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Cyclic peptide alkaloids from the bark of *Discaria americana*

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In memory of Professor Emilia C. M. Dessoy who passed away in August 2003.

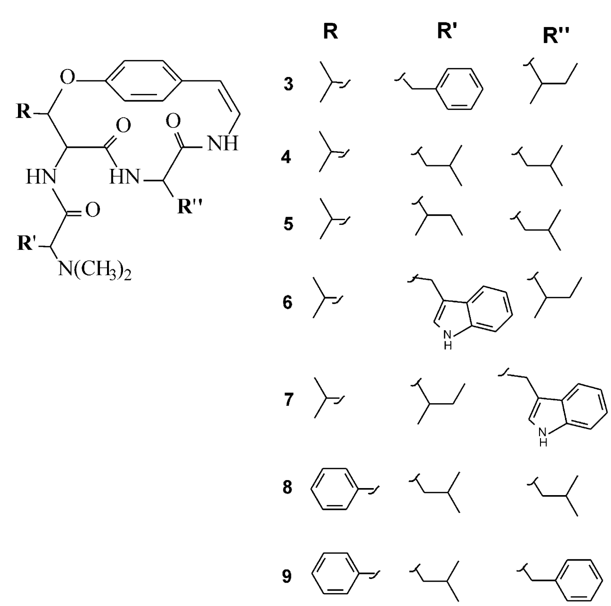
Abstract

The isolation and structure determination of cyclic peptide alkaloids, discarine-M and discarine-N, along with seven known cyclic peptide alkaloids, adouetine-Y′, franganine, frangulanine, discarines-A, -B, -C, and -D from the root bark of *Discaria americana* are described. Structures were determined spectroscopically, especially using 2D NMR spectroscopic analysis. The crude methanol extract, the basic ether extract, and the alkaloids **6** and **7** also weakly inhibited growth of gram-negative and gram-positive bacteria.

Keywords: *Discaria americana*; Rhamnaceae; Cyclic peptides; Antibacterial activity

1. Introduction

Plants of the genus *Discaria*, family Rhamnaceae, are widely used in Brazilian traditional medicine for the treatment of diabetes, skin disorders, stomach disorders, as a fever lowering agent, and as a tonic (Reissek and Martius, 1861; Bandoni et al., 1976; Correa, 1978; Davyt et al., 1991; Záchia and Moraes, 1999). Several cyclic peptide alkaloids have been isolated previously from plants of the genus *Discaria* (Mascaretti et al., 1972; Silva et al., 1974; Tscheche et al., 1980; Digel et al., 1983; Morel et al., 1984, 1985, 1995; Herzog et al., 1984a,b; Hennig et al., 1986; Machado et al., 1995). Recently, the isolation and structural elucidation of two new cyclic peptides from a 1997 collection of the root bark of *Discaria americana* was reported (Giacomelli et al., 2001). In continuation of this work, the isolation and structural elucidation of discarine-M (**1**) and discarine-N (**2**), together with seven known cyclic peptide alkaloids (**3**–**9**), is reported from plant material from a different location. Cyclic peptides **1** and **2** and alkaloids **5** and **6** were not found in the previously collected material. The antibacterial activities of the methanolic and basic ether extracts, of cyclic peptide alkaloids **1** and **2**, and of alkaloids **3**–**9** were determined by direct bioautography (Rahalison et al., 1991), against selected gram-positive and gram-negative bacteria.



