Pergamon

Tetrahedron 55 (1999) 9559–9574

TETRAHEDRON

[Hysp^2] and [Hap^2]didemnin B, two new [Hip^2]-modified Didemnin B from the Tunicate *Tridemnum cyanophorum*

Bernard Banaigs *, Eliane Abou Mansour, Isabelle Bonnard ☒, Anna Boulanger ☒, Christian Francisco.

Centre de Phytopharmacie, URA CNRS 461, Université de Perpignan, 66860 Perpignan cedex (France).

Received 4 January 1999; accepted 11 June 1999

Abstract : The structures of two new didemnins ([Hysp^2]didemnin B and [Hap^2]didemnin B) from the Aplousobranch ascidian *Tridemnum cyanophorum* (Didemnidae) are described. Structures are determined by a combination of mass spectrometry and one and two-dimensional high-field NMR techniques. Complete ^{1}H and ^{13}C NMR spectral assignments and conformational informations are presented. The cytotoxicities of the compounds towards drug-sensitive and drug-resistant tumor cell lines are compared with those of didemnins A and B.

Keywords : *Marine metabolites ; depsipeptides ; mass spectrometry ; NMR.*

INTRODUCTION

Tunicates are an increasingly popular target for study since the discovery, in 1981, of the antiviral and antitumor didemnins.[1] In a previous publication,[2] we described the complete structure and a conformational study of didemnin B [**1**]. This compound, a cyclic depsipeptide, originally characterized by K.L. Rinehart *et al.* [3] from a Caribbean tunicate *Tridemnum solidum* , was isolated as the major compound in the symbiotic association between a didemnid ascidian *Tridemnum cyanophorum* (Didemnidae) and an unicellular alga (Cyanophyta) *Synechocystis trididemni* .[4]

The unique pharmacological activities of didemnin B[5] as anticancer, antiviral and immunosuppressive agent has stimulated the interest of different groups in the total synthesis of this natural product [6] and several other analogues.[7] Recent Phase I and Phase II clinical and pharmacological studies indicate that didemnin B has little or no significant antitumor activity in some of the common carcinomas and showed significant toxic side effects such as nausea, vomiting and neuromuscular toxicity.[8] Despite the actually limited clinical promise of didemnin B as anticancer agent, there is still widespread interest in didemnins. Didemnin B has several potent biological activities apparently mediated by distinct mechanisms ; C.M. Crews et *al.* have begun to dissect the mechanisms involved in the cytostatic and immunosuppressive activities of didemnin B, observed at low concentrations.[9] A detailed knowledge of the structures and conformations of didemnin analogs is needed to develop the understanding of the molecular basis of their action and the vast differences in activity between the various members of the family.

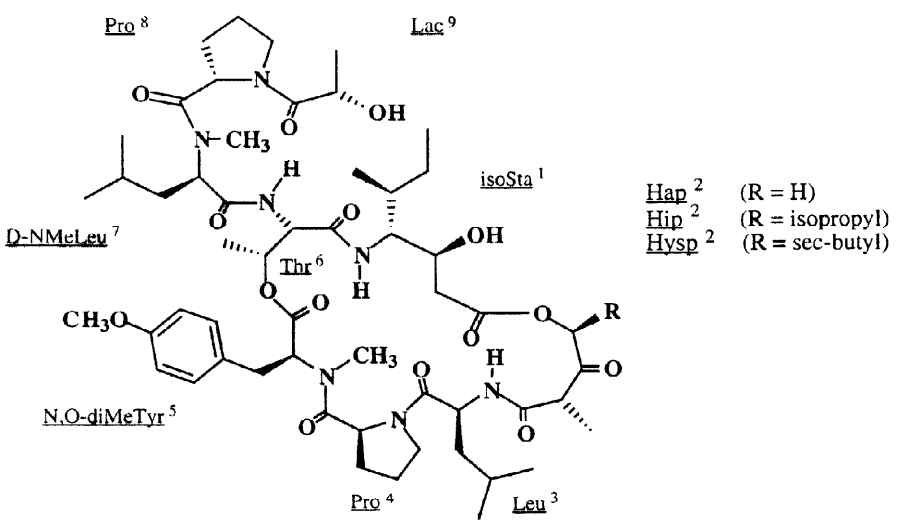
* Corresponding author : Dr Bernard Banaigs ; Fax : (33) 468662223 ; E-mail : banaigs@univ-perp.fr
☒ Present address : I. Bonnard : LCSNSA, Université de la Réunion, BP 7151, 97715 St Denis cedex 2 (France).
A. Boulanger : Université de Haute Alsace, Ecole Nationale Supérieure de Chimie de Mulhouse, 68093 Mulhouse (France)

0040-4020/99/$ - see front matter
PII: S0040-4020(99)00524-4

Didemnin B was also isolated and characterized by M. Guyot *et al.* [10] and T. McKee *et al.* [11] respectively from *Tridemnum cyanophorum* and *Tridemnum solidum*. These autors based the structural elucidation mainly on high-field NMR studies when the structural assignments of didemnins by K.L. Rinehart rested predominantly on high resolution FAB mass spectrometry.[12] Continued examination of a large *Tridemnum solidum* extract by K.L. Rinehart and co-workers provided three new congeners : didemnins G, X and Y, [13] whereas [pyruvyl^9]didemnin B [14] was curiously discovered in a Mediterranean tunicate *Aplidium albicans* (Polyclinidae). R. Sakai *et al.* [15] described didemnins M and N, nordidemnin N, epididemnin A_1 and acyclodidemnin A. Didemnin M was previously described as didemnin H by A. Boulanger *et al.* [16] whereas complete ^{1}H and ^{13}C NMR spectral assignments of [Tyr^5]didemnin B (described by R. Sakai as didemnin N) and [D-Pro^4]didemnin B were later reported by E. Abou-Mansour *et al.*.[17] M. Searle [18] and H. Kessler [19] fully determined the solution conformation of didemnins A and B by NMR spectroscopy and computer calculations, in comparison with the structure of didemnins A and B in the crystal obtained by an X-ray analysis.[20] The solution structure of [Me-L-Leu^7]didemnin B was compared to that of didemnin B [21] and an X-ray crystal analysis of the cyclic depsipeptide backbone of the didemnins was also obtained [22] and compared with that of didemnin B.

Some structure-activity relationships of didemnins were reported by Jouin and co-workers,[6] Kessler *et al*[21] and Joullié and co-workers.[23] More recently the Illinois group [24] reported systematic studies on the bioactivities of 42 didemnin congeners in which the authors discussed the structural requirements of these peptides regarding cytotoxicity, immunosuppression and antiviral activities. In this work three [Hip^2]-modified didemnins were described and evaluated.

We now wish to report the structure elucidation of [Hysp^2]didemnin B [**2**] and [Hap^2]didemnin B [**3**] (Figure 1) by means of a combination of mass spectrometry and one and two-dimensional NMR techniques as well as complete ^{1}H and ^{13}C NMR spectral assignments and conformational informations. Using a drug-sensitive human leukemic cell line (CCRF-CEM) and sublines that express drug resistance associated with either Pgp (CEM/VLB$_{100}$) or altered DNA topoisomerase II (CEM/VM-1), we also compare the growth-inhibitory properties of these two [Hip^2]-modified didemnins with that of didemnin B and [Hip^2-oxime]didemnin B [**4**] to examine the structure-activity relationships in this region of the molecule. The aim of this work was to generate structural information that would be useful in defining the relationship between structure, conformation and activity.



[**1**] = didemnin B : R = isopropyl
[**2**] = [Hysp^2]didemnin B : R = sec-butyl
[**3**] = [Hap^2]didemnin B : R = H

Figure 1 : Structures of didemnin B, [Hysp^2]- and [Hap^2]didemnin B.

Isolation

Collection and extraction of *Tridemnum cyanophorum* and initial separation of the Et_2O extract were described in the previous papers.[2,16,17] Further examination of the 29 silica gel chromatography (hexane, Et_2O, MeOH) fractions obtained from the Et_2O extract, by C8 RP HPLC (75/25 MeOH/H_2O 1‰ TFA) yielded in fraction 22 two HPLC pure peaks. NMR studies indicated that peak 1 was didemnin B (short form : did.B) (retention time : 16.06 min) and peak 2 was [Hysp^2]didemnin B (short form : [Hysp^2]did.B) (7 mg, retention time : 20.30 min). [Hap^2]didemnin B (short form : [Hap^2]did.B) was isolated (4 mg) from fraction 23 peak 1 (retention time : 10.08 min) whereas peaks 2 (retention time : 11.42 min) and 3 (retention time : 12.05 min) yielded nordidemnin A and didemnin A. [Hysp^2]did.B and [Hap^2]did.B were obtained as colourless amorphous solids and were negative to ninhydrin test suggesting a blocked N-terminus.

