

# Bisphosphonate-functionalized cyclic Arg-Gly-Asp peptidomimetics

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**Dedicated to Professor Richard R. Schmidt on the occasion of his 78th anniversary**

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## Abstract

We report the synthesis of three new conjugates between a cRGD integrin ligand and alendronic acid as a bisphosphonate anchor. The selected ligand is an RGD peptidomimetic, carrying the conformationally constrained RGD sequence on an azabicycloalkane scaffold and endowed with high affinity for integrin  $\alpha_v\beta_3$ . Since integrin  $\alpha_v\beta_3$  is involved in the adhesion of osteoprogenitor cells and of osteoblasts, these constructs could prove useful as new tools for biofunctionalization of materials in biomedical application and for development of modern implants.

**Keywords:** Arg-Gly-Asp, integrins, bisphosphonate, peptidomimetics

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## Introduction

Osseointegration of implants is known to be a biological process that occurs by formation of a direct structural and functional connection between ordered living bone and the surface of a load carrying implant, without intervening soft tissue.<sup>1</sup> Surface modifications have been shown to enhance osseointegration at early implantation times in terms of both velocity and intensity of bone formation. For example, rough surfaces can stimulate differentiation, growth and attachment of bone cells, and increase mineralization. The main methods reported in the literature to create implant roughness are acid etching, sandblasting, titanium plasma spraying and hydroxyapatite (HA) coating.<sup>2</sup> Furthermore, the biofunctionalization of implant surfaces, by adding different substances to improve their biological characteristics, appears an important tool to enhance the capacity of osseointegration.<sup>3</sup> A common theme in this field is the modification of the material in order to promote selective interactions with a specific cell type through

biomolecular recognition events. Typically, peptides containing the cell-binding domains found in the extracellular matrix proteins are immobilized on the material to promote cell adhesion via ligand receptor interaction.<sup>4</sup> Integrins are an example of cell adhesion receptors that bind to specific amino acid sequences, such as the RGD (Arg-Gly-Asp) sequence that is found in type I collagen, fibronectin, osteopontin and bone sialoprotein. Studies of collagen-coated surfaces<sup>5</sup> have confirmed the improved ability of extra cellular matrix-like surfaces to integrate implants into tissues, and the relevance of the RGD-motif in the adhesion of osteoprogenitor cells and of osteoblasts. The results have prompted research for the incorporation of RGD features into synthetic polymers suitable for implant coating<sup>6</sup> and also for the direct linking of RGD-carrying sequence to the implant material,<sup>7</sup> overcoming technological and biological problems of natural proteins. In particular, some papers explore the use of non-peptidic integrin ligands,<sup>8</sup> or of cyclic peptide structure carrying phosphonate groups on titanium or bone surfaces.

Recently, we have designed and synthesized cyclic peptidomimetic ligands which contain the RGD recognition sequence embedded into novel azabicycloalkane scaffolds.<sup>9</sup> These studies have produced some specific ligands with nanomolar affinity for  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  integrins, which are currently being profiled as anti-angiogenetic agents.<sup>10</sup> Among them, compound DB58 (Figure 1) contains a site suitable for conjugation to different functional units,<sup>11</sup> which has been derivatized for application in medical diagnosis and therapy.<sup>12</sup>

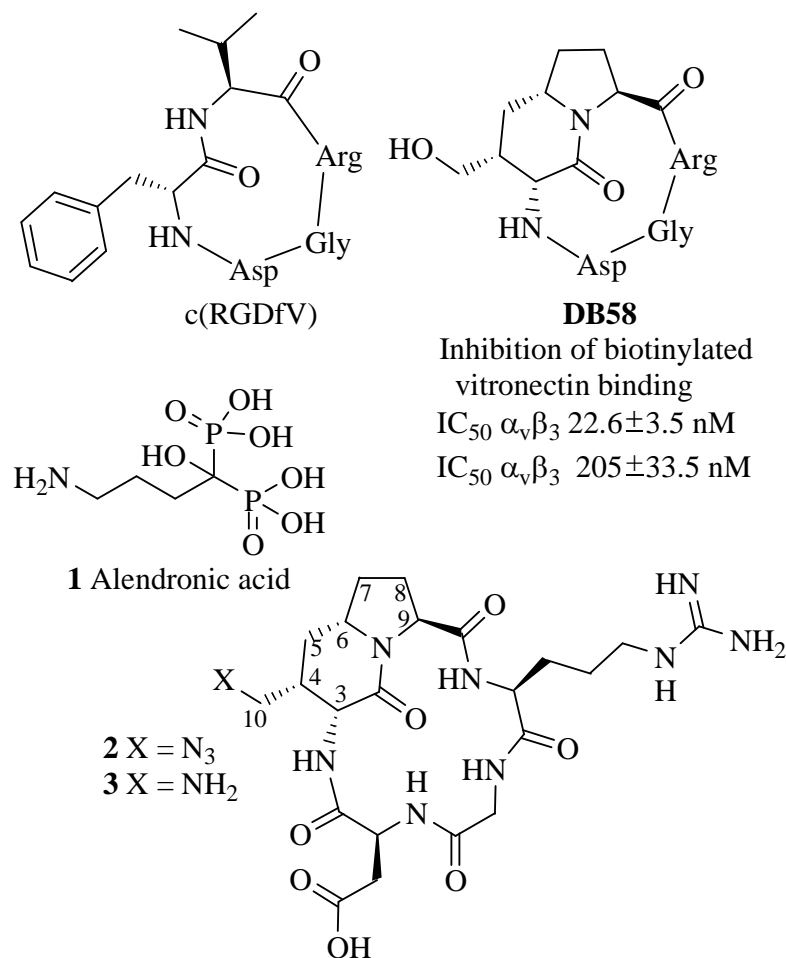
Our aim was the development of new coatings able to conjugate the  $\alpha_v$ -specific cyclic-RGD peptide to titanium implants or to hydroxyapatite-like objects or bone cements, using a methylene-bis-phosphonate moiety as anchor system. For practical reasons, such as commercial availability, biological characterization, and the presence in the same molecule of both the anchor and the conjugable functionalities, we chose alendronic acid (alendronate) as source of diphosphonic moiety. Sodium alendronate is a commonly used antiresorptive pharmacological agent that has been shown to be effective in osteopenic women for reducing bone resorption, increasing bone density, and decreasing fracture incidence.<sup>13</sup> Furthermore, studies on the effect of alendronate on implant surface has been already reported in the literature.<sup>14</sup> The capacity of phosphonic acid groups to bind strongly over a large pH range (pH 1-9) to  $\text{TiO}_2$ ,<sup>15</sup> and the presence of two phosphonate groups can improve the binding of the coating molecule to the Ti surface.

In this experimental study the alendronate will be bridged with the cyclic-RGD peptide by spacers that differ for length, hydrophilicity or hydrophobicity, flexibility, as well as the type of conjugation.

## Results and Discussion

The cyclic RGD peptidomimetic DB58 (Figure 1) which binds to  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  integrin receptors was developed by our group previously.<sup>11b</sup> The replacement of the D-Phe-Val dipeptide in the lead structure cyclo(RGDfV)<sup>16</sup> with an azabicycloalkane scaffold showing reverse-turn mimetic

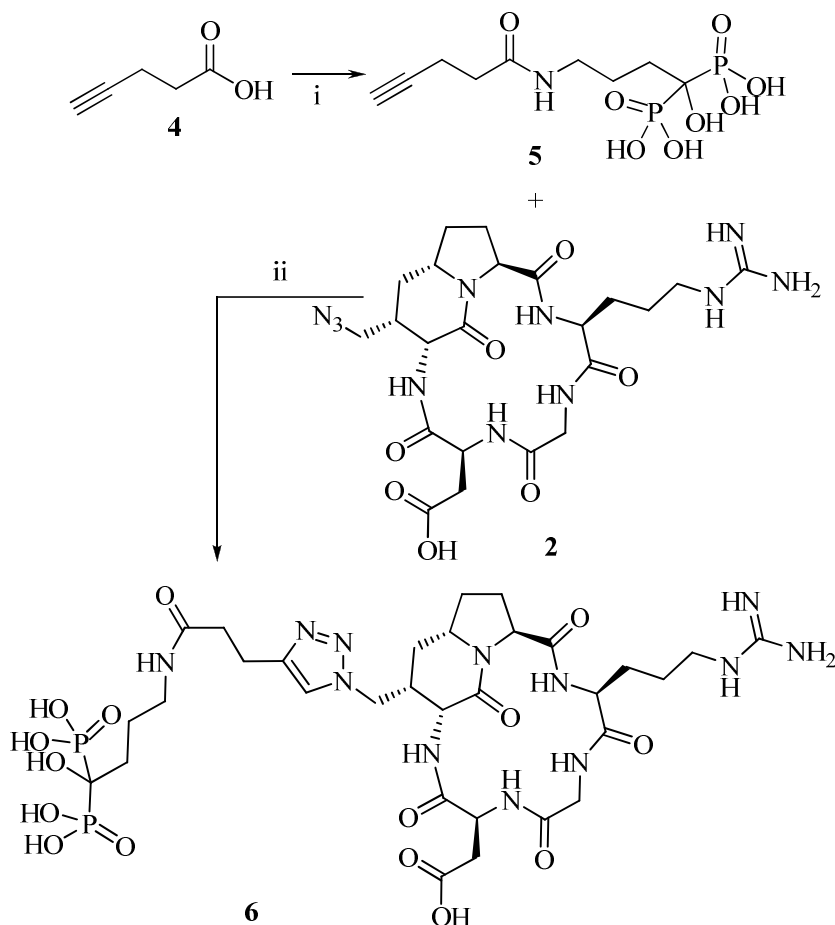
properties, constrains the RGD sequence into pre-organized conformations and provides the required affinity and selectivity for integrin antagonism. The functionalization of the scaffold with an heteroalkyl substituent enabled us to conjugate the RGD containing ligand with different diagnostic or therapeutic entities without losing activity.<sup>12</sup>



