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[Tyr^5]didemnin B and [D-Pro^4]didemnin B ; Two New Natural Didemnins with a Modified Macrocycle.

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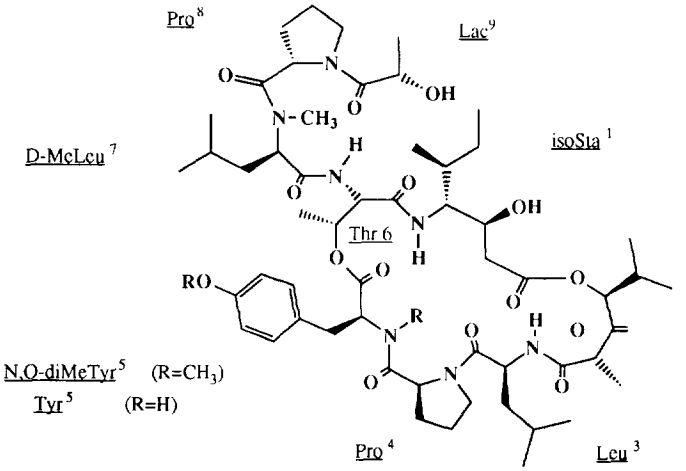
Abstract : The structures of the two natural didemnins with a modified macrocycle, [Tyr^5]didemnin B [2] and [D-Pro^4]didemnin B [3], isolated from the Aplousobranch ascidian *Tridemnum cyanophorum* (Didemnidae) are described. Structures were determined by FABMS and NMR spectroscopy, absolute configurations by Marfey analysis of the acid hydrolisates. Conformational informations are given and cytotoxic activities against human lymphoblastic leukemia cell lines evaluated.

The didemnins are a class of marine natural cyclodepsipeptides with a highly antineoplastic activity, isolated from a symbiotic association[1] between an ascidian (*Tridemnum solidum*[2] *or T. cyanophorum*[3]) and a cyanophyta (*Synechocystis trididemni*). Didemnin B [**1**], the major component among a dozen of already described structurally related compounds, was considered as one of the most potent derivatives of this class. The phase II NCI trials, temporarily closed to patient accrual in january 1990, were reactivated with a higher recommended phase II dose[4]. In addition, didemnin B is active against a variety of DNA and RNA viruses[5] (but unfortunately ineffective against HIV) and its immunosuppressive properties were demonstrated[6]. Characterization of the exact site and mechanism of action of this drug are not yet elucidated.

Didemnin B is composed of a lactyl-Pro-D-MeLeu dipeptide chain branched to a hexadepsipeptide ring structure with nonproteinogenic units. In our continuing search for potential anticancer agents from marine sources, we isolated didemnin B[3b], didemnin H[7] in addition to several already described congeners. Further examination of the Et_2O extract, obtained from *T. cyanophorum* collected in Guadeloupe Island, provided two new didemnins. This report concerns isolation and characterization of [Tyr^5]didemnin B [**2**] and [D-Pro^4]didemnin B [**3**] which differ from didemnin B in modified macrocyclic rings.



[**1**] = didemnin B R=CH_3
[**2**] = [Tyr^5]didemnin B R=H

Table I. NMR assignments of did.B, [Tyr^5]did.B [**2**] and [D-Pro^4]did.B [**3**] in C_5D_5N (ppm).

