THE SYNTHESIS OF THE ^{2}H, ^{3}H, AND ^{14}C-ISOTOPOMERS OF 2'-DEOXY-2',2'-DIFLUOROCYTIDINE HYDROCHLORIDE, AN ANTI-TUMOR COMPOUND

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SUMMARY

The ^{2}H, ^{3}H, and ^{14}C-isotopomers of 2'-deoxy-2',2'-difluorocytidine hydrochloride (**1a**, gemcitabine hydrochloride) have been synthesized in two radiochemical steps from the reaction of *bis*-trimethylsilylcytosine-[2-^{14}C] and 3,5-O-*bis*-benzoyl-1-O-methanesulfonyl-2- deoxy-2,2-difluororibose (**2b**). A mixture of anomers of 3',5'-dibenzoyl-2'-deoxy-2',2'-difluorocytidine (**4a**) or its ^{14}C-isotopomer (**4b**) were obtained which were readily separated by crystallization from ethyl acetate. Deprotection using methanolic ammonia yielded the target compound. The ^{2}H and ^{3}H-isotopomers were prepared by deuterium (or tritium) gas hydrogenolysis of 5-iodo-2'-deoxy-2',2'-difluorocytidine (**5**).

Key words: gemcitabine hydrochloride, carbon-14, tritium, deuterium, anti-tumor

INTRODUCTION

Preliminary reports of the synthesis[1] and anti-viral activity[2] of 2'-deoxy-2',2'-difluorocytidine hydrochloride (**1a**, gemcitabine hydrochloride) and related nucleosides were first described by Kroin *et al.* ; the synthesis was later described in greater detail by Hertel *et al.*[3] This antimetabolite of deoxycytidine initially showed promising activity in tissue culture against a variety of DNA and RNA virus strains.[2] Subsequent studies have shown that **1a** also has interesting *in vivo* anti-tumor activity, particularily against solid tumors.[4] Since **1a** is an agent for potential use in humans, ^{14}C-labeled material was needed for studies designed to determine its metabolism and disposition in laboratory animals. In addition, high specific activity ^{3}H-labeled material was needed for *in vitro* mechanism studies at the molecular level. In this report, we have described the synthesis of the ^{2}H, ^{3}H, and ^{14}C- isotopomers of **1a.**

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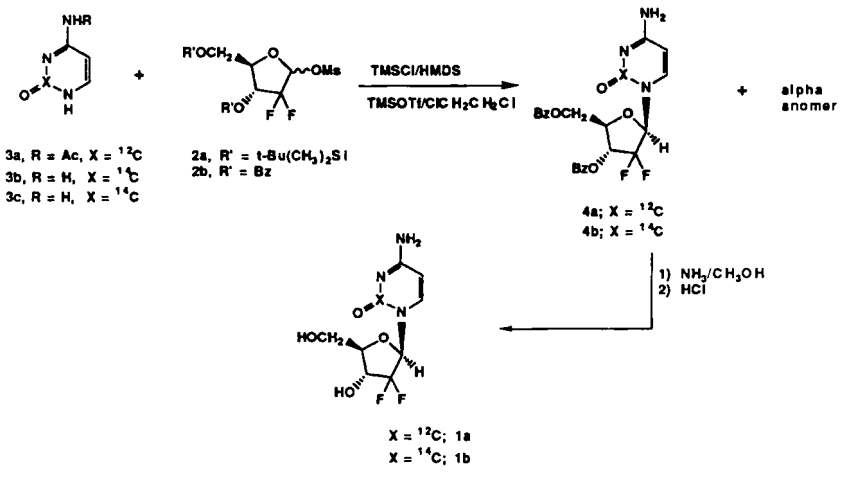
RESULTS AND DISCUSSION

