Preparation and Evaluation of Glycosylated Arginine–Glycine–Aspartate (RGD) Derivatives for Integrin Targeting

Brian H. M. Kuijpers,[§] Stan Groothuys,[§] Annemieke C. Soede,[†] Peter Laverman,[†] Otto C. Boerman,[†] Floris L. van Delft,[§] and Floris P. J. T. Rutjes*[,§]

Synthetic Organic Chemistry, Institute for Molecules and Materials, Radboud University Nijmegen, Toernooiveld 1, 6525 ED, Nijmegen, The Netherlands, and Department of Nuclear Medicine, Radboud University Nijmegen Medical Center, P.O. Box 9101, HB Nijmegen 6500, The Netherlands. Received April 27, 2007; Revised Manuscript Received August 9, 2007

Arginine–glycine–aspartate (RGD) derivatives were prepared by a combination of solid-phase and solution-phase synthesis for selective targeting of $\alpha_v\beta_3$ integrin expressed in tumors. In order to evaluate the value of a triazole moiety as a proposed amide isostere, the side chain glycosylated cyclic RGD (*c*RGD) peptides were synthesized with either a natural amide linkage or a triazole. Affinity of the *c*RGD constructs for the $\alpha_v\beta_3$ integrin was determined in a solid-phase competitive binding assay, showing strong similarity in binding affinity for each of the compounds under evaluation. Furthermore, the in vivo tumor targeting potential of glycosylated *c*RGD peptides, linked via amide or triazole, was investigated by determining the biodistribution of ^{125}I-labeled derivatives in mice with tumors expressing $\alpha_v\beta_3$. All of the cyclic RGD derivatives showed preferential uptake in the subcutaneous tumors, with the highest tumor-to-blood ratio measured for the triazole-linked glycosylated derivative. The results of the present study are a clear indication of the value of the triazole moiety as a suitable amide isostere in the development of glycosylated peptides as pharmaceuticals.

INTRODUCTION

In a recent communication, we showed (*1*) that the Cu(I)-catalyzed [3 + 2] cycloaddition (*2, 3*) of an organic azide and an acetylene, one of the most reliable click reactions (*4*), could be used for the synthesis of triazole-linked glycoamino acids. The incorporation of a triazole as a bridging moiety in a glycoamino acid is of particular interest because the triazole function has been postulated as a useful amide isostere (*4, 5*) in terms of electronic properties and atom placement, and the resulting cyclic triazolyl RGD peptides may therefore be of value for a variety of biological or medicinal applications. Thus, it was demonstrated by us, and by others (*6*) that subjection of 1-azidosugars with acetylene-containing amino acids to the standard protocol for copper-catalyzed Huisgen addition leads to triazolylglycoamino acids, with a nitrogen-linked sugar (^{N}TGA), in high yields (Figure 1). Similarly, application of the procedure to 1-acetylenosugar and azide-containing amino acid afforded the corresponding carbon-linked triazole-linked glycoamino acids (^{C}TGA).

The applicability of the triazole-linked glycoamino acids for the preparation of glycopeptides isosteres was inter alia demonstrated by us by incorporation of TGAs into a range of glycopeptides either via standard solution-phase peptide coupling protocols or under the influence of alcalase (*7*). In addition we have shown the possibility of preparing 5-bromo-substituted triazolylglycoamino acids by applying a modified procedure of the Cu(I)-catalyzed Huisgen cycloaddition to azides and bromoacetylenes (*8*).

After suitable technology for the preparation of stable glycopeptides isosteres was established, the present study aimed to investigate the biological relevance of a triazole function as an amide isostere. Some precedence for the isosteric value of a triazole has been provided for true peptide analogues (*4, 5, 9*),

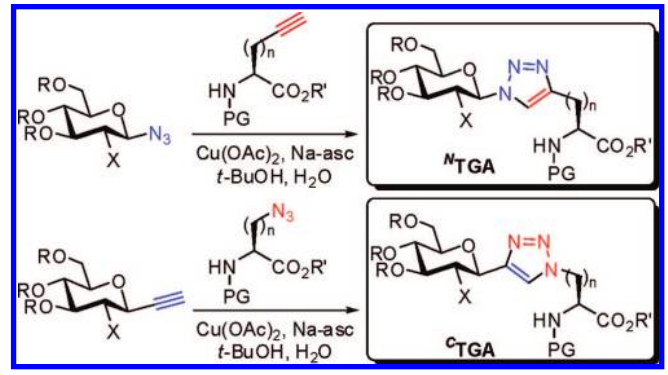


Figure 1. Synthesis of triazolyl glycoamino acids ^{N}TGA and ^{C}TGA.

but the synthesis of triazole-linked glycopeptides with relevant biological activity has hitherto not been reported. Moreover, little was known about the chemical stability of a glycosyl triazole linkage under basic or acidic conditions, with the exception of typical carbohydrate chemistry conditions (*10*). We became intrigued by the covalent attachment of carbohydrates to the so-called arginine–glycine–aspartate (RGD) peptides (*11*), found in proteins of the extracellular matrix such as vitronectin, fibrinogen, and laminin. The RGD motif is specifically recognized by heterodimeric transmembrane proteins called integrins. Integrins link the intracellular cytoskeleton with the extracellular matrix, thereby playing an important role in cell-signaling, cell–cell adhesion, apoptosis, and cell–matrix interactions. More specifically, the $\alpha_v\beta_3$ integrin is expressed on endothelial cells and modulates cell migration and survival during angiogenesis, while the $\alpha_v\beta_3$ integrin expressed on carcinoma cells potentiate metastasis by facilitating extravasation and tissue invasion. As a consequence, the integrin RGD binding site is an attractive pharmaceutical target (*12*) and a large number of RGD analogues have been prepared over the years. For example, Kessler et al. (*13*) showed that cyclo[Arg-Gly-Asp-D-Phe-Val] is one of the most active and selective antagonists for the $\alpha_v\beta_3$

* E-mail: F.Rutjes@science.ru.nl.
[§] Radboud University Nijmegen.
[†] Radboud University Nijmegen Medical Center.

