

METABOLIC PRODUCTS OF MICROORGANISMS. 224<sup>†</sup>  
BAFILOMYCINS, A NEW GROUP OF MACROLIDE ANTIBIOTICS  
PRODUCTION, ISOLATION, CHEMICAL STRUCTURE  
AND BIOLOGICAL ACTIVITY

GERHARD WERNER and HANSPAUL HAGENMAIER\*

Institut für Organische Chemie der Universität Tübingen  
Auf der Morgenstelle 18, D-74 Tübingen, G. F. R.

HANNELORE DRAUTZ, ANGELIKA BAUMGARTNER and HANS ZÄHNER

Institut für Biologie II, Lehrstuhl für Mikrobiologie I der Universität Tübingen,  
Auf der Morgenstelle 18, D-74 Tübingen, G. F. R.

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The bafilomycins A<sub>1</sub>, A<sub>2</sub>, B<sub>1</sub>, B<sub>2</sub>, C<sub>1</sub> and C<sub>2</sub>, 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<sup>1)</sup>. A change in culture conditions leads to the production of the Mg-complex of tirandamycin A<sup>2)</sup> 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*<sup>3,4)</sup>.

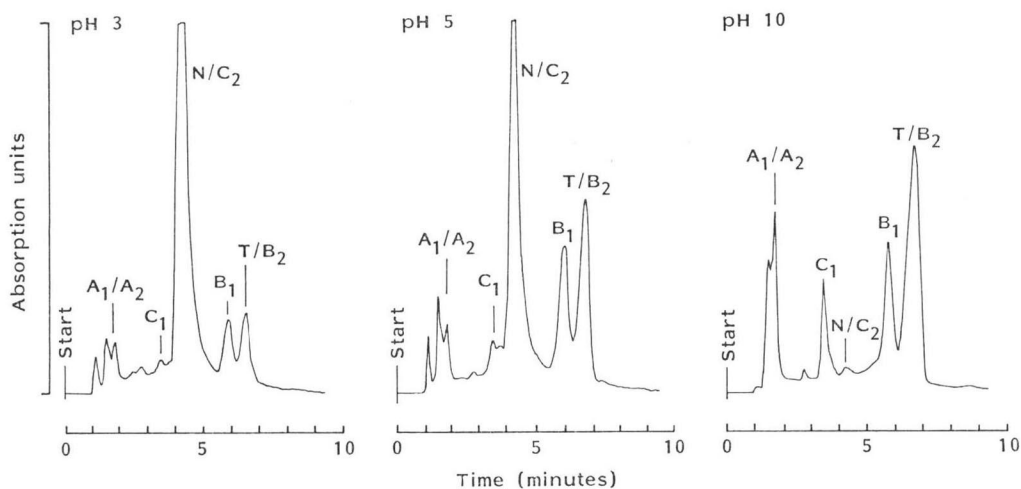
#### Fermentation Studies

*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<sub>3</sub> (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. 2. High pressure liquid chromatograms of culture media extracts at different pH values.  
 HPLC conditions: Lichrosorb RP-18 5  $\mu$ m, 125  $\times$  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  $\mu$ liters 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<sub>1</sub>, A<sub>2</sub> and B<sub>2</sub>, 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<sub>1</sub>, A<sub>2</sub> and B<sub>2</sub> respectively were pooled. The solvent was removed under reduced pressure to yield 760 mg of bafilomycins A<sub>1</sub>, A<sub>2</sub> and 60 mg of bafilomycin B<sub>2</sub>.

Separation of bafilomycins A<sub>1</sub> and A<sub>2</sub> 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  $\mu$ m, 250  $\times$  16 mm  $\phi$ ) 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<sub>1</sub> and 36 mg A<sub>2</sub>.

Fraction III, containing mainly bafilomycins B<sub>1</sub> and B<sub>2</sub>, 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<sub>1</sub> and B<sub>2</sub>. On the basis of TLC-analysis the fractions containing bafilomycins B<sub>1</sub> and B<sub>2</sub> 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<sub>1</sub> and 41 mg B<sub>2</sub>.

