An Integrated Chemo-enzymatic Route for Preparation of β-Thymidine, a Key Intermediate in the Preparation of Antiretrovirals

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Abstract:

A chemo-enzymatic method for production of β-thymidine, an intermediate in the synthesis of antiretrovirals, is described. Guanosine and thymine were converted by means of enzymatic transglycosylation to yield 5-methyluridine (5-MU), which was reproducibly synthesised at a 10−20-L scale in 85% yield at a final product concentration of ~80 g·L^{-1}. A downstream processing (DSP) protocol was designed to remove reaction components interfering with the subsequent synthetic step. The crystallised 5-MU produced in the biocatalytic reaction was found to behave similarly to commercially available 5-MU, and the integration of the initial biocatalytic and subsequent three-step chemical process to β-thymidine was successfully demonstrated at bench scale.

Introduction

β-Thymidine is required in multitonne quantities as a precursor to the anti-AIDS drugs stavudine (d4T)[1] and zidovudine (AZT).[2] There are four broad approaches to nucleoside production: extraction from natural sources, chemical synthesis, fermentation, and biocatalysis.

β-Thymidine may be obtained from natural sources through hydrolysis of DNA. Companies such as Yamasa Shoyu Co. (Tokyo, Japan) and Reliable Biopharmaceuticals Corp. (St. Louis, U.S.A.) have digested hundreds of tonnes of salmon milt each year to isolate tonne quantities of individual nucleosides, including β-thymidine. However, the use of natural resources is inefficient as typically 100 tonnes of salmon only yields a total of approximately 55 kg of the above deoxynucleosides in roughly equal amounts.[3]

Traditional chemical methodologies for nucleoside production suffer from numerous disadvantages, such as the use of toxic metal reagents, like silver and tin, which are used to activate substrates.[4] In addition, often both the α and β anomers are generated, and the desired product needs to be separated from the mixture.

Fermentation processes have been used for β-thymidine production, but generally low thymidine concentrations are achieved (0.5−7 g·L^{-1}),[5] resulting in additional processing costs for product isolation. The low concentration of β-thymidine produced may be attributed to tight metabolic control that is difficult to overcome.[6]

Methods of nucleoside synthesis involving use of biocatalysts[4] have also been developed. Mitsui Chemicals produce deoxynucleosides by chemical conversion of D-glucose to deoxyribose phosphate, followed by the enzyme-catalyzed addition of the required base.[3] Chemo-enzymatic routes allow for the increased volumetric productivity of chemical methods to be combined with the selectivity of biological methods.

Hori et al.[7] published extensive work in which they applied enzymes in the transglycosylation of inosine with thymine to yield 5-methyluridine (5-MU), a compound that can be used as an intermediate in the synthesis of β-thymidine.[8] Unfortunately, the reported yields and productivities were low. However, Ishii et al.[9] (1989) showed that by using guanosine as the ribose donor and whole cells of *Erwinia carotovora* as a biocatalyst, it was possible to produce 5-MU in a 74% yield at increased starting substrate concentrations of 300 mM guanosine [8.5 percentage mass guanosine per mass of reaction mixture (% m·m^{-1})], albeit over a 24−48 h period. More recently we have demonstrated guanosine conversions of greater than 95% and improved 5-MU yields of 85% with a volumetric productivity of up to 10 g·L^{-1}·h^{-1}.[10] From a commercial supply perspective, guanosine has the benefit of being available cheaply and in large quantities as it can be derived from disodium

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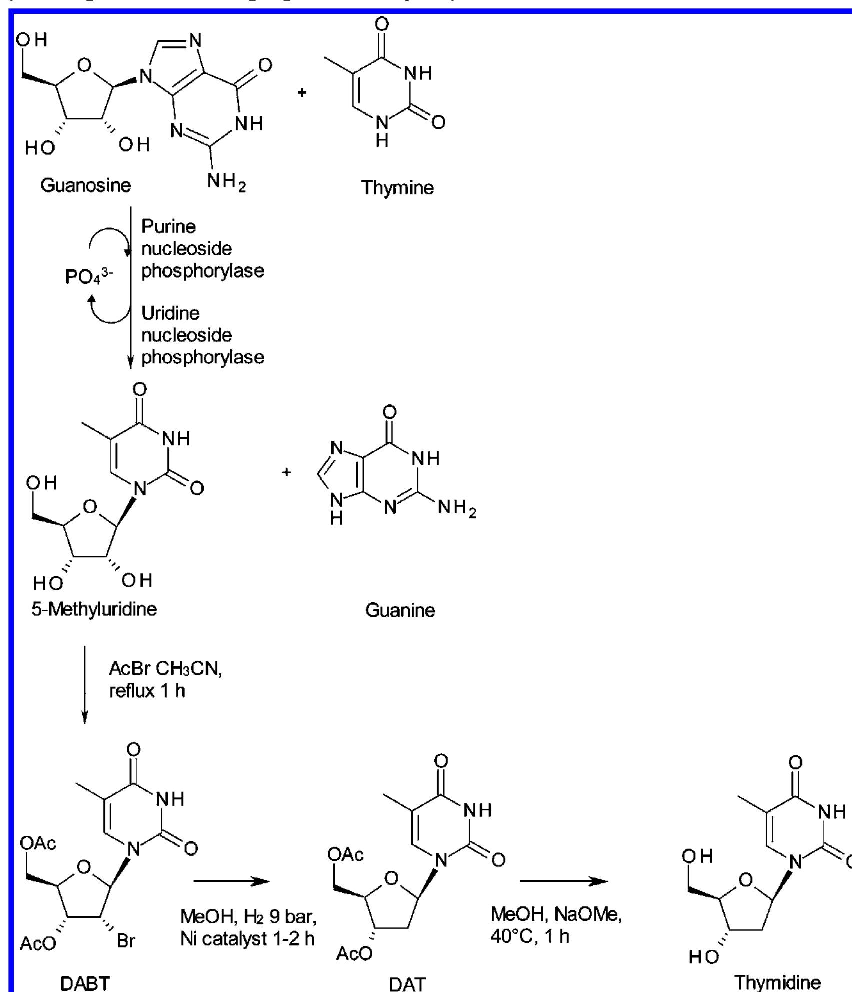
Scheme 1. **Chemo-enzymatic process for the preparation of β-thymidine**



guanosine-5′-monophosphate which is manufactured in Japan by yeast fermentation as a flavourant.[11]

