Synthesis of an organomercury hapten and conjugation of the hapten to proteins and peptides is described. Starting with allyl amine, synthesis of the organomercury hapten was completed in five steps using readily available and inexpensive reagents. The key transformation in the synthesis, intramolecular oxymercuration, was achieved in good yield and under mild conditions. Hapten conjugation was afforded via di-succinimide active ester coupling chemistry, and the resulting conjugates were analyzed by Matrix Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-MS). In order to exploit the accurate mass measuring capabilities of MALDI-MS, the conjugates were digested with trypsin prior to analysis. The masses of the peptides resulting from tryptic digestion of the organomercury conjugates were accurately measured, and five hapten attachments were identified in the mass range of 1000-2200m/z.

INTRODUCTION

Mercury has played a rich role and has made diverse contributions to the field of bioconjugate chemistry. For example, mercury's unique electronic properties have proved useful in designing electrochemical based thiol assay methods (1). Mercury metal has aided electron microscopy characterization of proteins (2) and has been incorporated into fluorescent peptide and protein tags (3). More recently, there has been interest in developing anti-mercury mAbs for their potential use as diagnostic tools capable of providing highly specific, inexpensive, easy to use mercury detection assays (4,5). Our interest in this area prompted us to design and prepare a synthetic organomercury hapten that is amenable to protein conjugation methods. Mercury metal, mercury salts, and organomercury compounds are ubiquitous and persistent environmental toxins, and the associated health hazards to humans, mammals, and aquatic systems are of keen interest (6).

The preparation of metal containing bioconjugates for the purpose of generating mAbs capable of binding metals and metal ions has been the focus of numerous research efforts over the last 20 years
Generally, the approach taken to generate metal-based bioconjugates has been to chemically modify carriers by covalently attaching EDTA or other structurally similar metal chelating agents. The chelating species facilitates encapsulation of a metal in a cage-like structure on the carrier's surface via formation of coordination bonds between the electron rich atoms of the chelate and the electron deficient metal atom. The earliest examples of bioconjugates prepared with the metal chelation methodology have resulted in several useful substrates. These substrates have found practical applications as metal-labeled therapeutic antibodies, magnetic resonance imaging substrates, and radioimaging techniques. One recent extension of the EDTA chelation methodology has been the synthesis of a fluorescent optical sensor designed to detect Hg$^{2+}$ ions. In this work, Hg$^{2+}$ is trapped in a cage-like structure arising from a porphyrin dimer. Preliminary work has shown that the chemical sensor is capable of detecting Hg$^{2+}$ at concentrations of $10^{-4}$ - $10^{-7}$ M with high degrees of specificity preferentially over other divalent metal ions.

One shortcoming of the chelation methodology is that EDTA is known to form stable coordination complexes with virtually all of the transition metals. Due to the nonselective nature of EDTA-metal complexes, efforts to develop immunoassays based on mAbs elicited from EDTA-metal bioconjugates have been thwarted by poor sensitivity and lack of metal specificity. Additionally, mAbs generated via EDTA-encapsulated metal bioconjugates often display poor selectivity between the metal-chelate analyte and metal-free chelate.

Currently, there are only a few examples of protocols that have yielded metal binding antibodies from methodologies other than chelate complexation. Benkovic and coworkers have chemically modified the binding site of a specific antibody to accommodate complexation of Zn$^{2+}$ ion. The catalytic hydrolysis rate of the resulting Ab-Zn$^{2+}$ substrate was observed to increase relative to the native enzyme. In another approach, Wylie and co-workers covalently attached glutathione, a tripeptide of glutamic acid, cysteine, and glycine, to KLH via carbodiimide chemistry. Mercuric
chloride (HgCl$_2$) was then attached to the glutathione-modified carrier by sulfur mediated ligand exchange. Mercury ions have a particularly high affinity for thiols; the binding constant between cysteine and mercury is on the order of 10$^{14}$ (21).

Our overall strategy was to synthesize a structurally simple, but chemically robust, organomercury hapten, which was obtained via an intramolecular oxymercuration reaction. The synthetic hapten was covalently bonded to BSA and a model peptide through an amine positioned at a site removed from the carbon-mercury bond. MALDI/FT-ICR MS$^1$ was used to unequivocally characterize the resulting bioconjugates. The short-term goals of this project have been completed and we are currently preparing to initiate a mAb development program with the organomercury bioconjugate resulting from this work. In this paper we describe the complete synthesis of the organomercury hapten, conjugation of the hapten, and the results obtained from MALDI-MS characterization of the bioconjugates.

EXPERIMENTAL PROCEDURES

Materials and Methods

All reagents were purchased from Sigma-Aldrich at the highest purity available and used without further purification, except where noted. Allyl amine, di-tert-butyl dicarbonate, mCPBA, and benzyl bromide were purchased from Acros. BS$^1$ was purchased from Pierce and C-18 Zip Tips were purchased from Millipore.

$^1$H NMR and $^{13}$C NMR were recorded on a Bruker Avance 400 Fourier transform spectrometer at 400 MHz for hydrogen and 100 MHz for carbon using tetramethylsilane (TMS) as an internal standard. HiResMALDI-FTMS (IonSpec, Irvine, CA) equipped with a 4.7 T actively shielded superconducting magnet was used to record all MALDI-MS. The MALDI source uses a 337-nm nitrogen laser and a 10-faceted sample probe. Flash chromatography was carried out on a SiO$_2$ column (32-63 µm particle size) at an elution rate of 2"/min. GC/MS was done on a Hewlett Packard Series.
5988A equipped with an EC-1 column, 30 m, 0.25 mm i.d., 0.25 µm film thickness (Alltech, Deerfield, IL), injector 250°C, detector 280°C, oven 40°C, programmed at a rate of 10°C/min to 280°C. EI, Cl (CH₄), and Cl (NO/CH₄) MS were collected. Infrared spectroscopy was carried out on a Nicolet 210 FTIR. Melting points were determined in open glass capillary tubes with a Mel-Temp II Melting Point Apparatus and were not corrected.

