METABOLIC PRODUCTS OF MICROORGANISMS. 224†

BAFILOMYCINS, A NEW GROUP OF MACROLIDE ANTIBIOTICS

PRODUCTION, ISOLATION, CHEMICAL STRUCTURE AND BIOLOGICAL ACTIVITY

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The bafilomycins A_1, A_2, B_1, B_2, C_1 and C_2, a new type of macrolide antibiotics with a 16-membered lactone ring, were isolated from the fermentation broth of three *Streptomyces griseus* strains (TÜ 1922, TÜ 2437, TÜ 2599) by ethyl acetate extraction and column chromatography on silica gel. The bafilomycins exhibit activity against Gram-positive bacteria and fungi. Physico-chemical data, chemical structures and biological activities are reported.

The strain, *Streptomyces griseus* sp. *sulphurus* (TÜ 1922) is a good example for the ability of microorganisms to produce various secondary metabolites, depending on the culture conditions. The recognition of these metabolites depends on the screening method used for their detection. Under certain culture conditions *Streptomyces griseus* produce mainly the iron-complex of tirandamycin B as biologically active compound[1]. A change in culture conditions leads to the production of the Mg-complex of tirandamycin A[2] and the bafilomycins as biological active compounds and pyrrol-2-carbonic acid, detected in a chemical screening procedure. In this paper we describe the fermentation (strain TÜ 1922), isolation, purification, chemical structure and biological properties of the bafilomycins, a new class of 16-membered macrolides.

Materials and Methods

Bacterial Strains

The standard strains for the activity spectrum of the bafilomycins were obtained from the stock culture collection in our laboratories or from ATCC. The antibiotic-producing microorganisms (TÜ 1922, TÜ 2437, TÜ 2599) were new soil isolates, classified according to HÜTTER and BERGEY as *Streptomyces griseus*[3,4].

Fermentation Studies

S. griseus was cultured for 96 hours at 27°C in a medium (100 ml in a 500-ml Erlenmeyer flask with one intrusion) consisting of 2% meat meal, 2% malt extract, 1% $CaCO_3$ (NL 111). The pH was adjusted to 7.2 before autoclaving. These cultures were used as inoculum for the 10-liter fermentor. Bafilomycins were produced in a 10-liter fermentor under the following culture conditions: 5% inoculum was

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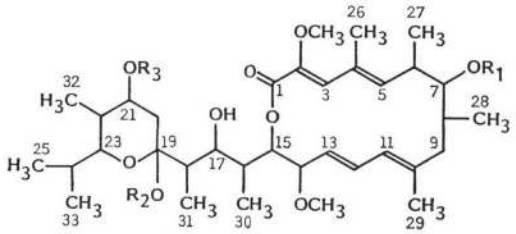
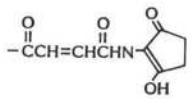
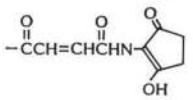
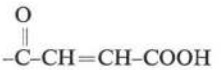
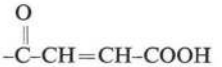
Fig. 1. Structures of bafilomycins.



Bafilomycin	R_1	R_2	R_3
A_1	H	H	H
A_2	H	CH_3	H
B_1	H	H	
B_2	H	CH_3	
C_1	H	H	
C_2	H	CH_3	
Monoacetyl-A_1	H	H	
Diacetyl-A_1		H	
Diacetyl-A_2		CH_3	

transferred to a 10-liter fermentor containing 9.5 liters medium (NL 111) and run at 27°C for 60 hours with 240 rpm agitation and 4 liters/minute aeration. As inoculum for the 100-liter fermentor (Model F-130, New Brunswick Scientific Co., New Brunswick, USA) one 10-liter fermentor (2 liters/minute aeration), 48 hours old, was used. Maximum production was reached after about 60 hours. Fermentation conditions were: agitation 200 rpm, aeration 50 liters/minute, incubation temperature 27°C. To prevent foaming, silicone antifoam (Merck) was added.

