Labeling of Proteins by a Triosmium Carbonyl Cluster via a Bolton−Hunter-like Procedure

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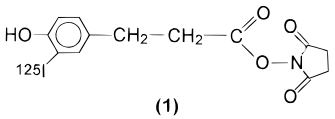
Received February 7, 1996[⊗]

The labeling of proteins employing succinimido 4-pentynoate or pyrylium derivatives of mono- and dinuclear organometallic complexes is now a well-established procedure. We have covalently linked a 5d trimetallic cluster fragment, namely "$Os_3(CO)_{10}$", to a model amine (i.e. benzylamine), to model amino acids (i.e. β-alanine and Nα-protected lysines), and finally to bovine serum albumin (BSA). The complete reaction sequence, starting from $Os_3(CO)_{10}(\mu_3\text{-}\eta^2$-succinimido 4-pentynoate) as the Bolton−Hunter-like reagent, was verified at the molecular level by spectroscopic techniques. The average coupling ratio (CR) between the Os_3 marker and BSA is fairly high, i.e. around 20. This result highlights the potential use of the Os_3-labeling procedure for improving the X-ray structural determination and transmission electron microscopy (TEM) definition of proteins.

Introduction

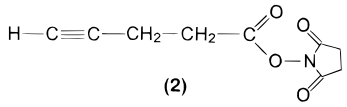
Radioisotopic labeling (^{125}I, ^{3}H, ^{14}C, ...) is a very sensitive method often employed in biological and clinical chemistry.[2] Among the conjugation protocols used for the production of ^{125}I proteins with high specific activities, the Bolton−Hunter reagent succinimido 3-(5-^{125}I-4-hydroxyphenyl)propionate, **1**, plays an important role. This acylating agent promptly reacts with terminal and side-chain amino groups (i.e. lysine residues) to form amide bonds.[3]



(1)

Owing to legal and technical problems associated with radioactivity, the optimization of "cold" labeling procedures has captured the interest of many research groups in the last two decades.[4] In 1977, Cais et al.[5] proposed a nonisotopic metalloimmunoassay (MIA) procedure, for which organometallic complexes were chemically bonded to biomolecules and the resulting conjugates quantified in virtue of their metallic content by atomic absorption spectroscopy (AAS). More recently, Jaouen et al. labeled biomolecules with metal carbonyl fragments and detected the conjugates by FT-IR spectroscopy in the ν_{CO} region (2150−1800 cm^{-1}), where proteins do not absorb. This protocol has been defined as carbonylmetalloimmunoassay (CMIA).[6]

Although proteins are polyfunctional molecules with several reactive sites, there is not at the moment a suitable method to covalently bind organometallic fragments to them. The synthesis of succinimido 4-pentynoate, **2**, represented a new entry in the well-know alkyne-cluster chemistry.[7]



(2)

The σ/π coordination of polymetallic fragments to the triple bond of **2** provided complexes which proved to be stable under biological conditions. These complexes are also able to conjugate proteins and can be easily detected by FT-IR spectroscopy.

In this way, the "$Co_2(CO)_6$" unit has been incorporated in **2**, and the resulting acylating reagent was employed for labeling peptides,[8] drugs,[9] and antibodies.[10]

In the present study it was attempted to form complexes between **2** and a large (and expensive) Os_3 cluster. This is justified by the hypothesis that the presence of third-row metal cores (of known nuclearity, geometry, and reactivity) in protein crystals could represent an important improvement in the resolution of their 3-D structure by X-ray diffraction.[11] So far, nonspecific labeling with inorganic salts has been generally employed for the isomorphous replacement method. A further advantage of the present labeling

[⊗] Abstract published in *Advance ACS Abstracts,* June 1, 1996.

