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Synthesis, structural aspects and cytotoxicity of the natural cyclopeptides yunnanins A, C and phakellistatins 1, 10

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Abstract—Yunnanins A and C, two cyclic heptapeptides occurring in the roots of *Stellaria yunnanensis*, and phakellistatins 1 and 10, a hepta- and an octacyclopeptide first isolated from marine sponges of the genus *Phakellia*, were efficiently synthesized using a combination of solid and solution-phase techniques. Structural analysis on the synthetic members of the yunnanin series showed that the synthetic sample of yunnanin A exhibited a configurational pattern at the Pro peptide linkages identical to the natural product (*trans*-Pro^3, *trans*-Pro^5), while yunnanin C was obtained as a complex mixture of geometric/conformational isomers; the major isomer (*trans*-Pro^3) was indistinguishable from the natural cyclopeptide and co-occurred along with lower amounts of a mixture (1:1 ratio) of two different rotamers, both displaying *cis* geometry at the Pro^3 linkage. In the phakellistatin series, the synthetic phakellistatin 1 (determined as *cis*-Pro^1, *cis*-Pro^3, *cis*-Pro^5) was identical to the natural one, while two different isomeric products of phakellistatin 10 could be obtained: a major one (*trans*-Pro^1, *trans*-Pro^4, *trans*-Pro^6) showing spectral properties superimposable with the natural metabolite, and a minor geometric isomer of the natural cyclopeptide. Interestingly, the synthetic cyclopeptides, although found to be chemically identical with their natural counterparts, did not display the same biological properties (in vitro cytotoxicity against a panel of cancer cell lines), leaving presently open the question whether or not the potent bioactivity reported in the literature should really be attributed to these natural cyclic peptides.
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1. Introduction

Cyclic peptides and depsipeptides are very common among natural products and exhibit a wide range of biological activities.[1] In particular, a number of compounds belonging to this family of secondary metabolites have recently showed to interfere with cell proliferation and differentiation, thus attracting great interest as potential candidates for the development of novel anticancer drugs.[2] An emerging class of bioactive cyclopeptides is represented by 'proline rich' compounds, a series of natural products occurring especially in the marine environment,[3] but found also in higher plants,[4] named after their unusual high content of proline residues, and characterized by remarkable similarity in their amino acid composition and sequence motif pattern.

As part of our on-going research program on bioactive marine metabolites as potentially useful models in the discovery of new and more effective pharmaceuticals,[5] we have recently focused our attention on several members belonging to the proline-rich cyclopeptides family. Recent reports from our laboratory include our work on hymenamide C, a homodetic proline rich heptacyclopeptide, showing an inhibitory effect on human neutrophil elastase degranulation release. In this context, we described its solid-phase synthesis,[6] a detailed structural and pharmacological study of the natural product itself along with a small library of its Ala-modified analogues.[7]

The present investigation was directed toward the total synthesis of other members of the family of proline rich cyclopeptides: yunnanins A and C, two cyclic heptapeptides isolated from the roots of *Stellaria yunnanensis*,[8] and phakellistatins 1 and 10, a hepta- and octacyclopeptide respectively, first isolated from marine sponges of genus *Phakellia*[9] and then found to be also present in other taxonomically unrelated sponge specimens (Fig. 1).[10]

Greatly encouraged by the potent cell growth inhibitory effects exhibited by the above members of proline rich cyclopeptides, we decided to undertake their total synthesis in order to increase the supply for further biological investigation and, ultimately, to ascertain whether the synthetic products may display the same biological properties exhibited by their natural counterparts. Interestingly, other cases have recently been reported of closely related

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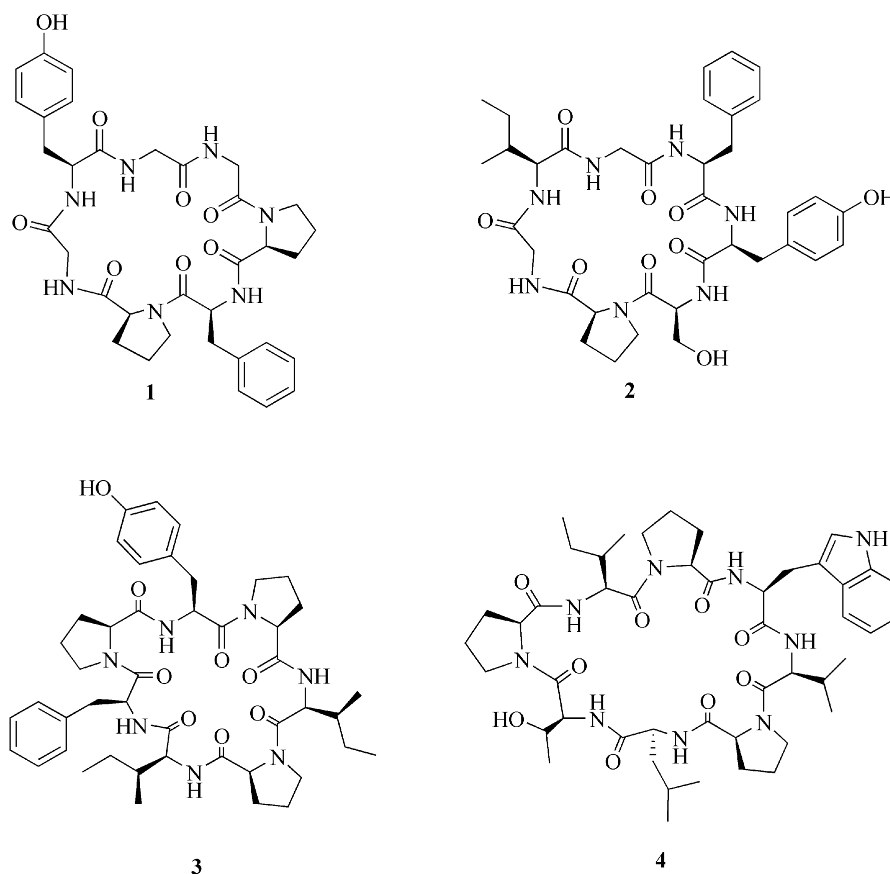

Figure 1. Chemical structures of natural products **1**–**4**.

cyclopeptides being synthesized and whose biological properties greatly differed from those found for the same chemical entities obtained from natural sources. For instance, Pettit and coworkers in their paper describing phakellistatin 5 solution and solid-phase synthesis raised the question whether it may be hypothesized that an unbearable amount of an extremely potent cytotoxin must co-occur with these natural cyclopeptides, in an attempt to explain the chemical albeit not biological equivalence of natural and synthetic samples.[11]

