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Petrosifungins A and B, Novel Cyclodepsipeptides from a Sponge-Derived Strain of *Penicillium brevicompactum*[§]

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A strain of *Penicillium brevicompactum* derived from a specimen of the Mediterranean sponge *Petrosia ficiformis* was investigated for its secondary metabolites. Using fast centrifugal partitioning chromatography (FCPC) two previously unknown cyclodepsipeptides, petrosifungins A (**1**) and B (**2**), were isolated, both containing two neighboring units of the nonproteinogenic amino acid L-pipecolinic acid. The absolute configurations of all amino acids were established to be L using GITC derivatization and Marfey's method. Furthermore, the known metabolite brevianamide A (**3**) was isolated. The likewise known compounds mycophenolic acid (**4**) and asperphenamate (**5**) were identified from their spectroscopic properties directly in the extract using HPLC-UV, -MS, and -NMR coupling.

Marine-derived fungi are a rich source of structurally new natural products with a wide range of biological activities.[1,2] Fungi isolated from various organisms in the marine environment, e.g., from mangroves,[3] algae,[4] and particularly from sponges,[5,6] have been examined for their secondary metabolites. The present study is part of an interdisciplinary program aimed at the identification of novel bioactive natural products from sponge-derived microorganisms, with the application of hyphenated HPLC techniques.[7] Within the scope of this project we have investigated a strain of *Penicillium brevicompactum* Dierckx (Trichocomaceae) isolated from a specimen of the Mediterranean sponge *Petrosia ficiformis* Poiret (Petrosiidae). *Penicillium* strains from sponges other than *P. ficiformis* had previously yielded diverse natural products.[6,8,9] The sponge *P. ficiformis* itself is known to contain a number of highly toxic and anti-HIV active polyacetylenes, among them the petrosyformynes[10] and petrosynol.[11]

In this paper, we report on the isolation of two new cyclopentadepsipeptides, petrosifungins A (**1**) and B (**2**), along with the known fungal metabolite brevianamide A (**3**).[12] A highly efficient and rapid purification of these compounds was achieved using fast partitioning centrifugal chromatography (FCPC), a newly developed technique of liquid−liquid chromatography. The known natural products mycophenolic acid[13] (**4**) and asperphenamate[14,15] (**5**) were also identified in the crude extract by applying HPLC-UV, -MS, -NMR, and, for the chiral compound **5**, HPLC-CD, and by comparing their spectral data with those from databases and from the literature.[7]

Results and Discussion

HPLC-UV, -MS, and -NMR analyses of the CH_2Cl_2−MeOH extract of the fungus, grown in static liquid culture, gave the molecular masses, the UV spectra, and the ^1H NMR spectra of the main compounds. These data, acquired online without isolation of the compounds, largely facilitated a fast dereplication of known metabolites by comparison with those from databases such as Antibase[16] and in the literature.[17] Using this method, two known metabolites were easily identified in the extract without the necessity of being isolated: mycophenolic acid (**4**), a well-

