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Trifunctional Reagents for Derivatizing Sulfhydryl Groups

FRANCES M. FINN,[1] KEITARO YAMANOUCHI,[2] GAIL TITUS, AND KLAUS HOFMANN

Protein Research Laboratory and the Department of Medicine, University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania 15261

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The syntheses of four trifunctional reagents for alkylating sulfhydryl groups in proteins are described: *N*-γ-maleimidobutyrylbiocytinyl-tyramine (compound I) and its sulfone, *N*-γ-maleimidobutyrylbiocytinyltyrosine (compound II), and N^{α}-4-maleimidobutyrylbiocytinamido-2′-[*p*-(hydroxyphenyl)-propionamido] ethane (compound III). Each reagent contains a maleimide function capable of reacting with SH groups, a *p*-hydroxyphenyl group that can be iodinated, and a "biotin handle" to facilitate purification of the derivatized proteins or peptides derived from them by biotin–avidin affinity chromatography. Detailed conditions for obtaining the pure di-iododerivatives of the compounds have been developed. The biotin is attached to all the reagents via the ε-amino group of lysine (biocytin) to provide sufficient space for optimum binding to avidin. The half-times ($t_{1/2}$) for dissociation of compound I from succinoyl avidin (36.7 days), its monoiodo (26.1 days) and di-iodo derivatives (21.4 days), and compound I sulfone (29.8 days), demonstrate that iodination does not significantly interfere with binding of the biotin residue to succinoyl avidin and that these reagents can be used effectively as affinity ligands. Remarkably, all the reagents can be iodinated without loss of their sulfhydryl alkylating capacity. Alkylation of highly purified human placental insulin receptor with the di-iodo derivatives of the reagents resulted in significant incorporation of ^{125}I into the β-subunit of the receptor and the alkylation was prevented by prior exposure of the receptor to NEM. The advantages of these reagents over those previously available are that the parent molecules (1) are inexpensive to prepare, (2) are solids that can be stored indefinitely without degradation, (3) and can be radiolabeled to specific activity levels over seventy times higher with ^{125}I than the specific activity available for ^{3}H derivatives.

INTRODUCTION

Recent advances in protein purification and sequencing methods have made possible the isolation and structure determination of subnanomolar quantities of a number of biologically interesting proteins. Often these proteins are present in very low copy numbers in the cell. Insulin receptor is a typical case. The purified receptor is available only in microgram quantities. In attempts to perform structure–function studies with this protein we recognized the need for group-specific

[1] To whom correspondence should be addressed.

[2] Presents address: Department of Veterinary Physiology, Veterinary Medical Science, University of Tokyo, Tokyo, Japan.

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reagents that contained a potential affinity ligand and that could be labeled to high specific radiactivity.

Specifically the problem we wish to address is to locate a particular sulfhydryl group in insulin receptor. Exposure of the insulin receptor to *N*-ethylmaleimide (NEM)[3] abolishes the tyrosine phosphorylation activity of the receptor. We have previously shown that NEM alkylation occurs only on a single sulfhydryl group in the receptor (*4*) and that alkylation is inhibited when the ATP binding site is occupied with either ATP-Mg or AMP-PMP-Mg (*5*). This suggests that this sulfhydryl group is located at, or near, the ATP binding site.

Derivatizing functionally important thiol groups in proteins and determining the site of derivatization still represent formidable undertakings. The commercial reagents available to solve these problems fall into two categories, radiolabeled compounds, e.g., [^{3}H]*N*-ethylmaleimide and the newer, unlabeled maleimide (*6*) and pyridyldithio reagents containing biotin. We realized that a reagent incorporating both these features, radioactivity and biotin, could have broad application in protein modification. The maleimide function has been used extensively in derivatizing thiols and the biotin facilitates purification of derivatized molecules through biotin–avidin affinity chromatography. Having a site that can be iodinated is an especially attractive feature as the specific activity of the reagent can be varied and higher specific activity can be achieved with either ^{125}I or ^{131}I than is the case with commercially available ^{3}H- or ^{14}C-labeled reagents. Thus, we designed a class of maleimide reagents whose structure incorporates all of these features.

In this communication we describe the syntheses of four "trifunctional" reagents (Fig. 1) containing a maleimide function to alkylate SH groups, a *p*-hydroxyphenyl group that can be iodinated, and a biotin to facilitate purification. The utility of these reagents is established by their ability to alkylate purified insulin receptor specifically.

MATERIALS AND METHODS

Materials

Thin layer chromatography plates (0.25 mm silica gel) were obtained from Brinkman Instruments, Inc. (Westbury, NY). Biotin-OSu, biocytin, carbonic anhydrase, NEM, and *p*-(dimethylamino) cinnamaldehyde were from Sigma; IRA-

[3] Abbreviations used: Boc, tert-butyloxycarbonyl; biocytin, N^{ϵ}-biotinyllysine; CMC metho-*p*-toluene sulfonate, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulfonate; DCC, *N*, *N'*-dicyclohexyl-carbodiimide; DIPEA, diisopropylethylamine; DMF, dimethylformamide; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); NEM, *N*-ethylmaleimide; HPLC, high performance liquid chromatography; HOBt, 1-hydroxybenztriazole; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; Msc, 2-(methylsulfonyl)ethyloxycarbonate; MscOSu, 2-(methylsulfonyl)ethyl succinimidyl carbonate; OSu, *N*-hydroxysuccinimide ester; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SHPP, succinimidyl-3-(4-hydroxyphenyl) propionate; Sulfo-SHPP, sulfosuccinimidyl-3-(4-hydroxyphenyl) propionate; TLC, thin-layer chromatography; TEA, triethylamine; TFA, trifluoroacetic acid.

