Carbohydrate Research, 124 (1983) 313–318
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

Note

Preparation of glycoconjugates for use as artificial antigens: A simplified procedure‡

B. MARIO PINTO* AND DAVID R. BUNDLE
Division of Biological Sciences, National Research Council of Canada, Ottawa, Ontario K1A OR6 (Canada)

(Received June 16th, 1983; accepted for publication, August 6th, 1983)

The chemical synthesis of antigenic determinants related to O-antigens of the enterobacteriaceae is a major activity in this laboratory. The intention of this research is to furnish natural and modified structures to probe the molecular and stereochemical basis of antibody specificity[1]. These same structures permit a significant streamlining of the hybridoma technique, especially for generating precise, O-factor antibodies, which, owing to their uniformity, are ideal for the aforementioned specificity studies[1,2]. Recent publications[3–10] from this laboratory described the elaboration of a large number of carbohydrate haptens suitably derivatized to permit covalent attachment to proteins, cell surfaces, and immunoabsorbent supports. We report here the preparation of the corresponding glycoconjugates by a simplified, acyl azide methodology[11,12].

The strategy of the synthetic work is based upon the procedure of Lemieux and co-workers[11,12], namely, the synthesis of haptens as their 8-(methoxycarbonyl)octyl glycosides and their coupling, by way of the corresponding acyl azides, to suitable carrier molecules. According to this method, the esters are first converted into hydrazides, and these are treated with nitrous acid in order to generate the activated, acyl azide intermediate. The generation of nitrous acid is achieved *via* hydrolysis of *tert*-butyl nitrite in *N,N*-dimethylformamide (DMF) by addition of a molar solution of hydrochloric acid in 1,4-dioxane. When conversion into acyl azide is complete, the excess of nitrous acid is decomposed by addition of an appropriate amount of sulfamic acid. This procedure, therefore, requires accurate preparation of three solution, namely, 4M hydrochloric acid in anhydrous 1,4-dioxane, *tert*-butyl nitrite in DMF, and sulfamic acid in DMF. The method is essentially that of Honzl and Rudinger[13] as used by Inman and co-workers[14] for the coupling of tripeptide haptens to proteins and cell surfaces. As an alternative to nitrous acid, nitrosyl chloride may be used to convert hydrazides into acyl azides[13].

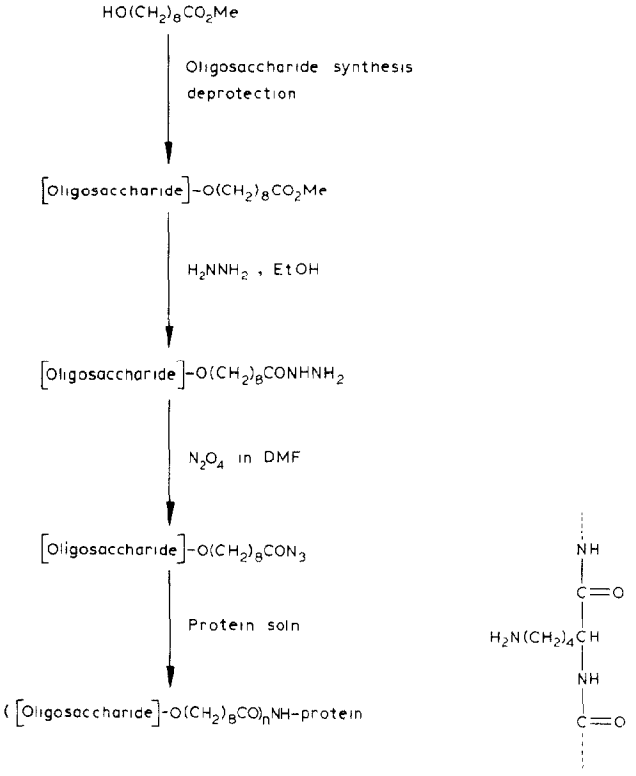
We have now developed a useful modification of the procedure in which di-

‡NRCC No. 22603.
*NRCC Research Associate, 1982–1983.

