# Amine-Reactive Forms of a Luminescent Diethylenetriaminepentaacetic Acid Chelate of Terbium and Europium: Attachment to DNA and Energy Transfer Measurements

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An isothiocyanate form of a lanthanide chelate which is highly luminescent when bound to terbium or europium has been synthesized. The chelate consists of diethylenetriaminepentaacetic acid (DTPA) covalently joined to a chromophore, 7-amino-4-methyl-2(1*H*)-quinolinone (cs124), and to L-*p*-aminophenylalanine, in which the aromatic amine was further converted to an isothiocyanate group. Ethylenediamine was also used in place of aminophenylalanine, but the isothiocyanate formed from the aliphatic amine was significantly less reactive. Site-specific attachments to triglycine and to the 5′ ends of amine-modified DNA oligomers have been made. In addition, as an alternative method of coupling to macromolecules, DTPA anhydride−cs124 can be used to react specifically with a 5′ amine group on base-deprotected synthetic DNA oligomers. Synthesis and purification is relatively straightforward in both cases, and luminescent properties are favorable for several applications, including as nonisotopic labels, as long-lifetime alternatives to fluorophores in imaging and diagnostics and particularly as donors in luminescence resonance energy transfer. Energy transfer measurements are consistent with previously reported measurements using different attachment mechanisms.

## INTRODUCTION

Luminescent lanthanide chelates have highly unusual spectral characteristics that make them useful nonisotopic alternatives to radioactive probes (*1*−*5*), as alternatives to organic fluorophores, particularly where there are problems of background autofluorescence (*6*−*9*), and as donors in luminescence resonance energy transfer (*10*−*16*). These characteristics include millisecond lifetime, sharply spiked emission spectrum (<10 nm fwhm), large Stokes shifts (>150 nm), no self-quenching (*17*, *18*), high quantum yield for lanthanide luminescence (∼1), and excellent solubility.

A commonly used luminescent lanthanide chelate is diethylenetriaminepentaacetic acid (DTPA),[1] covalently attached to an organic chromophore, the latter acting as an antenna or sensitizer to absorb the excitation light and overcome the weak absorbance of the lanthanides (*11*, *19*−*22*). We have previously shown that carbostyril 124 bound to DTPA (*11*, *12*) or to other polyaminocarboxylate chelates (*23*) can sensitize both europium and terbium, and the complex is an excellent donor in resonance energy transfer experiments.

Conjugation of DTPA−chromophore moieties is most often done through the dianhydride form of DTPA (*24*), where one anhydride reacts with an amine-containing chromophore and the other with amine-containing biomolecules (*11*, *17*, *19*, *21*, *22*). For conjugation, however, the dianhydride has several disadvantages: (1) The anhydride is nonspecific in which nucleophilic acyl substitution reactions occur readily. When reacting with DNA, this concern led us to use base-protected DNA in our previous work (*11*, *12*). However, base deprotection required strongly alkaline conditions, which we found cause significant cleavage of the DTPA−cs124 amide bond, particularly on longer DNAs, and more mild conditions led to questions of incomplete deprotection (*12*). (2) The anhydride is water-labile. (3) The dianhydride can lead to a number of products, including DTPA disubstituted with chromophore, DTPA attached to the macromolecule with no sensitizer, and macromolecules cross-linked by DTPA. (4) The length of the linker arm between DTPA and the macromolecule is fixed.

A number of groups have made isothiocyanate forms attached to the backbone of DTPA (without a sensitizer), in part to overcome these problems (*25*−*28*). The synthesis, however, involves many steps and is time-consuming, and its extension to DTPA-modified with a sensitizer is not necessarily straightforward. We have synthesized an isothiocyanate form of DTPA−cs124 by utilizing one of the carboxyl groups: caDTPA is reacted with cs124, followed by addition of diamino-compound, and one of the amines is then converted to an isothiocyanate group (*29*, *30*). The synthesis is straightforward, and the isothiocyanate group formed from an aromatic amine is highly reactive and does not interfere with lanthanide luminescence. Furthermore, the amide-bond formed from one of the carboxyl groups is able to maintain coordination to the lanthanide (*31*) and the binding constant is sufficiently high for most practical purposes. We have also revisited the reaction of DTPA−cs124 anhydride with DNA (*11*, *12*) and have found conditions in which base-*deprotected* DNA can be conjugated without side reactions.