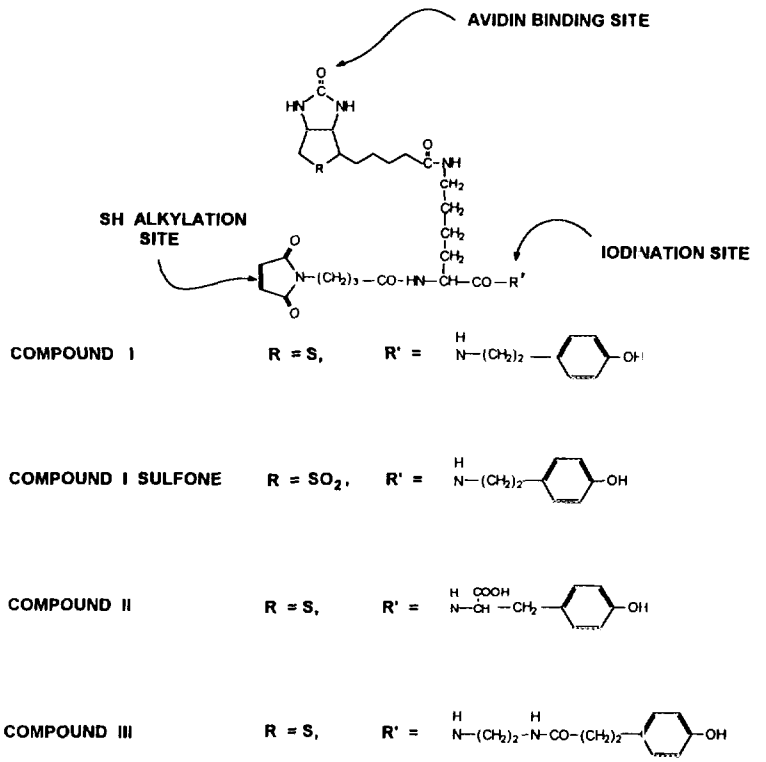


FIG. 1. Simplified structures of the reagents.

400 resin and *N*-γ-maleimidobutyryl-OSu from CalBiochem-Novabiochem; DTNB, Msc, HOBt, and CMC (metho-*p*-toluenesulfonate) from Aldrich Chemical Co.; Fluorescamine from Roche Diagnostics; Iodogen, sulfo SHPP, and SHPP from Pierce; and Disposaflex columns were from Kontes. Succinoyl avidin was prepared as previously described (*5*).

General Methods

Thin layer chromatography was performed in the following systems: (R_f^{I}) 1-butanol–glacial acetic acid–water, 60 : 20 : 20; (R_f^{II}) 1-butanol–pyridine–glacial acetic acid–water, 30 : 20 : 6 : 24. Compounds were visualized on thin-layer plates by fluorescamine (*7*) and the biotin reagent, *p*-(dimethylamino) cinnamaldehyde (*8*).

Analytical HPLC was performed with a Waters instrument. A linear gradient from 0–100% pump B was performed over a period of 35 min at 1 ml/min (pump A solvent, 0.1% trifluoroacetic acid; pump B solvent, 70 : 30 acetonitrile : water, uv adjusted to match the trifluoroacetic acid solution). Preparative HPLC was performed with a Waters 600 Multisolvent Delivery System. Waters μBondapak C_{18} columns were employed both for analytical and preparative chromatography.

The same solvent composition was used for preparative HPLC. The gradient was the same as for analytical separations unless otherwise stated; 18 ml/min pumping speed.

Carbonic anhydrase (6 mg, 207 nmol) was reacted with NEM (3.75 mg, 30 μmol) in 50 mM Hepes buffer, pH 7.6 (1 ml), for 1 h. Unreacted NEM was removed by chromatography on Sephadex G-100 in 5% acetic acid. Fractions corresponding to the protein were combined, neutralized with ammonium hydroxide, and lyophilized.

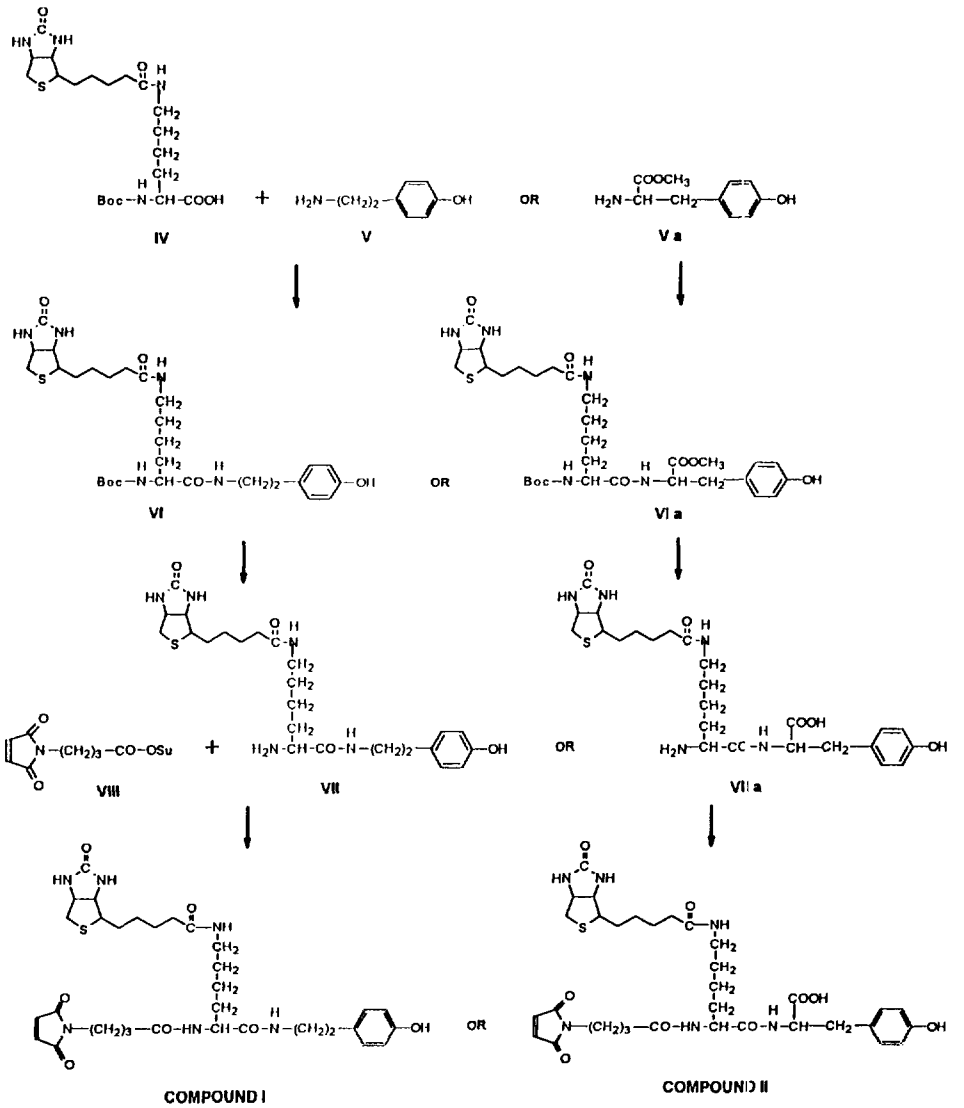
Thiol alkylating ability was assessed by titrating an excess of glutathionine. Unreacted glutathione was measured by reaction with DTNB according to the procedure of Riddles *et al.* (*9*).

