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Cyclopeptide alkaloids from *Scutia buxifolia* Reiss and their antimicrobial activity

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Abstract

The present study reports a cyclopeptide alkaloid, scutianine M, isolated from the methanolic root bark extract of *Scutia buxifolia* Reiss (Rhamnaceae) along with six known compounds, scutianines-B, -C, -D, -E, -F, and scutianene D. Its structure was established on the basis of spectroscopic analyses, including application of 2D NMR spectroscopic techniques. As part of a study of the bioactive compounds of medicinal plants from southern Brazil, we also compared the antimicrobial activity of the isolated compounds towards Gram (+), Gram (−) bacteria, and yeasts.

Keywords: *Scutia buxifolia*; Rhamnaceae; Cyclopeptide alkaloids; Antimicrobial activity

1. Introduction

As part of our ongoing search for new antimicrobial compounds from Brazilian plants, we have studied cyclopeptide alkaloids of plants belonging to the Rhamnaceae family collected in Rio Grande do Sul, South Brazil. In previous investigations, we examined the root bark of *Scutia buxifolia* (Menezes et al., 1995; Morel et al., 1979, 1998), *Discaria americana* [=*Discaria longispina*] (Morel et al., 1995; Giacomelli et al., 2001, 2004), and *Condalia buxifolia* (Morel et al., 2002). These studies resulted in the isolation of various cyclopeptide alkaloids. In the present paper, we describe the isolation and structural elucidation of a new cyclopeptide alkaloid, scutianine M (**1**) together with 6 known compounds, scutianines-B (**2**), -C (**3**), -D (**4**), -E (**5**), -F (**6**), and scutianene D (**7**) [=scutianene C (Sierra et al., 1974; Merkuza et al., 1974; Morel et al., 1979)] from some new *Scutia buxifolia* material collected at Sao Sepé, midwest Rio Grande do Sul, in 2003. Because antimicrobial activity is one of several interesting biological activities attributed to cyclopeptide alkaloids (Gournelis et al., 1997), the present paper deals with the relation between structure and antimicrobial activity of this class of compounds. For this purpose, the structures scutianine D (**2**), a diastereoisomer from scutianine E (**6**), the new alkaloid scutianine M (**1**), a diastereoisomer from condaline A (**8**) previously isolated from *Condalia buxifolia* (Morel et al., 2002), scutianines-B (**2**), -C (**3**), -F (**6**), and the neutral compound scutianene D (**7**) were selected for investigation. Scutianine D (**4**) differs from scutianine E (**5**) by the configuration at C-3, C-4, C-7 and C-30, while scutianine M (**1**) differs from condaline A (**8**) only by the configuration at C-3.

2. Results and discussion

Scutianine M (**1**), m.p. 257–259 °C, showed an LSIMS-mass $[M + H]^+$ at m/z 555, which in combination with ^{13}C NMR spectroscopy and elemental analysis, indicated the molecular formula $C_{33}H_{38}N_4O_4$. The IR spectrum exhibited bands corresponding to peptide linkages.

The ^1H NMR spectrum ($CDCl_3$, 400MHz) of **1** showed only two methyl groups: a methyl triplet at δ 0.83 and a

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methyl doublet at δ 0.70, which were assigned to the C-32 and C-33 methyl protons, respectively. In the COSY (H–H) spectrum, the doublet at δ 0.70 showed a cross-peak with the signal at δ 2.18 (1H, m), which corresponds to H-30, whereas the triplet at δ 0.83 showed a cross peak with the resonances at δ 1.0/1.24 (2H, m), which correspond to H_2-31. The cross-peak of H-30 with the resonance at δ 4.08 was assigned to the C-7 methine proton. This spin-system confirmed isoleucine as the α-amino acid of the ring. The C-3 and C-4 methine protons appeared as a doublet at δ 6.16 and as a double doublet at δ 4.70, respectively. In the COSY spectrum, a cross-peak between H-3 and H-4, and of the latter with H-21 at δ 7.40, confirmed β-phenylserine as the hydroxylated amino acid of the macrocyclic ring.

The C-24 methylene protons were observed as two double doublets at δ 1.58 and 2.78, whereas the C-23 methine proton appeared as a double doublet at δ 2.90. In the COSY spectrum, H_2-24 and H-23 showed internal cross-peaks. In addition, in the HMBC experiment, a singlet at δ 1.90 (3H), assigned to the protons of the *N*-methyl group (H_3-29), showed a cross-peak with C-23. This spin system confirmed *N*-methyl phenylalanine as the side-chain amino acid. The ^{1}H NMR spectroscopic assignments and the proton coupling constants of **1** are summarized in Table 1.

