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Asterobactin, a New Siderophore Group Antibiotic from *Nocardia asteroides*

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During continuing studies on biologically active compounds from clinical isolates of *Nocardia* species[1~4], one antitumor compound called asterobactin (**1**), was isolated from the mycelial cake of a clinically isolated *Nocardia asteroides* IFM 0959 strain.

Structural elucidation suggested that **1** is a new siderophore group antibiotic (Fig. 1). In this paper, we report the production, isolation, physicochemical properties, structural characteristics and biological activity of the antibiotic.

The seed broth was prepared by inoculating mycelial elements of the producing strain (*Nocardia asteroides* IFM 0959, a clinical isolate from a nocardiosis patient in Japan) grown on Sabouraud dextrose agar (Difco, Detroit) in 10 ml of brain heart infusion broth (BHI, Difco) with 2% glucose in 50-ml Erlenmeyer shake flask. The culture was incubated on a rotary shaker at 250 rpm for 96 hours. Ten percent inoculum was transferred to a 500-ml Erlenmeyer flask containing 150-ml of the production medium composed of meat extract 0.5%, peptone 0.5%, glycerol 2.0% and antifoam 0.05%. The pH was adjusted to 7.4. The culture was incubated on a rotary shaker at 250 rpm for 6 days. After the incubation, each volume of methanol was added to the culture broth and further incubated for 3 hours to kill the *Nocardia* and to extract the active substance from the mycelia. Thereafter the broth was filtered and evaporated under vacuum. The crude aqueous solution (150 ml) from 2 liters cultures was extracted with ether. After evaporation, the residue (about 5 g) was then subjected to silica gel chromatography (10 mm×10 cm) using Hexane, $CHCl_3$, and then $CHCl_3$-MeOH (30:1, 20:1, 10:1). Active fractions from the $CHCl_3$-MeOH (20:1) eluates were further purified by silica gel chromatography using ethyl acetate - MeOH (20:1) after purification activity was tested against HL-60 tumor cell line. From 2 liters cultures, 7 mg of asterobactin was obtained.

The structure of asterobactin (**1**) as shown in Fig. 1 was proposed on the basis of the spectroscopic and mass spectrometric data. The physico-chemical properties of **1** are shown in Table 1. The IR absorbances at 1623 , 1685, 1733, 3265 and 3406 cm^{-1} (film; FT-IR with ATR) suggested the presence of an aromatic system, carbonyls, OH and NH groups (Table 1). The molecular weight of 769 Da and the chemical formula $C_{34}H_{55}N_7O_{13}$ were readily inferred by positive ion ESI-MS (Quattro, VG Biotech; *m/z* 770.2 $(M+H)^+$; *m/z* 792.6 $(M+Na)^+$) and HRESI-MS (Finnigan MAT 95XL; *m/z* 770.3926 $(M+H)^+$, calcd. 770.3936 for $C_{34}H_{56}N_7O_{13}$). The negative ion ESI-MS showed *m/z* 804.6 $(M+Cl)^-$ as a further characteristic

Fig. 1. Chemical constitution of asterobactin (**1**).

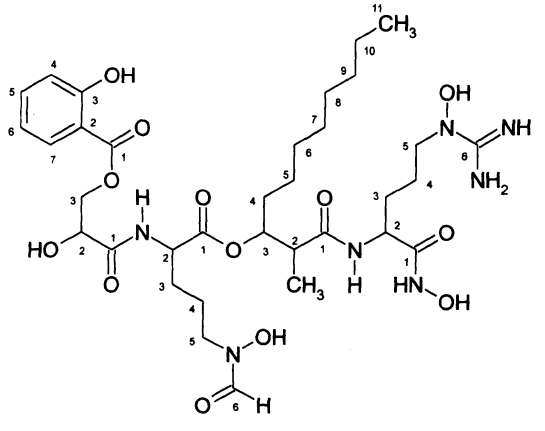

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Table 1. Physico-chemical properties of asterobactin (**1**).

