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Penaramides, Which Inhibit Binding of ω-Conotoxin GVIA to N-type Ca^{2+} Channels, from the Marine Sponge *Penares* aff. *incrustans*[1]

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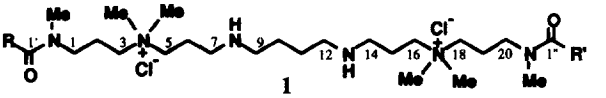
Abstract: Penaramides which inhibit binding of ω-conotoxin GVIA to N-type Ca^{2+} channels have been isolated from the marine sponge *Penares* aff. *incrustans* and their structures determined by spectral and chemical methods to be bis-amides of an unusual polyamine. The proposed structures have been confirmed by a total synthesis of the simplest component, penaramide A.

Calcium plays vital roles in physiological processes, ranging from cell division to muscle contraction and neurotransmission. Cytosolic Ca^{2+} is regulated by Ca^{2+} channels. In neurons, the major voltage-gated Ca^{2+} channels are distinguished as L-, N-, P-, and T-types.[2] A number of their agonists and antagonists have been developed mainly for therapeutic purposes. ω-Conotoxin GVIA (ω-CgTx GVIA), a 27-residue peptide from the venom of the marine snail, *Conus geographus*, binds to a specific site of the N-type channels, resulting in blockage of the channels.[3] To date, this is the only known N-type specific natural antagonist; more agonists or antagonists are required to understand the function of the N-type channels. In our search for biomedically important metabolites from Japanese marine invertebrates, the extract of the marine sponge *Penares* aff. *incrustans* strongly inhibited the binding of ^{125}I-ω-CgTx GVIA to rat brain synaptic plasma membranes.[4] Bioassay-guided isolation afforded an inseparable mixture of active principles named penaramides whose structures were elucidated by spectral and chemical methods and by total synthesis of panaramide A.

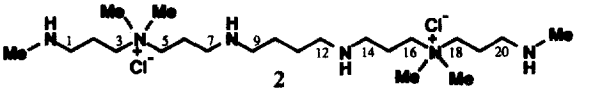
The EtOH extract of the frozen sponge (1 kg) was partitioned between H_2O and Et_2O, and the aqueous layer was further extracted with *n*-BuOH. The *n*-BuOH soluble portion was triturated with MeOH; the MeOH-soluble material was successively separated by gel filtration on Sephadex LH-20 (MeOH), ODS flash chromatography (aq MeOH), and CPC [EtOAc/*n*-BuOH/25% AcOH (4:1:4)]. The active fraction was subjected to HPLC on Asahipak GS-320P with CH_3CN/H_2O/TFA (20:80:0.05) followed by ODS HPLC with MeOH/H_2O/TFA (78:22:0.1) to yield a mixture of penaramides (**1**, 20.3 mg, 2.0×10^{-3}% yield) as a yellowish oil. Further attempts to separate penaramides by a variety of stationary and mobile phases in HPLC were unsuccessful.[5]



1

R,R'= $(CH_2)_9CH_3$ / $(CH_2)_7CH(CH_3)_2$ / $(CH_2)_6CH(CH_3)CH_2CH_3$ / $(CH_2)_2CH(CH_3)(CH_2)_5CH_3$ / $CH_2CH(CH_3)(CH_2)_6CH_3$

