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Efficient Synthesis of Complex Glycopeptides Based on Unprotected Oligosaccharides

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Received August 27, 2002

N-Glycopeptides containing 1 to 4 trisaccharide chains, with the carbohydrates vicinal to each other in the multivalent glycopeptides, were efficiently synthesized by using the glycosylated Fmoc-asparagine as a key building block. While the couplings of amino acids with glycopeptides could be achieved in the homogeneous solutions in *N*-methylpyrrolidinone (NMP) to give excellent yields, all products were conveniently isolated from the reaction mixtures through a precipitation method by using the free carbohydrate chains as phase tags. Commercially available pentafluorophenyl (Pfp) esters of amino acids were employed for the glycopeptide elongation. Longer glycopeptides were constructed by means of a highly convergent synthetic design that is based on the coupling of glycopeptide/peptide fragments. Hydrogen bond interactions between free oligosaccharides were proposed to explain the exceptionally high efficiency of the couplings between two glycosylated building blocks.

Introduction

Glycoproteins play an important role in various biological and pathological functions.[1,2] To meet the growing demand for synthetic glycoproteins and/or glycopeptides, many useful synthetic methods have been developed in the past two decades,[3] such as solution-phase and solid-phase syntheses with appropriately protected oligosaccharides, as well as the elegant chemo-enzymatic synthesis[4−9] and chemoselective ligation method[10−12] based on free carbohydrates. For chemo-enzymatic syntheses of glycopeptides, both the enzymatic transglycosylation and the enzymatic peptide and/or carbohydrate chain elongation have been exploited. Another interesting but inadequately addressed approach is the solid-phase synthesis of glycopeptides with unprotected glycosyl amino acids as building blocks.[13] Nevertheless, owing to the remarkable diversity and complexity of glycopeptide structures, it is difficult for any individual method to meet the demands of all kinds of synthetic endeavors, even though the established methods have been successfully applied to the preparation of numerous glycopeptides. Thus, alternative methodologies are still desired.

A special property shared by all glycopeptide synthetic targets, which may be utilized in organic synthesis, is that they contain free carbohydrate chains possessing a number of polar hydroxyl groups. The free hydroxyl groups of carbohydrates did not affect the peptide coupling reactions as indicated by the successful application of glycosyl amino acids of unprotected monosaccharides and disaccharides to conventional solid-phase synthesis of glycopeptides.[13] However, the high polarity of unprotected carbohydrate conjugates makes them only soluble in very polar organic solvents but not in less polar ones. It is thus possible to use the unprotected oligosaccharides as "phase tags" to facilitate the product isolation in the solution-phase synthesis of glycopeptides, i.e., the reactions used to elongate the glycopeptide chains may be performed in a polar organic solvent under homogeneous conditions, while the product of each reaction can be easily separated from the side products by adding a less polar solvent to the reaction for precipitating the polar glycopeptide after the reaction is accomplished. The synthesis can take advantage of the benefits of both

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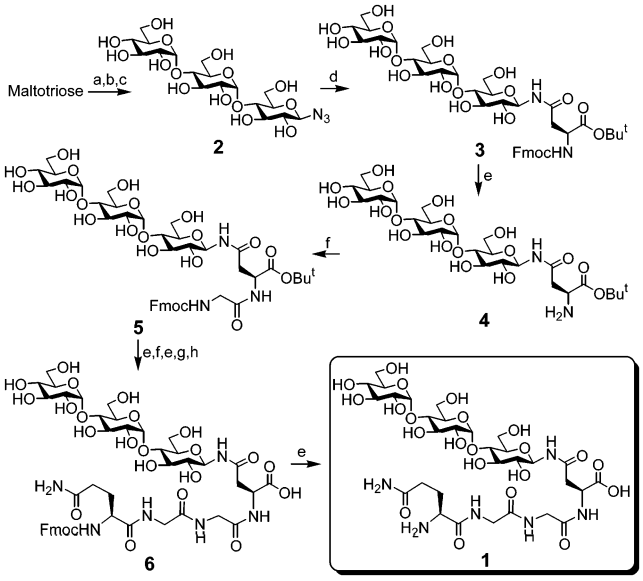
10.1021/jo020570c CCC: $25.00
Published on Web 02/21/2003

solution-phase and solid-phase syntheses, namely the faster and more efficient reactions in a homogeneous solution and the convenient product isolation through the precipitation method. The principle and the high efficiency of solution-phase organic synthesis with solid-phase workups have been proved by numerous recently designed phase tags.[14−21] The uniqueness of using unprotected carbohydrates as phase tags in glycopeptide synthesis is that they are also an integral part of the synthetic targets, so it is not necessary to cut these phase tags off after the syntheses are finished. Furthermore, the carbohydrate chains can be repeatedly used as phase tags without limit until the final synthetic targets are obtained. Another important advantage of this approach is that it can avoid the final carbohydrate deprotection, which is a necessary but sometimes problematic step in a majority of other synthetic methods. Consequently, a strategy for glycopeptide synthesis based on unprotected oligosaccharides can be especially useful for the synthetic targets containing acid-sensitive oligosaccharides and/or reduction-liable peptides, as well as for those with bulky side chains that may affect the coupling reactions.

Recently, we exploited the synthesis of glycopeptides with unprotected oligosaccharides as building blocks and as phase tags.[22] The reactions to elongate the glycopeptide chains were carried out in the homogeneous solutions of *N*-methylpyrrolidinone (NMP), and the expected glycopeptide products were easily isolated from the reaction mixtures via the precipitation method by adding diethyl ether to the reaction and isolating the precipitates via filtration or centrifugation. In our preliminary studies, *N*-hydroxybenzotriazole and *N*,*N*-dicyclohexylcarbodiimine (HOBt/DCC) were used as the condensation reagents. The new synthetic strategy was very successful for several simple glycopeptides containing a monosaccharide or a disaccharide chain by elongation of the glycopeptides at both termini.

