Available online at www.sciencedirect.com
SCIENCE DIRECT®
C. R. Chimie 7 (2004) 981–988
COMPTES RENDUS CHIMIE

Full paper / Mémoire

Annomuricatin C, a novel cyclohexapeptide from the seeds of *Annona muricata*

Alassane Wélé, Yanjun Zhang, Christelle Caux, Jean-Paul Brouard, Jean-Louis Pousset, Bernard Bodo *

Laboratoire de chimie des substances naturelles, ESA 8041 CNRS, Muséum national d'histoire naturelle, 63, rue Buffon, 75005 Paris, France

Received 4 August 2003; accepted 21 December 2003

Available online 02 September 2004

Abstract

A new cyclohexapeptide, annomuricatin C, *cyclo*(Pro[1]–Gly[2]–Phe[3]–Val[4]–Ser[5]–Ala[6]–) (**1**) has been isolated from the methanol extract of the seeds of *Annona muricata*. The structure was elucidated on the basis of the MS/MS fragmentation using a Q-TOF mass spectrometer equipped with an ESI source, chemical degradation and extensive 2D-NMR. ***To cite this article: A. Wélé et al., C. R. Chimie 7 (2004)***.

Résumé

L'annomuricatine C, un nouveau cyclohexapeptide isolé des graines d'*Annona muricata*. Un nouveau cyclohexapeptide, l'annomuricatine C, *cyclo*(Pro[1]–Gly[2]–Phe[3]–Val[4]–Ser[5]–Ala[6]–) a été isolé de l'extrait méthanolique des graines d'*Annona muricata*. La structure a été déterminée en se basant sur la fragmentation MS/MS en utilisant un spectromètre Q-TOF équipé d'une source d'ionisation par électronébulisation (ESI), la dégradation chimique et la RMN-2D hétéronucléaire. ***Pour citer cet article : A. Wélé et al., C. R. Chimie 7 (2004)***.

Keywords: Cyclic peptide; *Annona muricata*; Annomuricatin C

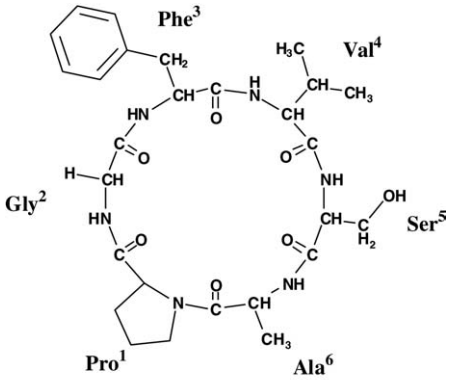
Mots clés : Peptide cyclique ; *Annona muricata* ; Annomuricatine C

* Corresponding author.
E-mail address: bodo@mnhn.fr (B. Bodo).

1631-0748/$ - see front matter © 2004 Académie des sciences. Published by Elsevier SAS. All rights reserved.
doi:10.1016/j.crci.2003.12.022

1. Introduction

Annona muricata L. (Annonaceae), known as corossol, is a widespread small tree native in Central America, which has now a widespread pantropical distribution. The sample we studied was collected in the south of Senegal, where it is cultivated for its fruits. There, traditional medicine also uses this species, named *ndélesor*, in the treatment of many diseases, especially as antiseptic, healing substance and also against dermatosis and malaria fever [1]. In previous studies, we described the structure elucidation of cyclic peptides from *Jatropha* species, some of them having antimalarial activity [2,3]. Continuing our investigation of the cyclopeptides from plants, we here report on the isolation from the seeds of *Annona muricata* and the structural elucidation, based on tandem mass spectroscopy and 2D NMR, of the novel cyclic peptide, annomuricatin C (**1**).



1

Most of the previous phytochemical studies on this species are related to acetogenins, as more than 50 compounds of this class were reported [4], and the two cyclopeptides annomuricatins A and B were also described [5,6]. Annomuricatin C was found to be cytotoxic against tumoural KB cells, with an IC_{50} 1.0 µM.

