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New spirocyclic sesquiterpenes from the marine sponge *Geodia exigua*

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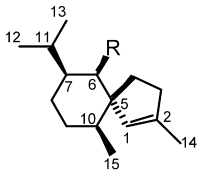
Abstract—Three new spirocyclic sesquiterpenes designated exiguamide (**1**), exicarbamate (**2**) and exigurin (**3**), together with (−)-10-*epi*-axisonitrile-3 (**4**), have been isolated from the marine sponge *Geodia exigua*. All four compounds possess the spiro[4.5]decene skeleton and their structures were determined on the basis of spectroscopic data. The structure of **1** was confirmed by X-ray crystallographic analysis and the absolute configuration was determined by applying the modified Mosher's method on its amine derivative. Exiguamide (**1**) inhibited cell fate specification during sea urchin embryogenesis at a minimum inhibitory concentration of 0.4 μM.

1. Introduction

The sea urchin embryo provides an excellent model to study the molecular mechanisms of cell fate specification.[1] In the fourth cleavage during the early development of sea urchins, eight blastomeres of the same size divide unequally into four larger macromeres, four smaller micromeres, and eight mesomeres. Micromeres give rise to spiculogenetic primary mesenchyme cells at later developmental stages. In the course of our search for selective inhibitors of echinoderm embryonic development from marine organisms,[2–10] we found that the methanolic extract of *Geodia exigua* Thiele inhibited the formation of micromeres in sea urchin embryos. A preliminary examination of the bioactive hexane-soluble extract led to the isolation of a new spirocyclic sesquiterpene designated exiguamide (**1**).[11] Further investigation of the extract has resulted in the isolation of two new analogous sesquiterpenes designated exicarbamate (**2**) and exigurin (**3**) in addition to (−)-10-*epi*-axisonitrile-3 (**4**). We now report on the isolation, structure elucidation and biological activities of these sesquiterpenes.



1 R : -NHCHO
2 R : -NHCO$_2$CH$_3$
3 R : -NHCOCH$_2$N(CH$_3$)CH$_2$COOCH$_3$
4 R : -NC
5 R : -NH$_2$

2. Results and discussion

The MeOH extract of the sponge *G. exigua* was partitioned between hexane and H_2O. Subjection of the bioactive hexane-soluble fraction to ODS column chromatography with MeOH–H_2O gradient mixtures afforded several fractions. Further individual purification of three fractions on silica gel column employing 0–100% EtOAc in hexane as eluent furnished compounds **1**–**4**.

Exiguamide (**1**) was obtained as colorless crystals (mp 139–140°C) upon crystallization from MeOH–H_2O. Its molecular formula, $C_{16}H_{27}NO$, was determined by high resolution FAB mass spectrometry (HRFABMS) (*m/z* 250.2174 $[M+H]^+$, Δ +0.3 mmu). The IR spectrum showed the presence of NH (3323 cm^{-1}) and amide and C=C (1657 cm^{-1}) functionalities. Analysis of the ^1H NMR

Keywords: biologically active compounds; natural products; spiro compounds; sponges.

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Table 1. ^{1}H NMR spectral data of **1**–**4**