[Hysp^2]didemnin B : structure elucidation

This new metabolite exhibited very similar spectral data (^1H, ^{13}C NMR and FABMS) to didemnin B but differed by 14 amu's due to a supplementary methylene group in the Hip residue - namely Hysp = α-(α-hydroxysecbutylacetyl) propionyl -.

Mass spectrometry : The positive ions in FAB mass spectrum of [Hysp^2]didemnin B gave structural informations which clearly showed the close relationship between [Hysp^2]did.B and didemnin B. Nearly all of the abundant ions could be assigned structures which resulted directly from cleavage of the peptide backbone. Generalizations about typical fragment ions, were drawn from an investigation of spectra of didemnins A, B, C, D, E, H, nordidemnin B, [D-Pro^4]did.B and [Tyr^5]did.B and applied to interpreting the spectrum of [Hysp^2]did.B. FAB mass spectral data for didemnins A, B, nordid.B, [Tyr^5]did.B, [Hysp^2]did.B and [Hap^2]did.B are summarized in Table 1. Nomenclature [25] and mechanisms of formation [26] for the peptide fragment ions observed have been already discussed. A pattern of cyclic peptides fragmentation was established [27] that was described by the initial protonation of an amide nitrogen, scission of the N-acyl bond and subsequent fragmentation by loss of amino acid residues (successively or competitively) from the ring-opened acylium ion.

The parent ion at m/z 1126 $(M+H)^+$, one of the most prominent ions in the spectrum, was consistent with the formula $C_{58}H_{91}N_7O_{15}$ (one extra methylene group as compared with didemnin B). In the higher mass region, ions resulting from the losses of H_2O and CO (characteristic of a cyclic peptide) were observed in very weak abundance (<2%).

Owing to the increased basicity of the N-alkylated amide nitrogen, such as Pro^4, Pro^8, D-NMeleu^7 and N,O-diMeTyr^5 in [Hysp^2]did.B, protonation and cleavage of these amide bonds were a highly favoured process. Thus protonation on the Pro^4 peptide nitrogen was followed by ring cleavage to form the linear acylium ion (Figure 2) with Pro^4 at the N-terminus. This acylium ion underwent a C-terminus fragmentation via a double hydrogen rearrangement (Mc Lafferty + 1 rearrangement, characteristic decomposition of esters), leading to the fragment ion m/z 861 (4 %), a useful structural information corresponding to the loss of Hysp—Leu. Moreover this assignment could be verified by observing mass shifts in the fragmentation pattern of the didemnin homologues.

The N-terminus fragmentation led to two acylium ions series [*]. The first one that defined the side chain of [Hysp^2]didemnin B, began at m/z 1126 (15 %), and displayed successive losses of Lac^9 m/z 1054 (2 % ; Y''_8 fragment), Pro^8 m/z 957 (5% ; Y''_7 fragment) and D-NMeLeu^7 m/z 830 (2 % ; Y''_6 fragment). The corresponding B_1 (Lac^9), B_2 (Lac^9—Pro^8) and B_3 (Lac^9—Pro^8—D-NMeLeu^7) acylium series were observable

[*] Cleavage of these bonds can occur by another pathway : direct protonation of the side chain of [Hysp^2]didemnin B.

at m/z 73 (4 %), 170 (15 %) and 297 (7 %). The second one began at m/z 307 and determined the Pro^4—N,O-diMeTyr^5 .

Table 1 : Sequence Ions Observable in the FAB Mass Spectra of Didemnins : m/z (relative intensity %)

Fragment	Did.A	Did.B	Nordid. B	[Tyr^5]did.B	[Hap^2]did.B	[$Hysp^2$]did.B
$(M+H)^+$	943 (45)	1112 (10)	1098 (12)	1084 (5)	1070 (47)	1126 (15)
$(M+H)^+ - H_2O$	—	1094 (1)	1080 (2)	—	1052 (1)	1108 (1)
$(M+H)^+ - CO$	915 (3)	—	1070 (1)	1056 (2)	1042 (1)	—
Y''_8	—	1040 (2)	1026 (2)	1012 (1)	998 (2)	1054 (2)
Y''_7	—	943 (2)	926 (8)	915 (2)	901 (2)	957 (5)
Y''_6	816 (3)	816 (3)	802 (2)	789 (1)	774 (2)	830 (2)
$(M+H)^+$ - (Hip—Leu)	692 (5)	861 (10)	847 (2)	833 (1)	861 (2)	861 (4)
B_3	—	297 (25)	297 (6)	297 (28)	297 (28)	297 (7)
B_2	—	170 (50)	170 (70)	170 (29)	170 (19)	170 (15)
Pro—N,O-diMeTyr	307 (25)	307 (5)	307 (10)	279 (6)	307 (10)	307 (2)
Acyloxy N,O-diMeTyr	210 (20)	210 (6)	210 (13)	182 (6)	210 (2)	210 (3)
Acylium N,O-diMeTyr	192 (15)	192 (3)	192 (10)	168 (4)	192 (1)	192 (2)
Iminium N,O-diMeTyr	164 (55)	164 (20)	164 (55)	136 (4)	164 (6)	164 (7)
Methoxybenzyl	121 (30)	121 (20)	121 (45)	107 (2)	121 (5)	121 (8)
Iminium Lac—Pro	—	142 (80)	142 (65)	142 (34)	142 (3)	142 (21)
Acylium NMeLeu	128 (10)	128 (5)	—	128 (6)	—	—
Iminium NMeLeu	100 (100)	100 (40)	100 (75)	100 (36)	100 (16)	100 (22)
Acylium Leu	114 (20)	114 (5)	—	114 (4)	114 (6)	—
Iminium Leu	86 (55)	86 (45)	86 (80)	86 (29)	86 (13)	86 (18)
Acylium Pro	98 (30)	—	—	98 (6)	98 (4)	—
Iminium Pro	70 (85)	70 (100)	70 (100)	70 (100)	70 (78)	70 (100)
B_1	—	73 (6)	73 (11)	73 (16)	73 (100)	73 (4)

In the medium mass region (from 310 to 890) no significant peak was observed. In the low mass region, the amide bond labilization due to the presence of N-alkylated amino acids (resulting in a weakening of both amide bonds adjacent to the N-alkylated residue) was one of the reasons for the strong abundance of iminium Leu m/z 86 (18 %) and iminium Pro m/z 70 (base peak), MeLeu m/z 100 (22 %), Lac—Pro m/z 142 (21 %) and N,O-diMeTyr m/z 164 (7 %) ions.

As the fragment ion $(M+H)^+$ - (Hysp—Leu) at m/z 861 was found at the same mass in didemnin B and in [$Hysp^2$]didemnin B, the Y''_6 ion was shifted to higher mass by 14 units. The variable residue could be either Hip or Leu, but the presence of an iminium Leu seemed to indicate that the change had taken place on the Hip residue.

Some ambiguities remained in the Hip residue related to the position of the supplementary methylene group on the C2, C4, C5, or C6 Hip and this was solved by a NMR approach.

Table 2 : NMR Assignments of [Hysp^2]did.B (**2**) and [Hap^2]did.B (**3**) Compared With Those of Did.B (**1**) in C_5D_5N (ppm).

