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Solid phase synthesis of the fibronectin glycopeptide V(Galβ3GalNAcα)THPGY, its β analogue, and the corresponding unglycosylated peptide

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The fibronectin fragment VTHPGY and the corresponding glycopeptides V(Galβ3GalNAcα)THPGY and V(Galβ3GalNAcβ)THPGY were synthesized by the FMOC/solid phase approach. FMOC derivatives of threonine, carrying O-linked, peracetylated Galβ3GalNAc chains were used for introduction (HOBt-mediated coupling) of the disaccharide moieties.

Keywords: glycopeptide, synthesis, fibronectin

Abbreviations: FMOC, 9-fluorenylmethyloxycarbonyl; DMTST, dimethyl(thiomethyl)sulfonium triflate; DMF, dimethylformamide; BOC, butoxycarbonal.

In glycoprotein biosynthesis, oligosaccharides are attached to the polypeptide chain post-translationally. The glycosylation of the protein greatly influences its biological properties. It is known, for example, that glycoprotein hormones such as luteinizing hormone, follicle-stimulating hormone and erythropoietin are less active or inactive if not properly glycosylated [1–8]. It is also known, that the extent and type of glycosylation influences the half-life of proteins in serum [9]. This knowledge has emerged from comparison of entire glycoproteins with their unglycosylated counterparts obtained by chemical or enzymatic deglycosylation of the parent glycoprotein. It has been suggested [10] that one of the reasons that glycosylation alters the chemical properties of a protein is that the presence of glycosyl residues alters the preferred conformation of the peptide chain. An attractive way to test this hypothesis is to prepare model glycopeptides and compare their conformational properties with their unglycosylated analogues. Synthetic techniques for glycopeptides are, however, still under development [11]. Several preliminary [12–15] and one more detailed [16] communication indicate that solid phase synthesis of glycopeptides using glycosylated amino acids is possible. However, some workers [15, 16], but not others [14], have indicated difficulties in obtaining quantitative coupling yields when larger sugars are attached to the activated amino acid. This communication reports, with full experimental details, conditions suitable for solid phase O-glycopeptide syntheses, giving, in the examples studied, high coupling yields throughout. We have chosen the V(Galβ3GalNAcα)THPGY glycopeptide (**3**) as a target. It is a partial structure of fibronectin, and a monoclonal antibody raised against oncofetal fibronectin binds to this minimum peptide sequence only when it is glycosylated [10].

In addition to the target structure V(Galβ3GalNAcα)-THPGY, we have synthesized the corresponding β analogue (**4**), and the unglycosylated parent peptide (**5**) (a preliminary account of parts of this work has appeared [12]). For synthesis of the glycopeptides, properly protected or glycosylated FMOC-amino acids [17] were used in solid phase peptide synthesis, followed by deprotection and purification of the obtained crude products. The unglycosylated parent peptide was prepared by the conventional BOC technique [18]. The NMR spectra of the glycopeptides could be assigned in great detail. Conformational studies of these two glycopeptides and the corresponding unglycosylated peptide will be reported in a separate paper.

Results and discussion

As reported earlier by us [17] and others [13, 14, 19], FMOC-threonine derivatives, carrying *O*-linked, protected mono- or disaccharides can be prepared in reasonable yields. Repeated work with preparations of such derivatives (**1b** or **2b**) according to the published procedure [17] suggested that the yield of these glycosides could be improved. The increased yields obtained were mainly due to the use

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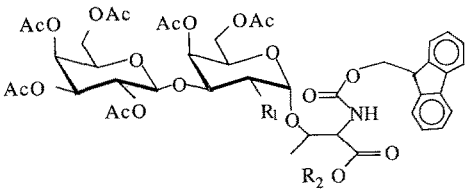
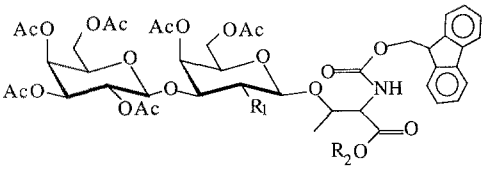


