Synthesis, Conformational Analysis, and Cytotoxicity of New Analogues of the Natural Cyclodepsipeptide Jaspamide†

Stefania Terracciano,§ Ines Bruno,§ Giuseppe Bifulco,§ Jean E. Copper,‡ Charles D. Smith,‡ Luigi Gomez-Paloma,§ and Raffaele Riccio*,§

Dipartimento di Scienze Farmaceutiche, Università degli Studi di Salerno, Via Ponte Don Melillo, 84084 Fisciano (SA), Italy, and Department of Pharmacology, Penn State College of Medicine, 500 University Drive, Hershey, Pennsylvania 17033

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Three analogues of the natural bioactive cyclodepsipeptide jaspamide (**3**−**5**) were efficiently synthesized using a combination of solid and solution phase techniques. The preliminary design of the molecules has involved the rational substitution and/or simplification of the most critical structural features of the lead compound. The synthetic products were subjected to pharmacological assays, and the conformational properties were investigated by MM (molecular mechanics) and MD (molecular dynamics) calculations, to describe the potential pharmacophoric core responsible for the observed activities.

The cytoskeleton is considered to be one of the most strategic subcellular targets of anticancer chemotherapeutics, as it plays a crucial role in the functioning of the cell.[1−3] Agents affecting the cytoskeleton, in fact, usually possess a potent antiproliferative activity and therefore are considered promising candidates for application in chemotherapy as well as useful tools for studying cellular systems.[4] It is noteworthy that, although both microfilaments of actin and microtubules are considered valid anticancer targets, a number of inhibitors of microtubule dynamics and function are currently used in cancer therapy, while no drug actin targeting is used in clinics. In this perspective, jaspamide (jasplakinolide) (**1**), a natural cyclodepsipeptide isolated from marine sponges of *Jaspis* genus,[5−8] is a very promising member of actin modifying agents possessing an effective inhibitory action on breast and prostate cancer cell growth.[9,10]

Its mechanism of action has been elucidated in detail, and it was reported that it can disrupt actin filaments in vivo and can induce polymerization of monomeric actin into amorphus masses.[11] In addition, in vitro data also suggested that jaspamide could have the same binding properties on F-actin as phalloidin, a well-known fungal toxin affecting actin dynamics.[12] On the basis of these considerations, we focused our attention on this natural molecule composed of a peculiar tripeptide unit joined to a 12-carbon polyketide chain. In particular, to identify the key structural elements that contribute to the biological activity of jaspamide, we decided to undertake a project aimed at the exploration of the chemistry and biology of the natural cyclopeptide through the design and synthesis of a focused library of analogues. Our first effort was a preliminary attempt to obtain a small array of simplified structural analogues, with the ultimate goal of reducing the complexity of the synthetic enterprise needed to approach the preparation of the natural product. Therefore, compounds **3**−**5** were efficiently obtained through established procedures of solid and solution phase chemistry and then subjected to pharmacological screening.

In developing jaspamide analogues we took into account the recent results obtained on a group of related natural cyclodepsipeptides, such as doliculide (**2**), chondramide C (**6**), and phalloidin, which were found to display the same kind of biological effects as jaspamide on the actin cytoskeleton.[13]

Despite their similar bioactivity, these molecules show different structural features both in their oligopeptide composition and in the variable-length polyketide fragment upon which the size of the macrocycle depends. Therefore, to gain insight into a possible common pharmacophoric core, they were subjected to a molecular modeling study. In more detail, the three-dimensional structure of doliculide was compared with the above-mentioned molecules, and the results obtained indicated that the segment of doliculide that best overlapped with the other cyclodepsipeptides is represented by its phenyl and isopropyl side chains as well as the intervening macrocyclic peptide backbone. The analysis of the 3D structure alignment of doliculide and jaspamide showed that the phenyl ring and the isopropyl group of doliculide occupy the same region of space as the indole and the phenyl rings of jaspamide, respectively. On the basis of these considerations, in the design of the analogues **3**−**5** of jaspamide we decided to replace the two unusual amino acids (D)-*N*-methyl-2-bromotryptophane and (D)-β-tyrosine with D-tryptophane and L-valine, respectively, and to substitute the polypropionate portion with a simplified carbon chain linker. Specifically, in cyclopeptide **3** we joined β-alanine and 5-aminopentanoic acid to the tripeptide, affording a 19-membered macrocycle featuring the bioisosteric substitution of the Δ^4-*trans* double bond, present in the natural jaspamide, with a peptide linkage. In the analogue **4** a residue of 6-aminohexanoic acid was employed as a spanned chain to form a 16-membered cyclopeptide, and in the analogue **5** an 8-aminooctanoic acid was used to connect the N- and C-termini of our tripeptidic segment, thus generating an 18-membered macrocycle.

