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Fluorescence resonance energy transfer terminators for DNA sequencing

Satyam Nampalli, Mahesh Khot and Shiv Kumar*

Nucleic Acid Chemistry, Amersham Pharmacia Biotech, 800 Centennial Avenue, Piscataway, NJ 08855, USA

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Abstract

A four-colour set of fluorescence resonance energy transfer dideoxy nucleotide terminators (**10**–**13**), have been synthesised using a rigid and linear tri-functional molecule (**2**), synthesised via Heck coupling reaction of *t*-Boc-L-4-iodophenylalanine (**1**) with *N*-TFA-propargylamine. Evaluation of the terminators in DNA sequencing reactions, in combination with Thermo Sequenase™ II DNA polymerase, demonstrated them to be excellent reagents for high-throughput DNA sequencing.

Development of new DNA polymerases[1] and fluorescence resonance energy transfer (FRET) dyes have helped improve dideoxy terminator DNA sequencing.[2] FRET dyes comprised of a common donor dye and different acceptor dyes are superior to single-dye-labels because they generate well-separated, enhanced fluorescence signals for the four kinds of DNA sequencing fragments to be detected using a single laser for analysis.[3] To date, there are a number of examples of energy transfer in DNA sequencing either by using fluorescence energy transfer dye-labelled primers[4] or terminators[5] with various linkers separating the donor and acceptor dyes. This work was directed towards finding FRET labelled terminators with improved brightness, sequence read-length, accuracy, and reactivity with Thermo Sequenase™ II DNA polymerase.

As part of the research program directed towards developing novel FRET dideoxy nucleotide terminators and DNA polymerases for high-throughput DNA sequencing, we have undertaken a novel synthetic approach to arrive at a four-colour set of fluorescent dye-labelled terminators. The objective was the design and synthesis of four FRET dye-labelled cassettes, which could be coupled with alkynylaminodideoxynucleoside-5′-triphosphates or other biological molecules of interest. For this purpose, the FRET cassettes **6**–**9** (Scheme 1) with a rigid and yet linear linker were envisaged to be synthesised from a common donor dye cassette **5**, derived from a