**Organomercury Hapten Synthesis**

**Tert-butyl allylcarbamate.** To a solution of allylamine (1) (4.87 mL, 65 mmol) in 80 mL of diH₂O was added 40 mL of 1.5 M Na₂CO₃, followed by 13.9 mL of di-tert-butylcarbonate (63.7 mmol), and the solution was stirred overnight at room temperature. The desired product, a white precipitate, was filtered and recrystallized from water. If precipitate did not form, the reaction mixture was extracted with CH₂Cl₂ and the combined extracts were washed with HCl (1M), brine, dried over Na₂SO₄, and concentrated *in vacuo*. Regardless of purification method, the product was obtained in 85% as a white solid: mp 30-32°C; ¹H NMR (CDCl₃) δ 1.40 (s, 9H, CH₃), 3.75 (m, 2H, CH₂), 4.65 (br s, 1H, NH), 5.13 (m, 2H, =CH₂), 5.85 (m, 1H, =CH); ¹³C NMR (CDCl₃) δ 28.6 (CH₃), 43.3 (CH₂), 79.6 (quaternary C), 115.9 (=CH₂), 135.1 (CH=), 156.0 (C=O); FTIR cm⁻¹ 3450 (νNH), 3056 (ν=CH₂), 2976 (νCH), 1710 (νC=O), 1502 (νC-N), 1272 (νC-O); GC/MS CI (CH₄) m/z 142 [(M+41)-56]⁺, 130 [(M+29)-56]⁺, 102 [(M+1)-56]⁺.

**Tert-butyl(oxiran-2-ylmethyl)carbamate (2).** To a solution of the protected amine (5 g, 31.9 mmol) in 60 mL of CH₂Cl₂ excess dry *m*CPBA (8.77g, 51 mmol) was slowly added, allowing the *m*CPBA to dissolve between additions. The reaction was stirred with a magnetic stir bar overnight at room temperature. To precipitate unreacted *m*CPBA and by-product *m*-chlorobenzoic acid, the reaction mixture was placed in the refrigerator overnight, filtered and washed with cold CH₂Cl₂. The filtrate was stirred magnetically for 1 h in 1M Na₂CO₃ and the aqueous layer was extracted with CH₂Cl₂. The pooled CH₂Cl₂ layers were washed with brine, dried over Na₂SO₄, and concentrated *in vacuo*, to obtain
a clear, colorless slightly viscous oil (84%): ¹H NMR (CDCl₃) δ 1.45 (s, 9H, CH₃), 2.59 (dd, 1H, CH₂O), 2.78 (dd, 1H, CH₂O), 3.08 (m, 1H, NHCH₂), 3.20 (m, 1H, ring CH), 3.51 (m, 1H, NHCH₂), 4.89 (br s, 1H, NH); ¹³C NMR (CDCl₃) δ 28.7 (CH₃), 42.0 (CH), 45.4 (CH₂O), 51.2 (NHCH₂), 80.0 (quaternary C), 156.3 (C=O); IR cm⁻¹ 3438 (νNH), 3046 (νCH₂ ring), 2978 (vsp3CH), 1707 (νC=O), 1509 (νC-N), 1263 and 1168 (νC-O-C); GC/MS CI (CH₄) m/z 202 [M+29]⁺, 174 [M+1]⁺, 158 [(M+41)-56]⁺, 146 [(M+29)-56]⁺, 118 [(M+1)-56]⁺.

**Tert-butyl(2-hydroxy-5-hexen-1-yl)carbamate (3).** Allylmagnesium bromide (1 M in ether, 57 mL, 57.8 mmoles), was transferred *via* a cannula to a round bottom flask equipped with reflux condenser, magnetic stir bar, and nitrogen inlet. The ether was evaporated under nitrogen flow to yield a white solid paste. Dry dioxane (100 mL) was added *via* cannula to the dried allylmagnesium bromide, and the mixture was stirred magnetically under nitrogen for 2.5 h to precipitate MgBr₂. A solution of 2 (2 g, 11.6 mmol) in 5 mL of dioxane was added and the reaction was refluxed for 2 h. A slight excess of 1M NH₄Cl(aq) was added, and the reaction was stirred magnetically overnight at room temperature. Dioxane and H₂O were removed *in vacuo* to yield a yellow oil (3) and white solid. The product was extracted with CH₂Cl₂ and diH₂O. The combined organic layers were washed with brine, dried over Na₂SO₄, and concentrated *in vacuo* to yield a yellow viscous oil (80%). GC analysis of the crude product indicates a 20:1 ratio of regioisomers. The crude product was purified by flash chromatography (10% acetone:CH₂Cl₂) to yield a pale yellow viscous oil (65%): ¹H NMR (CDCl₃) δ 1.45 (s, 9H, CH₃), 1.55 (q, 2H, HOCHCH₂), 2.16 (m, 2H, allylic CH₂), 2.72 (br s, 1H, OH), 3.04 (m, 1H, NHCH₂), 3.30 (m, 1H, NHCH₂), 3.71 (br s, 1H, NH), 4.98 (m, 2H, =CH₂), 5.06 (m, 1H, CHOH), 5.84 (m, 1H, CH=); ¹³C NMR (CDCl₃) δ 28.8 (CH₃), 30.2 (allylic CH₂), 34.2 (HOCHCH₂), 47.0 (NHCH₂), 71.5 (CHOH), 80.0 (quaternary C), 115.5 (=CH₂), 138.6 (=CH), 157.2 (C=O); FTIR cm⁻¹ 3339 (δOH), 3069 (ν=CH), 2978 (vsp³CH), 2923 (vsp³CH), 1687 (νC=O), 1513 (νC-N), 1362 (νOH), 1255 and 1160 (νC-O); GC/MS CI m/z 216 [M+1]⁺, 200 [(M+41)-56]⁺, 188 [(M+29)-56]⁺.

