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Unprecedented Occurrence of Isoaspartic Acid in a Plant Cyclopeptide

Damien Lacroix,[†] Soizic Prado,[†] Dennis Kamoga,[‡] John Kasenene,[‡] Séverine Zirah,[†] and Bernard Bodo*,[†]

Molécules de Communication et Adaptation des Micro-Organismes, UMR 7245 CNRS, Muséum National d'Histoire Naturelle, 63 rue Buffon 75005 Paris, France, and Department of Botany, Makerere University, PO BOX 7062, Kampala, Uganda

bodo@mnhn.fr

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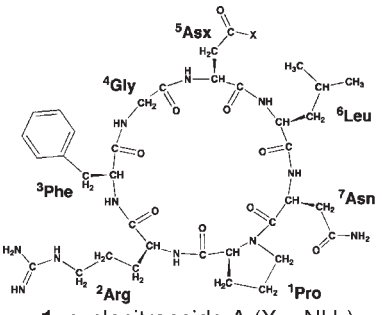
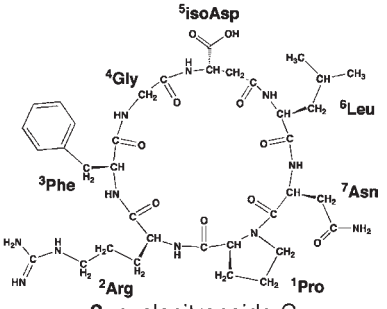
ABSTRACT



1: cyclocitropside A (X = NH_2)
2: cyclocitropside B (X = OH)



3: cyclocitropside C

Three structurally related cycloheptapeptides, cyclocitropsides A−C, have been isolated from a MeOH extract of the root bark of *Citropsis articulata*, a medicinal plant in Uganda. Their sequences were elucidated on the basis of their MS/MS fragmentation, extensive 2D-NMR, chemical degradation, and biochemical modifications. Surprisingly, the sequence of cyclocitropside C differed from that of cyclocitropside B only by an Asp[5]/isoAsp[5] substitution. This is the first report of an isoAsp residue in a plant cyclic peptide.

Citropsis articulata is a widespread small tree of the Rutaceae family and endemic in the tropical rain forest of Africa. It is well known as African cherry orange and named omuboro in Uganda. The root is used in East Africa as a powerful aphrodisiac for men.[1] The plant is also used to treat malaria, epilepsy and AIDS symptoms.[2] Several alkaloids and phenyl propanoids have been isolated.[3,4] In continuation of our programs on medicinal plants from Uganda and on cyclopeptides from plants,[5,6] we have investigated the root bark of *C. articulata* and isolated three novel cycloheptapeptides, named cyclocitropsides A−C (**1**−**3**). We report their sequence determination based on tandem MS, 2D-NMR and enzymatic specific reactions.

The dried and ground root bark of *C. articulata* was extracted with solvents of increasing polarity, and from the MeOH extract, cyclocitropsides A (**1**), B (**2**), and C (**3**), were purified by silica gel column chromatography and C_{18} RP-HPLC. Positive reaction with chlorine/*o*-tolidine reagent suggested that they were peptides and the absence of coloration with ninhydrin, that they were cyclic. The acidic hydrolysates of **1**−**3** were derivatized by the Marfeys's method, then analyzed by HPLC and the retention times compared with those of standards.[7] All the chiral amino acids have the L configuration. In addition, the hydrolysates analysis indicated the same composition

[†] Muséum National d'Histoire Naturelle.
[‡] Makerere University.

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for the three peptides: Asx (2), Arg (1), Gly (1), Leu (1), Phe (1), Pro (1). The two Asx residues in the hydrolysate of **1** were identified from NMR data as two Asn, whereas they were one Asn and one Asp for both **2** and **3**.

