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Synthesis, solution structure, and bioactivity of six new simplified analogues of the natural cyclodepsipeptide jaspamide

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Abstract—Recently, we described the synthesis and the biological evaluation of three modified analogues of jaspamide (**1**), a natural cyclodepsipeptide possessing a potent antitumor activity as a consequence of its ability to interfere with actin cytoskeleton. To obtain additional information on the potential pharmacophoric core of the target molecule, which is of fundamental importance to discover new and more effective anticancer products, we decided to explore the biological effects of further structural modifications carried out on the parent molecule. The synthesis and the chemical characterization of six jaspamide analogues (**2**–**7**) are reported and their conformational and biological properties are described.

1. Introduction

Jaspamide (**1**), a cyclodepsipeptide isolated from marine sponges of *Jaspis* genus,[1–4] represents an attractive molecular target for the development of a novel class of antimitotic agents with improved pharmacological profile. The natural compound showed ability to interact with actin filaments and in particular to produce structural and biophysical changes of cytoskeletal dynamics, resulting in a potent antiproliferative activity.[5–11] Despite the great interest reawakened by this potentially therapeutic agent, much of the information needed for a detailed understanding of the structural requirements essential for the activity is still missing. On the other hand, such information is absolutely necessary to perform a rational design of new actin-targeted inhibitors. With the aim of shedding more light on the potential pharmacophoric core of this natural lead compound, three modified analogues of jaspamide (**8**–**10**) (Fig. 1) were recently synthesized and their biological and conformational properties were described.[12] Actually, our project was mainly inspired by the shared conviction that fundamental to the bioactivity was the Ala-*N*-Me-2BrTrp-β-Tyr tripeptidic portion,[13] with the polyketide chain acting as an appropriate linker, presumably adding further conformational constrains to the macrocyclic system, thus providing further assistance to its intrinsic propensity to fold into a β-turn motif. In the course of such study, we learned by fact that even quite subtle structural differences in the arrangement of the putative pharmacophoric region, easily translate into loss of target selectivity of the intact natural product, even though compounds **8**–**10** were nevertheless

Abbreviations: Boc, *tert*-butyloxycarbonyl; ClTrt-Cl, 2-chlorotrityl-chloride resin; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DCM, dichloromethane; DIEA, *N*,*N*-diisopropylethylamine; DMAP, 4-dimethylaminopyridine; DMF, *N*,*N*-dimethylformamide; EBA, ethyl bromoacetate; Fmoc, 9-fluorenylmethyloxycarbonyl; Fmoc-5-Ava-OH, *N*-α-Fmoc-5-aminopentanoic acid; Fmoc-8-Aoc-OH, *N*-α-Fmoc-8-aminooctanoic acid; Fmoc-Ala-OH, *N*-α-Fmoc-L-alanine; Fmoc-D-Trp(Boc)-OH, *N*-α-Fmoc-N^{in}-Boc-D-tryptophan; Fmoc-Val-OH, *N*-α-Fmoc-L-valine; Fmoc-ε-Ahx-OH, *N*-α-Fmoc-6-aminohexanoic acid; Fmoc-β-Ala-OH, *N*-α-Fmoc-L-β-alanine; Fmoc-Phg-OH, *N*-α-Fmoc-L-phenylglycine; Fmoc-Tyr(tBu)-OH, *N*-α-Fmoc-*O*-*t*-butyl-L-tyrosine; HATU, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo-[4,5-*b*]pyridin-1-yl-methylene]-*N*-methyl-methanaminium hexafluorophosphate *N*-oxide; HBTU, *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; Hepa, 3-(2-hydroxyethyl)-phenoxyacetic acid; HOBt, *N*-hydroxybenzotriazole; NovaSyn® TGA resin, hydroxymethylphenoxyacetic acid linker; NMM, *N*-methylmorpholine; PyBop, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; TIS, triisopropylsilane.

Keywords: Jaspamide; Cyclopeptides; Solid-phase synthesis; Actin cytoskeleton; Cytotoxic.

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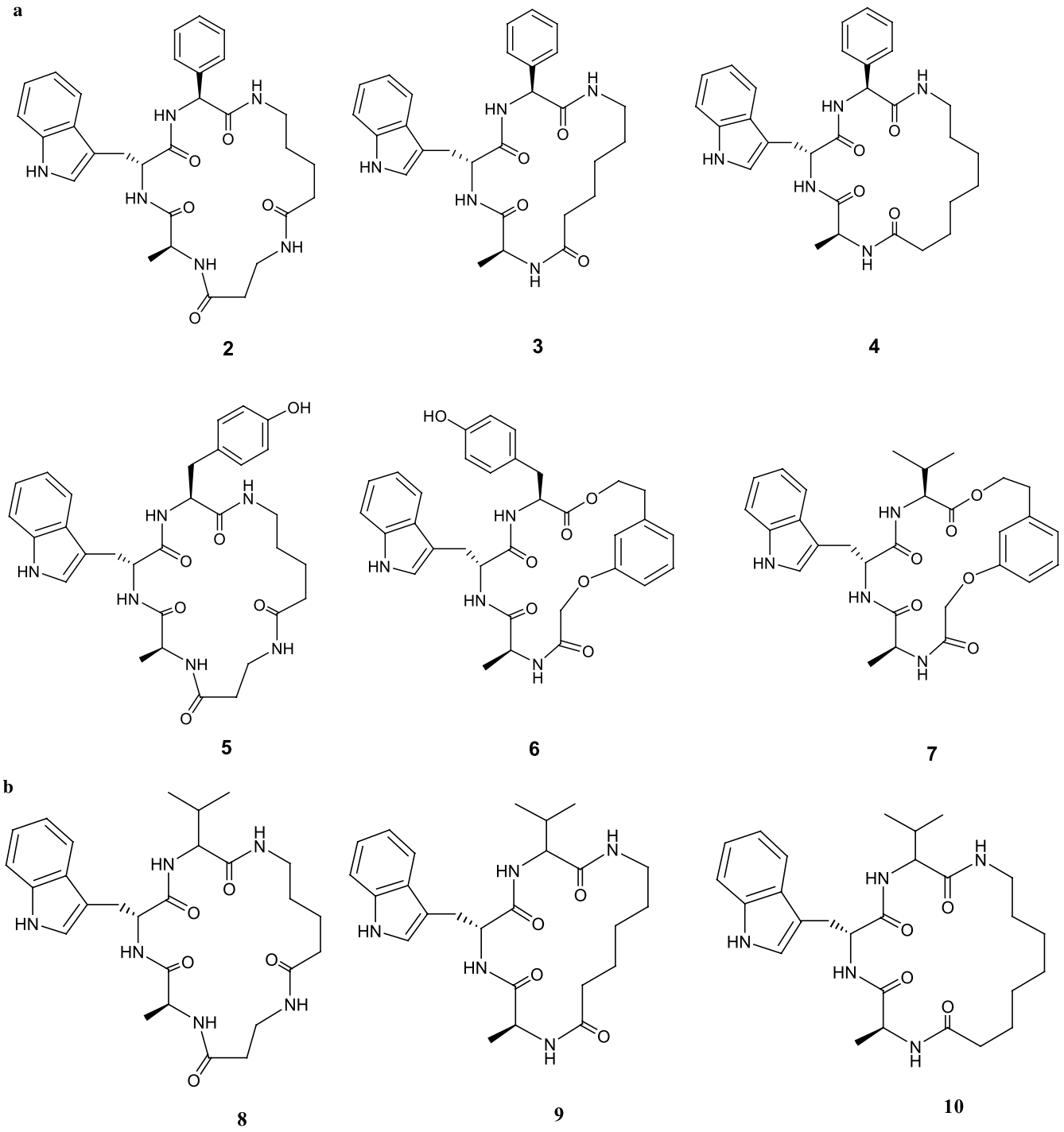
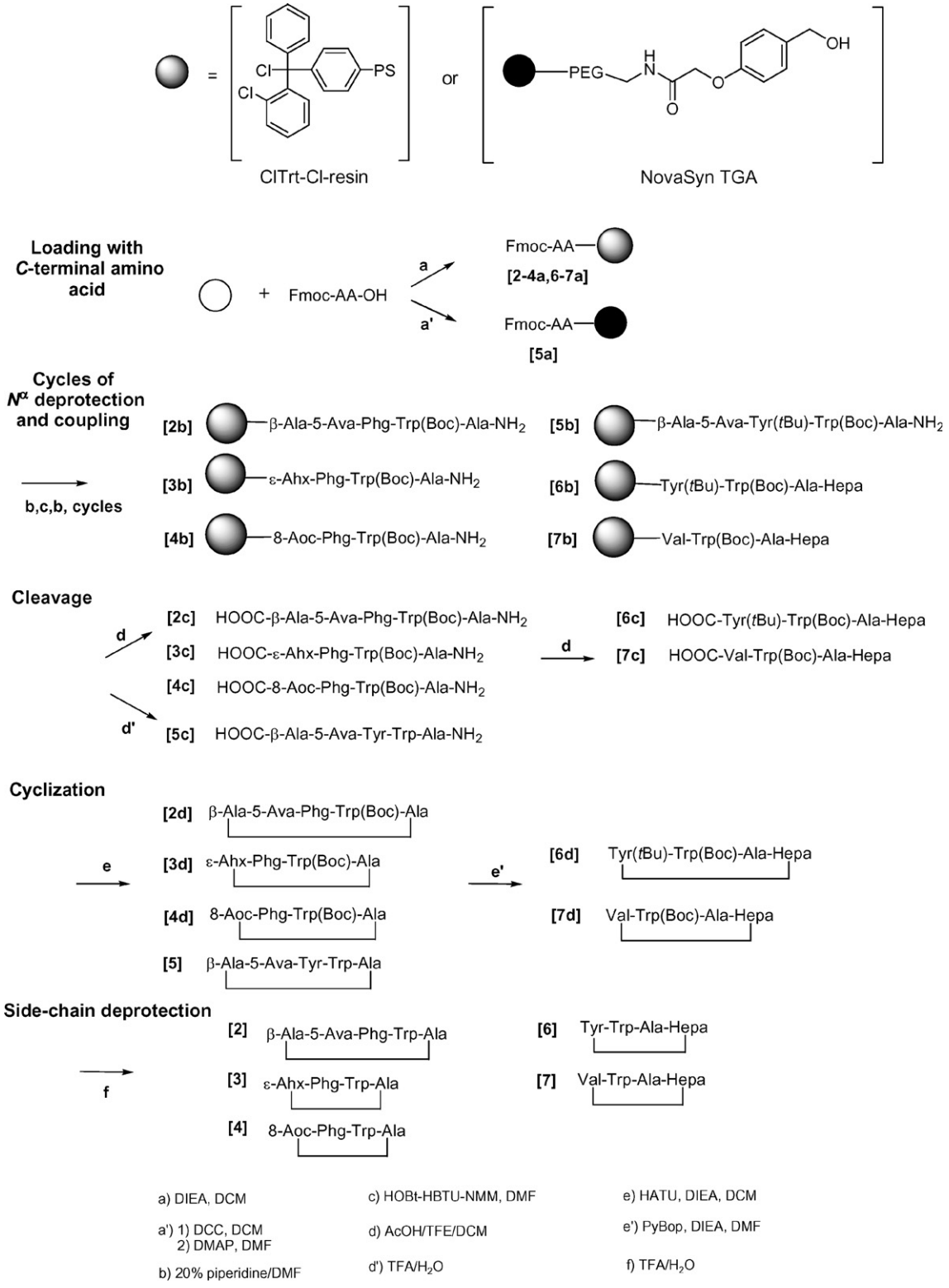


