METABOLIC PRODUCTS OF MICROORGANISMS. 200*

ISOLATION AND CHARACTERIZATION OF NIPHITHRICINS A, B, AND ELAIOPHYLIN, ANTIBIOTICS PRODUCED BY *STREPTOMYCES VIOLACEONIGER*

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Fermentations of *Streptomyces violaceoniger* TÜ 905 produce the antifungal antibiotics niphithricins A, B, elaiophylin and nigericin. The niphithricins have been characterized as new macrolide antibiotics, and the previously unknown structure of elaiophylin was determined to be a macrodiolide. The niphithricins were biologically active against Gram-positive bacteria and fungi. The mode of action is attributed to an alteration of the membrane permeability.

In the course of screening for antibiotics active against *Botrytis cinerea*, two new antifungal antibiotics were isolated in addition to the known antibiotics elaiophylin[1] and nigericin[2], from the fermentation broth of *Streptomyces violaceoniger* TÜ 905. These antibiotics were designated niphithricins A and B.

In this paper the authors describe the production, isolation and chemical properties of niphithricins and elaiophylin, and in addition the biological properties of the niphithricins.

Material and Methods

Fermentation

The fermentor inoculum was prepared by multiple-stage submerged culture in a medium containing 1% mannitol and 1% soybean meal in tap water (final pH 7.5). The initial stage was grown in 500 ml Erlenmeyer flasks containing 100 ml of media. They were incubated at 27°C on a rotary shaker at 140 rpm.

For the production of niphithricins a 10-liter fermentor (NB 10, New Brunswick) containing 9 liters of media was inoculated with 1 liter of the shaking cultures and incubated for 48 hours at 27°C, 150 rpm and aeration of 120 liters/hour. The production fermentor (NB 100, New Brunswick) containing 90 liters of media was incubated after inoculation with the 10-liter fermentor for 110~120 hours at 27°C, 120 rpm and aeration of 1,275 liters/hour.

For the production of elaiophylin and nigericin, a 20-liter fermentor (b 20, Giovanola) equipped with an intensor and containing 18 liters of media was inoculated with 2 liters of the shaking cultures

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and incubated for 72 hours at 27°C, 800 rpm and aeration of 180 liters/hour. The production fermentor (b 200, Giovanola) equipped with an intensor and containing 180 liters of media was incubated after inoculation with the 20-liter fermentor for 140 hours at 27°C, 800 rpm and aeration of 3,000 liters/hour.

Isolation and Purification

Hyflo super-cel (2%) was added to the fermentation broth (180 liters) which was then filtered. The antibiotics in the culture filtrate were adsorbed on Amberlite XAD-2 (column dimensions 15× 150 cm). Inactive impurities were washed out with water - methanol (1: 1) and water - acetone (6: 4). The desorption of the antibiotics was brought about by elution with acetone. The eluent was concentrated *in vacuo*.

The mycelia cake was extracted four times with methanol, the extract was concentrated and the combined concentrates were extracted four times with methylene chloride. The aqueous phase which contained the niphithricins A and B, was then extracted four times with *n*-butanol and the remaining inactive aqueous layer discarded.

Elaiophylin: The methylene chloride extracts containing elaiophylin and nigericin were evaporated *in vacuo* to a brown syrup (230 g). This was dissolved in 500 ml of ethyl acetate, whereupon a white crystalline precipitate was formed. After 48 hours the crystals (18.2 g) were collected by filtration. By recrystallization from ethyl acetate, pure crystals of elaiophylin were obtained, which gave a fine powder (m.p. 199°C) after drying in high *vacuo* over P_2O_5.

Niphithricins A and B: The aqueous solution after the extraction of elaiophylin and nigericin still showed antifungal activity, which was not extractable with methylene chloride. It was extracted with *n*-butanol and the extract evaporated under reduced pressure to yield 140 g of a brown resin. This was adsorbed on a silica gel column (10×30 cm). After washing with chloroform and chloroform - methanol (3: 1), the antibiotics were eluted with chloroform - methanol (1: 1) and (1: 3). The active eluate (71 g) was separated by counter-current distribution (500 ml funnels, 70 transfers) with ethyl acetate - *n*-butanol - methanol - water (7: 3: 1: 9). The active fractions were combined, concentrated (40 g residue) and further purified by chromatography on Sephadex LH-20 (5×150 cm). The antibiotics were eluted with methanol and yielded 25 g of a yellow powder. This consisted predominantly of niphithricin A, Rf=0.20, and niphithricin B, Rf=0.16 on silica TLC plates with *n*-butanol - acetic acid - water (67: 23: 10). Several faint spots with higher Rf values were also obtained when pure niphithricin A or B was left standing in solution, and apparently originate from the decomposition of the antibiotics.

