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Antibacterial cyclopeptide alkaloids from the bark of *Condalia buxifolia*

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Abstract

The cyclopeptide alkaloid, named condaline-A, was isolated from the root bark of *Condalia buxifolia* Reissek (Rhamnaceae), along with the known compounds adouetine-Y′, scutianine-B, and scutianine-C. Their structures were determined by spectroscopic analyses, with their antibacterial activities being evaluated by use of a direct bioautography method.

Keywords: *Condalia buxifolia*; Rhamnaceae; Cyclopeptide alkaloid; Condaline-A; Antimicrobial activity

1. Introduction

As a continuation of investigations on peptide alkaloids of plants belonging to the family Rhamnaceae (Morel et al., 1979, 1984, 1985, 1998; Machado et al., 1995; Menezes et al., 1995), the isolation of peptide alkaloids from the methanol extract of a plant of the genus *Condalia* is reported. The genus *Condalia* includes 18 American species distributed all over the continent, from North to South America (Johnston, 1962). Five of them are found in South America, one in Brazil (Johnston and Soares, 1972). *Condalia buxifolia* Reissek is a tree up to 4 m high that grows in the wild in southern Brazil, Uruguay, and Argentina, where it is used by local populations as a febrifuge, for its antiinflammatory properties, and against dysentery (Bastos, 1989). Since its chemistry has not previously been investigated, the root bark of the plant was examined to afford a new peptide alkaloid, condaline-A (**1**), together with three known peptide alkaloids, adouetine-Y′ (=myrianthine-B, **2**) (Marchand and Monseur, 1968), scutianine-B (**3**) (Tschesche et al., 1971, 1974), and scutianine-C (**4**) (Sierra et al., 1974). A combination of LSIMS mass spectrometry, ^{1}H and ^{13}C NMR spectroscopy, and some chemical transformations indicated structure **1** for condaline-A. Peptide alkaloids possess a variety of biological activities, including sedative (Lee et al., 2001; Han et al., 1989), antifungal (Tschesche et al., 1974; Tschesche and Ammermann, 1974; Gournelis et al., 1997), and antibacterial (Tschesche et al., 1974; Tschesche and Ammermann, 1974; Gournelis et al., 1997) properties. In this work, the antibacterial activity of each alkaloid was determined by direct bioautography (Rahalison et al., 1991), against Gram-positive (*Staphylococcus aureus*, *Staphylococcus epidermidis* and *Micrococcus luteus*) Gram-negative (*Klebsiela pneumoniae*, *Salmonella setubal* and *Escherichia coli*) bacteria.

2. Results and discussion

The methanol extract of the root bark of *C. buxifolia* was fractionated as described in the Experimental section. The main component, condaline-A (**1**) was separated by conventional chromatographic methods as a colorless crystalline material. Its LSIMS-mass spectrum displayed a prominent $[M+H]^+$ at m/z 555 which, in combination with ^{13}C NMR spectroscopy and elemental analysis data, suggested that **1** had the molecular formula $C_{33}H_{38}N_4O_4$.