The ^{13}C NMR spectrum (100.6 MHz, $CDCl_3$) provided strong support for the structure proposed. 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 of known cyclopeptide alkaloids (Morel et al., 1998, 1999, 2002).

Comparison of ^{1}H NMR spectroscopic data of scutianine M (**1**) and condaline A (**8**) revealed only small differences in chemical shifts and coupling constants, except for the hydrogen attached to C-3 of the phenylserine moiety. Other distinctive signals can be found in the ^{13}C NMR spectra of these alkaloids. The most striking difference is the chemical shift of C-3, which is deshielded ca. 5 ppm in scutianine M (δ 81.4 ppm) as compared to condaline A (δ 86.3 ppm).

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) with 3-pentyl-2,6-dimethyl-β-cyclodextrin (Konig et al., 1990) as the stationary phase. In scutianine M (**1**), both *N*-methyl phenylalanine and isoleucine 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 figure below). The vicinal coupling constant of ca. 8.0 Hz of the methine protons of β-phenylserine (H-3/H-4) indicates an *erythro* relative configuration for this residue (Morel et al., 1979). In addition, scutianine M shows a signal in ^{13}C NMR spectra at δ 81.4 ppm for C-3, which confirms that the β-phenylserine moiety possesses the *L-erythro* (*3S*, *4S*) configuration. H-3 did not show a cross-peak with H-4 in the NOESY spectrum of **1**. In turn, H-4 exhibited a cross-peak with NH-6, while the latter did not show a cross-peak with H-7. This evidence is consistent with the *L-erythro* (*3S*/*4S*) configuration of the β-phenylserine of scutianine M.

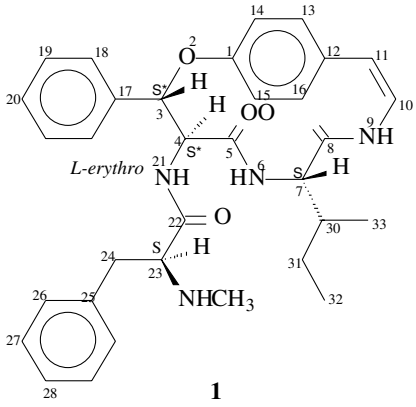
Table 1
^{1}H and ^{13}C NMR spectroscopic data for Scutianine-M (**1**) (in DMSO-d_6, 400/100 MHz)[a]

H/C	δ^1H (*J*, Hz)	δ^{13}C ppm	HMBC correlations	
			$^2J_{CH}$	$^3J_{CH}$
01	–	155.21	–	–
03	6.16; $J_{3,4} = 7.7$	81.4		C-5
04	4.70 (*dd*); $J_{4,3} = 7.7$, $J_{4,21} = 9.0$	56.39	C-3, C-5	–
05	–	171.39	–	–
06	6.36 (*d*); $J_{6,7} = 8.2$	–	–	–
07	4.08 (*dd*) $J_{7,6} = 8.2$; $J_{7,30} = 3.8$	59.52	C-8	C-5
08	–	167.08	–	–
09	6.60 (*d*); $J_{9,10} = 10.0$	–	–	–
10	6.72 (*d*); $J_{10,11} = 7.0$, $J_{10,9} = 10.0$	125.54	C-11	–
11	6.41 (*d*) $J_{11,10} = 7.0$	116.20	C-10	–
12				
13				
14				
15				
16	7.00–7.60			
17				
18				
19				
20				
21	7.40 (signal overlapped)	–	–	–
22	–	173.24	–	
23	2.90 (*dd*); $J_{23,24} = 9.4$, $J_{23,24'} = 3.6$	65.82	–	C-29
24	2.78 (*dd*), $J_{24,23} = 9.4$; $J_{24,24'} = 14.0$	37.96	C-23	–
	1.58 (*dd*), $J_{24',23} = 3.6$, $J_{24',24} = 14.0$	–	–	–
25		–	–	–
26	7.0–7.6	–	–	–
27		–	–	–
28		–	–	–
29	1.90 (*s*)	35.28	–	–
30	2.18 (*m*)	35.03	–	–
31	1.24/1.0 (*m*)	23.80	–	C-33
32	0.83 (*t*) $J_{32,31} = 7.0$	15.24	C-31	C-30
33	0.70 (*d*) $J_{33,30} = 7.0$	12.02	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.

The ^{1}H and ^{13}C spectroscopic data and the optical rotation of scutianine M (**1**) suggests that it is a stereoisomer of condaline A, isolated as the major alkaloid of *Condalia buxifolia* (Morel et al., 2002), and of aralioline-B, isolated as the minor alkaloid from the leaves of *Araliorhamnus vaginatus* (Tscheche et al., 1970).

