Notes

Chrysosporide, a Cyclic Pentapeptide from a New Zealand Sample of the Fungus *Sepedonium chrysospermum*

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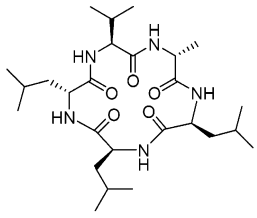
A new cyclic pentapeptide, chrysosporide (**1**), was isolated from a New Zealand sample of the mycoparasitic fungus *Sepedonium chrysospermum* by bioactivity-guided fractionation. The planar structure was deduced by detailed spectroscopic analysis, and the absolute configurations of the amino acid residues were defined by Marfey's method. As both enantiomers of Leu occurred in chrysosporide, molecular mechanics calculations were applied to the analysis to distinguish between the possible structural isomers. Only the lowest energy conformers of the cyclo-(L-Val-D-Ala-L-Leu-L-Leu-D-Leu) isomer were in agreement with the observed NOEs, suggesting that this was the most probable amino acid sequence for chrysosporide (**1**).

Mycoparasitic fungi are a diverse and prolific source of compounds with potential therapeutic value.[1] In our continuing search for new, bioactive metabolites from New Zealand fungi a strain of *Sepedonium chrysospermum* (CANU E609) (Hypocreaceae) was investigated, as the culture extracts were cytotoxic against the P388 murine leukemia cell line.

The genus *Sepedonium* Link comprises seven anamorphic species living as parasites on basidiomycetes.[2] The development of the modern infrageneric classification of this genus led to the splitting of the variable strains of the species *S. chrysospermum* (Bulliard) Fries into new taxa.[2] Accordingly, *S. chrysospermum sensu sticto* comprises yellow-pigmented strains distinguished from closely related species by microscopic features of their spores (aleurioconidia).[2]

Metabolites previously isolated from *S. chrysospermum* include the polyketide sepedonin and its derivatives[3] and the antifungal compound chrysodin,[4] while quinone derivatives and peptaibols have been isolated from *S. microspermum* Besl. and *S. ampullosporum* Damon, two species initially included in *S. chrysospermum sensu lato*.[5]

S. chrysospermum (CANU E609) was isolated from a degraded basidiomycete sporophore collected in a podocarp forest in the Bay of Plenty, New Zealand. When this strain was grown on liquid and solid media, different HPLC profiles were obtained for the extracts. In further studies fermentations were scaled up on both media, and bioactivity-guided fractionation of the liquid medium extract (IC_{50} 1.7 μg mL^{-1}) led to the isolation of a new cyclic pentapeptide, named chrysosporide (**1**), along with the known compound 3,6-dimethyl-β-resorcylaldehyde (**2**). In contrast, the metabolites obtained from the solid medium extract (IC_{50} 9.8 μg mL^{-1}) were 2,4-dihydroxy-3-methyl-6-(2-oxopropyl)benzaldehyde (**3**) and a mixture of phomalactone (**4**) and musacin D (**5**). This is the first reported isolation from a natural source of **3**, previously known only as an intermediate in the synthesis of the azaphilones.[6] Compounds **2**[7,8] and **4**[9,10] have previously been reported from several filamentous fungi. Compound **5** was probably an artifact, as it has been shown that under acidic conditions **4** is converted into **5**.[10]



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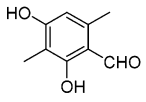

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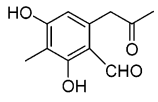

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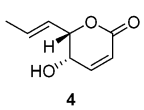

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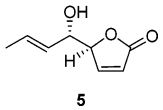