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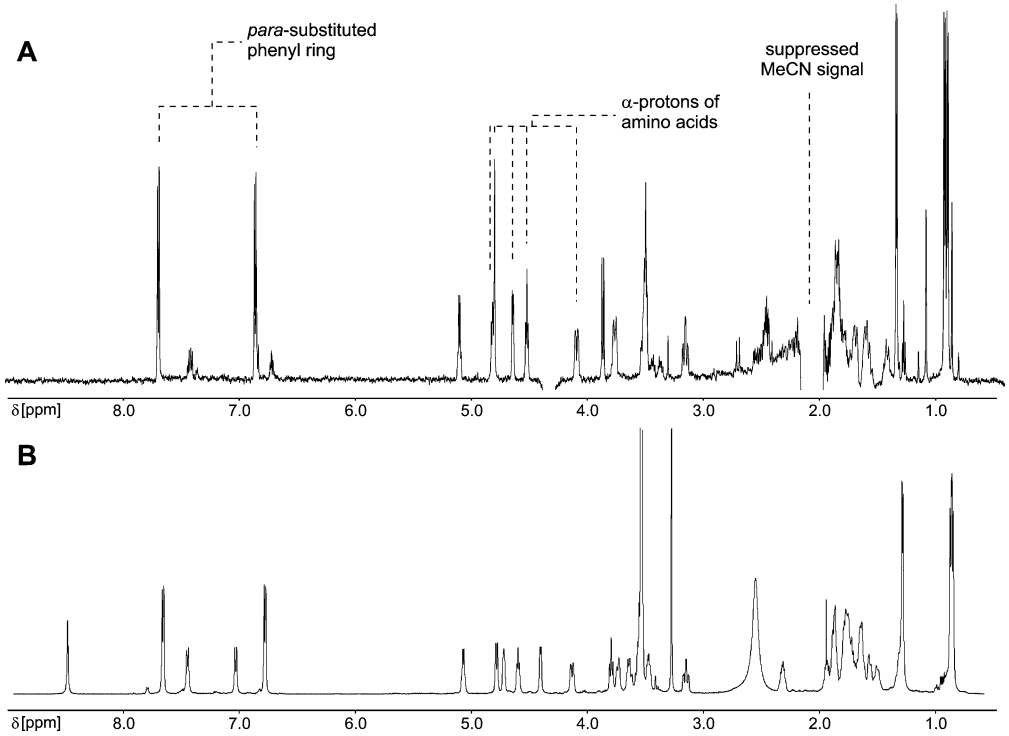
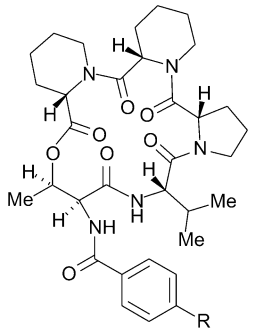


Figure 1. ^{1}H NMR spectra of petrosifungin B (**2**): (A) acquired with HPLC-NMR in the "stopped-flow" mode; (B) spectrum of the isolated compound in dioxane-d_8.

known immunosuppressive agent;[13] and asperphenamate (**5**), a fungal metabolite[14] also known to be produced by plants.[15] Of the latter compound even the absolute configuration could be established by LC-CD coupling and comparison with the published CD data.[18]



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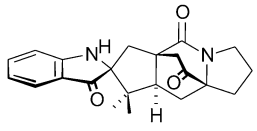

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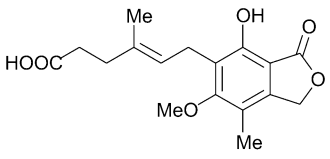

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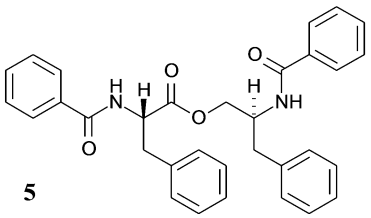

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Two compounds in the extract, not identified as known natural products in the database search, attracted our special attention. The similarity of the ^{1}H NMR spectra acquired online using HPLC-NMR suggested that these secondary metabolites were structurally related to each other. From the HPLC-NMR spectrum of the more polar compound (Figure 1) some hints at the structure were gained. Two doublets (8.9 Hz) between 7 and 8 ppm indicated a *para*-disubstituted phenyl ring, while several doublets, and doublets of doublets, near 4 ppm were attributed to α-protons of amino acids, suggesting a peptidic structure of the molecule. The HPLC-MS spectra of these compounds showed pseudomolecular ions *m/z* 640 [M + H]$^+$ for the more polar and *m/z* 624 [M + H]$^+$ for the less polar compound.

Since no known fungal peptides possessing these masses were found in the databases, we isolated the compounds in order to elucidate the full structures and to perform bioactivity tests. Purification of the compounds was achieved using FCPC.[19] By measuring the partition of the components of the extract between the upper and the lower phases of different mixtures of *n*-heptane−ethyl acetate−methanol−water using HPLC-UV, a two-phase solvent system suitable for this separation was established. For a 2:8:2:8 mixture of these four solvents, partition constants of 1.3 and 4.1 (concentration in the upper phase divided by concentration in the lower, more polar phase) were observed for the more polar and the less polar of the two peptides, respectively, while mycophenolic acid (**4**) and asperphenamate (**5**) had significantly less polar constants (28 and 101, respectively). The FCPC run was carried out using the upper phase as the stationary phase and applying about 1 g of extract at a time. The order of elution of the different compounds corresponded well with the partition constants measured earlier.