		Didemnin B		[Tyr^5]didemnin B		[D-Pro^4]didemnin B	
		^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
isoSta^1	NH	7.56		7.72		8.50	
	C4H	4.70	55.8	4.70	55.8	4.72	55.5
	C5H	2.56	34.3	2.47	34.3	2.41	34.4
	$C6H_2$	1.46 / 1.72	28.3	1.40 / 1.65	28.1	1.44 / 1.66	28.6
	$C7H_3$	1.10	12.4	1.03	12.3	0.98	11.9
	CH_3-C5	1.17	14.6	1.18	14.3	1.36	15.1
	C3H	4.79	67.0	4.85	67.1	4.87	70.1
	$C2H^R/C2H^S$ *	3.06 / 4.34	41.1	3.09 / 4.26	40.8	3.18 / 4.08	49.6
	CO		172.6		172.5		172.7
Hip^2	C4H	5.65	80.6	5.65	80.6	5.42	84.0
	C5H	2.48	30.4	2.45	30.4	2.60	31.2
	$C6H_3$	0.83	19.0	0.80	19.0	0.96	18.9
	CH_3-C5	0.88	16.9	0.84	16.5	1.10	19.1
	C3		205.6		206.0		205.0
	C2H	4.72	49.5	4.70	49.5	4.44	50.4
	CH_3-C2	1.75	16.0	1.74	15.8	1.70	15.3
	CO		169.9		170.2		169.6
Leu^3	NH	8.47		8.60		8.45	
	$C_\alpha H$	5.19	49.9	5.18	49.8	5.34	49.5
	$C_\beta H_2$	1.55 / 1.85	42.1	1.61 / -	42.0	1.81 / 1.90	42.0
	$C_\gamma H$	1.79	24.2	1.80	25.1	1.65	25.2
	$C_\delta H_3$	0.88	23.7	0.87	24.0	0.95	23.7
	$C_{\delta'}H_3$	0.98	21.1	0.96	21.0	1.06	21.3
	CO		171.1		171.7		173.2
Pro^4	$C_\alpha H$	4.75	57.6	4.60	56.7	4.92	57.5
	$C_\beta H_2$	1.65 / 1.82	28.7	1.65 / 1.81	28.7	1.79 / 1.87	27.2
	$C_\gamma H_2$	1.53 / 1.71	26.0	1.49 /1.79	25.9	1.60 / 2.00	24.9
	$C_\delta H_2$	3.37 / 3.55	47.6	3.35 / 3.51	47.2	3.71 / 3.94	47.1
	CO		170.9		171.6		170.9
N,OMe$_2$Tyr^5	NCH_3 / NH	2.64	38.6	9.18		2.74	38.5
Tyr^5	$C_\alpha H$	4.20	65.8	4.50	60.1	4.24	65.7
	$C_\beta H_2$	3.52 / 3.60	34.6	3.5 / 3.60	34.8	3.55 / 3.59	34.4
	C_γ		130.6		129.7		130.4
	C_dH	7.25	131.0	7.25	131.5	7.30	130.8
	C_eH	7.00	114.4	7.00	116.6	7.02	114.2
	C_ζ		159.1		158.1		159.0
	OCH_3 / OH	3.72	55.2	10.00		3.72	55.1
	CO		169.3		170.5		169.4
Thr^6	NH	8.25		8.27		8.41	
	$C_\alpha H$	5.07	58.8	5.22	58.5	4.95	58.6
	$C_\beta H$	5.81	71.0	5.71	71.3	5.83	70.9
	CH_3-C_β	1.80	17.1	1.83	16.8	1.80	17.0
	CO		169.7		169.5		170.2
D-MeLeu^7	NCH_3	3.24	31.3	3.25	31.1	3.19	31.2
	$C_\alpha H$	5.80	55.0	5.78	55.0	5.75	54.8
	$C_\beta H_2$	1.72 / 2.06	36.6	2.01 / 1.91	36.6	1.68 / 2.02	36.3
	$C_\gamma H$	1.56	24.9	1.89	24.9	1.52	24.9
	$C_\delta H_3$	0.86	23.6	0.87	23.7	0.82	23.3
	$C_{\delta'}H_3$	0.97	21.4	0.98	21.3	0.94	21.4
	CO		172.6		172.6		172.3
Pro^8	$C_\alpha H$	4.72	56.7	4.72	56.5	4.75	56.7
	$C_\beta H_2$	1.91 / 2.02	27.7	1.96 / 1.98	28.1	1.94 / 2.07	28.7
	$C_\gamma H_2$	1.84 / 2.06	24.8	1.89 / 2.03	24.8	1.81 / 2.05	24.9
	$C_\delta H_2$	3.67 / 3.89	47.0	3.67 / 3.91	47.6	3.90 / 3.62	47.4
	CO		173.7		173.8		173.6
Lac^9	C_2H	4.53	66.9	4.51	66.8	4.54	66.8
	CH_3-C2	1.49	20.3	1.49	20.2	1.50	20.0
	CO		173.6		173.6		173.6

* R = pro-R, S = pro-S. The diastereotopic differentiation pro-R / pro-S is performed by interpretation of nOes in the ROESY spectrum.