Figure 1. Chemical structures of (a) the natural jaspamide (**1**) and its simplified analogues **2**–**7** and (b) the previously reported simplified analogues **8**–**10**.

cytotoxic with IC_{50} values at micromolar levels.[12] In the design of these analogues, we intended to simplify those structural features of the parent compound that were considered most critical in terms of the challenge posed by their chemical synthesis. On the basis of this criterion, selected modifications, both on the polyketide and on the tripeptide fragments, were implemented. Accordingly, we first decided to replace the two unusual amino acids D-*N*-methyl-2-bromotryptophan and L-β-tyrosine, with D-tryptophan and L-valine or L-phenylglycine, respectively (affording two series of analogues named 'cycloval' and 'cyclophg'). The rational behind such two amino acidic substitutions was to take into due account the diverse structural solutions that *Nature* has used to fashion other effective agents capable of eliciting this kind of biological properties. Therefore, besides jaspamide, dolyculide and chondramide C structures were also considered a useful source of inspiration for this task. Instrumental for our design was a recent report describing a computational approach based on 3D alignments of microfilament-disrupting natural agents, aimed at identifying a hypothetical array

of molecular determinants that, if correctly oriented in the space, should be capable to evoke target selectivity and thus, ultimately, bioactivity.[14] Hence, the valine isopropyl side chain of dolyculide and the phenyl ring of the Phg residue of chondramide C can be both aligned with the phenyl ring of the β-Tyr of jaspamide, suggesting that these replacements are likely to be conservative in the context of these chemical agents. Under these premises, we have undertaken an additional exploration on the chemistry of these jaspamide analogues, in the hope of discovering new simplified actin-targeted cytotoxic products, or at least a series of molecules capable to maintain as much as possible the potent antiproliferative effects displayed by this class of natural products.



Scheme 1. Synthetic scheme followed to obtain analogues **2**–**7**.

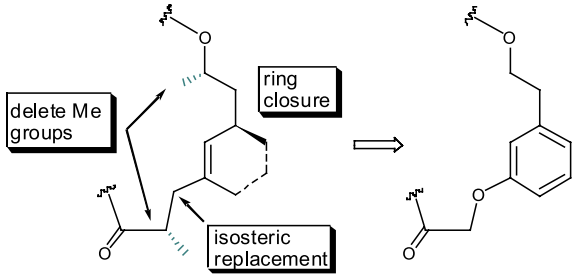
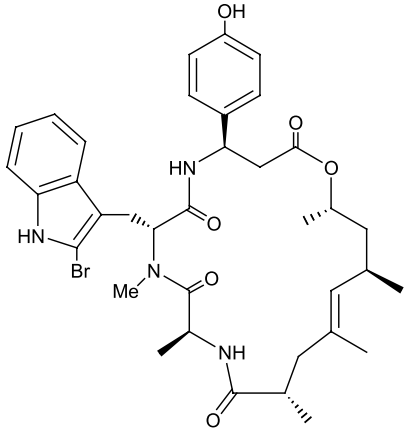


Chart 1. The replacement of the polyketide fragment of jaspamide with the *meta*-substituted aromatic portion used for analogues **6** and **7**.

To this end, we prepared by a combination of solution- and solid-phase synthesis (Scheme 1) six more simplified analogues (**2**–**7**) (Fig. 1) that from a structural viewpoint can be divided in two groups: the first series of compounds **2**–**4** share a common peptidic portion composed by alanine, D-tryptophan, and phenylglycine while differ for the variable-length open-chain linkers, in analogy with the already reported compounds **8**–**10**. In particular, in cyclopeptide **2** the β-alanine is joined together with 5-aminopentanoic acid to the tripeptide, affording a 19-membered macrocycle. In the analogue **3**, a residue of 6-aminohexanoic acid was employed as a spanned chain to form a 16-membered cyclopeptide, and in the analogue **4** an 8-aminooctanoic acid was used to connect the N- and C-termini of our tripeptidic segment, thus generating an 18-membered macrocycle. As concerning the analogue **5**, the polyketide-mimetic fragment composed of β-alanine and 5-aminopentanoic acid is hooked to a tripeptide moiety formed by alanine, D-tryptophan, and α-tyrosine. The second series of analogues is based on a more incisive modification at the polyketide level: both compounds share, in fact, the same 3-(2-hydroxyethyl)-phenoxyacetic acid spacer, synthesized in turn in three steps from commercially available 3-(2-hydroxyethyl)-phenol, to join the tripeptide Ala-D-Trp-Tyr (analogue **6**) and the Ala-D-Trp-Val peptidic moiety (analogue **7**), respectively. The choice of this peculiar linker was made in an attempt of mimicking the geometry of the central portion of the ω-hydroxy-4-octenoic acid polyketide found in jaspamide with a somewhat more rigid linker (Chart 1). Thus, we envisioned the replacement of the five carbons constituting the unsaturated dimethyl-C_3 segment with a suitable *meta*-disubstituted phenyl ring, which had to be elongated to form a 3-(2-hydroxyethyl)-phenoxyacetic acid to maintain in our analogues the same number of atoms present in jaspamide.



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2. Results and discussion

2.1. Synthesis

A convenient combination of solid- and solution-phase synthetic techniques was used for the synthesis of the six analogues **2**–**7**. As concerning solid-phase chemistry, the Fmoc/tBu protection scheme was adopted on a 2-chlorotritylchloride resin solid support (analogues **2**–**4** and **6**–**7**) and on NovaSyn®TGA (analogue **5**). Using the 2-chlorotritylchloride resin, the first Fmoc-protected amino acid was anchored to the linker by diisopropylethylamine (DIEA) treatment under anhydrous conditions, followed by capping of the unreacted trityl groups with methanol, according to the general procedure a (see Section 3). On the other hand, when working on the NovaSyn®TGA resin, the attachment of the first residue to the 4-hydroxymethylphenoxyacetic acid linker was achieved by a DMAP-catalyzed esterification with the appropriate symmetrical anhydride, according to the general procedure a′. The resulting loading degree was determined by UV spectrophotometric analysis using the general procedure a″. The resin was then submitted to several coupling-deprotection cycles to build the protected linear peptides as precursors of the cyclic analogues. All the Fmoc-protected amino acids were activated by hydroxybenzotriazole/*O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HOBt/HBTU) in the presence of *N*-methylmorpholine (NMM), as described in the general procedure c; the progress of the amino acid coupling was checked through the Kaiser and TNBS tests. Fmoc deprotection, before each coupling step, was achieved by treatment of the resin-anchored peptide with a 20% solution of piperidine in *N*,*N*-dimethylformamide (DMF), according to the general procedure b. After each linear peptide was obtained, the Fmoc-protecting group was removed from the N-terminal residue and the protected peptide was cleaved from the 2-ClTrt resin by using a 2:2:6 acetic acid/2,2,2-trifluoroethanol/dichloromethane (AcOH/TFE/DCM) solvent mixture, according to the procedure d (analogues **2**–**4** and **6**–**7**); when using the NovaSyn®TGA resin (analogue **5**), the cleavage was performed by treatment with TFA 95% (general procedure d′) with concurrent side-chain deprotection. The cyclization reactions of the linear precursors were allowed to proceed in solution using *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) or PyBop and DIEA in DCM/DMF, following general procedure e or e′. Finally, after removing the protecting groups, purification on semipreparative RP-HPLC yielded the pure cyclopeptides **2**–**7**.

2.2. Biological activity

Biological evaluation of the synthetic compounds **2**–**7** against a minipanel of four cancer cell lines (T24, MCF-7,[15] NCI/ADR,[15] and A-10) showed cell growth-inhibitory activity with IC_{50} value ranging from 60 to 150 μg/mL only for the analogue **7**. Quite surprisingly, no significant cytotoxicity toward any of the above cell lines was found for the other compounds. From these preliminary results, it is not easy to draw a clear-cut correlation between the structural simplifications/modifications and the biological activity. Nevertheless, a few observations can be made. It seems that the substitution of β-Tyr with α-Tyr or Phg produced a loss of activity which otherwise was retained in the cycloval analogues (**8**–**10**) in which the β-Tyr was replaced with Val. This consideration would be confirmed by the fact that the only active analogue (compound **7**) of the two series described herein contains a valine in its tripeptidic portion; a comparison of the biological properties of **7** with those shown by the previously described compound **10**, another cycloval analogue displaying the same macrocyclic size, suggests that the substitution of the 8-aminooctanoic acid with the less flexible 3-(2-hydroxyethyl)-phenoxyacetic acid as polyketide linker caused an improvement of the cytotoxicity.

