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Actinoflavoside, A Novel Flavonoid-Like Glycoside Produced by a Marine Bacterium of the Genus *Streptomyces*.

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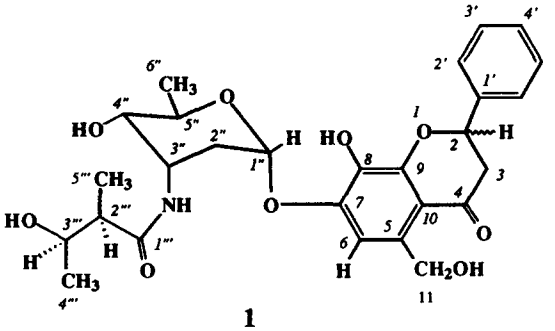
Abstract: Actinoflavoside (1), a novel glycoside composed of a rare 2, 3, 6-trideoxy-3-amino-ribo-pyranoside (ristosamine) aminosugar and an aglycone reminiscent of the plant flavonoids, was isolated from the culture broth of a marine *Streptomyces* sp. The structure of actinoflavoside was established by spectroscopic analysis and by chemical degradation. © 1997 Elsevier Science Ltd.

As part of a program to develop the medical applications of marine microorganisms, we have dedicated considerable efforts to investigate members of the bacterial order Actinomycetales found in saline environments.[1] In this connection, our attention was drawn to an estuarine isolate, CNB-689,[2] identified as a *Streptomyces* sp. by FAME analysis. The saline fermentation broths of this actinomycete showed modest antibacterial activity against *Bacillus subtilis*, hence we undertook isolation of the active constituent which we identify here as actinoflavoside (**1**), a molecule of an unprecedented structure class. Actinoflavoside resembles the common plant-derived flavonoid glycosides, but because of additional alkylation at C-5, and the general conclusion that prokaryotes do not produce this class of compounds, its origin via the flavonoid biosynthetic pathways seems questionable.



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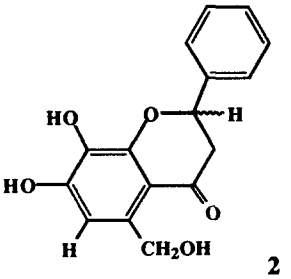

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Culture filtrates of strain CNB-689 were extracted using a solid phase method and the crude organic material was fractionated by silica gel flash chromatography.[3] Fractions which showed antibacterial activity were pooled and further fractionated by repeated RP C-18 HPLC (60-65% MeOH/water) ultimately leading to the isolation of actinoflavoside (**1**).[4] The new compound was obtained as a colorless, non-crystalline solid which analyzed for $C_{27}H_{33}NO_9$ by high resolution FABMS ($[M+H]^+$ *m/z* obsd 516.2231, -0.5 ppm dev.). Isocratic HPLC analyses of **1**, using various solvents and adsorbents, indicated good purity. However, the overall NMR data showed inconsistencies which could only be accommodated by a diastereomeric mixture. The ^1H NMR spectrum of **1**, for example, showed two sets of partially overlapping ABX spin systems in the vicinity of 3.0 and 5.5 ppm. In addition, the ^{13}C NMR spectrum showed two methine carbon signals, at δ 80.5 and 80.6 which, on the basis of the formula for **1**, could only account for one carbon. Production of a perbenzoate derivative, followed by gradient, RP C-18 HPLC analysis using diode array detection illustrated conclusively that **1** was a 50/50 mixture of diastereomers.[5] With the exception of the above, all other proton and carbon signals of the mixture appeared at identical chemical shifts (Table). Acid hydrolysis of **1** yielded the aglycone **2**, which clarified the NMR spectrum considerably.[6] The overlapping ABX spin systems of **1** were observed, in **2**, as a clean ABX pattern (from the C-2 and C-3 protons) which showed that **2** was racemic, and that actinoflavoside was racemized at the only aglycone chiral carbon (C-2).

