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On-resin cyclization of a head-to-tail cyclopeptide using an allyldimethylsilyl polystyrene resin pre-loaded by metathesis

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Abstract—Here, we report the solid-phase synthesis of a 17-mer cyclopeptide which is expected to have anti-angiogenic properties. The peptidic synthesis is performed on an allyldimethylsilyl polystyrene support loaded by metathesis with a conveniently functionalized D-Tyrosine amino acid. The linear peptide was assembled by standard Fmoc chemistry and on-resin cyclization was enabled after selective deprotection of the C-terminal group with 2% hydrazine/DMF at room temperature. Final cleavage was realized under mild acidic conditions allowing to obtain a cyclopeptide under partially protected form.

1. Introduction

Synthesis of head-to-tail cyclopeptides has attracted a considerable interest since the antibiotic gramicidin S was found to be a cyclic decapeptide.[1] Many antibiotics and toxins are also known to be cyclic peptides. Cyclization of amino acid sequences results in increased metabolic stability, potency, receptor selectivity and bioavailability.[2–4] Thus, cyclic peptides present suitable properties for investigating ligand–receptor interactions and structure–activity relationships[5,6] as well as for developing drugs with increased metabolic stability and receptor selectivity.[7–9]

Classical methods used to prepare cyclic peptides involve the synthesis of partially protected linear precursors either in solution or on solid-phase, their subsequent cleavage, and cyclization in solution; this one has to be performed under high dilution to minimize the formation of cyclodimers and oligomers. This synthetic procedure present some disadvantages, such as the necessity to isolate the desired peptide from the excess reagents, which leads in some cases to a considerable loss of product. An attractive alternative includes solid support linkage to an amino acid side-chain and solid-phase chain assembly of the linear sequence, followed by cyclization while the peptide still remains anchored to the resin. The pseudo-dilution attributed to the solid-phase favours intramolecular reactions over intermolecular reactions.[10] So far, this method has been applied to aspartic and glutamic acid,[11,12] lysine,[13] tyrosine[14] and recently to serine and threonine[15] in the Fmoc strategy with different C-terminal protecting groups. To date, general and commercial synthesis of head-to-tail cyclic peptides has used only Allyl group (All) for temporary protection of the α-COOH group of resin-bound amino acid.[16] The selective removal of the Allyl ester requires complex mixtures such as $Pd(PPh_3)_4$–AcOH–$CHCl_3$-*N*-methylmorpholine (NMM) over an extended period of 2 h. Moreover, the use of tetrakis(triphenylphosphine)palladium ($Pd(PPh_3)_4$) requires inert atmosphere and degazed solvents. To overcome these drawbacks, a novel 4-{*N*-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]-amino} benzyl alcohol protecting group called Dmab has been described and used in the synthesis of a model cyclic heptapeptide[17] using standard Fmoc/*t*Bu procedures.[18] Recently, the use of this Dmab group as a temporary α-COOH protecting group has been reported for automated solid-phase synthesis of an expanded (29-mer) cyclic peptide.[19]

Keywords: Solid-phase peptidic synthesis; Cyclic peptides; Metathesis; On-resin cyclization; Mitsunobu reaction.
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2. Results and discussion

In a previous work, we have reported the structural and the biological properties of a 17-mer cyclopeptide.[20] This 17-amino acid molecule, described as cyclic vascular endothelial growth inhibitor (cyclo-VEGI), revealed to be an attractive candidate for the development of novel angiogenesis inhibitor molecules useful for the treatment of cancer and other angiogenesis-related diseases, now under preclinical development. Angiogenesis allows the establishment of a vascular supply, which is fundamental not only for organ development but also for important processes in the adults such as wound healing and reproductive functions.[21,22] Angiogenesis is also implicated in the pathogenesis of a variety of disorders: proliferative retinopathies, age-related macular degeneration, tumors, rheumatoïd arthritis and psioriasis. A number of angiogenesis regulators such as Vascular Endothelial Growth Factors (VEGFs) and Fibroblast Growth Factors (FGFs) have been identified.[23–26] Cyclo-VEGI encompasses residues 79–93 of VEGF which are involved in VEGF–VEGF type2 receptor interactions.

Efforts have been made to conveniently functionalize one of the cyclo-VEGI side-chain's group in order to broaden its biological properties.[27] Indeed, introduction of a terminal olefin could allow different ways of functionalization on cyclo-VEGI with the aim to enhance its anti-angiogenic properties. However, cyclo-VEGI basic side-chains (i.e., Arg, Lys and His) should be kept free due to their key role in VEGF–receptor interaction.

