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Synthesis of Photostable Amine-Reactive Fluorescent Dyes by Postsynthetic Conversion of Bromide Dithienothiophene Derivatives

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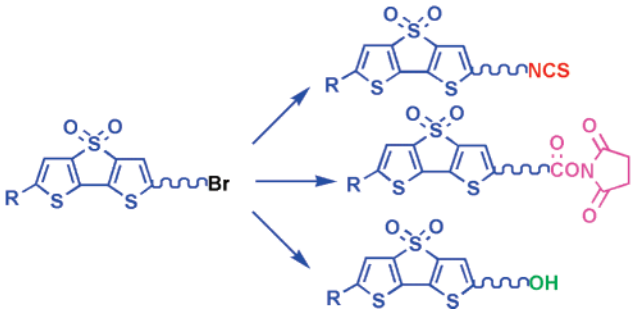
Received March 8, 2007



The synthesis, purification, and spectral properties of new dithienothiophene-based fluorescent dyes with a terminal alkyl bromide group are described. The bromides were easily converted to isothiocyanates and *N*-succinimidyl esters by appropriate chemical transformations with sodium thiocyanate or *N*-hydroxysuccinimide. The new fluorophores exhibited intense fluorescence emission and high photostability. Their suitability for bioanalytical applications was evaluated through conjugation with amine-reactive polystyrene microspheres and IgG anti-CD3 monoclonal antibody.

Introduction

Fluorescence-based techniques are indispensable tools in numerous fields of modern medicine and science, including molecular biology, biophysics, biochemistry, clinical diagnosis, and analytical and environmental chemistry.[1] These techniques are resulting in major improvements in bioanalytical applications because of their extraordinary sensitivity and selectivity.[2,3]

Many important biomolecules (oligonucleotides, aminoacids, peptides, etc.) are nonfluorescent or weakly fluorescent and the use of fluorescent labeling can provide the means for numerous in vitro assay procedures.[4] For instance, fluorescently tagged antibodies can be used to probe cells and tissues for the presence of particular antigens, and then be detected through the use of fluorescence microscopy techniques. Since each probe has its own fluorescence characteristics, more than one labeled molecule—each tagged with a different fluorophore—can be used at the same time to detect two or more target molecules.[2,3] Among the reactive fluorescent dyes, amine-reactive dyes are most often used to prepare various bioconjugates for immunochemistry, histochemistry, fluorescence in situ hybridization (FISH), cell tracing, receptor binding, and other biological applications.[4] An amine-coupling process can be used for the conjugation of nearly all protein or peptide molecules as well as to any other amine-containing macromolecule.[1−5]

Earlier papers from our laboratory introduced a new class of fluorescent labeling reagents based on oligothiophenes functionalized with isothiocyanate and succinimidyl ester as amine-reactive groups.[6−9] Thiophene-based fluorophores show high fluorescence quantum yields, large Stokes shifts, and high

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10.1021/jo070488n CCC: $37.00
Published on Web 06/01/2007

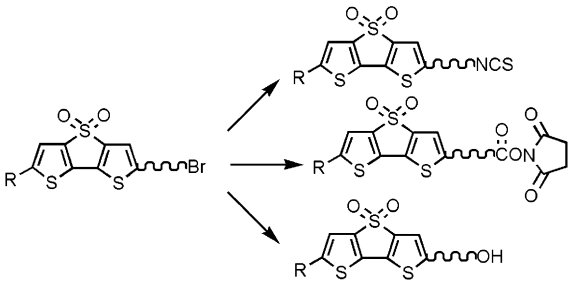

FIGURE 1. Graphical representation of the newly synthesized DTTO-based dyes.

photostability and chemical stability. Furthermore, they are amenable to easy structural modifications for the tuning of the emission color, are insensitive to pH, and have fluorescence spectra covering the entire visible range.[6−9]

The present work is an extension of our research aimed at synthesizing novel and more useful oligothiophene dyes with attractive photoluminescence characteristics for biomedical applications.

We made use of the dithieno[3,2-*b*:2′,3′-*d*]thiophene (DTT) "rigid core", which has emerged as a useful building block in the synthesis of a wide variety of compounds with functional properties,[10] including active materials for white-emitting electroluminescent diodes[11] and light-emitting transistors.[12]

In this article, we describe a series of oligothiophene dyes based on the corresponding oxidized derivative, DTTO, with alkyl bromide end groups to be converted into amine-reactive or other useful functionalities (Figure 1).

The new fluorescent dyes were evaluated for use in bioanalytical applications by conjugation reactions with amino functionalized polystyrene microspheres and the anti-CD3 monoclonal antibody. Surface-labeled microspheres are suited for mimicking biological samples and for testing the dyes photostability. A study of the photostability of the dyes conjugated to polystyrene spheres is reported.

Results and Discussion

I. Synthesis. Scheme 1 shows the routes taken to prepare DTTO-based compounds **3**, **6**, **8**, and **10** terminated with a bromide group. The dithienothiophene moiety (**1**) was synthesized according to a recently described method.[13]

For the synthesis of dye **3**, we first prepared product **2** by Friedel−Crafts acylation of DTT **1** with 4-bromobutyryl chloride in dichloromethane in the presence of anhydrous aluminum chloride as catalyst. Product **2** was further reacted with MCPBA in dichloromethane at room temperature to afford compound **3** (70%). Compound **6** was synthesized through Stille coupling of the bromo derivative of compound **3** with tributyl(5-(octylthio)thiophen-2-yl)stannane **5** in 71% yield. The bromo derivative **4** was obtained by the bromination of compound **3** with NBS, under exclusion of light, in a mixture of dichloromethane and acetic acid in 82% yield.