0008-6215/83/$ 03.00

nitrogen tetraoxide is used as the nitrosating agent[15]; the method is straightforward, and merely involves the direct addition of a solution of dinitrogen tetraoxide in dichloromethane to a solution of the hydrazide in DMF. The N_2O_4 solution, which can be stored at $-10°$ in a freezer, is prepared by transferring the gas or liquid (b.p. $+21.2°$) into dichloromethane at $-78°$ (acetone–Dry Ice). The reaction of N_2O_4 with hydrazide can be monitored by t.l.c. on silica gel, using 6:3:1 (v/v/v) ethyl acetate–methanol–water as the eluant, and additional reagent may be added if necessary. As the reaction progresses, t.l.c. indicates the disappearance of the starting hydrazide and the appearance of a less-polar component (presumably the acyl azide). The azide intermediate is then immediately caused to react with the carrier protein in buffer solution, in order to provide the glycoconjugate in similar fashion to the earlier procedure[11,13] (see Scheme 1).

The generality of the procedure is evident from the variety of glycoconjugates prepared in this way (see Table I). The azide-coupling procedure utilizes one of the most efficient and mild reactions for introducing carbohydrate haptens to proteins that contain lysine residues. A level of hapten incorporation of 15–60% was achieved, and was dependent on the molar ratio of hapten:protein used in the



Scheme 1. Synthesis of artificial antigen by the acyl azide coupling reaction

TABLE I

GLYCOCONJUGATES FOR USE AS ARTIFICIAL ANTIGENS

Antigen[b]	*Incorporation (%)*	*References*[a]
Shigella flexneri Y		
[β-D-GlcNAc-(1→2)-α-L-Rha-X]$_{34}$BSA′	57	3
[α-L-Rha-(1→3)-β-D-GlcNAc-X]$_{15}$BSA′	26	3
[α-L-Rha-(1→2)-α-L-Rha-X]$_{34}$BSA′	56	3
[α-L-Rha-(1→3)-α-L-Rha-X]$_{19}$BSA′	32	5
[α-L-Rha-(1→3)-β-D-GlcNAc-(1→2)-α-L-Rha-X]$_{25}$BSA′	41	4
[α-L-Rha-(1→3)-α-L-Rha-(1→3)-β-D-GlcNAc-X]$_{35}$BSA′	58	7
[β-D-GlcNAc-(1→2)-α-L-Rha-(1→2)-α-L-Rha-X]$_{30}$BSA′	50	6
[α-L-Rha-(1→2)-α-L-Rha-(1→3)-α-L-Rha-X]$_{22}$BSA′	36	5
[α-L-Rha-(1→2)-α-L-Rha-(1→3)-α-L-Rha-(1→3)-β-D-GlcNAc-X]$_{18}$BSA′	29	7
[β-D-GlcNAc-(1→2)-α-L-Rha(1→2)-α-L-Rha-(1→3)-α-L-Rha-X]$_{21}$BSA′	34	6
[α-L-Rha-(1→3)-α-L-Rha-(1→3)-β-D-GlcNAc-(1→2)-α-L-Rha-X]$_{17}$BSA′	29	1
Streptococcus A and A-variant		
[β-D-GlcNAc-(1→3)-α-L-Rha-X]$_{31}$BSA′	51	8
[β-D-GlcNAc-(1→3)-α-L-Rha-X]$_{48}$KLH′	40	8
[α-L-Rha-(1→2)-α-L-Rha-(1→3)-α-L-Rha-(1→2)-α-L-Rha-X]$_{24}$BSA′	41	8
[α-L-Rha-(1→2)-α-L-Rha-(1→3)-α-L-Rha-(1→2)-α-L-Rha-X]$_{33}$KLH′	27	8
Salmonella adelaide, E. coli 0111		
[α-Col-(1→6)-α-D-Glc-X]$_{18}$BSA′ 3 ↑ 1 α-Col	31	9
Salmonella serogroups A, B, D_1		
(α-Tyv-X)$_{20}$BSA′	34	10
[α-D-Gal-(1→2)-α-D-Man-X]$_{27}$BSA′	46	10
[α-D-Gal-(1→2)-α-D-Man-X]$_{10}$BSA′ 3 ↑ 1 α-Tyv	17	10
[α-D-Gal-(1→2)-α-D-Man-X]$_{10}$BSA′ 3 ↑ 1 α-Par	17	10
[α-D-Gal-(1→2)-α-D-Man-X]$_{10}$BSA′ 3 ↑ 1 α-Abe	17	16

