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TETRAHEDRON

Eurypamide A, a cyclic tripeptide from the Palauan sponge *Microciona eurypa*

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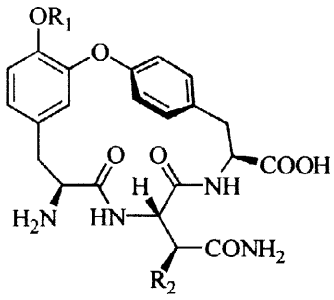
Abstract: The Palauan sponge *Microciona eurypa* contains the cyclic isodityrosine tripeptide, eurypamide A (**5**), and an inseparable mixture of three related tripeptides, eurypamides B - D (**6-8**) that can be identified but not characterized. Eurypamide A contains (2*S*,3*S*,4*R*)-3,4-dihydroxyarginine, which has not been described previously.

In 1986, Sano and co-workers isolated a group of four isodityrosine-derived 17-membered cyclic tripeptides, OF4949 I-IV (**1-4**), as potent aminopeptidase B inhibitors from the culture broth of the fungus *Penicillium rugulosum*.[1] They were later shown to exhibit immunopotentiating activity and confirmed antitumor activity, but they lacked classical cytotoxicity.[2] This led to the synthesis of both the natural products and a number of analogues in which the non-tyrosine amino acid was varied.[3] In our search for new marine natural products, we have developed a collection strategy that gives priority to soft-bodied sessile organisms that have no obvious physical defenses. The sponge *Microciona eurypa* was selected for study because it is an extremely delicate and fragile sponge that is highly successful in competing for space in certain densely populated marine lakes and is not fouled by other organisms. We were somewhat disappointed when we found that it lacked activity in all of our initial screens but we proceeded with a chemical study on the basis of interesting peaks in the ^{1}H NMR spectrum of the crude extract.



1 R_1 = Me, R_2 = OH
2 R_1 = H, R_2 = OH
3 R_1 = Me, R_2 = H
4 R_1 = R_2 = H

The sponge *Microciona eurypa* (de Laubenfels, 1954) was collected by hand in shallow water in Ngeruktabel Marine Lake in Palau and was quickly frozen. The water-soluble material from a methanolic extract was

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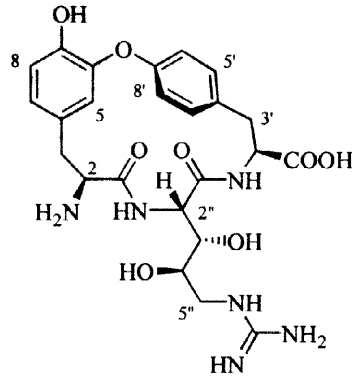
desalted and chromatographed on reversed phase supports to obtain eurypamide A (**5**) and an inseparable mixture of three related isodityrosine tripeptides, eurypamides B - D (**6-8**).



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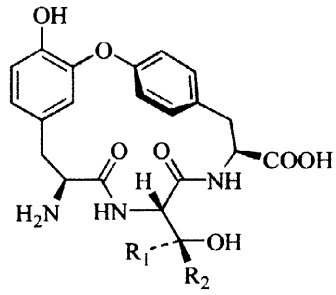


