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Synthesis of the cyclic heptapeptide axinellin A

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ABSTRACT

The first synthesis of the naturally occurring cyclic peptide axinellin A has been achieved. Cyclization and subsequent deprotection of linear precursors containing either a *t*-butyl protected Thr residue or a Thr($\Psi^{Me,Me}$pro) derivative gave a cyclic peptide, identical in all respects to the naturally occurring material, with the exception that the synthetic peptide does not exhibit the cytotoxic activity reported for the natural product.

1. Introduction

Naturally occurring cyclic peptides have generated much interest in recent years owing to their intriguing chemical structures and potent biological activity. An emerging class of proline-rich cyclic peptides, isolated mainly from South Pacific marine organisms, are characterized by the presence of seven or eight amino acids with the presence of two or three proline residues. Examples of such natural products include the axinastatins,[1] the phakellistatins,[2–4] the hymenamides,[5,6] stylostatin 1,[7] the rollamides,[8] and the axinellins.[9,10] Compounds from this class are frequently isolated as mixtures of geometrical isomers, with the *cis*-and *trans*-amide conformers unable to interconvert due to steric constraints. This makes these structures demanding targets for synthesis. To date, the synthesis of a handful of cyclic peptides from this 'proline-rich' class of natural products has been achieved,[11–17] however the head-to-tail cyclizations of the representative linear peptides are generally low yielding operations. This is not surprising as the cyclization of small linear peptides (three to eight amino acids) is notoriously difficult, often resulting in epimerization of the C-terminal amino acid and/or the formation of linear and cyclic oligomers.[18,19] We recently reported on the use of pseudoprolines ($\Psi^{Me,Me}$pro) as removable turn-inducers to facilitate head-to-tail peptide cyclization.[20,21] Incorporation of these Thr-derived azaacetals into linear peptides was found to substantially increase the head-to-tail cyclization yields compared to those for the same peptides having *O*-TBS-protected Thr residues.[20] After cyclization the turn-inducers were readily removed to reveal free Thr residues. Having successfully utilized this methodology in the synthesis of the heptapeptide mahafacyclin B[21] it was of interest to investigate whether it could be used to improve the synthesis of the more conformationally constrained proline-rich peptides. In addition, we wanted to investigate whether the incorporation of a *cis*-amide inducing Thr($\Psi^{Me,Me}$pro) residue would affect the conformation of the cyclic peptide obtained after the removal of this protecting group. We herein report on the application of this methodology to the total synthesis of the naturally occurring proline-rich cyclic peptide, axinellin A. Axinellin A (**1**) is a cyclic heptapeptide [*cyclo*(Asn-Pro-Phe-Thr-Ile-Phe-Pro)], which was isolated together with axinellin B from the marine sponge Axinella carteri (Axinellidae).[9] The structure and conformation of axinellin A were elucidated by Randazzo et al., however, to date the structure has not been confirmed by synthesis. The natural product was reported to have moderate in vitro antitumor activity against a human bronchopulmonary non-small cell carcinoma cell line (IC_{50}=3.0 μg/mL against NSCLC-N6 cells).

2. Results and discussion

Axinellin A contains a single threonine residue, which makes it an ideal target on which to test our pseudoproline-assisted cyclization methodology. In order to place the Thr($\Psi^{Me,Me}$pro) residue near the center of the linear precursor, and to avoid epimerization of the C-terminal amino acid we chose a point of cyclization between Asn and Pro to give the linear precursor **2** with Asn as the N-terminal amino acid and Pro as the C-terminal amino acid (Scheme 1).

