CHEMMEDCHEM

DOI: 10.1002/cmdc.200700025

Synthesis and Structure–Activity Relationship of Cytotoxic Marine Cyclodepsipeptide IB-01212 Analogues

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Several recently discovered marine products have remarkable in vitro and in vivo anticancer profiles against a wide range of tumor cell lines. Some of these compounds are currently in clinical trials. These compounds show complex structures and mechanisms of action of interest. Herein, we describe the preparation of a series of totally synthetic molecules that are structurally related to the natural marine product IB-01212 and evaluated them as antitumor agents. For this, total solid-phase syntheses of the products were performed in parallel by two distinct routes: linear synthesis and convergent synthesis. Structural modifications were introduced in several residue positions to afford 21 IB-01212 analogues for structure–relationship studies. An increase in the number of methyl groups in the macrocycle enhanced cytotoxic activity. Also, the replacement of an ester bond by an amide bond favored antitumor activity against several human cell lines. In addition, the L *configuration analogues were more active against all the tumor cell lines than those containing the* D *configuration. A significant increase in the size and asymmetry of the macrocycle diminished biological activity with respect to that of IB-01212. These results are of great value for the discovery of new and more effective anticancer agents.*

Introduction

Marine organisms use chemical means for defense, capture of prey, mediation of interspecies competition for food, or territory. By default, the structurally complex, water soluble natural products used for these functions are extremely potent, as upon expulsion into an aqueous environment they are rapidly diluted before interacting with a target organism. Drugs derived from marine organisms have recently inspired a new era in human therapy, offering vast potential for the treatment of a myriad diseases for which new, alternative therapies are desperately needed. Numerous marine compounds have been evaluated for cancer treatment, including ecteinascidin-743,[1] aplidine,[2–5], variolin B,[6,7] lamellarins,[8] dolastatin 10,[9] jasplakinolide,[10,11] and kahalalide F.[12–14]

In many cases, these promising compounds are peptides or depsipeptides with complex structures that favor conformational diversity. Specifically, rare residues such as D-amino acids, *N*- or *C*-alkylated amino acids, α,β-didehydroamino acids, hydroxyl acids, and structurally elaborate amino acids, such as the reverse prenyl (rPr) of two residues of Ser and Thr in Trunkamide A.[15] The presence of these residues could improve pharmacokinetic and pharmacodynamic profiles, information that can be then be applied to the design of new and more efficacious drugs.

These compounds of marine origin must go through several phases to become a drug: 1) extraction from the natural source,[16] 2) evaluation of biological activity,[17] 3) confirmation of chemical structures by numerous techniques[18] (NMR, MS, AAA, RP-HPLC, etc.), 4) chemical synthesis,[19] 5) structure–activity relationship (SAR) studies for drug optimization,[20,21] and finally, 6) assurance of a homogeneous supply of the compound for preclinical and clinical trials.[22,23] Each of these stages is crucial in the development of a drug.

The cyclodepsipeptide IB-01212 has successfully passed some of these stages. IB-01212 was isolated from the mycelium extract of the marine fungus *Clonostachys pytirodes*.[24,25][1] The amino acid sequence of the compound was determined by spectroscopic techniques, and the absolute configuration was found as reported above, in which all amino acids are in the L-configuration.[26] Also, several effective strategies for the solid-phase synthesis of IB-01212 have been developed by our group.[27] IB-01212 is a C2 symmetric octapeptide featuring a six-membered cyclic core, with two residues each of L-*N*,*N*-

[1] *The filamentous fungus.* Clonostachys sp. *ESNA-A009 was isolated from an unidentified marine sponge collected in Japan using oatmeal agar supplemented with 100% seawater. A culture of the strain has been deposited in the "Colección Española de Cultivo Tipo" at the University of Valencia, Spain, under the accession number CECT 20477.*

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Me_2Leu, L-Ser, L-*N*MeLeu and L-*N*MePhe (Figure 1). Herein, we report the design and synthesis of 21 new IB-01212 analogues and the evaluation of their antitumor activity against several human cancer cell lines.

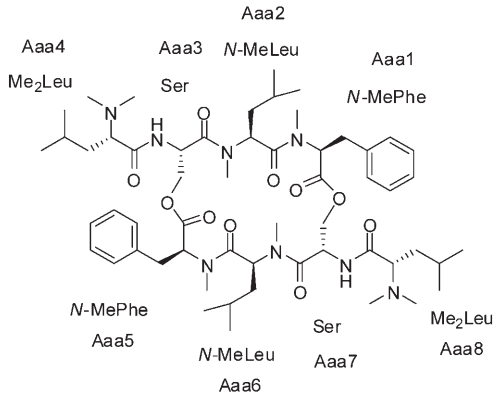


Figure 1. Structure coded of IB-01212.

Results and Discussion

Design and synthesis of IB-01212 analogues

In previous work, IB-01212 was synthesized by three solid-phase routes:[27] 1) dimerization of heterodetic fragments, 2) linear synthesis, and 3) convergent synthesis. The convergent and the linear strategies provided IB-01212 in acceptable yields, and were therefore used to obtain a diversity of new analogues quickly and efficiently. Syntheses of the analogues were performed by solid-phase methods in groups of between 2 and 8 compounds simultaneously.

The new analogue nomenclatures refer to the residues that were replaced by others in the coded positions of the IB-01212 natural structure (Figure 1).

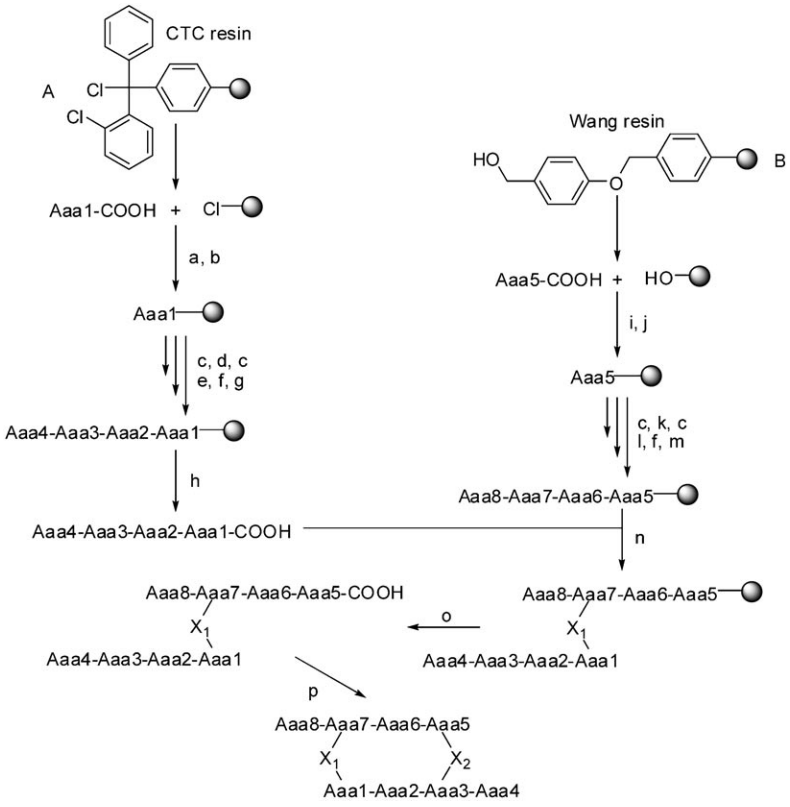
Several chemical modifications were studied such as: stereochemistry, *N*-methylation at Aaa3,4,7,8, substitution of the ester bond, and the size of the macrocycle, through the number of methylene groups in Aaa3 and Aaa7.

