furodysinin (3). $C_{16}H_{27}NO$, colourless oil, $[\alpha]_D^{20}$ −21.2 (c 0.56, $CHCl_3$); synthetic prod.[12] $[\alpha]_D^{20}$ −54 (c 0.5, $CHCl_3$). Selected ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.21 (1H, d, J=1.8 Hz, H-1), 6.23 (1H, d, J=1.8 Hz, H-2), 5.62 (1H, br s, H-7), 1.66 (3H, s, H_3-15), 1.20 (3H, s, H_3-13 or H_3-14), 1.18 (3H, s, H_3-13 or H_3-14). EIMS m/z 216 (M$^+$), 201, 122, 107.

4.4.4. Compound 4. $C_{21}H_{34}O_4$, white foam, UV λ_{max}^{MeOH}, nm: 204.0 (log ε 3.45), $[\alpha]_D^{20}$ −11.8 (c 0.13, $CHCl_3$). CD $[\theta]_{220.6}$ (EtOH) −3270. IR (liquid film) ν_{max} 3446, 2949, 1740 cm^{-1}. ^{1}H and ^{13}C NMR spectral values are given in Table 2. ESIMS m/z 723.67 ([2M+Na]$^+$), 373.23 ([M+Na]$^+$). HRESIMS m/z 373.2340 (calcd for $C_{21}H_{34}O_4Na$, 373.2355).

Acknowledgements

This research work was financially supported by 'National Marine 863 Project' (No. 2006AA09Z412), STCSM Project (No. 054307062), and CNR (Italy)/CAS (China) Joint International Project 2005/2007. The authors thank Mr. F. Castelluccio for his precious technical assistance, Mr. C. Iodice for spectrophotometric measurements and Mr. R. Turco for drawing. The NMR spectra were recorded at the ICB-NMR service, the staff of which is acknowledged.

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