**Figure 1.** Integrin ligands and bisphosphonate anchor moiety.

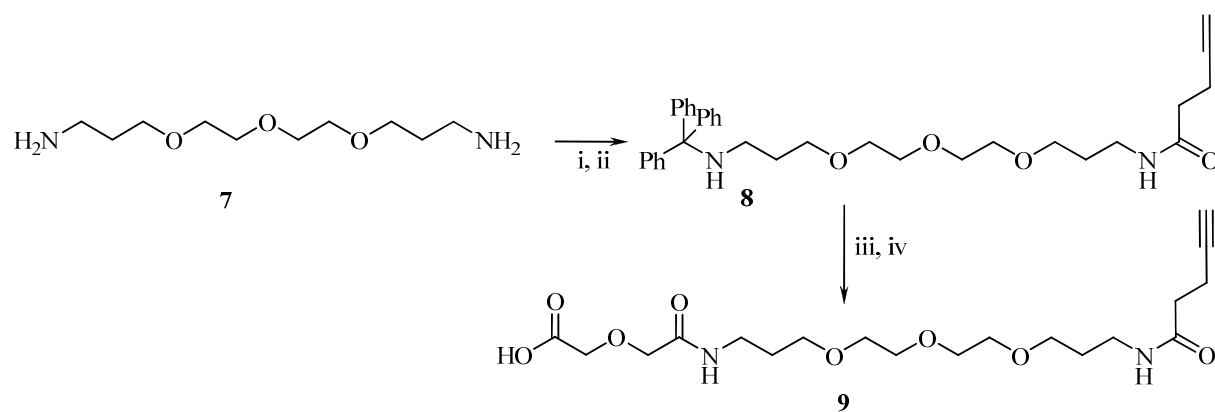
As mentioned above, we selected alendronic acid **1** as the anchor and prepared three compounds differing for the distance between the anchor moiety and the targeting groups, for the type of conjugation and for the hydrophilicity of the linkers. In compound **6** the anchor group is directly conjugated to the RGD-carrying moiety, whereas in compounds **12** and **16** two linkers of different length are introduced. Compound **6** is accessible in a three step synthesis starting from 4-pentynoic acid **4**, activated as succinimidyl ester followed by coupling with alendronic acid **1**. The regioselective copper-catalyzed Huisgen 1,3-dipolar cycloaddition between an azide and a terminal alkyne, often referred to as “click reaction”, has been reported to proceed in very good yields in *t*-butanol/water, a solvent mixture that appeared ideal for dissolution of both linker and

cyclopeptide. Thus, the “click” reactions of azide **2**, with the terminal alkyne present in compound **5**, ( $\text{CuSO}_4$ , Na L-ascorbate, *t*-BuOH,  $\text{H}_2\text{O}$ )<sup>17</sup> proceeded without drawback reaching completion within 18 hours to give the corresponding triazole conjugates **6** in 80 % yield after RP-HPLC purification. (Scheme 1)

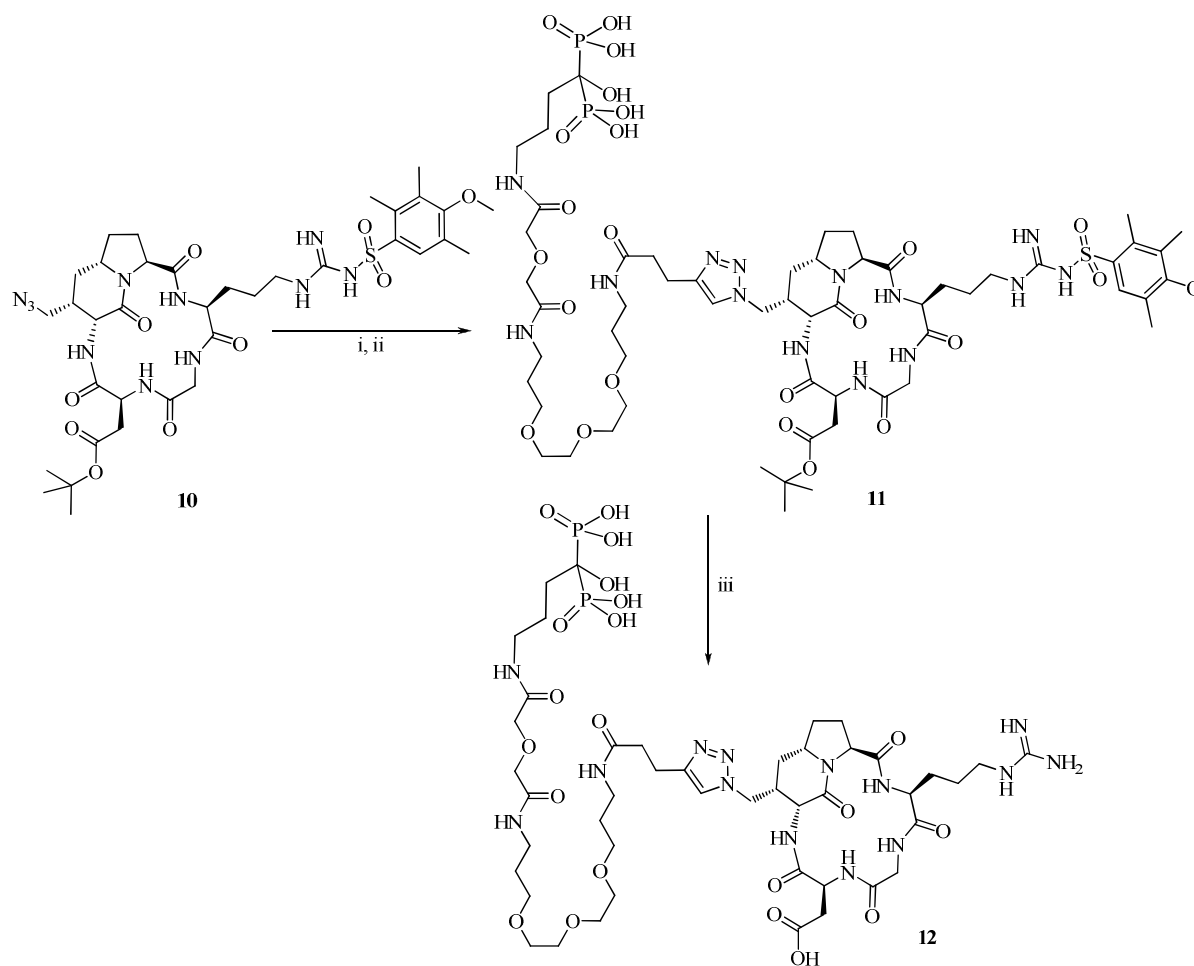


**Scheme 1.** i) NHS, DIC, DCM dry, r.t. 18 h, **1**,  $\text{H}_2\text{O}$ ,  $\text{CH}_3\text{CN}$ , NaOH until pH 7.2-7.4, 0 °C, 18 h, 70% over two steps; ii) Na-L-ascorbate, *t*-BuOH,  $\text{H}_2\text{O}$ ,  $\text{CuSO}_4$ , r.t., 18 h, 80%.