The separation of the two major compounds by counter-current distribution (Craig apparatus, 1700 transfers), chromatography on silica gel or reversed-phase chromatography was unsatisfactory. Better results were obtained with droplet counter-current chromatography[8,9]. The 300 tubes of an apparatus model DCC-A (Tokyo Rikakikai) were filled with the lower phase of a solvent system consisting of chloroform - methanol - water (35: 65: 40, v/v). A crude mixture of 628 mg of niphithricins was introduced with 4 ml each of both phases. The upper phase was pressed through the system with 5~6 bar (12 ml/hour, 25°C). Fractions of 6 ml each were collected over 3 days. Appropriate fractions were combined and evaporated under reduced pressure (Table 1). The pure fractions of niphithricins A and B respectively were precipitated four times from methanol - water and finally dried over P_2O_5 in high *vacuo*, yielding colorless amorphous powders.

Table 1. Droplet counter-current chromatography of niphithricins.

Fraction	Amount	Remarks
6~30	220 mg	polar impurities
52~66	37 mg	pure niphithricin B
67~89	41 mg	mixture
90~112	144 mg	pure niphithricin A
134~144	28 mg	side component, mixture
Stat. phase	110 mg	nonpolar impurities
Total	580 mg	92% recovery

Chemical Characterization

Niphithricin A: $[\alpha]_D^{25}+14°$ (*c* 1.23, MeOH). TLC on silica gel plates F_{254} with *n*-butanol - acetic acid - water (67: 23: 10), Rf=0.20. TLC on Whatman RP-18 plates with methanol - water

(10: 3), Rf=0.14. Potentiometric titration in methyl cellosolve - water (8: 2), *pKa* 4.91, equivalent weight found: 1,128. UV spectrum in ethanol $\lambda_{infl.}$ 215 nm (log ε 4.2). IR spectrum in KBr see Fig. 4. ^{1}H NMR spectrum (360 MHz) in DMSO-d_6 see Fig. 3. ^{13}C NMR spectrum in CD_3OD see Fig. 2a.

Anal. Calcd. for $C_{54}H_{95}N_3O_{17}\cdot H_2O$: C 60.25, H 9.08, N 3.90%. M.W. 1,076.35
Found: C 60.31, H 9.09, N 4.08%. M.W. 1,006*
C 60.54, H 9.16, N 3.86%.

Niphithricin B: $[\alpha]_D^{25}+15°$ (*c* 0.85, MeOH). TLC on silica gel plates F_{254} with *n*-butanol - acetic acid - water (67: 23: 10), Rf=0.16. TLC on Whatman RP-18 plates with methanol - water (10: 3), Rf=0.20. Potentiometric titration in methyl cellosolve - water (8: 2), *pKa* 4.88, equivalent weight found: 1,155. UV spectrum in ethanol $\lambda_{infl.}$ 215 nm (log ε 4.2). IR spectrum practically superimposable with that of niphithricin A. ^{1}H NMR spectrum nearly identical with that of niphithricin A. ^{13}C NMR spectrum in CD_3OD see Fig. 2b.

Anal. Calcd. for $C_{54}H_{95}N_3O_{17}\cdot 2H_2O$: C 59.26, H 9.12, N 3.83%. M.W. 1,094.37
Found: C 59.36, H 9.08, N 3.35%. M.W. 996*
C 59.03, H 9.16, N 4.41%.

Interconversion of Niphithricins A and B: Pure niphithricin A (2 mg) in 1 ml chloroform - methanol (1: 1) was kept at room temperature for 42 days. TLC on silica gel showed the presence of two major compounds with the Rf values of niphithricins A and B (ratio approx. 1: 1), and several minor components with slightly higher Rf values, apparently decomposition products of niphithricin and identical with minor contaminants of crude niphithricin.

When 2 mg of pure niphithricin B was treated under the same conditions, an identical mixture of niphithricin A and B and decomposition products was obtained.

Elaiophylin: $[\alpha]_D^{25}-46.9°$ (*c* 0.83, $CHCl_3$). TLC on silica gel plates F_{254} with chloroform - methanol (9: 1), Rf 0.25. UV spectrum in ethanol λ_{max} 253 nm (log ε 4.83).

Anal. Calcd. for $C_{54}H_{88}O_{13}\cdot\frac{1}{2}H_2O$: C 62.70, H 8.67, O 28.62%.
Found: C 62.66, H 8.57, O 28.51%.

The compound was identified as elaiophylin[1] by TLC and IR spectra. The ^{13}C NMR spectrum in DMSO-d_6 is shown in Fig. 5.

Biological Characterization

Lysis of EDTA-lysozyme Spheroplasts of *Escherichia coli*: Spheroplasts of *E. coli* K 12W *1485* (Köln) were prepared according to OSBORN *et al.*[3] and diluted with 0.25 M sucrose - 3 mM tris-HCl pH 7.8 to an optical density at 578 nm (A_{578}) of 0.5. Different amounts of niphithricin A (in 10 μl of methanol) were added to 1 ml of the spheroplast suspension and the lysis was monitored by plotting the A_{578} with a spectrophotometer (Zeiss PMQ 2) connected to a recorder.

