Malonylniphimycin: Macrolide Antibiotic from *Streptomyces hygroscopicus* B-7: Physico-chemical Properties and Structure Elucidation

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In the course of our screening program for active substances of microbial origin, an antifungal antibiotic complex was isolated from the culture broth of a strain of *Streptomyces hygroscopicus* B-7 and designated AK-B7. It is a complex of non-polyenic antifungal antibiotics, such as scopafungin[1], niphimycin[2~4], copiamycin[5], neocopiamycin[6], guanidylfungins A and B[7], azalomycin F_{3a}, F_{4a}, F_{5a}[8,9], amycin A[10] and others. In the previous paper[11], taxonomy of the producing organism *S. hygroscopicus* B-7 and biological activity of the antibiotic complex were presented.

In this report we describe the production, isolation, physico-chemical properties and the structure elucidation of the non-polyenic antibiotic AK-B7-3 (malonylniphimycin) (Fig. 1), through the use of two-dimensional NMR techniques.

The mature slant culture of *S. hygroscopicus* B-7 isolated from a soil sample collected in Bulgaria was inoculated into Erlenmeyer flasks, containing 150 ml of seed medium consisting of glucose 1.5%, soybean meal 1.5%, maize extract 0.5%, NaCl 0.5%, and $CaCO_3$ 0.2%, pH 6.8 before autoclaving. The flasks were cultivated on a rotary shaker at 325 rpm for 48 hours. The seed culture was inoculated into a 10-liter fermentor containing 6.0 liters of the production medium consisting of glucose 1.0%, soybean meal 1.0%, NaCl 0.5% and $CaCO_3$ 0.1%, pH 7.0 before autoclaving.

After 120 hours of cultivation at 30°C, the culture broth (4.0 liters) was centrifuged. The mycelium was extracted three times with MeOH, while the filtrate with *n*-BuOH. The active solvent extracts were combined and evaporated to dryness *in vacuo*. The crude products were dissolved in a small amount of MeOH, combined, filtered and precipitated with Me_2CO-ether, 10:1 (v/v). An amount of 2.60 g crude powder was obtained after dryness *in vacuo*. A methanolic solution of 0.70 g of the powder was chromatographed on a silica gel 60 (70~325 mesh) column equilibrated with chloroform. The malonylniphimycin was eluted from the column by isocratic mode with a solvent mixture consisting of $CHCl_3$-MeOH-H_2O 175:125:50 (v/v/v), lower phase. The active eluates were combined and evaporated to dryness *in vacuo*. The residue was dissolved in MeOH and Me_2CO-ether 10:1 (v/v) was added to precipitate malonylniphimycin, 96 mg after filtration and dryness *in vacuo*. The complete separation and purification of the antibiotic 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, Rt = 5.49. The active fractions were concentrated and desalted on a Sephadex LH-20 column (eluant-MeOH). A final purification of the malonylniphimycin 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,

Fig. 1. Structure of malonylniphimycin.

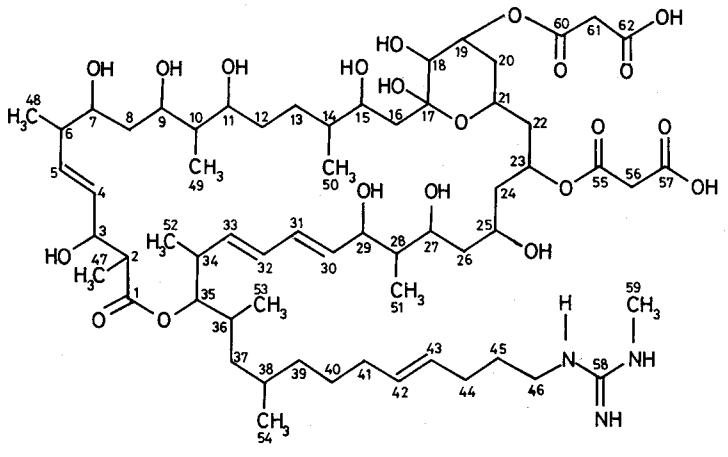


Table 1. Comparison of the ^{1}H-NMR data between malonylniphimycin (**1**) and niphimycin (**2**) in MeOH-d_4 (400 MHz).

