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# Tyrosine modified analogues of the α4β7 integrin inhibitor biotin-$R_8$ERY prepared via Click Chemistry: Synthesis and biological evaluation

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ABSTRACT

Our continuing programme aiming at developing inhibitors of integrin α4β7, a key mediator of various inflammatory diseases, led us to synthesise a library of cell-permeable peptides based on the biotin-$R_8$ERY* template, wherein the tyrosine residue has been modified by using the CuAAC reaction. The peptidomimetics were evaluated in a cell adhesion assay and shown to inhibit $Mn^{2+}$-activated adhesion of mouse TK-1 T cells to mouse MAdCAM-1. Two of the synthesised peptidomimetics, analogues **11** and **14**, are more active than our previously reported lead compound biotin-$r_9$YDRREY at concentrations of 100 and 50 μM, with **14** exhibiting an $IC_{50}$ of less than 10 μM.



## 1. Introduction

Integrins are a widely expressed family of cell surface adhesion receptors that play a major role in cell attachment to extracellular matrices, and also in the mediation of important cell–cell adhesion events.[1]

The α4 integrins α4β7 (LPAM-1) and α4β1 (VLA-4) are heterodimeric cell surface receptors expressed on most leukocytes[2,3] and consist of noncovalently-associated α and β transmembrane subunits with comparatively large extracellular and short cytoplasmic domains. The most important ligands of the α4 integrin subfamily include fibronectin (Fn), vascular cell adhesion molecule-1 (VCAM-1), and mucosal addressin cell adhesion molecule-1 (MAdCAM-1).[4–6] Integrin α4β1 preferentially recognises VCAM-1, whereas MAdCAM-1 is an exclusive ligand for α4β7 under physiological conditions.[3,7–9] Lymphocytes bind via α4β7 to MAdCAM-1 expressed on high endothelial venules, enabling their extravasation from blood to gut mucosal tissues of gut-associated lymphoid tissues (GALT) such as Peyer's patches, mesenteric lymph nodes (MLNs) and the lamina propria (LP).[10] In contrast, the αEβ7 receptor which is formed by association of the β7 subunit with the integrin αE subunit, mediates the binding of intestinal intraepithelial lymphocytes (iIEL) to E-cadherin expressed on gut enterocytes.[11]

The two β7 integrins α4β7 and αEβ7 play key roles in regulating gut immunity and are involved in the pathogenesis and progression of inflammatory bowel diseases, such as Crohn's disease, ulcerative colitis,[12–14] and intestinal graft-versus-host diseases (GVHD).[11,15] Moreover, α4β7 integrins contribute to the infiltration of leukocytes into the islets of Langerhans in type 1 diabetes[16] and the central nervous system in demyelinating diseases such as multiple sclerosis.[17] Clearly appropriate control of β7 integrins is crucial in many disease states and for this reason, the development of selective drugs that preclude the homing of lymphocytes to chronically inflamed tissues but with minimal effects on normal immune surveillance would be highly desirable. To date, several pharmaceutical companies have produced a number of anti-inflammatory therapies based on small molecule α4β1 and/or α4β7 antagonists that prevent the binding of integrins to their extracellular ligands.[7,18–20]

A very different strategy for inhibiting β7 integrin mediated extravasation of leukocytes was developed by Krissansen and co-workers in 2006. They demonstrated that the L- and D-enantiomers of biotin-$r_9$YDRREY inhibited adhesion of $Mn^{2+}$-activated T cells to β7 integrin ligands. The hexapeptide YDRREY corresponds to residues 735–740 of the cytoplasmic tail of the β7 subunit, a polyarginine tag and a biotin moiety were appended to the N-terminus in order to minimize their effect on the biological activity of the hexapeptide. The polyarginine tag serves as a cationic carrier enabling its penetration into the cytoplasm of activated

