Malonyl-4,5-dihydroniphimycin: A New Polyol Macrolide Antibiotic, Produced by *Streptomyces hygroscopicus*

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Z. Naturforsch. **2007**, *62b,* 1187 – 1192; received January 22, 2007

Malonyl-4,5-dihydroniphimycin was isolated as a new antibiotic from the mycelium of *Streptomyces hygroscopicus* **15**. The chemical constitution was elucidated from the physico-chemical properties, using NMR techniques and mass spectrometry, to be a 36-membered macrolide related to non-polyenic antibiotics. Malonyl-4,5-dihydroniphimycin displays activity against filamentous fungi and Gram-positive bacteria.

Key words: Streptomyces hygroscopicus 15, Malonyl-4,5-dihydroniphimycin, Antifungal and Antibacterial Activity

Introduction

During the course of our screening programme for novel antifungal agents of microbial origin, we found that the *Streptomyces hygroscopicus* 15 produced a new antifungal congener of the niphimycin group.

Non-polyene antibiotics described in the literature, such as azalomycins F [1, 2], scopafungin [3], niphimycin [4 – 6], copiamycin [7], guanidilfungins A and B [8], kanchanamycin C [9], antibiotic RS-22 A, B and C [10], malolactomycins [11], amycins A and B [12], malonylniphimycin [13], dihydroniphimycin [14] and others have similar chemical structures and antimicrobial spectra. Their structures show a macrocyclic polyhydroxyl lactone and a 6-membered intermolecular hemiacetal ring. They are malonylesters with a side chain bearing a terminal guanidine.

Here we describe the isolation, physico-chemical properties, structure elucidation and antimicrobial activity of a novel antifungal antibiotic, malonyl-4,5-dihydroniphimycin (**1**) (Fig. 1), produced by *Streptomyces hygroscopicus* 15.

Materials and Methods

Organism and growth conditions

The producing strain *Streptomyces 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 procedures for growth conditions have been described in previous reports [14, 15].

General methods

UV spectra in methanol were scanned on a Perkin-Elmer (Lambda 9) UV-spectrometer; IR spectra were measured on a Bruker (IFS-66) FT-IR spectrometer in KBr tablets. FAB-MS (3-nitrobenzyl alcohol as matrix) was carried out on a Varian Mat 311-A mass spectrometer. ^{1}H and ^{13}C NMR spectra were recorded in [D_4]MeOH on a Bruker AMX-400 NMR spectrometer at 400 MHz (^{1}H) and 100.62 MHz for (^{13}C). ^{13}C multiplicity data were obtained from JMOD experiments. Chemical shifts were expressed in ppm with TMS as an internal standard. The 2D NMR spectra were obtained by conventional methods. The optical rotation was measured at 25 °C using a Perkin-Elmer 141 polarimeter with a 1-dm cell.

Extraction and separation

After 140 h of cultivation at 30 °C, the culture broth (5.0 L of a glucose/soybean meal/NaCl/$CaCO_3$ medium) of *Streptomyces hygroscopicus* 15 was centrifuged. The supernatant fluid was discarded and the mycelium was extracted three times with 2.5 L of ethanol. The solvent extracts were concentrated to 400 mL and the aqueous solution was extracted with *n*-BuOH (2 : 1, v/v). The organic layer was concen-

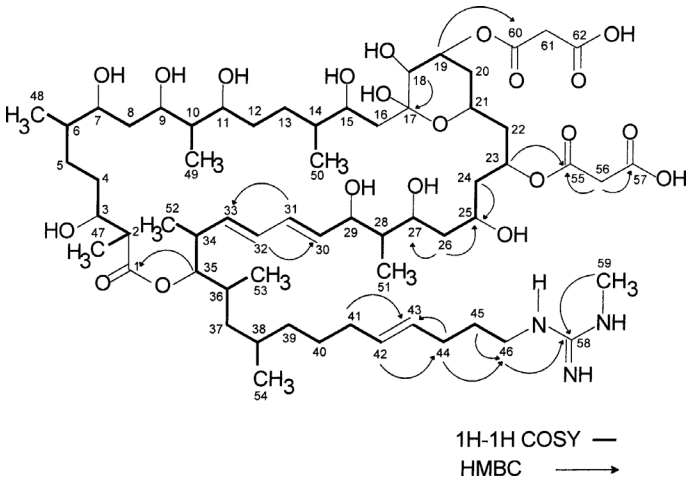

Fig. 1. Structure of malonyl-4,5-dihydroniphimycin (**1**).

