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PHOTO-AFFINITY LABELLING OF CARBOHYDRATE-BINDING SITES IN PROTEINS SYNTHESIS OF POTENTIAL INHIBITORS

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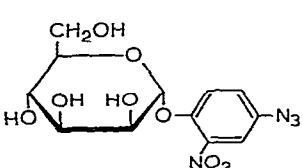
ABSTRACT

The synthesis and properties of 4-azido-2-nitrophenyl α-D-mannopyranoside, *N*-diazoacetyl-β-D-glucopyranosylamine, and 4-diazoacetamidophenyl α-D-mannopyranoside are described These compounds are potential reagents for photo-affinity labelling of carbohydrate-binding sites in proteins

INTRODUCTION

Photo-affinity labelling of ligand-binding sites in proteins involves the photolytic generation of a chemically reactive, radical species from a suitable precursor function attached to the ligand; the radical then undergoes covalent reaction with an amino acid side-chain at the binding site[1]. It represents an extension of the usual affinity-labelling method to those binding sites which do not contain nucleophilic side-chains liable to attack by alkylating functions 4-Azido-2-nitrophenyl α-D-mannopyranoside (**1**), *N*-diazoacetyl-β-D-glucopyranosylamine (**2**), and 4-diazoacetamidophenyl α-D-mannopyranoside (**3**) were accordingly selected as potential, specific labels for concanavalin A, since attempts to affinity-label this protein by a range of conventional reagents had failed[2] The aryl α-D-mannosides **1** and **3** could be expected to bind strongly to concanavalin A, and the substituted β-D-glucosylamine **2** was considered as a compound which could bind, but in which the potential carbene function was closer to the pyranoid ring Azido and diazoacetyl groups are known to give nitrene and carbenoid species on photolysis[3].



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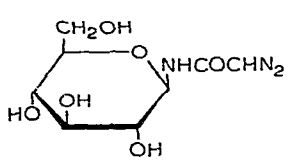

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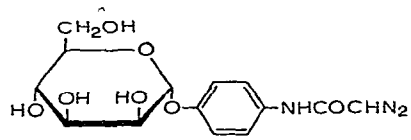

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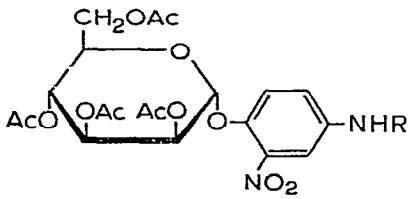
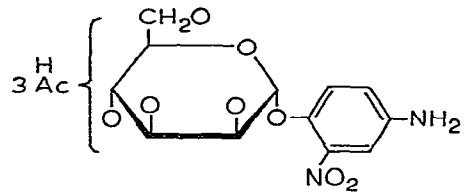
RESULTS AND DISCUSSION

Compound **1** proved to be accessible by a route which could easily be adapted to a radiochemical synthesis using D-mannose-*t*. Condensation of 4-(benzyloxycarbonylamino)-2-nitrophenol with tetra-*O*-acetyl-α-D-mannopyranosyl chloride under alkaline conditions gave the α-D-mannoside **4** as expected[4]. Removal of the benzyloxycarbonyl group with hydrogen bromide in acetic acid was accompanied by partial *O*-deacetylation Acetylation of the resulting amine **5** gave 4-acetamido-2-nitrophenyl α-D-mannopyranoside tetra-acetate (**6**), which was also synthesised by an independent route involving zinc chloride–catalysed fusion of 4-acetamido-2-nitrophenol and α-D-mannopyranose Treatment of diazotised **5** with sodium azide gave the 4-azido derivative, deacetylation of which gave **1**



