An Antifungal Tetrapeptide from the Culture of *Penicillium canescens*

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A new tetrapeptide D-Phe-L-Val-D-Val-L-Tyr (**1**), along with three known diketopiperazines and pseurotin A, were isolated from the culture of *Penicillium canescens*, collected from pollen from beehives, in a screening for new antimicrobial products from unexplored sources. The structure of the tetrapeptide, which exhibits antifungal activity comparable with that of the commercial product benomyl against the soybean phytopathogen *Fusarium virguliforme*, was determined by spectroscopic (2D-NMR, and MS and MS/MS) and chemical methods, and the sequence was confirmed by comparison with authentic synthetic isomeric peptides.

Introduction. – In the pharmaceutical and agrochemical industries, thousands of chemicals must be evaluated before finding a potentially useful compound. To improve this search, particularly when looking for natural bioactive microbial products, the spectrum of biodiversity is being explored for new sources. Previously unexplored environments have their own ecosphere, interactions, and evolution that might contain new producer organisms. Environments such as the open sea and marine sediment, and even extreme environments such as hot springs, demonstrate that the diversity of microorganisms is far greater than anticipated, and a growing number of new natural products are being isolated from them [1].

In this context, fungal strains in pollen from beehives were investigated for antimicrobial metabolites [2][3]. In this study, we describe the isolation and identification of bioactive metabolites, including a previously undescribed tetrapeptide together with other compounds, from a strain of *Penicillium canescens* whose organic extract showed antimicrobial activity.

Penicillium is known as a genus of rich metabolite producers, and, in particular, several *P. canescens* strains were reported to yield the antifungals griseofulvin, canescin, curvulinic acid, and Sch642305, as well as tryptophan-containing alkaloids [4].

Results and Discussion. – 1. *Chemistry.* The *P. canescens* isolate BAFC 3291 was obtained from beehive pollen and cultured in malt extract medium. The medium was solid-phase-extracted and subjected to reversed-phase (RP) chromatography and HPLC to yield compounds **1**–**5**. The purification process was guided by antimicrobial assays on the collected fractions. The spectroscopic data (2D-NMR, EI-MS, and optical rotation) of compounds **3**–**5** were identical to those of the diketopiperazines

aurantiamine [5], cyclo(L-phenylalanyl-*trans*-4-hydroxy-L-proline), and cyclo(L-phenylalanyl-L-proline) [6], respectively, while compound **2** was identical to pseurotin A [7] (*Fig. 1*).