The positive ESI-TOF MS of cyclocitropside A (**1**) displayed the $[M + H]^+$ ion at *m/z*: 799.4237 corresponding to the molecular formula $C_{36}H_{54}N_{12}O_9$ (calcd for $C_{36}H_{55}N_{12}O_9$: 799.4215), in agreement with the amino acid composition: Asn (2), Arg (1), Gly (1), Leu (1), Phe (1) and Pro (1). Cyclopeptides are not easily sequenced by MS due to multiple and indiscriminate ring-opening reactions which occur during the CID making the interpretation difficult. However, when a proline is present, a favored fragmentation occurs at the peptidyl-prolyl amide bond, leading to a main linear peptide ion which undergoes further fragmentation, generating series of acylium (b_n) ions from which the sequence can be deduced.[5,6] The $[M + H]^+$ ion of **1** at *m/z* 799 was subjected to CID experiment and a main linear peptide was generated (Figure 1I). Its fragmentation formed a series of adjacent b_n ions at *m/z*: 685, 572, 458, 401 and 254, corresponding to the successive loss of Asn, Leu, Asn, Gly, Phe and yielding the terminal dipeptide ion [H-Pro-Arg]$^+$, from which the sequence was deduced. The related (a_n) ion series was also observed.

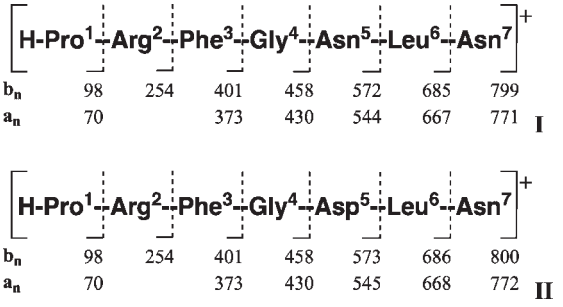

Figure 1. Main ion fragments observed in the mass spectra of **1** (I), **2** and **3** (II).

Similarly, when analyzed by MS, the $[M + H]^+$ ion of cyclocitropside B (**2**) was depicted at *m/z*: 800.4054 and that of cyclocitropside C (**3**) at *m/z*: 800.4044, both in agreement with the formula $C_{36}H_{54}N_{11}O_{10}$ (calcd 800.4051), and also with the amino acid composition: Asn (1), Asp (1), Arg (1), Gly (1), Leu (1), Phe (1) and Pro (1). The two peptides contained each one Asn and one Asp residue. The CID spectra of the $[M+H]^+$ ion at *m/z* 800 showed, for **2** and **3**, a main series of adjacent b_n ions at *m/z* 686, 573, 458, 401, and 254, corresponding to the successive loss of Asn, Leu, Asp, Gly, and Phe, yielding the terminal dipeptide ion [H-Pro-Arg]$^+$ and suggesting the same sequence [H-Pro^1-Arg^2-Phe^3-Gly^4-Asp^5-Leu^6-Asn^7]$^+$ for the two linearized peptides (Figure 1II). Here also, an a_n ion series was depicted. The proposed sequences for cyclocitropsides B (**2**) and C (**3**) were thus *cyclo*(Pro^1-Arg^2-Phe^3-Gly^4-Asp^5-Leu^6-Asn^7), both having the Asp residue at position 5, and the Asn residue at position 7. Nevertheless the two peptides were different, with distinct retention times on HPLC. Their difference cannot be due to the presence of a D residue instead of a L one, because all the chiral residues were shown to be L. The proposed sequences were thus verified by 2D-NMR analyses.

The ^{1}H NMR spectrum of cyclocitropside A (**1**) in DMSO-d_6 (Table 1) showed only one stable conformational state (> 95%). Six amide protons together with the four amine protons of the Arg lateral chain and the five aromatic protons of the Phe residue, were clearly depicted at low field. Analysis of the ^{13}C NMR, COSY, TOCSY, HSQC and HMBC spectra, agreed with a cyclic heptapeptide structure including one Pro and two Asn. The sequence determination and assignments of the two Asn were deduced from the correlations observed in the HMBC spectrum of the CO of Leu^6 (δ_C 172.1) and the NH of Asn^7 and of the CO of Gly^4 (δ_C 168.6) and the NH of Asn^5 (Figure 2I).[8] These two CO were previously assigned to the related Asn from their intraresidue correlations with their respective α-H and β-CH_2.

