METABOLITES OF MICROORGANISMS. 247† PHENAZINES FROM *STREPTOMYCES ANTIBIOTICUS*, STRAIN TÜ 2706

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From a strain of *Streptomyces antibioticus* seven yellow phenazines were isolated. The antibacterially most active antibiotic was identified as (−)-saphenamycin, a second one with compound DC-86-Y (saphenic acid). Three compounds were new: Saphenic acid methyl ether, 6-acetylphenazine-1-carboxylic acid and an inseparable mixture of fatty acid esters of saphenic acid. Two simple phenazines were phenazine-1-carboxylic acid (tubermycin B) and unsubstituted phenazine, which was isolated for the first time from a microorganism.

The most striking property of the actinomycete strain Tü 2706 was the dark green color of its extracts. The chemistry of the green pigments, the esmeraldines, will be described in a forthcoming paper. In addition to the esmeraldines a series of yellow pigments were present in the cultures. Since the chemical and spectroscopic properties of these compounds are the basis of the structural elucidation of the esmeraldines, we describe them in this paper.

The strain Tü 2706 could be identified by the classification of HÜTTER[2] and NONOMURA[3] as *Streptomyces antibioticus*.

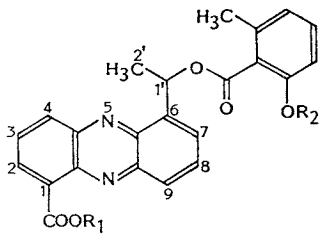
Most of the yellow pigments discernible by TLC in the crude extracts could be isolated in pure state by repeated chromatographic procedures under various conditions (see Experimental part). The following compounds were isolated and characterized:

Saphenamycin (1)

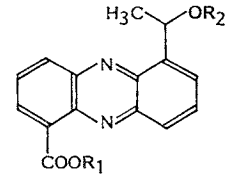
The only compound possessing high antibacterial activity was identical in its analytical and spectroscopic properties with saphenamycin (**1**), an antibiotic described by UMEZAWA's group. Its structure follows from an X-ray study[4]. An X-ray structural elucidation carried out independently in our laboratory[5] gave identical results. We have further characterized saphenamycin by the preparation of the methyl ester (**2**) and the methyl ether methyl ester (**3**) which were formed from **1** by reaction with diazomethane. The dimethylated product (**3**) showed the same chromatographic and spectroscopic properties as a racemic synthetic sample[6].

From UMEZAWA's paper one could have the impression that natural saphenamycin is a racemic compound ($[\alpha]_D$ 0° in chloroform). Indeed our compound also did not show optical activity when measured in chloroform. However, in DMSO it is optically active, $[\alpha]_D^{24}$ −3°. The esters **2** and **3**

† Preceding communication see ref 1.



1	R_1=H	R_2=H
2	R_1=CH_3	R_2=H
3	R_1=CH_3	R_2=CH_3



4	R_1=H	R_2=H
5	R_1=CH_3	R_2=H
6	R_1=H	R_2=CH_3
7	R_1=CH_3	R_2=CH_3
11	R_1=H	R_2=Fatty acid residues (Table 4)

particularly show considerable optical activities. Our sample is therefore certainly not a racemic mixture. On the other hand the considerably higher mp of UMEZAWA's preparation could indicate that it was racemic†.

Saphenic Acid (4)

A second compound showed the same analytical and spectroscopic properties as compound DC-86-Y (**4**), isolated by TAKAHASHI *et al.*[7,8] from an actinomycete. It was also obtained by hydrolysis of the antibiotic DC-86-M, the glycolic acid ester of **4**. Since this compound is the basical structure of a series of natural (this work) and synthetic antibiotics[6], we named it saphenic acid. It is optically active and has the same optical rotation as TAKAHASHI's sample. The acid **4** in virtually racemic form was obtained by alkaline hydrolysis of saphenamycin (**1**) and other compounds of this series (see Experimental part). A synthesis of racemic saphenic acid will be described in an accompanying paper[6]. With CH_2N_2 it gave the methyl ester **5**.

Saphenic Acid Methyl Ether (6)

A third component isolated from the culture was isomeric with methyl saphenate (**5**), $C_{16}H_{14}N_2O_3$. The ^1H NMR spectrum is closely related to that of **5**. However, the signal of 2-H is found at 8.9 ppm, as in all compounds of this series with a free carboxyl group at C-1 (see Table 1). In methyl esters (*e.g.* **5**) this signal is shifted to *ca.* 8.4 ppm. The signal of the acidic proton is found at 15.5 ppm as a broad singlet, and the OCH_3 signal is present at 3.4 ppm (in methyl saphenate and related esters at *ca.* 4.1 ppm). It follows from these observations that the new compound is the methyl ether **6** of saphenic acid. It is optically active, but whether its chirality is the same as that of the co-occurring (−)-saphenic acid is not yet known. With CH_2N_2 the methyl ester **7** is formed.

