Labeling of Oligopeptides with Luminescent Lanthanide(III) Chelates on Solid Phase

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Abstract: Synthesis of a building block based on 6,6'-(1*H*-pyrazole-1,3-diyl)bis(pyridine) (**3**) that allows introduction of photoluminescent lanthanide(III) chelates to the synthetic oligopeptides on solid phase using the standard Fmoc chemistry is described. Since the ligand based on (**3**) forms luminescent chelates with Eu^{3+}, Sm^{3+}, Tb^{3+} and Dy^{3+} ions, the ligand is highly useful for multiparametric homogenous assays simplifying considerably the preparation of chelates needed in these applications.

Keywords: Lanthanide(III) chelates, oligopeptides, solid phase synthesis, labeling.

INTRODUCTION

The use of long life-time emitting lanthanide(III) chelate labels or probes together with time-resolved fluorometry in detection provides a method to generate sensitive bioaffinity assays. Indeed, time-resolved fluorescence based on lanthanide(III) chelates has become a successful detection technology, and it has been used in *in vitro* diagnostics for over two decades [1]. For example, time-resolved fluorescence quenching assays based on energy transfer from a lanthanide(III) chelate to a nonfluorescent quencher have been applied in various assays of hydrolyzing enzymes [2-5] as well as for nucleic acid detection [4,6,7]. The different photochemical properties of europium, terbium, dysprosium and samarium chelates have proved to be suitable for the development of multiparametric homogenous assays [5].

Biomolecule conjugates used in many applications, such as homogenous quenching assays, have to be extremely pure, because even small amounts of fluorescent impurities can increase the luminescence background and thereby reduce detection sensitivity. Thus, it is desirable to perform solid phase conjugation of biomolecules because most impurities can be removed by washing while the biomolecule is anchored to the solid support. Once the biomolecule conjugate is released into the solution, only a single chromatographic purification is required.

We have already reported a solid phase method for the labeling of oligopeptides [8,9] and oligonucleotides [10-12] with photoluminescent lanthanide(III) chelates. The approach involves synthesis of oligonucleotide and oligopeptide building blocks, which can be introduced to the biomolecule structure using commercial oligonucleotide and oligopeptide synthesizers by 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. For solid phase oligopeptide derivatization, only europium(III) chelates have been employed. We report here synthesis of an oligopeptide labeling reactant based on 6,6'-(1*H*-pyrazole-1,3-diyl)bis(pyridine) which extends our labeling strategy to the preparation of synthetic oligopeptides labeled with photoluminescent terbium, dysprosium and samarium chelates.

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RESULTS AND DISCUSSION

Stable luminescent lanthanide(III) chelates generally include a ligand with a reactive group for covalent conjugation to bioactive molecules, an aromatic structure, which absorbs the excitation energy and transfers it to the lanthanide ion and additional chelating groups such as carboxylic or phosphonic acid moieties and amines. Unlike organic chromophores, these molecules do not produce unwanted Raman and Rayleigh scattering or suffer from concentration quenching. Although organic chelators and their substituents have a significant effect on the photophysical properties of lanthanide(III) chelates, no general rules for the estimation of these effects are available.

Lanthanide(III) chelates based on 2,6-bis(*N*-pyrazolyl)pyridine [13-18] (**A**; Chart (**1**)) and 6,6'-(1*H*-pyrazole-1,3-diyl)bis(pyridine) [12,18,19] (**B**) are among the best luminescent terbium(III) chelates synthesized: they have excellent photophysical properties including high luminescent quantum yield and relatively high energy of their lowest triplet state. Unfortunately, biomolecule labeling reactants based on 2,6-bis(*N*-pyrazolyl)pyridine have insufficient kinetic stabilities in the presence of high concentrations of EDTA thus making them unsuitable for bioanalytical assays [5]. In contrast, chelates based on 6,6'-(1*H*-pyrazole-1,3-diyl)bis(pyridine) have been shown to be highly suitable to these applications [5-7].

