Video Article

Modification and Functionalization of the Guanidine Group by Tailor-made Precursors

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Abstract

The guanidine group is one of the most important pharmacophoric groups in medicinal chemistry. The only amino acid carrying a guanidine group is arginine. In this article, an easy method for the modification of the guanidine group in peptidic ligands is provided, with an example of RGD-binding integrin ligands. It was recently demonstrated that the distinct modification of the guanidine group in these ligands allows for the selective modulation of the subtype (*e.g.,* between the subtypes αv and α5). Moreover, a formerly unknown strategy for the functionalization via the guanidine group was demonstrated, and the synthetic approach is reviewed in this document. The modifications described here involve terminally (N_ω) alkylated and acetylated guanidine groups. For the synthesis, tailor-made precursor molecules are synthesized, which are then subjected to a reaction with an orthogonally deprotected amine to transfer the pre-modified guanidine group. For the synthesis of alkylated guanidines, precursors based on *N*,*N*'-Di-Boc-1*H*-pyrazole-1-carboxamidine are used to synthesize acylated compounds, the precursor of choice being a correspondingly acylated derivative of *N*-Boc-*S*-methylisothiourea, which can be obtained in one- and two-step reactions.

Video Link

The video component of this article can be found at https://www.jove.com/video/54873/

Introduction

Among the most abundant pharmacophoric groups in natural ligands is the guanidine group, which is involved in multiple interactions[1,2]. For example, it serves as a potential four-fold hydrogen donor in hydrogen bond interactions and is involved in electrostatic interactions, such as salt bridges or cation-π interactions. In medicinal chemistry, this group is often found in drugs and drug candidates[4], although very often as guanidine mimetics[5,6]. The reason for the development of guanidine mimetics is the removal of the ubiquitous, positively charged guanidine group, as well as the adjustment of the lipophilicity of the ligand. In peptidic ligands, the only guanidine group-containing amino acid is arginine, which is therefore often found in the bioactive region of peptidic ligands.

A very prominent example for an arginine-containing ligand family is the subfamily of the RGD-binding integrins. In general, integrins are a class of cell adhesion receptors, which take over important functions in all higher organisms. Some of these functions involve cell adhesion, migration, and cell survival. Thus, they are also involved in pathological indications, such as cancer and fibrosis. Integrins are transmembrane heterodimeric proteins consisting of an α- and a β-subunit that form 24 currently known integrin subtypes; 8 of them recognize the tripeptide sequence Arg-Gly-Asp (=RGD) in their ligands[7]. The binding region is located at the interface between these two subtypes in the extracellular part, the so-called integrin head group[8]. RGD is recognized by two common interactions: the metal-ion-dependent adhesion site (MIDAS) region, which is located in the beta subunit and which binds the carboxylic acid in the ligands (side chain of Asp); and the guanidine group in the ligands, which is located in the alpha subunit. Most of the integrin subtypes are promiscuous and share at least a part of their natural extracellular matrix (ECM) ligands[9]. Thus, for the development of artificial integrin ligands, the major focus is, besides a high binding affinity, the subtype selectivity. Recently, we were able to unveil a key element for the generation of subtype-selective ligands: the guanidine group. Through distinct modifications, biselective ligands for the αv- and α5-containing integrin subtypes can be turned into selective compounds by simple modifications on the guanidine group, which can then discriminate the different α-subunits[10].