6-Acetylphenazine-1-carboxylic Acid (8)

This acidic component has two H atoms less than saphenic acid (**4**). The signals of the methyl group are shifted to 32.9 ppm in the ^{13}C NMR spectrum (Table 2) and to 3.09 ppm in the ^1H NMR spectrum (Table 1), where it appears as a singlet. The conclusion that the new compound is 6-acetylphenazine-1-carboxylic acid (**8**) was later proven by synthesis[6].

Phenazine (9)

Phenazine was present in very low amount in an early fraction of chromatography. It was iden-

† In a series of racemic synthetic compounds in this family we have observed higher mp's than for the corresponding natural compounds[6].

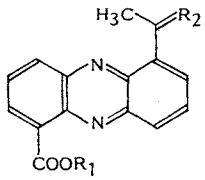
Table 1. ^{1}H NMR of natural phenazines, from strain Tü 2706 (300 MHz, J in Hz).

Saphenic acid ($CDCl_3$)	6-Acetylphenazine-1-carboxylic acid (pyridine-d_5)	Saphenic acid methyl ether ($CDCl_3$)	Saphenamycin ($CDCl_3$)	Assignment
15.37 (1H, br s, exch)	9.2~7.4 (1H, br, exch)	15.5 (1H, br s, exch)	15.40 (1H, s, exch)	COOH
8.97 (1H, dd, J=7.1, 1.4)	8.83 (1H, dd, J=7.0, 1.4)	8.97 (1H, dd, J=7.1, 1.4)	8.99 (1H, dd, J=7.1, 1.4)	2-H
8.51 (1H, dd, J=8.7, 1.4)	8.41 (1H, dd, J=8.8, 1.4)	8.55 (1H, dd, J=8.7, 1.4)	8.59 (1H, dd, J=8.7, 1.4)	4-H
8.17 (1H, dd, J=7.4, 2.8)	8.37 (1H, dd, J=8.7, 1.3)	8.19 (1H, dd, J=8.0, 2.2)	8.24 (1H, m)	7-H
8.04 (1H, dd, J=8.7, 7.1)	7.95 (1H, dd, J=8.8, 7.0)	8.09~8.00 (3H, m)	8.06 (1H, dd, J=8.7, 7.1)	3-H
7.99~7.95 (2H, m)	8.28 (1H, dd, J=7.0, 1.3)		8.00 (2H, m)	8-H
	7.89 (1H, dd, J=8.7, 7.0)			9-H
5.86 (m, after exch; q, J=6.6)		5.78 (1H, q, J=6.4)	7.47 (1H, q, J=6.6)	1′-H
4.03 (1H, br d, J=3.9, exch)		3.44 (3H, s)		OH, OCH_3
1.80 (3H, d, J=6.6)	3.09 (3H, s)	1.65 (3H, d, J=6.4)	1.97 (3H, d, J=6.6)	2′-H
			11.11 (1H, s, exch)	Signals of 6-methyl
			7.29 (1H, t-like, J=7.9)	salicylic acid
			6.83 (1H, br d, J=8.2)	residue
			6.76 (1H, br d, J=7.5)	
			2.73 (3H, s)	

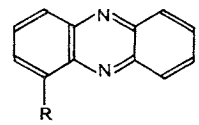
exch: Exchangeable.

Table 2. ^{13}C NMR spectra of phenazines from strain Tü 2706 (75 MHz).

Saphenic acid (**4**) ($CDCl_3$)	6-Acetylphenazine-1-carboxylic acid (**8**) (pyridine-d_5)	Saphenic acid methyl ether (**6**) ($CDCl_3$)	Saphenamycin (**1**) ($CDCl_3$)
23.9 q	23.9 q	23.4 q	22.3 q
—	—	56.9 q	24.5 q
67.8 d	200.0 s	73.9 d	70.1 d
—	—	—	112.3 s
—	—	—	115.9 d
—	—	—	123.1 d
124.7 s	124.8 s	124.5 s	124.8 s
127.0 d	130.7 d	126.4 d	127.5 d
127.8 d	131.3 d	127.0 d	127.7 d
130.4 d	132.2 d (2C)	129.8 d	130.4 d
133.3 d		133.2 d	132.9 d
134.9 d	135.0 d	135.1 d	134.5 d
—	—	—	135.5 d
137.4 d	137.8 d	137.0 d	137.7 d
139.7 s	139.1 s	139.4 s	139.7 s
140.2 s	139.2 s	139.6 s	140.0 s
141.6 s	139.6 s	141.9 s	141.0 s
142.2 s	141.2 s	142.2 s	141.2 s (2C)
143.9 s	142.6 s	143.3 s	142.6 s
			163.0 s
165.8 s	165.2 s	165.7 s	165.8 s
			170.7 s



8 R_1=H R_2=O
12 R_1=CH_3 R_2=H_2



9 R=H
10 R=COOH

tified by comparison of the spectra (IR, ^1H NMR, MS) with those of a commercial sample. To our knowledge this is the first time that unsubstituted phenazine has been isolated from a microorganism.

