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Peptide-Based Inhibitors of Hepatitis C Virus Full-Length NS3 (Protease-Helicase/NTPase): Model Compounds Towards Small Molecule Inhibitors

Karin Oscarsson,[a] Anton Poliakov,[b] Stefan Oscarson,[a] U. Helena Danielson,[b] Anders Hallberg[c] and Bertil Samuelsson[a,d,*]

[a]*Department of Organic Chemistry, Arrhenius Laboratory, Stockholm University, S-106 91 Stockholm, Sweden*
[b]*Department of Biochemistry, Uppsala University, BMC, Box 576, SE-751 23 Uppsala, Sweden*
[c]*Department of Organic Pharmaceutical Chemistry, BMC, Uppsala University, Box 574, S-751 23 Uppsala, Sweden*
[d]*Medivir AB, Lunastigen 7, S-141 44 Huddinge, Sweden*

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Abstract—From L-α-aminobutyric acid (Abu) a set of electrophilic and non-electrophilic replacements for the P1 cysteine of substrate and product inhibitors of hepatitis C virus full-length NS3 (protease-helicase/NTPase) serine protease have been synthesised and coupled to a model pentapeptide furnishing a set of hexapeptide inhibitors. Promising inhibitory activities with K_i values of 0.18 μM (**11b**, P1 electrophilic α,β-unsaturated ketone), 0.46 μM (**12e**, P1 electrophilic alkyl ketone) and 0.98 μM (**10e**, P1 non-electrophilic alkenyl alcohol as diastereomeric mixture). The reference hexapeptide product inhibitor had a K_i value of 1.54 μM (**14**, P1 Abu–OH). The electrophilic inhibitors exhibit increased potency as compared with the corresponding product inhibitor, and notably also the non-electrophilic P1 alkenyl alcohol **10e**. This represents the first example of non-electrophilic inhibitors that are not P1 amides or product inhibitors.

Introduction

Hepatitis C virus (HCV) was identified 1989 as the major etiological agent of non-A, non-B hepatitis.[1] An estimated 175 million people worldwide are affected from HCV infection, which becomes chronic in about 50% of cases leading to secondary diseases such as liver cirrhosis and hepatocellular carcinoma.[2–5] The prevalence of chronic hepatitis C is of such magnitude that patients with the disease constitute the highest proportion of candidates for liver transplantation.[5] Current therapies involve treatment with interferon-α (IFN-α) either alone or in combination with ribavirin. These treatment regimes although effective in selective patients show overall disappointing efficacy as many patients do not respond to therapy or tolerate therapy poorly underscoring the need for improved therapeutic options.[6,7]

One of the most studied molecular targets for antiviral therapy against HCV is the NS3 serine protease.[8,9] The NS3 is a multifunctional protein which contains a serine protease domain and a RNA helicase domain.[10] The protease cofactor NS4A, which is a relatively small protein, is absolutely required for enhanced serine protease activity.[9,11,12] The NS3 serine protease is essential in the viral lifecycle and therefore presents a prime target for development of new hepatitis C drugs. For inhibitor design, both X-ray crystal structure information,[13–15] consensus cleavage sites of the HCV polyprotein and cleavage kinetics of different peptide substrates have been used to develop a first generation of inhibitors.[6,9,16] From analysis of the substrate binding site as revealed by X-ray crystal structure, it has been shown that the binding site of the NS3 protease is remarkably shallow and solvent exposed making small molecule inhibitor design a challenge. NS3 protease inhibitors in general fall into two structural classes characterised by either an electrophilic carbonyl P1 group, acting as classical serine-trap or warhead for the catalytic serine of the NS3 protease active site,[9,17–24] or

*Corresponding author. Tel.: +46-8-608-3104; fax: +46-8-608-3199; e-mail: bertil.samuelsson@medivir.se

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by having a P1 carboxylic acid characterised as product based inhibitors.[9,17,18,25–29] Recently, we suggested, supported by modelling, that the helicase domain participates in the binding of the inhibitors to the native full-length NS3 protein (protease-helicase/NTPase).[30,31] A series of inhibitors of the full-length protease with K_i in nanomolar range were identified.[30,31]

We have been intrigued by the possibility to use potent serine-trap based inhibitors as a starting point for optimisation of small-molecule drug-like non-electrophilic inhibitors targeting the non-truncated protease. As part of this program we have also discovered P1 alkenyl alcohols as a potentially new class of non-electrophilic inhibitors. In this report we disclose our initial findings from the optimisation of a new P1 serine-trap and new P1 alkenyl alcohols based on Abu (α-aminobutyric acid) which is incorporated into a model P1-P6 spanning peptide, Ac-Asp-D-Glu-Leu-Ile-Cha-P1. Ingallinella et al. recently reported on the potent hexapeptide Ac-Asp-D-Glu-Leu-Ile-Cha-Cys-OH (Cha, β-cyclohexyl-Ala) which inhibits NS3 proteases with an $IC_{50} = 0.015\,\mu M$.[28] We have for comparison synthesised the corresponding P1 Abu peptide (**14**) which in our hand exhibited a K_i value of 1.54 μM. Furthermore, Ingallinella et al. reported that Ac-Asp-Glu-Met-Glu-Glu-Cys-OH and the corresponding P1 Abu showed an inhibitory IC_{50} potency of 1 and 5.8 μM, respectively.[28] These data consistently show that a substantial reduction of inhibition potency can be expected from replacing P1 Cys with Abu.[18] However, cysteine is generally not considered as a tractable P1 for drug discovery purposes, being chemically reactive and unstable, so for this model study we have chosen to use the chemically stable Abu as a starting point for P1 modifications. Numerous other stable and potent P1 Cys replacements have been disclosed that further underscores the rationale behind this methodology. 2-Amino-4,4-difluoro-butyric acid (difluoroAbu) was recently developed and successfully incorporated into potent tripeptide α-ketoacid inhibitors.[21] Moreover, 1-aminocyclopropane-1-carboxylic acid has been introduced as a chirally stable and potent P1 amino acid.[25,26,29] This line of work was further explored resulting in 1-amino-vinylcyclopropane-1-carboxylic acid as P1 amino acid, which yielded potent product based inhibitors.[32,33] There are, thus, several currently available chemically stable drugable P1 substituents, which can be used in place of the model P1 Abu used in this study to further optimise this set of inhibitors. Several other serine traps, for example boronic acids,[34] α-diketones,[20] α-ketoamides,[20,23] and α-ketoesters,[20] have previously been disclosed to show activity against HCV NS3 protease.

