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The Complete Spectral Assignment of Didemnin H, a New Constituent of The Tunicate *Trididemnum Cyanophorum* .

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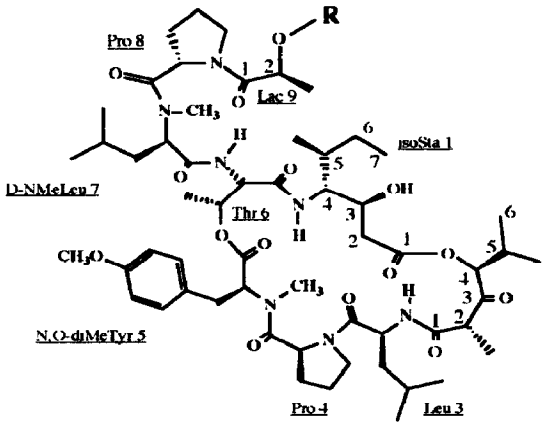
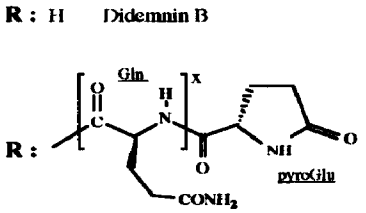
Key words : tunicate, didemnidae, *Trididemnum cyanophorum* , depsipeptide, didemnin H, NMR assignments.

Abstract : Complete ^{1}H and ^{13}C spectral assignments by 2D NMR means are presented for a new cyclodepsipeptide namely didemnin H, isolated from *Trididemnum cyanophorum* (Didemnidae).

Didemnins are a class of depsipeptides isolated from Caribbean tunicates of the Didemnidae family showing antitumor[1] and antiviral[2] properties and a potent immunosuppressive activity[3]. Didemnin B, the most prominent member of this peptidic group, was first isolated by Rinehart and his coworkers in 1981[4], then later by Van der Helm[5] and Fenical[6] from *Trididemnum solidum* and by Guyot[7] and us[8] from *Trididemnum cyanophorum* (an ascidian living in symbiotic association with an unicellular alga - Cyanophyta - *Synechocystis trididemni*)[9]. This family of cyclodepsipeptides has numerous members such as didemnins A, C , D, E and nordidemnins [4,10]. More recently, continued examination provided three new congeners from





Structures of didemnins B, H, E and D.

Trididemnum solidum : didemnins G, X, Y[11,12] and, from *Aplydium albicans* (Polyclinidae), a Mediterranean tunicate, dehydrodidemnin B[13,14]. The structural elucidation of most of these compounds was mainly based on HRFAB mass spectrometry. We report here the structural elucidation of didemnin H and the complete ^{1}H and ^{13}C NMR spectral assignments by means of a combination of mass spectrometry and one and two-dimensional NMR experiments.

Didemnin H (7 mg, 0.0011 % dry weight), was isolated from continued examination of the *Trididemnum cyanophorum* ether extract. This compound was purified by lowbar silica gel chromatography (hexane/ether/methanol) followed by C_{18} RP HPLC - 24/76 H_2O, CH_3COONH_4 (0.05 M) / MeOH -.

The molecular formula $C_{67}H_{102}N_{10}O_{19}$ is consistent with the molecular ion $(M+H)^+$ at m/z 1351 (FAB) together with ^{13}C NMR spectrum. The ^{13}C and DEPT spectra of didemnin H, show 67 unique resonances with supplementary resonances of four methylenes, two methines and four carbonyl carbons in comparison with didemnin B. FAB mass spectral analysis shows close similarities to those of didemnin B up to and including a positive ion at m/z 1112 (didemnin B + H). The N-terminus fragmentation leads to an useful acylium ions serie that defined the side chain. This serie begins at 1351 amu's and displays successive losses of 111 amu's (m/z 1240), 128 (m/z 1112), followed by well-known losses[15] of 72 (m/z 1040), 97 (m/z 943) and 127 (m/z 816) respectively for Lac, Pro and NMeLeu residues. The consecutive losses of 111 and 128 amu's can be ascribed to respectively cyclized glutamyl - pyroglutamyl (pyroGlu) - and glutamine (Gln) residues, as already described by J. B. Gloer[10] for didemnins E and D.

