Supplementary Material

Materials and methods

Enzyme assay

The enzymatic activity of β-glucosidase toward salicin was measured with the Miller method (Miller, 1959) using glucose as the standard. A total of 200 μl of reaction mixture containing 0.5% salicin and enzyme (10 μl diluted stored-enzyme in 50 mM HEPES and 100 mM NaCl, pH 8.0) in 100 mM phosphate-citrate buffer was incubated at pH 5.5 at 313 K for 30 minutes. Next, 3,5-dinitrosalicylic acid reagent (200 μl) was added, and the solution was incubated at 368 K for five minutes. Finally, 600 μl distilled water was added, and the absorbance was measured at 540 nm using a Lambda Bio 40 spectrometer (Perkin Elmer). The enzymatic activity of β-glucosidase toward cellobiose was measured according to the protocol of an Amplex red glucose/glucose oxidase assay kit (Invitrogen) using glucose as the standard. The total reaction mixture was 200 μl and contained 0.5% cellobiose and enzyme (10 μl diluted stored-enzyme in 50 mM HEPES and 100 mM NaCl, pH 8.0) in 100 mM phosphate-citrate buffer, and this was incubated at pH 5.5 at 313 K for 30 minutes; subsequently, the solution was incubated at 368 K for five minutes to stop the reaction. Finally, the solution was diluted and manipulated according to the protocol of an Amplex red glucose/glucose oxidase assay kit and monitored at excitation and emission wavelengths of 570 nm and 585 nm, respectively, with a Fluorolog-3 spectrofluorometer (Horiba). One unit of enzyme activity corresponded to one and two μmol of glucose per minute in the reaction for *Nk*Bgl toward salicin and cellobiose, respectively. Protein concentrations were determined as described by Bradford (1976) using the Bio-Rad Protein Assay Kit with BSA as the standard. The experiments were performed in triplicate using a single prepared protein sample.

Table S1. Alternative conformations in the crystal structures of *Nk*Bgl.

	Crystals	WT (Gluconolactone + HEPES)	WT (1-Deoxynojirimycin + HEPES)	WT (Glycerol)	WT (Bis-Tris)	E193A (Glucose)	E193S (Cellobiose)	E193S (Salicin)	E193D (HEPES-1-glucoside) (Cellobiose)	E193D (HEPES-1-glucoside) (Salicin)	E193D (EPPS-1-glucoside) (Salicin)	E193D (Opipramol-1-glucoside) (Salicin)
Residues	No.	17	15	10	10	16	19	14	20	15	22	17
E34	1									O		
E66	1	O										
I79	3	O	O			O						
E89	1						O					
K92	1						O					
E96	4						O			O	O	O
V101	11	O	O	O	O	O	O	O	O	O	O	O
R103	3						O	O	O			
E114	8	O	O		O		O	O		O	O	O
Q122	6					O	O	O	O	O	O	
L189	1					O						
D199	6	O				O	O	O			O	O
E204	1									O		
I205	1								O			
M207	10	O	O	O	O	O	O	O	O	O		O
I211	9	O	O	O	O	O			O	O	O	O
R231	8	O	O	O	O	O			O		O	O
Q238	6	O	O				O	O	O		O	
E243	2		O							O		
K247	3	O					O		O			
E265	1						O					
E271	2				O				O			
L280	9	O	O	O		O	O	O	O		O	O
E288	1											O
L296	10	O	O		O	O	O	O	O	O	O	O
S301	10	O	O	O		O	O	O	O	O	O	O
K343	2								O		O	
K344	2								O		O	
R353	10	O	O	O	O	O	O	O		O	O	O
Q363	11	O	O	O	O	O	O	O	O	O	O	O
S373	1										O	
K390	2					O					O	
N412	1										O	
T414	10	O	O	O		O	O	O	O	O	O	O
S445	2								O			O
M447	11	O	O	O	O	O	O	O	O	O	O	O
I475	2										O	O
K491	1								O			
E494	1										O	

Table S2. The root-mean-square differences (Å) among *Nk*Bgl models.

