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SAR Directed Design and Synthesis of Novel β(1-4)-Glucosyltransferase Inhibitors and Their In Vitro Inhibition Studies

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Abstract—This paper describes SAR directed design and synthesis of novel β(1-4)-glucosyltransferase (BGT) inhibitors. The designed inhibitors **1**–**5** provide conformational mimicry of the transition-state in glucosyltransfer reactions. The compounds were tested for in vitro inhibitory activity against (BGT) and the inhibition kinetics were examined. Three of the designed molecules were found to be potential inhibitors of BGT having IC_{50} values in micromolar (μM) range. Useful structure–activity relationships were established, which provide guidelines for the design of future generations of inhibitors of BGT.

The specific binding of complex carbohydrates forms the basis of vital intercellular recognition processes. Thus, biosynthesis of complex oligosaccharides and other glycosylated cellular metabolites is of immense importance, as these molecules are directly involved in numerous biological and cellular processes.[1–5] Hence, in glycobiology, one of the central questions raised is understanding the fundamental basis of the incredibly diverse set of glycan structures observed in nature.[6] There are a myriad of glycosyltransferases, each with its sugar specific donor and with acceptor and linkage specificity, which are responsible for the biosynthesis of these remarkably complex structures. Moreover, the role played by glycosyltransferases in the etiology of disease, as well as their potential role as therapeutic targets, are only now being appreciated.[7,8] However, the molecular basis of many processes are often still uncertain despite tremendous progress made in this field.[9] The glycosyltransferases of the Leloir pathway are key catalysts for the synthesis of oligosaccharides and glycoconjugates in vivo.[10–14] These enzymes transfer activated monosaccharide units in the form of their nucleotide mono- or diphosphate derivatives to a specific free hydroxy group of the acceptor molecule. Inhibition or modulation of this transfer reaction provides an excellent opportunity for intervention of the oligosaccharide biosynthesis and to obtain a more complete understanding of the structure–activity relationship (SAR) of oligosaccharides on a molecular basis.

β(1-4)-Glucosyltransferase (BGT) is an enzyme encoded by a number of bacteriophages belonging to the T-even group. It catalyses the transfer of glucose from uridine diphosphoglucose (UDP-Glc) to 5-hydroxymethylcytosine (5-HMC) bases in double-stranded DNA.[15] Glucosylation protects the infecting viral DNA from host restriction enzymes.[16] In addition to its protective role, the glucosylation has also been implicated in the control of phage-specific gene expression by influencing transcripton in vivo and in vitro.[17] Another example of DNA modification by glucosylation has been found in *Trypanosoma brucei*[18] where glucosylation is believed to be involved in regulating the expression of variant surface glycoproteins, used by the parasite for protection from immune recognition.[19] BGT is one of two enzymes involved in the glucosylation of T4 phage DNA. In contrast to its counterpart α-glucosyltransferase (AGT), which forms α-glycosidic linkages, BGT catalyses the formation of β-glycosidic linkages, between the Glc and the modified 5-HMC base. The T4 glucosyltransferases

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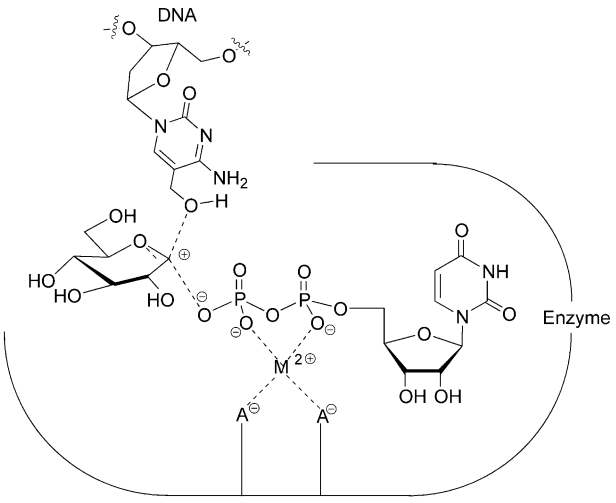


Scheme 1. Postulated transition-state for the sugar transfer catalysed by BGT.

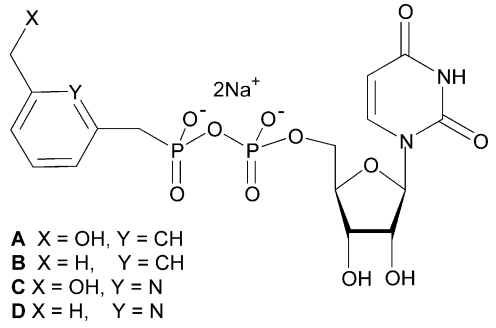


Figure 1. Reported inhibitors **A**–**D**.

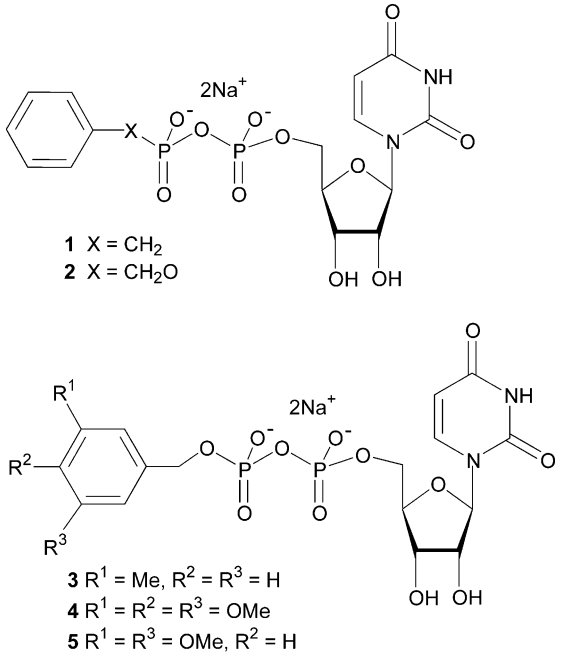


Figure 2. Structures of designed inhibitors **1**–**5**.