Table I (continued)

[α-D-Gal-(1→2)-α-D-Man-(1→4)-α-L-Rha-X]$_{25}$BSA′ 3 ↑ 1 α-Abe	41	17
[α-D-Gal-(1→2)-α-D-Man-(1→4)-α-L-Rha-X]$_{31}$BSA′ 3 ↑ 1 α-Par	52	18
[α-D-Gal-(1→2)-α-D-Man-(1→4)-α-L-Rha-X]$_{30}$BSA′ 3 ↑ 1 α-Tyv	50	18
[α-D-Gal-(1→2)-α-D-Man-(1→4)-α-L-Rha-X]$_{23}$BSA′ 3 ↑ 1 α-Asc	38	18
[α-D-Gal-(1→2)-α-D-Man-(1→4)-α-L-Rha-X]$_{15}$BSA′ 3 ↑ 1 α-R	25	18
[α-D-Gal-(1→2)-α-D-Man-(1→4)-α-L-Rha-X]$_{27}$BSA′ 3 ↑ 1 α-R′	45	18
Other structures		
[α-β-GalNAc-(1→3)-α-L-Rha-X]$_{32}$BSA′	53	8
[α-D-Glc-(1→3)-α-L-Rha-X]$_9$BSA′	15	19
[β-D-Glc-(1→3)-β-D-Glc-X]$_{34}$BSA′ 6 ↑ 1 β-D-Glc	56	20
[α-D-Gal-(1→3)-α-D-Glc-X]$_{35}$ 6 ↑ 1 α-D-Gal	58	9

[a]For synthesis of the 8-(methoxycarbonyl)octyl glycoside. [b]Key: X = the bridging arm [-O(CH$_2$)$_8$CO-]; Tyv = 3,6-dideoxy-D-*arabino*-hexopyranose, tyvelose; Par = 3,6-dideoxy-D-*ribo*-hexopyranose, paratose; Abe = 3,6-dideoxy-D-*xylo*-hexopyranose, abequose; Asc = 3,6-dideoxy-L-*arabino*-hexopyranose, ascarylose; Col = 3,6-dideoxy-L-*xylo*-hexopyranose, colitose; R = 2,3,6-trideoxy-D-*threo*-hexopyranose; and R′ = 3,4,6-trideoxy-D-*erythro*-hexopyranose

reaction. With bovine serum albumin (BSA) as carrier, a hapten:protein ratio of 40:1 typically gave glycoconjugates possessing 25–30 haptens per molecule of protein. The two examples employing keyhole-limpet haemocyanin (KLH) as the carrier molecule required higher (3 times) hapten:lysine ratios in order to achieve a 10% lower incorporation than in the corresponding cases employing BSA as the carrier. A typical procedure is described in the Experimental section.

The present method, by virtue of its simplicity, is a useful modification of the established procedure[11,12], and should find general application in the coupling of haptens (including haptens having spacers other than the nine-carbon unit, $CO(CH_2)_8$, employed here) to soluble proteins, cell surfaces, and immunoabsorbent support-materials.

EXPERIMENTAL

General methods. — Crystalline bovine serum albumin (BSA) was obtained from Miles Laboratories, Inc. Keyhole-limpet haemocyanin (KLH) was obtained from Calbiochem. The buffer was 0.35M $KHCO_3$ in 0.08M $Na_2B_4O_7$ (pH 8.9–9.0). The N_2O_4 solution was prepared by condensing gaseous N_2O_4, and transferring the liquid to a given volume of anhydrous dichloromethane[15]. The molarity of this solution was verified gravimetrically and by titration.