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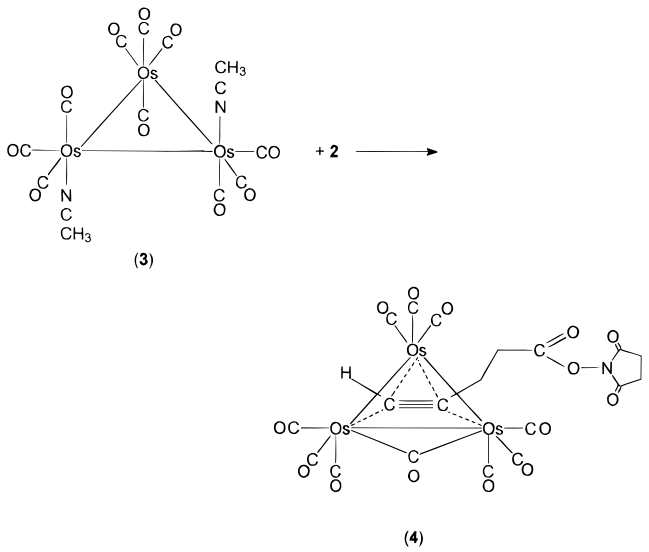
S0276-7333(96)00088-X CCC: $12.00

Scheme 1



method is that the binding of several electron-dense heavy metal clusters to the proteins is likely to allow better visualization of biological compounds *in vitro* by means of transmission electron microscopy (TEM). Up to now, the widespread use of gold colloids only permits nonspecific labeling. A precedent use of a tetrairidium cluster can be found in the literature.[12]

Results and Discussion

Synthesis and Characterization of Model Compounds. The parent cluster $Os_3(CO)_{12}$ is quite inert toward CO substitution. In contrast, the "light-activated" $Os_3(CO)_{10}(NCMe)_2$ derivative **3** is highly reactive, due to the presence of labile acetonitrile ligands.[13] As a matter of fact, **3** undergoes fast reaction with **2** in THF at room temperature giving $Os_3(CO)_{10}[\mu_3\text{-}\eta^2\text{-}(HC{\equiv}C\text{-}(CH_2)_2C(O)ONS)]$ (NS = *N*-succinimidyl radical), **4**, with an excellent yield (ca. 80%) (Scheme 1). This compound was characterized by elemental analysis, IR and ^{1}H- and ^{13}C-NMR spectroscopy, and desorption chemical ionization (DCI) mass spectrometry[14] (see Experimental Section). Noteworthy, compounds **4**−**8**, which have fairly high molecular weights (about 1000-1200 Da) and moderate to low polarity, are much more effectively characterized by the DCI technique than both electron impact (EI, ionizing energy = 70 eV) or fast atom bombardment (FAB, glycerol matrix) ionization methods. Among these techniques, only DCI provides clean spectra, excellent sensitivity, and well-defined isotopic patterns for the parent ions $[M + H]^+$ and the fragment ions $[M + H - CO]^+$ of the complexes under investigation (Figure 1).

Once the synthesis and characterization of **4** was completed it was still necessary to verify that the coordination by "$Os_3(CO)_{10}$" had not altered the acylating property of **4**, since inactivation of NS toward nucleophiles has occasionally been observed in some

