

## Thiol-Reactive Luminescent Lanthanide Chelates: Part 2

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Luminescent lanthanide complexes have unusual spectroscopic characteristics, including millisecond excited-state lifetime and sharply spiked emission spectra. These characteristics make them valuable alternatives to conventional organic fluorescent probes in detection applications and for measuring nanometer-scale conformational changes in biomolecules via resonance energy transfer. Our group has previously reported the syntheses and application of various luminescence complexes that have polyaminocarboxylate chelates coupled to a carbostyryl antenna and thiol or amine-reactive groups. Here we report the syntheses of new thiol-reactive forms of DTPA-cs124 chelates, including two iodoacetamide forms (phenylalanine-iodoacetamide and ethylenediamine iodoacetamide) and two methanethio-sulfonate forms (ethylmethanethiosulfonate and carboxyethylmethanethiosulfonate). In addition, we have developed an improved synthesis of a previously reported maleimide form.

### INTRODUCTION

Luminescent lanthanide chelate complexes are of great interest in time-resolved fluorescence assays (1–3). These complexes typically involve a chelate to bind and protect the lanthanide from solvent-quenching effects, and a covalently attached organic chromophore to act as an antenna to absorb excitation light. Their spectral characteristics include millisecond lifetime, sharply spiked emission spectra, large Stokes shifts, high quantum yield, and good solubility. These properties make them useful alternatives to radioactive probes and to organic fluorophores, particularly where there are problems of background autofluorescence (4, 5), and as donors in luminescence resonance energy transfer experiments (3, 6, 7). One group of such chelate complexes, developed in our laboratory, is the chelate diethylenetriaminepentaacetic acid covalently coupled to a chromophore, 7-amino-4-methyl-2-(1*H*)-quinolinone (carbostyryl 124, cs124). While still maintaining their high binding constants, a reactive group for attachment to biomolecules can be added to the chelate: amine reactive forms via either an anhydride or isothiocyanate (8), or thiol reactive forms via maleimide, bromoacetamide, pyridyldithio (9), or iodoacetamide (10) groups. Other thiol-reactive luminescent lanthanide chelates have been developed (11). The reactive group can be also placed on the chromophore. For example, pyridyldithio forms of DTPA and triethylenetetraaminehexanoic acid chelates, which are luminescent with europium, have been made previously by placing the pyridyldithio group on a coumarin chromophore (12).

Our group has previously reported the syntheses of thiol-reactive maleimide, bromoacetamide, and pyridyldithio forms of DTPA-cs124 chelates (9). The maleimide form of chelate has been used in energy transfer experiments on the muscle protein myosin (13–16) and on ion channels (17). The Ebright group has made a DTPA-

cs124 iodoacetamide utilizing a diaminobenzene linker, which they have used in protein–DNA studies (10). The syntheses are two- or three-step reactions. One anhydride of DTPA dianhydride is reacted with the 7-amino group of cs124; the other anhydride is reacted with a heterobifunctional compound containing an amino group on one end and a thiol-reactive group on the other end. Here we present the syntheses of additional thiol-reactive DTPA-cs124 chelates, including three iodoacetamides, two methanethiosulfonates (MTS), and an improved synthesis of a maleimide form.

### EXPERIMENTAL METHODS

**Chemicals and Materials.** The following were purchased from Sigma-Aldrich: diethylenetriaminepentaacetic acid dianhydride (caDTPA), 7-amino-4-methyl-2-(1*H*)-quinolinone (carbostyryl 124, cs124), iodoacetic anhydride, anhydrous dimethyl sulfoxide (DMSO, in sure seal bottle), piperidine, ethylenediamine (redistilled), and triethylamine (for reaction). DMSO and triethylamine were dried over activated molecular sieves before use. Glacial acetic acid, methanol, acetonitrile and triethylamine (for making TEAA buffer) were purchased from Fisher Scientific.  $\beta$ -Maleimidopropionic acid hydrazide·TFA was purchased from Molecular Biosciences (Boulder, CO). 2-Aminoethyl methanethiosulfonate, hydrobromide, and (*R*)-2-amino-2-carboxyethylmethanethiosulfonate were purchased from Toronto Research Chemicals, Inc. (North York, Ontario, Canada). Fmoc-*p*-aminophenylalanine (Fmoc-*p*-amino-Phe-OH) and *p*-nitro-phenylalanine (H-*p*-nitro-phe-OH) were purchased from Bachem Bioscience Inc. (King of Prussia, PA). Maltose binding protein (MBP) was a gift from Panvera Inc. (Madison, WI). Distilled and deionized (18 M $\Omega$  cm<sup>-1</sup>) water was used throughout. All glassware was washed with a mixed acid solution and thoroughly rinsed with deionized, distilled water. All plastic labware was purchased from Bio-rad (metal-free). All chemicals were of the purest grade available.

**Purification.** Most commonly, reverse-phase high-performance liquid chromatography was performed at room temperature on a Waters model 600 system with a Dynamax 60 Å C<sub>18</sub> column (10 or 25 mm i.d. × 250 mm,

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Rainin, at 8 mL/min, respectively) using a linear gradient (solvent A = 0.1 M triethylammonium acetate, pH 6.5; solvent B = methanol, or acetonitrile). Sep-Pak C<sub>18</sub> cartridges (Waters) were used for purifying the iodoacetamide chelates. Reaction mixtures were loaded on Sep-Pak cartridges, washed with H<sub>2</sub>O, and then 1:1 MeOH/H<sub>2</sub>O. The process was monitored by shining UV light ( $\lambda = 365$  nm) on the cartridge. The fluorescent fractions were dried under vacuum at room temperature, and the powder was stored at  $-80$  °C.