Syntheses

N^{α}-Boc-biocytin (IV). DIPEA (68 μl, 0.4 mmol) was added to a solution of N^{α}-Boc-L-lysine (98 mg, 0.4 mmol) and biotin-OSu (136 mg, 0.4 mmol) in 80% aqueous DMF (10 ml) and the solution was stirred at room temperature for 20 h. The solvent was evaporated to leave a crystalline residue (Boc-biocytin DIPEA salt). For conversion to the title compound the salt was triturated with ice-cold 0.1 N HCl (10 ml) to give a crystalline material which was washed with three portions of ice water (5 ml each) and dried. Yield 143 mg (76%); TLC behavior, R_f^{I} 0.61, R_f^{II} 0.65, single biotin positive, fluorescamine negative spot. Single biotin positive, fluorescamine negative peak at 21.8 min on HPLC.

N^{α}-Boc-biocytinyltyramine (VI) (*Scheme 1*). 1,1′-Carbonyl-di-imidazole (24 mg, 0.15 mmol) was added to a solution of N^{α}-Boc-biocytin (48 mg, 0.1 mmol) in dry DMF, (2.5 ml) and the solution was stirred for 2 h at room temperature. Tyramine (14 mg, 0.1 mmol) was added and the mixture was stirred at room temperature for 20 h. The DMF was removed *in vacuo* and the product was purified by HPLC. The desired fractions were pooled, TFA ions were exchanged for acetate ions on IRA-400, and the solution was evaporated to dryness. Yield 51.5 mg (85%). Single biotin positive, fluorescamine negative peak at 24.4 min on HPLC.

N^{α}-Boc-biocytinyltyrosine (VIa) (*Scheme 1*). 1,1′-Carbonyl-di-imidazole (49 mg, 0.3 mmol) was added to a solution of N^{α}-Boc-biocytin (96 mg, 0.2 mmol) in dry DMF, (5 ml) and the solution was stirred for 2 h at room temperature, and then L-tyrosine methyl ester hydrochloride (47 mg, 0.2 mmol) was added and stirring was continued for 20 h at room temperature. The DMF was removed *in vacuo*. Methanol (2.5 ml) and 1 N NaOH (1.0 ml) were added, the solution was stirred for 6 h at room temperature, acidified with 1 N HCl to Congo blue, and the solvent was removed *in vacuo*. The residue was dissolved in 50% aqueous acetonitrile (8 ml), the solution was diluted to 50 ml with 0.1% TFA, and the material was purified by preparative HPLC to give 64 mg (49%) of Boc-biocytinyltyrosine. TLC behavior, R_f^{I} 0.63, R_f^{II} 0.65, single biotin positive, fluorescamine negative spot. Single biotin positive, fluorescamine negative peak at 22.7 min on HPLC. Shorter saponification times resulted in contamination of the product with a material eluting 2 min after the desired product on HPLC. This was identified by high resolution mass spectrometry as the methyl ester of VIa. The value for the $MH^{\oplus}$ ion, deter-



SCHEME 1. Synthetic route to compounds I and II.

mined by high resolution mass spectrometry, agreed with the molecular weight expected for $C_{34}H_{47}O_9N_6S$: Calculated, 715.3125: Found, 715.3092.

Biocytinyltyramine TFA salt (VII). N^{α}-Boc-biocytinyltyramine (138 mg, 0.23 mmol) was dissolved in 10 ml of 95% aqueous TFA and the solution was kept at room temperature for 1 h. The TFA was evaporated, the product was precipitated with ice-cold ether, and the suspension was kept at −20°C for 1 h in a freezer. The solid was collected, washed with ether, and dried *in vacuo*. Yield 129 mg

(93%). TLC behavior, R_f^I 0.43, R_f^{II} 0.68, single biotin positive, fluorescamine positive spot. Single biotin positive, fluorescamine positive peak at 17.8 min on HPLC.

Biocytinyltyrosine TFA salt (VIIa). The HPLC purified Boc-biocytinyltyrosine was deprotected with TFA in the usual manner: yield 50 mg of the TFA salt (VIIa). TLC behavior, R_f^I 0.44, R_f^{II} 0.57, single biotin positive, fluorescamine positive spot. Single biotin positive, fluorescamine positive peak at 17.7 min on HPLC.

N-γ-Maleimidobutyrylbiocytinyltyramine (I). To an ice-cold solution of the TFA salt VII (59 mg, 0.1 mmol) in 0.5 ml of DMF was added DIPEA (10% solution in DMF, 171 μl, 0.1 mmol), followed by *N*-γ-maleimidobutyryl-OSu (VIII) (28 mg, 0.1 mmol), and the clear solution was stirred at ice-bath temperature for 2 h. After 1 h of stirring, the solution formed a gel which was triturated with ether to give a colorless powder. This was washed with several portions of ether and dried. Yield 58 mg (88%). This material was purified by HPLC. TLC behavior, R_f^I 0.48, single biotin positive, fluorescamine negative spot. In system II, some fluorescamine positive tailing was observed presumably due to maleimide ring opening under these basic conditions. The major biotin positive spot was R_f^{II} 0.71. Single biotin positive, fluorescamine negative peak at 21.5 min on HPLC. The value for the $MH^{\oplus}$ ion, determined by high resolution mass spectrometry, agreed with the molecular weight expected for $C_{32}H_{45}N_6O_7S$: Calculated, 657.3070; Found, 657.3099.