Nevertheless, the applicability of this new strategy to more complex structures remains to be proved. Moreover, for wider uses its protocols may need optimization. For example, though the above peptide coupling protocol gave excellent results, it is necessary for the amino acids to be activated just before the coupling reactions, and the freshly prepared active esters in solution were directly used without purification. Meanwhile, the acidic conditions used to deprotect carboxyl groups in the process of *C*-terminal elongation may affect the carbohydrate structures of the glycopeptides, especially if a synthesis involves many steps of *C*-terminal elongation and the synthetic target contains acid-sensitive glycosyl linkages. Thus, a more convenient protocol for peptide coupling and a synthetic design that involves minimum *C*-terminal elongations are desirable.

SCHEME 1[a]



[a] Reagents and conditions: (a) Ac_2O, NaOAc, reflux, 94%. (b) Et_2O-BF_3, $TMSN_3$, DCM, rt, 80%. (c) NaOMe, MeOH, rt, quant H_2, Pd/C. (d) Fmoc-Asp(OBt)-OBu^t, NMP, rt, 2 h, 42%. (e) (i) 20% piperidine, NMP, rt, 0.5 h; (ii) product precipitation by Et_2O. (f) (i) Fmoc-Gly-OPfp, NMP, rt, 2 h; (ii) product precipitation by Et_2O. (g) (i) Fmoc-Gln(Trt)-OPfp, NMP, rt, 2 h; (ii) product precipitation by Et_2O. (h) 25% TFA, DCM, 2 h.

Results and Discussion

Glycopeptide Chain Elongation with the Pentafluorophenyl (Pfp) Esters of Amino Acids. Trying to establish a more convenient protocol for glycopeptide elongations, this research was focused on the one that employs the Pfp esters of amino acids for the peptide coupling,[23,24] as it has been successfully used in the preparation of glycopeptides by other methods.[25−28] More importantly, the relatively stable Pfp esters are commercially available, and if this protocol is applicable to the new strategy, the commercial active esters can be directly used to construct glycopeptides. In this case the only side product of the coupling reactions will be pentafluorophenol that is soluble in the mixture of NMP and ether and can be easily removed by diethyl ether. It was therefore anticipated that the Pfp-protocol should be especially helpful for the new synthetic strategy.

The elongation of glycopeptide chains by means of Pfp esters was then investigated in the synthesis of glycopeptide **1** (Scheme 1), a simple model containing a maltotriose. Though this is an artificial synthetic target,

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the methods established herein will be applicable to natural *N*-glycopeptides, and the results should be of general significance. Actually, the protocols established in the synthesis of **1** and other glycopeptides in this work may also be adapted for *O*-linked glycopeptides. The key building block **3**, which has the unprotected oligosaccharide linked to the side chain of Fmoc-Asn-OBu^t, was prepared by reductive coupling between the azido derivative of maltotriose (**2**) and Fmoc-Asp-OBu^t as described before.[22] This product was purified by silica gel column chromatography, and its ^{1}H NMR spectrum proved the β-configuration of the newly formed glycosidic linkage (H-1: δ 4.92, $^3J_{1,2}$ = 9.2 Hz). Selective deprotection of the Fmoc group in **3** by 20% piperidine in NMP and then precipitation of the product by diethyl ether afforded **4** quantitatively. To the solution of **4** in NMP was added the Pfp ester of Fmoc-Gly (4 equiv) with stirring at room temperature. The reaction was monitored by TLC and reversed-phase HPLC with a C_{18} column (Discovery 250 × 2.5 mm), which showed that the reaction finished within 1 h and only one coupling product was observed, whereupon diethyl ether (ca. 10 times the volume of NMP) was added to precipitate the glycodipeptide product, and the precipitate was isolated on centrifugation. Washing of the product with diethyl ether, which was followed by drying under vacuum, gave **5** in a quantitative yield. Both HPLC and NMR showed that the crude product was homogeneous, and its NMR and MS spectra agreed with the expected structure. These results proved that the peptide coupling by using the Pfp-activated esters of Fmoc-Gly was clean and effective.

Following the same two-step protocol, i.e., (1) deprotection of the Fmoc group in a glycopeptide by 20% piperidine in NMP and isolation of the product by the precipitation method and (2) coupling of the glycopeptide with the next amino acid in NMP (rt, 2 h) by using its Pfp ester (3−4 equiv) and isolation of the product by the precipitation method, the peptide chain of **5** was further elongated with Fmoc-Gly-OPfp and Fmoc-Glu(Trt)-OPfp sequentially to afford a glycotetrapeptide which, on treatment with 25% trifluoroacetic acid (TFA) in dichloromethane (DCM), was transformed to **6** bearing a free *C*-terminus. The reactions were monitored and analyzed by TLC and HPLC, while the reaction products were characterized by means of NMR and MS. The final synthetic target **1** was eventually obtained upon the deprotection of **6** with 20% piperidine in NMP. The NMR spectra and the HPLC diagram of **1** showed that its purity was no less than 95%. These results suggested that the Pfp ester protocol was very convenient and that the coupling reactions were very efficient for glycopeptide elongations. Nonetheless, further purification of the product by gel filtration with a Bio Gel P4 column and then by reversed-phase HPLC on a semipreparative C_{18}-column (25 × 1 cm) afforded **1** in an overall yield of 75% (based on **3**). The lower than perfect yield was mainly due to the loss of products in the precipitation, analysis, and material transfer processes in each step of the glycopeptide synthesis, even though all the operations were performed within the same reaction vessel to minimize the product loss.