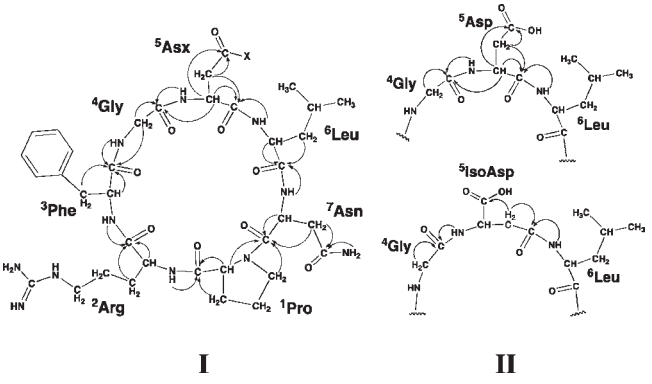

Figure 2. Main observed HMBC correlations (H→C) for cyclocitropsides A (X = NH_2) and B (X = OH) **(I)**, and for Asp in B and isoAsp in C (**II**).

The chemical shift assignments for cyclocitropsides B (**2**) and C (**3**) were obtained from 2D-NMR data (Table 1) and those for 2 were close to those of cyclocitropside A. Analysis of the HMBC correlations confirmed the sequence deduced from the mass fragmentations, and confirmed the sequence *cyclo*(Pro^1-Arg^2-Phe^3-Gly^4-Asp^5-Leu^6-Asn^7-) for (**2**). The CO of Gly^4 was correlated to the NH of Asp^5 and the CO of Leu^6 to the NH of Asn^7. The γ-CO of Asp^5 at δ_C 171.9 was only correlated with the α and β protons of this residue. Although the NMR chemical shifts for cyclocitropside C (**3**) were somewhat different, analysis of the HMBC spectrum, especially of the CO region, gave the same correlations and suggested again the same sequence.

A possible explanation that the difference between **2** and **3** could be due to two distinct conformations strongly

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Table 1. ^{13}C- and ^{1}H-NMR Data for Cyclocitropsides A–C (**1**–**3**)[a]

