Chrysospermins, New Peptaibol Antibiotics from *Apiocrea chrysosperma* Ap101

KLAUSJÜRGEN DORNBERGER*, WOLFGANG IHN, MICHAEL RITZAU, UDO GRÄFE, BRIGITTE SCHLEGEL and WERNER F. FLECK

Hans-Knöll-Institut für Naturstoff-Forschung,
Beutenbergstr. 11, D-07745 Jena, Germany

JÖRG W. METZGER

Institut für Organische Chemie der Universität,
Auf der Morgenstelle 18, D-72076 Tübingen, Germany

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Four new members of peptaibol antibiotics, designated as chrysospermins A, B, C, and D, were isolated from the mycelium of *Apiocrea chrysosperma* Ap101 by solvent extraction, silica gel chromatography and preparative recycling HPLC. Their structures as new nonadecapeptides were settled by detailed spectroscopic analysis and chemical degradation experiments. The chrysospermins display antibacterial and antifungal activity, and induce pigment formation by the fungus *Phoma destructiva*.

In a screening for new inducers of microbial differentiation[1] the fungus *Apiocrea chrysosperma* Ap101 was found to produce novel antifungal peptides, chrysospermins A (**1**), B (**2**), C (**3**), and D (**4**), as new members of the peptaibol class of linear lipophilic peptide antibiotics[2]. Peptaibols such as aibellin[3], alamethicins[4], antiamoebins[5,6], emerimicins[7], paracelsin[8], saturnisporins[9], suzukacillin[10], trichorzianins[11], trikoningins[12], and zervamicins[13] are of considerable biological interest because they facilitate the transport of ions across membranes *via* mechanism involving pore formation[14~16]. In contrast to other peptaibols, such as alamethicin A, the chrysospermins promote pigment formation by surface cultures of *Phoma destructiva* in a manner similar to the known immunosuppressant cyclosporin A[1].

In this report, we describe the production and isolation of the chrysospermins as well as their physico-chemical properties, structure elucidation and antimicrobial activities.

Materials and Methods

Microorganism

The producing strain Ap101 from the strain collection of the Hans-Knöll-Institut für Naturstoff-Forschung was identified as *Apiocrea chrysosperma* due to its morphological characteristics. This fungus was deposited in the DSM strain collection (Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany) with the accession No. DSM 7444[17].

Chromatography

Thin-layer chromatography was performed using Silica gel 60 F_{254} precoated TLC aluminium sheets (Merck, Darmstadt, Germany) by development with $CHCl_3$ - MeOH (90 : 10, v/v). The components could be vizualized by spraying with 3% vanillin - H_2SO_4.

Analytical and semi-preparative HPLC were performed on a Shimadzu LC-8A large-scale HPLC system consisting of two LC-8A pumps equipped with 150 ml/minute pump heads, a SCL-8A system controller, an SIL-8A auto injector, a FCV-100B fraction collector, a SPD-6AV variable UV/VIS-detector, a C-R4AX CHROMATOPAC data processor, a Rheodyne column switching valve, a manual Rheodyne 7125 sample injector and an FCV-120AL automatic recycle valve. The analytical HPLC was performed on the same system by column switching to an analytical column.

Fermentation

A small piece of a mature slant culture of the strain AP101 grown on malt extract agar (malt extract 4%, yeast extract 0.4% and agar 1.5%, deionized water, pH 6.0) was used to inoculate 500-ml Erlenmeyer flasks containing 100 ml of the producing medium consisting of malt extract 2%, glucose 1%, yeast extract 0.1%, and $(NH_4)_2SO_4$ 0.5%, pH 6.0. These surface cultures were incubated for 15 days at 28°C.

Monitoring of chrysospermin production was performed by analytical HPLC analysis of ethyl acetate extracts of culture samples under the following conditions: column, 4 mm i.d. × 250 mm, Nucleosil C18, 5 μm; solvent, CH_3CN - H_2O (50 : 50); flow rate, 1 ml/minute; detection, 210 nm. Under these conditions the retention times of **1**, **2**, **3**, and **4** were 12.8, 13.5, 18.1, and 19.1

minutes, respectively (Fig. 1).

Isolation

A typical 5 liter culture of *A. crysosperma* was extracted twice with equal volumes of ethyl acetate at pH 7.0 for 24 hours. The residue of evaporated ethyl acetate extract was dissolved in a small volume of $CHCl_3$ (50~100 ml) and the chrysospermins were precipitated by addition of 500 ml *n*-hexane (yield 750 mg). Thereafter, the crude chrysospermins were subjected to column chromatography (25 × 400 mm) on Silica gel 60 (0.063~0.2 mm, Merck, Darmstadt Germany) using stepwise $CHCl_3$ and $CHCl_3$ - MeOH (80 : 20, v/v) as eluents. The fractions showing one reddish spot on TLC after staining with vanillin - H_2SO_4 were combined and concentrated *in vacuo* to yield 250 mg. This residue was fractionated to yield mixtures **1/2** and **3/4**, respectively, by semi-preparative HPLC on a 20 mm i.d. × 250 mm Nucleosil C18, 7 μm column, equipped with a 30 mm guard column (Macherey-Nagel, Düren, Germany) using an CH_3CN - H_2O (1 : 1, v/v) isocratic solvent system and a flow rate of 20 ml/minute. Sample size: 10 mg/ml MeOH (25 injections for 250 mg total sample). Separation time: 40 minutes. Detection: UV-210 nm. Upon concentration and lyophilization of all separations peaks having retention time of 21 minutes afforded 30 mg of chrysospermins A/B mixture and peaks having retention time of 30 minutes yielded 145 mg of chrysospermins C/D mixture.

For final separation, mixtures of chrysospermins A/B

Fig. 1. HPLC chromatogram of the chrysospermins.

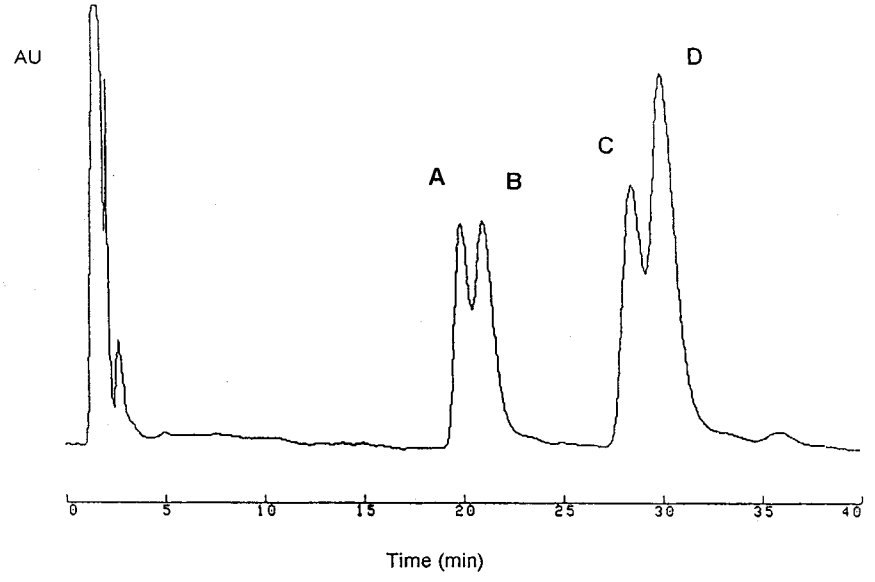
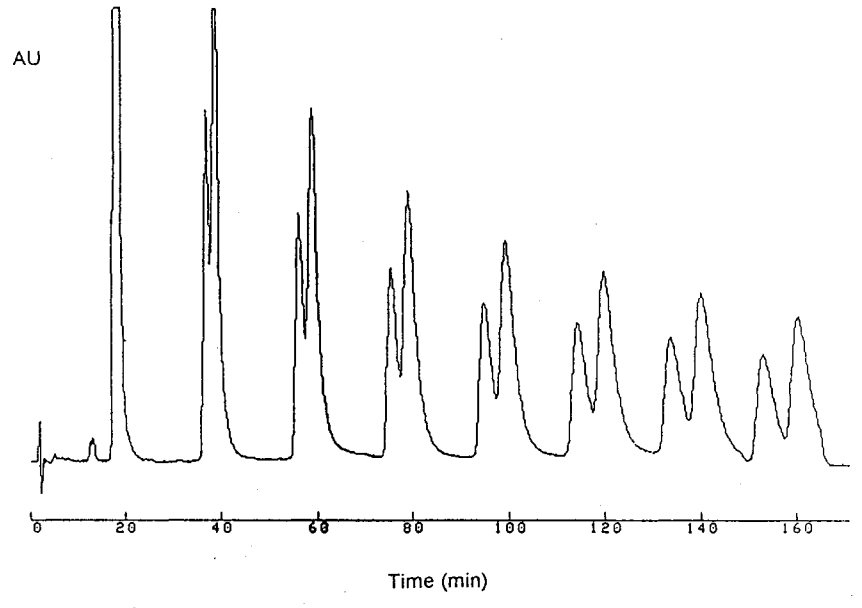