**Spectroscopy.** NMR spectra were recorded on a Varian Unity 400 spectrometer. Time-resolved and gated luminescence measurements were made on a laboratory-built spectrometer described previously, employing a 5-ns excitation pulse at 337 nm followed by time-resolved detection of lanthanide emission (18). The number of waters in the primary coordination sphere of the lanthanide was determined via the method of Horrocks and Sudnick (19): no. of waters =  $q(\tau_{\text{H}_2\text{O}}^{-1} - \tau_{\text{D}_2\text{O}}^{-1})$ , ( $q = 1.05$  for Eu, 4.2 for Tb). The overall quantum yield ( $Q_{\text{overall}}$ ), i.e., the probability of the lanthanide emitting given that the antenna was excited, was measured via an intensity comparison of a reactive chelate to either Tb<sup>3+</sup>- or Eu<sup>3+</sup>-DTPA-cs124, whose  $Q_{\text{overall}}$ 's have been measured to be 0.32 and 0.1, respectively (20). The efficiency of energy transfer from the antenna to the lanthanide ( $Q_{\text{transfer}}$ ) (20) was determined by comparing the number of excited lanthanides in the sample of interest to a reference sample, shortly after the excitation pulse. The reference is Tb<sup>3+</sup>- or Eu<sup>3+</sup>-DTPA-cs124, where  $Q_{\text{transfer}}$  was previously determined to be 0.67 and 0.59, respectively (20). Finally, the quantum efficiency of lanthanide emission,  $Q_{\text{Ln}}$ , i.e., the probability that the lanthanide will emit a photon given that the lanthanide was excited, was measured by comparing the lifetimes of the samples and the reference.  $Q_{\text{Ln}}$  for the reference Tb<sup>3+</sup>- and Eu<sup>3+</sup>-DTPA-cs124 was previously found to be 0.482 and 0.167, respectively (20).

**Syntheses (Figures 1 and 2). DTPA-cs124-EDA-Iodoacetamide.** DTPA-cs124-EDA (8) (5 mg, 8.5  $\mu\text{mol}$ ) was dissolved in 3 mL of 10% NaHCO<sub>3</sub>. The solution was stirred in an ice bath for 15 min. Iodoacetic anhydride (14.5 mg, 42.5  $\mu\text{mol}$ ) was added to the solution. The reaction mixture was stirred at 0 °C for 3 h. Cold 2.0 M H<sub>2</sub>SO<sub>4</sub> was added to the solution slowly to adjust the pH to the range of 4–5. The solution was loaded on a Sep-Pak C18 column, washed with H<sub>2</sub>O, and then 1:1 H<sub>2</sub>O/CH<sub>3</sub>OH. The elution process was monitored by shining UV 356 nm light on the cartridge. The fluorescent fraction was collected and dried under vacuum. The product was confirmed by mass spectroscopy (758, M<sup>-</sup> - 1, ESI).

**DTPA-cs124-Fmoc-*p*-NH<sub>2</sub>-Phenylalanine.** DTPA dianhydride (50 mg, 141  $\mu\text{mol}$ ) was dissolved in the mixture of 0.5 mL of DMSO and 0.4 mL of triethylamine under N<sub>2</sub>. A total of 17 mg of cs124 (97.7  $\mu\text{mol}$ ) in 0.5 mL of DMSO was added to the above solution dropwise via a syringe. The reaction was stirred at room temperature for 1 h. The solution was transferred to a flask containing 75 mg (187  $\mu\text{mol}$ ) of Fmoc-*p*-aminophenylalanine. The reaction mixture was stirred at room temperature for 2 h. A total of 10 mL of 0.1 M TEAA was added to quench the reaction. The reaction mixture was purified by HPLC with a linear 20–40% ACN/0.1 M TEAA (pH 6.5) gradient over 40 min. The product was eluted at 33.5 min (18 mg, ~20% yield; MS: 932, M<sup>-</sup> - 1; ESI).

**DTPA-cs124- $\alpha$ -NH<sub>2</sub>-Phenylalanine.** The aqueous solution of DTPA-cs124-Fmoc-*p*-NH<sub>2</sub>-phenylalanine was concentrated, and added to 5% piperidine aqueous solu-

tion. The solution was stirred at room temperature for 2 h. Acetic acid was added to adjust the pH to ~6.5. The reaction mixture was purified by HPLC with a linear 30–50% MeOH/0.1 M TEAA (pH 6.5) gradient over 40 min. The product was eluted at 21 min (~60% yield, estimated based on its HPLC profile; MS: 710, M<sup>-</sup> - 1; ESI). <sup>1</sup>H NMR in D<sub>2</sub>O: 7.75 (1H, s), 7.06 (1H, d), 6.94 (2H, d), 6.88 (1H, d), 6.68 (2H, d), 6.00 (1H, s), 3.61–3.72 (8H, m), 3.46 (4H, s), 3.26–3.38 (3H, b), 3.22 (2H, s), 3.16 (2H, s), 2.76 (1H, dd), 2.67 (3H, s), 2.60 (1H, dd).

