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Sanglifehrins A, B, C and D, Novel Cyclophilin-binding Compounds Isolated from *Streptomyces* sp. A92-308110

II. Structure Elucidation, Stereochemistry and Physico-chemical Properties

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A novel class of macrolides, the sanglifehrins, was discovered by screening of actinomycete strains with a cyclophilin-binding assay. The chemical structures and absolute stereochemistries of the sanglifehrins A, B, C and D were determined unambiguously by NMR-techniques and by X-ray crystallography of the complex with cyclophilin A. Sanglifehrin A consists of a 22-membered macrocycle containing a tripeptide subunit and features in position 23 a chain of nine carbon atoms bearing a spirocyclic substituent. Sanglifehrins A and B are genuine metabolites whereas sanglifehrins C and D are artefacts.

Screening for cyclophilin binding metabolites from actinomycete strains led to the discovery of a novel class of compounds, which were named sanglifehrins. The taxonomy, fermentation, isolation and biological activity are described in the foregoing paper[1].

In this part we describe the structure elucidation mainly done by NMR-analysis, and give the chemical characteristics of these new microbial compounds.

Methods

Spectroscopy

The ^{1}H and ^{13}C NMR spectra of the sanglifehrins were recorded in DMSO-d_6 on a Bruker Avance DMX-500 spectrometer with TMS as internal standard. The ^{1}H, ^{13}C and ^{15}N NMR shifts are listed in Table 2 and Table 3. The ^{15}N-shift of N-6 was deduced from a feeding experiment with ^{15}N labelled ornithine.

Other spectral data were recorded on the following instruments: IR spectra as KBr pellets on a FT-IR spectrometer Bruker IFS 66, UV spectra in methanol on Perkin Elmer Lambda 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 in order to establish the molecular ion peak

X-ray Crystallography

Sanglifehrin A was cocrystallized with cyclophilin A (CypA). X-ray intensity data to a resolution of 1.6 Å, at 20°C, have been collected at the Swiss-Norwegian-Beam-Line of the ESRF in Grenoble, France. The wavelength used was 0.873 Å, and the detector was a MAR image plate. Diffraction images were recorded with an exposure time of 80 seconds for 0.5° rotation per image, at a crystal-to-detector distance of 130 mm. Data processing was done with the programs DENZO 1.5.11 & Scalepack[2], and the CCP4 3.0-package[3]. The overall R_{sym} on intensities for a total of 179130 measurements of 41552 independent reflections in the resolution range 15 Å ~ 1.6 Å (completeness 99.2%) was 7.5%. The structure was solved by molecular replacement and refined with the program X-PLOR, Version 3.1[4].

Results

Sanglifehrins A and B are true natural products as supported by analytical HPLC control during the fermentation (data not shown) whereas sanglifehrins C and D are artefacts formed during the isolation process respectively from sanglifehrins A and B by addition of methanol.

The molecular formulas of the sanglifehrins were revealed by FAB-MS and elementary analysis to be $C_{60}H_{91}N_5O_{13}$ for sanglifehrin A, $C_{60}H_{89}N_5O_{12}$ for B, $C_{61}H_{93}N_5O_{13}$ for C and $C_{61}H_{91}N_5O_{12}$ for D. The UV and IR spectra of the 4 compounds resembled one another suggesting their structural similarity. The IR spectra exhibited characteristic and main absorption at $1645 \sim 1650\,cm^{-1}$ giving incidence for several amide bonds.

The chemical structure of the four sanglifehrins is given in Figure 1. The physico-chemical properties of the sanglifehrins are summarised in Table 1.

NMR Spectra

The NMR data are given in Table 2 and Table 3. Three parts, namely the 22 membered cyclic macrolide from C-1 to C-23, the linker part from C-24 to C-32 and the unique spiro ring system from C-33 to N-42 constitute the complex structure of the sanglifehrins.