Phenazine-1-carboxylic Acid (**10**)

This simple phenazine derivative was isolated in very small amount, and could be isolated only when large cultures (200 liters) were worked up. This compound (tubermycin B) is well known from various microorganisms and could be identified by comparison of spectra with those reported in the literature[9~11].

Saphenyl Fatty Acid Esters (S-7 Fraction) (**11**)

A large fraction isolated as an amorphous yellow powder gave a single spot in TLC. However, numerous signals of low and variable intensities in the ^{13}C NMR spectrum clearly indicated that it was a mixture of closely related compounds. The UV spectrum is identical with that of the other saphenic acid derivatives of this series. The ^1H NMR spectrum in the 7~9 ppm range is the same as that of saphenamycin, but the signals of the 6-methylsalicylic acid residue are absent. The signal

of the carboxylic acid proton (15.5 ppm) and the position of 2-H at 8.9 ppm prove that the carboxylic group at C-1 is free. A large number of CH_2 signals (and a few CH signals) in the 20~40 ppm range of the ^{13}C NMR spectrum and an intense broad singlet at 1.3~1.5 ppm in the ^1H NMR spectrum suggested S-7 to be a mixture of fatty acid esters (**11**) of saphenic acid. Alkaline hydrolysis of S-7 yielded saphenic acid (**4**), identified as its methyl ester (**5**), which — as in the hydrolysis of saphenamycin — was obtained in its racemic form. The fatty acid mixture of the hydrolysate was analyzed by GC-MS of the methyl esters prepared with CH_2N_2. By a computer search (best fit of MS, see Experimental part) the esters of the following acids were identified (in parentheses percentage according to GC): Tetradecanoic acid (0.7%), hexadecanoic acid (17.2%), 12-methyltridecanoic acid (8.6%), 14-methylpentadecanoic acid (24.2%), 16-methylheptadecanoic acid (1.5%), 12-methyltetradecanoic acid (16.4%), 14-methylhexadecanoic acid (3.8%).

Table 3. Antibacterial activity of saphenamycin.

Microorganisms	MIC (μg/ml)
Bacillus brevis[a]	0.01
B. cereus	0.07
B. subtilis[a]	0.001
Corynebacterium glutamicum	0.2
Streptomyces glaucescens	—
S. viridochromogenes	0.35
Proteus mirabilis	<0.01
P. vulgaris	0.005
Xanthomonas campestris	<0.001

[a] Defined media; all others were complex media.

Three minor components could not be identified, because their peaks in the GC were too close to more intense neighboring peaks for an unambiguous MS identification. One of the major peaks (20.5%) corresponded to an unsaturated C_{18} acid, but the MS did not allow an unambiguous identification. The presence of olefinic components in the S-7 mixture is confirmed by a multiplet (*ca.* 0.8H) at 5.33 ppm in the ^1H NMR spectrum of fraction S-7.

As usual in bacteria and actinomycetes several of the saturated acids have branched chains with *iso* or *anteiso* arrangements of the side chain methyl groups.

In the experimental part a hydrogenolysis of saphenamycin methyl ester to methyl 6-ethylphenazine-1-carboxylate (**12**) is described, because the analogous reaction will play an important role in the structure elucidation of the esmeraldines. As an unexpected side product saphenic acid methyl ether methyl ester (**7**), identical with the methylation product of genuine saphenic acid methyl ether (**6**), was obtained by this reaction carried out in methanol - ethyl acetate.

Biological Activities

All isolated metabolites were tested against various microorganisms by the agar plate diffusion method described by ZÄHNER and MAAS[12], but only saphenamycin showed good antibacterial activities. The determined MIC values are listed in Table 3. Characteristic of saphenamycin was a high activity against Gram-positive bacteria. The determination of ID_{50} values against the eucariotic tumor cell line CCRF/CEM will be described separately[13] and gave 0.6 μg/ml for saphenamycin and 0.4 μg/ml for esmeraldine B.

Experimental

Isolation of Phenazines from Strain Tü 2706

The production of phenazines from Tü 2706 was carried out in a medium consisting of 2% Soyameal and 5% mannitol (pH was adjusted to 7.3~7.5). Fermentations in shake flasks and stirred jars will be described in a forthcoming paper[13]. At the end of a fermentation (144~168 hours) the culture broth was separated into filtrate and mycelium. The latter was extracted with MeOH or a

mixture of MeOH - acetone (1 : 1), the green crude extract was concentrated and extracted twice with EtOAc at pH 2~4. The organic phase was then concentrated *in vacuo* and gave a deep green tar.

