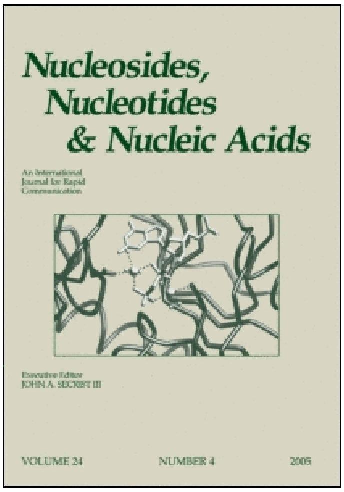

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A CONVENIENT HIGH YIELD SYNTHESIS OF N^4-ISOBUTYRYL-2'-O-METHYLCYTIDINE AND ITS MONOMER UNITS FOR INCORPORATION INTO OLIGONUCLEOTIDES

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ABSTRACT

We designed an efficient three step procedure for the synthesis of N^4-isobutyryl-2'-O-methylcytidine. This protected nucleoside was then used to prepare a methylphosphonamidite monomer for incorporation into oligonucleotides. Transamination at the C^4 position of cytidine using ethylenediamine, which has been reported for the N^4-benzoyl cytidine, was not observed with N^4-isobutyryl protected 2'-O-methylcytidine .

Introduction

Cytidine methylphosphonamidites protected with benzoyl at the N^4 position are conventionally used in oligonucleotide synthesis[1]. The N^4-benzoyl protecting group of cytidine undergoes unwanted nucleophilic displacement when treated with bases used for deprotection[2]. The reagent of choice for the deprotection of natural (phosphodiester) oligonucleotides is ammonium hydroxide in which the nucleophile is ammonia, therefore yielding natural product. In the case of methylphosphonate oligonucleotides, deprotection with ammonium hydroxide results in severe backbone cleavage; therefore, ethylenediamine (EDA) is preferably used for deprotection[3]. We found that up to 15% transaminated (EDA) adduct per base can be obtained when N^4-benzoyl protected cytidine is used in methylphosphonate oligonucleotide synthesis and deprotected with EDA[4]. We resolved this transamination problem by protecting the N^4 position of cytidine with isobutyryl instead of benzoyl. In this paper we report an efficient and simple procedure for the synthesis of 5'-O-dimethoxytrityl-N^4-isobytyryl-2'-O-methylcytidine-3'-O-(methyl-N,N-diisopropylaminophosphine) with the N^4-isobutyryl protecting group.

