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PREPARATION OF A WELL-DEFINED SUGAR-PEPTIDE CONJUGATE: A POSSIBLE APPROACH TO A SYNTHETIC VACCINE AGAINST *NEISSERIA MENINGITIDIS*

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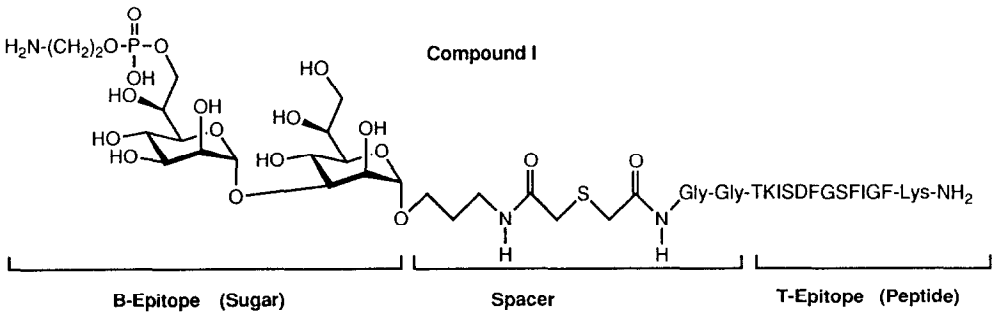
ABSTRACT: A phosphorylated disaccharide from the inner core region of *Neisseria meningitidis* immunotype lipopolysaccharide **6** could be conjugated via an artificial spacer to an elongated peptide sequence of a meningococcal outer membrane protein.

The structure of several lipopolysaccharide (LPS) immunotypes of the Gram-negative bacterium *Neisseria meningitidis*, the infection of which may *inter alia* lead to severe brain damage in humans, has been elucidated[1]. It was further illustrated that the inner core (IC) region of meningococcal LPS showed microheterogenicity. In addition, immunological studies[2] indicated that the determinants responsible for LPS immunotype specificity and cross reactivity resided largely in the IC regions and that the presence of ethanolamine phosphate substituents in native LPS contributes significantly to the antigenic specificity[3].

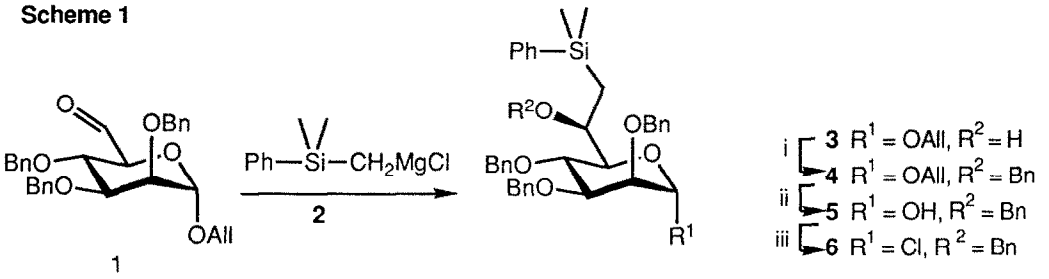
As part of a program to develop a broadly protective synthetic vaccine against *N. meningitidis*, we here report the preparation of the sugar-peptide conjugate **I** in which two essential immunological domains (*i.e.* the B- and T-epitopes responsible for antibody specificity and T-helper activity, respectively) are covalently



anchored by an artificial spacer. On the basis of its expected strong immunogenic response[4], we selected a fragment of the IC region of the LPS immunotype 6 to function as the B-epitope. Further, in order to elicit a homologous T-helper response (*e.g.* memory), we choose a T-cell epitope-containing peptide (sequence 47-59) of a meningococcal outer membrane protein[5] as the T-epitope.

