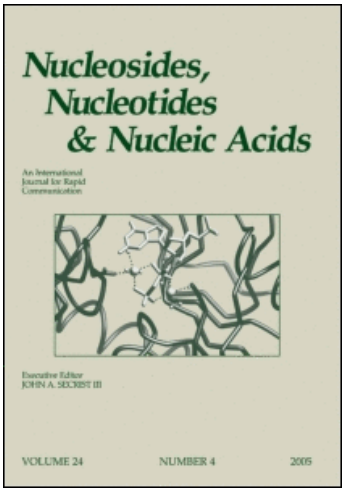

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FLUORESCENCE RESONANCE ENERGY TRANSFER DYE NUCLEOTIDE TERMINATORS: A NEW SYNTHETIC APPROACH FOR HIGH-THROUGHOUT DNA SEQUENCING

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ABSTRACT

Fluorescence resonance energy transfer (FRET) based dye-nucleotide terminators (**10**–**13**) were designed, synthesized, and formulated with Thermo Sequenase™ II DNA polymerase into a robust kit for high throughput DNA sequencing. The key energy transfer (ET) rigid and linear linker (**2**), required for the syntheses of energy transfer cassettes (**6**–**9**) was synthesized via Heck coupling reaction on t-Boc-L-4-iodo-phenylalanine (**1**) with N-TFA-propargylamine.

INTRODUCTION

Since the development of dideoxy terminator DNA sequencing (1), there have been new developments and advances in DNA polymerases (2), fluorescence DNA sequencing technology (3), and automated sequencers. Fluorescence resonance energy transfer (FRET) based dye terminator DNA sequencing is the most preferred method over the radio-isotope labeled one (poses severe problems in storage, handling, and health risks) or the single/ET dye-primer sequencing (requires four-lane sequencing). FRET dyes comprising a common donor dye and four different acceptor dyes are superior to single dye labels in DNA sequencing by virtue of

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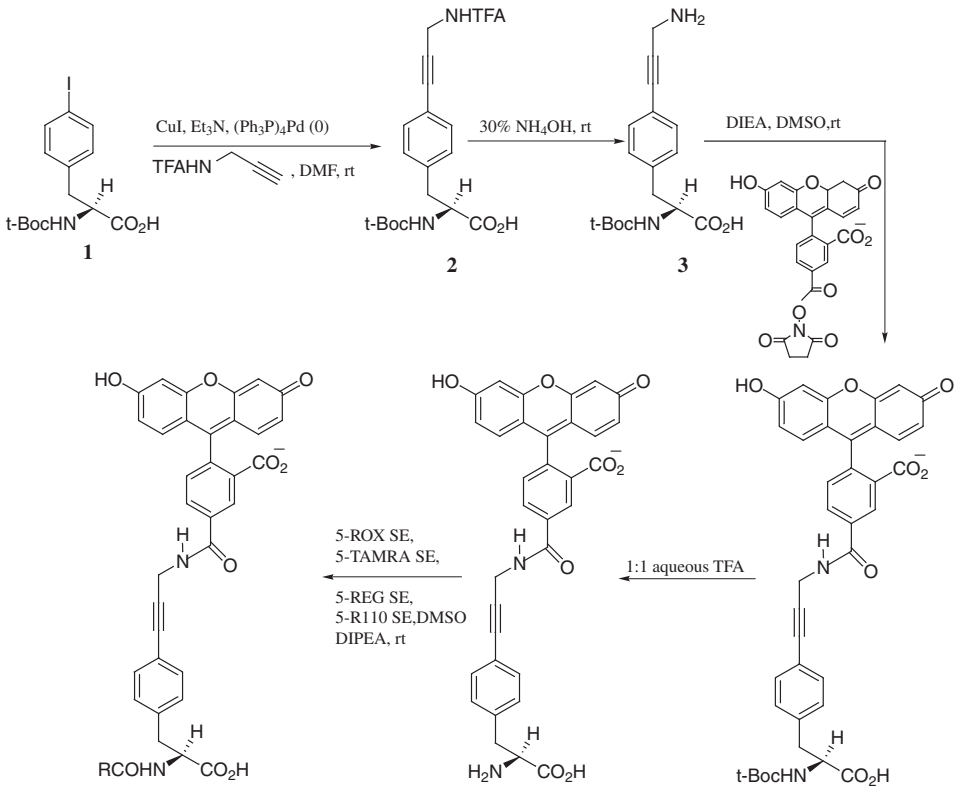
generating, well separated, enhanced fluorescence signals for the detection/analysis of four kinds of DNA sequencing fragments (4). To date, there are a number of examples of FRET DNA sequencing either by using FRET dye-labeled primers (5) or terminators (6) with various ET linkers separating the donor and acceptor dyes. As the Human Genome Project needs high-speed, high throughput sequencing for deciphering the whole genome, further improvements in the sequencing technology are necessary to help expedite the effort.