The NMR spectra of didemnin H are taken at 400 MHz in Pyr-D_5 which gives the best dispersion for ^1H NMR analysis (see table 1). One set of resonance is observed for each residue from which we conclude that one conformation is strongly dominant in this solvent. Structural elucidation of didemnin H spin systems is obtained through a study of 2D homo and heteronuclear NMR experiments. The DQF-COSY allows a straightforward identification of spin systems of the didemnin B amino-acid residues (isoSta, Hip, Leu, two Pro, N,O-diMeTyr, Thr and NMeLeu). Two additional spin systems corresponding to Gln and pyroGlu are also evident from this spectrum ; starting at the NH Gln resonance at 9.48 ppm leads to the subsequent cross-peak at 5.02 ppm (CαH), the CβH and CβH' resonances at 2.42 and 2.57 ppm and finally the $C\gamma H_2$ cross-peak at 2.70 ppm. Likewise starting with the NH pyroGlu at 9.09 ppm, one can follow the trace to the CαH cross-peak at 4.48 ppm, the $C\beta H_2$ at 2.33 ppm and the CγH and CγH' respectively at 2.22 and 2.50 ppm. The NH-NH cross-peak between the Gln NH_2 protons (7.79 and 8.17 ppm) is also observed in the same spectrum. In the 2D HOHAHA spectrum, recorded in phase sensitive mode with a mixing time of 100 ms, the spin systems of Pro 4, Pro 8, Gln 10 and pyroGlu 11 can be discriminated. Slices taken at the chemical shift of the α proton of these four residues give subspectra that identify the associated β and γ protons (16 protons in a range of 1.8 to 2.7 ppm) allowing unambiguously their assigments. The sharpened resonance assigned to the CH-O proton of Lac is shifted downfield relatively to its position in didemnin B (5.38 vs 4.53 ppm), while the shift of the CH-O proton signal of isoSta is unchanged (4.76 vs 4.79 ppm) establishing confidently the acylation of the hydroxyl group of the Lac residue. Coupling constants mentioned in table 1 are extracted from 1D ^1H NMR and 2D J resolved spectra.

Applying the inverse H,C-COSY, HMQC for direct $^1J_{H\text{-}C}$ connectivities and HMBC for long range $^nJ_{H\text{-}C}$ (optimized for J = 8 Hz) couplings, we assigned all C-resonances and especially carbonyl, NMe and OMe carbon resonances. Long range $^2J_{H\text{-}C}$ and $^3J_{H\text{-}C}$ correlations NH-Cα, NH-Cβ, NH-Cγ, Hα-CO, Hα-Cβ, Hγ-Cα, Hγ'-Cα, Hγ-CδO and Hγ'-CδO inside the pyroGlu residue confirm the structure and the ^{13}C assignments of this terminal amino-acid. In the same way NH-Cα, Hα-CO, Hα-Cβ, Hα-Cγ, Hγ-CδO correlations are observed in the Gln residue. A cross-peak correlation between Hα Lac and CO Gln and a second one between NH Gln and CO pyroGlu give sequential informations. The sequence of the cyclic peptide is found to be identical to didemnin B by ROESY experiment (performed with a 250 ms mixing time in a phase sensitive mode). For instance, ROE cross-peaks CH_3 Lac - Hβ Gln and NH Gln - Hα pyroGlu confirm the

Table 1. ^{1}H and ^{13}C NMR Chemical Shifts of didemnin H in C_5D_5N (293 K).

Residue Group		^{13}C-RMN (ppm)	^{1}H-RMN (ppm)	mult	J (Hz)
isoSta 1	NH	-	7 63	d	9.5
	C4H	56.09	4.66	m	-
	C5H	34.40	2.51	m	-
	$C6H_2$	28 22	1.44 . 1.71	m	-
	$C7H_3$	12 37	1 09	dt	7 7.5
	CH_3-C5	14 67	1.17	d	7
	C3H	67.03	4 76	m	-
	$C2H_{a/b}$	40 89	3.03 / 4.27	dd / d	18 10 / 18
	C1O	172 50	-	-	-
Hip 2	C4H	80 69	5.66	d	7
	C5H	30.40	2.46	m	-
	$C6H_3$	19 00	0.81	d	7
	CH_3-C5	16.83	0.85	d	7
	C3O	205.51	-	-	-
	C2H	49.56	4 66	d	7
	CH_3-C2	16.06	1 74	d	7
	C1O	169.92	-	-	-
Leu 3	NH	-	8.57	d	9 5
	CαH	49.89	5 20	ddd	9.5 10.5 4
	$CβH_2$	41.94	1 81 / 1 57	m / m	-
	CγH	25 17	1.80	m	-
	CδH_3	23.69	0 81	d	7
	Cδ'H_3	21.09	0.97	d	6 5
	CO	171 20	-	-	-
Pro 4	CαH	57 65	4.74	m	-
	$CβH_2$	27.75	1 68 / 1 85	m / m	-
	$CγH_2$	24.76	1.51 / 1.84	m / m	-
	$Cδ H_2$	47.08	3.37 / 3.53	m / m	-
	CO	170.81	-	-	-
N,O-diMeTyr 5	CH_3-N	38.55	2.59	s	-
	CαH	65.88	4.22	dt	10.5 4.5
	$CβH_2$	34.57	3.49 / 3.60	m / m	-
	Cγ	130.67	-	-	-
	CδH	131.04	7.29	d	9
	CεH	114 41	7.01	d	9
	Cζ	159.05	-	-	-
	CH_3-O	55.20	3.71	s	-
	CO	169.17	-	-	-

