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PSEUDOMONINE, AN ISOXAZOLIDONE WITH SIDEROPHORIC ACTIVITY FROM *PSEUDOMONAS FLUORESCENS* AH2 ISOLATED FROM LAKE VICTORIAN NILE PERCH

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ABSTRACT.—A siderophore, pseudomonine, and sodium salicylate were isolated from the culture broth of iron-deficient cultures of *Pseudomonas fluorescens* AH2 isolated from the surface of spoiled Nile Perch from Lake Victoria.

Microbial siderophores, iron-chelating metabolites expressed as a consequence of a deficiency of this element, have been studied thoroughly (1,2). Although the majority of studies are concerned with pathogenic or soil-inhabiting bacteria, there has recently been an increased interest in marine microorganisms, inasmuch as contemporary evidence indicates that iron availability controls microorganism productivity and biomass in the marine environment (3). Siderophores are produced both by open-ocean bacteria (4,5) and phytoplankton (6,7). The presence of a siderophore with exceptionally high iron affinity isolated from *Alteromonas luteoviolaceae* from oligotrophic and coastal waters has led to the suggestion that this feature provides a competitive advantage over other microorganisms (5–8). Siderophores from marine bacteria associated with plants, animals (9), and sediments (10–12) are less effective probably because of easier access to iron from the substratum (13). Although similar considerations may apply for freshwater ecosystems, the scarcity of chemical investigations precludes firm conclusions.

We wish to report the isolation and structure elucidation of an unusual isoxazolidone-imidazole alkaloid with siderophoric activity from cultures of the fluorescent *Pseudomonas fluorescens* (strain AH2) isolated from spoiled Nile perch (*Lates niloticus*) from Lake Victoria (14). Under iron limitation, this strain produces siderophores and inhibits growth of a number of bacteria (15).

Repeated fractionation of the fermentation broth gave pure pseudomonine with the molecular formula $C_{16}H_{18}N_4O_4$ corresponding to 10 sites of unsaturation. The structure was derived from analysis of the ^{1}H- and ^{13}C-nmr spectra (HMQC, HMBC) in different solvents. The base peak in the mass spectrum at *m/z* 120 characteristic of salicylic acid derivatives indicated the presence of the C-1–C-7 fragment supported by long-range couplings and the spin-systems of the aromatic protons. Comparison with ^{1}H- and ^{13}C-nmr spectra of authentic salicylamide identified this fragment as an *N*-substituted salicylamide previously found in siderophores (16–18). Of the remaining three aromatic carbon atoms, two (135.3 and 118.2 ppm) had protons with very small coupling constants (J_{HH}<0.5 Hz) attached and exhibited C-H coupling constants of 228 and 200 Hz, respectively, indicating the presence of an imidazole ring. The long-range couplings, the coupling pattern of H-15–H-16 with

magnetic non-equivalent H-15 protons, and comparison with the nmr spectra of authentic histamine, confirmed the presence of an ω-*N*-substituted histamine fragment as in the anguibactins (9). This left two sites of unsaturation, pinpointing the remaining carbonyl group to a ring system.The chemical shifts, spin-spin pattern and long-range couplings identified a CH_3-CH(O)-CH(N) fragment as part of a cyclothreonine ring incorporating (long-range coupling between H-15 and C-10) the ω-*N*-histamine nitrogen. The relative stereochemistry around the C-9 to C-11 bond was tentatively assigned as *trans* (i.e., as derived from *allo*-threonine) by comparison of the coupling constant (10.9 Hz) with those reported (19) from the cyclothreonines (J_{cis}=7.0 Hz and J_{trans}=8.7 Hz in D_2O). The synthesis of pseudomonine and elucidation of its absolute stereochemistry are underway and will be published elsewhere.

Sodium salicylate, identified by comparison with an authentic sample, was present in the broth from *P. fluorescens*. The Chrome-Azurol-S (CAS) reaction (20) indicated both compounds to have low siderophoric activity in agreement with the hypothesis of iron availability from the substratum (13). The microbial interaction mediated by iron sequestering systems of different iron affinities is well studied in the rhizosphere where the siderophores of fluorescent pseudomonads are believed to play a role in plant protection (21). Because *Pseudomonas* spp. are frequently isolated from fresh and spoiled fish of marine and fresh-water origin, a similar mechanism might be operative here. The present compounds have no chemical relationship to the catecholic siderophores from fresh-water aeromonads (22), but pseudomonine incorporates an *N*-aroylthreonine like the uraumycins produced by a *Streptomyces* sp. isolated from an unidentified marine sponge (23). The salicylamide moiety is encountered in pyochelin and the aeroginoic acids of terrestrial *Pseudomonas fluorescens* strains (24).