		Didemnin B		[Hysp^2]didemnin B		[Hap^2]didemnin B	
		^1H	^{13}C	^1H	^{13}C	^1H	^{13}C
isoSta^1	NH	7.56		7.61		8.09	
	C4H	4.70	55.83	4.70	55.89	4.57	55.58
	C5H	2.56	34.32	2.56	34.36	2.32	34.46
	$C6H_2$	1.46 / 1.72	28.31	1.46 / 1.71	28.31	1.34/1.55	27.98
	$C7H_3$	1.10	12.45	1.11	12.45	0.94	11.98
	CH_3-C5	1.17	14.63	1.18	14.66	1.21	15.13
	C3H	4.79	67.02	4.82	67.02	4.74	66.09
	$C2H^r/C2H^s$	3.06 / 4.34	41.09	3.08 / 4.36	41.04	3.03/3.90	41.33
	CO		172.64		172.61		173.41
Xxx^2	C4H	5.65	80.65	5.70	80.56	4.64/4.93	68.07
	C5H	2.48	30.36	2.22	37.29		
	$C6H_3$	0.83	19.05	1.23 / 1.44	24.79		
	CH_3-C5	0.88	16.86	0.82	15.83		
	$C7H_3$			0.76	12.15		
	C3		205.63		205.77		203.86
	C2H	4.72	49.54	4.78	49.59	4.22	50.20
	CH3-C2	1.75	15.98	1.76	16.10	1.59	14.32
	CO		169.95		169.91		170.63
Leu^3	NH	8.47		8.53		8.48	
	CαH	5.19	49.89	5.19	49.93	5.13	49.75
	$C\beta H_2$	1.55 / 1.85	42.06	1.55 / 1.84	42.10	1.48/1.71	42.03
	CγH	1.79	25.24	1.83	25.25	1.82	25.42
	$C\delta H_3$	0.88	23.72	0.86	23.81	0.78	23.65
	$C\delta'H_3$	0.98	21.12	0.97	21.14	0.90	21.18
	CO		171.14		171.19		171.42
Pro^4	CαH	4.75	57.60	4.76	57.63	4.70	57.65
	$C\beta H_2$	1.65 / 1.82	27.74	1.66 / 1.85	27.76	1.56/1.84	27.49
	$C\gamma H_2$	1.53 / 1.71	24.78	1.47 / 1.88	24.79	1.66/1.94	25.06
	$C\delta H_2$	3.37 / 3.55	47.03	3.33 / 3.52	47.05	3.44/3.55	47.09
	CO		170.92		170.92		170.96
Me_2Tyr^5	NCH_3	2.64	38.57	2.62	38.58	2.64	38.65
	CαH	4.20	65.83	4.23	65.86	4.16	65.89
	$C\beta H_2$	3.52 / 3.60	34.62	3.51 / 3.62	34.63	3.41/3.54	34.47
	Cγ		130.63		130.67		130.67
	CδH	7.25	131.03	7.26	131.04	7.21	131.05
	CεH	7.00	114.43	7.01	114.45	6.94	114.47
	Cζ		159.07		159.08		159.09
	CH_3-O	3.72	55.21	3.73	55.22	3.68	55.29
	CO		169.34		169.36		169.40
Thr^6	NH	8.25		8.34		8.29	
	CαH	5.07	58.78	5.07	58.77	4.94	58.62
	CβH	5.81	71.03	5.82	71.03	5.69	71.22
	CH_3-Cβ	1.80	17.06	1.81	17.06	1.68	17.08
	CO		169.67		169.71		170.04
DMeLeu^7	NCH_3	3.24	31.33	3.25	31.34	3.13	29.96
	CαH	5.80	54.99	5.81	55.01	5.65	55.03
	$C\beta H_2$	1.72 / 2.06	36.59	1.73 / 2.04	36.63	1.65/1.92	36.45
	CγH	1.56	24.94	1.48	24.98	1.45	25.06
	$C\delta H_3$	0.86	23.57	0.89	23.61	0.77	23.45
	$C\delta'H_3$	0.97	21.43	0.99	21.44	0.87	21.49
	CO		172.58		172.59		172.97
Pro^8	CαH	4.72	56.73	4.73	56.73	4.59	56.79
	$C\beta H_2$	1.91 / 2.02	28.75	1.91 / 2.01	28.75	1.68/2.02	28.73
	$C\gamma H_2$	1.84 / 2.06	26.04	1.68 / 2.03	26.05	1.83/1.99	26.03
	$C\delta H_2$	3.67 / 3.89	47.56	3.66 / 3.92	47.58	3.61/3.84	47.55
	CO		173.75		173.77		173.70
Lac^9	C2H	4.53	66.91	4.52	66.92	4.49	66.90
	CH_3-C2	1.49	20.33	1.50	20.35	1.41	20.41
	CO		173.64		173.68		173.70

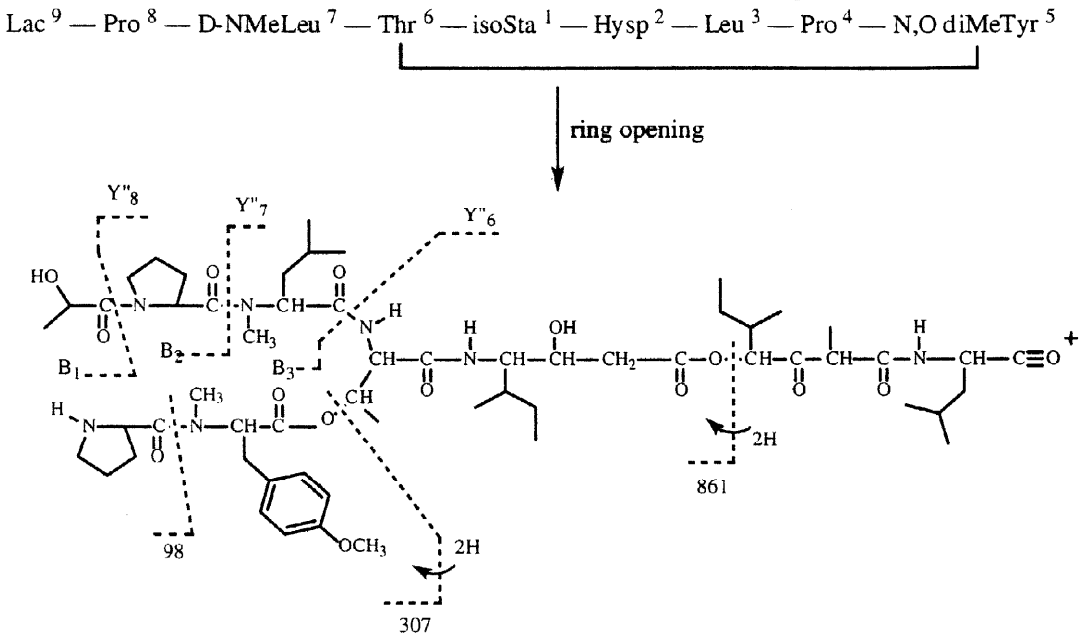


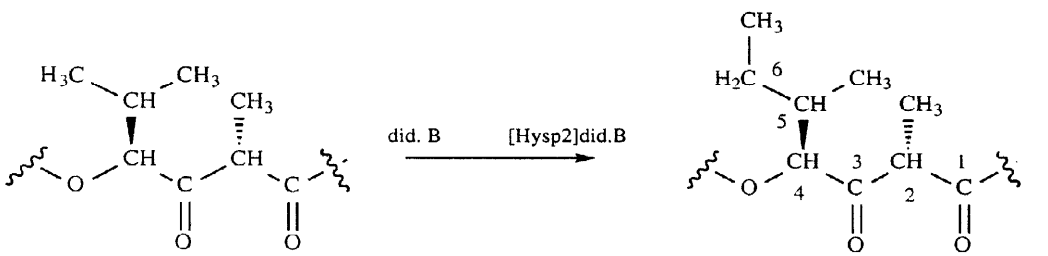
Figure 2 : Acylium ion formation and fragmentation of [Hysp^2]didemnin B

Assignment of ^{1}H NMR spectra : The ^{1}H NMR spectra of [Hysp^2]didemnin B were taken at 400 MHz in Pyr D_5. It was obvious from these well resolved spectra that one conformation strongly dominated in this solvent. Some minor peaks specially in the N-methyl region (2.5 - 4 ppm) were apparent indicating the presence of another conformation in slow exchange.

The assignment of almost all ^{1}H resonances (Table 2), mainly based on connectivity information transmitted via a double quantum filtered (DQF) ^{1}H-^{1}H COSY spectrum, was made by comparison with didemnin B spectra. In the 1D ^{1}H NMR spectrum, the close structural relationship between the two peptides was clear and only two significant differences were found out : the chemical shift of the H-C_4Hip and the replacement of the doublet methyl signal at δ 0.88 in didemnin B by a triplet methyl at δ 0.76 in [Hysp^2]did.B.
HOHAHA spectrum then allowed an unambiguous assignment of all the aliphatic side chain protons and provided some redundant informations to increase the reliability. DQF ^{1}H-^{1}H COSY experiments identified spin systems corresponding to Leu, NMeLeu, Thr, N,O-diMeTyr, 2 Pro, Lactate and isoSta residues. Due to small coupling constants, the H-Cα/H-CβLeu and H-C_4/H-C_5isoSta correlations were not observable, these two connectivities were established by the heteronuclear long-range correlation spectrum (HMBC). The COSY spectrum showed an additional spin system corresponding to a *sec*-butyl group. Beginning with the sharp doublet resonating at δ 5.70, we noted a coupling to one proton at δ 2.22 which in turn correlated with 5 other protons of a diastereotopic methylene (δ 1.23 and 1.44) and a methyl (δ 0.82). The methylene protons could finally be linked to a triplet methyl at δ 0.76. HOHAHA data confirmed this connectivity network and ROESY spectrum, performed with a 250 ms mixing time, exhibited ROE enhancements for the signals at δ 5.70 and 4.78, thus defining the α-(α-hydroxysecbutylacetyl) propionyl (Hysp) residue (Figure 3).