In the pocket of αv, the guanidine group interacts *side-on* via a bidentate salt bridge with Asp218[11,12]. This interaction can also be observed in α5β1 (here, with Asp227 in α5), but additionally, an *end-on* interaction of the guanidine group with a Gln residue (Gln221) is observed there[13]. Thus, we modified the guanidine group in two opposite ways: in one case, by blocking the *side-on* interaction with the methylation of the N_δ of the guanidine group, and in the other case, with the methylation of the guanidine N_ω, blocking the *end-on* interaction. Surprisingly, this small modification led to a complete selectivity shift in the ligands. In addition to the alkylation, a new functionalization method was introduced in this publication. The classical functionalization method for this type of pentapeptidic ligand is through the side-chain conjugation of an amino acid not involved in binding (*e.g.,* K in *c*(RGDfK))[14,15]. Here, we show that functionalization is also possible by modifying the guanidine — which is crucial for binding — with an acyl or alkylated linker. The positive charge that is essential for binding is retained, and models suggest that the long chain

points out of the binding pocket, thus providing an ideal possibility for the attachment of further linkers and labeling units (*e.g.,* a fluorescent label or a chelator for molecular imaging).

In this work, we concentrate on the preparative steps for the modification of the guanidine group in arginine-containing ligands. This involves the synthesis of N_{ω}-methylated species, as well as guanidines with longer linker units. The different modifications comprise acyl and alkyl groups.

Protocol

Note: All reagents and solvents were obtained from commercial suppliers and were used without further purification.

Caution: Please consult all relevant material safety data sheets (MSDS) before use. Please use all appropriate safety equipment when performing chemical syntheses (*e.g.,* fume hood, safety glasses, gloves, lab coat, full-length pants, and closed-toe shoes).

1. Synthesis of the Guanidinylation Precursors

1. **Alkylated species**
    1. **Methylated species**
        1. Dissolve 1.0 g of *N,N'*-di-Boc-1*H*-pyrazole-1-carboxamidine (3.2 mmol, 1 eq.) and 1.0 g of triphenylphosphine (PPh_3, 3.8 mmol, 1.2 eq.) in 10 mL of dry tetrahydrofuran (THF) in a 50-mL round-bottom flask in an inert atmosphere using a rubber septum at room temperature (RT). Add 194 µL of dry methanol using a syringe (4.8 mmol, 1.5 eq.) and let it stir at RT.
        2. Separately, prepare a solution with 747 µL of diisopropyl azodicarboxylate (DIAD, 3.8 mmol, 1.2 eq.) in 2 mL of dry THF in a small glass vial. While stirring, add the solution of DIAD in THF dropwise to the solution of *N,N'*-di-Boc-1*H*-pyrazole-1-carboxamidine, PPh_3, and methanol using a syringe (over 15 min). Let the solution stir for 2 h at RT.
        3. Remove the solvent under reduced pressure using a rotary evaporator and dissolve the crude product in 1 mL of dichloromethane (DCM). Load a flash column (2.5 cm x 20 cm) with 100 g of silica in 10% ethyl acetate (EtOAc) in a pentane solution. Load the dissolved crude product on the column and add the solvent under pressure (1.5 bar).
        4. Collect the fractions (solvent system: isocratic 10% EtOAc in pentane), identify the desired compound spot by thin layer chromatography (TLC), and collect it (R_f = 0.4, EtOAc/pentane 1:9, UV). Combine the desired fractions and evaporate the solvent under reduced pressure using a rotary evaporator to obtain 0.85 g of the title compound as a yellow, viscous oil (2.6 mmol, 81% yield). Store the yielded compound at RT for further use.

    2. **Hexylamine-modified species**
        1. Dissolve 1.0 g of *N,N'*-Di-boc-1*H*-pyrazole-1-carboxamidine (3.2 mmol, 1.0 eq.), 0.9 g of Dde-6-aminohexanol (3.8 mmol, 1.2 eq.), and 1.0 g of PPh_3 (3.8 mmol, 1.2 eq.) in 10 mL of dry THF in a 50-mL round-bottom flask in inert atmosphere using a rubber septum at RT.
        2. Separately, prepare a solution with 747 µL of DIAD (3.8 mmol, 1.2 eq.) in 2 mL of dry THF in a small glass vial. While stirring, add the solution of DIAD in THF dropwise to the solution of *N,N'*-di-Boc-1*H*-pyrazole-1-carboxamidine, PPh_3, and Dde-6-aminohexanol using a syringe (over 15 min). Let the solution stir for 2 h at RT.
        3. Remove the solvent under reduced pressure using a rotary evaporator and take the crude product up in 1 mL of DCM. Load a flash column (2.5 cm x 20 cm) with 100 g of silica in a solution of pentane/EtOAc (2:1). Apply the dissolved crude product on the column and add solvent under pressure (1.5 bar).
        4. Collect the fractions (solvent system: isocratic 2:1 pentane/EtOAc), identify the desired fractions with TLC, and collect them (R_f = 0.66, 2:1 pentane/EtOAc, UV). Combine the desired fractions and evaporate the solvent under reduced pressure using a rotary evaporator to obtain 1.6 g of the title compound as a yellow, viscous oil (2.8 mmol, 88% yield). Store the yielded compound at RT for further use.