Synthesis of the novel oligopeptide labeling reactant is depicted in Scheme **1**. Reaction of Fmoc-glutamic acid allyl ester with the amine (**1**) [17] in the presence of HATU and DIPEA gave the protected ligand (**2**) in moderate yield. Purification was easily performed on silica gel column chromatography. Palladium-catalyzed allyl cleavage using resin-bound sodium *p*-toluenesulfinate produced (**3**) in 2h at RT. The work up procedure was very simple including

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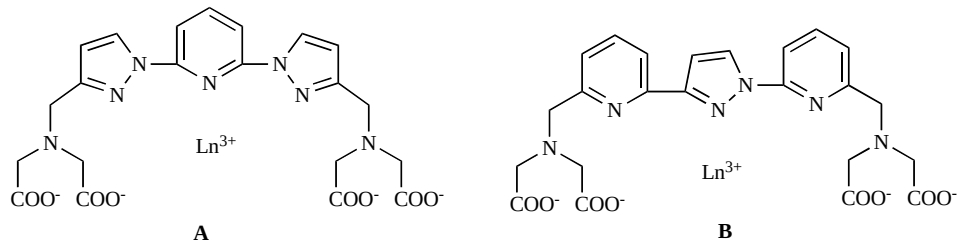
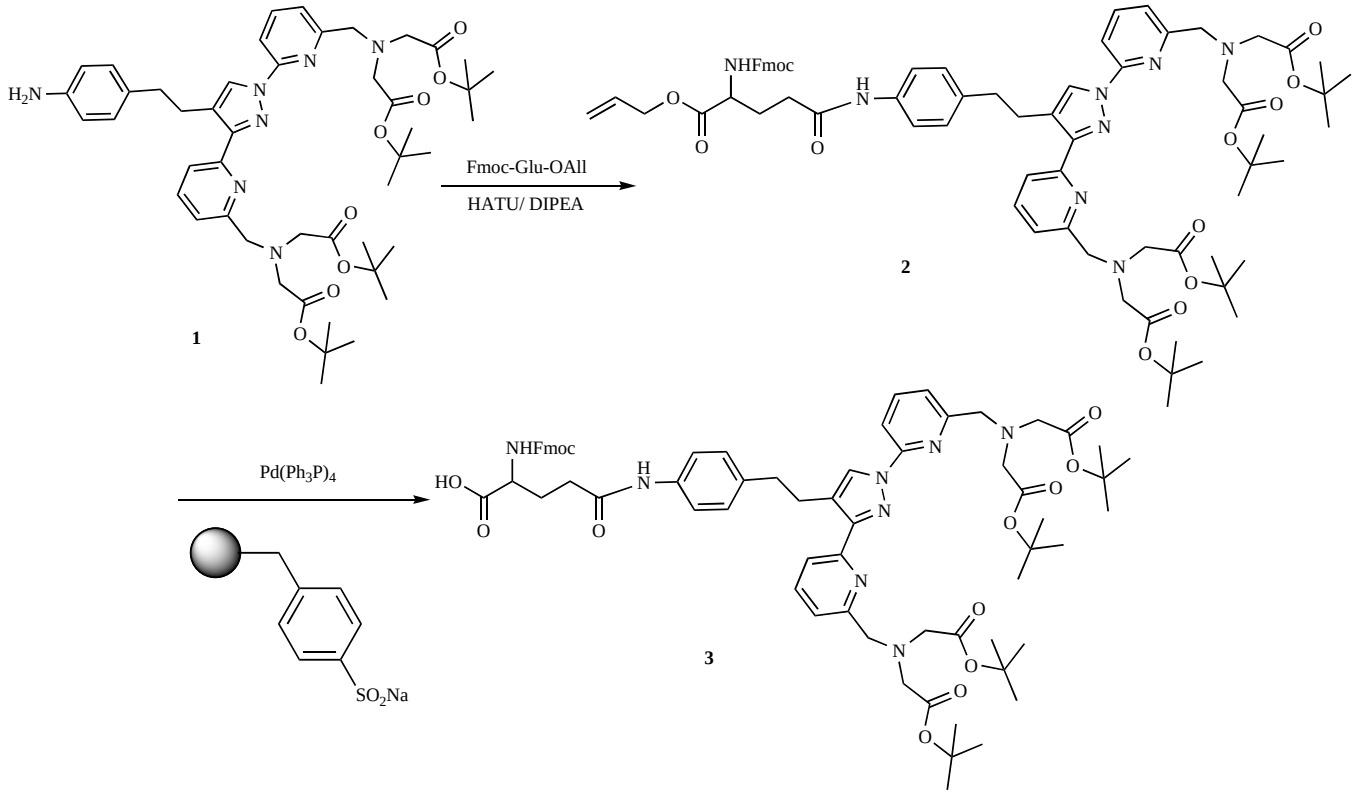