Preparation of IB-01212 analogues using a convergent strategy

As depicted in Scheme 1, the syntheses of the peptide fragments were performed on two resins. Thus, the ester bond on the CTC resin allowed selective cleavage of the protected tetrapeptide, whereas the ester bond on Wang resin was stable to the removal of high acid labile side-chain protecting groups. Later, the convergent synthesis of the protected peptide fragment was performed by incorporation of the partially protected tetrapeptide (Fragment A) synthesized on CTC resin onto the tetrapeptide (Fragment B) synthesized on Wang resin. Finally, the macrocycle was obtained by cyclization in solution.

The synthesis of Fragment A consisted of: 1) incorporation of a Fmoc-amino acid (Aaa1) onto a solid support, thereby forming an ester bond, 2) elongation of the peptide chain with three amino acids (Aaa2, Aaa3, Aaa4) using an Fmoc/*t*Bu strategy (when Aaa3 is Fmoc-Ser(*t*Bu)-OH, $X_1 = O$ and when Aaa3 is Fmoc-Cys(Trt)-OH, $X_1 = S$), and 3) cleavage of the protected peptide fragment from resin.

The synthesis of Fragment B consisted of: 1) incorporation of a Fmoc-amino acid (Aaa5) onto a solid support, 2) elongation of the peptide chain with three amino acids (Aaa6, Aaa7, Aaa8) using a Fmoc/*t*Bu strategy (when Aaa7 is Fmoc-Ser(Trt)-



Scheme 1. Convergent (4 + 4) solid-phase synthesis of IB-01212 analogues. a) Aaa1, DIEA, DCM; b) MeOH; c) piperidine-DMF (2:8) and piperidine/DBU/toluene/DMF (5:5:20:70); d) Aaa2, PyBOP/HOAt/DIEA, DMF; e) Aaa3/DIPCDI (2:1); f) piperidine-DMF (2:8); g) Aaa4, PyBOP/HOAt/DIEA, DMF; h) TFA-DCM (1:99) for Trt of Ser or TFA-TES-DCM (2:5:93) for Mmt of Cys; i) Aaa5, MSNT/NMI/DIEA, DMF; j) Ac_2O/NMI/DIEA; k) Aaa6, PyBOP/HOAt/DIEA, DMF; l) Aaa7/DIPCDI (2:1); m) Aaa8, PyBOP/HOAt/DIEA, DMF; n) MSNT/NMI/DIEA, DMF; o) TFA-H_2O-TES (95:2.5:2.5); p) MSNT/NMI/DIEA, DCM-DMF.

OH, $X_2 = O$ and when Aaa7 is Fmoc-Cys(Mmt)-OH, $X_2 = S$), and 3) selective deprotection of side-chain group (Trt or Mmt groups) while the ester-linked peptide remains on the Wang resin.

The convergent synthesis consisted of: 1) incorporation of the protected tetrapeptide Fragment A onto the tetrapeptide anchored onto Wang resin, 2) cleavage from the support of the lineal octapeptide and deprotection of side-chain groups, 3) cyclization of peptide in solution, and 4) purification and characterization of the target product.

The synthetic process was conducted as follows: the synthesis of tetrapeptide A was carried out by standard Fmoc/*t*Bu chemistry. The Fmoc-Aaa1 was incorporated onto CTC resin by esterification with DIEA and the remaining chloride groups were capped by addition of MeOH. The Fmoc-protecting group was removed with 20% of piperidine in DMF during 20 min and piperidine/DBU/toluene/DMF (5:5:20:70) for 10 min to assure complete removal.[28,29] Then, the Fmoc-Aaa2-OH (2 equiv) was carried out with an equimolar amount of PyBOP/HOAt and a double amount of DIEA in DMF for 1 h. This method

ology is one of the most effective for the coupling of Fmoc-Aaa-OH on *N*MeAaa because it combines a reactive stand-alone coupling reagent (PyBOP)[30] with an excellent leaving group (HOAt).[31] Furthermore, PyBOP is relatively inexpensive compared to PyAOP,[32] which is the phosphonium salt derived from HOAt. After the coupling, the chloranil test[33] was carried out and when positive the coupling was repeated in the same conditions, otherwise the process was continued. Washings between deprotection, coupling, and, again, deprotection steps were done with DMF and DCM using 10 mL solvent/g resin each time. Incorporation of Fmoc-Aaa3-OH (4 equiv) was carried out with DIPCDI (2 equiv) to form the symmetric anhydride. This strategy reduces the risk of diketopiperazine formation by intramolecular aminolysis.[34] The Aaa4 was coupled with PyBOP (2 equiv), HOAt (2 equiv), and DIEA (6 equiv). Protected peptide fragments were released from the CTC resin by cleavage with TFA-DCM (1:99).

Tetrapeptide B was also synthesized by Fmoc/*t*Bu chemistry on Wang resin. The Fmoc-Aaa5-OH was anchored on the resin by esterification with MSNT/NMI and DIEA. The remaining hydroxyl functions were acetylated with acetic anhydride, NMI, and DIEA. Tetrapeptide elongation was performed following the experimental procedures described above, except for the coupling of the third amino acid, where the semi-permanent protecting group (*t*Bu for Ser and Trt for Cys) of Fmoc-Aaa7-OH was replaced by a more labile group (Trt for Ser and Mmt for Cys), removed selectively by treatment with low TFA content (2%) in presence of TES in DCM while the peptide remained anchored on the Wang resin.

The free carboxyl extreme terminal of the protected fragment B (Aaa4-Aaa3(*t*Bu/Trt)-Aaa2-Aaa1-OH) obtained from the CTC resin was coupled onto the free hydroxyl group of the Aaa7 (Aaa8-Aaa7-Aaa6-Aaa5-Wang resin) with MSNT/NMI/DIEA or TBTU/HOAt/DIEA for 24 h.

For all analogues, final deprotection was carried out with TFA-TES-H2O (95:2.5:2.5) for 2 h and cyclization was performed with MSNT/NMI/DIEA in DCM:DMF (9:1). Thirteen analogues (**1**–**13** analogues) were synthesized using the convergent strategy (4 + 4) as shown in Table 1.

