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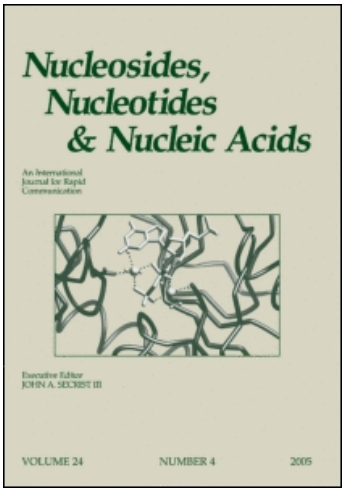

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Efficient Large-Scale Synthesis of 5'-*O*-Dimethoxytrityl-N^4-Benzoyl-5-Methyl-2'-Deoxycytidine

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EFFICIENT LARGE-SCALE SYNTHESIS OF 5′-*O*-DIMETHOXYTRITYL-*N*^{4}-BENZOYL-5-METHYL-2′-DEOXYCYTIDINE

Bruce S. Ross, Mingming Han, and Vasulinga T. Ravikumar □ *Isis Pharmaceuticals, Inc., Carlsbad, California, USA*

□ *An efficient process to synthesize 5′-O-dimethoxytrityl-N^4-benzoyl-5-methyl-2′-deoxycytidine in high yield and quality is described. Final benzoylation was improved by developing a method to selectively hydrolyze benzoyl ester impurities. This inexpensive approach was scaled up to multi-kilogram quantities for routine use in oligonucleotide therapeutics.*

Keywords Oligonucleotides; Antisense; 5′-*O*-DMT-N^4-Benzoyl-5-Methyl-2′-Deoxycytidine; Deoxynucleoside

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INTRODUCTION

Phosphorothioate-linked nucleic acid analogs have found widespread application in therapeutic drug development and molecular biology.[1–3] Increased resistance to nuclease digestion displayed by phosphorothioate diester DNA and RNA analogs has prompted use of these molecules for current and investigational antisense treatment of a variety of diseases.[4,5] Several antisense phosphorothioate oligodeoxyribonucleotides (ODN) are currently undergoing clinical evaluation and an antisense drug for treatment of CMV retinitis (Vitravene) has reached the market. Although phosphorothioate ODN are showing excellent promise as safe and effective therapeutic agents, their profiles are not yet ideal. Reported high-dose effects of phosphorothioate ODN include immune cell stimulation, complement activation, and introduction of blood clotting abnormalities. Although these effects are seen only at doses of oligonucleotide above those required for pharmacological activity, investigations of chemical modifications to enhance therapeutic index and facilitate delivery have been pursued. Chemical modification of antisense oligonucleotides can confer additional

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resistance to nucleases, longer serum half-life, and reduced toxicity. Modifications can also increase affinity of an antisense oligonucleotide for its complementary target RNA, resulting in enhanced potency and specificity. One among various modifications to be largely explored is the use of 5-methyl cytosine and its deoxy analog. 5-Methyl-2′-deoxycytidine has been shown to possess interesting biological properties that have been reviewed extensively.[6–8]

Although 5-methyl-2′-deoxycytidine can be isolated from salmon sperm DNA, the quantity is limited due to low percentage content. The previously reported[9–13] routes involve drastic conditions such as reflux at 140°C for 72 h or the use of sodium hydride, which is not desirable in large-scale synthesis. Recently an efficient amination method was reported by Komatsu *et al.*[14] A much simpler alternative method involving amination of 5′-*O*-DMT thymidine is described here. In addition, use of DMT thymidine as starting material (current procedure) instead of thymidine (literature procedure) for the amination step affords easy organic extraction of product. Synthesis of the title compound is shown in Scheme 1.

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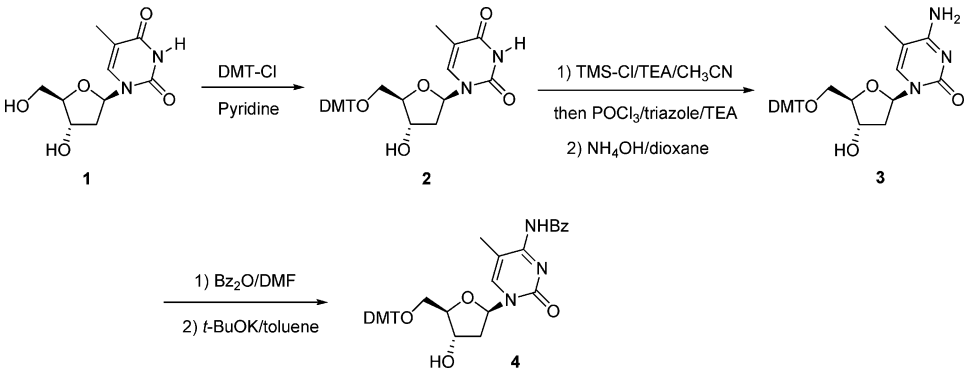
EXPERIMENTAL