Hertel *et al.* reported that the reaction of 3,5-*bis*-O-(*tert.*-butyldimethylsilyl-1-O-methanesulfonyl)-2- deoxy-2,2-difluororibose (**2a**) with the *bis*-trimethylsilyl ether of N-acetylcytosine (**3a**) in the presence of trimethylsilyl triflate afforded the desired nucleoside **1a** (after hydrolytic deprotection of the intermediate silylated nucleoside) as well as the corresponding alpha anomer in a 4:1 ratio (alpha/beta).[3] Chou and his co-workers later showed that the anomeric ratio of the protected nucleoside could be improved to 1:1 by use of the 3,5-O-(*bis*-benzoyl-1-O-methanesulfonyl)-2- deoxy-2,2-difluororibose (**2b**).[5] In both cases, the anomeric ratio of the protected mesylate was *ca.* 1:1; Chou *et al.* have also shown that a mixture of anomers (1:1) was obtained even when a single anomer of the mesylate **2b** was used in the glycosylation reaction.[5]

It was desireable to reduce the total number of steps in the synthesis, hence we planned to by-pass the preparation of N-acetylcytosine. In addition, the *bis*-trimethylsilylcytosine was prepared *in situ*.[6] Thus cytosine (**3b**) (or its ^{14}C-isotopomer, **3c**) was reacted with mesylate **2b**[5] in the presence of trimethylsilyl chloride, hexamethyldisilazane, and trimethylsilyltriflate in dichloroethane (**Scheme 1**). After work-up, the product mixture (TLC, with autoradiography where appropriate, using 9:1 chloroform/methanol showed the presence of both anomers of **4a,b** in approximately equal quantities) was re-dissolved in ethyl acetate, whereupon a crystalline precipitate formed which was shown to be a single anomer by TLC and subsequently shown to be the desired *beta*-anomer **4a,b**, by conversion to **1a** (*vida supra*). An additional quantity of **4a,b** was obtained by flash chromatography of the mother liquors from the crystallization. Although the yield in this step was low (16.8%), the method was convenient because the pure *beta* anomer was obtained prior to the final step. Chou *et al.* found that it was necessary to separate the isomers after the last step by repeated re-crystallization of the *alpha/beta* mixture of the hydrochloride salt. In addition, the synthesis of **3a** and its subsequent conversion to the

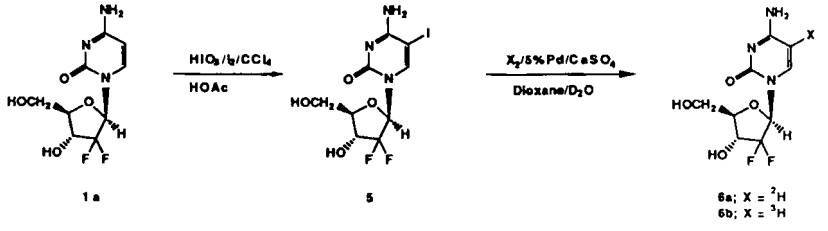
SCHEME 1



bis-TMS ether was eliminated, as well as its purification by vacuum distillation.[5] Conversion of **4a,b** to the desired product (**1a,b**) by reaction with methanolic ammonia, followed by conversion to the hydrochloride salt and its subsequent recrystallization from water was uneventful and proceeded in reasonable yield (63%). Thus **1b** was prepared in 10.2% overall yield; radio-HPLC and TLC-autoradiography showed that the radiochemical purity (RCP) was ≥99.2% (the *alpha/beta* anomeric ratio was 0.2:99.8). The specific activity was 178.4 μCi/mg (53.3 mCi/mmol).

Iodinated nucleoside **5** was chosen to serve as a substrate for both the $Pd/CaSO_4$ catalysed deuterio- and tritio-deiodination. The synthesis of **5** from 5-iodocytosine was fraught with difficulties. Separation of the mixture of *alpha*- and *beta*-anomers obtained from the reaction of the *bis*-TMS ether of N-acetyl-5-iodocytosine with **2b** was impractical, as was the attempted separation of the unprotected nucleosides.[7] To obviate the above problems, **1a** was converted to the 5-iodo analog, using a procedure described by Bobek *et al* for the preparation of 5-iodo-1-(2,3,5-tri-O-acetylarabinofuranosyl)cytosine.[8] Reaction of **1a** with iodic acid/I_2 in a mixture of acetic acid and CCl_4 provided **5** in 58% yield (**Scheme 2**).

