Versatile solid-phase thiolytic reduction of azido and *N*-Dts groups in the synthesis of haemoglobin (67–76) *O*-glycopeptides and photoaffinity labelled analogues to study glycan T-cell specificity

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A series of *O*-glycosylated peptides and photoaffinity labelled glycopeptide analogues of the mouse haemoglobin-derived decapeptide Hb (67–76), VITAFNEGLK, which binds well to the MHC class II E^k molecule and is non-immunogenic in CBA/J mice, was synthesized by multiple-column peptide synthesis employing the glycosylated building blocks 1–4 and 7–21. The non-immunogenic peptide VITAFNEGLK was converted into an immunogen by introducing different tumour-associated carbohydrate moieties [β-D-GlcNAc-*O*-Ser/Thr, α-D-GalNAc-*O*-Ser/Thr (T_N-antigen) core 1 (T-antigen), core 2, core 3 and core 4] to the central position Asn-72 in the decapeptide. Previous studies suggest that T cells may be capable of recognizing epitopes which are partially defined by glycans and may be in direct contact with the T-cell receptor. In order to study the specificity of glycan interactions with the T-cell receptor a series of corresponding glycopeptides labelled with 2-azidobenzamide on the carbohydrate amino function was synthesized. The glycan structure was varied with respect to *O*-GlcNAc, T and T_N-antigen moieties and anomeric configuration. Throughout, efficient reduction of the *N*-dithiasuccinyl- and azido-functionality-containing building blocks 1, 2, 7, 8, 11, 12, 13, 16, 18 and 20 could be achieved either (i) in solution by utilizing simultaneous *in situ* reduction with Zn in THF–HOAc–Ac_2O or (ii) on solid-phase upon treatment with diisopropylethylamine and an excess of dithiothreitol or α-mercapto-*N*-methylacetamide. N-Acetylation of the resin-bound glycopeptides furnished the *O*-glycopeptides 24, 25 and 31–36. No further modification of the carbohydrate moiety on the solid phase was required when utilizing the N-acetylated building blocks 3, 4, 9, 10, 14, 15, 17, 19 and 21. In addition, comparative studies with solid-phase reduction were conducted for the syntheses of the O-linked glycopeptides 24, 25 and 31–36 by employing any of the building blocks 1–4 and 7–21. The photoaffinity labelled glycopeptides 39–45 were synthesized by employing building blocks 1, 2, 7, 8 and 11–13 by reduction of azido or *N*-Dts functionalities by thiolysis with dithiothreitol and subsequent coupling of the activated photoaffinity label 38 to the glycanamino group of the resin-bound glycopeptides. The synthesized mucin *O*-glycopeptides 24, 25 and 31–36 and the photoaffinity labelled analogues 39–45 were fully characterized by 1D and 2D ^1H NMR spectroscopy and by electrospray mass spectrometry.

Introduction

Glycosylation is the most common modification of proteins. Carbohydrates are bound to almost all the proteins on the cell membrane and many microorganisms express glycosylated protein antigens. The presence of a glycan modulates the properties of the protein such as its solubility, protease resistance and immunogenicity.[1] Aberrant glycosylation of glycoproteins, due to incomplete synthesis of carbohydrate chains and accumulation of their precursors, is associated with oncogenesis and has been implicated in metastasis. Among such glycoconjugates, T_N (α-GalNAc-Ser/Thr) and sialyl-T_N (α-Neu5Ac-2→6-GalNAc-Ser/Thr) antigens, which belong to mucin-type (O-linked) core carbohydrate antigens, are known to be cancer-associated antigens that are expressed frequently in many tumours but rarely in normal tissues.[2] A glycoprotein is generally a heterogeneous mixture of different glycoforms: protein molecules which differ only in the structure of the bound carbohydrate. Heterogeneous glycosylation seems to be a subtle mechanism of biological control. In O-linked glycosylation the oligosaccharide chains are attached to the side chain of Ser or Thr through an α-anomeric linkage of a core α-D-GalNAc residue which can be further elongated by Gal, GlcNAc, Fuc or sialic acids to built the different core structures.[3] Recently a new form of O-glycosylation has been identified in which a single GlcNAc residue is attached *via* a β-O-linkage to serine or threonine on proteins within the nuclear and cytoplasmic compartments of eukaryotic cells.[4] This novel type of post-translational glycosylation is both an abundant and transient modification. The addition of *O*-GlcNAc appears to be highly dynamic and responsive to extracellular stimuli in a fashion analogous to that of protein phosphorylation.[5] Aberrant glycosylation on tumour cells is one of the most characteristic traits of malignancy.[6] It is therefore of significant interest to study whether glycopeptide epitopes, mimicking post-translational modification of proteins, can be recognized by T-cells. An important event in the generation of an immune response is the activation of T-cells by peptides bound to the class II proteins of the major histocompatibility complex (MHC).[7] The MHC proteins have a unique binding motif that enables them to bind to a variety of peptides. Antigenic peptides are derived from exogenous proteins, which are processed into peptide fragments of 10–25 amino acids and bound to MHC class II molecules by antigen-presenting cells. The peptide–MHC class II complex is then transported to the cell surface where they may be recognized by CD-4 positive T-cells which then trigger the immune

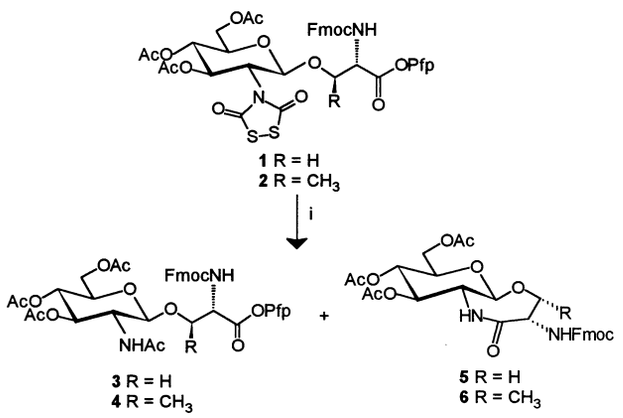
response. Although the majority of eukaryotic proteins are glycosylated, it has not yet been demonstrated whether the glycan part is mainly hydrolysed or remains attached during antigen processing. It was recently demonstrated that the glycan can be directly involved in the interaction with the T-cell receptor.[8] In the present study a peptide fragment of CBA/J mouse haemoglobin Hb (67–76), VITAFNEGLK, has been selected to investigate the influence of different glycosylations on T-cell response. This peptide is non-immunogenic itself in CBA/J mouse, but it is known to bind very well to the MHC class-II molecule, E^k, expressed by the antigen-presenting cell (APC) of the CBA/J mouse.[9] By introducing glycan structures at the position of the Asn residue the peptide was converted into an immunogen.[10] Preliminary studies using synthetic glycopeptide analogues of this epitope VITAFNEGLK have shown that glycosylation does not affect binding to MHC-class II and that glycopeptide can elicit a strong T-cell response, which is glycopeptide specific.[8d] In order to investigate further the fine specificity of T-cell response against synthetic glycopeptides, a series of glycopeptides containing *O*-glycan moieties such as β-GlcNAc, α-GlcNAc, α-GalNAc and core 1–4 structures **24**, **25** and **31**–**36** and corresponding photoaffinity labelled glycopeptide analogues **39**–**45** of immunogenic epitope structures has been synthesized during this study. These glycopeptide analogues may enable us to investigate the binding of contact residues of the epitope to the T-cell receptor binding sites. A detailed knowledge of the mechanisms by which glycopeptides are processed in the antigen-processing pathway, presented by the antigen-presenting cell and finally recognized by the T-cell receptor is important for the design of peptide-based vaccines. The most efficient and reliable approach to the synthesis of a wide range of *O*-glycopeptides is the use of suitable protected *O*-glycosylated serine and threonine amino acids as building blocks in the stepwise assembly of glycopeptides.[11] Direct glycosylation of active esters derivatives, N^{α}-Fmoc-Ser-OPfp and N^{α}-Fmoc-Thr-OPfp has been developed which alleviates the manipulation of protecting groups in the glycosyl amino acids and allows direct use in the multiple-column solid-phase synthesis.[12] Solid-phase methods utilizing N^{α}-Fmoc-protected glycosylated amino acid building blocks are the most flexible and reliable way to prepare a series of glycopeptides.[13] In the 'active ester' approach the preactivated Fmoc-AA-OPfp can be glycosylated directly to provide building blocks for multiple-column peptide synthesis (MCPS).[11]

This report describes the efficient multiple-column glycopeptide synthesis of a series of *O*-glycopetide analogues **24**, **25** and **31**–**36** employing either the use of the acetamido building blocks **3**, **4**, **9**, **10**, **14**, **15**, **17**, **19** and **21** or alternatively the use of the *N*-dithiasuccinyl (*N*-Dts)- and azido (N_3)-containing building blocks **1**, **2**, **7**, **8**, **11**–**13**, **16**, **18** and **20**. The syntheses of the *N*-Dts- and N_3-containing building blocks **1**, **2**, **7**, **8**, **11**–**13**, **16**, **18** and **20** and the *N*-acetylated building blocks **9**, **10**, **14**, **15**, **17**, **19** and **21** has been reported previously.[14,15] The reduction of the amino group precursors N_3 and *N*-Dts groups are important transformations in the solid-phase synthesis of glycopeptides to introduce the amino functions. The *N*-Dts functionality can be cleaved efficiently by thiolytic reduction on a solid phase by employing dithiothreitol (DTT) or β-mercaptoethanol (BME) in the presence of diisopropylethylamine (DIPEA) as a catalyst.[15,16] The parent amino functions can be successfully modified on the solid phase to the corresponding *N*-acetyl glycopeptide.[15,16] Azides, on the other hand, are, in general, readily reduced in solution to their corresponding amines by a range of reducing agents [*e.g.* $LiAlH_4$, H_2–Lindar catalyst, Zn–HCl, PPh_3–water, H_2S–pyridine, Bu_3SnH–azoisobutyronitrile (AIBN), $NaBH_4$–phase-transfer catalyst, thioacetic acid]. Most of these methods unfortunately suffer from disadvantages in relation to general applicability, selectivity and particularly convenience for reductions on a solid phase where homogeneous conditions are required. It has been shown that azido sugars, on the other hand, can be reduced by dithiols under weakly basic conditions on a solid phase.[17] As for the mechanism, initial attack by a thiolate anion on the azide with subsequent cyclization to a cyclic disulfide and the amine, respectively, is proposed. Reduction of azides with thioacetic acid and simultaneous N-acetylation is cumbersome and inconvenient due to the long reaction times required and the inherent formation of *N*-thioacetates as impurities. In order to circumvent these difficulties different thiols were investigated for the reduction of azide- and *N*-Dts-containing glycopeptides on a solid phase in the present report. A different strategy for the *in situ* reduction and N-acetylation of azido and *N*-Dts groups by means of Zn-reduction in tetrahydrofuran (THF)–Ac_2O–HOAc to yield the *N*-acetylated building blocks **9**, **10**, **14**, **15**, **17**, **19** and **21** for the MCPS of mucin core 1, 2, 3 and 4 *O*-glycopeptides has previously been described.[14] The 2-azido and the 2-*N*-Dts group have been employed as amino group precursors to obtain, stereoselectively, the α- (azido-approach) or β-linked (*N*-Dts approach) amino sugars, respectively. The synthetic utility of DTT or *N*-methyl-α-mercaptoacetamide (MCA) for the rapid and mild reduction of the azide- and the *N*-Dts functionalities both in solution and on solid phase in the MCPS of the glycopeptides **24**, **25** and **31**–**36** is described. Furthermore, MCPS and characterization of the seven photoaffinity labelled glycopeptide analogues **39**–**45** is reported. The MCPS was successfully achieved *via* the incorporation of the previously described *N*-Dts and azido building blocks **1**, **2**, **7**, **8** and **11**–**13** into standard MCPS, followed by DTT-reduction on the solid phase and coupling of the photoaffinity label **38** to the free amino group of the glycan during the MCPS procedure. The general application of glycosylated building blocks with thiol reduction of azido and *N*-Dts groups in the synthesis of a range of *O*-glycopeptides is described. In addition we report the ^{1}H NMR and electrospray mass spectrometry (ESMS) characterization of all the *O*-glycopeptides **24**, **25** and **31**–**36** and **39**–**45**.

