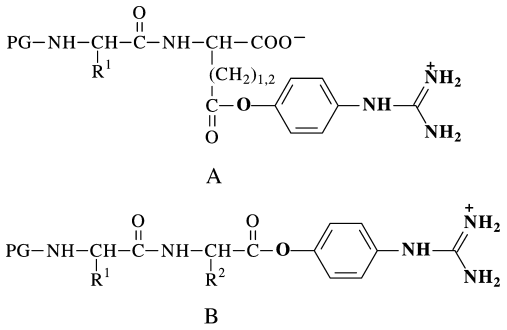
Synthesis of Neo-Peptidoglycans: An Unexpected Activity of Proteases**

Nicole Wehofsky, Reik Löser, Andrij Buchynskyy, Peter Welzel, and Frank Bordusa*

Glycations represent one of the most widespread "post-synthesis" modifications of polypeptides modulating the structure, stability, and biological activity of the carrier molecule. The involvement of glycopeptides in adhesion, differentiation, and growth of cells or the association with cancer-cell lines results in a great biological and pharmaceutical interest in these compounds and their analogues.[1] Isolation from natural sources or preparation by recombinant DNA techniques, however, usually leads to conjugates with an altered or inhomogeneous carbohydrate content. Currently, only chemical and chemoenzymatic strategies provide access to well-defined carbohydrate–peptide conjugates. Despite the large number of useful synthesis methods, the high complexity, hydrophilicity, and acid- and, in some cases, base-lability of the carbohydrate part, however, make synthesis of these compounds a vast challenge that is far from any routine.[2]

Herein we report a biocatalytic method allowing the selective acylation of N-glycans with amino acids and peptides. Like the known transglutaminase approach,[3] this novel strategy enables glutamine (Gln) moieties to be modified with structurally diverse carbohydrates. Moreover, the approach is not restricted to Gln, but is also effective for asparagine (Asn) derivatives. Even conjugates with the carbohydrate moiety linked to the C terminus of the peptide can be prepared by this method. The hallmark of the approach is the use of an ordinary protease as the biocatalyst, for example, clostripain from *Clostridium histolyticum*, which is combined with a novel iso-type of substrate mimetics which are used as the amino acid or peptide precursors. Similar to the known linear-type substrate mimetics,[4] the iso-type analogues bear a site-specific ester leaving group, for example, the 4-guanidinophenyl ester moiety (OGp), that mediates the acceptance of non-specific acyl residues by the original highly Arg-specific protease. However, to direct the intrinsic synthesis activity of the enzyme to the side chain of Asp and Glu, the OGp group is linked to the ω-carboxylate of the two amino acids instead of being at the C terminus of the peptide (Scheme 1). This different architecture was found to shift the synthesis activity of the biocatalyst from the α-carboxy group of the peptide towards that of Asp and Glu residues of the side chains enabling the formation of a broad spectrum of N-linked neo-peptidoglycans.



Scheme 1. General structure of iso-type (A) and the classical linear-type substrate mimetics (B). The site-specific 4-guanidinophenyl ester moiety (OGp) is highlighted in bold letters. PG = protecting group; R^1, R^2, individual side chains.

The capability of clostripain to acylate N-glycans was investigated. For this purpose, reactions with simple monomeric N-glycans, such as, D-glucosamine (**1a**), D-galactosamine (**2**), and muramic acid (**3**), and the classical linear-type substrate mimetic Boc-Phe-Gly-Gly-OGp (Boc = *tert*-butoxycarbonyl) acting as the acyl donor were performed. The OGp moiety was selected because of its known mimic function which was shown to mediate the acceptance of non-specific (non Arg containing) peptides by clostripain.[4c, 5] The reactions were performed in buffered aqueous solution at pH 8.0 containing 10% DMF and concentrations of peptide, carbohydrate, and enzyme of 2 mM, 20 mM, and 20 μM, respectively.[6] As revealed by HPLC, incubation times between 30 and 40 min led to complete ester consumption in all cases finally resulting in product yields of 68% (for **1a**), 70% (**2**), and 64% (**3**). The synthesis products have been identified by saponification experiments, mass spectrometry, and NMR spectroscopy following their isolation.[7] Importantly, only single-acylated carbohydrates were found in all instances. Accordingly, except hydrolyzed peptide ester no further side products could be detected. That the coupling failed with the amine-blocked *N*-acetyl-D-glucosamine (**1b**) indicates an exclusive *N*-acylation of the carbohydrates by the enzyme which is in agreement with the NMR spectroscopy data of the products and their resistance towards saponification as well.