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The key new compound from this study on *S. chrysospermum*, chrysosporide (**1**), was isolated as an optically active solid. Using HRESIMS in combination with the ^{13}C NMR data, the molecular formula $C_{26}H_{47}N_5O_5$ was established for **1**. The molecular formula and NMR data suggested that chrysosporide (**1**) was a peptide. In the ^{1}H NMR spectrum five amide (NH), five alpha (Hα), and high field signals due to methine, methylene, and methyl protons were observed, leading to the conclusion that **1** was a pentapeptide, containing only aliphatic amino acids. Even though several overlapping signals were present (δ 1.60−1.43; 0.97−0.89), the identities of the amino acids were unambiguously revealed by detailed analysis of the 1D and 2D NMR data (Table 1). The NH proton at δ 7.76 correlated in the COSY spectrum to an Hα proton at δ 4.23, which in turn was coupled to an isopropyl group. Thus, a Val residue was identified. The NH proton at δ 8.56 showed a cross-peak in the COSY spectrum with an Hα proton at δ 4.29, followed by a coupling to a methyl group at δ 1.23, defining the spin system for an Ala unit. The analysis of the COSY, TOCSY, and HSQC data of the remaining three amino acid residues disclosed similar substructures, pointing out the probable presence of isomeric Leu and/or Ile residues. The long-range H,C-correlations observed in the CIGAR-HMBC experiment (Table 1) between methyl protons

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Table 1. NMR Data (500 Mhz, Dmso-d_6 at 23 °C) for Chrysosporide (**1**)

	δ_C, mult[a]		δ_H (J in Hz)	CIGAR	ROESY
Val					
NH			7.76 d (9.4)	Leu3-CO[c]	Leu3-Hα, Hα, Hβ, Hγ,γ'
α	57.1	CH	4.23 dd (9.4, 7.2)	CO,[c] Cβ, Cγ'	Ala-NH, NH, Hβ, Hγ,γ'
β	30.3	CH	1.95 m	Cγ, Cγ'	NH, Hα, Hγ,γ'
γ	19.7	CH_3	0.94[b]	Cα, Cβ, Cγ'	NH, Hα, Hβ
γ'	18.3	CH_3	0.91[b]	Cα, Cβ, Cγ	NH, Hα, Hβ
CO	171.5	qC			
Ala					
NH			8.56 d (6.1)	Val-CO[c]	Val-Hα, Hα, Hβ
α	48.3	CH	4.29 m	CO,[c] Cβ	Leu1-NH, NH, Hβ
β	16.4	CH_3	1.23 d (6.9)	CO,[c] Cα	NH, Hα
CO	172.7	qC			
Leu1					
NH			8.69 d (7.3)	Ala-CO[c]	Leu2-NH, Ala-Hα, Hα, Hβ
α	52.8	CH	4.06 m	CO[c]	NH, Hβ, Hδ
β	39.9	CH_2	1.62 m, 1.52 m	Cα, Cγ, Cδ	NH, Hα, Hδ,δ'
γ	24.4	CH	1.70 m		
δ	19.7	CH_3	0.97[b]	Cβ, Cγ, Cδ'	Hα, Hβ
δ'	21.2	CH_3	0.88[b]	Cβ, Cγ, Cδ	Hα, Hβ
CO	171.6	qC			
Leu2					
NH			7.19 d (6.9)	Leu1-CO,[c] CO[c]	Leu1-NH, Leu3-NH, Hα, Hβ
α	51.6	CH	4.36 m		Leu3-NH, NH, Hβ, Hδ
β	40.6	CH_2	1.65 m, 1.46 m	CO, Cδ	NH, Hα, Hδ,δ'
γ	24.8	CH	1.41 m		
δ	21.5	CH_3	0.96[b]	Cβ, Cγ, Cδ'	Hα, Hβ
δ'	22.0	CH_3	0.93[b]	Cβ, Cγ, Cδ	Hα, Hβ
CO	171.1	qC			
Leu3					
NH			8.88 d (7.7)	Leu2-CO[c]	Leu2-NH, Leu2-Hα, Hα, Hβ
α	50.2	CH	4.42 m		Val-NH, NH, Hβ, Hδ
β	36.7	CH_2	1.62 m, 1.52 m	CO, Cγ, Cδ, Cδ'	NH, Hα, Hδ,δ'
γ	24.0	CH	1.63 m		
δ	22.9	CH_3	0.96[b]	Cβ, Cγ, Cδ'	Hα, Hβ
δ'	21.1	CH_3	0.87[b]	Cβ, Cγ, Cδ	Hα, Hβ
CO	171.8	qC			