In the case of compound **12**, an appropriate linker has been synthesized. The commercially available 4,7,10-trioxa-1,13-tridecanediamine **7** has been monoprotected at the nitrogen atom as the trityl derivative which was reacted with 4-pentynoic acid **4** using standard coupling procedures to give **8** in 55% yield over two steps. Compound **8** was treated with TFA in the presence of  $\text{iPr}_3\text{SiH}$  affording the deprotected compound. The resulting free amine was coupled with diglycolic anhydride to give the carboxylic acid **9** in 71% yield over two steps (Scheme 2).

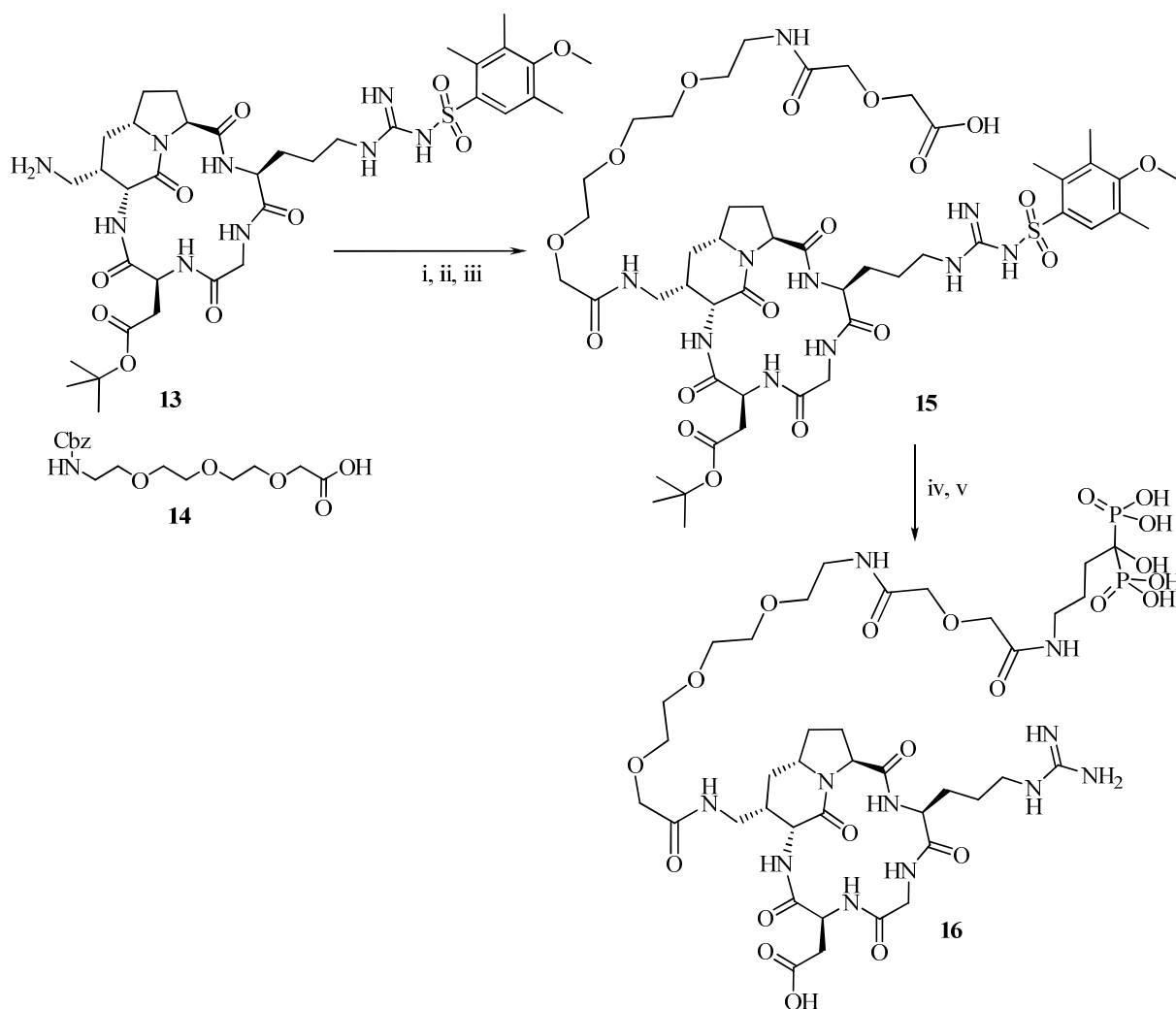


**Scheme 2.** i)  $\text{TrCl}$ ,  $\text{Et}_3\text{N}$ , DCM dry, r.t., 18 h; ii) **4**, DIC, DCM dry, r.t., 18 h, 55% over two steps; iii)  $\text{iPr}_3\text{SiH}$ , TFA, r.t., 4 h; iv) diglycolic anhydride, pyridine, DMF, r.t., 4 h, 71% over two steps.



**Scheme 3.** (i) **9**, Na L-ascorbate,  $t\text{-BuOH}$ ,  $\text{H}_2\text{O}$ ,  $\text{CuSO}_4$ , r.t., 18 h; (ii) NHS, DIC, DCM dry, r.t., 18 h, **1**,  $\text{CH}_3\text{CN}$ ,  $\text{H}_2\text{O}$ , NaOH until pH 7.2-7.4,  $0^\circ\text{C}$ , 18 h, 55% over two steps; (iii) TFA, thioanisole,  $\text{iPrSiH}$ , ethanediol, phenol, water, r.t., 18h, 84%.

With compound **9** in hand, the next task was to couple the linker and the integrin ligand to create the desired triazolyl-linked conjugates. An attempt to perform the click reaction between compound **9** and the unprotected integrin ligand **2** afforded the desired product in poor yield. Better results were obtained using the protected integrin ligand. Thus, completely protected azide **10** has been “clicked” with linker **9** to give the corresponding triazolyl conjugate. Activation of the carboxylic acid with *N*-hydroxysuccinimide (NHS) and *N,N'*-diisopropylcarbodiimide (DIC) followed by the addition of alendronate **1** gave the desired conjugate protected compound **11** in 55% over three steps. Finally, the side chain protecting group has been removed by TFA in presence of scavengers (thioanisole, phenol, *i*Pr<sub>3</sub>SiH) to give the desired compound **12** in 84% yield (Scheme 3).



**Scheme 4.** (i) **14**, HOBt, EDC, DIPEA, DCM dry, r.t. 2h; (ii) H<sub>2</sub>, Pd/C, MeOH, r.t., 2h; (iii) diglycolic anhydride, DMF, pyridine, r.t., 4h, 52% over three steps; (iv) NHS, DIC, DCM dry, r.t., 18h, **1**, CH<sub>3</sub>CN, H<sub>2</sub>O, NaOH until pH 7.2-7.4, 0°C, 18 h, 84% over two steps; (v) TFA, thioanisole, *i*Pr<sub>3</sub>SiH, ethandiol, phenol, water, r.t., 18h, 93%.

In the case of the last compound, the known linker **14**<sup>12d</sup> was linked with the amino derivative integrin ligand **13**<sup>11b</sup> using *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), 1-hydroxybenzotriazole (HOBt) and *N,N*-diisopropylethylamine (DIPEA) as coupling agents, followed by hydrogenation on Pd/C under standard condition to remove the benzyloxy carbonyl protecting group on the nitrogen. The resulting free amine was reacted with diglycolic anhydride to obtain the carboxylic acid **15** in 52% yield over three steps, ready for following reactions. Coupling of the intermediate **15** and alendronic acid **1** using standard reagents, followed by side chain protecting groups removal by TFA in presence of scavengers, gave the final product **16** in 78% yield over three steps and after RP-HPLC purification (Scheme 4).

### Receptor binding assay

The derivatives **6**, **12**, and **16** were examined *in vitro* for their abilities to inhibit the binding of biotinylated vitronectin to the isolated, immobilized  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  integrin receptors (Table 1). Affinities of commercially available compound c(RGDfV) and our starting compound DB58 were determined as references, in the same assays. The functionalized cyclic pentapeptides **6**, **12**, and **16** displayed a binding affinity toward  $\alpha_v\beta_3$  integrin in the nanomolar range, comparable or slightly lower than unconjugated DB58.

Although some differences in the binding affinities, probably due to the different nature and length of the linkers, all the three synthesized compounds still interact well with integrin  $\alpha_v\beta_3$ , thus confirming that the functionalization of the scaffold does not influence the binding activity.

