Dynamic combinatorial chemistry was applied to find inhibitors of glucosyltransferase (GTF) 180. GTFs are the major producers of extracellular polysaccharides, which are important factors in the initiation and development of cariogenic dental biofilms. These biofilms are also known as dental plaque and are causative for caries. The work described herein focusses on the design and synthesis of building blocks for DCC, the analysis of the dynamic combinatorial libraries (DCLs) via UPLC-MS and evaluation of the synthesised hits by activity assays and SPR measurements.


A. M. Hartman was involved in the analysis of the DCC, synthesis and analysis of hit compounds and preparation of the manuscript. V. R. Jumde designed and performed the DCC, and was involved in synthesis. W. A. M. Elgaher assisted in the SPR measurements and E. M. te Poele performed the activity assay. L. Dijkhuizen and A. K. H. Hirsch were involved in the preparation of the manuscript and supervision of the project.
4.1 Introduction
Cariogenic dental biofilms, also known as dental plaque, are causative for dental caries. Important factors for the initiation and development of this oral disease are the fermentation of dietary carbohydrates, of which sucrose is considered most cariogenic. It is a substrate for the synthesis of extracellular (EPS) and intracellular (IPS) polysaccharides, which form the biofilm, having glucan as one of the main components. The biofilm hosts bacteria and can promote their adhesion to the tooth enamel. Glucosyltransferases (GTFs) are the major producers of EPS, and are secreted by different strains of bacteria. These GTFs, also known as glucansucrases, are therefore potential targets in order to inhibit biofilm formation and therefore prevent dental caries.[1-3]

Glucansucrases are enzymes which are part of the glycoside hydrolase family GH70, consisting of four catalytically important conserved sequence motifs. To the superfamily of GH-H also belong the glycoside hydrolase families 13 and 77.[4] Cocrystal structures, containing the catalytic and C-terminal domains, of glucansucrase of Lactobacillus reuteri 180 were previously reported and provided evidence for an α-retaining double displacement mechanism using one nucleophilic residue.[5] After the glycosidic bond is cleaved, the glucosyl moiety can elongate a glucan chain, or it can be transferred to a water molecule or an acceptor substrate such as maltose (Figure 1).

![Figure 1](image_url)

**Figure 1.** Reaction scheme of the proposed catalytic mechanism of GTF-180 via an α-retaining double displacement, leading to retention of the stereochemistry.[5,6]

Glucansucrases can be inhibited by small-molecules, as well as natural compounds. Natural inhibitors can for example be found in culture broths of
bacteria, as was the case for acarbose (Figure 2). In 1977, researchers of Bayer published the finding of α-amylase inhibitors from broths of Actinoplanes strains SE 50, SE 82 and SB18, of which BAY g 5421 (acarbose) was the most potent. Schmidt et al. postulated that acarbose could be a transition-state anologue.\cite{7} Since then, acarbose has been used as an antidiabetic drug throughout the world, and was found to have cardiovascular benefits.\cite{8–10} Newbrun et al. showed that acarbose also inhibits glucansucrases.\cite{11}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Acarbose, an \(\alpha\)-glucosidase inhibitor acting as transition-state analogue. Identified from culture broths of Actinoplanes.\cite{7}}
\end{figure}

To identify hit compounds, dynamic combinatorial chemistry (DCC) has become an attractive strategy.\cite{12–14} One of the most frequently used reversible reactions in DCC, in order to find bioactive molecules, is the acylhydrazone formation. DCC allows a target protein to alter the equilibrium of a product mixture, also known as the dynamic combinatorial library (DCL). Due to the change in equilibrium by the protein, good binders get amplified and will therefore be found as hits (Scheme 1).

\begin{scheme}
\centering
\includegraphics[width=\textwidth]{scheme1.png}
\caption{Schematic illustration of target-directed DCC using the acylhydrazone linkage, formed by the reaction between a glucose derived aromatic aldehyde and representative hydrazides. The protein target causes a change in equilibrium, which leads to the amplification of the hit compounds.}
\end{scheme}
4.1.1 Dynamic combinatorial library design

We chose the acylhydrazone formation reaction for DCC. It is a reversible reaction between an aldehyde and a hydrazide. Inspired by the structure of acarbose, we designed the basic scaffold of our molecules. An aryl aldehyde linked to glucose and maltose (A-1 and A-2, Scheme 2). We then selected eighteen chemically different and commercially available hydrazides (H-1–H-18) and divided them into two separate groups (Figure 3). Each aldehyde (A-1 and A-2) was reacted separately with the two sets of hydrazides (Hydrazides-I and Hydrazides-II), resulting in four different DCLs (DCL-1–4, Scheme 2) which were analysed via UPLC-MS. DCL-1 is the library formed by aldehyde A-1 with the first set of hydrazides (A1HI). DCL-2 is the library formed by A-2 with first set of hydrazides (A2HI). DCL-3 is the library formed by A-1 with the second set of hydrazides (A1HII), and DCL-4 is the library formed by A-2 with the second set of hydrazides (A2HII).

Scheme 2. Each aldehyde was reacted separately with the two hydrazide libraries, resulting in the formation of four dynamic combinatorial libraries (A1H1–8 (DCL1), A2H1–8 (DCL2), A1H9–18 (DCL3) and A2H9–18 (DCL4)).

Figure 3. Hydrazide groups used in the DCC experiments.
Figure 3 continued. Hydrazide groups used in the DCC experiments.

