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Direct Composition Analysis of a Dynamic Library of Imines in an Aqueous Medium

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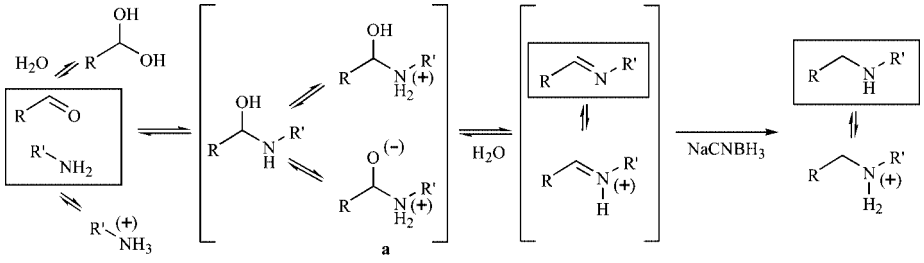
Dynamic Combinatorial Chemistry: Direct detection of imines in water and their adaptive re-equilibration in the presence of a target are possible by simple HPLC analysis. The equilibrating mixture is fixed by a fast drop in the pH during the analytical step.

Imines have been shown to be particularly well suited for dynamic combinatorial chemistry[1] in an aqueous medium, and their libraries were among the first dynamic combinatorial libraries (DCL) used to explore biological targets.[2,3] Simple mixing of aldehydes[2a,2b] or ketones[2c] with amines generates an equilibrating library of imines that form and hydrolyze rapidly. Addition of a target to such a dynamic mixture may shift the equilibrium towards the formation of any component interacting with this target. These mixtures were considered to be too unstable for a direct composition analysis and, in order to allow HPLC study, the redistribution of imines had to be typically "fixed" by a reductive step which can lead to a stable library of amines[2] (Scheme 1).

This procedure, necessary to evaluate the composition of such mixtures, implies that the amplified imines stabilized by the target are freely accessible to the reducing species that fix the composition. However, the rate of this reduction step for a given imine constituent may differ in solution and in the enzyme's binding site thereby possibly providing a distribution of the observable amine products that is different from the distribution of the imine components. Moreover, the reducing agent, present at the beginning of the incubation experiments that last from several hours to several days,[2] may alter the protein structure. Therefore, a strategy allowing direct chromatographic analysis of imine distributions would be highly desirable. We now report a simple alternative, which relies on the "freezing" of the equilibrating mixture *at the end* of the incubation experiments by a fast drop in the pH during the analytical step.

The formation and hydrolysis of imines in an aqueous medium follow a complex series of equilibria, with some of the most relevant species presented in Scheme 1. This has been extensively studied in the past and it was established that the rate-limiting step of imine hydrolysis is highly pH dependent.[4]

At low pH, typically below 4, a continuous rate decrease is observed with the lowering of the pH. This is explained



Scheme 1. Some equilibria involved in imine formation/hydrolysis, followed by reductive "fixation".

by the fact that in this pH range, the rate-determining step is the decomposition of the intermediate hemiaminal, which needs to be in zwitterionic form **a** "*in order to obtain sufficient driving force to expel the free amine*".[4a] With a decrease in pH, this form becomes less represented, which leads to a lower overall hydrolysis rate. This effect is more

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pronounced in the case of imines derived from aliphatic[4b] or electron-rich aromatic[4a] aldehydes. Given this observation and the fact that reverse-phase HPLC analysis is commonly performed in relatively acidic conditions (0.1% TFA in H_2O/CH_3CN mixtures, pH ca. 1.7), we anticipated the possibility of sufficiently slowing down the hydrolysis rate to allow direct HPLC detection of a library of well-chosen imines.

In a recent article,[5] we have shown that hen egg white lysozyme (HEWL) is able to select an inhibitor from a library of imines obtained by the reaction of monosaccharide derived amines with a series of aromatic aldehydes. In these imines, the aromatic moiety is presumed to mimic a second carbohydrate unit that interacts with the enzyme's binding site. This system was analyzed following the reductive procedure reported above and we considered that it might be adapted to illustrate direct imine detection by HPLC.

Amine **A** was incubated for 30 min with aldehydes **1**–**6** (Scheme 2). This mixture was directly analyzed by RP-HPLC-UV with a TFA-containing mobile phase (Figure 1). The resulting chromatogram showed, as expected, amine **A** and the set of aldehydes (Figure 1a). Attentive examination of the HPLC trace also revealed the presence of the six expected imines in the mixture[6] (see the framed part of Figure 1a and Figure 1b). Their assignments have been confirmed by HPLC injection of the individual imines prepared in a nonaqueous medium. Longer reaction times showed no further modifications in product distribution, which confirms that imine formation and equilibration was complete within 30 min.

