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SYNTHESIS OF 0-(2-0-α-D-GLUCOPYRANOSYL)-β-D-GALACTOPYRANOSIDE OF OPTICALLY PURE δ-HYDROXY-L-LYSYLGLYCINE AND δ-HYDROXY-L-LYSYLGLYCYL-L-GLUTAMYL-L-ASPARTYLGLYCINE

COMPONENTS OF THE GLOMERULAR BASEMENT MEMBRANE

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Abstract—The application of the levulinyl group as a temporary blocking group for the 2-OH function of galactose proved to be very efficient not only for the introduction of a β-linkage between galactose and the optically pure δ-hydroxy-L-lysylglycine, but also to link galactose, via an α-interglycosidic bond, with glucose. The partially protected glycodipeptide (**7b**) served as the starting product for the preparation of the naturally occuring glycopentapeptide (**9b**). The preparation of a conjugate between bovine serum albumin and the glycodipeptide (**7c**) will also be reported.

Glomerular Basement Membrane (GBM) is composed of at least two carbohydrate-rich protein moieties, one a collagen and the other a non-collagen glycoprotein.[1]

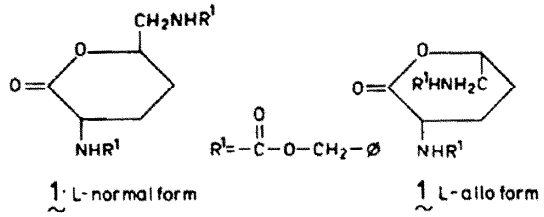
Careful analysis by Spiro *et al.* of the carbohydrate moiety of the collagen residue indicated the presence of the disaccharide 2-0-α-D-glucopyranosyl-D-galactopyranose, which is linked via a β-glycosidic bond with the alcoholic function of δ-hydroxy-L-lysine (Hyl).[2] A small glycopeptide composed of the same disaccharide and the peptide Hyl-Gly-Glu-Asp-Gly was also isolated[3] from GBM and reported to constitute the antigenic site of anti-Gbm autoantibody, which is present in some patients with glomerulonephritis and Goodpasture's syndrome.[4] In this paper we wish to describe in detail[5] the synthesis of the 0-(2-0-α-D-glucopyranosyl)-β-D-galactopyranoside of optically pure δ-hydroxy-L-lysylglycine (**7c**) and of the pentapeptide L-Hyl-Gly-L-Glu-L-Asp-Gly (**9b**).

Further, the coupling of glycodipeptide **7c** to bovine serum albumin (BSA, **10**) to form a synthetic antigen will be reported.

RESULTS AND DISCUSSION

(A) *Preparation of optically pure δ-hydroxy-L-lysylglycine* (**3**)

One prerequisite in the total synthesis of the naturally occuring glycoproteins **7c** and **9b** is the availability of optically pure δ-hydroxy-L-lysylglycine (**3**, normal form) with a free OH function (Scheme 1). The strategy we adopted for the synthesis of dipeptide **3** consists of the following steps: (i) enzymatic resolution of α - chloroacetyl - ε - carbobenzoxy - δ - hydroxy - DL - lysine (normal and allo forms); (ii) carbobenzoxylation of the so obtained L-mixture giving the dicarbobenzoxylated lactone **1** (normal and allo forms), followed by (iii) separation of the diastereomers of **1** by flash-chromatography affording **1** (normal, L-form); (iv) finally, aminolysis of the lactone **1** (normal L-form) with the t-butyl ester of glycine (**2**) which results in the formation of the required dipeptide **3**.



1: L-normal form **1** L-allo form

Thus, δ-hydroxy-DL-lysine monohydrochloride (normal and allo forms) was prepared as described by Izumiya *et al.*[6] The resolution of the amino acid, involving the L-directed hydrolytic action of acylase I on the corresponding α-chloroacetyl-ε-carbobenzoxy derivative, as described by Fones,[7] afforded ε-carbobenzoxy-δ-hydroxy-L-lysine (normal and allo forms). As already reported by Fones, α-chloroacetyl-ε-carbobenzoxy-δ-hydroxy - L - lysine turned out to be a poor substrate for acylase I and only a small quantity (20%) of the desired product could be isolated. On the other hand, most of the required material (76%) existed in the normal-form, as followed from the comparison of the specific rotations of the diastereomeric mixture with those of the individual isomers (Table 1); the same ratio of normal to allo was derived from the specific rotations of the corresponding dicarbobenzoxylactones (Table 1). In applying the above enzymatic resolution procedure, we obtained, starting from a mixture containing nearly equal amounts of the normal- and allo-form, a higher yield of the normal isomer: the latter indicates that the normal-form of α - chloroacetyl - ε - carbobenzoxy - δ - hydroxy - L - lysine is a better substrate for acylase I than the allo-form. Treatment of ε-carbobenzoxy-δ-hydroxy-L-lysine (normal and allo forms) with carbobenzoxychloride and 1N NaOH at pH 9.8 gave, after adjustment of the pH to 2.5 and work-up, crystalline L-lactone **1** (normal and allo forms) in 87% yield. Separation of the normal and allo isomers of L-lactone **1** was achieved by flash-chromatography[8] to afford crystalline δ-hydroxy-L-lysine

Table 1. Specific rotation and composition (normal vs allo form of several δ-hydroxy-lysine residues

COMPOUND	$[\alpha]_D^{\delta}$	COMPOSITION, %	
		normal	allo
δ-hydroxy-DL-lysine (normal and allo forms)		45[a]	55[a]
ε-carbobenzoxy-δ-hydroxy-L-lysine (normal and allo forms)[b]	+ 11.4 (c 1 6N HCL)		
ε-carbobenzoxy-δ-hydroxy-L-lysine (normal form)	+ 8.8[c] (c 1 6N HCL)	76	24
ε-carbobenzoxy-δ-hydroxy-L-lysine (allo form)	+ 19.8[c] (c 1 6N HCL)		
dicarbobenzoxy-δ-hydroxy-L-lysine lactone (1; normal and allo forms)[d]	+ 6.3 (c 1 $CHCl_3$)		
dicarbobenzoxy-δ-hydroxy-L-lysine lactone (1; normal form)[e]	+ 67.2 (c 1 $CHCl_3$)	71	29
dicarbobenzoxy-δ-hydroxy-L-lysine lactone (1; allo form)[e]	- 18.3 (c 1 $CHCl_3$)		

a. Composition determined after partial saponification of the diastereomeric mixture of dicarbobenzoxy-δ-hydroxy-DL-lysine lactone as described by Izumiya *et al.*[6]
b. Diastereomeric mixture obtained after the enzymatic resolution procedure (see Text).
c. See Fones, ref. 7a.
d. Diastereomeric mixture obtained after carbobenzoxylation of ε-carbobenzoxy-δ-hydroxy-L-lysine (normal and allo form, see Text and Experimental).
e. Obtained from a diastereomeric mixture after separation by flash-chromatography.

lactone **1** (normal form) in 39% yield. The use of flash-chromatography in the last separation step is based on the following observation.

Separation of the normal from the allo-form of lactone **1** by using short column chromatography[9] on Kieselgel 60 led to a considerable decomposition of the normal-form of **1**. However, we found that the decomposition of the normal form of **1** could be decreased by applying flash-chromatography.[8] The latter technique reduces the time to separate the normal from the allo-form to only 15 min: for comparison, the same separation, using short column chromatography is 8 hr. The same phenomenon was also observed in an attempt to analyse a mixture containing the normal- and allo-form of **1** by HPLC (solid phase: Partisil 5, eluens THF-hexane). X-ray analysis of **1** (normal-form) confirmed, as predicted by Witkop,[10a] unambiguously the trans configuration of normal δ-hydroxy-L-lysinelactone (see Fig. 1).[10b]

Finally, aminolysis[11] of lactone **1** with the t-butyl ester of glycine,[12] **2**, in dry dioxan (Scheme 1), during 20 hr at 70° gave, after work-up, the desired dipeptide **3** as a crystalline product in 72% yield. ^{13}C NMR data confirmed the proposed structure (Experimental).