Previously, we have defined the process-operating window for the biocatalytic process using 1-L scale reactions.[10c] This contribution describes the development of a kilogram-scale chemo-enzymatic process to produce β-thymidine. This process comprises a biocatalytic step to synthesise 5-methyluridine, followed by chemical transformations to yield β-thymidine. However, the integration of biocatalytic reactions into chemical synthesis is a critical aspect in technology adoption.[12] Hence, the aim of the current study was to successfully scale the biocatalytic reaction to 10−20 L and integrate this step with the chemical process for the production of β-thymidine as depicted in Scheme 1.

Results and Discussion

Biocatalytic Synthesis of 5-MU. The biocatalytic synthesis of 5-MU was carried out by transglycosylation of guanosine and thymine. In this reaction a ribose moiety is in effect transferred from guanosine, a purine nucleoside, to thymine, a pyrimidine nucleoside, to yield 5-MU and guanine. Ribose-1-phosphate is generated *in situ* by phosphorolysis of guanosine, and this then couples to thymine in a synthesis step to yield 5-MU. The reaction is catalysed by the enzymes purine nucleoside phosphorylase (PNP) and uridine phosphorylase (UP) for the phosphorolysis and synthesis steps, respectively. This is a coupled two-step reaction performed in one pot (Scheme 1). At laboratory scale the highest yield of 5-MU attained was 85%, in a 1-L glass baffled reactor at a productivity of 10 $g \cdot L^{-1} \cdot h^{-1}$.[10c] The substrate thymine (4.7% $m \cdot m^{-1}$) is used in slight excess to the cosubstrate guanosine (9.3% $m \cdot m^{-1}$) in a mole ratio of 1.15:1, and the two enzymes required (PNP and UP) were added at 2000 units each. Even at the reaction temperature of 60 °C, this is a slurry reaction due to low substrate solubility, and effective mixing is thus important. However, during the small laboratory-scale optimisation of the biocatalytic reaction, a crucial factor influencing the success of the reaction was avoidance of excessive agitation.[10c]

On scale-up to 10 L, a glass-lined 16-L reactor with anchor stirrer configuration (CR-16, Büchi, Switzerland) was selected to investigate whether this met the requirements. Reaction reproducibility and robustness were evaluated by performing three runs under identical conditions. The results obtained (Table 1) show that the biocatalytic reaction was both scalable and reproducible with respect to guanosine conversion and 5-MU yield.

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Table 1. **Biocatalytic reaction reproducibility**

reaction	guanosine conversion (%)	5-MU yield (%)	mole balance (%)	normalised 5-MU yield (%)[a]
rxn 1	92.9	92.2	109	84.6
rxn 2	94.3	91.4	108	84.9
rxn 3	96.9	81.1	95.2	85.1
average (%)	94.7	88.2	104	84.9
SD	2.03	6.21	7.61	0.30
RSD[b]	2.14	7.04	7.32	0.36

[a] 5-MU yield normalised to a mole balance of 100%. The reaction was performed at 60 °C over 26 h. Reaction conditions: 9.3% $m \cdot m^{-1}$ guanosine, and 4.7% $m \cdot m^{-1}$ thymine (a molar ratio of 1 to 1.15), 1600 U of each enzyme per litre. [b] RSD: relative standard deviation, (SD/X) × 100.

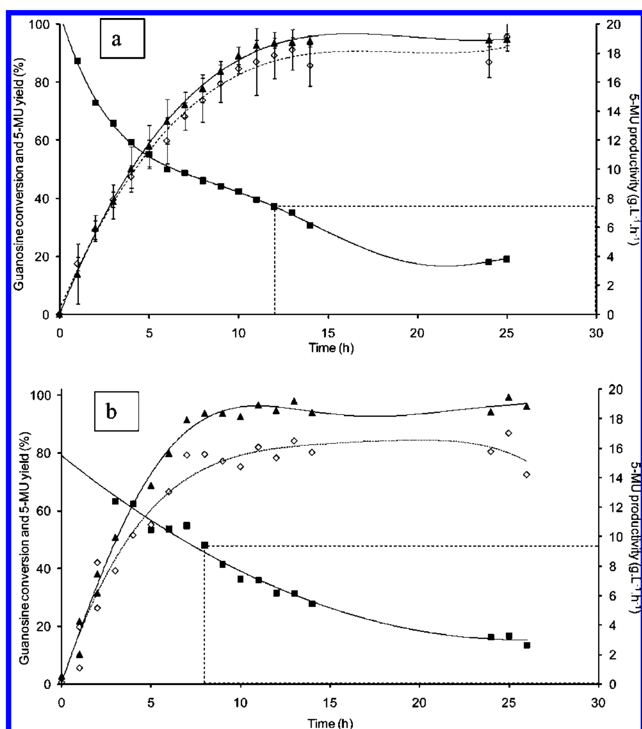

Figure 1. **Guanosine conversion (▲), 5-MU yield (◇), and productivity (■) on (a) a 10-L scale in a CR-16 reactor (triplicate reactions) with anchor stirrer and (b) on a 20-L scale using a CR-26 with a retreat blade impeller (single reaction).**

However, the reaction completion (the point at which no further conversion was observed) was delayed and took 10 h, compared with 7 h at 1-L scale. As a result the 5-MU productivity decreased to 7 $g \cdot L^{-1} \cdot h^{-1}$ (Figure 1a). Lab-scale studies (1 L) conducted using an anchor stirrer with and without baffles showed that proper mixing under low-shear conditions was crucial for optimum reaction performance (results not shown). It was therefore anticipated that the slightly lower productivity could be compensated for by reactor engineering and agitation optimisation. Subsequent replacement of the anchor stirrer with a retreat blade impeller in a 20-L reaction (CR-26 Büchi, Switzerland) returned the productivity to approximately 10 $g \cdot L^{-1} \cdot h^{-1}$ (Figure 1b).

