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Heterobifunctional Reagents for Cross-Linking of Sugar with Protein

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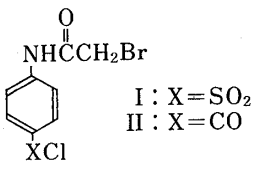
N-Bromoacetylsulfanilyl chloride and *p*-bromoacetamidobenzoyl chloride were synthesized as heterobifunctional reagents for the cross-linking of sugar with protein. These reagents react with the primary hydroxyl group of a sugar at the acid chloride group and with amino and imidazole groups of protein at the bromoacetyl group. Methyl α-D-glucoside, selected as a model sugar, reacts with these reagents to form methyl 6-*O*-(*N*-bromoacetylsulfanilyl)-α-D-glucoside and methyl 6-*O*-(*p*-bromoacetamidobenzoyl)-α-D-glucoside, respectively. These sugar derivatives combine with bovine serum albumin at weakly alkaline pHs. More than 10 of the sugar derivatives can be incorporated into one molecule of the albumin.

Keywords——*N*-bromoacetylsulfanilyl chloride; *p*-bromoacetamidobenzoyl chloride; heterobifunctional reagent; cross-linking; sugar; protein; methyl α-D-glucoside; bovine serum albumin

Preparation of a protein–hapten conjugate is a key step in the immunoassay of low molecular compounds, such as natural products which have a sugar moiety. Several methods have been developed for binding sugar to protein.[1-6] In most of these methods, a hydroxyl group of the sugar moiety[1-4] or its vicinal hydroxyl groups[5,6] are utilized for binding. The former approach can utilize the free hydroxyl group at the C-1 position of the sugar, while the latter requires periodate oxidation, which results in the destruction of the sugar structure. A bifunctional reagent for the binding of sugar with protein has not yet been reported. Such a bifunctional reagent which would not block the C-1 hydroxyl group of sugar and would not destroy the sugar structure should be very useful.

We have synthesized *N*-bromoacetylsulfanilyl chloride (I) and *p*-bromoacetamidobenzoyl chloride (II) as new heterobifunctional reagents (Chart 1). The acid chloride group can react with a hydroxyl group of a sugar to form an ester, and the haloacetyl group permits alkylation of sulfhydryl,[7] amino[8] and imidazole groups[8,9] in protein. Such reactions of these reagents have been investigated by using methyl α-D-glucoside (α-MeGlc) as a simple model of a sugar-containing natural product and bovine serum albumin (BSA) as a representative hapten-carrier.



I : X=SO_2
II : X=CO

Chart 1

Experimental

Materials and Apparatus——BSA (fraction V) was purchased from Miles Laboratories (Elkhart, U.S.A.). Other chemicals were of the highest commercial grade available unless otherwise noted.

Infrared (IR) spectra were measured in KBr pellets with a Hitachi 215 infrared spectrophotometer. Amino acid analyses were performed on a Hitachi 835 amino acid analyzer after hydrolysis of the protein samples in 6 N HCl *in vacuo* at 110 °C for 24 h. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were measured with JEOL PS-100 and JEOL FX-100 spectrometers at 100 and 25 MHz, respectively, using approximately 10% D_2O or dimethylsulfoxide-d_6 (DMSO-d_6) solutions of samples containing approximately 1% tetramethylsilane (TMS) or sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as an internal standard. Splitting patterns were designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Values of coupling constants (J) are reported in Hz. In the ^{13}C-NMR spectra, signals were assigned by both the complete decoupling and off-resonance decoupling techniques.