Position	**1**[a]	**2**[b]	**3**[b]	**4**[b]
1	5.58 br s	5.53 br s	5.67 br s	5.25 br s
2				
3a	2.34 ddd (15.8, 8.8, 7.5)	2.74 ddd (15.8, 8.7, 7.6)	2.82 m	2.11 ddd (16.4, 8.1, 8.1)
3b	1.99 br dd (15.8, 8.8)	2.09 ddd (15.8, 8.7, 2.1)	2.14 ddd (15.6, 8.8, 2.5)	1.95 ddd (16.4, 9.0, 2.9)
4a	1.80 ddd (12.6, 7.5, 2.1)	2.27 ddd (12.5, 7.6, 2.1)	2.23 ddd (12.2, 7.5, 2.5)	2.28 ddd (13.0, 8.1, 2.9)
4b	1.42 ddd (12.6, 8.8, 8.8)	1.60 ddd (12.5, 8.7, 8.7)	1.64 m	1.82 ddd (13.0, 9.0, 8.1)
6	3.82 dd (10.5, 3.5)	4.08 dd (10.5, 3.5)	4.48 dd (11.1, 3.5)	3.51 br s
6-NH	7.62 br d (10.5)	4.59 d (10.5)	7.56 d (11.1)	
7	1.11 m	1.26 m	1.36 m	0.96 m
8ax	1.31 m	0.79 qd (13.5, 3.5)	1.02 m	1.49 m
8eq	1.56 m	1.46 m	1.66 m	1.49 m
9ax	1.74 tt (13.5, 5.4)	1.69 tt (13.5, 3.5)	1.84 m	1.56 m
9eq	1.44 m	1.32 m	1.43 m	1.42 m
10	1.47 m	1.57 m	1.71 m	1.55 m
11	1.30 m	1.40 m	1.55 m	1.72 m
12	0.86 d (6.5)	0.88 d (6.6)	0.98 d (6.6)	0.86 d (6.6)
13	0.68 d (6.5)	1.05 d (6.6)	1.12 d (6.6)	0.82 d (6.6)
14	1.68 br s	1.62 br s	1.68 br s	1.51 br s
15	0.95 d (7.5)	0.87 d (7.6)	1.14 d (7.5)	1.29 d (7.8)
16	8.08 br s			
16-OCH_3		3.55 s		
17			3.05 s (2H)	
17-NCH_3			2.09 s	
18			2.85 s (2H)	
19-OCH_3			3.28 s	

Coupling constants, J_{H-H} (in Hz), are given in parentheses.
[a] Data measured in DMSO-d_6 at 500 MHz.
[b] Data measured in C_6D_6 at 500 MHz.

(Table 1), ^{13}C NMR (Table 2), ^{1}H–^{1}H COSY, HMQC and HMBC spectral data revealed **1** to be a spiro[4.5]decene having the formylamino, isopropyl and two methyl groups, thus allowing the gross structure of **1**. The relative stereochemistry of **1** was elucidated on the basis of difference NOE experiments and confirmed by X-ray crystallographic analysis (Fig. 1).[11]

Exicarbamate (**2**) had a molecular formula of $C_{17}H_{29}NO_2$, which was determined by (+)-HRFABMS (*m/z* 280.2267 $[M+H]^+$, Δ −0.9 mmu). Analyses of the ^{1}H and ^{13}C NMR spectral data revealed that **2** possessed the same spiro[4.5]-decene skeleton as **1** differing only on the functional group attached to C-6 and that the formylamino moiety in **1** was replaced by the methoxycarbonylamino functionality which was supported by IR absorption bands at 3366 (N–H) and 1711 cm^{-1} (C=O). Detailed analysis of the ^{1}H–^{1}H COSY, HMQC and HMBC (Fig. 2) spectral data allowed determination of the gross structure of **2**. The relative stereochemistry of **2** was elucidated on the basis of difference NOE experiments. An enhancement of signals of three protons on C-15 and one of the protons on C-8 (H-8ax) upon irradiation of the NH proton indicated that they were all in *cis* 1,3-diaxial positions relative to one another around the six-membered ring, and defined a chair conformation for the cyclohexane ring, as shown in Figure 3. Irradiation of the olefinic proton (H-1) enhanced the signals of H-6, H-7, H-9ax, H-10 and H_3-14, placing these protons on the same face of the cyclohexane ring. These

