Pergamon

Tetrahedron Letters 40 (1999) 719–722

TETRAHEDRON LETTERS

Sinulamide: an H,K-ATPase Inhibitor from a Soft Coral *Sinularia* sp.[1]

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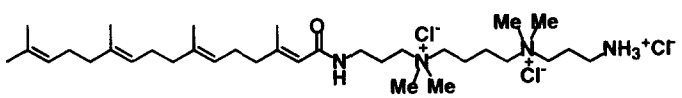
Received 19 October 1998; accepted 11 November 1998

Abstract: Sinulamide (**1**), a new tetraprenylated spermine derivative, has been isolated from a soft coral *Sinularia* sp. as an H,K-ATPase inhibitor. The structure was assigned on the basis of spectroscopic data and confirmed by a total synthesis.

Keywords: Coelenterates; Enzyme inhibitors; Biologically active compounds; Natural products

Polyamine metabolites are reported in increasing numbers from marine sources [2]. Soft corals of the genus *Sinularia* often contain acylated spermidine derivatives [3-5]. In our screening of Japanese marine invertebrates for inhibitors of gastric H,K-ATPase [6-8], the hydrophilic extract of *Sinularia* sp. collected in western Japan exhibited potent activity. Bioassay-guided isolation afforded an active substance named sinulamide, which was initially assigned structure **1** based on spectral data [9]. We now have succeeded in refining its structure by a combination of spectral analysis and total synthesis. This paper describes isolation and structure elucidation.



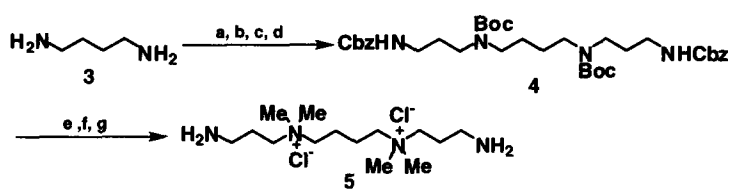
1

The water-solubles of the 70% EtOH extract of *Sinularia* sp. (500 g wet weight) collected off Tatsukushi on Shikoku Island were chromatographed on TSK G3000S gel with H_2O, MeOH/H_2O (1:1), MeOH/H_2O (7:3), MeOH, and acetone. The three active late fractions were combined and gel-filtered on Toyopearl HW-40 with MeOH followed by ODS HPLC with MeOH/H_2O/AcOH (45:55:0.1) to yield 120 mg of sinulamide (**1**) as a colorless gum. Sinulamide (**1**) showed ion peaks at *m/z* 581 $(M\text{-}Cl)^+$ and *m/z* 651 $(M+Cl)^-$ in the positive and

0040-4039/99/$ - see front matter
PII: S0040-4039(98)02438-1

negative mode FAB mass spectra, respectively. Considering ^{13}C NMR data as well as distribution of isotope peaks in the mass spectra, a molecular formula of $C_{34}H_{66}Cl_2N_4O$ was established. The IR spectrum revealed the presence of conjugated amide (1660 and 1640 cm^{-1}) and amine (3350, and 3250 cm^{-1}) functions. The ^1H and ^{13}C NMR spectra exhibited characteristic signals for a linear diterpenoid unit, e.g. five olefinic methyls [δ_H 1.77, 1.62, 1.55 and 1.53 (6H); δ_C 26.0, 25.3, 18.5, 16.2 (2C)], four tri-substituted olefins [δ_H 5.72, 5.12, 5.08 (2H); δ_C 154.1s, 134.4s, 133.8s, 129.9s, 123.4d (2C), 123.2d, 117.6d], and allylic methylenes (δ_H 1.90-2.12). One of the olefins was vicinal to a polar group (δ_C 154.1 and 117.6), thereby suggesting the terpenoid portion terminated in a carbonyl function. This was supported by fragment ions at *m/z* 388, 319, 251, 184, and 103 in the positive ion FABMS. The ^1H and ^{13}C NMR spectra also contained two exchangeable protons [δ 8.62 (br, 2H) and 8.27 (br, 1H)], four *N*-methyls [δ_H 3.06 (6H, s) and 3.08 (6H, s); δ_C 49.7 (4C)], six nitrogen-bearing methylenes [δ_C 64.4 (2C), 63.6, 62.0, 36.9 and 34.1], and four additional methylenes [δ_C 23.9, 22.1 and 20.6 (2C)], which indicated that a N^2,N^2,N^3,N^3-tetramethylspermine unit was linked to the diterpenoid unit through an amide bond. The downfield shifts for four nitrogen-bearing methylene carbons indicated that the two middle nitrogens were both *N,N*-dimethylated to form ammonium salts. The presence of a primary amide was confirmed by formation of a monoacetamide [*m/z* 623 $(M\text{-}Cl)^+$; δ_H 1.81; δ_C 22.5 and 167.4] upon treatment with Ac_2O/pyridine. The ^{13}C NMR chemical shift (δ_C 26.0) for one of the olefinic methyls suggested the presence of a *Z*-olefin. Unfortunately, further spectral studies were hampered due to decomposition during storage in an NMR tube.