(B) Preparation of glycodipeptide **7c**

The synthesis and the removal of protective groups from the fully protected glycoprotein **7** to give the naturally occuring compound **7c**, is illustrated in Scheme 1.

Both glycopeptides **7c** and **9b** contain one galactose unit the anomeric centre of which is β-linked to the δ-hydroxy-L-lysyl residue, while the neighbouring OH group is α-linked to glucose. For the introduction of the β-linkage we started[13] from the properly protected galactose bromide derivative **4** (R^3 = Ac, R^4 = Lev). Compound **4** is protected with three acetyl and one levulinyl group, the former functions as a persistent and the latter as a temporary blocking group. The use of the levulinyl group not only favors, under the conditions of Helferich,[14] the formation of a β-linkage with the partially protected dipeptide **3**, but also opens the way to extend the galactose derivative **5** in the 2-direction with the glucose derivative **6**. The alcoholic functions of the glucose derivative **6** (Scheme 1) are protected with the persistent benzyl groups, while the anomeric centre is functionalized with the N-methyl-acetimidyl group.

The nature of the latter group must ensure[15] the for-

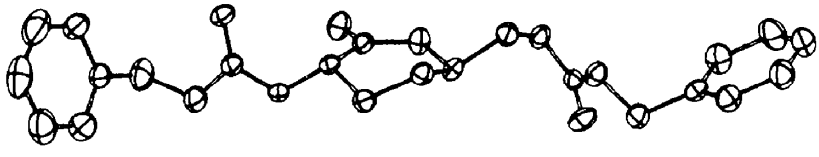
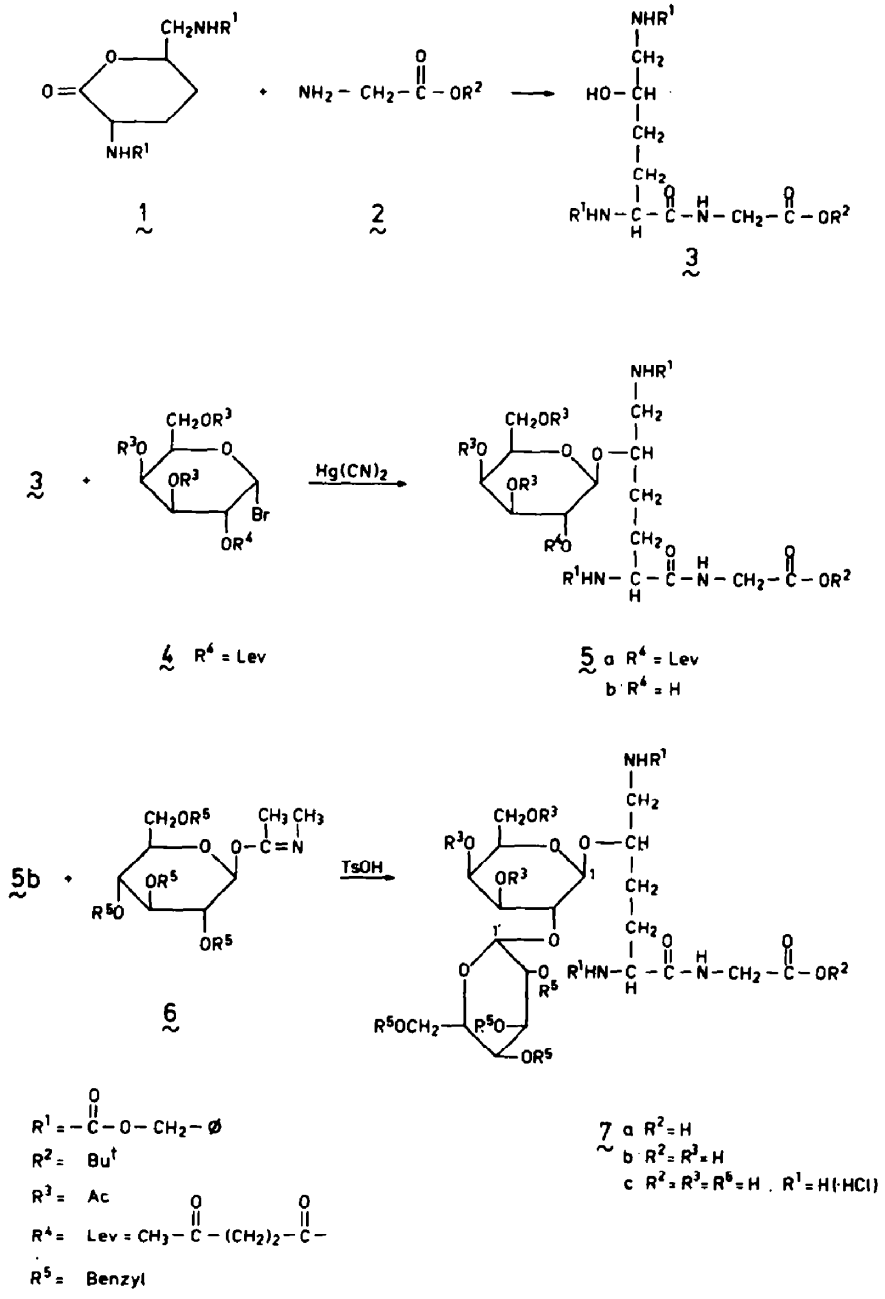


Fig. 1. ORTEP projection of dicarbobenzoxy-δ-hydroxy-L-lysine lactone, **1** (normal-form).



Scheme 1.

mation of the required α-linkage between the galactose and glucose moieties (e.g. compounds **5b** and **6**, respectively). For the introduction of the β-glycosidic bond we chose, as mentioned before, the conditions of Helferich. Thus, condensation of the bromo derivative **4** with the dipeptide **3** in dry toluene in the presence of Hg $(CN)_2$ afforded, after work-up and column chromatography, crude glycopeptide **5a** (R^4 = Lev) as a glass. The latter material was mainly (≈30%) contaminated with 2 - 0 - levulinyl - 3,4,6 - tri - 0 - acetyl - α/β - D - galactopyranose, which was formed during the hydrolysis of excess **4**. Unfortunately, this impurity had almost the same R_f-value as the required compound **5a** (R^4 = Lev), which made further purification by column chromatography ineffective. However, acetylation of the mixture with acetic anhydride, followed by work-up, afforded after purification by column chromatography, **5a** (R^4 = Lev; 31% yield) as a homogeneous glass.

The β-configuration of the glycosidic bond of **5a** was established by ^{1}H NMR spectroscopy. Removal of the levulinyl group was performed[13] by treating **5a** (R^4 = Lev) with a solution of hydrazine hydrate (1.0 M) in pyridine/acetic acid. Quenching of the reaction mixture with pentane-2,4-dione afforded, after work-up and purification by column chromatography, **5b** (R^4 = H) as a homogeneous glass in 64% yield. ^{1}H NMR and ^{13}C NMR data confirmed the proposed structure (Experimental). The final step in the synthesis of the fully-protected glycodipeptide **7**, which consisted of the introduction of the α-glycosidic bond between C-1′ of the glucose derivative **6** and the OH function at C-2 of the galactose derivative **5b**, was accomplished by applying the

methodology originally devised by Sinaÿ *et al.*[15] Thus, a solution of **5b** (R^4 = H) in dry nitromethane was treated with **6** (R^5 = benzyl) and anhydrous *p*-toluenesulphonic acid in the presence of powdered 4 Å molecular sieves. After 18 hr at 20°, tlc-analysis (solvent F) showed *ca* 50% conversion of starting material **5b** into the disaccharide derivative **7**.