Chemical Synthesis of *β*-Thymidine from 5-MU. *Synthesis of DABT from 5-MU.* The first step in the conversion of 5-MU to *β*-thymidine is reaction with acetyl bromide to produce 3′,5′-di-*O*-acetyl-2′-bromothymidine (DABT, Scheme 1). Conversion of DABT into 3′,5′-di-*O*-acetylthymidine (DAT) is achieved by hydrogenolysis catalysed by sponge nickel in methanol. Subsequent removal of the acetyl protecting groups in the presence of sodium methoxide gives the desired *β*-thymidine. These reactions were all initially evaluated at small scale, starting with commercially available 5-methyluridine and then repeated with our biocatalytically produced 5-MU, before being scaled to bench scale.

Investigation of Factors Influencing DABT Yield. A key to the success of this process was integration of the biocatalytic reaction and the subsequent chemical transformations. The first step was to identify any factors negatively influencing the yield of DABT from 5-MU using commercially available 5-methyluridine. This has direct bearing on the purity requirements of the material entering the first chemical step and was used to define the DSP for the biocatalytic reaction.

The general process followed from 5-MU to DABT was first described by Mansuri et al.[8] This reaction proceeds *via* an anhydro intermediate and can result in the formation of the byproducts thymine, 2′,3′,5′-tri-*O*-acetylmethyluridine (TAMU), and 3′-*O*-acetyl-2′,5′-dibromothymidine (ADBT), as shown in Scheme 2.[13] One of the possible factors leading to byproduct formation is the presence of water or alcohol which interferes with acetylation at the 5′-position, allowing bromination to occur at this position instead. On the basis of the proposed reaction mechanism,[13] *in situ* water formation is shown to take place, and Mansuri et al.[8] used an excess of acetyl bromide to 5-MU over and above that required by reaction stoichiometry in order to compensate for this. The formation of thymine due to acidic cleavage of the nucleoside while undergoing acetylation has also been reported.[14]

In order to define the specification of the 5-MU feed required for reasonable DABT yields we experimented with the DABT synthesis reaction conditions. Reaction parameters such as reaction solvent and substrate concentration were altered, and the effects of typical contaminants following biocatalytic reactions, such as water and phosphate salts, were also evaluated. The results of this investigation were used to define the DSP requirements of the 5-MU produced in the biocatalytic step (see below).

Table 2 shows the yield of DABT obtained according to the different conditions tested. The base case small-scale reaction (experiment 1) used for these investigations, based on the method of Mansuri et al.,[8] was performed in acetonitrile at 2.5% $m \cdot m^{-1}$ 5-MU under reflux conditions using an excess of acetyl bromide to 5-MU (mole ratio of >4.5 to 1). The solvent and acetyl bromide were distilled from the mixture after reaction, followed by partitioning between dichloromethane and water. The workup was slightly modified compared to the original procedure in that a saturated brine wash, instead of a water wash, was applied to minimise transfer of organics into the aqueous layer. A 92% yield was obtained using this procedure. This yield is lower than that reported by Mansuri et al.,[8] but higher than that obtained by Shiragami et al.[13] (48%), who commented on their failure to reproduce the original yield reported. Huang and Chu[15] modified the method by introducing acetic acid and HBr and were able to achieve a 63% yield.

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Scheme 2. **Preparation of DABT from 5-MU**

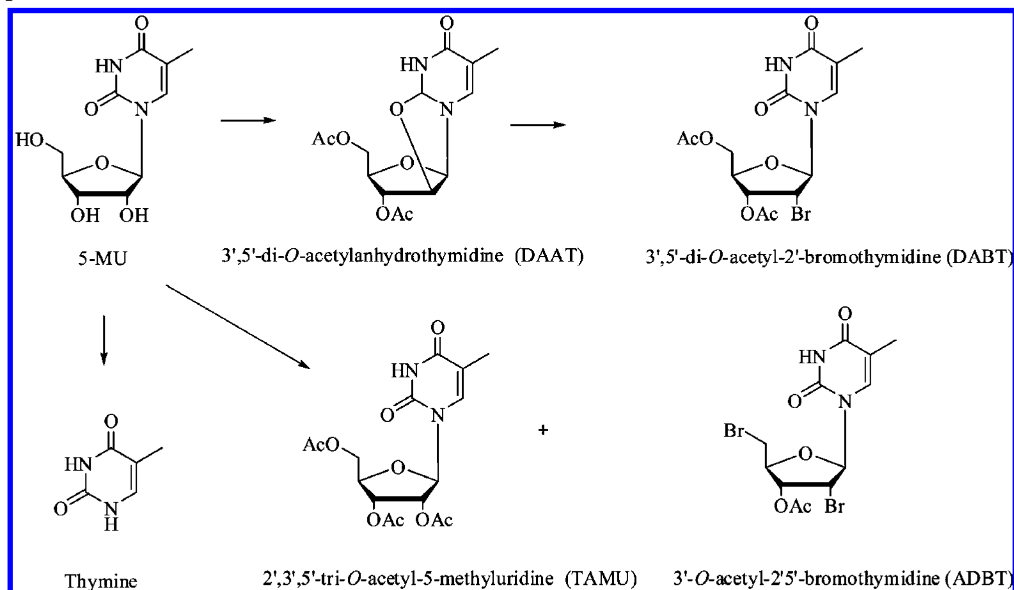

Table 2. **Factors influencing DABT yield**

experiment	parameter altered	DABT yield (%)
1	base case reaction	92
2	quench and neutralisation	84
3	reaction solvent (ethyl acetate)	59
4	water addition	73
5	phosphate addition	52
6	substrate conc. increased to 7.5% $m \cdot m^{-1}$	60

