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Dihydroniphimycin: New Polyol Macrolide Antibiotic Produced by *Streptomyces hygroscopicus* 15

Isolation and Structure Elucidation

VENETA IVANOVA, VICTORIA GESHEVA and MARIANA KOLAROVA

The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Acad. G. Bonchev-str., 26, 1113 Sofia, Bulgaria

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A new 36-membered ring macrolide antibiotic dihydroniphimycin (Fig. 1) which shows preventive effect to fungal infection of plants, has been isolated from the mycelium of a *Streptomyces hygroscopicus* 15. Besides the antifungal antibiotic, the strain *S. hygroscopicus* 15 produces also the nigericin complex and antibiotic AN-15[1].

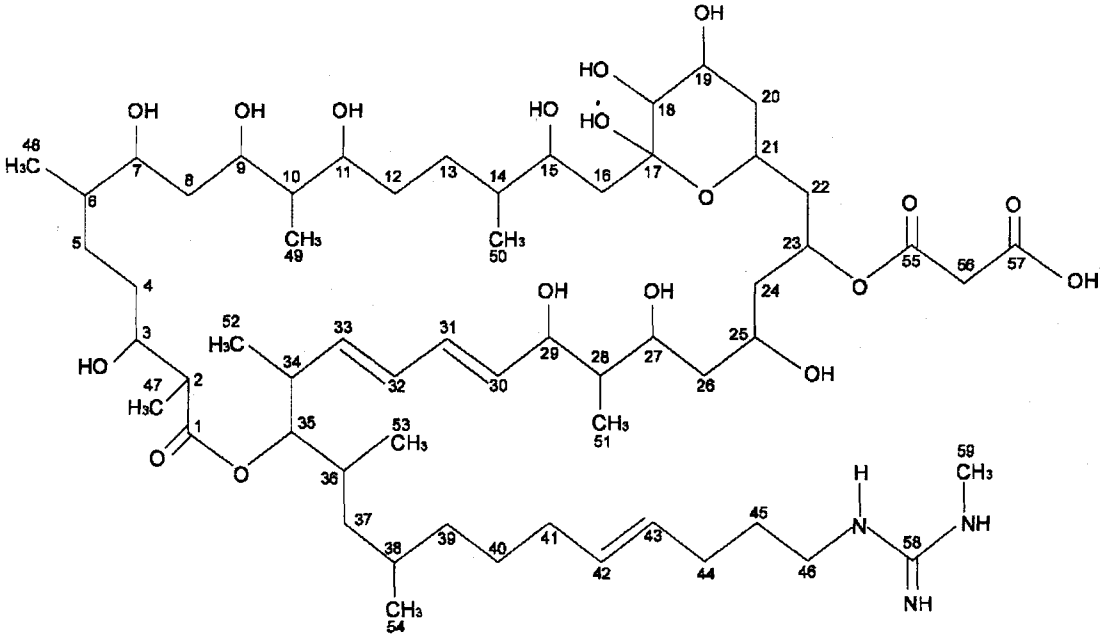
The producing strain *S. hygroscopicus* 15 has been deposited in the Bulgarian National Collection of Industrial Microorganisms and Cell Cultures. The taxonomic study of the producing strain and the biological activity of the antibiotic are described in the preceding papers[2, 3].

In this paper, we report the production, isolation, physico-chemical properties and the structure elucidation of the antibiotic AK-15-2 (dihydroniphimycin).

The mature slant culture of *S. hygroscopicus* 15 was inoculated into Erlenmeyer flasks (1000 ml), containing 500 ml of seed medium consisting of glucose 1.0%, soybean meal 1.0%, NaCl 0.5%, and $CaCO_3$ 0.1%, pH 6.8 before autoclaving. The flasks were cultivated on a rotary shaker at 325 rpm for 40 hours. The seed culture (5%) was inoculated into a 10-liter fermentor containing 6.0 liters of the production medium consisting of glucose 1.5%, soybean meal 1.5%, NaCl 0.5% and $CaCO_3$ 0.3%, pH 7.0 before autoclaving.