Release of Cellular Constituents from *Saccharomyces cerevisiae* FL 200: *Saccharomyces cerevisiae* was grown in a medium consisting of 0.4% yeast extract, 1% malt extract, and 0.4% glucose at an initial pH of 7.3. Cells of late logarithmic phase were spun down, washed three times with distilled water and finally resuspended in the original volume with distilled water to give a suspension of approximately 5×10^8 cells/ml (viable counts). After incubation for 100 minutes at 27°C with different amounts of niphithricin A, the release of amino acids and potassium was determined in the supernatant after removing the cells by centrifugation (according to ROSEN[4], and by atomic absorption spectroscopy, respectively).

Release of Entrapped Glucose Marker from Liposomes: Liposome preparation; liposomes were prepared by sonicating a suspension of 0.5 g L-α-phosphatidylcholine (from soy beans) suspended in 11 ml of 10 mM MOPS, pH 6.5 - 0.25 mM EDTA for a total of 10 minutes (at 4°C, N_2-atmosphere). The liposomes were spun down (100,000 *g*, 1 hour), the pellet was resuspended in 10 ml of 0.5 M glucose, incubated for 30 minutes at 40°C and dialyzed after cooling to 4°C against 0.25 M NaCl (3×2 liters, for 18 hours) to remove most of the untrapped marker.

Spectrophotometric assay of glucose release; release of glucose marker was detected enzymatically by a NAD^+-dependent reaction (Glc-DH-method, Merckotest, Merck, Darmstadt). The test

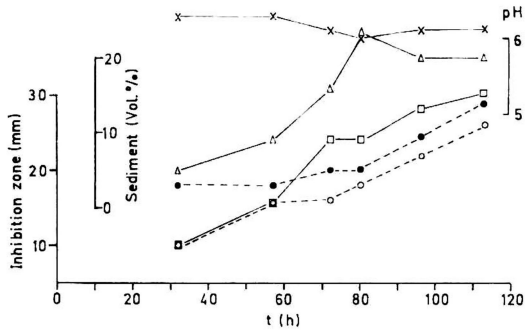
* Vapour pressure osmometry in ethanol.

Table 2. Dependence of antibiotic production on fermentor type.

Fermentor	Niphithricins A and B	Elaiophylin and nigericin
NB 100	20 g (33%)	40 g (66%)
b 200	0.2 g (0.2%)	100 g (99.8%)

Fig. 1. Time course of antibiotic production by *Streptomyces violaceoniger* in a 100-liter fermentor (NB 100). Bioassay against *Botrytis cinerea*.

(□) Biological activity of the fermentation broth, (○) biological activity of the culture filtrate, (●) biological activity of the mycelium, (△) mycelial growth, (×) pH.



was performed at room temperature. To 1 ml of enzyme-buffer solution (Merckotest No. 3389, containing glucose dehydrogenase and mutarotase), 10 μl of liposome suspension was added.

Results

Production

The production of the niphithricins, elaiophylin and nigericin was dependent on the type of fermentor which was used (Table 2). In a fermentor with a stirring vessel (NB 100), a large amount of niphithricins was produced. In a fermentor containing an intensor (b 200), the production of niphithricins was greatly reduced in favour of elaiophylin and nigericin. The different aeration may be responsible for this phenomenon.

The time course of a typical fermentation is shown in Fig. 1.

