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Generation of a dynamic combinatorial library using sialic acid aldolase and in situ screening against wheat germ agglutinin

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Abstract—This paper describes the generation of a dynamic combinatorial library of sialic acid analogues using sialic acid aldolase. Addition of wheat germ agglutinin to the equilibrating libraries results in selective amplification of one or more members.

1. Introduction

1.1. Dynamic combinatorial chemistry—the concept

Dynamic combinatorial chemistry (DCC) is a rapidly emerging field which offers a possible alternative to the approach of traditional combinatorial chemistry (CC).[1] Whereas CC involves the use of irreversible reactions to efficiently generate static libraries of related compounds, DCC relies upon the use of reversible reactions to generate dynamic mixtures. Selective binding of one member of the dynamic combinatorial library (DCL) to a molecular trap, such as the binding site of a protein, is expected to cause a shift in equilibrium to favour the formation of that member (Fig. 1). Comparison of the equilibrium product distribution of the 'perturbed' library with that of a control DCL will therefore indicate which members of the library are interacting with the trap.

The DCC approach offers in situ screening of combinatorial libraries, without the need for separation or deconvolution steps. The members of a DCL need not be produced with equal efficiency, since even members formed in trace amounts may still be amplified sufficiently for identification. In the extreme case, where amplification results in the collapse of the library to a single species, the method may be considered a tool for preparative synthesis.

Although attractive, the DCC approach presents significant experimental difficulties which have hindered its development. In particular, the choice of reversible reaction suitable for DCC is severely limited:

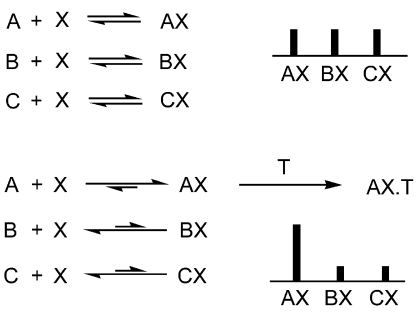

Figure 1. The DCC concept; reversible reactions performed with a limiting amount of **X** generate a mixture of compounds **AX**, **BX**, **CX**. Binding of **AX** to molecular trap **T** causes perturbation of the equilibria involving **A** and **X**, giving overall amplification of **AX** at the expense of the other library members.

- The reaction must accept a broad range of reacting components, in order to maximise the diversity of the library formed.
- It must proceed reversibly and efficiently under conditions which are appropriate to the recognition stage, and do not lead to a loss of binding activity in the trap. For drug discovery, in which the trap is usually envisaged as a protein or other biomolecule, this requirement translates to the use of near-physiological aqueous conditions.
- Once the system has reached equilibrium it must be possible to stop the reaction, converting the dynamic library to a static library for analysis.

Very few reversible reactions meet the above requirements, and consequently only a limited number of successful DCC experiments (involving small libraries) have been reported.

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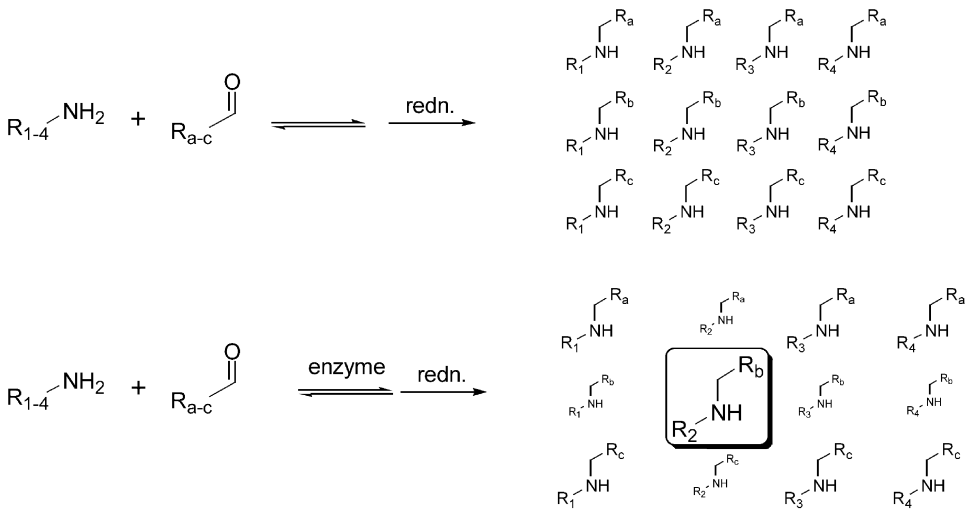


Scheme 1. Lehn's DCC experiment. When a (4×3) DCL of imines was prepared in the presence of an enzyme with binding affinity for one member, the product distribution was changed; the amine derived from the binding imine was amplified at the expense of other members derived from common components.

The discovery (or re-discovery) of reaction systems which are well-suited to DCC is therefore of prime importance to the future development of the method.