Results and discussion

The present paper describes the synthesis of a large variety of *O*-glycopeptides and their photoaffinity-labelled analogues of the Hb (67–76) peptide. One of the most efficient methods for the synthesis of such a series of glycopeptides is MCPS performed in a Teflon block with several synthesis columns employing glycosylated amino acids as building blocks. The protecting group patterns of the building blocks has previously been shown to be suitable for the solid-phase synthesis of *O*-glycopeptides. The *O*-glycopeptides were assembled by Fmoc-based solid-phase synthesis. The use of Fmoc allows mild deprotection with piperidine in *N*,*N*-dimethylformamide (DMF) without β-elimination of the carbohydrate. O-Acetyl groups were employed for protection of carbohydrate hydroxy groups, allowing mild deprotection with sodium methoxide in methanol. The carboxy group is activated as its pentafluorophenyl (Pfp) ester for aminolysis and formation of the peptide bonds. The reactivity of the Pfp esters was further enhanced by addition of 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine (DHBT-OH). The use of DHBT-OH as an auxiliary nucleophile allows the progress of formation of the amide bond to be followed visually. The peptide syntheses were performed on Macrosorb resin, derivatized by the acid-labile hydroxymethylphenoxyacetic acid (HMPA) linker, using a two-step reaction cycle (coupling and Fmoc deprotection). The linker was coupled to the resin by activation with *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) and *N*-ethylmorpholine (NEM). The first amino acid, lysine, was coupled as Fmoc-Lys(Boc)-OH by activation with 1-mesitylsulfonyl-3-nitro-1,2,4-triazole (MSNT) and *N*-methylimidazole. Unchanged amino groups were capped by 20% acetic anhydride in DMF. The resin was washed thoroughly

after each Fmoc-removal and acylation step. Fmoc cleavages were effected by treatment with 20% piperidine in DMF. The successive amino acids were coupled as Pfp esters. The glycosylated Fmoc-amino acid-OPfp esters were coupled as usual amino acids in MCPS by using extended reaction times and only a small excess of the building block. Acidolytic cleavage and deprotection of the peptide moiety of the completed glycopeptides with 95% aq. trifluoroacetic acid (TFA) and final deacetylation with sodium methoxide in methanol gave the desired glycopeptides. The crude glycopeptides were then purified by semipreparative C-18 HPLC and analysed by ESMS and ^{1}H NMR spectroscopy.



Scheme 1 Synthesis of the β-D-*O*-GlcNAc building blocks **3** and **4**; *reagents and conditions:* i, Zn in THF–Ac_2O–HOAc (3:2:1)

Synthesis of the β-*O*-GlcNAc glycopeptides 24 and 31

The *N*-Dts-concept afforded the stereocontrolled synthesis of the β-*O*-GlcNDts-containing building blocks **1**, **2**, **16**, **18** and **20** and the *N*-Dts group could be removed either (i) by zinc reduction in THF–acetic anhydride–acetic acid with simultaneous N-acetylation in solution or (ii) by thiolysis with DTT or BME on the solid phase. By treatment of the *N*-Dts-protected derivatives **1** and **2** with zinc in THF–acetic anhydride–acetic acid (3:2:1) the *N*-acetylated β-D-GlcNAc building blocks **3** and **4** were obtained after silica gel chromatography and recrystallization in 70 and 76% yield, respectively. Formation of the bicyclic lactams **5** and **6** as minor by-products (less than 5%) was observed during the conversion (see Scheme 1). The bicyclic lactams **5** and **6** are formed by intramolecular displacement of the Pfp ester by the amino group on C-2. This side reaction, forming a seven-membered ring, is a common problem in the selective removal of C-2 amino-protecting groups in the presence of adjacent active esters. The *N*-Dts and acetamido building blocks **1**, **2**, **3** and **4**, respectively, were then used in comparative studies for the MCPS of the β-*O*-GlcNAc glycopeptides **24** and **31**. Comparison of the thiolytic solid-phase reduction conditions employing different thiols (see Table 1) showed that rapid and quantitative cleavage of the *N*-Dts amino protecting group could be achieved by thiolysis with any of the thiols such as DTT, MCA, BME or propane-1,3-dithiol (PDT). Concerning yields, the use of DTT and MCA is superior to the less expensive but less efficient use of BME and PDT (see Table 1). Employing the N-acetylated β-*O*-GlcNAc building blocks **3** and **4** served the same purpose to synthesize the corresponding β-*O*-GlcNAc glycopeptides **24** and **31**. The comparative results (see Table 2) showed that, due to the omission of further deprotection and N-acetylation steps on the solid phase, generally higher yields were obtained by use of the β-*O*-GlcNAc building blocks **3** (80% compared with 78%) and **4** (80% compared with 68%). However, these yields do not take into account the loss of compound during reduction of **1** and **2**

Table 2 Comparative results obtained by employing the *O*-linked building blocks **1**–**4** and **7**–**19** in the synthesis of *O*-glycopeptides **24**, **25** and **31**–**36**. Expected and observed molecular masses for the individual glycopeptides **24**, **25** and **31**–**36** analysed by ESMS

Compound	Molecular formula	Relative molecular mass	ESMS[a]	Building block	Yield (%)[b]
24	$C_{57}H_{94}N_{12}O_{20}$	1267.46	1267.63	**1**	78[c]
24			1267.65	**3**	80
31	$C_{58}H_{96}N_{12}O_{20}$	1281.49	1281.72	**2**	68[c]
31			1281.78	**4**	80
25	$C_{57}H_{94}N_{12}O_{20}$	1267.46	1267.79	**7**	82[c]
25			1267.64	**9**	85
32	$C_{58}H_{96}N_{12}O_{20}$	1281.49	1267.63	**8**	63[c]
32			1267.72	**10**	75
33	$C_{64}H_{106}N_{12}O_{25}$	1443.76	1444.43	**13**	52[c]
33			1444.43	**15**	76
34	$C_{72}H_{119}N_{13}O_{30}$	1646.83	1647.35	**16**	45[c]
34			1647.38	**17**	64
35	$C_{66}H_{109}N_{13}O_{25}$	1484.68	1485.22	**18**	45[c]
35			1485.28	**19**	62
36	$C_{74}H_{122}N_{14}O_{30}$	1687.88	1688.35	**20**	35[c]
36			1688.36	**21**	55

[a] Glycopeptides were detected as $[glycopeptide + H]^+$ ions. [b] Obtained after preparative reversed-phase HPLC. [c] Thiolysis conditions: DTT (0.2 mol dm^{-3}), DIPEA (0.1 mmol dm^{-3}) in dichloromethane and subsequent N-acetylation with 20% Ac_2O in DMF.

Table 1 Reduction of *N*-Dts- and azido group-containing glycopeptides **22** and **23** with subsequent N-acetylation to obtain the glycopeptides **24** and **25** by employing different thiolytic reagents and DIPEA as a catalyst (see Scheme 2)

Entry	Building block	Glycopeptide	Thiol (0.2 M)	Base	Reaction time on solid phase	Yields (%)
1	*N*-Dts **1**	**24**	DTT	DIPEA (0.1 mM)	2 × 10 min	93,[a] 78[b]
2	*N*-Dts **1**	**24**	McA	DIPEA (0.3 M)	2 × 10 min	92,[a] 74[b]
3	*N*-Dts **1**	**24**	BME	DIPEA (0.3 M)	2 × 10 min	89,[a] 67[b]
4	*N*-Dts **1**	**24**	PDT	DIPEA (0.3 M)	2 × 10 min	87,[a] 62[b]
5	Azide **7**	**25**	DTT	DIPEA (0.1 mM)	2 × 2 h	99,[a] 82[b]
6	Azide **7**	**25**	McA	DIPEA (0.3 M)	2 × 2 h	98,[a] 78[b]
7	Azide **7**	**25**	BME	DIPEA (0.3 M)	2 × 2 h	65,[a] 48[b]
8	Azide **7**	**25**	PDT	DIPEA (0.3 M)	2 × 2 h	15,[a] 7[b]

[a] Yields were determined from chromatographic data (analytical HPLC and percentage of product determined by absorption at 220 nm). [b] Yields after preparative HPLC (based on 50 mg resin with the loading 0.2 mmol g^{-1} specified by the supplier).

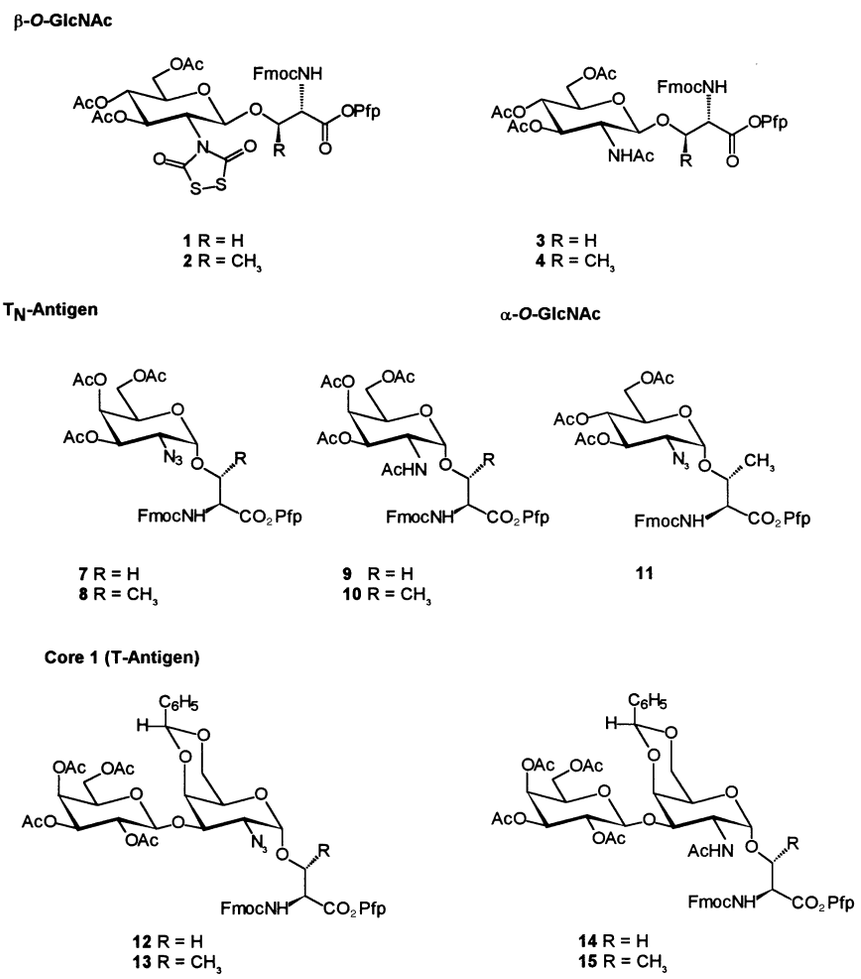
to **3** and **4**, respectively. This loss becomes more important with increasing complexity of the building blocks, *e.g.* **16** and **20**.

