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THE INCORPORATION OF SUGAR MOIETIES TO NEUROPEPTIDES: COMPARATIVE STUDY OF DIFFERENT METHODS

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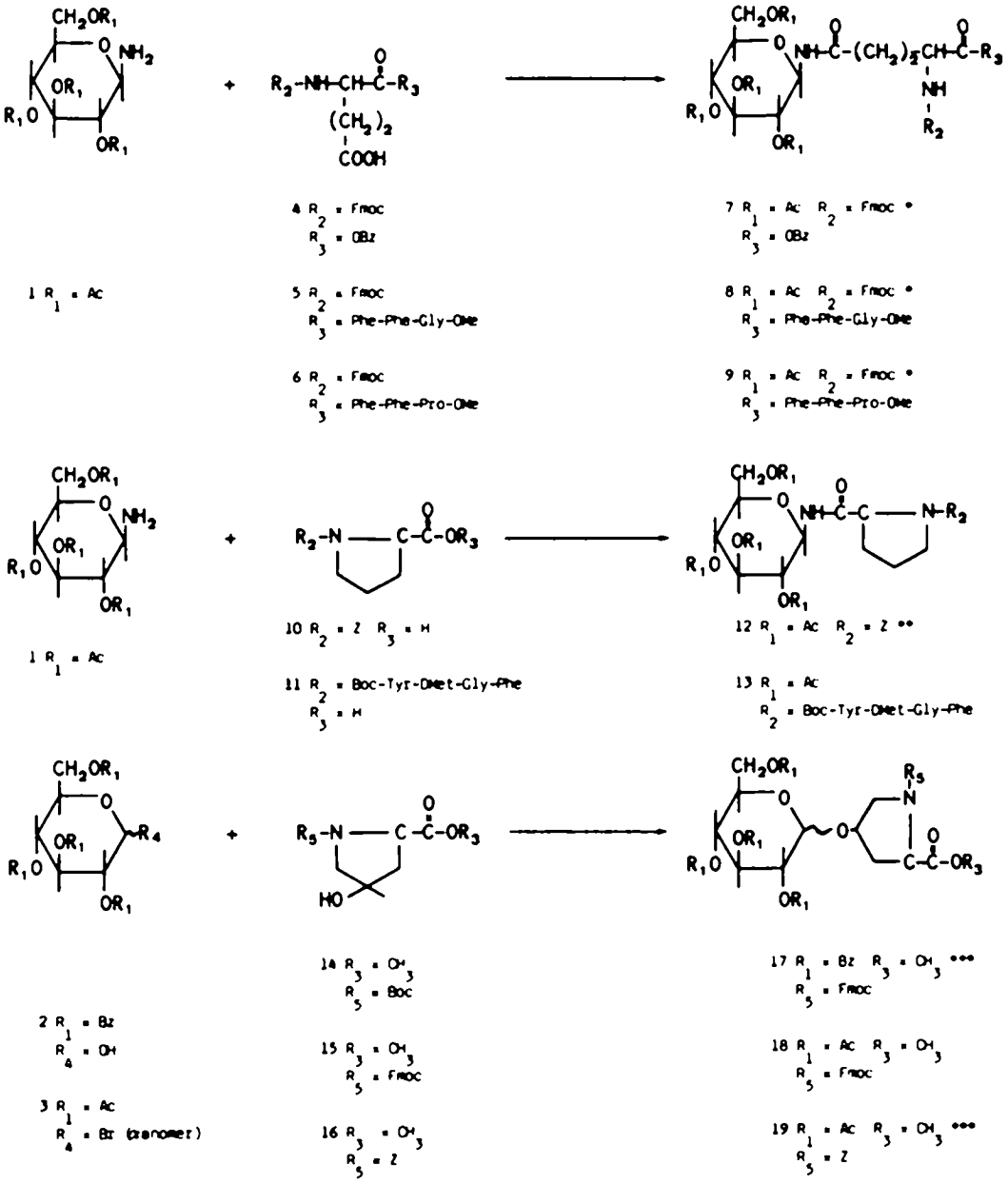
Abstract - By using both β-N-glycosylation and β-O-glycosylation procedures, different methods for the incorporation of glucose moieties to proline, hydroxyproline or glutamic acid containing protected neuropeptides have been examined. As far as glycosylation of Glu and Hyp containing fragments is concerned, the incorporation of either γ-β-N-glucosylated glutamic acid or 4-β-O-glucosylated hydroxyproline to the rest of the peptide have been chosen. However, in the case of C-terminal proline containing peptide fragments, direct β-N-glucosylation of the full peptide has been preferred. Acetyl protecting groups on the sugar moiety led to better yields than the bulkier benzyl groups.