Appearance	colorless wax
Molecular formula	$C_{34}H_{55}N_7O_{13}$
HR FAB-MS m/z $(M+H)^+$	770.3926; calcd 770.3916 for $C_{34}H_{56}N_7O_{13}$
$[\alpha]_D^{25}$ (c 1.0; MeOH)	-14.5 °
IR (film) ν cm^{-1}	7757, 868, 927, 967, 1035, 1053, 1125, 1136, 1182, 1202,1258, 1340, 1365, 1405, 1465, 1486, 1549, 1591, 1623,1675, 1733, 2852, 2921, 3265, 3406
UV λ_{max}^{nm} in MeOH	218, 255
R_f (TLC) Merck	
a) Silica gel sheets RP_{18}; MeCN-H_2O 83:17, 0.5 % TFA	0.85
b) silica gel sheets $CHCl_3$/MeOH 9:1 (reddish staining by 1 % vanillin/conc. H_2SO_4)	0.2

feature. The chemical composition $C_{34}H_{55}N_7O_{13}$ thus indicated the presence of 10 double bonds or rings in the molecule. The occurrence of seven nitrogens is compatible with the presence of a guanidino group in moiety E.

The chemical constitution of **1** was further elucidated on the basis of 1D and 2D NMR measurements (^1H, ^{13}C, DEPT-135, COSY, TOCSY, HSQC, HMBC; see Table 2). The ^1H NMR spectrum displayed a series of aromatic protons in the range of 6.5~7.7 ppm appearing as doublets and triplets. Proton signals in the range of 3.5~5.2 ppm showed the presence of methine and methylene groups bound to heteroatoms such as oxygen and nitrogen. In the range of 0.89~2.9 ppm a series of aliphatic protons was visible. The presence of a salicyclic acid structure (moiety A) was confirmed by the observed $^1H,^1H$- couplings of an *ortho*-disubstituted benzene ring in the COSY spectrum. The proton signals at 0.89 ppm (triplet) and 1.18 ppm (doublet) were attributable to methyl groups which were coupled to CH_2 and CH groups, respectively. The broadband proton-decoupled ^{13}C NMR spectrum displayed six aromatic carbons (109.3, 115.7, 122.2, 129.1, 135.8, 165.2 ppm), and six carbonyl signals (163.8, 165.8, 169.8, 170.5, 171.0, 172.0 ppm) attributable to ester and amide structures. An additional carbon signal at 153.2 ppm coupled to a singlet proton signal at 7.7 ppm was assigned to the *N*-formyl carbon in moiety C (see Fig. 1). Moreover, the ^{13}C and DEPT spectral data suggested the presence of 15 methylene groups whereby three were bound to oxygen or nitrogen. In addition, seven methine and/or methyl groups were recognized.

The chemical constitution of moiety B (2,3-dihydroxy propionic acid) and its location within the molecule of **1** was assigned unambiguously due to the ^1H and ^{13}C chemical shifts and proton couplings (Table 2). ^{13}C, ^1H long-range heteronuclear correlations in the HMBC spectrum between H_{3A} and H_{3B} of moiety B with the carbonyl carbon (C-1) of moiety A, at the one side, and of the amide proton of moiety C with C-1 of moiety B at the other, settled the sequence of moieties A, B and their connection with C. Similarly, the constitution and sequence of moieties C, D and E was elucidated.

Assignments of moiety C as N5-hydroxy-N5-formyl-ornithine, of moiety D as 2-methyl-3-hydroxy-undecanoic acid and of moiety E as N5-hydroxyarginine-N1-hydroxyamide were based on the COSY and HMBC coupling data. Thereby couplings of the amide protons (9.20 ppm (d; moiety C) and 7.21 ppm (d; moiety E)) and of H-2 in the moieties B, C, D and E with neighboured protons and/or carbons *via* $^3J_{H,H}$ and $^3J_{H,C}$ couplings, respectively, supplied conclusive evidence (Fig. 2). The presence of the hydroxyl group at N-5 of the guanidino group of moiety E was suggested by the observed