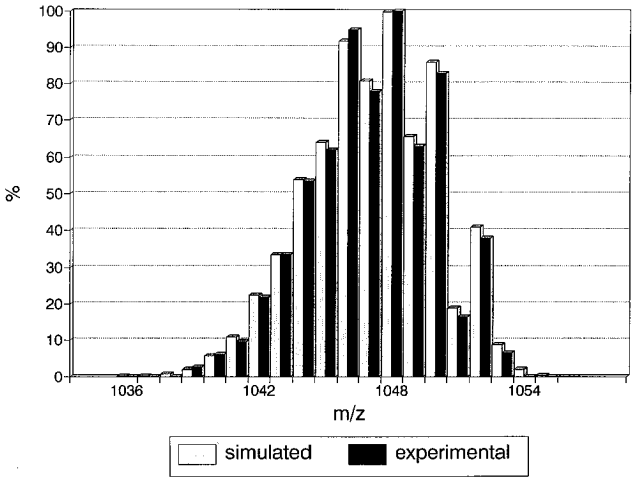
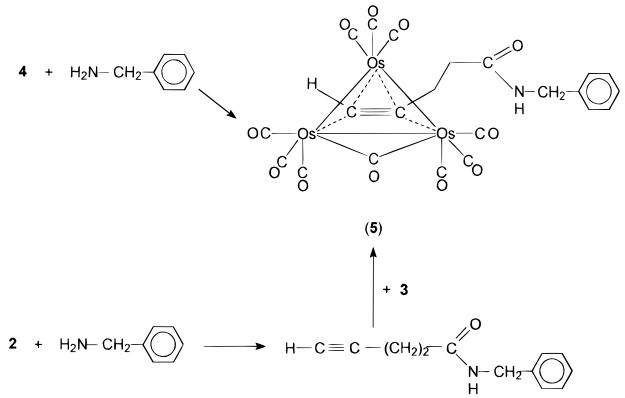


Figure 1. Experimental and simulated DCI mass spectra of **4** in the region corresponding to the $[M + H]^+$ ion. The two isotopic patterns have been compared by setting at 100% intensity the most abundant peaks at $m/z = 1048$ in each spectrum.

Scheme 2



organometallic derivatives.[15] Therefore, we tested the reactivity of **4** toward a model amine (i.e. benzylamine) and two model amino acids (i.e. β-alanine and lysine).

The reaction of **4** with benzylamine proceeded smoothly and completely within 24 h at ambient temperature giving $Os_3(CO)_{10}[\mu_3\text{-}\eta^2\text{-}HC{\equiv}C(CH_2)_2C(O)N(H)CH_2Ph]$, **5**, in ca. 60% yield (Scheme 2). Interestingly, the identical product **5** was obtained by following an alternative route, i.e. first coupling **2** with benzylamine and then reacting the resulting organic ligand with **3** (Scheme 2).

Similarly, **4** reacts with β-alanine ethyl ester to produce $Os_3(CO)_{10}[\mu_3\text{-}\eta^2\text{-}HC{\equiv}C(CH_2)_2C(O)N(H)(CH_2)_2C(O)OEt]$, **6**, in ca. 40% yield (Scheme 3).

It is important to recall that the IR spectra of derivatives **4**−**6** are quite similar in the ν_{CO} region; in other words, the local symmetry of the heavy "$Os_3(CO)_{10}$" moiety is only slightly perturbated when the organic chain is varied. Therefore, IR spectra in the ν_{CO} (2150−1800 cm^{-1}) range represent good fingerprints for the presence of the Os_3 marker in the conjugate.

Also UV−vis spectra of derivatives **4**−**6** are quite similar and exhibit an absorption band around 430 nm (they all appear yellow). This represents a second fingerprint of the presence of the Os_3 marker in the

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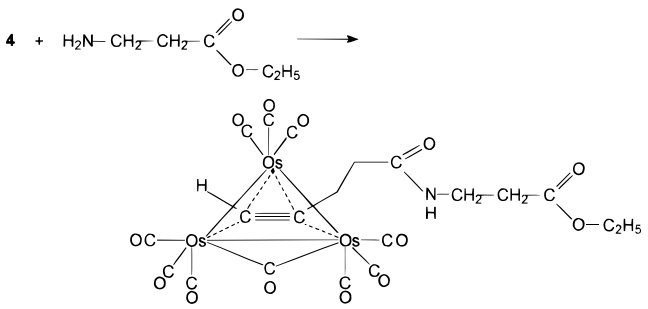
Scheme 3



(**6**)

conjugate. In contrast, in the higher frequency region of the spectrum, strong overlap occurs between bands due to the aromatic side chains of proteins and the metal-to-ligand charge-transfer (MCLT) transition [d(Os) → π^*(CO)] due to the Os_3 marker.[16]

