

# Cu-Catalyzed Formation of Triazole-Linked Glycoamino Acids and Application in Chemoenzymatic Peptide Synthesis

Brian H. M. Kuijpers,<sup>†,‡</sup> Stan Groothuys,<sup>†,‡</sup> Christine Hawner,<sup>§</sup> Jeroen ten Dam,<sup>†</sup> Peter J. L. M. Quaedflieg,<sup>§</sup> Hans E. Schoemaker,<sup>§</sup> Floris L. van Delft,<sup>†</sup> and Floris P. J. T. Rutjes<sup>\*,†</sup>

*Institute for Molecules and Materials, Radboud University Nijmegen, Toernooiveld 1, NL-6525 ED Nijmegen, The Netherlands, DSM Pharmaceutical Products - Advanced Synthesis, Catalysis & Development, P.O. Box 18, NL-6160 MD Geleen, The Netherlands*

## Abstract:

Novel stable triazole-linked glycoamino acids have been prepared, with the heterocyclic moiety being established by efficient Cu-mediated cycloaddition between the corresponding azido and acetylene moieties. Selected reactions were scaled up and successfully subjected to chemoenzymatic peptide-coupling reactions involving the proteolytic enzyme alcalase, resulting in several glycosylated di- and tripeptide structures. Since chemoenzymatic approaches have several advantages over chemical peptide coupling, especially concerning large-scale peptide synthesis, these results may be regarded as initial steps in the direction of production via fully chemoenzymatic peptide synthesis.

Naturally occurring glycosylated peptides play an important role in various biological processes and are therefore relevant lead molecules for the preparation of new drugs.<sup>1</sup> Numerous synthetic derivatives of glycopeptides have been prepared, and several of them have been reported to possess improved properties relative to the naturally occurring glycopeptides. The synthesis of natural glycopeptides is generally hampered by the sensitivity of the glycosidic linkage (viz. **1a** and **b**) toward acidic and basic conditions. In addition, the *O,O*- and *N,O*-acetal linkages in glycopeptides are prone to enzymatic cleavage of the carbohydrate moiety. A search for new, more stable glycopeptide mimics led to the development of *C*-linked isosteres,<sup>2</sup> providing excellent chemical and enzymatic stability without

negatively influencing the biological properties.<sup>3</sup> Our research in this field led to an expedient, high-yielding synthesis of triazole-linked glycopeptides via Cu(I)-catalyzed 1,3-dipolar cycloaddition (CuAAC)<sup>4,5</sup> of azido-functionalized glycosides and acetylenic amino acids, resulting in the novel class of stable glycopeptide mimics **2a**.<sup>6–8</sup> Inversely, we prepared similar glycopeptide derivatives **2b** using acetylenic sugars and azido-containing amino acids,<sup>6</sup> which were independently reported at the same time by the Dondoni group as well.<sup>9</sup> We also showed that replacement of the amide linkage in a glycoRGD peptide that selectively binds to tumor-associated  $\alpha_v\beta_3$ -integrin receptors can be readily replaced with a triazole-mimic without losing its biological activity.<sup>10</sup> In conjunction with these efforts and inspired by the possibilities for application of our molecules in medicinal research, we disclose recent efforts to expand the scope of the triazole-linking approach, including larger-scale preparation of new building blocks and initial studies toward the chemoenzymatic synthesis of selected oligopeptides containing triazole-linked glycoamino acids.

In order to enlarge the scope of the Cu-catalyzed 1,3-dipolar cycloaddition to triazole-linked glycoamino acids, we initially focused on making new combinations of azidosugars and acetylenic amino acids in which the protecting groups were varied (Table 1). First of all, 1-azido-2,3,4,6-tetra-*O*-acetyl- $\beta$ -D-glucopyranose (**3**) was condensed with Cbz-protected (*S*)-

\* Author for correspondence. E-Mail: F.Rutjes@science.ru.nl.

<sup>†</sup> Radboud University Nijmegen.

<sup>‡</sup> DSM Pharmaceutical Products.

<sup>§</sup> Both authors equally contributed to this manuscript.

- (1) (a) Bertozzi, C. R.; Kiessling, L. L. *Science* **2001**, *291*, 2357. (b) Davis, B. G. *Chem. Rev.* **2002**, *102*, 579. (c) Guo, Z. W.; Nakahara, Y.; Nakahara, Y.; Ogawa, T. *Carbohydr. Res.* **1997**, *303*, 373. (d) Kihlberg, J.; Ahman, J.; Walse, B.; Drakenberg, T.; Nilsson, A.; Soderbergahlm, C.; Bengtsson, B.; Olsson, H. *J. Med. Chem.* **1995**, *38*, 161. (e) Kobata, A. *Glycoprotein Glycan Structures in Comprehensive Glycoscience*, Kamerling, J. P., Ed.; Elsevier: Dordrecht, 2007, Vol. 1, pp 39–72.
- (2) (a) Dondoni, A.; Marra, A. *Chem. Rev.* **2000**, *100*, 4395. (b) Dondoni, A.; Mariotti, G.; Marra, A.; Massi, A. *Synthesis* **2001**, 2129. (c) Mizuno, M. *Trends Glycosci. Glycotechnol.* **2001**, *13*, 11. (d) Palomo, C.; Oiarbide, M.; Landa, A.; Gonzalez-Rego, M. C.; Garcia, J. M.; Gonzalez, A.; Odriozola, J. M.; Martin-Pastor, M.; Linden, A. *J. Am. Chem. Soc.* **2002**, *124*, 8637. (e) Turner, J. J.; Leeuwenburgh, M. A.; van der Marel, G. A.; van Boom, J. H. *Tetrahedron Lett.* **2001**, *42*, 8713. (f) Vincent, S. P.; Schleyer, A.; Wong, C. H. *J. Org. Chem.* **2000**, *65*, 4440. (g) Westermann, B.; Walter, A.; Florke, U.; Altenbach, H. *J. Org. Lett.* **2001**, *3*, 1375. (h) Xu, X.; Fakha, G.; Sinou, D. *Tetrahedron* **2002**, *58*, 7539.

- (3) (a) Bertozzi, C. R.; Bednarski, M. D. *Tetrahedron Lett.* **1992**, *33*, 3109. (b) Bertozzi, C. R.; Cook, D. G.; Kobertz, W. R.; Gonzalezscarano, F.; Bednarski, M. D. *J. Am. Chem. Soc.* **1992**, *114*, 10639.
- (4) (a) For original publications on the CuAAC, see: Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2002**, *41*, 2596. (b) Tornøe, C. W.; Christensen, C.; Meldal, M. *J. Org. Chem.* **2002**, *67*, 3057.
- (5) (a) For reviews, see Bock, V. D.; Hiemstra, H.; van Maarseveen, J. H. *Eur. J. Org. Chem.* **2005**, *51*. (b) Wu, P.; Fokin, V. V. *Aldrichimica Acta* **2007**, *40*, 7. (c) Dirks, A. J.; Cornelissen, J. J. L. M.; van Delft, F. L.; van Hest, J. C. M.; Nolte, R. J. M.; Rowan, A. E.; Rutjes, F. P. J. T. *QSAR Comb. Sci.* **2007**, *26*, 1200.
- (6) Kuijpers, B. H. M.; Groothuys, S.; Keereweere, A. R.; Quaedflieg, P. J. L. M.; Blaauw, R. H.; van Delft, F. L.; Rutjes, F. P. J. T. *Org. Lett.* **2004**, *6*, 3123.
- (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.; Rutjes, F. P. J. T. *Synthesis* **2006**, 3146.
- (8) Kuijpers, B. H. M.; Dijkmans, G. C. T.; Groothuys, S.; Quaedflieg, P. J. L. M.; Blaauw, R. H.; van Delft, F. L.; Rutjes, F. P. J. T. *Synlett* **2005**, 3059.
- (9) Dondoni, A.; Giovannini, P. P.; Massi, A. *Org. Lett.* **2004**, *6*, 2929.
- (10) Kuijpers, B. H. M.; Groothuys, S.; Soede, A. C.; Laverman, P.; Boerman, O. C.; van Delft, F. L.; Rutjes, F. P. J. T. *Bioconjugate Chem.* **2007**, *18*, 1847–1854.

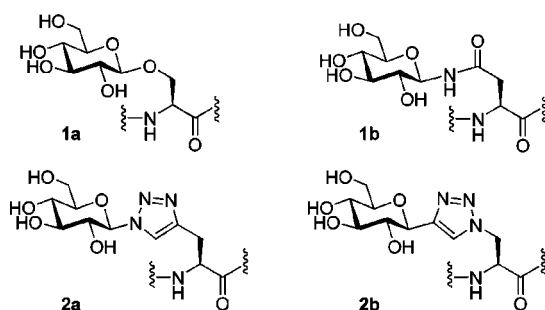
**Table 1.** Scope of CuAAC to prepare *N*-triazole-linked glycosidic amino acids and peptides

entry	glycosyl azide	amino acid derivative	glycopeptide <sup>a,b</sup> (yield)
1	3: X = OAc Y = OAc	6	13 (50%)
2	3	7	14 (87%)
3	4: X = OH Y = NHAc	8	15 (n.d.)
4	4	9	16 (53%)
5	3	10	17 (0%)
6	5: X = OAc Y = NHAc	11	18 (86%)
7	3	12	19 (55%) <sup>c</sup>

<sup>a</sup> Reagents and conditions: azidoglycoside (1 equiv), amino acid derivative (1 equiv), Cu(OAc)<sub>2</sub> (0.2 equiv), sodium ascorbate (0.4 equiv), H<sub>2</sub>O/*tert*-BuOH 1:1 (v/v), rt, 16 h. <sup>b</sup> Yield of isolated product. <sup>c</sup> 2 equiv of azidoglycoside was used.

propargylglycine methyl ester (**6**),<sup>11</sup> affording the Cbz-protected *N*-triazolyl glycoamino acid **13** in a moderate yield (entry 1).