The capability of the approach to mediate the coupling of N-glycans to the side chain carboxy groups of Asp and Glu was investigated. For this goal, at least two essential conditions must be fulfilled; 1) overcoming the original Arg specificity of the protease and 2) direction of the intrinsic synthesis activity of the enzyme from the C^{α}-carboxylate (backbone) to that of the side chain of Asp and Glu. Both requisites might be achieved by shifting the OGp moiety from the C^{α}- to the ω-carboxylate of the peptide precursor. To test

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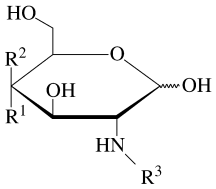

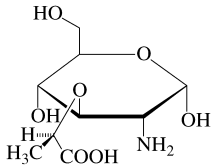

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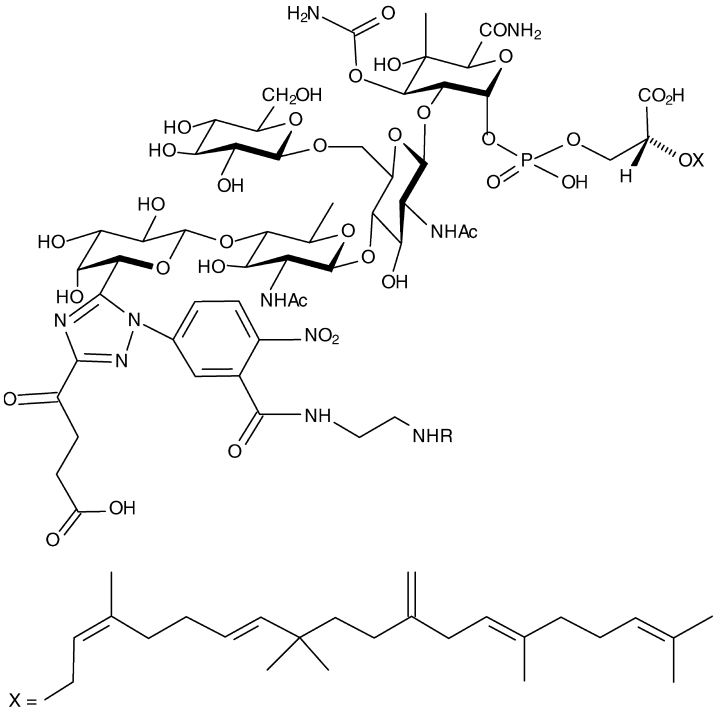
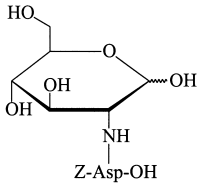
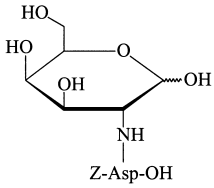
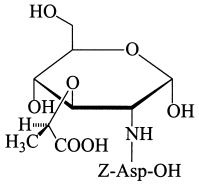
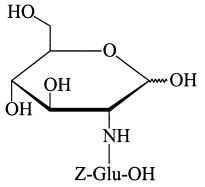
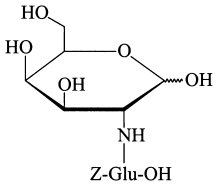
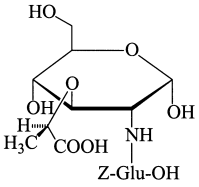
1a: R^1 = OH, R^2 = H, R^3 = H
1b: R^1 = OH, R^2 = H, R^3 = acetyl
2: R^1 = H, R^2 = OH, R^3 = H