During the whole fermentation course samples were taken for measuring the following parameters: pH, mycelium volume and estimation of the antibiotic concentration by disc diffusion assay using *Mucor miehei* and *Clostridium pasteurianum* as test organisms.

Biological Assay

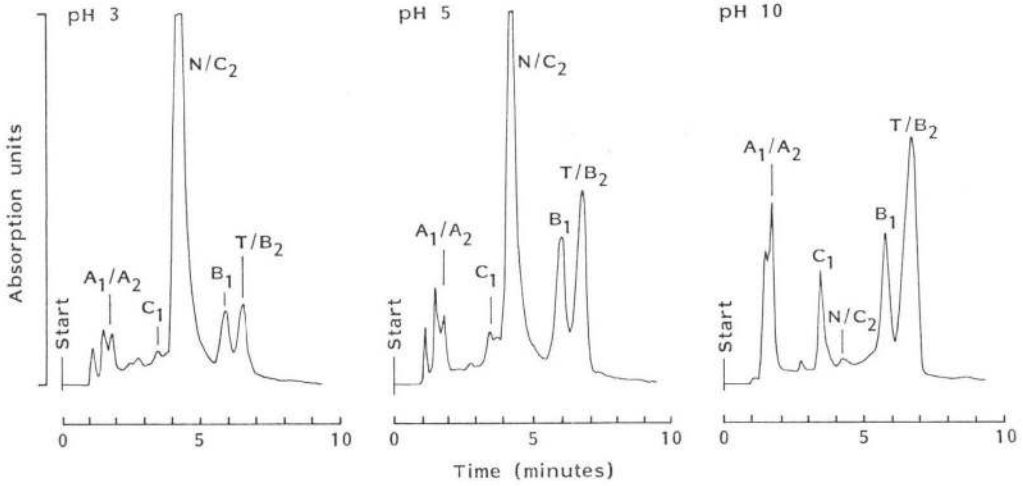
The disc diffusion assay was used for measuring the antibiotic content of the cultures and to determine the antibacterial and antifungal spectra of the bafilomycins.

Chemical Assay

The thin-layer chromatography (TLC) shows correlation of the antifungal activity (bioautogram) with a pink color reaction, after spraying TLC-plates with a solution of 18% methanolic HCl.

To determine the optimum pH value for the extraction of the culture media, aliquots of the fermentation broth were adjusted to pH 3, 5, 7, and 10 respectively and extracted with ethyl acetate. The organic layer was dried over sodium sulfate and evaporated to dryness by a stream of nitrogen. The

Fig. 2. High pressure liquid chromatograms of culture media extracts at different pH values.
HPLC conditions: Lichrosorb RP-18 5 μm, 125×4.6 mm.
Eluent: methanol 1 ml/minute, UV detection at 254 nm (0.032 AUFS).
N=pyrrole-2-carboxylic acid; T=tirandamycin A Mg-complex.



residues were dissolved in 1 ml of methanol and 10 μliters of this solution was analyzed by HPLC. Fig. 2 shows the chromatograms obtained for three different pH values at the extraction step. To correlate the peaks to the corresponding bafilomycins, the eluent was monitored biologically and by TLC. Best results for the extraction were obtained at pH 10.

Isolation

100-liter culture filtrate of *S. griseus* sp. *sulphurus*, adjusted to pH 10, was extracted twice with 20 liters of ethyl acetate. The extract was evaporated *in vacuo* to 2 liters. The concentrated solution was washed with 500 ml of water (pH 7.0), dried with sodium sulfate and concentrated to 500 ml. This solution was allowed to stand overnight at 18°C. The yellow precipitate of tirandamycin A Mg-complex was removed by filtration. This procedure was repeated twice. The remaining solution was evaporated *in vacuo* to dryness to obtain 4.67 g of a brown residue. This residue was dissolved in 30 ml of chloroform - methanol (1: 1) and applied to a column of 400 g of silica gel (Merck Si-60, 0.065~0.2 mm). The column was eluted with a mixture of chloroform - methanol (9: 1) to obtain, successively, fraction I (90 ml), fraction II (75 ml), fraction III (240 ml), fraction IV (210 ml) and fraction V (240 ml). The column was then eluted with methanol to obtain fraction VI (450 ml). The column eluate was monitored by TLC-analysis.

