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Covalent Binding of Proteins to Polysaccharides by Cyanogen Bromide and Organic Cyanates. II Model Studies With Methyl 4,6-*O*-Benzylidene-α-D-glucopyranoside and Cyanogen Bromide

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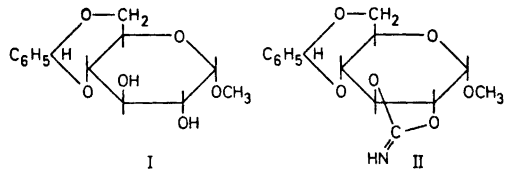
The reaction between methyl 4,6-*O*-benzylidene-α-D-glucopyranoside and cyanogen bromide afforded a mixture of products. The main component was identified as methyl 4,6-*O*-benzylidene-α-D-glucopyranoside 2,3-imidocarbonate. Small amounts of the corresponding 2,3-carbonate, 2- and 3-carbamate were also formed.

Enzymes and other proteins may be attached to soluble or insoluble carriers of polysaccharide nature by the cyanogen bromide method described by Axén and coworkers.[1,2] We have shown that cyanic acid esters can be used for similar coupling reactions.[3] It seems reasonable to assume that at least some of the active groups formed by these two reagents are identical.

In the original paper [1] on the cyanogen bromide method, it was suggested that the intermediate reactive groups as well as the final linkages consisted of imidocarbonic acid esters. It was also suggested that carbamate groupings might be of importance.

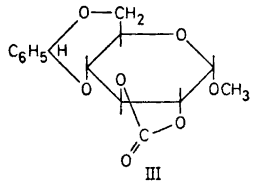
The molecular weight of dextran preparations increases on treatment with cyanogen bromide [4] or cyanates,[3] indicating the formation of cross-linkages. Since soluble dextran preparations containing 0.3 equiv. of nitrogen per glucose residue may be prepared, only a few of the incorporated groups are thus involved in cross-linkages. The formation of imidocarbonate structures, not involved in cross-linkages, has been proposed. In the case of D-glucose residues *trans*-fused 2,3- or 3,4-*O*-imidocarbonates may be postulated.

In order to study this possibility, the reaction between methyl 4,6-*O*-benzylidene-α-D-glucoside (I) and cyanogen bromide has been investigated. As reference compounds we also prepared the 2,3-carbonate (III) and 2- and 3-carbamate derivatives (IV, V) of I.

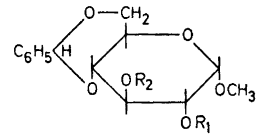


I

II



III



IV R_1 = $CONH_2$ R_2 = H

V R_1 = H R_2 = $CONH_2$

EXPERIMENTAL

Apparatus and methods. IR-spectra in KBr were recorded on a Unicam 200, and the NMR was measured in a Varian A 100 NMR spectrometer. An LKB 9000 mass spectrometer was employed for the mass spectrometric measurements.

Starting material I, and reference compounds III, IV and V were prepared as previously described.[5]

The reaction between cyanogen bromide (BrCN) and I. In a typical experiment I (5.0 g) was dissolved in hot water (250 ml). The solution was stirred vigorously and cooled rapidly to give a fine suspension. BrCN (10 g) dissolved in water (125 ml) was added with stirring during 75 min and the pH was kept at 10.7 by automatic addition of 2 M sodium hydroxide. After stirring for another 15 min, the suspension was neutralized with 2 M hydrochloric acid. The solid material was filtered off and dissolved in chloroform (50 ml) which was shaken with two small portions of water (2 × 10 ml), filtered and evaporated to dryness. The residue (3.0 g) was dried *in vacuo* at 50°C overnight. (Found: N 4.3. Calc. for $C_{15}H_{17}O_6N$: N 4.6.)

Treatment of the product of the reaction between I and BrCN with 0.1 M HCl. The title product (0.5 g) was shaken 4 h with 0.1 M hydrochloric acid (50 ml). The mixture was evaporated *in vacuo* at 50°C. The residue was suspended in water (20 ml) which was evaporated. The treatment with water was repeated once.

Treatment of methyl 4,6-O-benzylidene-α-D-glucopyranoside 2,3-carbonate with 0.1 M HCl. The same procedure as above was used.

Stability of the product of the reaction between I and BrCN. The title substance was dissolved in 99 % ethanol, chloroform, benzene, and methylene chloride. The solutions were kept at room temperature for certain periods of time. The solutions were then evaporated to dryness and the IR-spectra of the residues recorded.

RESULTS

On treatment of a dilute aqueous solution of methyl 4,6-*O*-benzylidene-α-D-glucoside (I) with cyanogen bromide an amorphous product was obtained, which because of its reactivity could not be crystallised or purified by other procedures. The main component of this product is assigned the structure methyl 4,6-*O*-benzylidene-α-D-glucoside 2,3-imidocarbonate (II) on the following evidence. A strong absorption in the IR, at 1710 cm^{-1}, was attributed to the carbon-nitrogen double bond. 2-Imino-1,3-dioxolone absorbs at 1710 cm^{-1}.[6]

Table 1. Partial interpretation of NMR-spectra of I, III, and I treated with BrCN.

Compound	Chemical shift δ-values (ppm)										Coupling constants (Hz)					
	Pyridine-d_5					$CDCl_3$					Pyridine-d_5			$CDCl_3$		
	H_1	H_2	H_3	H_4	B–H[a]	H_1	H_2	H_3	H_4	B–H[a]	J_{12}	J_{23}	J_{34}	J_{12}	J_{23}	J_{34}
I	5.03	4.05	4.45	3.91	5.71	4.63	~3.80	—	—	5.45	3.5	9.4	~9.0	3.2	—	—
III	5.39	4.67	5.18	4.40	5.84	5.12	4.18	4.83	3.93	5.55	~3.0	11.5	9.5	3.1	11.5	9.5
I treated with BrCN	5.28	4.33	4.86	4.3	5.76	5.14	4.05	4.69	—	5.57	3.0	~10.8	—	3.0	~10.8	~9.0

[a] Methine proton in the benzylidene group.

