SECONDARY METABOLITES BY CHEMICAL SCREENING. 8† DECARESTRICTINES, A NEW FAMILY OF INHIBITORS OF CHOLESTEROL BIOSYNTHESIS FROM *PENICILLIUM*

I. STRAIN DESCRIPTION, FERMENTATION, ISOLATION AND PROPERTIES

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A family of new 10-membered lactones was detected by chemical screening. Taxonomic studies and fermentation conditions of the producing organisms, which belong to the species *Penicillium simplicissimum* and *Penicillium corylophilum*, are presented. The isolation as well as physico-chemical data of the new compounds named decarestrictines A to D are reported. *In vitro* testing using the HEP-G2 cell assay showed the decarestrictines to be inhibitors of cholesterol biosynthesis, which could be confirmed *in vivo*. In addition to the decarestrictines from *P. corylophilum* epoxyagroclavine-I (**1**) was isolated.

Within our concept[2~5] to detect new secondary metabolites by chemical screening[6,7], we isolate and characterize microorganisms from various natural sources, *e.g.* soil samples, plant material, and foodstuff. The culture broths and the mycelia of the newly isolated and immediately cultivated strains were extracted by organic solvents. The obtained residues were dissolved in small amounts of methanol - water (1 : 1) and analyzed by TLC chromatography in different solvent systems by treatment with various staining reagents. This procedure renders a good visualization of the characteristic secondary metabolite pattern of each strain. Applying this method to various microorganisms it has been found that different *Penicillium* species (strains FH-A 6090, FH-A 6099, FH-A 6530, and FH-A 6360) exhibit a similar but unusual secondary metabolite pattern. The present paper deals with the description of the producing organisms, their fermentation conditions, isolation and purification procedures as well as the physico-chemical and biological properties of the new secondary metabolites, named decarestrictines A to D. In an accompanying paper[8], the structural elucidation of these novel 10-membered lactones will be presented in detail. In addition, from strain FH-A 6360 the ergot alkaloid epoxyagroclavine-I (**1**)[9,10] was isolated.

Description of the Producing Organisms

The decarestrictine-producing strains FH-A 6090, FH-A 6099, FH-A 6530, and FH-A 6360 were

† See ref 1.

Table 1. Characterization of the different decarestrictine-producing microorganisms (*Penicillium simplicissimum* and *Penicillium corylophilum*).

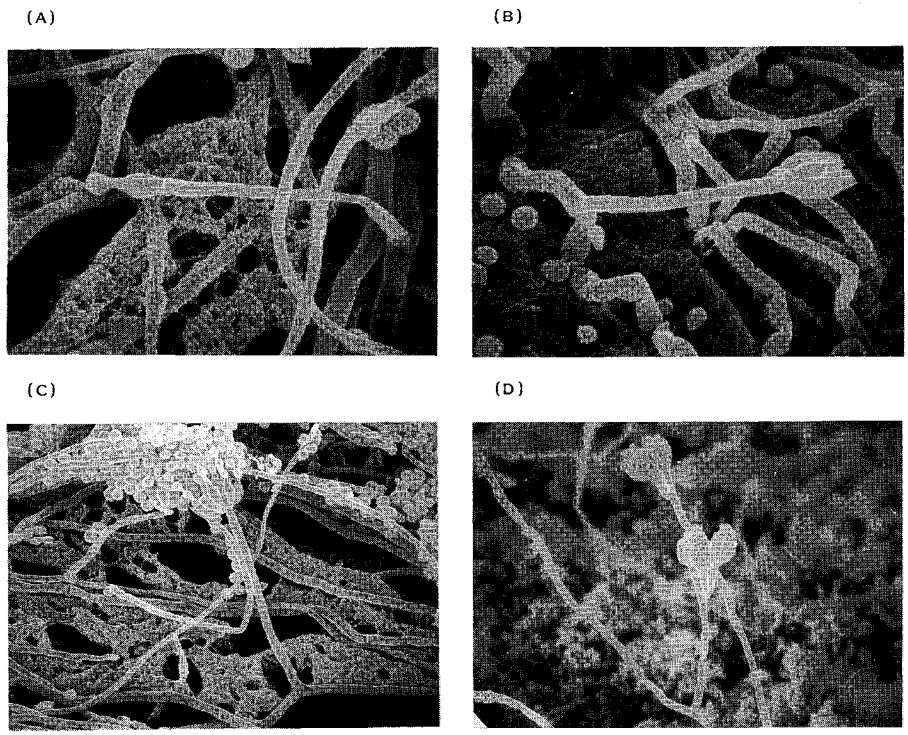
	P. simplicissimum		*P. corylophilum*	
Soil samples and isolates	Bryce Canyon, Utha, U.S.A. (FH-A 6090 and FH-A 6099) Oak Greek Canyon, Arizona, U.S.A. (FH-A 6530)		Aljezun, Portugal (FH-A 6360)	
	Medium A	Medium C	Medium A	Medium C
Colony growth				
7 days	15 mm	40 mm	20 mm	40 mm
20 days	30 mm	50 mm	25 mm	40 mm
Colony color	Green	Green/gray green	Light green	Gray/green/ dark green
Surface	Floccose	Smooth	Smooth	Floccose
Branching	Biverticillate	Monoverticillate	Biverticillate	Biverticillate
Size of metulae	20~30 μm	—	20~30 μm	20~30 μm
Number of metulae	2	—	2~4	2~4
Length of phialide	6~8 μm	6~8 μm	10~15 μm	10~15 μm
Number of phialide	6~9	6~9	12~15	12~15
Phialide shape	Ampulliform	Ampulliform	Flask-shaped	Flask-shaped
Diameter of conidia	2.5~3.5 μm	2.5~3.5 μm	2~2.5 μm	2~2.5μm
Surface of conidia	Smooth to spiny	Smooth to spiny	Smooth to warty	Smooth to warty
Shape of conidia	Subglobose	Subglobose	Ellipsoid	Ellipsoid

