Synthesis of the Marine Sponge Cycloheptapeptide Phakellistatin 5[1]

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Phakellistatin 5 (**1**), a constituent of The Federated States of Micronesia (Chuuk) marine sponge *Phakellia costada*, was synthesized by solution-phase and solid-phase techniques. Because the linear peptide bearing (*R*)-Asn resisted cyclization, the synthesis of this peptide was repeated using the PAL resin attachment proceeding from *N*-Fmoc-D-Asp-α-$OCH_2CH{=}CH_2$. After addition of the final unit (Ala), the allyl ester was removed under neutral conditions with Pd^o $[P(C_6H_5)_3]_4$. Removal of the final Fmoc-protecting group and cyclization with PyAOP provided (*R*)-Asn-phakellistatin 5 (**2**) in 28% overall yield. The same synthetic route from (*S*)-Asp led to natural phakellistatin 5 (**1**) in 15% overall recovery. The solution-phase and solid-phase synthetic products derived from (*S*)-Asp were found to be chemically but not biologically identical with natural phakellistatin 5 (**1**). This important fact suggested that a trace, albeit highly cancer-cell growth inhibitory, constituent accompanied the natural product or that there is a subtle conformational difference between the synthetic and natural cyclic peptides.

Early in our systematic investigation of marine invertebrates[2a] as new sources of structurally unique and potentially useful anticancer drugs, we uncovered the marine mollusk lead (in 1972) that led to discovery of the sea hare (*Dolabella auricularia*) dolastatin series of new linear and cyclic cancer cell growth-inhibitory peptides.[2b,c] At present, dolastatin 10[2d] has entered into a series of Phase II human cancer clinical trials under the auspices of the U.S. National Cancer Institute,[2e] and a structure modification, auristatin PE (or TZT-1027),[2f] is being prepared for that stage of clinical development. Furthermore, a structure modification of dolastatin 15, known as cemadotin,[2g,h] is also in Phase II human cancer clinical trials. Greatly encouraged by the extraordinary levels of antineoplastic activity exhibited by the most promising members of the dolastatin peptide series, we began to pursue selected peptide constituents in certain marine sponges (phylum Porifera). By 1985, this endeavor was well under way and led initially to the cyclooctapeptide hymenistatin 1.[3] That was followed by the isolation and structure elucidation of several other series of cyclic peptides,[4a−h] of which the cyclic decapeptides phakellistatins 7−9[4g,h] represent some of the more recent advances. Meanwhile, the overall research directed at isolation and characterization of cancer cell growth-inhibitory peptides contained by marine sponges has been steadily increasing, and the newest additions include cupolamide A,[5a] oriamide,[5b] and hemiasterlin,[5c] of which the latter has shown exceptional potency against a variety of cancer cell lines.

In 1985, we began to examine the antineoplastic constituents of the marine sponge *Phakellia costada* collected in the Federated States of Micronesia (Chuuk), and we isolated in 9.6×10^{-6}% yield the cyclic heptapeptide designated phakellistatin 5 (**1**).[6] In the NCI disease-oriented in vitro primary screen, phakellistatin 5 provided a mean panel GI_{50} value of 3 μM, and comparative analyses suggested the possibility of an uncommon mechanism of action. Results of the COMPARE analyses indicated the need for more detailed biological studies, and that required producing phakellistatin 5 by total synthesis. In addition, we later had reason to believe that the instrumental results leading to the (*R*)-Asn assignment rather than (*S*) was questionable, owing to a possible instrumental shortcoming. By this time, evidence was also accumulating that some of the sponge cyclic peptides can retain a chemically undetectable amount of a very potent antineoplastic substance that can only be revealed by biological techniques.[4a,b] To pursue these considerations, we undertook practical syntheses of (*S*)-Asn-phakellistatin 5 and (*R*)-Asn-phakellistatin 5.

Results

Because of the uncertainty concerning the (*R*)- vs (*S*)-Asn component of phakellistatin 5, a sequential amino acid addition and *N*-Fmoc/*tert*-butyl protection strategy[4a,b,7] was undertaken to obtain both Asn epimers of phakellistatin 5. Cleavage of Fmoc-protecting groups was easily realized using the classic diethylamine (DEA) procedure through the tetrapeptide stage and tris-(2-aminoethyl)amine (TAEA)[8a,b] through preparation of the heptapeptides. Formation of peptide bonds was performed in dichloromethane (DCM) utilizing diethylphosphorocyanidate (DEPC)[9] with diisopropylethylamine (DIEA) as base. The application of TAEA in reactions involving the larger peptides assisted in overcoming solubility constraints, thus easing purification procedures. Both introduction and elongation of the pentapeptide was easily accomplished without (cf. **9a**) or with *N*-trityl side-chain protection (**9b**), leading to comparable yields. Although solubility characteristics were enhanced, the trityl group did not offer any other advantages in purifying the linear hexapeptides and heptapeptides. However, with D-asparagine it became imperative to utilize the trityl protection to avoid dehydration to the corresponding nitrile.

Both the Asn-unprotected (**9a**) and *N*-trityl-protected (**9b**) heptapeptides containing the (*S*)-Asn unit were completely deprotected using trifluoroacetic acid in DCM (1:1) over 2 h at room temperature. After removal of solvent, the residue was dissolved in a minimal amount of dimethylformamide (DMF) and diluted with DCM to produce a 1.4 mM solution, whereupon TBTU (**10**)[10] and DIEA were added to effect cyclization. When the cyclization reaction was allowed to proceed for as much as two weeks, the yields of cyclo-heptapeptide **1** never exceeded 15%. In our experi-

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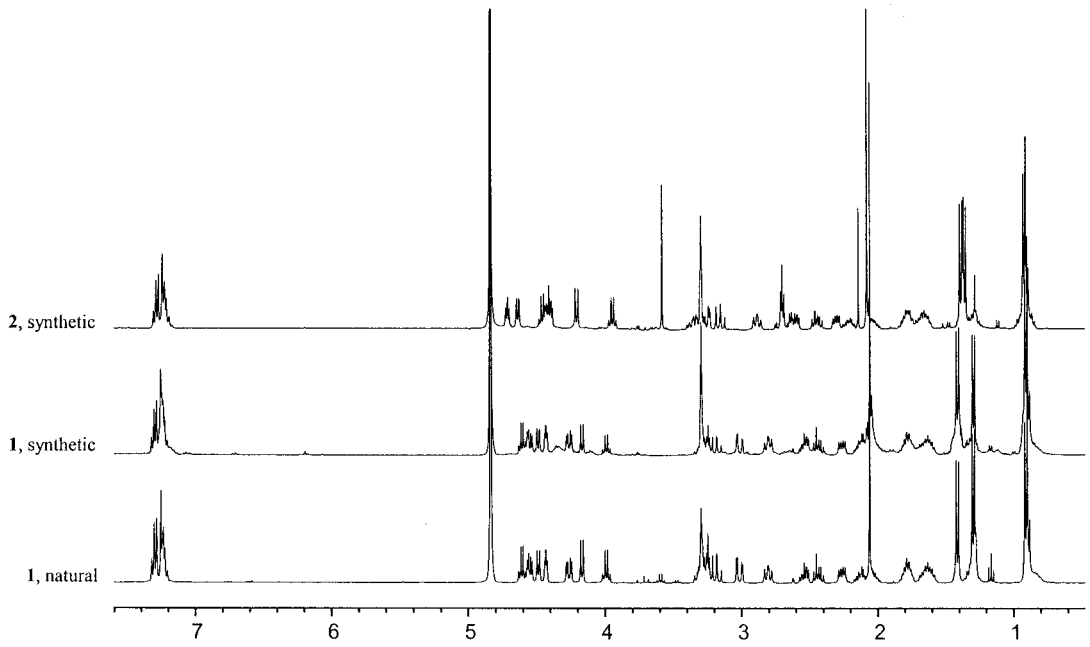


