[Chem. Pharm. Bull. 29(4)1130—1135(1981)]

Preparation and Characterization of Hetero-bifunctional Cross-linking Reagents for Protein Modifications

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(Received September 20, 1980)

Ten aromatic and aliphatic cross-linking reagents of hetero-bifunctional type were synthesized as part of a search for useful reagents of this type. All of the reagents possess two selectively reactive groups, a maleimide group which can combine with a thiol group *via* its double bond and an N-hydroxysuccinimidyl ester (one of the most hydrophilic active esters), which can react with an amine group. It was found that the active esters of the reagents tested were mostly more reactive with lysine than with leucine, and acylated amino acids more rapidly at pH 8.0 than at pH 7.0. It was also found that the stability of the maleimide group in the compounds tested depends largely upon the pH of the buffer used. The most stable pH was 5.0—6.0. To use one of the present compounds as a cross-linker, the crystalline reagent should be dissolved in tetrahydrofuran of dioxane and the solution used for cross-linking. The acylation step should be carried out first with thiol addition to the maleimide group as the second step. The optimum pH of the buffer used for the first step is slightly basic. The reaction time should be limited to less than 1 hr. When the first step is over, the pH of the reaction mixture should be changed to 5.0—6.0.

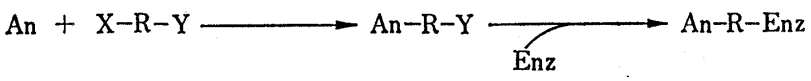
Keywords——cross-linker; hetero-bifunctional reagent; reactivity of N-hydroxysuccinimidyl ester; stability of maleimide residue; N-(maleimidobenzoyloxy)succinimide derivative; N-(maleimidoalkyloxy)succinimide derivative

Recent progress in immunochemistry has required the preparation of protein-protein or protein-hapten conjugates for analytical and preparative techniques. Thus, cross-linking reagents for the preparation of these conjugates have been studied extensively.[2] A general disadvantage of common cross-linkers of the homo-bifunctional type is a lack of the controlled incorporation of protein and hapten molecules into the resulting conjugate. Both groups of the cross-linkers react simultaneously, leading to a variety of complexes, for example in the enzyme labelling of antigen or antibody, as follows:

$$\mathrm{An} + \mathrm{X\text{-}R\text{-}X} + \mathrm{Enz} \longrightarrow (\mathrm{An})_x\text{-}(\mathrm{R})_y\text{-}(\mathrm{Enz})_z$$

Abbreviations used: An=antigen or antibody; X–R–X=homo-bifuctional reagent; Enz=enzyme; x, y, or z=0, 1, 2, 3, 4, ·····

Therefore, the yield of the desired product, An–R–Enz, is usually low, and its isolation and purification from the reaction mixture are not easy. In order to overcome these disaadvantages, we introduced a hetero-bifunctional cross-linker, N-(*m*-maleimidobenzoyloxy)-succinimide (MBS), and used it in a new method for the enzyme labelling of hormones and drugs[3,4] and in a new method for the preparation of antisera to drugs.[5] This reagent possesses two selectively reactive groups. One is the N-hydroxysuccinimidyl ester,[6] which reacts with the amino groups of antigen or antibody, and the other is the maleimide residue,[7] which reacts with the thiol groups of proteins. By using this reagent, the desired conjugation can be easily performed by a selective two-step process under aqueous mild conditions. The first step is acylation of amino groups of the antigen or antibody to introduce the maleimide residue (or residues) which is then conjugated with thiol groups of a protein in the second step.



Since MBS has been extremely useful, we have prepared ten reagents of this hetero-bifunctional type which possess various structures, including aromatic and aliphatic reagents. Their physico-chemical properties, especially the reactivities of their active esters with amino groups of amino acids, and the stabilities of their maleimide groups in aqueous solution were investigated to determine suitable conditions for cross-linking, in the hope of finding a practically useful reagent for protein-protein or protein-hapten conjugations. The present paper reports the results.