2. Results and discussion

2.1. Isolation and characteristics

The seeds of *Annona muricata* were successively extracted with cyclohexane and MeOH and the MeOH extract after concentration was dissolved in EtOAc. The EtOAc soluble fraction was purified by exclusion chromatography, silica-gel column chromatography and C_{18} reversed-phase-HPLC to yield a new cyclopeptide termed annomuricatin C (**1**). Positive reaction with chlore/*o*-tolidine reagent suggested it was a peptide and the absence of coloration of its TLC spot with ninhydrin that it was cyclic. Analysis of the total acidic hydrolysate, after derivatization, indicated the presence of Ala (1), Gly (1), Phe (1), Pro (1), Ser (1) and Val (1). The amino acids were converted into the *n*-propyl esters of their *N*-trifluoroacetyl derivatives, analysed by gas chromatography on a chiral capillary column ; their retention times compared with those of standards indicated that all the chiral amino acids were L.

2.2. Mass spectral analysis

The molecular weight 558 for annomuricatin C (**1**) was deduced from the positive ESI-QTOF spectrum, which displayed the $[M+N_a]^+$ adduct ion at *m/z* 581 and the protonated molecular $[M+H]^+$ ion at *m/z* 559. According to the amino acid composition, the molecular formula $C_{27}H_{38}N_6O_7$ was assigned to **1**.

Cyclopeptides are not easily sequenced even by mass spectrometry. The reason is that multiple and indiscriminate ring-opening reactions occur during the CID of cyclic peptides, resulting in the superimposition of random fragment ions and making the interpretation difficult [7,8]. However we have shown that when a proline is present in the sequence, a specific fragmentation occurs at the peptidyl–prolyl (Xaa–Pro) amide bond, leading to a linear peptide C-ended by an acylium ion (b_n), which undergoes further fragmentation, generating series of acylium ions from which the sequence could be deduced [2,9]. This specific fragmentation is explained by the more basic nature of the proline nitrogen relative to the other peptide-bond nitrogen atoms. In this way, the more basic site at proline level strongly directs the protonation, making the fragmentation less complex.

The protonated molecular ion $[M+H]^+$ of **1** at *m/z* 559 was subjected to CID experiment (Fig. 1). The ring opening began at the Ala-Pro amide bond level and a series of adjacent acylium ions (b_n) at *m/z* 488, 401, 302 and 155 was generated, from which the sequence

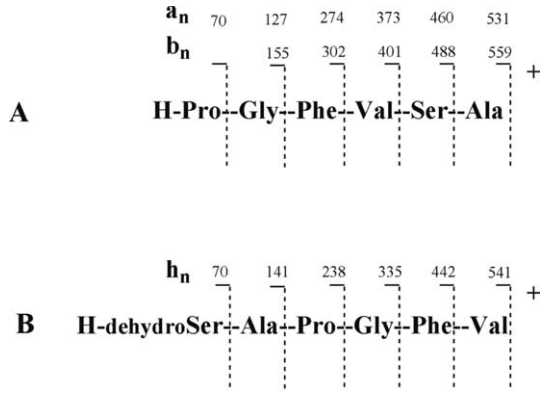


Fig. 1. CID fragmentation of the protonated annomuricatin C (**1**) ion.

could be deduced: amino acid residues were lost sequentially from the *C*-terminus to the *N*-terminus and for annomuricatin C (**1**) was observed the successive losses of Ala, Ser, Val, and Phe, yielding the *N*-terminal dipeptide Pro-Gly (Fig. 2). A second significant series of ions was observed at *m/z* 531, 460, 373, 274, 127 and 70, which were assigned to adjacent a_n ions related to the above b_n ion series. At *m/z* 541 was observed an abundant ion originating from the loss of a molecule of water from the protonated molecular ion $[M+H]^+$. It underwent fragmentation, yielding a third series of abundant ions here termed h_n (Fig. 1B), corresponding to the successive loss of Val, Phe, Gly, Pro and Ala, giving a final ion at *m/z* 70, which corresponded to a dehydrated Ser residue. This latter series of adjacent fragments confirmed the above sequence and was explained by a favoured cleavage of the Val–Ser amide bond after Ser dehydration; this cleavage could be due to the formation of a cyclic amino acid residue from Ser.