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2. Results and discussion

Discarine-M (**1**) was obtained as a white powder. Its positive FABMS displayed a prominent $[M+H]^+$ at *m/z* 456, which, in combination with the ^{13}C NMR spectroscopic and elemental analysis data (found: C 68.27%, H 8.09%, N 9.47%; calc.: C 68.54%, H 8.19%, N 9.22%), suggested a molecular formula $C_{26}H_{37}N_3O_4$. The ^1H NMR spectrum (DMSO-d_6, 400 MHz) of **1** showed six methyl doublets (δ 0.49, 0.69, 0.82, 0.94, 0.96, and 1.13). In the ^1H–^1H COSY spectrum, the doublets at δ 0.69 ($J=6.5$ Hz, CH_3-29) and 0.49 ($J=6.5$ Hz, CH_3-30) showed cross-peaks with H-28 at δ 1.25 (1H, m), which in turn showed cross-peaks with the methylene hydrogens H_2-27 at δ 1.31/1.43 (m). These protons also had cross-peaks with the signal at δ 3.78 (1H, m, H-7), and this signal, with NH-6 at δ 6.72. This spin-system confirms leucine as the α-amino acid of the ring. β-Hydroxyleucine was identified as the hydroxylated amino acid of the macrocyclic ring from a cross-peak between the doublets at δ 0.82 (Me-18) and 1.13 (Me-19), with the signal at δ 2.08 (1H, m), which corresponds to H-17. In turn, H-17 showed a cross-peak with H-3 at δ 4.89, and this resonance correlated with H-4 at δ 4.55. The proton H-4 exhibits another cross-peak with NH-20 at δ 8.42. The side-chain unit $[(CH_3)_2CHCH{=}CH–]$ was characterized as follows: the doublets at δ 0.94 ($J=6.7$ Hz, CH_3-25) and 0.96 ($J=6.7$ Hz, CH_3-26) show cross-peaks with the signal at δ 2.35 (1H, m, H-24), and this latter resonance with the signal at δ 6.61 (1H, d, $J=6.8$; 14.0 Hz, H-23). In turn, H-23 displayed a cross-peak with H-22, resonating at δ 5.84 (1H, d, $J=14.0$ Hz). The protonated carbons of **1** were assigned from the HMQC experiment, and the quaternary carbons were assigned with the aid of analysis of the HMBC spectrum. The NMR spectral data are in agreement with related structures previously studied (Morel et al., 1998; Pais et al., 1979; Giacomelli et al., 2001).

Discarine-N (**2**) had the composition $C_{32}H_{33}N_3O_5$, as suggested by positive FABMS, elemental analysis data (found: C 71.02%, H 6.04%, N 7.95%; calc.: C 71.22%, H 6.16%, N 7.79%.), with the support of ^{13}C NMR (DEPT, HMQC, and HMBC) analysis. The ^1H NMR spectrum of **2** showed two sets of doublets, at δ 1.14 and 0.86, which were assigned to the C-18 and C-19 methyl protons, respectively. The C-3 and C-4 methine protons appeared at δ 4.13 and 4.21, respectively, suggesting β-hydroxyleucine as an amino acid in the ring. The C-22 olefinic proton appeared as a doublet at δ 5.96 ($J=15.8$ Hz), whereas the other olefinic proton at C-23 gave a doublet at δ 6.85 indicating the presence of a side-chain unit [–CH=CH–Ph]. β-Phenylserine, the ring-bonded α-amino acid, was identified from cross-peaks between NH-6, H-7, and H-28. The proton NH-6 resonance at δ 7.19 showed cross-peaks with H-7, at δ 4.01. This proton (H-7) also exhibited cross-peaks with H-28, at δ 4.23 (dd, $J=6.4$, 4.4 Hz). The styrylamine moiety, characterized by the occurrence of a double doublet at δ 6.85, was assigned to H-10. It showed cross-peaks with H-11 at δ 6.44 and with NH-9 at δ 6.96.

The absolute stereochemistry of the ring-bonded α-amino acids, leucine in **1** and β-phenylserine in **2**, were determined as described previously (Giacomelli et al., 2001). In discarine-M (**1**), leucine has the L (*S*)-configuration, whereas in discarine-N (**2**), β-phenylserine has the L-*threo* (7*S*/28*R*)-configuration.

