# Synthesis of Undecagold Cluster Molecules as Biochemical Labeling Reagents. 2. Bromoacetyl and Maleimido Undecagold Clusters†

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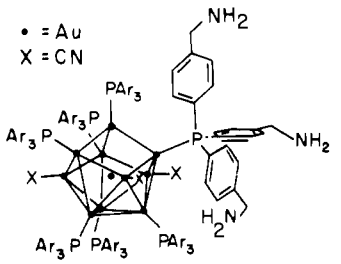
ABSTRACT: Derivatives of heptakis[4,4′,4″-phosphinidynetris(benzenemethanamine)]undecagold, **1**, molecular formula $Au_{11}(CN)_3[P(C_6H_4CH_2NH_3)]_7$, are described. These include undecagold complexes with a single free primary amino group, a single bromoacetyl group, and a single maleimido group per molecule. Hydrolysis of mono(*N*-phthalyl)icosa(*N*-acetyl)-**1** at pH 3.2 and 46 °C under anaerobic conditions and in the presence of $NaBH_3CN$ produces icosa(*N*-acetyl)-**1**. Partial acylation of **1** with 1.3 equiv of 2,3-dimethylmaleic anhydride followed by complete acetylation with acetic anhydride produces a mixture consisting largely of mono- and bis(dimethylmaleyl)peracetyl-**1**. Hydrolysis of 2,3-dimethylmaleimides at pH 3.2 for 1 h at 25 °C produces a mixture of icosa(*N*-acetyl)-**1**, with a single free amino group, and nondeca(*N*-acetyl)-**1**. This mixture can be quantitatively separated by cation-exchange chromatography at pH 7, giving homogeneous icosa(*N*-acetyl)-**1** in an overall yield of 55%. Icosa(*N*-acetyl)-**1** serves as the starting material for the synthesis of the alkylating derivatives mono(*N*-bromoacetyl)icosa(*N*-acetyl)-**1** and mono[*N*-(*p*-maleimidobenzoyl)]icosa(*N*-acetyl)-**1**. These derivatives can be used for alkylating proteins in preparation for electron microscopy.

Applications of tricyanoheptakis[4,4′,4″-phosphinidynetris(benzenemethanamine)]undecagold,[1] **1**, as an electron-dense labeling reagent to facilitate electron microscopic analysis of biological structures depend on the availability of derivatives that contain a single or well-defined small number of reactive functional groups. The parent compound contains 21 primary amino groups which, while reactive in alkylation, acylation, and imine formation, complicate the labeling process because of their large number. Derivatives of **1** containing a single carboxyl group can be prepared by procedures described in the preceding paper (Reardon & Frey, 1984).

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The primary amino group, because of a wide variety of chemical reactions it undergoes, is particularly attractive for use in preparing derivatives of **1** that contain biologically specific ligands or chemically selective functional groups. Many biological ligands can be prepared as derivatives with alkylating or acylating functional groups that would react with **1** to form stable amide or *N*-alkyl bonds linking the cluster compound and ligands. Amino groups can also be chemically modified by bifunctional reagents that link them covalently to alkylating or acylating functional groups. Such derivatives of **1** should be useful for selective chemical modifications of specific sites in multiprotein complexes and other biological structures. Derivatives of **1** containing a single or well-defined small number of primary amino groups should, therefore, be very useful for such studies.

In this paper we describe the synthesis of derivatives of **1** containing a single primary amino group per molecule, as well as methods for chemically modifying these amino groups with bifunctional reagents that introduce a single alkylating functional group per molecule. We also describe some chemical properties of derivatives of **1** that must be controlled during chemical manipulations.

## Experimental Procedures

*Materials.* Maleic anhyride and 2,3-dimethylmaleic anhydride were purchased from Aldrich Chemical Co. Phthalic anhydride was purchased from Fisher Scientific Co. and resublimed. Acetonitrile from Fisher Scientific was dried by stirring with $CaH_2$ for 24 h and then distilled into a receiver containing 4-Å molecular sieves. DMF from Fisher was dried over 4-Å molecular sieves and redistilled at reduced pressure at a temperature below 25 °C into a flask containing BaO. *N*-succinimidyl bromoacetate was synthesized by the procedure of Santi et al. (1974). Mono(*N*-phthalyl)icosa(*N*-acetyl)-**1** was synthesized as described by Reardon & Frey (1984), and henicosa(*N*-[$^{14}$C]acetyl)-**1** was prepared by substituting [$^{14}$C]acetic anhydride for unlabeled reagent in the procedure of Reardon & Frey (1984). **1** and 4,4′,4″-phosphinidynetris(benzenemethanamine) were synthesized by the procedures of Bartlett et al. (1978). *p*-Aminobenzoic acid was purchased from Aldrich Chemical Co. [*carbonyl*-$^{14}$C]Phthalic anydride

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[1] Abbreviations: **1**, tricyanoheptakis[4,4′,4″-phosphinidynetris(benzenemethanamine)]undecagold; henicosa(*N*-acetyl)-**1**, **1** with all 21 amino groups acetylated; icosa(*N*-acetyl)-**1**, **1** with 20 amino groups acetylated; nondeca(*N*-acetyl)-**1**, **1** with 19 amino groups acetylated; octadeca(*N*-acetyl)-**1**, **1** with 18 amino groups acetylated; mono(*N*-phthalyl)-icosa(*N*-acetyl)-**1**, **1** with 20 amino groups acetylated and 1 amino group phthalylated; mono[*N*-(bromoacetyl)]icosa(*N*-acetyl)-**1**, **1** with 1 amino group bromoacetylated and 20 amino groups acetylated; mono[*N*-(*p*-maleimidobenzoyl)]icosa(*N*-acetyl)-**1**, **1** with 1 amino group *p*-maleimidobenzoylated and 20 amino groups acetylated; DMF, dimethylformamide; NMR, nuclear magnetic resonance; $Me_2SO$, dimethyl sulfoxide.