Chemical Characterization

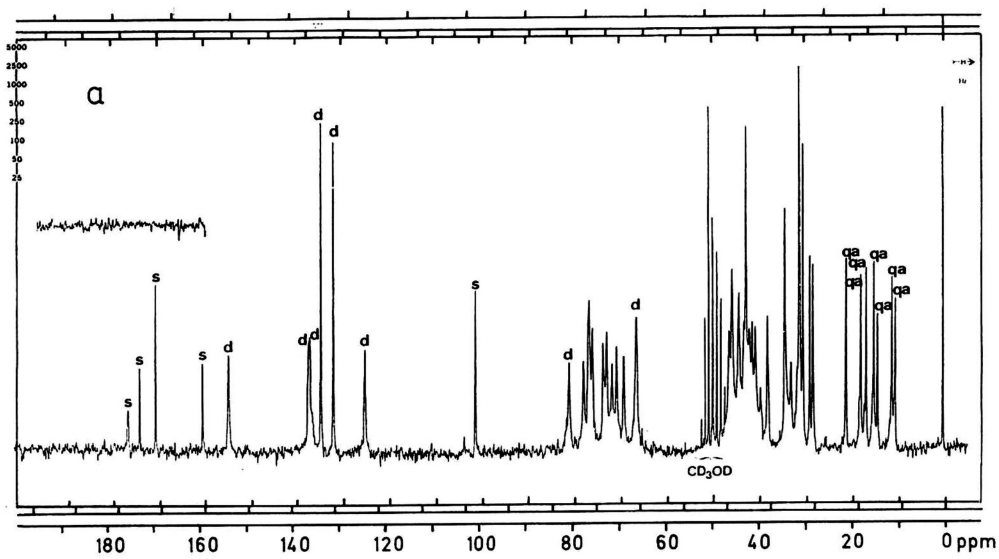
Niphithricins A and B

Both antibiotics were extractable from aqueous solutions with butanol, but not with less polar solvents such as ethyl acetate or chloroform. Partly purified niphithricin gave two spots on TLC which became visible by spraying with sulfuric acid, by exposure to iodine vapor or with reagents recommended for the determination of ureas and guanidines[5,6,7]. The preparative separation of the niphithricins A and B became possible with the new separation technique, droplet counter-current chromatography[8,9]. The compounds were obtained as colorless, amorphous powders, easily soluble in methanol, pyridine, dimethylsulfoxide and dimethylformamide, but not easily soluble or insoluble in water, ethyl acetate and chloroform. All attempts to crystallize the compounds have failed so far.

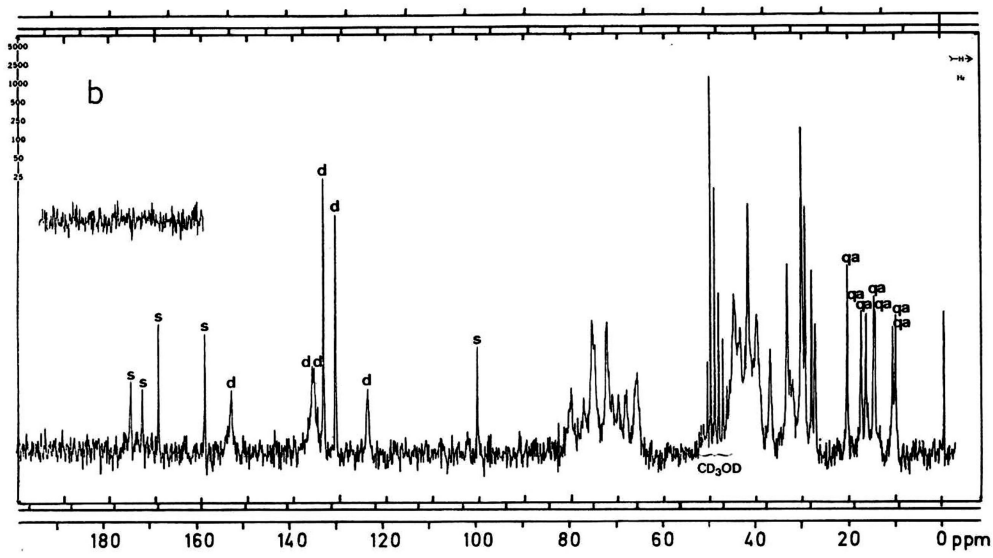
The elementary analyses do not allow an unambiguous deduction of the molecular formula. The formula $C_{54}H_{95}N_3O_{17}$ proposed in this paper for both niphithricins, is additionally based on ^{13}C NMR spectra and particularly on field desorption mass spectra. The ^{13}C NMR spectra of the two compounds (Fig. 2 a and b) are very similar. They display approximately 45 distinct peaks. However, the intensities of several of them indicate that they are due to more than one carbon atom. The spectra are in accordance with the presence of 54 carbon atoms. The potentiometric titration of niphithricin A in methyl cellosolve - water (8: 2) gave an equivalent weight of 1,128 (calcd. 1,057), whereas vapor pressure osmometry gave a molecular weight of 1,006. The corresponding values for niphithricin B were 1,155 and 996, respectively. The relatively high deviation from the calculated values is a result of water of crystallization.

Fig. 2. ^{13}C NMR spectra of niphithricins (25.2 MHz in CD_3OD).

a) niphithricin A



b) niphithricin B



The field desorption mass spectra of both niphithricins showed a base peak at 1,058 (calcd. 1,057). A smaller peak at m/z 1,080 ($M^+ + Na$) confirms the assumption that the base peak is a quasimolecular ion peak. The close resemblance of the niphithricins A and B in elementary composition and the identical behaviour in mass spectrometry indicate that they are isomeric. Niphithricin A is transformed into niphithricin B by standing in solution for several weeks and *vice versa*. The equilibrium composition is approximately 1:1 (probably with a slight predominance of A), roughly estimated from spot intensities on TLC. This equilibration is interpreted by us as an epimerization at a hemi-

Fig. 3. ^{1}H NMR spectrum of niphithricin A (360 MHz in DMSO-d_6).