(11) Throughout this article, trivial names of amino acids have been used; e.g. propargylglycine is 2-amino-4-pentynoic acid

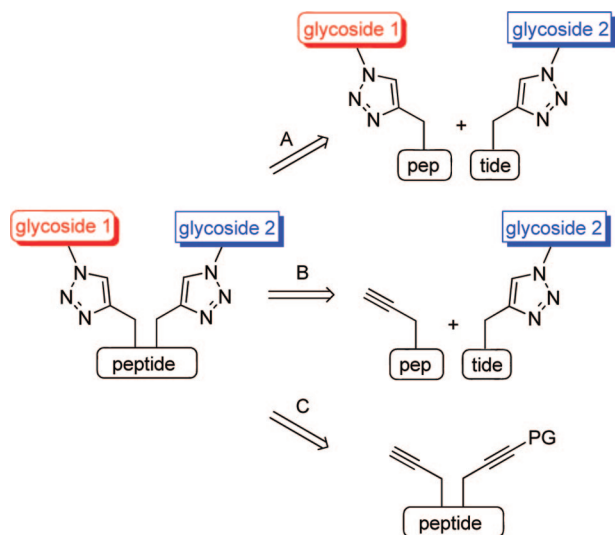


**Figure 1.** Triazole mimics **2** of acetal-linked glycopeptides **1**.

To verify the feasibility of scaling up, this reaction was also carried out on 5-g scale, providing product **13** in a similar yield. Not surprisingly, cycloaddition of racemic *N*s-protected propargylglycine *tert*-butyl ester **7** proceeded readily, affording **14** in a high yield of 87%. For large-scale application it was of interest to examine the use of unprotected carbohydrates. Thus, azidoglucosamine **5** was saponified using K<sub>2</sub>CO<sub>3</sub> in MeOH, affording the *O*-unprotected azidosugar **4**. Fortunately, Cu(I)-catalyzed cycloaddition of this with the unprotected sugar and (*R*)-*N*-Boc-propargylglycine methyl ester (**8**, entry 3) also proceeded smoothly, leading to quantitative formation of the desired compound **15** as indicated by TLC. However, due to its high water solubility, compound **15** could not be isolated in sufficiently purified form. The unprotected sugar **4** also reacted efficiently with the carboxylic acid-containing propargylglycine derivative **9**, again giving rise to full conversion to **16** as shown by TLC (entry 4). Purification of **16** was effected with ion-exchange chromatography, albeit in moderate yield (53%). In contrast, the free amino acid **10** (entry 5) could not be coupled under these conditions, probably due to bidentate coordination of copper(I) ions to the amino and carboxylate groups. This was underlined by the fact that subjection of protected glucosamine **5** to Boc-propargylglycine (**11**) proceeded readily, forming the desired glycosidic amino acid **18** in 86% yield (entry 6). For the assembly of more complex triazolyl glycopeptides, it is essential that CuAAC is not limited to single amino acids, but can be efficiently extended to the preparation of multiple carbohydrate-bearing glycopeptides. To this end, dipeptide **12** containing two adjacent propargylglycine moieties was reacted with azidoglucose derivative **3** (2 equiv) leading to the formation of the diglycosylated peptide **19** in a reasonable yield of 55% (entry 7).

Encouraged by these findings, we investigated whether the approach could be extended to introduce different glycosyl moieties into a peptide, particularly since more than 50% of the known proteins in nature are heavily glycosylated with different carbohydrate moieties.<sup>12</sup> Conceptually, three strategies can be envisaged to prepare peptides bearing multiple carbohydrates; these involve (A) peptide coupling of two separately synthesized triazole-linked glycopeptide building blocks (Figure 2), (B) incorporation of an acetylenic amino acid in a peptide already containing one glycoamino acid in the chain, followed by 1,3-dipolar cycloaddition, or (C) synthesis of a peptide containing both a terminal and a protected acetylene, allowing sequential 1,3-dipolar cycloaddition, acetylene deprotection, and another 1,3-dipolar cycloaddition.

(12) Dwek, R. A. *Chem. Rev.* **1996**, *96*, 683.



**Figure 2.** Strategies for the preparation of peptides containing multiple triazole-linked carbohydrate moieties.

While methods A and B may have advantages for preparing a small number of glycosylated oligopeptides, method C could be useful for combinatorial library synthesis. In that case, a large set of peptides could be prepared wherein a sequential protocol can be functionalized with an orthogonal set of azido-functionized glycoside derivatives.

The potential of a stepwise introduction of carbohydrates via 1,3-dipolar cycloaddition was investigated starting from the diacetylenic dipeptide **20** (Scheme 1). This dipeptide was prepared by condensing racemic Me<sub>3</sub>Si-protected propargylglycine methyl ester with commercially available Boc-(S)-propargylglycine via standard peptide-coupling chemistry (PyBOP, DiPEA). Initial cycloaddition with tetra-*O*-benzyl azidoglucoside under the typical Cu(OAc)<sub>2</sub>/Na-ascorbate conditions led, as anticipated, to a smooth and clear introduction of a single carbohydrate moiety, leaving the trimethylsilyl function fully unaffected. Subsequent desilylation with K<sub>2</sub>CO<sub>3</sub> in methanol proceeded readily. A second 1,3-dipolar cycloaddition, this time involving tetra-*O*-acetyl azidoglucose **3**, afforded the triazole-linked diglycosylated peptide **21** in a satisfactory overall yield of 77%. Currently, we aim at further expanding the scope of this particular application, more specifically by preparing libraries of glycosylated peptides via a solid-phase strategy.

Apart from the preparation of *N*-triazole-linked glycoamino acids **2a**, cycloaddition of acetylenic sugars with amino acid azides giving rise to glycoaminoacids of type **2b** was also feasible (Table 2). Thus, Cbz-protected L-azidoalanine methyl ester (**24**) was condensed with 1-ethynyl-2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose (**22**) producing glycoamino acid methyl ester **30** in a near quantitative yield (entry 1). In addition, coupling of 1-ethynyl-2,3,4,6-tetra-*O*-benzyl-β-D-glucopyranose (**23**) to Fmoc-protected L-azidoalanine (**25**) and the homologous L-4-azidobutylglycine (**26**) proceeded uneventfully without significant difference in yields between the two amino acid derivatives (entries 2 and 3). Interestingly, cycloaddition of benzyl-protected glycoside **23** with the diastereomeric pipecolic acid derivatives **27** and **28** was also feasible, although with lower efficiencies

(entries 4 and 5). The somewhat higher yield for **28** with respect to **27** may be rationalized by the respective equatorial or axial orientation of the azide, with the latter causing more steric hindrance.

Unfortunately, the 6-azidopipecolic acid derivative **29** failed to undergo cycloaddition, neither under the influence of Cu(OAc)<sub>2</sub>/sodium ascorbate nor in the presence of CuI and base. Presumably, steric hindrance from the neighboring Cbz protecting group in combination with 2,6-diaxial interaction of the methyl ester precluded sufficient access to the azide.

Chemical synthesis of glycopeptides becomes more complicated when carbohydrate and amino acid moieties become larger and the synthesis proceeds via multiple protection and deprotection steps, especially when processes have to be scaled up. In order to circumvent the problems that are associated with large-scale peptide synthesis, such as partial epimerization of stereocenters and use of high loadings of expensive coupling reagents, chemoenzymatic peptide synthesis could be a potential solution. As a result, there has been an increased interest in chemoenzymatic peptide synthesis over the past decade.<sup>13</sup> Chemoenzymatic synthesis is characterized by a number of specific advantages over chemical synthesis of peptides such as (a) chemoenzymatic peptide-coupling reactions are free from racemization, (b) side-chain protection can be mostly omitted, (c) the coupling reagent (enzyme) can often be recycled, (d) reactions proceed under mild conditions, and finally (e) conditions are environmentally friendly.