Materials and Methods

Equipment

All melting points were taken on Yanagimoto micromelting point apparatus and are uncorrected. NMR spectra were obtained in $CDCl_3$ solution on a Varian A-60 spectrophotometer and δ values are given in ppm downfield from the internal standard, tetramethylsilane. UV spectra were recorded on a Hitachi 124 spectrometer and amino acids were analyzed on a JEOL JLC-6 AH amino acid analyzer equipped with a JEOL DK-type integrator.

Chemicals

Amino acids used were bought from Kyowa Hakko Co. Ltd., (Tokyo) and mercaptoethanol and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) from Nakarai Chemicals Co. Ltd., (Kyoto). N-Hydroxysuccinimide and N,N′-dicyclohexylcarbodiimide were purchased from the Protein Research Foundation (Minoh) and silica gel plates from Merck Co., Ltd. (W. Germany). 2-Aminobenzoic acid, 3-aminobenzoic acid, 4-aminobenzoic acid, 5-amino-2-hydroxybenzoic acid, and 4-chloro-3-nitrobenzoic acid were bought from Nakarai Chemicals Co., Ltd. 4-Methoxy-3-nitrobenzoic acid and 3-methoxy-4-nitrobenzoic acid were prepared from the corresponding methoxybenzoic acids according to the methods of Wu and Herbst[8] and Rieche,[9] respectively. 4-Dimethylamino-3-nitrobenzoic acid was prepared from 4-chloro-3-nitrobenzoic acid according to the method of Wang.[10] 3-Amino-4-methoxybenzoic acid,[11] 3-amino-4-dimethylaminobenzoic acid[12] and 4-amino-3-methoxybenzoic acid[13] were prepared by catalytic hydrogenation from the corresponding nitro derivatives according to the methods cited.

Syntheses of Hetero-bifunctional Reagents

Maleanilic Acid Derivatives (I)——All of the maleanilic acid derivatives used in these experiments except for 3-carboxy-4-methoxymaleanilic acid were prepared from the corresponding aminobenzoic acid derivatives and maleic anhydride by a method similar to that of Parola.[14]

3-Carboxy-4-methoxymaleanilic Acid——3-Carboxy-4-hydroxymaleanilic acid (12.0 g), prepared from 5-amino-2-hydroxybenzoic acid by the method described above, was dissolved in 150 ml of 8% sodium hydroxide solution and allowed to react with 27 g of dimethyl sulfate at room temperature for 3.5 hr with stirring. The reaction mixture was then made acidic with 5 N hydrochloric acid and the precipitate formed was collected by filtration. Recrystallization from ethanol gave 3-carboxy-4-methoxymaleanilic acid, mp 209—211°, Yield, 5.5 g. *Anal.* Calcd for $C_{12}H_{11}NO_6$: C, 54.39; H, 4.18; N, 5.28. Found: C, 54.03; H, 4.10; N, 4.96.

General Procedure for the Preparation of Maleimide Acid Derivatives (II)——Cyclization of the maleamic function of maleanilic acids to maleimide derivatives was achieved by a mixed anhydride procedure. However, the aliphatic maleamic function could not be cyclized by this procedure and aliphatic maleimide derivatives were prepared according to Keller and Rudinger.[15]

A mixture of 4.0 g of maleanilic acid (I), 2.0 g of anhydrous sodium acetate, and 40 ml of acetic anhydride was heated at 90—100° for 2—3 hr and then poured into ice-water. The mixture was stirred for 2—3 hr at room temperature, then precipitated crystals were collected by filtration and recrystallized from an appropriate solvent to give N-(carboxyphenyl)maleimide.