During extraction and fractionation, several CAS-reactive fractions were encountered. Although pseudomonine is iron-chelating, other siderophores are likely to be produced and contribute to the antagonistic activity of the strain.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Mass spectra originated from a JEOL JMS-HX/HX 110A tandem mass spectrometer. Nmr spectra were recorded on Bruker 600 MHz and 250 MHz instruments.

MICROBIAL MATERIAL.—*Pseudomonas fluorescens* AH2 was isolated from spoiled iced Nile perch from Lake Victoria (14). This strain produced siderophores as determined on Chrome-Azurol-S-agar (20). The isolate produced siderophores on all substrates tested (asparagine-sucrose, M9 with glucose and casamino acids, and CAS basal medium), as opposed to other *Pseudomonas* strains that were often negative in the CAS basal medium.

EXTRACTION AND ISOLATION.—The strain was grown in aerated (150 rev/min) asparagine-sucrose broth (25) at 25° for 4 days where the siderophoric activity was high according to the spectrophotometric CAS assay (20). The culture was harvested by centrifugation (10,000×g for 15 min) and toluene (5 ml/liter supernatant) added to avoid further growth. The broth (1.5 liter) adjusted to pH 4.5 with phosphoric acid was thoroughly centrifuged, the excess sucrose (ca. 30 g) was eliminated by XAD chromatography (Merck XAD 4, elution with H_2O), and the crude alkaloid eluted with EtOH. Further fractionation (Merck RP-8), isocratic elution MeCN/H_2O in varying proportions with 0.1% TFA added) afforded pseudomonine (9 mg). One chromatographic fraction contained sodium salicylate identified by comparison (^1H, ^{13}C nmr, ir) with an authentic sample.

Pseudomonine [**1**].—Amorphous brownish solid; $[\alpha]_D$ −80.0° (c=0.041, H_2O); uv (H_2O) λ max (log ε) 298 (3.36), 237 (3.95), 203 (4.40) nm;

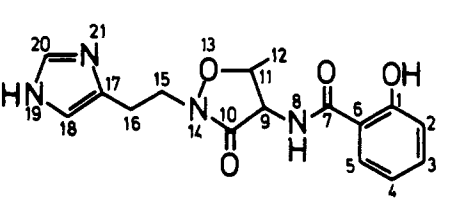

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TABLE 1. ^{1}H- and ^{13}C-Nmr Data of Pseudomonine [**1**].

Position	δ_C[a]	δ_H[a] (Multiplicity, J_{HH}, Hz)	δ_C[b]	J_{CH} (Hz)[b]
1	161.1		158.1	
2	118.5	6.92 (dd, 8.5, 1.1)	120.9	
3	135.3	7.40 (ddd, 7.5, 8.2, 1.5)	135.4	162.9
4	120.3	6.90 (ddd, 8.0, 7.3, 1.1)	118.0	165.8
5	129.2	7.78 (dd, 8.0, 1.5)	129.6	160.8
6	116.5		116.9	
7	171.1		170.7	
9	59.5	4.62 (d, 10.9)	59.2	142
10	169.1		168.4	
11	79.9	4.55 (dq, 10.9, 5.9)	79.5	153
12	17.2	1.46 (q, 5.9)	16.9	128
15	45.1	3.99 (td, 6.5, 14.5)	44.7	143
		3.89 (td, 6.5, 14.5)		
16	23.6	3.10 (d, 6.5)	22.5	133
17	132.6		130.9	
18	118.2	7.38 (d, <0.5)	117.6	200.4
20	135.3	8.62 (d, <0.5)	134.2	227.6

[a]In CD_3OD, chemical shifts relative to the solvent resonances at δ_C 47.07 and δ_H 3.35.
[b]In D_2O, chemical shifts relative to dioxan (δ_C 64.7).

