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Actinophyllic Acid, a Potent Indole Alkaloid Inhibitor of the Coupled Enzyme Assay Carboxypeptidase U/Hippuricase from the Leaves of *Alstonia actinophylla* (Apocynaceae)

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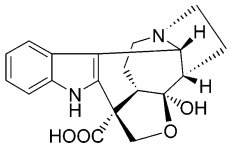

Bioassay-guided fractionation of the aqueous extract of the leaves of *Alstonia actinophylla* with use of a coupled enzyme assay, CPU/hippuricase, to detect carboxypeptidase U inhibitors led to the isolation of a novel indole alkaloid, actinophyllic acid (**1**). The structure of **1** was determined from detailed 2D NMR studies. Actinophyllic acid was found to be a potent inhibitor of the coupled enzyme assay with an IC_{50} of 0.84 μM. Actinophyllic acid possesses a unique 2,3,6,7,9,13c-hexahydro-1*H*-1,7,8-(methanetriyloxymethano)-pyrrolo[1′,2′:1,2]azocino[4,3-*b*]indole-8(5*H*)-carboxylic acid skeleton.

Fibrinolysis is the process the body uses to remove small blood clots from circulation. Central to this process is a cascade of enzymatic reactions that ultimately results in the degradation of fibrin by plasmin. Plasmin degradation of fibrin exposes carboxy-terminal lysine residues which bind strongly to the precursor of plasmin, plasminogen. Plasminogen is cleaved to produce plasmin by two plasminogen activators, tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). This cleavage process is more highly activated if plasminogen is bound to fibrin. Thus, fibrinolysis is accelerated by the initial exposure of the fibrin to plasmin through positive feedback.[1]

One of the endogenous inhibitors to fibrinolysis is carboxypeptidase U (CPU). CPU is formed during coagulation and fibrinolysis from its precursor proCPU by the action of proteolytic enzymes, such as thrombin, thrombin−thrombomodulin complex, or plasmin. CPU cleaves basic amino acids at the carboxy-terminal of fibrin fragments. The loss of carboxy-terminal lysines and thereby of lysine binding sites for plasminogen then serves to inhibit fibrinolysis. By inhibiting the loss of lysine binding sites for plasminogen and thus increasing the rate of plasmin formation, effective inhibitors of carboxypeptidase U are expected to facilitate fibrinolysis.[1]

As part of a search for novel natural products as useful leads for the discovery of therapeutic agents to treat cardiovascular disorders 40 000 extracts from Australian plants and marine organisms were screened against a coupled enzyme assay CPU/hippuricase designed to discover CPU inhibitors.[2] The water and methanol extracts of *Alstonia actinophylla* (A. Cunn.) K. Schum. were targeted for further investigation since they showed 100% inhibition at 50 μg/mL (dry weight). This paper reports on the bioassay guided fractionation and structure determination of the bioactive alkaloid, actinophyllic acid (**1**), which was isolated from the leaves of *A. actinophylla*. This is the first reported phytochemical investigation of the plant *A. actinophylla*.

The leaves of *A. actinophylla* were extracted sequentially with CH_2Cl_2, MeOH, and water and the three extracts were tested for inhibitory activity against the coupled enzyme assay CPU/hippuricase. The MeOH and water extracts each showed significant inhibition and were combined. Partitioning of the combined MeOH/water extracts between CH_2Cl_2 and water led to enzyme inhibitory activity being found only in the water layer. The water layer was fractionated on C_{18} silica gel with a gradient elution from water to MeOH. Bioassay of the C_{18} silica gel fractions showed that fractions eluting in less than 20% MeOH/80% water were the only active fractions. These fractions were combined, dissolved in water, and filtered through a strongly acidic ion-exchange resin. The resin was washed sequentially with water followed by concentrated aq NH_3 solution. Bioassay of the water and NH_3 fractions indicated that enzyme inhibitory activity was solely found in the NH_3 fraction. The NH_3 fraction was evaporated, redissolved in water, and then filtered through a strongly basic ion-exchange resin. The resin was washed sequentially with water followed by 2 M HCl. Bioassay of the water and HCl fractions showed that enzyme inhibitory activity was found only in the HCl fraction. The HCl fraction was chromatographed on Sephadex LH20, elution with MeOH, and an enzyme inhibitory band eluted early off the column. Repeated C_{18} silica gel HPLC purification eluting with a gradient from water to 20% MeOH/80% water and bioassay of fractions yielded actinophyllic acid (**1**) as the only active component in 0.0072% yield. The chromatographic profile of actinophyllic acid suggested it was an amino acid.