	WT (Gluconolactone + HEPES)	WT (1-Deoxynojirimycin + HEPES)	WT (Glycerol)	WT (Bis-Tris)	WT (Tris)	E193A (Glucose)	E193S (Cellobiose)	E193S (Salicin)	E193D (HEPES-1-glucoside) (Cellobiose)	E193D (HEPES-1-glucoside) (Salicin)	E193D (EPPS-1-glucoside) (Salicin)	E193D (Opipramol-1-glucoside) (Salicin)	E193D (*p*NPG)
WT (Gluconolactone + HEPES)		0.034	0.052	0.037	0.048	0.068	0.119	0.101	0.072	0.067	0.055	0.073	0.148
WT (1-Deoxynojirimycin + HEPES)	0.034		0.050	0.041	0.051	0.072	0.122	0.107	0.075	0.067	0.058	0.079	0.149
WT (Glycerol)	0.052	0.050		0.051	0.066	0.076	0.126	0.111	0.076	0.065	0.060	0.092	0.154
WT (Bis-Tris)	0.037	0.041	0.051		0.057	0.074	0.124	0.109	0.081	0.071	0.061	0.082	0.149
WT (Tris)	0.048	0.051	0.066	0.057		0.065	0.125	0.102	0.085	0.073	0.060	0.079	0.151
E193A (Glucose)	0.068	0.072	0.076	0.074	0.065		0.113	0.082	0.087	0.082	0.060	0.081	0.168
E193S (Cellobiose)	0.119	0.122	0.126	0.124	0.125	0.113		0.067	0.106	0.098	0.108	0.098	0.141
E193S (Salicin)	0.101	0.107	0.111	0.109	0.102	0.082	0.067		0.102	0.094	0.092	0.081	0.152
E193D (HEPES-1-glucoside) (Cellobiose)	0.072	0.075	0.076	0.081	0.085	0.087	0.106	0.102		0.049	0.047	0.066	0.124
E193D (HEPES-1-glucoside) (Salicin)	0.067	0.067	0.065	0.071	0.073	0.082	0.098	0.094	0.049		0.043	0.062	0.115
E193D (EPPS-1-glucoside) (Salicin)	0.055	0.058	0.060	0.061	0.060	0.060	0.108	0.092	0.047	0.043		0.054	0.135
E193D (Opipramol-1-glucoside) (Salicin)	0.073	0.079	0.092	0.082	0.079	0.081	0.098	0.081	0.066	0.062	0.054		0.123
E193D (*p*NPG)	0.148	0.149	0.154	0.149	0.151	0.168	0.141	0.152	0.124	0.115	0.135	0.123	

Table S3. The distance (Å) between the Cα atom of residue-193 and the Cα atoms of Tyr337, Trp374, Glu402, Trp444, and Glu451 and the Cζ3 atom of Trp374.

The distance (Å) between 193Cα and	E402Cα	E451Cα	Y337Cα	W374Cα	W374Cζ3	W444Cα
WT (Gluconolactone + HEPES)	10.41	14.58	12.50	15.59	11.70	13.52
WT (1-Deoxynojirimycin + HEPES)	10.36	14.57	12.45	15.56	11.75	13.46
WT (Glycerol)	10.35	14.40	12.43	15.51	11.62	13.41
WT (Bis-Tris)	10.37	14.47	12.47	15.64	11.76	13.46
WT (Tris)	10.32	14.41	12.39	15.56	11.58	13.37
E193D (HEPES-1-glucoside) (Cellobiose)	10.26	14.94	12.41	15.94	11.84	13.56
E193D (HEPES-1-glucoside) (Salicin)	10.26	14.81	12.44	15.88	11.90	13.50
E193D (EPPS-1-glucoside) (Salicin)	10.25	14.73	12.44	15.87	11.90	13.43
E193D (Opipramol-1-glucoside) (Salicin)	10.19	14.87	12.44	15.92	11.97	13.49
E193D (*p*NPG)	10.14	15.01	12.48	16.24	12.38	13.66
E193S (Cellobiose)	9.82	14.53	11.97	15.40	11.31	13.07
E193S (Salicin)	9.90	14.28	12.03	15.37	11.26	13.11
E193A (Glucose)	10.10	14.24	12.22	15.52	11.51	13.21
Average	10.21	14.60	12.36	15.69	11.73	13.40

