SYNTHESIS OF 1'-[^{14}C]-STAVUDINE® (d4T)

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SUMMARY

1-(1'-[^{14}C]-2',3'-Dideoxy-β-D-glyceropent-2'-enofuranosyl)thymine (1'-[^{14}C]-d4T), was synthesized from 1-[^{14}C]-ribose, 1, in 7 steps in an overall yield of 29.3%. 1-[^{14}C]-Ribose was converted in one step to 1-*O*-methyl-2,3,5-tri-*O*-benzoylribofuranoside 2 in quantitative yield. Compound 2 was converted to its β-1-*O*-acetyltribenzoyl derivative, 3, which was coupled with thymine and subsequently deprotected to give 1'-[^{14}C]-5-methyluridine, 5, in 56.0% yield from 1. Compound 5 was converted to its 2',3',5'-tri-*O*-methanesulfonate derivative, 6, in quantitative yield. In a "one-pot" transformation involving three consecutive transformations, compound 6 was converted to 1'-[^{14}C]-5'-benzoyl-d4T, 10, in 94.5% yield. Methanolysis of 10 followed by flash chromatography gave the title compound in 79.0% yield (99.9% radiochemical purity, and 98.6% potency as determined by HPLC weight assay).

Key Words: d4T, stavudine®, nucleosides, AIDS, HIV

INTRODUCTION

Stavudine® (1-(2,3-dideoxy-β-D-glyceropent-2-enofuranosyl)thymine, (d4T, BMY-27857) is a nucleoside analog which possesses potent anti-human immunodeficiency virus (HIV) activity. The drug has been recently approved by the Food and Drug Administration. Metabolic studies uncovered that after initial glycoside bond cleavage to give thymine and the ribose moiety, thymine is further degraded to produce β-aminoisobutyric acid (BAIBA), carbon dioxide and ammonia.[1] To date, all in-house radioisotopic studies with d4T have been performed with the label in the thymine portion. In order to investigate the metabolic fate of the 2',3'-dideoxydehydroribose portion of stavudine®, the synthesis of the title compound was undertaken.

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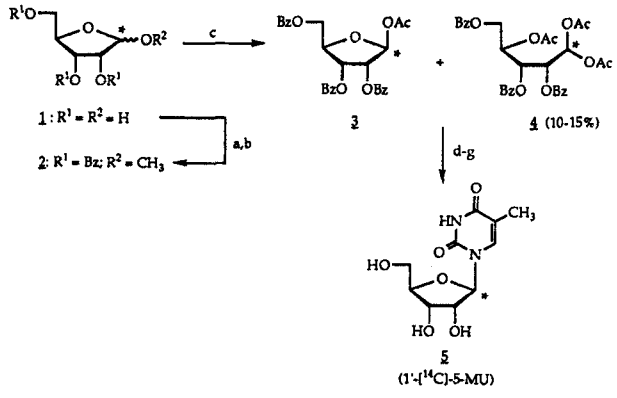
RESULTS AND DISCUSSION

The synthesis of 1'-[^{14}C]-d4T begins with the standard esterification of 1-[^{14}C]-ribose by the action of HCl/methanol to give the methylfuranoside (Scheme 1). Conversion of 1 to the tribenzoyl derivative 2 was accomplished in neat pyridine with benzoyl chloride. The major contaminants in the production of 2 were found to be methylbenzoate and benzoic anhydride. The former was produced from residual methanol remaining from the previous reaction. Attempts to remove the excess methanol by high vacuum only slightly reduced the amount of methyl benzoate produced. However, addition of toluene to the starting material followed by rotary evaporation (performed twice) removed all of the methanol azeotropically. When this procedure is performed prior to benzoylation, no methylbenzoate is produced. Benzoic anhydride was found to come from using impure benzoyl chloride. When a higher grade reagent was employed, the amount of benzoic anhydride produced decreased. The complete elimination of all benzoic anhydride was never realized.