10.1021/bc700154u CCC: $37.00
Published on Web 10/09/2007

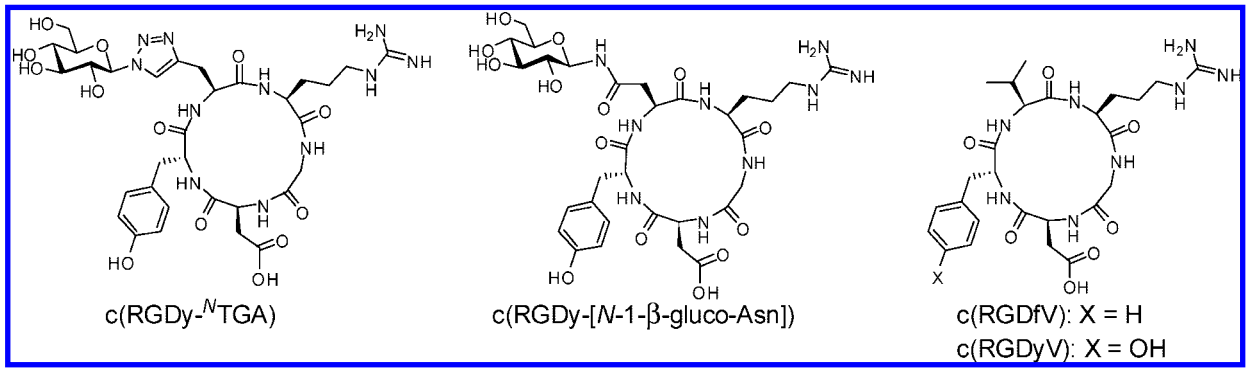
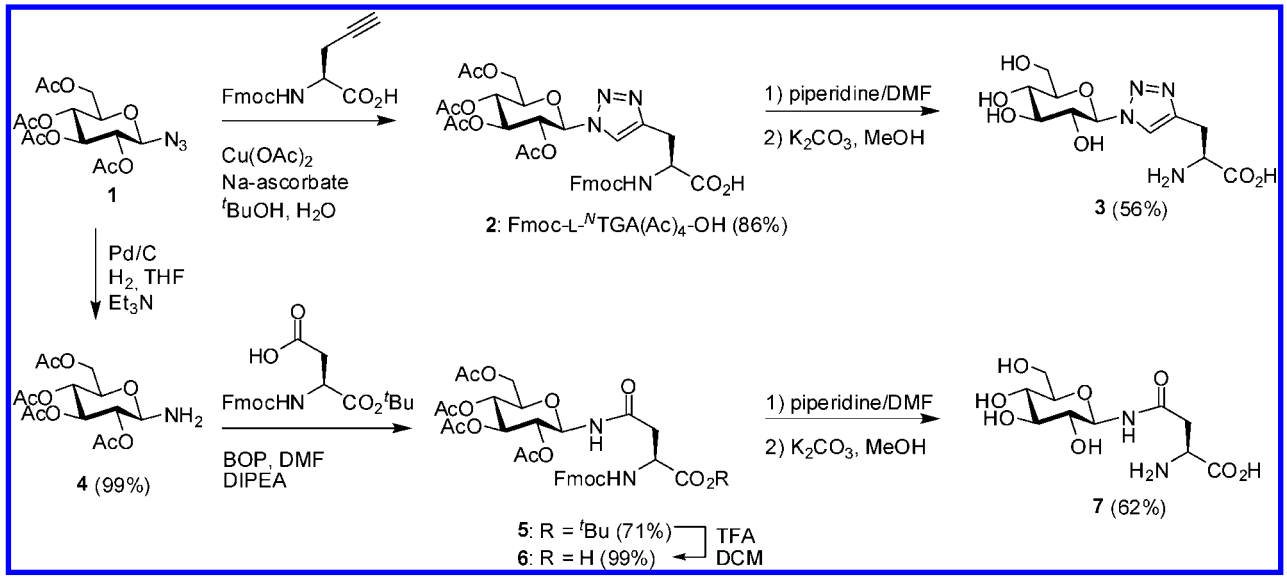


Figure 2. Cyclic RGD peptides for integrin targeting.

Scheme 1. Synthesis of Glycosylated Amino Acids



integrin. Later, it was shown that glycosylation of the side chains of the cyclic RGD (*c*RGD) peptides led to significant improvement of the pharmacokinetic properties of lipophilic peptides (*11, 14*). Various glycosylated *c*RGD peptides were prepared to elucidate the influence of various substitutions on the biological properties (*15*). On the basis of these studies, we hypothesized that triazole isosteres of such cyclic glyco-RGD peptides would display a comparable pharmaceutical profile. Here, we studied the stability of *N*-triazolylglycoamino acids and the preparation of a representative set of "normal" *c*RGD peptides as well as two glycosylated *c*RGD derivatives (Figure 2) synthesized by a combination of solid phase and solution phase techniques. The *in vitro* and *in vivo* $\alpha_v\beta_3$ binding characteristics of these compounds were investigated.

MATERIALS AND METHODS/EXPERIMENTAL PROCEDURES

All chemicals were used as supplied without further purification. The trityl chloride polystyrene (TCP) resin as well as the 9-fluorenylmethoxycarbonyl (Fmoc) amino acids were purchased from NovaBioChem (Bad Soden, Germany). 1-Hydroxybenzotriazole (HOBt) and diisopropylcarbodiimide (DIPCDI) were purchased from Aldrich (Steinheim, Germany). L-Propargylglycine was purchased from Chiralix (Nijmegen, The Netherlands). All other reagents were purchased from Acros (Geel, Belgium). Low-resolution mass spectra (LRMS) were obtained using a Thermo Scientific Advantage LCQ linear iontrap electrospray mass spectrometer (ESI-MS), and high-resolution mass spectra (HRMS) using electrospray ionization time-of-flight (ESI-TOF) were obtained using a JEOL AccuTOF mass spectometer. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Varian Inova 400 (400 MHz) or a Bruker DMX300 (300 MHz) spectrometer. The chemical shifts (δ) are given in ppm downfield from tetramethylsilane. Analytical reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on a Merck Hitachi HPLC using an analytical reversed-phase column (Alltech Adsorbosphere C18, 300 Å, 5 μm, 4.6 mm × 250 mm) and a Merck Hitachi (L-4000) UV detector operating at 215 nm. Elution was effected using an appropriate gradient from 0.1% trifluoroacetic acid (TFA) in H_2O/CH_3CN (95/5, v/v) to 0.1% TFA in CH_3CN/H_2O (95/5, v/v). Semipreparative HPLC was performed using an Alltech Adsorbosphere XL C18 column (250 mm × 10 mm, pore size of 100 Å, particle size of 10 μm) at a flow rate of 5.0 mL/min.

Synthesis of Sugar Derivatives (Scheme 1). *2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosylazide (**1**).* The glucopyranosylazide was prepared according to a literature procedure (*16*).

*2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosylamine (**4**).* The glucopyranosylamine was prepared according to a literature procedure (*17*) with slight modification. In an oven-dried Schlenck flask filled with N_2, the azidoglucose **1** (500 mg, 1.3 mmol), Et_3N (140 mg, 1.4 mmol), and Pd/C (14 mg, 0.1 mmol) were suspended in THF (3 mL). Next the Schlenk flask was subjected to a sequence of vacuum and flushing with H_2 (3×), after which it was vigorously stirred while H_2 was bubbled through the solution. After 30 min, the reaction was completed, the slurry was filtered over HyFlo, and THF was evaporated

under reduced pressure to yield **4** as a white solid (99%) sufficiently pure for further reactions.

Synthesis of Fmoc-Protected Glycosidic Amino Acids (Scheme 1). *Fmoc-L-^{N}TGA(Ac)$_4$-OH (2).* The triazole-linked sugar amino acid **2** was prepared according to a procedure reported earlier (*1*). To a solution of glycoside **1** (1 equiv) and Fmoc-protected L-propargylglycine (1 equiv) in *tert*-butanol (0.5 M) was added a solution of $Cu(OAc)_2$ (20 mol %) and sodium ascorbate (40 mol %) in H_2O (0.04 and 0.08 M, respectively). The mixture was stirred overnight, water was added, and the product was extracted with DCM (2×). The combined organic layers were washed with aqueous NaCl, dried over Na_2SO_4, and evaporated in vacuo. The product was purified by flash chromatography using EtOAc/heptane (2/1) to yield a white solid (86%). $R_f = 0.20$ (EtOAc/heptane, 2:1). IR (film): ν 3360, 2950, 2250, 1748, 1221 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (d, J = 7.6 Hz, 1H), 7.70 (s, 1H), 7.61–7.58 (m, 2H), 7.41–7.37 (m, 2H), 7.32–7.28 (m, 2H), 6.00 (d, J = 7.8 Hz, 1H), 5.86–5.83 (m, 1H), 5.48–5.35 (m, 2H), 5.31–5.19 (m, 1H), 4.80–4.67 (m, 1H), 4.38 (d, J = 7.2 Hz, 2H), 4.30 (ABdd, J = 12.6, 5.1 Hz, 1H), 4.23 (t, J = 7.2 Hz, 1H), 4.15–4.09 (m, 1H), 3.99 (ddd, J = 10.2, 4.9, 2.0 Hz, 1H), 3.45–3.30 (m, 2H), 2.10 (s, 3H), 2.07 (s, 3H), 2.02 (s, 3H), 1.84 (s, 3H). ^{13}C NMR (75 MHz, $CDCL_3$) δ 173.2, 170.7, 170.0, 169.8, 169.6, 156.2, 143.9, 143.6, 141.4, 127.9, 127.2, 125.3, 121.4, 120.1, 85.9, 75.4, 72.6, 70.6, 67.9, 67.4, 61.7, 53.4, 47.3, 27.9, 20.8, 20.7, 20.6, 20.2. HRMS (ESI) calculated for $C_{34}H_{37}N_4O_{13}$ $(M + H)^+$ 709.2344, found 709.2357.