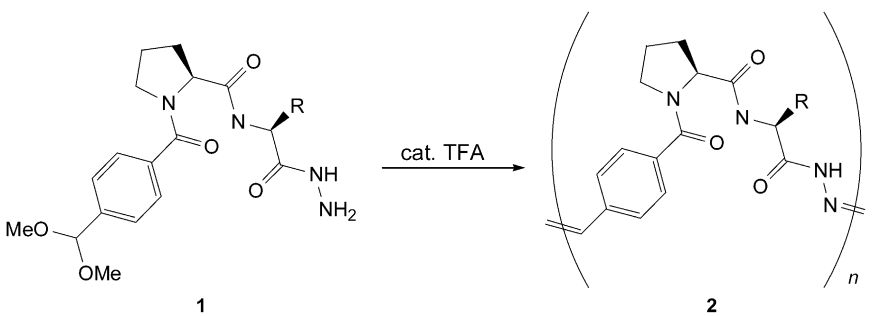
1.2. Examples of DCC

The DCC concept was clearly stated for the first time in 1996,[2] and shortly thereafter the first successful examples were reported. In 1997, Venton et al. reported the use of a protease (thermolysin) to generate a DCL, analysed by HPLC and sequencing, of at least fifteen short oligopeptides from an initial mixture of one dipeptide and one tripeptide.[3] A monoclonal antibody known to bind to one member of the DCL was used as the thermodynamic trap, and a modest amplification of the binding member (statistical analysis was necessary to verify that the variations recorded were significant) was observed. The use of enzyme catalysis enabled the generation of a DCL based on a transamidation reaction under physiological conditions; simple ultrafiltration removed the enzyme and reduced the DCL to a static library of robust compounds suitable for analysis.

A more impressive demonstration of DCC was provided by Lehn, also in 1997. Lehn employed imine formation as the reversible process, allowing the use of mild conditions in the generation of the DCL;[4] since the products were labile the library was 'frozen' by slow cyanoborohydride reduction, and the resulting mixture of amines analysed by HPLC. When a (4×3) library of twelve imines was prepared, both in the presence and in the absence of a stoichiometric amount of the enzyme carbonic anhydrase II (CA), clear qualitative differences were observed between the respective HPLC traces (Scheme 1). These differences indicated amplification of a strongly-binding imine (structurally similar to known inhibitors of CA) at the expense of the other library members sharing common amine or aldehyde components.

Several other examples of DCLs based on the formation and exchange of C=N bonds have been reported.[5,6] Systems based on transimination of hydrazones generated from amino acid-derived building blocks **1** have also been developed (Scheme 2),[7] in which TFA was employed both to deprotect the masked aldehyde function of the monomer, and to catalyse the formation of hydrazone-linked oligomers **2**. After generation of the DCL, the solution was adjusted to neutral pH, to give a static library of stable compounds for analysis. Again, the pseudopeptides produced in these experiments were screened against charged guest species. In one example, generation of the DCL in the presence of a stoichiometric amount of lithium iodide caused the collapse of the entire library to a single trimeric species, which was isolated in essentially quantitative yield.[7c] This efficient synthesis of a novel receptor for Li^+ from a diverse DCL provides a striking demonstration of the potential of (1→*n*) libraries.

Disulfide exchange also allows for the generation of DCLs



1 **2**

Scheme 2. In the presence of TFA, building blocks **1** were deprotected, and DCLs of hydrazone-linked oligomers **2** were generated.

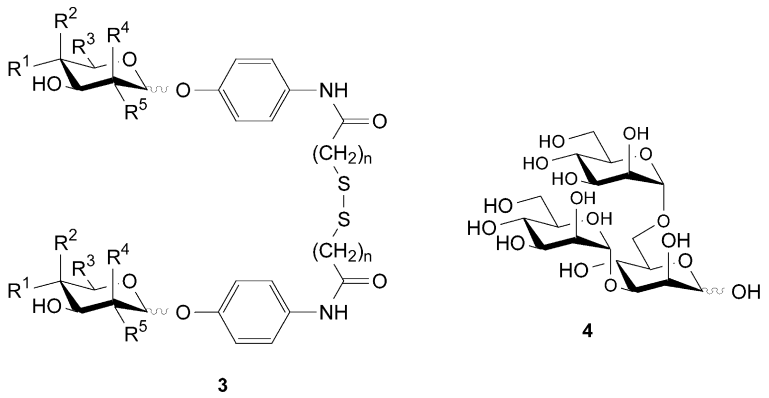


Figure 2. Disulfide exchange between 'bola-disacharides' **3** formed the basis for Lehn's DCL of carbohydrate mimetics. The library was screened against ConA, the preferred ligand for which is the trisaccharide **4**.

under mild conditions. Lehn investigated disulfide exchange as the basis for the generation of a DCL of 'bola-disaccharides' **3** (Fig. 2).[8] In the presence of dithiothreitol rapid disulfide exchange between a set of homodimers generated a DCL of up to twenty-one members, which was frozen by adjusting the solution to pH 4. This library was screened against Concanavalin A (ConA), a well-studied plant lectin whose preferred ligand is the mannose trisaccharide **4**.[9] Immobilised ConA was used to 'fish out' the binding species, which were eluted and analysed separately. The mannose homodimer was represented most strongly in the subset library of binding members; heterodimers containing one mannose residue were also bound. When the lectin was present throughout the scrambling process, some amplification of the mannose homodimer was observed.

1.3. Enzyme catalysis in DCC

As the above examples illustrate, the attractive features of DCC have been demonstrated in a number of successful experiments. Nevertheless, the development of DCC as a tool for drug discovery continues to be hindered by the requirement that, for this purpose, DCLs must be generated under physiological conditions.

We believe that enzyme catalysed reactions are ideally suited to the generation of DCLs for the following reasons:

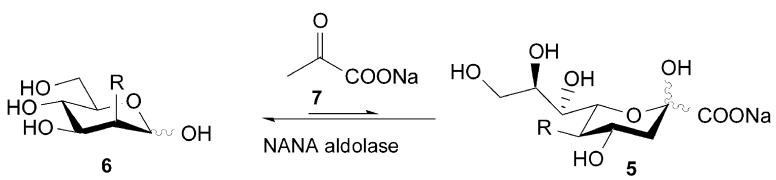
- They are characteristically reversible under aqueous, physiological conditions.
- The products of an enzyme catalysed reaction are usually stable compounds; simple removal or inactivation of the enzyme stops the reaction, reducing the dynamic mixture to a static library which may be analysed directly, without the need for a derivatisation step to freeze the product distribution.
- Many enzymes with broad specificity (required for library diversity) are already commercially available, and the application of modern techniques in directed evolution may be expected to increase their number.