trated *in vacuo* to give a crude oil. The crude product was dissolved in a small amount of MeOH, filtered and precipitated with Me_2CO/ether (10 : 1, v/v). An amount of 3.0 g crude product was obtained after evaporation of the solvent *in vacuo*. A methanolic solution of the powder (1.5 g) was chromatographed on a Silica gel 60 (70 – 325 mesh) column equilibrated with chloroform. Compound **1** was eluted from the column in the isocratic mode with a solvent mixture consisting of $CHCl_3$/MeOH/H_2O (175 : 125 : 50, v/v), as the lower phase. The complete separation and purification of **1** could be achieved only by preparative HPLC on a (250 × 5 mm) Lichroprep RP 18 column, using a gradient of 40 – 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 **1** 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, R_t = 2.09 min. The fractions were desalted on a LH-20 column by elution with MeOH. After evaporation of the solvents *in vacuo* 30 mg of pure malonyl-4,5-dihydroniphimycin (**1**) was obtained.

Analytical HPLC

Compound **1** was analysed by HPLC using a HP 85 apparatus with a HP 1040 A UV detector. Column: Lichrospher RP 18 (125 × 4 mm). Mobile phase: 0.01 M sodium phosphate buffer (pH = 4.0)/acetonitrile (65 : 35, v/v). Flow rate 1.20 mL/min; detection 220 nm; R_t = 2.55.

Thin-layer chromatography of compound **1** was carried out on Silica gel plates (Merck 60, F_{254}) with the following mobile phases: chloroform/methanol/water (2 : 2 : 1), lower phase, R_f = 0.25; chloroform/methanol/acetic acid (15 : 5 : 1), R_f = 0.27. The chromatographic spot was visualised by spraying with 3 % vanillin/sulphuric acid solution, Draggendorff's and Sakaguchi test and heating at 110 °C for 3 – 5 min.

Antimicrobial activity was determined by the agar diffusion test according to European Pharmacopoia [16].

*Malonyl-4,5-dihydroniphimycin (**1**).* Colourless amorphous solid. – $[\alpha]_D^{25} = +38$ (c = 0.5, MeOH). – IR (KBr): ν = 3401, 2962, 2932, 1727, 1602, 1461, 1381, 1292, 1142, 1049, 987, 915, 687, 588 cm^{-1}. – UV (MeOH): $\lambda_{max}(\varepsilon)$ = 226 sh, 232 (22000), 241 sh nm. – ^1H, ^{13}C NMR, HMBC: see Table 1. – ^1H-^1H COSY correlations: see Fig. 1. – MS ((+)-FAB): *m/z* = 1230.0 $[M+H]^+$. – HRMS ((+)-FAB): *m/z* = 1230.4900 (calcd. 1230.4860 for $C_{62}H_{108}N_3O_{21}$, $[M+H]^+$).

Results and Discussion

The mycelium of the fermentation broth of *Streptomyces hygroscopicus* 15 was extracted with ethanol and the antifungal extract was purified by silica gel-chromatography ($CHCl_3$/MeOH/H_2O,175 : 125 : 50 as the lower phase) followed by reversed-phase HPLC (sodium phosphate buffer (pH = 4.0)/acetonitrile = 53 : 47) to give malonyl-4,5-dihydroniphimycin (**1**).

Compound **1** is soluble in lower alcohols, pyridine, dimethyl sulfoxide and *N*,*N*-dimethylformamide,

Table 1. ^{1}H and ^{13}C NMR data of malonyl-4,5-dihydroniphimycin (**1**)[a].

[a] In [D_4]MeOH, 400 MHz and 100.62 MHz; δ in ppm, J in Hz.

