Dysinosins B−D, Inhibitors of Factor VIIa and Thrombin from the Australian Sponge *Lamellodysidea chlorea*†

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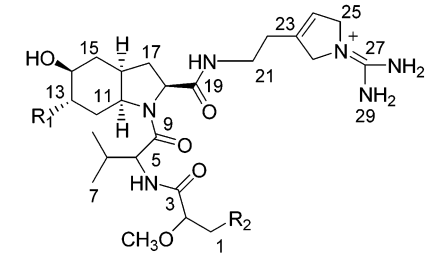
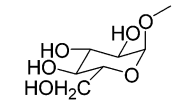
Received January 12, 2004

Three new marine natural products, dysinosins B−D (**1**−**3**), were isolated from the sponge *Lamellodysidea chlorea* and their structures determined by 1D and 2D NMR spectroscopy. These compounds are inhibitors of the blood coagulation cascade serine proteases factor VIIa and thrombin. These analogues, dysinosins B−D (**1**−**3**), allowed identification of two structural motifs within the structures that contribute to binding to the proteases, factor VIIa and thrombin.

Natural Product Discovery commenced a natural product screening program in 1994, and this program has been summarized on two occasions.[1,2] In a high-throughput campaign to find serine protease inhibitors, the sponge *Lamellodysidea chlorea* was studied. Bioassay-guided purification afforded three new peptides, dysinosins B−D (**1**−**3**). Dysinosin A (**4**) has been reported previously from another species of sponge from the family Dysideidae.[3] Dysinosins are related to the cyanobacterial compounds named aeruginosins.[4−6] Aeruginosin 298-A (**5**) was reported in 1994, as a thrombin and trypsin inhibitor from the cyanobacterium *Microcystis aeruginosa*.[4] The general structural features of dysinosins B−D (**1**−**3**) comprise a tetrahydroindoline-2-carboxylic acid, 3-aminoethyl 1-guanidylpyrroline, a glyceric acid, and valine, connected by three peptide bonds. The isolation and structure elucidation of compounds **1**−**3** are reported here, together with their inhibitor activity of the serine proteases factor VIIa and thrombin. As the crystal structure of dysinosin A in thrombin has been obtained,[3] the analogues may be useful to provide insight into the important interactions in factor VIIa relative to thrombin.



1 R_1 =  $R_2 = OSO_3^-$

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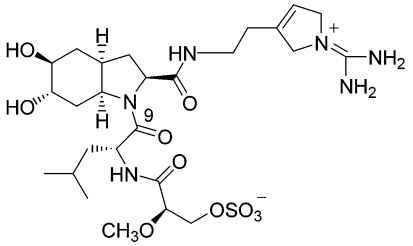

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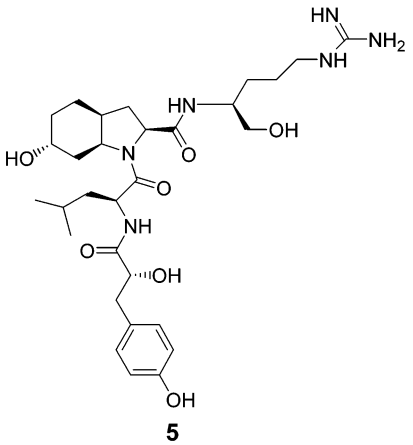

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Results and Discussion

The marine sponge *L. chlorea* was freeze-dried, then pulverized to give 13.76 g dry weight of material. This was extracted with H_2O/1% trifluoroacetic acid and then immediately chromatographed by reversed-phase C_{18} MPLC with final purification by reversed-phase C_{18} HPLC to yield the three compounds dysinosins B−D (**1**−**3**).

The molecular formula of **1** was determined to be $C_{31}H_{52}N_6O_{15}S$ by HRESIMS of the $[M - H]^-$ ion (*m*/*z* 779.3135, calcd 779.3138). The NMR assignments for compound **1** are listed in Table 1. Analysis of the ^1H and ^{13}C NMR data, together with the two-dimensional data, gCOSY, gHSQC, and gHMBC, revealed the following units: glyceric acid, methoxyl, valine, tetrahydro-5-hydroxy-6-glucopyranoside-dihydroxyindoline-2-carboxylic acid, and a 1-guanidylpyrroline. The ^1H NMR spectrum gave no indication of conformational isomers, as there was no signal doubling, unlike the ^1H NMR of dysinosin A (**4**),[3] and 31 discrete carbon signals were observed in the ^{13}C NMR spectrum. The vinylic proton at δ_H 5.62 (brs) (H-24) and the four-proton multiplet at δ_H 4.11 (2H-25 and 2H-30) are

† Dedicated to the late Dr. D. John Faulkner (Scripps) and the late Dr. Paul J. Scheuer (Hawaii) for their pioneering work on bioactive marine natural products.