Isolation

Further examination of lowbar silica gel chromatography (hexane, Et_2O, MeOH) fractions obtained from the Et_2O extract, by C8 RP HPLC (72/28 MeOH/H_2O 1‰ TFA) yields four HPLC pure peaks. NMR studies indicate that peak 1 is in reality a 1/1.5 mixture of closely related didemnins congeners. [Tyr^5]didemnin B (short form : [Tyr^5]did.B) is separated (5 mg) from this mixture by normal-phase (CH_2Cl_2/MeOH 9/1) column chromatography. [D-Pro^4]didemnin B (short form : [D-Pro^4]did.B) is isolated (4 mg) from peak 2 whereas peaks 3 and 4 yield nordidemnin B and didemnin B (short form : did.B). [Tyr^5]did.B and [D-Pro^4]did.B are obtained as colorless amorphous solids and are negative to ninhydrin test suggesting a blocked N-terminus.

Structural elucidation

The NMR spectra of [Tyr^5]did.B and [D-Pro^4]did.B are recorded at 400 MHz (^{1}H) and 100 MHz (^{13}C) in C_5D_5N which gives the best dispersion for ^{1}HNMR analysis; one set of resonance is observed for each residue from which we conclude that one conformation is strongly dominant in this solvent. Spectral data, including IR, UV, ^{1}H and ^{13}C NMR and FABMS, confirm that both peptides are closely related to didemnin B which as a model, allows to elucidate the structure and to describe the complete ^{1}H and ^{13}C NMR spectral assignments.

• ***[Tyr^5]didemnin B***

The molecular formula $C_{55}H_{85}N_7O_{15}$ is consistent with the pseudomolecular ion m/z 1084.4 ($M+H^+$) (base peak on the FAB MS spectrum) together with the ^{1}H and ^{13}C NMR spectra. The "N-terminus" fragmentation leads to an acylium ions series that defines the side chain. This series begins at 1084 amu's with the parent ion and displays successive didemnins well-known losses of 72 (m/z 1012), 97 (m/z 915) and 127 (m/z 788) respectively for Lac, Pro and NMeLeu residues, indicating that the change has taken place on the macrocyclic ring. Differences in the spectra, particularly the absence of the acylium serie determining the Pro^4–N,OdiMeTyr^5 (m/z 307 : acylium Pro–N,OdiMeTyr and m/z 210 : acyloxy N,OdiMeTyr) which is shifted to lower mass by 28 units with the ions m/z 279 and 182, suggest that the difference lies in the absence of the O- and N-methyl groups.