After work-up, the crude material thus obtained was treated once more with **6** in exactly the same way as described previously. After work-up and purification by column chromatography, the fully-protected glycodipeptide **7** was isolated as a homogeneous glass (yield: 58%). ^{13}C NMR data indicated that the newly introduced linkage had the α-configuration.[16] Moreover, the up-field shift (2.1 ppm) of C-1 of the galactosylunit after glycosylation of **5** (β-effect)[17,18] is indicative for the formation of a 1-2 glycosidic linkage (Experimental).

Complete deblocking of the fully-protected glycodipeptide **7** was performed in three distinct stages. Firstly, short trifluoroacetic acid treatment of **7** removed the t-butyl group to give homogeneous **7a** (R^2 = H) having a free carboxyl function. Secondly, deblocking of the base-labile acetyl groups from **7a** with triethylamine/methanol/water, afforded homogeneous **7b** (R^2 = R^3 = H). Finally, catalytic hydrogenolysis (Pd/C) removed the carbenzoxy (R^1) and benzyl (R^5) groups of **7b**. After purification by SP-Sephadex C25 ion-exchange chromatography using aqueous ammonium formate (pH 4.5) as the eluting solvent, followed by lyophilization of the pure fractions, the diformate salt of **7c** (R^2 = R^3 = R^5 = H; R^1 = H, HCOOH; normal, L-form) was obtained as a white powder in 80% yield (based on fully protected **7**). The identity and homogeneity of the glycodipeptide **7c** was confirmed by ^{13}C NMR spectroscopy, paper chromatography, paper electrophoresis, HPLC (Fig. 4) and amino acid analysis; finally the optical purity of **7** and **7c** obtained above was corroborated indirectly by comparison of (Experimental) the ^{13}C NMR data of the anomeric carbon atoms C-1 and C-1′ of these compounds with those obtained from the corresponding glycodipeptides **7** and **7c** which were synthesized starting from a racemic mixture of **1** (normal-form, Fig. 2 and 3, and allo-form). The appearance of two signals for C-1′ in the latter four glycoprotein derivatives is indicative for the optical impurity of these compounds.

The differences in ^{13}C chemical shifts, which we observed between the anomeric C atoms C-1 and C-1′ of the different optical isomers of the glycodipeptides **7** and **7c**, may be ascribed to non-bonded interactions between the glycosyl and peptidyl moieties. These interactions may lead to changes in the geometries of the glycosidic bonds. Similar effects (*exo*-anomeric effects) have been observed by Lemieux *et al.*[18] in a detailed study of the conformational properties of human blood-group substances.

(C) *Synthesis of glycopentapeptide* **9b**

The route we followed to attain the naturally occuring glycopentapeptide **9b** is illustrated in Scheme 2. It can be seen that the first step consists of condensing the partially-deblocked glycodipeptide **7b** (R^2 = R^3 = H), and not the glycopeptide **7a** which still contains the base-labile acetyl groups, with the properly-protected tripeptide[19] H-Glu (OBu^t)-Asp (OBu^t)-Gly (OBu^t) (**8**). The reason for this way of operating is prescribed by the extreme base-lability[20] of the peptide bond between the amino acids aspartic acid and glycine. In other words: in the synthesis

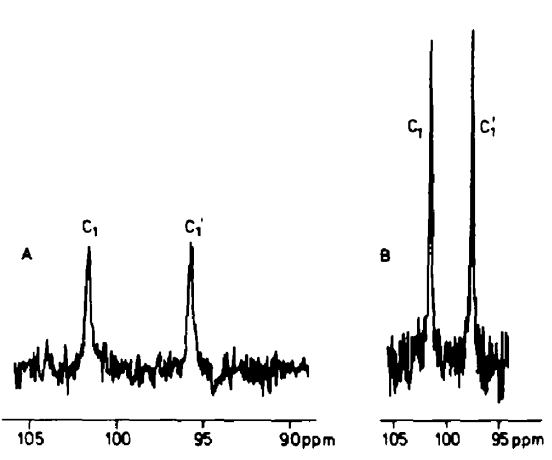


Fig. 2. Expanded parts of the ^{13}C NMR spectra of (A) the fully-protected glycodipeptide **7** and (B) the fully-unprotected glycodipeptide **7c** (R^1 = H, HOOCH), normal-L-form, which show the anomeric carbon atoms.

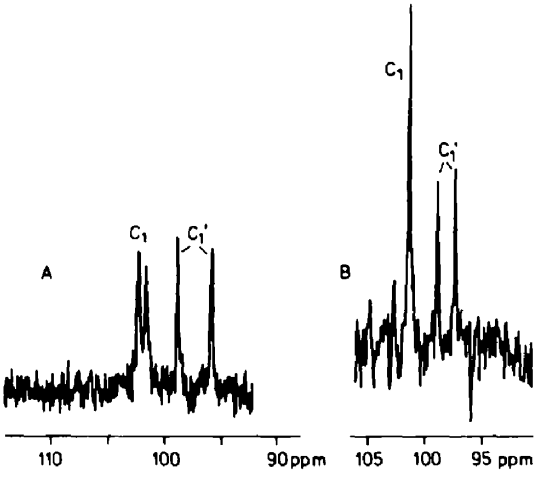
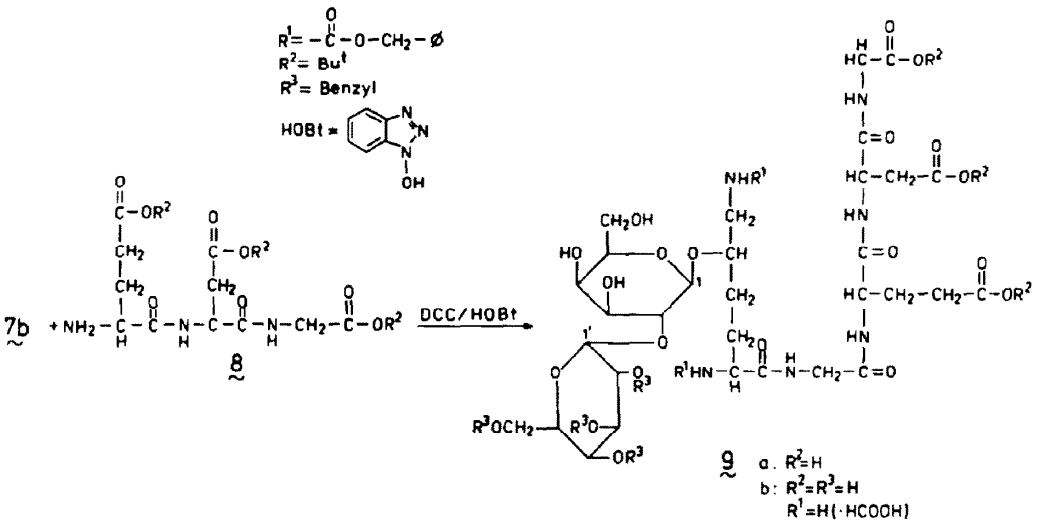


Fig. 3. Expanded parts of the ^{13}C NMR spectra of (A) the fully protected glycodipeptide **7** and (B) the fully-unprotected glycodipeptide **7c** (R^1 = H,HCOOH), normal-DL-form, which show the anomeric carbon atoms.

of the glycopentapeptide **9** the acetyl as well as the levulinyl protecting groups present in the galactose derivative **4** perform, contrary to the synthesis of the glycodipeptide **5a**, a temporary blocking function.

