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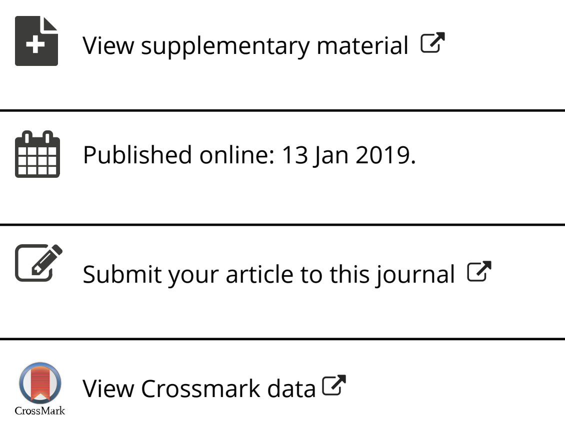

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Synthesis and anticancer activities of proline-containing cyclic peptides and their linear analogs and congeners

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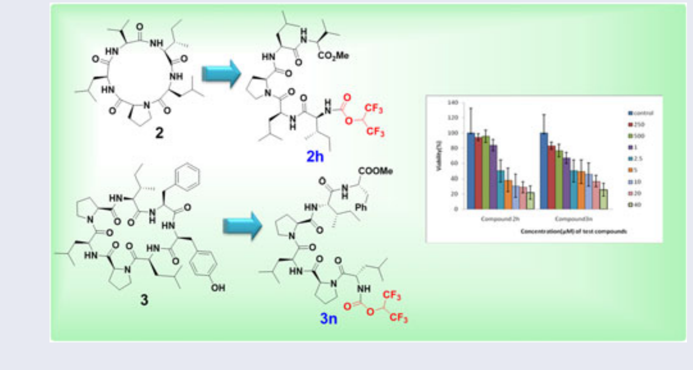
ABSTRACT

A solution phase method was adopted for the synthesis of proline-containing cyclic pentapeptide **2** and total synthesis of naturally occurring cyclic heptapeptide Reniochalistatin B **3**. For the synthesis of **3**, both divergent and convergent strategies were used to improve the overall yield from 12 to 25%. Different N and C terminal modified linear analogs and congeners of **2** and **3** were synthesized. Both cyclic peptides **2** and **3** and their linear analogs/congeners were evaluated for anti-cancer activity against HeLa cell line, among which pentapeptide **2h** and hexapeptide **3n** with N-terminal protected hexafluoroisopropyl carbamates (HFIPC) interestingly showed higher cytotoxicity with an IC_{50} of 2.73 and 4.3 μM, respectively compared to their Boc-protected analogs **2a** (IC_{50} 20 μM) and **3c** (IC_{50} 38.51 μM) and cyclic peptides **2** (>100 μM) and **3** (47 μM). These results were further validated by biological experiments such as colony formation and wound healing assays.

GRAPHICAL ABSTRACT



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Introduction

Peptide-based drugs are known for being highly selective, efficient and at the same time relatively safe, offering an alternative strategy in the field of rational drug design.[1,2]

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1 2 3

Figure 1. Chemical structure of the cyclic peptides.

Peptide drug moieties, mainly cyclized forms and their derivatives, often lead to the development of potential drug candidates than their linear counterparts and therefore continue to seek the attention of the drug industry. Though naturally occurring linear peptides as therapeutics are limited by their intrinsic weaknesses e.g. poor chemical, physical and proteolytic stability, however, in some cases linear peptides are considered better cancer targeting agents than their cyclic counterparts.[3] In general, cyclic peptides have been broadly exploited over the past 20 years in medicine as active ingredients of natural extracts (bacteria, fungi, plants, animal venoms). The lack of N and C termini keeps it from enzymatic degradation and physical denaturation which enhances the bioavailability.[4–6] Improved structural rigidity, receptor selectivity, biochemical stability are their characteristics features. Cyclic peptides usually have little to no toxicity due to their benign amino acid make-up.[7] Target specificity and extra stability makes them one of the most promising drug candidates or biochemical tools, a few of them being commercially available as drugs[4,8] e.g. bacitracin, daptomycin, cyclosporine, etc. Owing to its remarkable properties and growing demand, scientists are in pursuit of novel cyclic peptides and synthetic strategies for their production.[9–12]

In 2011, Wen Wu et al.[13] isolated a homodetic cyclic peptide **1** from the crude extract of an endolichenic *Xylaria sp,* which has shown antifungal activity and we describe here the synthesis of **2,** an analog of **1** where D-amino acids have been replaced by L-amino acids. In 2014, Kai-Xuan Zhan and et al.[14] isolated Reniochalistatin B (**3**), a cyclic heptapeptide from a marine sponge, *Reniochalina stalagmitis* having antitumor activities (Figure 1). While working on this molecule (**3**), Xu et al. have reported very recently the first total synthesis of **3** in solid phase method.[15] Reniochalistatin E, a cyclic octapeptide which is another member of Reniochalistatins family and its total synthesis was reported in both solid and solution phase methods. Solution phase synthesis was accomplished by Rafferty et al. in 15 steps with an overall 5.0% yield.[16] The best possible cyclization yield 15% was achieved in macrolactamization step when EDC/HOBt was used. On the other hand, Liang et al. have reported solid phase synthesis of Reniochalistatin E and its conformational isomers with 32% overall yield.[17] Reniochalistatin E showed cytotoxicity against HeLa cell line with IC_{50} of 16.9 μM (±1.9).[16] Fully deprotected linear form of Reniochalistatin E has also shown cytotoxicity with an almost equal potency having IC_{50} of 9.5 ± 2.1 μM in U937 cell line.[16] Herein, we report the second total synthesis of Reniochalistatin B (**3**) using solution phase method which can be used for large-scale synthesis. In this direction,

Scheme 1. Retrosynthetic analysis of the target cyclic peptide **2**.

cyclic peptides **2**, **3** and their several linear analogs and congeners have also been used to evaluate their anticancer activities.

