A New Process for Deprotection of Acetyl and Benzoyl Groups in Synthesis of Azacitidine

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4-Amino-1-β-D-ribofuranosyl-*s*-triazin-2(1*H*)-one or azacitidine is a promising DNA demethylation inhibitor used for the treatment of myloneplastic, bone cancer and breast cancer. An efficient, cost-effective and convenient manufacturing process for the synthesis of azacitidine is described. The present research relates to the synthesis, deprotection, isolation and purification of azacitidine (**1**). In this process, more particularly 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) is used as deprotection reagent for deprotection of *O*-acetyl, *O*-benzoyl to acquire azacitidine (**1**). The new process allows for the reliable and efficient production of drug substance similar overall yield. The new improved process has merits including enantiomeric purity, better crystallization and the product complies with the requirements of USP30.

Keywords: 5-Azacytosine, D-ribose, 5-Azacitidine, Vorbruggen condensation, Deprotection, DBU.

INTRODUCTION

4-Amino-1-β-D-ribofuranosyl-*s*-triazin-2(1*H*)-one or 5-azacytidine, a cytidine nucleoside present in DNA and RNA. Most of the cytidine nucleosides are used in the treatment of myelodysplastic syndrome [1]. Azacitidine is mainly employed in *in vitro* methyl group interactions from DNA. This may weaken the effects of gene silencing mechanisms that occurred prior to the methylation. Methylation events are therefore, believed to secure the DNA in a silenced state. Being a ribonucleoside, it has been shown effective biological activity against HIV and HTLV [2,3].

The synthesis of azacitidine was accomplished by Píscala [4] by using methanolic ammonia as deprotecting reagent of benzoyl groups at 0 °C. This route of synthesis was not suitable for large-scale material generation. The concentration and stability of methanolic ammonia leads to the failure of the classical method of synthesis. Different routes for the synthesis of compound **1** have been reported with low yield [4]. Azacitidine is commercially available as Vidaza™ for intravenous or subcutaneous treatment of acute forms of leukemia [5]. Vidaza is formulated at a pH of about 6.5 contains a small quantity of sodium hydroxide and delivers l00 mg of azacitidine in 10 mL of water when the lyophilized is reconstituted. This concentrate is further diluted with normal saline or Ringer's lactate to yield a finally administered concentration of 1 mg/mL [6]. The presence of sodium hydroxide in Vidaza also results in limited stability [7].

Winkly and Robins [8] reported a 5-azacitidine synthesis process that relies on the coupling of the 'bromo sugar' with a silyl derivative of 5-azacytosine. Niedballa and Vorbrueggen [9] reported the procedure that has been used historically for large scale synthesis of 5-azacitidine for the treatment of myclodysplastic syndromes. The industrial draw backs of the described process are (a) silylation of 5-azacytosine with more molar equivalents of hexamethyldisilazane (HMDS), more reaction duration time for silylation (b) more consumption of expensive stannic chloride (SnCl₄) and variable amounts of tin from one batch to another batch in lack of control of the tin level ensuing the procedure is not suitable for producing API for human use, (c) development of the emulsion in the work up procedure of coupled mixture. Vorbrüggen and Ruh-Pohlenz [10] have previously noted that silylated heterocycles and protected 1-*O*-acyl or 1-*O*-alkyl sugars in the presence of Fredal-Craft catalyst like SnCl₄ often form emulsion and colloids during work up d) expensive and unstable methanolic ammonia usage for deprotection of benzoylgroups e) very low yield 5 % from the 1a and the purity of the final product (98 %) did not comply with the requirement of USP 30 (> 99.8 %). Piskala *et al.* [11] have also reported a procedure for the ribosylation of silver salts of 5-azapyrimidine nucleobases. Specifically, the ribosylation of the silver salt of 5-azacytosine with 2,4,5-tri-*O*-benzoyl-β-D-ribosyl chloride gives a mixture of 5-azacitidine.

Herein, we report a method for preparing azacitidine by using 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as deprotecting reagent for carbohydrates which is suitable for the large

scale production of the material. This improved process has some industrial advantages such as convenient operation, economical material cost and moderate yield with the classical method of synthesis.