So as suggested by FABMS, the variable residue in didemnin B was the Hip unit which was replaced by a Hysp unit in [Hysp^2]did.B. The missing correlations in the COSY spectrum, H-Cα/H-CβLeu and H-C_4/H-C_5isoSta, were apparent in the HOHAHA spectrum. On the slice taken at the chemical shift of the NH Leu (δ 8.53), cross peaks were observed, corresponding to : H-Cα (δ 5.19), the degenerated system (strongly

overlapped) H-Cβ and H-Cγ at δ 1.83, H-Cβ' (δ 1.55), H-Cδ (δ 0.97) and H-Cδ' (δ 0.86). In the same way, starting at the NH isoSta resonance (δ 7.61), we could follow the trace to the cross peaks at δ 4.82 (H-C_3), 4.70 (H-C_4), 4.36 (H-C_2), 3.08 (H-C_2'), 2.56 (H-C_5) and 1.18 (H-C_8). Correlations to H-C_6 (δ 1.44), H-C_6' (δ 1.23) and H-C_7 (δ 0.76) could be found starting at another point of entry : H-C_5isoSta (δ 2.56). The HOHAHA spectrum was also employed to assign the complex spin systems of the two Proline residues. The N-methyl protons of the MeLeu and Me_2Tyr amino acids, protons without scalar coupling interactions, were assigned with the help of analysis of through-space interactions observed in ROESY experiment (Table 3) : the N-methyl resonance at δ 2.62 showing a ROE cross peak with the H-CαMe$_2$Tyr (δ 4.23) and the other one (δ 3.25) with H-CαNMeLeu (δ 5.81).



Hip = α-(α-hydroxyisovaleryl) propionyl

Hysp = α-(α-hydroxysecbutyl acetyl) propionyl

Figure 3 : Structure of Hysp residue.

Assignment of ^{13}C NMR spectra : In the broadband-decoupled ^{13}C NMR spectrum, taken at 100 MHz in Pyr D_5, almost all 58 carbon resonances were apparent. Only 3 signals overlapped : two aromatic C-resonances (δ 131.04 and 114.45) corresponding to respectively the Cδ, Cδ' and Cε, Cε' Me_2Tyr and one resonance at δ 24.79 representing two CH_2 groups. Nine carbonyl groups resonated in the range between 169 and 174 ppm ; the last one was found at 205.77 ppm. All the six aromatic carbons of Me_2Tyr were in the typical range 110-160 ppm, whereas 42 C-signals were detected in the aliphatic region. The multiplicity of each carbon resonance was determined with the use of the usual DEPT technique. Long range correlations between N-methyl group (δ 3.25) - CαN-MeLeu (δ 55.01) and N-methyl group (δ 2.62) - CαdiMeTyr (δ 65.86) confirmed the assignment made for these two N-methyl protons. They correlated also to the adjacent carbonyl C-atoms (respectively δ 173.77 and δ 170.92), providing the CO Pro signals assignment, but CO/H-CαPro cross peak was missing.

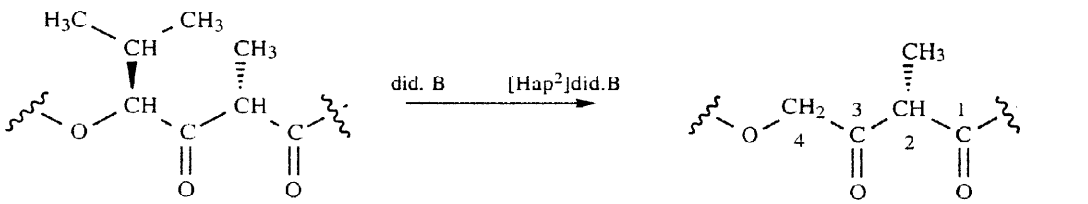
Sequence analysis and absolute configuration : Informations concerning the assignment of the two sets of ^{1}H and ^{13}C Pro signals to Pro^4 or Pro^8 were obtained by the ROESY experiment, with the cross peaks between H-CαLac^9/H-CδPro^8, H-CαPro^8/CH$_3$NMeLeu^7 and H-CαLeu^3/H-CδPro^4, H-CαPro^4/CH3NMeTyr^5. Sequential assignment of [Hysp^2]did.B, determined by interpretation of ROESY and HMBC data, was found to be identical to did.B.

Hydrolysis of [Hysp^2]did.B followed by Marfey's derivatization [28] and HPLC analysis assigned Leu, both Pro, Thr and N,O-diMeTyr as L, MeLeu as D and isoSta as 3S, 4R, 5S. The stereochemistry of the Lac residue was assumed to be L as in did.B, due to very similar ^{13}C and ^{1}H chemical shifts of this residue in both didemnins (maximum difference 0.04 ppm for COLac). In the same way, the stereochemistry of Hysp residue was presumed to be 2S, 4S as Hip in did.B : if we except the Hip-side chain, the maximum difference is 0.06

ppm in ^1H NMR for the H-C_2 and 0.14 ppm in ^{13}C NMR for the C_3 between the chemical shifts of [Hysp^2]did.B and did.B. This was conforted by the results obtained by R. Sakai [15] where significant differences in chemical shifts and coupling constants between didemnin A (2S, 4S Hip) and epididemnin A (2S, 4R Hip) were observed for the Thr-isoSta-Hip region - e.g., a difference of 0.60 ppm for the H-C_4 and 0.34 ppm for the H-C_2 the ^1H NMR and 1.6 ppm for the C_4 in ^{13}C NMR between these two epimers could be calculated -. Furthermore a detailed analysis of the ROESY spectrum showed similar Leu^{3}NH-Hysp^{2}C2H (strong), Hysp^{2}C2H-Hysp^{2}C4H (medium) and Hysp^{2}C4H-Hysp^{2}C5H (strong) correlations. The small value of the $^3J_{C4H-C5H}$ (3.5 Hz) observed for the *sec*-butyl side chain of Hysp suggested, as in didemnin B, a predominantly gauche configuration for the C4H-C5H bond but the lack of correlation between the $C6H_2$ or the $C7H_3$ and any other part of the molecule did not allow the stereochemistry determination of the C5 chiral center. The limited amount of this natural product prohibited further degradation to confirm the 2S, 4S stereochemistry and to determine the chirality at C5 of the Hysp residue.

[Hap^2]didemnin B : structure elucidation

Preliminary spectral data examination, including FAB MS, ^1H and ^{13}C NMR spectroscopy, showed that the new metabolite was a homologue of didemnin B. The molecular weight of [Hap^2]didemnin B (**3**), $(M+H)^+$ = 1070.6, agreed with a protonated molecular formula of $C_{54}H_{83}N_7O_{15}$ which was 42 amu's smaller due to the loss of the isopropyl side chain in the Hip residue - namely Hap = α-(α-hydroxyacetyl) propionyl -.



Hip = α-(α-hydroxyisovaleryl) propionyl

Hap = α-(α-hydroxyacetyl) propionyl

Figure 4 : Structure of Hap residue.

Mass spectrometry : The mass spectrum recorded on [Hap^2]didemnin B showed a $(M+H)^+$ pseudomolecular ion at m/z 1070 (Table 1). Comparison of this spectrum with those obtained for did.B and [Hysp^2]did.B showed the same B2 (m/z 170), B3 (m/z 297) and $(M+H)^+$-(Hip-Leu) (m/z 861) fragments. The Y"$_6$ ion, m/z 774, was shifted to a lower mass by 42 amu's in comparison to did.B, suggesting as in [Hysp^2]did.B, that the variable residue could be Hip. The presence of an acylium and iminium Leu seemed to indicate that no change has occurred on the Leu residue.

Assignment of ^1H NMR spectra : The ^1H NMR spectra of [Hap^2]didemnin B were taken at 400 MHz in Pyr D_5. One set of resonances was observed for each of the residues indicating that one conformation strongly dominates in this solvent. Some minor peaks specially in the N-methyl region (2.5 - 4 ppm) were apparent indicating the presence of another conformation in slow exchange. The proton resonances of [Hap^2]did.B could be assigned by using DQF ^1H-^1H COSY and HOHAHA by comparison with did.B spectra (Table 2). The assignment procedures were very similar to those described for [Hysp^2]did.B, so we shall concentrate on the differences between [Hap^2]did.B and did.B. The 1D spectrum revealed the absences of a sharp doublet signal (δ

5.65), of a multiplet signal (δ 2.48) and of a doublet methyl signal (δ 0.83 and 0.88), all assigned to the isopropyl side chain of the Hip residue in did.B. The DQF COSY experiment identified a new spin system corresponding to two mutually coupled one-proton doublets (δ 4.64 and 4.93, J = 16 Hz). This prochiral system was not assigned stereospecifically due to the lack of scalar and dipolar coupling. As suggested by FABMS, the variable residue in didemnin B was the Hip unit which was replaced by a Hap unit, α-(α-hydroxyacetyl) propionyl, in [Hap^2]did.B (Figure 4). We could also observe the downfield-shift of the amide NH resonance assigned to isoSta in did.B (δ 8.09 vs. 7.56) and a slightly upfield-shift of the H^{proS}-C2isoSta (δ 3.90 vs. 4.34).