[a] Chemical shifts are from HSQC, CIGAR-HMBC, and IMPRESS experiments. [b] Overlapping signals. Chemical shifts are from TOCSY experiment. [c] Correlation confirmed by IMPRESS experiment.

with Cβ, Cγ, and Cδ or Cδ' were characteristic for an isobutyl group (Leu) only, not for a *sec*-butyl moiety (Ile), allowing the remaining amino acid residues to be assigned as Leu.

Taking into account the amino acid composition, the molecular formula required chrysosporide (**1**) to be cyclic. The amino acid sequence of **1** was deduced from a detailed interpretation of ROESY and IMPRESS data.[11] The well-dispersed NH and Hα signals in the ^{1}H NMR spectrum of **1**, in contrast to the very similar chemical shifts of the carbonyl carbons, made use of the ROESY experiment more reliable. The key correlations in the ROESY spectrum were Hα(Val)/NH(Ala), Hα(Ala)/NH(Leu1), NH(Leu1)/NH(Leu2), Hα-(Leu2)/NH(Leu3), and Hα(Leu3)/NH(Val), establishing that **1** is a cyclic peptide with sequence cyclo-(Val-Ala-Leu1-Leu2-Leu3). The IMPRESS technique,[11] with its higher resolution in the F1 dimension, assisted in the assignment of the carbonyl signals of the amino acid residues and allowed confirmation of the gross structure of chrysosporide (**1**) (Table 1).

The absolute configurations of the amino acid residues were determined by acid hydrolysis of **1** followed by derivatization with Marfey's reagent (N$^\alpha$-(2,4-dinitro-5-fluorophenyl)-l-alaninamide)[12] and subsequent HPLC analysis. By comparing the chromatograms with those of derivatives of commercially available amino acids, it was found that chrysosporide (**1**) contained L-Val, D-Ala, L-Leu, and D-Leu in the ratio 1.1:1:2.2:1.2, showing the presence of two L-Leu and one D-Leu in the structure.

Since attempts to crystallize chrysosporide (**1**) were unsuccessful, the actual amino acid sequence of **1** was established from computer modeling and the ROESY NMR studies. A conformational search of cyclopentapeptides, containing L-Val, D-Ala, and all three possible combinations of two L-Leu and one D-Leu, was carried out with MacroModel in combination with the OPLS2003 force field using a GB/SA water solvent model. An ensemble of conformers was collected that lay within 12 kJ of the global minima.

The NOE is a function approximating to r^{-6} (r = H/H distance), which means for example that a H/H distance of 2 Å will give a much stronger NOE signal than a H/H distance of 3 Å. Boltzmann weighted r^{-6} total values were calculated for each of the inter-residue H/H distance pairs within each ensemble of conformations within a 12 kJ/mol window. These values were used to compare the ensembles of conformers of the three structures against the experimentally measured NOEs.

In the ROESY spectrum of **1** five strong inter-residue NOEs were observed. Table 2 lists the global minima and the Boltzmann-weighted average of H/H distances for each ensemble, along with the Boltzmann weighted r^{-6} values for each ensemble. Only cyclo-(L-Val-D-Ala-L-Leu-L-Leu-D-Leu) showed appropriate calculated distance data (relatively large values for the Boltzmann weighted r^{-6} total values) for the five NH/Hα and NH/NH combinations for which strong NOEs were observed. The other two structures have two or three calculated H/H values, which would not result in strong NOE signals (relatively small values for the Boltzmann weighted r^{-6} totals in Table 2). Therefore, the sequence of the isolated product was proposed to be cyclo-(L-Val-D-Ala-L-Leu- L-Leu-D-Leu).