Fraction I contains 0.7 g fatty compounds with no biological activity.

Fraction II, containing the bafilomycins A_1, A_2 and B_2, was evaporated under reduced pressure to yield 2.6 g of a crude, yellow powder, which was dissolved in 20 ml of chloroform and applied to a column of 300 g of silica gel (Woelm Si-60, 0.063~0.2 mm). Elution was carried out with a mixture of chloroform - methanol (95: 5). On the basis of TLC and HPLC analysis appropriate fractions containing bafilomycins A_1, A_2 and B_2 respectively were pooled. The solvent was removed under reduced pressure to yield 760 mg of bafilomycins A_1, A_2 and 60 mg of bafilomycin B_2.

Separation of bafilomycins A_1 and A_2 was carried out by column chromatography on 150 g of silica gel (Woelm Si-60, 0.063~0.2 mm) with a mixture of methyl ethyl ketone - chloroform (1: 1). Each compound was finally purified by HPLC on LiChrosorb RP-18 (Merck, 10 μm, 250×16 mm ϕ) with methanol - water (80: 20, pH 6.5) as eluent. Appropriate fractions were pooled on the basis of analytical HPLC control and the solvent was removed under reduced pressure. Yield: 45 mg bafilomycins A_1 and 36 mg A_2.

Fraction III, containing mainly bafilomycins B_1 and B_2, was evaporated under reduced pressure. The residue (710 mg) was redissolved in chloroform - methanol (9: 1) and applied to a column of 150 g

of silica gel (Merck Si-60, 0.063~0.2 mm) which was eluted with a mixture of chloroform - methanol (9: 1) to separate bafilomycins B_1 and B_2. On the basis of TLC-analysis the fractions containing bafilomycins B_1 and B_2 respectively were pooled and evaporated under reduced pressure.

The yellow residues were dissolved in chloroform - ethyl acetate (1: 1) and rechromatographed on 50 g of silica gel with a mixture of chloroform - ethyl acetate (1: 1) as eluent. Appropriate fractions were pooled on TLC control and the solvent was removed under reduced pressure. Yield: 92 mg bafilomycins B_1 and 41 mg B_2.

Fraction IV, containing no biologically active products, was discarded.

Fraction V, containing tirandamycin A Mg-complex, was evaporated under reduced pressure. The residue was combined with the previously obtained precipitate and recrystallized from acetone-water to yield 150 mg tirandamycin A Mg-complex.

Fraction VI, containing bafilomycins C_1 and C_2, was evaporated under reduced pressure. The crude powder was dissolved in methanol and applied to a column of 100 g of silica gel, eluted with chloroform - methanol (1: 1) containing 2% ammonia. The fractions containing bafilomycin C_1 and bafilomycin C_2 respectively, were pooled (based on TLC and HPLC-analysis) and the solvent was removed under reduced pressure to yield 120 mg bafilomycin C_1 and 24 mg of bafilomycin C_2.

Analytical Procedures

Melting points were determined with a Büchi melting point apparatus and are uncorrected. UV absorption spectra were measured in methanol with Beckmann DB-G. The IR spectra were taken in KBr pellets using Perking-Elmer PE 210 and 221 IR spectrometers. ^1H and ^{13}C NMR spectra were recorded with Bruker WH 90 and Bruker WM 400 spectrometers. The mass spectra were obtained on a Varian MAT 711. The EI-spectra were measured at 75 eV using a direct inlet system.

