Journal of Peptide Science
J. Peptide Sci. 2006; **12**: 199–205
Published online 1 August 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.697

JOURNAL OF PEPTIDE SCIENCE

Labeling of proteins and oligopeptides with luminescent lanthanide(III) chelates

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Received 15 March 2005; *Revised 15 May 2005*; *Accepted 2 June 2005*

Abstract: Synthesis of a building block that allows introduction of photoluminescent europium(III) and samarium(III) chelates to synthetic oligopeptides on solid phase using standard Fmoc chemistry is described. Upon completion of the oligopeptide synthesis, these conjugates were converted to the corresponding lanthanide(III) chelates by treatment with appropriate lanthanide(III) salt. Also synthesis of a new terpyridine-based europium(III) chelate designed for solution phase protein labeling is demonstrated.

Keywords: lanthanide(III) chelates; labeling; solid phase; solution phase

INTRODUCTION

Time-resolved fluorometry exploits the unique fluorescence properties of lanthanide(III) chelates [1–4]. The long fluorescence decay after excitation of these molecules allows time-delayed signal detection. This eliminates background signal originating, e.g. from microplates or buffer components. The large Stokes shift (i.e. the difference in the chelate's excitation and emission lines), in turn, results in a high signal-to-background ratio. These unique properties of lanthanide(III) chelates can be exploited particularly in homogeneous assays, when the use of conventional chromophores causes very high background. The different photochemical properties of europium, terbium, dysprosium and samarium chelates enable even development of multiparametric homogeneous assays [5–7].

Most commonly bioactive molecules are labeled in solution. Accordingly, an amino or mercapto group of a bioactive molecule is allowed to react with isothiocyanato, haloacetyl or 3,5-dichloro-2,4,6-triazinyl derivatives of the label molecule. Since in all the cases the reaction is performed with an excess of an activated label, laborious purification procedures cannot be avoided. Especially, when attachment of several label molecules, or site-specific labeling in the presence of several functional groups of similar reactivities is required, the isolation and characterization of the desired biomolecule conjugate is extremely difficult, and often practically impossible. Thus, it is highly desirable to perform the biomolecule conjugation on solid phase; since most of the impurities can be removed by washings when the biomolecule is still anchored to the solid support and after release to the solution, only one chromatographic purification is required. Naturally, solution-phase labeling of large biomolecules, such as proteins cannot be avoided. In these cases, the labeling reaction has to be as selective, and purification of the protein conjugate as effective as possible.

We describe here synthesis of a building block which allows introduction of luminescent europium(III) and samarium(III) chelates to synthetic oligopeptides on solid phase, as well as the preparation of one europium chelate designed for solution phase protein labeling.

MATERIALS AND METHODS

Adsorption column chromatography was performed on columns packed with silica gel 60 (Merck) or basic aluminum oxide (150 mesh, Brockmann I; Merck). Reagents for oligopeptide synthesis were purchased from Nova Biochem and Applied Biosystems. The oligopeptides were assembled on an Applied Biosystems 433A instrument in 10-μmol scale using Fmoc chemistry and recommended protocols. NMR spectra were recorded on a Brucker 250 spectrometer operating at 250.13 MHz for ^{1}H. The signal of tetramethylsilane (TMS) was used as an internal reference. Coupling constants are given in Hertz. IR and UV spectra were recorded on PerkinElmer Spectrum One and Shimadzu UV-2100 instruments, respectively. Electrospray ionization-time of flight (ESI-TOF) mass spectra were recorded on an Applied Biosystems Mariner instrument. Luminescence measurements were measured with a PerkinElmer LS-55 luminescence spectrometer equipped with a Hamamatsu R928 red-sensitive photomultiplier tube. The measurements were carried out in tris-buffer containing 0.9% NaCl at pH 7.75. The luminescence yields were measured by a relative method described earlier [8,9].

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Tetra-(*tert*-butyl)-2,2′,2″,2‴-{(4-(4-methoxytrityl) aminobutylimino)bis(methylene)bis(4-bromo-pyridine-16,2-diyl)bis methylenenitrilo)} tetrakis(acetate), 2

4-Bromo-6-bromomethyl-2-pyridylmethylenenitrilobis(acetic acid) di(*tert*-butyl ester) (**1**, 2.06 g, 6.0 mmol) and 4-(4-methoxytrityl)butane-1,4-diamine (1.15 g, 3.2 mmol) were dissolved in dry DMF (50 ml). *N*,*N*-diisopropylethylamine (DIPEA) (5.2 ml, 30 mmol) was added, and the mixture was stirred for 2 h at room temperature and concentrated. Purification on silica gel (eluent: petroleum ether, bp 40–60 °C: ethyl acetate, from 10 : 1 to 5 : 2, v/v) yielded 1.30 g (33%) of compound **2**. ^{1}H NMR ($CDCl_3$): δ 7.73 (2H, d, J 1.8); 7.57 (2H, d, J 1.8); 7.46 (4H, m); 7.36 (2H, d, J 9); 7.29–7.16 (6H, m); 6.80 (2H, d, J 9); 3.99 (4H, s); 3.78 (4H, s); 3.72 (3H, s); 3.46 (8H, s); 2.62 (2H, q, J 6.9); 2.51 (2H, m); 2.09 (4H, t, J 7.4); 1.98 (1H, s). 1.46 (36H, s and 4H, m). ESI-TOF-MS for $C_{62}H_{82}Br_2N_6O_9$ $(M+H)^+$: calcd, 1213.46; found, 1213.53.