Actinophyllic acid (**1**) was obtained as a brown gum $[\alpha]_D$ −29. A pseudomolecular ion in the positive HRESIMS at *m/z* 341.1502 (Δ 1.8 ppm) allowed a molecular formula of $C_{19}H_{20}N_2O_4$ to be assigned to **1**. Strong absorption bands at 3407, 2928, and 1709 cm^{-1} in the IR spectrum suggested that **1** contained hydroxyl, amine,

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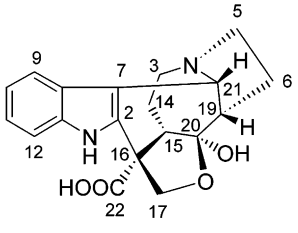

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and carboxylic acid functionalities. The ^{1}H NMR spectrum (Table 1) of **1** displayed signals for four aromatic methine protons at δ 6.91 (t, 7.8, 1H), 7.01 (t, 7.8, 1H), 7.44 (d, 7.8, 1H), and 7.51 (d, 7.8, 1H) and a sharp exchangeable proton at δ 11.70 (s, 1H). Analysis of the ^{13}C and DEPT spectra revealed that the molecule contained four aromatic methine, three aliphatic methine, five aliphatic methylene, a carboxylic acid carbonyl, an aliphatic quaternary, a dioxygenated quaternary, and four aromatic quaternary carbons. A gHSQC spectrum allowed all of the protonated carbons to be assigned. The chemical shifts observed for the methylenes assigned as 3-CH_2 (δ_C 45.4; δ_H 2.84) and 5-CH_2 (δ_C 48.6; δ_H 3.41, 3.54) and the methine assigned as 21-CH (δ_C 60.2; δ_H 5.26) suggested that each was attached to a nitrogen atom. Analysis of the COSY spectrum established that the molecule contained four proton spin systems which were represented by partial structures, C-9 to C-12, C-5−C-6−C-19−C-21, C-3−C-14−C-15, and an isolated oxygenated methylene, C-17. A large number of correlations were observed in the gHMBC spectrum and this provided evidence to assign the gross structure of **1**. The exchangeable proton H-1 showed correlations to four aromatic quaternary carbons, C-2, C-7, C-8, and C-13. The aromatic proton H-9 also showed $^3J_{CH}$ correlations to C-7 and C-13 as well as to the aromatic methine carbon C-11, and the aromatic proton H-12 showed $^3J_{CH}$ correlations to C-8 and C-10. These observations suggested that the molecule contained a 2,3-disubstituted indole.[3] The proton, H-21, was adjacent to C-7 since it correlated to C-2, C-7, and C-8. This methine proton, H-21, also correlated to C-3 indicating the connectivity of C-21 and C-3 via the nitrogen atom, N-4. The methylenes C-5 and C-3 were also connected through the same nitrogen atom, N-4, since H-5b correlated to C-7 in the gHMBC spectrum. A gHMBC correlation was not observed between H-21 and C-5 since the torsion angle between these atoms was ~80°. A correlation from H-21, H-6a, H-6b, and H-19 to the quaternary dioxygenated carbon C-20 indicated that C-20 was vicinal to the methine, C-19. A correlation from H-19 to C-15 and from H-15 to C-19 indicated that a seven-membered ring could be delineated by C-3−N-4−C-21−C-19−C-20−C-15−C-14−C-3. An eight-membered ring was delineated by C-7−C-21−C-19−C-20−C-15−C-16−C-2−C-7 since H-15 also showed correlations to the aliphatic quaternary carbon, C-16, and the indole quaternary carbon C-2. The carboxylic acid, C-22, and the oxygenated methylene, C-17, were both attached to C-16 since correlations were observed between H-17b and C-15 and C-22 and between H-17a and C-16, C-2, and C-15. Finally, a five-membered ring was delineated by C-20−C-15−C-16−C-17−O−C-20 since correlations were observed between H-17a and H-17b and C-20. Since the molecule contained four interconnecting ring systems only the relative stereochemistry displayed could be defined for actinophyllic acid (**1**). This stereochemistry was corroborated by analysis of the ^{1}H−^{1}H coupling constants. Even though well over 250 alkaloids have been isolated from species of *Alstonia* to date[4] actinophyllic acid (**1**) represents a new skeleton: 2,3,6,7,9,13c-hexahydro-1*H*-1,7,8-(methanetriyloxymethano)pyrrolo[1′,2′:1,2]azocino[4,3-*b*]indole-8(5*H*)-carboxylic acid.