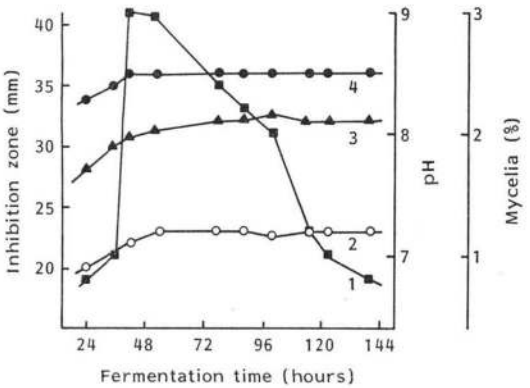
Results

Fermentation and Isolation

For the isolation of bafilomycins in larger amounts fermentations at a 10-liter and 100-liter scale were performed. Progress of the fermentation process is shown in Fig. 3. The production of all antibiotically active compounds starts very early in the logarithmical growing phase and is correlated with the increase of mycelia volume. The antibiotic activities are stable for more than 100 hours. The isolation were performed as described in the Figs. 4 and 5.

Fig. 3. Time course of the fermentation of *S. griseus* sp. *sulphurus*.

1) Growth (mycelia volume, %), 2) antifungal activity against *Mucor miehei* (inhibition zone, mm), 3) pH, 4) antibacterial activity against *Clostridium pasteurianum* (inhibition zone, mm).



Physical and Chemical Properties

Bafilomycins are all readily soluble in acetone, methanol and chloroform. Rf values on TLC (Silica Gel $60F_{254}$, E. Merck, Darmstadt, West Germany) are as follows: bafilomycin A_1 0.51; A_2 0.52; B_1 and B_2 0.43 and C_1 and C_2 0.1

Fig. 4. Extraction scheme of bafilomycins.

100 liters fermentor

adjusted to pH 10
extracted with 20 liters of ethyl acetate
concentrated to 2 liters

Extract (light yellow color)

washed with water - ammonium chloride
dried with Na_2SO_4
evaporated to 500 ml
precipitate: tirandamycin A Mg-complex (400 mg)
evaporated *in vacuo* to dryness

Raw material (brown color) 4.67 g

Fig. 5. Isolation course of the bafilomycins.