In the case of *o*-carboxymaleanilic acid, the reaction was carried out at room temperature for 90 hr. N-(2-Acetoxycarbonylphenyl)isomaleimide; mp 160—167°, was obtained as a precipitate. *Anal.* Calcd for $C_{13}H_9NO_5$: C, 60.23; H, 3.50; N, 5.40. Found: C, 60.58; H, 3.31; N, 5.52. NMR, 7.2—8.2 (4H, m, aromatic), 7.72 (1H, d, olefinic), 6.48 (1H, d, olefinic), 2.02 (3H, s, $-COCH_3$). The desired maleimide derivative was extracted from the aqueous layer with chloroform.

General Procedure for the Preparation of N-(Maleimidobenzoyloxy)succinimide Derivatives (III)——To a solution of maleimidobenzoic acid (II) (2 mmol) and 253 mg (2.2 mmol) of N-hydroxysuccinimide in 40 ml of dry tetrahydrofuran was added 453 mg (2.2 mmol) of N,N′-dicyclohexylcarbodiimide, and the mixture was stirred for 2—4 hr at room temperature. Precipitated N,N′-dicyclohexylurea was filtered off, then the filtrate was evaporated to dryness and the residue was chromatographed on a silica gel column with chloro-

form as an eluent. The eluate was evaporated to dryness *in vacuo* and the residue was recrystallized from ether–methylene chloride to afford N-(maleimidobenzoyloxy)succinimide (III).

Stability Test of the Maleimide Group of the Reagents in Aqueous Solution——Twenty μl of 10 mM sample solution in tetrahydrofuran was incubated in 0.5 ml of 0.05 M sodium phosphate buffer (pH 6.0, 7.0 or 8.0) or citrate buffer (pH 5.0) for 30 min at 30°. Next, 0.2 ml of 1 mM solution of mercaptoethanol was added. The whole was mixed well and 1.1 ml of 0.2 M tris-hydrochloride buffer (pH 8.2) containing 0.02 M ethylenediamine tetraacetate and 0.2 ml of 10 mM methanolic solution of DTNB were added. The absorbance value of test sample (A), a zero time blank value (B) (without the incubation) and the sample blank value (C) (20 μl of tetrahydrofuran instead of the sample solution) were obtained in a similar manner. The percentage decomposition of the maleimide residue was calculated as $(A-B)/(C-B)\times 100$.

The Reactivity of the N-Hydroxysuccinimidyl Ester Group of Reagents toward Amino Acids——A 10 mM solution of lysine or leucine (0.1 ml) was mixed with 0.9 ml of 0.05 M sodium phosphate buffer, pH 7.0, 7.5 or 8.0, and then 0.1 ml of a 1 mM solution of the reagent in tetrahydrofuran was added with vortex mixing. The solution was incubated for 30 min at 30°, then 1.4 ml of 0.01 N hydrochloric acid containing 100 nmol of a standard amino acid (histidine for assay of lysine and alanine for assay of leucine) was added. The amount of free lysine or leucine was measured in an amino acid analyzer by comparison with that of the corresponding standard amino acid.

Results and Discussion

Syntheses of Hetero-bifunctional Reagents

All the aromatic cross-linkers of hetero-bifunctional type were synthesized as shown in Fig. 1.

The chemical formulas of the cross-linkers thus prepared are shown in Fig. 2.

Elemental analyses and melting points of new N-(maleimidobenzoyloxy)succinimide cross-linkers (IIIa—IIIg)[16] are summarized in Table I and their NMR chemical shifts are listed in Table II.