For the sake of completeness, we also assayed the six new synthetic analogues on the cytoskeleton system. Hence, their activity on microfilaments and microtubules was examined in A-10 rat smooth-muscle cells. In these experiments, A-10 cells were treated with either jaspamide (**1**), as a positive control for microfilament depolymerizing activity, or micromolar amounts of compounds **2**–**7**. Jaspamide (**1**) very potently caused actin aggregation in these cells. This could be seen with doses as low as 0.85 nM, and at 23 nM, there was pronounced aggregation. Unfortunately, none of the jaspamide analogues seemed to affect either microfilaments or microtubules. Indeed, while binucleated cells were consistently and clearly present in samples treated with jaspamide, no cellular aberration was observed with compounds **2**–**7**. This further biological investigation suggests that the structural simplifications involved in these synthetic analogues gave rise to loss of target selectivity of the parent compound, implying an alternative mechanism of action for the observed cytotoxicity. In fact, in an attempt to gather more data on such a different mode of action underlying the cytotoxic activity displayed by analogue **7** (and very likely by analogues **8**–**10** as well), we investigated the induction of apoptosis exerted by this compound on Jurkat cells (Fig. 2), as already reported for jaspamide itself.[16,17]

Finally, with the aim of comparing the 3D features of jaspamide with those of the simplified analogues **2**–**7**, their conformational properties in solution were thoroughly studied by restrained molecular mechanics (MM) and molecular dynamics (MD) approaches using NMR-derived constraints as input data to correctly drive the calculations.

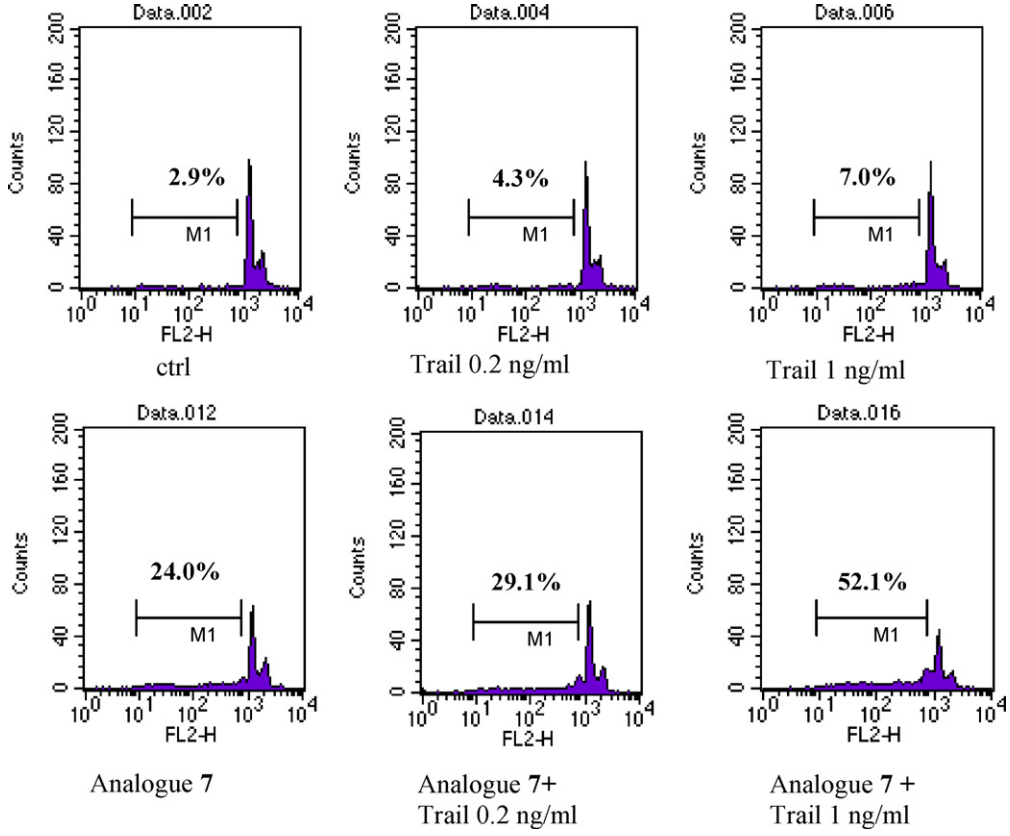

Figure 2. Induction of Jurkat cell apoptosis by compound **7**. Jurkat cells (1×10^6/mL) were incubated in 10% FCS-RPMI medium, in the presence of 100 μM of compound **7** and/or the indicated concentrations of recombinant TRAIL, for 24 h. Then, hypodiplody was analyzed by propidium iodide incorporation in permeabilized cells and flow cytometry.[18]

2.3. Conformational analysis of analogues 2–7

2.3.1. NMR spectroscopy and molecular mechanics and dynamics calculations. A detailed NMR analysis, based on a set of homonuclear and heteronuclear correlation 2D experiments (see Section 3), was carried out on analogues **2**–**7** to obtain the sequence-specific assignment of the ^{1}H and ^{13}C resonances of the six cyclopeptides (see Tables 1 and 2). Successively, a careful analysis of the ROESY data, based on a distance calibration (r^{-6}, two-spin approximation), was performed, to convert the most intense and significative ROESY cross-peak volumes into internuclear distances. The so-obtained distances were used as experimental restraints for the successive molecular mechanics and dynamics calculations.

A preliminary round of free molecular mechanics and dynamics calculations was run at 500 K to properly monitor the conformational space. Subsequently, MM and MD calculations were driven at 300 K by using the restraints collected during the NMR analysis, providing

Table 1. ^{1}H NMR data for analogues **2**–**7** (600 MHz, DMSO-d_6)

2		**3**		**4**		**5**		**6**		**7**	
Phg		Phg		Phg		Tyr		Tyr		Val	
α	5.30	α	5.40	α	5.23	α	4.17	α	4.18	α	3.88
β		β		β		β	2.57, 2.94	β	2.74, 2.90	β	1.92
γ	7.52[a]	γ	7.34[a]	γ	7.41[a]	γ		γ		γ_1-CH_3	0.77
δ	7.36[a]	δ	7.38[a]	δ	7.35[a]	δ	7.01[a]	δ	6.96	γ_2-CH_3	0.80
ε	7.32	ε	7.32	ε	7.30	ε	6.60[a]	ε	6.63	NH	8.12
NH	8.29	NH	8.29	NH	8.11	NH	8.52	NH	8.19		
Trp		Trp		Trp		Trp		Trp		Trp	
α	4.43	α	4.28	α	4.36	α	4.26	α	4.34	α	4.57
β	2.90, 3.72	β	2.94, 3.23	β	3.02, 3.19	β	2.68[a]	β	2.77, 3.01	β	2.87, 3.08
1-NH	10.84	1-NH	10.83	1-NH	10.86	1-NH	10.74	1-NH	10.80	1-NH	10.81
2	7.14	2	7.12	2	7.30	2	7.05	2	7.05	2	7.11
3		3		3		3		3		3	
4		4		4		4		4		4	
5	7.61	5	7.53	5	7.61	5	7.49	5	7.55	5	7.58
6	6.97	6	7.00	6	6.99	6	6.99	6	6.99	6	6.96
7	7.05	7	7.05	7	7.15	7	7.02	7	7.06	7	7.03
8	7.32	8	7.32	8	7.33	8	7.28	8	7.31	8	7.29
9		9		9		9		9		9	
NH	8.29	NH	8.64	NH	8.32	NH	8.09	NH	8.12	NH	8.14
Ala		Ala		Ala		Ala		Ala		Ala	
α	4.17	α	4.16	α	4.30	α	4.37	α	4.29	α	4.34
β	0.97	β	1.03	β	0.92	β	0.97	β	1.06	β	1.04
NH	8.16	NH	8.28	NH	8.03	NH	7.91	NH	8.26	NH	8.13
β-Ala		Ahx		Aoc		β-Ala		Hepa[b]		Hepa[b]	
α	2.28, 2.34	α	2.06, 2.20	α	2.00, 2.20	α	2.03, 2.43	α	4.47, 4.58	α	4.47, 4.58
β	3.22, 3.42	β	1.30, 1.63	β	1.10[a]	β	3.04, 3.25	1′		1′	
NH	7.31	γ	1.36, 1.44	γ	1.19[a]	NH	7.71	2′	6.74	2′	6.74
		δ	1.41[a]	δ	1.40, 1.60			3′		3′	
5-Ava		ε	2.85, 3.42	ε	1.30[a]	5-Ava		4′	6.84	4′	6.80
α	1.93, 2.15	NH	7.38	ζ	2.82, 3.17	α	1.91, 2.03	5′	7.16	5′	7.15
β	1.21, 1.42			NH	7.50	β	1.43, 1.49	6′	6.77	6′	6.75
γ	1.30[a]					γ	1.34[a]	1″	2.85[a]	1″	2.82, 2.87
δ	2.99, 3.08					δ	2.95, 3.06	2″	4.17, 4.31	2″	4.09, 4.40
NH	8.05					NH	7.42				

[a] 2H.