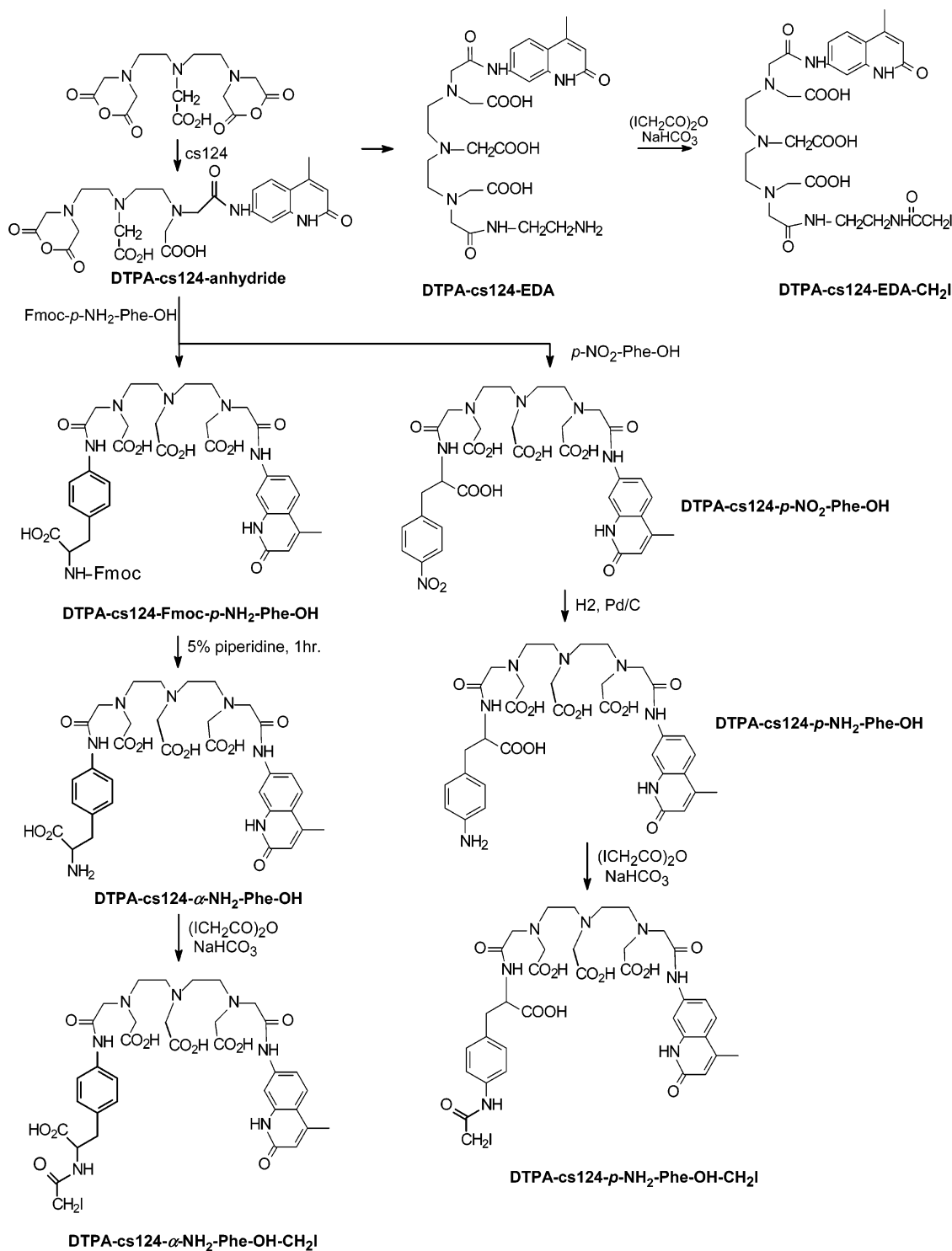
**DTPA-cs124- $\alpha$ -NH<sub>2</sub>-Phenylalanine-Iodoacetamide.** DTPA-cs124- $\alpha$ -NH<sub>2</sub>-phenylalanine (8 mg, 11  $\mu\text{mol}$ ) was dissolved in 3 mL of 10% NaHCO<sub>3</sub>. The solution was stirred in an ice bath for 15 min. Iodoacetic anhydride (8 mg, 23  $\mu\text{mol}$ ) was added to the solution. The reaction mixture was stirred at 0 °C for 3 h. Cold 2.0 M H<sub>2</sub>SO<sub>4</sub> was added to the solution slowly to adjust the pH to 2–3. A white precipitate formed. The white solid was collected and dried under vacuum (8.3 mg, 86%). The product was confirmed by its mass spectrum (878, M<sup>-</sup> - 1, ESI).