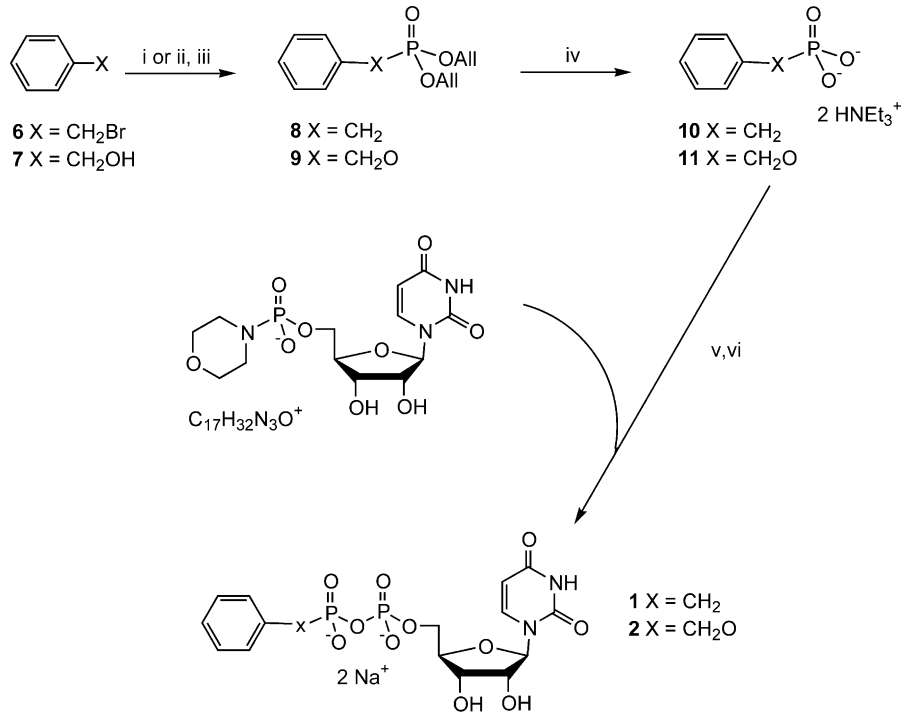
show little DNA sequence specificity but modify only 5-HMC bases, suggesting a mechanism of non-specific DNA binding combined with specific 5-HMC base recognition.[20,21] Inhibition or modulation of this transfer reaction provides an excellent opportunity for intervention of oligosaccharide biosynthesis and to obtain a more complete understanding of the SAR of oligosaccharides on a molecular basis.[22]

As part of an ongoing programme in our laboratory for the development of potential transition-state based glycosyltransferase inhibitors (Scheme 1), we had reported[23] the synthesis of a novel transition-state based analogue of galactosyltransferase, which exhibited high inhibition properties (K_i 6.20×10^{-5}) towards galactosyltransferases. Recently, we have successfully designed and synthesised[24] potential transition-state based analogues of sialyltransferases which showed very high affinity towards the enzyme in the nanomolar range.

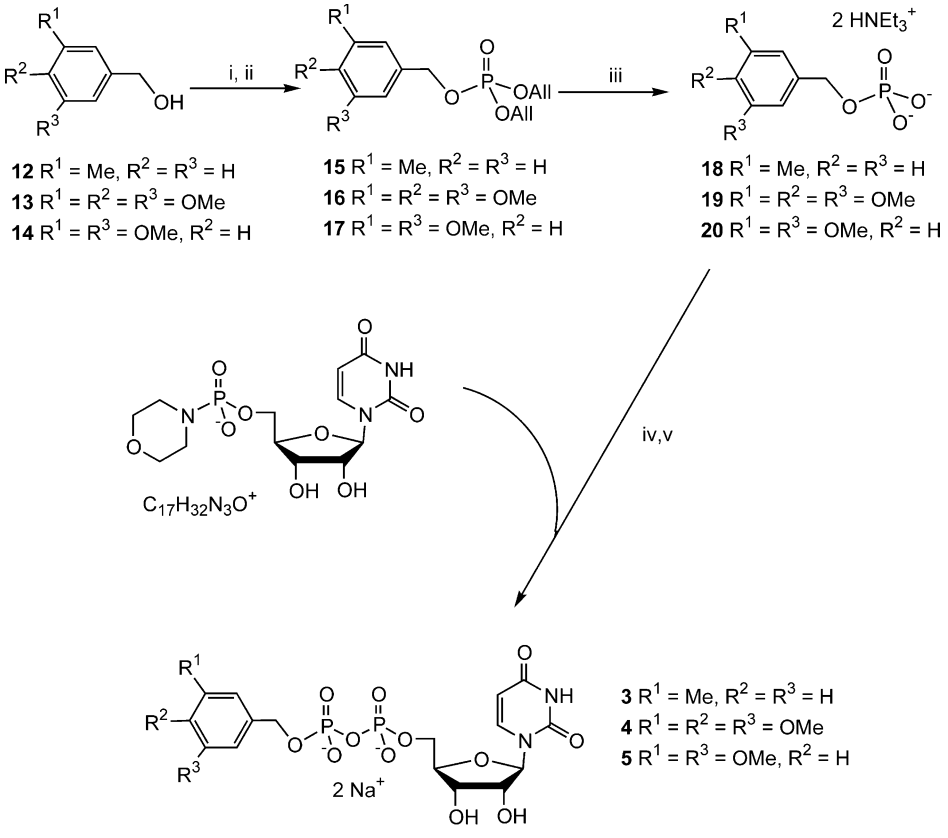
However, the aryl analogues mimicking the sugar of CMP-Neu5Ac turned out to be potent inhibitors exhibiting very high binding affinity to α(2-6)-sialyltransferase.[25] We have undertaken a programme on the SAR directed design and synthesis of potential β(1-4)-glucosyltransferases. Towards this goal, we have recently reported[26] design and synthesis of several substituted aryl/hetarylmethyl phosphonate–UMP derivatives **A**–**D** as glucosyltranferase inhibitors (Fig. 1), one of which showed inhibition properties in the micro molar range. Encouraged by these results, we have undertaken the present study in order to evaluate the substitution pattern on the aryl vis á vis their inhibitory activity. The earlier reported inhibitor **B** had shown an IC_{50} value of 1.9×10^{-3} M thereby raising the following questions: (i) is the methyl substitution on the aryl indispensable or can it be replaced by other analogous groups and, (ii) how many fold activity is raised or diminished by the addition of one more oxygen atom in the chain length? In order to address to the questions raised above, herein we describe the design, synthesis and in vitro inhibition studies of inhibitors **1**–**5** (Fig. 2).