Table 2. ^{13}C NMR spectral data of **1**–**4**

Position	**1**[a]	**2**[b]	**3**[b]	**4**[b]
1	131.77 d	131.62 d	131.82 d	130.20 d
2	139.29 s	140.88 s	140.81 s	141.67 s
3	34.67 t	35.38 t	35.48 t	34.69 t
4	33.76 t	34.48 t	34.85 t	34.99 t
5	56.16 s	56.96 s	57.10 s	55.14 s
6	50.33 d	55.33 d	52.39 d	59.43 d
7	43.37 d	44.36 d	44.77 d	44.07 d
8	19.41 t	19.92 t	20.36 t	19.70 t
9	29.42 t	29.75 t	30.15 t	29.62 t
10	36.34 d	37.11 d	37.24 d	37.12 d
11	28.99 d	29.87 d	30.20 d	30.02 d
12	21.05 q	21.21 q	21.51 q	20.78 q
13	20.36 q	21.15 q	21.22 q	19.98 q
14	16.78 q	16.99 q	17.07 q	16.72 q
15	15.87 q	16.59 q	16.54 q	16.31 q
16	161.16 d	157.22 s	168.98 s	161.44 s
16-OCH_3		51.78 q		
17			61.55 t	
17-NCH_3			43.19 q	
18			58.41 t	
19			170.64 s	
19-OCH_3			51.03 q	

[a] Data measured in DMSO-d_6 at 125 MHz.
[b] Data measured in C_6D_6 at 125 MHz.

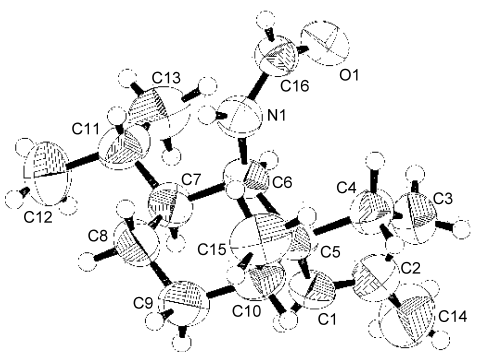

Figure 1. Perspective view of the crystal struture of **1**.

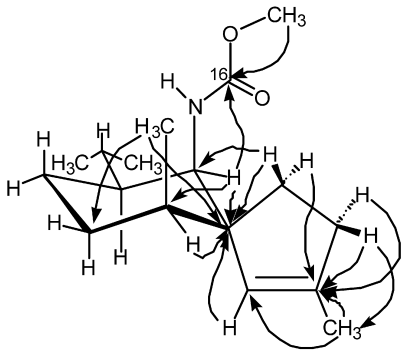

Figure 2. Key $^{1}H-^{13}C$ HMBC correlations observed for **2**.

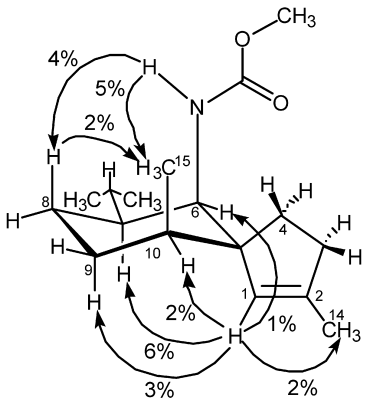

Figure 3. Key NOEs observed for **2**.

findings indicate that **2** possesses the same relative stereochemistry as **1**.

Exigurin (**3**) gave a molecular ion at *m/z* 364.2732 appropriate for the molecular formula $C_{21}H_{36}N_2O_3$ (Δ +0.6 mmu) in the HREIMS. Its IR spectrum indicated the presence of NH (3393 cm^{-1}), carbonyl ester (1742 cm^{-1}) and amide (1645 cm^{-1}) functionalities. Extensive analysis of the NMR data showed that **3** is a C-6 functional group analog of **1** and **2**. The HMBC spectral data (Fig. 4) indicated that the substituent at C-6 in **3** is a 2-(methoxy-carbonylmethyl-methyl-amino)-acetylamino group. The similarity of the ^{1}H and ^{13}C chemical shifts and $^{1}H-^{1}H$ coupling constants at C-1 to C-15 between **3** and both **1** and

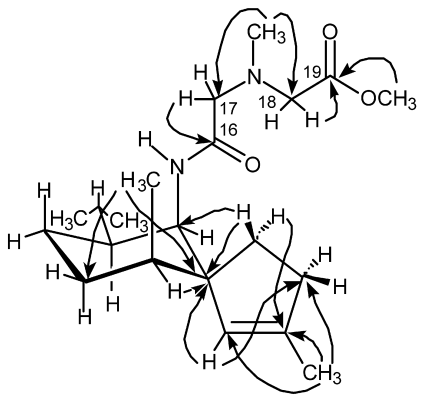

Figure 4. Key $^{1}H-^{13}C$ HMBC correlations observed for **3**.