**DTPA-cs124-*p*-NO<sub>2</sub>-Phenylalanine.** DTPA dianhydride (25 mg, 70  $\mu\text{mol}$ ) was dissolved in the mixture of 0.5 mL of DMSO and 0.3 mL of triethylamine under N<sub>2</sub>. A total of 10 mg of cs124 (57.5  $\mu\text{mol}$ ) in 0.5 mL of DMSO was added to the above solution dropwise via a syringe. The reaction was stirred at room temperature for 1 h under N<sub>2</sub>. The solution was transferred to a flask containing 17.3 mg (82.4  $\mu\text{mol}$ ) of *p*-nitrophenylalanine. The reaction mixture was stirred at room temperature for 2 h under N<sub>2</sub>. A total of 10 mL of 0.1 M TEAA was added to quench the reaction. The reaction mixture was purified by HPLC with a linear 30–50% MeOH/0.1 M TEAA (pH 6.5) gradient over 40 min. The product was eluted at 24 min. (18 mg, ~40% yield based on HPLC profile; MS: 740, M<sup>-</sup> - 1; ESI).

**DTPA-cs124-*p*-NH<sub>2</sub>-Phenylalanine.** A total of 1 mL of the concentrated HPLC elute (~5 mM) of DTPA-cs124-*p*-NO<sub>2</sub>-phenylalanine was mixed with 5 mg of 10% Pd/C, and degassed under vacuum. H<sub>2</sub> was introduced into the flask through a balloon. The reaction mixture was stirred at r.t. for 15 h. The Pd/C catalyst was filtered. The HPLC profile of the filtrate indicated the hydrogenation is 100% complete. The NMR spectrum has been previously published (8).

**DTPA-cs124-*p*-NH<sub>2</sub>-Phenylalanine-Iodoacetamide.** The HPLC-purified DTPA-cs124-*p*-NH<sub>2</sub>-phenylalanine solution was dried under vacuum. The solid residue was redissolved into 10% NaHCO<sub>3</sub>. Iodoacetic anhydride (5 mg, 14  $\mu\text{mol}$ ) was added to the above solution at 0 °C, and the reaction mixture was stirred at 0 °C for 30 min. Acetic acid was added to adjust the pH to ~5. The solution was then passed through a C18 Sep-Pak cartridge. The fluorescent elutes were collected and concentrated under vacuum. Mass spectroscopy confirmed DTPA-cs124-*p*-NH<sub>2</sub>-phenylalanine-iodoacetamide (878, M<sup>-</sup> - 1, ESI), and the product was used directly in labeling experiments without further purification.

**DTPA-cs124-MTSEA.** DTPA dianhydride (30 mg, 84  $\mu\text{mol}$ ) was dissolved in the mixture of 0.5 mL of DMSO and 0.2 mL of triethylamine under N<sub>2</sub>. A total of 10 mg of cs124 (57  $\mu\text{mol}$ ) in 0.3 mL of DMSO was added to the solution slowly via a syringe. The reaction mixture was stirred at room temperature for 1 h. 2-Aminoethyl methanethiosulfonate·HBr (52.4 mg, 111  $\mu\text{mol}$ ) in 0.5 mL of DMSO was added to the solution. The reaction mixture was stirred at room temperature for 2 h. TEAA buffer (4 mL, 0.1 M, pH 6.5) was then added to quench the reaction. The reaction mixture was purified via reverse