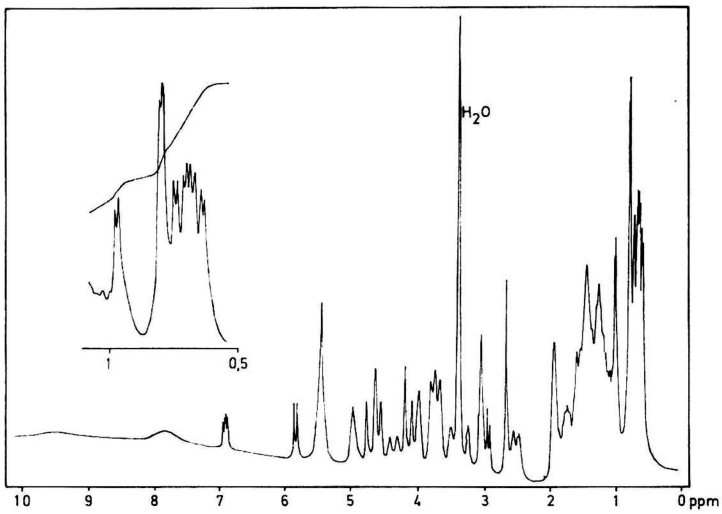
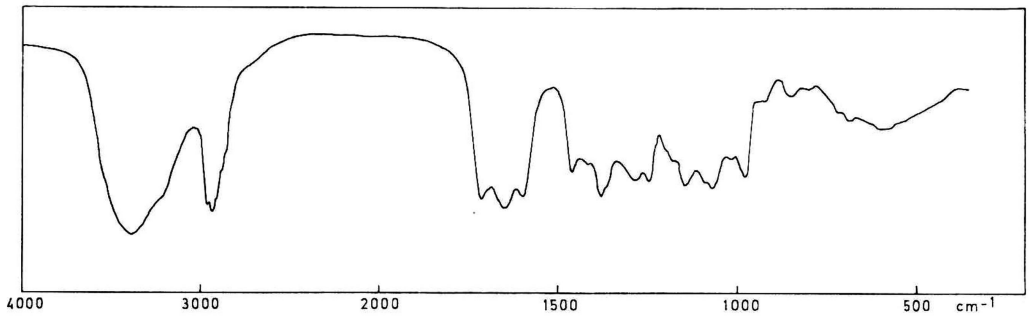


Fig. 4. IR absorption spectrum of niphithricin A (in KBr).



ketal carbon atom, whose presence is indicated by a signal at 99.5 ppm (singlet in the off-resonance spectrum) in the ^{13}C NMR spectrum[10].

The ^{13}C NMR spectrum indicates 7 C-methyl groups (off-resonance quartets from 10 to 20 ppm), whose presence is confirmed by 7 doublets (integral 3H) in the high field region of the ^{1}H NMR spectrum (Fig. 3). The region from 25 to 50 ppm (^{13}C NMR) showed a large series of signals of CH_2- and CH-carbon atoms. The off-resonance splitting cannot be interpreted in detail because of overlapping. The region from 60 to 82 ppm is difficult to interpret. It indicates the presence of approximately 12 carbinyl carbon atoms. The splitting pattern in the off-resonance spectrum is in accordance with the assumption that all carbinyl groups are secondary, however, the multiplicity is not completely discernible because of overlapping.

The niphithricins A and B contain 3 carbon-carbon double bonds, the signals of the olefinic carbon atoms (123~153 ppm) all being doublets in the off-resonance spectra. One of the double bonds is in the α, β-position of an ester (or lactone) group. The corresponding olefinic protons giving a doublet at 5.8 and a doublet of doublets at 6.9 ppm in the ^{1}H NMR spectrum. The proton resonances of the other 4 olefinic hydrogen atoms appear as a broad signal at about 5.3 ppm. The presence of an α, β-

unsaturated ester group is confirmed by the UV spectrum showing an absorption maximum at 215 nm (log ε 4.2) and an IR absorption band at 1710 cm^{-1} (Fig. 4).

A signal at 158.0 ppm in the ^{13}C NMR spectrum of the niphithricins is assigned to a guanidino group whose presence was deduced from a degradation product (to be published). Of the three singlets at 168.0, 171.5 and 174.0 ppm (^{13}C NMR), one is assigned to a conjugated lactone carbonyl group because there is a strong evidence (also from degradation studies) that the niphithricins belong to the macrolide family of antibiotics. The nature of the other two carbonyl groups is not yet clear.

Singlets integrating for 3 protons in the ^1H NMR spectra (2.70 ppm in DMSO-d_6; 2.82 ppm in CD_3OD) point toward the presence of an *N*-methyl group. In the ^{13}C NMR spectra the corresponding signal is hidden under the cluster of signals around 40 ppm. Subsequently, the existence of an *N*-methyl group was confirmed by an *N*-methylguanidino degradation product (to be published).

