Metabolites Produced by the Phytopathogenic Fungus *Rhizoctonia solani*: Isolation, Chemical Structure Determination, Syntheses and Bioactivity

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The isolation, structure determination, syntheses and biological activity of N_b-acetyltryptamine and three proline containing dioxopiperazines, *cyclo*(*S*-Pro-*S*-Leu) (**2**), *cyclo*(*S*-Pro-*S*-Ile) (**3**), and *cyclo*(*S*-Pro-*S*-Val) (**4**), from cultures of *Rhizoctonia solani* Kuhn are reported here for the first time. Despite the small amounts isolated, the absolute stereochemistry of these naturally occurring dioxopiperazines was established by ^{1}H NMR using for the first time the chiral solvating agent (*R*)-(−)-2,2,2-trifluoro-1-(9-anthryl)ethanol.

Key words: Dioxopiperazines, *Rhizoctonia solani*

Introduction

Rhizoctonia solani Kuhn, the mycelial state of *Thanatephorus cucumeris* (Frank) Donk, is an important pathogen associated with a wide host range worldwide, including Monocotyledoneae as cereals (Demirci, 1998) and Dicotyledoneae as crucifers (Kataria and Verma, 1992). Based on hyphal anastomosis behaviour, isolates of *R. solani* have been divided into 12 anastomosis groups (AG) denoted AG 1-11 and one binucleate (BI). Isolates AG 2-1 appear to affect mainly crucifers causing damping off and root rot of canola (*Brassica napus* L. and *B. rapa*) and brown mustard (*B. juncea*). Ongoing investigations of crucifer phytoalexins and their economically important pathogens showed that AG 2-1 isolates of *R. solani* were able to detoxify some of the strongest antifungal phytoalexins (Pedras and Ahiahonu, 2005). For example, camalexin was detoxified through oxidation of the indole ring to 5-hydroxycamalexin, and cyclobrassinin was detoxified to 5-hydroxybrassicanal A (Pedras and Ahiahonu, 2005). Interestingly, during investigation of these fungal biotransformations (Pedras and Liu, 2004), the presence of a number of compounds unrelated with the phytoalexins under study was detected in cultures of *R. solani*. Further detailed investigation of liquid cultures of *R. solani* led to the first isolation of N_b-acetyltryptamine (**1**) and three proline containing dioxopiperazines, *cyclo*(*S*-Pro-*S*-Leu) (**2**), *cyclo*(*S*-Pro-*S*-Ile) (**3**), and *cyclo*(*S*-Pro-*S*-Val) (**4**). Here we report the chemical structure determination, syntheses and biological activity of these compounds. The absolute stereochemistry of the naturally occurring dioxopiperazines was established by ^{1}H NMR using for the first time the chiral solvating agent (*R*)-(−)-2,2,2-trifluoro-1-(9-anthryl)ethanol [(*R*)-(−)-TFAE].

Results and Discussion

Examination of HPLC chromatograms of extracts of cultures of *R. solani* indicated the presence of several compounds not detectable in non-inoculated medium (UV detection at 206, 220, and 254 nm). A time-course HPLC analysis of culture extracts indicated that production of these compounds by *R. solani* increased up to seven days of incubation. These metabolites were isolated from larger scale cultures as described in Materials and Methods and their structures were established through analyses of the spectroscopic data and chemical syntheses, as described below.

Fungal cultures were grown for seven days, the broth was freeze-dried, extracted and concentrated to dryness. The residue was fractionated by flash column chromatography followed by preparative TLC. The structures of the new metabolites were first established through analyses of the spectroscopic data and further confirmed by syntheses. Compound **1** (R_t = 14.4 min) had a molecular formula of $C_{12}H_{14}N_2O$ (*m/z* found 202.1110 [M$^+$], calcd. 202.1106) and NMR spectroscopic data indicative of an indole nucleus, two methylene protons mutually coupled and an acetyl group. The protons of one of the methylene groups correlated with three aromatic carbon atoms, indicative of a CH_2-CH_2-group adjacent to the indole ring. Based on these structural features, the compound was identified as N_b-acetyltryptamine (**1**), identical in all respects to an authentic sample prepared from tryptamine. This is the first report of production of N_b-acetyltryptamine (**1**) by *R. solani* (Fig. 1).