When analysing the fragmentation of the cationated adduct $[M+Na]^+$ ion at *m/z* 581, it was observed that the energy collision needed for fragmentation should be higher (60 eV) and that the corresponding b_n series was not observed. The main observed fragmentation yielded an abundant series of a_n ion fragments at *m/z* 553, 482, 395, 296 and 149, in agreement with the above sequence. The base peak at *m/z* 563 was due to the loss of a water molecule from the $[M+Na]^+$ ion. These results suggested the sequence [H–Pro[1]–Gly[2]–Phe[3]–Val[4]– Ser[5]–Ala[6]]$^+$ for the linearised peptide ion derived from annomuricatin C and thus the structure **1** for the natural cyclohexapeptide.

2.3. NMR study

The ^{1}H-NMR spectrum of annomuricatin C (**1**) in DMSO-d_6 solution showed a main stable conformational state (> 85%) for which the five amide protons were clearly depicted, as well as the presence of six carbonyl groups in the ^{13}C-NMR spectrum, in agreement with a hexapeptide structure including a proline (Table 1). The peptide sequence determination was based on the HMBC experiment. This heteronuclear methodology was preferred to the homonuclear method described by Wüthtrich and based on $d_{NN(i,i+1)}$ and $d_{\alpha N(i,i+1)}$ connectivities from the ROESY/NOESY spectra [10,11], because for small size cyclic peptides,

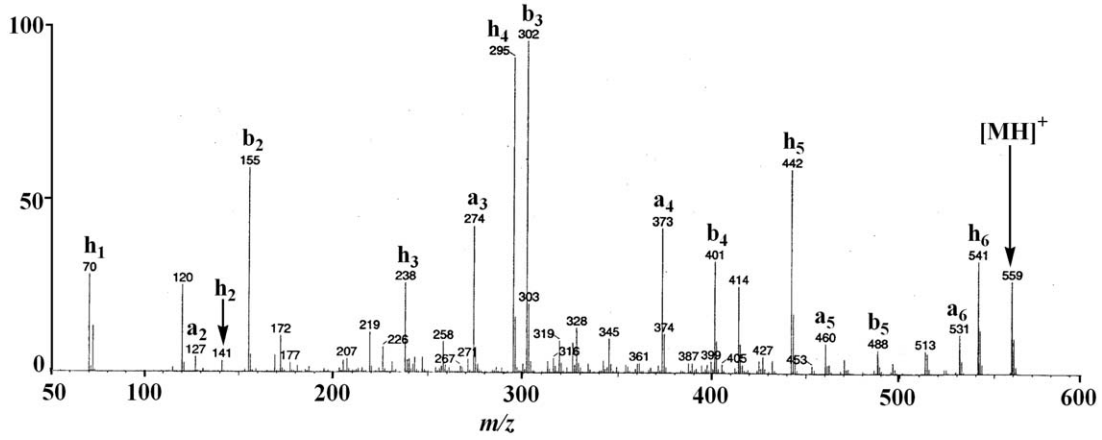


Fig. 2. MS/MS fragmentation of the protonated annomuricatin C (**1**) ion: (**A**) fragmentation at the Ala–Pro amide bond level; (**B**) fragmentation at the Val–Ser amide bond level.

