Synthesis of Phakellistatin 11: A Micronesia (Chuuk) Marine Sponge Cyclooctapeptide[1]

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The cyclic octapeptide phakellistatin 11 (**1**), a constituent of The Federated States of Micronesia (Chuuk) marine sponge *Phakellia* sp., was synthesized using solid-phase techniques. An initial solution-phase synthesis proved to be inadequate owing to spontaneous deprotection of the Fmoc group at the heptapeptide stage. Using the PAL resin attachment and proceeding from Fmoc-Glu-α-allyl ester, linear elongation of the octapeptide was performed until the final unit Pro was added. The allyl ester was removed using Pd⁰[P(C₆H₅)₃]₄. Cleavage of the final Fmoc group and cyclization with PyAOP provided phakellistatin 11 (**1**) in 17% overall yield. The synthetic specimen of phakellistatin 11 (**1**) was found to be chemically but not biologically (cancer cell lines) identical to the natural product. The result suggested a conformational difference or more likely the presence of a trace amount of a highly active antineoplastic agent that binds noncovalently to the natural cyclic octapeptide **1**.

From 1965 to 1966 onward[2] our group has pursued the isolation, structural elucidation, and synthesis of an array of marine animal constituents where certain members have proven to be remarkably active and clinically promising anticancer drugs.[3] The marine invertebrate phylum Porifera has been of continuous interest to us as a source of structurally unique and potentially important anticancer agents such as the spongistatins[4] and the halichondrins/halistatins.[5] Over fifty years ago the pioneering research of Bergmann[6] with marine sponge nucleoside constituents had already provided the scientific foundation for the now well-known anticancer and antiviral drugs ARA-C and ARA-A. Recent advances in this very productive area directed at sponge peptide constituents include the discovery by Boyd and colleagues[7] of a new and promising hemiasterlin (C) with very potent human cancer cell line inhibitory activity and the murine P388 lymphocytic leukemia active cyclodepsipeptides thiomycalolides A and B by the Fusetani group.[8] Other new marine sponge cyclodepsipeptides with biological activity (cytotoxic and/or antifungal or under study) include cyclolithistide A,[9a] keramides K and L,[9b] microsclerodermins C−E,[9c] geodiamolides H and I,[9d] and theopalanamide.[9e]

Over the past 15 years we have been isolating and elucidating the structures of marine sponge cyclic peptides. In the course of the research we have been examining the cancer cell line activity of these peptides versus the later synthetic specimens with focus on those from Federated States of Micronesia (Chuuk) marine sponge *Phakellia* sp. (phakellistatins 1−12). The present investigation was directed at phakellistatin 11 (**1**). Previously the natural cycloheptapeptides phakellistatin 2 (**2**)[10] and 5 (**3**)[11] exhibited anticancer cell line (P388 leukemia) activity with ED₅₀ 0.34 and 0.23 μg/mL, respectively. Subsequently the synthetic products were found to be inactive, exhibiting ED₅₀ values of 24 and >10 μg/mL.[12,13] Similar results were obtained with three of the axinastatins (**4a**−**c**),[14] isolated from the marine sponge *Axinella* sp., stylostatin 1 (**5**),[15] isolated from the marine sponge *Stylotella aurantium*, and stylopeptide 1 (**6**), isolated from both *Phakellia costata* and *Stylotella aurantium*.[16] Interestingly, both natural specimens of stylopeptide 1 (**6**) were found to be identical chemically (NMR, TLC, HPLC, and mp), but their ability
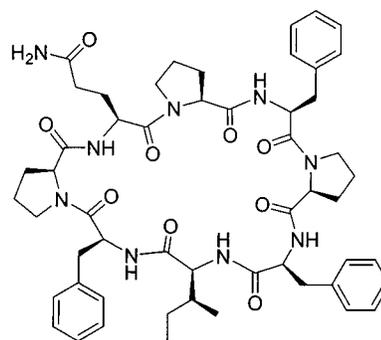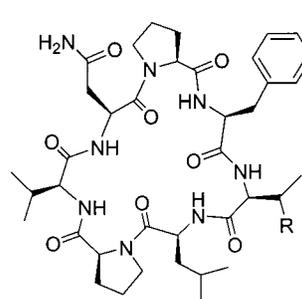

1, Phakellistatin 11, *cyclo*-(Ile-Phe-Pro-Gln-Pro-Phe-Pro-Phe)
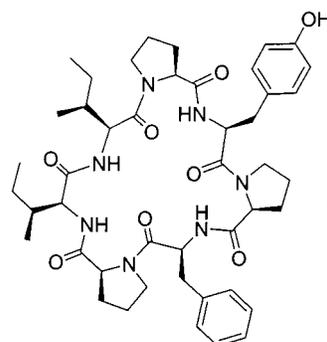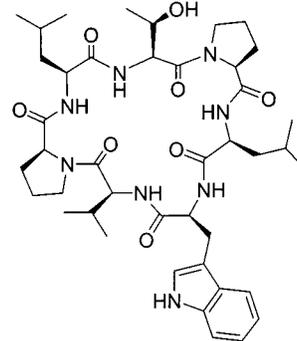

2, Phakellistatin 2
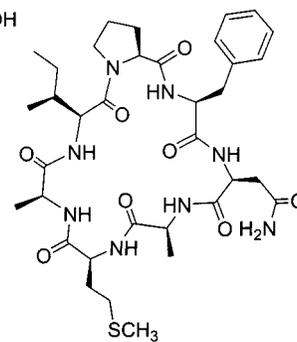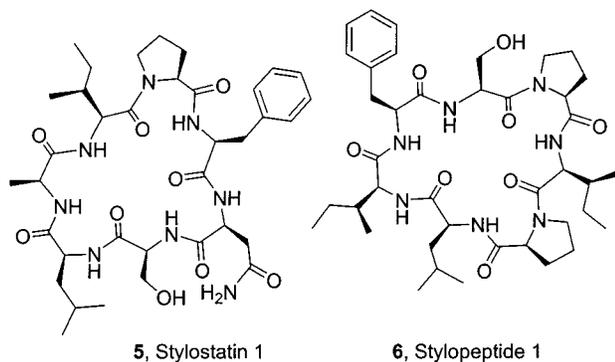

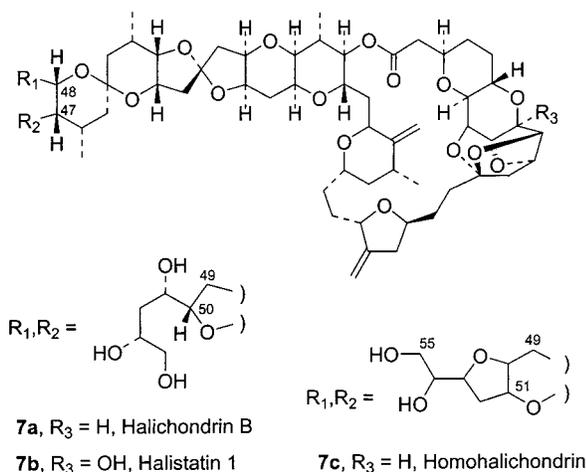**3**, Phakellistatin 5

4a, Axinastatin 2 (R = CH₃)

4b, Axinastatin 3 (R = CH₂CH₃)

4c, Axinastatin 4

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5, Stylostatin 1   **6**, Stylopeptide 1

to inhibit growth of the P388 lymphocytic leukemia cell line differed by more than 10-fold (ED$_{50}$ ~10 vs 0.1 μg/mL, respectively).[16] That result suggested the cell growth inhibitory specimens of stylopeptide 1 from *P. costata* might be transporting (by complex or other means), or are simply contaminated by, one or more of the extraordinarily active halistatin-type (cf., **7**)[5a] antineoplastic agents in a trace amount detectable only by biological means.[16] Furthermore, during the isolation of halichondrin B (**7a**)[5a] one of the major difficulties encountered was the separation of trace (~10^{-6}%) cyclic peptide constituents such as the heptapeptides axinastatins **4a**,**b**.[14]