The synthesis of tributyl(5-octylthio-thiophen-2-yl)stannane **5** was achieved by metalation of 2-(octylthio)thiophene, using BuLi at −20 °C followed by transmetalation with tributyltin chloride. The organotin compound **5** was obtained in 84% yield and was used in the Stille coupling reaction without further purification. The Stille reaction was performed in toluene under a nitrogen atmosphere and catalyzed by $Pd(Ph_3As)_4$ generated in situ.[14] Compound **8** was prepared by Friedel−Crafts reaction between DTT **1** and 4-bromobutyryl chloride in dichloromethane in the presence of anhydrous aluminum chloride as catalyst, followed by reduction of the keto group by addition of borane−*tert*-butylamine in the presence of anhydrous $AlCl_3$. The resulting product **7** was oxidated with MCPBA in dichloromethane at room temperature to give dioxide **8** in 71% yield. Compound **10** was obtained in 78% yield by Stille coupling of tributyl(5-(octylthio)thiophen-2-yl)stannane **5** and the bromo derivative of compound **8** under $Pd(Ph_3As)_4$ catalysis at 80 °C in toluene. Bromo derivative **9** was obtained by bromination with NBS in a solution of dichloromethane−acetic acid (1:1) in 66% yield.

To conjugate the above-described fluorophores to macromolecule or biopolymers it is necessary to convert them into a reactive form. This is accomplished by introducing a reactive group, isothiocyanate, *N*-hydroxysuccinimide ester, or hydroxyl moiety, by a nucleophilic substitution of the bromine end group (Scheme 2).

The bromo derivatives **3**, **6**, **8**, and **10** were converted to isothiocyanates **11**, **12**, **13**, and **15** respectively, by treatment with an excess of sodium thiocyanate in distilled acetone under vigorous stirring at 200 °C in a conical vial.[15] Under these conditions the reaction gave exclusively *N*-alkylation. The reaction gave in general good conversion (80−95%),[7] but for compounds **11** and **12** the yields were lower (60%), probably because of side reactions of the carbonyl group with strong nucleophile sodium thiocyanate.

The isothiocyanate group reacts with biomolecules almost exclusively through the ϵ-amino group of lysine residues and the *N*-terminal α-amino group to form thiourea bonds via an addition reaction.[5,16] The reaction involves attack of the nucleophile on the central, electrophilic carbon of the isothiocyanate group and offers the advantage of not generating any byproduct, thus simplifying the purification of the conjugate. Isothiocyanate compounds react best at alkaline pH values where the target amine groups are mainly unprotonated.[5] They are quite stable in aqueous solution and the optimum pH for conjugation is 9.0−9.5.[16]

Alcohol derivative **14** was synthesized in 89% yield from bromo derivative **8** by hydrolysis in a 15% (v/v) solution of water in *N*-methyl-2-pyrrolidinone (NMP) at 130 °C.[17] The direct displacement of the halogen end groups with hydroxyl groups was unsuccessful due to side reactions such as elimina-

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(15) The acetone is heated well above its boiling point so all necessary precautions should be taken when performing this experiment. Vessels designed to withstand elevated pressures must be used. After completion of an experiment, the vessel must be allowed to cool to a temperature below the boiling point of the solvent before opening to the atmosphere.

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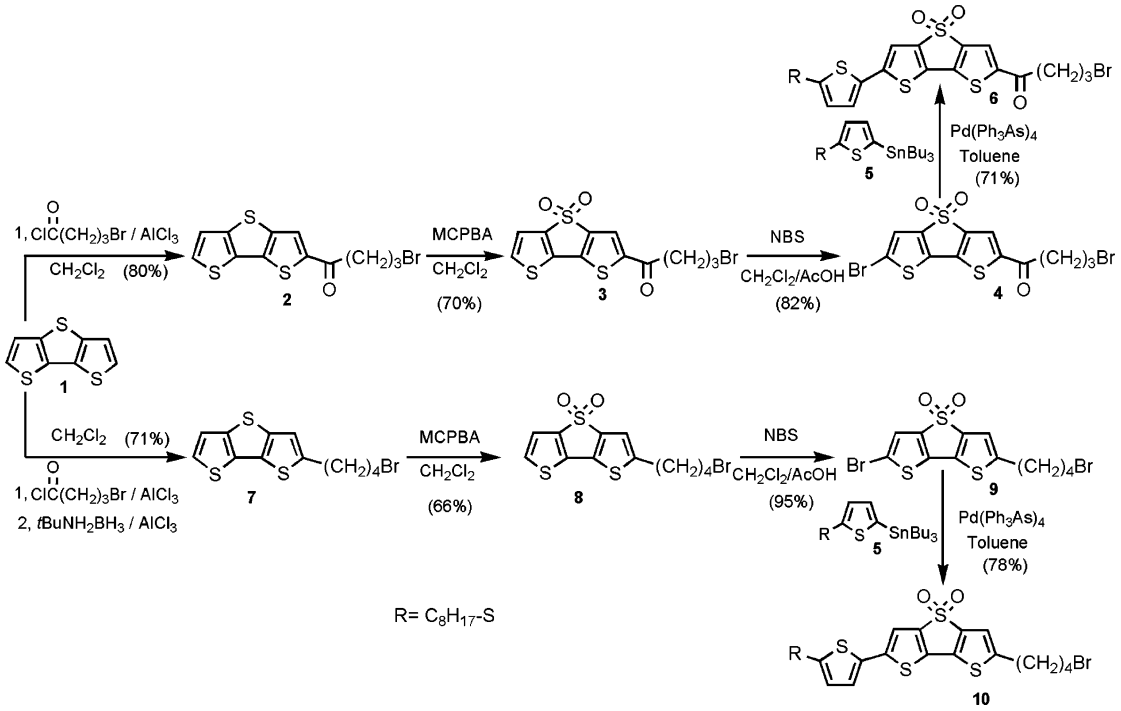
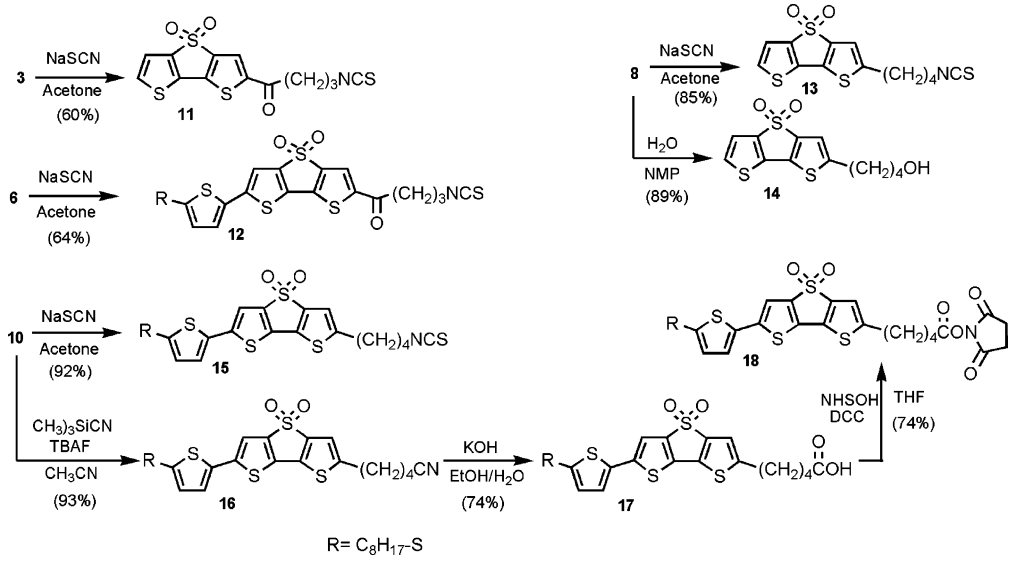
SCHEME 1. Synthesis of DTTO-Based Compounds 3, 6, 8, and 10