2. Results and discussion

The synthesis of yunnanin A [cyclo-(Gly-Gly-Pro^3-Phe-Pro^5-Gly-Tyr); (**1**)], yunnanin C [cyclo-(Tyr-Ser-Pro^3-Gly-Ile-Gly-Phe); (**2**)], phakellistatin 1 [cyclo-(Pro^1-Tyr-Pro^3-Ile-Pro^5-Ile-Phe); (**3**)], and phakellistatin 10 [cyclo-(Pro^1-Leu-Thr-Pro^4-Ile-Pro^6-Trp-Val); (**4**)] were accomplished using the Fmoc/*t*-Bu chemistry and a 2-chlorotritylchloride resin as solid support. The first Fmoc-protected amino acid was anchored to the linker by diisopropylethylamine (DIEA) treatment under anhydrous conditions, followed by capping of unreacted trityl groups with methanol according to general procedure A (see Section 3) (Fig. 2). The resulting loading degree was determined by UV spectrophotometric analysis using general procedure D. The resin was then submitted to six coupling–deprotection cycles to build the linear heptapeptides as precursors of the cyclic yunnanin A (**1**), yunnanin B (**2**) and phakellistatin 1 (**3**), and to seven coupling–deprotection cycles to build the N^{in}–Boc protected linear octapeptide as precursor of phakellistatin 10 (**4**). All the Fmoc-protected amino acids were activated by hydroxybenzotriazole/ *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HOBt/HBTU) in 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 ninhydrin 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 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. After HPLC analysis of the linear precursors, the cyclization reaction was allowed to proceed in solution using *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) and DIEA in DCM; finally, after removing the side-chain protecting groups with 95% aqueous trifluoroacetic acid (TFA), purification on semipreparative RP-HPLC yielded the final cyclopeptides.

A peculiar conformational feature of Pro-containing peptides is the *cis*–*trans* geometric isomerism at the peptide linkages connecting a proline residue with the preceding amino acid in the sequence (X-Pro). A wealth of data

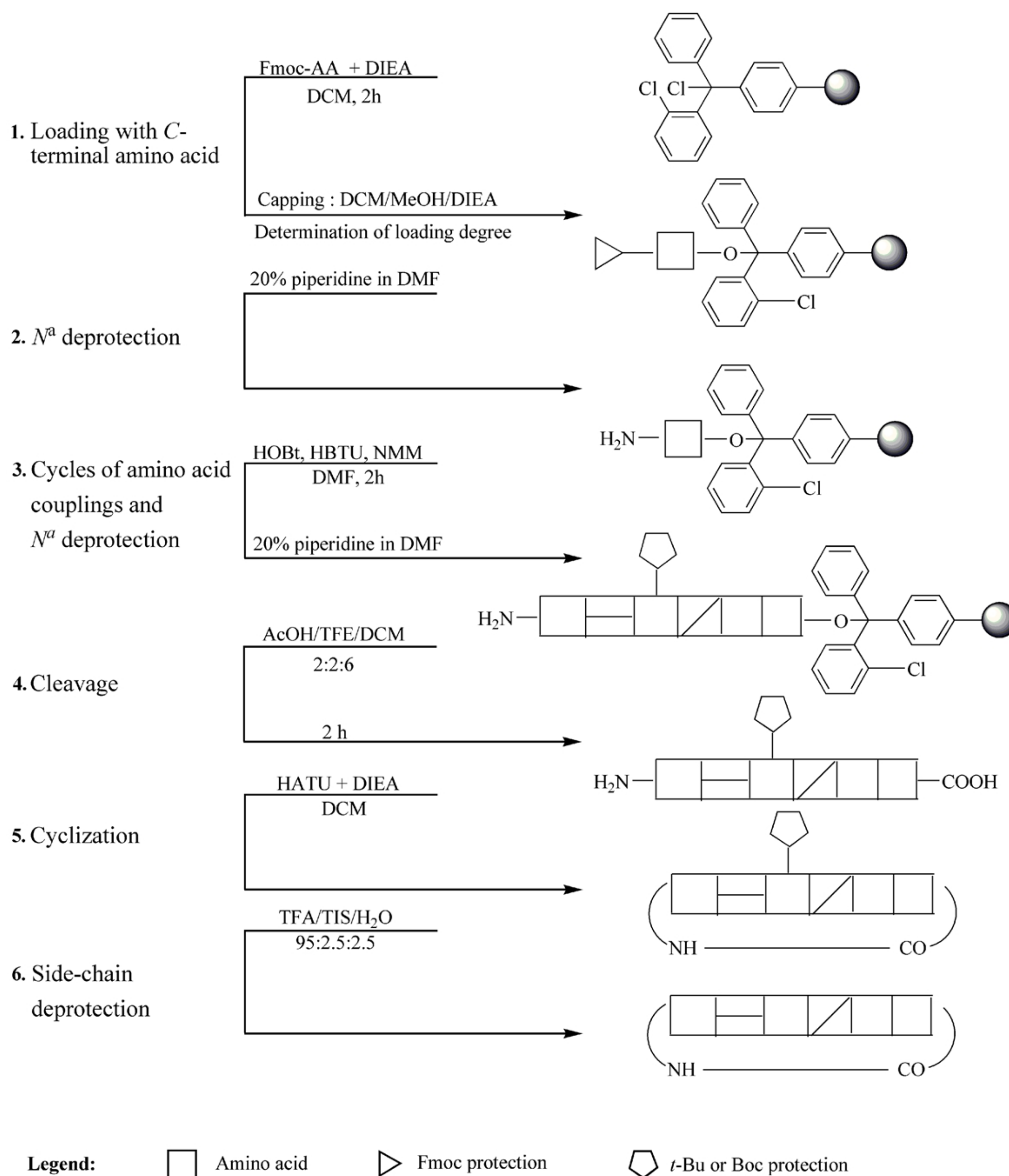

Figure 2. Synthetic scheme followed to obtain cyclopeptides **1–4**.

indicate that, in absence of structural constraints, Pro-containing peptide segments tend to adopt the more stable *trans* geometry at the Pro linkages, although often in times small but detectable amounts of *cis* X-Pro linkages are observed in dynamical equilibrium with the major *trans* species. This peculiar structural aspect makes more challenging the spectral analysis of proline-rich cyclopeptides, as two geometric isomers should be (at least in principle) expected for each proline occurring in the sequence; this kind of conformational equilibria become especially crucial in those cases where the size of the macrocycle permits greater flexibility of the peptide backbone, thus allowing the presence of multiple species slowly interconverting on the NMR time scale.