Tetra-*tert*-butyl 2,2′,2″,2‴-{(4-(4-methoxytrityl-aminobut-1-y1-imino)bis(methylene)bis(4-(2-furyl)pyridine-6,2-diyl))bis(methylenenitrilo)} tetrakis(acetate), 3

Compound **2** (1.11 g, 0.91 mmol) and 2-(tributylstannyl)furan (0.68 g, 1.9 mmol) were dissolved in dry DMF (35 ml) and deaerated with argon. $(Ph_3P)_4Pd$ (0.12 g, 0.10 mmol) was added, and the mixture was stirred at 90 °C for 2.5 h in dark. The mixture was cooled to room temperature and concentrated *in vacuo*. Purification was performed on silica gel (eluent: petroleum ether, bp 40–60 °C: ethyl acetate: triethylamine, from 5 : 1 : 1 to 5 : 3 : 1, v/v/v). Yield was 0.83 g (75%). ^{1}H NMR ($CDCl_3$): δ 7.84 (2H, d, J 1.6); 7.73 (2H, d, J 1.6); 7.47 (2H, dd, J 1.9 and <1); 7.44–7.40 (4H, m); 7.33 (2H, d, J 9); 7.25–7.14 (6H, m); 6.93 (2H, dd, J 3.4 and <1); 6.77 (2H, d, J 9); 6.49 (2H, dd, J 3.5 and 1.9); 4.04 (4H, s); 3.81 (4H, s); 3.76 (3H, s); 3.50 (8H, s); 2.69 (2H, m); 2.59 (2H, m); 2.09 (1H, m); 1.55 (4H, m); 1.45 (36 H, s). ESI-TOF-MS for $C_{70}H_{89}N_6O_{11}(M+H)^+$: calcd, 1189.66; obsd, 1189.71.

Tetra-*tert*-butyl 2,2′,2″,2‴-{(4-aminobut-1-yl-Imino) bis(methylene)bis(4-(2-furyl)pyridine-6,2-diyl))bis (methylenenitrilo)}tetrakis(acetate), 4

Compound **3** (0.95 g, 0.80 mmol) was dissolved in dichloromethane (25 ml). Trifluoroacetic acid (0.25 ml) was added, and the mixture was stirred for 2 h at room temperature before being washed with sat. $NaHCO_3$ (caution: foaming). The organic phase was dried over Na_2SO_4, concentrated in vacuo and purified on silica gel (eluent: petroleum ether, bp 40–60 °C: ethyl acetate: triethylamine, 2 : 5 : 1, v/v/v). Yield was 0.61 g (83%). ^{1}H NMR ($CDCl_3$): δ 7.82 (2H, s); 7.73 (2H, s); 7.50 (2H, m); 6.93 (2H, m); 6.51 (2H, m); 4.05 (4H, s); 3.83 (2H, s); 3.82 (2H, s), 3.51 (8H, s); 2.67–2.60 (4H, m); 1.60 (4H, m); 1.46 (36H, s). ESI-TOF-MS for $C_{50}H_{73}N_6O_{10}$ $(M+H)^+$: calcd, 917.54; obsd, 917.57.

Synthesis of the Oligopeptide Labeling Reactant 6

Compound **4** (0.57 g, 0.62 mmol) and Fmoc-Glu-OAll (0.30 g, 0.74 mmol) were dissolved in CH_2Cl_2 (20 ml). Dicyclohexylcarbodiimide (0.15 g, 0.74 mmol; predissolved in 3 ml of CH_2Cl_2) was added, and the mixture was stirred for 6 h at room temperature. The precipitate formed was removed by filtration, and the filtrate was concentrated *in vacuo*. Purification on silica gel (eluent: petroleum ether, bp. 40–60 °C: ethyl acetate from 10 : 1 to 2 : 5, v/v) yielded 0.49 g (60%) of compound **5** (ESI-TOF-MS for $C_{73}H_{94}N_7O_{15}$ $(M+H)^+$: calcd, 1308.62; obsd, 1308.75). It was then dissolved in dichloromethane (20 ml) and deaerated with argon for 15 min. $(Ph_3P)_4Pd$ (8 mg, 7 μmol) and $PhSiH_3$ (69 mg, 0.64 mmol) were added, and the mixture was stirred at room temperature in dark for 30 min, before being washed with 10% aq. citric acid and dried over 4-Å molecular sieves and concentrated. Yield was 0.43 g (91%). ESI-TOF-MS for $C_{70}H_{90}N_7O_{15}$ $(M+H)^+$: calcd, 1268.65; obsd, 1268.74.