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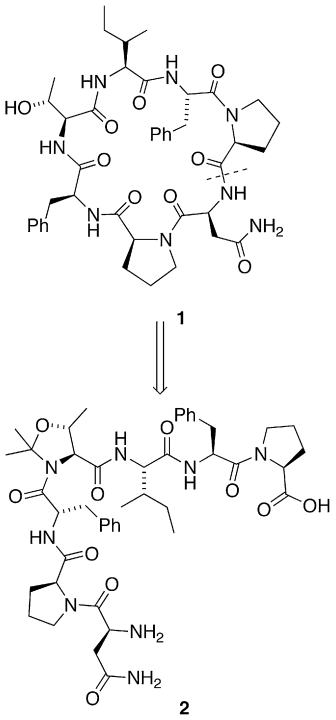
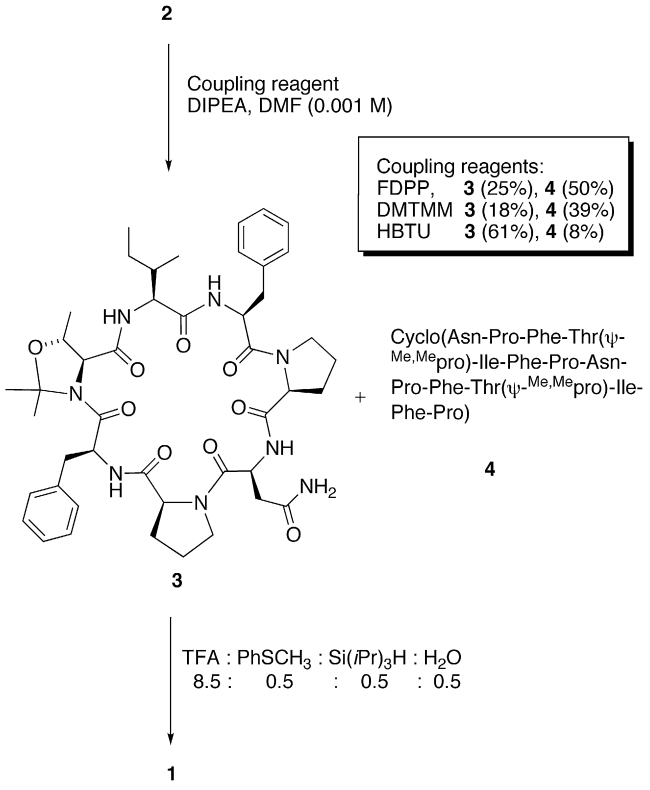


Scheme 1. Cyclization point and linear precursor for axinellin A (**1**).

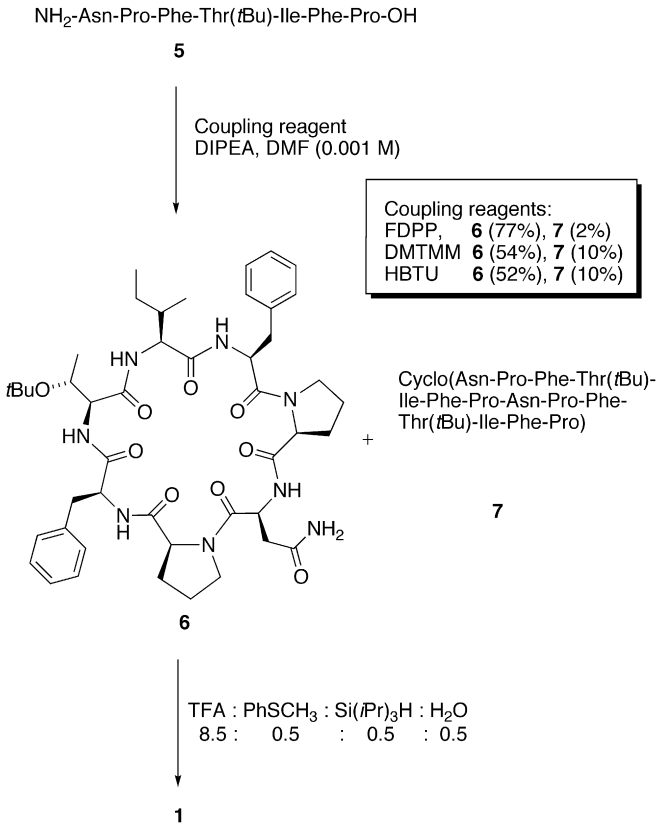


Scheme 2. Cyclization of pseudoproline containing linear precursor **2**.

The synthesis of the required linear peptide **2** was achieved by standard solid-phase peptide synthesis (SPPS), using Fmoc/HBTU chemistry on a PS3 automated peptide synthesizer, with the exception of the N-terminal Fmoc-Asn residue, which was coupled as the pentafluorophenyl ester to avoid side chain dehydration.[22] The 2-chlorotritylchloride resin was chosen as the solid support to enable cleavage of peptides from the solid phase with the side chain protecting groups intact. As anticipated, the cyclization of **2** was not straightforward. The reaction was attempted using a variety of coupling reagents under high dilution (0.001 M) in DMF. Using pentafluorophenyl diphenylphosphinate (FDPP), and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium tetrafluoroborate (DMTMM) as coupling reagents the corresponding cyclodimer **4** was obtained as the major product (50 and 39%, respectively) along with the desired cyclic peptide **3** as the minor product (25 and 18% yields, respectively). The best cyclization results were obtained using (*O*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) as the coupling reagent, and adding this together with **2** slowly over 24 h via syringe pumps. In this case a 61% yield of the desired **3** was obtained, along with a low yield of cyclodimer **4** (8%). Upon treatment of **3** with a solution of TFA: thioanisole: triisopropylsilane: H_2O (8.5:0.5:0.5:0.5) for four days, deprotection of the Thr($\Psi^{Me,Me}$pro) residue ensued to afford the cyclic peptide **1** in 75% yield (Scheme 2). This material was found to have physical and spectral properties identical to those reported for axinellin A, although the absolute optical rotation was found to be significantly higher {$[\alpha]_D$ −178.5 (*c* 0.2 MeOH)}, but of the same sign as that reported for the isolated material {$[\alpha]_D$ −98.2 (*c* 0.003 MeOH)}.[9] Notably, even though the pseudoproline containing precursor **3** is a mixture of conformers, as evidenced by doubling and/or broadening of signals in the ^1H and ^{13}C NMR spectra, the deprotected material has spectral data identical to the naturally occurring material, for which a single conformer is observed with *cis*-geometry about the Phe–Pro amide bond with all other amide bonds reported to adopt *trans*-geometries.[9] This indicates that, under the conditions required for pseudoproline deprotection, the cyclic peptide undergoes conformational changes to give the single conformer observed.