The structure of actinophyllic acid is unprecedented and its biogenesis warrants some discussion. A plausible biogenetic pathway was rationalized as shown in Scheme 1. The probable biogenetic precursors for actinophyllic acid, as with all monoterpene indole alkaloids isolated from plants of the family Apocynaceae, are tryptamine and (−)-secologanin glucoside. Condensation of these precursors could form geissoschizine (**2**), which could undergo rearrangement to precondylocarpine (**3**), an alkaloid previously isolated from *Vallesia dichrotoma* (Apocynaceae), via a stemmadenine iminium cation.[5] Oxidation of the C-20−C-19 double bond could yield the epoxide intermediate **i** which through proton migration produces the allylic alcohol **ii**. Intermediate **ii** could undergo further oxidation to form the epoxide intermediate **iii**. Formation of the iminium cation **iv** followed by ring closure would lead to the keto-alcohol **v**. Further oxidation of the alcohol would yield the C-18 carboxylic acid **vi**. Decarboxylation of the β-ketoacid **vi** would give the ketone intermediate **vii**. Re-aromatization of the indole could occur by elimination of C-6 to form the carbocation **viii**. Electrophilic attack on to the enol carbon, C-19, by the carbocation, C-6, would lead to the formation of the five-membered ring heterocycle **ix**. Hemiacetal formation via cyclization of the hydroxyl oxygen 17-OH onto the ketone carbonyl carbon C-20 and hydrolysis of the methyl ester would finally yield actinophyllic acid.

Actinophyllic acid inhibited the coupled, two-step enzymatic hydrolysis[2] of the substrate, 4-hydroxyhippurylarginine, by carboxypeptidase U to 4-hydroxyhippuric acid followed by hydrolysis of this product by hippuricase to 4-hydroxybenzoic acid with an IC_{50} of 0.84 μM. The control substance, Plummers inhibitor (**4**), showed an IC_{50} of 6.4 μM. A subsequent assay with only the hippuricase enzyme showed that actinophyllic acid did not inhibit the hydrolysis of 4-hydroxyhippuric acid. This suggested **1** was a selective inhibitor of CPU. However, further testing of actinophyllic acid in a CPU assay in which the substrate and product were detected by HPLC[2] indicated that **1** did not inhibit the hydrolysis of 4-hydroxyhippurylarginine to 4-hydroxyhippuric acid. Detection of enzyme activity for both the CPU/hippuricase assay and the hippuricase assay relied upon the generation of a red, quinoneimine dye formed by the oxidative coupling by sodium periodate of 4-aminoantipyrine (**5**) with 4-hydroxybenzoic acid. The CPU