Previous reports had indicated that loss of DABT by cleavage to thymine was possible during lengthy exposure to acidic conditions.[14] Thus, in experiment 2, in order to minimise exposure to acidic conditions, the reaction mixture was neutralized using solid sodium bicarbonate after initial quenching of excess acetyl bromide by the slow addition of methanol. The reaction mixture was then filtered to remove solids, and solvent was removed under vacuum. The crude organic residue was dissolved in dichloromethane (DCM) and washed with water to remove inorganic salts. The quench procedure led to a reduced yield of 84%. As no thymine was detected by HPLC, either with or without neutralisation, this step was not deemed necessary.

The use of a water-miscible solvent, such as acetonitrile, as reaction solvent increased the number of handling steps required for product isolation, and thus replacement with ethyl acetate was tested (experiment 3). Unfortunately, this resulted in increased byproduct formation, with an unidentified product and DABT being present in roughly equal quantities.

As discussed above, it has been reported[13] that the presence of water or an alcohol has an adverse effect on DABT formation, by preventing acetylation of the 5′-position and resulting in ADBT formation (see Scheme 2). Experiment 4 was therefore conducted in the presence of excess water (8.3% $m \cdot m^{-1}$ water on 5-MU) in order to investigate its influence on byproduct formation. This also has a bearing on the dryness necessary for the 5-MU produced in the biocatalytic reaction. The results show the anticipated decrease in DABT yield to 73%.

Phosphate is used in catalytic quantities in the biocatalytic reaction, where it also functions as a buffer. A synthetic mixture of 5-MU and phosphate buffer salts (roughly representing a typical biocatalytic reaction process stream) was prepared and subjected to the standard reaction conditions (experiment 5). The purpose of this experiment was to determine if the presence of phosphate salts had any detrimental effect on reaction performance with respect to DABT yield, and if separation of 5-MU from salts was required. The results showed that the presence of phosphate salts had an adverse effect on the reaction performance, as the amount of DABT produced was lower (52%) and the formation of a major byproduct was observed.

This reaction is particularly dilute and hence has a very low volumetric productivity. Therefore, the concentration of 5-MU in the reaction was increased from 2.5% $m \cdot m^{-1}$ to 7.5% $m \cdot m^{-1}$ in order to increase reactor productivity. Experiment 6 clearly shows that the increased concentration had a deleterious effect on DABT yield, with only 60% product being obtained.

Finally, 5-methyluridine produced in the biocatalytic reaction was used directly without purification in order to test the effect on DABT yield. Results from Table 2 suggested that the presence of water had a deleterious effect on DABT yield, and thus the material was thoroughly dried prior to use. HPLC analysis showed the purity of 5-MU to be 80% $m \cdot m^{-1}$, with the rest of the mass being attributable to inorganic salts, thymine, guanosine, and guanine. During addition of acetyl bromide to the reaction, the mixture turned very dark and remained turbid throughout the reaction. This was in contrast to reactions on pure 5-MU, where all solids dissolved during addition of acetyl bromide and the reaction mixture turned beige. Other than this, by HPLC monitoring, the course of the reaction was identical to that of the pure material. However, subsequent separation of the organic and aqueous layers during reaction workup proved difficult as salts precipitated at the solvent interface. On a small scale this problem could be ameliorated to some extent

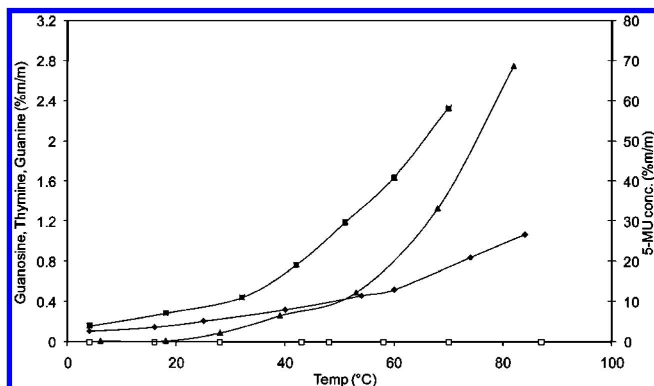

Figure 2. **Solubility of 5-MU (■), guanosine (▲), thymine (◆), and guanine (□) in phosphate buffer as a function of temperature.**

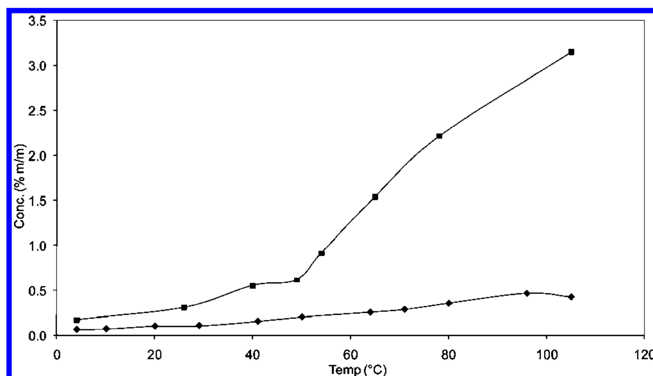

Figure 3. **Solubility of 5-MU (■) or (◆) thymine in isobutanol.**

by the use of a 50% brine solution, rather than saturated brine, but on scale-up this separation would present difficulties. Therefore, on balance, it was recommended that during DSP the 5-methyluridine be purified of salts before use in the first chemical step. On the basis of results from Table 2 and the above result, conditions chosen for scale-up were a 2.5% $m \cdot m^{-1}$ concentration of 5-MU in acetonitrile, with no quenching or neutralisation of the reaction mixture. In addition, it was determined that the 5-MU entering the reaction should be thoroughly dried and be free of salts.