There is a growing evidence of the crucial role played by carbohydrate residues in biological processes such as receptor recognition. Thus, an increasing number of results are pointing out the influence of the sugar moieties of glycopeptides in their biological activity and selectivity (1). Following this major tendency, one of the main goals of current research in our Laboratory is the improvement of the activity and selectivity of neuropeptides by means of the incorporation of sugar moieties to different positions on the peptide structure (2,3).

In the present paper we describe different methods for the incorporation of glucose moieties to some N and/or C-terminal protected neuroactive peptides. These peptide sequences are Boc-Tyr-DMet-Gly-Phe-Pro-OH, Boc-Tyr-DMet-Gly-Phe-Hyp-OMe (protected enkephalin related peptides), Fmoc-Glu-Phe-Phe-Gly-Leu-Met-OMe and Fmoc-Glu-Phe-Phe-Pro-Leu-Met-OMe (protected C-terminal hexapeptide analogues of Substance P). Structure-activity studies have shown that both position 5 of enkephalins and 6 of Substance P (6-11) allow modifications leading to more active agonists (4,5,6) whereas other positions such as Tyr^{1} of enkephalins and Phe^{7}, $Met\text{-}NH_2^{11}$ of Substance P have been recommended to remain unmodified (7,8,9). The glucose moieties have been incorporated either via β-N-glycosidic linkage between 2,3,4,6-tetra-O-acetyl-D-glucopyranosyl amine and the free α or γ-carboxyl group of Pro^{5} or Glu^{6} respectively or via β-O-glycosidic linkage between protected glucose and the hydroxyl group of Hyp^{5}.

An important goal of this work was the preparation of different glucosyl amino acid intermediates which could be used for the synthesis of glycosylneuropeptides. The preparation of the β-anomers of the glycosyl amino acids have been pursued since the β-glycosyl linkage is more usual than the α-linkage among the animal kingdom (10). Nevertheless, a method to obtain both α and β-glycosyl derivatives has been tested.



a) N-glycosylation by DCC/HOBt (compound 7,8,9,13) and mixed anhydride (compound 12)

b) O-glycosylation by trifluoromethanesulphonic anhydride (compound 17) or Helferich modification of the Koenigs-Knorr method (compounds 18,19)

* After cleavage of R_3 7 was linked to Phe-Phe-Gly-Leu-Met-OMe and Phe-Phe-Pro-Leu-Met-OMe to yield Fmoc [N^1-2,3,4,6-tetra-O-acetyl-β-glcp]Glu-Phe-Phe-Gly-Leu-Met-OMe (compound 20) and Fmoc[N^1-2,3,4,6-tetra-O-acetyl-β-glcp]Glu-Phe-Phe-Pro-Leu-Met-OMe (compound 21), chemical yield 25-30 % by DCC/HOBt procedure, 40 % by the mixed anhydride method. After saponification, compounds 8 and 9 were linked to Leu-Met-OMe by the DCC/HOBt procedure to yield compound 20 and compound 21 (5-10% chemical yield).

** After cleavage of R_2 compound 12 was linked to Boc-Tyr-OMet-Gly-Phe-OH by DCC/HOBt procedure to yield Boc-Tyr-OMet-Gly-Phe-[N^1-2,3,4,6-tetra-O-acetyl-β-glcp] Pro (compound 13, chemical yield 5 %).