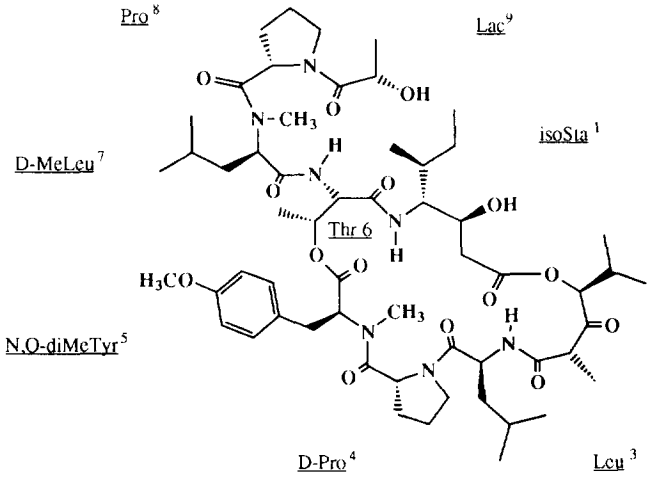
In the ^{1}H NMR spectrum of [Tyr^5]did.B the two CH_3-X, at 2.64 and 3.72 ppm, precedently assigned to CH_3-O and CH_3-N diMeTyr in did.B are lacking. On the other hand, this spectrum contains a fourth doublet signal at 9.18 ppm which slowly exchanges with D_2O. This new NH resonance is readly assigned to a Tyr residue replacing the N,OdiMeTyr^5, in the ^{1}H-^{1}H DQF COSY where cross peaks NH/H-C_αTyr (4.50 ppm), H-C_αTyr/H-C_βTyr (3.53 ppm) and H-C_αTyr/H'-C_βTyr (3.60 ppm) are observed. The ^{13}C NMR spectrum of **[2]** with 53 carbon resonances (2 aromatic C-resonances overlapped) is almost identical to did.B with only the disappearance of the two CH_3-O and CH_3-N diMeTyr resonances. DEPT and HMQC experiments establish the multiplicities of each carbon and all of the one bond ^{1}H-^{13}C correlations, in agreement with the molecular formula. The ^{1}H-^{1}H double-quantum-filtered COSY (^{1}H-^{1}H DQF COSY) spectrum recorded at first idendifies spin systems corresponding to isoSta, Leu, MeLeu, Thr, 2 Pro, Hip and lactate analogous to did.B, plus the spin system corresponding to the amino acid partial structure Tyr.

As pointed out by H. Kessler for didemnins A and B[8], H-C_4/H-C_5isoSta, H-C_β/H-C_γLeu and MeLeu correlations cannot be obtained in the COSY spectrum. These connectivities are observable in the HOHAHA and HMBC spectra. Cross sections of the HOHAHA spectrum taken at 4.70 ppm (H-C_4 isoSta), 5.18 ppm (H-C_α Leu) and 5.78 ppm (H-C_αMeLeu) exhibit the missing cross peak, which is of low intensity owing to the small coupling constants. In the same way a cross section taken at 9.18 ppm (HNTyr) exhibits cross peaks for coupling with the signals at 4.50 ppm (H-C_αTyr), 3.53 ppm and 3.60 ppm (H-C_β and H'-C_βTyr) defining the amino acid tyrosine. This spectrum leads finally to the assignment of all the protons (Table I). Only a few vicinal coupling constants are extracted (Table II) either directly from the one-dimensional spectrum or from the 2D-J resolved spectrum. So as suggested by FAB MS, the variable residue in comparison with did.B is the N,OdiMeTyrosine amino acid which is replaced by a tyrosine amino acid. The ^{13}C resonances are assigned (Table I) by HMQC and HMBC techniques. Sequential assignment of [Tyr^5]did.B, determined by interpretation of ROESY and HMBC data, is found to be identical to did.B.

Hydrolysis of [Tyr^5]did.B followed by Marfey's derivatization[9] and HPLC analysis assign Leu, both Pro, Thr and Tyr as L, MeLeu as D and isoSta as 3S, 4R, 5S. The stereochemistry of the Lac and Hip residues are assumed to be the same as in did.B: L-Lac and (2S, 4S)Hip, due to very similar ^{13}C and ^1H chemical shifts of these two units in both didemnins (maximum difference 0.4 ppm for $\underline{C}_3$Hip and 0.004 ppm for $C\underline{H}_3$-C_5Hip).

• ***[D-Pro^4]didemnin B***

The FABMS spectrum of [D-Pro^4]did.B shows a ($M+H^+$) pseudomolecular ion at m/z 1112.6 and a fragmentation pattern similar to that previously observed for did.B. The same N- and C-terminus fragmentations are visible but with lower abundance than with did.B. No difference in the amino acid compositions as well as in the amino acid sequence is detected in FABMS between [D-Pro^4]did.B and did.B.