2. **Acylated species (2 reaction steps)**
    1. Dissolve 1.4 g of *S*-methylisothiuronium hemisulfate (10 mmol, 1.0 eq.) and 2.2 g of di(*tert*-butyl) dicarbonate (10 mmol, 1.0 eq.) in a vigorously stirring biphasic solution of DCM/ *sat. aq*. $NaHCO_3$ and let it stir for 24 h at RT.
    2. Separate the layers in a separatory funnel and extract the aqueous phase three times with DCM. Combine the organic phases, dry them with Na_2SO_4, and remove the solvent under reduced pressure using a rotary evaporator. Take the crude product up in 1 mL of hexane.
    3. Load a flash column (2.5 cm x 20 cm) with 100 g of silica in a solution of 4:1 hexane/EtOAc. Load the dissolved crude product on the column. Add solvent under pressure. Identify the desired fractions with TLC (solvent system: 4:1 isocratic hexane/EtOAc (detection UV: 254 nm), and collect them (R_f = 0.30).
    4. Combine the desired fractions and evaporate the solvent under reduced pressure using a rotary evaporator to obtain 1.1 g of the title compound as a yellow, viscous oil (5.8 mmol, 58% yield). Store the reagent, *N*-(*tert*-butoxycarbonyl)-*S*-methylisothiourea, at RT for further use.
    5. Dissolve 250 mg of *N*-(*tert*-butoxycarbonyl)-*S*-methylisothiourea (1.3 mmol, 1.0 eq.) in 10 mL of dry *N,N*-dimethylformamide (DMF) in a 50-mL round-bottom flask in an inert atmosphere at RT. Add 440 µL of N, N-diisopropylethylamine (DIPEA) (2.6 mmol, 2 eq.) using a syringe. While stirring, add 245 µL of Ac_2O (5.2 mmol, 4.0 eq.) dropwise using a syringe and let it stir for 1 h at RT.
    6. Add an excess of methanol (2 mL) to the mixture using a syringe and let it stir for 15 min at RT. Remove the solvent under reduced pressure using a rotary evaporator. Take the crude product up in 1 mL of DCM.
    7. Load a flash column (2.5 cm x 20 cm) with 60 g of silica in a solution of 3:1 hexane/EtOAc 3:1. Apply the dissolved crude product to the column. Add solvent (3:1 hexane/EtOAc) and pressure (1.5 bar). Identify the desired fractions with TLC (3:1 hexane/EtOAc, UV: 220 nm), and collect them (R_f = 0.62).

8. Combine the desired fractions and evaporate the solvent under reduced pressure using a rotary evaporator to obtain 210 mg of the title compound as a white solid (0.9 mmol, 70% yield). Store the compound at RT for further use.

2. Synthesis of Cyclic Peptide Precursors

1. **Loading and capping TCP resin**
   1. Add 1.00 g of 2-chlortriyl chloride (CTC) resin (0.9 mmol/g) and 320 mg of Fmoc-Gly-OH (1.2 eq.) into a plastic 20-mL syringe with a frit. Prepare a solution of 313 µL of DIPEA (2 eq.) in 5 mL of dry DCM and add it to the syringe. Let it rotate for 1 h at RT using a rotary unit.
   2. Meanwhile, prepare a 2-mL solution of 5:1 methanol/DIPEA (v/v; capping solution). Add the capping solution to the syringe and let it rotate for another 15 min. Wash the resin with DCM (5x) and DMF (3x).

