Cymbimicin A and B, Two Novel Cyclophilin-binding Structures Isolated from Actinomycetes

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Two novel metabolites, cymbimicins A and B, were isolated from the culture broth of a strain of *Micromonospora* sp. by screening for cyclophilin binding metabolites from actinomycete strains. Cymbimicin A binds to cyclophilin A with a high affinity six fold lower than to that of cyclosporin A. The binding affinity of cymbimicin B is about 100 times lower. The taxonomy of the producing strain, fermentation, isolation, physical and biological properties and structure elucidation are described.

The fungal metabolite, cyclosporin A (CyA) exerts a powerful immunosuppressive activity directed mainly against T-lymphocytes. This natural compound has revolutionized the fields of solid organ and bone marrow transplantations. CyA binds to a family of cytosolic proteins named cyclophilins (*e.g.* Cyp A, B, C). The bimolecular complexes of CyA and cyclophilins block the T-cell signal transduction cascade by inhibiting the protein phosphatase activity of calcineurin. This results in blocking the transcription of early activation genes, including the genes coding for IL-2 and other cytokines[1]. The goal of this project was to discover novel compounds binding to Cyp A which might act on the same or different pathway than CyA.

Actinomycete strains selected from various environment samples were grown under three different culture conditions and the freeze-dried broths were extracted with methanol. Twelve thousand two hundred four methanol extracts were tested in the solid phase enzyme-linked cyclophilin binding assay (CBA)[2,3]. Three strains showed activity. One of these produced the metabolites cymbimicins A (**1**) and B (**2**). Only cymbimicin A (**1**) showed high binding activity similar to that of CyA. Despite a similar structure, cymbimicin B (**2**) shows a 100 times lower binding affinity. In this paper we report on the fermentation, isolation, structure determination and biological properties of cymbimicin A (**1**) and B (**2**).

Materials and Methods

General

Spectral data were recorded on the following instruments: NMR spectra were recorded either on a BRUKER AM-500 or a BRUKER AMX-400 spectrometer. All 2D experiments were carried out in phase sensitive mode with time proportional phase increments. Samples of 11 mg cymbimicin A in 0.5 ml DMSO and 5 mg of cymbimicin B in 0.5 ml DMSO under addition of 0.2 ml C_6D_6 were prepared. The addition of C_6H_6 resulted in a better resolution of the protons in the region between 3.3 and 3.6 ppm. The 1D- (^1H, ^{13}C) and 2D-spectra (COSY, HSQC, HMBC) of both samples were measured. IR spectra on a FT IR spectrometer BRUKER IFS 66 with KBr, UV spectra on Perkin Elmer Labmda 9 spectrometer and MS spectra on a VG-7044SE spectrometer, 8 keV Xenon with nitrobenzyl-alcohol as matrix operating in the FAB mode. For the mass spectrometry, the probe was mixed with LiI otherwise no molecular peak was detected.

Taxonomical characterization: The morphology of the strain was ascertained by light microscopy (Zeiss Axioplan). The analysis of diaminopimelic acid was performed on the hydrolysate of cells grown on BENNETT's agar medium.[4] The analysis of the fatty acids and the whole-cell sugars was determined by gas chromatography[5].

Cyclophilin Binding Assay

The ability of a compound to bind to cyclophilin was determined by means of a competitive binding assay (cyclophilin binding assay, CBA) as described previously[2,3]. Briefly, a D-Lys^8-cyclosporin-derivative was coupled to bovine serum albumin (BSA) and coated onto microtiter plate (2 μg/ml in phosphate buffered saline (PBS) for 2 hours at 37°C. After saturation of the plate

with 2% BSA in PBS (1 hour at 37°C) and washings with 0.05% Tween 20 containing PBS and three times with PBS, biotinylated recombinant cyclophilin A, B or C were incubated overnight at 4°C (75, 50 and 100 ng/ml, respectively, in 1%BSA-PBS). After washing, the amount of bound biotinylated cyclophilin was assessed by incubation with a streptavidin coupled to alkaline phosphatase (Jackson Immuno-research Labs, Inc, 1:6000 in 1%BSA-PBS, 2 hours at 37°C), followed by washing. The absorbance at 405nm was measured after hydrolysis of *p*-nitro-phenyl phosphate (1 mg/ml in diethanolamine buffer 1 M, pH 9.6, for 1~2 hours at 37°C).