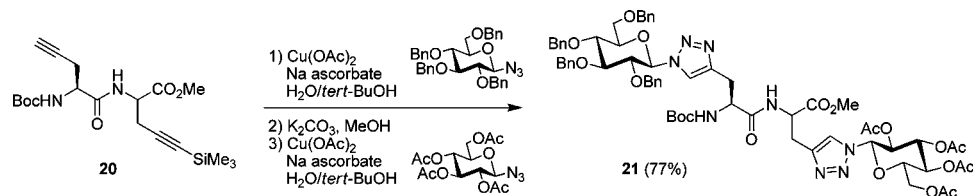
It was demonstrated by Chen et al. that alcalase, a cheap and commercially available proteolytic enzyme mixture produced by *Bacillus licheniformis*, containing subtilisin Carlsberg as the major enzyme component, can be conveniently applied for enzymatic peptide bond formation.<sup>14,15</sup> In addition, alcalase was found to be stable in a variety of organic solvents, retaining more than 65% of the original activity in *tert*-amyl alcohol after 10 days. On the basis of these results, we set out to investigate the chemoenzymatic synthesis of dipeptides via C-terminal elongation of 3-azidopropylglycine **36a**, propargylglycine **36b**, and a bulky glycosylated triazolyl-containing amino acid methyl ester **36c** with a proteinogenic amino acid amide (Table 3).<sup>7</sup> Much to our delight, alcalase-mediated coupling proceeded smoothly for both protected L-azidoornithine **36a** and L-propargylglycine **36b** with either phenylalaninamide or glycineamide, providing the desired dipeptides **37a,b** and **38a,b** in excellent yield (entries 1–2 and 4–5). Interestingly, the chemoenzymatic coupling reactions proceeded with considerably higher yields compared to the chemical peptide ligation, although it has to be noted that one equivalent of amide was added in the chemical synthesis, whereas 4 equiv was used in the chemoenzymatic ligation. On the other hand, for the triazole-linked glycosylated amino acid **36c** the chemical coupling procedure was required since chemoenzymatic coupling failed to afford the desired products **37c** and **38c** in good yields (entries 3 and

(13) Sergeeva, M. V.; Paradkar, V. M.; Dordick, J. S. *Enzyme Microb. Technol.* **1997**, *20*, 623.

(14) Chen, S. T.; Chen, S. Y.; Wang, K. T. *J. Org. Chem.* **1992**, *57*, 6960.

(15) (a) Chen, S. T.; Chen, S. Y.; Hsiao, S. C.; Wang, K. T. *Biotechnol. Lett.* **1991**, *13*, 773. (b) Chen, S. T.; Hsiao, S. C.; Wang, K. T. *Bioorg. Med. Chem. Lett.* **1991**, *1*, 445. (c) Chen, S. T.; Chen, S. Y.; Tu, C. C.; Chiou, S. H.; Wang, K. T. *J. Protein Chem.* **1995**, *14*, 205.

# Scheme 1. Preparation of a diglycosylated triazole linked glycopeptide



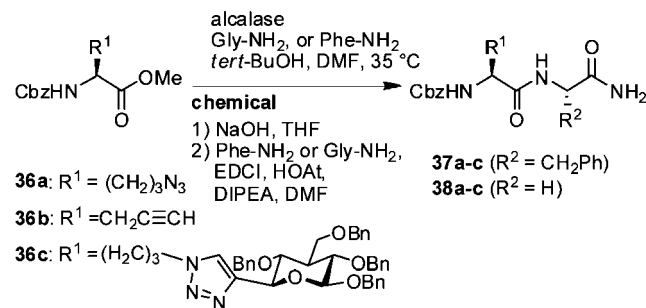
**Table 2.** Scope of CuAAC to prepare C-triazole-linked glycoamino acids

entry	gluco acetylene	amino acid derivative	glycopeptide <sup>a,b</sup> (yield)
1	22: X = OAc	24	30 (96%)
2	23: X = OBn	25	31 (69%)
3	23	26	32 (71%)
4	23	27	33 (49%)
5	23	28	34 (60%)
6	23	29	35 (0%) <sup>c</sup>

<sup>a</sup> Reagents and conditions: glucoacetylene (1 equiv); azidoamino acid derivative (1 equiv); Cu(OAc)<sub>2</sub> (0.2 equiv); sodium ascorbate (0.4 equiv); H<sub>2</sub>O/*tert*-BuOH, 1:1 (v/v); rt; 16 h. <sup>b</sup> Yield of isolated product. <sup>c</sup> Ligation was also attempted by subjection to CuI and 2,6-lutidine in acetonitrile.

6). In fact, HPLC analysis showed only 40% conversion after 8 days, resulting in an isolated yield of approximately 10%. Nevertheless, the findings that glycosylated triazole-linked amino acid acyl donors appeared substrates for chemoenzymatic ligation was taken as an encouragement, since it was anticipated that replacement of the bulky hydrophobic benzyl protecting groups of **36c** with less hindered acetyl functions (viz. **39**) would

**Table 3.** Chemical and chemoenzymatic coupling enzymatic



entry	acyl donor	nucleophile	chemical yield(%)	enzymatic yield(%)	product
1	<b>36a</b>	Phe-NH <sub>2</sub>	69	77	<b>37a</b>
2	<b>36b</b>		55	79	<b>37b</b>
3	<b>36c</b>		86	40 <sup>a</sup>	<b>37c</b>
4	<b>36a</b>	Gly-NH <sub>2</sub>	80	93	<b>38a</b>
5	<b>36b</b>		60	80	<b>38b</b>
6	<b>36c</b>		82	10	<b>38c</b>

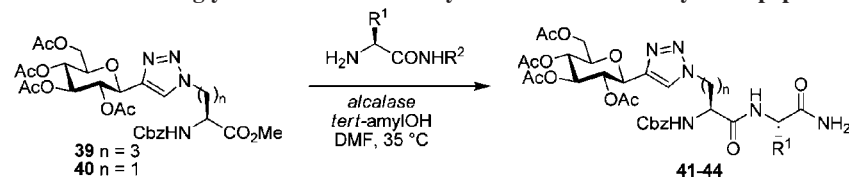
<sup>a</sup> Conversion of the acyl donor as determined by HPLC.

lead to an increase in enzyme activity. As expected, a small increase in yield compared to benzyl protection was accomplished (Table 4, entries 1 and 2), although the isolated yields were far from satisfactory. Much to our surprise, shortening of the side chain (**40**, *n* = 1) resulted in significantly enhanced coupling with glycine- and phenylalanine-amide to give the desired dipeptides **43** and **44** after prolonged stirring (entries 4 and 5). Interestingly, the coupling of phenylalanine-amide with glycoamino acid **40** proceeded in near quantitative yield to form **44**, while the coupling with glycineamide was slightly more troublesome, but still led to the desired dipeptide **43** in a satisfactory yield. The dipeptides **43** and **44** were isolated after crystallization in 65% and 98% yield, respectively. The chemoenzymatic coupling to **43** was monitored closely with RP-HPLC, which showed that in this particular case an unknown impurity accumulated in the reaction mixture during the 15 days stirring, eventually amounting to 34%.

In addition, application of *N*-triazole-linked glycoamino acid methyl esters (<sup>N</sup>TGA) **45** and **46** in chemoenzymatic coupling experiments with glycine- and phenylalanine amide also provided enhanced yields as compared to the bulky benzylated triazole-linked substrate **36c** (Table 5). We were delighted to find that upon ligation with glycineamide the desired dipeptide **47** was formed smoothly and isolated in nearly quantitative yield after 6 days reaction time (entry 1).

A less satisfactory result was obtained when the same methyl ester **45** was condensed with phenylalanine amide as a nucleophile, resulting in the isolation of dipeptide **48** in a rather poor 30% yield along with several byproducts. Unfortunately, the

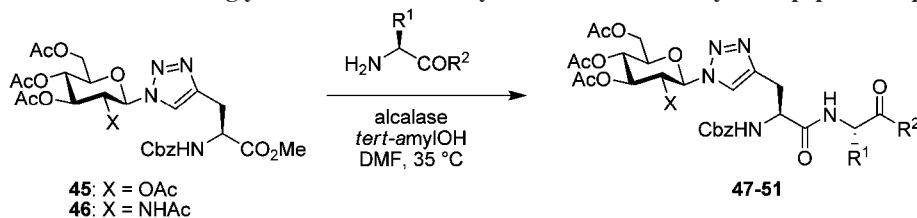
**Table 4.** Application of *C*-triazole-linked glycosidic amino acid acyl donors in chemoenzymatic peptide-coupling experiments



entry	R <sup>1</sup>	<i>n</i>	solvent	time(days)	product	HPLC yield <sup>a</sup> (%)	isolated yield (%)
1	H	3	<i>tert</i> -amylOH	9	<b>41</b>	34	n.d.
2	Bn	3	<i>tert</i> -amylOH	9	<b>42</b>	27	n.d.
4	H	1	<i>tert</i> -BuOH/DMF	15	<b>43</b>	66	65
5	Bn	1	<i>tert</i> -BuOH/DMF	6	<b>44</b>	94	98

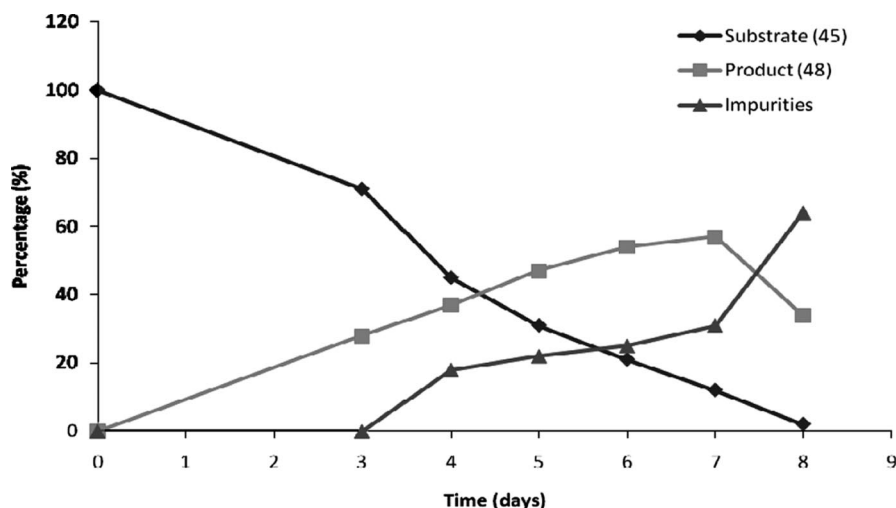
<sup>a</sup> Optimum HPLC yield.