*** After cleavage of R_5 compounds 17 and 19 were linked to Boc-Tyr-OMet-Gly-Phe-OH by the DCC/HOB procedure to yield Boc-Tyr-OMet-Gly-[O^1-2,3,4,6-tetra-O-benzyl-β-glcp] Hyp-OMe (compound 22 chemical yield 29 %) and Boc-Tyr-OMet-Gly-Phe-[O^1-2,3,4,6-tetra-O-acetyl-β-glcp] Hyp-OMe (compound 23 chemical yield 75 %).

SCHEME 1

Two condensation methods leading to α, β-O-glycosidic bonds have been assayed. Thus, the trifluoromethanesulphonic anhydride procedure (11) using O-benzylated sugar yielded mixtures of α and β-anomers whereas the $Hg(CN)_2$ catalysis method (12) using acetylated-glycosyl bromides gave, as the main isomer, the -anomer. In both cases further purification was necessary to obtain pure β-anomers. Concerning the -N-glycosylation, two common coupling methods in peptide synthesis have been used: DCC/HOBt and mixed anhydride (isobutylchloroformate). In this way, the β-N-glycosylation step could be performed before and after the incorporation of the corresponding amino acid to the rest of the peptide chain. In case of strategies involving the synthesis of simple glycosyl amino acid intermediates obtained by both O- and N-glycosylation, their incorporation to neuropeptide sequences have also been studied. Using both approaches, direct peptide glycosylation and through glycosyl amino acid intermediates, a wide range of the different possibilities of sugar incorporation to such neuropeptides has been achieved and the best routes, among those examined, have been determined.

Scheme 1 summarizes the different routes tested. In addition, the obtained chemical yields corresponding to the glycosylation steps are shown in Table 1.

TABLE 1

Chemical yields corresponding to the glycosylation step and analytical data of the resulting glycosyl amino acids and glycosyl peptide fragments.

Compound	Formula	Anal.	t.l.c. Rf_a	t.l.c. Rf_b	yield (%) β-anomer
7	$C_{41}H_{44}O_{14}N_2$	C,H,N	0.75	0.56[4]	72
8	$C_{55}H_{61}O_{17}N_5$	-	0.60	0.38[6]	50
9	$C_{58}H_{65}O_{17}N_5$	-	0.65	0.45[6]	57
12	$C_{27}H_{34}O_{12}N_2$	C,H,N	0.62	0.30[2]	55
13	$C_{49}H_{66}O_{17}N_6S$	-	0.50	0.20[5]	63
17	$C_{55}H_{55}O_{10}N$	C,H,N	0.60	0.60[1]	40 (84α+β)
18	$C_{35}H_{39}O_{14}N$	C,H,N	0.60	0.40[3]	50 (65α+β)
19	$C_{28}H_{35}O_{14}N$	C,H,N	0.60	0.30[2]	40 (60α+β)

t.l.c. solvent systems are (a) Chloroform/methanol/acetic acid (95:5:3) and (b) (1) Hexane/ethyl acetate (4:6), (2) Hexane/ethyl acetate (3:7), (3) Hexane/ethyl acetate (2:8), (4) Hexane/ethyl acetate (1:9), (5) Ethyl acetate, (6) 1-Heptane/tert-butanol/pyridine (7.5:1.5:1.5). Compounds 8,9 & 13, as well as compounds 20, 21, 22, 23 corresponding to Scheme 1, have been identified, after deprotection, by amino acid analysis and FAB-MS.