Glycopeptide Synthesis by Coupling of Glycopeptide/Peptide Fragments. To minimize the number of steps of *C*-terminal elongation in the preparation of

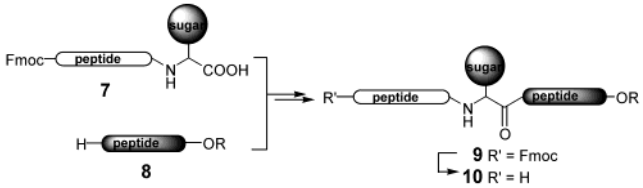

FIGURE 1. The convergent assembly of glycopeptides by coupling peptide and/or glycopeptide fragments.

complex glycopeptides, a convergent design for glycopeptide assembly (Figure 1) that is based on the coupling of a glycopeptide fragment with another peptide or glycopeptide fragment was investigated. This is an approach that has been commonly used in the synthesis of complex peptides but not fully exploited in glycopeptide synthesis. Glycopeptides built upon unprotected oligosaccharides are especially suitable for this convergent design, as their *N*- and *C*-termini can be easily and selectively exposed under mild conditions. For instance, after a glycopeptide is assembled by a procedure described above, it may be treated by 25% TFA in DCM to deprotect the *C*-terminus (Figure 1), and the resulting glycopeptide **7** is thus ready to be activated and coupled to a peptide or glycopeptide fragment **8** that bears a free *N*-terminus to give the complex glycopeptide **9**. Fragment **8** in turn can be prepared either by solid-phase synthesis, in the case of a simple peptide, or by this new synthetic strategy, in the case of a glycopeptide. Finally, complete deprotection of **9** will afford the synthetic target **10**.

This convergent method was examined in the preparation of a glycoheptapeptide **11** that could be achieved by coupling the glycotetrapeptide **6** to a tripeptide **12** bearing a free *N*-terminus (Scheme 2). The fragment **12** was prepared from a commercial tripeptide **13** in three conventional steps. On the other hand, the crude **6** obtained after condensation as described above was directly used for the coupling reaction without further purification. Thus, glycopeptide **6** was activated by reaction with DCC and HOBt in NMP for 2 h to give **14**, whereupon 5 equiv of **13** was added to the reaction mixture. After overnight stirring at room temperature, ether was added to the reaction, and the precipitate was isolated by centrifugation and analyzed by HPLC to show only one peptide/glycopeptide peak that was identified as the expected glycopeptide **15**. This suggests that the precipitation and washing process could efficiently remove the excess **13**. Final deprotection of glycopeptide **15** by 20% piperidine in NMP afforded the synthetic target **11** that was also isolated by the precipitation method. The crude product (89% yield) was already very pure as shown by both NMR and HPLC (≥95%).

It is worth mentioning that the synthetic target **11** contains a typrosine residue that was previously shown to be unstable to the reductive conditions used to remove benzyl groups in the solid-phase synthesis of glycopeptides based on benzyl-protected glycosyl amino acids.[29]

Synthesis of Multivalent Glycopeptides. Encouraged by the excellent results obtained above in regard to the new peptide coupling protocol as well as the fragment coupling method for glycopeptide synthesis, we

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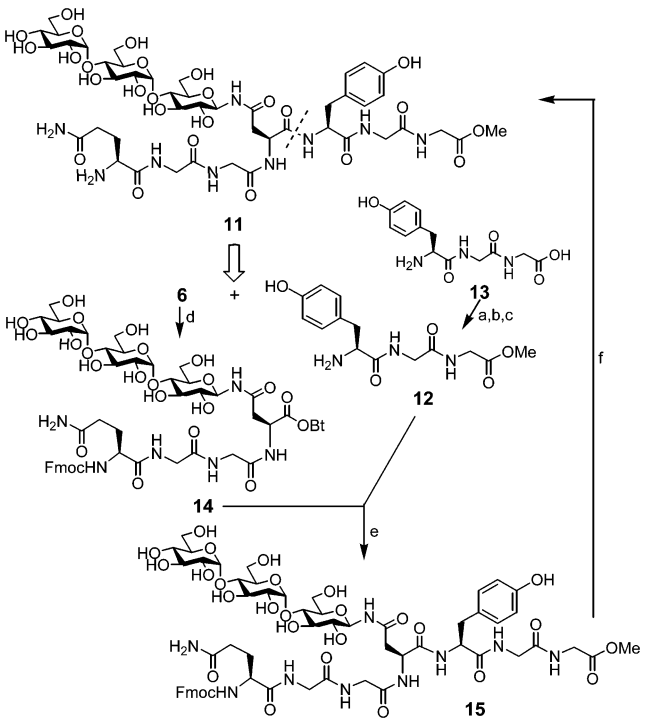
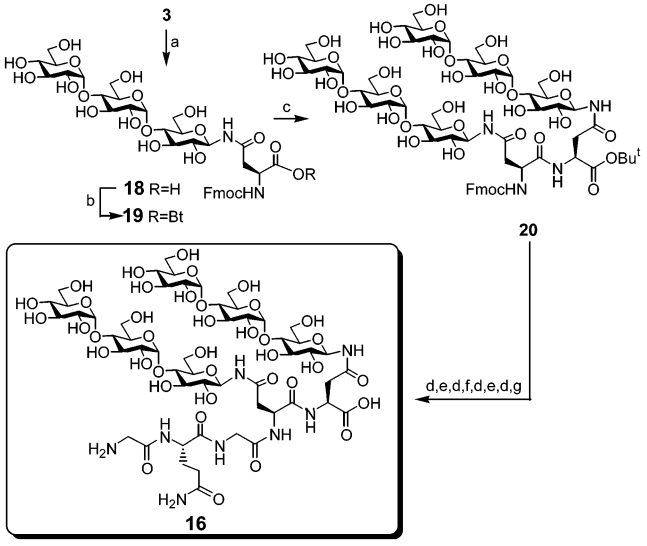
SCHEME 2[a]



[a] Reagents and conditions: (a) Fmoc-OBt, DCM, rt, 95%. (b) MeOH, $SOCl_2$, rt, 82%. (c) 20% piperidine, NMP, rt, 0.5 h, quant. (d) DCC, HOBt, NMP, rt, 2 h. (e) (i) rt, overnight; (ii) precipitation by Et_2O. (f) (i) 20% piperidine, NMP, rt, 0.5 h; (ii) precipitation by Et_2O, 89% (from **6**).