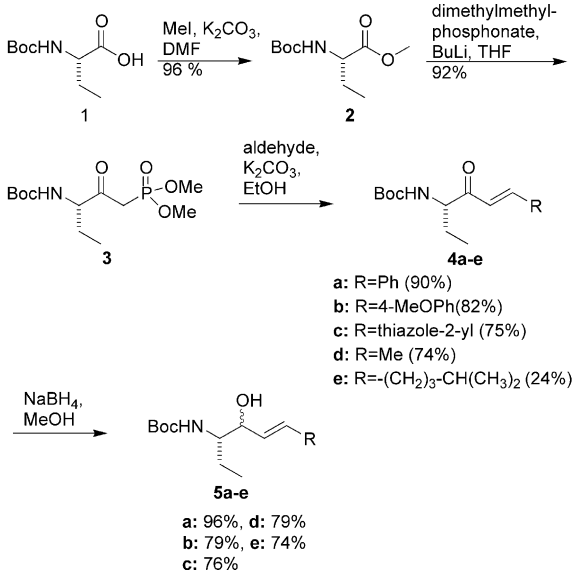
In this report, we present the synthesis of novel series of α,β-unsaturated alcohols (**10a–e**), α,β-unsaturated ketones (**11a–e**) and saturated ketones (**12a–e**) starting from the unnatural amino acid Abu. These three structurally related P1 inhibitor classes (**10a–e**, **11a–e** and **12a–e**) were assessed in an in vitro assay system comprising the native bifunctional full-length NS3 protein (protease-helicase/NTPase).[30,31,35] Several hexapeptides were identified exhibiting promising K_i values, for example, **11b** ($K_i = 0.18\,\mu M$) showing over 8-fold increase in potency over the corresponding product inhibitor **14**, and interestingly the non-electrophilic alcohol **10e** (diastereomeric mixture, $K_i = 0.98\,\mu M$) being substantially more potent than the corresponding product inhibitor **14**.

Chemistry and Structure–Activity Relationship

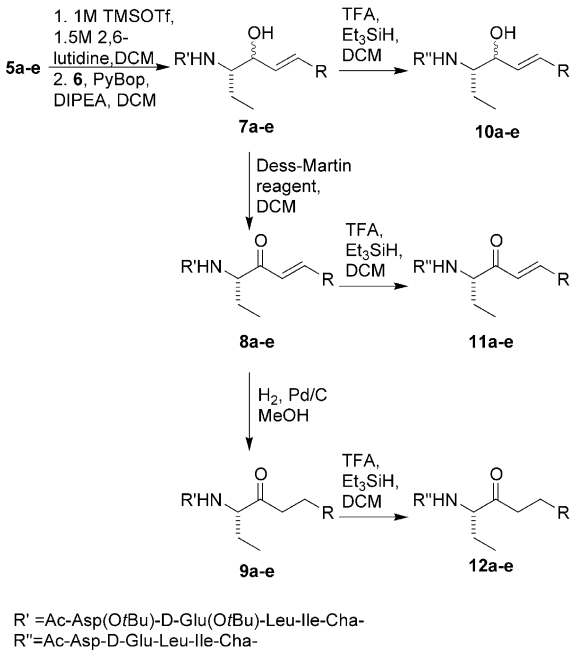
The target inhibitors (**10a–e**, **11a–e**, and **12a–e**) were synthesised starting from Boc protected L-α-aminobutyric acid (Boc-Abu-OH, **1**), which was transformed to phosphonate **3**, and reacted further in a Horner–Wadsworth–Emmons reaction to give the *trans*-enones. The ketones moiety of the *trans*-enones were subsequently reduced to the corresponding alcohols, the Boc groups cleaved off and the resulting amines coupled to the pentapeptide **6** giving **10a–e** after deprotection. Oxidation of the alcohols delivered the ketones **11a–e** after deprotection. The saturated ketones **12a–e** were prepared by chemo-selective hydrogenation of the C–C double bonds followed by deprotection.

The Boc protected amino acid **1** was converted to the methyl ester **2** in 96% yield, using methyl iodide and potassium carbonate in DMF (Scheme 1) and reacted further with 6 equiv of the lithium salt of dimethyl methylphosphonate at −78 °C in THF, according to Chakravarty et al.,[36] to yield the phosphonate **3** in 92% yield.

The *trans*-enones **4a–e** were obtained in good to moderate yields (24–90%) using Horner–Wadsworth–Emmons conditions, that is by reacting the phosphonate **3** with the appropriate aldehyde in dry ethanol using 1 equiv of potassium carbonate as base.[37] Initially, this reaction was accompanied by racemisation, and all attempts to suppress racemisation at the α-carbon of the *trans*-enones failed. Numerous different bases were explored, for example NaH in THF,[38] DIPEA



Scheme 1. Synthesis of key intermediates **5a–e**.



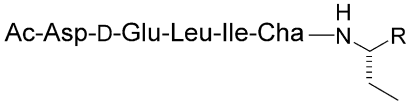
Scheme 2. Synthesis of target compounds **10–12a–e**.

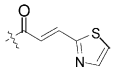
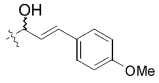
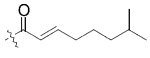
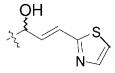
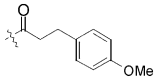
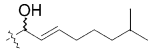
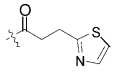
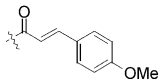
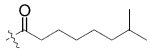
(*N*,*N*-ethyldiisopropylamine), Et_3N, DBU or diisopropylamine in CH_3CN with or without addition of LiCl/LiBr,[39–41] without success. However, with potassium carbonate as a base, the racemisation of the condensation products could be minimised. 5-Methylhexanal, used in the formation of condensation product **4e**, was synthesised by oxidation of 5-methyl-1-hexanol employing pyridinium dichromate (PDC) in CH_2Cl_2.[42] Cleavage of the Boc group on compounds **4a–e** was unsuccessful and therefore the ketones were reduced using sodium borohydride to produce the alcohols **5a–e** in 74–96% yield as diastereomeric mixtures. Synthesis of the pentapeptide, Ac-Asp(O*t*Bu)-D-Glu(O*t*Bu)-Leu-Ile-Cha-OH (**6**), was performed using a Fmoc solid phase methodology on SASRIN™[43] yielding the pure acid **6**.[44,45]

Boc deprotection was then performed by stirring **5a–e** with 1 M TMSOTf and 1.5 M 2,6-lutidine in CH_2Cl_2 to furnish the corresponding free amines (Scheme 2).[22]

Attempts to cleave off the Boc group using the standard conditions, for example TFA and HCl, resulted in decomposition of the alcohols **5a–e**. Subsequent coupling