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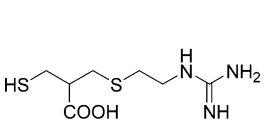
TABLE 1. ^{1}H (600 MHz), ^{13}C (150 MHz), HMBC, and COSY NMR Data for Actinophyllic Acid (1) in d_6-DMSO

atom	^{13}C (mult)[a]	^{1}H mult, J (Hz)	HMBC[b]	COSY[c]
1		11.70 (s, 1H)	2, 7, 8, 13	
2	143.7 (s)			
3	45.4 (t)	2.84 (m, 2H)		14a, 14b
5a	48.6 (t)	3.41 (ddd, 3.5, 9.0, 12.0, 1H)		5b, 6a, 6b
5b		3.54 (7.2, 12.0, 12.0, 1H)	3, 6	5a, 6a, 6b
6a	25.0 (t)	2.26 (ddd, 3.5, 12.0, 12.0, 1H)	20	5a, 5b, 6b, 19
6b		2.50 (m, 1H)	20	5b, 5a, 6a
7	100.2 (s)			
8	127.4 (s)			
9	117.2 (d)	7.51 (d, 7.8, 1H)	7, 11, 13	10, 11
10	118.6 (d)	6.91 (t, 7.8, 1H)	8, 12	9, 11, 12
11	120.8 (d)	7.01 (t, 7.8, 1H)	9, 13	9, 10, 12
12	111.5 (d)	7.44 (d, 7.8, 1H)	8, 10	10, 11
13	134.4 (s)			
14a	18.5 (t)	2.09 (bddd, 3.6, 12.0, 12.0, 1H)		3a, 3b, 14b
14b		2.20 (bdd, 2.1, 12.0, 1H)		3a, 3b, 14a, 15
15	53.4 (d)	2.89 (d, 2.0, 1H)	2, 3, 14, 16, 19, 20, 22	14b
16	57.7 (s)			
17a	75.4 (t)	3.36 (d, 7.2, 1H)	2, 15, 16, 20	17b
17b		3.94 (d, 7.2, 1H)	2, 15, 16, 20, 22	17a
19	51.6 (d)	2.94 (dd, 9, 6.6, 1H)	5, 6, 15, 20	6a, 21
20	106.7 (s)			
20-OH	-	5.90 (bs, 1H)		
21	60.2 (d)	5.26 (d, 6.6, 1H)	2, 3, 7, 8, 19, 20	19
22	170.8 (s)			

[a] Implied multiplicity determined by DEPT (s = C, d = CH, t = CH_2, q = CH_3). [b] Carbons that correlate to the proton resonance. [c] Protons that correlate to the proton resonance.



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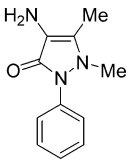

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assay in which the substrate and product were detected by HPLC contained no 4-aminoantipyrine. There is therefore a possibility that an adduct of actinophyllic acid and 4-aminoantipyrine formed in situ in the coupled enzyme assay could be responsible for the enzyme inhibition observed in this assay.