**Tert-butyl[(5-methyl mercuric chloride tetrahydrofuran-2-yl)methyl]carbamate (4).** Grignard product 3 (200 mg, 0.93 mmol) was dissolved in 5 mL of freshly distilled CH_3CN in a round bottom flask equipped with magnetic stir bar and argon gas inlet/outlet. Mercuric acetate (0.591 g, 1.9 mmol) was dissolved in 10 mL CH_3CN and added to the solution of 3. The mixture was stirred overnight at room temperature under argon gas, after which time the reaction mixture became clear and colorless. Brine was added and the resulting mixture was stirred 3 h at room temperature. Excess NaHCO_3 (250 mg, 3.0 mmol) was added to the crude mixture to neutralize the acetic acid formed. Acetonitrile was removed *in vacuo* and the remaining mixture was extracted with CH_2Cl_2. The organic layers were combined, washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. The desired product was purified by flash chromatography (2:1 hexane:ethyl acetate) to yield a viscous, yellow oil (270 mg, 65%): ¹H NMR (CDCl_3) δ 1.47 (s, 9H, CH_3), 1.65 (m, 2H, ring CH_2), 2.15 (m, 2H, ring CH_2), 2.19 (m, 1H, CH_2Hg) 2.32 (m, 1H, CH_2Hg), 3.06 (m, 1H, NHCH_2), 3.35 (m, 1H, NHCH_2), 4.15 (m, 1H, CHCH_2Hg), 4.23 (m, 1H, OCHCH_2), 5.08 (br s, 1H, NH); ¹³C NMR (CDCl_3) δ 28.8 (CH_3), 30.0 (ring CH_2), 36.8 (CH_2Hg), 38.6 (ring CH_2), 45.0 (NHCH_2), 77.9 (OCHCH_2), 78.7 (CHCH_2Hg), 78.8 (quaternary C), 156.1 (C=O); FTIR cm⁻¹ 3384 (br, vNH), 2975 (vs'CH), 2928 (vs'CH), 1752 (vC=O), 1703 (vC=O), 1505 (vC-N), 1265 and 1170 (vC-O-C); HRMS calcd for C_{11}H_{20}NO_3HgClNa 474.0729, found 474.0730.

**Tert-butyl[(5-methyltetrahydrofuran-2-yl)methyl]carbamate (6).** Crude 4 (60 mg, 0.113 mmol) dissolved in 2 mL of CH_2Cl_2, and 7.56 mg of NaBH_4 (0.2 mmol) dissolved in 1 mL of 10 M NaOH were added and stirred magnetically for 2 h. The resulting mixture was filtered through celite with CH_2Cl_2 to remove the mercury metal formed. The filtrate was extracted with diH_2O, and the organic layer was washed with brine, dried over Na_2SO_4, and concentrated *in vacuo* to yield a viscous yellow
oil: $^1$H NMR (CDCl$_3$) δ 1.19 (d, 3H, CH$_3$), 1.42 (s, 9H, CH$_3$), 1.52 (m, 2H, ring CH$_2$), 2.01 (m, 2H, ring CH$_2$), 3.12 (m, 1H, NHCH$_2$), 3.36 (m, 1H, NHCH$_2$), 4.05 (m, 1H, OCHCH$_2$), 4.07 (m, 1H CHCH$_3$), 5.04 (br s, 1H, NH); $^{13}$C NMR (CDCl$_3$) δ 21.4 (CH$_3$), 28.8 (CH$_3$ tBOC), 29.6 (ring CH$_2$), 34.2 (ring CH$_2$), 45.1 (NHCH$_2$), 76.2 (CHCH$_3$), 77.9 (OCHCH$_3$), 79.5 (quaternary C), 156.5 (C=O); GC/MS EI (diastereomer ratio 1:1.7; identical fragmentation patterns were seen for both diastereomers) m/z 159 [M-56]$^+$, 142 [M-56-H$_2$O]$^+$, 98 [M-117]$^+$ (117= tBOCOC(O)NHCH), 85 [M-130]$^+$ (130= tBOCOC(O)NHCH$_2$), 57 [t-butyl fragment]$^+$.

(5-Methylmercuric chloride tetrahydrofuran-2-yl)methylamine (5). A large excess of TFA (1.0 mL, 12.9 mmol) was added to 4 (100 mg, 0.22 mmol) in 1.0 mL of CH$_2$Cl$_2$ and the mixture was stirred magnetically overnight at room temperature. TFA and CH$_2$Cl$_2$ were removed in vacuo to yield a pale yellow viscous oil, which was extracted with CH$_2$Cl$_2$ and diH$_2$O. The aqueous layer was basified with NaOH (12.9 mmol) and CH$_2$Cl$_2$ was added to precipitate any remaining salts. The mixture was filtered and the filtrate was concentrated in vacuo to yield a viscous yellow oil (65%), which was used in the conjugation protocols without further purification.

