Design and Synthesis of FAJANU: a de Novo C_2 Symmetric Cyclopeptide Family

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A novel cyclic peptide has been designed from several potent marine cytotoxic peptides, including IB-01212, luzopeptin, triostin, and thiocoraline. The FAJANU scaffold maintains C_2 symmetry, cyclic structure, and the construction of aromatic and aliphatic character at the N- and C-terminal extremes. A first six-member family was previously synthesized and evaluated biologically. Several analogues presented greater activity than IB-01212. Furthermore, on the basis of the most active candidate, we have performed a more exhaustive synthetic and structural analysis: (i) structure−activity relationship provided clues about the key elements in the framework, (ii) NMR assignment confirmed C_2 symmetry, and (iii) confocal images revealed its penetration and cellular localization.

Introduction

During recent years, several families of antitumoral cyclic peptides with C_2 symmetry have been isolated from marine sources. All of these compounds show head-to-side chain heterodetic (ester for Ser or thioester for Cys) bonds linking the two backbones. Examples include the onchidin B,[1,2] thiocoraline,[3] triostin,[4] IB-01212,[5] sandramycin,[6] and luzopeptin A[7] families. Furthermore, these peptides usually contain *N*-methyl and D-amino acids in addition to other nonproteogenic amino acids and, in some cases, aromatic heterocycles, a few of which present a disulfide bridge, thereby converting them into bicyclic peptides.[8] Symmetric peptides with these characteristics have also been isolated from terrestrial organisms such as valinomycin, the related montanastatin, and korkormicin, all of which are from the actinobacteria phylum.[9]

This collection of symmetric natural peptides presents a wide range of biological activity. In some cases, symmetry has proved to be suitable for interaction with highly symmetric complex structures, as is the case of thiocoraline and triostin, which are DNA intercalators. In other cases, symmetry has been considered to be an instrument for the microorganisms themselves to become more complex bioactive structures. Furthermore, in the case of valinomycin, polar groups are oriented toward the center, thereby allowing the delivery of selected ions through the cell membrane.[10] Syntheses of these peptides present an enormous synthetic challenge, which can jeopardize the development of drug discovery programs or even their industrial production.[11–19] Here we explored a de novo C_2-symmetric cyclopeptide family, taking as the starting point the simplest cytotoxic IB-01212 (**1**)

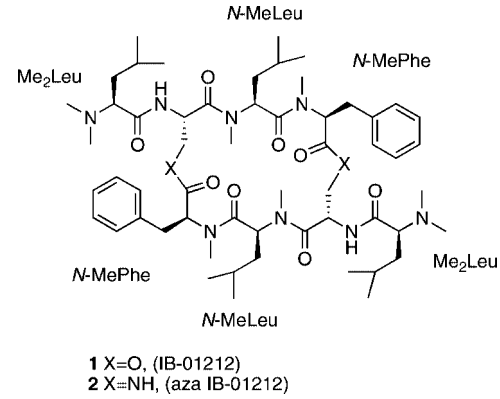

Figure 1. Structure of IB-01212 (**1**) and its aza analogue (**2**).

and its more active analogue **2** (Figure 1),[16,20] where both ester bonds were replaced by amide ones.

Results and Discussion

Design, Synthesis, and Biological Screening of the New FAJANU Peptide Family. Previous work by our group has described the preparation of **1** analogues, where the substitution of several amino acids using the same cyclic template, and the presence of the amide bond instead of the ester bond to close the cycle were examined.[29] The result of that study was the homodetic analogue **2**, which was more stable than **1** and showed a similar activity. In the present study, we designed a new structure by increasing the macrocycle size and by adding aromatic heterocycles with the purpose of improving antitumor activity. The new structure shares the chemical feasibility of IB-01212 analogues and the structural moieties relevant in highly cytotoxic compounds such as triostin or thiocoraline. *N*Me-Gly (Sar) [a], a common amino acid in this kind of peptide,

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[a] Abbreviations: Abbreviations used for amino acids and the designations of peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in *J. Biol. Chem.* **1982**, *247*, 977−983. The following additional abbreviations are used: aa, amino acid; Alloc, allyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; BPH, biphenyl-4-carboxylic acid; CHR, chromone carboxylic acid; CTC, chlorotrityl chloride (Barlos) resin; Dab, diaminobutiric acid; Dap, diaminopropyl carboxylic acid; DBU, 1,8-diazabicyclo-[5.4.0]undec-7-ene; DIPEA, *N,N*-diisopropylethylamine; DIPCDI, *N′,N″*-diisopropylcarbodiimide; DKP, diketopiperazine; DMF, *N,N*-dim-

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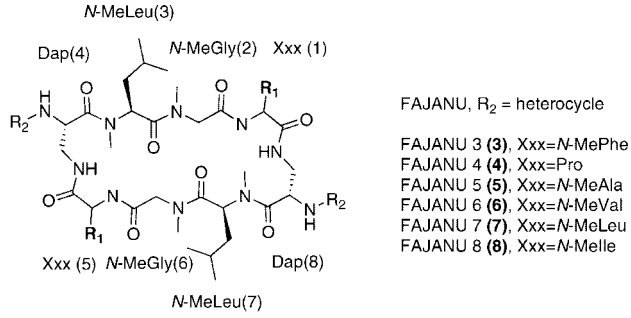

Figure 2. General structure of the FAJANU peptide family and first analogues synthesized.

was used to increase the size of the cycle, taking into account that the *N*Me group does not change the hydrogen bond map. In a first attempt, quinaldic acid, a heterocycle analogue present in other cytotoxic compounds, was incorporated as end-capping in order to mimic the aromatic ring of the Phe presented in **1**.

The new class of compounds, called FAJANU peptides, shows the following characteristics: (1) eight L-amino acid peptide backbone, (2) symmetric cyclic structure, (3) two antiparallel peptide chains linked by a homodetic amide bond, (4) *N*Me amino acids in the cyclic peptide backbone, and (5) two pharmacophore moieties linked to *N*-terminus chains. To explore the potential of the new FAJANU structures, a set of six peptides (Figure 2, see full structure in Figure 4) were synthesized and their biological activity was evaluated.

Synthesis of the FAJANU Peptide Family. The most convenient way to synthesize this kind of compound is by a combination of solid-phase and solution methods.[21–23] Three strategies can be used for this purpose (Scheme 1): (i) stepwise solid-phase synthesis, but with cyclization and introduction of the heterocycle in solution, (ii) total sequential stepwise solid-phase synthesis of the linear precursor and then cyclization performed in solution, and (iii) [4 + 4] convergent approach, taking advantage of the symmetry of the molecule, followed by cyclization and introduction of the heterocycle in solution.