2. **On-resin Fmoc deprotection**
   1. Treat the resin-bound Fmoc-Gly with 20% piperidine in DMF for 10 min and then for 5 min. Wash the resin with DMF (3x).

3. **On-resin coupling of Fmoc-Orn(Dde)-OH**
   1. Add 934 mg of Fmoc-l-Orn(Dde)-OH (2 eq.), 684 mg of 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) (2 eq.), and 245 mg of 1-hydroxy-7-azabenzotriazole (HOAt) (2 eq.) to a small glass vial and dissolve it in 5 mL of DMF. Add 814 µL of DIPEA.
   2. Add this solution to the Fmoc-deprotected, washed, and resin-bound Gly and let it rotate for 1 h. Wash the resin with DMF (3x).

4. **On-resin Fmoc deprotection**
   1. Treat the resin-bound Fmoc-Orn(Dde)-Gly with 20% piperidine in DMF for 10 min and then for 5 min. Wash the resin with DMF (3x).

5. **On-resin coupling of Fmoc-Val-OH**
   1. Add 610 mg of Fmoc-Val-OH (2 eq.), 684 mg of HATU (2 eq.), and 245 mg of HOAt (2 eq.) to a small glass vial and dissolve it in 5 mL of DMF. Add 814 µL of DIPEA.
   2. Add this solution to the Fmoc-deprotected, washed, and resin-bound Orn(Dde)-Gly and let it rotate for 1 h.

6. **On-resin Fmoc deprotection**
   1. Treat the resin-bound Fmoc-Val-Orn(Dde)-Gly with 20% piperidine in DMF for 10 min and then for 5 min. Wash the resin with DMF (3x).

7. **On-resin *N*- Methylation**
   1. Wash the resin with DCM (3x). Dissolve 887 mg of 2-nitrobenzenesulfonylchloride (*o*-NBS-Cl, 4 eq.) in DCM and add 1.2 mL of 2,4,6-collidine (10 eq.).
   2. Add the solution to the resin-bound peptide and let it incubate for 20 min at RT.
   3. Wash the resin with CH_2Cl_2 (3x) and with THF (5x). Prepare a solution with 1.18 g of PPh_3 (5 eq.) and 365 µL of methanol in THF. Add this solution to the syringe.
   4. Prepare a solution with 883 µL of DIAD in 2 mL of THF and add it to the syringe. Let it incubate for 15 min. Wash the resin with THF (5x) and with DMF (5x).

8. ***o* -Ns deprotection**
   1. Prepare a solution with 570 µL of mercaptoethanol (10.0 eq.) and 672 µL of diazabicycloundecen (DBU) in 2 mL of DMF. Add this solution to the syringe and let it incubate for 5 min.
   2. Repeat the deprotection step and then wash the resin with DMF (5x).

9. **On-resin coupling of Fmoc- d-Phe-OH**
   1. Add 697 mg of Fmoc-D-Phe-OH (2 eq.), 684 mg of HATU (2 eq.), and 245 mg of HOAt (2 eq.) to a small glass vial and dissolve it in 5 mL of DMF. Add 814 µL of DIPEA.
   2. Add this solution to the Fmoc-deprotected, washed, and resin-bound peptide and let it rotate for 1 h.

10. **On-resin Fmoc deprotection**
    1. Treat the resin-bound peptide with 20% piperidine in DMF for 10 min and then for 5 min. Wash the resin with DMF (3x).

11. **On-resin coupling of Fmoc-Asp(*Ot*Bu)-OH**
    1. Add 741 mg of Fmoc- Asp(*Ot*Bu)-OH (2 eq.), 684 mg of HATU (2 eq.), and 245 mg of HOAt (2 eq.) to a small glass vial and dissolve it in 5 mL of DMF. Add 814 µL of DIPEA.
    2. Add this solution to the Fmoc-deprotected, washed, and resin-bound peptide and let it rotate for 1 h.

