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The First Solid-phase Synthesis and Structural Studies on Phakellistatin 15

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Abstract: Phakellistatin 15 (**1**) is a cyclic octapeptide, containing three proline moieties, all in the *trans* form. It is a constituent of South China Sea sponge *Phakella fusca* and reported to have antitumor activity. This study describes the first solid-phase total synthesis of peptide **1** by using Kenner's *N*-acyl sulfonamide "safety-catch" linker. The structural analysis of peptide **1** was performed by mass, NMR and CD studies. The spectral data of synthetic peptide **1** was found to be identical to that reported for natural peptide. However, the synthetic peptide was found to be inactive on human glioblastoma (U-87), pancreatic cancer (PSN-1) and human non-small cell lung cancer (NCI-H460) cells.

Keywords: Cyclic peptide; phakellistatin; solid-phase synthesis; safety-catch linker. © 2016 ACG Publications. All rights reserved.

1. Introduction

Biologically active cyclic peptides are widely present in higher plants, microorganisms, and marine organisms [1,2]. They are membrane permeable molecules with faster membrane absorption and bioavailability than their linear counterparts. The syntheses of cyclic peptides by various approaches of solid-phase synthesis have been studied by many investigators in search of new bioactive agents [3-6].

Several cyclic peptides have been isolated from different species of marine sponges Phakella named as phakellistatins [7-13]. All members of phakellistatin family are reported as proline rich cyclic peptides with potent antitumor activity [7,10-12]. First member of this class was obtained from a marine sponge in the early nineties, named as phakellistatin 1 [10]. Until now 19 members of phakellistatins have been reported with potent antitumor activities. Previously phakellistatins 1 to 14 and 19 were successfully synthesized by using solid-phase/ solution-phase methodologies [7,9-22]. However, most of the synthesized phakellistatins have exhibited less or no activity against cancer cell lines [14,15,18-20].

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This biological incongruity has been proposed to be due the presence of cytotoxic impurities in the natural phakellistatins or a conformational discrepancy caused by the presence of many Pro residues in these small cyclic peptides [15]. This phenomenon has been exhibited by many other proline-rich cyclopeptides such as stylopeptides, axinastins and cherimolacyclopeptide E.

Recently, phakellistatins 15-18 were isolated from the South China Sea sponge *Phakella fusca.* Phakellistatin 15 is an octacyclopeptide containing three proline residues, all in the *trans* form. It has exhibited cancer cell growth inhibitory activity against P388 cell line (IC_{50} 8.5 μM). Phakellistatins 15 and 17 have analogous sequences, Pro-Trp-Val/Ile-Leu-Thr/Ile-Pro-Leu/Ile. They also structurally resemble with the cyclic peptide hymenamide H, reported from *Hymeniacidon* sp [13].

The current investigation was focused on the synthesis of phakellistatin 15 by using a safety-catch linker strategy, and its structural studies. Phakellistatin 15 was tested against different cancer cell line and it was found to be inactive.

2. Materials and Methods

2.1. Instruments and Materials

Bruker (Germany) 500 and 600 MHz NMR spectrometers were used for recording Proton and Carbon-13 NMR spectra. FAB/MS were recorded on JEOL JMS HX 110 mass spectrometer Japan. ESI mass spectra were recorded on QSTAR XL MS/MS systems (Applied Biosystem Spectrometer, USA). The capillary voltage was maintained between 5 and 5.5 kV. Silica gel 60 Å F-254 coated plates (0.25 mm thickness) (Merck, Germany) were used for thin-layer chromatography (TLC). Preparative recycling HPLC (Japan Analytical Industry Co., Ltd.) separation was performed by using a Jaigel ODS-MAT 80 (C18) column (Japan) using solvent system acetonitrile / water (60:40). Analytical HPLC profile was taken on Waters 2695 (Japan) by using column Poroshell 120 EC-C-18 (I.D. 3 mm, length 50 mm) particle size 2.7 µm and Optical rotation was recorded on JASCO DIP 360 polarimeter (Japan). 4-Sulfamylbutyryl AM resin was purchased from Novabiochem (Germany). Protected amino acids, PyBOP, Oxymapure and Fmoc amino acids were purchased from Chem-Impex (USA). All other chemicals and reagents were obtained from Aldrich and Novabiochem.