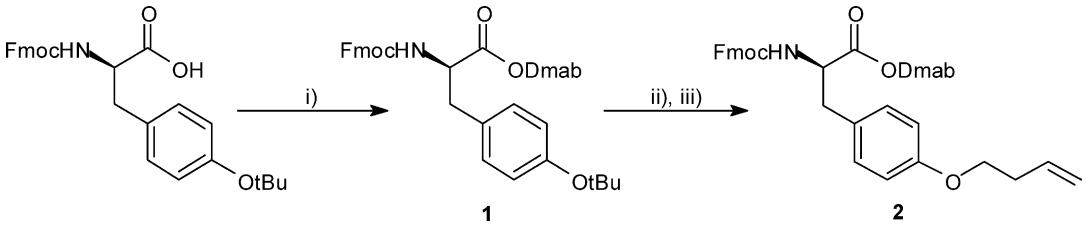
In this work, we describe a novel solid-phase peptide synthesis for such compounds which allows high functionalization potential. It takes advantage of an allyldimethylsilyl polystyrene support which could be loaded by cross metathesis with functionalized terminal olefins.[28] Then cyclo-VEGI was modified by substitution of *D*-Phenylalanine by D-Tyrosine, due to their structural homology. Hence, it affords a new side-chain group from which a terminal olefin could be introduced. Scheme 1 shows a synthetic way to conveniently functionalize the commercially available Fmoc-DTyr(O*t*Bu)-OH (Novabiochem) to realize solid-phase peptide synthesis and on-resin cyclization. The esterification of the D-Tyrosine derivative with Dmab-OH was accomplished by activation with diisopropylcarbodiimide (DIPCDI) to yield Fmoc-DTyr-(O*t*Bu)–ODmab **1**, which on treatment with trifluoroacetic acid (TFA) in CH_2Cl_2 gave the required Fmoc-DTyr-ODmab. Etherification has been attempted using K_2CO_3 and 4-bromo-1-butene in acetone, but it was unsuccessful. Then etherification with an alkenyl group was realized under Mitsunobu reaction conditions, that is, with triphenylphosphine/diethyl azodicarboxylate (PPh_3/DEAD) and but-3-en-1-ol, to yield the expected compound **2**. To protect the α-COOH of D-Tyrosine, Dmab group was preferred to allyl group, due to the metathesis step needed for resin loading. Indeed, allyl group could interfere with the terminal olefin of compound **2** during the loading step.

Compound **2** was then involved in our 17-mer cyclic peptide synthesis. Solid-phase peptide synthesis (Scheme 2) was performed on an allyldimethylsilyl polystyrene resin which has a silicon content of 1.3 mmol/g. Loading of the resin was achieved by cross-metathesis in refluxing CH_2Cl_2 using 10 mol% of Grubb's catalyst (first generation) and 1 mmol of **2** per gram of resin. After 18 h, the resin was filtered off and washed with DMF, CH_2Cl_2, MeOH and Et_2O. Residual diethyl ether was removed under vacuum, and a substitution level of 0.14 mmol/g was determined for this new preloaded resin. 0.1 mmol of this resin was then used for the linear peptide synthesis on a ABI-433A continuous-flow automated peptide synthesizer. Standard Fmoc chemistry was used throughout. Couplings were made with 10 M excess of the acylating amino acid by activation with a 0.45 M 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/hydroxybenzotriazole (HBTU/HOBt) in DMF solution as recommended in the automated synthesizer manual for 0.1 mmol scale peptide synthesis. After completion of the peptide assembly, the resin was treated with 2% hydrazine/DMF at room temperature for 3 min. The treatment was repeated two more times, and the partially protected resin was thoroughly washed with DMF. For the intramolecular cyclization 3 M equiv of each benzotriazol-1-yloxy-tris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP) and HOBt were used in the presence of 6 M equiv of diisopropylethylamine (DIEA) for 72 h.