Table 1
^{13}C and ^{1}H NMR data for annomuricatin C (**1**) (DMSO d_6, 318 K)

Residue	δ_C	δ_H	*m*	*J* (Hz)	$-\Delta\delta/\Delta T$ (10^{-3} ppm K^{-1})
Pro[1] CO	171.8	—			
α CH	61.4	4.12	dd	5.6, 5.6	
β CH_2	28.7	2.09	m		
	—	1.83	m		
γ CH_2	25.2	2.05	m		
	—	1.84	m		
δ CH_2	47.0	3.77	m		
	—	3.49	m		
Gly[2] CO	168.0	—			
NH	—	8.87	dd	8.6, 4.1	5.3
α CH_2	42.3	3.83	dd	17.1, 8.6	
	—	3.31	dd	17.1, 4.1	
Phe[3] CO	169.7	—			
NH	—	8.23	d	9.5	0.9
α CH	53.7	4.61	m		
β CH_2	38.7	3.26	m		
	—	2.62	dd	12.7, 11.0	
1′	137.5	—			
2′, 6′	129.7	7.23	m		
3′, 5′	127.9	7.21	m		
4′	128.8	7.22	m		
Val[4] CO	170.8	—			
NH	—	8.03	d	8.5	3.5
α CH	62.2	3.91	dd	9.8, 8.5	
β CH	29.4	2.07	m		
γ CH_3	19.5	0.86	d	6.5	
γ′ CH_3	19.3	0.88	d	6.6	
Ser[5] CO	168.8	—			
NH	—	8.12	d	9.1	3.5
α CH	54.8	4.28	m		
β CH_2	61.0	3.74	m		
	—	3.54	m		
Ala[6] CO	170.5	—			
NH	—	7.42	d	7.3	–0.3
α CH	46.3	4.59	dd	7.3, 6.9	
β CH3	17.5	1.23	d	6.9	

conformational information can interfere with sequential ones. All the amino acid spin systems were identified using scalar spin–spin couplings determined from the ^{1}H–^{1}H COSY and TOCSY experiments [12]. The ^{13}C-NMR assignments of the protonated carbons were obtained from the proton detected heteronuclear HSQC spectrum and combined with the HMBC experiment optimised for a long-range *J*-value of 7 Hz, for the non-protonated carbons. This experiment allowed the carbonyl groups to be assigned. By this way, the sequence determination was done from the connectivities between the carbonyl of residue *i* with the amide and/or α protons of residue *i* + 1 (Fig. 3). The $^{3}J_{CH}$, CO (*i*) to NH (*i*+1) correlations depicted on the HMBC spectrum are shown in Fig. 4. As examples, the CO group of Gly[2] at δ 168.0 was correlated to both αH and α'H of Gly[2] and NH of Phe[3], the CO of Ser[5] at δ 168.8 to the αH and NH protons of both Ser[5] and Ala[6], and the CO of Pro[1] at δ 171.8 to the α, α'-H and NH of Gly[2] (Figs. 3 and 4).

The ROESY spectrum clearly showed only two $d_{NN(i,i+1)}$ interactions between Gly[2] and Phe[3], and be-

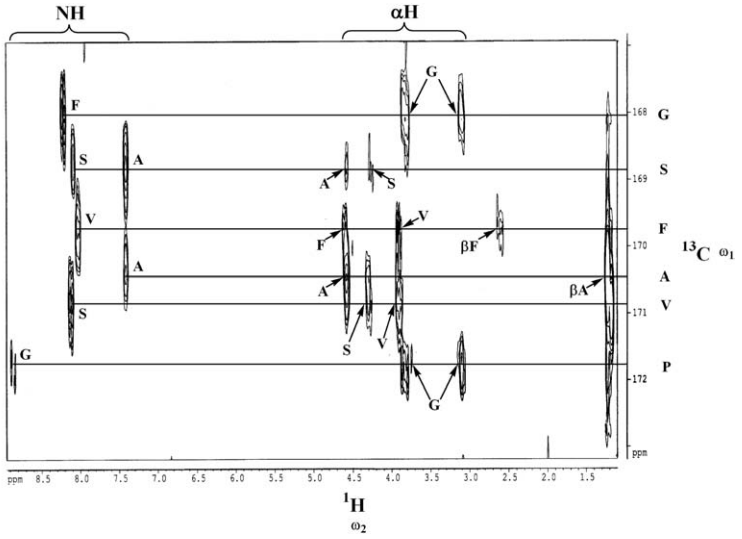


Fig. 3. HMBC spectrum of annomuricatin C (**1**) CO regio-correlations.