4.2 Results and Discussion

4.2.1 Synthesis of the building blocks
The aldehyde building blocks were synthesised according to the routes shown in Schemes 3 and 4. α-D-Glucose penta-acetate (1) was reacted with a solution of HBr in acetic acid, resulting in brominated glucose tetra-acetate (2). Without purification, 2 was coupled to 4-hydroxybenzaldehyde, using silver(I) oxide and yielding product 3.[15] Deprotection of the acetate groups by sodium methoxide using the classical Zemplen deacetylation gave aldehyde A-1 in quantitative yield (Scheme 3).[16] The same route was used for the synthesis of A-2, however β-maltose was acetylated first (Scheme 4).

**Scheme 3.** Synthetic route towards aldehyde A-1. Reagents and conditions: (a) Ac₂O, HBr in AcOH 33%, 0°C – rt, 15 h, 70%; (b) Ag₂O, MeCN, rt, overnight 40%; (c) NaOMe, MeOH, Amberlite H⁺ resin, quantitative yield.[15]

**Scheme 4.** Synthetic route towards aldehyde A-2. Reagents and conditions: (a) Ac₂O, HClO₄, AcOH, rt, 1 h; (b) 33% HBr in AcOH, 0°C – rt, 15 h, 52% yield over two steps; (c) MeCN, Ag₂O, rt, overnight, 62%; (d) NaOMe, MeOH, Amberlite H⁺ resin, 70%.[15,17]
4.2.2 Forming the DCLs
Each library consisted of one aldehyde, one group of hydrazides, aniline and DMSO in sodium acetate buffer. Aniline was added to enhance the rate at which the acylhydrazone formation reaches equilibrium, as it serves as nucleophilic catalyst to form Schiff bases with the corresponding aldehydes.\textsuperscript{[18]} The use of DMSO as a cosolvent allows the building blocks and products to be soluble, preventing an undesired shift in equilibrium due to precipitation. A desired shift in the equilibrium, also known as the template effect, was achieved by the addition of the target protein GTF-180. The protein was added after pre-equilibrium was reached in the blank library.

4.2.3 Monitoring the DCLs
The DCL was left shaking at room temperature and was frequently monitored via UPLC-MS. Samples for UPLC-MS were prepared by taking 100 µL of the corresponding library and diluting it with 100 µL acetonitrile. The pH was raised to > 8 by the addition of 8 µL NaOH (2 M), to freeze the equilibrium. The mixture was centrifuged at 10,000 rpm for 2 minutes, and the supernatant was analysed via UPLC-MS.

The formation of the acylhydrazones reached equilibrium within three hours. It was at this time point that we added the GTF enzyme and continued the analysis via UPLC-MS. The distribution of the products in the DCLs of the blank library versus the protein library can be compared by the relative peak areas from the UV-chromatograms (Figure 4 and Table 1, and Figures S1–S3 and Tables S1–3). We selected the most amplified structure of each library and synthesised these compounds (Figure 5). For two product members of DCL-2 (A2H2 and A2H8), the glycosidic bond between the two sugar rings was cleaved after 6 hours (Figure S1 and Table S1) resulting in A1H2 and A1H8. This indicates that these compounds enter the active site and get cleaved by the enzyme. Along with the hits, the cleaved products (A1H2 and A1H8) will also be tested for their activities.

![Figure 4. DCL-1. Dynamic combinatorial libraries of aldehyde A-1 with hydrazide library 1: a) UV-chromatogram at 290 nm of the blank reaction at 6 h; b) UV-chromatogram at 290 nm of the protein-templated reaction at 7 h. Data obtained from single experiment.](image-url)
Table 1. Amplification folds of the formed products in DCL-1; analysed via the relative surface areas of peaks in the UV-chromatograms of the protein-templated reaction (P) and blank reaction (B). Data obtained from single experiment.

<table>
<thead>
<tr>
<th>Compound</th>
<th>UV-retention time (min)</th>
<th>Amplification fold (%P/%B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1H1</td>
<td>9.6</td>
<td>0.9</td>
</tr>
<tr>
<td><strong>A1H2</strong></td>
<td><strong>8.1</strong></td>
<td><strong>1.8</strong></td>
</tr>
<tr>
<td>A1H3</td>
<td>7.5</td>
<td>1.1</td>
</tr>
<tr>
<td>A1H4</td>
<td>9.0</td>
<td>0.8</td>
</tr>
<tr>
<td>A1H5</td>
<td>5.8</td>
<td>0.8</td>
</tr>
<tr>
<td>A1H6</td>
<td>3.8</td>
<td>1.2</td>
</tr>
<tr>
<td>A1H7</td>
<td>4.5</td>
<td>0.9</td>
</tr>
<tr>
<td>A1H8</td>
<td>7.5</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Figure 5. Hit compounds from DCL-1 — DCL-4.
4.2.4 Binding studies by surface plasmon resonance (SPR)

We immobilised GTF-180 to a sensor chip via amine coupling and followed the change in surface plasmon resonance upon injecting our hit compounds. Firstly, we ran a measurement with acarbose as a positive control, which showed that the setup was working. We then injected our hit compounds and the data are given in Table 2. Most hit compounds showed solubility problems above 1.5 mM, when 5% DMSO was used as cosolvent. We therefore could not reach the plateaus and could only fit the available data. In addition, the responses for most of the compounds were relatively low, as can be seen in the sensorgrams at the end of the experimental section (S4 – S10). Due to a very low response, the sensorgram obtained for compound A2H6 could not be evaluated, and therefore its binding affinity could not be determined. As can be seen from Table 2, almost all compounds show weak binding, except for acarbose (10.7 μM). Compound A1H12 showed to be the most promising binder with micromolar affinity. Since the active site of GTF-180 is polar, it is a very challenging task to address the binding pockets. We were therefore pleased to find A1H12 as moderate binder.