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<sub>1</sub> and C<sub>2</sub>, 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<sub>1</sub> and bafilomycin C<sub>2</sub> respectively, were pooled (based on TLC and HPLC-analysis) and the solvent was removed under reduced pressure to yield 120 mg bafilomycin C<sub>1</sub> and 24 mg of bafilomycin C<sub>2</sub>.

#### 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. <sup>1</sup>H and <sup>13</sup>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.

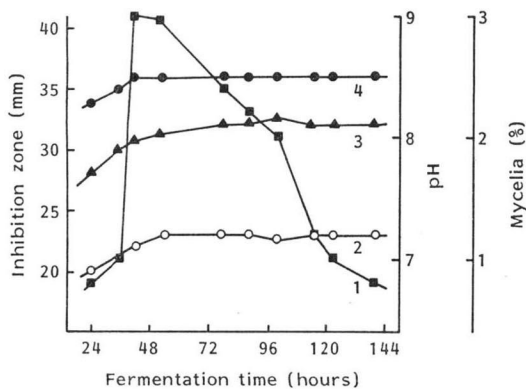
## Results

### 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 anti-biotically 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. R<sub>f</sub> values on TLC (Silica Gel 60F<sub>254</sub>, E. Merck, Darmstadt, West Germany) are as follows: bafilomycin A<sub>1</sub> 0.51; A<sub>2</sub> 0.52; B<sub>1</sub> and B<sub>2</sub> 0.43 and C<sub>1</sub> and C<sub>2</sub> 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<sub>2</sub>SO<sub>4</sub>  
 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.

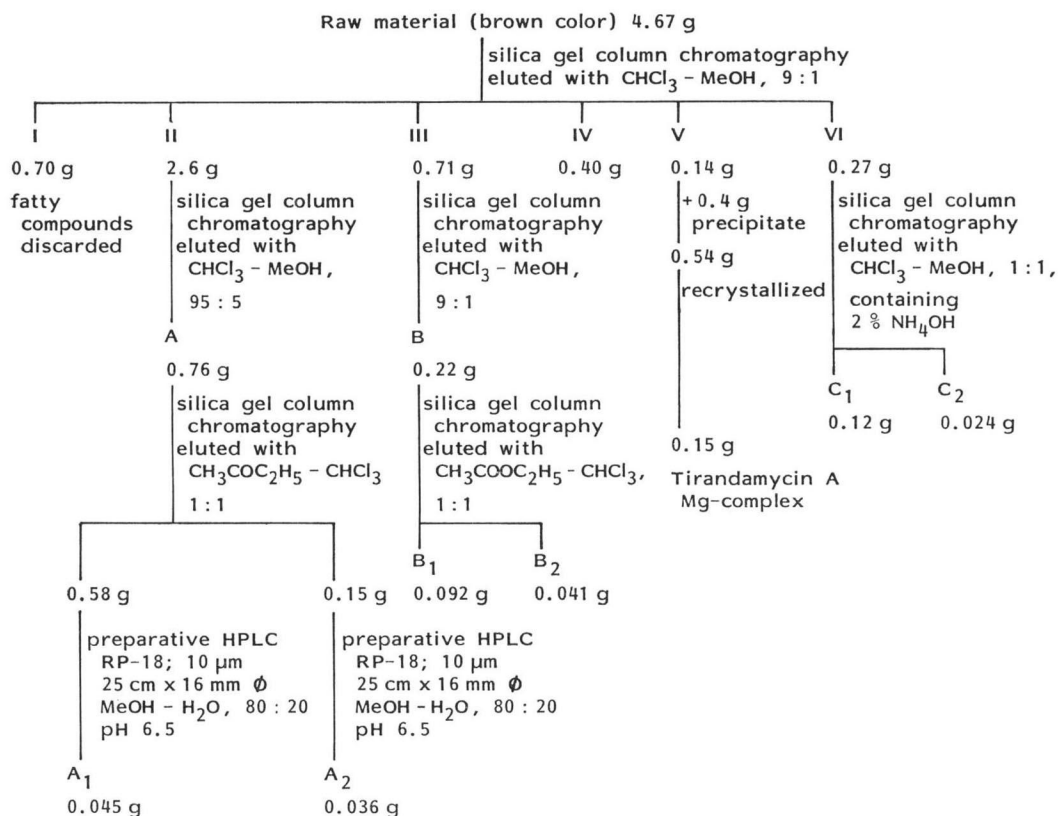


Table 1. Physico-chemical properties of the bafilomycins.