Figure 1. ^{1}H NMR overlay comparisons of natural phakellistatin 5 (**1**) and (R)-phakellistatin 5 (**2**)

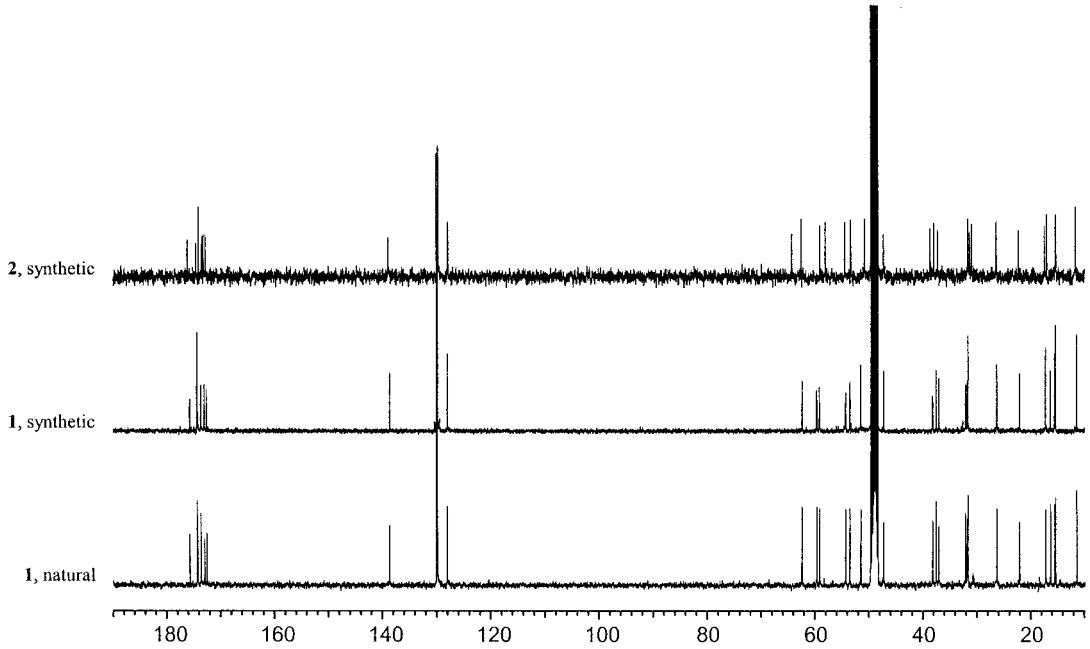


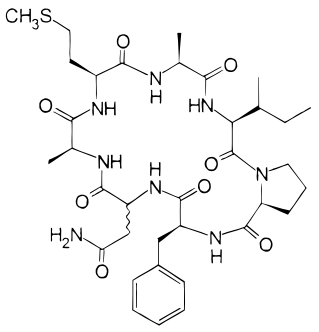
Figure 2. ^{13}C NMR overlay comparisons of natural phakellistatin 5 (**1**) and (R)-phakellistatin 5 (**2**)

ence with cyclization of related heptapeptides based on a C-terminal Pro unit, using TBTU for completion of the cyclization reaction, yields of 55% were consistently obtained.[11] When attempts were made to cyclize the heptapeptides **9c** and **9d** containing the (*R*)-Asn unit under analogous conditions (following total deprotection and application of TBTU for peptide bond formation) cycloheptapeptide **2** was not detected among the products. The same unpromising results were obtained using other coupling procedures such as TBTU/HOBt in DMF with DIEA, BOP−Cl[12] with DIEA in DCM, or DPPA with DEA in DMF. Apparently the superficially minor change in chirality exhibited by the (*R*)-Asn unit in heptapeptide **9c** caused a serious conformational change that greatly reduced availability for intramolecular cyclization.

The preceding challenges offered by the heptapeptide **9c** derived from D-Asn led to an abrupt change in strategy and adoption of the three-dimensional orthogonal solid-phase approach.[13] This relatively new approach for synthesizing cyclic peptides containing aspartic acid or glutamic acid derivatives holds many advantages, including side-chain anchoring to a resin, stepwise elongation of the linear sequence, and selective deprotection to liberate a free carboxyl group for the cyclization step, followed by intramolecular coupling with a liberated amino group, which benefits from the pseudo-dilution environment offered by the resin-bound peptide, and cleavage from the resin to release the cyclic peptide. Specific application of this general approach to synthesis of (*R*)-Asn-phakellistatin 5 (**2**) proved to be remarkably effective.

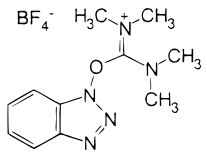
At the start, *N*-Fmoc-L-Asp-α-*O*-allyl (**11**) was condensed through the *β*-carboxyl group with the deprotected amino group derived from the 5-(4-Fmoc-aminomethyl-3,5-dimethoxyphenoxy)valeric acid-bonded resin (PAL). Here the α-allyl ester serves as the third dimension of protection and is easily cleaved without affecting the other protecting

groups by using Pd^0 $[P(C_6H_5)_3]_4$ under neutral conditions. The *N*-Fmoc-D-Asp-α-*O*-allyl was analogously attached to the PAL resin. Both solid-phase syntheses on the PAL resin proceeding to phakellistatin 5 (**1**) and (*R*)-Asn-phakellistatin 5 (**2**) were conducted analogously, employing Fmoc protection and deprotection with 20% piperidine[14] followed by peptide-bond formation using HATU (**12**).[15] After addition of the Ala 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 (**13**).[15] In each case, the cyclic peptide was cleaved from the polymer attachment with 90% trifluoroacetic acid in the presence of radical scavengers.

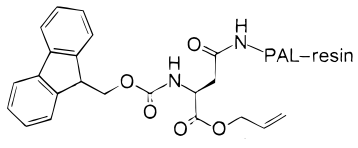


1, Phakellistatin 5, *cyclo*-(Phe-L-Asn-Ala-Met-Ala-Ile-Pro)

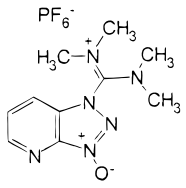
2, *cyclo*-(Phe-D-Asn-Ala-Met-Ala-Ile-Pro)



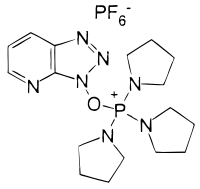
10, TBTU



11



12, HATU



13, PyAOP

Interestingly, detailed comparison of the synthetic L-Asn phakellistatin 5 specimens prepared by the solution-phase and solid phase syntheses showed them to be the same and also chemically (but not biologically) identical to the natural specimen of phakellistatin 5 (**1**) isolated from *Phakellia costada*.[6] Further comparison with (*R*)-Asn-phakellistatin 5 (**2**) provided unequivocal evidence (cf. Figures 1 and 2) that the natural phakellistatin 5 (**1**) was derived from L-Asn. In addition to the ^1H and ^{13}C NMR comparisons illustrated in Figures 1 and 2, where substantial chemical shifts attributed to Ala are quite apparent, other evidence was provided by detailed chromatographic studies including HPLC and TLC analyses. In the systems used, cycloheptapeptide **2** gave clearly higher R_f values than did phakellistatin 5 (**1**). The optical rotation values for the natural and synthetic specimens of phakellistatin 5 (**1**) also compared quite well. The most convincing chromatographic evidence was obtained by 6 N hydrochloric acid hydrolysis of both cycloheptapeptides **1** and **2**, along with the natural specimen (**1**), followed by comparative amino acid analyses with contemporary chiral chromatographic instrumentation. The retention times of L-Asp obtained from the natural and synthetic samples of phakellistatin 5 (**1**) very nicely matched, whereas the D-Asp derived from the solid-phase synthetic specimen of (*R*)-phakellistatin 5 (**2**) gave enough of an increase in retention time to remove any possible uncertainty.