Syntheses of I and II——I: Bromoacetanilide[10] (15 g, 0.7 mol) was added in small portions with stirring to 40.82 g (0.35 mol) of chlorosulfonic acid cooled in an ice bath. The mixture was warmed at 60 °C for 2 h. The resulting syrupy liquid was poured onto 150 g of ice with stirring. The solid that separated was filtered off and washed with H_2O to give I as a pale yellow powder (18.2 g, 83%), mp 125 °C. ^1H-NMR (in DMSO-d_6 with TMS) δ: 4.20 (2H, s), 7.45 (4H, s), 10.25 (1H, s). Mass spectrum (MS) m/z: 315 ($M^+ + 4$), 313 ($M^+ + 2$), 311 (M^+). IR (KBr): 1660 (C=O), 1165 (SO_2) cm^{-1}. *Anal.* Calcd for $C_8H_7NO_3SBrCl$: C, 30.74; H, 2.26; N, 4.48. Found: C, 30.75; H, 2.55; N, 4.43.

II: *p*-Bromoacetamidobenzoic acid[11] (10 g, 0.39 mol) was refluxed with 81.9 g (0.69 mol) of $SOCl_2$ for 4 h. Then excess $SOCl_2$ was completely removed *in vacuo* (below 40 °C) to give II quantitatively as a pale yellow powder, mp 214—215 °C. ^1H-NMR (in DMSO-d_6 with TMS) δ: 4.27 (2H, s), 7.53—7.93 (4H, m), 10.53 (1H, s). MS m/z: 279 ($M^+ + 4$), 277 ($M^+ + 2$), 275 (M^+). IR (KBr): 1680 cm^{-1} (C=O). *Anal.* Calcd for $C_9H_7NO_2BrCl$: C, 39.09; H, 2.55; N, 5.07. Found: C, 39.09; H, 2.45; N, 5.07.

Reaction of α-MeGlc with I (Chart 2)——α-MeGlc (100 mg, 0.52 mmol) and 1 ml of 2,6-lutidine were mixed, and a small amount (approximately 0.5 ml) of hexamethylphosphoric triamide (HMPA) was added to dissolve all α-MeGlc. The mixture was cooled in an ice bath, and a solution of I (323 mg, 1.03 mmol) in 1 ml of dry dioxane was added dropwise with stirring. The mixture was stirred at 4 °C for 1 h, then further I (300 mg, 0.96 mmol) dissolved in 1 ml of dry dioxane was added and the whole was stirred overnight at 4 °C. H_2O (30 ml) was added and the resulting mixture was washed 5 times with 50 ml of hexane and then extracted 6 times with 30 ml of ethyl acetate. The combined extract was concentrated to dryness *in vacuo* and the residue was chromatographed on a silica gel column (2.5 × 30 cm) with $CHCl_3$–MeOH (7.5 : 2.5, v/v). The main fraction was concentrated *in vacuo*, and the residue was dissolved in a small amount of H_2O then lyophilized to give methyl 6-*O*-(*N*-bromoacetylsulfanilyl)-α-D-glucoside (III) as a colorless powder (92 mg, 38%), mp 82—83 °C. MS m/z: 471 ($M^+ + 2$), 469 (M^+). IR (KBr): 3350 (NH), 1690 (C=O), 1170 (SO_2) cm^{-1}. For NMR data, see Tables I and II.

Reaction of α-MeGlc with II (Chart 2)——α-MeGlc (150 mg, 0.77 mmol) was dissolved in 1.5 ml of 2,6-lutidine and a small amount (approximately 0.75 ml) of HMPA. The mixture was cooled in an ice bath and a solution of II (400 mg, 1.45 mmol) in 1 ml of dry dioxane was added in small portions with stirring. After being stirred continuously overnight at 4 °C, the resulting mixture was treated in the same way as described for III to give methyl 6-*O*-(*p*-bromoacetamidobenzoyl)-α-D-glucoside (IV) as a colorless powder (53 mg, 35%), mp 82 °C, MS m/z: 435 ($M^+ + 2$), 433 (M^+). IR (KBr): 3400 (NH), 1690 (C=O) cm^{-1}. For NMR data, see Tables I and II.