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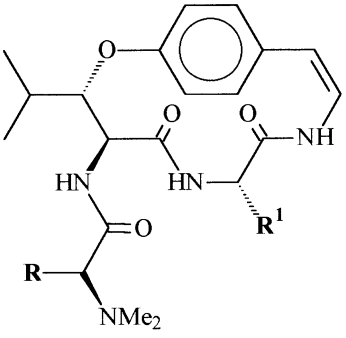
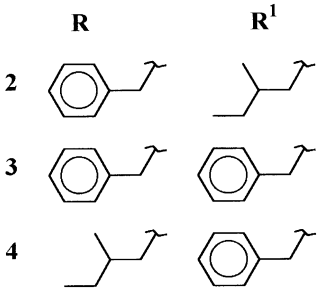
The ^{1}H NMR spectrum of **1** showed a methyl triplet at δ 0.83 ($J_{32,31}=6.5$ Hz) and a methyl doublet at δ 0.84 ($J_{33,30}=6.5$ Hz), which were assigned to the C-32 and C-33 methyl protons, respectively. In the COSY (H–H) spectrum, the doublet at δ 0.84 showed a cross peak with the signal at δ 1.40 (1H, *m*) which corresponds to H-30, whereas the triplet at δ 0.83 showed a cross peak with the resonance at δ 0.86/1.29 (2H, *m*) which corresponds to H_2-31. H-30 had a cross peak with the resonance at δ 3.90 (*dd*, $J_{7,6}=9.0$; $J_{7,30}=4.4$ Hz), which corresponds to H-7. This spin-system confirms isoleucine as the α-amino acid of the ring. *β*-Phenylserine, which is the hydroxylated amino acid of the macrocyclic ring, was identified from a cross peak between H-3, resonating at δ 5.81 (1H, *br s*), and H-4, which resonated at δ 4.58 (1H, *d*, $J_{4,21}=9.2$ Hz). The latter also exhibited a cross peak with H-21, which resonated at δ 8.03 (1H, *d*, $J_{21,4}=9.2$ Hz).





The C-24 methylene protons were observed as two double doublets at δ 2.38 ($J_{24',23}=5.6$; $J_{24,24'}=8.4$ Hz) and 2.52 ($J_{24,\ 23}=13.6$; $J_{24',\ 24}=8.4$ Hz), whereas the C-23 methine proton appeared as a double doublet at δ 3.02 ($J_{23,24}=5.6$ Hz; $J_{23,24'}=13.6$ Hz). In the COSY spectrum, H_2-24 and H-23 showed internal cross-peaks. In addition, a singlet at δ 2.08 (3H) was assigned to the protons of the *N*-methyl group (H-29). This spin system confirms *N*-methyl phenylalanine as the side-chain amino acid. The C-10 methine proton showed a double doublet at δ 6.03 ($J_{10,9}=9.0$; $J_{10,11}=7.6$ Hz), due to coupling with C-11 and NH-9, while the C-11 methine proton was observed at δ 6.98 ($J_{11,12}=7.6$ Hz). NH-9 was difficult to assign, due to the superimposition of this resonance with the aromatic protons. The ^{1}H NMR assignments of **1**, along with the proton coupling constants, are summarized in Table 1.

The ^{13}C NMR spectrum (100.6 MHz, $CDCl_3$) provided strong support for the proposed structure. The ^{13}C NMR chemical shifts of **1** (Table 1) were assigned from the analysis of DEPT spectra and 2D heteronuclear correlated spectra (HMQC and HMBC), together with a previous assignment for known cyclopeptide alkaloids (Morel et al., 1998, 1999).

The absolute stereochemistry of the C-7 amino acid (isoleucine) and of the *N*-methyl phenylalanine side-chain of **1** was determined by chiral phase gas chromatography (CPGC) using 3-pentyl-2,6-dimethyl-β-cyclodextrine (König et al., 1990) as stationary phase. The *N*-trifluoroacetylated methyl ester of the *D*, *L*-mixture and the pure *L*-form were used as CPGC standards. By comparing the R_ts of these standards with those of the corresponding amino acid from the hydrolysate of dihydrocondaline-A, it was possible to assign the absolute configuration unambiguously. In condaline-A, *N*-methyl phenylalanine and isoleucine both have the *L*(*S*)-configurations. The relative stereochemistry of the *β*-phenylserine unit (not found in the hydrolyzed alkaloid) was determined by analysis of ^{1}H NMR coupling constants and NOESY interactions (see Fig. 1). The H-3 proton was observed at δ 5.81 (1H, *br s*) and showed a COSY cross peak with H-4 which resonated at δ 4.58 (1H, *d*, $J_{4,21}=9.2$ Hz). The vicinal coupling constant of near zero of the methine protons of the *β*-phenylserine (H-3/H-4) indicates a *threo*-configuration (ϕ ca. 90°) for this residue (Morel et al., 1998). The coupling constant between H-3 and H-4 in the *erythro* configuration was ca. 14 Hz (ϕ ca. 180°) (Morel et al., 1998). The NOESY spectrum exhibited NOE cross peaks between H-3 and H-4. In turn, H-4 exhibits a cross peak with NH-6, while the latter did not show a cross peak with H-7. This evidence suggests that the β-phenylserine of condaline-A has an *L-threo* (3*R**/4*S**) configuration. The ^{1}H and ^{13}C spectral data and optical rotation of