Proton	^{1}H (ppm)		Proton	^{1}H (ppm)	
	1	**2**		**1**	**2**
H-2	2.51 (1H, m)	2.48 (1H, m)	H-31	6.22 (1H, dd, J=10.2, 15 Hz)	6.22 (1H, dd)
H-3	4.17 (1H, m)	4.14 (1H, m)	H-32	6.05 (1H, dd, J=10.7, 15 Hz)	6.10 (1H, dd)
H-4	5.46 (1H, dd, J=8.8, 14.9 Hz)	5.46 (1H, dd)	H-33	5.54 (1H, dd, J=8.8, 15.1 Hz)	5.56 (1H, dd)
H-5	5.77 (1H, dd, J=10.4, 15.0 Hz)	5.74 (1H, dd)	H-34	2.53 (1H, m)	2.58 (1H, m)
H-6	2.34 (1H, m)	2.36 (1H, m)	H-35	4.81 (1H, dd, J=4.1, 8.2 Hz)	4.79 (1H, dd)
H-7	3.78 (1H, m)	3.79 (1H, m)	H-36	1.92 (1H, m)	1.93 (1H, m)
H-8	1.56/1.77 (2H, m)	1.55/1.76 (2H, m)	H-37	0.97/1.39 (2H, m)	0.97/1.41 (2H, m)
H-9	3.83 (1H, m)	3.79 (1H, m)	H-38	1.67 (1H, m)	1.64 (1H, m)
H-10	1.60 (1H, m)	1.55 (1H, m)	H-39	1.38/1.67 (2H, m)	1.38/1.64 (2H, m)
H-11	3.95 (1H, m)	3.93 (1H, m)	H-40	1.38/2.01 (2H, m)	1.38/2.02 (2H, m)
H-12	1.40/1.69 (2H, m)	1.40/1.66 (2H, m)	H-41	2.00 (2H, m)	2.01 (2H, m)
H-13	1.10/1.34 (2H, m)	1.12/1.35 (2H, m)	H-42	5.49 (1H, dt)	5.52 (1H, dt)
H-14	1.62 (1H, m)	1.62 (1H, m)	H-43	5.43 (1H, dt)	5.45 (1H, dt)
H-15	3.94 (1H, m)	3.91 (1H, m)	H-44	2.07 (2H, m)	2.11 (2H, m)
H-16	1.73/1.90 (2H, m)	1.73/1.90 (2H, m)	H-45	1.64 (2H, m)	1.69 (2H, m)
H-18	3.70 (1H, dd, J=9.1 Hz)	3.38 (1H, dd)	H-46	3.12 (2H, m)	3.20 (2H, m)
H-19	5.27 (1H, m)	3.91 (1H, m)	H-47	1.07 (dd, J=6.80 Hz)	1.08 dd
H-20	1.41/2.02 (2H, m)	1.36/1.96 (2H, m)	H-48	1.12 (dd ,J=6.87 Hz)	1.12 dd
H-21	4.16 (1H, m)	4.14 (1H, m)	H-49	0.96 (dd, J=6.80 Hz)	0.94 dd
H-22	1.72/1.77 (2H, m)	1.74/1.82 (2H, m)	H-50	0.90 (dd, J=6.82 Hz)	0.92 dd
H-23	5.32 (1H, m)	5.24 (1H, m)	H-51	0.89 (dd, J=6.82 Hz)	0.90 dd
H-24	1.72/1.77 (2H, m)	1.74/1.82 (2H, m)	H-52	1.01 (dd, J=6.85 Hz)	1.06 dd
H-25	3.95 (1H, m)	3.93 (1H, m)	H-53	0.97 (dd, J=6.80 Hz)	0.96 dd
H-26	1.46/1.67 (2H, m)	1.46/1.65 (2H, m)	H-54	0.99 (dd, J=6.80 Hz)	0.98 dd
H-27	4.14 (1H, m)	4.16 (1H, m)	H-56	3.30 (2H, m)	3.30 (2H, m)
H-28	1.57 (1H, m)	1.58 (1H, m)	H-59	2.80 (3H, s)	2.83 (3H, s)
H-29	4.11 (1H, m)	4.11 (1H, m)	H-61	2.75 (2H, m)	—
H-30	5.66 (1H, dd, J=10.7, 15.0 Hz)	5.69 (1H, dd)			