In particular, yunnanin A was obtained as single product which, on the basis of ROESY cross-peaks' pattern, possessed *trans* geometries at Gly-Pro^3 and Phe-Pro^5 linkages (ROESY cross-peak Hα-Gly/H_2δ-Pro^3 Hα-Phe/H_2δ-Pro^5), resulting to be identical to the natural yunnanin A[8] (^{1}H and ^{13}C NMR spectra of synthetic and natural samples were virtually superimposable; Table 1). Yunnanin C, instead, was obtained as two main products, the major one being indistinguishable from the natural cyclopeptide,[8] and featuring *trans* geometry at the Ser-Pro^3 peptide bond (ROESY cross-peak Hα-Ser/H_2δ-Pro^3) (Table 2). The minor geometric isomer was identified as a mixture of two conformational isomers, slowly interconverting on the NMR time scale, both possessing a *cis* configuration at the Pro^3 peptide linkage. As expected, the ratio of conformers showed to be dependent on the temperature of the sample. In fact, variable temperature

Table 1. ^{1}H and ^{13}C NMR data for yunnanin A (600 MHz, CD_3OD)

Position	^{1}H^{a}	^{13}C^{a}	Position	^{1}H^{a}	^{13}C^{a}
Gly^{1}			Pro^{5}		
α	3.67, 4.46	42.55	α	4.30	62.72
NH	8.37		β	1.99, 2.30	30.49
Gly^{2}			γ	2.01, 2.21	26.17
α	3.67, 4.46	42.55	δ	3.90, 4.44	49.70
NH	8.90				
Pro^{3}			Gly^{6}		
α	4.46	62.06	α	3.54, 4.23	43.42
β	1.71, 1.95	30.48			
γ	0.96, 1.68	24.07	Tyr		
δ	3.57	48.41	α	4.75	57.81
	3.81		β	2.88	39.76
Phe				3.11	
α	5.18	52.89			
β	3.17, 3.53	38.20	2,6	7.09	131.33
			3,5	6.69	116.25
2,6	7.40	130.67	NH	8.08	
3,5	7.34	129.73			
4	7.26	128.25			
NH	7.88				

^{a} δ in ppm.

NMR experiments in the 25–70°C range showed that coalescence of selected resonances could be achieved at high temperature. The Gly^{4}-Hα protons, observed as splitted signals resonating at δ 3.63–4.26 and δ 3.96–4.02 at room temperature merged at 70°C into a broad resonance centered at δ 3.63–4.26. In the phakellistatin series the synthetic route yielded phakellistatin 1 as a predominant isomer characterized by *cis* geometries at the Phe-Pro^{1}, Tyr-Pro^{3}, Ile-Pro^{5} peptide bonds (ROESY cross-peaks: Hα-Phe/Hα-Pro^{1}, Hα-Tyr/Hα-Pro^{3}, Hα-Ile/Hα-Pro^{5}). Since the spectral data of synthetic phakellistatin 1 were superimposable with those reported for the natural product[9] (Table 3), we deduced that the same pattern of geometries must apply to the natural product too, though such kind of stereochemical assignment was not performed at the stage of structural characterization. In the case of phakellistatin 10 synthesis, we obtained a major product, spectrally indistinguishable from the natural one,[9] identified as the all *trans* isomer at Val-Pro^{1}, Thr-Pro^{4}, Ile-Pro^{6} peptide linkages (ROESY cross-peaks: Hα-Val/H_2δ-Pro^{1}, Hα-Thr/H_2δ-Pro^{4}, Hα-Ile/H_2δ-Pro^{6}, Table 4). The minor synthetic phakellistatin 10 isomer exhibited the same electrospray mass spectrum (ESIMS), but slightly different ^{1}H NMR spectral data. Unfortunately, owing to the very low amount available, we were not able in this case to identify the geometries at the peptide linkages of Pro residues.

Stereochemical integrity of the amino acid residues of the synthetic cyclopeptides was secured by chiral GC–MS on their hydrolysate (HCl 6 M, 140°C, for 12 h in a sealed

Table 2. ^{1}H and ^{13}C NMR data for yunnanin C (300 MHz, CD_3OD)

Position	^{1}H^{a}	^{13}C^{a}	Position	^{1}H^{a}	^{13}C^{a}
Tyr			Ile^{5}		
α	4.41	57.63	α	4.23	59.07
β	3.03, 3.28	36.70	β	1.97	36.69
			CH_3	0.98	15.98
2,6	7.11	130.87	γ	1.11, 1.52	25.53
3,5	6.77	116.12	CH_3	0.90	10.71
NH	8.08		NH	8.44	
Ser			Gly^{6}		
α	4.85	54.46	α	3.61, 4.07	43.51
β	3.68, 3.79	62.73			
NH	7.53		NH	8.28	
Pro^{3}			Phe		
α	4.30		α	4.29	57.24
β	1.98, 2.25	62.99	β	2.77, 2.98	37.14
γ	1.97, 2.12	29.88			
δ	3.81, 3.85	25.99	2,6	7.04	129.61
Gly^{4}		49.93	3,5	7.27	129.30
α	3.67, 4.24	43.46	4	7.24	127.47

^{a} δ in ppm.

Table 3. ^{1}H and ^{13}C NMR data for phakellistatin 1 (600 MHz, CD_3OD)

Position	^{1}H^{a}	^{13}C^{a}	Position	^{1}H^{a}	^{13}C^{a}
Pro^{1}			Pro^{5}		
α	3.56	61.48	α	4.48	62.50
β	1.38, 1.76	32.15	β	2.08, 2.5	32.11
γ	1.43, 1.62	22.25	γ	1.68, 1.98	22.7
δ	3.22, 3.50	46.94	δ	3.50, 3.61	47.56
Tyr			Ile^{6}		
α	4.60	54.05	α	3.98	59.62
β	3.03, 3.07	37.27	β	1.96	35.72
			CH_3	0.83	15.18
2,6	6.66	114.79	γ	1.18, 1.46	26.55
3,5	6.83	130.87	CH_3	0.89	9.64
Pro^{3}			Phe		
α	4.52	59.30	α	4.65	53.10
β	1.96, 2.08	31.32	β	2.94 3.06	39.45
γ	1.87, 2.11	22.34			
δ	3.41	47.87	2,6	7.22	129.40
	3.60		3,5	7.28	127.32
Ile^{4}			4	7.34	128.80
α	4.04	58.32			
β	1.81	37.36			
CH_3	1.01	14.27			
γ	1.38, 1.77	26.17			
CH_3	1.00	10.03			

^{a} δ in ppm.