^{N}TGA (3). Fmoc-L-^{N}TGA(Ac)$_4$-OH (**2**) (500 mg, 0.70 mmol) was treated with 20% piperidine in DMF (v/v, 5 mL) and stirred for 20 min before concentration of the solvent in vacuo. The crude product was dissolved in MeOH (5 mL), and a catalytic amount of K_2CO_3 was added. The mixture was stirred for 1 h. Purification of the product with a basic ion exchange column (IRA-410) afforded the triazole-linked glycosidic amino acid **3** (125 mg, 56%) as a white solid. IR (film): ν 3309, 2362, 2327, 1623 cm^{-1}. ^{1}H NMR (400 MHz, D_2O) δ 5.01 (d, J = 8.4 Hz, 1H), 4.04 (s, 1H), 3.89 (d, J = 12.2 Hz, 1H), 3.74 (dd, J = 13.0, 4.8 Hz, 1H), 3.46–3.39 (m, 2H), 3.44 (m, 2H), 3.14–2.80 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.3, 141.7, 123.3, 86.9, 78.4, 75.4, 71.7, 68.4, 59.9, 53.7, 25.7. HRMS (ESI) calculated for $C_{34}H_{37}N_4O_{13}$ $(M + H)^+$ 709.2357, found 709.2344.

Fmoc-(N-1-Glu(Ac)$_4$-Asn)-OH (6). The triazole-linked sugar amino acid was prepared from glucopyranosylamine **4** and Fmoc-Asp-O^{t}Bu via a procedure reported by van Ameijde et al. (*18*) involving peptide coupling catalyzed by BOP followed by deprotection using TFA/DCM (4 h, room temp) to afford the *N*-glycosylasparagine **6** as a white solid in 71% overall yield. $R_f = 0.12$ (EtOAc/heptane, 2:1). IR (film): ν 3313, 2250, 1740, 1506 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, J = 7.5 Hz, 2H), 7.58 (d, J = 7.4 Hz, 2H), 7.40 (t, J = 7.4 Hz, 2H), 7.31 (t, J = 7.4 Hz, 2H), 6.70 (d, J = 8.8 Hz, 1H), 6.16 (d, J = 7.6 Hz, 1H), 5.33 (t, J = 9.5 Hz, 1H), 5.26 (t, J = 9.1 Hz, 1H), 5.08 (t, J = 9.7 Hz, 2H), 4.94 (t, J = 9.5 Hz, 1H), 4.67–4.53 (m, 1H), 4.47–4.34 (m, 2H), 4.30 (dd, J = 12.6, 4.4 Hz, 1H), 4.22 (t, J =7.04 Hz, 1H), 4.08 (dd, J =12.6, 2.0 Hz, 1H), 3.82 (ddd, J =10.1, 4.1, 2.0 Hz, 1H), 2.90 (dd, J =16.3, 2.8 Hz, 1H), 2.76 (dd, J =16.3, 5.2 Hz, 1H), 2.06 (s, 5H), 2.04 (s, 3H), 2.02 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 176.1, 173.9, 171.4, 170.9, 170.2, 169.8, 156.6, 143.7, 141.3, 127.9, 127.2, 125.3, 120.1, 78.0, 73.8, 272.9, 70.8, 68.2, 67.6, 61.8, 50.5, 47.1, 37.7, 20.8, 20.7, 20.7, 20.6. HRMS (ESI) calculated for $C_{33}H_{36}N_2NaO_{14}$ $(M + Na)^+$ 707.2051, found 707.2064.

N-1-Gluco-Asn (7). Fmoc-(*N*-1-Glu(Ac)$_4$-Asn)-OH (**5**) (500 mg, 0.73 mmol) was treated with 20% piperidine in DMF (v/v, 5 mL) and stirred for 20 min before concentration of the solvent in vacuo. The crude product was dissolved in MeOH (5 mL), and a catalytic amount of K_2CO_3 was added. The mixture was stirred for 1 h. Purification of the product with a basic ion exchange column (IRA-410) afforded the amide-linked glycosidic aspargine **7** (133 mg, 62%) as a white solid. ^{1}H NMR (400 MHz, D_2O) δ 8.14 (s, 1H), 5.77 (d, J = 9.2 Hz, 1H), 4.10 (t, J = 5.0 Hz, 1H), 4.02 (t, J = 9.2 Hz, 1H), 3.93 (d, J = 11.4 Hz, 1H), 3.82–3.71 (m, 3H), 3.64 (t, J = 9.1 Hz, 1H), 3.43–3.34 (m, 1H). HRMS (ESI) calculated for $C_{33}H_{36}N_2O_{14}$ $(M + H)^+$ 707.2064, found 707.2051.

Table 1. Stability Tests for ^{N}TGA and *N*-1-β-Glyco-Asn, Respectively

			stability	
conditions	temperature	time	^{N}T GA (**3**)	*N*-1-β-glyco-Asn (**7**)
2 M HCl (aq)	room temp	3 d	++	++
2 M HCl (aq)	reflux	2 h	++	+
4 M HCl (aq)	reflux	3 h	++	− −
2 M NaOH (aq)	room temp	3 d	++	−
2 M NaOH(aq)	reflux	3 h	++	− −
2.6 M HCl/EtOAc	room temp	3 d	++	+
Et_3N	reflux	3 h	++	+

Stability Tests. ^{N}TGA (**3**) (50 mg, 0.07 mmol) or *N*-1-gluco-Asn (**7**) (50 mg, 0.07 mmol) was dissolved in 3 mL of 2 M HCl (aq), 4 M HCl (aq), 2 M NaOH (aq), 2.6 M HCl/EtOAc, or Et_3N and stirred for the time and temperature indicated in Table 1. The relative stability of the substrates was determined by TLC analysis and integration of the ^{1}H NMR signals of the side products and starting material.

General Procedures for the Preparation of the Cyclic RGD Peptides (Scheme 2). Loading of the trityl resin, synthesis of the pentapeptides, cleavage, subsequent cyclization, and deprotection of the cyclic RGD peptides were carried out following standard Fmoc peptide chemistry protocols. Side chains were protected with 2,2,5,7,8-pentamethylchroman (Pmc) for arginine and *tert*-butyl (^{t}Bu) for aspartic acid and tyrosine.

Removal of the Side Chain Protection of the Peptides. The peptides were treated with a solution of 95% trifluoroacetic acid (TFA), 2.5% water, and 2.5% ethanedithiol (EDT) for 4 h at ambient temperature. Next, the glycopeptides were precipitated in diethyl ether. The crude product was isolated by centrifugation and washed three times with diethyl ether.