1a $R_1 = N_3$ $R_2 = PhCOCH_2$

1b $R_1 = NHAc$ $R_2 = H$



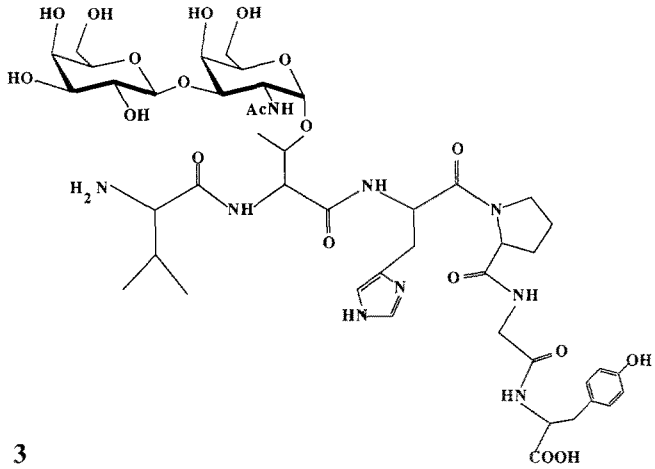
2a $R_1 = N_3$ $R_2 = PhCOCH_2$

2b $R_1 = NHAc$ $R_2 = H$

of thioglycoside/DMTST [20] instead of glycosyl bromide/silver triflate as donor/promoter in the glycosidation of threonine. The improved conditions for this transformation are given in the Materials and methods part.

The derivative **1b** or **2b** was used in FMOC solid phase peptide syntheses. The FMOC peptide synthesis protocol is well suited to glycopeptide synthesis, since it avoids repetitive use of strongly acidic reagents that could damage the acid-sensitive glycosidic bonds. Starting with FMOC-*O*-benzyltyrosine coupled to Sasrin resin, FMOC-Gly, FMOC-Pro, and FMOC-*N*-benzyl-His were successively coupled to the resin, then compound **1b** or **2b** was coupled, followed by FMOC-Val. FMOC-proline and FMOC-valine were coupled as symmetrical anhydrides, whereas the other amino acids were coupled as hydroxybenzotriazole (HOBt) esters (attempts to couple glycine as the symmetrical anhydride resulted in double incorporation). The extent of coupling was monitored by ninhydrin tests [21]. After coupling of histidine, this test could not be used, however. Therefore, as an extra monitoring method, a portion of the resin, after coupling of histidine or **1b**/**2b**, was treated with acid and the released material was analysed by FAB-MS. Although it is risky to use peak areas in FAB-MS spectra for quantification, this method indicated the presence of only traces of deletion sequences, and thus the coupling yields were high also with histidine or **1b**/**2b**.

After completion of the solid phase synthesis, the glycopeptides were removed from the resin by treatment with 1% trifluoroacetic acid in dichloromethane. The released protected glycopeptides were treated with methanolic sodium methoxide (to remove *O*-acetyl groups) and then with hydrogen and palladium on charcoal (to remove *O*- and *N*-benzyl groups). The hydrogenolysis step required large catalyst quantities, elevated pressure, and long reaction times to go to completion. The crude preparations were purified by gel filtration on Sephadex G-15, and then by FPLC. No racemization or β-elimination products were observed. The yield of pure **3** was 18%, and that of pure **4** was 27% (calculated from the original amount of tyrosine on the resin). The NMR data for **3**, **4** and **5** are shown in Tables 1–3.



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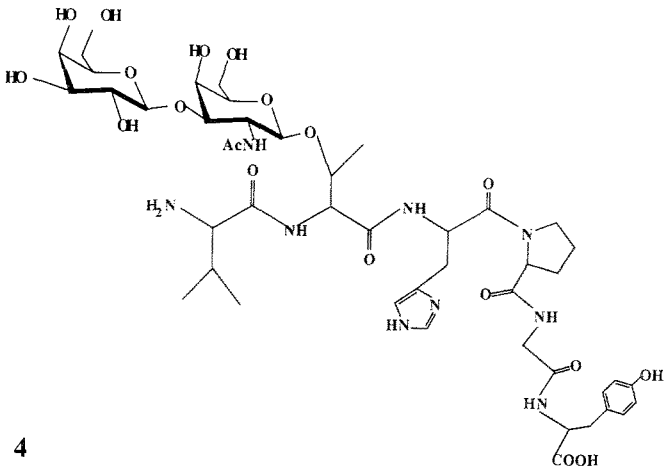

4

5 VTHPGY

Materials and methods

Evaporations were performed at $<40°C$ bath temperature. NMR spectra were recorded at 25°C for solutions in ^2H_2O unless otherwise stated, using a Bruker AM 500 spectrometer (acetone $\delta_H = 2.225$, external TMS $\delta_C = 0.00$). The FAB-MS spectra were recorded with a VG ZAB-SE mass spectrometer. The primary beam consisted of xenon atoms with a maximum energy of 8 keV. The samples were dissolved in thioglycerol and the positive ions were extracted and accelerated over a potential of 10 kV. Silica gel 60 F-254

Table 1. Proton shifts of V(Galβ3GalNAcα)THPGY (**3**) Val(Galβ3GalNAcβ)THPGY (**4**) and VTHPGY (**5**).