2.2. Peptide Synthesis

4-Sulfamylbutyryl-aminomethyl resin (0.8 mmol/g) was used as solid support in the synthesis. Solid-phase synthesis was accomplished manually in 10 mL polypropylene syringe fitted with filter disc and agitation was performed on an orbital shaker. The resin loading level was determined by UV method [5,16,23].

2.3. Loading Conditions for the First Amino Acid

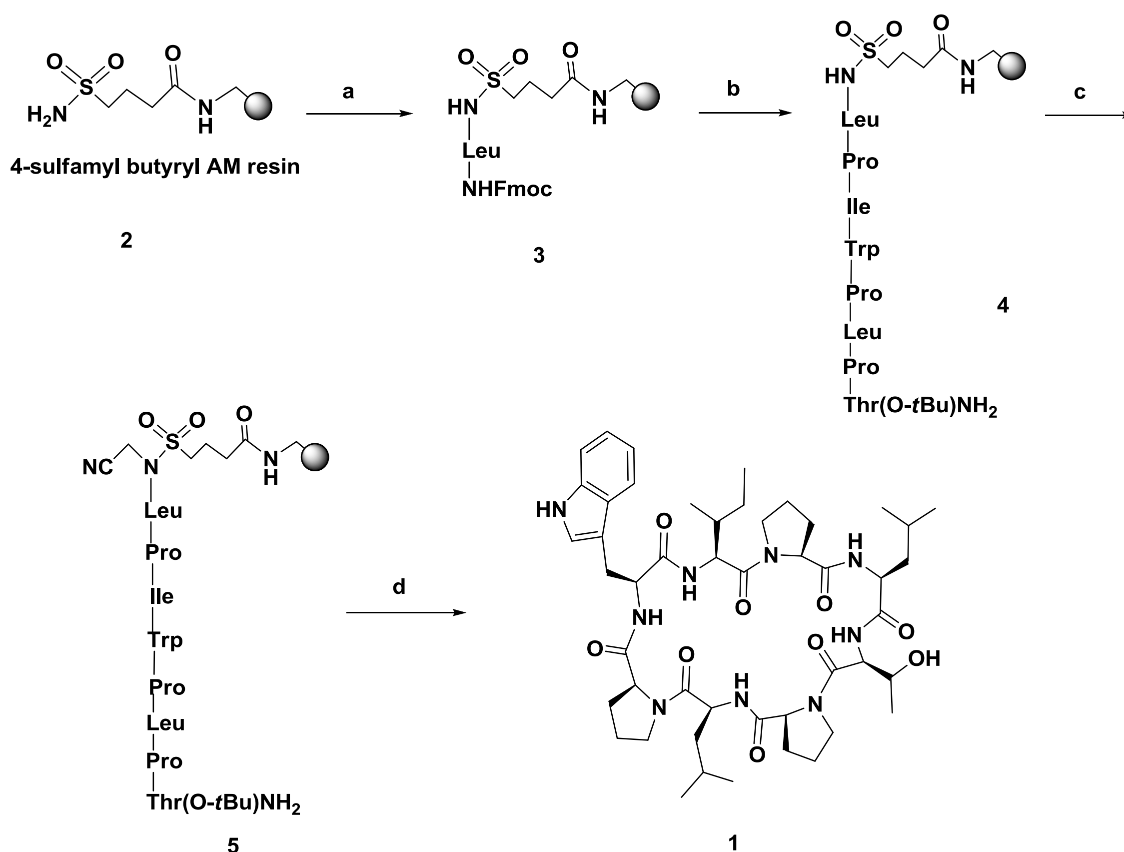
Fmoc-Leu-OH (4 mmol, 5 equiv) was attached to the resin by employing PyBOP (4 mmol, 5 equiv) and DIEA (6.4 mmol, 8 equiv) for 24 h at 0 °C in DCM / DMF. This step was repeated thrice to achieve maximum loading of first residue on resin. The Fmoc group was removed by the addition of 4-methylpiperidine / DMF (1:4) and the reaction mixture was shaked for about 20 minutes. After washing with DMF and DCM (3 × 25 mL each), the resin was dried completely in *vacuo*.

