A Tetramethyl Rhodamine (Tamra) Phosphoramidite Facilitates Solid-Phase-Supported Synthesis of 5′-Tamra DNA

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5-Carboxy Tamra **1** was conjugated to 4-hydroxypiperidine with BOP and *N*-methylmorpholine, and the resulting 5-(*N*-pipyridyl-4-hydroxy)-Tamra carboxamide **2** was treated with 2-cyanoethyl tetraisopropylphosphorodiamidite to give 5-[*N*-pipyridyl-4-*O*-(2-cyanoethyl diisopropylphosphoramidite)]-Tamra carboxamide **3**. Solutions of **3** were coupled onto the 5′-hydroxyl of solid-phase-supported DNA fragments with standard amidite coupling techniques. Cleavage and deprotection with aqueous *tert*-butylamine cocktail gave 5-Tamra-functionalized DNA as well as an additional compound without the Tamra chromophore. A mass spectrum of this product showed the incorporation of *tert*-butylamine. The extra product was completely suppressed by including a 5 min acetylation step after coupling. A model study of **3** coupled onto thymidine-functionalized CPG showed similar results. NMR and mass spectra of cleaved products confirmed the addition of *tert*-butylamine to the minor product. Coupling a Tamra active ester onto T CPG which was previously coupled with *N*-(4-methoxytrityl)piperidyl-4-*O*-(2-cyanoethyl diisopropylphosphoramidite) **4** produced the same major Tamra-bearing product, which coeluted on reverse phase HPLC with the major product generated with **3**.

Introduction

Development of high performance oligonucleotide labeling methods which are robust enough to work well on automated synthesizers still presents a significant challenge. Attachment of labels to the 5′ terminus of DNA has been traditionally done in solution[1−3] with nitrogen or sulfur nucleophiles pendant on the DNA previously incorporated as 5′ modifications.[4−6] A variety of activation chemistries have been developed to couple labels to these nucleophiles.[7−9] The reactions are generally slow and require chromatography to remove excess labeling reagents from the desired products. The added ease of fluorophore coupling to DNA still anchored on a solid support, as well as obviation of the 5′-amine amidite coupling, has been accomplished in the case of fluorescein[10,11] by the use of fluorescein amidites. Although these compounds have become widely used, the analogous rhodamine compounds have yet to become commercially available. The concept of rhodamine amidites has been put forth in the patent literature;[12] however, little information regarding the synthesis and use of the compounds was divulged. More recently, a paper from this same group[13] gainsays the utility of these compounds because of stability problems, and presents a technique for Tamra free acid coupling to DNA, again functionalized first with an amine phosphoramidite, on solid supports.

We present the synthesis and techniques for use of a stable Tamra phosphoramidite, which is compatible with modern automated oligonucleotide synthesis methods. The material is as easy to make and use as the analogous fluorescein amidite.[10]

Experimental Section

Materials and Methods. Pyridine, methanol (MeOH), ethyl acetate (EtOAc), dimethylformamide (DMF), dichloromethane (CH_2Cl_2), and acetonitrile (CH_3CN) were Omnisolve grade from VWR. Tetrahydrofuran (THF), sodium bicarbonate ($NaHCO_3$), *N*-methylimidazole, *N*-methylmorpholine, 4-hydroxypiperidine, ammonium acetate, chromatographic alumina, acetic anhydride (Ac_2O), *tert*-butylamine, concd HCl, 4-methoxytrityl chloride, diisopropylamine, and magnesium sulfate ($MgSO_4$) were from Aldrich; 2-cyanoethyl tetraisopropylphosphorodiamidite was obtained from Chemgenes. Benzotriazol-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate (BOP) was obtained from Chem Impex. 5- and 6-Carboxy Tamra and 5-carboxy Tamra-OSu active ester were made and purified in house and can also be obtained from Molecular Probes. Tetrazole and S-ethyltetrazole were obtained from AIC. DNA syntheses were performed on a Bioseearch 8750 synthesizer with Cruachem DNA amidites. All other DNA synthesis reagents were the same as those previously reported.[14] NMR was performed by Acorn NMR (Livermore, CA). Elemental analyses were performed by Desert Analytics (Tucson, AZ). High-resolution mass spectra were performed at the UC Berkeley Department of Chemistry MS lab. MALDI mass spectra were performed in-house on a Finnigan LaserMat.