	Val			*Thr*			*His*			*Pro*			*Gly*			*Tyr*			*Carbohydrate protons*		
	3	4	5	3	4	5	3	4	5	3	4	5	3	4	5	3	4	5		3	4
α_1	4.05	3.90	3.90	4.57	4.42	4.33	4.96	5.01	5.02	4.42	4.44	4.42	3.92	3.90	3.89	4.66	4.58	4.65	1	4.76	4.53
α_2														3.91	3.94				2	4.24	3.98
β_1	2.28	2.21	2.22	4.30	4.15	4.08	3.08	3.10	3.08	1.88	1.89	1.88				3.02	2.97	3.00	3	3.99	3.86
β_2							3.22	3.22	3.19	2.29	2.28	2.27				3.16	3.15	3.16	4	4.21	4.19
γ_1	1.02	0.96	0.978	1.27	1.12	1.17				2.02	2.02	2.01							5	4.02	N.D.
γ_2	1.05	0.98	0.982							2.02	2.02	2.01							6	≈3.75	N.D.
δ_1										3.60	3.63	3.62							1′	4.42	4.45
δ_2										3.70	3.78	3.77							2′	3.52	3.52
Ar-1							7.33	7.35	7.32							6.82	6.80	6.81	3′	3.62	3.62
Ar-2							8.60	8.58	8.59							7.13	7.12	7.12	4′	3.91	3.92
																			5′	3.64	N.D.
																			6′	≈3.75	N.D.

N.D., not detected.

Table 2. Proton coupling constants for V(Galβ3GalNAcα)THPGY (**3**), V(Galβ3GalNAcβ)THPGY (**4**) and VTHPGY (**5**).

	Val			*Thr*			*His*			*Pro*			*Gly*			*Tyr*			*Carbohydrate protons*		
	3	4	5	3	4	5	3	4	5	3	4	5	3	4	5	3	4	5		3	4
$J_{\alpha,\beta}$	5.8		6.1	1.9	6.0	5.6	8.3	7.7	8.4	6.1	6.0	6.1		N.D.	17[a]	8.2	8.2	8.3	1, 2	4.0	8.5
J_{α,β_2}							5.3	5.7	5.4	8.3	8.2	8.4				5.6	5.2	5.3	2, 3	11.5	10.8
$J_{\beta,\gamma}$	6.9	6.9	6.9	6.4	6.3	6.4	15.6[a]	15.5[a]	15.6[a]	13.0[a]	12.6[a]	13.0[a]				14.2[a]	N.D.	13.0[a]	3, 4	3.0	3.2
J_{β,γ_2}	6.9	6.9	6.9							N.D.		N.D.							4, 5	<0.5	<0.5
$J_{\gamma,\delta}$										N.D.		6.7							1′, 2′	7.7	7.7
J_{γ,δ_2}												6.9							2′, 3′	9.9	10.0
$J_{Ar\text{-}1}$							1.1	1.2	1.0							8.5	8.5	9.7	3′, 4′	3.4	3.4
$J_{Ar\text{-}2}$							1.4	1.1	1.4							8.5	8.6	8.6	4′, 5′	<0.5	<0.5

N.D., not detected. [a] Geminal coupling.

Table 3. Carbon shifts of V(Galβ3GalNAcα)THPGY (**3**), V(Galβ3GalNAcβ)THPGY (**4**) and VTHPGY (**5**).