Table 4. ^{1}H and ^{13}C NMR data for phakellistatin 10 (600 MHz, CD_3OD)

Position	^{1}H^{a}	^{13}C^{a}	Position	^{1}H^{a}	^{13}C^{a}
Pro^{1}			Pro^{6}		
α	4.20	62.35	α	3.98	63.21
β	1.88, 2.31	30.89	β	1.90, 2.05	30.27
γ	1.99, 2.14	25.74	γ	1.92, 2.07	30.28
δ	3.75, 4.09	49.48	δ	3.42, 4.10	48.69
Leu			Trp		
α	3.73	54.99	α	4.40	56.78
β	2.40, 1.79	37.26	β	3.43, 3.74	48.74
γ	1.58	26.02			
CH_3	0.95	20.92	2	7.02	
CH_3	0.97	23.55	3		
Thr			4		
α	5.09	58.08	5	7.60	118.90
β	4.31	69.22	6	7.07	119.66
CH_3	1.19	19.43	7	7.14	122.24
Pro^{4}			8	7.37	112.24
α	4.59	61.07	Val		
β	1.99, 2.32	29.96	α	4.70	58.24
γ	1.96, 2.03	25.90	β	2.27	29.60
δ	3.70, 3.83	48.70	CH_3	0.82	19.08
Ile			CH_3	0.94	20.10
α	4.12	56.41			
β	1.89	36.27			
CH_3	0.51	15.14			
γ	1.06, 1.49				
CH_3	0.85	10.33			

^{a} δ in ppm.

tube), after derivatisation with *N-tert*-butyldimethylsylyl-*N*-methyltrifluoroacetamide, in comparison with authentic amino acid samples treated under the same experimental conditions.

Biological evaluation of the synthetic yunnanins and phakellistatins against a minipanel of three cancer cell lines showed cell growth inhibitory activity with IC_{50} values always <100 μg/ml (Table 5). A detailed analysis of these IC_{50} values indicated that, on average, the synthetic compounds were 100–1000-fold less active than their natural counterparts which exhibited antiproliferative activity with IC_{50} values ranging from 2.1 to 7.5 μg/ml. As stated before, this result, albeit puzzling and presently rather difficult to explain in a satisfactory fashion, was not fully unexpected. Indeed, in the context of proline rich natural peptides there have been other recent reports claiming strong disagreements between the bioactivities of synthetic and natural samples.

Table 5. In vitro *anti*-proliferative activity of proline rich cyclopeptides on a minipanel of three cancer cell lines

Cyclopeptides	IC_{50} (M)[a] J774.A1[b]	WEHI-164[c]	HEK-293[d]
Yunnanin A	3.0×10^{-3}	2.5×10^{-4}	1.7×10^{-3}
Yunnanin C major product	2.22×10^{-3}	2.9×10^{-3}	1.61×10^{-3}
Yunnanin C minor product	1.14×10^{-3}	1.2×10^{-3}	1.23×10^{-3}
Phakellistatin 1	2.6×10^{-3}	9.98×10^{-4}	3.9×10^{-3}
Phakellistatin 10 major product	5.6×10^{-4}	5.3×10^{-4}	5.7×10^{-4}
Phakellistatin 10 minor product	1.12×10^{-3}	3.8×10^{-3}	8.6×10^{-4}

[a] The IC_{50} value is the concentration of compound that affords 50% reduction in cell growth (after 3 days incubation).
[b] J774.A1, murine monocyte/macrophage cell line.
[c] WEHI-164, murine fibrosarcoma cell line.
[d] HEK-293, human epithelial kidney cell line.

Indeed, our results match the biological data reported by Pettit and co-workers.[12] It remains still open whether these cyclic peptides of natural origin may retain a chemically undetectable amount of strongly active cytotoxic agents. An alternative explanation has to do with the presence in these metabolites of proline residues playing an important role in defining their conformational motif. In this view, the biological difference between natural and synthetic samples might result from subtle conformational changes stemming from diverse arrangements of the proline units. A recent report, describing the case of phakellistatin 2,[13] would indeed favour this latter hypothesis. In our case, however, we subjected to biological testing also minor (geometrical) isomers without finding evidence of a relevant difference in bioactivity of theirs. On the other hand, it should be considered that our synthetic approach could not produce all the possible conformations around the Pro linkages and therefore, at least in principle, the hypothesis that among them there may be the bioactive molecule responsible for the cytotoxicity observed on natural samples, cannot be excluded.

3. Experimental

3.1. General methods

Unless specified, solvents were reagent grade. They were purchased from Aldrich or Fluka or Carlo Erba and were used without further purification. DCM and DMF used for solid-phase reactions were synthesis grade (dried over 4 Å molecular sieves), CH_3CN was HPLC grade. 2-chlorotritylchloride resin was purchased from Novabiochem (loading capacity 1.08 mmol/g or 1.04 mmol/g). The Fmoc-L-amino acids and the coupling reagents (HOBt, HBTU, HATU) were supplied by Novabiochem or Fluka and used without further purification. Solid-phase reactions were carried out on a polypropylene ISOLUTE SPE column on a VAC MASTER system (a manual parallel synthesis device purchased from Stepbio, Bologna, Italy) and using the Fmoc/*t*-Bu protocol. For quantification of the Fmoc amino acids on the resin, absorbance at 301 nm was read employing a Shimadzu UV 2101 PC spectrophotometer. Melting points were determined on an Electrothermal 9100 apparatus. The ^1H and ^{13}C ($^1H-^1H$ and $^1H-^{13}C$) spectra were recorded using a Bruker Avance 600 MHz spectrometer using CD_3OD or d_6-DMSO as solvents. The ^1H NMR temperature coefficients experiments were conducted at 27, 30, 35, 40, 45, 50, 55, 60, 65 and 70°C. A LCQ Thermoquest mass spectrometer was used to record the ESIMS spectra.