Table 2. Comparison of the ^{13}C-NMR data between malonylniphimycin (**1**) and niphimycin (**2**) in MeOH-d_4 (100.62 MHz).

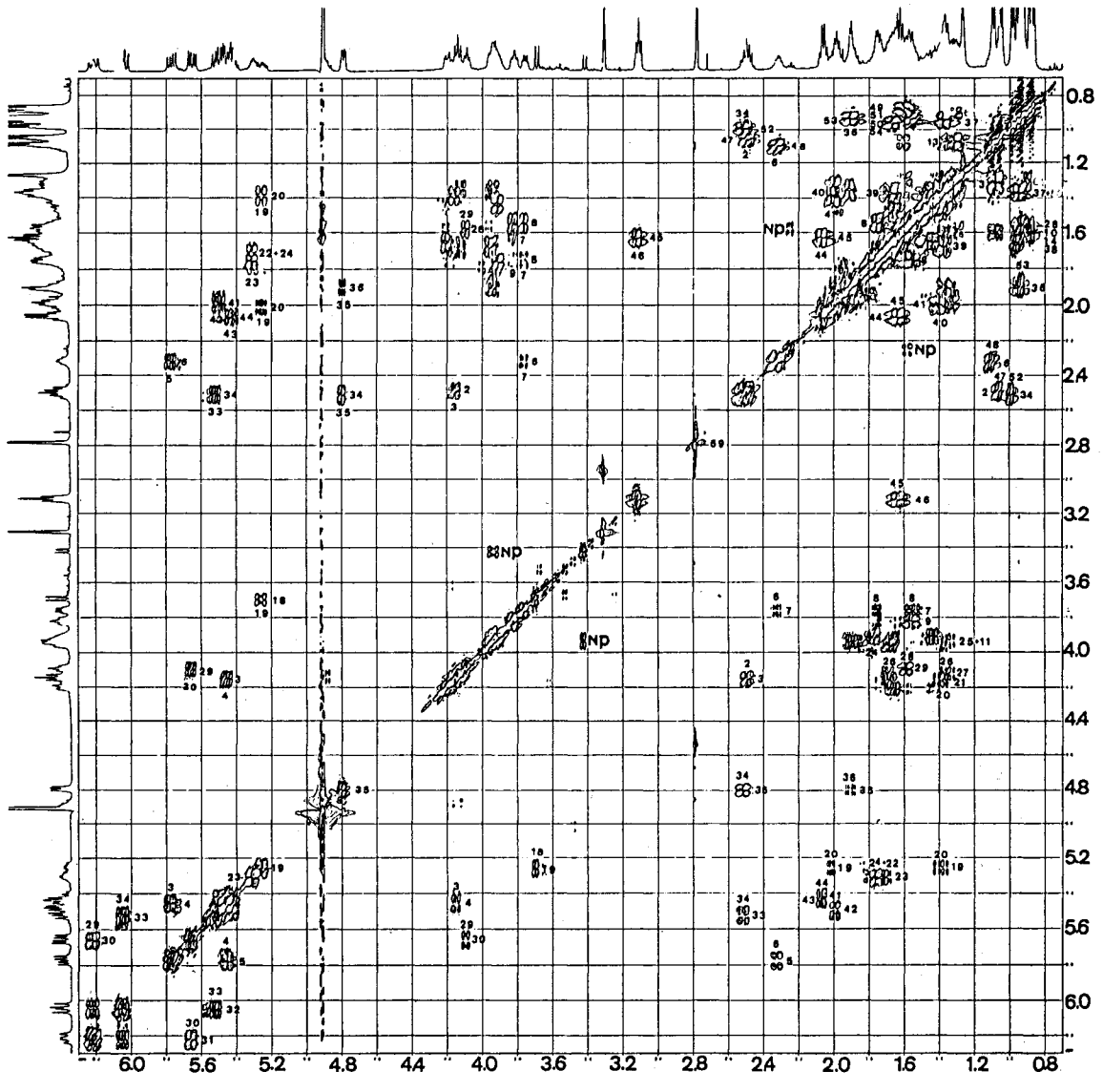
Carbon	^{13}C (ppm)		Carbon	^{13}C (ppm)	
	1	**2**		**1**	**2**
C-1	176.80 s	176.60 s	C-32	131.90 d	131.80 d
C-2	47.96 d	48.00 d	C-33	137.10 d	136.80 d
C-3	65.50 d	65.80 d	C-34	40.76 d	40.70 d
C-4	132.50 d	132.30 d	C-35	79.78 d	79.70 d
C-5	136.50 d	136.40 d	C-36	32.53 d	32.60 d
C-6	43.39 d	43.30 d	C-37	42.52 t	42.30 t
C-7	76.01 d	75.70 d	C-38	40.70 d	40.70 d
C-8	39.31 t	39.10 t	C-39	40.70 t	40.70 t
C-9	75.14 d	75.20 d	C-40	27.81 t	27.70 t
C-10	45.34 d	44.20 d	C-41	33.89 t	33.80 t
C-11	72.37 d	72.20 d	C-42	133.00 d	129.80 d
C-12	33.46 t	33.40 t	C-43	129.90 d	132.90 d
C-13	37.56 t	37.50 t	C-44	30.67 t	30.60 t
