[Chem. Pharm. Bull. 35(5)1991—1997(1987)]

New Heterobifunctional Cross-Linking Reagents for Protein Conjugation, *N*-(Bromoacetamido-*n*-alkanoyloxy)succinimides

KIYOSHI ZAITSU,[a] MIKIO OHNISHI,[b] HIROYUKI HOSOYA,[a] HIROYUKI SUGIMOTO,[b] and YOSUKE OHKURA*,[a]

Faculty of Pharmaceutical Sciences, Kyushu University 62,[a] Maidashi Higashi-ku, Fukuoka 812, Japan and Wakunaga Pharmaceutical Co., Ltd.,[b] Koda-cho, Takata-gun, Hiroshima 729-64, Japan

(Received October 3, 1986)

Five heterobifunctional reagents, *N*-(bromoacetamido-*n*-alkanoyloxy)succinimides having a glycine or ω-amino acid (β-alanine, γ-aminobutyric acid, δ-aminovaleric acid or ε-aminocaproic acid) residue, were synthesized. Bromoacetamido-*n*-alkanoyl groups could be introduced into horseradish peroxidase and hen egg-white lysozyme and its reduced and *S*-3-(trimethylated amino)propylated product by using the reagents. The number of bromoacetamido-*n*-alkanoyl groups introduced into the proteins could be successfully estimated from the amount of glycine or ω-amino acid (non-protein amino acid) produced from the introduced groups by acid hydrolysis. *N*-(β-Bromoacetamido-*n*-propionoyloxy)succinimide, one of the reagents, was examined as a reagent for the preparation of horseradish peroxidase–insulin conjugate. A single β-bromoacetamido-*n*-propionoyl group was first introduced into Gly^{A1}, Phe^{B1}-dicitraconylinsulin through the ε-amino group of its Lysine^{B29} residue and the product was subjected to decitraconylation with dilute acetic acid to obtain Lys^{B29}-(β-bromoacetamido-*n*-propionoyl)insulin. The insulin was reacted with thiolated horseradish peroxidase to give peroxidase–insulin conjugate (molar ratio of 1 : 1).

Keywords——heterobifunctional cross-linking reagent; protein conjugation; *N*-(bromoacetamido-*n*-alkanoyloxy)succinimide; ω-amino acid; amino acid analysis; enzyme label; horseradish peroxidase; insulin

Bifunctional cross-linking reagents are essential for the preparation of protein–protein conjugates, protein–low molecular compound conjugates, ligand-linked solid matrices and immobilized enzymes, which have been used in biological, physiological, pharmacological and analytical researches. Among these reagents, heterobifunctional reagents are especially useful to bind either the same or different proteins with each other without the undesirable linking inherent in the use of homobifunctional reagents.[1,2] A currently available heterobifunctional reagent, *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP)[3] has been used in the preparation of enzyme-labeled Fab' of immunoglobulin G (IgG),[4,5] enzyme-labeled deoxyribonucleic acid (DNA),[6] calmodulin-linked thiol-Sepharose[7] and antibody-linked liposomes.[8] Combinations of functional groups in other heterobifunctional cross-linking reagents were maleimidyl and succinimidyl groups,[8,9] and chloroformyl and bromoacetyl groups.[10]

This paper describes the synthesis and application of a new series of heterobifunctional cross-linking reagents, *N*-(bromoacetamido-*n*-alkanoyloxy)succinimides (II(*n*), Chart 1). The reagents can react with primary amino group(s) of a protein at the *N*-succinimidyl ester side to yield the protein carrying a bromoacetamido-*n*-alkanoyl group(s), and then with sulfhydryl group(s) of another protein through the introduced bromoacetyl group(s) to give a protein-protein conjugate. These reagents have a glycine residue (II(1)) or one of the ω-amino acid (II(2)—II(5); β-alanine,[11] γ-aminobutyric acid, δ-aminovaleric acid and ε-aminocaproic acid, respectively) residues (Chart 1). This enabled us to develop a powerful method for the estimation of the number of bromoacetamido-*n*-alkanoyl group(s) introduced into protein.

and the molar ratio of protein to protein in a conjugate prepared with II(n) except for II(1).

To verify the utility of the reagents, we investigated the introduction of bromoacetamido-n-alkanoyl groups of II(n) into horseradish peroxidase (HRP), hen egg-white lysozyme and its reduced and *S*-3-(trimethylated amino)propylated product (RT-lysozyme),[12] and we also prepared an HRP–insulin conjugate using II(2) as an example of protein–protein conjugate preparation.