2.4. Activation of Linker and Synthesis of Linear Peptidyl Resin 4

The peptidyl resin **4** was synthesized by Fmoc chemistry by using oxymapure / DIC as coupling agents. In each coupling step, the Fmoc protected amino acid (Fmoc-AA) (5 equiv) was activated with oxymapure (4 mmol, 5 equiv) and DIC (4 mmol, 5 equiv) in DMF / DCM. The

activated amino acid solution was added to the resin in 10 mL polypropylene syringe and then agitated for 3 to 4 h on orbital shaker. After each coupling step, Fmoc deprotection was achieved with 4-methylpiperidine / DMF (1:4) followed by washing of resin with DMF and DCM.

The Fmoc deprotection of terminal amino acid of the resin-bound peptide **4** was achieved with 4-methylpiperidine / DMF (1:4). The free amino group was re protected by treating the resin with trityl chloride (4 mmol, 5 equiv) and DIEA (6.4 mmol, 8 equiv) in DCM. After washing the resin-bound peptide was soaked in NMP. A mixture of iodoacetonitrile (6.4 mmol, 8 equiv), and DIEA (6.4 mmol, 8 equiv) in NMP (5 mL) was added to the resin in a nitrogen atmosphere. The resin mixture was protected from light and shake for 24 h. It was then subjected to filtration and washing with NMP, DMF and DCM (5 ×25 mL). The activation step was performed thrice. Removal of trityl group was achieved by treating the resin mixture with 5% TFA/DCM + 2 drops TIS for 1 h. The resin was then thoroughly washed with THF.



Reagents and reaction conditions: PyBOP method (a) resin (0.8 mmol/g), Fmoc-L-Leu (5 equiv.), DIEA (8 eq.), PyBOP (5 equiv.); (b) (i) 4-methylpiperidine / DMF (1:4); (ii) Fmoc-L-Pro-OH, DIC, Oxymapure (5 equiv. each); (iii) 4-methylpiperidine / DMF (1:4); (iv) Fmoc-L-Ile-OH, DIC, Oxymapure (5 equiv. each); (v) 4-methylpiperidine / DMF (1:4); (vi) Fmoc-L-Trp-OH, DIC, Oxymapure (5 equiv. each); (vii) 4-methylpiperidine / DMF (1:4); (viii) Fmoc-L-Pro-OH, DIC, Oxymapure (5 equiv. each); (ix) 4-methylpiperidine / DMF (1:4); (x) Fmoc-L-Leu-OH, DIC, Oxymapure (5 equiv. each); (xi) Fmoc-L-Pro-OH, DIC, Oxymapure (5 equiv. each); (xii) 4-methylpiperidine / DMF (1:4); (xiii) Fmoc-L-Thr(O-*t*Bu-OH, DIC, Oxymapure (5 equiv. each); (xiv) 4-methylpiperidine / DMF (1:4); (c) (i) Trt-Cl (5 eq.), DIEA (8 eq.); (ii) ICH_2CN (8 eq.), DIEA (8 equiv.); (iii) 5% TFA/CH_2CL_2/TIS; (d) (i) DIEA / THF (1:4), (ii) TFA/H_2O/TIS (9.5:2.5:2.5).

Figure 1. Solid-Phase total synthesis of Phakellistatin 15.

2.5 Cleavage of Peptides from Resin after Cyclization

Peptide resin **5** was soaked in THF. It was then treated with DIEA / THF (1:4) for 24 h in nitrogen atmosphere. The filtrate was collected in a glass vessel and solvent was removed under reduced pressure, the crude product was precipitated with cold diethyl ether as gummy material.

Removal of t-Bu Protecting Group

The removal of the *t*-butyl group from the protected peptide was carried out by treatment of 5 mL TFA/TIS/H_2O (9.5:2.5:2.5) mixture (v/v) for 1 h. The solvent was removed under reduced pressure and the crude peptide was lyophilized and further purified on RPHPLC.