In the competitive assay, biotinylated cyclophilins were incubated in the presence of the microbial extracts or compounds (overnight at 4°C). Free cyclosporin A was used as a reference compound. After washings to remove the unbound cyclophilin, the assay continued as above. Binding of a compound to the biotinylated cyclophilin results in a decrease in the amount of cyclophilin that can bind to the immobilised cyclosporin derivative coated on the plate and thus in a decrease in the final absorbance. The competition obtained in the presence of test compound was calculated as the percent inhibition of the control reaction between cyclophilin and the coated cyclosporin in the absence of inhibitor. 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 cyclophilin to the immobilized cyclosporin derivative (IC_{50}).

Producing Organism

Cymbimicin A and cymbimicin B are produced by fermenting a strain of the genus *Micromonospora* sp. A92-313709, which was isolated from a soil sample collected in Bromo, Indonesia. This strain has been deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany under the accession number DSM 8594.

The following taxonomic observations indicate that the strain A92-313709 belongs to the genus *Micromonospora*. On various agar media, the strain A92-313709 produces single non-motile spores in clusters. The mycelium is formed of thin hyphae which do not fragment. No aerial mycelium is formed on the traditionally used agar media. Analysis of the cell wall hydrolysates of the strain revealed that the cell walls contained *meso*-diaminopimelic acid. The fatty acid 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.

Fermentation

Cells from mature agar slants of the strain A92-313709 grown on a NZ-Amine-yeast extract-starch medium were suspended in 10 ml saline, and 20 ml of this suspension were inoculated into each 2 liters Erlenmeyer-flask containing 1 liter seed medium composed of glycerol 0.75%, glucose 0.75%, malt extract liquid (Wander) 0.75%, starch 0.75%, soya protein (siber Hegner) 0.25%, NZ-Amine type A (Sheffield) 0.25%, L-asparagine 0.1%, yeast extract (BBL) 0.135%, $CaCO_3$ 0.005%, KH_2PO_4 0.025%, K_2HPO_4 0.050%, $MgSO_4 \cdot 7H_2O$ 0.010%, NaCl 0.005%, trace element solution (Fe 1.5 ppm, Zn 1 ppm, Mn 1 ppm, Cu 0.04 ppm, Co 0.2 ppm, B 0.02 ppm, I 0.04 ppm), agar 0.1%, in deionized water (pH adjusted to pH 7.0 before sterilization). The inoculated flasks were incubated on a rotary shaker (200 rpm) at 27°C for 4 days. 2.5 liters of seed cultures were inoculated each in 75 liters bioreactor containing each 50 liters of the same medium. The cultures are fermented for 72 hours at 27°C. The broth were rotated at 120 rpm and air was introduced at a rate of 0.5 liter per minute per liter medium. 50 Liters of these intermediate cultures were inoculated in 750 liters bioreactors containing each 500 liters of the production medium composed of glucose 2.0%, malt extract liquid (Wander) 0.2%, yeast extract (Bacto) 0.2%, soytone (Bacto) 0.2%, KH_2PO_4 0.020%, K_2HPO_4 0.040%, $MgSO_4 \cdot 7H_2O$ 0.020%, $CaCl_2 \cdot 2H_2O$ 0.005%, NaCl 0.005%, trace element solution (as for the starter culture), in deionized water (pH adjusted to 6.3 before sterilisation). The main fermentation was carried out for 7 days at 24°C with agitation 80 rpm and aeration 0.8 liter per minute per liter medium.

Isolation of Cymbimicins

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 Westfalia separator. The combined extracts were evaporated to dryness under reduced pressure. The crude extracts from 5 fermentations of 500 liters were collected to give 2.2 kg and defatted by separation with 3 times 20 liters of methanol-water 9:1 and 3 times 20 liters of hexane. The methanol-water phases were combined and under addition of water the methanol was distilled off under reduced pressure. The residue was extracted twice with 10 liters ethyl acetate. Evaporation to dryness under reduced pressure gave 315 g defatted extract. The extract