Down-Stream Processing of 5-MU. Integration of the 5-MU synthesis reaction with later synthetic steps required removal of interfering reaction components after the biocatalytic step. Since the presence of water impacted negatively on DABT yield (Table 2), the 5-MU prepared in the biocatalytic reaction for use in the subsequent β-thymidine preparation was required to be free of moisture, and removal of phosphate salts was also considered essential.

The first DSP step involved the removal of guanine. As a result of the low guanine solubility at any temperature (Figure 2), this component could be removed by filtration. Hence, on reaction completion the reaction mixture was heated to 90 °C to maximise solubility of all other reaction components and was filtered hot to recover guanine as a byproduct.

Although any guanosine present would undergo acetylation during the preparation of DABT, the biocatalytic conversion of guanosine was ~95%, and guanosine solubility was low; hence, a negligible amount of guanosine was measurable in the process stream after filtration. It was anticipated that guanosine levels less than 0.1% $m \cdot m^{-1}$ would not have an adverse effect on the β-thymidine process.

Finally, removal of thymine was desirable to enhance 5-MU purity. Removal of unreacted thymine could be achieved by cold filtration at 4 °C, as 5-MU remains in solution at this temperature for short periods. Residual thymine at low levels (0.2% $m \cdot m^{-1}$), could be effectively removed during the brine wash step of the DABT DSP. Thereafter, recovery of 5-MU was possible by maintaining the reaction mixture at low temperature to allow crystallisation from this aqueous solution. However, a significant proportion of the 5-MU was not recovered in this step and remained in solution. A number of different solvents were tested for their suitability to separate the residual 5-MU from inorganic salts. Isobutanol was identified as the most promising on the basis of the relative solubilities of reaction components in phosphate buffer versus isobutanol (Figures 2 and 3). Isobutanol has low water miscibility, while also providing the advantage of relative ease of drying compared to water. Hence, residual 5-MU could be recovered from the crystallisation liquor by removal of water, followed by recrystallisation from isobutanol in order to eliminate inorganic salts.

The final DSP, as depicted in Figure 4, gave 84% recovery of 5-MU at a purity of >90% $m \cdot m^{-1}$. Losses of 5-MU observed during the guanine recovery could potentially be avoided through further washing of the guanine filter cake, where 10% of the 5-MU is lost. This would increase the overall 5-MU recovery to 94%. It is anticipated that further optimisation of the water crystallisation step and mother liquor recycling to recover 5-MU would decrease the proportion of material requiring isobutanol purification.

Integration of Biocatalytic Step into the Multistep Bench-Scale Synthesis of *β*-Thymidine. There is a growing application of biocatalysis in the synthesis of pharmaceutical compounds and intermediates.[16] Crucial to the success of these processes is successful integration with the chemical steps. 5-MU *ex.* biocatalytic reaction and purified according to the method shown in Figure 4 was used to evaluate the DABT yield at small scale on biocatalytically produced material. The results obtained showed the 5-MU to behave identically to commercially available material. The chemical steps were subsequently scaled up, and the bench-scale preparation of β-thymidine from 5-methyluridine produced by biocatalysis was successfully demonstrated. 5-MU ranging in purity from 87 to 95% $m \cdot m^{-1}$ purity was converted into β-thymidine in good yield. In general, yields obtained were similar to those obtained at laboratory scale, indicating no significant problems with scale-up (Table 3). The only significant variation occurred in the preparation of DABT, where we achieved a DABT yield of 92% in small-scale reactions, and only 78% at bench scale. It is considered likely that this reduction in yield at bench scale was the result of loss of material to the aqueous layer during DSP, mediated by water-miscible acetonitrile still present in the product residue. Thymidine recovery was not optimal and recoveries of 70−75% were obtained after collection of a single crop of crystals, with no recycling of the mother liquor. However, based on solubility data of thymidine in methanol, thymidine recovery can be significantly improved to 90% with recycling of the mother liquor. The overall yield of β-thymidine from guanosine was 69% for laboratory scale and 56% for

