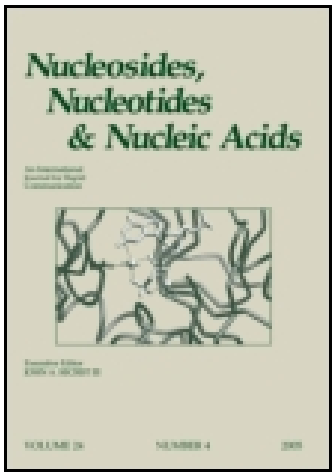

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RECENT HIGHLIGHTS ON PHOTOLITHIC OLIGONUCLEOTIDE ARRAY IN SITU SYNTHESIS

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RECENT HIGHLIGHTS ON PHOTOLITHIC OLIGONUCLEOTIDE ARRAY IN SITU SYNTHESIS

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▫ *Light directed synthesis of high-density oligonucleotide microarrays is currently performed using either ortho-nitro-benzyl-type [MeNPOC] (Pease, A.C.; Solas, D.; Sullivan, E.J.; Cronin, T.M.; Holmes, C.P.; Fodor, S.P.A. Proc. Natl. Acad. Sci. U.S.A.* ***1994****, 91, 6333.) or ortho-nitrophenyl-ethyl-type [NPPOC] (Hasan, A.; Stengele, K.P.; Giegrich, H.; Cornwell, P.; Isham, K.R.; Sachleben, R.A.; Pfleiderer, W.; Foote, R.S. Tetrahedron* ***1997****, 53, 4247.) protecting groups as the 5′-O-carbonate ester of the phosphoramidite building block. The synthesis cycle uses a combinatorial approach attaching one specific base per cycle, thus as many as 100 cycles need to be run to make an array of 25-mers. Time needed for deprotection/activation of the growing oligo chain determines overall manufacturing time and consequently also cost. In this report we demonstrate the development of photoprotected phosphoramidite monomers for light directed array synthesis with increasing sensitivity to the UV light used. If combined with maskless array synthesis, this technology allows for synthesis of arrays with >780,000 different 25-mer oligonucleotides in about one hour and allows for high flexibility in array design and reiterative redesign. The arrays synthesized show high quality and reproducibility in our standard hybridization based assay.*

INTRODUCTION

High-density oligonucleotide microarrays are preferably synthesized by UV light-directed photolithic synthesis by either a mask bearing or micro-mirror device[1] approach, the latter enabling highly flexible iteration of design, synthesis, hybridization, evaluation, and redesign.

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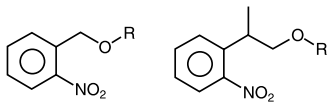

FIGURE 1 Nitrobenzyl-type (as in MeNPOC) and nitrophenylethyl-type (as in NPPOC) photolabile protective groups.

With combinatorial light-pattern projections, a typical synthesis of an array consisting of as many as 780,000 35-mer oligonucleotides may need up to 140 consecutive chemistry and illumination cycles. It is therefore important top cut down on synthesis time per cycle, especially if only one array is made at a time per instrument. In addition, the recent increase in interest in experiments requiring long-mers, 60 to 70 nucleotides,[2] make faster chemistry cycles a very attractive target for development.

RESULTS

We have reported earlier[3] on improvements of NPPOC-type photolabile protecting groups for photolithic synthesis mainly by mechanistic driven hypotheses of improving either molar absorption coefficient for the (n-pi*)-transition of the aromatic nitro group or enhancing quantum yield for the photocleavage reaction from the excited triplet state[4] (Figure 1).

Briefly, a stabilization of the benzylic radical intermediate of NPPOC by appropriate substituents on the phenyl ring led to favorable radical recombination, benzylic anion formation, and fragmentation to the nitro styrene, carbon dioxide and release of the nucleosidic alcohol. At the same time, the oxidation of the benzylic position was repressed, which would lead to a non-cleavable photoproduct in the case of NPPOC.

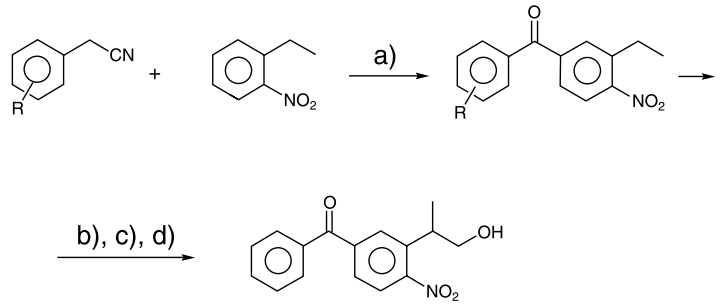

FIGURE 2 Preparation of benzoyl-NPPOC-type photolabile groups. a) KOH/methanol, then hydrogen peroxide; b) propylene glycol, toluene sulfonic acid (kat.), toluene (azeotropic dist.); c) paraformaldehyde, TRITON-B, DMSO; d) HCl(aq)/methanol.