For the synthesis of sugar mimetics **1**–**5** the appropriate phosphonate **8** and phosphates **9**, **15**, **16** and **17** had to be synthesised first. The phosphonate[27] **8** was synthesised in quantitative yield by treating benzyl bromide with triallyl phosophite in dry toluene at 95 °C (Scheme 2) whereas phosphates **9**,[28] **15**, **16** and **17** were prepared (Schemes 2 and 3) from their corresponding alcohols **7**, **12**, **13** and **14** by coupling the latter with bis(allyloxy)(diisopropylamino)phosphine in dry dichloromethane/acetonitrile (1:1) activated by 1*H*-tetrazole and then oxidising the resulting phosphites to phosphates **9**, **15**, **16** and **17** with *tert*-butylhydroperoxide in 82–92%, respectively, as colorless oils.

The deprotection of allyl groups and subsequent preparation of *bis*-triethylammonium salts were achieved in a one-pot procedure. The deprotection of allyl groups were carried out by treating the allylesters **8**, **9**, **15**, **16** and **17** with dimedone in THF at room temperature catalysed by *tetrakis*-(triphenylphosphine) palladium.[29]



Scheme 2. Reagents and conditions: (i) $(AllO)_3P$, toluene, 95 °C, quant (for **8**); (ii) $(i\text{-}Pr_2N)P(OAll)_2$, 1*H*-tetrazol, CH_2Cl_2/CH_3CN (1:1), rt, 2.5 h; (iii) *t*-BuOOH, 0 ° C, 1.5 h, 92% (for **9**); (iii) dimedone, $Pd(PPh_3)_4$, THF, Et_3N, rt, 1.5 h, 50% (for **10**), 60% (for **11**); (iv) 1*H*-tetrazol, py, rt, 3 days; RP-18; (v) Amberlite IR-120 (Na^+-form), 88% (for **1**), 77% (for **2**).



Scheme 3. Reagents and conditions: (i) $(i\text{-}Pr_2N)P(OAll)_2$, 1*H*-tetrazol, CH_2Cl_2/CH_3CN (1:1) rt, 2.5 h; (ii) *t*-BuOOH, 0 °C, 1.5 h, 91% (for **15**), 82% (for **16**), 82% (for **17**); (iii) dimedone, $Pd(PPh_3)_4$, THF, Et_3N, rt, 1.5 h, 59% (for **18**) 70% (for **19**), 44% (for **20**); (iv) 1*H*-tetrazol, py, rt, 3 days; RP-18; (v) Amberlite IR-120 (Na^+-form) 69% (for **3**), 22% (for **4**), 30% (for **5**).

Table 1. IC_{50} values of transition state analogues **1**–**5**[a]

Inhibitors	IC_{50} [mM]
1	>4
2	>4
3	1.60±0.06
4	1.58±0.06
5	0.71±0.03
UDP	1.80±0.07

[a]For details, see the Experimental.

After completion of the reaction, NEt_3 was added to the reaction mixture and stirred further for 15 min at room temperature. *bis*-Triethylammonium salts **10**, **11**, **18**, **19**, and **20** were obtained after flash chromatography ($CHCl_3$/MeOH, 6:4 + 1% NEt_3) as colorless oils in 44–70%, respectively.

The synthesis of mimetics **1**–**5** was performed following a recent procedure[30a] which is an improved variant of the Moffat–Khorana phosphomorpholidate coupling method.[30b] The phosphonophosphate and diphosphates were isolated in 22–88% yield. The ^1H NMR and ^{13}C NMR spectra showed the expected signals and total assignment could be performed by 2D NMR experiments. The ^{31}P NMR spectra also showed the characteristic signals associated with the diphosphate-/phosphonophosphate-moiety. Reaction of bis-triethylammonium salts **10, 11, 18, 19,** and **20** with uridine-5′-morpholidophosphate as activated UMP derivative in the presence of 1*H*-tetrazole in pyridine for 3 days at room temperature afforded **1**, **2**, **3**, **4** and **5** as white powders, respectively, after isolation and purification by HPLC, ion exchange [Amberlite IR-120 (Na^+ form)] and lyophilisation.

Biological Results

The compounds synthesised were tested for in vitro inhibitory activity with β(1-4)-glucosyltransferase, which was isolated as previously reported.[31] The biological studies reveal strong SAR for the binding of inhibitors **1**–**5** to BGT. The IC_{50} values (concentration of inhibitor at which 50% activity of the enzyme is observed) of inhibitors **1**–**5** were determined and are summarised in Table 1. Inhibitor **3** showed an IC_{50} value of 1.60×10^{-3} M whereas compounds **1** and **2** showed no inhibition ($>4\times10^{-3}$ M) thereby implying that the methyl substitution on the the aryl group is mandatory in order to develop potential inhibitors from this series. Diphosphate **3** showed slightly better inhibition than its phosphonophosphate analogue **B** (IC_{50} 1.9×10^{-3} M). However, better inhibition was shown by dimethoxy substituted compound **5** (IC_{50} 7.1×10^{-4} M) followed by compound **4** (IC_{50} 1.58×10^{-3} M). X-ray studies with some of these compounds bound to BGT will be reported in due course.

In conclusion, our inhibition studies clearly indicate that: (i) introduction of an extra oxygen atom in the chain length appreciably increases the inhibition properties as could be concluded from the postulated transition-state (Scheme 1), (ii) inactivity shown by compounds **1** and **2** suggest the necessity of substitution on the aryl group mimicking the side chain, (iii) replacement of methyl substitution by methoxy groups on aryl greatly enhances the activity. Our studies have revealed useful SARs which provide further guidelines for the design of future generations of inhibitors of BGT.

Experimental

General

Solvents were purified according to the standard procedures. NMR measurements were recorded at 22 °C on a Bruker AC250 Cryospec or Bruker DRX 600 spectrometers. Tetramethyl silane (TMS) or the resonance of the deuterated solvent was used as an internal standard; solvents: $CDCl_3$, δ 7.24; D_2O, δ = 4.63; DMSO-d_6, δ 2.49. For ^{31}P NMR, phosphoric acid was used as an external standard; ^{31}P NMR spectra were broadband ^1H-decoupled. MALDI-mass spectra were recorded on a Kratos Kompact Maldi 1 and 2,5-dihydroxybenzoic acid (DHB) or 6-aza-2-thiothymine (ATT) were used as matrices. FAB mass spectra were measured on a Finnigan MAT312/AMD 5000 spectrometer. Thin-layer chromatography was performed on Merck silica gel plastic plates $60F_{254}$ or Merck glass plates RP-18; compounds were visualised by treatment with a solution of $(NH_4)_6Mo_7O_{24}\cdot4H_2O$ (20 g) and $Ce(SO_4)_2$ (0.4 g) in 10% sulfuric acid (400 mL). Flash chromatography was performed on J.T. Baker silica gel 60 (0.040–0.063 mm) at a pressure of 0.3 bar. Preparative HPLC separations were performed on an Autochrom System with a Shimadzu LC8A preparative pump and a Rainin Dynamax UV 1 Detector at 260 nm. The column used was a Lichrosorb RP-18, 7 μm, 250×16 mm (Knauer, Germany). Mixtures of acetonitrile and 0.05 M triethylammonium bicarbonate (TEAB) (pH 7.0–7.2) were used as the mobile phase. Elemental analysis were carried out at the Microanalysis Unit, Fachbereich Chemie, Universität Konstanz, Konstanz.