Sulfone of compound I. N^{α}-Boc-biocytin (IV) was converted to the sulfone with H_2O_2 in glacial acetic acid (*10*) and the sulfone was reacted with tyramine as follows: DCC (45 mg, 0.22 mmol) was added to a solution of N^{α}-Boc-biocytin sulfone (100 mg, 0.2 mmol), HOBt (31 mg, 0.2 mmol), tyramine (28 mg, 0.2 mmol), and DIPEA (34 μl, 0.2 mmol) in DMF (5 ml) and the mixture was stirred for 20 h at room temperature. The DMF was evaporated and the crude product purified by HPLC. Fractions containing the desired material were pooled, TFA ions were exchanged for acetate ions on IRA-400, and the solvent was removed: yield 116 mg (93%). TLC behavior, R_f^I 0.38, R_f^{II} 0.54, single biotin positive, fluorescamine negative spot. Single biotin positive, fluorescamine negative peak at 21.6 min on HPLC. The Boc group was removed with 95% TFA (5 ml) for 1 h at room temperature: yield 92 mg (78%) of the TFA salt. TLC behavior, R_f^I 0.33, R_f^{II} 0.54, single biotin positive, fluorescamine positive spot. Single biotin positive, fluorescamine positive peak at 16.1 min on HPLC. The TFA salt (64 mg, 0.1 mmol) was acylated with *N*-γ-maleimidobutyryl-OSu in the manner described for the preparation of compound I and the crude product was purified by HPLC: yield 58 mg (84%). TLC behavior, R_f^I 0.47, R_f^{II} 0.54, single biotin positive, fluorescamine negative spot. Single biotin positive, fluorescamine negative peak at 19.1 min on HPLC. The value for the $MH^{\oplus}$ ion, determined by high resolution mass spectrometry, agreed with the molecular weight expected for $C_{32}H_{45}N_6O_9S$: Calculated, 689.2969; Found, 689.2997.

N-γ-Maleimidobutyrylbiocytinyltyrosine (II). To an ice-cold solution of the TFA salt VIIa (53 mg, 0.08 mmol) in 0.5 ml of DMF was added DIPEA (27 μl, 0.16 mmol, 10% in DMF), followed by N-γ-maleimidobutyryl-OSu (VIII) (23 mg, 0.08 mmol), and the clear solution was stirred at ice-bath temperature for 2 h. Ether

(10 ml) was added and the resulting sticky precipitate was washed with several portions of ether. The material was purified by preparative HPLC. The purified compound was lyophilized from dilute acetic acid: yield 48 mg (86%). TLC behavior, R_f^I 0.53, single biotin positive, fluorescamine negative spot; R_f^{II} 0.57, biotin positive with tailing (see compound I). Single biotin positive, fluorescamine negative peak at 20.7 min on HPLC. The value for the $MH^{\oplus}$ ion, determined by high resolution mass spectrometry, aggreed with the molecular weight expected for $C_{33}N_{45}N_6O_9S$: Calculated, 701.2969; Found, 701.2945.

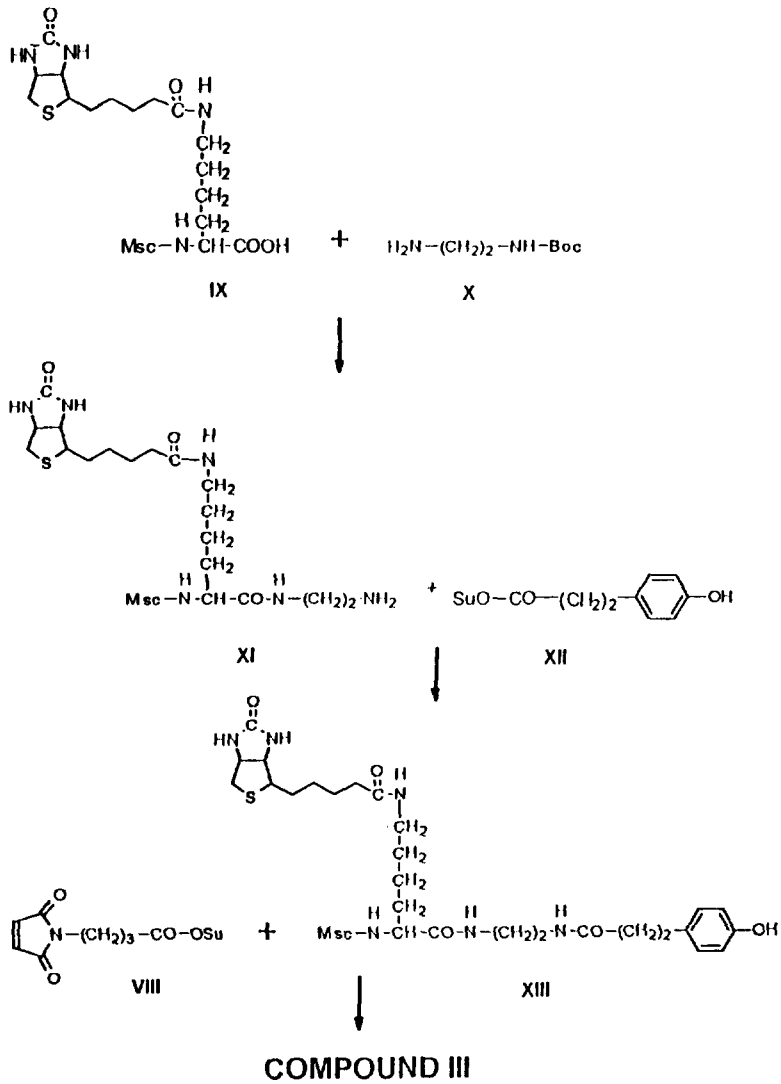
N^{α}-Msc-Biocytin (IX). TEA (276 μl, 2.0 mmol) was added to a solution of biocytin (745 mg, 2.0 mmol) and MscOSu (584 mg, 2.2 mmol) in 50% aqueous acetonitrile (26 ml). The solution was stirred at room temperature for 16 h when the product was precipitated with 2 N $KHSO_4$ (250 μl). Methanol (5 ml) was added and the suspension was filtered. The precipitate was dried *in vacuo* over P_2O_5. Yield, 971 mg (93%); TLC behavior R_f^I 0.22, R_f^{II} 0.55, single biotin positive, fluorescamine negative spot. Single biotin positive, fluorescamine negative peak at 18.8 min on HPLC. $MH^{\oplus}$ ion determined by mass spectrometry was 523. This value agrees with the molecular weight expected for $C_{20}H_{35}N_4O_8S_2$.