After 140 hours of cultivation at 30°C, the culture broth (5.00 liters) of *Streptomyces hygroscopicus* 15 was centrifuged at 5°C for 30 minutes at 5000 rpm. The supernatant fluid was discarded and the mycelium was extracted three times with 2.50 liters of ethanol. The active solvent extracts were combined and concentrated to 350 ml and the aqueous solution was extracted with *n*-BuOH 2 : 1 (v/v). The organic layer was concentrated *in vacuo* to give crude oil. The crude product was dissolved in a small amount of MeOH, filtered and precipitated with Me_2CO-ether 10 : 1 (v/v). The precipitate was allowed to stand for 24 hours at 5~10°C, filtered and washed with acetone and ether. An amount of 3.00 g crude powder was obtained after dryness *in vacuo*. A methanolic solution of the powder (1.50 g) was chromatographed on a Silica gel 60 (70~325

Fig. 1. Structure of dihydroniphimycin.



mesh) column equilibrated with chloroform. Dihydroniphimycin was eluted from the column by isocratic mode with a solvent mixture consisting of $CHCl_3$ - MeOH - H_2O 175 : 100 : 50 (v/v/v), lower phase. The active eluates were evaporated to dryness *in vacuo*. The complete separation and purification of the compound could be achieved only by preparative HPLC on a (250×5 mm) Lichroprep RP 18 column, using an gradient of 40% to 70% acetonitrile in 0.01 M sodium phosphate buffer, pH 4.0 and monitoring at 220 nm. The active fractions were concentrated and desalted on a Sephadex LH-20 column (eluant-MeOH). A final purification of the dihydroniphimycin was achieved under the same conditions on Lichroprep RP 18, using an 0.01 M sodium phosphate buffer, pH 4.0 - acetonitrile 53 : 47 (v/v), isocratic solvent system, Rt=5.35. The active fractions were desalted on a LH-20 by elution with MeOH. After concentration and dryness *in vacuo* 160 mg of pure dihydroniphimycin was obtained.

Dihydroniphimycin was isolated as colorless powder with melting point of 126~128°C. It is optically active with $[\alpha]_D^{25}$ value of +28.0° (c=0.5, MeOH). The antibiotic is soluble in lower alcohols, pyridine, dimethyl sulfoxide, *N,N*-dimethylformamide but insoluble in ethyl acetate, chloroform, ether, *n*-hexane and water. On thin-layer plates dihydroniphimycin demonstrated positive colour reactions to 3% vanillin-sulphuric acid solution, iodine vapour, Dragendorf and Sakaguchi test. It gave a single spot on TLC (silica gel; $CHCl_3$ - MeOH - H_2O 2 : 2 : 1 (v/v/v), lower phase, Rf=0.29). The UV spectrum in methanol showed absorption maxima at 225sh, 231 (ε=28033), 241 sh nm. The structure of two conjugated double bonds was easily asertained to be a dienic system on the precise wavelength of the absorption band at 231 nm. The IR spectrum (KBr) of dihydroniphimycin showed the expected stretching absorption of hydroxy groups (3391), 2935, lactone carbonyl group (1723), C=C double bonds (1646), 1597, 1460, 1419, 1381, 1293, 1242, 1142, 1066, 988, 688 and 597 cm^{-1}.

The high resolution fast atom bombardment mass spectrometry of dihydroniphimycin gave $(M+H)^+$ *m/z* 1144.5200 (Calcd. for $(C_{59}H_{105}N_3O_{18}+H)^+$ 1144.5130). A series of fragment ions are observed at *m/z* 1126 $(MH-H_2O)^+$, 1100 $(MH-CO_2)^+$, 1058 $(MH-OOCCH_2-CO)^+$, 1040 $(M-HOOCCH_2COO)^+$, 1022, 946, 836, 448, 386, 376, 346, 294, 282, 252, 224, 210, 185, 168, 155, 101, 95, 93, 87, 81, 69, 57, 43, 30. The fragment ions at *m/z* 1100, 1058 and 1040 suggested the presence in the structure of dihydroniphimycin a one malonate residue.

The ^{13}C NMR (100.62 MHz) and DEPT 135 spectra showed 59 carbon signals in which one guanidino carbon at 158.17 ppm, three carbonyl carbons at 176.80, 174.08 and 171.60 ppm and six olefinic carbons at 129.90, 131.90, 132.15, 132.72, 135.36 and 136.97 ppm.

Analyses of ^1H-^{13}C COSY spectrum clarified the presence of 8 methyl carbons. The signal at 28.34 ppm (singlet) corresponds to a –N–CH_3 group. A 18 methylene carbons, and 27 methine carbons including 6 olefinic and 13 oxygenated methine carbons and one quaternary hemiacetal carbon at 99.68 ppm were identified. Since three overlapped peaks at 72.28, 41.48 and 40.85 ppm were observed.