(1) Khanna, P. L.; Ullman, E. F. *Anal. Biochem.* **1980**, *108*, 156.
(2) Curtis, S. K.; Cowden, R. R. *Histochemistry* **1980**, *68*, 23.
(3) Urdea, M. S.; Warner, B. D.; Running, J. A.; Stempien, M.; Clyne, J.; Horn, T. *Nucleic Acids Res.* **1988**, *16*, 4937.
(4) Sinha, N. D.; Cook, R. M. *Nucleic Acids Res.* **1988**, *16*, 2659.
(5) Connolly, B. A.; Rider, P. *Nucleic Acids Res.* **1985**, *13*, 4485.
(6) Chollet, A.; Kawashima, E. H. *Nucleic Acids Res.* **1985**, *13*, 1541.
(7) van der Laan, A. C.; Brill, R.; Kuimelis, R. G.; Kuyl-Yeheskiely, E.; van Boom, J. H.; Andrus, A.; Vinayak, R. *Tetrahedron Lett.* **1997**, *38*, 2252.
(8) Adamczyk, M.; Fishpaugh, J. R.; Heuser, K. J. *Bioconjugate Chem.* **1997**, *8*, 255.
(9) Mujumdar, S. R.; Mujumdar, R. B.; Grant, C. M.; Waggoner, A. S. *Bioconjugate Chem.* **1996**, *7*, 356.
(10) Brush, C. US Patent 5,371,241, 1994.
(11) Adamczyk, M.; Chan, C.; Fino, J.; Mattingly, P. *J. Org. Chem.* **2000**, *65*, 596.
(12) Woo, S. L.; Menchen S. M.; Fung, S. US Patent 5,231,191**,** 1993.
(13) Vinayar, R. *Tetrahedron Lett.* **1999**, *40*, 7611.
(14) Wang, W. W.; Lyttle, M. H.; Borer, P. N. *Nucleic Acids Res.* **1990**, *18*, 3347.

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5-(*N*-Pipyridyl-4-hydroxy)-Tamra carboxamide 2. 5-Carboxy Tamra **1**, 11 g (26 mmol), was dissolved in 300 mL of DMF. BOP (13 g, 29 mmol) was added, along with 4 mL of *N*-methylmorpholine. The solution was magnetically stirred for 15 min, and 4-hydroxypiperidine (11 g, 109 mmol) was added. The deep red solution was stirred for 3 h, and 20 g of NH_4OAc dissolved in 50 mL of water was added. The DMF was removed by rotary evaporation, with a bath temp < 40 °C. The residue was dissolved in 700 mL of CH_2Cl_2 and washed with 700 mL of 1 N HCl. The organic phase was dried over $MgSO_4$, filtered, and evaporated. A column 7 × 30 cm of alumina, previously wetted with 7 wt % water, was packed in 5% methanol in CH_2Cl_2. The crude product was dissolved in 200 mL of this solvent mixture and loaded onto the column. A gradient to 15% MeOH was run over 6 L of mobile phase, and deeply colored fractions were checked by TLC. Those containing pure product (0.5 R_f, 20% MeOH/2% pyridine/CH_2Cl_2) were pooled and evaporated to give 6.2 g (46% yield) of **2** as a dark solid. ^{1}H NMR (DMSO-d_6, δ): 8.0 (d, 1H), 7.7 (d, 1H), 7.2 (s, 1H), 6.5 (m, 6H), 4.75 (s, 1H), 3.9 (m, 1H), 3.7 (m, 1H), 3.3 (s, 12H), 3.2−3.0 (m, 2H), 1.75 (m, 1H), 1.55 (m, 1H), 1.35 (M, 1H), 1.2 (m, 1H). MALDI *m*/*e* calcd 513.6, found 512.1. Anal. Calcd for $C_{22}H_{31}N_3O_5 \cdot H_2O$: C, 67.78; H, 6.25; N, 7.90. Found: C, 68.18; H, 5.85; N, 8.07.

5-[*N*-Pipyridyl-4-*O*-(2-cyanoethyldiisopropylphosphoramidite)]-Tamra Carboxamide 3. Compound **2** (5.3 g, 10.3 mmol) was dried by rotary evaporation with 100 mL of dry pyridine. A high vacuum was then applied to the material for 1 h. A solution of 3 g (10 mmol) of 2-cyanoethyl tetraisopropylphosphorodiamidite and 180 mg of tetrazole were mixed in 40 mL of dry acetonitrile, and the solution was added to the flask containing **2**. After swirling briefly to dissolve solids, the deep red solution was allowed to stand 3 h. An aliquot for TLC was prepared with about 1 mL of CH_2Cl_2 and 1 mL of aqueous $NaHCO_3$. A few drops of the reaction mixture were added, and the lower layer was spotted on a TLC plate and eluted with 20% MeOH/CH_2Cl_2/2% pyridine. Complete conversion was observed (product $R_f = 0.7$). The solution was stripped and dissolved in 200 mL of CH_2Cl_2 and then washed with 100 mL of satd aqueous $NaHCO_3$. The organic phase was dried over $MgSO_4$, filtered, and evaporated. The solid was further dried by coevaporation with 100 mL of dry pyridine followed by 100 mL of dry acetonitrile. The yield was 5.3 g, 72%. An analytical sample was prepared by chromatography with the same conditions as those used to purify **2**, except that the mobile phase included 2% pyridine. ^{31}P NMR (PPM, $CDCl_3$:) 147.4, 147.1. MALDI *m*/*e* calcd: 713.6, Found: 713.7. Anal. Calcd for $C_{39}H_{49}N_5O_6P$: C, 65.53; H, 6.91; N, 9.80. Found: C, 65.83; H, 6.60; N, 10.19.

