Synthesis of Water Soluble Undecagold Clusters for Specific Labelling of Proteins

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Water Soluble Undecagold Cluster, Ligand Exchange

A highly water soluble undecagold cluster was prepared using a N-hydroxyethyl-N-acetyl-derivative of 4,4′,4″-phosphinidynetri(benzenemethanamine). Monofunctional clusters were synthesized in high yield by the exchange of ligands with phosphines having one free amino group. The clusters can be used for heavy atom labelling of proteins after introduction of a reactive maleimido group.

Introduction

Water soluble undecagold clusters have been used successfully for the labelling of proteins and the subsequent visualization of the label by electron microscopy [1, 2, 3]. In addition, undecagold clusters have also been used as heavy atom labels in X-ray crystallography of very large proteins [4]. The monofunctional clusters needed for these experiments can be prepared by blocking all but one of the 21 amino groups of the water soluble undecagold cluster first described by Bartlett *et al.* [5, 6] or by use of a mixture of appropriately substituted phosphines for the synthesis of the gold complex [7]. However, in addition to the monofunctional clusters formed by these methods, a relatively large amount of the precious starting material is converted into clusters which do not have a functional group or have more than one and are thus unsuitable for specific labelling. For experiments in electron microscopy usually only trace amounts of the cluster samples are sufficient, whereas in crystallographic work relatively high amounts of monofunctional clusters are required. In this paper a more effective method of synthesis of monofunctional undecagold clusters is described.

In the experiments described below, a gold cluster having only identical, hydrophilicly substituted ligands is used as the starting material. Heating a solution of this cluster and a phosphine having one free amino group leads to an exchange of ligands. The desired mono-amino derivative can be easily separated from the reaction mixture and further substituted by a maleimido group, giving a cluster which displays a high affinity for the SH groups of proteins. Since all products of the ligand exchange reaction can be re-used, the yield of monofunctional cluster is theoretically quantitative.

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Results

Synthesis of phosphines

Phosphine ligands used for cluster synthesis were derivatives of the tris-[*p*-(aminomethyl)phenyl]phosphine **4** first described by Bartlett *et al.* [5]. The ligands of the gold cluster used as starting material were N-hydroxyethyl derivatives of **4,** prepared by reducing the Schiff base formed from 4,4′,4″-phosphinidynetri(benzaldehyde) (**1**) and monoethanolamine. Remaining NH groups were acetylated to give a hydrophilic phosphine **3** without free amino groups.

The phosphines used for exchange were prepared from **4** by acylation of two out of the three amino groups using a *t*-butyloxycarbonyl substituent (BOC) as an intermediate blocking group. Acetyl groups or succinyl groups were used as substituents, depending whether or not a negative charge of the final cluster derivative is desirable (see experiments).

Synthesis of the gold cluster used as starting material

The reduction of the complex formed from **3** and AuCN by $NaBH_4$ leads to a mixture of clusters, from which at least four major fractions can be isolated by ion exchange chromatography (Fig. 1). Part of the material does not bind to the acidic ion exchanger (fraction A). The other components (fraction B, C and D) can be eluted by a buffer of increasing ionic strength. The amount of a particular cluster species

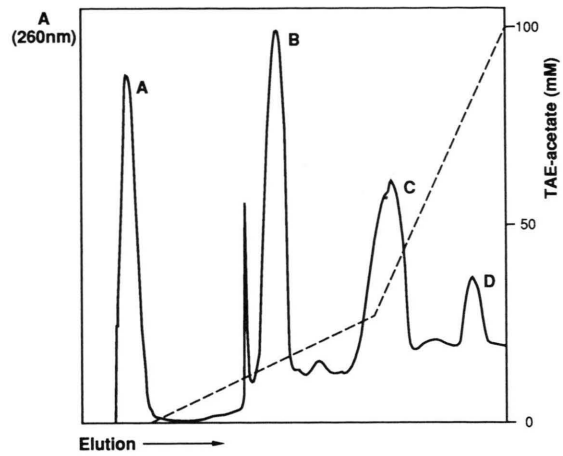


Fig. 1. Ion exchange chromatography of the mixture of cluster A, B, C, and D on a Sephadex SP C50 column (see experiments). The UV absorbance of the eluate and the TEA acetate concentration (broken line) were plotted. The total elution vol was about 2.2 l.

present in the reaction mixture depends strongly on the conditions of the reaction. The ratio between fractions A and B changes in favor of B if the reduction of the gold complex is done in water/alcohol mixtures at high pH (see experiments). Standing of the primary reaction mixture in air for several hours leads to a strong decrease of the yield of fractions C and D but shows very little influence on the yield of fractions A and B. This effect is accompanied by a change in colour of the reaction mixture from dark brown to ruby red.

Characterization of the reaction products by electrophoresis in a pH gradient

It was found that a sensitive analytical characterization of these clusters and their derivatives is possible on isoelectric focusing gels. Clusters without negative charges (having only their intrinsic positive charge) move by electrophoresis in the pH gradient, while clusters with additional negative charges move to the site of their isoelectric point. The term "isoelectric focusing" (IEF) will be used here, regardless of whether or not the separation is due to real electrofocusing or to electrophoresis.