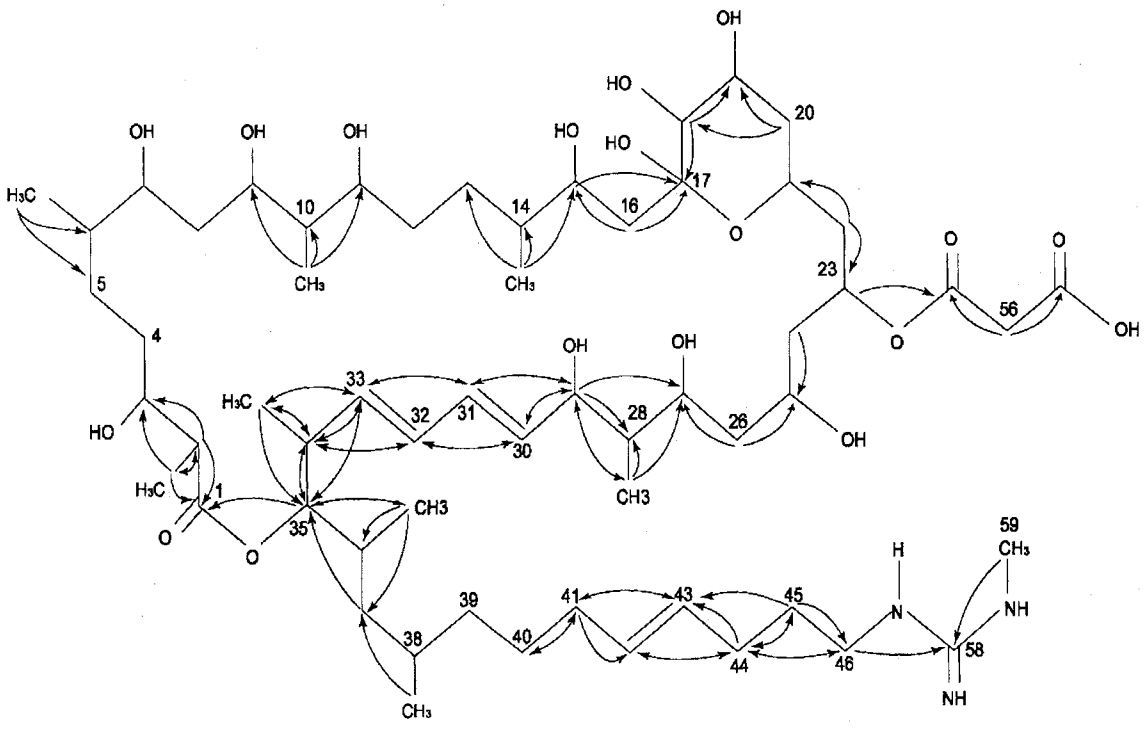
The signals between 3.30 and 5.30 ppm and the signal at 7.51 ppm in the 1D-proton spectrum with no corresponding cross peaks in the ^1H-^{13}C COSY were assigned to hydroxy protons and one NH proton.

The structure of dihydroniphimycin was deduced by ^1H-^1H COSY, ^1H-^{13}C COSY and HMBC (Table 1). On the ^1H-^1H COSY spectrum the resonances of the protons in the part of the dienic system were at δ 6.18 (1H, dd, H-31), 6.05 (1H, dd, H-32), 5.63 (1H, dd, H-30), 5.51 (1H, dd, H-33). The other two olefinic protons were distinguishable at 5.48 (1H, dt, H-42) and 5.44 (1H, dt, H-43). The overlapping signals (H-42 and H-43) could be unraveled in ^1H-^1H COSY and HMBC spectra. The protons H-34, H-29, H-44 and H-41 directly coupled to the olefinic ones, could be assigned without ambiguities, as well. The proton H-34 at δ 2.52 was clearly coupled to the methyl protons at δ 1.02 (H-52) and to the methine proton that on the basis of the chemical shift at δ 4.74 and the lack of other couplings, was assigned to H-35 adjacent to the carboxylic group.

The sequential assignments of C-2 to C-15 was deduced from ^1H-^1H COSY. The sequential assignments for C-15 to C-26, C-29 to C-37 and C-40 to guanidinomethyl were also determined by the analyses of ^1H-^1H COSY and HMBC (Table 1). The assignments of the six membered hemiacetal was executed by comparison with the NMR data for kanchanamycin C[4], copiamycin[5], malolactomycins[6], shurimycins[7], azalomycins[8], niphimycin A[9~11] and amycins[12,13].