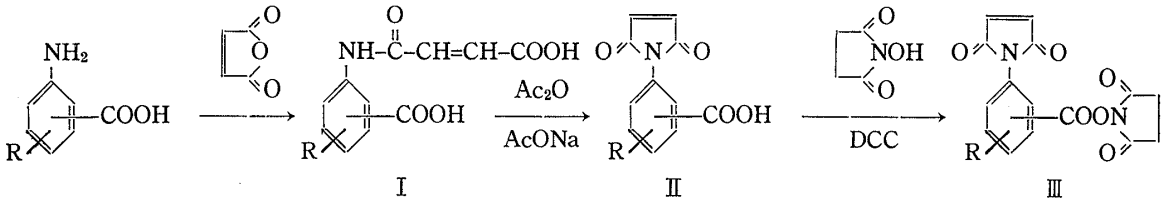


Fig. 1. Scheme for the Preparation of Aromatic Cross-linkers of Hetero-bifunctional Type

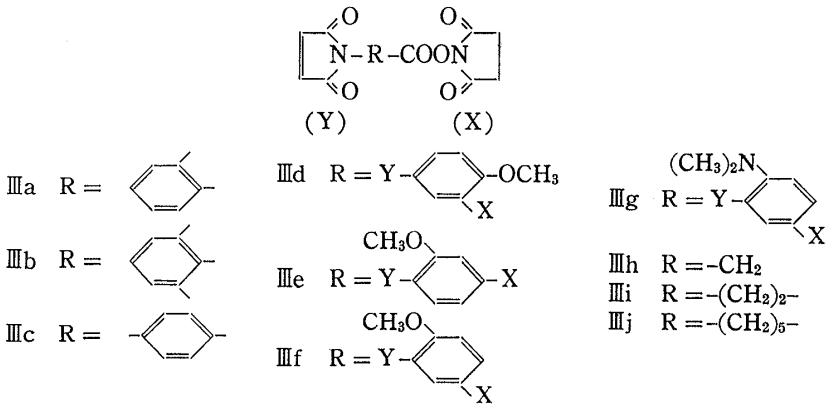


Fig. 2. Chemical Structures of Hetero-bifunctional Reagents used in the Present Experiments

Properties of Hetero-bifunctional Reagents

Hetero-bifunctional reagents that possess selectively high reavctivties to two different functional groups are already available. However, they are easily decomposed, especially in aqueous solution. Therefore, to use the reagents effectively as cross-linkers, their chemical properties should be understood especially in regard to the reactivity of the active ester group and the stability of the maleimide group.

Table I. Physico-chemical Properties of N-(Maleimido benzoyloxy)-succinimides and Their Analogs

	R	Ester	Yield %	mp, °C	Formula	*Anal.* Calcd C	H	N	Found C	H	N
IIIa	H	2-	27	125—128	$C_{15}H_{10}N_2O_6$	57.33	3.21	8.92	57.29	2.95	8.85
IIIb	H	3-	31	182—185	$C_{15}H_{10}N_2O_6$	57.33	3.21	8.92	57.49	3.09	8.84
IIIc	H	4-	28	189—200	$C_{15}H_{10}N_2O_6$	57.33	3.21	8.92	57.56	3.09	8.84
IIId	4-MeO	3-	21	164—166.5	$C_{16}H_{12}N_2O_7$	55.82	3.51	8.14	55.55	3.38	7.83
IIIe	2-MeO	4-	27	229—232	$C_{16}H_{12}N_2O_7$	55.82	3.51	8.14	55.62	3.31	8.04
IIIf	2-MeO	5-	14	206—210	$C_{16}H_{12}N_2O_7$	55.82	3.51	8.14	55.99	3.33	7.72
IIIg	2-Me_2N	5-	16	185—187	$C_{17}H_{15}N_3O_6$	57.14	4.23	11.76	56.91	4.26	11.59

Table II. NMR Chemical Shifts of N-(Maleimidobenzoyloxy)-succinimides and Their Analogs

