A New Cyclopeptide and Other Constituents from the Leaves of *Zanthoxylum rigidum* HUMB. & BONPL. ex WILLD. (Rutaceae)

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A new cyclopeptide, cyclozanthoxylane A (**1**), the lignans *cis*- and *trans*-methylpluviatolide, the flavonoid isoquercitrin, along with a mixture of benzoic and cinnamic acid derivatives, were isolated from the MeOH extract of the leaves of *Zanthoxylum rigidum* (Rutaceae). The structures of the compounds were determined on the basis of 1D- and 2D-NMR, and MS analysis. It is the first time that a natural cyclic peptide has been isolated from the genus *Zanthoxylum*.

Introduction. – *Zanthoxylum* genus (Rutaceae) comprise more than 200 species widely distributed around the world. This genus has been characterized by the presence of alkaloids, coumarins, flavonoids, lignans, and mono-, sesqui-, and triterpenes [1]. Many pharmacological activities such as antiplasmodic [2], anti-HIV [3], anti-inflammatory [4], and antihelmintic [5] properties of *Zanthoxylum* species have been reported. *Zanthoxylum rigidum* is popularly known as '*mamica de porca* (*pig's nipple*)', or '*mamica de cadela* (sow's nipple)'. There is only one phytochemical study reported on *Zanthoxylum rigidum*, in which the isolation and identification of lupeol, campesterol, stigmasterol, sitosterol, sucrose, the flavonoid hesperidine, and the alkaloids *N*-methylatanine and 6-acetonyldihydrochelerythrine from the roots were described [1]. This is the first phytochemical study of the extracts from the leaves of *Z. rigidum*, describing the isolation and structure determination of a new cyclic peptide, named cyclozanthoxylane A (**1**), two lignans, *cis*- and *trans*-methylpluviatolide, the flavonoid isoquercitrin, along with a mixture of benzoic and cinnamic acid derivatives, methyl (*E*)-4-hydroxycinnamate, methyl (*Z*)-4-hydroxycinnamate, methyl 4-hydroxybenzoate, and the methyl 4-hydroxyphenylacetate. Cyclic peptides are widely distributed in many higher plants, exhibiting many biological activities, including some of the mentioned above, and they have often been used as models for studies of structural features of proteins [6][7]. A small number of cyclic peptides from Rutaceae plants such as evolidine and citrusins from *Citrus* species [8], and clausenain I and clausenain B from *Clausena anisum-olens* [6][9] have been reported. This is the first time that a natural cyclic peptide has been isolated from the *Zanthoxylum* genus.

Besides other compounds, the structure of this new peptide was elucidated by ^{1}H- and ^{13}C-NMR and MS analysis, and comparison with literature data.

Results and Discussion. – The chromatographic fractionation of the MeOH extract of the leaves of *Z. rigidum* afforded methyl (*E*)-4-hydroxycinnamate [10], methyl (*Z*)-4-hydroxycinnamate [10], methyl 4-hydroxybenzoate [11], methyl 4-hydroxyphenylacetate [12], *cis*- and *trans*-methylpluviatolide [13], and isoquercitrin [14], besides a new cyclopeptide, named cyclozanthoxylane A (**1**). The known compounds were identified by GC/MS, 1D- and 2D-NMR spectra, and comparison with literature data [10–14].

Compound **1** was isolated as white amorphous powder with a molecular formula of $C_{23}H_{33}N_5O_5$, on the basis of its quasimolecular ion detected by HR-ESI-MS (positive-ion mode) at *m*/*z* 460.2557 ($[M+H]^+$; calc. for $C_{23}H_{34}N_5O_5^+$, 460.2554), corresponding to ten degrees of unsaturation. The IR absorption bands were characteristic of amine (3306 cm^{-1}) and amide C=O (1652 cm^{-1}) groups. The DEPTQ-NMR spectrum exhibited signals of five amide CO groups (δ(C) 173.2, 172.5, 171.9, 170.5, 169.6), and four CH groups δ(C) (55.6, 54.2, 50.1, 48.4) and one CH_2 group δ(C) (43.8), which