Macrolide

Valine, *meta*-tyrosine and 1,2-piperazine-3-carboxylic acid form a peptide part. The sequence is based on sequential NOE's. An inverse ^1H-^{15}N-HSQC confirms the hydrazide group of the 1,2-piperazine-3-carboxylic acid by the ^{15}N shift of NH-6′ at 83 ppm . All other NH's have typical amide shifts in the range of $115 \sim 130$ ppm.

An extended spin system from C-13 to C-23, consisting of a conjugated (*E*, *E*)-diene, two secondary OH-, a secondary methyl- and a methylene-group, concludes the macrolide. The carbonyl group C-13 forms an amide with valine and the oxygen of C-23 forms an ester with the acyl group of 1,2-piperazine-3-carboxylic acid. The attached butan-2-one moiety at C-14 in sanglifehrin A and B forms a ketal with methanol and the 15-OH group in sanglifehrin C and D.

C24-C32 Linker

The C24-C32 linker connects the macrolide with the spirobicyclic ring system. A methyl-substituted conjugated (*E*, *E*)-diene subunit is followed by a C28 to C32 aliphatic part, which bears a methyl and a hydroxy substituent in positions 30 and 31, respectively.

Spiro Ring System

This subunit consists of two 6-memebered rings, fused as a spirobicyclic system at C-37, which resonates at 86.9 ppm. One of these rings bears an ether linkage and the other one forms a lactam. In sanglifehrin A and C, the ether ring is in a chair conformation with two equatorial methyl- and an axial OH-group. Sanglifehrins B and D possess a C-35, C-36 double bound, formally arising from dehydratation of the C-35 hydroxyl of sanglifehrin A and C, respectively. The lactam ring is suggested to be in a boat conformation to allow the methyl group at C-38 and the ethyl group at C-40 to be equatorial.

Absolute Configuration

The absolute configurations of sanglifehrin A were determined by X-ray crystallography of the cyclophilin A/sanglifehrin A complex at 1.6 Å resolution, as shown in the Figure 1. The full details of this structure are going to be published in due time. The absolute configuration of sanglifehrins B, C and D were deduced by spectroscopic and chemical correlation with sanglifehrin A.

Sanglifehrins are a novel type of microbial metabolites of high molecular complexity. The 22-membered macrocycle of the molecules contains not only a polyketide chain but also a tripeptide subunit. This tripeptide consists of valine and two rather unusual amino acids, piperazic acid and *meta*-tyrosine. In contrast to other naturally occurring peptides which contain piperazic acid, in the present case the β-nitrogen (N6) of this amino acid is involved in amide bond formation, and not the α-nitrogen (N6′). The macrocycle features in position 23 a chain of nine carbon atoms bearing a spirocyclic substituent as its terminus. The differences in the four presented sanglifehrins are located in position 35 or 53 or 35 and 53. Additional natural sanglifehrin analogues will be described later.

Fig. 1. Structure of sanglifehrins A, B, C, and D.