Assignment of ^{13}C NMR spectra : In the broadband-decoupled ^{13}C NMR spectrum, taken at 100 MHz in Pyr D_5, 49 out of the 54 carbon resonances were apparent. The 5 overlapped signals were the two aromatic C-resonances (δ 131.0 and 114.4) corresponding to respectively the Cδ, Cδ' and Cε,Cε' Me_2Tyr, one resonance at δ 66.0 representing two CH (C2Lac and CαMe_2Tyr), one resonance at δ 34.5 for CH (C5isoSta) and CH_2 (CβMe_2Tyr) and one resonance at δ 26.0 for two CH_2 (CγNMeLeu and CγPro^4). The multiplicity of each carbon resonance was determined with the use of the DEPT technique and assignments (Table 2) were performed by using HMQC and HMBC experiments. The new methylene C-atom (C4Hap) was found at δ 68.1.

Sequence analysis and absolute configuration : Informations concerning the assignment of the two sets of ^{1}H and ^{13}C Pro signals to Pro^4 or Pro^8 were obtained by the ROESY experiment. Sequential assignment determined by interpretation of ROESY and HMBC data, was found to be identical to did.B.

As for [Hysp^2]did.B, hydrolysis of [Hap^2]did.B followed by Marfey's derivatization and HPLC analysis assigned Leu, both Pro, Thr and N,O-diMeTyr as L, MeLeu as D and isoSta as 3S, 4R, 5S. The stereochemistry of the Lac residue was assumed to be the same as in did.B: L-Lac, due to very similar ^{13}C and ^{1}H chemical shifts of this residue in both didemnins. In the same way, the stereochemistry of Hap residue was presumed to be 2S as Hip in did.B.

Conformational informations

Conformational informations were given by comparison of ^{1}H and ^{13}C chemical-shift values, vicinal ^{1}H-^{1}H coupling constants and temperature dependence of NH chemical-shift values between [Hysp^2]did.B/did.B and [Hap^2]did.B/did.B in C_5D_5N.

Chemical shifts and J ^{1}H-^{1}H couplings comparison : Both new depsipeptides showed very similar NMR chemical shifts to did.B for the macrocyclic part as well as for the side chains, indicating very closely related conformations.

An indication of this homology could be seen graphically in Figure 5 where the ^{1}H and ^{13}C main chain and side chain resonances of [Hysp^2]did.B were substracted from the equivalent ones of did.B. With the exception of the structurally modified valine/isoleucine side chain of Hip/Hysp residue, all the ^{1}H and ^{13}C resonances had similar chemical shifts in both did.B and [Hysp^2]did.B : the maximum difference observed was less than 0.20 ppm in ^{1}H and less than 0.10 ppm in ^{13}C.

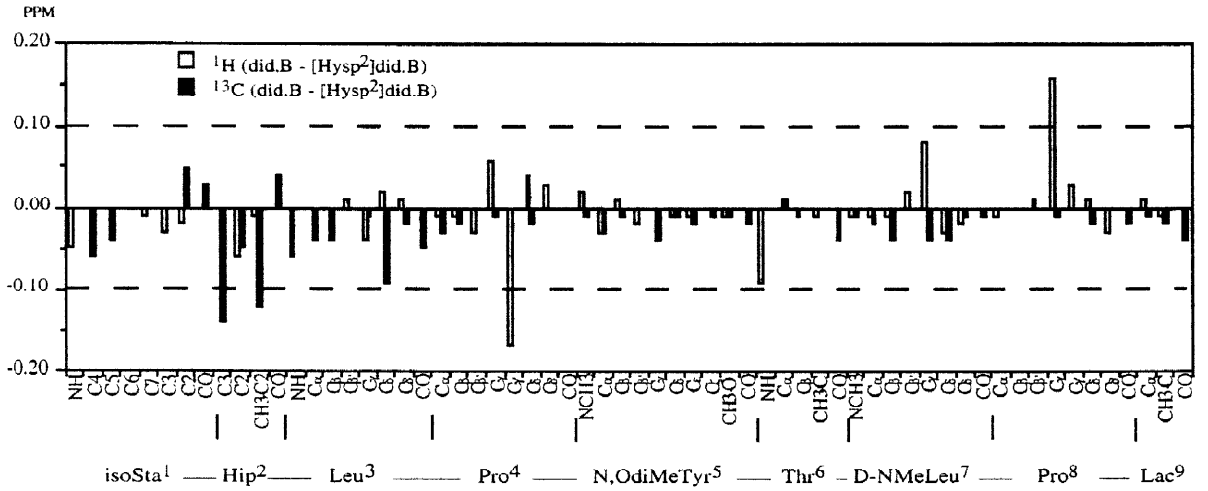


Figure 5 : Chemical shift changes, Δδ, for [Hysp^{2}]didemnin B relative to didemnin B.

Δδ is calculated by substracting the chemical shift of ^{1}H and ^{13}C resonances of did. B from that of [Hysp^{2}]did. B.

The vicinal coupling constant ^{3}J(NHCH) is related to the dihedral angle between NH and CHα by a Karplus-type relationship.[29] For [Hysp^{2}]did.B the ^{3}J(NHCH) together with ^{3}J(C2H^{r}-C3H)isoSta, ^{3}J(C3H-C4H)isoSta and ^{3}J(CHα-CHβ)Thr, useful for the backbone conformation, were extracted either directly from the 1D 400 MHz spectrum or from a 2D-J resolved experiment. All the values were similar to the corresponding ones for did.B (Table 3).

In the same way ^{1}H and ^{13}C NMR resonances were compared between [Hap^{2}]did.B and did.B (Figure 6). Except for the structurally modified Hip/Hap residue, some minor but significant differences in ^{1}H (2 protons shifted in the range from 0.3 to 0.6 ppm) and ^{13}C (3 carbons shifted in the range from 0.5 to 1.5 ppm) NMR

Table 3 : ^{3}J (H,H) Coupling Constants of Did. B, [Hysp^{2}]did. B and [Hap^{2}]did. B

	did. B	**[Hysp^{2}]did. B**	**[Hap^{2}]did. B**
^{3}J(NH-C4H)isoSta^{1}	9	9.5	11
^{3}J(C2H^{r}-C3H)isoSta^{1}	0	0	0
^{3}J(C3H-C4H)isoSta^{1}	10	10	-
^{3}J(NH-CαH)Leu^{3}	9	10	9.5
^{3}J(NH-CαH)Thr^{6}	6	5	5.5
^{3}J(CαH-CβH)Thr^{6}	1.5	2	2

resonances occurred between the two peptides. The largest changes occurred mainly for the isostatine residue (NH, Δδ -0.53 ; C3, Δδ 0.93 ; C2H^{s}, Δδ 0.44 ; CO, Δδ -0.77) which was implicated with Hap in the non peptidic backbone part of the macrocycle. In this residue, we could also note a larger value for the vicinal ^{3}J(NH-C4H) coupling constant (11 Hz in [**3**] vs. 9 Hz in [**1**]), reflecting a reinforced *trans* configuration for the NH—C4H isoSta bond. Another significant and unexplained change, in the linear chain residue D-NMeLeu^{7},

(NCH_3, $\Delta\delta$ 1.37) was also found.

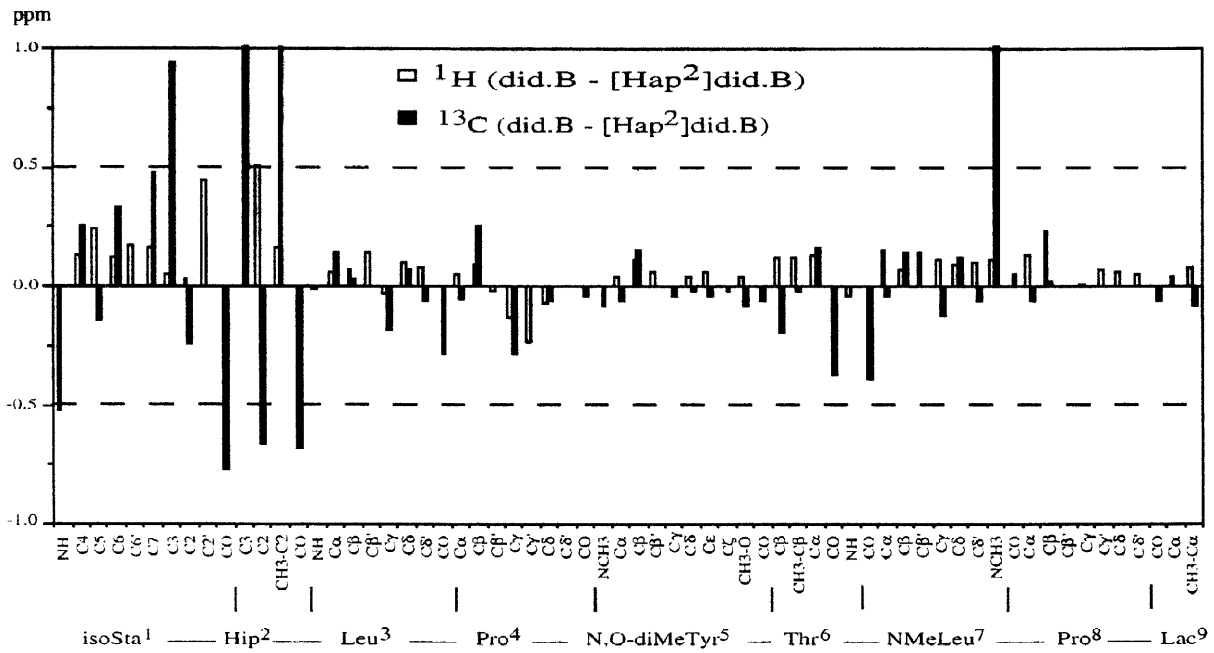


Figure 6 : Chemical shift changes, $\Delta\delta$, for [Hap^2]didemnin B relative to didemnin B.