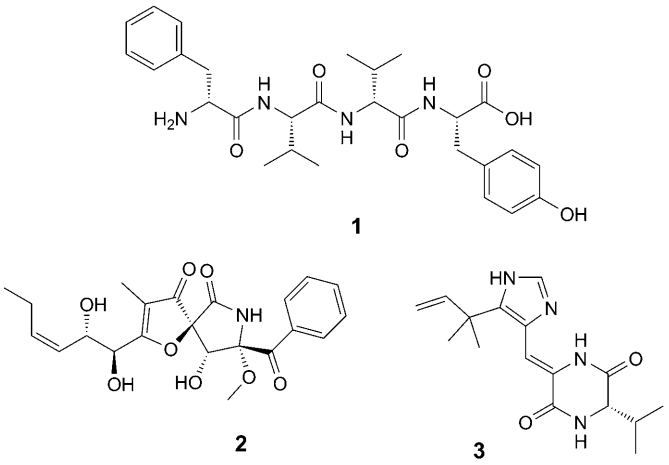

Fig. 1. *Compounds isolated from* Penicillium canescens

The molecular formula of compound **1** was determined as $C_{28}H_{38}N_4O_6$ on the basis of its HR-MS (ESI/APCI; *m/z* 527.2857 $[M+H]^+$). The ^{1}H-NMR ((D_6)DMSO) spectrum displayed signals corresponding to two aromatic rings, one monosubstituted with H-atom signals at 7.20–7.29 ppm, and the other *para*-disubstituted with shielded H-atom signals at 6.59 and 6.94 ppm; three signals at 4.25, 4.14 (2 H) and 3.89 ppm; ten other signals for aliphatic H-atoms, and three exchangeable H-atom signals at 8.31, 8.25, and 7.58 ppm. By the COSY spectrum, four partial structures were identified: two NH–CH–$CHMe_2$ and two CH–CH_2. Together with the two aromatic rings, these data suggested that **1** contained two valines, a phenylalanine, and a tyrosine. The ^{13}C-NMR, HSQC, and HMBC data supported these assumptions, allowing the full assignment of the four amino acid components (*Table*). The sequence of the amino acids in the peptide was revealed by ESI-MS/MS. The ESI-MS/MS of the $[M+H]^+$ ion, used as precursor, yielded peaks of product ions at *m/z* 247.1, 219.1 and 281.1 as major ions, and at 182.1 and 120.1 for minor ones. Two of the major product ions, b_2 and a_2, can be assigned to phenylalanine and valine residues, respectively, while the other three ions corresponded to Val-Tyr (y_2), Tyr (y_1), and the immonium ion of phenylalanine, suggesting the sequence Phe-Val-Val-Tyr (*Fig. 2*). This sequence was confirmed by the NOESY spectrum where the α-H-atom of Phe at δ 3.89 correlated with the N*H*-Val_1 at δ 8.25, and the α-H-atom of Val_1 at δ 4.25 ppm correlated with the N*H*-Val_2 at δ 8.31. Other NOE correlations are shown in *Fig. 3*.

To determine the absolute configuration, compound **1** was hydrolyzed with 6N HCl to obtain the corresponding amino acids, which were analyzed by GC and GC/MS as the *N*-trifluoroacetyl isopropyl ester derivatives in a *Chirasil-val* column. The result showed the presence of D-Phe, L- and D-Val, and L-Tyr in tetrapeptide **1**. The two possible structures for **1**, D-Phe-L-Val-D-Val-L-Tyr and D-Phe-D-Val-L-Val-L-Tyr were

Table 1. *NMR Data for the Natural Tetrapeptide* **1**, *and the Synthetic Peptides* $\mathbf{1}_{synth}$ *and* **6**. In (D_6)DMSO; δ in ppm, J in Hz. Assignments based on ^{1}H,^{1}H-COSY, HSQC, HMBC, and NOESY experiments.