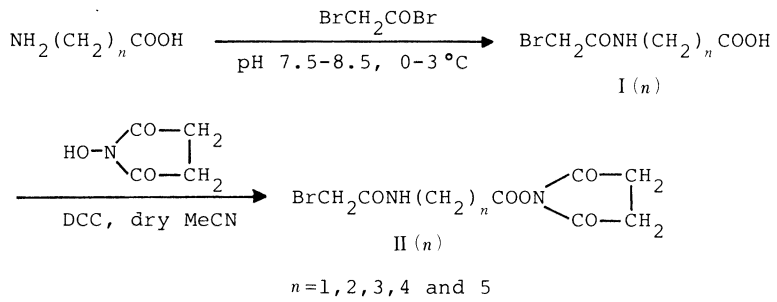

Chart 1. Synthesis of II(n)

II(1), *N*-(bromoacetamidoacetoxy)succinimide; II(2), *N*-(β-bromoacetamido-n-propionoyloxy)succinimide; II(3), *N*-(γ-bromoacetamido-n-butyryloxy)succinimide; II(4), *N*-(δ-bromoacetamido-n-valeroyloxy)succinimide; II(5), *N*-(ε-bromoacetamido-n-caproyloxy)-succinimide.

Experimental

Materials and Apparatus——Deionized water was used, which was filtered through a Milli-QII (Japan Millipore Ltd., Tokyo, Japan) before use. Diisopropyl ether (iso-Pr_2O) was passed through a column of neutral alumina AG 7 (100—200 mesh; Bio-Rad, Richmond, U.S.A.) to remove peroxide, which was checked with Peroxide Test (Japan Merck, Tokyo, Japan). Aqueous urea was passed through an Amberlite MB 3 (mixed resin 20—50 mesh; Bio-Rad) column. HRP (285 purpurogallin units/mg, type VI; Sigma, St. Louis, U.S.A.). Hen egg-white lysozyme and RT-lysozyme[12] (both from Seikagaku Kogyo, Tokyo, Japan) were used. Gly^{A1},Phe^{B1}-Dicitraconylinsulin (porcine) was prepared as described previously.[13] All other chemicals were of reagent grade.

Melting points were measured on a Yanagimoto micromelting point apparatus. Absorbances were measured with a Hitachi 150-20 spectrophotometer in semimicro-quartz cells (10-mm optical path length, 1 ml). Electron impact mass spectra were taken with a JEOL JMS-DX300 mass spectrometer. Fluorescence intensities in the microassay of HRP activity[14] were measured with a Hitachi MPF-4 spectrofluorimeter in semimicro-quartz cells (10-mm width parallel to the excitation beam, 3-mm length parallel to the emission beam, 1 ml).

Chromatographic Conditions——Thin layer chromatography (TLC) was performed on Silica gel F-254 precoated aluminum-backed sheets (Japan Merck) with $CHCl_3$–MeOH (19 : 1, v/v; solvent A) and (4 : 1, v/v; solvent B) as developing solvents. Fractions on the plates were detected by fluorescence quenching under ultraviolet light or by the use of ninhydrin (1% in butanol) spray followed by heating at 50 °C for 1 min after successive treatments with ammonia vapor and AcOH vapor. *N*-Hydroxysuccinimide esters were detected by the ferric chloride method.[15]

Adsorption column chromatography was carried out on a Silica gel 60 (Japan Merck) column (400 × 15 mm i.d.) with stepwise elution using 100 ml of $CHCl_3$ and 200 ml each of $CHCl_3$ solutions containing 0.5, 1, 2, and 3% (v/v) MeOH.

Anion-exchange high-performance liquid chromatography (HPLC) was carried out on a TSK gel DEAE-2SW column (300 × 7.8 mm i.d.; Toyo Soda, Tokyo, Japan) with a Hitachi 655 liquid chromatograph equipped with a 655 proportioning valve pump for gradient elution, a 650—60 recording processor, a Rheodyne 7125 syringe-loading sample injector valve (100-μl loop) and a Toyo Soda UV-8 model II spectrophotomonitor operated at 278 nm. Elution with an NaCl concentration gradient during 40 min was done at a flow rate of 1.4 ml/min with eluant A (0.05 M Na-K phosphate buffer (pH 7.0) containing 4 M urea) and eluant B (eluant A containing 0.5 M NaCl). The NaCl gradient was as follows: the initial eluant contained 90% of eluant A and 10% of eluant B. The content of eluant B was increased to 30% linearly during 20 min and to 100% linearly during another 20 min. The used column was regenerated by passing eluant B for 20 min and then the initial eluant for 20 min. Chromatogram peaks were identified by the 1-fluoro-2,4-dinitro-benzene method.[13,16]