## EXPERIMENTAL METHODS

**Chemicals.** The following chemicals were purchased from Aldrich: diethylenetriaminepentaacetic acid dian-

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[1] Abbreviations: DTPA, diethylenetriaminepentaacetic acid; caDTPA , dianhydride of DTPA; cs124 or carbostyril 124, 7-amino-4-methyl-2(1*H*)-quinolinone; DMSO, dimethyl sulfoxide; DMF, dimethylformamide; EDA, ethylenediamine; HPLC, high-performance liquid chromatography.

S1043-1802(96)00085-7 CCC: $14.00 

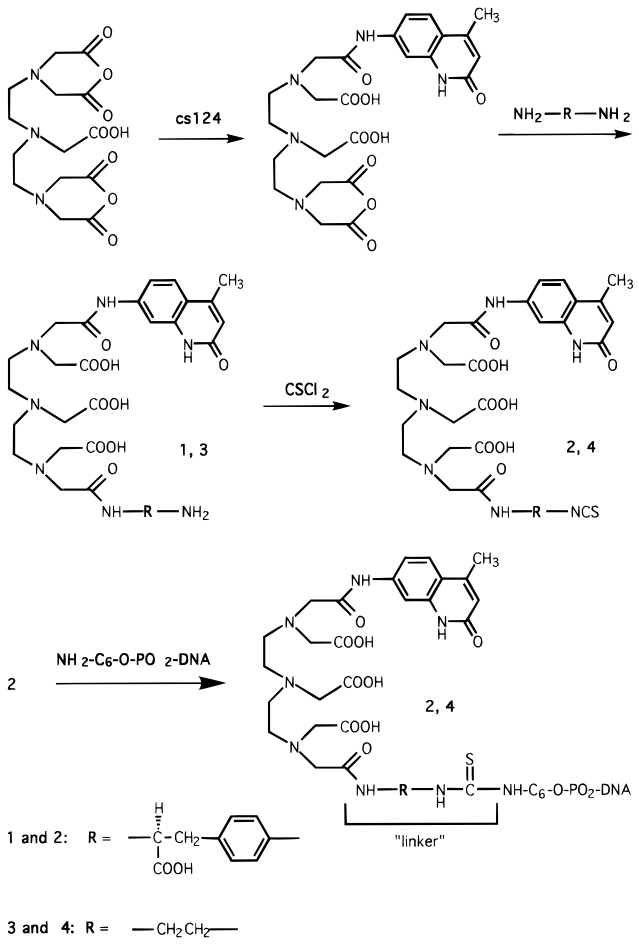


**Figure 1.** Synthesis of isothiocyanate formed from DTPA-cs124 attached to ethylenediamine (**4**) or L-*p*-aminophenylalanine (**2**) and conjugation with amino-modified DNA. When using the anhydride-based conjugation (see text) with an amine-containing compound, the indicated "linker" is absent.

hydride; 7-amino-4-methyl-2(1*H*)-quinolinone; anhydrous *N*,*N*-dimethylformamide; anhydrous dimethyl sulfoxide; triethylamine; ethylenediamine; thiophosgene; europium chloride hexahydrate (99.99%); terbium chloride hexahydrate (99.999%). L-*p*-Aminophenylalanine was purchased from Sigma. Distilled and deionized water (18 MΩ $cm^{-1}$) was used throughout. All glassware was washed with a mixed acid solution and thoroughly rinsed with deionized, distilled water (*32*). All plastic (metal-free) labware was purchased from Bio-Rad. All chemicals were of the purest grade available.

**Purification.** High-performance liquid chromatography was performed at room temperature on either a Beckman Model 100 system or a Waters 600E with a Dynamax $C_{18}$ column (Rainin) for reversed-phase purification and a GenPak Fax (Waters) 4.6 × 100 mm column for anion exchange purification. The UV-absorbing fractions were detected at 328 nm for cs-124 compounds and at 260 nm for DNA oligos.