This first FAJANU family **3** to **8** (**3**–**8**) (Figure 2) was synthesized using the stepwise linear method with cyclization and heterocycle introduction in solution (Scheme 1i). In the preparation of all cyclic peptides, the choice of cyclization point (the carboxylic component in the cyclization step should be the starting residue in the solid-phase synthesis) is of paramount importance[24] and is more crucial when the synthesis of peptides containing *N*Me amino acids is targeted. In FAJANU (**3**–**8**), the choice of *N*Me-Gly (**2**) as the C-component at the cyclization and solid-phase start point avoids the risk of racemization during cyclization and favors the parallel synthesis of all six analogues, because diversity Xxx (1) is introduced at the third step.

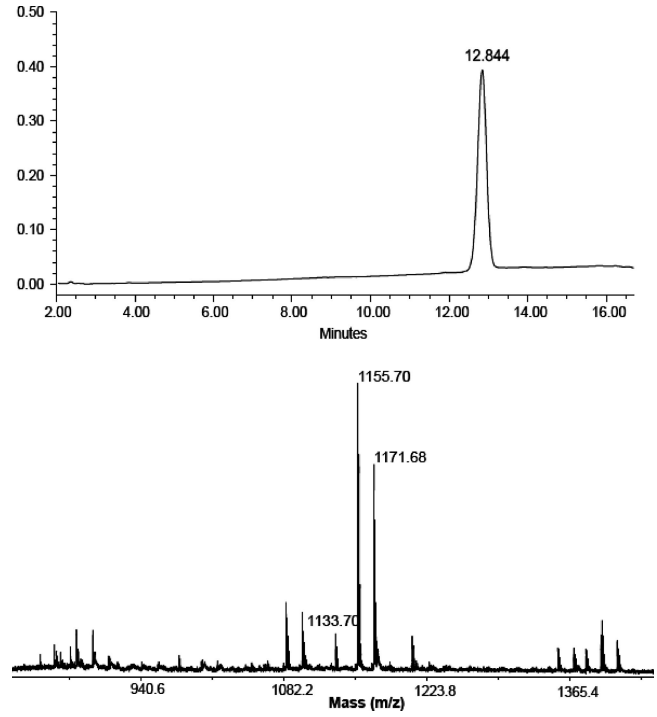

Figure 3. RP-HPLC profile (H_2O-ACN 5:5 to 0:10 in 15 min) and MALDI-TOF (*m/z* calcd for $C_{60}H_{84}N_{12}O_{10}$, 1132.6; found 1133.7 $[M + H]^+$, 1155.7 $[M + Na]^+$, 1171.7 $[M + K]^+$) of **7**.

Stepwise elongation was performed following methodologies previously developed by our group for the synthesis of other cyclic peptides.[25–27] For the preparation of *N*Me-containing peptides, the Fmoc strategy as temporary amino-protecting group is more convenient than the Boc strategy. In this case, the formation of a Boc-oxazolonium ion can occur by decomposing into an *N*-carboxyanhydride derivative, which could react and thereby lead to polymerization.[28] Removal of the Fmoc group from secondary amines is more difficult than from primary ones, thus an extra DBU treatment was carried out after standard treatment with piperidine.[29,30] The β-amino function of the Dap was also protected with the Fmoc group, reserving the Boc for the protection of the α-amino function, which had been removed before the heterocycle was introduced in solution at the end. At this point, the above-mentioned side-reaction cannot occur.

We chose the CTC (chlorotrityl chloride, Barlos)[31] resin as solid support for several reasons, the main ones being: (i) its lability in very mild acid conditions (1% TFA, HFIP, TFE),[32] because the use of higher TFA conditions during cleavage could cause the loss of the *N*-Ac-*N*Me amino acid terminus and cleavage of the *N*Me—*N*Me amino acid bond;[33] (ii) the absence of racemization during the incorporation of the first residue into the resin, because this is done through nucleophilic substitution and not through the activation of the carboxylic group required for anchoring to hydroxyl-based resins; (iii) this resin minimizes the formation of diketopiperazine (DKP).[34,35] This is of relevance in this case because of the presence of several consecutive *N*Me amino acids,

ethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; DMSO, dimethyl sulfoxide; GI_{50}, 50% growth inhibition; h, hour; HATU, 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo-[4,5-b] pyridinium hexafluorophosphate 3-oxide; HFIP, hexafluoroisopropanol; HOAt, 1-hydroxy-7-azabenzotriazole; HOBt, 1-hydroxybenzotriazole; HQX, hydroxy quinoline carboxylic acid; MALDI, matrix-assisted laser desorption ionization; MeCN, acetonitrile; MeOH, methanol; MS, mass spectrometry; 1OH-NPH, 1-hydroxy-2-naphthoic acid; 3OH-NPH, 3-hydroxy-2-naphthoic acid; *N*Me-aa, *N*-methylated amino acid; Orn, ornithine; PIC, 2-picolinic acid; pip, piperidine; PyAOP, (1*H*-7-azabenzotriazol-1-yloxy)tripyrrolidin-1-ylphosphonium hexafluorophosphate; PyBOP, (1-Hydroxyhydroxy-1*H*-benzotriazolato-O)tri-1-pyrrolidinylphosphorus hexafluorophosphate; pip, piperidine; PYR, pyrrole-2-carboxylic acid; QNL, quinaldic acid; QNX, 2-quinoxalinecarboxylic acid; RP-HPLC, reverse phase high performance liquid chromatography; SAR, structure–activity relationship; SPPS, solid-phase peptide synthesis; SRB, sulforhodamine B; TIS, triisopropyl silane; TFA, trifluoroacetic acid; TFE, trifluoroethanol; TOF, time-of-flight. Amino acid symbols denote L-configuration. All reported solvent ratios are expressed as v/v, unless otherwise stated.