Conjugation of Organomercury Hapten 5 and MALDI-MS Characterization

Prior to MALDI-MS analysis samples were purified using a C-18 zip tip and were eluted with 50% CH$_3$CN in 0.1% TFA. The purified samples (0.5 µL) were added to the matrix (0.5 µL of 40 mg DHB in 250 µL CH$_3$CN and 250 µL 0.1%TFA/H$_2$O stock solution) and the 1 µL mixture was placed on the MALDI probe. Protein concentrations were typically 120 µg/mL.

BSA-Benzyl Amine Model Conjugates. A 5 mg/mL stock solution of BSA was prepared in 20 mM phosphate buffer, pH 7.5. Just prior to use, 15 mg of BS$^3$ (2.62X10$^{-2}$ mmol) was dissolved in 5 mL of 20 mM phosphate buffer, pH 7.5. To the BS$^3$ solution, 2.28 µL of benzyl amine (2.08X10$^{-2}$ mmol) was added, and 1.7 mL aliquots of this solution were removed at time 0, 1, and 5 min and added to 200 µl aliquots of the BSA solution. The three reactions were stirred for 45 min at room temperature,
quenched with benzyl amine (1 µL, 9.15X10^{-3} mmol), and stirred for an additional 1.0 h. Each conjugate was dialyzed exhaustively (dialysis membrane tubing MWCO 10,000) against 20 mM phosphate buffer, pH 7.5.

**Tryptic Digestion (45) of BSA-Benzyl Amine Model Conjugates.** Each of the above conjugates (60 µg) was dissolved in 100 µL of urea (6 M in 25 mM NH₄HCO₃). To each mixture 5 mL of DTT (200 mM in 25 mM NH₄HCO₃) was added, the reactions were vortexed, and then gently rotated for 1 h at room temperature. After adding 20 µL of iodoacetamide (200 mM in 25 mM NH₄HCO₃), the reactions proceeded for 1 h at room temperature in the dark. To consume unreacted iodoacetamide, 20 µL of DTT (200 mM in 25 mM NH₄HCO₃) was added, followed by 900 µl of 25 mM NH₄HCO₃. The resulting mixtures were enzymatically digested with 20 µL of trypsin (0.1 µg/µL in 25 mM NH₄HCO₃) and incubated overnight at 37°C. Each sample was concentrated to approximately 0.5 mL. Identical fragmentation patterns were observed in the MALDI-MS for all three conjugates. MALDI/FT-ICR MS: Unconjugated BSA peptides were observed at m/z 1283.725, 1439.835, 1479.805 and 1567.716. These unconjugated peptides were also identified in the digested sample of conjugate 9 (corresponding to residues 361-371, 360-371, 421-433, and 347-359, respectively). Peptides coupled to one equivalent of benzyl amine were observed at m/z 1062.638 (452-459), 1092.654 (242-248), 1236.758 (210-218), 1246.735 (233-218) and 1886.138 (437-451). Numbers in parenthesis indicate the tryptic peptide residue that is coupled to benzyl amine. Residues 452-459, 242-248, and 437-451 were also observed as hapten attachment points in the MALDI-MS of digested conjugate 9.

**Model Peptide and Organomercury Hapten 5 Conjugations.** A 5 mg/100 µL stock solution of hapten 5 was prepared in 20 mM phosphate buffer at pH 7.5. Just prior to use, BS₃ was dissolved in 20 mM phosphate buffer at pH 7.5 to yield a 0.3 mg/100 µL solution. To 400 µL of the BS₃ solution (2.02X10^{-3} mmol), 7.0 µL of hapten stock solution was added (5:BS₃ 0.5:1 molar ratio), and the solution was mixed for 5 min. A 0.5 mg/100 µL peptide solution was prepared in 20 mM phosphate
buffer at pH 7.5, and 200 µL was added to the 5/BS³ solution. The reaction was magnetically stirred for 1 h at room temperature, quenched with a 0.5 molar equivalent of the hapten 5 stock solution, and stirred another 1 h at room temperature. The sample volume was reduced to approximately 0.5 mL in vacuo. MALDI/FT-ICR MS m/z: 1440.740 [MH-HCl]^+, 987.616 (uncoupled peptide), 1143.698 (peptide and BS³ only); with CsI added to the matrix m/z 1568.604 [M+H]^+ of peptide-mercuric iodide conjugate, 1440.740 [MH-HI]^+.

**BSA-Organomercury Hapten Conjugation (9).** To 100 µL (1.42X10⁻² mmol) of a 50 mg/mL hapten 5 stock solution in distilled water, 400 µL (2.99X10⁻⁵ mmol) of a 5 mg/mL BSA solution in 20 mM phosphate buffer at pH 7.5 was added. The pH of this solution was adjusted to 7.5. Immediately after preparing, 340 µL (1.75X10⁻² mmol) of a 30 mg/mL BS³ stock solution in 20 mM phosphate buffer at pH 7.5, was added to the hapten 5/BSA mixture, and the reaction was stirred magnetically for 1 h at room temperature. The reaction was quenched with 0.5 equivalents of the hapten stock solution (50 µL, 7.14X10⁻³ mmol) and stirred magnetically for 30 min.