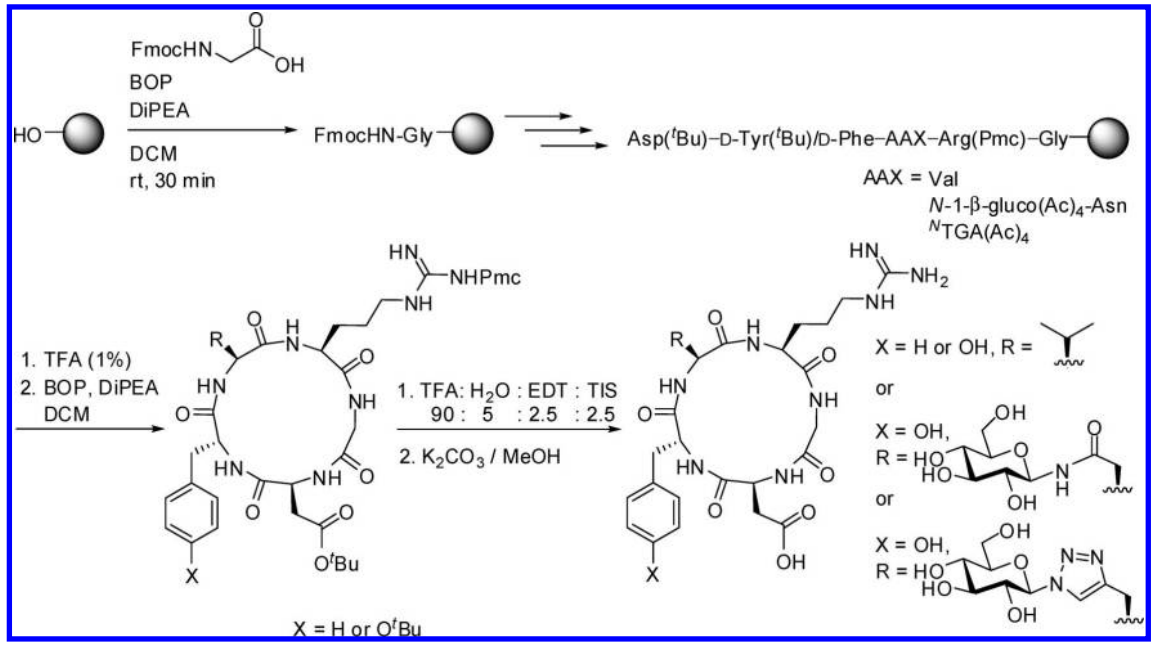
Removal of the Acetyl Protections of the Carbohydrate Bearing Peptides. The crude product was dissolved in MeOH. A catalytic amount of K_2CO_3 was added, and the mixture was stirred for 1 h. Ion exchange resin (Amberlyte IR-120) was added until the solution was neutral, and the ion exchange resin was filtered off. A final purification was done via semipreparative RP-HPLC as described above.

c(RGDfV). Retention time (t_R) (min): 8.1. HRMS (ESI) calculated for $C_{26}H_{39}N_8O_7$ $(M + H)^+$ 575.2914, found 575.2942. For additional data, see ref *14*.

c(RGDyV). t_R (min): 8.2. HRMS (ESI) calculated for $C_{26}H_{39}N_8O_8$ $(M + H)^+$ 591.2891, found 591.2891. For additional data, see ref *19*.

c(RGDy-N-[1-β-gluco(OAc)$_4$]-Asn). t_R (min): 6.3. ^{1}H NMR (400 MHz, MeOD) δ 7.02 (d, J = 8.6 Hz, 2H), 6.70 (d, J = 8.6 Hz, 2H), 5.32 (t, J = 9.5 Hz, 1H), 5.23 (t, J = 9.4 Hz, 1H), 5.04 (t, J = 10.0 Hz, 1H), 4.95 (t, J = 9.5 Hz, 1H), 4.73 (t, J= 6.9 Hz, 1H), 4.57–4.52 (m, 1H), 4.44–4.39 (m, 1H), 4.28–4.23 (m, 3H), 4.11 (dd, J = 12.4, 2.2 Hz, 1H), 3.92 (ddd, J = 10.2, 4.6, 2.4 Hz, 1H), 3.25–3.13 (m, 2H), 3.00–2.95 (m, 1H), 2.86–2.76 (m, 2H), 2.68 (dd, J = 16.1, 6.9 Hz, 1H), 2.57 (dd, J = 16.5, 6.8, 1H), 2.48 (dd, J = 16.1, 5.3 Hz, 1H), 2.03 (s, 3H), 2.03 (s, 3H), 1.99 (s, 3H), 1.98 (s, 3H), 1.98–1.55 (m, 4H). LRMS calculated for $C_{39}H_{54}N_9O_{18}$ $(M + H^+)$ 936.4, found 936.4.

Scheme 2. Preparation of the *c*RGD Peptides



c(RGDy-^{N}TGA(OAc)$_4$). t_R (min): 7.9. ^{1}H NMR (400 MHz, MeOD) δ 7.94 (s, 1H), 6.99 (d, J = 8.5 Hz, 2H), 6.69 (d, J = 8.5 Hz, 2H), 6.09 (d, J = 9.2 Hz, 1H), 5.63 (t, J = 9.4 Hz, 1H), 5.53 (t, J = 9.4 Hz, 1H), 5.27 (t, J = 9.8 Hz, 1H), 4.71 (dd, J = 8.3, 5.7 Hz, 1H), 4.58–4.50 (m, 2H), 4.35–4.24 (m, 3H), 4.17 (dd, J = 12.3, 1.7 Hz, 1 H), 4.13–4.09 (m, 1H), 3.38 (d, J = 15.4 Hz, 1H), 3.26–3.18 (m, 3H), 3.12–3.05 (m, 1H), 2.99–2.94 (m, 1H), 2.84–2.77 (m, 2H), 2.56 (dd, J = 16.6, 5.8 Hz, 1H), 2.06 (s, 3H), 2.00 (s, 3H), 2.00 (s, 3H), 1.83 (s, 3H), 2.00–1.52 (m, 4H). LRMS calculated for $C_{40}H_{54}N_{11}O_{17}$ ($M + H^+$) 960.4, found 960.4.

c(RGDy-N-[1-gluco]-Asn). Removal of the acetyl protecting groups as described above afforded 50 mg (0.06 mmol) of the product, 18% relative to the resin. ^{1}H NMR (400 MHz, D_2O/MeOD) δ 7.01 (d, J = 8.4 Hz, 2H), 6.68 (d, J = 8.2 Hz, 2H), 4.88 (ap d, J = 9.5, 1H), 4.70 (t, J = 7.2 Hz, 1H), 4.50 (dd, J = 6.8, 5.0 Hz, 1H), 4.45–4.38 (m, 2H), 4.24 (d, J = 14.5 Hz, 1H), 3.89 (ap d, J = 11.1 Hz, 1H), 3.74 (dd, J = 12.4, 5.1 Hz, 1H), 3.58 (t, J = 9.2, 1H), 3.53–3.40 (m, 3H), 3.38–3.31 (m, 1H), 3.26–3.15 (m, 2H), 2.98 (dd, J = 13.3, 5.9 Hz, 1H), 2.89–2.83 (m, 2H), 2.77 (dd, J = 16.4, 7.2 Hz, 1H), 2.65 (dd, J = 15.6, 6.5 Hz, 1H), 2.55–2.49 (m, 2H), 1.96–1.87 (m, 1H), 1.76–1.67 (m, 1H), 1.65–1.53 (m, 2H). HRMS (ESI) calculated for $C_{31}H_{46}N_9O_{14}$ $(M + H)^+$ 768.3101, found 768.3164.

c(RGDy-^{N}TGA). Removal of the acetyl protections as described above afforded 39 mg (0.05 mmol) of the product, 14% yield relative to the resin. ^{1}H NMR (400 MHz, D_2O) δ 7.83 (s, 1H), 7.01 (d, J = 8.4 Hz, 2H), 6.68 (d, J =8.4 Hz, 2H), 5.72 (d, J = 9.2 Hz, 1H), 4.68 (t, J =7.1 Hz, 1H), 4.51 (dd, J = 9.9, 5.9 Hz, 1H), 4.43 (t, J = 6.9 Hz, 1H), 4.34 (dd, J = 8.9, 5.6 Hz, 1H), 4.21 (d, J = 14.9 Hz, 1H), 3.99 (t, J = 9.2 Hz, 1H), 3.94 (d, J = 10.4 Hz, 1H), 3.68–3.55 (m, 3H), 3.64 (t, J = 9.3 Hz, 1H), 3.50 (d, J = 14.9 Hz, 1H), 3.09–2.95 (m, 3H), 3.04 (dd, J = 15.3, 6.5 Hz, 1H), 2.94 (dd, J = 13.4, 5.9 Hz, 1H), 2.86 (dd, J = 13.2, 10.2 Hz, 1H), 2.67 (dd, J = 15.8, 7.0 Hz, 1H), 2.53 (dd, J = 15.7, 7.3 Hz, 1H), 1.75–1.63 (m, 1H), 1.55–1.42 (m, 1H), 1.35–1.23 (m, 2H). HRMS (ESI) calculated for $C_{32}H_{46}N_{11}O_{13}$ $(M + H)^+$ 792.3210, found 792.3277.