6 R_1 = Me, R_2 = H
7 R_1 = R_2 = H
8 R_1 = H, R_2 = Me

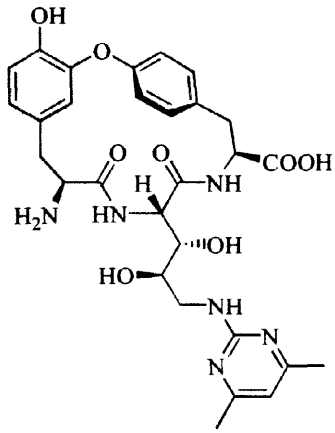
Eurypamide A (**5**), $[\alpha]_D$ -21.5, was isolated as a colorless white powder, mp. 171-175 °C. The molecular formula, $C_{24}H_{30}N_6O_8$, was derived from HRMS data. The ^{13}C NMR spectrum indicated that only 18 protons were attached to carbon, leaving 12 attached to heteroatoms. The IR spectrum contained broad bands at 3310 (-OH, -NH, COOH) and 1665 cm^{-1} (amide, guanidine), that suggested the peptidic nature of the molecule. The UV spectrum contained absorptions at λ_{max} 203 (ε 45 000), 231 (sh, ε 9670), 271 (ε 2450) and 278 nm (sh, ε 2380), which were shifted on addition of base to 203 (ε 75 000), 240 (ε 8300), 280 (ε 3100) and 297 nm (ε 3200), indicating the presence of a phenolic residue. The ^1H NMR spectrum (Table 1) contained signals at δ 5.95 (d, 1 H, J = 2 Hz), 6.68 (dd, 1 H, J = 8, 2 Hz), and 6.85 (d, 1 H, J = 8 Hz), due to a trisubstituted aromatic ring system, and at 6.89 (dd, 1 H, J = 8, 2 Hz), 7.05 (dd, 1 H, J = 8, 2 Hz), 7.22 (dd, 1 H, J = 8, 2 Hz), and 7.43 (dd, 1 H, J = 8, 2 Hz), that can only be assigned to a 1,4-disubstituted phenyl ring that cannot undergo free rotation so that each hydrogen experiences a different magnetic environment. An asymmetrical 1,4-disubstituted aromatic ring is typically found in isodityrosine cyclic tripeptides. In addition to the ABX systems assigned to α and β protons of the two tyrosine units, the ^1H NMR spectrum contained a series of mutually coupled proton signals at δ 4.70 (d, 1 H, J = 4.5 Hz), 3.70 (dd, 1 H, J = 8, 4.5 Hz), 3.87 (ddd, 1 H, J = 8, 7, 2 Hz), 3.47 (dd, 1 H, J = 12.5, 2 Hz) and 3.35 (dd, 1 H, J = 12.5, 7 Hz) that, after correlation with their respective ^{13}C NMR signals using the HMQC data, were assigned to a 3,4-dihydroxyarginine (DHA) unit. The presence of the guanidine group was confirmed by treatment of eurypamide A (**5**) with 2,4-pentanedione and sodium bicarbonate in ethanol at 100 °C for 14 hr. to obtain the corresponding dimethylpyramidyl derivative **9**. The HMBC data (Table 1) confirmed these assignments and allowed the connectivities through the amide bonds in the 17-membered ring to be defined. Thus, eurypamide A (**5**) is a new member of the OF4949 group of isodityrosine cyclic tripeptides.

The absolute configurations at C-2 of the Tyr and DOPA units were determined to be *S* by ozonolysis

Table 1. NMR data (300 MHz, MeOH-d_4) for eurypamide A (5).

C#	δ_C	δ_H	mult, J (Hz)	HMBC	NOESY
DOPA					
1	168.5				
2	54.2	4.15	d, 6	C-1, C-4	H-3a, H-3b
3	36.8	2.98	dd, 15,6	C-1, C-2, C-4, C-5	H-3b, H-9
		3.20	d, 15	C-4	H-3a, H-5
4	124.8				
5	117.0	5.95	d, 2	C-3, C-6, C-7, C-9	H-3b, H-6', H-8'
6	150.0				
7	147.6				
8	117.4	6.85	d, 8	C-4, C-6, C-7	H-9
9	125.3	6.68	dd, 8, 2	C-3, C-5, C-7	H-3a, H-8
Tyr					
1'	175.3				
2'	55.5	4.78	dd, 12.5, 3	C-1', C-1", C-3', C-4'	H-3', H-5'
3'	39.9	2.70	t, 12.5	C-1', C-2', C-4', C-5', C-9'	H-3'b, H-5'
		3.46	dd, 12.5, 3	C-2', C-4', C-5', C-9'	H-2', H-3'a, H-5'
4'	136.0				
5'	131.9	7.43	dd, 8, 2	C-7', C-9'	H-2', H-3'b, H-6'
6'	123.6	7.05	dd, 8, 2	C-4', C-7', C-8'	H-5, H-5'
7'	155.1				
8'	122.8	6.89	dd, 8, 2	C-4', C-6', C-7'	H-5, H-9'
9'	133.3	7.22	dd, 8, 2	C-5', C-7'	H-3'a, H-8', H-2"
DHA					
1"	169.3				
2"	55.7	4.70	d, 4.5	C-1, C-1", C-3", C-4"	H-9', H-3"
3"	75.2	3.70	dd, 8, 4.5	C-1", C-2", C-4", C-5"	H-2", H-4"
4"	71.4	3.87	ddd, 8, 7, 2		H-3", H-5"a,b
5"	45.8	3.35	dd, 12.5, 7	C-4", C-6"	H-4", H-5"b
		3.47	dd, 12.5, 2	C-6"	H-4", H-5"a
6"	159.7				

