Organic Process Research & Development

Communication

pubs.acs.org/OPRD

An Improved and Scalable Process for the Synthesis of 5-Azacytidine: An Antineoplastic Drug‖

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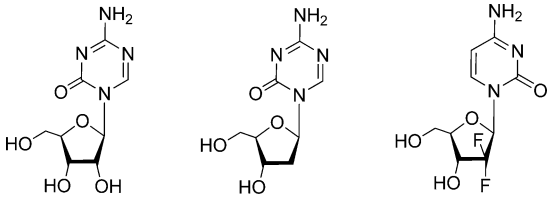
ABSTRACT: An improved, practical, and scalable process for the manufacture of antineoplastic drug, 5-azacytidine (**1**), is described. A thorough understanding of the reaction parameters and stability of the reaction intermediates led us to the development of a robust process. The challenges in the isolation and systematic approach used to streamline the process into a very robust and practical manufacturing process are described.

INTRODUCTION

5-Azacytidine (**1**) is a nitrogen bioisostere of cytidine conceived as a potential inhibitor of nucleic acid biosynthesis and was isolated from the culture filtrates of *Streptoverticillium ladakanus*. It is an an investigational antineoplastic agent that can be incorporated into the RNA and DNA during transcription and replication, respectively.[1] 5-Azacytidine is a nucleoside antimetabolite used in the treatment of different cancers to slow or stop the growth of abnormal cells.[2] Specifically it has been used for the treatment of acute nonlymphoblastic leukemia. It has received approval from the U.S. Food and Drug Administration (FDA) for the treatment of myelodysplastic syndrome (MDS) and is sold under the trade name Vidaza.[3] Other derivatives of 5-azacytidine, decitabine, and gemcitabine, were found to be active in the treatment of lung, pancreas, bladder, and breast cancers.[4]



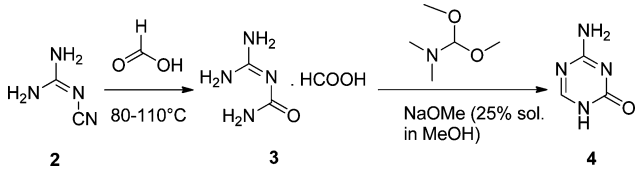
The noteworthy activity of **1** in biological systems and antitumor assays encouraged the synthetic chemists to explore efficient approaches for the synthesis of 5-azacytidine. Pisaka and co-workers[5] developed the first synthesis of 5-azacytidine using 1-glycosyl isocyanates. Subsequently, other groups[6] have synthesized 5-azacytidine using different strategies which include the following: (i) coupling of 1-bromo-2,3,5-tri-*O*-acetyl-D-ribofuranose with silylated 5-azacytosine; however, this process takes longer reaction times and gives poor yields (11%). (ii) Coupling of 1-chloro-2,3,5-tri-*O*-acetyl-D-ribofuranose with silylated 5-azacytosine, where preparation of 1-chloro-2,3,5-tri-*O*-acetyl-D-ribofuranose is difficult. (iii) Coupling of 1,2,3,5-tetra-*O*-acetyl-*β*-D-ribofuranose with silylated 5-azacytosine by using $SnCl_4$ and trimethylsilyl trifluoromethanesulfonate; however, this process has several disadvantages such as lengthy workup procedure and formation of emulsions and colloids during workup that have higher heavy metal and sulphated ash content which are noncompliant with pharmacopoeial requirements.

Herein, we present our efforts for the development of a viable industrial process for 5-azacytidine using triflic acid as a glycosylating agent.[7] Having understood the challenges associated with the stability of the product, an efficient workup process has been developed to obtain a product with good quality and yield.

RESULTS AND DISCUSSION

Our synthesis starts with the silylation of 5-azacytosine (**4**) with hexamethyldisilazane (HMDS) in the presence of ammonium sulfate to give the silylated 5-azacytosine (trimethylsilanyl-(4-trimethylsilanyloxy-[1,3,5]triazin-2-yl)-amine) **5**. The excess HMDS was removed by distillation, and the crude silylated 1,3,5-triazinone **5** was coupled with 1,2,3,5-tetra-*O*-acetyl-*β*-D-ribofuranose (**6**) in the presence of triflic acid at 40−45 °C to yield 2,3,5-tri-*O*-acetyl-5-azacytidine (**7**) (Schemes 1 and 2).

