Antascomicins A, B, C, D and E

Novel FKBP12 Binding Compounds from a *Micromonospora* Strain

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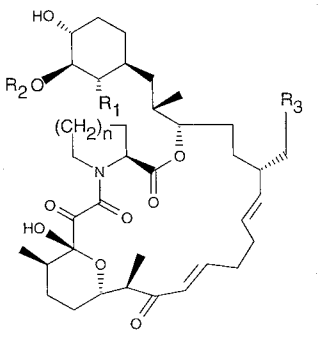
5 novel ascomycin-like compounds, antascomicins A, B, C, D and E were isolated from a strain of *Micromonospora*. The antascomicins bind strongly to the FK506-binding protein FKBP12 and antagonize the immunosuppressive activity of FK506 and rapamycin. The strain description, fermentation, structure elucidation and biological activity of these compounds are described.

Ascomycin, FK506 and rapamycin are powerful immunosuppressive macrolides. They bind with high affinity to the abundant intracellular binding protein FKBP12 (FK506-binding protein; also called macrophilin). These compounds are not immunosuppressive *per se*. It is rather the complex formed between the respective macrolide and FKBP12 which binds to and inhibits certain components of intracellular signalling cascades (for a review see ref. 1). Thus, although not sufficient, the ability to bind to FKBP12 is a prerequisite for immunosuppressive activity. With this in mind we conducted a screening program based on a macrophilin-binding assay (MBA), aiming at the discovery of novel immunosuppressive metabolites from Actinomycetes. About 1% of the strains were found to produce macrophilin-binding metabolites. Besides rapamycin, we identified as the main macrophilin-binding compound the macrolide 91-261402/A-1[2]. This compound was found in more than 60 rapamycin-producing and non-producing strains (*i.e.*, in about 65% of the positive strains which were further analyzed). Macrolide 91-261402/A-1 was recently published by others as meridamycin[3]. Only in one strain among more than 12,000 strains screened we found macrophilin-binding metabolites other than rapamycin, ascomycin, and meridamycin[4]. This strain produces five novel macrolides which we named antascomicins. Antascomicins are structurally related to FK506, bind strongly to FKBP12, but do not show immunosuppressive activity. Here we report on the fermentation, isolation, structure determination, and biological properties of antascomicin A, B, C, D, and E.

Fig. 1. Structures of antascomicins.



Antascomicin A	R_1=H	R_2=H	R_3=H	n=2
Antascomicin B	R_1=OH	R_2=H	R_3=H	n=2
Antascomicin C	R_1=OH	R_2=CH_3	R_3=H	n=2
Antascomicin D	R_1=H	R_2=H	R_3=H	n=1
Antascomicin E	R_1=H	R_2=H	R_3=OH	n=2

Materials and Methods

General

Spectral data were recorded on the following instruments: ^1H and ^{13}C NMR on a Bruker Avance DMX-500 spectrometer at 500 MHz in DMSO solution with TMS as internal standard, IR-spectra on a FT-IR spectrometer Bruker IFS 66 with KBr, UV-spectra on Perkin Elmer Lamda 9 spectrometer and MS-spectra on a VG-7044SE spectrometer, 8 keV Xenon with nitrobenzyl-alcohol as matrix operating in the FAB mode.

Macrophilin Binding Assay

The ability of a compound to bind to FKBP12 (macrophilin 12) is determined by means of a competitive binding assay (macrophilin binding assay, MBA). In this assay, FK506 is coupled to bovine serum albumin which is then used to coat microtiter wells. Biotinylated recombinant human FKBP12 is allowed to bind in the

presence or absence of the microbial extract or tested compound to the immobilized FK506. After washing (to remove FKBP12 which is not specifically bound to the immobilized FK506), the amount of bound biotinylated FKBP12 is assessed by incubation with a streptavidin-alkaline phosphatase conjugate, followed by washing and subsequent addition of p-nitrophenyl phosphate as a substrate. The read out is the OD at 405 nm. Any binding of a compound to the biotinylated recombinant human FKBP12 results in a decrease in the amount of FKBP12 that is bound to the immobilized FK506 and thus in a decrease in the OD 405. Testing serial dilutions of the microbial extracts or the test compound allows determination of the concentration resulting in 50% inhibition of binding of the biotinylated FKBP12 to the immobilized FK506 (IC_{50}).