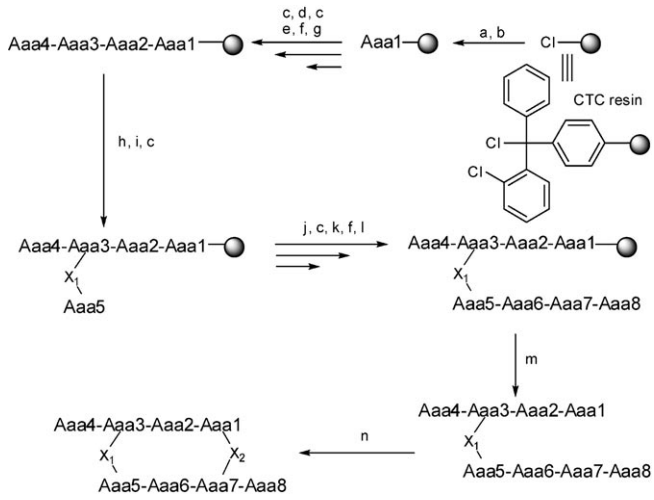
Preparation of IB-01212 analogues using a linear strategy

The remaining analogues were synthesized using a linear/stepwise solid-phase strategy.

Scheme 2 depicts the linear synthesis of cyclodepsipeptide analogues, which was performed on the CTC resin. This allowed the total linear synthesis of the octapeptide, and mini-

Table 1. IB-01212 analogues synthesized by convergent and linear approaches.

#	analogues	Aaa8	Aaa7	Aaa6	Aaa5	Aaa4	Aaa3	Aaa2	Aaa1	Bond
1	IB-01212	Me_2Leu	Ser	MeLeu	MePhe	Me_2Leu	Ser	MeLeu	MePhe	ester
2	2[Me_2DLeu^{4,8}]	Me_2DLeu				Me_2DLeu				ester
3	2[DSer^{3,7}]		DSer				Dser			ester
4	2[MeDLeu^{2,6}]			MeDLeu				MeDLeu		ester
5	2[MeDPhe^{1,5}]				MeDPhe				MeDPhe	ester
6	2[Leu^{2,6},Phe^{1,5}]			Leu	Phe			Leu	Phe	ester
7	2[Phe^{1,5}]				Phe				Phe	ester
8	2[Leu^{2,6}]			Leu				Leu		ester
9	2[MeSer^{3,7}]		MeSer				MeSer			ester
10	2[Me_3Leu^{4,8}]	Me_3Leu				Me_3Leu				ester
11	2[AcMeLeu^{4,8}]	AcMeLeu				AcMeLeu				ester
12	[AcMeLeu^{8}]	AcMeLeu								ester
13	2[Cys^{3},Cys^{7}]		Cys				Cys			thioester
14	2[Dap^{3,7}]		Dap				Dap			amide
15	[Dap^{3}]						Dap			amide, ester
16	2[Dab^{3},Dab^{7}]		Dab				Dab			amide
17	[Dab^{3}]						Dab			amide, ester
18	2[Orn^{3},Orn^{7}]		Orn				Orn			amide
19	[Orn^{3}]						Orn			amide, ester
20	2[Lys^{3},Lys^{7}]		Lys				Lys			amide
21	[Lys^{3}]						Lys			amide, ester
22	2[Dap^{3},Cys^{7}]		Cys				Dap			amide, thioester



Scheme 2. Linear solid-phase synthesis of IB-01212 analogues. a) Aaa1, DIEA, DCM; b) MeOH; c) piperidine-/DMF (2:8) and piperidine//DBU//toluene//DMF (5:5:20:70); d) Aaa2, PyBOP/HOAt/DIEA, DMF; e) Aaa3/DIPCDI (2:1), DCM//DMF; f) piperidine-/DMF (2:8); g) Aaa4, PyBOP/HOAt/DIEA, DMF; h) Pd-$(PPh_3)_4$/$PhSiH_3$; i) Aaa5, HOAt/DIPCDI, DMF; j) Aaa6/PyBOP/HOAt/DIEA, DMF; k) Aaa7/DIPCDI (2:1), DCM//DMF; l) Aaa8/DIPCDI/HOAt, DMF; m) TFA-DCM (1:99); n) PyAOP/HOAt/DIEA, DCM-DMF.

mization of diketopiperazine formation,[35] which is favored by the presence of *N*-alkylated amino acids, such as those contained in the IB-01212 peptide.[36] This linear synthesis on solid-phase is more convenient for this second group of analogues, which contains at least an amide bond (X_1) and even in some cases the two (X_1 and X_2). The amino side chain of Aaa3 was protected with the Alloc group. This group can be orthogonally removed in almost neutral conditions [Pd^0], which are compatible with the use of CTC resin. The general methodology of synthesis consisted of: 1) incorporation of the first Fmoc-amino acid (Aaa1) onto a solid support, thereby forming an ester bond, 2) elongation of the peptide chain with three amino acids (Aaa2, Aaa3, Aaa4) using a Fmoc/*t*Bu/Alloc strategy. Thus, Aaa3 was incorporated using Fmoc-Aaa(Alloc)-OH, 3) the Alloc group was removed with $Pd(PPh_3)_4$ in the presence of $PhSiH_3$ under an atmosphere of argon, 4) the elongation of the peptide sequence was continued through the free side chain with the following amino acids (Aaa5, Aaa6, Aaa7 (protected with acid labile protecting group), and Aaa8), 5) peptide cleavage and deprotection of the side chain; 6) peptide cyclizaton in solution, and finally 7) the purification and characterization of target product.

The synthetic process was performed basically as described for Fragment A and the Alloc group was removed with a catalytic amount of $Pd(PPh_3)_4$ in the presence of $PhSiH_3$ under an atmosphere of argon. Later the Fmoc-Aaa5-OH was introduced through the side chain of Aaa3 using DIPCDI and HOAt methods. For some analogues, the Alloc-protecting lateral group was completely removed by treatment with $Pd(PPh_3)_4$ in the presence of $PhSiH_3$ in DCM under an atmosphere of argon. Cleavage from the CTC-resin was performed with TFA-DCM (1:99).

Each analogue (crude linear peptide) was cyclized with PyAOP/HOAt/DIEA in DCM-DMF. The mixture was allowed to stir until RP-HPLC (linear gradient of 5 to 100% of CH_3CN (+0.036% TFA) to H_2O (+0.045% TFA)) indicated that the cyclization was done. The solvent was removed by evaporation under reduced pressure and each cyclic peptide was purified by RP-HPLC.

Summary of IB-01212 analogues

The IB-01212 analogues described in Table 1 were synthesized following the experimental procedures for convergent (analogues **1**–**13**, Scheme 1) and linear (analogues **14**–**22**, Scheme 2) approaches described above. Structural changes are indicated in the columns, where residue(s) (Aaa) found in IB-01212 were replaced by other(s).