Solid Phase $\alpha_v\beta_3$ Binding Assay. The affinity of *c*(RGDfV), *c*(RGDyV), *c*(RGDy-[*N*-1-β-gluco-Asn]), and *c*(RGDy-^{N}TGA) for the $\alpha_v\beta_3$ integrin was determined in a competitive binding assay using the dimeric peptide ^{111}In-DOTA-E-[*c*(RGDfK)]$_2$ as a tracer. The ^{111}In-labeled peptide (3 MBq/μg) was prepared as described earlier (*20*). Microtiter 96-well vinyl assay plates (Corning B.V., Schiphol-Rijk, The Netherlands) were coated with 100 μL/well of a solution of purified human integrin $\alpha_v\beta_3$ (150 ng/mL) (Chemicon International, Temecula, CA) in coating buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 1 mM $MnCl_2$) for 17 h at 4 °C. The plates were washed twice with binding buffer (0.1% bovine serum albumin (BSA) in coating buffer). The wells were blocked for 2 h with 200 μL of blocking buffer (1% BSA in coating buffer) at room temperature. The plates were washed twice with binding buffer. Then 100 μL of binding buffer containing 11 kBq of ^{111}In-DOTA-E-[*c*(RGDfK)]$_2$ and appropriate dilutions of non-labeled *c*(RGDfV), *c*(RGDyV), *c*(RGDy-[*N*-1-β-gluco-Asn]), and *c*(RGDy-^{N}TGA) in binding buffer were incubated in wells at 37 °C for 1 h. After incubation, the plates were washed three times with binding buffer. The retained radioactivity in each well was determined in a γ-counter. IC_{50} values of the *c*RGD peptides were calculated by nonlinear regression using GraphPad Prism (GraphPad Prism 4.0 software, San Diego, CA). Each data point is the average of three determinations.

Radiolabeling of the *c*RGD Peptides. The peptides containing tyrosine, *c*(RGDyV), *c*(RGDy-[*N*-1-β-gluco-Asn]), and *c*(RGDy-^{N}TGA) were labeled with ^{125}I using the iodogen method. The peptide (10–20 μg) was dissolved in 100 μL of phosphate-buffered saline (PBS) (pH 7.4) in a 1.5 mL polypropylene vial coated with 100 μg of iodogen, and [^{125}I]NaI (37 MBq) was added to the vial. After 10 min, the solution was transferred to another vial to terminate the iodination. A sample of the mixture was analyzed by HPLC. Radiochemical purity was always higher than 98%.

Biodistribution Studies. In the right flank of 6–8 week old female nude BALB/c mice, 0.2 mL of a suspension of 15–10^6 cells/mL SK-RC-52 cells was injected subcutaneously (sc). Two weeks after inoculation of the tumor cells, mice were randomly divided into four groups. Mice were injected with ^{125}I-*c*(RGDfV) (0.5 MBq, 0.5 μg), ^{125}I-*c*(RGDyV) (0.5 MBq, 0.5 μg), ^{125}I-*c*(RGDy-[*N*-1-β-gluco-Asn]) (0.5 MBq, 0.5 μg), or ^{125}I-*c*(RGDy-^{N}TGA) (0.5 MBq, 0.5 μg) via the tail vein. Mice were euthanized by CO_2 asphyxiation 2 h postinjection (pi) (four mice per group). Blood, tumor, and all the major organs and tissues were dissected, weighed, and counted in a well-type γ-counter.

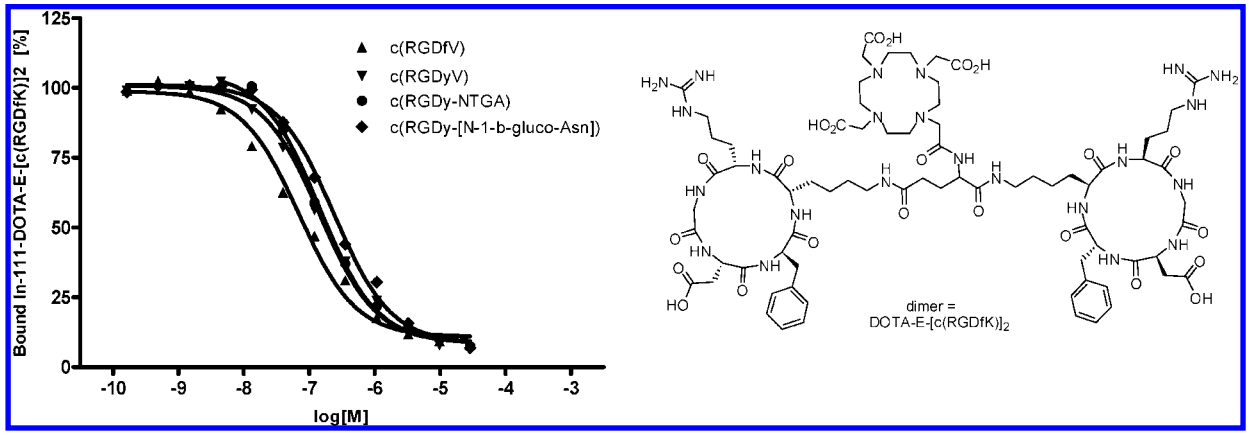


Figure 3. Competition of specific binding of ^{111}In-DOTA-E-[*c*(RGDfK)]$_2$ with *c*(RGDfV) (▲), *c*(RGDyV) (▼), *c*(RGDy-^{N}TGA) (●), and *c*(RGDy-[*N*-1-β-gluco-Asn]) (◆).

The percent injected dose per gram (%ID/g) was determined for each sample. To investigate whether the uptake of each of the four *c*RGD peptides (in the dissected tissues) was $\alpha_v\beta_3$-mediated, a separate group of mice was coinjected with an excess (50 μg) of nonradiolabeled DOTA-E-[*c*(RGDfK)]$_2$ to saturate all the $\alpha_v\beta_3$ integrin receptors, and the biodistribution of the radioiodinated *c*RGD peptide conjugates was determined as described above. All animal experiments were approved by the local animal welfare committee in accordance with the Dutch legislation and carried out in accordance with their guidelines.

RESULTS AND DISCUSSION

Synthesis of Glycoamino Acids. Cyclic RGD peptides represent valuable ligands for selective targeting of $\alpha_v\beta_3$ integrins. Consequently, large numbers of RGD analogues have been prepared (*20–22*) and extensively tested. We have shown that 1-azido sugars and acetylene-modified amino acids readily undergo [3 + 2] cycloaddition via a standard protocol for the copper-catalyzed Huisgen addition to form in high yields triazole isosteres of glycoamino acids (*1*). Although synthetically of interest, here we explored the potential value of triazolyl cyclic glyco-RGD peptides as targeting agents. In this respect, the first relevant question to answer is to what extent the triazole analogues of glycoamino acids have increased stability with respect to the natural compounds. Second, the medicinal value of the triazole compounds as glycoamino acid mimics can only be evaluated by incorporation in biologically active compounds. For this reason, a set of cyclic RGD peptides was chosen to evaluate the binding affinities for $\alpha_v\beta_3$ integrins, including the amide-linked glyco-RGD *c*[RGDy(*N*-1-β-glyco-Asn)], the triazole-linked *c*(RGDy-^{N}TGA) and *c*(RGDyV) and *c*(RGDfV) for reference purposes. D-Tyr was incorporated instead of D-Phe for *c*[RGDy(*N*-1-β-glyco-Asn)], *c*(RGDy-^{N}TGA), and *c*(RGDyV), enabling subsequent iodination for tumor imaging as reported by Haubner et al. (*14*).