12. **Cleavage of the linear peptide from the resin**
    1. Wash the resin with DCM (3x) and subsequently prepare 10 mL of a solution of hexafluoroisopropanol (HFIP) in DCM (1:4; v/v).
    2. Add the solution to the resin and let it rotate for 15 min. Collect the solution in a round-bottom flask. Repeat the cleavage and evaporate the solvent using a rotary evaporator.

13. **Cyclization of the linear peptide**
    1. Dissolve 384 mg of $NaHCO_3$ (5.0 eq.) and 582 µL of diphenylphosphoryl azide (DPPA) (3.0 eq.) in 50 mL of DMF and add it to the crude product. Let it stir overnight or until no linear peptide is observed with ESI-MS (10 h)[17].
    2. Under reduced pressure, reduce the solvent to a small volume using a rotary evaporator. Prepare a saturated aqueous solution of NaCl (brine). Using a Pasteur pipette, add the cyclized peptide dropwise to 40 mL of saturated aqueous solution of NaCl in a centrifuge tube.

3. Centrifuge the suspension (5,000 x g, 5 min) and wash the precipitate with HPLC-grade water (2x). Lyophilize the product.

3. Guanidinylation and Deprotection in Solution

1. **Guanidinylation with the tailor-made precursors**
   1. Dissolve a small amount (*e.g.,* 25 mg) of the orthogonally deprotected cyclic peptide in a small volume of DMF (*e.g.,* 2 mL). Add 2 eq. of the guanidinylation precursor and 2 eq. of DIPEA to the solution and let it stir for 2 h at RT.
   2. Monitor the progress of the reaction with HPLC-MS. After the reaction is complete, remove the solvent, re-dissolve the guanidinylated cyclic peptide in acetonitrile, and perform semipreparative HPLC purification[16].

2. **Removal of acid labile side chain protecting groups**
   1. Prepare 2 mL of a solution containing trifluoroacetic acid (TFA), water, and triisopropylsilane (TIPS) (95/2.5/2.5; v/v/v). Add this solution to the purified product in a small glass vial and let it stir at RT for at least 1 h. Observe the complete deprotection with ESI-MS[17].
   2. Evaporate the solvent under reduced pressure to a small volume. Prepare ice-cold ether and add 10 mL in a 50-mL centrifuge tube. Precipitate the deprotected peptide in ice-cold ether using a Pasteur pipette by adding it dropwise. Centrifuge the suspension (5,000 x g, 5 min) to obtain a pellet.
   3. Wash the precipitate with ice-cold ether and centrifuge it (5,000 x g, 5 min, 2x). Dissolve the product in 2 mL of HPLC-grade water and purify it with semipreparative HPLC[16].

4. Analytical Data and Parameters for Purification

1. **Analytical HPLC-ESI-MS**
   1. Perform analytical HPLC-ESI-MS with an LCQ mass spectrometer using a C18 column (12-nm pore size, 3-µm particle size, 125 mm × 2.1 mm) or a C8 column (20-nm pore size, 5-µm particle size, 250 mm × 2.1 mm), with H_2O (0.1% v/v formic acid)/acetonitrile (0.1% v/v formic acid) as eluents.

2. **Semi-preparative HPLC**
   1. Perform semi-preparative HPLC using a preparative HPLC instrument with an ODS-A column (20 x 250 mm, 5 µm), a flow rate of 8 mL/min, and linear gradients of H_2O (0.1% v/v TFA) and acetonitrile (0.1% v/v TFA).

Representative Results

The cyclic peptide precursor was synthesized as a linear peptide, cyclized, and orthogonally Dde-deprotected. After the precipitation, the purity of the compound was analyzed with HPLC-MS (**Figure 1**). To monitor the progress of the reaction, an HPLC analysis was performed after the 2-h reaction time (**Figure 2**).