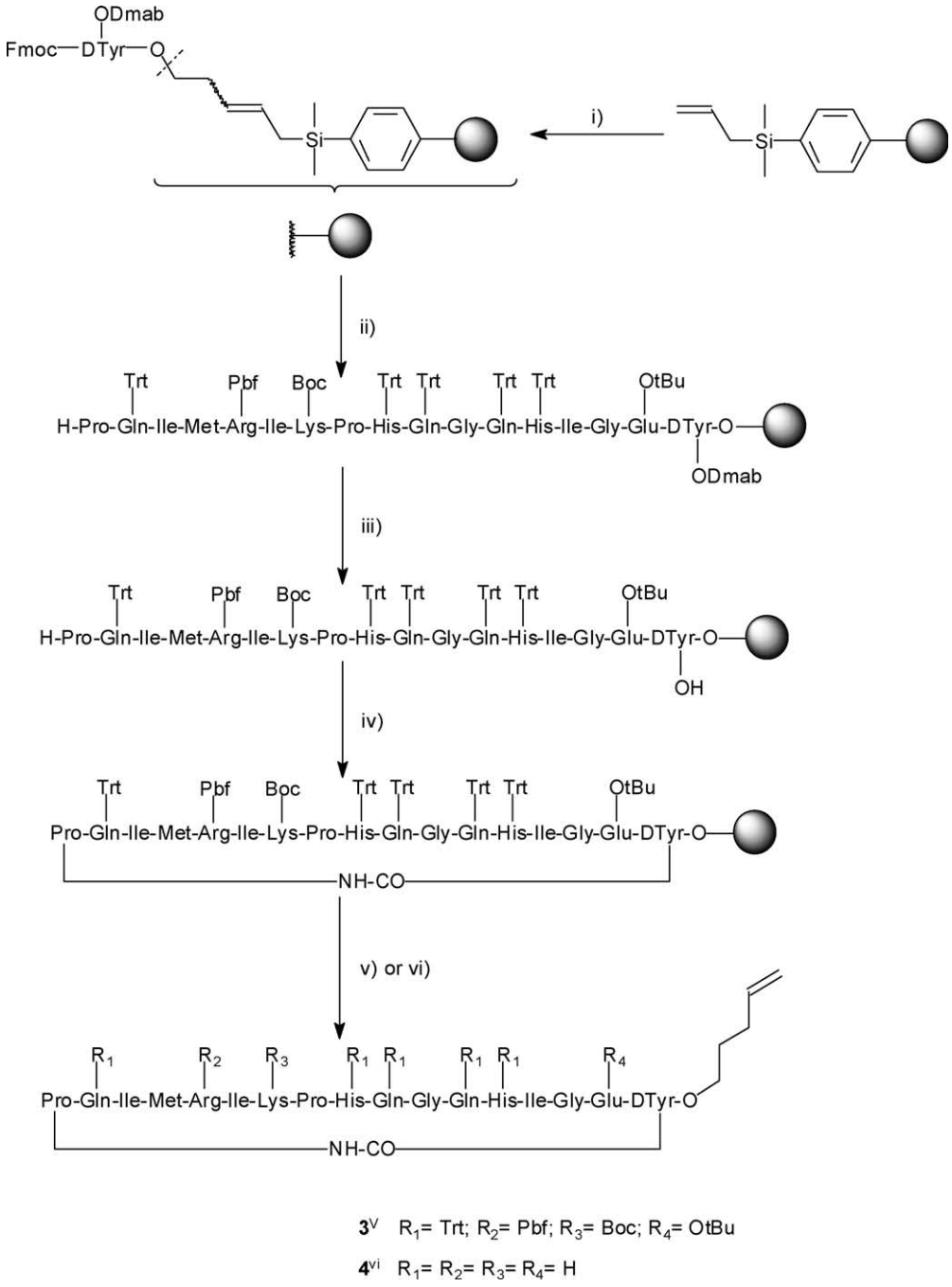
Peptide was cleaved from the resin with a solution of 1% TFA in CH_2Cl_2 affording a protected modified cyclo-VEGI. A portion of this peptide was taken for final deprotection step employing a mixture of trifluoroacetic acid (TFA) in the presence of suitable scavengers to obtain the functionalized free cyclic peptide. Peptides were purified by reverse-phase high performance liquid chromatography (RP-HPLC) and MALDI mass spectrometry analysis gave the expected mass results.