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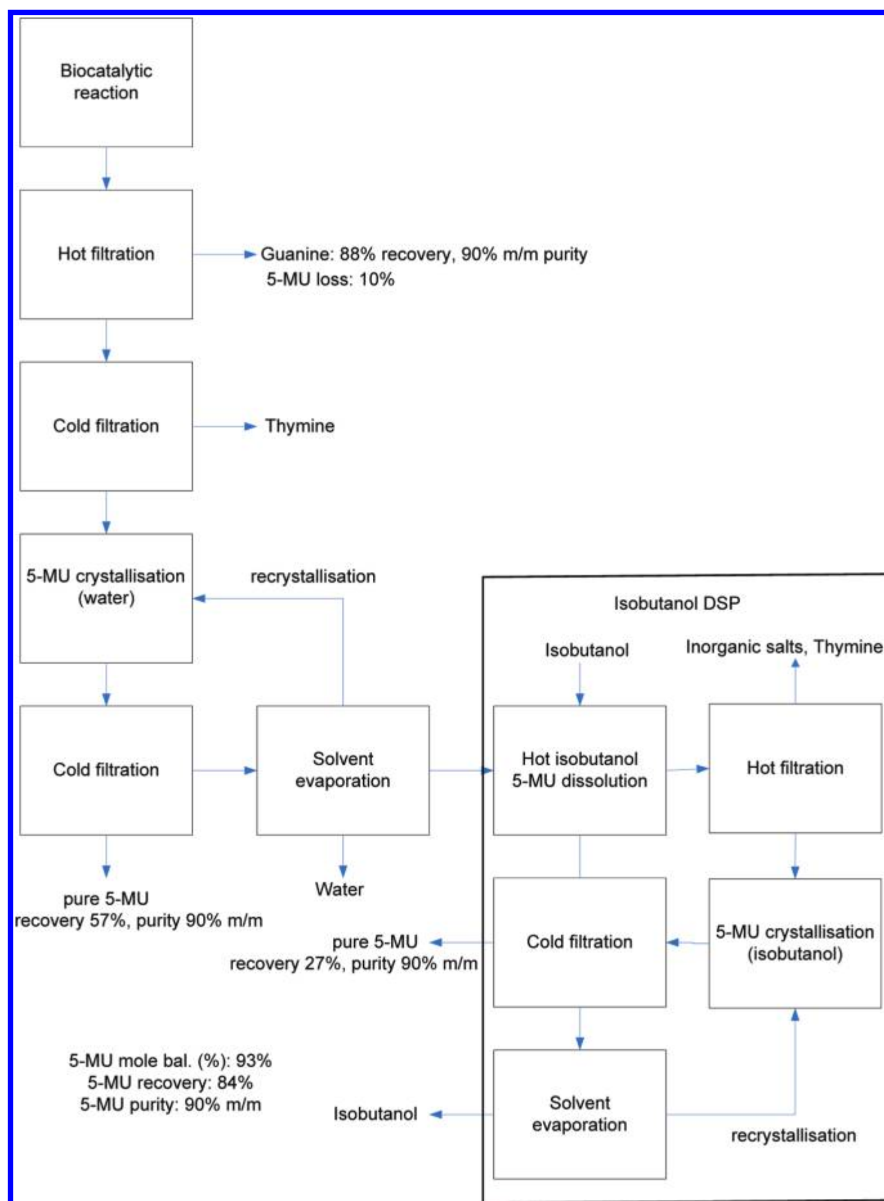

Figure 4. **Isolation and recovery of 5-MU and guanine.**

Table 3. **Yields obtained in the preparation of *β*-thymidine from 5-MU**

reactions	lab-scale yield (%) *ex.* biocat	bench-scale yield (%) *ex.* biocat
5-MU	90	85
DABT	92	78
DAT	90	85
β-thymidine	92	100

bench scale, not taking into account recoveries of 5-MU and thymidine, as these were not optimised. Working on a recovery of 90% for each of these products, the overall yield becomes 56% for laboratory scale and 45% for bench scale. This compares favorably to the yield of 28% obtained for a chemical method from tetra-*O*-acetylxylofuranose at small scale.[17]

The use of a biocatalytic step ensured formation of only the *β*-anomer of 5-MU, thereby maximising the overall yield and avoiding a complicated isolation step. This enabled a cost-effective overall synthesis of *β*-thymidine to be demonstrated at bench scale.

(17) Alla, V. R. R.; Mukund, K. G.; Sista, V. S. L. U.S. Patent 5,596,087, 1997.

Conclusions

Integration of biocatalytic 5-MU production at bench scale with preparation of an advanced pharmaceutical intermediate such as *β*-thymidine has been successfully demonstrated for the first time. Guanosine conversions of 95% and 5-MU reaction yields of 85% were achieved at an average 5-MU productivity of 10 $g \cdot L^{-1} \cdot h^{-1}$. 5-MU from the biocatalytic reaction was isolated and purified using a combination of filtration, crystallisation, evaporation, and isobutanol extraction and was recovered in 84% yield at a purity of 90% $m \cdot m^{-1}$. The material was demonstrated to be of a suitable quality for the subsequent preparation of *β*-thymidine without adversely affecting the chemical transformations.

Experimental Section

General Methods. Guanosine and guanine standards were purchased from Sigma-Aldrich (Germany), while thymine and

5-methyluridine standards were purchased from NSTU Chemicals (China). These compounds were quantitatively analysed by HPLC, using a Waters Alliance model 2609 instrument with a Synergi 4 μm Max-RP 150 mm $\times$ 4.6 mm column and a UV detector at 260 nm. Samples were prepared by dissolving the required amount of sample in sodium hydroxide (10 M, 1 mL) and then made up to the required volume using deionised water so as to ensure the sample concentration was within the linear region of the calibration curve. The injection volume was 10 μL, and elution was achieved with ammonium acetate, (25 mM, pH 4.0) at a flow rate 1 mL$\cdot$min^{-1} and at ambient temperature.

Analytical standards of DABT[8] and DAT[17] were prepared and used for quantitative analysis and for determining product yields. β-Thymidine was purchased from NSTU Chemicals (China) for use as a standard. HPLC analysis of these compounds was carried out on a Hewlett-Packard series 1100 instrument using a 5 μm Waters Spherisorb ODS2 4.6 mm $\times$250 mm column under a gradient elution program at a flow rate of 1 mL$\cdot$min^{-1}. The UV detector was set to 254 nm. Gradient elution was performed, with ammonium acetate (50 mM) decreasing from 90 to 20% as methanol increased from 10 to 80%. Retention times were 5-MU 5 min, thymidine 6.5 min, DAT 8 min, and DABT 10 min. NMR spectra were recorded using a 400 MHz Varian INOVA instrument.

