Solid-Phase Total Synthesis of Cyclic Decapeptide Phakellistatin 12

Liaqat Ali, Syed Ghulam Musharraf, and Farzana Shaheen*

H. E. J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan

Received November 14, 2007

Pakellistatin 12 (**1**) is a new cancer cell growth inhibitory (P388 ED_{50} 2.8 μg/mL) cyclodecapeptide that was isolated from a marine sponge *Phakellia* sp. The first total synthesis of compound **1** is reported here using solid-phase methodology with safety-catch linker strategy. For the sequence of amino acids in the cycle, the peptide product was analyzed with a MALDI TOF/TOF instrument, and the structure of the synthetic product was found to be chemically and spectroscopically identical to the natural substance.

Marine natural products have been a rich source of new medicinal agents for the treatment of cancer, AIDS, etc. Dolastatins,[1] didemnin B,[2] bryostatin 1,[3] aplidin,[4] and kahalalide F[5] are some of the naturally occurring cyclic peptides that have recently undergone clinical trials. Cyclic peptides have several advantages as potential drug candidates. Unlike linear peptides, they do not have charges at the peptide amino and carboxyl termini and lack zwitterionic character. Therefore they are more lipophilic and membrane permeable. Oral bioavailability is increased by faster membrane absorption in the digestive tract, and cyclic peptides have much greater half-lives *in vivo* than linear peptides. Because of these advantages, many laboratories have focused on the syntheses of cyclic peptides and cyclic depsipeptides, especially by solid-phase peptide synthesis methodologies.[6]

Homodetic cyclopeptides of the "proline-rich" class are mainly distributed in marine environments, but are found also in higher plants.[7] They have attracted great interest owing to their remarkable pharmacological activities, such as antiproliferative and cytotoxic effects, and also due to their peculiar structural aspects that make more challenging their spectroscopic analysis as well as their chemical synthesis. It is therefore of interest to synthesize these compounds and to generate molecular diversity based on these peptides in a search of new biological activities or to optimize their original activities.[8]

Over the past 20 years, several anticancer cyclic peptides have been isolated from the Western Pacific Ocean (Federated States of Micronesia-Ckuuk) marine sponge *Phakellia* sp.[9–14] As a result of these studies, a series of 14 members of phakellistatin (phakellistatin 1−14) have been isolated in addition to many other linear and cyclic peptides. Phakellistatin 12 is a new cancer cell growth inhibitory (P388, ED_{50} 2.8 μg/mL) cyclodecapeptide isolated by Pettit et al. in 2003, and the structure was assigned as *cyclo*-Ile-Phe-Thr-Leu-Pro-Pro-Tyr-Ile-Pro-Pro employing high-field NMR techniques.[15] Previously the same group has reported the isolation of the natural cyclooctapeptide phakellistatin 11 and cycloheptapeptides phakellistatins 2 and 5, which exhibited anticancer activity (P388 leukemia) with ED_{50} values of 0.2, 0.34, and 0.23 μg/mL, respectively. Subsequently the synthetic products were found to be inactive, exhibiting ED_{50} values between 10 and 20 μg/mL.[11–14] Phakellistatin 12 (**1**) has a close resemblance to an already reported cyclic decapeptide, phakellistatin 8, with a difference at the Thr^3 position, which is the valine residue in the case of phakellistatin 8.[7] Herein, we report for the first time the solid-phase total synthesis of the cytotoxic natural product phakellistatin 12 (**1**) using a safety-catch linker strategy.

* To whom correspondence should be addressed. E-mail: afnan.iccs@gmail.com. Tel: +92-21-4824930. Fax: +92-21-4819018, 19.