Scheme 1. Synthesis of 5-azacytosine (4)

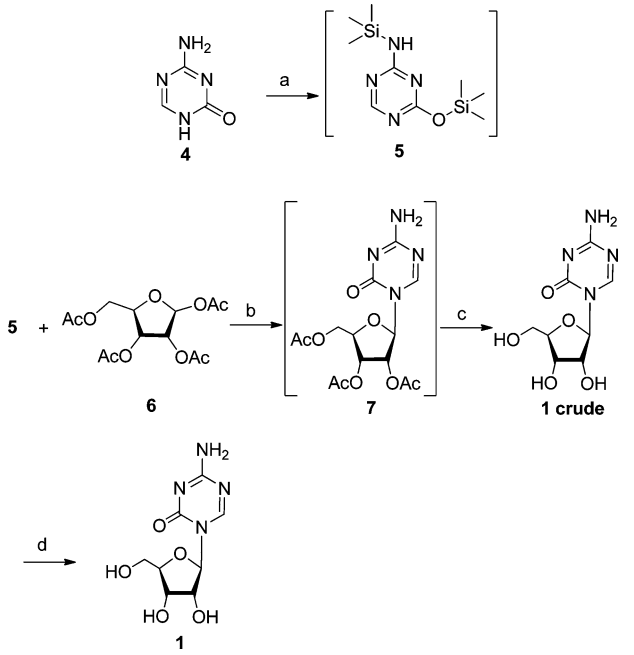


The ratio of 5-azacytosine, triflic acid, and 1,2,3,5-tetra-*O*-acetyl-*β*-D-ribofuranose plays an important role to obtain the product in good yield and purity (Table 1). The optimized conditions for the reaction are 1.2 (mole ratio) of triflic acid and 0.95 (mole ratio) of **6** with respect to 5-azacytosine. According to Baker's rule,[8] the 2-acetyl group controls the

Received: July 17, 2012
Published: January 2, 2013

dx.doi.org/10.1021/op300192e | *Org. Process Res. Dev.* 2013, 17, 303−306

Scheme 2. Synthesis of 5-azacytidine (1)[a]



[a]Reagents and conditions: a) HMDS, ammonium sulfate, acetonitrile, 80 °C. b) Triflic acid, ethyl acetate, 45 °C, 0.24 N HCl, 10% Na_2CO_3 solution. c) *n*-Butylamine, MeOH, 65 °C, d) DMSO, toluene.

Table 1. Ratio of triflic acid and 6 on the yield and purity of 1

entry	4 (mole ratio)	triflic acid (mole ratio)	6 (mole ratio)	yield (%)	purity of 1 by HPLC (%)
1	1	1.0	0.95	25.7	97.7
2	1	1.2	0.8	29.4	97.1
3	1	1.2	0.95	49.4	97.5
4	1	1.2	1.0	46.0	97.5
5	1	1.2	1.4	17.4	92.2
6	1	1.3	1.0	35.0	98.0
7	1	1.5	0.95	23.0	98.2

stereochemistry of the substitution; hence, the β-anomeric product was formed exclusively. The workup of this reaction is very crucial since the triazine ring is highly sensitive to water which undergoes a rapid hydrolytic degradation. Probably the inclusion of an extra nitrogen atom into the cytosine nucleus increases its chemical reactivity towards water.[9]

In order to understand the reaction completely, it becomes necessary to investigate all the reaction parameters in the process development. We have extensively studied the reaction mixture at the glycosylation stage, including the quantities of triflic acid and **6**, and the concentrations and addition times of HCl and Na_2CO_3 solution during the workup. After completion of the glycosylation the reaction mixture was concentrated, the crude residue was dissolved in dichloromethane, and aqueous workup was continued. Ethyl acetate was replaced with dichloromethane as ethyl acetate can hold more water (3.0%) than dichloromethane; moreover, we have minimized the hydrolytic degradation of triazine by choosing the dichloromethane as solvent to obtain the required quality and yield.

