Synthesis of Water Soluble Tetrairidium Clusters Suitable for Heavy Atom Labelling of Proteins

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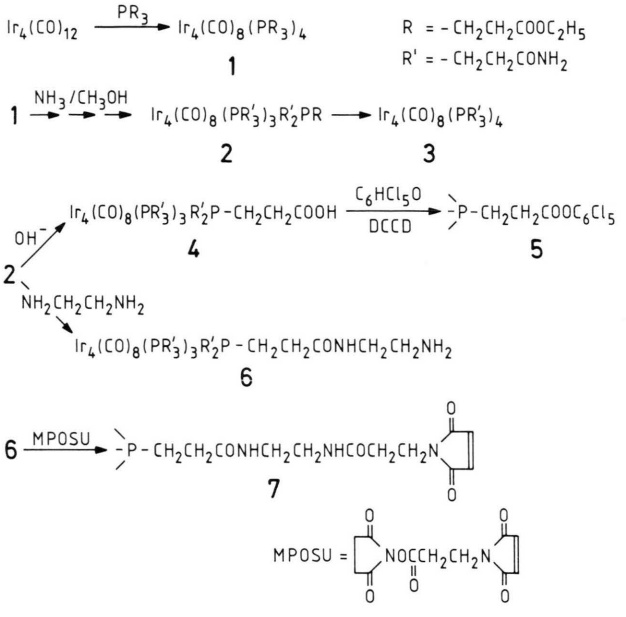
Water Soluble Tetrairidium Cluster

The substitution of 4 CO ligands in $Ir_4(CO)_{12}$ by tris-(2-carbethoxyethyl)phosphine and subsequent reaction with ammonia yielded water soluble Ir clusters which when modified further can be used for heavy atom labelling of proteins.

Current methods of protein structure determination by X-ray diffraction of protein crystals depend on the availability of heavy metal derivatives for solving the "phase problem". Usually the derivatives needed contain a single heavy metal atom at any specific site. For the analysis of complex biological structures such as ribosome crystals [1] or actin gels [2] the effect of a single heavy metal atom on X-ray diffraction may be too small. In this case the use of metal clusters should be of advantage. Large heavy metal clusters have often been used as electron dense labels for electron microscopy [3]. For X-ray experiments with a structural resolution higher than the size of these clusters smaller clusters are needed.

In preliminary experiments we found that water soluble triosmium clusters were useful labelling agents for F actin gels [2]. Further investigation revealed that tetrairidium clusters were even more suitable and easier to prepare.



Results and Discussion

A simple way to make water soluble tetrairidium clusters is by substitution of CO groups in dodecacarbonyl tetrairidium by hydrophilic phosphine ligands. We used tris-(2-carbethoxyethyl)phosphine for this purpose. This phosphine is readily available [4] and offers the possibility of further chemical modifications.

Heating of $Ir_4(CO)_{12}$ with tris-(2-carbethoxyethyl)-phosphine in toluene leads to substitution of four CO groups by this ligand, to give the dodecaester **1** (see formula). Subsequent treatment with methanolic ammonia gives a crystalline water soluble dodecaamide **3.** When stopping this reaction before all the ester groups have reacted, the monoester-undecaamide **2** is obtained. Evidence for the presence of one ester group in this material comes from the reaction with ammonia which gives the dodecaamide **3** without the appearance of any intermediate product (as checked by TLC). The ester **2** can be converted into an acid **4** by treatment with NaOH and into a mono-amino derivative **6** by reaction with ethylenediamine. A water soluble tetrairidium cluster with a maleimido group is synthesized from **6** and a maleimido acid N-hydroxysuccinimid ester [5]. Reaction of the acid **4** with pentachlorophenol/dicyclohexylcarbodiimide leads to an activated pentachlorophenylester **5.** This derivative is almost instantane-

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ously converted into the dodecaamide **3** by treatment with ammonia and into **6** by reaction with ethylenediamine.

The general formulae of the clusters described above are based on CHN analysis of the crystalline dodecaamide **3.** Substitution of four carbonyls in $Ir_4(CO)_{12}$ by phosphine ligands was assumed by comparison with analogous reactions described in the literature [6] and confirmed by the CHN analysis of the crystalline monoester **2.** It may be assumed, that the cluster **3** is not a completely symmetrical molecule, and that the monoester **2** will be a mixture of isomers differing only in the position of the ligand which bears the ester group. This may be the reason why it is difficult to prepare **2** in a crystallized form (see experimental part). The other derivatives did not crystallize.