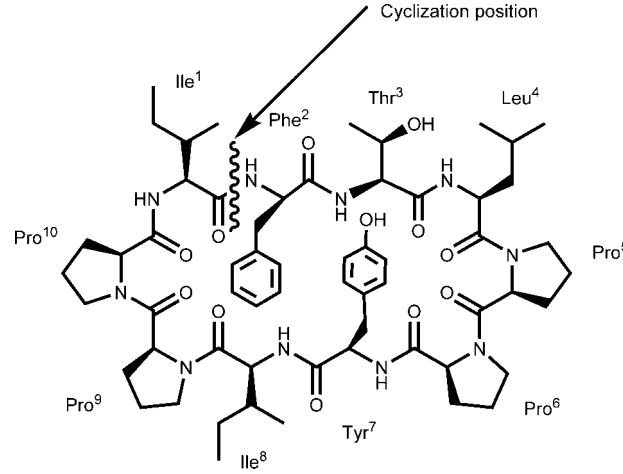


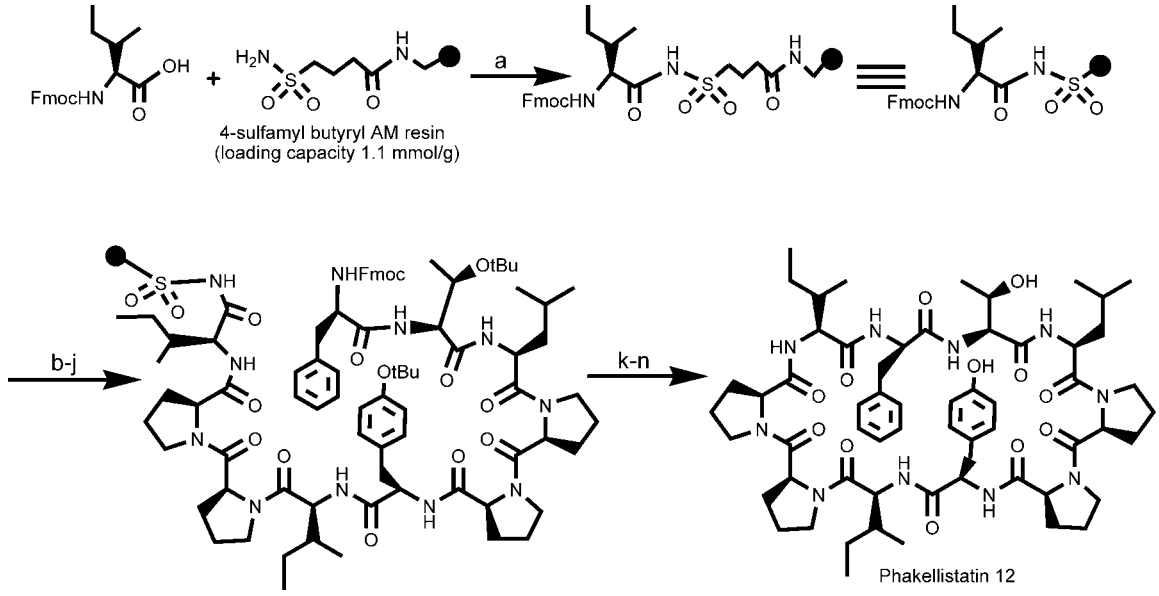
Figure 1. Phakellistatin 12 (**1**).

Cyclic head-to-tail connected peptides can easily be synthesized in good purity using standard procedures and orthogonally protected amino acid residues. However, the cyclization step is critical and can be very long depending on the peptide sequence, structural constraints, and the resulting ring size. A number of studies have been developed to improve this crucial step and to obtain cyclic peptides in good yield with minimum side reactions.[16,17]

In the present study the synthesis of phakellistatin 12 (**1**) was approached by solid-phase chemistry, using a Fmoc/tBu protecting scheme and 4-sulfamylbutyryl AM resin as solid support. The starting Fmoc-protected amino acid (Fmoc-Ile-OH) was loaded on 4-sulfamylbutyryl AM resin (Figure 1) by treatment with Hunig's base and the benzotriazole-1-yloxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBop) method.[18,19] The resulting loading degree was found to be 45%, determined by UV spectrophotometric analysis.[8] For the remaining linear peptide synthesis the standard Fmoc protocol was followed,[20] and the resin was submitted to coupling−deprotection cycles to build the linear decapeptide as the precursor for the cyclic phakellistatin 12 (**1**). All of the Fmoc-protected amino acids were activated by hydroxybenzotriazole/diisopropylcarbodiimide (HOBt/DIC) in the presence of *N,N*-dimethylformamide (DMF) before the next coupling was carried out. The progress of the amino acid coupling was checked through the ninhydrin colorimetric test (Kaiser test).[21] Fmoc deprotection before each coupling step was achieved by treatment of the resin-bound peptide with a 20% solution of piperidine in DMF. After the linear decapeptide was obtained, the Fmoc group from the N-terminal amino acid was removed and the N-terminal amine was reprotected with a trityl group followed by activation of the 4-sulfonamide linker by cyanomethylation. The resin was then

