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Synthesis of a phosphoramidate pro-drug of 6-thio-7-deaza-2′-deoxyguanosine (TDG): a regioselective phosphorylation

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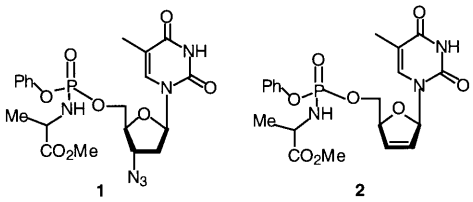
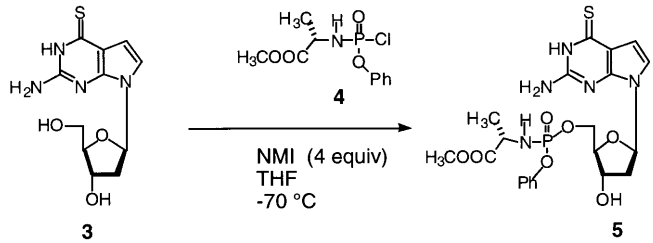
Abstract—6-Thio-7-deaza-2′-deoxyguanosine-5′-triphosphate (TDG-TP) is a potent inhibitor of human telomerase. Regioselective synthesis of the 5′-phenyl methoxyalaninyl phosphate pro-drug of 6-thio-7-deaza-2′-deoxyguanosine (TDG) has been achieved in good yields by the reaction of TDG with phenyl methoxyalaninyl phosphochloridate in the presence of *N*-methylimidazole at −70°C. This pro-drug of TDG is effective in producing measurable levels of TDG-TP in A549 cells.

There has been continued interest in the synthesis and biological evaluation of nucleoside analogs capable of delivering the corresponding nucleotides inside cells.[1] Of particular interest are the (aryloxy)phosphoramidate nucleotide monophosphate pro-drugs pioneered by McGuigan and co-workers.[2] The (aryloxy)phosphoramidates of AZT (**1**) and d4T (**2**) bypass the dependence of the parent nucleosides on thymidine kinase-mediated activation, displaying full activity in thymidine kinase-deficient cells.[2,3] We have recently shown that 6-thio-7-deaza-2′-deoxyguanosine-5′-triphosphate (TDG-TP) is a very potent inhibitor of human telomerase, with an IC_{50} of 60 nM (Fig. 1).[4] Here we report the regioselective synthesis of the 5′-(phenyl methoxyalaninyl)-phosphate derivative of 6-thio-7-deaza-2′-deoxyguanosine (TDG), and the ability of this TDG pro-drug, when incubated with A549 lung cancer cells, to afford detectable levels of intracellular TDG-TP.

TDG (**3**)[6,7] (2-amino-9-(2′-deoxy-β-D-erythro-pentofuranosyl)-7H-pyrrolo[2,3-d]pyrimidin-6-thione; 6-thio-7-deaza-2′-deoxyguanosine) was prepared from 3′,5′-ditoluyl protected intermediate as previously reported.[4] Phenyl methoxyalaninyl phosphochloridate (**4**) as a 1:1 diastereoisomeric mixture was prepared as reported[2] by the reaction of *L*-alanine methyl ester hydrochloride with phenylphosphorodichloridate in the presence of triethylamine in dry dichloromethane. While previous workers have employed this material after precipitation of the triethylamine hydrochloride with ether,[2] we have found that the regioselective coupling described here proceeds much better when the phenyl methoxyalaninyl phosphochloridate prepared in this fashion is further purified by rapid flash silica gel column chromatography using dichloromethane as the eluant. The purified material was characterized by ^{1}H, ^{13}C, ^{31}P NMR and HRMS and was stable for weeks when stored under an inert atmosphere in the freezer.[8]





