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A cyclic PNA-based compound targeting domain IV of HCV IRES RNA inhibits in vitro IRES-dependent translation

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Abstract—A cyclic molecule **1** constituted by a hepta-peptide nucleic acid sequence complementary to the apical loop of domain IV of hepatitis C virus (HCV) internal ribosome entry site (IRES) RNA has been prepared via a 'mixed' liquid-phase strategy, which relies on easily available protected PNA and poly(2-aminoethylglycinamide) building blocks. This compound **1** has been elaborated to mimic 'loop–loop' interactions. For comparison, its linear analog has also been investigated. Although preliminary biological assays have revealed the ability of **1** to inhibit in vitro the HCV IRES-dependent translation in a dose-dependent manner, the linear analog has shown a slightly higher activity.

1. Introduction

The internal ribosome entry site (IRES) located in the 5′-untranslated region (UTR) of the hepatitis C virus (HCV) genomic RNA mediates the cap-independent initiation of viral translation and seems to be involved in RNA replication. HCV IRES is a phylogenetically highly conserved RNA sequence[1] which adopts an ion-dependent tertiary fold including four major conserved secondary structure subdomains.[2] These overall secondary and tertiary structures were shown to be crucial for specific recognition and binding of several cellular components such as the eukaryotic initiation factor eIF3 and the ribosomal subunit 40S, to form the ribosomal initiation complex that, consequently initiates translation.[3,4] Thus, the subdomain sequences involved in such interactions represent targets of interest for inhibiting viral replication. In this context, the stem–loop of domain IV (Fig. 1) is particularly attractive as it contains in the apical loop the start codon AUG which pairs with the initiator tRNA during the translation initiation process.[5,6]

Among various approaches used to inhibit IRES function, the antisense strategy using synthetic DNA or RNA analogs complementary to different IRES sequences has been widely studied and seems to be an attractive tool both to understand IRES mechanisms and to develop anti-HCV drugs.

Thus, natural oligonucleotides,[7–11] as well as modified analogs such as phosphorothioates,[7,8,12–18] alpha-ano-

Figure 1. Structure of domain IV of HCV IRES RNA.

Abbreviations: Alloc, allyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; DIPEA, diisopropylethylamine; DMF, dimethylformamide; HATU, *O*-(7-aza-1-benzotriazolyl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexa-fluorophosphate; HOAt, 1-hydroxy-7-azabenzotriazole; Mmt, monomethoxytrityl; PyBop, (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; TFA, trifluoroacetic acid; TFMSA, trifluoromethane sulfonic acid; THF, tetrahydrofuran.

Keywords: Cyclic PNA; HCV IRES; Domain IV; Loop–Loop interaction.

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mer[9] and 2′-methoxyethoxy phosphodiesters,[14,18] methylphosphonates,[7] benzylphosphonates,[7,19] morpholinos,[20] (phenylalkyl)phosphonates,[21] 2′-*O*-methyl,[22] and recently peptide nucleic acids (PNAs),[23,24] have been developed to target different IRES regions. These studies showed that linear natural and modified oligonucleotides (containing, in general, 15–25 residues) could bind to domain IV very strongly, thus inhibiting in vitro the initiation of IRES-dependent translation.

Another interesting approach to inhibit stem–loop (hairpin) structures is to mimic intermolecular loop–loop interactions that are widely present in RNA–RNA associations and serve a diverse range of biological functions. Due to the increased accessibility of the bases in hairpin loops, intermolecular loop–loop interactions are particularly well adapted to trigger molecular recognition and to induce RNA–RNA annealing. Several loop–loop complexes are well described in the literature,[25–29] and one can cite as an example the interaction between two self-complementary dimerization initiation signal (DIS) loops responsible for the dimerization of the genomic HIV RNA.[30–33]

We previously reported the liquid-phase synthesis as well as the biological activity of two cyclic PNA molecules[34,35] and one of them was shown in vitro to interfere with the dimerization process of the HIV-1 genome. These 'loop-like' compounds, constituted by short PNA fragments (i.e., six residues), should afford several advantages over linear analogs, such as a greater selectivity, or over classical antisense oligomers, such as a lower molecular weight.

In this context, we designed compound **1**, which contains an antisense heptameric PNA moiety complementary to the seven residues of the domain IV loop and a spacer tethering the C- and N-terminal extremities of the PNA (Fig. 2). A molecular modeling study allowed us (i) to assess the length of the spacer in order to optimize the loop–loop interaction and (ii) to introduce a phenylalanine (F) residue at the PNA C-terminal extremity to increase stability of the complex via a π-stacking interaction between the phenyl moiety and the adjacent uracil nucleobase (Figs. 2 and 3).

In this paper, we report the molecular design and the liquid–phase synthesis of cyclic PNA [UCAUGGU]F_c

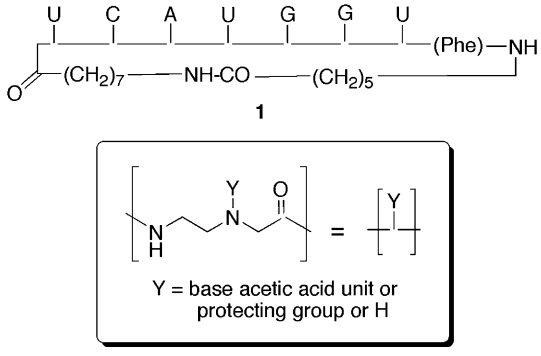


Figure 2. Structure of cyclic PNA [UCAUGGU]Fc (**1**).

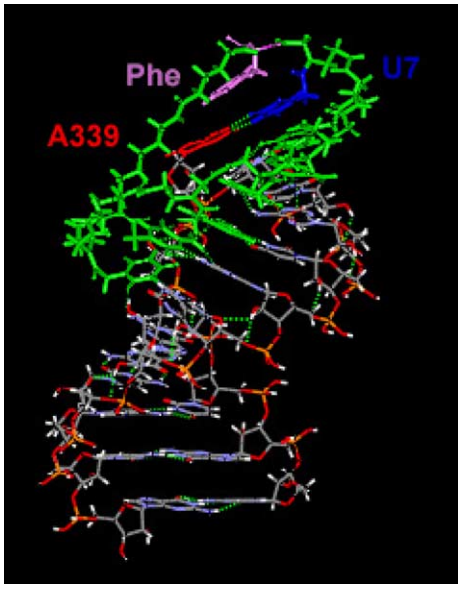


Figure 3. Molecular model of interaction between compound **1** and domain IV of HCV IRES RNA. Compound **1** is in green with its U7 residue in blue and its Phe residue in purple. A339 of domain IV is in red.

1, as well as the ability of **1** and of its linear PNA analog 5′-UCAUGGU-3′ (synthesis not shown) to inhibit in vitro IRES-dependent translation.

2. Results and discussion

2.1. Molecular modeling studies

In the molecular model of interaction between compound **1** and domain IV of HCV IRES RNA, the RNA stem and the annealed section constituted by the RNA loop and the PNA fragment, form two stable double helices (Fig. 3). The axes of these helices are collinear as previously reported for the interaction between a cyclic PNA and the DIS RNA of HIV-1.[35] The residue A339 is not strictly aligned with the other RNA residues, but this break ensures the continuity of the RNA sequence without making a kink in the double helix structure. The seven nucleobases of the RNA loop form Watson–Crick interactions with the complementary nucleobases of the PNA fragment. The presence of a phenylalanine (F) residue on compound **1** allows to stabilize the last Watson–Crick base pair interaction (A339–U7).