Gel-permeation HPLC was done on a TSK gel G3000SW column (600 × 7.5 mm i.d.; Toyo Soda) with a Waters

M-45 liquid chromatograph equipped with a Rheodyne 7125 syringe-loading sample injector valve (200-μl loop) and a Waters 440 absorbance detector operated at 254 and 405 nm. The mobile phase was 0.05 M Na phosphate buffer (pH 7.0) containing 0.15 M NaCl, and the flow rate was 0.7 ml/min.

Amino Acid Analysis——Performed on a Hitachi 835 amino acid analyzer after hydrolysis of protein samples in 6 M HCl *in vacuo* at 110 °C for 24 h, using a column (150 × 4 mm i.d.) packed with a Hitachi 2619 custom ion-exchanger resin with the sodium citrate buffer system as the mobile phase. The column temperature was 53 °C. The stepwise elution of amino acids was carried out as described in the handling manual[17] except that the concentrations of benzyl alcohol in eluants III and IV described in the manual (0 and 5% (v/v), respectively) were changed to 5 and 10% (v/v), respectively.

Determination of Bromoacetamido-*n*-alkanoyl Groups Introduced into Proteins——The number of bromoacetamido-*n*-alkanoyl groups ($BrCH_2CONH(CH_2)_nCO$–, $n=1$, 2, 3, 4 or 5) incorporated into proteins was determined from the amount of glycine (calculated by subtracting the amount of glycine in intact protein), β-alanine, γ-aminobutyric acid, δ-aminovaleric acid or ε-aminocaproic acid, respectively; the chromatogram peaks of these ω-amino acids (non-protein amino acids) were observed in that order between the peaks for tyrosine and phenylalanine, phenylalanine and lysine, lysine and ammonia, and histidine and arginine, respectively. When the peak area obtained with glycine was taken as 100, those with β-alanine, γ-aminobutyric acid, δ-aminovaleric acid and ε-aminocaproic acid were 51.2, 98.1, 81.1 and 60.1, respectively.

Synthesis of *N*-(Bromoacetamido-*n*-alkanoyloxy)succinimides (II(*n*)) *via* Bromoacetamido-*n*-alkylcarboxylic Acids (I(*n*))——I(1)—I(3) and I(5) (Chart 1) were synthesized by the previous method.[18] I(4) (Chart 1) was prepared by bromoacetylation of δ-aminovaleric acid with bromoacetyl bromide in the same way as reported previously, with some modifications.[18] The reaction was carried out at pH 7.5—8.5 in an ice-water bath. I(1) was obtained in sufficiently pure form. I(2)—I(5) were purified by adsorption chromatography. Fractions (40 ml each) containing I(2)—I(5) were collected, checked by TLC (*Rf* values (solvent A, solvent B): I(2) (0.10, 0.49); I(3) (0.11, 0.57); I(4) (0.13, 0.63); I(5) (0.18, 0.66)), concentrated to dryness and recrystallized from tetrahydrofuran (THF)–iso-Pr_2O. The melting points of I(1)—I(3) were in agreement with reported data.[18] That of I(5) was 78—79 °C, though it was reported to be 69—70 °C.[18] I(4) (mp 77—78 °C, colorless powder, yield 20%) was characterized by the following data. *Anal.* Calcd for $C_7H_{12}BrNO_3$: C, 35.31; H, 5.08; N, 5.88. Found: C, 35.03; H, 5.03; N, 5.86. Mass spectrum (MS) *m/z*: 238 (M^+H), 240 (M^+H+2).

I(*n*) (1.0 mmol) and *N*-hydroxysuccinimide (1.0 mmol) were dissolved in 15 ml of dry MeCN, and then 1.0 mmol of *N*,*N*′-dicyclohexylcarbodiimide (DCC) in 5 ml of dry MeCN was added. The mixture was stirred under protection from moisture at 4 °C overnight. The resulting precipitates were filtered off, the filtrate was concentrated to dryness, and the residue was recrystallized from THF–iso-Pr_2O to give II(*n*) (all colorless needles). The melting points, yields, elemental analyses and MS data are summarized in Table I.