Results and Discussion

Synthesis. A combination of solid phase and solution phase synthetic techniques was used for the synthesis of the three analogues **3**−**5**. For solid phase chemistry, the Fmoc/tBu protection scheme was adopted on a 2-chlorotrityl chloride resin solid support. The first Fmoc-protected amino acid was anchored to the linker by diisopropyleth-

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* Corresponding author. Tel: +39-089-962818. Fax: +39-089-962828. E-mail: riccio@unisa.it.

§ Università degli Studi di Salerno.

‡ Penn State College of Medicine.

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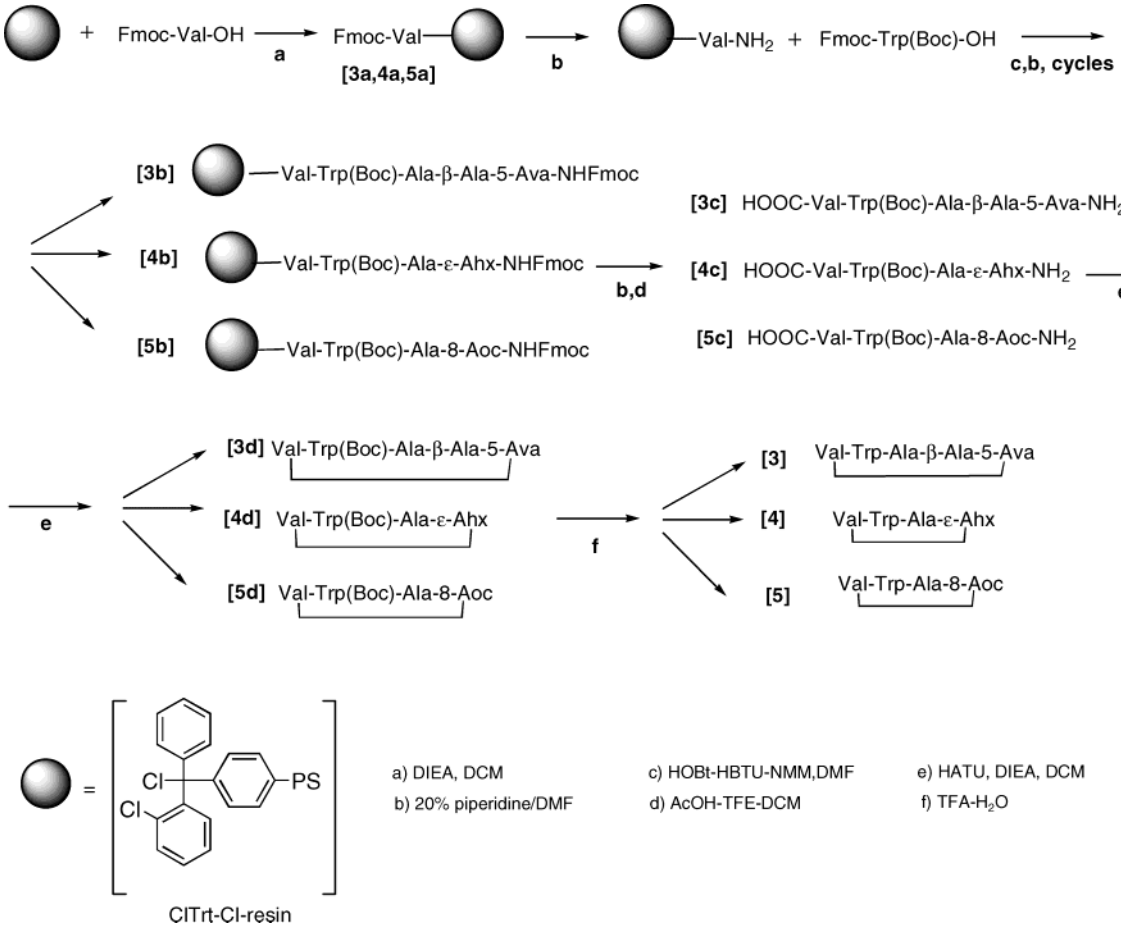
Scheme 1. Synthetic Scheme Followed to Obtain Analogues **3**−**5**



lyamine (DIEA) treatment under anhydrous conditions, followed by capping of unreacted trityl groups with methanol, according to general procedure **a** (see Experimental Section) (Scheme 1). The resulting loading degree was determined by UV spectrophotometric analysis using general procedure **a′**. The resin was then submitted to several coupling−deprotection cycles to build the linear N^{in}-Boc 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 test (the ninidrine colorimetric test). 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 general procedure **b**. After each linear peptide was obtained, the Fmoc protecting group was removed from the *N*-terminal residue and the (N^{in}-Boc protected) peptide was cleaved from the resin by using a 2:2:6 acetic acid/2,2,2-trifluoroethanol/dicholoromethane (AcOH/TFE/DCM) solvent mixture, according to procedure **d**. The cyclization reaction of the linear precursors was allowed to proceed in solution using *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) and DIEA in DCM following general procedure **e**. Finally, after removing the N^{in}-Boc protecting group with 95% aqueous trifluoroacetic acid (TFA), purification on semipreparative RP-HPLC yielded the pure cyclopeptides **3**−**5**.