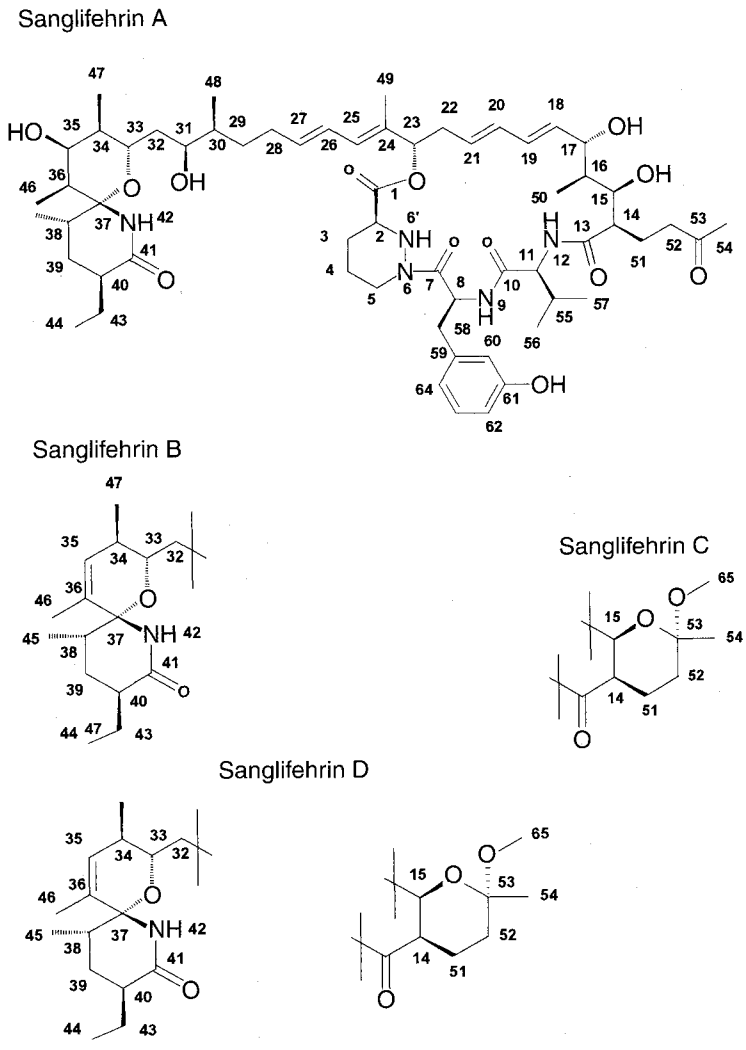

Table 1. Physico-chemical properties of sanglifehrins A~D.

	A	B	C	D
Appearance	White powder amorphous	White powder amorphous	White crystals crystalline	White powder amorphous
$[\alpha]_D^{20}$ (MeOH)	$-67.3°$ ($c=0.99$)	$-52.8°$ ($c=1.1$)	$-35.6°$ ($c=0.74$)	$-23.2°$ ($c=0.98$)
mp (°C)	142~145	117~121	165~170	137~142
Molecular formula	$C_{60}H_{91}N_5O_{13}$	$C_{60}H_{89}N_5O_{12}$	$C_{61}H_{93}N_5O_{13}$	$C_{61}H_{91}N_5O_{12}$
Molecular weight	1090.42	1072.40	1104.45	1086.43
FAB-MS (m/z)	1096 $(M+Li)^+$	1078 $(M+Li)^+$	1110 $(M+Li)^+$	1092 $(M+Li)^+$
	1102 $(M+2Li\text{-}H)^+$	1084 $(M+2Li\text{-}H)^+$	1116 $(M+2Li\text{-}H)^+$	1098 $(M+2Li\text{-}H)^+$
UV (MeOH) λ_{max} nm (ε)	275 (1962)	273 (4395)	275 (1876)	273 (3194)
	242 (54500)	242 (50600)	242 (51557)	242 (47584)
IR (KBr) ν_{max} cm^{-1}	1734, 1718, 1645, 1521, 1458, 1236, 1164, 986	1734, 1718, 1648, 1519, 1457, 1236, 1163, 990	1735, 1646, 1522, 1458, 1236, 1227, 1164, 983	1735, 1650, 1522, 1457, 1379, 1226, 1164, 989

Table 2. NMR-data of sanglifehrins A and B.