Experimental Section

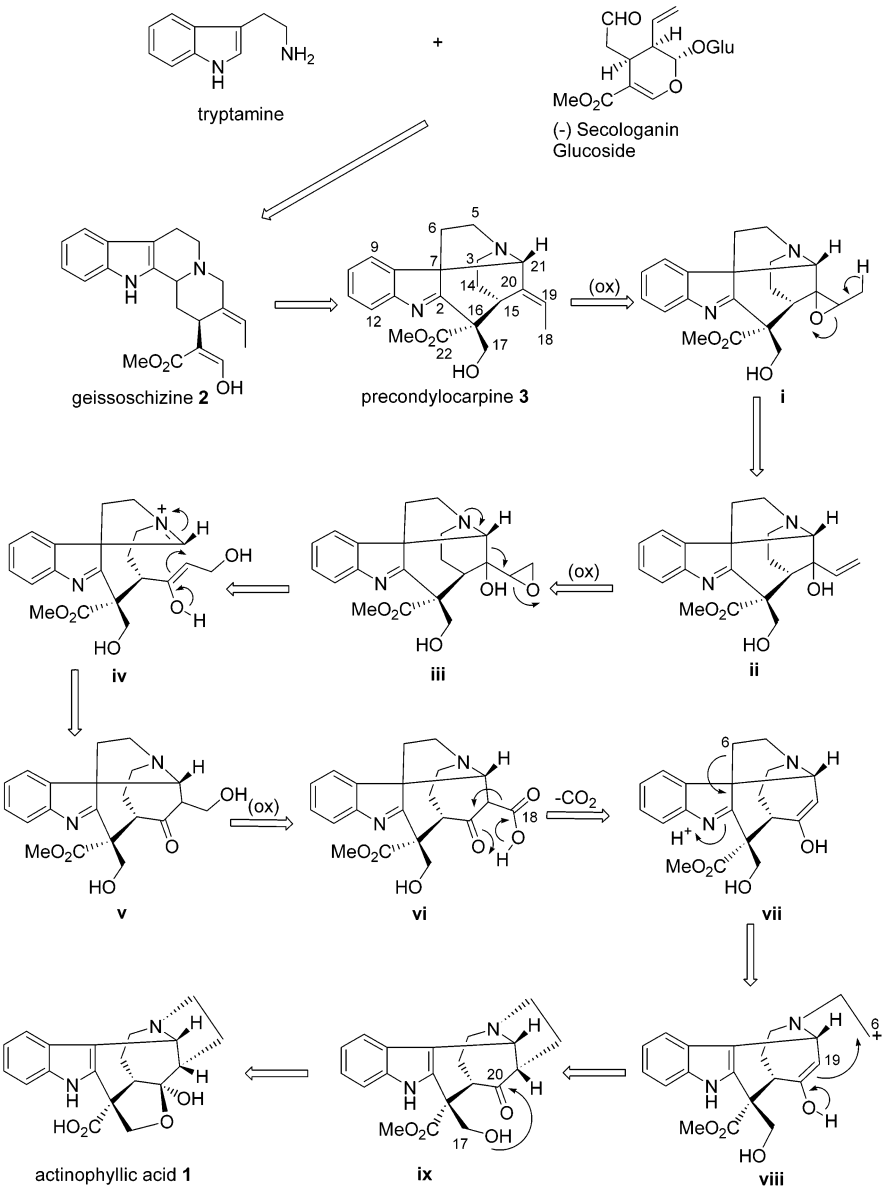
Plant Material. *A. actinophylla* was collected and identified by Paul I. Forster, Queensland Herbarium, in December 1993 at Possum Scrub on the road to Stone Crossing from Weipa on Cape York Peninsula, Far North Queensland. A voucher specimen, AQ 600495, is deposited in the Queensland Herbarium, Brisbane.

