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An enzymatic glycosylation of nucleoside analogues using β-galactosidase from *Escherichia coli*

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ABSTRACT

A new enzymatic method for the synthesis of β-galactosides of nucleosides and acyclic nucleoside analogues has been developed, using β-galactosidase from *Escherichia coli* as a catalyst and lactose as a sugar donor. The method is very rapid, feasible and last but not least inexpensive. Its applicability has been proven for a broad variety of possible substrates with respect to its scaling up for preparative use. Five new compounds from a series of nucleoside and acyclic nucleoside analogues have been prepared on a scale of several hundred milligrams, in all cases revealing very good results of the method concerning the reproducibility of the reaction yields and simplicity of the purification process.

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1. Introduction

A prodrug can be defined as a drug derivative that undergoes biotransformation enzymatically or nonenzymatically inside the body before exhibiting its therapeutic effect. In an ideal case, the prodrug is converted to the original drug as soon as the derivative reaches the site of action.

The main purpose for the synthesis of prodrugs is bioavailability enhancement or a specific targeting of the drug. Prodrugs are usually more soluble and more stable in the living organism; moreover, they often have a specific affinity to some biological structures. Glycosylation is an established method for the synthesis of prodrugs. A typical example is a targeting of glycosylated drugs on the colon, where they are specifically hydrolysed by the local intestinal anaerobic microflora.[1] Glycosylation has been effectively used as a tool for the enhancement of the therapeutic potential for the preparation of prodrugs of doxorubicin,[2] daunorubicin,[2,3] and pyrrolo[2,1-*c*][1,4]benzodiazepine.[4] On this basis, a series of compounds for antibody-directed enzyme prodrug therapy (ADEPT) and prodrug monotherapy (PMT) was developed. Also glycosylation of linear peptidic opioids[5–8] (enkephalin and leu-enkephalin) improved their bioavailability owing to their better stability towards enzymatic attack, enhanced transport through the membranes and lowered clearance. Another characteristic feature of the application of glycoside prodrugs can be a reduction of the side effects of some drugs as shown for example, in the case of morphin-β-6-glucuronide.[9] It was shown that the 6-glucuronide is not only more than three times more active than an equimolar subcutaneous dose of morphine in mice,[10] but recent clinical studies in cancer patients have indicated that its analgesic effects are achieved with an absence of the nausea and vomiting,[11] which is often caused by morphine itself.[10,11]

For the glycosylation of the drugs, the classical methods of organic synthesis are usually used, but this approach truly brings some complications as multistep syntheses involve the introduction and removal of protecting groups and sometimes also difficult purification of the product. Additionally, the requirements of stereoselectivity multiply the disadvantages of the conventional synthesis. Thus, enzymatic catalysis can be a good solution to the problem in many cases. There are a number of easily available enzymes with tranglycosylation activity, which are able to form a glycosidic bond stereospecifically and in most cases regioselectively. Recently, the utilisation of glycosidases for the preparation of oligosaccharides,[12–17] glycosides of amino acids[18,19] and nucleosides[20–22] has been described.

Nitrophenyl glycosides are frequently used as sugar donors owing to the function of the nitrophenyl group as a good leaving group. However, these compounds are of limited solubility and stability as well as being expensive. On the other hand, the utilisation of disaccharides as glycosyl donors is more advantageous because of their low costs, good solubility and high stability.

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In this work, we present an enzymatic method for the synthesis of β-galactosides from nucleosides and acyclic nucleoside analogues using the β-galactosidase from *Escherichia coli* as a catalyst[23] and lactose as a sugar donor. The reaction is a two steps process (Fig. 1). In the first step, the enzyme cleaves the glycoside bond in the lactose and an enzyme–galactose complex is formed. In the second step, the hydroxyl group of the acceptor molecule breaks the complex; new glycoside is formed and leaves the active site of the enzyme. This mechanism leads to retention of configuration of the glycosidic bond; therefore, all this way formed products have the β-configuration.

Since the starting compounds belong to a class of biologically active compounds and some[24–26] of them exhibit significant antiviral and/or antitumor activities, the potential utilisation of galactosides formed in this way is aimed at their serving as prodrug candidates for PMT and ADEPT therapy. The method itself can be a useful tool for the routine laboratory synthesis of glycosides.