**Synthesis (Figure 1).** *DTPA−cs124−*L*-p-Aminophenylalanine (**1**).* To a solution of caDTPA (200 mg, 560 μmol) in 5 mL of DMF and 300 μL of triethylamine was added dropwise cs124 (115.5 mg, 1.2 × 560 μmol) in 2 mL of DMF. After 45 min, the reaction mixture was added dropwise to a solution of L-*p*-aminophenylalanine (121 mg, 1.2 × 560 μmol) in 5 mL of DMSO and 100 μL of triethylamine. After 3 h of stirring, the solvent was evaporated to dryness under reduced pressure and the residue was redissolved in 5 mL of 1 M triethylammonium acetate, pH 6.5. The product was purified by reversed-phase HPLC using a 21.4 × 250 mm $C_{18}$ column (Dynamax 60 Å) with a flow rate of 12.5 mL/min. A 15-min linear gradient, from 10% to 15% solvent B (solvent A, 0.1 M triethylammonium acetate, pH 6.5; solvent B, $CH_3CN$), was used. The peak at retention time 14.5 min was collected and dried to give **1** as a light yellow solid: yield, 115 mg, 29%; $^1$H NMR δ ($D_2O$, pD = 6.5) 2.16 (3H, s), 2.7−3.4 (16H, m), 3.50(2H, s), 3.60(2H, s), 4.23(1H, m), 6.13(1H, s), 6.88 (2H, d), 6.95 (2H, d), 7.08 (1H, d), 7.33 (1H, d), 7.35(1H, s); FAB-MS, *m/e* 712 ($M + H^+$).

*DTPA−cs124−*L*-(p-Isothiocyanato)phenylalanine (**2**).* Compound **1** (50 mg, 70 μmol) in 40 mL of 0.5 M HCl was added to 30 mL of $CSCl_2$ (85% in $CCl_4$) at room temperature. The reaction was stirred vigorously for 1 h. The aqueous phase was washed with chloroform (5 × 5 mL) to remove excess $CSCl_2$ and then purified by reversed-phase HPLC using a 21.4 × 250 mm $C_{18}$ column (Dynamax 60 Å) with a flow rate of 12.5 mL/min. A 25-min linear gradient, from 10% to 50% solvent B (solvent A, 0.1% $CF_3COOH$ in $H_2O$; solvent B, $CH_3CN$), was used. The major peak at retention time 24 min was collected and dried to give **2** as a white solid: yield, 16 mg, 30%; $^1$H NMR δ ($CD_3OD$) 2.42 (3H, s), 3.06 (2H, t), 3.15 (2H, t), 3.38 (8H, m), 3.51 (2H, s), 3.61 (2H, s), 3.92 (2H, s), 4.56 (1H, m), 6.35 (1H, s), 6.02 (2H, d), 7.09 (2H, d), 7.35 (1H, d), 7.65 (1H, d), 7.90 (1H, s); FAB-MS, *m/e* 754 ($M + H^+$).

*DTPA−cs124−EDA (**3**).* To a solution of caDTPA (500 mg, 1.4 mmol) in 30 mL of DMF and 1 mLof triethylamine was added dropwise cs124 (240 mg, 1.4 mmol) in 4 mL of DMF and stirred. After 30 min, 5 mL (75 mmol) of ethylenediamine (EDA) was added, forming a white precipitate. The reaction was stirred for 2 h at room temperature and then stored in a refrigerator overnight, forming a brown precipitate. The precipitate turned white after several washes with 2-propanol and was finally washed with ether and dried overnight under vacuum. To remove excess EDA, the solid was redissolved in $H_2O$, loaded on a $C_{18}$ Sep-Pak (Waters), washed with 12 mL of 0.4 M NaOAc, pH 6, and 6 mL of $H_2O$, and eluted with 50% MeOH/$H_2O$. HPLC analysis [30-min linear gradient from 15% to 60% solvent B (solvent A, 0.1% $CF_3COOH$ in $H_2O$; solvent B, $CH_3CN$), 3 mL/min with 10 × 250 mm $C_{18}$ Dynamax 60 Å column] showed two well-resolved peaks. The first peak at 16 min was confirmed to be DTPA−cs124−EDA: FAB-MS, *m/e* 592 ($M + H^+$). The second peak at retention time 19 min was DTPA−$(cs124)_2$.