isolated from four different soil samples. These isolates were classified by taxonomical investigations as two different species of the genus *Penicillium*.

Three of these (FH-A 6090, FH-A 6099, and FH-A 6530), which were isolated from soil samples collected in Bryce Canyon (Utah, U.S.A.) and Oak Greek Canyon (Arizona, U.S.A.), are similar in growth, differentiation, morphology, and secondary metabolite pattern (see Table 1). On CZAPEK's agar (medium A), colonies of these three strains grew slowly, attaining a size of 3.0 cm i.d. after 20 days at 25°C. The colonies are light to grayish green. The conidiophores show a typical biverticillate branching. The phialides are ampulliform and the conidia subglobose. The surfaces of the conidia varied from smooth to spinous at different ages. On malt extract agar (medium C), the colonies grow fast reaching 4 to 5 cm i.d. in 48 to 72 hours. In addition, differentiation on medium C starts early forming conidiophores at the second day. Unexpectedly, in younger parts of the colonies we observed conidiophores exhibiting only one phialide, which carried a complete small spore chain (see Fig. 1A). Therefore, we analyzed colonies during different stages of growth by scanning electron microscopy. After 24 to 48 hours of growth on medium C at 25°C, the three strains formed stipes exhibiting small *Aspergillus*-like vesicles. Later, a single apical phialide appeared and the formation of the first conidium started. Simultaneously, in one or two steps, the conidiophores were completed around the first apical phialide (Fig. 1B). Young spores exhibited a smooth surface, while the adult spores appeared to be spiny. After 2 weeks of growth, the conidiophore of the isolates look like a monoverticillate *Penicillium* (Fig. 1C), which is typical for *Penicillium simplicissimum*[11].

In contrast, strain FH-A 6360, which was isolated from a soil sample in Portugal (Aljezun), exhibited characteristic morphological formation which differed from the other decarestrictine-producing strains by both, the obtained secondary metabolite patterns, and its morphology (Table 1). On CZAPEK's agar (medium A), the colonies of strain FH-A 6360 grew up to 2 cm i.d. after 7 days, exhibiting white to gray mycelium.

Fig. 1. Scanning electron micrographs of the decarestrictine-producing strains cultivated on malt extract agar (medium C) at 25°C.