Thus treatment of compound **7b** (0.12 mmol) with the HCl-salt of tripeptide **8** (0.1 mmol) in a mixture of DMF and THF in the presence of 1-hydroxy-benzotriazole (HOBt), N-ethylmorpholine (NEM) and DCC afforded, after work-up, glycopentapeptide **9** as homogeneous precipitate in 94% yield. Deblocking of glycopentapeptide **9** was performed by treating the compound with acid followed by hydrogenolysis. Regarding the acid deprotection, it is important to explain the reason for the use of the -Bu^t protecting groups in the peptidyl moiety **8**. The presence of the Asp-Gly sequence in the peptide to be deblocked necessitated the use of trifluoroacetic acid (TFA) for deprotection; since this particular sequence has been shown[21] to give rise to side products (e.g. succinimido derivatives) using acids other than TFA. The crude deblocked glycopeptide was purified by SP-



Scheme 2.

Sephadex C-25 ion-exchange chromatography using aqueous ammonium formate (pH 3.1) as the eluting solvent followed by lyophilization of the pure fractions to afford the di-formate salt of **9b** ($R^2 = R^3 = H$; $R^1 = H$, HCOOH) as a white powder in 35% yield (based on fully-protected **9**). The identity and homogeneity of glycopentapeptide **9b** was confirmed by ^{13}C NMR spectroscopy, paper chromatography, paper electrophoresis, HPLC (Fig. 4) and amino acid analysis.

(D) *Preparation of the glycodipeptide-BSA conjugate* (**17**)

As mentioned before, the glycopeptide containing 2-0-α-D-glucopyranosyl-0-β-D-galactopyranosyl-δ-hydroxy-L-lysine is reported[3] to be the antigenic site of anti-GBM autoantibody. Covalent attachment of synthetic glycopeptides to proteins or other polymers may provide artificial antigens which generate, after immunization of animals, antibodies specific to the glycodipeptide determinant.[22] These monospecific antibodies may have important immunological applications, e.g. to establish the antigenic site on kidney tissues.

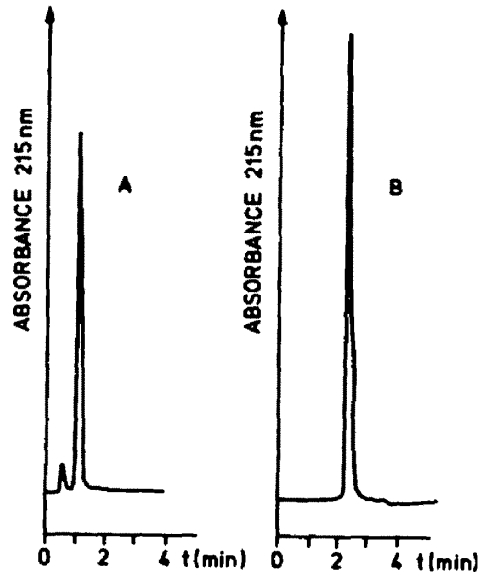


Fig. 4. HPLC-analysis of (A) the fully-unprotected glycodipeptide **7c** (R^1 = H, HCOOH) and (B) glycopentapeptide **9b**.

We now wish to describe the conjugation of glycopeptide **7c** to bovine serum albumin (BSA **10**), with the reagent N-succinimidyl-3-(2 pyridylthio)-propionate (SPDP, **11**).

The properties and applications of this heterobifunctional reagent in the preparation of conjugates has been described by Carlsson *et al.*[23] The strategy we adopted for the synthesis of the glycodipeptide—BSA conjugate **17** is outlined in Scheme 3 and consists of the following steps: (i) introduction of 2-pyridyl disulphide structures into BSA **10**, with SPDP affording **12**; (ii) preparation of the protein-bound thiol compound **14** by reduction of protein **12** with dithiothreitol (DTT); (iii) aminolysis of SPDP with **7c** which affords the 2-pyridyl-disulphide containing glycodipeptide **16**; (iv) finally, the reaction of **16** with protein **14** to afford the desired disulphide-linked conjugate **17**.

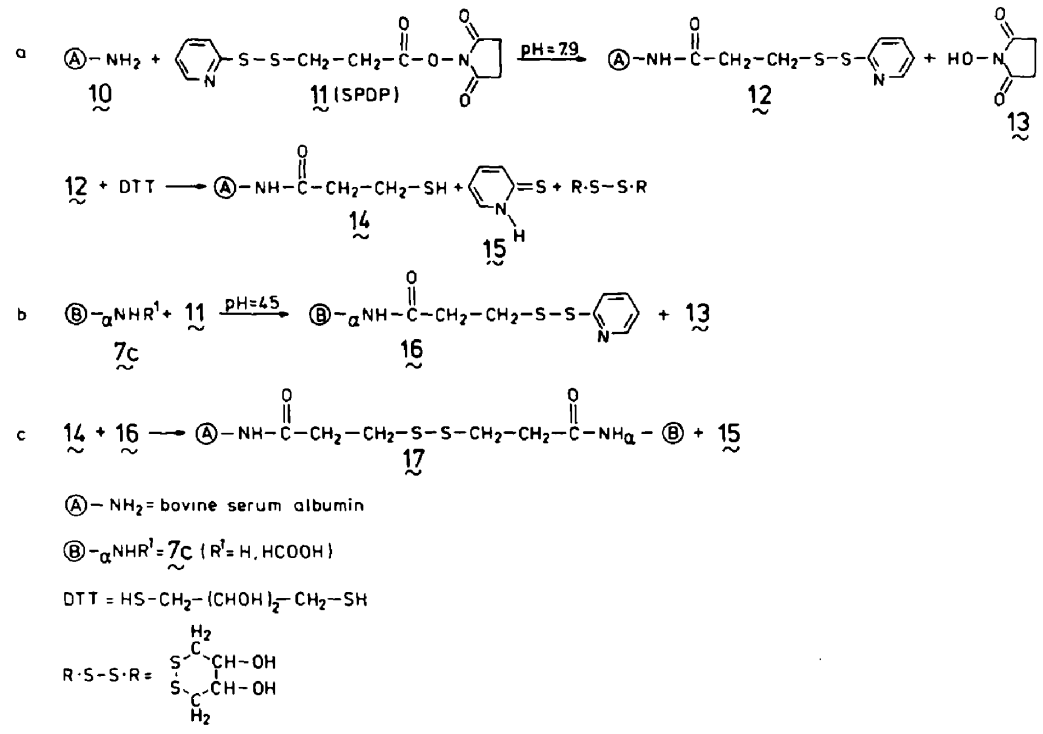
To be an useful antigen in immunological studies, conjugate **17** should contain as many glycodipeptide residues as possible. Therefore, most of the free ε-amino groups of the lysyl residues present in BSA (total amount 57) were converted to 2-pyridyl disulphide derivatives and subsequently to thiol groups.

Thus, treatment of a solution of BSA in 0.1 M N-ethylmorpholine-acetate buffer (NEM/HOAc) pH 7.9 with a solution of SPDP in THF afforded, after work-up, an aqueous solution of **12**. The number of 2-pyridyl disulphide structures introduced was *ca* 40 mol per mol BSA.

The latter quantity was determined spectrophotometrically by measuring the amount of pyridine-2-thione (**15**) released during the reaction of a sample of the modified BSA **12** with a large excess of DTT (Experimental). Compound **12** was reduced to the BSA-bound thiol compound **14** by treatment with DTT for 1 hr at pH 7.5. A gel filtration† of the reaction mixture afforded **14** in 45% yield (based on 10).