Table 2. Binding study results obtained from surface plasmon resonance (SPR). Data obtained from single experiments.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$R_{\text{max}}$ (RU)</th>
<th>$k_{\text{on}}$ (M⁻¹ s⁻¹)</th>
<th>$k_{\text{off}}$ (s⁻¹)</th>
<th>$K_D$ (mM)</th>
<th>Res sd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acarbose</td>
<td>1.36 ± 0.03</td>
<td>1.8 ± 0.1 × 10³</td>
<td>0.0191 ± 0.0008</td>
<td>0.0107 ± 0.0008</td>
<td>0.41</td>
</tr>
<tr>
<td>A1H2</td>
<td>4.7 ± 0.2</td>
<td>1.7 ± 0.2 × 10²</td>
<td>0.25 ± 0.02</td>
<td>1.4 ± 0.1</td>
<td>0.41</td>
</tr>
<tr>
<td>A1H8</td>
<td>32 ± 4</td>
<td>18 ± 3</td>
<td>0.142 ± 0.004</td>
<td>8 ± 1</td>
<td>0.53</td>
</tr>
<tr>
<td>A1H12</td>
<td>8.7 ± 0.3</td>
<td>3.4 ± 0.2 × 10²</td>
<td>0.112 ± 0.004</td>
<td>0.33 ± 0.02</td>
<td>0.54</td>
</tr>
<tr>
<td>A2H2</td>
<td>40 ± 20</td>
<td>8 ± 5</td>
<td>0.099 ± 0.006</td>
<td>12 ± 8</td>
<td>0.74</td>
</tr>
<tr>
<td>A2H12</td>
<td>1000.0 ± 3000</td>
<td>1 ± 3</td>
<td>0.126 ± 0.003</td>
<td>100 ± 300</td>
<td>0.7</td>
</tr>
</tbody>
</table>

$R_{\text{max}}$: maximum analyte binding capacity; $k_{\text{on}}$: association rate constant; $k_{\text{off}}$: dissociation rate constant; $K_D$: equilibrium dissociation constant; Res sd: residual standard deviation.
4.2.5 GTF-180 activity assay
The activity of the protein can be followed via glucose oxidase/peroxidase (GOPOD) analysis. We followed the hydrolysis of sucrose, which forms glucose, and also monitored the transferase product fructose. The enzyme was incubated for half an hour at 37 °C with the compounds before sucrose was added. Figure 6 shows the results of the analysis of the hit compounds in the GTF activity assay. As can be seen from this figure, hardly any inhibition was observed. We used acarbose as a positive control, however it should be mentioned that acarbose is a moderate to weak inhibitor of GTF-180. The assay condition contained 20% of DMSO, which could have a bad influence on protein–ligand binding.[19] We have screened the binding affinities via SPR at 5% DMSO and the compounds were binding with low mM affinities. These results are in line with each other and therefore, repeating the activity assay at lower DMSO percentages will most probably not change the outcome.

**Figure 6.** GTF-180 activity assay based on the hydrolysis of sucrose. No significant inhibition was observed for the hit compounds at 500 µM, and moderate inhibition by acarbose at 500 µM.
4.3 Conclusions

In this work, we showed the first application of DCC to glucansucrases. We have designed our compounds to bear a glucose or maltose head group, which resembles natural substrates, as a proper starting point. These molecules were then expected to grow into the pocket by eighteen different aromatic or aliphatic tails via DCC. By separating the complex libraries into four individual sublibraries, we were able to analyse the DCC experiments over time. Interestingly, we observed in the presence of enzyme that the glycosidic bond between the two glucose molecules in maltose was cleaved, indicating that it could also serve as a substrate for glucansucrases. Maltose is formerly known to be an acceptor for the glucosyl moiety. No cleavage was observed for the glycosyl moiety in the glucose derivative.

LC-MS analysis of the sublibraries resulted in six hit compounds, which were synthesised and analysed for their biophysical and biochemical properties via SPR and a GTF-activity assay. In comparison to acarbose, the hit compounds showed only moderate to low affinities or activities as inhibitors of GTF-180. The results of the SPR measurements are in line with the activity assay, even though they were performed at different concentrations of DMSO. In both SPR as the activity assay, acarbose was used as a positive control. It showed moderate binding, as well as moderate activity.

The first DCC approach for the quest of hits for GTFs leads to some interesting findings concerning solubility issues, active site of the enzyme and design of the scaffold. We may have failed in reaching strong binders since the active site is rather polar. This makes it difficult for compounds to enter and inhibit the active site. The cleavage of the glycosydic bond is a good indication that our compounds could enter the active site, but most likely they are not binding strong enough to inhibit the enzyme. This could be due to the fact that acylhydrazones are rather rigid and flat, possibly hindering the cavity to be filled properly.

Future work should focus on making these products more soluble to be able to test their activity with the least amount of DMSO possible. Moreover, mimicking the transition state, like acarbose does, might be the most rewarding strategy to achieve stable inhibitors. Furthermore, compounds should be flexible enough to be able to cover most of the cavity of the binding pocket.
4.4 Experimental section

4.4.1 Materials and methods
See section 3.4.1.

4.4.2 General procedure for DCC experiments
The reaction mixture composition for each sublibrary was obtained by adding the hydrazides (each 3 µL, stock solutions 100 mM in DMSO) and the aldehyde (3 µL, stock solutions 100 mM in DMSO) to a sodium acetate buffer (590.5 µL, 0.1 M, pH 5.2). Aniline (5.55 µL, stock solution 1.8 M) was added as well as DMSO, to reach a final concentration of DMSO in the DCL of 10%. Protein (309.5 µL, stock solution 96.93 µM) was added accordingly after 3 h of equilibration. Final concentrations in the DCL were: aniline (10 mM), aldehyde (300 µM), hydrazides (300 µM each), protein (30 µM) and DMSO (10%). The DCL was left shaking at room temperature and was frequently monitored via UPLC-MS. After 6–7 h of shaking with protein, the mixture was analysed via UPLC-MS.

