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Novel Photoacid Generators for Photodirected Oligonucleotide Synthesis

Pawel J. Serafinowski*,† and Peter B. Garland‡

Contribution from the Cancer Research UK Centre for Cancer Therapeutics and Section of Molecular Carcinogenesis, The Institute of Cancer Research, Sutton, Surrey SM2 5NG, UK

Received November 29, 2001; E-mail: p.serafinowski@icr.ac.uk

Abstract: Photodirected oligonucleotide synthesis uses either direct or indirect light-dependent 5′-deprotection. Both have been reported to give lower stepwise synthetic yields than conventional methods. The deficiency appears to be due to incomplete deprotection at the oligonucleotide 5′-position and, additionally in the case where photodirection is indirect and uses photogenerated photoacid to effect 5′-detritylation, the depurinating effects of strong acid. We have developed novel photosensitive-2-nitrobenzyl esters that on irradiation with near UV light generate α-chloro-substituted acetic acids, such as trichloroacetic acid, which are widely and successfully used in conventional solid-phase oligonucleotide synthesis. α-Phenyl-4,5-dimethoxy-2-nitrobenzyltrichloroacetate and α-phenyl-4,5-dimethoxy-2,6-dinitrobenzyltrichloroacetate showed appropriate photochemical characteristics and were used for photodirected synthesis of a variety of oligonucleotides, including $(T)_5$, TATAT, TGTGT, $(T)_{10}$, $(AT)_5$, $(CT)_5$ $(GT)_5$, and $(TGCAT)_2$ on a modified Millipore Expedite DNA synthesizer. The outcomes were compared with those obtained by use of directly added trichloroacetic acid (conventional synthesis). The stepwise yields for the two methods were essentially identical.

Introduction

High-density arrays of oligonucleotides chemically synthesized in situ on planar surfaces are powerful tools for research and other applications. Contact photolithography and direct photochemical deprotection of blocked groups have been successfully used for array fabrication,[1,2] but there are two main drawbacks: (a) the cost and inflexibility of photolithographic masks restrict their wider use, and (b) the stepwise yields for synthesis on glass surfaces are reported to be lower than those obtained for conventional synthesis using the acid-removable dimethoxytrityl group (DMTr) as the 5′-protecting agent.[3]

The drawbacks of photolithographic masks can be circumvented by using patterned light from a computer-controlled spatial light modulator,[4] but unfortunately the higher stray light content of the projected beam may cause inaccuracies due to extraneous addition of monomeric units appearing as insertions in the final product.[5]

Indirect photodeprotection, using photogenerated acid to remove acid-labile blocking groups, has been proposed[6−8] and should have several advantages over direct photodeprotection. For example, the required nucleotides are commercially available, the effects of stray light can be neutralized,[5] constraints associated with the use of powerful UV sources could be removed by photoacid generators sensitive to visible light, and the high stepwise synthetic yields obtained in conventional synthesis[9] could possibly be matched. However, currently available photoacid generators as developed primarily for the semiconductor industry may generate strong acids and liberate free radical species, and when used for the synthesis of oligonucleotides can have undesirable effects on the synthetic yield[7−9] and therefore on the achievable oligonucleotide length and abundance of the desired product among truncated chains. The most commonly observed undesired acid-dependent events include cleavage of the glycosidic bond of N^6-benzoyl-deoxyadenosine and N^2-isobutyryldeoxyguanosine residues. Furthermore, photochemically generated reactive species may re-block deprotected groups. On the other hand, the use of DMTr as the 5′-OH protecting group in combination with 2−3% chloroacetic acids in dichloromethane as deprotection agents minimizes artifacts in oligonucleotide synthesis.[10]

We reasoned that photoacid generators that release chloroacetic acids could have advantages over generators of stronger acids. In addition, we anticipated that photoacid generators that proceed via an intramolecular rearrangement would lower the

† Cancer Research UK Centre for Cancer Therapeutics.
‡ Section of Molecular Carcinogenesis.