2.6. Characterization of Synthetic Phakellistatin 15

(Cyclo Pro^1-Trp-Ile-Pro^2-Leu^1-Thr(OH)-Pro^3-Leu^2) (**1**). Yield 4.5%; FAB/MS *m/z* 918 $[M + H]^+$, *m/z* 940 $[M + Na]^+$; MS/MS (ESI+), Figure 1; ^{1}H NMR and ^{13}C NMR (Supporting information, Table S1); $[\alpha]^{25}_D$ -152 (*c* 0.002, MeOH); HR-TOF-ESIMS *m/z* 940.5239 $[M + Na]^+$ (calcd for $C_{48}H_{71}N_9O_9Na$, 940.5272).

2.7. CD and DLS Analysis

Circular dichroism (CD) conformational analysis of synthetic phakellistatin 15 (**1**) was performed at 25 °C in J-810 spectropolarimeter (JASCO, Japan) using a quartz cell of 0.1 cm path length. Sample was dissolved in DMSO and further diluted with sodium phosphate buffer, pH 7.4 (10 mM), 50% TFE and 50% ACN, at a final concentration of 10 µM**.** The far-UV (190-250 nm) CD spectra were acquired with bandwidth of 2 nm, response 2 sec, and scanning speed 50 nm/min. Each spectrum was the average of four scans and solvent spectra (i.e. PB7.4, 50% TFE and 50% ACN, respectively) were subtracted, followed by baseline correction and smoothing. Aggregation properties of the peptides under identical peptide and buffer/solvent concentrations were measured either by CD using high concentration of sample (30 µM) and/or by dynamic-light scattering (DLS) using Laser-Spectroscatter 201 (RiNA GmbH, Germany) as described earlier [24].

2.8. Cytotoxicity Assay

The MTT assay was used to determine the cytotoxicity of phakellistatin 15 on three cell lines from different origin i.e. pancreatic cancer cell line (PSN-1), glioblastoma cell line (U-87 MG) and lung cancer cell line (NCI-H460). This colorimetric assay utilizes the tetrazolium dye (3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT) that can be reduced only by live cells into insoluble formazan crystals [25] The cells were cultured in transparent 96-wells plate (1.0×10^4 cells/well) in DMEM containing 10% FBS and placed at 37 °C in 5% CO_2. Next day, cells were treated with different concentrations of phakellistatin 15 (25, 50, and 100 µM) and DMSO as a vehicle. Untreated cells were incubated with complete medium only. After 24 h of treatment, the culture medium was removed and 200 µL of fresh medium containing MTT reagent (5 mg/mL) was added to the wells, and the plate was further incubated at 37 °C for 4 h. The medium was then discarded and the formazan crystals were dissolved in dimethyl sulfoxide (DMSO). The absorbance was measured at 550 nm using spectrophotometer and the percentage inhibition was calculated. All the measurements were carried out in triplicate.
Percentage inhibition was calculated by using formula:
% Inhibition = (untreated – treated) x 100 /untreated

Table 1. ^{1}H (600 MHz, δ / ppm) and ^{13}C (150 MHz, δ / ppm) NMR data for Natural and Synthetic phakellistatin 15 (1).