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and [U-$^{14}$C]cysteine were purchased from Amersham and sodium [1,2-$^{14}$C]acetate was from New England Nuclear. [*carbonyl*-$^{14}$C]Phthalic anhydride was diluted with carrier and resublimed. *N*-Acetyl[U-$^{14}$C]cysteine was prepared from [U-$^{14}$C]cysteine. [U-$^{14}$C]Cysteine (100 μmol, $8.19 \times 10^4$ cpm mol$^{-1}$) was dissolved in 100 μL of water at pH 7.0. Acetic anydride (150 μmol) was added to this solution. After 30 min at 25 °C, an analysis for amino groups using the ninhydrin method (Moore & Stein, 1954) verified 97% acetylation. SP-Sephadex C-25 and QAE-Sephadex Q-25 were purchased from Sigma Chemical Co. Inorganic salts were reagent-grade chemicals purchased from commercial suppliers.

*General Methods.* Solutions of undecagold complexes were analyzed spectrophotometrically by measuing $A_{415}$ and calculating concentrations using the extinction coefficient $2.95 \times 10^4$ M$^{-1}$ cm$^{-1}$ (Reardon & Frey, 1984). Radioactive samples in aqueous solutions were radiochemically assayed by adding 15 mL of Aquasol to 1-mL samples and counting in a Beckman LS-100C liquid scintillation spectrometer. Proton NMR spectra were obtained by using a Bruker WH-270 NMR spectrometer field frequency locked on the deuterium resonance of 99.8% $D_2O$ or $Me_2SO$-$d_6$. A 0.1% tetramethylsilane external standard was used as a reference assigned a chemical shift of zero. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN. Dilute solutions of undecagold complexes were concentrated by rotary evaporation in vacuo using a Büchi apparatus with the bath temperature maintained at or below 30 °C. Linear gradients for elution of ion-exchange columns were generated by placing equal volumes of NaCl and water in two flasks connected by a tube filled initially with water. The flasks were placed side by side at the same level, and the contents of the water-containing flask were stirred efficiently while the gradient was drawn from this flask to feed the column.

*Conversion of Mono(N-phthalyl)icosa(N-acetyl)-1 to Icosa(N-acetyl)-1.* Mono(*N*-phthalyl)icosa(*N*-acetyl)-**1** (1–30 $A_{415}$ units) was dissolved in 1 mL of water and adjusted to pH 3.2 by addition of either formic or acetic acid. $NaBH_3CN$ (0.05 μmol) was added to this solution, which was then flushed with argon for 30–120 min. The solution was heated at 46 °C under argon for 65 h. At 8–12-h intervals during heating, 0.05 μmol of $NaBH_3CN$ was added, the solution flushed for 30 min with argon, and heating continued. After 65 h the solution was diluted to 2 mL with 10% ethanol in water (v/v) and desalted by gel filtration through a 1.5 × 48 cm column of Bio-Gel P-6 equilibrated and eluted with 10% ethanol in water. Fractions were collected and analyzed for undecagold complexes by measurements of $A_{415}$. Fractions containing $A_{415}$ were pooled, concentrated to 1.5 mL by rotary evaporation in vacuo, and stored at −70 °C. This procedure was also used to remove phthalyl groups from mono(*N*-phthalyl)icosa(*N*,*N*-dimethyl)-**1** and mono(*N*-phthalyl)icosa[*N*-methyl-*N*-(carboxymethyl)-**1**.