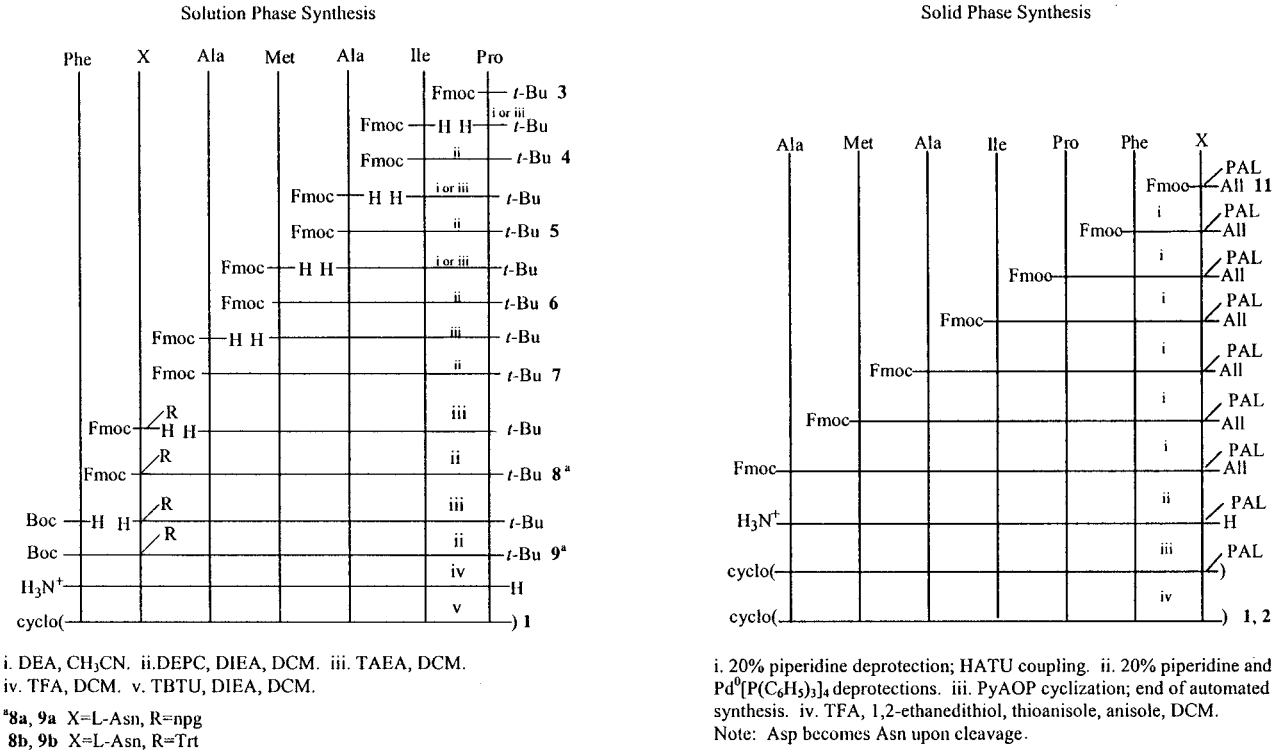
Discussion

With the correct structure of phakellistatin 5 established and its supply brought to an adequate level by means of the solid-phase synthesis (for example, 0.10 g corresponding to a 15% overall yield vs only a 15% yield in the last step by the solution-phase route), the initial human cancer cell line results were reexamined by means of the P-388 murine lymphocytic leukemia and the human cancer cell lines representing ovarian (OVCAR-3), CNS (SF-295), lung (NCI−H460), prostate (DU-145), colon (KM20L2), and melanoma (SK-MEL-5) cancers. Again, the natural specimen exhibited significant cell growth-inhibitory activity, with GI_{50} values ranging from 0.14 to 0.74 μg/mL. However, against the same cancer cell lines, the synthetic samples of phakellistatin 5 (**1**) and the (*R*)-Asn-phakellistatin 5 (**2**) gave results greater than 10 μg/mL and were considered inactive. These important results further confirm our earlier observations[4] that certain types of cyclic peptides derived from marine animal fractions with cancer cell growth-inhibitory activity can retain a chemically undetectable amount of a very potent antineoplastic substance whose presence is only revealed by biological techniques. Whether this retention is simply by contamination or a more subtle complexing or conformational change is presently unknown. In the present instance the COMPARE analysis seems to have excluded the halichondrin/halistatin and spongistatin types of extraordinary cancer cell growth-inhibitory sponge constituents and instead seems to be pointing to an as yet unknown, albeit powerfully active, antineoplastic constituent in *Phakellia costada*. Doubtlessly, such a constituent of *Phakellia costada* would have to be present in barely detectable quantities and require greatly improved isolation techniques. Perhaps phakellistatin 5 or some of the other sponge cyclic peptides we have found to retain exceptionally potent cancer cell growth inhibitory constituents might actually become part of a new affinity-type chromatographic method (perhaps by applying phakellistatin 5 bound to the PAL polymer) for concentrating such vanishingly small amounts of these complex mixture components. The affinity chromatography aspect will be pursued along with further biological evaluations of phakellistatin 5. For now it is important to confirm further the biological activity of such cyclic peptides by means of total syntheses.

Experimental Section

General Experimental Procedures. Each solvent was redistilled. The Fmoc L-amino acids and Boc−Phe were supplied by NovaBiochem and Sigma-Aldrich and used as received, except for *N*-Fmoc-L-Asp-α-allyl ester, which was purchased from PerSeptive Biosystems, and *N*-Fmoc-D-Asp-α-*O*-allyl, which was best prepared by the allyl bromide route.[13] Pyridine and triethylamine were redistilled and stored over potassium hydroxide pellets. Methylene chloride and acetonitrile were dried over 4 Å molecular sieves. DMF was redistilled and stored over 4 Å molecular sieves. Organic extracts of

Scheme 1. Syntheses of phakellistatin 5 (**1**) and (*R*)-Asn-phakellistatin 5 (**2**)



i. DEA, CH_3CN. ii.DEPC, DIEA, DCM. iii. TAEA, DCM.
iv. TFA, DCM. v. TBTU, DIEA, DCM.

[a] **8a**, **9a** X=L-Asn, R=npg
8b, **9b** X=L-Asn, R=Trt
8c, **9c** X=D-Asn, R=npg
8d, **9d** X=D-Asn, R=Trt
npg=no protecting group

i. 20% piperidine deprotection; HATU coupling. ii. 20% piperidine and $Pd^0[P(C_6H_5)_3]_4$ deprotections. iii. PyAOP cyclization; end of automated synthesis. iv. TFA, 1,2-ethanedithiol, thioanisole, anisole, DCM.
Note: Asp becomes Asn upon cleavage.

aqueous solutions were dried over sodium sulfate. Solvents were removed using a rotary evaporator with a water bath at ≤30 °C. Solid samples and oils were dried under high vacuum (0.05 mmHg, 25 °C) unless otherwise noted. Flash column chromatography was performed on ASTM Si gel from EM Science (230−400 mesh). The solid-phase syntheses were performed with a 9050 Plus PepSynthesizer following procedures recommended by PerSeptive Biosystems Biosearch Products. The PAL resin was also obtained from PerSeptive Biosystems.