Dynamic Behavior of Model Compounds. At room temperature the methylene groups of ≡CCH_2CH_2-CO− chain exhibit two complex multiplets in the ^{1}H-NMR spectra of the derivatives **4**−**6**, indicating a diastereotopic character. Indeed, each proton pair within a methylene group is rendered inequivalent because of the intrinsic chiral nature of the cluster (the addition of an unsymmetrical alkyne to an Os_3 triangle breaks the molecular mirror plane and lowers the overall symmetry to C_1). However, the well-known "windshield wiper" process, by which the alkyne migrates over the metal triangle, should lead to racemization of the entire cluster.[17] We have performed a VT-NMR study of **5**, which exhibit multiplets for the methylene protons in a region of the spectrum where other resonances do not overlap. For this study we employed a low-field (90 MHz) instrument in order to fit a suitable range of temperature (Figure 2). As the temperature is increased, the diastereotopic character of the methylene protons is lost and the −CH_2CH_2− system gives rise to two well-resolved triplets centered at 2.67 and 2.25 ppm, respectively. At the same temperature the virtual triplet (ABX system) assigned to the −NHCH_2Ph group simplifies to a doublet centered at 4.35 ppm. An activation energy of about 62 kJ mol^{-1} can be roughly estimated from the coalescence temperature.[18] This activation energy fits well with that obtained for $Os_3(CO)_{10}(\mu_3$-η^2-EtC≡CEt) by complete line-shape analysis of the VT ^{1}H- and ^{13}C-NMR spectra (i.e. 60 kJ mol^{-1}).[19]

Labeling Tests of Lysine with 4. We have employed Nα-protected lysines since a previous study[20] using similar Bolton−Hunter-like reagents with L-lysine reported the possible formation of mixtures of mono- and dicondensation derivatives (*i.e.* also the less basic Nα functionality can be acylated).

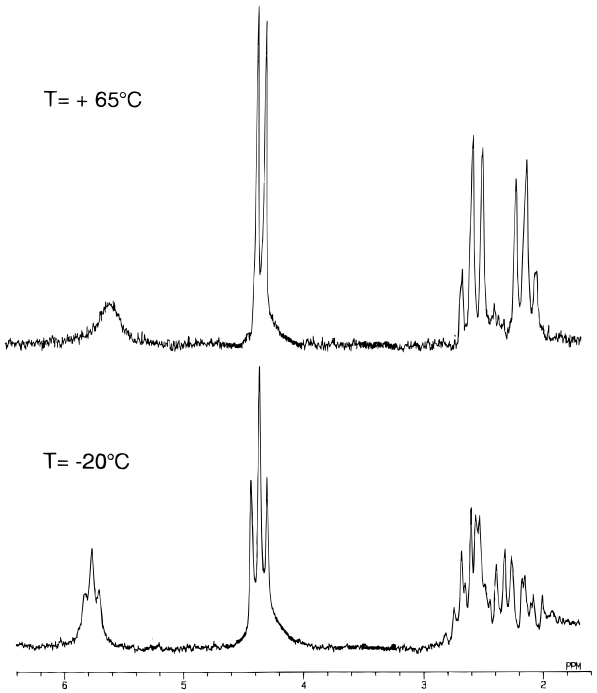


Figure 2. VT-^{1}H-NMR spectra (90 MHz, $CDCl_3$) of **5** at +65 °C (top) and −20 °C (bottom).

Samples of **4** reacted with Nα-*t*-BOC-L-lysine or Nα-CBZ-L-lysine (*t*-BOC = *tert*-butoxycarbonyl and CBZ = carbobenzoxy groups) giving rise to $Os_3(CO)_{10}[\mu_3$-η^2-HC≡C$(CH_2)_2$C(O)N(H)$(CH_2)_4$CH(NHCOOCMe$_3$)(COOH)], **7**, and $Os_3(CO)_{10}[\mu_3$-η^2-HC≡C$(CH_2)_2$C(O)N(H)$(CH_2)_4$CH(NHCOOC$H_2C_6H_5$)(COOH)], **8**, respectively. The crude products were identified by means of IR spectroscopy and mass spectrometry (see Experimental Section).