**Table 1.** Inhibition of biotinylated vitronectin binding to  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  receptors<sup>a</sup>

| Entry | Compound  | IC <sub>50</sub> (nM) $\pm$ SD for $\alpha_v\beta_3$ | IC <sub>50</sub> (nM) $\pm$ SD for $\alpha_v\beta_5$ |
|-------|-----------|--|--|
| 1     | c(RGDfV)  | 3.0 $\pm$ 1.6  | 17.4 $\pm$ 3.3                                       |
| 2     | DB58      | 22.6 $\pm$ 3.5                                       | 205 $\pm$ 33.5                                       |
| 3     | <b>6</b>  | 50.3 $\pm$ 13.4                                      | 475 $\pm$ 44   |
| 4     | <b>12</b> | 242.6 $\pm$ 62.9                                     | 1979 $\pm$ 835                                       |
| 5     | <b>16</b> | 20.5 $\pm$ 15.3                                      | 721 $\pm$ 99   |

<sup>a</sup> IC<sub>50</sub> values were calculated as the concentration of compound required for 50% inhibition of biotinylated vitronectin binding as estimated by GraphPad Prism 5 program. All values are the mean ( $\pm$  standard deviation) of triplicate determinations.

### Conclusions

In conclusion: we have synthesized three new conjugates between an integrin ligand and a methylene-bis-phosphonate anchor, choosing alendronic acid, to improve binding to the surface

of appropriate materials, such as titanium or hydroxyapatite. On the basis of the results obtained in the solid phase receptor binding assay, the conjugation of a biologically relevant entity on the appendage of the azabicycloalkane scaffold does not affect the affinity of the RGD ligand toward the integrin receptors.

Thus, these new conjugates could be used for biofunctionalization of materials in biomedical applications, and could find application in the development of modern implants. Biological evaluation *in vitro* and *in vivo* of these new compounds will be reported in due course.

## Experimental Section

**General.** All chemicals and solvents were of reagent grade and were used without further purification. Solvents were dried by standard procedures and reactions requiring anhydrous conditions were performed under nitrogen or argon atmosphere.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded at 300 K on a Bruker Avance-600 or Bruker Avance-400 spectrometer. Chemical shifts  $\delta$  for  $^1\text{H}$  and  $^{13}\text{C}$  are expressed in ppm relative to internal  $\text{Me}_4\text{Si}$  as standard.  $^{31}\text{P}$  NMR spectra were recorded on a 400 MHz spectrometer operating at 162 MHz, with complete proton decoupling.  $^{31}\text{P}$  NMR spectroscopic chemical shifts are reported in ppm ( $\delta$ ) relative to external 85%  $\text{H}_3\text{PO}_4$  at 0 ppm. Signals were abbreviated as s, singlet; bs, broad singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Mass spectra were obtained with Agilent 1100 analytical HPLC equipped with diode array detector and Bruker ion-trap Esquire 3000+ with ESI. Thin layer chromatography (TLC) was carried out with pre-coated Merck  $\text{F}_{254}$  silica gel plates. Elemental analyses were performed by the staff of the microanalytical laboratory of our department. Flash chromatography was carried out with Macherey-Nagel silica gel 60 (230-400 mesh) or using SP1 (single-column) Biotage flash purification system (with C18 cartridges). Preparative HPLC was performed using MS-based preparative HPLC system Waters. Semi-preparative HPLC was carried out on a Waters SymmetryPrep  $\text{C}_{18}$ -7 $\mu\text{m}$  7.8  $\times$  300 mm column or a Waters Atlantis  $\text{C}_{18}$  OBD 5 $\mu\text{m}$  19 mm  $\times$  10 cm.

**Synthesis of compound 5.** 4-Pentynoic acid (98.1 mg, 1.0 mmol) was dissolved in dry  $\text{CH}_2\text{Cl}_2$  (10 ml), and then *N*-hydroxysuccinimide (NHS) (115.09 mg, 1.0 mmol) and *N,N'*-diisopropylcarbodiimide (DIC) (387  $\mu\text{l}$ , 2.5 mmol) were sequentially added. The reaction mixture was stirred at room temperature overnight. After reaction completion, the solvent was removed under reduced pressure. The crude was left under high vacuum. The crude product was diluted in  $\text{CH}_3\text{CN}$  (20 ml) and the corresponding solution was slowly added to a solution of alendronic acid trihydrate (975 mg, 3 mmol) (solubilized with water (8 ml) and the pH of the corresponding solution was adjusted to pH 7.2-7.4 adding a 0.1 M water solution of NaOH, finally to the mixture was added  $\text{CH}_3\text{CN}$  (24 mL)) at 0  $^\circ\text{C}$  using a syringe pump (40  $\mu\text{l}/\text{min}$ ). The reaction mixtures was left under stirring 18 h at 0  $^\circ\text{C}$ . After reaction completion, the crude product was precipitated by addition of ethanol to obtain **5** as a dirty white solid (277 mg, 0.84



mmol, 70 %).  $^1\text{H}$  NMR (400 MHz,  $\text{D}_2\text{O}$ ):  $\delta$ : 1.85-1.75 (m, 2H), 1.95-1.85 (m, 2H), 2.34 (t,  $J$  2.0 Hz, 1H), 2.41 (m, 4H), 3.18 (t,  $J$  6.8 Hz, 2H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ): 174.6, 83.6, 70.3, 40.2, 34.6, 31.2, 23.4, 14.6. Anal. Calcd for  $\text{C}_9\text{H}_{17}\text{NO}_8\text{P}_2$  (329.04): C, 32.84; H, 5.21; N, 4.26%. Found: C, 32.88; H, 5.30; N, 4.26%.

**Synthesis of compound 6.** A 0.9 M water solution of sodium ascorbate (45  $\mu\text{l}$ , 0.4 mmol) and a 0.3 M water solution of  $\text{CuSO}_4$  (65  $\mu\text{l}$ , 0.02 mmol) were sequentially added to stirred solution of compound **5** (32.9 mg, 0.10 mmol) and compound **2** (56.4 mg, 0.10 mmol) in a 1:1 mixture of  $\text{H}_2\text{O}/t\text{-BuOH}$  (300  $\mu\text{l}$ ). The reaction mixture was stirred overnight at room temperature and then the solvent was removed under reduced pressure. Finally, the residue was purified by chromatography on a  $\text{C}_{18}$  reversed phase semi-preparative hplc column and then lyophilized. HPLC eluant conditions: from 98% of  $\text{H}_2\text{O}$  (0.1% TFA) and 2% of  $\text{CH}_3\text{CN}$  (0.1% TFA) to 70% of  $\text{H}_2\text{O}$  (0.1% TFA) and 30% of  $\text{CH}_3\text{CN}$  (0.1% TFA), flow rate 12 ml/min., 30 min. runs, giving pure **6** (71.4 mg, 0.08 mmol, 80%).

$^1\text{H}$  NMR (400 MHz,  $\text{D}_2\text{O}$  + 5% TFA):  $\delta$ : 1.12 (m, 1H,  $H\text{-}5$ ), 1.32-1.57 (m, 4H, 2  $H\gamma\text{-Arg}$ ,  $H\beta\text{-Arg}$ ,  $H\text{-}7$ ), 1.63 (m, 2H,  $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{C}(\text{OH})(\text{PO}_3\text{H}_2)_2$ ), 1.68-1.98 (m, 4H,  $H\text{-}8$ ,  $\text{CH}_2\text{CH}_2\text{-C}(\text{OH})(\text{PO}_3\text{H}_2)_2$ ,  $H\beta\text{-Arg}$ ), 2.22-2.40 (m, 3H,  $H\text{-}5$ ,  $H\text{-}7$ ,  $H\text{-}8$ ), 2.45-2.60 (m, 3H,  $\text{COCH}_2\text{CH}_2\text{-triazole}$ ,  $H\beta\text{-Asp}$ ), 2.91 (m, 1H,  $H\beta\text{-Asp}$ ), 2.93-3.10 (m, 6H,  $\text{COCH}_2\text{CH}_2\text{-triazole}$ , 2  $H\delta\text{-Arg}$ ,  $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{C}(\text{OH})(\text{PO}_3\text{H}_2)_2$ ), 3.25 (m, 1H,  $H\text{-}4$ ), 3.36 (d,  $J$  16 Hz, 1H,  $H\alpha\text{-Gly}$ ), 3.91 (m, 1H,  $H\text{-}6$ ), 4.10 (d,  $J$  16 Hz, 1H,  $H\alpha\text{-Gly}$ ), 4.15 (m, 1H,  $H\text{-}9$ ), 4.18-4.36 (m, 3H, 2  $H\text{-}10$ ,  $H\alpha\text{-Asp}$ ), 4.38-4.47 (m, 2H,  $H\text{-}3$ ,  $H\alpha\text{-Arg}$ ), 8.10 (s, 1H,  $H$  triazole);  $^{13}\text{C}$  NMR (100 MHz,  $\text{D}_2\text{O}$ ):  $\delta$ : 174.7, 174.0, 173.0, 171.7, 169.3, 156.8, 62.2, 55.8, 52.9, 51.6, 51.3, 42.4, 40.4, 39.7, 36.2, 35.2, 32.7, 32.2, 30.1, 27.0, 24.4, 21.1;  $^{31}\text{P}$ -NMR (161.9 MHz,  $\text{D}_2\text{O}$ ):  $\delta$ : 21.4. Anal. Calcd for  $\text{C}_{31}\text{H}_{50}\text{N}_{12}\text{O}_{15}\text{P}_2$  (892.30): C, 41.71; H, 5.65; N, 18.83%. Found: C, 41.75; H, 5.64; N, 18.84%; MS ( $\text{ESI}^+$ )  $m/z$ : 893.5 ( $\text{M}+\text{H}^+$ ), 447.3 ( $\text{M}+2\text{H}^+$ ).