Checking of the products of cluster synthesis by this method reveals, that the material of fraction A (cluster A) behaves like an almost uncharged compound. It moves very slowly to the anode (Fig. 2). The other fractions move to the cathode corresponding to their increasing positive charge, in accordance with the elution profile on the ion exchange column. Fraction B forms a homogeneous band (cluster B) while fractions C and D usual display several bands, depending on the time interval between chromatography and IEF.

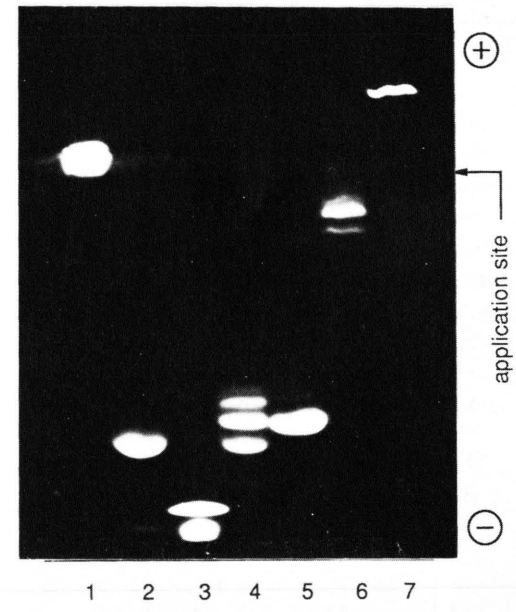


Fig. 2. Characterization of cluster species on an isoelectric focusing gel (contract print). Lane 1: fraction A, lane 2: fraction B (undecagold cluster), lane 3: fraction C and D, lane 4: equilibrium mixture after ligand exchange between **9** and **8** (in the original an additional band is visible), lane 5: purified **11,** lane 6: maleimido derivative **12,** lane 7: **12** after reaction with mercaptopropionic acid.

Characterization of cluster species by UV/VIS spectra

Clusters A and B display a high variability of their UV/VIS spectra, depending on the details of the preparative procedure. An investigation of factors which influence the spectra showed, that these cluster species are sensitive at low pH to oxidation (*e.g.* by *m*-chloroperbenzoic acid, *m*CPB) and at high pH to the effect of reducing agents (*e.g.* $NaBH_4$ or SH-reagents). Furthermore, similar or identical spectral changes can be induced by strong acids (HBF_4 or HCl, which mimic the effect of *m*CPB) or by complexing agents (CN^-, phosphines; same effect as $NaBH_4$), respectively. Surprisingly, the main products of oxidation of cluster A or B cannot be distinguished by IEF from the corresponding reduced forms of the same clusters. However, in addition to the main products there appear by-products after oxidation by *m*CPB displaying the spectrum of the ox-

idized form of the cluster but having an electrophoretic mobility different from the corresponding reduced form. In view of these observations, the terms "oxidized" or "reduced" will be used here in a technical sense only to describe those two forms of cluster A (or B, respectively) which differ in their UV/VIS spectra but show identical electrical charge on IEF. The description will also be restricted to results relevant for the synthesis of a monospecific undecagold cluster.

The clusters prepared by the method described below, usually display spectra of a mixture of "oxidized" and "reduced" forms. In some experiments the reaction products are in a completely "oxidized" state. The spectrum of cluster A in the "oxidized" form (Fig. 3) is almost identical to the spectrum of an Au_{13} species already described [8]. Addition of $NaBH_4$ or of NaCN converts this spectrum almost instantaneously to the "reduced" form (Fig. 3). After addition of a free phosphine the spectrum of cluster A changes slowly (time scale 30 min to several hours) to the spectrum of cluster B.

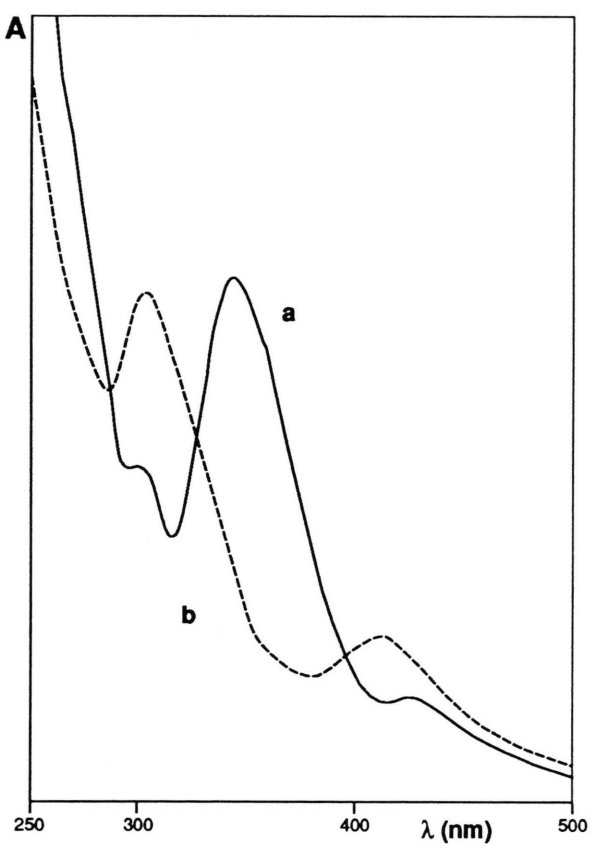


Fig. 3. UV/VIS spectrum of cluster A in water/0.1% TEA. a: "oxidized" form, b: "reduced" form, recorded 2 min after addition of 5 mM NaCN to the cuvette.