Residue	^{13}C		^{1}H		**Residue**	^{13}C		^{1}H	
	Isolated	**Synthetic**	**Isolated**	**Synthetic**		**Isolated**	**Synthetic**	**Isolated**	**Synthetic**
Pro^{1}					γ	24.44 CH_2	24.38	a: 2.00 m	1.99 m
CO	171.13 C	171.31						b: 1.85 m	1.844 m
α	60.51 CH	60.56	3.85, t	3.83 t	δ	47.91 CH_2	48.04	a: 3.99 m	3.983 m
β	28.71 CH_2	28.86	a: 1.81 m	1.809 m				b: 3.59 dt	3.60 dt
			b: 1.63 m	1.628 m	**Leu^{1}**				
γ	24.38 CH_2	24.38	a: 1.99 m	1.99 m	NH			8.76 d	8.79 d
			b: 1.81 m	1.809 m	CO	169.74 C	169.87		
δ	46.66 CH_2	46.79	a: 3.75 m	3.746 m	α	52.96 CH	52.98	3.46 m	3.49 m
			b: 3.46 m	3.46 m	β	35.82 CH_2	35.85	a: 2.16 m	2.16 m
Trp								b: 1.66 m	1.66 m
NH			7.91 d	7.99 d	γ	24.6 CH	24.68	1.47 m	1.44 m
CO	170.30 C	170.40			δ	20.92 CH_3	21.00	0.87 d	0.868 d
α	55.66 CH	55.78	3.94 m	3.94 m	δ'	23.28 CH_3	23.50	0.85 d	0.856 d
β	24.58 CH_2	24.60	a: 3.47 m	3.464 m	**Thr**				
			b: 3.36 dd	3.36 m	NH			7.49 d	7.49 d
1	110.96 C	111.07			CO	168.77 C	168.88		
2	123.38 CH	123.55	6.97 br s	6.99 s	α	56.12 CH	56.19	4.87 dd	4.87 dd
3			10.76 br s	10.799 br s	β	67.65 CH	67.77	4.13 m	4.125 m
4	136.05 C	136.13			γ	18.99 CH_3	19.12	1.03 d	1.026 d
5	111.3 CH	111.45	7.32 d	7.32 d	OH			5.22 d	5.23 d
6	120.76 CH	120.93	7.05 t	7.049 t	**Pro^{3}**				
7	118.1 CH	118.26	6.97 t	6.97 t	CO	170.93 C	171.07		
8	117.93 CH	118.09	7.46 d	7.46 d	α	59.07 CH	59.16	4.46 dd	4.45 dd
9	127.06 C	127.13			β	28.31 CH_2	28.49	a: 2.06 m	2.06 m
Ile								b: 1.92 m	1.92 m
NH			7.40 d	7.40 d	γ	24.54 CH_2	24.70	a: 1.83 m	1.83 m
CO	170.11 C	170.17						b: 1.72 m	1.719 m
α	54.19 CH	54.29	4.67 t	4.67 t	δ	46.92 CH_2	47.04	a: 3.68 dt	3.68 m
β	33.01 CH	33.02	2.02 m	2.003 m				b: 3.54 dt	3.54 m
	23.31 CH_2	23.42	a: 1.36 m	1.357	**Leu^{2}**				
γ			b: 1.03 m	1.026	NH			8.13 d	8.14 d
δ	9.52 CH_3	9.68	0.76 t	0.761 t	CO	170.87 C	170.92		
β-Me	15.07 CH_3	15.23	0.78 d	0.798 d	α	48.14 CH	48.24	4.44 m	4.44 m
Pro^{2}					β	38.02 CH_2	38.02	a: 1.70 m	1.70 m
CO	170.55 C	170.68						b: 1.05 m	1.026 m
α	60.32 CH	60.41	4.10 t	4.09 t	γ	24.36 CH	24.47	1.54 m	1.46 m
β	29.61 CH_2	29.78	a: 2.15 m	2.149 m	δ	20.86 CH_3	20.92	0.81 d	0.83 d
			b: 1.71 m	1.705 m	δ'	23.02 CH_3	23.24	0.86 d	0.86 d

3. Results and Discussion

3.1. Peptide Synthesis

During cyclic peptide synthesis, the head-to-tail bonding of amino acids leading to linear peptide precursor is an easy step in solid phase synthesis, while the cyclization between the two ends of linear peptidyl precursor is a challenging step to obtain the desired cyclic peptide. A number of procedures have been developed for on-resin cyclization in good yields with minimum side products [26,27]. Previously, Kenner's sulphonamide safety-catch linker strategy has been successfully used in the synthesis of several cyclic peptides [23,28-32]. This strategy has also been used by us in the synthesis of phakellistatin 12 [16], as well as cherimolacyclopeptide E and its alanine-substituted analogues [5] In present studies, we successfully applied this strategy in the synthesis of phakellistatin 15 (**1**) by using 4-sulfamylbutyryl AM resin as a solid support. Benzotriazole-1-yloxy-*tris*-pyrrolidinophosphonium hexafluorophosphate (PyBop) / DIEA was employed as the coupling system to load Fmoc-Leu-OH on 4-sulfamylbutyryl AM resin. The loading extent was determined as 53% by using UV spectrophotometric method [5,16,23].