* Corresponding author. Fax: 732-457-8353; e-mail: shiv.kumar@am.apbiotech.com

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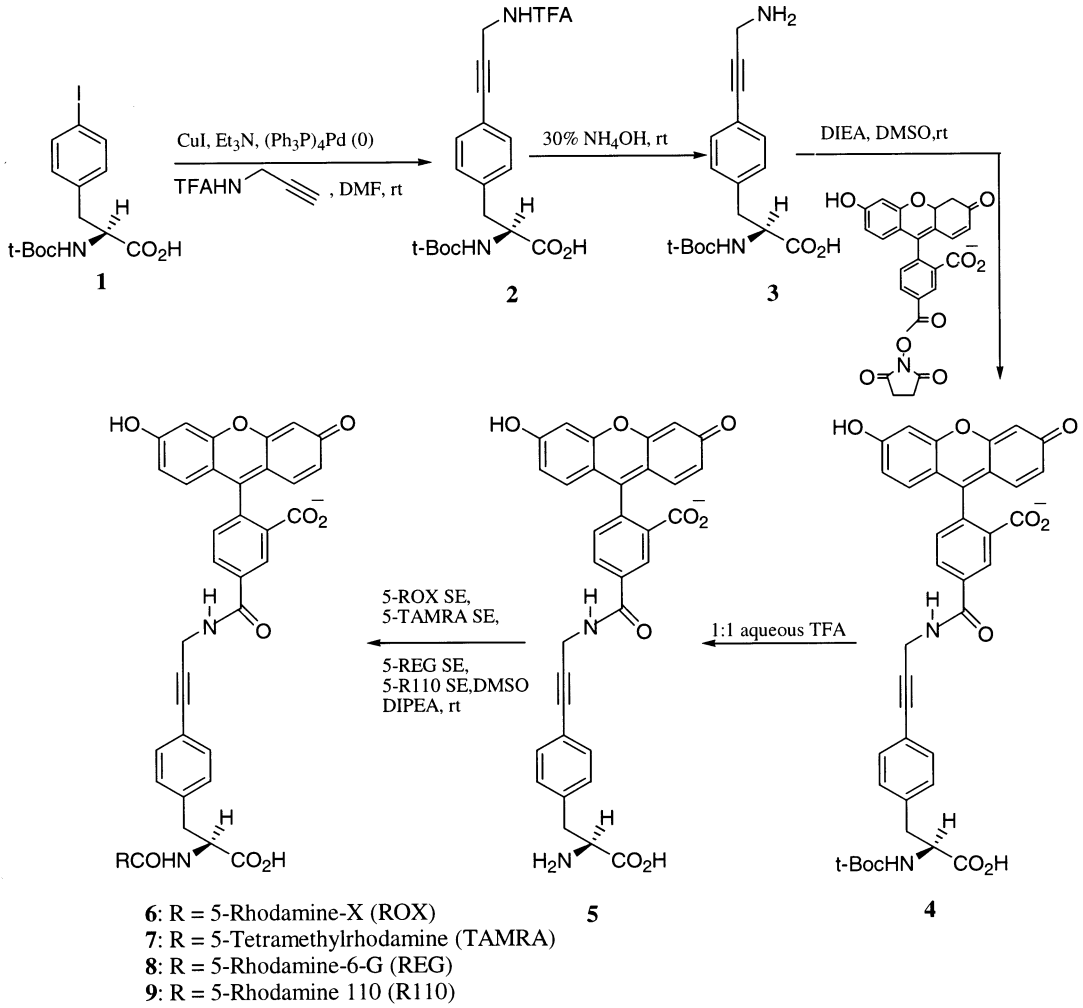
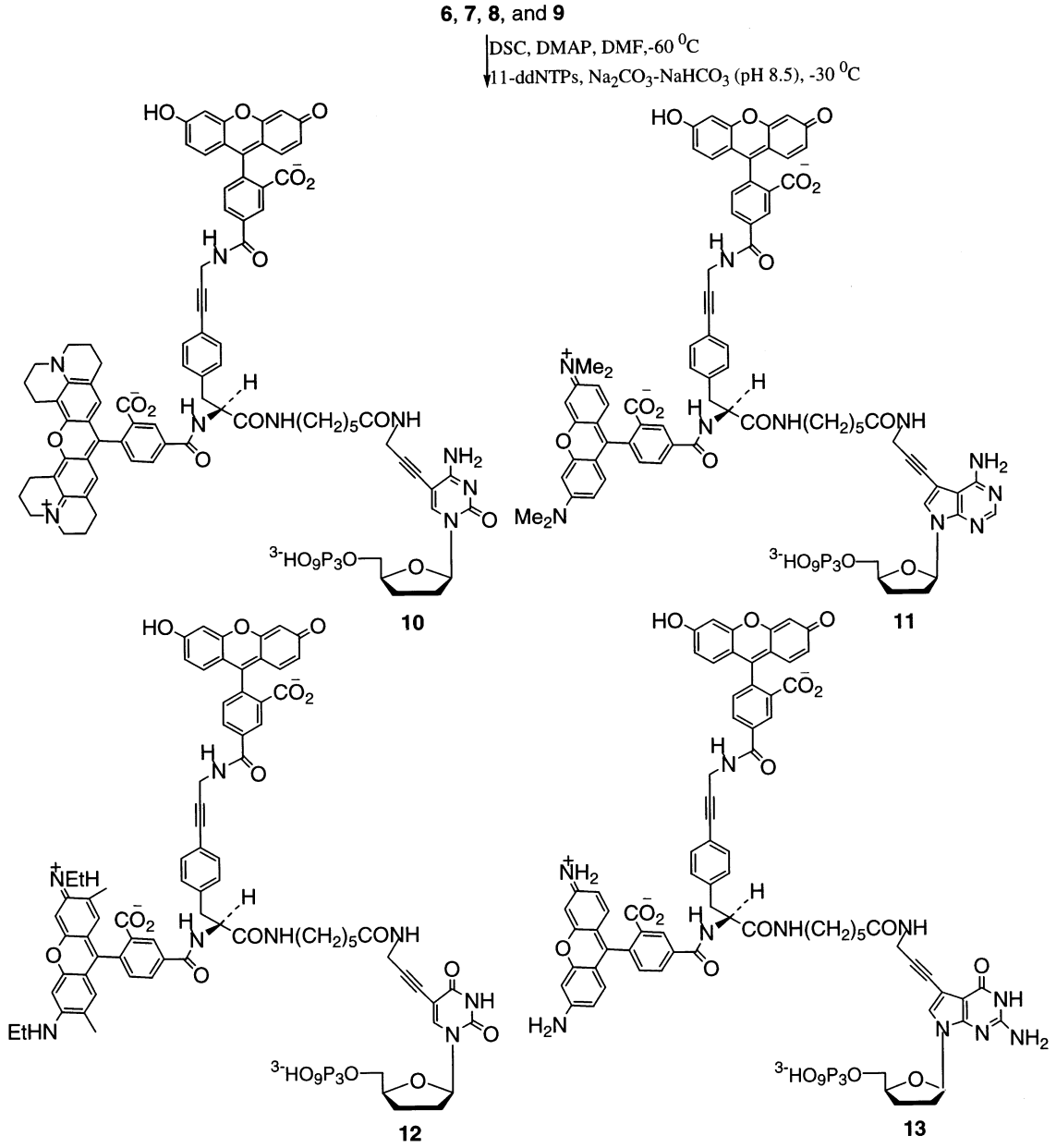
tri-functional molecule, *t*-Boc-L-4-iodophenylalanine **1** via a Heck coupling reaction with *N*-TFA-propargylamine.