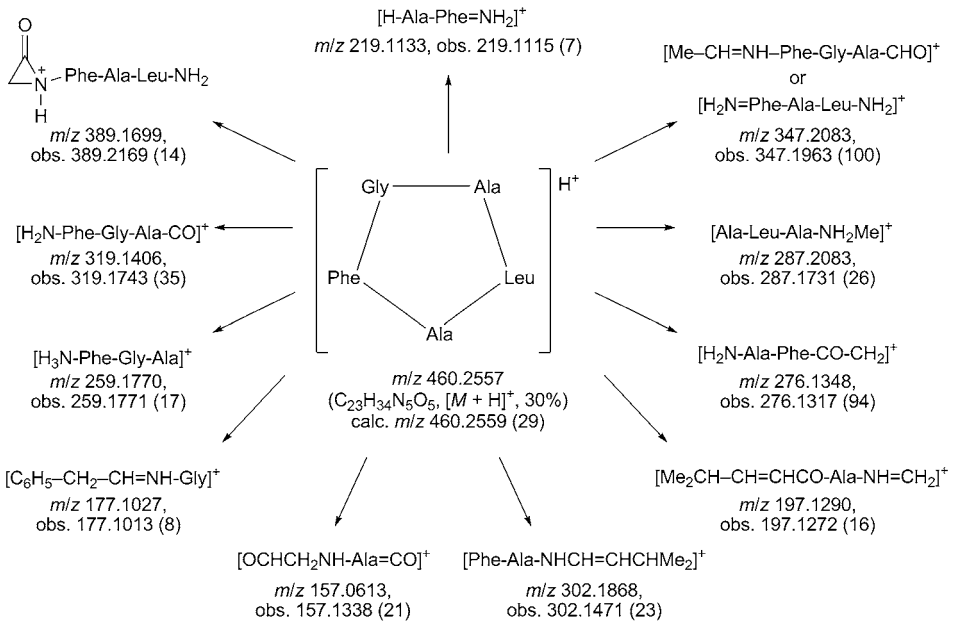
Table. *NMR Data of Cyclozanthoxylane A* (**1**). Recorded in (D_6)DMSO; δ in ppm, *J* in Hz.

	Position	δ(H)	δ(C)	HMBC
Gly	$CH_2(\alpha)$	3.89 (*dd*, *J* = 14.4, 6.4), 3.24 (*dd*, *J* = 14.4, 5.2)	43.8 (*t*)	CO(Ala^1), CO(Gly)
	NH	8.67 (*t*, *J* = 5.6)		CO(Ala^1)
	CO		169.6 (*s*)	
Ala^1	H–C(α)	4.19–4.24 (*m*)	48.4 (*d*)	CO(Ala^1), CO(Leu)
	Me(β)	1.20 (*d*, *J* = 7.2)	18.2 (*q*)	CO(Ala^1), CH(α)(Ala^1)
	NH	7.93–7.96 (*m*)		CO(Ala^1), CO(Leu)
	CO		173.2 (*s*)	
Leu	H–C(α)	3.98–4.03 (*m*)	54.2 (*d*)	CO(Leu)
	$CH_2(\beta)$	1.68–1.75 (*m*), 1.47–1.53 (*m*)	40.0 (*t*)	CO(Leu)
	H–C(γ)	1.56–1.61 (*m*)	25.0 (*d*)	
	Me(δ)	0.91 (*d*, *J* = 6.0)	23.2 (*q*)	$CH_2(\beta)$(Leu)
	Me(δ')	0.86 (*d*, *J* = 6.4)	22.0 (*q*)	$CH_2(\beta)$(Leu)
	NH	8.02 (*d*, *J* = 7.6)		CO(Ala^2)
	CO		172.5 (*s*)	
Phe	H–C(α)	4.34–4.40 (*m*)	55.6 (*d*)	CO(Phe), CO(Gly)
	$CH_2(\beta)$	3.12 (*dd*, *J* = 14.0, 5.2), 2.85 (*dd*, *J* = 14.2, 10.0)	37.2 (*t*)	CO(Phe), CH(α)(Phe), C(1′), C(2′), C(6′)
	1′		138.2 (*s*)	
	2′, 6′	7.18–7.30 (*m*)	129.3 (*d*)	
	3′, 5′	7.18–7.30 (*m*)	128.6 (*d*)	
	4′	7.18–7.30 (*m*)	126.8 (*d*)	
	NH	8.21 (*d*, *J* = 8.8)		CO(Gly)
	CO		170.5 (*s*)	
Ala^2	H–C(α)	4.19–4.24 (*m*)	50.1 (*d*)	CO(Ala^2), CO(Phe)
	Me(β)	1.31 (*d*, *J* = 7.2)	17.5 (*q*)	CO(Ala^2), CH(α)(Ala^2)
	NH	7.93–7.96 (*m*)		CO(Phe)
	CO		171.9 (*s*)	