	Val			*Thr*			*His*			*Pro*			*Gly*			*Tyr*			*Carbohydrate carbons*		
	3	4	5	3	4	5	3	4	5	3	4	5	3	4	5	3	4	5		3	4
α	59.1	59.1	59.3	58.0	58.8	60.2	51.2	51.2	51.3	61.5	61.5	61.7	43.3	43.1	43.3	55.0	55.2	55.2	1.	99.61	100.93
β	31.1	30.8	30.9	76.5	75.7	68.0	27.0	26.8	27.0	30.3	30.2	30.4				36.7	36.6	36.8	2.	49.25	52.06
γ_1	17.6	16.9	17.7	19.4	18.4	19.9				25.7	25.4	25.6							3.	78.12	80.61
γ_2	18.7	17.4	18.5																4.	69.57	68.62
δ										48.9	48.9	49.0							5.	72.02	75.54
Ar-1							118.6	118.6	118.6							116.3	116.2	116.4	6.	62.04	61.76
Ar-2							129.1	129.2	129.1							128.9	128.7	129.3	1′	105.72	105,61
Ar-3							134.5	134.3	134.4							131.5	131.4	131.5	2′	71.55	71.42
Ar-4																155.4	155.2	156.0	3′	73.49	73.31
																			4′	69.75	69.39
																			5′	75.98	75.84
																			6′	62.21	61.84

(Merck, Darmstadt, Germany) was used for TLC, detection by UV, 1% ninhydrin in ethanol or by charring with 8% sulfuric acid. Column chromatography was performed on silica gel (Matrex Silica Si, 60 Å, 35–70 μm, Amicon, Danvers, MA, USA). Toluene was dried with metallic sodium, and solvents (DMF, dichloromethane) over 4 Å molecular sieves (heated to 200°C for >2 weeks, Kebo, Stockholm, Sweden). Powdered molecular sieves (4 Å: Fluka, Buchs, Switzerland, pre-heated as above) were used when indicated. For gel filtrations Sephadex G-15 (Pharmacia, Uppsala, Sweden) was used. Double distilled water and p.a. acetonitrile (Merck) were used for FPLC or HPLC purifications. Amino acid derivatives were purchased from Bachem Feinchemikalien AG (Buchs, Switzerland) or synthesized according to established procedures. Resins were from Bachem Feinchemikalien AG or Bissendorf Biochemicals GmbH (Hannover, Germany).

N-(9-Fluorenylmethyloxycarbonyl)-O-[4,6-di-O-acetyl-2-azido-2-deoxy-3-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-α-D-galactopyranosyl]-L-threonine phenacylester (**1a**) *and N-(9-Fluorenylmethyloxycarbonyl)-O-[4,6-di-O-acetyl-2-azido-2-deoxy-3-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-β-D-galactopyranosyl]-L-threonine phenacylester* (**2a**)

Dimethyl(thiomethyl)sulfonium triflate [20] (2.56 g, 9.93 mmol) was added to a stirred solution of *N*-(9-fluorenylmethyloxycarbonyl)-L-threonine phenacyl ester (1.70 g, 3.72 mmol) and 4-methylphenyl 4,6-di-*O*-acetyl-2-azido-2-deoxy-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-1-thio-β-D-galactopyranoside [17] (1.80 g, 2.48 mmol) in dichloromethane (15 ml) and toluene (10 ml) containing powdered molecular sieves (2 g) at 20°C. The mixture was stirred for 1 h and then filtered. The filtrate was diluted with diethyl ether (200 ml) and washed with aqueous sodium hydrogencarbonate and water, dried (magnesium sulfate) and concentrated. Column chromatography (toluene/ethyl acetate, 6/4 by vol) gave **1a** (1.28 g, 1.21 mmol, 49%) and **2a** (0.62 g, 0.59 mmol, 24%). The NMR data were as reported earlier [17]. Conversion into **1b** and **2b** was done as described [17].

H_2N-Val-(Galβ3GalNAcα)Thr-His-Pro-Gly-Tyr-OH (**3**)

FMOC-Tyr(Bzl)-Sasrin resin (0.64 g, 0.5 mmol g^{-1}, 0.32 mmol) was allowed to swell in DMF for 10 min, then the resin was treated with 20% piperidine in DMF for 2 min, then washed with DMF three times, then treated again for 10 min with 20% piperidine, and finally the resin was washed 10 times with DMF. FMOC-Gly-OH (0.30 g, 1.01 mmol) and hydroxybenzotriazole (HOBT, 0.14 g, 1.04 mmol) were dissolved in DMF (2 ml) and diisopropylcarbodiimide (DIPCDI, 158 μl, 1.02 mmol) was added. The mixture was added to the resin which was stirred by a stream of nitrogen. The coupling reaction was monitored for completion with the ninhydrin test [21]. When the test was negative, the above cycle was repeated using the chemicals and quantities shown in Table 4.