C-14	30.60 d	30.60 d	C-45	29.90 t	29.90 t
C-15	65.81 d	65.60 d	C-46	42.03 t	42.00 t
C-16	42.03 t	41.90 t	C-47	15.00 q	15.00 q
C-17	100.00 s	99.80 s	C-48	16.95 q	16.80 q
C-18	74.23 d	77.40 d	C-49	10.58 q	10.60 q
C-19-O-Mal.	74.23 d	69.70 d	C-50	11.30 q	11.30 q
C-20	38.20 t	41.20 t	C-51	20.40 q	20.40 q
C-21	76.00 d	75.90 d	C-52	17.88 q	17.88 q
C-22	41.66 t	41.70 t	C-53	14.99 q	14.90 q
C-23-O-Mal.	71.57 d	71.00 d	C-54	15.10 q	15.10 q
C-24	41.66 t	41.70 t	C-55	170.00 s	171.40 s
C-25	72.38 d	72.30 d	C-56	49.85 t	49.60 t
C-26	43.16 t	43.20 t	C-57	172.20 s	174.20 s
C-27	69.30 d	69.40 d	C-58	158.30 s	158.20 s
C-28	44.46 d	45.00 d	C-59	28.34 q	28.30 q
C-29	75.60 d	75.90 d	C-60	169.50 s	
C-30	135.20 d	135.20 d	C-61	44.50 t	
C-31	132.00 d	131.90 d	C-62	171.80 s	