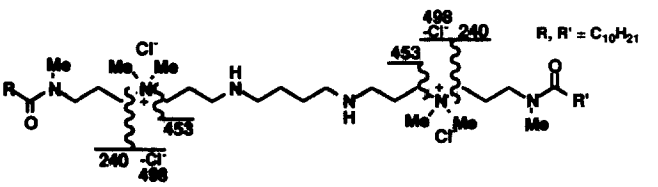
The mixture of penaramides reacted positive to both ninhydrin and Dragendorff reagents, suggesting their basic nature. The ^1H and ^{13}C NMR spectra exhibited two NH signals [δ_H 9.43 (2H, brs). D_2O exchangeable] and two types of *N*-methyl groups [$\delta_{H/C}$ 2.96 (6H, s) / 34.2 q and 3.07 (12H, s) / 49.8 q] in addition to numbers of alkane signals. Interpretation of the HOHAHA spectrum indicated that penaramides are acylated polyamines. Although the NMR signals of the polyamine portion were homogeneous, the high-field alkyl signals were associated with many small peaks, indicating the heterogeneity of the acyl portion. Thus, we hydrolyzed the penaramides with 2N HCl at 110℃ followed by solvent partitioning to afford a polyamine, penaramine (**2**), and a mixture of fatty acids.[6] Penaramine (**2**) gave the highest ion peak at *m/z* 437 in the positive FAB mass spectrum, which was eventually assigned to an $(M\text{-}Cl)^+$ ion. The ^{13}C NMR spectrum displayed 11 signals, three *N*-methyls and eight methylenes. Interpretation of the HOHAHA and HMBC[7] spectra led to the assignment of half of the molecule, thereby suggesting the symmetrical nature of **2**, which was also supported by FABMS data. GC-MS analysis of the methylated fatty acid fraction disclosed the presence of five C_{11} fatty acids.[6]



2

With partial structures of penaramides in hand, we set out to elucidate the entire molecule.[8] Penaramides exhibited FABMS peaks at *m/z* 723, 773, and 851, the third of which contained one $CF_3CO_2^-$ residue, since it disappeared after conversion to the HCl salt. The peak at *m/z* 773 contained one Cl atom, which was readily suggested by 3:1 intensity of ions at *m/z* 773 and 775. The fragment ion at *m/z* 723 was generated by a loss of one *N*-methyl group from the molecule, leaving one net positive charge.[9] Accurate measurement of the ion at *m/z* 773 led to a molecular formula of $C_{44}H_{94}Cl_2N_6O_2$ [$(M\text{-}Cl)^+$ *m/z* 773.7121, Δ -0.6 mmu], in accordance with the structures of the bisamides of penaramine (**2**) containing two C_{11}-fatty acids, which agreed with NMR data. This was supported by an MS/MS experiment of the $(M\text{-}Cl)^+$ ion at *m/z* 773, which resulted in four major daughter ions at *m/z* 240, 453, 498, and 723 (Scheme 1).

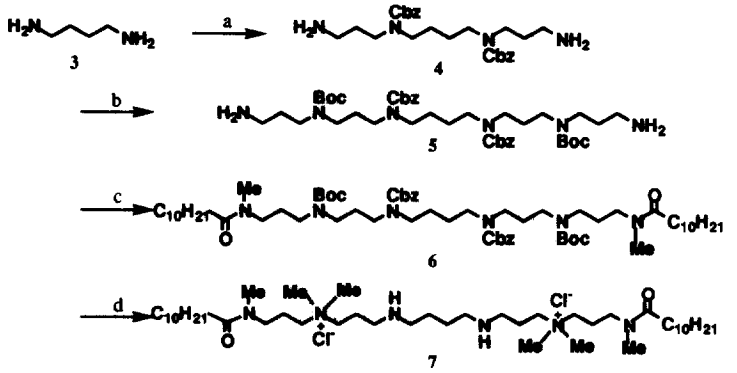
Scheme 1



In order to confirm the proposed structures of penaramides, the simplest derivative containing two linear C_{11} fatty acids, penaramide A, has been synthesized (Scheme 2). *N,N'*-(Dicyanoethyl)-1,4-diaminobutane was