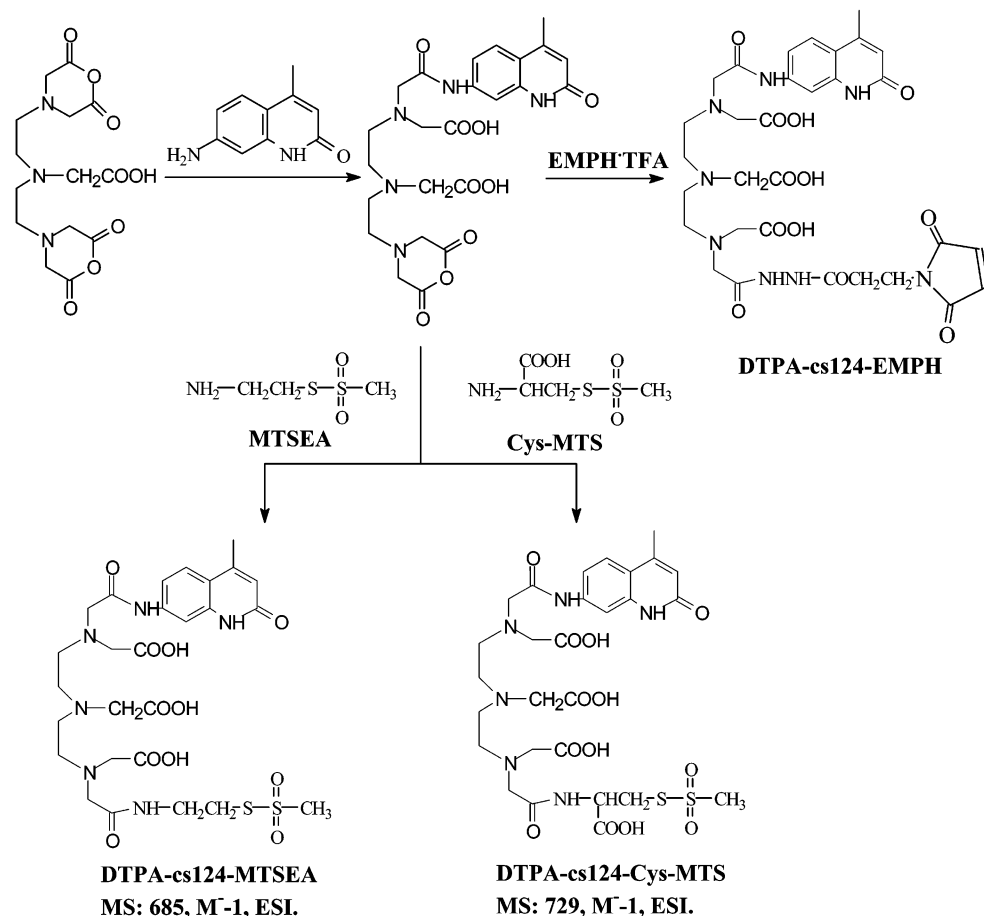


**Figure 1.** Synthetic scheme for iodoacetamide forms of DTPA-cs124.

phase HPLC with a 20–40% MeOH/TEAA (0.1 M, pH 6.5) linear gradient over 40 min. The product, DTPA-cs124-MTSEA, along with DTPA-bis-cs124, eluted at 18.5 min ( $M^-1$ : 685, DTPA-cs124-MTSEA, ESI;  $M^-1$ : 704, DTPA-bis-cs124, ESI).

**DTPA-cs124-Cys-MTS.** DTPA dianhydride (36 mg, 101  $\mu\text{mol}$ ) was dissolved in a mixture of 0.5 mL of DMSO and 0.2 mL of triethylamine under  $\text{N}_2$ . (*R*)-2-amino-2-carboxyethylmethanethiosulfonate (8.9 mg, 45  $\mu\text{mol}$ ) in 0.3 mL of DMSO was added slowly to the solution via a syringe. The reaction mixture was stirred at room temperature for 1 h. Carbostyryl 124 (27.3 mg, 157  $\mu\text{mol}$ ) in

0.5 mL of DMSO was added to the solution and the reaction mixture stirred at room temperature for 2 h. TEAA buffer (4 mL, 0.1 M, pH 6.5) was added to quench the reaction. The reaction mixture was purified via reverse phase HPLC with a 25–40% MeOH/TEAA (0.1 M, pH 6.5) linear gradient over 40 min. The product, DTPA-cs124-Cys-MTS, was eluted at 23 min ( $M^-1$ : 729, ESI).  $^1\text{H}$  NMR in  $\text{D}_2\text{O}$ : 7.74 (1H, s), 7.23 (1H, d), 6.96 (1H, d), 6.06 (1H, s), 4.37 (1H, dd), 3.72 (2H, s), 3.67 (2H, s), 3.63 (2H, s), 3.52 (2H, s), 3.50 (2H, s), 3.42 (1H, dd), 3.33 (1H, dd), 3.15–3.3 (b) and 3.26 (s) (11H total), 2.67 (3H, s).



**Figure 2.** Synthetic scheme for maleimide and methanethiosulfonate forms of DTPA-cs124.

**DTPA-cs124-EMPH.** DTPA dianhydride (25 mg, 70.5  $\mu\text{mol}$ ) was dissolved in a mixture of 0.5 mL of DMSO and 0.2 mL of triethylamine under  $\text{N}_2$ . A total of 10 mg of cs124 (57.5  $\mu\text{mol}$ ) in 0.5 mL of DMSO was added to the above solution slowly via a syringe. The reaction was stirred at room temperature for 1 h. 3-Maleimidopropionic acid hydrazide·TFA (EMPH, 17 mg, 57.2  $\mu\text{mol}$ ) in 0.5 mL of DMSO was then added to the solution, and the mixture was stirred at room temperature for 2 h. A total of 5 mL of 0.1 M TEAA pH 6.5 was added to quench the reaction. The reaction mixture was purified via reverse phase HPLC with a 25–40% MeOH/0.1 M TEAA pH 6.5 linear gradient over 40 min. DTPA-cs124-EMPH eluted at 22 min (~30% yield, estimated by HPLC profile.  $\text{M}^-$ -1: 713, ESI). The byproduct,  $\text{DTPA-(cs124)}_2$ , eluted at 23.5 min.

**Addition of Metals.**  $\text{TbCl}_3$  or  $\text{EuCl}_3$  was added to the chelate in a 0.9:1 molar ratio at usually >0.5 mM concentration at pH 6–7, usually in 0.1 M TEAA, pH 6.5 buffer, and allowed to stand for 30 min at room temperature before use.

**Coupling to Biomolecules.** The labeling conditions for conjugation to muscle protein (HMM, skeletal Heavy-meromyosin, or S1 fragment of smooth muscle myosin containing a single cysteine at position 208) have been described previously (9, 13, 16). The procedure for labeling Shaker ion channels in *Xenopus* oocytes has also been published (17). The iodoacetamide and methanethiosulfonate chelates were coupled to HMM, or to maltose binding protein (MBP, a 65-kDa protein containing a single cysteine) at a typical ratio of 10:1 with the protein concentration ~15  $\mu\text{M}$ . The coupling reaction was carried out on ice overnight in 20 mM Mops, pH 7.0, 5 mM

$\text{MgCl}_2$ . The excess lanthanide chelates were removed by passing the reaction mixture through a Sephadex G-50 size exclusion column.