Fmoc protocol was followed for the formation of peptidyl resin **4**. During the synthesis, Fmoc amino acids were activated by treatment with ethyl (hydroxyimino) cyanoacetate (Oxymapure) / *N,N*-diisopropylcarbodiimide (DIC) in DMF. The progress of the amino acid linking was periodically monitored through the Kaiser test [33]. Deprotection of Fmoc group prior to each coupling step was accomplished by the treatment with 4-methylpiperidine / DMF (1:4) solution.

After the formation of linear octapeptide, the Fmoc group from the last amino acid was replaced with trityl group. The sulfonamide linker was activated by treating peptidyl resin with iodoacetonitrile in presence of DIEA, followed by removal of trityl group. On-resin cyclization and cleavage of peptide from resin was carried out in THF by treatment with base DIEA. Finally, the tertiary butyl group was removed by treatment with TFA/TIS/H_2O (9.5:2.5:2.5).
The isocratic solvent system (60:40 acetonitrile/water) was used on reverse phase HPLC with 3 mL / min flow rate at 254 nm wavelength. C18 column Jaigel ODS-MAT 80, length 250 mm, inner diameter 20 mm was used in the purification of product.

a_n(+1) 579 680
b_n(+1) 284 397 494 607 708 805 918 +
H-Pro^1--Trp-Ile-Pro^2-Leu^1-Thr-Pro^3-Leu^2
y_n(+1) 635 522 312 211

Figure 2. Fragmentation of protonated phakellistatin 15 (1).

3.2. Structure elucidation

The purified peptide exhibited molecular ion in FAB/MS in positive mode at *m/z* 918 $[M+H]^+$ and *m/z* 940 $[M+Na]^+$. An MS/MS study of molecular ion at *m/z* 918 was carried out on ESIQTOF QSTAR XL MS/MS system. The synthetic peptide **1** exhibited a similar fragmentations pattern (Fig. 1) as reported for the natural peptide [13].

^{1}H NMR studies were performed on Bruker 500 MHz, while ^{13}C and 2D NMR were recorded on Bruker 600 MHz NMR spectrometer. DMSO d_6 was used as a solvent for NMR experiments. The comparative studies of proton and carbon-13 NMR of natural and synthetic compound **1** are shown in Table 1. The ^{1}H NMR data of **1** showed 5 amide protons at δ 7.99 (1H, d, Trp-NHCO), 7.40 (1H, d, Ile-NHCO), 8.79 (1H, d, Leu^1-NHCO), 7.49 (1H, d, Thr-NHCO) and 8.14 (1H, d, Leu^2-NHCO), whereas the alpha protons were resonated at δ 3.83 (1H, t, Pro^1-CH), 3.91 (1H, m, Trp-CH), 4.64 (1H, t, Ile-CH), and 4.09 (1H, t, Pro^2-CH), 3.49 (1H, m, Leu^1-CH), 4.87 (1H, dd, Thr-CH), 4.46 (1H, m, Pro^3-CH), and 4.44 (1H, m, Leu^2-CH). Five signals were observed in aromatic region due to the aromatic protons of Trp, at δ 6.96, 7.32, 7.049, 7.46, and 6.96**.**

Carbonyl carbon in ^{13}C NMR spectrum were observed at δ 171.3 (Pro^1-CO), 170.4 (Trp-CO), 170.2 (Ile-CO), 170.7 (Pro^2-CO), 169.9 (Leu^1-CO), 168.9 (Thr-CO), 171.1 (Pro^3-CO) and 170.9 (Leu^2-CO) (Supporting information, Table S1). There are three proline residues in phakellistatin 15; all exist in *trans* forms in natural product [13]. The synthetic counterpart of **1** was also found to have all proline residues in *trans* configuration, as shown by the small ^{13}C NMR chemical shift difference [5,34,35] Pro^1, $\Delta\delta$ Cβ−Cγ = 4.48 ppm, Pro^2, $\Delta\delta$ Cβ−Cγ = 5.34 ppm and Pro^3, $\Delta\delta$ Cβ−Cγ = 3.79 ppm). NMR Spectral data of synthetic peptide **1** was similar to the reported spectral data of the natural product (Table 1).