Introduction of Bromoacetamido-*n*-alkanoyl Groups into HRP——HRP (62.5 nmol, 2.5 mg) was dissolved in 500 μl of 0.1 M Na phosphate buffer (pH 7.5), and a 10 to 100-fold molar excess of II(*n*) in 100 μl of MeCN was added under stirring. The mixture was kept at 23 °C for 1 h, dialyzed against 1 l of water (7 times) at 4 °C and lyophilized. The number of bromoacetamido-*n*-alkanoyl groups introduced into HRP was calculated from the amino acid analysis data.

TABLE I. Data for II(*n*)

Compound[a]	mp[b] (°C)	Yield (%)	Formula Molecular weight	Elemental analysis Calcd (Found) (%)			MS peak[c] (*m/z*)		*Rf* in TLC Solvent[d] A	*Rf* in TLC Solvent[d] B
II(1)	119—121	76.1	$C_8H_9BrN_2O_5$ 293.1	32.78 (32.69	3.09 3.14	9.55 9.44)	293 (M^+H)	295 (M^+H+2)	0.21	0.73
II(2)	104—106	50.6	$C_9H_{11}BrN_2O_5$ 307.1	35.19 (35.32	3.61 3.66	9.12 9.32)	307 (M^+H)	309 (M^+H+2)	0.25	0.73
II(3)	98—100	53.3	$C_{10}H_{13}BrN_2O_5$ 321.1	37.40 (37.87	4.08 4.27	8.72 8.70)	321 (M^+H)	323 (M^+H+2)	0.25	0.73
II(4)	78—79	52.4	$C_{11}H_{15}BrN_2O_5$ 335.2	39.42 (39.39	4.51 4.51	8.35 8.42)	334 (M^+) 336 (M^++2)	335 (M^+H) 337 (M^+H+2)	0.27	0.73
II(5)	89—90	69.8	$C_{12}H_{17}BrN_2O_5$ 349.2	41.28 (41.08	4.90 5.03	8.02 7.91)	348 (M^+) 350 (M^++2)	349 (M^+H) 351 (M^+H+2)	0.35	0.76

a) Stable for 3 months when kept dry under protection from light. *b*) Uncorrected. *c*) Assigned by analogy with the peak for M^+H+2 of II(1) (found, *m/z* 294.9777; calcd for $C_8H_{10}{}^{81}BrN_2O_5$, *m/z* 294.9753) in the high-resolution MS. *d*) See the experimental section.

Introduction of Bromoacetamido-*n*-alkanoyl Group into Lysozyme and RT-Lysozyme——Lysozyme or RT-lysozyme (22.5 nmol; 320 and 350 μg, respectively) was dissolved in 360 μl of 0.1 M Na phosphate buffer (pH 7.5) and a 10 to 100-fold molar excess of II(*n*) in 20 μl of dry MeCN was added under stirring. The mixture was maintained at 23 °C for 1 h, dialyzed against 1 l of water (5—7 times) at 4 °C and lyophilized. The number of introduced bromoacetamido-*n*-alkanoyl groups was calculated from the amino acid analysis data.

Preparation of Lys^{B29}-(β-Bromoacetamido-*n*-propionoyl)insulin (BAP-insulin)——A solution of II(2) (17 μmol, 5.2 mg) in 100 μl of MeCN was added to a solution of Gly^{A1},Phe^{B1}-dicitraconylinsulin (0.17 μmol, 1 mg) in 1.0 ml of 0.1 M Na phosphate buffer (pH 7.5) containing 0.1 M NaCl at 30 °C under stirring, and stirring was continued for 1 h. The resulting mixture containing Gly^{A1},Phe^{B1}-dicitraconyl-BAP-insulin was dialyzed against 1 l of 1 M AcOH (pH 2.5) (3 times) at 4 °C. The dialyzate was allowed to stand at 30 °C for 20 h to decitraconylate, dialyzed against 1 l of water (3 times) at 4 °C and lyophilized. The resulting powder was dissolved in 200 μl of 0.1 M Na phosphate buffer (pH 7.0) and subjected to anion-exchange HPLC. The BAP-insulin fraction was dialyzed against 1 l of water (4 times) at 4 °C and lyophilized. The colorless powder (0.5 mg) thus obtained was stored at −20 °C. The molar ratio of BAP group to insulin moiety was estimated by the amino acid analysis data.