Apart from the early work by Venton,[3] the use of enzyme catalysis has been largely overlooked in the approaches to DCC published to date. In the current study the generation of DCLs of carbohydrate-related compounds, and their screening against commercially available lectins, was investigated. The results of this work are presented in Section 2.[10]

2. Results and discussion

Initially, the enzyme-catalysed generation of a simple ($m\times n$) DCL, to be screened against a protein with known binding affinity, was undertaken. *N*-Acetylneuraminic acid aldolase (NANA aldolase, EC 4.1.3.3) was chosen as a suitable enzyme for this purpose. NANA aldolase catalyses the cleavage of *N*-acetyl neuraminic acid (sialic acid, **5a**) to *N*-acetyl mannosamine (ManNAc, **6a**) and pyruvate **7** (Scheme 3).[11a]

In the presence of excess pyruvate the equilibrium may be driven in the direction of aldol product formation. For this 'forward' reaction the enzyme requires sodium pyruvate as the nucleophilic equivalent, but will accept a range of reducing sugars as the electrophilic component. This synthetic activity has been exploited in the enzymatic



6a R = NHAc
6b R = OH

5a R = NHAc
5b R = OH

Scheme 3. NANA aldolase catalyses the cleavage of sialic acid **5a** to ManNAc **6a** and pyruvate **7**; in the presence of excess pyruvate, aldol products **5a** and **5b** may be generated from the respective substrates **6a** and **6b**.

preparation of a number of sialic acid analogues.[11] It was envisaged that a (1×2) DCL (the smallest possible) of aldol products **5a** and **5b** (ketodeoxynonulosonic acid, KDN) would be produced through the action of NANA aldolase on a mixture of the corresponding substrates **6a** and **6b** (D-mannose).

Wheat germ aglutinin (WGA), a well-studied and readily available plant lectin,[12,13] was chosen as the molecular trap. WGA is known to specifically bind sialic acid with modest (mM) affinity, where the diequatorial C-4 hydroxy and C-5 acetamido groups of sialic acid form the primary recognition motif. Thus, amplification of **5a** over **5b** was expected when the DCL was generated in the presence of WGA.

2.1. Library generation

Generation of the two-component DCL proved straightforward; a mixture containing equimolar amounts of the two substrates **6a** and **6b**, with 2 equiv. of sodium pyruvate, was incubated overnight in the presence of NANA aldolase. After thermal inactivation of the enzyme and filtration, the mixture was analysed by high-field ^{1}H NMR. A 1:1 mixture of the aldol products, resulting from 40% conversion from the respective substrates, was indicated, confirming that the enzyme accepted both substrates with similar affinities.

In order to generate the 'perturbed' library, the incubation was to be performed in the presence of a stoichiometric amount of WGA binding sites, based on the observed equilibrium concentration of **5a**. At neutral pH WGA exists as a dimer of four-domain monomers, total molecular weight 43 kDa, with four independent binding sites. The reported affinity constants for sialic acid binding to WGA are based on whole-cell agglutination experiments, which must be interpreted with caution, but these indicate 50% saturation of WGA in 5 mM sialic acid. The incubations were therefore scaled up to produce ca. 1 μmol of each aldol product (at ca. 1–5 mM) at equilibrium. At this scale ^{1}H NMR was no longer practical, and all analyses were performed by ion-exchange HPLC. An HPLC trace of a control incubation is shown below (Fig. 3); the aldol product peaks are clearly resolved and have roughly equal areas.

Longer incubations produced the same product distribution, indicating that the reaction had reached equilibrium overnight. However, the composition of the diluted aliquots did change on standing; in these solutions, a slow drift toward the substrates was observed, indicating that some residual enzyme activity remained after heat treatment. When the denaturation step was made more stringent (5 min at 95 °C), some decomposition of the aldol products was observed. The use of molecular weight cut-off filters to remove the enzyme by filtration was also investigated, but there were concerns that, in the presence of WGA, some lectin-bound sialic acid might be retained on the filter. The most direct solution to these problems was adopted; aliquots of the incubation mixtures were diluted and immediately injected for HPLC analysis. Although somewhat crude, this procedure did prove successful in 'freezing' the DCL without decomposition, and ensuring quantitative recovery of bound species for analysis. Using this method, and provided that aliquots of the incubation mixtures were much diluted before injection, consistent and linear response factors were observed in the peaks assigned to the aldol products.

By reference to stock solutions made up from commercial samples, a ca. 15% conversion from substrate was estimated for each product. A solution was made up with the estimated equilibrium composition; this solution produced an HPLC trace very similar to those observed for 'real' incubation mixtures, and on incubation with NANA aldolase the composition changed only in that it moved closer to the observed equilibrium mixture.

That the enzyme does indeed catalyse both aldol formation and cleavage on the timescale employed was demonstrated by re-equilibrating a mixture of **5a** and **6b** in the presence of NANA aldolase; after incubation overnight the mixture contained, in addition to the initial components, **5b** and **7** which could only arise through a retro-aldol cleavage of **5a** followed by aldol formation of **6b**.

Having confirmed that NANA aldolase was able to accept both **6a** and **6b** as substrates with similar affinity, that roughly equimolar mixtures of the corresponding aldol products could be readily generated and analysed, and that the mixtures produced were truly dynamic, incubations in the presence of WGA were attempted. Since the incubations contained no internal standards, and hence could not be normalised to account for variations in aliquot volumes, equilibrium product distributions were expressed as fractions of the total area of the peaks assigned to aldol products (% Total Aldol Product Area, %TAPA). Relative amplification was then determined by comparing %TAPAs determined in the presence and absence of WGA.