was dissolved in 1.5 liters methanol and added to a column (diameter 30 cm) of 25 kg Sephadex LH20 in methanol. By elution with 25 liters methanol, fractions of 2.1 liter were collected and analyzed by TLC, HPLC and tested for there binding affinity to Cyp A. Fractions 9 to 13 (80 g) were the most active ones and were taken together to give 80 g of enriched material. This material was dissolved in methanol and 200 g Silicagel H were added. The mixture was evaporated to dryness and the impregnated Silicagel was added to a column of 200 g Silicagel H for further chromatographic separations on a column of 200 g Silicagel H (Merck). The column was eluted with 4 liters of hexane - acetone 4 : 1, 6 liters 1 : 1 and 10 liters in a gradient going from 1 : 1 to 100% acetone. The fractions with high binding affinity to Cyp A, 3 to 5 were taken together (23 g) and chromatographed on a column (diameter 8 cm) of 3 kg Lichroprep RP18 (Merck) 40 ~ 63 μm with methanol - water 8 : 2. Fractions of 1 liter were collected. The fraction 8 (3 g) and the most active fractions 13 and 14 (2 g) were further processed. Purification of fraction 13 and 14 on a column (diameter 8 cm) of 1 kg Sephadex LH20 in methanol solution, once more on a column of 3 kg Lichroprep RP18 with methanol - water 4 : 1 and a column of 100 g Silicagel H with hexane - acetone 3 : 2 yielded 290 mg 90% pure amorphous cymbimicin A (**1**), as determined by HPLC at 240 nm.

The fraction 8 (3 g) was chromatographed on a column of 250 g Silicagel H with hexane - acetone 1 : 1 and fractions of 250 ml were collected. Fraction 14 to 16 were taken together to give 790 mg amorphous cymbimicin B (**2**).

Cellular assays

The two-way mouse mixed-lymphocyte reaction and the stimulation of murine spleen cells with concanalavin A were performed according to standard procedures[6~8]. The IL-2 reporter gene assay and the cellular uptake assay have been described elsewhere[9,10].

Results and Discussion

Structure Elucidation

The IR-spectra of the two compounds are very similar, showing strong absorptions at 3400 cm^{-1} for OH- and NH-vibrations and for C-H bonds at 2967, 2934 and 2877 cm^{-1}. The strong carbonyl absorption at 1787 cm^{-1} indicates a gammalactone and the very strong peak at 1653 cm^{-1} is in agreement with peptide linkage. FAB-MS without addition of LiI give no results. Under addition of LiI the molecular ions $(M+Li)^+$ of 1059 resp. 1025 were detected. Together with the elemental analysis, the molecular formula was deduced as $C_{59}H_{92}N_2O_{14}$ for cymbimicin A and $C_{58}H_{86}N_2O_{13}$ for cymbimicin B. These formula were confirmed from proton and carbon counts by the spectral analysis of ^{1}H-NMR and ^{13}C-NMR. The NMR spectra show partial similarity. The region between 3.3 and 3.6 ppm is very complex for both molecules. It was solved, when the identical parts of both molecules were detected. All protons and carbons were unambiguously assigned as shown in Fig. 1 for cymbimicin A and Fig. 2 for cymbimicin B. The double bond configurations are all E and were determined either by the coupling constant or by the NOE. The relative configurations of the ring systems were deduced from ROESY. Two terminal ring structures of cymbimicin A and B are identical and Fig. 3 (a) and (b) show the relative configuration of them. The relative configuration of the ring in the middle of the molecules is shown in Fig. 3 (c) and (d), the arrows indicating the NOE's.

Biological Properties

Cymbimicin A binds to cyclophilin A with a high affinity, showing a relative affinity roughly sixfold lower

Table 1. Physico-chemical properties of cymbimicin A and B.

	Cymbimicin A	Cymbimicin B
Appearance	Amorphous	Amorphous
MP (dec)	78 ~ 83°C	n.d.
$[\alpha]_D^{22}$ (MeOH)	−6.6° (c = 0.558)	n.d.
Molecular weight	1053.4	1019.3
Molecular formula	$C_{59}H_{92}N_2O_{14}$	$C_{58}H_{86}N_2O_{13}$
FAB-MS $(M+Li)^+$	1059	1025
UV λ_{max} nm (log ε)	233 (4.69), 279 (3.44) 318 (3.44)	240 (4.40)
IR (KBr) cm^{-1}	1787, 1651, 1540, 1548, 1381, 1317, 1226, 1153, 1100, 987	1786, 1653, 1597, 1576, 1535, 1459, 1380, 1317, 1159, 1101, 987

Table 2. NMR shifts of cymbimicin A and cymbimicin B.