of eurypamide A (**5**), hydrolysis of the residue and derivitization of the amino acids produced to obtain L-aspartic acid as the only standard amino acid. The relatively rigid ring system of eurypamide A (**5**) resulted in strong nOe correlations between H-5 of the DOPA unit and H-6 and H-8 of the Tyr unit, between H-9 of the Tyr unit and H-2 of the DHA unit and between H-5 and H-2 in the Tyr unit, but did not give any useful information about the stereochemistry of the dihydroarginine side chain. Treatment of eurypamide A (**5**) with



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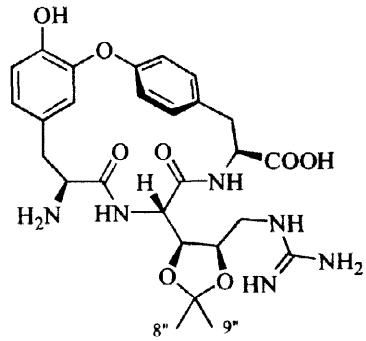

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Table 2. ^{1}H NMR data for the acetonide 10.

C#	δ_H mult., J (Hz) (MeOH-d_4)	δ_H mult., J (Hz) (DMSO-d_6)	NOESY (DMSO-d_6)
DOPA			
2	4.19 d, 6	4.01 br s	H-3, NH"
3	2.98 dd, 15, 6	2.90 m, 2 H	H-2, H-5, H-9, NH"
	3.20 d, 15		
5	5.95 d, 2	5.76 d, 2	H-3, H-6', H-8'
8	6.84 d, 8	6.79 d, 8	H-9, OH-7
9	6.66 dd, 8, 2	6.67 dd, 8, 2	H-3, H-8
OH-7		9.32 s	H-8
Tyr			
2'	4.65 dd, 12.5, 3	4.52 ddd, 13, 10, 4	H-3'b, H-5'
3'	2.69 t, 12.5	2.66 br t, 13	H-3'b, H-9'
	3.39 dd, 12.5, 3	3.26 dd, 13, 4	H-2', H-3'a, H-5'
5'	7.43 dd, 8, 2	7.34 dd, 8, 2	H-2', H-3'b, H-6'
6'	7.05 dd, 8, 2	6.97 dd, 8, 2	H-5, H-5'
8'	6.89 dd, 8, 2	6.70 dd, 8, 2	H-5, H-9'
9'	7.24 dd, 8, 2	7.25 dd, 8, 2	H-3'a, H-8', NH'
NH'		7.99 d, 10	H-9', H-2"
DHA			
2"	4.56 d, 9.5	4.46 t, 9	H-5", NH'
3"	4.28 dd, 9.5, 5.5	4.21 dd, 9, 4.5	H-4", H-8", NH"
4"	4.30 m	4.15 m	H-3", H-8"
5"	3.30 m, 2 H	3.15 m, 2 H	H-2"
8"	1.37 s, 3 H	1.26 s, 3 H	H-3", H-4", H-9"
9"	1.57 s, 3 H	1.45 s, 3 H	H-8"
NH"		8.37 d, 9	H-2, H-3, H-3"

dimethoxypropane and *p*-toluenesulfonic acid in DMF produced the corresponding acetonide **10**, which was used to determine the stereochemistry of the DHA side chain. Both H-3 and H-4 showed nOe correlations (Table 2) to the same acetonide methyl group, which requires that the DHA unit be assigned the (3*S*,4*R*) or (3*R*,4*S*) stereochemistry. The relatively large (9 Hz) coupling constant between H-2 and H-3 and the lack of an nOe between these signals suggested an absence of free rotation about the 2,3 bond. Molecular modeling of all potential diastereoisomers using PC Model indicated a preferred stereochemistry for the (2*S*,3*S*,4*R*) conformation of the DHA side chain that placed the guanidinium group of the DHA side chain adjacent to the carboxylic acid of the Tyr unit, as might be expected if there is an electrostatic interaction between the two functional groups. All of the expected nOe correlations for the (2*S*,3*S*,4*R*) conformation were observed and all other possible streochemistries were eliminated. The amino acid (2*S*,3*S*,4*R*)-3,4-dihydroxyarginine has not been reported previously.

