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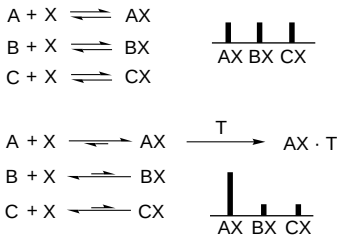
Enzymatic Generation and In Situ Screening of a Dynamic Combinatorial Library of Sialic Acid Analogues**

Roger J. Lins, Sabine L. Flitsch,* Nicholas J. Turner,* Ed Irving, and Stuart A. Brown

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. The binding of one member of the dynamic library to a molecular trap (such as the binding site of a protein) is expected to perturb the library in favor of the formation of that member (Scheme 1). Comparison of the "perturbed" library with that generated in the absence of the trap should indicate which members of the library are interacting with the trap, which effectively offers in situ screening of the combinatorial library.

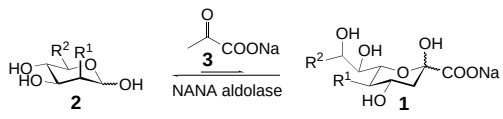
The DCC concept has already been proven through the elegant experiments by several research groups, including those of Lehn and Sanders.[1,2] However, significant experimental challenges remain before the method may be considered a practical complement to traditional CC. In particular, the in situ screening, which is such an attractive feature of DCC, demands the use of conditions amenable both to the library-formation and -trapping stages. If the trap is envisaged as a protein or other biomolecule, the system is limited to aqueous and near-physiological conditions, with which few covalent bond forming reactions are compatible. Thus, for example, Lehn and co-workers have employed both imine and disulfide exchange reactions in DCC experiments involving biomolecular traps,[2a] but to our knowledge no DCC experiment involving the formation of carbon–carbon bonds under physiological conditions has been performed.[3]



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

We believe that enzyme-catalyzed reactions, which are characteristically reversible under physiological conditions, are ideally suited to the generation of dynamic combinatorial libraries. 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. The products of an enzyme-catalyzed reaction are usually stable compounds; simple removal or inactivation of the enzyme stops the reaction, thus reducing the dynamic mixture to a static library which may be analyzed directly, without the need for a derivatization step to freeze the product distribution. Herein we present the first demonstration of DCC using enzyme catalysis for the generation of a dynamic library.

In considering the application of enzyme catalysis to DCC, we were encouraged by the thermodynamic resolution of a dynamic mixture of aldol products by Whitesides and co-workers through the use of a broad-specificity aldolase to effect reversible formation of carbon–carbon bonds under mild conditions.[4] For the current investigation we chose a related enzyme, *N*-acetylneuraminic acid aldolase (NANA aldolase, EC 4.1.3.3), which catalyzes the cleavage of *N*-acetylneuraminic acid (sialic acid, **1a**) to *N*-acetylmannosamine (ManNAc, **2a**), and sodium pyruvate **3** (Scheme 2). In



2a R^1 = NHAc, R^2 = CH_2OH **1a** R^1 = NHAc, R^2 = CH_2OH
2b R^1 = OH, R^2 = CH_2OH **1b** R^1 = OH, R^2 = CH_2OH
2c R^1 = OH, R^2 = H **1c** R^1 = OH, R^2 = H

Scheme 2. NANA aldolase catalyzes the cleavage of sialic acid **1a** to ManNAc **2a** and sodium pyruvate **3**; in the presence of excess sodium pyruvate, aldol products **1a–c** are generated from the respective substrates **2a–c**.

[*] Prof. S. L. Flitsch, Prof. N. J. Turner, Dr. R. J. Lins
Department of Chemistry
Edinburgh Protein Interaction Centre
The University of Edinburgh, King's Buildings
West Mains Road, Edinburgh EH9 3JJ (UK)
Fax: (+44) 131-650-4717 or (+44) 131-650-4743
E-mail: s.flitsch@ed.ac.uk
n.turner@ed.ac.uk
Dr. E. Irving, Dr. S. A. Brown
Ultrafine
Synergy House, Guildhall Close
Manchester Science Park, Manchester M15 6SY (UK)

[**] We are grateful to Ultrafine for a postdoctoral fellowship (R.J.L.) and to the Wellcome Trust for financial support.

the presence of excess sodium pyruvate the equilibrium may be driven toward the formation of an aldol product, in which the enzyme will accept a range of reducing sugars as the electrophilic component. This stereoselective aldol reaction has been successfully employed in the efficient enzymatic syntheses of sialic acid and several related compounds.[5] We envisaged the generation of a small dynamic library of aldol products **1a**, **1b** (ketodeoxynonulosonic acid, KDN), and **1c** (ketodeoxyoctulosonic acid, KDO) through the action of NANA aldolase on a mixture of the corresponding substrates **2a**, **2b** (D-mannose), and **2c** (D-lyxose).