***N*-(4-Methoxytrityl)piperidyl-4-*O*-(2-cyanoethyldiisopropylphosphoramidite) 4.** 4-Hydroxypiperidine (8 g, 79 mmol) was coevaporated twice with 100 mL of dry pyridine and redissolved in 150 mL of dry CH_2Cl_2 under argon. Diisopropylamine (26.5 mL) was added, followed by dropwise addition of 4-methoxytrityl chloride (25 g, 80 mmol) dissolved in 150 mL of dry CH_2Cl_2. After overnight stirring, the solution was checked by TLC (1:2 acetone/petroleum ether, product R_f 0.75 with 10% H_2SO_4 spray and heat), and the conversion was found to be complete. The mixture was washed three times with 300 mL of satd aqueous $NaHCO_3$ and then 400 mL of brine. The organic phase was dried over $MgSO_4$ and reduced to a foam by rotary evaporation. Column chromatography was performed on a 20 × 5 cm bed of silica initially packed with 2 L of 1:2 EtOAc:petroleum ether with 1% TEA, and then eluted with 2 L of 1:2 EtOAc:petroleum ether with 1% TEA, and finally 2:1 EtOAc:petroleum ether with 1% TEA. Pure fractions were pooled and evaporated to give 22.5 g (76% yield) of *N*-(4-methoxytrityl)-4-hydroxypiperidine. To all of this was added a premixed solution of 18 g (60 mmol) of 2-cyanoethyl tetraisopropylphosphorodiamidite and 1.08 g of tetrazole in 300 mL of dry acetonitrile. The solution was allowed to stand for 3 h, whereupon TLC (5:25:70 TEA:petroleum ether:EtOAc, R_f product 0.66, starting alcohol 0.5) showed complete conversion. The solution was reduced to a tar by rotary evaporation, and then the residue was redissolved in 700 mL of CH_2Cl_2. The solution was washed twice with 600 mL of satd $NaHCO_3$ and once with 600 mL of brine. The organic phase was dried over $MgSO_4$ and evaporated to a foam. Column chromatography was performed on a 25 × 5 cm bed of silica initially packed with 5:5:90 TEA:EtOAc:petroleum ether, and then eluted with 1 L of 5:5:90 TEA:EtOAc:petroleum ether, with 1 L of 5:25:70 TEA:EtOAc:petroleum ether, with 1L 5:50:45 TEA:EtOAc:petroleum ether, then finally with 5:70:25 TEA:EtOAc:petroleum ether. Pure fractions were pooled and evaporated to give 26.5 g (77% yield) of **4**. ^{1}H NMR ($CDCl_3$, δ): 7.5 (d, 4H), 7.35 (d, 2H), 7.2 (m, 4H), 7.1 (dd, 2H), 6.8 (d, 2H), 3.8 (s, 3H), 3.7 (m, 1H), 3.5 (m, 1H), 2.5 (m, 1H), 2.0−1.8 (m, 6H), 1.2−1.0 (dd, 12H). ^{31}P NMR (PPM, $CDCl_3$:) 146.2. MALDI *m*/*e* (fluorescein matrix) calcd 573.5; found 573.5. Anal. Calcd for $C_{39}H_{49}N_5O_6P$: C, 71.18; H, 7.73; N, 7.32. Found: C, 71.22; H, 7.79; N, 7.30.

Preparation of Model Compounds

Generation of T Mononucleoside Tamra Adduct 6 Using 3 (See Figure 4). T CPG, 10 g, was placed in a 150 mL coarse frit sintered glass funnel atop a 1 L sidearm flask. Dichloroacetic acid (3%) in CH_2Cl_2 (100 mL) was poured in, and the support was agitated briefly with a spatula. The solution became brightly orange. After 2 min, the solution was drained, and the step was repeated twice with 100 mL of fresh 3% dichloroacetic acid in CH_2Cl_2. The support was then washed three times with 100 mL of CH_2Cl_2, twice with 100 mL of CH_3CN, and once with 100 mL of pyridine. The support was transferred into a 250 mL round-bottom flask, and 1 g of **3** was added. Dry pyridine (40 mL) was added, and the solvent was removed by rotary evaporation. A plug of glass wool was used to keep the CPG from leaving the flask. Once dry, high vacuum was applied to the flask for 18 h. A solution of 100 mL of 0.4 M s-ethyltetrazole in dry acetonitrile was prepared, and enough of this was added to the flask containing the CPG to make a slurry. The slurry was allowed to stand for 15 min and was poured into a sintered glass funnel. The support was washed three times with 100 mL of CH_3CN, and then 60 mL of amidite oxidizer solution[14] was added. After 5 min, the support was washed three times with 100 mL of CH_3CN and dried. For cleavage of the T mononucleotide conjugate from the support, the support was placed in a 500 mL round-bottom flask fitted with a condenser, and a mixture of 20 mL water and 60 mL of *tert*-butylamine was added. The mixture was heated to a gentle reflux overnight. The solution was cooled and filtered, and the dark purple solution was concentrated by rotary evaporation. The two component products were separated by reverse phase column chromatography as below. *tert*-Butylamine phosphate counterions were removed by treatment with Dowex 50WX8-400 anion exchanger which had been previously treated with aqueous ammonia. High-resolution mass spectrum of early, major product **6** calcd for MH^+ $C_{40}H_{45}N_5O_{12}P$ = 818.28036; found 818.280730. The later product **6**, containing *tert*-butylamine, lost an OH^- upon ionization, calcd for M+ ($-OH^-$) $C_{44}H_{54}N_6O_{11}P$ = 873.358812; found 873.358970.