Biological Activity. Biological evaluation of the synthetic compounds **3**−**5** against a minipanel of four cancer cell lines (T24, MCF-7,[14] NCI/ADR,[14] and A-10) showed cell growth inhibitory activity with IC_{50} values ranging from 3 to 24 μg/mL for the analogue **3** and from 1.8 to 12.5 μg/mL for the analogue **4**. No significant cytotoxicity toward any of the above cell lines was found for the compound **5** (Table 4).

These preliminary results cannot be easily rationalized in terms of the main structural and chemical features of

Table 1. ^{1}H and ^{13}C NMR Data for Analogue **3**, c-[Ava-β-Ala-Ala-Trp-Val] (600 MHz, DMSO-d_6)

position	δ_H	δ_C	position	δ_H	δ_C
Val			β-Ala		
α	3.94	59.0	α	2.06, 2.48	35.7
β	2.04	29.8	β	3.09, 3.30	36.2
γ_1-CH_3	0.62	19.7	NH	7.79	
γ_2-CH_3	0.70	18.2			
NH	8.14				
Trp			Ava		
α	4.56	54.5	α	1.95, 2.02	35.9
β	2.95, 3.10	27.9	β	1.42, 1.48	23.9
1-NH	10.82		γ	1.35[a]	28.9
2	7.20	124.5	δ	3.00, 3.09	39.2
3			NH	7.58	
4					
5	7.58	119.3			
6	6.97	118.8			
7	7.05	121.6			
8	7.30	111.8			
9					
NH	8.23				
Ala					
α	4.44	48.0			
β	1.06	19.4			
NH	7.97				

[a] 2 H.

Table 2. ^{1}H and ^{13}C NMR Data for Analogue **4**, c-[Ahx-Ala-Trp-Val] (300 MHz, DMSO-d_6)

position	δ_H	δ_C	position	δ_H	δ_C
Val			Ahx		
α	3.84	59.4	α	2.01, 2.12	35.4
β	1.95	27.0	β	1.52[a]	25.5
γ_1-CH_3	0.54	19.7	γ	1.06[a]	25.8
γ_2-CH_3	0.57	17.9	δ	1.31[a]	29.3
NH	8.00		ε	2.92, 3.15	38.8
			NH	7.37	
Trp					
α	4.53	54.8			
β	2.96	27.7			
1-NH	10.81				
2	7.16	124.4			
3					
4					
5	7.55	119.27			
6	6.94	118.9			
7	7.01	121.5			
8	7.29	119.7			
9					
NH	8.32				
Ala					
α	4.45	47.7			
β	1.11	18.0			
NH	8.03				

[a] 2 H.

Table 3. ^{1}H and ^{13}C NMR Data for Analogue **5**, c-[Aoc-Ala-Trp-Val] (600 MHz, DMSO-d_6)

position	δ_H	δ_C	position	δ_H	δ_C
Val			Aoc		
α	3.83	59.7	α	1.93, 2.18	35.5
β	1.90	29.6	β	1.52, 1.36	25.6
γ_1-CH_3	0.68	19.8	γ	1.14, 1.25	25.7
γ_2-CH_3	0.70	18.9	δ	1.11[a]	27.9
NH	8.21		ε	1.30[a]	28.4
			ζ	1.27, 1.35	28.9
Trp			η	2.76, 3.23	38.6
α	4.59	54.1	NH	7.59	
β	2.95, 3.05	28.7			
1-NH	10.79				
2	7.13	124.6			
3					
4					
5	7.57	119.3			
6	6.94	118.7			
7	7.02	121.5			
8	7.29	111.7			
9					
NH	8.23				
Ala					
α	4.49	47.6			
β	0.99	19.0			
NH	7.92				

[a] 2 H.

Table 4. In Vitro Antiproliferative Activity of Analogues **3**−**5**

	IC_{50} (μg/mL)[a]			
analogue	T24	MCF-7	NCI/ADR	A-10
3	19 ± 4	3 ± 0	24 ± 1	4.3 ± 0.2
4	10.3 ± 1.6	1.8 ± 0.2	12.5 ± 0	2 ± 0
5	>100	>100	>100	>100

[a] The IC_{50} is defined as the concentration of compound that inhibited the proliferation of the cells by 50%, in a 48 h treatment assay.

the three tested compounds, since all of them contain the same tripeptidic portion and similar spanned chains. On the other hand, not even a clear correlation between the macrocycle size and the biological activity has emerged, as the largest and the smallest sized cyclopeptides were active, while the medium sized one proved to be completely ineffective, thus leaving open the question of the role played in the bioactivity by the conformational motif of the macrocycles. Additionally, since the IC_{50}'s for the NCI/ADR cells are somewhat higher than the IC_{50}'s for the MCF-7 cells, all of them are probably substrates for P-glycoprotein, the drug transporter associated with MDR (multidrug resistance).