Table II. ^1H NMR ($CDCl_3$, 500 MHz) chemical shifts (ppm) and multiplicities (*J* in Hz) of dioxopiperazines **2**–**4**.

H	**2**	**3**	**4**
3	3.56, 1H, m 3.62, 1H, m	3.55, 1H, m 3.64, 1H, m	3.55, 1H, m 3.65, 1H, m
4	1.93, 1H, m 2.10, 1H, m	1.19, 1H, m 2.05, 1H, m	1.90, 1H, m 2.05, 1H, m
5	2.14, 1H, m 2.37, 1H, m	2.05, 1H, m 2.32, 1H, m	2.05, 1H, m 2.38, 1H, m
6	4.13, 1H, dd (8.2, 8.2)	4.08, 1H, dd (7.7, 8.0)	4.08, 1H, dd (7.8, 7.7)
N–H	5.73, 1H, br s	5.92, 1H, br s	6.28, 1H, br s
9	4.03, 1H, dd (3.1, 9.4)	3.98, 1H, s	3.94, 1H, s
10	1.54, 1H, ddd (14.5, 9.5, 5.0) 2.04, 1H, m	2.39, 1H, m	2.64, 1H, m
11	1.77, 1H, m	1.19, 1H, m 1.44, 1H, m	–
12	0.97, 3H, d (6.5)	0.94, 3H, t (7.4)	0.92, 3H, d (6.7)
13	1.02, 3H, d (6.5)	1.07, 3H, d (7.2)	1.08, 3H, d (7.2)

The HRMS of compound **2** ($R_t = 6.5$ min) together with NMR spectroscopic data suggested a molecular formula of $C_{11}H_{18}N_2O_2$. The ^{13}C NMR spectrum (Table I) of compound **2** showed 11 signals, two of which resonated at 170.7 and 166.6 ppm, indicating the presence of two carbonyl groups. The remaining nine carbon atoms were all shown to be sp^3 carbon atoms, three of which were adjacent to heteroatoms (59.4, 53.8, 45.9 ppm). The ^1H NMR spectrum (Table II) showed a broad D_2O exchangeable singlet at 5.73 ppm, indicative of an NH group, coupled to a methine proton at 4.13 ppm, which was part of a spin system suggestive of a leucyl residue. An additional spin system of three methylene protons suggested the presence of a prolyl residue. The spectroscopic features of compound **2** suggested a dioxopiperazine with the structure of *cyclo*(Pro-Leu). The HRMS of a slightly more polar compound, metabolite **3** with $R_t = 5.7$ min, indicated it to be a structural isomer of **2**. The ^1H NMR spectrum of **3** showed the expected coupling pattern of a prolyl residue, whereas the leucyl residue of **2** was replaced with an isoleucyl residue in **3**. Therefore, compound **3** was determined to be *cyclo*(Pro-Ile). An additional but similar metabolite, compound **4**, was isolated and deduced to be *cyclo*(Pro-Val) from analyses of its NMR and HRMS data. To the best of our knowledge this is the first time that *R. solani* is reported to produce dioxopiperazines.