**Synthesis of compound 8.** Compound **7** (11.02 g, 50.0 mmol) was dissolved in dry  $\text{CH}_2\text{Cl}_2$  (50 ml), and then TEA (209  $\mu\text{l}$ , 1.5 mmol) and  $\text{TrCl}$  (278.8 mg, 1.0 mmol) were sequentially added. The reaction mixture was stirred at room temperature 18 h. After reaction completion, the mixtures was washed with a saturated solution of  $\text{NaHCO}_3$  (3 x 20 ml). The organic layer was dried over  $\text{Na}_2\text{SO}_4$ , and then the solvent removed under reduced pressure. The crude product was purified by flash chromatography (Biotage<sup>TM</sup> eluant conditions: 1% of MeOH and 99% of  $\text{CH}_2\text{Cl}_2$  to 10% of MeOH and 90% of  $\text{CH}_2\text{Cl}_2$ ). Yield 85% (393 mg, 0.85 mmol) of pure compound. Anal. Calcd for  $\text{C}_{29}\text{H}_{38}\text{N}_2\text{O}_3$  (462.29): C, 75.29; H, 8.28; N, 6.06%. Found: C, 75.28; H, 8.30; N, 6.05%.

The compound (462.6 mg, 1.0 mmol) was dissolved in dry  $\text{CH}_2\text{Cl}_2$  and then pentynoic acid (147.2 mg, 1.5 mmol) and DIC (310  $\mu\text{l}$ , 2.0 mmol) were sequentially added. The reaction mixture was stirred at room temperature overnight. After reaction completion, the solvent was removed under reduced pressure and the crude product was purified by flash chromatography (Biotage<sup>TM</sup> eluant conditions: 1% of MeOH and 99% of  $\text{CH}_2\text{Cl}_2$  to 10% of MeOH and 90% of  $\text{CH}_2\text{Cl}_2$ ). Yield 65% (353 mg, 0.65 mmol) of pure **8**.

$^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$ : 1.71 (m, 4H,  $-\text{CH}_2\text{CH}_2\text{CH}_2-$ ), 1.88 (s, 1H,  $\text{C}\equiv\text{CH}$ ), 2.12 (bs, 2H,  $\text{Ph}_3\text{NHCH}_2-$ ), 2.27 (t,  $J$  7.2 Hz, 2H,  $-\text{CH}_2\text{C}\equiv\text{CH}$ ), 2.44 (m, 2H,  $-\text{CH}_2\text{CO}-$ ), 3.31 (dd,  $J$  12.0, 5.6 Hz, 2H,  $-\text{CH}_2\text{NHCO}-$ ), 3.6-3.35 (m, 12H,  $-\text{CH}_2-$ ), 6.30 (bs, 1H,  $-\text{NHCO}$ ), 7.10 (t,  $J$  7.2 Hz, 3H, Ph), 7.20-7.15 (m, 6H, Ph), 7.39 (d,  $J$  7.6 Hz, 6H, Ph);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$ : 170.8, 146.3, 128.7, 127.7, 126.2, 83.1, 70.6, 70.3, 70.3, 70.1, 70.0, 69.1, 41.0, 38.2, 38.0, 35.5, 35.4, 30.6, 28.7, 14.9. Anal. Calcd for  $\text{C}_{34}\text{H}_{42}\text{N}_2\text{O}_4$  (542.31): C, 75.25; H, 7.80; N, 5.16%. Found: C, 75.35; H, 7.79; N, 5.15%. MS ( $\text{ESI}^+$ )  $m/z$ : 543.4 ( $\text{M}+\text{H}^+$ ).

**Synthesis of compound 9.** Compound **8** (543 mg, 1.0 mmol) was treated with  $i\text{Pr}_3\text{SiH}$  (410  $\mu\text{l}$ , 2.0 mmol) and TFA (153  $\mu\text{l}$ , 2.0 ml). The reaction mixture was stirred at room temperature for 2-4 hours. After reaction completion, TFA was removed under reduced pressure and the crude product was diluted with water and the mixture was extracted with  $i\text{Pr}_2\text{O}$  (3 X 20 ml). Water was removed under reduced pressure and the crude product was used on the following step without any further purification.

The crude product was dissolved in DMF and then diglycolic anhydride (232 mg, 2.0 mmol) and pyridine (323  $\mu\text{l}$ , 4.0 mmol) were sequentially added. The reaction mixture was stirred at room temperature for 4 hours. After reaction completion, water was added to the reaction mixture. Finally, the solvent was removed under reduced pressure and the crude product was purified by flash chromatography on Biotage using a  $\text{C}_{18}$  reverse column (Biotage<sup>TM</sup> eluant conditions: 1% of  $\text{CH}_3\text{CN}$  and 99% of  $\text{H}_2\text{O}$  to 100% of  $\text{CH}_3\text{CN}$  and 0% of  $\text{H}_2\text{O}$ ). Yield 71% (295 mg, 0.71 mmol) of pure **9**.

$^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$ : 1.82 (m, 4H,  $-\text{CH}_2\text{CH}_2\text{CH}_2-$ ), 2.02 (m, 1H,  $\text{C}\equiv\text{CH}$ ), 2.46 (m, 2H,  $-\text{CH}_2\text{C}\equiv\text{CH}$ ), 2.54 (m, 2H,  $-\text{CH}_2\text{CO}$ ), 3.41 (m, 4H,  $-\text{CH}_2\text{NH}$ ), 3.67-3.55 (m, 12H,  $-\text{CH}_2-$ ), 4.11 (d,  $J$  3.6 Hz, 2H,  $-\text{CO}-\text{CH}_2\text{O}-$ ), 4.19 (d,  $J$  4 Hz, 2H,  $-\text{CO}-\text{CH}_2\text{O}-$ ), 6.77 (bs, 1H,  $-\text{NH}$ ), 7.64 (bs, 1H,  $-\text{NH}$ );  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$ : 172.0, 171.8, 169.7, 82.9, 71.7, 70.4, 70.3, 70.2, 70.0, 69.9, 69.5, 69.4, 69.3, 42.6, 38.2, 36.9, 35.3, 29.0, 28.7, 14.9. Anal. Calcd for  $\text{C}_{19}\text{H}_{32}\text{N}_2\text{O}_8$  (416.22): C, 54.80; H, 7.74; N, 6.73%. Found: C, 54.85; H, 7.75; N, 6.72%. MS ( $\text{ESI}^+$ )  $m/z$ : 417.3 ( $\text{M}+\text{H}^+$ ).

**Synthesis of compound 11.** A 0.9 M water solution of sodium ascorbate (45  $\mu\text{l}$ , 0.4 mmol) and a 0.3 M water solution of  $\text{CuSO}_4$  (65  $\mu\text{l}$ , 0.02 mmol) were sequentially added to stirred solution of compound **10** (83.2 mg, 0.10 mmol) and of the alkyne **9** (41.6 mg, 0.10 mmol) in a 1:1 mixture of  $\text{H}_2\text{O}/t\text{BuOH}$  (300  $\mu\text{l}$ ). The reaction mixture was stirred 18 h at room temperature and then the solvent was removed under reduced pressure. Finally, the residue was purified by Biotage<sup>TM</sup> flash chromatography on a  $\text{C}_{18}$  reverse column (Biotage<sup>TM</sup> eluant conditions: 1% of  $\text{CH}_3\text{CN}$  and 99% of  $\text{H}_2\text{O}$  to 100% of  $\text{CH}_3\text{CN}$  and 0% of  $\text{H}_2\text{O}$ ). Yield 65% (81.2 mg, 0.65 mmol).