The next step was the exploration of the effect of the three synthetic analogues on the cytoskeleton system, and therefore 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 **3**−**5**. 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 **3**−**5**. 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 their observed cytotoxicity. In an attempt to rationalize these biological data, we decided to investigate the conformational properties of the synthetic compounds by restrained MM and MD analysis using NMR-derived data as constraints for the calculations.

Conformational Analysis of Analogues 3−5. NMR Spectroscopy. An extensive NMR analysis was carried out on analogues **3**−**5** in order to obtain assignment of the ^{1}H and ^{13}C resonances of the three cyclopeptides using a set of homonuclear and heteronuclear two-dimensional experiments (see Experimental Section). Successively, to collect a set of experimental restraints to be used in the course of molecular mechanics and dynamics calculations, a careful analysis of the ROESY data was performed, based on a distance calibration (r^{-6}, two-spin approximation) to convert the most intense and significative ROESY cross-peaks volumes into internuclear distances.

Molecular Mechanics and Dynamics Calculations. The three-dimensional structural characterization of analogues **3**−**5** required a careful investigation of the conformational space given the high flexibility expected for 16−19-membered cyclic compounds. Moreover, analogues **3**−**5** were characterized by the progressive increase in the linker chains, further reducing the rigidity of the macrocycle. For this reason, a preliminary round of molecular mechanics and dynamics calculations was run at 500 K in order to properly monitor the conformational space. Successively, MM and MD calculations were driven at 300 K by using the restraints collected during the NMR analysis, providing the final structures representing analogues **3**−**5**.

Structure−Activity Relationships for Analogues 3−5. To gain additional insight into the mechanism of action on the actin hyperpolymerization at the molecular level, and to determine which key elements in jaspamide (**1**) are responsible for antiproliferative activity, a careful analysis of the three-dimensional structures of compounds **3**−**5** was performed. In detail, after a preliminary, qualitative inspection of the similarities of **3**−**5** with regard to the X-ray structure of jaspamide, a detailed structural analysis followed, based on the comparison of **3**−**5** with the X-ray

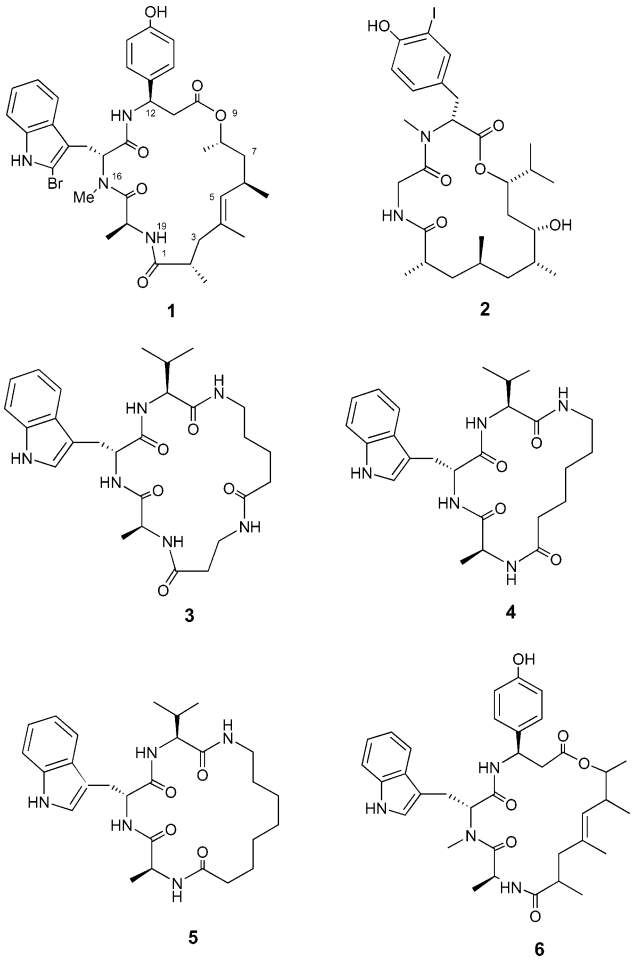


Figure 1. Structures of jaspamide (**1**), (−)-doliculide (**2**), jaspamide analogues (**3**−**5**), and chondramide C (**6**).