*DTPA−cs124−EDA−Isothiocyanate (**4**).* Approximately 10 mg of the precipitate containing **3** was dissolved in 3 mL of $H_2O$. Three milliliters of $CSCl_2/CCl_4$ (85%) was added [precipitation of DTPA−$(cs124)_2$ was observed], followed by addition of solid $CaCO_3$. After 30 min of vigorous stirring, HPLC analysis of the supernatant showed that the DTPA−cs124−EDA peak at retention time 16 min (see **3**) was quantitatively converted to a peak with retention time 22 min, later shown to be **4**. The solution was extracted with $CHCl_3$, and the precipitate containing DTPA−$(cs124)_2$ was removed by centrifugation. The material in the water phase was purified by HPLC using a 15-min linear gradient, from 15% to 40% solvent B (solvent A, 0.1% $CF_3COOH$ in $H_2O$; solvent B, $CH_3CN$) with a 21.4 × 250 mm $C_{18}$ Dynamax 60 Å column at a flow rate of 12.5 mL/min: FAB-MS, *m/e* 634 ($M + H^+$).

*Conjugation of **2** to DNA Oligo.* Thirty microliters of 0.1 M $NaHCO_3$, pH 7, was added to dried synthetic DNA oligo [10-mer with C6-amino linker at 5′ end, 50 nmol; sequence: $NH_2$-CCT-AGA-GTG-G (*12*)]. The solution was mixed with compound **2** (20 × 50 nmol, dried by

SpeedVac prior to use). The pH of solution was adjusted to 9−10 using $Et_3N$ aqueous solution. The mixture was incubated at 37 °C overnight and then passed through a P-6 column. The fraction was further purified on a reversed-phase HPLC using a 10 × 250 $C_{18}$ column (Dynamax, 300 Å) with a flow rate of 3 mL/min. A 60-min linear gradient, from 10% to 25% solvent B (solvent A, 0.1 M triethylammonium acetate, pH 6.5; solvent B, $CH_3CN$) was used. Peak at 37 min was collected to give the conjugate. Unconjugated DNA eluted at 18 min. Yield: 40−80% based on HPLC profiles. Mass (by time-of-flight MALDI): 4006 (calcd 4008). A second peak, not fully resolved, eluted less than 1 min later and was found to contain an approximately equal mixture of product and a compound of 56 extra mass units. Further purification by anion exchange [120-min gradient from 20% to 55% B with a flow rate of 0.5 mL/min (solvent A, 25 mM Tris HCl, pH 8.0, in 10% $CH_3CN$; solvent B, 25 mM Tris HCl, pH 8.0, and 1 M NaCl in 10% $CH_3CN$)] yielded two well-resolved peaks at 48 and 50 min, with the first peak containing twice the area and corresponding to the expected mass and the second peak corresponding to the species of 56 extra mass units. Conjugation with a 14-mer with C6-amino linker at 5′ end, $NH_2$-CCT-AGC-AGC-AGT-GG, was over 90% on the basis of HPLC profiles. Conjugation of **2** with the same DNA lacking a 5′ amine showed no reaction.

*Conjugation of caDTPA−cs124 to DNA Oligo.* To a solution of ca-DTPA (7.1 mg, 20 μmol) in 100 μL of DMSO and 11 μL $Et_3N$ was added dropwise cs124 (5.1 mg, 1.5 × 20 μmol) in 60 μL of DMSO at room temperature. After a 0.5 h, 43 μL of reaction solution was mixed with synthetic DNA oligos (e.g., 14-mer or 20-mer with C6-amino linker at 5′ end, 10 nmol, reversed-phase HPLC purified) in 60 μL of DMSO and 26 μL of 0.1 M $Et_3NOAc$, pH 8. The mixture was incubated at room temperature overnight and then passed through a P-6 column. The fraction was further purified on a reversed-phase HPLC using a 10 × 250 $C_{18}$ column (Dynamax, 300 Å) with a flow rate of 3 mL/min. A 60-min linear gradient, from 5% to 25% solvent B (solvent A, 0.1 M triethylammonium acetate, pH 6.5; solvent B, $CH_3CN$) was used. The peak at 44 min was collected and dried to give the conjugate: yield, 30−80% based on HPLC profiles; mass, 5011 (14-mer, calcd 5010), 6906 (20-mer, calcd 6906). Further anion exchange purification (see above) displayed a major single peak, with a second small (<10%) peak of 56 extra mass units. Conjugation of caDTPA−cs124 with the same DNA lacking a 5′ amine showed no reaction.