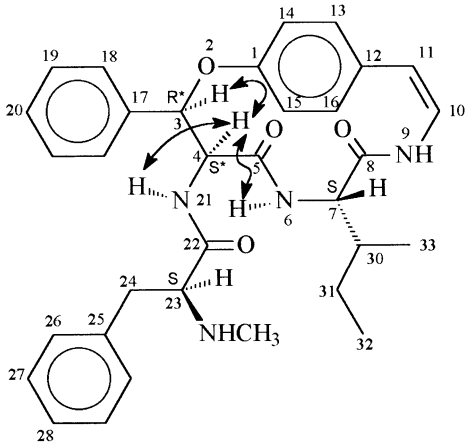


Fig. 1. Structure and selected NOESY correlations in condaline-A (**1**).

Table 1
^{1}H and ^{13}C NMR spectral data for condaline-A (**1**) (in DMSO-d_6, 400/100 MHz)[a]

H/C	δ ^{1}H (J, Hz)	δ ^{13}C ppm	HMBC correlations	
			$^{2}J_{CH}$	$^{3}J_{CH}$
01	–	158.6	–	–
03	5.81(*br s*)	86.3	C-17	C-1
04	4.58 (*d*) $J_{4,21}=9.2$	55.3	C-5	–
05	–	167.0	–	H-7, H-1
06	8.03 (*d*) $J_{6,7}=9.0$	–	–	–
07	3.90 (*dd*) $J_{7,6}=9.0$; $J_{7,30}=4.4$	56.8	C-8	C-5
08	–	169.2	–	–
09	–	–	–	–
10	6.03 (*d*) $J_{10,11}=7.6$; $J_{10,9}=9.0$	131.4	C-11	C-12
11	6.98 (*d*) $J_{11,10}=7.6$	126.9	C-10, C-12	–
12	–	132.4	–	–
13	6.99	129.4	–	C-1
14	6.93	124.4	–	C-12
15	7.15	119.8	–	C-12
16	6.81	121.7	–	C-1
17	–	139.7	–	-
18	7.58	126.0	C-19, C-20	C-3
19	7.36	127.6	C-18	C-17
20	7.25	127.0	–	C-18
21	8.03 (*d*) $J_{21,4}=9.2$	–	–	C-5
22	–	176.6	–	–
23	3.02(*dd*) $J_{23,24}=5.6$; $J_{23,24'}=13.6$	65.5	C-22, C-24	C-25
24	2.52 (*dd*) $J_{24,23}=13.6$; $J_{24,24'}=8.4$	39.2	C-23, C-25	C-22, C-26
	2.38 (*dd*) $J_{24',23}=5.6$; $J_{24,24'}=8.4$	–	–	–
25		138.2	–	–
26	7.0–7.5	128.0	–	C–24
27		129.9	–	C–25
28			–	–
29	2.08 (*s*)	37.6	–	C–23
30	1.40 (*m*)	34.2	–	–
31	1.29/0.86 9 (*m*)	24.4	–	–
32	0.83 (*t*) $J_{32,31}=6.5$	15.2	–	C–30
33	0.84 (*d*) $J_{33,30}=6.5$	10.8	C–30	C–7, C–31

[a] Assignments were obtained by 2D ^{1}H–^{1}H COSY, NOESY, DEPT 135° and 2D ^{1}H–^{13}C COSY (HMQC, HMBC) experiments.

condaline-A suggest that it is a stereoisomer of aralioline-B, isolated as the minor alkaloid from the leaves of *Araliorhamnus vaginatus* (Tschesche et al., 1970).