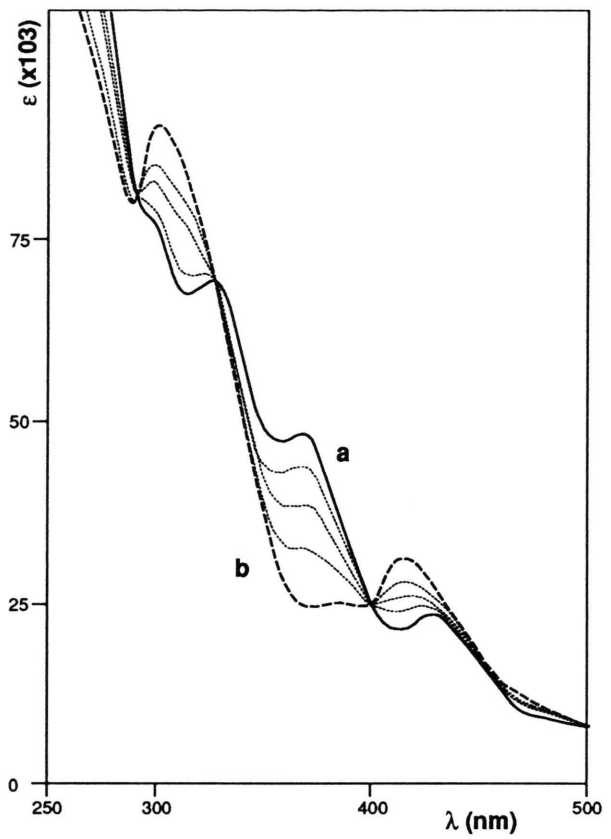


Fig. 4. UV/VIS spectrum of the undecagold cluster, dissolved in water/0.1% TEA. a: "oxidized" form, b: "reduced" form, recorded 60 min after addition of 10 mM $NaBH_4$ to the cuvette. The dotted lines represent intermediate states recorded 2, 8 and 16 min, respectively, after addition of $NaBH_4$.

The spectrum of cluster B after treatment with $NaBH_4$ at high pH is identical to the known [8] spectrum of the undecagold cluster (Fig. 4) and was used for the identification of this reaction product. The treatment of the undecagold cluster with $NaBH_4$ gives rise to the appearance of small amounts of other cluster species. For this reason the isosbestic points usually are not absolutely sharp. A quantitative conversion of the "oxidized" form to the "reduced" form is possible with phosphines (see below). Addition of NaCN to a solution of the undecagold cluster induces a similar spectral change as $NaBH_4$.

However, there is a superposition by a simultaneous interconversion of the undecagold cluster to cluster A (not shown). The "oxidized" form of the undecagold cluster forms spontaneously during storage of the cluster (dry substance or solution) at room temperature for a few weeks. It can be prepared also by reaction with *m*CPB in the presence of acetic acid. Prolonged action of this reagent leads to the appearance of the spectrum of the "oxidized" form of cluster A (not shown) and finally to complete destruction of the cluster.

The UV/VIS spectra of fractions C and D (not shown) which resemble spectra of gold clusters of lower nuclearity [8] and display a sensitivity to reducing agents, too, were not investigated further.

Preparation of undecagold clusters from fractions C and D

In fractions C and D various cluster species form spontaneously on storage at room temperature (in solution or in the dry state) including the undecagold cluster. The amount of undecagold cluster increases in the presence of low concentrations of cyanide (see experiments). In this way about 50% (by weight) of the amount of fractions C and D can be converted into the undecagold cluster.

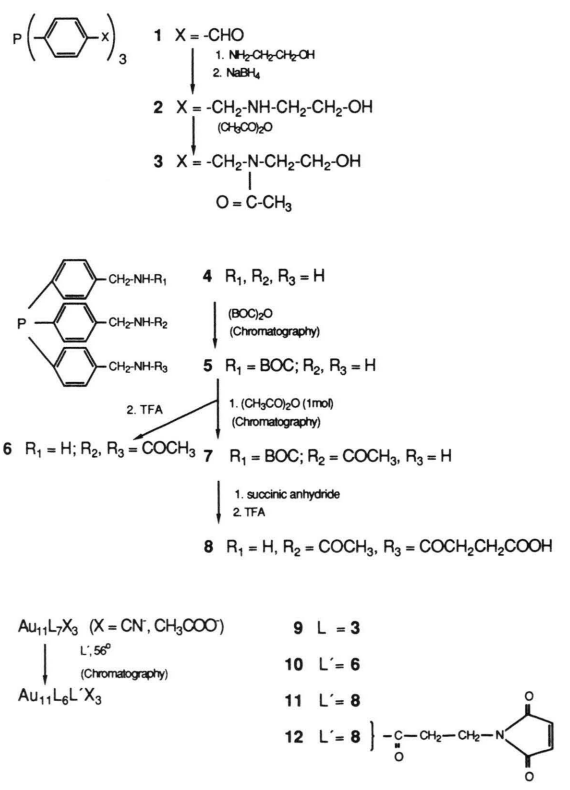
Ligand exchange

Heating a solution of the undecagold cluster **9** and any phosphine of type **4** in water (methanol, dimethylformamide or dimethylsulfoxide may be used as well) leads to an exchange of ligands. This can be easily demonstrated by IEF if the phosphine used is substituted by any basic or acidic group, which gives rise to a change of the electrical charge of the cluster after exchange (Fig. 2). The relative intensity (and number) of the bands formed due to ligand exchange depends on the ratio of phosphine to cluster used. The composition of the equilibrium mixture corresponds within the errors of measurement (photometry of bands eluted from the IEF gel) to the binomial distribution expected for a simple statistical exchange of ligands. At 56 °C complete equilibrium between free and attached ligands is obtained within 3 to 6 hours (molar ratio between cluster and ligands 1:1, concentration 5 to 10 mM). At 95 °C the reaction is completed within 1 hour.