Scheme 1.

Thus, a convenient Heck[6] coupling reaction of **1** with *N*-TFA-propargylamine using $(Ph_3P)_4Pd(0)/CuI/Et_3N/DMF$ afforded the rigid-linear cassette linker **2** in 87% yield. The *para*-propargylamino, *t*-Boc-α-amino, and the free carboxylic acid functional groups in **2** were preconceived to be conjugated sequentially with a donor dye, acceptor rhodamine dyes, and ddNTPs, respectively. Removal of the TFA protecting group with 30% NH_4OH gave compound **3** (quantitative yield), which upon conjugation with 5-carboxyfluorescein succinimidylester (5-FAM, SE) in anhydrous DMSO in the presence of DIPEA provided 5-FAM-PAPhe (propargylamino phenylalanine) cassette **4** in 88% yield. Removal of *t*-Boc protecting group by treating with ice-cold 1:1 aqueous TFA at rt afforded the TFA salt of **5** in 88% yield,[7] ready for preparing energy transfer cassettes. The very first energy transfer cassette synthesised was 5-FAM-PAPhe-ROX **6** (81%), resulting from the conjugation of FAM-cassette **5** with 5-carboxy-X-rhodamine succinimidyl ester (5-ROX, SE)/DIPEA in anhydrous DMSO. This ET

cassette **6** when excited at 488 nm, showed four times enhanced ROX emission to that of single ROX dye. In order to have a four-colour set, FAM cassette **5** was conjugated independently with 5-TAMRA (5-carboxytetramethylrhodamine), 5-REG (5-carboxyrhodamine 6G) and 5-R110 (5-rhodamine green carboxylic acid, trifluoroacetamide) NHS esters to provide 5-FAM–PAPhe–TAMRA **7** (78%), 5-FAM–PAPhe–REG **8** (84%), and 5-FAM–PAPhe–R110 **9** (69%) ET cassettes, respectively.



Scheme 2.

