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Lembehyne A, a Novel Neuritogenic Polyacetylene, from a Marine Sponge of *Haliclona* sp.

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Abstract—Lembehyne A (**1**), a novel long chain polyacetylene, which induces neurite outgrowth, was isolated from a Indonesian marine sponge of *Haliclona* sp. The total structure of **1** was determined by spectroscopic study, chemical degradation, and modified Mosher's method. Lembehyne A induced neuritogenesis in pheochromocytoma PC12 cells and neuroblastoma Neuro 2A cells at 2 and 0.1 μg/mL, respectively. © 2000 Elsevier Science Ltd. All rights reserved.

Introduction

Nerve growth factor (NGF) was found to regulate differentiation and survival of nerve cells.[1,2] NGF has potential as a therapeutic drug for a variety of neurological diseases including senile dementia such as Alzheimer's disease.[3,4] However, the peripheral administration of NGF incurs a problem in that its size impedes passage through the blood–brain barrier. Therefore, a smaller molecule exhibiting NGF-like activity might be a drug candidate for various neuronal diseases instead of NGF. The pheochromocytoma PC12 cell line has been well studied as a model of neurotrophic factors such as NGF. PC12 cells differentiate into sympathetic-like neurons characterized by neurite outgrowth by NGF treatment.[5] In the course of our study of bioactive substances from marine organisms,[6] we started to search for compounds which exhibit neuritogenic effect against PC12 cells, and found a novel polyacetylene named lembehyne A (**1**) from an Indonesian marine sponge of *Haliclona* sp. In this paper, we describe the details of the structure elucidation of lembehyne A (Chart 1).

Results and Discussion

The MeOH extract of the titled sponge (dried, 100 g) was partitioned into an AcOEt–water mixture. The AcOEt-soluble portion was further partitioned into an *n*-hexane-90% aq. MeOH mixture. The *n*-hexane-soluble portion, which showed neuritogenic activity against PC12 cells at 10 μg/mL concentration, was subjected to bioassay-guided separation. Thus, the *n*-hexane-soluble portion was purified by SiO_2 column chromatography [*n*-hexane–AcOEt] and ODS HPLC [$CHCl_3$/MeOH] to furnish lembehyne A (**1**) (30 mg).

Lembehyne A (**1**) was obtained as a colorless powder. The positive FAB MS of **1** gave a quasi-molecular ion [$(M+H)^+$] peak at *m/z* 511 and the molecular formula of **1** was determined as $C_{36}H_{62}O$ by HR-positive FAB MS and NMR analysis. The ^1H NMR spectrum of **1** showed the presence of one terminal methyl proton (δ 0.89, 3H, t, *J*=7.0 Hz), a long alkyl chain (δ ca 1.26), one oxymethine proton (δ 4.38, 1H, td, *J*=6.6, 2.2 Hz), and four olefinic protons (δ 5.36, 5.38, 5.44, 5.45). The presence of two acetylenic groups was readily revealed by the characteristic ^{13}C NMR signals in the region of δ_c 70–85 and absorption bands at 2360 and 2330 cm^{-1} in the IR spectrum. The IR spectrum of **1** also showed an absorption band due to a hydroxyl group (3420 cm^{-1}). The characteristic proton and carbon signals of **1** were assigned by NMR (DEPT

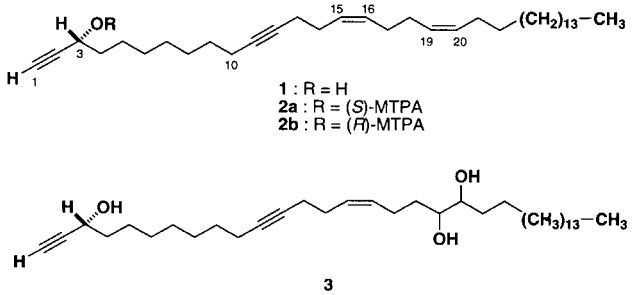

Chart 1.

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Table 1. ^{1}H- and ^{13}C NMR data for Lembehyne A (**1**) (600 MHz in $CDCl_3$)

No.	^{13}C δ_c	^{1}H δ (mult., J (Hz))	No.	^{13}C δ_c	^{1}H δ (mult., J (Hz))
1	72.8 (d)	2.46 (d, 2.2)	15	128.5 (d)	5.44 (m)
2	85.0 (s)		16	130.4 (d)	5.45 (m)
3	62.3 (d)	4.38 (td, 6.6, 2.2)	17	27.5 (t)	2.10 (br s)
4	37.6 (t)	1.72 (m)	18	27.3 (t)	2.10 (br s)
5	25.0 (t)	1.48 (m)	19	129.0 (d)	5.38 (m)
6–8	28.7–29.7 (t)	1.26–1.38 (m)	20	130.5 (d)	5.36 (m)
9	29.0 (t)	1.48 (m)	21	27.3 (t)	2.02 (td, 6.9, 6.8)
10	18.7 (t)	2.14 (tt, 7.1, 2.2)	22–33	28.7–29.7 (t)	1.26–1.38 (m)
11	80.4 (s)		34	31.9 (t)	1.26 (m)
12	79.7 (s)		35	22.7 (t)	1.28 (m)
13	19.2 (t)	2.21 (m)	36	14.1 (q)	0.89 (t, 7.0)
14	27.1 (t)	2.22 (m)			