$\Delta\delta$ is calculated by substracting the chemical shift of ^{1}H and ^{13}C resonances in did. B from that in [Hap^2]did. B.

Temperature dependence of NH chemical-shift values :

The macrocycle conformation of didemnin B is stabilized by three intramolecular NH---O hydrogen bonds [18,19] : the transannular hydrogen bond between the isostatine amide group and the leucine carbonyl group stabilizing the ring structure, the H-bond linking the Leu^3 amide group to the MeLeu^7 carbonyl group folding the linear moiety back to the macrocycle and the H-bond involved in the βII turn within the linear part of the peptidic chain between the Thr^6 amide group and the Lac^9 carbonyl group.

Indications of the solvent accessibility of the NH protons were obtained from the temperature dependence of their resonances over the range of 294-334 K at 5 K intervals in (D_6)DMSO. In C_5D_5N the effects led to the same conclusions but were less clear because aromatic solvent-induced shifts (ASIS) might cause additional effects. C-chemical-shift parameters and nOe gave evidences that the conformations of [Hysp^2]did.B and [Hap^2]did.B in C_5D_5N and the major conformer in DMSO were similar. As for did.B, [Hysp^2]did.B and [Hap^2]did.B were not homogeneous in DMSO on the time scale of the NMR experiment, [Hysp^2]did.B exhibiting two conformers in a ratio of 3/1 and [Hap^2]did.B three conformers in a ratio of 6/4/1.

The chemical shifts of the amide protons all showed linear upfield shifts as the temperature increased, indicating that no major conformational changes occurred over this temperature range. Temperature coefficients of $> 4 \times 10^{-3}$ ppm/K are indicative of readily exchanging (solvent accessible) protons, whereas values of $< 2 \times 10^{-3}$ ppm/K suggest involvement in hydrogen bonding.[30] Intermediate values suggest some reduction in solvent accessibility presumably due to a weak strained H-bonding interaction.

The temperature coefficients for the three amide proton chemical shifts of **[1]**, **[2]** and **[3]** are listed in Table 4. As for did.B **[1]**, the temperature coefficients in **[2]** and **[3]** suggest a strong hydrogen bond to the Leu^{3}NH and isoSta^{1}NH while that of the Thr^6 is lying in the intermediate range.

Table 4 : Temperature Dependence in DMSO-d_6 of the NH Chemical Shifts of **2** and **3** Compared With Those of **1**, Given as $-\Delta\delta/\Delta T$ [ppb/K].

	isoSta[1]	Leu[3]	Thr[6]
$-\Delta\delta/\Delta T$ for **1**	0.5	1.8	4.3
$-\Delta\delta/\Delta T$ for **2**	0.6	2.1	4.3
$-\Delta\delta/\Delta T$ for **3**	2.0	1.9	4.2

In comparison with did.B [**1**], both depsipeptides [**2**] and [**3**] showed very similar NMR data for the macrocyclic and linear parts of the molecule. So, as expected, no backbone conformational changes were detected between [**1**] and [**2**] on the basis of chemical shift values (Figure 5 and 6), scalar coupling constants (Table 3) and temperature dependence of NH (Table 4). This similarity is not very surprising considering that both peptides differ only by one methyl group. Only slight differences were observed between [**3**] and [**1**]. The temperature gradient of the isoSta[1] NH chemical shift in [**3**] (2.0 ppb/K) was not as small as the one for didemnin B (0.5 ppb/K), indicating the presence of a less stronger hydrogen bond isoSta[1]NH---OCLeu[3] and a less tighter macrocycle in [**3**]. This observation was supported by the corresponding downfield chemical shifts of isoSta[1] NH ($\Delta\delta$ -0.53) and Leu[3] CO ($\Delta\delta$ -0.28). In addition, the minor but significant changes occurring mainly within the isostatine residue confirmed a slightly modified conformation in this part of the macrocycle. Nevertheless it seems that the loss of the valine side chain in the Xaa[2] residue between did. B and [Hap[2]]did. B does not affect the structural rigidity of the non-peptidic part (isoSta[1]-Hap[2]) of the macrocyclic ring. The ^3J(NH-CH4)isoSta[1] and ^3J(C2H^r-C3H)isoSta[1] coupling constant values of respectively 11 Hz and about 0 Hz, were indicative of a single dominant conformation with a trans configuration for NH—C4H bond and a synclinal arrangement of the C2H^r and C3H protons. Furthermore, on both sides of the ester bond linking isoSta to Hap, the two geminal protons of the methylene groups ($C2H_2$ isoSta[1] and $C4H_2$ Hap[2]) were diastereotopic and the large chemical shift differences (respectively 0.87 and 0.29 ppm) between the geminal protons was indicative of conformational homogeneity in this part of the macrocycle.

So as in did.B, where Searle *et al.*[18] observed correlation times in the range $2.1\text{-}1.6\cdot10^{-10}$ s for all the backbone carbons of the depsipeptide ring, the [Hap[2]]did.B depsipeptide ring seems to be rigidly constrained. The limited amount of this natural product has prohibited the recording of T_1 relaxation time measurements of ^{13}C resonances as a probe of molecular flexibility.

Cytotoxicity

We screened [Hysp[2]]didemnin B and [Hap[2]]didemnin B against drug-sensitive and multidrug-resistant cell lines. In order to compare our results with those of others, the cytotoxicities of the two lead compounds didemnin A and B were determined on the same system together with another [Hip] modified did.B : [Hip[2]-oxime]did.B [**4**]. This latter compound, previously described by Sakai *et al.*,[24] was obtained by treatment of [**1**] with hydroxylamine.

The cytotoxicity of the different didemnins was evaluated for the parent drug-sensitive CCRF-CEM human leukemic lymphoblasts and sublines that express drug resistance associated with either overexpression of the plasma membrane protein P-glycoprotein (Pgp) for CEM/VLB_{100} cells (MDR phenotype) [31] or altered DNA

topoisomerase II for the CEM/VM-1 cells (usually referred to as atypical MDR cells).[32] The IC_{50} values for adriamycin were determined in these cell lines to provide a standard with which didemnins could be compared.

Growth-inhibitory effect of didemnins on sensitive human tumor cell lines :

The didemnins caused dose-dependent decreases in cell proliferation. The IC_{50} values (Table 5) ranged from 18 nM to 184 nM making about a 10-fold difference. [Hysp^2]did.B had an IC_{50} value of 21nM, similar to that of did.B (18nM), and [Hap^2]did.B an IC_{50} value of 184 nM. The substitution of the valine side chain on C4Hip in did.B by an isoleucine side chain in [Hysp^2]did.B induced only a minor change in the cytotoxicity whereas the loss of the side chain in [Hap^2]did.B resulted in a less toxic compound.

Growth-inhibitory effect of didemnins on multidrug-resistant cell lines :

The abilities of didemnins to inhibit the growth of the two resistant cell lines were summarized in Table 5. The resistance factors for CEM/VM-1 and CEM/VLB$_{100}$ cells to each of the compounds (calculated as IC_{50} for drug-resistant cells/IC_{50} for parental cells) were also indicated. The didemnins caused dose-dependent inhibition of the proliferation of both cell lines. They were almost as toxic to the CEM/VM-1 cells selected for resistance to teniposide as they were to the parent CEM cells. Didemnins were apparently a substrate for Pgp since the CEM/VLB$_{100}$ cells, selected for resistance to vinblastine, expressed cross-resistance to the didemnins with a range from 5 to 80-fold. Moreover it seemed that modifications on the Hip residue maked didemnins a better substrate for Pgp.

Table 5 : *InVitro* Cytotoxicity of Didemnins Against Drug-Sensitive and Multidrug-Resistant Tumor Cell Lines.