NMR-shifts of sanglifehrin A ^1H and ^{13}C: 10 mg/0.5 ml DMSO						NMR-shifts of sanglifehrin B ^1H and ^{13}C: 5 mg/0.5 ml DMSO					
Pos.	Group	δ ^{13}C* δ ^{15}N	δ ^1H**	Multiplicity***	Coupling const. Hz	Pos.	Group	δ ^{13}C* δ ^{15}N	d ^1H**	Multiplicity***	Coupling const. Hz
1	CO	170.6				1	CO	170.6			
2	CH	58.0	1.92	m		2	CH	58.0	1.92	m	
3	CH_2	27.4	1.45	m		3	CH_2	27.4	1.45	m	
4	CH_2	22.5	1.65/1.30	2xm		4	CH_2	22.5	1.65/1.30	2xm	
5	CH_2	40.5	4.20/2.65	2xm		5	CH_2	40.5	4.20/2.65	2xm	
6	N	^{15}N: 140.8				6′	NH		4.50	d	12
6′	NH	^{15}N: 83.0	4.82	d	12	7	CO	172.0			
7	CO	172.0				8	CH	48.9	5.70	m	
8	CH	48.9	5.70	m		9	NH		8.12	d	8.2
9	NH	^{15}N: 125.0	8.15	d	8.2	10	CO	170.1			
10	CO	170.1				11	CH	57.8	4.35	dxd	
11	CH	57.8	4.35	dxd		12	NH		7.51	d	9
12	NH	^{15}N: 116.5	7.50	d	9	13	CO	174.7			
13	CO	174.7				14	CH	51.4	2.18	m	
14	CH	51.4	2.18	m		15	CH	71.5	3.80	m	
15	CH	71.5	3.80	m		15	OH		5.43		
15	OH		5.43			16	CH	40.8	1.64	m	
16	CH	40.8	1.64	m		17	CH	74.4	3.92	m	
17	CH	74.4	3.92	m		17	OH		4.78		
17	OH		4.78			18	CH	134.4	5.75	dxd	15\6
18	CH	134.4	5.75	dxd	15\6	19	CH	130.6	6.35	dxd	15\11
19	CH	130.6	6.35	dxd	15\11	20	CH	131.3	6.10	dxd	15\11
20	CH	131.3	6.10	dxd	15\11	21	CH	129.7	5.72	m	15\7\8
21	CH	129.7	5.72	m	15\7\8	22	CH_2	36.5	2.55/2.30	2xm	
22	CH_2	36.5	2.55/2.30	2xm		23	CH	77.8	5.03	dxd	11\2
23	CH	77.8	5.03	dxd	11\2	24	Cq	132.9			
24	Cq	132.9				25	CH	126.1	5.95	d	11
25	CH	126.1	5.95	d	11	26	CH	125.6	6.20	dxd	15\11
26	CH	125.6	6.20	dxd	15\11	27	CH	136.3	5.70	m	15\7\7
27	CH	136.3	5.70	m	15\7\7	28	CH_2	30.9	2.10/1.90	2xm	
28	CH_2	30.9	2.10/1.90	2xm		29	CH_2	33.8	1.50/1.12	2xm	
29	CH_2	32.8	1.50/1.12	2xm		30	CH	38.5	1.45	m	
30	CH	38.7	1.35	m		31	CH	70.6	3.50	m	
31	CH	69.6	3.52	m		31	OH		4.02	d	
31	OH		4.05	d		32	CH_2	38.3	1.58/1.30	2xm	
32	CH_2	38.1	1.50/1.20	2xm		33	CH	70.7	3.42	m	
33	CH	66.6	3.70	m		34	CH	34.9	1.82	m	
34	CH	40.8	1.35	m		35	CH	132.5	5.42	s	
35	CH	73.3	3.57	m		36	Cq	132.2			
35	OH		5.58	d		37	Cq	85.2			
36	CH	37.6	1.72	m		38	CH	31.2	1.95	m	
37	Cq	86.9				39	CH_2	28.5	2.00/1.42	2xm	
38	CH	30.7	1.90	m		40	CH	41.5	2.10	m	
39	CH_2	28.7	1.90/1.40	2xm		41	CO	175.0			
40	CH	41.0	2.05	m		42	NH		8.05	s	
41	CO	174.1				43	CH_2	26.0	1.70/1.50	2xm	
42	NH	^{15}N: 129.1	7.90	s		44	CH_3	12.5	0.91	d	
43	CH_2	25.8	1.65/1.45	2xm		45	CH_3	15.0	0.75	d	
44	CH_3	12.4	0.85	d		46	CH_3	18.0	1.59	d	
45	CH_3	15.2	0.80	d		47	CH_3	16.5	0.85	d	
46	CH_3	13.5	0.85	d		48	CH_3	14.6	0.80	d	
47	CH_3	14.8	0.75	d		49	CH_3	12.0	1.70	s	
48	CH_3	14.6	0.80	d		50	CH_3	11.0	0.60	d	
49	CH_3	13.0	1.68	s		51	CH_2	23.2	1.75/1.70	2xm	
50	CH_3	15.4	0.85	d		52	CH_2	40.4	2.33/2.25	m	
51	CH_2	23.2	1.75/1.70	2xm		53	Cq	208.1			
52	CH_2	40.4	2.33/2.25	m		54	CH_3	30.1	2.05	d	
53	Cq	208.1				55	CH	30.5	1.95	m	
54	CH_3	30.1	2.05	d		56	CH_3	20.2	0.85	d	
55	CH	30.5	1.95	m		57	CH_3	17.9	0.75	d	
56	CH_3	20.2	0.85	d		58	CH_2	39.3	2.80/2.70	2xm	
57	CH_3	17.9	0.75	d		59	Cq	138.4			
58	CH_2	39.3	2.80/2.70	2xm		60	CH	116.7	6.52	s	
59	Cq	138.4				61	Cq	157.4			
60	CH	116.7	6.52	s		61	OH		9.21	s	
61	Cq	157.4				62	CH	113.3	6.60	d	
61	OH		9.21	s		63	CH	130.0	7.07	t	
62	CH	113.3	6.60	d		64	CH	120.2	6.58	d	
63	CH	130.0	7.07	t							
64	CH	120.2	6.58	d							