tween Ser^5 and Ala^6. A stretch of $d_{\alpha N(i,\ i+1)}$ sequential connectivities was depicted from Pro^1 to Ala^6 (Fig. 4). The α proton of Ala^6 gave a strong NOE correlation with the δ proton (3.77) of Pro^1 and a weaker with the geminal δ' proton (3.49), closing the cyclohexapeptide ring and indicating that the Ala^6-Pro^1 amide bond was *trans*. Chemical shifts of the β and γ carbons of Pro^1 at 28.7 and 25.2 ppm, respectively, gave further evidence for the presence of a *trans*-Pro amide bond [13]. The prochiral assignment of the β, γ and δ protons of Pro resulted of the observed NOEs in the ROESY spectrum. In addition to the sequential NOEs, some other interactions with conformational significance were depicted: the NH proton of Ser^5 was strongly correlated to the β-proton of Val^4, and the β-methyl of Ala^6 to the δ'-proton of Pro. The whole data agreed with the cyclic structure **1** for annomuricatin A, the sequence of which was thus determined as *cyclo*(Pro^1-Gly^2-Phe^3-Val^4-Ser^5-Ala^6-).

Cyclic hexapeptide backbone rings are constrained with two β-turns generally stabilised by two transannular hydrogen bonds involving the two residues joining the β-turns and forming a short antiparallel β-sheet arrangement. Analysis of the NMR data of annomuricatin C suggested a constrained structure, leading to a main conformation.

All the $^3J_{NH-H\alpha}$ coupling constants had high values, between 7.3 and 9.5 Hz, in agreement with β-turn and β-sheet structures. In a β-turn consisting of four residues numbered i to i+3, a *trans*-Pro^1 can only be located at the *i*+1 position, and as a result the following residue (Gly^2) at the *i*+2 position. This is corroborated by the NOEs observed between NH–Phe^3 (*i*+3 position) and both NH- and αH-Gly^2, and between NH–Gly^2 and αH–Pro^1. This first β-turn with Pro^1 and Gly^2 at the corners has thus the characteristics of a type II β-turn (Fig. 5). The second β-turn has Val^4 and Ser^5 at the corners and can be defined as a type-I β-turn. This is deduced from the strong $d_{Ni,\ Ni+1}$ and $d_{\alpha i,\ Ni+1}$ interactions observed between Ser^5 and Ala^6 and the NOEs observed between NH-Ser^5 and α-H and β-H of Val^4. Confirmation of the conformation shown in Fig. 5, arose from the NOESY correlations between NH–Val^4 and α-H and β-H of Phe^3, and between NH–Ala^6 and β-H of Phe^3.

The temperature coefficients $\Delta\delta/\Delta T$ were measured between 298 and 328 K in DMSO-d$_6$ (Table 1). The observed variations were linear and indicated that two amide groups (Phe^3 and Ala^6) with temperature coefficients between –0,9 and +0,5 × 10^{-3} ppm K^{-1} were involved in intramolecular hydrogen bonding, whereas the three other, especially that of Gly^2 ($\Delta\delta/\Delta T$: 10^{-3} ppm K^{-1}) were solvent exposed, in agreement with two hydrogen bonds between CO–Ala^6 and NH–Phe^3 and between CO–Phe^3 and NH–Ala^6. The conformation of annomuricatin C, as typically observed for the cyclohexapeptide rings, appeared to be formed by a short antiparallel β sheet, stabilised by two hydrogen bond-