NMR-shifts of Cymbimicin A. 1H and 13C : 11 mg/0,5ml DMSO						NMR-shifts of Cymbimicin B. 1H : 5mg/0.5mlDMSO+0.2mlC6D6; 13C: 20 mg/0.5 ml DMSO					
Pos.	Group	δ 13C *	δ 1H**	multiplicity***	coupling const. Hz	Pos.	Group	δ 13C *	δ 1H**	multiplicity***	coupling const. Hz
1	CH2	66.1	3.44\3.36	t+dxd		1	CH2	65.6	3.48/3.45	t+dxd	
3	CH	72.9	3.5	q		3	CH	73	3.52	q	
4	Cq	70.5				4	Cq	70.4			
4	OH		4.12	s		4	OH		4.21	s	
5	CH	75.3	3.37	dxd		5	CH	74.9	3.45	dxd	
5	OH		4.69	d		5	OH		4.8	d	
6	CH	43.5	2.54	m		6	CH	43.7	2.7	m	
7	CH	130.2	5.5	dxd	15\9	7	CH	138.5	5.96	dxd	14\8
8	CH	128.3	6.3	dxd	15\11	8	CH	128.3	6.59	dxd	14\11
9	CH	125.1	5.78	d	10	9	CH	140.3	6.54	d	11
10	Cq	137.1				10	Cq	132.7			
11	CH2	35.5	2.03\1.93	2xm		11	CH	147.2	7.25	d	16
12	CH2	34.5	1.75\1.55	2xm		12	CH	125.8	6.23	d	16
13	Cq	101.1				13	CO	198.5			
13	OCH3	47.2	3.04	s		14	CH2	44.9	2.82\2.61	dxd+dxd	
14	CH2	38.6	2.10\1.10	dxd+t		15	CH	77.1	3.72	m	
15	CH	73.2	3.55	m		15	OCH3	56.5	3.2	s	
15	OCH3	55.2	3.22	s		16	CH2	37.9	2.28	m	
16	CH2	36.5	2.05\1.01	dxd+t		17	CH	129.9	5.64	dxt	15\7\7
17	CH	69.5	3.99	m		18	CH	132.7	6.07	dxd	15\10
18	CH	131.5	5.57	dxd	17\7	19	CH	131.5	6.14	dxd	15\10
19	CH	131.3	6.2	dxd	16\11	20	CH	132.7	5.51	dxd	15\7
20	CH	129.6	6	dxd	15\11	21	CH	77	3.79	dxd	
21	CH	138.3	5.78	dxd	15\8	22	CH	41.4	1.33	m	
22	CH	40	2.4	m		23	CH	67.4	3.67	m	
23	CH	75.7	3.39	m		23	OH		4.48	d	
23	OH		4.73	d		24	CH	46.8	1.28	m	
24	CH	47.3	1.38	m		25	CH	74.9	3.48	m	
25	CH	71.9	3.72	m		26	CH2	31	2.25\2.05	2xm	
25	OH		4.58	d		27	CH	120.5	5.44	t	7\7
26	CH2	32.6	2.24\2.00	2xm		28	Cq	139.8			
27	CH	122	5.44	t	7\7	29	CH	72.5	4	m	
28	Cq	139.6				29	OH		4.57		
29	CH	72.5	3.96	m		30	CH2	36.8	1.51\1.28	2xm	
29	OH		4.47	d		31	CH	79.2	3.57	m	
30	CH2	37.9	1.50\1.25	2xm		31	OCH3	57.8	3.29	s	
31	CH	79.2	3.57	m		32	CH	43.4	2.71	m	
31	OCH3	57.8	3.27	s		33	CO	174.1			
32	CH	43.3	2.7	m		34	NH		7.89	d	9.1
33	CO	174.2				35	CH	58.1	4.21	dxd	
34	NH		7.88	d	9.1	36	CO	171.9			
35	CH	58.1	4.17	dxd		37	NH		8.48	d	8.4
36	CO	172				38	CH	56.1	4.48	dxd	
37	NH		8.45	d	8.4	39	CO	173.9			
38	CH	56.1	4.46	dxd		41	CH	84	5.07	d	10
39	CO	174				42	CH	44.2	2.52	m	
41	CH	84	5.05	d	10	43	CH3	14.5	1.02	d	
42	CH	44.3	2.5	m		44	CH3	18.8	1.05	s	
43	CH3	14.5	0.99	d		45	CH3	12.6	1.85	s	
44	CH3	18.9	1.02	s		46	CH3	14.7	0.79	d	
45	CH3	16.9	1.71	s		47	CH2	19.4	1.25	m	
46	CH3	19.1	0.99	d		48	CH3	11.3	0.86	t	
47	CH2	19.8	1.37\1.28	2xm		49	CH3	12.2	1.49	s	
48	CH3	12.2	0.82	t		50	CH3	12.8	0.9	d	
49	CH3	12.5	1.49	s		51	CH	30.9	2.01	m	
50	CH3	12.8	0.9	d		52	CH3	18.6	0.9	d	
51	CH	30.9	2	m		53	CH3	19.6	0.9	d	
52	CH3	18.7	0.9	d		54	CH3	13.4	0.99	d	
53	CH3	19.6	0.91	d		1'	Cq	138.2			
54	CH3	13.4	0.99	d		2'\6'	CH	127.1	7.43	m	
1'	Cq	138.2				3'\5'	CH	129.1	7.43	m	
2'\6'	CH	127.1	7.43	m		4'	CH	129.2	7.43	m	
3'\5'	CH	129.1	7.43	m							
4'	CH	129.3	7.43	m							
*13C-shifts relative DMSO-d6 = 39.9 ppm, **1H shifts relative DMSO-d6 = 2.5 ppm											
***s = singlet, d = doublet, T = Triplet, q = quartet, m = multiplet											