Generation of T Mononucleoside Tamra Adduct 6 Using 4 Followed by 5-Tamra OSu (see Figure 4). T CPG (10 g) was detritylated and washed as above, and 1 g of **4** was coupled onto the support in the same fashion as **3**. The detritylation procedure was repeated to remove the MMT group, and the support was washed with two 100 mL washes of 10% triethylamine in CH_2Cl_2 to generate the unprotonated amine. 5-Tamra OSu (1 g) was mixed with 80 mL of DMF and added to the CPG in a

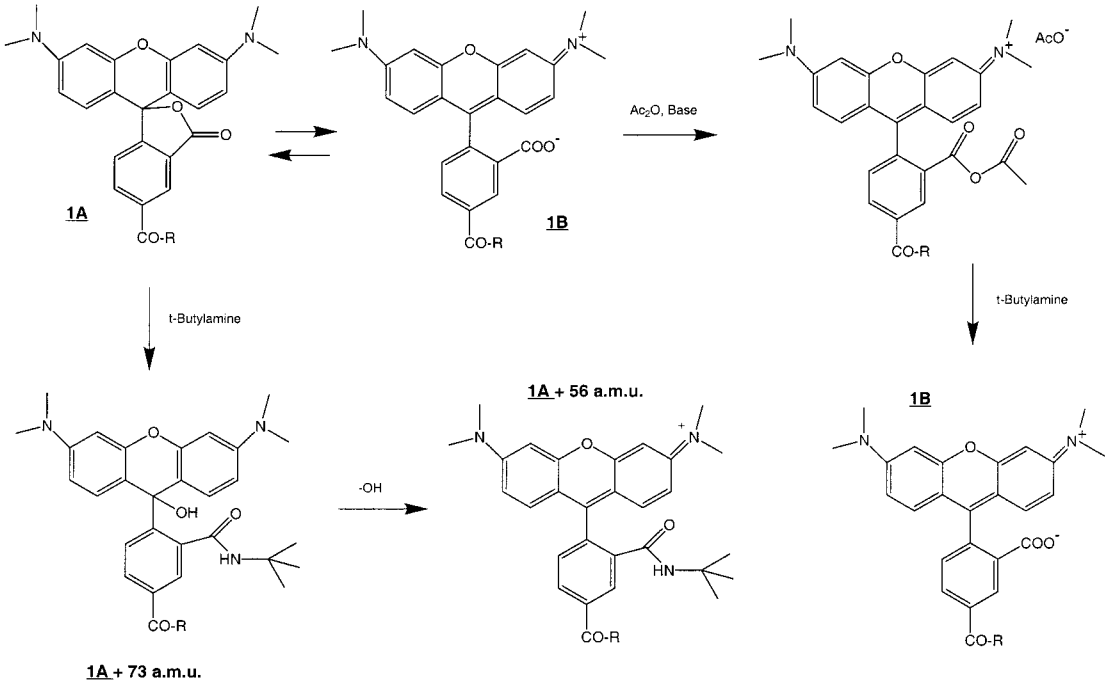

Figure 1. 5-Tamra isomers with 3-carboxylate in lactone form (**1A**) or as carboxylate (**1B**). Schematic for reaction of *tert*-butylamine with **1A** and possible mechanism for prevention by acetylation step. R = *N*-piperidyl-4-*O*-phosphoryl 5′ DNA or thymidine.

250 mL Erlenmeyer flask with stopper. After 18 h, the slurry was poured into 150 mL coarse sintered glass funnel atop a 1 L sidearm flask. The support was washed three times with 100 mL of CH_3CN and dried. Cleavage proceeded as above.