Fig. 2. Preparative recycling separation of the chrysospermins A/B (**1/2**) mixture.



25 mg were injected.

and C/D were recycled in the same system for eight times as shown in Fig. 2 for **1** and **2**. Fraction 1 was shaved from the front of the peak to obtain pure **1**, which could be pooled with subsequent fractions of comparable purity from this and later runs. In the same way, pure **2** (fraction 3) could be obtained from the tail of the peak. Fraction 2 will be saved until other runs are made, then combined with fractions of comparable purity and recycled again.

Instrumental Analysis

Mass Spectrometry

Electrospray mass spectrometry: The chrysospermin mixtures were dissolved in MeOH-1% aq. HCOOH (1:1, 0.2 mg/ml). Positive ion ES mass spectra were recorded on a triple-quadrupol mass spectrometer API III equipped with a nebulizer assisted electrospray (='ion spray')[18] source (Sciex, Thornhill, Ontario, Canada). The solutions were infused with a medical infusion pump (Model 22, Harvard Apparatus, Southnatick, U.S.A.) at a flow rate of 5 μl/minute. ES mass spectra and CID (daughter ion) spectra were acquired at unit resolution scanning with a step size of 0.1 u and a dwell time of 2 msec. Three to five spectra were summed. The potential of the spray needle was held at +4.8 kV. Spectra were recorded at an orifice voltage of +140 kV to enhance fragmentation[19]. Accuracy of mass assignment was $\pm$0.2 u.

Tandem mass spectrometry: CID experiments were performed using argon at a target gas thickness of about 5×10^{14} atoms cm^{-2} with collision energies of *ca.* 50 eV. The CID spectra were an average sum of *ca.* 20 scans. Cesium iodide was used to calibrate both quadrupoles Q_1 and Q_3.

Positive ion FAB mass spectra were recorded on an AMD 402 double focusing instrument of BE geometry (AMD Intectra, Harpstedt, Germany). Ions were produced by fast ion bombardment with a 12 keV Cs^+ ion beam generated in a Cs^+ gun (Liquid SIMS system, AMD Intectra). Peptide solutions were mixed with m-nitrobenzylalcohol as matrix on the FAB probe tip. Exact mass measurements were performed using the peak matching technique (PEG as standard).

NMR Spectroscopy

NMR spectra were recorded at 300 K in DMSO-d_6 on a Bruker AMX 600 spectrometer with sample concentrations of 12.8 mM, 6.3 mM, 12.4 mM, and 5.9 mM for **1**~**4**, respectively. Chemical shifts are given in ppm relative to internal TMS (^{1}H) or DMSO-d_6. ^{1}H NMR and ^{1}H-detected two dimensional NMR spectra were obtained with a recycle delay of 1.8 seconds by solvent presaturation. NOESY (mixing time 400 ms), TOCSY (spin lock time 80 ms) and HMQC data were obtained in phase sensitive mode using TPPI. The HMBC spectra were optimized on a long range coupling constant of 7 Hz (τ=71 ms).

Amino Acid Analysis

Total acidic hydrolysis of the chrysospermins was carried out in sealed tubes with 6 N HCl at 110°C for 18 hours. Identification of the amino acids and determination of their chirality was accomplished after derivatization to the N-trifluoroacetyl-*n*-propyl esters by GC using Chirasil-L-Val (N-propionyl-L-valine-ter.-butyl amide) polysiloxane quartz capillary column (Chrompak-k, L=25 m, i.d.=0.2 mm). Alkaline hydrolysis for the characterization of tryptophanol was performed with barium hydroxide according to a procedure published previously[11]. A separation of L- and D-tryptophanol was achieved after derivatization of the amino alcohol DL-tryptophanol (L-tryptophanol as reference) with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide on TLC plates (Silica gel F_{254}, Merck, Darmstadt, Germany). The Rf values of the yellow MARFEY's adducts of D-Trpol were Rf=0.25 and of L-Trpol Rf=0.32 (ethyl acetate-dichloromethane 4:1, v/v).

Determination of Biological Activities

Antibiotic Activity

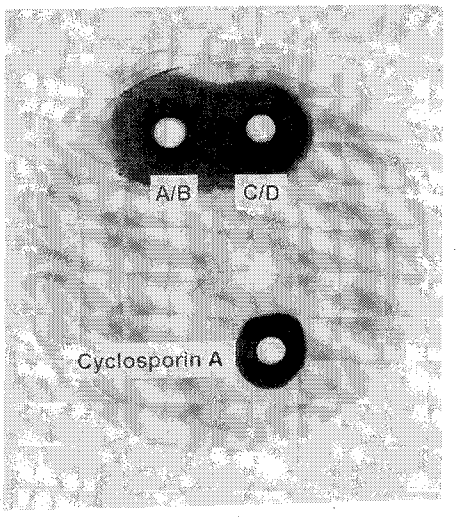
The minimum inhibitory concentrations (MIC) were determined by means of a standardized agar diffusion plate assay[20]. The following media were used for strain cultivation and agar diffusion assay: Standard I Nutrient Agar (SERVA, Germany), Sabouraud-2%-Glucose-Agar (DIFCO) and malt agar (malt extract 4%, yeast extract 0.4%, agar 1.5%).

Effect on Cytodifferentiation of the Fungus *Phoma destructiva*

Malt agar plates with vegetative mycelium of the fungus *Phoma destructiva* were prepared in a manner similar to the standardized agar diffusion assay[20]. 50 μl of methanolic solutions of chrysospermin samples were

Fig. 3. Bioassay of cytodifferentiation of *P. destructiva*.

A/B: 100 μg of chrysospermins A/B mixture, C/D: 100 μg of chrysospermins C/D mixture, Cyclosporin A: 2.5 μg of cyclosporin A.



applied to agar wells (9 mm i.d.). 48~72 hours after incubation at 25°C the diameter of brownish pigmented halo around the wells was a quantitative measure of these antibiotics (Fig. 3).

Results

Description of the Producing Strain

The diagnostic microscopic features on the wild-type strain of *A. chrysosperma* DSM 7444 are illustrated in Fig. 4. The strain forms aerial mycelium and conidia on malt agar.