SCHEME 3[a]



[a] Reagents and conditions: (a) 25% TFA, DCM, rt, 2 h. (b) (i) DCC, HOBt, NMP, rt, 2 h; (ii) precipitation by Et_2O. (c) (i) **4**, NMP, rt, 12 h; (ii) precipitation by Et_2O, quant. (d) (i) 20% piperidine, NMP, rt, 0.5 h; (ii) precipitation by Et_2O. (e) (i) Fmoc-Gly-OPfp, NMP, rt, 2 h; (ii) precipitation by Et_2O. (f) (i) Fmoc-Gln(Trt)-OPfp, NMP, rt, 2 h; (ii) precipitation by Et_2O, (g) 25% TFA, DCM, 2 h.

further studied their application to more complex synthetic targets. The synthetic models were two *N*-linked glycopeptides **16** and **17** (Schemes 3 and 4) that were decorated by two and four vicinal maltotriose glycans, respectively.

All these syntheses used glycosyl amino acid **3** as the key building block. As shown above, the Fmoc in **3** could be selectively removed by 20% piperidine in NMP to give **4** with the *N*-terminus exposed. On the other hand, its *C*-terminus was selectively deprotected by treatment with 25% TFA in DCM to give **18** which, after condensation to dryness under vacuum, was directly used in the next step of the reaction.

The coupling of **18** to **4** was achieved via its Bt-ester **19** formed by reaction with HOBt and DCC (Scheme 3). Thus, after **18** was stirred with DCC and HOBt (2.0 equiv) in NMP at room temperature for 2 h, **4** (1.0 equiv) was added to the solution. This mixture was stirred at room temperature for another 12 h. The glycopeptide product was then precipitated out and washed with diethyl ether to afford **20** that was characterized by NMR and MS. The HPLC analysis (using a Waters Spherisorb 5 μm NH_2 analytic column, 250 × 4.6 mm) of this product indicated that it contained only one glycopeptide peak and all the starting materials were consumed. It did not need any purification for further transformations. This coupling reaction was also studied by utilizing different ratios of **18** to **4**, and it was found that each experimental entry produced a quantitative coupling in terms of the minor substrate. Therefore, it is suggested that a 1:1 ratio of the substrates should be employed for this coupling reaction, as in such cases the intermediate purification can be avoided.

Once the glycodipeptide **20** was obtained, its peptide chain was elongated stepwise (Scheme 3) by the protocols described in the synthesis of glycopeptide **1** to yield the divalent glycopeptide **16**. All the coupling reactions, which were monitored by NMR and HPLC, gave excellent results. In regard to the product isolation by the precipitation method, it was proved to be more convenient to work with these divalent structures than to work with the monovalent glycopeptides, as the divalent glycopeptides could be more easily precipitated from the reaction mixtures. Crude **16** (quantitative yield from **3**) obtained from the final precipitation showed only one glycopeptide peak by HPLC. Its ^1H NMR spectrum also indicated that this crude product was rather pure, except for being contaminated by a small amount of piperidine (Figure 2a). The crude **16** was finally purified by a Bio Gel P4 column and then reversed-phase HPLC to afford **16** in an overall yield of 93% (from **18**). The structure of **16** was supported by its NMR (Figure 2b,c) and MS.

The tetravalent glycopeptide **17** (Scheme 4) was similarly synthesized. First, the glycodipeptide **20** obtained above was converted to **21** and **22** by selective deprotection of its Bu^t and Fmoc groups with 25% TFA/DCM and 20% piperidine/NMP, respectively. The workup procedures for these reactions followed exactly that described for **18** and **4**. After **21** was activated by DCC/HOBt in NMP, the two glycodipeptide fragments (**21**:**22** 1:1) were coupled under the conditions described for **20** to give the glycotetrapeptide containing four vicinal glycans, which was precipitated and washed by diethyl ether. HPLC analysis of this reaction indicated that the coupling efficiency was about 85%. The product was then *N*-deprotected by the standard protocol to afford **23**. A part of **23** was purified by gel filtration chromatography and HPLC to get an isolated coupling yield of 75%, and the structure of **23** was fully characterized. As shown, this

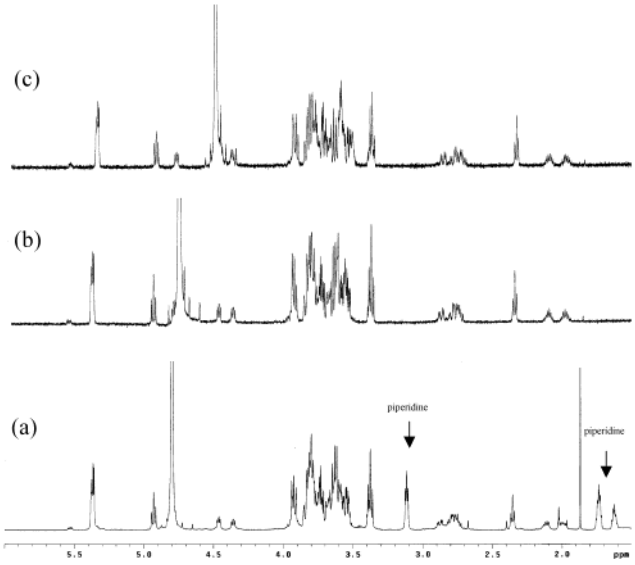


FIGURE 2. The ^{1}H NMR (D_2O, 600 MHz) spectra of **16**: (a) the crude product at 25 °C; (b) the purified product (after Bio Gel P4 column chromatography and HPLC) at 25 °C; and (c) the purified product at 50 °C.