Scheme 1. Three Methods Used for the Preparation of FAJANU Cyclopeptides: (i) Solid and Solution Strategy, (ii) Linear Strategy, and (iii) 4 + 4 Convergent Strategy

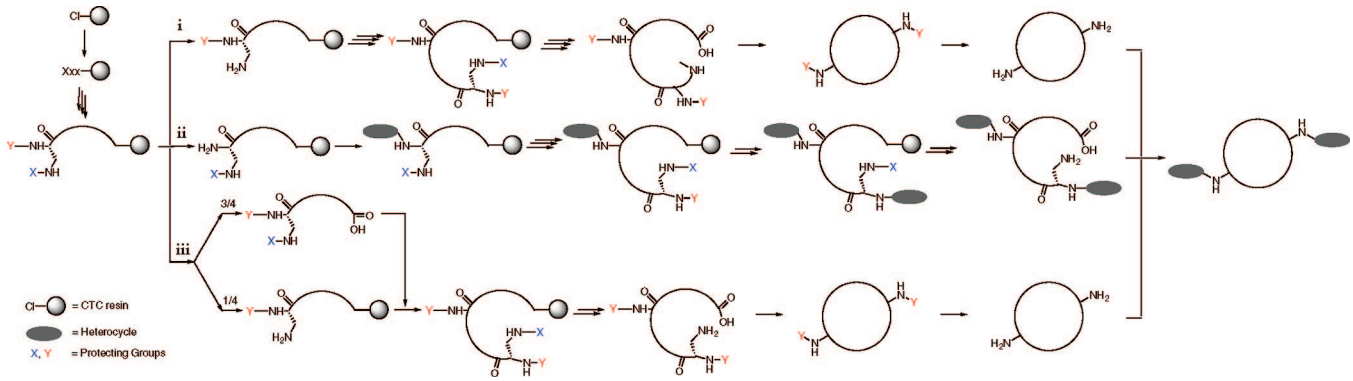

Table 1. Cytotoxic Activity of the FAJANU Family against Several Tumor Cell Lines (GI_{50} 0.1μM)[43]

		activity (GI_{50}) in vitro		
compound	structure	breast[44] MDA-MB-231 10^{-7}	lung A549 10^{-7}	colon HT29 10^{-7}
1		12.3	29.0	6.4
2		7.1	28.6	6.5
3	{[QNL-Dap(&^{1,2})-(Me)Phe-(Me)Gly-(Me)Leu(&^{2,1})]$_2$}		41.6	40.8
4	{[QNL-Dap((&^{1,2})-Pro-(Me)Gly-(Me)Leu(&^{2,1})]$_2$}			
5	med>{[QNL-Dap(&^{1,2})-(Me)Ala-(Me)Gly-(Me)Leu(&^{2,1})]$_2$}	53.4		35.3
6	{[QNL-Dap(&^{1,2})-(Me)Val-(Me)Gly-(Me)Leu(&^{2,1})]$_2$}			
7	{[QNL-Dap(&^{1,2})-(Me)Leu-(Me)Gly-(Me)Leu(&^{2,1})]$_2$}	**6.4**	5.7	**5.0**
8	{[QNL-Dap(&^{1,2})-(Me)Ile-(Me)Gly-(Me)Leu(&^{2,1})]$_2$}	41.5	28.2	35.3

which are highly prone to form this stable cycle before the incorporation of the third residue, thereby resulting in the loss of the two first amino acids.[36]

Another important issue in solid-phase synthesis is the choice of coupling reagents. Thus, peptides containing *N*Me amino acids, the amine of which has lower nucleophilicity, require the concourse of the most efficient coupling reagents such as those based on 7-aza-1-hydroxybenzotriazole (HOAt).[37] Thus, when an amino acid was incorporated into a secondary amine, the HATU–HOAt–DIPEA strategy was preferred.[37] These *N*Me amino acids should be taking into account for the incorporation of the third residue in order to minimize the formation of DKPs.[38] Thus, Fmoc removal from the second amino acid is carried out in less time and the coupling of the third amino acid is performed with DIPCDI-HOAt in the absence of bases, which would otherwise increase the risk of DKP formation.[39] Another critical step is the cyclization method in solution, the PyAOP–HOAt–DIPEA[40,41] at 1 mM concentration was chosen as the most effective method to obtain the desired cyclic peptide to avoid the guanidinylation that may occur when aminium salts, such as HATU, are used.[42] Finally, after Boc deprotection, quinaldic acid was introduced using PyBOPHOAt–DIPEA. Using this strategy, the overall yield for **7** after RP-HPLC purification was 3.6%, with 99% purity as shown by RP-HPLC. Figure 3 shows, as an example, the purity of one of the analogues (**7**).

Biological Activity of the FAJANU Family. We evaluated the cytotoxic activity of this first FAJANU family against several tumor cell lines (Table 1). The closest analogue to **1** was active but did not show improved activity, while cyclic proline (**2**) was noncytotoxic. Surprisingly, *N*Me-Val (**6**) was not active, while the other hydrophobic residues were (**5**, **7**, and **8**). Moreover, **7** [Xxx (1 and 5) = *N*Me-Leu] was the most active.

Synthesis and Biological Evaluation of a Second FAJANU-Based Library. On the basis of the most appropiate candidate, peptide **7**, we designed, synthesized, and evaluated the biological activity of a new chemical library.

Exploring Synthetic Strategies. Prior to the generation of a second library of FAJANU peptides based on **7**, other synthetic approaches were explored. To compare the results, **7** was used as a model. In the second strategy (Scheme 1ii), where the heterocycle was introduced on solid-phase, the Fmoc group was used for the α-amine and the Alloc was chosen for the β-amine side-chain. The overall yield for **7** was slightly lower (2.1% yield, 92.1% purity) than that obtained when strategy 1i was used. For the convergent approach (Scheme 1iii), the reagent for the coupling of the fragments must be chosen carefully. The phosphonium salt PyAOP offers the possibility to run long coupling reactions without the formation of undesired byproduct caused by the excesses presented. In this approach, the starting point is changed in order to facilitate fragment coupling. Thus, the synthesis began with the *N*Me-Leu (**1**) and the Dap was introduced, well protected by N^{α}-Boc and N^{β}-Fmoc. Once the four-amino-acid sequence was elongated in solid phase, $^3/_4$ were cleaved from the resin and coupled to the $^1/_4$ remaining anchored to the resin after removal of the Fmoc group.[45] At the end of the synthetic process, compound **7** was obtained with an overall yield of 3.5% and 98% of purity.