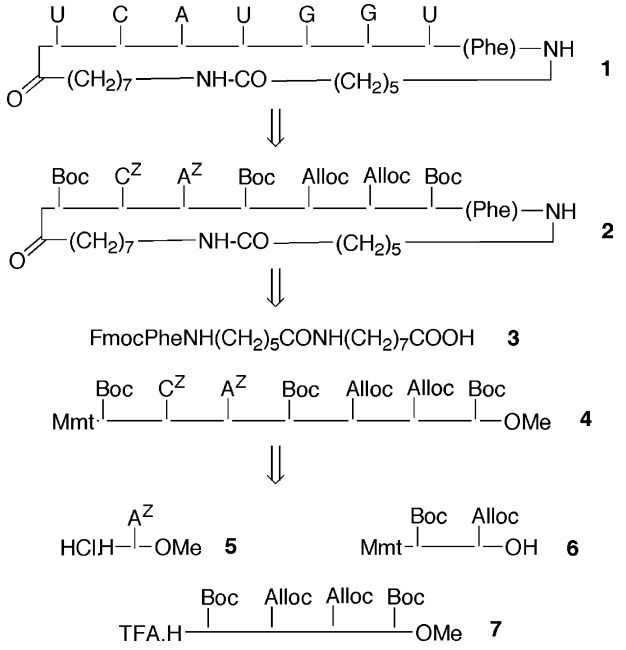
In the first step, a complex resulting from the interaction between the linear heptameric PNA bound with the phenylalanine residue and the domain IV RNA loop was built using Insight II molecular modeling package and the complex was energetically minimized with the CFF forcefield. In a second step, a linker of optimal length was added to close the structure. After an energetic minimization, the model was put under rectangular water box filled with TIP3P water molecules. The global charge was neutralized with Mg^{2+} counter ions. A 6 ns molecular dynamics simulation was computed at con-

stant pressure (1 bar) and at constant temperature (300 K). The simulation was performed on a SGI Origin 3800 supercomputer located at the 'Centre Informatique National de l'Enseignement Supérieur' (CINES) with the Amber 6 molecular modeling package. The shake algorithm was selected with 2 fs as integration time. During the first 800 ps, the temperature was linearly increased starting from 100 to 300 K. A conformation was sampled every 20 ps, and a close analysis of the trajectory revealed that all Watson–Crick interactions remained, and that the length of the linker was optimal. The phenylalanine residue protects the interaction between A339 and U7, since the angle between these two bases is comparable to the other base-pairs, angles in the RNA/PNA helix.

2.2. Chemistry

To synthesize compound **1**, we applied a 'mixed' strategy recently elaborated in our laboratory.[34] It relies on the protected PNA fragments and protected poly(2-aminoethylglycinamide) building blocks. This strategy has been shown to be a good alternative to the fully protected backbone (FPB) strategy[35,36] when the PNAs to prepare contain four different nucleobases, because it allows to circumvent the difficulty to work with a combination of at least eight orthogonal protecting groups.

The retrosynthetic pathway to compound **1** is illustrated in Scheme 1. This compound results from the cyclic precursor **2**, a 'mixed' heptameric structure that contains two PNA units (C^Z and A^Z) and a protected penta(2-aminoethylglycinamide) unit. At the C- and N-terminal extremities, a spacer constituted by a 8-aminooctanoic acid, a 6-aminocaproic acid, and a phenylalanine (F), allows to close the molecule. The secondary amino functions of the penta(2-aminoethylglycinamide) moiety



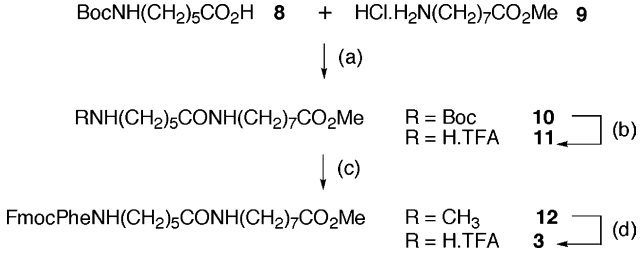
Scheme 1. Retrosynthetic route to compound **1**.

are protected by allyl and Boc protecting groups which are subsequently replaced, respectively with guanine and uracil acetic acid units in the last steps of the synthesis. Compound **2** is synthesized from spacer **3** and heptameric 'mixed' compound **4**, which is prepared from three key synthons previously described:[37] PNA fragment **5** and the protected di- and tetra-(2-aminoethylglycinamide) units (respectively, **6** and **7**).

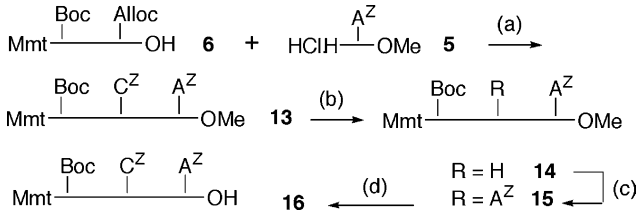
The synthesis of linker **3** is detailed in Scheme 2. It was prepared in four steps (70% overall yield) from commercially available *N*-Boc-aminocaproic acid **8**, methyl 8-aminooctanoate **9**, and *N*-α-Fmoc-L-phenylalanine.

The preparation of heptameric 'mixed' compound **4** (cf. Scheme 1) required the synthesis of 'mixed' compound **16** in four steps from compounds **5** and **6** (Scheme 3). In a first step, condensation of *N*-Z-adenine PNA monomer **5** with **6** by means of Bop reagent led to trimer **13** (80%). Then, a selective cleavage of the Alloc protecting group with $Pd[PPh_3]_4/Et_2NH$ afforded **14**, onto which was condensed a *N*-Z-cytosine acetic acid unit via HATU/HOAt activation, to give **15** in 68% overall yield. Finally, acid **16** was obtained by saponification using 1 M LiOH.

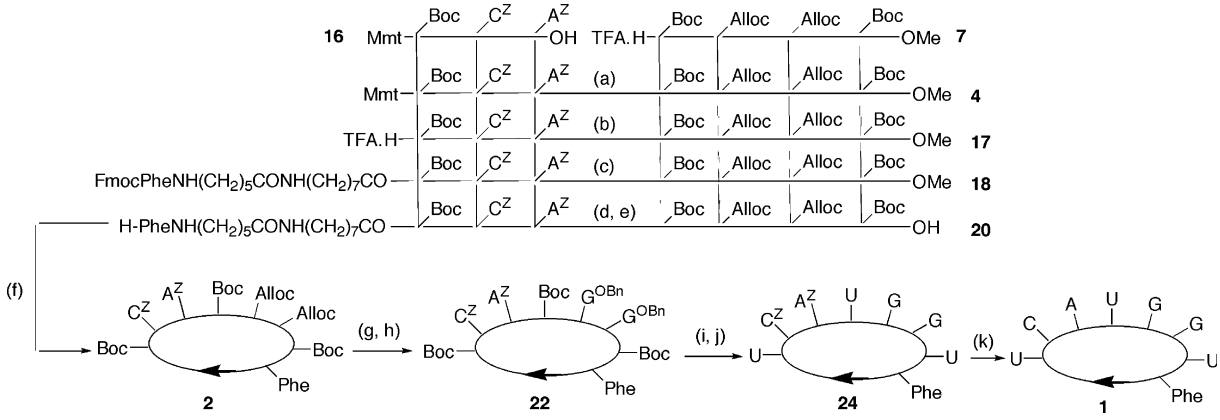
The condensation of **16** with protected tetramer **7** afforded 'mixed' heptameric compound **4** (Scheme 4). After cleavage of the Mmt protecting group by means of a solution of 2% TFA in CH_2Cl_2, the resulting compound **17** was coupled with spacer **3** to give compound **18**. The two condensation steps described above were performed via a PyBop activation, respectively, in 79% and 91% yields. The next steps were consisted removal of Fmoc from **18** with a Et_2NH/CH_2Cl_2 solution, to get **19**



Scheme 2. Synthesis of compound **3**. Reagents: (a) PyBrop, NMM, DMF (89%); (b) TFA, CH_2Cl_2 (90%); (c) Fmoc-Phe-OH, Bop, DIPEA, DMF (86%); (d) Dioxane, HCl (12 N), (5:1, v/v), reflux (100%).