	CCRF-CEM IC $_{50}$ (nM)[a]	CEM/VLB100 IC $_{50}$ (nM) [a]	Resistance factor[b]	CEM/VM-1 IC $_{50}$ (nM)[a]	Resistance factor[b]
Adriamycin	56 ± 16	3710 ± 1300	65	554 ± 142	10
Didemnin A	115 ± 37	6540 ± 320	57	65 ± 8	<1
Didemnin B	18 ± 5	101 ± 18	5.6	12 ± 5	<1
[Hysp^2]didemnin B	21 ± 8	274 ± 18	13	n.t. [c]	
[Hap^2]didemnin B	184 ± 14	4150 ± 130	22.5	310 ± 14	1,7
[Hip^2-oxime]didemnin B	52 ± 9	4000 ± 60	77	40 ± 8	<1

[a] 50% inhibitory concentration in a 48h growth inhibition assay ± SD. Values shown are means of 3 separate experiments.

[b] Resistance factors calculated as IC 50 for drug-resistant cells / IC 50 for drug-sensitive cells

[c] (n.t. = not tested)

Experimental section

General Instrumentation : Nuclear magnetic resonance (NMR) spectra were recorded on a Jeol EX 400 spectrometer. IR and UV spectra were recorded respectively on a Perkin-Elmer 1600 FTIR spectrometer and a Perkin-Elmer 551 spectrometer. Liquid secondary ion mass spectra were performed on a Autospec instrument (Fisons, VG analytical, Manchester, UK). The Cesium gun worked at 30 kV, the ion source voltage being 8 kV. Samples were dissolved in a few microliters of 20 % aqueous acetic acid and 1 microliter was mixed on the target with 1 microliter of a 1:1 glycerol thioglycerol mixture acidified by 1 microliter of 1 % trichloroacetic acid in water. High performance liquid chromatography (HPLC) was performed with Jasco 880-PU pumps, 7125 Rheodyne injectors and either a Merck (LMC systeme) differential refractometer detector or a Waters 996

photodiode array detector.

Isolation of [Hysp^2]didemnine B [2] and [Hap^2]didemnine B [3] : A sample (≈ 5 kg) of *Tridemnum cyanophorum* collected by scuba at a depth of -10 to -40 m off the coast of Guadeloupe in 1988 was stored in EtOH until workup. The extract obtained by repetitive steeping in $CHCl_3$/EtOH was separated by solvent partition, flash chromatography and silica gel column chromatography. Pure [Hysp^2]did.B (7 mg) was obtained from a medium polar fraction together with did.B (120 mg) by repetitive reverse phase HPLC (RP C-8 column, 250 x 10 mm, 5 µm particule size, flow rate 2 ml/min., UV detection at 280 nm) using MeOH-H_2O-TFA (80:20:0.1). [Hap^2]did.B (3.5 mg) was obtained from a more polar fraction together with did.A (52 mg) and [Tyr^5]did.B (5 mg) using MeOH-H_2O-TFA (75:25:0.1).

NMR measurement conditions : All spectra were obtained with a NM-40TH5 dual ^1H, ^{13}C probe in a JEOL EX400 operating at 400 MHz for proton and 100.53 MHz for carbon-13 at 298 K. ^1H and ^{13}C NMR chemical shifts are referenced to solvent peaks : δ_H 7.19 (residual C_5HD_4N), δ_C 123.5 for C_5D_5N and δ_H 2.49 (residual DMSO-D_5H) δ_C 39.5 for DMSO-D_6. [Hysp^2]did.B (7 mg) and [Hap^2]did.B (3.5 mg) were dissolved in a 5 mm tube in 0.75 ml of C_5D_5N or DMSO-D_6. The temperature dependence of the amide proton resonances was determined by five measurements over a range of 293-333 K in DMSO-D6.

Two-dimensional (2D) homonuclear correlated experiments DQF-COSY, HOHAHA and ROESY were all acquired using standard procedures with a spectral width of ca. 4000 Hz in both columns F1 and F2. HOHAHA and ROESY were acquired in the phase sensitive mode. The time domain matrix consisted of 256 points in t_1 and 1024-2048 points in t_2 with 64-128 acquisitions for 256 experiments in t_1. Data sets were zero-filled to 512 points in t_1 prior to Fourier transformation to obtain a frequency domain matrix of 512 x 1024-2048 real data points. Squared sine bell apodization functions were used. The HOHAHA spectra were recorded with a mixing time of 100 ms. ROESY spectra were measured with mixing times of 150, 250 and 350 ms. Heteronuclear correlated experiments were performed in ^1H-detected mode using the standard pulse programs HMQC and HMBC with a spectral width of ca. 20000 Hz in F1 and 4000 Hz in F2. The time domain matrix consisted of 256 points in t_1 and 2048 points in t_2 with 128 acquisitions for 256 experiments in t_1. Data sets were zero-filled to 512 points in t_1 prior to Fourier transformation to obtain a frequency domain matrix of 512 x 2048 real data points. The evolution delay was set to optimize 140 Hz couplings for HMQC and 8 Hz couplings for HMBC. Squared sine bell apodization functions were used. The J-resolved spectra were recorded using a standard pulse sequence to obtain a 4096 x 256 matrix in t_2 and t_1, respectively, with a spectral width of 50 Hz in the t_1 dimension.

[Hysp^2]didemnin B [2] : White amorphous solid; IR ($CHCl_3$) 3670, 3600, 2975, 2935, 2884, 1730, 1655, 1605 cm^{-1}; UV (MeOH) λ_{max} 227 (ε 13800), 275 (ε 7010); FABMS m/z and NMR data are shown respectively in Tables 1 and 2.

[Hap^2]didemnin B [3] : White amorphous solid; IR ($CHCl_3$) 3690, 3327, 3016, 2929, 1734, 1654, 1630, 1601, 1377 cm^{-1}; UV (MeOH) λ_{max} 228 (ε 5250), 278 (ε 1888), 284 (ε 1888); FABMS m/z and NMR data are shown respectively in Tables 1 and 2.

Hydrolysis of didemnins : [Hysp^2]did.B or [Hap^2]did.B (0.2 mg) in 0.5 ml 6N HCl was heated at 110° for 16 h in a sealed vial. The cooled reaction mixture was evaporated to dryness, and traces of HCl were removed from the residual hydrolysate by repeated evaporation from H_2O.

Amino acid analysis : For the FDAA (Marfey's reagent = 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide) derivatization procedure, the precedently obtained crude hydrolysate or a small amount of standard free amino acid, in 50 µl of H_2O/acetone was mixed with 100 µl of a 1% solution of FDAA (purchased from Sigma)

in acetone. 1.0 M sodium bicarbonate solution (20 µl) was added to this mixture and the resulting solution was heated at 40°C for one hour and then allowed to cool. After addition of 10 µl of 2 M HCl, the resulting solution was evaporated, dissolved in 0.5 ml of DMSO and then analyzed by HPLC. The HPLC analysis used the following conditions : solvent A, 0.05 M Et_3N in water, H_3PO_4, pH 3 + 5 % acetonitrile; solvent B, acetonitrile; gradient with flow rate of A + B at 1 ml/min., 80/20 to 60/40 in 10 min. and from 60/40 to 20/80 in 30 min.; column, Interchim Spherisorb OD2 5µ , 250 mm x 4 mm; UV detector at 340 nm. The peaks were identified by co-injection with a DL-mixture of standard amino acids. Retention times (min) are given in parentheses : L-Thr (6.09), L-Pro (11.29), (3S, 4R, 5S)Ist (25.41), L-Leu (26.69), D-NMeLeu (32.59), L-N,O-diMeTyr (34.71).

Cytotoxic evaluation : The acute lymphoblastic leukemia CCRF-CEM, the subline CEM-VLB100 selected for resistance to vinblastine and the subline CEM/VM-1 selected for resistance to teniposide (VM-26), were obtained from Dr. W.T. Beck, St. Jude Children's Research Hospital, Memphis, Tennessee, USA. All cell lines were grown in plastic tissue culture flasks using RPMI 1640 medium supplemented with 10% foetal calf serum (v:v) and antibiotics (penicillin 1,000U/ml; streptomycin 100 µg/ml) in an humidified atmosphere of 5% carbon dioxide in air at 37°C. Serial dilutions of didemnins were prepared in the culture medium. The drug at the appropriate concentration was added to cell cultures (2 10^5 cells/ml) for two days without renewal of the medium. Cells were then enumerated using a Coulter counter model ZME. Assays were carried out in triplicate and the results averaged. The concentration of drugs required to inhibit growth of cells by 50% (IC_{50}) in 48h was determined for each cell line. Cross-resistance was calculated by dividing the IC_{50} of a drug in the resistant cell line by that in the parent cells.

Acknowledgements :

This work was supported in part by research grants from INSERM and FNCLCC (Fed. Nat. des Centres de Lutte Contre le Cancer). I. Bonnard is supported by La Ligue Nationale Contre le Cancer. The authors are indebted to Mr and Mme Promé for accurate mass measurements. The acute lymphoblastic leukemia cell line CCRF-CEM was obtained from Dr. W.T. Beck, St. Jude Children's Research Hospital, Memphis, Tennessee, USA.