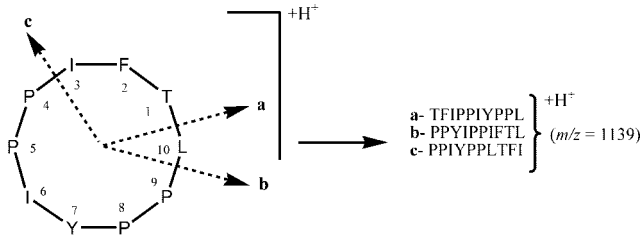
10.1021/np070648q CCC: $40.75
Published on Web 05/20/2008

Scheme 1. Synthesis of Phakellistatin 12[a]



[a] Reagents and conditions: (a) PyBop, DIEA, Fmoc-Ile-OH/DMF, 0 °C, 12 h, repeated twice; (b) (i) 20% piperidine/DMF, 1 h (ii) Fmoc-Pro-OH/DMF, DIC, HOBt, 4 h; (c) (i) 20% piperidine/DMF, 1 h (ii) Fmoc-Pro-OH/DMF, DIC, HOBt, 4 h; (d) (i) 20% piperidine/DMF, 1 h (ii) Fmoc-Ile-OH/DMF, DIC, HOBt, 4 h; (e) (i) 20% piperidine/DMF, 1 h (ii) Fmoc-Tyr(tBu)-OH/DMF, DIC, HOBt, 4 h; (f) (i) 20% piperidine/DMF, 1 h (ii) Fmoc-Pro-OH/DMF, DIC, HOBt, 4 h; (g) (i) 20% piperidine/DMF, 1 h (ii) Fmoc-Pro-OH/DMF, DIC, HOBt, 4 h; (h) (i) 20% piperidine/DMF, 1 h (ii) Fmoc-Leu-OH/DMF, DIC, HOBt, 4 h; (i) (i) 20% piperidine/DMF, 1 h (ii) Fmoc-Thr(tBu)-OH/DMF, DIC, HOBt, 4 h; (j) (i) 20% piperidine/DMF, 1 h (ii) Fmoc-Phe-OH/DMF, DIC, HOBt, 4 h; (k) (i) 20% piperidine/DMF, 1 h (ii) trityl chloride, DIEA; (l) ICH_2CN, DIEA, NMP, 20 h; (m) (i) 5%TFA/DCM, 0.5 h, (ii) DIEA, 20 h; (n) TFA/TIS/H_2O 9.5:2.5:2.5.

Scheme 2. Cleavage Sites of Protonated Phakellistan 12 (**1**) by MALDI-TOF-TOF-MS



Phakellistatin 12 (m/z = 1139)

deprotected, and cyclization and cleavage were accomplished simultaneously by using DIEA in tetrahydrofuran (THF). The side chain deprotection was carried out by treatment with TFA/TIS/H_2O (9.5:2.5:2.5) for 1 h with stirring.

Finally, the purification was carried out on recycling preparative reversed-phase HPLC (RP-HPLC) using a Jaigel Polyamine column and 1:1 ratio of water/acetonitrile as solvent system. The FABMS spectrum of the major peak in HPLC showed the molecular ion peak of the cyclized product at 1139.89 $[M + H]^+$.