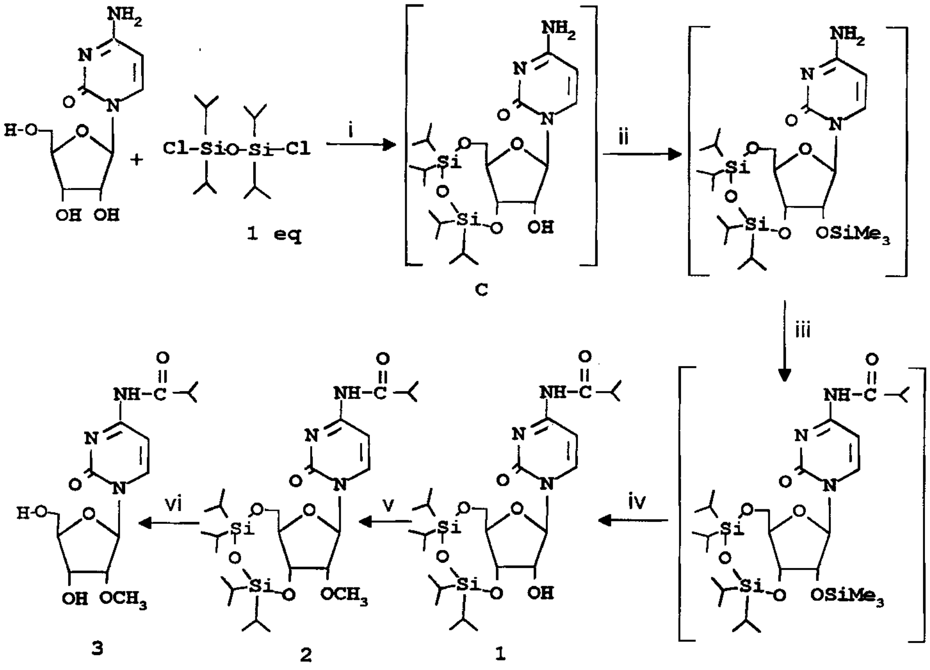
Results

N^4-Isobutyryl-3',5'-O-(tertaisopropyldisiloxane-1,3-diyl)-cytidine (**1**) (Scheme 1) was prepared by condensation of cytidine with one equivalent of dichloro-1,1,3,3-tertaisopropyldisiloxane (DCTPS) at room temperature for 2 hours[5]. To this mixture, 0.5 equivalent of hexamethyldisilazane (HMDS) was added and stirred for 1 hr. Quantitative trimethylsilylation of the 2'-hydroxyl was achieved as determined by TLC. This mixture reacted with 2 equivalents of isobutyryl chloride and 2 equivalents of triethylamine to give quantitative blocking of the exocyclic amine as observed by TLC. The 2'-silyl group was then removed by treatment with methanol/water. The product was purified to give 89% yield of **1** which was >95% pure and was used in the next reaction without further purification.

Methylation of the 2'-hydroxyl was accomplished by using methyl iodide/silver oxide according to the procedure of Inoue, H. *et al*[6] which, after column purification, yielded 97% of N^4-isobutyryl-2'-O-methyl-3',5'-O-(tertaisopropyldisiloxane-1,3-diyl)-cytidine (**2**) which was 98% pure as determined by analytical HPLC. A one-pot synthesis of N^4-benzoyl-2'-O-methylcytidine is reported in which mainly 2'-position and to some extent 3'-position was methylated by diazomathane and after crystalization of the 2'-methoxy analog gave 57% overal yield[7]. By our procedure methylation only occures at the 2'-position and with higher overall yield.

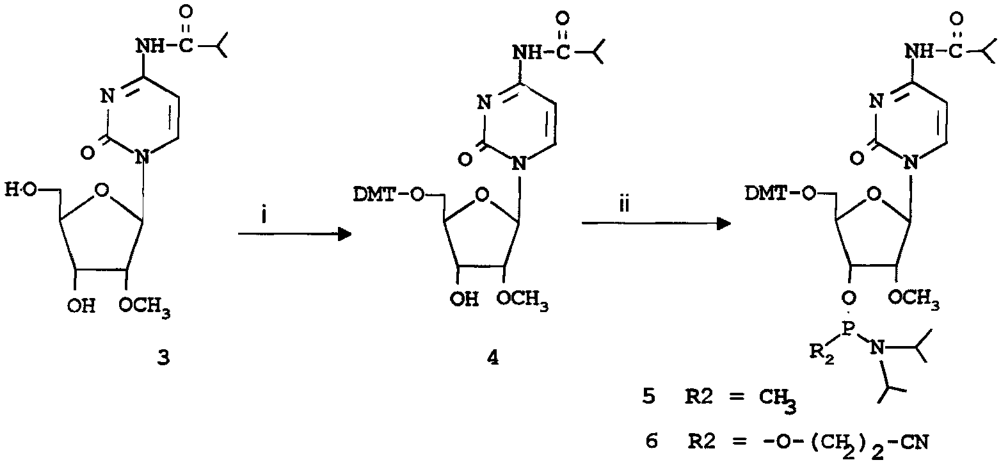
Disiloxane was then removed from compound **2** with a freshly prepared 1M solution of triethylamine hydrofluoride in dioxane/-tetrahydrofuran (1:1) pH 7[8]. N^4-Isobutyryl-2'-O-methylcytidine (**3**) was analyzed by NMR and elemental analysis.