Results and discussion

Chemistry

To achieve the formal synthesis of the desired cyclic peptide **2**, we started with cheap and easily available L- amino acids. Accordingly, the D-isoleucine and D-leucine in **1** were replaced by their L- isomers. The retrosynthetic analysis of **2** was outlined in Scheme 1. Cyclic peptide was obtained by macrolactamization of linear pentapeptide **2a**. L-Valine was disconnected at the C- terminal to get linear tetrapeptide **2b.** Further disconnection at the N-terminal and C-terminal gave tripeptide **2c** and dipeptide **2d,** respectively. These peptides can be synthesized by stepwise peptide synthesis method starting from L-proline and L-leucine, respectively (Scheme 1).

The forward synthesis commenced with the condensation of L-proline methyl ester hydrochloride with Fmoc-L-Leu-OH to yield the linear dipeptide [18] **2d** in 95% yield which was purified by a flash column chromatography and used in the next step. The methyl ester of dipeptide **2d** was then hydrolyzed under buffered conditions in order to keep the Fmoc intact. LiOH.H_2O mediated hydrolysis in presence of excess $CaCl_2$[19,20] afforded the desired product. The corresponding free acid was subsequently treated with L-leu-OMe.HCl to give the tripeptide **2c** in 79% yield. The Fmoc group in the linear tripeptide **2c** was deprotected using 20% piperidine in DMF to yield the free amine which was then subjected to another coupling with N-Boc-L-Ile yielded the linear tetrapeptide **2b** in 81% yield. Methyl ester of tetrapeptide **2b** was hydrolyzed using LiOH, following another coupling with L-Val-OMe yielded the desired linear pentapeptide analog **2a** of **2** in 73% yield. The linear precursor **2a** was then treated with LiOH to yield the corresponding free carboxylic acid. The crude carboxylic acid was then treated with 20% TFA in DCM to yield the NBoc deprotected compound. The deprotected compound underwent an intramolecular condensation in presence of $NaHCO_3$ and BOP in

Scheme 2. Synthesis of cyclic peptide **2**. Reagents and conditions: (**a**) DIPEA, Fmoc-L-Leu-OH, HOBt, EDC, DCM, 12 h, 95%; (**b**) (**i**) LiOH, $CaCl_2$, $^iPrOH/THF/H_2O$ (10:3:4), 1 h; (**ii**) L-Leu-OMe.HCl, DIPEA, EDC, HOBt, DCM, 12 h, 79%; (**c**) (**i**) 20% Piperidine in DMF; (**ii**) Boc-L-Ile-OH, EDC, HOBT, DCM, 12 h, 81%; (**d**) (**i**) LiOH, $THF/H_2O/MeOH$ (10:4:1), 1 h; (**ii**) L-Val-OMe.HCl, DIPEA, EDC, HOBT, DCM, 12 h, 73%; **e**) (**i**). LiOH, $THF/H_2O/MeOH$ (10:4:1), 1 h; (**ii**) TFA (20%) in DCM; (**iii**) $NaHCO_3$, BOP, DMF, RT, 14 h, 61%.

Scheme 3. Retrosynthetic analysis of the naturally occurring cyclic peptide **3**.

DMF[21] to yield the cyclic peptide **2** in 61% yield (Scheme 2). The overall yield of **2** in the five steps was 27%.

The retrosynthetic analysis of Reniochalistatin B (**3**) has been shown in scheme 3. Cyclic peptide can be obtained by macrolactamization of linear heptapeptide **3a** or **3b3b**. Linear peptide **3a** and **3b** can be synthesized from three major fragments **3c**,

Scheme 4. Synthesis of linear heptapeptide presursor **3a**. Reagents and conditions: (**a**) L-Pro-OMe.HCl, DIPEA, HOBt, EDC, DCM, 12 h, 85%; (**b**) (**i**) LiOH, THF/H_2O/MeOH (10:4:1), 1 h; (**ii**) L-Ile-OMe.HCl, DIPEA, HOBt, EDC, DCM, 12 h, 62%; (**c**) (**i**) LiOH, THF/H_2O/MeOH (10:4:1), 1 h; (**ii**) L-Phe-OMe.HCl, DIPEA, HOBt, EDC, DCM, 12 h, 92%; (**d**) (**i**) LiOH, THF/H_2O/MeOH (10:4:1), 1 h; (**ii**) HOBt, EDC, DCM, 12 h, 64%.

3d, and **3e.** Other amino acids are easily recognized as L-Tyrosine, L-Phenylalanine, L-Isoleucoine, and L-proline. Tripeptide **3e** is same as **2c** except the change in terminal protections, hence **3e** was synthesized following the procedure of **2c** as described in Scheme 2 (SI Scheme S1).