Chart 1.



Scheme 1. Synthesis of the oligopeptide labeling reactant (**3**).

removal of the resin by filtration, washing of the filtrate with 10% citric acid and drying over 4Å molecular sieves.

To demonstrate the applicability of the building block (**3**) for oligopeptide derivatization, a caspase-3 substrate (DEVDK) was synthesized in 10 μmol scale using standard Fmoc chemistry and recommended protocols. The block (**3**) was coupled to the amino terminus using a prolonged coupling time (2 h instead of 30 min). HBTU/HOBt was used as an activator and 3.5 equivalents of (**3**) was used relative to the resin (the amount of standard amino acids was 5 equivalents); higher concentrations of (**3**) did not give any advantages. According to fulvene-piperidine assay, coupling efficiency of (**3**) was comparable to natural amino acids. After completion of the oligopeptide synthesis, the peptide was cleaved from the resin with TFA in the presence of scavengers followed by precipitation with diethyl ether. Treatment of the deprotected oligopeptide with terbium(III) citrate converted the oligopeptide conjugate to the desired terbium(III) chelate (Scheme **2**). The oligopeptide synthesized was purified on reversed phase column and characterized on ESI-TOF mass spectrometry. A HPLC trace of a crude reaction mixture is shown in Fig. (**1**) demonstrating the success of the coupling reaction. The observed molecular weight was in accordance with the proposed structure.

The excitation and emission wavelengths as well as the decay time (τ) of the oligopeptide conjugate (**4**) were measured in tris-buffer containing 0.9% NaCl at pH 7.75. The main emission line was centered as usual at 544 nm. The excitation maximum was at 329 nm. The decay time, 2.34 ms, although slightly shorter than that of the corresponding unconjugated terbium chelate (Table **1**) was normal for a nonadentate chelate.

Brunet *et al.* [17] have demonstrated that lanthanide chelates based on 2,6-bis(*N*-pyrazolyl)pyridine have reasonably good photophysical properties even with Eu^{3+}, Sm^{3+} and Dy^{3+}. We verified that this is the case also with chelates based on 6,6'-(1*H*-pyrazole-1,3-diyl)bis(pyridine) (Table (**1**)). The observed emission spectra were typical for lanthanide chelates corresponding to the $^5D_0 \rightarrow {}^7Fj$, $^4G5/2 \rightarrow$