For larger residues on the guanidine group, the reaction time of 2 h is often not enough. In this case, the reaction was continued and monitored with LC-MS. After the reaction and final deprotection, the compounds were purified using semipreparative HPLC equipment (yields typically in the low-mg range). The final compounds were analyzed with HPLC-MS to evaluate the purity (see **Figure 3**).

A small amount was weighed into a microcentrifuge tube and diluted with DMSO to obtain a stem solution for the biological evaluation of the compound in an ELISA-like, solid-phase binding assay. The results are depicted in **Figure 4**. The standard molecule Cilengitide (unmodified guanidine group) is included as a reference. All compounds possess a relative high affinity for the integrin subtype αvβ3 and high selectivity against α5β1.

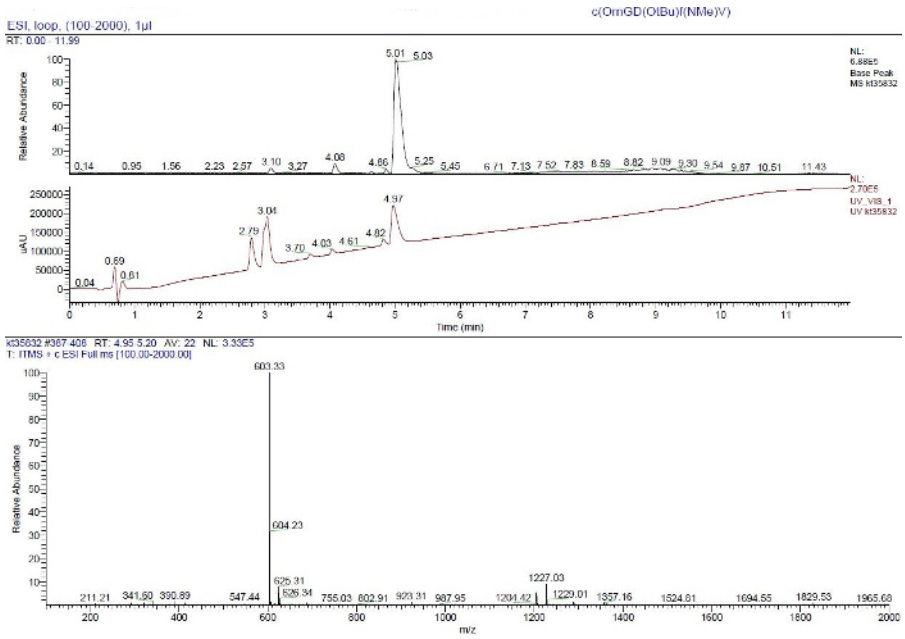

Figure 1: HPLC-MS spectrum (gradient: 10-90% acetonitrile (ACN) in a biphasic solvent system with H_2O and ACN) of the orthogonally Dde-deprotected derivative *c*(OrnGD(*Ot*Bu)f(*N*Me)V) (calculated mass: 602.34 g/mol), as obtained after the cyclization of the linear peptide, subsequent Dde-deprotection, and precipitation. Please click here to view a larger version of this figure.