7a, R$_3$ = H, Halichondrin B

7b, R$_3$ = OH, Halistatin 1

7c, R$_3$ = H, Homohalichondrin

In 1986−1987 the yellow-orange sponge *Phakellia* sp. (class Demospongiae, order Axinellida) was collected by scuba (500 kg wet wt, at depths of 25 to 40 m), and we isolated 34 mg of phakellistatin 11 (**1**) (6.8 × 10^{-6}%)[17] along with phakellistatin 10 and the already known, from the closely related *P. costata*, phakellistatins 7−9.[18] Cyclooctapeptide **1** was found to significantly inhibit growth of the murine P388 lymphocytic leukemia (ED$_{50}$ 0.20 μg/mL).[17] Furthermore phakellistatin 11 (**1**) was tested (10^{-5} M high test concentration; log$_{10}$ dilutions) in the NCI's 60-cell line human tumor in vitro screen,[19a−c] and a variety of data analyses[19b−d] were performed.[17] Phakellistatin 11 (**1**) gave overall panel-averaged GI$_{50}$ concentrations of (1.32 ± 0.49) × 10^{-7} M.[17] TGI-COMPARE correlation analyses[19d] of the differential cytotoxicity of cyclic octapeptide **1** showed Pearson correlation coefficients of 0.83, 0.96, and 0.87 with profiles of phakellistatin 4, phakellistatin 10, and vinblastine, respectively.[17] In a continuing effort to explore the biological activity of cyclic peptides isolated from *Phakellia*

sp., the total synthesis of phakellistatin 11 (**1**) was undertaken.

Results and Discussion

An initial investigation of a synthetic approach to phakellistatin 11 (**1**) involved the solution-phase sequential amino acid addition and *N*-Fmoc/*tert*-butyl protection strategy[20] similar to one we used to obtain both axinastatins **4a** and **4b** and stylopeptide **6**.[14,16a] The *tert*-butyl ester[21] for *C*-terminal protection was selected because they do not readily undergo nucleophilic attack and are therefore useful to minimize diketopiperzine (DKP) formation.[21d,22] For *N*-terminal blocking the 9-fluorenylmethoxycarbonyl (Fmoc) group[21d,23] was chosen, as cleavage is easily accomplished with diethylamine[22a,b] or tris(2-aminoethyl)-amine (TAEA).[24] Formation of peptide bonds was performed in either dichloromethane (DCM) or dimethylformamide (DMF) utilizing diethylphosphorocyanidate (DEPC)[25] (**8**) with diisopropylethylamine (DIEA) as the base. Solu-

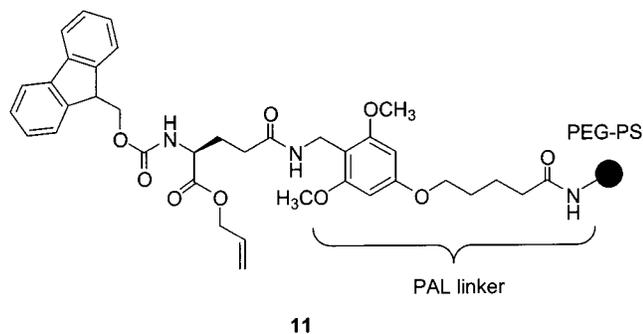**8**, DEPC   **9**, HATU   **10**, PyAOP
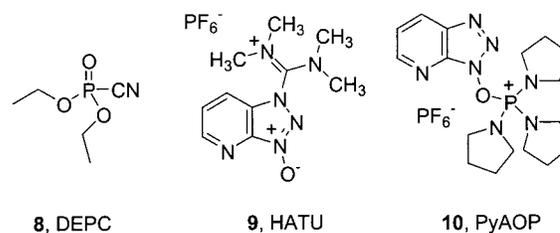
tion-phase synthesis of phakellistatin 11 (**1**) proceeded without problem until the heptapeptide stage (**18**). While most of the *N*-Fmoc peptide *tert*-butyl esters seem to lose the Fmoc group over time, heptapeptide **18** seemed particularly fragile since dibenzylfulvene (DBF) was observed by TLC after a few days and the insoluble DBF polymer formed (Scheme 1).

As a result of the unstable nature of heptapeptide **18**, isolated from solution-phase coupling, a change in the synthetic strategy was adopted in which a three-dimensional orthogonal (Fmoc/*tert*-butyl/allyl) solid-phase approach was attempted.[26−28] The mild conditions used to remove the allyl groups are compatible with classical Fmoc/*t*Bu methods for solid-phase peptide synthesis (SPPS).[26,28] The α-allyl ester serves as the third dimension of protection and is easily cleaved without disrupting the other protecting groups by using Pd^0[P(C$_6$H$_5$)$_3$]$_4$ under neutral conditions.[26,28] Allyl-based protecting groups have been used extensively in organic synthesis and have recently been applied to nucleotide, carbohydrate, and peptide syntheses.[27] The use of allyl protection for the α-carboxyl group of glutamic acid in combination with classical Fmoc and *t*Bu strategy allowed for synthesis of the cyclic octapeptide directly on the resin. In addition, by choosing the correct solid-phase resin, in this case the PAL polymer, cleavage of the bound peptide with trifluoroacetic acid (TFA) resulted in formation of the corresponding amide of glutamic acid (glutamine) required for phakellistatin 11 (**1**). Once the glutamic acid was successfully anchored to the resin with the *C*-terminus protected with the allyl group and the amino portion protected with the Fmoc group, the carbamate was removed and stepwise elongation performed until finally both allyl and terminal Fmoc groups were removed and cyclization effected (Scheme 1).

Initially, *N*-Fmoc-L-Glu-α-*O*-allyl was anchored to the deprotected amino group of the 5-(4-Fmoc-aminomethyl-

3,5-dimethoxyphenoxy)valeric acid-bonded (PAL) resin through the glutamic acid β-carboxyl group (**11**). Solid-
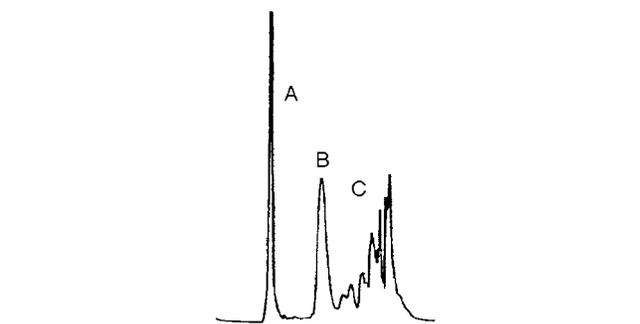

