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Dysinosin A: A Novel Inhibitor of Factor VIIa and Thrombin from a New Genus and Species of Australian Sponge of the Family Dysideidae

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We report here the isolation and structure determination of a new marine natural product dysinosin A **1**, from a new genus and species of sponge of the family Dysideidae found near Lizard Island, North Queensland, Australia. Dysinosin A, which is related to the cyanobacterial compounds belonging to the aeruginosins,[1] is a potent inhibitor of the blood coagulation cascade factor VIIa[2] and an inhibitor of the serine protease thrombin.[3] Among the distinctive features of dysinosin A are the presence of a 5,6-dihydroxy-octahydroindole-2-carboxylic acid, 3-amino-ethyl 1-*N*-amidino-Δ-3-pyrroline,[4,5] a sulfated glyceric acid, and D-leucine, assembled through three peptidic linkages.

Dysinosin A (**1**) (Figure 1) was assigned a molecular formula $C_{26}H_{44}N_6O_{10}S$ by ESIMS mass measurement of the $[M + Na]^+$ ion (655.2707: requires 655.2732). The ^{1}H NMR spectrum of dysinosin A in d_6-DMSO (Supporting Information) was very complex since nearly every signal had a lower intensity double in a ratio of 3:1. Chemical exchange correlations observed between the high- and low-intensity signals in a NOESY spectrum indicated the presence of conformational isomers and not structural isomers. All 26 carbons for the major conformer were observed in the ^{13}C NMR spectrum (Supporting Information) and a DEPT experiment established the presence of 36 carbon-bound protons (3 methyls, 9 methylenes, and 9 methines). Analysis of the gCOSY and TOCSY experiments, in conjunction with a gHMQC experiment, indicated that the molecule contained a glyceric acid, a leucine, a methoxyl group, a trisubstituted double bond ($RCH_2CH{=}C(CH_2R_1)$-CH_2CH_2NH-), a guanidine, and a highly functionalized cyclohexane (substituted by two hydroxyl groups, an ethyl group, and an amino group).

A gHMBC experiment indicated that the cyclohexane was part of a 5,6-dihydroxyoctahydroindole-2-carboxylic acid since a correlation between H12 and C19 provided evidence for the octahydroindole moiety. Chlorinated or mono-oxygenated octahydroindole-2-carboxylic acid derivatives have been reported previously from several cyanobacterial cultures.[1] A weak correlation from 26-CH_2 and 31-CH_2 to the guanidino carbon C28 indicated that the trisubstituted double bond was part of a 2-aminoethyl-(1-*N*-amidino-Δ-3-pyrroline). This partial structure was confirmed from correlations observed from the guanidine protons 29-NH_2 and 30-NH_2 to N29 and N30 in the ^{1}H/^{15}N gHMQC spectrum and from correlations from H25, 29-NH_2, and 30-NH_2 to N27 and from 29-NH_2 and 30-NH_2 to N30 and N29 in the ^{1}H/^{15}N gHMBC spectrum. The octahydroindole and the 1-*N*-amidino-Δ-3-pyrroline were linked

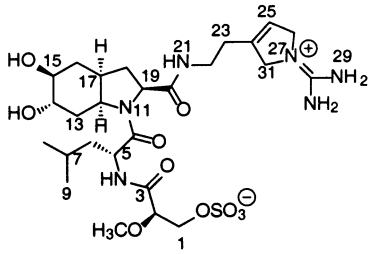

Figure 1. Structure of dysinosin A.

by an amide bond between C20 and N21 since correlations were observed from H18b, H19, H21, and 22-CH_2 to the carbonyl carbon C20. The glyceric acid was O-methylated at C2 since a correlation was observed between H2 and the methoxyl carbon (2-OCH_3) and from the methoxyl proton signal (δ 3.30) and C2. An amide bond linked the leucine to the glyceric acid since the leucine amide proton H4 showed a correlation to the glyceric acid carbonyl carbon C3. Finally a correlation from the octahydroindole proton H19 to the leucine carbonyl carbon, C10, indicated an amide bond linked the octahydroindole nitrogen N11 to the leucine carbonyl carbon C10; thus, the carbon backbone of **1** was delineated. The structure of dysinosin A includes a sulfate ester at C1 (the only oxygenated position not showing correlations to exhangeable protons) since the positive electrospray mass spectrum showed an $[MH - SO_3]^+$ ion peak at *m*/*z* 553.

The relative stereochemistry for the major conformer of dysinosin A was established from ROESY correlations and ^{1}H NMR coupling constants (see Supporting Information). In particular, correlations from 14-OH to H12 and from 15-OH to H18b indicated that the two hydroxyl groups and H12 on the octahydroindole were axial. This was consistent with the small couplings observed between H15 and H14, H16a, and H16b and between H14 and H13a and H13b. The diaxial arrangement of H19 and H18b was supported by the observation of a large coupling (12 Hz) between these two protons. Correlations between H12 and H17 indicated a cis ring junction. The C10−N11 amide bond was trans relative to C5 and C19 since correlations were observed between H5 and H12. The relative stereochemistry of the leucine was as shown since correlations were observed from H5 and H6a to H12. The relative stereochemistry at C2 could not be assigned from the ROESY experiment.