Melting points were determined using an Electrothermal 9100 unit and are uncorrected. TLC was performed with Analtech Si gel GHLF plates. All compounds were visible under short-wave UV light (254 nm) and stained with 2% ceric sulfate in ethanol. Electron impact mass spectra were recorded using a Varian MAT 312 (70 eV) instrument, while the FABMS measurements were made at the Midwest Center for Mass Spectrometry, University of Nebraska, Lincoln, NE. The ^1H and ^{13}C NMR spectra were recorded employing Varian Gemini 300, Varian Gemini 400, or Varian VXR-500S instruments using a deuterated solvent and were referenced either to TMS or the solvent. The UV spectra were obtained employing a Perkin−Elmer Lambda 3B spectrometer interfaced with an IBM PC running PECSS data acquisition software or a UV−vis−NIR Scanning Spectrophotometer interfaced with a Shimadzu UV-3101PC. The IR spectra were obtained with a Mattson Instrument 2020 Galaxy Series FTIR instrument using NaCl plates. Combustion analyses were determined by the Galbraith Microanalytical Laboratory, Inc., Knoxville, TN.

***N*-Fmoc-Pro-*O*-Bu^t (3).** The previously described *tert*-butylation procedure[4a] was employed to give protected amino acid **3** (31.5 g, 94%) after purification on a column of Si gel (3:1 hexanes−ethyl acetate as eluent; 3-in pad) and recrystallization from methanol: mp 109−110 °C; R_f 0.40 (3:1 hexanes−ethyl acetate).

General Deprotection Procedure Using DEA. The following method was applied in the formation of the dipeptides,tripeptides,tetrapeptides, and pentapeptides **4**−**7**, respectively. A solution of the *N*-Fmoc amino acid or peptide *tert*-butyl ester in 0.2 M DEA−acetonitrile (1:1) was stirred under argon until TLC analysis revealed no starting material (usually 1−2 h). The reaction mixture was concentrated in vacuo and twice washed with methylene chloride and concentrated. The residue was placed under high vacuum for at least 1 h prior to the coupling reaction.

General Deprotection Method Using TAEA.[8] For preparation of the hexa- and heptapeptides **8** and **9**, the following method was used. To a stirred solution (or suspension) of *N*-Fmoc peptide *tert*-butyl ester in DCM (0.5 M, under argon) was added TAEA (20−50 equiv, depending on reaction size). The resultant homogeneous yellow solution was stirred, while the disappearance of starting material and formation of dibenzofulvene were monitored by TLC. Starting material disappeared within 15 min, while the TAEA−dibenzofulvene complex appeared over 1−3 h. The reaction mixture was washed (2 ×) successively with brine and 10% potassium phosphate (monobasic/dibasic) pH 5.5 buffer solution. The combined aqueous solution was back-extracted with DCM (1 ×), and the combined organic extract was dried, filtered, and concentrated. The residue was dried under vacuum for at least 1 h.

General Procedure for Peptide-Bond Formation Using DEPC.[4a] The amino acid or peptide *tert*-butyl ester was dissolved in methylene chloride (0.1 M under argon), whereupon the *N*-Fmoc-amino acid (1.0 eq.) was added followed by DIEA (1.0 equiv at room temperature). Upon dissolution, DEPC 1.0 equiv) was added (dropwise) over a 1-min period. Stirring was continued with monitoring by TLC (ninhydrin indicator). Concentration of the reaction mixture (except for **7**, **8a**, and **8b**, see below) in vacuo was followed by dissolution of the residue in ethyl acetate and successive washing (once each) with 0.1 *N* hydrochloric acid, water, 10% sodium bicarbonate, water, and brine. The organic layer was dried and filtered, and the solvent was removed to give the crude peptide, which was purified as described in the following typical experiment.

***N*-Fmoc-Ile-Pro-*O*-Bu^t (4).**[3] The yellow oily residue was subjected to flash chromatography (3:1 hexane−ethyl acetate)

to give dipeptide **4** (20 g, 79%) as a foamy white solid: mp 58−60 °C; R_f 0.23 (3:1 hexane−ethyl acetate).

***N*-Fmoc-Ala-Ile-Pro-*O*-Bu^t (5).** Purification via chromatography (1:1 hexane−ethyl acetate) gave tripeptide **5** (3.3 g, 73%) as a tan foamy solid that was recrystallized from ethyl acetate-hexane: mp 170−171 °C; R_f 0.18 (1:1 hexane−EtOAc); $[\alpha]^{24}_D$ −72° (*c* 0.10, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ϵ) 223 (4.25), 228 (4.25), 268 (4.54) nm; IR (Nujol) ν_{max} 3270, 2924, 2855, 1721, 1632, 1539, 1454 cm^{-1}; EIMS *m/z* (int) 577 (3%, M^+), 504 (1%, $[M-t\text{-BuO}]^+$), 178 (100%, dibenzofulvene), 70 (60%); *anal.* C 68.51%, H 7.56%, N 6.89%, calcd for $C_{33}H_{43}N_3O_6$, C 68.61%, H 7.50%, N 7.27%.

***N*-Fmoc-Met-Ala-Ile-Pro-Bu^t (6).** Flash chromatographic separation (1:1 DCM−ethyl acetate) and recrystallization from toluene−hexane afforded peptide **6** (3.4 g, 91%) as a colorless powder: mp 115−117 °C (the hemi-hydrate); R_f 0.15 (1:1 DCM−ethyl acetate); $[\alpha]^{24}_D$ −63° (*c* 0.10, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ϵ) 209 (4.50), 213 (4.51), 236 (4.43), 249 (4.75), 264 (4.65), 289 (4.81), 300 (4.02) nm; IR (Nujol) ν_{max} 3281, 2922, 1724, 1631, 1535, 1452 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.86 (3 H, t, J = 7.2 Hz, Ile δ), 0.98 (3 H, d, J = 6.6 Hz, Ile β-CH_3), 1.04−1.14 (1 H, m, Ile γ H_a), 1.32 (3 H, d, J = 6.6 Hz, Ala β), 1.42 (9 H, s, *t*-Bu), 1.48−1.57 (1 H, m, Ile γ H_b), 1.78−2.17 (7 H, m, Pro β, Pro γ, Ile β, Met β), 2.07 (3 H, s, Met δ), 2.48−2.60 (2 H, m, Met γ), 3.57−3.66 (1 H, m, Pro δ H_a), 3.72−3.80 (1 H, m, Pro δ H_b), 4.20 (1 H, t, J = 7.0 Hz, Fmoc CH), 4.32−4.46 (2 H, m, Ala α, Ile α), 4.36 (2 H, d, J = 7.0 Hz, Fmoc CH_2), 4.50−4.65 (2 H, m, Pro α, Met α), 5.80 (1 H, d, J = 8.1 Hz, Met NH), 6.76 (1 H, d, J = 6.9 Hz, amide NH), 6.87 (1 H, d, J = 9.6 Hz, amide NH), 7.28 (2 H, t, J = 7.0 Hz, Fmoc ArH), 7.37 (2 H, t, J = 7.0 Hz, Fmoc ArH), 7.56 (2 H, d, J = 7.0 Hz, Fmoc ArH), 7.73 (2 H, d, J = 7.0 Hz, Fmoc ArH); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 11.1 (Ile δ), 15.2 (Met β or Ile β-CH_3), 15.4 (Met δ or Ile β-CH_3), 18.5 (Ala β), 24.2 (Pro γ), 24.8 (Ile γ), 27.9 (*t*-Bu CH_3), 29.0 (Met β), 30.0 (Met γ), 32.1 (Pro β), 37.9 (Ile β), 47.1 (Fmoc CH), 47.4 (Pro δ), 49.0 (Ala α), 53.7 (Met α), 54.9 (Ile α), 59.9 (Pro α), 67.0 (Fmoc CH_2), 81.3 (*t*-Bu-C), 120.0 (Fmoc ArCH), 125.1 (Fmoc ArCH), 127.0 (Fmoc ArCH), 127.7 (Fmoc ArCH), 141.3 (Fmoc ArC), 143.8 (Fmoc ArC), 156.1 (urethane CO), 170.2 (ester or amide CO), 170.8 (ester or amide CO), 171.0 (ester or amide CO), 171.6 (ester or amide CO); EIMS *m/z* (int) 707 (3%, M^+), 634 (1%, $[M - t\text{-BuO}]^+$), 178 (100%, dibenzofulvene), 116 (60%), 86 (72%); *anal.* C 63.53%, H 7.49%, N 7.67%, S 4.54%, calcd for $C_{38}H_{52}N_4O_7S\cdot{}^1/_2H_2O$, C 63.58%, H 7.44%, N 7.80%, S 4.47%.