Synthesis of the cyclic peptide **3** commenced with the coupling of tripeptide **3e** and L-Pro-OMe.HCl to yield the protected tetrapeptide **3d**. The methyl ester deprotection of **3d** with LiOH followed by EDC coupling with L-Ile-OMe.HCl resulted in the pentapeptide **3f**. Repeating the sequence of methyl ester hydrolysis and subsequent couplings with L-Phe-OMe.HCl and L-Tyr-O^{t}Bu (the Fmoc group in L-Fmoc-Tyr-O^{t}Bu was deprotected using 20% piperidine in DMF) prior to coupling with **3c** yielded the desired linear heptapeptide **3a,** the precursor of cyclic peptide **3** (Scheme 4). The notable drop in yields during the synthesis of **3f** and **3a**, compelled us to adopt a convergent route for the total synthesis of Reniochalistatin B (**3**). The linear precursor heptapeptide was synthesized using the convergent route described in Scheme 5.

In this case, heptapeptide precursor **3b** was prepared in methyl ester form instead of *tert*-butyl ester **3a** because of cheap and easily available of L-Tyr-OMe than L-Tyr-O^{t}Bu. Moreover, preparation of *tert*-butyl ester from Fmoc-L-Tyr-OH was not clean to yield Fmoc-L-Tyr- O^{t}Bu in 40% yield (SI Scheme S2).

Scheme 5. Synthesis of linear tripeptide 3 h and heptapeptide **3b**. Reagents and conditions: **(a)** L-Phe-OMe.HCl, DIPEA, HOBt, DCC, DCM, 12 h, 88%; **(b)** **(i)** LiOH, THF/H_2O/MeOH, (10:4:1), 1 h; **(ii)** L-Tyr-OMe.HCl, DIPEA, HOBt, EDC, DCM, 12 h, 96%; (**c**) LiOH, THF/H_2O/MeOH, (10:4:1), 1 h; **d**) TFA (20%) in DCM; (**e**) DIPEA, HOBt, EDC, DCM, 88%.

Accordingly, **3b** was prepared from **3d** and **3h** (Scheme 5). Tripeptide **3h** was prepared as follows. DCC-mediated coupling of Boc protected L-isoleucine with L-phenylalanine methyl ester hydrochloride to give the dipeptide **3g**. Methyl ester hydrolysis of **3g** using LiOH followed by coupling with L-Tyr-OMe.HCl in presence of HOBt/EDC the linear tripeptide **3 h** was obtained in 84% overall yield. The amine generated by TFA mediated Boc deprotection of tripeptide **3h** was subjected to coupling with free acid obtained by methyl ester hydrolysis of tetrapeptide **3d** to provide the linear heptapeptide precursor **3b** (Scheme 5).

Cyclization of 3a or 3b to achieve 3

The Boc group and *tert*-butyl ester group of linear heptapeptide **3a** were simultaneously deprotected by treating with 20% TFA in DCM. Marcolactamization of the deprotected compound is done in presence of $NaHCO_3$ and BOP in DMF[21] to yield the cyclic peptide **3** in 39% yield (Scheme 6). Overall yield was 12% from **3e**.

On the other hand, the linear precursor **3b** was treated with LiOH to yield the corresponding free carboxylic acid. The crude carboxylic acid is then treated with 20% TFA in DCM to yield the Boc deprotected compound. The fully deprotected compound after macrolactamization yields the cyclic peptide **3** in 40% yield (Scheme 6). Overall yield was 25% from **3e**.

Synthesis of linear analogs of 2

After first phase screening of **2** and its four linear analogs such as **2a**, **2e**, **2f** and **2g**, compound **2a** was found to be the most potent with an IC_{50} value of 20 μM and

realized that the N-terminal protection has a role in biological activities, hence N-terminal *tert*-Boc of **2a** was replaced by hexafluoroisopropyl carbamate (HFIPC) to obtain the peptide **2h** (Scheme 7). Hexafluoroisopropyl carbamate is chosen because fluorine is known to improve the lipophilic character, cell-permeability and pharmacokinetics of a

Scheme 6. Synthesis of Reniochalistatin B (**3**). Reagents and conditions: (**a**) (**i**) TFA (20%) in DCM, (**ii**) $NaHCO_3$, BOP, DMF, RT, 14 h, 39%; (**b**) (**i**) LiOH, THF/H_2O/MeOH, (10:4:1): then (**iii**) TFA (20%) in DCM, then cyclization using (**ii**), 40%.

Scheme 7. Synthesis of analogs of **2**. Reagents and conditions: (**a**) TFA (20%) in DCM, 90%; **b**) trisphosgene, Pyridine, HFIP, Et_3N, 15 h, 40%; (**c**) LiOH, THF/H_2O/MeOH, (10:4:1), 1 h, 92%; (**d**) TFA (20%) in DCM, 95%.

Scheme 8. Reagents and conditions: (**a**) LiOH, THF/H_2O/MeOH, (10:4:1), 1 h, 94%; (**b**). TFA (20%) in DCM, 91%; (**c**) TFA (20%) in DCM, 97%; (**d**) Trisphosgene, Pyridine, HFIP, Et_3N, 15 h, 62%; **e**) AllocCl, Pyridine, 0 °C, 16 h, 87%; (**f**) LiOH, THF/H_2O/MeOH, (10:4:1), 1 h, 95%; (**g**) Fmoc- L-Tyr-OtBu, Piperidine, HOBt, EDC, DCM,12 h, 78%.

medicinal compound[22–25] HFIPC protected **2h** was obtained by reaction of **2e** with trisphosgene and hexafluoroisopropanol (HFIP) in presence of pyridine.[26,27]

Synthesis of linear analogs and congeners of 3

Similarly, several linear heptapetide analogs and other short peptides (congeners) were synthesized based on cyclic peptide **3**.