are compatible to C(α) of α-amino acids. The ^{1}H-NMR spectrum (*Table*) displayed five signals at δ(H) 8.67 (*t*, 1 H), 8.21 (*d*, 1 H), 8.02 (*d*, 1 H), and a *multiplet* at 7.93–7.96 (2 H) that was identified as two *doublet*s according the TOCSY experiment. The HMQC spectrum analysis led to the assignment of these signals to the amide NH H-atoms, compatible with a cyclic system containing five amino acids, including one glycine, as described above. At higher field, four signals corresponding to Me groups were present. The analysis of ^{1}H,^{1}H-COSY and selective TOCSY (selecting the H–N signals) spectra allowed identification of the coupling signals of a glycine (Gly), two alanine (Ala^1 and Ala^2), one leucine (Leu), and one phenylalanine (Phe) (see the *Table*). The following IMPACT-HMBC correlations of the five CO group chemical shifts were observed: 169.6 (Gly) with NH(Phe) and NCH$_2$(Gly); 170.5 (Phe) with NH(Ala^2) and CH$_2$(Phe); 171.9 (Ala^2) with NH(Leu), NCH(Ala^2) and Me(Ala^2); 172.5 (Leu) with NH(Ala^1); 173.2 (Ala^1) with NH(Gly), NCH$_2$(Gly), NCH(Ala^1), and Me(Ala^1). Additional analysis of HMBC and NOESY spectra (*Table* and *Fig.*) allowed us to propose the sequence of Phe-Gly-Ala^1-Leu-Ala^2 in the cyclic system. The detailed MS analysis (*Scheme*) was used to confirm the structure with this amino acid sequence. The *Table* contains the complete ^{1}H- and ^{13}C-NMR assignments of compound **1** that was elucidated as cyclo(-Phe-Gly-Ala^1-Leu-Ala^2-) and named cyclozanthoxylane A.

The absolute configurations of the amino acids present in **1** were determined by the adapted *Marfey* method [15][16]. HPLC analysis of hydrolyzated derivatives indicated four types of amino acid present in the mixture, which were identified as glycine, L-alanine, L-phenylalanine, and L-leucine by comparation with the derivatives of authentic samples.

Scheme. *Proposed Fragments for the Principal Peaks Detected in HR-ESI-MS of* **1**



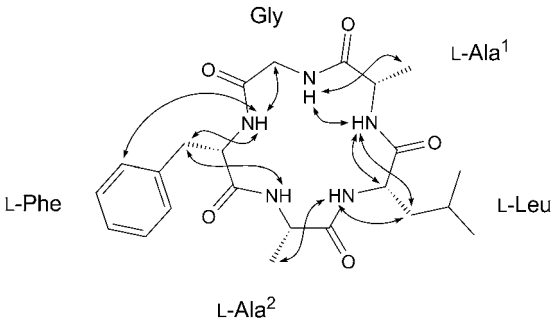


Figure. *Key NOESY correlations of* **1**

Experimental Part

General. TLC: silica gel plates *60 F254* (*Whatman*). Column chromatography (CC): silica gel (SiO_2; 73–230 mesh; *Silicycle*), *Sephadex LH-20* (*Sigma–Aldrich*). HPLC: *Shimadzu Prominence LC-20AT*, equipped with a PDA (photodiode array) detector, *RP-18* (4.6 × 250 mm, 5 μm; *Thermo*); t_R in min. UV Spectra: (λ in nm). IR Spectra: *Vertex-70 FT-IT*; in KBr discs; $\tilde{\nu}$ in cm^{-1}. 1D- and 2D-NMR spectra: *Bruker Avance II* 400 MHz; δ in ppm, with the residual solvent peak as the internal standard, *J* in Hz. ESI-MS: *QP2010 Plus Shimadzu.* HR-ESI-MS (positive-ion mode): *Bruker Daltonics*, in *m/z* (rel. %).

Plant Material. Leaves of *Zanthoxylum rigidum* were collected in Poconé, Mato Grosso State, Brazil, in June 2008. A voucher specimen (No. 38648) is deposited with the herbarium of Universidade Federal de Mato Grosso, Mato Grosso state, Brazil.