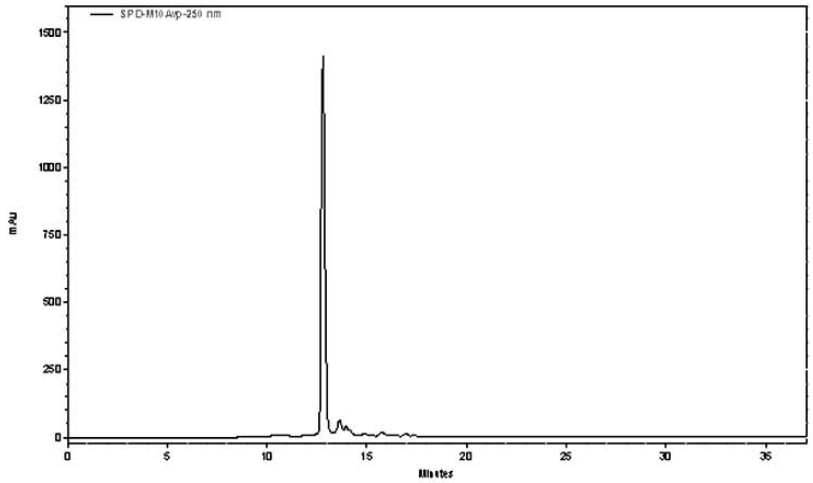
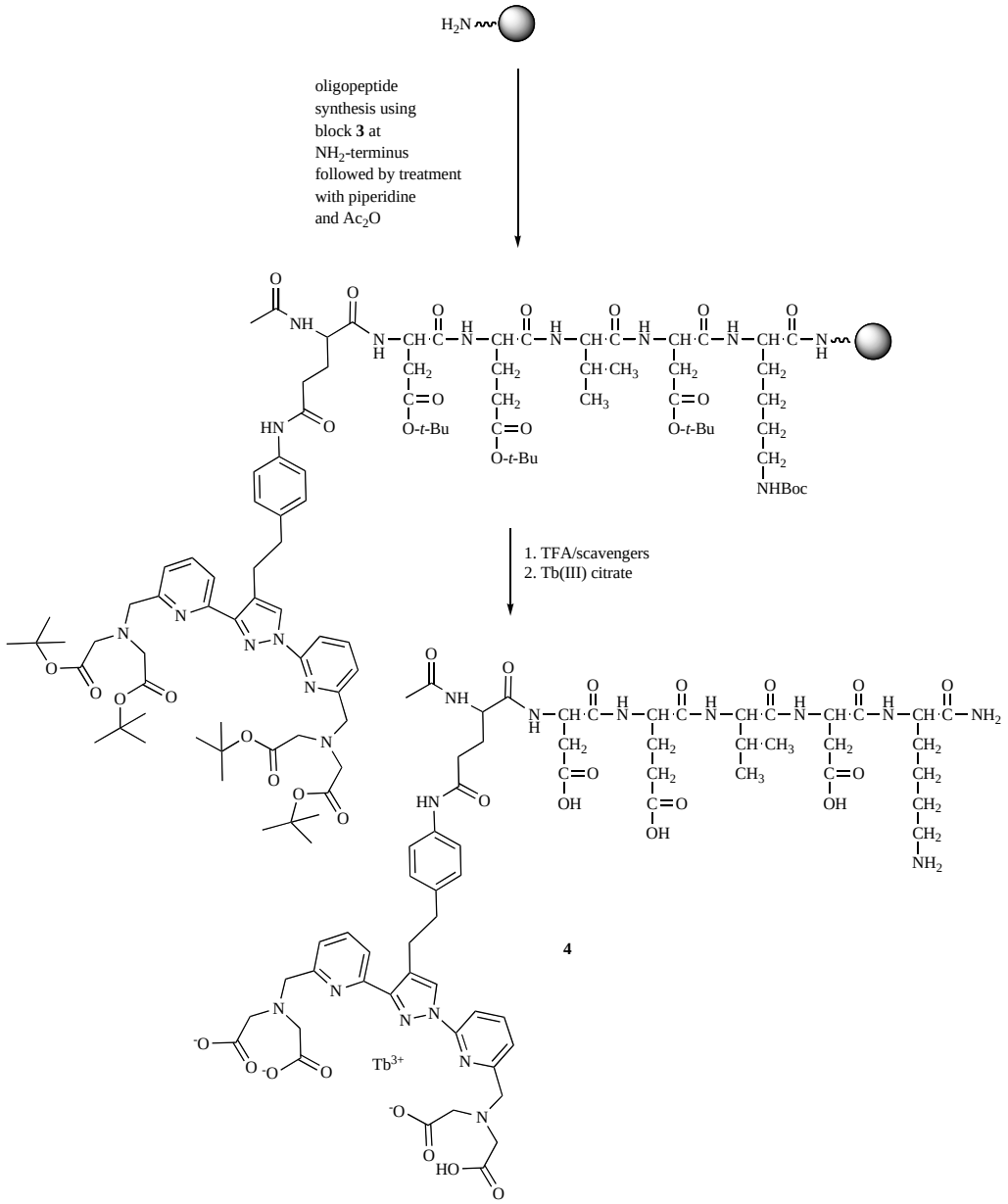


Fig. (1). Reversed phase HPLC trace of the oligopeptide conjugate (**4**) (crude reaction mixture). The main peak is the desired product as judged by ESI-TOF MS.