11

Figure 1. HPLC chromatogram of the crude peptide mixture: (A) MeOH; (B) phakellistatin 11 (**1**); (C) undesired products; see Experimental Section for solvent gradient system.
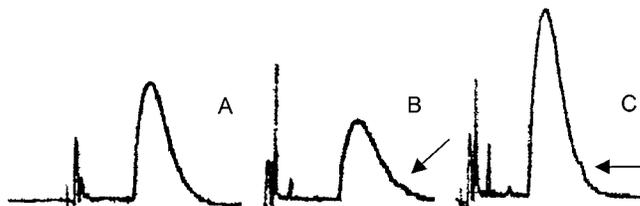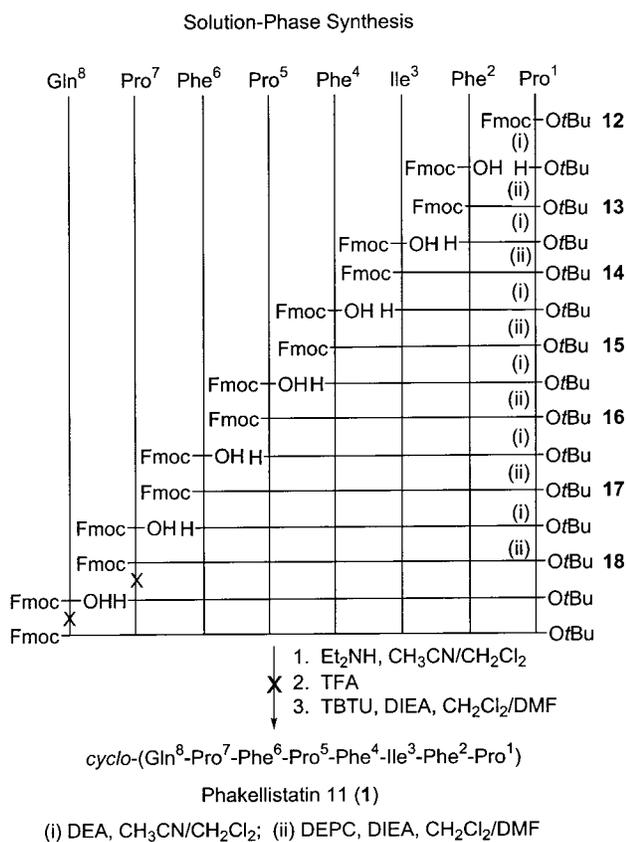

Figure 2. HPLC chromatogram of phakellistatin 11 (**1**): (A) synthetic; (B) natural; (C) mixture; see experimental for solvent system. Arrow points to a conformer or possible impurity in the natural product.

phase peptide synthesis of phakellistatin 11 (**1**) employed Fmoc deprotection with 20% piperidine[29] followed by peptide-bond formation using HATU (**9**).[30] After addition of the Pro unit, the Fmoc amine protecting group was removed, and the allyl ester was cleaved by means of the Pd^0 reagent. The cyclization reaction on the resin was performed with PyAOP (**10**) in order to minimize possible guanidinium formation that may occur in the potentially slow cyclization coupling step.[30] Phakellistatin 11 (**1**) bound to the resin was then cleaved from the polymer attachment with 90% TFA in the presence of radical scavengers. Isolation of the resulting product was performed using HPLC (Figure 1).

Comparison of the synthetic and natural specimens of phakellistatin 11 (**1**) illustrated that the two compounds were identical chemically but not biologically. The synthetic sample of phakellistatin 11 (**1**) was shown to have a P388 leukemia ED$_{50}$ value of >10 μg/mL, whereas the natural product ED$_{50}$ was 0.2 μg/mL. In addition, the optical

rotation value of the synthetic sample was significantly less negative ($[\alpha]_D^{25}$ $-130°$ vs $-163°$), which points to the possibility of the natural product being associated with a chemically undetected contaminant or complex or representing a different conformer during cancer cell line evaluation. HPLC analyses of the synthetic, natural, and mixture samples are presented in Figure 2. Each specimen was shown to have the same retention time.

Scheme 1. Solid-Phase Synthesis and Partial Solution-Phase Synthesis of Phakellistatin 11 (**1**)

Solution-Phase Synthesis

cyclo-(Gln^8-Pro^7-Phe^6-Pro^5-Phe^4-Ile^3-Phe^2-Pro^1)

Phakellistatin 11 (**1**)

(i) DEA, CH$_3$CN/CH$_2$Cl$_2$; (ii) DEPC, DIEA, CH$_2$Cl$_2$/DMF

1. Et$_2$NH, CH$_3$CN/CH$_2$Cl$_2$
2. TFA
3. TBTU, DIEA, CH$_2$Cl$_2$/DMF

Solid-Phase Synthesis

(i) 20% piperidine deprotection; HATU coupling; (ii) 20% piperidine and Pd^0[P(C$_6$H$_5$)$_3$]$_4$ deprotections; (iii) PyAOP cyclization; end of automated synthesis; (iv) TFA, 1,2-ethanediol, thioanisole, anisole, CH$_2$Cl$_2$.
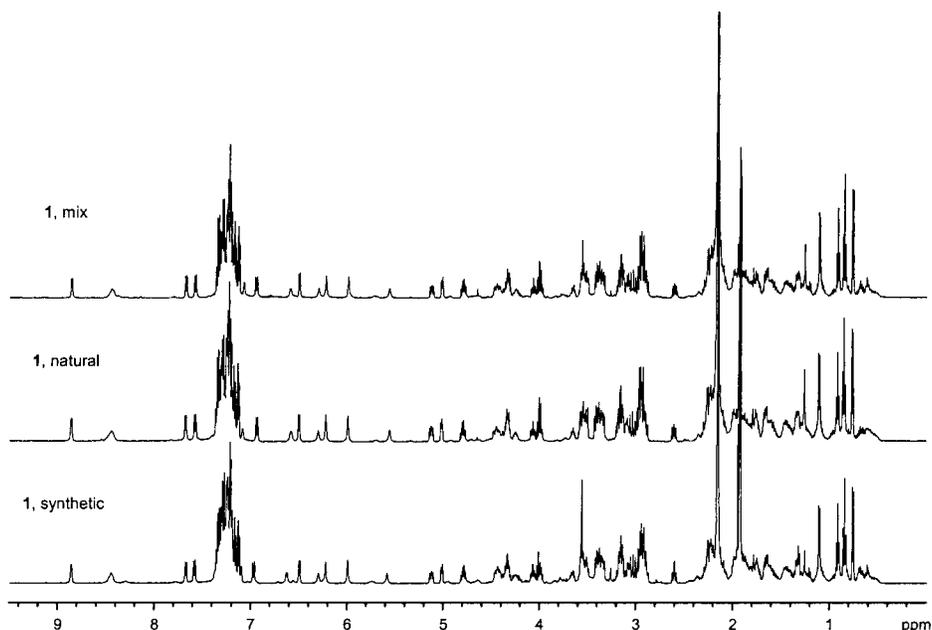
Note: Glu yields Gln upon cleavage.

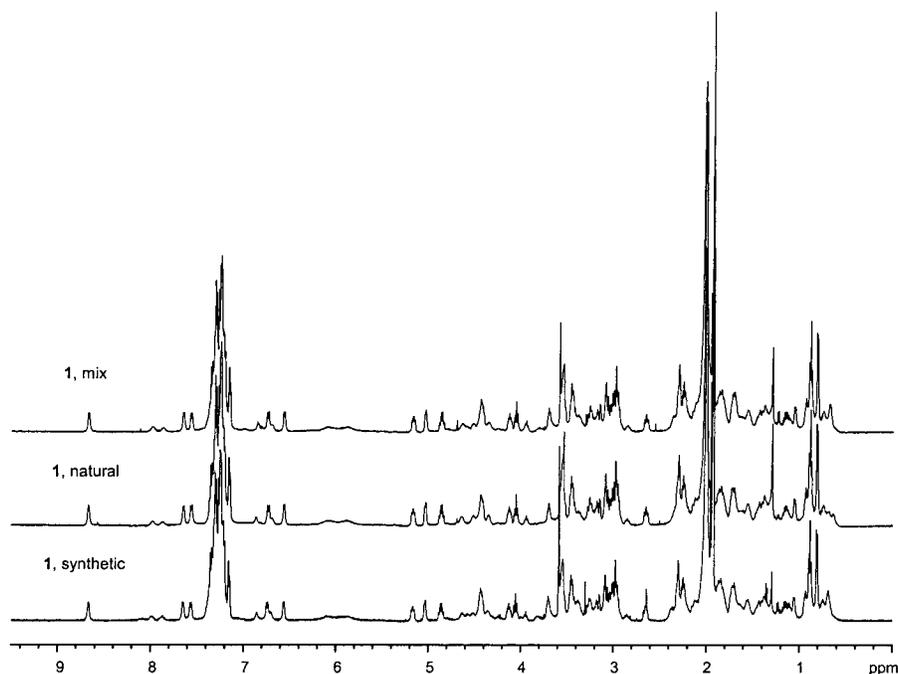**Figure 3.** ^{1}H NMR overlay of phakellistatin 11 (**1**) at 25 °C.