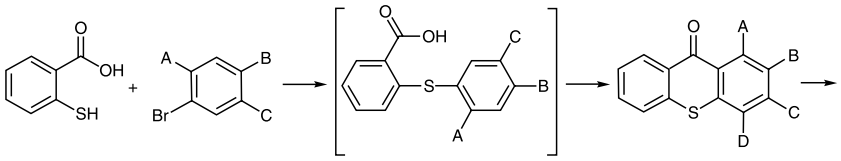

FIGURE 3 Synthesis of nitrothioxanthonepropanols. A = H; B = Nitro-; C = Ethyl-.

Preferred substituents and positions were reported in Bühler et al.[3] Furthermore, we now have introduced a benzoyl-group *meta*- to the benzylic position, which according to[5] led to a very strong benzylic effect.

Synthesis of the new benzophenones was achieved by coupling of appropriate substituted benzyl cyanides with *ortho*-nitro ethylbenzene and subsequent oxidative decarboxylation according to Davis et al.[6] in moderate yields for electron rich and good yields for electron deficient benzylcyanides. Subsequent hydroxymethylation of the ethyl group led to the parent alcohol of the photolabile group. In most cases we could improve the yield for the hydroxymethylation reaction by intermittently protecting the benzophenone by ketalization with propylene glycol (Figure 2).

Another previously reported photolysis improvement, triplet sensitization[7] of NPPOC chromophore with thioxanthone, led us to the design of an entity having both *meta*-benzoyl- and thioxanthone structure elements in an NPPOC molecule.

We synthesized diphenylsulfide molecules by Heck type reaction of bromo nitro ethylbenzene with thiosalicylic acid under copper catalysis,[8] which underwent cyclization upon heating in polyphosphoric acid with little to no stereoselectivity and moderate yield, in most cases less than 20% for a single isomer. Hydroxymethylation with paraformaldehyde then led to the parent alcohol in again only about 10–20% yield due to the strong activation and presumably oligomerization under the basic reaction conditions (Figures 3 and 4).

Photolysis studies of newly protected nucleosides analyzed by HPLC indicated the extremely fast photocleavage kinetics (half-lives) of NTXPOC (0.65 s) over the current gold standard MeNPOC and NPPOC (6–7 s) groups. Benzoyl-NPPOC (3.5 s) also showed favorable kinetics over NPPOC but was significantly slower than NTXPOC and slower than previously reported Phenyl-NPPOC (2 s) in our analytical test set-up, essentially similar to[9] but with higher light intensity (approximately 300 mW/cm^2) (Figure 5).

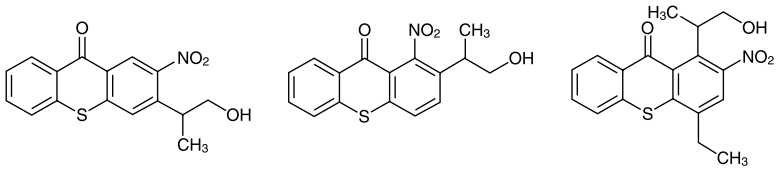

FIGURE 4 Structural isomers of nitrothioxanthonepropanols.

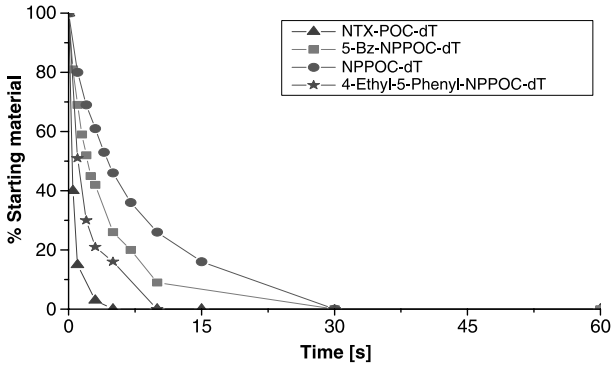

FIGURE 5 Photolysis kinetics of different NPPOC-type photolabile groups.