3.3. Circular dichroism (CD)

Circular dichroism (CD) frequently employed to investigate the peptide conformations in solution [36]. As very little is known about the CD spectra of phakellistatin family, conformational analysis of the purified phakellistatin 15 (1) was also performed in 10 mM sodium phosphate buffer, pH 7.4 at 25 °C (Figure 2). CD Spectra of 1 at 10 and 30 μM concentrations showed identical band pattern (i.e. 218 nm negative band, followed by positive band at 196 nm), suggesting that there is no self-aggregation. Dynamic-light Scattering (DLS) studies under identical conditions also revealed no signal for any peptidyl aggregation.

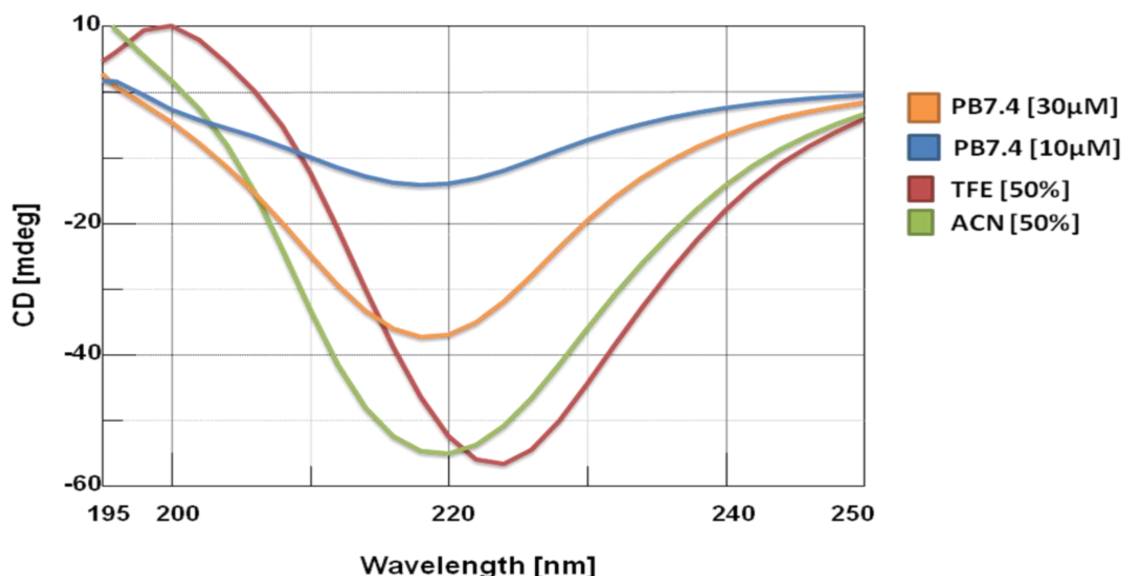


Figure 3. Circular dichroism spectra of phakellistatin 15 (10 μM and 30 μM) recorded in 10 mM sodium phosphate buffer, pH7.4. Effect of 50% ACN and 50% TFE in 10 μM phakellistatin 15 dramatically increases the ellipticity and induces blue and red shift transitions, respectively, suggesting the induction of possible secondary structure conformations.

Optical features observed near physiological conditions revealed predominantly beta-sheet and/or beta-turn structures as expected in case of short cyclo-peptides [37,38]. Furthermore addition of 50% ACN and 50% TFE in 10 μM phakellistatin 15 (**1**) dramatically increased the ellipticity and induced slight blue shift (i.e. λ 216 nm minima followed by λ 196 nm maxima for typical antiparallel beta-sheet), and significant red shift (i.e. λ 224 nm minima followed by λ 200 nm maxima for beta-turn and/or helical conformation), respectively, suggesting the possible transitions of phakellistatin 15 (**1**) into secondary structure conformations.

3.4. Cytotoxic effects of phakellistatin 15

Tetrazolium dye MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay was used to evaluate the cytotoxicity of phakellistatin 15 (**1**) against human glioblastoma (U-87), pancreatic (PSN-1), and human non-small cell lung cancer (NCI-H460) cell lines. Three different concentrations, along with vehicle (DMSO) and negative control (untreated), were assayed for 24-hours in triplicate. Absorbance data obtained from spectrophotometer clearly indicated that peptide **1** had no or less cytotoxic effects against these tumor cell lines.