Anal. Calcd for  $\text{C}_{55}\text{H}_{85}\text{N}_{13}\text{O}_{18}\text{S}$  (1247.59): C, 52.91; H, 6.86; N, 14.59%. Found: C, 52.95; H, 6.87; N, 14.61%. MS ( $\text{ESI}^+$ )  $m/z$ : 1249.1 ( $\text{M}+\text{H}^+$ ), 625.4 ( $\text{M}+2\text{H}^+$ ).

The above compound (81.2 mg, 0.065 mmol) was dissolved in dry  $\text{CH}_2\text{Cl}_2$  (1 ml), and then NHS (7.5 mg, 0.065 mmol) and DIC (25.2  $\mu\text{l}$ , 0.1625 mmol) were sequentially added. The reaction mixture was stirred at room temperature overnight. After reaction completion, the solvent was removed under reduced pressure. The crude was left under the high vacuum. The crude product

was diluted in CH<sub>3</sub>CN (2 ml) and the corresponding solution was slowly added to a solution of alendronate (63.4 mg, 0.195 mmol, was solubilized with 4 mL of water and the pH of the corresponding solution was adjusted to pH 7.2-7.4 adding a 0.1 M water solution of NaOH, finally to the mixture were added 12 mL of CH<sub>3</sub>CN) at 0 °C using a syringe pump (40 µl/min). The reaction mixtures was left under stirring over night at 0 °C. After reaction completion, the solvent was removed under reduced pressure and the crude product was purified by HPLC using a C<sub>18</sub> reverse column (HPLC eluant conditions: from 90% of H<sub>2</sub>O (0.2% TFA) and 10% of CH<sub>3</sub>CN (0.2% TFA) to 0% of H<sub>2</sub>O (0.2% TFA) and 100% of CH<sub>3</sub>CN (0.2% TFA), flow rate 12 ml/min., 30 min. runs). Yield 84% (80.8 mg, 0.0546 mmol) of pure protected compound.

<sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O): δ: 1.24 (m, 1H, *H*-5), 1.32-1.48 (m, 12H, 2 *H*<sub>γ</sub>-Arg, *H*<sub>β</sub>-Arg, COC(CH<sub>3</sub>)<sub>3</sub>), 1.58 (m, 1H, *H*-7), 1.64 (m, 2H, OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH), 1.71-1.87 (m, 6H, OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH, NHCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>C(OH)(PO<sub>3</sub>H<sub>2</sub>)<sub>2</sub>, *H*-8, *H*<sub>β</sub>-Arg), 1.97 (m, 2H, NHCH<sub>2</sub>CH<sub>2</sub>-CH<sub>2</sub>C(OH)(PO<sub>3</sub>H<sub>2</sub>)<sub>2</sub>), 2.06 (s, 3H, CH<sub>3</sub> Mtr), 2.25-2.38 (m, 3H, *H*-5, *H*-7, *H*-8), 2.50 (s, 3H, CH<sub>3</sub> Mtr), 2.53-2.64 (m, 6H, CH<sub>3</sub> Mtr, COCH<sub>2</sub>CH<sub>2</sub>-triazole, *H*<sub>β</sub>-Asp), 2.92 (dd, *J* 7.6 Hz, *J* 16.4 Hz, 1H, *H*<sub>β</sub>-Asp), 2.98 (m, 2H, COCH<sub>2</sub>CH<sub>2</sub>-triazole), 3.07-3.19 (m, 4H, 2 *H*<sub>δ</sub>-Arg, OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH), 3.19-3.23 (m, 5H, *H*-4, NHCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>C(OH)(PO<sub>3</sub>H<sub>2</sub>)<sub>2</sub>, OCH<sub>2</sub>CH<sub>2</sub>-CH<sub>2</sub>NH), 3.43 (t, *J* 6.2 Hz, 2H, OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH), 3.48 (m, 1H, *H*<sub>α</sub>-Gly), 3.51 (t, *J* 6.2 Hz, 2H, OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH), 3.54-3.66 (m, 8H, OCH<sub>2</sub>), 3.81 (s, 3H, OCH<sub>3</sub> Mtr), 3.95 (m, 1H, *H*-6), 4.05 (s, 2H, OCH<sub>2</sub>CO), 4.06 (s, 2H, OCH<sub>2</sub>CO), 4.12-4.17 (m, 3H, *H*-9, *H*-10, *H*<sub>α</sub>-Gly), 4.32-4.43 (m, 4H, *H*-10, *H*<sub>α</sub>-Asp, *H*-3, *H*<sub>α</sub>-Arg), 6.75 (s, 1H, *H* Mtr), 7.85 (s, 1H, *H* triazole); <sup>13</sup>C NMR (100 MHz, D<sub>2</sub>O): δ: 174.7, 173.7, 172.0, 171.6, 169.2, 112.8, 83.1, 70.0, 69.6, 69.4, 68.6, 68.4, 62.2, 55.8, 52.9, 52.1, 51.3, 42.5, 39.4, 36.4, 36.3, 36.2, 34.9, 34.7, 32.7, 32.3, 30.8, 29.7, 28.3, 27.3, 23.3, 23.1, 20.7, 11.4. Anal. Calcd for C<sub>59</sub>H<sub>96</sub>N<sub>14</sub>O<sub>24</sub>P<sub>2</sub>S (1478.59): C, 47.90; H, 6.54; N, 13.25%. Found: C, 47.87; H, 6.55; N, 13.28%; MS (ESI<sup>+</sup>) *m/z*: 1480.0 (M+H<sup>+</sup>), 741.0 (M+2H<sup>+</sup>).

**Synthesis of compound 12.** A mixture composed of TFA (5 ml), 5% of thianisole (250 µl) 5% triisopropylsilane (250 µL), 5% ethandiol, 5% Phenol (275 mg) and 5% water (250 µL) was added to a stirred solution of compound **11** (80.8 mg, 0.0546 mmol). The reaction mixtures were left stirring at room temperature overnight and then concentrated under reduced pressure. The crude product was solubilized with water (10 ml) and washed with EtOAc (3 X 10 ml), Et<sub>2</sub>O (3 X 10 ml), *i*Pr<sub>2</sub>O (3 × 10 ml), hexane (3 × 10 ml), DCM (3 × 10 ml), CHCl<sub>3</sub> (3 × 10 ml), *i*Pr<sub>2</sub>O (3 × 10 ml). Finally, water was removed under reduced pressure. When the crude was not sufficiently pure, the residue was purified by chromatography on a C<sub>18</sub> reversed phase semi-preparative hplc column and then lyophilized. HPLC eluant conditions: from 98% of H<sub>2</sub>O (0.1% TFA) and 2% of CH<sub>3</sub>CN (0.1% TFA) to 70% of H<sub>2</sub>O (0.1% TFA) and 30% of CH<sub>3</sub>CN (0.1% TFA), flow rate 12 ml/min., 30 min. runs. Yield 84% (55.5 mg, 0.0459 mmol) of pure **12**.

<sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O): δ: 1.26 (m, 1H, *H*-5), 1.42-1.60 (m, 4H, 2 *H*<sub>γ</sub>-Arg, *H*<sub>β</sub>-Arg, *H*-7), 1.64 (m, 2H, OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH), 1.70-1.88 (m, 5H, OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH, NHCH<sub>2</sub>CH<sub>2</sub>-CH<sub>2</sub>-C(OH)(PO<sub>3</sub>H<sub>2</sub>)<sub>2</sub>, *H*-8), 1.90-2.08 (m, 3H, NHCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>C(OH)(PO<sub>3</sub>H<sub>2</sub>)<sub>2</sub>, *H*<sub>β</sub>-Arg), 2.27-2.47 (m, 3H, *H*-5, *H*-7, *H*-8), 2.54 (m, 2H, COCH<sub>2</sub>CH<sub>2</sub>-triazole), 2.66 (dd, *J* 17.2 Hz, *J* 6.8 Hz, 1H, *H*<sub>β</sub>-Asp), 2.95 (m, 2H, COCH<sub>2</sub>CH<sub>2</sub>-triazole), 3.02, (dd, *J* 17.2 Hz, *J* 7.6 Hz, 1H, *H*<sub>β</sub>-Asp), 3.07-