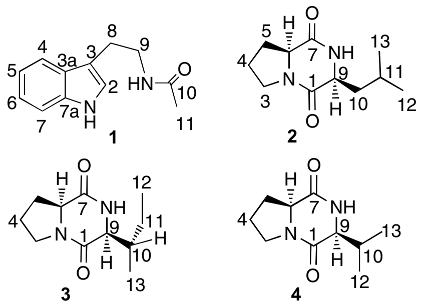


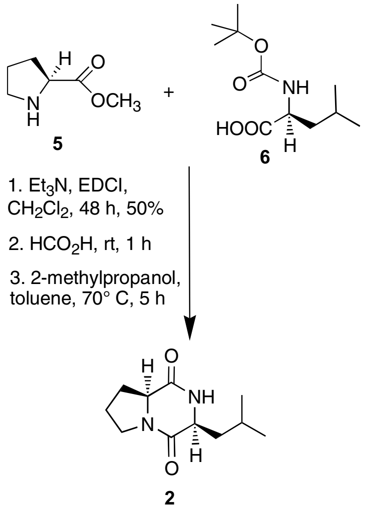
Fig. 1. Chemical structures of metabolites produced by *Rhizoctonia solani*: N_b-acetyltryptamine (**1**), *cyclo*(*S*-Pro-*S*-Leu) (**2**), *cyclo*(*S*-Pro-*S*-Ile) (**3**), and *cyclo*(*S*-Pro-*S*-Val) (**4**).

Table I. ^{13}C NMR ($CDCl_3$, 500 MHz) chemical shifts (ppm) of dioxopiperazines **2**–**4**.

C	**2**	**3**	**4**
1	170.7	170.4	169.4
3	45.9	45.5	44.4
4	23.1	22.7	21.6
5	28.5	28.9	27.8
6	59.4	59.2	58.1
7	166.6	165.4	164.2
9	53.8	60.9	59.7
10	39.0	35.7	27.7
11	25.1	24.4	–
12	23.7	12.5	15.3
13	21.6	16.3	18.5

Although dioxopiperazines **2**–**4** were reported previously, due to the very small amounts isolated from *R. solani*, the relative and absolute configurations of dioxopiperazines **2**–**4** were established through synthesis and ^1H NMR spectroscopic data obtained in the presence of (*R*)-(–)-TFAE, as follows. *S,S*-Dioxopiperazines **2**–**4**, their *R,R*-enantiomers **2a**–**4a**, and the diastereomers *cyclo*(*R*-Pro-*S*-Leu) (**2b**) and *cyclo*(*S*-Pro-*R*-Ile) (**3b**) were synthesized by condensation of the methyl ester of the corresponding proline (**5**) with the corresponding *N*-protected amino acid [*t*-Boc-Leu (**6**), *t*-Boc-

Ile, or *t*-Boc-Val], as shown in Scheme 1 for *cyclo*(*S*-Pro-*S*-Leu) (**2**) (Férezou *et al.,* 1980). As expected, the ^{1}H NMR spectra of synthetic dioxopiperazines **2**–**4** and **2a**–**4a** were identical to the corresponding natural products, whereas the ^{1}H NMR spectra of diastereomers **2b** and **3b** were clearly different from those of **2**/**2a** and **3**/**3a**. Subsequently, to determine the absolute configuration of the natural dioxopiperazines, the ^{1}H NMR spectra of synthetic **2**, **3**, and **4** were obtained separately in the presence of the NMR chiral solvating agent (*R*)-(−)-TFAE. Furthermore, the ^{1}H NMR spectra of a 3:1 mixture of synthetic enantiomeric pairs **2**/**2a**, **3**/**3a**, and **4**/**4a**, obtained in the presence of (*R*)-(−)-TFAE as well, indicated that each enantiomer could be clearly differentiated by the chemical shift of H-9 (for **3** and **4**) or the N–H (for **2**) (Table III). Finally, the ^{1}H NMR spectrum of each natural product obtained in the presence of (*R*)-(−)-TFAE at identical concentrations, was identical to those of the *S,S*-enantiomers. Furthermore, the ^{1}H NMR spectrum of the natural products **2**, **3** and **4** spiked with the *R,R*-enantiomers **2a**, **3a** and **4a**, respectively, displayed distinct signals for the *S,S*- and *R,R*-enantiomers, as summarized in Table III. For example, *cyclo*(*S*-Pro-*S*-Leu) (**2**) and *cyclo*(*R*-Pro-*R*-Leu) (**2a**) mixed in a 3:1 ratio showed separate signals corresponding to the *S,S*- and *R,R*-enantiomers for the N–H, whereas the mixtures **3**/**3a** and **4**/**4a** showed separate signals for the H-9. These results confirmed that the absolute configuration of the three natural products **2**–**4** was *S,S*.