Alloc-protected (Alloc-NH-Leu-Pro-Leu-Pro-OMe) **3i** and (Alloc-NH-Leu-Pro-Leu-Pro-Ile-OMe) **3j** were synthesized from (Boc-NH-Leu-Pro-Leu-Pro-OMe) **3d** and (Boc-NH-Leu-Pro-Leu-Pro-Ile-OMe) **3f**, respectively using literature procedure[28] (SI Schemes S3 and S4).

In order to know the role of N-terminal and C-terminals in anticancer activities, several linear hexapeptides and heptapeptide were synthesized. Methyl ester hydrolysis of the linear hexapeptide **3c** using LiOH yielded the corresponding free carboxylic acid **3k**. The free acid was then treated with 20% TFA in DCM to afford fully deprotected linear hexapeptide **3 l**. Again Boc group of the linear peptide **3c** was deprotected by 20% TFA in DCM to afford the free *N*-terminal compound **3m**. The compound **3m** on reaction with trisphosgene and hexafluoroisopropanol in presence of pyridine yielded hexafluoroisopropyl carbamate[26,27] derivative **3n**.

N-Alloc derivatives of linear hexapeptide **3c** and heptapeptide **3a** were also synthesized. Alloc carbamate **3o** was synthesized by reacting free N-terminal compound **3m** with Alloc chloride and pyridine. Methyl ester hydrolysis of N-Alloc protected hexapeptide **3o** on treatment with LiOH yielded free acid **3p**. Coupling of carboxylic acid **3p** with L-Tyr-O^{t}Bu (obtained by Fmoc deprotection of L-Fmoc-Tyr-O^{t}Bu using 20% piperidine in DMF) in presence of HOBt/EDC afforded the heptapeptide **3q** (Scheme 8).

Table 1. IC_{50} values of the cyclic and linear peptides against HeLa cells.

Sl. No.	Compound name	Sequence	IC_{50} (μM)
1	2	c-(Ile-Leu-Pro-Leu-Val)	>100
2	2a	Boc-NH-Ile-Leu-Pro-Leu-Val-OMe	20 ± 0.11
3	2e	NH_2-Ile-Leu-Pro-Leu-Val-OMe	>60
4	2f	Boc-NH-Ile-Leu-Pro-Leu-Val-OH	>60
5	2g	NH_2-Ile-Leu-Pro-Leu-Val-OH	>60
6	**2h**	**HFIP-NH-Ile-Leu-Pro-Leu-Val-OMe**	**2.73 ± 0.26**
7	3	c-(Leu-Pro-Leu-Pro-Ile-Phe-Tyr)	47.17 ± 0.39
8	3a	Boc-NH-Leu-Pro-Leu-Pro-Ile-Phe-Tyr-OtBu	>100
9	3b	Boc-NH-Leu-Pro-Leu-Pro-Ile-Phe-Tyr-OMe	50.63 ± 0.48
10	3c	Boc-NH-Leu-Pro-Leu-Pro-Ile-Phe-OMe	38.51 ± 0.26
11	3d	Boc-NH-Leu-Pro-Leu-Pro-OMe	>100
12	3f	Boc-NH-Leu-Pro-Leu-Pro-Ile-OMe	50.79 ± 0.19
13	3i	Alloc-NH-Leu-Pro-Leu-Pro-OMe	>100
14	3j	Alloc-NH-Leu-Pro-Leu-Pro-Ile-OMe	51.52 ± 0.38
15	3k	Boc-NH-Leu-Pro-Leu-Pro-Ile-Phe-OH	>60
16	3l	TFA.NH_2-Leu-Pro-Leu-Pro-Ile-Phe-OH	>60
17	3m	TFA.NH_2-Leu-Pro-Leu-Pro-Ile-Phe-OMe	>60
18	**3n**	**HFIP-NH-Leu-Pro-Leu-Pro-Ile-Phe-OMe**	**4.3 ± 0.19**
19	3o	Alloc-NH-Leu-Pro-Leu-Pro-Ile-Phe-OMe	22.59 ± 0.17
20	3p	Alloc-NH-Leu-Pro-Leu-Pro-Ile-Phe-OH	>60
21	3q	Alloc-NH-Leu-Pro-Leu-Pro-Ile-Phe-Tyr-OtBu	>100

The bold values highlight the best results obtained for IC50.

Biological activity

Though, cyclic peptide **1** is known to have anti-fungal activity however, the anticancer activity of its analog **2** has been evaluated as some antifungal compounds have been found to be potential anti-cancer agents in literature.[29–32] Accordingly, the anti-cancer activity of both the compounds **2** and **3** and their linear analogs was investigated against HeLa cells (cervical cancer cell line) by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method. The experiment was repeated in triplicates for each compound. From the preliminary evaluation, it was found that cyclic peptide **2** was almost inactive up to 100 μM dose whereas its linear analog pentapeptide **2a** (Boc-NH-Ile-Leu-Pro-Leu-Val-OMe) was active with an inhibitory concentration (IC_{50}) of 20 μM. Neither partially or fully deprotected pentapeptides **2e**, **2f** and **2g** showed any cytotoxicity with the cell survivability of up to 80–90% (Table 1, Figure S1). In the case of **3** and its linear peptides, it was found that hexapeptides **3c** (Boc-NH-Leu-Pro-Leu-Pro-Ile-Phe-OMe) and **3o** (Alloc-NH-Leu-Pro-Leu-Pro-Ile-Phe-OMe) became more cytotoxic than **3** against the cancer cells with $IC_{50}\sim$ of 38.51 ± 0.26 μM (**3c**), 22.59 ± 0.17 μM (**3o**) and 47.17 ± 0.39 μM (**3**), respectively (Table 1, and Figure S2). When Boc protection was changed to alloc protection, then **3o** became more potent than **3c**. In compare to earlier report,[16] Reniochalistatin B was much more less potent than Reniochalistatin E. Similarly, in the case of free amine or carboxyl terminal or fully deprotected linear peptides namely **3k, 3 l**, **3 m** and **3p** became less potent (IC_{50} 60 μM or even more) than the linear form of Reniochalistatin E. Neither heptapeptides (**3a**, **3b**, **3q**) nor tetra (**3d**, **3i**) and pentapeptides (**3f**, **3j**) were active even at 50 μM dose.