Preparation of Gly^{A1},Phe^{B1}-Dicitraconyl-BAP-insulin and Phe^{B1}-Citraconyl-BAP-insulin——Gly^{A1},Phe^{B1}-Dicitraconylinsulin (0.85 μmol, 5 mg) was reacted with II (2) (85 μmol, 26 mg) in the same way as described above using 5 volumes of the solvents. The resulting mixture was dialyzed against 1 l of water (3 times) at 4 °C and lyophilized. The lyophilizate was dissolved in 300 μl of 0.1 M AcOH. This solution was allowed to stand at 4 °C for 10 h, then 3 ml of water was added, and the mixture was neutralized with 2.5 M NaOH. The neutralized mixture was dialyzed against 1 l of water (3 times) at 4 °C and lyophilized again. The resulting powder was dissolved in 400 μl of eluant A. A portion (40 μl) of the solution was subjected to anion-exchange HPLC. The eluates corresponding to the peaks of Gly^{A1},Phe^{B1}-dicitraconyl-BAP-insulin, Phe^{B1}-citraconyl-BAP-insulin and BAP-insulin were collected. This procedure was repeated 10 times and the combined individual eluates were dialyzed against 1 l of water (5 times) at 4 °C and lyophilized. Gly^{A1},Phe^{B1}-dicitraconyl-BAP-insulin (0.3 mg) and Phe^{B1}-citraconyl-BAP-insulin (1.5 mg) were obtained along with BAP-insulin (0.3 mg).

Preparation of Thiolated HRP (HRP-SH)——A 250 μl aliquot of 0.1 M *S*-acetylmercaptosuccinic anhydride in dimethylformamide was added to 5 mg (125 nmol) of HRP in 2.5 ml of 0.1 M Na phosphate buffer (pH 7.5) containing 0.1 M NaCl under stirring at 30 °C, and the mixture was stirred continuously for 1 h. The resulting mixture was dialyzed against 1 l of 0.1 M Na phosphate buffer (pH 6.0, evacuated) at 4 °C. To the dialyzate (2.7 ml), 540 μl of 0.1 M Tris–HCl buffer (pH 7.0, evacuated), 54 μl of 0.1 M ethylenediaminetetra acetic acid (EDTA)·2Na in the Tris–HCl buffer and 190 μl of 1 M hydroxylamine·HCl in the Tris–HCl buffer were added. Immediately after the addition, the pH of the solution was adjusted to 7.3 with 2.5 M NaOH (evacuated) at 30 °C and the mixture was stirred continuously for 20 min. The resulting mixture was dialyzed against 1 l of 0.1 M Na phosphate buffer (pH 6.0) containing 0.05 M NaCl and 5 mM EDTA·2Na (evacuated) (4 times) at 4 °C. A 3.5 ml aliquot of HRP-SH solution (35.7 nmol/ml) was obtained. The number of sulfhydryl groups in the HRP-SH was determined as 2.15 by the 4,4′-dithiodipyridine method.[19]

Preparation of HRP–Insulin (1 : 1) Conjugate——BAP-insulin (150 nmol, 0.9 mg) was dissolved in 700 μl of the HRP-SH solution (evacuated) and the pH of the mixture was adjusted to 8.0 with 2.5 M NaOH (evacuated) under stirring at 30 °C. The mixture was kept at 30 °C for 1 h. The resulting mixture was dialyzed against 1 l of water (3 times) at 4 °C and lyophilized. The resulting powder was dissolved in 200 μl of 0.1 M Na phosphate buffer (pH 7.0) and subjected to gel-permeation HPLC. The eluate from the peak of HRP–insulin was collected, and dialyzed against 1 l of water (2 times) at 4 °C. The dialyzate was concentrated to 2.0 ml (10.3 nmol/ml HRP–insulin conjugate) under vacuum, and the solution was stored at −20 °C. The molar ratio of HRP to insulin in the conjugate was estimated from the amino acid analysis data. The concentration of the HRP–insulin conjugate was calculated from the absorbance at 403 nm, where the absorption is only due to the HRP moiety. The molar absorptivity of HRP at 403 nm was taken as $1.02 \times 10^5\ M^{-1}\ cm^{-1}$.[20]

Results and Discussion

The *N*-succinimidyl ester moiety in the reagents undergoes nucleophilic attack by a primary amine to form an amide bond.[21] Because HRP and its derivatives have been widely used in biological investigations, the introduction of bromoacetamido-*n*-alkanoyl groups into HRP was examined.