SCHEME 2



Hydrogenolysis of **5** with deuterium or tritium gas in 50:50 dioxane/D_2O in the presence of 5% $Pd/CaSO_4$ at atmospheric pressure, yielded 2',2'-deoxy-2',2'-difluorocytosine-[5-^{2}H] (**6a**) or the corresponding 5-^{3}H-isotopomer(**6b**) respectively[9], following purification by HPLC. The RCP of **6b** was ≥99% by radio-HPLC and autoradiography; the specific activity was 100 mCi/mg (26.5 Ci/mmol).

EXPERIMENTAL

The cytosine-[2-^{14}C] was purchased from Amersham Corporation; the tritiation of **5** and the subsequent purification of **6b**, was conducted at Amersham Corporation in the manner similiar to that described for **6a**. Mesylate **2b** was obtained from 4-(benzoyloxy)-5-[(benzoyloxy)methyl]-3,3-difluoro-D-arabino-2(3H)-furanone *via* a two step sequence as described by Chou *et al.*[5] The NMR spectra were obtained on a General Electric QE-300 nuclear magnetic resonance spectrometer at 300 MHz. Chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane. Field desorption mass spectra were run on a Varian Associates MAT 731 mass spectrometer; direct chemical ionization spectra were recorded on a Nermag R30-10 triple stage quadrupole mass spectrometer. Microanalytical data were provided by the Physical Chemistry Research Department of the Lilly Research Laboratories.

Flash chromatography was conducted as described by Still *et al.*,[10] using E.M. Science silica gel 60 (230-400 mesh).

Radiochemical purity (RCP) was assessed by TLC/autoradiography employing E. Merck silica gel F-254 TLC plates and Kodak X-ray film BB-5. In addition, RCP was determined using HPLC and counting one minute fractions of the eluate after dilution with PCS™ scintillation cocktail (Amersham Corp.).

3',5'-Dibenzoyl-2'-deoxy-2',2'-difluorocytidine, 4a:

Cytosine (0.846 g, 7.62 mmol) was suspended in dichloroethane (15 mL) and treated sequentially with hexamethyldisilazane (1.125 mL), chlorotrimethylsilane (0.675 mL), and trimethylsilyltriflate (3.6 mL) and stirred for 10 min. A dichloroethane solution (10 mL) of **2b** (3.46 g, 7.62 mmol) was added and the mixture was stirred at reflux for 48 hr, then allowed to cool to room temperature. The dichloroethane was removed *in vacuo* and the residue was redissolved in ethyl acetate (50 mL). The solution was washed with saturated aqueous sodium bicarbonate (2 x 10 mL) and saturated brine (2 x 10 mL), dried (anhydrous $MgSO_4$) and concentrated. The residue was crystallized from ethyl acetate to yield **4a** (0.490, 15.6% yield): NMR ($CDCl_3$+DMSO/d_6) δ 4.66 (2H, m, 4'-H, 5'-H), 5.75 (1H, d, J = 7.5 Hz, 3'-H), 5.82 (2H, bs, NH_2, exchanges in D_2O), 6.28 (1H, m, 1'-H), 7.68 (10H, m, aromatic), 7.90 (1H, d, J = 7.2 Hz, 5-H), and 8.00 ppm (1H, d, J = 7.2 Hz, 6-H); DCI-MS $(M+H)^+$ 472; UV λ_M 232 nm (ε_M 32300).

Anal calc'd for $C_{23}H_{19}F_2N_3O_6$: C, 58.60; H, 4.06; N, 8.91. Found: C, 58.83; H, 4.13; N, 8.64.

3',5'-Dibenzoyl-2'-deoxy-2',2'-difluorocytidine-[2-^{14}C], 4b:

Cytosine-[2-^{14}C] (250 mCi, specific activity 54mCi/mmol, 4.63 mmol) was suspended in dichloroethane (5 mL) and stirred at room temperature under argon. Hexamethyldisilazane (0.685 mL), followed by chlorotrimethylsilane (0.410 mL), and finally trimethylsilyltriflate (2.19 mL) were added (the cytosine dissolved after the addition of the TMS-triflate) and the resulting mixture was stirred for 10 min.