A. chrysosperma, the teleomorph of *Sepedonium chrysospermum* Link[21] is characterized by the white, later dark yellow pigmented mycelium, and by generally round, blunt-warty conidia with 10.5~14 μm diameter. The teleomorph form was not observed in cultivation.

Isolation and Characterization of the Chrysospermins A (1), B (2), C (3) and D (4)

The isolation of the chrysospermins was carried out according to the scheme given in Fig. 5. Immediate separation of the four chrysospermins from one another was not possible because of the similar retention times in reversed phase HPLC of **1/2** and **3/4** mixtures, respectively. Therefore time consuming recycling chromatography of the two chrysospermin mixtures was necessary to obtain the pure peptaibols.

Difficult-to-separate components can be separated well by allowing the sample to recycle through the column many times until the sample being separated occupies the total volume of the column. A technique for making recycle HPLC an even more effective tool is peak shaving. In this method, portions of pure sample components of interest from peak fronts and tails are collected and the remainder of incompletely separated sample mixtures is recycled back through the column. The determination of

Fig. 4. Digital scanning micrograph of hyphae and conidia of the strain *A. chrysosperma*.

Bar represents 20 μm.

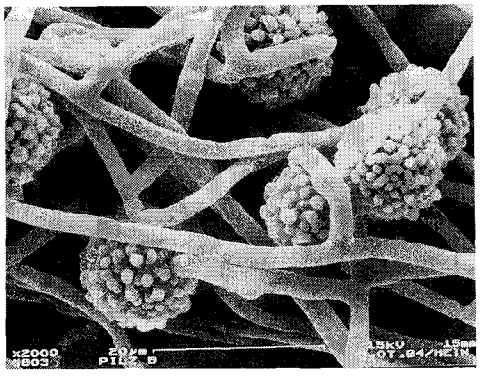


Fig. 5. Isolation procedure of the chrysospermins.

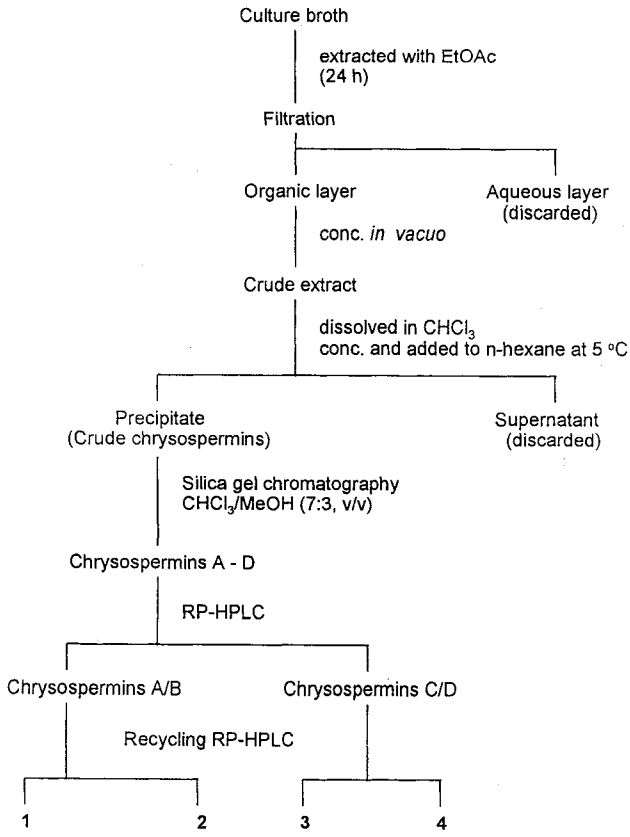


Table 1. Physico-chemical properties of chrysospermins A (**1**), B (**2**), C (**3**), and D (**4**).

	1	2	3	4
Appearance	White powder	White powder	White powder	White powder
MP	250°C (dec)	250°C (dec)	250°C (dec)	250°C (dec)
MW (monoisotopic mass)	1897	1911	1911	1925
Molecular formula	$C_{90}H_{140}N_{22}O_{23}$	$C_{91}H_{142}N_{22}O_{23}$	$C_{91}H_{142}N_{22}O_{23}$	$C_{92}H_{144}N_{22}O_{23}$
UV λ_{max}^{MeOH} ($E_{1cm}^{1\%}$)	274 (5,400)	274 (5,430)	274 (5,600)	274 (5,650)
	282 (5,760)	282 (5,800)	282 (6,270)	282 (6,310)
	290 (5,040)	290 (5,070)	290 (5,350)	290 (5,390)
Solubility				
Soluble	MeOH, DMSO	MeOH, DMSO	MeOH, DMSO	MeOH, DMSO
Insoluble	H_2O	H_2O	H_2O	H_2O

fraction purity was made by use of analytical HPLC. The benefits of peak shaving recycle of chrysospermins A/B are shown in Fig. 2. After eight recycles, a resolution of R = 1.1 was achieved, the latter being slightly more abundant (60%) than the former (40%).

Chrysospermins C/D were separated in the same fashion. The physico-chemical properties of **1**, **2**, **3**, and **4** are shown in Table 1.

Structural Elucidation

The structures of **1**~**4** as shown in Table 2 were elucidated by detailed spectroscopic investigations and amino acid analysis.

Supporting evidence of the peptide nature of the chrysospermins was first obtained from the IR data indicated by absorption bands at 3300 (NH), 1660 (amide I, CO), and 1525 cm^{-1} (amide II, NH). Moreover, characteristic NH signals at 6.7~8.5 ppm in the ^1H NMR spectra (DMSO-d_6) and signals in the CO and C_α region in the ^{13}C NMR spectra (DMSO-d_6) gave full support to this conclusion.

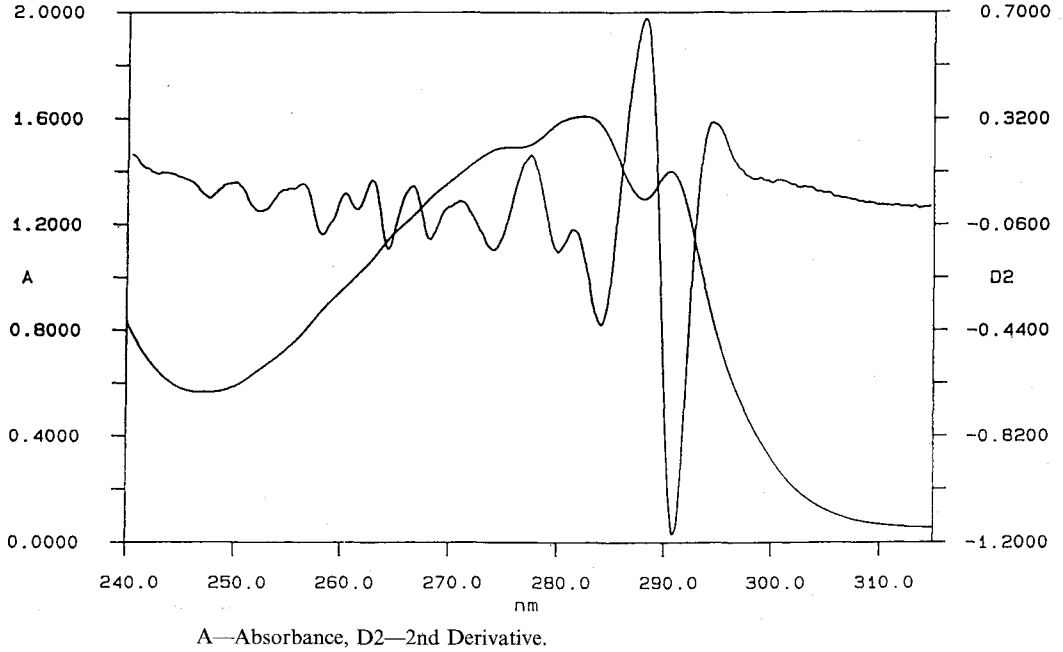
Amino acid analysis after total hydrolysis by GC on a chiral phase revealed the presence of Gly (1×), L-Ala (2×), L-Ser (1×), L-Pro (1×), L-Leu (1×), L-Glx (2×) and L-Phe (1×) in all chrysospermins. The distinction between the four peptaibols arose from the presence of additional α-aminoisobutyric acid (Aib) (9×) in **1**, Aib (8×) and D-Isovaline (Iva) (1×) in **2** and **3** and Aib (7×) and D-Iva (2×) in **4**. By comparison of the Rf values of tryptophanol (Trpol) in the basic hydrolysate with