Table II shows the number of bromoacetamido-*n*-alkanoyl groups introduced per HRP under the described conditions. The maximum number was 2.1—3.2. A similar result was observed in the introduction of the 3-(2-pyrydyldithio)propionoyl or ε-maleimidocaproyl group into HRP using SPDP or *N*-(ε-maleimidocaproyloxy)succinimide (EMCS),[22] re-

TABLE II. Number of Bromoacetamido-*n*-alkanoyl Groups Introduced into HRP[a]

Reagent	Reagent/HRP (mol/mol) in the reaction				
	10	15	25	50	100
II(1)	0.5	0.6	1.0	1.6	2.3
II(2)	0.8	1.1	1.5	1.8	2.1
II(3)	1.3	1.6	2.2	2.5	2.6
II(4)	1.2	1.8	2.3	2.9	3.2
II(5)	1.3	1.8	2.1	2.6	2.6

a) Based on 23 residues of alanine in an HRP molecule.

TABLE III. Number of II(*n*) Moieties Introduced into Lysozyme and RT-Lysozyme[a]

Reagent	Lysozyme or RT-lysozyme[b]	Reagent/lysozyme or RT-lysozyme (mol/mol) in the reaction				
		10	15	25	50	100
II(1)	L	0.7	1.2	1.3	4.5	5.8
	RT-L	1.2	1.6	2.7	6.0	7.6
II(2)	L	5.5	6.8	7.7	8.1	10.9
	RT-L	6.1	7.8	9.1	9.7	11.1
II(3)	L	3.0	4.1	5.0	7.1	8.7
	RT-L	4.0	5.0	7.3	8.5	9.6
II(4)	L	3.4	4.5	5.1	6.4	7.9
	RT-L	4.7	6.7	7.5	8.5	9.6
II(5)	L	4.5	5.7	6.5	8.8	10.0
	RT-L	6.4	8.1	8.7	9.9	10.5

a) Based on 12 residues of alanine in a lysozyme molecule. *b*) L, lysozyme; RT-L, RT-lysozyme.

spectively (data not given). There is no evidence of alkylation of lysine or histidine residues of HRP, indicating that the reaction occurred only at the *N*-succinimidyl ester side of II(*n*). These observations suggest that II(*n*) can be used for controlled introduction of bromoacetamido-*n*-alkanoyl groups into protein amino groups.

The reactions of II(*n*) with lysozyme and RT-lysozyme were also investigated (Table III). Lysozyme is useful to examine various reactions because the protein contains all the kinds of amino acid residues that occur in natural proteins. The number of II(*n*) moieties introduced into RT-lysozyme was greater than that for lysozyme. This can be explained on the basis of the unfolded structure of RT-lysozyme. The introduction of more than 7 II(*n*) moieties was observed, though lysozyme and RT-lysozyme both have 7 amino groups. The reason for this result is not known.

In general, haloacyl groups readily react with the thiolate ion, and under certain conditions, they also react with other nucleophilic sites (amino, imidazole and thioether groups) of proteins.[23] Thus, II(*n*) should be usable for cross-linking between a protein without a reactive sulfhydryl group and a protein with a reactive sulfhydryl group.

Because of our interest in the use of enzymelabeled insulin in an assay of insulin receptor, we prepared HRP–insulin conjugate by using II(2) (Chart 2). HRP was selectively linked to the ε-amino group of the lysine^{B29} residue of insulin. In the first step, a single BAP group was introduced into Gly^{A1},Phe^{B1}-dicitraconyl-insulin to obtain Gly^{A1},Phe^{B1}-dicitraconyl-BAP-insulin. Then, the citraconyl groups of Gly^{A1},Phe^{B1}-dicitraconyl-BAP-