The assembly of the sugar B-epitope **18** (Scheme 3), suitable for the selective conjugation with the functionalised peptide T-epitope **21** (see Scheme 3), commences with the synthesis of the properly protected L-*glycero*-D-*manno*-heptopyranose (LD-Hep*p*) donor **6**, the design of which is based[6] on the reaction of (phenyldimethylsilyl)methyl magnesium chloride (**2**) with the easily accessible[7] allyl 2,3,4-tri-*O*-benzyl-α-D-*manno*-hexodialdo-1,5-pyranoside (**1**). Thus, addition (Scheme 1) of **2** in 1,3-dioxolane to **1** in

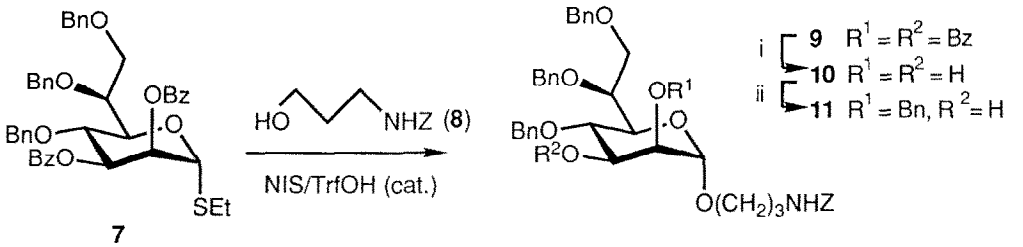
Scheme 1



Reagents and conditions: (i) BnBr/ NaH/ nBu_4NI/ DMF, (ii) a) $Ir(COD)(PMePh_2)_2$.] PF_6, b) HgO/ $HgCl_2$/ acetone/ water, (iii) chlorodimethylformamidinium chloride/acetonitrile/CH_2Cl_2

the same solvent proceeded with high diastereofacial selectivity to afford the α-hydroxysilane adduct **3**[8] (d.p.≥ 95%) in 77% yield. Benzylation[9] of **3**(→**4**) and two-step deallylation[10] gave homogeneous **5**[8] in 68% overall yield. Treatment of **5** with the Vilsmeier reagent[11] furnished the glycosyl chloride donor **6** which will be coupled in a later stage (see Scheme 3) with the spacer containing LD-Hep*p* acceptor **11**, the synthesis of which is outlined in Scheme 2. Glycosylation of 3-(benzyloxycarbonylamino)-1-propanol[12] (**8**) by the ethyl