Table 1. Percent inhibition at 10 and 1 μM and inhibition constant against full-length NS3 (protease-helicase/NTPase) protease



R	Compd	% inhib at 10 μM	% inhib at 1 μM	K_i (μM)	R	Compd	% inhib at 10 μM	% inhib at 1 μM	K_i (μM)
	**14**	60	17	1.54±0.16		**11c**	95	50	0.32±0.03
	**10a**	8	nm	nm		**11d**	96	54	0.20±0.01
	**10b**	7	nm	nm		**11e**	95	42	0.39±0.02
	**10c**	47	nm	2.30±0.21		**12a**	82	25	0.98±0.1
	**10d**	7	nm	nm		**12b**	81	30	0.88±0.05
	**10e**	64	nm	0.98±0.09		**12c**	64	nm	nm
	**11a**	96	58	0.21±0.03		**12d**	80	40	0.65±0.04
	**11b**	100	64	0.18±0.01		**12e**	91	31	0.46±0.07

nm, not measured.

of the corresponding amines with the acid **6** was accomplished using benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP) and DIPEA in CH_2Cl_2 furnishing **7a** (66%), **7b** (87%), **7c** (50%), **7d** (52%) and **7e** (64%) as diastereomeric mixtures.[46]

Oxidation of compound **7a–e** to the ketones **8a** (69%), **8b** (53%), **8c** (73%), **8d** (78%) and **8e** (96%) was performed using Dess–Martin periodinane reagent in CH_2Cl_2.[47]

The unsaturated ketones **8a–e** were hydrogenated over palladium on carbon in EtOAc delivering the saturated ketones **9a–e** in quantitative yields. Finally, the *t*-butyl esters were cleaved in compounds **7a–e**, **8a–e** and **9a–e**, using TFA in CH_2Cl_2 containing Et_3SiH as scavenger, to afford the target compounds **10a–e** (as diastereomeric mixtures), **11a–e** and **12a–e** in quantitative yields (no racemisations were observed).[48]

The reference hexapeptide **14**, Ac-Asp-D-Glu-Leu-Ile-Cha-Abu-OH was synthesised by coupling of *t*-butyl L-α-aminobutyrate (H-Abu-O*t*Bu) with pentapeptide **6** using PyBOP and DIPEA in CH_2Cl_2 yielding the protected hexapeptide Ac-Asp(O*t*Bu)-D-Glu(O*t*Bu)-Leu-Ile-Cha-Abu-O*t*Bu **13** in 52% yield. The *t*-butyl esters were then cleaved off, as above, furnishing unprotected **14** in quantitative yield.

From examination of the three classes of inhibitors in Table 1, the α,β-unsaturated alcohol, **10e** (diastereomeric mixture) shows promising inhibitory activity with a 1.6-fold increased activity as compared with the reference product inhibitor **14**. The most potent group of inhibitors are the α,β-unsaturated ketones, **11a–e**, all showing promising K_i values in the range 0.18–0.39 μM, with potency selectivity of 4–8.5 over the product inhibitor **14**. The potency of the α,β-unsatureted reduced ketones appears especially promising in view of the activity of the alkyl ketones **12d** and **12e** with $K_i = 0.65$ and 0.46 μM, respectively, and anticipating reversible kinetics.

Conclusion

Potent inhibitors of the HCV NS3 protease have been obtained from a limited set of hexapeptides containing a P1 modified Abu showing K_i values in the 0.1–2.0 μM range. Preliminary SAR has been obtained, and simple P1 alkenyl alcohols and alkyl ketones appear to hold much promise for further optimisations towards small molecule inhibitors. Interestingly the non-electrophilic alkenyl alcohol **10e** have superior potency over the corresponding product based inhibitor **14**, thus providing a novel entry of non-electrophilic non-product based inhibitors.

Experimental

Inhibition assay

The inhibition of full-length hepatitis C NS3 was measured essentially as described elsewhere.[35] Briefly, the hydrolysis of a depsipeptide substrate, Ac-DED(Edans)EEAbuψ[COO]ASK(Dabcyl)-NH_2 (AnaSpec, San José, USA), was measured spectrofluorometrically in the presence of a peptide cofactor, KKGSVVIVGRIVLSGK (Åke Engström, Department of Medical Biochemistry and Microbiology, Uppsala University, Sweden).[49] The enzyme (1 nM) was incubated in 50 mM HEPES, pH 7.5, 10 mM DTT, 40% glycerol, 0.1% *n*-octyl-β-D-glucoside, with 25 μM cofactor and inhibitor at 30 °C for 10 min, whereupon the reaction was initiated by addition of 0.5 μM substrate. Inhibitors were dissolved in DMSO, sonicated for 30 s and vortexed. The solutions were stored at −20 °C between measurements. The final concentration of DMSO in the assay sample was adjusted to 3.3%. The rate of hydrolysis was corrected for inner filter effects according to published procedures.[50] K_i values were estimated by non-linear regression analysis (GraFit, Erithacus Software, Staines, UK), using a model for competitive inhibition and a fixed value for K_m (0.15 μM). A minimum of two replicates was performed for all measurements.

General

All glassware was dried over an open flame before use in connection with an inert atmosphere. Concentrations were performed under reduced pressure at <40 °C (bath temperature). Thin layer chromatography was performed using silica gel 60 F-254 plates with detection by UV and charring with ninhydrine or $KMnO_4$. Silica gel (0.040–0.063 mm) was used for column chromatography. Me_4Si (0.0 ppm) was used as an internal standard in ^1H NMR and Me_4Si or $CDCl_3$ (77.0 ppm) were used in ^{13}C NMR. MALDI-TOF spectra were recorded on a Bruker Biflex III using 2′,4′,6′-trihydroxy-acetophenone monohydrate (THAP) as matrix. Unless stated otherwise, all materials were obtained from commercial suppliers and used without further purification. The preparative RP-HPLC was performed on a Wares-Micromass: 600 controller and ZMD mass on a Xterra MS C8 (5 μm, 19×100 mm), using an acetonitrile/H_2O gradient with 10 mM NH_4HCO_3, using MS detection (positive ionisation with ES). The analytical RP-HPLC was performed on a Waters-Micromass: 2790 Waters pump, 996 DAD Waters, and Micromass ZMD mass on a Phenomex C18 Synergi Max Rp-80Å, (4 μ, 50×4.6 mm), using an acetonitrile/H_2O gradient with 10 mM NH_4HCO_3, using UV (DAD and 214 and 254 nm) and MS detection (positive ionisation with ES).