Overnight incubations performed in the presence of WGA

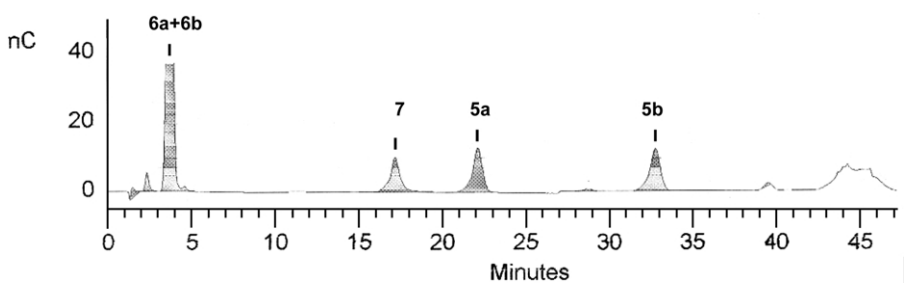


Figure 3. HPLC trace for the (1×2) control DCL. Substrates **6a** and **6b** co-elute, but the aldol products **5a** and **5b** are well-resolved and give reproducible relative areas. This product distribution was present after 24 h incubation, and remained unchanged for at least the following 6 days.

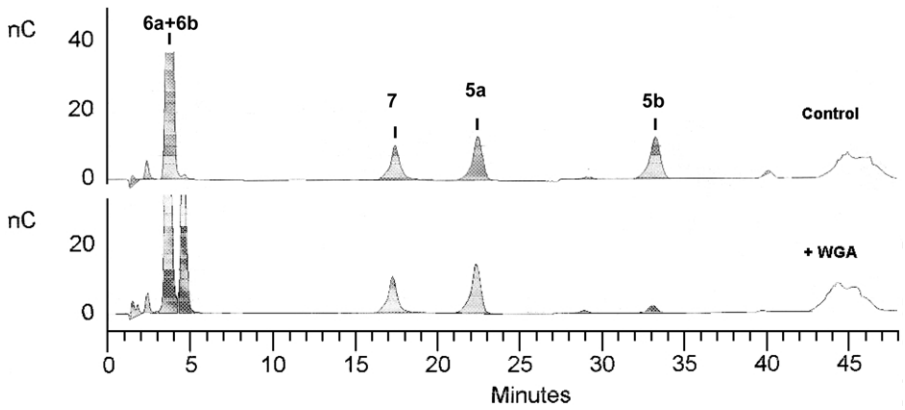


Figure 4. Comparison of control and 'perturbed' (1×2) libraries after 7 days incubation. In the presence of WGA, the production of nonbinding member **5b** has been almost completely suppressed in favour of WGA-binding member **5a**.

showed reproducible but small (ca. 10%) amplification of **5a** over **5b**, but the extent of amplification increased with increasing reaction time (Fig. 4). The system appears initially to approach a similar distribution to that observed in control incubations, but thereafter slowly to deviate toward a new equilibrium position. After 7 days incubation the peak assigned to **5a** had risen from 49 to 85% TAPA, a 73% amplification, while the peak assigned to **5b** had dropped from 51 to 15% TAPA, a 71% suppression.

In order to prove that the observed changes were not caused by a selective aldolase activity associated with the lectin preparation, a mixture approximating the equilibrium distribution of the control incubation was prepared from commercial samples, and incubated in the presence of WGA (but not NANA aldolase). No change from the initial distribution was observed, demonstrating that WGA affects the DCL by acting as a thermodynamic trap, and not as a selective catalyst for KDN degradation.

Microcalorimetry experiments were undertaken to provide independent confirmation of the observed WGA binding affinities (Fig. 5); although **5a** binding to WGA is weak, a binding affinity of 172 M^{-1}, corresponding to 50% binding at 5.8 mmol L^{-1}, was determined, while no detectable binding affinity was observed for **5b**.