Structure–Activity Relationship (SAR). Using the peptide **7** structure, we synthesized a second generation of cyclic peptides using the strategies described above (characterization is available in the Supporting Information). All the suitable diversity points were examined taking into account symmetry preservation. These analogues were divided into three groups depending on the modification (Figure 4): (i) cycle length, (ii) backbone amino acids, and (iii) aromatic moiety. Table 3

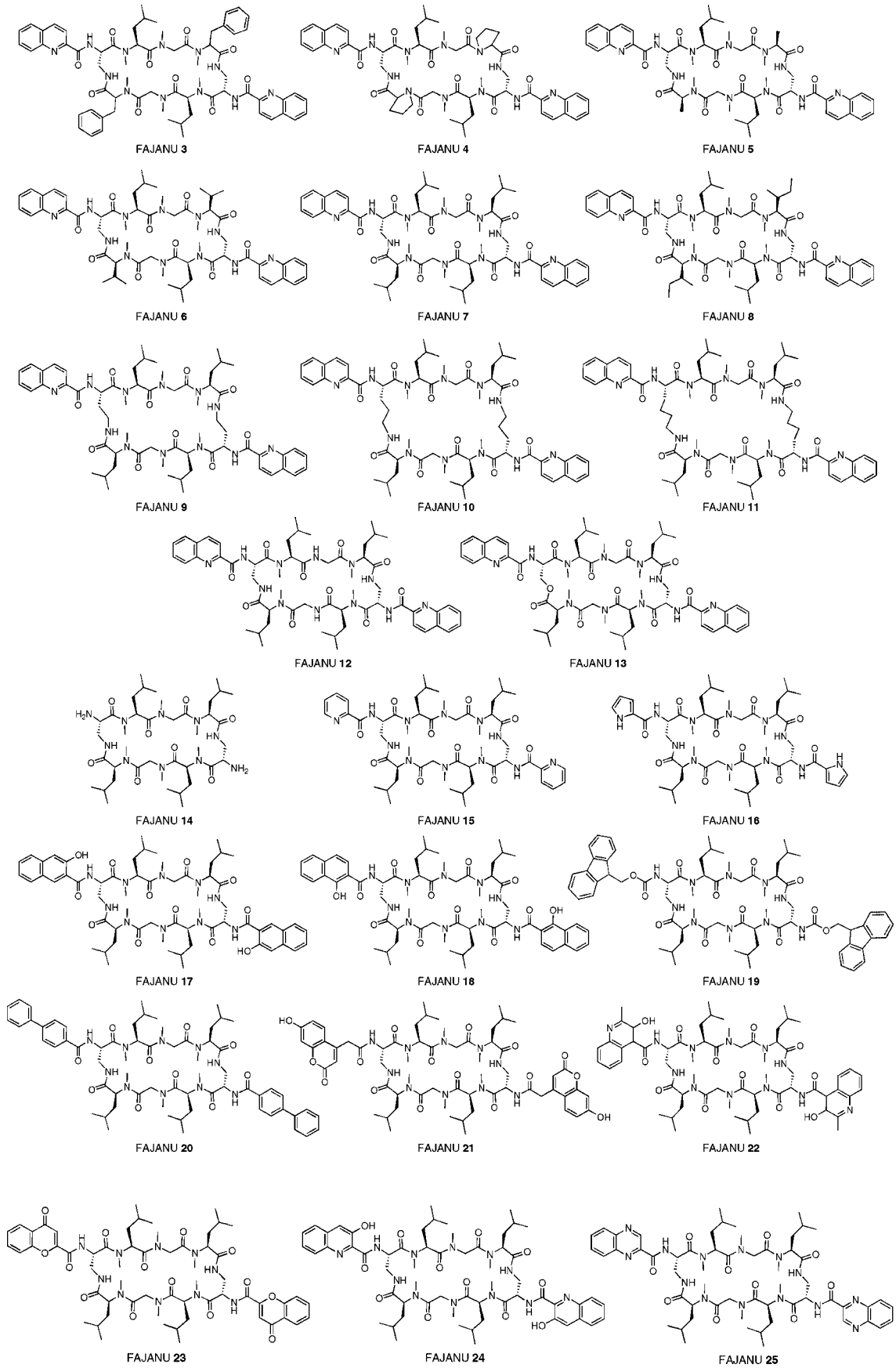

Figure 4. Structural scheme of the first and second generation of the FAJANU family.

summarizes the library and indicates the code, synthesis strategy, and GI_{50} in three tumor cell lines in comparison with the peptide of origin.

Analogues **9–11**, which have a larger macrocycle than the reference compound, differed in their activity. While **10** (2 Orn) and **11** (2 Lys) were less active against all the cell lines, **9** (2

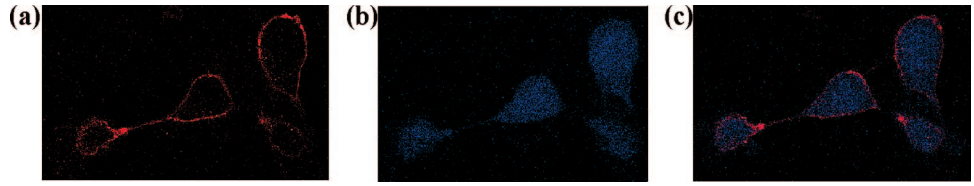

Figure 5. Live-cell confocal images. Peptide **7** internalization at 50 μM after 30 min incubation. (a) Cell membrane is marked in red (HeNe 543); (b) identical field under UV (UV 350 nm), FAJANU **7** is shown in blue; (c) overlapping of both channels (control performed without peptide does not show autofluorescence).

Table 2. Cytotoxic Activity of the FAJANU Family (GI_{50} 0.1 μM) against Several Tumor Cell Lines