	1		$\mathbf{1}_{synth}$		**6**[a])	
	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)
D-Phe						
1	169.9		169.8		170.6[b])	
2	54.1	3.89 (br. *t*, *J* = 7.0)	54.0	3.90 (br. *t*, *J* = 7.1)	55.9	3.60 (br. *s*)
3	38.8	2.95 (*dd*, *J* = 13.4, 7.2), 2.79 (*dd*, *J* = 13.4, 7.4)	38.5	2.96 (*dd*, *J* = 13.4, 7.1), 2.79 (*dd*, *J* = 13.4, 7.4)	40.1	3.02 (*dd*, *J* = 13.6, 4.4), 2.69 (*dd*, *J* = 13.6, 8.4)
4	136.6		136.5		137.8	
5, 9	129.5	7.21–7.27 (*m*)	129.6	7.22–7.28 (*m*)	129.7	7.21–7.25 (*m*)
6, 8	128.5	7.24–7.29 (*m*)	128.5	7.25–7.30 (*m*)	128.6	7.24–7.29 (*m*)
7	126.8	7.19–7.23 (*m*)	126.9	7.19–7.23 (*m*)	126.7	7.17–7.21 (*m*)
Val-1						
1	171.3		171.3		170.8[b])	
2	57.7	4.25 (*t*, *J* = 8.0)	57.6	4.26 (*d*, *J* = 8.2)	57.8	4.18 (*dd*, *J* = 8.6, 6.3)
3	30.6	1.71–1.78 (*m*)	30.9	1.72–1.79 (*m*)	30.7	1.97–2.04 (*m*)
4	19.2	0.71 (*d*, *J* = 6.7)	19.2	0.70 (*d*, *J* = 6.7)	19.6	0.83 (*d*, *J* = 6.7)
5	18.1	0.57 (*d*, *J* = 6.7)	18.0	0.56 (*d*, *J* = 6.7)	18.2	0.78 (*d*, *J* = 6.7)
NH		8.25 (br. *d*, *J* = 8.0)		8.26 (br. *d*, *J* = 8.2)		8.08–8.11 (*m*)[b])
Val-2						
1	170.7		170.9		170.8[b])	
2	57.3	4.12–4.17 (*m*)	57.2	4.15–4.20 (*m*)	57.5	4.37 (br. *t*, *J* = 7.4)
3	28.9	1.91–1.96 (*m*)	29.4	1.89–1.96 (*m*)	31.5	1.89–1.96 (*m*)
4	19.8	0.73 (*d*, *J* = 6.7)	19.7	0.72 (*d*, *J* = 6.7)	19.6	0.82 (*d*, *J* = 6.7)
5	17.9	0.68 (*d*, *J* = 6.7)	17.8	0.68 (*d*, *J* = 6.7)	18.2	0.80 (*d*, *J* = 6.7)
NH		8.31 (br. *d*, *J* = 8.3)		8.31 (br. *d*, *J* = 8.5)		8.10–8.13 (*m*)[b])
L-Tyr						
1	173.5		173.5		173.1	
2	54.8	4.12–4.17 (*m*)	54.6	4.17–4.22 (*m*)	54.6	4.21–4.26 (*m*)
3	37.0	2.89 (*dd*, *J* = 13.6, 4.8), 2.69 (*dd*, *J* = 13.6, 8.4)	36.9	2.90 (*dd*, *J* = 13.8, 4.9), 2.68 (*dd*, *J* = 13.8, 8.5)	36.6	2.88 (*dd*, *J* = 13.9, 5.5), 2.79 (*dd*, *J* = 13.9, 8.1)
4	128.6		128.7		127.9	
5, 9	130.2	6.94 (*d*, *J* = 8.3)	130.2	6.94 (*d*, *J* = 8.5)	130.3	6.98 (*d*, *J* = 8.4)
6, 8	114.9	6.59 (*d*, *J* = 8.3)	114.9	6.59 (*d*, *J* = 8.5)	115.2	6.61 (*d*, *J* = 8.4)
7	155.8		155.8		155.9	
NH		7.58 (br. *s*)		7.60 (br. *s*)		7.99 (br. *d*, *J* = 8.3)

[a]) Compound **6** did not show any NOESY correlations using different mixing times from 0.3 to 0.9 s.
[b]) Superimposed signals.

evaluated by spectroscopic comparison with the two synthetic tetrapeptides D-Phe-L-Val-D-Val-L-Tyr ($\mathbf{1}_{synth}$) and D-Phe-D-Val-L-Val-L-Tyr (**6**), which were prepared for this purpose. In this way, the complete structure of D-Phe-L-Val-D-Val-L-Tyr was determined for **1** (*Table*).

2. *Bioactivity.* Compounds **1**, **3**, and **5** were responsible for the bioactivity of the extract. Compound **1** showed good, although specific, antifungal activity against *Fusarium virguliforme*, the causal agent of sudden-death syndrome of soybean, with an