Most of the analogues, except **12**, **15**, **17**, **19**, **21**, and **22**, were modified symmetrically (Table 1). In addition to Me_2Leu, which was prepared in solution by catalytic reductive condensation with formaldehyde, Me_3Leu (analogue **10**) was obtained directly on solid-phase during the peptide synthesis. Me_3Leu-OH was prepared by trimethylation of fragments A and B with an excess of methyl iodure and DIEA after the final coupling with Fmoc-Leu-OH and removal of the Fmoc-protecting group. Analogues **11** and **12** were also obtained by modification during the solid-phase procedure. The residues of fragments A and B were acetylated on solid phase using Ac_2O and DIEA after incorporation of Fmoc-MeLeu-OH and removal of the Fmoc-protecting group. The acetylations were allowed to react until a negative ninhydrin test result was obtained.

For analogues **2**–**5**, L-amino acids were replaced with D-amino acids to increase biological activity and metabolic stability to proteases, a common strategy in the design of peptidic drugs.

As methyl groups can influence the conformation and rigidity of a target molecule, we designed analogues **6**–**12**, which have varying degrees of *N*-methylation of Aaa 3,4,7,8 with respect to IB-01212, to study the relationship between methylation and biological activity.

Analogues **14**–**21** varied in the size of the macrocycle, through the number of methylene groups in Aaa3,7, and the bond that forms the cyclodepsipeptide. These compounds were synthesized using commercially available reagents such as Fmoc/Alloc Dap, Dab, Orn, and Lys.

Finally, compounds **13** and **22** had either one (**22**) or two (**13**) thioester bonds instead of the original ester bond between the two fragments that form the macrocycle.

Antitumor activity

The cyclodepsipeptide analogues of IB-01212 were designed from a chemical and structural point of view, specifically to assess the roles of stereochemistry, methylation of the terminal Leu, the bridge bonds (ester, thioester, amide), and macrocycle size in biological activity.

The analogues were evaluated for cytotoxicity against a panel of several human tumor cell lines. A conventional colori-

metric assay was set up to estimate GI_{50} values, that is, the drug concentration which causes 50% cell growth inhibition after 72 h continuous exposure to the test molecules. These assays provided preliminary medicinal chemistry information on the IB-01212 analogues and should be used for the design of new analogues. The results of the cytotoxicity assay are shown in Table 2, which includes the synthetic IB-01212 as a reference. Several general observations can be made.

Table 2. Cytotoxic activity against several tumor cell lines (GI_{50}, μM).[a]

#	Analogues	Prostate DU-145	Ovary IGROV	Breast SK-BR-3	Melanoma SK-MEL-28	Lung A-549	Leukemia K-562	Pancreas PANC-1	Colon HT-29
1	IB-01212 Synthetic	2.90	1.27	1.23	1.05	2.90	7.69	0.65	0.64
2	2[Me_2DLeu^{4,8}]	1.93	2.18	1.36	1.74	2.19	0.41	1.70	0.49
3	2[DSer^{3,7}]	2.25	3.00	1.47	2.25	2.91	1.81	1.97	1.42
4	2[MeDLeu^{2,6}]	9.67	6.18	3.69	6.28	9.68	4.09	4.31	5.32
5	2[MeDPhe^{1,5}]	2.35	2.73	1.02	2.04	2.48	2.68	1.80	1.21
6	2[Leu^{2,6},Phe^{1,5}]	10.21	12.40	23.00	10.24	20.26	50.26	12.92	10.23
7	2[Phe^{1,5}]	2.37	2.94	2.94	2.52	4.35	2.41	3.87	3.33
8	2[Leu^{2,6}]	9.95	5.97	4.89	9.95	9.95	6.39	9.95	9.95
9	2[MeSer^{3,7}]	1.58	0.79	0.35	0.38	1.28	0.50	0.78	5.90
10	2[Me_3Leu^{4,8}]	10.40	9.43	9.40	12.40	11.40	9.46	14.40	12.95
11	2[AcMeLeu^{4,8}]	10.05	15.65	12.75	13.65	12.45	17.64	14.09	18.73
12	[AcMeLeu^{8}]	2.08	2.88	1.27	2.01	2.84	2.16	1.94	1,94
13	2[Cys^{3},Cys^{7}]	–	–	–	–	8.63	–	–	9.83
14	2[Dap^{3,7}]-CH	2.49	1.01	0.71	0.41	2.86	0.54	0.52	0.65
15	[Dap^{3}]	0.16	0.20	–	0.17	0.31	0.16	0.29	0.24
16	2[Dab^{3},Dab^{7}]	0.51	4.24	0.11	0.32	0.54	0.88	0.34	0.55
17	[Dab^{3}]	2.87	2.87	2.38	1.20	2.87	1.79	2.87	2.87
18	2[Orn^{3},Orn^{7}]	2.39	2.,76	2.76	0.84	2.11	0.63	1.50	0.65
19	[Orn^{3}]	2.07	1.81	1.89	1.07	2.66	1.84	2.11	1.00
20	2[Lys^{3},Lys^{7}]	–	2.52	–	–	2.52	–	–	–
21	[Lys^{3}]	2.77	2.79	1.95	1.10	2.61	1.25	2.03	1.29
22	2[Dap^{3},Cys^{7}]	2.86	2.86	1.93	1.14	2.86	1.05	1.93	1.61

[a] The lines that are overshadowed indicate that the compound presents biological activity of significance in comparison with the synthetic IB-01212. All assays were carried out in triplicate at 10 concentrations. The 30 points obtained were used for curve calculation and hence GI_{50} values determination using LIMS (Laboratory Information Management Systems) software from PharmaMar.

The D-amino acid analogues **3**–**5** were less active than synthetic IB-01212, except analogue **2** that is almost as active as analogue **1** (IB-01212 synthetic natural). This finding demonstrates the importance of the L configuration in each position of the cyclodepsipeptide, and confirms the stereochemical configuration established for IB-01212 in the previous experiments.[26]

The demethylated analogues **6**–**8** were also inactive. In contrast, analogue **9** (*N*MeSer), which has more *N*-methyl groups than synthetic IB-01212, was highly cytotoxic against all the tumor cell lines assayed. This result demonstrates the importance of the methyl groups for the conformation of the molecule. Activity was lost in analogue **10**, in which a third methyl group was incorporated in the *N*-terminal of the molecule. This loss of activity may be explained by the fact that three methyl groups on the *N*-terminal of the Leu generate two global positive charges in the molecule.

On the other hand, replacement of the methyl group at the Leu *N*-terminal (Aaa4 and Aaa8 for analogues **11** and **12**, respectively) with an acetyl group also caused a decrease in antitumor activity. This observation indicates the importance of dimethylation of the Leu at the *N*-terminal.

Substitution of Ser with Dap at positions Aaa3, as in Aaa7, led to the azacompounds. Analogue **14** (2 Dap), which contains an amide bond in place of an ester bond, and analogue **15** (Ser, Dap), which has both an amide and an ester bond, were more active than the synthetic IB-01212.