2 indicates that the relative stereochemistry of **3** is the same as **1** and **2**.

Compound **4** {$[\alpha]_D^{25}$ −43.5°} was obtained as a colorless oil that gave a molecular ion at *m/z* 231.1980 corresponding to the molecular formula $C_{16}H_{25}N$ (Δ −0.7 mmu) in the HREIMS. The ^{1}H and ^{13}C NMR spectral data of **4** were identical to those of 10-*epi*-axisonitrile-3,[12] indicating that **4** has the same relative structure as 10-*epi*-axisonitrile-3, the absolute configuration of which has not been determined.

(−)-10-*epi*-Axisonitrile-3 (**4**) was converted into **1** by hydration. Exiguamide (**1**) was hydrolyzed to an amine derivative **5** with 10% HCl. The absolute configuration at C-6 of **5** was elucidated by the application of the modified Mosher's method.[13] Two portions of **5** were treated separately with (−)- and (+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPACl) in pyridine to afford the (*S*)- and (*R*)-α-methoxy-α-(trifluoromethyl)-phenylacetyl (MTPA) derivatives (**6a** and **6b**), respectively. The $\Delta\delta$ ($\delta_S-\delta_R$) values are shown in Figure 5. The $\Delta\delta$ values for H-1, H_2-3, H_2-4, H-10, H_3-14 and H_3-15 were negative. The values for H-7, H_2-8, H-11, H_3-12 and H_3-13 were positive. In accordance with the modified Mosher model, the absolute configuration of C-6 was determined to be *R*. Thus, the absolute stereochemistry of exiguamide (**1**) and (−)-10-*epi*-axisonitrile-3 (**4**) was elucidated to be 5*S*,6*R*,7*S*,10*S*.

Due to the paucity of material, the absolute stereochemistry of **2** and **3** has not been experimentally determined. However, because **1**–**4** were derived from the same origin, it is likely that **2** and **3** share the same absolute stereochemistry (5*S*,6*R*,7*S*,10*S*) as that of **1** and **4**.

Chemical investigation of marine sponges belonging to the Geodiidae family has led to the isolation of interesting new bioactive compounds which include a series of sterols[14] and steroids,[15] geodiastatins,[16] barettin,[17] geodiamolides,[18,19] geodiatoxins,[20]geodisterol,[21] geodin A magnesium salt,[22] stelleferin riboside[23] and stellettins A and B.[23] Recently, geoditins A and B were isolated from *G. japonica*.[24] However, to the best of our knowledge, there have been no reports of sesquiterpenes bearing the spiro[4.5]decene skeleton from this genus. Such a carbon skeleton has only been found within the genera *Axinella*,[25] *Acanthella*[26] and *Topsentia*.[27]It has been shown by previous studies,[25,28–32] that isocyanides/isonitriles often co-exist with the corresponding thiocyanates and formamides in a single animal species. In some cases, the corresponding amines or

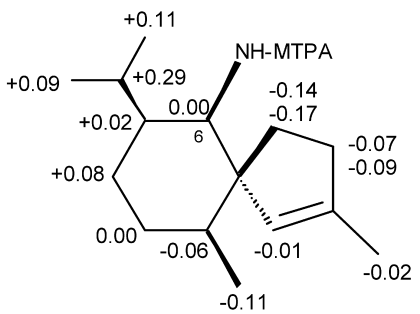

Figure 5. $\Delta\delta$ values [($\delta_S-\delta_R$) in ppm] for MTPA esters **6a** and **6b**.