N^{α}-Msc-Biocytinamido 2-aminoethane (XI) (Scheme 2). To a solution of Msc-biocytin (IX) (392 mg, 0.75 mmol) in 50% aqueous acetonitrile (7.5 ml) was added mono Boc-1,2-ethanediamine (X) (*11*), (132 mg, 0.83 mmol), TEA (114 μl 0.83 mmol), HOBt (126 mg, 0.83 mmol), and CMC metho-*p*-toluene sulfonate (352 mg, 0.83 mmol). The solution was stirred at room temperature for 16 h, the solvent was evaporated, and the residual oil was washed with three portions of ethyl acetate. The residue was dissolved in 1-butanol–pyridine–glacial acetic acid–water (30 : 20 : 6 : 24) (2 ml) and was applied to a silica gel column (52 × 3 cm) equilibrated with the same solvent system. Fractions free from starting material were combined and evaporated to dryness. The residue, in 50-mg portions, was purified by preparative HPLC. Yield, 348 mg (70%); TLC behavior, R_f^I 0.16, R_f^{II} 0.69, single biotin positive, fluorescamine positive spot. Single biotin positive, fluorescamine positive peak at 22.5 min on HPLC. $MH^{\oplus}$ ion determined by mass spectrometry was 665. This value agrees with the formula weight for $C_{27}H_{49}N_6O_9S_2$.

The material was deprotected with TFA in the usual manner and TFA ions were exchanged for acetate ions on IRA-400. TLC behavior R_f^{II} 0.51, single biotin positive, single fluorescamine positive spot. Single biotin positive, fluorescamine positive peak at 16.9 min on HPLC.

N^{α}-Msc-Biocytinamido-2'-[p-(hydroxyphenyl)-propionamido] ethane (XIII). Compound XI (0.1 mmol) was dissolved in water (2.5 ml) and SHPP (33.4 mg, 0.12 mmol) in acetonitrile (2.5 ml) and DIPEA (17.1 μl, 0.1 mmol) were added. The solution was stirred at room temperature for 20 h. For purification by HPLC the following gradient was used: 0–20% pump B, 7 min; 20–49% pump B, 20 min; 49–100% pump B, 18 min (at 18 ml/min pumping speed). Fractions corresponding to the desired product were collected and evaporated to dryness; Yield 52 mg (73%). TLC behavior, R_f^I 0.41, R_f^{II} 0.56, single biotin positive, fluorescamine negative spot. Single biotin positive, fluorescamine negative peak at 20.5 min on HPLC.

N^{α}-4-Maleimidobutyryl-biocytinamido-2'-[p-hydroxyphenyl)-propionamido] ethane (III). The Msc group was cleaved from compound XIII (52 mg, 0.07 mmol)



SCHEME 2. Synthetic route to compound III.

by the procedure of Tesser and Balvert-Geers (*12*). TLC behavior, R_f^{I} 0.38, R_f^{II} 0.59, single biotin positive, fluorescamine positive spot. Single biotin positive, fluorescamine positive peak at 18.7 min on HPLC.

Deprotected compound XIII was dissolved in 0.3 M sodium phosphate buffer, pH 7.4 (1.5 ml), and *N*-γ-maleimidobutyryl-OSu (VIII) (22.6 mg, 0.08 mmol) in 1.5 ml acetonitrile was added. The solution was stirred for 1 h at room temperature and then it was acidified with 1 *N* HCl to Congo blue, diluted with 0.1% TFA to 50 ml, and applied to preparative HPLC. Fractions corresponding to the product

were pooled and TFA ions were exchanged for acetate ions on IRA-400. Yield 48 mg (95%). TLC behavior, R_f^I 0.45, single biotin positive, fluorescamine negative spot; R_f^{II} 0.69, biotin positive with tailing (see compound I). Single biotin positive peak at 21.7 min on HPLC. The value for the $MH^{\oplus}$ ion, determined by high resolution mass spectrometry, agreed with the molecular weight expected for $C_{35}H_{50}N_7O_8S$: Calculated, 728.3441; Found, 728.3433.

Iodinations

Iodination with ^{127}I. Compound III, isolated by preparative HPLC (0.026 mmol), in acetonitrile : 0.1% TFA (16 ml) was added together with NaI (0.025 mmol) to a flask coated with Iodogen (0.025 mmol). The solution was stirred for 5 min and then it was centrifuged, filtered, diluted with 0.1% TFA to 60 ml, and applied to preparative HPLC.

Iodination with ^{125}I. The reagents were iodinated by the transfer method (*13*) as follows. A 2 mM solution of compound I in 25% aqueous acetic acid (10 μl) was evaporated to dryness in a micro centrifuge tube. Another micro centrifuge tube was coated with Iodogen (2 μmol) and was washed with 100 μl of 0.1 M sodium borate buffer, pH 7.8. A fresh portion of borate buffer (100 μl) was added followed by $Na^{125}I$ (1 mCi, Sp Act 48 mCi/μmol) and the solution was incubated for 5 min and transferred to the micro centrifuge tube containing compound I. The reaction mixture was incubated at ice-bath temperature for 5 min and applied to a μBondapak C_{18} column. Products were separated by reverse phase HPLC using the gradient described for analytical HPLC. Fractions (0.25 ml) were collected and aliquots (5 μl) were evaluated for ^{125}I content. Fractions containing di-iodinated material were combined and diluted to a specific activity of 9.5 μCi/nmol with the corresponding unlabeled material. Portions (5 nmol) were evaporated to dryness *in vacuo* after addition of glacial acetic acid (10 μl). Compound I sulfone and compounds II and III were iodinated in an identical manner.