2. Results and discussion

In the kinetic study outlined in Table 1, we investigated the dependence of the yield of the enzymatic reaction on the reaction time for 20 diverse compounds—18 acyclic nucleoside analogues (alcohols or diols) with a different sterical hindrance of the reacting hydroxyl group and two nucleosides with a different nucleobase. Following these data, we estimated the optimal reaction times to perform reactions on the preparative scale. Some relationships between the structure and the kinetic data are discussed below. The reaction mixture consisted of sodium phosphate buffer (0.1 M; pH 7.8; 1 mM of $MgCl_2$), 1% (m/w) acceptor molecule, 500 mM α-lactose and 33 nkat/mL of the enzyme. The reaction was carried out at 25 °C for 24 h. The progress of the reaction was measured in the first 9 h of the reaction time to estimate a reaction maximum yield, after 24 h a sample was taken to evaluate some further changes in the reaction mixture during longer time periods (meaning, if the product disappear or not).

The sample composition was determined by HPLC performed on a Waters High-Performance Carbohydrate Cartridge Column. By this analytical method, the acceptor leaves the column first, with retention times about 4 min (for example compound **2**: 3.6 min; compound **6**: 3.5 min and compound **16**: 4.3 min), followed with monoglycosylated compounds with retention times about 7 min (for example for the same compounds 6.8 min; 6.4 min and 7.1 min). Diglycosides, if produced, leave the column as the last, with retention times about 12 min. Different structure of the possible products results in different interaction forces on the column, and so in the formation of one or two peaks with near the same retention times (for example for the same compounds: 11.3 + 12.5 min—two products; 12.7 min; 10.8 + 12.5 min—two products). These results are in a good agreement with the data from column producer, given for separation of mono-, di- and trisaccharides. The structure of the compounds was assigned by the comparison of UV-spectra of the peaks—because of the occurrence of the nucleobase in the molecule, the UV-spectra are characteristic and sufficient for this method.

2.1. Reaction yields and their dependence on the structure of acceptor

As shown in Table 1, the steric hindrance of the reaction centre by an adjacent nucleobase plays a crucial role in the reaction rate. For example, the enzymatic galactosidation of the 2-pyrimidone derivative **1**, whose nucleobase is too close to the primary hydroxy group, afforded the desired glycoside in only a 9% yield. An extension of the aliphatic chain by one carbon atom (compounds **7** and **8**) increased the yield of the glycosides to 43% and 35%, respectively. The same effect is manifested by a comparison of the reaction rates of derivatives **2**, **3**, **4** and **9** (Table 1). While the surroundings of the reacting hydroxyl group in compounds **2** and **3** are nearly the same, leading thus to the same yields, compounds **4** and **9** with longer aliphatic chains exhibit reaction yields which are 13–15% higher.

The influence of the proximity to another hydroxyl group is also interesting. Although the reaction yields were found mostly similar or slightly lower, the reaction rate is usually higher. For most of the alcohols, no direct reaction yield maximum could be observed and the reaction could be stopped after 24 h. A further prolongation of reaction time had no influence on the yield and, interestingly, neither was any hydrolysis of the corresponding products observed (the data are not shown). In all diols, a very good and reproducible reaction yield maximum was achieved in the range of 3–9 h. After this time, the yields of the products were lowered by hydrolysis, the formation of digalactosides, or remained the same.

A small dependence of the reaction yield on the absolute configuration of the diols was also observed. In most cases (with the exception of compounds **10** and **11** Table 1), the (*R*)-isomer afforded better results than its (*S*)-counterpart. This effect can be explained by a comparison of the lactose, that is, the substrate of the hydrolytic reaction, and the product formed. The product with the (*R*)-configuration in the diol part has the same structural pattern as lactose. As shown by crystallographic studies,[21] the glucose hydroxyl, which lies in the superposition with the hydroxyl group of the product, creates a hydrogen bond with the enzyme active site. We can assume that this hydrogen bond can thus facilitate the transglycosylation formation. As shown in Table 1, however, this contribution is very small and perhaps can be overbalanced by the entropic effect and free rotation of the aliphatic chain.