Synthesis and Purification of the Oligopeptide Conjugates

Oligopeptide synthesis and introduction of the block **6** to the growing oligopeptide chain was performed using methods described previously [10]. Upon completion of the chain assembly, the oligopeptide conjugates were released from the resin, deprotected and treated with samarium(III) or europium(III) citrate [10]. The crude oligopeptide conjugates were purified on HPLC using a PerkinElmer Series 2000 instrument and a reversed phase column (LiChrocart 125-3 Purospher RP-18e 5 μm). Mobile phase: (Buffer A): 0.02 M-triethylammonium acetate (pH 7.0); (Buffer B): A in 50% (v/v) acetonitrile. Gradient: from 0 to 1 min 95% A, from 1 to 21 min from 95% A to 100% B. Flow rate was 0.6 ml min^{-1}.

4′-(4-(4-Nitrophenyl)butoxy))-2,2′ : 6′-2″-terpyridine 1,1″-dioxide, 8

Compound **7** (4.2 g, 9.86 mmol) was dissolved in dichloromethane (130 ml). *m*-Chloroperbenzoic acid, 8.5 g) was added, and the mixture was stirred overnight at room temperature, washed with sat. $NaHCO_3$ and dried over Na_2SO_4. Purification on silica gel (eluent: MeOH in CH_2Cl_2, from 5 : 95 to 1 : 10, v/v) yielded 3.0 g (67%) of compound **8**. ^{1}H NMR ($CDCl_3$) δ 8.60 (2H, s); 8.35 (2H, dd, J 6.4 and <1); 8.25 (2H, dd, J 8.2 and 2.4); 8.15 (2H, d, J 8.6); 7.37 (6H, m); 4.20 (2H, t, J 5.8); 2.81 (2H, t, J 7.0); 1.88 (4H, m). ESI-TOF-MS for $C_{25}H_{23}N_4O_5$ $(M+H)^+$: calcd, 459.17 obsd, 459.17.

4′-(4-(4-Nitrophenyl)butoxy))-2,2′ : 6′,2″-terpyridine 6,6″-dicarbonitrile, 9

Compound **8** (3.00 g, 6.55 mmol) was dissolved in dichloromethane (120 ml). Trimethylsilyl cyanide (8.3 ml, 66.5 mmol) was added and the mixture was stirred for 5 min at room temperature, after which benzoyl chloride (3.1 ml, 26.7 mmol) was added dropwise during 20 min and the mixture was stirred for an hour room temperature. An aqueous 10% solution of K_2CO_3 (80 ml) was added, and stirring was continued for 1 h. The organic layer was separated, and the aqueous phase was extracted twice with dichloromethane. The combined dichloromethane layers were dried over Na_2SO_4. Purification on basic alumina (eluent 1% MeOH in CH_2Cl_2, v/v) yielded the title compound (2.4 g, 76%). ^{1}H NMR ($CDCl_3$) δ 8.81 (2H, dd, J <1 and 8.0); 8.08 (2H, s); 8.00 (2H, t, J 7.8); 7.75 (2H, dd, J 1 and 7.5); 4.26 (2H, t, J 6.4); 3.71 (2H, t, J 6.3); 1.93 (2H, p, J

6.5); 1.64 (4H, m). IR (KBr): 2237 cm^{-1} ν(C≡N). ESI-TOF-MS for $C_{23}H_{22}N_5O_2^+$ $(M+H)^+$: obsd, 400.1769; calcd, 400.1768.

Tetra-*tert*-butyl 2,2′,2″,2‴-(4-(4-nitrophenyl)butoxy))-2,2′ : 6′,2″-terpyridine-6,6″-diyl)bis(methylenenitrilo) tetrakis(acetate) 11

