SECONDARY METABOLITES BY CHEMICAL SCREENING†

II††. AMYCINS A AND B, TWO NOVEL NIPHIMYCIN ANALOGS ISOLATED FROM A HIGH PRODUCER STRAIN OF ELAIOPHYLIN AND NIGERICIN

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Two novel natural niphimycin analogs, amycins A (**5**) and B (**3**) were isolated from the culture broth of the *Streptomyces* sp. DSM 3816 by chemical screening methods. In addition this strain produces the antibiotics niphimycin (**4**), elaiophylin (**2**) and nigericin (**1**). Fermentation, isolation, structure elucidation and biological activity of the amycins are described.

Chemical screening is an efficient method for detecting secondary metabolites of microorganisms with different types of structures and biological activities[1~4]. This type of screening can also lead to biological inactive products, which are important for the understanding of metabolic pathways used by a microorganism. Furthermore novel natural products can be detected which might be synthetic intermediates. In our chemical screening of actinomycetes, two *Streptomyces* strains were isolated showing a remarkable productivity of various compounds.

The strain DSM 3816 produces nigericin (**1**)[5,6], elaiophylin (**2**)[7~11], niphimycin (**4**) and two novel niphimycin analogs, amycins A (**5**) and B (**3**). Niphimycin (**4**) was isolated first from *Streptomyces hygroscopicus* B-255 in 1967[12]. The structure was elucidated in 1983[13,14]. Furthermore, it could be proved that scopafungin, which was first isolated 1971 from *S. hygroscopicus* var. *enhygrus*[15,16], is identical with niphimycin (**4**)[17~19].

Closely related to niphimycin (**4**) are the azalomycins F_3, F_4 and F_5[20,21], copiamycin, neocopiamycin[22,23] and guanidylfungins A and B[24,25]. All antibiotics mentioned are active against Gram-positive bacteria and fungi[26,27]. Compound **4** is an inhibitor of the oxidative phosphorylation in mitochondria[19].

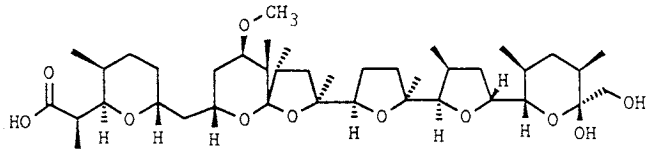
Characterization of the Producing Strains

The producing strains DSM 3816 and DSM 4137 belong to the genus *Streptomyces*. The strain DSM 3816 was isolated from a soil sample from Kypcerissia, Greece and the strain DSM 4137 was a laboratory isolate. The strains are characterized as follows in Table 1.

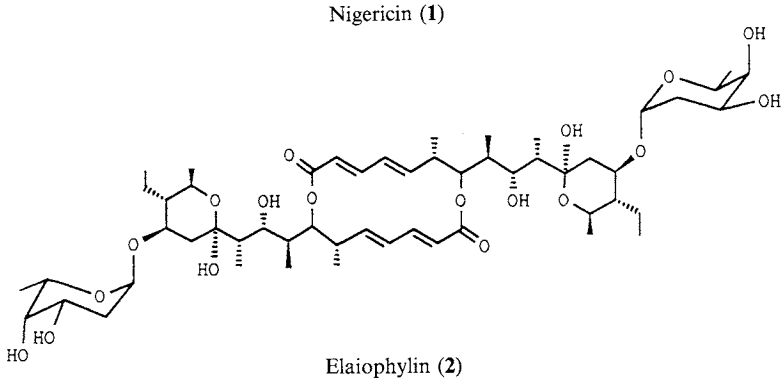
Fermentation and Isolation

The metabolite pattern and the yields of the different antibiotics is dependent on the culture medium