Position	δ (^{1}H)	HMBC J	HMBC H → C	δ (^{13}C)	Groups
1				176.80 s	C_q
2	2.31 (1H, m)		C-1, C-3, C-47	47.96 d	CH
3	3.63 (1H, m)			73.20 d	HC-O-
4	1.47 (2H, m)			37.56 t	CH_2
5	1.25/1.44 (2H, m)			29.47 t	CH_2
6	1.50 (1H, m)			40.00 d	CH
7	3.55 (1H, m)			77.30 d	HC-O-
8	1.42/1.75 (2H, m)			37.45 t	CH_2
9	3.73 (1H, m)			76.38 d	HC-O-
10	1.49 (1H, m)			44.80 d	CH
11	3.80 (1H, m)			72.30 d	HC-O-
12	1.36/1.59 (2H, m)			33.99 t	CH_2
13	0.99/1.21 (2H, m)			37.77 t	CH_2
14	1.56 (1H, m)			30.50 d	CH
15	3.79 (1H, m)		C-17, C-50	65.78 d	HC-O-
16	1.60/1.75 (2H, m)		C-15, C-17	41.92 t	CH_2
17				99.97 s	C_q
18	3.53 (1H, dd)	8.9	C-17, C-19	74.20 d	HC-O-
19-Mal	5.10 (1H, m)		C-60	74.20 d	HC-O-
20	1.21/1.81 (2H, m)		C-18, C-19	38.25 t	CH_2
21	4.15 (1H, m)			71.02 d	HC-O-
22	1.60/1.72 (2H, m)		C-21, C-23	41.66 t	CH_2
23-Mal	5.16 (1H, m)		C-55	71.00 d	HC-O-
24	1.60/1.72 (2H, m)		C-25	41.66 t	CH_2
25	3.77 (1H, m)			72.38 d	HC-O-
26	1.45/1.60 (2H, m)		C-25, C-27	43.46 t	CH_2
27	4.02 (1H, m)			69.05 d	HC-O-
28	1.53 (1H, m)			45.15 d	CH
29	3.94 (1H, m)		C-27, C-28, C-30, C-51	75.60 d	HC-O-
30	5.55 (1H, dd)	10.6, 15.0	C-29, C-32	135.20 d	HC=
31	6.07 (1H, dd)	10.2, 15.0	C-29, C-33	132.00 d	HC=
32	5.99 (1H, dd)	10.6, 15.0	C-30, C-34	131.90 d	HC=
33	5.45 (1H, dd)	8.9, 15.1	C-31, C-35, C-52	137.15 d	HC=
34	2.47 (1H, m)		C-32, C-33, C-35, C-52	40.76 d	CH
35	4.65 (1H, dd)	4.0, 8.1	C-1, C-34, C-53	79.78 d	HC-O-
36	1.83 (1H, m)			32.53 d	CH
37	0.82/1.26 (2H, m)		C-35	42.52 t	CH_2
38	1.56 1H, m)			40.70 d	CH
39	1.29/1.57 (2H, m)			40,70 t	CH_2
40	1.29/1.92 (2H, m)		C-41	27.81 t	CH_2
41	1.92 (2H, m)		C-40, C-42, C-43	33.80 t	CH_2
42	5.37 (1H, dt)		C-44	132.10 d	HC=
43	5.34 (1H, dt)		C-41	129.90 d	HC=
44	1.99 (2H, m)		C-42, C-43, C-45, C-46	30.67 t	CH_2
45	1.60 (2H, m)		C-43, C-44, C-46	29.90 t	CH_2
46	3.09 (2H, t)		C-44, C-58	42.03 t	CH_2
47	1.120 (3H, dd)	6.8	C-1, C-2, C-3	13.15 q	CH_3
48	0.890 (3H, dd)	6.8	C-5, C-6	15.51 q	CH_3
49	0.900 (3H, dd)	6.8	C-9, C-10, C-11	10.31 q	CH_3
50	0.865 (3H, dd)	6.8	C-13, C-14, C-15	11.10 q	CH_3
51	0.870 (3H, dd)	6.8	C-27, C-28, C-29	20.41 q	CH_3
52	1.000 (3H, dd)	6.8	C-33, C-34, C-35	17.84 q	CH_3
53	0.885 (3H, dd)	6.8	C-35, C-36, C-37	14.89 q	CH_3
54	0.867 (3H, dd)	6.8	C-37	14.55 q	CH_3
55				170.62 s	C_q
56	3.23 (2H, m)		C-55, C-57	46.00 t	CH_2
57				172.20 s	C_q
58				158.30 s	C_q
59	2.80 (3H, s)		C-58	28.34 q	-N-CH_3
60				169.50 s	C_q
61	2.74 (2H, m)			44.48 t	CH_2
62				171.80 s	C_q

Table 2. Comparison of the ^{1}H and ^{13}C NMR data of malonyl-4,5-dihydroniphimycin (**1**), malonylniphimycin (**2**) and dihydroniphimycin (**3**)[a].