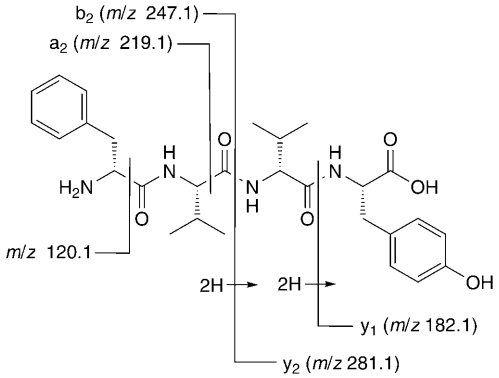

Fig. 2. *Product ions observed in the ESI-MS/MS spectrum of* **1**

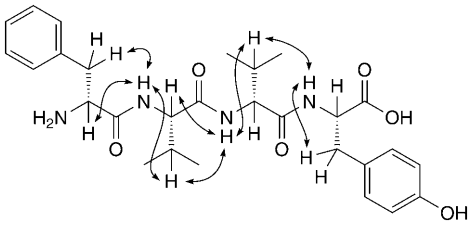

Fig. 3. *Important NOESY correlations for* **1**

inhibition halo diameter of 20 mm, whereas the commercial fungicide benomyl exhibited an inhibition halo diameter of 30 mm, both at 25 μg/pt. Compound **1** also showed moderate antibiotic activity against *Bacillus subtilis.* Compounds **3** and **5** exhibited moderate antifungal activities against the phytopathogenic fungi *Colletotrichum truncatum* and *Fusarium lateritium*, with inhibition halo diameters of 8 and 12 mm, respectively. The bioactivity of synthetic peptide $\mathbf{1}_{synth}$ was the same as **1**, and **6** proved to be inactive in all the tested bioassays.

3. *Discussion.* Small peptides are known to possess a wide range of bioactivities. A family of tetrapeptides containing a C-terminal tyrosine have shown antiopiate activity with considerable selectivity for the μ-opiate binding site. They are transported across the blood–brain barrier and exert more than one central action at the brain [8].

Antimicrobial peptides have also been proposed as new pesticides, because they are short-sequence peptides, produced by microorganisms, and they cover a broad spectrum of bioactivities taking into account the increasing necessity for plant-disease protection that satisfies the new requirements based on toxicology, traceability, and environmental impact [9]. Synthetic peptides were prepared using natural compounds as models, and a tetrapeptide, a pentapeptide, and several hexapeptides with antifungal activities were reported. All these peptides have at least a basic amino acid in their structure [9].

Although the cyclic peptides cyclo(L-phenylalanyl-*trans*-4-hydroxy-L-proline) and cyclo(L-phenylalanyl-L-proline) were previously reported to be both antibacterial and antifungal [10][11], the fungal strains tested were different from the current work. It is noteworthy that the antifungal activity of cyclo(L-Phe-L-Pro) in bacteria has been proposed to be a secondary effect, and the primary reason for its presence might be related to quorum sensing [10][12]. Other bioactivities such as antiviral and antitumoral properties have been reported for these cyclic peptides. In particular, cyclo(Phe-Pro) has been shown to inhibit cancer cell growth and induce apoptosis in HT-29 colon cancer cells [13].

More than 700 metabolites have been reported from the genus *Penicillium* [14], many of them having useful properties that can be exploited for developing new pharmaceuticals [4]. The presence of some metabolites has also assisted on the fungal strain classification process. An extensive analysis of extrolites of the *Penicillium* subgenus *Penicillium* has been performed [15], and the *Penicillium* subgenus *Furcatum* has been revised with the aid of secondary metabolites.

Griseofulvin, canescin, trytophan-containing alkaloids (fellutamines, isorugulosuvine, meleagrin, roquefortine), curvulinic acid, and compound Sch642305 were previously detected in *P. canescens* strains [4]. Although the compounds isolated in this work were not previously reported from this species, pseurotin A and related compounds were isolated and identified from a *Penicillium* sp. isolated from driftwood on the foreshore of a beach in Canada [7], and diketopiperazines, including aurantiamine, were isolated from related *Penicillium* strains.

The tetrapeptide **1** might have been identified here and missed in previous analyses, because the usual extraction method does not allow the extraction and analysis of very polar molecules like amino acids and peptides, so small bioactive peptides can be overlooked [15].

Conclusions. – In summary, a previously undescribed tetrapeptide, D-Phe-L-Val-D-Val-L-Tyr, with antifungal activity, three bioactive diketopiperazines, *i. e.*, cyclo(L-Phe-*trans*-4-hydroxy-L-Pro), cyclo(L-Phe-L-Pro), and aurantiamine, together with pseurotin A, were isolated and identified from a culture of the fungus *P. canescens*. This is the first report on the isolation of these compounds from this species.

We thank Universidad de Buenos Aires, *CONICET*, and *ANPCYT* for partial financial support.