Compound **9** (1.0 g, 2.1 mmol) was dissolved in dry tetrahydrofuran (THF) (20 ml), and the mixture was deaerated with argon. Borane THF complex (25 ml; 1 M) was added during 5 min and the mixture was stirred overnight at room temperature. The excess of borane was destroyed by careful addition of methanol (100 ml), and all volatiles were removed *in vacuo*. The residue was suspended in sat. methanolic HCl (50 ml), stirred for 4 h at room temperature and concentrated. The residue was triturated with diethyl ether and filtered to give compound **10**. It was then suspended in dry DMF (15 ml). DIPEA (5.7 ml), *tert*-butyl bromoacetate (0.89 ml, 6.0 mmol) and potassium iodide (0.4 g) were added, and the mixture was stirred overnight at room temperature and concentrated. Purification on silica gel (eluent: petroleum ether bp 40–60 °C: ethyl acetate: triethylamine 5 : 2 : 1, v/v/v) yielded compound **11** (1.2 g, 67%). ^{1}H NMR ($CDCl_3$): δ 8.49 (2H, dd, J <1 and 7.2); 8.02 (2H, s); 7.83 (2H, t, J 7.7); 7.67 (2H, dd, J <1 and 6.1); 4.23 (2H, t, J 6.4); 4.14 (4H, s); 3.69 (2H, t, J 6.3); 3.55 (8H, s); 1.86 (4H, m); 1.65 (4H, m); 1.48 (36H, s). ESI-TOF-MS for $C_{47}H_{70}N_5O_{10}$ $(M+H)^+$: obsd, 864.512; calcd, 864.512.

Tetra-*tert*-butyl 2,2′,2″,2‴-{4′-(4-(4-aminophenyl) butoxy)-2,2′ : 6′,2″-terpyridine-6,6″-diylbis (methylenenitrilo)}tetrakis(acetate) 12

Compound **11** (0.62 g, 0.66 mmol) was dissolved in dry methanol (15 ml) containing 10% Pd/C (70 mg). $NaBH_4$ (50 mg, 1.26 mmol) was added, and the mixture was stirred for 3 h at room temperature, filtered through Celite and neutralized with 1 M HCl. All volatiles were removed *in vacuo*, and the crude product was purified on silica gel (eluent: petroleum ether, bp 40–60 °C: ethyl acetate: triethylamine 5 : 1 : 1, v/v/v) to give compound **12** (0.35 g, 56%). ^{1}H NMR ($CDCl_3$) δ 8.49 (2H, d, J 7.0); 8.02 (2H, s); 8.02 (2H, s); 7.83 (2H, t, J 7.6); 7.67 (2H, d, J 7.0); 7.02 (2H, d, J 8.3); 6.65 (2H, d, J 8.3); 4.22 (2H, t, J 6.1); 4.14 (4H, s); 3.55 (8H, s); 2.62 (2H, t, J 7.3); 1.86 (4H, m); 1.48 (36H, s). ESI-TOF-MS for $C_{53}H_{75}N_6O_{10}$ $(M+H)^+$: obsd, 955.55; calcd, 955.55.

2,2′,2″,2‴-{4′-(4-(4-aminophenyl)butoxy)-2,2′ : 6′,2″-terpyridine-6,6″-diylbis(methylenenitrilo)}tetrakis (acetate) europium(III), 13

Compound **12** (0.30 g, 0.29 mmol) was dissolved in trifluoroacetic acid (10 ml), and the mixture was stirred for 3 h at room temperature. All volatiles were removed in vacuo. The residue was dissolved in water (1.5 ml). pH of the solution was adjusted to 6.5 with solid $NaHCO_3$. Europium(III) chloride (0.29 mmol) was added, and the mixture was stirred for 1 h at room temperature, after which pH was adjusted to 8.5 with sodium hydroxide (1.0 M). The precipitate formed was removed by centrifugation. After phenol extraction, the chelate, **13**, was isolated by precipitation with THF. Yield was 0.24 g (94%). IR (KBr) 1621 and 1395 cm^{-1}(C–O–O). λ_{max} (H_2O) 302 nm. ESI-TOF-MS for $C_{37}H_{38}N_6O_{10}$ Eu $(M-H)^-$: obsd, 879.19; calcd, 879.19.

2,2′,2″,2‴-{4′-(4-(4-Isothiocyanatophenyl)butoxy)-2,2′ : 6′,2″-terpyridine-6,6″-diylbis(methylenenitrilo)} tetrakis(acetate) europium(III), 14

An aqueous solution (1.5 ml) of compound **13** (100 mg) was added over 15 min to a mixture of thiophosgene (50 µl), $NaHCO_3$ and chloroform (1 ml). After the mixture was stirred vigorously for 1.5 h, the phases were separated and the water phase was washed with chloroform. The aqueous layer was extracted with phenol, and the phenol phase was treated with water and diethyl ether. The aqueous phase was separated and washed diethyl ether. Precipitation from acetone gave the activated chelate, **14**. ESI-TOF-MS for $C_{38}H_{36}N_6O_{10}SEu^-$ $(M-H)^-$: obsd, 921.12; calcd, 921.14.