[b]

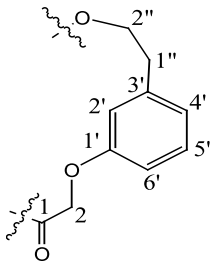

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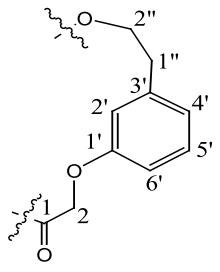
Table 2. ^{13}H NMR data for analogues **2–7** (600 MHz, DMSO-d_6)

2		**3**		**4**		**5**		**6**		**7**	
Phg		Phg		Phg		Tyr		Tyr		Val	
α	58.1	α	58.2	α	58.0	α	55.3	α	55.1	α	59.3
β	138.8	β	139.2	β	138.6	β	36.7	β	36.0	β	29.8
γ	127.8	γ	128.3	γ	127.6	γ	129.2	γ		γ_1-CH_3	19.20
δ	128.7	δ	128.6	δ	128.4	δ	130.6	δ	130.9	γ_2-CH_3	19.17
ε	128.1	ε	129.2	ε	127.3	ε	115.3	ε	115.6		
Trp		Trp		Trp		Trp		Trp		Trp	
α	54.4	α	55.0	α	54.8	α	54.9	α	54.24	α	53.8
β	28.0	β	26.7	β	27.2	β	27.6	β	27.69	β	29.2
2	124.5	2	124.2	2	124.5	2	124.7	2	121.54	2	124.0
3		3		3		3		3		3	
4		4		4		4		4		4	
5	119.0	5	119.0	5	118.3	5	119.0	5	118.90	5	120.4
6	118.8	6	118.9	6	118.5	6	118.9	6	118.73	6	120.0
7	121.9	7	121.2	7	123.8	7	121.4	7	121.41	7	121.46
8	110.9	8	111.0	8	110.6	8	111.9	8	111.92	8	111.7
9		9		9		9		9		9	
Ala		Ala		Ala		Ala		Ala		Ala	
α	49.4	α	50.0	α	48.36	α	47.81	α	48.50	α	49.5
β	17.1	β	17.5	β	17.84	β	19.12	β	18.24	β	20.0
β-Ala		Ahx		Aoc		β-Ala		Hepa[a]		Hepa[a]	
α	35.8	α	35.0	α	35.5	α	35.7	α	67.80	α	69.1
β	35.5	β	24.4	β	25.2	β	35.9	1′	158.5	1′	158.5
		γ	27.5	γ	28.2			2′	116.1	2′	115.1
5-Ava		δ	27.6	δ	28.6	5-Ava		3′	141.0	3′	140.6
α	36.2	ε	38.4	ε	38.4	α	35.8	4′	121.8	4′	121.8
β	23.5			ζ	37.5	β	23.7	5′	129.8	5′	129.7
γ	29.4					γ	28.7	6′	114.1	6′	114.0
δ	38.2					δ	39.3	1″	34.6	1″	34.6
								2″	65.41	2″	65.3

[a]



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the final ensembles of structures representing analogues **2–7**.

2.3.2. Conformational analysis on analogues 2–7. In a previous study, we carried out a comparative structural analysis of analogues **8–10** with respect to jaspamide to gain insight into its possible molecular determinants underlying the induced actin hyperpolymerization, and hence to determine what may be the key elements of the parent compound ultimately responsible for its potent antiproliferative activity. To this end, we first compared the three above-mentioned derivatives to the X-ray structure of jaspamide. Subsequently, since jaspamide was reported to contain in its D-Trp-β-Tyr region a β-turn of type II[13] considered critical to its bioactivity, the corresponding regions of the NMR structures of analogues **8–10** were inspected. Actually, the torsion angle values found for those compounds were not in agreement with a β-turn of type II, resulting in values quite different from the corresponding angles observed for jaspamide, suggesting, considering that analogues **8–10** do not affect the cytoskeleton, a crucial role of this β-turn type II region for specifically targeting actin microfilaments.

In analogy with the above approach, we have also compared the NMR solution structures of compounds **2–7** with the X-ray structure of jaspamide (Figs. 3–8). The results indicate that among all of these simplified derivatives, only compounds **5** and **7** reveal a good superimposition of the backbone in the segment Ala-D-Trp, even if a quite poor correlation is observed for the region in

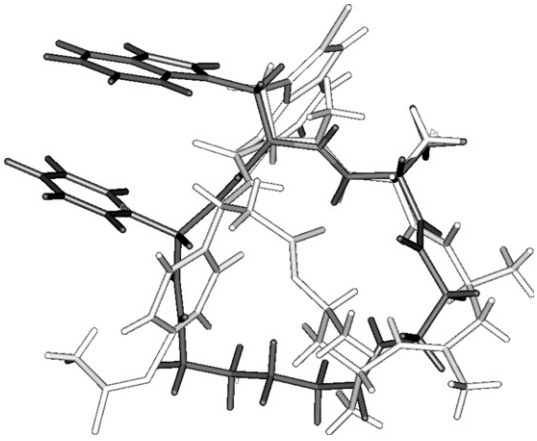


Figure 3. Superposition of the NMR solution structure of analogue **2** (gray) and the X-ray structure of jaspamide (black).

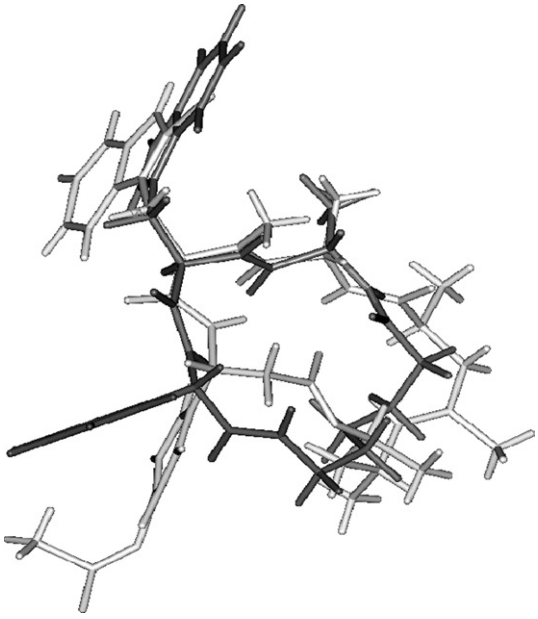


Figure 4. Superposition of the NMR solution structure of analogue **3** (gray) and the X-ray structure of jaspamide (black).

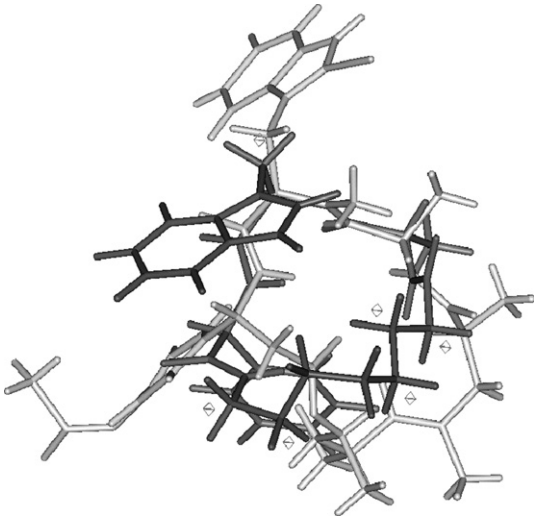


Figure 5. Superposition of the NMR solution structure of analogue **4** (gray) and the X-ray structure of jaspamide (black).

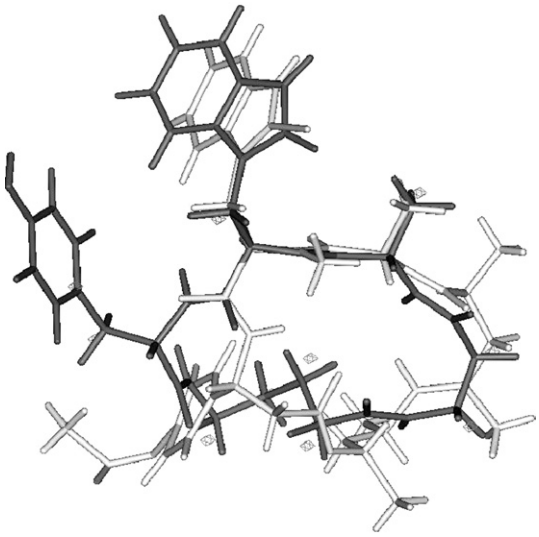


Figure 6. Superposition of the NMR solution structure of analogue **5** (gray) and the X-ray structure of jaspamide (black).

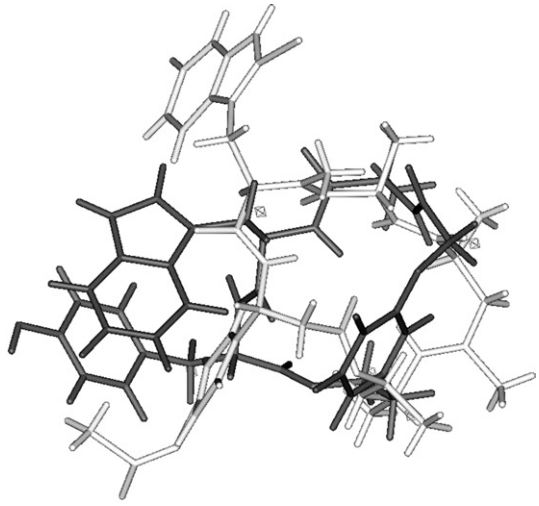


Figure 7. Superposition of the NMR solution structure of analogue **6** (gray) and the X-ray structure of jaspamide (black).

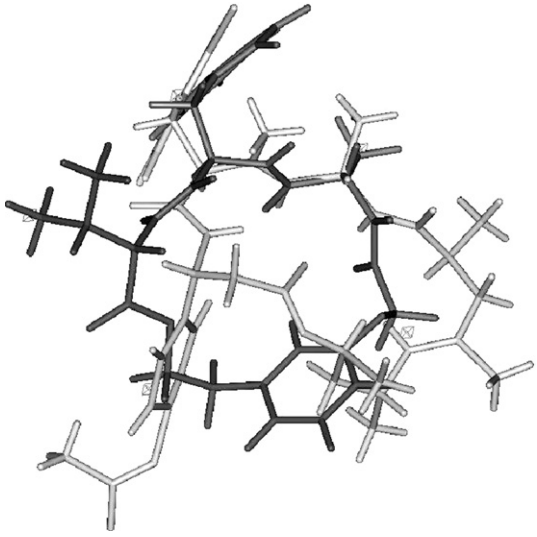


Figure 8. Superposition of the NMR solution structure of analogue **7** (gray) and the X-ray structure of jaspamide (black).