To determine if the Thr($\Psi^{Me,Me}$pro) residue was assisting **2** to adopt a conformation amenable to cyclization, we prepared and cyclized the linear peptide **5**, in which the Thr($\Psi^{Me,Me}$pro) residue was replaced with a *t*-butyl protected Thr residue. Linear heptapeptide **5** was synthesized in an analogous manner to **2**, and the head-to-tail cyclization was investigated using the same range of coupling reagents. We found that, in all cases, the desired cyclic peptide **6** was obtained as the major product in good yield and as a single conformer. Furthermore, the best cyclization results were obtained using FDPP as the coupling reagent. In this case 77% of cyclic peptide **6** was obtained, along with trace amounts of cyclodimer **7** (2%). The *t*-butyl protecting group in **6** was removed in an analogous manner to the *N*,*O*-isopropylidene group in **3** to afford axinellin A (**1**) in 80% yield (Scheme 3). The material obtained via this synthetic route was identical in all respects to that obtained above.

The cytotoxicity of synthetic axinellin A (**1**) and synthetic intermediates **3**, **4**, **6** and **7** was evaluated against the human bronchopulmonary non-small cell lung cancer lines NSCLC-N6 and A549 employing the same procedures and cell line (NSCLC-N6) as used previously for the naturally derived axinellin A (**1**). In all cases, the synthetic peptides were found to be inactive (all IC_{50}s>90 μM). Although difficult to explain, these results were not entirely unexpected. In fact, there have been many reports of large disagreements between the bioactivities of synthetic and natural proline-rich cyclic peptide samples.[11–14,16,17] Given that our synthetic sample of **1** was identical in every way to that of the natural material, including in the conformations about the amide bonds, perhaps the lack of activity can be explained by the naturally derived cyclic peptide retaining a chemically undetectable amount of strongly active cytotoxic compound, as has previously been suggested for the phakellistatins.[16,17]



Scheme 3. Cyclization of linear peptide **5**.

3. Conclusions

In summary, we have successfully completed the first total synthesis of the naturally occurring cyclic peptide, axinellin A from two linear precursors, and hence confirmed the elucidated structure. Contrary to our previous work in this area,[21] it appears that the introduction of a single pseudoproline turn-inducer into the linear precursor **2** does not enhance the head-to-tail cyclization yield, compared to that for linear precursor **5**, in which the Thr residue is protected by a *t*-butyl group. In the case of linear peptide **2** it appears that, while the incorporation of the Thr($\Psi^{Me,Me}$pro) residue results in the adoption of a predominantly *cisoid* conformation at the preceding amide bond, this does not provide an overall linear peptide conformation that is more favorable for cyclization than that adopted by the linear precursor **5**, and that the introduction of this conformational constraint instead favors the formation of the cyclodimer **4**. Nevertheless, good yields were obtained for the cyclizations of both the linear heptapeptide containing the pseudoproline residue, **2**, and that containing a *t*-butyl protected Thr residue, **5**, and the material obtained after removal of the Thr protecting groups from both the pseudoproline containing **3** and *t*-butyl protected **6** was identical. This indicates that, in the case of axinellin A, the incorporation of a *cis*-amide inducing pseudoproline residue in the linear precursor does not affect the geometry of the amide bonds in the deprotected cyclic product.

The total synthesis of **1** has confirmed the structure of the natural product as originally reported[9] and provided further material for biological testing. The lack of cytotoxic activity in our synthetic material, as previously observed for similar compounds from this class,[11–14,16,17] provides further evidence that the naturally derived proline-rich cyclic peptides may be masking small quantities of a highly cytotoxic compound.