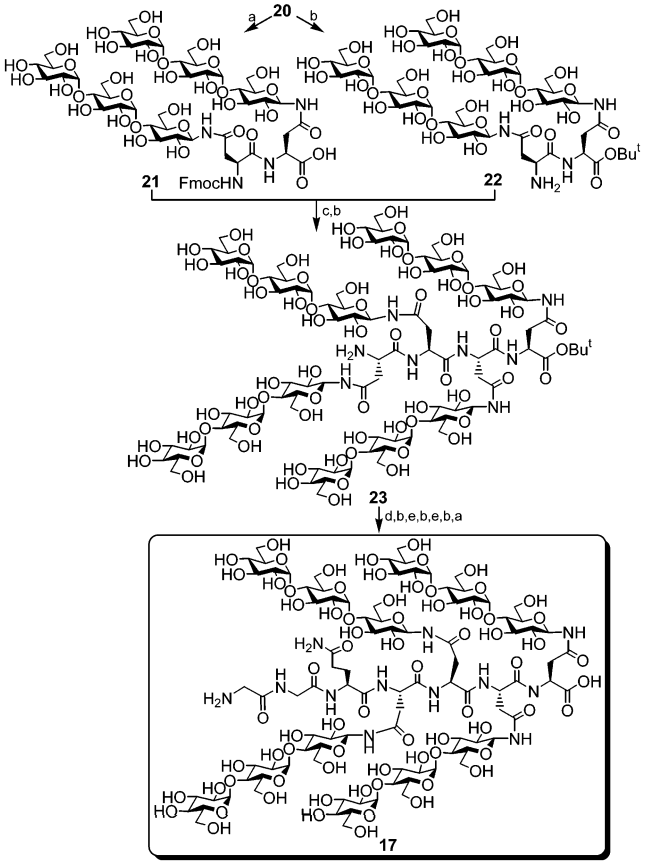
coupling reaction turned out to be less efficient than that between **18** and **4**. However, the result was still quite impressive if the extremely bulky structures of **21** and **22** are taken into consideration.

For further elongation of the peptide chain of **23**, the crude product was applied without purification, since the expected final product **17**, a tetravalent glycopeptide, should be rather easily separated from the divalent side products. Therefore, the peptide chain of **23** was elongated by the established protocols. Final *N*- and *C*-terminal deprotection of the resulting glycopeptide and then purification by gel filtration chromatography and RP HPLC afforded **17** in a 68% overall yield (from **20**). The ^{1}H NMR spectra of **17** at different temperatures are shown in Figure 3.

As shown in Schemes 1−4, several glycopeptides were synthesized by the new strategy in excellent yields. The TLC, HPLC, and NMR analyses showed that the peptide coupling reactions based on the Pfp esters of amino acids (3−4 equiv) could complete within 1 h to give the expected products in perfect conversion, whereas most coupling reactions were kept for at least 2 h to ensure the complete coupling. Moreover, the purification of intermediates by column chromatography or other sophisticated separation methods was not necessary in these syntheses, except for the building block **3**. The crude final products, which were obtained after many steps of deprotection, peptide coupling, and intermediate precipitation, were already pure. These results also supported the high efficiency of the peptide coupling reactions and that of the precipitation method for product isolation/purification. Thus, using commercial Pfp esters of amino acids for the glycopeptide elongation was proved to be a very convenient and efficient protocol, and the convergent method for glycopeptide synthesis by coupling glycopeptides with peptide or glycopeptide fragments was also proved to be highly feasible.

Compared to the coupling reaction between a benzyl-protected glycosyl amino acid and a peptide on the solid-

SCHEME 4[a]



[a] Reagents and conditions: (a) 25% TFA, DCM, 2 h, quant. (b) (i) 25% piperidine, NMP, rt, 0.5 h; (ii) precipitation by Et_2O. (c) (i) DCC, HOBt, NMP, rt, overnight, quant.; (ii) precipitation by Et_2O. (d) (i) Fmoc-Gln(Trt)-OPfp, NMP, rt, 2 h; (ii) precipitation by Et_2O. (e) (i) Fmoc-Gly-OPfp, NMP, rt, 2 h; (ii) precipitation by Et_2O.

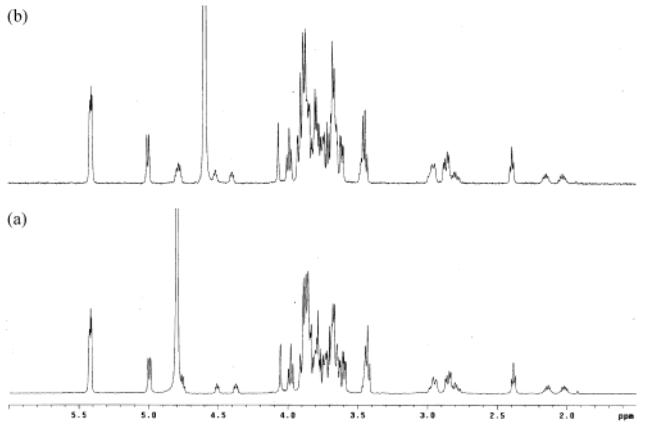


FIGURE 3. The ^{1}H NMR (D_2O, 600 MHz) spectra of **17** at different temperatures: (a) 25 and (b) 45 °C.

phase support,[29] it was interesting to notice the extremely high coupling efficiency of the bulky glycopeptides during the synthesis of multivalent glycopeptides **16** and **17**. Several factors may be accountable for the outcome. For instance, it is anticipated that achieving the coupling reactions in a homogeneous NMP solution may significantly improve the reaction efficiency over that performed under the two-phase conditions. Moreover, the decreased

size of the unprotected oligosaccharide chains, in comparison to that of the protected ones, may also contribute to the high coupling efficiency, as it may reduce the steric interactions between the glycans of two glycosylated substrates. Meanwhile, the results may also suggest potential hydrogen bonding between the free carbohydrate chains, as this can help to bring together the two substrates involved and thus facilitate their coupling. To prove this hypothesis, more detailed studies are necessary. Nevertheless, it is generally accepted, as well as observed,[30] that hydrogen bonds between free oligosaccharides are feasible, especially in organic solvents that interact with carbohydrates less effectively than water.

This research has demonstrated that the new strategy can be efficient and useful for the synthesis of a variety of complex *N*-glycopeptides, including ones that contain multiple vicinal oligosaccharide chains. To the best of our knowledge, this is the first report of the chemical synthesis of a glycopeptide with 4 vicinal complex oligosaccharides. Additionally, the successful preparation of **11** also suggests that the synthetic method based on unprotected glycosyl amino acids can be especially suitable for glycopeptides containing vulnerable peptide sequences.