Adouetine-Y′ (**2**), scutianine-B (**3**), and scutianine-C (**4**) were identified by direct comparison (TLC) with authentic samples and by comparison of their NMR spectral data with analogous alkaloids (Haslinger, 1978; Pais et al., 1979; Morel et al., 1997, 1999). This is the first isolation of these alkaloids from this plant.

The antibacterial activity of the methanol extract and of alkaloids **1**–**4** was evaluated by means of direct bioautography using a TLC bioassay (Rahalison et al., 1991), against standard bacterial strains *S. aureus*, *S. epidermidis*, *M. luteus*, *K. pneumoniae*, *S. setubal*, and *E. coli*. The methanol extract showed modest activity (>100 μg), while condaline-A (**1**) and scutianine-B (**3**) were much more active (12.5–3.12 μg) against the strains, than the methanol extract. Alkaloids **2** and **4** were found to be inactive against these bacteria. Detection limits of alkaloids **1** and **3** are shown in Table 2, and the highest sample amount tested was 50 μg. Amoxicillin (0.5 μg) was used as a control.

3. Experimental

3.1. General

Mps are uncorr. IR spectra were recorded in KBr pellets on a Brüker IFS 28 FT spectrophotometer. LSIMS MS were obtained on a Quattro LC instrument from Micromass (UK) equipped with a mega flow electrospray probe. Optical rotations were taken on a Perkin Elmer 341 digital polarimeter. ^{1}H and ^{13}C NMR spectra were recorded at 400 and 100.6 MHz, on a Brüker DPX-400 spectrometer. Chemical shifts are given as δ (ppm) using TMS as an internal standard. Chiral GLC analyses were carried out with a Varian 3800 Gas

Table 2
Bioautography assay results of condaline-A (**1**) and scutianine-B (**3**), against bacteria[a]

Micro-organisms[b]		Antibacterial activity[c] (μg)	
		1	**3**
Staphylococcus aureus	ATCC 6538p	12.5	3.12
Staphylococcus efidermidis	ATCC 12228	3.12	6.25
Micrococcus luteus	ATCC 9341	12.5	12.5
Klebsiella pneumoniae	ATCC 10031	3.12	6.25
Salmonella setubal	ATCC 19196	6.25	6.25
Escherichia coli	ATCC 11103	6.25	6.25

[a] Positive control: amoxicillin (0.5 μg).
[b] ATCC (American type culture collection).
[c] Minimum amount required for inhibition of bacterial growth on TLC plates.

Chromatograph with a flame ionization detector using an 0.25 i.d. fused silica capillary coated with 3-Pe-2,6-Me-β-CD (König et al., 1990), diluted with the polysiloxane OV 1701. Thin layer chromatography was performed on pre coated TLC plates (Merk, silica 60 F-254).

3.2. Plant material

Condalia buxifolia was collected in December, 1998 in a suburb of Piratini, State of Rio Grande do Sul, Brazil. Nelci R. Bastos identified the plant, and a voucher specimen (3296) is deposited in the Herbarium SMDB of the University of Santa Maria.