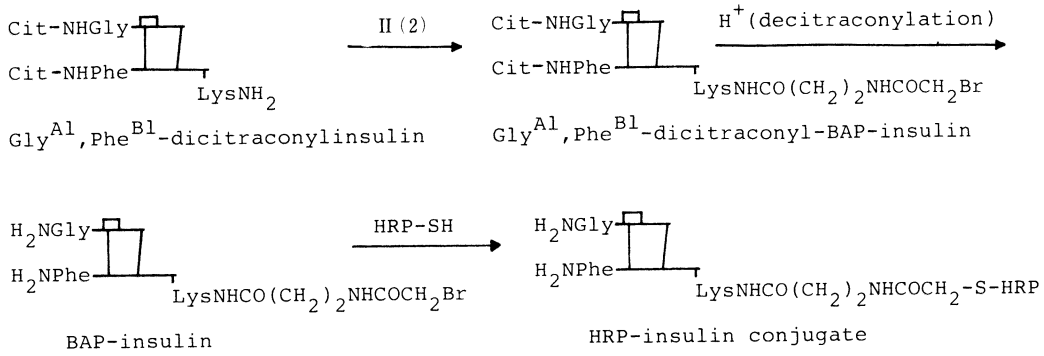

Chart 2. Introduction of a Single BAP Group into Gly^{A1},Phe^{B1}-Dicitraconylinsulin and Preparation of HRP–Insulin Conjugate

Cit: citraconyl.

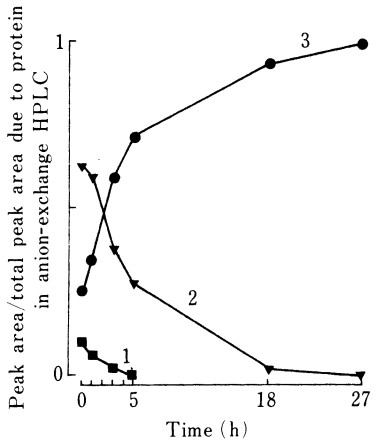

Fig. 1. Time-Course of Decitraconylation of Gly^{A1},Phe^{B1}-Dicitraconyl-BAP-Insulin and Phe^{B1}-Citraconyl-BAP-Insulin

The dialyzate obtained after the reaction of II (2) with Gly^{A1},Phe^{B1}-dicitraconylinsulin in the procedure for the preparation of BAP-insulin was allowed to stand at 30 °C for various periods (1—27 h) in the presence of 1 M acetic acid. Portions (10 μl) of the dialyzate and the mixtures at various reaction periods were subjected to anion-exchange HPLC. Curves: 1, Gly^{A1},Phe^{B1}-dicitraconyl-BAP-insulin; 2, Phe^{B1}-citraconyl-BAP-insulin; 3, BAP-insulin.

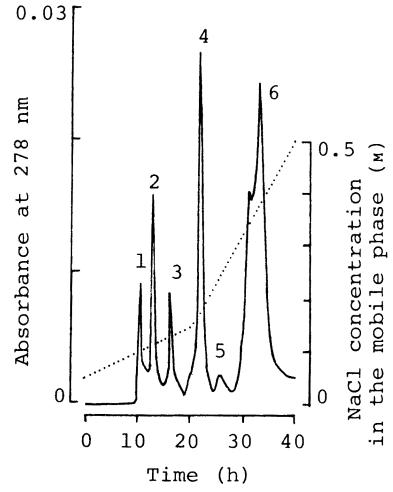

Fig. 2. Chromatogram of Insulin Derivatives

A portion (60 μl) of the neutralized mixture obtained in the preparation of Gly^{A1},Phe^{B1}-dicitraconyl-BAP-insulin and Phe^{B1}-citraconyl-BAP-insulin was subjected to anion-exchange HPLC. Peaks: 1, insulin; 2, BAP-insulin; 3, citraconic acid; 4, Phe^{B1}-citraconyl-BAP-insulin; 5, Gly^{A1},Phe^{B1}-dicitraconyl-BAP-insulin; 6, unknown (non-protein).

insulin were eliminated with 1 M acetic acid to obtain BAP-insulin. The time-course of the decitraconylation could be followed by anion-exchange HPLC (Fig. 1). A chromatogram of the neutralized mixture obtained in the preparation of Gly^{A1},Phe^{B1}-dicitraconyl-BAP-insulin and Phe^{B1}-citraconyl-BAP-insulin is shown in Fig. 2. The decitraconylation had to be carried out prior to the conjugation with HRP, otherwise a marked deactivation of HRP was observed. The BAP group of the insulin was stable in the acidic medium. On the other hand, when EMCS was used instead of II(2), the ε-maleimidocaproyl group introduced into Gly^{A1},Phe^{B1}-dicitraconylinsulin was destroyed by the acid treatment (data not given). These

observations suggest that the acid-resistant nature of the bromoacetyl group can be effectively employed in the course of preparation of protein conjugates.