Fig. 1. Cymbimicin A.

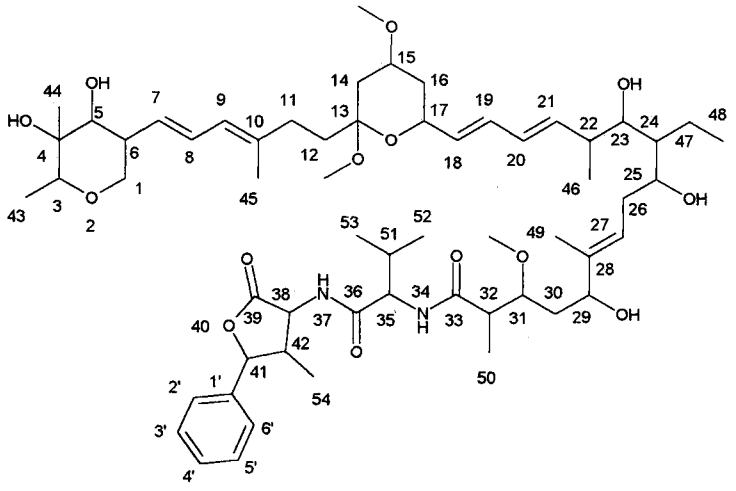


Fig. 2. Cymbimicin B.

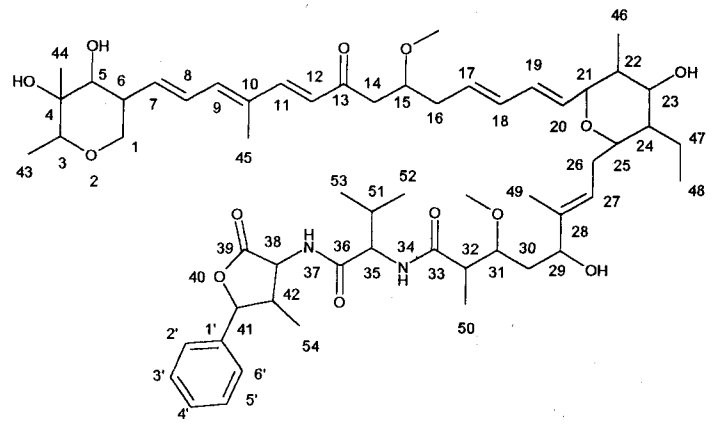


Fig. 3. Relative configuration in the ring systems based on NOE's.