General method for the preparation of 5a–e. Oven dried K_2CO_3 (268 mg, 1.94 mmol) was added to a stirred solution of phosphonate **3**[36] (600 mg, 1.94 mmol) and the appropriate aldehyde (2 mmol) in absolute EtOH (15 mL). After 16 h the reaction mixture was filtered and the filtrate was neutralised by glacial acetic acid. The solvent was removed and the residue was portioned between EtOAc and saturated aqueous $NaHCO_3$. The aqueous phase was extracted with EtOAc and the combined organic phases were dried over Na_2SO_4 and concentrated. The crude product was purified by silica gel column chromatography (solvent system A; toluene; toluene–EtOAc 20:1, solvent system B; toluene–EtOAc

3:1) providing the ketones **4a–e**. $NaBH_4$ (1 equiv) was added at 0 °C in small portions over 10 min, to a stirred solution of **4a–e** (1 equiv) in MeOH. After 2.5 h at 0 °C, the solution was neutralised with glacial acetic acid, the solvent was removed and the residue was partitioned between EtOAc and saturated aqueous $NaHCO_3$. The aqueous phase was extracted with EtOAc and the combined organic phases were dried over Na_2SO_4 and concentrated. The crude product was purified by silica gel column chromatography (solvent system C; toluene; toluene–EtOAc 6:1, solvent system D; toluene–EtOAc 1:1) providing the alcohol **5a–e** as a diasteromeric mixture.

(4*S*)-4-[*N*-(*tert*-Butyloxycarbonyl)amino]-3-hydroxy-1-benzyl-1-hexen 5a. The title compound was prepared in a two-step synthesis according to general procedure, vide supra. The intermediate **4a** was prepared in 90% yield (507 mg, 1.8 mmol) according to general procedure, vide supra (solvent system A). ^{13}C NMR (75 MHz, $CDCl_3$) δ 197.8, 155.3, 143.8, 134.0, 130.5, 128.7, 128.2, 122.5, 79.5, 58.8, 28.3, 25.3, 9.20. The title compound was prepared in 96% yield (289 mg, 1.0 mmol) according to general procedure, vide supra (solvent system C). ^1H NMR (300 MHz, $CDCl_3$) δ 7.44–7.26 (m, 4H), 6.64, 6.16 (d, 1H), 6.26–6.16 (m, 1H), 4.82, 4.7, (d, 1H), 4.37, 4.28 (bs, 1H), 3.73–3.46 (m, 1H), 3.47 (bs, 1H), 1.72–1.31 (m, 2H), 1.40, 1.43 (s, 9H), 0.96 (t, 3H) ^{13}C NMR (75 MHz, $CDCl_3$) δ 157.3, 136.9, 131.5, 131.9, 128.8, 128.7, 128.5, 127.9, 126.8, 126.7, 80.0, 75.5, 74.5, 57.6, 57.0, 28.6, 23.6, 11.1, 11.0. Anal. calcd for ($C_{17}H_{25}NO_3$): C, 70.1; H, 8.7; N, 4.8. Found: C, 69.7; H, 8.6; N, 4.8.

(4*S*)-4-[*N*-(*tert*-Butyloxycarbonyl)amino]-3-hydroxy-1-(4-methoxybenzyl)-1-hexen 5b. The title compound was prepared in a two-step synthesis according to general procedure, vide supra. The intermediate **4b** was prepared in 82% yield (590 mg, 1.6 mmol) according to general procedure, vide supra (solvent system A). ^{13}C NMR (75 MHz, $CDCl_3$) δ 198.2, 162.1, 155.7, 144.2, 130.5, 127.2, 120.7, 114.7, 79.7, 59.0, 55.6, 28.6, 25.9, 9.4. The title compound was prepared in 79% yield (283 mg, 0.88 mmol) according to general procedure, vide supra (solvent system C). ^1H NMR (300 MHz, $CDCl_3$) δ 7.34–7.28 (m, 2H), 6.86–6.81 (m, 2H), 6.55, 6.57 (d, 1H), 6.11–6.00 (m, 1H), 4.62, 4.72 (bd, 1H), 4.33, 4.24 (m, 1H), 3.8 (s, 3H), 3.76–3.53 (m, 2H), 3.13 (bd, 1H), 1.67–1.31 (m 11H). 0.96 (t, 3H), ^{13}C NMR (75 MHz, $CDCl_3$) δ 159.5, 157.3, 131.5, 129.7, 128.0, 127.9, 126.1, 114.2, 114.2, 80.0, 75.7, 74.8, 57.3, 55.5, 28.6, 23.7, 11.1, 10.9. Anal. calcd for ($C_{18}H_{27}NO_4$): C, 67.3; H, 8.5; N, 4.4. Found: C, 67.3; H, 8.5; N, 4.3.

(4*S*)-4-[*N*-(*tert*-Butyloxycarbonyl)amino]-3-hydroxy-1-(thiazol-2-yl)-1-hexen 5c. The title compound was prepared in a two-step synthesis according to general procedure, vide supra. The intermediate **4c** was prepared in 75% yield (500 mg, 1.7 mmol) according to general procedure, vide supra (solvent system B). ^{13}C NMR (75 MHz, $CDCl_3$) δ 197.9, 163.6, 155.4, 145.3, 134.8, 126.3, 122.3, 80.0, 59.7, 28.5, 25.2, 9.5. The title compound was prepared in 76% yield (382 mg, 1.3 mmol) according to general procedure, vide supra (solvent system D). ^1H NMR (300 MHz, $CDCl_3$) δ 7.70, 7.68 (d, 1H), 7.17, 7.19 (d, 1H), 6.90 (d, 1H), 6.55, 6.60 (d, 1H), 4.9, 4.8 (d, 1H), 4.38 (m, 1H). 3.8–3.4 (m, 2H), 1.68–1.33 (m, 11H), 0.94 (t, 3H), ^{13}C NMR (75 MHz, $CDCl_3$) δ 166.6, 157.3, 143.3, 135.7, 124.4, 118.6, 80.1, 74.6, 73.0, 57.4, 56.6, 28.5, 23.6, 11.1, 11.0. Anal. calcd for ($C_{14}H_{22}N_2O_3S$): C, 56.4; H, 7.4; N, 9.4. Found: C, 56.3; H, 7.5; N, 9.1.