Figure 4. ^{1}H NMR overlay comparisons of phakellistatin 11 (**1**) at 70 °C.

Interestingly, the HPLC of the natural product exhibited a small shoulder peak (possible conformer or impurity) not displayed by the synthetic sample. In addition, phakellistatin 11 (**1**) was found to exist as two conformers in solution. The ratio of conformers is dependent on the solvent, temperature, and concentration of sample. Three proline amide bonds are present in phakellistatin 11 (**1**), which have an ability to give rise to conformational changes owing to the low rotational energy barrier that needs to be overcome. Initial ^{1}H NMR studies performed in deuterated acetonitrile (Figure 3) showed the presence of two conformers, which prompted us to conduct an NMR experiment in which the temperature was raised from 25 °C to 70 °C in an effort to force the conformational equilibrium to one conformer. Figure 4 illustrates the ^{1}H NMR spectra collected at 70 °C for the synthetic, natural, and mixture specimens of phakellistatin 11 (**1**). The data suggest that,

upon a rise in temperature, the cyclic octapeptide adopts almost exclusively one spatial orientation. Specifically diagnostic of this fact is the ^{1}H NMR spectrum of the Ile side-chain methyl groups for the mixture specimen (Figure 5). Over the course of the temperature experiment one conformer obviously is more stable relative to the other. As a final check of the correct ^{1}H NMR spectrum of the synthetic versus the natural specimens of phakellistatin 11 (**1**), spectra were taken in deuterated DMSO and the peaks were shown to be identical with those reported in our earlier report.[17]

To study the contamination of cyclic peptides isolated from marine natural products with biologically potent compounds, some detailed experiments were undertaken. Synthetic stylopeptide 1 (**6**) was mixed with diminishing small quantities of homohalichondrin (**7c**). The ^{1}H NMR and biological data (P388 cell line) are illustrated in Figure
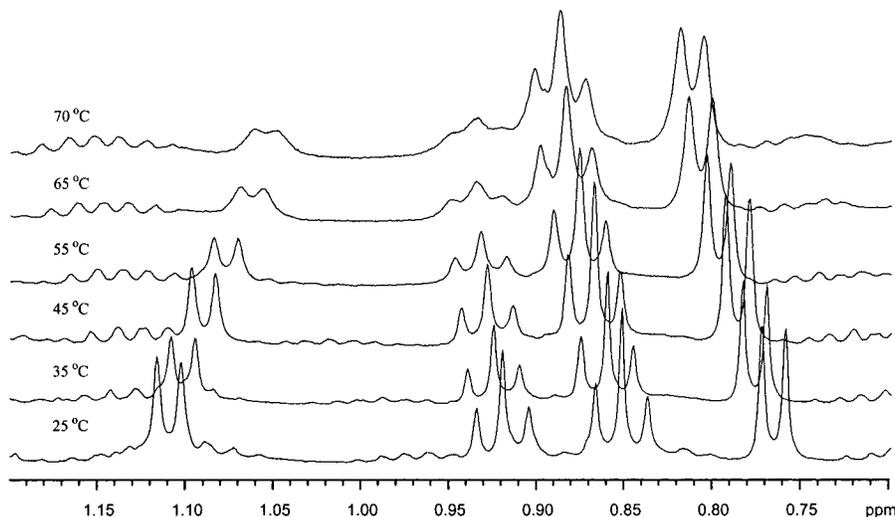
Figure 5. Temperature-dependent ^{1}H NMR spectra for the isoleucine side chain of phakellistatin 11 (**1**).
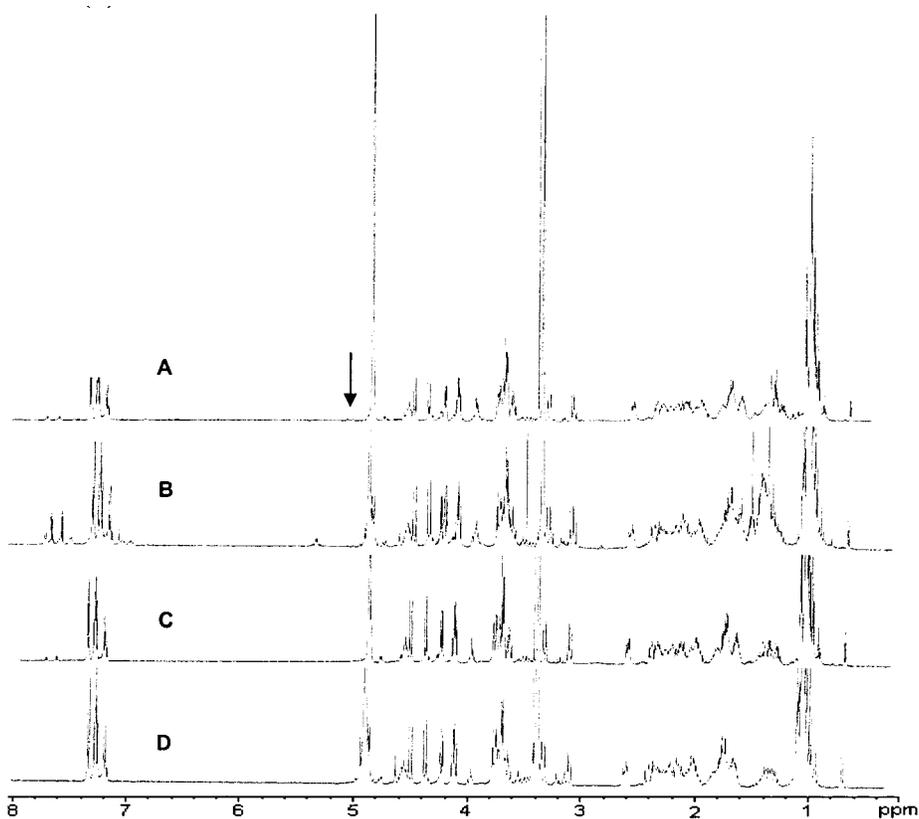

Figure 6. ^{1}H NMR biological activity study of stylopeptide contaminated with homohalichondrin (see Table 1 and text for explanation of data).

Table 1. NMR Biological Activity Study of Stylopeptide (1 mg) Contaminated with Homohalichondrin (see Figure 6 for NMR data)

experiment	NMR	homohalichondrin (μg)	wt %	P388 cell line (ED$_{50}$ μg/mL)
1	A	100	10	0.005
2	B	50	5	0.032
3	C	10	1	0.041
4		1.0	0.1	0.51
5		0.1	0.01	4.5
6	D			6.0

6 and Table 1, respectively. The results show that when 100 μg of homohalichondrin (**7c**) is added to a 1 mg sample of synthetic stylopeptide (**6**), the impurity is almost non-detectable by ^{1}H NMR (Figure 6, A) yet biologically the

specimen becomes extremely potent (P388 cell line ED$_{50}$ 0.005 μg/mL; Table 1). The remaining data illustrate the results of chemically undetectable amounts of the anticancer drug that are readily detected biologically using the murine (P388) leukemia cell line.