After further purification by gel filtration on Sephadex LH-20, the peptides were examined by one- and two-dimensional NMR experiments and mass spectrometry. For the less polar compound, HREIMS gave a molecular mass of *m/z* 623.3317, suggesting the molecular formula $C_{33}H_{45}N_5O_7$ (calcd 623.3319). The NMR data verified the proposed peptidic structure of the compound. By COSY, HMQC, and HMBC spectra, starting from the respective α-protons, the amino acids present in the molecule were shown to be threonine, valine, proline, and two pipecolinic acid units. In addition, the spectra indicated the presence of a benzoyl group (*Bz*). The sequence of these building blocks in the

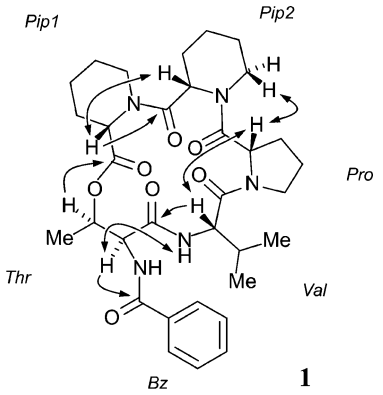

Figure 2. HMBC and ROESY interactions of petrosifungin A (**1**) indicative of the amino acid sequence.

peptide was established by HMBC correlations, *Pip1*-α → *Pip2*-CO, *Val*-α → *Thr*-CO, *Val*-NH → *Thr*-CO, and *Thr*-α → *Bz*-CO, and by ROESY correlations, *Pip2*-α ↔ *Pip1*-α, *Pro*-α ↔ *Pip2*-ϵ, *Pro*-β ↔ *Pip2*-ϵ, *Val*-α ↔ *Pro*-α, and *Thr*-α ↔ *Val*-NH (Figure 2). The ester bond between one of the pipecolinic acids (*Pip1*) and the β-oxygen of threonine, as indicated by the HMBC correlation *Thr*-β → *Pip1*-CO, showed that the compound was a cyclodepsipeptide of the constitution **1** shown.

The absolute configurations of the amino acid units were determined by acid hydrolysis followed by derivatization with a chiral reagent and subsequent HPLC analysis, comparing the chromatograms with those of derivatives of commercially available amino acids. Using 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC)[20] as the derivatizing reagent, valine, proline, and the pipecolinic acids were all shown to possess the L-configuration. Since two of the GITC derivatives of the four possible threonine stereoisomers could not be separated by HPLC under the conditions used, another chiral reagent, N^{α}-(2,4-dinitro-5-fluorophenyl)- L-alaninamide (FDAA; Marfey's reagent),[21] was used. By using this method, the L-configuration for the α-position, and the "normal" *R*-configuration at the second stereogenic center were established for threonine, while the configurations of the other amino acids were reconfirmed to be all L, resulting in the overall structure **1**. With regard to the sponge from which the fungus had been isolated, the name petrosifungin A (**1**) was given to this new compound.

The second, more polar peptide isolated displayed a molecular ion in HREIMS at *m*/*z* 639.3264, suggesting the formula $C_{33}H_{45}N_5O_8$ (calcd 639.3268). The NMR data revealed its structure to be very similar to that of petrosifungin A (**1**), the only difference being the presence of a *para*-hydroxybenzoyl (*pOH-Bz*) group. Again, the configurations of all amino acids were determined to be L, by using the same methods as for petrosifungin A. Because of its close relationship to petrosifungin A, the second peptide, also a previously unknown natural product **2**, was named petrosifungin B.

Several examples of fungal cyclodepsipeptides are known from the literature, of which the largest group, with more than 30 compounds, are the destruxins, insecticidal and phytotoxic hexadepsipeptides.[22] Other bioactive fungal cyclodepsipeptides such as the ionophore beauvericin[23,24] and the neurokinin antagonist Sch 217048[25] have also been reported. A noteworthy structural feature of the petrosifungins is the array of the three neighboring cyclic amino acids proline and twice pipecolinic acid, which in this sequence has never been observed before in a natural cyclopeptide.