EXPERIMENTAL

Reactions involving oxygen and/or moisture sensitive reagents were carried out under an atmosphere of nitrogen or argon using anhydrous solvents. Anhydrous solvents were obtained in the following manner: THF was dried over sodium/benzophenone and distilled, methanol was dried over activated molecular sieves (3 Å) and degassed. Acetonitrile was dried over P_2O_5 and distilled, and DMF was dried over activated molecular sieves (4 Å) and degassed. All the other solvents were of technical quality and distilled prior to use and deionized water was used throughout the process. Column chromatography was carried out on silica gel 60-120 mesh (0.040-0.063 mm) and 230-400 mesh under flash conditions. Thin layer chromatography (TLC) was performed on aluminum plates precoated with silica gel 60 F_{254} and Rf's were determined using the solvent system used to elute the column unless otherwise specified. Solvent system are reported in vol:vol ratios. Visualization of the spots was carried out using UV light (254 nm) and/or stanning under heating (H_2SO_4 stanning solution. Preparation – 4 g of vanillin, 25 mL of conc. H_2SO_4, 80 mL of acetic acid and 680 mL of MeOH. $KMnO_4$ solution, preparation- 1 g of $KMnO_4$, 6 g of K_2CO_3, 1.5 mL of 1.25 NaOH solution and 100 mL of water.

Analytical HPLC was performed on a standard system with a diode array UV detector and equipped with a Zorbax column (5 μm) containing reversed-phase silica gel purospher RP 18 (5 μm).

Method-I: Eluent A water (0.1 % TFA), eluent B MeCN; 0-15 min gradient of B (3-30 %, 15-20 min 100 % B, 20-22 min gradient eluent A water.

^{1}H NMR spectra were recorded at 400 MHz. ^{13}C NMR spectra were recorded at 100 MHz and were ^{1}H-decoupled. All spectra were measured at room temperature except some samples in DMSO-d_6 and D_2O (standard 35 °C). All the NMR spectra were referenced internally to solvent reference frequencies wherever possible. All chemical shifts (δ) are quoted in ppm and coupling constants (*J*) values are reported in Hz. Assignment of signals was carried out using ^{1}H, ^{1}H-COSY, NOESY spectra.

Infrared spectroscopy (IR) was either performed on a spectrometer equipped with an ATR unit or on a machine lacking the ATR unit, with solids being measured as KBr Palates. Peaks are given as wave numbers (ν) in cm^{-1}. UV/visible spectroscopy: wavelengths of maximum absorption (λ_{max}) are reported in nm with the corresponding logarithmic molar extinction coefficient (log (ε, dm^3 mol^{-1} cm^{-1})) given in parentheses. Melting points (m.p.) are determined by the capillary method on a Buchi melter apparatus and are not corrected. The specific optical rotations were measured with Perkin Elmer 241 polarimeter using a sodium lamp (589 nm) at room temperature. Mass spectra were recorded with an Agilent system using electron spray-ionization (ESI) technique.

2,3,5-Tri-*O*-acetyl-1-*O*-methyl-D-ribofuranose (2): To a solution of 1-*O*-methyl-β-D-ribofuranose (1.0 Kg) in diisopropyl ether (400 mL) was added and cooled to 4 °C. Then acetic anhydride (80.0 g) and acetic acid (48.0 g) were added followed by pyridine (25.0 g). The reaction mixture was cooled to 0 °C and added concentrated sulphuric acid (88 g). The reaction mixture was stirred for 24 h at 0 °C and added sodium acetate (216.5g). Ethyl acetate (1.2 L) was added and stirred for 5 min followed by the addition of saturated sodium bicarbonate to adjust the pH up to 7.3. Separated the layers and aqueous layers is extracted with ethylaceate (1.0 L) and combined organic layer dried with $MgSO_4$ and concentrated to obtain crude 2,3,5-tri-*O*-acetyl-1-*O*-methyl-L-ribofuranose (1.5 Kg). Crystallization done with ethanol by heating to 45 °C and ramp cooling to room temperature to obtain 950 g of 2,3,5-tri-*O*-acetyl-1-*O*-methyl-D-ribofuranose. ^{1}H NMR (400MHz, CDCl$_3$, TMS = 0 ppm): 2.08 (s, 3H), 2.09 (s 3H), 2.10 (s, 3H), 2.13 (s, 3H), 4.12-4.19 (m, 1H), 4.30-4.40 (m, 2H), 5.32-5.38 (m, 2H), 6.17 (s, 1H) ppm.