When this mixture was equilibrated in the presence of lysozyme (1 equiv.), a significant amplification of the peaks corresponding to imines **A·1** and **A·2** was observed (Figure 1c).[7,8] This supports our previous experiments with a reductive treatment of the library,[5] in which amine **A1**, resulting from **A·1** reduction, had been shown to be amplified by lysozyme. The addition of more lysozyme (2 more equiv.) to this equilibrated mixture led to higher amplification of these peaks (Figure 1d), which proves that amplification is dependent on the concentration of lysozyme.

To confirm that this amplification was due to the interaction with the lysozyme's active site, chitotriose (3 equiv.), a

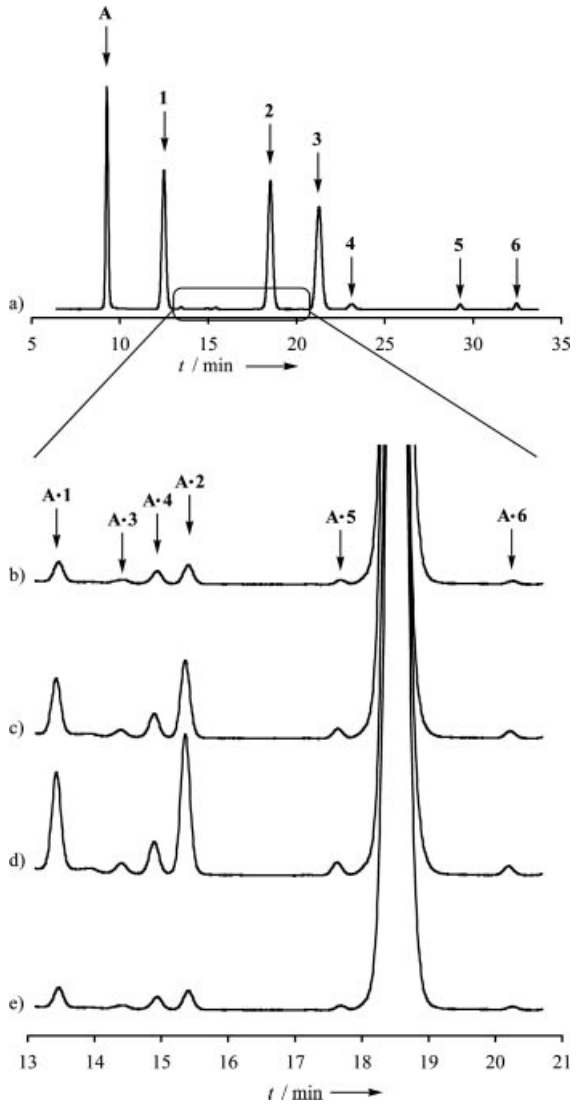
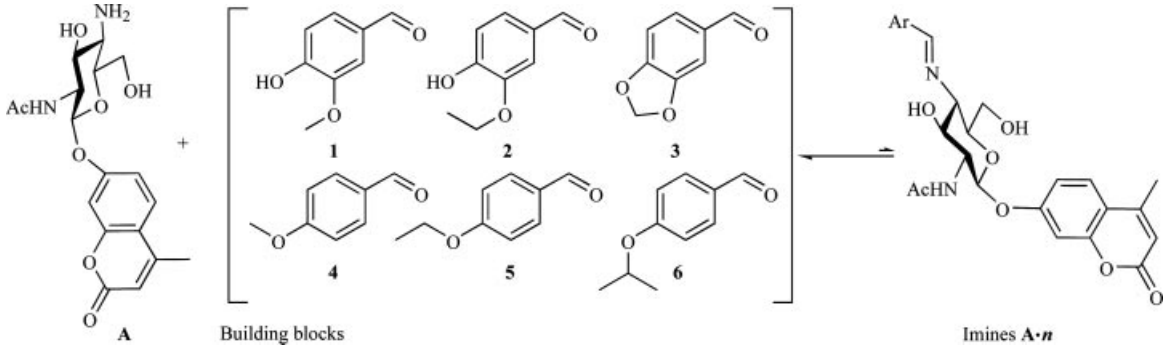


Figure 1. HPLC-UV (322 nm) chromatogram of the DCL made from: a) amine **A** and aldehydes **1**–**6**, and selected portion of the chromatograms obtained, b) in the absence of HEWL, c) in the presence of HEWL (1 equiv.), d) in the presence of HEWL (3 equiv.), e) in the presence of HEWL (1 equiv.) and chitotriose (3 equiv.).