By using FCPC and gel filtration, a further compound was isolated, which showed a bright yellow-green fluorescence in daylight. By comparison with spectral data from the literature,[12] it was found to be identical to brevianamide A (**3**), an insecticidal fungal metabolite.[26]

The metabolites isolated or identified by hyphenated HPLC techniques support the taxonomic identification of the fungus as *P. brevicompactum*. The presence of mycophenolic acid (**4**), asperphenamate (**5**), and especially brevianamide A (**3**) is characteristic of this fungal species.[27] Strains of *P. brevicompactum* are also known to produce the cyclodepsipeptide brevigellin,[28] which contains a benzoyl group attached to a ring-closing threonine, but is otherwise different from **1** and **2**.

The fungus investigated here was found to grow and sporulate on both saline and nonsaline medium. It can therefore be considered a facultative marine species. Fungal growth in the sponge tissue itself has not been observed.

Experimental Section

General Experimental Procedures. Melting points were determined on a Kofler hot-stage microscope and are uncorrected. Optical rotations were measured using a Perkin-Elmer 241MC polarimeter (25 °C, 10 cm cell) and CD spectra (25 °C, MeOH, 0.05 cm cell) on a Jasco J-715 spectropolarimeter. IR spectra were taken on a Jasco FT/IR-410 spectrometer, and ^{1}H NMR (400 MHz, 600 MHz) and ^{13}C NMR (100 MHz, 150 MHz) spectra were measured on Bruker AMX 400 or on DMX 600 instruments, using CD_3OD (δ 3.31 and 49.15) and dioxane-d_8 (δ 3.53 and 66.66) as the solvents and internal ^{1}H and ^{13}C standards. Proton-detected, heteronuclear correlations were measured using HMQC (optimized for $^1J_{HC}$ = 145 Hz) and HMBC (optimized for $^nJ_{HC}$ = 7 Hz or $^nJ_{HC}$ = 3.5 Hz) pulse sequences. ROE effects were measured using a standard pulse sequence from the standard Bruker pulse program library. EIMS (70 eV) and HREIMS (70 eV) were determined on Finnigan MAT 8200 and Finnigan MAT 90 instruments. HPLC-ESIMS was performed with a triple-stage quadrupole TSQ 7000 mass spectrometer (temperature of capillary: 210 °C, ESI-voltage: 3.5 kV, N_2 as sheath and auxiliary gas, positive mode). HPLC-CD measurements were done on a Jasco J-715 spectropolarimeter with a standard flow cell. UV detection was performed with a JASCO MD-1510 photodiode array detector. For HPLC-UV and HPLC-ESIMS, a Symmetry C_{18} HPLC column was used (Waters, 2.1 × 150 mm, 5 μm); solvents (A) H_2O, (B) MeCN (0.05% trifluoroacetic acid); linear gradient, 0 min 10% B, 25 min 50% B, 35 min 97% B, 45 min 97% B, flow rate 0.4 mL/min. For FCPC, an apparatus from Kromaton was used, equipped with a 200 mL rotor.[19] Marfey's reagent and GITC were purchased from Aldrich and Fluka, respectively. Organic solvents were dried and distilled prior to use.

Fungus. The fungus, morphologically identified as *Penicillium* cf. *brevicompactum* Dierckx (Trichocomaceae), was isolated from the interior of a marine sponge, *Petrosia ficiformis* Poiret (Petrosiidae), collected at a depth of 11−14 m at Capo S. Andrea, Elba/Italy, in May 2000. For chemical investigations the fungus was grown as a standing culture in 30 1 L Erlenmeyer flasks on 300 mL of WS liquid medium[29] each. After a growth period of 14 days, 30 mL of EtOAc was added to each flask and the mixture was kept at −80 °C until extraction. The strain was deposited in the "Kulturensammlung Marine Pilze Bremerhaven (KMPB)" under the accession no. E-00-2/6a.

Extraction. The fungus was separated from the culture medium and extracted exhaustively with CH_2Cl_2−MeOH (1:1). The medium was extracted three times with 1 L of EtOAc each. Both extracts were separately dried and partitioned between petroleum ether and MeOH−H_2O (9:1). The methanol phases were dried and again partitioned between EtOAc and