Table 3. Inhibitory effects of **1**–**4** on developmental events of sea urchin embryos

Event	Minimum inhibitory concentration (μM)			
	1	**2**	**3**	**4**
Formation of micromeres	0.4	>360	>270	>430
Formation of spicule	0.4	>360	>270	>430

ammonium chloride salt forms were also isolated.[33] We believe this is the first occurrence of an isonitrile co-existing with 2-(methoxycarbonylmethyl-methyl-amino)-acetyl-amino, methoxycarbonylamino, and formylamino groups.

When fertilized eggs of the sea urchin, *Hemicentrotus pulcherrimus*, were cultured in the presence of 0.4–12.0 μM exiguamide (**1**), they divided equally to form 16-cell embryos that were comprised of sixteen cells of the same size. In a control experiment, normal embryos formed four macromeres, four micromeres, and eight mesomeres at the same 16-cell stage. After passing through the blastula and then gastrula stages, the **1**-treated embryos developed to form spicule-deficient plutei (Table 3). There are very few substances having the biological activity.[34] Compounds **2**–**4** did not exhibit such activity. Therefore, it is concluded that the difference of functional groups attached at the C-6 of these compounds reflects the difference of the inhibitory activities. Exiguamide (**1**) is considered to be a useful tool for elucidating the mechanism of cell fate specification during sea urchin embryogenesis.[35]

3. Experimental

3.1. General

Melting point was recorded on a Yanagimoto micromelting point apparatus and is uncorrected. IR spectra were obtained on a JASCO FT/IR-5300 spectrometer. Optical rotations were measured on a JASCO DIP-370 digital polarimeter. NMR spectra were recorded on JEOL GSX500 and LA500 spectrometers at 25°C. Mass spectra were obtained from a JEOL SX102A spectrometer.

3.2. Bioassay for developmental inhibitors using sea urchin embryos

Experiments were performed at 20°C, and filtered seawater was used throughout. Eggs and sperm were collected by electric stimulation of 10 V to the mature female and male sea urchin, respectively, allowing them to shed into beakers filled with filtered seawater. After washing three times with seawater, eggs were fertilized by the addition of the diluted sperm suspension. Fertilized eggs were washed three times with seawater, then allowed to develop in seawater until they reached desired developmental stages. The DMSO solution of sample to be tested was added to the suspension of embryos to give final concentrations of DMSO less than 0.1% in seawater. DMSO had no effects on the phenomena observed at the concentrations tested. To assay for embryogenesis, 2 or 4-cell stage embryos were placed into serially diluted sample solutions. After having been cultured until the 16-cell stage, sample-treated embryos were transferred to seawater. They were periodically observed for any cytological changes.

3.3. Sponge sample

The marine sponge *G. exigua* Thiele (Order Astrophorida, Family Geodiidae) was collected off Oshima, Kagoshima Prefecture, Japan in July 2001 and was identified by Professor Patricia R. Bergquist of the University of Auckland, New Zealand. A voucher specimen is kept in the laboratory of the authors (S. O.).

3.4. Extraction and isolation

The sponge sample (160 g, wet weight) was cut into small pieces and soaked in MeOH (500 mL×3) for 3 d in the refrigerator. The pooled MeOH extract was concentrated in vacuo to afford a residue which was partitioned between H_2O (200 mL) and hexane (150 mL×3). The hexane soluble extract (1.1 g; 0.68% wet weight) was then subjected to medium pressure column chromatography on ODS employing solvent gradient MeOH–H_2O to give nine fractions. Individual purification of three fractions on a silica gel column using varying ratios of EtOAc–hexane solvent system afforded **1** (8.0 mg; 0.005% wet weight), **2** (2.1 mg; 0.001% wet weight), **3** (0.4 mg; 0.0003% wet weight) and **4** (63.8 mg; 0.04% wet weight), respectively.