## RESULTS AND DISCUSSION

**Syntheses of Compounds.** Figures 1 and 2 show the synthetic pathways for the various thiol-reactive chelates. Their correct structures were judged by mass spectroscopy, UV–Vis absorption, lanthanide luminescence properties, the reactivity with thiol groups, and NMR for DTPA-cs124-Cys-MTS and DTPA-cs124- $\alpha$ - $\text{NH}_2$ -phenylalanine-iodoacetamide. Depending on the commercial availability of starting materials, the syntheses were either two-step (one-pot) reactions as for DTPA-cs124-MTSEA, DTPA-cs124-Cys-MTS, and DTPA-cs124-EMPH, or multiple-step reactions as for DTPA-cs124-EDA- $\text{CH}_2\text{I}$  and the two forms of DTPA-cs124-Phe- $\text{CH}_2\text{I}$ . The syntheses of DTPA-cs124-MTSEA and DTPA-cs124-Cys-MTS are straightforward. DTPA dianhydride was reacted with either cs124 or the corresponding amino-methanethiosulfonate reagents first, followed by the addition of the other reagents to complete the reaction. By using a combination of methanol and TEAA buffer, DTPA-cs124-Cys-MTS is well-separated from byproducts by HPLC, while DTPA-cs124-MTSEA is coincidental with the  $\text{DTPA-(cs124)}_2$  byproduct.

We previously reported on the synthesis of DTPA-cs124-EMPH via a two- and three-step (one-pot) reaction, but found low yields for the two-step reaction (9). We now report conditions for the two-step (one-pot) synthesis of DTPA-cs124-EMPH that gives a satisfactory (30%) yield. In addition, by using a linear gradient of 25–40%

**Table 1. Life Times of Thiol-Reactive Tb<sup>3+</sup> Chelates Labeled to Proteins**

lanthanide	chelates	proteins	lifetime (ms)		
Tb <sup>3+</sup>	DTPA-cs124-Phe- $\alpha$ -NH <sub>2</sub> -COCH <sub>2</sub> I	HMM <sup>a</sup>	34% 0.81		
		MBP	66% 1.57		
			57% 1.30		
			12% 1.65		
			31% 0.22		
	DTPA-cs124-EDA-COCH <sub>2</sub> I	HMM	43% 0.82		
		MBP	57% 1.68		
			36% 1.65		
			37% 0.78		
	DTPA-cs124-MTSEA	myosin S1	16% 0.31		
			HC208 <sup>b</sup>	84% 1.41	
		MBP	56% 1.26		
			44% 0.58		
		Shaker K <sup>+</sup> channels in oocyte cells <sup>c</sup>	66% 1.20		
			34% 0.55		
			DTPA-cs124-Cys-MTS	myosin S1	90% 1.42
				HC208	10% 0.55
		MBP		79% 1.29	
		Shaker K <sup>+</sup> channels in oocyte cells <sup>c</sup>	21% 0.60		
	90% 1.16				
10% 0.47					
none	89% 1.53				
DTPA-cs124-Phe- <i>p</i> -NH <sub>2</sub> -COCH <sub>2</sub> I	MBP	11% 0.44			
		74% 1.46			
	DTPA-cs124-EMPH	26% 0.37			
		89% 1.92			
			11% 0.79		

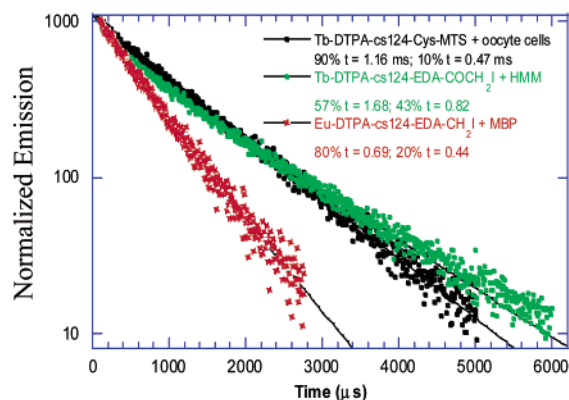
<sup>a</sup> Heavy meromyosin fragment (350 kD) of skeletal myosin.

<sup>b</sup> Subfragment 1 (100 kD) of smooth muscle myosin with a single cysteine at position 208 of the heavy chain. <sup>c</sup> Details of ion channel expression can be found at ref 17.

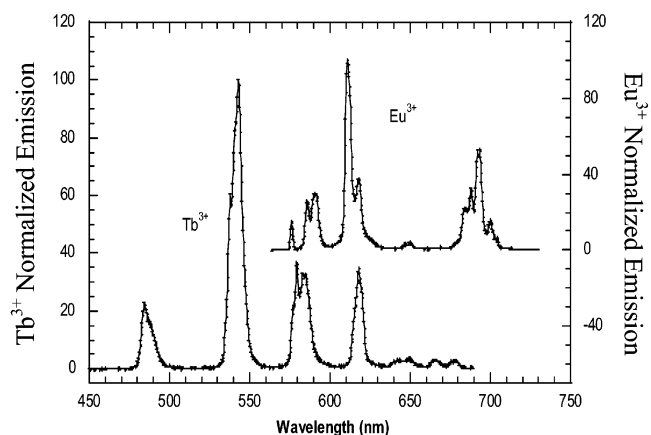
methanol/TEAA buffer, the DTPA-cs124-EMPH product is well separated from the DTPA-(cs124)<sub>2</sub> byproduct.