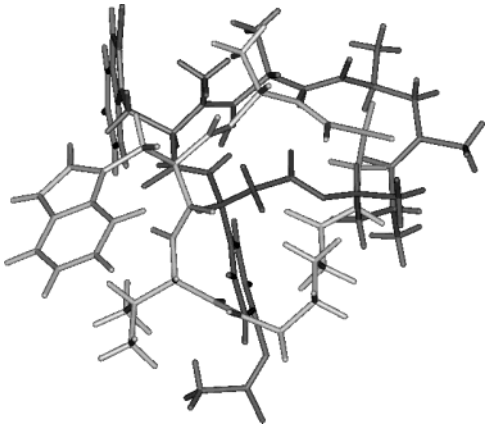


Figure 2. Superposition of the NMR solution structure of analogue **3** (lgray) and the X-ray structure of jaspamide (black). In the superposition criterion, the backbone atoms of the L-Ala-D-Trp segment were considered.

structure and the NMR-derived conformational ensemble of jaspamide proposed by Inman and Crews.[16]

The preliminary analysis of analogue **3**, compared with the X-ray structure of jaspamide (Figure 2), revealed a good superimposition of the backbone in the segment Ala-D-Trp, while a poorer correlation is observed for the region in which the β-Tyr is replaced by a L-Val. Analogue **4** has a 16-membered macrocycle, in analogy with the doliculide ring system, and hence smaller with respect to jaspamide. This cyclopeptide showed a good superimposition of the backbone in the segment Ala-D-Trp, and differently from

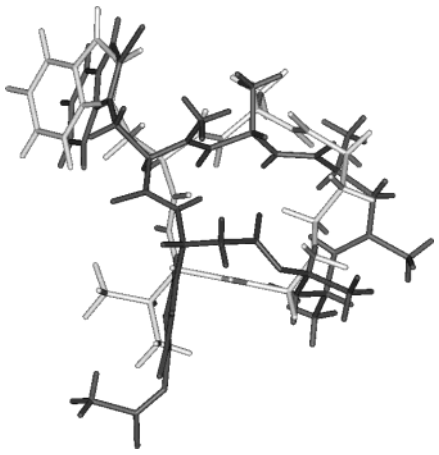


Figure 3. Superposition of the NMR solution structure of analogue **4** (gray) and the X-ray structure of jaspamide (black). In the superposition criterion, the backbone atoms of the L-Ala-D-Trp segment were considered.

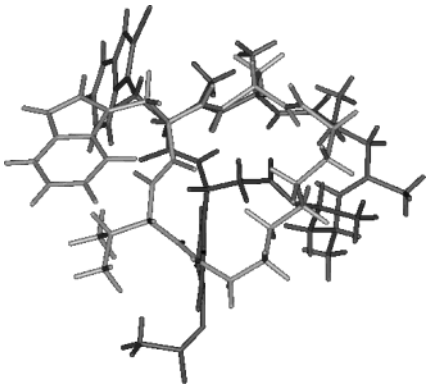


Figure 4. Superposition of the NMR solution structure of analogue **5** (gray) and the X-ray structure of jaspamide (black). In the superposition criterion, the backbone atoms of the L-Ala-D-Trp segment were considered.

what is observed for analogue **3**, a better positioning of the Val residue was observed in the hydrophobic region represented by the β-Tyr in the parent compound (Figure 3). Analogue **5** has an 18-membered macrocycle, in analogy with chondramide. The structural analysis of **5** revealed a poor alignment with jaspamide in both the tripeptide and linker regions (Figure 4), and not surprisingly, in this case both the activity on the cytoskeleton and the antitumor activity are missing.

The above observations may preliminarily suggest an involvement of the β-Tyr residue in exerting the specific activity on the cytoskeleton displayed by jaspamide, a structural feature missing in all analogues **3**−**5**. On the contrary, the conserved segment Ala-D-Trp may be responsible for the cytotoxic activity of **3** and **4** on the four cell lines tested.

Furthermore, jaspamide was reported to contain a type II β-turn in the D-Trp-β-Tyr region of the molecule.[15] An analysis on the corresponding regions of the NMR structures of analogues **3**−**5** was carried out, in particular by measuring φ and ψ angles describing the torsions of the backbone in the region L-Ala-D-Trp-L-Val. Subsequently, the dihedral angles of analogues **3**−**5** were compared to the corresponding angles measured for the X-ray structure of jaspamide (**1x**, see Table 5),[15] and for the six predominant conformers (**1i**−**1vi**, see Table 5) of jaspamide found by means of NMR and molecular mechanics and dynamics calculations.[16] Angles φ_{i+1} and ψ_{i+1}, characteristic of the D-Trp-β-Tyr region of jaspamide, are indeed reminiscent