General Methods

Melting points (uncorrected) were recorded using a Mel-Temp apparatus. TLC was performed on Silica Gel 60 precut plates (240–400; Merck). Reagent grade trimethylsilyl chloride, phosphorous oxychloride, 1,2,4-triazole, 4,4′-dimethoxytrityl chloride, pyridine, sodium hydrogen carbonate, ammonium hydroxide, dichloromethane, triethylamine, ethyl acetate, hexanes, methanol, and anhydrous *N*,*N*-dimethylformamide were purchased from Aldrich Chemical Company, Inc., and used without further purification. Thymidine was purchased from Samchully Pharmaceuticals,



SCHEME 1 Synthesis of 5′-*O*-DMT-N^4-benzoyl-2′-deoxycytidine.

South Korea, and benzoic anhydride was purchased from Chem Impex Inc. Low water acetonitrile (<50 ppm) was purchased from Burdicken and Jackson and used without further purification. Note; Trimethylsilyl chloride and phosphorous oxychloride are corrosive and the reaction should be conducted in a well-ventilated hood.

5′-O-Dimethoxytritylthymidine (2). A 50-L glass reactor equipped with air stirrer and argon gas line was charged with thymidine (1.0 kg, 4.13 mol) and anhydrous pyridine (6 L) at ambient temperature. 4,4′-Dimethoxytrityl chloride (1.47 kg, 4.34 mol) was then added as a solid in four portions over 1 h. After an additional 30 min, TLC indicated about 95% product, 2% thymidine, 5% DMT chloride and by-products, and 2% 3′,5′-*bis-O*-dimethoxytritylthymidine (Rf in ethyl acetate 0.45, 0.05, 0.98, and 0.95, respectively.). Saturated aqueous sodium bicarbonate solution (4 L) and dichloromethane were added with stirring. An additional 18 L of water was added. After stirring and allowing the phases to separate, the organic layer was transferred to a second 50-L vessel. The aqueous layer was extracted with additional dichloromethane (2 × 2 L). The combined organic layer was washed with water (10 L) and then concentrated in a rotary evaporator to a foamy material. This foam was redissolved in dichloromethane (3.5 L), and added to the reactor followed by water (6 L) and hexanes (13 L). The mixture was vigorously stirred and seeded to give a fine white suspended solid at the interface. After stirring for 1 h, the suspension was removed by suction through a 1/2-inch-diameter Teflon tube into a 20-L suction flask. From the suction flask the suspension was poured onto a 25-cm Coors Buchner funnel, washed with water (2 × 3 L) and a mixture of hexanes-dichloromethane (4:1, 2 × 3 L), and allowed to air dry overnight in stainless steel pans (1-inch deep). The product was further dried in a vacuum oven (75°C/0.1 mm) for 48 h to a constant weight (2.07 kg; 93%) of a white solid; mp 122–124°C. TLC indicated trace contamination by 3′,5′-*bis-O*-dimethoxytritylthymidine.

5′-O-Dimethoxytrityl-2′-deoxy-5-methylcytidine (3). A 50-L Schott glass-lined steel reactor equipped with an mechanical stirrer, reagent addition pump (connected to an addition funnel), heating/cooling system, internal thermometer, and argon gas line was charged with 5′-*O*-dimethoxytritylthymidine (3.00 kg, 5.51 mol), anhydrous acetonitrile (25 L), and triethylamine (12.3 L, 88.4 mol). The mixture was chilled with stirring to −10°C. Trimethylsilyl chloride (2.1 L, 16.5 mol) was added over 30 min while maintaining the temperature below −5°C, followed by a wash of anhydrous acetonitrile (1 L). The reaction was mildly exothermic and copious hydrochloric acid fumes were liberated over the course of addition. The reaction was allowed to warm to 0°C and the progress confirmed by TLC (Rf of starting material to trimethylsilyl product was 0.43 to 0.84 in ethyl acetate–hexanes 4:1). Upon completion, 1,2,4-triazole (3.05 kg, 44 mol) was added to reaction mixture that was cooled to −20°C. Phosphorous