Analogues **16**–**21**, which have a larger macrocycle than the reference compound, differed in their activity. Whereas analogues **16** (2 Dab) and **18** (2 Orn) were active against all the cell lines, analogues **17** (Ser, Dab), **19** (Ser, Orn), **20** (2 Lys), and **21** (Ser, Lys) were less active. The lack of inactivity of these four larger analogues indicates that size is important for activity. The activity of **16** and **18** (also larger cycle sizes) can be interpreted by the presence of the two amide bonds, which counterparts the increase in macrocycle size. This general loss of cytotoxic activity with increased macrocycle size could be explained by a decrease in intramolecular hydrogen bonding of the macrocycle. This may generate a greater degree of freedom in the molecule, which results in more conformations and a decrease in the proportion of the most active conformation. Another possible explanation is an improvement in the optimal macrocycle size and its conformation requirements with the receptor binding site. These results indicate that the size and a high degree of symmetry in the molecule are required for biological activity.

Finally, analogues **13** and **22**, inspired in the cyclothiodepsipeptide thiocoraline,[37] in which Cys residues were placed at positions Aaa3 and Aaa7, were also less active than the reference compound. The higher lability of the thioester bonds may cause the decrease in their biological activity.

Conclusions

Several IB-01212 analogues were synthesized on solid-phase using convergent or linear approaches depending on synthetic feasibility. The convergent method, using CTC and Wang resins, was used to synthesize analogues with ester and/or thioester linkages in the macrocycle (analogues **1**–**13**). Linear synthesis, using just CTC resin, was used to obtain analogues with amide and/or amide ester bond linkages in the macrocy-

cle (analogues **14**–**21**). All compounds were obtained with an appropriate purity for the antitumor activity assays and were characterized by RP-HPLC and MS.

SAR studies were performed on a series of IB-01212 analogues, focusing on the number of *N*Me groups, amino acid configuration, macrocycle size, and type of bridge. The antitumor activity against a broad panel of tumor cell lines of these analogues varied greatly. D-amino acid analogues were less active than those of the L configuration. An increase in the number of *N*Me groups favored activity in the internal amino acid (Ser). Macrocycle size and linkage type were crucial factors to optimize biological activity: amide or amide and ester bonds favored activity compared to the original compound. Larger and asymmetric macrocycles were less active. These results are currently being used for the design of a new series of cyclo(depsi)peptides based on the same scaffold.

Experimental Section

Materials and reagents. CTC-resin, protected Fmoc-amino acid derivatives, HOAt, PyBOP, and MSNT were purchased from Luxembourg Industries (Tel-Aviv, Israel), Neosystem (Strasbourg, France), NovaBiochem (Läufelfingen, Switzerland), and Applied Biosystems (Framingham, USA). DIEA, DIPCDI, piperidine, TFA, and CH_3CN (HPLC grade) were purchased from Merck (Darmstadt, Germany), Scharlau (Barcelona, Spain), and Panreac (Barcelona, Spain). DMF, DCM, and methanol were obtained from SDS (Peypin, France). All commercial reagents and solvents were used as received, with the exception of DCM, which was passed through an alumina column to remove acidic contaminants. *N*,*N*Me$_2$Leu was synthesized as described in a previous manuscript.[26] The synthetic IB-01212 was obtained previously in our group.[27]

Analysis by RP-HPLC. Cyclodepsipeptide analogue samples were analyzed on Symmetry C_{18} reverse-phase HPLC columns (4.6× 150 mm, 5 μm) (Waters, Ireland). Analytical HPLC was carried out on a Waters instrument 996 photodiode array detectors, equipped with a Waters 2695 separation module and Millennium software. UV detection was performed at 220 nm, and a linear gradient of 5 to 100% of CH_3CN (+0.036% TFA) to H_2O (+0.045% TFA) was run over 15 min at a flow rate of 1.0 mL min^{-1}.

Analysis by HPLC–MS. HPLC–MS analyses were performed on an Alliance system (Waters 2795 Separation Module, USA) coupled to a double wavelength UV detector (Waters 2487 dual λ Absorbance Detector, USA) and a ZQ4000 mass spectrometer (Waters, Micromass ZQ, USA). UV detection was performed at 220 nm, and linear gradients from 5 to 100% of CH_3CN (+0.07% formic acid) to H_2O (+0.1% formic acid) was run at 1.0 mL min^{-1} flow rate. Data were processed by Masslynx version 4.0.

Purification. The cyclodepsipeptide analogues were purified by semi-preparative RP-HPLC on a Waters 2487 Dual λ absorbance detector equipped with a Waters 2700 sample manager, a Waters 600 controller, a Waters fraction collector, and a Symmetry® column (C_{18} reverse-phase column, 5 μm, 30×100 mm). A linear gradient of 5 to 60% of CH_3CN (+0.1% TFA) to H_2O (+0.1% TFA) over 30 min at a flow rate of 20 mL min^{-1} was used. For all compounds, the injection volume was about 5 mL of a solute aqueous solution. Data were processed by Millenium software.

Mass spectrometry. MALDI-TOF analyses of cyclodepsipeptide samples were performed on an Applied Biosystems Voyager DE RP using 2,5-dihydroxybenzoic acid (DHB) matrix.

Synthesis of new IB-01212 analogues using convergent (4 + 4) solid-phase methods.

Fragment A synthesis. CTC resin (0.6 g, 1.6 mmol g^{-1}) was placed in a 10 mL polypropylene syringe fitted with a polyethylene filter disk. The resin was then washed with DCM (5×2 min), DMF (5× 2 min) and DCM (5×2 min). A solution of Fmoc-Aaa1-OH (0.6 mmol) and DIEA (1 equiv) in DCM (2 mL) was added, and the mixture was stirred for 5 min. DIEA (2 equiv) was added and the mixture was stirred for 1 h. The reaction was quenched by the addition of MeOH (400 μL) with stirring for 15 min. The Fmoc-Aaa1-O-Trt-Cl-resin was then washed with DCM (3×1 min) and DMF (3× 1 min), and treated with piperidine-DMF (1×1 min, 2×10 min) and DBU/piperidine/toluene/DMF (5:5:20:70, 1×1 min, 2×5 min). In general, the loading calculated by Fmoc determination was about 0.8 mmol g^{-1}. Fmoc-Aaa2-OH (2 equiv) was then added using PyBOP (2 equiv), HOAt (2 equiv), and DIEA (6 equiv) in DMF. The Fmoc group was removed as described above and Fmoc-Aaa3-OH (4 equiv) was coupled sequentially with DIPCDI (2 equiv) using the asymmetric anhydride method. The Fmoc group was removed by treatment with piperidine-DMF (1×1 min, 2×10 min). Next, Fmoc-Aaa4-OH (2 equiv) was coupled with PyBOP (2 equiv), HOAt (2 equiv) and DIEA (6 equiv) for 2 h. The course of reaction was verified by the ninhydrin test.[38]

Tetrapeptide fragments were cleaved from the resin with TFA-DCM (1:99) (5×1 min). TFA was evaporated under reduced pressure and the residue was dissolved in CH_3CN-H_2O (1:1) and lyophilized. Each compound was identified by RP-HPLC and MALDI-TOF.