Labeling of BSA with 4. Bovine serum albumin (BSA) is a 67 kDa globular, acidic protein which is responsible for ligand transport in biological fluids and whose primary structure is well-established.[21] The three-dimensional structure of the human homologue HSA has been recently solved by X-ray diffraction.[22] BSA consists of 582 amino acids, including 59 lysines and one N-terminal residue all being potentially available for the coupling. In nondenaturing conditions (low temperature, almost neutral pH, and low amount of organic cosolvent) 30−40 lysines are believed to be accessible to coupling reagents.[23] Since **4** is totally insoluble in water, a limited amount of an organic solvent, such as methanol (typically 10% v/v), was added necessary to carry out the coupling reaction. In separate experiments, we checked the stability of **4** in 10% methanol−water solution. When kept under stirring at room temperature for 2 h, **4** was recovered almost quantitatively from the solution. The following procedure was adopted: **4** (dissolved in methanol) was reacted with BSA (dissolved in a basic buffer, pH 9.0) at a molar ratio 30:1. After incubation at 4 °C for 5 h, the mixture was filtered and chromatographed on a gel filtration column. Several fractions were collected; those in which BSA was quantitatively detected by Coomassie Brilliant Blue test[24] were gathered. The

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Table 1. Coupling of BSA with 4: Calculation of the Coupling Ratio CR of the Labeled Protein by Different Methods

species	concn	CR
BSA	[P] = 6.7 μM[a]	
Os_3 marker		
IR (2103 cm^{-1})	[M] = 147 μM[b]	22.0
UV−vis (426 nm)	[M] = 116 μM[c]	17.3
bound amino groups	[M] = 130 μM[d]	19.4

[a] Measured by the Bradford method at 595 nm. [b] $k = A_{2103}$/[M] = 13.8. [c] $\epsilon = 1800$. [d] Measured at 420 nm after overnight reaction with TNBS at pH 9.3.

protein content in the resulting sample was measured by the Bradford method at 595 nm,[24] and the Os_3-marker content by both UV−visible spectroscopy at 426 nm and IR spectroscopy at 2103 cm^{-1}. Furthermore, the number of the residual free amino functions in the labeled BSA was evaluated by the reaction of 2,4,6-trinitrobenzene-1-sulfonic acid (TNBS).[25] All these measurements allowed us to calculate the coupling ratio (CR) as follows: CR = nmol of bound marker/nmol of protein in sample. The results are reported in Table 1. The methods based on direct detection of the marker (i.e. V−vis and IR spectroscopy) gave the same CR value, within the experimental error, as the method based on an indirect detection (TNBS reacts with amino groups of BSA unlabeled by the Os_3 marker). This proves that the coupling of the Bolton−Hunter-like reagent with BSA is not accompanied by decomplexation of the triosmium cluster.

The total recovery of BSA was found to be around 60%, and no free marker was detected after chromatography of the incubation mixture.

The IR spectrum (recorded in the ν_{CO} region) of the labeled protein deposited onto a nitrocellulose membrane is shown in Figure 3, along with the spectrum of **4** recorded under the same conditions. While the number of bands is identical for both samples, their positions are slightly different, except for the totally symmetrical vibration at 2103 cm^{-1}, which was selected for the measurement of marker content (see above).

Conclusions

Succinimido 4-pentynoate reacts with $Os_3(CO)_{10}(NCMe)_2$ in high yield giving a Bolton−Hunter-like reagent. This proved capable to introduce triosmium clusters into biological compounds by a selective acylating reaction with free amino functions. The triosmium acylating reagent reacts with bovine serum albumin in mixed organic/aqueous medium to give a conjugate bearing an average of 20 clusters per protein. These results open the way to the new possible application of organometallic chemistry in the structure elucidation of biomolecules.