4 R=CO OCH$_2$Ph
6 R=Ac



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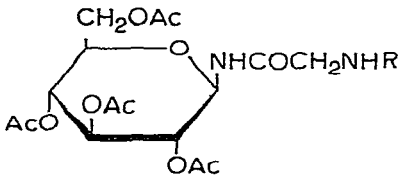
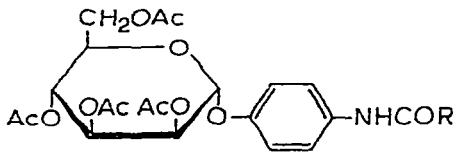
The absorption maximum (355 nm) of **1** enables photolysis to be effected by radiation (~350 nm) which should not damage the protein Aqueous solutions of **1** rapidly developed a brown colour on irradiation at 360 nm which was discharged by dithionite and was presumably due to the azo compound **11** Addition of concanavalin A to irradiated solutions of **1** gave a brown precipitate which redissolved immediately on adding methyl α-D-mannopyranoside Precipitation probably involves the formation of a linear, insoluble aggregate between concanavalin A [below pH 6, the concanavalin molecule (mol wt. $\sim 55 \times 10^3$) is composed of two sub-units, each with a discrete, carbohydrate-binding site] and **11**, which is bifunctional with respect to the α-D-mannopyranosyl moiety Photolytic conversion of aromatic azides into azo compounds is well documented[5], and is presumed to involve nitrene species Experiments are in progress with ^{3}H-labelled **1** in order to detect any covalent binding to concanavalin A on photolysis Preferential formation of azo compounds may limit the usefulness of aromatic azido compounds in photo-affinity labelling, particularly in the case of weaker complexes

Compounds **2** and **3** are potential carbene precursors Compound **2** was obtained by the following reaction sequence Condensation of *N*-benzyloxycarbonylglycine with β-D-glucopyranosylamine, followed by acetylation, gave **7** Hydrogenolysis of **7** gave the glycyl derivative **8**, which, on treatment with sodium nitrite followed by Zemplén deacetylation, was converted into the diazoacetyl compound **2**

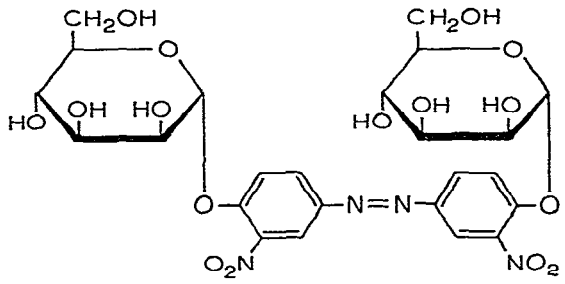
Acetylation of 4-nitrophenyl α-D-mannopyranoside was followed by catalytic reduction. Coupling of the resulting amine with *N*-benzyloxycarbonylglycine and



7 R=CO OCH$_2$Ph
8 R=H HCl



9 R=CH$_2$NH$_2$·HCl
10 R=CHN$_2$



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hydrogenolysis of the product gave **9** Treatment of **9** with sodium nitrite, followed by deacetylation of the product **10**, gave the diazoacetyl compound **3**

Compounds **2** and **3** were stable in aqueous solution at 23° for at least 24 h. Decomposition occurred within 15 min at 253 7 nm, and within 3 h when irradiated at 350 nm Hence, unless unusually susceptible to Wolff rearrangement (giving the corresponding *N*-carboxymethyl compounds), **2** and **3** seem to be suitable reagents for photo-affinity labelling and, being diazoketones, they could also be useful as conventional affinity-labelling reagents

That compounds **1–3** bound reversibly and specifically to concanavalin A was shown qualitatively by the fact that they all re-dissolved the insoluble aggregate formed from concanavalin A and 1,3,5-tris[*p*-(β-D-glucopyranosyloxy)phenylazo]-2,4,6-trihydroxybenzene[6] Only carbohydrates which bind to concanavalin A can dissociate and thereby solubilize the aggregate Although the binding constants of **1–3** were not measured, they can be expected[7] to range from 10^3 to 5×10^4. In the labelling experiments, an appreciable molar excess of protein over ligand can be used in order to ensure significant binding of ligand.

EXPERIMENTAL

4-Amino-2-nitrophenol and 4-nitrophenyl α-D-mannopyranoside were obtained from Aldrich and Koch–Light Chemicals, respectively β-D-Glucopyranosylamine was prepared by a literature procedure[8].