Scutianines-B (**2**), -D (**4**), -E (**5**), -F (**6**) and scutianene D (**7**) were identified by direct comparison (TLC) with authentic samples and by comparison of their NMR spectroscopic data with those of analogous alkaloids (Haslinger, 1978; Pais et al., 1979; Morel et al., 1995, 1999, 2002). Condaline



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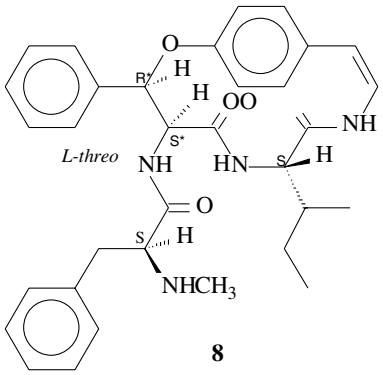

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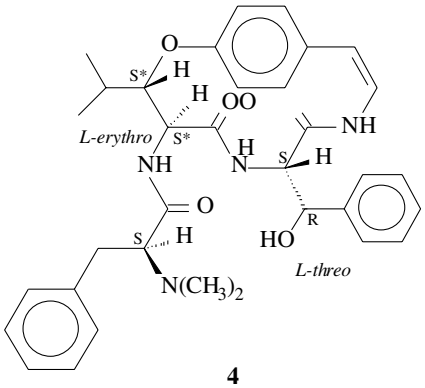

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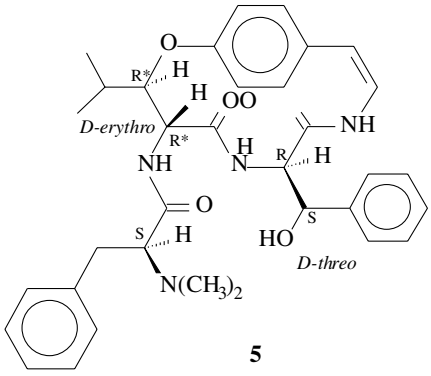

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A (Morel et al., 2002) was used for the sake of comparison with the antimicrobial activity of the diastereomeric alkaloid scutianine M extracted in this work.

The antimicrobial activities of compounds **1**–**8** were evaluated by direct bioautography using a TLC bioassay (Rahalison et al., 1991) against standard bacterial strains *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Micrococcus luteus* (Gram positive) *Klebsiella pneumoniae*, *Salmonella setubal, Escherichia coli* (Gram negative) and two yeasts, *Saccharomyces cerevisae* and *Candida albicans*. Table 2 summarizes the antimicrobial activity of the tested compounds. From this, the compound with the widest activity spectrum was condaline A (**8**), followed by scutianine E (**5**). It was observed that scutianine D (**4**), a diastereoisomer of **5**, showed only modest antibacterial activity against *M. luteus*, *S. epidermidis*, and *E. coli*, while scutianine M (**1**), a diastereoisomer of **8**, was inactive against all strains. Scutianine B (**2**) was only active against *E. coli* (12.5 μg), while scutianines C (**3**) and F (**6**) and scutianene D (**7**) were inactive against all strains tested. This finding suggests that the stereochemistry and the presence of the β-phenylserine residue in the structure of the alkaloids have an influence on bioactivity. Furthermore the importance of the *N*,*N*-dimethyl (or *N*-methyl) group in the structures was demonstrated by the total lack of activity of scutianene D (**7**) when compared to scutianine D (**4**). None of the tested compounds showed antifungal activity against the largest sample amount tested (100 μg) of *S. cerevisiae* and *C. albicans*.

3. Experimental

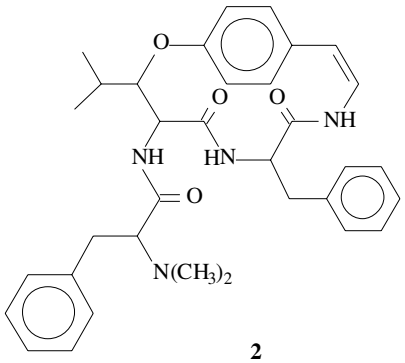
3.1. General

Melting points were determined with an "MQAPF-301" apparatus and are uncorrected. Optical rotations were

Table 2
Bioautography assay results (μg) of compounds **2**, **4**, **5** and **8**

Microorganisms		**2**	**4**	**5**	**8**	Control (μg)[a]
Staphylococcus aureus	ATCC 6538p	–	–	25.0	12.5	0.7
Staphylococcus epidermidis	ATCC 12228	–	50.0	6.25	3.12	0.7
Micrococcus luteus	*ATCC* 9341		25.0	6.25	12.5	0.7
Salmonella setubal	ATCC 19796	–	–	–	6.25	0.7
Escherichia coli	ATCC 25792	6.25	50.0	6.25	6.25	0.5
Klebsiella pneumoniae	ATCC 10031	–	–	12.5	3.12	0.5

[a] Standard antibiotic chloramphenicol.