All the clusters described display the same UV/VIS spectrum (Fig. 1). The spectrum of **5** is a little different, however, due to the absorption of the pentachlorophenyl group.

It was found that **3** was very stable in aqueous solution at pH 6. After standing at room temperature for 10 days no changes could be detected by TLC or spectroscopic measurements. After one month at room temperature traces of by-products could be traced on thin layer plates. On the basis of these experiments the cluster should be suitable for labelling of proteins. The successful attachement of a tetrairidium cluster to a ribosomal protein will be described elsewhere [1].

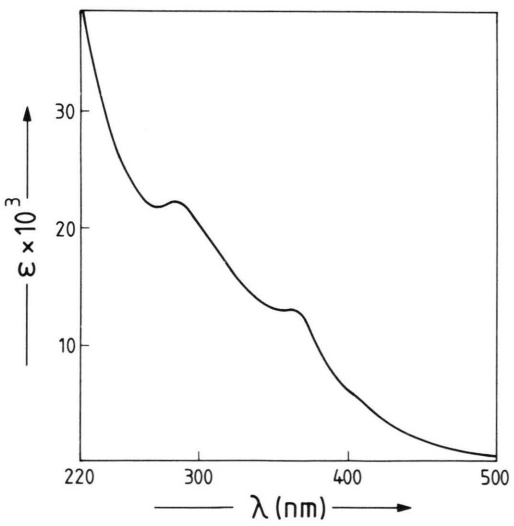

Fig. 1. UV/VIS spectrum of **3** in water.

Experimental

Tris-(2-carbethoxyethyl)phosphine was prepared from tris(cyanoethyl)phosphine* according to Rauhut *et al.* [4]. The reaction with $Ir_4(CO)_{12}$ was carried out under argon. All the reaction products described were insensitive to air and did not need protection by inert gas. Thin layer chromatography (TLC) was performed on silica gel on glass plates (MERCK, Darmstadt) using solvent A (10% water in methanol, v/v) or solvent B (10% aqueous ammonia (25%) in methanol, v/v). The concentration of clusters was estimated on the basis of the absorption at 365 nm using an extinction coefficient = 13000 (measured with **3** in water, see Fig. 1). Optical units (OD) at 365 nm were used as a measure for the amount of clusters.

Synthesis of **1**

To the boiling solution of 0.3 g (0.27 mmol) dodecacarbonyltetrairidium in 250 ml toluene 0.5 ml (about 0.55 g; 1.6 mmol) freshly prepared tris-(2-carbethoxyethyl)phosphine were added. Boiling was continued for 2 h. The toluene was removed under reduced pressure and the residue was dissolved in about 2.5 ml diethylether and 2.5 ml dichloromethane. Any insoluble material was removed by centrifugation. The supernatant was mixed with 2.5 ml petrolic ether and applied to a silica gel column (Kieselgel 60, 70–230 mesh, MERCK, Darmstadt; 2×36 cm) equilibrated with dichloromethane/diethylether/petrolic ether 1/1/1 (v/v). The column was eluted with the same solvent. The red band of the main product emerged after about 200 ml of solvent. Fractions containing the cluster were evaporated to dryness *in vacuo*. Yield 2600 OD (0.2 mmol; 74%).

Synthesis of **2** *and* **3**

0.2 mmol **1** were dissolved in 50 ml saturated methanolic ammonia and kept for 30 h at room temperature. The solvent was evaporated *in vacuo* and the residue was dissolved in about 8 ml water by gentle warming. This solution was applied to a reversed phase silica gel column (Lobar Fertigsäule RP8, 2.5×31 cm; MERCK) which had been previously equilibrated with water. The column was eluted first with a gradient formed by 300 ml each of water and 20% methanol/water (v/v) followed by isocratic elution with 20% methanol/water. Traces of yellow material which eluted first were discarded.

* We are grateful to Hoechst AG. for a gift of this compound.

The first and the second intense orange coloured band were collected separately. All other coloured material remaining on the column was eluted with 100% methanol. Evaporation of the solvent gave the dodecaamide **3** (first band, about 500 OD) and the monoester **2** (second band, about 700 OD). The material eluted with methanol was again dissolved in methanolic ammonia, kept for 8 h at room temperature and worked up in the same way to give additional 200 OD of the monoester. Total yield of **2** 900 OD (35%).

3 crystallized from water (no characteristic F.P., decomp. 220–230 °C). R_f (solvent A) 0.21.

$Ir_4C_{44}H_{72}N_{12}O_{20}P_4$ (1981)
Calcd C 26.65 H 3.66 N 8.49,
Found C 26.41 H 3.44 N 8.40.