Hydrazides. — The 8-(methoxycarbonyl)octyl glycoside (0.09 mmol) was dissolved in absolute ethanol (0.3 mL) and hydrazine hydrate (0.1 mL). After 18 h at 20°, the solution was evaporated to a syrup, which was co-evaporated twice with water (2 mL). T.l.c. on silica gel, using the solvent system 6:3:1 (v/v/v) ethyl acetate–methanol–water, indicated that all of the ester had been converted into a single, slower-moving component. The product was dissolved in distilled water (2 mL), the solution lyophilized, and the compound used directly in the coupling reaction without further manipulation.

Antigens. — A stirred solution of the hydrazide (29 μmol) in freshly distilled *N,N*-dimethylformamide (0.4 mL) was cooled to −40 to −50°, and a standardized, stock solution of dinitrogen tetraoxide in dichloromethane (81 μL; 37 μmol, 0.46M) was added by means of a microlitre syringe precooled in Dry Ice. The temperature was maintained for 15 min at −20 to −10° and the mixture was then added to a stirred solution of the carrier protein in buffer (0.75 μmol; 10 mg/mL) at 0°. After being kept overnight at 0°, the mixture was dialyzed against at least five changes of de-ionized water in an Amicon ultrafiltration cell equipped with a PM-10 membrane, and then lyophilized, to provide the glycoconjugate as a white powder. Incorporation levels were determined on the basis of carbohydrate content, determined as previously described[11].

REFERENCES

1 D. R. BUNDLE, M. A. J. GIDNEY, S. JOSEPHSON, AND H.-P. WESSEL, *Am. Chem. Soc. Symp. Ser.*, 231 (1983) 49–63.

2 D. R. BUNDLE, M. A. J. GIDNEY, N. KASSAM, AND A. F. R. RAHMAN, *J. Immunol.*, 129 (1982) 678–682.
3 D. R. BUNDLE AND S. JOSEPHSON, *Can. J. Chem.*, 57 (1979) 662–668.
4 D. R. BUNDLE AND S. JOSEPHSON, *J. Chem. Soc., Perkin Trans. 1*, (1979) 2736–2739.
5 S. JOSEPHSON AND D. R. BUNDLE, *J. Chem. Soc., Perkin Trans. 1*, (1980) 297–301.
6 S. JOSEPHSON AND D. R. BUNDLE, *Can. J. Chem.*, 57 (1979) 3073–3079.
7 D. R. BUNDLE AND S. JOSEPHSON, *Carbohydr. Res.*, 80 (1980) 75–85.
8 T. IVERSEN, S. JOSEPHSON, AND D. R. BUNDLE, *J. Chem. Soc., Perkin Trans. 1*, (1981) 2379–2385.
9 T. IVERSEN AND D. R. BUNDLE, *Can. J. Chem.*, 60 (1982) 299–303.
10 T. IVERSEN AND D. R. BUNDLE, *Carbohydr. Res.*, 103 (1982) 29–40.
11 R. U. LEMIEUX, D. R. BUNDLE, AND D. A. BAKER, *J. Am. Chem. Soc.*, 97 (1975) 4076–4083.
12 R. U. LEMIEUX, D. A. BAKER, AND D. R. BUNDLE, *Can. J. Biochem.*, 55 (1977) 507–512.
13 J. HONZL AND J. RUDINGER, *Collect. Czech. Chem. Commun.*, 26 (1961) 2333–2344.
14 J. K. INMAN, B. MERCHANT, L. CLAFLIN, AND S. E. TACEY, *Immunochemistry*, 10 (1973) 165–174.
15 E. H. WHITE, *J. Am. Chem. Soc.*, 77 (1967) 6008–6010; *Org. Synth.*, 47 (1967) 44-47.
16 B. M. PINTO AND D. R. BUNDLE, unpublished results.
17 M. MELDAL AND K. BOCK, *Acta Chem. Scand., Ser. B*, in press.
18 M. MELDAL AND K. BOCK, *Acta Chem. Scand., Ser. B*, in press.
19 D. R. BUNDLE AND S. JOSEPHSON, unpublished results.
20 D. R. BUNDLE AND T. IVERSEN, unpublished results.