*Abbreviations:* CuAAC, copper catalyzed azide-alkyne cycloaddition; FAK, focal adhesion kinase; FBS, fetal bovine serum; GALT, gut-associated lymphoid tissues; GnHCl, guanidine hydrochloride; GVHD, graft-versus-host disease; HBSS, Hank's buffered salt solution; HMP, 4-(hydroxymethyl)phenoxyacetic acid; iIEL, intestinal intraepithelial lymphocytes; LPAM-1, lymphocyte Peyer's patch adhesion molecule-1; MAdCAM-1, mucosal addressin cell adhesion molecule-1; NSAIDs, non-steroidal anti-inflammatory drugs; PBS, phosphate buffered saline; $r_9$, (D-Arginine)$_9$; SPPS, solid phase peptide synthesis; src, sarcoma; TFA, trifluoroacetic acid; VCAM-1, vascular cell adhesion molecule-1; VLA-4, very late activating antigen-4.

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T cells,[21–23] whereas the biotin facilitates detection of peptide uptake by cells as required. Biotin-$r_9$YDRREY is presumably acting as a competitive substrate for src, FAK, and other tyrosine kinases and signalling molecules.[24] It is thereby proposed to interrupt the normal phosphorylation of the intracellular β7 subunit, and the association of α4β7 with intracellular signalling molecules and cytoskeletal elements. It suppresses MAdCAM-1-induced clustering of α4β7 receptors on the surface of T cells and thereby disrupts cell adhesion (Fig. 1).

Recently, we showed that biotin-$R_8$ERY which displays a shorter active unit, namely tripeptide ERY, exhibited similar activity to that of the biotin-$r_9$YDRREY analogue. We initially synthesised a small library of biotin-$R_8$ERY* peptides using a number of commercially available tyrosine analogues and we established that the analogue biotin-$R_8$ERY* (Y* = 4-chlorophenylalanine) possessed activity approaching that of the biotin-$r_9$YDRREY. We therefore envisaged using the well-known Cu(I)-catalysed azide–alkyne 'click' cycloaddition (CuAAC) reaction to generate a new library of complementary biotin-$R_8$ERY* analogues derived from peptide **6** (Y* = *O*-propargyl-L-tyrosine), which, we trusted, would further improve the activity. The choice of azide component was dictated by the availability of certain azides in our laboratory. It was hoped that the range of azides incorporated into the peptide scaffold would lead to an effective cell adhesion inhibitor.

Click chemistry was introduced by Sharpless and co-workers in 2001[25] and subsequently they and Meldal et al. independently discovered a dramatic acceleration of reaction rate between terminal alkynes and azides by the addition of catalytic amounts of Cu(I) one year later.[26,27] Consequently, although several types of reactions fall under the term click chemistry,[28] the CuAAC has assumed a particularly important role in organic synthesis over the last decade owing to the stability of azides under a large number of reaction conditions.[29–31] Additionally, the Cu(I)-catalysed cyclisation unites azides and terminal alkynes regiospecifically, giving only 1,4-disubstituted 1,2,3-triazoles. The CuAAC reaction can be easily combined with peptide chemistry as the reaction remains unaffected by the presence of other functional groups such as those present on the peptide side-chains.