As part of the research program directed towards developing novel FRET dideoxy nucleotide terminators for high-throughput DNA sequencing with improved brightness, sequence read-length, accuracy, and reactivity with new DNA polymerases, we have undertaken a new synthetic approach (7) to arrive at a four-color set of fluorescent dye-labeled terminators.



1 2 3 4 5

6: R = 5-Rhodamine-X (ROX)
7: R = 5-Tetramethylrhodamine (TAMRA)
8: R = 5-Rhodamine-6-G (REG)
9: R = 5-Rhodamine 110 (R110)

Scheme 1.

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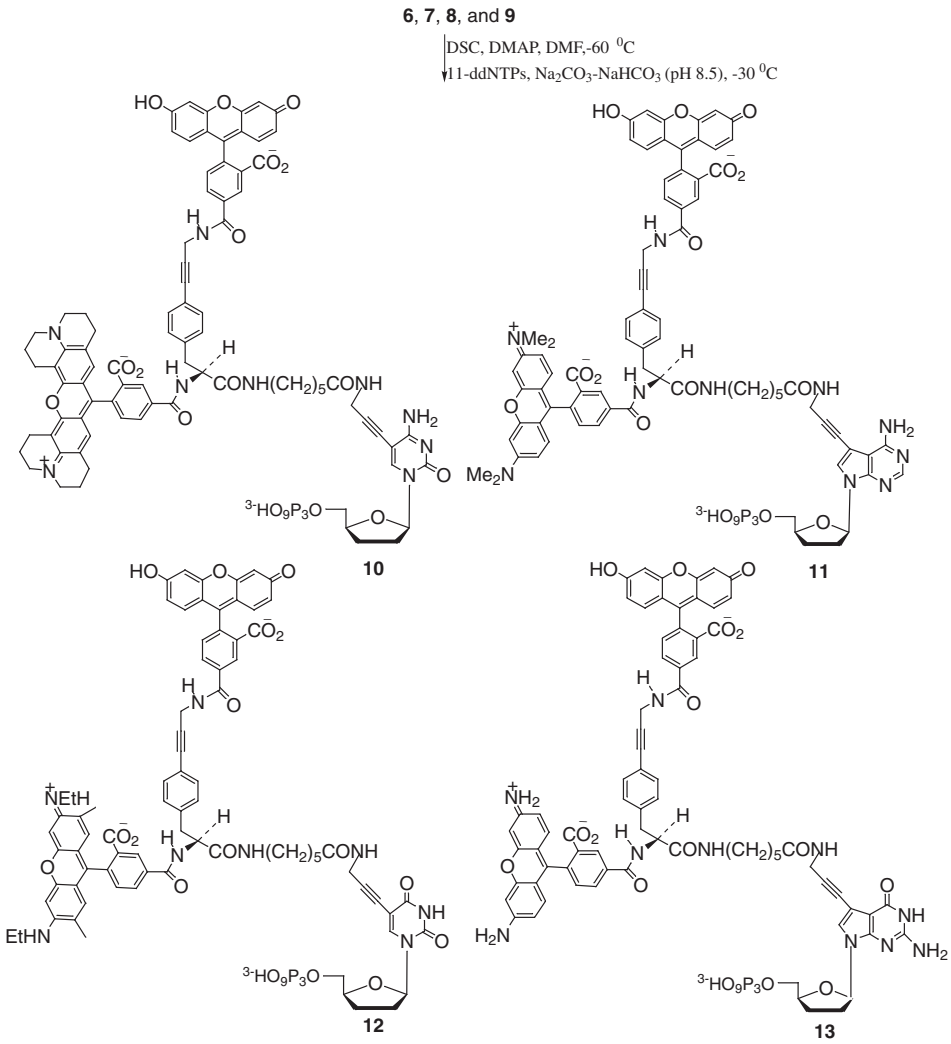
SYNTHESIS AND RESULTS