Scheme 3. Synthesis of compound **16**. Reagents: (a) Bop, DIPEA, DMF (80%); (b) $Pd[PPh_3]_4$, Et_2NH, CH_2Cl_2 (91%); (c) $C^ZCH_2CO_2H$, HATU, HOAt, DIPEA, DMF (75%); (d) 1 N LiOH, THF (99%).



Scheme 4. Synthesis of compound **1**. Reagents: (a) PyBop, DIPEA, DMF (79%); (b) TFA, CH_2Cl_2 (0.02:1, v/v) (88%); (c) Fmoc-PheNH$(CH_2)_5$CONH$(CH_2)_7CO_2H$ **3**, PyBop, DIPEA, DMF (91%); (d) Et_2NH, CH_2Cl_2 (97%); (e) 1 M LiOH, THF (93%); (f) HATU, HOAt, DIPEA, DMF (65%); (g) $Pd[PPh_3]_4$, Et_2NH, CH_2Cl_2 (95%); (h) $G^{OBn}CH_2CO_2H$, HATU, HOAt, DIPEA, DMF (95%); (i) TFA, CH_2Cl_2, TIS (93%); (j) UCH_2CO_2H, HATU, HOAt, DIPEA, DMF (88%); (k) TFMSA, TFA, thioanisole (100%).

(97%), which was saponified using a 1 M LiOH aqueous solution (93%) to afford **20**. At last, a head-to-tail cyclization of **20** via a HATU/HOAt activation and semi-high dilution conditions (10 mM) yielded cyclic 'mixed' precursor **2** in 65% yield.

The last stage for the synthesis of **1** consists in the introduction of the uracil and guanine acetic acid units to generate the heptameric PNA moiety (Scheme 4). To avoid unpredictable side reactions that uracil nucleobases are suspected to induce,[31] we introduced first the guanine acetic acid units. Thus, a selective cleavage of the two Alloc protecting groups by means of $Pd(PPh_3)_4/Et_2NH$, afforded the coupling of two OBn–guanine acetic acid units onto the two free amino functions of **21**, via a HATU/HOAt activation, to give derivative **22** in 90% overall yield. Then, treatment of **22** with TFA/CH_2Cl_2/TIS led to the removal of the three Boc protecting groups as well as to the cleavage of the benzyl guanine exocyclic protecting groups. HATU-mediated condensation of three uracil acetic acid units onto **23** was then successfully achieved (88% yield) despite the presence of the free exocyclic amine functions onto the guanine residues. Finally, simultaneous Z removal of cytosine and adenine nucleobases of **24** by means of a TFMSA/TFA/thioanisole solution allowed to obtain compound **1** which was isolated after semi-preparative HPLC (16% yield). Its purity was determined by HPLC analyses and its structure was confirmed by MALDI-TOF experiments.

2.3. Inhibitory effect on HCV in vitro translation

To investigate the inhibitory action of compound **1** and of its linear counterpart (data not shown) on HCV IRES dependent translation, coupled transcription and translation experiments were performed (Fig. 4). Different concentrations of **1** were mixed with linearized plasmid pFl-HCV IRES-hRl leading, after transcription by T7 polymerase, to the expression of a single bicistronic transcript and, after translation, to the expression of luciferase proteins. The expression of firefly luciferase

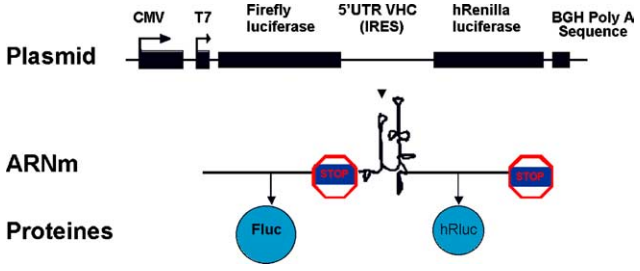


Figure 4. Model used for transcription and translation experiments.

is driven by a cap-dependent mechanism and should not be affected by the addition of **1**. In contrast, the expression of hRenilla luciferase is under the control of HCV IRES; its activity should be inhibited upon binding of **1** to IRES sequence(s), if the interaction is adequate to disrupt the association of proteins.

A dose-dependent inhibition of viral translation was observed (Fig. 5). Maximal effect was obtained with 30 μM

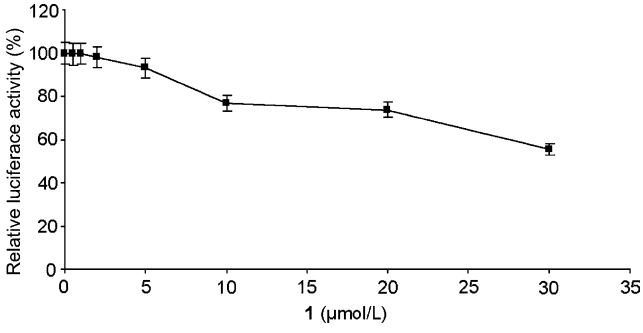


Figure 5. Inhibition assay of HCV-IRES dependent in vitro translation by **1**. Varying concentrations of **1** (μmol L^{-1}) were mixed with a rabbit reticulocyte lysate (Quick Master Mix) and 0.5 μg of linearized plasmid pFl-HCV IRES-hRl. Effect of **1** on the inhibition of IRES dependent translation of hRenilla luciferase was observed. hRenilla activity was normalized relative to firefly luciferase activity. Percentages are calculated relative to luciferase activity in the absence of **1**. All experiments were performed at least in triplicate. The percentage mean values ± SD are given on the curve.

of **1**, which corresponds to 45 ± 5% of IRES-mediated translation inhibition. Higher concentrations (up to 60 μM) showed increasing inhibition of hRenilla luciferase and also a non-specific inhibition of firefly luciferase. Similar results were obtained when compound **1** was assayed directly with RNA transcripts on rabbit reticulocyte lysate (decoupling translation–transcription). The same experiments on the linear analog also revealed a dose-dependent inhibition of translation, of about 5 μM (data not shown).

The lower activity of cyclic PNA [UCAUGGU]Fc **1** compared with its linear analog could be explained in the light of the work reported by Toulmé and co-workers.[29,38–40] Actually, they identified by in vitro selection the RNA aptamers directed against several RNA hairpins through loop–loop interactions. In all the cases, the loops of the aptamers showing the highest affinities are not strictly complementary to the targeted apical loops. Thus, the aptamers targeting domain IV of the HCV IRES contain in their loops a consensus sequence 5′-**AUCAUGG**-3′ in which six residues (in bold) interact with six of the seven residues of the apical loop of domain IV.[29] This result seems to indicate that the structure of cyclic PNA **1** is not well adapted for a high-affinity interaction. The molecular modeling as well as the synthesis of a cyclic PNA containing the consensus sequence is currently under progress.

3. Conclusions

A cyclic PNA-based compound (**1**), containing the complementary heptameric sequence of the apical loop of domain IV of HCV IRES, has been designed in order to mimic highly stable loop–loop complexes. For comparison, its linear counterpart has also been investigated. The cyclic structure, of lower molecular weight than classical antisense oligomers, should be more selective than the linear analog.