In contrast to the acyclic nucleoside analogues, nucleosides **19** and **20** used in this study were not found to be appropriate compounds for this method. For example, enzymatic galactosylation performed with ribavirin afforded two reaction products, both in a very low yield. The second nucleoside, the α-anomer of 2′-deoxy-5,6-dihydro-5-azacytidine (**19**), also formed two products, although the yields were much better. This reactivity of all of the hydroxyl groups present neither corresponds to our expectations, nor does it agree with the information in the literature for ribavirine,[20] floxuridine[21] and 2′-deoxynucleosides.[22]

In general, only a sterical non-hindered hydroxyl group can take part in reaction. So, in case of acyclic nucleoside analogues occurring in a diol form, only the primary hydroxyl group undergoes this reaction. It is possible that the product of the glycosylation can subsequently serve as a reactant in the next reaction cycle. This re-

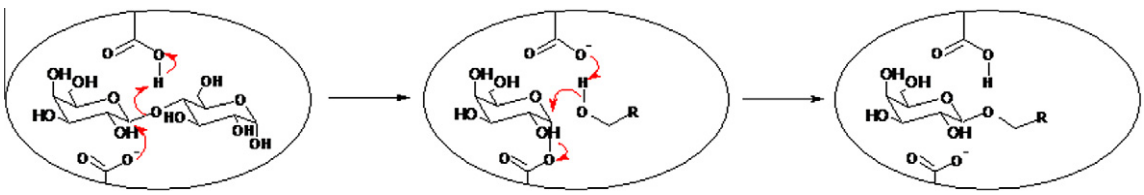
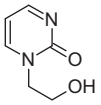
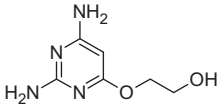
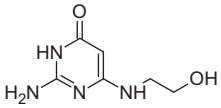
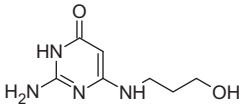
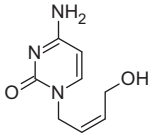
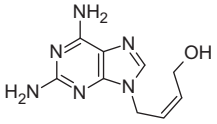
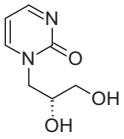
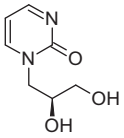
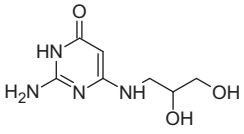
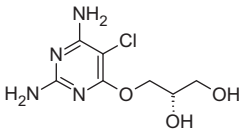
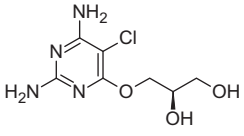
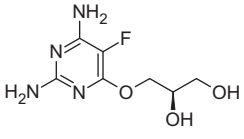


Figure 1. Reaction mechanism of the glycosylation reaction.

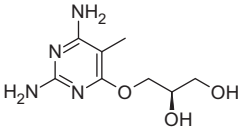
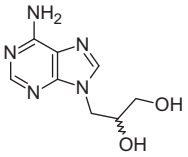
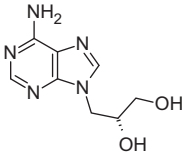
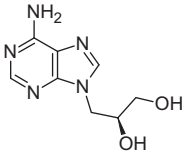
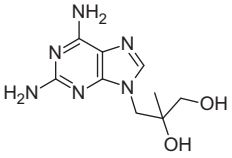
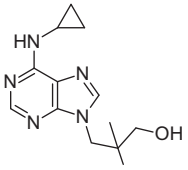
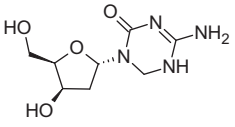
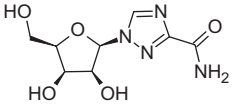
sults in the formation of digalactosides (Fig. 2). Theoretically, four new compounds can be thus formed, but practically maximum of two such products were detected; due to their low yields we did not solve structures of these compounds.