prepared from putrescine (**3**) by symmetric dicyanoethylation.[10] After protection of the secondary amines with Cbz groups, the cyano groups were reduced by $NaBH_4/CoCl_2$ yielding diamine **4**,[11] which was successively subjected to cyanoethylation, protection with Boc, and reduction to furnish diamine **5**.[11] The diamine **5** was acylated with *n*-undecanoyl chloride, followed by treatment with MeI/NaH to afford *N,N'*-dimethylamide **6**. Deprotection of the Boc group, permethylation with $MeI/KHCO_3$ in MeOH,[12] removal of the Cbz group with AcOH/HBr (3:1), and conversion to the dichloride resulted in penaramide A (**7**).[13] Its ^1H and ^{13}C NMR spectra were superimposable on those of the natural penaramide mixture, except for alkyl region of acyl moieties. The FAB mass spectrum of **7** was indistinguishable from that of the chloride salts of the natural penaramides.

Scheme 2



(a) i. CH_2=CHCN, MeOH, 25°C, 92%, ii. CbzCl, 0.2N NaOH-Et_2O, 25°C, 70%, iii. $NaBH_4$ / $CoCl_2$, MeOH, 25°C, 80%; (b) i. CH_2=CHCN, MeOH, 25°C, 98%, ii. Boc_2O, Et_3N-MeOH 1:9, 25°C, 67%, iii. $NaBH_4$ / $CoCl_2$, MeOH, 25°C, 80%; (c) i. $C_{10}H_{21}COCl$,Et_3N-CH_2Cl_2, 91%, ii. MeI / NaH, THF, 77%; (d) i. TFA, 95% ii. MeI / $KHCO_3$, MeOH, 46%, iii. HBr / AcOH, ion exchange, 45%

The natural penaramides (**1**) and the synthetic material **7** inhibited the binding of ^{125}I-ω-CgTx GVIA to N-type Ca^{2+} channels with IC_{50} values of 1.3 μM and 5.8 μM, respectively. The penaramides are the first nonpeptidic natural products which inhibit the binding of ω-CgTx GVIA to the N-type channels. More significantly, they also possess an unprecedented polyamine structure.