Compound **1** has been successfully prepared via a 'mixed' liquid-phase strategy which relies on easily available protected PNA and poly(2-aminoethylglycinamide) building blocks. This procedure offers the advantage over the FPB strategy of requiring fewer orthogonal protecting groups, and enables the preparation of PNA containing the four nucleobases A, C, G, and U. Moreover, to avoid side-reactions due to the uracil moiety, this nucleobase must be introduced at the last stages of the synthesis.

Preliminary biological assays have revealed the ability of compound **1** and of its linear counterpart to interfere in vitro with the HCV IRES-dependent translation in a dose–dependent manner. This result suggests for the first time that a cyclic PNA targeting the HCV 5′-UTR is able to specifically downregulate HCV IRES-directed translation. The lower activity of the cyclic compound compared with its linear analog could be explained by the results obtained by Toulmé and co-workers. Further experiments are under progress to confirm the binding site as well as the mode of action of these compounds, to evaluate their selectivity and to optimize the cyclic PNA structure.

4. Experimental (chemistry)

4.1. General

Analytical thin-layer chromatography was conducted on Merck precoated silica gel $60F_{254}$ plates and the compounds were visualized with ninhydrin test and/or by visualization under ultraviolet light (254 nm). Chromatography was performed on Merck Silica gel 60 (230–400 mesh ASTM) using the solvent systems (volume ratios) indicated below. Analytical HPLC chromatograms were obtained using a Waters HPLC system (600E system controller, 996 photodiode array detector or 2487 dual wavelength absorbance detector) and a Merck Lichrospher 100 (250×4 mm^2) RP-18 (5 μm) column. The HPLC flow rate was 1 mL/min, and the elution solvents were water (0.1% TFA) as solvent A, and acetonitrile (0.1% TFA) as solvent B (pH 6). ^{1}H and ^{13}C NMR spectroscopies were performed using a Bruker AC 200 or AC 500 Fourier Transform spectrometer. Chemical shifts (δ) are reported in parts per million (ppm) (in ^{1}H NMR descriptions s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and bs = broad peak). ESI mass spectra were recorded with an INCOS 500^E FINNIGAN MAT or a TSQ 7000 FINNIGAN MAT. MALDI-TOF-MS spectra were recorded with a MALDI-TOF 'DE PRO' Applied biosystem.

4.2. Synthesis

4.2.1. (UCAUGGU)F$_c$·4TFA (1). Compound **24** (55 mg, 21.7 μmol) was dissolved in 1.5 mL of TFMSA/thioanisole/TFA (1:0.4:0.3, v/v/v) at 0 °C. The mixture was stirred at room temperature for 1 and the deprotected product was precipitated out by Et_2O (5 mL). The crude product (79 mg) is obtained after washing with MeOH/Et_2O (1:1, v/v) and drying, and it was further purified on a reverse phase preparative HPLC column (Prep Nova-Pack, HR-C18, 100×40 mm^2) and eluted with 20% acetonitrile in water (0.1% TFA) at a flow rate of 5 mL/min to give **1** (10 mg, 16%) as a white solid after lyophilization. HPLC (A/B 80:20 to 0:100 over 30 min) t_R = 9.7 min (λ_{max} = 258.4 nm). MS (ESI+) calcd for $C_{96}H_{123}N_{41}O_{26}$ $[(M+2H)/2]^+$: 1134.0; found: 1134.0 $[(M+2H)/2]^+$. MALDI-TOF-MS Calcd for $C_{96}H_{123}N_{41}O_{26}$ $[M+H]^+$: 2267.9; found: 2267.7 $[M+H]^+$.

4.2.2. (BocC^{Z}A^{Z}BocAllocAllo cBoc)F$_c$ (2). To a cold solution (0 °C) of **20** (67 mg, 30.2 μmol) and DIPEA (34 μL, 196.2 μmol, 6.5 equiv) in DMF (3 mL), were added HATU (17 mg, 45.9 μmol, 1.5 equiv) and HOAt (8 mg, 60.4 μmol, 2 equiv). The mixture was stirred for 2 h at room temperature. The solvent was evaporated under reduced pressure. The residue was taken up in $CHCl_3$ and washed successively with a 1 M aqueous $KHSO_4$ solution, a saturated aqueous $NaHCO_3$ solution, brine, and finally dried over Na_2SO_4. The solvent was then removed in vacuo and the residue was purified by column chromatography ($CHCl_3$/MeOH 9:1 to 8:2) to afford **2** (43 mg, 65%) as

a white amorphous powder. TLC ($CHCl_3$/MeOH 9:1) $R_f = 0.50$. HPLC (A/B 80:20 to 0:100 over 30 min) $t_R = 21.1$ min ($\lambda_{max} = 241.8$ and 275.0 nm). ^1H NMR (500 MHz, CD_3OD) δ 8.80–6.60 (m, 26H, (19CH, 7NH)); 6.30–5.65 (m, 4H, (2CH, 2NH)); 5.30–4.40 (m, 16H, ($8CH_2$)); 4.40–2.80 (m, 49H, (1CH, $24CH_2$)); 2.20–1.10 (m, 47H, ($10CH_2$, $9CH_3$)). MS (ESI+) calcd for $C_{103}H_{145}N_{25}O_{27}$ $[(M+Na+H)/2]^+$: 1094.0; found: 1093.9 $[(M+Na+H)/2]^+$.

4.2.3. FmocPheNH$(CH_2)_5$CONH$(CH_2)_7CO_2H$ (3). Compound **12** (829 mg) was dissolved in 16 mL of a dioxane/HCl (12 N) (5:1, v/v) solution. The mixture was stirred at reflux for 8 h. The solvent was evaporated under reduced pressure and the residue was precipitated and triturated in water. Compound **3** (808.0 mg, 100%) was obtained as a white solid after washing, filtering, and drying in vacuo. TLC (EtOAc/MeOH 8:2) $R_f = 0.40$. HPLC (A/B 80:20 to 0:100 over 30 min) $t_R = 22.4$ min ($\lambda_{max} = 269.8$ and 303.8 nm). ^1H NMR (200 MHz, $CDCl_3$) δ 7.80–7.75 (d, 2H, (2CH)); 7.55–7.51 (d, 2H, (2CH)); 7.50–7.10 (m, 9H, (9CH)); 4.40–4.00 (m, 4H, (2CH, CH_2)); 3.25–2.75 (m, 6H, ($3CH_2$)); 2.40–2.05 (m, 4H, ($2CH_2$)); 1.70–1.10 (m, 16H, ($8CH_2$)). MS (ESI+) calcd for $C_{38}H_{47}N_3O_6$ for $[M+Na]^+$: 664.3; found: 664.4 $[M+Na]^+$.