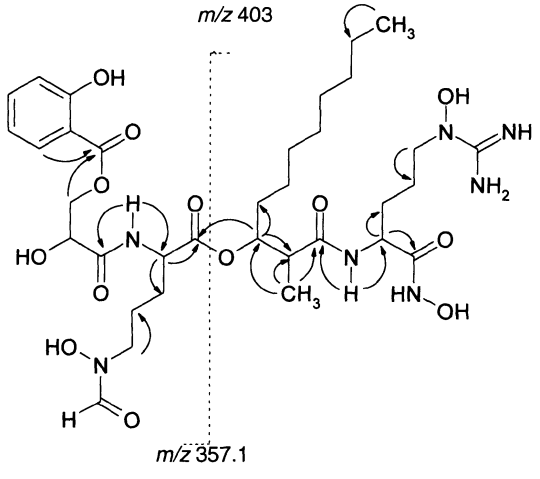
Table 2. Assignment of 500 MHz ^{1}H and 125 MHz ^{13}C NMR data of asterobactin (**1**).

Moiety	position	δ_C	δ_H	$^{1}H,^{1}H$ COSY
A	1	172.0 (s)	-	-
(salicylic acid)	2	109.9 (s)	-	-
	3	165.2 (s)	-	-
	4	129.1 (d)	7.61 d; 7.9; 1.5	H-5, H-6
	5	115.7 (d)	6.61 dd; 7.9, 7.1	H-4, H-6,
	6	135.8 (d)	7.35 dd; 7.1, 8.9	H-5, H-7
	7	122.2 (d)	6.91 d; 8.9, 1.6	H-6, H-5
B	1	169.8 (s)	-	-
(2,3-dihydroxy-	2	66.8 (d)	4.49,dd, 4.1,3.5	H-3
propionic acid)	3	71.1 (t)	4.58, dd; 8.3, 3.5; 5.08 dd ; 8.3, 4.1	H-2, H-3_B H-2; H-3_A
C	1	170.5 (s)	-	-
(N5-hydroxy-	2	53.4 (d)	4.43 dd; 4.2, 1.8; 9.6 (NH) d; 4.2	H-3 H-2
N5-formyl-				
ornithine	3	29.4 (t)	1.26 m	n.r.
	4	25.2 (t)	2.01 m	n.r.
	5	47.9 (t)	3.68 dd; 17.3, 3.9; 3.81 m	H-4; H-5_B H-4; H-5_A
	6	153.2 (s)	-	-
D	1	171.0 (s)	-	-
(2- methyl-	2	44.6 (d)	2.48 m	H-3
hydroxy-undeca	3	76.5 (d)	5.16 dd; 8.2, 1.3, 0.6	H-2, H-4, H-11
- noic acid)	4	26.7 (t)	1.80 m, br	H-3, H-5
	5	29.5 (t)	1.26 m, br	n.r.
	6	29.7 (t)	1.26 m, br	n.r.
	7	29.7 (t)	1.16 m, br	n.r.
	8	29.7	1.16 m, br	n.r.
	9	29.7 (t)	1.16 m, br	n.r.
	10	25.5 (t)	1.80 m, br	n.r.
	11	14.1 (q)	0.89 t; 6.9	H-9
	12	13.2 (q)	1.15 d; 8.2	H-2
E	1	165.2 (s)	-	-
(N5-hydroxy-	2	49.9 (d)	4.7 ddd; 4.5, 6.8, 5.0 7.20 (NH) d; 5.0	H-3; NH-2 H-2
arginine-N1-				
hydroxyamide)	3	32.3 (t)	1.26 m	n.r.
	4	29.5 (t)	1.78 m	H-5
	5	53.5 (t)	3.80 m 4.00 dd,1.8, 11.3	H-5_B H-5_A, H-4
	6	163.8 (s)	-	-