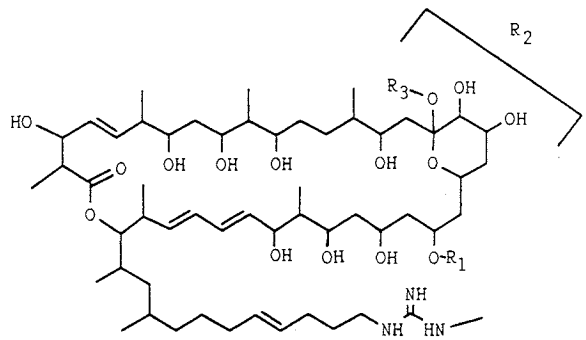
† This paper is dedicated to Professor Dr. WOLFGANG HILGER on the occasion of his 60th birthday.
†† See ref 1.



Nigericin (**1**)



Elaiophylin (**2**)



Amycin B (**3**)	$R_1=R_2=R_3=H$	
Niphimycin (**4**)	$R_1=COCH_2COOH$	$R_2=R_3=H$
Amycin A (**5**)	$R_1=R_2=COCH_2COOH$	$R_3=H$
Methylamycin B (**6**)	$R_1=R_2=H$	$R_3=CH_3$

Table 1. Characterization of the producing strains DSM 4137 and DSM 3816.

Strain	DSM 4137	DSM 3816
Aerial mycelium	Grey	Grey
Spore chain	spirals	spirals
Spore surface	Warty	Warty
Utilization of sugars:		
Sucrose, *m*-inositol, mannitol, L-rhamnose, raffinose, adonitol, melibiose, dextran	+	+
D-Fructose, xylose, arabinose, melanin	–	–
Product spectrum (mg/liter)		
Niphimycin (**4**)	100	175
Amycin A (**5**)	—	250
Amycin B (**3**)	—	6
Nigericin (**1**)	—	100
Elaiophylin (**2**)	1,700	1,200

Fig. 1. Scanning electron micrograph of the streptomyces strains DSM 3816 (left: magnification 1,500) and DSM 4137 (right; magnification 7,500) on soybean-meal agar.

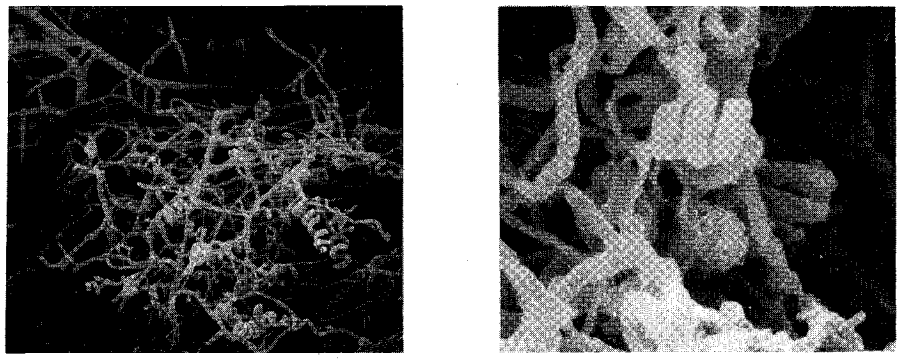
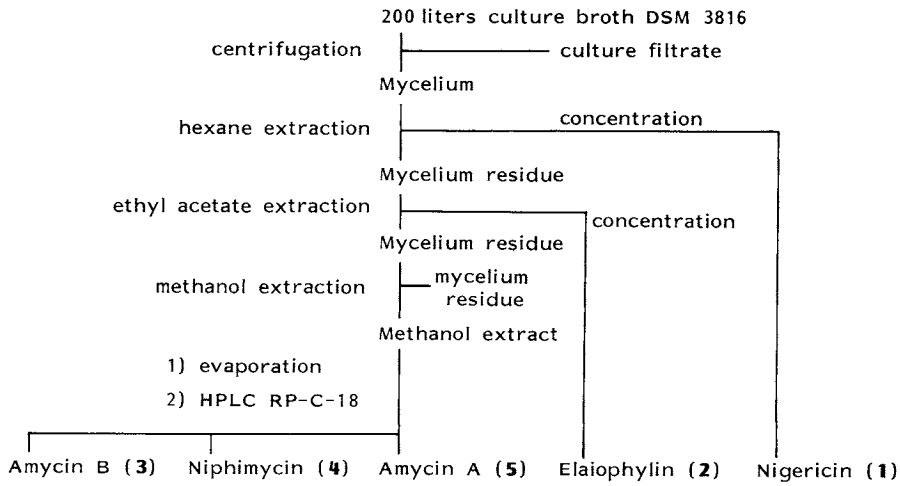