The requisite building blocks for solid-phase synthesis, 1-azido-2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose **1** (*16*) was efficiently condensed with Fmoc-L-propargylglycine under the influence of Cu(OAc)$_2$ and sodium ascorbate in a mixture of ^{t}BuOH and H$_2$O to synthesize fully protected *N*-triazolylglycosidic alanine (*2*), which we refer to as Fmoc-L-^{N}TGA(Ac)$_4$-OH, in a 91% yield. The amide-linked glycoamino acid was prepared by hydrogenation of **1** with hydrogen in the presence of Pd/C and Et$_3$N in MeOH to afford 1-aminoglucose derivative **3**. Subsequent coupling to Fmoc-aspartic acid *tert*-butyl ester yielded acetyl and Fmoc protected glycosidic asparagine *tert*-butyl ester **5** in a yield of 87%. Next, the *tert*-butyl ester was treated with TFA in DCM (1/1, v/v) to afford the free acid Fmoc-[*N*-1-β-glyco(Ac)$_4$-Asn](OH) in near-quantitative yield.

Evaluation of Stability. In order to investigate the chemical stability of the substrates ^{N}TGA(Ac)$_4$-OH, as well as *N*-1-glycosyl(Ac)$_4$-Asn(OH), the protected glycoamino acids **2** and **6** were subjected to treatment with 20% piperidine in DMF (v/v) followed by a saponification of the acetyl esters by K$_2$CO$_3$ in MeOH. The resulting glycoamino acids ^{N}TGA (**3**) and *N*-1-β-glyco-Asn (**7**) were subjected to various acidic and basic conditions as summarized in Table 1. The triazole-linked ^{N}TGA was found to be completely compatible with a range of basic and acidic conditions, whereas the natural amide-linked *N*-1-β-glyco-Asn did not withstand some of the conditions tested. From these results, it can be concluded that triazole-linked substrates are versatile building blocks for the preparation of stable analogues of the common asparagine-linked *N*-glycoproteins.

Solid-Phase Synthesis. For the synthesis of the cyclic RGD peptides, a solid-phase strategy was chosen, followed by cleavage from the resin and cyclization. Thus, in order to avoid racemization of the C-terminal amino acid during the last activation step, Fmoc-glycine was elected as the C-terminal amino acid and coupled to trityl resin with DiPEA in DCM. Subsequent elongation of the peptide was executed by application of standard Fmoc chemistry involving HOBt/DIPCDI-mediated coupling followed by piperidine-induced Fmoc deprotection. Both Fmoc-L-^{N}TGA(Ac)$_4$-OH and Fmoc-[*N*-1-β-glyco(Ac)$_4$]-Asn(OH) were compatible with the conditions applied, although the amide-linked glycoamino acid was incorporated at a much slower rate. After completion of the synthesis, the fully protected pentapeptides were cleaved from the resin using a 1% solution of TFA in DCM and purified by silica gel column chromatography. Next, cyclization of the linear RGD peptides was effected under the influence of BOP to give the protected cyclic peptides in good overall yields (14–18%). Finally, protecting groups were removed under basic conditions and the final product was purified by semipreparative RP-HPLC as described above.

Solid-Phase $\alpha_v\beta_3$ Binding Assay. The affinities of *c*(RGDfV), *c*(RGDyV), *c*(RGDy-[*N*-1-β-gluco-Asn]), and *c*(RGDy-^{N}TGA) for the $\alpha_v\beta_3$ integrin as determined in the competitive binding assay are summarized in Figure 3.

From Figure 3, it becomes clear that binding of ^{111}In-DOTA-E-[*c*(RGDfK)]$_2$ (*23*) to $\alpha_v\beta_3$ was inhibited by each compound in a concentration-dependent manner. In fact, only relatively small differences between the different peptides can be observed, with IC$_{50}$ values of 65 nM for *c*(RGDfV), 144 nM for *c*(RGDyV), 238 nM for *c*(RGDy-[*N*-1-β-gluco-Asn]), and 144 nM for *c*(RGDy-^{N}TGA), indicating that side chain modification had only limited effect on the $\alpha_v\beta_3$ binding affinity of the compounds, which is in agreement with earlier reports (*16, 24, 25*).

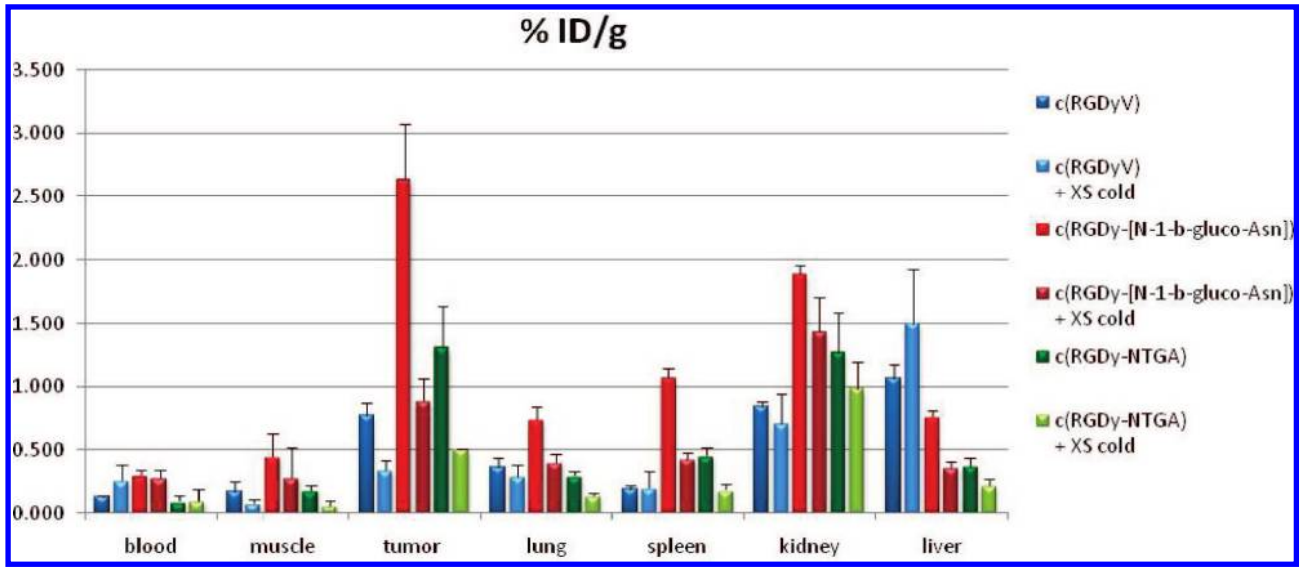


Figure 4. Biodistribution of *c*RGD peptides in athymic mice.

Table 2. Biodistribution Data of ^{125}I-*c*(RGDyV), ^{125}I- *c*(RGDy-[*N*-1-*β*-gluco-Asn]), and ^{125}I-*c*(RGDy-^{N}TGA) in the Presence and Absence of an Excess of Unlabeled DOTA-E-[*c*(RGDfK)]$_2$ in Athymic Mice with sc SK-RC-52 Tumors 2 h after Injection[a]

	^{125}I-*c*(RGDyV)		^{125}I-*c*[RGDy-(*N*-1-*β*-gluco-Asn)]		^{125}I-*c*(RGDy-^{N}TGA)	
		nonspecific uptake		nonspecific uptake		nonspecific uptake
blood	0.13 ± 0.01	0.26 ± 0.12	0.30 ± 0.04	0.28 ± 0.06	0.09 ± 0.04	0.10 ± 0.10
muscle	0.17 ± 0.08	0.08 ± 0.03	0.45 ± 0.18	0.28 ± 0.24	0.17 ± 0.04	0.06 ± 0.04
tumor	0.77 ± 0.10	0.34 ± 0.07	2.63 ± 0.45	0.89 ± 0.17	1.31 ± 0.33	0.51 ± 0.01
lung	0.36 ± 0.08	0.29 ± 0.09	0.74 ± 0.1	0.40 ± 0.07	0.29 ± 0.03	0.13 ± 0.02
spleen	0.19 ± 0.02	0.20 ± 0.13	1.07 ± 0.07	0.43 ± 0.05	0.46 ± 0.06	0.18 ± 0.04
kidney	0.84 ± 0.04	0.71 ± 0.24	1.89 ± 0.07	1.43 ± 0.27	1.27 ± 0.32	0.99 ± 0.20
liver	1.07 ± 0.11	1.49 ± 0.44	0.76 ± 0.05	0.36 ± 0.05	0.37 ± 0.07	0.22 ± 0.05
intestine	1.93 ± 1.62	1.82 ± 0.36	2.10 ± 0.44	1.15 ± 0.78	0.8 ± 0.20	0.30 ± 0.13

[a] The organ uptake is expressed as %ID/g.