Reactions were followed by t l c on silica gel G (Merck), using either ethyl acetate–acetone (for acetates) or methanol–acetone (for free sugars) Column chroma-

tography was performed with Kieselgel (0 05–0 2 mm, Merck) or neutral alumina (Woelm)

Melting points are uncorrected

Photolyses were effected with either a Mazda 250W ME/D lamp fitted with a 2-cm filter of 10% aqueous sodium nitrite, or directly (in quartz curvettes) with a Hanovia "Mineralite" source

1,3,5-tris[*p*-(*β*-D-glucopyranosyloxy)phenylazo]-2,4,6-trihydroxybenzene was prepared by the procedure of Yariv *et al* [6], and used as a saturated solution in 0 05M sodium acetate–0 2M sodium chloride (pH 5 2) On addition of concanavalin A (in saturated, aqueous sodium chloride, available from Miles–Yeda Chemicals), immediate precipitation of the red aggregate took place, this was centrifuged and washed free of azo dye by repeated resuspension in pH 5 2 buffer and centrifugation Solid sugars were added to 1 ml of aggregate suspensions prepared in pH 5 2 buffer, followed by vibratory mixing Only those sugars which specifically bound to concanavalin A dissolved the aggregate, giving a clear, red solution

4-(N-Benzyloxycarbonylamino)-2-nitrophenol — To a solution of 4-amino-2-nitrophenol (4 5 g, 30 mmoles) in *p*-dioxane (150 ml) was added potassium hydrogen carbonate (120 mmoles) dissolved in water (50 ml) With stirring at 5°, benzyloxycarbonyl chloride (5 ml) was added dropwise over 30 min After stirring at 23° for 2 h, the mixture was poured into water (250 ml) and acidified with M sulphuric acid The product was extracted with ethyl acetate (3 portions), and then back-extracted into M potassium hydroxide Acidification and cooling of the aqueous phase gave the crystalline phenol, which was recrystallised from ether–hexane to give material (5 8 g, 64%) having m p 103°.

Anal Calc for $C_{14}H_{12}N_2O_5$ N, 9 7 Found N, 9 6

4-Acetamido-2-nitrophenyl α-D-mannopyranoside — 4-Acetamido-2-nitrophenol (2 6 g), α-D-mannopyranose penta-acetate (3 5 g), and anhydrous zinc chloride (1 3 g) were fused and stirred at 160°/20 mmHg After 30 min, the reaction mixture was cooled and dissolved in chloroform, and the solution was twice extracted with water, followed by M potassium hydroxide until the extracts were colourless The material remaining in the chloroform crystallised from 1-propanol to give **6** (0 6 g, 13%), m p 230° (dec), $[\alpha]_D^{23}$ +85 5° (*c* 0 67, chloroform), which was homogeneous by t l c

Conventional deacetylation of **6** gave the title product, m p 211° (from ethanol), $[\alpha]_D^{20}$ +68° (*c* 0 43, water)

Anal Calc for $C_{14}H_{18}N_2O_9$ C, 46 9, H, 5 0 Found C, 46 7; H, 5 2

4-(Benzyloxycarbonylamino)-2-nitrophenyl α-D-mannopyranoside tetra-acetate (**4**) — 4-(Benzyloxycarbonylamino)-2-nitrophenol (3 3 g) was dissolved in M sodium hydroxide (17 ml) and acetone (30 ml) at 5° Tetra-*O*-acetyl-α-D-mannopyranosyl chloride (3 7 g) dissolved in acetone (20 ml) was added, and the mixture was left at 5° for 12 h The mixture was then poured into water (200 ml) and extracted five times with chloroform Unreacted phenol was recovered from the chloroform extracts by six extractions with M potassium hydroxide The chloroform layer was then washed with dilute acid, dried, and evaporated to give a mixture of the desired mannoside

and mannosyl chloride Elution of the mixture from neutral alumina with chloroform gave the mannosyl chloride, and then with chloroform–ethyl acetate (1 1) gave **4** (0 7 g, 11% based on the mannosyl chloride), m p 188° (from 1-propanol), $[\alpha]_D^{23}$ +68° (*c* 0 2, chloroform)

Anal Calc for $C_{28}H_{30}N_2O_{14}$ C, 54.4, H, 4 85 Found C, 54 8, H, 4 9

4-Azido-2-nitrophenyl α-D-*mannopyranoside* (**1**) — To a suspension of **4** (2 3 g) in dry chloroform (10 ml), a 45% solution (30 ml) of hydrogen bromide in glacial acetic acid was added After storage at 23° with vigorous stirring for 15 min, the yellow solution was poured into chloroform (200 ml) which was in turn poured on to crushed ice (200 ml) The chloroform layer was separated, washed with ice-cold, aqueous sodium hydrogen carbonate until neutral, dried, and evaporated The resulting, orange solid was freed from benzyl bromide by trituration with a small volume of ice-cold chloroform The yellow residue (1 3 g) was almost homogeneous by t l c , and gave a positive diazo reaction for aromatic amines Acetylation (pyridine–acetic anhydride) gave **6**, which was identified by m p and R_F value *N*-Acetylation of the amine with acetic anhydride in methanol gave a substance of slightly lower mobility than **6** on t l c , which gave a negative diazo reaction, and was presumably a tri-*O*-acetyl derivative