N-glycosylation

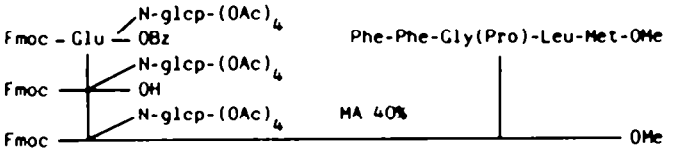
The best chemical yield (72%) for the β-N-glycosylation of Glu, was obtained by the DCC/HOBt procedure, when 1 was joined to the protected amino acid 4 to yield 7. However, the same reaction between 1 and the peptide fragments 5 & 6 resulted in lower chemical yields. Moreover, the coupling between saponified compound 7 and Phe-Phe-Gly or Pro-Leu-Met-OMe yielded the final protected Substance P (6-11) analogues 20, 21, with 40% chemical yield (MA, isobutylchoroformate) whereas the fragment condensation between saponified compounds 8,9 and Leu-Met-OMe yielded the same final analogues, with only 5-10% chemical yield. In consequence, the β-N-glycosylation of Glu and further incorporation of the glucosyl amino acid to the rest of the peptide structure resulted to be the preferred method for the synthesis of glycosylated SP(6-11) analogues. On the contrary, for the enkephalin related sequences, the route concerning

β-N-glycosylation of Pro was less effective and the glycosylation step led to lower yields. Moreover, only 5% chemical yield was obtained in the incorporation of the previously deprotected compound 12 to the rest of the peptide fragment. In summary, the obtained chemical yields have been shown to be strongly dependent on the nature of the glycosylated amino acid.

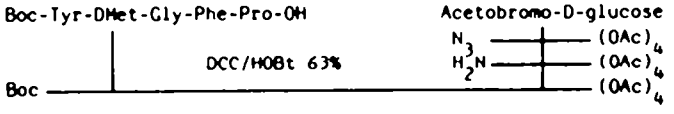
β-O-glycosylation

For SP(6-11) analogues, both glycosylation methods have led to similar chemical yields although slightly depending on the N-protecting group used. The best O-glycosylation assay yielding 40% of the β-anomer of compound 17 has been conducted with trifluoromethanesulphonic anhydride, benzylated sugar and Fmoc as N-protecting group. Moreover, by $Hg(CN)_2$ catalysis the β-glucosyl derivative of 18 was obtained from 3 and Fmoc-Hyp-OMe (15) with 50% chemical yield, whereas from 3 and L-Hyp-OMe (16) 40% chemical yield was achieved.

O-glucosyl enkephalin sequences have been obtained by coupling the glycosylated hydroxyprolines 17 & 19 with the peptide fragment Boc-Tyr-DMet-Gly-Phe-OH. However, non-compatibility of piperidine treatment for the cleavage of Fmoc protecting group with acetyl groups on the glucose moiety, made routes such as the one involving compound 18 unpractical. It is worth noting that acetylated sugar moieties such as 17, after N-deprotection, coupled to identical peptide sequence with higher yield (77%) than the benzylated compound 19 (29%). In addition, significant yield differences have been observed for proline derivatives containing sugar moieties on the amino acid side chain (compound 19) or at the N-carboxyl group (compound 12) when condensing them to the same enkephalin sequence. In this way, peptides 23 & 13 were obtained with 75% and 5% yield respectively.



(a)



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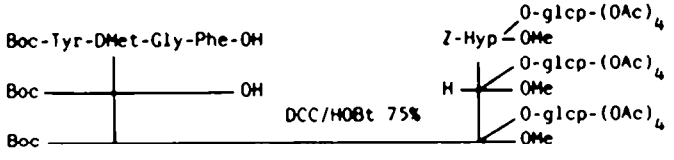

(c)

SCHEME 2

In summary, the best routes among those tested are shown in Scheme 2. They involve the incorporation by β-N-glycosylation of 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl amine 1 both (a) to N-α & C-α-protected glutamic acid of Substance P (6-11), (b) to Pro^5 of the enkephalin related sequence and (c) the β-O-glycosylation of benzyloxycarbonyl protected hydroxyproline using 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl bromide. The direct O-glycosylation of tyrosine and methionine containing peptide fragments was always avoided because of either the strong acidic conditions or high temperatures involved in the O-glycosylation methods.