Table 1
The reaction yields and other reaction details of the enzymatic galactosylation of diverse types of acyclic nucleoside analogues and nucleosides with β-galactosidase from *E. coli*

No.	Structure	Yield[a] (%)	Time of the maximum yield (h)	Formation of digalactosides[b]	Hydrolysis
Alcohols					
1		9	–	–	–
2		24	9	<5%	–
3		24	–	<2%	–
4		39	–	5%	–
5		34	–	<3%	–
6		31	–	<3%	–
Diols					
7		43	3	<5%	+
8		35	7.5	<5%	+
9		36	8.5	<2%	–
10		31	9	10%	–
11		32	9	11%	–
12		28	9	6%	+

(*continued on next page*)

Table 1 (*continued*)

No.	Structure	Yield[a] (%)	Time of the maximum yield (h)	Formation of digalactosides[b]	Hydrolysis
13		33	—	11%	–
14		35	6	<5%	+
15		40[c]	5[c]	<5[c]	+
16		31	8	<5%	+
17		29	8.5	<5%	+
18		32	7	<3%	+
Nucleosides					
19		29	7	<3%	+
20			—	—	–

[a] The reaction yield of the product was estimated from a HPLC-chromatogram by a comparison of the areas corresponding with the starting compound, product and digalactosides.
[b] The overall yield of digalactosides after 24 h.
[c] The data calculated from the experimental data of (*RS*)-DHPA and (*S*)-DHPA.

2.2. Studies on glycosylation of the selected nucleoside analogues in analytical scale

As described below, we have obtained some pattern from the kinetic studies. Simple alcohols lacking a neighbouring hydroxyl group did not exhibit any reaction maximum during the first 9 h and the reaction could be terminated after 24 h. Some measurements performed after a longer time revealed no significant increase of the reaction yield. This pattern is shown in Figure 3 as a point set for compounds **21**, **23** and **24**. Another pattern could be obtained for diols, as is shown in the case of compound **22** (Fig. 3). In this case, a well reproducible significant maximum was observed. After 2 or 3 h, the reaction yield was only lowered by hydrolysis or by the formation of digalactosides.

The kinetic studies have shown that digalactoside synthesis can occur after a relatively long time, mostly after four reaction hours or later. This fact was attributable to the necessity of an appropriate concentration of the pre-formed monogalactoside. In general, after a conversion of approximately 20% of the starting aglycone molecule, a subsequent rapid formation of digalactosides could occur and the glycosylation of the monoglycosylated product take place. Therefore, for syntheses that require a long time we have to take into account a possible lowering of the reaction yield by the formation of digalactosides. Fortunately, in most cases, the overall yield of digalactosides was very small. Only in four cases did digalactoside formation significantly affected the reaction yield with the overall yield of digalactoside in the range of 6–11%. The real problem of the formation of digalactosides is the prolongation

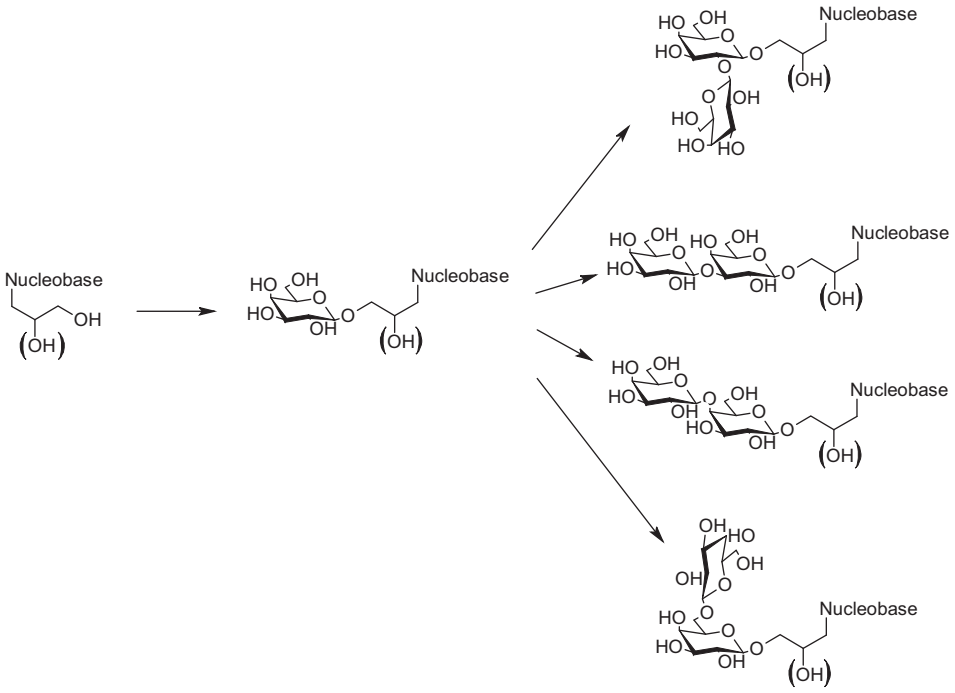


Figure 2. Formation of the monoglycosylated product and his further reactions.