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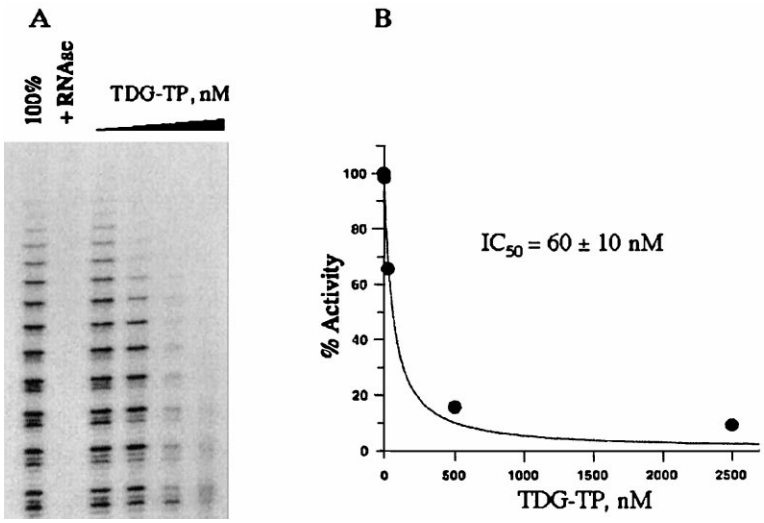

Fig. 1. Inhibition of human telomerase by TDG-TP. A. PAGE of telomerase reactions[5] employing 5′-biotinylated $d(TTAGGG)_3$ primer, dATP, dTTP, [α-^{32}P]dGTP, and crude telomerase extract from HeLa cells in the presence of RNase or increasing concentrations of TDG-TP. B. Plot of telomerase inhibition data from A.

To a solution of 50 mg of **3** in 10 ml THF and 4 equiv. of *N*-methylimidazole (NMI) at −70°C was slowly added a solution of 1.2 equiv. of **4** in 10 ml THF. Immediate evaporation of the reaction mixture followed by column chromatography of the residue afforded the phosphoramidate derivative **5** in 60% yield. The phosphoramidate **5** was isolated as an inseparable 2:1 mixture of diastereoisomers due to the phosphate stereocenter.[9] The direct conversion of **3** to **5** is noteworthy in that the selective coupling of the 5′-hydroxyl group proceeds in better yields than for previously reported 5′-selective couplings involving 3′-unprotected pyrimidine nucleosides,[10,11] despite the presence of other potentially nucleophilic sites on the purine base in the case of **3** (e.g. 6-thio and 2-amino groups). The clean conversion of **3** to **5** could only be achieved at −70°C; reactions performed at room temperature or 0°C resulted in the formation complex mixtures of products including the 3′,5′-bis(phosphoramidate) along with small amounts of **5**. We also attempted the preparation of **5** by treating a THF/pyridine solution of **3** with 2 equiv. of *t*BuMgCl in THF at room temperature, followed by the addition of 1 equiv. of **4**; however, this procedure only led to very low (ca. 9%) and variable yields of the desired phosphoramidate **5**.

We incubated 2×10^6 A549 human lung cancer cells with various concentrations (20–100 μM) of TDG (**3**) for 4–24 h to determine the amount of TDG-TP formed intracellularly. After incubation, the cells were rinsed (PBS), trypsinized, collected, and lysed in $HClO_4$ followed by centrifugation. HLPC analysis (Partisil-10 SAX anion exchange, ammonium phosphate buffer gradient pH 2.8–3.7, UV detection 344 nm) of the neutralized supernatant showed no measurable intracellular TDG-TP. In contrast, A549 cells treated with 20–100 μM of **5** showed measurable levels of TDG-TP after 4–24 h of incubation (Fig. 2).

We are currently investigating the biological effects of the TDG pro-drug **5** in vitro and in vivo, and will report on this work in due course.