Alkylation of Insulin Receptor

Purified human placental insulin receptor (*4*) was added (5 pmol, 20 μl) to microfuge tubes containing the desired di-iodinated reagents (250 μM) and the resulting solutions were incubated for 1 h at room temperature. The reaction was terminated by addition of NEM (200 nmol, 10 μl) dissolved in 50 mM Hepes, pH 7.6. NEM-Deactivated carbonic anhydrase (20 μg) was added as a carrier and the solution was extracted with chloroform/methanol according to the method of Wessel and Flügge (*14*). The precipitate was dissolved in reducing buffer (*15*), heated at 50°C for 15 min, and subjected to SDS–PAGE using a 4% stacking and a 7.5% resolving gel. An autoradiogram of the samples was obtained with Kodak XAR-5 film at −70°C. Receptor (5 pmol) incubated with NEM (2.5 mM) for 30 min at room temperature prior to exposure to ^{125}I-labeled compound I was used to demonstrate the SH specificity of labeling.

Ligand Displacement Studies

Complexes were prepared by incubating succinoylavidin (*16*) (13.3 μM with respect to biotin binding sites) in 50 mM Tris–HCl, pH 7.6, with the desired ligand

(32 μM) for 16 h. [^{14}C]Biotin (3.75 μCi/μmol) (667 μM) was added and aliquots of this solution were subjected to gel filtration on Sephadex G-50 (0.9 × 54-cm column) immediately after mixing or following incubation at room temperature (23–25°C) for specified times. The radioactivity in the protein-containing eluates provided a measure of the rate of dissociation.

Those compounds containing a maleimide function were incubated with glutathione (a twofold excess) in the manner described for determining thiol alkylating ability (*9*) prior to measuring their dissociation behavior.

RESULTS AND DISCUSSION

Synthetic Strategy

The compounds whose syntheses are described in (Fig. 1) contain three reactive groups: (1) a biotin residue that binds to avidin and streptavidin and contains a thioether function that can be subject to oxidation, (2) a *p*-hydroxyphenyl group that can be iodinated, and (3) a maleimide function that alkylates SH groups and is highly sensitive to ring opening under alkaline conditions. Careful attention was given to these facts when devising synthetic routes to the desired compounds.

The original plan was to synthesize *N*-γ-maleimidobutyryl-biocytinamido 2-aminoethane and radiolabel this compound with [^{125}I]sulfo-SHPP (*17*, *18*). However, acylation of the maleimide derivative with sulfo-SHPP did not proceed in good yield despite a number of attempts. Finding that coupling via a Bolton–Hunter (*17*) approach was not successful, we prepared compound I and its sulfone and compounds II and III, all containing the readiliy labeled *p*-hydroxyphenyl group. The maleimide group was introduced in the last step of the syntheses.

Iodination with ^{127}I

When the products of iodination of compound III were separated by preparative HPLC, peaks corresponding to the mono- and di-iodinated products appeared as dublets. Since it is well recognized that thioethers can undergo oxidation to their sulfoxides under the conditions used for iodination (*13*), we suspected that the dublets were the result of oxidation to biotin sulfoxides. We established that biotin itself is converted to its *dl*-sulfoxides[4] under conditions identical to those used for iodinating compound III. The HPLC retention times for biotin sulfoxides were established with an authentic sample of the sulfoxides (*8*). The identity of the monoiodo sulfoxide was confirmed by high resolution mass spectrometry. The value for the $MH^{\oplus}$ ion agrees with the expected molecular weight for $C_{35}H_{49}N_7O_9SI$: Calculated, 870.23588; Found, 870.23637.

[4] The terms biotin *d*-sulfoxide or biotin (+) sulfoxide found in older literature refer to the molecule in which the oxygen is equatorial (S stereochemistry at sulfur). The absolute configuration was established both by NMR (*1*, *2*) and by X-ray analysis (*3*).

Iodination with ^{125}I

To determine the conditions necessary to produce primarily one iodinated species, iodination of compound III was performed using several concentrations of Iodogen at a basic pH where sulfoxide formation is less favored (Fig. 2).

The products of the iodination reaction were examined with analytical HPLC. The ratios of Iodogen : NaI : compound III employed were 1 : 1 : 1, 10 : 1 : 1, and 100 : 1 : 1. The HPLC profiles depicted in Fig. 2 show that as the Iodogen concentration is increased, the amount of uniodinated material decreases from 50 to 25 to 6% of the total and amount of di-iodinated compound III increases from 31 to 51 to 66%.

The retention times for uniodinated (21.2 min), monoiodinated (23.6 min) and di-iodinated compound III (25.6 min) show that each iodine incorporated into compound III increases the hydrophobicity. The retention times for the sulfoxides of each species are typically shifted to a position 1.3 to 1.7 min earlier than the corresponding thioether. For example, on Fig. 2C the sulfoxides of di-iodinated compound III appear at 23.9 and 24.3 min. Each of the sulfoxides migrates as a clearly discernible peak using the gradient described for analytical HPLC. In view of these findings we performed the large scale iodination with ^{127}I described above using micromolar amounts of compound I so that the di-iodinated derivative could be isolated and used for adjusting the specific activity of the ^{125}I-labeled material. We consider that it is essential to use a pure iodinated derivative for protein labeling to avoid partitioning (based on iodine content) on HPLC of the peptides isolated from derivatized proteins.

Eliminating sulfoxide formation altogether would be the best solution to the problem since Garlick and Giese (*19*) have shown that the *l*- and *d*-sulfoxides of biotinylamidoethyl-3-(4-hydroxy-3-iodophenyl)-propionamide bind to avidin with different affinities. The $t_{1/2}$ for dissociation of the *d*-sulfoxide was 1.6 times faster than the thioether, whereas the $t_{1/2}$ for the *l*-isomer was 446 times faster.

The problem of sulfoxide formation during iodination can be eliminated by the use of the sulfone of compound I. In this case iodination has no effect on the oxidation state of the biotin sulfur and the resulting HPLC profile of the iodinated compound contains only the uniodinated, mono-iodo and di-iodo derivatives.

Ligand Dissociation Studies

Succinoyl avidin was employed for ligand dissociation studies rather than avidin because the latter binds nonspecifically to glass, Sephadex, and other surfaces. This property makes it inconvenient to use as a matrix for affinity chromatography. In addition we have previously shown that $t_{1/2}$ for dissociation of biotin from succinoyl avidin is not markedly different from that for avidin (*16*, *20*). It should be mentioned that each dissociation curve is a combination of two rates, an initial faster rate, the so-called "anomalous dissociation," (*19*) accounts for 5–10% of the sites, followed by a slower rate that remains constant for as long as the dissociation is studied. The same is true for the dissociation of biotin itself and other analogs that have been studied (*21*).