*Hybridization Conditions.* DNA oligomers were hybridized to their complement in 10 mM Tris, pH 8.0, 10 mM $MgCl_2$, and 150 mM NaCl by heating to 65−70 °C and cooling over a period of 15−60 min to either room temperature or 5 °C. The solvent was either $H_2O$ or $D_2O$ (99.9%; Aldrich). For energy transfer measurement, and an increasing amount of acceptor-labeled strand [tetramethylrhodamine (TMR) is the acceptor for terbium; Cy5 is the acceptor for europium] was added to lanthanide-labeled DNA at approximately 0.25 μM. Complementary DNA (labeled or unlabeled) was synthesized and purified as previously described (*11*, *12*).

*Spectroscopy.* Absorption measurements were made on a Hewlett-Packard 8452A spectrometer. Time-resolved and gated luminescence measurements were made on a laboratory-built spectrometer used previously (*11*, *12*, *23*) utilizing 337-nm pulsed excitation (5 ns, 40 Hz) and photon-counting detection (GaAs PMT and multichannel scalar with 2-μs resolution). Terbium emission was monitored at 546 nm and europium emission at 617 nm. Data were curve-fit to one or two exponentials using TableCurve software (Jandel Scientific).

*Metal Binding.* All of the chelate-conjugated compounds were added in 1:1 molar ratio with a solution of $TbCl_3$ or $EuCl_3$ in triethylammonium acetate, pH 5. The solutions were incubated at room temperature for 30 min and diluted in the appropriate spectroscopy buffer.

## RESULTS AND DISCUSSION

**Proof of Structure.** Figure 1 shows the synthesis pathways of two isothiocyanate forms of DTPA−cs124, **2** and **4**. Several lines of evidence confirm the synthesis and purity of the compounds. Single well-resolved HPLC peaks were found for the products, with starting materials running at different retention rates—conversion of the amine to an isothiocyanate resulted in significantly extended retention; mass spectroscopy confirmed the expected mass; NMR showed the expected peaks with no significant impurity components; both isothiocyanate forms reacted quantitatively with an excess of triglycine as determined by a shift in retention time in HPLC and by mass spectroscopy analysis; products were highly luminescent with terbium and europium, whereas starting materials mixed with lanthanides showed no significant lanthanide luminescence; absorption spectra showed characteristic spectra of carbostyril as a 7-amide (maximum at 328 nm with secondary maximum at 342 nm (*23*).

**Reactivity Testing of −NCS Group.** Compounds **2** and **4** were each mixed with ∼50-fold excess of triglycine in a solution of 0.1 M triethylammonium acetate, pH 9, for 0.5 h at room temperature. HPLC analysis [30-min linear gradient 15−60% solvent B (solvent A, 0.1% $CF_3COOH$ in $H_2O$; solvent B, $CH_3CN$; 3 mL/min with 10 × 250 mm $C_{18}$ Dynamax 60 Å column] of the reaction mixtures showed a peak corresponding to **2** at retention time 23 min converted quantitatively to a peak at 13 min (FAB-MS, *m/e* 943 [M + $H^+$]) and a peak of **4** at retention time 16 min was quantitatively converted to a peak at 11 min, respectively. Under conditions appropriate for labeling amine-modified DNA (up to a 50-fold excess of **4** over DNA; see above), we found that **2** but not **4** was sufficiently reactive. This is in agreement with the results of Mujumdar et al., who found that an isothiocyanate of cyanine dyes formed from an aromatic—but not aliphatic—amine was sufficiently reactive with amine-containing biomolecules (*33*).