Fig. 2. Isolation of the metabolites from strain DSM 3816.



as well as on the culture conditions. For production of the antibiotics nigericin (**1**), elaiophylin (**2**), niphimycin (**4**) and the amycins A (**5**) and B (**3**) in a 200-liter Braun fermenter, we used the strain DSM 3816 with a medium containing soybean meal 2% and mannitol 2%. The production of the antibiotics during the fermentation was followed by TLC. After incubation for 120 hours at 30°C the amount of the antibiotics has reached its maximum and the culture was harvested.

From both strains the metabolites could be isolated easily (Fig. 2). The mycelium (1.8 kg) of DSM 3816 was separated from the culture filtrate by centrifugation. After drying it was extracted with 3×15 liters hexane, after concentrating the organic phase nigericin (**1**) of high purity crystallized in almost quantitative yield. The mycelium residue was extracted three times with ethyl acetate (15 liters). After concentrating (4 liters) elaiophylin (**2**) crystallized in high purity. Further extraction of the mycelium with 3×15 liters methanol yields a mixture of niphimycin and the amycins. This crude product was separated by HPLC on a RP-C-18 column with MeOH-water (8:2) as eluent. If 1 g was separated on preparative column, 250 mg amycin A, 175 mg niphimycin, and 6 mg amycin B were obtained. The total yield out of a 200-liter fermentation was approx 50 g amycin A (**5**), 35 g niphimycin (**4**), 1.2 g amycin B (**3**), 20 g nigericin (**1**) and 240 g elaiophylin (**2**).

The work up procedure of DSM 4137 was carried out as described for DSM 3816. Extraction with hexane removes some non-polar impurities which would disturb the crystallization process of elaiophylin from ethyl acetate. Niphimycin was also extracted with methanol and chromatographed with HPLC on RP-C-18 with the same solvent system.

Structure Elucidation

Nigericin (**1**)[6], elaiophylin (**2**)[11] and niphimycin (**4**)[14], were identified by comparison of the NMR spectra with data given as reference.

The amycins have Rf values on TLC silica gel in the solvent system acetic acid - butanol - water (1 : 4 : 5) similar to niphimycin (**4**). Furthermore they show identical color reactions with anisaldehyde - sulforic acid exhibiting a blue colored spot. Compounds **2** and **5** were only soluble in lower alcohols, *e.g.*, methanol, ethanol, butanol. The UV spectra (λ_{max} 231 nm) were in all these compounds almost identical. It can be assumed that these compounds are niphimycin analogs. This can be verified by comparison of the FAB mass spectra of **3** and **5** (Fig. 3). Amycin A (**5**) exhibits a molecular ion at *m/z* 1,229 $(M+H)^+$ and amycin

Fig. 3. FAB mass spectra of **5** and **3**.

(A) **5**, (B) **3**.