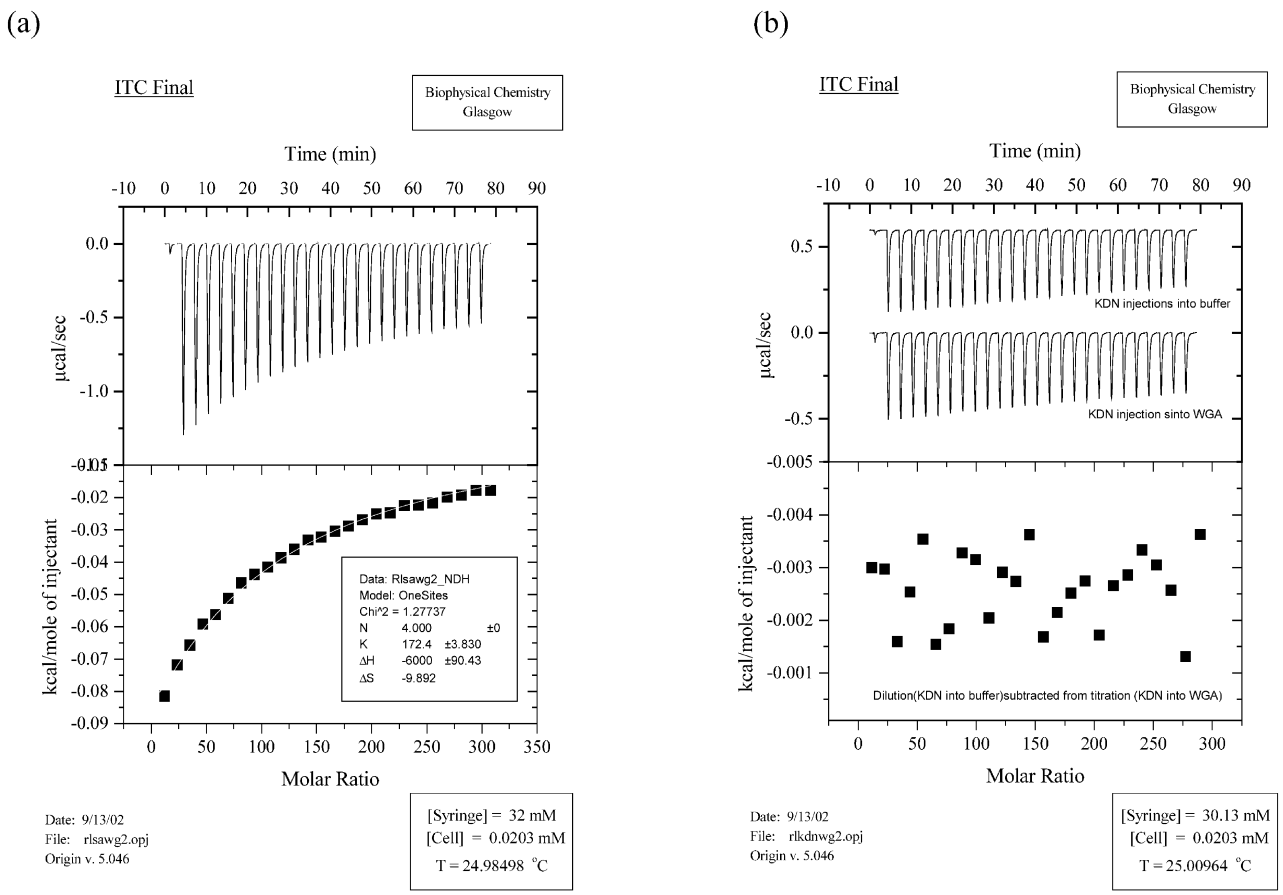
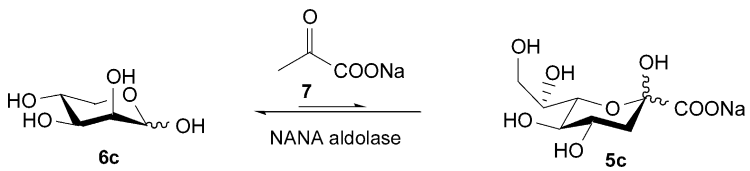


Figure 5. Isothermal titration curves for the binding of WGA to (a) **5a** and (b) **5b**.



Scheme 4. KDO **5c**, the third library member, is produced from D-lyxose **6c**.

2.2. Three components

D-Lyxose **6c** was chosen as a third substrate for the generation of a (1×3) DCL (Scheme 4). Despite having the same C2–C4 configuration as **6b**, **6c** has been shown to be a relatively poor substrate for NANA aldolase.[11a] The corresponding ketodeoxyoctulosonic acid **5c** (KDO) is the eight carbon analogue of **5b**, and was thus not expected to bind to WGA.

Incubations performed with the three substrates **6a**–**c** gave 1:1:2 mixtures (in terms of peak area) of the respective aldol products **5a**–**c**. **5c** is not available commercially, so a quantitative analysis of the mixture was not attempted, but it was assumed that the response factor for this compound was unlikely to differ greatly from those of **5a** and **5b**. Conversion from **6c** to **5c** was thus estimated to be in the range 15–30%. When the incubation was performed in the presence of WGA, a similar pattern of amplification of **5a** (53%) with suppression of **5b** (72%) was observed. The peak assigned to KDO was not suppressed, but showed a small relative amplification (10%).

This observation that **5b** and **5c** were not suppressed to the same extent might be due to WGA having a weak binding affinity for **5c**, such that the latter species may also experience some measure of relative amplification during incubation in the presence of the lectin. In effect, the extent of relative amplification observed in the above experiment might suggest a ranking order of the three components based on the relative affinities of WGA for each of them. In this case, the two-component DCC experiment involving only **5b** and **5c** should demonstrate some degree of amplification of **5c**, while that involving only **5a** and **5c** should show amplification of **5a** over **5c**. When these two-component experiments were performed, the results were not conclusive; the predicted amplifications were observed, but with much smaller magnitude than those involving the **5a**–**5b** pair. If **5c** has appreciable binding affinity for WGA there should be significant amplification of **5c** over **5b** (which has no binding affinity), whereas if **5c** has only very small binding affinity for WGA then the amplification of **5a** acid over **5c** should be significant (Table 1).

An alternative explanation for the observed results is that the equilibrium amount of **5c** is not sensitive to **5a** binding to WGA. When **5a** binds to WGA the resulting changes in product distribution are mediated by NANA aldolase. If the rate of **5c**–**6c** interchange is slow compared to the rate of **5b**–**6b** interchange, then the latter couple might be expected to bear the brunt of re-equilibration.

Table 1. Summary of the results from two-component libraries after 7 days incubation

Components	Control %TAPA		Perturbed %TAPA		% Amplification	
5a+**5b**	49	51	85	15	+73	−71
5a+**5c**	36	64	40	60	+11	−6
5b+**5c**	19	81	15	85	−21	+5

Timecourses for the three-component DCLs were measured in order to investigate the relative rates of formation of the library members. The graph below (Fig. 6) shows the development of the control library in terms of absolute peak areas for each aldol product (a) and in terms of %TAPA (b). The data clearly indicate that **5a** is formed most rapidly, while **5c** is generated relatively slowly. After 24 h incubation the system is at equilibrium, and the distribution is then stable for at least the next 6 days.