References

1 Rinehart, K. L.; Gloer, J. B.; Hughes, R. G.; Renis, H. E.; McGovren, J. P.; Swynenberg, E. B.; Stringfellow, D. A.; Kuentzel, S. L. and Li, L. H. *Science* **1981**, *212*, 933-935.

2 Banaigs, B.; Jeanty, G.; Francisco, C.; Jouin, P.; Poncet, J.; Heitz, A.; Cave, A.; Prome, J.C.; Wahl, M. and Lafargue, F. *Tetrahedron* **1989**, *45*, 181-190.

3 Rinehart, K. L.; Gloer, J. B. and Cook, J. C. *J. Am. Chem. Soc.* **1981**, *103*, 1857-1859.

4 Lafargue, F. and Duclaux, G. *Ann. Inst. Oceanogr.* **1979**, *55*, 163-184.

5 a) National Cancer Institute, Bethesda, MD, Division of Cancer Treatment, Annual Report to the Food and Drug Administration, Didemnin B, NSC 325319, **1994**.
b) SirDeshpande, B. V. and Toogood, P. L. *Biochemistry* **1995**, *34*, 9177-9184.

6 a) Rinehart, K. L.; Kishore, V.; Nagarajan, S.; Lake, R. J.; Gloer, J. B.; Bozich, F. A.; Li, K.; Maleczka, R. E.; Todsen, W. L.; Munro, M. H.; Sullins, D. W. and Sakai, R. *J. Am. Chem. Soc.* **1987**, *109*, 6846-6848.
b) Schmidt, U.; Kroner, M. and Griesser, H. *Tetrahedron Letters* **1988**, *29*, 4407-4408.
c) Hamada, Y.; Kondo, Y.; Shibata, M. and Shioiri, T. *J. Am. Chem. Soc.* **1989**, *111*, 669-673.
d) Jouin, P.; Poncet, J.; Dufour, M.; Pantaloni, A.; Castro, B. *J. Org. Chem.* **1989**, *54*, 617-627.
e) Ewing, W. R.; Harris, B. D.; Li, W. and Joullié, M. M. *Tetrahedron Letters* **1989**, *30*, 3757-3760.
f) Li, W. R.; Ewing, W. R.; Harris, B. D. and Joullié, M. M. *J. Am. Chem. Soc.* **1990**, *112*, 7659-7672.

7 a) Jouin, P.; Poncet, J.; Dufour, M.; Aumelas, A.; Pantaloni, A.; Cros, S. and François, G. *J. Med. Chem.* **1991**, *34*, 486-491.

b) Mayer, S. C.; Pfizenmayer, A. J.; Cordova, R.; Li, W. R. and Joullié, M. M. *Tetrahedron Asymmetry* , **1994**, *5*, 519-522.
c) Jou, G.; Gonzalez, I.; Albericio, F.; Lloyd-Williams, P. and Giralt, E. *J. Org. Chem.* **1997**, *62*, 354-366.
8 a) Shin, D. M.; Holoye, P. Y.; Forman, A.; Winn, R.; Perez-Soler, R.; Dakhil, S.; Rosenthal, J.; Raber, M. N. and Hong, W. K. *Investigational New Drugs* **1994**, *12*, 243-249.
b) Weiss, R. B.; Peterson, B. L.; Allen, S. L.; Browning, S. M.; Duggan, D. B. and Schiffer, C. A. *Investigational New Drugs* **1994**, *12*, 41-43.
c) Malfetano, J. H.; Blessing, J. A.; Homesley, H. D.; Look, K. Y. and McGehee, R. *Am. J. Clin. Oncol.* **1996**, *19*, 184-186.
9 a) Crews, C. M.; Collins, J. L.; Lane, W. S.; Snapper, M. L. and Schreiber, S. L. *J. Biol. Chem.* **1994**, *269*, 15411-15414.
b) Crews, C. M.; Lane, W. S. and Schreiber, S. L. *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 4316-4319.
10 Guyot, M.; Davoust, D. and Morel, E. *C. R. Acad. Sci. Paris* **1987**, *305*, 681-686.
11 Mc Kee, T. C.; Ireland, C. M.; Lindquist, N. and Fenical, W. *Tetrahedron Letters* **1989**, *30*, 3053-3056.
12 Rinehart, K. L.; Cook, J. C.; Pandey, R. C.; Gaudioso, L. A.; Meng, H.; Moore, M. L.; Gloer, J. B.; Wilson, G. R.; Gutowsky, R. E.; Zierath, P. D. and Shield, L. S. *Pure & Appl. Chem.* **1982**, *54*, 2409-2424.
13 Rinehart, K. L.; Sakai, R. and Stroh, J. G. *Chem. Abstr.* **1991**, *114*, 214413h.
14 a) Rinehart, K. L. *U.S. Patent Application* P-82,663, December 13, 1990 and British Patent Application 8922026.3, filed Sept. 29, **1989**.
b) Rinehart, K. L.; Lithgow-Bertelloni, A. M. *PCT Int. Pat. Appl.* WO 91.04985, Apr.18, **1991**.
15 Sakai, R.; Stroh, J. G.; Sullins, D. W. and Rinehart, K. L. *J. Am. Chem. Soc.* **1995**, *117*, 3734-3748.
16 Boulanger, A.; Abou-Mansour, E.; Badre, A.; Banaigs, B.; Combaut, G. and Francisco, C. *Tetrahedron Letters* **1994**, *35*, 4345-4348.
17 Abou-Mansour, E.; Boulanger, A.; Badre, A.; Bonnard, I.; Banaigs, B.; Combaut, G. and Francisco, C. *Tetrahedron* **1995**, *51*, 12591-12600.
18 Searle, M. S.; Hall, J. G.; Kyratzis, I. and Wakelin, L. P. G. *Int. J. Peptide Protein Res.* **1989**, *34*, 445-454.
19 Kessler, H.; Will, M.; Antel, J.; Beck, H. and Sheldrick, G. M. *Helvetica Chimica Acta* **1989**, *72*, 530-555.
20 a) Hossain, M. B.; van der Helm, D.; Antel, J.; Sheldrick, G. M.; Sanduja, S. K. and Weinheimer, A. J. *Proc. Natl. Acad. Sci. USA* **1988**, *85*, 4118-4122.
b) Hossain, M. B.; van der Helm, D.; Antel, J.; Sheldrick, G. M.; Weinheimer, A. J. and Sanduja, S. K. *Int. J. Peptide Protein Res.* **1996**, *47*, 20-27.
21 Kessler, H.; Mronga, S.; Will, M. and Schmidt, U. *Helvetica Chimica Acta* **1990**, *73*, 25-47.
22 Mayer, S. C.; Carroll P. J. and Joullié M. *Acta Cryst.* **1995**, *C51*, 1609-1614.
23 Mayer, S. C.; Ramanjulu, J.; Vera, M. D.; Pfizenmayer, A. J. and Joullié, M. M. *J. Org. Chem.* **1994**, *59*, 5192-5205.
24 Sakai, R ; Rinehart, K. L.; Kishore, V.; Kundu, B.; Faircloth, G.; Gloer, J. B.; Carney, J. R.; Namikoshi, M.; Sun, F.; Hughes, R. G.; Gravalos, D. G.; Dequesada, T. G.; Wilson, G. R.; Heid, R. M. *J. Med. Chem.* **1996**, *39*, 2819-2834.
25 Roepstorff, P. and Folhman, J. *Biomed. Mass Spectrom.* **1984**, *11*, 601.
26 Hunt, D. F.; Yates, J. R.; Shabanowitz, J.; Winston, S.; Hauer, C. R. *Proc. Natl. Acad. Sci. USA* **1986**, *83*, 6233-6237.
27 a) Eckart, K.; Schwarz, H.; Tomer, K. B. and Gross, M. L. *J. Am. Chem. Soc.* **1985**, *107*, 6765-6769.
b) Eckart, K.; Schwarz, H.; Chorev, M. and Gilon, C. *Eur. J. Biochem.* **1986**, 157, 209-216.
28 Marfey, P. *Calsberg Res. Commun.* **1984**, *49*, 591-596.
29 Bystrov, V. F. *Prog. Nucl. Magn. Reson. Spectrosc* .; Emsley, J. W.; Feeny, J.; Sutcliffe, L. H., Eds.; Pergamon Press : Oxford, **1976**, *10*, pp 41-82.
30 Kessler, H. *Angew. Chem. Int. Ed. Engl.* **1982**, *21*, 512-523.
31 Beck, W. T.; Mueller, T. J. and Tanzer L. R. *Cancer Research* **1979**, *39*, 2070-.2076.
32 Danks, M. K.; Yalowich, J. C. and Beck, W. T. *Cancer Research* **1987**, *47*, 1297-.1301.
33 Granzen, B.; Graves, D. E.; Baguley, B. C.; Danks, M. K. and Beck, W. T. *Oncology Research* **1992**, *4*, 489-496.