Scheme 1. Synthesis of *cyclo*(*S*-Pro-*S*-Leu) (**2**).

To establish the phytotoxicity of **1** and dioxopiperazines **2**–**4**, **2b** and **3b**, bioassays were carried out with three different plant species of known resistance to isolates AG 2-1 of *R. solani*: *Brassica napus* cv. Westar (susceptible), *B. juncea* cv. Cutlass (susceptible), and *Sinapis alba* cv. Ochre (resistant), as described in Materials and Methods. None of the dioxopiperazines **2**–**4**, **2b** and **3b**, nor N_b-acetyltryptamine (**1**) caused detectable necrosis or chlorosis on the leaves of test plants.

Conclusion

Four secondary metabolites were isolated for the first time from the liquid cultures of *R. solani,* N_b-acetyltryptamine (**1**) and three dioxopiperazines, *cyclo*(*S*-Pro-*S*-Leu) (**2**), *cyclo*(*S*-Pro-*S*-Ile) (**3**), and *cyclo*(*S*-Pro-*S*-Val) (**4**). Dioxopiperazines are common secondary metabolites produced by a variety of organisms of both marine and terrestrial origin. For example, compounds **2**–**4** were reported to be produced by bacteria (Yang *et al.,* 2002; Jayatilake *et al.,* 1996) and marine sponges (Wegerski *et al.,* 2004; Bull *et al.,* 1998; Adamczeski *et al.,* 1995). Interestingly, N_b-acetyltryptamine (**1**) was first reported from a natural source, *Prosopis nigra* an Argentine medicinal plant, in 1975 (Moro *et al.,* 1975). Since then N_b-acetyltryptamine (**1**) was isolated from various organisms including *Penicillium chrysogenum* and *P. expansum* isolated on board the Mir space station (Kozlov-

Table III. ^{1}H NMR (CDCl$_3$, 500 MHz) selected chemical shifts (ppm) and multiplicities of dioxopiperazines **2**–**4** (10^{-6} mol/500 μl) in the presence of the chiral solvating agent (*R*)-(−)-TFAE.

H	**2** + **2a** (3:1)	**3** + **3a** (3:1)	**4** + **4a** (3:1)
N–H	5.51, br s + 5.54, br s[a]	5.57, br s	5.69, br s
9	3.72, overlapping m	3.58, s + 3.62, s[b]	3.78, s + 3.80, s[c]

[a] Containing 7 equivalents of (*R*)-(−)-TFAE.
[b] Containing 2 equivalents of (*R*)-(−)-TFAE.
[c] Containing 4 equivalents of (*R*)-(−)-TFAE.

skii *et al.,* 2002), as well as *P. vitale* (Vinokurova *et al.,* 2000). The secondary metabolites from *R. solani* reported until now are phenylacetic acid, *m*-hyroxyphenylacetic acid, and *p*-hyroxyphenylacetic acid, which were considered to be phytotoxins due to their toxicity to roots of sugar beet (Aoki *et al.,* 1963). Nonetheless, these AG 2 isolates of *R. solani* isolated from crucifers produced only traces of phenylacetic acid and *p*-hyroxyphenylacetic acid. To the best of our knowledge no other metabolites have been reported from crucifer isolates of *R. solani*; however several fungal pathogens of crucifers are known to produce phytotoxins (Pedras and Chumala, 2005; Pedras *et al.,* 2005). Although dioxopiperazines **2**–**4** showed no toxicity to leaves of canola or mustard, the dioxopiperazines polanrazines C and E produced by the blackleg fungus [*Leptosphaeria maculans* (Desm.) Ces. et de Not., asexual stage *Phoma lingam* (Tode ex Fr.) Desm.] showed moderate but selective toxicity on brown mustard leaves (Pedras and Biesenthal, 2001). Nonetheless, further work has to be carried out to investigate the potential ecological significance of dioxopiperazines in the interaction of crucifer isolates of *R. solani* with their host plants.