4. Experimental

4.1. General procedures

Most reagents were commercially available reagent grade chemicals and were used without further purification. Dimethylformamide was dried over 4 Å molecular sieves prior to use. Dichloromethane was dried over CaH_2 and distilled prior to use. Melting points were determined using a Gallenkamp melting point apparatus and are reported in degrees Celsius (uncorrected). ^{1}H Nuclear magnetic resonance spectra were recorded at a frequency of 300 MHz or at a frequency of 400 MHz and are recorded as parts per million (ppm) downfield shift with deuteromethanol (δ_H 3.34), deuteroacetonitrile (δ_H 1.95) or deuteropyridine (δ_H 8.71, 7.55, 7.19) as internal references, unless otherwise stated. ^{13}C Nuclear magnetic resonance spectra were recorded at a frequency of 75.47 MHz or at a frequency of 100.62 MHz and are reported as parts per million (ppm) downfield shift with deuteromethanol (δ_C 49.00), deuteroacetonitrile (δ_H 118.2, 1.3) or deuteropyridine (δ_H 149.9, 135.5, 123.5) as an internal reference, unless otherwise stated. Optical rotations were measured on a dual wavelength polarimeter in a 2.5 cm cell at 22 °C using the indicated spectroscopic grade solvents. Analytical reverse phase high performance liquid chromatography (RP-HPLC) was performed using a Sunfire™ C_{18} column (5 μm, 2.1×150 mm ID). Flow rate was maintained at 0.2 mL/min over a linear gradient from 0 to 100% solvent B (solvent A: 100:0.01 v/v Milli-Q water/TFA, solvent B: 100:0.01 v/v acetonitrile/TFA) over 30 min and eluent monitored from 210 to 320 nm. Preparative RP-HPLC was performed using a Sunfire™ PrepC_{18} OBD™ column (5 μm, 19×150 mm ID). Flow rate was maintained at 7.0 mL/min over a linear gradient from 0% to 100% solvent B (solvent A: 100:0.01 v/v Milli-Q water/TFA, solvent B: 100:0.01 v/v acetonitrile/TFA) over 45 min and eluent monitored at wavelengths 214 and 280 nm.

4.2. General procedure for the synthesis of linear heptapeptides using solid-phase peptide synthesis (SPPS)

4.2.1. Loading of the resin. Under anhydrous conditions, 2-chlorotrityl chloride resin (1.0 g, loading 1.4 mmol/g) was swollen in CH_2Cl_2 (10 mL) for 10 min, then a solution of Fmoc-Pro-OH (1.42 g, 4.2 mmol) and *N*,*N*-diisopropylethylamine (DIPEA) (2.2 mL, 12.6 mmol) in CH_2Cl_2 (10 mL) was added. Dry N_2 was bubbled through the mixture for 1 h, then the mixture was gently agitated overnight. The solvent was drained and the resin was washed sequentially with CH_2Cl_2:MeOH:DIPEA (17:2:1, 3×10 mL), CH_2Cl_2 (2×10 mL), DMF (2×10 mL), CH_2Cl_2 (2×10 mL), and ether (2×10 mL). The resin was dried under vacuum overnight, to give the Fmoc-proline loaded resin (0.4–0.7 mmol/g). The proline loaded 2-chlorotrityl chloride resin (1.0 g, 0.4–0.7 mmol/g) was then placed in a PS3 automated peptide synthesizer, and the resin was washed with DMF (3×30 s).

4.2.2. Fmoc deprotection. The Fmoc group was removed with 20% piperidine in DMF (2×5 min), and then the resin was washed with DMF (6×30 s).

4.2.3. Peptide coupling. Fmoc-amino acid (2 equiv) and HBTU (2 equiv) were activated by treatment with a DIPEA/DMF mixture for 30 s, before addition to the reaction vessel. After 40 min the resin was washed with DMF (3×30 s).

4.2.4. Final coupling to form heptapeptide. The hexapeptide loaded 2-chlorotrityl chloride resin was removed from the peptide synthesizer and suspended in DMF. Fmoc-Asn-pentafluorophenyl ester (2 equiv) was added and dry N_2 was bubbled through the reaction vessel for 1 h. The solution was then drained and the resin washed

sequentially with DMF (2×10 mL), CH_2Cl_2 (2×10 mL), and ether (2×10 mL). A solution of 20% piperidine in DMF (10 mL) was then added to the resin and dry N_2 was bubbled through the mixture for 5 min, then the resin was washed with DMF (3×10 mL).

4.2.5. Cleavage from the resin. A mixture of hexafluoroisopropanol:trifluoroethanol:CH_2Cl_2 (1:2:7, 20 mL) was added to the resin bound heptapeptide and N_2 was bubbled through the vessel for 30 min. The resin was washed with CH_2Cl_2 (3×10 mL) and the filtrate was collected. Concentration in vacuo gave the crude heptapeptide, which was purified by RP-HPLC.