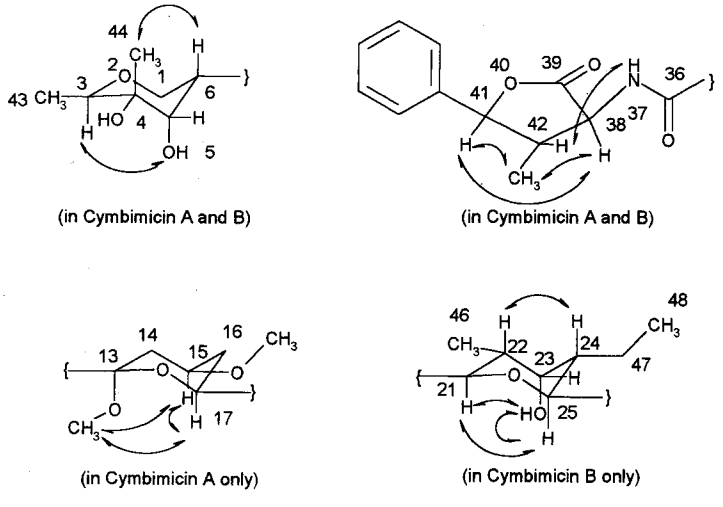
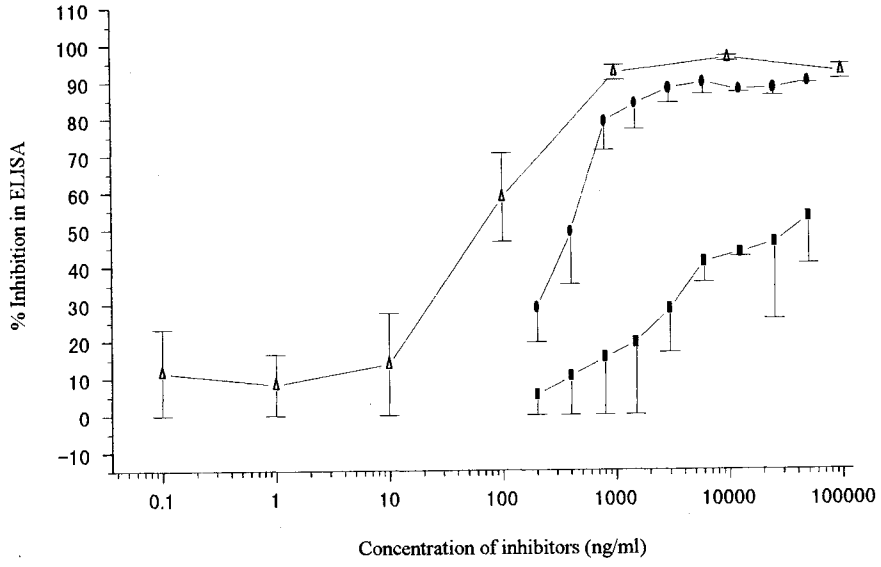


Fig. 4. Inhibition by cymbimicin A and B of the reaction between cyclosporin A and cyclophilin A determined in competitive ELISA.

■ Cymbimicin B, ● cymbimicin A, △ cyclosporin A.



Cyclosporin A conjugated to BSA was immobilized onto the solid phase and allowed to react with cyclophilin A in the presence of increasing concentrations of free cyclosporin A in solution (triangles), cymbimicin A (circles) and cymbimicin B (squares) as described in Methods. The results are expressed as percent inhibition of the control reaction between immobilised cyclosporin A and cyclophilin A in the absence of inhibitor and are the mean ± SEM from 2~3 independent experiments.

than that of cyclosporin A in competitive binding assay (IC_{50} of 400 ng/ml versus 70 ng/ml, *e.g.* 380 nM versus 58 nM for cymbimicin A and cyclosporin A, respectively; Fig. 4). Cymbimicin B binds very poorly to cyclophilin A (IC_{50} >30 μM; Fig. 4). The ~100-fold difference in relative affinity of cymbimicin A and B indicates either that the region of the molecule essential for the interaction of cymbimicin A with cyclophilin A comprises the residues C11 to 25, which are different in cymbimicin B, or that the overall structure of the cymbimicin A molecule, *e.g.* the respective orientation of the two terminal ring structures, should be preserved for binding to cyclophilin A. Binding of cymbimicin A and B to cyclophilin isomers B and C was also measured and found similar to the binding to cyclophilin A (Table 3).