The preparation of 1-*O*-acetyl-2,3,5-tribenzoyl-β-D-ribofuranose, 3 was accomplished selectively through the use of acetic acid and acetic anhydride in the presence of concentrated sulfuric acid catalyst.[2] The desired product was produced along with a 10 - 15% impurity, which was determined by ^{1}H NMR to be compound 4. It was decided that this impurity would be carried through the following step as it was reasoned that this compound would also lead to the desired intermediate, 5.

Scheme 1



a: CH_3OH, CH_3COCl, 25°C, 3h. b: Pyridine (18.6 eq), PhCOCl (3.6 eq), 0°C to 45°C, 3h. c: CH_3COOH, $(CH_3CO)_2O$, -5°C, conc. H_2SO_4. d: Thymine, HMDS, TMSCl, MeCN, add to substrate, then F_3CSO_3H cat. 85°C, 3h. e: NaOMe/MeOH, Dowex(50X8-200) H^+. f: flash chromatography, silica, 100% CH_2Cl_2 to 10% MeOH/CH_2Cl_2. g: Crystallization from MeOH, -20°C.

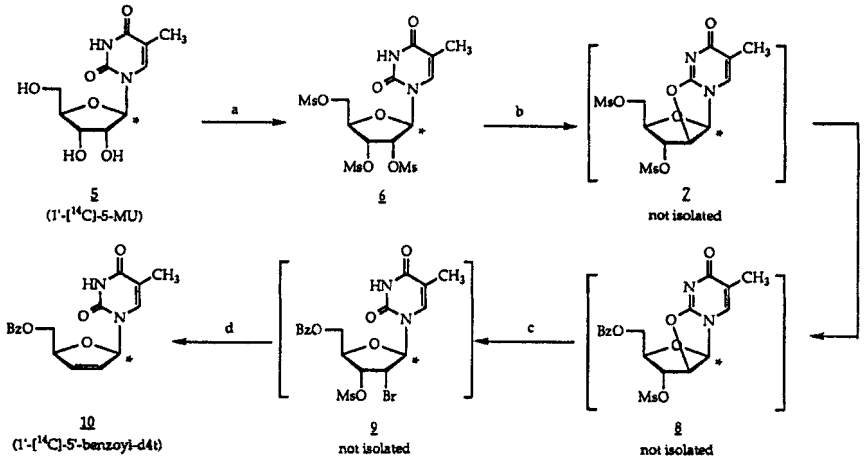
The condensation of thymine with the mixture of 3 and 4 was performed using a modification of the Hilbert-Johnson reaction[3] developed by Vorbrüggen and co-workers.[4] Although other chemical and enzymatic routes to prepare ribonucleosides have been reported,[5,6,7] the method of Vorbrüggen appears most efficient. Silylation of thymine with HMDS/chlorotrimethylsilane in acetonitrile, followed by Friedel-Crafts condensation with 3 and subsequent removal of the benzoyl groups gave the desired compound 5 (1'-[^{14}C]-5-methyluridine, 5-MU) in good yield.

Due to the presence of the 10-15% impurity (4) in the reaction, ~5-7% of the α-anomer was produced along with the desired β-anomer. However, these compounds were able to be separated by a combination of flash chromatography and crystallization to give anomerically pure 5 in 56.0% yield from 1.

The preparation of [^{14}C]-d4T from 5-MU was accomplished by a modification of the procedures set forth by Chen, et al.[8] Compound 5 was converted to the trismesyl intermediate 6, by the action of methanesulfonyl chloride in neat pyridine. In the original procedure,[8] the product was crystallized directly from the reaction mixture. On our scale, this procedure gave low yields due to loss of product in the mother liquor. In place of this procedure, an extractive workup with dilute aqueous acid was employed and the yield was increased to nearly quantitative.