DNA Synthesis. DNA was made on Biosearch 8750 DNA synthesizers with standard conditions as previously described.[14] dG^{DMF} was used to ensure complete deprotection of dG during deprotection with the Tamra deprotection cocktail, which was 1:3 water:*tert*-butylamine. Test sequences 5′-TTTTTTTTTT-3′, 5′-CGATCTGAATAGCTT-3′ and 5′-TCCAGCTCATCGAGGTCATA-3′ were made at 0.2, 1.0, and 1.0 μmol synthesis scales, respectively, on standard CPG supports. The final DMT was removed, and after thorough washing a solution of 200 μL of 50 mg/mL of **3** in dry acetonitrile and 200 μL of 0.4 M S-ethyltetrazole in dry acetonitrile were introduced onto the column by the DNA synthesizer. After 10 min, the coupling solution was washed off the column with acetonitrile, and oxidation was performed. Next, a mixture of acetic anhydride, *N*-methylimidazole and THF (1:1:8) was automatically introduced to the column. After 5 min exposure, the acetylation solution was rinsed with acetonitrile and the CPG containing the 5′-Tamra-labeled DNA was washed thoroughly with acetonitrile. The CPG was placed into screw cap eppendorf tubes and 1 mL of the Tamra deprotection cocktail added to each. The tubes were heated at 55° C for 18 h and cooled and the purple solutions evaporated.

Reverse phase separation of 5′-Tamra-TTTTTTTTTT-3′ and additional products made without the post oxidation acetylation step was accomplished by first equilibrating a Biosearch Micropure II cartridge with acetonitrile and then water and then loading a solution of 30 O.D. units crude DNA onto the column in 0.5 mL of water. Acetonitrile (20%) in water eluted the first product as a bright pink solution, and then 50% acetonitrile in water gave the second, less-colored band containing the byproduct. For the mononucleotide model compounds, a 5 × 20 cm column of Toso−Haas Amberchrom 50−100 μm packing was used with the same sequence of mobile phase elutions. MALDI Mass spectra of the T-10 products were performed according to Ball and Packmann.[15]

Anion-exchange HPLC analyses were performed as follows: 2−20 μL of the aqueous samples, depending on the concentration, was injected onto a Dionex anion-exchange column (4.6 × 250 mm); samples were eluted at 2 mL/min with aqueous buffers of (A) 0.025 M Tris·HCl and 0.01 M Tris, and (B) 0.025 M Tris·HCl, 0.01 M Tris, and 1.0 M NaCl using a linear gradient of 1:0 to 0:1 over 20 min, with UV detection at 260 nm. The detector was a Waters 996 photodiode array. Samples for base composition analysis were treated as previously described,[16] with analysis by reverse phase HPLC as follows: 20 μL of the aqueous sample was injected onto a HAISIL HL C18 5 μm column (4.6 × 150 mm); samples were eluted at 1 mL/min with buffers of (A) 0.1M TEAA, 5% acetonitrile, (B) acetonitrile, with a linear gradient of 1:0 to 0:1 over 20 min. UV detection at 260 nm.

Results and Discussion

The key to understanding the complex chemistry exhibited by the rhodamine dyes lies in the disposition of the 3-carboxylate. The various species present at various pHs probably follow the scheme established for fluorescein[17] and is either strongly violet colored or not depending on the disposition of the 3-carboxylate[18] (see Figure 1, **1A** and **1B**). The two forms also exhibit marked differences in solubility characteristics, in our hands. The

(15) Ball, R. W.; Packman, L. C. *Anal. Biochem.* **1997**, *246*, 185.
(16) Wheatly, J. B.; Lyttle, M. H.; Hocker, M. D.; Schmidt, D. E. *J. Chromatogr. A.* **1996**, 77.
(17) Sun, W.; Gee, K. R.; Klaubert, D. H.; Haugland, R. P. *J. Org. Chem.* **1997**, *62*, 6469.
(18) Ramette, R. W.; Sandell, E. B. *J. Am. Chem. Soc.* **1956**, *78*, 4872.

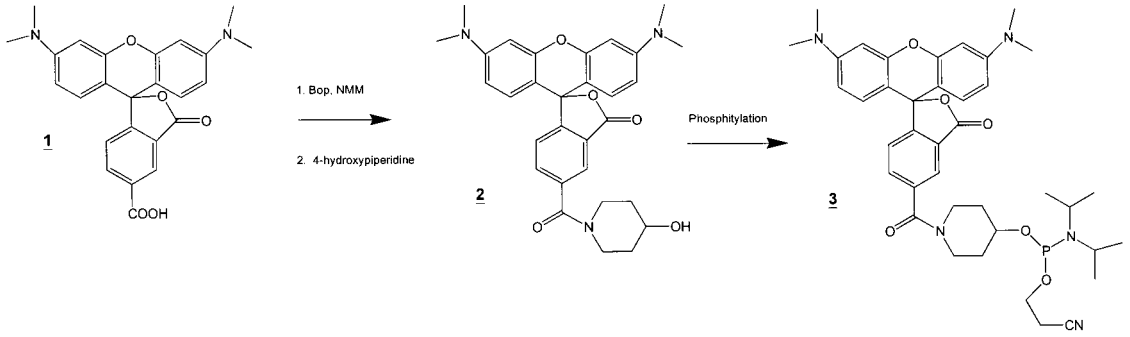

Figure 2. Synthesis of Tamra amidite **3**.

reactivity of the 3-carboxylate toward reagents used to functionalize the 5- or 6-carboxylates and deprotect Tamra-tagged DNA probably determines the quantity and character of side reaction products formed. We believe that the synthesis and use of the Tamra derivative below demonstrate these principles in action.