Rt = 4.60. The active fractions were desalted on a LH-20 by elution with MeOH. After concentration and dryness *in vacuo*, 25 mg of pure malonylniphimycin was obtained.

The antibiotic gave a single spot on TLC (silica gel; $CHCl_3$ - MeOH - H_2O 2:2:1 (v/v/v), lower phase, Rf = 0.27; $CHCl_3$ - MeOH - AcOH 15:5:1 (v/v/v), Rf = 0.29). Malonylniphimycin was isolated as a colorless powder with melting point of 114~116°C. It is optically active with $[\alpha]_D^{25}$ value of +36.5° (c = 0.5, MeOH). It is soluble in lower alcohols, dimethylsulfoxide, *N*,*N*-dimethylformamide but insoluble in ethyl acetate, chloroform and water. It gave positive reactions to potassium permanganate, 3% vanillin-sulphuric acid solution, Dragendorf and Sakaguchi test. The UV spectrum in MeOH, showed absorption maximum at 232 nm (ε 2200). The IR spectrum (KBr) indicated the following absorption bands: 3401, 2962, 2932, 2876, 1727, 1644, 1602, 1461, 1381, 1292, 1142, 1049, 987, 915, 687, 588 cm^{-1}. According to the data of FAB-MS, malonylniphimycin possesses a protonated molecular ion peak at m/z 1228.9 $(M+H)^+$ and empiric formula $C_{62}H_{105}N_3O_{21}$ (MW 1227.9). A series of fragment ions are observed at m/z 1210 $(MH-H_2O)^+$; 1184 $(MH-CO_2)^+$; 1142 $(MH-OOCCH_2CO)^+$; 1124 $(1142-H_2O)^+$; 1056 $(1142-OOCCH_2CO)^+$; 1038 $(1056-H_2O)^+$; 1080 $(1124-CO_2)^+$. The protonated molecular ion peak of the niphimycin at m/z 1142.9 $(M+H)^+$, molecular weight of niphimycin was reported to be 1141.9, which differs in 86 mass units from malonylniphimycin. This difference might be explained by the presence of another malonyl residue in malonylniphimycin.

The ^{13}C-NMR (100.62 MHz) and JMOD spectra showed the following structural elements: in the range

Fig. 2. ^1H-^1H COSY spectrum of malonylniphimycin in MeOH-d_4.