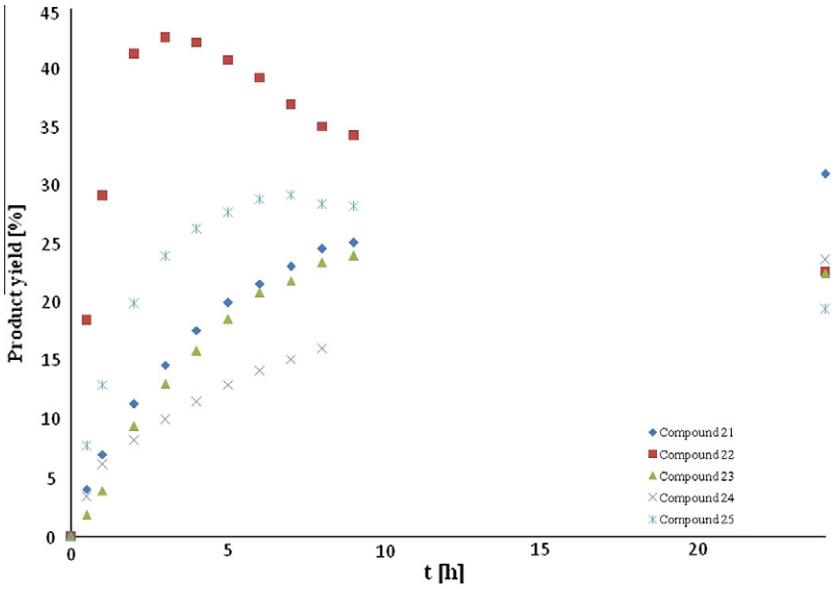


Figure 3. Profile of formation of the glycosylated products.

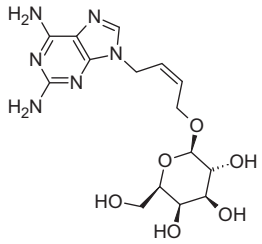
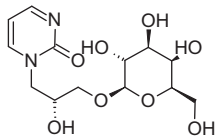
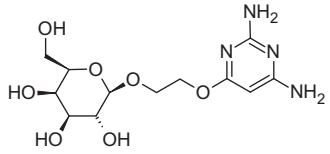
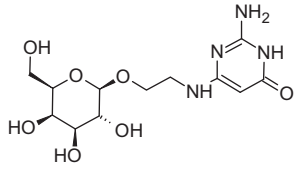
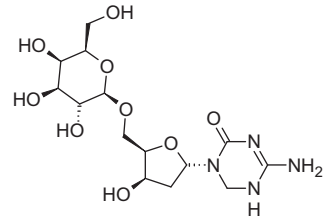
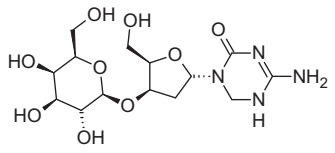
of the purification time owing to their long retention time on the HPLC column. This leads to a higher consumption of the solvents and higher costs.

2.3. Preparative synthetic method

We proved the suitability of our method for practical synthetic use by the synthesis of five diverse galactosides (Table 2). The synthesis of galactosides in the described way is very fast, cheap and leads to high or satisfactory yields. The galactose donor, lactose, can be afforded in large quantities; moreover, it is inexpensive and soluble enough to achieve high concentrations (compared for example with ONPG, or other nitrophenyl galactosides normally used for the same purposes). The aqueous medium ensures the good solubility of the acceptor molecule, which enhances the relative yield of galactoside as against the volume of the reaction mixture. The presence of water limits the universal applicability of this protocol and because of this fact, simple optimisations of the reaction conditions for each single compound are necessary.