4.3. NH_2-Asn-Pro-Phe-Thr($\psi^{Me,Me}$pro)-Ile-Phe-Pro-OH (2)

Preparation was in accordance to that described in the general procedure for the SPPS of heptapeptides. Heptapeptide **2** (265 mg) was obtained as a colorless solid: mp 145–146 °C, $[\alpha]_D$ −88.5 (*c* 0.1 in MeOH); ^{1}H NMR (400 MHz, CD_3OD) δ 0.89 (d, *J* 6.2 Hz, 3H), 0.95 (d, *J* 6.8 Hz, 3H), 1.03–1.12 (m, 1H), 1.13–1.23 (m, 2H), 1.28 (s, 3H), 1.39–1.49 (m, 3H), 1.53 (s, 3H), 1.50–1.73 (m, 3H), 1.74–1.87 (m, 1H), 1.88–2.09 (m, 5H), 2.10–2.32 (m, 2H), 2.61–2.72 (m, 1H), 2.79–3.21 (m, 5H), 3.35–3.55 (m, 1H), 3.57–3.71 (m, 2H), 3.72–3.88 (m, 2H), 4.10–4.24 (m, 1H), 4.34–4.61 (m, 3H), 7.13–7.36 (m, 12H), 8.05 (d, *J* 7.0 Hz, 1H), 8.13 (m, 1H), 8.20 (d, *J* 7.0 Hz, 1H), 8.26 (m, 1H), NH_2 (Asn) not observed; ^{13}C NMR (100 MHz, CD_3OD) δ 11.7, 15.9, 19.6, 23.1, 24.3, 25.8, 25.9, 26.5, 30.1, 30.8, 35.4, 37.7, 38.0, 38.5, 40.0, 40.1, 41.2, 50.1, 53.8, 59.9, 60.5, 61.8, 67.7, 77.1, 98.1, 127.8, 128.2, 129.4, 129.6, 130.5, 130.8, 138.2 (2×ArC), 168.4, 170.0, 170.7, 171.1, 171.9, 172.4, 172.9, 175.2; MS (ESI) *m*/*z* 875 ($[MH]^+$, 100), 897 ($[MNa]^+$, 20%); HRMS *m*/*z*=875.4539 $[MH]^+$, 875.4400 calcd for $C_{45}H_{62}N_8O_{10}$.

4.4. *cyclo*[-Asn-Pro-Phe-Thr($\psi^{Me,Me}$pro)-Ile-Phe-Pro] (3)

Linear peptide NH_2-Asn-Pro-Phe-Thr($\psi^{Me,Me}$pro)-Ile-Phe-Pro-OH (**2**) (0.10 g, 0.11 mmol) and HBTU (0.13 g, 0.33 mmol) were each dissolved in DMF (10 mL) and added dropwise via syringe pumps (0.5 mL/h) to a vigorously stirred solution of DIPEA (0.10 mL, 0.56 mmol) and DMF (114 mL, 1 mM). The reaction mixture was stirred for two days, after which time the solvent was removed under reduced pressure and the crude residue was purified by RP-HPLC to afford two fractions, A (t_R 26.4 min) and B (t_R 31.6 min). Concentration of fraction A gave cyclic heptapeptide **3** (0.060 g, 61%) as a colorless solid: mp 179–182 °C, $[\alpha]_D$ −94.7 (*c* 0.1 in MeOH); ^{1}H NMR (400 MHz, CD_3CN) δ 0.78 (d, *J* 7.9 Hz, 3H), 0.82 (t, *J* 7.6 Hz, 3H), 1.08 (d, *J* 6.9 Hz, 3H), 1.59 (s, 3H), 1.62 (s, 3H), 1.66–1.86 (m, 2H), 2.03–2.14 (m, 1H), 2.50 (dd, *J* 14.2, 11.2 Hz, 1H), 2.66 (dd, *J* 14.2, 3.4 Hz, 1H), 2.86–3.00 (m, 4H), 3.01–3.14 (m, 4H), 3.29–3.38 (m, 2H), 3.39–3.56 (m, 4H), 3.61–3.72 (m, 1H), 3.82 (t, *J* 9.0 Hz, 1H), 4.08–4.21 (m, 2H), 4.24–4.32 (m, 1H), 4.38 (dd, *J* 8.3, 4.4 Hz, 1H), 4.50 (d, *J* 7.6 Hz, 1H), 4.54–4.62 (m, 1H), 4.63–4.70 (m, 1H), 4.74–4.84 (m, 1H), 7.15–7.41 (m, 10H), NH and NH_2 signals not observed; ^{13}C NMR (75 MHz, $CDCl_3$) δ 4.7, 10.3, 15.2, 18.6, 24.9, 25.9, 29.47, 29.52, 33.1, 36.41, 36.44, 37.0, 38.3, 47.7, 49.4, 53.1, 53.9, 56.1, 57.1, 61.2, 68.6, 77.2, 77.8, 104.3, 126.8, 127.0, 128.3, 128.5, 129.0, 129.6, 136.2, 137.3, 169.4, 170.3, 170.6, 171.2, 173.0, 173.2, 173.4 (1 signal obscured or overlapping); MS (ESI) *m*/*z* 857 ($[MH]^+$, 100), 879 ($[MNa]^+$, 10%); HRMS *m*/*z*=879.4219 $[MNa]^+$, 879.4300 calcd for $C_{45}H_{60}N_8O_9Na$.