Synthesis of Tamra Amidite 3. The first reaction with Tamra was the formation of an amide with a bifunctional amine containing a hydroxyl for later attachment of the phosphoramidite group. Linear variants, 6-amino-1-hexanol and 2-(2-aminoethoxy)ethanol, both gave many products, according to TLC. 4-Hydroxypiperidine gave much better results; mostly a single product was formed. Extraction of the Tamra amide **2** proved tricky; usually one extracts organic solvent solutions of crude BOP amide formation products[19] with mild base such as satd $NaHCO_3$ to remove the hydroxybenzotriazole and other unwanted aqueous base soluble materials. In this case, the product **2** was quite soluble in the aqueous layer. Extraction instead with 1 N HCl gave satisfactory results; very little Tamra amide **2** was observed in the aqueous layer. Column chromatography on damp alumina gave 46% yield of **2**, which was quite pure by NMR and had a good elemental analysis. After thorough drying, the Tamra amide alcohol **2** was converted into the amidite **3** by reaction with a stoichiometric amount of 2-cyanoethyl tetraisopropylphosphorodiamidite and a catalytic amount of tetrazole. Unreacted compound **2** was easily removed by washing an ethyl acetate solution of the product with aqueous $NaHCO_3$. The product amidite **3** was dried by stripping from dry pyridine and then acetonitrile and high vacuum. The yield was over 70% for this step. Chromatography of the final product was unnecessary; no increase in activity was seen upon purification (see Figure 2).

5′-Tamra DNA Synthesis. Satisfactory (over 95%) coupling efficiencies were obtained either by manual syringe coupling methods[20] or with automated coupling. Ten milligrams of **3** dissolved in 200 μL of acetonitrile was used. When the CPG immobilized sequence 5′-TTTTTTTTTT-3′ was treated with an acetonitrile solution of **3** and activator, followed by oxidation and then cleavage from the CPG and deprotection in *tert*-butylamine and water, two products were observed by AX HPLC, as shown in Figure 3 (upper panel). When a 5 min *acetylation* step (Ac_2O, base) was included after the amidite oxidation, cleavage, deprotection, and AX HPLC analysis revealed complete suppression of the minor

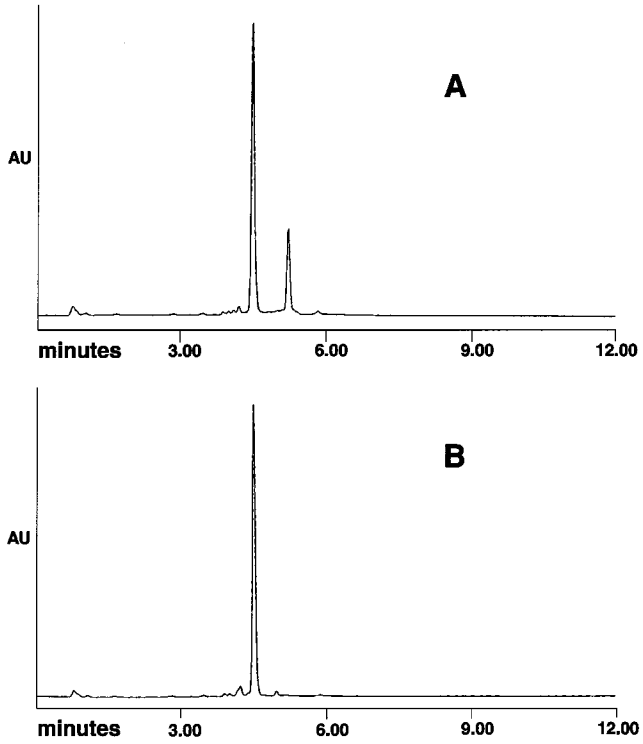

Figure 3. Anion exchange (AX) HPLC of: A, 5′-Tamra-TTTTTTTTTT-3′ without post-coupling acetylation step; B, same sequence with 5 min with post-coupling acetylation step.

product peak with the major product present in about 90% purity (see Figure 3, lower panel).

The unacetylated DNA sample containing the two products was subjected to reverse phase chromatography (see Figure S1), and mass spectral analyses were performed on each separated component. The earlier, major peak had the correct mass for the desired conjugate (calcd 3553.6, found 3555.6 amu), while the later, minor peak had a molecular weight of the desired product plus 73.1 amu (calcd 3626.8, found 3628.3), within experimental error (±0.2%) for the addition of *tert*-butylamine, the amine present in the deprotection cocktail, as demonstrated in Figure S2.