The methodology for the isolation and purification of the desired product from the reaction mixture was optimised and a very fast and cheap technique was finally developed. This protocol is based on the retention of the studied heterocyclic molecules on acidic ion exchange resin (Dowex 50 in H^+ form), where all of the carbohydrates are first washed out with water while the starting nucleoside or nucleoside analogues and the desired product are

Table 2
The structures of the prepared selected galactosides

Compound	Structure	Yield (%)	
		Before purification[a]	After purification[b]
21		30	27
22		39	22
23		24	23
24		25	24
25[c]	 and 	29	20

[a] According to the HPLC-analysis of the reaction mixture.
[b] Isolated yields.
[c] The product was found as a mixture of two isomers.

subsequently eluted by aqueous ammonia. The resultant mixture is separated by preparative HPLC. The desired product can be obtained in very high purity, free of the starting compounds, buffer components and di- and trigalactosides, and almost without losses as is shown in Table 2. The only problem can occur in the case of low absorbance of the nucleobase, which makes detection of the product during purification difficult. This problem was observed in the case of compound **25**. For purification, a fresh Dowex is strictly recommended. An old and many times recovered Dowex is not suitable because of its low capacity and its lowered affinity to the purified compounds. Structurally similar isomers (e.g., in the case of compound **25**) can be purified in the form of monogalactosides; for further isolation of pure isomers an additional purification step is necessary.

The enzyme used in our study, β-galactosidase from *E. coli*, is commercially available and relatively inexpensive. It has been known for a long time, and therefore a great deal of information can be found in the literature concerning its optimisation and further development.

3. Conclusions

The described method is a powerful tool for the preparation of glycosides derived from acyclic nucleoside analogues, which can find utilisation as prodrugs. The rapidness of the whole process, its easy usage and the relatively high yields of the products make the method very applicable for the preparation and screening of various galactoside prodrugs of diverse compounds of biological interest.

4. Experimental

4.1. General

The chemicals were purchased from Sigma Aldrich and were of analytical grade. β-Galactosidase from *E. coli* (EC; Grade) was purchased from Sigma Aldrich. ^{1}H and ^{13}C NMR spectra were recorded in DMSO-d_6 at 27 °C using the Bruker Avance II 600 and/or Bruker Avance II 500 spectrometers operating at 600.0 or 500.0 MHz in ^{1}H and 150.9 or 125.7 MHz in ^{13}C. The proton and carbon spectra were referenced to the residual solvent signals (δ 2.50 ppm and 39.7 ppm for DMSO). For the description of the spectroscopic data, we used superscripts to denote the atoms or groups in a nucleobase, the carbon chain of the acceptor and the carbon atoms in the galactose and to refer to the individual sugar residues and aglycones, respectively. If they are not possible to simplify, they are serially indicated beginning with the nucleobase without a superscript, following by the side-chain of the aglycone indicated with (′) and ending by galactose indicated with (″).

4.2. Analytical enzymatic reactions

To the reaction mixture containing 6 mg of the acceptor molecule (1% (m/w)) in 580 μL with 500 mM of lactose in 0.1 M of a sodium phosphate buffer (pH 7.8; 1 mM of $MgCl_2$) heated to 25 °C, 20 μL of an enzyme stock solution (enzyme activity of 990 nkat/ml) in the same buffer was added to reach an overall enzyme activity of 33 nkat/mL. The volume of the reaction mixture was 600 μL in all cases and the reaction proceeded at 25 °C. In time intervals of 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 24 h an aliquot of 50 μL was sampled and the reaction was stopped by boiling for 3 min. The sample composition was determined by HPLC performed on a Waters High-Performance Carbohydrate Cartridge Column (acetonitrile/water 3:1, flow 1 mL/min). The compounds were detected by UV-absorption using the Waters 2996 Photodiode Array Detector. For the analysis, the wave length of the absorption maximum of the starting compound was chosen.

4.2.1. Preparative enzymatic reactions

The reaction mixture (50 mL) containing an acceptor molecule (500 mg, 1% m/w) and of α-lactose monohydrate (9 g, 500 mM) in a sodium phosphate buffer (pH 7.8; 1 mM $MgCl_2$) was preheated to 25 °C. An enzyme lyophilisate was added to this mixture to reach an overall enzyme activity of 33 nkat/mL (the amount of the enzyme was calculated from the producer data). The mixture was gently mixed until the enzyme was completely dissolved. The reaction proceeded at 25 °C. The reaction time was determined from analytical measurements as the time necessary to achieve the maximum reaction yield. No further optimisation of the reaction time was necessary.