**Table 5.** Application of *N*-triazole-linked glycosidic amino acid acyl donors in chemoenzymatic peptide-coupling reactions



entry	substrate	R <sup>1</sup>	R <sup>2</sup>	time (days)	product	HPLC yield (%)	isolated yield (%)
1	<b>45</b>	H	NH <sub>2</sub>	6	<b>47</b>	100 <sup>a,b</sup>	98
2	<b>45</b>	Bn	NH <sub>2</sub>	8	<b>48</b>	57 <sup>a</sup> (34) <sup>b</sup>	30
3	<b>45</b>	Bn	Val-NH <sub>2</sub>	15	<b>49</b>	53 <sup>a</sup>	n.d.
4	<b>46</b>	H	NH <sub>2</sub>	11	<b>50</b>	31 <sup>a</sup>	n.d.
5	<b>46</b>	Bn	NH <sub>2</sub>	21	<b>51</b>	73 <sup>a</sup>	n.d.

<sup>a</sup> Optimum HPLC yield. <sup>b</sup> HPLC yield before quenching and isolation.

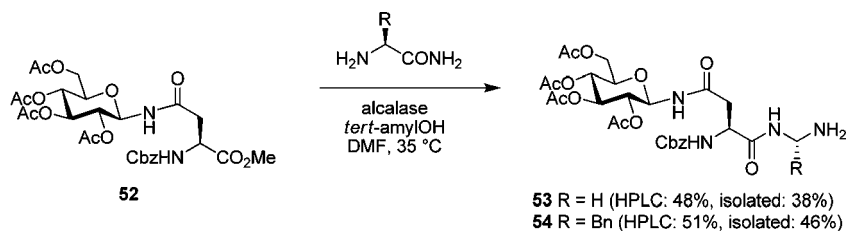


**Figure 3.** Reaction profile of triazole-linked glycosidic amino acid **45** with glycine.

identity of the impurities could not be established, although it was clear that enzymatic hydrolysis of the starting material was not an issue. Since glycine was added to the reaction mixture in excess, formation of a tripeptide (<sup>N</sup>TGA-Gly-Gly-NH<sub>2</sub>) might be one of the possibilities.

A closer inspection of the reaction profile with HPLC, however, showed that the reaction initially advanced slowly leading to a maximum yield of 57% after 7 days, along with 30% of unidentified side products (Figure 3). Only after this time, the formation of side products increased rapidly with

concomitant decrease in yield of dipeptide **48** to 34% as judged by HPLC resulting in the 30% isolated yield (Table 5, entry 2). Next, a slightly more challenging ligation was carried out, involving addition of the dipeptide nucleophile H-Phe-Val-NH<sub>2</sub> to methyl ester **45** in order to prepare tripeptide **49** (entry 3). It was rewarding to find that alcalase-mediated ligation led to an HPLC yield of tripeptide **49** of 53%, thereby clearly showing that the chemoenzymatic coupling is not restricted to formation of dipeptides. Next, the fully acetylated glucosamine-containing triazolyamino acid **46** was utilized as acyl donor. Interestingly,

**Scheme 2.** Application of amide-linked glycosidic amino acid acyl donors in chemoenzymatic peptide-coupling experiments

contrasting results were encountered with respect to condensation of glycynamide (entry 4) and phenylalaninamide (entry 5) as compared to the glucose-containing donor **45**. While compound **51** employing phenylalanine amide resulted in an HPLC yield of 73% (entry 5), reaction of **50** with glycynamide as the nucleophile led to a poor yield of 31% (entry 4). Although some solubility problems were encountered during the latter chemoenzymatic ligations, we currently have no satisfactory explanation for the unexpected reversal in yield between substrates **45** and **46**.

Finally, a condensation of the naturally occurring amide-linked glycoamino acid **52** with glycynamide and phenylalaninamide was performed under identical conditions in the presence of alcalase (Scheme 2). In the case of this particular substrate, both nucleophiles reacted similarly, reaching an optimum yield of approximately 50% (HPLC) for both dipeptides **53** and **54**. The respective dipeptides were eventually isolated in yields that were only slightly lower than the indicated HPLC values.

In conclusion, we have shown that the Cu-mediated cycloaddition between azides and acetylenes can be readily extended to a large variety of suitably protected and partially unprotected sugar and amino acid derivatives, resulting in the corresponding glycoamino acid derivatives. In addition, for selected substrates we have shown that the cycloaddition step can be scaled up to prepare larger, multigram quantities of the glycoamino acids without notable problems. Moreover, a stepwise introduction of azidosugars onto an orthogonally acetylene-protected peptide was successfully achieved providing the desired differentially diglycosylated peptide in a high yield. This proof of concept shows that this sequential approach may also be successful in the generation of larger libraries of multiple glycosylated peptide structures. Currently, we aim at further expanding the scope of the latter application, more specifically by preparing the glycosylated peptides on a resin. As a starting point to large-scale chemoenzymatic peptide synthesis, various alcalase-catalyzed peptide-coupling reactions were performed, involving several triazole-linked glycoamino acids as acyl donors and glycynamide, phenylalaninamide, and a dipeptide as nucleophiles. Although the coupling reactions proceeded relatively slowly, presumably due to low solubility of the substrates and/or steric factors, the glycoamino acid derivatives could be successfully incorporated to form the corresponding di- and tripeptides.

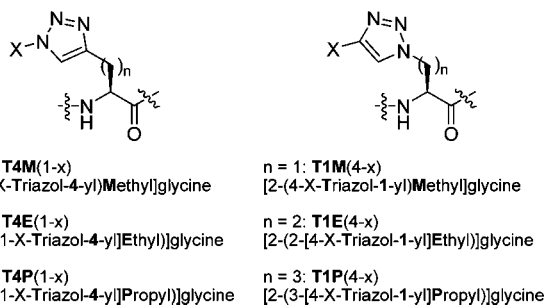
## Experimental Section

**General Information.** Thermal analysis of representative azide-containing compounds has shown that upon warming exothermic decomposition may take place at onset temperatures

lying between 70 and 150 °C. As an example, DSC measurements showed that compound **3** has an onset temperature of 140 °C, at which a strongly exothermic reaction takes place. Therefore, special caution has to be taken when handling these compounds.

Reactions were followed, and  $R_f$  values were obtained using thin layer chromatography (TLC) on silica gel-coated plates (Merck 60 F254) with the indicated solvent mixture. Detection was performed with UV light, and/or by charring at ~150 °C after dipping into a solution of either 2% anisaldehyde in ethanol/ $\text{H}_2\text{SO}_4$  or  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$  (25 g/L) and  $(\text{NH}_4)_4\text{Ce}(\text{SO}_4)_4 \cdot 2\text{H}_2\text{O}$  (10 g/L) in 10%  $\text{H}_2\text{SO}_4$ . IR spectra were recorded on an ATI Mattson Genesis Series FTIR spectrometer, or a Bruker Tensor 27 FTIR spectrometer. NMR spectra were recorded on a Bruker DMX 300 (300 MHz), and a Varian 400 (400 MHz) spectrometer. Chemical shifts are given in ppm with respect to tetramethylsilane (TMS) as internal standard. Coupling constants are reported as  $J$  values in hertz. Column or flash chromatography was carried out using ACROS silica gel (0.035–0.070 mm, and ~6 nm pore diameter). High-resolution mass spectra were recorded on a JEOL AccuTOF (ESI) or a MAT900 (EI, CI, and ESI). Alcalase type DX PLN04842 was purchased from Novozymes (Denmark) as a brown liquid with a specific activity of 2.5 U/mL. For the hydrolysis experiments this enzyme solution was used as such. For the (anhydrous) peptide-coupling reactions the water in the alcalase solution was removed using the method of Chen et al.<sup>14</sup> As a matter of fact, the aqueous alcalase (0.25 mL) and absolute ethanol (0.5 mL) were mixed in an Eppendorf cup, and the resulting suspension was agitated on a Vortex mixer for 5 min and centrifuged (3000 rpm) for 10 min to spin down the enzyme. The supernatant was decanted and the enzyme resuspended in absolute EtOH (0.5 mL), agitated for 5 min on a Vortex mixer, and spun down by centrifugation. This procedure was repeated once with absolute EtOH (0.5 mL) and finally once with dry *tert*-amylOH (0.5 mL). The resulting enzyme was resuspended in *tert*-amylOH (1.0 mL) for use in the chemoenzymatic peptide-coupling reactions. All other commercially available reagents were used as received. HPLC analyses were performed with an Inertsil RP-18 column (25 cm  $\times$  0.46 cm, RP-18, 5  $\mu\text{m}$ ).