(5*S*)-5-[*N*-(*tert*-Butyloxycarbonyl)amino]-4-hydroxy-2-hepten 5d. The title compound was prepared in a two-step synthesis according to general procedure, vide supra. The intermediate **4d** was prepared in 74% yield (335 mg, 1.4 mmol) according to general procedure, vide supra (solvent system A). ^{13}C NMR (75 MHz, $CDCl_3$) δ 198.1, 155.6, 144.8, 128.6, 79.6, 58.3, 28.4, 25.5, 18.5, 9.2. The title compound was prepared in 79% yield (222 mg, 0.94 mmol) according to general procedure, vide supra (solvent system C). ^1H NMR (300 MHz, $CDCl_3$) δ 5.70 (m, 1H), 5.44 (m, 1H), 4.74, 4.63 (bd, 1H), 4.06, 3.98 (m, 1H), 3.52–3.38 (m, 2H), 3.13 (bs, 1H), 1.66 (d, 3H), 1.61–1.19 (m, 11H), 0.90 (t, 3H) ^{13}C NMR (75 MHz, $CDCl_3$) δ 157.2, 129.9, 128.7, 128.5, 79.7, 75.4, 74.4, 57.3, 56.8, 28.6, 23.5, 18.0, 18.0, 10.9, 10.8. Anal. calcd for ($C_{12}H_{23}NO_3$): C, 62.9; H, 10.11; N, 6.1. Found: C, 62.7; H, 10.1; N, 5.9.

(3*S*)-3-[*N*-(*tert*-Butyloxycarbonyl)amino]-4-hydroxy-10-methyl-5-undecaen 5e. The title compound was prepared in a two-step synthesis according to general procedure, vide supra. The intermediate **4e** was prepared in 24% yield (140 mg, 0.47 mmol) according to general procedure, vide supra (solvent system A). ^{13}C NMR (75 MHz, $CDCl_3$) δ 198.3, 155.7, 149.9, 127.1, 79.6, 58.4, 38.6, 33.1, 28.5, 28.0, 26.0, 25.8, 22.7, 9.3. The title compound was prepared in 74% yield (104 mg, 0.35 mmol) according to general procedure, vide supra (solvent system C). ^1H NMR (300 MHz, $CDCl_3$) δ 5.64–5.55 (m, 1H), 5.38–5.30 (m, 1H), 5.00–4.80 (m, 1H), 3.98–3.90 (m, 1H), 3.48–3.30 (m, 3H), 1.95–1.90 (m, 2H), 1.34 (s, 9H), 1.52–1.13 (m, 4H), 0.83 (t, 3H), 0.77 (s, 3H), 0.75 (s, 3H), ^{13}C NMR (75 MHz, $CDCl_3$) δ 157.2, 133.8, 133.5, 139.8, 128.6, 79.6, 75.0, 74.0, 57.3, 56.8, 39.0, 38.6, 32.7, 28.4, 27.9, 27.1, 23.2, 22.6, 10.8, 10.6. Anal. calcd for ($C_{17}H_{33}NO_3$): C, 68.2; H, 11.1; N, 4.7. Found: C, 68.4; H, 11.1; N, 4.4.

Ac-Asp(O*t*Bu)-D-Glu(O*t*Bu)-Leu-Ile-Cha-OH 6. The peptide was synthesised using standard Fmoc solid-phase peptide synthesis techniques. Fmoc-Cha-OH (5 eqiuv.) was loaded to the SASRIN™ (1 equiv) using 2,6-dichlorobenzoyl chloride (5 equiv) and pyridine (5.5 equiv) in DMF with a loading time of 16 h. The resin was washed with MeOH, DMF, and DCM. Removal of the Fmoc group was achieved by reaction with 20% piperidine in DMF for 10 + 10 min. Peptide elongation was performed using Fmoc-AA-OH (2 equiv), DIC (2 equiv) and HOBt (2 equiv) in DCM. The peptide was cleaved from the resin using 3% TFA in DCM and Et_3SiH as scavenger, producing **6**. ^{13}C NMR (75 MHz, $CDCl_3$) δ 175.2, 174.4, 167.9, 172.6, 172.0, 171.9, 171.7, 171.5, 57.4, 53.0, 52.1, 49.9, 49.9,

40.0, 38.7, 36.4, 35.2, 33.7, 33.2, 31.9, 29.9, 26.0, 25.8, 25.6, 25.5, 24.3, 24.3, 22.4, 21.7, 20.6, 14.6, 10.0.

General method for the preparation of 7a–e

Compound **5a–e** was added to a solution of TMSOTf (1 M, 1 mL) and 2,6-lutidine (1.5 M, 1 mL) in CH_2Cl_2. After 6 h of stirring the mixture was evaporated. PyBOP (1 equiv), the pentapeptide **6** (1 equiv) and DIPEA (2 equiv) was added to a stirred solution of the residue in CH_2Cl_2. After 16 h the reaction mixture was washed with aqueous 5% $KHSO_4$ (2×) and aqueous 5% $NaHCO_3$ (2×), dried over Na_2SO_4 and concentrated. The residue was purified by silica gel column chromatography (CH_2Cl_2; CH_2Cl_2–MeOH 40:1) to give the target compound **7a–e**.

(4*S*)-4-[*N*-(Ac-Asp(O*t*Bu)-D-Glu(O*t*Bu)-Leu-Ile-Cha)-amino]-3-hydroxy-1-benzyl-1-hexen 7a. The title compound was prepared in 66% yield (161 mg, 0.17 mmol) according to general procedure, vide supra. ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.9, 173.4, 172.7, 172.1, 172.0, 171.9, 170.2, 136.8, 131.2, 121.2, 128.7, 128.4, 128.4, 127.4, 126.4, 81.9, 81.0, 74.7, 59.0, 56.3, 53.4, 53.1, 53.0, 51.5, 40.1, 39.9, 36.4, 36.3, 33.9, 33.5, 31.7, 31.6, 29.6, 27.8, 26.3, 26.1, 25.8, 25.0, 24.7, 22.7, 22.3, 22.0, 21.3, 15.2, 11.0, 10.5; MS (MALDI-TOF) *m*/*z* mass calcd for: $C_{52}H_{84}N_6O_{11}$, 968.62 found: 1007.58 (MK^+) 998.58 (MNa^+).

(4*S*)-4-[*N*-(Ac-Asp(O*t*Bu)-D-Glu(O*t*Bu)-Leu-Ile-Cha)-amino]-3-hydroxy-1-(4-methoxybenzyl)-1-hexen 7b. The title compound was prepared in 87% yield (217 mg, 0.22 mmol) according to general procedure, vide supra. ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.9, 173.5, 172.6, 172.1, 172.1, 172.0, 170.0, 169.8, 159.1, 140.9, 129.7, 127.6, 126.6, 113.7, 113.7, 74.9, 58.9, 56.2, 54.9, 54.6, 53.3, 53.0, 50.7, 42.6, 40.0, 36.3, 36.3, 33.8, 33.5, 31.5, 27.6, 26.0, 25.7, 25.0, 24.6, 22.5, 22.0, 21.0, 17.9, 16.5, 15.0, 12.2, 10.8, 10.3; MS (MALDI-TOF) *m*/*z* mass calcd for: $C_{53}H_{86}N_6O_{12}$, 998.63 found: 1037.60 (MK^+) 1021.61 (MNa^+).