		activity (GI_{50}) in vitro		
compound	structure	Breast MDA-MB-231[44] 10^7	Lung A549 10^7	Colon HT29 10^7
1		12.3	29.0	6.4
2		7.1	28.6	6.5
7	{[QNL-Dap(&^{1,2})-MeLeu-MeGly-MeLeu(&^{2,1})]$_2$}	**6.4**	**5.7**	**5.0**
9	{[QNL-Dab(&^{1,2})-MeLeu-MeGly-MeLeu(&^{2,1})]$_2$}	n.d.	n.d.	n.d.
10	{[QNL-Orn(&^{1,2})-MeLeu-MeGly-MeLeu(&^{2,1})]$_2$}	58.0	29.4	24.4
11	{[QNL-Lys(&^{1,2})-MeLeu-MeGly-MeLeu(&^{2,1})]$_2$}	n.d.	39.4	n.d.
12	{[QNL-Dap(&^{1,2})-MeLeu-Gly-MeLeu(&^{2,1})]$_2$}	5.5	11.8	6.2
13	{[QNL-Dap(&^{1})-MeLeu-MeGly-MeLeu(&^{2})] [QNL-Ser(&^{2})-MeLeu-MeGly-MeLeu(&^{1})]}	11.6	27.6	12.3
14	{[H-Dap(&^{1,2})-MeLeu-MeGly-MeLeu(&^{2,1})]$_2$}	n.d.	n.d.	n.d.
15	{[PIC-Dap(&^{1,2})-MeLeu-MeGly-MeLeu(&^{2,1})]$_2$}	n.d.	n.d.	n.d.
16	{[PYR-Dap(&^{1,2})-MeLeu-MeGly-MeLeu(&^{2,1})]$_2$}	n.d.	n.d.	n.d.
17	{[3OH-NPH-Dap(&^{1,2})-MeLeu-MeGly-MeLeu(&^{2,1})]$_2$}	n.d.	n.d.	n.d.
18	{[1OH-NPH-Dap(&^{1,2})-MeLeu-MeGly-MeLeu(&^{2,1})]$_2$}	n.d.	n.d.	n.d.
19	{[Fmoc-Dap(&^{1,2})-MeLeu-MeGly-MeLeu(&^{2,1})]$_2$}	n.d.	n.d.	n.d.
20	{[BPH-Dap(&^{1,2})-MeLeu-MeGly-MeLeu(&^{2,1})]$_2$}	n.d.	n.d.	n.d.
21	{[OH-COU-Dap(&^{1,2})-MeLeu-MeGly-MeLeu(&^{2,1})]$_2$}	n.d.	n.d.	n.d.
22	{[3-OH-Qui-Dap(&^{1,2})-MeLeu-MeGly-MeLeu(&^{2,1})]$_2$}	n.d.	n.d.	n.d.
23	{[CHR-Dap(&^{1,2})-MeLeu-MeGly-MeLeu(&^{2,1})]$_2$}	23.1	n.d.	29.1
24	{[HQX-Dap(&^{1,2})-MeLeu-MeGly-MeLeu(&^{2,1})]$_2$}	n.d.	24	34.3
25	{[QNX-Dap(&^{1,2})-MeLeu-MeGly-MeLeu(&^{2,1})]$_2$}	16.7	14.1	12.3

Dab) showed no activity. This general loss of cytotoxic activity with increased macrocycle size could be explained by a major flexibility freedom. These results indicate that appropriate molecule size is required for biological activity. Consecutive change included the incorporation of nonmethylated amino acids into the structure. When *N*Me-Gly (**2** and **6**) was replaced by Gly (**12**), the structure was less rigid, thereby resulting in maintained but lower cytotoxicity. Our results showed that *N*Me-Gly is crucial and both *N*Me-Leu amino acids can be replaced but at the expense of one order of activity. In addition to the above, we synthesized a depsipeptide. Thus, an ester bond was added to the structure (**13**), which resulted in a lower order of activity.

The quinaldic acid was replaced, which provided important clues about the role of the heterocycle. As shown in analogue **14**, heterocycle presence is crucial for antitumor activity. When one-ring heterocyclic moiety (**15** and **16**) was introduced, no cytotoxic activity was observed against any of the tumor cell lines. Similar results were obtained when the heterocyclic was replaced by a bicyclic moiety but with no heteroatom in the rings (**17**, **18**, and **20**) or more distance between the amide bond and the aromatic ring (**19** and **21**). Antitumor activity was detected when the quinaldic acid was replaced by chromone-2-acid (**23**), hydroxyquinaldic acid (**24**), and quinoxaline acid (**25**), but not when a methyl-substituted heterocycle, such as analogue **22**, was introduced. Although further studies are required, on the basis of these preliminary results, we can conclude that a heteroatom-containing bicycle is the preferred moiety for the ability to maintain cytotoxicity.

NMR Study of Peptide 7. NMR spectra were recorded at 400 MHz in DMSO. Spectra showed several sets of peaks from minor conformations as a result of cis–trans isomers of the *N*Me amino acids. Major conformation was assigned (Table 3) by interpretation of the ^{1}H and ^{13}C 2D-NMR spectra: HSQC, HMBC, TOCSY, and NOESY. Symmetry was rendered by a duplicate numbering of peaks.

Internalization Study. Internalization study of **7** was shown by confocal microscopy (Figure 5) with recording of optical sections. Using a nonfixed A549 lung cell line, we studied capacity of 50 μM of the cyclopeptide to penetrate the cell after a 30 min incubation. The cell membrane was marked in red by WGA (Figure 5a) and peptide **7** was detected in UV (Figure 5b, λ_{max} 450 nm). Overlapping of both channels (Figure 5c) showed a nonmembrane effect and the internalization of **7** in the cytosol. On the basis of this result and on experiments that demonstrated that our candidate **7** does not act as DNA intercalator (please refer to Supporting Information), we propose that, in contrast to other marine cyclic peptides,[3] peptide **7** is not a DNA bisintercalator.

Conclusions

Here we designed a novel hybrid cyclopeptide, FAJANU, from potent cytotoxic cyclopeptides. Three strategies were followed based on the solid-phase approach: the first was elongation of the peptide sequence on solid-phase, where the group was introduced in solution, the second was performed entirely on solid-phase, and the third variant was based on a convergent synthesis 4 + 4. In all cases, the desired peptide was obtained; the solid and solution combination approach being the most effective. The most active cyclopeptide, **7**, was successfully characterized by HPLC–MS, MALDI–TOF, exact mass, and NMR. The SAR analysis detected key features of cytotoxic activity, which could be extrapolated for use in the design of more potent

Table 3. NMR Assignment of **7**

		^{13}C δ (ppm)	^{1}H δ (ppm)	J (Hz)
*N*Me-Leu (1 and 5)	CαH	55.2	5.07 (t)	5.6
	CβH$_2$	38.6	1.35–1.65 (m)	
	CγH	38.7	1.89–1.91 (m)	
	CδH$_3$	22.6	0.79 (2d)	5.2
	Cδ'H$_3$	22.6	0.81 (2d)	5.2
	N-CH$_3$	30.4	3.06 (s)	
	CO	172.5		
*N*Me-Gly (2 and 6)	CαH$_2$	49.6	4.59/4.62 (2s)	
	N-CH$_3$	37.0	3.07 (s)	
	CO	171.6		
*N*Me-Leu (3 and 7)	CαH	51.3	5.11 (t)	5.6
	CβH$_2$	37.3	1.35–1.65 (m)	
	CγH	38.7	1.89–1.91 (m)	
	CδH$_3$	23.5	0.86 (2d)	5.2
	Cδ'H$_3$	23.5	0.91 (2d)	5.2
	N-CH$_3$	30.8	2.72 (s)	
	CO	169.5		
Dap (4 and 8)	CαH	49.5	5.68 (t)	5.6
	CβH$_2$	49.5	3.75/3.79 (d)	13.6
	NH		9.02 (s)	
	CO	169.2		
QNL	C$_2$	163.2		
	C$_3$H	119.0	8.57–8.62(m)	
	C$_4$H	138.5	8.15–8.17 (m)	
	C$_{4a}$	131.0		
	C$_5$H	128.8	7.71–7.74 (m)	
	C$_6$H	128.7	8.09–8.12 (m)	
	C$_7$H	131.2	7.87–7.91 (m)	
	C$_8$H	129.8	8.16 (d)	6.8
	C$_{8a}$	146.5		