The principal differences in the spectra of the minor conformer compared with those of the major conformer were the ^{1}H NMR signals associated with the octahydroindole and leucine moieties. This was consistent with isomerism about the C10−N11 amide bond. ROESY correlations corroborated this hypothesis since cross-peaks were observed between H5 and H19.

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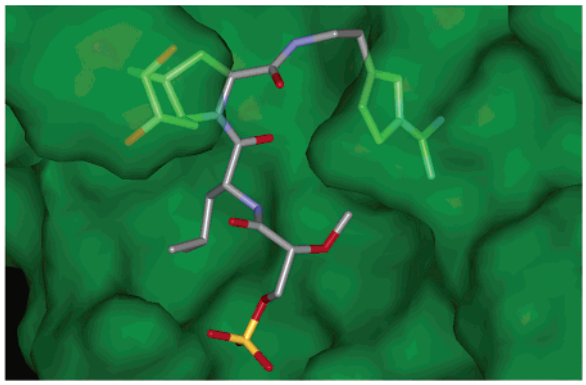

Figure 2. Dysinosin A−thrombin complex. Dysinosin A is shown in a stick representation, and the thrombin transparent solvent-accessible surface is shown in green at a radius of 1.4 Å.

Acid hydrolysis of dysinosin A followed by derivatization with Marfeys reagent (dinitroflurophenylalaninamide) and comparative HPLC/mass spectral analysis of the acid hydrolysate established that dysinosin A contained D-leucine. Thus, the configuration of the chiral centers in dysinosin A were C5 (*R*), C12 (*S*), C14 (*S*), C15 (*S*), C17 (*R*), and C19 (*S*) which were corroborated by X-ray analysis.

The ternary complex of dysinosin A−thrombin−hirugen was obtained by soaking overnight a single crystal of human α-thrombin−hirugen.[7] The crystallization of human α-thrombin in complex with hirugen was carried out as published previously and refined starting from the previously published hirugen−α-thrombin structure.[8] The X-ray crystal analysis of dysinosin A infused into a crystal of thrombin confirmed the structure of dysinosin A and allowed the glyceric acid to be assigned *R* stereochemical configuration.

Dysinosin A occupies the $P_1-P_2-P_3$ pocket of thrombin (Figure 2). Dysinosin A is positioned with the guanidino group buried deep in the P1 pocket. The inhibitor is covered by the surface of thrombin. The 5,6-dihydroxy-octahydroindole is underneath tryptophan 86 with the two hydroxyl groups parallel to the tryptophan aromatic ring. Glutamic acid 232 is positioned above the 1-*N*-amidino-Δ-3-pyrroline. In Figure 2, the protein solvent-accessible surface is shown transparent to allow the inhibitor to be viewed. The D-leucine side chain is pointing out to the surface, and the sulfate group is between two arginines on the surface of the protein. It appears that the protein would have to undergo a conformation change after binding to accommodate dysinosin A.

The hydrogen bonding network of the dysinosin A−thrombin complex showing short hydrogen bonds lengths (<2.5 Å) is shown in Figure 3. The guanidino group is strongly bonded to aspartic acid 229. The carbonyl oxygen and amide hydrogen of the D-leucine in dysinosin A, hydrogen bonds to the corresponding acceptor and donor of gylcine 258 in thrombin. The 5,6-dihydroxyoctahydroindole group does not appear to have hydrogen-bonding interactions even though the P2 pocket has histidine, tyrosine, typtophan, and lysine amino acids. The methoxyl oxygen on carbon 2 has a strong interaction with the amide of gylcine 260 whose carbonyl also has a weak hydrogen bond to one of the NH_2 of the guanidino group. The sulfate is interacting with arginines 208 and 263 with several hydrogen bonds, of which two are less that 2.5 Å in length and are shown in Figure 3.

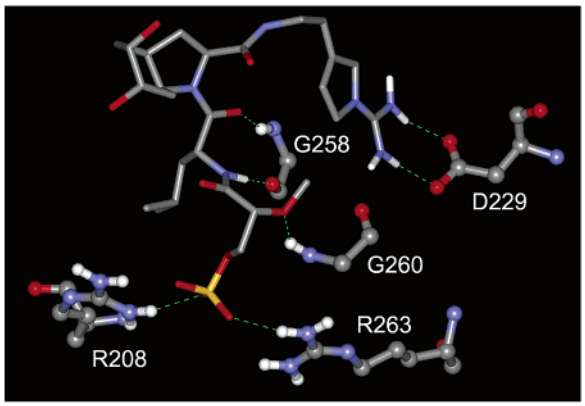

Figure 3. Hydrogen-bonding pattern of dysinosin A−thrombin complex. Dysinosin A and thrombin are represented as capped stick and ball-and-stick, respectively. The green dashed lines show the hydrogen bonds which are less than 2.5 Å.

Dysinosin A inhibited factor VIIa at a K_i of 108 nM and thrombin at a K_i of 452 nM. The identification of the 1-*N*-amidino-Δ-3-pyrroline and 5,6-dihydroxy-octahydroindole-2-carboxylic acid as P_1 and P_2 moieties respectively, should pave the way for the design and synthesis of new structure-based inhibitors. The total synthesis of dysinosin A is communicated in the following paper.[9]

Supporting Information Available: Details of the isolation, the NMR data, and the crystallographic data of **1** (PDF). This information is available free of charge via the Internet at http://pubs.acs.org.

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