***N*-Fmoc-L-Ala-L-Met-L-Ala-L-Ile-L-Pro-Bu^t (7).** The required pentapeptide (**7**) precipitated from the reaction mixture (2.2 g, 99%) as a colorless flaky solid that was collected and washed with hexane: mp 234−235 °C (dec); R_f 0.53 (1:1:1 acetone−hexane−ethyl acetate); $[\alpha]^{25}_D$ −23° (*c* 0.15, DMSO); *anal.* C 62.53%, H 7.37%, N 8.44%, calcd for $C_{41}H_{57}N_5O_8S$, C 63.14%, H 7.37%, N 8.98%.

***N*-Fmoc-Asn-Ala-Met-Ala-Ile-Pro-*O*-Bu^t (8a).** The reaction mixture was partitioned with an equal volume of water and the layers separated. The aqueous layer was extracted with DCM (2 ×), and the combined organic extract was concentrated in vacuo. Ethyl acetate was added and the organic solution was processed as stated in the general procedure above. Recrystallization from acetone−hexane provided the hexapeptide (0.75 g, 68%) as a translucent, flaky, colorless solid. Peptide **8a** was used without further purification in the next step and at this stage gave: mp 204−208 °C (dec); R_f 0.31 (1:1:1 acetone−hexane−ethyl acetate); $[\alpha]^{25}_D$ +118° (*c* 0.11, DMSO); IR (Nujol) ν_{max} 3389, 3308, 2955, 2856, 1745, 1697, 1629, 1462 cm^{-1}; ^{1}H NMR (DMSO, 400 MHz) δ 0.82 (3 H, t, J = 7.5 Hz, Ile δ), 0.89 (3 H, d, J = 7.0 Hz, Ile β-CH_3), 1.03−1.11 (1 H, m, Ile γ H_a), 1.18 (3 H, d, J = 7.0 Hz, Ala^2 β), 1.22 (3 H, d, J = 7.0 Hz, Ala^1 β), 1.38 (9 H, s, *t*-Bu), 1.45−1.55 (1 H, m, Ile γ H_b), 1.70−1.96 (6 H, m, Met β H_a, Ile β, Pro β, Pro γ), 2.02 (3 H, s, Met δ), 2.09−2.18 (1 H, m, Met β H_b), 2.37−2.60 (4 H, m, Met γ, Asn β), 3.51−3.57 (1 H, m, Pro δ H_a), 3.69−3.79 (1 H, m, Pro δ H_b), 4.13−4.37 (9 H, m, Pro α, Ile α, Ala^1 α, Ala^2 α, Met α, Asn α, Fmoc CH, Fmoc CH_2), 6.96 (1 H, s, amide NH), 7.33 (2 H, t, J = 7.5 Hz, Fmoc ArH), 7.39 (1 H, s, amide NH), 7.42 (2 H, t, J = 7.5 Hz, Fmoc ArH), 7.53 (1 H, d, J = 7.5 Hz, amide NH), 7.71 (2 H, t, J = 7.5 Hz, Fmoc ArH), 7.77 (1 H, d, J = 8.5 Hz, amide NH), 7.81 (1 H, d, J = 7.0 Hz, amide NH), 7.89 (2 H, d, J = 7.5 Hz, Fmoc ArH), 8.01 (1 H, d, J = 8.5 Hz, amide NH), 8.18 (1 H, d, J = 6.0 Hz, amide NH); ^{13}C NMR (DMSO, 100 MHz) δ 10.8 (Ile δ), 14.5 (Ile β-CH_3), 14.9 (Met δ), 16.7 (Ala^1 β or Ala^2 β), 16.8 (Ala^1 β or Ala^2 β), 22.2 (Pro γ), 24.5 (Ile γ), 27.6 (*t*-Bu CH_3), 28.7 (Met γ), 29.6 (Pro γ), 31.5 (Met γ), 36.3 (Ile β), 37.3 (Asn β), 46.6 (Fmoc CH), 46.8 (Pro δ), 47.9 (Ala^1 α), 48.7 (Asn α), 51.7 (Ala^2 α), 54.3 (Met α), 58.9 (Ile α), 59.4 (Pro α), 65.7 (Fmoc CH_2), 80.2 (*t*-Bu−C), 120.1 (Fmoc ArCH), 125.3 (Fmoc ArCH), 127.0 (Fmoc ArCH), 127.6 (Fmoc ArCH), 140.7 (Fmoc ArC), 143.7 (Fmoc ArC), 156.2 (urethane CO), 166.4 (ester or amide CO), 169.7 (ester or amide CO), 170.9 (ester or amide CO), 172.2 (ester or amide CO), 172.3 (ester or amide CO), 172.6 (ester or amide CO), 172.9 (ester or amide CO), 174.4 (ester or amide CO); HRFABMS *m/z* 916.4273 (calcd for $C_{45}H_{63}N_7O_{10}SNa$, 916.4254); *anal.* C 60.30%, H 7.28%, N 9.77%, S 3.52%, calcd for $C_{45}H_{53}N_7O_{10}S$, C 60.45%, H 7.10%, N 10.97%, S 3.59%.

***N*$^\beta$-Trt-*N*$^\alpha$-Fmoc-Asn-Ala-Met-Ala-Ile-Pro-*O*-Bu^t (8b).** Subjection of the residue to flash column chromatography (1:1:1 hexane−acetone−ethyl acetate) followed by recrystallization from ethyl acetate−hexane afforded peptide **8b** (0.78 g, 75%) as a flaky, colorless solid: mp >150 °C (dec); R_f 0.40 (1:1:1 hexane−acetone−ethyl acetate); HRFABMS *m/z* 1158.5391 (calcd for $C_{64}H_{77}N_7O_{10}SNa$, 1158.5350); *anal.* C 68.23%, H 7.37%, N 8.14%, S 2.87, calcd for $C_{64}H_{77}N_7O_{10}S$, C 67.64%, H 6.83%, N 8.63%, S 2.82%.

***N*-Fmoc-D-Asn-Ala-Met-Ala-Ile-Pro-*O*-Bu^t (8c).** Concentration in vacuo of the solution followed by crystallization from 2-propanol−hexane and recrystallization from ethyl acetate−hexane provided hexapeptide **8c** (0.78 g, 75%) as a flaky, beige solid: mp >200 °C (dec); R_f 0.67 (1:1 acetone−DCM); HRFABMS *m/z* 894.4407 (calcd for $C_{41}H_{59}N_5N_8S$, 894.4435); *anal.* C 55.54%, H 7.86%, N 10.07%, S 3.42%, calcd for $C_{45}H_{63}N_7O_{10}S\cdot 4\ H_2O$, C 55.94%, H 7.41%, N 10.15%, S 3.32%.