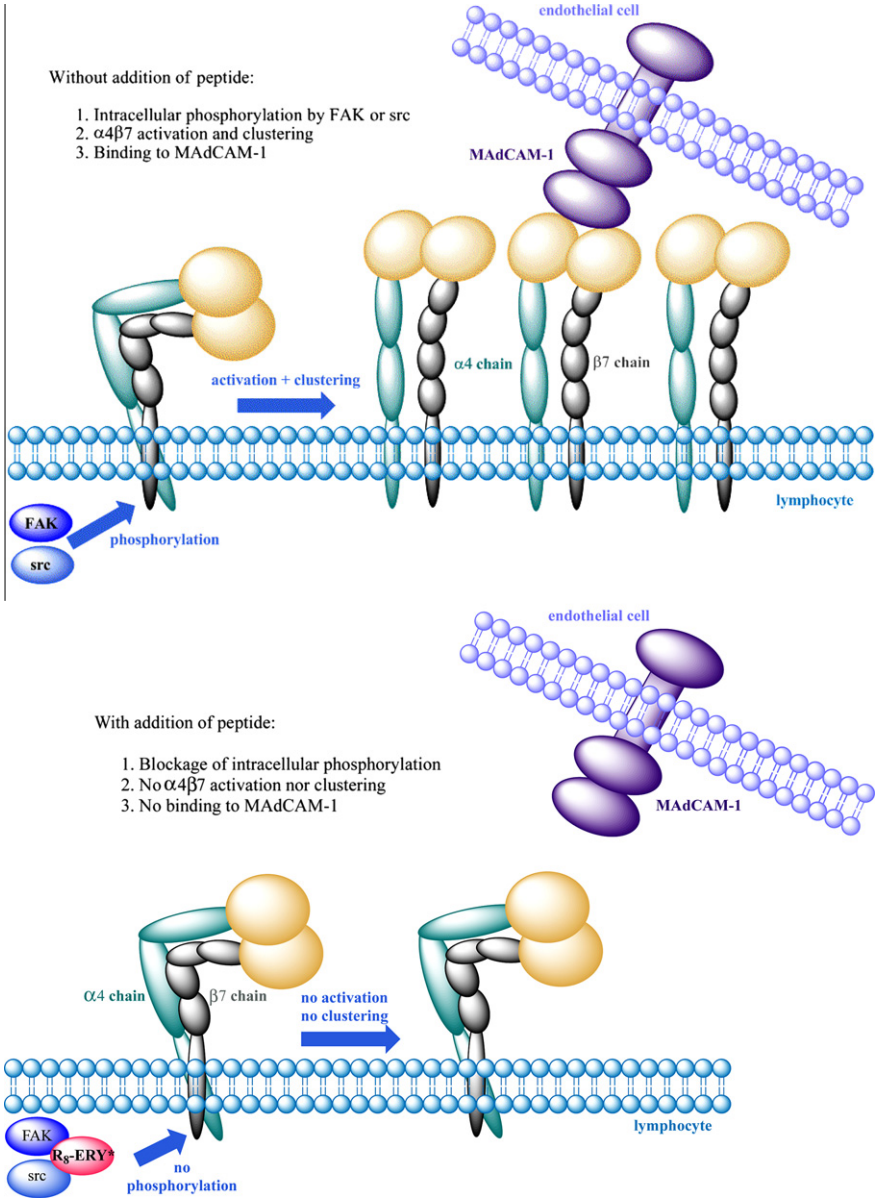
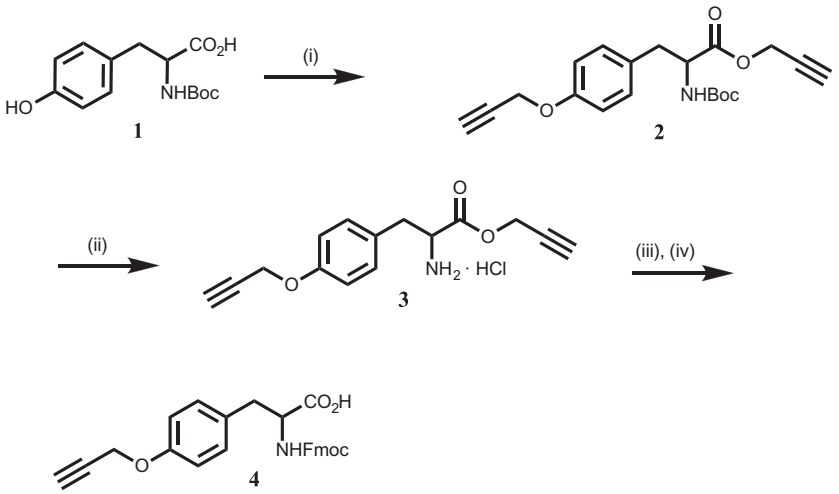