Experimental Section

General Methods. Thin-layer chromatography (TLC) was performed on silica gel GF_{254} with an eluent of MeOH, DCM, and AcOH (3:6:1 to 6:3:1), and the detections were achieved by charring with 2% H_2SO_4/EtOH. Commercial anhydrous solvents were used without further purification.

***N*-(9-Fluorenylmethoxycarbonyl)-*N*-[α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl]-L-asparagine *tert*-Butyl Ester (3).** To a solution of α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→**4**)-β-D-glucopyranosyl azide (**2**, 1.4 g, 2.64 mmol) in 20 mL of dry DMF was added 10% Pd/C (100 mg) under H_2 atmosphere. The mixture was stirred at room temperature for 2 h. TLC showed that the azide was completely transformed to the glycosylamine. The catalyst was then filtered off and the resulting solution was directly used in the next step without other treatment.

In the meantime, Fmoc-Asp(OBt)-OBu^t was prepared by reaction of Fmoc-Asp-OBu^t (1.7 g, 4.1 mmol) with HOBt (0.68 g, 5.0 mmol) and DCC (1.0 g, 5.0 mmol) in CH_2Cl_2 (20 mL) and DMF (1 mL) at room temperature for 2 h. The resulting side product (DCU) as a precipitate was filtered off and the solution was concentrated into syrup under high vacuum. To this syrup was added the above solution of glycosylamine, and the solution was stirred at room temperature overnight. The reaction mixture was concentrated to a small volume under vacuum, and the product was purified with a silica gel column to give pure **3** as a white solid (1.0 g, 1.12 mmol, yield: 42%). $[\alpha]_D$ +31.8 (*c* 0.6, MeOH). TLC: R_f 0.35 (eluent: MeOH, DCM, and AcOH, 3:6:1). FABMS: calcd for $C_{41}H_{56}N_2O_{20}$ 896.4, found 897.4 ($M + H^+$), 919.3 ($M + Na^+$). HRFABMS: calcd for $C_{41}H_{56}N_2O_{20}Na$ ($M + Na^+$) 919.3324, found 919.3371. ^{1}H NMR (CD_3OD, 300 MHz) δ 7.80 (d, J = 7.7 Hz, 2 H), 7.67 (d, J = 7.7 Hz, 2 H), 7.40 (t, J = 7.2 Hz, 2 H), 7.32 (t, J = 7.2 Hz, 2 H), 5.18 (d, J = 3.8 Hz, 1 H), 5.16 (d, J = 3.8 Hz, 1 H), 4.92 (d, J = 9.2 Hz, 1 H), 4.48 (t, J = 6.0 Hz, 1 H), 4.20−4.40 (m, 3 H), 2.78 (br s, 2 H), 1.46 (s, 9 H).

A Typical Procedure for the *N*-Terminal Deprotection (of Fmoc). A glycosyl Fmoc-amino acid or *N*-terminal protected glycopeptide (56 μmol) was dissolved in a 2-mL mixture of piperidine and NMP (1:4). After 1.5 h of stirring at room temperature, 20 mL of diethyl ether was added to the reaction. Following centrifugation at 3000 rpm for 5 min, the solvents were poured off carefully, while the precipitate remained in the reaction flask. This crude product was then suspended in 20 mL of diethyl ether with vigorous shaking on a vortex mixer, which was followed by centrifugation. The ether solution was removed from the vessel, and the remaining solid was washed by ether 2 to 3 more times. The precipitate was finally dried under vacuum to afford the product that was directly used for the next step of the reaction.

A Typical Procedure for the Peptide Coupling with Pfp Esters of Amino Acids. A glycosyl amino acid or glycopeptide with an exposed *N*-terminus (56 μmmol) was dissolved in NMP (2 mL), and to the solution was added the Pfp ester of an Fmoc-amino acid (3−4 equiv) with stirring at room temperature. The reaction process was monitored by TLC and HPLC (on a C_{18} or an NH_2 column). The reaction usually took about 1 h to finish. Then, 20 mL of diethyl ether was added to precipitate the product, and the precipitate was collected following centrifugation at 3000 rpm for 5 min. This crude product, after being washed 4 times with diethyl ether (as described above) and dried under vacuum, was directly applied to the next step of the reaction.

A Typical Procedure for the *C*-Terminal Deprotection (of Bu^t). After the *tert*-butyl ester of a glycosyl amino acid or a glycopeptide (33 μmol) was treated with a mixture of TFA and DCM (1:3, 2 mL) at room temperature for 2 h, the reaction mixture was condensed to dryness. The residue was washed by diethyl ether 3 to 4 times as described above to afford the desired product that was used for the next step of the reaction or to afford the final product that was then thoroughly purified by HPLC.

L-Glutaminyl-glycyl-glycyl-{*N*-[α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl]}-L-asparagine (1). After *N*-terminal deprotection of **3** (50 mg, 56 μmol) following the typical procedure shown above, the resulting glycosyl amino acid **4** (38 mg, quantitative) was dissolved in NMP (2 mL) and treated with Fmoc-Gly-OPfp (107 mg, 230 μmol) at room temperature for 2 h to accomplish the coupling reaction. Precipitation followed by washing and drying of the product as described above eventually afforded the glycodipeptide **5** (53 mg) in quantitative yield.

Similarly, *N*-terminal deprotection of **5** and treatment of the deprotected product with Fmoc-Gly-OPfp (200 μmol), which was followed by sequential *N*-deprotection and coupling to Fmoc-Glu(Trt)-OPfp (200 μmol) of the resulting glycotripeptide, afforded the glycosyl tetrapeptide that was then treated with 20% TFA/DCM as described to give **6** (64 mg, overall yield 82%). FABMS: calcd for $C_{46}H_{62}N_6O_{24}$ 1082.4, found 1083.4 ($M + H^+$). ^{1}H NMR (CD_3OD, 600 MHz) δ 7.77 (d, J = 7.2 Hz, 2 H), 7.66 (t, J = 7.2 Hz, 2 H), 7.37 (t, J 7.2 = Hz, 2 H), 7.30 (t, J = 7.2 Hz, 2 H), 5.14 (d, J = 3.8 Hz, 1 H), 5.13 (d, J = 3.8 Hz, 1 H), 4.86 (d, J = 9.0 Hz, 1 H), 4.75 (t, J = 6.0 Hz, 1 H), 4.40 (m, 2 H), 4.21 (t, J = 6.6 Hz, 1 H), 4.09 (t, J = 7.2 Hz, 1 H), 3.88 (s, 4 H), 2.80 (m, 2 H), 2.29 (t, J = 7.8 Hz, 2 H), 2.07 (m, 1 H), 1.94 (m, 1 H).