Reduction of the azido group on the solid phase

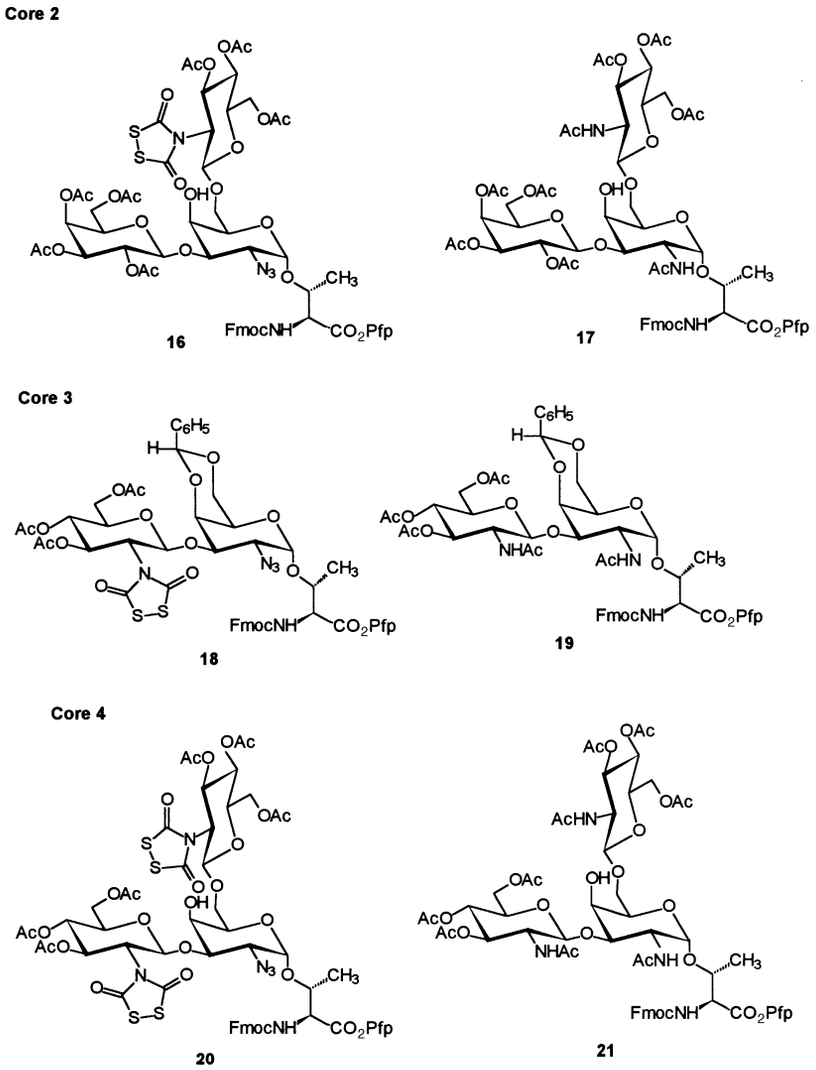
The azido approach, on the other hand, offers the possibility of synthesizing predominantly the α-linked *O*-GalNAc-serine and -threonine building blocks. Glycosylation of Fmoc-Ser-OPfp or Fmoc-Thr-OPfp esters with 2-azido-2-deoxygalactosyl donors affords, with high stereoselectivity and good yields, the corresponding α-linked building blocks. The azido group can be reduced and N-acetylated in the same way as the *N*-Dts group either (i) prior to incorporation of the building blocks to MCPS by zinc reduction in THF–acetic anhydride–acetic acid or (ii) after incorporation on the solid phase by employing DTT or MCA as reducing reagents. In Table 1 are listed both the analytical and the isolated yields for the reduction of the resin-bound azido group-containing glycopeptide by means of the different thiolytic reagents. The results demonstrate the ease and applicability of the thiolytic azido-reduction procedure on the solid phase. The rate of reduction varies considerably with the employed thiol. High reduction yields were obtained by using DTT and MCA (99 and 98%). In contrast, employment of the less reactive thiolytic reagents BME and PDT gave yields which were low(er) (65 and 15%). Due to the higher yields of the obtained glycopeptide **25** (see Table 1) the azido-reduction procedures employing the DTT–DIPEA and MCA–DIPEA thiolysis procedure were preferred and consequently used in the following glycopeptide syntheses.

Selective reduction of *N*-Dts in the presence of azido groups in solution and on the solid phase

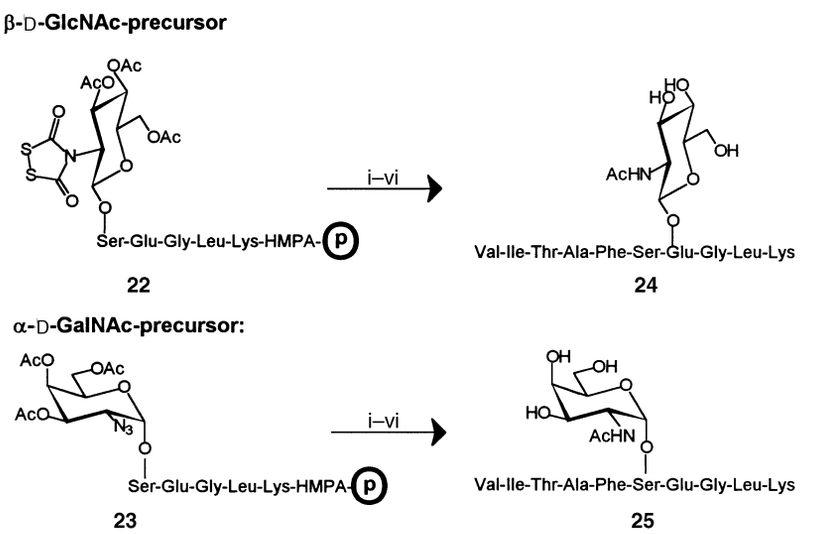
In the course of studies concerning the development of orthogonal protecting-groups schemes for solid-phase glycopeptide and glycopeptide analogue synthesis, the selectivity between the reduction of *N*-Dts and azido functionalities in solution and on the solid-phase was investigated. Therefore, different conditions for deprotection of the Dts group in the presence of azides were studied. The previously described compound **26** [18] was therefore treated with different thiols. It could be shown that it is possible to reduce both the *N*-Dts and azido group simultaneously, utilizing DTT or MCA as reducing reagent and DIPEA as catalyst. After N-acetylation the corresponding 1,2-bisacetamido compound **28** could be obtained in almost quantitative yield (Scheme 3). By use of the less reactive PDT in the presence of DIPEA it was possible to reduce selectively the *N*-Dts group of substrate **26** without affecting the azido group. After N-acetylation the β-azide **27** was obtained in 94% yield. Furthermore, this selective reduction was used for selective removal of *N*-Dts in the presence of azido groups on the solid phase. To study this reaction the resin-bound glycopeptide **29** was synthesized on the solid phase by employing the previously described building block **16** containing both an *N*-Dts and an azido group. Our results concerning reduction of azides on the solid phase (see Table 1) showed that utilizing PDT as reducing agent, even at prolonged reaction times, only reduces the azido group slightly (15% after 4 h) while the *N*-Dts group was completely cleaved after only about 10 min. By employing PDT as reducing reagent for the glycopeptide **29**, thiolytic removal of *N*-Dts could be obtained selectively in the presence of the azido group. After acetylation, cleavage and HPLC separation the glycopeptide **30** was obtained in 76% yield (Scheme 4). No reduction of the azido group was observed. The use of the less reactive PDT as thiolytic reagent allowed the selective reduction of *N*-Dts in the presence of the less reactive azido group. The successful thiolysis of the azide can be easily detected by ^{1}H NMR spectroscopy. Upon transformation of azide to acet-



Building blocks **1**–**4** and **7**–**15** employed in the MCPS of α- and β-*O*-GlcNAc, T-antigen and T_N-antigen glycopeptides



Building blocks **16**–**21** employed in the MCPS of core 2, core 3 and core 4 glycopeptides



Scheme 2 Reduction of the *N*-Dts glycopeptide **22** and the azido glycopeptide **23** in the MCPS of the glycopeptides **24** and **25**, employing different thiols (see Table 1); *reagents:* i, thiol, DIPEA; ii, Ac_2O, DMF; iii, SPPS; iv, piperidine, DMF; v, 95% aq. TFA; vi, NaOMe

amide the H-1 proton characteristically shifts upfield while H-2 shifts downfield from δ ~3.84 (**30**) to δ ~4.29 (**34**), respectively (see Table 3). Another indication for the conversion of azide into acetamide is the resonance for C-2 in the ^{13}C NMR spectrum shifting characteristically from δ_C 59.2 (**30**) to δ_C 47.1 (**34**).

Syntheses of the mucin *O*-glycopeptides 25 and 32–36

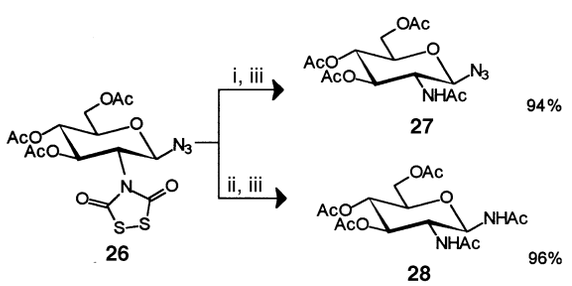
Due to the high yields obtained in stereospecific α-glycosylations using 2-azido-2-deoxyglycosyl donors the T- and T_N-antigen building blocks **7**–**10** and **12**–**15** were conveniently used to synthesize the T- and T_N-glycopeptides **25**, **32** and **33**.

Table 3 ^{1}H NMR data for the *O*-linked glycopeptides **24**, **25** and **30**–**36** measured in CD_3CO_2D–D_2O (1:1) at 600 MHz at 300 K

