Graphical Abstract

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Ietrochamide A
 $IC_{50} = 3.4 \mu M$ (*T. b. brucei*)

Ietrochamide B
 $IC_{50} = 4.7 \mu M$ (*T. b. brucei*)

Antitrypanosomal iotrochamides A and B from the Australian marine sponge *Iotrochota* sp.

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ABSTRACT

Bioassay-guided isolation of the CH₂Cl₂/MeOH extract from the Australian sponge *Iotrochota* sp. resulted in the purification of two new *N*-cinnamoyl-amino acids, iotrochamides A (**1**) and B (**2**). The chemical structures of **1** and **2** were determined by 1D/2D NMR and MS data analyses. Compounds **1** and **2** were shown to inhibit *Trypanosoma brucei brucei* with IC₅₀ values of 3.4 and 4.7 μM, respectively.

Human African Trypanosomiasis (HAT) is a fatal disease caused by two subspecies of a protozoan parasite *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense*. *T. b. rhodesiense* is the agent of the acute form of the disease, prevailing in Eastern and Southern Africa and *T. b. gambiense* causes the chronic form of the disease in Western and Central Africa. The World Health Organization (WHO) has recently estimated a cumulative rate of around 50 to 70 thousand cases of the disease.¹ The registered drugs suramin and pentamidine are not effective against both stages of the disease. The second stage effective drug melarsoprol has associated toxicity which has been reported as lethal in up to 12% of cases.² There have also been reports of incidence of drug resistance in HAT cases.^{2,3} These factors highlight the need for the development of new, safer and more effective drugs to fight African Sleeping Sickness.

Natural products have traditionally played a highly significant role in drug discovery and development,⁴ particularly in the areas of cancer and infectious diseases.⁴ The basis for this can be explained by the similarity of the biosynthetic imprint, the molecular recognition between substrate and biosynthetic enzyme, and the natural product binding motif, the molecular recognition between a ligand and its therapeutic target that led to the concept of protein fold topology (PFT).⁵ PFT describes cavity recognition points unrelated to fold and sequence similarity, and defines a natural product's ability to recognize biology space.⁶⁻⁸ In our ongoing search for new lead compounds for neglected diseases,⁹⁻¹³ a 384-well fluorescence based trypanosomal high throughput screening (HTS) assay¹⁴ against *T. b. brucei* was used to screen a pre-fractionated natural product library consisting of 202,983 fractions.¹⁵ *T. b. brucei* is routinely used in screening for antitrypanosomal compounds with potential to be developed into HAT drugs.¹⁶ In efforts to identify drug-like natural products following HTS campaigns, we recently reported a procedure to capture natural products with LogP less than 5 in extracts.¹⁵ The extracts of over 18,000 marine and terrestrial extracts were then fractionated with 11 fractions collected per sample. From the eleven fractions derived from the Australian sponge *Iotrochota* sp., a single fraction was identified that showed activity against *T. b. brucei* and desirable selectivity against a human embryonic kidney cell line HEK293 (>10 fold). Bioassay-guided

fractionation of the CH₂Cl₂/MeOH extract afforded two new *N*-cinnamoyl-amino acids, iotrochamides A (**1**) and B (**2**), which both displayed activity towards *T. b. brucei*.

The Australian sponge *Iotrochota* sp. (Order: Poecilosclerida, Family: Iotrochotidae) was collected from the west side of Curacao Island, Queensland, at a depth of 30 m by SCUBA diving in 1999. A voucher sample (G314368) is stored at the Queensland Museum, Brisbane, Australia. The freeze-dried and ground *Iotrochota* sp. (10 g) was sequentially extracted with *n*-hexane, CH₂Cl₂/MeOH (4:1), and MeOH. The CH₂Cl₂/MeOH extracts were combined and chromatographed using C₁₈ bonded silica HPLC (MeOH/H₂O/0.1% TFA). Following biological evaluation, the active fractions were combined and further purified by C₁₈ HPLC (MeOH/H₂O/0.1% TFA) to yield two new *N*-cinnamoyl-amino acids, iotrochamides A (**1**, 1.8 mg, 0.018% dry weight) and B (**2**, 1.4 mg, 0.014% dry weight).