Although we were unable to separate and characterize the three components of a mixture of isodityrosine cyclic peptides, eurypamides B - D, we were able to determine the structures as **6-8**. In the ^{1}H NMR spectrum of the mixture, the signals in the aromatic region clearly indicated the presence of isodityrosine derivatives and some idea of the complexity of the mixture was apparent from the observation of three signals associated with H-5 of the DOPA unit at δ 5.94, 5.97, and 5.99 in a 3:1:1 ratio. Methyl signals at δ 1.16 (d, 3 H, J = 6.5 Hz) and 1.13 (d, 3 H, J = 6.5 Hz) in a 3:1 ratio, which were coupled to signals at 4.18 and 4.09, respectively, suggested that two of the cyclic peptides contained threonine or allothreonine residues. The FABMS spectrum contained a peak at *m/z* 444.1753, which corresponds to the molecular formula $C_{22}H_{26}N_3O_7$ $[M+H]^+$ for an isodityrosine tripeptide containing threonine or allothreonine, and a second peak at *m/z* 430.1620, which corresponds to the molecular formula $C_{21}H_{24}N_3O_7$ $[M+H]^+$ for an isodityrosine tripeptide containing serine. Using low resolution negative ion MS/MS, both molecular ion peaks at *m/z* 442 and 428 gave rise to fragment peaks at 398, due to loss of the variable side chain, and at 354, resulting from a further loss of the carboxylate group, which is identical to the pattern arising from similar treatment of eurypamide A (**5**). Hydrolysis of the peptide mixture followed by formation and analysis of the *N*-pentafluoropropionyl isopropyl ester derivatives by GC-MS using a Chirasil-Val column revealed that the major amino acid was L-threonine, from eurypamide B (**6**) and the minor amino acids were L-serine and L-*allo*-threonine, from eurypamides C (**7**) and D (**8**) respectively. Ozonolysis of the peptide mixture followed by hydrolysis, derivatization and GC-MS analysis again showed that L-aspartic acid was produced from the tyrosine residues.

Eurypamides A - D (**5-8**) were not cytotoxic or antimicrobial in our in house assays and showed no activity in two antiinflammatory assays.[4,5] Although eurypamides A - D (**5-8**) have not been reported previously as natural products, eurypamide C (**7**) has been synthesized.[6]

EXPERIMENTAL SECTION

Animal Material: A sample of the fragile coffee colored sponge *Microciona eurypa* (de Laubenfels, 1954) was collected by hand in shallow water (-1 m) at Ngeruktabel Marine Lake in Palau. A specimen of the sponge (Collection # 96-030) has been deposited in the SIO Benthic Invertebrate Collection (# P-1171).

Extraction and Isolation: The frozen sponge (880 g wet weight) was diced and extracted with MeOH (2 x 1L) at room temperature. The methanol extract was concentrated to an aqueous suspension (50 mL) that was diluted with H_2O (50 mL) and extracted with CH_2Cl_2 (2 x 250 mL). The aqueous extract was freeze dried and the residue was desalted by repeatedly washing with MeOH and removing the salt by filtration to obtain an oily residue (4.8 g) that was dissolved in H_2O (15 mL) and loaded onto a preparative C_{18} cartridge (10 mL). Elution with a gradient of 0-100% MeOH in 0.05% aqueous TFA solution gave fractions that were monitored by TLC on C_{18} plates. The peptide mixture (185 mg), which gives a yellow spot with ninhydrin, eluted with 5% MeOH in 0.05% aqueous TFA. Chromatography of this fraction by HPLC on a C_{18} support using 20% acetonitrile in 0.1% aqueous TFA as eluant gave eurypamide A (**5**, 36 mg, 0.0041% wet weight) and a mixture of isodityrosine cyclic peptides, eurypamides B - D (**6-8**, 18 mg, 0.0021% wet weight), that defied all attempts at separation.