4a: R = H
4b: R = phenylacetyl

this hypothesis, we initially used the simple amino acid derivatives Z-Glu(OGp)-OH and Z-Asp(OGp)-OH as precursors (Z = benzyloxycarbonyl). Synthesis of the esters can be achieved in a similar way to that of the C^{α}-esterified analogues by using an inverse protecting-group strategy for the carboxy groups of Asp and Glu.[8] The enzymatic reactions themselves were performed under exactly the same conditions and with the reactant concentrations as described above for Boc-Phe-Gly-Gly-OGp. Similarly, HPLC, NMR spectroscopy, mass spectrometry, and saponification experiments were utilized for product analysis. In addition, the blocked amino component **1b** was used as a probe to verify the selectivity of syntheses. The yields of reactions are summarized in Table 1 and document that both amino acid esters show productive binding at the active site of the enzyme which results in the formation of appropriate N-linked neopeptidoglycans. Only reactions with **1b** gave no products, which indicates an exclusive N-acylation as has been verified by NMR spectroscopy and saponification experiments. In the case of all other carbohydrates (**1a**, **2**, **3**), product yields within

Table 1. Product yields of the clostripain-catalyzed carbohydrate – amino acid coupling reactions.[a]

Acyl donor	Carbohydrate acceptor	Product	Yield [%]
Z-Asp(OGp)-OH	**1a**		72
Z-Asp(OGp)-OH	**2**		73
Z-Asp(OGp)-OH	**3**		71
Z-Glu(OGp)-OH	**1a**		67
Z-Glu(OGp)-OH	**2**		68
Z-Glu(OGp)-OH	**3**		67

[a] Conditions were as described in ref. [6]. Concentrations: [donor] = 2 mM, [acceptor] = 20 mM, [clostripain] = 20 μM.

a range of 67 – 73 % were obtained corresponding with those found for Boc-Phe-Gly-Gly-OGp.

Owing to the apparent independence of this approach to the structure and type of the acyl donor, we investigated its ability to modify Asp-derived peptides instead of a single Asp moiety. As a model, the Leu-enkephalin sequence Z-*Asp*-Tyr-Gly-Gly-Phe-Leu-OH was selected. Its biological relevance is because the glycosylation of a related peptide drastically increases its affinity to opiate receptors.[9] The precursor peptide Z-*Asp(OGp)*-Tyr-Gly-Gly-Phe-Leu-OH was synthesized by standard solid-phase peptide synthesis on Wang resin employing the Fmoc strategy (Fmoc = 9-fluorenylmethoxycarbonyl). To allow ester modification, Z-Asp(OGp($Boc)_2$)-OH was used in the last coupling step. Cleavage of the peptide

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from the resin by trifluoroacetic acid (TFA) treatment simultaneously deprotected the Tyr side chain and the OGp moiety leading to the peptide ester ready for enzymatic synthesis. An enzyme concentration of 20 μM and an incubation time of less than 15 min with all other reaction conditions and reactant concentrations unchanged led to complete peptide ester consumption resulting in product yields of 70% (for **1a**), 69% (**2**), and 66% (**3**). Except hydrolyzed peptide ester no further side products could be detected while the selectivity of reactions was verified as described above.

Finally, we demonstrated the ability of the method to couple carbohydrates of higher complexity. For this purpose, reactions with a synthetic amino-functionalized moenomycin A analogue **4a**[10] and the four amino acid and peptide precursors were performed. For synthesis-economy reasons the latter have been used in 1.25-fold excess over **4a** in concentrations of 25 mM and 20 mM, respectively. All other reaction conditions were kept unchanged including the low content of organic solvent. After 1 h and complete ester consumption the reactions were terminated and analyzed by HPLC and mass spectrometry. Figure 1 shows selected HPLC profiles that illustrate the well-defined course of catalysis for the reaction of the peptide precursor with **4a**. Similar to reactions using the monomeric carbohydrates, only the formation of single-acylated **4a** could be observed. As for the efficiency of synthesis, for reactions with Boc-Phe-Gly-Gly-OGp 24%, with Z-*Asp(OGp)*-Tyr-Gly-Gly-Phe-Leu-OH and Z-Glu(OGp)-OH 26%, and with Z-Asp(OGp)-OH 29% of the desired product could be obtained. On the contrary, *N*-phenylacetyl-moenomycin derivative (**4b**), which was used to verify the selectivity of the enzyme, gave no products. Compared to the yields of about 70% for the monomeric carbohydrates (**1a**, **2**, **3**), the use of the bulky moenomycin derivative clearly decreases the efficiency of synthesis. However, moenomycin is not only a complex carbohydrate, but also an amphiphilic compound with a strong tendency to form micelles (critical micelle concentration (CMC) of non-modified moenomycin: 0.5 mM at pH 6.8).[11] At present, it remains an open question whether the formation of micelles or the increase in complexity of the carbohydrate scaffold contributes to this effect. Assuming that micelle formation decreases at least the rate of diffusion and, thus, hinders the moenomycin attack of the acyl–enzyme intermediate, these results indicate to a rather broad acceptance by the enzyme of highly complex and usually non-micelle N-glycans.