*Direct Synthesis of Icosa(N-acetyl)-1.* A 1-mL aqueous solution of **1** (10.2 μmol) at pH 7.4 previously freed of $CO_2$ was magnetically stirred inside a 50-mL pear-shaped flask while 13.2 μmol of 2,3-dimethylmaleic anyhydride dissolved in 0.1 mL of acetonitrile was added. Stirring was continued during acylation. The pH was maintained between 7.0 and 7.5 by microliter additions of 1 M NaOH. After the pH became stable (<5 min), the solution was diluted with distilled water to 30 mL. Acetic anydride (100 μL, 1 mmol) was added to this solution, which was stirred and maintained between pH 7 and 7.5 by additions of 4 M NaOH until the pH became stable. The addition of acetic anhydride was repeated four additional times. The pH of the solution was then adjusted to 3.2 by addition of acetic acid. After 60 min at 25 °C, the pH was readjusted to 7.0 by addition of 4 M NaOH. The solution was concentrated by rotary evaporation in vacuo to between 3 and 4 mL and desalted by gel filtration through a 4 × 25 cm column of Sephadex G-10 equilibrated and eluted with water. Elution of the orange-red band of gold complexes was monitored visually and by measurements of $A_{415}$. Fractions containing gold complexes were pooled and diluted with water to an $A_{415}$ of 2. This solution was flushed with bubbling $N_2$ gas for 30 min to remove $CO_2$ and then passed through a 1.5 × 13 cm column of SP-Sephadex C-25 in the $Na^+$ form. Cationic species of gold complexes absorbed at the top of this column in a narrow band were eluted at pH 7 by a linear gradient of NaCl increasing in concentration from 0 to 0.4 M and formed from 400 mL of each component. Fractions 4.2 mL in volume were collected at a flow rate of 1 mL/min. Icosa(*N*-acetyl)-**1** appeared in fractions 23–31 (see Figure 1) which were pooled, concentrated by rotary evaporation, and desalted by gel filtration through Sephadex G-10 as described previously. The desalted product was concentrated to 5 mM and stored at −70 °C. The yield of icosa(*N*-acetyl)-**1** was 55–60%. Lesser amounts of nondeca(*N*-acetyl)-**1** were also isolated from fractions 44–53 (see Figure 1).

*Characterization of Icosa(N-acetyl)-1.* A sample of icosa(*N*-acetyl)-**1** that had been stored for 5 months at −70 °C was thawed and rechromatographed through a column of SP-Sephadex C-25. The sample consisted of 0.76 μmol of icosa(*N*-acetyl)-**1** dissolved in 7.5 mL of water. It was absorbed to a 1.5 × 9 cm column of SP-Sephadex C-25 and eluted by a linear gradient of NaCl at pH 7, increasing in concentration from 0 to 0.4 M and formed from 400 mL of each component. Fractions 3.6 mL in volume were collected, and $A_{415}$ of each fraction was measured. Icosa(*N*-acetyl)-**1** appeared as a single sharp band in fractions 17–19, with quantitative recovery. The fractions were pooled, concentrated by rotary evaporation, and desalted by gel filtration through a 1.5 × 27 cm column of Sephadex G-10.

An aliquot of the desalted compound (0.05 μmol) was freed by $CO_2$ by bubbling a stream of $N_2$ through the solution for 30 min. It was then adjusted to pH 9.9 by addition of NaOH and concentrated to dryness by rotary evaporation. The residue was dissolved in 300 μL of dry DMF and acylated by addition of 0.25 μmol of [*carbonyl*-$^{14}$C]phthalic anhydride ($2.78 \times 10^5$ cpm μmol$^{-1}$). After 2.5 h at 25 °C, 200 μL of 0.2 M potassium phosphate at pH 7.0 was added and the solution passed through a 1.5 × 27 cm column of Sephadex G-10 equilibrated and eluted with water. The desalted sample was absorbed to 1.5 × 9 cm column of QAE-Sephadex Q-25 in the $Cl^-$ form. The column was eluted by using a linear gradient of NaCl at pH 7 increasing in concentration from 0 to 0.4 M and formed from 400 mL of each component. Measurements of $A_{415}$ and radioactivity showed that 25% of the gold compounds appeared in the flow through and contained no radioactivity. This was presumably unreacted icosa(*N*-acetyl)-**1**. The remaining 75% of the material was eluted in fractions 19–24 (4-mL fractions). That this was mono(*N*-[$^{14}$C]phthalyl)icosa(*N*-acetyl)-**1** was confirmed by $A_{415}$ and radiochemical measurements showing that the specific radioactivity of this compound was $2.84 \times 10^5$ cpm μmol$^{-1}$, the same as that of the [*carbonyl*-$^{14}$C]phthalic anhydride used.

Fractions 19–24 were pooled, concentrated by rotary evaporation to 0.03 mM, and adjusted to pH 3.2. After 0.15 mM $NaBH_3CN$ was added to the solution inside a small flask, the flask was capped and the solution flushed with argon for

30 min. The solution was heated to 46 °C for 65 h, with periodic additions of $NaBH_3CN$ as described above, to remove phthalyl groups. The solution was concentrated by rotary evaporation, adjusted to pH 7, and desalted by gel filtration through a 1.5 × 27 cm column of Sephadex G-10. The desalted compound was rechromatographed through a column of SP-Sephadex C-25 as described above for icosa(*N*-acetyl)-**1**. The column flow through contained 31% of the 415 nm absorbing material as mono(*N*-[$^{14}$C]phthalyl)icosa(*N*-acetyl)-**1**, while the remaining 69% appeared in a single band eluted in fractions 17–20, corresponding exactly to the elution position of icosa(*N*-acetyl-**1**).