Labeling of an Antibody with an Isothiocyanato-activated Chelate, 13

About 2 mg of **14** was dissolved in water (50 µl). Anti-mouse IgG antibody (DAKO; 1.4 mg ml^{-1} in 50 mM Tris-HCl containing 0.1% sodium azide) was prepurified using NAP columns (Amersham) to remove Tris-HCl and azide, which interfere labeling. After a concentration step in Centricon 30 device (Amicon) antibody concentration was 2.4 mg ml^{-1} in 0.9% NaCl. One milligram of antibody (6.25 nmol) was labeled using 470 nmol of the dissolved Eu chelate, **14**. Reaction was started by adding 50 µl of 1 M sodium carbonate pH 9 to adjust pH to 9–9.3. Total reaction volume was 500 µl. After overnight reaction at +4 °C the labeled antibody was purified from the unreacted chelate with Sephadex G-50/Sepharose 6B column (10 cm + 35 cm, diameter 1 cm) and TSA buffer (50 mM Tris-HCl pH 7.8 containing 0.9% sodium chloride and 0.05% sodium azide) as the eluent; UV detection at 254 nm. The labeled antibody was characterized and labeling yield was 6.5 Eu chelates per antibody.

RESULTS AND DISCUSSION

Labeling of Oligopeptides on Solid Phase

We have already reported a solid phase method for the labeling of oligopeptides [10] and oligonucleotides [11–13]. The approach involves synthesis of oligonucleotide and oligopeptide building blocks, which can be introduced to the biomolecule structure using commercial oligonucleotide and oligopeptide synthesizers with phosphoramidite and Fmoc chemistries, respectively. Upon completion of the chain assembly, the oligomers are deprotected and finally treated with the appropriate lanthanide(III) citrate producing the desired biomolecule conjugates. Recently, we have demonstrated that lanthanide(III) chelates based on furylpyridine subunits are among the brightest europium(III) and samarium(III) chelates synthesized [5]. Also their applicability to oligonucleotide [13] and

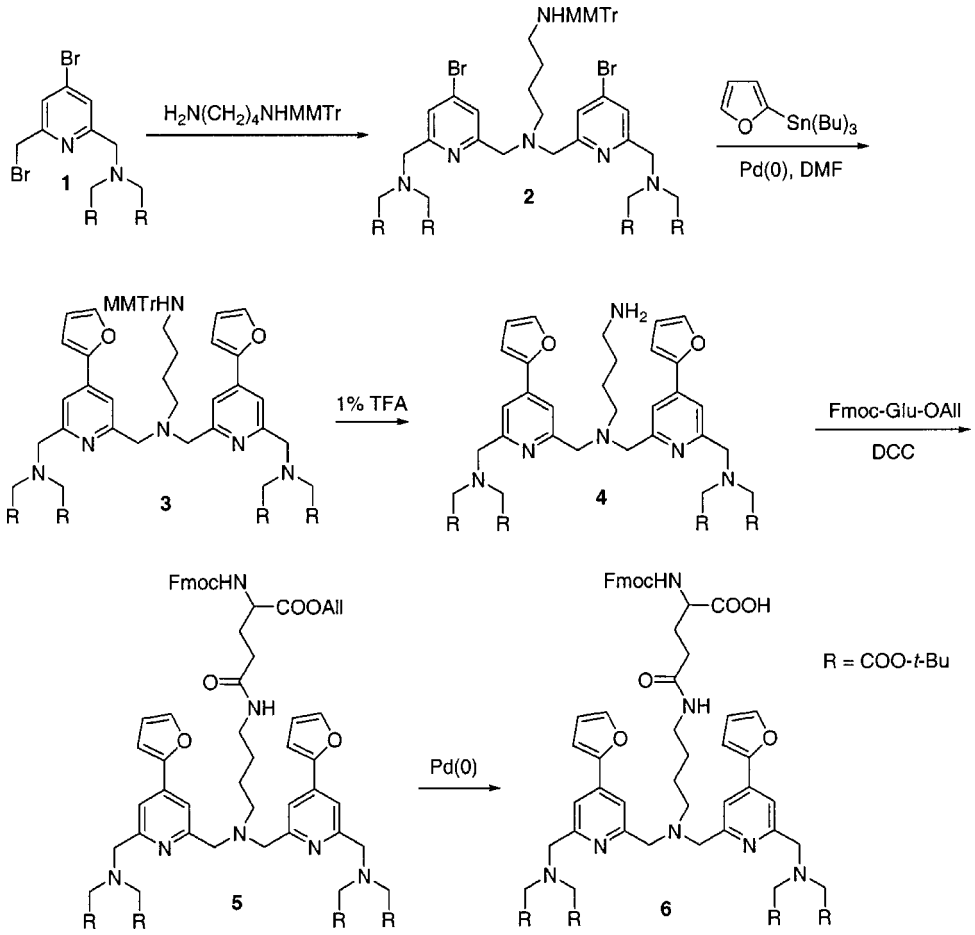
oligopeptide labeling [5] on solid and solution phase, respectively, was demonstrated. Here, the synthetic strategy of the ligand was modified to fulfill the requirements of standard solid phase oligopeptide synthesis (Scheme 1). Accordingly, 4-bromo-6-bromomethyl-2-pyridylmethylene-nitrilobis(acetic acid) di(*tert*-butyl ester), **1** [14] was allowed to react with MMTr-protected butane-1,4-diamine to give **2**, reaction of which with tributylstannylfuran in the presence of Pd(0) yielded **3**. After removal of the amine protection, the ligand **4** was converted to the corresponding glutamic acid derivative, **5** using DCC as the promoter. Palladium-catalyzed allyl cleavage using phenylsilane as the allyl scavenger yielded the oligopeptide labeling reagent, **6**.