In addition, these data showed that only a slight difference was noticeable between the carbohydrate containing *c*RGD peptides (*c*[RGDy-^{N}TGA]) and *c*(RGDy-[*N*-1*β*-gluco-Asn]), which is a first indication that the glycoamino acid binding characteristics are nearly unchanged upon substitution of the amide linkage for a triazole linkage.

Biodistribution Studies. Side chain glycosylation can improve the pharmacological properties of hydrophobic/lipophilic peptides (*26*), which has also been demonstrated for *c*RGD (*14*). In order to validate the potential of the glycoamino acids **3** and **7** for incorporation into glyco-RGD peptides, the biodistribution of ^{125}I-*c*(RGDyV), ^{125}I-*c*(RGDy-[*N*-1-*β*-gluco-Asn]), and ^{125}I-*c*(RGDy-^{N}TGA) in athymic mice with sc $\alpha_v\beta_3$-expressing tumors was determined (Figure 4). The results of the biodistribution studies of ^{125}I-RGDyV, ^{125}I-*c*(RGDy-[*N*-1-*β*-gluco-Asn]), and ^{125}I-*c*(RGDy-^{N}TGA) in athymic mice with SK-RC-52 tumors at 2 h pi are summarized in Table 2. In order to determine the nonspecific uptake of the peptides, the biodistribution was also determined in the presence of excess unlabeled *c*RGD compound, i.e., dimeric DOTA-E-[*c*(RGDfK)]$_2$.

All peptides ^{125}I-*c*(RGDyV), ^{125}I-*c*(RGDy-[*N*-1-*β*-gluco-Asn]), and ^{125}I-*c*(RGDy-^{N}TGA) rapidly cleared from the blood because blood levels at 2 h pi did not exceed 0.3% ID/g. Two hours after injection, the concentration of ^{125}I-*c*(RGDy-[*N*-1-*β*-gluco-Asn]) and ^{125}I-*c*(RGDy-^{N}TGA in the tumor was the highest of all tissues examined. Coinjection of excess unlabeled DOTA-E-[*c*(RGDfK)]$_2$ (50 *μ*g) along with ^{125}I-*c*(RGDyV), ^{125}I-*c*(RGDy-[*N*-1-*β*-gluco-Asn]), and ^{125}I-*c*(RGDy-^{N}TGA) indicated that for each of the three compounds, the major part of the uptake in the tumor was $\alpha_v\beta_3$-mediated. In this mouse model, ^{125}I-*c*(RGDy-^{N}TGA) revealed the highest tumor-to-blood ratio (18 at 2 h pi). The carbohydrate-bearing *c*RGD peptides, ^{125}I-*c*(RGDy-[*N*-1-*β*-gluco-Asn]) and ^{125}I-*c*(RGDy-^{N}TGA), also showed $\alpha_v\beta_3$-mediated uptake in nontarget organs like lung, spleen, and intestine. Specific uptake of *c*RGD analogues in normal tissues has been reported in previous studies and is probably due to $\alpha_v\beta_3$ expression in these tissues (*27, 28*). Furthermore, the conducted studies once more show that tumor uptake is not solely dependent on the binding affinity but relies on many more factors such as blood residence time, molecular weight, structure, charge, etc. These aspects may explain the fact that ^{125}I-*c*(RGDy-[*N*-1-*β*-gluco-Asn]) displayed the highest tumor uptake while the corresponding monomer failed to provide the highest binding affinity. Although ^{125}I-*c*(RGDy-^{N}TGA) showed the highest tumor-to-blood ratio, it showed a lower tumor uptake than ^{125}I-*c*(RGDy-[*N*-1-*β*-gluco-Asn]). Therefore, it may be concluded that the high tumor uptake of ^{125}I-*c*(RGDy-[*N*-1-*β*-gluco-Asn]) can only partially be explained by the presence of the carbohydrate moiety.

CONCLUSION

A high-yielding route for the preparation of Fmoc-L-^{N}TGA(Ac)$_4$-OH starting from azidoglucose and Fmoc-protected propargylglycine is described by application of our technology for the synthesis of triazolylglycoamino acids (*1*). The ^{N}TGA building block was found to display significantly improved chemical stability with respect to the "natural" amide-linked glycoamino acid and is fully compatible with basic and acidic conditions. The incorporation of the Fmoc-

protected ^{N}TGA derivative in an *c*RGD peptide proceeded without incidence, in fact more smoothly than the amide-linked glycoamino acids, with the final yield of the substrate being comparable to the yields of the other cyclic RGD substrates. The solid-phase binding assay revealed no significant differences between the different peptides under evaluation, confirming that modification of the side chain of the cyclic RGD peptide did not affect affinity for $\alpha_v\beta_3$. The ^{125}I-labeled cyclic peptides accumulated specifically in SK-RC-52 tumors. The uptake of the cyclic RGD substrates in the tumor, lung, spleen, and intestine was $\alpha_v\beta_3$-mediated. The biodistribution studies indicated that the side chain glycosylated *c*RGD peptide ^{125}I-*c*(RGDy-^{N}TGA) showed improved pharmacologic properties compared to ^{125}I-*c*(RGDyV), with reduced liver uptake and the highest uptake in the tumor, in accordance with earlier literature reports (*14, 15*) for different glycosylated cyclic RGD peptides. Moreover, the in vitro and in vivo tests indicated that *c*(RGDy-^{N}TGA) to some extent has the same pharmacologic properties as *c*(RGDy-[*N*-1-β-gluco-Asn]), which may be interpreted as one of the first examples of the value of triazoles as amide isosteres. The ease of synthesis of the triazolylglycoamino acids, the improved stability and behavior in solid-phase peptide synthesis, and the similar pharmacologic properties are a demonstration of the potential of ^{N}TGA building blocks as isosteres of amide-linked glycoamino acids for the synthesis of pharmaceutically relevant glycopeptide mimics.

ACKNOWLEDGMENT

SenterNovem (Ministry of Economic Affairs, The Netherlands) is gratefully acknowledged for providing financial support.

Note Added after ASAP Publication: This manuscript was released ASAP on October 9, 2007 with an incorrect definition for (RGD). The correct version was posted on October 17, 2007.

LITERATURE CITED

(1) Kuijpers, B. H. M., Groothuys, S., Keereweer, A. R., Quaedflieg, P. J. L. M., Blaauw, R. H., van Delft, F. L., and Rutjes, F. P. J. T. (2004) Expedient synthesis of triazole-linked glycosyl amino acids and peptides. *Org. Lett. 6*, 3123–3126.

(2) Tornøe, C. W., Christensen, C., and Meldal, M. (2002) Peptidotriazoles on solid phase: 1,2,3-triazoles by regiospecific copper(I)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides. *J. Org. Chem. 67*, 3057–3064.

(3) Rostovtsev, V. V., Green, L. G., Fokin, V. V., and Sharpless, K. B. (2002) A stepwise Huisgen cycloaddition process: copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes. *Angew. Chem., Int. Ed. 41*, 2596–2599.