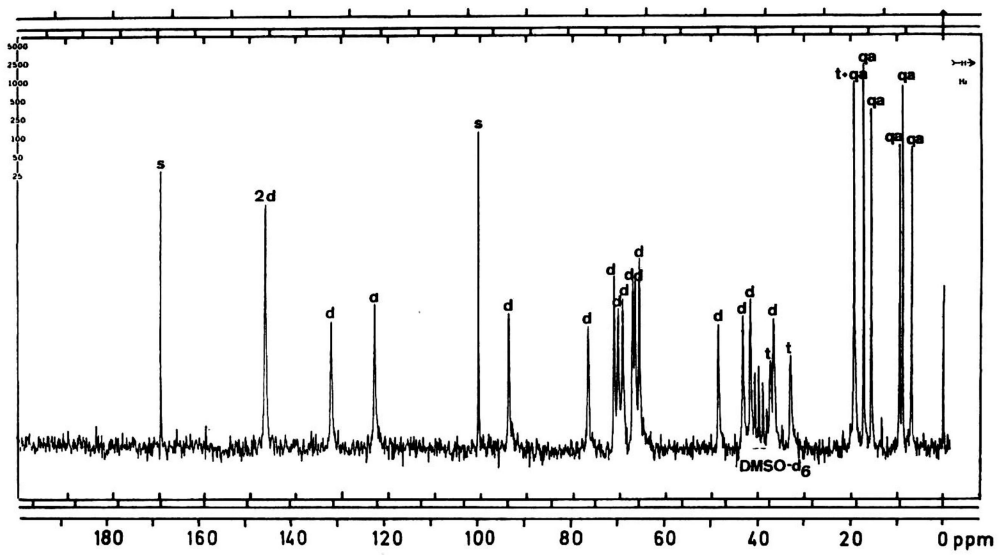
Elaiophylin

This compound was first isolated more than 20 years ago[1]. A sample of the original compound, kindly provided by Dr. ARCAMONE (Milan, Italy) allowed us to prove the identity by direct comparison (TLC, IR and UV spectra). Obviously identical with elaiophylin is azalomycin B[11,12]. This conclusion in not only based on the comparison of our spectra (IR, UV and ^1H NMR) and other physical and biological properties with those published in the literature, but also on several identical degradation products[13,14,15]. These will be described in a forthcoming paper[16].

Closely related to, or identical with elaiophylin, is also the antibiotic sapromycetin A[17].

ARCAMONE assigned the compound a rational formula $(C_6H_{10}O_2)_n$ which is (when n=9) nearly identical with the molecular formula $C_{54}H_{88}O_{18}$ proposed for the niphithricins. Furthermore TAKAHASHI's formula of $C_{56}H_{92}O_{19}$, based on elemental analysis and molecular weight determinations of azalomycin B, gives nearly the same calculated percentage of the constitutive elements. The analysis of our samples fit best with a rational formula $C_{27}H_{44}O_9$ which is in good agreement with the ^{13}C NMR spectrum (Fig. 5) which displays 25 distinct signals. However, a broadened signal at 19.1 ppm is

Fig. 5. ^{13}C NMR spectrum of elaiophylin (25.2 MHz in DMSO-d_6).



split in the off-resonance spectrum into a quartet and a triplet, and accounts therefore, for two carbon atoms. A signal of increased intensity at 144.9 ppm (Fig. 5) must be assigned to two olefinic carbon atoms.

The mass spectrum of elaiophylin, although lacking a molecular ion peak, shows fragment peaks up to *m/z* 800 (calcd. for $C_{27}H_{44}O_9$: 512), and a molecular weight determination by vapor pressure osmometry gave a value of 1,036. The C_{27}-formula should therefore be doubled to $C_{54}H_{88}O_{18}$ (calcd. 1,025.24). The ^{13}C NMR spectrum with signals of 27 carbon atoms shows that the molecule is composed of two identical halves.

Each half of the molecule contains 6 C-methyl groups (signals between 6 and 20 ppm), 3 methylene groups (triplets at 19.1, 32.7 and 37.0 ppm), 4 methine carbon atoms (4 doublets between 36 and 48 ppm), and 7 carbon atoms attached to alcohol, ether or ester oxygen atoms. The doublet at 92 ppm is assigned to C(1) of a 2-deoxy-L-fucose residue, which was identified as a part of elaiophylin (azalomycin B) by TAKAHASHI *et al.*[14] The presence of 2-deoxy-L-fucose was confirmed by our own degradation studies which will be reported in a forthcoming paper[16]. A singlet at 99.2 ppm must be assigned to a hemiketal carbon atom since this signal is also present in octahydro-elaiophylin and can therefore not belong to an olefinic carbon atom. Hemiketal formation has been observed in several polyene macrolide antibiotics, the hemiketal carbon atoms giving signals in this region[10,18].

From the UV spectrum (λ_{max} 252 nm), TAKAHASHI *et al.*[13] postulated an $\alpha, \beta, \gamma, \delta$-unsaturated ester group which is in agreement with 4 signals of olefinic carbon atoms in the range of 120~145 ppm in the ^{13}C NMR spectrum (Fig. 5) and corresponding signals of olefinic hydrogen atoms in the ^1H

Table 3. Antimicrobial spectrum of niphithricins A and B.