For monitoring via UPLC-MS, 100 µL of the corresponding library was diluted in 100 µL acetonitrile, the pH was raised to pH > 8 by adding 8 µL NaOH (2.0 M) to freeze the reaction equilibrium. The mixture was centrifuged at 10,000 rpm for 2 min, and the supernatant was analysed via UPLC-MS.

4.4.3 Binding studies by surface plasmon resonance (SPR)
See section 3.4.6. Instead of 14-3-3, GTF-180 (5 µM, 117 kDa) was immobilised on the surface.

4.4.4 GTF-180 activity assay
In an 8-well PCR strip, 50 µL sodium acetate buffer (100 mM, pH 4.7, 8 mM CaCl₂), 40 µL DMSO, inhibitor 20 µL (5 mM), 20 µL GTF-180ΔN (4.5 µM) and 70 µL Milli-Q H₂O were added and incubated for 30 min at 37 °C. To the wells of a 96-wells PCR plate was added 12.5 µL NaOH (0.4 M). The wells of a different 8-well PCR strip were filled with 200 µL sucrose solution (100 mM) and incubated for 5 min before the start of the assay.

To start the assay, 20 µL of the 100 mM sucrose stock was added to the wells containing inhibitor and enzyme. Every 30 seconds, 25 µL sample was taken and mixed with 12.5 µL NaOH (0.4 M). After the last time point at 3.5 min, 12.5 µL HCl (0.4 M) was added to neutralise the samples.

For the glucose oxidase/peroxidase (GOPOD) analysis, 12.5 µL sample was mixed with 187.5 µL GOPOD. The glucose standard ranged from 25 to 0.195 mM.
4.4.5 Synthesis

**General procedure for acylhydrazone formation (GP1):**

To the hydrazide (1 eq.) dissolved in MeOH, the corresponding aldehyde (1.2 eq.) was added. The reaction mixture was stirred at room temperature or refluxed until completion. After cooling to room temperature, the reaction mixture was concentrated *in vacuo*. Purification of acetylated products was performed by column chromatography and deprotected sugars were purified by preparative high-performance liquid chromatography, affording the corresponding acylhydrazone in 60% to quantitative yield. For acylhydrazones, some $^{13}$NMR signals doubled due to presence of two isomers ($E$ & $Z$).

**General procedure for the deprotection of the acetyl groups (GP2):**

The classical Zemplén deacetylation method of the $O$-acetyl protecting groups with sodium methoxide in methanol at room temperature was used. The $O$-acetyl protected sugar was dissolved in methanol (0.01 M), and a catalytic amount of sodium methoxide (0.15 eq) was added. The reaction mixture was stirred at room temperature until complete deprotection was achieved.

**tert-Butyl 4-(2-(4-(((2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2$H$-pyran-2-yl)oxy)benzylidene)hydrazine-1-carbonyl)piperidine-1-carboxylate (A1H2)**

The acylhydrazone was synthesised according to GP1 by using 1-Boc-isonipecotic acid hydrazide (26 mg, 0.1 mmol) in methanol (1.0 mL) and para-glucose-benzaldehyde (14.5 mg, 0.05 mmol). After purification, the acylhydrazone was obtained as a mixture of $E$ and $Z$ isomers ($E$:$Z = 7:3$) as a white solid (12 mg, 46%). $^1$H-NMR (500 MHz, MeOD) $\delta = 8.06$ (s, 1H, $E$), 7.90 (s, 1H, $Z$), 7.72 (d, $J = 8.8$ Hz, 1H), 7.62 (d, $J = 8.8$ Hz, 1H), 7.13 (dd, $J = 8.8$, 4.0 Hz, 2H), 5.00 – 4.95 (m, 1H), 4.14 (d, $J = 13.3$ Hz, 2H), 3.93 – 3.86 (m, 1H), 3.71 (dd, $J = 12.1$, 5.6 Hz, 1H), 3.52 – 3.44 (m, 3H), 3.44 – 3.37 (m, 1H), 2.47 (tt, $J = 11.4$, 3.7 Hz, 1H), 1.89 – 1.76 (m, 2H), 1.75 – 1.56 (m, 2H), 1.47 (s, 9H), 1.36 – 1.26 (m, 1H), 1.15 (d, $J = 6.2$ Hz, 1H), 0.94 – 0.83 (m, 1H); $^{13}$C-NMR (126 MHz, MeOD) $\delta = 178.6$, 173.9, 161.0, 160.6, 156.4 (d, $J = 10.1$ Hz), 149.3, 145.5, 130.2, 129.8, 129.5, 117.9 (d, $J = 10.7$ Hz), 101.9 (d, $J = 6.1$ Hz), 81.1 (d, $J = 12.8$ Hz), 78.2 (d, $J = 3.5$ Hz), 77.9 (d, $J = 4.2$ Hz), 74.8, 71.3 (d, $J = 3.1$ Hz), 62.5, 42.7, 39.5, 29.5, 28.9, 28.7; HRMS (ESI) calcd for C$_{24}$H$_{35}$N$_{3}$O$_{9}$ [M+H]$^+$: 510.2446, found 510.2432

The acylhydrazone was synthesised according to GP1 by using 1-boc-isonipecotic acid hydrazide (38 mg, 0.15 mmol) in methanol (1.0 mL) and para-maltose-benzaldehyde (22 mg, 0.05 mmol). After purification, the acylhydrazone was obtained as a mixture of E and Z isomers (E:Z = 3:2) as a white solid (9.1 mg, 28%).