(4) Horne, W. S., Stout, C. D., and Ghadiri, M. R. (2003) A heterocyclic peptide nanotube. *J. Am. Chem. Soc. 125*, 9372–9376.

(5) Bock, V. D., Speijer, D., Hiemstra, H., and van Maarseveen, J. H. (2007) 1,2,3-Triazoles as peptide bond isosteres: synthesis and biological evaluation of cyclotetrapeptide mimics. *Org. Biomol. Chem. 5*, 971–975.

(6) Dondoni, A., Giovannini, P. P., and Massi, A. (2004) Assembling heterocycle-tethered C-glycosyl and alpha-amino acid residues via 1,3-dipolar cycloaddition reactions. *Org. Lett. 6*, 2929–2932.

(7) Groothuys, S., Kuijpers, B. H. M., Quaedflieg, P. J. L. M., Roelen, H. C. P. F., Wiertz, R. W., Blaauw, R. H., van Delft, F. L., and Rutjes, F. P. J. T. (2006) Chemoenzymatic synthesis of triazole-linked glycopeptides. *Synthesis* 3146–3152.

(8) Kuijpers, B. H. M., Dijkmans, G. C. T., Groothuys, S., Quaedflieg, P. J. L. M., Blaauw, R. H., van Delft, F. L., and Rutjes, F. P. J. T. (2005) Copper(I)-mediated synthesis of trisubstituted 1,2,3-triazoles. *Synlett* 3059–3062.

(9) Brik, A., Alexandratos, J., Lin, Y. C., Elder, J. H., Olson, A. J., Wlodawer, A., Goodsell, D. S., and Wong, C. H. (2005) 1,2,3-triazole as a peptide surrogate in the rapid synthesis of HIV-1 protease inhibitors. *ChemBioChem 6*, 1167–1169.

(10) Wilkinson, B. L., Bornaghi, L. F., Poulsen, S. A., and Houston, T. A. (2006) Synthetic utility of glycosyl triazoles in carbohydrate chemistry. *Tetrahedron 62*, 8115–8125.

(11) Kessler, H., Diefenbach, B., Finsinger, D., Geyer, A., Gurrath, M., Goodman, S. L., Holzemann, G., Haubner, R., Jonczyk, A., Muller, G., vonRoedern, E. G., and Wermuth, J. (1995) Design of superactive and selective integrin receptor antagonists containing the RGD sequence. *Lett. Pept. Sci. 2*, 155–160.

(12) Kantlehner, M., Finsinger, D., Meyer, J., Schaffner, P., Jonczyk, A., Diefenbach, B., Nies, B., and Kessler, H. (1999) Selective RGD-mediated adhesion of osteoblasts at surfaces of implants. *Angew. Chem., Int. Ed. 38*, 560–562.

(13) Gurrath, M., Muller, G., Kessler, H., Aumailley, M., and Timpl, R. (1992) Conformation activity studies of rationally designed potent antiadhesive RGD peptides. *Eur. J. Biochem. 210*, 911–921.

(14) Haubner, R., Wester, H. J., Burkhart, F., Senekowitsch-Schmidtke, R., Weber, W., Goodman, S. L., Kessler, H., and Schwaiger, M. (2001) Glycosylated RGD-containing peptides, tracer for tumor targeting and angiogenesis imaging with improved biokinetics. *J. Nucl. Med. 42*, 326–336.

(15) Lohof, E., Planker, E., Mang, C., Burkhart, F., Dechantsreiter, M. A., Haubner, R., Wester, H. J., Schwaiger, M., Holzemann, G., Goodman, S. L., and Kessler, H. (2000) Carbohydrate derivatives for use in drug design: cyclic α_v-selective RGD peptides. *Angew. Chem., Int. Ed. 39*, 2761–2764.

(16) Shiozaki, M., Mochizuki, T., Hanzawa, H., and Haruyama, H. (1996) Synthesis of a tetrahydropyrano[2,3-*d*]oxazole analogue of trehazolin. *Carbohydr. Res. 288*, 99–108.

(17) Ichikawa, Y., Nishiyama, T., and Isobe, M. (2001) Stereospecific synthesis of the alpha- and beta-D-glucopyranosyl ureas. *J. Org. Chem. 66*, 4200–4205.

(18) van Ameijde, J., Albada, H. B., and Liskamp, R. M. J. (2002) A convenient preparation of several N-linked glycoamino acid building blocks for efficient solid-phase synthesis of glycopeptides. *J. Chem. Soc., Perkin Trans. 1* 1042–1049.

(19) Haubner, R., Wester, H. J., Reuning, U., Senekowitsch-Schmidtke, R., Diefenbach, B., Kessler, H., Stocklin, G., and Schwaiger, M. (1999) Radiolabeled $\alpha_v\beta_3$ integrin antagonists: a new class of tracers for tumor targeting. *J. Nucl. Med. 40*, 1061–1071.

(20) Dijkgraaf, I., Kruijtzer, J. A. W., Liu, S., Soede, A. C., Oyen, W. J. G., Corstens, F. H. M., Liskamp, R. M. J., and Boerman, O. C. (2007) Improved targeting of the alpha(v)beta(3) integrin by multimerisation of RGD peptides. *Eur. J. Nucl. Med. Mol. Imaging 34*, 267–273.

(21) Dijkgraaf, I., Kruijtzer, J. A. W., Frielink, C., Corstens, F. H. M., Oyen, W. J. G., Liskamp, R. M. J., and Boerman, O. C. (2007) $\alpha_v\beta_3$ integrin-targeting of intraperitoneally growing tumors with a radiolabeled RGD peptide. *Int. J. Cancer 120*, 605–610.

(22) Zhang, X. Z., and Chen, X. Y. (2007) Preparation and characterization of Tc-99m(CO)(3)–BPy–RGD complex as $\alpha_v\beta_3$ integrin receptor-targeted imaging agent. *Appl. Radiat. Isot. 65*, 70–78.

(23) Dijkgraaf, I., Kruijtzer, J. A. W., Frielink, C., Soede, A. C., Hilbers, H. W., Oyen, W. J. G., Corstens, F. H. M., Liskamp, R. M. J., and Boerman, O. C. (2006) Synthesis and biological evaluation of potent $\alpha_v\beta_3$-integrin receptor antagonists. *Nucl. Med. Biol. 33*, 953–961.

(24) Haubner, R., Finsinger, D., and Kessler, H. (1997) Stereoisomeric peptide libraries and peptidomimetics for designing selective inhibitors of the $\alpha_v\beta_3$ integrin for a new cancer therapy. *Angew. Chem., Int. Ed. Engl. 36*, 1375–1389.

(25) Haubner, R., Gratias, R., Diefenbach, B., Goodman, S. L., Jonczyk, A., and Kessler, H. (1996) Structural and functional aspects of RGD-containing cyclic pentapeptides as highly potent and selective integrin $\alpha_v\beta_3$ antagonists. *J. Am. Chem. Soc. 118*, 7461–7472.

(26) Sinclair, A. M., and Elliott, S. (2005) Glycoengineering: the effect of glycosylation on the properties of therapeutic proteins. *J. Pharm. Sci. 94*, 1626–1635.

(27) Janssen, M. L., Oyen, W. J., Dijkgraaf, I., Massuger, L. F., Frielink, C., Edwards, D. S., Rajopadhye, M., Boonstra, H., Corstens, F. H., and Boerman, O. C. (2002) Tumor targeting with radiolabeled $\alpha_v\beta_3$ integrin binding peptides in a nude mouse model. *Cancer Res. 62*, 6146–6151.

(28) Max, R., Gerritsen, R. R. C. M., Nooijen, P. T. G. A., Goodman, S. L., Sutter, A., Keilholz, U., Ruiter, D. J., and DeWaal, R. M. W. (1997) Immunohistochemical analysis of integrin $\alpha_v\beta_3$ expression on tumor-associated vessels of human carcinomas. *Int. J. Cancer 71*, 320–324.

BC700154U