Residue Group		^{13}C-RMN (ppm)	^{1}H-RMN (ppm)	mult.	J (Hz)
Thr 6	NH	-	8.02	d	6.5
	CαH	58.31	5.17	dd	6.5 2.5
	CβH	71.14	5.51	dq	6.5 2.5
	CH_3-Cβ	16.83	1.81	d	6.5
	CO	169.88	-	-	-
D-NMeLeu 7	CH_3-N	31.22	3.17	s	-
	CαH	54.94	5.74	dd	11 3
	$CβH_2$	36.62	1.73 / 1 97	m / m	-
	CγH	24.86	1 47	m	-
	CδH_3	23.50	0.86	d	7
	Cδ'H_3	21.40	0.94	d	7
	CO	172.46	-	-	-
Pro 8	CαH	56.79	4.81	m	-
	$CβH_2$	28 79	1.87 ' 2.03	m m	-
	$CγH_2$	25.77	1.79 / 2.00	m / m	-
	$Cδ H_2$	47.46	3.57 / 3 79	m / m	-
	CO	173.45	-	-	-
Lac 9	C2H	69.46	5 38	q	6.5
	CH_3-C2	15.88	1 44	d	6 5
	C1O	169.41	-	-	-
Gln 10	NH	-	9 48	d	6.5
	CαH	53.00	5 02	m	-
	$CβH_2$	27.18	2.42 / 2.57	m / m	-
	$CγH_2$	32 17	2.70	m	-
	CδNH_2	175.09	8.17 / 7.79	bs / bs	-
	CO	172.19	-	-	-
pyroGlu 11	NH	-	9.09	bs	-
	CαH	57.01	4.48	dd	13 1.5
	$CβH_2$	26.21	2.33	m	-
	$CγH_2$	29.92	2.22 / 2.50	m / m	-
	Cδ O	178.40	-	-	-
	CO	173.70	-	-	-

terminal part of the side chain - Lac - Gln - pyroGlu. The NH Gln - Hα Gln correlation observed in the ^1H-^1H DQF-COSY and confirmed by selective irradiation ($^3J_{NH-H\alpha}$ = 6.5 Hz) together with a negative ninhydrin test for didemnin H establish the Gln peptide linkage as α type (a γ linkage would lead to a primary amine giving a positive ninhydrin reaction).

Hydrolysis of didemnin H followed by Marfey's derivatization[16] and HPLC analysis assign Leu 1, Pro 4, N,O-diMeTyr 5, Thr 6, Pro 8 as L, N-MeLeu 7 as D and isoSta 1 as 3S, 4R, 5S, as for didemnin B. A L configuration is found for the additional pyroGlu and Gln residues by the same workup. This result is established by the occurence of two equivalent of L glutamic acid which arise for Gln and pyroGLu residues upon hydrolysis. The strereochemistry of the Hip 2 residue is assumed to be the same as in didemnin B (2S, 4S) due to very similar ^{13}C and ^1H chemical shifts of the Hip unit in both didemnins (maximum difference 0.12 ppm for C3 Hip and 0,06 ppm for C2H Hip).

Didemnin H (0,74 μM) elicits a positive response with the HPLC procedure described by Pezzuto[17] to detect compounds capable of interacting with DNA. The new didemnin is found to reduce (60% - 70 %) the DNA peak while didemnin A (1,06 μM) and B (0,90 μM) show negative responses. Conformational informations will be described in a following paper as well as more detailed biological activity.

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