Concentration of fraction B gave the cyclic dimer **4** (0.008 g, 8% yield) as a colorless solid: mp 150–158 °C, $[\alpha]_D$ −105.5 (*c* 0.1 in MeOH); ^{1}H NMR (400 MHz, CD_3CN) δ 0.61 (d, *J* 6.8 Hz, 2H), 0.81–0.99 (m, 18H), 1.14–1.22 (m, 6H), 1.37–1.45 (m, 6H), 1.48–1.63 (m, 8H), 1.64–1.98 (m, 16H), 1.20–1.25 (m, 2H), 2.41 (dd, *J* 14.5, 4.1 Hz, 2H), 2.53 (dd, *J* 12.9, 10.6 Hz, 2H), 2.80–3.19 (m, 4H), 3.20–3.29 (m, 2H), 3.29–3.54 (m, 4H), 3.54–3.78 (m, 6H), 3.79–3.85 (m, 2H), 3.89–3.99 (m, 2H), 4.01–4.11 (m, 2H), 4.21–4.26 (m, 2H), 4.27–4.44 (m, 4H), 4.49–4.55 (m, 2H), 4.57–4.70 (m, 2H), 4.75–4.92 (m, 2H), 4.95–5.10 (m, 2H), 7.08–7.34 (m, 20H), NH and NH_2 signals not observed; MS (ESI) *m*/*z* 1713 ($[MH]^+$, 100), 1735 ($[MNa]^+$, 10%); HRMS *m*/*z*=1735.8838 $[MNa]^+$, 1735.8859 calcd for $C_{90}H_{120}N_{16}O_{18}Na$.

4.5. NH_2-Asn-Pro-Phe-Thr(^{t}Bu)-Ile-Phe-Pro-OH (5)

Preparation was in accordance to that described in the general procedure for the SPPS of heptapeptides. Heptapeptide **5** (700 mg) was obtained as a colorless solid: mp 135–136 °C, $[\alpha]_D$ −57.2 (*c* 0.1 in MeOH); ^{1}H NMR (400 MHz, CD_3OD) δ 0.85–0.94 (m, 9H), 1.03 (d, *J* 6.2 Hz, 3H), 1.21 (s, 9H), 1.38–1.58 (m, 2H), 1.61–1.91 (m, 4H), 1.92–2.02 (m, 2H), 2.06–2.26 (m, 2H), 2.70–2.80 (m, 1H), 2.84–2.94 (m, 1H), 2.96–3.09 (m, 2H), 3.10–3.25 (m, 2H), 3.44–3.50 (m, 1H), 3.59–3.67 (m, 1H), 3.70–3.80 (m, 1H), 4.00–4.10 (m, 1H), 4.22–4.29 (m, 1H), 4.35–4.43 (m, 2H), 4.44–4.51 (m, 2H), 4.63–4.70 (m, 1H), 7.16–7.37 (m, 10H), 7.74 (d, *J* 9.0 Hz, 1H), 7.83 (m, 1H), 8.18 (d, *J* 7.9 Hz, 1H), one NH and two NH_2 (Asn) not observed; ^{13}C NMR (100 MHz, CD_3OD) δ 11.7, 16.0, 19.0, 25.4, 25.8, 25.9, 28.70 (^{t}Bu), 28.74 (2×CH_2), 30.1, 30.5, 38.2, 38.5, 38.8, 53.9, 56.1, 59.0, 59.1, 60.6, 61.9, 68.3, 68.4, 76.2, 76.3, 127.8, 127.9, 129.45 (2×CH), 129.54 (2×CH), 130.45 (2×CH), 130.52 (2×CH), 138.0, 138.6, 168.7, 171.1, 171.7, 172.8, 173.0, 173.1, 173.8, 175.2; MS (ESI) *m*/*z* 891 ($[MH]^+$, 10), 913 ($[MNa]^+$, 100%); HRMS *m*/*z*=891.4990 $[MH]^+$, 891.4980 calcd for $C_{46}H_{67}N_8O_{10}$.