Scheme 1

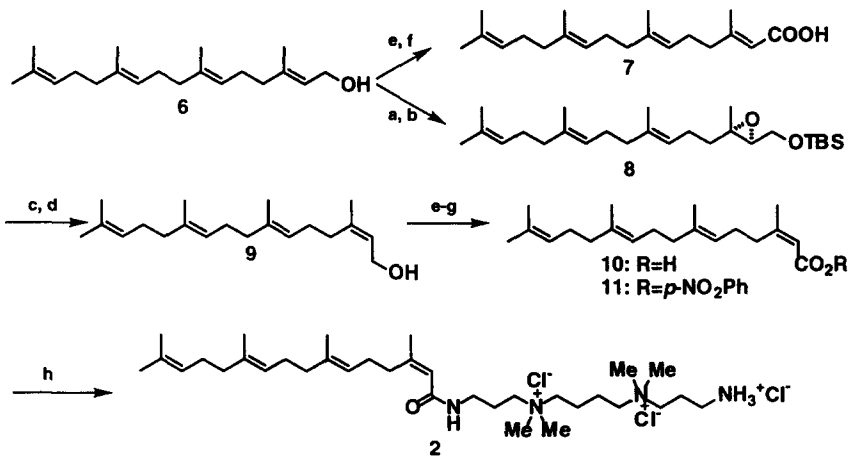


(a) CH_2=CHCN, MeOH, 92%; (b) Boc_2O, Et_3N, 98%; (c) $NaBH_4$ / $CoCl_2$, MeOH, 80%; (d) CbzCl, 1N NaOH-Et_2O, 58%; (e) TFA, CH_2Cl_2, 91%; (f) MeI, $KHCO_3$, MeOH, 80%; (g) HBr-AcOH 1:3, 100%

In order to confirm the structure of sinulamide we attempted to synthesize the all-*E* isomer **1**. Synthesis of N^2,N^2,N^3,N^3-tetramethylspermine (**5**) was begun with dicyanoethylation [10] of putrescin (**3**) which was followed by Boc protection, reduction with $NaBH_4/CoCl_2$ [11], and Cbz protection to furnish **4**. Fully protected spermine **4** was treated with TFA to generate two secondary amines which were methylated with MeI/KOH-K_2CO_3 [12]. The deprotection of the Cbz group with HBr/AcOH afforded the desired compound **5**

(Scheme 1). Geranylgeranoic acid (**7**) was prepared from geranylgeraniol (**6**) by two oxidations with MnO_2 [13] and then with $NaClO_2$ [14]. Coupling of **5** and **7** was catalyzed by DCC (Scheme 2). Although the major product was the digeranylgeranoyl amide, we were able to isolate the desired product **1** [15] by HPLC. Synthetic **1** showed NMR data almost superimposable on those of the natural product except for the chemical shift of the olefinic methyl in the conjugated olefin (natural product: δ_H 1.77, δ_C 26.0; synthetic product: δ_H 2.11, δ_C 18.6). Therefore, it was concluded that sinulamide had *Z*-geometry of the pertinent olefin, *i.e.* geranyl*neroyl*amide of N^2,N^2,N^3,N^3-tetramethylspermine.

Scheme 2



(a) TBHP, L-(+)-DET, $Ti(O^iPr)_4$, CH_2Cl_2, -20°C, 83% (b) TBSCl, Et_3N, DMAP, CH_2Cl_2, 98%; (c) LDP, THF, and then MeI, 95%; (d) TBAF, THF, 100%; (e) MnO_2, hexane, 79 and 87% for *E* and *Z*; (f) $NaClO_2$, NaH_2PO_4, 2-methyl-2-butene, H_2O-*tert*-BuOH 4:15, 87 and 85% for *E* and *Z*; (g) *p*-nitrophenol, DCC, CH_2Cl_2, 76% for *Z*; (h) 5, Et_3N, MeOH, 30% for *Z*

We then tried to prepare geranylneroic acid (**10**), which could be prepared from **6** by inversion of the terminal olefin and oxidation of the primary alcohol. Geranylgeraniol was subjected to Sharpless epoxidation [16] followed by protection with TBS to furnish **8** which was treated with lithium diphenylphosphide and then with MeI; deprotection of TBS group afforded geranylnerol (**9**) [17]. Oxidation of **9** as described above furnished **10** which was converted to the *p*-nitrophenyl ester **11** which was coupled with **5** to yield a compound indistinguishable from natural sinulamide. Therefore, the structure of sinulamide is **2** [18].

Natural sinulamide (**2**) not only inhibits H,K-ATPase with an IC_{50} value of 5.5 μM, but also is cytotoxic against L1210 and P388 with IC_{50} values of 3.1 and 4.5 μg/mL, respectively. It is the first acylated spermine derivative of soft coral origin; however, acylated spermidines are known from soft corals of the same genus [3-5].