analogues. Structural determination by NMR revealed that **7** presented C_2 symmetry. In addition, internalization of the peptide was shown by a confocal technique.

Experimental Section

General. Protected L-*N*-methyl amino acids were obtained from Luxembourg Industries (Tel Aviv, Israel), Neosystem (Strasbourg, France), Calbiochem-Novabiochem AG (Laüfelfingen, Switzerland), Bachem AG (Bubendorf, Switzerland), and Iris BioTech GmbH (Germany). PyBOP was supplied by Calbiochem-Novabiochem AG. CTC resin was obtained from Rohm & Haas (PA). DIPCDI was obtained from Fluka Chemika (Buchs, Switzerland), TBTU and HOBt from Albatross Chem. Inc. (Montreal, Canada), HOAt and PyAOP were supplied by Applied Biosystems (Foster City, CA), and solvents for peptide synthesis, RP-HPLC, and HPLC-MS were obtained from Scharlau (Barcelona, Spain). Trifluoroacetic acid was supplied by KaliChemie (Bad Wimpfen, Germany). Other chemicals used in this study were obtained from Aldrich (Milwaukee, WI) and were of the highest purity available. HPLC was performed on a Waters Alliance 2695 chromatographic system (Waters, MA) with a PDA 995 detector and a reverse-phase Symmetry C18 column (4.6 mm × 150 mm, 5 μm). Linear gradients of 0.045% TFA in H_2O and 0.036% in MeCN were run at a flow rate of 1.0 mL/min, and the gradient went from from 5:5 to 0:10 MeCN in H_2O unless indicated otherwise. HPLC-MS was performed on a Waters Alliance 2796 with UV/vis detector 2487 and an ESI-MS Micromass ZQ chromatographic system (Waters), using a reverse-phase Symmetry 300 C_{18} column (3.9 mm × 150 mm, 5 μm). Then 0.1% formic acid in H_2O and 0.07% formic acid in MeCN were used as eluents. Mass spectra were recorded on a MALDI Voyager DE RP time-of-flight (TOF) spectrometer (PE Biosystems, Foster City, CA) using AcH as matrix. Exact mass spectra were recorded by the mass spectrometry unit at the University of Santiago de Compostela.

Purification was performed in a semipreparative RP-HPLC (reverse-phase Symmetry C_{18} column, linear gradient from 3:7 to 0:10 of MeCN (0.05% TFA) in H_2O (0.1% TFA) for 30 min, 20 mL/min, detection at 220 nm).

^{1}H NMR and ^{13}C NMR spectroscopy was performed on a Varian Mercury 400 apparatus.

Chemical Synthesis. General Methods. Manual solid-phase peptide elongation and other solid-phase manipulations were carried out in polypropylene syringes, each fitted with a polyethylene porous disk. Solvents and soluble reagents were removed by suction. Fmoc removal was carried out with piperidine-DMF (1:5) (1 × 1 min, 2 × 10 min), and for removal of the Fmoc group in a secondary amine, the piperidine protocol used was piperidine-DMF (1:5) (1 min, 2 × 15 min) and DBU-piperidine-DMF-toluene (5:5:70:20) (2 × 5 min). Previous washings of the resin were performed with CH_2Cl_2 (5 × 1 min), DMF (5 × 1 min), and CH_2Cl_2 (1 × 15 min). Washings between deprotection, coupling, and subsequent deprotection steps were carried out with DMF (5 × 0.5 min), CH_2Cl_2 (5 × 0.5 min), and DMF (5 × 0.5 min) using 10 mL of solvent/g of resin each time. After the first amino acid coupling, the remaining chloride functions were methoxylated by treatment with MeOH (1 mg of resin:1μL of MeOH) for 30 min.

The couplings of the amino acids were verified by several tests. The ninhydrin test was used for primary amines and the De Clercq[46] or Chloranil[47] tests for secondary amines, the former being more sensitive.

Cleavage was performed by TFA-CH_2Cl_2 (1:99) (5 × 1 min). Filtrate was collected on H_2O (1 mL/100 mg resin) in 50 mL centrifuge tubes, and the solvents were partially removed under pressure. The peptides were precipitated by adding cold *tert*-butylmethyl ether and centrifuged (5 min × 4000 rpm); the solution was then decanted and the solid was triturated with cold *tert*-butylmethyl ether, which was again decanted. This process was repeated twice. Next, the peptide was solved in H_2O-MeCN (1:1) and the solution was lyophilized.

Solution reactions were performed in round-bottomed flasks followed by convenient workup. Organic solvent extracts were dried over anhydrous $MgSO_4$, followed by solvent removal under reduced pressure at temperatures below 40 °C.