The conversion of compound 6 to the penultimate compound 10, consisted of three steps (four chemical transformations), but was performed as a "one-pot" transformation (Scheme 2). Hence, a solution of the

Scheme 2



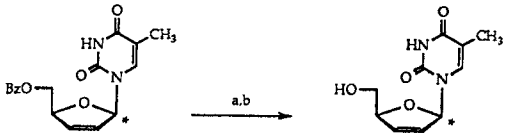
a: CH_3SO_2Cl, pyridine; b: $PhCO_2Na$ (2.4 eq), DMF, 90°C; c: CH_3COBr, 80°C, then HBr/CH_3CO_2H
d: Zn°, RT to 65°C to RT

starting material in DMF at 90°C was treated with milled[9] sodium benzoate to initially produce 2,2'-anhydro-3',5'-bismesyl-5-MU, 1'-[^{14}C], 7. Upon further exposure to sodium benzoate, intermediate 7 was transformed to 2,2'-anhydro-3'-mesyl-5'-benzoyl-5-MU, 1'-[^{14}C], 8, by direct displacement of the 5'-mesylate. Treatment of 8 with HBr/AcOH (30 - 32% w/v) at ~ 85°C produced 9 cleanly. Formation of the 2',3'-olefin to produce 1'-[^{14}C]-5'-benzoyl-d4T, 10, was accomplished by reductive elimination of the 2',3'-bromomesylate with finely divided zinc metal.

It was found that this reaction was greatly accelerated by sonication (10 - 15 min vs. 1 - 2 h). Excess zinc metal was removed by treatment with 1,2-dibromoethane. This three-step, one-pot reaction is highly efficient, producing the penultimate compound, 1'-[^{14}C]-5'-benzoyl-d4T, 10, in 94.8% from 6.

Compound 10 was transformed to the title compound, 11, with sodium methoxide in methanol and was complete in 1.5 h (Scheme 3).

Scheme 3



10
1'-[^{14}C]-5'-Benzoyl-d4T

11
1'-[^{14}C]-d4T

a: NaOMe/MeOH, 1.5 h, then Dowex resin (50X8-200, H^+ form) to pH $\leq$4.0; b: Flash chromatography, 100% CH_2Cl_2 gradient to 4% MeOH/CH_2Cl_2.

The reaction was acidified with Dowex resin (50X8-200, H^+) and the crude product was purified by flash chromatography to give 506.7 mg, 2.26 mmol, of the title compound in 79% yield, 29.3 % overall from ribose (99.2% radiochemical purity, and 98.6% potency as determined by HPLC weight assay).

EXPERIMENTAL

^{1}H NMR spectra were recorded on a Bruker AM360 spectrometer. Chemical shifts are expressed on the δ scale downfield of tetramethylsilane internal standard. Thin layer chromatography plates (silica gel GF) were purchased from Analtech, Inc., Newark, DE. Developed TLC plates were viewed under short

wave UV light. Flash chromatography silica gel (32-63 μm, 60Å) was purchased from ICN, Costa Mesa, CA. Radiochemical measurements were obtained on a Beckman LS9000 liquid scintillation counter. Radiochemical purities were determined on an IN/US Systems Model 2 β-RAM HPLC detector. 1-[^{14}C]-ribose was purchased from Vitrax Co., Placentia, CA, and was used as received.

1-[^{14}C]-1-*O*-Methyl-2,3,5-tri-*O*-benzoylribofuranoside , 2:

To a 250 mL single neck round bottomed flask, under nitrogen atmosphere, was charged anhydrous methanol (20 mL) via syringe. To this was added acetyl chloride (541 μL) via syringe. This was allowed to stir at ambient temperature (~25°C) for 10 min. Ribose (1.043 g, 6.95 mmol) was added to the reaction mixture. 1-[^{14}C]-ribose[10] (114.3 mg, 0.753 mmol, 56.7 mCi/mmol, 96.2% pure), which was received as a solution in anhydrous methanol (6 mL), was transferred to the reaction vessel via syringe. The vial was rinsed with anhydrous methanol (~2 mL) and the washings were transferred to the reaction vessel. The reaction was stirred at room temperature for 3 h.