**Tryptic Digestion of Conjugate (9).** The protocol previously described for the digestion of the BSA-benzyl amine conjugate was not suitable because of potential coupling between DTT and the mercury. Consequently, the digest of the BSA-hapten conjugate 9 was done without DTT. The conjugate was purified on a Sephadex G-25 column (20 mM phosphate buffer, pH 7.2) and was concentrated in vacuo. A 0.125 mg/mL solution was prepared by adding 0.5 mL of 100 mM NH₄HCO₃ to the purified conjugate. To 100 µL of the conjugate solution, 100 µL of trypsin (10 µg/mL in 25 mM NH₄HCO₃) was added and reacted overnight at 37°C. The sample was concentrated to dryness in vacuo, and the resulting fragmented conjugate was taken up in 5 µL of 0.1% TFA. MALDI-MS Unconjugated BSA peptides were observed at m/z 847.5 (161-167), 1283.7 (361-371), 1439.8 (360-371), 1479.8 (421-433), 1567.7 (347-359), 1639.9 (437-451), and 2045.1 (168-183). Peaks at m/z 1283.7, 1439.8, 1479.8, and 1567.7 were also observed in the digested benzyl amine
conjugate. BSA-hapten conjugate peptides were observed at m/z 1270.6 (452-459), 1300.6 (242-248), 1646.7 (25-34), 1702.7 (35-44), 1802.6 (24-34), and 2093.2 (437-451) all corresponding to [MH-HCl]+. Loss of water [MH-HCl-H_2O]^+ from peaks at m/z 1646.7, 1702.7, and 2093.2 lead to peaks at m/z 1628.7, 1684.7, and 2075.2, respectively.

RESULTS AND DISCUSSION

Organomercury Hapten Synthesis

Synthesis of the organomercury hapten 5 (Scheme 1) was initiated by protecting allyl amine with t-butyl carbamate (t-Boc). Using standard protocols, the protection proceeded cleanly at room temperature in CH_2Cl_2 (22). Recrystallization of the crude reaction mixture provided t-Boc-allylamine, which was converted directly to epoxide 2 by reaction with mCPBA in CH_2Cl_2 under standard conditions (23). The desired epoxide was routinely prepared in greater than 80% yield and was used without further purification. GC data of the crude reaction mixture indicated pure product, as did the sharp melting point of the crystalline solid. Both the amine protection reaction and the epoxidation reaction were readily amenable to scale up (>5 g batch size) without further optimization.
Initially the nucleophilic ring opening reaction of epoxide 2 in the presence of allyl magnesium bromide in THF appeared to provide the desired hydroxy alkene 3, albeit in poor yield. Upon further characterization, it was found that the desired product was contaminated by a significant amount of bromohydrin by-product 7 (Scheme 2). Several purification methods were attempted in order to isolate the desired hydroxy alkene (3) from the crude reaction mixture, but under all conditions investigated, bromohydrin contamination remained unacceptable.

Scheme 2

\[
\begin{align*}
\text{Grignard Reagent Used} & \quad 3 \text{ (75%)} & 7 \text{ (25%)} & 8 \text{ (trace)} \\
\text{MgBr / THF} & \quad 3 \text{ (>99%)} & 7 \text{ (<1%)} & 8 \text{ (trace)} \\
\text{Mg}_2 / \text{dioxane (anh)} & \quad & & \\
\end{align*}
\]

Etheral solutions of Grignard reagents tend to exist as equilibrium mixtures of alkyl magnesium bromide, magnesium bromide and dialkyl magnesium (24). When magnesium bromide is present in solution, epoxide 2 is susceptible to bromide ion mediated nucleophilic ring opening reactions, thus forming the bromohydrin as a by-product. Hydroxy alkene 3 was prepared successfully upon addition of anhydrous dioxane to allyl magnesium bromide prior to reacting with epoxide 2. Dioxane promotes precipitation of magnesium bromide, shifting the equilibrium toward higher concentrations of diallylmagnesium (25,26). When the resulting enriched solution of diallylmagnesium was reacted with epoxide 2, the desired hydroxy alkene (3) was formed without the problematic bromohydrin present. GC analysis of the crude reaction mixture indicated that the product consisted of a 20:1 mixture of regioisomeric ring-opened products (3 and 8).
With hydroxy alkene 3 in hand, optimization of the intramolecular oxymercuration cyclization, the key synthetic transformation in the synthesis, was explored. The organomercury precursor (3) was designed to readily accommodate intramolecular mercury mediated cyclization to generate a mercurio cyclic ether. Oxymercuration reactions have been extensively studied and the intramolecular reaction of γ-hydroxy alkenes was well suited for preparing the target organomercury hapten (27-30) because these reactions are fast, high yielding, and occur under extremely mild conditions (28). The reaction is also highly versatile, accommodating numerous alkene structures, and tolerant of many organic functional groups (29,30). The resulting carbon-mercury bond is especially chemically and thermally stable (28). Additionally, organomercury compounds are air stable and therefore amenable to routine purification and characterization techniques.

The oxymercuration reaction proceeded smoothly upon addition of mercuric acetate to a solution of hydroxy alkene 3 in acetonitrile. The organomercury compounds prepared in this study were not amenable to routine GC-MS analysis, presumably due to their low volatility, therefore the course of the cyclization reaction was followed via TLC. When starting material was no longer observed, the reaction mixture was quenched with NaCl to facilitate mercury ligand exchange, yielding cyclic mercuric chloride 4 (Scheme 1). The organomercury substrate was purified via column chromatography and was isolated in good yield. The exact mass of mercuric chloride 4 was determined by high resolution MALDI/FT-ICR MS. The experimental exact mass found for the sodiated parent ion of 4 ([M+Na]+) was 474.0730 m/z and the calculated mass was 474.0729 m/z. The isotopic distribution of Hg was observed in the MS and proved to be a very useful diagnostic tool in characterizing the organomercury hapten and the bioconjugates coupled with the hapten.

A small sample of the crude oxymercuration reaction mixture was reduced with NaBH₄ to generate cyclic ether 6 (Scheme 1). GC analysis of the reaction mixture after reduction revealed a 1:1.7 mixture of cyclic diastereomers. In the proton NMR of a purified sample of the reduced product
two methyl doublets were observed, providing additional evidence that the mercuric chloride (4) was formed as a pair of diastereomers.