Wheat germ agglutinin (WGA), a well-studied and readily available plant lectin,[6] was chosen as the molecular trap. WGA is known to specifically bind sialic acid with modest (mM) affinity, with the diequatorial C-4 hydroxy and C-5 acetamido groups of sialic acid forming the primary recognition motif. We thus expected amplification of sialic acid to occur when the dynamic library containing sialic acid was generated in the presence of WGA.

Generation of the dynamic library proved straightforward; a mixture containing equimolar amounts of the three substrates **2a–c** and two equivalents of sodium pyruvate was incubated in the presence of NANA aldolase. Aliquots of the incubation mixture were withdrawn at intervals and analyzed by ion-exchange HPLC (Figure 1a, bottom). The three

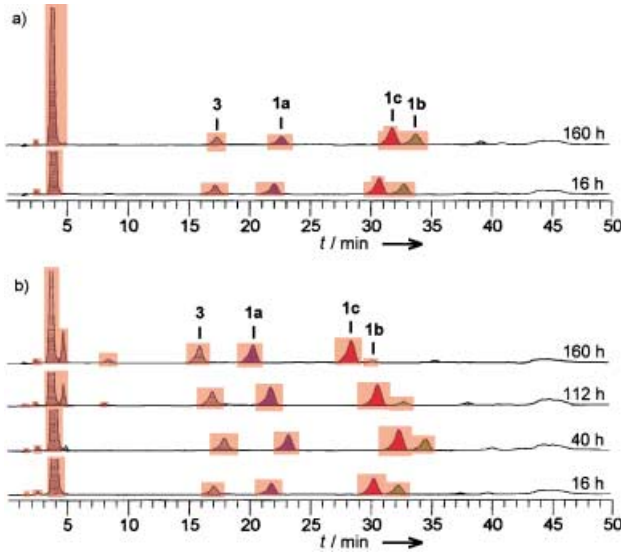


Figure 1. HPLC traces showing the change in concentration of sialic acid (**1a**, blue), KDN (**1b**, green), and KDO (**1c**, red) over time, for a) control incubation, b) incubation with WGA.

substrate sugars co-eluted with low retention times and did not give reproducible peak areas, presumably because of overlap with other variable-intensity peaks with low R_f values. The peak assigned to sodium pyruvate (**3**) was also variable in intensity; this peak was presumed to arise from the homoaldol product of sodium pyruvate, which was not expected to be stable under the elution conditions. The three aldol products gave reproducible peak areas, although absolute retention times were somewhat variable. As expected, the use of a small excess of sodium pyruvate resulted in low (15–40%) conversion to the aldol products,[7] and gave 2–5 mM concentrations of each. It was evident that the aldol reaction had reached equilibrium after 16 hours incubation, since the product distribution changed very little after this time.

Evidence that the enzyme does indeed catalyze both aldol formation and cleavage on the timescale employed was demonstrated by re-equilibrating a mixture of sialic acid and D-mannose in the presence of the NANA aldolase;[8] after incubation overnight the mixture contained, in addition to the initial components, KDN and sodium pyruvate which could only arise through a retroaldol cleavage of sialic acid followed by aldol formation of KDN.

When the incubations were performed in the presence of WGA (sufficient lectin was added to provide at least one equivalent of binding sites, based on the amount of sialic acid formed in the control experiments) the product distribution was observed to change dramatically over time (Figure 1b). The system appears initially to approach a similar distribution to that observed in the control incubation, but thereafter the relative concentration of sialic acid is seen to increase, with a corresponding drop in the relative concentration of KDN. A plot of the percentage amplification in the relative concentration (as estimated from relative peak area) of each aldol product over controls is shown in Figure 2; the peak assigned

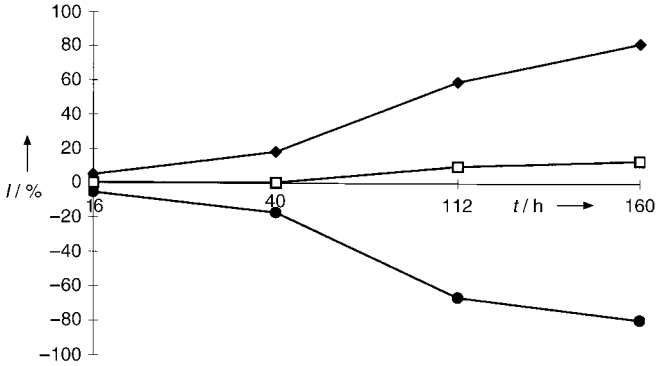


Figure 2. Time course for a three-component system showing the relative amplification of sialic acid (◆), KDO (□), and KDN (●).

to sialic acid contributed approximately 40% of the total aldol product peak area after 160 hours in the presence of WGA, but only 22% of the total aldol product peak area in the control mixture at the same time point. This difference corresponds to a relative amplification of about 80%. Conversely, the contribution of KDN to the total aldol product peak area after 160 hours was 31% in the control incubation, but only 6% in the presence of WGA; this difference coresponds to a relative suppression of 80%.