Small-Scale Synthesis. *3′,5′-Diacetyl-2′-bromothymidine (DABT).* To 5-methyluridine (10.0 g, 39 mmol), in a three-necked round-bottomed flask fitted with a thermometer, reflux condenser, and dropping funnel, was added acetonitrile (389 g). This slurry was heated to reflux, and 5.7 equiv of acetyl bromide (27.32 g, 222 mmol) was added dropwise over 30 min. During the course of addition, the solids dissolved to leave a yellow solution. The reaction was heated for an additional 30 min, and then solvent was removed under reduced pressure through two NaOH traps to remove HBr. The residue was taken up in dichloromethane, and this was washed twice with saturated brine. The organic layer was dried over $MgSO_4$ (2 g), and filtered, and the solvent was removed to yield a brown residue (16.9 g) containing DABT (14.6 g, 92% yield, as adjudged by HPLC).

3′,5′-Diacetylthymidine (DAT). Without further purification, the 16.9-g residue obtained above containing DABT (14.6 g, 36 mmol) was dissolved in MeOH (63.4 g) and transferred to a Parr reactor. Sodium bicarbonate (4.6 g, 1.5 equiv) and Ni catalyst A-5000 (4.5 g, $\sim$15% m$\cdot$m^{-1} catalyst loading, assume 50% wet) were added. The reaction was warmed to 35 °C, and the H_2 pressure was set at 9 bar. After 30 min the temperature was raised to 40 °C for the remainder of the reaction. After 2 h the reaction mixture was filtered through Celite 535 to remove catalyst. Solvent was removed *in vacuo*, and the residue was taken up in ethyl acetate (90 mL). Precipitated salts were removed from the ethyl acetate solution by filtration, after which solvent was removed *in vacuo* to yield a brown residue (12.7 g) containing DAT (10.6 g, 90% yield, by HPLC).

β-Thymidine. Without further purification, DAT prepared as described above (9.3 g residue containing 7.4 g DAT, 22.7 mmol) was dissolved in MeOH (29.2 g), and NaOMe (1.4 g, 1.1 equiv) was added. The reaction was warmed at 40 °C for 1 h. After this time additional MeOH (119 g) and Amberlite resin (IR-120, 30 g, Rohm and Haas, U.S.A.) were added and stirred at room temperature for 30 min. The beads were filtered off and washed with MeOH (3 $\times$ 40 g). Solvent was removed *in vacuo* from the reaction mixture and combined methanol washings to yield a white solid containing thymidine (5.1 g, 92% by HPLC). Thymidine was purified by a single recrystallisation from MeOH, with a recovery of 75%.

Bench-Scale Biocatalytic 5-MU Synthesis. *Substrate Preparation.* Guanosine, 9.3% m$\cdot$m^{-1} (1040 g, 3.67 mol), and 50 mM sodium phosphate buffer solution (9 600 g, pH 7.5) were charged to a 16-L reactor (CR-16 Büchi, Switzerland) while stirring at 100 rpm (anchor stirrer). To this was added 4.8% m$\cdot$m^{-1} thymine (535 g, 4.24 mol) as a prewetted paste with phosphate buffer. The reactor was then heated to 60 °C, while stirring, and the reaction mixture was sampled (in triplicate, 1−3 g) prior to enzyme addition for determination of initial concentrations. A portion of phosphate buffer solution (300 g, 50 mM, pH 7.5) was retained and used to dissolve and quantitatively transfer the enzymes to the reactor. The reaction mixture was maintained at 60 °C, while stirring at 100 rpm and then sampled at intervals.

Enzyme Preparation. Lyophilized powders of enzyme, purine nucleoside phosphorylase (PNP) *ex Bacillus halodurans* overexpressed in *Escherichia coli* and uridine phosphorylase (UP) *ex E. coli* overexpressed in *E. coli*, were produced in-house according to our procedures as described by Visser et al.[10a,18] and stored at 4 °C. Approximately 16 kU each of PNP (3.114 g, at a specific activity 5.14 U$\cdot$mg^{-1}) and UP (3.758 g, at a specific activity 4.27 U$\cdot$mg^{-1}) were then dissolved in 100 mL of phosphate buffer prior to addition to the reaction mixture.

Isolation and Recovery of 5-MU (DSP). *Isolation and Recovery of Guanine (Byproduct).* Immediately after biocatalytic reaction (at 60 °C) the mixture was heated to 80−90 °C and then filtered hot to remove guanine. The hot reaction mixture was centrifuged at 80 °C, using a basket centrifuge (10-μm mesh filter cloth, 1000 rpm, 30 min). The filter cake was washed or slurried in hot deionised water (90 °C, 2−5 L) and dried at 1500 rpm for 20 min. The filter-cake was initially air-dried overnight in a fume-cupboard and then dried further in a vacuum oven at 55 °C. From this process 464 g of guanine, with a purity of 90% m$\cdot$m^{-1}, was obtained.

5-MU Crystallisation. The filtrate (*ex* hot filtration) was allowed to cool down from 80 to 30 °C over 4 h, followed by cooling down from 30 to 4 °C over 2 h. The suspension was then cold filtered to remove thymine. The reaction mixture was then maintained at 4 °C for 24−48 h. During the cooling process, the solution was stirred at 200−300 rpm using an anchor stirrer. The 5-MU crystallised and was recovered by conducting a cold filtration at 4 °C. The filter-cake was placed in a fume cupboard, air-dried overnight, and then dried further in a vacuum oven at 55 °C until dry. This crystallization yielded 486 g of 5-MU (a recovery of 57%), at an average purity of between 88% m$\cdot$m^{-1} and 95% m$\cdot$m^{-1}.