The FMOC group was removed by two successive treatments with 50% morpholine in DMF (2 and 10 min, respectively) and then the resin was washed five times with DMF and 10 times with dichloromethane. The resin-bound peptide was cleaved from the resin by repetitive treatment with 1% trifluoroacetic acid in dichloromethane, collecting the filtrate and evaporating the solvent. The residue was then dissolved in methanol (8 ml) and methanolic sodium methoxide (2 ml, 0.5 M) was added. After 1 h, the mixture was neutralized with acetic acid (2 ml), transferred to a Parr flask containing 10%Pd/C (0.80 g), and hydrogenated at 60 lb in^{-2} for 18 h. The reaction mixture was filtered to remove the Pd/C and the filtrate was evaporated. The residue was dissolved in aqueous ammonium bicarbonate (3 ml, 0.1 M). The solution was purified by Sephadex G-15 gel filtration, using 0.1 M aqueous ammonium bicarbonate as eluant. The UV absorbance of the eluate was monitored (235 and 280 nm). Two pools gave NMR and FAB-MS spectra showing the presence of the desired glycopeptide **3** plus impurities. The major pool (87.6 mg) was further purified by reversed phase FPLC chromatography using a Waters RCM 25 × 10 radial compression unit, a μ-Bondapak 10 μm/125 Å Guardpak pre-column, and a μ-Bondapak C-18 10 μm/125 Å column. The sample (10 mg portions) was dissolved in water containing 0.05% TFA and eluted

Table 4. Sequence of reagents used in the synthesis of H_2N-Val-(Galβ3GalNAcα)-Thr-His-Pro-Gly-Tyr-OH.

Amino acid	*Quantity*	*HOBT*	*DIPCDI*	*Deblock*
FMOC-Pro-OH	0.69 g 2.05 mmol	—	158 μl 1.02 mmol	20% Piperidine in DMF
FMOC-His(Bzl)-OH	0.48 g 1.03 mmol	0.14 g 1.04 mmol	158 μl 1.02 mmol	20% Piperidine in DMF
FMOC-Thr(Galβ3-GalNAcα)OH (**1b**)	0.51 g 0.53 mmol	0.073 g 0.54 mmol	84 μl 0.54 mmol	20% Piperidine in DMF
FMOC-Val-OH	0.69 g 2.05 mmol	—	158 μl 1.02 mmol	50% Morpholine in DMF

Table 5. Sequence of reagents used in the synthesis of H_2N-Val-(Galβ3GalNAcβ)Thr-His-Pro-Gly-Tyr-OH.

Amino acid	*Quantity*	*HOBT*	*DIPCDI*	*Deblock*
FMOC-Gly-OH	0.11 g 0.37 mmol	0.051 g 0.38 mmol	58 µl 0.37 mmol	20% Piperidine in DMF
FMOC-Pro-OH	0.26 g 0.77 mmol	—	61 µl 0.39 mmol	20% Piperidine in DMF
FMOC-His(Bzl)-OH	0.175 g 0.37 mmol	0.051 g 0.38 mmol	58 µl 0.37 mmol	20% Piperidine in DMF
FMOC-Thr(Galβ3-GalNAcβ)OH (**2b**)	0.241 g 0.25 mmol	0.033 g 0.25 mmol	39 µl 0.25 mmol	20% Piperidine in DMF
FMOC-Val-OH	0.339 g	—	78 µl	50% Morpholine in DMF

(6 ml min^{-1}) with a gradient of 0–40% acetonitrile in water containing 0.05% TFA. The UV absorbance of the eluate was monitored (280 nm). Appropriate fractions were pooled, concentrated, dissolved in water and lyophilized to give **3** (63.4 mg, 0.061 mmol, 18%). FAB-MS showed a dominant M + 1 ion at *m/z* 1038. The NMR parameters are shown in Tables 1–3.