* ^{13}C-shifts relative DMSO-d_6 = 39.9 ppm. ** ^1H-shifts relative DMSO-d_6 = 2.5 ppm. *** s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. ^{15}N-shifts: Relative urea = 75 ppm.

Table 3. NMR-data of sanglifehrins C and D.

NMR-shifts of sanglifehrin C ^1H and ^{13}C: 10 mg/0.5 ml DMSO						NMR-shifts of sanglifehrin D ^1H and ^{13}C: 10 mg/0.5 ml DMSO					
Pos.	Group	δ ^{13}C* δ ^{15}N	δ ^1H**	Multiplicity***	Coupling const. Hz	Pos.	Group	δ ^{13}C* δ ^{15}N	δ ^1H**	Multiplicity***	Coupling const. Hz
1	CO	170.6				1	CO	170.6			
2	CH	59.1	2.15	m		2	CH	58.0	1.92	m	
3	CH_2	26.7	1.45	m		3	CH_2	27.4	1.45	m	
4	CH_2	22.8	1.65/1.30	2xm		4	CH_2	22.5	1.65/1.30	2xm	
5	CH_2	40.5	4.20/2.65	2xm		5	CH_2	40.5	4.20/2.65	2xm	
6′	NH		4.82	d	12	6′	NH		4.82	d	12
7	CO	172.2				7	CO	172.0			
8	CH	48.9	5.70	m		8	CH	48.9	5.70	m	
9	NH		8.28	d	8.2	9	NH		8.28	d	8.2
10	CO	171.2				10	CO	170.1			
11	CH	57.1	4.35	dxd		11	CH	57.8	4.35	dxd	
12	NH		6.95	d	9	12	NH		6.95	d	9
13	CO	173.5				13	CO	174.7			
14	CH	44.4	2.30	m		14	CH	44.4	2.30	m	
15	CH	73.7	3.62	m		15	CH	73.7	3.62	m	
16	CH	40.2	2.38	m		16	CH	40.2	2.38	m	
17	CH	73.2	3.70	m		17	CH	73.2	3.70	m	
17	OH		4.65			17	OH		4.65		
18	CH	135.1	5.75	dxd	15\6	18	CH	135.1	5.75	dxd	15\6
19	CH	130.7	6.35	dxd	15\11	19	CH	130.7	6.35	dxd	15\11
20	CH	132.7	6.10	dxd	15\11	20	CH	131.3	6.10	dxd	15\11
21	CH	129.8	5.72	m	15\7\8	21	CH	129.7	5.72	m	15\7\8
22	CH_2	36.5	2.55/2.30	2xm		22	CH_2	36.5	2.55/2.30	2xm	
23	CH	78.8	5.03	dxd	11\2	23	CH	77.8	5.03	dxd	11\2
24	Cq	133.4				24	Cq	132.9			
25	CH	126.6	5.95	d	11	25	CH	126.1	5.95	d	11
26	CH	126.0	6.20	dxd	15\11	26	CH	125.6	6.20	dxd	15\11
27	CH	136.6	5.70	m	15\7\7	27	CH	136.3	5.70	m	15\7\7
28	CH_2	30.9	2.10/1.90	2xm		28	CH_2	30.9	2.10/1.90	2xm	
29	CH_2	32.8	1.50/1.12	2xm		29	CH_2	33.8	1.50/1.12	2xm	