The final step in the synthesis of organomercury hapten 5, deprotection of cyclic mercuric chloride 4, was carried out in excess TFA, which was removed in vacuo prior to quenching the crude reaction mixture with one equivalent of NaOH. The free amine organomercury hapten 5 was coupled directly to BSA without further purification (Scheme 3). In designing the organomercury hapten, we had intended to prepare a water-soluble substrate to facilitate protein conjugation protocols. Hapten 5 was moderately water soluble upon deprotection and basification to the free amine.

**Conjugation of Organomercury Hapten**

Prior to conjugating organomercury hapten 5 to BSA, hapten carrier coupling conditions were optimized with benzyl amine serving as a model hapten. Benzyl amine was selected as a suitable model hapten because it is an inexpensive, water-soluble aliphatic amine, and has a pKₐ similar to hapten 5. Additionally, benzyl amine would be a useful analyte for testing the feasibility of MALDI/FT-ICR MS as a viable method for characterizing the resulting bioconjugates.

The bioconjugate prepared in this work constitutes a novel approach to achieving an organometallic-protein complex, in which the metal is covalently bound to the protein. BS³, a commercially available homobifunctional (di-succinimide ester) cross-linking reagent that targets amine functional groups and forms stable amide linkages (31,32), was utilized to covalently couple organomercury hapten 5 to BSA and in all the model conjugates prepared in this study. Upon coupling, a linker arm distance of 14 atoms separates the mercury atom from the protein in bioconjugate 9 (Scheme 3). We propose that the hydrophobic, non-peptide character of the linker arm will provide more optimal immunological response and result in mAbs consisting of unique recognition capabilities, compared to the anti mercury Ab previously prepared (20).
Succinimide esters are commonly used amine reactive cross-linking reagents, but can be problematic because undesirable ester hydrolysis becomes favorable at pH levels most suited for promoting nucleophilic substitution by unprotonated amines. Active ester coupling reactions are typically preformed in pH ranges of 7.0-7.5 (33). In this pH range the concentration of unprotonated amine is low, but the rate of the nucleophilic substitution reaction is sufficiently fast (10 min) relative to the succinimide ester hydrolysis rate, which is on the order of hours. The reaction conditions examined for the model coupling consisted of incubating a 1:2 molar ratio of benzyl amine and BS\(^3\) for varying times (0 min, 1 min, and 5 min) in pH 7.5 phosphate buffer prior to coupling with BSA. The preincubation was intended to maximize monosubstitution of BS\(^3\) with benzyl amine and limit protein cross coupling. BSA-phosphate buffer solution was added to each reaction mixture after the BS\(^3\)-benzyl amine preincubation and the solutions were allowed to react an additional 45 min at room temperature. The conjugates were dialyzed and enzymatically digested with trypsin. MALDI/FT-ICR MS analysis of the model conjugates revealed that preincubating BS\(^3\) with benzyl amine at different time intervals did not significantly affect the molar ratio of benzyl amine to protein in the bioconjugates.

Since the model conjugation reactions with benzyl amine indicated that the extent of hapten coupling was not sensitive to incubation times, organomercury hapten 5 was covalently coupled to
BSA via BS$_3^*$ cross-linking reagent under slightly modified reaction conditions. BSA and hapten 5 were dissolved initially in pH 7.5 phosphate buffer. A concentrated stock solution of BS$_3^*$ was prepared in pH 7.5 phosphate buffer and immediately added to the hapten-carrier solutions. After incubating for 1 h, each reaction was quenched with an additional aliquot of hapten and stirred for 30 more minutes at room temperature.

**MALDI Characterization of Conjugates**

The techniques suitable for characterizing the bioconjugates prepared in this study were limited. UV spectroscopy, a very common bioconjugate characterization method, was not possible because hapten 5 does not contain any UV detectable chromophores. Another example of a routine method for characterizing carrier:hapten ratios is TNBS colorimetric assays (34), which quantify the number of lysine residues that have been modified upon conjugation of a hapten to a protein carrier. Hampered by competitive hydrolysis of BS$_3^*$, there is the possibility that the hydrolyzed linker alone occupies some of the lysine sites. Consequently, the results of a TNBS assay would not distinguish between lysine residues that had been modified by covalent attachment of hapten-BS$_3^*$ linker from those that had been modified only by the addition of hydrolyzed linker.

MALDI-MS techniques have been shown to be very powerful and accurate tools for analyzing complex bioconjugate structures (35-40). For example, in a comparative study, Adamczyk and coworkers demonstrated that MALDI-MS is a significantly more accurate analytical tool than either UV spectroscopy or TNBS colorimetric assays for determining the extent of coupling of low molecular weight haptens to protein carriers (37). MALDI-MS is a sensitive ionization technique (detection limits in the low attomole range have been demonstrated) that generates primarily singly charged [M+H]$^+$ ions for peptides and proteins (35,40). These ions undergo limited subsequent fragmentation, and are ideally suited for characterization of mixtures of structurally complex substrates (39). MALDI is most commonly used in combination with TOF mass analyzers; however, the MALDI/FT-MS used...
in this study has ultra-high mass resolution, coupled with ppm mass accuracy. In addition, MS/MS techniques can be used for structural characterization (40,41).