To demonstrate the applicability of the building block **6** for oligopeptide derivatization, two model sequences (Angiotensin 2 and Substance P) were synthesized in 10-μmol scale using Fmoc chemistry. The block **6** was coupled either to the amino or carboxyl terminus of the coding sequence using prolonged coupling time (2 h instead of 30 min) but otherwise standard conditions (3.5 equiv. of **6**; HBTU/HOBt as an activator). According to fulvene–piperidine assay, coupling efficiency of **6** was comparable to natural amino acid analogs. After completion of the oligopeptide synthesis, the oligopeptides were released from the resin and deprotected followed by precipitation from ether. Treatment of the deblocked oligomers with europium(III) or samarium(III) citrate converted the oligopeptide conjugates to the corresponding lanthanide chelates (Scheme 2).

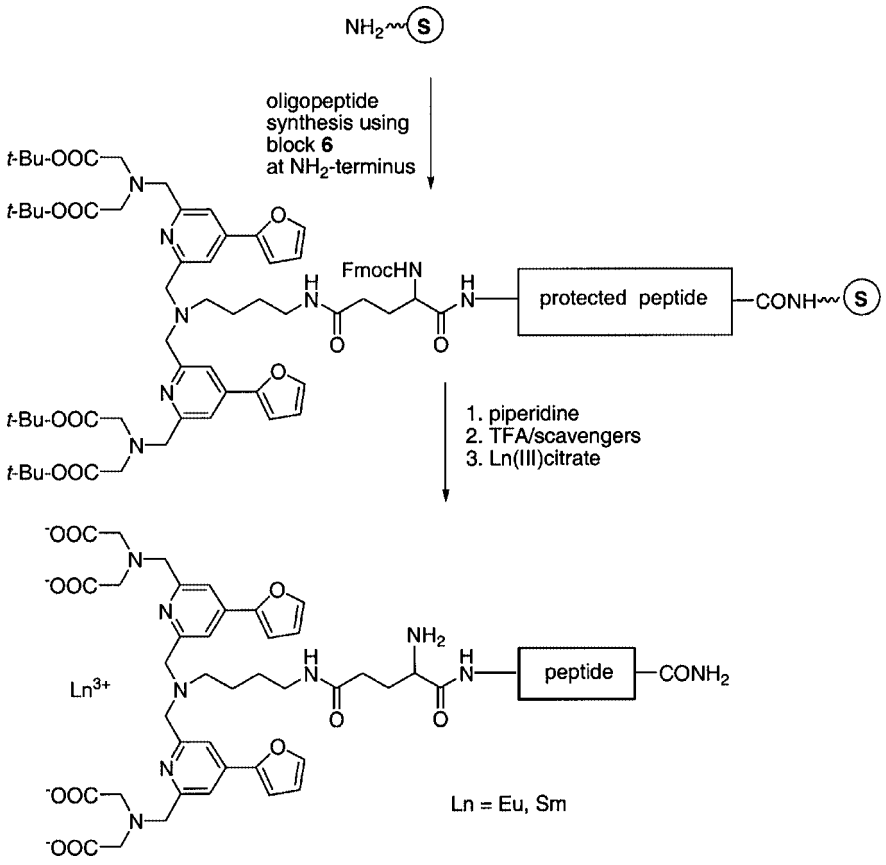
The oligopeptides synthesized were purified on reversed phase HPLC and characterized on ESI–TOF mass spectrometry. A typical HPLC profile of the purified oligopeptide conjugate is shown in Figure 1. In all the cases, the observed molecular weights were in accordance with the proposed structures.

Labeling of Proteins in Solution

The metal chelating ability of terpyridines is well established. In fact, lanthanide(III) chelates based on terpyridine structure are among the best luminescent europium(III) chelates synthesized [8,15,16] (representative structures are shown in Chart 1), some of which are even commercially available. This type of labels has certain drawbacks: Firstly, the amino substitution of the phenyl ring at the aromatic part



Scheme 1 Synthesis of a labeling reactant designed for solid phase introduction of luminescent lanthanide(III) chelates to oligopeptides.