Extraction and Isolation. Dried and powered leaves of *Z. rigidum* (1.3 kg) were extracted with hexane and MeOH using a *Soxhlet* apparatus for 10 h. The extracts were concentrated under vacuum to furnish the residues from hexane (46.6 g) and MeOH (205.9 g). Part of the MeOH extract (100.0 g) was dissolved in MeOH/H_2O 7:3 and partitioned with hexane, $CHCl_3$, and AcOEt to yield the fractions *ZRFH* (35.8 g), *ZRFC* (1.7 g), *ZRFE* (3.1 g), and *ZRFM* (57.1 g). *Fr. ZRFC* (1.5 g) was subjected to CC (SiO_2; gradient CH_2Cl_2/AcOEt and AcOEt/MeOH): Fractions *A–J. Fr. C* (80 mg) was submitted to CC (SiO_2; gradient CH_2Cl_2/AcOEt): *Frs. C1–C8. Fr. C3* (30 mg) was fractioned by CC (SiO_2; gradient CH_2Cl_2/MeOH): mixture (6 mg) of *methyl 4-hydroxybenzoate* (3.91%), *methyl (*E*)-4-hydroxycinnamate* (87.51%), *methyl (*Z*)-4-hydroxycinnamate* (6.55%), and *methyl 4- hydroxyphenylacetate* (2.01%). *Fr. C5* (5 mg) was submitted to CC (SiO_2; $CHCl_3$/MeOH 19:1): mixture (3 mg) of cis- (16.38%) and trans-*methylpluviatolide* (83.61%). *Fr. H* (526 mg) was submitted to CC (SiO_2; gradient CH_2Cl_2/MeOH): *cyclozanthoxylane A* (**1**; 4 mg). *Fr. ZRFE* (1.8 g) was subjected to CC (SiO_2; gradient $CHCl_3$/AcOEt and AcOEt/MeOH): *Frs. 1–8. Fr. 4* (523 mg) was subjected to CC (SiO_2; gradient $CHCl_3$/MeOH): *Frs. 4.1–4.7. Fr. 4.5* (219 mg) was submitted to CC (SiO_2; gradient $CHCl_3$/MeOH): *Frs. 4.5A–4.5F.* Separation of *Fr. 4.5C* (47 mg) by CC (*Sephadex LH-20*; MeOH): *isoquercitrin* (16 mg).

Acid Hydrolis of Cyclozanthoxylane A (**1**). Compound **1** (1 mg) was dissolved in 6N HCl (1 ml) and heated at 110° for 14 h in a reaction vial. After cooling, the soln. was evaporated to dryness under reduced pressure and redissolved in 100 μl of H_2O.

Preparation and Analysis of Marfey *Derivatives.* To 50 μl of a 50 mM soln. of the respective amino acid (or to 50 μl of the acid hydrolysate soln.) was added 100 μl of a 1% (*w*/*v*) soln. of L-FDAA (*Marfey*'s reagent, N^{α}-(5-fluoro-2,4-dinitrophenyl)-L-alaninamide) in acetone. After addition of a 1M $NaHCO_3$ soln. (20 μl), the mixture was incubated for 1 h at 40°. The reaction was stopped by the addition of 10 μl of 2N HCl. The solvents were evaporated to dryness, and the residue was redissolved in MeOH. An aliquot (20 μl) was analyzed by reversed-phase (RP) HPLC (4.6 × 250-mm C_{18} column (5 μm), linear gradient of MeCN (*A*) and aq. CF_3COOH (*B*) (pH 3.1) from 28 to 55% *A* over 35 min; flow rate, 1 ml/min; detection, at 340 nm). Peaks in the chromatogram were identified by comparing the t_R values with those of the L-FDAA derivatives of the authentic amino acids. The standards derivatives gave the

following t_R values [min]: 9.30 (Gly-L-FDAA), 10.94 (L-Ala-L-FDAA), 14.56 (D-Ala-L-FDAA), 15.51 (L-FDAA), 26.92 (L-Phe-L-FDAA), 27.22 (L-Leu-L-FDAA), 29.52 (D-Phe-L-FDAA), 30.83 (D-Leu-L-FDAA). The L-FDAA derivatives of the liberated amino acids from **1** showed peaks at 9.45 (Gly-L-FDAA), 11.10 (L-Ala-L-FDAA), 15.79 (L-FDAA), 27.11 (L-Phe-L-FDAA), 27.37 (L-Leu-L-FDAA).

Cyclozanthoxylane A (*Cyclo(*L*-alanyl-*L*-leucyl-*L*-alanyl-glycyl-*L*-phenylalanyl)*; **1**). White amorphous powder. IR (KBr): 3306, 2953, 1652, 1533, 1384, 746. ^{1}H- and ^{13}C-NMR: see the *Table*. HR-ESI-MS: 460.2557 ($[M+H]^+$, $C_{23}H_{34}N_5O_5^+$; calc. 460.2554).

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