4.2.4. Mmt(Boc$C^Z$$A^Z$BocAllocAllocBoc)OMe (4). To a cold solution (0 °C) of **16** (555 mg, 0.43 mmol), DIPEA (226 μL, 1.30 mmol, 3 equiv) and **7** (446 mg, 0.49 mmol, 1.13 equiv) in DMF (5 mL), was added PyBop (271 mg, 0.52 mmol, 1.2 equiv). The mixture was stirred for 10 min at this temperature then allowed to warm to room temperature (ca. 1 h). The solvent was then evaporated under reduced pressure. The residue was taken up in $CHCl_3$ and washed successively with a saturated aqueous $NaHCO_3$ solution, brine, and finally dried over Na_2SO_4. The solvent was then removed in vacuo and the residue was purified by column chromatography ($CHCl_3$/MeOH 9:1) to afford **4** (709 mg, 79%) as a white amorphous powder. TLC ($CHCl_3$/MeOH 9:1) R_f 0.46. HPLC (A/B 80:20 to 0:100 over 30 min) $t_R = 28.2$ min ($\lambda_{max} = 246.5$ and 275.7 nm). ^1H NMR (500 MHz, $CDCl_3$) δ 8.80–6.50 (m, 36H, (28CH, 8NH)); 6.00–5.70 (m, 2H, (2CH)); 5.40–4.30 (m, 16H, ($8CH_2$)); 4.30–2.00 (m, 49H, ($21CH_2$, $2CH_3$, NH)); 1.60–1.00 (m, 27H, ($9CH_3$)). ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.0–163.0 (10C); 158.0–150.0 (11C); 149.3 (2C); 146.3 146.2 (3C); 138.1 (1C); 135.7 135.3 (2C); 132.8 (2C); 129.9 (2C); 128.6 128.4 128.0 (18C); 126.4 (2C); 121.7 (1C); 117.9 117.4 (2C); 113.3 (2C); 95.7 (1C); 81.1 80.8 80.6 (3C); 70.5 (1C); 67.6 (2C); 66.6 66.5 (2C); 55.3 (1C); 52.5 (1C); 52.0–36.0 (23C); 28.4 28.3 (9C). MS (ESI+) calcd for $C_{101}H_{130}N_{22}O_{26}$ $[(M+2H)/2]^+$: 1034.5; found: 1034.5 $[(M+2H)/2]^+$.

4.2.5. BocNH$(CH_2)_5$CONH$(CH_2)_7CO_2Me$ (10). To a cold solution (0 °C) of BocNH$(CH_2)_5CO_2H$ **8** (181.8 mg, 0.79 mmol), NMM (548 μL, 3.15 mmol, 4 equiv) and HCl. $H_2N(CH_2)_7CO_2Me$ **9** (200 mg, 0.96 mmol, 1.1 equiv) in DMF (2 mL), was added PyBrop (440.2 mg, 0.94 mmol, 1.2 equiv). The mixture was stirred for 3 h at room temperature. The solvent was evaporated under reduced pressure. The residue was taken up in CH_2Cl_2 and washed successively with a 1 M aqueous $KHSO_4$ solution, a saturated aqueous $NaHCO_3$ solution, brine and finally dried over Na_2SO_4. The solvent was then removed in vacuo and the residue was purified by column chromatography (EtOAc 100%) to afford **10** (270.6 mg, 89%) as a colorless resin. TLC (EtOAc 100%) R_f 0.51. HPLC (A/B 80:20 to 0:100 over 30 min) $t_R = 17.9$ min. ^1H NMR (200 MHz, $CDCl_3$) δ 5.57 (br s, 1H, (NH)); 4.58 (br s, 1H, (NH)); 3.65 (s, 3H, (CH_3)); 3.30–3.00 (m, 4H, ($2CH_2$)); 2.29 (t, 2H, (CH_2), $^3J_{2-3} = 7.4$ Hz); 2.15 (t, 2H, (CH_2), $^3J_{11-12} = 7.4$ Hz); 1.75–1.20 (m, 25H, ($8CH_2$, $3CH_3$)). ^{13}C NMR (50.3 MHz, $CDCl_3$) δ 174.5 (1C); 172.8 (1C); 156.0 (1C); 79.5 (1C); 51.5 (1C); 39.5 (2C); 36.6 34.0 (2C); 29.8 29.6 29.0 28.8 (4C); 28.5 (3C); 26.7 26.4 25.4 24.8 (4C).

4.2.6. TFA·$NH_2(CH_2)_5$CO NH$(CH_2)_7CO_2Me$ (11). Compound **10** (706.0 mg, 1.83 mmol) was dissolved in 4 mL of TFA/CH_2Cl_2 (1:1, v/v). The mixture was stirred at room temperature for 1 h. The solvent was concentrated in vacuo and on addition of Et_2O, **11** (656 mg, 89%) was obtained as a white solid after filtration and drying. ^1H NMR (200 MHz, $CDCl_3$) δ 6.52 (t, 1H, (NH)); 3.65 (s, 3H, (CH_3)); 3.15 (m, 2H, (CH_2)); 2.93 (t, 2H, (CH_2)); 2.30 (t, 2H, (CH_2), $^3J_{2-3} = 7.4$ Hz); 2.14 (t, 2H, (CH_2), $^3J_{11-12} = 7.4$ Hz); 1.65–1.25 (m, 18H, ($9CH_2$)). ^{13}C NMR (50.3 MHz, $CDCl_3$) δ 174.5 (1C); 173.5 (1C); 51.5 (1C); 39.6 (1C); 35.8 (1C); 34.0 (2C); 30.0–24.0 (9C).

4.2.7. FmocPheNH$(CH_2)_5$CONH$(CH_2)_7CO_2Me$ (12). To a cold solution (0 °C) of FmocPheOH (570.0 mg, 1.47 mmol), DIPEA (768 μL, 4.41 mmol, 3 equiv) and **11** (587.8 mg, 1.47 mmol, 1.1 equiv) in DMF (4 mL), was added Bop (780.5 mg, 1.76 mmol, 1.2 equiv). The mixture was stirred for 2 h at room temperature. The solvent was evaporated under reduced pressure. The residue was taken up in $CHCl_3$ and washed successively with a 1 M aqueous $KHSO_4$ solution, a saturated aqueous $NaHCO_3$ solution, brine and finally dried over Na_2SO_4. The solvent was then removed in vacuo and the residue was purified by column chromatography ($CHCl_3$/MeOH 95:0.5) to afford **12** (829 mg, 86%) as a white amorphous powder. TLC (EtOAc 100%) R_f 0.27. HPLC (A/B 80:20 to 0:100 over 30 min) $t_R = 25.2$ min ($\lambda_{max} = 265.0$ and 299.4 nm). ^1H NMR (200 MHz, $CDCl_3$) δ 7.77–7.73 (d, 2H, (2CH)); 7.55–7.51 (d, 2H, (2CH)); 7.48–7.10 (m, 9H, (9CH)); 6.22 (br s, 1H, (NH)); 5.70 (m, 1H, (NH)); 4.50–4.00 (m, 4H, (2CH, CH_2)); 3.65 (s, 3H, (CH_3)); 3.30–2.90 (m, 6H, ($3CH_2$)); 2.28 (t, 2H, (CH_2),$^3J_{2-3} = 7.4$ Hz); 2.10 (t, 2H, (CH_2), $^3J_{11-12} = 7.2$ Hz); 1.80–1.00 (m, 16H, ($8CH_2$)). ^{13}C NMR (50.3 MHz, $CDCl_3$) δ 174.3 (1C); 172.8 (1C); 170.8 (1C); 155.9 (1C); 143.8 (2C); 141.3 (2C); 136.7 (1C); 129.3 (2C); 128.7 (2C); 127.8 (2C); 127.1 127.0 (3C); 125.0 (2C); 120.0 (2C); 67.0 (1C); 56.5 (1C); 51.5 (1C); 47.2 (2C); 39.5 39.1 (2C); 36.3 (1C); 34.0 (1C); 29.7 29.6 (2C); 29.0 26.9 (2C); 26.7 (1C); 26.2 (1C); 24.9 24.8 (2C). MS (ESI+) calcd for $C_{39}H_{49}N_3O_6$ for $[M+Na]^+$: 678.4; found: 678.4 $[M+Na]^+$.