The results of this and our previous research in this area illustrate that certain natural cyclic peptides are able to complex with or otherwise carry trace (too small for usual NMR and chromatographic detection) amounts of exceptionally potent antineoplastic compounds such as the sponge halichondrins/halistatins.[14] Alternatively, albeit much less likely, there may be a conformational explanation under conditions of the biological experiments. We are now currently engaged in attempting to isolate these trace contaminants. Clearly, it is important to confirm the

biological activity of such cyclic peptides by means of total syntheses.

Experimental Section

General Experimental Procedures. All solvents were redistilled except for acetonitrile (HPLC grade, EM Science) and DMF (anhydrous, Aldrich). All coupling reactions were conducted under argon (solution-phase) or nitrogen (solid-phase). The L-proline *tert*-butyl ester, diethyl phosphorocyanidate (DEPC, 93%), diisopropylethylamine (DIEA), diethylamine (Et$_2$NH), and trifluoroacetic acid (TFA) were used as received from Sigma-Aldrich Co. The peptide-bond-forming reagents used in the solid-phase synthesis, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-yl-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU) and 7-azabenzotriazol-1-yloxy)tris(pyrrolidino)phosphonium hexafluorophosphate (PyAOP), were purchased from PerSeptive Biosystems. The *N*-Fmoc-L-amino acids were supplied by NovaBiochem or the Sigma-Aldrich Co. and used as received except for the *N*-Fmoc-L-Glu-α-allyl ester, which was purchased from PerSeptive Biosystems. Flash column chromatography employed 230–400 mesh, 0.040–0.063 mm silica gel 60 (Merck). The solid-phase syntheses were performed with a 9050 Plus PepSynthesizer following procedures recommended by PerSeptive Biosystems Bioresearch Products. The PAL resin was also obtained from PerSeptive BioSystems. Organic extracts of aqueous solutions were dried over sodium sulfate. Solvents were removed using a rotary evaporator.

Melting points were determined using an Electrothermal 9100 unit and are uncorrected. Thin-layer chromatography was performed using silica gel GHLF Uniplates (Analtech), and the plates were visualized by UV light and/or ceric sulfate–sulfuric acid (by heating 2–3 min). All compounds were visible under short-wave UV light. Optical rotation data were collected using a Perkin-Elmer 241 polarimeter (1 mL, 1 dm cell) at the sodium D line (589 nm at indicated temperatures). IR spectra were obtained on a Nicolet FT-IR MX-1 using a thin film of compound evaporated from DCM on a NaC1 plate. ^{1}H NMR and ^{13}C NMR spectra were recorded employing Varian Gemini 300, Varian Unity 400, or Varian VXR-500S instruments using a deuterated solvent and were referenced to either TMS or the solvent. Analytical samples were dried in vacuo (Abderhalden over P$_2$O$_5$ at CH$_3$OH or water reflux temperatures for several hours). Elemental analyses were done by Galbraith Laboratories, Inc. (Knoxville, TN). Atmospheric pressure chemical ionization high-resolution mass spectrometry (ACPIHRMS) was recorded using a LC-mate JEOL LCMS system. Fast-atom bombardment high-resolution mass spectroscopy (FABHRMS) was done at the Midwest Center for Mass Spectrometry, Department of Chemistry, University of Nebraska (Lincoln, NE 68588-0362).

General Deprotection Procedure. A solution of the *N*-Fmoc *tert*-butyl ester in 1:1 CH$_3$CN–Et$_2$NH (~0.2 M) was stirred at room temperature for 2 h. We avoided the use of DCM as solvent in the deprotection step because it was found to be a variable that adversely affected the yield of final product. Deprotection was observed to be complete by TLC after several minutes, but longer times were used to ensure maximum deprotection. Reaction of ninhydrin by mild heating of the TLC plate indicated the free amino *tert*-butyl ester near the origin. Solvents were removed by rotary evaporation for several minutes followed by evaporation of CH$_3$CN in portions (2 × 5 mL) to yield viscous amber syrups. Further removal of trace solvent was also done in vacuo (typically ~0.3 Torr, 30 min). The free amino *tert*-butyl ester thus obtained was used immediately in couplings without further purification.

Amino Acid Coupling Procedure. A solution of the crude amino *tert*-butyl ester in DCM (~0.5 M) containing DIEA (1 equiv) at room temperature was transferred slowly under positive argon pressure via cannula to a solution of *N*-Fmoc-amino acid (1 equiv) and DEPC (1 equiv) in DCM (~0.3 M) at −10 °C. (The calculated volume of 93% pure DEPC was added to give 1 equiv). Transfer was completed via cannula with

small portions of DCM, so the final concentration with respect to the amino *tert*-butyl ester was ~0.1–0.2 M. The reaction mixture was usually stirred under argon for 3–4 h between −10 and 0 °C. TLC monitoring showed dibenzofulvene (DBF) byproduct(s) and the desired *N*-Fmoc *tert*-butyl ester.

***N*-Fmoc-Pro-O-Bu^t (12).** A 500 mL pressure bottle was charged with a magnetic stirrer and *N*-Fmoc-Pro (23.8 g, 70.6 mmol, and 150 mL of DCM) and then sealed with a rubber septum secured tightly with wire. The bottle was cooled in an *i*-PrOH/dry ice bath while condensed isobutylene (70 mL) was transferred via cannula into the bottle held in the cold bath. After about 20 mL of isobutylene was added, it was followed by H$_2$SO$_4$ (0.7 mL), and the addition of isobutylene was continued. The cold reaction mixture was stirred for 2 h. After stirring for an additional 16 h at room temperature, the bottle was cooled in an *i*-PrOH/dry ice bath and opened carefully. The reaction mixture was allowed to degas fully by being stirred for several minutes open to the air at room temperature. Saturated aqueous NaHCO$_3$ (100 mL) was carefully added to the reaction mixture, and it was stirred for 2 h at room temperature. The pH of the aqueous layer was about 8. (Addition of several milliliters of water removed an emulsion that sometimes formed in the neutralization.) The aqueous layer was washed with DCM (50 mL). The combined DCM extract was washed with saturated aqueous NaHCO$_3$ (3 × 20 mL), water (3 × 20 mL), and saturated aqueous NaCl (20 mL). The organic layer was dried (MgSO$_4$) and filtered. Evaporation of the solvent gave 29.1 g of syrup, from which 23.9 g of a crude powder was precipitated using hexane/minimal EtOAc (86% yield after one crop). TLC (1:1 EtOAc–hexane) of this powder showed two spots: *N*-Fmoc-Pro-O-Bu^t, R_f 0.6, and an unidentified minor impurity, R_f 0.45. (Crystallization was precluded in the presence of this impurity, which was always present from the acid-catalyzed *tert*-butyl esterification of *N*-Fmoc-Pro, but was obtained in larger amounts when excess TsOH was used instead of catalytic H$_2$SO$_4$.) Silica gel chromatography (1:1:1 hexane–EtOAc–DCM eluent) of the crude powder gave fractions that recrystallized readily (from hexane–minimal EtOAc). This sequence of precipitation/chromatography/recrystallization gave colorless crystals in 84% overall isolated yield from several reactions (38.33 g of *N*-Fmoc-Pro was converted to 37.74 g of *N*-Fmoc-Pro-O-Bu^t): mp 108–109 °C; R_f 0.63 (EtOAc); $[\alpha]^{25}_D$ −80.6° (*c* 1.0, CHCl$_3$); UV λ_{max} nm, (log ε) 235 (3.8); 265 (4.3); 289 (3.7); 300 (3.8); IR (NaCl thin film) ν_{max} cm^{-1} 3070, 2976, 2882, 1740, 1707, 1451, 1416, 1350, 1154, 1119, 1090, 741; ^{1}H NMR (500 MHz, CDCl$_3$) conformers δ 1.44, 1.47 (2S, 9H total), 1.88, 2.10 (2m, 3H), 2.24 (m, 1H), 3.53 (m, 1H), 3.66 (m, 1H), 4.28 (m, 1H), 4.23, 4.32 (2m, 2H), 4.44 (m, 1H), 7.32 (t, *J* = 6.9, 2H), 7.40 (t, *J* = 6.9, 2H), 7.64 (m, 2H), 7.77 (d, *J* = 7.7, 2H); ^{13}C NMR (APT, 100.6 MHz, CDCl$_3$) conformers δ 23.2, 24.2, 27.9, 29.8, 31.0, 46.4, 46.9, 47.2, 47.3, 59.5, 59.8, 67.3, 67.5, 81.2, 81.3, 119.8, 125.1, 125,3, 127.0, 127.6, 141.1, 141.2, 143.6, 143.9, 144.1, 144.3, 154.7, 155.4, 171.7, 171.8; EIMS *m/z* 393 [M]$^+$; *anal.* C 73.73%, H 6.93%, N 3.56%, calcd for C$_{24}$H$_{27}$NO$_4$ C 73.22%, H 6.92%, N 3.56%.