Position	δ (^{1}H)			δ (^{13}C)		
	1	**2**	**3**	**1**	**2**	**3**
3.	3.63 (1H, m)	4.17 (1H, m)	3.72 (1H, m)	73.20 d	65.50 d	72.96 d
4.	1.47 (2H, m)	5.46 (1H, dd)	1.50 (2H, m)	37.56 t	132.50 d	37.40 t
5.	1.25/1.44 (2H, m)	5.77 (1H, dd)	1.31/1.50 (2H, m)	29.47 t	136.50 d	29.38 t
6.	1.50 (1H, m)	2.34 (1H, m)	1.55 (1H, m)	40.00 d	43.39 d	39.91 d
7.	3.55 (1H, m)	3.78 (1H,m)	3.65 (1H,m)	77.30 d	76.01 d	77.17 d
18.	3.53 (1H, dd)	3.70 (1H, dd)	3.53 (1H, dd)	74.20 d	74.23 d	77.07 d
19.	5.10 (1H, m)	5.27 (1H, m)	3.87 (1H, m)	74.20 d	74.23 d	69.65 d
20.	1.21/1.81 (2H, m)	1.41/2.02 (2H, m)	1.30/1.90 (2H, m)	38.25 t	38.20 t	41.22 t
21.	4.15 (1H, m)	4.16 (1H, m)	4.07 (1H, m)	71.02 d	76.00 d	65.40 d
22.	1.60/1.72 (2H, m)	1.72/1.77 (2H, m)	1.66/1.80 (2H, m)	41.66 t	41.66 t	41.48 t
23.	5.16 (1H, m)	5.32 (1H, m)	5.25 (1H, m)	71.00 d	71.57 d	70.66 d
24.	1.60/1.72 (2H, m)	1.72/1.77 (2H, m)	1.66/1.80 (2H, m)	41.66 t	41.66 t	41.48 t
25.	3.77 (1H, m)	3.95 (1H, m)	3.85 (1H, m)	72.38 d	72.38 d	72.28 d
55.				170.62 s	170.00 s	171.60 s
56.	3.23 (2H, m)	3.30 (2H, m)	3.25 (2H, m)	46.00 t	49.85 t	46.00 t
57.				172.20 s	172.20 s	174.08 s
58.				158.30 s	158.30 s	158.17 s
59.	2.80 (3H, s)	2.80 (3H, s)	2.83 (3H, s)	28.34 q	28.34 q	28.34 q
60.				169.50 s	169.50 s	
61.	2.74 (2H, m)	2.75 (2H, m)		44.48 t	44.50 t	
62.				171.80 s	171.80 s	

[a] In [D_4]MeOH, 400 MHz and 100.62 MHz; δ in ppm.

but insoluble in ethyl acetate, chloroform, ether, *n*-hexane, and water. Compound **1** showed UV maxima at 226 (sh), 232, and 241 (sh) nm. The structure of two conjugated double bonds was easily ascertained to be a dienic system by the precise wavelength of the absorption band at 232 nm. Structural assignment of **1** was achieved by interpretation of MS and NMR data, including two-dimensional ^{1}H-^{1}H COSY, ^{1}H-^{13}C COSY, HMBC, and by comparison with the data of dihydroniphimycin [14] and malonylniphimycin [13]. The positive FAB-MS spectrum of **1** indicated an intense peak at *m/z* = 1230.0 $[M+H]^+$. The molecular formula of **1** was established as $C_{62}H_{107}N_3O_{21}$ by HRFAB-MS analysis (*m/z* = 1230.4900 $[M+H]^+$). The chemical formula of **1** suggested the presence of eleven double bond equivalents. A series of fragment ions were observed at *m/z* = 1186 $[MH–CO_2]^+$, 1144 $[MH–OOCCH_2CO]^+$, 1126 $[M–HOOCCH_2COO]^+$, 448, 386, 282, 252, 224. The protonated molecular ion peak of dihydroniphimycin [14] at *m/z* = 1144 $[M+H]^+$ differs in 86 mass units from the protonated molecular ion of **1** at *m/z* = 1230 $[M+H]^+$. This difference might be explained by the presence of another malonyl residue in **1**.