Table 1. NMR Data of Petrosifungin A (**1**)[a]

position	^{13}C [ppm]	^{1}H [ppm]	COSY	HMBC	ROESY
Pip1					
CO	169.74				
α	54.25	4.36	*β*	*β*, *γ*, *ϵ*, CO, *Pip2*-CO	*Pip2*-α, *Thr*-NH
β	27.50	1.74/1.86	α		
γ	19.28	∼1.5−1.7	*δ*		
δ	24.65	1.30/1.80	*ϵ*, *γ*		
ϵ	39.19	3.14/4.13	*δ*	*γ*, *δ*	
Pip2					
CO	174.26				
α	51.18	4.71	*β*	*β*, *γ*, *ϵ*, CO, *Pro*-CO	*Pip1*-α, *Val*-α
β	26.29	1.76/1.88	α, *γ*		
γ	19.47	∼1.5−1.7	*β*		
δ	24.93	1.65/1.80	*ϵ*		
ϵ	42.90	3.65/3.73	*δ*		*Pro*-α, *Pro*-*β*
Pro					
CO	171.36				
α	59.10	4.58	*β*	*β*, *γ*, *δ*, CO	*Pip2*-*ϵ*, *Val*-α, *Val*-*γ*′
β	30.37	1.92/2.31	α, *γ*	α, *γ*, *δ*, CO	*Pip2*-*ϵ*
γ	23.45	1.72/1.82	*ϵ*, *β*	α, *β*, *δ*	
δ	47.31	3.47/3.56	*γ*	*β*	
Val					
CO	171.32				
α	57.38	3.79	NH, *β*	*β*, *γ*, *γ*′, CO, *Thr*-CO	*γ*, *γ*′, *Pip2*-α, *Pro*-α
β	33.82	1.85	α, *γ*, *γ*′	α, *γ*, *γ*′	NH
γ	19.12	0.84	*β*	α, *β*, *γ*′	α
γ′	18.87	0.86	*β*	α, *β*, *γ*	α, NH, *Pro*-α
NH		7.29	α	α, CO, *Thr*-CO	*β*, *γ*′, *Thr*-α, *Thr*-*β*
Thr					
CO	168.86				
α	56.05	4.77	NH, *β*	*γ*, CO, *Bz*-CO	*γ*, *Val*-NH
β	73.46	5.07	α, *γ*	*γ*, CO, *Pip1*-CO	NH,*Val*-NH
γ	17.29	1.29	*β*	α	α, NH, *Bz*-(2, 6)
NH		7.19	α	CO, *Bz*-CO	*β*, *γ*, *Bz*-(2, 6), *Pip1*-α
Bz					
CO	167.49				
1	135.63				
2, 6	127.90	7.78	(3, 5)	(2, 6), 4, CO	*Thr*-NH, *Thr*-*γ*
3, 5	128.80	7.45	(2, 6), 4	1, (2, 6), CO	
4	131.63	7.5	(3, 5)	(2, 6)	

[a] All spectra were recorded in dioxane-d_8.

Table 2. NMR Data of Petrosifungin B (**2**)[a]

position	^{13}C [ppm]	^{1}H [ppm]	COSY	HMBC	ROESY
Pip1					
CO	170.16				
α	54.58	4.40	*β*	*β*, *γ*, *ϵ*, CO, *Pip2*-CO	*Pip2*-*a*
β	27.84	1.73/1.87	α	α, CO	
γ	19.64	1.56/1.64	*δ*		
δ	25.05	1.30/1.77	*γ*, *ϵ*		
ϵ	39.58	3.14/4.13	*δ*	α, *γ*, *δ*	
Pip2					
CO	174.71				
α	51.55	4.72	*β*	*β*, *γ*, CO	*Pip1*-α, *Val*-α
β	26.65	1.73/1.87	α		
γ	19.81	1.50/1.64			
δ	25.23	1.63/1.76	*ϵ*		
ϵ	43.35	3.63/3.72	*δ*	α, *γ*, *δ*	*Pro*-*β*, *Pro*-α
Pro					
CO	171.73				
α	59.50	4.60	*β*	*β*, *γ*, *δ*	*Pip2*-*ϵ*, *Val*-α, *Val*-*γ*′
β	30.72	1.93/2.31	α, *γ*	α, *γ*, *δ*, CO	*Pip2*-*ϵ*
γ	23.80	1.70/1.80	*β*, *δ*	α, *β*, *δ*	
δ	47.70	3.47/3.56	*γ*	α, *β*, *γ*	
Val					
CO	171.67				
α	57.85	3.79	NH, *β*	*β*, *γ*, *γ*′, CO, *Thr*-CO	*γ*, *γ*′, *Pro*-α, *pOH-Bz*-OH, *Pip2*-α
β	34.17	1.86	α, *γ*, *γ*′	α, *γ*, *γ*′	NH
γ	19.45	0.84	*β*	α, *β*, *γ*′	α, NH
γ′	19.20	0.86	*β*	α, *β*, *γ*	α, *Pro*-α
NH		7.45	α	CO, *Thr*-CO	*β*, *γ*, *Thr*-α, *Thr*-*β*
Thr					
CO	169.85				
α	56.34	4.78	NH	*γ*, CO, *pOH-Bz*-CO	*γ*, *Val*-NH
β	73.89	5.07	*γ*	*γ*, *Pip1*-CO	*Val*-NH
γ	17.62	1.27	*β*	α, *β*	α, NH, *pOH-Bz*-(2,6)
NH		7.03	α	α, *pOH-Bz*-CO	*γ*, *pOH-Bz*-(3, 5)
pOH-Bz					
CO	167.32				
1	126.98				
2, 6	130.16	7.66	(3, 5)	(3, 5), 4, CO	OH, *Thr*-*γ*
3, 5	115.75	6.78	(2, 6)	1, 4	*Thr*-NH
4	160.93				
OH		8.49		(3, 5), 4	(2, 6), *Val*-α