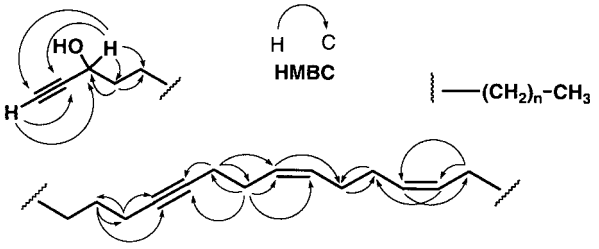

Figure 1. Partial structures with key HMBC correlations.

and HMQC) analysis of **1** (Table 1), and three partial structures in **1** were clarified by interpretation of COSY and HMBC (Fig. 1). The geometries of the Δ^{15} and Δ^{19} double bonds were assigned as *Z* on the basis of the chemical shifts of the allylic carbons (δ_c 27.1 and 27.5, 27.3 and 27.3), respectively.

The connectivity of each partial structure and the length of each alkyl chain were not revealed from the COSY and HMBC spectra of **1**, since each partial structure was linked with a long alkyl chain, of which the NMR signals overlapped each other. Firstly, ozonolysis of **1** followed by $NaBH_4$ treatment gave 1-heptadecanol, which was identified with an authentic sample by GC–MS. Thus, the length of the terminal alkyl chain was determined as $-(CH_2)_{15}CH_3$. Secondly, the double bonds of **1** were oxidized by microencapsulated osmium tetraoxide (MC OsO_4)[7] to afford two major compounds, one of which was confirmed as a 19,20-dihydroxyl derivative **3** by COSY analysis. The other one was deduced to be a 15,16-dihydroxyl derivative. $NaIO_4$ oxidation of **3** followed by $NaBH_4$ treatment also gave 1-heptadecanol. This evidence elucidated the connection of the Δ^{19} double bond and the terminal alkyl chain. Consequently, the planar structure of lembehyne A (**1**) was confirmed as shown.

Next, we elucidated the configuration at C-3 in **1** by application of modified Mosher's method.[8] Thus, **1** was treated with *S*-(+)- or *R*-(−)-2-methoxy-2-phenyl-2-trifluoromethylacetic acid (MTPA), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI), and *N*,*N*-dimethylaminopyridine (DMAP) in CH_2Cl_2 at room temperature to furnish the 3-*O*-*S*-(+)-MTPA ester **2a** and 3-*O*-*R*-(−)-MTPA ester **2b**, respectively. The characteristic protons of both compounds were assigned and the 3*R* configuration was confirmed by comparison of the $\Delta\delta$ values (Fig. 2). Thus, the absolute stereostructure of lembehyne A was determined as a C_{36} linear diacetylene alcohol **1**.

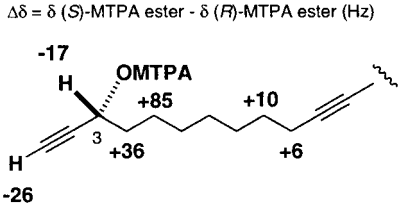

Figure 2. Application of modified Mosher's method.

Many linear polyacetylenes have been isolated from marine sponges and most of them have been found to exhibit various biological activities such as antifungal, cytotoxic, antiviral, enzyme-inhibitory activities.[9–19] However, there is no report of linear polyacetylene having neuritogenic activity. Lembehyne A (**1**) induced neurite outgrowth of rat pheochromocytoma PC12 cells at 2 μg/mL as minimum concentration. Lembehyne A also induced neuritogenesis in mouse neuroblastoma Neuro 2A cells at 0.1 μg/mL. Treatment with cytochalasin B, an inhibitor of actin polymerization, or cycloheximide, an inhibitor of protein synthesis in eukaryotes, blocked neurite outgrowth induced by **1**. This finding indicates that the morphological change induced by **1** is dependent upon actin polymerization and de novo protein synthesis.