	30	**24**	**31**	**25**	**32**	**33**	**34**	**35**	**36**
Val									
NH									
α-H	3.93	3.94	3.94	3.95	3.92	3.91	3.95	3.95	3.95
β-H	2.23	2.23	2.23	2.23	2.17	2.20	2.23	2.22	2.21
γ-H_3	1.01	1.01	0.99	1.01	1.01	1.00	1.00	1.01	1.00
γ-H_3	1.00	0.99	1.01	1.00	0.99	0.99	1.01	1.00	1.00
Ile									
NH	8.36	8.37	8.37	8.39	8.40	8.41	8.36	8.37	8.37
α-H	4.32	4.34	4.32	4.33	4.28	4.30	4.33	4.33	4.33
β-H	1.83	1.83	1.82	1.82	1.83	1.81	1.83	1.82	1.80
γ-H_2	1.51/1.15	1.52/1.15	1.51/1.15	1.52/1.17	1.51/1.15	1.50/1.15	1.51/1.15	1.51/1.14	1.51/1.14
γ-H_3	0.87	0.89	0.87	0.85	0.86	0.87	0.88	0.89	0.88
δ-H_3	0.85	0.86	0.85	0.87	0.84	0.84	0.85	0.85	0.84
Thr									
NH	8.14	8.12	8.13	8.11	8.14	8.14	8.14	8.13	8.12
α-H	4.42	4.43	4.42	4.39	4.35	4.37	4.43	4.41	4.44
β-H	4.17	4.20	4.17	4.19	4.17	4.15	4.17	4.16	4.15
γ-H_3	1.12	1.14	1.11	1.14	1.12	1.12	1.12	1.19	1.20
Ala									
NH	8.00	8.01	7.98	8.01	8.20	8.03	8.00	7.99	7.99
α-H	4.39	4.43	4.40	4.30	4.39	4.33	4.38	4.36	4.38
β-H_3	1.29	1.27	1.29	1.25	1.23	1.29	1.28	1.27	1.26
Phe									
NH	8.00	7.98	8.04	7.90	8.08	8.04	8.00	7.99	7.98
α-H	4.81	4.65	4.72	4.60	4.75	4.79	4.82	4.81	4.82
β-H_2	3.17/2.98	3.15/2.91	3.15/3.01	3.06/2.91	3.16/3.01	3.18/3.00	3.17/2.99	3.18/2.99	3.17/2.98
1-H	7.31	7.25	7.26	7.28	7.31	7.31	7.30	7.30	7.29
2-H	7.26	7.22	7.22	7.23	7.25	7.25	7.25	7.25	7.25
3-H	7.23	7.10	7.16	7.15	7.24	7.23	7.23	7.23	7.23
Ser or **Thr**									
NH	8.26	8.06	7.90	8.03	7.99	8.26	8.27	8.16	8.23
α-H	4.59	4.56	4.49	4.56	4.39	4.55	4.59	4.56	4.57
β-H/β-H′	4.23	4.07/3.93	4.26	3.98/3.84	4.29	4.28	4.24	4.26	4.26
γ-H_3	1.20		1.10		1.29	1.23	1.20	1.20	1.20
Glu									
NH	8.20	8.07	7.95	8.11	8.23	8.19	8.20	8.07	8.19
α-H	4.45	4.41	4.41	4.38	4.36	4.42	4.46	4.54	4.48
β-H_2	2.12/1.99	2.17/1.99	2.16/1.99	2.05/1.89	2.12/1.97	2.12/1.96	2.13/1.98	2.12/1.95	2.13/1.96
γ-H_2	2.49	2.48	2.49	2.41	2.49	2.49	2.50	2.46	2.47
Gly									
NH	8.24	8.06	8.08	8.12	8.27	8.27	8.23	8.13	8.21
α-H_2	4.06/3.88	3.98	3.98	3.91/3.86	3.96/3.84	4.01/3.87	4.06/3.88	4.39	4.04/3.98
Leu									
NH	7.99	7.84	7.85	7.87	7.98	8.01	7.98	8.13	8.02
α-H	4.40	4.40	4.40	4.39	4.36	4.38	4.39	4.43	4.42
β-H_2	1.60	1.60	1.60	1.59	1.60	1.60	1.60	1.61	1.60
γ-H	1.61	1.61	1.62	1.62	1.60	1.62	1.61	1.61	1.61
δ-H_3	0.88/0.91	0.90/0.85	0.88/0.84	0.90/0.85	0.90/0.87	0.86/0.90	0.88/0.91	0.87/0.92	0.88/0.91
Lys									
NH	8.15	8.10	8.11	8.14	8.20	8.21	8.14	8.15	8.15
α-H	4.43	4.43	4.43	4.40	4.54	4.40	4.43	4.42	4.43
β-H_2	1.91/1.75	1.90/1.74	1.90/1.75	1.90/1.75	1.90/1.75	1.90/1.76	1.91/1.74	1.91/1.72	1.91/1.73
γ-H_2	1.45	1.44	1.44	1.45	1.44	1.43	1.45	1.44	1.44
δ-H_2	1.70	1.70	1.69	1.70	1.68	1.69	1.70	1.70	1.70
ε-H_2	3.03	3.02	3.02	3.03	3.01	3.01	3.03	3.03	3.03
NH_2	7.46	7.46	7.46	7.48	7.46	7.49	7.47	7.47	7.46
GalNAc									
1-H	5.01			5.08 (2.14)	5.13 (1.98)	4.92 (2.41)	4.91 (2.35)	4.86 (2.27)	4.90 (2.41)
2-H	3.84			4.47	4.37	4.27	4.29	4.23	4.27
3-H	3.95			4.02	4.38	3.96	3.96	3.95	3.95
4-H	4.02			4.04	4.05	4.24	4.02	4.06	4.06
5-H	4.35			3.85	3.88	3.73	4.31	4.26	4.32
6-H	4.36			4.03	4.02	4.01	4.35	4.38	4.42
6-H′	4.35			3.92	3.86	4.01	4.35	4.38	4.38

Table 3 (*contd.*)

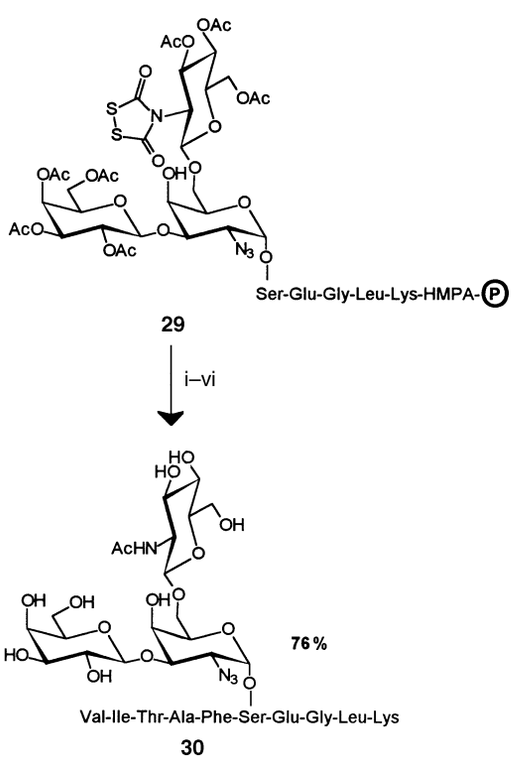
	30	24	31	25	32	33	34	35	36
GlcNAc									
1-H	4.56	4.57 (7.95)	4.56 (8.00)				4.52 (7.98)	4.66 (8.05)	4.53 (7.88) 4.67 (8.03)
2-H	3.76	3.76	3.73				3.74	3.69	3.72, 3.69
3-H	3.45	3.61	3.62				3.45	3.64	3.47, 3.56
4-H	3.58	3.47	3.45				3.57	3.52	3.53, 3.44
5-H	3.59	3.47	3.45				3.57	3.41	3.48, 3.45
6-H	3.89	3.96	3.93				3.94	3.89	3.90, 3.94
6-H′	3.90	3.76	3.93				3.94	3.78	3.88, 3.92
Gal									
1-H	4.43					4.44	4.45		
2-H	3.58					3.55	3.57		
3-H	3.64					3.61	3.62		
4-H	3.95					3.92	3.93		
5-H	3.64					3.60	3.64		
6-H	3.82					3.79	3.81		
6-H′	3.80					3.75	3.78		
NH NHAc	2.03	2.03	2.03	2.02	2.02	2.02	2.03, 2.02	2.02	2.03, 2.03, 2.01



Scheme 3 Reduction of compound **26** containing *N*-Dts and azido functionalities; *reagents and conditions:* i, 0.2 mol PDT, 0.3 mol DIPEA in CH_2Cl_2; ii, 0.2 mol DTT, 0.1 mmol DIPEA in CH_2Cl_2; iii, Ac_2O–pyridine (1:2)

Employing the azido building blocks **7**, **8**, **12** and **13** the azido groups could be efficiently reduced on the solid phase by treatment with DTT–DIPEA in dichloromethane on the solid phase. After N-acetylation with Ac_2O in DMF the T- and T_N-glycopeptides **25**, **32** and **33** were obtained in fairly high yields [**25** (68%), **32** (63%) and **33** (52%), respectively]. Utilizing the alternative route of reduction and simultaneous *in situ* N-acetylation of the azido building blocks prior to incorporation onto solid-phase, gave in general higher yields [**25** (85%), **32** (75%) and **33** (76%), respectively]. However, taking into account the loss of compounds during the additional synthetic step in the synthesis of the building blocks **9**, **10** and **12** the use of azide-containing building blocks is a valuable alternative because of the effectiveness of the mild reduction procedure carried out on the solid phase. Even though azides can be reduced and transformed into the *N*-acetamido function with thioacetic acid, the DTT reduction method offers other advantages. The reaction times are generally shorter and no inherent formation of impurities like *N*-thioacetates was observed. The DTT method is extremely mild and generally applicable for azide reduction on the solid phase.

Efficient and reliable procedures for the synthesis of mucin core 1, 2, 3 and 4 *O*-glycopeptides, the synthesis of the corresponding building blocks **12**–**21** for solid-phase peptide synthesis (SPPS) of *O*-glycopeptides was reported.[14] Simultaneous *in situ* reduction of *N*-Dts and azido functionalities in precursors **12**, **13**, **16**, **18** and **20** with zinc dust in THF–acetic acid in the presence of acetic anhydride offered a method for the synthesis of the N-acetylated building blocks **14**, **15**, **17**, **19** and **21**. These

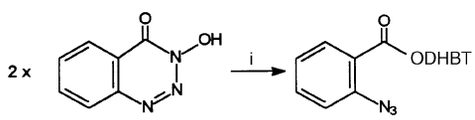


Scheme 4 Selective reduction of *N*-Dts in the presence of an azido group on the solid phase; *reagents and conditions:* i, 0.2 mol PDT, 0.3 mol DIPEA in CH_2Cl_2, 2 × 20 min, room temp.; ii, 15% Ac_2O in DMF, 2 × 10 min; iii, SPPS; iv, 20% piperidine in DMF; v, 95% aq. TFA; vi, 1% NaOMe in MeOH (pH 8.5)

building blocks were used directly in MCPS of mucin core 1, 2, 3 and 4 *O*-glycopeptides, omitting the need for further reduction steps on the solid phase. For comparative studies the building blocks **12**–**21** were then used in MCPS of the MHC class II binding glycopeptides **33**–**36**. For the synthesis of the glycopeptides **33**–**36** the *N*-Dts- and azide-containing building blocks **12**, **13**, **16**, **18** and **20** and the corresponding *N*-acetamido building blocks **14**, **15**, **17**, **19** and **21** were used. As the key step the simultaneous reduction of *N*-Dts and azido functionalities was achieved by thiolysis with DTT (0.1 M)–DIPEA (0.1 mM) in dichloromethane. After subsequent N-acetylation with acetic anhydride in DMF the peptide synthesis was continued. Due to instability of the *N*-Dts group towards secondary amines to form urea derivatives, it is necessary to remove the *N*-Dts group before resuming the peptide synthesis using piperidine or morpholine for removal of Fmoc. *The* N-*Dts group has to be removed before continuation of the peptide synthesis!* After coupling of the last amino acid, Fmoc deprotection, cleavage from the resin with 95% TFA, O-deacetylation and preparative HPLC purification, the pure *O*-glycopeptides were obtained in yields of 35–64% based on the loading of the resin (Table 2). Depending on the method of synthesis, reduction on the building-block stage or reduction during peptide assembly, the mucin core 1, 2, 3 and 4 *O*-glycopeptides **33**–**36** could be obtained in 52 or 76% (**33**); 45 or 64% (**34**); 45 or 62% (**35**) and 35 or 55% (**36**) yield, respectively. The purity of the glycopeptides **33**–**36** was excellent as indicated by analytical HPLC. In the case of the azide- and *N*-Dts-containing building blocks the corresponding glycopeptides were obtained without any problems during assembly or reduction when utilizing building block **20** with two *N*-Dts and one azido functionality. This shows that the azido and *N*-Dts building blocks **12**, **13**, **16**, **18** and **20** and the acetamido building blocks **14**, **15**, **17**, **19** and **21** can be used alternatively and independently in the assembly of *O*-glycopeptides on a solid phase. Compounds **33**–**36** were fully characterized by ESMS and 1D and 2D ^{1}H NMR spectroscopy (see Table 3). The chemical shifts of the anomeric carbohydrate protons vary significantly in comparison with the other carbohydrate protons. Connectivities of the α, β, γ, δ and ε protons were accessed by ^{1}H–^{1}H 2D homonuclear chemical shift correlation (COSY) and nuclear Overhauser enhancement in rotating frame (ROESY) experiments.