Table 5. Peptide Backbone Dihedral Angles Observed for the L-Ala-D-Trp-L-Val Segment in the NMR Structures of Analogues **3**−**5** 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
3	−83.0	+140.9	+92.6	−141.1
4	−89.9	+105.2	+86.8	−117.5
5	−98.6	+101.2	+78.1	−135.2

of a type II β-turn in the X-ray structure of **1**, being around +90° and 0°, respectively; larger values for ψ_{i+1} are observed for conformers **1i**−**1vi** (Table 5). In analogues **3**−**5** angles φ_{i+1} are always around 90° (the smallest value is observed for analogue **5**, i.e., +78.1°), while angles ψ_{i+1} range from −117.5° (analogue **4**) to −141.1° (analogue **3**). These values are not in agreement with a type II β-turn, and they are quite different from the corresponding angle observed for the X-ray structure of jaspamide (**1x**) and for the NMR structures **1i**−**1vi**. Considering that analogues **3**−**5** do not affect the cytoskeleton, the above analysis may suggest a crucial role of the backbone arrangement in the D-Trp-β-Tyr region not only for its insecticide activity,[5] as already reported, but also for targeting actin microfilaments.

Experimental Section

Abbreviations: Boc, *tert*-butyloxycarbonyl; ClTrt-Cl, 2-chlorotrityl chloride-resin; DIEA *N*,*N*-diisopropylethylamine; DCM, dichloromethane; DMF, *N*,*N*-dimethylformamide; 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; HATU, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo-[4,5-*b*]pyridin-1-yl-methylene]-*N*-methylmethanaminium hexafluoroposphate *N*-oxide; HBTU, *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, *N*-hydroxybenzotriazole; NMM, *N*-methylmorpholine; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol.

General Experimental Procedures. All the NMR spectra (^{1}H, HMBC, HSQC, TOCSY, COSY, ROESY with t_{mix} = 0.4s) were recorded either on a Bruker Avance DRX600 or on a Bruker Avance 300 MHz spectrometers at T = 298 K. The three cyclopeptides (**3**−**5**) were dissolved in 0.5 mL of 99.95% d_6-DMSO (Carlo Erba, 99.95 atom % D) (^{1}H, δ = 2.50 ppm; ^{13}C δ = 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 ThermoQuest (San Josè, CA) 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 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). Water and CH_3CN were HPLC grade. 2-Chlorotrityl chloride resin (100−200 mesh), 1% DVB (ClTrt-Cl, loading level: 1.04 mmol/g), Fmoc-Ala-OH, Fmoc-D-Trp(Boc)-OH, Fmoc-Val-OH, HOBt, and HBTU were purchased from Novabiochem. Fmoc-β-Ala-OH, Fmoc-5-Ava-OH, Fmoc-ϵ-Ahx-OH, and Fmoc-8-Aoc-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.

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 temperature (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-peaks 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 CVFF (consistent-valence force field)[17] 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.[18]

General Procedures for the Synthesis of Compounds 3−5. (a) Loading of the Resin. 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, DCM 2 × 3 mL (1.5 min each).

(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 (∼2.5 mg for compound **3**, ∼4.3 mg for compound **4**, ∼4.4 mg for compound **5**) 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 shaken, and the absorbance of the samples versus the reference solution was measured at 301 nm. The substitution level (expressed in mmol of amino acid/g of resin) was calculated from the following equation: mmol $g^{-1} = (A_{301}/7800) \times (25$ mL g^{-1} 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, 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. 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.

(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.

(f) Final Deprotection. Final deprotection was carried out with TFA/H_2O (95:5; 100 μL × 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.

Synthesis of Analogues 3−5. Anchoring of *N*-Fmoc-L-Val-OH to the Resin [3a−5a]. This step was accomplished by using the general procedure **a** described above. Following are reported the amounts, loading levels, and other experimental details employed.

To the ClTrt-Cl resin (524.6 mg, 1.04 mmol/g loading level, **3a**; 489.0 mg, 1.04 mmol/g loading level, **4a**; 468.0 mg, 1.04 mmol/g loading level, **5a**) were added *N*-α-Fmoc-L-valine (78.06 mg, 0.23 mmol, **3a**; 57.7 mg, 0.17 mmol, **4a**; 55.1 mg, 0.16 mmol, **5a**) in DCM (2.5 mL) and DIEA (160.2 μL, 0.92 mmol, **3a**; 118.4 μL, 0.68 mmol, **4a**; 111.4 μL, 0.64 mmol, **5a**) to obtain a lower substitution level. The reaction was quenched with DCM/MeOH/DIEA (17:2:1), and the resin was then filtered, washed, and dried under vacuum over KOH for 24 h according to procedure **a**. The substitution level of Fmoc-Val-O−ClTrt resins, determined spectrophotometrically by Fmoc cleavage, following protocol **a′** outlined in the general procedures, was 0.43 mmol/g (**3a**), 0.27 mmol/g (**4a**), and 0.30 mmol/g (**5a**).