[3] = [D-Pro^4]didemnin B

Detailed NMR studies including ^{1}H-^{1}H COSY, HOHAHA, HMQC and HMBC result in identification of spin systems corresponding to isoSta, Leu, MeLeu, Thr, 2 Pro, N,OdiMeTyr, Hip and Lactate as in did.B. But comparison of ^{1}H NMR spectra reveals some significant or small ($0.9 > \Delta\delta > 0.2$ ppm) resonance shifts which occur exclusively in the isoSta^1–Hip^2–Leu^3–Pro^4 region. NHisoSta^1 (8.50 vs 7.56 ppm), CH_3-C_5Hip^2 (1.10 vs 0.88 ppm), H-C_βLeu^3 (1.81 vs 1.55 ppm), H_4-C_γPro^4 (2.00 vs 1.71 ppm) and H_2-C_δPro^4 (3.71/3.94 vs 3.37/3.55 ppm) are shifted downfield relatively to their positions in did.B, while those assigned to H^R-C_2isoSta^1 (4.08 vs 4.34 ppm), H-C_4Hip^2 (5.42 vs 5.65 ppm) and H-C_2Hip^2 (4.44 vs 4.72 ppm) are upfield shifted. Only four ^{13}C resonances are significantly ($\Delta\delta > 1$ ppm) shifted: C_3isoSta^1 (70.1 vs 67.0 ppm), C_4Hip^2 (84.0 vs 80.6 ppm), CH_3-C_5Hip^2 (19.1 vs 16.8 ppm) and COLeu^3 (173.2 vs 171.4 ppm). Again the amino acid sequence is determined by interpretation of ROESY and HMBC data and found to be identical to did.B.

Chirality of amino acid residues is determined by Marfey analysis of the acid hydrolysis: all amino acids except one Pro have absolute configurations identical with those in did.B. One Pro is determined to be D when the other remains L. Mild alkaline hydrolysis (1% NaOH in MeOH, 1h, RT) gives the two peptidic fragments Lac–Pro–MeLeu–Thr–isoSta [**4**] and Hip–Leu–Pro–N,OdiMeTyr [**5'**] as major products. HPLC analysis of the two fragments as protected methyl ester derivative in comparison with those obtained from saponification of did.B reveals that the modified fragment is Hip–Leu–Pro–N,OdiMeTyr : as fragment [**4**] from both origins gives the same HPLC retention time, peptide [**5**] from did.B shows a longer retention (0.25 min) time than [**5'**] from [D-Pro^4]did.B. The location of D-Pro residue is additionally confirmed by complete acid hydrolysis of the small amounts of HPLC purified fragments [**4**] and [**5'**] followed by derivatization with Marfey's reagent and gradient HPLC analysis. Peptide [**4**] gives L-Pro, D-MeLeu, L-Thr and isoSta as 3S, 4R, 5S while [**5'**] is analysed for L-Leu, L-N,OdiMeTyr and D-Pro. So Pro^4 is shown to be of D configuration and Pro^8 to be of L configuration. The stereochemistry of Lac is assumed to be the same as in did.B (L-Lac) due to very similar ^{13}C and ^{1}H chemical shifts in both didemnins (maximum difference 0.3 ppm for $\underline{C}H_3$-C_2 and 0.03 ppm for $C\underline{H}_3$-C_2). The determination of the chirality of Hip is inconclusive and not easily ascertained only by NMR analysis. In comparison with did.B, Hip residue shows some ^{1}H (CH_3-C_5, H-C_4 and H-C_2) and ^{13}C (CH_3-C_5) significant chemical shift differences, but a detailed analysis of the ROESY spectrum shows the same correlation (NHLeu^3/H-C_2Hip^2) and the same lack of correlation between the isopropyl side chain of Hip residue and any other part of the molecule. The limited amount of this natural product prohibits further degradation to confirm or infirm the 2S, 4S stereochemistry of Hip residue.