Hence, based on the preliminary screening of cytotoxic potential, modification at the N-terminal of **2a** and **3o** were made with the incorporation of hexafluoroisopropyl carbamate (HFIPC) moiety (compounds **2h** and **3n**), which is expected to have higher cell penetrating property because of fluorine as described previously.[22–25] Interestingly, these new compounds **2h** and **3n** were found to be promising and showed strikingly higher cytotoxicity against HeLa cell line with remarkably low IC_{50} values of 2.73 ± 0.26

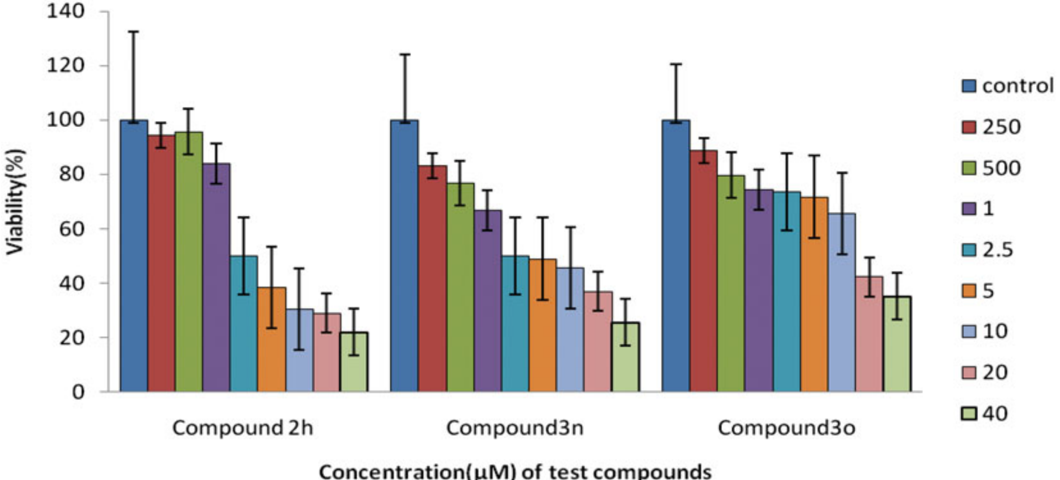

Figure 2. Cell viability of compounds **2h**, **3n** and **3o** against HeLa cells. Cells were treated with the compounds at the indicated concentrations for 48 h.

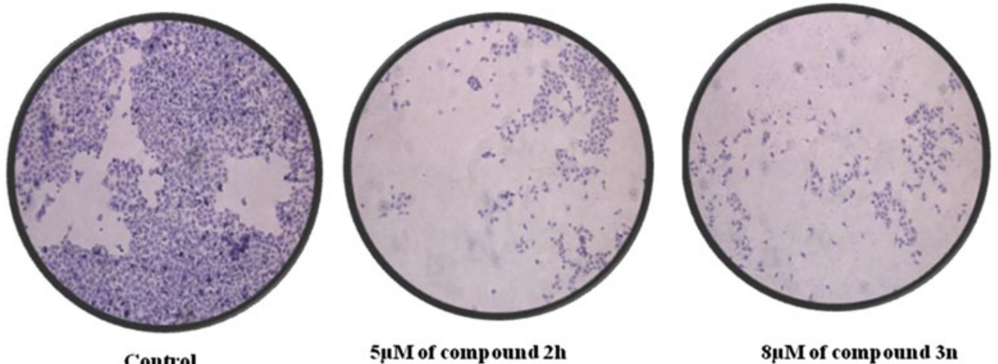

Figure 3. Colony formation of HeLa cells after treatment with compounds **2h** (5 µM) and **3n** (8 µM). A representative figure from triplicate experiments is shown.

µM and 4.3 ± 0.19 µM, (Table 1 and Figure 2) respectively. Compared to the parent compounds **2a** and **3o**, these two compounds were thus found to be having 7.3 and 5.25 times more activity, respectively.

Colony formation assay

On the basis of the IC_{50} values found in the cytotoxic assay of the compounds, the colony formation ability[33] of HeLa cells in presence of compounds **2h** and **3n** was checked at double concentration of 5 µM and 8 µM, respectively. With respect to the control, lesser colonies were formed in **2h** and **3n** as envisioned (Figure 3).