3. Conclusion

To sum up, we have presented here, a new way to synthesize a cyclic peptide with one side-chain selectively functionalized which was obtained directly after cleavage from the resin. To our knowledge this is the first time that a peptide synthesis was performed on allyldimethylsilyl polystyrene resin. Thus this 17 amino-acid anticancer cyclic peptide



Scheme 1. Functionalization of Fmoc-DTyr(O*t*Bu)-OH. Reagents and conditions: (i) 1.5 equiv DIPCDI and HOBt, 3 equiv DIEA, CH_2Cl_2, rt, 18 h, 80%; (ii) TFA/CH_2Cl_2 (95/5 v/v), rt, 3 h, 92%; iii) 1.5 equiv of each PPh_3-DEAD-but-3-en-1-ol, CH_2Cl_2, rt, 48 h, 52%.



Scheme 2. Solid phase synthesis of the 17-mer cyclopeptide. Reagents and conditions: (i) **2**, 10% mol $Cl_2(PPh_3)_2Ru{=}CHPh$, CH_2Cl_2; (ii) 16 cycles of Fmoc/*t*Bu solid-phase peptide synthesis a—20% piperidine/*N*-methylpyrrolidone (NMP), b—Fmoc-AA-OH, HBTU/HOBt/NMP, DIEA with final deprotection; (iii) 2% hydrazine/DMF; (iv) PyBOP, HOBt, DIEA, NMP; (v) TFA/triisopropylsilane (Tis)/thioanisole/water/phenol; (vi) 3% TFA/CH_2Cl_2.

could be obtained either under protected or unprotected form. Our synthetic approach opens the door to subsequent functionalizations such as radio-labelling or dimerization of cyclopeptide **3** and study in order to investigate the structural and biological properties of these new compounds.

4. Experimental

4.1. General information

MALDI mass spectra were run using a MALDI-TOF Reflex III Bruker apparatus, and HRMS were run on a LCT premier from Waters. For the NMR spectra, a Bruker Avance 300 was used. Chemicals shifts are reported in parts per million relative to tetramethylsilane as an internal standard (in NMR description s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet, br=broad peak). UV measurements were taken on a Genesys 5 Spectronic Instruments spectrophotometer. For column chromatography, 63–200 mesh silica gel 60 (VWR International) was used as the stationary phase.

The *N*-(9-Fluorenylmethoxycarbonyl, Fmoc) protected

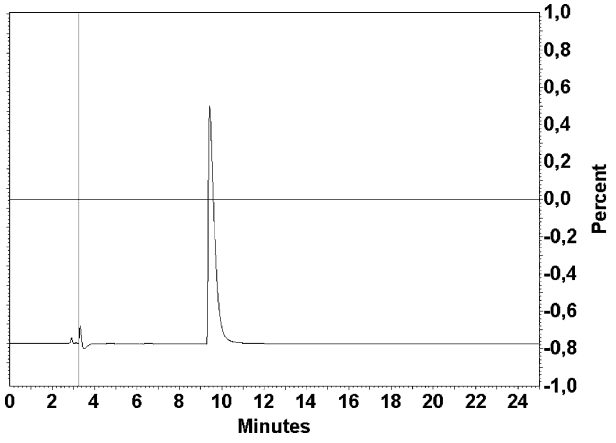
natural aminoacids and allyldimethylsilyl polystyrene resin were purchased from Advanced Chemtech. All trifunctional aminoacids were suitably protected. The α-carboxyl group of D-Tyrosine was protected with the 4-{*N*-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]-amino} benzyl alcohol (Dmab) group. The ε-amino group of lysine was protected with the (*tert*-butoxy)carbonyl (Boc) group. The histidine imidazole group and the amide group of glutamine were protected with the triphenylmethyl (Trityl, Trt) group. The guanidino function of arginine was protected with the 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) group and the ω-carboxyl group of glutamic acid was protected with the *tert*-butyl (*t*Bu) group. Solution of 0.45 M 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) in *N*-hydroxybenzotriazole (HOBt) was purchased from Applied Biosystems. Diethyl azodicarboxylate (DEAD), diisopropylethylamine (DIEA), dimethylformamide (DMF), phenol, thioanisole, trifluoroacetic acid (TFA), triisopropylsilane and triphenylphosphine were purchased from Aldrich. Diethyl ether, piperidine and potassium hydroxide were purchased from Avocado. Hydrazine was purchased from Acros and Grubb's 1st generation catalyst was purchased from Strem chemicals. Absolute ethanol, dichloromethane, ethyl acetate, *n*-hexane and methanol were purchased from J. T. Baker.