Experimental Section

$Os_3(CO)_{12}$ and $Os_3(CO)_{10}(NCCH_3)_2$ were synthesized according to the literature methods.[13b] Succinimido 4-pentynoate, **2**, was prepared according to a published procedure.[9] IR and NMR spectra were recorded on a Perkin−Elmer 580 B and on a JEOL-EX 400 spectrometer, respectively. DCI-MS spectra were recorded on a Finnigan-MAT 95Q instrument with magnetic and electrostatic analyzers. Isobutane was used as the reagent gas at 0.5 mbar pressure. The ion source temperature was kept at 50 °C, the electron emission current at 0.2 mA, and the electron energy at 200 eV. Positive ion spectra were collected. A computer program provides a digital readout of peak intensity and their comparison with the simulated isotopic pattern. The fit was satisfactory for all compounds under investigation.

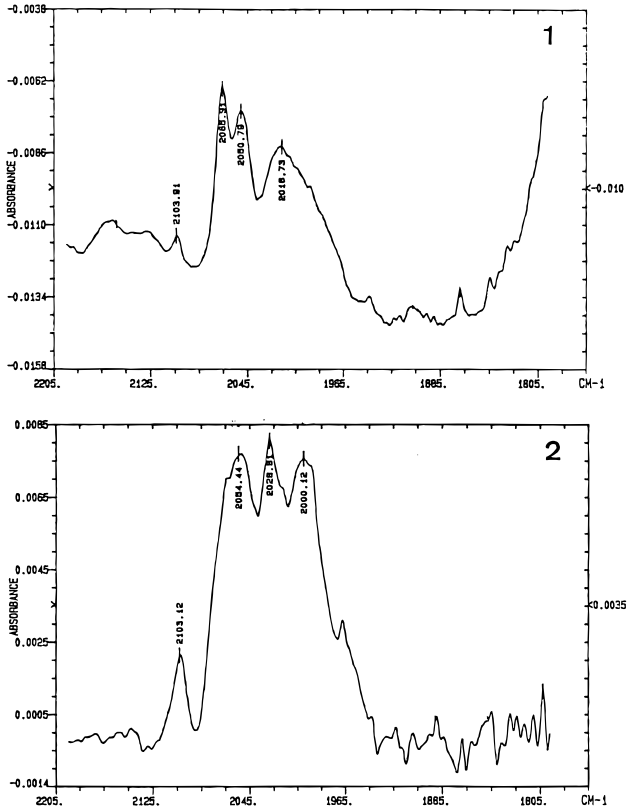

Figure 3. IR spectrum (ν_{CO} region) of BSA labeled with **4** (1). The sample was prepared by deposition of 10 μL of protein solution on a nitrocellulose filter. IR spectrum (ν_{CO} region) of reagent **4** (2). The sample was prepared by deposition of 10 μL of a chloroform solution on a nitrocellulose filter. Spectra were recorded on a Bomem MB100 FT-IR spectrometer equipped with a InSb detector (recording time 1 min).