The ^{13}C NMR and JMOD spectra showed in the range of 10.31 to 20.41 ppm the peaks of eight methyl groups. The signal at 28.34 ppm has been assigned to an -HN–CH_3 unit. The presence of 8 methine and 19 methylene groups was indicated in the range from 19 to 50 ppm, and the presence of 13 HC–O- units by resonances in the range from δ = 65.78 to 79.78. The signal at 99.97 ppm showed the presence of the hemiacetal carbon. In the 129.90 to 137.15 ppm range 6 signals of olefinic carbon atoms were observed. The singlet at 158.30 ppm could be ascribed to the guanidino carbon, while the singlet at 176.80 ppm indicated the presence of a lactone carbonyl. Compound **1** exhibits two sets of signals due to the malonyl groups at 170.62 and 169.50 ppm (COOR), and at 172.20 and 171.80 ppm (COOH).

In the ^{1}H-^{1}H COSY spectrum, the resonances of the protons in the dienic system were found at δ = 6.07 (1H, dd, 31-H), 5.99 (1H, dd, 32-H), 5.55 (1H, dd, 30-H) and 5.45 (1H, dd, 33-H). The other two olefinic protons were distinguishable at δ = 5.37 (1H, dt, 42-H) and 5.34 (1H, dt, 43-H). The protons 34-H, 29-H, 44-H and 41-H are directly coupled to the olefinic protons (33-H, 30-H, 43-H and 42-H). The proton 34-H at δ = 2.47 was clearly coupled to the methyl protons at δ = 1.00 (52-H_3), and the methine proton at δ = 4.65, with a lack of other couplings, was assigned to 35-H adjacent to the carboxylic group. The absolute interpretation of ^{1}H-^{1}H COSY spectrum is demonstrated in Fig. 1.

The positions of two malonyl groups of **1** were determined for C-23 and C-19 the protons of which appeared at δ = 5.16 and 5.10. The ^{1}H-^{1}H COSY spectrum also showed multiplets at δ = 5.16, 5.10 and 3.77, 3.53 which accounted for the methine protons attached to these two malonyl groups (C-23, C-19), and two hydroxyl groups at C-25 and C-18, respectively. A signal at δ = 3.77 (25-H) showed cross peaks with the multiplets at δ = 1.45/1.60 (26-H_2) and 1.60/1.72 (24-H_2), while a signal at δ = 5.16 (23-H) was correlated only to the signals at 1.60/1.72 (22-H_2, 24-H_2). The proton 18-H appeared as a doublet at δ = 3.53 due to the hemiacetal moiety at C-17. A signal at δ = 5.10 (19-H) was correlated to the signals at δ = 1.21/1.81 (20-H_2) and 3.53 (18-H). These observations confirmed the correct positions of the malonyl groups at C-23 and C-19. The ^{1}H-^{1}H COSY spectrum of **1** revealed connectivities of C-2∼C-16, C-18∼C-31, C-32∼C-42, and C-43∼C-46. The signals between 3.40 and 4.20 ppm with no corresponding cross peaks in the ^{1}H-^{13}H COSY spectrum were assigned to hydroxyl protons.

The stereochemistry of the double bonds of malonyl-4,5-dihydroniphimycin were established on the basis of the coupling constants in the ^{1}H NMR spectrum. The configuration of the diene system at C-30 and C-32 was determined as 30E, 32E from the large *trans* coupling constants of 30-H (J = 10.6, 15.0 Hz), 31-H (J = 10.2, 15.0 Hz), 32-H (J = 10.6, 15.0 Hz) and 33-H (J = 8.9, 15.1 Hz). Though the signals of 42-H and 43-H of the malonyl-4,5-dihydroniphimycin are poorly resolved in the normal spectrum because of the overlapping of these signals with 33-H. The stereochemistry of the double bond C-42,C-43 could not be established.