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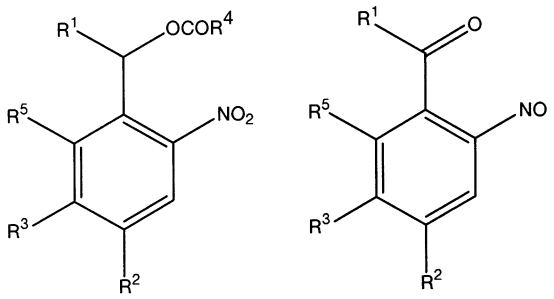
Chart 1. Photolabile Esters and Their Photoproducts



R^1= H, CH_3, C_6H_5, (p) m-$MeOC_6H_4$; R^2 = H, OCH_3; R^3= H, OCH_3, $N(CH_3)_2$; R^4= CH_3, $ClCH_2$, Cl_2CH, Cl_3C, R_5=NO_2,H

risk of free radical modification of freshly unblocked oligonucleotide 5′-OH groups. We therefore chose to explore esters based on 2-nitrobenzyl alcohol,[11] a well-known photosensitive blocking group that can be modified to give desired photochemical properties.

There are alternative but less well-known photosensitive groups that could also be used for photoacid generators, such as 2,5-dimethylphenacyl[12] and 2-(2-nitro-phenyl)ethyl.[13] Nevertheless, whatever group is chosen, it must still meet the same performance criteria, not least of which is a suitably high extinction coefficient at 330 nm or greater (to be clear of the absorption bands of the oligonucleotide bases) and a useful quantum yield. Other design criteria include straightforward synthesis in high yield, stability, solubility in organic solvents, absence of basic sites in the ester or its photoproduct, formation of preferably a single photoproduct and certainly none that may modify oligonucleotides, and ability when used for photodirected oligonucleotide synthesis to give the same stepwise yields as obtained in conventional synthesis.

Experimental Section

Full details are provided in the Supporting Information. Descriptions given here are to indicate general strategy.

Synthesis of Photolabile Esters. Literature searches showed that various photoacid generators based on 2-nitrobenzyl esters have been synthesized,[14] but only a few with carboxylic acids.[15] Two general features of 2-nitrobenzyl esters are (a) the quantum yield for photolysis is enhanced by α-substitution with a methyl or phenyl group[16] and also by introduction of a second nitro group at the 6-position,[17] and (b) extinction coefficients in the near UV region can be increased by suitable benzyl ring substitutions.[18]

We have therefore synthesized a series of substituted 2-nitrobenzyl esters of trichloroacetic acid (TCA), using condensation of trichloroacetic anhydride with appropriate 2-nitrobenzyl alcohols. This method can also be used for synthesis of mono- or di-chloroacetyl esters of 2-nitrobenzyl alcohol (see Supporting Information) (Chart 1). Esters with useful extinction coefficients above 330 nm were tested for their photosensitivity to light at 350 or 365 nm light, using spectrophotometry, TLC, and HPLC analysis to follow photolysis. Quantum yields were determined from the ratio of the amount of ester photolyzed to the light energy absorption over a given time. Acid release was measured using titration with alkali and an indicator. Other photoproducts were isolated by column chromatography on silicagel or HPLC or both, and characterized by ^{1}H and ^{13}C NMR, HRMS, and UV spectroscopy.

Two esters of particular promise were tested for their ability on photolysis to detritylate DMTr-T-cpg. They were then examined for their ability to replace TCA in automated solid-phase oligonucleotide synthesis. The key issue was whether the stepwise synthetic yields observed with the conventional method using directly added TCA could be matched by a photodirected method using TCA that was photogenerated in situ. For both methods, overall % yields of synthesized 5′-DMTr-oligonucleotides in the reaction products released from cpg with ammonia and analyzed by reverse phase HPLC with spectrophotometric flow detection at 254 and 280 nm were equated with the % areas under each peak. Average stepwise yields for N-mers were calculated as the *N*th root of the overall yield. In comparing the yields of photodirected to conventional synthesis we have used the ratios of the two, thereby attaching less significance to the absolute values.

Results and Discussion

Evaluation of Photolabile Esters. Solutions of the synthesized esters, in dichloromethane or acetonitrile,[19] were irradiated at 350 or 365 nm using either a semimicro photochemical reactor with a 4-watt UV lamp, or spectrophotometer cuvettes (or flasks) with a 100-W high-pressure mercury arc lamp. These experiments demonstrated that each ester could be converted into the corresponding photoproduct, substituted 2-nitroso-benzaldehyde (R^1 = H), 2-nitrosoacetophenone (R^1 = CH_3), or 2-nitrosobenzophenone (R^1 = C_6H_5) with the formation of the appropriate carboxylic acid (see Supporting Information and Chart 1).