Conjugation of BSA to III or IV (Chart 3, Table III)——A mixture of BSA (5—50%, w/v) and III or IV (29—100 times the molar quantity of BSA) was stirred in 0.4—1.5 ml of 0.16—1.45 M $NaHCO_3$ solution (pH approximately 8.5) or 0.01 M borate buffer (pH 9.18) at 4 °C or 23 °C for 12 h. The reaction mixture (with approximately 2.5 mg of BSA) was poured onto a column (1.5 × 100 cm) of Sephadex G-25 (35 g) and eluted with 10% (v/v) acetic acid. The protein fraction in the void volume was collected and subjected to amino acid analysis. The amount of III or IV incorporated into BSA was estimated from the decrease in the number of histidine and lysine residues in the amino acid composition of the modified BSA as compared with that of intact BSA.

Results and Discussion

Reagents I and II were synthesized by the chlorosulfonation of bromoacetanilide and the reaction of *p*-bromoacetamidobenzoic acid with thionyl chloride, respectively. Reagent I or II reacted with α-MeGlc in dry dioxane in the presence of 2,6-lutidine and a small amount of HMPA at low temperature (4 °C), though the reaction required a long time (about 12 h). Both reactions gave single products, III and IV, respectively.

Compounds III and IV each have an ester linkage between α-MeGlc and the reagent, as

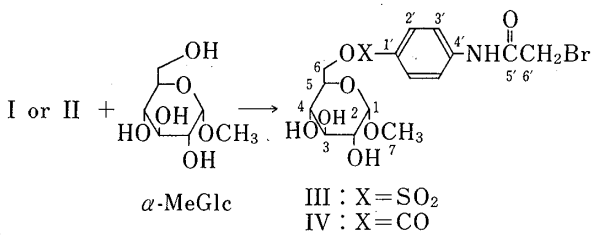


Chart 2

TABLE I. ^{1}H-NMR Data for III and IV[a)]

Compound	Proton	Chemical shift (δ)	
III	OCH_3	3.30	(s, 3H)
	CH_2	4.30	(s, 2H)
	Sugar H	3.32—4.40	(m, 7H)
	Aromatic H	7.70	(d, 2H, J=9.0)
		7.85	(d, 2H, J=9.0)
IV	OCH_3	3.26	(s, 3H)
	CH_2	4.30	(s, 2H)
	Sugar H	3.30—4.56	(m, 7H)
	Aromatic H	7.74	(d, 2H, J=9.0)
		7.92	(d, 2H, J=9.0)

a) III: in D_2O with DSS; IV: in DMSO-d_6 with TMS.

TABLE II. ^{13}C-NMR Data for III and IV[a)]

Numbering of carbon[b)]	III		IV	
	Chemical shift (δ)	Multiplicity	Chemical shift (δ)	Multiplicity
1	105.2	d	99.6	d
2	76.9	d	71.7	d
3	78.9	d	73.1	d
4	75.1	d	69.5	d
5	75.1	d	64.1	d
6	75.8	t	70.3	t
7	61.1	q	54.3	q
1′	148.5	s	142.7	s
2′	135.1	d	130.2	d
3′	126.7	d	118.7	d
4′	135.4	s	124.6	s
5′	173.9	s	165.1	s
6′	49.1	t	43.5	t
O–C=O			165.1	s

a) III: in D_2O with DSS; IV: in DMSO-d_6 with TMS.
b) For numbering of the carbons, see Chart 2.

confirmed by the mass and ^{1}H-NMR spectral data (Table I). In the ^{13}C-NMR spectra of III and IV (Table II), significant downfield shifts were observed only at the C-6 carbon signal in the α-MeGlc moiety for both compounds. These observations indicate that the ester linkage is formed between the C-6 hydroxyl group (the only primary hydroxyl group) of α-MeGlc and reagent I or II. α-MeGlc is not selectively acetylated[12)] but tosylation occurs selectively at the