SCHEME 2. Nucleophilic Substitutions of Bromine End Group



tion, but the combination of water and polar aprotic solvent, NMP, provided a potent and selective source of nucleophilic oxygen.[17] The hydroxyl moiety can be phosphitylated to yield the phosphoramidite derivative suitable for incorporation into oligonucleotides by automated synthesizers.[18] A detailed study in this direction will be published elsewhere.

The synthesis of NHS derivative **18** was achieved in three steps. The nitrile derivative **16** was synthesized in 93% yield from the bromo derivative **10** via a nucleophilic substitution with trimethylsilyl cyanide (TMS-CN) in the presence of tetrabutylammonium fluoride (TBAF) in acetonitrile at room temperature.[19] The hydrolysis of nitrile **16** under basic conditions afforded carboxylic acid **17**, which was used after crystallization from diethyl ether for the next conversion. The carboxyl group was converted to the *N*-succinimidyl ester **18** by esterification, in the presence of NHS and an activator, dicyclohexylcarbodiimide, in THF.[20] Also *N*-hydroxysuccinimide ester derivatives react with biomolecules through the ϵ-amino group of lysine residues, however, via a substitution reaction with release of NHS leaving group to form an amide bond, known for its stability.[16,21] The optimum pH for reaction in aqueous systems is 8.0−9.0. One limitation relies on the susceptibility of the

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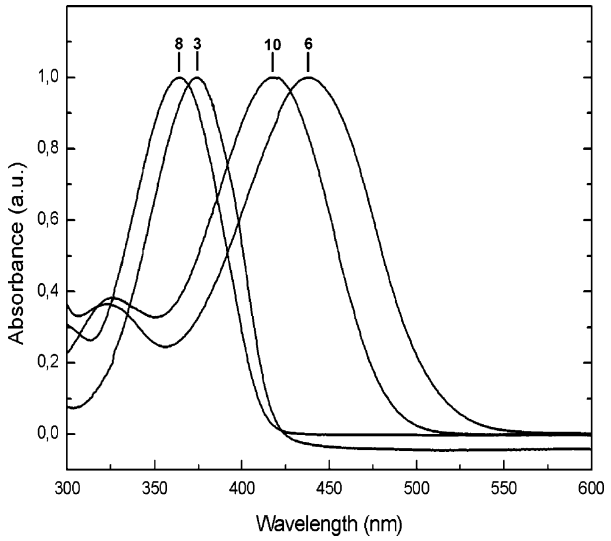

FIGURE 2. Normalized absorption spectra of bromoderivatives **3**, **6**, **8**, and **10** in CH_2Cl_2.

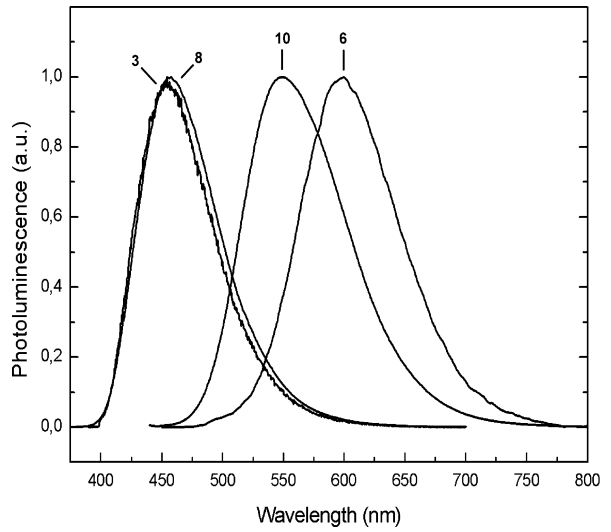

FIGURE 3. Normalized photoluminescence spectra of bromoderivatives **3**, **6**, **8**, and **10** in CH_2Cl_2.

TABLE 1. Maximum Absorption and Photoluminescence Wavelengths of Bromoderivatives 3, 6, 8, and 10 in CH_2Cl_2

compd	λ_{max} (nm)	λ_{PL} (nm)	Stokes shifts (cm^{-1})
8	363	457	5666
3	374	454	4712
10	421	550	5571
6	440	600	6061

amide bond to hydrolysis that competes with aminolysis, a side reaction that is usually slow below pH 9.[16]

II. Optical Properties. The normalized absorption and photoluminescence spectra of bromoderivatives **3**, **6**, **8**, and **10**, taken in CH_2Cl_2, are shown in Figures 2 and 3, respectively, while the λ_{max}, λ_{PL}, and Stokes shift values are summarized in Table 1. Note that postfunctionalization does not alter the optical features.

The introduction of electron-withdrawing or electron-donating groups affects the absorption and emission characteristics of the dyes, which cover the visible spectrum from blue to orange. The absorption spectra of the compounds in Figure 2 showed broad and structureless $\pi-\pi^*$ transition bands.