***N*-Fmoc-Phe-Pro-O-Bu^t (13).** Using the above general procedures, *N*-Fmoc-Pro-O-Bu^t (5.00 g, 12.71 mmol) was *N*-deprotected in 2:2:1 CH$_3$CN–Et$_2$NH–DCM and coupled with *N*-Fmoc-Phe. After coupling, the mixture was kept at 0 °C for 16 h and then subjected to chromatography (2:1 hexane–EtOAc), which gave crystals (4.5 g, 66%) from hot EtOAc–hexane or hexane–DCM by slow evaporation.

Alternatively, a solution of pure Pro-O-Bu^t (5.0 g, 29.20 mmol) and DIEA in DCM at room temperature was transferred via cannula to a mixture of *N*-Fmoc-Phe-DEPC in DCM and DMF (10 mL, added first to *N*-Fmoc-Phe for better solubility) at −10 °C under argon. After coupling, chromatography (2:1 hexane–EtOAc) yielded 15.4 g (98%) of colorless crystals (from hexane–EtOAc). This method was repeated on the same scale to yield an additional 14.6 g (93%) of pure dipeptide: mp 142.5–143.5 °C; R_f 0.24–0.28 (2:1 hexane–EtOAc); $[\alpha]^{26}_D$ −40.2° (*c* 1.41, CHCl$_3$); IR (NaCl thin film) ν_{max} cm^{-1}: 3279, 3063, 3028, 3005, 2978, 2936, 2884, 1734, 1717, 1653, 1645, 1636, 1541, 1506, 1449, 1368, 1248, 1152, 1040, 758, 741, 700; ^{1}H NMR (300 MHz, CDCl$_3$) δ minor 1.37, 1.49

(2s, C(C*H$_3$*)$_3$, 9H); minor 1.47, 1.66, 1.97 (3 m, Pro γ-C*H$_2$*, 2H); 1.85, 2.15 (2m, Pro β-C*H$_2$*, 2H); 2.95 (dd, *J* = 13.7, 6.0, Phe β-C*H$_2$*, 1H); 3.17 (dd, *J* = 13.7, 6.0, Phe β-C*H$_2$*, 1H); minor 3.30, 3.37, minor 3.54, 3.66 (4 m, Pro δ-C*H$_2$*, 2H); 4.16, 4.23 (2m, Fmoc β-C*H$_2$*, 2H); 4.32 (m, Fmoc α-C*H*, 1H); 4.41 (m, Pro α-C*H*, 1H); Phe α-C*H*, 1H, minor 4.45 (m), 4.74 (q, *J* = 7.3); Phe N*H*, 1 H: 5.62 (d, *J* = 8.2), 5.69 (d, *J* = 9.3), 5.80 (d, *J* = 8.2); Phe Ar H: 7.20−7.33 (m, 5H); Fmoc Ar*H*: 7.20−7.33 (2H), 7.39 (2H), 7.55 (2H), 7.75 (2H); ^{13}C NMR (APT, 75.5 MHz, CDCl$_3$) δ minor 22.1, 24.8 (Pro γ-*C*H$_2$); 27.7 (Pro C(*C*H$_3$)$_3$); 28.0, minor 29.0, minor 30.5 (Pro β-*C*H$_2$); 38.6, minor 40.4 (Phe β-*C*H$_2$); minor 45.9, 46.9 (Pro δ-*C*H$_2$); 47.0 (Fmoc α-*C*H); 53.5, minor 54.5 (Phe α-*C*H); 59.7 (Pro α-*C*H); 67.0 (Fmoc β-*C*H$_2$); 81.3 (Pro *C*(CH$_3$)$_3$); 119.9, 125.1, 127.0, 127.6 (Fmoc Ar *C*H); 128.3, minor 128.5, minor 129.4, 129.8 (Phe Ar*C*H); 136.0, minor 136.3 (Phe Ar *C*q); 141.2, 143.8 (Fmoc Ar *C*q); 155.7 (urethane *C*O); 170.0 (Phe *C*O); 171.0 (Pro *C*O) FABHRMS *m/z* 541.2697 [M + H]$^+$ (calcd for C$_{33}$H$_{37}$N$_2$O$_5$, 541.2702); *anal.* C 73.79%, H 6.96%, N 5.08%, calcd for C$_{33}$H$_{36}$N$_2$O$_5$ C 73.31%, H 6.71%, N 5.18%.

N-Fmoc-Ile-Phe-Pro-O-Bu^t (14). By application of the general methods *N*-Fmoc-Phe-Pro-O-Bu^t (19 g, 35.07 mmol) was *N*-deprotected and coupled with *N*-Fmoc-Ile in DCM and DMF (12 mL, added first). Chromatography (5:4 hexane−EtOAc) yielded a foam in 90% yield (21.3 g): mp 72−74 °C (foam); *R$_f$* 0.19−0.22 (2:1 hexane−EtOAc); [α]^{27}$_D$ −41° (*c* 1.01, CHCl$_3$); IR (NaCl thin film) ν$_{max}$ cm$^{−1}$ 3295, 3061, 2969, 2936, 2878, 1724, 1640, 1537, 1451, 1368, 1240, 1154, 1034, 760, 740, 700; ^{13}C NMR (APT, 75.5 MHz, CDCl$_3$) δ 11.3 (Ile δ-*C*H$_3$); 15.4 (Ile γ-*C*H$_3$); minor 22.0, 24.4 (Pro γ-*C*H$_2$); 24.7 (Ile γ-*C*H$_2$); 27.9 (Pro C(*C*H$_3$)$_3$); 28.9, minor 30.6 (Pro β-*C*H$_2$); 37.4 (Ile β-*C*H); 38.2, minor 40.8 (Phe β-*C*H$_2$); minor 46.0, 46.9 (Pro δ-*C*H$_2$); 47.1 (Fmoc α-*C*H); 51.7 (Ile α-*C*H); 52.9 (Phe α-*C*H); 59.6, minor 60.3 (Pro α-*C*H); 66.9 (Fmoc β-*C*H$_2$); 81.3, minor 82.3 (Pro *C*(CH$_3$)$_3$); 119.9, 125.1, minor 126.8, 127.0, 127.6 (Fmoc Ar *C*H); 128.2, minor 128.4, minor 129.3, 129.7 (Phe Ar *C*H); 135.8, minor 136.1 (Phe Ar *C*q); 141.2, 143.8 (Fmoc Ar *C*q); 156.0 (urethane *C*O); 169.4, minor 169.8 (Phe *C*O); 170.5 (Ile *C*O); 170.9 (Pro *C*O); FABHRMS *m/z* 654.3542 [M + H]$^+$ (calcd for C$_{39}$H$_{48}$N$_3$O$_6$ 654.3543); *anal.* C 71.75%, H 7.57%, N 6.38%, calcd for C$_{39}$H$_{47}$N$_3$O$_6$ C 71.65%, H 7.25%, N 6.43%.