Fragment B synthesis. Wang resin (0.2 g, 0.82 mmol g^{-1}) was placed in a 10 mL polypropylene syringe fitted with a polyethylene filter disk. The resin was washed with DMF (5×1 min) and DCM (5× 1 min). The hydroxyl groups (Wang resin) were esterified with a solution of Fmoc-Aaa5-OH (2 equiv), MSNT (2 equiv), NMI (2 equiv), and DIEA (4 equiv) in DMF. The remaining hydroxyl functions were acetylated by treatment with Ac_2O-NMI-DIEA-DMF (2:1:1:6) for 30 min. The loading calculated by Fmoc determination was approximately 0.6 mmol per gram of resin. Next, Fmoc-Aaa6-OH (2 equiv) was added using PyBOP (2 equiv), HOAt (2 equiv), and DIEA (6 equiv) in DMF. After 1 h of coupling, the reaction was verified by the chloranil test.[33] The Fmoc group was removed and Fmoc-Aaa7-OH (8 equiv) was coupled with DIPCDI (4 equiv) by means of the asymmetric anhydride method. The last amino acid, Fmoc-Aaa8-OH (2 equiv), was coupled with PyBOP (2 equiv), HOAt (2 equiv), and DIEA (6 equiv). Washings between deprotection and coupling were carried out with DMF (5×1 min) and DCM (5× 1 min) using 10 mL solvent g^{-1} resin for each treatment. Finally, the Trt- or Mmt-protecting groups were removed completely by treatment with TFA-TES-DCM (2:5:93) for 2 h. The TFA salt was neutralized by washing it with DIEA-DCM (81:19). An aliquot of each peptide resin was treated with TFA-TES-H_2O (95:2.5:2.5) and analyzed by RP-HPLC and MALDI-TOF.

Convergent Synthesis of Fragments A and B. Crude peptide (fragment A, 2 equiv) was coupled to the free hydroxyl group of the Aaa7 (fragment B, 1 equiv) using MSNT (4 equiv), NMI (4 equiv), and DIEA (8 equiv) in DMF. The reaction was monitored by taking an aliquot of the peptide resin every 4 h, cleaving it with TFA, and analyzing the resulting crude product by RP-HPLC and MALDI-TOF or HPLC–MS. Simultaneous cleavage of each peptide from the resin and deprotection of side chain protecting group was accomplished with TFA-TES-H_2O (95:2.5:2.5, 10 mL) for 2 h. The cleavage solution was filtered into 50 mL centrifuge tubes containing 30 mL of cold tert-butyl methyl ether. After centrifugation (5 min at 4000 rpm) and decantation, the precipitates were washed four times by addition of cold ether. The peptide was dissolved in CH_3CN-H_2O (1:1, 20 mL) and lyophilized.

For the cyclization, each crude linear peptide (40 mg, 0.038 mmol) was dissolved in DCM-DMF-NMI (9:0.8:0.2, 40 mL), and MSNT (45 mg, 0.152 mmol, 4 equiv) and DIEA (79 mL, 0.456 mmol, 12 equiv) was added. The mixture was allowed to stir until the cyclization was shown by RP-HPLC to be complete. The solvent was removed by evaporation under reduced pressure. The cyclic peptide was dissolved in CH_3CN-H_2O (1:1) and purified by semi-preparative RP-HPLC as described above. Each sample was analyzed by RP-HPLC and MALDI-TOF.

The analogues described in Table 3 were synthesized following experimental procedures by a convergent strategy, as described above. For each analogue, the changes were indicated into the line, where original residue(s) (A) were replaced by other(s) (B).

Table 3. Analogues obtained by a convergent strategy.

#	Analogues	Aaa	Residue replaced A	Residue incorporated B
2	2[Me_2-D-$Leu^{4,8}$]	Aaa8	Me_2-L-Leu^8-OH	Me_2-D-Leu^8-OH
		Aaa4	Me_2-L-Leu^4-OH	Me_2-D-Leu^4-OH
3	2[D-$Ser^{3,7}$]	Aaa7	Fmoc-L-Ser(*t*Bu)^7-OH	Fmoc-D-Ser(*t*Bu)^7-OH
		Aaa3	Fmoc-L-Ser(Trt)^3-OH	Fmoc-D-Ser(Trt)^3-OH
4	2[Me-D-$Leu^{2,6}$]	Aaa6	Fmoc-Me-L-Leu^6-OH	Fmoc-Me-D-Leu^6-OH
		Aaa2	Fmoc-Me-L-Leu^2-OH	Fmoc-Me-D-Leu^2-OH
5	2[Me-D-$Phe^{1,5}$]	Aaa5	Fmoc-Me-L-Phe^5-OH	Fmoc-Me-D-Phe^5-OH
		Aaa1	Fmoc-Me-L-Phe^1-OH	Fmoc-Me-D-Phe^1-OH
6	2[$Leu^{2,6}$,$Phe^{1,5}$]	Aaa6	Fmoc-Me-L-Leu^6-OH	Fmoc-L-Leu^6-OH
		Aaa5	Fmoc-Me-L-Phe^5-OH	Fmoc-L-Phe^5-OH
		Aaa2	Fmoc-Me-L-Leu^2-OH	Fmoc-L-Leu^2-OH
		Aaa1	Fmoc-Me-L-Phe^1-OH	Fmoc-L-Phe^1-OH
7	2[$Phe^{1,5}$]	Aaa5	Fmoc-Me-L-Phe^5-OH	Fmoc-L-Phe^5-OH
		Aaa1	Fmoc-Me-L-Phe^1-OH	Fmoc-L-Phe^1-OH
8	2[$Leu^{2,6}$]	Aaa6	Fmoc-Me-L-Leu^6-OH	Fmoc-L-Leu^6-OH
		Aaa2	Fmoc-Me-L-Leu^2-OH	Fmoc-L-Leu^2-OH
9	2[Me-$Ser^{3,7}$]	Aaa7	Fmoc-L-Ser(*t*Bu)^7-OH	Fmoc-Me-L-Ser(*t*Bu)^7-OH
		Aaa3	Fmoc-L-Ser(Trt)^3-OH	Fmoc-Me-L-Ser(Trt)^3-OH
10	2[Me_3-$Leu^{4,8}$]	Aaa8	Me_2-L-Leu^8-OH	Me_3-L-Leu^8-OH
		Aaa4	Me_2-L-Leu^4-OH	Me_3-L-Leu^4-OH
11	2[AcMe-$Leu^{4,8}$]	Aaa8	Me_2-L-Leu^8-OH	Ac-Me-Leu^8-OH
		Aaa4	Me_2-L-Leu^4-OH	Ac-Me-Leu^4-OH
12	[Ac-Me-Leu^8]	Aaa8	Me_2-L-Leu^8-OH	Ac-Me-Leu^8-OH
13	2[Cys^3-Cys^7]	Aaa7	Fmoc-L-Ser(*t*Bu)^7-OH	Fmoc-Cys(Mmt)^7-OH
		Aaa3	Fmoc-L-Ser(Trt)^3-OH	Fmoc-Cys(Trt)^3-OH