The bromo derivative **8** has an absorption maximum at 363 nm and an emission maximum at 457 nm. Despite the presence of the carbonyl group, compound **3** displays spectra quite similar to that of compound **8**, with a small bathocromic shift of the absorption maximum and the same emission maximum. Compound **10** exhibits a bathochromic shift compared with dyes **8** and **3** as a result of an extra thienylic ring and electron-donating effect of the thio-octyl chain. Compound **6** with both donor and acceptor groups has a push−pull effect and exhibits a greater red shift with photoluminescence in the orange. Compounds **6** and **10** show broad and structureless bands probably due to the intramolecular change transfer (ICT) absorption.[22]

All compounds exhibit very large Stokes shifts (difference between the spectral positions of the band maxima of the absorption and emission), from 4712 cm^{-1} for **3** to 6061 cm^{-1} for compound **6**.

III. Labeling of Polystyrene Microspheres. Surface-labeled polymeric microspheres are most commonly used in biology as controls and standards in a wide number of existing and emerging applications utilizing fluorescence detection, like flow cytometry, confocal microscopy, immunoassays, etc.[4,23]

We used for our study nominally 6.31 μm amino-modified polystyrene microspheres, with about 134 μequiv/g amino groups attached to the surface of the particles by alkyl chains. All amine-reactive dyes were covalently bound to microspheres in dry DMSO. The dyes and the microspheres were vortexed and incubated for 2 h at room temperature on a rotary mixer. At the end of this reaction period, the DTTO-conjugated microspheres were removed by microfiltration through a 0.45 μm syringe filter and washed with DMSO and Millipore water several times until all unbound dye molecules were removed. The particles were redispersed in PBS buffer and before microscopic examination the samples were vortexed for 2 min, sonicated for 5 min, and vortexed again to disperse the microspheres. As shown in Figure 4, the microspheres labeled with DTTO dyes (Figure 4b−e) have superior photostability compared to microspheres labeled with fluorescein, prepared for comparison, with the same modalities (Figure 4a). Microspheres labeled with dye **18** (data not reported) have the same color and photostability as the particles labeled with dye **15**.

All DTTO-tagged microspheres were not visibly photobleached under illumination with a fluorescent microscope excitation source for 60 s, while under the same illumination conditions nearly complete degradation occurred for fluorescein-labeled spheres after only 30 s of exposure.

Photostability of the fluorescent microparticles during prolonged exposure by high-intensity irradiation at excitation wavelengths is particularly important when using the labeled microparticles for fluorescence microscopy. It is also important in fluorescence immunoassays, because the loss of fluorescence ultimately decreases the sensitivity of the assay.

IV. Labeling of Monoclonal Antibody Anti-CD3. Compound **13** was tested in the labeling of the monoclonal antibody anti-CD3 (purified from mouse ascitic fluid) em-

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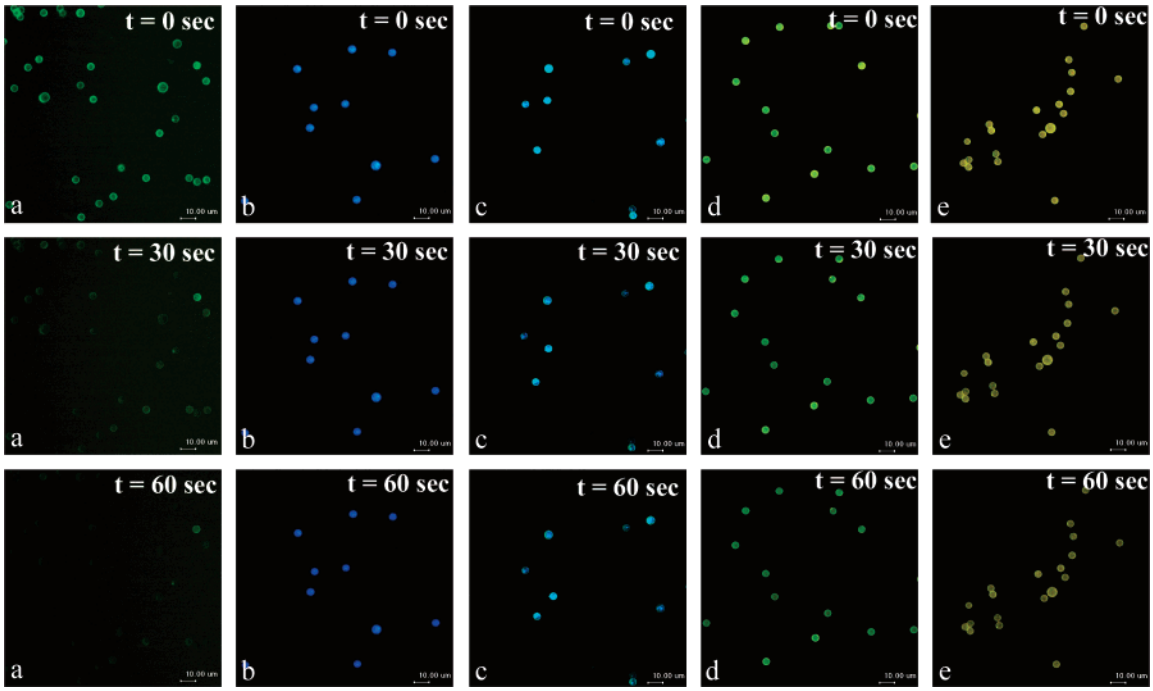

FIGURE 4. Fluorescence microscopy imaging of labeled microspheres at time zero and after 30 s and 60 s under continuous light irradiation: spheres labeled with (a) fluorescein, (b) dye **13**, (c) dye **11**, (d) dye **15**, and (e) dye **12**.