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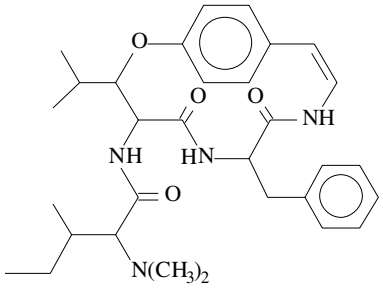

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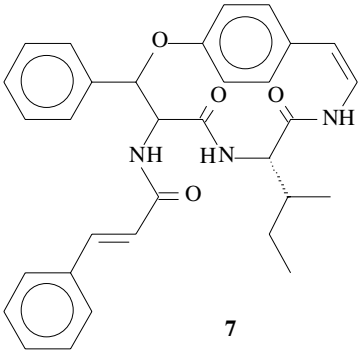

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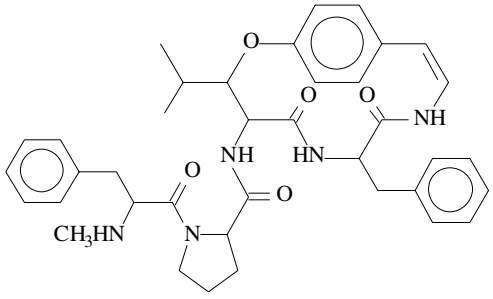

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taken on a Perkin Elmer 341 digital polarimeter. High-resolution ESI mass spectra were recorded on a Bruker Bio Apex 70eV FT-ICR (Bruker Daldonis, USA) instrument. Low resolution MS were recorded on a Varian 3800 operating in the ionization potential mode at 70 eV. ^{1}H- and ^{13}C NMR spectra were recorded at 400.1/100.6 MHz on a Bruker DPX-400 spectrometer with $CDCl_3$ as solvent and TMS as an internal standard. Thin layer chromatography was performed on pre-coated TLC plates (Merk, silica 60 F-254) sprayed with Dragendorff's reagent and 10% H_2SO_4, and followed by heating.

3.2. Plant material

The root bark of *S. buxifolia* was collected in January 2003 at São Sepé, RS, Brazil (29°45′30″S, 54°20′33″W) and authenticated by Prof. Adelino Alvarez Filho from the Botany Department of Universidade Federal de Santa Maria, RS, Brazil, and where a specimen samples (SMD-B146) is retained.

3.3. Extraction and isolation

Dried ground bark (4.0 kg) of *S. buxifolia* was exhaustively extracted with methanol at room temperature. The resulting MeOH extract was concentrated in vacuo to obtain a crude residue (400 g). This extract was dissolved in H_2O (250 mL) and acidified with 2 N HCl to pH 2–3. After exhaustive extraction with Et_2O, the acidic solution was made basic with NH_4OH to pH 8–9 and extracted with Et_2O to yield the basic ether extract (4.0 g). The alkaloid mixture (1 g) was fractionated on a SiO_2 chromatographic column (90 g) eluted with $CHCl_3$ containing increasing amounts of MeOH (up to 30%) to give 20 fractions of 100 mL each. Fractions 4–5 ($CHCl_3$:MeOH 99:1) were combined (45 mg) and submitted to preparative TLC ($CHCl_3$:MeOH 99:1) to yield **1** (18 mg) and **7** (23 mg). Fractions 8–9 ($CHCl_3$:MeOH 98:2) were combined (150 mg) and submitted to preparative TLC ($CHCl_3$:MeOH 98:2, two elutions) to yield **2** (70 mg) and **3** (55 mg). Fraction 11 ($CHCl_3$:MeOH 98:2) consisted of one alkaloid and was concentrated in vacuo to give **6** (70 mg). Fractions 12–14 ($CHCl_3$:MeOH 97:2) were combined (150 mg) and resubmitted to a silica gel column (15 g) eluted with $CHCl_3$ containing increasing amounts of MeOH (up to 4%) to give **4** (80 mg) and **5** (20 mg).