EXPERIMENTAL

Thin layer chromatography was performed on silica gel plates (0.25 mm) from Merck. Spots were detected by reaction with ninhydrin or chlorine followed by tolidine solution. The sugar was detected by the Orcinol-HCl reagent. Amino acid analysis were performed in a Beckman 119 C instrument or a Biotronik LC 5001. ^{1}H and ^{13}C-NMR spectra were obtained with a Bruker (80 MHz) or a Varian XL200 (200 MHz) spectrometer in $CDCl_3$. Chemical shifts are reported in δ-units, using tetramethylsilane as internal standard. All the solvents used were analytical grade. They were distilled and stored over molecular sieve when necessary.

Microanalysis were performed by the Microanalytical Laboratory, Department of Biological Organic Chemistry, C.I.D. Barcelona. FAB-Mass spectra were determined with a MS9-V6 updated system equipped with a V6-11 250 unit by the Laboratory of Mass Spectrometry, C.I.D. Barcelona.

Isolation and purification of the protected glycosyl derivatives of amino acids and peptides were carried out by "Flash Chromatography" (13), using Silica gel (40-63 μm), 15x2 cm or 15x5 cm columns and eluting with the appropiate solvent systems at a flow rate of 5 cm/min.

The purity of the protected glycosyl amino acids or peptides were checked by HPLC-reversed-phase (ODS, 5 μm column, H_2O 0.05% TFA/CH_3CN gradient elution from 9 to 100 % CH_3CN at a linear rate of 3.5% CH_3CN/min) Detection by absorbance rationing measurements at several wavelengths.

2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl amine (1) has been obtained from acetobromo-D-glucose (14). The resulting product was characterized by ^{1}H-NMR. Melting point 129°. Yield 54 % .

Three different amino acid N-protecting groups have been used, 9-fluorenylmethyloxycarbonyl (Fmoc), Benzyloxycarbonyl (Z) and tert.-butyloxycarbonyl (Boc). Fmoc groups were removed by treatment with piperidine/CH_2Cl_2 (1:1) 1 hour at room temperature. Z groups were cleaved by hydrogenolysis in the presence of 10 % palladium on charcoal (0.1 g/g) in methanol. Removal of Boc groups was achieved with TFA/CH_2Cl_2 (1:1) containing a few drops of 2-mercaptoethanol, 30 minutes at room temperature with a ratio of 10 mL acid-solvent mixture to 1 mmol of peptide or amino acid derivatives.

To illustrate the different glycosylation and peptide bond formation coupling methods used in this work, one example of each type of reaction is given. Purification conditions and n.m.r. characterization of all new or previously described compounds prepared following a given procedure are also provided. ^{1}H-n.m.r. chemical shifts are reported avoiding repetition of common signals corresponding to amino acid residues already included in similar given spectra. All the glycosyl derivatives have proved to be homogenous on TLC and HPLC.

All optically active amino acids were of the L configuration with the exception of D-methionine, as indicated throughout the text.

The following abbreviations according to the IUPAC-IUB Comission were used: THF Tetrahydrofuran, DMF N,N-dimethylformamide, NMM N-methylmorpholine, HOBt, 1-hydroxybenzotriazole, DCC, N,N'-dicyclohexylcarbodiimide, MA mixed anhydride procedure, Boc tert.-butyloxycarbonyl, Fmoc 9-fluorenylmethyloxycarbonyl, Z benzyloxycarbonyl.

The nomenclature of glycopeptides used in this paper follows the IUPAC-IUB Joint Commission rules which are due to be published in brief.

1.- β-N-glycosylation. DCC/HOBt procedure Synthesis of N-9-fluorenylmethyloxycarbonyl-[N^1 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl] glutamic acid Benzyl Ester 7.