Iotrochamide A (**1**) was obtained as an optically active glassy solid.¹⁷ HRESIMS measurement on [M+Na]⁺ ion (*m/z* 364.1146), in combination with ¹H and ¹³C NMR spectroscopic data (Table 1), supported the molecular formula of C₁₉H₁₉NO₅ with 11 double bond equivalents. The ¹H NMR spectrum of **1** displayed signals due to two exchangeable protons (δ_H 9.18 and 8.20), ten aromatic/olefinic protons (δ_H 6.59 - 7.64), one methine (δ_H 4.49), one methylene (δ_H 2.96 and 3.05) and one methoxy group (δ_H 3.49). Fifteen carbons were observed in the ¹³C NMR spectrum, including two carbonyl (δ_C 162.9 and 172.7), ten sp² and three sp³ carbons.

The mono-substituted benzene ring was evident by the coupling patterns of five aromatic protons, including one doublet of doublet of doublets (δ_H 7.32, 1H, *J* = 7.8, 7.8, 1.2, Hz), and two doublet of doublets (δ_H 7.37, 2H, *J* = 7.8, 7.8 Hz; δ_H 7.64, 2H, *J* = 7.8, 1.2 Hz). An enamide group was established by the HMBC correlations from an olefinic proton (δ_H 6.59) and an exchangeable amide proton to a conjugated carbonyl carbon (δ_C 162.9) (Figure 1). The attachment of the enamide functionality to the benzene ring was elucidated by the HMBC correlation from the same olefinic proton to the aromatic carbon (δ_C 129.2). Further HMBC correlations from the olefinic proton and methoxy protons (δ_H 3.49) to a sp² quaternary carbon (δ_C 148.7) established the formation of an enol methyl ether moiety. A *para*-substituted benzene ring was indicated by the presence of two coupled aromatic doublets (δ_H 7.05, 2H, *J* = 8.4 Hz and δ_H 6.65, 2H, *J* = 8.4 Hz). HMBC correlations from the aromatic doublets and an exchangeable phenolic proton (δ_H 9.18) to an aromatic oxygenated quaternary carbon (δ_C 155.4) suggested that this benzene ring was a 4-hydroxyphenyl group. A 2-carboxy ethyl moiety was established by the COSY correlation between the methine (δ_H 4.49) and methylene (δ_H 2.96 and 3.05) protons in the ethyl group, and the HMBC correlations from the ethyl protons to the carboxylic acid carbon (δ_C 172.7) (Figure 1). The 2-carboxy ethyl unit was directly attached to the *para*-substituted benzene ring since HMBC correlations were observed from the ethyl protons to a quaternary aromatic carbon (δ_C 127.6). Finally, the HMBC

The figure displays two chemical structures, labeled 1 and 2, with curved arrows indicating the direction of electron flow or conjugation. Structure 1 is a complex molecule featuring a central benzene ring substituted with a hydroxyl group (HO), a carbonyl group (C=O), and a hydroxyl group (OH). It is connected to a side chain containing a carbonyl group (C=O), a nitrogen atom (NH), and a methoxy group (OMe). Structure 2 is similar to structure 1 but includes a bromine atom (Br) and a nitrogen atom (HN) on the side chain. Both structures show extensive conjugation, with arrows indicating the movement of electron pairs through the various functional groups and rings.

The geometry of the $\Delta^{2,3}$ double bond in **1** was established by the characteristic ^{13}C chemical shift of C-3 in comparison with structurally similar botryllamides, such as botryllamide A (**3**). Previous study suggested that botryllamides possess a $\Delta^{2,3}$ Z double bond when the C-3 resonates at $\delta_{\text{C}} > 120$ in CDCl_3 , and a $\Delta^{2,3}$ E double bond when C-3 resonates at $\delta_{\text{C}} < 110$ in acetone- d_6 and DMSO- d_6 .^{18, 19} The C-3 in compound **1** resonates at δ_{C} 117.4 in DMSO- d_6 and 120.9 in CDCl_3 , in agreement with a $\Delta^{2,3}$ Z double bond. This assignment was supported by the ROESY correlation between H-5/9 (δ_{H} 7.64) and 2-OMe (δ_{H} 3.49) (Figure 1). The absolute configuration of the amino acid residue in **1** was determined by Marfey's method.²⁰ The acid hydrolysis of compound **1** followed by