Finally, *N*-terminal deprotection of **6** by the general protocol described above afforded the free glycopeptide **1**. After precipitation and washing with ether, the precipitate product was further purified by gel filtration column chromatography on a Bio Gel A4 column (2.5 × 35 cm) with distilled water as the eluent and then by HPLC on a semipreparative Discovery C_{18} column (25 × 1 cm) with an eluent of 0.2% 2-propanol in water at a 2.0 mL/min flow rate to produce the pure product **1** (36 mg, 91%). HPLC: RT = 6.20 min. FABMS: calcd for $C_{31}H_{52}N_6O_{22}$ 860.3, found 861.4 ($M + H^+$). ^{1}H NMR (D_2O, 600 MHz) δ 5.42 (d, J = 3.8 Hz, 1 H), 5.41 (d, J = 3.8 Hz, 1 H), 4.98 (d, J = 9.0 Hz, 1 H), 4.75 (t, J = 6.0 Hz, 1 H), 4.57 (dd, J = 7.2, 4.2 Hz, 1 H), 4.03 (s, 2 H), 3.99 (s, 2 H), 2.87 (dd, J = 15.6, 4.8 Hz, 1 H), 2.73 (dd, J = 15.6, 8.4 Hz, 1 H), 2.37 (t, J = 7.2 Hz, 2 H), 1.98 (m, 1 H), 1.93 (m, 1 H).

(30) Blanzat, M.; Turrin, C.-O.; Perez, E.; Rico-Lattes, I.; Caminade, A. M.; Majoral, J.-P. *Chem. Commun.* **2002**, 1864−1865.

L-Glutaminyl-glycyl-glycyl-{*N*-[α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-*β*-D-glucopyranosyl]}-L-asparaginyl-L-tyrosyl-glycyl-glycine Methyl Ester (11). After the mixture of **6** (5 mg, 4.6 μmol), HOBt (1.9 mg, 13.8 μmol), and DCC (2.8 mg, 13.8 μmol) in 0.5 mL of NMP was stirred at room temperature for 2 h, **12** (7.1 mg, 23 μmol) was added to the solution. The stirring was continued for another day, and then diethyl ether was added to precipitate the product. The precipitate was collected following centrifugation, washed with ether, and dried under vacuum to afford the crude product **11** (4.7 mg, 89%). The product was studied with HPLC (on a Discovery C_{18} 25 × 1 cm column with an eluent of 8% 2-propanol in water at a 2.0 mL/min flow rate), which did not show any impurity peak. HPLC: RT = 5.18 min. FABMS: calcd for $C_{45}H_{69}N_9O_{26}$ 1151.4, found 1152.4 ($M + H^+$), 1174.3 ($M + Na^+$), 1190.4 ($M + K^+$). ^{1}H NMR (D_2O, 600 MHz) δ 7.11 (d, *J* = 8.4 Hz, 2 H), 6.81 (d, *J* = 8.4 Hz, 2 H), 5.37 (d, *J* = 3.6 Hz, 1 H), 5.36 (d, *J* = 3.6 Hz, 1 H), 4.91 (d, *J* = 9.0 Hz, 1 H), 4.70 (d, *J* = 6.6 Hz, 1 H), 4.53 (t, *J* = 7.8 Hz, 1 H), 4.00 (s, 2 H), 3.97 (s, 2 H), 3.88 (s, 4 H), 3.72 (s, 3 H), 3.06 (dd, *J* = 13.8, 7.2 Hz, 1 H), 2.94 (dd, *J* = 13.8, 8.4 Hz, 1 H), 2.79 (dd, *J* = 15.2, 6.0 Hz, 1 H), 2.70 (dd, *J* = 15.2, 7.2 Hz, 1 H), 2.37 (t, *J* = 7.8 Hz, 2 H), 2.11 (m, 1 H), 1.99 (m, 1 H).

Glycyl-L-glutaminyl-glycyl-{*N*-[α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-*β*-D-glucopyranosyl]}-L-asparaginyl-{*N*-[α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-*β*-D-glucopyranosyl]}-L-asparagine (16). After a solution of **3** (30 mg, 33 μmol) in 2 mL of 25% TFA/DCM was stirred at room temperature for 2 h, the solvent was evaporated under reduced pressure. The residue was washed with ether 3 times to give the expected product **18** (25 mg, 90%). Its ^{1}H NMR spectrum indicated the complete removal of the *tert*-butyl group. This crude product was stirred with DCC (18.3 mg, 89 μmol) and HOBt (12.0 mg, 89 μmol) in NMP (2 mL) at room temperature for 2 h. To this reaction mixture was then added **4** (22 mg, 33 μmol), and the solution was stirred at room temperature overnight. The product was precipitated by addition of ether (20 mL) and the precipitate was washed with ether (3 × 20 mL) to give the crude **20** (49 mg, quantitative). The product was analyzed by HPLC and characterized by NMR and MS. HPLC: RT = 7.42 min (on a Waters Spherisorb 5 μm NH_2 250 × 4.6 mm analytic column with an eluent of 75% acetonitrile in water at a 2.0 mL/min flow rate). FABMS: calcd for $C_{63}H_{92}N_4O_{37}$ 1497.5, found 1498.4 ($M + H^+$), 1520.4 ($M + Na^+$). ^{1}H NMR (D_2O, 600 MHz) δ 7.40−7.95 (m, 8 H), 5.41 (m, 4 H), 4.98 (m, 2 H), 4.65 (m, 2 H), 4.56 (m, 1 H), 4.40 (m, 2 H), 4.00 (m, 4 H), 2.80 (m, 4 H), 1.43 (s, 9 H).