Scheme 2 Introduction of luminescent lanthanide(III)chelates to oligopeptides in the aid of block **6**.

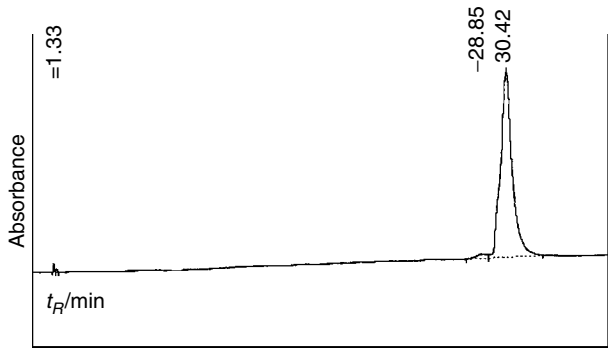
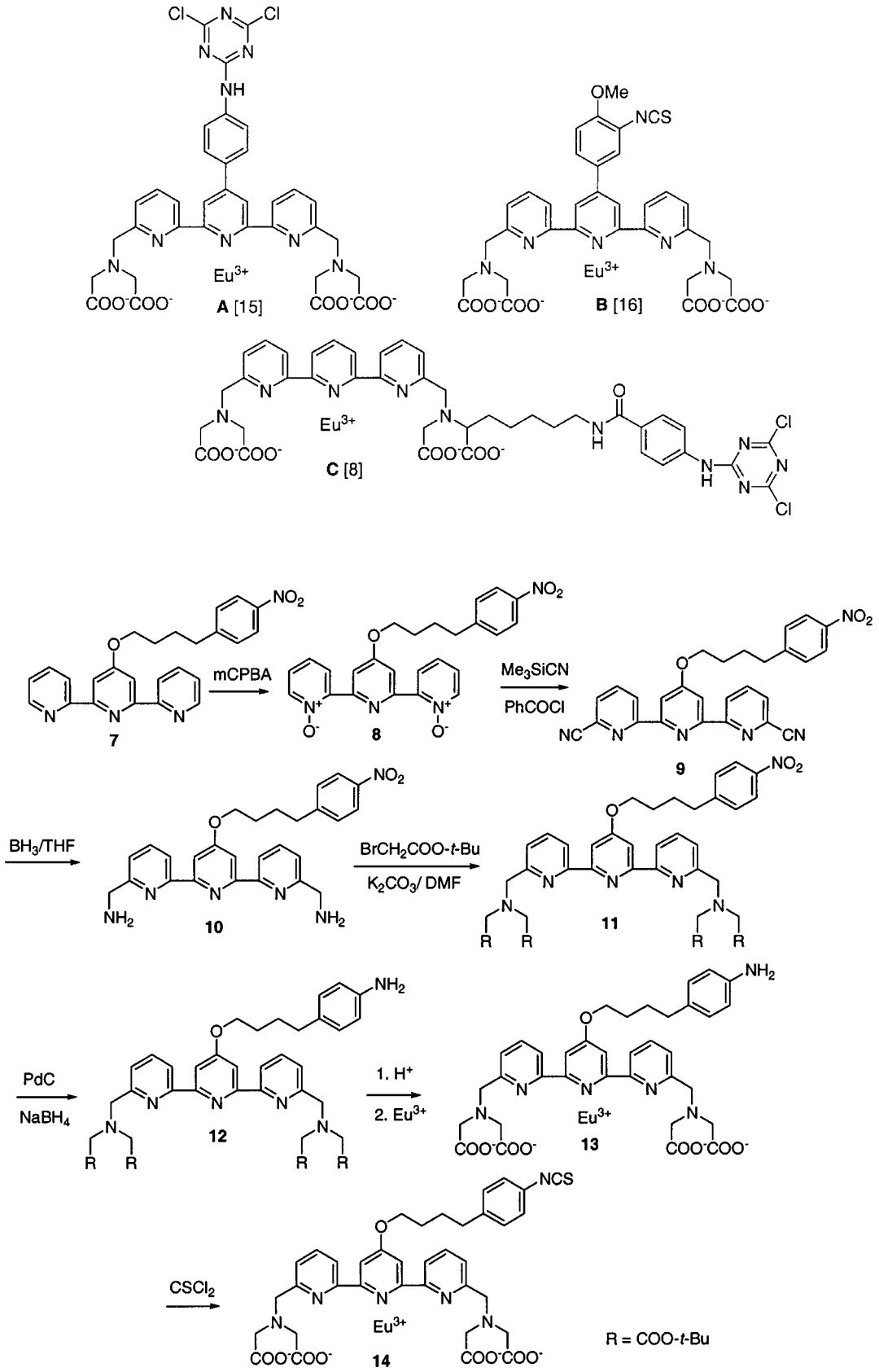