Due to the two free amino groups (α and ε) in the δ-hydroxy-L-lysine residue, two 2-pyridyl disulphide

†Gel filtrations were performed in collaboration with the Department of Nephrology, University Hospital, Leiden.



Scheme 3.

structures can be introduced in **7c** to give different conjugates by coupling with **14**. Fortunately, however, the differences in pK values (≈2.5) between the two amino groups enabled us to achieve a reasonable degree of specificity by performing the aminolysis at a lower pH value.[24] Thus treatment of glycodipeptide **7c** in 0.1 M NEM/HOAc pH 4.7 with SPDP gave (tlc, solvent G) *ca* 40% conversion of **7c** into glycodipeptide derivative **16** without serious degree of disubstitution. Finally, the desired conjugate **17** was obtained by the reaction of BSA derivative **14** with the glycodipeptide derivative **16** at pH 7.5 for three days at 0°; spectrophotometric analysis of the reaction mixture indicated complete conversion of starting material **14** into the disulphide-linked conjugate **17**.

Successive gel filtration followed by dialyzation and lyophylization afforded **17** in 78% yield (based on **14**) as a fluffy product. Charring with 20% conc sulfuric acid in methanol of the base-line material, after tlc-analysis of **17** (solvent G), was indicative for the presence of the sugar residue in the coupling product. The preparation of glycopentapeptide-BSA conjugate, which will be prepared in the same way as reported for the glycodipeptide, as well as the characterization of rabbit antibodies raised against both conjugates are presently under investigation.

EXPERIMENTAL

General methods and materials. All amino acids were of the L-configuration unless otherwise stated. δ-Hydroxy-DL-lysine monohydrochloride (normal and allo forms) **6**, α-chloroacetyl-ε - carbobenzoxy - δ - hydroxy - DL -lysine (normal and allo forms),[7b] glycine t-butyl ester (**2**),[12] 1 - 0 - (N - methyl) - acetimidyl - 2,3,4,6 - tetra - 0 - benzyl - β - D - glucopyranose (**6**)[25] and 2 - 0 - levulinyl - 3,4,6 - tri - 0 - acetyl - α - D - galactopyranosyl bromide (**4**)[13] were prepared in accordance with published procedures.

M.ps were determined with the apparatus designed by Tottoli and are uncorrected. The optical rotations (room temp.) were measured photoelectrically with a Perkin-Elmer instrument model P-141. Tlc were run on silicagel GF-254 (Merck) or on Schleicher & Schüll DC Fertig folien F 1500 LS 254, paper chromatograms on Whatman No. 4 or 1 filter paper (descending technique) for 72 hr in the following solvents: (A) $CHCl_3$–MeOH (9:1; v/v); (B) $CHCl_3$–MeOH – AcOH (85:10:5; v/v); (C) $CHCl_3$–n-Me_2CO(7:3); v/v); (D) BuOH-AcOH-water (4:1:1; v/v); (E) $CHCl_3$–Me_2CO (3:1; v/v); (F) EtOAc–hexane (1:1; v/v); (G) n-BuOH–AcOH–pyridine–water (15:3:10:12; v/v). Paper electrophoresis was carried out with a refrigerated Pherograph, type Mini 65 on MN paper No. 214 at 1500 V and pH 1.7 (AcOH–formic acid–water, 3:7:16; v/v) and pH 4.8 (AcOH–pyridine–water, 3:4.1:993). On chromatography and electrophoresis (50–150 μg amounts were spotted) the compounds were detected by UV-light (254 nm, UV) and/or by spraying with the following reagents: ninhydrin (N), Reindel-Hoppe[26] after chlorination (RH) and 15% conc H_2SO_4 in MeOH, charring at 120° (H_2SO_4). Paper chromatographic mobilities are given relative to δ-hydroxy-lysine; electrophoretic mobilities relative to phenylalanine (at pH 1.6–1.8), lysine (pH 4.8) and δ-hydroxy-lysine (pH 6.5). UV absorption spectra were measured with a Cary C 14 recording spectrophotometer. Column chromatography was performed on Kieselgel H or Kieselgel 60 (230–400 mesh) from Merck. Ion-exchange chromatography was performed with SP-Sephadex C-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) using an LKB 1130 Ultrograd Gradient Mixer and an LKB 2089 Uvicord III. Gel filtration chromatography was performed with Sephadex G-25. Hydrogenolyses were carried out with Pd 10% on C at atmospheric pressure and room temp. unless otherwise stated.

^1H NMR spectra were measured at 100 MHz with a JEOL JNMPS 100 spectrometer, the shifts are given in ppm (δ) relative to TMS as internal reference. ^{13}C NMR spectra were measured with a JEOL JNMPS 100 spectrometer, equipped with an EC-100 computer, operating in the Fourier transform mode. High-performance liquid chromatogrraphy (hplc) of the compounds **7c** (R^1 = H, HCOOH) and **9b** (Fig. 4) were carried out on a Micromeritics 7000 B liquid chromatography equipped with an UV absorption detector (215 nm). Hplc-analysis was performed on a column (12.5 × 0.46 cm) packed with Nucleosil 5 C_{18}. Elution was

effected with 0.01 M phosphate-buffer, pH 6, at a flow of 2 ml per min. The enzyme acylase I was purchased from Serva, 1-hydroxy-benzotriazole (HOBt) from Aldrich, N-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) from Pharmacia Fine Chemicals (Uppsala, Sweden), N-ethylmorpholine (NEM) from Fluka, bovine serum albumin (BSA) from Sigma and mercuric cyanide from Baker. Evaporations were carried out under reduced pressure (15 mm or 0.5 Hg) at bath temp below 40°.

Dicarbobenzoxy-δ-hydroxy-L-lysine lactone, 1 (*normal-from*)

(a) *Resolution procedure of α-chloroacetyl - ε - carbobenzoxy-δ - hydroxy - DL - lysine.* Treatment of α - chloroacetyl - ε - carbobenzoxy - δ - hydroxy - DL - lysine (normal and allo forms; 53.1 g, 0.15 mol) with the enzyme acylase I (2.35 g) was performed analogously to the lit procedure.[7] When a further addition of the enzyme appeared to produce no more hydrolysis of the substrate (tlc, solvent D; UV, N, RH), the pH of the solution was adjusted to 5 with AcOH and the mixture evaporated *in vacuo* to about 150 ml. After centrifugation of the so obtained mixture the remaining soln was acidified to pH 3 and extracted with EtOAc (3 × 100 ml).

The pH of the aqueous layer was adjusted to 7.6 with 2 N LiOH and adjusted to pH 5 with AcOH and concentrated to dryness. The residue was recrystallized twice from a minimum amount of water to afford ε-carbobenzoxy-δ-hydroxy-L-lysine (normal and allo forms) as a crystalline product. Yield 4.4 g (20%), homogeneous on tlc (solvent D; UV, N, RH), $[\alpha]_D$ + 11.4° (*c* 1 6N HCL).