A solution of the amine tri-acetate (500 mg) in methanol–acetic acid (1 1, 10 ml) was cooled to 0° and isopentyl nitrite (0 5 ml) was added After 30 min, M hydrochloric acid (2 ml) was added, followed by sodium azide (1 mol) portionwise After stirring for 60 min, the mixture was poured into excess water and extracted with chloroform

The extract was washed, dried, and evaporated to yield a cream-coloured solid, which was homogeneous by t l c and had ν_{max} 2120 cm^{-1} (N_3) Conventional deacetylation of this product gave **1** (100 mg, 26%, after recrystallisation from water) as pale-yellow, light-sensitive needles, m p 132–135°, $[\alpha]_D^{20}$ +130° (*c* 0 22, water), $\lambda_{max}^{H_2O}$ 355 nm (ε 1900), ν_{max} 2100 cm^{-1} (N_3)

Anal Calc for $C_{12}H_{14}N_4O_8$ C, 42 1, H, 4 1. Calc for $C_{12}H_{14}N_4O_8$ H_2O C, 40.0, H, 4 4 Found C, 40.3, H, 4 6

*2,3,4,6-Tetra-*O*-acetyl-*N*-(*N*-benzyloxycarbonylglycyl)-β-*D*-glucopyranosylamine* (**7**) — To an ice-cold solution of *N*-benzyloxycarbonylglycine (420 mg) in dry *N,N*-dimethylformamide (3 ml), re-distilled triethylamine (0 28 ml) was added, followed by isobutyl chloroformate (0 26 ml) with stirring After storage at 0° for 30 min, the precipitated triethylamine hydrochloride was collected and washed with cold *N,N*-dimethylformamide (1 ml) β-D-Glucopyranosylamine (360 mg) was added to the filtrate and washings, it dissolved completely within 30 min, with evolution of gas After storage at 23° for 12 h, excess of dry ether was added, and the mixture was left at 4° for 12 h The ether was then decanted, and the residue was acetylated conventionally with pyridine–acetic anhydride to give **7** (740 mg, 68%; after crystallisation from ethyl acetate–hexane), m p 123–125°. The optical rotation (*c* 0.53, water) was too low for accurate measurement.

Anal Calc for $C_{24}H_{30}N_2O_{12}$ C, 53 5; H, 5 6 Found C, 53.4; H, 5 7.

2,3,4,6-Tetra-O-acetyl-N-glycyl-β-D-glucopyranosylamine hydrochloride (**8**) — Hydrogenolysis of **7** (540 mg) dissolved in methanol (20 ml) containing M hydrochloric acid (1 ml) and 5% palladium/charcoal (100 mg) as catalyst gave **8** in quantitative yield, with m p 136° (from methanol–ether), $[\alpha]_D^{23}$ +1 7° (*c* 1.17, water)

Anal Calc for $C_{16}H_{25}ClN_2O_9 \cdot H_2O$ C, 41 9, H, 5 9 Found C, 41 4; H, 5 7

2,3,4,6-Tetra-O-acetyl-N-diazoacetyl-β-D-glucopyranosylamine — A solution of **8** (100 mg) in 2M sodium acetate (2 ml) at 0° was treated with sodium nitrite (50 mg) and glacial acetic acid (0 5 ml) After storage at 0° for 5 h, the product (49 mg, 52%) precipitated as fine needles, which were collected, washed with cold water, and dried *in vacuo* over phosphorus pentaoxide It appeared homogeneous on t l c, and had m p 153–154° Recrystallisation from ethyl acetate–hexane gave the title compound, m p. 160–161°, $[\alpha]_D^{23}$ −32° (*c* 1, chloroform), λ_{max}^{MeOH} 253 nm (ε 14 2 × 10^3)

Anal Calc for $C_{16}H_{21}N_3O_{10}$ C, 46 3, H, 5 1 Found C, 46 6; H, 5 0.