Bafilomycin	mp °C (dec)	$\lambda_{\text{max}}^{\text{MeOH}}$ nm ( $\epsilon$ )	$\nu_{\text{max}}^{\text{KBr}}$ ( $\text{cm}^{-1}$ )
A <sub>1</sub>	103~106	245 (25,000), 280 (12,100)	3350, 2920, 1680, 1245, 1100, 915
A <sub>2</sub>	116~119	245 (25,000), 280 (12,100)	3400, 2920, 1680, 1245, 1100
B <sub>1</sub>	89~96	248 (35,000), 285 (18,500), 355 (2,500)	3450, 3200, 2900, 1720, 1690, 1350, 1240, 1170, 1100
B <sub>2</sub>	—	—	—
C <sub>1</sub>	145~150	245 (22,000), 280 (12,000), 335 (4,800), 350 (2,500)	3450, 2900, 1715, 1690, 1250, 1100, 1000
C <sub>2</sub>	—	—	—

— Not determined.

( $\text{CHCl}_3$  - 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  $3300 \sim 3500 \text{ cm}^{-1}$ ,  $1670 \sim 1690 \text{ cm}^{-1}$ , and at  $1240 \sim 1250 \text{ cm}^{-1}$ . The IR spectra of bafilomycins B and C show additional bands due to an ester group at  $1710 \sim 1730 \text{ cm}^{-1}$  and at  $1220 \text{ cm}^{-1}$  (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  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectroscopy. Conventional proton spin decoupled spectra, single frequency off-resonance spectra, internuclear double resonance spectra

Table 2. Mass spectra of bafilomycins.

Bafilomycin	M <sup>+</sup> (FD) m/z	Fragment ion (EI) m/z									Molecular formula		
A <sub>1</sub>	622	568	525	399	368	338	209	169	137	109	C <sub>35</sub> H <sub>55</sub> O <sub>9</sub>		
A <sub>2</sub>	636	568	525	399	368	338	209	169	137	109	C <sub>36</sub> H <sub>60</sub> O <sub>9</sub>		
B <sub>1</sub>	(815*)	568	525	399	368	338	211	169	137	113	109	C <sub>44</sub> H <sub>85</sub> O <sub>13</sub> N	
B <sub>2</sub>	829											C <sub>45</sub> H <sub>87</sub> O <sub>13</sub> N	
C <sub>1</sub>	(720)	692	568	525	400	368	338	209	169	137	123	109	C <sub>39</sub> H <sub>60</sub> O <sub>12</sub>
C <sub>2</sub>	734												C <sub>40</sub> H <sub>62</sub> O <sub>12</sub>
Monoacetyl-A <sub>1</sub>	664	646	568	525	399	368	338	209	169	137	109		C <sub>37</sub> H <sub>60</sub> O <sub>10</sub>
Diacetyl-A <sub>1</sub>	706	610	568	525	430	376	333	217	191	137	109		C <sub>39</sub> H <sub>62</sub> O <sub>11</sub>

( ): Not detected.

\* In the FD-MS appears m/z 839 (M+H+Na).

Table 3. <sup>1</sup>H and <sup>13</sup>C NMR chemical shifts and coupling constants of bafilomycin A<sub>1</sub>.

Carbon	δ <sub>H</sub> (ppm)	Multiplicity	J (Hz)	δ <sub>C</sub> (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		
9b	1.94	dd	14.0/11.5	41.3	t
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		
20b	1.16	ddd	11.9/11.0/2.2	43.5	dd
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 <sub>3</sub>	3.63	s		59.9	q
C14-OCH <sub>3</sub>	3.24	s		55.5	q