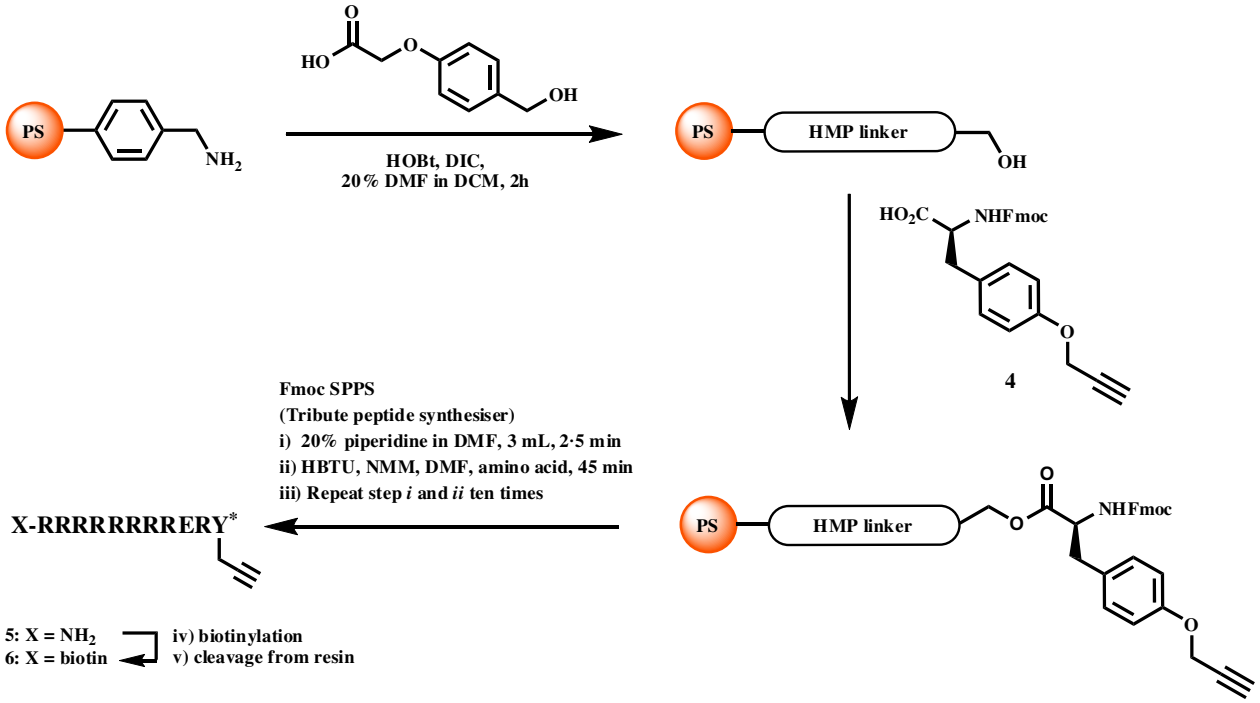