Eurypamide A (5): white powder, mp. 171-175 °C; $[\alpha]_D$ -21.5 (*c* 0.23, MeOH); IR (AgCl) 3310 (br), 1665 (br), 1515, 1440, 1270, 1200 cm^{-1}; UV (MeOH) 203 (ε 45 000), 231 (sh, ε 9670), 271 (ε 2450) and 278 nm (sh, ε 2380), (MeOH + NaOH) 203 (ε 75 000), 240 (ε 8300), 280 (ε 3100) and 297 nm (ε 3200); ^1H NMR (300 MHz, MeOH-d_4) see Table 1; ^{13}C NMR (100 MHz, MeOH-d_4) see Table 1; ^1H NMR (300 MHz, DMSO-d_6) δ 2.65 (br t, 1 H, *J* = 13 Hz, H-3'), 2.88 (br s, 2 H, H-3), 3.15 (m, 1 H, H-5"), 3.35 (m, 1 H, H-3'), 3.38 (m, 1 H, H-5"), 3.40 (m, 1 H, H-3"), 3.79 (m, 1 H, H-4"), 4.02 (br s, 1 H, H-2), 4.58 (dd, 1 H, *J* = 9, 3.5 Hz, H-2"), 4.64 (m, 1 H, H-2'), 5.18 (br s, 1 H, OH-3"), 5.63 (br s, 1 H, OH-4"), 5.75 (d, 1 H, *J* = 2 Hz, H-5), 6.67 (dd, 1 H, *J* = 8, 2 Hz, H-9), 6.70 (dd, 1 H, *J* = 8, 2 Hz, H-8'), 6.80 (d, 1 H, *J* = 8 Hz, H-8), 7.00 (dd, 1 H, *J* = 8, 2 Hz, H-6'), 7.24 (dd, 1 H, *J* = 8, 2 Hz, H-9'), 7.38 (dd, 1 H, *J* = 8, 2 Hz, H-5'), 7.38 (br s, 1 H, NH-5"), 7.74 (d, 1 H, *J* = 10 Hz, NH'), 7.84 (br s, 2 H, NH_2), 8.38 (d, 1 H, *J* = 9 Hz, NH"), 9.37 (s, 1 H, OH-7); HRFABMS *m/z* 531.2190 $(M+H)^+$ (calcd for $C_{24}H_{31}N_6O_8$, 531.2203); LRMS/MS *m/z* 428 → 398, 354.

Mixture of eurypamides B - D (6-8): Colorless powder; HRFABMS *m/z* 430.1620 $(M+H)^+$ (calcd for $C_{21}H_{24}N_3O_7$, 430.1614), *m/z* 444.1753 $(M+H)^+$ (calcd for $C_{22}H_{26}N_3O_7$, 444.1771); LRMS/MS *m/z* 428 → 398, 354, *m/z* 442 → 398, 354.

Preparation of dimethylpyrimidyl derivative 9 of eurypamide A: A solution of 2,4-pentanedione (0.2 mL) in EtOH (2 mL) was slowly added to a solution of eurypamide A (**5**, 5 mg, 9.4 μmol) and $NaHCO_3$ in H_2O (0.5 mL) and the mixture was heated under reflux for 14 hr. using an oil bath heated to 100 °C. The solvent

was evaporated under vacuum and the residue dissolved in H_2O (5 mL), then washed with Et_2O (2 x 8 mL) and CH_2Cl_2 (2 x 8 mL). The aqueous phase was brought to pH 7 by addition of 0.1 N HCl and the solvent was lyophilized. The residue was chromatographed on a C_{18} reversed phase cartridge (3 mL) using 15% CH_3CN in 0.1% aqueous TFA as eluant to obtain the dimethylpyramidyl derivative **9** (4 mg, 71% yield): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42 (dd, 1 H, J = 8, 2 Hz), 7.22 (dd, 1 H, J = 8, 2 Hz), 7.05 (dd, 1 H, J = 8, 2 Hz), 6.89 (dd, 1 H, J = 8, 2 Hz), 6.84 (d, 1 H, J = 8 Hz), 6.66 (dd, 1 H, J = 8, 2 Hz), 6.60 (s, 1 H), 5.95 (d, 1 H, J = 2 Hz), 4.85 (dd, 1 H, J = 12.5, 3 Hz), 4.80 (d, 1 H, J = 4.5 Hz), 4.10 (dd, 1 H, J = 6, 2 Hz), 3.93 (td, 1 H, J = 8, 4 Hz), 3.69 (br d, 2 H, J = 4 Hz), 3.66 (dd, 1 H, J = 8, 4 Hz), 3.44 (dd, 1 H, J = 12.5, 3 Hz), 3.22 (dd, 1 H, J = 15, 2 Hz), 2.95 (dd, 1 H, J = 15, 6 Hz), 2.70 (br t, 1 H, J = 12.5 Hz), 2.36 (s, 6 H).