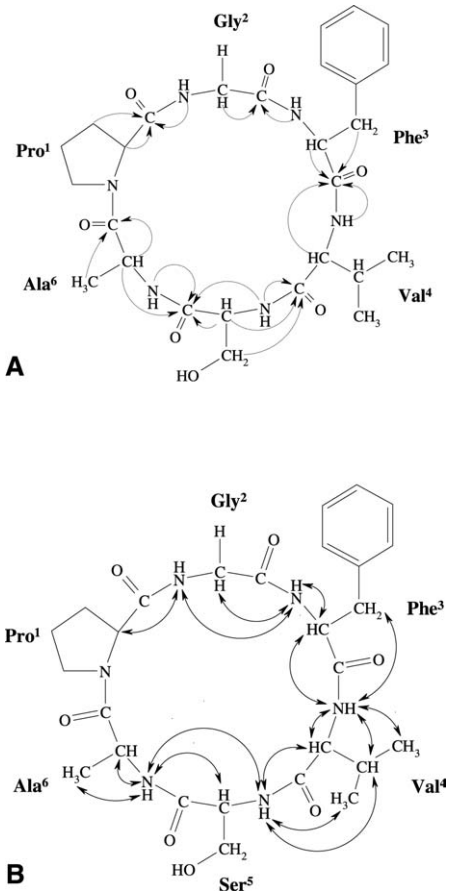


Fig. 4. (**A**) HMBC and (**B**) NOEs correlations for annomuricatin C (**1**).

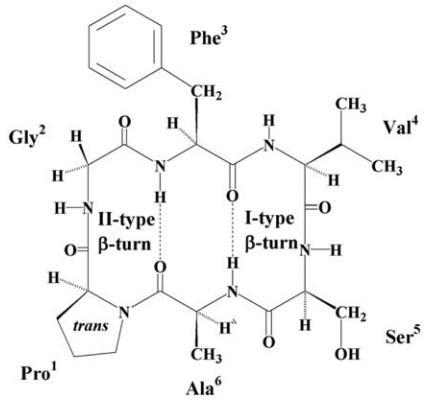


Fig. 5. Solution conformation suggested for annomuricatin C (**1**).

ing and two β-turns, a type-II turn depicted for Pro[1]-Gly[2] and a type-I turn for Val[4]–Ser[5] (Fig. 5).

It is remarkable that the peptides isolated from the Annonaceae family, hexa- to nonapeptides, enclose at least one proline residue. As usual, this residue induces, in such small-size cyclic peptides, conformational constraints. In addition, its presence is useful, as it favours the formation of one linearised peptide, the mass fragmentation of which allows the sequence to be unambiguously determined.

3. Experimental section

Optical rotation was measured with a Perkin-Elmer model 341 Polarimeter and the $[\alpha]_D^{22}$ values are given in deg cm^2 g^{-1}. The melting point was determined on a Büchi melting point B-545 apparatus. ^{1}H and ^{13}C NMR spectra were recorded with either (1D ^{13}C) a Bruker AC 300 spectrometer, equipped with an Aspect 3000 computer using DISNMR software or (2D spectra) a Bruker Advance 400 spectrometer operating at 400.13 MHz (2D spectra). The coupling constant used to establish the necessary delay for the selection of the proton coupled to the carbon in the HSQC spectrum was 135 Hz, corresponding to a delay of 3.7 ms; the delay for the HMBC spectra was 70 ms, corresponding to a long-range coupling constant of 7 Hz. The phase-sensitive ROESY experiments were obtained with mixing times of 150 ms. Mass spectra were recorded on a MALDI-TOF or an API Q-STAR PULSAR i of Applied Biosystems. For the CID spectra, the collision energy was 40 to 60 eV and the collision gas was nitrogen.

3.1. Plant material

Seeds of *Annona muricata* L. (Annonaceae) were collected near Dakar (Senegal) in January 2000. Samples were immediately washed with distilled water and dried at room temperature. A voucher has been deposed at the National Museum of Natural History (Paris).