In summary, we have described an efficient method for the synthesis of a wide variety of N-linked neo-peptidoglycans by exploiting the protease/substrate mimetics-based methodology. The approach allows selective coupling of carboxylate moieties derived from Asp and Glu side chains as well as the C-terminus of peptides with both simple monomeric and highly complex acid- and base-labile carbohydrate derivatives under extraordinary mild reaction conditions with yields ranging between 24 and 73%. Because of the known independence of the classical substrate mimetics approach of the individual acyl moiety,[4] a high flexibility of the synthesis method towards the sequence of the peptide precursor, at least of those derived from the linear-type derivatives can be expected. Only the presence of specific Arg residues within the peptide may lead to undesired cleavage reactions.[12] In such an instance the use of a side chain protected Arg moiety seems to offer a promising escape. In any case, to our knowledge there is no other enzyme system that shows a similar synthetic flexibility towards both the peptide and carbohydrate part. These characteristics make the new approach a powerful and rather general one for the synthesis of N-linked neo-peptidoglycans and amino acid–carbohydrate building blocks.

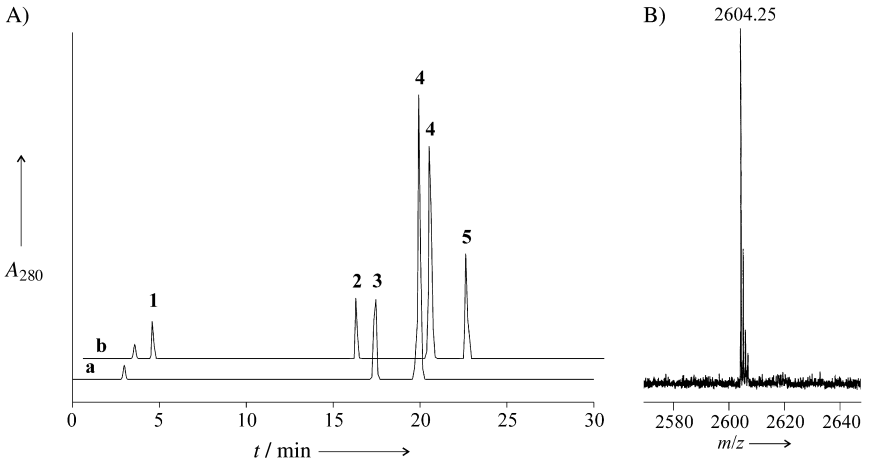

Figure 1. Analysis of the clostripain-catalyzed coupling of Z-Asp(OGp)-Tyr-Gly-Gly-Phe-Leu-OH with moenomycin A (**4a**) by HPLC (A) and MALDI-TOF mass spectrometry (B).[7] A) a) Before addition of enzyme; b) after 30 min. 1) released 4-guanidinophenol; 2) Z-Asp-Tyr-Gly-Gly-Phe-Leu-OH; 3) Z-Asp(OGp)-Tyr-Gly-Gly-Phe-Leu-OH; 4) moenomycin A; 5) Z-Asp(moenomycin)-Tyr-Gly-Gly-Phe-Leu-OH. A_{280} = absorbance at 280 nm. B) Mass calcd: 2602.96.