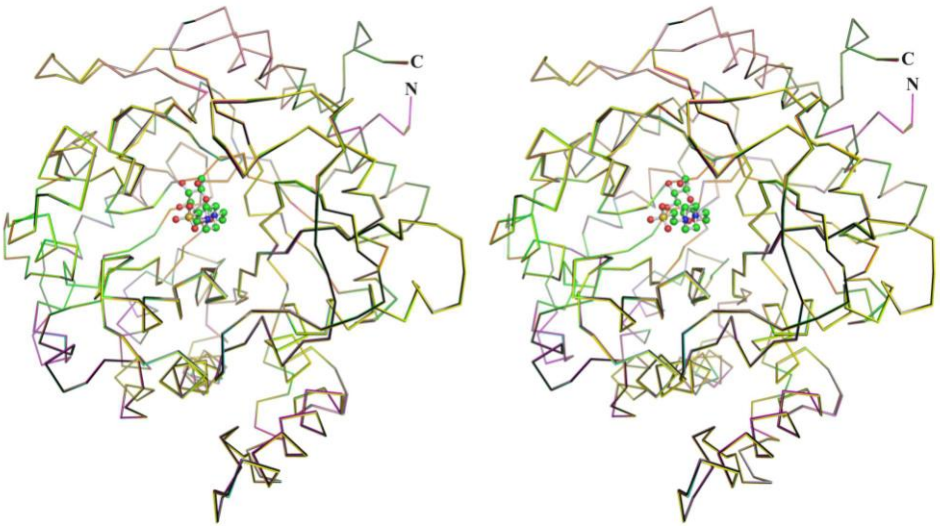
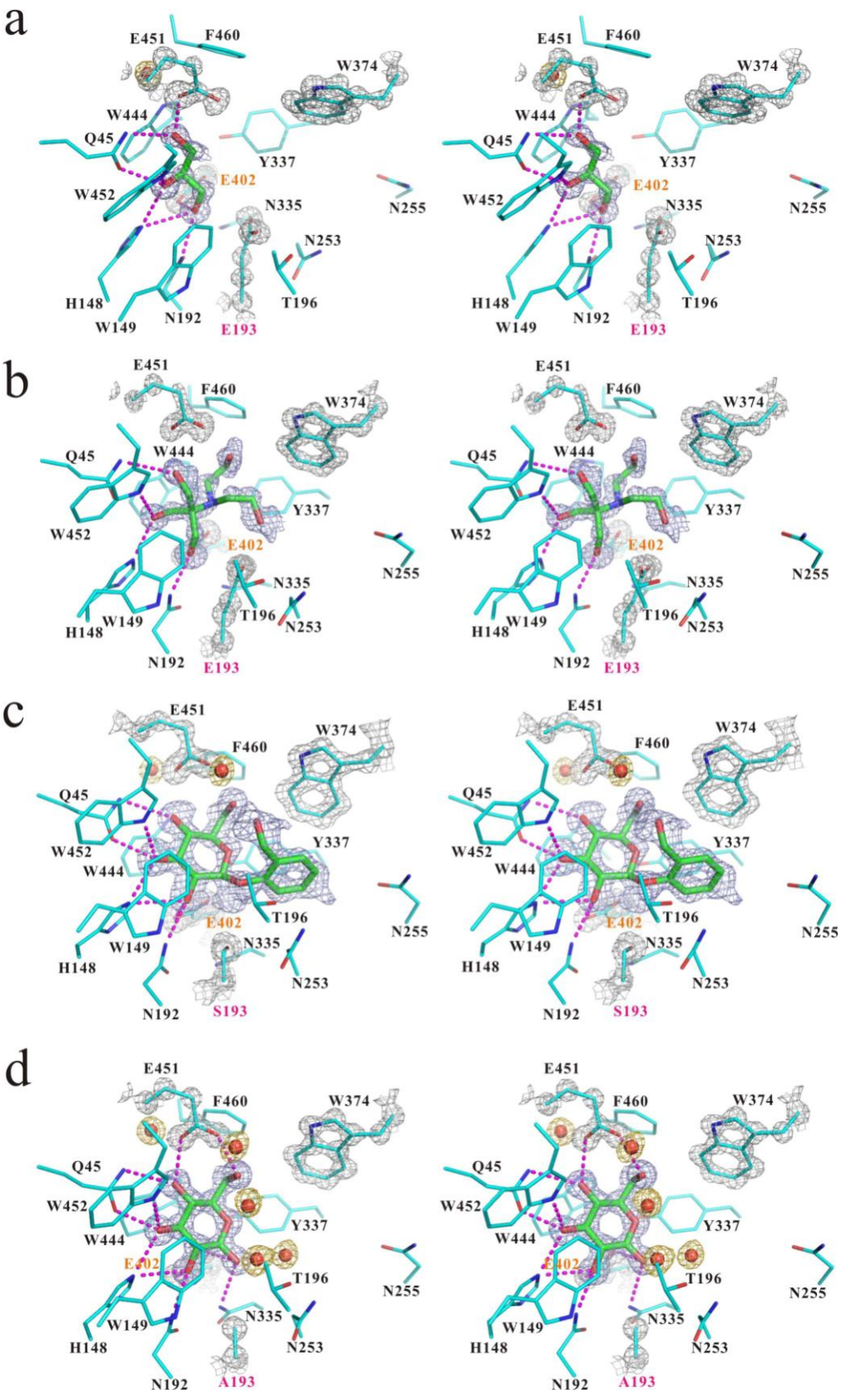