Wound healing assay

Similarly, based on IC_{50} values evident in the cytotoxic assay of the compounds, the migration ability[34] of the HeLa cells were tested in presence of compounds **2h** and **3n**

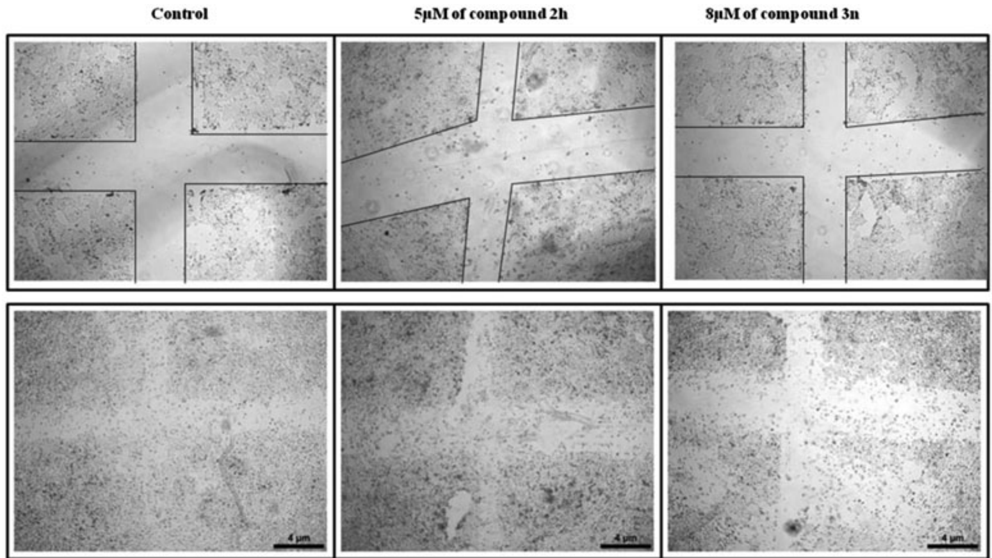

Figure 4. Wound healing assay in HeLa cells treated with Compounds **2h** (5 μM) and **3n** (8 μM) with respect to control.

at concentrations of 5 μM and 8 μM, respectively. Here also the migration of HeLa cells in both the cases was found to be lesser with respect to control (Figure 4).

Conclusion

In conclusion, we have developed concise routes for the synthesis of cyclic peptides **2** and total synthesis of Reniochalistatin B (**3**). The low yielding linear route for the synthesis of Reniochalistatin B (**3**) was replaced by a high yielding convergent route. Though conventionally, cyclization of peptides confers better therapeutic efficacies by enhancing selective receptor binding due to constrained geometry, improved stability, etc, however, cyclization cannot always ensure to produce an improved version of its linear counterpart. In certain instances, the biological activities of the cyclic and linear conjugates may be equivalent and even linear peptides with artificial amino acids substitution, hybridization, N- or C- terminal modification can achieve desired rigid conformation and could be more potent compared to its cyclized forms. In this direction, the linear peptides synthesized in this study have shown more cytotoxicity against HeLa cell lines owing to the attachment of different groups perhaps due to the better cell-penetrating capacity. Interestingly, N-terminal protected with hexafluoroisopropyl carbamate became more potent and showed IC_{50} values as low as 2.73 ± 0.26 (**2h**) and 4.3 ± 0.19 μM (**3n**). The linear peptide **3n** is a new finding which has not been disclosed earlier.[15] Even the colony formation ability and migration property of HeLa cells were significantly inhibited by these linear analogs which strongly supports their candidature as a potential therapeutic anticancer agent. In summary, here we report total syntheses of **2** and **3**, several analogs and congeners of cyclic peptides and evaluated their respective action as anti-cancer agents in *in-vitro* cell culture model. Further mechanistic investigation of these peptides is under study and their therapeutic potency in cancer

treatment needs to be further validated in suitable animal models followed by clinical trials.

Experimental section

All reagents were purchased from commercial sources and used without further purification, unless otherwise stated. Petroleum ether (PE) refers to the fraction of petroleum boiling between 60–80 °C. The following abbreviations are used for DCM = dichloromethane, DIPEA = *N*,*N*-diisopropylethylamine, MeOH = methanol, TFA = trifluoroacetic acid, EDC =1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, HOBt =1-hydroxybenzotriazole, DMF = dimethylformamide, EtOAc = ethyl acetate, THF = tetrahydrofuran, BOP = (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate, HFIP = hexafluoroisopropanol, AllocCl = allyl chloroformate. All reactions were carried out in oven-dried glassware under an argon atmosphere using anhydrous solvents, standard syringe and septum techniques unless otherwise indicated. Organic extracts were dried over anhydrous Na_2SO_4 and then filtered prior to removal of all volatiles under reduced pressure on rotary evaporation. Chromatographic purification of products was accomplished using column chromatography on silica gels (mesh 100 ∼ 200). Thin-layer chromatography (TLC) was carried out on aluminum sheets, Silica Gel 60 F254 (Merck; layer thickness 0.25 mm). Visualization of the developed chromatogram was performed by UV light and/or Ceric Ammonium Molybdate (CAM), ninhydrin stains. Optical rotations were measured using a sodium (589, D line) lamp and are reported as follows: $[\alpha]_D{}^{25}$ (c (mg/100 mL), solvent) ^{1}H and ^{13}C NMR spectra were recorded at 300 or 400 MHz and 75 or 100 MHz, respectively using $CDCl_3$ or MeOH-d_4 or DMSO-d_6 as solvent. Chemical shifts (δ) are given in ppm relative to the solvent residual peak or TMS as internal standard. The following abbreviations are used for multiplicity of NMR signals: s = singlet, d = doublet, t = triplet, m = multiplet, br = broad. High Resolution Mass Spectra (HRMS) were measured in a QTOF I (quadrupole-hexapole-TOF) mass spectrometer with an orthogonal Z-spray-electrospray interface on Micro (YA-263) mass spectrometer (Manchester, UK).