3.2. Extraction and isolation

The dried and powdered seeds of *A. muricata* (1.3 kg) were macerated three times with cyclohexane

(3 l) and the combined extracts yielded an oil (344 g) that was discarded. The seeds were then extracted three times with MeOH (3 l) at room temperature to give after concentration under reduced pressure the MeOH extract (89.7 g), which was partitioned between EtOAc and water. The organic phase was concentrated to dryness and the residue (15.8 g) was dissolved in MeOH and chromatographed on Sephadex LH-20 column with MeOH. The peptide fraction (15.8 g) was then repeatedly subjected to silica gel column chromatography (Kieselgel 60 H Merck) and eluted with CH_2Cl_2 containing increasing amount of MeOH from 5% to 20%.

The peptide purification was monitored by TLC (silica gel 60 F_{254} Merck) with CH_2Cl_2/MeOH 9:1 as eluent system and the peptides were detected with the Cl_2/*o*-tolidine reagent, exhibiting a characteristic blue spot with R_f 0.38. The corresponding peptide mixture was finally purified by isochratic reversed phase HPLC (Kromasil C_{18}, 250 × 7.8 mm, 5 µm, AIT France; flow rate 2 ml min^{-1}, detection 220 nm) using MeOH/H_2O: 65/35 with 1% TFA to yield annomuricatin C (**1**, t_R 7.2 min, 23.1 mg).

3.3. Absolute configuration of amino acids

Solution of **1** containing 1 mg of peptide, in 6 N HCl (1 ml), was heated at 110 °C for 24 h in sealed tubes. After cooling, each solution was concentrated to dryness. The hydrolysate was dissolved in anhydrous solution of 3 N HCl in 2-propanol and heated at 110° C for 30 min. The reagents were evaporated under reduce pressure and the residue dissolved in CH_2Cl_2 (0.5 ml) and 0.5 ml trifluoracetic anhydride was added. The mixture was kept in a screw-capped at 110 °C for 20 min, the reagents were evaporated and the mixture analysed on a Chirasil-L-Val (*N*-propionyl-L-valine-*tert*-butylamide polysiloxane) quartz capillary column with helium (1.1 bar) as carrier gas and temperature program of 50–130 °C at 3 °C min^{-1} and 130–190 °C at 10 °C min^{-1}, with a Hewlett Packard series 5890 apparatus. Comparison of R_t values with those of standards amino acids was used: L-Ala (11.6), L-Val (13.9), Gly (14.6), L-Thr (15.2), L-Pro (18.2), L-Ser (18.8), L-Leu 19.2) and L-Tyr (31.9).

3.4. Annomuricatin C (*1*)

Colourless solid, Mp 284–285 °C (MeOH), $[\alpha]_D^{22}$ –2.7° (c 0.1, MeOH) ; ^1H and ^{13}C NMR (see Table 1); ESI-QTOF, *m/z*: 597.23 $[M+K]^+$, 581.26 $[M+Na]^+$, 559.28 $[M+H]^+$; ESI-QTOF MS/MS on *m/z* 559 $[M+H]^+$ (ce 40 eV) *m/z* (%): 559 (28), 541 (34), 531 (12), 513 (9), 488 (7), 470 (4), 460 (9), 442 (61), 427 (4), 414 (28), 401 (33), 374 (11), 373 (44), 345 (10), 328 (13), 326 (9), 319 (10), 302 (100), 295 (96), 274 (44), 258 (11), 238 (27), 226 (7), 219 (12), 172 (10), 155 (61), 141 (4), 127 (5), 120 (26), 72 (13), 70 (30). ESI-QTOF MS/MS on $[M+Na]^+$ (ce 60 eV) *m/z* (%): 581 (96), 563 (100), 553 (93), 535 (73), 523 (49), 507 (20), 492 (42), 482 (75), 466 (51), 464 (49), 454 (26), 436 (36), 422 (17), 405 (4), 396 (25), 395 (95), 368 (21), 367 (93), 324 (22), 306 (7), 297 (20), 296 (91), 276 (13), 234 (11), 220 (6), 202 (6), 163 (4), 149 (2).