**Nomenclature of the Triazole-Linked Glycosidic Amino Acids.** For the sake of clarity the names of the peptides were abbreviated, applying the standard three-letter codes for natural amino acids. For the nonproteinogenic amino acids we derived a code as depicted in Figure 4. For example, T4M stands for a triazol-4-yl-methylglycine-containing amino acid and triazol-1-yl-butylglycine was abbreviated as T1B. Any substituents are presented in brackets.



**Figure 4.** Nomenclature of the triazole-linked glycosidic amino acids.

**General Procedure A.** To a solution of the acetylene (1 equiv) and azide derivative (1 equiv) in *tert*-butyl alcohol (0.5 M) was added a mixture of  $\text{Cu}(\text{OAc})_2$  (20 mol %) and sodium ascorbate (40 mol %) in  $\text{H}_2\text{O}$  (0.04 and 0.08 M, respectively). The reaction was stirred overnight, water was added, and the product was extracted with  $\text{EtOAc}$  (2 $\times$ ). The combined organic layers were washed with water, dried ( $\text{Na}_2\text{SO}_4$ ), and concentrated. The product was purified by flash chromatography using  $\text{EtOAc}$ /heptane mixtures.

**Chz-L-T4M(1-[ $\beta$ -D-Glc(Ac)<sub>4</sub>])OMe (13).** Preparation according to general procedure A afforded **13** (5.23 g, 8.25 mmol, 50%) as a white solid. FTIR (ATR):  $\nu$  3373, 2950, 1740, 1212  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.55 (s, 1H), 7.38–7.28 (m, 5H), 5.82 (d,  $J = 9.1$  Hz, 2H), 5.44–5.30 (m, 2H), 5.21 (dd,  $J = 10.1$ , 9.2 Hz, 1H), 5.09 (s, 2H), 4.75–4.65 (m, 1H), 4.28 (dd,  $J = 12.6$ , 5.0 Hz, 1H), 4.12 (dd,  $J = 12.6$ , 2.1 Hz, 1H), 3.98 (ddd,  $J = 10.1$ , 5.0, 2.1 Hz, 1H), 3.73 (s, 3H), 3.27 (d,  $J = 4.9$  Hz, 2H), 2.05 (s, 3H), 2.05 (s, 3H), 2.00 (s, 3H), 1.81 (s, 3H).  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ):  $\delta$  171.5, 170.5, 169.9, 169.4, 169.2, 156.0, 143.5, 136.4, 128.6, 128.2, 120.5, 85.8, 75.2, 72.6, 70.3, 67.8, 67.1, 61.6, 53.3, 52.7, 28.1, 20.8, 20.6, 20.6, 20.1. HRMS (ESI): calculated for  $\text{C}_{28}\text{H}_{34}\text{N}_4\text{O}_{13}\text{Na}$  ( $M + \text{Na}$ )<sup>+</sup> 657.2020, found 657.2025.

**Ns-T4M(1-[ $\beta$ -D-Glc(Ac)<sub>4</sub>])O-*tert*-Bu (14).** Preparation according to general procedure A afforded **14** (66 mg, 0.09 mmol, 87%) as a mixture of isomers (1:1).  $R_f = 0.26$  ( $\text{EtOAc}$ /heptane, 1/1). FTIR (ATR):  $\nu$  3369, 1735, 1709, 1221  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  8.31 and 8.29 (ap d,  $J = 9.1$  Hz, 2H), 8.02 and 8.00 (ap d,  $J = 9.1$  Hz, 2H), 9.79 and 7.61 (s, 1H), 6.01 and 5.91 (d,  $J = 9.7$  and 9.0 Hz, 1H), 5.83 and 5.78 (d,  $J = 9.1$  and 8.9 Hz, 1H), 5.47–5.33 (m, 2H), 5.24 (dd,  $J = 19.4$ , 9.3 Hz, 1H), 4.31 and 4.28 (dd,  $J = 12.7$ , 4.9 Hz, 1H), 4.24–4.20 (m, 1H), 4.19–4.14 (m, 1H), 4.02 and 4.01 (ddd,  $J = 7.5$ , 5.0, 1.9 and 6.5, 5.2, 1.9, 1H), 3.35 and 3.25 (ABdd,  $J = 14.6$ , 4.1 and 15.3, 5.3, 1H), 3.20 and 3.14 (ABdd,  $J = 14.6$ , 4.7 and 15.3, 4.8, 1H), 2.09 (s, 3H), 2.06 (s, 3H), 2.06 (s, 3H), 2.06 (s, 3H), 2.04 (s, 3H), 2.03 (s, 3H), 2.02 (s, 3H), 1.98 (s, 3H), 1.86 (s, 3H), 1.27 (s, 9H), 1.26 (s, 9H).  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ):  $\delta$  170.3, 170.2, 170.0, 169.6, 169.6, 169.3, 169.0, 169, 168.9, 167.9, 149.9, 149.8, 145.9, 145.9, 142.5, 142.0, 128.4, 124.1, 124.0, 121.7, 120.7, 85.9, 85.8, 83.5, 83.4, 75.4, 75.3, 72.5, 72.1, 70.9, 70.7, 68.0, 67.8, 61.7, 61.6, 60.6, 55.6, 55.6, 30.1, 29.9, 28.0, 28.0, 21.0, 21.0, 20.9, 21.8, 20.5, 20.4, 14.5, 14.5. HRMS (ESI): calculated for  $(\text{C}_{29}\text{H}_{37}\text{O}_{15}\text{N}_5)_2\text{Na}$  ( $2M + \text{Na}$ )<sup>+</sup> 1477.3911, found 1477.3820.

**Boc-L-T4M(1-[ $\beta$ -D-GlcNAc])OH (16).** Preparation according to general procedure A afforded **16** (22 mg, 0.05 mmol,

53%) as a white solid. FTIR (ATR):  $\nu$  3347, 2971, 2933, 2358, 2340, 2111  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.92 (s, 1H), 5.77 (d,  $J = 9.8$  Hz, 1H), 4.44–4.37 (m, 1H), 4.17 (t,  $J = 9.9$  Hz, 1H), 3.89 (dd,  $J = 12.3$  Hz, 1H), 3.78–3.67 (m, 3H), 3.61–3.43 (m, 2H), 3.22 (dd,  $J = 14.9$  Hz, 1H), 3.12 (dd,  $J = 14.9$  Hz, 1H), 1.79 (s, 3H), 1.42 (s, 9H).  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ):  $\delta$  175.3, 173.5, 157.7, 144.8, 123.0, 88.0, 81.3, 80.6, 75.6, 71.5, 62.4, 56.9, 54.9, 29.1, 28.7, 22.6. LRMS calculated for  $\text{C}_{18}\text{H}_{29}\text{N}_5\text{O}_9$  ( $M - \text{H}$ )<sup>+</sup>: 458.2, found 458.3.

**Boc-L-T4M(1-[ $\beta$ -D-GlcNAc(Ac)<sub>3</sub>])OH (18).** Preparation according to general procedure A afforded **18** (1.25 g, 2.14 mmol, 86%) as a white amorphous solid. FTIR (ATR):  $\nu$  1744, 1364, 1213  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.75 (s, 1H), 6.48 (br d,  $J = 9.6$  Hz, 1H), 5.95 (d,  $J = 10.0$  Hz, 1H), 5.62 (d,  $J = 8.0$  Hz, 1H), 5.39 (dd,  $J = 10.8$ , 10.0 Hz, 1H), 5.24 (t,  $J = 9.6$  Hz, 1H), 4.71–4.55 (m, 2H), 4.30 (dd,  $J = 12.8$ , 4.8 Hz, 1H), 4.18–4.10 (m, 1H), 4.07–4.01 (m, 1H), 3.34 (dd,  $J = 15.2$ , 5.2, 1H), 3.23 (dd,  $J = 15.2$ , 4.8, 1H), 2.08 (s, 3H), 2.07 (s, 3H), 2.06 (s, 3H), 1.80 (s, 3H), 1.45 (s, 9H).  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ):  $\delta$  171.6, 170.7, 170.4, 170.3, 169.1, 155.4, 142.9, 121.7, 86.2, 80.0, 75.1, 72.3, 68.2, 61.9, 53.7, 53.3, 52.6, 28.8, 28.6, 23.0, 21.0, 21.0, 20.9. HRMS (ESI): calculated for  $\text{C}_{24}\text{H}_{35}\text{O}_{12}\text{N}_5\text{Na}$  ( $M + \text{Na}$ )<sup>+</sup> 608.2180, found 608.2192.