To synthesize an iodoacetamide chelate, one NH<sub>2</sub> group in a diamine compound was reacted with DTPA-cs124 mono anhydride to form DTPA-cs124-diamine compound in an in situ reaction. The remaining NH<sub>2</sub> group in the DTPA-cs124-diamine compound was then reacted with iodoacetic anhydride in a sodium bicarbonate solution to form the corresponding iodoacetamide chelate. All reaction mixtures were loaded onto Sep-Pak columns, and washed with H<sub>2</sub>O and 1:1 MeOH/TEAA buffer. The elution with 1:1 MeOH/TEAA buffer was dried down under vacuum and redissolved in TEAA buffer for labeling purpose without further purification. DTPA-cs124-EDA was synthesized using a published method (8). DTPA-cs124- $\alpha$ -NH<sub>2</sub>-Phe-OH was synthesized by the reaction of DTPA dianhydride with cs124 and Fmoc-*p*-NH<sub>2</sub>-Phe-OH. The Fmoc protecting group was subsequently removed by hydrolysis with 5% piperidine to form DTPA-cs124- $\alpha$ -NH<sub>2</sub>-Phe-OH. DTPA-cs124-*p*-NH<sub>2</sub>-Phe-OH was synthesized by the reaction of DTPA dianhydride with cs124 and *p*-NO<sub>2</sub>-Phe-OH. The NO<sub>2</sub> group was subsequently reduced to NH<sub>2</sub> by catalytic hydrogenation process.

**Spectroscopy of Compounds.** Tables 1 and 2 list the excited-state lifetimes of Tb<sup>3+</sup> and Eu<sup>3+</sup> measured for their complexes with the chelates labeled to proteins. Figure 3 shows three representative lifetime decays of thiol-reactive chelates labeled to HMM, MBP, and to Shaker K<sup>+</sup> expressed in an *Xenopus* oocyte. Figure 4 shows the characteristic spiked emission spectra of Tb<sup>3+</sup> and Eu<sup>3+</sup> coordinated to DTPA-cs124-Cys-MTS. All of the



**Figure 3.** Representative lifetimes of Tb<sup>3+</sup> and Eu<sup>3+</sup> complexes attached to HMM, MBP, and Shaker K<sup>+</sup> channels in oocytes.



**Figure 4.** Normalized Tb<sup>3+</sup> and Eu<sup>3+</sup> emission spectra in DTPA-cs124 Cys-MTS in 0.1 M TEAA pH 6.5 at a concentration of 1  $\mu$ M. Tb<sup>3+</sup> and Eu<sup>3+</sup> display their characteristic sharply spiked spectra. Pulsed excitation was at 40 Hz, 3.5  $\mu$ J/pulse for Eu<sup>3+</sup>, 3.0  $\mu$ J/pulse for Tb<sup>3+</sup>; integration time is 1 s for Tb<sup>3+</sup> and 10 s for Eu<sup>3+</sup>.

**Table 2. Life Times of Thiol-Reactive Eu<sup>3+</sup> Chelates Labeled to Proteins**

lanthanide	chelates	proteins	lifetime (ms)
Eu <sup>3+</sup>	DTPA-cs124-Phe- $\alpha$ -NH <sub>2</sub> -COCH <sub>2</sub> I	MBP	31% 0.70
			69% 0.08
			80% 0.69
	DTPA-cs124-EDA-COCH <sub>2</sub> I	MBP	20% 0.44
			76% 0.62
	DTPA-cs124-MTSEA	MBP	24% 1.05
			77% 0.70
	DTPA-cs124-Cys-MTS	MBP	23% 0.16
			94% 0.58
			6% 0.96
	DTPA-cs124-Phe- <i>p</i> -NH <sub>2</sub> -COCH <sub>2</sub> I	MBP	59% 0.96
			41% 0.33
DTPA-cs124-EMPH	MBP	14% 1.07	
		37% 0.32	
			49% 0.043

thiol-reactive chelates can quickly and efficiently react with thiol groups in proteins. As expected, the lifetime for both lanthanides is in the millisecond range. Although all the lifetimes are either biexponential or triexponential, the percentage of long or short lifetime components varies upon the nature of chelates and the attached proteins. In general, a high percentage of long lifetime component (0.6 ms for Eu, 1.5 ms for Tb) is advantageous. Among them, the methanethiosulfonate (MTS) chelates show biexponential fitting with a major population (typ.

**Table 3. Photophysics Data of Tb<sup>3+</sup>- and Eu<sup>3+</sup>-DTPA-cs124-Cys-MTS and Their Glutathione Adduct<sup>a</sup>**

Tb <sup>3+</sup> (Eu <sup>3+</sup> ) complexes	no. of coordinated H <sub>2</sub> O <sup>b</sup>	Q <sub>overall</sub>	Q <sub>transfer</sub>	Q <sub>Ln</sub>	τ (ms)
DTPA-cs124	1.1 (1.26)	0.324 (0.099)	66.6% (59.1%)	0.482 (0.167)	1.55 (0.62)
DTPA-cs124-Cys-MTS (n = 3)	1.0 (1.0)	0.21 ± 0.003 (0.075 ± 0.004)	52% ± 1% (38% ± 3%)	0.42 ± 0.01 (0.12 ± 0.002)	1.35 ± 0.02 (0.73 ± 0.02)
DTPA-cs124-Cys-MTS-Glu (n = 3)	1.02 (1.28)	0.25 ± 0.02 (0.074 ± 0.003)	54% ± 5% (44% ± 2%)	0.46 ± 0.006 (0.10 ± 0.001)	1.48 ± 0.01 (0.60 ± 0.003)

<sup>a</sup> All quantum yields (Q) and excited-state lifetimes (τ) are in H<sub>2</sub>O-based buffer. Uncertainties are reported as ± one standard deviation.