Measured in CDCl<sub>3</sub> 99.95% at 400 MHz (<sup>1</sup>H NMR) and in CDCl<sub>3</sub> at 100.62 MHz (<sup>13</sup>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 <sub>1</sub>	B <sub>1</sub>	C <sub>1</sub>	Organism	A <sub>1</sub>	B <sub>1</sub>	C <sub>1</sub>
<i>Achromobacter geminiani</i>	—	—	—	<i>Streptomyces prasinus</i> TÛ 30	—	tr	tr
<i>Agrobacterium tumefaciens</i>	—	—	—	<i>S. lavendulae</i> TÛ 35	—	—	—
<i>Escherichia coli</i> K 12	—	—	—	<i>S. glaucescens</i> TÛ 49	—	—	tr
<i>E. coli</i> K 12*	—	—	—	<i>Candida albicans</i> TÛ 565	—	tr	tr
<i>Salmonella typhimurium</i>	—	—	—	<i>C. albicans</i> ampicillin resistant	—	—	—
<i>Pseudomonas aeruginosa</i>	—	—	—	<i>Candida membranaefaciens</i>	—	—	—
<i>Pseudomonas fluorescens</i>	—	—	—	<i>Hansenula anomala</i>	—	13	14
<i>Proteus mirabilis</i>	—	tr	10	<i>Lipomyces lipofer</i>	—	—	tr
<i>Proteus vulgaris</i>	—	—	tr	<i>Nadsonia fulvescens</i>	15	26	28
<i>Bacillus brevis</i>	—	9	10	<i>Rhodotorula rubra</i>	tr	16	18
<i>Bacillus subtilis</i> ATCC 6051	—	—	—	<i>Saccharomyces cerevisiae</i> TÛ 125	—	—	—
<i>B. subtilis</i> ATCC 6051*	—	11	15	<i>S. cerevisiae</i> FL 200	—	13	15
<i>B. subtilis</i> A 14	—	—	9	<i>Saccharomyces</i> sp. FL 599-1B	13	21	23
<i>B. subtilis</i> F-24-2-WA	—	—	tr	<i>Schizosaccharomyces pombe</i>	—	23	17
<i>Clostridium pasteurianum</i>	—	9	14	<i>Wingea robertsii</i>	tr	10	14
<i>Staphylococcus aureus</i>	—	—	—	<i>Alternaria mali</i> polyoxin resistant	26	>30	>30
<i>Micrococcus luteus</i>	—	—	—	<i>A. mali</i> polyoxin sensitive	24	>30	>30
<i>Micrococcus luteus</i>	—	12	14	<i>Aspergillus niger</i>	—	20	19
<i>Micrococcus luteus</i> W 45	—	14	19	<i>Aspergillus terreus</i>	—	19	18
<i>Corynebacterium insidiosum</i>	—	10	12	<i>Botrytis cinerea</i>	22	27	24
<i>Corynebacterium rathayi</i>	—	—	tr	<i>Coprinus cinereus</i>	14	>30	>30
<i>Arthrobacter aureus</i>	—	10	11	<i>Mucor hiemalis</i> TÛ 179/180	14	32	31
<i>Arthrobacter crystallopoietes</i>	—	13	14	<i>Mucor miehei</i> TÛ 284	13	27	25
<i>Arthrobacter globiformis</i>	—	14	15	<i>M. miehei</i> TÛ 284*	19	40	39
<i>Arthrobacter pascens</i>	—	14	16	<i>Neurospora crassa</i> Arg <sup>-</sup>	—	12	14
<i>Brevibacterium sterolicum</i>	—	—	—	<i>Paecilomyces varioti</i> TÛ 137	10	22	22
<i>Streptomyces antibioticus</i> TÛ 4	—	—	—	<i>Penicillium puberulum</i>	—	21	21
<i>S. griseus</i> TÛ 17	—	—	tr	<i>Phythium debaryanum</i>	—	18	22
<i>S. diastatochromogenes</i> TÛ 20	—	—	—	<i>Rhizoctonia solani</i>	23	29	29
<i>S. violaceoruber</i> TÛ 22	—	—	—	<i>Saprolegnia asterophora</i>	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<sub>2</sub>, B<sub>2</sub> and C<sub>2</sub> are not native, and are formed during the isolation procedure.

The structure of hygrolidin shows some similarity to bafilomycin C<sub>1</sub><sup>6)</sup>. 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<sup>7,8)</sup>.

The recently published adenosine triphosphatase inhibitors, L-681,110 A<sub>1</sub> and L-681,110 A<sub>2</sub><sup>9)</sup>, are identical with the bafilomycins C<sub>1</sub> and C<sub>2</sub> 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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