**Proof of Conjugation to DNA.** **2** and caDTPA−cs124 were each reacted with base-deprotected and HPLC-purified synthetic DNA oligomers containing a 5′ amine. After both reversed-phase and anion exchange HPLC, well-resolved peaks of the expected product mass were isolated. Control reactions with DNA lacking a 5′ amine showed no reaction of either **2** or caDTPA−cs124 with the amines of the (deprotected) DNA bases. For similar reaction conditions, no significant reaction of **4** with DNA was observed. With **2** there was a minor but significant second peak of 56 extra mass units which was not well resolved by reversed-phase HPLC alone but was resolved after additional anion exchange. The source of this extra mass is unknown, though it is possible that the chelate bound some trace heavy metals. The two peaks displayed identical luminescent properties after lanthanides were added, and both hybridized approximately equally well to complementary DNA. Nevertheless, we routinely purified the conjugated DNA by both reversed-phase and anion exchange HPLC to isolate the product of proper molecular weight and found that this gave the best hybridization results.

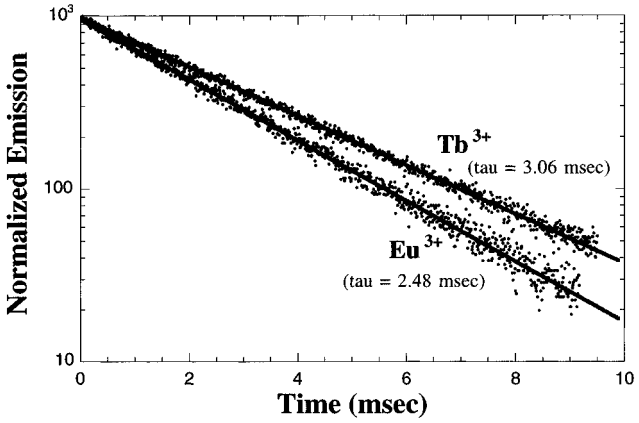


**Figure 2.** Lifetime of $Tb^{3+}$ and $Eu^{3+}$ bound to **2** attached to the 5′ amine group of synthetic DNA. The Tb data are for DNA hybridized to an unlabeled complement. Solution conditions are the same as in Table 1. Data were acquired every 2 $\mu$s, digitally binned every 10 $\mu$s, and curve fit to a single exponential which showed no residual structure and $r^2 > 0.99$.

**Table 1. Lanthanide Excited State Lifetime Attached to DNA**[a]

| sample | $H_2O$; RT (ms) | $D_2O$; RT (ms) | $D_2O$; 5 °C (ms) |
|---|---|---|---|
| Tb-anhydride | 1.5 (1.25) | 2.38 (2.0) | 2.98 (2.1−2.5) |
| Tb-ITC | 1.43 (1.35) | 2.50 (2.19) | 3.06 |
| Eu-anhydride | 0.63 (0.63) | (2.38) | 2.41 (2.46) |
| Eu-ITC | (0.60) | 2.39 | 2.45 |

[a] The table shows the excited state lifetime of terbium or europium attached to DNA either via **2** or via the anhydride reaction. The numbers in parentheses correspond to unhybridized DNA, those not in parentheses to lanthanide-labeled DNA hybridized to an unlabeled complement. Data were taken at 10 mM Tris, pH 8.0, 10 mM $MgCl_2$, 150 mM NaCl. In some cases, particularly the single-stranded DNA, there was a minor component (<10%) with a highly quenched lifetime (a few hundred microseconds), likely caused by interaction of the chelate with the DNA bases. The relatively large spread in the data for the Tb-anhydride unhybridized data is due to variability in the temperature regulation of the cuvette.

**Luminescence Measurements.** *Triglycine Complexes.* Terbium and europium luminescence of both **2** and **4** conjugated to triglycine had lifetimes and intensities unchanged from the unmodified DTPA-cs124. This indicates that modification of the chelate complex with these isothiocyanate groups does not alter the luminescence properties of the bound lanthanide.