**Figure 1.** An hypothesis: Interruption of cell adhesion of lymphocytes to MAdCAM-1 by competitive binding of biotin-$R_8$ERY* to FAK and src.




**Scheme 1.** Reagents and conditions: (i) $K_2CO_3$, propargyl bromide, DMF, 18 h, rt; (ii) AcCl, MeOH, 4 h, 0 °C to rt; (iii) NaOH, MeOH, 1.5 h, HCl, $H_2O$, 4 °C, 18 h; (iv) $NaHCO_3$, FmocOSu, dioxane, $H_2O$.




**Scheme 2.** Fmoc SPPS of the propargylated peptide on amino methylated PS resin.

We have synthesised nine analogues of peptide biotin-$R_8$ERY*6 (Y* = *O*-propargyl-tyrosine) by using the CuAAC reaction. The constituent azido-substrates were reacted with the propargylated peptide in solution phase and the activities of the resulting products were evaluated in a cell adhesion assay.

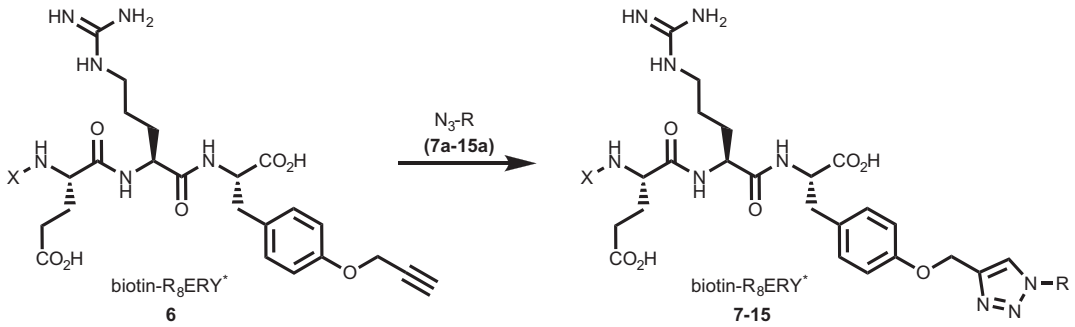
## 2. Results and discussion

### 2.1. Chemistry

First of all, the key propargylated tyrosine derivative **4** was prepared. Precursor H-L-Tyr(*O*-propargyl)-OH was synthesised according to the procedure published by Schultz et al.[32] from Boc-L-tyrosine-OH **1** and subsequently Fmoc-protected according to the method of Essen et al.[33] to afford the desired building block Fmoc-*O*-propargyl-L-tyrosine **4** for Fmoc SPPS (Scheme 1).

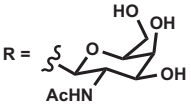
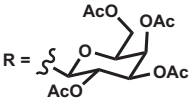
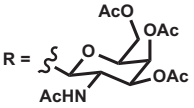
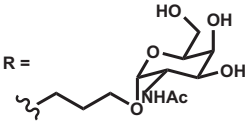
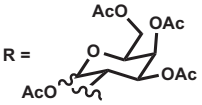
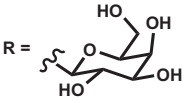
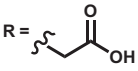
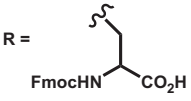
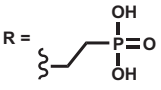
The monopropargylated peptide $R_8$ERY* **5** was synthesised on a Tribute™ peptide synthesiser under standard Fmoc SPPS conditions, using compound **4**. The N-terminus of peptide **5** was derivatised with biotin and cleaved from resin to afford the desired product **6** in 47% yield (99% purity) after RP-HPLC (Scheme 2).

The required azido components 2-acetamido-2-deoxy-β-D-galactopyranosyl azide (**7a**),[34] 1-β-azido-2,3,4,6-tetra-*O*-acetyl-D-galactose (**8a**),[35] 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-galactopyranosylazide (**9a**),[34] 3′-azido-1′-propyl-2-acetamido-2-deoxy-α-D-galactopyranoside (**10a**),[36,37] 1,3,4,6-tetra-*O*-acetyl-2-azido-2-deoxy-D-galactose (**11a**),[38] 1-deoxy-β-D-galactopyranosyl azide (**12a**),[39,40] 2-azidoacetic acid (**13a**),[41] Fmoc-L-Ala($N_3$)-OH