Another important aspect is that, for the ignition test of the final API, the residue of sulphated ash content should have ROI less than 0.1% w/w (of final API); therefore, it is necessary to treat the reaction mixture with aqueous HCl to remove the traces of silicon impurities. Since silicon is a metalloid, which will be reflected in the residue on ignition, aqueous acidic treatment during workup will remove the silicon from the organic phase, but here the problem is the moderate stability of the triazine ring at acidic pH.[9] After treating the reaction mixture with 0.24 N HCl, the 2,3,5-tri-*O*-acetyl-5-azacytidine (**7**) was found in both aqueous and organic phases. In order to extract the product **7** into the organic phase, 10% Na_2CO_3 solution was added, and the aqueous phase was separated immediately. Since triazine ring is sensitive to water, the longer exposure of the organic layer to the aqueous layer was avoided by maintaining the reaction mixture for less time after the addition of HCl and Na_2CO_3 (Tables 2 and 3). With this modification the ROI of the 5-azacytidine was less than 0.1% w/w, the quality and yield were good, and the results were consistent.

Table 2. Influence of HCl quantity and treatment time on yield, purity, and ROI of 1

entry	4 (g)	normality of HCl (10 vol wrt 4)	HCl treatment time (min)	ROI (w/w %)	yield (%)	purity of 1 by HPLC (%)
1	5.0	0.12	45	0.25	27.5	98.5
2	5.0	0.24	90	0.07	27.8	91.0
3	5.0	0.24	45	0.06	45.4	97.6

Table 3. Influence of Na_2CO_3 quantity and addition time on yield and purity of 1

entry	4 (g)	Na_2CO_3 (vol wrt 5)	Na_2CO_3 soln. addn. time (min)	yield (%)	purity of 1 by HPLC (%)
1	5.0	20	25	23.0	98.3
2	5.0	10	25	19.3	94.0
3	5.0	15	25	45.4	97.6
4	100.0	15	65	32.8	97.1

After completion of the workup at glycosylation stage, the organic phase was concentrated and codistilled with methanol to get unstable foamy residue 2,3,5-tri-*O*-acetyl-5-azacytidine (**7**). Here ensuring the removal of traces water is important hence codistillation with methanol is mandatory to get consistent yield and purity. The obtained residue was treated with *n*-butylamine in methanol at reflux temperature to get the crude 5-azacytidine in 50% yield. During the acetyl deprotection the methanol volume and temperature play a key role in getting the required quality and yield (Table 4). The byproducts obtained in the deprotection step are washed off into filtrate. The crude 5-azacytidine, which has the ROI <0.05% w/w and purity of about 98.0% exclusively as 100% β-isomer was recrystallized in DMSO/toluene to get 99.9% pure 5-azacytidine.

The hurdle for the large-scale production of the 5-azacytidine is the availability of 4-amino-1,3,5-triazin-2-one (**4**) with required purity. Known synthesis for the preparation of **4**[10] has given poor yields and purity. Some of the processes reported in the literature gave the purity of <50%, and the LR grade materials are also not available with required quality. Thus, we have modified the reported process and developed the scalable process for the large-scale preparation of **4** (5-azacytosine). We have observed that the reaction is exothermic during the scale-up, and the temperature was controlled

dx.doi.org/10.1021/op300192e | *Org. Process Res. Dev.* 2013, 17, 303−306

Table 4. Influence of methanol quantity, maintenance time, and temperature on quality of 1

entry	4 (g)	methanol (vol wrt 5)	temp. range (°C)	time (h)	yield (%)	purity of 1 by HPLC (%)
1	5	10	40−45	4	38.6	94.0
2	5	15	40−45	1	40.4	93.1
3	5	25	40−45	1	41.3	92.6
4	5	35	40−45	1	42.5	94.4
5	5	25	60−65	1	42.8	95.5
6	5	45	60−65	1	32.1	98.0
7	5	35	60−65	1	47.6	98.1
8	5	35	60−65	2	34.9	98.5
9	5	35	60−65	3	29.4	98.4
10	5	35	60−65	4	27.5	99.0
11	5	35	60−65	5	25.7	98.6

effectively by thorough understanding of calorimetric studies and obtained 91% yield with 99% purity.