The extract of the mycelium from 20 liters of culture fluid (64.5 g), dissolved in 50 ml $CHCl_3$ was filtered through cotton and evaporated under reduced pressure. The dark brownish green residue was flash chromatographed in two batches on 800 g of silica gel each. $CHCl_3$ and mixtures of $CHCl_3$ - EtOAc up to 1 : 1 eluted first a large amount of fatty material and then a multiplicity of yellow to brown metabolites. The dark green fractions containing saphenic acid and the esmeraldines were eluted after the addition of 0.05% HCOOH to the eluent. The green fractions were again chromatographed (700 g silica gel; $CHCl_3$ - EtOAc, 1 : 1) and gave 1.32 g of a brownish yellow oil, containing saphenic acid. The green esmeraldines were again eluted after the addition of HCOOH. The further purification of these compounds will be described in a forthcoming paper.

Saphenic Acid (4)

The yellow fraction was chromatographed on Sephadex LH-20 ($CHCl_3$ - MeOH, 1 : 1). The fractions containing a yellow compound with Rf 0.32 (TLC, Silica gel Merck F_{254}; hexane - acetone, 1 : 1) were combined and further purified on 110 g of silica gel ($CHCl_3$ - hexane - MeOH, 10 : 1 : 1) and then on 30 g of silica gel (benzene - MeOH, 9 : 1) resulting in 129 mg of pure saphenic acid. From impure side fractions an additional 75 mg of pure compound **4** were obtained after further chromatography. Saphenic acid was recrystallized from acetone - hexane and gave yellow needles which turned brown after standing several hours in day light. However, in the dark it could be stored unaltered for several weeks: MP 202~204°C; $[\alpha]_D^{24}$ −11.4° (*c* 0.57, $CHCl_3$), +55° (*c* 0.16, DMSO); UV λ_{max}^{EtOH} nm (ε) 365 (13,200), 255 (58,100); IR and ^1H NMR, coincident with spectra in the literature[5]; electron impact mass spectra (EI-MS) *m/z* (relative intensity) 268 (26, M^+), 254 (16), 253 (100), 226 (10), 225 (57), 224 (39), 209 (9), 207 (5), 206 (10), 205 (20), 182 (8), 181 (42), 180 (14), 179 (28), 153 (7), 152 (6), 104 (8), 103 (5), 102 (7), 90 (5), 77 (8), 76 (9), 75 (10), 63 (6), 52 (6), 51 (9), 50 (6), 43 (10). The spectra and Rf were identical with those of a racemic synthetic sample[6].

Anal Calcd for $C_{15}H_{12}N_2O_3$ (268.27): C 67.16, H 4.51, N 10.44.
Found: C 67.23, H 4.44, N 10.68.

Methyl Saphenate (5)

From 34 mg saphenic acid in 5 ml $CHCl_3$ - MeOH (5 : 1) and CH_2N_2 in 2 ml ether the methyl ester was prepared (5 minutes, room temp) and purified by chromatography (30 g silica gel; hexane - acetone, 2 : 1). The yellow powder was recrystallized from acetone - hexane: MP 151~152°C; $[\alpha]_D^{24}$ −26.9° (*c* 0.42, $CHCl_3$); UV λ_{max}^{EtOH} nm (ε) 365 (14,200), 252 (74,100); ^1H NMR ($CDCl_3$) δ 8.35 (1H, dd, *J*=8.7 and 1.5 Hz), 8.24 (1H, dd, *J*=7.0 and 1.5 Hz), 8.23 (1H, dd, *J*=8.2 and 1.9 Hz), 7.86 (1H, dd, *J*=8.7 and 7.0 Hz), 7.82~7.75 (2H, m), 5.71 (1H, quintet, *J*=6.4 Hz; after exchange q, *J*=6.4 Hz), 4.78 (1H, br d, *J*=6.1 Hz, exchangeable), 4.11 (3H, s), 1.80 (3H, d, *J*=6.6 Hz); EI-MS *m/z* (relative intensity) 283 (10), 282 (54, M^+). The spectra are identical with those of a racemic synthetic sample[6].

Anal Calcd for $C_{16}H_{14}N_2O_3$ (282.29): C 68.08, H 5.00, N 9.92.
Found: C 67.87, H 5.03, N 10.17.