The extinction of the reaction mixture, measured at an isosbestic point of the cluster (398 nm), does not change during the exchange, indicating that no cluster material is lost. If the "oxidized" form of **9** is used as starting material, the conversion of the spectrum to the "reduced" form, mentioned above, is already completed within 5 min at 56 °C, when the ligand exchange is still far from equilibrium. The pH of the reaction medium (range 5 to 9) is without detectable influence on the ligand exchange. A pH above 7 is recommended, however, to avoid partial re-oxydation of the cluster during the reaction.

On a preparative scale the newly formed cluster species can be easily isolated by ion exchange chromatography (see experiments). It may be assumed, that the cluster which elutes from the cation exchange column just behind the starting material, represents the cluster having one amino group. This can by unequivocally confirmed by partial acetylation, using a substoichiometric amount of acetic anhydride. IEF of the reaction products reveals the appearance of only one new band (experiments not shown).

Ligand exchange takes place not only between free phosphine and cluster, but between clusters too. This effect, which cannot be detected by simple chemical



methods in a cluster with identical ligands, gives rise to an instability of the monofunctional clusters. Fortunately the exchange rate at room temperature is low. Standing at room temperature for three days in solution (about 2 mM), the amount of the mono-amino gold cluster **11** diminishes by about 10%, due to partial redistribution of the ligands (the theoretically expected amount in the equilibrium mixture is 40% of the starting value). At elevated temperature the rate of exchange is in the same range as measured in the corresponding experiments with free phosphine ligands. The ligand exchange between clusters is the basis for the re-use of all by-products having more than one amino group.

The rate of ligand exchange is considerably increased in the presence of cyanide. In a 0.1 M NaCN solution the equilibrium distribution of ligands between cluster **9** and **6** is obtained already within 10 min at room temperature (checked by IEF, not shown). For preparative purposes, however, this effect is of little advantage due to the appearance of small amounts of by-products (other cluster species).

Cluster A transforms to the undecagold cluster by treatment with phosphines, as revealed by spectroscopic measurements (see above). Since the phosphine used becomes a ligand in the newly formed undecagold cluster, this reaction can be used for the synthesis of monofunctional undecagold clusters as well. In contrast to the ligand exchange reaction, there is little change in the "oxidation state" of the reacting clusters if not more than approximately one equivalent phosphine is used. Starting the reaction *e.g.* with the "oxidized" form of cluster A yields the "oxidized" form of the undecagold cluster (see experiments).

Introduction of reactive groups

For the attachment of a cluster to a protein it is first necessary to introduce any substituent with a reactive group which is able to form a covalent bond to a protein. For this purpose a maleimido group, which binds with a high affinity to SH groups, can be used [9].

The mono-amino clusters **10** or **11** react at pH 7.8 within seconds with maleimido propionic acid succinimidyl ester [13]. The presence of a reactive maleimido group in the reaction product can be easily demonstrated by treatment of a sample with mercapto-proprionic acid (ammonium salt). IEF of the reaction product reveals the appearance of only one band, which is more acidic than the starting material, due to the change of the electrical charge of the cluster by introducing an additional carboxylic group (see Fig. 2). The activated maleidoyl cluster **12** displays on IEF usually several bands (not seen in Fig. 2, due to overloading), obviously due to reaction with ampholines in the gel.

Discussion

The experiments presented here describe the synthesis of an undecagold cluster, which even when substituted with uncharged ligands displays an extremely high water solubility. Solutions of a concentration higher than 0.1 M can be prepared (molecular weight of the cluster 6500). However, the most important advantage of the procedure described above, as compared to known methods [6, 7], is the efficiency of the synthesis of the monofunctional cluster. The method of ligand exchange allows an almost complete stepwise conversion of a cluster without reactive groups to a monofunctional one. In addition, cluster species which form as by-products during the synthesis of the starting material can be transformed, at least in part, to the undecagold cluster (fraction C and D) or directly used as starting material for the synthesis of the monofunctional undecagold cluster (cluster A).

Fortunately, the ligand exchange in solution is slow at room temperature and will not seriously interfere with the final aim of labelling proteins. The observation of a fast ligand exchange at elevated temperature is in agreement with other investigations in gold cluster chemistry [10]. In contrast, Yang *et al.* [9] describe a very slow rate of ligand exchange in water soluble gold clusters, possibly due to problems of the analytical technique.

The intention of this investigation is not the complete elucidation of the complex reactions involved in the synthetic methods used. Thus, the by-products of synthesis were studied only from the point of their use for the preparation of the undecagold cluster. The interconversion of clusters is a common method in gold cluster synthesis [8, 10, 11].