A dichloroethane solution (10 mL) of **2b** (2.11 g, 4.63 mmol) was added and the mixture was stirred at reflux for 48 hr, then allowed to cool to room temperature. The dichloroethane was removed *in vacuo* and the residue was redissolved in ethyl acetate (50 mL). The ethyl acetate solution was washed succesively with 10 mL each of saturated aqueous sodium bicarbonate, water and saturated brine, then dried (anhydrous $MgSO_4$) and concentrated *in vacuo*. The resulting amorphous foam was redissolved in ethyl acetate (15 mL) and subsequently crystallized to yield **4b** (0.292 g) after filtration, washing with cold ethyl acetate and drying *in vacuo*.. TLC-autoradiography (chloroform/methanol 9:1) showed **4b** to be pure and free of the *alpha*-anomer. The mother liquors contained the

corresponding *alpha*-anomer as well as additional **4b**, unreacted cysosine, and two unknown materials of higher R_f. The residue remaining after evaporation of the solvent was purified by flash chromatography over silica gel. The product was eluted with 90:10 $CHCl_3/CH_3OH$ in 10 mL fractions. Fractions 13-20 were concentrated and the residue was crystallized from ethyl acetate to yield an additional 0.065 g of **4b** (combined yield 16.3%). This material co-eluted with authentic **4a** on TLC (9:1 $CHCl_3/CH_3OH$); DCI-MS $(M + H)^+$ 474.

2'-Deoxy-2',2'-difluorocytidine-[2-^{14}C], Hydrochloride Salt, 1b:

A suspension of **4b** (0.357 g, 0.755 mmol) in methanol (40 mL) was chilled in ice and treated with anhydrous ammonia (bubbled through the solution for 5 min). The mixture was allowed to warm to room temperature and stirred overnight. The solvent was removed *in vacuo* and the residue was triturated with ethyl acetate, then redissolved in 2-propanol (3 mL) and acidified with concentrated hydrochloric acid (0.144 mL, 2 equivalents), whereupon crystallization occurred. The crystals were collected by filtration, washed with 2-propanol, and air dried at room temperature (0.1758 g). HPLC (Zorbax C-18, 0.01 M NH_4OAc at 1.5 mL/min with UV detection at 290 nm) showed that the material contained 97.8% of the *beta*-anomer.

The crude beta-anomer **1b** was dissolved in water (0.97 mL), warmed to 80°C, and treated dropwise with acetone (12 mL). A crystalline precipitate began to form after 10 mL of acetone was added. The mixture was chilled, filtered, washed with fresh acetone and dried to yield **1b** (0.144 g, 63% yield). Radio-HPLC as described above showed the RCP to be 99.3%; the material contained 99.8% of the desired *beta*-isomer by UV-HPLC peak areas. Autoradiography on TLC (acetonitrile/water with 0.1% sodium laurylsulfate/formic acid, 20:4:1) corfirmed that **1b** co-eluted with **1a** and had a RCP ≥99.4%. The specific activity was 178.4 μCi/mg (53.3 mCi/mmol). The overall radiochemical yield was 10.2%.

5-Iodo-2'-deoxy-2',2'-difluorocytidine, 5:

A mixture of acetic acid (2.01 mL), carbon tetrachloride (2.01 mL), and iodine (0.0865 g, 0.34 mmol) was stirred until the color was evenly distributed between the two phases. 2'-Deoxy-2',2'-difluorocytidine (0.230 g, 0.875 mmol) was added and it dissolved immediately. The reaction mixture was warmed to 45°C and treated with a solution of iodic acid (0.0865 g, 0.492 mmol) in water (0.23 mL). After stirring at 45°C for 17 hr, TLC (8:2 $CHCl_3/CH_3OH$) showed that the reaction was virtually complete, so the mixture was allowed to cool to room temperature. The mixture was concentrated *in vacuo*; the residue was diluted with water (10 mL), and made basic with saturated sodium bicarbonate and purified by preparative HPLC on RP C-18 reversed phase silica gel. The product was eluted at 250 mL/min, using 10% methanol in water as the mobile phase. After analysis by analytical HPLC, the appropriate fractions were combined, concentrated, and dried overnight *in vacuo* to yield **5** as a white crystalline solid (0.203 g, 58% yield): mp >290°C;