Table II. ^{3}J (H,H) coupling constants of did.B, [Tyr^5]did.B and [D-Pro^4]did.B in C_5D_5N.

	did.B	**[Tyr^5]did.B**	**[D-Pro^4]did.B**
^{3}J (HN,HC$_4$) isoSta	9	10,5	10
^{3}J (HC$_3$,HC$_4$) isoSta	10	10	8,5
^{3}J (H^R C$_2$,HC$_3$) isoSta	0	0	1,5
^{3}J (HN,HC$_\alpha$) Leu	9	9	9
^{3}J (HN,HC$_\alpha$) Thr	6	5	5
^{3}J (HC$_\alpha$,HC$_\beta$) Thr	1,5	3	2,5
^{3}J (H_α,H_β) Tyr	-	6	-

Conformational informations

Conformational informations are given by comparison of ^{1}H and ^{13}C chemical shift values, vicinal ^{1}H-^{1}H coupling constants, nOe measurements and temperature dependance of NH chemical shift values between [Tyr^{5}]did.B or [D-Pro^{4}]did.B and did.B. Due to the limited amounts of these two natural didemnins only intraresidual and sequential nOe are visible. Transannular nOes are not sufficient for unequivocal and complete backbone conformational studies. Temperature dependence of NH chemical shifts ($-\Delta\delta/\Delta T$) is undertaken (table III) in (D_6)DMSO solution to identify external or internal NH orientations. In C_5D_5N the effects lead to the same conclusions but are less clear because aromatic solvent induced shifts (ASIS) may cause additional effects. C-chemical-shift parameters and nOe give evidence that the conformations of [Tyr^{5}]did.B and [D-Pro^{4}]did.B in C_5D_5N and the major conformer in DMSO are similar. Similarly to did.B, [Tyr^{5}]did.B and [D-Pro^{4}]did.B are not homogeneous in DMSO on the time scale of the NMR experiment ; [Tyr^{5}]did.B exhibits two conformers in a ratio of 2/1 and [D-Pro^{4}]did.B three conformers in a ratio of 8/1/1.

• ***[Tyr^{5}]didemnin B***

Chemical shift differences reflect changes in conformation and/or the local environment of a spin. The maximum differences in ^{1}H chemical shifts between [Tyr^{5}]did.B and did.B are 0.15 ppm (H-C_αThr^{6}) and 0.4 ppm (C_3Hip^{2}) in ^{13}C if we except the shifts induced directly by the structural change in the Tyr residue. The vicinal coupling constants ^{3}J(NH,HC_αThr^{6}), ^{3}J(HC_α, HC_βThr^{6}), ^{3}J(H$^{R}C_2$, HC_3 isoSta^{1}) and ^{3}J(HC_3, HC_4isoSta^{1}) are related to dihedral angles that restrain the backbone conformation. These values (table II) are similar to those of did.B. For all the amide bonds a nOe between NH or NCH_3 and the α-proton of the preceding amino acid is observed. This confirms their trans-conformation. Carbon chemical shifts of the β- and γ-carbons of the proline rings confirm the trans conformation about Leu^{3}–Pro^{4} and Lac^{9}–Pro^{8} bonds [$\Delta\delta(C_\beta\text{-}C_\gamma) < 6$ ppm]. The $-\Delta\delta/\Delta T$ quotients indicate similar H-bonding pattern in [Tyr^{5}]did.B as in did.B. No significant difference in the temperature dependence of the isoSta, Leu and Thr chemical shifts is observed between the two compounds in the range from 293K to 333K. The NHTyr^{5} in [Tyr^{5}]did.B is shifted to high field by about 6 ppb/K indicating that this NH group is not involved in internal H-bridge. Further more the NH protons undergo H-D exchanges in (D_6)DMSO upon addition of D_2O in the following order Tyr^{5} > Thr^{6} > Leu^{3} > isoSta^{1} confirming the precedent results. So with all these results it seems that [Tyr^{5}]did.B and did.B have very closely related conformations.