It is not clear, what the change of UV/VIS spectra of clusters under the influence of oxidizing or reducing agents, respectively, really represents. The fact that NaCN, for example, induces almost identical

changes as $NaBH_4$ suggests, that a substitution of complexing ligands (CN-/acetate?) may be involved.

For practical reasons the reactivity of the "oxidized" form of the undecagold cluster requires further attention, if the SH group of oxidation sensitive proteins need to be labelled. The problem of oxidation by the goldcluster will not however be a serious one, because firstly any monofunctional undecagold cluster prepared by ligand exchange is in an almost completely "reduced" state (due to the reaction with a phosphine in the last step of the synthesis) and secondly, the "reduction" of the cluster by SH groups is very slow compared to the high reactivity of the maleimido group used for the attachment to proteins (experiments not shown). The successful attachment of an undecagold cluster prepared by the method described above, to a ribosomal protein [4] and to the light chain of myosin will be described elsewhere [12].

Experiments

Reversed phase (RP) column chromatography was done on a RP8 column (Lobar-Fertigsäule, RP8, 2×24 cm; MERCK, Darmstadt). Sephadex(R) SP C25 or C50 was used for ion exchange chromatography. The ion exchange gels were pre-washed with 1 M triethylamine acetate (TEA acetate) pH 5.4 and subsequently with water. The same buffer was used for elution. Thin layer chromatography (TLC) was done on silicagel on glass plates (MERCK), using solvent A (*n*-butanol/water/acetic acid, 4/1/1, v/v) or solvent B (50% saturated methanolic ammonia/diethylether/dichloromethane, 1/1/1, v/v). For isoelectric focusing Servalyt(R) precotes(R) 3–10 were used (SERVA, Heidelberg). Testsubstances were placed about 3 cm apart from the anode strip. Running conditiones were according to the suggestions of the manufacturer. Most substances were estimated by absorption measurements giving the amount as optical units (OD) at the wavelength specified. A molar extinction coefficient of $14 \cdot 10^3$ at 260 nm was used for derivatives of **4.** The extinction coefficient estimated for the undecagold cluster **9** at the isosbestic point (398 nm) was $25 \cdot 10^3$ (mean from 4 measurements).

4,4′,4″-Phosphinidynetri(benzaldehyde) (**1**) was prepared according to [5] with minor modifications. The reaction of the Li derivative of *p*-bromobenzaldehyde-diethylacetal with PCl_3 was done below −40 °C to reduce the amount of by-products formed. The trialdehyde **1,** which is hardly soluble in diethylether, was taken up in dichloromethane. The crude **1** crystallized in part (about 80% of the total yield) from acetone by cooling an approximately 20% (w/w) solution to −70 °C. The material remaining in the mother liquor was purified by chromatography on silica gel (Kieselgel 60, MERCK), using *n*-hexan/diethylether/dichloromethane (1/1/1, v/v) as solvent.

Tris-[*p*-(aminomethyl)phenyl]phosphine (4,4′,4″-phosphinidynetri(benzenemethanamine)) (**4**) was prepared by reduction of the corresponding tris-O-methyloxime using $LiAlH_4$ instead of borane. To a solution of 25 g of tris-O-methyloxime in 300 ml tetrahydrofuran (THF) in a 4 l flask was added an excess of $LiAlH_4$ (10 g). A vigorous reaction started spontaneously or after refluxing the mixture for 15 to 30 min. A solid foam formed, filling all the flask. After cracking this material and addition of more THF (500 ml) the mixture was refluxed for an additional 30 min. Excess $LiAlH_4$ was carefully destroyed using THF/water and finally NaOH (35 g NaOH in 500 ml water). The THF layer was decanted from the aqueous phase containing the precipitated $Al(OH)_3$. THF was evaporated *in vacuo*. The residue was dissolved in CH_2Cl_2. Washing of this solution with water and evaporation gave **4** as a syrup (19 g) which crystallized on standing in the refrigerator. This material was used immediately or converted to the tris-tosylate. This substance was identical with a sample prepared according to the original procedure [5].

Tris-[p-(N-acetyl-N-hydroxyethyl-aminomethyl)phenyl]-phosphine (**3**)

1.73 g (5 mmol) **1** were added to a mixture of 20 ml dry ethanol and 3 g (about 50 mmol) monoethanolamine. The suspension was stirred for 20 min at room temperature. 0.4 g $NaBH_4$ (10.5 mmol) were added to the clear solution and stirring was continued for 1 to 2 hours. Liquids were distilled off *in vacuo* (bath temperature up to 90 °C). Excess $NaBH_4$ was destroyed by addition of 10 ml acetic acid. Most of the solvent was evaporated *in vacuo* and the residue was dissolved in 1% acetic acid/water (v/v). This solution (total vol about 15 ml) was applied to a RP column, equilibrated with 1% acetic acid/water. By elution with the same solvent most of the salts emerged first (within a volume of about 130 ml) followed by the N-hydroxyethylphosphine **2** (total elution vol about 200 ml). Fractions containing **2** (checked by TLC; RF 0.3, solvent B) were evaporated *in vacuo* and the residue (about 42,000 OD_{260}; 3 mmol, still containing monoethanolamine acetate) was dissolved in 20 ml methanol and 10 ml triethyl-