(A) Stipe with an apical phialide and two conidia from a 2-day old culture of *Penicillium simplicissimum* (strain FH-A 6090; ×3,500). (B) Stipe exhibiting one ring of phialides from a 5-day old culture of *P. simplicissimum* (strain FH-A 6090; ×3,500). (C) Stipe with an adult group of phialides as well as young conidia from a 7-day old culture of *P. simplicissimum* (strain FH-A 6090; ×5,000). (D) Biverticillate conidiophores from a 7-day old culture of *P. corylophilum* (strain FH-A 6360; ×750).

Later, the color of the colonies changed to light green (Table 1). In contrast, on malt extract agar (medium C) the strain grew moderately (4.0 to 4.5 cm i.d.) in 7 days and the color is gray-green to dark green. The conidia appeared to be biverticillate (Fig. 1D). The stipe is smooth walled and terminated from 2 to 4 metulae. The phialides are flask-shaped and the conidia are ellipsoid. The surface of the conidia appeared to be smooth to rugose in texture. The behavior of FH-A 6360 is typical for *Penicillium corylophilum*[11] (Fig. 1D). Using different classification keys, the two species described above belong to the same group *Asymmetrica divaricata*[11] or subgenus *Furcatum*[12].

Detection of Secondary Metabolites

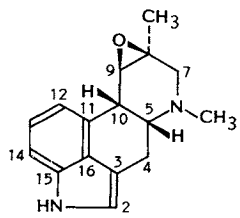
In order to examine the secondary metabolite pattern, the strains were cultivated on a rotary shaker in 300-ml Erlenmeyer flasks containing 100 ml of various media at 25°C. The mycelium of each strain was separated by filtration or centrifugation. The organic compounds present in the culture broths were adsorbed on Amberlite XAD-16 and eluted with methanol-water (4:1). The concentrated eluates were chromatographed on TLC silica gel plates using butanol-acetic acid-water (4:1:5, upper phase) or chloroform-methanol (9:1) as solvent systems. The metabolite pattern of each strain was analyzed by means of color reactions carried out directly on the TLC plates using different staining reagents. On

Table 2. Rf values, color reactions, and characterization of the decarestrictines A to D and epoxyagroclavine-I (**1**).

	A	B	C	D	Epoxy-agroclavine-I (**1**)
Solvent systems and staining reagents:					
BuOH - acetic acid - H_2O (4 : 1 : 5, upper phase)	0.95	0.80	0.80	0.80	0.25
$CHCl_3$ - MeOH (9 : 1)	0.55	0.55	0.35	0.30	0.30
EtOAc - *n*-hexane (3 : 1)	0.75	0.56	0.25	0.19	—
Anisaldehyde - H_2SO_4	Brown	Blue-brown	Beige	Dark brown	Red-violet
EHRLICH's reagent	None	Orange	None	None	Blue-green
Orcinol reagent	Brown	Gray-brown	Brown	Gray	Violet
Blue tetrazolium reagent	Violet	Violet	None	None	Blue
Physico-chemical properties:					
Molecular formula	$C_{10}H_{14}O_4$	$C_{10}H_{14}O_5$	$C_{10}H_{16}O_4$	$C_{10}H_{16}O_5$	$C_{16}H_{18}N_2O$
MW	198	214	200	216	254
FAB-MS ($(M+H)^+$, *m/z*)	199	215	201	217	—
Anal Calcd for	$C_{10}H_{14}O_4$: C 60.60, H 7.12	$C_{10}H_{14}O_5$: C 56.07, H 6.54	$C_{10}H_{16}O_4$: C 60.00, H 8.00	$C_{10}H_{16}O_5$: C 55.55, H 7.41	$C_{16}H_{18}N_2O$: 254.1419
Found:	C 60.28, H 7.04	C 56.16, H 6.52	C 60.40, H 8.09	C 55.73, H 7.48	254.1419[a]
$[\alpha]_D^{20}$ (*c* 1.0, MeOH)	−11°	−49°	−39°	−62°	−45.9°[b]
UV λ_{max}^{MeOH} nm (ε)	End absorption	End absorption	End absorption	End absorption	[c]
IR (KBr) cm^{-1}	1715, 1680	1740, 1700	1720, 1700 (sh)	1700, 1640	1615, 1610 (weak)

[a] HREI-MS.
[b] *c* 0.14, MeOH.
[c] 211 (sh, 14,630), 225 (25,500), 276 (sh), 282 (5,470), 292 (4,560).

medium C, all strains described were shown to have similar metabolite patterns and the main metabolites, named decarestrictines, were present in each analyzed strain. The color reactions and Rf values of the decarestrictines A to D are summarized in Table 2. Some minor components of the decarestrictine complex were still detectable by TLC analysis.