3.18 (m, 4H, 2  $H\delta$ -Arg,  $OCH_2CH_2CH_2NH$ ), 3.18-3.30 (m, 5H,  $H$ -4,  $NHCH_2CH_2CH_2C(OH)(PO_3H_2)_2$ ,  $OCH_2CH_2CH_2NH$ ), 3.40 (t,  $J$  6.2 Hz, 2H,  $OCH_2CH_2CH_2NH$ ), 3.47 (m, 1H,  $H\alpha$ -Gly), 3.51 (t,  $J$  6.2 Hz, 2H,  $OCH_2CH_2CH_2NH$ ), 3.54-3.66 (m, 8H,  $OCH_2$ ), 4.00 (m, 1H,  $H$ -6), 4.05 (s, 2H,  $OCH_2CO$ ), 4.06 (s, 2H,  $OCH_2CO$ ), 4.14-4.28 (m, 3H,  $H$ -9,  $H$ -10,  $H\alpha$ -Gly), 4.33-4.47 (m, 3H,  $H$ -10,  $H\alpha$ -Asp,  $H$ -3), 4.54 (m, 1H,  $H\alpha$ -Arg), 7.81 (s, 1H,  $H$  triazole);  $^{13}C$  NMR (100 MHz,  $D_2O$ ):  $\delta$ : 174.7, 174.6, 174.5, 173.9, 173.0, 171.6, 169.2, 156.8, 124.5, 70.0, 69.6, 69.4, 68.5, 68.3, 62.2, 55.8, 52.9, 51.8, 51.7, 51.3, 42.4, 40.4, 39.4, 36.4, 36.3, 36.2, 34.9, 33.3, 32.7, 32.3, 30.9, 30.1, 28.3, 27.0, 24.4, 23.3, 20.8;  $^{31}P$ -NMR (161.9 MHz,  $D_2O$ ):  $\delta$ : 20.4. Anal. Calcd for  $C_{45}H_{76}N_{14}O_{21}P_2$  (1210.48): C, 44.63; H, 6.33; N, 16.19%. Found: C, 44.68; H, 6.34; N, 16.24%; MS (ESI $^+$ )  $m/z$  1211.7 ( $M+H^+$ ), 606.7 ( $M+2H^+$ ).

**Synthesis of 15.** Compound **13** (34.1 mg, 0.1 mmol) was dissolved in dry  $CH_2Cl_2$  (1 ml), and then EDC (23 mg, 0.12 mmol), HOBt (16.2 mg, 0.12 mmol) and DIPEA (70  $\mu$ l, 0.4 mmol) were sequentially added. The reaction mixture was stirred at room temperature for 15 min. before adding the compound **12** (100.7 mg, 0.125 mmol). The reaction mixture was stirred at room temperature overnight, and then the solvent was removed under reduced pressure, and the residue was purified by Biotage<sup>TM</sup> flash chromatography on a  $C_{18}$  reverse column (Biotage<sup>TM</sup> eluant conditions: 1% of  $CH_3CN$  and 99% of  $H_2O$  to 100% of  $CH_3CN$  and 0% of  $H_2O$ ). Yield 74% (83.6 mg, 0.074 mmol).

$^1H$  NMR (400 MHz,  $CDCl_3$ ):  $\delta$ : 8.13 (bs, 1H), 7.69 (bs, 1H), 7.27-7.15 (m, 5H), 7.00 (bs, 1H), 6.45 (s, 1H), 6.32 (m, 1H), 5.57 (m, 1H), 5.02 (m, 2H), 4.57 (m, 1H), 4.38 (m, 2H), 4.19 (m, 2H), 4.13 (m, 2H), 3.75 (s, 3H), 3.57 (m, 6H), 3.44 (m, 2H), 3.41 (m, 1H), 3.31 (m, 2H), 3.26 (m, 1H), 3.12 (m, 2H), 2.90 (m, 1H), 2.60 (s, 3H), 2.52 (s, 3H), 2.48 (m, 1H); 2.32 (m, 3H), 2.04 (s, 9H), 1.87 (m, 3H), 1.70-1.45 (m, 4H), 1.35 (s, 9H), 1.15 (m, 1H);  $^{13}C$  NMR (100 MHz,  $CDCl_3$ ):  $\delta$ : 173.2, 171.4, 170.6, 169.8, 136.6, 128.5, 128.1, 111.8, 83.1, 70.9, 70.5, 70.3, 70.0, 66.7, 62.5, 55.7, 55.5, 40.8, 36.2, 34.9, 33.5, 30.4, 28.3, 28.0, 24.1, 18.3, 12.0. Anal. Calcd for  $C_{52}H_{76}N_{10}O_{16}S$  (1128.52): C, 55.31; H, 6.78; N, 12.40%. Found: C, 55.40; H, 6.77; N, 12.41%.

Catalytic amount of 10% Pd-C was added to solutions of the above compounds (56.5 mg, 0.05 mmol) in MeOH (3 ml). The resulting mixture was stirred at room temperature for ca. 2 hours under hydrogen (1 atm). After reaction completion, the mixture was filtered through a Celite pad, and then washed with MeOH ( $3 \times 1$  ml). The combined organic solution was concentrated under reduced pressure. The crude product was dissolved in DMF dry (1 mL) and then diglycolic anhydride (23.2 mg, 0.2 mmol) and pyridine (32  $\mu$ l, 0.4 mmol) were sequentially added. The reaction mixture was stirred at room temperature for 4 hours. After reaction completion, water was added to the reaction mixture. Finally, the solvent was removed under reduced pressure and the crude product was purified by flash chromatography on Biotage using a  $C_{18}$  reverse column (Biotage<sup>TM</sup> eluant conditions: 1% of  $CH_3CN$  and 99% of  $H_2O$  to 100% of  $CH_3CN$  and 0% of  $H_2O$ ). Yield 70% (38.9 mg, 0.035 mmol) of pure **15**.

$^1H$  NMR (400 MHz,  $CDCl_3$ ):  $\delta$ : 1.27 (m, 1H,  $H$ -5), 1.43 (s, 9H,  $COC(CH_3)_3$ ), 1.43-1.68 (m, 4H, 2  $H\gamma$ -Arg,  $H\beta$ -Arg,  $H$ -7), 1.79-1.97 (m, 2H,  $H$ -8,  $H\beta$ -Arg), 2.05 (s, 3H,  $CH_3$  Mtr), 2.20-2.40 (m, 3H,  $H$ -5,  $H$ -7,  $H$ -8), 2.40-2.58 (m, 4H,  $CH_3$  Mtr,  $H\beta$ -Asp), 2.60 (s, 3H,  $CH_3$  Mtr), 2.75 (m, 1H,

*H*-4), 2.92 (dd, *J* 6.6 Hz, *J* 16.6 Hz, 1H, *H* $\beta$ -Asp), 3.00-3.12 (m, 2H, *H*-10, *H* $\delta$ -Arg), 3.18 (m, 1H, *H* $\delta$ -Arg) 3.27 (m, 1H, *H*-10), 3.33-3.48 (m, 3H, OCH<sub>2</sub>CH<sub>2</sub>NH, *H* $\alpha$ -Gly), 3.52 (t, *J* 4.8 Hz, 2H, OCH<sub>2</sub>CH<sub>2</sub>NH), 3.54-3.69 (m, 8H, OCH<sub>2</sub>), 3.75 (s, 3H, OCH<sub>3</sub> Mtr), 3.92 (s, 2H, OCH<sub>2</sub>CO), 3.98-4.09 (m, 3H, *H*-6, OCH<sub>2</sub>CO), 4.04 (s, 2H, OCH<sub>2</sub>CO), 4.11-4.24 (m, 2H, *H*-9, *H* $\alpha$ -Gly), 4.39 (m, 1H, *H*-3), 4.49 (m, 1H, *H* $\alpha$ -Asp), 4.55 (m, 1H, *H* $\alpha$ -Arg), 6.10-6.41 (m, 3H, NH guanidinium), 6.45 (s, 1H, *H* Mtr), 7.15 (m, 1H, NHCH<sub>2</sub>azabicycloalkane), 7.30 (m, 1H, NHazabicycloalkane), 7.41 (m, 1H, NH-Arg), 7.60 (m, 1H, NH linker), 7.74 (m, 1H, NH-Gly), 8.29 (m, 1H, NH-Asp); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):  $\delta$ : 173.2, 171.5, 170.6, 169.8, 136.6, 111.8, 83.1, 71.2, 70.9, 70.4, 70.3, 70.2, 70.0, 69.6, 69.0, 62.4, 55.7, 55.5, 38.8, 36.0, 34.9, 33.5, 30.4, 28.3, 24.1, 18.3, 12.0. Anal. Calcd for C<sub>48</sub>H<sub>74</sub>N<sub>10</sub>O<sub>18</sub>S (1110.49): C, 51.88; H, 6.71; N, 12.60%. Found: C, 51.87; H, 6.72; N, 12.61%. MS (ESI<sup>+</sup>) *m/z* 1111.9 (M+H<sup>+</sup>).