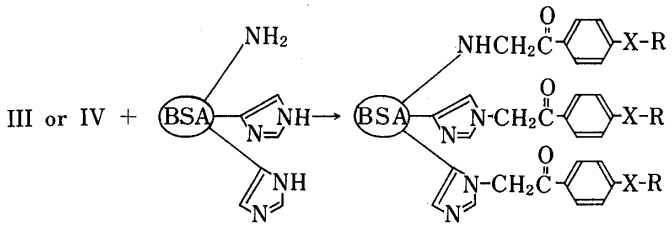


Chart 3

TABLE III. Reactivities of III and IV with BSA

Run	Reaction conditions					Reacted amino acid residues of BSA		Number of III or IV incorporated into BSA
	Molar ratio to BSA		Solvent[a] (M)	BSA (%, w/v)	Temperature (°C)	Lysine	Histidine	
	III	IV						
1	29		A	5.0	4	0.7	2.9	3.6
2	29		A	5.0	23	1.7	1.8	3.6
3	100		B (1.45)	50.0	23	4.6	6.2	10.8
4		32	A	5.0	4	1.0	2.9	3.9
5		32	A	5.0	23	2.7	2.0	4.7
6		32	B (0.16)	12.5	23	2.8	2.9	5.7
7		100	B (0.39)	13.3	23	9.8	5.5	15.3

a) A: 0.01 M borate buffer (pH 9.18); B: 0.16—1.45 M $NaHCO_3$ (pH approximately 8.5).

C-6 hydroxyl group.[13] The tosyl group is much bulkier than the acetyl group, in common with reagents I and II. Therefore, the selectivity of the reagents may be due to their bulkiness.

When pyridine or triethylamine was used as a base in place of 2,6-lutidine in the reaction of I or II with α-MeGlc, the bromoacetyl moiety reacted with the base to give the corresponding pyridinium or quaternary ammonium salt, which delays the reaction. However, in the absence of a base, the reaction proceeds very slowly. Thus, 2,6-lutidine is preferred as a solvent for the reaction.

In general, the reactivity of the haloacetyl group with protein increases with increase of pH.[8] Various reaction conditions for III or IV with BSA were examined at weakly alkaline pHs. The reaction mixture was chromatographed on a column of Sephadex G-25 and the fraction of BSA incorporating III or IV was subjected to amino acid analysis. In every case, considerable decreases in lysine and histidine residues were observed in the amino acid composition of the modified BSA as compared with that of intact BSA. In the chromatogram obtained in the amino acid analysis, three peaks were detected as shoulders near the peaks of proline, alanine and valine. The retention times of these peaks were consistent with those of 1-carboxymethylhistidine, 3-carboxymethylhistidine and N^{ε}-carboxymethyllysine, respectively.[8,12] No other changes in the amino acid composition were noted. No evidence for the reaction of III or IV with sulfhydryl groups in BSA could be found under the conditions tested in the present study. This is probably because BSA has only one cysteine residue (0.7 reactive sulfhydryl group) in the molecule.[14] These results clearly indicate that ε-amino groups of lysine residues and imidazole nitrogens of histidine residues in BSA were the sites of alkylation by the bromoacetyl moiety of III or IV (Chart 3).

No hydrolysis of the ester bonds of III and IV was found under the reaction conditions

examined. Thus, the number of III or IV moieties incorporated into one BSA molecule could be estimated from the decrease of lysine and histidine residues (Table III).

The molar ratio of III or IV to BSA in the reaction affected the number of III or IV moieties introduced. The maximum numbers were attained at the molar ratio of 100, and 11 and 15 mol of III and IV were bound per mol of BSA, respectively. The reaction temperature hardly influenced the number of III or IV moieties introduced (4 or 23 °C).

The above observations show that the new heterobifunctional reagents I and II should be useful for the preparation of conjugates between proteins and natural products containing sugar moieties (such conjugates are required for the immunoassay of low molecular natural products), and also for the preparation of artificial glycoproteins.

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