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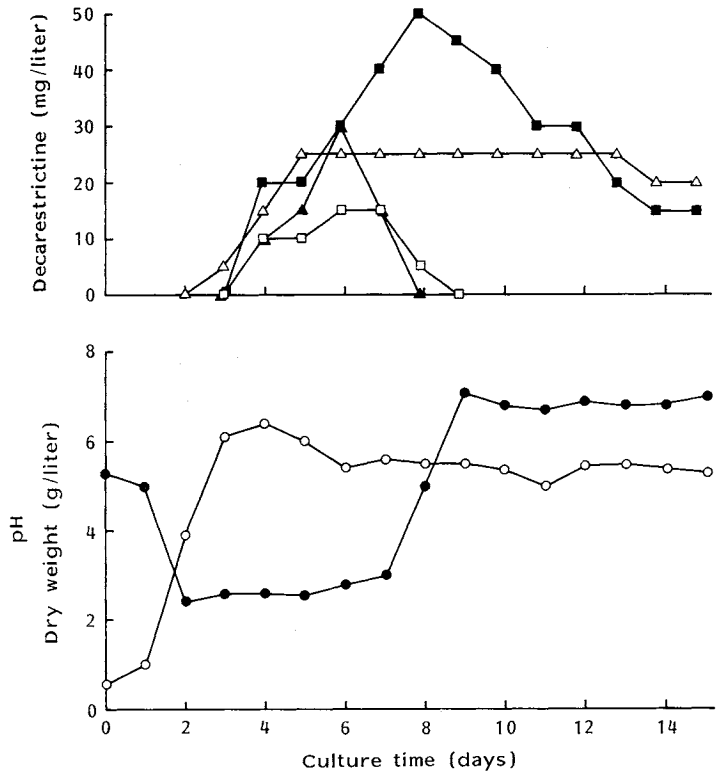
On medium C, *Penicillium corylophilum* strain FH-A 6360 produced a supplementary minor compound, whose coloration on TLC plates, after staining, deviated from the decarestrictines (see Table 2). In addition, a shift in the produced secondary metabolite pattern of strain FH-A 6360 was observed on medium E. Accompanied by a drastic decrease of the amounts of the decarestrictines the ergot alkaloide epoxyagroclavine-I (**1**)[9,10] was found to be the main secondary metabolite.

Fermentation

In 10-liter fermenters the different strains were cultivated at 25°C with medium C to produce the decarestrictines and with medium E to produce epoxyagroclavine-I (strain FH-A 6360). During the fermentation, mycelium dry weight, pH and the amount of decarestrictines produced were measured. As an example, the fermentation parameters of strain *Penicillium simplicissimum* (FH-A 6090) are presented

Fig. 2. Time course of a fermentation of *Penicillium simplicissimum* (FH-A 6090) and production of the decarestrictines (concentrations estimated by TLC).

○ Dry weight of the mycelium (g/liter), ● pH of the culture broth, ▲ decarestrictine A, ■ decarestrictine B, △ decarestrictine C, □ decarestrictine D.