(4*S*)-4-[*N*-(Ac-Asp(O*t*Bu)-D-Glu(O*t*Bu)-Leu-Ile-Cha)-amino]-3-hydroxy-1-(thiazol-2-yl)-1-hexen 7c. The title compound was prepared in 50% yield (164 mg, 0.17 mmol) according to general procedure, vide supra. ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.7, 172.6, 172.5, 172.3, 172.2, 172.1, 172.0, 170.2, 170.1, 167.4, 142.4, 142.3, 136.7, 123.5, 118.6, 118.5, 81.9, 80.9, 73.5, 59.6, 55.9, 55.8, 55.7, 53.9, 53.8, 53.2, 51.7, 51.6, 50.7, 42.8, 36.4, 34.0, 31.6, 31.5, 27.8, 26.3, 26.3, 26.2, 26.1, 25.8, 25.2, 24.7, 22.5, 22.3, 21.3, 18.2, 16.7, 15.2, 12.4, 11.1, 11.0,10.5, 10.4; MS (MALDI-TOF) *m*/*z* mass calcd for: $C_{49}H_{81}N_7O_{11}S$, 975.57 found: 1014.45 (MK^+) 998.49 (MNa^+) 976.51 (MH^+).

(5*S*)-5-[*N*-(Ac-Asp(O*t*Bu)-D-Glu(O*t*Bu)-Leu-Ile-Cha)-amino]-4-hydroxy-2-hepten 7d. The title compound was prepared in 52% yield (140 mg, 0.15 mmol) according to general procedure, vide supra. ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.2, 173.6, 172.6, 172.3, 172.2, 172.2, 172.1, 170.3, 130.2, 127.8, 81.9, 80.9, 74.9, 59.4, 56.5, 54.8, 53.9, 53.4, 51.7, 50.9, 46.4, 46.3, 42.9, 40.1, 39.1, 36.5, 36.3, 34.1, 33.8, 31.8, 31.7, 28.0, 26.5, 26.4, 26.0, 25.4, 24.8, 22.8, 22.5, 22.5, 21.8, 18.4, 17.8, 16.9, 15.4, 12.6, 11.2, 11.2, 10.7; MS (MALDI-TOF) *m*/*z* mass calcd for: $C_{47}H_{82}N_6O_{11}$, 906.60 found: 945.58 (MK^+) 929.63 (MNa^+) 907.65 (MH^+).

(3*S*)-3-[*N*-(Ac-Asp(O*t*Bu)-D-Glu(O*t*Bu)-Leu-Ile-Cha)-amino]-4-hydroxy-10-methyl-5-undecaen 7e. The title compound was prepared in 64% yield (200 mg, 0.20 mmol) according to general procedure, vide supra. ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.3, 173.7, 172.6, 172.3, 172.1, 172.1, 170.4, 170.3, 132.7, 128.7, 82.0, 81.0, 74.9, 59.5, 56.7, 54.9, 54.0, 53.4, 53.4, 51.8, 50.9, 43.0, 40.1, 39.1, 38.7, 36.6, 36.3, 34.2, 33.8, 32.7, 31.9, 31.8, 28.0, 26.5, 25.4, 26.1, 25.4, 25.3, 24.9, 22.8, 22.6, 22.6, 21.6, 18.5, 17.0, 15.4, 12.7, 11.3, 10.8; MS (MALDI-TOF) *m*/*z* mass calcd for: $C_{52}H_{92}N_6O_{11}$, 976.68 found: 1015.64 (MK^+) 999.68 (MNa^+).

General method for the preparation of 8a–e

Dess–Martin reagent (1 equiv) was added to a stirred solution of the alcohol **7a–e** in CH_2Cl_2 and the mixture was stirred for 16 h at room temperature. The reaction mixture was diluted with CH_2Cl_2 and washed with $Na_2S_2O_3$ (7 equiv) in aqueous saturated $NaHCO_3$ (2×) and water (1×). The combined aqueous phases were washed with CH_2Cl_2 (2×) and the combined organic phases were dried over Na_2SO_4 and concentrated. The residue was purified by silica gel column chromatography (CH_2Cl_2; CH_2Cl_2–MeOH 40:1) and on preparative RP-HPLC producing the target compound **8a–e**.

(4*S*)-4-[*N*-(Ac-Asp(O*t*Bu)-D-Glu(O*t*Bu)-Leu-Ile-Cha)-amino]-1-benzyl-1-hexen-3-one 8a. The title compound was prepared in 69% yield (70 mg, 0.07 mmol) according to general procedure, vide supra. ^{13}C NMR (75 MHz, $CDCl_3$) δ 198.2, 172.9, 172.3, 172.2, 172.1, 171.8, 170.2, 144.8, 134.5, 131.07, 129.1, 128.8, 122.4, 81.9, 81.1, 59.6, 58.6, 58.5, 53.1, 51.5, 51.4, 50.6, 40.7, 39.4, 36.7, 34.1, 33.7, 32.5, 31.9, 28.0, 26.5, 26.3, 26.2, 26.1, 25.0, 24.9, 24.6, 22.9, 22.5, 21.5, 15.5, 11.0, 9.7; MS (MALDI-TOF) *m*/*z* mass calcd for: $C_{52}H_{82}N_6O_{11}$, 966.60 found: 1005.65 (MK^+) 989.68 (MNa^+); RP-LC MS (EI) found: 966.57 (MH^+).

(4*S*)-4-[*N*-(Ac-Asp(O*t*Bu)-D-Glu(O*t*Bu)-Leu-Ile-Cha)-amino]-1-(4-methoxybenzyl)-1-hexen-3-one 8b. The title compound was prepared in 53% yield (60 mg, 0.06 mmol) according to general procedure, vide supra. ^{13}C NMR (75 MHz, $CDCl_3$) δ 198.3, 173.6, 173.5, 172.9, 172.3, 172.3, 172.1, 172.0, 170.2, 162.2, 144.8, 130.7, 127.2, 120.1, 114.6, 81.8, 81.18, 58.6, 58.5, 58.4, 55.4, 53.2, 51.4, 50.7, 40.7, 39.4, 36.7, 34.1, 33.7, 32.4, 32.9, 29.7, 27.9, 26.5, 25.3, 26.2, 26.1, 25.0, 24.9, 24.7, 24.7, 22.9, 22.4, 21.4, 15.4, 11.0, 9.7; MS (MALDI-TOF) *m*/*z* mass calcd for: $C_{53}H_{84}N_6O_{12}$, 996.61 found: 1035.63 (MK^+) 1019.54 (MNa^+) RP-LC MS (EI) found: 997.64 (MH^+).