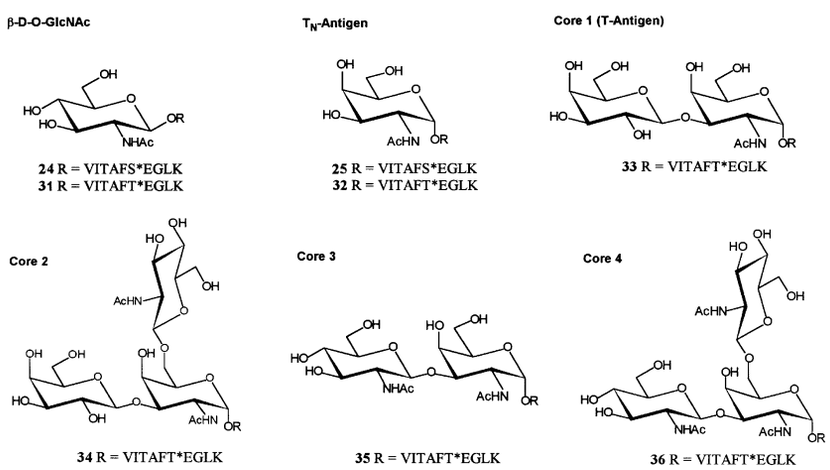
Synthesis of the photoaffinity labelled glycopeptides 39–45

It has been demonstrated that the glycans in the Hb (67–76) glycopeptide analogues are in direct contact with the T-cell receptor and specific recognition of the glycan on the glycopeptide was observed. In order to identify more specifically the binding site of the T-cell receptor, corresponding photoaffinity labelled glycopeptides have been synthesized. These analogues are modified on the carbohydrate amino group by the photoaffinity tag **38** which can be activated to produce a reactive nitrene. The photoaffinity labelling technique requires the synthesis of photolabile reagents to produce reactive intermediates, such as carbenes or nitrenes, which insert rapidly into the amino acid residues of the biomolecule and tag the target molecule for isolation and structural determination. Several photolabile probes such as diazo ketones, diazoacetates and aromatic azides have been used. The most frequently employed are aromatic azides. Thus, aromatic azides produce long lived nitrene intermediates, have remarkable chemical stability, a long-wavelength absorption upon photolysis and they are readily synthesized. The DHBT-activated 2-azidobenzoic acid **38** was selected for the present investigation. The synthesis of the photoaffinity tag **38** is depicted in Scheme 5. Treatment of the commercially available compound **37** with 0.5 mol equiv. of dicyclohexylcarbodiimide (DCCI) in DMF at room temperature for 12 h afforded the DHBT-active ester **38**, in a

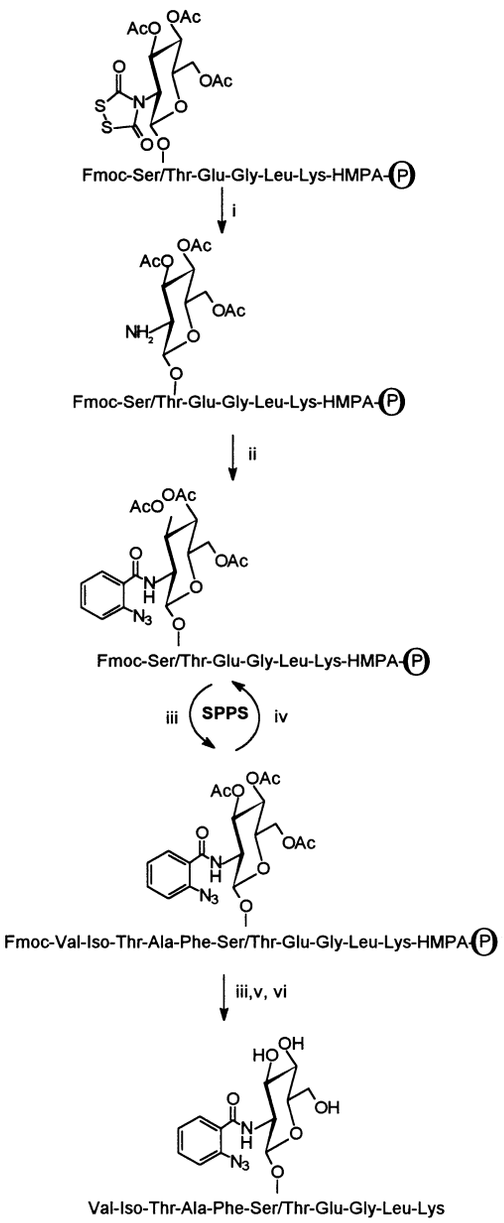


Scheme 5 Synthesis of the photoaffinity label **38**; *reagents and conditions:* i, DCCI, DMF, 24 h, room temp.

high yield of 93% after silica gel chromatography, as yellowish crystals. MCPS, performed in a Teflon block with several synthesis columns, was found to be efficient in the synthesis of the photoaffinity labelled glycopeptides **39**–**45**. The synthesis was carried out on Macrosorb resin modified with the acid-labile HMPA linker using acylation reactions utilizing stock solutions of preactivated Fmoc-amino acid-OPfp esters with DHBT-OH catalysis and subsequent Fmoc deprotection with 20% piperidine in DMF. The glycosylated Fmoc-amino acid-OPfp esters **1**, **2**, **7**, **8**, **11**–**13** were coupled as usual amino acids in MCPS using extended reaction times and only a small excess of the building block (1.6 mol equiv.). The use of DHBT-OH allows the acylation reaction to be monitored visually due to the yellow ion-pair formed with residual amine. The key step in the present strategy is the DTT reduction of the resin bound *N*-Dts and azido glycopeptides and subsequent coupling of the photoaffinity label **38** to the corresponding amino groups on the solid phase (see Schemes 6 and 7). For the stereoselective introduction of amino glycans into the glycopeptides the azido and the *N*-Dts groups were employed to obtain stereoselectively α- and β-linked amino sugar glycopeptides, respectively. The previously described *N*-Dts **1** and **2** and the azido building blocks **7**, **8**, **11**–**13** were used. For the reduction of the amino group precursors on the solid phase, DTT was used. The Mac-

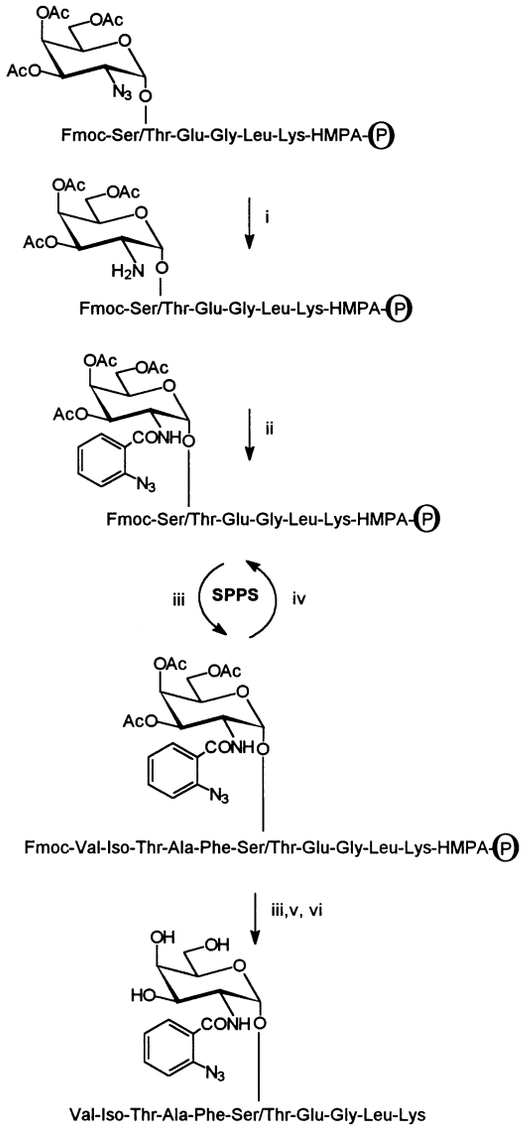


O-Glycopeptides **29**–**36** synthesized by multiple-column solid-phase synthesis. The asterisk * indicates site of glycosylation.



Scheme 6 Principle of SPPS of photoaffinity labelled glycopeptides **39** and **40** utilizing compounds **1** and **2**; *reagents and conditions:* i, DTT (0.2 mol), DIPEA (0.1 mmol) in CH_2Cl_2; ii, **38** in DMF, 6 h; iii, 20% piperidine in DMF; iv, Fmoc-AA-OPfp (3 mol equiv.), DHBT-OH (1 mol equiv.); v, 95% aq. TFA; vi, 1% NaOMe in MeOH (pH 9)

rosorb resin (100 mg) was, after incorporation of the building blocks **1**, **2**, **7**, **8**, **11**–**13**, thoroughly washed with dichloromethane. The azido and *N*-Dts reduction was then performed by treatment of the resin with a solution of DTT (0.2 M)–DIPEA (0.1 mM) in dichloromethane for 6 h (1 × 2 min, 2 × 3 h). The transformation of the azido group into the corresponding amino function was followed by IR spectroscopy by observation of complete disappearance of the azide absorption band at ν_{max} 2117 cm^{-1}. In addition the Kaiser test was used to follow the formation of amino groups on the solid phase. After complete conversion into the amino function, the resin was washed successively with dichloromethane and DMF. Further modification of the amino group with the photoaffinity label was achieved by adding a solution of 3 mol equiv. of the active-DHBT ester **38** dissolved in DMF to the resin. Coupling of ester **38** was complete after 6 h. After Fmoc-deprotection, peptide synthesis was continued by employing Fmoc-amino acid-OPfp esters and DHBT-OH. After the addition of the last amino acid residue, the Fmoc group was removed and the glycopeptide was deprotected and cleaved from the resin by treatment with

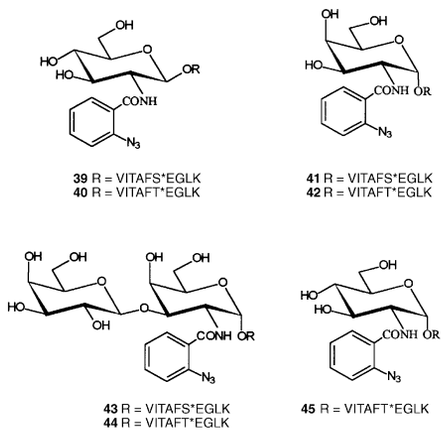


Scheme 7 Principle of SPPS of photoaffinity labelled glycopeptides **41**–**45** utilizing compounds **7**, **8**, **11**–**13**; *reagents and conditions:* i, DTT (0.2 mol), DIPEA (0.1 mmol) in CH_2Cl_2; ii, **38** in DMF, 6 h; iii, 20% piperidine in DMF; iv, Fmoc-AA-OPfp (3 mol equiv.), DHBT-OH (1 mol equiv.); v, 95% aq. TFA; vi, 1% NaOMe in MeOH (pH 9)

TFA–water (95:5). Removal of the acetyl groups from the carbohydrate moiety was achieved with sodium methoxide (1%) in methanol at pH 8.5 (pH paper). The labelled glycopeptides **39**–**45** were then subjected to semipreparative HPLC purification before being characterized by ESMS and ^{1}H NMR spectroscopy. After lyophilization the pure labelled glycopeptides were obtained in yields from 61 to 78% based on the loading of the resin (see Table 4). The synthesis was achieved without any problems concerning the reduction of *N*-Dts and azide groups and subsequent acylation with the photoaffinity label **38**. This reaction procedure can be generally applied in the synthesis of modifications of the amino function of amino sugars in glycopeptides.