***N*-Boc-Phe-Asn-Ala-Met-Ala-Ile-Pro-*O*-Bu^t (9a).** Purification using column chromatography (1:1 acetone−DCM) led to an off-white solid (**9a**) that was recrystallized from acetone−hexane (1.9 g, 37%): mp 76−77 °C; R_f 0.23 (1:1 acetone−DCM); ^{1}H NMR (CD_3OD, 500 MHz) δ 0.85−0.91 (1 H, m, Pro γ H_a), 0.89 (3 H, t, J = 7.5 Hz, Ile δ), 0.99 (3 H, d, J = 6.5 Hz, Ile β−CH_3), 1.10−1.19 (1 H, m, Ile γ H_a), 1.35 (3 H, d, J = 7.5 Hz, Ala^2 β), 1.37 (9 H, s, Boc−CH_3), 1.40 (3 H, d, J = 7.0 Hz, Ala^1 β), 1.43 (9 H, s, *t*-Bu), 1.57−1.65 (1 H, m, Ile γ H_b), 1.83−2.06 (4 H, m, Pro β H_a, Pro γ H_b, Ile β, Met β H_a), 2.08 (3 H, s, Met δ), 2.10−2.23 (2 H, m, Pro β H_b, Met β H_b), 2.45−2.54 (1 H, Met γ H_a), 2.58−2.64 (1 H, Met γ H_b), 2.69 (2 H, dd, J = 4.8, 15.8 Hz, Phe β H_a), 2.74−2.87 (2 H, m, Asn β), 3.10 (1 H, dd, J = 5.2, 13.8 Hz, Phe β H_b), 3.61−3.66 (1 H, m, Pro δ H_a), 3.83−3.88 (1 H, m, Pro δ H_b), 4.17 (1 H, q, Ala^2 α), 4.23−4.30 (3 H, m, Phe α, Ile α, Asn α), 4.40 1 H, dd, J = 4.2, 10.2 Hz, Met α), 4.43 (1 H, d, J = 9 Hz, Pro α), 4.57−4.63 (1 H, m, Ala^2 α), 7.21−7.32 (5 H, m, Phe−CH); ^{13}C NMR (CD_3OD, 125 MHz) δ 11.3 (Ile δ), 15.3 (Ile β−CH_3), 15.6 (Met δ), 17.3 (Ala^1 β), 17.5 (Ala^2 β), 25.2 (Pro γ), 25.6 (Ile γ), 28.2 (*t*-Bu), 28.6 (Boc−CH_3), 30.7 (Met γ), 31.3 (Pro β), 31.4 (Met β), 37.4 (Asn β), 37.8. (Ile β), 38.6 (Phe β), 50.2 (Pro δ), 51.2 (Ala^1 α), 51.8 (Asn α), 53.9 (Ala^2 α), 56.4 (Met α), 57.5 (Ile α), 61.3 (Phe α), 64.6 (Pro α), 80.8 (*t*-Bu−C), 82.3 (Boc−C), 127.8 (Phe−ArCH), 129.4 (Phe−ArCH), 130.3 (Phe−ArCH), 138.3 (Phe−ArC), 157.5 (urethane CO), 171.9 (ester or amide CO), 172.7 (ester or amide CO), 173.2 (ester or amide CO), 173.3 (ester or amide CO), 173.9 (ester or amide CO), 174.0 (ester or amide CO), 174.4 (ester or amide CO), 175.0 (ester or amide CO); HRFABMS *m/z* 919.4932 (calcd for $C_{44}H_{71}N_8O_{11}S$, 919.4962); *anal.* C 57.10%, H 7.90%, N 11.59%, calcd for $C_{44}H_{70}N_8O_{11}S\cdot 1/2\ H_2O$, C 56.94%, H 7.71%, N 12.07%.

***N*-Boc-L-Phe-*N*$^\beta$-Trt-Asn-Ala-Met-Ala-Ile-Pro-*O*-Bu^t (9b).** The crude peptide was subjected to flash chromatography (eluent, ethyl acetate) to yield a colorless, flaky solid (**9b**, 0.60 g, 74%); R_f 0.15 (100% ethyl acetate); HRFABMS *m/z* 1161.6139 (calcd for $C_{63}H_{85}N_8O_{11}S$, 1161.6058); *anal.* C 68.23%, H 7.37%, N 8.14%, S 2.87%, calcd for $C_{64}H_{77}N_7O_{10}S$, C 67.64%, H 6.83%, N 8.63%, S 2.82%.

***N*-Boc-Phe-D-Asn-Ala-Met-Ala-Ile-Pro-*O*-Bu^t (9c).** The reaction mixture was diluted with water and the layers separated. The organic layer was concentrated to an oily residue that was dissolved in ethyl acetate, and the product was isolated as described in the general procedure. The TLC analysis (1:1 acetone−DCM) showed two products; the linear heptapeptide (**9c**) and a presumed asparagine dehydration derivative (evidenced by MS and IR results) at R_f 0.30 and R_f 0.65, respectively. After separation by column chromatography, the two products were isolated as powdery solids (0.10 g, 28% for **9c**; 0.08 g, 22% for the dehydration product).

Peptide 9c: mp 98−99 °C; $[\alpha]^{25}_D$ +8.0° (*c* 0.15, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.80−0.88 (1 H, m, Pro γ H_a), 0.93 (3 H, t, J = 7.2 Hz, Ile δ), 0.97 (3 H, d, J = 6.4 Hz, Ile β−CH_3), 1.10−1.19 (1 H, m, Ile γ H_a), 1.37 (9 H, s, Boc−CH_3), 1.41 (9 H, s, *t*-Bu), 1.46 (3 H, d, J = 7.2 Hz, Ala^1 β), 1.50 (3 H, d, J = 7.6 Hz, Ala^2 β), 1.60−1.70 (1 H, m, Ile γ H_b), 1.79−2.06 (4 H, m, Pro β H_a, Pro γ H_b, Ile β, Met β H_a), 2.09 (3 H, s, Met δ), 2.10−2.23 (2 H, m, Pro β H_b, Met β H_b), 2.34−2.46 (1 H, Met γ H_a), 2.50−2.59 (1 H, Met γ H_b), 2.88 (2 H, dd, J = 8.8, 14 Hz, Asn β), 2.90 (2 H, dd, J = 6.8, 16 Hz, Phe β H_a), 3.16 (1 H, dd, J = 6.0, 14.0 Hz, Phe β H_b), 3.60−3.66 (1 H, m, Pro δ H_a), 3.93−4.04 (1 H, m, Pro δ H_b), 4.20−4.29 (2 H, m, Ala^2 α, Ile α), 4.45 (1 H, t J = 8.4 z, Asn α), 4.47−4.55 (3 H, m, Pro α, Met α, Phe α), 4.59−4.64 (1 H, m, Ala^2 α), 5.09 (1 H, s, amide NH), 6.13 (1 H, s, amide NH), 7.04−7.36 (9 H, m, Phe-CH, 4 amide NH's), 7.42 (1 H, d, J = 6.8 Hz, amide NH), 7.62 (1 H, d, J = 4.4 Hz, amide NH); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 11.1 (Ile δ), 15.1 (Ile β−CH_3), 15.3 (Met δ), 15.9 (Ala^1 β), 17.0 (Ala^2 β), 24.8 (Pro γ), 24.9 (Ile γ), 27.9 (*t*-Bu), 28.2 (Boc-CH_3), 29.2 (Met γ), 29.3 (Pro β), 29.7 (Met β), 31.8 (Asn β), 35.5 (Ile β), 37.1 (Phe β), 47.6 (Pro δ), 50.4 (Ala^1 α), 51.5 (Asn α), 52.7 (Ala^2 α), 53.8 (Met α), 55.3 (Ile α), 60.3 (Phe α), 69.5 (Pro α), 81.2 (*t*-Bu−C), 81.3 (Boc−C), 127.4 (Phe−ArCH), 129.0 (Phe−ArCH), 135.7 (Phe−ArC), 156.3 (urethane CO), 171.2 (ester or amide CO), 171.3 (ester or amide CO), 171.4 (ester or amide CO), 172.1 (ester or amide CO), 172.5 (ester or amide CO), 172.6 (ester or amide CO), 172.9 (ester or amide CO), 173.9 (ester or amide CO); HRFABMS *m*/*z* 919.4968 (calcd for $C_{44}H_{71}N_8O_{11}S$ 919.4962).