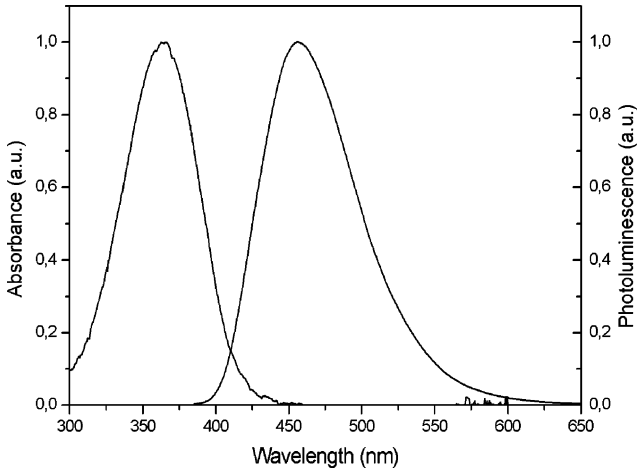

FIGURE 5. Normalized absorption and emission spectra of dye **13** in CH_2Cl_2.

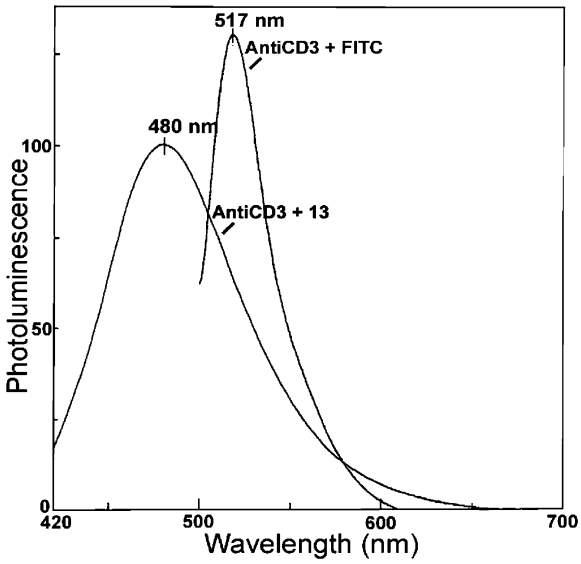

FIGURE 6. Photoluminescence spectra of the anti-CD3 MoAb labeled with dye **13** and with FITC.

ploying the same experimental procedure previously described.[7] Anti-CD3 is a IgG monoclonal antibody (MoAb) that recognizes the corresponding antigen present on the membrane of T-lymphocyte cells.[24] For comparison, the antibody was also labeled with fluorescein isothiocyanate (FITC, one of the most commonly used reagents in fluorescence-based analyses).[4]

Figures 5 and 6 report respectively the absorption and photoluminescence spectra of compound **13** and the photoluminescence spectra of anti-CD3 MoAb labeled with dye **13** and with FITC.

The fluorophore:MoAb ratio, derived from the absorption spectrum,[8] was 10 for the bioconjugate with dye **13** and 12 for the bioconjugate with FITC. To obtain the spectra of Figure 6, with comparable photoluminescence intensities for the two bioconjugates, the solution of anti-CD3 MoAb labeled with FITC was 32 times more dilute than that labeled with dye **13**.

Tests by an indirect method on flow cytometry with use of a second-step antibody conjugated with FITC showed that the biological activity of the antibody marked with dye **13** was completely preserved.

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Conclusions

In this paper we have described a series of novel dithienothiophene-based fluorescent dyes with an alkyl bromide end group. Synthetic procedures were developed to permit easy conversion in isothiocyanate or succinimidyl ester groups to bind them to the ϵ-amino group of monoclonal antibodies or amine-functionalized polystyrene microspheres or with hydroxyl groups for binding to oligonucleotides. The dyes are characterized by absorption maxima in the range 374−440 nm and photoluminescence maxima in the range 454−600 nm with large Stokes shifts (4700−6000 cm^{-1}).The high photostability displayed by the dyes conjugated to polystyrene microspheres and the preservation of biological activity of antibody marked with dye **13** suggest the possibility of using these labels in bioimaging applications.[4]

Experimental Section

General Procedures. Organic solvents were dried by standard procedures. Analytical thin-layer chromatography (TLC) was carried out with 0.2 mm sheets of silica gel 60 F_{254} or 0.2 mm sheets of aluminum oxide 60 F_{254} neutral. Preparative column chromatography was performed on glass columns of different sizes packed with silica gel 60 (particle sizes 0.040−0.063 mm) or aluminum oxide 90 standardized (particle sizes 0.063−0.200 mm). Petroleum ether refers to the fraction with bp 40−70 °C. The reactions allowing the introduction of the isothiocyanate group were performed in 5 mL conical vials made from low extractable borosilicate glass.[15]

NMR spectra were recorded at 400 MHz for ^{1}H and100 MHz for ^{13}C. Chemical shifts are reported in parts per million referenced to residual $CHCl_3$ at 7.26 for ^{1}H and 77.0 for ^{13}C.

All commercial reagents were used as obtained without further purification. Amino functionalized polystyrene particles were commercially available. $Pd(AsPh_3)_4$[14] and dithieno[3,2-*b*;2′,3′-*d*]-thiophene (**1**)[6,25] were prepared according to literature procedures.

4-Bromo-1-dithieno[3,2-*b*;2′,3′-*d*]thiophen-2-ylbutan-1-one (2). 4-Bromobutyryl chloride (2.35 mmol, 0.27 mL) was added to a solution of aluminum chloride (2.82 mmol, 375 mg) in 20 mL of dichloromethane at 0 °C. The mixture was stirred for 1 h and then was added dropwise to a solution of dithieno[3,2-*b*;2′,3′-*d*]thiophene (**1**) (2.35 mmol, 460 mg) dissolved in 25 mL of dichloromethane at 0 °C. The mixture was stirred overnight at room temperature before being quenched with a solution of 0.1 M hydrochloric acid. The product was extracted with ether and dichloromethane, and the combined organic layers were dried over sodium sulfate. The solvent was removed by rotary evaporation. The residue was washed several times with warm hexane yielding 649 mg (80%) of the title product as a pale green powder: mp 104 °C; MS *m*/*e* 346 ($M^{\bullet+}$); FTIR (neat) ν_{CO} 1649 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.957 (s, 1H), 7.525 (d, ^3J = 5.2 Hz, 1H), 7.322 (d, ^3J = 5.2 Hz, 1H), 3.559 (t, ^3J = 6.4 Hz, 2H), 3.162 (t, ^3J = 6.8 Hz, 2H), 2.345 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 191.8, 145.2, 143.7, 141.2, 137.2, 130.8, 129.2, 125.9, 120.9, 36.8, 33.4, 27.2.