Materials and Methods

General procedures

All chemicals were purchased from Sigma-Aldrich Canada Ltd./Oakville, ON). All solvents were HPLC grade and used as such, except for CH_2Cl_2 and $CHCl_3$ that were redistilled.

HPLC analysis was carried out with a high performance liquid chromatograph equipped with a quaternary pump, automatic injector, and diode array detector (wavelength range 190–600 nm), degasser, and a Hypersil ODS column (5 μm particle size silica, 4.6 i.d. × 200 mm), equipped with an in-line filter; mobile phase: 80% H_2O/20% CH_3CN for 10 min, followed by gradient elution 80% H_2O/20% CH_3CN to 60% H_2O/40% CH_3CN for 10 min, 60% H_2O/40% CH_3CN to 25% H_2O/75% CH_3CN for 10 min, and finally 25% H_2O/75% CH_3CN to 100% CH_3CN for 10 min, with a flow rate 1.0 ml/min. Semi-preparative HPLC was used for isolating compounds **2**–**4**: Hypersil ODS column (10 μm particle size silica, 10 i.d. × 250 mm) gradient elution with H_2O (100) to CH_3CN/H_2O (25:75) for 20 min and a flow rate of 5.0 ml/min. NMR spectra were recorded on Bruker Avance 500 spectrometers; for ^1H NMR (500 MHz), δ values were referenced as follows: $CDCl_3$ ($CHCl_3$ 7.27 ppm), CD_3OD (CD_2HOD 3.31 ppm); for ^{13}C NMR (125.8 MHz) δ values were referenced to $CDCl_3$ (77.23 ppm), CD_3OD (49.15 ppm). Mass spectra (MS) were obtained on a VG 70 SE mass spectrometer. Specific rotations, $[\alpha]_D$, were determined at ambient temperature on a Rudolph DigiPol DP 781 polarimeter using a 1 ml, 10 cm path length cell; the units are 10^{-1} deg cm^2 g^{-1} and the concentrations are reported in g/100 ml. Fourier transform infrared spectra (FTIR) were recorded on a Bio-Rad FTS-40 spectrometer and were measured by the diffuse reflectance method on samples dispersed in KBr.

Fungal cultures and plant assays

R. solani isolate AG 2-1 was obtained from Agriculture and Agri-Food Canada Research Station, Saskatoon, Saskatchewan. The fungus was grown on potato dextrose agar (PDA) plates at (24 ± 2) °C, under constant light. Solid cultures were started by placing a plug of mycelium (6 mm diameter) at the centre of the plate containing the agar. Liquid cultures were initiated by cutting plugs of mycelium from the edges of 5-day-old cultures and placing them in PDB medium (5 plugs per 100 ml of medium).

Plants were grown in a growth chamber for three weeks, with 16 h light (fluorescent and incandescent, 450–530 mmol s^{-1} m^{-2})/8 h dark, at (24 ± 2) °C. Three different species of known resistance to V isolates of *Phoma lingam* were employed in the bioassays: *Brassica napus* cv. Westar (susceptible) and *B. juncea* cv. Cutlass (susceptible), *Sinapis alba* cv. Ochre (resistant). Pure compounds (10^{-3} and 5×10^{-4} M) where dissolved in $MeOH/H_2O$ (1:1). Leaves were punctured with a needle (6 punctures per leaf) and 10 μl droplets (6 droplets per leaf) were applied on punctured sites. Control leaves were treated similarly employing a 1:1 $MeOH/H_2O$ solution. Plants were incubated in a growth chamber as reported above [16 h light/8 h dark, at (24 ± 2) °C]; leaves were observed over a 1-week period and the diameter of each lesion was measured after 7 d.