3.4.1. Exiguamide (1). Colorless crystals; mp 139–140°C; $[\alpha]_D^{25}$ +31.7° (*c* 0.08, $CHCl_3$); IR (KBr) ν_{max} 3323, 1657 cm^{-1}; ^1H NMR, see Table 1; ^{13}C NMR, see Table 2; (+)-HRFABMS *m/z* 250.2174 $[M+H]^+$ (calcd for $C_{16}H_{28}NO$, 250.2171); (−)-FABMS *m/z* 248 $[M-H]^-$.

3.4.2. Exicarbamate (2). Colorless oil; $[\alpha]_D^{25}$ +28° (*c* 0.02, $CHCl_3$); IR (film) ν_{max} 3366, 1711 cm^{-1}; ^1H NMR, see Table 1; ^{13}C NMR, see Table 2; (+)-HRFABMS *m/z* 280.2267 $[M+H]^+$ (calcd for $C_{17}H_{30}NO_2$, 280.2276).

3.4.3. Exigurin (3). Colorless oil; $[\alpha]_D^{25}$ −32° (*c* 0.03, $CHCl_3$); IR (film) ν_{max} 3393, 1742, 1645 cm^{-1}; ^1H NMR, see Table 1; ^{13}C NMR, see Table 2; HREIMS *m/z* 364.2732 $[M]^+$ (calcd for $C_{21}H_{36}N_2O_3$, 364.2726); EIMS *m/z* (rel. intensity) 364 $[M]^+$ (19), 305 $[M-COOCH_3]^+$ (6), 248 $[M-CH_2N(CH_3)CH_2COOCH_3]^+$ (4), 116 (100).

3.4.4. (−)-10-*epi*-Axisonitrile-3 (4). Colorless oil; $[\alpha]_D^{25}$ −43.5° (*c* 0.64, $CHCl_3$); IR (film) ν_{max} 2132, 1653, 1456 cm^{-1}; ^1H NMR, see Table 1; ^{13}C NMR, see Table 2; HREIMS *m/z* 231.1980 $[M]^+$ (calcd for $C_{16}H_{25}N$, 231.1987); EIMS *m/z* (rel. intensity) 231 $[M]^+$ (32), 216 $[M-CH_3]^+$ (77), 204 $[M-HCN]^+$ (38), 188 (50), 161 (100).

3.5. Single crystal X-ray analysis of 1

Crystal data: $C_{16}H_{27}NO$, M=249.40, monoclinic, space group $C2$ (no. 5), Z=8, a=24.4390 (8), b=8.8390 (4), c=16.8350 (8) Å, β=115.786 (2)°, V=3274.5 (2) $Å^3$, $F(000)$=1104, μ(Mo Kα)=0.62 cm^{-1}, D_c=1.012 g cm^{-3}, T=295 K. The reflection data were collected on a Mac Science DIP 2030 imaging plate area detector with graphite monochromated Mo Kα radiation (λ=0.71069 Å) from a

colorless crystal of approximate dimensions 0.7×0.4×0.2 mm. A total of 3076 independent reflections were collected of which 1568 were considered to be observed [$I>2.90\sigma(I)$]. The structure was solved by direct methods[36] and expanded using Fourier techniques.[37] The non-hydrogen atoms were refined anisotropically by full-matrix least-squares refinement. Hydrogen atoms were refined isotropically. The structure was finally refined to R=0.056 (R_w=0.076). Crystallographic data for the structure in this paper has been deposited with the Cambridge Crystallographic Data Centre as supplementary publication number CCDC 185123. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK (fax: +44-1223-336033; e-mail: deposit@ccdc.cam.ac.uk).

3.6. Conversion of 4 into 1

To a solution of **4** (29 mg) in EtOH was added acetic acid (2 mL) and the mixture was stirred at room temperature for 18 h. After the solvent was removed, the residue was purified by silica gel column chromatography (eluted with 20% EtOAc–hexane) to give 28 mg of the product. The IR, ^{1}H NMR and MS spectral data and $[\alpha]_D$ of the product were identical to those of **1**.