4-Bromo-1-(4,4-dioxo-dithieno[3,2-*b*;2′,3′-*d*]thiophen-2-yl)butan-1-one (3). A solution of 3-chloroperbenzoic acid (0.867 mmol, 194.4 mg) in 5 mL of dichloromethane, previously dried over magnesium sulfate, was added dropwise to a solution of 4-bromo-1-dithieno[3,2-*b*;2′,3′-*d*]thiophen-2-ylbutan-1-one (**2**) (0.289 mmol, 0.1 g) in 10 mL of dichloromethane. The mixture was stirred at room temperature overnight, washed sequentially with water, 10% aqueous KOH, and 10% aqueous $NaHCO_3$, and extracted with dichloromethane. The organic layer was dried over sodium sulfate, and no further purification was necessary to yield 76 mg (70%) of a pale orange powder. Anal. Calcd for $C_{12}H_9BrO_3S_3$: C, 38.2; H, 2.4. Found: C, 38.5; H, 2.43. Mp 187 °C; MS *m*/*e* 378 ($M^{\bullet+}$); FTIR (neat) ν_{SO_2} 1303, 1141 cm^{-1}, ν_{CO} 1646 cm^{-1}; λ_{max} (CH_2Cl_2) 374 nm; λ_{em} (CH_2Cl_2) 454 nm; ϵ (CH_2Cl_2) 14 000 $M^{-1}\cdot cm^{-1}$; ^{1}H NMR ($CDCl_3$) δ 7.788 (s, 1H), 7.534 (d, ^3J = 5.2 Hz, 1H), 7.296 (d, ^3J = 5.2 Hz, 1H), 3.536 (t, ^3J = 6.0 Hz, 2H), 3.117(t, ^3J = 6.8 Hz, 2H), 2.318 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 191.1, 147.5, 145.0, 143.2, 141.9, 135.1, 132.4, 123.6, 120.7, 36.726, 32.9, 26.6.

4-Bromo-1-(4,4-dioxo-6-bromodithieno[3,2-*b*;2′,3′-*d*]thiophen-2-yl)-butan-1-one (4). Under exclusion of light, *N*-bromosuccinimide (0.39 mmol, 69.5 mg) was added to a solution of 4-bromo-1-(4,4-dioxo-dithieno[3,2-*b*;2′,3′-*d*]thiophen-2-yl)butan-1-one (**3**) (0.13 mmol, 49 mg) dissolved in 4 mL of a 1:1 mixture of dichloromethane:acetic acid at −20 °C. The mixture was allowed to warm to room temperature and stirred overnight before being quenched with water. The aqueous layer was extracted with dichloromethane, and the combined organic layers were washed with 10% aqueous KOH and 10% aqueous $NaHCO_3$. After drying over sodium sulfate, the solvent was evaporated and no further purification was necessary to obtain 48.8 mg of the desired product (82% yield) as a yellow solid: Mp 201 °C; MS *m*/*e* 456 ($M^{\bullet+}$); λ_{max} (CH_2Cl_2) 385 nm; FTIR (neat) ν_{SO_2} 1311, 1156 cm^{-1}, ν_{CO} 1651 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.777 (s, 1H), 7.292 (s, 1H), 3.526 (t, ^3J = 6.4 Hz, 2H), 3.106 (t, ^3J = 6.8 Hz, 2H), 2.311 (m, 2H);

Tributyl(5-octylthiothiophen-2-yl)stannane (5). To a solution of *t*-BuOK (1.81 g, 0.0162 mol) in ethyl alcohol (5 mL) was added dropwise a solution of 2-thienyl hydrosulfide (1.252 g, 0.0108 mol) in CH_2Cl_2 (2 mL) at 0 °C. After 30 min, 1.86 mL (0.0108 mol) of 1-bromooctane was added. The reaction mixture was then refluxed for 2 h, treated with water (3 × 10 mL), extracted with CH_2Cl_2 (2 × 10 mL), dried, and evaporated. The crude product was chromatographed on a silica gel column with hexane/CH_2Cl_2 (9:1) as eluent to give 2-octylthiothiophene as a colorless oil (750 mg, 60% yield): MS *m*/*e* 228 ($M^{\bullet+}$); ^{1}H NMR ($CDCl_3$) δ 7.313 (dd, ^4J = 1.2 Hz, ^3J = 5.2 Hz, 1H), 7.090 (dd, ^4J = 1.2 Hz, ^3J = 3.6 Hz, 1H) 6.958 (dd, ^3J = 3.6 Hz, ^3J = 5.6 Hz, 1H), 2.774 (t, ^3J = 7.6 Hz, 2H), 1.592 (m, 2H), 1.301 (m, 10H), 0.866 (m, 3H); ^{13}C NMR ($CDCl_3$) δ 134.9, 133.2, 128.8, 127.4, 38.9, 31.8, 29.4, 29.14, 29.08, 28.4, 22.6, 14.1.

To a solution of 2-(octylthio)thiophene (2.19 mmol, 500 mg) in 15 mL of dry ether at −20 °C was added a 2.5 M solution of BuLi in hexane (2.30 mmol, 0.92 mL). After 1 h, 785 mg (2.41 mmol) of tributyltin chloride was added dropwise. The reaction was stirred overnight, hydrolyzed with water, extracted with ether, dried over Na_2SO_4, and evaporated. A total of 954 mg (84% yield) as a colorless oil was isolated: MS *m*/*e* 517 ($M^{\bullet+}$); ^{1}H NMR ($CDCl_3$) δ 7.175 (d, ^3J = 3.6 Hz, 1H,), 7.024 (d, ^3J = 3.2 Hz, 1H), 2.803 (t, ^3J = 7.2 Hz, 2H), 1.576 (m, 8H), 1.330 (m, 16H), 1.089 (m, 6H), 0.892 (m, 12H); ^{13}C NMR ($CDCl_3$) δ 141.6, 139.8, 135.6, 133.5, 38.8, 31.8, 29.4, 29.153, 29.1, 28.9, 28.5, 27.2, 22.6, 14.1, 13.6, 10.8.