Diallyl benzylphosphonate (8). A mixture of **6** (1.0 g, 5.88 mmol) and triallyl phosphite (1.01 mL, 5.88 mmol) in dry toluene (5 mL) was heated under argon to 95 °C for 4 h to afford **8**[27] in quantitative yield. The formed allyl bromide was distilled off under slightly reduced pressure. The residue was used as such in the next step without further purification. $R_f = 0.14$ (pentane/diethyl ether, 1:1); ^1H NMR (600 MHz, $CDCl_3$): δ 3.20 (d, $J = 20.2$ Hz, 2H, CH_2–Ar), 4.41–4.58 (m, 4H, CH_2=CH–CH_2), 5.18–5.39 (m, 4H, CH_2=CH–CH_2), 5.83–5.95 (m, 2H, CH_2=C*H*–CH_2), 7.25–7.31 (m, 5H, Ar–H); ^{31}P (243 MHz, $CDCl_3$): δ 28.39 (s, 1P); MS (EI): *m*/*z* 252 $[M]^+$ for $C_{13}H_{17}O_3P$ (252.3).

General procedure for the synthesis of (9), (15), (16) and (17). To a solution of **7, 12, 13** or **14** (1.4–4.6 mmol) in a mixture of dry CH_2Cl_2 and dry CH_3CN (each 10 mL) under argon, vacuum dried 1*H*-tetrazole (2.5–12.5 mmol) was added at room temperature. *bis*(Allyloxy)

(diisopropylamino)phosphine (1.5–5.7 mmol) was added dropwise to the reaction mixture through a syringe under argon. After stirring for 2.5 h at rt, the reaction mixture was cooled to 0 °C and 5.5 M *tert*-BuOOH solution in nonane (1.5–5.7 mmol) was added dropwise and on completion of the addition, reaction mixture was further stirred for 0.5–1.5 h at rt. After completion of the reaction (TLC), $Na_2S_2O_3$ solution (50 mL) was poured into the reaction mixture. The organic phase was washed successively with NaCl solution, $NaHCO_3$ solution and once again with NaCl solution (each 50 mL). The aqueous solution was extracted with CH_2Cl_2 (2×25 mL) and added to the organic phase. The pooled organic phase was dried ($MgSO_4$) and evaporated to dryness. The residue after flash chromatography afforded **9, 15, 16** or **17** as a colorless oil.

Diallyl benzylphosphate (9). A mixture of **7** (500 mg, 4.63 mmol), 1*H*-tetrazole (875 mg, 12.5 mmol), *bis*(allyloxy) (diisopropylamino)phosphine (1.5 mL, 5.74 mmol) in dry CH_2Cl_2/CH_3CN was treated as per the general procedure described above. After 2.5 h, 5.5 M *tert*-BuOOH solution in nonane (1.04 mL, 5.74 mmol) was added dropwise and after workup afforded a residue which on flash chromatography (pentane/diethyl ether, 1:1) afforded **9**[28] as a colorless oil (1.14 g, 92%). $R_f = 0.11$ (pentane/diethyl ether, 1:1); ^{1}H NMR (600 MHz, $CDCl_3$): δ 4.51 (m, 4H, CH_2=CH–CH_2), 5.09 (d, $J = 8.2$ Hz, 2H, CH_2–Ar), 5.24–5.34 (m, 4H, CH_2=CH–CH_2), 5.88–5.92 (m, 2H, CH_2=CH–CH_2), 7.33–7.39 (m, 5H, Ar–*H*); ^{31}P NMR (243 MHz, D_2O): δ 0.34 (s, 1P); MS (EI): *m*/*z* 268 [M^+] for $C_{13}H_{17}O_4P$ (268.25).

Diallyl 3-methylbenzylphosphate (15). A mixture of **12** (400 mg, 3.28 mmol), 1*H*-tetrazole (620 mg, 8.86 mmol), bis(allyloxy)(diisopropylamino)phosphine (1.12 mL, 4.29 mmol) in dry CH_2Cl_2/CH_3CN (10 mL each) was treated as per the general procedure described above. After 2.5 h, 5.5 M *tert*-BuOOH solution in nonane (782 μL, 4.30 mmol) was added dropwise and after work up afforded a residue which on flash chromatography (pentane/diethyl ether, 1:1) afforded **15** as a colorless oil (841 mg, 91%). $R_f = 0.12$ (pentane/diethyl ether, 1:1); ^{1}H NMR (600 MHz, $CDCl_3$): δ 2.36 (s, 3H, CH_3), 4.52 (m, 4H, CH_2=CH–CH_2), 5.05 (d, $J = 8.0$ Hz, 2H, CH_2–Ar), 5.23–5.35 (m, 4H, CH_2=CH–CH_2), 5.89–5.93 (m, 2H, CH_2=CH–CH_2), 7.14–7.27 (m, 4H, Ar–*H*); ^{13}C NMR (151 MHz, $CDCl_3$, assignment by HMQC): δ 21.27 (1C, *C*H_3), 68.01 (2C, CH_2=CH–*C*H_2), 69.27 (1C, *C*H_2–Ar), 118.17 (2C, *C*H_2=CH–CH_2), 124.93, 128.42, 128.59, 129.21 (4C, Ar), 132.39 (2C, CH_2=CH–CH_2); ^{31}P NMR (243 MHz, $CDCl_3$): δ 0.29 (s, 1P); MS (EI): *m*/*z* 282 [M^+] for $C_{14}H_{19}O_4P$ (282.28).

