Next-generation sequencing (NGS) technologies have facilitated important biomedical discoveries, yet high error rates and slow cycle times warrant further improvements in the chemistry.[1a] Such technologies that employ the cyclic reversible termination (CRT) method[1a,b] typically utilize 3’-O-blocked reversible terminators.[2a–c] Recently, we described a novel 3’-OH-unblocked reversible terminator based on 2-nitrobenzyl-modified 5-hydroxymethyl-2’-deoxyuridine (HOMedU) 5’-triphosphate.[3] Our study revealed that the proximity of the 2-nitrobenzyl group to the nucleobase and the size of the alkyl group attached to its α-methylene carbon are important structural features that confer the unique properties of single-base termination, efficient incorporation, and high nucleotide selectivity (i.e., high fidelity) to these 3’-OH-unblocked nucleotides.[3] These properties have the potential to improve accuracy and read-lengths in the CRT method. As HOMedU is a naturally found hypermodified nucleoside,[4a] we set out to identify other such examples. 5-Hydroxymethyl-2’-deoxycytidine (HOMedC) is found naturally in the genomes of T-even bacteriophages[4a,b] and mammals.[5] Pyrrolopyrimidine (7-deazapurine) is also found naturally in nucleoside antibiotics[6] and tRNAs.[7] Thus, various analogues of 2-nitrobenzyl-modified 7-deaza-7-hydroxymethyl-2’-deoxyadenosine (C7-HOMedA),[8] HOMedC, 7-deaza-7-hydroxymethyl-2’-deoxyguanosine (C7-HOMedG),[9] and HOMedU were synthesized with the goal of developing a complete set of reversible terminators (Figure 1).

Ideally, these terminators should exhibit fast nucleotide-incorporation kinetics, single-base termination, high nucleotide selectivity, and rapid terminating group cleavage. For the latter, the degree to which the rate of photochemical cleavage is altered depends on numerous factors including substitution of the benzylic carbon,[10a–c] attachment of functional group(s) to the benzyl ring,[10b–d] and nature of the leaving group,[10a] as well as pH,[10a,d,e] solvent,[10c,f,g] and light intensity.[10e,g] One
property, however, that has not been studied is stereochemistry, whereby substitution of 2-nitrobenzyl’s benzylic or α-carbon results in a chiral center. For the case of nucleotide synthesis, coupling of a racemic α-substituted 2-nitrobenzyl alcohol would result in two diastereomers, which differ only by the absolute configuration (R or S) at the benzylic carbon (+−a) in Figure 1). Here, we describe our efforts toward improving the photochemical-cleavage properties by examining various ring-substituted, stereospecific α-isopropyl- and α-tert-butyl-2-nitrobenzyl-modified reversible terminators.

Unlike our work with α-substituted HOMedU analogues,[3] we identified chromatographic conditions to separate C-HOMedA analogues into single diastereomeric nucleotides, with the first eluting isomer denoted as ds1 and the second as ds2. To evaluate photochemical-cleavage effects, three 2-nitrobenzyl-modified C-HOMedA analogues were synthesized and separated into single diastereomers, dA.III.a (α-isopropyl), dA.III.b (α-isopropyl-4-OMe), and dA.III.c (α-isopropyl-6-NO₂), along with the parent dA.I (see the Supporting Information). These C-HOMedATP analogues were applied in incorporation assays followed by photochemical-cleavage experiments in sodium azide solution (Table 1). In all cases, the ds2 isomers of dA.III.a, dA.III.b, and dA.III.c showed faster photochemical cleavage rates (i.e., lower DT50 values) by factors of 2.0, 6.4, and 1.2, respectively, compared to their ds1 counterparts. Interestingly, the ds1 isomers exhibited similar (dA.III.c) or higher rate increases of 3.6-fold and 4.4-fold, respectively, suggesting that the stereospecific tert-butyl group enhances the effect of the 5-OMe substituent, Hasan et al. reported a rate increase of only 1.2-fold for a 5-Ome-2-nitrobenzyl analogue over its corresponding parent.[10b] Comparison of ds1 and ds2 isomers of dG.V.c with dG.V.a revealed higher rate increases of 3.6-fold and 4.4-fold, respectively, suggesting that the stereospecific tert-butyl group enhances the effect of the 5-Ome group. With four-color CRT applications, this combination provides good flexibility for the utility of the ring system, as a linker can be attached to the 4-position to create dye-labeled analogues.[1a]

To determine the stereochemistry of these α-tert-butyl C-HOMedG analogues, the (1S)-camphanate of (R/S)-1-(5-methoxy-2-nitrophenyl)-2,2-dimethyl-1-propanol was resolved into its enantiopure S alcohol by fractional crystallization[13] (Figure S1 in the Supporting Information). This S alcohol and (S)-α-tert-butyl-2-nitrobenzyl alcohol[9] were each coupled to C-HOMedG (Figure 1). RP-HPLC analysis of their corresponding triphosphates revealed that both ds2 isomers of dG.V.a and dG.V.c have peak retention times identical to those of dG.V and dG.VI, respectively, thus indicating that both ds2 isomers have the same S configuration at the α-carbon. By inference, the corresponding ds1 isomers of dG.V.a and dG.V.c have been assigned the R configuration.