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10.1021/np049968p CCC: $27.50
Published on Web 07/09/2004

Table 1. ^{1}H (600 MHz), ^{13}C (150 MHz), and gHMBC NMR Data for Dysinosin B (**1**) in DMSO-d_6[a,b]

position	^{13}C	^{1}H	HMBC
1a	66.1 t	3.97 (dd, J = 11.0, 2.5 Hz)	3
1b		3.77 (dd, J = 11.0, 6.6 Hz)	
2	80.3 d	3.92 (dd, J = 6.6, 2.5 Hz)	1, 3, 2-OMe
3	169.1 s		
4		7.56 (d, J = 7.5 Hz)	3, 9
5	55.2[c] d	4.38 (t, J = 7.5 Hz)	6, 7, 8, 9
6	30.7 q	1.86 (oct., J = 7.5 Hz)	7, 8
7	18.4[d] q	0.90[d] (d, J = 7.5 Hz)	6, 7
8	18.3[d] q	0.86[d] (d, J = 7.5 Hz)	6, 8
9	169.3 s		
10			
11	53.8 d	4.33 (dt, J = 12.0, 6.0 Hz)	16, 17, 18
12α	23.0 t	1.90 (brt, J = 12.0 Hz)	
12β		2.15[e] (m)	
13	72.5 d	3.77 (m)	
14	65.7 d	3.66 (m)	
15α	27.1 t	1.62 (brd, J = 13.4 Hz)	11, 13, 14
15β		2.05[e] (m)	
16	35.3 d	2.12[e] (m)	11
17α	33.3 t	2.35[e] (m)	19
17b		2.07[e] (m)	
18	59.5 d	4.07 (t, J = 10.0 Hz)	17
19	171.1 s		
20		7.57 (t, J = 6.0 Hz)	19
21a	36.4 t	3.28[e] (m)	
21b		3.09[e] (m)	19, 22, 23
22a	28.4 t	2.29[e] (m)	21, 23, 24
22b		2.22[e] (m)	21, 23, 24
23	136.1 s		
24	119.1 d	5.62 (brs)	25
25	54.0[c] t	4.11 (m, 2H)	
26			
27	154.1 s		
28		7.18 (m, 2H)	
29		7.18 (m, 2H)	
30	55.3[c] t	4.11 (m, 2H)	
2-OMe	57.2 q	3.30[e] s	2
14-OH		4.89 (d, J = 3.0 Hz)	
1′	95.1 d	5.02 (d, J = 3.6 Hz)	
2′	71.8 d	3.28[e] (m)	
3′	73.1 d	3.46 (m)	
4′	70.3 d	3.11[e] (m)	
5′	73.0 d	3.38 (m)	
6a′	60.9 t	3.59 (m)	
6b′		3.46 (m)	
2′-OH		4.68 (d, J = 6.5 Hz)	1′, 2′
3′-OH		4.64 (brd, J = 3.5 Hz)	
4′-OH		4.83 (brd, J = 4.8 Hz)	
6′-OH		4.49 (brt, J = 6.0 Hz)	

[a] Assignments confirmed by two-dimensional experiments (gCOSY, ROESY, gHSQC, and gHMBC). [b] Mutiplicities were determined by DEPT experiments. [c,d] Signals within the same column are interchangeable. [e] Chemical shifts derived from gCOSY/gHSQC/gHMBC experiments.