The reaction mixture was applied onto a column of Dowex 50 W (H^+, 10 g). The column was washed with water (500 mL) and then eluted with 10% of aqueous ammonia. The eluate was evaporated and the residue dissolved in the minimum amount of water. The resulting mixture was separated by preparative HPLC (Luna 10μ NH_2 100 Å 250 × 21 column, 20 mm, acetonitrile/H_2O 3:1, flow 10 mL/min). The compounds were detected at the absorption maximum of the starting compound. The product-containing fraction was evaporated, the residue dissolved in the minimum amount of water and lyophilised.

4.2.2. 9-[4-*O*-(β-D-Galactopyranosyl)-(2*Z*)-(2-butenyl)]-2,6-diamino-(9*H*)purine (21)

The reaction proceeded for 24 h. The wave length used for detection during purification was 254 nm. The purification and lyophilisation afforded 232.5 mg (0.6 mmol; 27%) of the product; ^{1}H NMR (DMSO-d_6): 7.69 (1H, s, H-8), 6.67 (2H, br s, 6-NH_2), 5.78 (2H, br s, –NH_2), 5.67–5.74 (2H, m, CH═CH), 4.67 (2H, m, N-CH_2), 4.44 (1H, m, 1′-O-CH_2 a), 4.32 (1H, m, 1′-O-CH_2 b), 4.17 (1H, d, $J_{1'-2'}$ = 7.5, H-1′), 3.64 (1H, dd, $J_{4'-3'}$ = 3.2, $J_{4'-5'}$ = 1,1, H-4′), 3.50–3.55 (2H, m, H-6′), 3.36 (1H, td, $J_{5'-6'}$ = 6.2, $J_{5'-4'}$ = 1.1, H-5′), 3.33 (1H, dd, $J_{2'-3'}$ = 9.5, $J_{2'-1'}$ = 7.4, H-2′), 3.29 (1H, dd, $J_{3'-2'}$ = 9.5, $J_{3'-4'}$ = 3.3, H-3′). 13C NMR (DMSO-d_6): 160.5 (C-2), 156.3 (C-6), 151.7 (C-4), 137.3 (C-8), 129.7 and 127.7 (CH═CH), 113.3 (C-5), 102.8 (C-1′), 75.6 (C-5′), 73.6 (C-3′), 70.7 (C-2′), 68.4 (C-4′), 63.9 (1′-O-CH_2), 60.7 (C-6′), 39.8 (N-CH_2). HRMS (ESI) *m/z* $C_{15}H_{23}O_6N_6$ $[M+H]^+$ calcd: 383.1674, found: 383.1674.

4.2.3. 1-[(2*R*)-2′-Hydroxy-3′-*O*-(β-D-galactopyranosyl)propyl] pyrimidin-2(1*H*)-one (22)

The reaction proceeded for 4 h. The wave length used for detection during purification was 305 nm. The purification and lyophilisation afforded 210 mg (0.63 mmol; 22%) of the product; ^{1}H NMR (DMSO-d_6): 8.53 (1H, dd, J_{4-5} = 4.2, J_{4-6} = 2.8, H-4), 8.05 (1H, dd, J_{6-5} = 6.5, J_{6-4} = 2.8, H-6), 6.40 (1H, dd, J_{5-6} = 6.5, J_{5-4} = 4.2, H-5), 5.14 (1H, br s, OH), 4.90 (1H, br s, OH), 4.74 (1H, br s, OH), 4.60 (1H, br s, OH), 4.39 (1H, br s, OH), 4.17 (1H, dd, J_{gem} = 13.0, $J_{1'-2'}$ = 3.6, H-1′a), 4.12 (1H, d, $J_{1''-2''}$ = 7.5, H-1″), 3.97 (1H, m, H-2′), 3.73 (1H, dd, J_{gem} = 10.5, $J_{3'a-2'}$ = 5.3, H-3′a), 3.59–3.64 (2H, m, H-1′b,4″), 3.47–3.55 (2H, m, H-6″), 3.47 (1H, dd, J_{gem} = 10.5, $J_{3'b-2'}$ = 5.3, H-3′b), 3.31–3.36 (2H, m, H-2″, 5″), 3.28 (1H, dd, $J_{3''-2''}$ = 9.5, $J_{3''-4''}$ = 3.3, H-3″). 13C NMR (DMSO-d_6): 166.1 (C-4), 156.0 (C-2), 151.4 (C-6), 104.1 (C-1″), 103.5 (C-5), 75.5 (C-5″), 73.5 (C-3″), 71.4 (C-3′), 70.8 (C-2″), 68.4 (C-4″), 66.5 (C-2′), 60.7 (C-6″), 54.2 (C-1′). HRMS (ESI) *m/z* $C_{13}H_{20}O_8N_2Na$ $[M+Na]^+$ calcd: 355.11119, found: 355.11111.