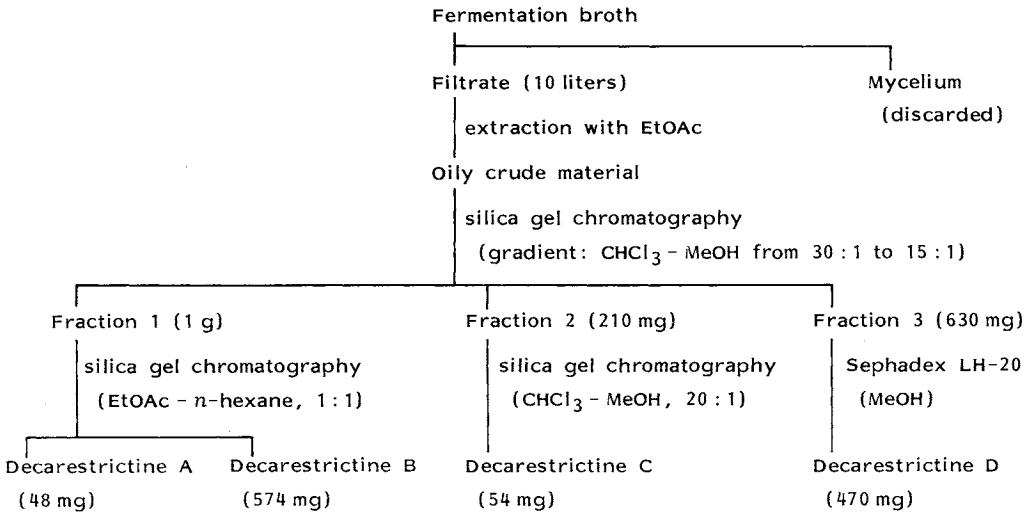
in Fig. 2. Decarestrictine production started 24 to 36 hours after inoculation. The maximum amount of decarestrictines A and C were achieved after 6 days followed by a rapid decrease during further fermentation. The main metabolite decarestrictine B appeared at the 4th day and reached its maximum amount after 9 days. Decarestrictine D reached its highest yield 5 days after inoculation and remained fairly constant until the end of the fermentation. Similarly, nearly identical fermentation behaviors have been observed for the other decarestrictine-producing strains.

In addition, fermentations of strain FH-A 6090 were performed in medium C using 10-liter fermenters at constant pH-values. Analysis of the yield of the two main compounds, decarestrictines B and D, indicated that an optimum of both metabolites had been produced at pH 3.5. Remarkably, below pH 2.5, the main product is decarestrictine D, whereas fermentations at pH 7 resulted in an insufficient growth of the microorganism.

Isolation and Purification

In order to purify the decarestrictines from the extracellular medium the fermentation broths (medium C) of 10-liter fermenters were separated from the mycelium by filtration. The culture filtrates were extracted with ethyl acetate yielding an oily, brownish concentrate after evaporation. Further purification was carried out by column chromatography in two steps. The obtained oily crude products were chromatographed on silica gel in chloroform - methanol (gradient elution) leading to three main fractions.

Scheme 1. Isolation of the decarestrictines A to D.



Rechromatography of fractions 1 and 2 on silica gel, as well as fraction 3 on Sephadex LH-20 (in MeOH) resulted in the decarestrictines A to D (Scheme 1).

Properties

The decarestrictines A to D are readily soluble in methanol, ethyl acetate or chloroform, but insoluble in water or *n*-hexane. With the exception of the crystalline decarestrictine D the compounds appear to be colorless oils. Based on elemental analysis and mass spectra the molecular formulae were determined and the physico-chemical properties are summarized in Table 2. The NMR data will be presented in the accompanying paper[8] resulting in the structure elucidation of the decarestrictines. The data of the known ergot alkaloid epoxyagroclavine-I (**1**)[9,10] are summarized in Table 2, because of their incomplete description in the primary literature.

Biological Activities

The decarestrictines show interesting activity in cell line tests with HEP-G2 liver cells[13,14] due to an inhibitory effect on cholesterol biosynthesis (Table 3A). This was tested *via* sodium acetate incorporation into cholesterol. The 10^{-7} mol/liter concentration of each decarestrictine resulted in inhibition effects of about 40% (decarestrictine A), 20% (B), 30% (C), and 50% (D), respectively. In this test the IC_{50} for the standard compound lovastatin is 2.4×10^{-8}. In normolipemic rats after oral application of 10 mg/kg daily for a total of 7 days the most potent decarestrictine D showed hypolipidemic activity, which is equivalent to the commercial product clofibrate by an application of 100 mg/kg daily (Table 3B). Due to the lack of pathologic changes of defined safety parameters (Table 3C), decarestrictine D revealed a good tolerability.

However, the described decarestrictines A to D exhibit no significant antibacterial, antifungal, antiprotozoal, and antiviral activity.