Diallyl 3,4,5-trimethoxybenzyl phosphate (16). A mixture of **13** (369 mg, 1.87 mmol), 1*H*-tetrazole (280 mg, 4.0 mmol), *bis*(allyloxy)(diisopropylamino)phosphine (740 μL, 2.80 mmol) in dry CH_2Cl_2/CH_3CN (10 mL each) was treated as per the general procedure described above. After 2.5 h, 5.5 M *tert*-BuOOH solution in nonane (545 μL, 3.0 mmol) was added dropwise and after workup afforded a residue which on flash chromatography (toluene/acetone, 9:1) afforded **16** as a colorless oil (552 mg, 82%); $R_f = 0.22$ (toluene/acetone, 9:1); ^{1}H NMR (250 MHz, $CDCl_3$): δ 3.81 (s, 3H, *p*-OCH_3), 3.84 (s, 6H, *m*-OCH_3), 4.47–4.53 (m, 4H, CH_2=CH–CH_2), 4.99 (d, $J_{C,P} = 8.5$ Hz, 2H, CH_2–Ar), 5.20–5.36 (m, 4H, CH_2=CH–CH_2), 5.82–5.97 (m, 2H, CH_2=CH–CH_2), 6.59 (s, 2H, Ar–*H*); $C_{16}H_{23}O_7P$ (358.3) calcd C 53.63, H 6.47; found C 53.43, H 6.66.

Diallyl 3,5-dimethoxybenzyl phosphate (17). A mixture of **14** (240 mg, 1.45 mmol), 1*H*-tetrazole (200 mg, 2.86 mmol), *bis*(allyloxy)(diisopropylamino)phosphine (389 μL, 1.45 mmol) in dry CH_2Cl_2/CH_3CN (10 mL each) was treated as per the general procedure described above. After 2.5 h, 5.5 M *tert*-BuOOH solution in nonane (264 μL, 1.45 mmol) was added dropwise and after workup afforded a residue which on flash chromatography (toluene/acetone, 6:1) afforded **17** as a colorless oil (390 mg, 82%); $R_f = 0.32$ (toluene/acetone, 4:1); ^{1}H NMR (250 MHz, $CDCl_3$): δ 3.76 (s, 6H, OCH_3), 4.47–4.55 (m, 4H, CH_2=CH–CH_2), 4.99 (d, $J = 8.0$ Hz, 2H, CH_2–Ar), 5.19–5.36 (m, 4H, CH_2=CH–CH_2), 5.82–5.98 (m, 2H, CH_2=CH–CH_2), 6.39 (t, $J = 2.3$ Hz, 1H, 4-H), 6.50 (d, $J = 2.3$ Hz, 2H, 2-H, 6-H); MS (MALDI, positive mode, matrix: DHB, dioxane): *m*/*z* 351 [$M + Na^+]^+$, 367 [$M + K^+]^+$ $C_{15}H_{21}O_6P$ (328.3) calcd C 54.87, H 6.45; found C 54.85, H 6.46.

General procedure for the synthesis of bis-triethylammonium salts (10), (11), (18), (19) and (20). To a solution of **8, 9, 15, 16** or **17** (0.5–1.4 mmol) in dry THF (6–30 mL), dimedone (2.7–7.0 mmol) and *tetrakis*-(triphenylphosphine)-palladium (0.11–0.24 mmol) was added and stirred at room temperature under argon protected from light. After 1.5 h when the TLC showed no starting material, NEt_3 (0.15 mL) was added and further stirred for 15 min at room temperature. The reaction mixture was evaporated and the residue was purified over flash chromatography to furnish **10**, **11**, **18**, **19** or **20** in 44–70%.

***bis*-Triethylammonium benzylphosphonate (10).** A mixture of **8** (150 mg, 0.60 mmol), dimedone (420 mg, 3.0 mmol) and *tetrakis*-(triphenylphosphine)-palladium (139 mg, 0.12 mmol) in dry THF (6 mL) was treated as per the general procedure described above. After evaporation of the solvent, the residue was purified over flash chromatography ($CHCl_3$/MeOH, 6:4 + 1% NEt_3) to afford **10** as a colorless oil (110 mg, 50%); $R_f = 0.29$ ($CHCl_3$/MeOH, 6:4 + 1% NEt_3); ^{1}H NMR (600 MHz, $CDCl_3$): δ 1.08 (t, $J = 7.3$ Hz, 18H, NCH_2CH_3), 2.79 (q, $J = 7.3$ Hz, 12H, NCH_2CH_3), 2.92 (d, $J = 21.5$ Hz, CH_2–Ar), 7.20–7.40 (m, 5H, Ar–*H*); ^{13}C NMR (151 MHz, $CDCl_3$): δ 8.30 (6C, NCH_2CH_3), 35.90 (d, $J = 134$ Hz, CH_2P), 45.28 (6C, NCH_2CH_3), 125.63, 127.92, 130.03, 130,07, 135.20, 135.26 (6C, Ar); ^{31}P NMR (243 MHz, $CDCl_3$): δ 21.7 (s, 1P); MS (FAB, positive mode, matrix: NBA/THF): *m*/*z* 274 [$M - HNEt_3^+ + 2H^+]^+$, 375 [$M + H^+]^+$ for $C_{19}H_{39}N_2O_3P$ (374.5).

***bis*-Triethylammonium benzylphosphate (11).** A mixture of **9** (250 mg, 0.93 mmol), dimedone (651 mg, 4.65 mmol) and *tetrakis*-(triphenylphosphine)-palladium

(219 mg, 0.19 mmol) in dry THF (10 mL) was treated as per the general procedure described above. After evaporation of the solvent, the residue was purified over flash chromatography ($CHCl_3$/MeOH, 6:4 + 1% NEt_3) to afford **11** as a colorless oil (216 mg, 60%); $R_f = 0.18$ ($CHCl_3$/MeOH, 6:4 + 1% NEt_3); ^{1}H NMR (600 MHz, $CDCl_3$): δ 1.24 (t, $J = 7.3$ Hz, 18H, NCH_2CH_3), 2.96 (q, $J = 7.3$ Hz, 12H, NCH_2CH_3), 4.98 (d, $J = 6.2$ Hz, 2H, CH_2–Ar), 7.20–7.40 (m, 5H, Ar–*H*); ^{13}C NMR (151 MHz, $CDCl_3$): δ 8.50 (6C, NCH_2CH_3), 45.30 (6C, NCH_2CH_3), 66.67 (d, $J_{C,P} = 17.4$ Hz, 1C, CH_2-Ar), 127.01–139.26 (6C, Ar); ^{31}P NMR (243 MHz, $CDCl_3$): δ 3.11 (s, 1P); MS (FAB, positive mode, matrix: NBA/THF): *m*/*z* 290 [M–$HNEt_3^+ + 2H^+$]$^+$, 391 [M + H^+]$^+$ for $C_{19}H_{39}N_2O_4P$ (390.5).