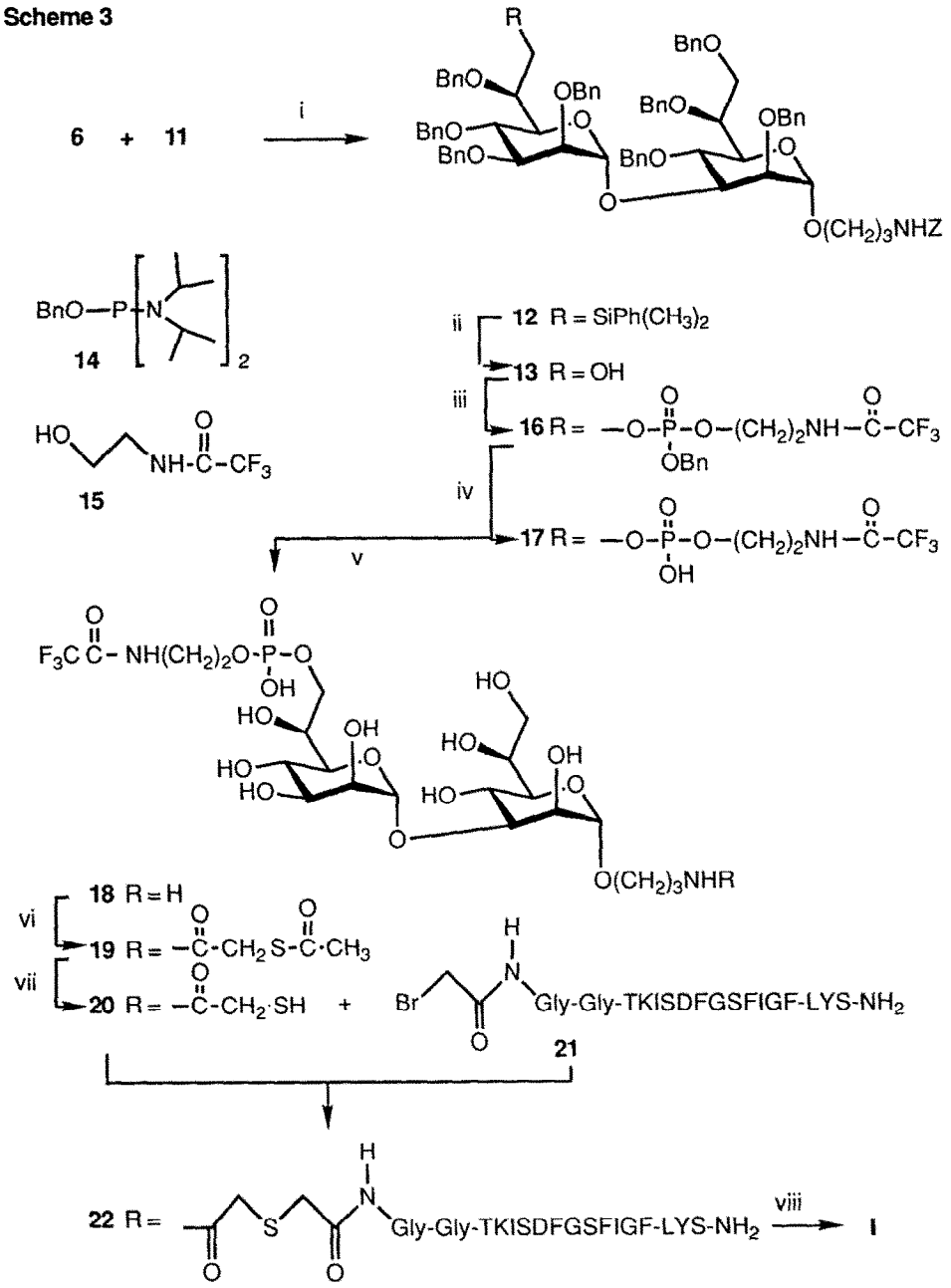
Scheme 2



Reagents and conditions. (i) NaOMe / MeOH (ii) NaOH / H_2O, CH_2Cl_2, n-$(Bu)_4NI$, BnBr.

1-thio-α-LD-Hep*p* derivative **7**, which was prepared in eight steps[13] from 2,3,4,6,7-penta-O-acetyl-L-*glycero*-D-*manno*-heptopyranosyl bromide[14], in the presence of N-iodosuccinimide (NIS) and catalytic trifluoromethane sulfonic acid (TfOH)[15] gave the α-linked derivative **9**[8] in 80% yield. Zemplén deacylation of **9**(→**10**) and subsequent regioselective benzylation under phase-transfer conditions[16] gave **11**[8] in 85% overall yield. Stereospecific 1,2-trans-glycosylation of acceptor **11** by excess donor **6** could be accomplished (see Scheme 3) using the promoter silver triflate[17] to give, after work up and purification by silica gel chromatography, homogeneous disaccharide **12**[8] in 92% yield (based on **6**). In this respect it is of interest to note that the dimethyl(phenyl)silyl masking group in donor **11** was compatible with the glycosidation conditions.

Scheme 3



Reagents and conditions: (i) AgOTrf, CH_2Cl_2 , 18 h. (ii) NaBr, HOOAc in AcOH / NaOAc, 1.5 h, (iii) a) **13** /**14** / 1-H-tetrazole; b) **15** /1-H- tetrazole; c) t-BuOOH (iv) 10 eq. NaI / acetone, 18 h, (v) Pd/C, H_2, MeOH. (vi) pentafluorophenyl S-acetylmercaptoacetate in 0.1 M phosphate buffer (pH 7.8), 1h. (vii) H_2NOH HCl in phosphate buffer (pH 7.2), 18 h. (viii) NH_4OH (25%), 1h