From the temperature gradients of the NH signals ($\Delta\delta/\Delta T$: Val: 2.4×10^{-3} ppm/K, Ala: 4.6×10^{-3} ppm/K, Leu1: 5.6×10^{-3} ppm/K, Leu2: 0.4×10^{-3} ppm/K, Leu3: 6.8×10^{-3} ppm/K) along with significant differences between the $J_{NH,H\alpha}$ couplings of the amino acid residues (Table 1), it was concluded that **1** fulfilled the requirements for conformational homogeneity.[13] The values of the NH temperature gradients indicated that NH-Val and NH-Leu2 were involved in intramolecular hydrogen bonding.[14] Examination of the modeled low-energy conformers of cyclo-(L-Val- D-Ala-L-Leu-L-

Table 2. Selected Inter-residue H/H Distances[a] in Diastereoisomeric Structures of Chrysosporide (**1**) Generated by Molecular Modeling

		Hα(Ala)/ NH(Leu1)	NH(Leu1)/ NH(Leu2)	NH(Leu2)/ NH(Leu3)	NH(Val)/ Hα(Leu3)	Hα(Leu2)/ NHLeu3
cyclo-(L-Val-D-Ala-L-Leu-L-Leu-D-Leu)	GMD[b]	2.21	2.58	2.54	2.68	3.23
	BWA[c]	2.22	2.71	3.04	2.6	2.86
	BWr^{-6}[d]	2.5	0.96	0.69	1.31	1.05
cyclo-(L-Val-D-Ala-L-Leu-D-Leu-L-Leu)	GMD	2.52	4.32	4.51	3.36	2.17
	BWA	2.5	4.13	4.46	3.42	2.2
	BWr^{-6}	0.53	0.03	0.02	0.08	1.05
cyclo-(L-Val-D-Ala-D-Leu-L-Leu-L-Leu)	GMD	3.48	4.03	4.47	3.56	2.13
	BWA	3.26	3.42	3.95	3.53	2.65
	BWr^{-6}	0.42	0.35	0.11	0.1	1.13

[a] Inter-residue NOEs between these protons were experimentally observed. [b] GMD - Global minima distance (Å). [c] BWA − Boltzmann-weighted average distance in ensemble (Å). [d] BWr^{-6} − Boltzmann-weighted r^{-6} total (r = H/H distance)

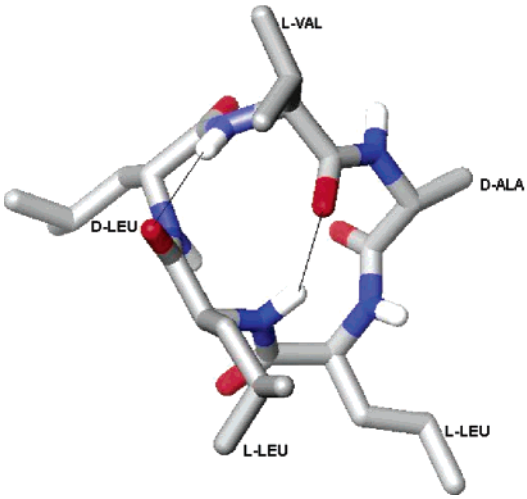

Figure 1. Low-energy conformer of cyclo-(L-Val-D-Ala-L-Leu-L-Leu-D-Leu) showing intramolecular H-bonds.

Leu-D-Leu) showed intramolecular hydrogen bonding involving NH-Val and NH-Leu2 (Figure 1). However, this was a supportive, not a definitive observation helping to resolve between the isomers, as all of the low-energy conformers of each possible structure formed the two H-bonds with equal frequency.

Chrysosporide (**1**) exhibited weak cytotoxic activity against the murine P388 murine leukemia cell line with an IC_{50} of 33.4 μM. In the same assay compounds **2**−**4** showed IC_{50} values of 126, 19.7, and 40.9 μM, respectively.