Preparation of acetonide 10 from eurypamide A: 2,2-Dimethoxypropane (400 μL) was added to a stirred solution of eurypamide A (**5**, 6 mg, 11 μmol) and *p*-toluenesulfonic acid (2 mg, 10 μmol) in dry DMF (2 mL) and the solution was heated at 50-60 °C for 4 hr. under an atmosphere of dry N_2. Pyridine (20 μL) was added and the excess reagent was removed in a stream of dry N_2. The residue was transferred to a C_{18} cartridge (3 mL) and eluted with 33% MeOH in H_2O to obtain the acetonide **10** as an oil: ^{1}H NMR (300 MHz, MeOH-d_4 and DMSO-d_6) see Table 2.; HRFABMS *m/z* 571.2516 $(M+H)^+$ (calcd for $C_{27}H_{35}N_6O_8$, 571.2516).

Hydrolysis of peptides and analysis of hydrolysates by chiral GC-MS: A solution of the mixture of cyclic peptides **6-8** (800 μg) in 6N HCl (500 μL) was heated in a sealed conical vial at 110 °C for 15 hr. The solvent was removed in a stream of N_2 and the residue was dissolved in *i*-PrOH (400 μL) to which was added acetyl chloride (100 μL). The vial was resealed and the solution was heated at 100 °C for 1 hr. Excess reagents were removed under N_2 and the residue was redissolved in CH_2Cl_2 (400 μL) and pentafluoropropionic anhydride (400 μL). The reaction mixture was again sealed and heated at 100 °C for 15 min. The reagents were again removed under N_2 and the residue was redissolved in CH_2Cl_2 (400 μL). The derivatized hydrosylates were analysed by GC-MS using a Chirasil-Val capillary column to obtain peaks that corresponded in both retention times and mass specta to L-threonine, L-serine and L-*allo*-threonine.

Ozonolysis of eurypamide A (5) and peptide mixture 6-8: In separate experiments, a stream of ozone in O_2 was bubbled through cooled solutions of eurypamide A (**5**, 1 mg) or the peptide mixture **6-8** (1 mg) in MeOH (0.5 mL) at -78 °C for 1 hr. Hydrogen peroxide (50%, 5 drops) was added to the reaction mixtures which were then allowed to stand at room temperature for 1 hr. The solvent was removed under a stream of N_2 and the ozonolysis products were hydrolyzed and derivatized as described above. The derivatized hydrosylates were analysed by GC-MS using a Chirasil-Val capillary column to obtain L-aspartic acid, as judged by the retention time and mass spectrum.

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screening data. We also thank the Republic of Palau and the State of Koror for Marine Research Permits. This research was supported by grants from the National Science Foundation (CHE95-27064), and the National Institutes of Health (CA 49084). The Varian Inova 300 MHz NMR spectrometer was purchased using a grant from the National Science Foundation (CHE95-23507).

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4. Eurypamide A (**5**) was assayed against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans* and phospholipase A_2. It was also assayed in the PQ-37 assay, the NCI 60 cell line panel, the brine shrimp lethality assay, the mouse ear adema assay and for its ability to disrupt basic cellular processes. All assays gave negative results.
5. We were unable to locate a laboratory that was performing the aminopeptidase B inhibition assay and, on the advice of Prof. Dale Boger, we have not attempted to perform the assays in house.
6. Itokawa, H.; Watanabe, K.; Kawaoto, S.; Inoue, T. *Jpn. Kokai Tokkyo Koho* JP 63,203,671 [CA 110:213362w]