Discussion

Chemical screening for secondary metabolites applied to Fungi imperfecti led to the isolation of some strains of the genus *Penicillium*, which attracted attention by producing a family of metabolites called

Table 3. Biological activity of decarestrictine D.

(A) Incorporation of ^{14}C-acetate into the sterol fraction of HEP-G2 cell-cultures and LDH-release into culture media:

Compound	Concentration		Incorporation of ^{14}C-acetate nmol/mg cell protein per 3 hours		LDH-release U/liter ($n=3$)	
	mol/liter	n	$\bar{x}$	s	$\bar{x}$	s
Decarestrictine D	10^{-5}	3	8.80	1.18	34.3	1.15
	10^{-7}	3	10.16	1.10	33.9	0.92
Control	—	6	19.25	3.79	33.7	0.58

n: Number of Assays.

(B) Hypolipidemic activity of decarestrictine D in rats after 7 days of oral application:

Number of animals	Compound	Dose (mg/kg/day)	Total cholesterol[a]			Protein[a]		Antiatherogenic index		Body weight (g)			
			VLDL	LDL	HDL	VLDL	LDL	HDL-Cholesterol: LDL-Cholesterol	% of control	Initial value		Final value	
										$\bar{x}$	s	$\bar{x}$	s
10	Clofibrate	100	17	68	437	129	149	6.43	129	266	17	283	21
5	Decarestrictine D	10	34	70	601	157	127	8.59	172	266	6	287	12
19	Control		45	125	624	175	196	4.99	100	266	11	279	12

[a] All units in μg per ml serum.

(C) Activity of decarestrictine D in rats after 7 days of oral application:

Number of animals	Compound	Dose (mg/kg/day)	Parameters of tolerance																	
			Serum enzymes												Bilirubin (μmol/liter)				Creatinine (μmol/liter)			
			GOT (U/liter)				GPT (U/liter)				aP (U/liter)											
			Initial value		Final value		Initial value		Final value		Initial value		Final value		Initial value		Final value		Initial value		Final value	
			$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
5	Decarestrictine D	10	53	3	41	5	22	4	23	5	395	67	328	22	2.5	0.4	2.1	0.5	61	2	58	3
19	Control		57	8	55	6	26	6	26	5	429	82	364	55	2.4	0.4	2.5	0.4	61	6	62	6

decarestrictines. By taxonomic investigations the strains have been classified to be the members of the species *Penicillium simplicissimum* and *Penicillium corylophilum*, which can be combined in the subgenus *Furcatum*. Some compounds of the decarestrictine complex have been isolated and it is considered that these metabolites are structurally related to each other based on their physico-chemical properties. Successful attempts to increase the yield of the decarestrictines were performed by fermentations using defined pH-values. Repeated fermentations of the producing organisms with standardized conditions showed slight variations of the detected metabolite patterns. The isolation of minor components is still under investigation. Pharmacological interest is based on the biological activities of the decarestrictines, especially on component D. This metabolite appears to resemble a potent inhibition of the cholesterol biosynthesis, which yields favorable effects on lipid metabolism *in vivo*. It is remarkable that besides the ability to produce the decarestrictines, only *Penicillium corylophilum* strain FH-A 6360 is also able to produce the ergot alkaloid epoxyagroclavine-I (**1**).

Experimental

General

IR spectra in pressed KBr discs were recorded on Perkin-Elmer Model 297 spectrometer, and the UV spectra on a Kontron Uvikon 860 spectrometer. Optical rotations were recorded with a Perkin-Elmer 241 polarimeter. FAB mass spectra were taken with a Finnigan MAT 8230. Morphological observations were done by using a scanning electron microscope, Jeol Model JSM T20. Drying of the strain samples at the critical point were performed by using Technics Inc. CPA II, and covered with a gold layer by a Humer Junior chamber (Technics Inc.). Preparative ultracentrifugation was performed on Kontron TGA 65, rotor Beckmann 50.4 Ti. Malt and yeast extract, casein, bacto, and meat peptone were purchased from Difco Ltd., and all chemicals from Riedel de Haen. Fermentation was carried out in a 10-liter fermenter (Biostat E) from Braun Dissel (Melsungen, FRG). TLC was performed on silica gel plates (Merck, HPTLC-Fertigplatten, Silica gel $60F_{254}$ on aluminium foil or glass) and column chromatography on Silica gel 60 (0.040~0.063 mm, Merck) or Sephadex LH-20 (Pharmacia).