In order to introduce the 2-trifluoroacetamidoethyl phosphate function at C-7', the $PhMe_2Si$ group in **12** was unmasked[18] oxidatively in the dark to afford **13**[8] in 91% yield. Phosphorylation of the free hydroxyl group in **13** to give the fully protected phosphorylated disaccharide **16** could be accomplished by the following three-step one-pot procedure. Thus 1-*H*-tetrazole assisted phosphitylation of **13** with the bifunctional reagent **14**[19] gave an intermediate phosphoramidite, which was condensed *in situ* and under similar conditions with excess 2-trifluoroacetamido ethanol (**15**)[20]. The phosphite triester (δp 142.0 and 142.2 ppm) thus obtained was oxidized[21] immediately to afford the phosphotriester derivative **16** (δp-3.1 and -3.2 ppm) in 81% overall yield[22]. Partial deblocking of **16** to furnish **18** (overall yield 56%), the free amino function of which is amenable to further elaboration, could be realized in two distinct steps. Firstly, ionic debenzylation[23] of the phosphotriester function gave the charged derivative **17** (δp 0.42 ppm). Secondly, hydrogenolysis of the benzyloxycarbonyl (Z) and benzyl groups afforded **18**, the ^1H- and ^{13}C NMR data[8] of which were in accord with the proposed structure.

The last stage in the assembly of the target sugar-peptide conjugate **I** comprises a two-step conversion of **18** into the thiol derivative **20**, followed by its *in situ* condensation with the bromoacetyl-peptide **21**, and subsequent removal of the trifluoroacetyl group from **22**. Thus treatment of **18** with the reagent pentafluorophenyl S-acetylmercaptoacetate[24] gave, after purification (Sephadex G-25), homogeneous **19**[8]. To an oxygen-free and buffered solution containing **19** and the peptide derivative **21**, prepared via a solid phase approach[25], was added hydroxylamine. HPLC-analysis of the crude reaction mixture, after 48 h at 20°C, revealed *inter alia* the presence of two main products. The faster eluting product was purified by HPLC and, thereafter, subjected to FAB MS analysis[26]. The FAB mass spectrum showed a signal of the protonated molecular ion (MH^+) at m/z 2353.2, which is in good agreement with the proposed structure of the partially protected conjugate **22**. Ammonolysis of **22**, followed by purification (HPLC), gave a homogeneous product which showed, as evidenced by FAB mass spectroscopy, a protonated molecular ion at m/z 2257.0: thus confirming the complete removal of the trifluoroacetyl group and the identity of target molecule **I**.

In conclusion, the results presented in this paper illustrate for the first time the feasibility to prepare a complex and well-defined sugar-peptide conjugate (*e.g.* **I**), the immunological properties of which may be of great value for the future design and development of a broadly protective synthetic vaccine against *N. meningitidis*.