**Scheme 3.** CuAAC reactions on peptide **6**.

**Table 1**
biotin-$R_8$ERY* peptides and their percentage of inhibition in a cell adhesion assay relative to biotin-$r_9$YDRREY

| No | Y*= | Yield (mg, %) (purity) | MS ($^{4+}$ ion) calcd/obsd[b] | % Inhibition (50 μM) | % Inhibition (100 μM) |
|---|---|---|---|---|---|
| **6** | Fmoc-*O*-propargyltyrosine | 8.20 mg, 47% [a] (99%) | 496.1/496.0 | 39.77 | 39.75 |
| **7** | R =  | 0.80 mg, 15% (99%) | 557.6/557.6 | 28.03 | 39.63 |
| **8** | R =  | 1.46 mg, 27% (94%) | 617.9/617.8 (+TFA) | 47.38 | 52.46 |
| **9** | R =  | 2.78 mg, 93% (93%) | 617.7/617.6 (+TFA) | 40.02 | 46.53 |
| **10** | R =  | 2.46 mg, 46% (99%) | 600.7/600.6 (+TFA) | 41.47 | 42.36 |
| **11** | R =  | 1.36 mg, 25% (96%) | 617.9/617.8 (+TFA) | 104.50 | 104.76 |
| **12** | R =  | 1.64 mg, 32% (86%) | 547.4/547.3 | 60.17 | 62.73 |
| **13** | R =  | 2.53 mg, 52% (98%) | 521.4/521.3 | 56.14 | 34.85 |
| **14** | R =  | 2.12 mg, 39% (96%) | 584.2/584.1 | 114.48 | 110.55 |
| **15** | R =  | 2.09 mg, 42% (99%) | 533.9/533.8 | 60.30 | 65.39 |

[a] Purified yield was based on calculated resin loading, see Supporting information for further details.
[b] ESI-MS, see Supporting information for further details.

(**14a**),[42,43] and 2-azidoethylphosphonic acid (**15a**)[44] were synthesised according to published procedures.

With the propargylated peptide **6** and nine different azides (**7a**–**15a**) in hand, attention focused on developing suitable conditions for the Cu(I)-catalysed click reaction in solution phase. Initial attempts to carry out the CuAAC reaction using catalytic amounts of $CuSO_4$ and sodium ascorbate in a $H_2O$/$^t$BuOH solvent mixture were unsuccessful, even at elevated temperatures (microwave irradiation, 80 °C, 120 W). Changing the reaction conditions by adding more catalyst, modifying the solvent system ($H_2O$/$^t$BuOH/DCM) or allowing longer reaction times also proved to be unsuccessful. Previous studies of click reactions in our group[34,36] involving unprotected peptides and azido sugars suggested a need for greater than equimolar amounts of Cu(I) species per propargyl group, possibly due to chelation of Cu ions by the peptide. Lee et al.[36] recently published a much milder strategy for Cu(I)-catalysed click