Figure S1. A stereoview of the Cα traces of thirteen superimposed *Nk*Bgl structures. The view is from the entrance of the active site pocket.


a
E451
F460
W374
W444
Q45
Y337
E402
W452
N335
N255
N253
H148
T196
W149
N192
E193
b
S193
c
d
A193

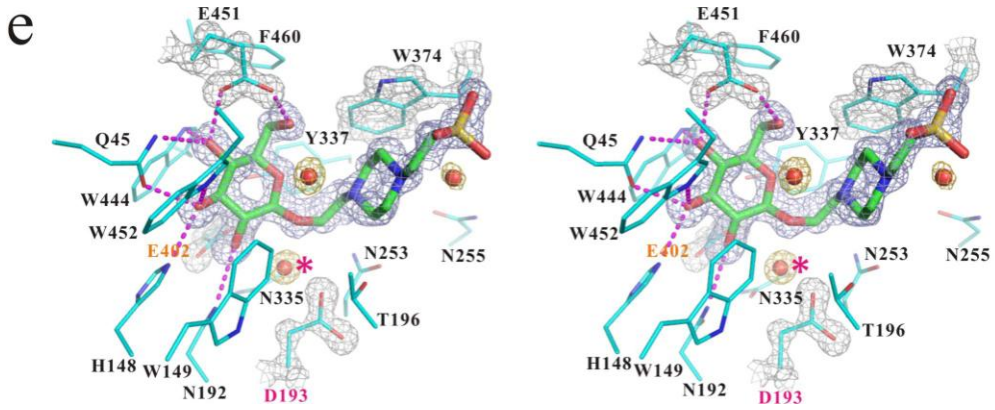


Figure S2. A stereoview of the ligand molecules and their surrounding residues in the active sites of *Nk*Bgl structures. The wild-type *Nk*Bgl is in complex with (a) glycerol, and (b) Bis-Tris. (c) The *Nk*Bgl-E193S mutant is in complex with salicin. (d) The *Nk*Bgl-E193A mutant is in complex with glucose. (e) The *Nk*Bgl-E193D mutant is in complex with HEPES-1-glucoside, which is a new adduct generated from salicin mixing with HEPES. |Fo| - |Fc| difference Fourier maps for the ligand molecules contoured at 3.0σ are shown in light blue. 2|Fo| - |Fc| maps for the side chains of selective residues contoured at 2.0σ are shown in gray, whereas water molecules contoured at 1.5σ are shown in yellow-orange. The side chains of residues around the active site of the *Nk*Bgl are shown as line models. Ligand molecules are shown as stick models. The E402 residue labeled in orange is the catalytic nucleophile of *Nk*Bgl. The acid-base residues of wild-type or mutated *Nk*Bgl are labeled in magenta. Hydrogen-bond interactions between residues and ligand molecules are labeled in magenta dotted lines. Carbon atoms of proteins and ligand molecules are shown in cyan and green, respectively. Protein oxygen atoms are shown in red, nitrogen in blue and sulfur in gold. Next to the carboxyl group of Asp193, a water molecule, presumably involved in both the hydrolysis and transglycosylation reactions is indicated with the magenta asterisk.