(b) *Synthesis of dicarbobenzoxy-δ-hydroxy-L-lysine lactone (normal and allo forms).* To a stirred and cooled (ice-water bath) soln of ε-carbobenzoxy-δ-hydroxy-L-lysine (normal and allo forms; 4.3 g, 14.5 mmol) in water (15 ml) was added dropwise carbobenzoxychloride (18 mmol, 2.7 ml), the pH being maintained at 9.8 with 1 N NaOH by means of a pH-stat. One hour after addition, tlc (solvent D; UV, N, RH) showed complete conversion of starting material into 1 (normal and allo forms). The solution was acidified to pH 2.5 with N HCl and extracted with EtOAc (3 × 100 ml). The EtOAc soln was washed with half-sat NaClaq (100 ml), dried (Na_2SO_4) and evaporated. Toluene (100 ml) was added to the remaining oil and the mixture was heated under reflux for 3 hr, the liberated water being removed azeotropically using a Dean and Stark distilling apparatus. The soln was evaporated to dryness. After the addition of hexane, the remaining oil crystallized to give 5.2 g (87%) crystalline 1 (diastereomeric mixture; *ca* 71% normal form, based on the $[\alpha]_D$-values obtained after the separation procedure (see c.); $[\alpha]_D$ of the mixture + 6.3° (*c* 1 $CHCl_3$); R_f normal-isomer 0.65 and R_f allo-isomer 0.80 (solvent A; UV, RH).

(c) *Separation of the diastereomers of* 1. The diastereomeric mixture of 1 (1.2 g, 2.9 mmol) was dissolved in $CHCl_3$–Me_2CO (92:8; v/v, 7 ml) and applied to a column of Kieselgel 60 (400–230 mesh, 120 g) suspended in the same solvent. Elution of the column with this solvent under the conditions described by Still *et al.*[8] and evaporation of the appropriate fractions (tlc, solvent A; UV, RH) afforded pure allo-isomer (140 mg, 12%) m.p. 145–146°, $[\alpha]_D$ + 67.2° (*c* 1 $CHCl_3$), pure normal-isomer (470 mg, 39%) m.p. 145–147°, $[\alpha]_D$ − 18.3° (*c* 1 $CHCl_3$) and a mixture of both isomers (280 mg, 24%). The products were homogeneous on tlc (solvent A, B, C and D; UV, RH).

Separation of the diastereomers of DL-lactone 1 was performed by exactly the same chromatographic procedure as described above for the optical pure diastereomers of 1.

Dicarbobenzoxy-δ-hydroxy-L-lysylglycine t-butyl ester (3)

A soln of 1 (normal form 1.4 g, 3.4 mmol) and 2 (524 mg, 4.0 mmol) in dry dioxan (7 ml) was allowed to stand at 70°. After 20 hr, when tlc (solvent A) indicated that no lactone remained, the mixture was concentrated to an oil. The latter was dissolved in EtOAc (100 ml) and successively washed with $KHSO_4/K_2SO_4$ (pH 2) soln (25 ml), half-sat NaClaq (25 ml), 2.2 M K_2CO_3 (25 ml) and half-satd NaClaq (25 ml).

The dried (Na_2SO_4) organic layer was concentrated to a glass, which was crystallized from disopropyl ether to afford 3 in 72% yield. A sample (200 mg) was recrystallized from EtOAc-hexane (yield 75%); m.p. 98–99°, homogeneous on tlc (solvent A, B, C and D; RH), $[\alpha]_D$ − 9.8°(*c* 1 $CHCl_3$). ^{13}C NMR ($CDCl_3$, TMS as internal reference): δ 172.39 (–NHC=O), 168.94 (O=C–OBu^t), 157.17 and 156.50 (2 × –C=O, carbobenzoxy), 128.41 and 127.95 (aromatic C atoms), 27.97 (t-Butyl C atoms). (Found: C, 61,48, H, 6.74; O. 23,4; N, 7.78. Calc. for. $C_{28}H_{37}O_8N_3$: C, 61.86; H, 6.86: O, 23,54; N, 7.73%).

Dicarbobenzy - δ - hydroxy - L - lysyl - 0 - t - butylglycyl - 0 - 3,4,6 - tri - 0 - acetyl - β - D - galactopyranoside (5b)

To a stirred soln of 3 (1.3 g, 2.4 mmol) and 4(2,2 g, 4.8 mmol) in dry toluene (30 ml) mercuric cyanide (1.36 g, 5.4 mmol) was added. After 2 hr at 40°, when tlc (solvent E; UV, RH) indicated that no dipeptide was left, the mixture was filtered off and evaporated to dryness. The residue was diluted with $CHCl_3$ (100 ml) and the soln was washed with 1 M KBr (3 × 50 ml) and water (50 ml). The combined organic layers dried ($MgsO_4$) and concentrated to a glass. This material, dissolved in $CHCl_3$–Me_2CO (82:18; v/v, 4 ml) was applied to a column of Kieselgel 60 (60 g) suspended in the same solvent. Elution of the column with the same solvent afforded 5a as a glass (1.09 g) which was contaminated with 2-0-levulinyl-3,4,6-tri-0-acetyl-α/β-D-galactopyranose (*ca* 30%). This mixture was treated with Ac_2O (3 ml) and pyridine (6 ml). After 2 hr at 20°, when tlc (solvent E; H_2SO_4) showed the acetylation of 2 - 0 - levulinyl - 3,4,6 - tri - 0 - acetyl - α/β - D - galactopyranose to be complete, the mixture was concentrated to an oil and coevaporated with toluene to remove the last traces of pyridine.

The oil thus obtained was dissolved in $CHCl_3$ (100 ml) and washed with 10% $NaHCO_3$aq (25 ml) and water (25 ml). The dried ($MgSO_4$) organic layer was concentrated to a small volume and applied to a column of Kieselgel 60 (25 g) suspended in $CHCl_3$–Me_2CO (84:16, v/v). Elution of the column with the same solvent and evaporation of the appropriate fractions gave 5a as a glass (670 mg, 31% based on 3), homogeneous on tlc (solvent E; RH, H_2SO_4); ^1H NMR ($CDCl_3$): δ 7.33 (aromatic protons), 5.09 (–CH_2–fenyl), 4.49 (H–1, d, J 7.5 Hz), 2.7–2.4 (2 CH_2, levulinyl 2,16–1,95 (4 CH_3, s), 1.46 (t-Bu protons, s).

Removal of the levulinyl group of 5a

A soln of hydrazine-hydrate (0.35 g, 7 mmol) in pyridine-AcOH (3:2; v/v, 7 ml) was added to a soln of 5a (670 mg, 0.72 mmol) in pyridine (7 ml). After 10 min at 20°, pentane-2,4-dione (1.4 ml) was added to the mixture and the flask was immersed into an ice-water bath. After a further 2 min, the soln was diluted with toluene and coevaporated with the same solvent to remove the last traces of pyridine. The resulting residue was dissolved in EtOAc (100 ml) and washed successively with $KHSO_4/K_2SO_4$ (pH2) soln (25 ml), half-sat. NaClaq (25 ml), 10% $NaHCO_3$ aq (25 ml) and half-sat. NaClaq (25 ml). The dried ($MgSO_4$) organic layer was concentrated to an oil. The latter was dissolved in $CHCl_3$–Me_2CO (4:1; v/v, 4 ml) and applied to a column of Kieselgel 60 (30 g) suspended in the same solvent. Elution of the column with the same solvent gave 5b as a glass (380 mg, 64%), homogeneous on tlc (solvent E; RH, H_2SO_4); ^1H NMR ($CDCl_3$): δ 7.32 (aromatic protons), 5.06 (CH_2-fenyl, s), 4.37 (H–1, d, J 7.5 Hz), 2.06 and 1.98 (3 × CH_3, s), 1.44 (t-Butyl protons, s); ^{13}C NMR ($CDCl_3$, TMS as internal ref): δ 172.26 (–NHC=O), 170.27 and 170.12 (–C=O, acetyl), 168.84 (O=C–O–Bu^t), 156.71 and 156.41 (2 × –C=O, carbobenzoxy), 128.47 and 128.10 (aromatic C atoms), 103.75 (C–1, β-configurations), 27.97 (t-Bu C atoms).