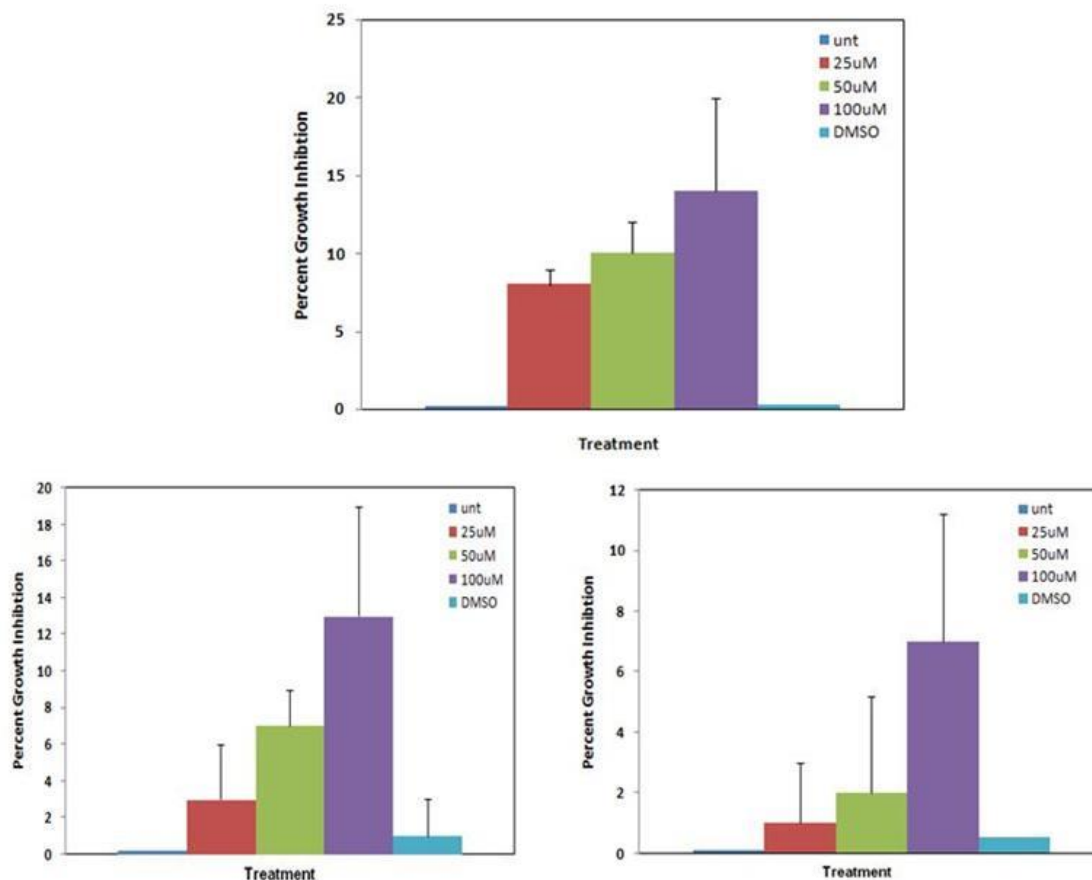


Figure 4. Cytotoxic effects of phakellistatin 15
(A) human glioblastoma U-87 cells, (B) pancreatic cancer cells (PSN-1) and (C) human non-small cell lung cancer (NCI-H460) cell lines assessed by MTT assay. Results indicates that phakellistatin 15 had very little cytotoxic effects against these cancer cells upto 100 µM concentration. The values in the graph are shown as mean percent inhibition ± standard deviation.

4. Conclusion

In conclusion, the first solid-phase total synthesis of phakellistatin 15, a natural cyclic octapeptide constituent of a Sea sponge *Phakella fusca*, was completed through 19 steps. Safety-catch linker strategy was employed in the synthesis which afforded 4.5% overall yield of phakellistatin 15. The synthetic phakellistatin 15 was found non-cytotoxic for human glioblastoma (U-87), pancreatic (PSN-1), and human non-small cell lung cancer (NCI-H460) cell lines. While the original natural phakellestatin 15 was reported to be cytotoxic for P388 murine lymphocytic leukemia cells.

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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