Comprehensive analysis of the COSY, HMQC and HMBC NMR spectra for **1** defined three substructures, a 2, 3, 6-trideoxyaminosugar, a 2-methyl-3-hydroxy-butyramide, and an aglycone possessing a 5-hydroxymethyl-7, 8-dihydroxyflavonoid structure. HMBC correlations were effective in providing the substitution pattern of the pentasubstituted aromatic ring and in connecting substructures. Correlations from the C-6 proton (δ 6.87) to the C-5, -7, -8, -10 and -11 carbons (δ 135.8, 147.6, 133.8, 114.1 and 64.9, repectively), from the C-11 methylene protons to the C-5, -6 and C-10 carbons (δ 135.8, 110.4 and 114.1), and from the C-8 phenolic proton (δ 6.48) to the C-7, -8 and -9 carbons (δ 147.6, 133.8 and 151.1), served to position free hydroxyl and hydroxymethyl substituents. The connection of the ketone fragment to the C-10 carbon (δ 114.1) was indicated by a correlation from the C-3b proton (δ 2.89). The C-3b proton also correlated with C-1', thus leading to placement of the phenyl substituent at C-2. The linkage of the glycoside at the C-7 hydroxyl was shown by the correlation of the anomeric proton (C-1", δ 5.75) to the C-7 carbon at δ 147.6. Furthermore, the sugar was confirmed to be in the pyranose configuration by HMBC correlations from the anomeric proton to the C-5" carbon.

Complete analysis of the NMR signals from the aminosugar led to its assignment as ristosamine,[7] a rare deoxyamino sugar first observed as a component of the vancomycin-related antibiotic, ristomycin.[8] The C-3" axial amine, however, was functionalized as an amide with 2-methyl-3-hydroxybutyric acid. This was apparent from HMBC correlations from the C-3" proton (δ 4.58) to the C-1"' amide carbonyl carbon (δ 178.3). The relative stereochemistry of the ristosamine moiety was determined by coupling constant analysis complemented with single frequence decoupling experiments. The small couplings (2.5 and 4.0 Hz) of the anomeric proton to the C-2" methylene protons showed the C-1" proton to be equatorial, thus the glycoside was in the α-configuration. Complex couplings to the C-3" proton rendered analysis difficult. However, the coupling constants for the C-4" proton were clear (3.5 and 10.0 Hz). Irradiation of the C-3" proton signal resulted in a simplified doublet, with J = 10 Hz, for the C-4" proton. Therefore, the 3.5 Hz coupling constant placed the C-3", C-4" and C-5" protons in equatorial, axial and axial configurations, respectively.

The relative stereochemistry of the 2-methyl-3-hydroxybutyramide was assigned as *erythro* by comparison to model compounds. Maskens and Polgar, who synthesized all four enantiomers of 2-methyl-3-hydroxybutyric acid, reported a *J* value of 4 Hz between the C-2 and C-3 protons in *erythro*-2-methyl-3-hydroxybutyric acid methyl ester.[9] In contrast, the *threo* isomer showed *J* = 7 Hz. Irradiation of the C-5"' methyl group simplified the C-2"' signal to a slightly broadened singlet with *J*= <3 Hz, indicating that actinoflavoside was composed of the *erythro*-2-methyl-3-hydroxybutyramide isomer.

NMR Data for Actinoflavoside (1) and the Corresponding Aglycone 2.*

	actinoflavoside (1)			aglycone 2
Carbon#	^{1}H NMR δ (m, *J* in Hz)	^{13}C NMR (#Hs)	HMBC	^{1}H NMR δ (m, *J* in Hz)
2	5.49 (m)	80.5, 80.6 (CH)	1', 2', 4	5.49 (dd, 13.5, 2.5)
3a	3.17(m)	45.3 (CH2)	1', 2, 4	3.12 (dd, 16.5, 13.5)
3b	2.89		1', 4, 10	2.85 (dd, 16.5, 3.0)
4		193.4 (C)		
5		135.8 (C)		
6	6.87(s)	110.4 (CH)	5, 7, 8, 10, 11	6.69 (s)
7		147.6 (C)		
8		133.8 (C)		
9		151.1 (C)		
10		114.1 (C)		
11	4.68(m)	64.9 (CH2)	5, 6, 10	4.67 (m)
1'		137.6 (C)		
2'	7.4-7.5(m)	126.4 (CH)		7.4-7.5 (m)
3'	7.4-7.5(m)	128.9 (CH)		7.4-7.5 (m)
4'	7.4-7.5(m)	129.3 (CH)		7.4-7.5 (m)
8-OH	6.48(s)		7, 8, 9	
11-OH	4.24(t, 7.5)			4.39 (t, 8.0)
1"	5.75(dd, 4.0, 2.5)	95.2 (CH)	5", 7	
2"a	2.24(dt, 15.0, 4.0)	32.8 (CH2)		
2"b	2.21(br.d, 15.0)			
3"	4.58(m)	46.4 (CH)	1", 2", 4", 5", 1"'	
4"	3.50(dd, 10.0, 3.5)	73.6 (CH)		
5"	3.76(m)	65.7 (CH)	1"	
6"	1.1-1.2(overlapping ds)	17.4 (CH3)		
NH	7.79(d, 8.0)		2", 3", 1"'	
4"-OH	3.81(dd, 6.5, 2.5)			
1"'		178.3 (C)		
2"'	2.43(m)	46.1 (CH)	1"'	
3"'	4.01(m)	68.4 (CH)	1"'	
4"'	1.1-1.2(overlapping ds)	19.4 (CH3)		
5"'	1.1-1.2((overlapping ds)	11.5 (CH3)	1"'	