Dicarbobenzoxy - δ - hydroxy - L - lysyl - 0 - t - butylglycyl - 0 - [2- 0 - (2,3,4,6 - tetra - 0 - benzyl) α - D - glucopyranosyl] - 3,4,6-tri - 0 - acetyl - β - D - galactopyranoside (7)

A soln of 6 (490 mg, 0.82 mmol) and 5b (374 mg, 0.45 mmol) in dry nitromethane (7.5 ml) was vigorously stirred in the presence of powdered 4 Å molecular sieves under an atmosphere of dry N_2. After 3 hr at 20°, a soln of anhydrous *p*-toluene-sulfonic acid (86 mg, 0.5 mmol) was added under an atmosphere of dry N_2. After 18 hr, tlc-analysis (solvent F; UV, RH, H_2SO_4) of the crude mixture showed *ca* 50% conversion of starting material 5b into 7. After addition of Et_3N (1 ml), the mixture was filtered off and evaporated to an oil. This material was diluted with $CHCl_3$

(100 ml) and the soln was washed with 10% $NaHCO_3$aq (50 ml) and water (50 ml). The dried ($MgSO_4$) organic layer, was concentrated to a glass which was kept *in vacuo* (P_2O_5) for 24 hr.

Without further purification, the above glass was treated for a second time with **6** (490 mg, 0.82 mmol) and anhydrous *p*-toluenesulphonic acid (86 mg, 0.5 mmol) in nitromethane (7.5 ml). After 18 hr at 20°, tlc-analysis (solvent F; UV, RH, H_2SO_4) of the crude mixture showed *ca* 80% conversion of starting material **5a** into **7**. After addition of Et_3N (1 ml) and work-up, in the same way as described above, a soln of the glass in EtOAc–hexane (1:1; v/v, 4 ml) was chromatographed on the column of Kieselgel H (60 g) suspended in the same solvent.

Elution of the column with the same solvent and pooling of the appropriate fractions afforded the fully-protected **7** as a glass (350 mg, 58% based on **5b**); homogeneous on tlc (solvent A, C, D and F: UV, RH, H_2SO_4): ^{1}H NMR ($CDCl_3$): δ7.28 (aromatic protons), 5.06, 4.74 and 4.49 (-CH_2-fenyl), 1.92 (3 × CH_3, acetyl), 1.44 (t-Bu protons, s); ^{13}C NMR ($CDCl_3$, TMS as internal ref): δ 171.51 (-NH-C=O), 170.18, 170.05 and 169.69 (3 × -C=O, acetyl), 168.51 (O=C-O-But^t), 157.10 and 156.26 (2 × -C=O, carbobenzoxy), 138.42, 138.27, 138.05, 137.31, and 136.20 (6 × CH_2-fenyl), 128.38–127.38 (aromatic C atoms), 101.65 (C-1 β-configuration), 95.83 (C-1′, α-configuration), 28.00 (-t-Bu C atoms), 20.48 (3 × CH_3 acetyl)

Deblocking of the fully-protected glycodipeptide **7**

(a) *Removal of the acid-labile t-Bu group.* The fully protected **7** (338 mg, 0.25 mmol) was partially deblocked by exposure to 5 ml TFA for 30 min. After dilution with toulene, the mixture was concentrated to an oil and coevaporated with the same solvent, to remove the last traces of TFA, to afford **7a** as an oil, homogeneous on tlc (solvent A, D: UV, RH, H_2SO_4).

(b) *Removal of the base-labile acetyl groups.* To a stirred soln of 7a in MeOH (2.7 ml) Et_3N (0.54 ml) and water (2.7 ml) was added. The soln was allowed to stand at 0–5°. After 18 hr tlc (solvent B; UV, RH, H_2SO_4) showed the deacetylation to be complete and after addition of 10% $NaHCO_3$ aq (5 ml) the MeOH and Et_3N were evaporated *in vacuo*. The resulting soln was diluted with water (15 ml) and extracted with ether (3 × 15 ml). After evaporation of last traces of ether and addition of EtOAc (20 ml), the pH of the mixture was adjusted to 2.5 with N HCl. The aqueous phase was extracted with EtOAc (3 × 20 ml), and after washing with half-sat NaClaq the combined dried (Na_2SO_4) organic layers were concentrated to give **7b** as an oil, homogeneous on tlc (solvent B, D; UV, RH, H_2SO_4).

(c) *Removal of the carbobenzoxy and benzyl groups.* Removal of the carbobenzoxy-groups was accomplished by catalytic hydrogenolysis of **7b** (0.112 mmol) in 20 ml DMF containing Pd/C (10%, 100 mg) in the presence of 0.1 N HCl (2.24 ml, pH 1.8). After 3 hr at 20°, tlc (solvent G; N, H_2SO_4) showed complete removal of the carbobenzoxy-groups (pH = 5). After filtration of the mixture and addition of water (4 ml) and AcOH (1 ml), the removal of the benzyl groups was accomplished in 4 hr at 20° by catalytic hydrogenolysis (Pd/C; 10%, 100 mg) using a Parr-apparatus (2 atm.; tlc, solvent G; N, H_2SO_4). The filtered solution was lyophilized affording the fully-deprotected glycodipeptide **7c** as a white powder (82 mg). This material was dissolved in 0.04 M ammonium formate pH 3.8 and applied to a column (1.1 × 25 cm) of SP-Sephadex C-25 (NH_4^+). The column was eluted with an ammonium formate gradient (0.04 M NH_4 formate to 0.15 M NH_4 formate pH 4.5). Fractions of 3 ml were collected.

Those containing the desired glycodipeptide (UV.-absorption detection, 206 nm) were pooled and repeatedly lypohilized to yield 66 mg (85% based on **7**) of **7c** (R^1 = H, HCOOH; normal, L-form), homogeneous on electrophoresis: $E^{4.8}_{Lys}$ 0.56, $E^{1.6}_{Phe}$ 1.28 (UV, N), on paper chromatography: R_f Hyl 0.78 (solvent G; UV, N), R_f Hyl 0.29 (solvent D; UV, N) and on tlc: R_f 0.08 (solvent G; UV, N, H_2SO_4), $[\alpha]_D$ + 53.3° (*c* 0.8 H_2O). ^{13}C NMR (D_2O, dioxan as external ref): 101.47 (C-1 β-configuration), 97.47 (C-1′, α-configuration). Amino acid ratios: HYL 1.03; Gly 0.97.

In exactly the same way as described above for the synthesis of **7** and **7c** (normal; L-form), the compounds **7** and **7c** (normal, DL-form) and the compounds **7** and **7c** (allo, DL-form) were prepared starting from a racemic mixture of **1** (normal-form) and **1** (allo-form), respectively. ^{13}C NMR (H_2O, dioxan as external ref) compound **7** (normal, DL-form): δ 102.35 and 101.71 (C-1, β-configuration), 98.89 and 95.86 (C-1′, α-configuration); compound **7c** (normal, DL-form): δ 101.44 (C-1, β-configuration), 98.98 and 97.44 (C-1′, α-configuration); compound **7** (allo, DL-form): δ 102.08 (C-1, β-configuration), 98.95 and 96.22 (C-1′, α-configuration); compound **7c** (allo, DL-form): 101.47 (C-1, β-configuration), 98.95 and 97.44 (C-1′, α-configuration).

0 - (2 - 0 - α - D - glucopyranosyl) - β - D - galactopyranoside of δ-hydroxy-lysygly-cyl-glutamylaspartylglycine (**9b**)

(a) The HCl-salt of the tripeptide Glu (OBu^t)–Asp (OBu^t)–Gly–OBu^t(**8**) was prepared in accordance with published procedures[18] and crystallized from methanol-ether. m.p. 171–172°, $[\alpha]_D$-9.4 (*c* 1 MeOH), homogeneous on tlc (solvent A, B and D; N, RH) and electrophoresis: $E^{4.8}_{Lys}$ 0.59 and $E^{1.8}_{Phe}$ 0.72.