The ESI-QTOF-MS of compound **1** displayed a protonated molecular ion peak $[M + H]^+$ at m/z 1139.6541, corresponding to the molecular formula $C_{60}H_{87}N_{10}O_{12}$ (calculated 1139.6499). Similarly, MALDI-TOF-TOF-MS of compound **1** showed $[M + H]^+$ at m/z 1139. Under Lift experiment[22] (MS/MS), it produced ions at m/z 1122 $[M + H - NH_3]^+$ and at m/z 1111 $[M + H - CO]^+$. Moreover, three peptides a, b, and c (Scheme 2) were produced by the cleavage of T^1-L^{10}, $L^{10}-P^9$, and P^4-I^3, respectively. Peptide "a" produced a main series of adjacent b_n peaks at m/z 1026, 929, 832, 669, and 556 corresponding to the successive loss of Leu, Pro, Pro, Tyr, and Ile, respectively. Similarly, y_n ions were detected at m/z 211, 308, 471, 584, 681, and 778 (Figure 2). Peptide "b" showed b'_n ions at m/z 1026, 925, and 778, while peptide "c" showed b''_n ions at m/z 1026, 879, and 778 (Figure 2).

The structure was further confirmed through NMR spectroscopy (see Experimental Section), and it was found to be spectroscopically identical with that of the natural product.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a JASCO DIP 360 polarimeter at the sodium D line (path length 50 mm). Proton NMR spectra were recorded on a Bruker NMR spectrometer operating at 600 MHz. Proton chemical shifts are reported in ppm. Data are reported as follows: chemical shift (multiplicity [singlet (s), doublet (d), triplet (t), quartet (q), and multiplet (m)], coupling constants [Hz], integration). Carbon NMR spectra were recorded on a Bruker NMR spectrometer operating at 600 (150 MHz), with complete proton decoupling. NMR data were collected at 25 °C. FAB mass spectra were recorded on JEOL JMS HX 110 mass spectrometers. ESI mass spectra were recorded on QSTAR XL (Applied Biosystem). The capillary voltage was maintained between 5 and 5.5 kV. Analytical thin-layer chromatography (TLC) was performed using silica gel 60 Å F-254 precoated plates (0.25 mm thickness). Visualization was accomplished after spraying with cerric sulfate reagent. Preparative reversed-phase HPLC separation was performed using a polyamine column using elution with 1:1 acetonitrile/water.

Materials. Protected amino acids, 4-sulfamylbutyryl AM resin, PyBOP, and HOBt were purchased from Novabiochem. All other reagents and chemicals were purchased from Novabiochem, Fluka, and Aldrich, and were used without further purification. Anhydrous organic solvents were obtained either from Aldrich or distilled from the drying agents: Na or CaH_2. All reactions were carried out under a N_2 atmosphere employing oven- or flame-dried glassware. All solvents were either distilled or obtained from passing through activated alumina.

Peptide Synthesis and Screens. For all reactions, commercially available 4-sulfamylbutyryl-aminomethyl resin (loading level; 1.1 mmol/g) from Novabiochem was used. Peptide synthesis was accomplished manually using a stepwise solid-phase procedure. Agitations were achieved with an orbital shaker or magnetic stirrer. The yield and scale of the solid-phase reactions are given with regard to the amount of the first amino acid coupled to the resin. The resin loading was determined by measuring the Fmoc groups remaining on the resin by the established UV method. For this, a small amount of resin (~1 mg) was treated with 20% piperidine/DMF solution (10 mL) for 30 min, and the UV absorption of the solution at 301 nm ($\varepsilon = 7800\ M^{-1}\ cm^{-1}$) was determined.