*DNA Complexes.* Figure 2 shows luminescence of **2** bound to $Eu^{3+}$ or $Tb^{3+}$, conjugated to DNA, and hybridized to its DNA complement in a $D_2O$-based buffer. Table 1 summarizes the lifetime of both **2** and the anhydride DTPA−cs124 conjugated to DNA under a variety of solvent, temperature, and hybridization conditions. The Eu lifetime is relatively insensitive to environmental conditions, except for the well-known quenching effect of $H_2O$ when ligated in the primary coordination sphere (*34*−*36*). The Eu lifetime of 2.48 ms shown in Figure 2 is very similar to that of the unconjugated, unmodified Eu−DTPA−cs124 [2.42 ms (*23*)] and to that of the Eu−DTPA−cs124 complexed to DNA via the anhydride reaction [2.48 ms, this work; 2.5 ms (*11*)]. As reported previously for the anhydride form (*11*), the Eu lifetime for both the isothiocyanate and the anhydride reactions changes <5% between single- and double-stranded DNA.

The terbium excited state lifetime when the chelate is bound to DNA is more sensitive to environmental conditions—temperature, salt, and hybridization conditions—than europium under similar conditions. The terbium lifetime in DTPA−cs124 *not* attached to a macromolecule is insensitive to these conditions (*23*). It is likely that the origin of this greater sensitivity is due to strong interaction between the chelate and the DNA bases, and this interaction affects the excited state lifetime of $Tb^{3+}$ more than $Eu^{3+}$ (likely because of the greater energy of the terbium excited state). The 3.063 ms terbium lifetime in Figure 2 is somewhat longer than that of the unmodified Tb−DTPA−cs124 [2.63 ms (*23*)] and very similar to the lifetime of Tb−DTPA−cs124 complexed to DNA via the anhydride reaction and hybridized [2.98 ms, this work; 2.81 ms (*12*)]. (The small difference appears to be due to slightly lower temperature used in the current work, although both are reported as "5 °C".) As we found previously for the anhydride reaction (*12*), the lifetime of the terbium when bound to DNA via the isothiocyanate increases upon hybridization (2.19 ms for single-stranded, 2.50 ms for double-stranded; both at room temperature and in $D_2O$ buffer) and decreases with temperature (2.50 ms in $D_2O$ at room temperature; 3.06 ms in $D_2O$ buffer at 5 °C for the double-stranded, isothiocyanate-form) and increases slightly with salt. The emission intensity of the lanthanide complexes also decreases upon attachment to DNA (roughly 5−10-fold). In contrast, the terbium lifetime in the unreacted form is temperature and salt independent (*23*). These observations imply that the chelate interacts strongly with the DNA, and this interaction is modulated by hybridization, temperature, and salt. More specifically, hybridization increases the terbium lifetime, which is consistent with the symmetry in the crystal field surrounding the terbium increasing upon hybridization. In addition, particularly in the single-stranded form, the cs124 may be able to interact with bases, forming a new energy level which can lead to nonradiative deexcitation. We find, for example, that in the single-stranded case, terbium (but not europium) can have a small (typically ≤10% amplitude) component of a few hundred microseconds, indicating a species which is quenched. The amplitude of this component generally decreases with increasing salt and with hybridization.

**Energy Transfer Measurements: ITC-Based Reactions.** We have performed energy transfer measurements by hybridizing the chelate-labeled DNA to a complementary DNA containing an acceptor dye attached to the 5′ end, in analogy to our previous work (*11*−*13*). For Eu, we used Cy5 as the acceptor, and for Tb, we used tetramethylrhodamine (TMR) as the acceptor. Addition of 0.25 Cy5-labeled DNA per Eu−DTPA−cs124−ITC-14-mer−DNA yielded a biexponential donor lifetime at 617 nm of 22% exp(−$t$/786 $\mu$s) + 78% exp(−$t$/2405 $\mu$s), indicating 67% energy transfer (1−786 $\mu$s/2.41 ms) and a distance of 62.2Å ($R_0$ = 70 Å) for the hybridized species in $D_2O$ buffer. As expected, addition of further amounts of Cy5 increased the amplitude of the short-time component, without changing its lifetime (see also below). For Eu bound via the anhydride, we found 70% energy transfer (60.8 Å) for the hybridized species (see below). These distances are in excellent agreement with the 61.2 Å found previously using the Tb-anhydride chelate attached to the same 14-mer (*12*).