3.3. Extraction and isolation

Dried ground root bark of *C. buxifolia* (920 g) was exhaustively extracted with MeOH in a Soxhlet apparatus for 12 h. The resulting MeOH extract was filtered and concentrated in vacuum to obtain a crude residue (181 g). This residue was dissolved in H_2O (50 ml) and acidified to pH 2–3. The acidic solution was exhaustively extracted with Et_2O (5×50 ml) to yield the acidic ether extract (10 g). The aqueous solution was then made basic (pH 8–9) to yield the basic ether extract (2.5 g), mainly consisting of one alkaloid. A portion of the basic ether extract (1.0 g) was applied to a silica gel column (80 g) and eluted with $CHCl_3$ containing increasing amounts of MeOH (up to 20%) to give 10 fractions. Fractions 2–3 ($CHCl_3$:MeOH, 99:1) were combined (20 mg) and submitted to preparative TLC ($CHCl_3$:MeOH, 99:1, two elutions) to yield **2** (12 mg). Fractions 4–5 ($CHCl_3$:MeOH, 99:2) were recombined and concentrated in vacuo to give a yellow solid material (50 mg) which was applied to a silica gel column (2.0 g) eluted with $CHCl_3$ containing increasing amounts of MeOH (up to 5%) to give **3** (15 mg), and **4** (10 mg). Fractions 7–8 ($CHCl_3$:MeOH, 95:5), consisting of one alkaloid, were combined and concentrated in vacuo to give **1** (850 mg).

3.4. Condaline-A (*1*)

Needles from $CHCl_3$–MeOH, mp 115–116 °C; $[\alpha]_D^{25}$: −73° (*c* 0.08, MeOH); IR ν_{max} cm^{-1}: 3400–3200 (NH), 2800 (C–O-arylether), and 1685–1635 (C=O); LSIMS (positive) *m*/*z*: 555 [$C_{33}H_{38}N_4O_4$]; (Found: C, 71.38; H, 6.89; N,10.06. Calcd. for $C_{33}H_{38}N_4O_4$: C, 71.46; H, 6.91; N,10.10); ^1H and ^{13}C NMR spectral data: see Table 1.

3.5. Dihydrocondaline-A

The hydrogenation of condaline-A (10 mg), under the conditions described for peptide alkaloids (Morel et al., 1979) (MeOH, 5 ml; 10% Pd/C, 5 mg; 15 h; 1 atm) yielded the corresponding dihydroalkaloid (ca. 8 mg). Mp: 236–238 °C. IR ν_{max} cm^{-1}: 3400–3200 (NH), 2805 (C–O-arylether), and 1680–1630 (C=O). ^1H NMR (DMSO-d_6): δ 0.63 (3H, *d*, J=6.8 Hz, CH_3-33), 0.73 (3H, *t*, J=6.8 Hz, CH_3-32), 1.29 (2H, *m*, H-31), 1.31 (1H, *m*, H-30), 2.09 (3H, *s*, CH_3-29), 2.40/2.83 (2H, *m*, H-24), 2.71 (2H, *m*, H-11), 3.63 (1H, *dd*, J=10.0; 5.0 Hz, H-7), 3.89 (2H, *m*, H-10), 4.57 (1H, *d*, J=9.0 Hz, H-4), 5.75 (1H, *br s*, H-3), 6.75 (1H, *dd*, J=8.4; 2.2 Hz, H-16), 6.85 (1H, *dd*, J=8.0; 2.2 Hz, H-14), 6.95 (1H, *dd*, J=8.0; 2.2 Hz, H-13), 7.05 (1H, *dd*, J=8.4; 2.2, H-15), 6.50–7.60 (10H, superimposition of aromatic hydrogens of β-phenylserine and *N*-methyl phenylalanine), 7.11 (1H, *m*, H-9), 7.90 (1H, *d*, J=10.0 Hz, H-6), 8.12 (1H, *br*, H-21); ^{13}C (DMSO-d_6): δ 11.1 (C-33), 15.2 (C-32), 24.4 (C-31), 34.0 (C-30), 34.2 (C-11), 34.4 (C-24), 38.7 (C-29), 39.3 (C-10), 56.3 (C-4), 56.9 (C-7), 68.1 (C-23), 85.5 (C-3), 119.0 (C-16), 120.9 (C-14), 130.0 (C-15), 131.3 (C-13), 133.6 (C-12), 139.6 (C-17), 157.8 (C-1), 166.6 (C-8), 169.8 (C-5), 169.9 (C-22), 120.0–129.0 (10 unidentified carbons).