3.2. General procedures for solid-phase reactions

(A) 2-Chlorotritylchloride resin loading: Fmoc-AA-OH (0.5 equiv.), DIEA (2 equiv.) in DCM (10 ml/g of resin), 2 h; capping with 20 ml of DCM, MeOH, DIEA (17:2:1); washings (1.5 min each): 3×3 ml of DCM, 2x3 ml of DMF, 2×3 ml of DCM.

(B) Fmoc deprotection: 20% piperidine in DMF (3 ml), 1.5 min; 3 ml of 20% piperidine in DMF, 10 min; washings (1.5 min each): 2×3 ml of DMF, 3×3 ml of DCM, 2×3 ml of DMF.

(B1) Fmoc deprotection: 20% piperidine in DMF (3 ml), 5 min; washings (1.5 min each): 2×3 ml of DMF, 3×3 ml of DCM, 2×3 ml of DMF.

(C) Peptide coupling conditions: HOBt (4 equiv.), HBTU (4 equiv.), Fmoc-amino acid (4 equiv.) NMM (5 equiv.) in DMF (500 μl/100 mg of resin), 2 h; washings (1.5 min each): 3×3 ml of DMF, 3×3 ml of DCM.

(D) Spectrophotometric analysis of the Fmoc chromophore: the assay was performed on duplicate samples. 0.4 ml of piperidine and 0.4 ml of DCM were added to two dried samples of the resin-bound peptide (~6 mg) in two 10 ml volumetric flasks. The reaction was allowed to proceed for 30 min at room temperature in the sealed flasks. 1.6 ml of MeOH were added and the solutions were diluted to 10 ml volume with DCM. A reference solution was prepared in a 10 ml volumetric flask using 0.4 ml of piperidine, 1.6 ml of MeOH and DCM to volume. The solutions were shook and the absorbance of the samples versus the reference solution was measured at 301 nm. The substitution degree (in mmol of amino acid/g of resin) was calculated from the equation: mmol/g=$(A_{301}/7800)\times$(10 ml/g of resin).

3.3. Synthesis of the linear side chain protected peptides of yunnanin A and C, phakellistatin 1 and 10 (1–4)

2-Chlorotritylchloride resin was placed into a 25 ml

Table 6. Data of the linear protected peptides

	Yunnanin A	Yunnanin C	Phakellistatin 1	Phakellistatin 10
Resin amount	0.600 g	0.600 g	0.439 g	0.800 g
Initial loading level	1.04 mmol/g	1.04 mmol/g	1.08 mmol/g	1.04 mmol/g
AA C-terminal	Fmoc-Gly-OH, 92.8 mg, 0.31 mmol	Fmoc-Phe-OH, 120.9 mg, 0.31 mmol	Fmoc-Phe-OH, 91.8 mg, 0.237 mmol	Fmoc-Val-OH, 141.19 mg, 0.42 mmol
Loading level	0.40 mmol/g	0.35 mmol/g	0.31 mmol/g	0.465 mmol/g
1° coupling	Fmoc-Gly-OH, 285.4 mg, 0.96 mmol	Fmoc-Gly-OH, 249.7 mg, 0.84 mmol	Fmoc-Ile-OH, 192.2 mg, 0.54 mmol	Fmoc-Trp(Boc)-OH, 452.9 mg, 1.5 mmol
2° coupling	Fmoc-Tyr(OtBu)-OH, 441.2 mg, 0.96 mmol	Fmoc-Ile-OH, 296.8 mg, 0.84 mmol	Fmoc-Pro-OH, 183.5 mg, 0.54 mmol	Fmoc-Pro-OH, 502.05 mg, 1.5 mmol
Loading level	0.33 mmol/g	0.35 mmol/g	0.31 mmol/g	0.465 mmol/g
3° coupling	Fmoc-Gly-OH, 235.5 mg, 0.79 mmol	Fmoc-Gly-OH, 249.7 mg, 0.84 mmol	Fmoc-Ile-OH, 192.2 mg, 0.54 mmol	Fmoc-Ile-OH, 525.8 mg, 1.5 mmol
4° coupling	Fmoc-Pro-OH, 267.2 mg, 0.79 mmol	Fmoc-Pro-OH, 283.4 mg, 0.84 mmol	Fmoc-Pro-OH, 183.5 mg, 0.54 mmol	Fmoc-Pro-OH, 502.05 mg, 1.5 mmol
5° coupling	Fmoc-Phe-OH, 306.8 mg, 0.79 mmol	Fmoc-Ser(OtBu)-OH, 322.1 mg, 0.84 mmol	Fmoc-Tyr(OtBu)-OH, 250 mg, 0.54 mmol	Fmoc-Thr(OtBu)-OH, 591.8 mg, 1.5 mmol
6° coupling	Fmoc-Pro-OH, 267.2 mg, 0.79 mmol	Fmoc-Tyr(OtBu)-OH, 459.6 mg, 0.84 mmol	Fmoc-Pro-OH, 183.5 mg, 0.54 mmol	Fmoc-Leu-OH, 525.8 mg, 1.5 mmol
7° coupling	/	/	/	Fmoc-Pro-OH, 502.05 mg, 1.5 mmol

polypropylene ISOLUTE column on a VAC MASTER system, swelled for 1 h with 3 ml of DMF by a N_2 stream and then washed with 2x3 ml of DCM. The resin was loaded with corresponding C-terminal Fmoc-AA-OH, in order to obtain a lower substitution level, according to procedure (A). Unreacted trityl groups were capped with methanol. The resin was dried under vacuum over KOH and the resulting substitution level was determined spectrophotometrically according to the general procedure (D). After Fmoc-AA-*O*-2ClTrt-Cl swelling (45 min with 3 ml of DMF), Fmoc protecting group removal was obtained according to general procedure (B). The resin was subsequently submitted to the following series of coupling–deprotection cycles: (i) peptide coupling according to general procedure (C) with appropriate amino acid, followed by Fmoc deprotection according to general procedure (B1) to avoid the formation of DKP. The result of the Fmoc removal was monitored according to general procedure (D); (ii) peptide coupling according to general procedure (C) with appropriate amino acid, followed by determination, according to general procedure (D), of the degree of substitution of the resin bound peptide. In the cases of low DKP formation it was chosen to perform the following peptide couplings according to general procedure (C), while, when it was found an highest amount of DKP formation, couplings were performed according to the new loading level. The Fmoc deprotection was performed according to general procedure (B); (iii) peptide coupling according to general procedure (C) with appropriate amino acid, followed by Fmoc deprotection according to general procedure (B), up to obtain the expected linear protected peptide. The ninhydrin test was performed after each amino acid coupling step and the coupling repeated if necessary (Table 6).