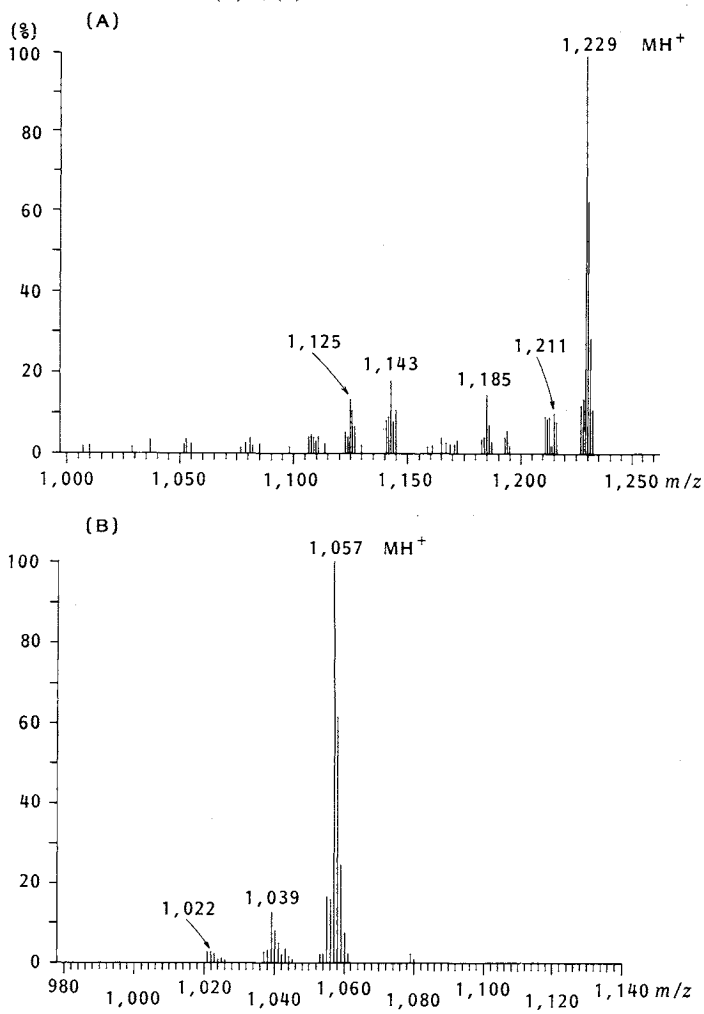
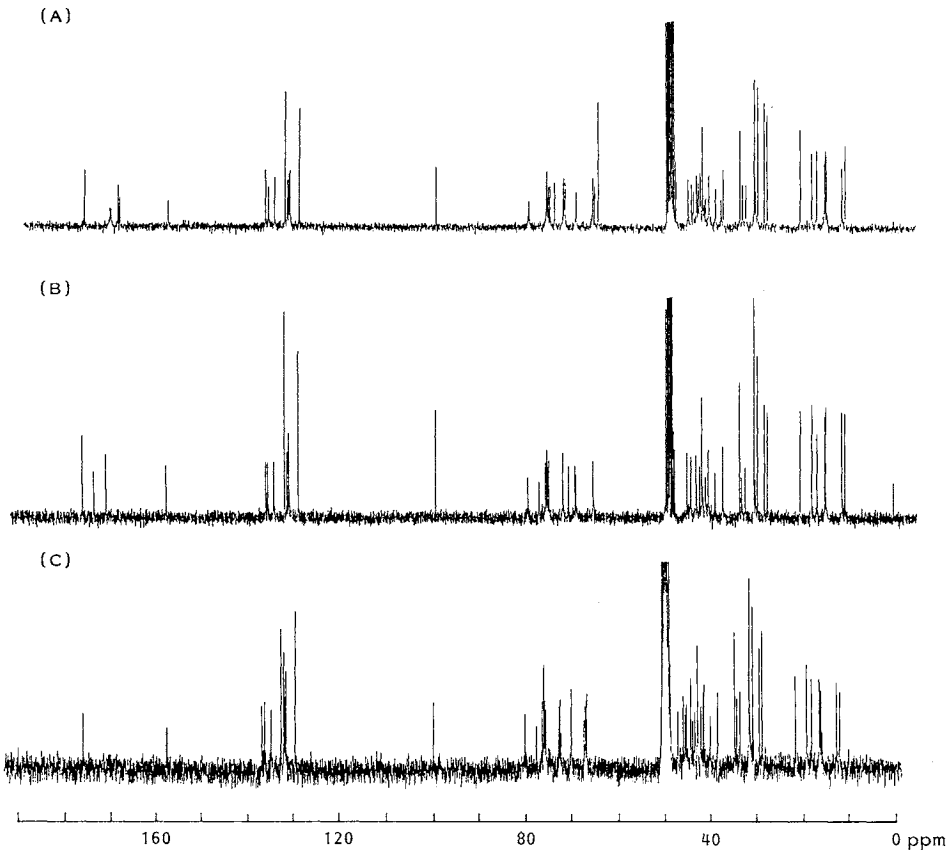