Anhydrous pyridine (1.39 mL, 17.6 mmol) was added to the reaction mixture and the methanol was removed by rotary evaporator (bath temperature 45°C). The crude syrup was contacted with anhydrous toluene (80 mL) and was evaporated via rotary evaporator (bath temperature 45°C). This step was repeated once more with another 80 mL portion of toluene. The crude syrup (pale yellow) was placed on high vacuum for 0.5 h at room temperature.

Anhydrous pyridine (11.6 mL, 143 mmol, 18.6 eq) was added to the crude syrup via syringe and was stirred until the mixture became homogenous. The reaction was cooled to 0°C (ice bath) and was treated with benzoyl chloride (3.25 mL, 28.0 mmol, 3.6 eq), via addition funnel, over a 0.5 h period. The reaction mixture was stirred at 0°C for an additional 10 min and was analyzed by TLC.[11] At this point, the reaction was nearly complete but small amounts of the mono- and bis-benzoylated products remained. The reaction mixture was warmed to 45°C in a water bath for 3 h. Analysis[11] of the reaction mixture at this time showed a single product spot at Rf 0.5 (desired compound), and a faint spot at Rf 0.65 which corresponded to benzoic anhydride.

The reaction was cooled to room temperature and was quenched by the addition of several chips of ice. Deionized water (20 mL) was added followed by methylene chloride (40 mL). The biphasic solution was transferred to a separatory funnel and the layers separated. The aqueous phase was extracted with

methylene chloride (1 x 20 mL). The combined organic phases were treated with 1.5 *M* H_2SO_4 (2 x 30 mL). The organic phase was treated with sat. aq. sodium bicarbonate (50 mL) and the phases were separated. The organic layer was dried over anhydrous magnesium sulfate, filtered and the solvent was removed via rotary evaporator. The product was placed on high vacuum for 1 h. The crude material (~3.8 g) was used directly in the following step.

1-[^{14}C]-1-*O*-Acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranoside, 3:

The crude material from the previous step was dissolved with acetic acid (glacial, 1.86 mL). Gentle warming was necessary to reach complete solution. To this was added acetic anhydride (4.32 mL). The reaction mixture was cooled to -5°C (ice-salt bath) under an inert atmosphere and concentrated sulfuric acid (225 μL) was added via syringe over a 20 min period. After 0.5 h at this temperature, the reaction solidified. The ice bath was removed and stirring resumed in ~ 5 min. TLC[11,12] analysis of a reaction aliquot showed complete consumption of the starting material.

Water (40 mL, 0°C) was added to the reaction mixture followed by ethyl acetate (75 mL) and the biphasic solution was stirred for 5 min. The solution was transferred to a separatory funnel and the organic phase was collected. The organic phase was washed with water (2 x 25 mL) followed by sat. aq. sodium bicarbonate (2 x 30 mL), and finally brine (1 x 25 mL). The organic phase was transferred to a 500 mL single neck round bottom flask and was concentrated to a clear yellow oil. Analysis of the crude oil by TLC showed one major spot and a minor impurity. A ^{1}H NMR was obtained on the crude product which was received as an off-white foam after high vacuum (1 h), showed the desired compound[13] along with 10-15% of impurity 4.[14] The product was used without purification in the following reaction.

1-(1'-[^{14}C]-2',3',5'-Trihydroxy-β-D-ribofuranosyl)thymine (1'-[^{14}C]-5-methyluridine), 5:[4]

A dry 100 mL two neck round bottom flask under inert atmosphere was charged with thymine (714 mg, 5.66 mmol, 1.05 eq) in dry acetonitrile (20 mL). To this was added 1,1,1,3,3,3-hexamethyldisilazane (1.19 mL, 5.66 mmol, 1.02 eq) followed by chlorotrimethylsilane (698 μL, 5.50 mmol, 1.05 eq). The reaction was stirred for 35 - 40 min. To this was added the crude starting sugar in acetonitrile (10 mL). This was immediately followed by the addition, of trifluoromethanesulfonic acid (0.5 mL) in one portion. Upon addition of the acid catalyst the reaction immediately clarifies. The solution was heated to 82 - 85°C on an oil bath. After 3 h, TLC analysis indicated that the reaction was complete.[15]