amine. Acetic anhydride (2.5 ml, about 25 mmol) was added to the stirred solution. After 20 min most of the solvents were evaporated *in vacuo*. The residue (about 6 ml) was mixed with water giving a total vol of 15 ml. This solution was applied to a RP column equilibrated with 10% methanol/water (v/v). The column was eluted first with 100 ml 10% methanol followed by a gradient formed from 400 ml each of 10% methanol/water and methanol. **3** emerged at a methanol concentration of about 60%. Fractions containing the pure **3** (as checked by TLC) were evaporated *in vacuo* and the dry material (about 36,000 OD_{260}; 2.6 mmol; 52%) was used immediately or stored at −26 °C for a few weeks. The substance crystallized very slowly from a methanolic solution (about 0.3 M) after standing for several weeks at −26 °C.

RF 0.8 (solvent B); FP 165−166 °C.

$C_{33}H_{42}N_3O_6P$ (607)

Calcd	C 65.23	H 6.92	N 6.92,
Found	C 65.51	H 7.11	N 6.82.

Mono-N-t-butyloxycarbonyl-tris-[p-(aminomethyl)-phenyl]phosphine (mono-BOC-tris-[p-(aminomethyl)-phenyl]phosphine) (**5**)

1,7 g (5 mmol) **4** (corresponding to 4.6 g of the **4**-tosylate) were dissolved in 20 ml dioxane and 20 ml water. 1.1 g (5 mmol) di-*t*-butyldicarbonate dissolved in 5 ml dioxane were added within 5 min to the stirred solution. After 10 min 20 ml acetic acid and 100 ml water were added and the solution was extracted once with 100 ml dichloromethane. The mixture was centrifuged, if necessary, to get phase separation. All the mono-BOC derivate and the unreacted starting material remained in the aqueous phase, while most of the bis- and tris-BOC derivates dissolve in the CH_2Cl_2-phase. The aqueous layer was evaporated *in vacuo* to a vol of about 10 ml and applied to a RP column equilibrated with 1% acetic acid/water (v/v). The column was eluted first with 100 ml 1% acetic acid followed by a gradient formed from 500 ml each of the same solvent and methanol. The unreacted **4** (about 14,000 OD_{260}; 1 mmol) emerged in the elution vol between 90 and 280 ml. **5** (about 17,000 OD_{260}; 1.2 mmol) emerged in the vol between 410 and 560 ml. Traces of bis- and tris-BOC-product were eluted from the column by methanol. Fractions containing **5** (as checked by TLC) were evaporated *in vacuo* and the remaining material was dissolved in 5 ml dimethylformamide (DMF) and 0.5 ml triethylamine. **5** crystallized after standing overnight in the refrigerator. Yield of crystalline product 0.62 g (1.1 mmol). An analytical sample was recrystallized from DMF. Fp. 153−155 °C.

$C_{26}H_{32}N_3O_2P \cdot 2(CH_3COOH)$ (569)

Calcd	C 63.2	H 7.03	N 7.37,
Found	C 63.14	H 7.12	N 7.23.

RF (solvent A) **5:** 0.46, bis-BOC derivative: 0.74, tris-BOC derivate: 0.95, **4:** 0.04.

The material from the dichloromethane phase was dissolved in trifluoroacetic acid and kept for 30 min at room temperature. Evaporation of the solvent, treatment of the residue with excess NaOH and extraction with dichloromethane gave part of the starting material back. Total yield of **4** recovered was about 1.1 g (3.1 mmol).

Bis-N-acetyl-tris-[p-(aminomethyl)phenyl]phosphine (**6**) *and mono-N-acetyl-mono-N-succinyl-tris-[p-(aminomethyl)phenyl]phosphine* (**8**)

0.57 g (1 mmol) **5** were dissolved in 10 ml methanol and 0.3 ml triethylamine. 0.1 g (1 mmol) acetic anhydride dissolved in 0.8 ml dioxane were added with stirring within a few seconds. After 5 min solvents were evaporated *in vacuo* and the residue was dissolved in a mixture of 1 ml acetic acid, 30 ml water and 30 ml dichloromethane. The emulsion formed after mixing was centrifugated for phase separation. The aqueous phase, containing starting material **5** and the mono-acetyl-BOC derivative (**7**) was evaporated to a vol of about 5 ml. This solution was applied to a RP column equilibrated with 1% acetic acid/water. The column was eluted with a gradient formed from 500 ml each 1% acetic acid/water and methanol. Fractions were checked by TLC. Unchanged starting material **5** (3500 OD_{260}) emerged at a methanol concentration of 30 to 36%, followed by the monoacetyl derivative **7** (3000 OD_{260}; RF 0.75, solvent A) at a methanol concentration of 46 to 54%. The solvent was evaporated and **7** was dissolved in 10 ml methanol and 0.2 ml triethylamine and treated with 40 mg succinic acid anhydride. Excess anhydride was destroyed after 15 min by addition of a few ml methanolic ammonia and the material obtained after evaporation of solvents was treated for 30 min with trifluoroacetic acid (TFA). The crude **8** obtained after evaporation of TFA was dissolved in 5 ml water/10% methanol/5% acetic acid, (v/v) and purified on a RP column under the same conditiones as described above. **8** emerged at a methanol concentration of 39 to 46%.