Iotrochamide B (**2**) was also obtained as an optically active glassy solid.²² LRESIMS showed a cluster of two isotopic ions at m/z 443 and 445 (1:1), suggesting the presence of one bromine atom in the molecule. HRESIMS measurement on $[M+Na]^+$ ion (m/z 465.0416), in combination with 1H and ^{13}C NMR spectroscopic data (Table 1), supported the molecular formula of $C_{21}H_{19}BrN_2O_4$ with 13 double bond equivalents. Comparison of the 1H and ^{13}C NMR spectroscopic data between **1** and **2** (Table 1) showed that **2** had an identical *N*-cinnamoyl moiety as **1**. The only difference between **1** and **2** was that the two aromatic doublets from the *L*-tyrosine residue in **1** were replaced by signals characteristic of a 3-(6-bromo)-indole system in **2**. These included three doublets (δ_H 7.52, d, J = 8.4 Hz; 7.50, d, J = 1.8 Hz; 7.20, d, J = 2.4 Hz), one doublet of doublet (7.10, dd, J = 1.8 and 8.4 Hz;) and one broad indole-NH exchangeable proton (δ_H 10.97). The placement of the bromine atom at the 6-position of the indole ring was determined by the ROESY correlation between H-17 (δ_H 7.50) and the indole-NH, and confirmed by the detailed analysis of the HMBC correlations (Figure 1).

[illegible]

^aSpectra were recorded at 600 MHz for ¹H and 150 MHz for ¹³C at 30 °C.

Comparison of the C-3 chemical shift between compound **1** and **2** (δ_{C} 117.4 and 117.3 respectively, in DMSO- d_6) indicated that compound **2** also had a $Z \Delta^{2,3}$ double bond. This assignment was confirmed by the ROESY correlation between H-5/9 (δ_{H} 7.62) and 2-OMe (δ_{H} 3.46) (Figure 1). The Marfey's method was not performed on compound **2** as *L*- and *D*-6-bromotryptophan standards are not available. However, compound **2** had the same sign of optical rotation ($[\alpha]_{\text{D}}^{24}$ -12.5°, *c* 0.1, MeOH) as that of *L*-tryptophan ($[\alpha]_{\text{D}}^{24}$ = -54.3°, *c* 0.2, MeOH) and compound **1** ($[\alpha]_{\text{D}}^{24}$ -13.6°, *c* 0.1, MeOH), suggesting a *L*-6-bromotryptophan in **2**. Iotrochamide B was therefore assigned as *N*-(*Z*)-2-methoxycinnamoyl-*L*-6-bromotryptophan.

Marine sponges belonging to the genus *Iotrochota* have been reported to produce fatty acids, bromotyrosine derivatives, indole and pyrrole alkaloids.²³ Of the compounds isolated so far, iotrochidoside A²⁴ and matemone²⁵ displayed cytotoxic activity, while purpurone²⁶ exhibited ATP-citrate lyase inhibition. Cinnamoyl amides have been frequently isolated from plants of different genera.²³ They have also been reported from marine organisms such as ascidians belonging to the genus *Botrylloides* sp.^{18, 27, 28} Iotrochamides A (**1**) and B (**2**), however, are the first cinnamoyl amino acids reported from the marine sponge *Iotrochota* sp. Biological study from three experimental replicates (*n*=3) showed that both compounds exhibited moderate activity against *T. b. brucei* with IC₅₀ values of 4.7 and 3.4 μM , respectively. A preliminary cytotoxicity study using a human embryonic kidney cell line, HEK293, showed that compounds **1** and **2** had 85% inhibition at 50 μM and 100% inhibition at 70 μM , respectively. These results indicated that both compounds **1** and **2** showed moderate selectivity towards *T. b. brucei*.

In conclusion, two new cinnamoyl amino acids, iotrochamides A (**1**) and B (**2**) were isolated from the marine sponge *Iotrochota* sp. Compounds **1** and **2** were active against *T. b. brucei* with single digit μM IC₅₀ values.

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Supplementary Material

Supplementary data available: 1D and 2D NMR spectra for compounds **1** and **2**, general experimental details, collection and identification of the sponge, extraction and isolation procedures, amino acid analysis of compound **1**, *T. b. brucei* Alamar Blue™ viability assay, and HEK293 cytotoxicity assay. Supplemental data associated with this article can be found, in the online version at doi:

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