H_2N-Val-(Galβ3GalNAcβ)Thr-His-Pro-Gly-Tyr-OH (**4**)

FMOC-Tyr(Bzl)-Sasrin resin (0.25 g, 0.5 mmol g^{-1}, 0.125 mmol) was treated as described above for the α-isomer **3** with the chemical and amounts as shown in Table 5. The FMOC group was removed by treatment with 50% morpholine in DMF for 2 and 10 min respectively, and then the resin was washed five times with DMF and 10 times with dichloromethane. The resin-bound glycopeptide was cleaved from the resin, deprotected and purified as described for the α-isomer **3** to give **4** (35 mg, 0.034 mmol, 27%). FAB-MS showed a dominant M + 1 ion at *m/z* 1038. The NMR parameters are shown in the tables.

H_2N-Val-Thr-His-Pro-Gly-Tyr-OH (**5**)

BOC-Gly-OH, BOC-Pro-OH, BOC-His(Dnp)-OH, BOC-Thr(Bn)-OH, and BOC-Val-OH were coupled, successively, to BOC-Tyr(2,6-dichlorobenzyl)-PAM resin (0.56 mmol) using the conventional BOC technique [15]. Each step was monitored for completion with the ninhydrin test [21]. After removal of the DNP group with thiophenol [18], the peptide was released and deblocked with anhydrous HF (0°C, 60 min). The peptide was then precipitated with ether, taken up in 10% aqueous acetic acid and lyophilized to give crude **5** (186 mg). The material was purified by HPLC using an LKB ultrapac TSK ODS 120T, (10 µm, 7.8 × 300 mm) column, eluted with a gradient of 0–40% acetonitrile in water containing 0.1% TFA. The UV absorbance of the eluate was monitored (280 nm). Appropriate fractions were pooled, concentrated, dissolved in water and lyophilized to give **5** (147 mg, 0.219 mmol, 39%). FAB-MS showed a dominant M + 1 ion at *m/z* 673. The NMR parameters are shown in Tables 1–3.

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References

1. Ryan RJ, Charlesworth MNC, McCormick DJ, Milius RP, Keutmann HT (1988) *FASEB J* 2:2661–70.
2. Kobata A (1988) *J Cell Biochem* **37**:79–91.
3. Paulson JC (1989) *Trends Biochem Sci* **14**:272–6.
4. Bhalla VK, Sairam MR, Browne ES (1990) *Pure Appl Chem* **62**:1427–33.
5. Takeuchi M, Takasaki S, Shimada M, Kobata A (1990) *J Biol Chem* **265**:12127–31.
6. Tsuda E, Kawanishi G, Ueda M, Masuda S, Sasaki R (1990) *Eur J Biochem* **188**:405–11.
7. Smith PL, Kaetzel D, Nilson J, Baenzinger J (1990) *J Biol Chem* **265**:874–82.
8. Takeuchi M, Inoue N, Strickland TW, Kubota M, Wada M, Shimizu R, Hoshi S, Kozutsumi H, Takasaki S, Kobata A (1989) *Proc Nat Acad Sci USA* **86**:7819–23.
9. Ashwell G, Hartford J (1982) *Ann Rev Biochem* **51**:531–44.
10. Matsuura H, Takio K, Titani K, Greene T, Levery SB, Salyan MEK, Hakomori S (1988) *J Biol Chem* **263**:3314–22.
11. Kunz H (1987) *Angew Chem Int Ed Engl* **26**:294–308.
12. Lüning B, Norberg T, Tejbrant J (1989) *J Chem Soc Chem Commun* 1267–8.
13. Meldal M, Jensen K (1990) *J Chem Soc Chem Commun* 483–5.
14. Jansson AM, Meldal M, Bock K (1990) *Tetrahedron Lett* **31**:6991–4.
15. Urge L, Kollat E, Hollosi M, Laczko I, Wroblewski K, Thurin J, Otvos L Jr (1991) *Tetrahedron Lett* **32**:3445–8.
16. Paulsen H, Merz G, Peters S, Weichert U (1990) *Liebigs Ann Chem* 1165–73.
17. Lüning B, Norberg T, Tejbrant J (1989) *Glycoconjugate J* **6**:5–19.
18. Barany G, Merrifield RB (1988) In *The Peptides* (Gross E, Meienhofer J, eds), pp. 3–254. New York: Academic Press.
19. Paulsen H, Adermann K (1989) *Leibigs Annln Chem* 751.
20. Fügedi P, Garegg PJ (1986) *Carbohydt Res* **149**:C9–C12.
21. Sarin V, Kent S, Tam J, Merrifield RB (1981) *Anal Biochem* **117**:147–51.