[a] All spectra were recorded in dioxane-d_8.

H_2O. The resulting EtOAc phases were concentrated to yield 1.18 and 0.80 g of extract from the mycelium and the culture medium, respectively.

HPLC-NMR. Mycelial extract (50 mg) was dissolved in 1 mL of MeOH-d_4−MeCN (1:1). A 100 μL portion of this solution was subjected to HPLC on an analytical reversed-phase column (Waters Symmetry C_{18}, 4.6 × 250 mm, 5 μm) with the solvents (A) D_2O (0.05% trifluoroacetic acid) and (B) MeCN as the eluents (linear gradient, 0 min 20% B, 30 min 100% B, flow rate 1 mL min^{-1}). ^{1}H NMR spectra were acquired in the stop-flow mode with a 60 μL *z*-gradient flow probe (Bruker) in a 14.1 T magnet (600 MHz, Bruker) using the WET sequence[30] for suppression of solvent signals. The MeCN signal served as the internal standard for calibration (2.0 ppm). The compounds brevianamide A (**3**), petrosifungin B (**2**), petrosifungin A (**1**), mycophenolic acid (**4**), and asperphenamate (**5**) were eluted, and their ^{1}H NMR spectra were acquired, after 11.5, 12.2, 14.5, 17.0, and 23.5 min, respectively. The ^{1}H NMR spectra measured for mycophenolic acid (**4**) and asperphenamate (**5**) as well as their molecular masses obtained from HPLC-ESIMS corresponded well to the structures and to the data previously published.[14] In LC-CD a negative Cotton effect for **5** at 227 nm was observed, corresponding to the known enantiomer[18] of asperphenamate.

Isolation. The extracts of the mycelium and the culture medium were subjected to FCPC, using the two-phase solvent system heptane−EtOAc−MeOH−H_2O (2:8:2:8). The upper phase served as the stationary phase (flow rate 7 mL min^{-1}, rotation speed 1300 min^{-1}). After 124 min the stationary phase was flushed out backwards with MeOH. Petrosifungin A (**1**), petrosifungin B (**2**), and brevianamide A (**3**) were eluted after 140−154 min, 44−64 min, and 80−110 min, respectively. Each compound was further purified on a Sephadex LH-20 column eluted with MeOH, yielding 39.8 mg of petrosifungin A (**1**), 35.0 mg of petrosifungin B (**2**), and 34.0 mg of brevianamide A (**3**).

Petrosifungin A (1): white amorphous solid; mp 162−164 °C; $[\alpha]^{25}_D$ −56.2° (*c* 0.5, MeOH); CD (MeOH) $\Delta\epsilon_{199}$ +20.3, $\Delta\epsilon_{225}$ −6.6, $\Delta\epsilon_{252}$ +2.2; IR (KBr) ν_{max} 3436, 2939, 2871, 1749, 1649, 1523, 1447, 1265, 1235, 1169, 1030, 715, 647 cm^{-1}; for NMR data, see Table 1; ESIMS *m*/*z* 624 $[M + H]^+$; EIMS *m*/*z* 623 $[M]^+$ (37), 540 (7), 345 (44), 287 (54), 84 (100); HREIMS *m*/*z* 623.3317 (calcd for $C_{33}H_{45}N_5O_7$, 623.3319).