N-Fmoc-^{4}Phe-^{3}Ile-^{2}Phe-^{1}Pro-O-Bu^t (15). By application of the general procedures, *N*-Fmoc-Ile-Phe-Pro-O-Bu^t (23.7 g, 36.29 mmol) was *N*-deprotected and coupled with *N*-Fmoc-Phe in DCM and DMF (12 mL, added first for better solubility). After coupling, the mixture was stored 16 h at −25 °C. Chromatography (1:1 hexane−EtOAc) yielded a slightly impure foam, which required additional chromatography. Yield: 28.7 g (99%); *R$_f$* 0.27−0.30 (1:1 hexane−EtOAc); [α]^{25}$_D$ −39.6° (*c* 0.72, CHCl$_3$); ^{13}C NMR (APT, 75.5 MHz, CDCl$_3$) δ 11.4 (Ile δ-*C*H$_3$); minor 15.0, 15.2 (Ile γ-*C*H$_3$); minor 22.0, 24.7, minor 24.8 (Pro γ-*C*H$_2$, Ile γ-*C*H$_2$); 27.9 (Pro C(*C*H$_3$)$_3$); 29.0, minor 30.6 (Pro β-*C*H$_2$); 37.5 (Ile β-*C*H), minor 38.1, 38.3, minor 41.0 (^{2,4}Phe β-*C*H$_2$); minor 46.0, 46.9 (Pro δ-*C*H$_2$); 47.1 (Fmoc α-*C*H); 51.8 (Ile α-*C*H); minor 53.0, 56.1, minor 57.5, 57.7 (^{2,4}Phe α-*C*H); minor 59.4, 59.6 (Pro α-*C*H); 67.0 (Fmoc β-*C*H$_2$); 81.3, minor 82.3 (Pro *C*(CH$_3$)$_3$); 119.9, 125.1, minor 126.8, minor 126.9, 127.0, 127.6 (Fmoc Ar *C*H); 128.3, minor 128.5, 128.7, 129.3, 129.8 (^{2,4}Phe Ar *C*H); 136.0, minor 136.2, 136.5 (^{2,4}Phe Ar *C*q); 141.2, 143.8 (Fmoc Ar *C*q); 156.0 (urethane *C*O); 169.5, minor 169.8, 170.1, minor 170.4, minor 170.6, 170.7 (^{2,4}Phe *C*O, Ile *C*O); 171.0 (Pro *C*O); FABHRMS *m/z* 801.4204 [M + H]$^+$ (calcd for C$_{48}$H$_{57}$N$_4$O$_7$ 801.4227); *anal.* C 71.72%, H 7.36%, N 7.02, calcd for C$_{48}$H$_{56}$N$_4$C$_7$ C 71.98%, H 7.05%, N 6.99%.

N-Fmoc-^{5}Pro-^{4}Phe-^{3}Ile-^{2}Phe-^{1}Pro-O-Bu^t (16). With the general procedure (see above) *N*-Fmoc-Phe-Ile-Phe-Pro-O-Bu^t (28.8 g, 35.91 mmol) was *N*-deprotected and coupled with *N*-Fmoc-Pro. The reaction was stored for 5 days at −25 °C. Chromatography (4:1 hexane−EtOAc) yielded fractions from which a 29.2 g precipitate (90%) was obtained. The precipitate was very difficult to redissolve in EtOAc (used as eluent), but easily dissolved in DCM. Deprotection occurred slowly and unpredictably in some fractions, evidenced by the formation of insoluble material and verified by TLC: mp 132.5−

134.5 °C; *R$_f$* 0.23 (1:4 hexane−EtOAc), 0.46 (EtOAc); [α]^{26}$_D$ −77.6° (*c* 0.87, CHCl$_3$); ^{13}C NMR (APT, 75.5 MHz, CDCl$_3$) δ 11.3 (Ile δ-*C*H$_3$); 15.3 (Ile γ-*C*H$_3$); 22.0, 24.5, 24.7 (^{1,5}Pro γ-*C*H$_2$, Ile γ-*C*H$_2$); 27.9 (Pro C(*C*H$_3$)$_3$); 28.7, 29.0, minor 30.6 (^{1,5}Pro β-*C*H$_2$); 36.8 (Ile β-*C*H), 38.1, minor 40.9 (^{2,4}Phe β-*C*H$_2$); minor 46.0, 46.9 (^{1,5}Pro δ-*C*H$_2$); 47.1 (Fmoc α-*C*H); 51.7, 52.9, 54.2, 57.7, 58.2, 59.4, 59.7, 60.8 (^{1,5}Pro α-*C*H, ^{2,4}Phe α-*C*H, Ile α-*C*H); 67.8 (Fmoc β-*C*H$_2$); 81.1, minor 82.4 (Pro *C*(CH$_3$)$_3$); 120.0, 125.0, 126.6, 126.9, 127.1, 127.8 (Fmoc Ar *C*H); 128.3, 128.5, 128.6, 129.1, 129.4, 129.7 (^{2,4}Phe Ar *C*H); 136.5 (^{2,4}Phe Ar *C*q); 141.3, 143.6 (Fmoc Ar *C*q); 156.0 (urethane *C*O); 169.4, 169.8, 170.1, 170.4, 171.1, 171.8 (^{1,5}Pro *C*O, ^{2,4}Phe *C*O, Ile *C*O); FABHRMS *m/z* 898.4781 [M + H]$^+$ (calcd for C$_{53}$H$_{64}$N$_5$O$_8$ 898.4755); *anal.* C 70.86%, H 7.08%, N 7.89%, calcd for C$_{53}$H$_{63}$N$_5$O$_8$ C 70.88%, H 7.07%, N 7.80%.