Isolation of metabolites

Time-course experiment

Erlenmeyer flasks (1 l) containing 500 ml of PDB were inoculated with 25 plugs of 6-day-old solid cultures of *R. solani* isolate AG 2-1 and were incubated at (23 ± 2) °C on a shaker at 150 rpm. Samples (50 ml) were taken from each flask after 3 d inoculation. Subsequently, 50 ml samples were taken every 24 h up to 9 d. Samples were extracted immediately with ethyl acetate (50 ml), dried over Na_2SO_4 and concentrated in a rotary evaporator. The residue was dissolved in 0.5 ml of acetonitrile and analysed by HPLC.

Scale up experiments. Erlenmeyer flasks (1 l) each containing 750 ml of PDB were inoculated with 38 plugs of *R. solani* isolate AG 2-1, as reported above. After 7 d of incubation, the liquid cultures were combined, filtered, concentrated to one third of the initial volume by freeze-drying and extracted with ethyl acetate, as reported above. The residue was fractioned using flash column chromatography (FCC) as follows.

Isolation of N_b-acetyltryptamine (**1**) and dioxopiperazines **2**–**4**

The residue obtained from 10 l of culture (75 mg) was fractionated by FCC (silica gel ca. 40 ml, gradient elution CH_2Cl_2/MeOH, 95:5 to 90:10, 20 ml fractions). HPLC chromatograms showed that the peaks of interest were present in fractions 1 (5 mg) and 3–7. Fraction 1 was further purified by PTLC (toluene/ethyl acetate/acetic acid, 6:3:1) to yield N_b-acetyltryptamine (**1**) (R_t = 14.4 min, 3 mg); fractions 3–7 (24 mg) were combined and further fractionated by PTLC (toluene/ethyl acetate/acetic acid, 6:3:1) to yield weakly active UV bands which corresponded to compounds with HPLC R_t = 2.9, 5.7 and 6.5 min. This mixture was further purified by HPLC (semi-preparative method) to yield pure compounds **2** (1.5 mg, R_t = 6.5 min), **3** (1.2 mg, R_t = 5.7 min) and **4** (1 mg, R_t = 2.9 min).

General procedure for synthesis of dioxopiperazines

The synthesis of dioxopiperazines **2**–**4** followed a previously reported method used for synthesis of *cyclo*(*S*-tyrosine-*S*-serine) (Férezou *et al.,* 1980). A general description is reported below.

Proline methyl ester

Thionyl chloride (49 μl, 0.65 mmol) was added to a solution of proline (49.5 mg, 0.43 mmol) in methanol (5 ml) at room temperature. The mixture was refluxed for 8 h, allowed to cool to room temperature and the solvent was removed under vacuum. The residue was rinsed three times with methylene chloride to yield the proline methyl ester quantitatively.

t-Boc-protected AA

The amino acid (AA; 0.44 mmol) was added to a mixture of NaOH (1 M, 0.9 ml) and dioxane (0.9 ml) at –5 °C, followed by addition of di-*tert*-butyl dicarbamate (161.8 mg, 0.74 mmol). The mixture was allowed to warm to room temperature and then stirred overnight. The volume of the mixture was reduced to half under vacuum, diluted with water and then extracted with diethyl ether followed by acidification to pH 3 and extraction with ethyl acetate. The ethyl acetate extract solution was dried over Na_2SO_4 and the solvent was removed under vacuum to yield quantitatively the *t*-Boc-protected AA.

t-BocAA-proline methyl ester

A solution of the *t*-BocAA (0.20 mmol) and proline methyl ester (32 mg, 0.19 mmol) were dissolved in methylene chloride (2.5 ml), cooled to –15 °C, followed by addition of triethylamine (31 μl, 0.21 mmol) and EDCI (38 mg, 0.20 mmol). The reaction mixture was stirred 1–2 h and then kept in a freezer at –15 °C for 48 h. The reaction mixture was transferred into a separatory funnel using methylene chloride (25 ml), and was washed successively with water (10 ml), $NaHCO_3$ (1 M, 10 ml), citric acid (1 N, 10 ml) and finally again with water (10 ml). The organic layer was dried over Na_2SO_4, then concentrated to dryness under vacuum to give the *t*-BocAA-proline methyl ester in ca. 80% yield.