Petrosifungin B (2): white amorphous solid; mp 210−212 °C; $[\alpha]^{25}_D$ −37.9° (*c* 0.5, MeOH); CD (MeOH) $\Delta\epsilon_{202}$ +13.2, $\Delta\epsilon_{222}$ −7.4, $\Delta\epsilon_{253}$ +0.7; IR (KBr) ν_{max} 3431, 2944, 2872, 1747, 1638, 1535, 1501, 1448, 1266, 1237, 1169, 1028, 50, 768 cm^{-1}; for NMR data, see Table 2; ESIMS *m*/z 640 $[M + H]^+$; EIMS *m*/z 639 $[M]^+$ (2), 303 (7), 203 (16), 137 (19), 121 (100); HREIMS *m*/z 639.3264 (calcd for $C_{33}H_{45}N_5O_8$, 639.3268).

Brevianamide A (3): yellow amorphous solid; mp 230 °C (subl) (lit.[12] 220−250 °C (subl)); $[\alpha]^{25}_D$ +381° (*c* 0.1, MeOH) (lit.[12] +413°, EtOH); MS and NMR data in agreement with the structure and with published data.[12]

Acid Hydrolysis of 1 and 2. Petrosifungins A and B (**1** and **2**) were hydrolyzed by heating 3 mg of the respective peptide in 2 mL of 6 M HCl at 110 °C for 24 h. After cooling, the solution was evaporated to dryness and redissolved in 250 μL of H_2O.

Preparation and Analysis of GITC Derivatives. To 50 μL of the acid hydrolysate solution were added 25 μL of Et_3N solution (50 mg/mL MeCN) and 40 μL of GITC solution (10 mg/mL MeCN). After 40 min at room temperature, 30 μL of the solution was subjected to HPLC. For the amino acid standards, 20 μL of the amino acid solution (100 μg/mL H_2O), 20 μL of the Et_3N solution, and 40 μL of the GITC solution were combined, and 10 μL of the mixture was injected into the HPLC. For HPLC, a Symmetry C_{18} column was used (Waters, 4.6 × 250 mm, 5 μm); solvents (A) H_2O (0.05% trifluoroacetic acid), (B) MeCN (0.05% trifluoroacetic acid); linear gradient, 0 min 28% B, 50 min 45% B, flow rate 1 mL min^{-1}. Retention times (min) in parentheses were as follows: L-Thr (23.5), D-Thr (24.5), DL-allo-Thr (22.9 and 23.5), L-Pro (24.5), D-Pro (25.8), L-Val (30.3), D-Val (31.4), L-Pip (29.5), D-Pip (30.5).

Preparation and Analysis of Marfey Derivatives. To 50 μL of a 50 mM solution of the respective amino acid (or to 50 μL of the acid hydrolysate solution) was added 100 μL of a 1% (w/v) solution of FDAA (Marfey's reagent, N^{α}-(2,4-dinitro-5-fluorophenyl)-L-alaninamide)[21] in acetone. After addition of 1 M $NaHCO_3$ solution (20 μL), the mixture was incubated for 1 h at 40 °C. The reaction was stopped by addition of 10 μL of 2 M HCl, the solvents were evaporated to dryness, and the residue was redissolved in 1 mL of MeOH−H_2O (1:1). An aliquot of this solution (5 μL) was analyzed by HPLC (for column and solvents see previous paragraph); linear gradient, 0 min 23% B, 45 min 48% B, flow rate 1 mL min^{-1}. Retention times (min) in parentheses were as follows: L-Thr (12.7), D-Thr (16.2), DL-allo-Thr (12.7 and 14.1), L-Pro (18.9), D-Pro (20.5), L-Val (27.6), D-Val (34.3), L-Pip (28.6), D-Pip (26.2). A better resolution of the L-Thr and the DL-allo-Thr derivatives was achieved in an isocratic run with 20% B: L-Thr (25.8), DL-allo-Thr (27.2 and 33.6).

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