quite characteristic for the 1-guanidylpyrroline unit in these peptides.[3] The indoline and the 1-guanidylpyrroline are linked by an amide bond between the carbonyl attached to the indoline (C-19) and the amino group at the end of the ethyl side chain attached to 1-guanidylpyrroline (N-20). Correlations in the gHMBC experiment from both H-17α and H-21b to the carbonyl carbon C-19 revealed this (Table 1). The difference between **1** and the previously isolated dysinosin A (**4**) is the presence of a valine amino acid residue in **1** instead of leucine, which is present in dysinosin A (**4**). Also **1** has a 6-glucopyranoside instead of a 6-hydroxyl in dysinosin A (**4**). The glyceric acid has a sulfate at C-1 {δ_C 66.2 (t); δ_H 3.97 (dd, J = 11.0, 2.5 Hz), 3.77 (dd, J = 11.0, 6.6 Hz} and is *O*-methylated at C-2 {δ_C 57.2 (q); δ_H 3.30 (s)}. The correlation between the proton at N-4 {δ_H 7.56 (d, J = 7.5 Hz)} and the carbonyl C-3 identifies the amide bond between the valine and glyceric acid moiety. The planar structure for **1** is clearly completed by an amide bond linking the indoline nitrogen N-10 and the valine carbonyl C-9.

The relative stereochemistry of **1** was established from correlations in a ROESY experiment and ^{1}H NMR coupling constants. To help in the interpretation, the solvent was changed from DMSO-d_6 to pyridine-d_6. The shape of the signals for H-13 and H-14 suggested small couplings between H-14 and H-13, H-15α and H-15β and between H-13 and H-14, H-12α and H-12β. This information together with data from that of dysinosin A (**4**)[3] implies that the two hydroxyl groups on the indoline are axial. The ROESY correlation between H-11 and H-16 indicates a *cis* ring junction, while there is a weak correlation between H-18 and H-16, giving minor evidence that these two atoms are on the same side of the indoline. The amide bond C-9−N-10 is *trans* relative to C-5 and C-18 since correlations were seen between H-5 and H-11. The relative stereochemistry of the valine and at C-2 could not be assigned from the ROESY experiment. The α-linkage of the sugar was determined on the basis of the ^{1}H NMR coupling constant of the anomeric proton (3.6 Hz) and the ^{13}C NMR chemical shift of the anomeric carbon (δ_C 95.1).[7] Thus, the relative stereochemistry of the sugar and indoline was determined. There were no unambiguous ROESY correlations between the sugar and the indoline so that the ring systems could be related to each other. The above information indicates that dysinosin B has structure **1**.

The molecular formula of **2** was established as $C_{25}H_{42}N_6O_{10}S$ by high-resolution negative electrospray mass spectroscopic analysis (m/z 617.2610 [M − H]$^-$). This information together with its NMR data (Table 2) indicates that **2** is related to **1**. The structural difference is that in **2** there is a secondary alcohol at C-13 {δ_H 3.68 (m); δ_C 68} replacing the α-glucose in **1**. Dysinosin C was therefore assigned structure **2**.

A molecular formula of $C_{25}H_{43}N_6O_7$ was assigned to **3** from its HRESIMS (m/z 539.3192 [M]$^+$). Compound **3** is also clearly related to **1** and **2** (Tables 1 and 2), and comparing the molecular formula of **2** and **3** indicates that **3** is the desulfated derivative of **2**. The only significant differences in the NMR data are related to the oxygenated methylene in the glyceric acid unit (C-1). Thus, the C-1 protons have shifted upfield by approximately 0.3 ppm {δ_H 3.66 (m), 3.51 (m)} and C-1 by 5 ppm (δ_C 61) in **3** when compared to **2**. Dysinosin D was assigned structure **3**. The absolute stereochemistry of dysinosins B−D (**1**−**3**) was not established, but on biogenetic grounds is likely to be the same as dysinosin A (**4**).

Thrombin and factor VIIa are serine protease enzymes in the blood coagulation cascade.[8] The inhibitory activity of dysinosins A−D (**1**−**4**) against these serine proteases is shown in Table 3. The related peptide dysinosin A (**4**) is an inhibitor of factor VIIa and thrombin as previously reported.[3] The aeruginosin class of linear peptides, which contain octahydroindole systems similar to the dysinosins, are known to inhibit various serine proteases such as thrombin, trypsin, and plasmin.[4−6] Dysinosins B−D (**1**−**3**) are further examples of linear peptides containing octahydroindole systems exhibiting serine protease inhibitory activity.