Synthesis of Linear Peptide on the Solid Support [3b−5b]. 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-Val-O-ClTrt in DMF (3 mL, 1 h), the Fmoc protecting group was removed by treatment with 20% piperidine in DMF according to general procedure **b**.

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

[3b]: First coupling: Fmoc-D-Trp(Boc)-OH (331.1 mg, 0.63 mmol), HOBt (96,4 mg, 0.63 mmol), HBTU (239.0 mg, 0.63 mmol), and NMM (92.3 μL, 0.84 mmol). After incorporation of Fmoc-D-Trp(Boc)-OH, the Fmoc protecting group was removed according to general procedure **b**.

Second coupling: Fmoc-Ala-OH (196.1 mg, 0.63 mmol), HOBt (96,4 mg, 0.63 mmol), HBTU (239.0 mg, 0.63 mmol), and NMM (92.3 μL, 0.84 mmol), followed by *N*-α-Fmoc deprotection.

Third coupling: Fmoc-β-Ala-OH (196.1 mg, 0.63 mmol), HOBt (96,4 mg, 0.63 mmol), HBTU (239.0 mg, 0.63 mmol), and NMM (92.3 μL, 0.84 mmol), followed by *N*-α-Fmoc deprotection.

Fourth coupling: Fmoc-5-Ava-OH (214.0 mg, 0.63 mmol), HOBt (96,4 mg, 0.63 mmol), HBTU (239.0 mg, 0.63 mmol), and NMM (92.3 μL, 0.84 mmol).

[4b]: First 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-Fmoc deprotection accomplished with 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-Fmoc deprotection

Third coupling: Fmoc-ϵ-Ahx-OH (169.6 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).

[5b]: First 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-Fmoc deprotection according to general procedure **b**.

Second 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-Fmoc deprotection.

Third coupling: Fmoc-8-Aoc-OH (213.6 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).

After the peptide chain was completed, a small scale cleavage of the peptide−resin was performed to characterize it as follows. A small amount (∼5 mg) of the dried peptide−resin was treated with 100 μL × 1 mg of resin (∼0.5 mL) of the cleavage solution (AcOH/TFE/DCM, 2:2:6) for 2 h. Then the resin was filtered off and washed three times with cleavage mixture. After addition of hexane (15 times volume) to remove acetic acid, the filtrate was condensed, lyophilized, and characterized by analytical RP HPLC and ES-MS.

The crude product was analyzed by a 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 a 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.

[3b]: t_R 25.18 min; ES-MS *m/z* 867.0 $[M + H]^+$, 889.2 $[M + Na]^+$, 905.1 $[M + K]^+$; $C_{47}H_{58}N_6O_{10}$.

[4b]: t_R 26.80 min; ES-MS *m/z* 810.1 $[M + H]^+$, 848.0 $[M + Na]^+$, 832.1 $[M + K]^+$; $C_{45}H_{55}N_5O_9$.

[5b]: t_R 27.90 min; ES-MS *m/z* 838.1 $[M + H]^+$, 860.0 $[M + Na]^+$, 876.2 $[M + K]^+$; $C_{47}H_{59}N_5O_9$.

The peptide−resins **[3b−5b]** were swollen in DMF (3 mL, 1 h), and then the Fmoc protecting group was removed by treatment with 20% piperidine in DMF according to general procedure **c**. The free amine content on the resin was controlled by Kaiser test.

Cleavage of the Protected Peptide from the Resin [3c−5c]. After chain assembly, the partially protected peptide was cleaved from the resins according to general procedure **d**. The dried peptide resin was treated for 2 h with the cleavage mixture AcOH/TFE/DCM (2:2:6) (6.6 mL, **3b**, 5.6 mL, **4b**, 5.5 mL, **5b**) 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 and lyophilized to give the crude product [**3c**, **4c**, **5c**].

Synthesis of the Cyclopeptides [3d−5d] and Final Deprotection to Obtain 3−5. The cyclization step was accomplished, for each peptide, by following procedure **e** described above.