Dissociation behavior of compound I and its mono- and di-iodinated derivatives

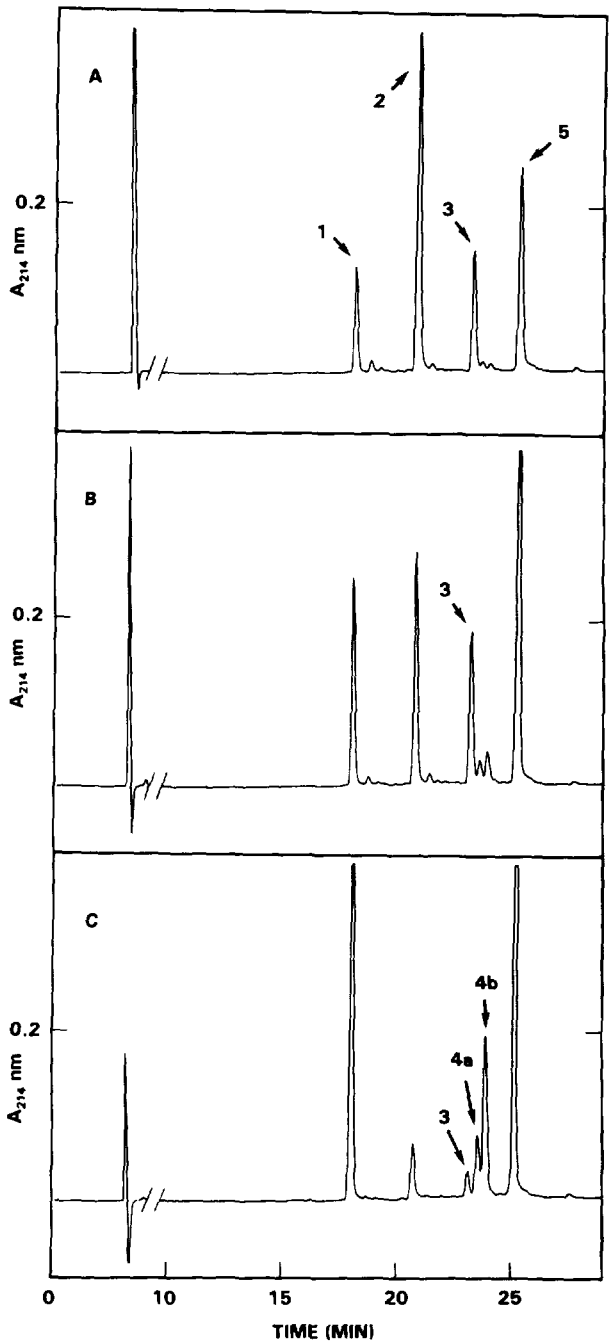


FIG. 2. Effect of varying Iodogen concentration on the iodination of compound III. Compound III (20 nmol) was iodinated with Iodogen and analyzed on HPLC as described under Materials and Methods. The ratios of Iodogen : NaI : compound III are (A) 1 : 1 : 1; (B) 10 : 1 : 1; (C) 100 : 1 : 1. Peak 1, side product from Iodogen; peak 2, uniodinated compound III; peak 3, monoiodinated compound III; peaks 4a and 4b, *dl*-sulfoxides of di-iodinated compound III; peak 5, di-iodinated compound III.

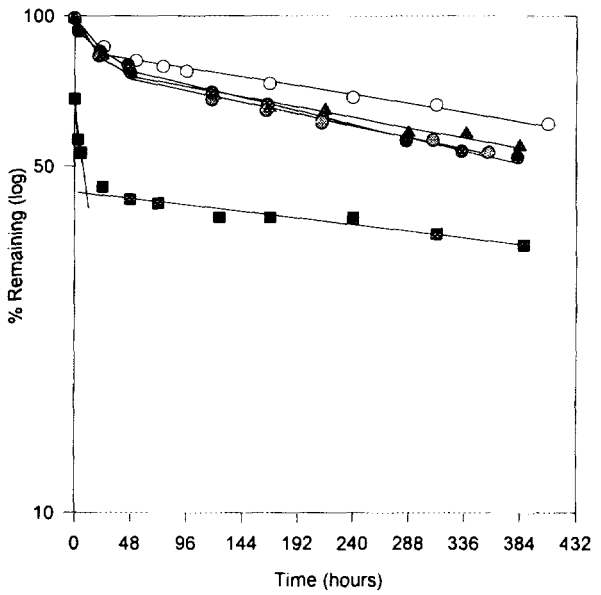


FIG. 3. Rate of dissociation of succinoylavidin complexes with compound I (○), mono-iodo compound I (◎), di-iodo compound I (●), compound I sulfone (▲), and di-iodo compound III *dl*-sulfoxides (■). Dissociation experiments were performed as described (Materials and Methods).

was measured (Fig. 3) over a period of 17 days. To insure that the maleimide ring was not opened by exposure to the basic conditions used for the dissociation studies, the maleimide reagents were reacted with glutathione prior to use in dissociation reactions. This precaution has the added advantage of better simulating the situation in which these reagents will be reacted with sulfhydryl groups in peptides from proteolytic digests of modified proteins.

The $t_{1/2}$ values for compound I and its iodinated derivatives are essentially the same: 36.7 days for the uniodinated and 26.1 and 21.4 days for the mon-iodo and di-iodo derivatives of compound I. These dissociation times clearly demonstrate that both iodinated species will function efficiently as ligands for affinity chromatography of derivatized materials. The binding is sufficiently strong to permit the affinity columns to be washed exhaustively to remove contaminants and still allow retrieval of the derivatized material from the affinity column without resorting to drastic measures.

Dissociation of the sulfoxides of compound III follow a predictable course based on the findings of Garlick and Giese (*19*) who measured the avidin binding of the sulfoxides of biotinylamidoethyl-3-(4-hydroxy-3-[^{125}I]iodophenyl)propionamide. These investigators observed that the two sulfoxide isomers had considerably different affinities for avidin. The *d*-sulfoxide, in which the oxygen is equatorial, dissociated approximately 10 times more slowly than the *l*-sulfoxide. When the dissociation behavior of a mixture of the *d*- and *l*-sulfoxides of di-iodo compound III was measured, a complex curve was obtained with faster dissociation occurring

TABLE 1

Alkylating Capacity of the Maleimide Reagents

Compound	Alkylating capacity[b] %
Compound I	91.6 ± 7.4
Compound II	91.8 ± 4.5
Compound III	79.9 ± 2.2
Compound III[a]	74.3 ± 0.8
Mono-iodo compound III	82.4 ± 8.0
Di-iodo compound III	88.1 ± 12.8

Note. Assays were done in triplicate. NEM served as the control.