hreims $[M]^+$ *m/z* 330.1330 ($C_{16}H_{18}N_4O_4$, Δ +0.6 ppm); ^{1}H- and ^{13}C-nmr data, see Table 1; selected ^{1}H-nmr data from DMSO-d_6 solution: OH, 12.00 ppm, br; NH-19, 13.75 ppm, br; NH-8, 9.10 ppm, J=8 Hz; ^{1}H-nmr data from D_2O solution relative to Me_2CO (δ_H 2.225): δ 6.96 (1H, d, J=8.3 Hz, H-2), 7.45 (1H, dd, J=7.8 and 8.3 Hz, H-3), 6.97 (1H, dd, J=7.4 and 7.8 Hz, H-4), 7.69 (1H, d, J=7.8 Hz, H-5), 4.61 (1H, br s, H-9), 4.61 (1H, br s, H-11), 1.43 (1H, br s, H-12), 3.86, 3.96 (1H, dd, J=6.3 and 14.8 Hz, H-15), 3.08 (1H, t, J=6.3 Hz, H-16), 7.32 (1H, s, H-18), 8.54 (1H, s, H-20); HMBC data for **1** in D_2O: (a) Salicylamide moiety: two CH (δ_H 6.96–6.97) exhibited long-range couplings with δ_C 129.6, 158.1; CH (7.45) with 158.1, 129.7, 116.9; CH (7.69) with 170.7, 158.1, 135.4. (b) Histamine moiety: CH (δ_C 134.2) exhibited long-range couplings with δ_C 130.9, 117.6; CH (7.32) with δ_C 134.2; CH_2 (3.08) with δ_C 130.9, 117.6, 44.7, CH_2 (δ_H 3.86+3.96) with δ_C 168.4, 130.9. (c) Cyclothreonine moiety: two CH (δ_H 4.61) exhibited long-range couplings with δ_C 170.7, 168.4, 16.9.

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LITERATURE CITED

1. L.L. Barton and B.C. Hemming, Eds., "Iron Chelation in Plants and Soil Microorganisms." Academic Press, San Diego, 1993.
2. G. Winkelmann, Ed., "CRC Handbook of Microbial Iron Chelates." CRC Press, Boca Raton, FL, 1991.
3. Z.S. Kolber, R.T. Barber, K.H. Coale, S.E. Fitzwater, R.M. Greene, K.S. Johnson, S. Lindley, and P.G. Falkowski, *Nature*, **371**, 145 (1994).
4. C.G. Trick, *Current Microbiol.*, **18**, 375 (1989).
5. R.T. Reid, D.H. Live, D.J. Faulkner, and A. Butler, *Nature*, **366**, 455 (1993).
6. C.G. Trick, R.J. Andersen, N.M. Price, A. Gillam, and P.J. Harrison, *Marine Biol.*, **75**, 9 (1983).
7. C.G. Trick, R.J. Andersen, A. Gillam, and P.J. Harrison, *Science*, **219**, 306 (1983).
8. R.T. Reid and A. Butler, *Limnol. Oceanogr.*, **36**, 1783 (1991).
9. M.A.F. Jalal, M.N. Hossain, D. van der Helm, J. Sanders-Loehr, L.A. Actis, and J.H. Crosa, *J. Am. Chem. Soc.*, **111**, 292 (1989).
10. T. Kameyama, A. Takahashi, S. Kurasawa, M. Ishizuka, Y. Okami, T. Takeuchi, and H. Umezawa, *J. Antibiot.*, **40**, 1664 (1987).
11. T. Nishio, N. Tanaka, J. Hiratake, Y. Katsube, Y. Ishida, and J. Oda, *J. Am. Chem. Soc.*, **110**, 8733 (1988).
12. D.K. Hancock, B. Coxon, S.-Y. Wang, E. White, D.J. Reeder, and J.M. Bellama, *J. Chem. Soc., Chem. Commun.*, 468 (1993).
13. A.V. Pham, S. Nearhos, and G.H.G. Davis, *Microbiol. Lett.*, **30**, 79 (1985).
14. L. Gram, C. Wedell-Neergaard, and H.H. Huss, *Int. J. Food Microbiol.*, **10**, 303 (1990).
15. L. Gram, *Appl. Environ. Microbiol.*, **59**, 2197 (1993).

16. T. Peterson, K.-E. Falk, S.A. Leong, M.P. Klein, and J.B. Neilands, *J. Am. Chem. Soc.*, **102**, 7715 (1980).
17. W. Keller-Schierlein, L. Hagmann, H. Zähner, and W. Huhn, *Helv. Chim. Acta*, **71**, 1528 (1988).
18. G.H. Tait, *Biochem. J.*, **146**, 191 (1975).
19. K.F. Turchin, *J. Struct. Chem. USSR*, **12**, 918 (1971).
20. B. Schwyn and J.B. Neilands, *Anal. Biochem.*, **160**, 47 (1987).
21. J.E. Loper and J.S. Buyer, *Mol. Plant Microbe Interact.*, **4**, 5 (1991).
22. J.R. Telford, J.A. Leary, L.M.G. Tunstad, B. Rowe-Byers, and K.N. Raymond, *J. Am. Chem. Soc.*, **116**, 4499 (1994).
23. N. Imamura, M. Nishijima, K. Adachi, and H. Sano, *J. Antibiot.*, **46**, 241 (1993).
24. R. Carmi, S. Carmeli, E. Levy, and F.J. Gough, *J. Nat. Prod.*, **57**, 1200 (1994).
25. F.M. Scher and R. Baker, *Phytopathol.*, **72**, 1567 (1982).

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