Chrysosporide (**1**) is the first cyclic peptide isolated from a fungus of the genus *Sepedonium*. Other peptides, belonging to the peptaibol group of linear peptides, have been reported from the related *S. ampullosporum*.[15]

Experimental Section

General Experimental Procedures. Optical rotations were measured with a Perkin-Elmer 341 polarimeter. NMR spectra were recorded on a Varian INOVA AS-500 spectrometer (500 and 125 MHz for ^{1}H and ^{13}C NMR, respectively), using the signals of the residual solvent protons and the solvent carbons as internal references (δ_H 2.60 and δ_C 39.6 ppm for DMSO-d_6). The spectra were run at 23 °C except for the variable-temperature experiments, where the temperature coefficients of the amide proton chemical shifts ($\Delta\delta/\Delta T$) were measured at five different temperatures in 5° steps. HRESIMS were acquired using a Micromass LCT TOF mass spectrometer. Solvents used for extraction and isolation were distilled prior to use. Cytotoxicity against P388 cells was measured using a standard protocol.[16]

Fungus. The *Sepedonium chrysospermum* fungus was isolated from a degraded basidiomycete sporophore collected in a podocarp forest in the Kamai Ranges near Katikati, Bay of Plenty, and identified on the basis of the characteristic yellow mold-like mycelium and globose, tuberculate, bright yellow aleurioconidia. A culture has been deposited in the culture collection of the School of Biological Sciences, University of Canterbury (CANU E609). For chemical investigation the fungus was grown for 28 days at 26 °C both in static liquid culture (6 × 500 mL of half-strength Sabouraud dextrose yeast broth (SDY))[17] and on solid SDY medium (24 Petri dishes).

Extraction. A. Liquid Culture. The mycelium was separated from the culture medium, macerated, and extracted with EtOAc (300 mL). The culture broth (3 L) was extracted with EtOAc (3 × 1.5 L). The combined extracts were dried to yield the crude extract (993 mg), which was partitioned between petroleum ether and MeOH/H_2O (19:1, v/v). The methanol phase was dried and again partitioned between EtOAc and H_2O. The resulting EtOAc phase was concentrated (448 mg, IC_{50} 1.7 μg mL^{-1}).

B. Agar Culture. The mycelium and agar were macerated and extracted with EtOAc (350 mL) to yield the crude extract (203 mg), which was defatted and desalted by solvent/solvent partitioning as described above, to yield a concentrated extract (49 mg, IC_{50} 9.8 μg mL^{-1}).

Isolation of Compounds 1−5. The EtOAc extract of A (448 mg) was fractionated on a flash RP-18 column (LiChroprep RP-18, 40−63 μm, Merck), using a sharp, stepped gradient from 20% MeOH/H_2O to MeOH and CH_2Cl_2.[18] The fractions eluted with 100% MeOH, MeOH/CH_2Cl_2 (1:1, v/v), and 100% CH_2Cl_2 (IC_{50} 4.8, 10.8, 44.1 μg/mL, respectively) were combined and further chromatographed using a semipreparative HPLC column (Phenomenex Luna C18, 10 × 250 mm, 5 μm) under isocratic conditions (H_2O containing 0.05% (v/v) TFA (A), MeCN (B); isocratic: 50% B; flow: 5 mL min^{-1}; UV detection at 210 nm) to yield **1** (10 min, 2.4 mg). In all following purification procedures by semipreparative HPLC the same solvents, column, and flow rate were used and the compounds were detected at 210 nm. The fraction eluting with 80% MeOH (IC_{50} 0.32 μg mL^{-1}) was further purified by semipreparative HPLC under gradient conditions (0 min 10% B, 14 min 75% B) to yield **2** (12.9 min, 7.2 mg).

The EtOAc extract of B was purified by semipreparative HPLC under gradient conditions (0 min 10% B, 30 min 60% B). Mixtures of **3** and **4** (3.8 mg) and compound **5** (1.4 mg) were eluted at 7.5 and 18.8 min, respectively.