Loading Conditions for the First Amino Acid. Fmoc-Ile-OH (1.555 g) was coupled to the resin using PyBOP (2.289 g) and DIPEA (1.5 mL) for 12 h at 0 °C in DMF. This procedure was repeated twice for effective loading of the building blocks. The Fmoc group was removed

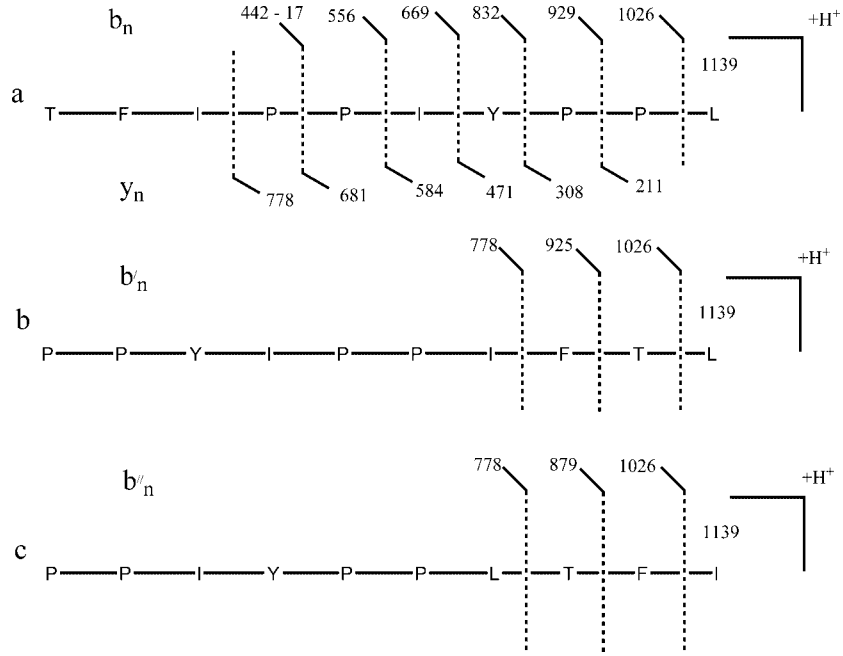


Figure 2. Fragmentation of protonated phakellistan 12 (**1**).

by the addition of a 20% solution of piperidine/DMF and shaking the reaction mixture on an orbital shaker for about 1 h. The resulting solution was filtered and washed sequentially with DMF, DCM, and DMF (3 × 25 mL each). After washing the resin was dried in a vacuum overnight.

General Methods for the Coupling of Further Amino Acids. After the first amino acid had been loaded, all protected amino acids were coupled by using HOBt/DIC chemistry. Typically, the amino acid (AA) (3 equiv) was treated for 2 min with HOBt (505 mg, 3 equiv) and DIC (520 μL, 3 equiv) in DMF. The solution was added to the resin, which was then agitated for 4 h at room temperature. After coupling and Fmoc deprotection with 20% piperidine/DMF the resin was washed three times with DMF and DCM, respectively. Every step was carried out under a N_2 atmosphere except for the washings.

General Procedure for the Activation with Iodoacetonitrile. The amino group of the resin-bound linear peptide was protected by a trityl group after deprotection of the Fmoc group from the terminal amino acid. The reaction was carried out by treating it with trityl chloride (1.227 g, 4 equiv) in the presence of DIEA (1.5 mL, 8 equiv). The resin-bound *N*-acylsulfonamide was then soaked in DMF and washed three times with NMP (3 × 25 mL). After filtration through an alumina basic plug prior to use, a mixture of iodoacetonitrile (0.8 mL, 10 equiv) and DIEA (2.3 mL, 12 equiv) in NMP (4 mL) was added to the resin in a N_2 atmoshphere, and the reaction flask was shielded from light. The resin was agitated for 20 h, filtered, and successively washed with NMP (5 × 25 mL) and DCM (5 × 25 mL). The activation step was repeated. The trityl group was deprotected by treating the reaction mixture with 25 mL of 5% TFA/DCM for 0.5 h followed by washing with THF (3 × 25 mL).

Cyclization and Cleavage of Peptides from the Solid Support. Immediately after washing, the activated *N*-acylsulfonamide was soaked in THF for 1 h and treated with DIEA (565 μL, 3 equiv) for 20 h under a N_2 atmosphere. The resin was filtered and washed with THF and DCM (3 × 25 mL each). The solvents were evaporated under reduced pressure, and the crude residue was dissolved in a small quantity of MeOH, recrystallized several times with cold diethyl ether, and dried *in vacuo*. The cyclization process was repeated twice.