Compound **3** was blocked at the 5' position using dimethoxytrityl chloride according to published procedures[9]. The 5'-O-dimethoxytrityl-N^4-isobutyryl-2'-O-methylcytidine (**4**) was then phosphinylated at the 3'-position with chloro-N,N-diisopropylaminomethylphosphine to make 3'-(N,N-diisopropylaminomethylphosphonamidite)-5'-O-dimethoxytrityl-N^4-isobutyryl-2'-O-methylcytidine (**5**) (Scheme 2). Reaction of **4**



Scheme 1

(i) Pyridine, room temp; (ii) 0.5 eq. hexamethyldisilazane; (iii) 2 eq. triethylamine, 2 eq. isobtyryl chloride r.t.; (iv) Methanol/water (1:1); (v) Benzene, CH_3I, Ag_2O, r.t. 2hr; (vi) Triethylamine hydrofluoride, THF, r.t. overnight.



Scheme 2

(i) Pyridine, dimethoxytrityl chloride; (ii) CH_2Cl_2, diisopropylethylamine, $[(CH_3)_2CH]_2NP(CH_3)Cl$.

with 2-cyanoethoxy-N,N-diisopropylamino-chlorophosphine under similar conditions could produce the phosphoramidite analog of N^4-isobutyryl-2'-O-methylcytidine (**6**) for phosphodiester DNA synthesis.

Compound **5** was then incorporated into oligonucleotides using an automated DNA-synthesizer.[10] The coupling efficiency of this monomer was comparable to those of commercial monomers used for DNA synthesis.

Experimental

Materials

Solvents were dried or distilled before use. Chloro-N,N-diisopropylaminomethylphosphine was from JBL Scientific, San Louis Obispo, CA. TLC was done on precoated silica gel 60 F_{254} plates from EM Sciences, Gibbstown, NJ. Column chromatography was done with Silica gel 60 (230-400 mesh) from EM Sciences. U.V. spectrum was obtained on a Milton Roy Spectronic 3000 and the NMR spectra were obtained on a Bruker 360 mHz instrument. Analytical HPLC was done with a Beckman System Gold with 126 pump and Whatman RAC II C18 reverse-phase column (4.6x10cm).

Method

N^4-Isobutyryl-3',5'-O-(tertaisopropyldisiloxane-1,3-diyl)-cytidine (**1**). In a 1 l round bottom flask, 30 g (123 mmole) of cytidine was dried by co-evaporation with pyridine and dissolved in 350 ml of anhydrous pyridine. To this solution 39 g (123 mmole, 1eq.) of 1,3-dichloro-1,1,3,3-tertaisopropyldisiloxane (DCTPS) was added slowly and the reaction mixture was stirred at r.t. until the completion of the reaction (2 hr) was observed by TLC (5% methanol in dichloromethane). To this mixture, 9.96 g hexamethyldisilazane (HMDS) (62 mmole, 0.5 eq.) was added and stirred for 1 hr. Quantitative trimethylsilylation of 2'-hydroxyl was achieved as observed by TLC (60% ethyl acetate in heptane). To this reaction mixture, 2 equivalents of triethylamine and 2 equivalents of isobutyryl chloride (26.2 g) was added and stirring continued at r.t. for another 2 hr. Quantitative blocking of the exocyclic amine was observed by TLC (60% ethyl acetate in heptane). The 2'-silyl group was then removed by addition of 60 ml of methanol/water (1:1) in one hour. The solvents were removed by rotary evaporator and the residue was dissolved in 300 ml of dichloromethane. The mixture was extracted with 2x50 ml of saturated sodium hydrogen carbonate solution and washed with 50 ml of water. The organic solution was dried ($MgSO_4$) and evaporated to give 63 g of a product which was >95% pure by TLC using 5% methanol in dichloromethane (Rf = 0.4) (89% yield). A 2 g sample of this material was purified on a flash silica gel column for analytical purpose and the bulk of the product was used in the next reaction without further purification . ^{1}H-NMR ($CDCl_3$): 8.48 (s, 1H N-H), 8.19 (d, 1H), 7.43 (d, 1H, $J_{5,6}$ = 7.44 Hz), 5.84 (s 1H, H1'), 4.30-3.99 (m 5H, 2',3',4' and 5'H), 3.14 (s 1H, OH), 2.64 (m, 1H. C-H of ibu), 1.24 (d 6H, CH_3 of ibu, $J_{CH3,CH}$ =7.9 Hz), . Elemental analysis calc. for $C_{25}H_{45}N_3O_7Si_2$: C, 54.02; H, 8.16; N, 7.56. Found: C, 54.21; H, 8.33; N, 7.29.