Table 2. Primary structures of chrysospermin A (**1**), B (**2**), C (**3**), and D (**4**) diagnostic ions observed in electrospray tandem MS and FAB-MS (the *m/z* values correspond to the nominal masses).

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
1	AcPhe-	Aib-	Ser-	Aib-	**Aib**-	Leu-	Gln-	Gly-	Aib-	Aib-	Ala-	Ala-	Aib-[c]	Pro-	**Aib**-	Aib-	Aib-	Gln-	Trpol
2	AcPhe-	Aib-	Ser-	Aib-	**Aib**-	Leu-	Gln-	Gly-	Aib-	Aib-	Ala-	Ala-	Aib-	Pro-	**Iva**-	Aib-	Aib-	Gln-	Trpol
3	AcPhe-	Aib-	Ser-	Aib-	**Iva**-	Leu-	Gln-	Gly-	Aib-	Aib-	Ala-	Ala-	Aib-	Pro-	**Aib**-	Aib-	Aib-	Gln-	Trpol
4	AcPhe-	Aib-	Ser-	Aib-	**Iva**-	Leu-	Gln-	Gly-	Aib-	Aib-	Ala-	Ala-	Aib-	Pro-	**Iva**-	Aib-	Aib-	Gln-	Trpol
	Acylium ion, *m/z*																		
1		275	362	447	532	645	773	830	915	1000	1071	1142	1227		183[a]	268	353	481	671
2		275	362	447	532	645	773	830	915	1000	1071	1142	1227		197[b]	282	367	495	685
3		275	362	447	546	659	787	844	929	1014	1085	1156	1241		183[a]	268	353	481	671
4		275	362	447	546	659	787	844	929	1014	1085	1156	1241		197[b]	282	367	495	685

[a] The fragment ion *m/z* 183 could be formulated as H-Pro-Aib.
[b] The fragment ion *m/z* 197 could be formulated as H-Pro-Iva.
[c] Preferential cleavage site observed in MS.
Aib—α-aminoisobutyric acid, Iva—isovaline, Trpol—tryptophanol

Fig. 6. Zero- and second-order derivative spectra of **1**.



A—Absorbance, D2—2nd Derivative.

L-Trpol on TLC plates, the presence of the L-isomer in **1**~**4** has unambiguously confirmed.

Moreover, the occurrence of aromatic residues was indicated by UV absorption bands in the range 270~290 nm. Enhancement of resolution by second-order derivative spectroscopy[22] revealed minima at 264 and 292 nm (Fig. 6), the latter being characteristic for the tryptophanyl residues in the C-terminal amino alcohol Trpol. The second minimum at 264 nm could be ascribed to the phenylalanyl residues. Tandem mass spectrometry showed that this residue was acetylated and constituted the N-terminal amino acid.

This finding was supported by a sharp singulet at 2 ppm in the ^{1}H NMR spectra of **1**~**4** as a characteristic of an acetyl group.

The peptaibols reacted neither with diazomethane nor acetic anhydride and gave no color reaction with ninhydrin, indicating that a free carboxy and N-terminal amino group were not present. Hence, sequence determination by means of Edman-degradation was impossible.

Most peptaibols rich in Aib residues, adopt a predominant α-helical structure in solution. In accord the circular dichroism (CD) spectra of **1**~**4** (Fig. 7) show three Cotton effects around 226 nm (−), 208 nm (−) and 198 nm (+) typical for α-helical conformation[23].

Mass and Tandem Mass Spectrometry

Because the chrysospermins are not susceptible to usual peptide sequencing techniques and hardly separable, their amino acid sequences were determined by electrospray mass spectrometry (ES-MS)[18] in the state of the mixtures **1**/**2** and **3**/**4**.

In the ES-MS spectrum of the chrysospermins A/B mixture the singly and doubly protonated molecular ions of two peptaibols with relative molecular masses of 1898 and 1912 were observed. The chrysospermins C/D mixture constituted two peptaibols with masses of 1912 and 1926 (Fig. 8). The observed mass difference of 14 units between all of these components suggested that the chrysospermins were a mixture of closely related peptaibols differing in one CH_2-group.

In addition to the $[M+H]^+$- and $[M+2H]^{2+}$-ions, abundant ions were observed caused by fragmentation of the peptaibols into a N-terminal and C-terminal part.

Fig. 7. CD spectra of the chrysospermins A (**1**), B (**2**), C (**3**), and D (**4**).