2 was used as amorphous material. For the analysis a sample was crystallized from water after standing for several weeks (no characteristic F.P., sintering at 125–130 °C).

$Ir_4C_{46}H_{75}N_{11}O_{21}P_4 \cdot 4\,H_2O$ (2082) R_f (solvent A) 0.27
Calcd C 26.51 H 4.01 N 7.40,
Found C 26.39 H 3.56 N 7.42.

Synthesis of **4**

0.25 mmol (3250 OD) monoester **2** were dissolved in 6 ml water. The pH was adjusted to 12.4 by addition of about 0.2 ml 2 M NaOH. After 5 min at room temperature the solution was made acidic (pH 4.5) by addition of acetic acid and diluted to 20 ml. This mixture was loaded on a Sephadex QAE A25 column (2×12 cm) previously washed with 2 M acetic acid and water. The column was eluted with water to remove unreacted starting material, followed by a gradient formed from 250 ml each of water and 0.5 M acetic acid. The main reaction product emerging from the column after about 150 ml of the gradient was collected. The solvent was evaporated *in vacuo*. Yield about 2100 OD (65%) R_f (solvent A) 0.48. The dry material was stored at −20 °C. The water solution of this compound was not stable. Within a few days at room temperature another species was formed, displaying a differnt UV spectrum.

Synthesis of **5**

50 μmol (650 OD) **4** and 0.1 g pentachlorophenol-dicyclohexylcarbodiimid complex [7] in 2 ml dimethylformamide were stirred until a clear solution was obtained. After standing for 3 d at room temperature the cluster was precipitated by addition of acetone (approx. 20 ml) and collected by centrifugation. The crude material was dissolved in 5 ml 50% methanol/water (v/v) and applied to a Sephadex LH20 column (2×90 cm) equilibrated with methanol. Elution with methanol yielded a broad peak, the front of which contained unreacted starting material and was collected separately (checked by TLC). Yield of pure pentachlorophenylester **5** 460 OD (70%), R_f (solvent A) 0.34.

5 is only sparingly soluble in water. It is easily soluble in dimethylsulfoxide/water mixtures. The substance reacted with methanolic ammonia to give **3** and with ethylenediamine to give **6.** Treatment with diluted NaOH in methanol gave the starting material **4** (checked by TLC).

Synthesis of **6**

0.2 mmol **2** (2600 OD) were dissolved in 10 ml methanol and 5 ml ethylenediamine and heated for 30 min to 50 °C. Liquids were evaporated *in vacuo*. The residue was dissolved in 100 ml water, the pH adjusted to 4.5 using 2 M acetic acid and the solution applied to a Sephadex SP C25 column (2×12 cm) previously washed with 1 M triethylamine acetate pH 6.2 followed by water. The column was eluted with a gradient from 0 to 120 mM triethylamine acetate (pH 6.2), total volume 600 ml. The monoamino cluster **6** emerged at 60 to 80 mM buffer concentration, followed by a minor peak of unknown material. The main fractions were concentrated *in vacuo* to a volume of 3 to 4 ml, and the product was precipitated with 50 ml acetone and collected by centrifugation. Yield about 2000 OD (76%), R_f (solvent B) 0.13.

Synthesis of **7**

50 μmol (650 OD) **6** were dissolved in 0.5 ml 0.4 M HEPES/triethylamine buffer (pH 7.8), 75 μM (20 mg) maleimidopropionic acid N-succinimidyl ester (5) dissolved in 0.6 ml acetonitrile were added and the pH was kept between 7.6 and 7.8 for 10 min by careful addition of 10% triethylamine/water (v/v). The reaction was stopped by adjusting the pH to 6.0 using acetic acid. The cluster was precipitated by addition of 40 ml acetone. The material was collected by centrifugation, dissolved in 1 ml water and applied to a reversed phase silica gel column (Lobar Fertigsäule RP8, 1×24 cm; MERCK) previously equilibrated with water. The column was eluted with 150 ml water followed by a gradient formed from 300 ml each of water and methanol. The yellow band of **7** emerged at a methanol concentration of about 20%. Fractions were concentrated *in vacuo* to about 2 ml and the cluster was precipitated by addition of

30 ml acetone. Yield 325 OD (50%), R_f (solvent A) 0.21.

The substance was used for labelling experiments immediately or stored for a few days in liquid nitrogen. The reactivity of the maleimido group was checked by addition of mercapto propionic acid (aqueous neutralized solution; R_f of the reaction product: 0.48, solvent A).

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