	1[b]		**2**[b]		**3**[c]	
amino acid	δ_C	δ_H mult. *J* (Hz)	δ_C	δ_H mult. *J* (Hz)	δ_C	δ_H mult. *J* (Hz)
[1]Pro CO	172.0		171.3		170.3	
α CH	61.9	4.19 *dd* 8.6; 6.1	61.8	4.18 *dd* 7.9; 7.0	61.5	4.08 *m*
β CH_2	29.6	2.23 *m* and 1.81 *m*	29.5	2.23 *m* and 1.76 *m*	29.6	2.14 *m* and 1.79 *m*
γ CH_2	24.6	1.89 *m* and 1.89 *m*	24.5	1.87 *m* and 1.80 *m*	24.4	1.95 *m* and 1.80 *m*
δ CH_2	47.9	3.66 *ddd* 9.9; 6.9; 6.9	47.5	3.84 *ddd* 9.5; 6.5; 6.5	47.7	3.97 *m*
		3.90 *ddd* 9.9; 6.7; 6.7		3.61 *m*		3.58 *m*
[2]Arg CO	171.5		171.3		171.9	
NH		7.91 *d* 8.1		8.00 *d* 8.7		7.73 *d* 9.9
α CH	53.8	4.00 *m*	53.3	4.07 *m*	53.5	4.10 *m*
β CH_2	27.9	1.43 *m* and 1.43 *m*	27.8	1.51 *m* and 1.37 *m*	28.7	1.33 *m* and 1.22 *m*
γ CH_2	25.2	1.31 *m* and 1.23 *m*	25.0	1.29 *m* and 1.18 *m*	24.7	1.15 *m* and 0.79 *m*
δ CH_2	40.7	2.96 *m*	40.1	2.96 *m*	40.0	2.82 *ddd* 6.5; 6.5; 4.5
ε NH		8.03 brs		7.51 *t* 5.3		7.56 *t* 4.5
ζ C	157.2		156.6		156.7	
η, *η*′ NH		7.29 brs		7.05 brs		7.05 brs
[3]Phe CO	170.8		170.6		171.1	
NH		7.51 *d* 9.5		7.56 *d* 9.0		7.61 *d* 9.0
α CH	54.6	4.48 *ddd* 9.5; 9.2; 5.0	54.7	4.48 *ddd* 9.0; 8.6; 5.8	54.7	4.43 *ddd* 12.0; 9.0; 3.1
β CH_2	38.7	3.18 *dd* 14.0; 5.0	38.6	3.11 *dd* 13.5; 5.8	38.5	3.25 *dd* 12.6; 3.1
		2.69 *m*		2.58 *dd* 13.5; 8.6		2.32 *dd* 12.6; 12.0
1′-C	138.0		137.7		137.4	
2′,6′-CH	129.1	7.12 *m*	128.9	7.12 *m*	128.9	7.23 *m*
3′,5′-CH	128.2	7.23 *m*	128.0	7.23 *m*	128.3	7.14 *m*
4′-CH	126.3	7.16 *m*	126.2	7.16 *m*	126.5	7.18 *m*
[4]Gly CO	168.6		168.6		169.9	
NH		7.91 *m*		8.15 *dd* 5.0; 5.0		7.91 *dd* 8.4; 4.4
α CH_2	42.8	3.97 *m*	42.6	3.92 *dd* 16.3; 5.0	41.0	4.25 *dd* 17.0; 8.4
		3.44 *dd* 16.5; 4.7		3.44 *m*		3.32 *dd* 17.0; 4.4
[5]Asx CO	170.9	**Asn**	170.2	**Asp**	170.0	**isoAsp**
NH		8.05 *d* 7.9		8.39 *dd* 7.7		8.62 *d* 9.5
α CH	50.6	4.34 *ddd* 7.9; 5.9; 5.9	50.5	4.28 *ddd* 7.7; 7.0; 6.1	49.0	5.17 *ddd* 12.1; 9.5; 3.1
β CH_2	36.1	2.66 *m*	35.2	2.80 *dd* 17.0; 6.1	40.1	2.90 *dd* 13.2; 3.1
		2.66 *m*		2.69 *dd* 17.0; 7.0		2.19 *dd* 13.2; 12.1
γ CO	171.8		171.9		169.9	
δ NH_2 *syn*		6.84 brs				
anti		7.38 brs				
[6]Leu CO	172.1		171.5		170.0	
NH		8.09 *d* 7.3		8.17 *d* 8.0		8.38 *d* 8.1
α CH	54.0	4.00 *m*	53.2	4.03 *m*	51.2	4.20 *m*
β CH_2	39.5	1.77 *m*	39.2	1.78 *m* and 1.56 *m*	40.6	1.62 *m* and 1.45 *m*
		1.54 *ddd* 13.4; 8.7; 5.2				
γ CH	24.3	1.67 *m*	24.1	1.65 *m*	24.2	1.56 *m*
δ CH_3	23.0	0.88 *d* 6.4	22.9	0.88 *d* 6.4	23.3	0.86 *d* 6.3
δ′ CH_3	21.4	0.82 *d* 6.4	21.2	0.79 *d* 6.4	20.9	0.78 *d* 6.3
[7]Asn CO	171.6		171.1		172.2	
NH		7.88 *d* 8.5		7.67 *d* 8.0		7.18 *d* 7.9
α CH	47.4	4.87 *ddd* 10.1; 8.5; 4.0	47.5	4.84 *ddd* 8.8; 8.0; 3.5	48.4	4.80 *ddd* 12.4; 7.9; 3.5
β CH_2	37.6	2.99 *dd* 14.5; 10.1	37.3	2.96 *m*	38.1	3.37 *dd* 13.2; 12.4
		2.63 *dd* 14.5; 4.0		2.64 *dd* 11.6; 3.8		2.65 *dd* 13.2; 3.5
γ CO	172.9		172.7		174.4	
δ NH_2 *syn*		7.41 *s*		7.44 brs		8.56 brs
anti		7.98 *s*		8.09 brs		7.99 brs

[a] Recorded in DMSO-d_6. [b] 298 K. [c] 318 K, *δ*: ppm, ^{13}C 75.47 MHz, ^{1}H 400.13 MHz.

stabilized for example by an ionic bridge between the negatively charged carboxylate group of Asp^5 and the positively charged guanidinium group of Arg^2 was given up, as when their NMR spectra were recorded at different temperatures, pH and solvents (DMSO-d_6, pyridine-d_5), the spectra always remained significantly different. It was puzzling that, whatever the solvent was, the β-CH_2 protons of Asn^7 for the three peptides and of Asp^5 for **2**, were almost equivalent, whereas those of Asp^5 for **3** were inequivalent. In addition, the ^3J couplings between α-CH and β,β'-CH_2 in Asx^5, were significantly different when comparing **3** to both **1** and **2**: the ^3J for **1** and **2** had average values with $^3J_{\alpha\beta} \approx {}^3J_{\alpha\beta'} \approx 6-7$ Hz, whereas they were very different for **3** in DMSO-d_6 ($^3J_{\alpha\beta}$ = 12.1 and $^3J_{\alpha\beta'}$ = 3.1 Hz) as well as in pyridine-d_5 ($^3J_{\alpha\beta}$ = 12.5 and $^3J_{\alpha\beta'}$ = 3.5 Hz).