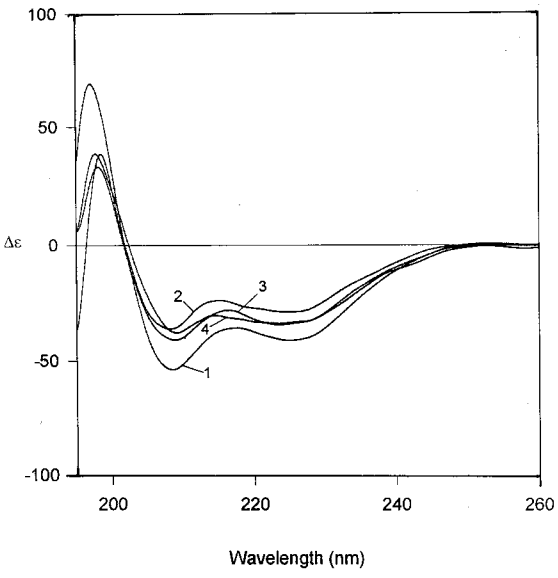
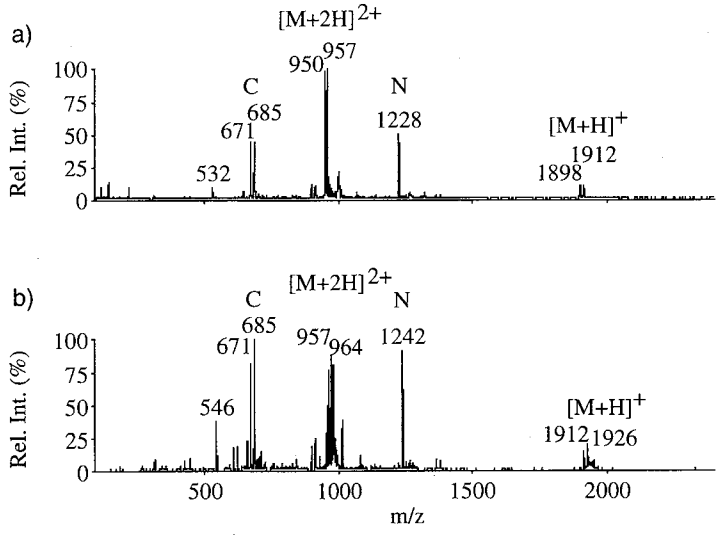
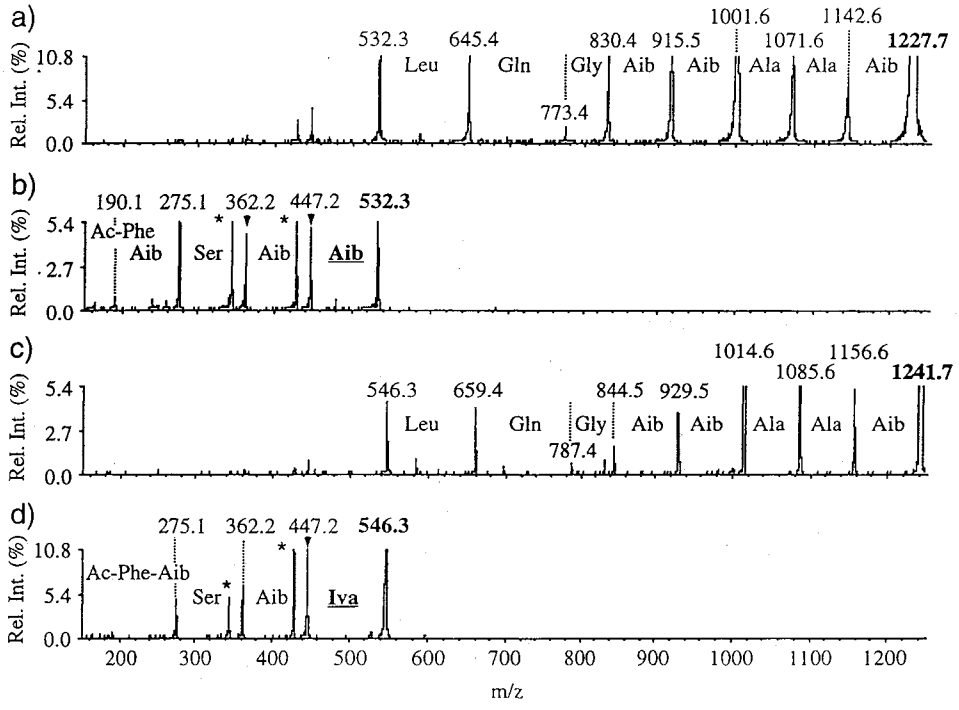


Fig. 8. ES mass spectra of a mixture of a) **1**/**2** and b) **3**/**4**.



The spectra show the singly and doubly protonated molecular ions and N- and C-terminal fragment ions formed by cleavage of the labile Aib-Pro in the transport region of the ES ion source.

Fig. 9. ES tandem mass spectrometry of the mixtures of **1**/**2** and **3**/**4**, respectively.



Grand daughter ions of the N-terminal fragment ions at a) *m/z* 1228 and b) *m/z* 532 (corresponding to **1** and **2**) and c) *m/z* 1242 and d) *m/z* 546 (corresponding to **3** and **4**).

Fragmentation, which occurs in the transport region of the ES ion source, can be enhanced by raising the voltage at the sampling orifice of the ion source[19]. Hydrophobic Aib-containing peptides and peptaibols (*e.g.* alamethicin) have a high tendency to fragment and generally form fragment ions of the B- and Y″-series according to the nomenclature of ROEPSTORFF *et al.*[24]. Thereby, MS/MS like spectra are obtained, from which valuable sequence information can be derived. The ES spectra of **1**/**2** and **3**/**4** contained abundant fragment ions at *m/z* 671 and 685, which later turned out to correspond to the C-terminus of the two peptaibols present in each fraction. The fragment ions at *m/z* 1228 in the spectrum of **1**/**2** and at *m/z* 1242 in the spectrum of **3**/**4** correspond to the N-terminal part of the peptaibols.

Table 3. Exact mass measurements of diagnostic ions of chrysospermins by HRFAB-MS.

Mass		Elemental composition
Found:	Calcd.:	
275.1416	275.1396	$C_{15}H_{19}N_2O_3$
362.1698	362.1716	$C_{18}H_{24}N_3O_5$
447.2242	447.2244	$C_{22}H_{31}N_4O_6$
532.2800	532.2771	$C_{26}H_{38}N_5O_7$
645.3612	645.3860	$C_{32}H_{49}N_3O_8$
671.3898	671.3801	$C_{33}H_{51}N_8O_7$
685.3924	685.3954	$C_{34}H_{53}N_8O_7$
830.4456	830.4412	$C_{39}H_{60}N_9O_{11}$
915.4928	915.4940	$C_{43}H_{67}N_{10}O_{12}$
1000.5439	1000.5468	$C_{47}H_{74}N_{11}O_{13}$
1071.5796	1071.5838	$C_{50}H_{79}N_{12}O_{14}$
1142.5967	1142.6210	$C_{53}H_{84}N_{13}O_{15}$
1227.6914	1227.6738	$C_{57}H_{91}N_{14}O_{16}$

Collision induced dissociation (CID) spectra of the doubly protonated molecular ions (daughter ion spectra) and of N-terminal and C-terminal fragment ions already formed in the transport region of the ion source (grand daughter spectra) allowed the complete sequence determination of **1**, **2**, **3**, and **4**. Fig. 9 shows the CID spectra of the fragment ions at *m/z* 1228 (N-terminus of **1** and **2**) and at *m/z* 1242 (N-terminus of **3** and **4**). Mass differences of 99, 113 and 128 are attributable to isovaline, leucine and glutamine residues, respectively, because the amino acid analysis excluded corresponding isobaric residues of the same mass, such as valine, isoleucine and lysine, to be present. The grand daughter spectra of the smaller fragment ions were particularly helpful for the determination of the N-terminal sequences. CID of the fragment ions at *m/z* 532 (**1** and **2**) and at *m/z* 546 (**3** and **4**) revealed N-acetylphenylalanine (Ac-Phe) to be the N-terminal residue of **1**～**4**. These spectra also clearly indicated the Aib/Iva replacement in position 5 (Fig. 9). In addition, the presence of serine in these N-terminal fragments can be deduced from characteristic 'double

Table 4-1. ^{1}H NMR assignments of chrysospermins A (**1**), B (**2**), C (**3**), and D (**4**) in DMSO-d_6 (600 MHz, in ppm relative to internal TMS).