6-Acetylphenazine-1-carboxylic Acid (8)

A side fraction of the purification of saphenic acid (188 mg) contained a second yellow compound visible on TLC. Chromatography on 13 g silica gel impregnated with oxalic acid gave 74 mg saphenic acid and 43 mg of the new compound. An additional chromatography through Sephadex LH-20 (2×70 cm; $CHCl_3$ - MeOH (1 : 1) in the dark) gave the pure compound, which was recrystallized from acetone to give yellow needles turning to brown in day light: MP 219~221°C; UV λ_{max}^{EtOH} nm (ε) 367 (18,640), 252 (77,570); ^1H NMR Table 1; ^{13}C NMR Table 2; EI-MS *m/z* (relative intensity) 266 (26, M^+), 251 (17), 222 (100), 207 (12), 206 (8), 194 (7), 179 (31), 153 (5), 152 (9), 151 (5), 103 (6), 102 (5), 89 (5), 76 (9), 75 (14), 74 (8), 63 (9), 52 (4), 51 (7), 50 (7), 43 (15), 39 (4). The spectra are in agreement with those of a synthetic sample[6].

Anal Calcd for $C_{15}H_{10}N_2O_3$ (266.26): C 67.67, H 3.79, N 10.52.
Found: C 67.38, H 3.75, N 10.42.

Phenazine (9)

An early fraction of the first chromatography, 1.03 g, was again chromatographed on 600 g silica gel. With hexane - acetone (3 : 1) a fraction (387 mg) was eluted containing fatty material and a single yellow compound. The latter was isolated by chromatography on Sephadex LH-20 (1×53 cm; acetone - MeOH, 1 : 1) as a powder, yielding 11 mg of yellow needles by recrystallization from acetone, mp 175~176.5°C. The spectra (UV, IR, ^{1}H NMR and MS) were identical with those of commercial phenazine†.

Anal Calcd for $C_{12}H_8N_2$ (180.21): C 79.98, H 4.47, N 15.54.
Found: C 79.73, H 4.42, N 15.55.

Saphenic Acid Methyl Ether (6)

Several fractions of the above mentioned chromatography eluted with hexane - acetone (2 : 1) contained a yellow compound with Rf 0.28 (TLC, hexane - acetone, 2 : 1). After additional purification (Sephadex LH-20, acetone) 31 mg of a yellow powder was obtained which gave needles by recrystallization from acetone - hexane: MP 158~159°C (dec); $[\alpha]_D^{24}$ +9.1° (*c* 0.54, $CHCl_3$); UV λ_{max}^{EtOH} nm (ε) 365 (14,900), 255 (91,200); IR ($CHCl_3$) cm^{-1} 3400 (br), 1735 (s), 1625 (m), 1605 (m), 1570 (m), 1535 (s); ^{1}H NMR Table 2; EI-MS *m/z* (relative intensity) 282 (18, M^+), 268 (18), 267 (100), 252 (16), 251 (59), 238 (20), 208 (15), 207 (12), 206 (14), 205 (35), 180 (11), 179 (12), 111 (7), 103 (5), 77 (7), 75 (5), 43 (7), 15 (10).

Anal Calcd for $C_{16}H_{14}N_2O_3$ (282.30): C 68.08, H 5.00, N 9.92.
Found: C 68.04, H 4.92, N 9.89.

Methyl Ester of Saphenic Acid Methyl Ether (7)

This ester was prepared from 20 mg **6** in 10 ml $CHCl_3$ and CH_2N_2 in 3 ml ether at room temperature. After chromatography on 12 g silica gel and recrystallization from acetone - hexane 15 mg ester **7** was obtained as fine yellow needles: MP 139°C; $[\alpha]_D^{24}$ +6.5° (*c* 0.49, $CHCl_3$); UV λ_{max}^{EtOH} nm (ε) 365 (15,650), 252 (97,530); ^{1}H NMR ($CHCl_3$) δ 8.40 (1H, dd, *J*=8.4 and 1.5 Hz), 8.24 (1H, dd, *J*=7.0 and 1.5 Hz), 8.23 (1H, dd, *J*=8.9 and 1.8 Hz), 7.96 (1H, dd, *J*=7.2 and 1.8 Hz), 7.89 (1H, dd, *J*=8.4 and 7.0 Hz), 7.84 (1H, dd, *J*=8.9 and 7.2 Hz), 5.79 (1H, q, *J*=6.4 Hz), 4.11 (3H, s), 3.41 (3H, s), 1.62 (3H, d, *J*=6.4 Hz); EI-MS *m/z* (relative intensity) 296 (12, M^+), 281 (100).