N-Fmoc-^{6}Phe-^{5}Pro-^{4}Phe-^{3}Ile-^{2}Phe-^{1}Pro-O-Bu^t (17). Using the general procedures summarized above, *N*-Fmoc-Pro-Phe-Ile-Phe-Pro-O-Bu^t (29.0 g, 32.29 mmol) was *N*-deprotected and coupled with *N*-Fmoc-Phe in DCM and DMF (12 mL, added first for better solubility). For chromatography, EtOAc as eluent was too polar and required another chromatographic pass (1:4 hexane−EtOAc) due to poor resolution. Precipitation occurred with some difficulty from hexane−minimal EtOAc to yield 28.6 g (85%) of the hexapeptide: mp 118−121 °C; *R$_f$* 0.37 (4:1 EtOAc−hexane); [α]^{25}$_D$ −50.6° (*c* 0.16, CHCl$_3$); ^{13}C NMR (BB, 75.5 MHz, CDCl$_3$) δ 11.3 (Ile δ-*C*H$_3$); 15.3 (Ile γ-*C*H$_3$); 22.6, 24.7 (^{1,5}Pro γ-*C*H$_2$, Ile γ-*C*H$_2$); 27.9 (Pro C(*C*H$_3$)$_3$); 29.0 (^{1,5}Pro β-*C*H$_2$); 37.1 (Ile β-*C*H); 38.8 (^{2,4,6}Phe β-*C*H$_2$); 46.9 (^{1,5}Pro δ-*C*H$_2$); 47.0 (Fmoc α-*C*H); 51.8, 54.9, 58.0, 59.4, 59.6, 60.3 (^{1,5}Pro α-*C*H, ^{2,4,6}Phe α-*C*H, Ile α-*C*H); 67.0 (Fmoc β-*C*H$_2$); 81.3 (Pro *C*(CH$_3$)$_3$); 119.9, 125.2, 126.8, 127.0, 127.7 (Fmoc Ar *C*H); 128.4, 128.7, 128.9, 129.4, 129.7 (^{2,4,6}Phe Ar *C*H); 136.3 (^{2,4,6}Phe Ar *C*q); 141.2, 143.8 (Fmoc Ar *C*q); 155.9 (urethane *C*O); 169.7, 170.2, 170.5, 171.0, 171.2 (^{1,5}Pro *C*O, ^{2,4,6}Phe *C*O, Ile *C*O); FABHRMS *m/z* 1045.5453 [M + H]$^+$ (calcd for C$_{26}$H$_{73}$N$_6$O$_9$ 1045.5439); *anal.* C 70.84%, H 7.30%, N 7.77%, calcd for C$_{62}$H$_{72}$N$_6$O$_9$ C 71.24%, H 6.94%, N 8.04%.

N-Fmoc-^{7}Pro-^{6}Phe-^{5}Pro-^{4}Phe-^{3}Ile-^{2}Phe-^{1}Pro-O-Bu^t (18). By means of the above general procedures, *N*-Fmoc-Phe-Pro-Phe-Ile-Phe-Pro-O-Bu^t (27.8 g, 26.58 mmol) was *N*-deprotected and coupled with *N*-Fmoc-Pro. After coupling, the mixture was stored 2 days at −25 °C. Chromatography (20:1 EtOAc−*i*-PrOH) yielded 28 g (92%) of crude heptapeptide, which was subjected to another chromatographic separation owing to inadequate resolution and purity: the pure heptapeptide exhibited *R$_f$* 0.34 (20:1 EtOAc−*i*-PrOH), 0.21 (EtOAc); [α]^{25}$_D$ −71° (*c* 0.10, CHCl$_3$); ^{13}C NMR (BB, 75.5 MHz, CDCl$_3$) δ 11.4 (Ile δ-*C*H$_3$); 15.4, minor 15.5 (Ile γ-*C*H$_3$); minor 21.1, minor 22.0, minor 24.2, 24.4, 24.7 (^{1,5,7}Pro γ-*C*H$_2$, Ile γ-*C*H$_2$); 27.9 (Pro C(*C*H$_3$)$_3$); 29.0, 30.1, 30.6 (^{1,5,7}Pro β-*C*H$_2$); 36.8 (Ile β-*C*H), 38.2, 41.5 (^{2,4,6}Phe β-*C*H$_2$); 46.9 (^{1,5,7}Pro δ-*C*H$_2$); 47.1 (Fmoc α-*C*H); 51.9, 53.8, 54.4, 56.0, 57.6, 58.2, 59.4, 59.7, 60.6 (^{1,5,7}Pro α-*C*H, ^{2,4,6}Phe α-*C*H, Ile α-*C*H); 67.8, 68.5 (Fmoc β-*C*H$_2$); 81.1 (Pro *C*(CH$_3$)$_3$); 120.1, 120.4, 124.5, 124.9, 126.7, 127.0, 127.3 (Fmoc Ar *C*H); 128.3, 128.4, 128.8, 129.0, 129.2, 129.4, 129.8 (^{2,4,6}Phe Ar *C*H); 136.1, 136.4 (^{2,4,6}Phe Ar *C*q); 141.0, 141.5, 145.1, 145.9 (Fmoc Ar *C*q); 158.0 (urethane *C*O); 169.3, 169.4, 169.9, 170.2, 170.4, 170.6, 170.8, 171.2, 171.4 (^{1,5,7}Pro *C*O, ^{2,4,6}Phe *C*O, Ile *C*O); *anal.* C 68.86%, H 7.28%, N 9.04, calcd for C$_{67}$H$_{79}$N$_7$O$_{10}$ C 70.44%, H 6.94%, N 8.58%.

Cyclo-(Pro-Phe-Pro-Phe-Ile-Phe-Pro-Gln) [phakellistatin 11] (1). General Procedure for the Solid-Phase Synthesis. The Fmoc-protected PAL polymer (1.1 g, 0.26 mmol scale, 0.24 mmol/g), swelled in DMF, was added to a glass column (15 × 1 cm). Eight of 10 vials were prepared to contain the necessary amino acids (3 equiv) and HATU (2.9 equiv) in the following order: Fmoc-Glu-α-*O*-allyl, Fmoc-Pro, Fmoc-Phe, Fmoc-Ile, Fmoc-Phe, Fmoc-Pro, Fmoc-Phe, Fmoc-Pro. To the ninth and tenth vials were added Pd^0[P(C$_6$H$_5$)$_3$]$_4$ (0.48 g) and PyAOP (3 equiv), respectively. Both the resin amino group and the amino acid units were deprotected with 20% piperidine in DMF (15 min). Peptide bond formation was

allowed to proceed for 30 min using DIEA (3.0 equiv) in DMF. Allyl deprotection using palladium(0) complex was recycled through the column for 2 h, and cyclization of the polypeptide was accomplished in a 1 h period. After the final cycle (dichloromethane wash), the resin was removed and dried under high vacuum for 14 h (0.01 mmHg). A 20 mL (10 mL/ 0.5 g resin) deprotecting solution of TFA (90%), thioanisole (5%), 1,2-ethanedithiol (3%), and anisole (2%) was freshly prepared and added to the peptide-bound resin. The slurry was stirred for 2 h under argon, followed by filtration of the solution and concentration to an oil. The oil was dissolved in DCM and product precipitated with hexane. Separation of the impurities and isolation of the desired product were accomplished through the use of reversed-phase HPLC on a C8 column in acetonitrile−water HPLC gradient: at $t = 0-40$ min, 45% A, 55% B; at $t = 50$ min, 80% A, 20% B; at $t = 60$ min, 45% A, 55% B, where solvent A is HPLC grade acetonitrile and solvent B is distilled water (flow rate 6 mL/min), to afford 42 mg (17%) of a colorless solid (**1**): mp 188−189 °C (lit. 194−196 °C); $[\alpha]^{25}_D$ −130° (c 1.00, CH$_3$OH) (lit. $[\alpha]^{25}_D$ −163°, c 0.08, CH$_3$OH); APCIHRMS m/z 974.5140 (calcd for C$_{53}$H$_{68}$N$_9$O$_9$, 974.5132); *anal.* C 60.29%, H 7.14%, N 11.80%, calcd for C$_{53}$H$_{68}$N$_9$O$_9$·4^1/$_2$H$_2$O.

Comparison of the natural and synthetic specimens of phakellistatin 11 (**1**) by ^{1}H and ^{13}C NMR in DMSO gave identical NMR signals.

HPLC Analysis of the Synthetic, Natural, and Mixture Specimens of Phakellistatin 11 (1). HPLC analysis of the synthetic, natural, and mixed specimens of phakellistatin 11 (**1**) was carried out on a C18 column using a 35% acetonitrile−65% water solvent system and a flow rate of 1 mL/min.

References and Notes

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