N^4-Isobutyryl-2'-O-methyl-3',5'-O-(tertaisopropyldisiloxane-1,3-diyl)-cytidine (**2**). In a 1l round bottom flask 55 g of **1** (0.1 mole) was dissolved in 350 ml of benzene. To this solution 284 g of methyl iodide (20 eq., 2 moles) and 69.5 g of silver oxide (3 eq., 0.3 mole) were added and stirred at r.t. for 2 hours. The progress of the reaction was monitored by TLC. Quantitative methylation was observed in 2 hours. The solvent was removed by evaporation and the residue was taken up in 200 ml of methylene chloride and filtered through a layer of celite. The clear solution was extracted with 2x50 ml of water. The organic phase was separated and dried ($MgSO_4$) and loaded on a layer of 230-400 mesh silica gel and washed with 2% methanol in dichloromethane. A trace of silver oxide was identified in this product which was removed by extraction with 0.5 M ammonium chloride solution (2x50 ml extractions). The solution was then evaporated to dryness which gave 56.9 g of 98% pure **2** by TLC (5% MeOH/CH_2Cl_2, Rf = 0.5). Yield 97%. This product was taken to the next step without further purification. A 2 g sample was further purified on a silica gel column for analytical evaluation. ^{1}H-NMR ($CDCl_3$)8.53 (s, 1H N-H), 8.23 (d, 1H, $J_{5,6}$ = 7.4 Hz, H6), 7.41 (d, 1H, $J_{5,6}$ = 7.4 Hz, H5), 5.85 (s 1H, H1'), 4.31-3.76 (m 5H, 2',3',4' and 5'H), 3.76 (s, 3H, 2'-OCH_3), 2.62 (m, 1H. C-H of ibu), 1.25 (d 6H, CH_3 of ibu, $J_{CH3,CH}$ =7.9 Hz), 1.11-0.92 (28H, all of the tertaisopropyl-disiloxane protons). Analysis calc. for $C_{26}H_{47}N_3O_7Si_2$: C, 54.8; H, 8.31; N, 7.37. Found: C, 54.84; H, 8.47; N, 7.11.

N^4-Isobutyryl-2′-O-methylcytidine (**3**). Triethylamine hydrofluoride was freshly prepared according to the procedure of Markiewics et al[7]. To 52 g of **2** above 200 ml of the fluoride solution was added and stirred at r.t. overnight. TLC showed a major product and two minor spots which ran faster on silica gel. The solvent was removed by rotary evaporator and the product was purified on a flash silica gel column which was eluted with 5% methanol in dichloromethane to give 22.4 g of product (yield 75%). This compound was pure by TLC (5% methanol in dichloromethane, Rf = 0.15). ^{1}H-NMR ($CDCl_3$)8.51 (s, 1H N-H), 8.28 (d, 1H, $J_{5,6}$ = 7.41 Hz, H6), 7.41 (d, 1H, $J_{5,6}$ = 7.41 Hz, H5), 5.83 (s 1H, H1'), 5.02 (t, 1H, $O\underline{H}_{5'}$), 4.66 (d, 1H, $O\underline{H}_{3'}$), 4.68-3.65 (m, 4H, 2',3',4' and 5'H), 3.54 (s, 3H, 2'-$OC\underline{H}_3$), 2.66 (m, 1H. C-$\underline{H}$ of ibu), 1.05 (d 6H, $J_{CH3,CH}$ =7.9 Hz). Analysis calc. for $C_{14}H_{21}N_3O_6$: C, 51.37; H, 6.47; N, 12.84. Found: C, 51.24; H, 6.52; N, 12.78.