The X-ray structure of dysinosin A (**4**) in thrombin has been determined.[3] The introduction of a sugar at C-13 in dysinosin B (**1**) gave a slight increase in inhibition of factor VIIa compared to dysinosins A (**4**) and C (**2**) (90 nM

Table 2. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR Data for Dysinosins C (**2**) and D (**3**) DMSO-d_6[a,b]

	2		**3**	
position	^{13}C	^{1}H	^{13}C	^{1}H
1a	66[e] t	3.99 (dd, J = 11.0, 2.5 Hz)	61[e] t	3.66[e] (m)
1b		3.78 (dd, J = 11.0, 6.6 Hz)		3.51[e] (m)
2	80[e] d	3.92 (dd, J = 6.6, 2.5 Hz)	83[e] d	3.68[e] (m)
3	*f*		*f*	
4		7.60 (d, J = 7.5 Hz)		7.32 (d, J = 7.6 Hz)
5	55[e] d	4.34 (t, J = 7.5 Hz)	54[e] d	4.43 (t, J = 7.6 Hz)
6	*f*	1.91[e] (m)	31[e] d	1.89 (oct., J = 7.6 Hz)
7	18[d,e] q	0.90[d,e] (m)	17[e] q	0.87[c] (d, J = 7.6 Hz)
8	19[d,e] q	0.87[d,e] (m)	19[e] q	0.86[c] (d, J = 7.6 Hz)
9	*f*		*f*	
10				
11	54[e] d	4.16[e] (m)	54[e] d	4.14[e] (m)
12	27[e] t	1.96 (m, 2H)	28[e] t	1.98 (m, 2H)
13	68[e] d	3.68 (m)	68[e] d	3.68 (m)
14	67[e] d	3.56 (m)	67[e] d	3.57[e] (m)
15α	*f*	1.58 (d, J = 14.0 Hz)	26[e] t	1.58 (d, J = 14.0 Hz)
15β		1.94[e] (m)		1.94*e* (m)
16	*f*	2.11[e] (m)	35[e] d	2.13 (m)
17α	*f*	2.36[e] (m)	33[e] t	2.36[e] (m)
17β		2.07[e] (m)		2.04[e] (m)
18	59[e] d	4.08 (t, J = 10.0 Hz)	59[e] d	4.07 (t, J = 10.0 Hz)
19	*f*		*f*	
20		7.48 (t, J = 6.0 Hz)		7.65 (t, J = 7.3 Hz)
21a	*f*	3.28[e] (m)	36[e] t	3.19[e] (m)
21b		3.06[e] (m)		3.12[e] (m)
22a	28[e] t	2.26[e] (m)	28[e] t	2.25[e] (m)
22b		2.22[e] (m)		2.23[e] (m)
23	*f*		136[e] s	
24	119[e] d	5.62 (brs)	119[e] d	5.62 (brs)
25	54[d,e] t	4.11 (m, 2H)	54[d,e]t	4.11 (m, 2H)
26				
27	*f*		*f*	
28		7.14 (brs, 2H)		7.23 (brs, 2H)
29		7.14 (brs, 2H)		7.23 (brs, 2H)
30	55[d,e] t	4.11 (m, 2H)	55[d,e] t	4.11 (m, 2H)
2-OMe	58[e] q	3.32[e] (s)	57[e] q	3.32[e] (s)
1-OH				4.80 (t, J = 6.0 Hz)
13-OH				4.84 (d, J = 3.6 Hz)
14-OH				4.69 (d, J = 3.0 Hz)

[a] Assignments confirmed by two-dimensional experiments (gCOSY, ROESY, gHSQC, and gHMBC). [b] Mutiplicities were determined by gHSQC experiments. [c,d] Signals within the same column are interchangeable. [e] Chemical shifts derived from gCOSY/gHSQC/gHMBC experiments. [f] Chemical shift could not be determined.

Table 3. Factor VIIa and Thrombin Inhibitory Activity for Dysinosins A–D (**1**–**4**)

compound	factor VIIa K_i (μM)	thrombin K_i (μM)
dysinosin B (**1**)	0.090	0.170
dysinosin C (**2**)	0.124	0.550
dysinosin D (**3**)	1.320	>5.1
dysinosin A (**4**)	0.108	0.452

compared to 108 and 124 nM, respectively), while selectivity relative to thrombin decreased to 1.9 compared to 4.2 and 4.4 respectively for dysinosin A (**4**) and C (**2**). In the thrombin–dysinosin A structure the hydroxyl at C-13 lies within the P2 pocket. Increased P2 bulk is tolerated and results in slight increase in affinity, indicating that this region should be explored to increase selectivity for factor VIIa over thrombin.