*N*-[*4*-[(*Succinimidooxy*)*carbonyl*]*phenyl*]*maleimide*. Maleic anhydride (0.98 g, 10.0 mmol) was dissolved in 10.0 mL of dry acetonitrile. *p*-Aminobenzoic acid (1.37 g, 10.0 mmol) in 25.0 mL of acetonitrile was added dropwise over a 15-min period. After the addition was complete, the reaction mixture was stirred in an ice–water bath for 30 min. The product, *N*-(4-carboxyphenyl)maleamic acid, was collected by vacuum filtration and dried overnight in a vacuum desiccator to give 2.17 g (94% yield) of a yellow powder: mp 219–220°C; $^1$H NMR (270 MHz, $Me_2SO$-$d_6$) $\delta$ 10.61 (singlet, 1 H), 7.94 (doublet, 2 H), 7.73 (doublet, 2 H), 6.41 (doublet of doublets, 2 H, AB, $J$ = 12 and 46 Hz.

*N*-(4-Carboxyphenyl)maleamic acid (1.00 g, 4.25 mmol) was placed in 9.0 mL of acetic anhydride. Sodium acetate (0.90 g) was added and the solution stirred while the temperature was increased to 100 °C. After the solution became homogeneous, the reaction mixture was stirred for an additional 15 min at 100 °C. The solution was cooled to room temperature and poured into a separatory funnel containing 30.0 mL of water. The aqueous mixture was extracted 2 times with 20.0-mL portions of chloroform. The organic extracts were dried over $MgSO_4$ and concentrated by rotary evaporation under reduced pressure. The residue was dissolved in 5.0 mL of acetonitrile and added dropwise to 150 mL of 50% acetonitrile–water. The pH was maintained at 6.0 by addition of 0.50 M sodium bicarbonate. When the solution became homogeneous and stable at pH 6.0, the pH was adjusted to 3.0 with 1.0 N HCl. The solution was extracted twice with 20.0-mL portions of $H_2O$. The $CHCl_3$ layer was then dried over $MgSO_4$ and concentrated to a solid by rotary evaporation under reduced pressure. The crude *N*-(4-carboxyphenyl)-maleimide was recrystallized from ethanol to give 735 mg (80% yield) of pale yellow needles (mp 233–236 °C): $^1$H NMR (270 MHz, $Me_2SO$-$d_6$) $\delta$ 10.64 (singlet, 1 H), 8.05 (doublet, 2 H), 7.50 (doublet, 2 H), 7.22 (singlet, 2 H).

*N*-(4-Carboxyphenyl)maleimide (651 mg, 3.0 mmol) and *N*-hydroxysuccinimide (345 mg, 3.0 mmol) were placed in 25.0 mL of acetonitrile. The mixture was stirred in an oil bath at 50 °C to dissolve the reactants. Dicyclohexylcarbodiimide (618 mg, 3.0 mmol), dissolved in 5.0 mL of acetonitrile, was added to the solution. The reaction mixture was removed from the oil bath and stirred for 60 min. The dicyclohexylurea was removed by vacuum filtration and the filtrate concentrated to a syrup by rotary evaporation in vacuo. The syrup crystallized upon standing. The solid was recrystallized from cyclohexane–ethyl acetate to give 810 mg (86% yield) of long pale yellow-white needles (mp 192–194 °C): $^1$H NMR (270 MHz, $Me_2SO$-$d_6$) $\delta$ 8.23 (doublet, 2 H), 7.70 (doublet, 2 H), 7.25 (singlet, 2 H), 2.91 (singlet, 4 H). Anal. Calcd for $C_{15}H_{10}N_2O_6$: C, 57.33; H, 3.21; N, 8.91; O, 30.55. Found: C, 56.93; H, 3.55, N, 8.83; O, 30.77.

*Reaction of Icosa*(*N-acetyl*)-**1** *with N*-[*4*-[(*Succinimidooxy*)*carbonyl*]*phenyl*]*maleimide*. Icosa(*N*-acetyl)-**1**, 0.1 μmol, was dissolved in 20 μL of water inside a small pear-shaped flask, and 1 μL of 0.1 M NaOH was added. The solution was concentrated to dryness by rotary evaporation. The residue was dissolved in 50 μL of dry DMF, and 0.8 μmol of *N*-[4-[(succinimidooxy)carbonyl]phenyl]maleimide dissolved in dry acetonitrile was added. The solution was stirred for 4 h and filtered through a 1.5 × 18 cm column of Sephadex G-10 equilibrated and eluted with water adjusted with HCl to pH 3.5 to remove excess reagents and organic solvents.

In one experiment the gel-filtered product was concentrated to 100 μL by rotary evaporation and 0.5 μmol of *N*-acetyl-[U-$^{14}$C]cysteine in 100 μL of 0.2 M potassium phosphate buffer at pH 7.4 added. After 30 min at 25 °C the solution was filtered through a 1.5 × 18 cm column of Sephadex G-10 equilibrated and eluted with 50 mM potassium phosphate buffer at pH 7. $A_{415}$ and radiochemical assays on fractions collected were used to calculate specific radioactivity of gold complexes. Selected fractions were combined with 2 M hydroxylamine at pH 7.4 for 1 h at 25 °C and again gel filtered. Radiochemical and $A_{415}$ measurements showed that 30% of the radioactivity originally associated with the product was removed by reaction with hydroxylamine.