The mass range of the MALDI/FT-MS is lower than that of a MALDI/TOF and analysis of the intact BSA-conjugates was not possible (41-43). Consequently, the conjugates were digested with trypsin to produce peptides of an appropriate mass for MALDI/FT-MS analysis (39). With this approach, modifications to specific regions of BSA can be identified through the accurate measurement of the masses of the tryptic peptides. As a test of this approach, a sample of unmodified BSA and the benzyl amine model bioconjugates were enzymatically digested with trypsin (39). The monoisotopic masses of the peptides resulting from tryptic digestion of the benzyl amine bioconjugates were compared to native BSA’s tryptic peptides. A MALDI/FT-MS (mass range 1000-2000m/z) spectrum of a digested benzyl amine-BSA conjugate is shown in Figure 1A. The spectrum shows that five benzyl amine-coupled peptides are detected along with four peptides without the amine attached. Unconjugated BSA peptides are observed at m/z 1283.7, 1439.8, 1479.8 and 1567.7. Peptides coupled to one equivalent of benzyl amine are observed at m/z 1062.6, 1092.7, 1236.8, 1246.7 and 1886.1 (Figure 1A). This experiment revealed that the BS₃ coupling protocol was effective in covalently attaching the aliphatic amine model hapten to BSA, and that tryptic digestion coupled with MALDI-MS would provide a convenient method for accurately measuring hapten attachment sites in the bioconjugates.

In order to investigate the behavior of peptides containing organomercury moieties under MALDI-MS conditions, the synthetic hapten (5) was coupled via BS₃ to a commercially available peptide, nine amino acids in length and containing a terminal lysine (KKRAARATS-amide). Upon examination of the MS data, the [M+H]⁺ peak (m/z 1476.7) was not detected, however, several peaks containing mercury were apparent. The base peak in the spectrum, containing one mercury, corresponds to [MH-HCl]⁺ at m/z 1440.7. The mass spectra of organomercury halides are often
dominated by loss of the halide, and the facile loss of HCl in this case may prevent detection of the intact \([M+H]^+\) peak. When cesium iodide was added to the matrix/peptide-conjugate mixture, iodine mediated ligand exchange with the mercuric chloride hapten was favorable, and a peak at m/z 1568.6 corresponding to the \([M+H]^+\) parent ion of the peptide-mercuric iodide conjugate was observed (Figure 2). A distinct peak, clearly containing mercury, at m/z 1440.7 was also observed in this spectrum. This peak arises from the parent ion minus HCl or HI ([MH-HCl]^+, [MH-HI]^+), respectively. MS/MS of the m/z 1568.6 \([M+H]^+\) ion showed a peak at m/z 1440 resulting from loss of HI, demonstrating that the m/z 1440 peak results from an ionization-induced fragmentation of the protonated parent ion. The \([MH-HCl]^+\) ion was found to be characteristic of the other peptide-organomercury conjugates. Other peaks observed in the spectrum included the unreacted peptide (m/z 987.6) and peptide coupled to hydrolyzed BS\(^3\) (m/z 1143.7) and a peak at m/z 1240.8 arising from loss of HgCl from the \([M+H]^+\) parent ion. (Figure 2). The MS/MS experiment indicated that m/z 1240.8 is also an ionization-induced fragment.

Confident that MALDI-MS was a viable tool for analyzing peptides covalently bound to mercury, BSA-organomercury bioconjugate \(9\) was digested with trypsin and the resulting peptide fragments were analyzed by MALDI-MS. Analysis of the model peptide-organomercury conjugates had revealed that the peak of key diagnostic value arises from loss of HCl from the parent ion ([MH-HCl]^+). There are five hapten attachment sites apparent in the MS of the tryptic fragments of conjugate \(9\) in the range of 1000 - 2200m/z.

The exact mass of all the peptide fragments expected upon tryptic digestion of BSA and of each peptide coupled to the organomercury hapten through a BS\(^3\) linker were calculated by the Web version of Protein Prospector (44) and compared to the experimental masses measured in the digested sample of \(9\). Several tryptic peptide fragments without any hapten attachment are observed at m/z 847.5, 1283.7, 1439.8, 1479.8, 1567.7, 1639.9, and 2045.1. Four of these peptides are consistent with the
peptides identified in the MS of the digested benzyl amine model conjugate. The calculated monoisotopic masses of each of these hapten free peptide fragments were compared to the experimentally measured mass values and are shown in Table 1. The peaks at m/z 1270.6, 1300.6, 1646.7, 1702.7, and 2093.1 all correspond to a BSA tryptic peptide coupled to organomercury hapten 5 ([MH-HCl]^+). The calculated monoisotopic mass of these peptides compared to the measured mass is also presented in Table 1. Fragmentation of water from peaks at m/z 1646.7, 1702.7, and 2093.1 gives rise to the peaks observed at m/z 1628.7, 1684.7, and 2075.2, respectively. The peak at m/z 1802.6 was initially interpreted as a sixth hapten attachment site, as it clearly displays the mercury isotopic pattern evident in other peaks and matched the calculated mass (1802.8266 m/z) for hapten 5 coupled to lysine 28 in the peptide containing amino acids 24-34 (RDTHKSEIAHR). This peak, however, is a result of BSA’s multiple possible tryptic cleavage points, which can generate peptide fragments containing overlapping amino acid sequences. The peak at m/z 1646.7, which arises from hapten 5 coupled to the peptide composed of amino acids 25-34 (DTHKSEIAHR), already accounts for coupling between the hapten and lysine 28. The m/z 1893.1 peak is consistent with the loss of Hg from the m/z 2093.5 peak, but in this case a MS/MS experiment did not identify this peak as arising from ionization-induced fragmentation. Surprisingly, there were no peaks corresponding to peptides coupled to hydrolyzed BS3 in the mass range investigated.