Chrysosporide (1): light brown solid, 0.24% of dry wt of the crude extract; $[\alpha]^{20}_D$ −7.1 (*c* 0.24, MeOH); for ^{1}H and ^{13}C NMR, see Table 1; ESIMS *m/z* (%) 532 $[M + Na]^+$ (8), 510 $[M + H]^+$ (10); ESIMS *m/z* (%) 554 $[M + HCOOH - H]^-$ (40), 508 $[M - H]^-$ (51); HRESIMS *m/z* 554.3557 $[M + HCOOH - H]^-$ (calcd for $C_{27}H_{48}N_5O_7$, 554.3554).

Compounds 2−5. ^{1}H NMR, ^{13}C NMR, and ESIMS data and results from CIGAR, COSY, and NOESY experiments were consistent with the reported data for **2**,[19] **3**,[6] **4**, and **5**.[10]

Preparation and Analysis of Marfey Derivatives. Chrysosporide (**1**) (0.5 mg) was hydrolyzed by heating in HCl (6 M; 1 mL) at 110 °C for 24 h. After cooling, the solution was evaporated to dryness and redissolved in H_2O (50 μL). To the acid hydrolyzate solution (or to 50 μL of a 50 mM solution of the respective amino acid) was added a 1% (w/v) solution (100 μL) of FDAA (Marfey's reagent, N^α-(2,4-dinitro-5-fluorophenyl)-L-alaninamide)[12] in acetone. After addition of $NaHCO_3$ solution (1 M; 20 μL) the mixture was incubated for 1 h at 40 °C. The reaction was stopped by the addition of HCl (2 M; 10 μL), the solvents were evaporated to dryness, and the residue was dissolved in MeOH/H_2O (1:1; 1 mL). An aliquot of this solution (20 μL for **1** and 10 μL for the standards) was analyzed by HPLC (Phenomenex Luna C18, 250 × 4.6 mm, 5 μm; linear gradient: 0 min 35% B, 30 min 45% B; 1 mL min^{-1}; 25 °C). Retention times (min) of the FDAA amino acid derivatives used as standards were as follows: L-Ala (6.42), D-Ala

(7.67), L-Val (10.15), D-Val (14.16), L-Leu (14.94), and D-Leu (20.58). Retention times (min) and relative peak areas (%) of the observed peaks in the HPLC trace of the FDAA derivatized hydrolysis product of **1** were as follows: D-Ala (7.70, 2.63%), L-Val (10.17, 2.34%), L-Leu (14.97, 5.05%), and D-Leu (20.60, 2.75%).

Molecular Modeling. The three possible combinations of cyclopentapeptides based on the structural analysis were constructed *in silico* using the Maestro build function of the 2005 Schrödinger molecular modeling suite. A Monte Carlo multiple minimum (MCMM) conformational search of each structure was carried out with MacroModel 9.0 using the OPLS2003 force field with the GB/SA water solvent model. The criteria for convergence of each conformational search were the generation of 3000 starting conformations and a maximum of 5000 iterations in the energy miminization routine (PRCG method) for each conformer, collecting the ensemble of conformers within 12 kJ of the global minima. A Boltzmann weighting for the conformers in each of the three ensembles was established using the following expression:

$$P_\alpha = \frac{\exp[-(E_\alpha/k_B T)]}{\sum_{\alpha^-}^{N_A} \exp[-(E_\alpha/k_B T)]}$$

The five NH/Hα and NH/NH distances, for which strong NOEs were observed, were measured for all conformers within each ensemble. The Boltzmann weighted r^{-6} total was calculated for these five H/H distances within each ensemble, which gave virtual NOE values. These were used to compare the modeled data with the experimental data in order to determine the amino acid sequence of chrysosporide (see Table 2).

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Supporting Information Available: ^{1}H NMR spectrum of **1**, HPLC analysis of the FDAA derivatized hydrolizate of **1** and amino acid standards, and full data for molecular mechanics calculations for the diastereosisomers of **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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