4.2. Anchoring of Fmoc-DTyr(O–$(CH_2)_2$–CH=CH_2)–ODmab to the allyldimethyl silyl polystyrene resin

Fmoc-DTyr(O–$(CH_2)_2$–CH=CH_2)–ODmab (540 mg, 0.7 mmol) was dissolved in degazed CH_2Cl_2 (25 mL). Then allyldimethylsilyl polystyrene resin (702 mg, 1.3 mmol g^{-1}) and Grubb's first generation catalyst (28.9 mg, 35.11 μmol) were successively added. The resulting suspension was refluxed under argon atmosphere overnight. Then Grubb's catalyst (28.9 mg, 35.11 μmol) was added again and the reaction mixture was maintained under refluxed for 12 h. The resin was filtered off and successively washed with DMF, CH_2Cl_2, MeOH and Et_2O (100 mL of each). The residual diethyl ether was removed under high vacuum. The substitution level was determined spectrophotometrically by Fmoc cleavage. Fmoc-DTyr-(resin)–ODmab (5.4 mg) was introduced into a test tube and a solution of 20% piperidine in DMF was added (0.5 mL). 20% piperidine in DMF (0.5 mL) was also added to an empty test tube to serve as a blank. Over the next 15 min, the test tube with the resin was swirled two or three times to make sure all the resin has come in contact with the piperidine solution. DMF was added to both tubes to bring a volume of 50 mL. The blank was used to zero the UV spectrophotometer at 301 nm. The absorbance of the solution is 0.126. The substitution level was calculated from the formula: $Abs_{301} \times Vol\ (mL)/(7800 \times m\ (g))$ and was determined to be 0.15 mmol/g.

4.3. Peptide synthesis

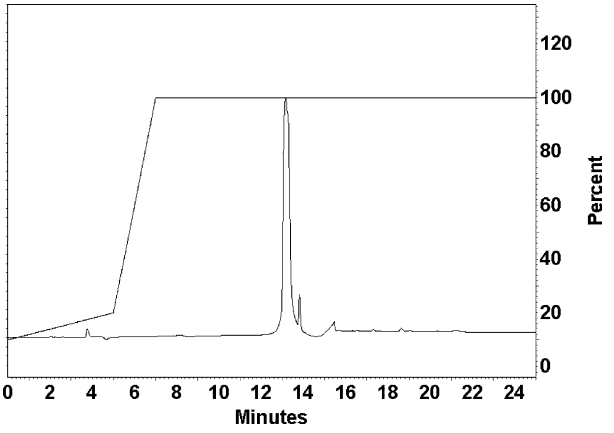
Cyclo(*D*YFPQIMRIKPHQGQHIGE) was synthesized by Fmoc/*t*-Bu batch solid phase synthesis on an Applied Biosystems 433A automated peptide synthesizer. Preloaded Fmoc-DTyr(resin)–ODmab was used for the linear chain assembly. Subsequent Fmoc aminoacids were coupled using a 4-fold excess of aminoacids activated as HOBt ester by means of a 0.45 M HBTU/HOBt solution. Removal of the Dmab protecting group was performed after N-terminal Fmoc deprotection. The peptidyl resin was weighed (1.12 g, 0.11 mmol) and placed on a frit in a syringe barrel and allowed to equilibrate for 5 min in DMF (20 mL/g of resin). The solvent was removed from the resin by applying a nitrogen pressure and the residue was resuspended in a solution of 2% hydrazine monohydrate in DMF (20 mL/g of resin). Reaction was allowed to proceed for 3 min with gentle manual agitation and the hydrazine treatment was repeated a further 2 times to ensure complete reaction. The peptide-resin was washed with DMF (5×20 mL/g of resin) and resuspended in a solution of DIEA in DMF (1/9 v/v; 20 mL/g of resin) for 10 min. Finally, the peptidyl resin was washed successively with DMF, MeOH, Et_2O and dried in vaccuo over KOH. On-resin cyclization was performed by mixing peptidyl resin (890 mg, 0.09 mmol) with a solution of PyBOP (140.5 mg, 0.27 mmol), HOBt (36.5 mg, 0.27 mmol) and DIEA (93.5 μL, 0.54 mmol) in 20 mL of NMP. The mixture was swelled at room temperature for 72 h. The peptidyl resin was washed with 50 mL of each NMP, CH_2Cl_2, MeOH and was dried under high vacuum. Final cleavage of cyclo(*D*YFPQIMRIKPHQGQHIGE) from the resin without loss of any side-chain protecting group was performed with a solution of 1% TFA in CH_2Cl_2 (10 mL/g of resin). Cyclopeptidyl resin was mixed with dilute TFA and shake for 4 min. Then the solution was filtered and filtrate was collected in a flask containing a solution of 10% pyridine in MeOH (2 mL/10 mL of 1% TFA). It was repeated 5 times and resin was washed with 3×30 mL of CH_2Cl_2, 3×30 mL of MeOH, 2×30 mL of CH_2Cl_2 and 3×30 mL of MeOH. Then filtrate was evaporated under reduced pressure to 5% of the volume. Thereafter, 40 mL of cold water were added to the residue to aid precipitation of the product which was isolated by filtration through a sintered glass funnel. Product was washed three times with fresh water, dissolved in a solution of CH_3CN/H_20 (70/30) with 0.1% TFA (eluant B) and then loaded onto a preparative Hibar Purosphere column C18. The elution was achieved using the following conditions: eluant A, 0.1% TFA in water; eluant B, 0.1% TFA in CH_3CN/H_2O (70/30); gradient: 10% of B at 0 min, 20% of B at 5 min, 100% of B at 7 min and 100% of B at 30 min;