In the presence of WGA, the formation of the DCL proceeds in the same manner, with slow generation of **5c** (Fig. 7). The product distribution does not stabilise after 24 h, but drifts in the direction of increased **5a** and decreased **5b**, due to WGA binding of the former. A plot of relative amplification over time for each component (Fig. 7c), shows these changes more clearly. The slight (10%) amplification observed for **5c** may not be significant considering the variations in %TAPA observed for this component over the first two hours incubation. Similarly, a small kinetic effect (amplification of **5a** in the first two hours of incubation) is recorded, but may not be significant.

2.3. Four components

In order to further investigate the behaviour of poor enzyme substrates in the DCL, a (1×4) library was investigated. The fourth substrate chosen was D-galactose **6d**, which is known to be a poor substrate for NANA aldolase.[11a] Further, the nonulosonic acid produced (Scheme 5, designated somewhat glibly as 7-*epi*-KDN **5d**) differs from **5b** only in the configuration at C-7, and thus is not expected to possess any greater binding affinity for WGA than does the latter. Thus, if in general poor enzyme substrates produce false positives in the presence of better substrates, then both **5c** and **5d** should show no suppression in the four-component DCL. If the observed behaviour of **5c** is due to a real binding affinity, the four-component DCL should show suppression of both **5b** and **5d**.

The HPLC traces for the control and perturbed four-component DCLs after 7 days incubation are shown below (Fig. 8). Although the peaks due to **5b** and **5d** were not fully resolved with the elution protocol used, and hence accurate peak areas were not available for these components individually, it can be seen that in the perturbed library **5b** has been greatly suppressed, while **5d** has remained relatively unchanged. This result is supported by the results from the two-component DCL formed from **5a** and **5d**

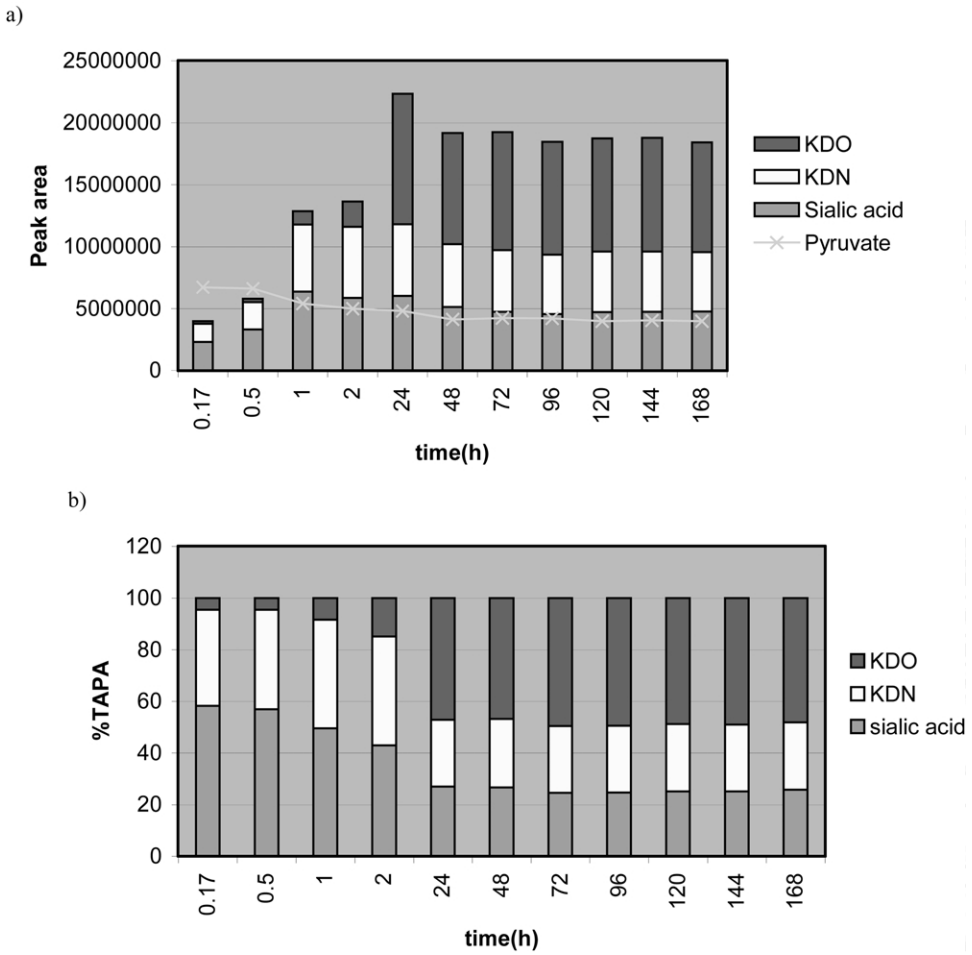


Figure 6. Timecourse for three-component control DCL, measured by (a) absolute peak areas and (b) %TAPA.

(Table 2), which again shows at best only slight amplification of **5a** over **5d**.

The experiments described above constitute the first clear demonstration of the application of enzyme catalysis to DCC. The use of an aldolase allowed for the straightforward preparation of a DCL through stereoselective carbon–carbon bond formation; the members of the library produced were complex molecules of biological interest which are difficult to prepare chemically. It is significant for the development of DCC in general that even the very weak binding affinity of WGA for sialic acid was still sufficient to produce a marked change in the equilibrium product distribution of the DCL; traps with greater affinity should allow for successful detection at lower concentrations.