Producing Organism

The antascomicins A, B, C, D and E are produced by fermenting a strain of the genus *Micromonospora* n. sp. A92-306401, which was isolated from a soil sample collected in China. The strain has been deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany under accession number DSM 8429. The strain A92-306401 can be assigned to the genus *Micromonospora* as a novel species, using the standard classification systems, *e.g.* as described in BERGEY's Manual (1989) and The Prokaryotes (1992). It was confirmed by the 16S rDNA sequence analyses done by DSM. The cell walls contain meso-diaminopimelic acid. The fatty acids are iso- and anteiso-branched straight and unsaturated and characteristically 18:0 (stearic acid). The sugar spectrum is composed of ribose, arabinose, xylose, mannose, galactose, glucose, meso-inositol and an unknown sugar which is not madurose. The mycelium is formed on thin hyphae and does not break down in fragments. No aerial mycelium is formed on the traditionally used media. Spores in most of the cases rare, non-motile and single.

Fermentation

Fermentations were carried out in a 750-liter fermentor containing 500 liters of the main culture medium. The composition of the main culture medium was (g/liter): mannitol 20, yeast extract 1.0 pharmatone 20, KH_2PO_4 0.10, $MgSO_4 \cdot 7H_2O$ 0.05, $CaCl_2 \cdot 6H_2O$ 0.02, trace element solution, agar 1 g and demineralized water for 1 liter. The pH is adjusted to 6.5. The medium is sterilized for 20 minutes at 121°C. The main culture is incubated for five days at 24°C. The fermentation vessel is rotated at 60 rpm during the first 24 hours and hereafter at 80 rpm. Air is introduced at 0.5 liter per minute per liter medium during the 24 first hours and thereafter at 0.8 liter per minute per liter medium.

Isolation of Antascomicins

500 liter fermentation broth was stirred twice with 500 liters of ethyl acetate during 2 hours in a Dispax apparatus and thereafter the two phases were separated in a separator. The combined extracts were evaporated to dryness under reduced pressure and the crude extract was defatted by separation with 3 times 10 liters of methanol - water 9 : 1 and 3 times 10 liter of hexane. The methanol/water phases were combined and the methanol was distilled off under reduced pressure. The residue was extracted twice with 5 liters ethyl acetate. Evaporation to dryness gave 180 g defatted extract. The extract was dissolved in 1 liter of ethyl acetate and 600 g of Silicagel were added. The solvent was evaporated to dryness under reduced pressure in a rotary evaporator. This impregnated silica gel was added to a column of 1 kg silica gel and the components of the extract were separated by chromatography using methyl-tertiary butylether (MTBE - hexane 1 : 1, 3 : 1, MTBE, MTBE 5% methanol, MTBE 10% methanol and methanol as eluent. Fractions of 2 liters were collected and analyzed by HPLC and MBA. Fractions 3 through 11 (75 g) showing good MBA-activity were combined and kept in methanol solution. A precipitate of 14.5 g inactive material was formed which was removed by filtration. The filtrate was separated on a column of 1 kg Sephadex LH20 in methanol by collecting fractions of 800 ml. The active fractions were combined and chromatographed on a column of 3 kg Lichroprep RP18 (Merck) in methanol - water 4 : 1 without pressure. The most active fractions were combined and chromatographed on a column of 300 g Silicagel H (Merck) with a gradient hexane - acetone 8 : 2 to 1 : 9. The most active fractions 3 (2.4 g) and 5

Table 1. Physico-chemical properties of antascomicins.