Residue	Proton	**1** δ_H (m, J (Hz))	**2** δ_H (m, J (Hz))	**3** δ_H (m, J (Hz))	**4** δ_H (m, J (Hz))
Ac	2	1.83 (s)	1.83 (s)	1.86 (s)	1.87 (s)
Phe[1]	NH	8.43 (br d, 6.1)	8.44 (br d, 6.2)	8.36 (br d, 6.1)	8.36 (br d, 5.9)
	α	4.37 (dd, 9.4, 5.6)	4.38 (dd, 9.3, 5.7)	4.34 (dd, 9.1, 5.7)	4.34 (dd, 9.0, 5.6)
	β_1	3.02 (dd, 13.2,5.6)	3.03 (dd, 13.8, 5.7)	3.02 (dd, 13.6, 5.7)	3.03 (dd, 13.6, 5.6)
	β_2	2.84 (dd, 13.2, 0.4)	2.84 (dd, 13.8, 9.3)	2.87 (dd, 13.6, 9.1)	2.87 (dd, 13.6, 9.0)
	2′, 6′	7.26 (m)	7.26 (m)	7.26 (m)	7.26 (m)
	3′, 5′	7.27 (m)	7.26 (m)	7.26 (m)	7.26 (m)
	4′	7.20 (obsc)	7.20 (obsc)	7.19 (obsc)	7.19 (obsc)
Aib[2]	NH	8.69 (br s)	8.70 (br s)	8.53 (br s)	8.51 (br s)
	β_1	1.36 (s)	1.36 (s)	1.37 (s)	1.36 (s)
	β_2	1.31 (s)	1.31 (s)	1.29 (s)	1.28 (s)
Ser[3]	NH	7.71 (br d, 6.6)	7.82 (br d, 6.8)	7.74 (br d, 6.7)	7.56 (br d, 6.8)
	α	3.95 (m)	3.97 (m)	3.97 (m)	3.96 (m)
	β_1	3.64 (m)	3.65 (m)	3.64 (m)	3.64 (m)
	β_2	3.72 (m)	3.73 (m)	3.73 (m)	3.72 (m)
Aib[4]	NH	8.11 (br s)	8.12 (br s)	8.11 (br s)	8.12 (br s)
	β_1	1.36 (s)	1.36 (s)	1.39 (s)	1.39 (s)
	β_2	1.32 (s)	1.32 (s)	1.32 (s)	1.32 (s)
Aib[5]/Iva[5]	NH	7.64 (br s)	7.72 (br s)	7.66 (br s)	7.66 (br s)
	β_1	1.36 (s)	1.37 (s)	1.23 (s)	1.22 (s)
	β_2	1.32 (s)	1.32 (s)	2.14 (m)	2.14 (m)
	$\beta_{2'}$	—	—	1.62 (dd, 14.0, 7.5)	1.62 (dd, 14.0, 7.4)
	γ	—	—	0.64 (t, 7.5)	0.63 (t, 7.4)
Leu[6]	NH	7.61 (d, 6.2)	7.60 (d, 6.3)	7.60 (d, 6.2)	7.61 (d, 6.2)
	α	3.95 (m)	3.95 (m)	3.94 (m)	3.95 (m)
	β_1	1.51 (m)	1.51 (m)	1.51 (m)	1.52 (m)
	β_2	1.81 (m)	1.81 (m)	1.81 (m)	1.82 (m)
	γ	1.77 (m)	1.78 (m)	1.77 (m)	1.78 (m)
	δ_1	0.91 (d, 6.4)	0.91 (d, 6.4)	0.91 (d, 6.4)	0.91 (d, 6.4)
	δ_2	0.82 (d, 6.4)	0.82 (d, 6.5)	0.83 (d, 6.4)	0.82 (d, 6.4)
Gln[7]	NH	7.72 (br d, 6.8)	7.72 (br d , 7.0)	7.73 (br d, 6.8)	7.72 (br d, 6.8)
	α	4.02 (m)	4.02 (m)	4.01 (m)	3.99 (m)
	β_1	2.02 (m)	2.03 (m)	2.02 (m)	2.02 (m)
	β_2	1.88 (m)	1.87 (m)	1.87 (m)	1.86 (m)
	γ_1	2.15 (m)	2.15 (m)	2.15 (m)	2.15 (m)
	γ_2	2.03 (m)	2.03 (m)	2.02 (m)	2.03 (m)
Gly[8]	NH	7.90 (t, 5.6)	7.88 (t, 5.7)	7.88 (t, 5.7)	7.88 (t, 5.7)
	α_1	3.69 (m)	3.70 (m)	3.70 (m)	3.70 (m)
	α_2	3.60 (dd, 15.8, 5.6)	3.61 (dd, 15.8, 5.7)	3.61 (dd, 15.6, 5.7)	3.61 (dd, 15.9, 5.7)
Aib[9]	NH	8.26 (br s)	8.25 (br s)	8.22 (br s)	8.22 (br s)
	β_1	1.39 (s)	1.40 (s)	1.40 (s)	1.40 (s)
	β_2	1.33 (s)	1.34 (s)	1.34 (s)	1.34 (s)
Aib[10]	NH	7.88 (br s)	7.88 (br s)	7.88 (br s)	7.88 (br s)
	β_1	~1.39 (s)	~1.39 (s)	~1.39 (s)	~1.39 (s)
	β_2	~1.32 (s)	~1.33 (s)	~1.32 (s)	~1.33 (s)
Ala[11]	NH	7.68 (d, ~6)	7.67 (d, ~6)	7.68 (d, ~6)	7.68 (d, ~6)
	α	3.89 (m)	3.90 (m)	3.90 (m)	3.91 (m)
	β	1.36 (d, ~7)	1.36 (d, ~7)	1.36 (d, ~7)	1.36 (d, ~7)
Ala[12]	NH	7.67 (d, ~7)	7.66 (d, ~7)	7.66 (d, ~7)	7.66 (d, ~7)
	α	4.22 (t, 7.5)	4.23 (t, 7.6)	4.22 (t, 7.5)	4.22 (t, 7.6)
	β	1.33 (d, ~7)	1.33 (d, ~7)	1.34 (d, ~7)	1.34 (d, ~7)
Aib[13]	NH	7.58 (br s)	7.59 (br s)	7.58 (br s)	7.58 (br s)
	β_1	1.44 (br s)	1.44 (br s)	1.45 (br s)	1.44 (br s)
	β_2	1.38 (br s)	1.38 (br s)	1.39 (br s)	1.38 (br s)
Pro[14]	α	4.16 (t, 7.6)	4.19 (t, 7.6)	4.17 (t, 7.6)	4.18 (t, 7.5)
	β_1	2.16 (m)	2.17 (m)	2.16 (m)	2.15 (m)
	β_2	1.62 (m)	1.63 (m)	1.61 (m)	1.61 (m)
	γ_1	1.81 (m)	1.80 (m)	1.81 (m)	1.80 (m)
	γ_2	1.89 (m)	1.88 (m)	1.88 (m)	1.89 (m)
	δ_1	3.45 (m)	3.49 (m)	3.46 (m)	3.49 (m)
	δ_2	3.63 (m)	3.65 (m)	3.68 (m)	3.66 (m)
Aib[15]/Iva[15]	NH	7.88 (br s)	7.78 (br s)	7.88 (br s)	7.74 (br s)
	β_1	~1.40 (s)	1.30 (s)	~1.41 (s)	1.31 (s)
	β_2	~1.33 (s)	2.13 (m)	~1.34 (s)	2.14 (dd, 13.9, 7.5)
	$\beta_{2'}$	—	1.69 (dd, 13.8, 7.5)	—	1.68 (dd, 13.9, 7.5)
	γ	—	0.76 (t, 7.5)	—	0.75 (t, 7.5)

Table 4-2. ^{1}H NMR assignments of chrysospermins A (**1**), B (**2**), C (**3**), and D (**4**) in DMSO-d_6 (600 MHz, in ppm relative to internal TMS).