Acknowledgment. We thank Professor P. J. Scheuer, University of Hawaii, for editorial comments. We are grateful to Drs. H. Shikama, A. Ohta, H. Nagano, and C. Nohara of the Central Research Laboratories, Yamanouchi Pharmaceutical Co., Ltd., for H,K-ATPase assay, to Dr. M. Inoue, Department of Chemistry, The University of Tokyo, for his valuable suggestion for preparation of the epoxide. The sample of geranylgeraniol was a gift from Kurare, Co. Ltd. A JSPS Research Fellowship for Young Scientists to N. U. S. is also acknowledged.

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[15] **1**: Colorless powder; TLC on cellulose, R_f 0.20 [*n*-BuOH/AcOH/H_2O (4:1:2)], positive to Dragendorff and ninhydrin reagents]; ^{1}H NMR (CD_3OD, 500 MHz) δ_H 5.69 (s, 1H), 5.12 (t, 1H, *J*=7.0 Hz), 5.09 (t, 1H, *J*=7.0), 5.08 (t, 1H, *J*=6.5), 3.40 (m, 4H), 3.14 (m, 8H), 3.10 (m, 8H), 3.05 (m, 4H), 2.17 (m, 2H), 2.14 (m, 2H), 2.11 (s, 3H), 2.07 (m, 6H), 1.98 (m, 6H), 1.85 (m, 4H), 1.65 (s, 3H), 1.61 (s, 3H), 1.58 (s, 6H); ^{13}C NMR (CD_3OD, 125MHz) δ_C 170.2s, 155.9s, 137.0s, 136.0s, 132.1s, 125.4d (2C), 124.5d, 118.9d, 64.8t, 64.5t, 63.7t, 62.2t, 51.5q (4C), 41.9t, 40.8t (2C), 37.6t, 37.0t, 27.8t, 27.6t, 27.2t, 25.9q, 24.1t, 22.1t, 20.6t (2C), 18.6q, 17.7q, 16.1q (2C); FABMS (glycerol) *m/z* (rel int.) 581 (5), 531 (0.5), 479 (1.5), 443 (3), 389 (2), 344 (37), 193 (28), 157 (18), 143 (17), 100 (100), 58 (68), 41 (28).

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[17] Vedejs E, Fuchs PL. J. Am. Chem. Soc. 1973;95: 822-825.

[18] **2**: Colorless powder; TLC on cellulose, R_f 0.20 [*n*-BuOH/AcOH/H_2O (4:1:2)]; IR (KBr) ν_{max} 3350 (br), 3250, 2900, 2800, 1660, 1640, 1525, 1480, 1440, 1370, 1250, 1170, 980, 895, 850, 750 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ_H 8.63 (brs, 2H), 8.22 (brt, 1H, NH), 5.70 (s, 1H), 5.11 (t, 1H, *J*=7.2 Hz), 5.06 (t, 1H, *J*=7.2), 5.05 (t, 1H, *J*=7.2), 3.53 (m, 2H), 3.39 (m, 4H), 3.34 (m, 2H), 3.13 (dt, 2H, *J*=5.8, 6.2), 3.07 (s, 6H, NMe_2), 3.04 (s, 6H, NMe_2), 2.85 (m, 2H), 2.58 (dd, 2H, *J*=7.7, 8.1 Hz), 2.12 (m, 2H), 2.06 (q, 2H, *J*=7.3), 2.03 (q, 2H, *J*=7.3), 2.01 (q, 2H, *J*=7.3), 1.91 (t, 2H, *J*=5.8), 1.90 (t, 2H, *J*=6.5), 1.85 (m, 2H), 1.77 (s, 3H), 1.75 (m, 4H), 1.62 (s, 3H), 1.55 (s, 3H), 1.53 (s, 6H); ^{13}C NMR (CD_3OD) δ_C 167.2s, 154.1s, 134.4s, 133.8s, 129.9s, 123.4d (2C), 123.2d, 117.6d, 64.4t (2C), 63.6t, 62.0t, 49.7q (4C), 40.8t (2C), 37.7t, 36.9t, 34.1t, 27.9t, 27.7t, 27.6t, 26.0q, 25.3q, 23.9t, 22.1t, 20.6t (2C), 18.5q, 16.2q (2C) (DMSO-d_6, 150 MHz) δ_C 165.8s, 152.3s, 134.6s, 134.3s, 130.6s, 124.1d, 123.9d (2C), 119.2d, 62.2t (2C), 61.5t, 60.0t, 50.4s (2C), 50.1s (2C), 39.1t (2C), 35.9t, 35.2t, 32.3t, 26.4t, 26.1t, 26.0t, 25.4q, 24.7q, 22.4t, 20.2t, 19.0t(2C), 17.5q, 15.7q (2C); FABMS (glycerol) *m/z* (rel int.) 581 (23), 488 (2), 443 (4), 389 (1), 344 (10), 100 (84), 69 (100), 58 (100), 41 (48), 30 (23); HRFABMS [$C_{34}H_{66}{}^{35}ClN_4O$ (M-Cl)$^+$ obsd. *m/z* 581.4930, Δ +0.5 mmu].