The relative stereochemistry of the β-hydroxyleucine unit (not found in the hydrolysate) was deduced by analysis of the NOESY spectrum (see Figs. 1 and 2). Since the stereochemistry of C-7 is absolute, the spatial position of H-7 was starting point for the assignment of the relative stereochemistry of C-3 and C-4. In the NOESY spectrum of **1** and **2**, H-7 does not show a cross-peak with H-6. In its turn, H-6 exhibited a nOe cross-peak with H-4, and this did not show a cross peak with H-3. This indicates that H-7 and H-3 are in the same plane (β), while H-6 and H-4 are oriented on the same face of the plane (α). This evidence suggests that the β-hydroxyleucine unit of **1** and **2** has an L-*erythro* (3*S**/4*S**) configuration. These results and the optical

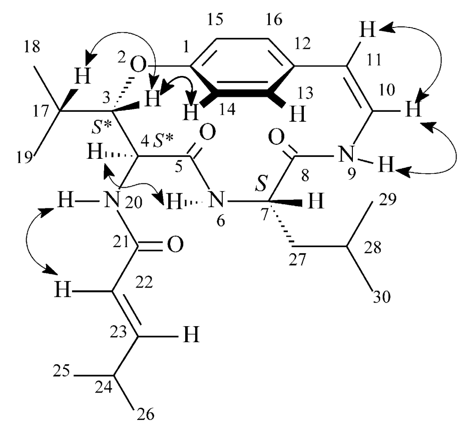

Fig. 1. Structure of discarine-M (**1**) and selected NOESY correlations.

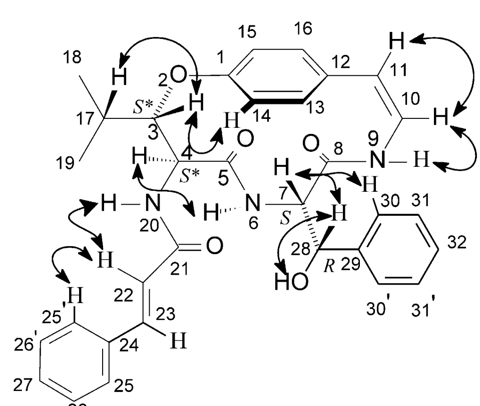

Fig. 2. Structure of discarine-N (**2**) and selected NOESY correlations.

Table 1
Bioautography assay results of MeOH extract, basic ether extract, discarine-A (**6**) and discarine-B (**7**), against bacteria

Test material (μg)[a]	Micro organism					
	S. aureus	*S. epidermidis*	*B. subtilis*	*K. pneumoniae*	*S. setubal*	*E. coli*
Discarine-A	NA	100	25	100	100	NA
Discarine-B	12.5	25	12.5	6.75	12.5	12.5
Basic ether extract	100	50	50	12.5	25	50
MeOH extract	125	31.2	62.5	31.2	62.5	31.2
Amoxicillin	0.16	0.32	0.48	0.32	0.48	0.48

NA: Not active.
[a] Minimum amount required for inhibition of bacterial growth on TLC plates.

rotation of **2** $\{[\alpha]_D^{20}$ +98.1° (*c* 0.11, MeOH:$CHCl_3$ 1:1)$\}$ suggest that it is a diastereoisomer of scutianene-C $\{[\alpha]_D^{20}$ +203° (*c* 0.12, MeOH:$CHCl_3$, 3:2)$\}$, isolated from the roots of *Scutia buxifolia* (Sierra et al., 1974).

Alkaloids **3–9** were determined to be adouetine-Y′ (**3**), franganine (**4**), frangulanine (**5**), discarines-A (**6**), -B (**7**), -C (**8**), and -D (**9**) by direct comparison with authentic samples, and based on their NMR spectral data (Pais et al., 1979).

The antibacterial activities of methanol and basic ether extracts, of cyclic peptide alkaloids **1** and **2**, and of alkaloids **3–9** was evaluated by means of direct bioautography using TLC biossay (Rahalison et al., 1991) against standard bacterial strains *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis*, *Klebsiela pneumoniae*, *Salmonella setubal*, and *Escherichia coli*. The methanol and basic ether extracts showed modest acivity against *S. aureus* (100–125 μg), but were much more active (62.5–12.5 μg) against the others strains. Discarine-A (**6**) also had modest activity (100 μg) against *S. epidermidis, S. setubal*, and *K. pneumoniae*, and moderate activity (25 μg) against *B. subtilis*. Discarine-B (**7**) was more active (25.0–6.75 μg) against all strains, than the methanol and basic ether extracts and discarine-A (**C**). Alkaloids **3–5**, **8**, and **9** were inactive against these bacteria. The detection limits are show in Table 1, and the highest sample amount tested was 200 μg for the extracts and 100 μg for compounds **1–9**.