These data suggested they these protons were involved in a more flexible structure, such as a lateral chain in **1** and **2** and in a more constraint one, such as the backbone in **3**. In addition, in the HMBC spectra, the α-proton of Asx^5 was strongly correlated to three CO (CO of Gly^4, αCO and γCO of Asx^5) for both **1** and **2**, and not for **3**, suggesting a variation in the Φ torsion angles [CO^4-NH-αC^5-αH^5] and [αH^5-αC^5-CH_2-γCO^5] (Figure 2II). The presence of an isoaspartic acid (isoAsp) instead of an Asp in the sequence of **3** was thus suspected. Their distinction by spectral methods is difficult.[9,10] Such an Asp/isoAsp substitution have been previously described as postranslational modification of proteins, especially during the deamidation of Asn to yield both Asp and mainly isoAsp and related to the pathology of Alzheimer's disease and more generally to protein aging.[11] Thus a biochemical method was used to solve the problem.

Both cyclocitropsides B and C were submitted to the ISOQUANT isoaspartate detection kit, which allows the specific methylation by *S*-adenosyl-L-methionine (SAM) in the presence of protein isoaspartyl methyltransferase (PIMT) of the lateral carboxylate group of isoAsp, and not that of the lateral methylene carboxylate group of Asp (Figure 3).[10,12−14] The reaction was performed separately with cyclocitropsides B and C and the reaction products were analyzed by HPLC. The chromatograms clearly showed the presence of *S*-adenosyl-L-homocysteine (SAH) originating from SAM and indicating cyclopeptide methylation in the experiment using cyclocitropside C and not in the case of cyclocitropside B. It was concluded that the two cycloheptapeptides **2** and **3** differed between themselves by an Asp^5/$isoAsp^5$ substitution.

When isoAsp occurs in proteins, its formation is explained by deamidation of an Asn via a succinimide intermediate which undergoes further rearrangement to form a mixture of isoAsp and Asp, the first one being preferentially generated in an about 3/1 ratio.[10,12] It is also described that the presence of a Gly at position i+1 favors the rearrangement, which is in addition catalyzed by an amino group.[10] In the case of cyclocitropsides B−C, the Gly is at position i−1, not i+1 and the isoAsp containing peptide is not the main one's in the plant (**3** relative to **2** ratio is here about 1/5). In addition, many cyclopeptides with Asn or Asp have been isolated from plants, fungi and marine sponges, but the presence of isoAsp have never been described until now.[15]

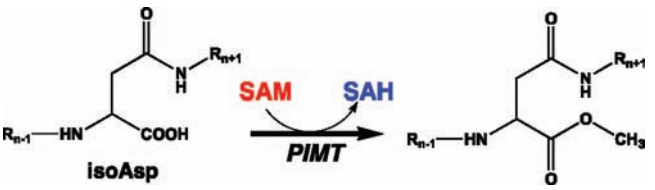

Figure 3. Enzymatic methylation of isoAsp by SAM in the presence of PIMT.

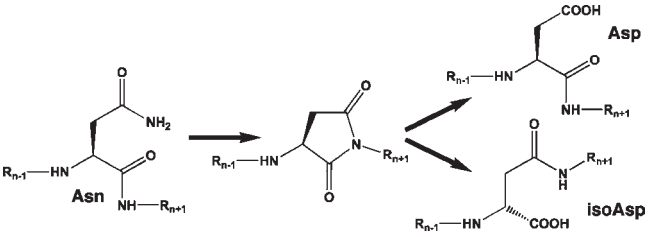

Figure 4. Plausible formation of isoAsp and Asp from Asn.

Cyclopeptides with basic residues are very rare in nature, and in the case of cyclocitropsides A−C, there is in the structure both an Asn/Asp and an Arg containing an amino group in its lateral chain. It is thus plausible that this Arg specifically catalyzed the deamidation of Asn^5 and the rearrangement of the resulting Asp^5 to form the unexpected $isoAsp^5$ residue (Figure 4).

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Supporting Information Available. NMR and MS spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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