The major limitation of enzyme catalysis in DCC was also highlighted in the example studied; the preparation of libraries of useful size requires an enzyme with broad specificity. In this example, only two components of the four-component DCL were good substrates for NANA aldolase, and only these two responded to the presence of WGA as a thermodynamic trap. It is important to note that the data still clearly indicates sialic acid **5a** as the best binder to WGA, but it would be of interest to repeat the aldolase/WGA experiments with a different aldolase for which **5a** was the poorer substrate. In such a system, sialic acid binding to WGA would still occur, and sialic acid amplification (and the subsequent suppression of other components) would be expected to follow, though at a lower rate. Provided that significant amplification may still be observed when the binding member is a poor substrate, the absence of suppression among the poorer response from some with binding affinity may still produce significant amplification In general, the problem of high enzyme specificity might be addressed by performing independent DCC experiments with enzymes of different specificity. Ultimately it is anticipated that the application of modern techniques in directed evolution will increase the number of available enzymes with truly broad specificity; there is evidence that increased activity through directed evolution is often associated with a loss of specificity.[14]

3. Experimental

HPLC analyses were performed on a Dionex DX-500 Chromatography system consisting of a LC30 Chromatography Module, GP40 Gradient Pump, ED40 Electrochemical Detector (gold electrode and amperometry cell), AS3500 Autosampler (stainless steel needle), controlled from a workstation running PeakNet v4.30. A CarboPac PA-100 column with guard column was used, flow rate 1 mL min^{-1}. Solvents used were 100 mM sodium hydroxide, 100 mM sodium hydroxide/1 M sodium acetate, water.

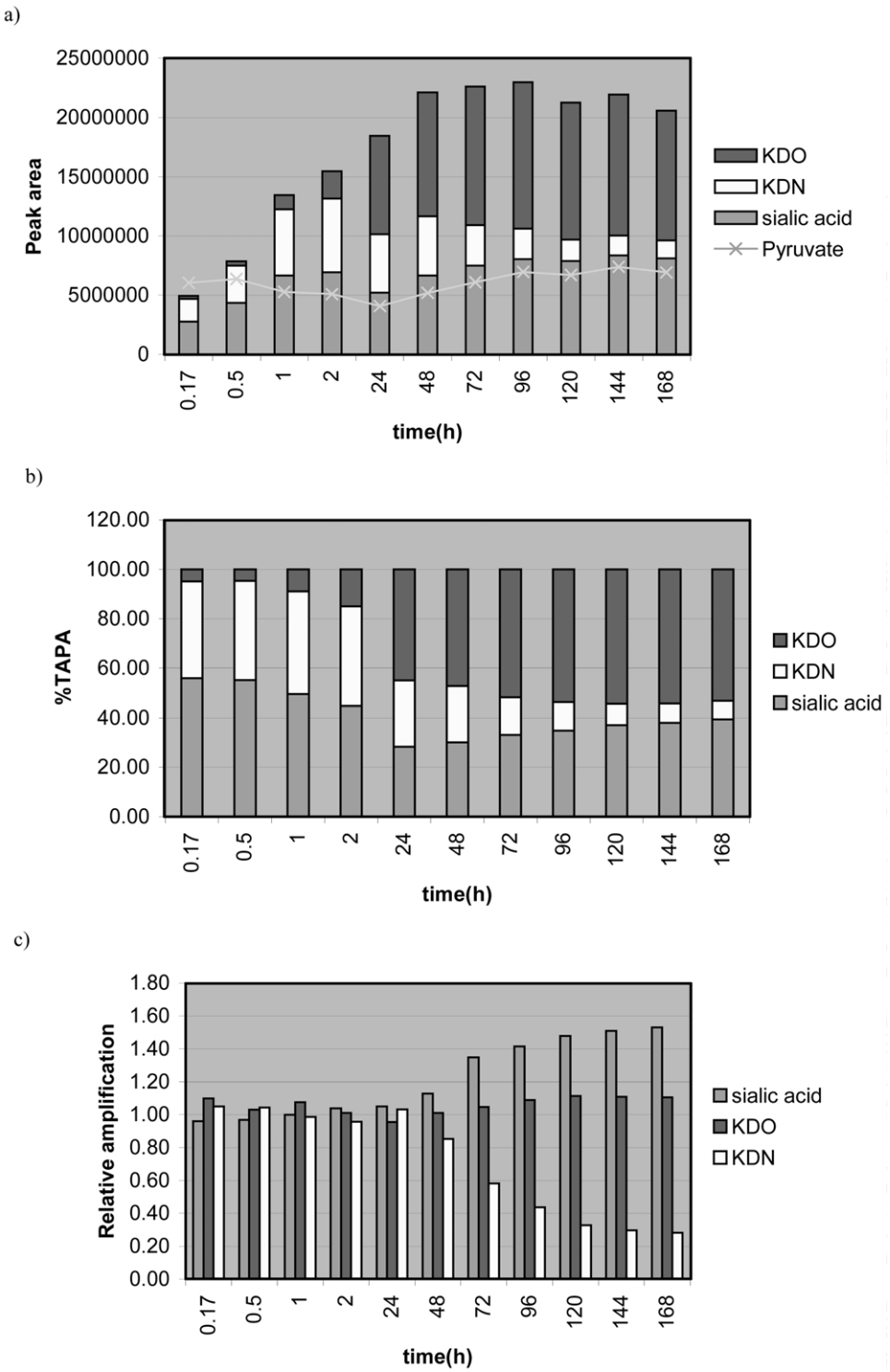
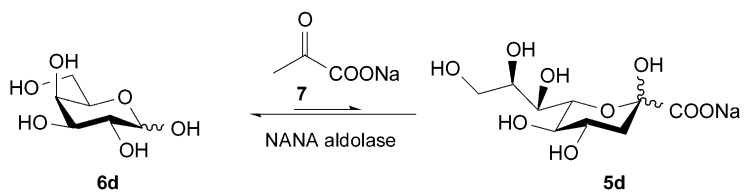


Figure 7. Timecourse for the three-component peturbed DCL, measured in (a) absolute peak areas, (b) %TAPA, and (c) relative amplifications over the control DCL.