***N*-Boc-L-Phe-D-Nβ-Trt-Asn-Ala-Met-Ala-Ile-Pro-*O*-Bu^t (9d).** Separation of the crude product by flash column chromatography (1:1 acetone−DCM) led to colorless powder **9d** (2.6 g, 88%): R_f 0.30 (1:1 acetone−DCM); ^{13}C NMR (DMSO, 125 MHz) δ 11.2 (Ile δ), 15.1 (Ile β-CH_3), 15.3 (Met δ), 18.2 (Ala^1 β), 18.2 (Ala^2 β), 24.3 (Pro γ), 24.9 (Ile γ), 28.0 (*t*-Bu), 28.2 (Box−CH_3), 29.1 (Met γ), 30.3 (Pro β), 32.1 (Met β), 36.1 (Asn β), 36.2 (Ile β), 37.7 (Phe β), 47.4 (Pro δ), 49.1 (Ala^1 α), 50.3 (Asn α), 51.7 (Ala^2 α), 52.7 (Met α), 54.8 (Ile α), 56.7 (Phe α), 59.7 (Pro α), 70.7 (*t*-Bu−C), 81.1 (Boc−C), 127.0 (Trt−ArCH), 127.2 (Phe−ArCH), 127.8 (Trt−ArCH), 128.8 (Trt−ArCH), 128.9 (Phe−ArCH), 129.1 (Phe−ArCH), 135.9 (Phe−ArC), 144.2 (Trt−ArC), 156.0 (urethane CO), 170.1 (ester or amide CO), 170.8 (ester or amide CO), 170.9 (ester or amide CO), 171.2 (ester or amide CO), 171.3 (ester or amide CO), 172.0 (ester or amide CO), 172.4 (ester or amide CO), 172.9 (ester or amide CO); LRFABMS *m*/*z* 1183 (calcd for $C_{63}H_{84}N_8O_{11}SNa$, 1183).

Cyclo-Phe-Asn-Ala-Met-Ala-Ile-Pro (1). General Method A, Solution-Phase Synthesis. The linear heptapeptide **9a** and its N$^{\beta}$-Trt-Asn derivative **9b** were each separately dissolved in trifluoroacetic acid−DCM (1:1, 0.1 M under argon). The resultant yellow-red solutions were each stirred for 2 h followed by removal of solvent in vacuo. Each residue was washed with dichloromethane. After evaporation, the crude solid was dissolved in the minimal amount of DMF, and enough DCM was added to produce a 1.4 mM solution. Next, TBTU (**10**, 5.0 equiv)[10] and DIEA (1% vol/total vol) were added at 0 °C. The reaction mixture was warmed to room temperature (25 °C) over a 6-h period, and the solution was stirred for 14 days. Concentration of the solvent led to a yellow oil that was dissolved in DCM and extracted successively with 10% citric acid, 10% sodium bicarbonate, and water. The organic phase was dried and filtered, and the solvent was removed to give a colorless solid in a yellow oil that was further fractionated by flash column chromatography (11:8:1 DCM−hexane−methanol). Concentration of the required fractions followed by recrystallization from ethyl acetate−hexane gave an off-white powder (**1**) that was identical with natural phakellistatin 5 (56 mg, 15%): mp 216−218 °C; R_f 0.30 (5:4:1 DCM−hexane−methanol); $[\alpha]^{24}_D$ −103° (*c* 1.67, MeOH); ^{1}H NMR (CD_3OD, 400 MHz) δ 0.80−0.90 (1 H, Pro γ H_a), 0.90 (3 H, t, J = 7.2 Hz, Ile δ), 0.91 (3 H, d, J = 7.2 Hz, Ile β−CH_3), 1.26−1.37 (1 H, m, Ile γ H_a), 1.30 (3 H, d, J = 6.8 Hz, Ala^1 β), 1.42 (3 H, d, J = 7.2 Hz, Ala^2 β), 1.57−1.70 (2 H, m, Ile γ H_b, Pro γ H_b), 1.74−1.84 (2 H, m, Pro β H_a, Ile β), 2.00−2.17 (2 H, m, Met β), 2.06 (3 H, s, Met δ), 2.27 (1 H, dd, J = 6.2, 12.6 Hz, Pro β H_b), 2.40−2.58 (2 H, m, Met γ), 2.81 (1 H, dd, J = 10.4, 10.4 Hz, Pro δ H_a), 3.01 (1 H, dd, J = 3.0, 15.4 Hz, Asn β H_a), 3.15−3.35 (2 H, m, Pro δ H_b,Asn β H_b), 3.99 (1 H, q, J = 7.2 Hz, Ala^2 α), 4.17 (1 H, d, J = 8.0 Hz, Ile α), 4.27 (1 H, dd, J = 4.2, 12.2 Hz, Phe α), 4.43 (1 H, dd, J = 3.4, 4.6 Hz, Asn α), 4.49 (1 H, d, J = 7.2 Hz, Pro α), 4.55 (1 H, dd, J = 4.6, 10.6 Hz, Met α), 4.61 (1 H, q, J = 6.4 Hz, Ala^1 α), 7.20−7.33 (5 H, m, Phe−ArH); ^{13}C NMR (CD_3OD, 125 MHz) δ 11.3 (Ile δ), 15.3 (Ile β−CH_3), 15.5 (Met δ), 16.3 (Ala^1 β), 17.2 (Ala^2 β), 22.1 (Pro γ), 26.3 (Ile γ), 31.7 (Met γ), 31.8 (Pro β), 32.2 (Met β), 37.1 (Asn β), 37.6 (Ile β), 38.3 (Phe β), 47.3 (Pro δ), 49.2 (Ala^1 α), 51.6 (Asn α), 53.6 (Ala^2 α), 54.4 (Met α), 59.2 (Ile α), 59.7 (Phe α), 62.4 (Pro α), 128.0 (Phe−ArCH), 129.8 (Phe−ArCH), 130.0 (Phe−ArCH), 138.7 (Phe−ArC), 172.6 (Pro−CO), 173.0 (Asn−CO), 173.6 (Ile−CO), 173.7 (Met−CO), 174.3 (Phe−CO), 174.4 (Ala^1−CO), 175.8 (Ala^2−CO); HRFABMS *m*/*z* 745.3707 (calcd for $C_{35}H_{53}N_8O_8S$, 745.3707); *anal*. C 54.68%, H 7.20%, N 13.94%, calcd for $C_{35}H_{52}N_8O_8S\cdot 1.5\ H_2O$: C 54.46%, H 7.18%, N 14.52%.