2,3,5-Tri-*O*-acetyl-β-D-ribofuranose-4-amino-1,3,5-triazine (3): To a suspension of 5-azacytocin (276.0 g), hexamethyldisilazane (2.5 L) and trimethylsilylchloride (100 mL), iodine (1.0 g) were added and heated to 125 °C to reflux for 2 h. 5-Azacytocin was dissolved and reflux is stopped after the compete gas evaluation. The excess HMDS is evaporated off in the vacuum to obtain off-white solid, which is silylated azacytocin. The above solid was diluted with ethyl acetate (5.0 L) and 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose (1.0 Kg) was added. Cooled the reaction mixture to 10 °C for 30 min and added SnCl$_4$ (600 mL) over a period of 10 min. Temperature raised for 24 °C. Reaction mixture became clear solution from suspension. Stirred for 2 h, after the reaction monitoring by HPLC added NaHCO$_3$ (2.0 Kg) and Na$_2$CO$_3$ (2.0 Kg) and to the reaction mixture. Added demineralized water (500 mL) slowly to the reaction mixture over a period of 15 min and filtered. Separated the organic layer and the aqueous layer is extracted with ethyl acetate (1.0 L). The organic layer is washed with cold 10 % sodium bicarbonate (2X1L). Organic layer is separated and washed with 10 % NaCl solution. Organic layer is separated and added charcoal (50 g) and heated to 50 °C for over 2 h and filtered through the celite bed. Celite bed is washed with ethyl acetate (500 mL). Organic layer is dried over magnesium sulphate. Silica gel (60-120 mesh) was added to the organic layer, stirred for 1 h at room temperature and filtered through the celite bed. Celite bed was washed with ethyl acetate (500 mL). Organic layer is concentrated in vacuum to dryness to obtain 1.25 Kg of compound **3** as a brown solid. Yield 85 %, ee 90 % (chiral purity).

4-Amino-1-β-D-ribofuranosyl-*s*-triazin-2(1*H*)-one or crude azacitidine (4): To a solution of 2,3,5-tri-O-acetyl-β-D-ribofuranose-4-amino-1,3,5-triazine (1.0 Kg) which is dissolved in DBU (5.0 L) was added methanol (40 L) and stirred for 6 h at 20-25 °C. Filtered the solid and washed with methanol (500 mL) and suck dried for 30 min and subjected for drying under vacuum tray drier to get crude 400 g of 4-amino-1-β-D-ribofuranosyl-s-triazin-2(1*H*)-one.

Purification of crude 4-amino-1-β-D-ribofuranosyl-*s*-triazin-2(1*H*)-one (1): To a solution of crude 4-amino-1-β-

D-ribofuranosyl-*s*-triazin-2(1*H*)-one (**4**) (400 g) in DBU (2.0 L) was heated to 90 °C and added methanol (10 L) and acetic acid (400 mL) slowly over a period of 30 min. Cooled the reaction mixture to room temperature, filtered the solid and washed the product with methanol (500 mL) to get pure 4-amino-1-β-D-ribofuranosyl-*s*-triazin-2(1*H*)-one (**I**) (390 g), *m/z* 244.4 (**Scheme-I**).