Experimental Part

General. Optical rotations: *Perkin Elmer* polarimeter *343*. The UV Spectra: *Hewlett Packard 8451A* diode-array spectrophotometer (λ_{max} in nm (log ε)). NMR Spectra: *Bruker Avance II* instrument (^{1}H: 500.13 and ^{13}C: 125.13 MHz). EI-MS: *Trio-2 VG Masslab* (Manchester, UK) mass spectrometer. FAB-MS: *ZAB BEqQ* (VG, Manchester, UK) mass spectrometer. ESI/APCI-MS and ESI-MS/MS: *Agilent LC-TOF 6210* with dual probe ESI/APCI (*UCR Mass Spectrometry Facility*, Riverside, CA, USA) or a *Bruker* MicrOTOF-Q II; in *m*/*z*.

D-Phe-L-Val-D-Val-L-Tyr and D-Phe-D-Val-L-Val-L-Tyr were purchased from *Instituto de Química y Fisicoquímica Biológicas* (UBA-CONICET) and prepared for this work purpose, using a peptide synthesizer (*Applied Biosystems* model *431A*). The Fmoc-amino acids activated with HOBt/DCC (1-hydroxybenzotriazole/dicyclohexylcarbodiimide) and an AB HMP resin ([*p*-(hydroxymethyl)phenoxy]-methyl polystyrene) were employed. The synthesized peptides were purified by HPLC (*RP C4*, *Vydac* 214TP510, gradient elution from 10 to 70% *B* in 40 min, 1.5 ml/min, *A:* H_2O, *B:* ^{i}PrOH/H_2O 8:2).

Fungal Strain. The fungus *Penicillium canescens* SOPP was isolated from pollen and classified by NP and deposited with the BAFC culture collection (Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires) under the accession number BAFC 3291. The pollen was collected from beehives of the apiary of Unidad Integrada INTA (Balcarce, Buenos Aires), Facultad de Ciencias Agrarias (Universidad de Mar del Plata), Argentina, and diluted with a sterile physiological soln. Dilutions were seeded on agar malt *Petri* dishes, from which individual colonies were isolated.

Fermentation. A well-grown agar slant of *P. canescens* was used to inoculate 250-ml *Erlenmeyer* flasks containing 75 ml of malt extract medium containing malt extract (30 g) and peptone (5 g/l). After a week, one *Erlenmeyer* media was employed to inoculate 1 l of the above media in 3-l *Erlenmeyer* flasks. The fermentation was carried out at 25° for 23 d under static conditions.

Extraction and Isolation. The fermentation broth (3 l) was filtered, and *Amberlite XAD-16* was added to the filtrate. After 10 h, the suspension was filtered, and the phase was washed with H_2O and then eluted with MeOH. The MeOH eluate was evaporated and subjected to vacuum chromatography on *RP-C18* using H_2O, and mixtures of H_2O and MeOH of decreasing polarity. *Fraction 3*, eluted with H_2O/MeOH 1:1, yielded on HPLC (column: *YMC C18*, 5 μm, 22.5 × 2.5 cm; H_2O/MeOH 65:35) the diketopiperazines cyclo(L-phenylalanyl-*trans*-4-hydroxy-L-proline) (**4**; 8.0 mg) and cyclo(L-phenylalanyl-L-proline) (**5**, 16 mg). *Fr. 4*, eluted with H_2O/MeOH 25:75, yielded on HPLC (column: *YMC C 18*, 5 μm, 22.5 × 2.5 cm; H_2O/MeOH 45:55) pseurotin A (**2**, 9.0 mg), aurantiamine (**3**, 6.7 mg), and D-*Phe*-L-*Val*-D-*Val*-L-*Tyr* (**1**; 11.6 mg).

Antibiotic Assay. The antibiotic activity was determined by the agar diffusion method using 50 μg of sample/6 mm disk against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, and *Escherichia coli* ATCC 25922. Compound **1** caused an inhibition zone of 20 mm against *B. subtilis*, whilst **4** showed an inhibition zone of 16 mm against *S. aureus*. Gentamicin, which was used as the positive test compound, showed inhibition halos of 30 mm at a concentration level of 25 μg/disk.