\[ \delta = 8.07 (s, 1H, E), 7.90 (s, 1H, Z), 7.73 (d, J = 8.8 Hz, 1H), 7.63 (d, J = 8.8 Hz, 1H), 7.13 (dd, J = 8.8, 3.5 Hz, 2H), 5.21 (d, J = 3.8 Hz, 1H), 5.01 (dd, J = 7.8, 1.9 Hz, 1H), 4.14 (d, J = 13.4 Hz, 2H), 3.95 – 3.42 (m, 13H), 2.47 (tt, J = 11.5, 3.8 Hz, 1H), 1.90 – 1.77 (m, 2H), 1.77 – 1.57 (m, 3H), 1.47 (s, J = 6.5 Hz, 9H); \]

\[ ^{13}\text{C NMR (126 MHz, MeOD)} \delta = 178.57 (s), 173.93 (s), 160.73 (d, J = 40.2 Hz), 156.45 (d, J = 10.2 Hz), 149.25 (s), 145.48 (s), 130.25 (s), 129.54 (d, J = 8.3 Hz), 117.84 (d, J = 11.2 Hz), 102.94 (s), 101.71 (d, J = 6.4 Hz), 81.14 (d, J = 12.5 Hz), 80.83 (d, J = 5.5 Hz), 77.66 (d, J = 3.9 Hz), 76.80 (d, J = 2.8 Hz), 75.08 (s), 74.85 (s), 74.44 (s), 74.18 (s), 71.50 (s), 62.76 (s), 61.90 (s), 42.69 (s), 39.54 (s), 29.51 (s), 28.67 (s); \]

HRMS (ESI) calcd for C₃₀H₄₅N₃O₁₄ [M+H]⁺: 672.2974, found 672.2958


The acylhydrazone was synthesised according to GP1 by using 2-(methylsulfonyl)acetic acid hydrazide (12.8 mg, 0.08 mmol) in methanol (0.8 mL) and para-maltose-benzaldehyde (30.2 mg, 0.07 mmol). After purification, the acylhydrazone was obtained as a mixture of E and Z isomers (E:Z = 3:2) as a white solid (4.1 mg, 10%).

\[ \delta = 8.10 (s, 1H, E), 7.95 (s, 1H, Z), 7.75 (d, J = 8.8 Hz, 1H), 7.66 (d, J = 8.8 Hz, 1H), 7.14 (d, J = 8.8 Hz, 2H), 5.21 (d, J = 3.8 Hz, 1H), 5.01 (dd, J = 7.7, 4.5 Hz, 1H), 4.15 (s, 1H), 3.94 – 3.43 (m, 13H), 3.20 (d, J = 7.9 Hz, 3H); \]

\[ ^{13}\text{C-NMR (126 MHz, MeOD)} \delta = 165.9, 161.1 (d, J}
\( = 15.2 \text{ Hz} \), 160.8, 150.8, 146.8, 130.5, 129.8, 129.4, 129.1, 117.9 (d, \( J = 6.4 \text{ Hz} \)), 102.9, 101.7 (d, \( J = 5.4 \text{ Hz} \)), 80.8 (d, \( J = 5.4 \text{ Hz} \)), 77.7 (d, \( J = 3.2 \text{ Hz} \)), 76.8, 75.1, 74.8, 74.4, 71.5, 62.8, 61.9 (d, \( J = 2.2 \text{ Hz} \)), 60.0, 57.4, 49.6, 49.5, 49.3, 49.2, 49.0, 48.8, 48.7, 48.5, 42.6, 42.0, 40.4; HRMS (ESI) calcd for \( \text{C}_{22}\text{H}_{32}\text{N}_{2}\text{O}_{14}\text{S} \) [M+H]⁺: 581.1647, found 581.1620.

\( (2\text{R},3\text{R},4\text{S},5\text{R},6\text{S})-2-\text{(Acetoxymethyl)}-6-\text{-((2-(2-(2-chlorophenoxy)acetyl)hydrazineylidene)methyl)phenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate} \) (Acetylated A1H12)