Taxonomy

Culture media (morphological and strain conservation was performed by adding 2% agar to the following media):

Medium A (CZAPEK-DOX medium[7]): Sucrose 3%, $NaNO_3$ 0.3%, $MgSO_4 \cdot 7H_2O$ 0.05%, KCl 0.05%, $FeSO_4$ 0.001%, K_2HPO_4 0.1%, pH 7.3 prior to sterilization.

Medium B (malt extract medium[7]): Malt extract 1%, glucose 2%, bacto peptone 0.1%, agar 2%, pH 4.7 prior to sterilization.

Medium C: Malt extract 2%, yeast extract 0.2%, glucose 1%, $(NH_4)_2HPO_4$ 0.05%, pH 6.0 prior to sterilization.

Medium D: K_2HPO_4 0.38%, KH_2PO_4 0.12%, $MgSO_4 \cdot 7H_2O$ 0.51%, NaCl 0.25%, $FeSO_4$ 0.005%, $MnSO_4 \cdot 4H_2O$ 0.005% and 1 ml/liter Tween 80, pH 7.0 prior to sterilization.

Medium E (SABOURAUD medium[15]): Casein peptone 0.5%, meat peptone 0.5%, glucose 1%, maltose 1%, pH 6.8 prior to sterilization.

Scanning Electron Microscopy

Preparations of the strains were performed using a modified method of VOBIS and KOTHE[16]. The strains were cultivated on malt extract agar (medium C) at 25°C. Over a period of 20 days, 1 cm^2-pieces of agar from these cultures were incubated in a solution of 4% glutaraldehyde in water for 24 hours. After washing with water (10 minutes, five times), the samples were dehydrated by incubation in 2-methylglycerol (10 minutes, three times). The methylglycerol was replaced by dry acetone (three times, 10 minutes) and the samples were dried at the critical point using CO_2. The samples were fixed on a brass cylinder and were covered with gold using a Humer Junior chamber.

Fermentation

A 1 cm^2 piece of agar from a 7-day old culture, which grew on medium C, was used to inoculate a

300-ml Erlenmeyer flask containing 100 ml of medium C. These flasks were cultivated for 5 to 7 days at 25°C on a rotary shaker (140 rpm). 300 ml of each culture was used to inoculate a fermenter (10 liters working volume) containing medium C (200 rpm, 7 days, aeration 5.0 liter/minute). The total fermentation process was analyzed using a cultivation time of 16 days. Mycelium dry weight was determined by drying the filter cake at 105°C and the pH-value by using a pH-electrode.

Isolation

After harvesting the culture, the fermentation broth of strain FH-A 6090 was filtered and the filtrate (10 liters) was extracted three times with 8 liters ethyl acetate. *In vacuo*, the organic layer was concentrated to dryness and the oily residue was chromatographed on a silica gel column (60 × 8 cm) using a gradient of $CHCl_3$ - MeOH from 30 : 1 to 15 : 1. Three fractions were collected containing decarestrictines A and B (fraction 1), decarestrictine C (fraction 2) and decarestrictine D (fraction 3). Fraction 1 was rechromatographed on a silica gel column (40 × 5 cm) with ethyl acetate - *n*-hexane (1 : 1) as the solvent system to obtain pure decarestrictines A (48 mg) and B (574 mg). Fraction 2 was chromatographed on a silica gel column (35 × 3 cm) using $CHCl_3$ - MeOH (20 : 1) as eluent to yield 54 mg pure decarestrictine C. A column chromatography of fraction 3 on Sephadex LH-20 (150 × 5 cm) in MeOH yielded 470 mg pure decarestrictine D. The physico-chemical data of the decarestrictines A to D are given in Table 1.