Acknowledgements

The French 'Ministère de la Coopération' (EGIDE) is gratefully acknowledged for a fellowship for one of us (AW), and the 'Région Île-de-France' for its generous contribution to the funding of the 400-MHz NMR and the ESI-TOF mass spectrometers. We wish to thank M. A. Blond and M. L. Dubost for NMR and mass spectra contributions, respectively.

References

[1] J. Kerharo, J.G. Adam, in: La pharmacopée sénégalaise traditionnelle, Éditions Vigot Frères, Paris, 1974, p. 144.

[2] C. Baraguey, C. Auvin-Guette, A. Blond, F. Cavelier, F. Lezenven, J.-L. Pousset, B. Bodo, Isolation, structure and synthesis of chevalierins A, B and C, cyclic peptides from the latex *Jatropha chevalieri*, J. Chem. Soc. Perkin Trans. 1 (1998) 3033.

[3] C. Baraguey, A. Blond, F. Cavelier, J.-L. Pousset, B. Bodo, C. Auvin-Guette, Isolation, structure and synthesis of mahafacyclin B, a cyclic heptapeptide from the latex of *Jatropha mahafalensis*, J. Chem. Soc. Perkin Trans. 1 (2001) 2098.

[4] C. Gleye, A. Laurens, O. Laprévote, R. Serani, R. Hocquemiller, Isolation and structure elucidation of sabadelin, an acetogenin from the roots of *Annona muricata*, Phytochemistry 52 (1999) 1403.

[5] C.-M. Li, N.-H. Tan, Y.-P. Lu, H.-L. Liang, Q. Mu, H.-L. Zheng, X.-J. Hao, Y. Wu, J. Zhou, Annomuricatin A, a new cyclopeptide from the seeds of *Annona muricata*, Acta Bot. Yunnanica 17 (1995) 459.

[6] C.-M. Li, N.-H. Tan, H.-L. Zheng, Q. Mu, X.-J. Hao, Y.-N. He, J. Zhou. Cyclopeptides from the seeds of *Annona muricata*, Phytochemistry 48 (1998) 555.

[7] M. Yuan, M. Namikoshi, A. Otsuki, K.L. Rinehart, K. Sivonen, M.F. Watanabe, Low-energy collisionally activated decomposition and structural characterization of cyclic heptapeptide microcystins by electrospray ionization mass spectrometry, J. Mass. Spectrom. 34 (1999) 33.

[8] S. Lin, G. Liehr, B.S. Cooperman, R.J. Cotter, Sequencing cyclic peptide inhibitors of mammalian ribonucleotide reductase by electrospray ionization mass spectrometry, J. Mass. Spectrom. 36 (2001) 658.

[9] C. Auvin-Guette, C. Baraguey, A. Blond, S.X. Xavier, J.-L. Pousset, B. Bodo, Pohlianins A, B and C, cyclic peptides from the latex of *Jatropha pohliana* spp. *molissima*, Tetrahedron 55 (1999) 11495.

[10] K. Wüthrich, G. Wider, G. Wagner, W. Braun, Sequential resonance assignment as a basis for determination of spatial protein structure by high resolution proton nuclear magnetic resonance, J. Mol. Biol. 155 (1982) 311.

[11] K. Wüthrich, M. Billeter, W. Braun, Polypeptide secondary structure determination by nuclear magnetic resonance observation of short proton-proton distances, J. Mol. Biol. 180 (1984) 715.

[12] G. Wagner, K.W. Akumar, Systematic application of two-bidimensional ^{1}H nuclear-magnetic-resonance technique for study of protein. 2. Combined use of correlated spectrocospy and nuclear Overhauser spectroscopy for sequential assignment of backbone resonances and elucidation of polypeptide secondary structures, Eur. J. Biochem. 114 (1981) 319.

[13] D.E. Douglas, F.A. Bovey, Carbon-13 magnetic resonance spectroscopy. The spectrum of proline in oligopeptides, J. Org. Chem. 38 (1973) 2379.