Mixture of Fatty Acid Saphenyl Esters (11) (Fraction S-7)

A fraction from the first chromatography eluted with $CHCl_3$ - EtOAc (3 : 2), 6.455 g of a brown oil, was again chromatographed on 500 g silica gel (flash chromatography). The elution solvents varied from hexane - acetone (3 : 1) to (1 : 1). The fractions containing a yellow compound with Rf 0.36 (TLC, hexane - acetone, 2 : 1) were combined and chromatographed on Sephadex LH-20 (4× 90 cm; $CHCl_3$ - MeOH, 1 : 1) and then on silica gel (hexane - acetone, 2 : 1), yielding 100 mg of a yellow glass, which was precipitated from acetone with hexane at 0°C as a fine powder: MP 91~92.5°C. The sample gave a single spot on TLC (Rf 0.28, hexane - acetone, 3 : 1); $[\alpha]_D^{24}$ +1.6° (*c* 0.56, $CHCl_3$), +5.4° (*c* 0.26, DMSO); UV λ_{max}^{EtOH} nm ($E_{1cm}^{1\%}$) 365 (227), 254 (1,413); IR ($CHCl_3$) cm^{-1} 3500~2500 (br), 1730 (s), 1625 (w), 1605 (w), 1570 (w); ^{1}H NMR ($CDCl_3$) δ 15.49 (1H, s, exchangeable), 8.89 (1H, dd, *J*=7.1 and 1.4 Hz), 8.56 (1H, dd, *J*=8.7 and 1.4 Hz), 8.22~8.17 (1H, m), 8.03 (1H, dd, *J*=8.7 and 7.1 Hz), 7.98 (2H, m), 7.22 (1H, q, *J*=6.6 Hz), 5.33 (*ca.* 0.8H, m); the part from 3.0~0.8 ppm is complex and shows several signals with varying integrals below 1H; 1.51~1.31 (*ca.* 24H, br s); ^{13}C NMR ($CDCl_3$) δ 172.98 (s)*, 165.96*, 142.56 (s)*, 142.56 (s)*, 142.21 (s)*, 139.91 (s)*, 138.68 (s)*, 137.58 (d)*, 135.54 (d)*, 133.02 (d)*, 130.20 (d)*, 130.06 (d), 129.95 (d), 129.71 (d), 128.12 (d), 127.90 (d), 127.22 (d)*, 126.93 (d)*, 124.80 (s)*, 67.52 (d)*, 39.01 (t), 34.63 (d), 31.96 (t), 30.04 (t), 29.98 (t), 29.72 (t), 29.63 (t), 29.52 (t), 29.35 (t), 29.30 (t), 29.24 (t), 29.14 (t), 28.00 (d), 27.45 (d), 27.26 (t), 27.22 (t), 27.13 (t), 25.09 (q)*, 22.64 (q), 22.38 (q), 14.09 (q) (signals with * show intensities of *ca.* 1C, all others are of reduced intensity).

Saphenamycin (1)

A fraction of the first chromatography eluted with $CHCl_3$ was nearly homogeneous (TLC) and

† Fluka AG, Buchs SG, Switzerland.

gave 423 mg of a brownish powder upon evaporation. After an additional chromatography (45 g silica gel, hexane - acetone, 1 : 1) and recrystallization from MeOH - $CHCl_3$ 253 mg saphenamycin was obtained as yellow prisms: MP 172~172.5°C (dec); $[\alpha]_D^{24}$ 0° (*c* 0.49, $CHCl_3$), −3.0° (*c* 0.33, DMSO); UV λ_{max}^{EtOH} nm (ε) 365 (13,730), 255 (86,650); ^1H NMR Table 1; according to a 2D COSY spectrum the following signals belong to coupling systems: 8.99-8.59-8.06 (ring A), 8.24-8.00 (2C) (ring C), 7.29-6.83-6.76 (6-methylsalicylic acid ring), 7.47-1.97 (CH_3CHO); ^{13}C NMR Table 2. The spectra (IR, ^1H NMR and ^{13}C NMR) are in good agreement with those reported in the literature[2].

Anal Calcd for $C_{23}H_{18}N_2O_5$ (402.41): C 68.65, H 4.51, N 6.96.
Found: C 68.45, H 4.51, N 6.92.

Saphenamycin Methyl Ester (**2**)

A solution of 70 mg saphenamycin in 15 ml $CHCl_3$ was reacted with CH_2N_2 in 5 ml ether (5 minutes, room temp). The reaction product gave after chromatography (35 g silica gel, hexane - acetone, 3 : 1) and recrystallization from acetone - hexane 58 mg fine yellow needles: MP 146~147°C; $[\alpha]_D^{24}$ +205.9° (*c* 0.17, $CHCl_3$); UV λ_{max}^{EtOH} nm (ε) 365 (14,800), 251 (83,540); IR (KBr) cm^{-1} 3325, 1730 (s), 1710 (s), 1660 (m), 1620 (w); ^1H NMR ($CDCl_3$) δ 11.18 (1H, s, exchangeable), 8.43 (1H, dd, *J*= 8.7 and 1.5 Hz), 8.28 (1H, dd, *J*=8.6 and 1.6 Hz), 8.24 (1H, dd, *J*=7.0 and 1.5 Hz), 7.90 (1H, dd, *J*=6.9 and 1.6 Hz), 7.85 (1H, dd, *J*=8.7 and 7.0 Hz), 7.84 (1H, dd, *J*=8.6 and 6.9 Hz), 7.47 (1H, q, *J*=6.6 Hz), 7.27 (1H, t-like, *J ca.* 7.9 Hz), 6.83 (1H, br d, *J*=8 Hz), 6.73 (1H, br d, *J*=7.4 Hz), 4.11 (3H, s), 2.68 (3H, s), 1.94 (3H, d, *J*=6.6 Hz); EI-MS *m/z* (relative intensity) 416 (7, M^+).