The acylhydrazone was synthesised according to GP1 by using 2-chlorophenoxyacetic acid hydrazide (26.6 mg, 0.13 mmol) in methanol (1.8 mL) and para-(O-acetylatedglucose)-benzaldehyde (50.0 mg, 0.11 mmol). After purification, the acylhydrazone was obtained as a mixture of \( E \) and \( Z \) isomers (\( E:Z = 3:2 \)) as a white solid (43 mg, 61%). \(^1\text{H}-\text{NMR} \) (500 MHz, MeOD) \( \delta = 8.19 \text{ (s, } 1\text{H}, E), 7.93 \text{ (s, } 1\text{H}, Z), 7.72 \text{ (dd, } J = 54.3, 8.8 \text{ Hz, } 2\text{H}), 7.40 \text{ (dd, } J = 24.3, 7.9, 1.6 \text{ Hz, } 1\text{H}), 7.31 - 7.20 \text{ (m, } 1\text{H}), 7.14 - 6.92 \text{ (m, } 4\text{H}), 5.44 - 5.35 \text{ (m, } 2\text{H}), 5.25 \text{ (s, } 1\text{H}), 5.20 - 5.08 \text{ (m, } 2\text{H}), 4.75 \text{ (d, } J = 6.6 \text{ Hz, } 1\text{H}), 4.31 \text{ (dd, } J = 12.3, 5.3 \text{ Hz, } 1\text{H}), 4.20 - 4.06 \text{ (m, } 2\text{H}), 2.10 - 1.95 \text{ (m, } 12\text{H}); \(^{13}\text{C}-\text{NMR} \) (126 MHz, MeOD) \( \delta = 171.9 \text{ (d, } J = 82.7 \text{ Hz, } 1\text{H}), 171.4 \text{ (s), 171.3 \text{ (s), 171.1 \text{ (s), 167.1 \text{ (s), 159.9 \text{ (d, } J = 44.7 \text{ Hz, } 1\text{H), 155.3 \text{ (d, } J = 74.1 \text{ Hz, } 1\text{H), 150.6 \text{ (s), 146.1 \text{ (s), 131.4 \text{ (d, } J = 19.4 \text{ Hz, } 1\text{H), 130.6 \text{ (s), 130.3 \text{ (s), 130.1 \text{ (s), 129.8 \text{ (s), 129.3 \text{ (s), 128.9 \text{ (s), 124.4 \text{ (s), 124.1 \text{ (s), 124.0 \text{ (s), 123.1 \text{ (s), 117.9 \text{ (d, } J = 10.9 \text{ Hz, } 1\text{H), 116.1 \text{ (s), 115.3 \text{ (s), 99.1 \text{ (d, } J = 6.4 \text{ Hz, } 74.1 \text{ (s), 73.0 \text{ (s), 72.6 \text{ (s), 69.7 \text{ (d, } J = 3.6 \text{ Hz, } 69.2 \text{ (s), 67.3 \text{ (s), 63.1 \text{ (d, } J = 4.4 \text{ Hz, } 20.9 - 20.1 \text{ (m); HRMS (ESI) calcd for } \text{C}_{29}\text{H}_{31}\text{ClN}_{2}\text{O}_{12} \) [M+H]⁺: 635.1638, found 635.1638.

\( 2-(2-\text{Chlorophenoxy})-N'-(Z)-4-(((2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)benzylidene)acetohydrazide \) (A1H12)

The acylhydrazone was synthesised according to GP2 by using acetylated A1H12 (26.1 mg, 0.04 mmol) in methanol (4 mL) and sodium methoxide (0.33 mg, 0.006 mmol). After purification, the acylhydrazone was obtained as a mixture of \( E \) and \( Z \) isomers (\( E:Z = 3:7 \)) as a white solid in quantitative yield (20 mg). \(^1\text{H}-\text{NMR} \) (500 MHz, DMSO) \( \delta = 11.53 \text{ (bs, } 1\text{H}), 8.22 \text{ (s, } 1\text{H}, E), 7.96 \text{ (s, } 1\text{H}, Z), 7.64 \text{ (t, } J = 8.4 \text{ Hz, } 2\text{H), 7.44 \text{ (ddd, } J = 12.5, 7.9, 1.5 \text{ Hz, } 1\text{H), 7.33 - 7.22 \text{ (m, } 1\text{H), 7.10 - 7.04 \text{ (m, } 2\text{H), 7.04 - 6.92 \text{ (m, } 2\text{H), 5.37 \text{ (bs, } 1\text{H}, \text{OH), 5.25 \text{ (s, } 2\text{H), 5.16 \text{ (bs, } 1\text{H}, \text{OH), 5.08 \text{ (bs, } 1\text{H),} \) 90}
91

OH, 4.96 – 4.91 (m, 1H), 4.74 (s, 1H), 4.58 (s, 1H), 3.69 (d, J = 11.3 Hz, 1H), 3.47 (s, 1H), 3.29 – 3.21 (m, 2H), 3.17 (t, J = 9.0 Hz, 1H); 13C-NMR (126 MHz, DMSO) δ = 168.4, 158.7, 153.7, 143.6, 130.1, 130.0, 128.6, 128.4, 128.3, 128.1, 127.7, 122.1, 121.5, 121.1, 116.4, 116.4, 114.1, 113.8, 100.0, 77.1, 76.6, 73.2, 69.7, 65.3, 60.6; HRMS (ESI) calcd for C21H23ClN2O8 [M+H]+: 467.1216, found 467.1205.

(2R,3R,4S,5R,6S)-2-(Acetoxymethyl)-6-(4-((Z)-(2-(cyclopentylacetyl)hydrazineylidene)methyl)phenoxy)tetrahydro-2H-pyran-3,4,5-triyl triacetate (Acetylated A1H8)

The acylhydrazone was synthesised according to GP1 by using 2-Chlorophenoxyacetic acid hydrazide (18.9 mg, 0.13 mmol) in methanol (1.8 mL) and para-(O-acetylatedglucose)-benzaldehyde (50.0 mg, 0.11 mmol). After purification, the acylhydrazone was obtained as a mixture of E and Z isomers (E:Z = 7:3) as a white solid (59 mg, 93%). 1H-NMR (500 MHz, MeOD) δ = 8.05 (s, 1H, E), 7.89 (s, 1H, Z), 7.69 (dd, J = 60.2, 8.8 Hz, 2H), 7.07 (d, J = 8.8 Hz, 2H), 5.45 – 5.34 (m, 2H), 5.22 – 5.06 (m, 2H), 4.37 – 4.25 (m, 1H), 4.21 – 4.08 (m, 2H), 2.74 (d, J = 7.4 Hz, 1H), 2.30 (d, J = 1.9 Hz, 2H), 2.08 – 1.96 (m, 1H, 12H), 1.85 (m, 2H, 1.74 – 1.64 (m, 2H), 1.63 – 1.54 (m, 2H), 1.34 – 1.18 (m, 2H); 13C-NMR (126 MHz, MeOD) δ = 172.3 (d, J = 12.7 Hz), 171.4 (d, J = 42.3 Hz), 171.1, 159.9, 148.6, 130.3, 129.5, 117.9, 117.8, 99.1, 74.1, 73.1, 72.7, 69.7, 63.1, 49.6, 49.5, 49.3, 49.2, 49.0, 48.8, 48.7, 48.5, 41.6, 39.4, 38.6, 38.0, 33.5, 33.4, 25.9, 20.6, 20.5; HRMS (ESI) calcd for C28H36N2O11 [M+H]+: 577.2392, found 577.2360.