Table III. NH chemical shift temperature dependencies : $-\Delta\delta/\Delta T$ [ppb/K]

	isoSta^{1}NH	Leu^{3}NH	Thr^{6}NH	Tyr^{5}NH
did.B	0.5	1.8	4.3	-
[Tyr^{5}]did.B	0.7	2.1	4.0	6.0
[D-Pro^{4}]did B	1.7	1.8	4.2	-

• ***[D-Pro^4]didemnin B***

As mentionned above some significant differences in C_5D_5N in ^1H (9 protons shifted in the range from 0.2 to 0.9 ppm) and ^{13}C (4 carbons shifted in the range from 1 to 3.4 ppm) NMR resonances occur between [D-Pro^4]did.B and did.B, all these differences occurring in the isoSta^1–Hip^2–Leu^3–Pro^4 region. The differences of the ^{13}C NMR resonances of the CO are smaller than 0.5 ppm with the exception of the COLeu^3 which differs by 2.1 ppm (173.2 vs 171.1 ppm). When the behaviour of the NH protons is compared, it appears that no shielding or unshielding occurs for NHLeu^3 and NHThr^6 while a large shift (8.50 vs 7.56 ppm) towards the downfield side is observed (from [D-Pro^4]did.B to did.B) for NHisoSta^1. No difference in the temperature dependance of NHLeu^3 and Thr^6 chemical shifts are observed, the $-\Delta\delta/\Delta T$ quotient of NHisoSta^1 (1.7 ppb/K) being not as small as the one for did.B (0.5 ppb/K). The NH isoSta^1 remains within the range of a "buried" NH group, but as judged from the aforementioned results - the deshielding of the COLeu^3 and of the NHisoSta^1, the larger $-\Delta\delta/\Delta T$ value of the NHisoSta^1 - the intramolecular isoStaNH ----O=CLeu hydrogen bond would rather be weak. The conformationally relevant coupling constants indicate just some minor changes. As for did.B and [Tyr^5]did.B a nOe between NH or NCH_3 and the α-proton of the preceding amino acid is observed, confirming the trans conformation of all the amide bonds. As an example ROESY cross peaks are found between H-C_δPro^4 and H-C_αLeu^3, H'-C_δPro^4 and H-C_αLeu^3 as well as between CH_3N N,OdiMeTyr^5 and H-C_αPro^4.

It seems that no drastic conformational change occurs when L-Pro^4 is substituted by a D-Pro residue. The overall conformation is retained with the three H-bonds : ThrNH ----- O=CLac involved in the linear moiety βII turn, isoStaNH ---- O=CLeu stabilizing the ring structure and LeuNH ---- O=CMeLeu responsible for the back folding of the linear moiety towards the ring system[8b]. It is quite reasonable to imagine that the βII turn where the Pro^4 (i+1) and the N,OdiMeTyr^5 (i+2) are involved, changes to a βII' turn about DPro^4–N,OdiMeTyr^5 in [D-Pro^4]did.B. The chirality of the amino acids involved in the β-bend (sequence LDLL) allows a type-II' conformation and D-Pro^4 residue stay in the (i+1) position, a favorable and preferred position for a D-Proline in βII' turn[10]. The minor changes observed in the isoSta^1–Hip^2–Leu^3 region may be caused by the influence of a difference in the orientation of the Leu^3–DPro^4 amide bond (consecutively to the βII/βII' change), with a weak spacing between isoSta^{1}NH and O=CLeu^3. Both epimers show very similar NMR data for the linear part of the molecule (DMeLeu^7–Pro^8–Lac^9) indicating very closely related conformations for this part.

Cytotoxic evaluation

Using a human lymphoblastic leukemia CCRF-CEM cell lines we compared the growth-inhibitory properties of [Tyr^5]did.B, [D-Pro^4]did.B and did.B. [Tyr^5]did.B shows a less pronounced cytotoxic activity with IC_{50} of 186 nM than did.B (IC_{50} = 9 nM) or [D-Pro^4]did.B (IC_{50} = 20 nM). Since no evident modifications in the conformation of the molecules could be deduced from the NMR data, we speculated that this 20-fold loss in activity is not the result of a gross change in conformation and can be explained by the fact that the non methylated tyrosine can be recognised and metabolised before reaching the actif site. When only residual potency is preserved by inversion of the D-MeLeu^7 chirality[11], to the contrary the substitution of the L-Pro^4 by a D-Pro residue preserves a similar cytotoxic activity.