Organism	Incubation temperature	Medium*	MIC (μg/ml)**	
			Niphithricin A	B
Bacillus subtilis Cohn ATCC 6051	37°C	1	30	100
Staphylococcus aureus Rosenbach TÜ 202	37°C	1	30	100
Streptococcus faecalis ATCC 9790	37°C	2	>100	>100
Mucor racemosus DSM 62 760	27°C	3	10	30
Mucor hiemalis Wehmer (−) TÜ 180	27°C	3	30	30
Saccharomyces cerevisiae Hansen FL 200	27°C	3	30	100
Saccharomyces cerevisiae Hansen FL 500–1 B	27°C	3	10	30
Candida albicans (Robin) Berkhout TÜ 164	27°C	3	10	100
Debaromyces hansenii DSM 70 238	27°C	3	10	30
Geotrichum candidum Link CBS 109.12	24°C	3	3	10
Cladosporium butyri Jensen CBS 159.54	24°C	3	3	10
Paecilomyces varioti Bainier TÜ 137	37°C	3	10	10
Aspergillus oryzae DSM 63 303	27°C	3	10	30
Aspergillus panamensis CBS 120.45	27°C	3	3	10
Ascoidea rubescens Brefeld TÜ 195	27°C	3	3	10
Botrytis cinerea Persoon ex Fries TÜ 157	24°C	3	10	30
Ustilago maydis TÜ 567	27°C	3	3	10

* 1=8 g nutrient broth, 5 g NaCl/liter water.
2=11 g glucose, 10 g tryptone, 5 g yeast extract, 13.7 g K_2HPO_4/liter water, pH 7.6.
3=4 g glucose, 10 g malt extract, 4 g yeast extract/liter water, pH 7.3.

** serial dilution test; size of inoculum: 10^5 cells (spores)/ml.

NMR spectrum[13]. From the coupling constants of these protons, we concluded that both double bonds are *trans*-disubstituted. Finally the singlet at 167.2 ppm (^{13}C NMR) is assigned to a conjugated ester carbonyl group in agreement with an IR absorption maximum at 1695 cm^{-1}.

Nigericin

From the mother liquors of elaiophylin, large amounts of nigericin could be isolated by known procedures. The compound was identified by direct comparison with an authentic sample (Eli Lilly & Co.) by TLC, IR, ^{1}H NMR and ^{13}C NMR spectra of the free acid and the sodium complex.

Biological Characterization

Niphithricins A and B showed a rather broad spectrum of antimicrobial activity (Gram-positive bacteria and fungi) with MIC's for most of the tested organisms between 3~30 μg/ml for niphithricin A and 10~100 μg/ml for niphithricin B in the serial dilution test (Table 3).

Spheroplasts of *E. coli* are sensitive to niphithricin A. Fig. 6 shows a rapid lysis of the spheroplasts after addition of the antibiotic to a final concentration of 10 μg/ml and more. *E. coli*, with an intact outer membrane, is not affected by niphithricin A; no inhibition of growth could be seen in the agar plate diffusion test, when up to 100 μg of the antibiotic were included per filter disk.

Fig. 6. Effect of niphithricin A on EDTA-lysozyme spheroplasts of *Escherichia coli*.

The arrow indicates the addition of the antibiotic (in 10 μl of MeOH) to 1 ml of spheroplast suspension to final concentrations of:

(a) 0 (control, no antibiotic added), (b) 2.5 μg/ml, (c) 5 μg/ml, (d) 10 μg/ml, (e) 25 μg/ml, (f) 50 μg/ml.

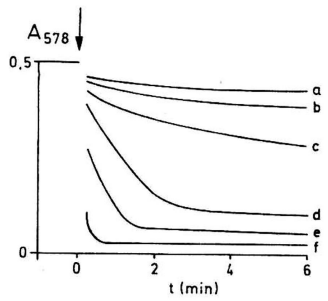
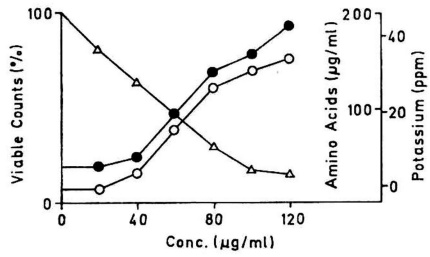


Fig. 7. Effect of niphithricin A on viable counts and on leakage of cellular constituents.

(△) Viable counts, (○) concentration of amino acids in the supernatant, (●) concentration of potassium in the supernatant.



By incubation of *Saccharomyces cerevisiae* with niphithricin A, a release of cellular constituents (amino acids, potassium) was observed (Fig. 7) which can be correlated to the number of killed cells. The decrease in viable counts also demonstrates the fungicidal action of niphithricin A.

Fig. 8. Effect of niphithricin A on liposome permeability.