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7. Compound **1** was prepared in 63% overall yield by the regioselective tritylation (trityl chloride/pyridine) of allyl α-D-mannopyranoside (see: Chernyak *et al.*, *Carbohydr. Res.*, **1984**, *128*, 269) followed by benzylation (BnBr/NaH), detritylation (trifluoroacetic acid/methanol/dioxane) and subsequent Swern oxidation.
8. Satisfactory elemental analytical data were obtained for compounds **3**, **4**, **5**, **6**, **9**, **11**, **12**, **13** and **17**. FAB MS of **19** gave a signal of MH^+ at m/z 795.2 (calcd. monoisotopic mass 794.2).
   Relevant ^1H NMR data ($CDCl_3$) of compound: **3**; δ 4.85 (d, 1H, H-1, $J_{1,2}$ = 1.8 Hz), 1.41 (dd, 1H, H7a), 0.92 (dd, 1H, H-7b). **4**; δ 4.84 (d, 1H, H-1, $J_{1,2}$ = 1.7 Hz), 1.42 (m, 2H, H7a,b). **11**; δ 4.94 (d, 1H, H-1, $J_{1,2}$ = 1.0 Hz), 3.98 (dt, 1H, H-3, $J_{2,OH}$ = $J_{3,4}$ = 9.2 Hz, $J_{2,3}$ = 3.4 Hz). **12**; 5.35 (d, 1H, H-1', $J_{1,2}$ = 2.0 Hz), 4.83 (bs, 1H, H-1), 1.48 (dd, 1H, H-7a), 0.90 (dd, 1H, H-7b). **13**; δ 5.28 (H-1', $J_{1,2}$ = 1.6 Hz), 4.85 (d, 1H, $J_{1,2}$ = 1.5 Hz). **17**; δ 5.32 (d, 1H, H-1', $J_{1,2}$ = 1.7 Hz), 4.87 (d, 1H, H-1, $J_{1,2}$ = 1.6 Hz), 4.87 (d, 1H, H-1, $J_{1,2}$ = 1.6 Hz), 3.66 (dd, 2H, OCH_2, PEA), 3.36 (m, 2H, NCH_2, PEA). **18**; δ 5.14 (d, 1H, H-1', $J_{1,2}$ = 1.6 Hz), 4.81 (d, 1H, H-1, $J_{1,2}$ = 1.7 Hz). **19**; 8 δ 5.12 (d, 1H, H-1', $J_{1,2}$ = 1.3 Hz), 4.80 (bs, 1H, H-1), 2.41 (s, 3H, CH_3, SAc).
   Relevant ^{13}C NMR data ($CDCl_3$) of compound: **3**; δ 97.3 (C-1), 21.7 (C-7). **4**; δ 97.0 (C-1), 17.1 (C-7). **5**; δ 91.0 (C-1), 16.3 (C-7). **9**; δ 97.4 (C-1), 64.4 (OCH_2, spacer), 38.2 (NCH_2, spacer), 29.4 (CH_2, spacer). **11**; δ 96.8 (C-1). **12**; 99.2, 97.8 (C-1, C-1'), 21.0 (C-7'). **13**; δ 99.2, 97.8 (C-1, C-1'), 64.6, (C-7), 61.1 (C-7'). **17**; 157.6 (q, C=O, TFA, $J_{C,F}$ 38 Hz), 114.5 (q, CF_3, TFA, $J_{C,F}$ 89 Hz), 99.4, 97.8 (C-1, C-1'), 68.1 (d, C-6', $J_{C-6,P}$ 5.8 Hz), 64.0 (d ,C-7', $J_{C-7,P}$ 5.9 Hz), 63.3 (C-7), 41.1 (41.1 (d, NCH_2, PEA). **18**; δ 103.0, 100.4 (C-1, C-1').
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13. Thus the bromide was first converted (EtSH/KOtBu/MeOH) into the deacetylated ethyl α-thioglycoside followed by di-acetonation (acetone/2,2-dimethoxypropane/*p*TSOH), benzylation (BnBr/NaH), regioselective deacetonation ($AcOH/H_2O$; 20°C), benzylation (BnBr/NaH), deacetonation ($AcOH/H_2O$; 60°C) and benzoylation (BzCl/pyridine) to give **7** in 13% overall yield.
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25. The *N*-bromoacetyl-Gly-Gly-elongated peptide sequence 47-59 of strain H44/76 class 1 outer membrane protein (*i.e.*, **21**) was prepared by continuous-flow solid-phase synthesis on a dimethoxybenzhydrylamino-resin unsing N^{α}-fluorenylmethoxycarbonyl (Fmoc)amino acid derivatives, according to standard procedures [Atherton, E. and Sheppard, R.C., solid phase peptide synthesis: a practical approach, IRL Press, Oxford University Press, 1989]. The *N*-terminal bromoacetyl group was introduced during the last cycle of the synthesis [Robey, F.A. and Fields, R.L., *Anal. Biochem.* **1989**, *177*, 373]. After side-chain deprotection and cleavage from the solid support (TFA/H_2O, 95/5, 1.5 h, r.t.), the peptide was purified by semi-preparative reverse phase HPLC. FAB mass analysis gave a signal of the protonated molecular ion (MH^+) at m/z 1680.0 (calcd. monoisotopic mass 1679.7).
26. The FAB mass spectrum of the slower running product was identical with that of the bromoacetyl-peptide **21**.