	Antascomicin A	Antascomicin B	Antascomicin C	Antascomicin D	Antascomicin E
Appearance	crystals	crystals	crystals	amorphous	amorphous
MP (dec)	154~156°C	139~142°C	128~132°C	88~92°C	--
$[\alpha]_D^{22}$ (MeOH)	-59.3°(c 0.575)	-84.7°(c 0.782)		-73°(c 0.534)	--
Molecular weight	659.9	675.9	689.9	645.8	675.9
Molecular Formula	$C_{37}H_{57}NO_9$	$C_{37}H_{57}NO_{10}$	$C_{38}H_{59}NO_{10}$	$C_{36}H_{55}NO_9$	$C_{37}H_{57}NO_{10}$
FAB-MS $(M-18)^+$	642	658	672	628	658
UV λ_{max}^{MeOH} nm(log ε)	222 (4.19)	222 (4.07)	222 (4.08)	222 (4.08)	222(4.05)
IR (KBr) cm^{-1}	1735,1649	1742,1659,1623	1742,1659,1622	1744,1698,1634	1736,1690,1647

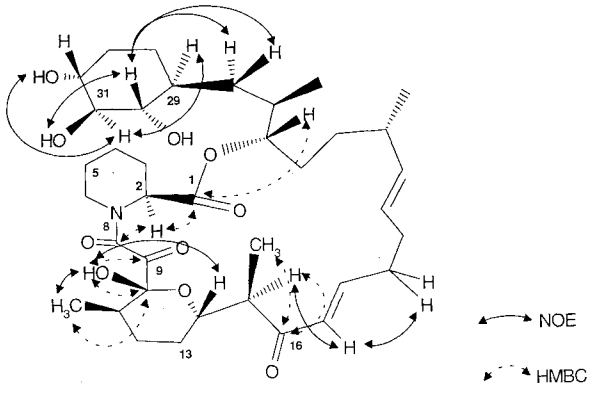
(2.5 g) were each chromatographed again on the column with 3 kg Lichroprep RP18 with methanol-water 4:1. From the chromatographed fraction 5, 490 mg pure antascomicin B could be isolated. The chromatography of fraction 3 revealed 118 mg antascomicin A crystallized from methanol of the fractions 8 to 11 and out of fractions 3 to 6 pure antascomicin C (3.5 mg) crystallized.

The fractionation of the mother liquor on 20 g Silicagel H with CH_2Cl_2-methanol-water 92:7.5:0.5 gave a fraction 4 containing 60 mg antascomicin D. Several active side fractions of the different chromatograms were taken together and chromatographed on a column of 3 kg Lichroprep RP18 40~63 μm with methanol-water 4:1. After elution of 4.8 liters of solvent a fraction of

Table 2. ^{13}C and ^{1}H NMR data of antascomicin A, B, D and E.