Conclusions

In the present work an efficient MCPS protocol which allows the simultaneous and parallel synthesis of a large variety of mucin *O*-glycopeptides and their photoaffinity labelled analogues has been described. Comparative studies demonstrated that any of the building blocks **1**–**4** and **7**–**21** are well suited for the MCPS of the *O*-glycopeptides **24**, **25** and **31**–**36**. The use of the acetamido building blocks **3**, **4**, **9**, **10**, **14**, **15**, **17**, **19** and **21** gave overall a similar yield when compared with the



39 R = VITAFS*EGLK
40 R = VITAFT*EGLK

41 R = VITAFS*EGLK
42 R = VITAFT*EGLK

43 R = VITAFS*EGLK
44 R = VITAFT*EGLK

45 R = VITAFT*EGLK

Photoaffinity labelled glycopeptides **39**–**45** synthesized by MCPS. The asterisk * indicates site of glycosylation.

Table 4 Results of the SPPS of the photoaffinity labelled glycopeptides **39**–**45** by use of the building blocks **1**, **2**, **7**, **8**, **11**–**13**. *N*-Dts and azido reduction[a] and coupling of the photoaffinity label **38** performed on the solid phase. Comparison of expected and observed molecular masses for the individual glycopeptides **39**–**45** analysed by ESMS

Compound	Molecular formula	Relative molecular mass	ESMS[b]	Yield (%)[c]
39	$C_{62}H_{96}N_{16}O_{19}$	1369.56	1370.76	64
40	$C_{63}H_{98}N_{16}O_{19}$	1383.58	1384.75	63
41	$C_{62}H_{96}N_{16}O_{19}$	1369.56	1370.82	78
42	$C_{63}H_{98}N_{16}O_{19}$	1383.58	1384.77	73
43	$C_{63}H_{98}N_{16}O_{19}$	1383.58	1384.53	71
44	$C_{68}H_{106}N_{16}O_{24}$	1531.69	1532.70	63
45	$C_{69}H_{108}N_{16}O_{24}$	1545.72	1546.61	61

[a] Reduction conditions: dithiothreitol (DTT) (0.2 mol dm^{-3}), diisopropylethylamine (DIPEA) (0.1 mmol dm^{-3}) in dichloromethane. [b] Glycopeptides were detected as $[glycopeptide + H]^+$ ions. [c] Yields were determined based on the loading of the resin (0.2 mmol g^{-1}) and obtained after semipreparative reversed-phase HPLC.

use of their precursors **1**, **2**, **7**, **8**, **12**, **13**, **16**, **18** and **20** with solid-phase reduction, in particular for the larger building blocks. The *N*-Dts- and azido group-containing building blocks possess, on the other hand, some favourable properties. Mild reduction conditions for the conversion of *N*-Dts and azido to the corresponding amino function by treatment with thiols (DTT and MCA) does not induce any decomposition of the glycopeptide and it offers the possibility of further modification of the sugar amino function into the acetamido or photoaffinity labelled glycopeptides **24**, **25**, **31**–**36** and **39**–**45**. Selective reduction of *N*-Dts in the presence of the more stable azido group can be achieved by treatment with the less reactive PDT both in solution and on the solid phase. For the introduction of the photoaffinity label onto the glycopeptides the readily available DHBT-activated 2-azidobenzoic acid **38** was selected. Incorporation of the building blocks **1**, **2**, **7**, **8** and **11**–**13**, subsequent reduction of the *N*-Dts or azido functionalities by treatment with DTT, and coupling of the photoaffinity label **38** onto the corresponding amino group affords excellent yields of the labelled glycopeptides **39**–**45**. The outlined protocol for affinity labelling proved to be advantageous in many respects. A difficult synthesis of the corresponding labelled building blocks prior to incorporation into the peptide was avoided because acylation of the glycosyl amino group with the photoaffinity label **38** could be effected efficiently on the solid phase. Similarly, a large variety of other labels can be attached to the amino function of amino sugars on the solid phase. Applying these MCPS protocols the *O*-GlcNAc and mucin *O*-glycopeptides **24**, **25** and **31**–**36** and the photoaffinity labelled glycopeptide analogous **39**–**45** could be obtained in excellent yields and purity (see Tables 2 and 4). The mild conditions used in the conversion of *N*-Dts and azide into the corresponding amino function by means of thiols shows that both groups are useful precursors of amines and that conversion can be employed selectively during synthetic procedures. Application of this new concept in other areas of peptide synthesis on a solid phase are currently under investigation. The corresponding immunological studies of all the glycopeptides **24**, **25**, **31**–**36** and **39**–**45** will be published elsewhere.

Experimental

General procedures

All solvents were purchased from Labscan Ltd. (Dublin, Ireland). Vacuum liquid chromatography (VLC) was performed on pre-dried Merck silica gel 60 H (0.040–0.060 mm, heating at 120 °C for 24 h) with solvents dried over molecular sieves. TLC was performed on Merck Silica Gel 60 F254 aluminium sheets with detection by charring with sulfuric acid and by UV light, when applicable. DMF was freshly distilled by fractional distillation at reduced pressure and kept over molecular sieves (3 Å). Dichloromethane was distilled from phosphorus pentaoxide and kept over molecular sieves (3 Å). Pyridine was distilled and kept over molecular sieves (3 Å). Light petroleum was the 60–80 °C fraction. Concentrations were performed under reduced pressure at temperatures below 40 °C. $MgSO_4$ was used to dry organic extracts. Suitably protected N^{α}-Fmoc-amino acid-OPfp esters were purchased from Bachem (Bubendorf, Switzerland) and NovaBiochem (Switzerland). Macrosorb SPR 250 resin from Sterling Organics (UK), NEM and DHBT-OH from Fluka, *threo*-1,4-dimercaptobutane-2,3-diol (DTT) from Aldrich, DIPEA, MCA and PDT from Sigma. Electrospray mass spectrometry was performed in the positive mode on a Fisons VG Quattro Instrument. MALDI-TOF MS was performed on a Finnigan MAT 2000 using a matrix of α-cyano-4-hydroxycinnamic acid. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM 600 MHz or DRX 250 MHz spectrometer. Chemical shifts are given in ppm and referenced to internal $SiMe_4$ (δ_H 0.00) or $CDCl_3$ (δ_C 77.00) at 300 K. *J*-Values are given in Hz. For spectra recorded in CD_3CO_2D–water the HOAc signal at δ_H 2.03 was used as internal reference. For the assignment of signals ^{1}H–^{1}H COSY, ^{1}H–^{1}H double-quantum-filtered phase-sensitive COSY and NOE in rotating frame (ROESY) experiments were used. Analytical and semipreparative reversed-phase HPLC separations were performed on a Waters HPLC system using analytical RCM (8 × 100 mm) and Delta PAK (15 μm; 300 Å; 25 × 200 mm) C-18 columns with a flow rate of 1 cm^3 min^{-1} and 10 cm^3 min^{-1}, respectively. Detection was at 215 and 280 nm with a photodiode array detector (Waters M 991). Solvent System A: 0.1% TFA in water; B: 0.1% TFA in 90% acetonitrile–10% water.

O-(2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-gluco-pyranosyl)-*N*-(fluoren-9-ylmethoxycarbonyl)-L-serine pentafluorophenyl ester 3

Compound **1** (1 g, 1.11 mmol) was dissolved in THF–acetic anhydride–acetic acid [50 cm^3 (3:2:1)] and zinc (~750 mg, activated with 2% aq. $CuSO_4$) was added. The mixture was stirred for 6 h at room temperature, diluted with THF (100 cm^3), filtered through Celite, rinsed several times with THF and concentrated. Purification by VLC-chromatography on dried silica gel [ethyl acetate–light petroleum (2:1)] and subsequent crystallization afforded the title compound **3** (633 mg, 70%) as crystals; NMR data, mp and optical rotation were in agreement with those previously reported.[19] A small fraction of the lactam **5** (less than 5%) was isolated from the purification by silica gel chromatography. $[\alpha]_D^{27}$ +17.5 (*c* 1.7, $CDCl_3$); $C_{31}H_{34}N_2O_{11}$ [MALDI-MS $(M + H)^+$ Calc. *m*/*z*, 596.53. Obs. *m*/*z*, 597.57]; δ_H(250 MHz, $CDCl_3$) 6.35 (1 H, NH), 5.86 (1 H, *J* 5.2, NH-Fmoc), 5.15 (1 H, 4-H), 5.06 (1 H, 3-H), 4.55 (1 H,

$J_{1,2}$ 7.73, 1-H), 4.43 and 4.37 (2 H, FmocCH_2), 4.25 and 4.15 (2 H, 6-H_2), 4.14 (1 H, FmocCH), 3.75 (1H, 5-H) and 3.68 (1 H, 2-H).

O-(2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-N-(fluoren-9-ylmethoxycarbonyl)-L-threonine pentafluorophenyl ester 4

Compound **2** (1 g, 1.09 mmol) was dissolved in THF–acetic anhydride–acetic acid [50 cm^3 (3:2:1)] and zinc (~750 mg, activated with 2% aq. $CuSO_4$) was added. The mixture was stirred for 6 h, diluted with freshly distilled THF (100 cm^3), filtered through Celite, rinsed several times with THF and concentrated. Purification on pre-dried silica gel [ethyl acetate–light petroleum (2:1)] and subsequent crystallization afforded the title compound **4** (693 mg, 76%) as crystals; NMR data, mp and optical rotation were in agreement with those previously reported.[19] A small fraction of the cyclic lactam **6** (less than 5%) was isolated from the silica gel purification, $[\alpha]_D^{27}$ +17.5 (*c* 1.7, $CDCl_3$); $C_{31}H_{34}N_2O_{11}$ [MALDI-MS $(M + H)^+$ Calc. *m*/*z*, 610.62. Obs. *m*/*z*, 611.57]; δ_H(250 MHz; $CDCl_3$) 6.25 (1 H, NH), 5.86 (1 H, $J_{NH,H\alpha}$ 5.2, NH-Fmoc), 5.14 (1 H, 4-H), 4.99 (1 H, 3-H), 4.74 (1 H, H^{α}-Thr), 4.69 (1 H, *J* 7.73, 1-H), 4.43 and 4.37 (2 H, Fmoc-CH_2), 4.35 (1 H, H^{β}-Thr), 4.21 and 4.11 (2 H, 6-H_2), 4.14 (1 H, Fmoc-CH), 3.67 (1 H, 5-H), 3.62 (1 H, 2-H) and 1.31 (3 H, *J* 6.02, H^{γ}-Thr); δ_C(62.9 MHz; $CDCl_3$) 96.05 (C-1), 72.97 (C-5), 72.89 (C-3), 71.92 (Fmoc-CH_2), 67.59 (C^{β}-Thr), 67.51 (C-4), 62.05 (C-6), 59.15 (C-2), 58.41 (C^{α}-Thr), 47.55 (Fmoc-CH), 21.14, 20.94, 20.93 (3 × OAc) and 12.83 (C^{γ}-Thr).