Table 3. Peptide backbone dihedral angles observed for analogues **2**–**10** in the segment corresponding to the L-Ala-D-*N*-Me-2BrTrp-β-Tyr region of jaspamide in comparison with the ones measured for the X-ray structure of jaspamide (**1x**) and for the NMR conformational ensemble **1i**–**1vi** in the corresponding tripeptidic segment

Compound	ϕ_i	ψ_i	ϕ_{i+1}	ψ_{i+1}
1x	−156.5	+160.0	+109.4	−26.7
1i	−162.0	+166.7	+87.6	−60.6
1ii	−160.2	+91.7	+90.4	−70.6
1iii	−156.6	+85.7	+88.5	−65.6
1iv	−162.3	+162.6	+84.2	−71.3
1v	−154.6	+161.1	+84.8	−61.2
1vi	−136.6	+86.3	+90.3	−92.0
8	−83.0	+140.9	+92.6	−141.1
9	−89.9	+105.2	+86.8	−117.5
10	−98.6	+101.2	+78.1	−135.2
2	−89.5	+142.3	+88.9	−122.8
3	−81.4	+130.9	+110.4	−69.2
4	−100.1	+70.8	+59.3	+47.0
5	−146.40	+137.8	+97.9	−138.7
6	−73.5	−31.0	+154.8	−105.0
7	+69.2	+101.6	+122.0	−130.2

which the β-Tyr is replaced by a α-Tyr or a L-Val, respectively.

In Table 3, the ϕ and ψ dihedral angles of analogues **2**–**7** describing the backbone torsions of the L-Ala-D-Trp-L-Val region are reported and compared to the corresponding angles measured for jaspamide in its X-ray structure and in its NMR-derived conformational ensemble proposed by Inman and Crews.[19] Angles ϕ_{i+1} and ψ_{i+1}, characteristic of the D-Trp-β-Tyr region of jaspamide, are indeed reminiscent of a β-turn of type II, being around +90° and 0°, respectively. In analogues **2**–**7**, the angles ϕ_{i+1} are found in a large range, from +59.3 of **4** to 154.8 of **6**, and only compounds **2**, **3**, and **5** present an angle of about 90°; the little superimposibility in the conformations of these analogues is also confirmed by the values of angles ψ_{i+1}, which range from +47.0° of analogue **4** to −138.7° of analogue **5**. These values are not in agreement with a β-turn of type II and, in addition, they are quite different from the corresponding angle observed for jaspamide.

Overall, such comparative analysis accounts for the rather poor bioactivity profile shown by these simplified analogues, emphasizing how small must be the region of chemical space, both in terms of geometrical/conformational features and allowed functional groups, in which macrocycles of this type are still endowed with the same bioactivity displayed by the parent compound. This consideration would in turn discourage further attempts of producing additional simplified molecules of this kind. However, to be fair and to put under the right perspective such unsuccessful (at least from a pharmaceutical viewpoint) attempts, one has also to take into account that no 3D model at the atomic level or any other chemical information is yet available on the forces intervening in the interaction between jaspamide and its biological target. A final consideration that emerges from the whole of our model studies and structural comparisons has to do with the relative importance of the peptide and the polyketide portions in eliciting bioactivity. If from one side it is likely true that the tripeptidic segment of the molecule may well contain the main functional determinants for exerting the bioactivity, it is also true that the role of the polyketide portion cannot be underestimated to the mere function of a linker that has only to join the two N- and C-termini of the tripeptide. In other words, the importance of the polyketide portion may have well been mistaken or misunderstood.

3. Experimental section

3.1. General experimental procedures

All the NMR spectra (^{1}H, HMBC, HSQC, TOCSY, COSY, and ROESY with $t_{mix} = 0.4$ s) were recorded on a Bruker Avance DRX600, on a Bruker Avance 300 MHz, or on a Bruker DRX 400 spectrometers at $T = 298$ K. The compounds (**2**–**7**) were dissolved in 0.5 mL of 99.95% DMSO-d_6 (Carlo Erba, 99.95 Atom % D) (^{1}H, $\delta = 2.50$ ppm; ^{13}C, $\delta = 39.5$ ppm). The NMR data were processed on a Silicon Graphic Indigo 2 workstation using UXNMR software.

Electrospray mass spectrometry (ES-MS) was performed on a LCQ DECA TermoQuest (San Josè, California, USA) mass spectrometer.

For estimation of Fmoc amino acids on the resin, absorbance at 301 nm was read employing a Shimadzu UV 2101 PC. Analytical and semipreparative reverse-phase HPLC was performed on a Jupiter C-18 column (250×4.60 mm, 5 μm, 300 Å; 250×10.00 mm, 10 μm, 300 Å, respectively).

All starting materials, reagents, and solvents were commercially available and used without further purification. Unless specified, solvents were of reagent grade; they were purchased from Aldrich, Fluka, and Carlo Erba. DCM and DMF used for solid-phase reactions were synthesis grade (dried over activated 4 Å molecular sieves). H_2O and CH_3CN were of HPLC grade. 2-Chlorotritylchloride resin (100–200 mesh), 1% DVB, (ClTrt-Cl, loading level: 1.04 and 1.4 mmol/g), NovaSyn® TGA resin (loading level: 0.24 mmol/g), Fmoc-Ala-OH, Fmoc-Tyr(*t*Bu)-OH, Fmoc-D-Trp(Boc)-OH, Fmoc-Val-OH, DCC, DMAP, HOBt, HBTU, and PyBop were purchased from Novabiochem. Fmoc-β-Ala-OH, Fmoc-5-Ava-OH, Fmoc-ε-Ahx-OH, Fmoc-8-Aoc-OH, and Fmoc-Phg-OH were obtained from Neosystem. HATU was purchased from Fluka. Solid-phase peptide syntheses, using the Fmoc-*t*-Bu strategy, were carried out on a polypropylene ISOLUTE SPE column on a VAC MASTER system, a manual parallel synthesis device purchased from Stepbio.

3.2. Computational details

A conformational search was performed for all the compounds by: (1) a step of free molecular dynamics at high temperature (500 K, 5.0 ps); (2) a round of restrained molecular dynamics and minimization at high tempera-

ture (500 K, 5.0 ps), the restraints were obtained using a distance calibration (r^{-6}, two-spin approximation) to convert the most intense and significant ROESY cross-peak volumes into internuclear distances; and (3) a final step of restrained molecular dynamics and minimization at 300 K (5.0 ps).

During the simulations, the consistent-valence force field (CVFF)[20] was applied. The effect of the solvent (DMSO) was incorporated in the calculations by considering it as a continuous dielectric medium, characterized by a dielectric constant of 47. Minimizations and molecular dynamics simulations were performed in the Discover module of Insight II.[21]

3.3. General procedures for the synthesis of compounds 2–7

(a) Loading of the resin (ClTrt-Cl). The ClTrt-Cl resin was placed in a 25 mL polypropylene ISOSOLUTE syringe on a VAC MASTER system, swollen in 3 mL of DMF for 1 h, and then washed with 2 × 3 mL of DCM.

A solution of Fmoc-AA-OH (1 equiv) and DIEA (4 equiv) in 2.5 mL of dry DCM was added and the mixture was stirred for 2 h with a N_2 stream. The mixture was then removed, and the resin was washed with 3× DCM/MeOH/DIEA (17:2:1), and sequentially with the following washing/treatments: DCM 3 × 3 mL, DMF 2 × 3 mL, and DCM 2 × 3 mL (1.5 min each).

(a′) Loading of the resin (NovaSyn® TGA). The resin was placed into a 25 mL polypropylene ISOSOLUTE syringe on a VAC MASTER system, swollen in 5 mL of DMF for 1 h. The symmetric anhydride of the first Fmoc-AA-OH (10 equiv) was prepared by dissolving it in dry DCM (12 mL) and DMF (few drops); DCC (5 equiv) was added at 0 °C and the mixture was stirred for 25 min. The reaction mixture was filtered and the filtrate was evaporated under reduced pressure to give the desired product. It was dissolved in DMF and added to the swelled polymeric support; DMAP (1 equiv) was added and the resulting mixture was stirred for 4 h. The support was washed with DMF 3 × 3 mL, DCM 3 × 3 mL, and Et_2O (1.5 min each) and then dried in vacuo over KOH.

(a″) Estimation of the level of first residue attachment. The loading of the resin was determined by UV quantification of the Fmoc-piperidine adduct.

The assay was performed on duplicate samples: 0.4 mL of piperidine and 0.4 mL of DCM were added to two dried samples Fmoc amino acid-resin in two volumetric flasks of 25 mL. The reaction was allowed to proceed for 30 min at rt and then 1.6 mL of MeOH was added and the solutions were diluted to 25 mL volume with DCM. A reference solution was prepared in a 25 mL volumetric flask using 0.4 mL of piperidine, 1.6 mL of MeOH and DCM to volume. The solutions were shaked and the absorbance of the samples versus the reference solution was measured at 301 nm. The substitution level (expressed in millimoles of amino acid per gram of resin) was calculated from the equation: mmol/g = (A_{301}/7800) × (25 mL/g of resin).

(b) Fmoc deprotection. 20% piperidine in DMF (3 mL, 1 × 1.5 min), 20% piperidine in DMF (3 mL, 1 × 10 min); washings in DMF 2 × 3 mL, DCM 2 × 3 mL, and DMF 2 × 3 mL (1.5 min each).

(c) Peptide coupling conditions. The coupling reaction was promoted by a HOBt/HBTU in DMF coupling protocol: Fmoc-amino acid (3–4 equiv), HOBt (3–4 equiv), HBTU (3–4 equiv), and NMM (4–5 equiv) were stirred under N_2 in 2.5 mL of DMF for 2 h. After each coupling, washings were carried out with DMF (3 mL, 3 × 1.5 min) and DCM (3 mL, 3 × 1.5 min).

(d) Cleavage (ClTrt-Cl). The dried peptide resin was treated for 2 h, under stirring, with the following cleavage mixture: AcOH/TFE/DCM (2:2:6; 10 μL × 1 mg of resin). Then, the resin was filtered off and washed with neat cleavage mixture (3 mL, 3 × 1.5 min). After addition of hexane (15 times volume) to remove acetic acid as an azeotrope, the filtrate was concentrated and lyophilized.