We found that α-phenyl-4,5-dimethoxy-2-nitrobenzyltrichloroacetate (**ester A**: R^1 = C_6H_5, R^2 = OCH_3, R^3 = OCH_3, R^4 = CCl_3) and α-phenyl-4,5-dimethoxy-2,6-dinitro-benzyltrichloroacetate (**ester D**: R^1 = C_6H_5, R^2 = OCH_3, R^3 = OCH_3, R^4 = CCl_3, R^5 = NO_2) gave the best fit to our design criteria. **Ester A** is nonionic, has an extinction coefficient at 365 nm of 3500 cm·M^{-1}, an acceptable quantum yield of 0.14, stability, ease of synthesis, and only one UV-absorbing product on photolysis (4,5-dimethoxy-2-nitrosobenzophenone: R^1 = C_6H_5, R^2 = OCH_3, R^3 = OCH_3) as analyzed by HPLC, TLC, MS, and NMR. Irradiation of the ester released close to 1.0 proton per molecule of ester photolyzed. **Ester D** is also nonionic and has an extinction coefficient at 365 nm of 2000 cm·M^{-1}, a higher quantum yield of 0.4, a similar stability and ease of synthesis, and again only one UV-absorbing product on photolysis (4,5-dimethoxy-2-nitroso-6-nitro-benzo-phenone: R^1 = C_6H_5, R^2 = OCH_3, R^3 = OCH_3, R^5 = NO_2).

Both the esters were assessed for their ability to effect detritylation when irradiated in solution with DMTr-protected nucleotides either attached or unattached to controlled porosity glass. The experiments demonstrated that complete detritylation occurred, given time, even with a relatively dilute solution of the ester, 10−30 mM, and a weak source of UV light.

Protocols for Photodirected Oligonucleotide Synthesis. To provide proof of principle for the use of photogenerated trichloroacetic acid in oligonucleotide synthesis, we developed protocols for automation with a Millipore Expedite DNA

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Table 1. Stepwise Yields for Photodirected Synthesis of a Variety of 5′-Dimethoxytrityl-oligonucleotides, and the Ratios of the Yields to Those for Conventional Synthesis, Using Trichloroacetic Acid Either from Photolysis of **Esters A** and **D** (Photodirected) or Directly Added (Conventional) to Effect 5′-Detritylation

synthetic oligonucleotide (5′-dimethoxytrityl)	stepwise synthetic yields (%) and their ratios					
	ester A			**ester D**		
	(i) photodirected	(ii) conventional	ratio (i)/(ii)	(iii) photodirected	(iv) conventional	ratio (iii)/(iv)
$(T)_5$	95.1	93.4	1.019	94.4	95.2	0.991
TATAT	89.8	89.7	1.001	-	-	-
TGTGT	88.0	86.6	1.014	93.6	94.5	0.990
$(AT)_5$	92.1	93.0	0.990	95.3	94.9	1.004
$(CT)_5$	94.3	94.2	1.001	96.6	96.4	1.002
$(GT)_5$	-	-	-	95.7	96.0	0.997
$(TGCAT)_2$	94.6	95.3	0.992	93.4	94.3	0.990
$(T)_{10}$	97.8	98.0	0.997	97.4	97.6	0.998
	mean of the ratios for **ester A** (±standard deviation)		**1.002 (±0.004)**	mean of the ratios for **ester D** (±standard deviation)		**0.996 (±0.002)**

synthesizer at the 0.5 μmol scale using controlled porosity glass (cpg), modified flow columns, a shuttered UV source (365 nm from a 100-W high-pressure Hg arc) to illuminate the column through a flexible light guide and suitable optical filters and lenses, and the ester (11−22 mM in dichloromethane) in place of directly added trichloroacetic acid (183 mM).