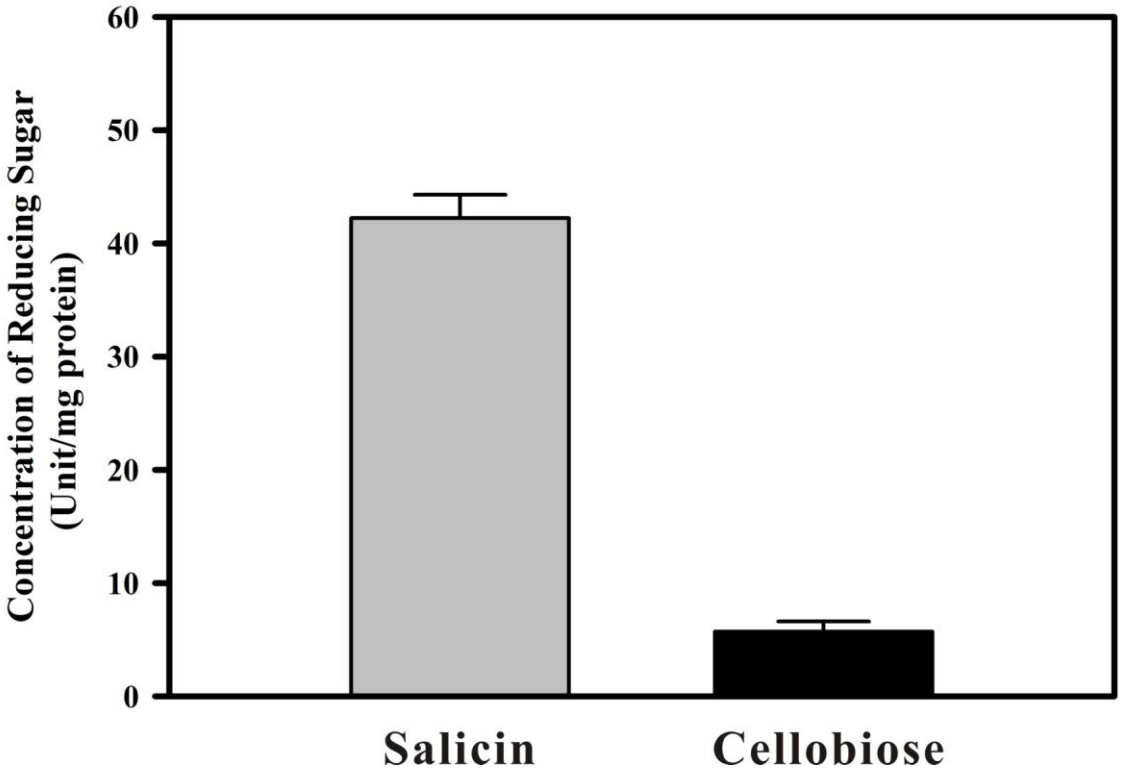


Figure S3. Activity assays of the wild-type *Nk*Bgl toward salicin or cellobiose substrates. The measurements were analyzed in triplicate with error bars indicated. Error bars represent the standard deviation of three replicates from a single prepared protein sample.

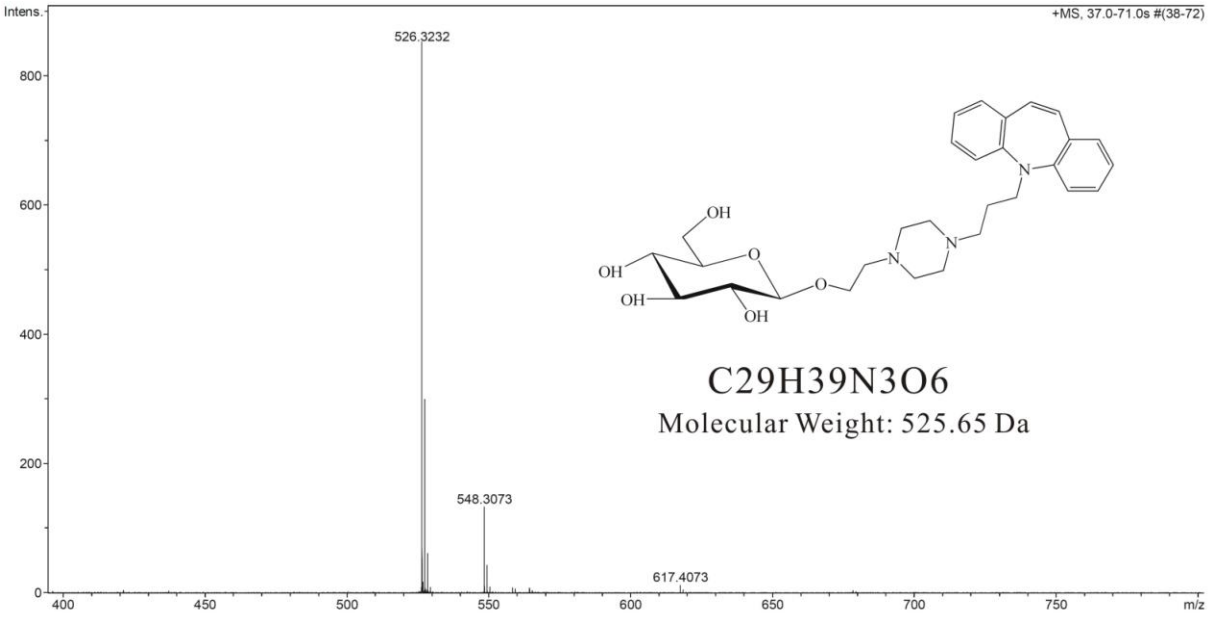


Figure S4. A MALDI mass spectrometric analysis of the new glucopyranosidic product "opipramol-1-glucoside".

References

Bradford, M. M. (1976). *Anal Biochem* **72**, 248-254.
Miller, G. L. (1959). *Anal Chem* **31**, 426-428.