In another experiment the acylation product was passed through a cation-exchange column. A 0.068-μmol sample of gel-filtered material was passed through a 0.4 × 4 cm column of SP-Sephadex C-25 equilibrated and eluted with water. $A_{415}$ measurements on the flow through showed that 0.042 μmol (63%) passed through the column without adhering. This was mono[*N*-(*p*-maleimidobenzoyl)]icosa(*N*-acetyl)-**1**. The remaining material, 37%, that was absorbed by SP-Sephadex C-25 was cationic alkylation product and unreacted icosa(*N*-acetyl)-**1**.

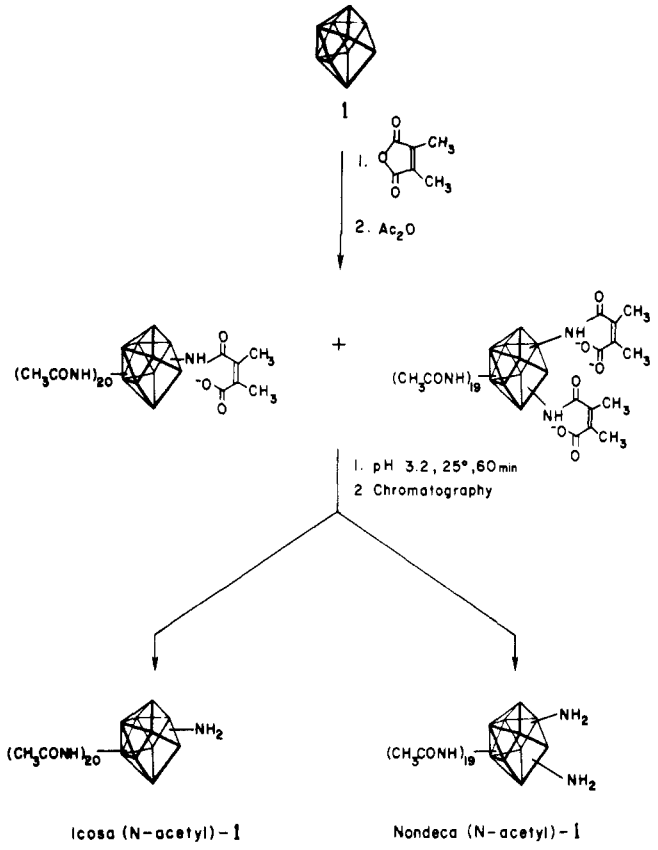
*Reaction of Icosa*(*N-acetyl*)-**1** *with N-Succinimidyl Bromoacetate*. Icosa(*N*-acetyl)-**1** (3.0 $A_{415}$ units, 0.1 μmol) was dissolved in 20 μL of water inside a 1-mL round-bottom flask. After 0.1 μmol of NaOH was added, the sample was dried by rotary evaporation in vacuo. The residue was dissolved in 50 μL of dry DMF. *N*-Succinimidyl bromoacetate (0.8 μmol dissolved in 8 μL of acetonitrile) was added in darkness and the solution stirred for 4 h at 25 °C. After 1 μL of 0.1 M solution of tri-*n*-butylamine in acetonitrile and 300 μL of $H_2O$ adjusted to pH 3.0 were added, the solution was passed through a 1.5 × 18 cm column of Sephadex G-10 equilibrated and eluted with water adjusted to pH 3.0 with HCl. Elution of the orange-red product was monitored visually and by $A_{415}$ measurements. Product-containing fractions were stored at −70 °C and used soon after being prepared. The product mixture was analyzed for acylation and alkylation products as described above. These products could be separated by cation-exchange chromatography as described above for mono[*N*-(*p*-maleimidobenzoyl)]icosa(*N*-acetyl)-**1**.

## Results

*Icosa*(*N-acetyl*)-**1** *from Mono*(*N-phthalyl*)*icosa*(*N-acetyl*)-**1**. Icosa(*N*-acetyl)-**1** is a derivative of **1** in which 20 of the 21 primary amino groups have been acetylated, leaving a single primary amino group available for reaction. Two methods have been devised for preparing this derivative.

The phthalyl group in mono(*N*-phthalyl)icosa(*N*-acetyl)-**1** can be removed by heating to 45 °C at pH 3.2 under anaerobic conditions in the presence of a modest excess of $NaBH_3CN$. This procedure removes about 80% of the phthalyl groups, producing about an 80% yield of icosa(*N*-acetyl)-**1**. This can be separated from unreacted mono(*N*-phthalyl)icosa(*N*-acetyl)-**1** by cation-exchange chromatography through a column of SP-Sephadex C-25 at pH 7. Icosa(*N*-acetyl)-**1** is

Scheme I



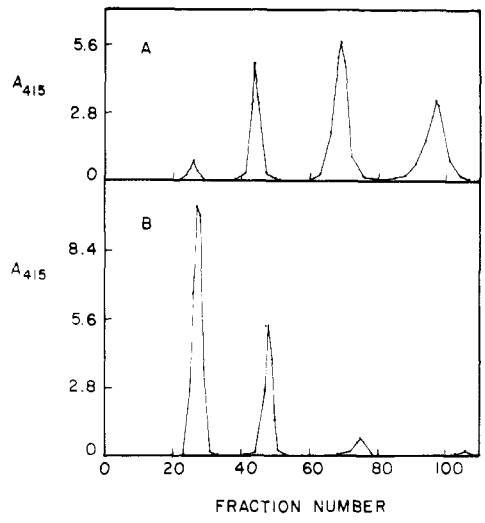




FIGURE 1: Purification of icosa(*N*-acetyl)-**1** and nondeca(*N*-acetyl)-**1**. The synthetic procedure and chromatography are described under Experimental Procedures. In part A the molar ratio of 2,3-dimethylmaleic anhydride to **1** in the syhthesis was 2.6:1.0. In part B this ratio was 1.3:1.0.