Fmoc-Glu-OBz (0.5 g, 1.09 mmol) and 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl amine (0.375 g, 1.09 mmol) were dissolved in 10 mL THF at -15°. HOBt (0.175 g, 1.09 mmol) and DCC (0.25 g, 1.31 mmol) dissolved in THF were added and the reaction mixture kept 1 h at -15° and overnight at room temperature. The mixture was filtered and the filtrate washed succesively with 5% citric acid (3x20 mL), 5% sodium bicarbonate (3x20 mL) and H_2O (3x20 mL). The organic layer was then dried over anhydrous Na_2SO_4 and the amorphous solid obtained after evaporation was purified by flash chromatography on Silica gel using hexane/ethyl acetate (1:9). ^{1}H-n.m.r. 7.2-7.9 arom Fmoc, OBz, m 4.25 C_αH Glu, m 1.70 C_βH Glu, m 2.13 C_γH Glu, d 5.66, $J_{1,2}$ =8.3 Hz H-1, 11t. (15) 8.5 Hz, 4.1-5.4 6H glucopyranose, s 2.13 (3H), s 2.07 (3H), s 2.03 (6H) OAc, ^{13}C-n.m.r. (20 MHz) 83.2 C-1 β-anomer.

N-9-fluorenylmethyloxycarbonyl [N^1-2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl] glutamyl, phenylalanyl, phenylalanyl, glycine Methyl Ester 8. Purification eluent, hexane/ethyl acetate (1:9). ^{1}H-n.m.r. 7.25 arom Phe, m 4.70 C_αH Phe, d 3.85 C_αH Gly, s 3.55 O-CH_3, m 3.1-2.65 C_βH Phe. N-9-fluorenylmethyloxycarbonyl [N^1-2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl] glutamyl, phenylalanyl, phenylalanyl, proline Methyl Ester 9. Purification eluent, hexane/ethyl acetate (1:9). ^{1}H-n.m.r. d 4.36 C_αH Pro, m 2.03 C_βH Pro, m 1.85 C_γH Pro, m 3.61 C_δH Pro.

N-tert.-butyloxycarbonyl tyrosyl, D-methionyl, glycyl, phenylalanyl, [N^1-2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl] proline 13. Purification eluent, ethyl acetate/methanol (8:2). ^{1}H-n.m.r. s 1.33 tert.-butyl, m 7.25 arom Phe, 7.02 & 6.72 arom Tyr, s 4.03 C_αH Tyr, m 4.32 C_αH DMet, d 3.85 C_αH Gly, m 4.70 C_αH Phe, m 4.36 C H Pro, m 2.89 C_βH Tyr, m 1.70 C_βH DMet, m 3.0 & 2.77 C_βH Phe, m 2.03 C_βH Pro, m 2.23 C_γH DMet, m 1.85 C_γH Pro, m 3.61 C_δH Pro, s 2.0 -S-CH_3 DMet, d 5.02 $J_{1,2}$=8.4 Hz H-1, 4.1-5.4 6H glucopyranose, s 2.10 (3H), s 2.07 (3H), s 2.03 (6H) OAc. ^{13}C-n.m.r. (20 MHz) 83.0 C-1 β-anomer.

2.- β-N-glycosylation. Mixed Anhydride, Isobutylchloroformate procedure Synthesis of N-benzyloxycarbonyl [N^1-2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl] proline 12.

Z-Pro-OH (0.125 g, 0.61 mmol) and NMM (0.067 mL, 0.61 mmol) were dissolved in 7 mL of DMF. The solution was cooled to -15° (dry ice/acetone) and isobutylchlorocarbonate (0.079 mL, 0.61 mmol) was added dropwise; the mixture was stirred at

-15° for 90 s. A precooled (-15°) solution of 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl amine (0.211 g, 0.61 mmol) and NMM (0.067 mL) in 5 mL of DMF was then added to the formed mixed anhydride. The reaction mixture was stirred for 1 h at -15° and 2 h at room temperature, filtered and after solvent evaporation the remaining oily residue was submitted to flash chromatography on Silica gel, 15x5 cm ID column eluted with 300 mL hexane/ethyl acetate 3:7. ^{1}H-n.m.r. s 7.25 5H arom Z, s 5.2, -CH_2-CO- Z, d 5.06 $J_{1,2}$=8.4 Hz H-1, 4.1-5.4 6H glucopyranose, s 2.10 (3H), s 2.07 (3H), s 2.03 (6H) OAc, m 4.36 C_αH Pro, m 2.03 C_βH Pro, m 1.85 C_γH Pro, m 3.61 C_δH Pro. ^{13}C-n.m.r. (20 MHz) 83.2 C-1 β-anomer.