(b) To a stirred mixture of **7b** containing δ-hydroxy-L-lysine (normal-form; 117 mg, 0.1 mmol), the HCl-salt of **8** (60 mg, 0.115 mmol) and HOBt (14 mg, 0.11 mmol) in THF (2 ml) and DMF (0.5 ml) cooled to −20°, was added a soln of 0.2 M NEM in THF (0.525 ml) and DCC (21 mg, 0.102 mmol). After 2 hr at 0 − 5° and 18 hr at room temp, the precipitated dicylohexylurea and NEM–HCl salts were filtered off and the THF was evaporated. The resulting soln was diluted with EtOAc (20 ml) and succesively washed with $KHSO_4/K_2SO_4$ (pH 2) soln (20 ml), half-satd NaClaq (20 ml), 2.2 M $KHCO_3$aq (20 ml) and half-satd NaClaq (20 ml). After drying (Na_2SO_4) and evaporation of the organic layer, the oily residue was treated with diisopropyl ether-hexane to afford a white precipitate of **9**. Yield 155 mg (94%); $[\alpha]_D$ + 11.0 (*c* 0.5 MeOH), homogeneous on tlc (solvent A, B, D and G; UV, RH, H_2SO_4).

Deblocking procedure

The procedure used for deblocking of **9** was the same as described above for the deblocking of **7b** and afforded 60 mg (70%, based on **9**) of the crude deblocked glycopentapeptide **9b** (R^1 = H, HCl). The compound was dissolved in 0.04 M ammonium formate pH 3.1 and applied to a column (1.1 × 25 cm) of SP-Sephadex C-25 (NH_4^+). The column was eluted with an ammonium formate gradient (0.04 M NH_4 formate to 0.14 MF.pH 3.1). Fractions of 3 ml were collected. Those containing the desired glycopentapeptide (UV absorption detection, 206 nm) were pooled and repeatedly lyophilized to yield 35 mg (35% based on **9**) of **9b** (R^1 = H, HCOOH). Homogeneous on electrophoresis $E^{4.8}_{Lys}$ − 0.2 g_1 $E^{1.7}_{Phe}$ 0,98 (U.V, N) and on paper chromatography. (Whatman No. 1): R_f Hyl 0.33 (solvent D; UV, N) and R_f Hyl 0.57 (solvent G; UV, N); $[\alpha]_D$ + 27.6° (*c*1 H_2O); ^{13}C NMR (D_2O, dioxan as external ref): δ101.68 (*C*-1, β-configuration), 97.65 (C-1′, α-configuration). Amino acid ratios: Hyl 1.09; Asp 0.99 · Gly 0.99; Gly 1.93.

Preparation of glycodipeptide **7c** *(normal, L-form)-BSA conjugate* (**17**)

(a) *Introduction of 2-pyridyl disulphide structures into BSA* (**10**) *by SPDP* (**11**). To a stirred soln of BSA (**10**; 50 mg, 7.5 × 10^{-4} mmol) in 0.1 M NEM HOAc pH 7.9 (1.2 ml) was added in portions (6 × 0.1 ml and 1 × 0.05 ml) a soln of SPDP (**11**; 18.7 mg, 6 × 10^{-2} mmol) in THF (0.65 ml) during 35 min at 20°. After another hr, tlc (solvent A; UV, N) showed the conversion of **10** into **12** to be complete. The pH of the soln was adjusted to 7.5 with 2 N-AcOH. After evaporation of the THF, the soln was successively diluted with water (0.3 ml), extracted with EtOAc (3 × 2 ml) and after evaporation of the last traces of EtOAc, diluted with water (0.25 ml). To estimate the content of 2-pyridyl disulphide structures in the BSA-2-pyridyl disulphide derivative **12**, 25 μl of the so obtained soln in 0.1 M NEM HOAc pH 7.5 (2.5 ml) was treated with 50 μl of a 0.1 M DTT soln (0.1 M NEM HOAc pH 7.5). The amount of pyridine-2-thione (**15**) released is equivalent to the content of 2-pyridyl disulphide groups in BSA and the concentration of **15** was determined by measuring the absorbance of **15** at 343 nm (molar extinction coefficient of **15** at 343 nm: 8.08 × 10^3 M^{-1} − cm^{-1}).[23] Yield: *ca* 40 mol 2-pyridyl disulphide groups/mol BSA (71% based on the total amount of free amino groups in BSA).

(b) *Reduction of 2-pyridyl disulphide groups of BSA derivative* **12** *affording* **14**. To the above obtained soln of BSA derivative **12** (part a.; 7.5×10^{-4} mmol, 1.75 ml) was added a soln of DTT (22.5 mg, 0.07 mmol) in 0.2 M NEM HOAc pH 7.5 (1.25 ml). After 1 hr the thiol groups containing BSA derivative **14** was separated from the low-molecular-weight material by Gel filtration over Sephadex G-25 in 0.1 M NEM HOAc pH 7.5 containing 0.1 M NaCl. Fractions containing the desired product **14** were pooled and concentrated by pressure filtration (AMICON, TYPE XM 50) to yield 23.4 mg (45% based on BSA **10**) of **14** in 0.1 M NEM HOAc pH 7.5 containg 0.1 M NaCl (3 ml).

(c) *Introduction of the 2-pyridyl disulphide structure in glycodipeptide* **7c** *(R^1 = H, HCOOH) with SPDP* (**11**). To a stirred soln of **7c** (R^1 = H, HCOOH; 15.9 mg, 2.5×10^{-2} mmol) in 0.1 M NEM HOAc pH 4.7 (0.25 ml) was added a soln of SPDP (**11**; 8.6 mg, 2.75×10^{-2} mmol) in THF (0.2 ml). After 18 hr at 0–5°, tlc (solvent G; UV, N, H_2SO_4) showed *ca* 40% conversion of starting **7c** into glycodipeptide derivative **16**. After evaporation of the THF and adjustment of the pH to 7.5 with 0.1 M-aq NEM, the soln was diluted with water (0.25 ml) and extracted with EtOAc (3 × 1 ml). The aqueous phase (0.5 ml) containing *ca* 10^{-2} mmol of **16** and *ca* 1.5 10^{-2} mmol not thiolated **7c** (and a trace, <5%, of dithiolated **7c**) was used without further purification for the synthesis of the conjugate **17**.

(d) *Reaction of thiol groups containing BSA* **14** *with glycodipeptide-2-pyridyl disulphide* **16** *affording* **17**. A soln of **14** (11.5 mg, 1.6×10^{-4} mmol; *ca* 40 mol thiol groups per mol BSA) in 0.1 M NEM HOAc pH 7.5 containing 0.1 M NaCl (1.5 ml) was mixed with a soln of **16** (10^{-2} mmol; 0.5 ml pH7.5) obtained previously (see part c.). After standing for three days, spectrophotometrically determination of pyridine-2-thione (**15**, see part a.), which was released during the reaction, indicated complete conversion of the thiol groups of BSA derivative **14**(0.64×10^{-2} mmol) into glycodipeptide-BSA conjugate **17**. After Gel filtration over Sephadex G-25 in 0.1 M NEM HOAc pH 7.5, containing 0.1 M NaCl, the appropriate fractions were successively pooled, dialyzed against distilled water and lyophilized to afford 11.7 mg (78% based on **14**) of **17** as a fluffy material.

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