Scheme 5. 7-*epi*-KDN **5d**, the fourth library member, is produced from D-galactose **6d**.

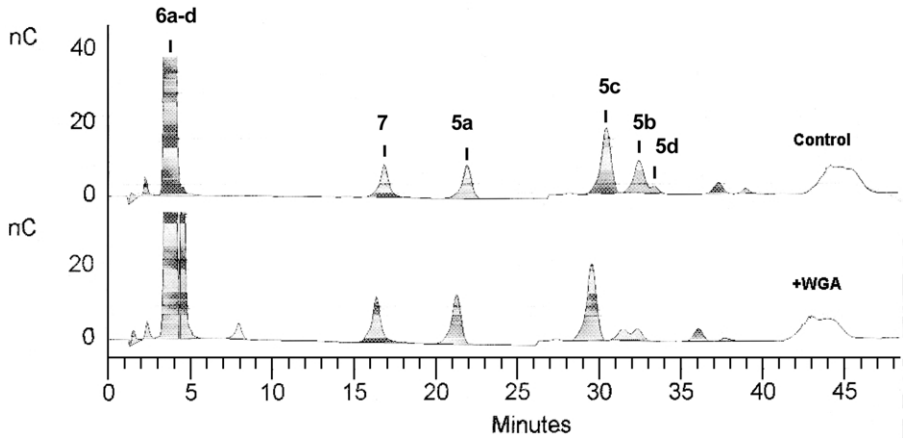


Figure 8. Comparison of perturbed and control (1×4) DCLs after 7 days incubation. **5a** has been amplified, but only **5b** shows significant suppression.

Table 2. Summary of results from libraries containing **5d**

Components	Control %TAPA			Perturbed %TAPA			% Amplification		
5a+**5c**+(**5b** and **5d**)	23	48	29	32	53	15	+39	+10	−48
5a+**5d**	75	25		76		24	+1		−4

N-Acetyl neuraminic acid aldolase (EC 4.1.3.3, cat. 153493, 22.2 U mg^{-1}) and wheat germ agglutinin (cat. 152266, lyophilised powder) were purchased from ICN Biomedicals. Substrate sugars and other chemicals were purchased from Sigma.

3.1. Large-scale preparation of two-component DCL

A stock solution was made up containing 29 mg **6b** (160 μmol), 38 mg **6a** (160 μmol), 35 mg **7** (320 μmol) and 1 mg sodium azide in 50 mM phosphate buffer, pH 7.6. To 50 μL of this stock was added 50 μmol NANA aldolase stock solution (2 U mL^{-1} in 50 mM phosphate buffer, pH 7.6), and the mixture incubated at 37 °C overnight. The solution was heated to 80 °C for 2 min, cooled, and diluted to give 600 μL of a 10% D_2O in water solution for NMR analysis.

δ_H (600 MHz) inter alia 8.02 (1H, br. d J=9.3 Hz, **5a** NH), 7.88 (1H, br d J=10.3 Hz, **6a** NH), 5.16 (1H, s, **6b** 1-H_α), 5.11 (1H, s, **6a** 1-H_α), 5.01 (1H, s, **6a** 1-H_β), 4.88 (1H, s, **6b** 1-H_β), 2.20 (1H, dd J=13.2, 4.9 Hz, **5a** 2-H_{ax}), 2.15 (1H, dd J=12.7, 4.9 Hz, **5b** 2-H_{ax}), 1.83 (1H, t J=12.2 Hz, **5a** 2-H_{eq}), 1.78 (1H, t J=12.2 Hz, **5b** 2-H_{eq}) ppm.

3.2. General procedure for the preparation of small-scale DCLs

Phosphate-buffered saline (PBS) contained 150 mM sodium chloride, 10 mM sodium phosphate, and 0.02% w/v sodium azide, adjusted to pH 7.5. NANA aldolase stock was 20 U mL^{-1} in PBS. WGA stock was 50 mg mL^{-1} in PBS. Substrate stock solutions contained 50 μmol of each substrate sugar (12 mg **6a**, 9 mg **6b**, 7.5 mg **6c**, and/or 9 mg **6d** as required) and 100 μmol **7** in 1 mL PBS. Control incubations contained 10 μL substrate stock solution, 20 μL PBS, and 10 μL aldolase stock. For the generation of perturbed DCLs 20 μL WGA stock was substituted for PBS. These mixtures were centrifuged for 1 min at 5000 rpm, 20 μL paraffin oil was added, the mixtures were centrifuged again, and incubated at 37 °C. At intervals 5 μL aliquots were withdrawn, added to 495 μL water, and the diluted solutions immediately analysed by HPLC.

3.3. Microcalorimetry

Isothermal Titration Calorimetry (ITC) was performed by staff at the BBSRC/EPSRC Biological Microcalorimetry Facility at the University of Glasgow, according to standard procedures. WGA (Sigma) was used without further purification and was dissolved in PBS (0.15 M NaCl, 0.1 M P_i, 0.02% NaN_3, pH 7.5) and degassed before use. Ligands **5a** and **5b** were dissolved in the same buffer. Protein concentrations in the ITC cell were determined from UV absorbance measurements at 280 nm using an absorption coefficient of 1.5 $mg^{-1}cm^2$.[15] Control experiments were performed under identical conditions by injection of ligand into buffer alone (to correct for heats of ligand dilution).

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