*NMR spectra were recorded at 500 MHz in chloroform-*d* solution. Assignments were aided by single frequency decoupling, DEPT, COSY, HMQC and HMBC experiments.

Flavonoids are extremely common in higher plants, but very rare (probably non-existent) as bacterial metabolites. Although flavonoids have been reported from bacterial culture broths, the origin appears to be in the nutrient media components, probably flavonoid glycosides, which are contaminants in commercial starch and corn steep liquor products.[10] Flavonoids are produced by selected fungi, but here too this seems rare.[10] Actinoflavoside possesses a 5-hydroxymethyl flavonone skeleton which is different from all other flavonones

known. Hence, the true origin of **1** in flavonoid biosynthesis must be considered in question. In final evaluation, actinoflavoside showed only weak antibacterial activity against Gram-positive bacteria (MIC values), including *Staphylococcus pneumoniae* (64 μg/ml), *S. pyrogenes* (64 μg/ml), *S. aureus* (64 μg/ml), and *Micrococcus luteus* (64 μg/ml).

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2. Actinomycete strain CNB-689 was isolated from a black, anaerobic intertidal sediment collected near Christchurch, New Zealand in 1994. The strain was initially isolated by serial dilution methods on solid agar (1.6% agar) using a medium composed of the following: soluble starch 1%, yeast extract 0.4%, peptone 0.2%, seawater 75%, deionized water 25%. FAME (Fatty Acid Methyl Ester) analysis (Microbial ID Inc., Newark, DE) resulted in a poor match (similarity index 0.436) with *Streptomyces halstedii.*

3. Strain CNB-689 was cultivated, in the medium described above, at the 50L scale using a New Brunswick, Fermatron FM-75 pilot scale fermenter, for 7 days at 23° C. The whole culture was filtered and the clear broth was extracted, using a new solid-phase method involving percolation over stearic acid impregnated charcoal. The filtered charcoal was washed with water and eluted with methanol to provide the crude culture extract (yield of **1** was ca. 3 mg/L).

4. Actinoflavoside showed the following spectral features: $[\alpha]_D$ = -110°, (c = 1.3, MeOH); IR υ_{max} (film, NaCl): 3375, 2960, 1650, 1602, 1533, 1508, 1300, 1296, 1144 and 947 cm^{-1}; UV λ_{max} (MeOH): 232 nm (ε 1.9 x 10^4), 282 (1.8 x 10^4) and 325 (4.5 x 10^3).

5. Actinoflavoside perbenzoate was prepared by treatment of **1** (3 mg) with a solution of 0.5 ml dry pyridine and 0.5 ml benzoyl chloride. The solution was stirred for 20 min at RT, cooled in ice, and hydrolyzed with dist. water. The pH was adjusted to ca. 8.0 and the organic products were extracted with EtOAc. Purification of the ester was accomplished by silica HPLC using 50% EtOAc in isooctane.

6. Aglycone **2** was prepared by hydrolysis of actinoflavoside. Actinoflavoside (6 mg) was dissolved in 1 ml MeOH and 1 ml 5N HCl and stirred at RT overnight. The solution was neutralized with Na_2CO_3 and the organic products were extracted by solid phase adsorption on RP C-18 resin. After elution of the reactions products with methanol, the aglycone was purified by silica HPLC (40% EtOAc/isooctane) to lead to pure **2** (2 mg).

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