The resin was removed from the polypropylene column, washed with methanol and dried under vacuum over KOH for 1 h. The overall resin-bound peptide was cleaved from the solid support by treatment with an AcOH/TFE/DCM (2:2:6) solution for 2 h under stirring. The cleavage mixture was filtered off and the resin was washed 2 times with the same solution. Hexane was added (15 times volume) and the solution was evaporated, adding further hexane if necessary. The crude peptide product was lyophilised and analysed by RP-HPLC on a Jupiter C-18 analytical column (250×4.60 mm, 5 μm, 300 Å), using a 31 min gradient from 5 to 100 of CH_3CN/H_2O (each containing 0.1% TFA) at a flow rate of 1.0 ml/min and UV detection at 220 nm. The HPLC analysis showed one peak that was identified as the linear side-chain protected peptide on the basis of an ESIMS experiment (Table 7).

Table 7. Analytical data of the linear protected peptides

		HPLC R_t (min)	Mass data[a]
1	Yunnanin A	16.33	750
2	Yunnanin C	18.57	852
3	Phakellistatin 1	20.35	903
4	Phakellistatin 10	24.03	1077

[a] ESIMS, *m/z* for $[M+H]^+$.

3.4. Cyclization of the linear side-chain protected yunnanin A (1)

The crude linear peptide (45.1 mg, 0.06 mmol) was dissolved in DCM (4×10^{-4} M) with HATU (45.6 mg, 0.12 mmol, 2 equiv.) and DIEA (26.1 μl, 0.15 mmol, 2.5 equiv.). The solution was stirred for 1 h on an ice bath and then allowed to warm at room temperature and kept at this temperature for 22.30 h. During this time DCM was gradually added until to 2.4×10^{-4} M to avoid side reactions such as oligodimerization.

The cyclization reaction was monitored via HPLC and ESIMS spectra. After 22.30 h the solvent was removed. Side-chain deprotection was obtained by treatment with TFA/TIS/H_2O=95: 2.5: 2.5 (100 μl×1 mg of resin) for 1 h under stirring. The cleavage mixture was evaporated and lyophilised, yielding 82.7 mg of crude cyclopeptide. It was analysed by RP-HPLC on a Jupiter C-18 analytical column (250×4.60 mm, 5 μm, 300 Å), using a 56 min gradient from 25:75 to 60:40 of CH_3CN/H_2O (each containing 0.1% TFA) at a flow rate of 1.0 ml/min and UV detection at 220 nm. The HPLC analysis showed one main peak (R_t=11.16 min; ESIMS, *m/z* 676 for $[M+H]^+$) identified as the *trans*-Pro[3], *trans*-Pro[5] cyclopeptide yunnanin A on the basis of ESIMS and ^{1}H NMR experiments. The crude cyclopeptide was then purified by semi-preparative RP-HPLC on a Jupiter C-18 column (250×10.00 mm, 10 μm, 300 Å), using a 24 min gradient from 20:80 to 35:65 of CH_3CN/H_2O (each containing 0.1% TFA) at a flow rate of 5.0 ml/min. The HPLC purification yielded, as white solid, 12.8 mg (yield=51.8%) of *trans*-Pro[3], *trans*-Pro[5] cyclopeptide yunnanin A (R_t=14.93 min; ESIMS, *m/z* 676 for $[M+H]^+$; mp 228–229°C).

3.5. Cyclization of the linear side-chain protected yunnanin C (2)

The crude linear peptide (61 mg, 0.072 mmol) was dissolved in DCM (4×10^{-4} M) with HATU (54.4 mg, 0.143 mmol, 2 equiv.) and DIEA (31.4 μl, 0.18 mmol, 2.5 equiv.). The solution was stirred for 1 h on an ice bath and then allowed to warm at room temperature and kept at this temperature for 22.30 h. During this time DCM was gradually added until to 2.4×10^{-4} M to avoid side reactions such as oligodimerization.

The cyclization reaction was monitored via HPLC and ESIMS spectra. After 22.30 h the solvent was removed. Side-chain deprotection was obtained by treatment with TFA/TIS/H_2O=95:2.5:2.5 (100 μl×1 mg of resin) for 1 h under stirring. The cleavage mixture was evaporated and lyophilised, yielding 90.6 mg of crude cyclopeptide. It was analysed by RP-HPLC on a Jupiter C-18 analytical column (250×4.60 mm, 5 μm, 300 Å), using a 31 min gradient from 5 to 100 of CH_3CN/H_2O (each containing 0.1% TFA) at a flow rate of 1.0 ml/min and UV detection at 220 nm. The HPLC analysis showed two main peaks; the major one was identified as the *trans*-Pro[3] yunnanin C, indistinguishable from the natural cyclopeptide (R_t=15.12 min; ESIMS, *m/z* 722 for $[M+H]^+$) on the basis of ESIMS and ^{1}H NMR experiments, while the minor one was identified as a mixture of two conformational isomers of yunnanin C (R_t=14.93 min; ESIMS, *m/z* 722 for $[M+H]^+$). The crude cyclopeptides were then purified by semi-preparative RP-HPLC on a Jupiter C-18 column (250×10.00 mm, 10 μm, 300 Å), using a 37 min gradient from 20:80 to 35:65 of CH_3CN/H_2O (each containing 0.1% TFA) at a flow rate of 5.0 ml/min and UV detection at 220 nm. The HPLC purification yielded, as white solids, 9.7 mg (yield=28.7%) of *trans*-Pro[3] cyclopeptide yunnanin C (R_t=12.48 min; ESIMS, *m/z* 722 for $[M+H]^+$; mp 255°C) and 4.8 mg (yield=14.2%) of the minor isomeric mixture of cyclopeptide yunnanin C (R_t=13.09 min; ESIMS, *m/z* 722 for $[M+H]^+$; mp 237–239°C).

3.6. Cyclization of the linear side-chain protected phakellistatin 1 (3)

The crude linear peptide (167.9 mg, 0.186 mmol) was dissolved in DCM (7.79×10^{-4} M) with HATU (140.1 mg, 0.368 mmol, 2 equiv.) and DIEA (80.8 μl, 0.465 mmol, 2.5 equiv.). The solution was stirred for 1 h on an ice bath and then allowed to warm at room temperature and kept at this temperature for 21.30 h. During this time DCM was gradually added until to 4×10^{-4} M to avoid side reactions such as oligodimerization.