Optimization of Triflic Acid and 1,2,3,5-Tetra-*O*-acetyl-β-D-ribofuranose (6) Mole Ratios during Synthesis of 1. The mole ratios of triflic acid and **6** play a pivotal role in the synthesis of **1**. Reactions were conducted using different mole ratios of triflic acid (1.0, 1.2, 1.3, and 1.5) and **6** (0.8, 0.95, 1.0, and 1.4). Experimental results obtained by using different mole ratios of triflic acid and **6**, as shown in Table 1, indicate that (a) the yield decreases by changing the mole ratio of triflic acid from 1.2 mol. (b) The yield decreased on increasing the mole ratio of **6** as the formation of impurities were greater during the glycosylation step, and these impurities were washing out into the filtrate during isolation of **1**. (c) By decreasing the mole ratio of **6**, yield decreases, and the unreacted **5** was removed by washing into the filtrate. (d) Yield was slightly lower when the reaction was carried out using a 1.2 mol ratio of triflic acid and a 1.0 mol ratio of **6**. (e) Optimum results were obtained when the reaction was carried out using 1.2 mol of triflic acid and 0.95 mol of **6** (Table 1, entry 3).

Optimization of HCl Quantity and Treatment Time during Synthesis of 1. Experimental results obtained by using different normalities of HCl (0.12 and 0.24) and by changing the treatment times (45−90 min) are summarized in Table 2. The data indicate that (a) the reaction with 0.12 N HCl for 45 min gave lower yield and more ROI due to the formation of degradation impurity; (b) when the reaction was treated with 0.24 N HCl for 90 min, it resulted in decreased yield due to the formation of RGU impurity, which was removed by washing into the filtrate; (c) when the reaction was carried out using 0.24 N HCl for 45 min, optimum results were obtained (Table 2, entry 3).

Optimization of Na_2CO_3 Quantity and Treatment Time during Synthesis of 1. Experimental results obtained by using different quantities of Na_2CO_3 (10, 15, and 20 vol) by changing the treatment time (25 to 65 min) are shown in Table 3, which indicates that (a) the reaction with 10 vol of Na_2CO_3 and 25 min of treatment time gave poor yield due to the loss of **3** in the aqueous layer. (b) The reaction with 20 vol of Na_2CO_3 and 25 min of treatment time gave poor yield due to the formation of degradation impurities which are removed by filtration. (c) Optimum results were obtained by treating with 0.24 N HCl for 45 min (Table 3, entry 3).

Optimization of Methanol Quantity, Maintenance Time, and Temperature during Synthesis of 1. Experimental results obtained by using different quantities of methanol (10, 15, 25, 35, and 45 vol) by maintaining at different temperatures (40−65 °C) are shown in Table 4. The results indicate that (a) the reactions with 10, 15, 25, and 35 vol of methanol and at 40−45 °C gave poor yields and lower HPLC purity which is due to incomplete hydrolysis of acetate groups of **3**; (b) when the quantity of methanol and the temperature were increased to 45 vol and 60−65 °C, respectively, the reaction gave lower yield due to loss of **1** in the filtrate; (c) when the reaction was maintained at 60−65 °C for more than 1 h, it resulted in the gradual decrease of the yield due to the formation of degradable impurities; 35 vol of methanol and 60−65 °C for 1 h were chosen as suitable reaction conditions for the hydrolysis of ester to **1** (Table 4, entry 7)

CONCLUSION

In conclusion, the improved and scalable process for the high-yielding synthesis of 5-azacytidine has been discussed. Simplification of the workup procedure by thorough understanding of the process and deprotection at reflux temperature to wash out all the impurities provided a robust and scalable process. The residue on ignition, yield, and purities were consistent from lab to scale-up (see Table 5).

Table 5. Experimental results by incorporating all optimal conditions for the synthesis of 1

entry	4 (g)	yield (%)	HPLC purity of 1 (%)
1	100	49.4	97.5
2	200	47.0	98.6
3	200	47.5	98.1

EXPERIMENTAL SECTION

Solvents and reagents were obtained from commercial sources and used without further purification. ^{1}H NMR spectra were recorded in DMSO-d_6 at room temperature on a Varian Mercury spectrometer plus 400 MHz using TMS as an internal standard. ^{13}C NMR spectra were obtained from a Varian Mercury plus 100 MHz spectrometer in DMSO-d_6 at room temperature. IR spectra were recorded in the solid state as KBr dispersion using a Perkin-Elmer 1650 FT IR spectrometer. The mass spectrum (70 eV) was recorded on an HP 5989 A LC−MS spectrometer. TLC analyses were performed on Merck silica gel 60_{F254} plates.