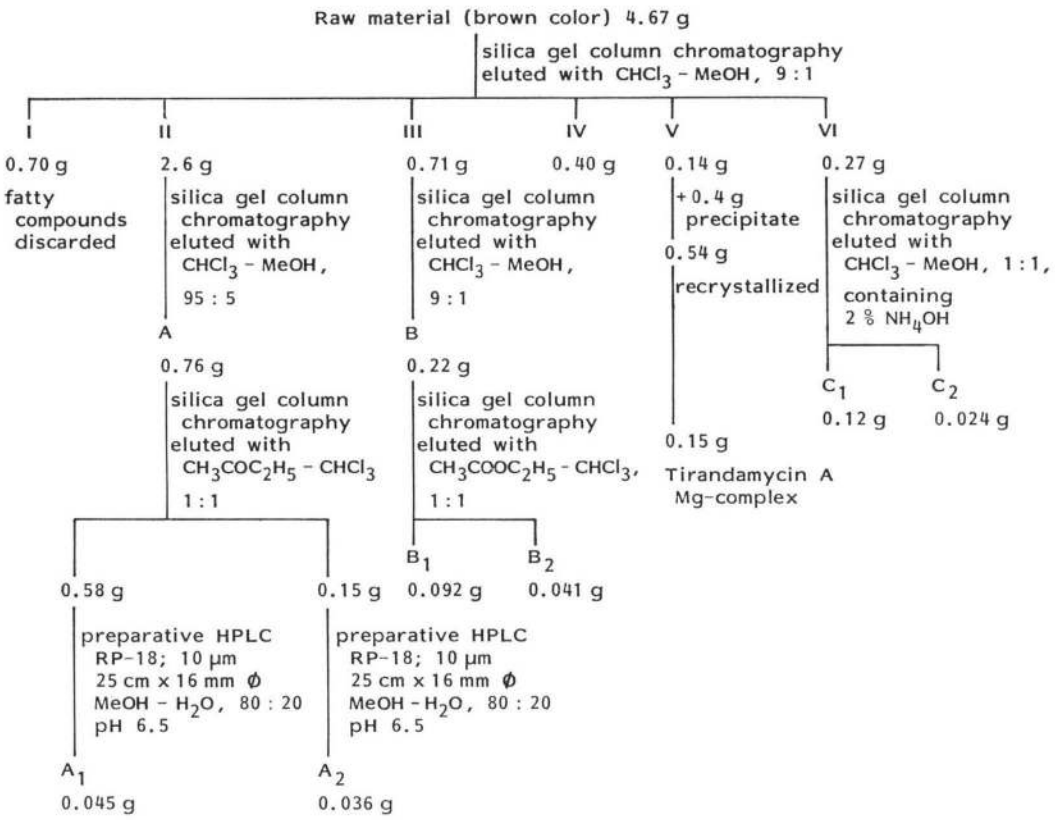


Table 1. Physico-chemical properties of the bafilomycins.

Bafilomycin	mp °C (dec)	λ_{max}^{MeOH} nm (ε)	ν_{max}^{KBr} (cm^{-1})
A_1	103~106	245 (25,000), 280 (12,100)	3350, 2920, 1680, 1245, 1100, 915
A_2	116~119	245 (25,000), 280 (12,100)	3400, 2920, 1680, 1245, 1100
B_1	89~96	248 (35,000), 285 (18,500), 355 (2,500)	3450, 3200, 2900, 1720, 1690, 1350, 1240, 1170, 1100
B_2	—	—	—
C_1	145~150	245 (22,000), 280 (12,000), 335 (4,800), 350 (2,500)	3450, 2900, 1715, 1690, 1250, 1100, 1000
C_2	—	—	—

— Not determined.

($CHCl_3$ - MeOH, 9: 1). The UV spectra of the bafilomycins show maximum absorptions at 242, 248 and 280 nm, bafilomycins B and C show in addition shoulders between 340 and 360 nm. In all IR spectra of bafilomycins, absorption bands due to the hydroxyl and lactone groups were observed at 3300~3500 cm^{-1}, 1670~1690 cm^{-1}, and at 1240~1250 cm^{-1}. The IR spectra of bafilomycins B and C show additional bands due to an ester group at 1710~1730 cm^{-1} and at 1220 cm^{-1} (Table 1). The EI-mass spectrum for each bafilomycin shows several intensive fragment ion peaks up to *m/z* 568, but no molecular ion peak. The molecular ion peaks appear in the FD-mass spectra (Table 2). The structure was elucidated by the extensive use of ^{1}H NMR and ^{13}C NMR spectroscopy. Conventional proton spin decoupled spectra, single frequency off-resonance spectra, internuclear double resonance spectra

Table 2. Mass spectra of bafilomycins.

Bafilomycin	M^+ (FD) m/z	Fragment ion (EI) m/z											Molecular formula
A_1	622		568	525	399	368	338	209	169	137		109	$C_{35}H_{58}O_9$
A_2	636		568	525	399	368	338	209	169	137		109	$C_{36}H_{60}O_9$
B_1	(815*)		568	525	399	368	338	211	169	137	113	109	$C_{44}H_{65}O_{13}N$
B_2	829												$C_{45}H_{67}O_{13}N$
C_1	(720)	692	568	525	400	368	338	209	169	137	123	109	$C_{39}H_{60}O_{12}$
C_2	734												$C_{40}H_{62}O_{12}$
Monoacetyl-A_1	664	646	568	525	399	368	338	209	169	137		109	$C_{37}H_{60}O_{10}$
Diacetyl-A_1	706	610	568	525	430	376	333	217	191	137		109	$C_{39}H_{62}O_{11}$

( ): Not detected.
* In the FD-MS appears m/z 839 (M+H+Na).

Table 3. ^1H and ^{13}C NMR chemical shifts and coupling constants of bafilomycin A_1.