2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl azide 27

The glycosyl azide **26** (500 mg, 1.1 mmol) was dissolved in dry dichloromethane (20 cm^3) and a solution of PDT (0.2 M) and DIPEA (0.3 M) in dichloromethane was added. The solution was stirred at room temperature until TLC [toluene–ethyl acetate (2:1)] showed complete disappearance of the starting material. The solution was concentrated and the residue was dissolved in pyridine (10 cm^3)–acetic anhydride (5 cm^3). The solution was kept at room temperature for 2 h, concentrated and co-concentrated with toluene. The product was purified by VLC on silica gel [toluene–ethyl acetate (2:1)] to yield the *title acetamide* **27** (408 mg, 94%) [Found: ESMS: $(M + H)^+$, 373.56. $C_{14}H_{20}N_4O_8$ requires M, 372.34]; δ_H(250 MHz; $CDCl_3$) 1.99 (3 H, s, NH*Ac*), 2.04 (6 H, s, Ac), 2.06 (3 H, s, Ac), 3.83 (1 H, ddd, $J_{4,5}$ 9.2, $J_{5,6a}$ 2.0, $J_{5,6b}$ 5.0, 5-H), 3.95 (1 H, ddd, $J_{1,2}$ 9.4, $J_{2,NH}$ 9.0, $J_{2,3}$ 10.0, 2-H), 4.21 (1 H, dd, $J_{6a,6b}$ 12.4, 6-H^a), 4.32 (1 H, dd, 6-H^b), 4.8 (1 H, d, 1-H), 5.14 (1 H, dd, $J_{3,4}$ 9.2, 4-H), 5.28 (1 H, dd, 3-H) and 5.65 (1 H, d, NH); δ_C(62.9 MHz; $CDCl_3$) (*inter alia*) 20.9, 21.0, 21.1 and 23.6 (COCH_3), 53.8 (C-2), 61.9 (C-6), 68.2 (C-4), 72.0 (C-3), 73.6 (C-5) and 88.2 (C-1).

1,2-Bisacetamido-3,4,6-tri-O-acetyl-1,2-dideoxy-β-D-glucopyranose 28

A solution of DTT (0.2 M) and DIPEA (0.1 mM) in dichloromethane was added to the β-azide **26** (500 mg, 1.1 mmol), dissolved in dry dichloromethane (20 cm^3). The mixture was stirred at ambient temperature for 30 min [TLC, chloroform–methanol (5:1)], concentrated and redissolved in a mixture of pyridine (10 cm^3) and acetic anhydride (5 cm^3). The mixture was kept for 2 h at room temperature, concentrated, co-concentrated with toluene and then purified by VLC on silica gel [toluene–ethyl acetate (2:1)] to yield *title compound* **28** (418 mg, 96%) [Found: ESMS: $(M + H)^+$, 389.57. $C_{16}H_{24}N_2O_9$ requires M, 388.38]; δ_H(250 MHz; $CDCl_3$) 1.99, 2.02, 2.10, 2.12 and 2.14 [15 H, 5 s, NHAc (2×) and Ac (3×)], 3.80 (1 H, ddd, $J_{4,5}$ 10.2, $J_{5,6a}$ 4.3, $J_{5,6b}$ 2.1, 5-H), 4.16 (1 H, ddd, $J_{1,2}$ 8.3, $J_{2,3}$ 10.2, $J_{2,NH}$ 8.0, 2-H), 4.12 (1 H, $J_{6a,6b}$ 12.5, 6-H^a), 4.33 (1 H, dd, 6-H^b), 5.08 (1 H, dd, $J_{3,4}$ 9.2, 4-H), 5.09 (1 H, dd, $J_{1,NH}$ 8.3, 1-H), 5.15 (1 H, dd, 3-H), 6.11 (1 H, d, NH) and 7.02 (1 H, d, NH); δ_C(62.9 MHz; $CDCl_3$) (*inter alia*) 20.6, 20.7, 20.8, 23.1 and 23.4 (COCH_3), 53.4 (C-2), 61.7 (C-6), 67.63 (C-3), 72.9 (C-4), 73.5 (C-5) and 80.34 (C-1).

3-(2-Azidobenzoyloxy)-4-oxo-3,4-dihydro-1,2,3-benzotriazine 38

DHBT-OH (3.26 g, 20 mmol) was dissolved in DMF (20 cm^3) and DCCI (2.07 g, 10 mmol) was added. The solution was stirred for 24 h at room temperature [TLC: light petroleum–ethyl acetate (1:1)], filtered through Celite and concentrated. The residue was washed with diethyl ether and purified by chromatography on silica gel [light petroleum–ethyl acetate (2:1)] to yield compound **38** (2.86 g, 93%) as yellowish crystals, mp 131 °C [Found: ESMS: $(M + H)^+$, 308.57. $C_{14}H_8N_6O_3$ requires M, 308.26]; δ_H(250 MHz; $CDCl_3$) 7.25–7.43 (2 H, m), 7.66–7.77 (1 H, ddd), 7.82–7.94 (1 H, ddd), 8.02–8.11 (1 H, ddd), 8.20–8.34 (2 H, m) and 8.38–8.48 (1 H, dd); δ_C(62.9 MHz; $CDCl_3$) 120.2, 125.1, 126.2, 129.5, 133.2 and 135.9 (arom. C).

General procedure for the simultaneous reduction and N-acetylation of the *N*-Dts and azido groups of the building blocks 1, 2, 7, 8, 11–13, 16, 18 and 20

The appropriate building block (50 mg) was dissolved in THF–acetic acid–acetic anhydride [5 cm^3 (3:1:2)]. Zinc dust (activated for 2 min in 2% aq. copper sulfate and washed twice with water) was added and the mixture was stirred for 2 h. The solution was filtered through Celite, washed with THF–acetic acid, concentrated and dried *in vacuo*. The residue was dissolved in dichloromethane (10 cm^3), washed three times with water, dried over $MgSO_4$ and concentrated to give the crude product. The acetamido building blocks were essentially pure by ^1H NMR spectroscopy and could be employed directly in SPPS without further purification. Alternatively the acetamido building blocks **3**, **4**, **9**, **10**, **14**, **15**, **17**, **19** and **21** were purified by chromatography on pre-dried silica gel for characterization.

Preparation of the Macrosorb SPR 250 resin

Syntheses of the *O*-glycopeptides were performed in DMF using the Macrosorb SPR 250 resin. The resin (5 g, loading 0.2 mmol g^{-1}) was packed into a 50 cm^3 disposable syringe (Discardit II, Beckton Dickinson) fitted with a Teflon filter. The syringe was connected to a suction flask through a Teflon tube with a manual 2-way Teflon valve and the resin was swelled in DMF (20 cm^3) for 30 min; excess of reagents, DMF *etc.* was removed by applying vacuum. The resin was derivatized with the hydroxymethylphenoxy acetic acid HMPA-linker: The HMPA-linker (455 mg, 2.5 mmol), TBTU (750 mg, 2.37 mmol) and NEM (315 mm^3, 2.5 mmol) were dissolved in DMF (20 cm^3) and after 5 min added to the resin. After 2 h the resin was washed carefully with DMF (5 × 20 cm^3) and dichloromethane (5 × 10 cm^3). N^{α}-Fmoc-Lys(Boc)-OH (1.75 mg, 3.75 mmol) and *N*-methylimidazole (231 mg, 223 mm^3, 2.81 mmol) were dissolved in dichloromethane (20 cm^3) and MSNT (1.11 g, 3.71 mmol) was added. After 5 min the solution was added to the resin and the mixture was kept for 3 h. The resin was then washed thoroughly with dichloromethane (5 × 10 cm^3) and DMF (5 × 10 cm^3), and unreactive amino groups of the resin were acetylated with a solution of 20% acetic anhydride in DMF (10 cm^3). After 20 min the resin was washed successively with DMF (5 × 10 cm^3) and diethyl ether (5 × 10 cm^3) and dried *in vacuo*.

General procedures for SPPS of the *O*-glycopeptides

The derivatized resin was transferred into a custom-made 20 column Teflon synthesis block (100 mg resin per column). N^{α}-Fmoc deprotection was effected by successive 2 min and 20 min treatments of the resin with 20% piperidine in DMF (1 cm^3). The washing procedure (10 times with DMF) was repeated after each coupling/Fmoc deprotection step. Each N^{α}-Fmoc-amino acid-OPfp ester (3 mol equiv.) and DHBT-OH (1 mol equiv.) was dissolved in DMF (0.6 cm^3). In the case of the glycosylated

building blocks only 1.6 mol equiv. were employed. The solutions were added to the resins and then left for 4 h. After each coupling step the resin was washed, the N^{α}-Fmoc group was removed and the resin was washed as described above.

Comparative studies for the reduction of *N*-Dts and azido groups with DTT, MCA, BME and PDT on a solid phase (see Table 1)

Comparative studies for the reduction of *N*-Dts and azido groups on a solid phase were performed in a custom-made Teflon block with 20 wells. The derivatized Macrosorb resin was transferred into the Teflon block (50 mg resin per column), Fmoc deprotection was effected by treatment with 20% piperidine in DMF, and amino acids were coupled as OPfp-esters (3 mol equiv.) with DHBT-OH (1 mol equiv.) added as auxiliary nucleophile. Incorporation of the glycosylated *N*-Dts and azido building blocks into the glycopeptides was accomplished by utilizing N^{α}-Fmoc-Ser(Ac_3-β-D-GlcNDts)-OPfp **1** and N^{α}-Fmoc-Ser(Ac_3-α-D-GalN_3)-OPfp **7** (1.6 mol equiv.) in the presence of DHBT-OH (1 mol equiv.). After coupling of the *N*-Dts- and azide-containing building blocks, reduction was performed on the solid phase by thiolysis, alternatively with DTT (0.2 M)–DIPEA (0.1 mM), MCA (0.2 M)–DIPEA (0.3 M), BME (0.2 M)–DIPEA (0.3 M) or with PDT (0.2 M)–DIPEA (0.3 M) in dichloromethane in three portions. The first portion was sucked quickly through the resin, followed by two additional portions, which were removed after 10 min (*N*-Dts reduction) and 2 h (azide reduction), respectively. After a thorough wash with dichloromethane (5 vol.) and DMF (10 vol.) the amino group was acetylated with acetic anhydride in DMF (20%). The residual amino acids were then coupled in the usual way. After completion of the syntheses the resin was washed with dichloromethane and dried. Cleavage of the glycopeptide was achieved with 95% TFA for 2 h, followed by filtration and concentration and precipitation by addition of diethyl ether. The glycopeptides were then O-deacetylated by treatment with 2% sodium methoxide in methanol and analysed by analytical and semipreparative HPLC, using a linear gradient 0 to 50% B for 30 min and then 50 to 100% for 10 min (analytical HPLC) and 0 to 50% B for 50 min and then 50 to 100% B for 10 min (semipreparative HPLC), respectively. Yields and comparative data are presented in Table 1. ^{1}H NMR data are presented in Table 3.

Selective reduction of *N*-Dts, in the presence of azide, on the solid phase

The *N*-Dts- and azido group-containing core 2 building block **16** (1.6 mol equiv.) was coupled to the peptide on the HMPA-derivatized Macrosorb resin. Selective *N*-Dts reduction was accomplished by two 10 min treatments of the resin with PDT (0.2 M) and DIPEA (0.3 M) in dichloromethane. After a thorough wash with dichloromethane (5 vol.) and DMF (10 vol.) the amino group was acetylated with acetic anhydride in DMF (20%). The synthesis of the glycopeptide **30** was then continued by the same procedure as described above. Purification of compound **30** was achieved by semipreparative HPLC to afford a 76% yield of the azido group-containing glycopeptide **30**. ^{1}H NMR data of compound **30** are presented in Table 3.