4.2.8. Mmt(BocAllocA^{Z})OMe (13). To a cold solution (minus 15 °C) of **6** (1.13 g, 1.68 mmol) and **5** (0.82 g, 1.72 mmol, 1.1 equiv) in DMF (20 mL) was added DIPEA (1.1 mL, 6.27 mmol, 3.7 equiv) and Bop (0.89 g, 2.01 mmol, 1.2 equiv). The mixture was stirred for 1 h at this temperature then allowed to warm to room temperature (ca. 1 h). The solvent was evaporated under reduced pressure. The residue was taken up in EtOAc and washed successively with a saturated aqueous $NaHCO_3$ solution, water and finally dried over Na_2SO_4. The solvent was then removed in vacuo and the residue was purified by column chromatography (EtOAc/MeOH 9:1) to afford **13** as a white amorphous powder (1.47 g, 80%). TLC (CH_2Cl_2/MeOH 95:05) R_f 0.31. ^{1}H NMR (200 MHz, $CDCl_3$) δ 9.20–7.05 (m, 22H, (19CH, 3NH)); 6.90–6.70 (m, 2H, (2CH)); 6.00–5.60 (m, 1H, (CH)); 5.40–4.90 (m, 6H, ($3CH_2$)); 4.60–4.30 (m, 2H, (CH_2)); 4.15–3.20 (m, 22H, ($2CH_3$, $8CH_2$)); 2.60–2.30 (m, 2H, (CH_2)); 1.60–1.35 (m, 9H, ($3CH_3$)). ^{13}C NMR (50.3 MHz, $CDCl_3$) δ 171.2 170.1 169.6 (3C); 167.1 (1C); 158.2 (1C); 156.4 155.9 (2C); 152.1 (1C); 151.8 (1C); 151.1 (1C); 149.2 (1C); 146.0–145.0 (3C); 137.2 (1C); 135.6 (1C); 132.2 (1C); 129.8 (2C); 129.0–127.7 (13C); 126.6 (2C); 121.5 (1C); 11.7 (1C); 113.3 (2C); 80.7 (1C); 70.7 (1C); 67.6 (1C); 66.9 (1C); 55.2 (1C); 54.0–48.0 (7C); 44.3 42.6 (2C); 38.3 36.8 (2C); 28.4 (3C). MS (ESI+) calcd for $C_{57}H_{67}N_{11}O_{12}$ $[M+H]^+$: 1098.5 and for $[M+Na]^+$: 1120.5; found: 1098.0 $[M+H]^+$ and 1120.3 $[M+Na]^+$.

4.2.9. Mmt(BocHA^{Z})OMe (14). Compound **13** (210 mg, 0.19 mmol) and Et_2NH (297 μL, 2.87 mmol, 15 equiv) were dissolved in CH_2Cl_2 (2 mL) and $Pd[P(Ph)_3]_4$ (22 mg, 19.1 μmol, 0.1 equiv) was added. The mixture was stirred for 1 h at room temperature. The solvent was concentrated under reduced pressure and the crude residue was purified by column chromatography ($CHCl_3$/MeOH 9.5:0.5 to 9:1) to afford **14** (190 mg, 98%) as a yellowish amorphous powder. TLC ($CHCl_3$/MeOH 95:05) R_f 0.28. HPLC (A/B 80:20 to 0:100 over 30 min) t_R = 21.4 min (λ_{max} = 273.3 nm). ^{1}H NMR (200 MHz, $CDCl_3$) δ 9.00–7.00 (m, 22H, (19CH, 3NH)); 6.90–6.70 (m, 2H, (2CH)); 5.30–4.95 (m, 4H, ($2CH_2$)); 4.32 (mi.) and 4.10 (ma.) (s, 2H, (CH_2)); 3.90–2.90 (m, 18H, ($2CH_3$, $6CH_2$)); 2.70–1.85 (m, 3H, (CH_2, NH)); 1.60–1.20 (m, 9H, ($3CH_3$)). ^{13}C NMR (50.3 MHz, $CDCl_3$) δ 173.1 (1C); 169.7 169.6 (2C); 166.6 (1C); 158.3 (1C); 156.3 (1C); 152.7 (1C); 151.7 (1C); 151.0 (1C); 149.4 (1C); 145.9 (2C); 144.3 (1C); 137.6 (1C); 135.6 (1C); 129.9 (2C); 128.7 128.6 128.1 (13C); 126.8 126.7 (2C); 121.5 (1C); 113.3 (2C); 81.2 (1C); 70.6 (1C); 67.8 (1C); 55.3 (1C); 53.5 53.1 52.8 52.6 49.6 48.3 47.9 (7C); 44.0 (1C); 42.6 (1C); 39.1 (1C); 37.3 (1C); 28.4 (3C). MS (ESI+) calcd for $C_{53}H_{63}N_{11}O_{10}$ $[M+H]^+$: 1014.5; found: 1014.0 $[M+H]^+$.

4.2.10. Mmt(BocC^{Z}A^{Z})OMe (15). A solution of **14** (631 mg, 0.62 mmol), $C^{Z}CH_2CO_2H$ (221 mg, 0.73 mmol, 1.17 equiv), DIPEA (326 μL, 1.87 mmol, 3 equiv) in DMF (3 mL) was stirred at 0 °C. HATU (284 mg, 0.75 mmol, 1.2 equiv) was then added. The mixture was stirred for 15 min at 0 °C then allowed to warm to room temperature and stirred for 1 h. The solvent was evaporated under reduced pressure and the crude residue was taken up in EtOAc and washed successively with a saturated aqueous $NaHCO_3$ solution, and with water and then dried over Na_2SO_4. The solvent was then removed in vacuo and the residue was purified by column chromatography ($CHCl_3$/MeOH 9.5:0.5) to afford **15** as a white amorphous powder (605 mg, 75%). TLC (EtOAc/MeOH 8:2) R_f 0.26. HPLC (A/B 80:20 to 0:100 over 30 min) t_R = 25.9 min (λ_{max} = 246.5 and 274.5 nm). ^{1}H NMR (200 MHz, $CDCl_3$) δ 10.40–6.60 (m, 31H, (28CH, 3NH)); 5.50–4.40 (m, 8H, ($4CH_2$)); 4.40–2.60 (m, 22H, ($8CH_2$, $2CH_3$)); 2.33 (br s, 2H, (CH2)); 1.60–1.00 (m, 9H, ($3CH_3$)). ^{13}C NMR (50.3 MHz, $CDCl_3$) δ 170.8 170.3 169.5 168.1 167.7 (5C); 163.5 (1C); 158.0 (1C); 156.0 (1C); 152.6 152.4 151.6 (5C); 150.3 149.4 (2C); 146.1 (2C); 145.2 (1C); 137.9 (1C); 135.8 135.2 (2C); 129.8 (2C); 128.5 128.4 128.3 128.0 (18C); 126.5 (2C); 121.4 (1C); 113.3 (2C); 95.5 (1C); 80.7 (1C); 70.4 (1C); 67.6 (2C); 55.3 (1C); 53.5 (1C); 52.6 52.0 50.8 50.2 48.3 47.6 (5C); 44.6 (1C); 42.5 (1C); 37.4 36.7 (2C); 28.4 (3C). MS (ESI+) calcd for $C_{67}H_{74}N_{14}O_{14}$ $[M+H]^+$: 1299.5 and for $[M+Na]^+$: 1321.5; found: 1299.0 $[M+H]^+$ and 1321.2 $[M+Na]^+$.