Carbon	δ_H (ppm)	Multiplicity	J (Hz)	δ_C (ppm)	Multiplicity**
1				167.3	s
2				141.3	s
3	6.71	d	0.7	141.3	d
4				143.0	s
5	5.79	dm	9.2/1.2	125.1	d
6	2.55	dqd	9.2/7.0/2.0	36.8	d
7	3.29	m		81.0	d
8	1.91	dq	11.5/6.5	40.1	d
9a	2.15	dm	14.0	41.3	t
9b	1.94	dd	14.0/11.5		
10				132.8	s
11	5.82	dm	10.5/1.2	142.7	d
12	6.53	dd	15.0/10.5	133.0	d
13	5.15	dd	15.0/9.5	127.0	d
14	3.89	dd	9.5/8.7	82.3	d
15	4.96	dd	8.7/1.2	76.8	d
16	2.12	dqd	11.0/6.7/1.2	37.2	d
17	4.13	ddd	11.0/4.1/2.0	70.6	d
18	1.76	qm	7.2/1~2	42.1	d
19				98.9	s
20a	2.30	dd	11.9/4.7	43.5	dd
20b	1.16	ddd	11.9/11.0/2.2		
21	3.69	ddd	11.0/9.9/4.7	70.8	d
22	1.33	ddq	10.2/9.9/6.5	41.0	d
23	3.48	dd	10.2/2.2	75.9	d
24	1.89	sept.d	6.7/2.2	27.9	d
25	0.90	d	6.7	12.2*	q
26	1.98	d	1.2	14.0	q
27	1.06	d	7.0	17.2	q
28	0.93	d	6.5	14.5*	q
29	1.93	s		20.1	q
30	0.83	d	6.7	9.8	q
31	1.04	d	7.2	7.0	q
32	0.94	d	6.5	21.7*	q
33	0.76	d	6.7	21.2*	q
C2-OCH_3	3.63	s		59.9	q
C14-OCH_3	3.24	s		55.5	q

Measured in $CDCl_3$ 99.95% at 400 MHz (^1H NMR) and in $CDCl_3$ at 100.62 MHz (^{13}C NMR spectrum).
* Cannot be exactly attached.
** Observed in off-resonance spectrum.

Table 4. The biological activity of the bafilomycins against various strains.

Organism	A_1	B_1	C_1	Organism	A_1	B_1	C_1
Achromobacter geminianii	—	—	—	*Streptomyces prasinus* TÜ 30	—	tr	tr
Agrobactererium tumefaciens	—	—	—	*S. lavendulae* TÜ 35	—	—	—
Escherichia coli K 12	—	—	—	*S. glaucescens* TÜ 49	—	—	tr
E. coli K 12*	—	—	—	*Candida albicans* TÜ 565	—	tr	tr
Salmonella typhimurium	—	—	—	*C. albicans* ampicillin resistant	—	—	—
Pseudomonas aeruginosa	—	—	—	*Candida membranaefaciens*	—	—	—
Pseudomonas fluorescens	—	—	—	*Hansenula anomala*	—	13	14
Proteus mirabilis	—	tr	10	*Lipomyces lipofer*	—	—	tr
Proteus vulgaris	—	—	tr	*Nadsonia fulvescens*	15	26	28
Bacillus brevis	—	9	10	*Rhodotorula rubra*	tr	16	18
Bacillus subtilis ATCC 6051	—	—	—	*Saccharomyces cerevisiae* TÜ 125	—	—	—
B. subtilis ATCC 6051*	—	11	15	*S. cerevisiae* FL 200	—	13	15
B. subtilis A 14	—	—	9	*Saccharomyces* sp. FL 599-1B	13	21	23
B. subtilis F-24-2-WA	—	—	tr	*Schizosaccharomyces pombe*	—	23	17
Clostridium pasteurianum	—	9	14	*Wingea robertsii*	tr	10	14
Staphylococcus aureus	—	—	—	*Alternaria mali* polyoxin resistant	26	>30	>30
Micrococcus luteus	—	—	—	*A. mali* polyoxin sensitive	24	>30	>30
Micrococcus luteus	—	12	14	*Aspergillus niger*	—	20	19
Micrococcus luteus W 45	—	14	19	*Aspergillus terreus*	—	19	18
Corynebacterium insidiosum	—	10	12	*Botrytis cinerea*	22	27	24
Corynebacterium rathayi	—	—	tr	*Coprinus cinereus*	14	>30	>30
Arthrobacter aurescens	—	10	11	*Mucor hiemalis* TÜ 179/180	14	32	31
Arthrobacter crystallopoietes	—	13	14	*Mucor miehei* TÜ 284	13	27	25
Arthrobacter globiformis	—	14	15	*M. miehei* TÜ 284*	19	40	39
Arthrobacter pascens	—	14	16	*Neurospora crassa* Arg$^-$	—	12	14
Brevibacterium sterolicum	—	—	—	*Paecilomyces varioti* TÜ 137	10	22	22
Streptomyces antibioticus TÜ 4	—	—	—	*Penicillium puberulum*	—	21	21
S. griseus TÜ 17	—	—	tr	*Phythium debaryanum*	—	18	22
S. diastatochromogenes TÜ 20	—	—	—	*Rhizoctonia solani*	23	29	29
S. violaceoruber TÜ 22	—	—	—	*Saprolegnia asterophora*	17	24	27