Synthesis of $Os_3(CO)_{10}(HC{\equiv}C(CH_2)_2C(O)ONS)$, 4. Solid **3** (200 mg, ca. 0.2 mmol) was added to a freshly distilled THF solution (10 mL) of **2** (42 mg, ca. 0.2 mmol) in a Schlenk tube equipped with a magnetic stirring bar and an inlet arm for purging the solution with nitrogen. The solution color turned immediately from yellow to orange. The mixture was stirred at room temperature for 1 h and chromatographed on a silica column. Toluene first eluted $Os_3(CO)_{12}$ (in the presence of adventitious CO, **3** always forms the parent carbonyl cluster), and then CH_2Cl_2 eluted the desired product, **4**, which was crystallized in 1:1 v/v toluene/petroleum ether at 0 °C, giving yellow microcrystals. Yield: 77%. IR (CH_2Cl_2): ν_{CO} 2101 s, 2063 vs, 2054 sh, 2023 vs, 2002 vs, 1840 w, 1819 w, 1791 w, 1745 s cm^{-1}. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 9.18 (s, 1H, ≡CH), 3.37 and 2.90 (both t, 4H, CH_2CH_2, $^3J_{HH}$ = 7.6 Hz), 2.72 (s, 4H, NH cycle). ^{13}C-NMR (100.4 MHz, $CDCl_3$): 177.19 (Os−CO in rapid exchange), 168.84 (CO, NS cycle), 167.34 (C=O), 157.20 (≡*C*CH_2), 107.88 (HC≡), 49.40 and 33.47 (CH_2CH_2), 25.52 (CH_2 NS cycle). MS spectra (DCI, isobutane): $[M + H]^+$ = *m/z* 1049 (for ^{191}Os). Anal. Calcd for $C_{19}H_9NO_{14}Os_3$: C, 21.82; H, 0.87. Found: C, 21.67; H, 0.92.

Synthesis of $Os_3(CO)_{10}(HC{\equiv}C(CH_2)_2C(O)NHCH_2Ph)$, 5. Solid **4** was added to a freshly distilled THF solution of benzylamine in 1:1 molecular ratio. The mixture was stirred overnight and then chromatographed on a silica column.

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Elution with CH_2Cl_2 collected the desired product, **5**. The yield was 58% on the crude product. IR (CH_2Cl_2): ν_{CO} 2101 s, 2063 vs, 2054 sh, 2023 vs, 2002 vs, 1840 w, 1677 m (amide) cm^{-1}. ^{1}H-NMR (90 MHz, $CDCl_3$, + 65 °C): δ 9.18 (s, 1H, ≡CH), 7.40−7.20 (m, 5H, Ph), 5.62 (s, broad, 1H, NH), 4.35 (d, 2H, CH_2N), 2.67 and 2.25 (each t, 4H, CH_2CH_2). ^{13}C-NMR (100.4 MHz, $CDCl_3$): δ 178.10 (Os−CO in rapid exchange), 170.71 (C═O), 159.82 (≡*C*CH_2), 137.93 (C_{ipso}, Ph), 128.7, 127.9 and 127.6 (Ph), 108.94 (HC≡), 50.23 (CH_2N), 43.75 and 38.83 (CH_2CH_2). MS spectra (DCI, isobutane): $[M + H]^+ = m/z$ 1041 (for ^{191}Os). Anal. Calcd for $C_{22}H_{13}NO_{11}Os_3$: C, 25.46; H, 1.26. Found: C, 25.56; H, 1.18.

Synthesis of $Os_3(CO)_{10}(HC{\equiv}C(CH_2)_2C(O)NH(CH_2)_2$-COOEt), 6. β-Alanine ethyl ester hydrochloride, dissolved in CH_2Cl_2, was N-deprotected with a slight excess of NEt_3, and then solid **4** was added in 1:1 molecular ratio. The mixture was stirred overnight at room temperature and chromatographed on a silica column. CH_2Cl_2 removed side products, and then acetone eluted the desired product **6** in 43% yield. IR (CH_2Cl_2): ν_{CO} 2097 s, 2063 vs, 2058 sh, 2023 s, 2002 vs, 1840 w, 1729 m, 1675 m (amide) cm^{-1}. ^{1}H-NMR (400 MHz, CD_2Cl_2): δ 9.19 (s, 1H, ≡CH), 6.09 (s, br, 1H, NH), 4.19 (q, 2H, −COOCH_2CH$_3$), 3.45, 3.02, 2.51 (m, 2H each, methylene groups), 1.25 (t, 2H, −COOCH$_2$CH_3). ^{13}C-NMR (100.4 MHz, CD_2Cl_2): δ 178.20 (Os−CO in rapid exchange), 172.54, 171.01 (*C*═O), 160.48 (≡*C*CH_2), 109.10 (HC≡), 60.86 (−COO*C*H_2CH$_3$), 50.41, 38.87, 35.12 and 32.91 (−CH_2−), 14.14 (−COOCH$_2$*C*H_3). MS spectra (DCI, isobutane): $[M + H]^+ = m/z$ 1051 (for ^{191}Os). Anal. Calcd for $C_{20}H_{15}NO_{13}Os_3$: C, 22.92; H, 1.44. Found: C, 23.02; H, 1.48.