Yield 1900 OD_{260}, RF: 0.5 (solvent A), 0.09 (solvent B).

The substance did not crystallize and was either used immediately or stored for up to a few weeks at −26 °C.

The dichloromethane phase obtained after the acetylation step contained mono- and bis-acetyl-

mono-BOC derivative. The CH_2Cl_2 was evaporated and the residue dissolved in 5 ml methanol and 0.2 ml TEA. 50 μl acetic anhydride were added, followed after 10 min by a few ml methanolic ammonia to destroy excess anhydride. The residue obtained after evaporation of solvents was dissolved in TFA (about 10 ml). After 30 min TFA was distilled off *in vacuo* and the residue was dissolved in 5 ml methanol/acetic acid/water, 1/1/8 (v/v). This solution was applied to a reversed phase column equilibrated with 1% acetic acid/water. The column was eluted with a linear gradient up to 100% methanol. Fractions were chacked by TLC. **6** emerged from the column at a methanol concentration of 38 to 46%. Evaporation of the solvent gave a noncrystalline material which was stored at −26 °C.

Yield 3000 to 4000 OD_{260}, RF: 0.5 (solvent A), 0.8 (solvent B).

If **6** was the only product of synthesis wanted, an excess acetic anhydride was used in the first acetylation step.

Synthesis of the gold cluster

1.2 mmol **3** (about 17,000 OD_{260}) in 30 ml 50% ethanol/water (v/v) were mixed with 270 mg (1.2 mmol) AuCN and stirred at room temperature for 60 min. Any unsoluble material was removed by centrifugation and the pH of the supernatant was adjusted to about 9.2 using TEA. Solid $NaBH_4$ (45 mg; 1 mmol) was added to the stirred solution. After 5 min 2 ml of acetic acid were added (CAUTION, HCN is liberated!) and the solution was evaporated *in vacuo* to a vol of about 8 ml. The dark brown solution was applied to a Sephadex LH20 column (2×100 cm) equilibrated and eluted with 20% methanol/0.5% acetic acid/water (v/v). The main peak of the orange red coloured material was collected separately from the slightly yellow and UV absorbing material which eluted immediately thereafter (tail fraction). Most of the solvent of the main fraction was evaporated *in vacuo,* the residue diluted to about 100 ml and applied to a Sephadex SP C50 column (18×2.5 cm). The column was first eluted with about 200 ml of water to remove unbound material (fraction A) followed by elution with a TEA acetate gradient (total vol 2 l) up to 0.1 M (see Fig. 1). A very small sharp peak which emerged immediately before the first main peak was discarded. The main peaks emerged from the column at about 12 mM (fraction B, the undecagold cluster), 30 mM (fraction C) and 100 mM (fraction D) salt concentration (Fig. 1). The particular fractions were evaporated *in vacuo* almost to dryness. The remaining material was mixed with about 3 ml methanol and precipitated by addition of 60 ml acetone to remove the TEA acetate. The desalted cluster substances were collected by centrifugation and dried in a vacuum exsiccator. Yield (results from 5 similar experiments): fraction A (cluster A) 150 ± 70 OD_{394}, fraction B (undecagold cluster) 970 ± 180 OD_{398}, fraction C+D 100 to 300 mg dry substance.

The tail fraction from the LH20 column was evaporated to dryness and the remaining gold complexes were dissolved in ethanol/water and treated with $NaBH_4$ after adjusting the pH to 9.2 as described above. Analogous work up gave additional 95 to 115 OD_{398} of undecagold cluster.

Recovery of undecagold cluster from fraction C and D

The dry material obtained from fraction C and D was collected together and stored at room temperature. 200 mg of this material and 4 mg NaCN were dissolved in 2 ml water and kept at room temperature overnight. Clusters were precipitated by 60 ml acetone and collected by centrifugation. The pellet was dissolved in about 60 ml water and the pH was adjusted to about 3.5. This solution was applied to a Sephadex SP C50 column as described above. Elution with a TEA acetate gradient gave 160 to 180 OD_{398} of undecagold cluster and about 100 mg of fraction C+D.

The total yield of **9** obtained from 1.2 mmol **3** including the material recovered, was about 1400 OD_{398} (56 μmol, 51%). Analysis of **9** (fraction B): $Au_{11}L_7X_3$ [L = **3**, $X_3 = (CN)_2$, CH_3COO].

$C_{235}H_{297}N_{23}O_{44}P_7Au_{11}$ (6526)

Calcd	C 43.21	H 4.55	N 4.93,
Found	C 43.55	H 4.58	N 5.01.

9 was stored as dry material at room temperature. The substance was highly soluble in water, methanol, dimethylformamide or dimethylsulfoxide. Solubility in water was higher than 50% (w/w). In 50% saturated aqueous $(NH_4)_2SO_4$ the maximal concentration was 0.7 mM.

If in the synthesis of gold clusters a higher yield of cluster A was wanted, the reduction of the AuCN/phosphine complex with $NaBH_4$ described above was done in 25 ml ethanol/1 ml triethylamine without addition of water. Yield under these conditiones (results from 5 similar experiments, starting from 1.2 mmol **3**): cluster A 430 ± 100 OD_{394}, undecagold cluster 390 ± 170 OD_{398}.