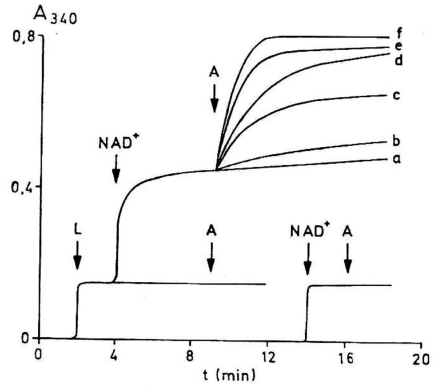
L: Addition of 10 μl liposome suspension.

A: Addition of niphithricin A (in 20 μl of methanol).

NAD^+: Addition of 20 μl 0.22 M NAD^+.

Niphithricin was added to final concentrations of:

(a) 0 (control, no antibiotic added), (b) 20 μg/ml, (c) 30 μg/ml, (d) 40 μg/ml, (e) 60 μg/ml, (f) 100 μg/ml.



Entrapped glucose marker was released from liposomes upon addition of the antibiotic (Fig. 8), so demonstrating a disorganization of the lipid bilayer membrane of the liposomes. The release of glucose is monitored by increase in A_{340} now available to enzymatic reaction. The figure does not show a kinetic of glucose release because of the rate-limiting step of enzymatic reaction. Addition of the liposome suspension caused an increase in A_{340} because of light dispersion. The increase in A_{340} after addition of NAD^+ solution was caused by traces of NADH in the NAD^+ solution and enzymatic oxidation of glucose not entrapped in liposomes.

Discussion

From a comparison of the ^{13}C and ^1H NMR spectra of the niphithricins with those of niphimycin[19], as well as from the very similar antibiotic properties, we can conclude that a close relationship exists between the two antibiotics. However, a clear difference in the UV spectra (the niphimycins contain a conjugated diene group giving a high absorption maximum at 233 nm) proves the non-identity. The niphimycins and niphithricins can also be distinguished by TLC. Minor differences are discernible in the ^1H and ^{13}C NMR spectra, although the general spectral patterns are very similar. Closely related to or identical with niphimycin, are the antibiotics melanosporin[1], antibiotic E-79[17], antibiotics 15565 A and B[20] and scopafungin[21].

Similarities also exist between the niphithricins and primycin[22~28] in their analytical properties as well as in their biological activities. However, primycin contains a glycosidally bound arabinose residue, whereas no sugar constituents are present in the niphithricins and niphimycins. Major differences in the structure of the macrolide rings follow from degradation studies (to be published).

The molecular formula $C_{54}H_{88}O_{18}$ of elaiophylin demands the presence of 11 rings and double bonds. Two rings are present in the sugar moieties and two in the hemiketal rings. From the NMR spectra the presence of four double bonds is evident. Finally, the compound contains two ester carbonyl groups. There remains a single parameter of unsaturation which must be assigned to a dilactone ring. Elaiophylin belongs therefore to the small family of macrodiolides, together with boromycin[29], the aplasmomycins[30], the antibiotically inactive conglobatin[31] and a few metabolites of lower fungi.

The lysis of spheroplasts suggested an interference of niphithricin A with the membrane organization. Similar effects were observed for detergents[32,33], polyene antibiotics[34], iturin[35], pyrrolnitrin[36], which cause membrane disorganization. This hypothesis was further enhanced by the release of cytoplasmic constituents of *Saccharomyces cerevisiae* after the addition of niphithricin A. The same was shown to be true for tyrocidines[37,38], polymyxin[39], nystatin[40], miconazole[41], azalomycin F[42], and others which also cause an alteration of membrane permeability of sensitive organisms.

The mode of action of niphithricin A was further supported by tests using liposomes, which are used as model systems for the investigation of permeability changes of membranes[43]. Release of a trapped marker demonstrates an alteration in liposome permeability as has been shown for polyenes[44,45], and imidazole antifungals[46]. Niphithricin A also causes the release of a trapped marker (glucose).

Therefore we propose that the cytoplasmic membrane is the target of niphithricin A action. This is in agreement with the observed relatively broad spectrum of antimicrobial activity and with the unspecific inhibition of the biosynthesis of macromolecules of *Bacillus subtilis* (DNA, RNA, protein and murein) and *Saccharomyces cerevisiae* (RNA and protein), as tested by the incorporation of precursors into trichloroacetic acid precipitable material.

With niphithricin B similar effects including the lysis of spheroplasts and permeability changes of liposomes and cells of *Saccharomyces cerevisiae*, could be detected. The concentrations needed to produce the same effect were approximately three times those of niphithricin A, as may be expected from the antimicrobial activity.

Niphimycin, closely related to niphithricins A and B, was also found in our investigations to cause lysis of spheroplasts and leakage of liposomes, comparable to niphithricin B. Primycin, an antibiotic with related structure, is also supposed to alter membrane permeability[47].

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