3.6. Hydrolysis

Hydrolysis of dihydrocondaline-A (5 mg) was performed in a sealed tube at 110° with 6 N HCl for 12 h,

the aq. acidic solvent was concentrated and the residue was used to identify the absolute stereochemistry of isoleucine (C-7 aminoacid) and of the *N*-methylphenylalanine side-chain as previously described (Silva et al., 1996).

3.7. N-Methyl amino acids

The methyl amino acids were prepared by methylation of the corresponding *N*-benzyloxycarbonyl derivatives (MacDermott and Benoiton, 1973). Z-*D,L*-Phe and Z-*L*-Phe (596 mg; 2 mmol) and methyl iodide (1 ml, 16 mmol) were dissolved in THF (10 ml) and the solution were cooled to 0 °C. A sodium hydride dispersion (264 mg; 6 mmol) was added cautiously with gentle stirring. The suspension was stirred at room temp. for 24 h, and the product was isolated by the general procedure of McDermott and Benoiton (1973). Finally, the benzyloxycarbonyl group was removed by catalytic hydrogenation in the presence of palladium on carbon, yielded 469.5 mg (ca. 75%) of *D,L*-MePhe and 407.0 mg (ca. 60%) of *L*-MePhe.

3.8. Amino acid derivatization

Acid catalyzed esterifications were carried out by addition of a 1.6 N anhydrous solution of HCl (gas) in MeOH and leaving the mixture at room temp. for 30 min (Bayer and König, 1969). After removal of the reagents in a stream of dry nitrogen, the samples were taken up in CH_2Cl_2 (200 μl) and trifluoroacetic anhydride (50 μl), the mixture was allowed to stand at room temp. for 30 min, and the reagent was removed in a stream of dry nitrogen.

3.9. GC analysis of N-methyl phenylalanine and isoleucine

The derivatized amino acids were analyzed by enantioselective capillary CPGC, employing modified cyclodextrin as the chiral stationary phase and by coinjection with authentic *L*- and *D,L*-amino acids (Silva et al., 1996). The absolute stereochemistry of *N*-methyl phenylalanine and of the ring bonded α-amino acid (isoleucine) was unambiguously established. In condaline-A (1), *N*-methyl phenylalanine and isoleucine have an *L* (*S*) configuration.

3.10. Antimicrobial activity

The antibacterial activity of alkaloids **1**–**4** against three Gram-positive bacteria: *Staphylococcus aureus* (ATCC 6538p), *Staphylococcus epidermidis* (ATCC 12228), *Micrococcus luteus* (ATCC 9341), and the three gram-negative bacteria: *Klebsiela pneumoniae* (ATCC 10031), *Salmonella setubal* (ATCC 19196) and *Escherichia coli* (ATCC 11103), were evaluated using a bioautography technique (Rahalison et al., 1991). Methanolic crude extract of the root bark of *Condalia buxifolia* (10 μl of solution corresponding to 100 μg) was used as reference crude plant extract. The microorganisms used in the antibacterial assay are maintained at the Departamento de Química of the Universidade Federal de Santa Maria, RS, Brazil. For the antimicrobial assay of **1**–**4**, samples of 50.0, 25.0, 12.5, 6.25, 3.12, 1.06 μg was applied to pre-coated TLC plates. TLC plates were developed with $CHCl_3$:MeOH (95:5) and dried for complete removal of solvents. The inoculum was prepared by culturing each organism in tryptone soya broth (TSb, Oxoid) at 37 °C to a turbidity equivalent to McFarland 0.5 standard (1.5×10^8 CFU/ml). One microliter of each diluted inoculum (10^4–10^6 CFU/ml) was added in 10 ml of Mueller–Hinton Agar medium (MHA-DIFCO), and distributed over developed TLC plates (5×5 cm). After solidification of the media, the TLC plates were incubated overnight at 37 °C (Saxena et al., 1995). Subsequently, bioautograms were stained with an aqueous solution of 2,3,5-triphenyltetrazolium chloride (TCC, 5 mg/ml). Amoxicillin (0.5 μg) was used as positive control.

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