4-Bromo-1-(4,4-dioxo-6-(5′-octylthiothiophen-2′-yl)dithieno-[3,2-*b*;2′,3′-*d*]thiophen-2-yl)butan-1-one (6). To a 2 mL toluene solution containing 0.0027 mmol $Pd(Ph_3As)_4$ formed in situ[14] was added 4-bromo-1-(4,4-dioxo-6-bromodithieno[3,2-*b*;2′,3′-*d*]thiophen-2-yl)butan-1-one (**4**) (0.055 mmol, 25 mg) in 3 mL of toluene. The mixture was warmed to reflux, and then tributyl(5-octylthiothiophen-2-yl)stannane (**5**) (0.060 mmol, 31.28 mg) was added dropwise. After this addition, the reflux was continued for 2 h. The solvent was removed by rotary evaporation, and the remaining residue was washed several times with warm pentane. The residue was purified by flash chromatography (aluminum oxide; cyclohexane/dichloromethane/ethyl acetate 8:1:1) to afford 23.4 mg (71% yield) of the title product as a green solid: Anal. Calcd for $C_{24}H_{27}BrO_3S_5$: C, 47.8; H, 4.5. Found: C, 48.1; H, 4.53. Mp 155 °C; MS *m*/*e* 604 ($M^{\bullet+}$); FTIR (neat) ν_{SO_2} 1306, 1140 cm^{-1}, ν_{CO} 1654 cm^{-1}; λ_{max} (CH_2Cl_2) 440 nm; λ_{em} (CH_2Cl_2) 600 nm; ϵ (CH_2Cl_2) 14187 $M^{-1}\cdot cm^{-1}$; ^{1}H NMR ($CDCl_3$) δ 7.777 (s, 1H), 7.239 (s, 1H), 7.128 (d, ^3J = 4.0 Hz, 1H), 7.023 (d, ^3J = 3.6 Hz, 1H), 3.533 (t,

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^3J = 6.8 Hz, 2H), 3.109 (t, ^3J = 7.6 Hz, 2H), 2.867 (m, 2H), 2.326 (m, 2H), 1.427 (m, 2H), 1.269 (br s, 10H), 0.875 (t, 6.4 Hz, 3H).

2-(4-Bromobutyl)dithieno[3,2-*b*;2′,3′-*d*]thiophene (7). 4-Bromobutyryl chloride (0.0056 mol, 0.65 mL) was added to a solution of aluminum chloride (0.0067 mol, 0.90 g) in 30 mL of dichloromethane at 0 °C. The mixture was stirred for 30 min and then was added dropwise to a solution of dithieno[3,2-*b*;2′,3′-*d*]thiophene (**1**) (0.0051 mol, 1.00 g) dissolved in 20 mL of dichloromethane at 0 °C. The mixture was stirred overnight at room temperature. This mixture was then added dropwise to a solution of aluminum chloride (0.015 mol, 2.04 g) and borane−*tert*-butylamine (0.031 mol, 2.66 g) in 30 mL of dichloromethane at 0 °C. The final solution was allowed to warm to room temperature and stirred for 4 h before quenching with a solution of 0.1 M hydrochloric acid. The product was extracted with diethyl ether and dichloromethane, and the combined organic layers were dried over sodium sulfate. The solvent was evaporated. The residue was purified by flash chromatography (aluminum oxide; pentane/dichloromethane 9:1) yielding 1.20 g (71%) of the title product as a pale yellow oil: MS *m*/*e* 332 ($M^{\bullet+}$); ^{1}H NMR ($CDCl_3$) δ 7.308 (d, ^3J = 4.8 Hz, 1H), 7.265 (d, ^3J = 4.8 Hz, 1H), 6.991 (m, 1H), 3.442 (t, ^3J = 6.4 Hz, 2H), 2.946 (t, ^3J = 7.2 Hz, 2H), 1.931 (m, 4H); ^{13}C NMR ($CDCl_3$) δ 145.8, 140.7, 140.2, 131.1, 128.9, 125.1, 120.7, 117.9, 33.3, 31.7, 30.2, 29.9.

2-(4-Bromobutyl)dithieno[3,2-*b*;2′,3′-*d*]thiophene 4,4-Dioxide (8). A solution of 3-chloroperbenzoic acid (5.438 mmol, 1.22 g) in 10 mL of dichloromethane, previously dried over magnesium sulfate, was added dropwise to a solution of 2-(4-bromobutyl)-dithieno[3,2-*b*;2′,3′-*d*]thiophene (**7**) (1.807 mmol, 0.6 g) in 25 mL of dichloromethane. The mixture was stirred at room temperature overnight before being washed sequentially with water, 10% aqueous KOH, and 10% aqueous $NaHCO_3$ and extracted with dichloromethane. The organic layer was dried over sodium sulfate, and the solvent was purified by flash chromatography (aluminum oxide; diethyl ether/dichloromethane/ethyl acetate 6:1:3) yielding 436 mg (66%) of a yellow solid: Anal. Calcd for $C_{12}H_{11}BrO_2S_3$: C, 39.7; H, 3.1. Found: C, 39.95; H, 3.39. Mp 110 °C; MS *m*/*e* 364 ($M^{\bullet+}$); FTIR (neat) ν_{SO_2} 1307, 1139 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.306 (d, ^3J = 5.2 Hz, 1H), 7.197 (d, ^3J = 5.2 Hz, 1H), 6.928 (m, 1H), 3.434 (t, ^3J = 6.4 Hz, 2H), 2.871 (t, ^3J = 6.4 Hz, 2H), 1.902 (m, 4H); ^{13}C NMR ($CDCl_3$) δ 150.6, 142.6, 142.1, 136.5, 133.4, 128.9, 120.2, 117.2, 32.9, 31.6, 29.7.