3. Experimental

3.1. General

Melting points were determined in a MQAPF-301 melting point apparatus and are uncorrected. IR spectra were recorded in KBr pellets on a Brüker IFS 28 spectrophotometer. Optical rotations were taken on a Perkin-Elmer 341 digital polarimeter. FABMS were obtained on a VG Analytical 70-150-S mass spectrometer equipped with a FAB ion source from a 3-nitrobenzylalcohol matrix. NMR spectra were acquired on a Brüker DPX-400 operating at 400 and 100 MHz, for ^1H and ^{13}C, respectively. Chemical shifts are given in δ (ppm) using TMS as internal standard. A 25 m fused silica column with heptakis (2,6-*di-O*-methyl-*3-O*-pentyl)-β-cyclodextrin (König et al., 1990) diluted with polysiloxane OV 1701 (1:1 w/w), and a column temperature of 85 °C was used in a Varian 3800 gas chromatograph, equipped with FID. TLC was performed on precoated silica gel 60 F_{254} plates (Merck) and detection was achieved by UV light (254 nm), by spraying with Dragendorff's reagent, and by spraying with 10% H_2SO_4, followed by heating.

3.2. Plant material

The root bark of *D. americana* was collected in São Sepé, Brazil (29° 45′ 30″ S, 54° 20′ 33″ W), in December 2000 and authenticated by Prof. Renato Záchia, Department of Botany, Universidade Federal de Santa Maria, RS, Brazil, where a specimen sample (SMDB 2688) is retained.

3.3. Extraction and isolation

Dried ground bark (3.0 kg) of *D. americana* was extracted with MeOH in a Soxhlet apparatus for 12 h. The resulting MeOH extract was filtered and concentrated in vacuo to obtain a crude residue (950 g). This residue was dissolved in H_2O (300 mL) and acidified with 2 N HCl to pH 2–3. The acidic solution was exhaustively extracted with Et_2O (5x 300 ml) to yield the acidic ether extract (32 g). The aqueous solution was made basic with NH_4OH (pH 8–9) and extracted with Et_2O to yield the basic ether extract (6 g). A portion of the basic ether extract (3 g) was applied to silica gel column (250 g) which was eluted with $CHCl_3$ containing increasing amounts of MeOH (up to 20%) to give 10 fractions (A–J). Fractions A and B ($CHCl_3$:MeOH, 99:1) were combined (60 mg) and submitted to preparative TLC ($CHCl_3$:MeOH, 99:1, two elutions) to yield **1** (53 mg). Fractions C and D ($CHCl_3$:MeOH, 98:2) were combined (60 mg) and submitted to preparative TLC ($CHCl_3$:MeOH, 98:2, two elutions) to yield **2** (53 mg). Fraction E ($CHCl_3$:MeOH, 98:2) afforded a mixture (900 mg) of three alkaloids. Further chromatography of this residue on silica gel ($CHCl_3$:MeOH, 99:1–97:3) yielded **3** (85 mg), **4** (428 mg), and **5** (326 mg). Fraction F ($CHCl_3$:MeOH, 98:3), consisting of one alkaloid, was evaporated to give **6** (412 mg). Fractions G and H ($CHCl_3$:MeOH, 97:4) were combined and evaporated to give a yellow solid (830 mg) which was subjected to flash chromatography with $CHCl_3$ containing increasing amounts of MeOH (up to 5%) to furnish **7** (691 mg). Fraction J ($CHCl_3$:MeOH, 95:5) afforded a mixture of two alkaloids (380 mg). Preparative TLC (SiO_2; $CHCl_3$:MeOH, 95:5) of the obtained residue furnished **8** (135 mg) and **9** (220 mg).