[3d]. The crude linear peptide (224.3 mg, 0.35 mmol) was dissolved in DCM (449.0 mL) with HATU (263.5 mg, 0.69 mmol, 1.98 equiv) and DIEA (152.3 μL, 0.87 mmol, 2.5 equiv). The solution was stirred for 1 h on an ice bath and then the mixture allowed to warm 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 (95:5) (100 μL × 1 mg of resin) for 1 h under stirring. The cleavage mixture was evaporated and lyophilized, yielding 470 mg of crude cyclopeptide. It was analyzed by 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 a flow rate of 4 mL/min and UV detection at 250 nm. The HPLC analysis showed a main peak at t_R = 16.82 min that was identified as the pure cyclopeptide **3** on the basis of ES-MS and ^{1}H NMR data. The minor peak eluting at t_R = 17.4 was shown to contain a mixture of **3** along with a diasteroisomeric compound (amounting to 3%), presumably formed through loss of stereochemical integrity at one C-α position. A portion of the crude cyclopeptide (80.0 mg) was purified by semipreparative RP-HPLC on a Jupiter C-18 column, under the conditions described above. The HPLC purification yielded the analogue **3**, c-[Ava-β-Ala-Ala-Trp-Val], as a white solid (9.0 mg; t_R = 16.82 min; ES-MS, *m/z* 527.1 $[M + H]^+$, 549.3 $[M + Na]^+$, 565.2 $[M + K]^+$; $C_{27}H_{38}N_6O_5$).

[4d]. The crude linear peptide (86.0 mg, 0.15 mmol) was dissolved in DCM (197.0 mL) with HATU (113.0 mg, 0.30 mmol, 1.98 equiv) and DIEA (65.3 μL, 0.37 mmol, 2.5 equiv). The solution was stirred for 1 h on an ice bath and then the mixture allowed to warm at room temperature for 19 h. The cyclization reaction was monitored via HPLC and ES-MS spectra. After 19 h the solvent was removed under reduced pressure. Side-chain deprotection was obtained by treatment with TFA/H_2O (95:5) (100 μL × 1 mg of resin) for 1 h under stirring. The reaction mixture was evaporated and lyophilized, yielding 198.0 mg of crude cyclopeptide that was analyzed by RP-HPLC on a Jupiter C-18 column (250 × 10.00 mm, 10 μm,

300 Å) using a 65 min gradient from 5:95 to 50:50 of CH_3CN/H_2O (each containing 0.1% TFA) at a flow rate of 4 mL/min and detecting at 250 nm. The HPLC chromatogram showed two main peaks of different intensity, as in the case of **3** (see above): the major one eluting at $t_R = 36.8$ min was identified as the pure cyclopeptide **4** on the basis of ES-MS and ^{1}H NMR data. The minor peak eluting at $t_R = 36.0$ was shown to contain a mixture of **4** along with a diasteroisomeric compound (amounting to 6%). A portion of this crude cyclopeptide (119.0 mg) was purified by semipreparative RP-HPLC on a Jupiter C-18 column, under the conditions described above. The purification yielded, as a white solid, the analogue **4**, c-[Ahx-Ala-Trp-Val] (14.0 mg; $t_R = 36.8$ min; ESI-MS, *m*/*z* 470.2 $[M + H]^+$, 492.3 $[M + Na]^+$; $C_{25}H_{35}N_5O_4$).

[5d]. The crude linear peptide (86.0 mg, 0.14 mmol) was dissolved in DCM (185.0 mL) with HATU (108.4 mg, 0.27 mmol, 1.98 equiv) and DIEA (63.0 μL, 0.35 mmol, 2.5 equiv). The solution was stirred for 1 h on an ice bath and then the mixture allowed to warm at room temperature for 18 h. The cyclization reaction was monitored via HPLC and ESI-MS spectra. After 18 h the solvent was evaporated under reduced pressure. Side-chain deprotection was carried out with TFA/H_2O (95:5) (100 μL × 1 mg of resin) for 1 h under stirring. The reaction mixture was evaporated and lyophilized, yielding 194.0 mg of crude cyclopeptide. This product was analyzed by RP-HPLC on a Jupiter C-18 column (250 × 10.00 mm, 10 μ, 300 Å) using a 70 min gradient from 5:95 to 50:50 of CH_3CN/H_2O (each containing 0.1% TFA) at a flow rate of 4 mL/min and detecting at 250 nm. The HPLC chromatogram showed two main peaks of different intensity as already seen for **3** and **4**: the major one at $t_R = 45.7$ min was identified as the pure cyclopeptide **5** on the basis of ES-MS and ^{1}H NMR data. The minor peak eluting at $t_R = 44.9$ was shown to contain a mixture of **5** along with a diasteroisomeric compound (9%). A portion of this crude cyclopeptide (110.0 mg) was then purified by semipreparative RP-HPLC on a Jupiter C-18 column (250 × 10.00 mm, 10 μm, 300 Å), under the conditions described above. The HPLC purification yielded, as a white solid, the analogue **5**, c-[Aoc-Ala-Trp-Val] (16.0 mg; $t_R = 45.7$ min; ESI-MS, *m*/*z* 498.2 $[M + H]^+$, 520.4 $[M + Na]^+$; $C_{27}H_{39}N_5O_4$).

Biological Tests. 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. 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 GibcoBRL (Grand Island, NY).

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 gentamycin 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.[19] The percentage of cell survival was calculated as the percentage of SRB binding as compared with control cultures.

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.[20,21] 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.

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