Strategy 1: Synthesis of Compound 7 Backbone. Heterocycle Addition in Solution (Scheme 1 Supporting Information). The first strategy started with the incorporation onto the CTC resin (1.0 mmol/g) of the Fmoc-*N*MeGly-OH (1 equiv) directly with DIPEA (1.6 equiv) in CH_2Cl_2. Next, 5 min later, more DIPEA (3.2 equiv) was added. The mixture was stirred for 1 h. The remaining chloride functions were methoxylated as described in the General Methods section. After washings and Fmoc removal, the Fmoc-*N*MeLeu-OH (3 equiv) was coupled using HATU–HOAt–DIPEA (3:3:9) in DMF. After 90 min of coupling, the Chloranil test was negative. Removal of Fmoc-protecting group and washings were carried out as described above for secondary amines. The Boc-Dap(Fmoc)-OH (3 equiv) was coupled with DIPCDI (3 equiv) and HOAt (3 equiv). After overnight coupling, the Fmoc-protecting group in the lateral chain was removed. The other amino remained protected by the Boc group until the end of the backbone formation. The fourth amino acid, Fmoc-*N*MeLeu-OH (3 equiv) was coupled with PyBOP–HOAt–DIPEA (3:3:9) in DMF. After 2 h of coupling, more PyBOP (3 equiv) was added and the reaction continued for 60 min more. Following Fmoc removal as described above for secondary amines, the second Fmoc-*N*MeGly-OH (3 equiv) was coupled with HATU–HOAt–DIPEA (3:3:9) in DMF for 90 min. The Fmoc was removed and the Fmoc-*N*MeLeu-OH (3 equiv) was coupled using HATU (3 equiv), HOAt (3 equiv), and DIPEA (9 equiv) in DMF for 90 min. To continue the backbone formation, the second Boc-Dap(Fmoc)-OH (3 equiv) was incorporated using PyBOP–HOAt–DIPEA (3:3:9) in DMF for 2 h after Fmoc deprotection. Subsequently, the last amino acid was added: Fmoc-*N*MeLeu-OH (3 equiv) was coupled with PyBOP (3 equiv) and HOAt (3 equiv) with DIPEA (9 equiv) as base in DMF. Again, after 2 h of coupling, more PyBOP (3 equiv) was added, and the reaction was left to stand for a further 60 min. After Fmoc removal, the peptide was cleaved from the resin as described in the General Methods section, thereby obtaining a solid-white peptide in a 70% yield, which was

characterized by RP-HPLC (t_R 10.48 min, 90%) and HPLC-MS (*m*/*z* calcd for $C_{50}H_{92}N_{10}O_{13}$, 1041.3; found 1040.6 $[M + H]^+$). Furthermore, the peptide was cycled using the PyAOP protocol (3 equiv), HOAt (3 equiv), and DIPEA (9 equiv) at a 1 mM concentration in CH_2Cl_2-DMF (9:1). Total coupling was followed by HPLC-MS (t_R 15.45 min, *m*/*z* calcd for $C_{50}H_{90}N_{10}O_{12}$, 1023.3; found 1024.9 $[M + H]^+$). Next, the Boc group was removed by TFA-H_2O-TIS (95:2.5:2.5), (1 mL of cocktail:50 mg of peptide) for 1 h and characterized by RP-HPLC (t_R 11.22 min, 76%) and HPLC-MS (*m*/*z* calcd for $C_{40}H_{74}N_{10}O_8$, 824.0; found 824.7 $[M + H]^+$, 413.2 $[M+2H]^{2+}$). After lyophilization by H_2O-MeCN (1:1), the quinaldic acid (3 equiv) was introduced using PyBOP (3 equiv), HOAt (3 equiv), and DIPEA (9 equiv) for 3 h in DMF-CH_2Cl_2 (1:9). The total incorporation was followed by HPLC-MS. Once the peptide was obtained, solvents were evaporated and the cyclic peptide was dissolved in MeCN-H_2O (1:1) and purified by semipreparative RP-HPLC (t_R 12.8 min) to give the title product (3.6% yield, 99%). MALDI-TOF-MS (*m*/*z* calcd for $C_{60}H_{84}N_{12}O_{10}$, 1132.6; found 1132.9 $[M + H]^+$, 1155.8 $[M + Na]^+$, 1171.8 $[M + K]^+$) and exact mass spectra ESI-TOF (*m*/*z* calcd for $C_{60}H_{84}N_{12}O_{10}$, 1132.6; found 1133.65, 1134.65, 1135.66, 1136.66, 1137.67) confirmed the desired peptide.

Strategy 2: Linear Synthesis of 7 (Scheme 2 Supporting Information). A solution of Fmoc-*N*MeLeu-OH (1 equiv) and DIPEA (1.5 equiv) in CH_2Cl_2 was added to the CTC resin (1.0 mmol/g), and after 5 min, more DIPEA (3 equiv) was added. The mixture was stirred for 1 h. The Fmoc-protecting group was removed, and the Fmoc-*N*MeGly-OH (2 equiv) was coupled using HATU (2 equiv), HOAt (2 equiv), and DIPEA (6 equiv) in DMF. After 1 h of coupling, the De Clercq test was negative. Removal of the Fmoc-protecting group and washings were carried out as described in the General Methods section. The Fmoc-*N*MeLeu-OH (4 equiv) was coupled with DIPCDI (2 equiv) and a minimum amount of DIPEA (DIPCDI-DIPEA/19:1) in CH_2Cl_2 using the asymmetric anhydride method. After overnight coupling, the De Clercq test was negative and Fmoc was removed. The fourth amino acid, Fmoc-Dap(Alloc)-OH (4 equiv), was coupled with HATU (2.5 equiv), HOAt (2.5 equiv), and DIPEA (5 equiv) in DMF for 1 h. Once the Fmoc group was removed, the heterocycle (quinaldic acid, 2 equiv) was coupled in the sequence with PyBOP–HOAt–DIPEA (2:2:6) in DMF overnight. Next, the Alloc group of the Dap was removed using a reduction procedure, which consisted of reduction by $PdPh_3$ (0.1 equiv) and $PhSiMe_3$ (10 equiv) in CH_2Cl_2 (3 × 15 min) under Ar atmosphere;[48] washings with CH_2Cl_2 followed each treatment. Once the Alloc had been removed, the other four amino acids were introduced into the sequence as follows: Fmoc-*N*MeLeu-OH (2 equiv) was added using PyBOP–HOAt–DIPEA (2:2:6) as coupling reagents, once again in DMF for 3 h. After Fmoc removal, Fmoc-*N*MeGly-OH was coupled as previously described. Once the Fmoc group had been removed, the Fmoc-*N*MeLeu-OH (2 equiv) was introduced using HATU–HOAt–DIPEA (2:2:6) as coupling reagents in DMF for 1 h. The Fmoc was removed, and the last amino acid was incorporated: Fmoc-Dap(Alloc)-OH (2 equiv) with HATU (2 equiv), HOAt (2 equiv), and DIPEA (6 equiv) in DMF during 2 h. Quinaldic acid incorporation and Alloc removal was done as described above. Cleavage from the CTC resin was performed as described in the General Methods section. Finally, the peptide was cyclized using PyAOP (2 equiv), HOAt (2 equiv), and DIPEA (6 equiv) in CH_2Cl_2–DMF (9:1) to give the desired peptide in a 2.1% yield of the title compound with a purity of 91% as analyzed by HPLC (t_R 12.4 min). MALDI-TOF-MS, *m*/*z* calcd 1132.6 for $C_{60}H_{84}N_{12}O_{10}$; found 1132.7 $[M + H]^+$, 1156.1 $[M + Na]^+$, 1172.1 $[M + K]^+$.