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[6] The enzymatic reactions were performed in a total volume of 0.5 mL containing 0.1 M *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (Hepes), pH 8.0, 0.1 M NaCl, 0.01 M $CaCl_2$, 10 % (*v*/*v*) dimethylformamide (DMF) at 25 °C. The amino acid and peptide esters were dissolved in 10 % aqueous DMF while the carbohydrates were suspended in the appropriate buffer. Readjusting to pH 8.0 was achieved by adding NaOH. After mixing the reactants the reactions were initiated by addition of pre-incubated enzyme (1 mM DL-dithiothreitol, 2.5 mM $CaCl_2$ in water for 2 h). After defined time intervals the reactions were stopped by addition of trifluoroacetic acid (1 % solution) and further analyzed as described in ref. [7]. To control for spontaneous reactions, parallel reactions without enzyme were analyzed in all cases. On the basis of these controls, non-enzymatic synthesis could be ruled out and the extent of spontaneous hydrolysis of the acyl donor esters was found to be less than 5 %.

[7] The reactions were analyzed under optimized conditions by reversed-phase HPLC (LiChrospher, 5 μm, 125 × 3 mm; Merck). Detection was at 254 nm (monomeric carbohydrates) or 280 nm (moenomycin, Leu-enkephalin derivative). Mass spectra were recorded for separated and lyophilized probes by using MALDI-TOF (MALDI 5 V5.1.2, Kratos Kompakt) or ESI (Apex II/7 Tesla, Bruker-Daltonics) ionization. NMR spectroscopy (GEMINI 300, Varian) was used to verify the identity of the reactants and products (except moenomycin and its conjugates). Saponification experiments were performed by incubating the synthesis products in 50 % aqueous methanol (pH 11) for 1 h and analysis by HPLC.

[8] For the synthesis of linear-type substrate mimetics see: N. Müller, F. Bordusa, *Anal. Biochem.* **2000**, *286*, 86 – 90, and references therein. The isomeric Z-Glu/Asp(OGp)-OH esters were prepared by condensation of Z-Glu/Asp-O*t*Bu and 4-[*N*′,*N*″-bis(Boc)-guanidino]phenol (ratio 1:2) using *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5b]pyridine-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU) as the coupling reagent, *N*-ethyldiisopropylamine as the general base, and DMF as the solvent at 0 °C. Treatment of the protected amino acid diester products with TFA resulted in the Boc/*t*Bu-cleavage leading to the final Z-Glu/Asp(OGp)-OH esters. The identity and purity of the esters were checked by analytical HPLC, NMR spectroscopy, and thermospray mass spectrometry. By using that optimized synthesis method, the isomeric esters were not formed.

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Investigation of Reactive Intermediates of Chemical Reactions in Solution by Electrospray Ionization Mass Spectrometry: Radical Chain Reactions**

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Dedicated to Professor Hans J. Schäfer on the occasion of his 65th birthday

The reaction mechanism is the detailed, step-by-step description of a chemical reaction. Most chemical reactions take place through a complex sequence of steps via reactive intermediates. The most important reactive intermediates in organic chemical reactions in solution are carbocations, carbanions, carbenes, and radicals. Of course, chemists have been able to detect these intermediates indirectly by chemical and physical methods, and spectroscopic methods are available to study them directly and in detail.[1] For example, Olah investigated carbocations in "magic acid" solutions under nonreaction conditions.[2] Transient carbocations that contained an appropriate chromophore were studied by using UV spectroscopy.[3, 4] Radicals were explored by ESR spectroscopy and in appropriate cases by CIDNP (chemically induced dynamic nuclear polarization), and UV spectroscopy.[5, 6] However, it seems to be most remarkable that these methods (there may be some exceptions) are not generally suited to detect and to study these reactive intermediates directly in reaction solutions, for example, of a radical chain reaction. Furthermore, substrates, intermediates, and final products cannot be monitored by using these methods. Additional measurements have to be applied to do so. Clearly, it would be of great importance to have a simple method available to study a reaction by monitoring substrates and all intermediates and final products formed, and especially to detect and characterize simultaneously and directly the reactive intermediates. Such a method could give new and important insights in our understanding of reactions and their mechanisms. Furthermore, the method should be applicable to micro amounts of substrates and should allow a high throughput, thus contributing to a sustainable development.[7]

Recently, electrospray ionization mass spectrometry (ESIMS)[8] has been successfully applied to the investigation of some chemical reactions in solution. The investigations were mostly performed offline, for example, with the oxidation of tetrahydropterins to radical cations,[9] homogeneously catalyzed reactions such as the Suzuki reaction,[10] and the

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