		Antascomicin A			Antascomicin B			Antascomicin D			Antascomicin E		
Position	group	$^{13}C_{JMOD}$ (ppm)	$^{1}H_{COSY}$ (ppm)	J(Hz)	$^{13}C_{JMOD}$ (ppm)	$^{1}H_{COSY}$ (ppm)	J(Hz)	$^{13}C_{JMOD}$ (ppm)	$^{1}H_{COSY}$ (ppm)	J(Hz)	$^{13}C_{JMOD}$ (ppm)	$^{1}H_{COSY}$ (ppm)	J(Hz)
1	C=O	170.3			170.3			171.6			170.2		
2	CH	51.6	5.03		51.8	4.98	d (5)	59.2	4.27	dxd 9/4	51.5	5.1	d 5
3	CH2	26.8	2.18		26.8	2.17	d 13	32.1	2.32		26.6	2.22	
			1.63	d 13		1.65			1.84			1.6	
4	CH2	21.3	1.77/1.28		21.2	1.75/1.28		24.9	1.92/1.86		21.2	1.69/1.34	
	5 CH2	24.7	1.64/1.38		24.6	1.64/1.38		47.4	3.58/3.32	m	24.6	1.56/1.38	
6	CH2	44.2	3.57	d 14	44	3.54	d 13				44	3.65	
			3.16	dxt 14/14/2		3.17	dxt 13/13/2					3.24	
7	N												
8	C=O	167.3			167.3			166			167.3		
9	C=O	199.4			199.4			199			199.4		
10	C	99.6			99.6			100			99.6		
11	CH	35.1	2.08		35.1	2.07		34.8	2.08		35	2.24	
12	CH2	26.3	1.46		26.5	1.47		26.4	1.48		26.3	1.55	
13	CH2	29.2	1.46		28.9	1.46		29.4	1.43		29.1	1.55	
			1.07			1.06			1.05			1.18	
14	CH	71	3.91	dxt 10/10/1	71.1	3.9	t 11/11	70.2	4.04	dxt 11/11/2	71	4.08	dxt 10/10/1
15	CH	45.7	3.01		45.5	3.03	q 7	45.8	3.01		45.6	3.14	
16	C=O	202.7			202.6			202.7			202.5		
17	HC=	131.6	6.01	d 16	131.6	5.98	d 16	131.4	5.97	d 16	131.7	6.06	d 16
18	HC=	147.9	6.8	m 16/5/9	147.9	6.79	m 16/5/9	148.3	6.8	m 16/10/6	147.4	6.85	m 16/5/9
19	CH2	31.4	2.35		31.5	2.36		31.5	2.34		31.6	2.34	
			2.28			2.28			2.24			2.26	
20	CH2	31.3	2.3		31.4	2.28		30	2.34		31.3	2.3	
			1.97			1.96			1.85			1.96	
21	HC=	127.7	5.45	m 16/5/9	127.6	5.44	m 15/5/9	128	5.43	m 15/10/4	129.7	5.52	m 16/5/9
22	HC=	137.4	5.21	dxd 16/8.5	137.5	5.2	dxd 15/9	137.8	5.11	dxd 15/3	133.5	5.27	dxd 16/8.5
23	CH	38.1	2.13		38	2.12		39.2	2.1		46.6	2.16	
24	CH2	33.5	1.26		33.6	1.27		34	1.2		27.4	1.6	
												1.29	
25	CH2	28.9	1.54		26.7	1.53		28.2	1.53		28.4	1.68	
													1.53
26	CH2	80.3	4.83	dxd 9/4/4	79.2	4.91	dxd 9/4/4	80.3	4.74	m 12/4/2	80.2	4.93	dxt 9/4/4
27	CH	34.1	1.71		34.1	1.78		33.7	1.79		33.6	1.78	
28	CH2	38.1	1.23		36.2	1.79		39.5	1.22		38.1	1.29	
			1.02			0.84			0.98			1.07	
29	CH	33.2	1.35		40.3	1.23		33.4	1.34		33.1	1.4	
30	CH2	38.4	1.78					39.5	1.75		38.9	1.92	
			0.7						0.72			0.83	
31	CH	74.2	3.09		80.1	2.89	dxt 9/9/4	74.4	3.08		74.3	3.24	
32	CH	74.9	3.03		72.4	3.12	m	75.2	3.03		75	3.2	
33	CH2	32.8	1.75		32.3	1.7		33.1	1.73		32.7	1.85	
			1.16			1.15			1.13			1.26	
34	CH2	32.2	1.55		27.2	1.65		31.9	1.59		32.1	1.56	
			0.87			0.86			0.85			0.94	
11-CH3	CH3	15.7	0.72	d 7	15.7	0.71	d 7	15.8	0.68	d 7	15.5	0.84	
15-CH3	CH3	16	1.08	d 7	15.9	1.08	d 7	16.3	1.08	d 7	15.7	1.2	
23-CH3	CH3	21.6	0.95	d 7	21.5	0.95	d 7	22.4	0.92	d 7			
27-CH3	CH3	15.8	0.84	d 7	16.7	0.86	d 7	15.6	0.82	d 7	15.9	0.87	
10-OH	OH		6.64	d 7		6.67	d 1		6.57	d 2		6.7	
31-OH	OH		4.48	d 7		4.66	d 4		4.45	d 4		4.56	
32-OH	OH		4.49	d 7		4.51	d 4		4.46	d 4		4.56	
30	CH				77.2	2.73	dxt 9/9/5						
30-OH	OH					4.43	d 5						
23CH_2OH	OH											4.56	
23-CH2O	CH2										65.2	3.45/3.35	m