Strategy 3: Convergent Synthesis of 7 (Scheme 3 Supporting Information). The third strategy parts from the same resin, which is divided at a later stage. The first four amino acids were coupled as follows: a solution of Fmoc-*N*Me-Leu-OH (1 equiv) and DIPEA (1 equiv) in CH_2Cl_2 was added to the CTC resin (1.0 mmol/g), and 5 min later, more DIPEA (2 equiv) was added. The mixture was stirred for 1 h. The remaining chloride functions were methoxylated. After washings, the Fmoc-protecting group was removed using the protocol described in the General Methods section. Next, the Fmoc-*N*MeGly-OH (2 equiv) was coupled using HATU (2 equiv), HOAt (2 equiv), and DIPEA (6 equiv) in DMF. After 1 h of coupling, the De Clercq test was negative. Removal of the Fmoc-protecting group and washings were carried out as described above. The Fmoc-*N*MeLeu-OH (3 equiv) was coupled with DIPCDI (3 equiv) and HOAt (3 equiv) in CH_2Cl_2, thereby avoiding DKP formation. After leaving the reaction to rest overnight, the De Clercq test was negative and Fmoc was removed. The fourth amino acid, the Boc-Dap(Fmoc)-OH (1.9 equiv), was coupled with HATU (1.9 equiv), HOBt (1.9 equiv), and DIPEA as base for 1 h in DMF. A second coupling was not necessary following the De Clercq test.

At this point the resin was divided into two parts, called fragments A and B:Fragment A. Three-quarters of the resin was cleaved using TFA-CH_2Cl_2 (1:99, 5 × 0.5 min). The solution was evaporated and precipitated as described in the General Methods section. After lyophilization with H_2O-MeCN (1:1), the fragment was characterized by RP-HPLC (t_R 8.63 min, 91%) and MALDI-TOF (*m*/*z* calcd for $C_{40}H_{58}N_6O_8$, 750.4; found 750.7 $[M + H]^+$, 774.7 $[M + Na]^+$, 790.7 $[M + K]^+$), which verified the desired fragment.Fragment B. In the remaining quarter of the resin, the Fmoc group was removed.Convergent Coupling of Fragment A and B. Fragment A (3 equiv) was coupled to the remaining quarter of the resin using PyAOP (3 equiv), HOAt (3 equiv), and DIPEA (9 equiv) in DMF overnight. The ninhydrin test confirmed the total coupling of *Fragment A onto B*. Next, the Fmoc group was removed in solid-phase. The peptide was cleaved from the resin as described above for fragment A and again lyophilized.Cyclization. The crude linear peptide was dissolved in CH_2Cl_2-DMF (9:1, 1 mM); next, PyAOP (3 equiv), HOAt (3 equiv), and DIPEA (9 equiv) were added. The mixture was allowed to stir until cyclization was completed, which was followed by RP-HPLC. The solvent was removed by evaporation under reduced pressure. The cyclic peptide was dissolved in H_2O-MeCN (1:1), the fragment was characterized by HPLC-MS (t_R 12.27 min, *m*/*z* calcd for $C_{50}H_{90}N_{10}O_{12}$, 1022.7; found 1022 $[M + H]^+$), which verified the desired cyclic peptide.Boc Removal. Boc was removed by TFA-H_2O-TIS (95:2.5:2.5, 1 mL of cocktail for each 50 mg of peptide) for 1 h. Peptide precipitation was performed as described in the General Methods section.Quinaldic Acid Coupling. The heterocycle was added in solution as follows: quinaldic acid (4 equiv) was coupled by PyBOP (4 equiv), HOAt (4 equiv), and DIPEA (12 equiv) until the reaction was completed, which was followed by RP-HPLC. The cyclic peptide was dissolved in H_2O–MeCN (1:1) and purified by semipreparative RP-HPLC (t_R 12.6 min, same conditions as described in the General Methods section) to give the title product (1.2% yield, 99%). MALDI-TOF-MS (*m*/*z* calcd for $C_{60}H_{84}N_{12}O_{10}$, 1132.6; found 1133.8 $[M + H]^+$, 1155.9 $[M + Na]^+$, 1171.9 $[M + K]^+$) verified the desired cyclopeptide.

Cell Growth Inhibition Assay. A colorimetric assay using sulforhodamine B (SRB) was adapted to perform a quantitative measurement of cell growth and viability, following a previously described method.[49] The cells were seeded in 96-well microtiter plates at 5×10^3 cells/well 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. Afterward, samples were added in aliquots of 5 μL (dissolved in DMSO–H_2O, 3:7). After 72 h of exposure, the antitumor effect was measured by the SRB methodology. Cells were fixed by adding 50 μL of cold 50% (wt/vol) 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 wt %/vol 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 1% acetic acid. 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 caused 50% growth inhibition (GI_{50} value) was calculated from the equation, $100 \times [(T - T_0)/C - T_0)] = 50$.

Confocal Microscopy. Lung tumor cell line A 549 was used as model for the confocal assay. A 549 was settled onto glass-bottom microwell dishes (35 mm Petri dish, 14 mm microwell, 1.5 cover glass). Cells were seeded at a concentration of 10^4 cells/dish. After 24 h, cells were incubated for 30 min with **7** and WGA (TRITC) C was added just before recording the image.

Confocal studies were performed on a LEICA UV microscope with a 63× objective lens. The peptide was excited at 350 nm UV laser and its emission was recorded at 450 nm. When no peptide was in the sample under identical conditions, no fluorescence was detected.

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Supporting Information Available: Characterization of the FAJANU family, gel electrophoresis analysis and synthetic strategy schemes. This material is available free of charge via the Internet at http://pubs.acs.org

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(43) 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 was done using LIMS (Laboratory Information Management Systems) software from PharmaMar.

(44) IB-01212 and its analogue were tested against breast cancer line SK-BR-3.

(45) Alternatively, in the 4 + 4 strategy, if the heterocycle was incorporated in solid-phase before the coupling of the protected segment, N^{α}-Alloc, N^{β}-Fmoc was required and FAJANU 7 was obtained with an overall yield of 0.4%. The most probable reason for this poor overall yield is the lower reactivity of the amine function in the lateral chain of the Dap when the heterocycle had been coupled.

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