Table 3. Binding of cymbimicin A and B to cyclophilin A, B and C, as determined in competitive ELISA.

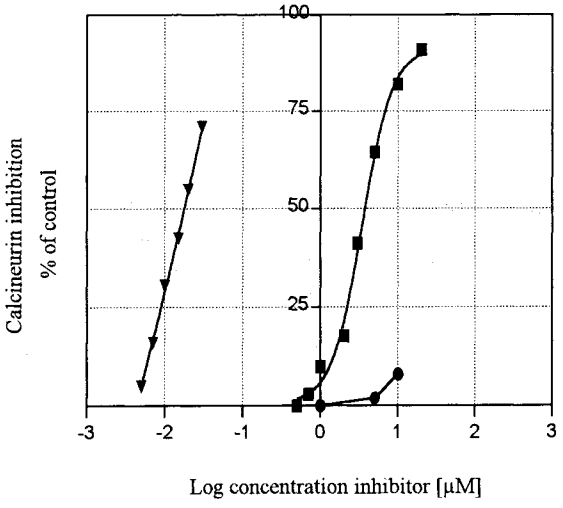
Compound	Cyclophilin A	Cyclophilin B	Cyclophilin C
Cyclosporin A	73±18	30±10	60±10
Cymbimicin A	400±115	325±75	630±270
Cymbimicin B	>33,000	>50,000	>40,000

The results are expressed as IC_{50} ± SEM (in ng/ml) determined in 2~3 independent competitive ELISA experiments.

In T-lymphocytes, binding of cyclosporin to cyclophilin results in an inhibition of calcineurin phosphatase activity[11] and subsequent inhibition of T-lymphocyte activation through inhibition of the expression of early activation genes which include the gene coding for lymphokines such as interleukine 2 (IL-2). The ability of the cymbimicin-cyclophilin complexes to inhibit calcineurin phosphatase activity was tested in a cell-free enzymatic assay using a 19 amino acid phospho peptide as substrate[12]. Neither of the two cymbimicins (at concentration up to 20 μM) complexed to cyclophilin A affected calcineurin activity measured by the calmodulin dependent dephosphorylation of the phospho peptide (A. ENZ, unpublished data) . However, cymbimicin A did inhibit calcineurin activity when complexed with cyclophilin B (IC_{50} 4 μM). Stronger calcineurin inhibition with cyclophilin B rather than cyclophilin A complexes was previously observed with cyclosporin[13] and some cyclosporin derivatives (A. ENZ, unpublished observation). Neither cymbimicin A nor B are active in cellular immunoassays measuring T-lymphocyte activation such as the mixed lymphocyte reaction or concanavalin A stimulated spleen cells. IC_{50} values were ≈8 μM for cymbimicin A and lower for cymbimicin B, compared

Fig. 5. Calcineurin inhibition by cymbimicin A, B and cyclosporin A complexed with cyclophilin B values represent means of two independent *in vitro* experiments.

▼ Cyclosporin A, ■ cymbimicin A, ● cymbimicin B.



with an IC_{50} in the 100 nM range for cyclosporin A. In contrast to cyclosporin A[9], cymbimicin A did not inhibit the transcription of the gene coding for IL-2 measured in an IL-2 reporter gene assay (IC_{50} 8.8 ng/ml and > 10,000 ng/ml, respectively). Moreover, a high excess of cymbimicin A (up to 1000-fold) was unable to reverse the activity of cyclosporin A in the IL-2 reporter gene assay (data not shown), indicating that it cannot act as an antagonist of cyclosporin.

The absence of activity in cellular assays can be due to a lack of cell membrane penetration. cymbimicin A was unable to compete for the binding of 3H cyclosporin A to cells, when added at a molar excess of 30-fold in a cellular uptake assay. Thus, the lack of immuno-suppressive activity of cymbimicin A in cellular assays could be attributed at least in part to the fact that they do not enter the cell.

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