The reaction of the HRP-SH with an approximately 6-fold molar excess of BAP-insulin gave HRP–insulin (1 : 1) conjugate. Excess BAP-insulin could be recovered easily by gel-permeation HPLC because the molecular size of BAP-insulin is much smaller than that of HRP–insulin conjugate. The molar ratio of HRP to insulin in the conjugate could be calculated as 1 : 1.01 from the results of amino acid analysis of the conjugate. In the calculation, the molar ratio of β-alanine to alanine in BAP-insulin (1 : 2) was used and the amount of alanine derived only from the HRP moiety of the conjugate could be estimated by using the amount of β-alanine. The principle of the method should be applicable for the estimation of the molar ratio of protein to protein in other protein–protein conjugates prepared with II(n) except for II(1).

The HRP–insulin conjugate retained 66.7% of native HRP activity. The actual use of the conjugate will be described elsewhere.

Acknowledgements We wish to thank Miss N. Tohjyo for superb technical assistance, Messrs. M. Shido and M. Abe for elemental analysis and Mr. R. Isobe for MS measurement. We also wish to thank Drs. T. Imoto and H. Yamada for their helpful suggestions and Toyo Soda Manufacturing Co., Ltd. for the generous gift of HPLC columns.

References and Notes

1) F. Wold, *Methods Enzymol.*, **28**, 623 (1972).
2) K. Peters and F. M. Richards, *Annu. Rev. Biochem.*, **46**, 523 (1977).
3) J. Carlsson, H. Drevin, and R. Axen, *Biochem. J.*, **173**, 723 (1978).
4) M. Imagawa, S. Hashida, E. Ishikawa, and A. Sumiyoshi, *J. Appl. Biochem.*, **4**, 400 (1982).
5) K. Sugimoto, K. Zaitsu, and Y. Ohkura, *Anal. Chim. Acta*, **169**, 133 (1985).
6) R. Fallon and A. Malcolm, *Biochem. Soc. Trans.*, **13**, 367 (1985).
7) R. L. Kincaid and M. Vaugham, *Biochemistry*, **22**, 826 (1983).
8) A. Goundalkar, T. Ghose, and M. Mezei, *J. Pharm. Pharmacol.*, **36**, 465 (1983).
9) T. Kitagawa and T. Aikawa, *J. Biochem.* (Tokyo), **79**, 233 (1976).
10) M. Ohnishi, H. Sugimoto, H. Yamada, T. Imoto, K. Zaitsu, and Y. Ohkura, *Chem. Pharm. Bull.*, **33**, 674 (1985).
11) M. Ohnishi, H. Sugimoto, H. Yamada, T. Imoto, K. Zaitsu, and Y. Ohkura; A report on the reagent II(2) was presented at the 103rd Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, April 1983.
12) K. Okazaki, T. Imoto, and H. Yamada, *Anal. Biochem.*, **145**, 87 (1985).
13) K. Zaitsu, H. Hosoya, Y. Hayashi, H. Yamada, and Y. Ohkura, *Chem. Pharm. Bull.*, **33**, 1159 (1985).
14) K. Zaitsu and Y. Ohkura, *Anal. Biochem.*, **109**, 109 (1980).
15) D. D. Pless, S. Roseman, Y. C. Lee, and R. L. Schnaar, *J. Biol. Chem.*, **258**, 2340 (1983).
16) Y. Nakagawa and G. E. Perlmann, *Arch. Biochem. Biophys.*, **149**, 476 (1972).
17) The handling manual for the Hitachi 835 amino acid analyzer, No. 835-1978, Hitachi Co., Tokyo, Japan.
18) H. Yamada, F. Uozumi, A. Ishikawa, and T. Imoto, *J. Biochem.* (Tokyo), **95**, 503 (1984).
19) D. R. Grassetti and J. F. Murray, Jr., *Arch. Biochem. Biophys.*, **119**, 41 (1967).
20) G. R. Schobaum and S. Lo, *J. Biol. Chem.*, **247**, 3353 (1972).
21) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Am. Chem. Soc.*, **86**, 1839 (1964).
22) O. Keller and J. Rudinger, *Helv. Chim. Acta*, **58**, 531 (1975).
23) A. Fontana and C. Toniolo, "The Chemistry of the Thiol Group," part 1, ed. by S. Pattai, John Wiley and Sons, Inc., New York, 1974, pp. 271—324.