Desulfated dysinosin D (**3**) is 10 times less potent against both factor VIIa and thrombin compared with dysinosins A (**4**), B (**1**), and C (**2**), indicating that the sulfate group contributes to both factor VIIa and thrombin binding; the X-ray structure for thrombin–dysinosin A shows hydrogen bonding between arginines 208 and 263 of thrombin and the sulfate residue in dysinosin A (**4**).

The three analogues dysinosins B–D (**1**–**3**) allowed identification of two structural motifs within the structures that contribute to binding to the proteases, factor VIIa and thrombin.

Experimental Section

General Experimental Procedures. Water was Millipore Milli-Q PF filtered, while all other solvents used were Omnisolv HPLC grade. A Hypersil BDS C_{18} 5 μm (10 mm × 250 mm i.d.) was used for semipreparative HPLC. A Waters 600 pump fitted with a 996 photodiode array detector and 717 plus autosampler was used for the semipreparative separations. NMR spectra were recorded at 30 °C on a Varian Inova 600 MHz NMR spectrometer. Samples were dissolved in DMSO-d_6 (residual ^{1}H δ 2.50 and ^{13}C δ 39.5 ppm). HRESIMS were measured on a Bruker BioAPEX 47e mass spectrometer equipped with a Bradford CT 06405 electrospray ion source. FTIR and UV spectra were recorded on a Thermo Nicolet-Nexus FTIR spectrophotometer and an Agilent 8453 UV/vis spectrophotometer, respectively. Optical rotations ($[\alpha]_D$) were measured on a Jasco P-1020 polarimeter. A molecular Devices Thermomax microtiter plate reader and a WALLAC Victor II were used as the spectrophotometric readers. Spectrophotometric assay materials: tris buffer, sodium chloride, calcium chloride, and bovine serum albumin were obtained from Sigma-Aldrich. Factor VIIa was purchased from Enzyme Research Laboratories Inc. and thrombin from Haematologic Technologies. The cofactor r-tissue factor was obtained from American Diagnostica Inc. Chromogenic substrate S-2366 was purchased from Helena Laboratories and spectrazyme chromogenic substrate #217L FVIIa from American Diagnostica Inc. Clear polystyrene 96-well microtiter plates were purchased from Nunc.

Animal Material. The sponge sample *Lamellodysidea chlorea* [phylum Porifera, class Demospongiae, order Dictyoceratida, family Dysideidae] was collected by scuba diving at a depth of 16 m on the southeast tip of Wooded Islet, Low Isles, Queensland, Australia (16.23.8′ S; 145.34.0′ E) in January 1997. A voucher sample, QMG307864, has been deposited at the Queensland Museum, South Brisbane, Queensland, Australia.

Extraction and Isolation. The freeze-dried sponge material, *Lamellodysidea chlorea*, was pulverized (13.76 g) and then extracted with H_2O/1% trifluoroacetic acid and chromatographed on a Waters AP-2 MPLC column packed with C_{18} and the solvent pumped through at 3 mL/min. The following conditions were used: H_2O/1% trifluoroacetic acid isocratic for 15 min followed by a H_2O/1% trifluoroacetic acid gradient to MeOH/1% trifluoroacetic acid in 120 min and then MeOH/1% trifluoroacetic acid isocratic for 148 min. Ninety-four test tubes were collected, and on the basis of screening results, 16 were combined (ca. 15% H_2O/85% MeOH), concentrated, and applied to a C_{18} HPLC column with the following solvent conditions: H_2O/0.1% trifluoroacetic acid (100%) gradient to H_2O/0.1% trifluoroacetic acid (100%) + MeOH/0.1% trifluoroacetic acid (60%) in 10 min. Three compounds were collected, dysinosin B (**1**), dysinosin C (**2**), and dysinosin D (**3**), with retention times of 4.5, 5.7, and 6.4 min, respectively.

General Assay Principle. When the serine protease (in the presence of co-enzyme in the case of factor VIIa) acts on the substrate, a yellow color is developed.