**Ts-L-T4M(1-[ $\beta$ -D-Glc(Ac)<sub>4</sub>])-L-T4M(1-[ $\beta$ -D-Glc(Ac)<sub>4</sub>])-OMe (19).** Preparation according to general procedure A afforded **19** (57 mg, 0.05 mmol, 55%) as a white solid.  $R_f$  0.17 ( $\text{EtOAc}$ /heptane, 5:1).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  8.57 (s, 1H), 7.69–7.68 (m, 3H), 7.58 (d,  $J = 5.8$  Hz, 1H), 7.32 (d,  $J = 8.0$  Hz, 2H), 6.24–6.19 (m, 1H), 5.86–5.82 (m, 2H), 5.67–5.63 (m, 2H), 5.50–5.45 (m, 1H), 5.44–5.36 (m, 2H), 5.30–5.26 (m, 1H), 4.79–4.75 (m, 1H), 4.34–4.29 (m, 2H), 4.27–4.22 (m, 1H), 4.20–4.14 (m, 2H), 4.11–4.05 (m, 1H), 4.02 (ddd,  $J = 10.2$ , 4.5, 1.8 Hz, 1H), 3.85 (s, 3H), 3.61 (ABdd,  $J = 14.7$ , 4.4 Hz, 1H), 3.53 (ABdd,  $J = 15.6$ , 2.0 Hz, 1H), 3.30 (ABdd,  $J = 14.7$ , 4.4 Hz, 1H), 2.78 (ABdd,  $J = 15.4$ , 6.8 Hz, 1H), 2.44 (s, 3H), 2.12 (s, 3H), 2.09 (s, 3H), 2.09 (s, 3H), 2.05 (s, 3H), 2.03 (s, 3H), 1.91 (s, 3H), 1.83 (s, 3H), 1.77 (s, 3H).  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ):  $\delta$  172.1, 170.7, 170.0, 169.5, 169.0, 168.6, 145.5, 143.3, 120.5, 85.8, 80.3, 75.3, 72.9, 70.5, 68.1, 61.8, 52.6, 51.7, 47.1, 28.6, 28.3, 24.2, 20.8, 20.7, 20.6, 20.3.

**Boc-T4M(1-[ $\beta$ -D-Glc(Bn)<sub>4</sub>])-T4M(1-[ $\beta$ -D-Glc(Ac)<sub>4</sub>])OMe (21).** To a solution of dipeptide **20** (103 mg, 0.26 mmol, 1:1 mixture of diastereoisomers) and tetra-*O*-benzylazidoglucose (149 mg, 0.26 mmol) in *tert*-butanol (4 mL) was added a mixture of  $\text{Cu}(\text{OAc})_2$  (16 mg, 21 mol %) and sodium ascorbate (21 mg, 41 mol %) in  $\text{H}_2\text{O}$  (4 mL). The reaction was stirred for 24 h at room temperature,  $\text{KHSO}_4$  (5% aq) was added, and the product was extracted with  $\text{EtOAc}$  (3 $\times$ ). The combined organic layers were washed with aqueous  $\text{NaHCO}_3$  and aqueous  $\text{NaCl}$ , dried over  $\text{Na}_2\text{SO}_4$ , and evaporated *in vacuo*. After purification by flash chromatography, the monoglycosylated peptide was dissolved in MeOH (4 mL), and a catalytic amount of  $\text{K}_2\text{CO}_3$  was added. The mixture was stirred for 4 h, Amberlite IR 120 plus was added until the solution was neutral, and the ion exchange was filtered off. Evaporation of the solvent *in vacuo* afforded the deprotected peptide. A subsequent cycloaddition (same conditions) using azidoglucose

**3** and workup as described above afforded **21** (264 mg, 0.20 mmol, 77%). FTIR (ATR): 3360, 1744, 1218  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.66 (s, 1H), 7.54 (s, 1H), 7.31–7.11 (m, 18H), 6.93–6.91 (m, 2H), 5.78–5.75 (m, 1H), 5.68 (d,  $J$  = 7.2 Hz, 1H), 5.58 (d,  $J$  = 9.0 Hz, 1H), 5.36 (dd,  $J$  = 6.6 Hz, 2H), 5.25–5.21 (m, 1H), 4.88–4.79 (m, 3H), 4.71 (d,  $J$  = 6.1 Hz, 1H), 4.57–4.39 (m, 6H), 4.27 (dd,  $J$  = 12.5 Hz, 1H), 4.09–4.06 (m, 1H), 4.02–3.92 (m, 2H), 3.89 (ddd,  $J$  = 9.9, 4.3, 1.5 Hz, 1H), 3.80–3.74 (m, 2H), 3.73–3.62 (m, 3H), 3.66 (s, 3H), 3.28–3.08 (m, 4H), 2.01 (s, 3H), 1.97 (s, 3H), 1.96 (s, 3H), 1.80 (s, 3H), 1.36 (s, 9H).  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ):  $\delta$  171.1, 171.1, 170.6, 170.0, 169.4, 169.3, 155.7, 143.6, 143.3, 138.3, 137.9, 137.9, 137.2, 128.6, 128.5, 128.5, 128.3, 128.1, 128.0, 127.9, 127.9, 127.8, 122.0, 121.1, 87.6, 85.8, 85.6, 81.0, 80.3, 78.0, 77.4, 75.9, 75.3, 75.2, 74.9, 73.6, 72.7, 70.7, 68.6, 67.8, 61.6, 53.7, 52.7, 51.9, 28.4, 27.9, 27.8, 20.7, 20.7, 20.6, 20.2. HRMS (ESI):  $m/z$  calculated for  $\text{C}_{64}\text{H}_{77}\text{N}_8\text{O}_{19}$  ( $\text{M} + \text{H}$ ) $^+$  1261.5305, found 1261.5354.

**Cbz-L-T1M(4-[ $\beta$ -D-Glc(Ac) $_4$ ])-OMe (30).** Preparation according to general procedure A afforded **30** (1.7 g, mmol, 96%) as a white solid.  $R_f$  (EtOAc) = 0.74.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3/\text{CD}_3\text{OD}$ )  $\delta$  7.75 (s, 1 H); 7.41–7.29 (m, 5 H); 5.37 (t,  $J$  = 9.4 Hz, 1 H); 5.25 (t,  $J$  = 9.7 Hz, 1 H); 5.18 (dd,  $J$  = 10.0, 9.4 Hz, 1 H); 5.12 (s, 2 H); 4.91–4.71 (m, 4H); 4.28 (dd,  $J$  = 12.5, 4.9 Hz, 1 H); 4.13 (dd,  $J$  = 12.5, 2.2 Hz, 1 H); 3.94 (ddd,  $J$  = 10.1, 4.8, 2.2 Hz, 1 H); 3.79 (s, 3 H); 2.07 (s, 3 H); 2.05 (s, 3 H); 2.02 (s, 3 H); 1.86 (s, 1H).  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  170.5, 170.0, 169.7, 169.4, 169.0, 155.6, 144.3, 135.8, 128.5, 128.2, 128.0, 123.2, 76.2, 73.7, 73.1, 71.3, 68.3, 67.3, 62.0, 53.9, 53.1, 50.7, 20.6, 20.5, 20.3. IR (film)  $\nu$  3356, 3140, 2950, 2885, 2250, 1748, 1532  $\text{cm}^{-1}$ . HRMS (ESI)  $m/z$  calculated for  $\text{C}_{28}\text{H}_{34}\text{N}_4\text{NaO}_{13}$  ( $\text{M} + \text{Na}$ ) $^+$  657.2020, found 657.1996.

**Fmoc-L-T1P(4-[ $\beta$ -D-Glc(Bn) $_4$ ])-OH (31).** Preparation according to general procedure A afforded **31** (626 mg, 0.695 mmol, 69%) as a white solid. FTIR (ATR):  $\nu$  3058, 3032, 2863, 2245, 1718  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.74 (d,  $J$  = 7.5 Hz, 2 H), 7.55–7.51 (m, 2 H), 7.39–6.94 (m, 24 H), 5.01–4.33 (m, 13 H), 4.18–4.03 (m, 2 H), 3.88–3.81 (m, 5 H), 3.58–3.42 (m, 1 H). HRMS (ESI):  $m/z$  calculated for  $\text{C}_{54}\text{H}_{52}\text{N}_4\text{NaO}_9$  ( $\text{M} + \text{Na}$ ) $^+$  923.3632, found 923.3629.

**Fmoc-L-T1B(4-[ $\beta$ -D-Glc(Bn) $_4$ ])-OH (32).** Preparation according to general procedure A afforded **32** (662 mg, 0.702 mmol, 71%) as a white amorphous solid. IR (film)  $\nu$  3058, 3021, 2924, 2859, 1714  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.75 (d,  $J$  = 7.5 Hz, 2 H), 7.61–7.55 (m, 2 H), 7.45–6.95 (m, 25 H), 5.40 (d,  $J$  = 6.9 Hz, 1 H), 4.92 (dd,  $J$  = 24.0, 11.1 Hz, 2 H), 4.82 (d,  $J$  = 10.8 Hz, 1 H), 4.67 (d,  $J$  = 10.9 Hz, 1 H), 4.59–4.23 (m, 10 H), 4.20 (t,  $J$  = 6.8 Hz, 1 H), 4.01 (t,  $J$  = 9.5 Hz, 1 H), 3.81 (t,  $J$  = 8.7 Hz, 1 H), 3.75–3.53 (m, 4 H), 2.02–1.78 (m, 2 H), 1.77–1.58 (m, 2 H), 1.39–1.22 (m, 1 H), 1.14–0.96 (m, 1 H). HRMS (ESI):  $m/z$  calculated for  $\text{C}_{57}\text{H}_{58}\text{N}_4\text{NaO}_9$  ( $\text{M} + \text{Na}$ ) $^+$  965.4101, found 965.4126.