To more accurately explore the *tert*-butylamine addition phenomena, **3** was coupled onto T CPG and the resulting mononucleotide 5′-Tamra product **5** cleaved, purified, and analyzed by reverse phase HPLC (see Figure 4). As with the 5′-Tamra T-10 study discussed above, two products were observed when the coupled CPG was treated with aqueous *tert*-butylamine and heat. When the CPG bearing the oxidized Tamra T mono-

(19) Hudson, D. *J. Org. Chem.* **1988**, *53*, 617.
(20) Lyttle, M. H.; Adams, H.; Hudson, D.; Cook, R. M. *Bioconjugate Chem.* **1997**, *8*, 193.

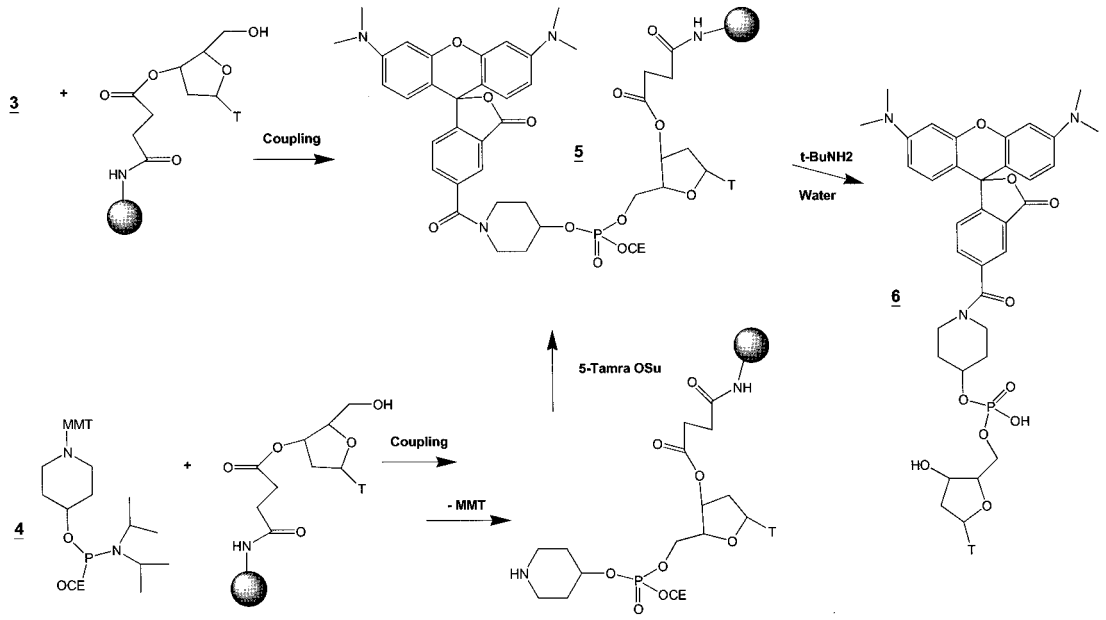

Figure 4. Solid-phase-supported synthesis of 5′-Tamra-linked Thymidine mononucleotide model compounds.

nucleotide **5** was subjected to a 5 min acetylation step, as above, only the major product was observed. The two components generated by the unacetylated synthesis were separated by reverse phase chromatography and subjected to NMR analysis. A large singlet resonance at δ 1.2 with an area of 9 protons was seen in the minor product whereas only minimal absorbances were seen in the major mononucleotide adduct in that region of the NMR spectrum, given in Figure S3. High-resolution mass spectra of each product confirm the molecular formulas, although the minor product appeared to lose an OH group during ionization (see Figure 1, where R = 5′-(4-*O*-piperidyl phosphate)thymidine). The NMR and mass spectra confirmed the addition of *tert*-butylamine to the minor product. The aromatic region of each NMR spectrum of both the major and minor Tamra mononucleotide model compounds was similar, with no loss of relative area, which dispels the possibility of aromatic substitution by *tert*-butylamine. As well, the minor byproduct containing the extra mass did not show the characteristic (λ_{max} 556 nm) Tamra spectrum in either the T-10 DNA or the model studies. An added *tert*-butylamine at the 3-carboxylate would force the lower ring out of the plane of the xanthene nucleus and disrupt the full conjugation needed to show the Tamra spectrum. The λ_{max} of the minor *tert*-butylated byproducts was ~240 nm, close to that of unsubstituted *N*,*N*-dimethylanaline. On the basis of the evidence above, we believe that *tert*-butylamine is adding to the lactone form of the 3-carboxylate during the cleavage and deprotection step as shown in Figure 1.