a monocation at pH 7 and is absorbed by the ion exchanger, whereas unreacted mono(*N*-phthalyl)icosa(*N*-acetyl)-**1** is a monoanion and passes straight through the column. Icosa-(*N*-acetyl)-**1** can be eluted with NaCl and desalted by gel filtration through Sephadex G-10.

It is critically important to carry out the removal of phthalyl groups under anaerobic conditions, preferably also in the presence of $NaBH_3CN$. Heating derivatives of **1** at pH 3.2 or even at pH 7 in the presence of air leads to marked, progressive changes in the visible absorption spectrum, which are detectable within a few minutes. The spectral change can be fully reversed by addition of $NaBH_4$, demonstrating that it results from an oxidative process. Accordingly, the exclusion of oxygen during heating at pH 3.2 prevents 90% or more of the decomposition. The additional presence of $NaBH_3CN$, which in contrast to $NaBH_4$ can be used at pH 3.2, completely prevents decomposition.

This procedure can also be used for preparing icosa(*N*,*N*-dimethyl)-**1** and icosa[*N*-methyl-*N*-(carboxymethyl)]-**1** from the corresponding mono(*N*-phthalyl) derivatives.

*Direct Synthesis of Icosa(N-acetyl)-1.* A convenient direct synthesis of isosa(*N*-acetyl)-**1** makes use of the ease with which 2,3-dimethylmaleamides undergo hydrolysis. The method is outlined in Scheme I. Partial acylation of **1** with a slight molar excess (1.3) of 2,3-dimethylmaleic anhydride produces a mixture of N-2,3-dimethylmaleylated derivatives of **1**, represented in Scheme I as a mixture of mono- and bis(2,3-dimethylmaleyl) clusters. Acetylation of this mixture with acetic anhydride converts the remaining free amino groups to *N*-acetyl groups. Adjustment of the pH to 3.2 for 60 min at 25 °C effects deacylation of all of the *N*-(2,3-dimethylmaleyl) sites, producing a mixture of icosa(*N*-acetyl)-**1**, which contains 20 *N*-acetyl groups and one primary amino group, and nondeca(*N*-acetyl)-**1**, with two primary amino groups, plus smaller amounts of other species. In neutral solutions the primary amino groups exist as ammonium ions. Icosa(*N*-acetyl)-**1** and nondeca(*N*-acetyl)-**1** are, therefore, monocations and dications, respectively, at pH 7 and can easily be separated by cation-exchange chromatography.

The separations are illustrated in Figure 1, which shows the results of two experiments in which the ratio of 2,3-dimethylmaleic anydride to **1** was varied. In part A the ratio was 2.6, resulting in the production of largely bis- and tris-(2,3-dimethylmaleyl) clusters and smaller amounts of mono- and tetrakis(2,3-dimethylmaleyl) species. After acetylation and removal of 2,3-dimethylmaleyl groups, the product mixture consisted largely of nondeca- and octadeca(*N*-acetyl)-**1**, i.e., with two and three free primary amino groups, respectively, and only a small amount of icosa(*N*-acetyl)-**1**, which appeared as the first band eluted from a column of SP-Sephadex C-25. In part B the ratio of 2,3-dimethylmaleic anhydride to **1** was 1.3, which led to largely icosa(*N*-acetyl)-**1** at a yield of 55% and a smaller amount of nondeca(*N*-acetyl)-**1**.

The cluster species separated in Figure 1 are homogeneous with respect to their acetylation states. Note the base-line separations. Homogeneity of icosa(*N*-acetyl)-**1** is verified in experiments that follow. The primary amino groups in icosa(*N*-acetyl)-**1** are not structurally equivalent since the benzylamines in **1** are not structurally equivalent. They are chemically equivalent, however, and for practical purposes in electron microscopic applications they are equivalent.

*Characterization of Icosa(N-acetyl)-1.* The spectroscopic properties of this compound are essentially the same as those of henicosa(*N*-acetyl)-**1**, so we characterized it by a chemical and radiochemical method outlined in Scheme II. The compound after storage for 5 months remained homogeneous by

Scheme II

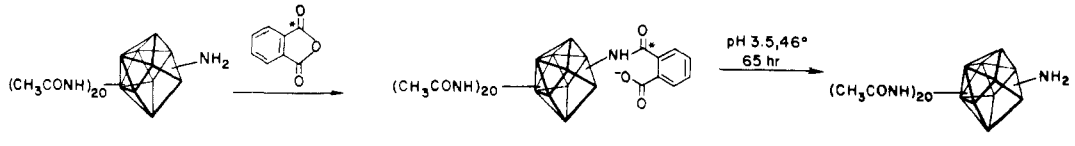


Table I: Exchange Stability of Triarylphosphine Ligands

| time (h) | specific radioactivity (cmp nmol$^{-1}$)[a] |
|---|---|
| 0 | 560 |
| 0.5 | 597 |
| 2.0 | 508 |
| 7.0 | 604 |
| 19.0 | 605 |
| 40.0 | 542 |