2-(4-Bromobutyl)-6-bromodithieno[3,2-*b*;2′,3′-*d*]thiophene 4,4-Dioxide (9). Under exclusion of light, *N*-bromosuccinimide (0.493 mmol, 87.7 mg) was added in small amounts over 10 min to a solution of 2-(4-bromobutyl)dithieno[3,2-*b*;2′,3′-*d*]thiophene 4,4-dioxide (**8**) (0.448 mmol, 163 mg) dissolved in 20 mL of a 1:1 mixture of dichloromethane:acetic acid at −20 °C. The mixture was allowed to warm to room temperature and stirred overnight before being quenched with water. The aqueous layer was extracted with dichloromethane, and the combined organic layers were washed with a 10% aqueous solution of KOH and a 10% aqueous solution of $NaHCO_3$. After drying over sodium sulfate, the solvent was evaporated and the remaining residue was crystallized from toluene/pentane giving 188 mg of the desired product (95% yield) as a dusty yellow solid: mp 165 °C; MS *m*/*e* 442 ($M^{\bullet+}$); ^{1}H NMR ($CDCl_3$) δ 7.196 (s, 1H), 6.930 (m, 1H), 3.434 (t, ^3J = 6.4 Hz, 2H), 2.868 (t, ^3J = 8.0 Hz, 2H), 1.903 (m, 4H); ^{13}C NMR ($CDCl_3$) δ 151.1, 141.4, 141.0, 136.3, 132.8, 122.6, 117.2, 115.8, 32.8, 31.5, 29.8, 29.7.

2-(4-Bromobutyl)-6-(5-octylthiothiophen-2-yl)dithieno[3,2-*b*;2′,3′-*d*]thiophene 4,4-Dioxide (10). To a flame-dried flask charged with a 5 mL toluene solution containing 0.011 mmol of $Pd(Ph_3As)_4$ formed in situ[14] was added 2-(4-bromobutyl)-6-bromodithieno[3,2-*b*;2′,3′-*d*]thiophene 4,4-dioxide (**9**) (0.424 mmol, 188 mg). The mixture was then heated to reflux, and tributyl(5-octylthiothiophen-2-yl)stannane (**5**) (0.488 mmol; 252 mg) was slowly added by syringe. After this addition the reflux was continued for 5 h, then the solvent was removed by rotary evaporation, and the residue was purified by column chromatography (silica gel; pentane/ethyl acetate/dichloromethane 6:3:1) and then recrystallized from isopropanol giving 194 mg (yield 78%) of orange microcrystals: Anal. Calcd for $C_{24}H_{29}BrO_2S_5$: C, 48.9; H, 5.0. Found: C, 49.11; H, 5.25. Mp 98 °C; MS *m*/*e* 590 ($M^{\bullet+}$); FTIR (neat) ν_{SO_2} 1302, 1134 cm^{-1}; λ_{max} (CH_2Cl_2) 418 nm; λ_{em} (CH_2Cl_2) 546 nm; ^{1}H NMR ($CDCl_3$) δ 7.172 (s, 1H), 7.049 (d, ^3J = 3.6 Hz, 1H), 7.002 (d, ^3J = 3.6 Hz, 1H), 6.934 (t, ^4J = 0.8 Hz, 1H), 3.438 (t, ^3J = 6.4 Hz, 2H), 2.859 (m, 4H), 1.907 (m, 4H), 1.641 (m, 2H), 1.399 (m, 2H), 1.267 (br s, 8H), 0.875 (t, 6.8 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 150.8, 142.5, 141.8, 141.2, 137.5, 137.2, 133.8, 133.5, 133.4, 125.1, 117.3, 115.6, 38.8, 32.9, 31.7, 31.6, 29.8, 29.7, 29.4, 29.13, 29.06, 28.4, 22.6, 14.1.

General Procedure for the Labeling of Particles. Dyes were dissolved in dry DMSO. The microspheres (0.5 mL, 5 wt % solids with 63 μequiv/g amino groups) were washed several times with Millipore water, suspended in dry DMSO, and sonicated for at least 5 min before use. The dye was slowly added dropwise at microspheres suspension at room temperature. After gentle shaking for 2 h at room temperature, the samples were filtered through a 0.45 μm filter syringe and washed with DMSO and copious amounts of Millipore water. The particles were redispersed in PBS buffer and before microscopic examination the samples were vortexed for 2 min, sonicated for 5 min, and vortexed again to disperse the microspheres.

Bioconjugation Methods. Monoclonal antibodies anti-CD3 were first concentrated via ultrafiltration under nitrogen on a micrometer membrane with a 10 kDa cutoff. They were then transferred to a 0.05 M carbonate buffer solution containing 0.05% Tween 20 (pH 9.5) with use of a buffer exchange column of Sephadex G25 equilibrated with 5 bed volumes of the same buffer. Fractions of 0.5 mL were collected. To these solutionswere added aliquots of dye **13** dissolved in DMSO (concentration 10 mg/mL) in the amount needed to reach the desired protein:fluorophore molar ratios. The solutions were incubated for 3 h at room temperature with stirring. Finally, the conjugates were chromatographed on a desalting 1.0 mL GH25 column in PBS (pH 7.4).

Absorption and Photoluminescence Measurements. Absorption and photoluminescence spectra were recorded with use of 10^{-5}−10^{-6} M solutions in CH_2Cl_2 or aqueous solvents (absorbance 0.1−0.2). The excitation wavelengths were those of the maximum absorption wavelengths of the fluorophores.

Photostability of the fluorescent microspheres was tested with a fluorescence microscope fitted with a 100 W mercury lamp. Fluorescence of microspheres tagged with fluorescein and dyes **12** and **15** was monitored by using 455 ± 35 excitation and 515 barrier bandpass filters and a 500 long-pass dichroic. Fluorescence of microspheres tagged with dyes **11** and **13** was monitored by using 330/380 excitation and 420 barrier bandpass filters and a 400 long-pass dichroic.

Acknowledgment. We thank Drs. Fabio Aruffo and Mario Benzi for the synthesis of some of the compounds described in this work. We are grateful to Dr. Alfredo Ventola for the preparation of the antiCD3-13 conjugate. We also thank Mr. Marco Ballestri, ISOF-CNR, for very helpful discussions about the microspheres conjugation. This work was partially funded by the project FIRB RBNE03S7XZ_005 (SYNERGY).

Supporting Information Available: ^{1}H and ^{13}C NMR spectral data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

JO070488N