A possible mechanism for the curtailment of byproduct formation by acetylation is that the two configurations of the Tamra 3-carboxylate are in rapid equilibrium, and that the free 3-carboxylate (structure **1B**, Figure 1) is acetylated, forming a mixed anhydride. The equilibrium is thus shifted until no more of the vulnerable lactone form is present. The mixed anhydride can then only interact with *tert*-butylamine to regenerate the unreactive **3** carboxylate anion; recall mixed anhydride amide-forming reactions in peptide chemistry where the least hindered side of a mixed anhydride is functionalized

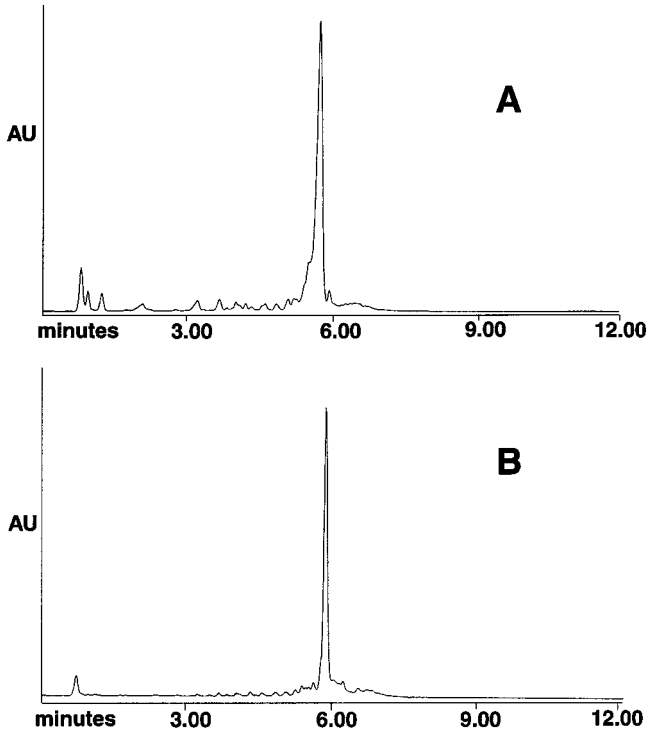

Figure 5. AX HPLC of unpurified 5′ Tamra DNA made with **3**. A = 5′-Tamra-CGATCTGAATAGCTT-3′, B = 5′-Tamra-TCCAGCTCATCGAGGTCATA-3′.

specifically by an amine nucleophile.[21] Further support for the structure of **6**, and also a comparison of the products generated through Tamra amidite coupling versus Tamra active ester addition, was given by the alternate synthesis shown in Figure 4. In this case *N*-(4-methoxytrityl)piperidinyl-4-*O*-(2-cyanoethyl diisopropyl-lphosporamidite) **4** was first added onto T CPG. After oxidation and washing, the MMT group was removed, and then Tamra-OSu was added to the amine. The major Tamra-containing products from both syntheses were

(21) Vaughn, J. R.; Osato, R. L. *J. Am. Chem. Soc.* **1951**, *73*, 5553.

isolated by chromatography, and HPLC analysis showed identical compounds by coelution (see Figure S4). This should allay any fears about dissimilar Tamra DNA conjugates being generated by amidite or active ester couplings.

Two heterogeneous sequences, 5′-CGATCTGAAT-AGCTT-3′ and 5′-TCCAGCTCATCGAGGTCATA-3′ were 5′ labeled on the CPG support with **3**, followed by oxidation and acetylation as described above. All manipulations were automatically executed by the DNA synthesizer. Figure 5 shows AX HPLC of these unpurified 5′-Tamra 15 and 20 mers. PDA (photodiode array) spectra of each major product peak showed the presence of Tamra in the products (See Figure S5). Tamra amidite solutions on the DNA synthesizer retained equivalent to fresh activity after 7 days. Following 18 h of heating (55 °C) in *tert*-butylamine/water and evaporation, a digest of each sequence with phosphodiesterase II, followed by alkaline phosphatase, showed the absence of any modified bases which could be due to the added treatments (data not shown). G^{DMF} was used to allow effective G deprotection[22] in the milder (than aqueous ammonia) Tamra cocktail.

(22) Vu, H.; McCollum, C.; Jacobsen, K.; Theilsen, P.; Vinayak, R.; Spiess, E.; Andrus, A. *Tetrahedron Lett.* **1990**, *31*, 7269.

Conclusion

Results have been presented regarding the preparation and use of a new Tamra amidite. Improved product DNA conjugate quality has been demonstrated by the use of a post-coupling acetylation step. Detailed spectral studies of DNA conjugates and model compounds show that *tert*-butylamine can add to the Tamra to generate a stable byproduct, and that the position of addition is most likely across the lactone ring. The studies also show that identical compounds can be generated from Tamra amidite and Tamra-OSu active ester couplings to the appropriate substrates.

Tamra amidite coupling to 5′-OH DNA immobilized on a solid support will be more efficient and result in cleaner products than those for 5′ labeling methods previously reported, i.e., that of using first a 5′ amine functionalization amidite followed by active ester coupling. We hope that these methods will facilitate the development and use of other rhodamine fluorophores in DNA labeling.

Supporting Information Available: Figures S1−S5 are available free of charge via the Internet at http://pubs.acs.org.

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