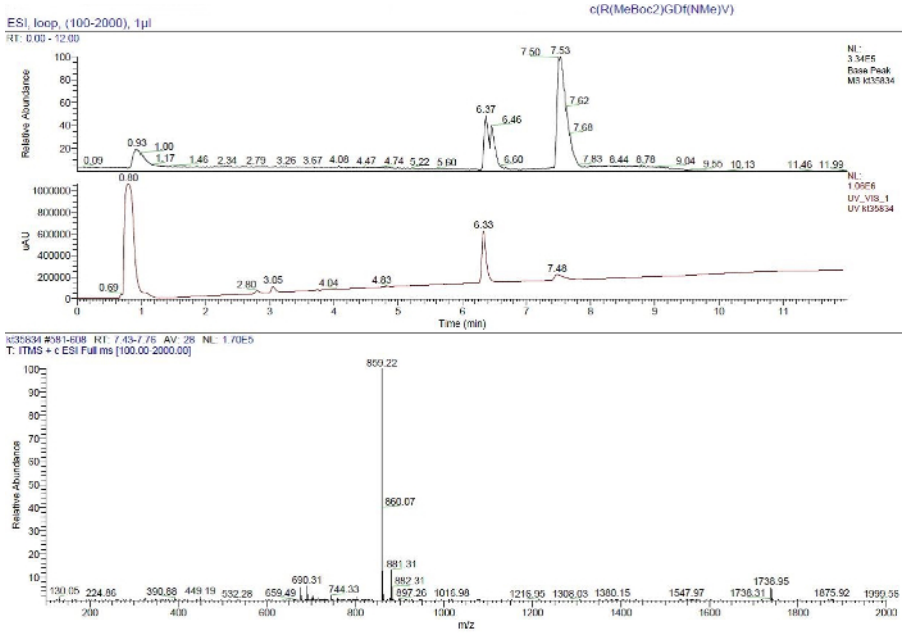

Figure 2: HPLC-MS spectrum (gradient: 10-90% ACN in a biphasic solvent system with H_2O and ACN) of the reaction mixture after the guanidinylation reaction of the orthogonally deprotected cyclic peptide and the methylated precursor for guanidinylation. Besides the product peak (R_t = 7.50 min, calculated mass = 858.49 g/mol), only the excess of guanidinylation precursor (R_t = 6.38 min) and the base DIPEA (R_t = 0.90 min) can be observed. Please click here to view a larger version of this figure.

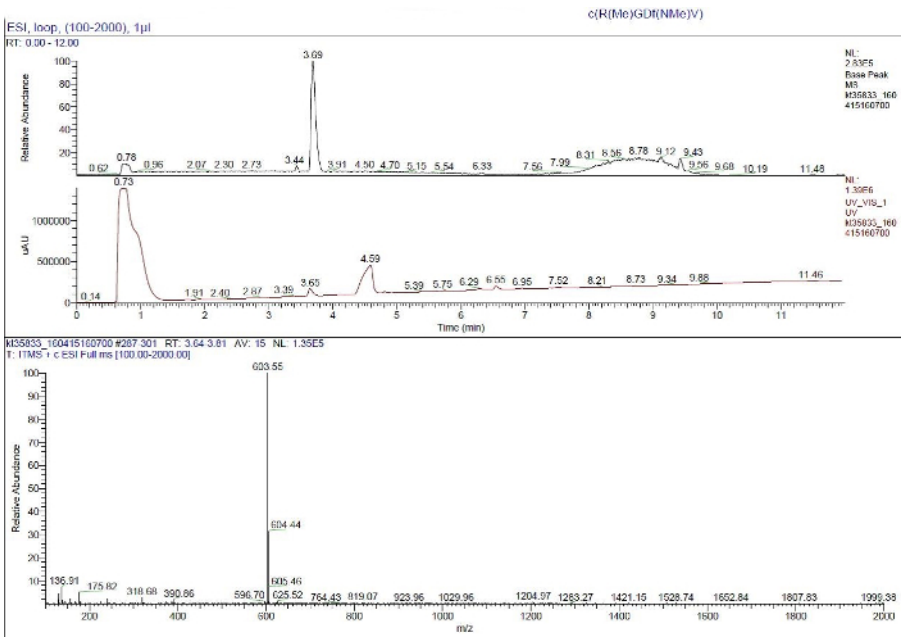

Figure 3: HPLC-MS spectrum (gradient: 10-90% ACN in a biphasic solvent system with H_2O and ACN) of the crude compound **2** (R_t = 3.69 min, calculated mass = 603.32 g/mol) after the deprotection of the acid labile side chains prior to the semipreparative purification. Please click here to view a larger version of this figure.