Cyclo(proline-AA)

t-BocAA-proline methyl ester (0.16 mmol) in formic acid (4.0 ml) was kept at room temperature for 2 h, then concentrated to dryness under vacuum and the residue rinsed with toluene. The remaining residue was dissolved in toluene (2.0 ml) and *sec*-butanol (6.0 ml) and heated in an oil bath at 70 °C for 5 h. The product was separated by preparative TLC to give *cyclo*(proline-AA) as a white solid in ca. 50% yield.

N_b-acetyltryptamine (**1**). HPLC: R_t = 14.4 min. – ^{1}H NMR (500 MHz, CD_3OD): δ = 7.34 (d, J = 8 Hz, H-7), 7.09 (ddd, J = 7.5, 7.5, 1 Hz, H-5), 7.08 (s, H-2), 7.57 (d, J = 8 Hz, H-4), 7.01 (ddd, J = 7.5,

7.5, 1 Hz, H-6), 3.48 (t, $J = 7.5$ Hz, H_2-9), 2.95 (t, $J = 7.5$ Hz, H_2-8), 1.92 (s, H_3-11). – ^{13}C NMR (125.8 MHz, CD_3OD): δ = 172.2 (C-10), 136.8 (C-7a), 127.8 (C-3a), 122.0 (C-2), 121.3 (C-6), 118.2 (C-4), 118.5 (C-5), 118.2 (C-7), 112.3 (C-3), 40.5 (C-9), 25.2 (C-8), 21.6 (C-11). – EI-HRMS *m/z* = 202.1110, $C_{12}H_{14}N_2O$, calcd. 202.1106. – FTIR: ν = 3395, 3281, 2928, 1648, 1553, 1457, 1439, 1367 cm^{-1}.

Cyclo(S-Pro-S-Leu) (**2**). HPLC: $R_t = 6.5$ min (A). – $[\alpha]_D^{25} = -133°$ (ethanol, $c = 0.30$) (Bull *et al.,* 1998, for *R,R*: $[\alpha]_D = 128°$, ethanol, $c = 0.28$). – EI-HRMS: *m/z* = 210.1367, $C_{11}H_{18}N_2O_2$, calcd. 210.1368. – FTIR: ν = 3229, 2959, 2871, 1685, 1434 cm^{-1}.

Cyclo(S-Pro-S-Ile) (**3**). HPLC: $R_t = 5.7$ min (A). – $[\alpha]_D^{25} = -153°$ (ethanol, $c = 0.15$) (Bull *et al.,* 1998, for *R,R*: $[\alpha]_D = 168°$, ethanol, $c = 0.13$). – EI-HRMS: *m/z* = 210.1367, $C_{11}H_{18}N_2O_2$, calcd. 210.1368. – FTIR: ν = 3236, 2965, 2878, 1672, 1432 cm^{-1}.

Cyclo(S-Pro-S-Val) (**4**). HPLC: $R_t = 2.9$ min (A). – $[\alpha]_D^{25} = -110°$ (ethanol, $c = 0.11$) (Bull *et al.,* 1998, for *R,R*: $[\alpha]_D = 120°$, ethanol, $c = 0.10$). – EI-HRMS: *m/z* = 196.1212, $C_{10}H_{16}N_2O_2$, calcd. 196.1211. – FTIR: ν = 3214, 3077, 2962, 2878, 1668, 1427 cm^{-1}.

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