HPLC (μ-Bondapak, CH_3OH/water 20:80 at 2 mL/min) showed that this material contained <0.7% of **1a** (r_T for **1a** = 1.31 min, r_T for **5** = 2.60 min); NMR (DMSO/d_6) δ 3.59 (1H, dd, J = Hz, 4'-H), 3.76 (2H, m, 5'-H), 4.17 (1H, m, 3'-H), 5.40 (1H, bs, 5'-OH), 6.05 (1H, t, J = Hz, 1'-H), 6.20 (1H, bs, 3'-OH), 6.82 and 8.05 (1H, bs, NH_2), and 8.19 ppm (1H, s, 6-H); FD-MS: M^+389.

Anal calc'd for $C_9H_{10}F_2IN_3O_4$: C, 27.78; H, 2.59; N, 10.80; I, 32.62. Found: C, 27.77; H, 2.68; N, 10.54; I, 32.39.

2'-Deoxy-2',2'-difluorocytidine-[5-^{2}H] Hydrochloride Salt, 6a:

5-Iodo-2-deoxy-2',2'-difluorocytidine (**5**, 0.100 g, 0.257 mmol) was dissolved in a mixture of dioxane (5 mL) and deuterium oxide (99.5 atom % deuterium, 5 mL) and added to 5% Pd/$CaSO_4$ (0.100 g); the resulting mixture was stirred under an atmosphere of deuterium gas overnight. TLC (chloroform/methanol 80:20) showed that no reaction had occurred. After an additional 6 hours, approximately 50% conversion had occurred. The mixture was stirred overnight (reaction was complete by TLC) and then filtered. The filtrate was concentrated *in vacuo*. The residue was re-dissolved in methanol (5 mL) and evaporated three times. The product was dissolved in 2-propanol (1.25 mL) and treated with slightly more than two equivalents of concentrated hydrochloric acid (0.040 mL) and chilled at 5°C, whereupon the product crystallized to yield **6a** (0.1236 g, 34.5% yield) as a white crystalline solid: NMR (DMSO/d_6) δ 3.56 (1H, dd, J = 3.32 and 12.84 Hz) and 3.74 (1H, d, J = 12.84 Hz) (5'-H), 3.85 (1H, m, 4'-H), 4.17 (1H, m, 3'-H), 6.05 (1H, t, J = 8.03 Hz, 1'-H), 6.28 (bs, exchanges with D_2O), 8.04 (1H, s, 6-H), 8.70 and 9.80 (s, exchanges with D_2O); MS (DCI) $(M+1)^+$ 265.
Anal calc'd for $C_9H_{11}{}^2HF_2ClN_3O_4$: C, 35.95; H, 3.99; N, 13.98. Found: C, 35.62; H, 3.64; N, 13.42.

2-Deoxy-2',2'-difluorocytidine-[5-^{3}H], 6b[8]:

5-Iodo-2-Deoxy-2',2'-difluorocytidine (**5**, 0.030 g, 0.070 mmol) was dissolved in dioxane, containing triethylamine (0.100 mL) and 10% Pd/C (0.020 g); was stirred under an atmosphere of tritium gas (10 Ci) for four hours. The mixture was filtered, acidified with hydrochloric acid, whereupon the labile tritium was removed by repeated evaporation with water. The product (**6b**) was purified by preparative TLC and HPLC.

The RCP of **6b** was assessed by autoradiography (chloroform/methanol ammonium hydroxide 80:20:1) and by radio-HPLC (Hypersil ODS, potassium dihydrogen phosphate/ methanol gradient) and shown to be >99.5% pure. The specific activity was 100 mCi/mg (26.5 Ci/mmol).

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