Fig. 4. ^{13}C NMR spectra of **5**, **4** and **3** (75.5 MHz, CD_3OD).
(A) **5**, (B) **4**, (C) **3**.



B (**3**) at *m/z* 1,057 $(M+H)^+$. In both cases the MW of niphimycin (**4**) (*m/z* 1,143, $(M+H)^+$) differs in 86 mass units. This difference might be explained by a malonyl residue. It can be assumed that amycin B (**3**) contains a malonyl-monoester less and amycin A (**5**) has one more than niphimycin.

This has been proved by the ^{13}C NMR spectra of **3**, **4** and **5** (Fig. 4). The ^{13}C NMR spectrum of niphimycin (**4**) shows the signals of C-1 at δ 176.8 and of the malonyl group at δ 168.9 (–COOR) and δ 170.9 (COOH). In the ^{13}C NMR spectrum of amycin B (**3**) no signals of the malonyl moiety can be detected. Amycin A (**5**) exhibits two signal groups of the malonyl group at δ 169.1 and 168.8 (COOR) and δ 171.0 and 170.6 (COOH).

It was not possible to completely assign the signals in the ^{13}C NMR spectra as well as the position of the second malonyl group in **5**. This was due to the overlay of signal groups in the 2D-NMR contour plots.

The malonyl-monoester groups of **5** could be hydrolyzed. Problems arise from the base sensitivity of these compounds, especially in the retro aldol-cleavage in position C-15/C-17 or C-19/C-17. So the saponification of **4** is described only by prior ketalization at C-17[28~30]. This was avoided by controlled reaction conditions with sodium methoxide in methanol at 0°C. Amycin A (**5**) was dissolved in methanol and kept in solution for 10 days at room temperature yielding niphimycin (**4**). When amycin A (**5**) or niphimycin (**4**) was treated with sodium methoxide in methanol for 12 hours amycin B (**3**) was obtained

Table 2. Antimicrobial activity of niphimycin (**4**) and its derivatives (MIC: μg/ml).

Organisms	**3**	**4**	**5**	**6**
Trichophyton mentagrophytes	3.91	3.91	7.81	15.6
T. rubrum	3.91	1.95	7.81	15.6
Microsporum canis	1.95	0.48	1.95	7.81
Candida albicans	3.91	7.81	31.2	31.2
Aspergillus niger	1.95	3.91	7.81	15.6
Staphylococcus aureus 6511	3.13	6.25	50.0	12.5
S. aureus 285	3.13	12.5	50.0	12.5
S. aureus 503	3.13	6.25	50.0	12.5
Streptococcus pyogenes 308	6.25	25.0	100.0	6.25
S. pyogenes 77 A	6.25	25.0	100.0	6.25
S. faecium D	6.25	50.0	—	50.0

in 80% yield. The ketalization of **3** to **6** with acidic methanol was carried out as described[28~30].

Biological Activities

Niphimycin is active against Gram-positive bacteria and fungi (Table 2). (The reaction sequence **5**→**4**→**3** without ketalization had an advantage concerning the biological activity). The demalonyl derivatives of methyl-copiamycin, methyl-guanidylfungin A and methyl-azalomycin F_4 showed higher activity against bacteria and fungi than the parent compounds[28~30]. Because of the β-hydroxy-keto moiety in all molecules, where a retro aldol-cleavage is possible, a protection has to take place before the saponification. These derivatives exhibit a decreased antifungal activity, which could be increased by saponification of the malonyl-monoester group. It can be assumed that the demalonyl compounds without the ketal group show the highest activities against fungi.