The primary goal was the design and synthesis of four FRET dye-labeled cassettes, which could be coupled with reactive alkynylamino-dideoxynucleotides or other biological molecules of interest. In order to achieve the set goal, the FRET cassettes **6–9** (Scheme 1) with a rigid and yet linear linker were envisioned to be synthesized from a common donor dye cassette **4**, derived from a tri-functional molecule, t-Boc-L-4-iodophenylalanine **1**. via Heck coupling reaction with N-TFA-propargylamine. Thus, commercially available starting material **1** was



Scheme 2.

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conveniently subjected to the Heck (8) coupling reaction with N-TFA-propargylamine using $(Ph_3P)_4$ Pd (0)/CuI/Et_3N/DMF to provide the rigid-linear cassette linker **2** in 87% yield. The base and acid sensitive protecting groups–TFA of *para*-propargylamine, t-Boc of α-amine–and the free carboxylic acid group in **2** were thought to be exploited in a sequential covalent attachment with a donor carboxyfluorescein, acceptor carboxy-rhodamine NHS esters, and alkynylamino-ddNTPs, respectively. Accordingly, removal of the base sensitiveTFA group in **2** with 30% NH4OH furnished 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 the acid sensitive t-Boc protecting group by treating with ice-cold 1:1 aqueous TFA at rt afforded the TFA salt of **5** (88%), setting the stage for preparing the energy transfer cassettes. The very first energy transfer cassette synthesized 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 4 times enhanced ROX emission to that of single ROX dye. In order to have a four-color set, FAM cassette **5** was conjugated independently with 5-TAMRA, 5-REG and 5-R110 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.

According to the MM^+/MO calculations, the relative spatial orientation of the donor to acceptor dyes in these ET cassettes was shown to be parallel, which should be optimal for FRET (9). ^{1}H-NMR, 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.

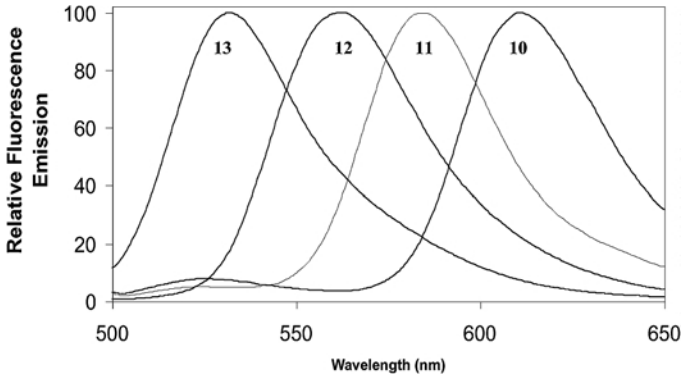

Figure 1. Blue Argon-laser (448 nm) excited, normalized fluorescence emissions of the four-color set **10**–**13** in 1 X TBE 8.0 M urea.