General procedure for *N*-Dts and azido reduction utilizing DTT/DIPEA on the solid phase

The appropriate Macrosorb SPR resin (100 mg) with the *N*-Dts- or the azido group-containing glycopeptides was washed thoroughly with dichloromethane (5×). The thiolytic reduction was then performed by treatment of the resin with a solution of DTT (0.2 M)–DIPEA (0.1 mM) in dichloromethane (1 × 2 min, 2 × 3 h). In the case of the *N*-Dts-containing building blocks the thiolysis procedure could be reduced to 2 × 10 min. The resin was then washed thoroughly with dichloromethane (5×) and DMF (10×) because traces of thiols left on the resin could lead to the undesired reduction of the azido function of the photoaffinity label **34** after coupling to the glycopeptide. This undesired side reaction was detected after the completion of the glycopeptide synthesis.

Multiple-column peptide syntheses of the *O*-GlcNAc and mucin *O*-glycopeptides 24, 25 and 31–36

The derivatized resin was transferred into a 20 column Teflon block (100 mg/column, loading 0.2 mmol g^{-1}). Throughout the syntheses the cleavage of the N^{α}-Fmoc moiety was achieved by treatment with 20% piperidine in DMF. All amino acids were then coupled to the peptide as their Pfp esters (3 mol equiv. each) with DHBT-OH (1 mol equiv.) added as an auxiliary nucleophile. Acylation times were 2–4 h throughout the syntheses. Incorporation of the glycosylated serine and threonine amino acids into the glycopeptide was achieved by utilizing either the building blocks **1**, **2**, **7**, **8**, **12**, **13**, **16**, **18** and **20** or the reduced and *N*-acetylated building blocks **3**, **4**, **9**, **10**, **14**, **15**, **17**, **19** and **21**. The appropriate building blocks (1.6 mol equiv.) were coupled in the presence of DHBT-OH (1 mol equiv.). The reaction time for the glycosylated building blocks was 8 h. After thorough washing of the resin with DMF (5 vol.) and dichloromethane (10 vol.) the reduction of the *N*-Dts and azido groups on the solid phase was achieved by adding DTT (0.2 M) and DIPEA (0.1 mM) in dichloromethane (1 × 2 min and 2 × 3 h in the case of azido reduction and 1 × 2 min and 2 × 10 min in the case of *N*-Dts reduction). The amino groups were then N-acetylated with 20% acetic anhydride in DMF after washing of the resin with DMF. After incorporation of the last amino acid, valine, the terminal N^{α}-Fmoc group was cleaved, and the resin was washed successively with DMF and diethyl ether and dried. The cleavage of the *O*-glycopeptides from the HMPA-linker and simultaneous amino acid deprotection were performed by treatment with 95% aq. TFA for 2 h. After cleavage the solution was filtered from the resin and the resin was washed three times with 95% aq. TFA. The combined filtrates were concentrated, co-concentrated with toluene and the glycopeptides were then precipitated by several triturations with diethyl ether. Residual solvent was removed under reduced pressure and the acetylated glycopeptides were dissolved in dry methanol (2 mg cm^{-3}) and O-deacetylated by addition of a catalytic amount of 2% sodium methoxide in methanol until pH paper indicated pH 8.5. The mixture was then stirred for 4 h at room temperature, neutralized with solid CO_2 and concentrated. The crude glycopeptides were dissolved in water and purified by semipreparative HPLC, using a linear gradient 0–50% B in 50 min and then 50–100% in 10 min. The purified glycopeptides **24**, **25** and **31**–**36** were then lyophilized from water and fully characterized by ESMS and ^{1}H NMR spectroscopy. Yields and ESMS data are presented and compared in Table 2. ^{1}H NMR data are presented in Table 3.

Multiple-column peptide syntheses of the photoaffinity labelled glycopeptides 39–45

The MCPS of the photoaffinity labelled glycopeptides was performed analogously to the synthesis of the *O*-glycopeptides. Incorporation of the glycosylated *N*-Dts and azido building blocks **1**, **2**, **7**, **8**, **11**, **12** and **13** (1.6 mol equiv.) in the presence of DHBT-OH (1 mol equiv.) and the subsequent thiolytic reduction were performed as previously described. Coupling of the photoaffinity label **38** to the free amino group was achieved, after thorough washing with dichloromethane (10×) and DMF (10×), by treatment with the active ester **38** (3 mol equiv.), dissolved in DMF (0.6 cm^3), for 6 h. After Fmoc cleavage with 20% piperidine in DMF the peptide synthesis was continued by utilizing the Fmoc amino acid OPfp esters (3 mol equiv.) and DHBT-OH (3 mol equiv.) in DMF (0.6 cm^3) for 3 h, respectively. After the final N^{α}-Fmoc deprotection, the resins were washed successively with DMF and diethyl ether and dried. The cleavage of the glycopeptides from the linker and simultaneous side-chain deprotection were performed by treatment with 95% aq. TFA (2 cm^3) for 2 h at room temperature, followed by filtra-

Table 5 ^{1}H NMR data of the photoaffinity labelled glycopeptides **39**–**45** measured in CD_3CO_2D–D_2O (1:1) at 600 MHz at 300 K

	39	**40**	**41**	**42**	**43**	**44**	**45**
Val							
NH							
α-H	3.95	3.94	3.95	3.93	3.95	3.93	3.95
β-H	2.22	2.23	2.23	2.22	2.23	2.23	2.22
γ-H_3	1.00	1.00	1.01	1.00	1.00	1.01	1.01
γ-H_3	1.00	1.00	1.00	0.99	1.01	1.00	1.00
Ile							
NH	8.37	8.38	8.39	8.38	8.39	8.39	8.40
α-H	4.36	4.33	4.33	4.32	4.34	4.32	4.33
β-H	1.81	1.82	1.82	1.80	1.80	1.80	1.81
γ-H_2	1.54/1.15	1.51/1.14	1.52/1.17	1.49/1.12	1.51/1.14	1.50/1.14	1.50/1.13
γ-H_3	0.89	0.87	0.85	0.88	0.86	0.86	0.86
δ-H_3	0.86	0.85	0.87	0.84	0.85	0.88	0.87
Thr							
NH	8.06	8.14	8.11	8.14	8.14	8.14	8.15
α-H	4.48	4.40	4.39	4.40	4.38	4.32	4.41
β-H	4.23	4.15	4.19	4.17	4.19	4.19	4.18
γ-H_3	1.17	1.09	1.14	1.10	1.12	1.11	1.12
Ala							
NH	8.06	7.96	8.01	7.99	8.03	8.01	8.02
α-H	4.44	4.39	4.30	4.31	4.30	4.33	4.30
β-H_3	1.24	1.26	1.25	1.26	1.24	1.26	1.25
β-H_3							
Phe							
NH	7.83	8.01	7.90	8.00	7.88	7.95	7.99
α-H	4.45	4.66	4.60	4.67	4.61	4.69	4.66
β-H_2	2.91/2.67	3.11/2.96	3.06/2.91	3.07/2.91	3.04/2.89	3.05/2.91	3.05/2.92
Ser or **Thr**							
NH	7.92	7,85	8.13	7.85	7.88	7.92	7.85
α-H	4.60	4.54	4.66	4.51	4.65	4.51	4.51
β-H/β-H′	4.19/4.02	4.35	3.98/3.84	4.28	4.01/3.87	4.28	4.30
γ-H_3		1.13		1.20		1.21	1.20
Glu							
NH	8.01	8.00	8.11	7.85	8.10	7.84	8.05
α-H	4.39	4.43	4.38	4.17	4.34	4.20	4.40
β-H_2	2.16/1.98	2.17/2.01	2.05/1.89	1.88/1.62	2.04/1.86	1.90/1.61	2.14/1.98
γ-H_2	2.47	2.52	2.41	2.25	2.40	2.27	2.47
Gly							
NH	8.01	8.10	8.12	8.08	8.11	8.03	8.08
α-H_2	3.97	3.97	3.91/3.86	3.93	3.89	3.95	3.94
Leu							
NH	7.79	7.84	7.87	7.92	7.88	7.94	7.91
α-H	4.39	4.39	4.39	4.41	4.38	4.42	4.40
β-H_2	1.59	1.58	1.59	1.59	1.59	1.60	1.60
γ-H	1.60	1.59	1.62	1.60	1.62	1.60	1.59
δ-H_3	0.88/0.82	0.84/0.89	0.90/0.85	0.92/0.87	0.88/0.84	0.87/0.91	0.87/0.91
Lys							
NH	8.08	8.11	8.14	8.15	8.16	8.18	8.15
α-H	4.41	4.41	4.40	4.40	4.42	4.42	4.43
β-H_2	1.91/1.75	1.91/1.72	1.90/1.75	1.90/1.73	1.90/1.76	1.91/1.71	1.90/1.72
γ-H_2	1.44	1.42	1.45	1.43	1.44	1.44	1.44
δ-H_2	1.69	1.68	1.70	1.69	1.68	1.69	1.70
ε-H_2	3.02	3.01	3.03	3.02	3.02	3.02	3.03
NH_2	7.45	7.46	7.48	7.48	7.48	7.48	7.48
GalNHR							
1-H			5.08 (3.14)	5.17 (3.4)	5.08	5.19	
2-H			4.47	4.41	4.62	4.58	
3-H			4.02	4.08	4.13	4.20	
4-H			4.04	4.08	4.02	4.35	
5-H			4.31	4.33	4.31		
6-H			4.39	4.40	4.35		
6-H′			4.39	4.39	4.35		
GlcNHR							
1-H	4.77 (8.28)	4.77 (8.26)					5.16 (3.24)

Table 5 (*contd.*)

	39	40	41	42	43	44	45
GlcNHR							
2-H	4.08	3.97					4.16
3-H	4.00	3.97					3.99
4-H	4.07	3.97					3.61
5-H	3.79	3.78					3.79
6-H	3.55	3.52					3.89
6-H′	3.55	3.52					3.83
Gal							
1-H					4.57	4.63	
2-H					3.62	3.67	
3-H					3.61	3.62	
4-H					3.93	3.95	
5-H					3.64	3.65	
6-H					3.81	3.81	
6-H′					3.78	3.76	
NH							
NHAc							
Phenyl	7.25, 7.22, 7.06	7.26, 7.22, 7.16	7.28, 7.23, 7.15	7.29, 7.24, 7.16	7.28, 7.26, 7.13	7.28, 7.24, 7.15	7.28, 7.24, 7.15
Azido-benzoyl	7.75, 7.54, 7.29, 7.22	7.70, 7.57, 7.33, 7.25	7.75, 7.56, 7.29, 7.25	7.77, 7.56, 7.31, 7.26	7.78, 7.57, 7.29, 7.24	7.78, 7.55, 7.30, 7.25	7.77, 7.57, 7.31, 7.26

tion and washing of the resin successively with 95% aq. TFA and acetic acid. After concentration the acetylated glycopeptides were freeze dried with water. The final removal of the acetyl groups from the glycopeptides was performed with sodium methoxide in methanol at a pH of 8.5. After 4 h the solutions were neutralized by addition of solid CO_2, concentrated, and lyophilized from water. The glycopeptides **39**–**45** were purified by semipreparative HPLC, using a linear gradient of 0–50% solvent B in 50 min and then 50–100% in 10 min. The pure compounds were lyophilized from water and fully characterized by ESMS and ^{1}H NMR spectroscopy. Yields and ESMS data are presented in Table 4, ^{1}H NMR data are presented in Table 5.

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