Cyclo-Phe-D-Asn-Ala-Met-Ala-Ile-Pro (2). General Method B, Solid-Phase Synthesis. The Fmoc-protected PAL polymer (5.0 g, 0.95 mmol scale, 0.190 meq/g), swollen in DMF, was loaded onto a glass column (1 in × 4 in). Seven of nine vials were prepared containing the necessary amino acids (2.0 equiv) and HATU (2.0 equiv) in the following order: Fmoc-D-Asp-α-*O*-allyl, Fmoc-Phe, Fmoc-Pro, Fmoc-Ile, Fmoc-Ala, Fmoc-Met, and Fmoc-Ala. The eighth and ninth vials were charged with Pd° $[P(C_6H_5)_3]_4$ (1.19 g) and PyAOP (2.0 equiv), respectively. The resin amino group and the amino acid units were deprotected with 20% piperidine in DMF (6 min). Peptide bond formation was allowed to proceed for 30 min, allyl deprotection (recycled) for 3 h, and cyclization for 3.5 h. After the final cycle (DCM wash), the resin was removed and dried under high vacuum for 16 h (0.02 mmHg). A 50-mL (0.5 mL/0.5 g resin) deprotecting solution compound of TFA (90%), thioanisole (5%), 1,2-ethanediol (3%), and anisole (2%) was freshly prepared and added to the peptide bound resin. The slurry was stirred for 4 h followed by filtration of the solution and concentration to an oil. The oil was dissolved in DCM and the product precipitated with hexane. The solid was collected and further purified by flash chromatography (11:8:1 DCM−hexane−methanol). Recrystallization from acetone−hexane gave a colorless powder (0.20 g, 28%): mp 175−178 °C; R_f 0.37 (5:4:1 DCM−hexane−methanol); $[\alpha]^{24}_D$ −130° (*c* 1.00, MeOH); ^{1}H NMR (CD_3OD, 400 MHz) δ 0.83−0.99 (1 H, Pro γ H_a), 0.91 (3 H, t, J = 7.2 Hz, Ile δ), 0.92 (3 H, d, J = 7.2 Hz, Ile β−CH_3), 1.24−1.33 (1 H, m, Ile γ H_a), 1.36 (3 H, d, J = 6.4 Hz, Ala^1 β), 1.39 (3 H, d, J = 7.2 Hz, Ala^2 β), 1.60−1.73 (2 H, m, Ile γ H_b, Pro γ H_b), 1.73−1.85 (2 H, m, Pro β H_a, Ile β), 1.99−2.06 (1 H, m, Met β H_a), 2.08 (3 H, s, Met δ), 2.17−2.26 (1 H, m, Met β H_b), 2.31 (1 H, dd, J = 6.2, 12.6 Hz, Pro β H_b), 2.41−2.49 (1 H, m, Met γ H_a), 2.57−2.65 (1 H, m, Met γ H_b), 2.89 (1 H, dd, J = 10.4, 10.4 Hz, Pro δ H_a), 3.12−3.40 (2 H, m, Pro δ H_b, Asn β H_b), 3.25 (1 H, dd, J = 4.2, 13.8 Hz, Asn β H_a), 3.95 (1 H, q, J = 7.2 Hz, Ala^2 α), 4.21 (1 H, d, J = 8.0 Hz, Ile α), 4.38−4.45 (2 H, m, Phe α, Asn α), 4.46 (1 H, q, J = 6.4 Hz, Ala^1 α), 4.64 (1 H, d, J = 7.2 z, Pro α), 4.71 (1 H, t, J = 5.2 Hz, Met α), 7.18−7.32 (5 H, m, Phe−ArH); ^{13}C NMR (CD_3OD, 125 MHz) δ 11.5 (Ile δ), 15.2 (Ile β−CH_3), 15.3 (Met δ), 16.9 (Ala^1 β), 17.4 (Ala^2 β), 22.2 (Pro γ), 26.4 (Ile γ), 31.0 (Met γ), 31.3 (Pro β), 31.7 (Met β), 37.3 (Asn β), 38.0 (Ile β), 38.7 (Phe β), 47.3 (Pro δ), 49.0 (Ala^1 α), 50.8 (Asn α), 53.4 (Ala^2 α), 54.5 (Met α), 58.1 (Ile α), 59.1 (Phe α), 62.6 (Pro α), 127.9 (Phe−ArCH), 129.7 (Phe−ArCH), 130.1 (Phe−ArCH), 138.9 (Phe−ArC),

172.8 (Pro−CO), 173.2 (Asn−CO), 173.4 (Ile−CO), 173.5 (Met−CO), 174.1 (Phe−CO), 174.6 (Ala[1]−CO), 176.1 (Ala[2]−CO); HRFABMS *m/z* 767.3514 (calcd for $C_{35}H_{52}N_8O_8SNa$, 767.3526); *anal.* C 55.09%, H 7.13%, N 14.11%, S 4.53%, calcd for $C_{35}H_{52}N_8O_8S{\cdot}H_2O$, C 55.10%, H 7.13%, N 14.69%, S 4.20%.

Under these same solid-phase reaction conditions phakellistatin 5 (**1**) was synthesized (0.10 g, 15%) and found to be identical with the solution-phase specimen prepared from heptapeptides **9a** and **9b** and with the sample of natural phakellistatin 5 (**1**).

Comparison Experiments with the Natural and Synthetic Specimens of Phakellistatin 5 (1). The comparative TLC R_f values (in 5:4:1 hexane−DCM−methanol), melting points, and optical rotations (in methanol) for the cyclopeptides were found to be as follows, respectively: cyclopeptide phakellistatin 5 (**1**, natural) 0.30, 197−199°, $[\alpha]^{25}_D$ −102 (*c* 2.28); (**1**, synthetic) 0.30, 216−218°, $[\alpha]^{25}_D$ −103 (*c* 1.67); and (*R*)-Asn-phakellistatin 5 (**2**, synthetic) 0.37, 175−178°, $[\alpha]^{25}_D$ −130 (*c* 1.00). Comparative ^{1}H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD) experiments were performed in parallel using 5 mg of phakellistatin 5 (**1**) (both synthetic and natural) and 4 mg of (*R*)-Asn-phakellistatin 5 (**2**). Overlay of the ^{1}H NMR spectra (Figure 1) reveals the pronounced differences between cyclic peptides **1** and **2**. The overlap of the doublets from the alanine units of peptide **2** corresponding to the downfield shift of Ala[1] β (1.36 ppm) and the slight upfield shift of Ala[2] β (1.39 ppm) is apparent, whereas the chemical shift separation of the alanine units in peptide **1** is almost 0.1 ppm. A slight downfield shift was observed for Asn β H_a (3.25 ppm) in peptide **1**. For peptide **2** the α protons exhibited better resolution mainly owing to Pro α, Met α, and Ala[1] α shifting downfield. The ^{13}C NMR spectrum of peptide **2** contained downfield shifts corresponding to Ala[1] β and Pro β (16.9 and 31.3 ppm, respectively) and upfield shifts for Met γ, Asn α, and Ile α (31.0, 50.8, and 58.1 ppm, respectively) compared to synthetic and natural **1**.

Hydrolyses and Chiral HPLC Amino Acid Analyses. Each 0.3-mg sample (natural phakellistatin 5, synthetic phakellistatin 5, and peptide **2**) was hydrolyzed in 6N HCl (0.6 mL) at 110 °C for 45 h in a sealed tube. After reaction, the remaining hydrochloric acid in each hydrolysate was removed under a stream of nitrogen. The hydrolysates were diluted with methanol, and the solvent was evaporated in vacuo (repeatedly). Finally, the samples were dried in vacuo for 5 h.

Authentic samples of L- and D-aspartic acid (Asp) were analyzed by chiral HPLC using a CHIREX 3126 columnn [4.6 × 50 mm, ligand-exchange type based on (*N*,*S*)-octyl-D-penicillamine complexed with copper(II), Phenomenex] eluted with 2 mM $CuSO_4$−CH_3CN (95:5, flow rate: 1 mL/min). The retention times of L- and D-Asp were 4.563 and 5.765 min, respectively. Chiral HPLC examination of the hydrolysates obtained from natural peptide **1**, synthetic peptide **1**, and peptide **2** were carried out under the same analytical conditions showing the retention times of L-Asp to be 4.767 min in natural peptide **1** and 4.497 min in synthetic peptide **1**, while that of D-Asp from peptide **2** was 5.828 min. Based upon these direct comparisons of retention times, the absolute configuration of the Asn unit in both natural and synthetic phakellistatin 5 (**1**) was identified as (*S*) and the Asn unit in synthetic peptide **2** as (*R*).

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