Fig. 2. Long range C-H coupling (HMBC) and NOE's of antascomicin B.



400 ml was collected which showed good binding activity and a new metabolite by HPLC analyses. Further purification on two columns of 100 g Silicagel H with acetone - hexane 7 : 3 and 1 : 1 respectively yielded 11 mg of antascomicin E.

Results and Discussion

Structure Elucidation

All five compounds gave the same UV-spectra with a maximum at 222 nm and an extinction of $\log \varepsilon = 4.1$ indicating that an α, β unsaturated carbonyl is present. Also the IR-spectra were nearly equal and showed some similarity with the IR-spectra of ascomycin. The conclusion could be made the five MBA active compounds are ascomycin-like macrolides. Elemental analyses together with the mass spectra revealed the molecular formulas $C_{37}H_{57}NO_9$ for antascomicin A, $C_{37}H_{57}NO_{10}$ for antascomicin B and E, $C_{38}H_{59}NO_{10}$ for antascomicin C and $C_{36}H_{55}NO_9$ for antascomicin D. In the ordinary FAB-MS with nitrobenzyl-alcohol as matrix the main peak was M^+-18. Addition of LiI gave as main peak $M^+ + Li$. Structure elucidation of the antascomicins was done with NMR-spectroscopy by interpretation of the proton-, ^{13}C- and COSY, ROESY and HCCORR spectra. The assignment of C-1, C-8, C-9, C-10 and C-16 was deduced from the HMBC-spectrum of antascomicin B and was transmitted to the others. The long range C-H coupling (HMBC) and NOE's of antascomicin B are shown in Fig. 2. The structure of antascomicin C was derived only from the proton NMR-spectrum. The structures of antascomicins are very similar to the macrolides ascomycin and FK506, except for the alpha-beta unsaturated ketone in the main ring system. They share with rapamycin, ascomycin and FK506 the region in the molecule which is responsible for the binding of the latter to FKBP12. Antascomicins bound to the binding protein could be cocrystallized. Only from the results of the X-ray studies of these complexes it was possible to determine the full stereochemistry of the antascomicins as it is shown in Fig. 1. The X-ray studies will be reported elsewhere.

Table 3. FKBP12 binding assay, IC_{50} values.

Compound	I_{50} (nMol)
FK506	1.1
Rapamycin	0.6
Antascomicin A	2.0
Antascomicin B	0.7
Antascomicin C	1.0
Antascomicin D	5.2
Antascomicin E	0.7

Biological Properties

The antascomicin macrolides are potent FKBP12-binders. Their ability to bind to FKBP12 is in the same range as that of FK506 and rapamycin, except for antascomicin D which binds 5 ~ 10 times less well (Table 3). FK506 and rapamycin are potent inhibitors of T cell proliferation in the mixed lymphocyte reaction, whereas the antascomicins are inactive. These novel macrolides do not inhibit the synthesis of interleukin-2, like FK506 does, or growth factor-driven cell proliferation, as is the case with rapamycin.

The antascomicins are antagonists of FK506 and rapamycin. A 100-fold molar excess of antascomicin B or C completely abrogates the inhibitory effects of FK506 in relevant *in vitro* assays; remarkably, only equimolar concentrations are needed to reverse the effect of rapamycin (W. SCHULER, manuscript in preparation). No other cellular activity of the antascomicins has been identified yet.

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