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oxychloride (1035 mL, 11.1 mol) was added over 1 h to maintain the temperature between −20°C and −10°C, followed by a wash of anhydrous acetonitrile (1 L). The reaction was warmed to 0°C and allowed to stir for 1 h. TLC indicated a complete conversion to the 1,2,4-triazole product (Rf 0.83 to 0.34 with the product spot glowing in long UV light). The reaction was cooled to −15°C and water (5 L) was slowly added at a rate to maintain the temperature below 10°C (caution—initially very exothermic) in order to quench the reaction and to form a homogenous solution. Approximately one half of reaction volume (22 L) was transferred by air pump to a first work-up vessel. Ethyl acetate (12 L) and water (8 L) were added and the mixture was stirred and the water layer transferred to a second work-up vessel. The organic layer was washed with additional water (8 L). The combined water layer was back-extracted with ethyl acetate (6 L). The water layer was discarded and the organic layers were combined and concentrated in a 20-L rotary evaporator to a foam. The foam was co-evaporated once with anhydrous acetonitrile (4 L) to remove ethyl acetate.[15] The second half of the reaction was treated in the same way and concentrated in a separate flask. Each residue was dissolved in 1,4-dioxane (3 L) and concentrated aqueous ammonium hydroxide (750 mL) was added. The solution became homogenous in a few minutes and the reaction was allowed to stand overnight.[16] TLC indicated the reaction to be complete (product Rf 0.35 in ethyl acetate-methanol 4:1). The reaction mixture was concentrated on a rotary evaporator to a dense foam, redissolved in ethyl acetate (4 L), transferred to a 50-L glass reactor vessel, and extracted with water (2 × 4 L) to remove 1,2,4-triazole. The water was back-extracted with ethyl acetate (2 L). The organic layers were combined, concentrated to about 8 kg total weight, and cooled to 0°C. After overnight standing, the first crop was collected on a 25-cm Coors Buchner funnel and washed repeatedly with ethyl acetate until a white free-flowing powder remained (3 × 3 L) and then with ethyl ether (2 × 3 L). The solid was transferred to stainless steel pans (1-inch deep) and allowed to dry overnight. The filtrate was concentrated to an oily product, redissolved in ethyl acetate (2 L), cooled, and seeded with a sample of pure compound. The second crop was collected and washed as before (with solvents proportional to those used for the first crop). The filtrate was extracted and seeded as before one more time to yield a third crop. After air drying, the three crops were separately dried in a vacuum oven (50°C, 0.1 mm, 24 h) to a constant weight of 1750, 600, and 200 g, respectively. TLC and ^{1}H NMR of the three crops were comparable and were combined for a total of 2.55 Kg (85%) as fine white crystals.[17] The mother liquor still contained product with minor impurities. If desired, the mother liquor may be purified by silica gel chromatography using a gradient of methanol (0–25%) in ethyl acetate to increase the yield.

Preparation of 5′-O-Dimethoxytrityl-2′-deoxy-N^4-benzoyl-5-methyl-2′-deoxycytidine (4). 5′-*O*-Dimethoxytrityl-5-methyl-2′-deoxycytidine (1500 g, 2.76 mol)

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was dissolved in anhydrous DMF (4.5 kg) at ambient temperature in a 50-L glass reactor vessel equipped with air stirrer and argon line. Benzoic anhydride (749 g, 3.31 mol) was added and the reaction was stirred at ambient temperature for 22 h. TLC (0.25 Rf, CH_2Cl_2-ethyl acetate 4:1) indicated about 98% complete reaction. The reaction mixture was diluted with toluene (9 L) and then triethylamine (722 mL, 5.52 mol) was added. After stirring for 10 min, water (10 L) was added. The mixture was stirred, allowed to separate, and the aqueous layer (14 L) was removed. The organic layer was washed two more times (2 × 10 L) in the same manner. Finally, the organic layer (ca. 11 L) was dried with anhydrous sodium sulfate (500 g for 0.5 h) and concentrated in a rotary evaporator (20 L) until about 7 L solvent was removed. The residue was transferred to a 12-L three-necked reaction flask equipped with a mechanical stirrer and diluted with 6 L toluene. Potassium *tert*-butoxide (500 g, 4.45 mol) was added over a period of 10 min. The reaction mixture was stirred for 15 h at ambient temperature under an argon atmosphere. A pink color developed during this period of time and the solution became viscous and difficult to stir. Another portion of potassium *tert*-butoxide (140 g, 1.25 mol) was added over a period of 5 min and the reaction mixture was stirred for an additional 24 h. The reaction mixture was washed with 10% citric acid solution (2 × 9 L), 5% aqueous sodium bicarbonate solution (8 L), and water (2 × 10 L). The organic layer was concentrated in a rotary evaporator flask (20 L) to remove about half of the solvent. The partially concentrated solution was run through a short silica gel column (2.5 kg of silica gel in a 6-L sintered glass funnel), washing with a mixture of ethyl acetate-hexane (1:1) until the eluate became nearly light and no polar impurity had come through. The eluate was concentrated until the formation of foam. The white foam (1460 g, 82% yield, 99.5% purity by HPLC) was obtained as the final product after drying (0.1 mmHg, 25°C) for 24 h.

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15. 1,4-Dioxane may be used instead of acetonitrile.
16. The reaction with ammonium hydroxide is usually complete in 1 h.
17. The product had a melting point between 215 and 217°C.

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