4-Amino-1,3,5-triazin-2-one (4). A mixture of cyanoguanidine (**2**) (16 kg, 190.5 mol) and formic acid (6.29 kg, 571 mol) were heated to 80 °C in 200 L reactor, whereupon the reaction started to boil vigorously; cooling was applied to the reactor. Even though, because of the exothermic reaction the temperature rises to 110 °C. After 15 min, the heterogeneous mixture was further cooled to 30 °C and diluted with 80 L isopropyl alcohol. Solid product was isolated by filtration and then dried at 70 °C to obtain 24 kg (85%) of guanylurea formate (**3**). The resultant **3** (24 kg, 162.2 mol) was dissolved in methanol (7.2 L) and sodium methoxide (10.5 kg, 48.6 mol of 25% solution in methanol). DMF-DMA (31.4 kg, 210 mol of 80% solution) was added to the resultant mixture and heated to 40−50 °C for 4 h. Water was (19.2 L) added, the product was isolated by filtration, and the wet cake was slurried in a solution of water and methanol (75 L in 1:1 ratio) and then dried at 70 °C to get **4** as a white powder with 90% yield (16.3 kg) having >99% HPLC purity.

dx.doi.org/10.1021/op300192e | Org. Process Res. Dev. 2013, 17, 303−306

4-Amino-1-β-D-ribofuranosyl-1,3,5-triazin-2(1*H*)-one (1, Crude). A mixture of 5-azacytosine (**4**, 4.0 kg, 38.74 mol), hexamethyldisilazane (HMDS) (8.64 kg, 53.57 mol), ammonium sulfate (200 g, 1.51 mol), and acetonitrile (12.0 L) were charged into GLR through isolator, and the resultant mixture was heated to reflux for 4−8 h. After observing the clear solution, the reaction mixture was concentrated to obtain the residue. Ethyl acetate (8 L) was co-distilled in two lots to remove HMDS completely to obtain the **5**, then dissolved in ethyl acetate (40 L), and cooled to 0−10 °C. To the above mixture was added 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose, and then triflic acid (6.42 kg, 42.82 mol) was added, and the resulting mixture was heated to 40−50 °C and stirred for 30 min. Then the reaction mixture was concentrated to obtain residue, the residue was dissolved in dichloromethane (40 L). Then 0.24 N hydrochloric acid (40 L) was added, and after 45 min stirring, 10% sodium carbonate solution (60 L) was added in 30 min. The organic layer was separated and transferred to SSR and then concentrated. Methanol (8 L) was co-distilled in two lots to obtain a foamy residue, **8**. The foamy residue was dissolved in methanol (140 L), and then *n*-butylamine (1.6 L, 16.2 mol) was added. The resultant mixture was heated to reflux and stirred for 60 min; the product was filtered at the same temperature in ANFD and dried to afford the crude 5-azacytidine with 49.3% yield (4.3 kg) having HPLC purity of >98%.

4-Amino-1-β-D-ribofuranosyl-1,3,5-triazin-2(1*H*)-one (1). To a stirred solution of 1.5 kg of **crude 1** and DMSO (6 L), was added 22.5 L of toluene. The resulting mixture was stirred for 5 h, and then product was filtered and dried to obtain the 5-azacytidine. Yield 1.13 kg (75.3%) with 99.89% HPLC purity. M + 1 = 245.08, ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.58 (s, 1H), 7.52 (bs, 2H, NH_2), 5.67 (d, J = 3.6 Hz, 1H), 5.42 (d, J = 4.8 Hz, 1H), 5.11 (t, J = 4.8 Hz, 1H), 5.02 (d, J = 6.0 Hz, 1H), 4.08 (q, J = 4.8 Hz, 1H), 4.01 (d, J = 5.2 Hz, 1H), 3.86 (m, 1H), 3.68 (m, 1H), 3.57 (m, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 165.9, 156.5, 153.5, 89.5, 84.4, 74.1, 69.1, 60.3; IR (KBr) cm^{-1} 3404, 2975, 1702, 1497, 1367; Anal. Calcd for $C_8H_{12}N_4O_5$: C 39.35, H 4.95, N 22.94. Found: C 39.43, H 4.87, N 22.88.

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Notes

[‖]Dr. Reddy's Communication no. IPDO IPM-00214

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank the management of Dr. Reddy's Laboratories Ltd. for supporting this work. Co-operation from the project colleagues of API-Process Research Department, Integrated Product Development Organization, Dr. Reddy's Laboratories Ltd. is highly appreciated.

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