The reaction mixture was transferred to a 500 mL single neck round bottom flask and was concentrated *in vacuo* to an oil. The crude oil was dissolved in methylene chloride (40 mL) and was washed with water (1 x 25 mL). It was further washed with sat. aq. sodium bicarbonate (1 x 20 mL) and brine (1 x 20 mL) and was dried over magnesium sulfate, filtered and concentrated. It was placed on high vacuum for 1 h. TLC analysis of the crude product showed the product, thymine, and a minor impurity.

A solution of sodium methoxide in methanol (604 mg Na/ 100 mL methanol) was prepared. The crude tribenzoyl-5-MU was dissolved in THF (40 mL). The sodium methoxide/methanol solution was added to the THF solution in one portion and the reaction was stirred at room temperature for 0.5 h. Analysis by TLC,[15] indicated that the reaction was complete. A pH probe was inserted into the reaction mixture (initial pH = ~ 12.0). Dowex H^+ resin (50X8-200) was added spatula wise (1 min between additions) until the pH was $\leq$ 4.0. The solution was filtered immediately and the resin was washed with methanol (~50 mL). The solution was concentrated on rotary evaporator to a brown residue. Attempts to crystallize this material by the addition of methylene chloride failed.

The crude product was purified by flash chromatography using silica gel in 100% methylene chloride. The column was flushed with methylene chloride until all methyl benzoate had eluted. The eluant was ramped to 5% methanol/methylene chloride until all of the mid-polar impurities eluted. The eluant was ramped further to 10 % methanol/methylene chloride and the spot corresponding to 5-MU was collected in Erlenmeyer flasks (1500 mL total). The solvent was concentrated on a rotary evaporator. The crude residue was further dried on high vacuum to give a tan foam, 1.44 g, 5.57 mmol. A ^{1}H NMR was obtained and the material was found to be a ~7:1 mixture of β- and α-5-MU.[16]

The material was dissolved with 10 mL of methanol. To this was added methylene chloride (~ 100 mL) dropwise over a 10 min period. It was allowed to stir for 30 min and in that time, a white crystalline precipitate forms. The flask was placed in the freezer at -20°C for 2.5 h. The crystals were filtered on a Büchner funnel and transferred quantitatively to a tared single neck round bottom flask. The crystals were further dried on high vacuum. A ^{1}H NMR analysis of the product indicated that the compound was nearly exclusively the desired β-anomer (~1% α-anomer). It was recovered in 47.4 % yield (660 mg, 2.55 mmol). The mother liquor was subjected to the identical crystallization method and after crystallizing at -20°C for 60 h, a second crop of crystals was recovered of identical purity (120 mg, 0.464 mmol) to give a combined total of 780 mg, 3.02 mmol, 56.0% yield from compound 3.

1-(1'-[^{14}C]-2',3',5'-Tri-*O*-methanesulfonyl-β-D-ribofuranosyl)thymine, 6: [8]

Purified 1-[^{14}C]-5-MU was dissolved in pyridine (15 mL) and the pyridine was removed by rotary evaporator to remove any residual methanol. The residue was re-dissolved in pyridine (5 mL) and the solution was cooled to 0°C in an ice bath. To this solution was added methanesulfonyl chloride, neat, via syringe, over a 7 - 8 min period. The solution yellowed slightly. The ice bath was removed after 20 min and the reaction was stirred at room temperature for 3 h.