To determine whether cyclic peptide alkaloids **1** and **2** were artifacts of the extraction and isolation procedure, the same plant material was extracted with MeOH at room temp. followed by extraction under neutral conditions. From this procedure cyclic peptides **1** and **2** were still present.

3.4. Discarine-M (1)

White amorphous powder, $[\alpha]_D^{20}$ −176.7° (*c* 0.2, MeOH:$CHCl_3$, 1:1). TLC 0.36 ($CHCl_3$–MeOH, 98:2). IR (KBr) ν_{max} 3280, 1625, 1234 cm^{-1}. ^1H NMR (DMSO-d_6, 400 MHz): δ 8.42 (1H, d, *J* 10.0 Hz, NH-20), 7.62 (1H, d, *J* 9.7 Hz, NH-9), 7.11 (1H, d, *J* 8.3 Hz, H-14), 7.08 (1H, d, *J* 8.7 Hz, H-15), 7.00 (1H, d, *J* 8.7 Hz, H-16), 6.93 (1H, d, *J* 8.3 Hz, H-13), 6.72 (1H, d, *J* 8.0 Hz, NH-6), 6.61 (1H, dd, *J* 6.8, 14.0 Hz, H-23), 6.51 (1H, dd, *J* 9.7, 7.5 Hz, H-10), 6.38 (1H, d, *J* 7.5 Hz, H-11), 5.84 (1H, d, *J* 14.0 Hz, H-22), 4.89 (1H, dd, *J* 7.6, 7.7 Hz, H-3), 4.55 (1H, dd, *J* 7.6, 10.0 Hz, H-4), 3.78 (1H, m, H-7), 2.35 (1H, m, H-24), 2.08 (1H, m, H-17), 1.43/1.31 (each 1H, m, H-27, H-27′), 1.25 (1H, m, H-28), 1.13 (3H, d, *J* 6.7 Hz, CH_3-19), 0.96 (3H, d, *J* 6.7 Hz, CH_3-26), 0.94 (3H, d, *J* 6.7 Hz, CH_3-25), 0.82 (3H, d, *J* 6.7 Hz, CH_3-18), 0.69 (3H, d, *J* 6.5 Hz, CH_3-29), 0.49 (3H, d, *J* 6.5 Hz, CH_3-30); ^{13}C NMR (DMSO-d_6, 100 MHz): δ 171.4 (C-5), 168.0 (C-8), 164.1 (C-21), 155.8 (C-1), 149.7 (C-23), 131.1 (C-12), 130.9 (C-13), 130.3 (C-16), 125.7 (C-10), 122.8 (C-15), 121.4 (C-14), 121.0 (C-22), 116.8 (C-11), 82.2 (C-3), 54.9 (C-4), 52.1 (C-7), 38.6 (C-27), 30.0 (C-24), 28.2 (C-17), 23.4 (C-28), 23.1 (C-29), 21.5 (C-26), 21.4 (C-25), 21.3 (C-30), 20.4 (C-19), 14.4 (C-18). FABMS *m/z* 456 $[M+H]^+$; anal. C 68.27%, H 8.09%, N 9.47%, calc. for $C_{26}H_{37}N_3O_4$, C 68.54%, H 8.19%, N 9.22%.