The acylhydrazone was synthesised according to GP2 by using compound acetylated A1H8 (23.0 mg, 0.04 mmol) in methanol (4 mL) and sodium methoxide (0.32 mg, 0.006 mmol). After purification, the acylhydrazone was obtained as a mixture of E and Z isomers (E:Z = 7:3) as a white solid (13 mg, 82%). 1H-NMR (500 MHz, MeOD) δ = 8.05 (s, 1H, E), 7.88 (s, 1H, Z), 7.73 (d, J = 8.8 Hz, 1H), 7.61 (d, J = 8.8 Hz, 1H), 7.13 (dd, J = 8.8, 1.9 Hz, 2H), 4.98 – 4.95 (m, 1H), 3.94 – 3.86 (m, 1H), 3.71 (dd, J = 12.1, 5.6 Hz, 1H), 3.52 – 3.44 (m, 3H), 3.44 – 3.36 (m, 1H), 2.74 (d, J = 7.5 Hz, 1H), 2.34 – 2.24 (m, 2H), 1.92 – 1.78 (m, 2H), 1.74 – 1.64 (m, 2H), 1.64 – 1.49 (m, 2H), 1.34 – 1.18 (m, 2H); 13C-NMR (126 MHz, MeOD) δ = 177.5, 172.3, 160.9, 160.6, 149.0, 145.1, 130.2, 91
The acylhydrazone was synthesised according to GP1 by using 2-Chlorophenoxyacetic acid hydrazide (8.9 mg, 0.04 mmol) in methanol (0.5 mL) and para-(O-acetylatedmaltose)-benzaldehyde (25.0 mg, 0.03 mmol). After purification, the acylhydrazone was obtained as a mixture of E and Z isomers (E:Z = 3:2) as a white solid (30 mg, 48%). 1H NMR (500 MHz, MeOD) δ = 8.19 (s, 1H, E), 7.92 (s, 1H, Z), 7.71 (dd, J = 53.8, 8.8 Hz, 2H), 7.45 – 7.36 (m, 1H), 7.32 – 6.91 (m, 4H), 5.47 – 5.34 (m, 4H), 5.24 (s, 1H), 5.07 (dt, J = 13.6, 9.4 Hz, 2H), 4.88 (dd, J = 9.9, 3.3 Hz, 1H), 4.76 (s, 1H), 4.58 – 4.51 (m, 1H), 4.30 (dd, J = 10.8, 8.3 Hz, 1H), 4.27 – 4.11 (m, 1H), 4.09 (d, J = 6.3 Hz, 2H), 2.23 – 1.89 (m, 21H); 13C NMR (126 MHz, MeOD) δ = 172.3 (d, J = 6.0 Hz), 171.9, 171.8, 171.6, 171.3, 171.2, 167.1, 160.1, 159.7, 155.5, 154.9, 154.8, 150.6, 146.1, 131.5, 131.4, 131.4, 130.6, 130.3, 130.0, 129.8, 129.3 (d, J = 6.5 Hz), 128.9, 124.4, 124.1, 124.0, 123.9, 123.1, 117.9, 117.8, 116.1, 115.7, 115.3, 98.7 (d, J = 4.7 Hz), 97.3, 76.4, 74.94 (d, J = 7.6 Hz), 73.6, 73.3, 71.7, 70.7, 69.9, 69.7, 69.2, 68.8, 67.3, 64.3, 63.1, 21.2, 20.8 (d, J = 3.5 Hz), 20.6 (d, J = 6.1 Hz), 20.6 (d, J = 1.4 Hz); HRMS (ESI) calcd for C₄₁H₄₇ClN₂O₂₀ [M+H]+: 923.2484, found 923.2446.

The acylhydrazone was synthesised according to GP2 by using compound acetylated A2H12 (27.0 mg, 0.03 mmol) in methanol (3 mL) and sodium methoxide (0.36 mg, 0.007 mmol). After purification, the acylhydrazone was obtained as a mixture of E and Z isomers (E:Z = 3:2) as a white solid (17.2 mg, 93%). 1H NMR (500 MHz, MeOD) δ = 8.19 (s, 1H, E), 7.93 (s, 1H, Z), 7.70 (dd, J = 56.2, 8.8 Hz, 2H), 7.40 (ddd, J = 25.3, 7.9, 1.6 Hz, 1H), 7.32 - 7.20 (m, 1H), 7.17 - 6.92 (m, 4H), 5.26 (s, 1H), 5.21 (d, J = 3.7 Hz, 1H), 5.01 (dd, J = 7.7, 5.7 Hz, 1H), 4.76 (s, 1H), 3.98 - 3.42 (m, 12H); 13C NMR (126 MHz, MeOD) δ = 171.36, 167.10, 161.10, 160.73, 155.57, 155.00, 150.95, 146.43, 131.51, 131.34, 130.48, 129.71, 129.30 (d, J = 5.5 Hz), 128.90, 124.43, 124.13, 124.02, 123.11, 117.85 (d, J = 7.0 Hz), 116.09, 115.24, 102.94, 101.70 (d, J = 5.6 Hz), 80.83 (d, J = 4.9 Hz), 77.67 (d, J = 3.1 Hz), 76.82, 75.09, 74.85, 74.45, 74.19, 71.51, 69.18, 67.30, 62.78, 61.92; HRMS (ESI) calcd for C21H23ClN2O8 [M+H]+: 629.1744, found 629.1744.