(d′) Cleavage (NovaSyn® TGA). The dried peptide resin was treated for 1 h, under stirring, with the following cleavage mixture: TFA/H_2O (95:5; 10 μL × 1 mg of resin). Then, the resin was filtered off and washed with neat cleavage mixture (3 mL, 3 × 1.5 min). The filtrate volume was reduced under a N_2 stream and then added with a 20-fold excess of cold diethyl ether. A white precipitate appeared soon after ether addition and ether peptide mixture was kept at 0 °C for 2 h. The precipitate was collected by filtration through a 0.45 μm PTFE membrane filter (PALL Corporation/Gelman Laboratory) installed on a glass 47 mm filter holder with a vacuum aspirator; the precipitated peptide was washed with cold ether. The filter was transferred to a glass vessel, the solid was removed by HPLC-grade water (some drops of glacial acetic acid) and ultrasounds, and the suspension was lyophilized.

(e) Cyclization. The cyclization step was performed in solution at a concentration of 7.7×10^{-4} M with HATU (2.0 equiv) and DIEA (2.5 equiv) in DCM. The solution was stirred at 4 °C for 1 h and then allowed to warm to room temperature overnight. The solvent was removed under reduced pressure.

(e′) Cyclization. The cyclization step was performed in solution at a concentration of 7.7×10^{-3} M with PyBop (3.0 equiv) and DIEA (6.0 equiv) in DMF. The solution was stirred at 4 °C for 1 h and then allowed to warm to room temperature overnight. The solvent was removed under reduced pressure.

(f) Side-chain deprotection. Final deprotection was carried out with TFA/H_2O/TIS (95:2.5:2.5; 100μL × 1 mg of resin) for 1 h, under stirring. The crude product was purified by semipreparative RP-HPLC and characterized by ES-MS and NMR spectra.

3.4. Synthesis of analogues 2–4, 6, and 7

3.4.1. Anchoring of *N*-Fmoc-AA-OH to the resin [2a–4a, 6a, 7a]. This step was accomplished by using the general procedure a described above. In the following are

reported the amounts, loading levels, and other experimental details employed.

To the ClTrt-Cl resin (427.9 mg, 1.04 mmol/g loading level, **2a**; 462.7 mg, 1.04 mmol/g loading level, **3a**; 442.6 mg, 1.04 mmol/g loading level, **4a**; 319.0 mg, 1.40 mmol/g loading level, **6a**; and 383.0 mg, 1.40 mmol/g loading level, **7a**) were added the corresponding C-terminal *N*-α-Fmoc-AA-OH (*N*-α-Fmoc-β-Ala-OH: 53.2 mg, 0.17 mmol, **2a**; Fmoc-ε-Ahx-OH: 65.3 mg, 0.18 mmol, **3a**; Fmoc-8-Aoc-OH: 67.1 mg, 0.17 mmol, **4a**; Fmoc-Tyr(*t*Bu)-OH: 59.7 mg, 0.13 mmol, **6a**; and Fmoc-Val-OH: 52.5 mg, 0.15 mmol, **7a**) in DCM (2.5 mL) and DIEA (118.4 μL, 0.68 mmol, **2a**; 125.4 μL, 0.72 mmol, **3a**; 122.6 μL, 0.70 mmol, **4a**; 90.5 μL, 0.52 mmol, **6a**; and 106.6 μL, 0.61 mmol, **7a**), in order to obtain a lower substitution level. The reaction was quenched with DCM/MeOH/DIEA (17:2:1), the resin was then filtered, washed, and dried under vacuum over KOH for 24 h according to the procedure a. The substitution level of Fmoc-AA-O-ClTrt resins, determined spectrophotometrically by Fmoc cleavage, following the protocol a″ outlined in general procedures, was: 0.38 mmol/g (**2a**), 0.33 mmol/g (**3a**), 0.31 mmol/g (**4a**), 0.32 mmol/g (**6a**), and 0.33 mmol/g (**7a**).

3.5. Synthesis of analogue 5

3.5.1. Anchoring of *N*-Fmoc-AA-OH to the resin [5a]. This step was accomplished by using the general procedure a′ described above. In the following are reported the amounts, loading levels, and other experimental details employed.

To the *N*-α-Fmoc-β-Ala-OH (532.3 mg, 1.71 mmol) in DCM (12 mL) and DMF (500 μL) was added at 0 °C DCC (176.4 mg, 0.85 mmol) in DCM (1 mL) and the mixture was stirred for 25 min. The reaction mixture was filtered and the filtrate was evaporated under reduced pressure. The symmetric anhydride was dissolved in DMF (4 mL) and added to the swelled NovaSyn® TGA resin (714.0 mg, 0.24 mmol/g loading level); DMAP (21.3 mg, 0.171 mmol) was added and the resulting mixture was stirred for 4 h according to the procedure a′. The substitution level of Fmoc-β-Ala-O-NovaSyn® TGA resin, determined spectrophotometrically by Fmoc cleavage, following the protocol a″ outlined in general procedures, was 0.20 mmol/g (**5a**).

3.5.2. Synthesis of linear peptide on the solid support [2b–7b]. The assembly of the linear peptides was performed manually, in the C → N direction, adopting the Fmoc protection scheme and using HOBt/HBTU as activation reagents. After swelling Fmoc-AA-O-resin in DMF (3 mL, 1 h), the Fmoc-protecting group was removed by treatment with 20% piperidine in DMF according to the general procedure b.

After the *N*-α-Fmoc deprotection, each amino acid was introduced by a single coupling step, using a 3- to 4-fold excess of the Fmoc amino acid and of the activation reagents, following the coupling protocol c. The Kaiser or TNBS test was used to assess coupling efficiency.

3.5.2.1. [2b]. First coupling: Fmoc-5-Ava-OH (154.0 mg, 0.45 mmol), HOBt (69.4 mg, 0.45 mmol), HBTU (172.0 mg, 0.45 mmol), and NMM (66.8 μL, 0.60 mmol). After incorporation of Fmoc-5-Ava-OH, the Fmoc-protecting group was removed according to the general procedure b.

Second coupling: Fmoc-Phg-OH (169.4 mg, 0.45 mmol), HOBt (69.4 mg, 0.45 mmol), HBTU (172.0 mg, 0.45 mmol), and NMM (66.8 μL, 0.60 mmol), followed by *N*-α-deprotection.

Third coupling: Fmoc-D-Trp(Boc)-OH (238.8 mg, 0.45 mmol), HOBt (69.4 mg, 0.45 mmol), HBTU (172.0 mg, 0.45 mmol), and NMM (66.8 μL, 0.60 mmol), followed by *N*-α-deprotection.

Fourth coupling: Fmoc-Ala-OH (141.2 mg, 0.45 mmol), HOBt (69.4 mg, 0.45 mmol), HBTU (172.0 mg, 0.45 mmol), and NMM (66.8 μL, 0.60 mmol), followed by *N*-α-deprotection.

3.5.2.2. [3b]. First coupling: Fmoc-Phg-OH (209.0 mg, 0.56 mmol), HOBt (86.0 mg, 0.56 mmol), HBTU (212.4 mg, 0.56 mmol), and NMM (77.0 μL, 0.70 mmol), followed by *N*-α-deprotection accomplished with the general procedure b.

Second coupling: Fmoc-D-Trp(Boc)-OH (294.8 mg, 0.56 mmol), HOBt (86.0 mg, 0.56 mmol), HBTU (212.4 mg, 0.56 mmol), and NMM (77.0 μL, 0.70 mmol), followed by *N*-α-deprotection.

Third coupling: Fmoc-Ala-OH (174.3 mg, 0.56 mmol), HOBt (86.0 mg, 0.56 mmol), HBTU (212.4 mg, 0.56 mmol), and NMM (77.0 μL, 0.70 mmol), followed by *N*-α-deprotection.

3.5.2.3. [4b]. First coupling: Fmoc-Phg-OH (179.2 mg, 0.48 mmol), HOBt (73.5 mg, 0.48 mmol), HBTU (182.1 mg, 0.48 mmol), and NMM (66.0 μL, 0.60 mmol), followed by *N*-α-deprotection according to the general procedure b.

Second coupling: Fmoc-D-Trp(Boc)-OH (253.0 mg, 0.48 mmol), HOBt (73.5 mg, 0.48 mmol), HBTU (182.1 mg, 0.48 mmol), and NMM (66.0 μL, 0.60 mmol), followed by *N*-α-deprotection.

Third coupling: Fmoc-Ala-OH (149.5 mg, 0.48 mmol), HOBt (73.5 mg, 0.48 mmol), HBTU (182.1 mg, 0.48 mmol), and NMM (66.0 μL, 0.60 mmol), followed by *N*-α-deprotection.

3.5.2.4. [5b]. First coupling: Fmoc-5-Ava-OH (175.1 mg, 0.51 mmol), HOBt (78.5 mg, 0.51 mmol), HBTU (194.6 mg, 0.51 mmol), and NMM (75.2 μL, 0.68 mmol), followed by *N*-α-deprotection according to the general procedure b.

Second coupling: Fmoc-Tyr(*t*-Bu)-OH (235.7 mg, 0.51 mmol), HOBt (78.5 mg, 0.51 mmol), HBTU

(194.6 mg, 0.51 mmol), and NMM (75.2 μL, 0.68 mmol), followed by *N*-α-deprotection.

Third coupling: Fmoc-D-Trp(Boc)-OH (270.1 mg, 0.51 mmol), HOBt (78.5 mg, 0.51 mmol), HBTU (194.6 mg, 0.51 mmol), and NMM (75.2 μL, 0.68 mmol), followed by *N*-α-deprotection.

Fourth coupling: Fmoc-Ala-OH (159.7 mg, 0.51 mmol), HOBt (78.5 mg, 0.51 mmol), HBTU (194.6 mg, 0.51 mmol), and NMM (75.2 μL, 0.68 mmol), followed by *N*-α-deprotection.