At this point, the reaction was found to be complete by TLC analysis. The reaction was quenched with water (20 mL) and was stirred for 5 min. To this was added ethyl acetate (50 mL) and the solution was transferred to a separatory funnel and the layers were separated. The aqueous phase was extracted with ethyl acetate (1 x 25 mL) and the combined organic layers were treated successively with 1.5 *M* H_2SO_4 (2 x 30 mL), sat. aq. sodium bicarbonate (1 x 25 mL) and brine (1 x 25 mL). The organic phase was dried over magnesium sulfate, filtered, and the cake washed with ethyl acetate. The solvent was removed by rotary evaporator and the compound was further dried on high vacuum (0.5 mm Hg, 50°C) for 1 h to give 1.59 g (>100% theory). ^{1}H NMR analysis of the compound showed the desired compound and some residual ethyl acetate and water. The product was used in the following reaction without further purification.

1-(1'-[^{14}C]-2,2'-Anhydro-5'-*O*-benzoyl-3'-*O*-methanesulfonyl-β-D-ribofuranosyl)thymine, 8:

A solution of the starting material (1.50 g, 3.04 mmol, estimated) in *N*,*N*-dimethylformamide (DMF, ~ 8 mL), was charged to a 100 mL 3 necked round bottom flask possessing a nitrogen inlet tube and an overhead stirrer. The solution, under inert atmosphere, was heated to 90°C in an oil bath. When the reaction temperature had stabilized, finely milled[9] sodium benzoate (total 1.05 g, 7.30 mmol) was added in four equal portions over a 1 h period. The slurry thickens. The reaction was monitored by HPLC[17] and was deemed complete in 7.5 h. The solution was cooled to 80°C. This solution was used in the next reaction without workup.

1-(1'-[^{14}C]-5'-*O*-Benzoyl-2'-bromo-3'-*O*-methanesulfonyl-β-D-ribofuranosyl)thymine, 9:

To the reaction mixture described in the above procedure was added acetyl bromide (~ 355 mg) via syringe, to destroy any residual water remaining in the reaction. To this was added a solution of HBr in acetic acid (30 - 32 % w/v, 735 µL) via syringe, over a 30 min period. The solution clarified and became more viscous. The reaction was again followed by HPLC[17] and was found to be complete in 3 h. The mixture was cooled to room temperature. Conversion to the next intermediate was performed directly, without workup.

1-(1'-[^{14}C]-5'-*O*-Benzoyl-2',3'-dideoxy-β-D-ribofuranosyl)thymine (1'-[^{14}C]-Bd4T): 10:

To the reaction mixture described in the above procedure was added zinc dust (258 mg, 3.95 mmol, 1.3 eq) in one portion. The solution initially turns a gray-green color, but as the zinc metal is consumed, the reaction becomes clear and nearly colorless. An additional amount of zinc dust (50 mg) was added to insure an excess of the reagent. The reaction was heated to 65°C and immediately cooled to room temperature. The reaction was again monitored by HPLC.[17] The reaction was deemed complete after reacting for 12 h. At this point, the elemental zinc was consumed by the addition of 1,2-dibromoethane (~600 μL) and was completely reacted in 0.5 h. The reaction was transferred to a 250 mL round bottom flask with an overhead stirrer. Water (distilled, 35 - 40 mL) was dripped into the reaction mixture over a 1 h period. After the addition was complete, the reaction was cooled in an ice bath for 2 h with stirring. The product was collected on a Büchner funnel and was further dried on high vacuum for 2 h. The product was received as an off-white solid (940 mg, 2.86 mmol, 94.8% from 6.

1-(1'-[^{14}C]-2',3'-Dideoxy-β-D-ribofuranosyl)thymine (1'-[^{14}C]-d4T), 11:

To a room temperature slurry of 10 (940 mg, 2.86 mmol) in methanol (10 mL), was added a previously prepared solution of sodium methoxide in methanol (200 mg sodium in 5 mL methanol). The reaction was monitored by HPLC[18] and was complete in 1.5 h. A pH probe was inserted into the reaction mixture (initial pH = ~ 12.0). Dowex H^+ resin (50X8-200)[19] was added spatula wise (1 min between additions) until the pH was ≤ 4.0. The solution was filtered immediately and the resin was washed with methanol (~50 mL). The solution was concentrated on rotary evaporator to a clear orange-brown residue.