4.6. *cyclo*[-Asn-Pro-Phe-Thr(^{t}Bu)-Ile-Phe-Pro] (6)

Linear peptide **5** (0.10 g, 0.11 mmol) and FDPP (0.13 g, 0.33 mmol) were each dissolved in DMF (10 mL) and added dropwise via syringe pumps (0.5 mL/h) to a vigorously stirred solution of DIPEA (0.10 mL, 0.56 mmol) and DMF (110 mL, 1 mM). The reaction mixture was stirred for three days, after which time the solvent was removed under reduced pressure and the crude residue was purified by RP-HPLC to afford two fractions, A (t_R 29.5 min) and B (t_R 40.4 min). Concentration of fraction A gave cyclic heptapeptide **6** (0.076 g, 77%) as a colorless solid: mp 168–170 °C, $[\alpha]_D$ −126.9 (*c* 0.1 in MeOH); ^{1}H NMR (400 MHz, CD_3OD) δ 0.85 (m, 1H), 0.98 (t, *J* 7.5 Hz, 3H), 1.18 (d, *J* 6.4 Hz, 3H), 1.23 (d, *J* 6.4 Hz, 3H), 1.32 (s, 9H), 1.37–1.49 (m, 2H), 1.59–1.69 (m, 2H), 1.69–1.89 (m, 4H), 1.90–2.01 (m, 2H), 2.58 (dd, *J* 14.6, 5.9 Hz, 1H), 2.81–2.96 (m, 2H), 3.05 (t, *J* 12.7 Hz, 1H), 3.17 (dd, *J* 12.7, 4.7 Hz, 1H), 3.26–3.40 (m, 4H), 3.58–3.67 (m, 1H), 3.92 (q, *J* 8.4 Hz, 1H), 4.04 (dd, *J* 7.5, 4.6 Hz, 1H), 4.17 (dd, *J* 8.3, 5.8 Hz, 1H), 4.27–4.39 (m, 2H), 4.40–4.50 (m, 2H), 4.74 (q, *J* 7.0 Hz, 1H), 7.16–7.36 (m, 10H), 7.74 (d, *J* 6.3 Hz, 1H), 7.85 (d, *J* 4.5 Hz, 1H), 8.53 (d, *J* 8.0 Hz, 1H), 8.59 (s, 1H), 8.88 (d, *J* 8.0 Hz, 1H), NH_2 (Asn) not observed; ^{13}C NMR (100 MHz, CD_3OD) δ 11.2, 15.9, 18.3, 22.7, 25.5, 26.5, 28.7, 30.5, 31.4, 37.4, 38.1, 38.9, 39.1, 47.5, 50.4, 55.8, 57.1, 58.9, 59.9, 62.1, 62.5, 67.1, 77.0, 86.8, 127.7, 128.7, 129.4, 130.1, 130.4, 130.8, 137.0, 139.6, 170.1, 172.6, 172.7, 173.0, 173.2, 173.6, 174.3, 174.8; MS (ESI) *m*/*z* 873 ($[MH]^+$, 50%), 895 ($[MNa]^+$, 100%); HRMS *m*/*z*=873.4875 $[MH]^+$, 873.4869 calcd for $C_{46}H_{65}N_8O_9$.

Concentration of fraction B gave the cyclic dimer **7** (0.002 g, 2% yield) as a colorless solid: mp 236–237 °C, $[\alpha]_D$ −100.5 (*c* 0.2 in MeOH); ^{1}H NMR (400 MHz, CD_3OD) δ 0.81 (d, *J* 6.9 Hz, 6H), 0.82 (t, *J* 6.9 Hz, 6H), 0.89–0.97 (m, 2H), 0.97–1.08 (m, 4H), 1.12 (d, *J* 6.3 Hz, 6H), 1.15–1.27 (m, 6H), 1.32 (s, 18H), 1.35–1.41 (m, 2H), 1.63–1.77 (m, 4H), 1.78–2.04 (m, 10H), 2.08–2.21 (m, 2H), 2.69–2.79 (m, 2H), 2.82–2.99 (m, 2H), 3.01–3.15 (m, 2H), 3.17–3.28 (m, 2H), 3.38–3.51 (m, 2H), 3.62–3.75 (m, 4H), 3.81–3.91 (m, 2H), 4.10–4.21 (m, 2H), 4.24–4.31 (m, 2H), 4.31–4.41 (m, 4H), 4.42–4.50 (m, 2H), 4.62–4.75 (m, 2H), 4.97–5.09 (m, 2H), 7.14–7.56 (m, 20H), 8.36 (d, *J* 9.2 Hz, 2H), six NH and two NH_2 (Asn) not observed; ^{13}C NMR (75 MHz, CD_3OD) δ 12.0, 16.2, 18.3, 24.8, 25.7, 26.0, 28.6, 28.7, 28.8, 37.7, 38.7, 53.6,