(4*S*)-4-[*N*-(Ac-Asp(O*t*Bu)-D-Glu(O*t*Bu)-Leu-Ile-Cha)-amino]-1-(thiazole-2-yl)-1-hexen-3-one 8c. The title compound was prepared in 73% yield (70 mg, 0.0.7 mmol) according to general procedure, vide supra. ^{13}C NMR

(75 MHz, $CDCl_3$) δ 197.5, 173.7, 173.3, 172.9, 172.3, 172.2, 172.1, 172.0, 170.2, 144.7, 134.6, 126.7, 122.6, 81.8.81.0, 59.3, 59.2, 58.6, 53.1, 51.4, 50.7, 40.6, 39.4, 36.7, 34.1, 33.7, 32.6, 31.8, 31.7, 29.7, 27.9, 26.5, 26.3, 26.1, 26.1, 25.0, 24.9, 24.0, 22.9, 22.3, 21.3, 15.4, 10.9, 9.7; MS (MALDI-TOF) *m/z* mass calcd for: $C_{49}H_{97}N_7O_{11}S$, 973.56 found: 1012.50 (MK^+) 996.54 (MNa^+) 974.57 (MH^+); RP-LC MS (EI) found: 974.59 (MH^+).

(5*S*)-5-[*N*-(Ac-Asp(O*t*Bu)-D-Glu(O*t*Bu)-Leu-Ile-Cha)-amino]-2-hepten-4-one 8d. The title compound was prepared in 78% yield (78 mg, 0.09 mmol) according to general procedure, vide supra. ^{13}C NMR (75 MHz, $CDCl_3$) δ 198.1, 198.0, 173.4, 172.9, 172.8, 172.3, 172.2, 172.0, 171.8, 145.5, 128.3, 81.8, 81.1, 58.9, 58.3, 58.1, 57.8, 53.3, 53.2, 53.1, 53.0, 52.7, 52.3, 50.6, 50.3, 40.1, 39.0, 36.8, 36.7, 35.9, 34.2, 34.1, 33.9, 33.7, 32.5, 31.8, 31.7, 29.8, 28.0, 26.5, 26.3, 26.1, 26.0, 25.2, 25.0, 24.9, 24.6, 24.3, 23.0, 22.9, 22.4, 21.4, 21.2, 18.4, 18.4, 16.9, 15.4, 15.3, 13.6, 10.9, 10.8, 10.0, 9.5; MS (MALDI-TOF) *m/z* mass calcd for: $C_{47}H_{80}N_6O_{11}$, 904.59 found: 943.65 (MK^+) 927.66 (MNa^+) 905.67 (MH^+); RP-LC MS (EI) found: 905.60 (MH^+).

(3*S*)-3-[*N*-(Ac-Asp(O*t*Bu)-D-Glu(O*t*Bu)-Leu-Ile-Cha)-amino]-10-methyl-5-undecaen-4-one 8e. The title compound was prepared in 96% yield (95 mg, 0.10 mmol) according to general procedure, vide supra. ^{13}C NMR (75 MHz, $CDCl_3$) δ 198.1, 173.4, 172.9, 172.7, 172.3, 172.2, 172.0, 171.8, 170.2, 150.4, 126.6, 81.8, 81.0, 58.3, 57.8, 53.1, 52.9, 51.3, 50.6, 40.7, 39.6, 39.4, 38.9, 38.6, 36.8, 36.7, 34.1, 33.7, 33.1, 32.5, 31.8, 28.9, 26.5, 26.3, 26.2, 26.1, 25.9, 24.9, 24.7, 22.9, 22.5, 22.4, 22.4, 22.4, 21.4, 15.4, 10.9, 9,5; MS (MALDI-TOF) *m/z* mass calcd for: $C_{52}H_{90}N_6O_{11}$, 974.67 found: 1015.64 (MK^+) 999.68 (MNa^+); RP-LC MS (EI) found: 975.70 (MH^+).

General method for the preparation of 9a–e

A catalytic amount of Pd/C (10%) was added to **8a–e** in MeOH. Hydrogen was added at atmospheric pressure to the system and the reaction mixture was stirred for 1 h. The suspension was then filtrated through Celite and the solvent was removed to give **9a–e**.

(4*S*)-4-[*N*-(Ac-Asp(O*t*Bu)-D-Glu(O*t*Bu)-Leu-Ile-Cha)-amino]-1-benzyl-3-hexenone 9a. The title compound was prepared in 100% yield (33 mg, 0.034 mmol) according to general procedure, vide supra. ^{13}C NMR (75 MHz, $CDCl_3$) δ 209.2, 173.9, 173.5, 172.8, 172.4, 172.4, 172.3, 172.2, 170.2, 141.0, 128.4, 128.4, 126.1, 81.9, 81.0, 60.3, 58.7, 53.3, 53.2, 51.3, 50.9, 40.8, 40.4, 39.1, 36.6, 36.6, 34.1, 33.7, 32.2, 31.7, 29.4, 27.8, 26.4, 26.3, 26.1, 25.9, 25.1, 24.9, 23.6, 22.7, 22.1, 21.2, 15.3, 10.9, 9.8; MS (MALDI-TOF) *m/z* mass calcd for: $C_{52}H_{84}N_6O_{11}$, 968.62 found: 1007.58 (MK^+) 998.58 (MNa^+).

(4*S*)-4-[*N*-(Ac-Asp(O*t*Bu)-D-Glu(O*t*Bu)-Leu-Ile-Cha)-amino]-1-(4-methoxybenzyl)-3-hexenone 9b. The title compound was prepared in 100% yield (36 mg, 0.036 mmol) according to general procedure, vide supra. ^{13}C NMR (75 MHz, $CDCl_3$) δ 209.3, 1173.8, 173.4, 172.8, 172.4, 172.4, 172.3, 172.2, 170.2, 158.1, 133.2, 129.4, 113.9, 81.7, 80.9, 60.3, 58.7, 55.0, 53.3, 53.2, 51.3, 50.9, 41.0, 40.4, 39.1, 36.6, 36.5, 34.1, 33.7, 32.3, 31.7, 28.5, 27.8, 26.4, 26.3, 26.1, 25.9, 25.1, 24.9, 23.6, 22.7, 22.1, 21.2, 15.3, 10.8, 9.8; MS (MALDI-TOF) *m/z* mass calcd for: $C_{53}H_{86}N_6O_{12}$, 998.63 found: 1037.65 (MK^+) 1021.68 (MNa^+).