Automated photodirected syntheses of the pentamer DMTr-$(T)_5$, commencing with a flow column containing 0.5 μmol of DMTrT attached through its 3′-position to cpg, gave satisfactory results for **ester A** when the detritylation step consisted of 300 pulses (a total volume of around 4.5 mL) of a 22 mM solution of the ester in dichloromethane passed through the irradiated column in 1800 s. These arrangements ensured adequate steady-state concentrations of photogenerated trichloroacetic acid (2−4 mM), and provided continuing but pulsatile flow during photolysis to avoid the effects of unstirred and unilluminated volumes. The higher quantum yield of **ester D** enabled the concentration, volume, and illumination time for its use to be halved to 11mM, 150 pulses, and 900 s respectively

Otherwise, conditions were as usual, with automated protocols for capping, oxidation, coupling, and washing. Inner filter effects and controlled porosity glass within the flow tube made for suboptimal optical conditions, but it was important to synthesize sufficient oligonucleotide for subsequent analysis. When inner filter effects are absent, as in a thin film on a glass microscope slide or in a dilute solution, the half-life for photolysis of the esters at an intensity of 25 mW/cm^2 is less than 20 s.

Synthetic Yields. The overall oligonucleotide synthetic yield for DMTr$(T)_5$ as estimated by MS and HPLC was at least 81.8%, which corresponds to 95.1% stepwise yield. An essentially identical yield was obtained when the pentamer was synthesized conventionally with directly added trichloroacetic acid (3% v/v in dichloromethane). We then used photodirected synthesis with **ester A**, or conventional synthesis for comparison, for a variety of oligonucleotides which included DMTr-$(T)_5$, DMTrTATAT, DMTrTGTGT, DMTr$(T)_{10}$, DMTr$(AT)_5$, DMTr$(CT)_5$, and DMTr$(TGCAT)_2$. The yields varied with the sequence, but for any one sequence the yields for the photodirected and conventional methods were very similar. The set of photodirected and conventional syntheses was repeated using **ester D** in place of **A**. The pattern of results for yields was similar. Yields for both sets are given in the Table 1.

Some differences between the two sets were observed and are due to the use of different batches of DMTrT-cpg. However, comparison of the individual and mean values of the ratios of the stepwise yields revealed only minor differences, scattered around a mean value of 1.0 irrespective of which ester was used or oligonucleotide synthesized. Because the stepwise yields were essentially the same for photodirected or conventional synthesis, we conclude that the photoproducts from **esters A** and **D** do not block the newly exposed 5′-OH group of the oligonucleotides. To our knowledge equality of yield between photodirected and conventional syntheses has not been reported, although the converse has.[7,8]

For further characterization, all the tritylated oligonucleotides listed in Table 1 were deprotected manually with 3% aqueous acetic acid to give their appropriate detritylated counterparts in quantitative yields, isolated by HPLC, and characterized by ESI MS. The anticipated expected molecular ions were observed in all cases (see Supporting Information).

The estimated stepwise yield for synthesis of DMTr$(T)_{10}$ in our hands was 97.8% for the photochemical method (using **ester A**) and 98% for the conventional method. Corresponding values for **ester D** were 97.4% and 97.6%, respectively. The yield for a TT coupling using the conventional method is generally considered to be in the region of 98−99%, and thus there is little, if any, room for improvement. Coupling efficiencies involving purine nucleosides, particularly guanosine, are usually lower, and our results were no exception.

We anticipate that photogeneration of trichloroacetic acid is likely to be the light-sensitive step of choice for photodirected oligonucleotide array synthesis using maskless projection photolithography. The small amounts of acid generated by stray light can be neutralized by the presence of low concentrations of suitable buffer or weak base.[5] In contrast, the effects of stray light on direct photodeprotection are probably nonpreventable except in the unlikely event of using dual-photon activation.

Encouraged by our results we have synthesized several examples of second-generation 2-nitrobenzyl-based trichloroacetate esters with absorption maxima at 380−395 nm and molar extinction coefficients approaching 8000 cm. M^{-1} at 405 nm. They can be photolyzed with violet light provided by light-emitting diodes and may facilitate the development of instrumentation for array fabrication by usefully widening the choice of light sources and therefore projection devices.

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Supporting Information Available: (1) Synthesis of photolabile esters, (2) photolysis of photolabile esters, (3) determination of photolytic quantum yields, (4) photodirected synthesis of oligonucleotides including retention times and molecular ions of synthesized oligonucleotides, (5) list of references (PDF). This material is available free of charge via the Internet at http://pubs.acs.org..

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