Antifungal Activity. Direct bioautography on TLC was employed as the method for detecting fungitoxic substances [16]. A concentration level of 25 μg/spot of each assayed compound was used, except for compounds **3**–**5**, where 50 μg/spot was tested. Benomyl, which was used as test compound, showed an inhibition zone of 30 mm at a conc. level of 25 μg/spot (0.086 μmol/spot) and 22 mm at 14 μg/spot (0.048 μmol/spot). Benomyl, which is used in several countries, is a systemic benzimidazole fungicide selectively toxic to microorganisms and to invertebrates with known concerns about health and environment protection. Compound **1** showed a diameter inhibition zone of 20 mm at 25 μg (0.048 μmol/spot) and a minimum inhibitory concentration (*MIC*) of 9.5×10^{-3} μmol, against *Fusarium virguliforme*, whilst **5** showed a halo of 12 mm against *Fusarium lateritium*, and **3** showed a halo of 8 mm against *Colletotrichum truncatum*.

Data of **1**. Amorphous powder. $[\alpha]_D^{25} = -124$ ($c = 0.05$, EtOH). UV (EtOH): 208 (3.03). ^{1}H- and ^{13}C-NMR: see *Table 1*. FAB-MS$^+$ (RA; glycerol matrix): 527 ($[M+H]^+$, 25), 72 (100). FAB-MS$^-$ (RA; glycerol matrix): 525 ($[M-H]^-$, 100); HR-ESI/APCI-MS: 527.2857 ($[M+H]^+$, $C_{28}H_{39}N_4O_6^+$; calc. 527.2864, Δ 0.2 ppm), 549.2675 ($[M+Na]^+$, $C_{28}H_{38}N_4NaO_6^+$; calc. 549.2684, Δ 1.6 ppm). ESI-MS/MS (527 u): 281.1 ($[y_2]^+$, 22), 247.1 ($[b_2]^+$, 95), 219.1 ($[a_2]^+$, 100), 182.1 ($[y_1]^+$, 13), 120.1 ($[H_2N{=}CHCH_2Ph]^+$, 5).

Absolute Configuration of **1**. Compound **1** (0.5 mg) was dissolved in MeOH, and 6N HCl (0.3 ml) was added, heated at 120° for 16 h and evaporated to dryness. Identification of the amino acids was accomplished by GC after derivatization [17]. Retention times of the *N*-trifluoroacetyl isopropyl ester derivatives were compared with those of standards.

The GC analyses of *N*-trifluoroacetyl isopropyl ester derivatives were carried out on a *Hewlett Packard 5890* gas chromatograph on a *Chirasil-val* cap. column (*Alltech*, 25-m length, 0.25-mm i.d.) with N_2 as carrier gas and a temp. programme: 60° (1 min) to 180°, 4°/min and 180° (10 min) to 200°, 10°/min. t_R (min): D-Val (11.0), L-Val (11.4), D-Phe (25.3), L-Phe (29.4), D-Tyr (29.8), L-Tyr (33.9), **1** (11.0, 11.4, 25.3, 33.9). Co-injections and GC/MS were realized to confirm the identities. GC/MS was recorded on a gas chromatograph *GC 17 A* coupled to a mass spectrometer *QP 5000* (*Shimadzu*).

Data of $\mathbf{1}_{synth}$. Amorphous powder. $[\alpha]_D^{25} = -137$ ($c = 0.05$, EtOH). ^{1}H- and ^{13}C-NMR: see *Table.* HR-ESI-MS: 527.2858 ($[M+H]^+$, $C_{28}H_{39}N_4O_6^+$; calc. 527.2864, Δ 1.1 ppm), 549.2710 ($[M+Na]^+$, $C_{28}H_{38}N_4NaO_6^+$; calc. 549.2684, Δ 4.9 ppm).

Data of **6**. Amorphous powder. $[\alpha]_D^{25} = -31$ ($c = 0.06$, EtOH). ^{1}H-NMR and ^{13}C-NMR: see *Table.* HR-ESI-MS: 527.2865 ($[M+H]^+$, $C_{28}H_{39}N_4O_6^+$; calc. 527.2864, Δ 0.2 ppm), 549.2688 ($[M+Na]^+$, $C_{28}H_{38}N_4NaO_6^+$; calc. 549.2684, Δ 0.8 ppm).

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Received November 24, 2008