	R	Ester	Chemical shift: Succinimide	Maleimide	Aromatic	R
IIIa	H	2-	2.82 (4H, s)	6.86 (2H, s)	7.28—7.95 (3H, m) 8.13—8.37 (1H, m)	
IIIb	H	3-	2.98 (4H, s)	6.89 (2H, s)	7.57—7.77 (2H, m) 8.03—8.73 (2H, m)	
IIIc	H	4-	2.90 (4H, s)	6.90 (2H, s)	7.65 (2H, d, J=8.8 Hz) 8.23 (2H, d, J=8.8 Hz)	
IIId	4-MeO	3-	2.88 (4H, s)	6.86 (2H, s)	7.12 (1H, d, J=9.0 Hz) 7.60 (1H, dd, J=9.0 and 2.9 Hz) 8.03 (1H, d, J=2.9 Hz)	3.96 (3H, s)
IIIe	2-MeO	4-	2.91 (4H, s)	6.88 (2H, s)	7.35 (1H, d, J=8.0 Hz) 7.74 (1H, d, J=1.8 Hz) 7.87 (1H, dd, J=8.0 and 1.8 Hz)	3.88 (3H, s)
IIIf	2-MeO	5-	2.89 (4H, s)	6.88 (2H, s)	7.12 (1H, d, J=8.6 Hz) 7.99 (1H, d, J=2.2 Hz) 8.25 (1H, dd, J=8.6 and 2.2 Hz)	3.90 (3H, s)
IIIg	2-Me_2N	5-	2.87 (10H, s)[a]	6.90 (2H, s)	7.00 (1H, d, J=9.1 Hz) 7.77 (1H, d, J=2.3 Hz) 8.04 (1H, dd, J=9.1 and 2.3 Hz)	2.87(10 H,s)[a]

[a] The protons of the succinimide moiety and the dimethylamino group show the same chemical shift.

I Reactivity of the N-Hydroxysucinimidyl Ester Group

The reactivities of N-hydroxysuccinimidyl ester groups of the reagents under various conditions are summarized in Table III.

Every active ester tested was more reactive with lysine than with leucine. Most esters acylated amino acids more rapidly at pH 8.0 than at pH 7.0. The difference in reactivities between pH 7.5 and 8.0 was small, but in some cases the reactivities at pH 8.0 were lower than at pH 7.5.

II Stability of the Maleimide Group

The stability of the maleimide groups of the reagents in several buffers was examined by measuring the decomposition. The maleimide content was determined by back titration of thiol according to the method of Sedlak and Lindsay[17] after addition of excess mercaptoethanol. The results are summarized in Table IV.

The stability of the maleimide group of a test sample depended largely upon the pH of the buffer used. The most stable pH for every sample was slightly acidic, pH 6.0. The rate of decomposition of the maleimide moiety of aromatic samples was greatly promoted by a

Table III. Acylation (%) of Leucine and Lysine by Hetero-bifunctional Cross-linkers in Twenty Minutes on 0.05 M Phosphate Buffers, pH 7.0, 7.5 and 8.0

	pH 7.0		pH 7.5		pH 8.0	
	Leu	Lys	Leu	Lys	Leu	Lys
IIIa	20.4	37.1	19.1	34.5	21.2	32.0
IIIb	21.0	40.9	31.5	44.9	36.0	46.0
IIIc	17.7	28.7	20.1	39.1	26.2	33.8
IIId			31.3	40.4		
IIIe	15.8	26.5	16.6	33.6	20.5	46.3
IIIf	17.5	31.0	33.2	46.5	38.8	48.0
IIIg	9.3	22.6	9.1	28.8	20.6	27.9
IIIh	5.1	21.1	11.3	29.0	14.9	26.0
IIIi	11.9	22.6	13.5	33.0	29.1	35.8
IIIj	9.5	24.0	14.1	27.1	25.3	38.3

Table IV. Decomposition (%) of Maleimide Groups of Hetero-bifunctional Cross-linkers in 30 Minutes in 0.05 M Phosphate (pH 6.0, 7.0 or 8.0) and Citrate (pH 5.0) Buffers at 30° and also in 20 Minutes in 0.05 M Phosphate Buffer, pH 7.5, at 30°