Synthesis of new IB-01212 analogues using linear solid-phase methods. CTC resin (0.6 g, 1.64 mmol g^{-1}) was placed in a 10 mL polypropylene syringe fitted with a polyethylene filter disk. The resin was then washed with DCM (5×2 min), and a solution of Fmoc-Aaa1-OH (0.6 mmol) and DIEA (1 equiv) in DCM (2 mL) was added, and the mixture was stirred for 5 min. DIEA (2 equiv) was added and the mixture was stirred for 1 h. The reaction was quenched by the addition of MeOH (400 μL) with stirring for 15 min. The Fmoc Aaa1-O-TrtCl-resin was then washed with DCM (3×1 min) and DMF (3×1 min), and treated with piperidine-DMF (1×1 min, 2×10 min) and DBU/piperidine/toluene/DMF (5:5:20:70, 1×1 min, 2×5 min). In general, the loading calculated by Fmoc determination was about 0.8–1.0 mmol g^{-1}. Fmoc-Aaa2-OH (2 equiv) was added using PyBOP (2 equiv), HOAt (2 equiv), and DIEA (6 equiv) in DMF. The coupling reaction was verified by the chloranil test. Fmoc-Aaa3-OH (4 equiv) was coupled with DIPCDI (2 equiv) using the asymmetric anhydride method. Next, Fmoc-Aaa4-OH (4 equiv) was coupled with PyBOP (2 equiv), HOAt (2 equiv), and DIEA (6 equiv). The Alloc group was removed with Pd-$(PPh_3)_4$ (0.1 equiv) in the presence of $PhSiH_3$ (10 equiv) under an atmosphere of argon, and Fmoc-Aaa5-OH (4 equiv) and HOAt (4 equiv) were dissolved in DMF (2 mL) and added to peptide resin. DIPCDI (4 equiv) was then added and the mixture was stirred until the ninhydrin test was negative. Next, Fmoc-Aaa6-OH (4 equiv) was added using PyBOP (4 equiv), HOAt (4 equiv), and DIEA (12 equiv) in DMF. The coupling reaction was verified by the chloranil test. Fmoc-Aaa7-OH (4 equiv) was coupled with DIPCDI (2 equiv) using the asymmetric anhydride method. The last amino acid, Fmoc-Aaa8-OH (4 equiv), was coupled with DIPCDI (4 equiv) and HOAt (4 equiv). When the lateral chain of the analogue was protected with the Alloc-protective group, it was removed by treatment with $Pd(PPh_3)_4$ (0.1 equiv) in the presence of $PhSiH_3$ (10 equiv) in DCM under an atmosphere of argon. Cleavage from the CTC resin was performed with TFA-DCM (1:99). The cleavage solution was removed by evaporation under reduced pressure. For the analogues with the *t*Bu group in the lateral chain, it was completely removed by treatment with TFA-H_2O-TES (95:2.5:2.5 *v/v/v*) for 2 h. All crude peptide products showed purity greater than 70% by RP-HPLC. Each compound was characterized by RP-HPLC and MALDI-TOF.

Each crude linear peptide (40 mg, 0.038 mmol) was dissolved in DCM-DMF (9:1, 40 mL), and PyAOP (39.5 mg, 0.076 mmol, 2 equiv), HOAt (10.3 mg, 0.076 mmol, 2 equiv), and DIEA (19.3 μL, 0.13 mmol, 4 equiv) were added. The mixture was allowed to stir

Table 4. Analogues obtained by linear synthesis.

#	Analogues	Aaa	Residue replaced A	Residue incorporated B
14	[Dap^3,Dap^7]	Aaa3	Fmoc-Ser(Trt)^3-OH	Fmoc-Dap(Alloc)^3-OH
		Aaa7	Fmoc-Ser(*t*Bu)^7-OH	Fmoc-Dap(Alloc)^7-OH
15	[Dap^3]	Aaa3	Fmoc-Ser(Trt)^3-OH	Fmoc-Dap(Alloc)^3-OH
16	[Dab^3,Dab^7]	Aaa3	Fmoc-Ser(Trt)^3-OH	Fmoc-Dab(Alloc)^3-OH
		Aaa7	Fmoc-Ser(*t*Bu)^7-OH	Fmoc-Dab(Alloc)^7-OH
17	[Dab^3]	Aaa3	Fmoc-Ser(Trt)^3-OH	Fmoc-Dab(Alloc)^3-OH
18	[Orn^3,Orn^7]	Aaa3	Fmoc-Ser(Trt)^3-OH	Fmoc-Orn(Alloc)^3-OH
		Aaa7	Fmoc-Ser(*t*Bu)^7-OH	Fmoc-Orn(Alloc)^7-OH
19	[Orn^3]	Aaa3	Fmoc-Ser(Trt)^3-OH	Fmoc-Orn(Alloc)^3-OH
20	[Lys^3,Lys^7]	Aaa3	Fmoc-Ser(Trt)^3-OH	Fmoc-Lys(Alloc)^3-OH
		Aaa7	Fmoc-Ser(*t*Bu)^7-OH	Fmoc-Lys(Alloc)^7-OH
21	[Lys^3]	Aaa3	Fmoc-Ser(Trt)^3-OH	Fmoc-Lys(Alloc)^3-OH
22	[Dap^3,Cys^7]	Aaa3	Fmoc-Ser(Trt)^3-OH	Fmoc-Dap(Alloc)^3-OH
		Aaa7	Fmoc-Ser(*t*Bu)^7-OH	Fmoc-Cys(Trt)^7-OH

until finalization of the cyclization, which was followed by RP-HPLC. The solvent was removed by evaporation under reduced pressure. Each cyclic peptide was dissolved in acetonitrile-water (1:1, *v/v*) and purified by semi-preparative RP-HPLC.

The analogues described in Table 4 were synthesized following experimental procedures by linear synthesis, as described above. For each analogue, changes were indicated into the line, where original residue(s) (A) were replaced by other(s) (B).

Characterization of the IB-01212 analogues synthesized. All macrocycle compounds were analyzed by RP-HPLC and MALDI-TOF. Table 5 shows the characterization of each cyclic peptide obtained in this study. The analysis by RP-HPLC allowed determination of the purity and retention time. The purity degree of the analogues was greater than 90%, which is acceptable for analyzing biological activity. MALDI-TOF and HPLC–MS were used for final characterization.