Energy transfer from terbium bound to a 10-mer DNA via the isothiocyanate also yielded results consistent with previous (and current) anhydride-based reactions. In $D_2O$ buffer, at room temperature, for example, an intermediate point in the titration with a TMR-labeled strand yielded a donor lifetime at 546 nm of 43% exp(−$t$/355 $\mu$s) + 67% exp(−$t$/2187 $\mu$s). Previously, we found 329 and 2101 $\mu$s, respectively (*12*).

**Energy Transfer Using Anhydride Reaction: Comparison to Previous Work.** We repeated our previ-

ously published energy transfer measurements (*11*, *12*) on the 10-mer DNA, which was based on a base-protected anhydride-coupling reaction, using our new anhydride-coupling procedure. The repeatability was excellent: In the current work, the terbium lifetime of terbium-labeled DNA hybridized to a TMR-labeled complement is 328−343 $\mu$s, corresponding to 88% energy transfer (previous value 328 $\mu$s, 88% energy transfer), and the terbium lifetime of the unhybridized donor strand is 2.19 ms (previous value 2.10 ms), although this latter value is fairly temperature-sensitive, increasing to as much as 2.50 ms if the temperature is not carefully controlled. The sensitized emission of the TMR is 333−356 $\mu$s (326 $\mu$s, previous value), in excellent agreement with the donor lifetime of the hybridized species.

Similar agreement is found with the Eu-anhydride−10-mer DNA hybridized to Cy5 complement (*11*). In this work, the unhybridized donor lifetime is 2.41 ms (previous value 2.40−2.52 ms) and the hybridized lifetime is 0.19 ms, corresponding to 92% energy transfer (previous values 0.22 ms, 91% energy transfer). The sensitized emission lifetime of Cy5 at 668 nm is 0.24 ms (previous value 0.25 ms).

**Energy Transfer: Hybridization Conditions.** Although no effort was made to achieve optimal hybridization conditions in this work, we generally found that our simple (but commonly used) hybridization procedure did *not* yield 100% hybridized donor strand, even with a 1.25-fold excess of acceptor strand and at 5 °C, which was well below the melting temperature of the DNAs. The amount of unhybridized donor strand varied from 10% to 50% (typically 10−20%). We generally found that DNA chelate purified by both reversed-phase and anion exchange yielded higher percentage of hybridization than those purified only by reversed phase, although this result is not rigorous and the reason for it is unclear. Since each strand was shown to be pure, we do not believe the unhybridized donor strand is due to some fraction of donor strand that cannot hybridize. Complete hybridization was achieved after gel purification to isolate the double-stranded complex under nondenaturing conditions (*37*): luminescence measurements on the DNA in gel fragments yielding a single exponential and quenched donor lifetime. Presumably kinetically trapped single-stranded DNA complexes were formed in solution in our simple hybridization procedures. We note that lanthanide-based energy transfer, with the capability to accurately measure multiexponential donor lifetimes, has the ability to detect even a relatively small percentage of unhybridized strands. Standard fluorescence resonance energy transfer does not have this ability. We believe, therefore, that caution should be exercised in assuming complete hybridization in FRET experiments and that gel purification of the double-stranded complex may be necessary, as others have found (*37*).

**Conclusion.** We have synthesized, purified, and spectrally characterized new isothiocyanate derivatives of DTPA−cs124 bound to small molecules and to DNA oligomers. We have also optimized conditions for the anhydride-based reaction of DTPA−cs124 with base-deprotected DNA and shown that specific reaction at a 5′ amine can be achieved without side reaction at the DNA bases. Finally, we have used these new products in luminescence resonance energy transfer measurements and have shown that the results are consistent with previously published measurements. The facile attachment to biomolecules of luminescent lanthanide chelates should increase the utility of these compounds.

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