3.4. Scutianine M (*1*)

White powder; m.p. 257–259 °C. TLC 0.70 ($CHCl_3$:MeOH 97:3), $[\alpha]_D^{25} = +120^\circ$ (*c* 0.018, $CHCl_3$); $IR_{\nu max}$ cm^{-1}: 3400–3200 (NH) and 1680–1640 (C=O); LSIMS (positive) *m*/*z*: 555; (Found: C, 71.42; H, 6.90; N, 10.09. Calc. for $C_{33}H_{38}N_4O_4$: C, 71.46; H, 6.91; N, 10.10. For ^{1}H NMR and ^{13}C NMR spectroscopic data, see Table 1.

3.5. Dihydroscutianine M

The hydrogenation of **1** (10 mg) under the conditions described for peptide alkaloids (Morel et al., 1979), [MeOH (5 mL), 10% Pd/C (6 mg), 15 h at 1 atm] yielded the corresponding dihydroalkaloid (ca.7 mg). M.p.: decomposition

at 280 °C. ^{1}H NMR (DMSO-d_6): δ 0.64 (3H, *d*, $j = 7Hz$, C-33), 0.70 (3H, *t*, $j = 7$ Hz, C-32), 1.31 (3H, *m*, C-30), 2.07 (3H, *s*, C-29), 2.30–2.80 (4H, *m*, C-11/C-24), 3.53 (1H, *dd*, $j = 8.5$; 4.5 Hz, C-7), 3.85 (1H, *m*, C-10), 6.15 (1H, *d*, $j = 6.6$ Hz, C-3), 4.30 (1H, *dd*, $j = 6.6$; 8.0, C-4), 6.50–7.80 (14H, superimposition of aromatic hydrogens), 6.80 (1H, *m*, NH-9), 6.50 (1H, *d*, $J = 8.5$ Hz, NH-6), 8.0 (1H, d, $J = 8.0$ Hz, NH-20).

3.6. Hydrolysis and amino acid derivatization

Hydrolysis of dihydroscutianine M (3 mg) was performed in a sealed tube at 110 °C with 6 N HCl for 14 1h as previously described (Silva et al., 1996). The acidic solution was concentrated and the residue was used to identify the absolute stereochemistry of isoleucine and *N*-methyphenylalanine. Acid-catalyzed esterification of the resulting amino acids (2 mg) was carried out by the addition of a 1.6 N anhydrous solution of HCl gas in MeOH for 30 min at room temperature (Bayer and Konig, 1969). After removal of the reagents under dry nitrogen stream, the sample was taken up in CH_2Cl_2 (200 μl) and trifluoroacetic anhydride (50 μl). The mixture was kept at room temperature for 30 min and the excess reagent was removed under dry nitrogen stream.

3.7. GC analysis of N-methyl phenylalanine and the ring-bonded amino acid isoleucine

The derivatized amino acids were analyzed by enantioselective capillary GC by employing modified cyclodextrin as chiral stationary phase and by coinjection with standard L- and D, L-amino acids (Bayer and Konig, 1969). The amino acid *N*-methyl phenylalanine was obtained as previously described (Mcdermott and Benoiton, 1973).

3.8. Antimicrobial activity

Antibacterial assays. The antibacterial activities of compounds **1**–**8** were assayed using the bioautography technique (Rahalison et al., 1991). The collection of eight microorganisms used included three Gram-positive bacteria: *Staphylococcus aureus* (ATCC 6538p), *Staphylococcus epidermidis* (ATCC 12228), *Micrococcus luteus* (ATCC 9341); three Gram-negative bacteria: *Klebsiella pneumoniae* (ATCC 10031), *Eschericchia coli* (ATCC 11103), and *Salmonella setubal* (ATCC 19196), and two yeasts: *Saccharomyces cerevisiase* (ATCC 2601) and *Candida albicans* (ATCC 10231). Standard microorganism strains were maintained at the Chemistry Department of the University of Santa Maria, RS, Brazil. For assay, the compounds were applied to pre-coated TLC plates in concentrations from 100 to 1.56 μg. Mueller–Hinton agar medium (MHA-Merck) was inoculated with microorganisms suspended in saline solution (10^5 CFU/mL) and distributed over TLC plates. Bacterium and yeast plates were incubated for 24 h at 37 °C and 72 h at 25 °C, respectively. Standard antibiotic chloramphenicol and nistatine were used to control the sensitivity of the microbial test. After incubation, the plates were stained with an aqueous solution of 2,3,5-triphenyl-tetrazolium chloride (TTC, 1 mg/mL). The appearance of inhibition zones was used to demonstrate the lesser sample amount that inhibited microorganism growth. Samples were tested in triplicate.

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