(3S,6S,9S,12S,17aS)-9-((S)-sec-Butyl)-3,12-diisobutyl-6-isopropyldodecahydro-1H-pyrrolo[1,2-a][1,4,7,10,13]pentaazacyclopentadecine-1,4,7,10,13-pentaone (2)

(24 mg, 0.036 mmol) was taken in THF: MeOH (1 ml: 0.1 ml) mixture. A solution of $LiOH.H_2O$ (15 mg, 0.36 mmol) in 0.3 ml H_2O was added dropwise to attain a molarity of 1.5 M. The reaction mixture was stirred for 30 min. The solvent was removed, acidified to pH 4 at 0 °C, extracted with ethyl acetate. The organic layer was washed with water and brine to yield the carboxylic acid as white foamy solid. The crude product so obtained was dissolved in 20% TFA (0.4 ml) in DCM (1.6 ml) at 0 °C and stirred for 2 h. Removal of DCM in vacuo yielded a brownish viscous liquid which was used in the next step without further purification.

The brownish solid obtained above (21 mg, 0.031 mmol) was dissolved in dry DMF (32 ml) to attain a concentration of 1 mM. To it $NaHCO_3$ (13 mg, 0.15 mmol) was added followed by the addition of BOP (20 mg, 0.05 mmol) at room temperature. The reaction mixture was then stirred overnight at rt. Removal of DMF followed by a flash

column chromatography (5% MeOH-DCM) yielded the desired cyclic peptide **2** as white solid (10 mg, 0.019 mmol) in 61% yield. M.p. 119–121 °C; ^{1}H NMR (300 MHz, DMSO-d$_6$) δ = 8.87 (d, J = 8.7 Hz, 1H), 7.75 (s, 1H), 7.39 (d, J = 9.9 Hz, 1H), 7.07 (d, J = 7.8 Hz, 1H), 4.46 (m, 1H), 4.28 (d, J = 7.2 Hz, 1H), 4.04–3.98 (m, 2H), 3.84 (t, J = 9.6 Hz, 1H), 3.57– 3.40 (m, 2H), 2.27–2.18 (m, 1H), 2.15–1.99 (m, 2H), 1.86 (br s, 2H), 1.77– 1.70 (m, 2H), 1.55 - 1.30 (m, 7H), 1.10–1.01 (m, 2H), 0.88–0.82 (m, 22H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$:CD_3OD (9:1)) δ = 172.4, 172.2, 171.7, 171.6, 171.1, 61.7, 59.7, 59.6, 51.7, 51.1, 47.0, 41.9, 41.4, 35.1, 32.2, 26.7, 25.2, 25.0, 24.7, 23.2,22.7, 22.3, 22.2, 22.1, 19.6, 18.5, 15.4, 10.5 ppm; HRMS (ESI): m/z $[M + Na]^+$ calcd for $C_{28}H_{49}N_5O_5Na$: 558.3632, found 558.3634.

(S)-Methyl 2-((S)-2-((2S,3S)-2-((S)-1-((S)-2-((S)-1-((S)-2-((tert-butoxycarbonyl)amino)-4-methylpentanoyl)pyrrolidine-2-carboxamido)-4-methylpentanoyl)pyrrolidine-2-carboxamido)-3-methylpentanamido)-3-phenylpropanamido)-3-(4-hydroxyphenyl) propanoate (3b)

The compound **3d** (202 mg, 0.365 mmol) was taken with THF (5.5 ml) and MeOH (0.6 ml). A solution of LiOH.H_2O (154 mg, 3.655 mmol) in H_2O (2.4 ml) was added dropwise ultimately attaining a molarity of 1.5 M. The reaction mixture was stirred for about 30 min. The solvent was removed, acidified to pH 4 at 0 °C, extracted with ethyl acetate. The organic layer was washed with water and brine to yield the acid (197 mg, 0.365 mmol) in quantitative yield.

The compound **3h** (203 mg, 0.365 mmol) was dissolved in dry DCM (4.8 ml) and 1.2 ml TFA was added at 0 °C and stirred for 2 h. Removal of DCM *in vacuo* yielded a brownish viscous liquid which was used in the next step without further purification.