MM+/MO calculations showed that the donor and the acceptor dyes in these ET cassettes have spatial orientations parallel to each other, which should be optimal for FRET.[8] ^{1}H NMR,[9] UV–vis, and TOF MS,[10] data confirmed the molecular structures of these ET cassettes. The ET linker, *para*-propargylamino phenylalanine **2**, has the versatility to be conjugated not only with widely used fluorescein and rhodamine dyes, but also with cyanines or any other fluorescent tags. It is also worth mentioning that these ET cassettes have a great potential to label modified primers, should one choose to do primer DNA sequencing.

Initial attempts to conjugate activated ET cassettes with alkynylamino 11-ddNTPs[11,12] (Scheme 2) using a large variety of activating reagents[13] such as TSTU/DIPEA, DCC/HOSu, HBTU, HATU, HOBt, PyBOP, PyBrOP, EDAC/HOSu, and TFA–HOSu, at different temperatures produced low yields of the desired ET terminators. However, using the optimised DSC/DMAP/DMF/−60°C conditions, we could convert **6**, **7**, **8**, and **9** to the corresponding NHS esters and in situ conjugate with 11-ddNTPs (11-ddCTP, 11-ddATP, 11-ddUTP, and 11-ddGTP) at −30°C to provide the desired ET dye terminators[14,15] **10**, **11**, **12**, and **13**, respectively, in yields ranging from 15–20%. The normalised fluorescence emission spectra for these ET terminators are given in Fig. 1. The fluorescence emission enhancement rates of these ET terminators compared to the corresponding single dye terminators were found to be 18 (ROX), 6.5 (TAMRA), 5 (REG), and 1.6 (R110).

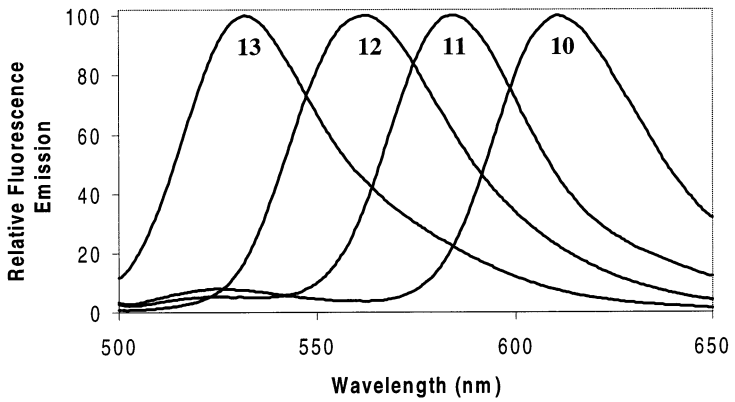


Figure 1. Argon-laser (488 nm) excited, normalised fluorescence emissions of the four-colour set: **10**–**13** in 1 X TBE (tris-boric acid EDTA) 8.0 M urea

Of the 64 possible combinations of ET terminators (8 carboxyrhodamine 5/6 regio-isomers×2 caboxyfluorescein 5/6 regio-isomers×4 alkynylamino 11-ddNTPs), 12 were synthesised and tested in the DNA sequencing experiments. DNA sequencing performed, employing the four-colour set of **10**–**13** with a variety of DNA templates and Thermo Sequenase™ II DNA polymerase, were found to be of high quality with read-lengths in excess of 800 bases.

In conclusion, a novel synthetic route for the four-colour set of FRET terminators has been developed involving a rigid-linear ET cassette linker chemistry, and formulated the set into a robust DNA sequencing kit with Thermo Sequenase II. Selection process for the best four-colour set, energy transfer efficiencies of the ET cassettes, and DNA sequencing results will be published elsewhere.