***bis*-Triethylammonium 3-methylbenzyl phosphate (18).** A mixture of **15** (150 mg, 0.53 mmol), dimedone (371 mg, 2.65 mmol) and *tetrakis*-(triphenylphosphine)-palladium (122 mg, 0.11 mmol) in dry THF (10 mL) was treated as per the general procedure described above. After evaporation of the solvent, the residue was purified over flash chromatography ($CHCl_3$/MeOH, 6:4 + 1% NEt_3) to afford **18** as a colorless oil (126 mg, 59%); $R_f = 0.21$ ($CHCl_3$/MeOH, 6:4 + 1% NEt_3); ^{1}H NMR (600 MHz, $CDCl_3$): δ 1.25 (t, $J = 7.3$ Hz, 18H, NCH_2CH_3), 2.29 (s, 3H, CH_3), 2.98 (q, $J = 7.3$ Hz, 12H, NCH_2CH_3), 4.95 (d, $J = 6.0$Hz, 2H, CH_2–Ar), 7.01–7.22 (m, 4H, Ar–*H*); ^{13}C NMR (151 MHz, $CDCl_3$): δ 8.47 (6C, NCH_2CH_3), 21.30 (1C, CH_3), 45.27 (6C, NCH_2CH_3), 66.66 (d, $J_{C,P} = 4.5$ Hz, 1C, CH_2–Ar), 124.49, 127.72, 127.83, 128.20, 137.46, 139.02 (6C, Ar); ^{31}P NMR (243 MHz, $CDCl_3$): δ 3.10 (s, 1P); MS (FAB, positive mode, matrix: CH_3CN/glycerol/0.1% TFA, 1:1:1): *m*/*z* 304 [M–$HNEt_3^+ + 2H^+$]$^+$, 405 [M + H^+]$^+$ for $C_{20}H_{41}N_2O_4P$ (404.53).

***bis*-Triethylammonium 3,4,5-trimethoxybenzyl phosphate (19).** A mixture of **16** (500 mg, 1.40 mmol), dimedone (980 mg, 7.0 mmol) and *tetrakis*-(triphenylphosphine)-palladium (176 mg, 0.15 mmol) in dry THF (30 mL) was treated as per the general procedure described above. After evaporation of the solvent, the residue was purified over flash chromatography ($CHCl_3$/MeOH/H_2O, 8:4:0.5 + 1% NEt_3) afford **19** as a colorless oil (470 mg, 70%). $R_f = 0.22$ ($CHCl_3$/MeOH/H_2O, 8:4:0.5 + 1% NEt_3); ^{1}H NMR (250 MHz, D_2O): δ 1.05 (t, $J = 7.3$ Hz, 18H, NCH_2CH_3), 3.00 (q, $J = 7.3$ Hz, 12H, NCH_2CH_3), 3.59 (s, 3H, *p*-OCH_3), 3.70 (s, 6H, *m*-OCH_3), 4.64 (d, overlap with HDO, 2H, CH_2–Ar), 6.67 (s, 2H, Ar–H); MS (MALDI, negative mode, matrix: ATT, H_2O): *m*/*z* 277 [M−2$HNEt_3^+ + H^+$]$^-$ for $C_{22}H_{45}N_2O_7P$ (480.6).

***bis*-Triethylammonium 3,5-dimethoxybenzyl phosphate (20).** A mixture of **17** (350 mg, 1.07 mmol), dimedone (750 mg, 5.36 mmol) and *tetrakis*-(triphenylphosphine)-palladium (138 mg, 0.12 mmol) in dry THF (20 mL) was treated as per the general procedure described above. After evaporation of the solvent, the residue was purified over flash chromatography ($CHCl_3$/MeOH/H_2O, 8:4:0.5 + 1% NEt_3). The product obtained after flash chromatography was lyophilised with water to afford **20** as a colorless powder (210 mg, 44%). R_f 0.29 ($CHCl_3$/MeOH/H_2O, 8:4:0.5 + 1% NEt_3); ^{1}H NMR (250 MHz, D_2O): δ 1.06 (t, $J = 7.3$Hz, 18H, NCH_2CH_3), 2.95 (q, $J = 7.3$ Hz, 12H, NCH_2CH_3), 3.62 (s, 6H, OCH_3), 4.64 (d, overlapping with D_2O peak, CH_2–Ar), 6.28 (t, $J = 2.3$ Hz, 1H, 4-H), 6.48 (d, $J = 2.3$ Hz, 2H, 2-H, 6-H); MS (MALDI, negative mode, matrix: ATT, H_2O): *m*/*z* 247 [M−2$HNEt_3^+ + H^+$]$^-$ for $C_{21}H_{43}N_2O_6P$ (450.6).

General procedure for the synthesis of (1), (2), (3), (4) and (5). A mixture of **10**, **11**, **18**, **19** or **20** (0.13–0.33 mmol) and 4 - morpholine - *N*,*N*′ - dicyclohexylcarbox - amidinium uridine 5′-monophosphomorpholidate (0.16–0.36 mmol) was evaporated with dry pyridine (3×1.5 mL). To this mixture, vacuum dried 1*H*-tetrazole (0.39–1.0 mmol) and dry pyridine (1–4 mL) were added under argon and the solution was stirred at rt for 3 days. The reaction mixture was evaporated and co-evaporated with water (2×1 mL). The residue was purified by preparative HPLC (0.05 M triethylammonium hydrogencarbonate buffer + 7.5–10% CH_3CN, flow rate: 10–15 mL/min). The fractions containing product were pooled and evaporated. The residue was dissolved in water (2 mL) and passed through a column (2.5×12 cm) of Amberlite IR-120 (Na^+ form). The column was washed with water (50 mL) and the collected fractions were evaporated. The residue thus obtained was dissolved in few drops of water and then precipitated with EtOH (5 mL). The suspended solution was centrifuged and the supernatant was carefully decanted to afford a white solid which was dissolved in water and lyophilised. After lyophilisation, **1**, **2**, **3**, **4** or **5** was obtained as a white powder in 22–88% yield.