Cell Growth Inhibition Assay Screening. A colorimetric assay using sulforhodamine B (SRB) was adapted to perform a quantitative measurement of cell growth and viability, following a previously described method.[39] This assay uses 96-well cell culture microplates of 9 mm diameter.[40] Most of the cell lines were obtained from the American Type Culture Collection (ATCC) and were derived from several human cancer types (Table 6). Cells were maintained in RPMI 1640 10% FBS, supplemented with 0.1 g L^{-1} penicillin and 0.1 g L^{-1} streptomycin sulfate and then incubated at 37 °C, 5% CO_2, and 98% humidity. For the experiments, cells were harvested from subconfluent cultures using trypsin and were then resuspended in fresh medium before plating. The cells were seeded in 96-well microtiter plates at 5×10^3 cells well^{-1} in aliquots of 195 μL of RPMI medium and were left to grow in a drug-free medium for 18 h to allow attachment to the plate surface. Afterwards, samples were added in aliquots of 5 μL in a ranging from 10 to 10^{-8} μg mL^{-1}, dissolved in DMSO-EtOH-PBS (0.5:0.5:99). After 72 h of exposure, the antitumor effect was measured by SRB methodology: Cells were fixed by adding 50 μL of cold 50% (*w/v*) trichloroacetic acid (TCA) and were incubated for 60 min at 4 °C. Plates were washed with deionized H_2O and dried; 100 μL of SRB solution (0.4% *w/v* in 1% acetic acid) was added to each microtiter well and incubated for 10 min at room temperature. Unbound SRB was removed by washing with HOAc-H_2O (1:99). Plates were air-dried, and the bound stain was solubilized with Tris buffer. Optical densities were read on an automated spectrophotometer plate reader at a single wavelength of 490 nm. Data analyses were automatically generated by LIMS implementation. Using control OD values (C), test OD values (T), and time zero OD values (T_0), the drug concentration that causes 50% growth inhibition (GI_{50}) values) was calculated from the equation: 100 X $[(T\text{-}T_0)/C\text{ -}T_0)] = 50$.

Table 5. Analysis of the IB-01212 analogues by RP-HPLC and MALDI-TOF.

#	Analogues	t_R (min)	Purity (RP-HPLC) (%)	Mass calc.	Mass exp. (*m/z*) $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$
1	IB-01212 Synthetic	8.90	98%	1032.66	1033.32/1056.28/1072.24
2	2[Me_2DLeu^{4,8}]	8.54	96%	1032.66	1033.26/1055.21/1074.19
3	2[DSer^{3,7}]	8.48	90%	1032.66	1032.90/1056.90/1073.80
4	2[MeDLeu^{2,6}]	8.45	91%	1032.66	1032.92/1055.84
5	2[MeDPhe^{1,5}]	8.60	98%	1032.66	1033.27/1055.25/1071.22
6	2[Leu^{2,6},Phe^{1,5}]	8.21	92%	976.60	978.05/1000.03/1017.0
7	2[Phe^{1,5}]	8.28	94%	1004.63	1005.50/1028.01
8	2[Leu^{2,6}]	8.23	97%	1004.63	1005.10/1027.80
9	2[MeSer^{3,7}]	7.50	97%	1060.69	1062.20/1084.62
10	2[Me_3Leu^{4,8}]	7.89	91%	1062.71	1063.21
11	2[AcMeLeu^{4,8}]	7.80	97%	1088.65	1089.96/1112.31
12	[AcMeLeu^{8}]	8.62	95%	1060.66	1061.20
13	2[Cys^{3},Cys^{7}]	7.21	92%	1064.62	1065.87/1087.88
14	2[Dap^{3},Dap^{7}]	8.32	91%	1030.69	1031.83/1053.81/1069.81
15	[Dap^{3}]	8.30	90%	1032.36	1033.27/1054.25/1070.00
16	2[Dab^{3},Dab^{7}]	7.40	95%	1058.73	1061.80/1083.10/1100.4
17	[Dab^{3}]	7.47	92%	1045.69	1046.69/1068.90/1084.88
18	2[Orn^{3},Orn^{7}]	7.59	95%	1086.76	1087.98/1109.94/1125.92
19	[Orn^{3}]	7.47	98%	1059.71	1061.00/1082.84
20	2[Lys^{3},Lys^{7}]	7.39	96%	1114.79	1115.92/1137.90/1153.88
21	[Lys^{3}]	7.62	92%	1073.73	1075.65/1098.69
22	2[Dap^{3},Cys^{7}]	7.39	95%	1047.66	1049.21/1072.20/1087.13

Table 6. The tumor cell lines tested.

Name	N° ATCC	Species	Tissue	Characteristics
A-549	CCL-185	human	lung	lung carcinoma "NSCL"
SK-MEL-28	HTB-72	human	melanoma	malignant melanoma
HT-29	HTB-38	human	colon	colon adenocarcinoma
DU-145	HTB-81	human	prostate	prostate carcinoma, not androgen receptors
SK-BR-3	HTB-30	human	breast	breast adenocarcinoma, Her2/neu+, (pleural effusion)
IGROV		human	ovary	ovary adenocarcinoma
K-562	CCL-243	human	leukemia	chronic myelogenous leukemia
PANC-1	CRL-1469	human	pancreas	pancreatic epitheloid carcinoma

Abbreviations

AAA, amino acid analysis, C_{18}, octadecylsilica; DIEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; DCM, dichloromethane; DIPCDI, *N,N'*-Diisopropylcarbodiimide; DBU, 1,8-Diazabicyclo-[5.4.0]-undec-7-ene; DHB, 2,5-dihydroxybenzoic acid; Fmoc, 9-fluorenylmethoxycarbonyl; HATU, 1-[bis(-dimethylamino)methylene]-1H-1,2,3-triazolo-[4,5-b]pyridinium hexafluorophosphate 3-oxide; HOBt, 1-hydroxybenzotriazole; HOAt, 1-hydroxy-7-azabenzotriazole (3-hydroxy-3*H*-1,2,3-triazolo-[4,5-*b*]pyridine); HPLC, high pressure liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization with time-of-flight analysis; MS, mass spectrometry; MSNT, 1-(mesitylene-2-sulfonyl)-3-nitro-1*H*-1,2,4-triazole; NMI, 1-methylimidazole; PyBOP, benzotriazol-1-yloxy-tris(pyrrolidino)phosphonium hexafluorophosphate; RP, reverse phase; TES, triethylsilane; TFA, trifluoroacetic acid; TBTU, 1-[bis(dimethylamino)methylene]-1H-benzotriazolium tetrafluoroborate 3-oxide.

Acknowledgements

This study was partially supported by PharmaMar, S. A., CICYT (CTQ2006-03794/BQU, PETRI), the

Generalitat de Catalunya (2005SGR 00662), and the Barcelona Science Park.

Keywords: combinatorial chemistry · IB-01212 · marine compounds · solid-phase synthesis · structure–activity relationships

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Received: February 5, 2007
Revised: April 16, 2007
Published online on May 18, 2007