Extraction and Isolation. The leaves, 50 g, were air-dried, milled, and extracted exhaustively with CH_2Cl_2 (5 × 150 mL) followed by MeOH (5 × 200 mL) followed by water (5 × 200 mL). The three extracts were tested for inhibitory activity against the coupled enzyme assay CPU/hippuricase and the MeOH and water extracts each showed significant enzyme inhibition and were combined yielding a brown gum (35 g). Partitioning of the combined extracts between CH_2Cl_2 (300 mL) and water (3 × 300 mL) led to enzyme inhibitory activity being found only in the water layer. The water fraction (900 mL) was fractionated on C_{18} silica gel (100 g) eluting with water (250 mL), 10% MeOH/90% water (200 mL), 20% MeOH/80% water (200 mL), 40% MeOH/60% water (200 mL), 60% MeOH/40% water (200 mL), and MeOH (200 mL). The six fractions were tested for enzyme inhibition and the three fractions eluting between water and 20% MeOH/80% water were shown to be inhibitory. The three bioactive fractions were combined to yield a brown gum (8.5 g). The gum was dissolved in water (200 mL) and filtered through Dowex WX8-400 strongly acidic ion-exchange resin (100 g). The resin was washed with water (500 mL) followed by a 27% aq NH_3 solution (200 mL). The water and NH_3 fractions were tested for enzyme inhibitory activity and only the NH_3 fraction was found to be active. The NH_3 fraction was evaporated to yield a brown gum (2.8 g). This fraction was resuspended in water (30 mL) and filtered through Dowex IX8-400 strongly basic ion-exchange resin (50 g). The resin was washed with water (300 mL), followed by 2 M HCl (100 mL). Bioassay of the water and HCl fractions obtained from the basic ion-exchange resin revealed inhibitory activity only in the acid fraction. This fraction (320 mg) was chromatographed on Sephadex LH20 (50 g), eluting with MeOH (180 mL). A band of activity eluted early off the column. This fraction (230 mg) was chromatographed on a C_{18} silica gel HPLC column, elution with a 14 min gradient from water to 40% MeOH/60% water. An enzyme inhibitory band eluted with 20% MeOH/80% water. This fraction (15 mg) was purified by C_{18} silica gel HPLC with a 15 min gradient from water to 20% MeOH/80% water. Activity was associated with a band eluting with 18% methanol, and characterized as actinophyllic acid (**1**).

Actinophyllic acid (**1**) was isolated as a brown gum (3.6 mg, 0.0072%). $[\alpha]_D$ −29 (*c* 0.001, MeOH); UV (MeOH) λ_{max} (ϵ) 217 (24 410), 280 nm (17 000); IR υ_{max} (film) 3407, 2928, 1709, 1659, 1594, 1444, 1377 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; (+)-HRESIMS *m/z* 341.1502 (calcd for $C_{19}H_{21}N_2O_4$ $[M + H]^+$ 341.1496).

CPU/Hippuricase Assay. Pro-CPU (20 μL) was activated by incubation with equal amounts of thrombin (20 μL, 12 nM) and thrombomodulin (20 μL, 48 nM) for 10 min at room temperature. The thrombin and thrombomodulin were then inhibited by addition of Ppack (H-D-Phe-Pro-Arg-chloromethyl ketone 2HCl) (20 mL, 20 nM) and incubation at room temperature for 10 min. CPU was standardized prior to each assay by performing the assay with serial dilutions of enzyme to achieve an A490 reading of approximately 0.3, which is equivalent to an enzyme concentration of 0.25 mU/20 μL. Assays were performed in 96-well microtiter plates containing final reagent concentrations in 100 μL: 4.5 mM 4-hydroxyhippurylarginine, 2.25 mM 4-aminoantipyrine, 1.2 U/mL hippuricase, and unknowns. Extracts, fractions, and compounds were constituted

SCHEME 1. Biogenetic Pathway Proposed for Actinophyllic Acid (1)



in neat DMSO and assayed at 1% DMSO, using diluent (100 mM HEPES, pH 7.4). The reaction was started by the addition of 20 μL of CPU. Assays were mixed for 5 min and incubated at 23 °C for 75 min after which absorbance at 490 nm (A_{490}) was measured. Blank control wells contained 20 μL of activation buffer in place of CPU. The reaction was quenched by the addition of 30 μL of stop solution (23 mM NaIO4 and 71.5 mM EDTA) and incubated for an additional 15 min for color development and a second absorbance measurement was made. Compound IC_{50} values (concentration required to increase A_{490} to 50% of control) were estimated from the average of triplicate measurements with use of nonlinear, least-squares analysis.

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Supporting Information Available: General experimental procedures and ^{1}H and ^{13}C NMR and two-dimensional NMR spectra of actinophyllic acid (**1**). This material is available free of charge via the Internet at http://pubs.acs.org.

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