Scheme 3. Protected peptide analytical HPLC performed on Hibar Purosphere C18 column with an isochratic of 1% TFA in CH_2CL_2 over a period of 25 min.

flow rate: 4 mL min^{-1}; detector: 214 nm. Then 130 mg (35%) of protected cyclo(*D*YFPQIMRIKPHQGQHIGE) were obtained. MALDI mass spectrometry analysis gave the expected result (theoretical value: 3716.64 Da; experimental value: 3716.94 Da) and analytical HPLC on Hibar Purosphere column C18, eluted with an isochratic gradient of CH_2Cl_2 with 0.1% TFA, gave the following profile: (Scheme 3)

A portion of the product was treated with 0.75 g of phenol in a TIS/thioanisole/H_20/TFA solution (1:2:2:40) for 3 h at room temperature. The product was precipitated from cold diethyl ether and filtered. Preparative Hibar Purosphere column C18 was achieved using the same conditions as below. Then MALDI mass spectrometry analysis gave the expected result (theoretical value: 2082 Da; experimental value: 2081.69 Da) and analytical HPLC on Hibar Purosphere column C18, eluted with a gradient between eluant A and B (see below), gave the following profile: (Scheme 4).



Scheme 4. Deprotected peptide analytical HPLC performed on Hibar Purosphere C18 column with a gradient of eluant A and B over a period of 25 min (eluant A: 1% TFA in water; eluant B: 1% TFA in CH_3CN/H_2O (70:30)).

4.4. Compound 1: *N*-α-Fmoc-O-*tert*butyl-D-Tyrosine-4-{*N*-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]-amino} benzyl ester

To a solution of Fmoc-Dtyr(*t*Bu)-OH (5 g; 10.88 mmol) in CH_2Cl_2 was added successively DIEA (5.65 mL; 32.64 mmol), DIC (2.5 mL; 16.32 mmol) and HOBt (2.21 g; 16.32 mmol). After complete dissolution, Dmab-OH (5 g; 15.23 mmol) was added. Thereafter, the solution was stirred at room temperature for 18 h. Then the reaction mixture was filtered and washed with water (4×60 mL). The organic layer was dried over $MgSO_4$ and filtrated. The solvent is evaporated under reduced pressure to give a greenish oil which was purified by silica gel chromatography (*n*-hexane/ethyl acetate, 60:40, 70:30). Then 6.68 g (80%) of compound **1** were obtained as a yellowish oil.