Disodium uridine 5′-phosphono benzylphosphate (1). A mixture of **10** (53 mg, 0.14 mmol) and 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidinium uridine 5′-monophospho-morpholidate (158 mg, 0.23 mmol) and 1*H*-tetrazole (27 mg, 0.39 mmol) in dry pyridine (1 mL) was treated as per general procedure described above. After 3 days, product was purified by HPLC (0.05 M triethylammonium hydrogencarbonate buffer + 8% CH_3CN, flow rate: 10 mL/min, t_R 11.1 min). Compound **1** was obtained as a white powder (36 mg, 88%) after ion-exchange and precipitation. ^{1}H NMR (600 MHz, D_2O): δ 3.04 (d, $J = 21.4$ Hz, 2H, 1a″-H, 1b″-H), 3.93 (m, 1H, 5a′-H), 3.97 (m, 2H, 3′-H, 5b′-H), 4.04 (m, 1H, 4′-H), 4.11 (m, 1H, 2′-H), 5.73 (d, $J_{5,6} = 7.8$ Hz, 1H, 5-H), 5.81 (d, $J_{1',2'} = 4.6$ Hz, 1H, 1′-H), 7.11–7.23 (m, 5H, Ar–*H*), 7.68 (d, $J_{6,5} = 7.8$ Hz, 1H, 6-H); ^{31}P NMR (243 MHz, D_2O): δ −9.95 (d, $J = 27.2$ Hz, $P(O)O_3$), 15.23 (d, $J = 27.2$ Hz, $CP(O)O_2$); MS (FAB, positive mode, matrix: CH_3CN/glycerol/0.1% TFA, 1:1:1): *m*/*z* 523 [M + H^+]$^+$, 545 [M + Na^+]$^+$ for $C_{16}H_{18}N_2O_{11}P_2Na_2$ (522.25).

Disodium benzyl uridine 5′-diphosphate (2). A mixture of **11** (107 mg, 0.27 mmol) and 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidinium uridine 5′-monophospho-morpholidate (261 mg, 0.38 mmol) and 1*H*-tetrazole (46 mg, 0.66 mmol) in dry pyridine (1 mL) was treated as per general procedure described above. After 3 days, product was purified by HPLC (0.05 M triethylammonium hydrogencarbonate buffer + 8% CH_3CN, flow

rate: 10 mL/min, t_R 13.8 min). Compound **2** was obtained as a white powder (64 mg, 77%) after ion-exchange and precipitation. ^{1}H NMR (600 MHz, D_2O): δ 4.02 (m, 1H, 5a′-H), 4.07 (m, 1H, 2′-H), 4.09 (m, 2H, 4′-H, 5b′-H), 4.13 (m, 1H, 3′-H), 4.86 (d, J = 6.3 Hz, 2H, 1a″-H, 1b″-H), 5.64 (d, $J_{5,6}$ = 7.9 Hz, 1H, 5-H), 5.79 (d, $J_{1',2'}$ = 4.6 Hz, 1H, 1′-H), 7.21–7.29 (m, 5H, Ar–*H*), 7.65 (d, $J_{6,5}$ = 7.9 Hz, 1H, 6-H); ^{13}C NMR (151 MHz, D_2O, assignment by HMQC): δ 64.8 (1C, 5′-C), 68.5 (1C, *C*H_2–Ar), 69.4 (1C, 3′-C), 74.3 (1C, 2′-C), 83.3 (1C, 4′-C), 89.3 (1C, 1′-C), 103.3 (1C, 5-C), 141.2 (1C, 6-C); ^{31}P NMR (243 MHz, D_2O): δ −10.21, −9.84 (2d, $^3J_{P,P}$ = 21.6 Hz, 2P(O)O_3); MS (FAB, positive mode, matrix: CH_3CN/glycerol/0.1% TFA, 1:1:1): m/z 539 $[M+H^+]^+$, 561 $[M+Na^+]^+$ for $C_{16}H_{18}N_2O_{12}P_2Na_2$ (538.25).

Disodium (3-methylbenzyl) uridine 5′-diphosphate (3). A mixture of **18** (55 mg, 0.14 mmol) and 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidinium uridine 5′-monophospho-morpholidate (158 mg, 0.23 mmol) and 1*H*-tetrazole (27 mg, 0.39 mmol) in dry pyridine (1 mL) was treated as per general procedure described above. After 3 days, product was purified by HPLC [0.05 M triethylammonium hydrogencarbonate buffer + 10% CH_3CN, flow rate: 10 mL/min, t_R 16.5 min]. Compound **3** was obtained as a white powder (52 mg, 69%) after ion-exchange [Amberlite IR-120 (Na^+ form)] and precipitation. ^{1}H NMR (600 MHz, D_2O): δ 2.18 (s, 3H, C*H*$_3$), 4.01 (m, 2H, 5a′-H, 2′-H), 4.07 (m, 2H, 5b′-H, 4′-H), 4.10 (m, 1H, 3′-H), 4.82 (d, J = 6.2 Hz, 2H, C*H*$_2$–Ar), 5.59 (d, J = 7.7 Hz, 1H, 5-H), 5.79 (d, J = 4.6 Hz, 1H, 1′-H), 7.03–7.15 (m, 4H, Ar–H), 7.57 (d, J = 7.7 Hz, 1H, 6-H); ^{13}C NMR (151 MHz, D_2O, assignment by HMQC)): δ 20.10 (1C, *C*H_3), 64.11 (1C, 5′-C), 67.28 (1C, *C*H_2–Ar), 68.69 (1C, 3′-C), 73.36 (1C, 2′-C), 81.87 (1C, 4′-C), 88.20 (1C, 1′-C), 102.40 (1C, 5-C), 123.97, 127.53, 128.01, 128.19 (4C, Ar), 139.57 (1C, 6-C); ^{31}P NMR (243 MHz, D_2O): δ −9.76, −9.41 (2d, J = 21.8 Hz, 2P(O)O_3); MS (FAB, positive mode, matrix: CH_3CN/glycerol/0.1% TFA, 1:1:1): m/z 553 $[M+H^+]^+$, 575 $[M+Na^+]^+$ for $C_{17}H_{20}N_2O_{12}P_2Na_2$ (552.28).