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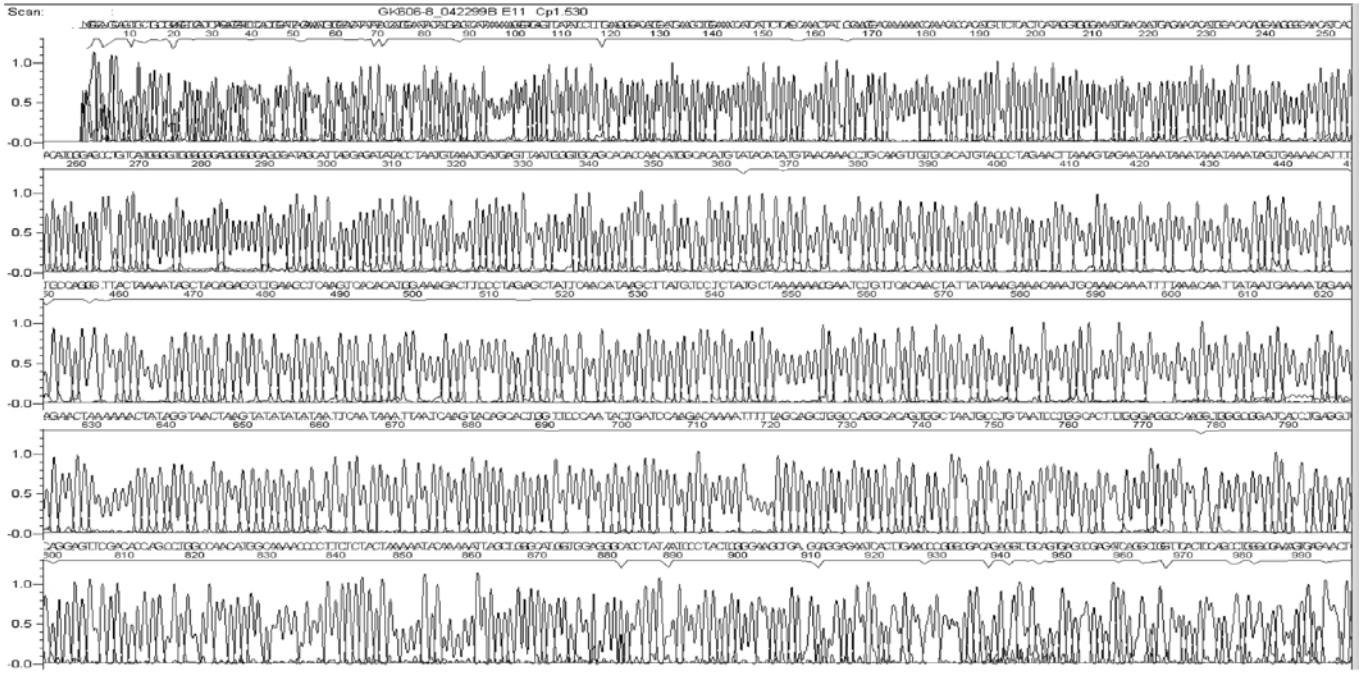

Figure 2. DNA Sequencing results of a plasmid sample using FRET terminators (**10**–**13**) and Thermo Sequenase II DNA polymerase on the MegaBACE sequencer, shows read-lengths in excess of 1000.base-pairs.

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Initial attempts to conjugate activated ET cassettes **6**–**9** (Scheme 2) with alkynylamino 11- ddNTPs (11,12) using a large variety of activating reagents 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, employing DSC/DMAP/DMF/−60°C conditions to generate NHS esters of the acid functionalities in **6**–**9**, and *in situ* conjugation with 11-ddNTPs (11-ddCTP, 11-ddATP, 11-ddUTP, and 11-ddGTP) at −30°C provided the desired ET dye terminators (13) **10**, **11**, **12**, and **13**, respectively, in yields ranging from 15–20%. The normalized fluorescence emission spectra for these ET terminators are given in Figure 1. The fluorescence emission enhancement rates of these ET terminators compared to the corresponding single dye terminators were found to be 18 (**10**), 6.5 (**11**), 5 (**12**), and 1.6 (**13**).

Of the many possible combinations of ET terminators, 6 different four-color sets were synthesized and tested in the DNA sequencing experiments. DNA sequencing performed employing the four-color set of **10**–**13** with a variety of DNA templates and Thermo Sequenase™ II DNA polymerase (14), found to be of high quality with read-lengths in excess of 1000 base-pairs (Fig. 2). The four-color FRET terminators' kit has the demonstrated ability to be easily used on capillary electrophoresis (APB's MegaBACE™ 1000 and ABI's 310 and 3700) and slab gel (ABI's 373 and 377) based automated DNA sequencers. In terms of high throughput sequencing results, using the capillary electrophoresis based MegaBACE (15), one can sequence about 864 DNA samples to identify in excess of 0.5 million base-pairs in 18 hrs.

CONCLUSION

A novel synthetic route for the four-color 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 DNA polymerase. In addition, the FRET cassettes developed in this study have the potential of labeling other reactive biological molecules, such as oligonucleotides and proteins.

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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).
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12. The number 11 preceding the ddNTPs, indicates the number of atoms in the linker arm attached at 5-position in pyrimidines and 7 in 7-deazapurines.
13. 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).
14. Thermo Sequenase is a trade mark of Amersham Pharmacia Biotech.
15. MegaBACE 1000 is a trademark of Molecular Dynamics-Amersham Pharmacia Biotech.

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