Sample	pH 5.0	pH 6.0	pH 7.0	pH 8.0	pH 7.5
IIIa	3.1	6.2	21.4	69.0	18.8
IIIb	2.9	2.5	7.1	43.8	9.4
IIIc	3.8	6.6	32.0	52.0	37.5
IIId	3.2	1.5	18.3	48.7	29.6
IIIe	17.0	15.2	26.9	44.0	
IIIf	3.0	0.7	2.0	41.0	8.3
IIIg	22.9	18.7	41.9	56.1	
IIIh	5.0	2.4	6.0	12.2	8.9
IIIi	4.7	2.0	6.3	7.5	8.0
IIIj	5.3	3.0	4.5	8.4	5.0

weak base even at pH 8.0, in contrast with that of aliphatic analogs. Marked differences in the decomposition rates of aromatic analogs between pH 7.5 and 8.0 were observed.

Addition of the thiol group of mercaptoethanol to the double bond of the maleimide group of every reagent in all buffers tested (pH 5.0—8.0) proceeded very rapidly, and no appreciable difference in rate was observed, in contrast with the reaction of the active ester.

III Structure-Reactivity Relationships of Cross-Linkers

Aromatic analogs showed remarkable differences both in the reactivities of their active esters and in the stabilities of their maleimide residues, depending upon their structures. Among *o*-, *m*- and *p*-derivative of N-(maleimidobenzoyloxy)succinimide (IIIa, IIIb and IIIc, respectively), the *m*-derivative (IIIb, MBS) possessed the most stable maleimide group, as shown in Table IV. Aliphatic analogs (IIIh—IIIj) did not show such remarkable differences in stability and possessed fairly stable maleimide groups. On the basis of these results an electron-attracting substituent, the active ester group, ortho or para to the maleimide group appears to destabilize the maleimide function and also decreases the activity of the active ester. Thus, compounds possessing an electron-releasing substituent such as a methoxy or dimethylamino group ortho or para to the maleimide moiety were prepared and the reactivities of their active esters toward amino acids and the stabilities of their maleimide residues in buffer solutions were tested. The results obtained are shown in Tables III and IV. Among them, IIIf possesses the most stable maleimide residue under neutral and

slightly acidic conditions. Its active ester is the most reactive one. The best reagent among the aromatic cross-linkers tested was thus IIIf, and IIIb possessed an almost equally advantageous properties. Comparing aromatic reagents such as IIIb and IIIf with aliphatic ones, we found that the former were advantageous in terms of the reactivities of their active esters and the stabilities of their maleimide residues under neutral or slightly acidic conditions, thought the latter showed high stabilities of their maleimide residues under slightly basic conditions (pH 8.0). Compound IIIf, which contains a methoxy substituent as an electron-releasing group possesses the advantages mentioned above, but it also has the disadvantage of water solubility.

IV Suitable Conditions for Use of the Reagents

All of the crystalline reagents of this hetero-bifunctional type tested can be kept safety at 4° for more than one year. All the reagents are unstable in aqueous solution, but are stable in organic solvents. Therefore, they can be dissolved in tetrahydrofuran or dioxane, which mixes freely with water, and the solution can be used immediately for cross-linking in buffer solutions, since none of them dissolve in aqueous solution immediately. The acylation step should be carried out first and the thiol addition step second, since thiol compounds react with the active ester gradually as well as undergoing rapid addition to the maleimide moiety. The pH of the buffer used for the first acylation step should be slightly basic, preferably pH 7.0 to 7.5 for aromatic reagents and 7.3 to 8.0 for aliphatic ones. The reaction time of the first step should be less than 1 hr to avoid decomposition of the reagent, we usually used conditions of 30 minutes at 30°. When the first step is over, the pH of the reaction mixture should be changed to 5.0—6.0 to reduce decomposition of the maleimide group. Addition of a thiol group to a maleimide double bond proceeds very rapidly in any buffer tested over the range of pH 5.0 to 8.0.

References and Notes

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