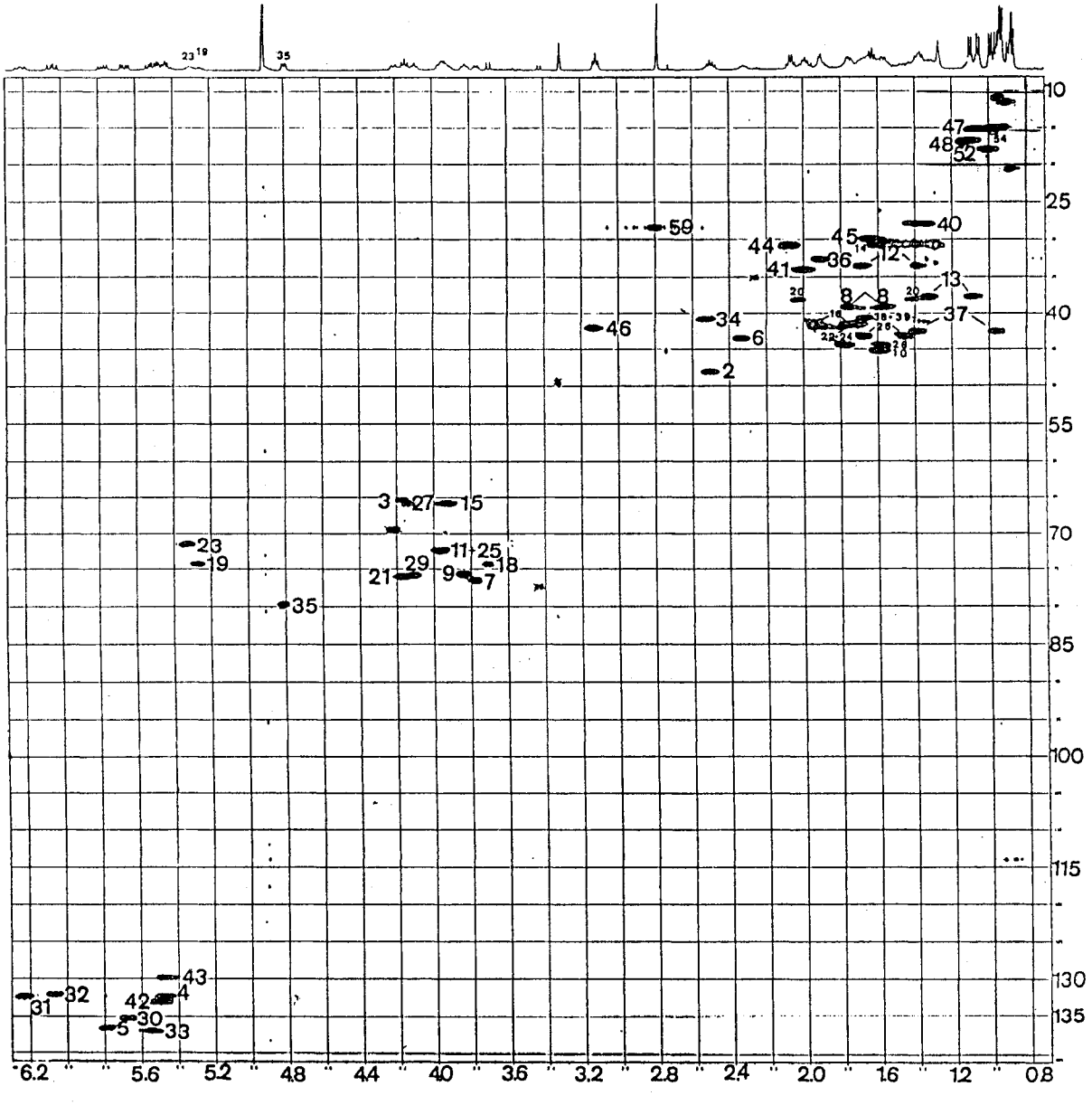


of 10.60 to 20.50 ppm, the peaks of eight methyl residues are indicated. The signal at 28.34 ppm assigned to a N–CH_3 residue. The presence of 8 methine and 17 methylene residues is indicated in the range from 27 to 50 ppm, while the presence of 13 CH–O residues was shown at 65 to 80 ppm.

The signal at 100.00 ppm showed the presence of 6-membered hemiketal ring. In the 129 to 138 ppm range 8 signals of olefinic carbon atoms are observed. The singlet at 158.30 ppm is ascribed to the guanidine–C–atom, while the singlet at 176.80 ppm indicated the presence of a lactone. Malonylniphimycin exhibits two sets of the signals due to the malonyl residues at 170.00 and 169.50 ppm (COOR), and 172.20 and 171.80 ppm (COOH).

All ^{13}C and ^{1}H signals of malonylniphimycin and niphimycin were completely assigned by further NMR studies including 2D experiments of ^{1}H-^{1}H and ^{1}H-^{13}C COSY difference spectra (Tables 1 and 2, Fig. 2 and Fig. 3). The positions of two malonyl residues of malonylniphimycin were determined to C-23 and C-19 of which protons appeared at 5.32 and 5.27 ppm. The ^{1}H-NMR (400 MHz) spectrum also showed multiplets at 5.32, 5.27 and 3.95, 3.70 which accounted for the methine protons attached to the two malonyl residues at (C-23; C-19), and two hydroxyl residues at C-25 and C-18, respectively. A signal at δ 3.95 (25-H) showed cross peaks with the multiplet at δ 1.46/1.67 (26-H) and 1.72/1.77 (24-H, 22-H), while a signal at δ 5.32 (23-H) was only correlated to the signals at δ 1.72/1.77. The 18-H proton appeared

Fig. 3. ^{1}H-^{13}C COSY spectrum of malonylniphimycin in MeOH-d_4.



as a doublet at δ 3.70 due to the hemiketal moiety at C-17, a signal at δ 5.27 (19-H) was corrlelated to the signals at δ 1.41/2.02 (20-H) and 3.70 (18-H) in Fig. 2. These observations confirmed the correct positions of the malonyl residues at C-23 and C-19. The assignments of the six membered hemiacetal were conducted by comparison with the NMR data of guanidylfungins A and B[7], copiamycin[5], azalomycins[8,9] and niphimycin[2~4].

Malonylniphimycin possesses biological activity against Gram-positive bacteria, fungi and yeasts, but lower activity than niphimycin A.

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