DIPEA (223 µL, 1.28 mmol) was added dropwise to a stirred suspension of free amine-TFA salt (0.365 mmol) in dichloromethane (16 mL) at room temperature under nitrogen. On dissolution, the solution was cooled to 0 °C and then the acid obtained above (197 mg, 0.365 mmol) and 1-hydroxybenzotriazole (55 mg, 0.402 mmol) were added successively, each in one portion. The suspension was stirred at 0 °C for a further 15 min, and then EDC (78 mg, 0.402 mmol) was added in one portion. The mixture was allowed to warm to room temperature over the course of 12 h, and the solvent was then evaporated in vacuo. The residue was taken up in ethyl acetate and washed with cold 0.1 N HCl followed by saturated aqueous sodium bicarbonate solution. The combined organic extracts were dried and evaporated in vacuo to leave the crude product which was purified by chromatography on silica using 3% MeOH-DCM as eluent to give the heptapeptide **3b** (312 mg, 0.319 mmol, 88%) as a white solid. M.p. 132–135 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.09 (s, 1H), 7.67 (s, 1H), 7.18–7.14 (m, 6H), 6.88 (d, J = 8.1 Hz, 2H), 6.70 (d, J = 8.4 Hz, 2H), 5.47 (s, 1H), 4.79–4.69 (m, 4H), 4.54–4.46 (m, 2H), 4.28 (s, 1H), 3.77–3.74 (m, 2H), 3.64–3.57 (m, 5H), 3.24 (m, 1H), 2.94–2.86 (m, 3H), 2.53 (s, 1H), 2.19–1.90 (m, 8H), 1.74–1.62 (m, 3H), 1.56–1.32 (m, 13H), 1.09 (s, 1H), 0.95–0.86 (m, 13H), 0.75–0.66 (m, 6H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ = 173.4, 172.6, 172.0, 171.7, 171.4, 171.1, 170.9, 156.0, 155.9, 136.9, 130.5, 129.4, 129.4, 128.5, 126.9, 126.8, 115.6, 79.8, 60.5, 59.8, 58.4, 54.2, 53.6, 52.3, 50.4, 49.4, 47.7, 47.4, 42.2, 41.6, 37.7, 37.2, 36.6, 28.5, 28.0, 27.6, 25.3, 24.6, 24.5, 24.3, 23.5, 23.4, 21.9, 21.7,

15.6, 11.4 ppm; $[\alpha]_D^{25}$ = −54.2 (*c* 1.44, $CHCl_3$); HRMS (ESI): *m*/*z* $[M + Na]^+$ calcd for $C_{52}H_{77}N_7O_{11}Na$ 998.5579; found 998.5577.

(S)-Methyl 2-((S)-2-((S)-1-((S)-2-((2S,3S)-2-((1,1,1,3,3,3-hexafluoropropan-2-yloxy)carbonylamino)-3-methylpentanamido)-4-methylpentanoyl)pyrrolidine-2-carboxamido)-4-methylpentanamido)-3-methylbutanoate (2h)

Triphosgene (96 mg, 0.323 mmol) was added portion wise to the solution of hexafluoroisopropanol (HFIP) (69 µL, 0.647 mmol) in dry DCM (3 mL) at 0 °C. Then pyridine (52 µL, 0.647 mmol) was added dropwise and the reaction mixture was stirred at rt for 3.5 h. Et_3N (136 µL, 0.97 mmol) was added slowly to reaction mixture at 0 °C and solution became red. A solution of compound **2e** (73.5 mg, 0.108 mmol) was added via cannula and reaction mixture was stirred for 12 h. Excess phosgene was removed by purging Argon and the solvent was then evaporated in vacuo. The residue was taken up in ethyl acetate and washed with water followed by brine solution. The combined organic extracts were dried and evaporated in vacuo to leave the crude product which was purified by chromatography on silica using 3% MeOH-DCM as eluent to give the pentapeptide **2 h** (33 mg, 0.043 mmol, 40%) as a white solid. M.p. 88–90 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.01 (d, *J* = 7.2 Hz, 1H), 6.60 (d, *J* = 8.7 Hz, 1H), 5.82 (td, *J* = 11.7, 5.7 Hz, 1H), 4.88 (dd, *J* = 12.1, 3.6 Hz, 1H), 4.56 (dd, *J* = 8.1, 2.7 Hz, 1H), 4.51–4.46 (m, 2H), 4.31 (m, 1H), 3.71 (s, 3H), 3.59 (t, *J* = 7.2 Hz, 2H), 2.74 (t, *J* = 11.4 Hz, 1H), 2.34 (m, 1H), 2.23–2.11 (m, 3H), 2.00(m, 1H), 1.89 (m, 1H), 1.80–1.71 (m, 2H), 1.61–1.41 (m, 6H), 1.02–0.86 (m, 24H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$): δ = 172.4, 171.8, 171.0, 169.0, 151.1, 146.7, 68.4 (q, *J* = 36 Hz), 62.4, 60.0, 57.2, 53.4, 52.4, 52.2, 47.5, 40.2, 36.6, 35.4, 31.4, 29.8, 26.9, 25.6, 25.5, 25.2, 24.6, 23.2, 21.6, 20.9, 19.0, 17.8, 13.1, 11.8 ppm; HRMS (ESI): *m*/*z* $[M + Na]^+$ calcd for $C_{33}H_{53}N_5O_8F_6Na$: 784.3696; found: 784.3698.

Anti-proliferative assay

The anticancer activities of the cyclic peptides, their analogs and congeners were screened against HeLa cells. They were grown to log phase in 10% fetal bovine serum, were seeded in 96 well plates at a density of 1.5×10^4 and grown to a confluency of 60% at 37 °C in a 5% CO_2 incubator. After 24 hrs, the compounds were added in their respective doses to 96- well plates in 0.5% FBS media and cultured at 37 °C for 48 h. The MTT solution was prepared in 0.5% FBS media (at a concentration of 1 mg/mL) and 100 µL of MTT solution was added to each well and incubated for 4 h at 37 °C for violet crystal formation. The suspension was then discarded and 100 µL of dimethyl sulfoxide (DMSO) was added to each well and the plates were shaken for 30 mins to dissolve the dark violet formazan crystals. The absorbance was measured at 570 nm wavelength using a microplate reader. The analysis was repeated for three times with reproducible results. The IC_{50} values were calculated from the graphs.

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