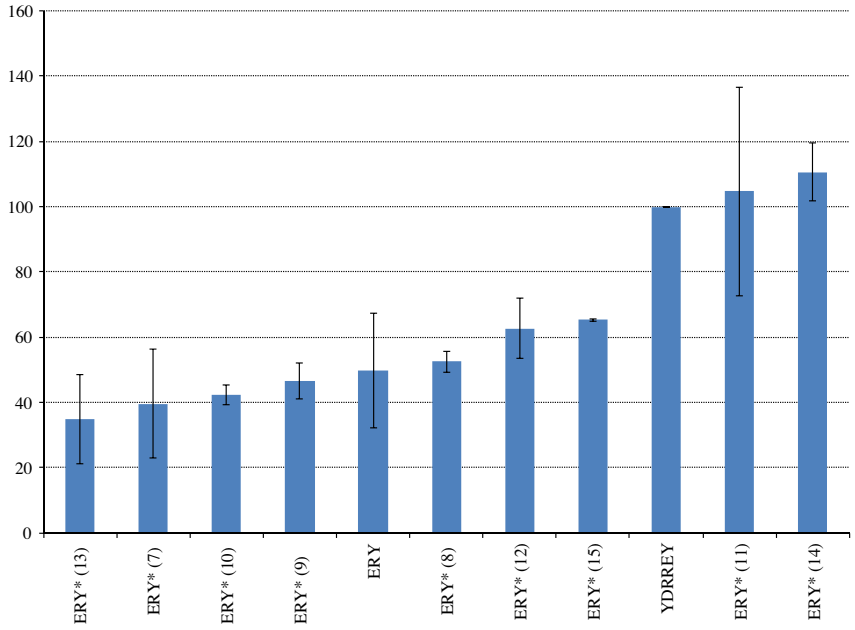



**Figure 2.** Percent of inhibition relative to biotin-r$_9$YDRREY of biotin-R$_8$ERY* peptides on $Mn^{2+}$-activated TK-1 cell adhesion to mouse MAdCAM-1-Fc (100 μM concentration).

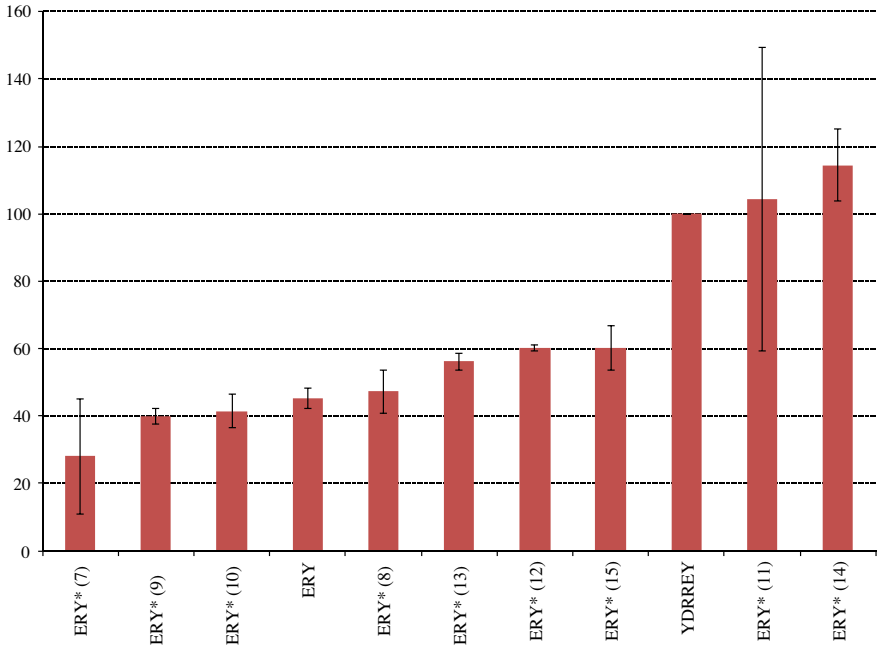



**Figure 3.** Percent of inhibition relative to biotin-r$_9$YDRREY of biotin-R$_8$ERY* peptides on $Mn^{2+}$-activated TK-1 cell adhesion to mouse MAdCAM-1-Fc (50 μM concentration).

reactions between unprotected propargylated peptides and sugar azides, using tris(carboxyethyl)phosphine (TCEP) as reducing agent, $CuSO_4$ in excess and a 6 M GnHCl/0.2 M $Na_2HPO_4$ solution. Applying these reaction conditions to the present case, propargylated peptide **6** was fully converted into click analogues **7**–**15** with the nine azides **7a**–**15a** within 30 min at 60 °C (microwave irradiation, 20 W) (Scheme 3). All products were obtained in sufficient purity and yield for biological studies after purification by RP-HPLC as shown in Table 1.

## 2.2. Biology

The α4β7$^+$TK-1 T cell line is unique in that it does not express β1 integrins and, hence can be used to measure binding to MAdCAM-1 independently of α4β1. In the present work, the synthetic β7 tripeptides were tested for their ability to block the adhesion of $Mn^{2+}$-activated mouse TK-1 cells to mouse MAdCAM-1 coated onto chamber slides at a peptide concentration of 50 and 100 μM (Figs. 2 and 3).