5-MU Crystallisation from Isobutanol. Residual 5-MU after crystallisation could be recovered from the mother liquor. Water

(18) Visser, D. F.; Hennessy, F.; Rashamuse, K.; Louw, M.; Brady, D. *Extremophiles* **2010**, *14*, 185–192.

was removed using a Büchi evaporator by gradually heating at 60−80 °C for 8−10 h. The crude 5-MU solid recovered was dried under vacuum. The crude 5-MU was dissolved in isobutanol (3 g 5-MU per 100 g of isobutanol) by heating the slurry to reflux (110 °C) for 2−3 h in a 15-L glass reactor (GR-15, Büchi, Switzerland). The slurry mixture was then filtered hot to remove the inorganic salts and insoluble organics such as thymine. The filtrate containing 5-MU was then allowed to crystallise from isobutanol, and 5-MU was recovered by carrying out a cold filtration at 4 °C. The material was dried further in a vacuum oven at 55 °C to remove residual solvent. The material was then resuspended in water and recrystallised. During this crystallization 230 g of 5-MU was recovered, which corresponds to 27% of the total 5-MU produced, at an average purity of between 88% $m \cdot m^{-1}$ and 95% $m \cdot m^{-1}$.

Bench-Scale Synthesis. *3′,5′-Diacetyl-2′-bromothymidine (DABT).* Acetonitrile (13.9 kg) was transferred into a 26-L glass-lined reactor (CR-26, Büchi, Switzerland), and stirring commenced. 5-MU (424 g at 90% purity, 1.48 mol) in solid form, produced in the biocatalytic reaction described above, was added to the reactor, and the reaction was heated to 75 °C. A peristaltic pump, primed with acetonitrile (0.5 kg), was used for acetyl bromide transfer. Acetyl bromide (1.042 kg, 8.48 mol) was added over a period of 0.5 h. After addition, the pump lines were rinsed with acetonitrile (0.5 kg). The reaction was sampled 30 min after addition of acetyl bromide, and HPLC showed the reaction to be complete with no residual 5-MU detectable. Solvent was removed by distillation to leave a brown residue inside the reactor. Dichloromethane (5.8 kg) was added, followed by a 50% brine solution (5.87 kg). The two layers were stirred for 10 min at 100 rpm and then transferred into a separating vessel. The layers were allowed to stand for 45 min before separation. The organic layer was transferred back to the reactor and an additional portion of 50% brine was added (5.87 kg). This was stirred for 10 min at 100 rpm and then transferred into a separating vessel, where separation was carried out after 15 min. Dichloromethane (983 g) was used to rinse the reactor, and this was added to the combined brine layers and allowed to stand overnight before separation. All organic layers were combined, and the solvent was removed *in vacuo* to leave a beige-coloured foam (575 g) containing DABT (468 g, 78% yield by HPLC). Multiple batches of DABT were combined to provide material for the subsequent reaction.

3′,5′-Diacetylthymidine (DAT). To DABT prepared as described above (1167 g of residue containing 914 g DABT, 2.26 mol) was added methanol (3.78 kg), and the residue was dissolved by mixing the material gently at 40 °C. Material was transferred to an 8-L Parr reactor. Sodium bicarbonate (270 g, 1.4 equiv) and Ni catalyst A-5000 (260 g, ∼15% $m \cdot m^{-1}$) (Johnson Matthey Catalysts, TN, U.S.A.) were added to the reaction. The reaction was warmed to 35 °C while stirring at 1000 rpm. When the reaction reached temperature, the H_2 pressure inside the Parr reactor was set to 9 bar (131 psi). The reaction was sampled hourly and stopped after 2 h, when no starting material remained. The reaction mixture was removed from the Parr reactor under positive pressure and filtered through two layers of Whatman No. 1 filter paper to retain the catalyst. Solvent was removed by distillation at 60 °C under reduced pressure in a 20-L rotary evaporator under rotation. To this residue was added ethyl acetate (7.7 kg), and once the residue had dissolved, $MgSO_4$ (120 g) was added for drying. The salts were removed by filtration through Whatman no. 1 filter paper, and a small amount of ethyl acetate was used to rinse the precipitated material. Solvent was removed on a 20-L rotary evaporator under reduced pressure to give a brown residue (908 g) containing DAT (628 g, 85% yield by HPLC).

β-Thymidine. Brown residue (839 g) containing DAT (562 g, 1.72 mol) obtained from hydrogenolysis was dissolved in MeOH (2.3 kg) in a 10-L rotary evaporator flask, and to this was added sodium methoxide (100 g, 1 equiv). The reaction was warmed while rotating the 20-L rotary evaporator at 44 °C for 1 h at atmospheric pressure, after which time HPLC analysis showed the reaction to be complete, with 100% conversion. Methanol (3.2 kg) was added, followed by 1 kg of Amberlite IR-120 resin (Rohm and Haas, U.S.A.) to adjust the alkaline reaction solution, and the resultant pH was found to be 5. The beads were removed by filtration through Whatman no. 1 filter paper and washed three times with methanol (total of 2.3 kg). The solvent fractions were combined, and the solvent was removed under vacuum on a 20-L rotary evaporator to leave a residue of mass 778 g, containing 417 g thymidine (100% yield) by HPLC. To this residue was added MeOH (3224 g), and the mixture was transferred to a glass reactor (GR-15) and heated for 2 h. The solution was cooled while stirring, and the slurry was transferred to a vessel fitted with an overhead stirrer, stirred at 4 °C overnight, and then filtered and washed with ice-cold MeOH. The recovered solid was dried under vacuum to give a white powdery material (295.6 g, 71% recovery). Analysis showed the material to have a purity of 99% $m \cdot m^{-1}$ by HPLC and a melting point of 185−186 °C. Proton NMR spectra and melting point are in agreement with previously reported data for β-thymidine.[19,20]

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