So far, it has been reported that various types of compounds induce neurite extension of PC12 or Neuro 2A, for example, protease inhibitor,[20,21] protein kinase inhibitor,[22] cyclic nucleotide phosphodiesterase inhibitor,[23] and proteasome inhibitor.[24] The molecular target of lembehyne A (**1**) is unclear at present, and more detailed biochemical study is needed for understanding the action mechanism of **1**.

Experimental

Isolation from a marine sponge of *Haliclona* sp. The titled sponge (100 g, dry weight) collected in July 1999 at Lembeh Island, Bitung, Indonesia, was extracted with MeOH (1 L) at room temperature 3 times. The residue obtained by evaporation of the solvent under reduced pressure was partitioned into an AcOEt–water mixture (1:1), and the AcOEt layer was evaporated to give the AcOEt-soluble portion (7 g). The AcOEt soluble portion

was further partitioned into an *n*-hexane-90% aq. MeOH mixture (1:1). The *n*-hexane layer was evaporated to give the *n*-hexane-soluble portion (2.8 g), which showed neuritogenic activity against PC12 cells at 10 μg/mL. The *n*-hexane-soluble portion was subjected to bioassay-guided separation. The *n*-hexane-soluble portion (369 mg) was purified by SiO_2 column chromatography (eluted with *n*-hexane: AcOEt=8:1→3:1→AcOEt→MeOH) to give eight fractions (Fr. A∼Fr. H). Finally, the active Fr. B (67 mg) was purified by HPLC (Cosmosil $5C_{18}$-AR, 10ϕ×250 mm, $CHCl_3$–MeOH=1:4) to give lembehyne A (**1**) (30 mg, 8.1% from the *n*-hexane ext.). **Lembehyne A (1):** colorless powder, $[\alpha]_D=+1.9°$ (c=0.50, $CHCl_3$, 22°C). IR ν_{max}. (KBr): 3420, 2360, 2330 cm^{-1}. Positive ion FAB MS (nitrobenzyl–alcohol): *m/z* 511 $(M+H)^+$. HR-FAB MS: *m/z* 511.4879: calcd for $C_{36}H_{63}O$. Found: *m/z* 511.4857. ^1H NMR (600 MHz, $CDCl_3$, δ) and ^{13}C NMR (150 MHz, $CDCl_3$, δc): as shown in Table 1.

Ozonolysis of lembehyne A (1). The CH_2Cl_2 solution (0.7 mL) of **1** (1.8 mg) was treated with ozone (O_3) for 1 min at −78°C and then $NaBH_4$ (1.3 mg) was added. The reaction mixture was stirred for 1 h at room temperature (25°C). After filtration through cotton, the crude product was subjected to GC–MS analysis. GC–MS conditions: ionization, EI; column, DB-5 (0.25 mm ϕ×25 m); mobile phase, N_2; column temperature, initial 100°C and +5°C/min gradient for 36 min from 2 min after injection. The product was determined to be 1-heptadecanol [t_R=17.3 min, *m/z* 238 $(M-H_2O)^+$], which was identified with an authentic sample.

Dihydroxylation and oxidation of lembehyne A (1). A solution of **1** (10 mg) in an H_2O–acetone–CH_3CN mixture (1:2:1, 5 mL) was treated with MC OsO_4 (5 mol%, 10 mg) and *N*-methylmorpholine *N*-oxide (23 mg). The reaction mixture was stirred at room temperature (25°C) for 1 week. After filtration through cotton, the filtrate was partitioned into an AcOEt–water mixture. The AcOEt soluble portion was purified by SiO_2 column chromatography (*n*-hexane–AcOEt) and SiO_2 HPLC (Cosmosil 5SL, 10ϕ×250 mm, *n*-hexane–Et_2O=1:2) to afford a 19,20-dihydroxyl derivative **3** (3.5 mg). **19,20-dihydroxyl derivative 3:** FAB MS: *m/z* 567 $(M+Na)^+$. HR-FAB MS: *m/z* 567.4754: calcd for $C_{36}H_{64}O_3Na$. Found: 567.4744. ^1H NMR (600 MHz, $CDCl_3$): δ 5.47 (2H, m, H-15, -16), 4.37 (1H, td, *J*=6.7, 2.0 Hz, H-3), 3.62 (1H, m, H-19), 3.60 (1H, m, H-20), 2.46 (1H, d, *J*=2.0 Hz, H-1), 2.27 (2H, m, H-14), 2.21 (2H, m, H-13), 2.18 (2H, m, H-17), 2.14 (2H, tt, *J*=7.0, 2.3 Hz, H-10), 1.72 (2H, m, H-4), 1.49 (2H, m, H-18), 1.47 (2H, m, H-9), 1.46 (2H, m, H-5), 1.43 (2H, m, H-21), 0.89 (3H, t, *J*=7.1 Hz, H-36). A solution of **3** (0.7 mg) in a Et_2O–H_2O mixture (1:1, 1.5 mL) was treated with $NaIO_4$ (3 mg). The reaction mixture was stirred at room temperature (25°C) for 20 h. After extraction with Et_2O, the product obtained by evaporation was further treated with $NaBH_4$ (1.0 mg) in CH_2Cl_2 (0.5 mL). After filtration through cotton, the product was subjected to GC–MS analysis to identify 1-heptadecanol.