Removal of tBu Protecting Groups. The removal of the tBu group was carried out by stirring the cyclized product with a 5 mL mixture (v/v) of TFA/TIS/H_2O (9.5:2.5:2.5) for 1 h at room temperature. The solvent was evaporated under reduced pressure, and the product was recrystallized from cold diethyl ether.

MALDI-TOF-TOF-MS Analysis of Compound 1. Matrix-assisted laser desorption/ionization was carried out on Ultraflex III TOF/TOF (Bruker Daltonics, Bremen, Germany) mass spectrometer. Compound **1** (5 pmol in MeOH/H_2O, 1:1, with 0.1% TFA) was mixed with 0.5 μL of the matrix (DHB) solution (saturated solution in 0.1% TFA/CH_3CN, 2:1) and deposited on a MALDI plate. Mass was recorded with the ion source 1 (IS1) set to 25.00 kV and ion source 2 (IS2) set to 21.50 kV and without delay extraction (DE). These experiments were performed with the laser energies of 70−80 μJ. The Lift experiment was performed on the same instrument with IS1 set to 8.00 kV and IS2 set to 7.15 kV, and Lift 1 set to 19.00 kV and without DE. The laser energies used were 75−85 μJ. The validation of data obtained, including baseline subtraction of the TOF data, external calibration using peptide calibration standard (Bruker Daltonics, Bremen, Germany), and all further data processing was carried out by using Flex analysis 2.0 post analysis software and for data acquisition by Flex control 2.0. Lift experiment on *m/z* 1139 $[M + H]^+$ *m/z* (%): 1122 (9), 1111 (100), 1026 (88), 929 (6), 925 (9),879 (1), 832 (31), 778 (7),669 (99), 556 (15).

Characterization of Cyclic Peptide Product, Phakellistatin 12 (1). [*cyclo*-(Ile[1]-Phe[2]-Thr(OH)[3]-Leu[4]-Pro[5]-Pro[6]-Tyr(OH)[7]-Ile[8]-Pro[9]-Pro[10]] was synthesized starting from 1 g of 4-sulfamylbutyryl AM resin (1.1 mmol). Overall yield = 5.2%; $[\alpha]_D^{30}$ −39 (*c* 0.007, MeOH); ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.79 (s, 1H), 7.69−7.67 (m, 2H), 7.59 (s, 1H), 7.51 (s, 1H), 7.49 (s, 1H), 7.43−7.35 (m, 3H), 7.41 (s, 1H), 7.33 (s, 1H), 6.82−6.72 (m, 4H), 3.85−3.52 (m, 23H), 1.91−1.71 (m, 19H), 2.48 (m, 1H), 2.28 (m, 1H), 2.24 (m, 1H), 2.54 (m, 1H), 2.15 (m, 1H), 2.18 (m, 1H), 1.29 (d, J = 6.8 Hz, 3H), 1.35 (d, J = 6.6 Hz, 3H), 1.39 (d, J = 6.6 Hz, 3H), 1.44 (d, J = 6.5 Hz, 3H), 1.12 (t, J = 6.1 Hz, 3H), 1.07 (d, J = 5.8 Hz, 3H), 0.85 (t, J = 7.0,6.8 Hz, 3H); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 174.4, 173.2, 173.1, 172.9, 172.1, 172.0, 171.9, 171.8, 171.7, 170.9, 162.1, 140.8, 140.1, 130.9, 129.1, 128.8, 127.9, 124.5, 118.7, 65.4, 61.4, 61.1, 55.2, 54.6, 53.5, 53.1, 51.9, 51.7, 49.6, 46.9, 46.8, 39.9, 39.5, 32.3, 24.0, 19.3, 18.2, 17.7, 17.3, 14.1, 11.5, 10.2; FABMS, *m/z* 1139.89 ($[M + H]^+$); ESI-QTOF-MS (+ve), *m/z* 1139.6541 $[M + H]^+$ ($C_{60}H_{87}N_{10}O_{12}$, calcd, for 1139.6499); MALDI-TOF-TOF-MS, *m/z* 1139 $[M + H]^+$.