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7. ^{1}H NMR of the common single dye cassette **5** (DMSO-d_6): δ 2.84 (2H, q, $J=6$, 15 Hz, benzylic), 3.11 (2H, dd, $J=3$, 18 Hz, propargylic), 4.36 (1H, s, chiral), 6.50 (4H, m, aromatic), 6.66 (2H, s, aromatic), 7.25 (2H, d, $J=9.0$ Hz, aromatic), 7.34 (2H, d, $J=9.0$ Hz, aromatic), 7.37 (1H, d, $J=6$ Hz, aromatic), 8.25 (1H, d, $J=6.0$ Hz, aromatic), 8.43 (1H, s, aromatic), 9.34 (1H, m, NH-).
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9. ^{1}H NMR of **6** (DMSO-d_6): δ 1.72 (8H, br s, ROX-dye rings′ homo-benzylic), 1.92 (8H, br s, ROX-dye rings′ benzylic), 2.83 (8H, br s, ROX-dye rings′ methylenes adjacent N), 3.05 (2H, m, benzylic), 4.32 (2H, br s, propargylic), 4.44 (1H, m, chiral), 6.49 (4H, m, aromatic), 6.62 (2H, s, aromatic), 7.16 (1H, d, $J=9.0$ Hz, aromatic), 7.28 (5H, m, aromatic), 7.96 (1H, d, $J=6.0$ Hz, aromatic), 8.25 (1H, d, $J=6.0$, aromatic), 8.34 (1H, s, aromatic), 8.46 (1H, s, aromatic). Compounds **7**, **8** and **9** showed satisfactory ^{1}H NMR data.
10. TOF MS ES m/z, cone 50v, 50% CH_3CN/H_2O: compound **6**: 1089.66 (MH-3); **7**: 985.83 (MH-3); **8**: 1013.89 (MH-3); **9**: 931.23 (MH-2).
11. Hobbs Jr., F. W.; Cocuzza, A. J. U.S. Patent 5 047 519, 1991.
12. The number 11 preceding the ddNTPs (dideoxynucleoside triphosphates), indicates the number of atoms in the linker arm attached at 5-position in pyrimidines and 7 in 7-deazapurines.
13. Glossary of terms; TSTU (*O*-(*N*-succinimidyl)-1,1,3,3-tetramethyluronium tetrafluoroborate), DIPEA (*N*,*N*-diisopropylethylamine), DCC (*N*,*N*′-dicyclohexylcarbodiimide), HOSu (*N*-hydoxysuccinimide), HBTU (2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate), HATU (2-(1H-azobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate), HOBt (*N*-hydroxybenzotriazole), PyBOP (benzotraizole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate), PyBroP (bromo-tris-pyrrolidino-phosphonium hexafluorophosphate), EDAC (1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide. HCl, TFA-HOSu (*N*-trifluoacetylhydroxysuccinimide), DMAP (4-*N*,*N*-dimethylaminopyridine).
14. Typical experimental procedure: To a stirred mixture of ET cassette 8 (100 mg), 91 (mol) and *N*,*N*′-disuccinimidyl carbonate (453 μmol, 5 equiv.) in anhydrous DMF (10 mL), was added anhydrous DMF solution of DMAP (454 μmol, 5 equiv.) at −60°C. TLC monitoring of the reaction within 5 min indicated complete conversion to the NHS ester, which, without isolation was treated with 0.1 M Na_2CO_3–$NaHCO_3$ buffer solution of 11-ddATP (74 μmol, 0.8 equiv.) at −30°C and stirred at rt for 1 h. The reaction mixture was subjected to a silica gel column (6 iso-PrOH:3 NH_4OH:1 H_2O) purification followed by Q-Sepharose FPLC (buffer A: 40% CH_3CN/0.1 M TEAB pH 7.5; buffer B: 40% CH_3CN/1.0 M TEAB pH 7.5) and C18 reversed-phase HPLC (buffer C: 0.1 M TEAB, pH 7.0; buffer D: 100% CH_3CN) to yield **11** (20%) as a pink fluffy solid. Similarly, compounds **10**, **12** and **13** were obtained in 15–20% yields.
15. TOF MS ES m/z, cone 100v, 20% CH_3CN/H_2O:ET terminator **10**: 1687.79 (MH-4); **11**: 1606.69 (MH-4); **12**: 1612.49 (M-4); **13**: 1569.23 (MH-1).