(4*S*)-4-[*N*-(Ac-Asp(O*t*Bu)-D-Glu(O*t*Bu)-Leu-Ile-Cha)-amino]-1-(thiazole-2-yl)-3-hexenone 9c. The title compound was prepared in 100% yield (23 mg, 0.024 mmol) according to general procedure, vide supra. ^{13}C NMR (75 MHz, $CDCl_3$) δ 207.9, 173.9, 173.6, 172.8, 172.4, 172.4, 172.4, 172.3, 170.2, 141.6, 119.0, 81.7, 80.9, 60.3, 58.3, 53.3, 53.2, 51.3, 50.9, 40.3, 39.1, 38.5, 36.6, 36.5, 34.1, 33.7, 32.1, 31.7, 27.8, 26.6, 26.4, 26.3, 26.1, 25.9, 25.2, 24.9, 23.6, 22.7, 22.1, 21.16, 15.3, 10.8, 9.8; MS (MALDI-TOF) *m/z* mass calcd for: $C_{49}H_{81}N_7O_{11}S$, 975.57 found: 1014.62 (MK^+) 998.67 (MNa^+) 976.67 (MH^+).

(5*S*)-5-[*N*-(Ac-Asp(O*t*Bu)-D-Glu(O*t*Bu)-Leu-Ile-Cha)-amino]-4-heptenone 9d. The title compound was prepared in 100% yield (25 mg, 0.0.28 mmol) according to general procedure, vide supra. ^{13}C NMR (75 MHz, $CDCl_3$) δ 210.4, 210.2, 173.9, 173.8, 173.2, 172.9, 172.8, 172.7, 172.4, 172.1, 172.0, 1720, 170.2, 170.1, 81.9, 81.7, 81.1, 60.2, 60.1, 59.1, 58.7, 53.4, 53.3, 53.2, 51.3, 51.2, 50.8, 41.4, 41.2, 40.6, 40.4, 39.1, 39.0, 36.8, 36.7, 36.6, 35.7, 34.2, 34.2, 33.9, 33.7, 32.2, 31.8, 27.9, 26.5, 26.4, 26.2, 26.1, 25.9, 25.3, 25.1, 25.9, 23.8, 23.6, 23.0, 22.8, 22.3,.21.3, 21.1, 16.9, 16.9, 15.4, 15.3, 13.5, 11.0, 10.2, 9.9; MS (MALDI-TOF) *m/z* mass calcd for: $C_{47}H_{82}N_6O_{11}$, 906.60 found: 945.65 (MK^+) 929.69 (MNa^+)

(3*S*)-3-[*N*-(Ac-Asp(O*t*Bu)-D-Glu(O*t*Bu)-Leu-Ile-Cha)-amino]-10-methyl-4-undecaenone 9e. The title compound was prepared in 100% yield (24 mg, 0.025 mmol) according to general procedure, vide supra. ^{13}C NMR (75 MHz, $CDCl_3$) δ 210.3, 173.8, 173.3, 172.9, 172.4, 172.2, 170.2, 81.8, 81.0, 60.1, 58.7, 53.3, 53.2, 51.3, 50.8, 40.4, 39.3, 39.2, 38.9, 36.7, 36.6, 34.1, 33.8, 32.2, 31.8, 29.5, 28.0, 27.9, 27.3, 26.5, 26.4, 26.1, 25.9, 25.1, 25.9, 23.8, 23.5, 22.9, 22.8, 22.4, 22.2, 21.3, 15.3, 10.9, 9.9; MS (MALDI-TOF) *m/z* mass calcd for: $C_{52}H_{92}N_6O_{11}$, 976.66 found: 1015.70 (MK^+) 999.72 (MNa^+).

General method for the preparation of 10a–e, 11a–e and 12a–e

Compound **7a–e, 8a–e** or **9a–e** was added to a stirred solution of TFA (30 equiv) and Et_3SiH (6 equiv) in CH_2Cl_2 (100 equiv). After 1 h, toluene (3 mL) was added and the mixture was concentrated to give the free acid **10a–e**, **11a–e** and **12a–e**. The peptides were analysed by MALDI-TOF and by analytical RP-HPLC. All the measured weight were within +0.13 to +0.19 mass unit of the calculated weight ($M+H^+$, $M+Na^+$, $M+K^+$), of the compounds.

Ac-Asp-D-Glu-Leu-Ile-Cha-Abu-OH 14. H-*N*-Abu-OtBu × HCl (23 mg, 0.14 mmol) was added to a mixture of

PyBOP (65 mg, 0.13 mmol), **6** (100 mg, 0.13 mmol) and DIPEA (47 μL, 0.27 mmol) in CH_2Cl_2. After 16 h stirring at rt the reaction mixture was washed with aqueous 5% $KHSO_4$ (2×) and aqueous 5% $NaHCO_3$ (2×), dried over Na_2SO_4 and concentrated. The residue was purified by silica gel column chromatography (CH_2Cl_2; CH_2Cl_2–MeOH 40:1) to give Ac-Asp(O*t*Bu)-D-Glu(O*t*Bu)-Leu-Ile-Cha-Abu-O*t*Bu **13** in 52% yield (68 mg, 0.08 mmol). ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.4, 172.9, 172.4, 172.3, 171.9, 171.6, 171.5, 170.9, 169.7, 81.4, 81.2, 80.5, 57.8, 54.0, 52.6, 52.4, 50.6, 50.3, 40.1, 38.9, 36.2, 33.7, 33.6, 33.3, 31.9, 31.2, 27.3, 27.2, 26.0, 25.8, 25.7, 25.6, 24.6, 24.4, 24.4, 22.3, 21.7, 20.7, 14.8, 10.2, 9.1. Compound **13** (25 mg, 0.03 mmol) was added to a stirred solution of TFA (85 μL, 1.14 mmol) and Et_3SiH (29 μL, 0.18 mmol) in CH_2Cl_2 (185 μL, 2.9 mmol). After 1 h, toluene (3 mL) was added and the solvent was removed to give the target compound **14** in quantitative yield (23 mg, 0.03 mmol). The peptide was analysed by MALDI-TOF *m*/*z* mass calcd for: $C_{36}H_{60}N_6O_{12}$, 768.43 found: 807.43 (MK^+) 791.46 (MNa^+) 769.46 (MH^+) and by analytical RP-HPLC: 791.45 (MNa^+) 769.48 (MH^+).

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