56.3, 59.0, 59.8, 61.7, 62.1, 67.4, 77.0, 127.8, 127.9, 129.5, 129.6, 130.4, 130.5, 138.2, 139.2, 171.3, 172.3, 172.9, 173.5, 173.9, 174.3, 174.4, 175.4; MS (ESI) m/z 1745 ($[MH]^+$, 100), 1767 ($[MNa]^+$, 60%); HRMS m/z=1767.9485 $[MNa]^+$, 1767.9485 calcd for $C_{92}H_{128}N_{16}O_{18}Na$.

4.7. Axinellin A (1)

A solution of TFA:thioanisole:triisopropylsilane:H_2O (8.5:0.5:0.5:0.5, 10 mL) was added to the cyclic heptapetide (**3**, 75 mg or **6**, 85 mg) at rt and the resulting reaction mixture was stirred for 4 d. Concentration in vacuo gave the crude residue, which was purified by RP-HPLC to give *cyclo*[Asn-Pro-Phe-Thr-Ile-Phe-Pro], Axinellin A (**1**) (75% from **3** and 80% from **6**) as a colorless solid: mp 115–116 °C, $[\alpha]_D$ −178.5 (*c* 0.2 in MeOH) [lit.[9] $[\alpha]_D$ −98.2 (*c* 0.003 MeOH)]; ^1H NMR (400 MHz, pyridine-d_5): δ 0.81 (t, *J* 7.63 Hz, 3H), 1.14–1.27 (m, 2H), 1.29 (d, *J* 6.8 Hz, 3H), 1.36–1.54 (m, 5H), 1.46 (d, *J* 6.42 Hz, 3H), 1.55–1.66 (m, 1H), 1.78–1.90 (m, 1H), 2.05–2.19 (m, 1H), 2.23–2.30 (m, 1H), 3.04 (dd, *J* 13.6, 3.2 Hz, 1H), 3.20 (dd, *J* 12.0, 4.4 Hz, 2H), 3.41 (t, *J* 14.4 Hz, 1H), 3.46 (d, *J* 8.4 Hz, 1H), 3.50–3.62 (m, 2H), 3.66 (dd, *J* 14.4, 3.2 Hz, 1H), 3.95 (dd, *J* 15.2, 5.4 Hz, 1H), 4.16 (t, *J* 3.6 Hz, 1H), 4.28 (dd, *J* 15.2, 6.4 Hz, 1H), 4.49 (dd, *J* 9.6, 4.0 Hz, 1H), 4.56 (br q, *J* 6.42 Hz, 1H), 4.62 (dd, *J* 10.0, 4.0 Hz, 1H), 4.98 (t, *J* 8.4 Hz, 1H), 5.19 (ddd, *J* 14.4, 8.8, 3.2 Hz, 1H), 5.22 (d, *J* 9.6 Hz, 1H), 5.35 (ddd, *J* 13.6, 8.4, 3.2 Hz, 1H), 7.11–7.46 (m, 10H), 7.94 (d, *J* 9.6 Hz, 1H), 8.53 (br s, 1H), 8.54 (br s, 1H), 9.81 (br s, 1H), 8.60 (d, *J* 8.8 Hz, 1H), 9.16 (d, *J* 8.4 Hz, 1H), 9.84 (br s, 1H); ^{13}C NMR (100 MHz, pyridine-d_5): δ 11.4, 15.4, 21.6, 22.5, 24.5, 25.7, 29.8, 31.4, 38.5, 39.0, 39.2, 48.0, 47.1, 49.1, 55.2, 56.7, 56.9, 58.4, 61.1, 62.0, 67.7, 127.7, 128.9, 128.6, 129.8, 129.8, 126.7, 136.3, 139.4, 169.8, 170.4, 171.7, 172.1, 172.2, 173.2; MS (ESI) m/z 817 ($[MH]^+$, 100%), 839 ($[MNa]^+$, 30); HRMS: m/z=817.4243, 817.4270 calcd for $C_{42}H_{57}N_8O_9$.

4.8. Cytotoxicity assays

Experiments were performed in 96-well microtiter plates (10^5 cells/mL for NSCLC-N6 and 2×10^4 cells/mL for A549). Cell growth was estimated by a colorimetric assay based on the conversion of tetrazolium dye (MTT) to a blue formazan product by live mitochondria.[23] Eight repeats were performed for each concentration. Control growth was estimated from eight determinations. Optical density at 570 nm corresponding to solubilized formazan was read for each well on Titertek Multiskan MKII.

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Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2009.11.090.

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