**(2S,4R)-N-Cbz-4-(4-[ $\beta$ -D-Glc(Bn) $_4$ ])- [1,2,3]triazol-1-yl]pipecolic Acid Methyl Ester (33).** Preparation according to general procedure A afforded **33** (39 mg, 0.045 mmol, 49%) as a white amorphous solid.  $R_f$  (1/1 EtOAc/heptane) = 0.26.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.45 (s, 1 H), 7.39–6.95 (m, 25 H), 5.24–5.08 (m, 3 H), 4.95 (d,  $J$  = 11.1 Hz, 1 H), 4.91 (d,

$J$  = 11.2 Hz, 1 H), 4.85 (d,  $J$  = 10.8 Hz, 1 H), 4.66–4.47 (m, 6 H), 4.39–4.25 (m, 2 H), 3.95 (t,  $J$  = 9.2 Hz, 1 H), 3.84–3.61 (m, 8 H), 3.21 (dt,  $J$  = 25.0, 12.5 Hz, 1 H), 2.84–2.67 (m, 1 H), 2.20–2.10 (m, 2 H), 2.00–1.86 (m, 1H).  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  170.7, 155.9, 145.8, 138.5, 138.0, 137.9, 136.1, 129.6, 128.4, 128.3, 128.3, 128.1, 128.0, 127.9, 127.7, 127.6, 127.5, 127.4, 121.8, 86.9, 81.7, 79.2, 78.1, 75.5, 75.0, 74.5, 74.0, 73.3, 69.1, 67.6, 52.8, 52.4, 52.1, 37.9, 30.9, 28.3. HRMS (ESI):  $m/z$  calculated for  $\text{C}_{51}\text{H}_{54}\text{N}_4\text{NaO}_9$  ( $\text{M} + \text{Na}$ ) $^+$  889.3789, found 889.3815.

**(2S,4S)-N-Cbz-4-(4-[ $\beta$ -D-Glc(Bn) $_4$ ])- [1,2,3]triazol-1-yl]pipecolic Acid Methyl Ester (34).** Preparation according to general procedure A afforded **34** (42 mg, 0.049 mmol, 60%) as a white amorphous solid.  $R_f$  (1/1 EtOAc/heptane) = 0.36.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.44 (s, 1 H), 7.34–7.03 (m, 25 H), 5.17–5.14 (m, 2 H), 4.92 (d,  $J$  = 2.8 Hz, 2 H), 4.85 (d,  $J$  = 10.9 Hz, 1 H), 4.73–4.47 (m, 6 H), 4.38 (d,  $J$  = 11.0 Hz, 1 H), 4.00–3.59 (m, 7 H), 3.43 (s, 2 H), 2.84–2.78 (m, 1 H), 2.42–2.35 (m, 2 H), 2.19–2.11 (m, 1 H).  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  170.7, 155.8, 145.4, 138.4, 137.9, 136.0, 129.6, 128.5, 128.3, 128.2, 128.2, 128.1, 128.0, 127.9, 127.8, 127.8, 127.7, 127.6, 127.5, 127.4, 120.9, 86.8, 81.6, 79.3, 78.1, 75.5, 75.0, 74.7, 73.9, 73.3, 69.0, 67.9, 55.0, 54.9, 53.7, 52.7, 40.5, 32.7, 31.8. HRMS (ESI):  $m/z$  calculated for  $\text{C}_{51}\text{H}_{54}\text{N}_4\text{NaO}_9$  ( $\text{M} + \text{Na}$ ) $^+$  889.3789, found 889.3812.

**General Procedure B for Chemoenzymatic Peptide Coupling.** The *N*-Cbz-protected amino acid methyl ester (0.39 mmol) was dried by coevaporation with DMF ( $2 \times 5$  mL) and dissolved in *tert*-amylOH (4.0 mL). After adding a solution of Phe-NH $_2$  or Gly-NH $_2$  (1.5 mmol) in *tert*-amylOH (2.0 mL), the reaction mixture was stirred at 37  $^\circ\text{C}$ . Subsequently, the dried alcalase suspension in *tert*-amylOH (1 mL) was added, and the reaction mixture was stirred at 37  $^\circ\text{C}$ . Samples were taken at regular time intervals and analyzed by HPLC. Upon (virtually) complete conversion, the reaction mixture was concentrated *in vacuo* to remove most of the volatiles. The residue was taken up in EtOAc (50 mL) and H $_2$ O (20 mL), to which a few drops of 1 N aqueous HCl were added. The aqueous layer was extracted with EtOAc ( $3 \times 30$  mL), and the combined organic phase was washed with aqueous KHCO $_3$  (1 M, 40 mL), aqueous HCl (1 N, 40 mL), and brine (40 mL), dried (Na $_2$ SO $_4$ ), and concentrated *in vacuo*. Analytically pure samples were obtained by recrystallization. A new load of enzyme (dried precipitate from 500  $\mu\text{L}$  of enzyme solution) was added three times a week. Quenching solution for HPLC samples: 50% KH $_2$ PO $_4$  (0.1 M, pH 5), 50% MeCN.

**Cbz-L-T1M(4-[ $\beta$ -D-Glc(Ac) $_4$ ])-Gly-NH $_2$  (43).** Applying general procedure B (with 0.37 mmol **30**, and 5 equiv of Gly-NH $_2$ ) for chemoenzymatic peptide coupling gave **43** (165 mg; 65%).  $R_f$  (EtOAc) = 0.25.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3/\text{CD}_3\text{OD}$ )  $\delta$  7.93 (s, 1 H), 7.33 (m, 5 H), 5.38 (t,  $J$  = 9.4 Hz, 1 H), 5.28 (t,  $J$  = 9.7 Hz, 1 H), 5.17 (t,  $J$  = 9.7 Hz, 1 H), 5.10–5.04 (m, 2 H), 4.90 (dd,  $J$  = 13.8, 4.5 Hz, 1 H), 4.81–4.64 (m, 3 H), 4.28 (dd,  $J$  = 12.4, 4.8 Hz, 1 H), 4.12 (dd,  $J$  = 12.4, 2.1 Hz, 1 H), 3.98 (ddd,  $J$  = 10.0, 4.8, 2.2 Hz, 1 H), 3.92 (d,  $J$  = 17.1 Hz, 1 H), 3.83 (d,  $J$  = 17.1 Hz, 1 H), 2.05 (s, 3 H), 2.03 (s, 3 H), 2.00 (s, 3 H), 1.85 (s, 3 H).  $^{13}\text{C}$  NMR (75 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  174.0, 172.3, 171.7, 171.3, 171.0, 145.4, 137.7, 129.5, 129.1,

129.0, 125.8, 77.1, 75.4, 73.6, 72.9, 69.8, 68.1, 63.4, 56.2, 51.7, 43.2, 20.6, 20.5. IR (film)  $\nu$  3330, 2955, 1744, 1671, 1524  $\text{cm}^{-1}$ . HRMS (ESI)  $m/z$  calculated for  $\text{C}_{29}\text{H}_{36}\text{N}_6\text{NaO}_{13}$  ( $\text{M} + \text{Na}$ )<sup>+</sup> 699.22380, found 699.22173.

**Cbz-L-T1M(4-[ $\beta$ -D-Glc(Ac)<sub>4</sub>])-L-Phe-NH<sub>2</sub> (44).** Applying general procedure B (with 0.39 mmol **30**, and 5 equiv of Phe-NH<sub>2</sub>) for chemoenzymatic peptide coupling gave **44** (300 mg; 98%).  $R_f$  (EtOAc) = 0.25. <sup>1</sup>H NMR (400 MHz,  $\text{CDCl}_3/\text{CD}_3\text{OD}$ )  $\delta$  7.81 (s, 1 H), 7.41–7.14 (m, 10 H), 5.37 (t,  $J$  = 9.4 Hz, 1 H), 5.24 (t,  $J$  = 9.7 Hz, 1 H), 5.17 (t,  $J$  = 9.7 Hz, 1 H), 5.06 (s, 2 H), 4.81–4.74 (m, 2 H), 4.66–4.60 (m, 3 H), 4.28 (dd,  $J$  = 12.5, 4.9 Hz, 1 H), 4.12 (dd,  $J$  = 12.4, 2.1 Hz, 1 H), 3.96 (ddd,  $J$  = 10.1, 4.7, 2.1 Hz, 1 H), 3.16 (dd,  $J$  = 13.9, 5.9 Hz, 1 H), 2.95 (dd,  $J$  = 13.9, 8.3 Hz, 1 H), 2.06 (s, 3 H), 2.03 (s, 3 H), 2.01 (s, 3 H), 1.85 (s, 3 H). <sup>13</sup>C NMR (75 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  175.1, 172.1, 171.5, 171.0, 170.8, 170.0, 157.5, 145.0, 137.7, 137.3, 130.0, 129.3, 129.3, 129.0, 128.8, 128.6, 127.6, 125.4, 76.9, 75.1, 73.5, 72.6, 69.5, 67.9, 63.2, 55.7, 55.4, 51.4, 38.5, 20.7, 20.6. IR (neat)  $\nu$  3417, 3283, 2950, 1748, 1697, 1636, 1532  $\text{cm}^{-1}$ . HRMS (ESI)  $m/z$  calculated for  $\text{C}_{36}\text{H}_{42}\text{N}_6\text{NaO}_{13}$  ( $\text{M} + \text{Na}$ )<sup>+</sup> 789.27075, found 789.26506.