Labeling Tests of Lysine with 4. Solid **4** was added to a freshly distilled THF solution of Nα-protected lysines (Nα-*t*-BOC-L-lysine or Nα-CBZ-L-lysine) in a 1:1 molecular ratio. The mixture was stirred overnight and then chromatographed on a silica column. Elution with acetone collected the desired products **7** and **8**, respectively. We were not able to obtain analytically pure products (i.e. completely free of solvent); however, they were characterized as follows.

$Os_3(CO)_{10}[\mu_3$-η^2-HC≡C(CH$_2$)$_2$C(O)N(H)(CH$_2$)$_4$CH-(NHCOOCMe$_3$)(COOH)], 7. IR (CH_2Cl_2): ν_{CO} 2101 s, 2062 vs, 2057 sh, 2023 vs, 2003 vs, 1839 w, 1789 w, 1722 vs, 1675 s cm^{-1}. MS spectra (DCI, isobutane): $[M + H - CO]^+ = m/z$ 1152 (for ^{191} Os).

$Os_3(CO)_{10}[\mu_3$-η^2-HC≡C(CH$_2$)$_2$C(O)N(H)(CH$_2$)$_4$CH-(NHCOOCH$_2$C$_6$H$_5$)(COOH)], 8. IR (CH_2Cl_2): ν_{CO} 2101 s, 2063 vs, 2055 vs, 2024 vs, 2004 vs, 1840 w, 1787 w, 1717 vs, 1675 w cm^{-1}. MS spectra (DCI, isobutane): $[M + H - CO]^+ = m/z$ 1186 (for ^{191}Os).

BSA Labeling with 4. Two standard solutions were prepared, the first containing 1.0 mg/mL (1.5×10^{-5} M) of BSA (grade V, Sigma) in borate buffer (0.1 M, pH 9.0) and the second containing 4.2 mg/mL (4×10^{-3} M) of **4** in methanol. A mixture of 2.7 mL of protein solution and 0.3 mL of marker solution (i.e. the resulting 10% methanol v/v solution has a 30:1 marker−protein molar ratio) was incubated for 5 h at 4 °C. The mixture was subsequently chromatographed on a gel filtration column (Econopac 10DG, Biorad), and the four initial mL fractions (which proved to contain BSA by positive response to the Coomassie Brilliant Blue reagent[24]) were collected together and analyzed. The protein concentration [P] was determined by the Bradford method[24] while the marker concentration [M] was measured by both UV−vis spectroscopy at 426 nm (ϵ = 1800 M^{-1} cm^{-1}) and IR spectroscopy at 2103 cm^{-1}, following a technique previously described.[20]

The number of free amino groups was measured by reaction of 2,4,6-trinitrobenzene-1-sulfonic acid with both native and labeled proteins by overnight incubation of TNBS at room temperature and pH 9.3, followed by spectroscopic analysis at 420 nm.[25]

The combination of all these data provided the ratio between marker concentration and protein concentration, namely the so-called coupling ratio CR = [M]/[P], as reported in Table 1.

Acknowledgment. We thank the European Union (EU) for financial support (Contracts ERBCIPAACT922266 and ERBCIPAACT930027). We also thank Johnson Matthey Ltd. for a generous loan of OsO_4 and Mr. P. A. Loveday (University Chemical Laboratory, Cambridge, U.K.) for the high-pressure synthesis of $Os_3(CO)_{12}$. We are indebted to Professor M. J. McGlinchey (McMaster University, Hamilton, Canada) for useful discussions.

OM960088I