[a] A solution consisting of 0.027 mM henicosa(*N*-[$^{14}$C]acetyl)-**1** and 1.9 mM 4,4′,4″-phosphinidynetris(benzenemethanamine) was maintained at pH 7.2 and 25 °C under anaerobic conditions. At selected times 20-μL aliquots were withdrawn and passed through a 0.7 × 10 cm column of Sephadex G-10 to separate dissociated ligands from the complex. The specific activities of the earliest fractions eluted were measured.

cation-exchange chromatography through SP-Sephadex. Acylation by [*carbonyl*-$^{14}$C]phthalic anhydride produced a high yield of mono(*N*-[$^{14}$C]phthalyl)icosa(*N*-acetyl)-**1**, which was purified by anion-exchange chromatography through a column of QAE-Sephadex Q-25 and shown to have the same specific radioactivity as that of the [*carbonyl*-$^{14}$C]phthalic anhydride. Deacylation by heating at 46 °C and pH 3.2 converted this compound back to icosa(*N*-acetyl)-**1**, which exhibited exactly the same properties upon cation-exchange chromatography as the starting compound.

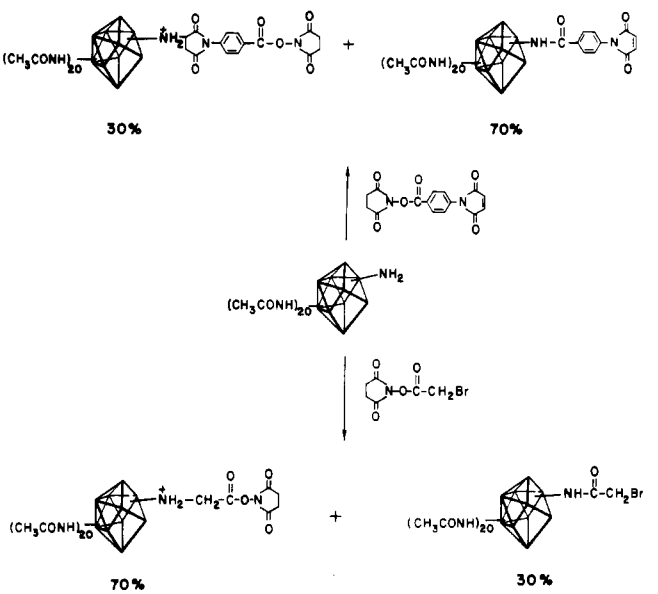
*Stability of Gold Complexes to Ligand Exchange.* To be practical as labeling reagents, monofunctionalized derivatives of **1** must not undergo ligand exchange reactions on a short time scale. The properties of icosa(*N*-acetyl)-**1** and henicosa(*N*-acetyl)-**1** demonstrate the stability of the triacylphosphine ligands. If these ligands were to undergo exchange at a significant rate, removal of phthalyl groups from mono(*N*-phthalyl)icosa(*N*-acetyl)-**1** by heating at pH 3.2 and 46 °C for 65 h should not lead exclusively to icosa(*N*-acetyl)-**1**, since ligand exchange would also produce nondeca(*N*-acetyl)-**1**. In our characterization of this compound in the preceding section the removal of [$^{14}$C]phthalyl groups from mono(*N*-[$^{14}$C]phthalyl)icosa(*N*-acetyl)-**1** produced no more than 0.5% nondeca(*N*-acetyl)-**1**.

In a further test of the exchange stability of ligands henicosa(*N*-[$^{14}$C]acetyl)-**1** was incubated with a large excess of 4,4′,4″-phosphinidynetris(benzenemethanamine), the ligand used in the synthesis of **1**. The specific radioactivity of the gold complex was measured at selected times over a period of 40 h. Any tris[4-[([$^{14}$C]acetylamino)methyl]phenyl]-phosphine ligands that dissociated would have been replaced by tris[4-(aminomethyl)phenyl]phosphine, resulting in a decrease in the specific radioactivity of the complex. As shown in Table I, no such decrease occurred during 40 h.

*Conversion of Icosa(N-acetyl)-1 to an Alkylating Reagent.* The single free primary amino group in icosa(*N*-acetyl)-**1** provides a chemically reactive site to which functional groups that react with proteins may be attached. We have attempted to prepare a derivative in which this amino group is attached to an alkylating functional group. Efforts to convert this amino group to a 2-bromoacetamido group have met with limited success. Reaction with bromoacetic anydride led to very rapid destruction of the gold complex, as shown by the disappearance of the visible absorption bands. Bromoacetyl bromide reacted apparently indiscriminately with other groups in the complex, most probably the acetamido groups.