<sup>b</sup> On the basis of lifetime measurements via method of Horrocks and Sudnik (see text, (19)) in H<sub>2</sub>O and D<sub>2</sub>O. For Tb<sup>3+</sup> (Eu<sup>3+</sup>) – DTPA-cs124-Cys-MTS, the lifetimes were 1.35 ms (0.70) in H<sub>2</sub>O, and 2.3 ms (2.2 ms) in D<sub>2</sub>O. For Tb<sup>3+</sup> (Eu<sup>3+</sup>) – DTPA-cs124-Cys-MTS-Glu, the lifetimes were 1.47 ms (0.59) in H<sub>2</sub>O, and 2.29 ms (2.16) in D<sub>2</sub>O.

80–90%) having a long lifetime (ms) when unconjugated or conjugated to biomolecules.

The iodoacetamide chelates showed significantly improved labeling efficiency compared to the bromoacetamide form previously made (9). (By using higher pH, however, conjugation of a DTPA-cs124-EDA-bromoacetamide to cysteines can be improved (21)). The iodoacetamide chelates generally show a nearly equal population of the long and short lifetime components, although the ratio appears to depend on the biomolecule it is conjugated to. (DTPA-cs124-EDA-Br, which forms the same product as DTPA-cs124-EDA-I after conjugation to a thiol, showed a single-exponential signal upon conjugation to a muscle protein, the regulatory light chain (9)). In some cases when MBP was used, it became triexponential. Tb-DTPA-cs124-EMPH was also labeled to MBP. The measurement shows 89% long lifetime at 1.92 ms and 11% short lifetime at 0.79. This result is similar to when Tb-DTPA-cs124-EMPH is attached to the regulatory light chain of myosin (13).

**Photophysics of MTS Lanthanide Chelates.** We have characterized the photophysics of the Tb<sup>3+</sup>- and Eu<sup>3+</sup>- DTPA-cs124-Cys-MTS and their glutathione adducts, DTPA-cs124-Cys-MTS-Glu (Table 3). The latter was formed by reaction of the MTS-chelate with the reduced form of glutathione at pH 7.2 and purified by HPLC. The number of waters in the primary coordination sphere of Tb<sup>3+</sup>- and Eu<sup>3+</sup>-DTPA-cs124-Cys-MTS and DTPA-cs124-Cys-MTS-Glu were found to be 1.0 (1.0) and 1.02 (1.28), respectively, based on lifetime measurements of the chelates' major component in H<sub>2</sub>O and D<sub>2</sub>O. The reference complexes, Tb<sup>3+</sup>- and Eu<sup>3+</sup>-DTPA-cs124, were previously found to contain 1.1 (1.26) H<sub>2</sub>O molecules in their coordination spheres (22). These data indicate that there is no extra ligation to the lanthanides in DTPA-cs124-Cys-MTS complexes and their glutathione adducts.

We also measured the overall quantum yield (Q<sub>overall</sub>), the transfer efficiency from antenna to lanthanide (Q<sub>transfer</sub>), and the quantum yield for lanthanide emission (Q<sub>Ln</sub>) of the DTPA-cs124-Cys-MTS and its glutathione adduct (Table 3). This is accomplished by comparing the overall lanthanide intensity, the intensity of the initially excited lanthanide ions, and the average lifetimes, respectively, of the MTS-chelates to Tb<sup>3+</sup>- and Eu<sup>3+</sup>-DTPA-cs124 standards (20). We find Q<sub>overall</sub> = 0.21 (0.075) for Tb<sup>3+</sup>-(Eu<sup>3+</sup>-)DTPA-cs124-Cys-MTS and Q<sub>overall</sub> = 0.25 (0.074) for Tb<sup>3+</sup>-(Eu<sup>3+</sup>-)DTPA-cs124-Cys-MTS-Glu.

**Conclusion.** We have synthesized new thiol-reactive iodoacetamide and methanethiosulfonate forms of the luminescent lanthanide chelate, DTPA-cs124, and improved the synthesis and separation method for DTPA-cs124-EMPH. All of these chelates can easily react with thiol groups in proteins under neutral or slightly acidic condition. Generally, their lifetimes after labeling to proteins are biexponential, with a major population displaying a long lifetime. A particularly promising

chelate is DTPA-cs124-Cys-MTS, especially for ion channel studies where MTS reagents are commonly used. In addition, the ethylenediamine iodoacetamide chelate is a more reactive form of the bromoacetamide chelate which has previously been synthesized and shown to be single exponential when bound to the regulatory light chain of myosin (9). Our results demonstrate the synthetic versatility of this kind of chelate and their capability for labeling cysteines on proteins. These compounds should be useful as donors in resonance energy transfer experiments.

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