The demalonyl product amycin B (**3**) which is now available by fermentation as well as by chemical degradation of **4** and **5**, had an increased activity against bacteria compared with amycin A (**5**) and niphimycin (**4**). The ketalization to **6** leads to a decreased activity against bacteria and fungi.

Discussion

Amycins A (**5**) and B (**3**) are two novel niphimycin analogs. Because of instability of the additional malonyl-monoester in **5** against nucleophiles in comparison with niphimycin (**4**), it can be assumed that the dimalonyl compound **5** is the original fermentation product. Niphimycin (**4**) and amycin B (**3**) are hydrolyzed products of **5**, which were generated by nucleophiles in the fermentation broth. Another natural product, which was described without a monoester group, is demalonylcopiamycin[28].

It was shown that the cleavage of the monoester in **5** to **4** and **3** leads to higher activities against bacteria, fungi and yeast. Niphimycin (**4**) exhibited the highest activity against fungi and amycin B (**3**) showed the highest antibacterial activity.

Experimental

General

IR spectra in pressed KBr disks were recorded on a Perkin-Elmer 197 spectrometer, UV spectra using a Perkin-Elmer 550 S UV Vis spectrophotometer. The NMR spectra were determined with a Bruker AM-300. Chemical shifts are expressed in values (ppm) with TMS as an internal standard. The FAB mass spectra were taken by a MS 50 Kratos Analytical (FAB-MS) with 3-nitrobenzyl alcohol as matrix.

TLC was carried out on Silica gel plates (Merck 60 F_{254}). HPLC was performed with a Waters M 6000 with a RP-C-18 (Merck) column.

Antifungal Testing

The test was performed as a serial dilution in the test tube. A predetermined concentration of the test compound was dissolved in a suitable solvent (*e.g.* DMSO) (12.5 mg compound + 1 ml organic solvent + 9 ml distilled water or 12.5 mg compound + 10 ml distilled water). The media (cutaneous fungi: Neopeptone - glucose broth; yeasts and moulds: Yeast - nitrogen base) were mixed with benzylpenicillin sodium and streptomycin sulfate (200 IU/liter each) to prevent bacterial growth, in the routine screening.

The starting tubes (cutaneous fungi: 2~24 tubes/strain; yeasts and moulds: 12 tubes/strain) are each filled with 5.4 ml medium, 0.6 ml compound solution was added to each, mixed and diluted in the ratio of 1 : 2 by transferring 3 ml from each tube into the following tube containing 3 ml.

Each tube was then inoculated with 1 drop of standardized organism suspension (= 160,000 micro-conidia/ml (cutaneous fungi)) or 1,000 conidia or cells/ml (yeasts and moulds).

There was also a growth control (inoculated, not medicated), a solvent control (inoculated, not medicated, containing solvent as in medicated tubes) and a negative control (not inoculated, not medicated, containing only medium). For compound **3** the antifungal tests were performed under the same conditions, without the antibiotics mentioned above, to verify that no synergism was observed in the routine test. After incubation for 10 days at 30°C, the tubes were inspected macroscopically for growth.

Antibacterial Testing

The sensitivity of bacteria was tested by means of the agar dilution test in Mueller-Hinton agar (Difco). When testing Streptococci, the agar was supplemented with 10% horse blood. Plates were inoculated with a Denley Multipoint inoculator which delivered 5×10^4 cfu of an overnight culture of the strain concerned. The MIC was taken as the lowest concentration at which no visible growth could be detected after 24 hours at 37°C. The MIC_{50} was calculated from the results.