Disodium 3,4,5-trimethoxybenzyl uridine 5′-diphosphate (4). A mixture of **19** (160 mg, 0.33 mmol) and 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidinium uridine 5′-monophosphomorpholidate (250 mg, 0.36 mmol) and 1*H*-tetrazole (70 mg, 1.0 mmol) in dry pyridine (4 mL) was treated as per general procedure described above. After 3 days, product was purified by HPLC [0.05 M triethylammonium hydrogencarbonate buffer + 7.5% CH_3CN, flow rate: 15 ml/min, t_R 12.7 min]. Compound **4** was obtained as a white powder (47 mg, 22%) after ion-exchange and precipitation. ^{1}H NMR (600 MHz, D_2O): δ 3.62 (s, 3H, *p*-OC*H*$_3$), 3.73 (s, 6H, *m*-OC*H*$_3$), 4.02 (m, 5a′-H), 4.09 (m, 2H, 2′-H, 4′-H), 4.15 (m, 2H, 3′-H, 5b′-H), 4.80 (d, J = 4.9 Hz, 2H, C*H*$_2$–Ar), 5.59 (d, $J_{5,6}$ = 8.1 Hz, 1H, 5-H), 5.73 (d, $J_{1',2'}$ = 4.6 Hz, 1H, 1′-H), 6.65 (s, 2H, Ar–*H*), 7.67 (d, $J_{6,5}$ = 8.1 Hz, 1H, 6-H); ^{13}C NMR (151 MHz, D_2O, assignment by HMQC): δ 55.47 (2C, *m*-O*C*H$_3$), 60.20 (1C, *p*-O*C*H$_3$), 64.11 (1C, 5′-C), 67.00 (1C, *C*H_2–Ar), 68.72 (1C, 3′-C), 73.39 (1C, 2′-C), 82.35 (1C, 4′-C), 87.98 (1C, 1′-C), 101.79 (1C, 5-C), 104.12 (2C, 2″-C, 6″-C), 133.57 (1C, 1″-C), 135.36 (1C, 4″-C), 140.72 (1C, 6-C), 150.86 (2C, 3″-C, 5″-C), 151.84, 165.23 (2C, 2-C, 4-C); ^{31}P NMR (243 MHz, D_2O): δ −10.20, −10.02 (2 bd, 2P(O)O_3); MS (MALDI, negative mode, matrix: ATT, H_2O): m/z 583.4 $[M-2Na^+H^+]^-$ for $C_{19}H_{24}N_2O_{15}P_2$ Na_2 (628.4).

Disodium 3,5-dimethoxybenzyl uridine-5′-diphosphate (5). A mixture of **20** (60 mg, 0.13 mmol) and 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidinium uridine 5′-monophosphomorpholidate (110 mg, 0.16 mmol) and 1*H*-tetrazole (27 mg, 0.39 mmol) in dry pyridine (2 mL) was treated as per general procedure described above. After 3 days, product was purified by HPLC [0.05 M triethylammonium hydrogencarbonate buffer + 10% CH_3CN, flow rate: 15 mL/min, t_R 17.0 min]. Compound **5** was obtained as a white powder (23 mg, 30%) after ion-exchange and precipitation. ^{1}H NMR (600 MHz, D_2O): δ 3.68 (s, 6H, OC*H*$_3$), 4.05 (m, 2H, 5a′-H, 2′-H), 4.10 (m, 1H, 4′-H), 4.16 (m, 2H, 3′-H, 5b′-H), 4.81 (d, J = 5.6 Hz, 2H, C*H*$_2$–Ar), 5.54 (d, $J_{5,6}$ = 8.1 Hz, 1H, 5-H), 5.71 (d, $J_{1',2'}$ = 4.1 Hz, 1H, 1′-H), 6.31 (s, 1H, 4″-H), 6.49 (s, 2H, 2″-H, 6″-H), 7.65 (d, $J_{6,5}$ = 8.1 Hz, 1H, 6-H); ^{13}C NMR (151 MHz, D_2O, assignment by HMQC): δ 54.84 (2C, O*C*H$_3$), 63.90 (1C, 5′-C), 66.67 (1C, *C*H_2–Ar), 68.42 (1C, 3′-C), 73.48 (1C, 2′-C), 82.14 (1C, 4′-C), 88.17 (1C, 1′-C), 98.78 (4″-C), 101.60 (1C, 5-C), 104.73 (2C, 2″-C, 6″-C), 139.66 (1C, 1″-C), 140.57 (1C, 6-C), 150.87 (1C, 2-C or 4-C), 159.61 (2C, 3″-C, 5″-C), 165.36 (1C, 2-C or 4-C); ^{31}P NMR (243 MHz, D_2O): δ −12.85,−12.51 (2 d, J = 20.9 Hz, 2P(O)O_3) MS (MALDI, negative mode, matrix: ATT, H_2O): m/z 553 $[M-2Na^+H^+]^-$ for $C_{18}H_{22}N_2O_{14}P_2$ Na_2 (598.4).

Inhibition studies of β-glucosyltransferase of phage T4. The activity of β-glucosyltransferase was monitored by the transfer of ^{14}C-glucose from the substrate ^{14}C-UDPG to 5-hydroxymethylcytosine (HMC) of unglucosylated T4*DNA. T4*DNA was obtained from progeny phage propagating in a $UDPG^-$ *Escherichia coli* K host strain (W4597). The reaction mixture used to determine the IC_{50} in the inhibition studies, contained in a final volume of 100 μL, 100 mM Tris–HCl, pH 7.9, 9 μg of T4*-DNA, 25 mM $MgC1_2$, 200 μM UDPG (Sigma), 5 μL of ^{14}C-UDPG (NEN, 330 mCi/mmol or 1.85 MBq, delivered in a total volume of 2 mL), 0.5μg BGT and different inhibitors, with concentrations varying between 0.5 and 2.5 mM. After 15 min of incubation at 30 °C, the reaction mixtures were transferred onto DEAE filter disks (Whatman DE81). The filters were washed three times with 5 mL of a buffer containing 0.5 M NaH_2PO_4, pH 7.0, dried and counted in a Beckman scintillation counter, model LS 6000 TA.

K_m and V_{max} values were determined in essentially the same buffer and reaction volume, however, the concentrations of the substrate mixture varied between 1.6 and 200 μM and the inhibitors were added at constant concentrations of 0.5, 1, and 2 mM, respectively. The different substrate concentrations were obtained by the appropriate dilution of a mixture containing UDPG and ^{14}C-UDPG in a ratio of 27.5:1. Radioactivity transferred from the substrate to the T4*DNA, again was measured as described above.

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