^{1}H NMR ($CDCl_3$) δ ppm: 0.75 (d, 6H, $(CH_3)_2$–CH, $^3J_{H-H}$=6.65 Hz), 1.06 (s, 6H, 2 CH_3), 1.29 (s, 9H, 3 CH_3), 1.82 (m, 1H, $(CH_3)_2$–CH), 2.38 (s, 2H, CH_2), 2.48 (s, 2H, CH_2), 2.99 (d, 2H, CH_2–CH, $^3J_{H-H}$=6.80 Hz), 3.06 (br s, 2H, CH_2 DTyr), 4.23 (t, 1H, CH Fmoc, $^3J_{H-H}$=6.69 Hz), 4.36 (br s, 2H, CH_2 Fmoc), 4.65 (m, 1H, CH DTyr), 5.12 (s, 2H, CH_2 Bzl), 5.27 (d, 1H, NH DTyr, $^3J_{H-H}$=8.07 Hz), 6.86 (d, 2H, CH_{Ar} DTyr, $^3J_{H-H}$=7.86 Hz), 6.94 (d, 2H, CH_{Ar} DTyr, $^3J_{H-H}$=7.86 Hz), 7.09 (d, 2H, CH_{Ar} Bzl, $^3J_{H-H}$=7.78 Hz), 7.27 (d, 2H, CH_{Ar} Bzl, $^3J_{H-H}$=7.78 Hz), 7.24–7.40 (m, 4H, CH_{Ar} Fmoc), 7.54 (d, 2H, CH_{Ar} Fmoc, $^3J_{H-H}$=7.13 Hz), 7.74 (d, 2H, CH_{Ar} Fmoc, $^3J_{H-H}$=7.13 Hz), 15.3 (s, 1H, NH Dmab).

^{13}C NMR ($CDCl_3$) δ ppm: 14.09, 22.56, 28.77, 29.54, 30.01, 37.63, 38.33, 47.08, 52.26, 53.75, 54.86, 66.24, 66.97, 78.45, 107.75, 119.96, 124.17, 125.01, 126.67, 127.01, 129.13, 129.70, 130.17, 134.59, 137.03, 141.28, 143.67, 154.56, 155.53, 171.44, 176.37, 196.37, 200.21.

IR (cm^{-1}): 3307; 2957; 2869; 1725; 1644; 1557; 1507; 1451; 1414; 1387; 1366; 1325; 1163; 1051; 898; 759; 741.

MALDI: expected 770.97; found 769.32.

HRMS (ES+): calculated for $C_{48}H_{55}N_2O_7$ 771.4009; found 771.3978.

$[\alpha]_D^{20}$ +23.14°, *c* 1.08 in CH_2Cl_2.

4.5. Deprotection of compound 1: *N*-α-Fmoc-D-Tyrosine-4-{*N*-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]-amino} benzyl ester

To a solution of compound **1** (6.13 g, 7.95 mmol) in CH_2Cl_2 (80 mL) was added TFA (80 mL). The reaction mixture was stirred at room temperature for 3 h. The solution was concentrated over reduced pressure to 5% of the volume. Cold water was added to the residue and a white precipitate appears. The solid was isolated by filtration and washed 3 times with cold water. Then it was dried in dessicator under high vacuum over KOH to give 5.22 g (92%) of deprotected compound **1** as a white solid (mp 92–94 °C).

^{1}H Nmr ($CDCl_3$) δ ppm: 0.76 (d, 6H, $(CH_3)_2$–CH, $^3J_{H-H}$=6.63 Hz), 1.06 (s, 6H, 2 CH_3), 1.84 (m, 1H, $(CH_3)_2$–CH), 2.42 (br s, 4H, 2 CH_2), 2.99 (m, 4H, CH_2–CH and CH_2 DTyr), 4.18 (t, 1H, CH Fmoc, $^3J_{H-H}$=6.59 Hz), 4.34–4.43 (m, 2H, CH_2 Fmoc), 4.65 (br s, 1H, CH DTyr), 5.13 (d, 1H, NH DTyr, $^3J_{H-H}$=8.07 Hz), 5.27 (s, 2H, CH_2 Bzl), 6.66 (d, 2H, CH_{Ar} DTyr, $^3J_{H-H}$=7.86 Hz), 6.83 (d, 2H, CH_{Ar} DTyr, $^3J_{H-H}$=7.86 Hz), 7.07 (d, 2H, CH_{Ar} Bzl, $^3J_{H-H}$=7.89 Hz), 7.26 (d, 2H, CH_{Ar} Bzl, $^3J_{H-H}$=7.89 Hz), 7.24–7.39 (m, 4H, CH_{Ar} Fmoc), 7.53 (d, 2H, CH_{Ar} Fmoc, $^3J_{H-H}$=7.32 Hz), 7.74 (d, 2H, CH_{Ar} Fmoc, $^3J_{H-H}$=7.32 Hz), 15.1 (s, 1H, NH Dmab).