*N*-Succinimidyl bromoacetate was partially effective (see Scheme III) in that the reaction product could be shown to bind *N*-acetyl[$^{14}$C]cysteine. However, 70% of the radioactivity was released from the complex upon further reaction with

Scheme III




hydroxylamine. This meant that *N*-hydroxysuccinimido bromoacetate reacted in two ways with the amino group, in part (30%) as an acylating agent converting the amino group to a bromoacetamido group and in part (70%) as an alkylating agent. The desired acylation product mono(*N*-bromoacetyl)icosa(*N*-acetyl)-**1** could be separated from the alkylation product because the former does not adhere to the cation exchanger SP-Sephadex C-25 while the alkylation product, which contains a secondary amine, is absorbed by such a column at pH 7. The yield of mono(*N*-bromoacetyl)icosa(*N*-acetyl)-**1** is low (30%), but amounts usable for alkylating proteins can conveniently be prepared.

*N*-[4-[(Succinimidooxy)carbonyl]phenyl]maleimide also reacts with icosa(*N*-acetyl)-**1** (see Scheme III). Again, both acylation and alkylation by this reagent are possible, but in this case acylation predominates. The product binds about 0.85 μmol of *N*-acetyl[$^{14}$C]cysteine/μmol of gold complex. About 30% of the $^{14}$C is released by reaction with hydroxylamine. This reflects the presence of 30% alkylation product since, as illustrated in Scheme III, this product is itself an acylating agent and would react with *N*-acetyl[$^{14}$C]cysteine to form a thio ester. Hydroxylamine cleaves the thio ester and thereby releases *N*-acetyl[$^{14}$C]cysteine bound in this way. The 70% of *N*-acetyl[$^{14}$C]cysteine not released by hydroxylamine reflects the acylation product. This product, illustrated in Scheme III, alkylates *N*-acetyl[$^{14}$C]cysteine, but $^{14}$C is not released by treatment with hydroxylamine. Again, both products are formed, with mono[*N*-(*p*-maleimidobenzoyl)-icosa(*N*-acetyl)-**1** predominating. The alkylation product is cationic at pH 7 and can be removed by passage of the product mixture through a small column of SP-Sephadex C-25, as described under Experimental Procedures.

## Discussion

The results of this and the preceding paper (Reardon & Frey, 1984) demonstrate that undecagold complex **1** can be chemically modified and manipulated in aqueous solutions between pH 3.2 and 12 as well as in organic solvents. Derivatives containing a single reactive carboxyl or amino group can be prepared, and these groups can be chemically activated or modified to prepare acylating and alkylating derivatives of undecagold clusters for use in labeling proteins.

The most serious experimental complication encountered in this work was the propensity of **1** to form carbamates in

the presence of $CO_2$. Solutions of **1** that have not been expressly freed of $CO_2$ contain carbamoylated species of **1** that seriously complicate and even undermine the chemical modifications and chromatographic purifications described here. One manifestation of the carbamate problem was the tendency of **1** to become cross-linked to dimers and higher multimers during various chemical modifications. These species could be detected by gel filtration through Bio-Gel P-6. The relationship between dimerization and a few carbamoylated sites undermined the process of blocking primary amino groups by alkylation or acylation of *N*-succinyl, *N*-phthalyl, or *N*-(2,3-dimethylmaleyl) derivatives. The gradual decarboxylation of such carbamates unmasked primary amino groups at later, sensitive times during further chemical modification processes, and these amino groups frequently reacted with acylating or alkylating derivatives of **1** to form dimers. The importance of freeing all samples of $CO_2$ before and during chemical manipulations cannot be overemphasized. It is advisable to carry out all chemical reactions of **1** in an inert atmosphere.

Gold clusters with a single reactive primary amino group per molecule can be prepared by removing phthalyl groups from the appropriate monophthalyl derivatives in which the remaining 20 amino groups have been stably acylated or alkylated to tertiary amines. By this means such complexes of any desired charge type can be synthesized. For most applications it is best for the overall charge on the complex to be small or zero; the ideal molecule then is icosa(*N*-acetyl)-**1**, which is most conveniently synthesized by the procedure outlined in Scheme I. The amino group in icosa(*N*-acetyl)-**1** is reactive in acylation and alkylation reactions as well as imine formation. It is in many ways an ideal anchoring point to which a broad range of biospecific ligands and chemically selective functional groups can be attached.

The procedure in Scheme I depends upon the special properties of 2,3-dimethylmaleic anydride as a protective group. It is quite effective for a short period of time, in this case during acetylation of unreacted amino groups. The 2,3-dimethylmaleamides are quite labile to hydrolysis, however, so that after the remaining primary amines have been converted to acetamido groups, hydrolysis of 2,3-dimethylmaleamides proceeds smoothly under mild conditions. The hydrolytic lability of 2,3-dimethylmaleamides has been reported by Dixon & Perham (1968).

The gold complexes described here show promise as protein labeling reagents in electron microscopic analysis of complex biochemical structures. The complexes can be visualized by scanning transmission electron microscopy (Wall et al., 1982; Safer et al., 1982). Single heavy atom labeling of biological specimens has shown some promise of producing high-resolution structural information, but problems remain because of radiation damage to biological structures at the high electron doses required to visualize single atoms (Beer & Ottensmeyer, 1979; Wall et al., 1978; Cole et al., 1977). It is conceivable that with the lower radiation doses required for **1**, somewhat improved structural analysis may be achieved.

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