Oxidation of cluster A

To the solution of 150 mg cluster A (about 650 OD_{394}) in 1 ml methanol were added 15 mg *m*-chloroperbenzoic acid (*m*CPB) dissolved in 200 μl

acetic acid. After 10 min at room temperature the reaction product was precipitated by addition of about 60 ml acetone and collected by centrifugation. The reaction product (about 500 OD_{394}) displayed the spectrum as shown in Fig. 3. A purer sample could be obtained by standing a solution of cluster A in water at room temperature for 2 to 3 weeks under exposure to air.

Synthesis of the mono-amino/mono-succinyl undecagold cluster (**11**)

To a solution of 50 μmol (1250 OD_{398}) undecagold cluster **9** in 5 ml water were added 840 OD_{260} (about 60 μmol) phosphine **8** dissolved in methanol (about 2 ml). The pH was adjusted to about 7.5 using TEA. The mixture was heated overnight (12 to 18 hours) to 56 °C (this temperature was chosen, because it was a fixed temperature on the heating block used). Thereafter the solution was diluted with 50 ml water and the pH was adjusted to about 3.5. Cluster species were separated on a Sephadex SP C25 column (1.7×15 cm). After application of the reaction mixture the column was first washed with 50 ml water, to elute the main part of unchanged starting material (270 OD_{398}). The remaining material was eluted with a TEA-acetate gradient from 0 to 0.1 M, pH 5.6 (total vol 1000 ml). An other part (86 OD_{398}) of unchanged **9** emerged at a 1 to 7 mM salt concentration followed by the mono-amino derivative **11** (440 OD_{398}) at 11 to 16 mM TEA acetate and the bis-amino derivative (229 OD_{398}) at 18 to 25 mM TEA acetate concentration. A very small and sharp peak of unknown material (about 21 OD_{398}) which emerged at the front of the peak of the mono-amino derivative was discarded. Material still remaining on the column (about 160 OD_{398}) was eluted with a small vol of 0.5 M TEA acetate. The particular fractions were evaporated *in vacuo* to a small vol (about 2 ml), diluted with 1 ml methanol and desalted by precipitation with acetone as described above. The pellets obtained after centrifugation were dried in a vacuum exsiccator for a few hours. The monofunctional cluster was either used immediately or stored in liquid nitrogen.

Total recovery of cluster material was about 1200 OD_{398} (96%). The clusters containing more than one amino group were mixed with cluster **9** and used for the ligand exchange again.

The preparation of the mono-amino undecagold cluster **10** was done in the same way, using phosphine **6** instead of **8** for the ligand exchange. The monofunctional cluster **10** was eluted from the SP C25 column at a TEA-acetate concentration of 30 to 50 mM. The yield and the total recovery of cluster material was in the same range as described for **11.**

Synthesis of the monofunctional undecagold cluster (**10**) *and* (**11**) *starting from cluster A*

To a solution of 100 mg cluster A (approximately 430 OD_{394}) in 5 ml water were added 220 OD_{260} (about 16 μM) phosphine **6** or **8,** respectively, dissolved in about 0.5 ml methanol. The mixture was kept at room temperature for 1 to 2 days. Alternatively, the solution was heated to 56 °C for three hours. The working up procedure was identical to the method described above. The total amount of undecagold cluster species formed from 100 mg cluster A was about 390 OD_{398} (15 μmol). The yield of monofunctional undecagold cluster (**10** or **11,** respectively) was about 160 OD_{398} (6.4 μmol).

Preparation of the mono maleidoyl undecagold cluster (**12**)

150 OD_{398} (6 μmol) cluster **11** were dissolved in 1 ml 0.2 M HEPES/TEA buffer (pH 7.9). 3.2 mg (12 μmol) 3-maleimido propionic acid succinimidyl ester [13] dissolved in about 100 μl acetonitril were added with stirring. The pH dropped within a few seconds to 7.8. After 10 min at room temperature the pH was adjusted to about 6.5 using acetic acid. The reaction product including part of the buffer substances was precipitated with 60 ml acetone and collected by centrifugation. The pellet was dissolved in about 1 ml methanol (part of the salts remained undissolved) and the precipitation was repeated once to remove excess of succinimidyl ester. Finally the pellet was dissolved in 1 ml water and this solution was applied to a Sephadex LH20 column (90×1.5 cm), equilibrated with water. The column was eluted with water, fractions of 3.6 ml were collected. A small first peak of cluster material (7.4 OD_{398}) appeared in fractions 16 to 22. The main peak of **12** (140 OD_{398}, 5.6 μmol) emerged in fractions 23 to 27. Small amounts of remaining cluster material were eluted from the column with 20% methanol/1% acetic acid/water (v/v). The main fraction was evaporated to a small vol (about 0.5 ml) and precipitated with acetone (50 ml). The cluster was stable in a frozen aqueous solution (5 to 10 μmol/ml) at −26 °C for a few days or in liquid nitrogen for at least several months.

The reactivity of the material was checked by IEF before and after addition of a trace of mercapto propionic acid (see Fig. 2).

The mono-amino cluster **10** could be used as starting material in the same way. Purification of the

reaction product was done in this case, however, by ion exchange chromatography on Sephadex SP C50. The corresponding mono maleimido derivative displayed the same binding characteristics to the ion exchanger as cluster **9.**

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