tr: Trace.
Inhibitory diameter (paper disc, 6 mm); concentration 1 mg/ml in ethanol.
*: Minimal media.

as well as nuclear overhauser effect spectra were measured and analyzed for their structure information (Table 3). The structure elucidation is described in detail in references 2 and 5.

The bafilomycins A_2, B_2 and C_2 are not native, and are formed during the isolation procedure.

The structure of hygrolidin shows some similarity to bafilomycin C_1[6]. These compounds have unsaturated 16-membered lactone rings with methoxyl groups. The structure of concanamycin is also an unsaturated macrolide, but has an 18-membered lactone ring[7,8].

The recently published adenosine triphosphatase inhibitors, L-681,110 A_1 and L-681,110 A_2[9], are identical with the bafilomycins C_1 and C_2 reported here.

Biological Activity

In the disc diffusion assay the sensibility of various bacteria and fungi were tested against the bafilomycins (Table 4). The results show that these antibiotics possess a broad activity spectrum including Gram-positive bacteria, fungi and yeasts. Gram-negative organisms seem to be insensitive. The antibacterial activity of the three bafilomycins described increase in order A<B<C. By the disc diffusion assay we found, that the antifungal activity of the compounds is by far more pronounced than

the antibacterial effects.

Acknowledgment

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References

1) HAGENMAIER, H.; K. JASCHKE, L. SANTO, M. SCHEER & H. ZÄHNER: Stoffwechselprodukte von Mikroorganismen. 158. Tirandamycin B. Arch. Mikrobiol. 109: 65~74, 1976
2) WERNER, G.: Strukturaufklärung neuer Antibiotika. Thesis Universität Tübingen, 1982
3) HÜTTER, R.: Systematik der Streptomyceten. Karger AG, Basel 1967
4) BUCHANAN, R. & N. GIBBONS: BERGEY's Manual of Determinative Bacteriology. The Williams & Wilkins Company, Baltimore, 1974
5) WERNER, G.; H. HAGENMAIER, K. ALBERT, H. KOHLSHORN & H. DRAUTZ: The structure of the bafilomycins, a new group of macrolide antibiotics. Tetrahedron Lett. 24: 5193~5196, 1983
6) SETO, H.; H. AKAO, K. FURIHATA & N. ŌTAKE: The structure of a new antibiotic, hygrolidin. Tetrahedron Lett. 23: 2667~2670, 1982
7) KINASHI, H.; K. SOMENO, K. SAKAGUCHI, T. HIGASHIJIMA & T. MIYAZAWA: Alkaline degradation products of concanamycin A. Tetrahedron Lett. 22: 3857~3860, 1981
8) KINASHI, H.; K. SOMENO, K. SAKAGUCHI, T. HIGASHIJIMA & T. MIYAZAWA: Structure of concanamycin A. Tetrahedron Lett. 22: 3861~3864, 1981
9) HENSENS, O. D.; R. L. MONAGHAN, L. HUANG & G. ALBERS-SCHÖNBERG: Structure of the sodium and potassium ion activated adenosinetriphosphatase inhibitor L-681,110. J. Am. Chem. Soc. 105: 3672~3679, 1983