4.2.11. Mmt(BocC^{Z}A^{Z})OH (16). Compound **15** (147 mg, 0.11 mmol) was dissolved in THF (3.5 mL). To the solution, were added three times, at 5 min intervals, 1 M aqueous LiOH (230 μL, 0.23 mmol, 2 equiv). The mixture was stirred for 15 min at room temperature, then the pH was adjusted to 7 with a 0.5 M HCl solution and the aqueous layer was extracted with $CHCl_3$. The organic extract was dried over Na_2SO_4 and concentrated in vacuo to dryness to afford **16** (140.0 mg, 99%) as a white amorphous powder. TLC (EtOAc/MeOH 7:3) R_f 0.28. HPLC (A/B 80:20 to 0:100 over 30 min) t_R = 24.4 min (λ_{max} = 246.5 and 275.7 nm). ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.80–6.50 (m, 31H, (28CH, 3NH)); 5.40–4.30 (m, 8H, ($4CH_2$)); 4.30–2.70 (m, 21H, ($8CH_2$, CH_3)); 1.60–1.00 (m, 9H, ($3CH_3$)). MS (ESI) calcd for $C_{66}H_{72}N_{14}O_{14}$ $[M-H]^-$: 1283.5; found: 1283.2 $[M-H]^-$.

4.2.12. TFA·H(BocC^{Z}A^{Z}BocAllocAllocBoc)OMe (17). Compound **4** (509 mg, 0.25 mmol) was dissolved in 3 mL of a 2% TFA/CH_2Cl_2 (v/v) solution. The mixture was stirred at room temperature for 2 h. The solvent was concentrated in vacuo and on addition of Et_2O, **17** (422 mg, 88%) was obtained as a yellowish solid after filtration and drying. HPLC (A/B 80:20 to 0:100 over 30 min) t_R = 20.6 min (λ_{max} = 247.6 and 274.5 nm). ^{1}H NMR (500 MHz, CD_3OD) δ 8.90–6.50 (m, 24H, (24CH)); 6.00–5.65 (m, 2H, (2CH)); 5.40–4.30 (m, 16H, ($8CH_2$)); 4.30–2.00 (m, 45H, ($21CH_2$, CH_3)); 1.60–1.10 (m, 27H, ($9CH_3$)). ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.0–163.0 (10C); 162.2 161.5 (1C (COO^- (TFA))); 158.0–150.0 (10C); 149.2 (2C); 145.2 (1C); 135.7 135.3 (2C); 132.7 (2C); 128.6 (10C); 121.1 (1C); 119.8 117.4 114.0 (1C (CF_3 (TFA))); 117.9 117.2 (2C); 95.7 (1C); 81.3 81.1 80.7 (3C); 67.8 (2C); 66.6 66.5 (2C); 54.0–35.0 (24C); 28.2 (9C). MS (ESI+) calcd for $C_{81}H_{114}N_{22}O_{25}$ $[M+H]^+$: 1795.8; found: 1795.5 $[M+H]^+$.

4.2.13. FmocPheNH$(CH_2)_5$CONH$(CH_2)_7$CONH(BocC^Z-A^ZBocAllocAllocBoc)OMe (18). To a cold solution (0 °C) of **3** (58 mg, 84.7 μmol, 1.1 equiv), DIPEA (43 μL, 0.25 mmol, 3 equiv) and **17** (158 mg, 82.4 μmol, 1 equiv) in DMF (0.8 mL), was added PyBop (52 mg, 99.2 μmol, 1.2 equiv). The mixture was stirred for 10 min at this temperature then allowed to warm to room temperature (ca. 1 h). The solvent was evaporated under reduced pressure. The residue was taken up in $CHCl_3$ and washed successively with a 1 M aqueous $KHSO_4$ solution, a saturated aqueous $NaHCO_3$ solution, brine and finally dried over Na_2SO_4. The solvent was then removed in vacuo and the residue was purified by column chromatography ($CHCl_3$/MeOH 9:1 to 8.5:1.5) to afford **18** (183 mg, 91%) as a white amorphous powder. TLC (EtOAc/MeOH 6:4) R_f 0.41. HPLC (A/B 80:20 to 0:100 over 30 min) t_R = 26.2 min (λ_{max} = 265.5 nm). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.62 (ma.) 8.60 (mi.) (s, 1H, (CH)); 8.11 (m, 1H, (CH)); 7.70 7.69 (d, 2H, (2CH)); 7.60–7.05 (m, 23H, (23CH)); 6.80–5.70 (m, 4H, (2CH, 2NH)); 5.30–4.43 (m, 16H, ($8CH_2$)); 4.43–2.40 (m, 55H, (2CH, $25CH_2$, CH_3)); 2.15–1.80 (m, 4H, ($2CH_2$)); 1.60–1.10 (m, 43H, ($8CH_2$, $9CH_3$)). ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.5–166.0 (12C); 163.3 (1C); 156.8–154.5 (6C); 152.8–150.0 (5C); 149.4 (2C); 144.8 (1C); 143.8 (2C); 141.3 (2C); 136.8 (1C); 135.7 135.3 (2C); 132.6 (2C); 129.3 (2C); 128.8-128.0 (12C); 127.7 (2C); 127.1 126.9); 125.1 125.0 (2C); 121.2 (1C); 120.0 (2C); 117.9 (2C); 95.6 (1C); 81.1 80.8 80.6 (3C); 67.7 67.4 (2C); 67.0 (1C); 66.6 66.4 66.3 (2C); 56.2 (1C); 53.8–36.6 (26C); 47.2 (2C); 36.5 (1C); 36.2 (1C); 29.7 29.3 (2C); 29.0 28.6 (2C); 28.4–27.7 (9C); 26.6 (1C); 26.2 (1C); 25.5 24.1 (2C). MS (ESI+) calcd for $C_{119}H_{159}N_{25}O_{30}$ $[M+Na]^+$: 2441.1; found: 2441.4 $[M+Na]^+$.

4.2.14. HPheNH$(CH_2)_5$CONH$(CH_2)_7$CONH(Boc$C^Z$$A^Z$-BocAllocAllocBoc)OMe (19). Compound **18** (352 mg, 0.15 mmol) was dissolved in CH_2Cl_2 (2 mL) and Et_2NH (2 mL, 19.33 mmol, 130 equiv) was added at 0 °C. The mixture was allowed to warm to room temperature and stirred for 1 h. The solvent was concentrated under reduced pressure. The residue was taken up in CH_2Cl_2 (1 mL) and precipitated with Et_2O at 0 °C. Compound **19** (320 mg, 97%) was obtained as a white amorphous powder after triturating in Et_2O and drying. TLC ($CHCl_3$/MeOH 8.5:1.5) R_f 0.55. HPLC (A/B 80:20 to 0:100 over 30 min) t_R = 20.8 min (λ_{max} = 241.8 and 273.8 nm). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.80–6.50 (m, 19H, (19CH)); 6.50–5.70 (m, 4H, (2CH, 2NH)); 5.40–4.30 (m, 16H, ($8CH_2$)); 4.30–2.50 (m, 52H, (CH, $24CH_2$, CH_3)); 2.30–2.00 (m, 4H, ($2CH_2$)); 1.60–1.00 (m, 43H, ($8CH_2$, $9CH_3$)). ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.1 174.5 174.0 171.2 170.0 169.5 168.7 168.3 168.0 167.7 164.5 164.3 (12C); 163.6 (1C); 156.8 (5C); 153.5–149.0 (7C); 146.0 (1C); 137.4 (1C); 135.7 135.2 (2C); 132.7 (2C); 129.3 (2C); 129.0-126.0 (13C); 121.6 (1C); 118.2–116.0 (2C); 95.7 (1C); 81.5–80.2 (3C); 67.6 67.5 (2C); 66.6 66.4 66.3 (2C); 56.2 (1C); 54.0–37.5 (27C); 36.6 (1C); 36.4 (1C); 30.3–27.5 (13C); 26.8–26.0 (2C); 25.7–24.9 (2C). MS (ESI+) calcd for $C_{104}H_{149}N_{25}O_{28}$ $[M+Na]^+$: 2219.1; found: 2219.5 $[M+Na]^+$.