Experimental

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 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 [Tyr^5]did.B and [D-Pro^4]did.B. A sample (≈ 5 kg) of *Trididemnum cyanophorum* collected by scuba at a deph 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 [Tyr^5]did.B (4 mg) and [D-Pro^4]did.B (5 mg) were obtained from a medium polar fraction together with nordid.B (64 mg) and did.B (18 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 (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 ppm (residual C_5HD_4N), δ_C 123.5 ppm for C_5D_5N and δ_H 2.49 ppm (residual DMSO-D_5H) δ_C 39.5 ppm for DMSO-D_6. [Tyr^5]did.B (4 mg) and [D-Pro^4]did.B (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 optimise 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 t2 and t_1, respectively, with a spectral width of 50 Hz in the t_1 dimension.

[Tyr^5]did.B. [2] White amorphous solid; IR ($CHCl_3$) 3525, 3425, 3328, 2960, 2922, 2854, 1740, 1724, 1657, 1631 cm^{-1}; UV (MeOH) λ_{max} 232 (ε 10700), 276 (ε 1000); FABMS m/z 1084, 1012, 974, 915, 856, 833, 788, 351, 297, 279, 182, 170, 142, 127, 100, 98, 93, 86, 70 ; NMR data (C_5D_5N) are shown in table I.

[D-Pro^4]did.B. [3] White amorphous solid; IR ($CHCl_3$) 3501, 3333, 2960, 2923, 2875, 1740, 1737, 1656, 1631 cm^{-1}; UV (MeOH) λ_{max} 226 (ε 10900), 284 (ε 3600); FABMS m/z 1112, 1094, 1040, 943, 861, 843, 816, 771, 703, 307, 210, 170, 164, 142, 100, 70 ; NMR data (C_5D_5N) are shown in table I.

Hydrolysis of didemnins [Tyr^5]did.B or [D-Pro^4]did.B (0.5 mg) in 0.5 ml 6N HCl was heated at 105° for 16 h in a sealed vial. The cooled reaction mixture was evaporated to dryness, and traces of HCl were removed from the residual hydrolyzate 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 in acetone. 1.0 M sodium bicarbonate solution (20 µl) was added to this mixture and the resultant 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 evapored, 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), D-Pro (14.46), L-Tyr (22.34), (S,R,S)Ist (25.41), L-Leu (26.69), D-NMeLeu (32.59), L-N,OdiMeTyr (34.71).

Mild alkaline hydrolysis of [D-Pro^4]did.B. A sample of [D-Pro^4]did.B. (1.2 mg) dissolved in 0.1 ml of methanol was hydrolysed with 0.5 ml of 1% sodium hydroxyde at room temperature. The disappearance of starting material was monitored by TLC. After 1h, the reaction was stopped by acidification with 1% hydrochloric acid. The reaction mixture was extracted with ethyl acetate (5 ml) and the organic phase was evaporated to give a colorless oil. The colorless oil was methylated with CH_2N_2 in ether. The resulting solution was evaporated and the residue analyzed by reverse phase HPLC (RP C-8 column, 250 x 5 mm, 5 µm particule size, flow rate 1 ml/min., photodiode array detection at 280, 254 and 226 nm) in a linear gradient of MeOH in H_2O (1‰TFA) 40 % to 80 % in 40 minutes. The two major peptidic fragments **[4]** and **[5']** were purified in the same HPLC conditions and were hydrolysed in 6N HCl at 105°C for 12 hours. This procedure was previously used with did.B (5 mg) to obtain the two reference peptidic fragments **[4]** and **[5]** and the corresponding acid hydrolysates. Amino acids analysis of these acid hydrolysates were then performed on the FDAA derivatives as previously reported for didemnins [**2**] and [**3**].

Cytotoxic evaluation The acute lymphoblastic leukemia CCRF-CEM cell line was 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 mg/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.

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