In the HMBC spectrum seven quaternary carbon atoms were detected, five in the carbonyl region (δ = 176.80, 172.20, 171.80, 170.62 and 169.50), one guanidino carbon (δ = 158.30) and one at δ = 99.97. The formation of a 36-membered lactone ring in **1** is proved unambiguosly by a strong cross peak between 35-H and C-1. Two strong cross peaks from the methylene protons of 56-H_2 to the carbonyl groups at δ = 170.62 (C-55) and 172.20 (C-57) were established. The position of the esterification is defined by cross peaks between 23-H and 19-H to the carbonyl groups at δ = 170.62 (C-55) and 169.50 (C-60). The cross peak between 46-H_2 (δ = 3.09) and C-58 (δ = 158.30) allowed to identify the guanidino moiety. A cross peak was also observed from the methyl singlet of 59-H_3 (δ = 2.80) to a quaternary C-58 (δ = 158.30). ^{1}H-^{1}H COSY and HMBC data implied that the methyl groups were attached at C-2, C-6, C-10, C-14, C-28, C-34, C-36 and C-38. The chemical shifts of all proton and carbon atoms are summarised in Table 1.

Table 3. Comparison of the antimicrobial activity of malonyl-4,5-dihydroniphimycin (**1**), malonylniphimycin (**2**), dihydroniphimycin (**3**) and niphimycin (**4**)[a].

Test organisms	**1**	**2**	**3**	**4**
Candida albicans	25.00	31.25	7.00	7.81
Aspergillus niger	6.25	7.81	3.13	3.91
Penicillium chrysogenum	7.81	7.81	6.25	6.25
Bacillus subtilis	50.00	50.00	15.62	15.62
Staphilococcus aureus	50.00	50.00	12.50	12.50
Micrococcus luteus	6.25	6.25	3.13	3.13
Microsporum canis	1.95	1.95	0.98	0.98
Streptococcus pyogenes	100.00	100.00	25.00	25.00
Escherichia coli	0.00	0.00	0.00	0.00

[a] MIC: μg mL^{-1}.

The malonyl-4,5-dihydroniphimycin (**1**) is a natural product, directly isolated from the mycelium of the strain *Streptomyces hygroscopicus* 15. Compound **1** is a new antibiotic [17, 18] having a 36-membered ring, two malonate and one monomethylguanidino groups. The positions of two malonyl residues were determined to be C-23 and C-19 the protons of which appeared at δ = 5.16 and 5.10. The antibiotic **1** is the most similar to malonylniphimycin [13]. The molecular weight of malonylniphimycin, 1227 Da ($C_{62}H_{105}N_3O_{21}$), differs by two mass units from the molecular weight of **1**, 1229 Da ($C_{62}H_{107}N_3O_{21}$). The structural difference between malonylniphimycin and malonyl-4,5-dihydroniphimycin (**1**) is the absence of one double bond at C-4 and C-5 in the unsaturated lactone moiety of **1** (Table 2). In the ^{1}H-^{1}H COSY spectrum of **1** the methylene protons at δ = 1.25/1.44 (5-H_2) are coupled with the methylene protons at δ = 1.47 (4-H_2), which directly couples to the methine proton of 3-H. It can be assumed that malonyl-4,5-dihydroniphimycin (**1**) has one more malonyl residue (δ = 169.50 s, C-60; 44.48 t, C-61; 171.80 s, C-62) at C-19 in comparison with the data of dihydroniphimycin. The chemical shifts of the proton and carbon atoms of different regions of malonyl-4,5-dihydroniphimycin (**1**), malonylniphimycin (**2**) and dihydroniphimycin (**3**) are summarised in Table 2.

The inhibitory effect of malonyl-4,5-dihydroniphimycin (**1**) on filamentous fungi, yeasts, Gram-positive and Gram-negative bacteria was investigated *in vitro* by comparison with the non-polyenic macrolide antibiotics such as malonylniphimycin (**2**), dihydroniphi-

mycin (**3**) and niphimycin (**4**) (Table 3). The malonyl-4,5-dihydroniphimycin (**1**) displayed an inhibitory effect against fungi, yeasts and Gram-positive bacteria comparable to the malonylniphimycin (**2**). The antibiotics (dihydroniphimycin and niphimycin), which possess one malonyl residue in their structures, showed a higher antimicrobial activity than the antibiotics with two malonyl groups.

Acknowledgement

The authors are grateful to Mrs. M. Ponelle, Novartis Pharma, Basel, for helpful discussions regarding the structure determination by 2D NMR spectroscopy.

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