3.- O-glycosylation. Trifluoromethanesulphonic Anhydride Procedure. Synthesis of N-9-fluorenylmethyloxycarbonyl [O^1-2,3,4,6-tetra-O-benzyl-α,β-D-glucopyranosyl] hydroxyproline Methyl Ester 17.

2,3,4,6-tetra-O-benzyl-glucopyranose (0.500 g, 0.94 mmol) and Fmoc-Hyp-OMe (0.850 g, 2.3 mmol) were dissolved in 10 mL of CH_2Cl_2. The solution was cooled to -70° (dry ice/acetone) and 0.223 mL (1.4 mmol) of trifluoromethanesulphonic anhydride were added, the mixture was stirred at -70° for 5 min and 1 h at room temperature. The solution was then cooled again to -70° and 80 mL of water were added. The resulting products were extracted with ethyl acetate (3x15 mL) and the organic layer was neutralized with a 5% bicarbonate solution and washed with water (3x15 mL), dried over Na_2SO_4 and evaporated. The oily resulting mixture was then purified on Silica gel, 15x5 cm I.D. column, eluted with hexane/ethyl acetate 4:6. ^{1}H-n.m.r. d 4.33 C_αH Hyp, m 2.12 C_βH Hyp, m 4.45 C_γH Hyp, m 3.82 C_δH Hyp, s 7.20 20H arom OBz, d 4.32 $J_{1,2}$=7.2 Hz H-1 β-anomer, s 3.55 O-CH_3. ^{13}C-n.m.r. (20 MHz) 103.0 C-1 β-anomer, 96.8 C-1 α-anomer, 58.5 C_α Hyp, lit. (11) 102.42 & 102.28 C-1 β-anomer, 96.7 & 96.08 C-1 α-anomer, 58.04 & 57.84 C_α Hyp.

4.- β-O-glycosylation. $Hg(CN)_2/C_6H_6$ Procedure. Synthesis of N-9-fluorenylmethyloxycarbonyl [O^1-2,3,4,6-tetra-O-acetyl-α,β-D-glucopyranosyl] hydroxyproline Methyl Ester 18.

Fmoc-Hyp-OMe (0.330 g, 0.9 mmol) was dissolved in 30 mL of dry benzene and 30 mL of nitromethane and heated under anhydrous conditions until about 15 mL of solvent had distilled off. To this solution a double equivalent amount of both 2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl bromide (0.737 g) and $Hg(CN)_2$ (0.461 g) were added in three portions: half of the given amounts at the beginning of the reaction and the rest in two equal portions after 2 h and 3 h respectively. The mixture was heated at 80° during a total time of 4 h. The solution was then cooled and diluted with ether (80 mL) and filtered to eliminate the precipitated mercuric salts. The filtrate was then washed with water, dried over Na_2SO_4 and evaporated. The oily resulting mixture was submitted to chromatography on Silica gel, 15x5 cm I.D. column eluted with hexane/ethyl acetate (2:8). ^{1}H-n.m.r. d 4.35 $J_{1,2}$=8.5 Hz H-1 β-anomer.

In addition, the final protected β-N-glucosylated or β-O-glucosylated peptide fragments 20, 21, 22 & 23 have been obtained by using either DCC/HOBt or MA Isobutylchloroformate as stated in Scheme 1. They have been identified, after deprotection, by Amino acid analysis and FAB-Mass Spectrometry and characterized by n.m.r.

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