30	CH	38.7	1.35	m		30	CH	38.5	1.45	m	
31	CH	69.9	3.52	m		31	CH	70.6	3.50	m	
31	OH		4.05	d		31	OH		4.02	d	
32	CH_2	38.1	1.50/1.20	2xm		32	CH_2	38.3	1.58/1.30	2xm	
33	CH	66.9	3.70	m		33	CH	70.7	3.42	m	
34	CH	40.8	1.35	m		34	CH	34.9	1.82	m	
35	CH	73.4	3.57	m		35	CH	132.5	5.42	s	
35	OH		5.60	d		36	Cq	132.2			
36	CH	37.6	1.72	m		37	Cq	85.2			
37	Cq	87.2				38	CH	31.2	1.95	m	
38	CH	30.7	1.90	m		39	CH_2	28.5	2.00/1.42	2xm	
39	CH_2	28.7	1.90/1.40	2xm		40	CH	41.5	2.10	m	
40	CH	41.0	2.05	m		41	CO	175.0			
41	CO	174.4				42	NH		8.08	s	
42	NH		7.90	s		43	CH_2	25.5	1.69/1.48	2xm	
43	CH_2	25.8	1.65/1.47	2xm		44	CH_3	12.5	0.91	d	
44	CH_3	12.4	0.85	d		45	CH_3	14.0	0.79	d	
45	CH_3	15.2	0.80	d		46	CH_3	18.0	1.58	d	
46	CH_3	13.5	0.85	d		47	CH_3	14.8	0.88	d	
47	CH_3	14.8	0.75	d		48	CH_3	15.1	0.88	d	
48	CH_3	14.6	0.80	d		49	CH_3	13.2	1.69	s	
49	CH_3	13.0	1.68	s		50	CG_3	15.2	0.75	d	
50	CH_3	15.4	0.85	d		51	CH_2	23.2	1.95/1.65	2xm	
51	CH_2	23.6	1.90/1.60	2xm		52	CH_2	31.8	1.60	m	
52	CH_2	32.0	1.62	m		53	Cq	98.5			
53	Cq	98.6				54	CH_3	24.0	1.27	d	
54	CH_3	24.4	1.21	d		55	CH	30.5	1.95	m	
55	CH	30.5	1.95	m		56	CH_3	20.2	0.89	d	
56	CH_3	20.2	0.85	d		57	CH_3	17.9	0.79	d	
57	CH_3	17.9	0.75	d		58	CH_2	39.3	2.80/2.70	2xm	
58	CH_2	39.3	2.80/2.70	2xm		59	Cq	138.4			
59	Cq	139.0				60	CH	116.7	6.59	s	
60	CH	117.1	6.59	s		61	Cq	157.4			
61	Cq	157.8				61	OH		9.21	s	
61	OH		9.22	s		62	CH	113.3	6.60	d	
62	CH	113.4	6.60	d		63	CH	130.0	7.07	t	
63	CH	129.9	7.07	t		64	CH	120.2	6.68	d	
64	CH	120.6	6.68	d		65	OCH_3	48.1	3.12	s	
65	OCH_3	48.1	3.12	s							

* ^{13}C-shifts relative DMSO-d_6 = 39.9 ppm. ** ^1H-shifts relative DMSO-d_6 = 2.5 ppm. *** s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. ^{15}N-shifts: Relative urea = 75 ppm.

Acknowledgments

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