In the HMBC spectrum five quaternary carbon atoms were detected, three in the carbonyl region of the spectrum (176.80 ppm, 174.08 ppm and 171.60 ppm), one guanidino carbon (158.17 ppm) and one at 99.68 ppm. A signal at δ (^{13}C) 99.68 corresponds to the hemiacetal carbon atom C-17. This assignment was confirmed by cross peaks to H-16 and H-18. The formation of a 36-membered lactone ring in dihydroniphimycin is proved unambiguously by a strong cross peak between C-1 and H-35. The carbonyl group at 176.80 ppm (C-1) show also cross peak to H-2. Two strong cross peaks from the methylene protons of H-56 to the

Fig. 2. HMBC contacts found in dihydroniphimycin.



Arrows point from proton to the corresponding carbon atom. Double arrows indicate contacts in both directions.

carbonyl groups at 171.60 ppm (C-55) and 174.08 ppm (C-57), established the presence of a malonyl residue in the structure of dihydroniphimycin. The position of the esterification is defined clearly by a cross peak between H-23 and the carbonyl group at 171.60 ppm (C-55). Cross peak between H-46 (3.16 ppm) and C-58 (158.17 ppm) allowed to identify the guanidino moiety. In the HMBC spectrum the methyl protons at 1.18 ppm (H-47) shows couplings to C-2 and C-3; the methyl protons at 0.91 ppm (H-48) to C-5 and C-6; methyl protons at 0.92 ppm (H-49) to C-9, C-10 and C-11; methyl protons at 0.875 ppm (H-50) to C-13, C-14 and C-15; methyl protons at 0.880 ppm (H-51) to C-27, C-28 and C-29; methyl protons at 1.02 ppm (H-52) to C-33, C-34 and C-35; methyl protons at 0.905 ppm (H-53) to C-35, C-36, C-37 and the methyl protons at 0.876 ppm (H-54) to C-37. By analysis of the HMBC spectrum, it was possible to determine connectivities in almost all areas of the molecule (Fig. 2). The chemical shifts of all proton and carbon atoms and the long-range coupling are summarized in Table 1.

The configuration of the diene system at C-30 and C-32 was determined as 30E, 32E from the large *trans* coupling constans of H-30 (J=7.3, 15.10 Hz), H-31 (J=10.40, 15.03 Hz), H-32 (J=10.41, 15.01 Hz) and H-33 (J=9.0, 15.0 Hz).

The dihydroniphimycin is a natural product, directly isolated from the mycelium of the strain *S. hygroscopicus* 15. The structure of the new macrolide antibiotic ($C_{59}H_{105}N_3O_{18}$, MW. 1143) has been determined by 1D and 2D NMR and FAB mass analyses. The antibiotic is related to polyol macrolide antibiotics such as kanchanamycin C[4], copiamycin[5], malolactomycins[6], shurimycins[7], azalomycins[8], niphimycin A[9~11], amycins[12,13], guanidylfungins[14], scopafungin[15], antibiotics RS-22 A, B and C[16,17], malonylniphimycin[18] and other.

Dihydroniphimycin is a new antibiotic having a 36-membered ring, a malonate and a monomethylguanidino group. The position of the one malonyl residue is at C-23. The antibiotic is the most similar to niphimycin A[18]. The molecular weight of niphimycin A, 1141 ($C_{59}H_{103}N_3O_{18}$), differs with two mass units from the molecular weight of dihydroniphimycin. The structure difference between niphimycin A and dihydroniphimycin is the absence of one

Table 1. ^{1}H and ^{13}C chemical shifts of dihydroniphimycin in MeOH-d_4 (400 MHz and 100.62 MHz).