¹H NMR (CDCl₃, 500 MHz): d 8.18 (s, 1H), 7.69 (s, 1H), 6.33 (s, 1H), 5.82 (d, 1H, *J* = 3.0 Hz), 5.54 (t, 1H, *J* = 4.0 Hz),

5.41 (t, 1H, *J* = 6.0 Hz), 4.30-4.41 (m, 3H), 2.11 (s, 3H), 2.10 (s, 3H) and 2.08 (s, 3H) ¹³C NMR (CDCl₃, 100 MHz) 170.3, 169.6, 169.5, 166.0, 156.0, 153.1, 89.7, 79.9, 73.7, 69.9, 62.8, 20.7, 20.4 and 20.3 ppm.

RESULTS AND DISCUSSION

1,2,3,5-Tri-*O*-acetyl-β-D-ribofuranose was prepared by the reported method [12] with acetyl chloride in the presence of acetic acid and pyridine. Silylation of 5-azacytoise was

Route of synthesis:
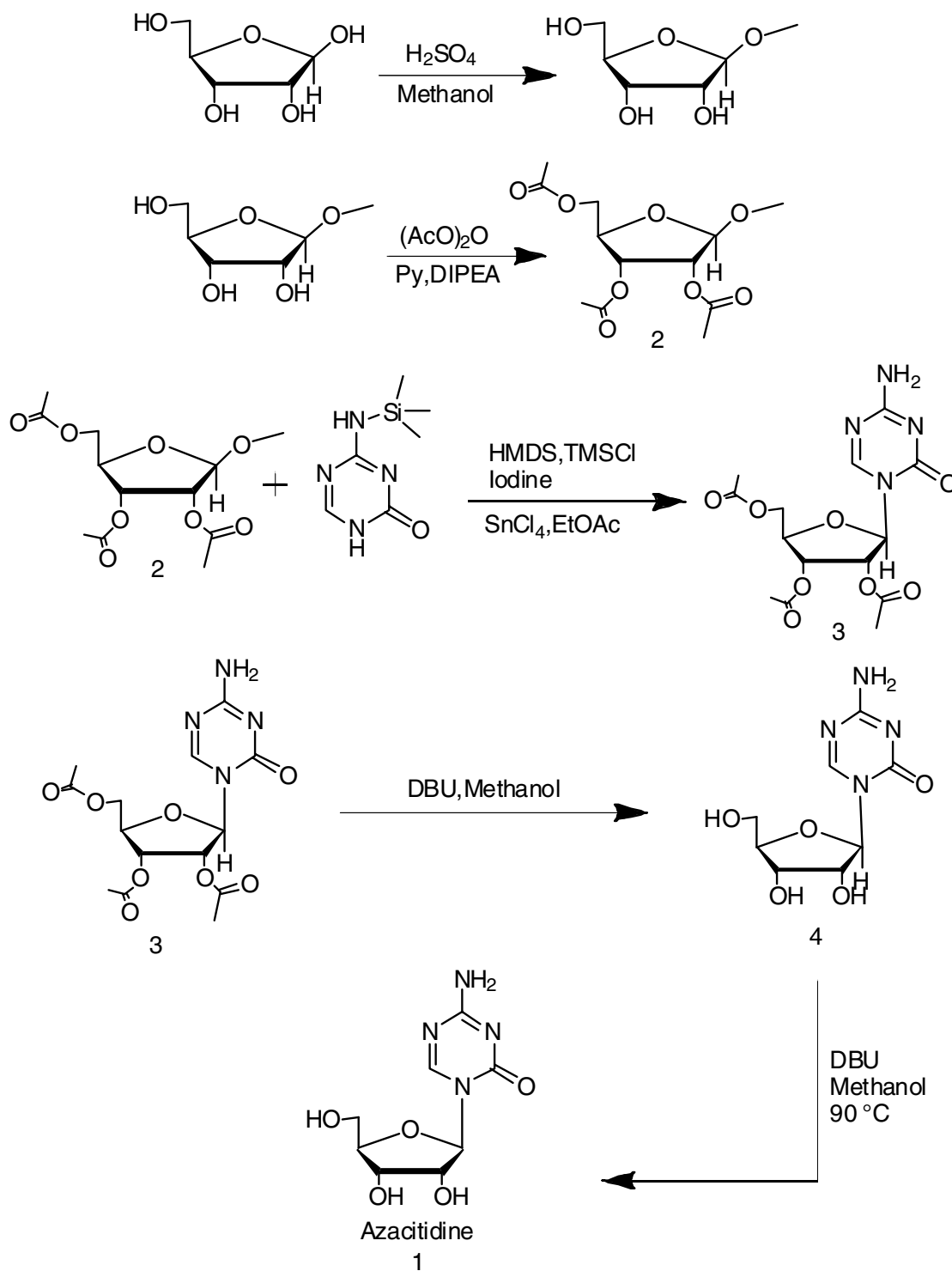
Scheme-I

performed with *N,N-bis*trimethylsilylacetamide (BSA) hexamethyldisilazane (HMDS) as reaction solvent. TMSCl, TMSI, $(NH_4)_2SO_4$ were tested as catalyst for the silylation reaction initiation. The coupling reaction between 5-azacytocin and protected carbohydrates carried out with different Lewis acids in various solvents (Table-1). Work up of coupled reaction furnished in the solution of ethyl acetate at ambient temperature with the good purity. The coupling reaction phenomenon indicates that the reaction proceeds through *via* SN^1 like path way, which is consistent with the hypothesis of Piskala. An expensive $SnCl_4$ molecular equivalent is studied here for the coupling reaction. The usage of class-1 solvents like benzene, carbon tetrachloride, carbon trichloride, ethylene dichloride was avoided for the coupling reaction by the contemplation of ICH guidelines. We have studied the number of solvents for the successful coupling reaction such as tetrahydrofuran, dichloromethane, acetonitrile, ethyl acetate. Finally, ethyl acetate was provided the clear reaction mixture without any emulsion at the time of work up. After the reaction completion, $SnCl_4$ was removed by the $NaHCO_3$ and Na_2CO_3 mixture (1:1). Charcoal treatment was done to improve the colour of the intermediate. Neutral alumina and charcoal mixture was used to make slurry of the coupled product and then filtered through the celite pad.