[a] Recovered from iodination reaction by preparative HPLC.

[b] ±SD.

until only 43% of the compound remained bound. Thereafter, the curve became linear with a $t_{1/2}$ of 43.0 days. In analogy with the findings of Garlick and Giese (*19*) it seems reasonable to predict that the *d*-sulfoxide is the slower dissociating isomer.

The $t_{1/2}$ for the sulfone of compound I, 29.8 days, was not anticipated. Based on their finding that the sulfoxide, in which the oxygen is axial, binds only weakly to avidin, Garlick and Giese (*19*) predicted that the sulfone, containing both an equatorial and an axial oxygen, would behave like the *l*-sulfoxide and dissociate more rapidly than the *d*-isomer. We measured the dissociation rate for biotin sulfone and found that it dissociated much more slowly from succinoyl avidin than would have been predicted from structural considerations. The $t_{1/2}$ (96.4 days) was longer than for either of the sulfoxides. We reasoned that the sulfone of compound I would behave similarly and thus provide a useful alternative to the oxidizable analogues. This was, indeed, the case. The sulfone of compound I dissociates from succinoyl avidin with a $t_{1/2}$ virtually identical to that of the parent compound.

Maleimide Function

From the outset of these studies we were mindful of the possibility that iodination of the completed reagent could destroy the sulfhydryl alkylating property of the maleimide group since it involved exposure to the oxidant Iodogen. To minimize exposure to Iodogen, we adopted the transfer iodination technique. We examined the ability to NEM to alkylate glutathione after exposure to the same conditions and found that the alkylating capability of NEM was not affected by this treatment (data not shown). Thus, it was reasonable to expect that our compounds could be iodinated without destruction of their alkylating potential and, as can be seen (Table 1), this is the case. Reaction of glutathione with compound III, recovered

from iodination, or with either of the iodinated derivatives showed that the maleimide function was still capable of alkylating SH groups (Table 1). The iodinated derivatives of compound I and its sulfone and of compound II can be expected to behave similarly. All three reagents had the capacity to alkylate insulin receptor (see discussion below) and the level of incorporation of ^{125}I was the same for each reagent.

Alkylation of Insulin Receptor

Human insulin receptor is a tetrameric glycoprotein composed of two heteromeric dimers each containing an α- and a β-subunit. Insulin binds to the α-subunit which is entirely extracellular while the β-subunit, which spans the plasma membrane, is the site of tyrosine kinase activity. The β-subunit contains an SH function whose alkylation with NEM inhibits tyrosine kinase activity. Wilden *et al.* (*22*) showed that [^{3}H]NEM was incorporated exclusively into the β-subunit in the absence of reducing agents. We have shown that the β-subunit contains a single SH group (*4*) near the ATP binding site.

Locating the position of the NEM-reactive cysteine residue within the β-subunit is not a trivial matter. The need for detergents to solubilize the receptor precludes the use of certain chromatographic techniques for purification of peptides obtained from proteolytic digests of derivatized receptor. The present reagents were designed to solve this problem. The presence of biotin in the reagents will facilitate purification of alkylated peptides and detergent removal through biotin–avidin affinity chromatography.

Alkylation of receptor with the di-iodo derivative of each of the three reagents[5] resulted in incorporation into the β-subunit (Fig. 4). To establish the specificity of labeling, receptor alkylated with a large excess of unlabeled NEM was also reacted with compound I and the level of incorporation of ^{125}I was negligible. This was also true of incorporation into the α-subunit. The finding that the di-iodo derivatives reacted with the receptor indicates that the SH group in the folded protein chain was accessible to the reagents even when two bulky iodine atoms are attached. Although the distance between the iodinated phenol and the maleimide group is 6Å less in compounds I and II than in compound III, there is no difference in the ability of any of the three reagents to interact with the SH group of the receptor in its native conformation.

In this paper we demonstrate that maleimide derivatives containing an iodination site such as the *p*-hydroxyphenyl group can be iodinated to high specific activity without loss of their SH-alkylating ability. This unanticipated finding adds a new dimension to the SH-alkylation procedure for identification of sulfhydryl groups in biological materials. The "biotin handle" contained in these reagents affords the opportunity to purify the derivatized materials using the powerful tool of biotin–avidin affinity chromatography.

[5] The di-iodo derivative of compound I sulfone also alkylated receptor with the same specificity (data not shown).

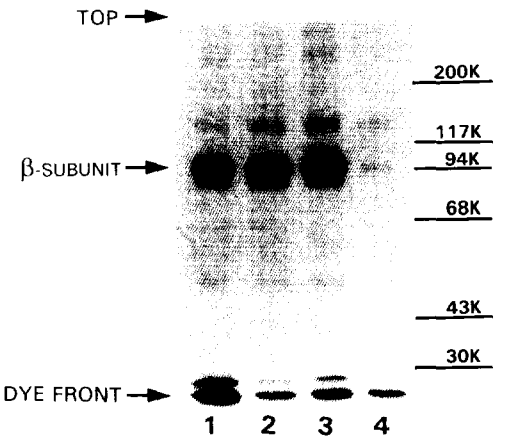


FIG. 4. Autoradiogram of SDS–PAGE containing insulin receptor alkylated with ^{125}I-labeled compounds I, II, and III. Identical results were obtained with compound I sulfone (data not shown). See Materials and Methods for details of the labeling procedure, the alkylation of the receptor, and SDS–PAGE. Lane 1, compound III; lane 2, compound II; lanes 3 and 4, compound I. In lane 4, insulin receptor was alkylated with NEM before incubating with compound I. Numbers on the right of the gel refer to the molecular masses of the standards (myosin, 200K; β-galactosidase, 117K; phosphorylase b, 94K; bovine serum albumin, 68K; ovalbumin, 43K; and carbonic anhydrase, 30K).

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