Further elongation of the peptide chain of **20** with Fmoc-Glu(Trt)-OPfp and Fmoc-Gly-OPfp in sequence, as well as final deprotection of the resulting glycopeptide, followed the standard procedures described above. The crude product was eventually obtained in quantitative yield. Its purity was determined by HPLC and NMR. Thorough purification of this product by gel filtration chromatography and then RP HPLC afforded the pure **16** in an isolated yield of 93%. HPLC: RT = 6.15 min (on a Discovery 25 × 1 cm C_{18} column with an eluent of 4% 2-propanol in water at a 2.0 mL/min flow rate). FABMS: calcd for $C_{53}H_{88}N_8O_{39}$ 1460, found 1461 ($M + H^+$). ^{1}H NMR (D_2O, 600 MHz) δ 5.42 (m, 4 H), 4.99 (d, *J* = 9.0 Hz, 1 H), 4.98 (d, *J* = 9.0 Hz, 1 H), 4.85 (dd, *J* = 7.2, 6.0 Hz, 1 H), 4.52 (dd, *J* = 6.0, 6.2 Hz, 1 H), 4.42 (dd, *J* = 8.4, 7.2 Hz, 1 H), 3.99 (s, 2 H), 3.98 (s, 2 H), 2.93 (dd, *J* = 15.6, 4.2 Hz, 1 H), 2.86 (dd, *J* = 15.0, 4.2 Hz, 1 H), 2.82 (m, 1 H), 2.80 (dd, *J* = 15.0, 7.2 Hz, 1 H), 2.41 (t, *J* = 7.2 Hz, 2 H), 2.17 (m, 1 H), 2.05 (m, 1 H).

Glycyl-glycyl-L-glutaminyl-{*N*-[α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-*β*-D-glucopyranosyl]}-L-asparaginyl-{*N*-[α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-*β*-D-glucopyranosyl]}-L-asparaginyl-{*N*-[α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-*β*-D-glucopyranosyl]}-L-asparagine (17). The *tert*-butyl group in **20** (20 mg, 13.4 μmol) was deprotected by treatment with 25% TFA/DCM (3 mL) at room temperature for 2 h. The reaction mixture was condensed and the residue was washed with ether to produce **21** that was directly used in the coupling reaction. At the same time, 20 mg of **20** was treated with 20% piperidine/NMP (2 mL) at room temperature for 2 h to give the *N*-terminal free product **22** that was isolated by the precipitation method. After **21** was stirred with DCC (8.3 mg, 40 μmol) and HOBt (5.4 mg, 40 μmol) in NMP (1 mL) for 2 h, **22** was added to the mixture. The reaction mixture was stirred at room temperature overnight, and then ether (10 mL) was added. The resulting precipitate was collected and washed by ether. Subsequent treatment of this precipitate with 20% piperidine/NMP (2 mL) and precipitation and washing of the product with ether (20 mL) afforded the crude **23** (33.0 mg, quantitative). A part of this product (4.0 mg) was purified by gel filtration chromatography and RP HPLC to produce pure **23** (3.0 mg). HPLC: RT = 5.58 min (on a Discovery 25 × 1 cm C_{18} column with an eleuent of 6% 2-propanol in water at a 2.0 mL/min flow rate). ESMS (negative mode): calcd for $C_{92}H_{154}N_8O_{69}$ 2476, found 2476 (M^-). ESMS (positive mode): 2499 ($M + Na^+$). ^{1}H NMR (D_2O, 600 MHz) δ 5.37 (m, 8 H), 4.94 (m, 4 H), 4.75−4.73 (m, 3 H), 4.70 (t, *J* = 6.0 Hz, 1 H), 3.98 (t, *J* = 9.6 Hz, 4 H), 2.60−2.90 (m, 8 H), 1.49 (s, 9 H).

Sequential elongation of the peptide chain of **23** with Fmoc-Glu(Trt)-OPfp and Fmoc-Gly-OPfp and final deprotection of the resulting glycopeptide following the standard procedures gave the crude product **17** in quantitative yield. Thorough purification of this product by gel filtration chromatography and then RP HPLC afforded the pure **17** (19.5 mg, 68% isolated yield from **20**). $[\alpha]_D$ +7.2 (*c* 0.1, H_2O). HPLC: RT = 11.9 min (on a Discovery 25 × 1 cm C_{18} column with an eluent of 0.4% 2-propanol in water at a 2.0 mL/min flow rate). MALDIMS: calcd for $C_{97}H_{160}N_{12}O_{73}$ 2662, found 2663 ($M + H^+$), 2685 ($M + Na^+$), 2771 ($M + K^+$). ^{1}H NMR (D_2O, 600 MHz) δ 5.41−5.43 (m, 8 H), 5.00 (m, *J* = 9.0 Hz, 4 H), 4.79 (t, *J* = 7.1 Hz, 1 H), 4.78 (t, *J* = 7.0 Hz, 1 H), 4.76 (t, *J* = 7.2 Hz, 1H), 4.51 (t, *J* = 6.0 Hz, 1 H), 4.38 (dd, *J* = 7.8, 6.0 Hz, 1 H), 4.05 (s, 2 H), 3.98 (t, *J* = 9.6 Hz, 4 H), 3.91 (s, 2 H), 2.94−2.99 (m, 3 H), 2.82−2.87 (m, 4 H), 2.79 (dd, *J* = 15.6, 7.8 Hz, 1 H), 2.39 (t, *J* = 7.2 Hz, 2 H), 2.14 (m, 1 H), 2.02 (m, 1 H).

Acknowledgment is made to the Research Corporation (Research Innovation Award RI 0663) and to the donors of the Petroleum Research Funds administered by the American Chemical Society (ACS PRF grant 37743-G) for their support of this research. The authors are grateful to Dr. S. Chen and Mr. H. Faulk for the MS measurements.

JO020570C