Scheme 2. Building blocks for the dynamic combinatorial library (DCL) and structure of the library components (***n*** in **A·*n*** designates the aldehyde number).

known lysozyme competitive inhibitor, was added to the experiment. Chromatographic analysis after 30 min showed complete inhibition of the amplification effect (Figure 1e) and confirms that the amplified imines are in competition with chitotriose for the lysozyme's binding site. This set of experiments also confirms that the system is dynamic and that simple HPLC analysis is able to "sense" its adaptive behaviour.

This novel procedure of directly analyzing the distribution of imines also provides the opportunity to better understand such systems. Because the concentration of each imine is accessible, one should be able to estimate the imine's apparent binding constant,[9] a figure difficult to obtain otherwise. Analysis of the chromatograms from experiments with amine **A** (0.4 mM) and only one aldehyde (**1**, 0.4 mM), in the presence and in the absence of lysozyme (0.4 mM), provided the concentration of all the species in solution [amine **A**, aldehyde **1** and imine **A·1**[10] (bound and free)]. From such experiments, the apparent binding affinity of imines **A·1** and **A·2** were estimated to be, respectively, around 0.15 and 0.12 mM (see Supporting Information). Interestingly, these affinities can also be estimated from the easily accessible amplification factor α only[11] by the following simplified equation: $K_i \approx [E]°/(\alpha - 1)$, where [E]° is the initial lysozyme concentration ($\alpha_{A\cdot1} = 3.5$, $K_{i\ A\cdot1} \approx 0.16$ mM; $\alpha_{A\cdot2} = 4.2$, $K_{i\ A\cdot2} \approx 0.13$ mM, see Supporting Information).[12]

This type of analysis also revealed that the reduction step used in our previous DCL experiments[5] and presumably in the others[2] is slow enough so that the equilibrating DCL of imines is not perturbed. When sodium cyanoborohydride was added to the above experiments, the imine and the resulting amine concentrations were accessible on the same chromatogram; hence, the rate of amine formation could be followed with time (see Figure 2 for imine **A·1** and amine **A1**). This shows that while imine formation is rapid (equilibrium is reached in less than 30 min as previously observed), the overall rate of amine formation is much slower.

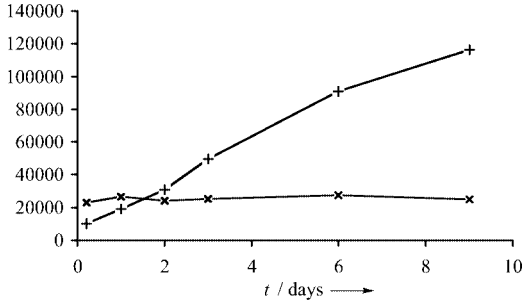


Figure 2. Evolution of the concentration (as determined by peak area) of imine **A·1** (x) and amine **A1** (+) as a function of time, when amine **A** (0.4 mM) and aldehyde **1** (0.4 mM) were equilibrated in an aqueous phosphate buffer (20 mM, pH 6.2) in the presence of sodium cyanoborohydride (3.6 mM).

Further experiments (not shown) allowed us to estimate the apparent rate of imine reduction to about 1% of the equilibrium rate of imine formation or hydrolysis under our conditions. Another important result is that the amplification factor measured for imines **A·1** (2.8) and **A·2** (4.0) in the presence of the enzyme (1 equiv., Figure 1c) was consistently higher than the amplification factor measured for the corresponding amines **A1** (1.8, 48 h) and **A2** (2.4, 48 h) when sodium cyanoborohydride was added to the same library. This probably indicates that reduction occurs at a slower rate inside the enzyme's active site, as would be expected for steric reasons. This provides an estimate of the bias introduced by the reducing step.[13]

Finally, a library derived from aliphatic aldehydes provided a set of imines stable enough at the acidic pH of the analytical step to allow HPLC detection (Figure 3), which shows the potential generality of this approach.[14]