The observation that KDN and KDO were not suppressed to the same extent might be a consequence of WGA having a weak binding affinity for KDO, 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 suggests 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 KDN and KDO should demonstrate some degree of ampli-

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fication of KDO, while that involving only sialic acid and KDN should show amplification of sialic acid over KDN, with the extent of amplification being greater in the latter experiment. The data from these two-component experiments are shown in Figure 3, bottom; they show dramatic relative

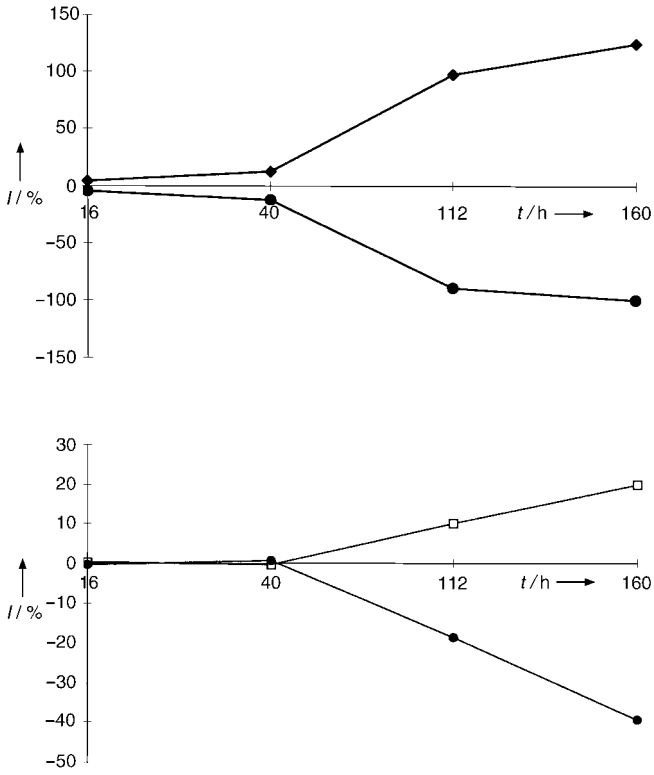

Figure 3. Time courses for two-component DCC experiments showing: top: relative amplification of sialic acid (◆) over KDN (●), bottom: relative amplification of KDO (□) over KDN (●).

amplification of sialic acid over KDN, but more modest relative amplification of KDO over KDN when sialic acid is not present. Since all three experiments showed supression of KDN, it was necessary to exclude the possibility that this component was being affected by a selective aldolase activity associated with the lectin preparation. Thus, a mixture of sodium pyruvate, mannose, manNAc, sialic acid, and KDN, in proportions approximating their equilibrium distribution in the two component control experiment, was prepared from commercial samples and incubated in the presence of WGA (but not NANA aldolase). No change from the initial distribution was observed, which demonstrates that the changes in product distribution observed in the DCC experiments were caused by WGA acting as a thermodynamic trap, and not as a selective catalyst for KDN degradation.

In conclusion, we have successfully demonstrated, for the first time, the generation and in situ screening of a dynamic mixture of biologically significant compounds, where enzyme catalysis has been used to effect reversible formation of carbon–carbon bonds under physiological conditions. We believe this work can be extended to larger libraries through the use of broad-specificity enzymes, which represent a powerful (and hitherto overlooked) tool for the development of dynamic combinatorial chemistry; we are currently looking to increase the size of the library, to adapt the system for the identification of novel ligands such as neuraminidase inhibitors, and to develop multienzyme systems for the generation of dynamic libraries with increased diversity.

Experimental Section

N-acetylneuramic acid aldolase (EC 4.1.3.3, 22.2 U mg^{-1}) and wheat germ agglutinin (lyophilized powder) were purchased from ICN Biomedicals.

The following stock solutions were prepared in 0.05 M potassium phosphate (KPi) buffer, pH 7.5: aldolase stock (20 U mL^{-1}); wheat germ agglutinin stock (50 mg mL^{-1}); sugar stock solutions containing sodium azide (0.5 g L^{-1}); 0.1 M sodium pyruvate; and 0.05 M each of ManNAc, D-mannose, and D-lyxose (solution A), ManNAc and D-mannose (solution B), or D-mannose and D-lyxose (solution C).

Incubation mixtures A–C contained enzyme stock (10 μL), WGA stock (20 μL), and sugar stock A, B, or C (10 μL), respectively. Controls contained enzyme stock (10 μL), 0.05 M KPi buffer (20 μL), and sugar stock A, B, or C (10 μL). After centrifugation (1 min@3000 rpm), the solutions were incubated at 37 °C without stirring. Aliquots (5 μL) were withdrawn at intervals, diluted to 500 μL with milli-Q water, and immediately analyzed by HPLC.

Received: April 4, 2002 [Z19042]

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