These S alcohols were then coupled to the remaining nucleosides to examine the effect of the nucleotide leaving group on the rate of photochemical cleavage. For example, photochemical-cleavage experiments revealed that DT50 values for the parent 2-nitrobenzyl analogues ranged from 2.0 s for dC.I to 9.2 s for dG.I, suggesting that the leaving group can influence the rate of photochemical cleavage (Figure 2). Substitution of the benzylic carbon with a tert-butyl group in the stereospecific S configuration, denoted simply as (S)-α-tert-butyl, resulted in increased cleavage rates by factors of 1.5–3.1, and the additional substitution with a 5-

Table 1: Rates of photochemical cleavage for C-HOMedA analogues.

| C-HOMedA Analogue | DT50 in 1 mm NaN3 | DT50 in 5 mm NaN3 |
|---|---|---|
| dA.I | 3.6 ± 0.1 | 3.5 ± 0.1 |
| dA.III.a ds1 | 4.5 ± 0.2 | 4.4 ± 0.2 |
| dA.III.a ds2 | 2.2 ± 0.1 | 2.1 ± 0.1 |
| dA.III.b ds1 | 7.0 ± 0.3 | 6.1 ± 0.4 |
| dA.III.b ds2 | 1.1 ± 0.1 | 1.0 ± 0.1 |
| dA.III.c ds1 | 3.4 ± 0.2 | 3.0 ± 0.2 |
| dA.III.c ds2 | 2.8 ± 0.2 | 2.5 ± 0.1 |
| dG.I | 9.2 ± 0.3 | 8.1 ± 0.2 |
| dG.IVa ds1 | 11.0 ± 0.4 | 10.7 ± 0.2 |
| dG.IVa ds2 | 3.6 ± 0.3 | 3.5 ± 0.3 |
| dG.IVb ds1 | 4.9 ± 0.3 | 4.6 ± 0.3 |
| dG.IVb ds2 | 1.1 ± 0.1 | 1.3 ± 0.2 |
| dG.IVc ds1 | 3.5 ± 0.3 | 3.0 ± 0.1 |
| dG.IVc ds2 | 0.8 ± 0.1 | 0.8 ± 0.1 |
| dG.IVd ds1 | 2.4 ± 0.1 | 2.3 ± 0.2 |
| dG.IVd ds2 | 0.8 ± 0.1 | 0.8 ± 0.1 |

[a] A DT50 value is defined as the point in time at which 50% of the 2-nitrobenzyl groups have been photochemically cleaved from the extended primer/template complex. Lower DT50 values indicate faster photochemical cleavage rates. DTT = dithiothreitol.
OMe group further increased rates by factors of 3.0–11.5 compared with the parent analogues. The greatest rate improvement was observed in the set of C7-HOMedG analogues, for which DT_{50} values were reduced from 9.2 to 0.8 s (Figure 2, blue bars). The complete set of (S)-α-tert-butyl-5-OMe reversible terminators showed a more narrow range of DT_{50} values from 0.6 to 0.8 s. These data suggest that the combined effects of the (S)-α-tert-butyl and 5-OMe groups play an important role in diminishing the variation in cleavage rates observed with particular nucleotide leaving groups, which has the practical application of providing normalized and faster cleavage conditions for the CRT cycle.

Following brief exposure to UV light, transient products were observed from incorporation assays for (S)-α-tert-butyl-5-OMe-C7-HOMedA, -HOMedC, and -HOMedU (Figure 3A), but not for C7-HOMedG. As the only difference was the just-incorporated nucleotide, we hypothesize that the faster cleaving (S)-α-tert-butyl-5-OMe-2-nitrobenzyl group produces a more reactive 2-nitrosoketone by-product, which attacks the 3-terminal nucleotide of the growing primer strand. To investigate conditions for quenching the nitroso intermediate, a number of amino and thiol agents were tested (Figure S2 in the Supporting Information). Of these, only dithiothreitol (DTT) eliminated the transient product (Figure 3B). To test rate effects, photochemical-cleavage experiments were repeated for all compounds in the presence of DTT, of which DT_{50} values for several parent and ds1 isomers were reduced (Tables 1 and 2, and Table S3). Corrie and colleagues proposed that DTT attacks the nitroso group with nucleaseic action, thereby, providing in our case protection against such undesired reactions.

We have demonstrated that the stereospecific S configuration of an α-tert-butyl group and the ring modification of a 5-OMe group are major determinants for creating a complete set of fast-cleaving reversible terminators with normalized rates. We believe this stereospecific effect, however, is not limited to just an α-tert-butyl group. We have shown several examples of α-isopropyl ds2 isomers, presumed to have S configuration, which also have faster rates for photochemical cleavage than their ds1 isomers. In the presence of DTT, the reactive nitrosoketone by-product can be eliminated effectively during photochemical cleavage. The complete set of (S)-α-tert-butyl-5-OMe-2-nitrobenzyl-modified nucleotides also exhibit single-base termination, fast incorporation kinetics, and high nucleotide selectivity (unpublished results). Thus, this work not only expands the repertoire of 2-nitrobenzyl modifications that yield faster-cleaving protecting groups, but when coupled with reversible terminators, yield faster cycle times for NGS instrument systems.

Keywords: cleavage reactions · nucleotides · photochemistry · reversible terminators

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