Saphenamycin Methyl Ether Methyl Ester (**3**)

In a second preparation of the methyl ester with somewhat longer reaction time in addition to **2** a second product was observed in TLC. By chromatography (silica gel, hexane - acetone, 3 : 1) 33 mg **2** and 20 mg of a brownish oil, a mixture of **2** and **3** was obtained. An additional chromatography gave 16 mg of pure methyl ether **3** as a yellow oil which crystallized during storing at −15°C: MP 127~129°C; $[\alpha]_D^{24}$ +25.4° (*c* 0.59, $CHCl_3$); IR ($CHCl_3$) cm^{-1} 1725 (s), 1625 (w), 1600 (br), no OH; ^1H NMR ($CDCl_3$) δ 8.44 (1H, dd, *J*=8.8 and 1.4 Hz), 8.25 (1H, dd, *J*=8.8 and 1.3 Hz), 8.24 (1H, dd, *J*=6.9 and 1.4 Hz), 8.02 (1H, dd, *J*=6.8 and 1.3 Hz), 7.85 (1H, dd, *J*=8.8 and 6.9 Hz), 7.84 (1H, dd, *J*=8.8 and 6.8 Hz), 7.50 (1H, q, *J*=6.5 Hz), 7.24 (1H, t-like, *J ca.* 8.0 Hz), 6.80 (1H, br d, *J*=7.6 Hz), 6.78 (1H, br d, *J*=8.2 Hz), 4.11 (3H, s), 3.80 (3H, s), 2.31 (3H, s), 1.87 (3H, d, *J*=6.5 Hz); EI-MS *m/z* (relative intensity) 430 (12, M^+), 399 (2), 281 (7.6). These spectra were identical with those of a racemic synthetic sample.

Phenazine-1-carboxylic Acid (**10**)

This compound was present in very small amount and could not be isolated in pure form from the 20-liter fermentations. However, during the isolation of esmeraldines from a 200-liter culture it was obtained after repeated chromatography (Sephadex LH-20, $CHCl_3$ - MeOH, 1 : 1; silica gel, $CHCl_3$ - hexane - MeOH, 10 : 1 : 1; silica gel, benzene - MeOH, 9 : 1; preparative TLC on Silica gel Merck F_{254}) and recrystallization from acetone - hexane - $CHCl_3$ as fine yellow needles (26 mg): MP 239~240°C; Rf 0.35 (TLC, benzene - MeOH, 9 : 1); UV λ_{max}^{EtOH} nm (ε) 365 (13,800), 252 (76,600); ^1H NMR ($CDCl_3$) δ 15.52 (1H, s, exchangeable), 8.98 (1H, dd, *J*=7.1 and 1.4 Hz), 8.53 (1H, dd, *J*=8.8 and 1.4 Hz), 8.37~8.26 (2H, m), 8.07~7.95 (3H, m); EI-MS *m/z* (relative intensity) 224 (1.4, M^+), 181 (13.6), 180 (100), 179 (20), 153 (7.4), 152 (5.0), 103 (5.8), 102 (8.0), 90 (8.1), 77 (7.1), 76 (14.8), 75 (13.6), 74 (7.8), 63 (9.3), 52 (9.4), 51 (11.7), 50 (16.7), 45 (5.5), 39 (5.2). The IR spectrum is in agreement with spectra from the literature[9].

Reactions of Phenazines from Strain Tü 2706

Hydrolysis of Fraction S-7

A suspension of 15 mg S-7 in 15 ml 2 N methanolic NaOH was refluxed 2.5 hours. The acidified solution (2 N HCl, pH 2) was extracted 3 times with $CHCl_3$ (10 ml each time). The extract was dried with Na_2SO_4 and evaporated *in vacuo*. The crude product in 5 ml $CHCl_3$ - MeOH (1 : 1) was esterified with CH_2N_2 in ether (5 minutes, room temp). Chromatography on 12 g silica gel (hexane - acetone, 2 : 1) gave 8 mg methyl saphenate and 10 mg of a mixture of fatty acid methyl esters.

Table 4. Identification of fatty acid methyl esters from saphenyl esters S-7.