Preparation of (*S*)-(+)- or (*R*)-(−)-MTPA ester of lembehyne A (1). A CH_2Cl_2 (0.5 mL) solution of **1** (1.0 mg) was treated with (*S*)-(+)-MTPA (2.3 mg), EDCI (1.9 mg), and DMAP (0.7 mg). The reaction mixture was stirred at room temperature (25°C) for 2 h under an N_2 atmosphere. The reaction was quenched by saturated aq. NaCl and the whole was extracted with AcOEt. The AcOEt phase was washed by 5% HCl, saturated aq. $NaHCO_3$, and saturated aq. NaCl. After evaporation of the solvent, the residue was purified by SiO_2 HPLC (Cosmosil 5SL, 10ϕ×250 mm, *n*-hexane–AcOEt=19:1) to afford (*S*)-(+)-MTPA ester **2a** (0.5 mg). A CH_2Cl_2 (0.5 mL) solution of **1** (1.0 mg) was similarly treated with (*R*)-(−)-MTPA (2.3 mg), EDCI (1.9 mg), and DMAP (0.7 mg) to afford the (*R*)-(−)-MTPA ester **2b** (0.6 mg).

(*S*)-MTPA ester 2a: FAB MS: *m/z* 749 $(M+Na)^+$. HR-FAB MS: *m/z* 749.5097: calcd for $C_{46}H_{69}O_3F_3Na$. Found: 749.5109. ^1H NMR (600 MHz, $CDCl_3$): δ 7.53 (2H, br d, *J*=7.7 Hz, ArH), 7.42–7.38 (3H, m, ArH), 5.51 (1H, td, *J*=6.6, 1.4 Hz, H-3), 5.45 (1H, m, H-16), 5.44 (1H, m, H-15), 5.38 (1H, m, H-19) 5.36 (1H, m, H-20), 3.55 (3H, s, OMe), 2.49 (1H, d, *J*=1.4 Hz, H-1), 2.22 (2H, m, H-14), 2.18 (2H, m, H-13), 2.13 (2H, m, H-10), 2.09 (4H, br s, H-17, -18), 2.02 (2H, td, *J*=6.8, 6.8 Hz, H-21), 1.86 (2H, m, H-4), 1.47 (4H, m, H-5, -9), 0.88 (3H, t, *J*=7.1 Hz, H-36).

(*R*)-MTPA ester 2b: FAB MS: *m/z* 749 $(M+Na)^+$. HR-FAB MS: *m/z* 749.5097: calcd for $C_{46}H_{69}O_3F_3Na$. Found: 749.5093. ^1H NMR (600 MHz, $CDCl_3$): δ 7.55 (2H, br d, *J*=5.5 Hz, ArH), 7.42–7.38 (3H, m, ArH), 5.54 (1H, td, *J*=6.5, 2.2 Hz, H-3), (1H, m, H-16), 5.44 (1H, m, H-15), 5.38 (1H, m, H-19) 5.36 (1H, m, H-20), 3.6 (3H, s, OMe), 2.53 (1H, d, *J*=2.2 Hz, H-1), 2.22 (2H, m, H-14), 2.18 (2H, m, H-13), 2.12 (2H, m, H-10), 2.09 (4H, br s, H-17, -18), 2.02 (2H, td, *J*=6.8, 6.8 Hz, H-21), 1.8 (2H, m, H-4), 1.45 (2H, quintet-like, *J*=7.4 Hz, H-9), 1.32 (2H, m, H-5), 0.88 (3H, t, *J*=7.0 Hz, H-36).

Assay for neuritogenic activity in PC12 cells and Neuro 2A cells. PC12 cells were grown on collagen-coated plates in Dulbecco's modified essential medium (DMEM) with 10% heat-inactivated horse serum and 5% fetal bovine serum (FBS). Neuro 2A cells were grown in DMEM with 10% FBS. The cells were kept in an incubator at 37°C with 5% CO_2. The cells were plated on 24-were plates at a density of 2×10^4 per well with 1 mL of the culture medium. After 24 h cultivation, the medium was changed to fresh medium and the testing sample as 10 μL of EtOH solution was added to each well. After 48 h incubation, morphological changes of the cells were observed under microscope. In a related experiment, 5 μM of cytochalasin B or 10 μM of cycloheximide was added 1 h before addition of **1**.

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