The material was re-dissolved in methanol and concentrated to an oil. It was purified by flash chromatography (silica gel, 100% methylene chloride gradient to 4% methanol/methylene chloride). Pure fractions were combined and concentrated on rotary evaporator. The product was further dried on high vacuum (0.5 mm Hg, 18 h) and gave 506.7 mg, 2.26 mmol, 79.0% yield from 10. [20]

ACKNOWLEDGMENTS

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9. Sodium benzoate was milled using an electric coffee grinder until the compound was a fine powder. Without milling, the reaction is sluggish and requires additional equivalents of reagent.
10. 1-[^{14}C]-D-Ribose was purchased from Vitrax Co., 660 S. Jefferson St., Placentia, CA, 92670. Material was received as a solution in anhydrous methanol, 6.17 mCi/mL, 96.2% radiochemical purity.
11. TLC conditions: 20% ethyl acetate/hexanes; Rf's: product 0.50; bisbenzoyl derivative 0.35; monobenzoyl derivative 0.18; benzoic anhydride 0.65.
12. Rf's: starting material 0.50; product 0.38.
13. ^{1}H NMR was identical to commercially available material.
14. TLC Conditions: 30% Ethyl acetate/hexanes; Rf's: 3 0.55; product 0.25.
15. TLC Conditions: 60% Ethyl acetate/hexanes; Rf's: 2',3',5'-tribenzoyl-5-MU 0.60; product 0.05.
16. ^{1}H NMR (CD_3OD): δ 1.81 (s, 3H); 1.83 (s, α-anomer); 3.72 (d, 1H + α-anomer); 3.81 (d, 1H + α-anomer); 4.0 (m, 1H + α-anomer); 4.18 (m, 2H + α-anomer); 5.80 (s, α-anomer); 5.85 (s, 1H); 7.55 (s, α-anomer), 7.82 (s, 1H).
17. HPLC conditions: . Column: Supelcosil LC-18-DB 25 cm x 4.6 mm, 254 nm, 10 μL injection, 1.8 mL/min. Samples prepared using 10-15 μL of a reaction aliquot dissolved in 1.5 - 2.0 mL of 50% A

and 50% B. Mobile phase A: 5% CH_3CN/0.01 *M* NH_4OAc; Mobile phase B: 50% CH_3CN/0.01 *M* NH_4OAc. Gradient schedule: 100%A for 5 min, linear gradient to 60% B over 5 min, hold at 60% B for 5 min, linear gradient to 100% B over 5 min, hold for 10 min, reverse linear gradient to 100% A over 2 min, equilibrate at 100% A for 10 min. Retention times: 6, 10.7 min.; 7, 7.7 min.; 8, 11.9 min.; 9, 18.3 min.; 10, 13.1 min.; benzoic acid, 2.9 min.

18. HPLC conditions: Mobile phase: 65% 0.01*M* NH_4OAc/35% CH_3CN, isocratic. Column: Supelcosil LC-18-DB 25 cm x 4.6 mm, 254 nm, 10 μL injection. . Samples prepared using 10-15 μL of a reaction aliquot dissolved in 1.5 - 2.0 mL of mobile phase. Retention times: 10, 4.3 - 4.5 min.; 11, 1.15 - 1.20 min.; methylbenzoate, 8.10 - 8.20 min.

19. Crude Dowex resin was conditioned by washing with deionized water and vacuum filtering followed by further rinsing of the resin cake with dry methanol.

20. Spectral properties were identicle to an authentic sample of d4T. ^{1}H NMR (DMSO-d_6): δ 1.71 (s, 3-H), 3.60 (br. s, 2-H), 4.78 (br. s., 1-H), 5.00 (AB t., 1-H), 5.90 (m. 1-H), 6.40 (m. 1-H), 6.80 (s, 1-H), 11.3 (br. s., 1-H).