Acknowledgment. One of us (L.A.) is thankful to the Higher Education Commission of Pakistan for financial support.

References and Notes

(1) Bai, R.; Verdier-Pinard, P.; Gangwar, S.; Stessman, C. C.; Mcclure, K. J.; Sausville, E. A.; Pettit, G. R.; Bates, R. B.; Hamel, E. *Mol. Pharmacol.* **2001**, *59*, 462–469.
(2) Baker, M. A.; Grubb, D. R.; Lawen, A. *Apoptosis* **2002**, *7*, 407–412.
(3) Mohammad, R. M.; Adsay, N. V.; Philip, P. A.; Pettit, G. R.; Vaitkevicius, V. K.; Sarkar, F. H. *Anticancer Drugs* **2001**, *12*, 735–740.
(4) Jimeno, J.; Faircloth, G.; Fernandez Sousa-Faro, J. M.; Scheuer, P.; Rinehart, K. *Mar. Drugs* **2004**, *2*, 14–29.

(5) Hamann, M. T.; Scheuer, P. J. *J. Am. Chem. Soc.* **1993**, *115*, 5825–5826.
(6) Gu, W.; Silverman, R. B. *J. Org. Chem.* **2003**, *68*, 8774–8779.
(7) Napolitano, A.; Bruno, I.; Riccio, R.; Gomez-Paloma, L. *Tetrahedron* **2005**, *61*, 6808–6815.
(8) Qin, C.; Bu, X.; Wu, X.; Guo, Z. *J. Comb. Chem.* **2003**, *5*, 353–355.
(9) Pettit, G. R.; Tan, R. *J. Nat. Prod.* **2005**, *68*, 60–63.
(10) Pettit, G. R.; Lippert, J. W., III; Taylor, S. R.; Tan, R.; Williams, M. D. *J. Nat. Prod.* **2001**, *64*, 883–891.
(11) Pettit, G. R.; Tan, R.; Williams, M. D.; Tackett, L.; Schmidt, J. M. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 2869–2874.
(12) Pettit, G. R.; Xu, J. P.; Cichacz, Z. A.; Williams, M. D.; Dorsaz, A. C.; Brune, D. C.; Boyd, M. R.; Cerny, R. L. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 2091–2096.
(13) Pettit, G. R.; Rhodes, M. R.; Tan, R. *J. Nat. Prod.* **1999**, *62*, 409–414.
(14) Pettit, G. R.; Toki, B. E.; Xu, J. P.; Brune, D. C. *J. Nat. Prod.* **2000**, *63*, 22–28.
(15) Pettit, G. R.; Tan, R. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 685–688.
(16) Yang, L.; Morriello, G. *Tetrahedron Lett.* **1999**, *40*, 8197–8200.
(17) Li, P.; Roller, P. P.; Xu, J. *Curr. Org. Chem.* **2002**, *6*, 411–440.
(18) Isied, S. A.; Kuehn, C. G.; Lyon, J. M.; Merrifield, R. B. *J. Am. Chem. Soc.* **1982**, *104*, 2632–2634.
(19) Osapay, G.; Profit, A.; Taylor, J. W. *Tetrahedron Lett.* **1990**, *31*, 6121–6124.
(20) Veber, D. F.; Saperstein, R.; Nutt, R. F.; Freidinger, R. M.; Brady, S. F.; Curley, P.; Perlow, D. S.; Paleveda, W. J.; Colton, C. D.; Zacchei, A. G.; Tocco, D. J.; Hoff, D. R.; Vandlen, R. L.; Gerich, J. E.; Hall, L.; Mandarino, L.; Cordes, E. H.; Anderson, P. S.; Hirschmann, R. *Life Sci.* **1984**, *34*, 1371–1378.
(21) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 595–598.
(22) Moneti, G.; Francese, S.; Mastrobuoni, G.; Pieraccini, G.; Seraglia, R.; Valitutti, G.; Traldi, P. *J. Mass Spectrom.* **2007**, *42*, 117–126.

NP070648Q