The expanded spectrum of the peak measured at m/z 2093.2 is compared to the theoretical isotopic distribution and the calculated monoisotopic mass for hapten 5 coupled to the tryptic peptide fragment containing amino acids 437-451 in Figure 3A and 3B, respectively. The experimentally measured mass, 2093.0579 m/z, has a mass accuracy of -2.1 ppm relative to the calculated mass value (2093.0623 m/z). The symmetrical nature observed in the peaks shown in Figure 3 was a reoccurring pattern found in all the peptide fragments containing an organomercury moiety. The peaks at m/z 2089.1-2093.1 and 2095.1 arise from the mercuric isotopes of mass 198-202 and 204, respectively.
Aided by MALDI-MS, we were able to validate our conjugation protocol through the examination of model conjugates, and specifically identify sites containing covalently linked organomercury hapten 5 by accurate mass measurements. It was also demonstrated that our approach to preparing a metal-protein complex via formation of a stable organometallic bond was successful. As MALDI-MS analysis of conjugates becomes a more widely exploited technique, it may provide the opportunity to correlate epitope density, hapten conformation and linker arm length and structure to immune response.

ACKNOWLEDGMENTS

This work was supported by CIB, (Center for Innovation in Biotechnology) in Augusta, ME, and by the University of Southern Maine Faculty Senate. The MALDI instrumentation used in this study was supported by the National Science Foundation under Grant No. CHE-0116416. The NMR(400MHz) instrumentation was supported by the National Science Foundation under Grant No. 9724321. The authors thank Professor Elizabeth Stemmler at Bowdoin College for her assistance in collecting and analyzing MALDI-MS data.

Table 1. Mass (measured vs. calculated) of BSA Peptide Fragments Observed in MALDI MS (Figure 1B) after Tryptic Digestion of Organomercury Bioconjugate 9.

<table>
<thead>
<tr>
<th>Measured Mass (m/z)</th>
<th>Calculated Mass (m/z)*</th>
<th>PPM</th>
<th>AA sequence in tryptic peptide</th>
<th>Residue number</th>
</tr>
</thead>
<tbody>
<tr>
<td>847.5141</td>
<td>847.5036</td>
<td>12.4</td>
<td>(R)LSQKFPA</td>
<td>161-167</td>
</tr>
<tr>
<td>1283.7059*</td>
<td>1283.7106</td>
<td>-3.7</td>
<td>(R)HPEYAVSVLLR</td>
<td>361-371</td>
</tr>
<tr>
<td>1439.8123*</td>
<td>1439.8117</td>
<td>0.4</td>
<td>(R)RHPEYAVSVLLR</td>
<td>360-371</td>
</tr>
<tr>
<td>1479.8036*</td>
<td>1479.7954</td>
<td>5.5</td>
<td>(K)LGEYGFQNALIVR</td>
<td>421-433</td>
</tr>
<tr>
<td>1567.7456*</td>
<td>1567.7427</td>
<td>1.8</td>
<td>(K)LGEYGFQNALIVR</td>
<td>347-359</td>
</tr>
<tr>
<td>1639.9261*</td>
<td>1639.9378</td>
<td>-7.1</td>
<td>(R)KVPQVSTPTLVEVSR</td>
<td>437-451</td>
</tr>
<tr>
<td>2045.1175</td>
<td>2045.0280</td>
<td>43.8</td>
<td>(R)RHPYFYAPELLYYANK</td>
<td>168-183</td>
</tr>
<tr>
<td>1270.6136</td>
<td>1270.6127</td>
<td>0.7</td>
<td>(R)SLGKVGR</td>
<td>452-459</td>
</tr>
<tr>
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<td>1300.6264</td>
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<td>242-459</td>
</tr>
<tr>
<td>1646.7043</td>
<td>1646.7249</td>
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<td>(R)DTHKSEIAHR</td>
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<tr>
<td>1702.7185</td>
<td>1702.7439</td>
<td>-14.9</td>
<td>(R)DTHKSEIAHR</td>
<td>35-44</td>
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<tr>
<td>2093.0579</td>
<td>2093.0623</td>
<td>-2.1</td>
<td>(R)KVPQVSTPTLVEVSR</td>
<td>437-451</td>
</tr>
</tbody>
</table>

Calculated monoisotopic masses are given. Unmodified lysine residues shown in italic. Calibration peaks are indicated by an asterisk. Lysine residues coupled to hapten 5 shown in bold.
Figure 1. MALDI MS of trypsin digested conjugates. A. Benzylamine-BSA conjugate. B. Organomercury hapten-BSA conjugate 9.
Figure 2. MALDI-MS of hapten 5 coupled to model peptide (KKRAARATS-amide), base peak ([MH-HCl]+) observed at m/z 1440.74.
Figure 3. Expansion of m/z 2093.0579 peak arising from hapten 5 coupled to tryptic peptide composed of amino acid sequence KVPQVSTPTLVEVSR (437-451). A. Experimentally measured peak, including isotopic distribution of mercury. B. Calculated monoisotopic mass and isotope distribution.
LITERATURE CITED


Abbreviations:
MALDI/FT-ICR MS, Matrix Assisted Laser Desorption Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectroscopy; EDTA, ethylene diamine tetraacetic acid; KLH, keyhole limpet hemocyanin; t-Boc, tert-butyl carbamate; mCPBA, meta-chloroperoxy benzoic acid; THF, tetrahydrofuran; DEP, Direct Exposure Probe; TFA, trifluoroacetic acid; BSA, bovine serum albumin; BS³, bis[sulfosuccinimidyl]suberate; DMSO, dimethyl sulfoxide; TNBS, Trinitrobenzene sulfonic acid; TOF, time of flight DTT, dithiothreitol; DHB, 2,5-dihydroxy benzoic acid.