Scheme 2. Labeling of an oligopeptide in solid phase using the labeling reactant (**3**).

Table 1. Emission Maxima, Luminescence Yields (εΦ) and Decay Times (τ) of Lanthanide(III) Chelates Based on 6,6'-(1*H*-pyrazole-1,3-diyl)bis(pyridine)

Ln	Emission/nm	εΦ	τ / ms
Tb^{3+}	544	9089	2.80
Eu^{3+}	616	1632	1.05
Sm^{3+}	603	45	0.014
Dy^{3+}	575	188	0.012

^6Hj, $^5D_4 \rightarrow {}^7Fj$ and $^4F_{9/2} \rightarrow {}^6Hj$ transitions for Eu^{3+}, Sm^{3+}, Tb^{3+} and Dy^{3+}, respectively. The main emission lines of chelates were centered as usual around 617 (Eu^{3+}), 603 (Sm^{3+}), 544 (Tb^{3+}) and 575 nm (Dy^{3+}). The decay times were normal for nonadentate chelates except that of the dysprosium chelate, which has surprisingly short decay time. This phenomenon was found also for the corresponding oligonucleotide conjugates [12].

MATERIALS AND METHODS

Adsorption column chromatography was performed on columns packed with silica gel 60 (Merck). Reagents for oligopeptide synthesis and Fmoc-Glu-Oall were purchased from Nova Biochem. Sodium sulfinate resin (200-400 mesh, 1% DVB, 1.3 mmol g^{-1}) was purchased from Tianjin Nankai Hecheng Science & Technology Company Limited (China). All dry solvents were from Merck and they were used as received. Lanthanide chelates of 2,2',2'',2'''-{[6,6'-(pyrazole-1,3''-diyl)bis(pyridine)-2,2'-diyl]bis(methyleneni-trilo)}-tetrakis(acetic acid) were synthesized as described previously [18]. The oligopeptides were assembled on an Applied Biosystems 433A instrument, using recommended protocols. HPLC purifications were performed using a Shimadzu LC 10 AT instrument equipped with a diode array detector, a fraction collector 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 31 min from 95% A to 100% B. Flow rate was 0.6 mL min^{-1}. ESI-TOF mass spectra were recorded on an Applied Biosystems Mariner instrument. Luminescence measurements were performed on a PerkinElmer LS-55 luminescence spectrometer equipped with a Hamamatsu R928 red-sensitive photomultiplier tube.

5-{*N*-{4'-{2''-{1''',3'''-bis{6''''-[*N*,*N*-bis(*tert*-butoxycar-bonylmethyl)aminomethyl]-2''''-pyridyl}-1*H*-pyrazo-4'''-yl}ethyl}phenyl}amino}-2-[*N*-(fluorenylmethoxycar-bonyl)amino]-5-oxopentanoic acid allyl ester, 2