Peptides **7**, **9** and **10** containing protected and unprotected galactosamine moieties showed the lowest activity in the cell adhesion assay at both concentrations and are less potent inhibitors than the unmodified biotin-R$_8$ERY sequence. Peptide **13**, synthesised by the addition of 2-azidoacetic acid exhibited inconsistent results: at a concentration of 100 μM it was the least active compound, whereas at 50 μM it showed activity superior to **7**–**10**. Compounds **8** and **12**, bearing a protected and unprotected galactose moiety respectively, and peptide **15** exhibited greater activity

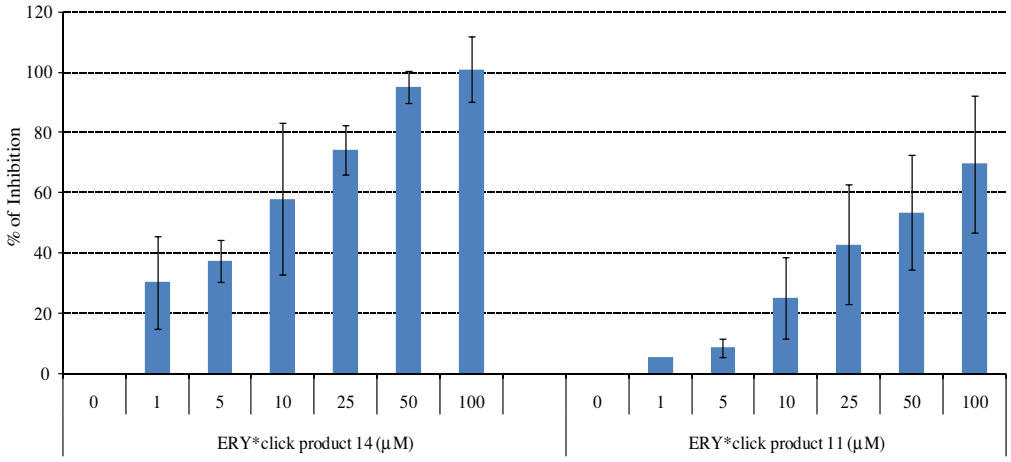



**Figure 4.** Dose response assays of peptides **11** and **14**.

than the unmodified biotin-$R_8$ERY, but were less active than biotin-$r_9$YDRREY at both concentrations. Finally, peptides **11** and **14** exhibited higher inhibition activity in the cell adhesion assay than all other compounds tested, with peptide **11** demonstrating a 5% higher level of inhibition than biotin-$r_9$YDRREY and approximately 60% greater activity than peptide **8** at 50 μM. The marked difference in activities of **8** and **11** appears to be due to the different points of attachment (C1 and C2 respectively) of the glycoside ring. The most active peptide **14** in this series, carrying an Fmoc protected alanine, displayed an improvement over biotin-$r_9$YDRREY by 15%. This is consistent with our previous SAR studies showing that large, non-polar residues connected to the 4-position of the tyrosine phenyl ring increase activity.

Dose response assays for the two most active peptides **11** and **14** have been carried out and indicate the $IC_{50}$ for **11** is 50 μM and that of **14** is <10 μM (Fig. 4).

## 3. Conclusion

A series of nine novel cell-permeable peptidomimetics incorporating the biotin-$R_8$ERY* motif were prepared from propargylated peptide **6** using the CuAAC reaction. The click analogues **7**–**15** were then tested for α4β7 antagonism in a lymphocyte cell-adhesion assay. Five of the peptidomimetics displayed potency greater than that of the unmodified biotin-$R_8$ERY peptide at a concentration of 100 μM and six at 50 μM. Two of the analogues, **11** and **14**, exhibited slightly higher potency than the biotin-$r_9$YDRREY at both concentrations tested, a dose response assay of the most active peptide **14** revealing an $IC_{50}$ value of less than 10 μM. Investigations to further improve the activity of the synthetic peptidomimetics building on these promising results are underway.

## Acknowledgment

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2012.02.035.

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