5′-O-(4,4′-Dimethoxytrityl)-N^4-isobutyryl-2′-O-methylcytidine (**4**). Compound **3** (14.5 g, 44 mmole) was dried by co-evaporation with pyridine and dissolved in 100 ml of dry pyridine. To the stirred solution of **3** at 4°C 16 g of 4,4'-dimethoxytrityl chloride (47 mmole, 1.07 eq.) in 100 ml CH_2Cl_2/pyridine (1:1) was added and stirred at room temperature for 45 minutes. The progress of the reaction was monitored by TLC. The reaction was quenched by MeOH (20 ml)and after stirring for 5 additional min. the solution was evaporated. The residue was dissolved in CH_2Cl_2 (50 ml) and extracted with saturated sodium hydrogen carbonate (2 x 50 ml) followed by water (30 ml). The organic phase was dried ($MgSO_4$) and filtered. The product **4** was purified on a flash silica gel column which was eluted with 2% MeOH in CH_2Cl_2 containing 0.5% triethylamine. The yield was 22.38 g (80%) which was pure by TLC (5% methanol in dichloromethane, Rf = 0.325). ^{1}H-NMR ($CDCl_3$) 8.53 (d, 1H, $J_{5,6}$ = 7.97, Hz, 6H), 7.97 (s, 1H N-H), 7.41 (d, 1H, $J_{5,6}$ = 7.97 Hz, 5H), 6.85-7.32 (m, 13 H trityl protons), 6.01 (s 1H, H1'), 4.40-3.55 (m, 5H, 2',3',4',5',), 4.01 (d, 1H, $O\underline{H}_{3'}$), 3.75 (s, 3H, 2'-$OC\underline{H}_3$), 2.53 (m, 1H. C-H of ibu), 1.23 (d 6H, CH_3 of ibu, $J_{CH3,CH}$ =7.9 Hz). Analysis calc. for $C_{35}H_{39}N_3O_8$: C, 66.76; H, 6.24; N, 6.67. Found: C, 66.42; H, 6.20; N, 6.52.

5′-O-(4,4′-Dimethoxytrityl)-N^4-isobutyryl-2′-O-methylcytidine-3′-(methyl-N,N-diisopropylphosphonamidite) (**5**). To dry **4** (16 g, 25.4 mmole) in anhydrous dichloromethane under dry argon, 1.5 eq. of diisopropylethylamine (15 g, 38 mmole) was added and the mixture cooled to 0°C in an ice bath. To this solution, 1.2 eq. (30 mmole) of chloro-N,N-diisopropylaminomethylphosphine $[(CH_3)_2CH]_2NP(CH_3)Cl$ was added dropwise and the reaction mixture was warmed to room temperature and stirred until the reaction was completed (45 minutes). The reaction was monitored by silica gel TLC which was developed by hexane/ethyl acetate (1:1) containing 1% triethylamine. Pre-treatment of the silica gel plate with triethylamine is required to reduce hydrolysis on the plate. The solution was evaporated and the residue was purified on a silica gel column. The column was packed with ethyl acetate/hexane (1:1) containing 5% triethylamine and washed with ethyl acetate/hexane (1:1) containing 1% triethylamine. The same solvent combination was used to elute the product from the column. The product was pooled and dried to give 14.0 g of **5** as foamy material (yield 75%) which was pure by TLC (Rf = 0.385). 31P-NMR (CD_3CN), 127.4 and 131.2 (two stereoisomers) referenced with external $PO(OH)_3$ in D_2O. ^{1}H-NMR ($CDCl_3$) also showed anomeric protons for two stereoisomers. Analysis calc. for $C_{42}H_{55}N_4O_8P$: C, 65.1; H, 7.15; N, 7.23; P, 4.00. Found: C, 64.87; H, 7.21; N, 7.18; P, 3.79.

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