The absorption of the hydroxy groups in the starting material at 3500 cm^{-1} had disappeared. It was replaced by a weak but fairly sharp band at 3400 cm^{-1}, assigned to the NH-group. In the mass spectrum of the above mentioned product, a peak at *m/e* 307 and a strong peak at *m/e* 264 are assigned to the molecular ion and to M – 43 of II, respectively. Weak peaks at *m/e* 308 and 325 indicate the presence of the *trans*-carbonate (III) and of the carbamates (IV and V). Authentic samples of these all gave strong (M – 43) ions, probably due to the elimination of the fragment C_2H_3O.

The NMR spectrum of I treated with BrCN (Table 1) differs from that of I but resembles that of III, which also contains a *trans*-fused five-membered ring. These differences may in part be ascribed to the distortion of the pyranose ring by the *trans*-fused five-membered ring in II and III.

The product containing II was not stable but decomposed during chromatography on silica gel or on standing in polar or non-polar solvents. The reactions in benzene solution could be followed by IR; the typical absorptions at 1710 cm^{-1} diminished and had disappeared after 60 h at room temperature. An absorption at 1670 cm^{-1}, which was observed after 15 h but not after 60 h, indicates the formation of an unstable intermediate. The final product showed absorption at 1570 cm^{-1} and 3500 cm^{-1}.

The average molecular weight of this product as determined by VP osmometry was 540. Mass spectrometry, using solid inlet, indicated the presence of at least two components. For the component appearing at 120°C, the heaviest ion was found at *m/e* 590, whereas that for the component appearing at 180°C had *m/e* 921. It is known that imidocarbonates are unstable and may give 2,4,6-trialkoxy-1,3,5-triazines in the absence of catalysts.[7,8] The formation of such triazines by trimerisation of II is indicated by the IR-absorption at 1570 cm^{-1},[9] and the appearance of *m/e* 921 (3 × 307) in the mass spectrum.

The product obtained from the reaction between cyanogen bromide and I was treated with dilute hydrochloric acid to remove the benzylidene groups and the behaviour of the resulting material was studied. The IR spectrum of the product was very similar to that obtained by similar treatment of methyl 4,6-*O*-benzylidene-α-D-glucopyranoside 2,3-carbonate. The product

from activated I showed additional bands at 3200 – 3100 cm^{-1} and 1410 cm^{-1}. This agrees well with the absorption of ammonium chloride which might be formed from II on acid hydrolyses. A rather broad carbonyl band at 1780 cm^{-1} was observed in both cases. It is known that diphenyl imidocarbonate in weakly acid media is transformed into the corresponding carbonate.[10]

DISCUSSION

From the results discussed above, it may be concluded that the main product formed when I is reacted with cyanogen bromide in aqueous alkali is the reactive cyclic imidocarbonate II. It seems reasonable to assume that the 2,3- or 3,4-linked cyclic imido carbonates are formed when cyanogen bromide or cyanates are allowed to react with dextran or cross-linked dextran (Sephadex). 4,6-Linked cyclic imidocarbonates at the terminal D-glucose residues and interchain imidocarbonates may also be formed. Cyanogen bromide activation has also been applied to agarose,[2,3] a polysaccharide composed of alternating (1→3)-linked β-D-galactopyranose and (1→4)-linked 3,6-anhydro-α-L-galactopyranose residues. This polysaccharide contains no vicinal hydroxyl groups and the reaction product is assumed to contain cyclic 4,6-imidocarbonate groups and interchain imidocarbonates.

It is known that acyclic imidocarbonates react with amino acid esters to give *N*-substituted imidocarbonates.[7] In agreement with this ammonia is liberated when alanine methyl ester is coupled to Sephadex by the cyanogen bromide method.[1] However, on coupling glycine to activated Sephadex, no ammonia was liberated.[4] Preliminary results also indicate that an isourea derivative is formed in the reaction between the product containing II and glycine.

The present results therefore strongly support the assumption that cyclic imidocarbonate groups are formed in reacting polysaccharides with cyanogen bromide and that they constitute the reactive groups in the subsequent reaction with proteins or other amino compounds. Further studies are required to establish the structure and properties of the linkages formed during the coupling reaction.

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REFERENCES

1. Axén, R., Porath, J. and Ernback, S. *Nature* **214** (1967) 1302.
2. Porath, J., Axén, R. and Ernback, S. *Nature* **215** (1967) 1491.
3. Kågedal, L. and Åkerström, S. *Acta Chem. Scand.* **24** (1970) 1601.
4. Kågedal, L. and Åkerström, S. *Acta Chem. Scand.* **25** (1971) 1855.
5. Doane, W. M., Sasha, B. S. and Stout, E. I. *Carbohyd. Res.* **11** (1969) 321.
6. Addor, R. W. *J. Org. Chem.* **29** (1964) 738.
7. Houben, J. and Zivadinovitsch, R. *Ber. Deut. Chem. Ges.* **B 69** (1936) 2352.
8. Jensen, K. A., Due, M., Holm, A. and Wentrup, C. *Acta Chem. Scand.* **20** (1966) 2091.
9. *Documentation of Molecular Spectroscopy*, Card No. 16531, Verlag Chemie and Butterworths, London 1967.
10. Nef, J. U. *Ann.* **287** (1895) 320.

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