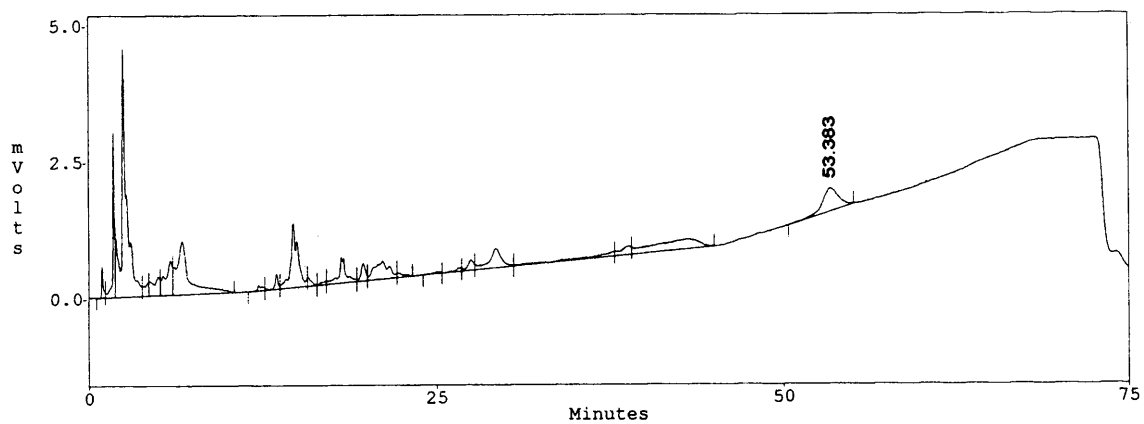

Fig. 2. HPLC chromatogram of the supernatant from A549 cells incubated with 80 μM 5 for 4 h demonstrating the presence of TDG-TP (peak at 53.38 min, confirmed by MS and co-elution with authentic material).

Acknowledgements

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8. Phosphochloridate **4** was isolated as a colorless oil in 70% yield: ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.37–7.32 (m, 2H), 7.25–7.18 (m, 3H), 4.44–4.36 (m, 1H), 4.23–4.10 (m, 1H), 3.77, 3.75 (s X 2, 3H), 1.49, 1.47 (dd X 2, 3H, J=7.1, 0.8 Hz). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 171.08–171.20, 149.66, 129.88, 125.96, 120.53–120.47, 52.75–52.82, 50.32–50.62, 20.47–20.54. ^{31}P NMR (121.5 MHz, $CDCl_3$): δ 8.74, 8.50. CIMS m/z: 278/280 (3:1, MH^+); CIHRMS m/z calc'd for $C_{10}H_{14}ClO_4P$: 278.0348, found 278.0344.
9. Compound **5** was isolated as a colorless glass: ^{1}H NMR (500 MHz, DMSO-d_6) [the resonances for the minor diastereomer, when resolved, are indicated by an asterisk]: δ 7.38–7.32 (m, 2H, C_6H_5 (m)), 7.21–7.13 (m, 3H, C_6H_5 (o,p)), 6.99, 6.97* (d X 2, 1H, J=3.8 Hz, 5-H), 6.61 (br s, 2H, NH_2), 6.39, 6.36* (d X 2, 1H, J=3.7 Hz, 6-H), 6.32–6.28 (m, 1H, 1′-H), 6.05–5.99 (m, 1H, NH-Cα), 5.39 (br s, 1H, 3′-OH), 4.32 (br s, 1H, 3′-H), 4.16–4.07, 4.04–4.00* (m X 2, 2H, 5′-H), 3.98–3.96, 3.95–3.92* (m X 2, 1H, 4′-H), 3.88–3.78 (m, 1H, HCα), 3.57, 3.56* (s X 2, 3H, OCH_3), 2.36–2.26 (m, 1H, 2′-H_a), 2.14–2.09 (m, 1H, 2′-H_b), 1.21, 1.18* (d X 2, 3H, J=7 Hz, CH_3). ^{13}C NMR (125.7 MHz, DMSO-d_6): δ 175.8 (CS), 173.6. 173.5* (CO), 152.2 (C2), 150.7 (C_6H_5(i)), 147.1 (C4), 129.5 (C_6H_5(m)), 124.5 (C_6H_5(p)), 120.0 (C_6H_5(o)), 119.8 (C8), 113.0 (C5), 104.5 (C7), 84.4 (4′), 82.3 (1′), 70.6 (3′), 66.1*, 66.0 (5′), 51.8 (OCH_3), 49.7, 49.6* (Cα), 39.8 (2′), 19.6*, 19.5 (CH_3). ^{31}P NMR (121.5 MHz, $CDCl_3$): δ 4.92, 4.78. CIMS m/z: 524 (MH^+); CIHRMS m/z calc'd for $C_{21}H_{27}N_5O_7PS$: 524.1368, found 524.1371.
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