Nucleotide-5′-carbonates of *o*-nitrobenzylalcohol, *o*-nitroveratryl-1-ethanol (MeNPOC) *o*-nitrophenyl-2-propanol (NPPOC), *o*-nitro(*m*-benzoylphenyl)-2-propanol, and *o*-nitrothioxanthone-2-propanol were then synthesized according to published methods by first reacting the alcohols with diphosgen and then the properly functionalized nucleosides generally in good yields. Further conversion to the beta-cyanoethyl-N-diisopropyl phosphoramidites furnished the array synthesis monomers, e.g., NTXPOC-amidites, as shown in Figure 6.

Arrays of a test oligonucleotide were then synthesized on a NimbleGen maskless array synthesizer as previously described.[7,10] Briefly, a 24-mer oligonucleotide complementary to a stretch of the calmodulin gene of rat was made in an arrangement where multiple groups of 20 × 20 mirror subsets 10 × 10 positive features (separated by one dark feature in each direction) were made with increasing light dose per each irradiation step for the full-length oligo from right to left columns, resulting in 10 identical lines per square of a dose-response curve based on hybridization signals obtained from complementary synthetic Cy-3-labeled oligonucleotide. It is herewith possible to determine optimum irradiation time for each chemistry chosen in a very straightforward and direct manner not having to extrapolate from "off-array" experiments. Best irradiation time at a given light intensity corresponds to the maximum of the hybridization signal (Figures 7–9).

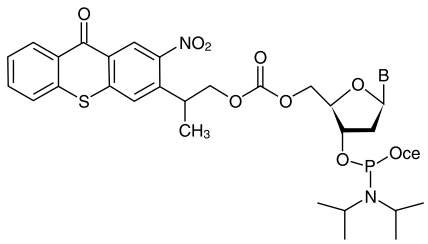

FIGURE 6 5′-[3-Nitrothioxanthone-2-(propyl-2-oxycarbonyl)]-nucleoside-3′-phosphoramidite.

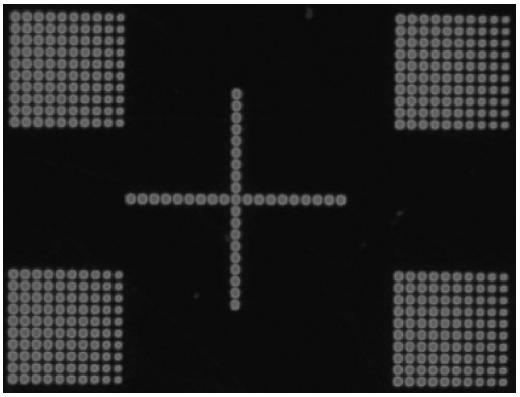

FIGURE 7 Dose matrix (3–21 s) synthesis using NTXPOC-amidites: 5 s best mode.

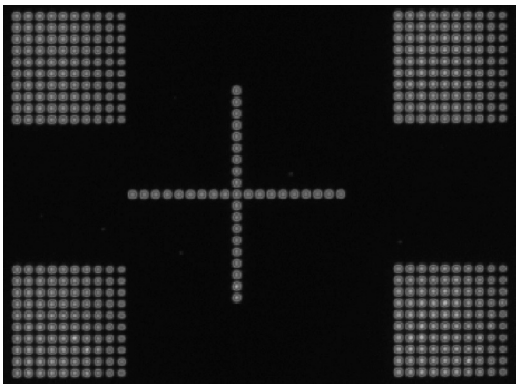

FIGURE 8 Dose matrix (15–60 s) benzoyl-NPPOC-amidites: 35 s best mode.

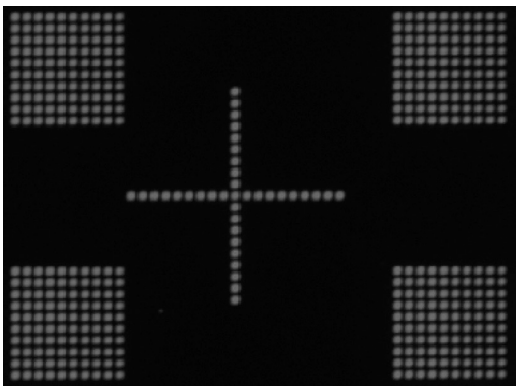

FIGURE 9 Dose matrix (35–80 s) MeNPOC-amidites: 60 s best mode.

SUMMARY

In summary we have developed new photolabile protective groups chemistry that will allow for about 10 times faster deprotection rates in the MAS synthesis cycle. The overall machine time for making a high-density array consisting of 70-mers may thus be reduced by up to 4 h, essentially cut in half. Experiments are now under way to improve synthesis of the new building blocks, optimization of chemistry cycles and validation in a number of different applications. Finally, we will qualify the new synthesis chemistry for routine production in the MAS systems.

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