TABLE-1
DIFFERENT LEWIS ACIDS FOR COUPLING REACTION

Entries	Batch size (g)	$SnCl_4$ equiv.	Solvents for glycosylation	Purity by HPLC, 4	Tin level[a] in 7
1	5.0	1.0	DCE	75	10
2	5.0	1.5	DCE	98	19
3	5.0	2.0	DCE	98	30
4	5.0	2.5	DCE	98	60
5	10.0	1.5	DCE	97	15
6	100.0	1.5	DCE	98	15
7	500.0	1.5	DCE	98	17

DCE = Dichloroethane

The deprotection of compound **4** performed with DBU, methanol was used as solvent with DBU to furnish crude azacitidine. The crude azacitidine was crystallized with DBU, methanol and acetic acid (5:10:1) to get pure azacitidine.

Comparing the previously reported result of Piskala procedure, it was inferred that the yield and purity obtained in the present study was almost similar (Table-2). In addition, the present synthetic process involves simple and user friendly methodology for the deprotection benzoyl, acetyl group. So that this approach could be applied for the large scale production.

TABLE-2
YILED AND PURITY OF FINAL PRODUCT

Entries	Silylating reagent	Temp. (°C)	Catalyst	Time (min)	Yield[d] (%) of 4
1	HDMS	125	TMSCl	180	60
2	HDMS	125	$(NH_4)_2SO_4$	180	65
3	HDMS	125	I_2	180	58
4	HDMS	125	TMSI	180	50
5	BSA	80	–	180	60

Conclusion

A new process for the preparation of azacitidine, including crystallization has been provided with similar yield over 3 steps. Because of the effect of *O*-acetyl ribose and *O*-benzoyl ribose, the resulting intermediates have some industrially favourable physical properties. For example, compound **1** can be simply separated from the crude and purified. We have successfully executed the same process to prepare the 8-bromo-3-methyl-7-(B-D-ribofuranosyl)purine-2,6-dione which is we have reported earlier [13]. The advantages of the new process ensure that the suggested approach is an efficient, cost effective and industrially convenient process. Therefore, this methodology could be employed for commercial production of azacitidine in large scale.

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