Epoxyagroclavine-I (**1**)

The culture broth of strain FH-A 6360 (10 liters), cultivated on medium E, was separated from the mycelium by filtration. The filtrate was extracted three times with 5 liters ethyl acetate and the organic layer was evaporated to dryness. The dark brown crude product was chromatographed in a fast-running column chromatography (silica gel, 35 × 4.5 cm; CH_2Cl_2 - MeOH, 12 : 1) leading to 2.3 g roughly purified product. Gel permeation chromatography on Sephadex LH-20 in MeOH (150 × 2.5 cm) and repurification on a silica gel column (60 × 3 cm; ethyl acetate - *n*-hexane, 1 : 1) yielded 30 mg/liter pure amorphous **1**: Rf values and characterization (see Table 2); mp 100 ~ 102°C; $[\alpha]_D^{20}$ −40.9° (*c* 0.3 in MeOH); EI-MS (70 eV) *m/z* (abundance, %) 254 (100, M^+), 239 (14, $M-CH_3$), 211 (22), 197 (11), 182 (13), 167 (16), 154 (24), 127 (11), 59 (23); ^1H NMR (200 MHz, $CDCl_3$) δ 1.41 (s, 17-H_3), 2.47 (s, N-CH_3), 2.89/3.11 (AB system, 4-H_2), 2.90/3.18 (AB system, 7-H_2), 3.18 (m, 5-H), 3.42 (d, J=2.2 Hz, 9-H), 3.65 (dd, J=5.0 and 2.2 Hz, 10-H), 6.83 (d, J=2.0 Hz, 2-H), 7.01 (m, 12-H), 7.18 (m, 13-H, 14-H), 7.95 (br, NH); ^{13}C NMR (50.3 MHz, $CDCl_3$) δ 17.7 (C-4), 22.5 (C-17), 39.2 (C-10), 42.3 (N-CH_3), 53.1 (C-7), 57.5 (C-5), 57.9 (C-8), 63.0 (C-9), 108.8 (C-14), 110.6 (C-3), 115.9 (C-12), 118.3 (C-2), 122.8 (C-13), 127.0 (C-16), 130.3 (C-11), 133.9 (C-15).

Inhibition of Acetate Incorporation into Cholesterol

Monolayers of HEP-G2 cells in RPMI-1640 medium (Flow) with 10% delipidated fetal calf serum were preincubated for 1 hour with suitable concentrations of the test compounds. After addition of ^{14}C-labeled sodium acetate, the incubation was continued for 3 hours. ^3H-Labeled cholesterol was added as an internal standard and an aliquot of the cells was saponified with alkali. The lipids were extracted with chloroform - methanol (2 : 1). After addition of carrier cholesterol, the lipid mixture was separated preparatively on TLC-plates using chloroform - acetone (9 : 1). The cholesterol zone was visualized with both iodine vapor and a TLC radioscanner and was scraped out. The amount of ^{14}C-labeled cholesterol was determined scintigraphically. Morphological examination gave no rise for toxic damage of the cells. In addition, a release of intracellular lactate-dehydrogenase into the culture medium was not observed.

Hypolipidemic Effect in Animals

Groups of male Wistar-rats were given the specified dose of the test compounds in PEG 400 once daily by stomach tube on 7 consecutive days, the final one being 24 hours before blood sampling and subsequent sacrificing. The control group was given PEG 400 only. During the study, the animals had free access to food and water. Food was withdrawn 24 hours before blood samples were taken retroorbitally under mild ether anesthesia. To estimate the serum lipoproteins, the serum of each rat group was pooled. The serum lipoproteins were separated on a preparative ultracentrifuge. The separation of the VLDL, LDL and HDL fractions was carried out as follows[17,18]: VLDL, native density of the serum (1.006), 16 hours at 40,000 rpm; LDL, 1.04, 18 hours at 40,000 rpm; HDL, 1.21, 18 hours at 40,000 rpm. Boehringer

Mannheim enzymatic test combinations were used to determine the content of cholesterol according to the CHOP-PAP high performance method[19], and protein content was measured by the method of LOWRY *et al.*[20]. The safety parameters in the serum of each animal (aspartate transaminase (ASAT/GOT), alanine transaminase (ALAT/GPT), alkaline phosphatase (aP), bilirubin, and creatine) were analyzed. With the tested compounds no effects on the safety parameters were found.

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