3.5.2.5. [6b]. First coupling: Fmoc-D-Trp(Boc)-OH (195.0 mg, 0.37 mmol), HOBt (56.6 mg, 0.37 mmol), HBTU (140.3 mg, 0.37 mmol), and NMM (50.7 μL, 0.46 mmol), followed by *N*-α-deprotection according to the general procedure b.

Second coupling: Fmoc-Ala-OH (115.2 mg, 0.37 mmol), HOBt (56.6 mg, 0.37 mmol), HBTU (140.3 mg, 0.36 mmol), and NMM (50.7 μL, 0.45 mmol), followed by *N*-α-deprotection.

Third coupling: Hepa (80.0 mg, 0.41 mmol), HOBt (58.7 mg, 0.38 mmol), HBTU (137.8 mg, 0.36 mmol), and NMM (50.7 μL, 0.46 mmol).

3.5.2.6. [7b]. First coupling: Fmoc-D-Trp(Boc)-OH (252.8 mg, 0.48 mmol), HOBt (73.5 mg, 0.48 mmol), HBTU (182.1 mg, 0.48 mmol), and NMM (66.0 μL, 0.60 mmol), followed by *N*-α-deprotection according to the general procedure b.

Second coupling: Fmoc-Ala-OH (149.4 mg, 0.48 mmol), HOBt (73.5 mg, 0.48 mmol), HBTU (182.1 mg, 0.48 mmol), and NMM (66.0 μL, 0.60 mmol), followed by *N*-α-deprotection.

Third coupling: Hepa (115.0 mg, 0.58 mmol), HOBt (73.5 mg, 0.48 mmol), HBTU (182.1 mg, 0.48 mmol), and NMM (66.0 μL, 0.60 mmol).

3.5.3. Cleavage of the protected peptide from the resin [2–4c, 6–7c]. After chain assembly, the partially protected peptide was cleaved from the resins according to the general procedure d.

The dried peptide resin was treated for 2 h with the cleavage mixture AcOH/TFE/DCM (2:2:6) under stirring, followed by an additional treatment with fresh reagent (3 mL, 3 × 1.5 min). Then, the resin was removed by filtration and the filtrate was condensed, lyophilized, and characterized by analytical RP-HPLC and ES-MS.

The crude product (117.0 mg **2c**; 100.0 mg **3c**; 98.0 mg **4c**; 100.0 mg **6c**; and 115.0 mg **7c**) was analyzed by RP-HPLC Jupiter C-18 column (250 × 4.60 mm, 5 μm, 300 Å) using the following gradient: from 5% B to 100% B over 30 min at flow rate of 1 mL/min. The binary solvent system (A/B) was as follows: 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B). The absorbance was detected at 240 nm.

3.5.3.1. [2c]. The HPLC analysis showed a main peak at t_R 18.0 min; ES-MS: *m/z* 679.1 $[M+H]^+$, 701.4 $[M+Na]^+$. The minor peak eluting at t_R 17.0 min was shown to contain a mixture of **2c** along with a diastereoisomeric compound.

3.5.3.2. [3c]. The HPLC analysis showed a main peak at t_R 20.1 min; ES-MS: *m/z* 622.2 $[M+H]^+$. The minor peak eluting at t_R 18.6 min was shown to contain a mixture of **3c** along with a diastereoisomeric compound.

3.5.3.3. [4c]. The HPLC analysis showed a main peak at t_R 20.7 min; ES-MS: *m/z* 650.1 $[M+H]^+$, 672.1 $[M+Na]^+$. The minor peak eluting at t_R 19.8 min was shown to contain a mixture of **4c** along with a diastereoisomeric compound.

3.5.3.4. [6c]. The HPLC analysis showed a main peak at t_R 25.21 min; ES-MS: *m/z* 773.0 $[M+H]^+$, 795.2 $[M+Na]^+$, 811.3 $[M+K]^+$.

3.5.3.5. [7c]. The HPLC analysis showed a main peak at t_R 22.39 min; ES-MS: *m/z* 653.2 $[M+H]^+$, 675.3 $[M+Na]^+$, 691.3 $[M+K]^+$.

3.5.4. Cleavage from the resin and side-chains deprotection [5c]. After chain assembly, the partially protected peptide was deprotected from Boc and cleaved from the resin according to the general procedure d′.

The crude product (89.1 mg) was identified as **5c**, NH_2-Ala-Tyr-5-Ava-β-Ala-COOH, on the basis of ES-MS (*m/z* 609.2 $[M+H]^+$, 631.1 $[M+Na]^+$, and 647.0 $[M+K]^+$) and ^1H NMR experiments.

3.5.5. Synthesis of the cyclopeptides [2–7d], and final deprotection to obtain 2–7. The cyclization step was accomplished, for each peptide, by following the procedure e (**2**–**4d**) or e′ (**6** and **7d**) described above.

3.5.5.1. [2d]. The crude linear peptide (117.0 mg, 0.17 mmol) was dissolved in DCM (220.0 mL) with HATU (128.0 mg, 0.34 mmol, 1.98 equiv) and DIEA (74.0 μL, 0.43 mmol, 2.5 equiv). The solution was stirred for 1 h on an ice bath and then the mixture was allowed to warm up at room temperature overnight. The cyclization reaction was monitored via HPLC and ES-MS spectra. After 20.0 h, the solvent was removed under reduced pressure.

Side-chain deprotection was carried out with TFA/H_2O/TIS 95:2.5:2.5 (100 μL × 1 mg of resin) for 1 h under stirring. The cleavage mixture was evaporated and lyophilized, yielding 254.0 mg of crude cyclopeptide. A portion of the crude cyclopeptide (160.0 mg) was purified by semipreparative RP-HPLC on a Jupiter C-18 column (250 × 10.00 mm, 10 μm, 300 Å), using a 55 min gradient from 10:90 to 45:55 of CH_3CN/H_2O (each containing 0.1% TFA) at flow rate of 4 mL/min and detecting at 250 nm. The HPLC purification yielded the analogue **2**, c-[Ala-Trp-Phg-5-Ava-β-Ala], as a yellow oil (17.1 mg; t_R = 33.10 min; ES-MS, *m/z*

561.1 $[M+H]^+$, 583.3 $[M+Na]^+$, and 599.2 $[M+K]^+$; $C_{30}H_{36}N_6O_5$).

3.5.5.2. [3d]. The crude linear peptide (100.0 mg, 0.16 mmol) was dissolved in DCM (205.0 mL) with HATU (122.0 mg, 0.32 mmol, 1.98 equiv) and DIEA (70.5 μL, 0.40 mmol, 2.5 equiv). The solution was stirred for 1 h on an ice bath and then the mixture was allowed to warm up at room temperature overnight. The cyclization reaction was monitored via HPLC and ES-MS spectra. After 20 h, the solvent was removed under reduced pressure. Side-chain deprotection was accomplished by using the general procedure f. The reaction mixture was evaporated and lyophilized, yielding 217.6 mg of crude cyclopeptide. A portion of this crude cyclopeptide (100.0 mg) was purified by semipreparative RP-HPLC on a Jupiter C-18 column (250 × 10.00 mm, 10 μm, 300 Å), using a 57 min gradient from 10:90 to 45:55 of CH_3CN/H_2O (each containing 0.1% TFA) at flow rate of 4 mL/min and detecting at 250 nm. The purification yielded, as a pale yellow oil, the analogue **3**, c-[Ala-Trp-Phg-ε-Ahx] (9.0 mg; t_R = 38.11 min; ESI-MS, *m/z* 504.2 $[M+H]^+$, 526.3 $[M+Na]^+$; $C_{28}H_{33}N_5O_4$).

3.5.5.3. [4d]. The crude linear peptide (98.0 mg, 0.15 mmol) was dissolved in DCM (199.0 mL) with HATU (116.7 mg, 0.30 mmol, 1.98 equiv) and DIEA (67.5 μL, 0.39 mmol, 2.5 equiv). The solution was stirred for 1 h on an ice bath and then the mixture was allowed to warm up at room temperature overnight. The cyclization reaction was monitored via HPLC and ESI-MS spectra. After 20 h, the solvent was evaporated under reduced pressure. Side-chain deprotection was carried out by using the general procedure f. The reaction mixture was evaporated and lyophilized, yielding 214.4 of crude cyclopeptide. A portion of this crude cyclopeptide (130.0 mg) was then purified by semipreparative RP-HPLC on a Jupiter C-18 column (250 × 10.00 mm, 10 μm, 300 Å), using a 73 min gradient from 10:90 to 55:45 of CH_3CN/H_2O (each containing 0.1% TFA) at flow rate of 4 mL/min and detecting at 250 nm. The HPLC purification yielded, as a pale yellow oil, the analogue **4**; c-[Ala-Trp-Phg-8-Aoc] (10.3 mg; t_R = 45.48 min; ESI-MS, *m/z* 532.3 $[M+H]^+$, 554.4 $[M+Na]^+$; $C_{30}H_{37}N_5O_4$).

3.5.5.4. [5]. The crude linear peptide (87.5 mg, 0.14 mmol) was dissolved in a DCM/DMF mixture (199.0 mL/50.0 mL) with HATU (105.4 mg, 0.28 mmol, 1.98 equiv) and DIEA (61.0 μL, 0.35 mmol, 2.5 equiv). The solution was stirred for 1 h on an ice bath and then the mixture was allowed to warm up at room temperature overnight. The cyclization reaction was monitored via HPLC and ESI-MS spectra. After 20 h, the reaction mixture was evaporated and lyophilized, yielding 191.0 of crude cyclopeptide. A portion of this crude cyclopeptide (150.0 mg) was then purified by semipreparative RP-HPLC on a Jupiter C-18 column (250 × 10.00 mm, 10 μm, 300 Å), using a 50 min gradient from 15:85 to 65:35 of CH_3CN/H_2O (each containing 0.1% TFA) at flow rate of 4 mL/min and detecting at 250 nm. The HPLC purification yielded, as a white solid, the analogue **5**; c-[Ala-Trp-Tyr-5-Ava-β-Ala] (10.7 mg; t_R = 16.09 min; ESI-MS, *m/z* 591.3 $[M+H]^+$, 613.4 $[M+Na]^+$; and 629.4 $[M+K]^+$; $C_{31}H_{38}N_6O_6$).