Fermentation

A loopful of a start culture of *Streptomyces* sp. DSM 3816 was inoculated into a 2-liter Erlenmeyer flask containing 500 ml of a medium containing 20 g/liter soybean meal and 20 g/liter mannitol. This culture was cultivated for 3 days at 20°C on a rotary shaker. Two liters of the incubated culture were transferred to a 200-liter fermenter with the same media. The fermentation was carried out at 30°C for 5 days stirring at 40 rpm and an flow of 200 liters per minute. The production of the antibiotic started 48 hours after inoculation and reached its maximum after 5 days. For the production of niphimycin and elaiophylin the strain DSM 4137 was used under the same conditions.

Isolation

200 liters fermentation broth was centrifugated or simply filtrated. The mycelium was dried *in vacuo* and then extracted with 3 × 15 liters hexane. After concentration of the organic layer up to 3 liters, 20 g nigericin (**1**) crystallized from the solution. Extraction of the mycelium with 3 × 15 liters EtOAc gave after evaporation up to 4 liters, 240 g elaiophylin (**2**). Finally the mycelium was extracted with 3 × 15 liters MeOH. Evaporation *in vacuo* gave 200 g syrup, which was further purified on a semi-preparative HPLC RP-C-18 column with the solvent system MeOH - water (8 : 2).

If 1 g was chromatographed and the solvent evaporated and dried in oil pump vacuum for 2 hours 250 mg amycin A (**5**), 175 mg niphimycin (**4**) and 6 mg amycin B (**3**) was obtained as white foams.

5: Positive FAB-MS *m/z* 1,229 ($(M+H)^+$, abundance 100%); ^{13}C NMR (75 MHz, CD_3OD) δ 176.6, 171.0, 170.7, 169.1, 168.8, 158.1, 137.0, 136.3, 135.0, 132.8, 132.3, 131.9, 137.7, 129.7, 99.9; $[\alpha]_D^{20}$ +36° (*c* 1, CH_3OH); Rf 0.26 (BuOH - HOAc - H_2O, 67 : 10 : 23); UV $\lambda_{max}^{CH_3OH}$ nm 231 (5,000).

Cleavage of the Malonyl Moiety; Amycin B (**3**) from **5**

1 g amycin A (**5**) was stirred in 100 ml MeOH with 100 mg NaH for 12 hours at 0°C. After neutralization with 5 N HCl, the solvent was removed by evaporation *in vacuo*. The resulting syrup was purified by HPLC (RP-C-18 column) with MeOH - water (8 : 2). Evaporation of the solvent *in vacuo* yielded 680 mg (70%) amycin B (**3**). Positive FAB-MS *m/z* 1,057 ($(M+H)^+$, abundance 100%); ^{13}C NMR (75 MHz, CD_3OD) δ 177.0, 158.0, 137.4, 136.8, 135.4, 133.2, 132.6, 132.4, 132.1, 130.1, 100.4; $[\alpha]_D^{20}$ +43.7° (*c* 1, MeOH); Rf 0.4 (BuOH - HOAc - H_2O, 67 : 10 : 23).

Niphimycin (**4**) from **5**

1 g amycin **5** was stirred in 100 ml MeOH for 10 days at 25°C. The work up procedure was done as described for amycin B. Positive FAB-MS *m/z* 1,143 (abundance 100%); ^{13}C NMR (75 MHz, CD_3OD) δ 176.8, 170.9, 168.9, 158.3, 137.2, 136.5, 135.0, 133.6, 133.0, 132.4, 131.4, 130.0, 100.0; Rf 0.32 (BuOH - HOAc - H_2O, 67 : 10 : 23).

Amycin B from **4**

1 g niphimycin (**4**) was stirred in 100 ml methanol for 12 hours at 0°C in the presence of 100 mg NaH. The work up procedure was carried out as described for amycin B.

Methylamycin B (**6**)[30]

1 g of amycin B was stirred with a mixture of 1.5 ml of acetyl chloride and 75 ml of methanol at room temperature for 15 minutes. After neutralization with 5 N NaOH, the mixture is concentrated *in vacuo* to a syrup. Chromatography by HPLC on RP-C-18 with MeOH - water (8 : 2) yields after evaporation of the solvent 800 mg (78%) **6**.

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