**Cbz-L-T4M(1-[ $\beta$ -D-Glc(Ac)<sub>4</sub>])-Gly-NH<sub>2</sub> (47).** Preparation according to the general procedure B afforded **47** (260 mg, 0.38 mmol, 98%) as a white solid.  $R_f$  (EtOAc) = 0.52. IR (KBr):  $\nu$  3292, 2948, 1753, 1687, 1535  $\text{cm}^{-1}$ . <sup>1</sup>H NMR (400 MHz,  $\text{CDCl}_3/\text{MeOD}$ ):  $\delta$  7.93 (s, 1 H), 7.42–7.25 (m, 5 H), 5.99–5.90 (m, 1 H), 5.53 (s, 1 H), 5.50–5.45 (m, 2 H), 5.34–5.23 (m, 1 H), 5.11 (d,  $J$  = 12.4 Hz, 1 H), 5.08 (d,  $J$  = 12.4 Hz, 1 H), 4.49 (t,  $J$  = 6.2, 6.2 Hz, 1 H), 4.32 (dd,  $J$  = 12.6, 4.8 Hz, 1 H), 4.18 (dd,  $J$  = 12.6, 2.1 Hz, 1 H), 4.16–4.10 (m, 1 H), 3.92 (d,  $J$  = 17.1 Hz, 1 H), 3.84 (d,  $J$  = 17.0 Hz, 1 H), 3.29–3.18 (m, 2 H), 2.09 (s, 1 H), 2.08 (s, 1 H), 2.04 (s, 1 H), 1.83 (s, 1 H). <sup>13</sup>C NMR (75 MHz,  $\text{CDCl}_3/\text{MeOD}$ ):  $\delta$  172.1, 171.5, 170.5, 169.7, 169.3, 168.9, 156.3, 135.6, 127.9, 127.6, 127.4, 121.6, 85.0, 74.2, 72.1, 70.2, 67.3, 66.6, 61.1, 54.1, 41.8, 27.1, 19.7, 19.6, 19.1. HRMS (ESI):  $m/z$  calculated for  $\text{C}_{29}\text{H}_{36}\text{N}_6\text{O}_{13}\text{Na}$  ( $\text{M} + \text{Na}$ ) 699.2238, found 699.2213.

**Cbz-L-T4M(1-[ $\beta$ -D-Glc(Ac)<sub>4</sub>])-L-Phe-NH<sub>2</sub> (48).** Preparation based on the general procedure B using 1.01 g (1.58 mmol) of **45** afforded **48** (300 mg, 0.46 mmol, 30%) as a white solid.  $R_f$  (EtOAc) = 0.60. <sup>1</sup>H NMR (400 MHz,  $\text{CDCl}_3/\text{MeOD}$ ):  $\delta$  7.74 (s, 1 H), 7.41–7.16 (m, 10 H), 5.96–5.87 (m, 1 H), 5.53 (s, 1 H), 5.49–5.41 (m, 2 H), 5.06 (s, 2 H), 4.63 (dd,  $J$  = 8.1, 5.9 Hz, 1 H), 4.42 (t,  $J$  = 6.4, 6.4 Hz, 1 H), 4.31 (dd,  $J$  = 12.6, 4.9 Hz, 1 H), 4.16 (dd,  $J$  = 12.6, 2.1 Hz, 1 H), 4.11 (ddd,  $J$  = 10.2, 4.8, 2.1 Hz, 1 H), 3.20–3.15 (m, 2 H), 3.05 (dd,  $J$  = 15.16, 6.86 Hz, 1 H), 2.96 (dd,  $J$  = 13.90, 8.27 Hz, 1 H), 2.09 (s, 3 H), 2.06 (s, 3 H), 2.04 (s, 3 H), 1.82 (s, 3 H). <sup>13</sup>C NMR (75

MHz,  $\text{CDCl}_3/\text{MeOD}$ ):  $\delta$  173.6, 170.7, 170.5, 169.8, 169.4, 168.9, 156.1, 143.1, 136.2, 135.6, 128.6, 128.5, 128.0, 127.7, 127.4, 126.3, 121.4, 85.0, 74.3, 72.2, 70.1, 67.3, 66.6, 61.2, 53.9, 53.6, 36.8, 27.2, 19.8, 19.7, 19.2. IR (KBr)  $\nu$  3351, 2942, 1748, 1693, 1666, 1524  $\text{cm}^{-1}$ . HRMS (ESI):  $m/z$  calculated for  $\text{C}_{36}\text{H}_{42}\text{N}_6\text{O}_{13}\text{Na}$  ( $\text{M} + \text{Na}$ ) 789.2708, found 789.2675.

**Cbz-L-(N-1-Glu(Ac)<sub>4</sub>-Asn)-Gly-NH<sub>2</sub> (53).** Preparation according to the general procedure B afforded **53** (98 mg, 0.15 mmol, 38%) as a white solid. FTIR (ATR):  $\nu$  3317, 1752, 1671  $\text{cm}^{-1}$ . <sup>1</sup>H NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.37 (bs, 5H), 7.04 (m, 1H), 6.65 (d,  $J$  = 9.0 Hz, 1H), 6.37 (s, 1H), 6.10 (d,  $J$  = 7.4 Hz, 1H), 5.46 (s, 1H), 5.31 (dd,  $J$  = 11.4 Hz, 2H), 5.16 (t,  $J$  = 12.0 Hz, 1H), 5.05 (t,  $J$  = 9.7 Hz, 1H), 4.90 (t,  $J$  = 9.6 Hz, 1H), 4.63–4.52 (m, 1H), 4.27 (dd,  $J$  = 12.5 Hz, 1H), 4.18–4.02 (m, 2H), 3.84–3.69 (m, 2H), 3.02 (dd,  $J$  = 16.4 Hz, 1H), 2.91 (d,  $J$  = 10.6 Hz, 1H), 2.62 (dd,  $J$  = 16.4 Hz, 1H), 2.02 (s, 3H), 2.01 (s, 3H), 2.08 (s, 3H), 2.07 (s, 3H). <sup>13</sup>C NMR (75 MHz,  $\text{CDCl}_3$ ):  $\delta$  171.5, 171.0, 171.0, 170.8, 170.1, 169.5, 169.0, 155.7, 135.2, 128.2, 128.0, 127.8, 77.5, 76.7, 73.2, 72.0, 69.9, 67.5, 67.2, 61.1, 51.0, 42.5, 20.2, 20.2, 20.1. HRMS (ESI):  $m/z$  calculated for  $\text{C}_{28}\text{H}_{36}\text{N}_4\text{O}_{13}\text{Na}$  ( $\text{M} + \text{Na}$ )<sup>+</sup> 675.2126; found 675.2116.

**Cbz-L-(N-1-Glu(Ac)<sub>4</sub>-Asn)-L-Phe-NH<sub>2</sub> (54).** Preparation according to the general procedure B afforded **54** (135 mg, 0.18 mmol, 46%) as a white solid. FTIR (ATR):  $\nu$  3399, 1753, 1631  $\text{cm}^{-1}$ . <sup>1</sup>H NMR (400 MHz,  $\text{D}_2\text{O}$ ):  $\delta$  7.43–7.16 (m, 10H), 6.88 (d,  $J$  = 7.9 Hz, 1H), 6.72 (d,  $J$  = 9.3 Hz, 1H), 6.16 (d,  $J$  = 7.9 Hz, 1H), 6.00 (s, 1H), 5.40 (s, 1H), 5.29 (t,  $J$  = 9.6 Hz, 1H), 5.16 (t,  $J$  = 9.2 Hz, 1H), 5.09–5.01 (m, 2H), 4.91 (t,  $J$  = 9.6 Hz, 1H), 4.60–4.51 (m, 1H), 4.50–4.40 (m, 1H), 4.26 (dd,  $J$  = 12.6 Hz, 1H), 4.16–4.07 (m, 2H), 3.78 (ddd,  $J$  = 10.1, 2.0 Hz, 1H), 3.17–3.01 (m, 2H), 2.76 (dd,  $J$  = 16.1 Hz, 1H), 2.59 (dd,  $J$  = 16.1 Hz, 1H), 2.07 (s, 3H), 2.05 (s, 3H), 2.03 (s, 3H), 2.02 (s, 3H). <sup>13</sup>C NMR (75 MHz,  $\text{CDCl}_3$ ):  $\delta$  172.7, 172.0, 171.2, 170.4, 196.7, 169.6, 169.4, 146.5, 136.0, 127.6, 127.3, 127.2, 76.9, 72.9, 72.9, 70.1, 67.7, 66.2, 61.3, 51.2, 41.6, 36.5, 29.0, 18.7, 18.7. HRMS (ESI):  $m/z$  calculated for  $\text{C}_{35}\text{H}_{42}\text{N}_4\text{O}_{14}\text{Na}$  ( $\text{M} + \text{Na}$ )<sup>+</sup> 765.2595; found 765.2605.

## Acknowledgment

SenterNovem (Ministry of Economic Affairs, The Netherlands) is gratefully acknowledged for providing financial support. Jean Andrien (DSM Resolve, Geleen, The Netherlands) is kindly acknowledged for analytical assistance.

Received for review November 1, 2007.

OP700249F