3.5. Discarine-N (2)

White powder; mp 233–235 °C, $[\alpha]_D^{20}$ +98.1° (*c* 0.092, MeOH:$CHCl_3$, 1:1). TLC 0.50 ($CHCl_3$:MeOH, 95:5). IR (KBr): ν_{max} 3260, 1648, 1277, 1261 cm^{-1}. ^1H NMR (DMSO-d_6, 400 MHz): δ 8.05 (1H, d, *J* 8.8 Hz, NH-20), 7.45–7.41 (5H, H-30, H-30′, H-31, H-31′, H-32), 7.19 (1H, d, *J* 11.5 Hz, NH-6), 7.11 (1H, d, *J* 8.1 Hz, H-16), 7.00 (1H, d, *J* 8.5 Hz, H-13), 6.85 (1H, dd, *J* 7.4, 8.8 Hz, H-10), 6.98–6.92 (5H, H-25, H-25′, H-26, H-26′, H-27), 6.44 (1H, d, *J* 7.4 Hz, H-11), 6.87 (1H, d, *J* 8.5, H-14), 6.85 (1H, d, *J* 15.8 Hz, H-23), 6.54 (1H, d, *J* 8.1 Hz, H-15), 6.09 (1H, d, *J* 8.8 Hz, NH-9), 5.96 (1H, d, *J* 15.8 Hz, H-22), 5.49 (1H, d, *J* 4.4, Hz, OH-28), 4.23 (1H, dd, *J* 6.4, 4.4 Hz, H-28), 4.21 (1H, dd, *J* 7.2, 8.8 Hz, H-4), 4.13 (1H, d, *J* 7.2 Hz, H-3), 4.01 (1H, m, H-7), 1.83 (1H, dd, *J* 6.7, 6.8 Hz, H-17), 1.14 (3H, d, *J* 6.7 Hz, CH_3-18), 0.86 (3H, d, *J* 6.8 Hz, CH_3-19); ^{13}C NMR (DMSO-d_6, 100 MHz): δ 168.9 (C-21), 166.9 (C-5), 163.3 (C-8), 158.6 (C-1), 142.2 (C-29), 139.4 (C-23), 134.7 (C-24), 132.0 (C-12), 131.2 (C-13), 129.7 (C-27), 129.4 (C-16), 129.1 (C-26), 127.9 (C-15), 127.7 (C-25), 127.3 (C-32). 127.0 (C-30), 126.9 (C-10), 126.8 (C-31), 121.7 (C-22), 121.2 (C-14), 117.6 (C-11), 86.5 (C-3), 73.3 (C-28), 57.4 (C-7), 56.0 (C-4), 28.0 (C-17), 20.4 (C-19), 14.9 (C-18). FABMS *m/z* 540 $[M+H]^+$; anal. C 71.02%, H 6.04%, N 7.95%, calc. for $C_{32}H_{33}N_3O_5$, C 71.22%, H 6.16%, N 7.79%.

3.6. Hydrolysis of 1 and 2

Total hydrolysis of **1** and **2** was performed by heating in a sealed tube at 110 °C with 6 N HCl for 12 h. The acidic solutions were concentrated and the residues were treated as described for amino acids (Silva et al., 1996).

3.7. Amino acid derivatization

Acid-catalyzed esterification was carried out by addition of a 1.6 N anhydrous solution of HCl (gas) in methanol 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.

3.8. GC analysis of leucine and β-phenylserine

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 the ring bonded α-amino acids, leucine in **1** and β-phenylserine in **2**, was unambiguously established as L(*S*) and L-*threo* (7*S*/28*R*), respectively.

3.9. Antimicrobial activity

The antibacterial activity of the crude methanol extract, the basic ether extract, of cyclic peptides **1** and

2, and of alkaloids **3–9** was performed by direct bioautography on TLC plates (Merck Si-gel 60 F_{254}, 0.25 mm thick) according to Rahalison et al. (1991). The following test bacteria were used: three gram-positive (*Staphylococcus aureus*, ATCC 6538p, *Bacillus subtilis* ATCC 6633, and *Staphylococcus epidermidis*, ATCC 12228), and three gram-negative (*Klebsiella pneumoniae*, ATCC 10031, *Salmonella setubal,* ATCC 19196, and *Escherichia coli*, ATCC 11103) bacteria. The inoculum was prepared by culturing each organism in tryptone soya agar (TSA, 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) was applied into Mueller Hinton Agar (MHA-DIFCO), and distributed over developed TLC plates (5 × 5). For the antimicrobial assay, 200.0, 150.0, 125.0, 100.0, 75.0, 62.5, 50.0, 31.2, 25.0, 12.5, 6.25 μg of the extracts, and 100.0, 50.0, 25.0, 12.5, 6.25, 3.12, 1.06 μg of **1–9** was applied to pre-coated TLC plates. 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 was used as positive control (see Table 1). Each assay was conduced in triplicate.

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