4.2.15. HPheNH$(CH_2)_5$CONH$(CH_2)_7$CONH(Boc$C^Z$$A^Z$-BocAllocAllocBoc)OH.HCl (20). Compound **20** was obtained from **19** (72 mg, 32.8 μmol), following the procedure described above for the preparation of **16**. After acidification, the mixture was concentrated in vacuo and the residue purified on a Sephadex LH-20 gel column in methanol to give compound **20** (68 mg, 93%) as a white amorphous powder. TLC ($CHCl_3$/MeOH 8.5:1.5) R_f 0.19. HPLC (A/B 80:20 to 0:100 over 30 min) t_R = 19.3 min (λ_{max} = 241.8 and 271.4 nm). ^{1}H NMR (500 MHz, CD_3OD) δ 8.65–6.65 (m, 19H, (19CH)); 6.00–5.80 (m, 2H, (2CH)); 5.40–4.30 (m, 16H, ($8CH_2$)); 4.30–2.70 (m, 49H, (CH, $24CH_2$)); 2.20–2.05 (m, 4H, ($2CH_2$)); 1.60–1.10 (m, 43H, ($8CH_2$, $9CH_3$)). MS (ESI+) calcd for $C_{103}H_{147}N_{25}O_{28}$ $[M+H]^+$: 2183.1; found: 2183.1 $[M+H]^+$.

4.2.16. (Boc$C^Z$$A^Z$BocHHBoc)$F_c$ (21). Compound **2** (135 mg, 62.5 μmol) and Et_2NH (291 μL, 2.81 mmol, 45 equiv) were dissolved in DMF (180 μL), and $Pd[P(Ph)_3]_4$ (14 mg, 12.5 μmol, 0.2 equiv) was added. The mixture was stirred for 2 h at room temperature. The solvent was concentrated under reduced pressure. The residue was taken up in methanol (0.1 mL) and precipitated with Et_2O at 0 °C. Compound **21** (100 mg, 95%) was obtained as a yellowish amorphous powder after triturating in Et_2O and drying. TLC ($CHCl_3$/MeOH 8:2) R_f 0.48. HPLC (A/B 80:20 to 0:100 over 30 min) t_R = 19.9 min (λ_{max} = 241.8 and 269.1 nm). MS (ESI+) calcd for $C_{95}H_{137}N_{25}O_{23}$ $[M+Na]^+$: 2019.0; found: 2019.1 $[M+Na]^+$.

4.2.17. (Boc$C^Z$$A^Z$BocG^{OBn}G^{OBn}Boc)$F_c$ (22). To a solution of **21** (100 mg, 50.1 μmol), $G^{OBn}CH_2CO_2H$ (45 mg, 150.3 μmol, 3 equiv), and DIPEA (87 μL, 500.1 μmol, 10 equiv) in DMF (150 μL), were added at 0 °C, HATU (57 mg, 150.3 μmol, 3 equiv) and HOAt (14 mg, 100.2 μmol, 2 equiv). After 2 h of stirring at room temperature, the solvent was evaporated under reduced pressure and the crude residue was taken up in $CHCl_3$ and washed successively with a saturated aqueous $NaHCO_3$ solution, water and dried over Na_2SO_4. The solvent was then removed in vacuo and the residue was purified by column chromatography ($CHCl_3$/MeOH 1:1) to afford **22** (122 mg, 95%) as a white amorphous powder. HPLC (A/B 80:20 to 0:100 over 30 min) t_R = 23.4 min (λ_{max} = 243.0 and 278.6 nm). MS (ESI+) calcd for $C_{123}H_{159}N_{35}O_{27}$ $[M+Na]^+$: 2581.2 and for $[(M+2Na)/2]^+$: 1302.1; found: 2581.2 $[M+Na]^+$ and 1302.0 $[(M+2Na)/2]^+$.

4.2.18. (HC^ZA^ZHGGH)F_c·5TFA (23). Compound **22** (147 mg, 57.3 μmol) was dissolved in 2 mL of a solution of TFA/CH_2Cl_2/TIS (1:1:0.25, v/v/v) at 0 °C. The mixture was stirred at room temperature for 30 min and the deprotected product was precipitated out by Et_2O (10 mL). Compound **23** (98 mg, 93%) was obtained as a yellowish solid after washing with Et_2O, filtration and drying. HPLC (A/B 80:20 to 0:100 over 30 min) t_R = 13.5 min (λ_{max} = 243.0 and 278.6 nm). MS (ESI+) calcd for $C_{94}H_{128}N_{35}O_{21}$ $[(M+2H)/2]^+$: 1040.0; found: 1040.0$[(M+2H)/2]^+$.

4.2.19. (UC^ZA^ZUGGU)F_c (24). To a solution of **23** (71 mg, 26.7 μmol), UCH_2CO_2H (18 mg, 106.7 μmol, 4 equiv) and DIPEA (93 μL, 533.5 μmol, 20 equiv) in

DMF (130 μL), were added at 0 °C, HATU (43 mg, 112.0 μmol, 4.2 equiv) and HOAt (14 mg, 106.7 μmol, 4 equiv). After stirring at room temperature for 3 h, the product was precipitated out by a saturated aqueous $NaHCO_3$ solution (10 mL). The precipitate was washed successively with water, methanol, and Et_2O. After drying, compound **24** (60 mg, 88%) was obtained as a white amorphous powder. HPLC (A/B 80:20 to 0:100 over 30 min) $t_R = 13.8$ min ($\lambda_{max} = 257.2$ nm). MS (ESI+) calcd for $C_{112}H_{135}N_{41}O_{30}$ $[(M+2H)/2]^+$: 1268.0 and for $[(M+3H)/3]^+$: 845.7; found: 1268.1 $[(M+2H)/2]^+$ and 845.6 $[(M+3H)/3]^+$.

5. Experimental (virology)

5.1. Plasmid construction

An original bicistronic expression vector, termed *pFl-HCV IRES-hRl* was used in this study. Details of the cloning procedure will be described elsewhere. Briefly, this construction contains the coding sequence of the firefly luciferase (Promega) under the control of cytomegalovirus and T7 promoter followed by HCV 5′-UTR genomic sequence (HCV nucleotides 19–355) corresponding to the internal ribosome entry site (IRES) and the coding sequence for the humanized Renilla luciferase (Promega) in the pcDNA 3.1 + vector (Invitrogen). HCV fragment (HCV IRES) was amplified by RT-PCR with RNA extracted from serum of a chronically HCV infected patient. No difference was observed between the sequence obtained compared with a standard 1a genotype HCV genome.

5.2. In vitro coupled transcription–translation assays

In vitro coupled transcription–translation assays were performed in 25 μL in a mixture containing 20 μL of TnT Quick Master Mix (Promega), 1 μL of methionine 1 mM, 1 μL of pFl-HCV IRES-hRl at a concentration of 0.5 μg/μL and 3 μL of PNA, diluted in water. Concentration from 0.5 to 60 μM were used. A 90 min incubation at 30 °C was performed. All this experiments were made in triplicates, at least twice.

5.3. Measurement of luciferase activity

The enzymatic activities of firefly and humanized Renilla were measured using dual luciferase reporter assay system (Promega), as recommended by the manufacturer. Results are the ratio of humanized Renilla luciferase activity compared to firefly luciferase activity, measured by light emission integrated over a period of 1 seconde in a Luminoskan Ascent.

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