Figure 1 Reversed phase HPLC trace of an oligopeptide (X-RPKPEEFFGLM-$CONH_2$) labeled with a luminescent europium(III) chelate synthesized in the aid of block **6** detected at 330 nm. X is the position of the label. ESI–TOF–MS for the peak at t_R 30.42 min: found, 1148.92 $(M-H)^-$, 2298.8 $(M-2H)^{2-}$. Calcd. for $C_{102}H_{139}EuN_{25}O_{25}S$ 1148.97 $(M-2H)^{2-}$; 2298.9 $(M-H)^-$ For chromatographic conditions, see *Materials and Methods*.

quenches the luminescence of Eu(III), although this effect is partially recovered upon further modification of the NH_2 group. The effect can be diminished by modifying the chelating part as in **C**, but synthesis of this kind of labeling reagents is more complicated [8]. Secondly, although the 4,6-dichloro-1,3,5-triazin-2-ylamino-activated chelates, **A,C** [8,15] are more suitable for protein labeling than the corresponding isothiocyanato derivatives (problems of the NCS-derivatives are associated to the removal of the unreacted chelate from the labeled protein), they imply the risk of overlabeling. Also, their water solubility is rather limited. Furthermore, the methoxy function of **B** [16] is rather labile, and is partially released during synthesis. In order to avoid these problems, we developed a simple route for the preparation of an alternative, more hydrophilic terpyridine chelate (Scheme 3). Accordingly, the terminal pyridine moieties of 4′-(4-nitrophenylbutoxy)-2,2′:6′,2″-terpyridine, **7** [17], were oxidized with 2-chloroperbenzoic acid to *N*,*N*″-dioxides to give rise to **8**. The modified Reissert–Henze reaction yielded the 6,6″-dicarbonitrile **9**. The reaction was completed in 1 h at room temperature. Borane reduction of **9** yielded the diamine, **10**, carboxymethylation of which with *tert*-butyl bromoacetate, gave the tetraester, **11**. Reduction of the nitro group to the amino function was performed with a mixture of Pd/C and sodium borohydride in dry methanol giving rise to **12**. The protecting groups were removed by acid catalyzed hydrolysis, and ligand formed was converted to the corresponding europium(III) chelates **13** by treatment with aqueous europium(III) chloride.

Lanthanide(III) chelates can be coupled to bioanalytical reagents after activation of their amino function



Scheme 3 Synthesis of a labeling reactant designed for solution phase introduction of luminescent lanthanide(III) chelates to proteins.

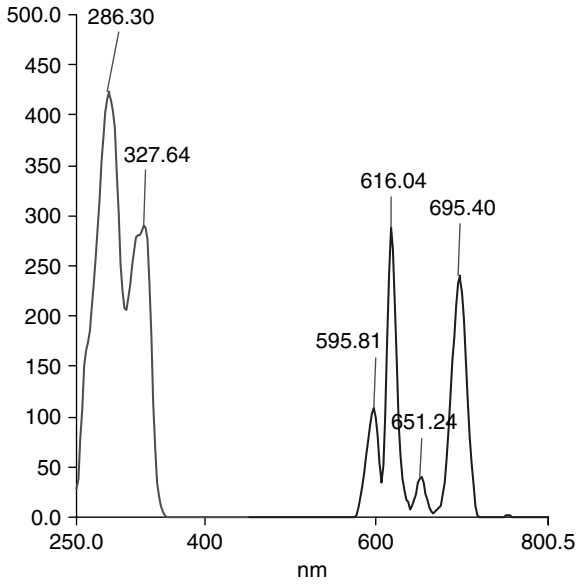


Figure 2 Emission and excitation spectra of the IgG fraction of a rabbit anti-mouse IgG labeled with the europium(III) chelate 14.

with, e.g. thiophosgene, 2,4,6-trichloro-1,3,5-triazine, or iodoacetic anhydride [18]. As an example, **13** was converted to the corresponding isothiocyanato derivative, **14**, by treatment with thiophosgene followed by precipitation from acetone.

To demonstrate the applicability of the chelate synthesized for biomolecule derivatization, **14** was used in the labeling of an antibody (the IgG fraction of a rabbit anti-mouse IgG; carbonate buffer, pH 9.3). The reaction proceeded smoothly, and the protein conjugate was easily and completely purified on standard gel chromatography.

The excitation and emission spectra of the protein conjugate is shown in Figure 2. The observed emission spectrum was typical for europium chelates corresponding to the $^5D_0 \rightarrow 7F_j$ transition. The main emission line of the chelate was centered as usual at around 617 nm. The luminescence yield of the protein conjugate was high (ε_ϕ 1684) and the decay time was long (τ 1.46 ms) and comparable to those recently observed for the corresponding oligonucleotide derivative [13].

We are currently studying the use of the biomolecule conjugates synthesized in multiparametric TruPoint assays. These results will be published in due course.

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