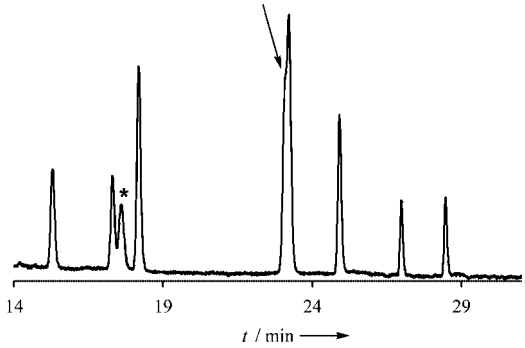


Figure 3. HPLC-UV (322 nm) chromatogram of the DCL of imines made from amine **A** and, in the elution order, propanal, isobutyraldehyde, butyraldehyde, phenylacetaldehyde (arrow), cyclohexanecarboxaldehyde, hexanal, heptanal and octanal (* residual 4-methyl umbelliferone).

This work shows that HPLC can be used to successfully analyze product distribution in a dynamic library of imines equilibrated in an aqueous medium. In the presence of a target, this approach allows direct detection of amplified imines and product re-equilibration when conditions are modified. The freezing of the equilibrating mixture is obtained by a simple and fast drop in pH during the analytical step. This method also allows estimation of the actual binding affinities of the transient library species. This strategy, which avoids a cyanoborohydride reduction step, might also prove useful in a situation where the topology of the enzyme's binding site precludes direct reduction of the bound imine.

Experimental Section

Solutions of amine **A** (33 μL, 12 mM, water), aldehydes (667 μL, 0.6 mM, 30 mM phosphate buffer, pH 6.2) and distilled water (300 μL) were introduced into an eppendorf tube equipped with a small magnetic stirring bar. This mixture (M_1, 1000 μL) was stirred for 30 min and directly analyzed. The solution was then fractioned and introduced (500 μL) into an eppendorf tube containing 1 equiv. (3.0 mg) of HEWL (mixture M_2). Another aliquot (250 μL) was introduced into an eppendorf tube containing 3 equiv. (4.5 mg) of HEWL (M_3, not shown in the main text). After stirring, the resulting DCLs were equilibrated at room temperature for 24 h and analyzed. Mixture M_2 (250 μL) was then withdrawn and introduced into an eppendorf tube containing 3 equiv. of chitotriose (5 μL, 60 mM/H_2O). The resulting mixture (M_4) was stirred and

analyzed 30 min later. HEWL (2 equiv.) was added to the remaining M_2, and the resulting DCL (M_5) was stirred, equilibrated at room temperature for 24 h and analyzed.

Analytical chromatography was performed with a JASCO LC-1500 system equipped with a Phenomenex LUNA C18 (2) 5 μ reverse-phase HPLC column (150 × 4.60 mm), with UV-detection at 322 nm. A binary solvent gradient (solvent A: 0.1% trifluoroacetic acid in H_2O 95%/CH_3CN 5%, solvent B: 0.1% trifluoroacetic acid in CH_3CN 95%/H_2O 5%) was optimized in order to separate most of the DCL compounds: A 90%/B 10% to A 70%/B 30% over 20 min, with a flow rate of 0.8 mL min^{-1}.

Supporting Information (see footnote on the first page of this article): Data used for the estimation of the dissociation constants.

Acknowledgments

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[6] In this study, the terms aldehyde and imine are used for any species in fast equilibrium with the true aldehyde and the true imine, respectively, including hydrate and hemiaminals. An NMR study performed at a higher concentration showed, however, the aldehyde and the imine as the sole detectable species.

[7] In this system, and in contrast with the analysis after reduction,[5] the other imines were also slightly amplified.

[8] The eluent contained 0.1% TFA, which resulted in a fast denaturation of the enzyme and the release of any bound product on the column. This precluded any composition analysis biased by the enzyme.

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[10] The molar extinction coefficient of imine **A·1** at 322 nm was estimated as approximately 1.6-fold the molar extinction coefficient of amine **A**.

[11] The amplification factor is defined as the ratio of the total (bound + free) imine concentration in the presence of the target versus the corresponding concentration in the absence of the target.

[12] Taking into account the affinity of starting amine **A** for the binding site of the lysozyme (around 5 mM),[5] slightly lower estimates can be calculated by numerical resolution, namely 0.13 and 0.10 mM.

[13] The observed difference might in theory result from the recognition by the active site of the target of a nonreducible species, such as a hemiaminal. As previously mentioned,[6] no hemiaminal could be detected by NMR, which suggests that the amplified species is indeed the corresponding imine.

[14] As anticipated,[5] no redistribution of the imines in the presence of the lysozyme was observed with such a library.

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