The cyclization reaction was monitored via HPLC and ESIMS spectra. After 21.30 h the solvent was removed, yielding 332.1 mg of crude side-chain protected cyclopeptide. Side-chain deprotection was obtained by treatment with TFA/TIS/H_2O=95:2.5:2.5 (100 μl×1 mg of resin) for 1 h under stirring. The cleavage mixture was evaporated and lyophilized. The crude cyclopeptide was analysed by RP-HPLC on a Jupiter C-18 analytical column (250×4.60 mm, 5 μm, 300 Å), using a 31 min gradient from 5 to 100 of CH_3CN/H_2O (each containing 0.1% TFA) at a flow rate of 1.0 ml/min and UV detection at 220 nm. The HPLC analysis showed one main peak (R_t=19.34 min; ESIMS, *m/z* 828 for $[M+H]^+$) identified as the *cis*-Pro[1], *cis*-Pro[3], *cis*-Pro[5] cyclopeptide phakellistatin 1 on the basis of ESIMS and ^{1}H NMR experiments. The crude cyclopeptide was then purified by semi-preparative RP-HPLC on a Jupiter C-18 column (250×10.00 mm, 10 μm, 300 Å), using a 32 min gradient from 25:75 to 45:55 of CH_3CN/H_2O (each containing 0.1% TFA) at a flow rate of 5.0 ml/min and UV detection at 220 nm. The HPLC purification yielded, as white solid, 9.6 mg (yield=22.3%) of *cis*-Pro[1], *cis*-Pro[3], *cis*-Pro[5] cyclopeptide phakellistatin 1 peak (R_t=21.54 min; ESIMS, *m/z* 828 for $[M+H]^+$; mp 240–242°C).

3.7. Cyclization of the linear side-chain protected phakellistatin 10 (4)

A portion of the crude linear peptide (150.0 mg, 0.14 mmol) was dissolved in DCM (2.4×10^{-4} M) with HATU (106.5 mg, 0.28 mmol, 2 equiv.) and DIEA (61.3 μl, 0.352 mmol, 2.5 equiv.). The solution was stirred for 1 h on an ice bath and then allowed to warm at room temperature and kept at this temperature for 7.30 h. The cyclization reaction was monitored via HPLC and ESIMS spectra. After 7.30 h the solvent was removed. Side-chain deprotection was obtained by treatment with TFA/TIS/H_2O=95:2.5:2.5 (100 μl×1 mg of resin) for 1 h under stirring. The cleavage mixture was evaporated and lyophilised,

yielding 283.9 mg of crude cyclopeptide. The crude cyclopeptide was analysed by RP-HPLC on a Jupiter C-18 analytical column (250×4.60 mm, 5 μm, 300 Å), using a 31 min gradient from 5 to 100 of CH_3CN/H_2O (each containing 0.1% TFA) at a flow rate of 1.0 ml/min and UV detection at 220 nm. The HPLC analysis showed two main peaks; the major one was identified as the *trans*-Pro[1], *trans*-Pro[4], *trans*-Pro[6] cyclopeptide phakellistatin 10 (R_t=22.42 min; ESIMS, *m/z* 903 for $[M+H]^+$) on the basis of ESIMS and ^{1}H NMR experiments, while the minor one was supposed to be a geometric isomer of phakellistatin 10 (R_t=22.88 min; ESIMS, *m/z* 903 for $[M+H]^+$). The crude cyclopeptides were then purified by semi-preparative RP-HPLC on a Jupiter C-18 column (250×10.00 mm, 10 μm, 300 Å), using a 56 min gradient from 25:75 to 60:40 of CH_3CN/H_2O (each containing 0.1% TFA) at a flow rate of 5.0 ml/min and UV detection at 220 nm. The HPLC purification yielded, as white solids, 6.3 mg (yield=16.4%) of *trans*-Pro[1], *trans*-Pro[4], *trans*-Pro[6] cyclopeptide phakellistatin 10 (R_t=34.70 min; ESIMS, *m/z* 903 for $[M+H]^+$; mp 215–218°C) and 4.8 mg (yield=12.5%) of the minor product (R_t=35.60 min; ESIMS, *m/z* 903 for $[M+H]^+$; mp 193–195°C).

3.8. Preparation of cells

J774.A1, murine monocyte/macrophage cells were grown in adhesion on Petri dishes and maintained with Dulbecco's modified Eagle's medium (DMEM) at 37°C in DMEM supplemented with 10% foetal calf serum (FCS), 25 mM HEPES, 2 mM glutamine, 100 u/ml penicillin and 100 μg/ml streptomycin. WEHI-164, murine fibrosarcoma cells were maintained in adhesion on Petri dishes with DMEM supplemented with 10% heat-inactivated FCS, 25 mM HEPES, 100 u/ml penicillin and 100 μg/ml streptomycin. HEK-293, human epithelial kidney cells were maintained and grown in adhesion on Petri dishes with DMEM supplemented with 10% FCS, 25 mM HEPES, 100 u/ml penicillin and 100 μg/ml streptomycin. All reagents for cell culture were from Hy-Clone (Euroclone, Paignton Devon, UK); [3-(4,5-dimethylthiazol-2-yl)-2,5-phenyl-2*H*-tetrazolium bromide] (MTT) and 6-mercaptopurine (6-MP) were from Sigma Chemicals (Milan, Italy).

3.9. Antiproliferative assay

J774.A1, WEHI-164 and HEK-293 (3.5×10^4 cells) were plated on 96-well microtiter plates and allowed to adhere at 37°C in 5% CO_2 and 95% air for 2 h.

Thereafter, the medium was replaced with 50 μL of fresh medium and a 75 μL aliquot of 1:4 serial dilution of each test compound was added and then the cells incubated for 72 h. In some experiments, serial dilutions of 6-MP were added. The cell viability was assessed through an MTT conversion assay.[14,15] Briefly, 25 μL of MTT (5 mg/ml) were added and the cells were incubated for an additional 3 h. Thereafter, cells were lysed and the dark blue crystals solubilised with 100 μL of a solution containing 50% (v:v) *N*,*N*-dimethylformamide, 20% (w:v) SDS with an adjusted pH of 4.5.[16] The optical density (OD) of each well was measured with a microplate spectrophotometer (Titertek Multiskan MCC/340) equipped with a 620 nm filter. The viability of each cell line in response to treatment with tested compounds and 6-MP was calculated as: % dead cells=100−(OD treated/OD control)×100. Table 5 shows the results obtained expressed as an IC_{50} value (μM), the concentration that inhibited cell growth by 50% as compared to the control.