Position	^{1}H (ppm)	HMBC H → C	^{13}C (ppm)	Groups
1			176.80 s	C
2	2.41 (1H, m)	C-1, C-3, C-47	46.89 d	CH
3	3.72 (1H, m)		72.96 d	CH-O
4	1.50 (2H, m)		37.40 t	CH_2
5	1.31/1.50 (2H, m)		29.38 t	CH_2
6	1.55 (1H, m)		39.91 d	CH
7	3.65 (1H, m)		77.27 d	CH-O
8	1.50/1.80 (2H, m)		37.37 t	CH_2
9	3.82 (1H, m)		76.34 d	CH-O
10	1.54 (1H, m)		44.48 d	CH
11	3.85 (1H, m)		72.28 d	CH-O
12	1.39/1.61 (2H, m)		33.96 t	CH_2
13	1.07/1.26 (2H, m)		37.72 t	CH_2
14	1.62 (1H, m)		30.59 d	CH
15	3.84 (1H, m)	C-17, C-50	65.53 d	CH-O
16	1.66/1.80 (2H, m)	C-15, C-17	41.90 t	CH_2
17			99.68 s	C
18	3.35 (1H, dd)	C-17, C-19	77.07 d	CH-O
19	3.87 (1H, m)		69.65 d	CH-O
20	1.30/1.90 (2H, m)	C-18, C-19	41.22 t	CH_2
21	4.07 (1H, m)		65.40 d	CH-O
22	1.66/1.80 (2H, m)	C-21, C-23	41.48 t	CH_2
23	5.25 (1H, m)	C-55	70.66 d	CH-O
24	1.66/1.80 (2H, m)	C-25	41.48 t	CH_2
25	3.85 (1H, m)		72.28 d	CH-O
26	1.50/1.64 (2H, m)	C-25, C-27	43.66 t	CH_2
27	4.16 (1H, m)		68.86 d	CH-O
28	1.57 (1H, m)		45.24 d	CH
29	4.03 (1H, m)	C-27, C-28, C-30, C-31, C-51	75.90 d	CH-O
30	5.63 (1H, dd)	C-29, C-32	135.36 d	C=
31	6.18 (1H, dd)	C-29, C-33	132.15 d	C=
32	6.05 (1H, dd)	C-30, C-34	131.90 d	C=
33	5.51 (1H, dd)	C-31, C-34, C-35, C-52	136.97 d	C=
34	2.52 (1H, m)	C-32, C-33, C-35, C-52	40.58 d	CH
35	4.74 (1H, dd)	C-1, C-34, C-53	79.44 d	CH-O
36	1.92 (1H, m)		32.53 d	CH
37	0.89/1.30 (2H, m)	C-35	42.52 t	CH_2
38	1.62 (1H, m)		40.85 d	CH
39	1.33/1.62 (2H, m)		40.85 t	CH_2
40	1.33/1.98 (2H, m)	C-41	27.80 t	CH_2

Table 1. Continued

Position	^{1}H (ppm)	HMBC H → C	^{13}C (ppm)	Groups
41	1.96 (2H, m)	C-40, C-42, C-43	33.78 t	CH_2
42	5.48 (1H, dt)	C-44	132.72 d	C=
43	5.44 (1H, dt)	C-41	129.90 d	C=
44	2.06 (2H, m)	C-42, C-43, C-45, C-46	30.65 t	CH_2
45	1.64 (2H, m)	C-43, C-44, C-46	29.90 t	CH_2
46	3.16 (2H, m)	C-44, C-58	42.00 t	CH_2
47	1.180 (3H, dd)	C-1, C-2, C-3	13.12 q	CH_3
48	0.910 (3H, dd)	C-5, C-6	15.51 q	CH_3
49	0.920 (3H, dd)	C-9, C-10, C-11	10.33 q	CH_3
50	0.875 (3H, dd)	C-13, C-14, C-15	11.05 q	CH_3
51	0.880 (3H, dd)	C-27, C-28, C-29	20.41 q	CH_3
52	1.020 (3H, dd)	C-33, C-34, C-35	17.84 q	CH_3
53	0.905 (3H, dd)	C-35, C-36, C-37	14.87 q	CH_3
54	0.876 (3H, dd)	C-37	14.55 q	CH_3
55			171.60 s	C
56	3.25 (2H, m)	C-55, C-57	46.00 t	CH_2
57			174.08 s	C
58			158.17 s	C
59	2.83 (3H, s)	C-58	28.34 q	N-CH_3
	7.51 (1H, m)			NH

double bond at C-4 and C-5 in the unsaturated lactone moiety of dihydroniphimycin.

The stereochemistries of the double bonds of niphimycin were established on the basis of coupling constants. In the ^{1}H-NMR spectrum of niphimycin, the configuration was determined from the large *trans* coupling constants of H-30 (J=7.4, 15.1 Hz), H-31 (J=10.4, 15.1 Hz), H-32 (J=10.4, 15.0 Hz), H-33 (J=9.0, 15.3 Hz), H-4 (J=7.0, 14.8 Hz) and H-5 (J=8.7, 15.4 Hz) as 30E, 32E and 4E. Though the signals of H-42 and H-43 by dihydroniphimycin and niphimycin are poorly resolved in the normal spectrum because of the overlapping of those signals with H-33. The stereochemistry of C-42=C-43 double bond could not be established.

Dihydroniphimycin showed broad antimicrobial activity against various fungi, yeasts and Gram-positive bacteria, but higher activity than niphimycin A.

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