3.7. Preparation of the (*S*)- and (*R*)-MTPA esters

To a solution of **1** (27 mg) in EtOH was added 10% HCl (1.5 mL). The mixture was allowed to react at 60°C for 4 d.[38] After removal of the solvent, the residue was treated through a short ODS column (eluted with 40% MeOH–H_2O to 100% MeOH) to give a crude fraction (13 mg) of the amine derivative **5** {(+)-FABMS *m/z* 222 $[M+H]^+$}. The fraction was used for the preparation of MTPA esters without further purification. To a solution of the amine derivative **5** (6 mg) in pyridine (dried over CaH_2) was added 4-dimethylaminopyridine and (−)-MTPACl at room temperature and stirred for 2.5 h. After evaporation of the solvent, the residue was passed through a silica gel column (5% EtOAc–hexane) to afford the (*S*)-MTPA ester (**6a**). The (*R*)-MTPA ester (**6b**) was prepared in a similar manner using (+)-MTPACl.

3.7.1. (*S*)-MTPA ester (6a). ^{1}H NMR ($CDCl_3$) δ 7.56 and 7.40 (5H, m, MTPA phenyl protons), 6.98 (1H, br d, J=10.5 Hz, NH), 5.48 (1H, br s, H-1), 3.98 (1H, dd, J=10.5, 3.5 Hz, H-6), 3.38 (3H, s, MTPA CH_3O−), 2.39 (1H, ddd, J=16.5, 8.5, 7.9 Hz, H-3a), 2.02 (1H, ddd, J=16.5, 8.5, 2.4 Hz, H-3b), 1.82 (1H, tt, J=13.0, 4.5 Hz, H-9ax), 1.76 (1H, m, H-8eq), 1.73 (1H, ddd, J=12.5, 7.9, 2.4 Hz, H-4a), 1.69 (3H, br s, H-14), 1.58 (1H, m, H-10), 1.52 (1H, m, H-9eq), 1.44 (1H, ddd, J=12.5, 8.5, 8.5 Hz, H-4b), 1.34 (1H, m, H-11), 1.26 (1H, m, H-7), 1.18 (1H, qd, J=13.0, 3.5 Hz, H-8ax), 0.92 (3H, d, J=6.5 Hz, H-12), 0.89 (3H, d, J=7.5 Hz, H-15), 0.83 (3H, d, J=6.5 Hz, H-13); EIMS *m/z* 437 $[M]^+$.

3.7.2. (*R*)-MTPA ester (6b). ^{1}H NMR ($CDCl_3$) δ 7.38 and 7.52 (5H, m, MTPA phenyl protons), 6.88 (1H, br d, J=10.5 Hz, NH), 5.49 (1H, br s, H-1), 3.98 (1H, dd, J=10.5, 3.1 Hz, H-6), 3.43 (3H, s, MTPA CH_3O–), 2.48 (1H, ddd, J=16.5, 8.5, 7.9 Hz, H-3a), 2.09 (1H, ddd, J=16.5, 8.5, 2.4 Hz, H-3b), 1.90 (1H, ddd, J=12.8, 7.9, 2.4 Hz, H-4a), 1.82 (1H, dddd, J=14.0, 12.8, 4.9, 4.3 Hz, H-9ax), 1.71 (3H, br s, H-14), 1.68 (1H, m, H-8eq), 1.58 (1H, ddd, J=12.8, 8.5, 8.5 Hz, H-4b), 1.51 (1H, m, H-9eq), 1.24 (1H, m, H-7), 1.10 (1H, m, H-8ax), 1.05 (1H, m, H-11), 1.00 (3H, d, J=7.6 Hz, H-15), 0.83 (3H, d, J=6.7 Hz, H-12), 0.72 (3H, d, J=6.7 Hz, H-13); EIMS *m/z* 437 $[M]^+$.

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