Residue	Proton	**1** δ_H (m, J (Hz))	**2** δ_H (m, J (Hz))	**3** δ_H (m, J (Hz))	**4** δ_H (m, J (Hz))
Aib[16]	NH	7.79 (s)	7.79 (s)	7.79 (s)	7.79 (s)
	β_1	1.38 (s)	1.38 (s)	1.38 (s)	1.38 (s)
	β_2	1.31 (s)	1.32 (s)	1.31 (s)	1.31 (s)
Aib[17]	NH	7.58 (s)	7.58 (s)	7.59 (s)	7.59 (s)
	β_1	1.39 (s)	1.39 (s)	1.39 (s)	1.39 (s)
	β_2	1.33 (s)	1.32 (s)	1.33 (s)	1.33 (s)
Gln[18]	NH	7.51 (br d, 7.6)	7.51 (br d, 7.6)	7.52 (br d, 7.6)	7.49 (br d, 7.5)
	α	3.94 (m)	3.94 (m)	3.93 (m)	3.94 (m)
	β_1	2.03 (m)	2.04 (m)	2.04 (m)	2.03 (m)
	β_2	1.81 (m)	1.81 (m)	1.82 (m)	1.80 (m)
	γ_1	2.15 (m)	2.15 (m)	2.16 (m)	2.15 (m)
	γ_2	2.04 (m)	2.04 (m)	2.04 (m)	2.03 (m)
Trpol[19]	NH	7.17 (d, 8.5)	7.18 (d, 8.4)	7.17 (d, 8.5)	7.17 (d, 8.4)
	α	3.92 (m)	3.93 (m)	3.93 (m)	3.94 (m)
	β_1	2.99 (dd, 14.5, 5.5)	2.99 (dd, 14.6, 5.5)	2.99 (dd, 14.5, 5.5)	2.99 (dd, 14.7, 5.4)
	β_2	2.68 (dd, 14.5, 7.6)	2.68 (dd, 14.6, 7.4)	2.69 (dd, 14.5, 7.7)	2.69 (dd, 14.7, 7.6)
	β_1	3.39 (m)	3.38 (m)	3.38 (m)	3.38 (m)
	β_2	3.37 (m)	3.37 (m)	3.36 (m)	3.36 (m)
	1′	10.74 (d, ~1)	10.73 (d, ~1)	10.73 (d, ~1)	10.73 (d, ~1)
	2′	7.11 (d, 2)	7.12 (d, 2)	7.11 (d, 2)	7.11 (d, 2)
	4′	7.56 (d, 7.9)	7.56 (d, 8.0)	7.56 (d, 7.9)	7.56 (d, 8.0)
	5′	6.95 (t, ~7.9)	6.94 (t, ~7.9)	6.95 (t, ~7.9)	6.93 (dd, 8.0, 7.5)
	6′	7.03 (t, ~7.9)	7.03 (t, ~7.9)	7.03 (t, ~7.6)	7.03 (t, 7.5)
	7′	7.29 (d, 7.5)	7.29 (d, 7.6)	7.29 (d, 7.6)	7.29 (d, 7.5)

peaks' with a mass difference of 18 units due to the elimination of water from these residues[19].

Comparison of the mass spectral data showed that the chrysospermins A~D are four homologous nona-decapeptides possessing either Aib or Iva in positions 5 and 15 (Table 2). These results explain the observed mass difference of 14 u between the molecular masses of **1** (Aib[5]/Aib[15]), **2**/**3** (Aib[5]/Iva[15]; Iva[5]/Aib[15]) and **4** (Iva[5]/Iva[15]) and also the fragmentation pattern in the MS/MS experiment. **1** and **2**, and also **3** and **4** have an identical N-terminus, whereas for all chrysospermins the C-terminus is identical. These structural differences are evident in the ES mass spectra of **1**/**2** and **3**/**4** and also in the corresponding tandem mass spectra. The collision induced cleavage of the labile Aib-Pro bond, which is characteristic for many peptaibols and is found also in the chrysospermins, leads to a N-terminal fragment ion containing Aib or Iva in position 5 (m/z 1228 and 1242, respectively) and a C-terminal fragment ion containing Aib or Iva in position 15 (m/z 671 and 685, respectively).

The pure chrysospermins A~D were investigated by high-resolution positive ion fast-atom-bombardment (FAB) mass spectrometry. This ionization technique also produces sequence ions and has been successfully applied to the structure elucidation of peptaibols[8,25,26].

The fragment ion peaks were assigned by HRFAB-MS as shown in Table 3. Using this technique the proposed sequences were confirmed and it was additionally proved that the two Glx residues found in the total hydrolysate were Gln residues in each of the four peptaibols.

NMR Spectroscopy

^{1}H and ^{13}C NMR assignments (DMSO-d_6) of the chrysospermins A~D (**1**~**4**) are summarized in Table 4 and 5, respectively. In order to obtain a complete ^{1}H and ^{13}C NMR data set the following strategy was applied. First, the multiplicities of the carbon atoms were assigned using DEPT spectra. The proton spin systems of the amino acids were elucidated by COSY and TOCSY experiments and correlated with the corresponding carbons by means of HMQC spectra. Although, the complete sequence of **1**~**4** were already elucidated by MS, NOESY and HMBC experiments were essential for the complete assignment of NMR data to the individual amino acids.

The spectra of **1** should be taken as examples and discussed in greater detail. According to the ^{13}C and DEPT spectra in **1** are ninety carbons, twenty three methyls, thirteen methylenes, twenty methines, thirteen quaternary carbons and twenty one carbonyles.

Nine Aib were easily detectable due to the occurrence of nine quaternary α-carbons between δ 55.71 and

Table 5-1. ^{13}C NMR assignments of chrysospermins A (**1**), B (**2**), C (**3**), and D (**4**) in DMSO-d_6 (150 MHz, δ in ppm relative to internal TMS).

Residue	Carbon	**1** δ_C	**2** δ_C	**3** δ_C	**4** δ_C
Ac	CO	170.4 s	170.5 s	170.4 s	170.5 s
	CH_3	22.4 q	22.4 q	22.3 q	22.4 q
Phe[1]	CO	172.1 s	172.0 s	172.0 s	172.0 s
	α	55.3 d	55.5 d	55.5 d	55.6 d
	β	36.7 t	36.6 t	36.5 t	36.6 t
	1′	137.6 s	137.4 s	137.3 s	137.4 s
	2′, 6′	129.2 d	129.1 d	129.0 d	129.1 d
	3′, 5′	128.0 d	128.0 d	127.9 d	128.0 d
	4′	126.3 d	126.3 d	126.2 d	126.3 d
Ser[3]	CO	171.7 s	171.7 s	171.6 s	171.6 s
	α	57.8 d	57.8 d	57.9 d	58.0 d
	β	60.6 t	60.6 t	60.4 t	60.5 t
Iva[5]	CO	—	—	176.3 s	176.4 s
	α	—	—	58.5 s	58.6 s
	β_1	—	—	22.4 q	22.4 q
	β_2	—	—	25.6 t	25.6 t
	γ	—	—	7.0 q	7.1 q
Leu[6]	CO	173.8 s	173.8 s	173.8 s	173.9 s
	α	53.6 d	53.6 d	53.5 d	53.7 d
	β	39.0 t	39.0 t	39.0 t	39.0 t
	γ	24.4 d	24.4 d	24.3 d	24.4 d
	δ_1	23.0 q	23.0 q	22.9 q	22.9 q
	δ_2	20.9 q	20.9 q	20.9 q	20.9 q
Gln[7]	CO	173.3 s	173.3 s	173.3 s	173.3 s
	α	54.1 d	54.0 d	54.1 d	54.1 d
	β	26.4 t	26.4 t	26.4 t	26.4 t
	γ	31.4 t	31.4 t	31.3 t	31.4 t
	δ	173.5 s	173.4 s	173.4 s	173.4 s
Gly[8]	CO	170.1 s	170.0 s	170.0 s	170.0 s
	α	43.6 t	43.6 t	43.6 t	43.6 t
Ala[11]	CO	172.8 s	172.8 s	172.7 s	172.8 s
	α	50.8 d	50.8 d	50.7 d	50.8 d
	β	16.5 q	16.4 q	16.3 q	16.4 q
Ala[12]	CO	172.7 s	172.6 s	172.6 s	172.6 s
	α	48.5 d	48.5 d	48.4 d	48.4 d
	β	16.9 q	16.8 q	16.7 q	16.8 q
Pro[14]	CO	173.2 s	173.2 s	173.2 s	173.1 s
	α	63.1 d	63.0 d	63.0 d	63.1 d
	β	28.1 t	28.1 t	28.0 t	28.1 t
	η	25.7 t	25.7 t	25.6 t	25.7 t
	δ	48.4 t	48.3 t	48.3 t	48.3 t
Iva[15]	CO	—	175.5 s	—	175.6 s
	α	—	58.7 s	—	58.7 s
	β_1	—	23.0 q	—	23.1 q
	β_2	—	26.1 t	—	26.1 t
	γ	—	7.5 q	—	7.5 q
Gln[18]	CO	171.2 s	171.1 s	171.1 s	171.1 s
	α	53.8 d	53.7 d	53.7 d	53.7 d
	β	26.8 t	26.8 t	26.7 t	26.8 t
	γ	31.8 t	31.9 t	31.7 t	31.9 t
	δ	173.3 s	173.3 s	173.2 s	173.3 s
Trpol[19]	α	51.9 d	51.8 d	51.7 d	51.8 d
	β_1	26.4 t	26.4 t	26.4 t	26.4 t
	β_2	62.9 t	62.8 t	62.8 t	62.8 t
	2′	123.0 d	123.0 d	122.9 d	123.0 d
	3′	111.5 s	111.4 s	111.4 s	111.4 s
	4′	118.4 d	118.3 d	118.2 d	118.3 d
	5′	118.1 d	118.0 d	117.9 d	118.0 d
	6′	120.7 d	120.6 d	120.6 d	120.6 d
	7′	111.2 d	111.1 d	111.0 d	111.1 d
	8′	127.5 s	127.5 s	127.4 s	127.5 s
	9′	136.1 s	136.0 s	135.9 s	136.0 s