**Synthesis of 16.** Compound **15** (111.1 mg, 0.1 mmol) was dissolved in dry CH<sub>2</sub>Cl<sub>2</sub> (1 ml), and then NHS (11.5 mg, 0.1 mmol) and DIC (39  $\mu$ l, 0.25 mmol) were sequentially added. The reaction mixture was stirred at room temperature 18 h. After reaction completion, the solvent removed under reduced pressure. The crude was left under the high vacuum overnight. The crude product was diluted in CH<sub>3</sub>CN (2 ml) and the corresponding solution was slowly added to a solution of alendronate **1** (97 mg, 0.3 mmol, was solubilized with water (4 ml) and the pH of the corresponding solution was adjusted to pH 7.2-7.4 adding a 0.1 M water solution of NaOH, finally to the mixture was added CH<sub>3</sub>CN (12 mL)) at 0 °C using a syringe pump (40  $\mu$ l/min). The reaction mixtures was left under stirring over night at 0 °C. After reaction completion, the solvent was removed under reduced pressure and the crude product was purified by HPLC using a C<sub>18</sub> reverse column. HPLC eluant conditions: from 90% of H<sub>2</sub>O (0.2% TFA) and 10% of CH<sub>3</sub>CN (0.2% TFA) to 0% of H<sub>2</sub>O (0.2% TFA) and 100% of CH<sub>3</sub>CN (0.2% TFA), flow rate 12 ml/min., 30 min. runs. Yield 84 % (124 mg, 0.084 mmol) of pure protected compound.

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$ : 7.65 (bs, 1H), 6.72 (s, 1H), 4.46 (t, *J* 7.2 Hz, 1H), 4.33 (m, 2H), 4.18 (m, 2H), 4.04 (d, *J* 4.4 Hz, 4H), 3.96 (s, 2H), 3.93 (m, 1H), 3.78 (s, 3H), 3.64 (d, *J* 6.0 Hz, 8H), 3.59 (t, *J* 5.2 Hz, 2H), 3.41 (m, 3H), 3.20 (t, *J* 6.8 Hz, 3H), 3.08 (m, 3H), 2.90 (m, 1H), 2.74 (m, 1H), 2.56 (s, 3H), 2.47 (s, 3H), 2.47-2.25 (m, 3H), 2.02 (s, 3H), 1.95 (m, 2H), 1.90-1.55 (m, 5H), 1.38 (s, 9H), 1.22 (m, 2H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):  $\delta$ : 174.8, 174.5, 173.7, 172.8, 172.0, 171.8, 171.6, 171.5, 170.1, 112.7, 83.1, 70.4, 70.0, 69.7, 69.6, 69.4, 68.8, 62.1, 55.9, 55.7, 52.9, 51.5, 42.5, 40.0, 39.3, 38.6, 35.7, 34.8, 33.3, 32.9, 32.5, 30.9, 30.2, 29.7, 27.3, 26.8, 23.3, 23.1, 17.6, 11.4.

A mixture composed of TFA (5 ml), 5% of thioanisole (250  $\mu$ l) 5% triisopropylsilane (250  $\mu$ L), 5% ethandiol, 5% Phenol (275 mg) and 5% water (250  $\mu$ L) was added to a stirred solution of protected compound (0.084 mmol). The reaction mixtures were left stirring at room temperature overnight and then concentrated under reduced pressure. The crude product was solubilized with water (2 ml) and washed with EtOAc (3  $\times$  2 ml), Et<sub>2</sub>O (3  $\times$  2 ml), *i*Pr<sub>2</sub>O (3  $\times$  2 ml), hexane (3  $\times$  2 ml), DCM (3  $\times$  2 ml), CHCl<sub>3</sub> (3  $\times$  2 ml), *i*Pr<sub>2</sub>O (3  $\times$  2 ml). Finally, water was removed under reduced pressure. When the crude was not sufficiently pure, the residue was purified by chromatography on a C<sub>18</sub> reversed phase semi-preparative hplc column and then lyophilized.

HPLC eluant conditions: from 98% of H<sub>2</sub>O (0.1% TFA) and 2% of CH<sub>3</sub>CN (0.1% TFA) to 70% of H<sub>2</sub>O (0.1% TFA) and 30% of CH<sub>3</sub>CN (0.1% TFA), flow rate 12 ml/min., 30 min. runs. Yield 93 % (83.8 mg, 0.078 mmol) of pure **16**.

<sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O):  $\delta$ : 1.26 (m, 1H, *H*-5), 1.42-1.70 (m, 4H, 2 *H* $\gamma$ -Arg, *H* $\beta$ -Arg, *H*-7), 1.73-1.89 (m, 3H, NHCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>C(OH)(PO<sub>3</sub>H<sub>2</sub>)<sub>2</sub>, *H*-8), 1.90-2.10 (m, 3H, NHCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>C(OH)(PO<sub>3</sub>H<sub>2</sub>)<sub>2</sub>, *H* $\beta$ -Arg), 2.34-2.51 (m, 3H, *H*-5, *H*-7, *H*-8), 2.69 (dd, *J* 17.0 Hz, *J* 7.0 Hz, 1H, *H* $\beta$ -Asp), 2.78 (m, 1H, *H*-4), 3.00-3.30, (m, 7H, *H* $\beta$ -Asp, 2 *H* $\delta$ -Arg, 2 *H*-10, NHCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>C(OH)(PO<sub>3</sub>H<sub>2</sub>)<sub>2</sub>), 3.37-3.53 (m, 3H, OCH<sub>2</sub>CH<sub>2</sub>NH, *H* $\alpha$ -Gly), 3.60 (m, 3H, OCH<sub>2</sub>CH<sub>2</sub>NH), 3.63-3.74 (m, 8H, OCH<sub>2</sub>), 3.92-4.04 (m, 3H, OCH<sub>2</sub>CO, *H*-6), 4.07 (s, 4H, OCH<sub>2</sub>CO), 4.20-4.32 (m, 2H, *H*-9, *H* $\alpha$ -Gly), 4.36 (d, *J* 8.4 Hz, 1H, *H*-3), 4.48 (t, *J* 7.0 Hz, 1H, *H* $\alpha$ -Asp), 4.55 (m, 1H, *H* $\alpha$ -Arg); <sup>13</sup>C NMR (100 MHz, D<sub>2</sub>O):  $\delta$ : 174.8, 174.5, 174.0, 173.0, 172.8, 171.9, 171.6, 170.2, 156.4, 70.3, 70.0, 69.7, 69.6, 69.4, 68.8, 62.2, 56.0, 53.0, 51.7, 51.5, 42.5, 40.4, 40.0, 39.4, 38.6, 35.7, 33.3, 32.9, 32.5, 30.8, 30.2, 26.9, 24.5, 23.3; <sup>31</sup>P-NMR (161.9 MHz, D<sub>2</sub>O):  $\delta$ : 20.7. Anal. Calcd for C<sub>38</sub>H<sub>65</sub>N<sub>11</sub>O<sub>21</sub>P<sub>2</sub> (1073.38): C, 42.50; H, 6.10; N, 14.35%. Found: C, 42.60; H, 6.11; N, 14.38%. MS (ESI<sup>+</sup>) *m/z*: 1074.6 (M+H<sup>+</sup>).

## Biology

### Solid-phase receptor binding assay

Purified  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  receptors (Chemicon International, Inc., Temecula, CA, USA) were diluted to 0.5  $\mu$ g/mL in coating buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM MnCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, and 1 mM MgCl<sub>2</sub>. An aliquot of diluted receptors (100  $\mu$ L/well) was added to 96-well microtiter plates (NUNC MW 96F Medisorp Straight) and incubated overnight at 4°C. The plates were then incubated with blocking solution (coating buffer plus 1% bovine serum albumin) for an additional 2 h at room temperature to block nonspecific binding, followed by 3 h incubation at room temperature with various concentrations (10<sup>-5</sup>–10<sup>-12</sup> M) of test compounds in the presence of biotinylated vitronectin (1  $\mu$ g/mL). Biotinylation was performed using an EZ-Link Sulfo-NHS-Biotinylation kit (Pierce, Rockford, IL, USA). After washing, the plates were incubated for 1 h at room temperature with biotinylated streptavidin–peroxidase complex (Amersham Biosciences, Uppsala, Sweden) followed by 30 min incubation with 100  $\mu$ L/well Substrate Reagent Solution (R&D Systems, Minneapolis, MN) before stopping the reaction with the addition of 50  $\mu$ L/well 2N H<sub>2</sub>SO<sub>4</sub>. Absorbance at 415 nm was read in a Synergy<sup>TM</sup> HT Multi-Detection Microplate Reader (BioTek Instruments, Inc.). Each data point represents the average of triplicate wells; data analysis was carried out by nonlinear regression analysis with GraphPad Prism software. Each experiment was repeated in triplicate.

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