4.2.4. 6-[2-*O*-(β-D-Galactopyranosyl)ethoxy]-2,4-diaminopyrimidine (23)

The reaction proceeded for 12 h. The wave length used for detection during purification was 265 nm. The purification and lyophilisation afforded 218 mg (0.66 mmol; 23%) of the product; ^{1}H NMR (DMSO-d_6): 6.01 (2H, br s, NH2), 5.87 (2H, br s, NH_2), 5.04 (1H, s, H-5), 4.22–4.24 (2H, m, 6-O-CH_2), 4.13 (1H, d, $J_{1'-2'}$ = 7.3, H-1′), 3.95 (1H, m, 1′-O-CH_2), 3.68 (1H, m, 1′-O-CH_2), 3.63 (1H, br d, $J_{4'-3'}$ = 3.0, H-4′), 3.44–3.56 (2H, m, H-6′), 3.33 (1H, td, $J_{5'-6'}$ = 6.2, $J_{5'-4'}$ = 1.1, H-5′), 3.24–3.31 (2H, m, H-2′,3′). ^{13}C NMR (DMSO-d_6): 170.0 (C-6), 166.2 (C-2), 163.1 (C-4), 103.9 (C-1′), 76.4 (C-5), 75.5 (C-5′), 73.6 (C-3′), 70.7 (C-2′), 68.3 (C-4′), 67.1 (1′-O-CH_2), 64.2 (6-O-CH_2), 60.6 (C-6′). HRMS (ESI) *m/z* $C_{12}H_{19}O_7N_4$ $[M+H]^+$ calcd: 331.1259, found: 331.1258.

4.2.5. 2-Amino-6-[(2-*O*-β-D-galactopyranosylethyl)amino] pyrimidin-4(3*H*)-one (24)

The reaction proceeded for 24 h. The wave length used for detection during purification was 265 nm. The purification and lyophilisation afforded 235 mg (0.71 mmol; 24%) of the product; ^{1}H NMR (DMSO-d_6): 9.67 (1H, br s, H-3), 6.15–6.44 (3H, m, NH, NH_2), 4.92 (1H, br s, OH), 4.75 (1H, br s, OH), 4.67 (1H, br s, OH), 4.40 (1H, br s, OH), 4.48 (1H, s, H-5), 4.09 (1H, d, $J_{1'-2'}$ = 7.4, H-1′), 3.76 (1H, dt, J_{gem} = *10.0,* $J_{CH2-CH2}$ = 5.9, 1′-O-CH_2a), 3.62 (1H, m, H-4′), 3.47–3.57 (3H, m, 1′-O-CH_2b, 6′), 3.20–3.37 (5H, m, H-2′, 3′, 5′, NH-CH_2). ^{13}C NMR (DMSO-d_6): 164.4 (C-6), 163.2 (C-4), 155.2 (C-2), 100.9 (C-1′), 75.5 (C-5, 5′), 73.5 (C-3′), 70.8 (C-2′), 68.4 (C-4′), 67.9 (1′-O-CH_2), 60,7 (C-6′), 41.0 (NH-CH_2).

4.2.6. 1-[2-Deoxy-5-*O*-(β-D-galactopyranosyl)-α-D-ribofuranosyl]-5,6-dihydro-5-azacytosine (25)

The reaction proceeded for 7 h. The wave length used for detection during purification was 235 nm. The purification and lyophilisation afforded 135 mg (0.33 mmol; 20%) of the product as a

mixture of two isomers. HRMS (ESI) *m/z* $C_{14}H_{25}O_9N_4$ $[M+H]^+$ calcd: 393.16160, found: 393.16153.

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