Figure S1. DCL-2. Dynamic combinatorial libraries of aldehyde A-2 with hydrazide library 1: a) UV-chromatogram at 290 nm of the blank reaction at 3 h; b) UV-chromatogram at 290 nm of the protein-templated reaction at 6 h. Data obtained from single experiment.
Table S1. Amplification folds of the formed products in DCL-2; analysed via the relative surface areas of peaks in the UV-chromatograms of the protein-templated reaction (P) and blank reaction (B). Data obtained from single experiment.

<table>
<thead>
<tr>
<th>Compound</th>
<th>UV-retention time (min)</th>
<th>Amplification folds (%P/%B)</th>
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</thead>
<tbody>
<tr>
<td>A2H1</td>
<td>9.2</td>
<td>0.8</td>
</tr>
<tr>
<td>A2H2</td>
<td>7.8</td>
<td>0.9</td>
</tr>
<tr>
<td>A2H3</td>
<td>7.3</td>
<td>0.8</td>
</tr>
<tr>
<td>A2H4</td>
<td>8.7</td>
<td>0.8</td>
</tr>
<tr>
<td>A2H5</td>
<td>5.6</td>
<td>0.7</td>
</tr>
<tr>
<td>A2H6</td>
<td>3.8</td>
<td>1.5</td>
</tr>
<tr>
<td>A2H7</td>
<td>4.5</td>
<td>1.1</td>
</tr>
<tr>
<td>A2H8</td>
<td>7.3</td>
<td>0.8</td>
</tr>
<tr>
<td>A1H2</td>
<td>8.1</td>
<td>16.4</td>
</tr>
<tr>
<td>A1H8</td>
<td>7.4</td>
<td>4.4</td>
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</table>

Figure S2. DCL-3. Dynamic combinatorial libraries of aldehyde A-1 with hydrazide library 2: a) UV-chromatogram at 290 nm of the blank reaction at 3 h; b) UV-chromatogram at 290 nm of the protein-templated reaction at 6 h. Data obtained from single experiment.

Table S2. Amplification folds of the formed products in DCL-3; analysed via the relative surface areas of peaks in the UV-chromatograms of the protein-templated reaction (P) and blank reaction (B). Data obtained from single experiment.

<table>
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<th>Compound</th>
<th>UV-retention time (min)</th>
<th>Amplification fold (%P/%B)</th>
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<tbody>
<tr>
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<tr>
<td>A1H10</td>
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<tr>
<td>A1H11</td>
<td>6.5</td>
<td>1.2</td>
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<tr>
<td>A1H12</td>
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<td>2.1</td>
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<tr>
<td>A1H13</td>
<td>9.2</td>
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<td>A1H14</td>
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<td>A1H15</td>
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<td>A1H16</td>
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<td>A1H17</td>
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<tr>
<td>A1H18</td>
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**Figure S3.** DCL-4. Dynamic combinatorial libraries of aldehyde A-2 with hydrazide library 2:(a) UV-chromatogram of the blank reaction at 3 h; b) UV-chromatogram of the protein-templated reaction at 6 h. Data obtained from single experiment.

**Table S3.** Amplification folds of the formed products in DCL-4; analysed via the relative surface areas of peaks in the UV-chromatograms of the protein-templated reaction (P) and blank reaction (B). Data obtained from single experiment.

<table>
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<th>Compound</th>
<th>UV-retention time (min)</th>
<th>Amplification fold (%P/%B)</th>
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<tbody>
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<td>A2H10</td>
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<tr>
<td>A2H15</td>
<td>-</td>
<td>-</td>
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<tr>
<td>A2H16</td>
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<tr>
<td>A2H17</td>
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<td>0.1</td>
</tr>
<tr>
<td>A2H18</td>
<td>8.4</td>
<td>1.2</td>
</tr>
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</table>
Figure S4. Sensorgram of the immobilisation procedure for GTF180-ΔN on CMD500M sensor chip: (1) Four injections of cleaning solution, (2) activation solution, (3) GTF180-ΔN, and (4) quenching solution. The blue, red, and magenta curves represent the left (active) channel, right (reference) channel, and the difference, respectively.

Figure S5. Overlay of sensorgrams (black) of acarbose at concentrations of 0.8–500 µM running over an immobilised GTF-180. Global fitting of the association and dissociation curves (red). Data obtained from single experiment.
Figure S6. Overlay of sensorgrams (black) of A1H2 at concentrations of 7.6–2000 µM running over an immobilised GTF-180. Global fitting of the association and dissociation curves (red). Data obtained from single experiment.

Figure S7. Overlay of sensorgrams (black) of A1H8 at concentrations of 7.6–2000 µM running over an immobilised GTF-180. Global fitting of the association and dissociation curves (red). Data obtained from single experiment.

Figure S8. Overlay of sensorgrams (black) of A1H12 at concentrations of 7.6–500 µM running over an immobilised GTF-180. Global fitting of the association and dissociation curves (red). Data obtained from single experiment.
Figure S9. Overlay of sensorgrams (black) of A2H2 at concentrations of 7.6–1500 µM running over an immobilised GTF-180. Global fitting of the association and dissociation curves (red). Data obtained from single experiment.

Figure S10. Overlay of sensorgrams (black) of A2H12 at concentrations of 7.6–1000 µM running over an immobilised GTF-180. Global fitting of the association and dissociation curves (red). Data obtained from single experiment.
4.5 References