Table 5-2. ^{13}C NMR assignments of chrysospermins A (**1**), B (**2**), C (**3**), and D (**4**) in DMSO-d_6 (150 MHz, δ in ppm relative to internal TMS).

Residue	Carbon	**1** δ_C	**2** δ_C	**3** δ_C	**4** δ_C
Aib 2, 4, (5), 9, 10, 13, (15), 16, 17	CO	172.5 s (13) 174.6 s (17) 175.2 s (2) 175.2 s (9) 175.3 s (4) 175.3 s (16) 175.6 s (15) 175.9 s (10) 176.1 s (5)	172.4 s (13) 174.5 s (17) 175.1 s (2) 175.2 s (9) 175.2 s (4) 175.3 s (16) 175.9 s (10) 176.0 s (5)	172.4 s (13) 174.4 s (17) 175.1 s (2) 175.1 s (9) 175.2 s (4) 175.2 s (16) 175.4 s (15) 175.8 s (10)	172.4 s (13) 174.5 s (17) 175.1 s (2) 175.1 s (9) 175.2 s (4) 175.2 s (16) 175.8 s (10)
	α	55.7 (×2), 55.8 (×2), 55.9 (×2), 56.1 (×2), 56.2	55.7 (×2), 55.8 (×3), 55.9, 56.1, 56.2	55.6 (×2), 55.7 (×2), 55.8, 55.9, 56.0, 56.1	55.7 (×2), 55.8 (×2), 56.0, 56.1 (×2)
	β_1	22.7, 23.0, 23.1, 23.2, 23.4 (×2), 23.5, 23.7, 24.3	22.7, 23.0, 23.2, 23.4 (x2), 23.5, 23.7, 24.2	22.6, 23.1, 23.2, 23.4 (×2), 23.6, 23.9, 24.3	22.7, 23.2, 23.3 (×2), 23.6, 23.7, 24.0
	β_2	25.3 (×2), 25.7 (×2), 25.8, 26.2 (×2), 26.5, 26.8	25.2, 25.3, 25.7 (×2), 26.1 (x2), 26.5, 26.8	25.2, 25.4, 25.6 (×2), 26.0, 26.1, 26.3, 26.7	25.2, 25.5, 25.7 (×2), 25.9, 26.0, 26.8

Table 6. Antibiotic activity of chrysospermins A (**1**), B (**2**), C (**3**), and D (**4**) in the agar diffusion assay.

Test organisms	MIC (μg/diffusion zone) chrysospermins			
	1	**2**	**3**	**4**
1. *Bacillus subtilis* ATCC 6633	5	10	5	25
2. *Staphylococcus aureus* SG511	5	5	5	10
3. *Mycobacterium smegmatis* SG 987	>100	5	10	10
4. *Mycobacterium phlei* SG346	>100	10	25	25
5. *Klebsiella pneumoniae* SG117	25	50	25	50
6. *Escherichia coli* SG 458	>100	>100	>100	>100
7. *Rhodotorula rubra* IMET 25030	>100	>100	>100	>100
8. *Sporobolomyces salmonicolor* SBUG 549	25	25	10	25
9. *Fusarium culmorum* JP 15	>100	>100	>100	>100
10. *Penicillium notatum* JP36	>100	>100	>100	>100
11. *Phoma destructiva*	25	25	12.5	12.5

1~6. Standard I nutrient agar (SERVA).
7~8. Sabouraud-2%-glucose-agar (DIFCO).
9~11. Malt-agar.

δ 56.19. The β_1 and β_2 methyls of these amino acids strongly overlapped in ^1H (δ 1.31~1.44) and ^{13}C spectra (δ 22.7~24.3 and 25.3~26.8), which aggravated the assignment especially in the case of two or three neighbouring Aib units.

The spin systems of the other amino acids and of Trpol were unambiguously settled by COSY and TOCSY experiments. Conformation of the peptide backbone as derived from the MS spectra was obtained from the NOESY spectrum. Especially the observed nuclear Oberhauser effects between the amide protons and the α-CH protons of the adjacent amino acid were of particular assistance.

HBMC data were also used for backbone assignment as well as for the analysis of the quaternary carbons. Together with the HMQC spectra most of the carbons present in **1** were assigned, with the exeption of the strongly overlapping α, β_1 and β_2 signals of Aib (see Table 4).

Biological Activity

The chrysospermins showed selective activity against some yeasts, filamentous fungi and bacteria (Table 6).

Discussion

The above physico-chemical data settle the structure of chrysospermins A, B, C, and D (**1~4**) as new homologous representatives of the peptaibol family.

Generally, these peptides contain a large portion of Aib and in some cases also D-Iva. The C-terminus of peptaibols is usually formed by an amino alcohol such as phenylalaninol (Pheol), valinol (Valol), leucinol (Leuol) or tryptophanol (Trpol), whereas the N-terminus is acetylated. Similar to other non-ribosomally synthesized antibiotics the peptaibols are characterized by a microheterogeneity due to the replacement or insertion of amino acid residues (*e.g.* Ala/Aib, Aib/Iva or Glu/Gln) at distinct positions of the molecule.

The chrysospermins display structural similarity to the trichorzianins isolated from *Trichoderma harzianum*, which also are 19-mers possessing a C-terminal Trpol and one labile Aib-Pro bond[11].

ES-MS in combination with tandem MS was shown to be an invaluable analytical tool of structural elucidation of peptaibols. Particular advantages are that only minimum amounts of sample were required, no partial hydrolysis was necessary for complete sequence determination and even mixtures could be analyzed.

Chrysospermins may be interesting as channel-forming ionophores, since it was shown that in planar lipid bilayers peptaibols containing C-terminal Trpol are very efficient regarding the voltage threshold and the length of the open channel life-times[27].

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