^{13}C NMR ($CDCl_3$) δ ppm: 22.59, 28.25, 29.53, 30.04, 37.44, 38.42, 47.19, 52.25, 54.93, 66.28, 67.03, 107.91, 115.47, 120.01, 125.02, 126.63, 127.75, 129.54, 130.44, 134.76, 141.34, 143.69, 155.11, 155.59, 171.44, 176.48, 196.61.

IR (cm^{-1}): 3340; 2958; 2868; 1704; 1616; 1553; 1515; 1450; 1414; 1326; 1248; 1170; 1103; 1051; 827; 759; 740.

MALDI: expected 714.86; found 715.30.

HRMS (ES+): calculated for $C_{44}H_{47}N_2O_7$ 715.3383; found 715.3384.

$[\alpha]_D^{20}$ +45.80°, *c* 1.31 in CH_2Cl_2.

4.6. Compound 2: *N*-α-Fmoc-*O*-butenyl-D-Tyrosine-4-{*N*-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]-amino} benzyl ester

To a solution of deprotected compound **1** (4.5 g, 6.29 mmol) in CH_2Cl_2 (150 mL) was added triphenylphosphine (2.47 g, 9.44 mmol). After complete dissolution the solution was cooled at 0 °C and DEAD (1.48 mL, 9.44 mmol) was added. Thereafter, the reaction mixture was warmed to room temperature and then but-3-en-1-ol (0.81 mL, 9.44 mmol) was added. The reaction mixture was stirred at room temperature for 48 h. Then the solvent was evaporated under reduced pressure. The residue was purified by silica gel chromatography (*n*-hexane/ethyl acetate, 70:30) to give 2.54 g (52%) of compound **2** as a yellowish solid (mp: 104–106 °C).

^{1}H NMR ($CDCl_3$) δ ppm: 0.75 (d, 6H, $(CH_3)_2$–CH, $^3J_{H-H}$=6.56 Hz), 1.06 (s, 6H, 2 CH_3), 1.26–1.28 (m, 2H, =CH_2), 1.82 (m, 1H, $(CH_3)_2$–CH), 2.38 (s, 2H, CH_2), 2.51 (s, 2H, CH_2), 2.98 (d, 2H, CH_2–CH, $^3J_{H-H}$=6.86 Hz), 3.04 (br s, 2H, CH_2 DTyr), 3.95 (t, 1H, CH Fmoc, $^3J_{H-H}$=6.57 Hz), 4.15–4.22 (m, 2H, O–CH_2 DTyr), 4.41 (m, 2H, CH_2 Fmoc), 4.76 (br s, 1H, CH DTyr), 5.06–5.16 (m, 4H, CH_2 Bzl and CH_2–CH=), 5.21 (d, 1H, NH DTyr, $^3J_{H-H}$=8.18 Hz), 5.85 (m, 1H, CH=CH_2), 6.76 (d, 2H, CH_{Ar} DTyr, $^3J_{H-H}$=7.99 Hz), 6.94 (d, 2H, CH_{Ar} DTyr, $^3J_{H-H}$=7.99 Hz), 7.08 (d, 2H, CH_{Ar} Bzl, $^3J_{H-H}$=8.03 Hz), 7.26 (d, 2H, CH_{Ar} Bzl, $^3J_{H-H}$=8.03 Hz), 7.24–7.41 (m, 4H, CH_{Ar} Fmoc), 7.53 (d, 2H, CH_{Ar} Fmoc, $^3J_{H-H}$=7.09 Hz), 7.74 (d, 2H, CH_{Ar} Fmoc, $^3J_{H-H}$=7.09 Hz), 15.3 (s, 1H, NH Dmab).

^{13}C NMR ($CDCl_3$) δ ppm: 22.41, 28.04, 29.40, 30.01, 33.60, 37.37, 38.33, 46.93, 52.26, 53.75, 54.88, 66.22, 66.96, 67.15, 107.74, 115.35, 117.06, 119.80, 124.92, 126.46, 126.87, 127.54, 129.01, 130.12, 134.23, 137.03, 141.27, 143.67, 155.52, 158.19, 171.46, 176.37, 196.36, 200.21.

IR (cm^{-1}): 3307; 2958; 1722; 1641; 1555; 1513; 1415; 1242; 1064; 761; 742.

MALDI: expected 768.95; found 769.03.

HRMS (ES+): calculated for $C_{48}H_{53}N_2O_7$ 769.3853; found 769.3832.

$[\alpha]_D^{20}$ +7.69°, *c* 1.30 in CH_2Cl_2.

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