General Assay Procedure. The standards, the controls, and the test substances are pipetted together with the assay-

buffer to a microtiter plate. Then the two reagents, the enzyme (and its co-enzyme in the case of factor VIIa) and the chromogenic substrate, are multidispensed to the plate. The plate is incubated for 2 h at room temperature. After incubation the plate is read at 405 nm and the percentage inhibition calculated for each well from the two standards, 0% and 100% inhibition.

Factor VIIa Spectrophotometric Assay Method. Stock solutions of factor VIIa enzyme and r-tissue factor co-enzyme were diluted to a working concentration of 40 and 90 nM, respectively, in assay buffer consisting of 50 mM Tris (pH 7.4 at room temperature), 100 mM NaCl, 5 mM $CaCl_2$, and 0.1% BSA made up fresh on the day of the experiment. The assay was performed in 96-well microtiter plates and consisted of the following: 1 μL of compound (stock 10 mM in DMSO), 59 μL of assay buffer, 6 μL of factor VIIa/co-enzyme solution (40/90 nM in assay buffer), 6 μL of spectrazyme FVIIa (2.5 mM in assay buffer). In-plate controls consisted of 100% activity (1 μL of DMSO) and 0% activity (1 μL of DMSO plus 20 μL of assay buffer in place of enzyme). All plates were incubated at room temperature (20−22 °C) for 2 h**,** and the absorbance was read at 405 nm on a WALLAC Victor II.

Thrombin Spectrophotometric Assay Method. Stock solutions of thrombin were diluted to a working concentration of 0.25 NIH U/mL in assay buffer consisting of 50 mM Tris (pH 7.4 at room temperature), 100 mM NaCl, 5 mM $CaCl_2$, and 0.1% BSA made up fresh on the day of the experiment. The assay was performed in 96-well microtiter plates and consisted of the following: 2 μL of compound (stock 10 mM in DMSO), 124 μL of assay buffer, 12 μL of thrombin solution (0.25 NIH U/mL in assay buffer), 12 μL of S-2366 (3.75 mM in assay buffer). In-plate controls consisted of 100% activity (1 μL of DMSO) and 0% activity (1 μL of DMSO plus 20 μL of assay buffer in place of enzyme). All plates were incubated at room temperature (20−22 °C) for 2 h, and the absorbance was read at 405 nm on a Molecular Devices Thermomax microtiter plate reader.

Percent inhibition for each extract was calculated as follows:

$$\text{percent inhibition} = 100 - \{[(\text{Abs}_{\text{compound}} - 0\%\ \text{control})/(100\%\ \text{control} - 0\%\ \text{control})] \times 100\}$$

Saturation binding experiments were performed with enzyme and increasing concentration of chromogenic substrate to determine the affinity of the substrate for the enzyme (k_d). IC_{50} values were converted to K_i values (to normalize between variations in assays) by using the following relationship:

$$K_i = IC_{50}/\{1 + ([\text{Substrate}]/k_d)\}$$

Dysinosin B (1): amorphous solid (2.25 mg, 0.016% dry weight); $[\alpha]^{25}_D$ +72° (*c* 0.019 in MeOH); UV (MeOH) λ_{max} (log ϵ) 203 (4.20) nm; IR (film) ν_{max} 3453, 2922, 2859, 1655 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; negative-HRESIMS *m/z* 779.3135 (calcd for $[M - H]^-$, $C_{31}H_{51}N_6O_{15}S$ 779.3138).

Dysinosin C (2): amorphous solid (0.95 mg, 0.007% dry weight); λ_{max} (log ϵ) 202 (3.92) nm; IR (film) ν_{max} 3414, 2922, 2844, 1672 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2; negative-HRESIMS *m/z* 617.2610 (calcd for $[M - H]^-$, $C_{25}H_{41}N_6O_{10}S$ 617.2624).

Dysinosin D (3): amorphous solid (0.85 mg, 0.006% dry weight); ^1H and ^{13}C NMR data, see Table 2; positive-HRESIMS *m/z* 539.3192 (calcd for $[M]^+$, $C_{25}H_{43}N_6O_7$ 539.3187).

Acknowledgment. We thank AstraZeneca for financial support. We are indebted to Dr. J. Hooper, Sessile Marine Invertebrate Section, Queensland Museum, for the collection of the sponge and to R. Willis, Australian Institute of Marine Science, Townsville, for the HRESIMS analyses.

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NP049968P