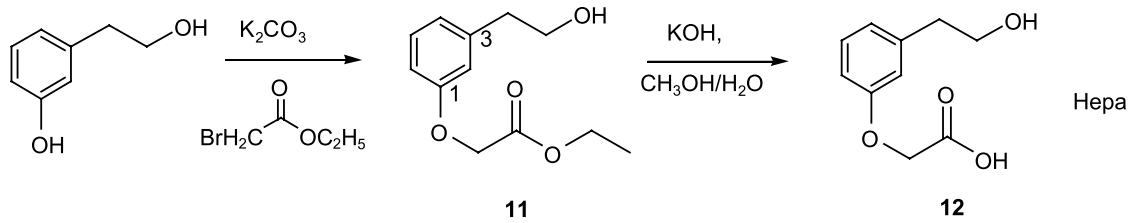
3.5.5.5. [6d]. The crude linear peptide (100.0 mg, 0.13 mmol) was dissolved in DMF (17.3 mL) with PyBop (210.7 mg, 0.40 mmol, 3.0 equiv) and DIEA (141.0 μL, 0.81 mmol, 6.0 equiv). The solution was stirred for 1 h on an ice bath and then the mixture was allowed to warm up at room temperature overnight. The cyclization reaction was monitored via HPLC and ESI-MS spectra. After 24 h, the solvent was evaporated under reduced pressure. Side-chain deprotection was carried out by using the general procedure f. The reaction mixture was evaporated and lyophilized; a portion of this crude cyclodepsipeptide (130.0 mg) was then purified by semipreparative RP-HPLC on a Jupiter C-18 column (250 × 10.00 mm, 10 μm, 300 Å), using a 85 min gradient from 10:90 to 70:30 of CH_3CN/H_2O (each containing 0.1% TFA) at flow rate of 4 mL/min and detecting at 260 nm. The HPLC purification yielded, as yellow oil, the analogue **6**; c-[Hepa-Ala-Trp-Tyr] (7.2 mg; t_R = 41.19 min; ESI-MS, *m/z* 599.3 $[M+H]^+$, 621.4 $[M+Na]^+$; $C_{33}H_{34}N_4O_7$).

3.5.5.6. [7d]. The crude linear peptide (115.0 mg, 0.18 mmol) was dissolved in DMF (24.2 mL) with PyBop (280.9 mg, 0.54 mmol, 3.0 equiv) and DIEA (188.0 μL, 1.08 mmol, 6.0 equiv). The solution was stirred for 1 h on an ice bath and then the mixture was allowed to warm up at room temperature overnight. The cyclization reaction was monitored via HPLC and ESI-MS spectra. After 24 h, the solvent was evaporated under reduced pressure. Side-chain deprotection was carried out by using the general procedure f. The reaction mixture was evaporated and lyophilized; a portion of this crude cyclodepsipeptide (240.0 mg) was then purified by semipreparative RP-HPLC on a Jupiter C-18 column (250 × 10.00 mm, 10 μm, 300 Å), using a 60 min gradient from 10:90 to 70:30 of CH_3CN/H_2O (each containing 0.1% TFA) at flow rate of 4 mL/min and detecting at 260 nm. The HPLC purification yielded, as yellow oil, the analogue **7**; c-[Hepa-Ala-Trp-Val] (7.0 mg; t_R = 38.22 min; ESI-MS, *m/z* 535.3 $[M+H]^+$, 557.4 $[M+Na]^+$; and 573.4 $[M+K]^+$; $C_{29}H_{34}N_4O_6$).

3.5.6. Synthesis of 3-(2-hydroxyethyl)-phenoxyacetic acid ethyl ester (11). To a stirred solution of commercially available 3-hydroxyphenethyl alcohol (0.640 g, 4.63 mmol) in 25 mL of acetone, potassium carbonate (3.20 g, 23.1 mmol) and, after 10 min, ethylbromoacetate (0.62 mL, 0.56 mmol) were added. The resulting mixture was refluxed overnight. Chloroform (20 mL) was added and potassium carbonate was filtered off and repeatedly washed several times with chloroform. The filtrate was concentrated in vacuo to afford **11** (1.01 g, 97%) as a pale yellow oil which was used in the next step without further purification.

R_f = 0.75 (30% petroleum ether in ethyl acetate).

^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.29 (3H, t, J = 7.1 Hz, –CH_2CH_3); 2.83 (2H, t, J = 6.5 Hz, Ar–CH_2CH_2–OH); 3.83 (2H, t, J = 6.5 Ar–CH_2CH_2–OH); 4.26 (2H, q,



Scheme 2. Synthetic scheme followed to obtain 3-(2-hydroxyethyl)-phenoxyacetic acid (**12**).

J = 7.1 Hz, $-CH_2CH_3$); 4.61 (2H, s, $ArOCH_2COOEt$); 6.76 (1H, br d, J = 8.0 Hz, H-6); 6.80 (1H, br s, H-2); 6.86 (1H, br d, J = 8.0 Hz, H-4); 7.23 (1H, t like, J = 8.0 Hz, H-5).

^{13}C NMR ($CDCl_3$, 400 MHz) δ: 14.1, 39.1, 61.3, 63.4, 65.3, 112.3, 115.6, 122.4, 129.6, 140.3, 158.0, 168.9.

3.5.7. Synthesis of 3-(2-hydroxyethyl)-phenoxyacetic acid (12). To a stirred solution of 3-(2-hydroxyethyl)-phenoxyacetic acid ethyl ester (**11**) (1.01 g, 4.51 mmol) in methanol (20 mL), a solution of potassium hydroxide (0.280 g, 5.00 mmol) in water (10 mL) was added, and the resulting mixture was stirred for 3 h. The reaction mixture was diluted with water (10 mL) and was neutralized with HCl (1 N). Methanol was evaporated in vacuo, and the aqueous layer was extracted with diethyl ether (3×20 mL). The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, and evaporated in vacuo to afford 3-(2-hydroxyethyl)-phenoxyacetic acid (**12**) (0.630 g, 0.321 mmol, 70%) as a pale yellow oil (see Scheme 2).

R_f = 0.11 (0.1% acetic acid in diethyl ether).

^1H NMR ($CDCl_3$, 400 MHz) δ: 2.85 (2H, t, J = 6.4 Hz, $Ar-CH_2CH_2-OH$); 3.86 (2H, t, J = 6.5 Hz, $Ar-CH_2CH_2-OH$); 4.68 (2H, s, $ArOCH_2COOH$); 6.79 (1H, br d, J = 8.0 Hz, H-6); 6.82 (1H, br s, H-2); 6.90 (1H, br d, J = 8.0 Hz, H-4); 7.26 (1H, t like, J = 8.0 Hz, H-5).

^{13}C NMR ($CDCl_3$, 400 MHz) δ: 39.0, 63.4, 64.8, 112.5, 115.5, 122.7, 129.7, 140.4, 157.0, 172.2.

3.6. Biological tests

3.6.1. Materials. MCF-7 breast carcinoma cells and NCI/ADR cells, an MDR line that overexpresses P-glycoprotein, were obtained from the Division of Cancer Treatment of the National Cancer Institute, USA. T24 human bladder carcinoma (HTB-4) and A-10 rat aortic smooth-muscle (CRL-1476) cells were from the American Type Culture Collection. Sulforhodamine B, antibodies against β-tubulin (T-4026), and antibodies against β-actin (A-2228) were obtained from Sigma Chemical Company (St. Louis, MO). RPMI-1640, α-MEM, and fetal bovine serum were from Gibco-BRL (Grand Island, NY).

3.6.2. Cytotoxicity assay. Cells were maintained in RPMI-1640 (MCF-7, NCI/ADR, and T24 cells) or α-MEM (A-10 cells) medium containing 10% fetal bovine serum and 50 μg/mL gentamicin at 37 °C in an atmosphere of 5% CO_2 and 95% air. To determine the cytotoxicities of the test compounds, cells were plated into 96-well tissue culture plates at approximately 15% confluency, and were allowed to attach and recover for 24 h. The cells were then treated in triplicate with varying concentrations of the test compound for 48 h, and cell survival was assayed using the sulforhodamine B (SRB) binding assay.[22] The percentage of cell survival was calculated as the percentage of SRB binding as compared with control cultures.

3.6.3. Immunofluorescence assays. A-10 cells were grown to near-confluency on glass coverslips in 24-well plates, and then treated with the test compounds for 24 h. Microtubules were incubated with monoclonal anti-β-tubulin, followed by fluorescein-conjugated anti-mouse IgG as previously described.[23,24] In similar experiments, microfilaments were incubated with monoclonal anti-β-actin, followed by fluorescein-conjugated anti-mouse IgG. Cytoskeletal structures were then visualized by epi-fluorescence and digital images were recorded of representative fields.

3.6.4. Analysis of hypodiploid (apoptotic) nuclei. The human lymphoblastoid cell line Jurkat was obtained from American Type Culture Collection (ATCC, LGC Promochem, Middlesex, UK) and cultured in RPMI medium supplemented with 10% heat-inactivated fetal calf serum (FCS). Recombinant TRAIL was obtained from Alexis Biochemicals, San Diego, CA. Apoptosis was analyzed by propidium iodide incorporation in permeabilized cells and flow cytometry as described.[18] Briefly, cells (5×10^5) were washed in PBS and resuspended in 500 μL of a solution containing 0.1% sodium citrate, 0.1% Triton X-100, and 50 μg/mL propidium iodide (Sigma Chemical Co., St. Louis, MO). Following incubation at 4 °C for 30 min in the dark, cell nuclei were analyzed with a Becton–Dickinson FACScan flow cytometer. Cellular debris was excluded from analysis by raising the forward scatter threshold, and the DNA content of the nuclei was registered on a logarithmic scale. The percentage of the elements in the hypodiploid region was calculated.

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