References

1. Faulkner, D. J. *Nat. Prod. Rep.* **1991**, 97–147. Bewley, A.; Faulkner, D. J. *Nat. Prod. Rep.* **1997**.
2. Brown, A. P.; Morrissey, R. L.; Faircloth, G. T.; Levine, B. S. *Cancer Chemother. Pharmacol.* **2002**, *50*, 333–340. Rinehart, K. L.; Gloer, J. B.; Hughes, R. G.; Renis, H. E.; McGovren, J. P.; Swynenberg, E. B.; Stringfellow, D. A.; Kuentzel, S. L.; Li, L. H. *Science* **1981**, *212*, 933–935. Jiang, T. L.; Liu, R. H.; Salmon, S. E. *Cancer Chemother. Pharmacol.* **1983**, *11*, 1–4. Tada, H.; Tozyo, T.; Terui, Y.; Hayashi, F. *Chem. Lett.* **1992**, *3*, 431–434.
3. Pettit, G. R.; Clewlow, P. J.; Dufresne, C.; Doubek, D. L.; Cerny, R. L.; Rutzler, K. *Can. J. Chem.* **1990**, *68*, 708–711. Pettit, G. R.; Srirangam, J. K.; Herald, D. L.; Erickson, K. L.; Doubek, D. L.; Schmidt, J. M.; Tackett, L. P.; Bakus, G. J. *J. Org. Chem.* **1992**, *57*, 7217–7220. Pettit, G. R.; Herald, C. L.; Boyd, M. R.; Leet, J. E.; Dufresne, C.; Doubek, D. L.; Schimdt, J. M.; Cerny, R. L.; Hooper, J. N. A.; Rutzler, K. C. *J. Med. Chem.* **1991**, *34*, 3339–3340. Kobayashi, J.; Tsuda, M.; Nakamura, T. i.; Mikami, Y.; Shigemori, H. *Tetrahedron* **1993**, *49*, 2391–2402. Tsuda, M.; Shigemori, H.; Mikami, Y.; Kobayashi, J. *Tetrahedron* **1993**, *49*, 6785–6796. Tsuda, M.; Sasaki, T.; Kobayashi, J. *Tetrahedron* **1994**, *49*, 4667–4680.
4. Morita, H.; Kobata, H.; Takeya, K.; Itokawa, H. *Tetrahedron Lett.* **1994**, *35*, 3563–3564. Morita, H.; Gonda, A.; Takeya, K.; Itokawa, H. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 767–770. Morita, H.; Kayashita, T.; Shishido, A.; Takeya, K.; Itokawa, H.; Shiro, M. *Tetrahedron* **1996**, *52*, 1165–1176. Morita, H.; Kayashita, T.; Kobata, H.; Gonda, A.; Takeya, K.; Itokawa, H. *Tetrahedron* **1994**, *50*, 6797–6804.
5. Verbist, J. F.; Minale, L.; Franz, G.; Sodano, G.; Riccio, R.; Chermann, J. C. et al. Third European Marine Science and Technology Conference (Lisbon, 23–27 May 1998), Vol. III Project Synopses, pp 1215–1229..
6. Napolitano, A.; Bruno, I.; Rovero, P.; Lucas, R.; Payà Peris, M.; Gomez-Paloma, L.; Riccio, R. *Tetrahedron* **2001**, *57*, 6249–6255.
7. Napolitano, A.; Bruno, I.; Rovero, P.; Lucas, R.; Payà Peris, M.; Gomez-Paloma, L.; Riccio, R. *J. Pept. Sci.* **2002**, *8*, 407–417.
8. Morita, H.; Shishido, A.; Kayashita, T.; Shimomura, M.; Takeya, K.; Itokawa, H. *Chem. Lett.* **1994**, 2415–2418. Morita, H.; Kayashita, T.; Shimomura, M.; Takeya, K.; Itokawa, H. *J. Nat. Prod.* **1996**, *59*, 280–282.
9. Pettit, G. R.; Cichacz, Z.; Barkoczy, J.; Dorsaz, A. C.; Herald, D. L.; Williams, M. D.; Doubek, D. L.; Schmidt, J. M.; Tackett, L. P.; Brune, D. C.; Cerny, R. L.; Hooper, J. N. A.; Bakus, G. *J. Nat. Prod.* **1993**, *56*, 260–267. Pettit, G. R.; Tan, R.; Ichihara, Y.; Williams, M. D.; Doubek, D. L.; Tackett, L. P.; Schmidt, J. M.; Cerny, R. L.; Boyd, M. R.; Hooper, J. N. A. *J. Nat. Prod.* **1995**, *58*, 961–965.
10. Pettit, G. R.; Cichacz, Z.; Barkoczy, J.; Dorsaz, A. C.; Herald, D. L.; Williams, M. D.; Doubek, D. L.; Schmidt, J. M.;

Tackett, L. P.; et al. *J. Nat. Prod.* **1993**, *56*, 253–260. Randazzo, A.; Dal Piaz, F.; Orrù, S.; Debitus, C.; Roussakis, C.; Pucci, P.; Gomez-Paloma, L. *Eur. J. Org. Chem.* **1998**, 2659–2665.
11. Pettit, G. R.; Toki, B. E.; Xu, J. P.; Brune, D. C. *J. Nat. Prod.* **2000**, *63*, 22–28.
12. Pettit, G. R.; Lippert, J. W., III; Taylor, S. R.; Tan, R.; Williams, M. D. *J. Nat. Prod.* **2001**, *64*, 883–891.
13. Tabudravu, J. N.; Jaspars, M.; Morris, L. A.; Kettenes-Van Den Bosch, J. J.; Smith, N. *J. Org. Chem.* **2002**, *67*, 8593–8601.
14. Setsuo, S.; Shgeya, S.; Norihiro, T.; Yiochi, N.; Keishi, N.; Madoka, I.; Isao, I. *Chem Pharm. Bull.* **1990**, *38*, 411–414.
15. Kinjo, J.; Uemura, H.; Nakamura, M.; Nohara, T. *Chem. Pharm. Bull.* **1994**, *42*, 1339–1341.
16. Mosman, T. *J. Immunol. Methods* **1983**, *65*, 55–63.