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5. Further purification was hampered due to the presence of numerous closely related compounds, leading to a broad peak in HPLC.
6. A portion of penaramides (**1**, 2.9 mg) was hydrolyzed with 2N HCl (1 mL) at 110°C for 15 h, and the reaction mixture was evaporated to dryness followed by partitioning between H_2O and Et_2O. The aqueous layer was purified on a cellulose column [*n*-BuOH/AcOH/H_2O, (4:1:2)] to yield **penaramine (2)**: TLC on cellulose, R_f 0.28 [*n*-BuOH/AcOH/H_2O (4:1:2), ninhydrin]; IR (KBr) ν_{max} 3450 (br), 2900, 2800, 1630, 1460, 1100 cm^{-1}; ^1H NMR (D_2O) δ 3.25 (8H, m), 2.95 (12H, s), 2.94 (12H, m), 2.52 (6H, s), 2.01 (8H, m), 1.59 (4H, m); ^{13}C NMR (D_2O) δ 61.2t (4C), 50.5q (4C), 47.1t (2C), 45.1t (2C), 44.0t (2C), 32.8q (2C), 22.6t (2C), 19.8t (2C), 19.6t (2C); FABMS (thioglycerol) *m/z* (rel. intensity, %) 437 (13), 387 (2), 330 (5), 321 (3), 285 (5), 186 (25), 112 (5), 84 (13), 72 (26), 58 (84); HRFABMS *m/z* 437.4066 calcd for $C_{22}H_{54}{}^{35}ClN_6$ $(M\text{-}Cl)^+$. Found: 437.4123. The ether layer of the hydrolysate was methylated with ethereal CH_2N_2; a hexane solution (0.6 mg/mL) was analyzed by GC/MS. The following C_{11} fatty acids were identified on the basis of fragment ions in the EIMS: *n*-undecanoic acid [*m/z* (rel intensity): 200 (26%), 157 (17), 143 (15), 87 (69)], 9-methyldecanoic acid [200 (12), 157 (31), 143 (22), 87 (62)], 8-methyldecanoic acid [200 (29), 143 (63), 87 (64)], 4-methyldecanoic acid [200 (9), 143 (26), 87 (100)], and 3-methyldecanoic acid [200 (10), 101 (48)].
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8. **A mixture of penaramides (1).** $[\alpha]^{20}_D$ -11° (*c* 0.39, $CHCl_3$/MeOH 1:1); IR ν_{max}(film) 3400(br), 2950, 2920, 2850, 1690, 1630, 1480, 1460, 1410, 1200, 1170, 1130, 750 cm^{-1}; ^1H NMR in DMSO-d_6/$CDCl_3$ (2:1) **polyamine portion:** δ 9.43 (2H, brs, 2×NH), 3.53 (4H, m, H-5,16), 3.32 (4H, m, H-1,20), 3.26 (4H, m, H-18), 3.07 (12H, s, H-4,17), 2.97 (4H, m, H-7,14), 2.96 (6H, s, 2×N-Me), 2.93 (4H, m, H-9,12), 2.16 (4H, m, H-6,15), 1.92 (4H, m, H-2,19), 1.79 (4H, m, H-10,11), **fatty acid portion:** 1.48 brs, 1.22 brs, 0.81 m; ^{13}C NMR in DMSO-d_6/$CDCl_3$ (2:1) **polyamine portion:** δ 172.0s (C-1',1"), 61.2t (C-3,18), 59.8t (C-5,16), 49.8q (C-4,17), 45.5t (C-9,12), 43.2t (C-1,20), 43.2t (C-7,14), 34.2q (2×N-Me), 21.9t (C-10, 11), 19.9t (C-2,19), 18.7t (C-6,15), **fatty acid portion:** δ 36.3t, 34.8t, 32.6d, 31.5t, 28.9t, 28.8t, 26.6t, 26.3t, 24.4t, 22.4t, 19.5q, 13.7q; FABMS (thioglycerol) *m/z* (rel. intensity, %) 773 (14), 723 (4), 498 (5), 453 (6), 285 (6), 240 (100), 112 (10), 84 (22), 58 (31), 44 (21); HRFABMS *m/z* 773.7127 calcd for $C_{44}H_{94}{}^{35}ClN_6O_2$ $(M\text{-}Cl)^+$. Found: 773.7121.
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13. **Penaramide A (7).** IR ν_{max}(film) 3400(br), 2920, 2850, 1690, 1630, 1480, 1465, 1420, 1200, 1170, 1130, 755 cm^{-1}; ^1H NMR in DMSO-d_6/$CDCl_3$ (2:1) δ 9.57 (2H, brs, 2×NH), 3.55 (4H, m), 3.33 (6H, m), 3.27 (2H, m), 3.06 (12H, s, 2×N-Me_2), 2.98 (6H, s, 2×N-Me), 2.95 (8H, m), 2.25 (4H, dt, *J*=15.0, 7.0), 2.19 (4H, m), 1.93 (4H, m), 1.83 (4H,m), 1.48 (4H, m), 1.22-1.24 (28H, m), 0.83 (6H, t, 6.6); ^{13}C NMR in DMSO-d_6/$CDCl_3$ (2:1) δ 172.2s (2C), 61.7t (2C), 60.2t (2C), 49.9q (4C), 45.9t (2C), 43.7t (2C), 34.8q (2C), 32.6t (2C), 31.1t (2C), 28.9t (10C), 28.6t (2C), 24.4t (2C), 22.3t (2C), 21.9t (2C), 20.3t (2C), 18.9t (2C), 13.7q (2C); FABMS (thioglycerol) *m/z* (rel. intensity, %) 773 (15), 723 (3), 498 (5), 453 (6), 285 (7), 240 (100), 112 (10), 84 (24), 58 (33), 44 (18); HRFABMS *m/z* 773.7127 calcd for $C_{44}H_{94}{}^{35}ClN_6O_2$ $(M\text{-}Cl)^+$. Found: 773.7103.

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