N-Diazoacetyl-β-D-glucopyranosylamine (**2**) — The foregoing tetra-acetate (0 5 g) was suspended in dry methanol (10 ml), and a small chip of sodium was added After dissolution, the yellow solution was kept at 4° for 2 h and then neutralised with solid carbon dioxide After evaporation to a small volume, 1-propanol (25 ml) was added, and the precipitate was collected and washed with a little 1-propanol T l c of the filtrate (acetone–methanol, 4 1) showed one component, and concentration to a small volume resulted in the precipitation of **2**, which was collected, washed with ice-cold 1-propanol and ether, and finally dried *in vacuo* The product (150 mg, 60%) had m p 60–65° (dec), $[\alpha]_D^{20}$ −27.5° (*c* 0.44, methanol), λ_{max}^{MeOH} 252 nm (ε 16 × 10^3) which disappeared on acidification, ν_{max} 2060 (CHN_2) and 1660 cm^{-1} (amide I); there was no absorption for ester C=O.

Anal Calc. for $C_8H_{13}N_3O_6$· C, 38 9; H, 5 3; N, 17 0 Found C, 38 3, H, 5 5; N, 16 8

4-N-Glycylaminophenyl 2,3,4,6-tetra-O-acetyl-α-D-mannopyranoside hydrochloride (**9**) — 4-Nitrophenyl α-D-mannopyranoside was conventionally acetylated with sodium acetate–acetic anhydride, and a methanolic solution of the product was hydrogenated with platinum oxide as a catalyst. The resulting 4-aminophenyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside was coupled with *N*-benzyloxycarbonylglycine as described for **7**, but using dichloromethane as solvent Hydrogenolysis (using 5% palladium on charcoal as catalyst) of the resulting, neutral product gave **9** as a hygroscopic powder, m p. 205° (dec), $[\alpha]_D^{20}$ +56 5° (*c* 0 95, water), which was homogeneous by t l.c (methanol), gave a positive ninhydrin test, and had ν_{max} 1750 (ester C=O) and 1690 cm^{-1} (amide I)

Anal Calc. for $C_{22}H_{29}ClN_2O_{11}$. C, 47 95; H, 5 3 Found. C, 48 2, H, 5.4.

4-Diazoacetamidophenyl 2,3,4,6-tetra-O-acetyl-α-D-mannopyranoside (**10**) — A solution of **9** (1 g) in 2M sodium acetate (50 ml) was treated dropwise with glacial acetic acid until a clear solution was obtained After cooling to 0°, sodium nitrite (1 g) was added with stirring, and the solution was left at 4° for 15 h. Extraction with ethyl acetate gave the crude product (0.9 g), which was eluted from Kieselgel G with

chloroform to give **10** Crystallisation from ethyl acetate–hexane gave material (200 mg, 20%), m p 155° (dec), $[\alpha]_D^{20}$ +75° (*c* 0 66, chloroform), λ_{max}^{MeOH} 275 nm (ε 2 35 × 10^4) replaced by λ_{max} 248 nm (ε 1 54 × 10^4) on acidification

Anal Calc for $C_{22}H_{25}N_3O_{11}$ C, 52 1, H, 4 9 Found C, 51 8, H, 5 15

4-Diazoacetamidophenyl α-D-mannopyranoside (**3**) — A solution of **10** (100 mg) in methanol (3 ml) was treated with a small chip of sodium After dissolution, the yellow solution was left at 4° for 3 h, then neutralised with solid carbon dioxide, and evaporated to a small volume Sufficient 1-propanol was added, with vigorous stirring, so that a definite, colourless, gelatinous precipitate remained in the solution, which was then stored at 4°. Complete precipitation of gelatinous material then occurred, leaving a greenish-yellow supernatant T l c (methanol–acetone 1 4) at this point showed the precipitate to contain material of low R_F value which did not have a diazo function (u v) The supernatant, which contained the desired product, was essentially homogeneous; it was concentrated and stored at 4° to give **3** as greenish-yellow prisms (38 mg, 55%), m p 160–163° (dec), $[\alpha]_D^{20}$ +82° (*c* 1 02, 1-propanol), λ_{max}^{MeOH} 275 nm (ε 1 5 × 10^4); ν_{max} 2100 (CHN_2), 1640 cm^{-1} (amide I)

Anal Calc for $C_{14}H_{17}N_3O_7$ C, 49.6, H, 5 0, N, 12 4 Found. C, 49 2; H, 5 4, N, 12 0

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