Fmoc-Glu-OAll (0.15 g, 0.37 mmol), HATU (0.14 g, 0.37 mmol), and DIPEA (65 μL, 0.37 mmol) were dissolved in dry DMF (3 mL), and the mixture was stirred for 30 min at room temperature. Tetra(*tert*-butyl) 2,2',2'',2'''-{{6,6'-{4''-[2-(4-aminophenyl)ethyl]-1*H*-pyrazole-1'',3''-diyl}bis (pyridine)-2,2'-diyl}bis(methylene-nitrilo)}tetrakis(acetate) (**1**) (0.32 g, 0.37 mmol) predissolved in dry DMF (2 mL) was added and the mixture was stirred for 1.5 h. The mixture was diluted with CH_2Cl_2 (20 mL), washed twice with 10% citric acid and dried over Na_2SO_4. Purification on silica gel (eluent 5% MeOH/CH_2Cl_2) gave 0.24 g (52%) of the product. ESI-TOF-MS for $C_{70}H_{86}N_8O_{13}$ $(M+H)^+$: calcd, 1247.64; found, 1247.64. ^{1}H-NMR (DMSO-d_6): 8.45 (s, 1H), 8.00-7.95 (m, 2H), 7.90-7.85 (m, 5H), 7.72 (d, 2H, *J*=7.6 Hz), 7.55 (d, 1H, *J*=7.7 Hz), 7.49 (d, 2H, *J*=8.8 Hz), 7.47 (d, 1H, *J*=8.8 Hz), 7.41 (t, 2H, *J*=7.7 Hz), 7.33 (t, 2H, *J*=7.4 Hz), 7.16 (d, 2H, *J*=8.2 Hz), 5.93-5.87 (m, 1H), 5.31 (d, 1H, *J*=17.3 Hz), 5.20 (d, 1H, *J*=10.3 Hz), 4.60 (d, 2H, J=5.0 Hz), 4.34-4.31 (m, 1H), 4.28-4.25 (m, 1H), 4.23-4.21 (m, 1H), 4.15-4.11 (m, 1H), 4.03 (s, 2H), 3.98 (s, 2H), 3.48 (s, 4H), 3.47 (s, 4H), 3.29 (t, 1H, *J*=5.6 Hz), 3.19 (t, 2H, *J*=8.0 Hz), 2.87 (t, 2H, *J*=7.9 Hz), 2.42 (t, 2H, *J*=7.3 Hz), 2.13-2.08 (m, 1H), 1.92-1.85 (m, 1H), 1.40 (s, 18H), 1.36 (s, 18H).

5-{*N*-{4'-{2''-{1''',3'''-bis{6''''-[*N*,*N*-bis(*tert*-butoxycar-bonylmethyl)aminomethyl]-2''''-pyridyl}-1*H*-pyrazo-4'''-yl}ethyl}phenyl}amino}-2-[*N*-(fluorenylmethoxycar-bonyl)amino]-5-oxopentanoic acid, 3

Compound (**2**) (0.22 g, 0.18 mmol) was dissolved in dry THF (2 mL), and the solution was deaerated with argon. $Pd(Ph_3P)_4$ (13 mg, 11 μmol) and sodium sulfinate resin (0.2 g) were added and the mixture was stirred overnight at RT. The resin was filtered off, washed with THF, and the filtrate was concentrated *in vacuo*. The residue was dissolved in CH_2Cl_2, washed twice with 10% citric acid, dried with 4Å molecular sieves and concentrated. Yield was 86 %. ESI-TOF-MS for $C_{67}H_{82}N_8O_{13}$ $(M+H)^+$: calcd. 1207.61; found, 1207.59.

Synthesis and Purification of Oligopeptide Conjugates

A model sequence (DEVDK) was synthesized on PAL™ Support for peptide amides (PE Biosystems) in 10 μmol scale using Fmoc chemistry and recommended protocols (coupling time 30 min for natural amino acid analogues, and 2 h for (**3**)) followed by treatment with piperidine and acetic anhydride. The resin was treated with the mixture of crystalline phenol (75 mg), ethanedithiol (25 μL), thioanisole (50 μL), water (50 μL) and trifluoroacetic acid (1 mL) for 4 h. The resin was removed by filtration, and the solution was concentrated *in vacuo*. The crude oligopeptide was precipitated with diethyl ether. The precipitate was redissolved in water and treated with terbium(III) citrate (5 equiv per ligand). Purification was performed on HPLC.

Yield was 68%. MS found: 1543.47, calcd for $C_{62}H_{77}N_{15}O_{22}Tb^{-}$ 1543.45.

CONCLUSION

We presented here a straightforward method to label oligopeptides with a luminescent terbium(III) chelate on solid phase using a standard oligopeptide synthesizer. Since 6,6'-(1*H*-pyrazole-1,3-diyl)bis(pyridine) based ligand forms luminescent chelates with four lanthanide(III) ions, the ligand simplifies considerably the preparation of chelates needed in bioanalytical applications. Although the decay times are shorter and luminescence yields much lower in the case of samarium and dysprosium chelates than in the case of europium and terbium chelates, these chelates are valuable when multiparametric assays are developed.

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