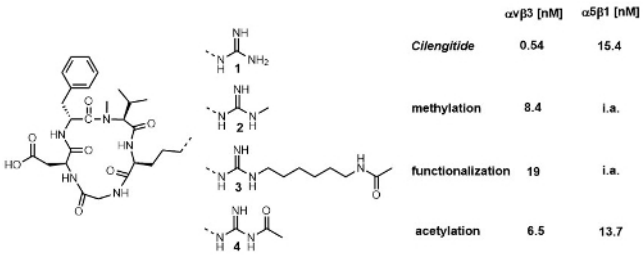

Figure 4: Functionalization of guanidines in solution and biological evaluation. Compound **1**, with an unaltered guanidine group, is Cilengitide. The modifications addressed in this manuscript are the methylated (**2**), functionalized (here, acetyl amino hexane; **3**), and acetylated (**4**) derivatives. The binding affinity was determined in a solid-phase binding assay using isolated proteins[18]. Please click here to view a larger version of this figure.

Discussion

The precursor for guanidinylation is an orthogonally deprotected cyclic peptide derivative, (*c*(OrnD(*Ot*Bu)Gf(*N*Me)V)), which is synthesized by a standard Fmoc protocol of solid-phase peptide synthesis (SPPS). Ornithin was used as the orthogonally protected derivative, (Fmoc-Orn(Dde)-OH), which can be deprotected with hydrazine in DMF after the cyclization of the peptide scaffold. The peptide precursor is purified by the precipitation of the compound and by the subsequent lyophilization.

Alkylated precursors for the guanidinylation can be obtained in good yields in a one-step reaction starting from the commercially available substance *N*,*N*′-di-Boc-1*H*-pyrazole-1-carboxamidine through an alkylation reaction under Mitsunobu conditions (PPh_3, DIAD, THF)[19,10]. With this reaction, a huge variety of precursors is accessible from the corresponding alcohols. The guanidinylation reaction with the alkylated precursors yields the defined product in a clean reaction. If complete conversion of the reactant is not observed after 2 h, the reaction should be continued. Under the conditions given in the protocol, only minor side products formed; however, higher amounts of impurities cannot be excluded for all, especially differently protected, substrates carrying larger moieties.

If using guanidine-functionalized peptides (longer linkers), the Dde protecting group on the terminal amine of the linker is removed and can be conjugated by amide coupling to a corresponding acid. The deprotection of Dde is performed in a solution of 2% hydrazine in DMF and yields the orthogonally deprotected peptide, which should be purified (*e.g.,* semipreparative HPLC) before further use. In this case, a simple acetylation reaction was performed *in situ*.

If using acetylated guanidines, a different precursor strategy should be applied. Starting from *S*-methylisothiourea, a two-step reaction sequence is required[20]. First, a mono Boc protection of *S*-methylisothiourea must be performed[21] before acetylation with an acid of choice (here, acetic acid). The guanidinylation reaction is a very clean and fast reaction, the final compound is obtained after the final deprotection of acid labile side chain protecting groups.

As already stated in the introduction, this method allows for the modification and functionalization of any peptidic guanidine group. We demonstrated this technique on integrin ligands, which allow, in this case, tuning of the subtype selectivity of the ligands. The unmodified integrin

antagonist Cilengitide is biselective for the αvβ3/α5β1 subtypes. Through the methylation of the terminal N_{ω} of the guanidine group, an αvβ3-selective ligand is yielded. This blocks an important end-on interaction that is uniquely observed in α5β1, thus inactivating the ligand for this integrin. The main interaction with the binding site of the αvβ3 subtype is an end-on interaction that is not disturbed by this interaction[10].

By modifying the ligand with longer linker units ("functionalization"), two goals can be reached in one go: selectivity is generated through breaking the end-on interaction with the α5β1 subtype and, on the other side, the linker points out of the binding pocket, allowing for conjugation to large entities (*e.g.,* chelators or fluorescent dyes for imaging techniques)[10].

Disclosures

The authors have nothing to disclose.

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