Peak	t_R (minutes)	%	MW (MS)	Formula	Identified as methyl ester of acid	FIT[a]
A	2.78	8.6	242	$C_{15}H_{30}O_2$	12-Methyltridecanoic	986
B	2.96	0.7	242	$C_{15}H_{30}O_2$	Tetradecanoic	911
C	3.34	4.5	—	—	Not identified	—
D	3.41	16.4	256	$C_{16}H_{32}O_2$	12-Methyltetradecanoic	981
E	4.08	24.2	270	$C_{17}H_{34}O_2$	14-Methylpentadecanoic	976
F	4.25	1.0	—	—	Not identified	—
G	4.39	17.2	270	$C_{17}H_{34}O_2$	Hexadecanoic	987
H	5.02	1.6	—	—	Not identified	—
I	5.14	3.8	284	$C_{18}H_{36}O_2$	14-Methylhexadecanoic	984
J	6.49	20.5	296	$C_{18}H_{36}O_2$	Unsaturated C_{18} acid	—
K	6.75	1.5	298	$C_{19}H_{38}O_2$	16-Methylheptadecanoic	920

[a] Perfect coincidence: FIT=1,000.
t_R: Retention time.

Racemic Methyl Saphenate (5)

Small yellow crystals from acetone - hexane: MP 161~162°C; $[\alpha]_D^{24}$ 0° (*c* 0.44, $CHCl_3$); Rf (TLC), IR ($CHCl_3$), ^1H NMR and EI-MS in agreement with the corresponding data of the optically active sample prepared from authentic saphenic acid.

Fatty Acid Methyl Esters

The mixture of methyl esters was analyzed by GC-MS (Carlo Erba Fractovap 2150 combined with MS Varian MAT 112). The MS were compared with literature spectra (Library Search, Finnigan Inocs data system, NBS library with 25,400 compounds). The results are given in Table 4.

Hydrolysis of Saphenamycin (1)

See Table 3. In the same manner 24 mg saphenamycin was hydrolyzed and the crude mixture methylated with CH_2N_2. Chromatography (20 g silica gel; hexane - acetone (3:1), flash method) gave 14 mg methyl saphenate and 9 mg methyl 6-methylsalicylate.

Methyl Saphenate (5)

After recrystallization from acetone - hexane: MP 161~162°C; $[\alpha]_D^{24}$ +2.3° (*c* 0.65, $CHCl_3$); identified with an authentic sample by TLC, IR, ^1H NMR and EI-MS.

Methyl 6-Methylsalicylate

Methyl 6-methylsalicylate was an oil, bp 60°C (0.1 mmHg), which crystallized after prolonged standing at −15°C. It was not distinguishable from a synthetic sample[14] by TLC, IR, ^1H NMR and EI-MS. ^1H NMR ($CDCl_3$) δ 11.22 (1H, 8s, exchangeable), 7.26 (1H, t-like, *J ca.* 7.9 Hz), 6.83 (1H, br d, *J*=0.0 Hz), 6.71 (1H, br d, *J*=7.6 Hz), 3.96 (3H, s), 2.54 (3H, s).

Catalytic Hydrogenolysis of Saphenamycin Methyl Ester

To a solution of 33 mg saphenamycin methyl ester in 6 ml MeOH - EtOAc (1:1) was added 5 mg Pd on charcoal (10%, Fluka AG). After stirring for 1 hour the catalyst was removed by filtration and the crude product after evaporation chromatographed on Sephadex LH-20 (2×70 cm, $CHCl_3$ - MeOH, 1:1). A first fraction contained 8.3 mg saphenic acid methyl ester methyl ether (7), identical with the sample described above (TLC, mp, IR, ^1H NMR and EI-MS). The second fraction gave after further purification (preparative TLC, hexane - acetone, 4:1) 6.8 mg methyl 6-ethylphenazine-1-carboxylate (12), yellow crystals from acetone - hexane: MP 80°C; UV λ_{max}^{EtOH} nm (ε) 364 (14,730), 253 (79,730); IR ($CHCl_3$) cm^{-1} 1730 (s), 1625 (w), 1605 (w), no OH; ^1H NMR ($CDCl_3$) δ 8.41 (1H, dd, *J*=8.7 and 1.5 Hz), 8.21 (1H, dd, *J*=6.9 and 1.5 Hz), 8.15 (1H, dd, *J*=8.7 and 1.3 Hz), 7.81 (1H, dd, *J*=8.7 and 6.9 Hz), 7.78 (1H, dd, *J*=8.7 and 6.8 Hz), 7.67 (1H, br dd, *J*=6.8 and 1.3 Hz), 4.11 (3H, s), 3.43 (2H, q, *J*=7.5 Hz), 1.45 (3H, t, *J*=7.5 Hz); EI-MS *m/z* (relative intensity) 266 (100, M^+), 251

(6), 238 (8), 235 (11), 208 (23), 207 (14), 206 (8), 205 (7), 192 (6), 180 (3), 179 (5), 117 (3), 104 (3), 103 (7), 96 (4), 77 (4), 75 (3).

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