In $CDCl_3$; δ in ppm, TMS as internal standard; J in Hz; Abbreviations: s: singlet, d: doublet, t: triplet, q: quartet, m: multiplet, br: broad, multiplicity in parentheses, n.r.: not resolved correlations in the $^{1}H,^{1}H$-COSY.

downfield shift of nitrogen-bound C-5 appearing at 53.6 ppm. Moreover, this suggestion was confirmed by the occurrence of L-N5-hydroxyarginine as an antibacterial metabolite in both bacteria and fungi[5,6]. However, due to many overlapping signals of methylene protons of moieties C, D and E the carbons of their aliphatic chains were not clearly distinguishable. Nevertheless, conclusive evidence for the structure of **1** was furnished by ESI-CID-MS and ESI-CID-MS^n. Thus collision-induced dissociation of $[M+H]^+$ in presence of ammonium acetate generated *m/z* 403.4 ([M-moieties A, B, C+H]$^+$, 85%) due to bond cleavage between C-1 of moiety C and O-3 of moiety D and *m/z* 357.1 ([M-moieties D, E; –CO; $+NH_4]^+$, 80%) due to cleavage between C-1 of moiety C and O-3 of moiety D (Fig. 2). Similarly ESI-CID-MS^2 of *m/z* 792 ($[M+Na]^+$) furnished *m/z* 426.3 (M-moieties A, B, C+Na]$^+$.

Fig. 2. Instructive C,H long-range couplings in the HMBC spectrum of **1** and diagnostic fragment ions observed in ESI-CID-MS/MS.



1 thus appears as a novel representative of the *N*-hydroxyamate family of microbial metabolites which are known as iron scavangers (siderophores), radical scavangers or pharmacologically active agents[7,8]. In accord with the hydroxamato structure compound **1** formed a brownish complex with Fe^{+3} ions in methanolic solution. The chemical structure of **1** is distinguishable from related molecules such as formobactin[9], nocobactin[10] and the mycobactins[11] by the absence of an oxazolin ring in moiety B and the presence of ornithine and arginine skeletons. As in case of the above related molecules IR and UV spectroscopy, polarimetry, mass spectrometry and NMR spectroscopy did not furnish informations about the stereochemistry of **1**. However, *S*-configuration could be proposed for the ornithine and arginine skeletons (moieties C and E) at least due to their obvious biosynthetic origin from the general pool of amino acids. This conclusion was supported by the observed negative optical rotation value (Table 1).

Mycobacterium and the related genus, *Nocardia*, are unique in possessing an intracellular siderophore, mycobactin and nocobactin, respectively[7~9]. The metabolic reason of this lipophilic compound in these bacteria may be connected with the thickness of the lipophilic cell envelope which is a characteristic of these organisms. Mycobactins and nocobactin are lipid-soluble, wholly intracellular siderophores that have a mixed type Fe(III) chelation center[8]. Their structures vary by the producing species although all have the same core nucleus. However, as stated above, asterobactin is clearly separated from nocobactin produced by the same species of *N. asteroides* due to the absence of an oxazolin ring and the presence of *N*-hydroxy ornithine and arginine skeletons.

Asterobactin shows antitumor activity: it was active against various cultured cell lines such as HL-60 and HeLa

Table 3. Antitumor activity of asterobactin (**1**) against various tumor cells.

IC_{50} values against tumor cells	Asterobactin (μg/ml)	
	drug only	with $FeCl_3$
HL-60	0.2	>100
K562	3.1	>100
MDA-MB-231	0.9	>100
A549	3.2	>100
HeLa	2.5	>100

* treatment with 0.1 mM $FeCl_3$ (saturation dose).

cells in concentration of 0.2 to 3.2 μg/ml (Table 3).

The activity was reduced in the presence of Fe^{3+} and their IC_{50} values after addition of Fe^{3+} are more than 100 μg/ml. This experiment confirmed the ability of the compound to chelate Fe^{3+} in *in vitro* experiment. Since the *N. asteroides* is an opportunistic pathogen, further studies on the role of asterobactin in pathogenesis of this bacterium are of interest.

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