

HPLC AND ^{13}C NMR FOR ANALYSIS OF TRANSAMIDATION IN A DYNAMIC COMBINATORIAL CHEMISTRY SYSTEM

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SUMMARY

The permanent demand for novel drugs by the pharmaceutical industry results in a variety of novel methods for drug discovery. Combinatorial methods are a novel direction of drug research based on synthesis of large number of compounds in the hope of finding interesting drug-like molecules. In dynamic combinatorial chemistry (DCC) an enzyme is used to control the synthetic path and increase the proportion of the drug-like molecules in the reaction mixture. Because the mixture formed by the reaction is complex, analytical procedures limit possible application of the method. In the work discussed in this publication HPLC was coupled with ^{13}C NMR for analysis of a DCC system. Transamidation controlled by use of *Candida rugosa* lipase was compared with the reaction without the enzyme. This enabled us to show that the enzyme-promoted reaction favoured the amine with the lowest nucleophilicity.

INTRODUCTION

Discovering drugs is a complex issue that still lacks a general approach. Combinatorial chemistry, a method for drug discovery developed in the nineteen-eighties, attempts to imitate Nature's strategy for molecular discovery by substantially increasing the number of molecules investigated. Traditional combinatorial approaches are based on completely random synthesis. Statistically, this does not increase the chances for finding a new drug, because we change the range of compounds tested and a pool of hundreds of thousands of molecules must be screened to find potential leads. The significant expansion of the range of compounds investigated in recent years has not, in fact, resulted in an increase in new drug approvals [1–3].

Novel process-driven approaches to combinatorial molecular discovery, dynamic combinatorial chemistry (DCC), are new experimental methods that combine the advantages of screening a large number of molecules with rational use of a target receptor structure [4,5]. In these approaches, which attempt in-vitro selection, an isolated receptor controls the direction of synthesis, selecting among many possible reagents, which increases the concentration of strong binders at the expense of the weak binding molecules [6]. This elegant, modern approach does, however, require advanced analytical methods suitable for mixtures of many interacting organic compounds.

Many reactants and products, and enzyme decomposition products, occur in a reaction mixture, which makes it extremely difficult to analyze. Chromatographic techniques are well known to be suitable for rapid and reliable determination of multi-component mixtures. In this paper we describe results obtained from use of HPLC techniques to identify components and monitor dynamic reactions in combinatorial systems. By use of patterns from previous syntheses we successfully determined the dynamic routes of the enzyme-promoted reactions.

EXPERIMENTAL

Lipase (*Candida rugosa*) was obtained from Aldrich and was used without further purification or treatment. All other chemicals were supplied by Fluka except 8-hydroxyquinoline-7-carboxylic acid benzylamide, which was synthesized by a published procedure [7].

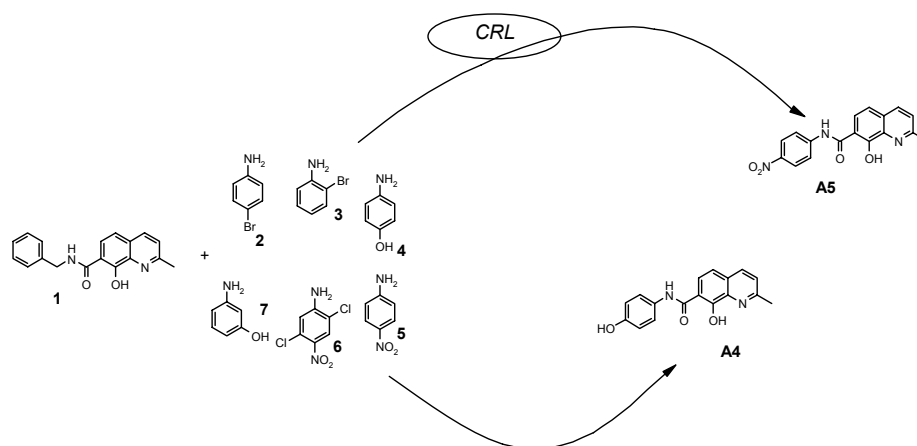
HPLC analysis of the mixtures studied was performed with Gynkotek equipment, with Chromelon 6.2 software and diode-array detection (DAD). Compounds were separated on a column packed with 5- μm LiChrosphere 100, RP-18 (Merck; #1.50983). The mobile phase was acetonitrile-water, 3:2, at a flow rate of 0.6 mL min⁻¹. ¹³C NMR spectra were recorded with a Bruker 400-MHz instrument. ¹³C NMR spectra were simulated by use of ACD Labs 7.0 software.

All experiments were performed in a buffered mixture containing 0.032 g lipase (CRL), 0.1 mmol 8-hydroxyquinoline-7-carboxylic acid benzylamide (**1**) and 0.2 mmol of each of the amines 4-bromoaniline (**2**), 2-bromoaniline (**3**), 4-hydroxyaniline (**4**), 4-nitroaniline (**5**), 2,5-dichloro-4-nitroaniline (**6**), and 3-hydroxyaniline (**7**) dissolved in 12 mL EtOH and 4 mL buffer. A reference experiment was performed with an identical mixture but without the enzyme. Reagents were incubated at 40°C and pH 6.9 (Na₂HPO₄ and KH₂PO₄) for 4 days. Samples were taken from the mixtures

every 24 h and frozen to stop enzyme-promoted reactions. The enzyme was then separated and the mixture was immediately analyzed by HPLC and NMR spectroscopy.

RESULTS AND DISCUSSION

Although the carboxamide bonds common in biopolymers are of rather low reactivity, transamidation can proceed efficiently. Metal complexes catalyzing a novel class of amide-exchange reaction under mild conditions have recently been reported [8]. Reaction also occurs under natural conditions – e.g. tissue transglutaminase can modify proteins by transamidation or deamidation of specific glutamine residues [9,10] and transamidation of non-activated amides can be achieved with an enzyme from *Candida antarctica*, the only active enzyme among 45 different lipolytic and proteolytic enzymes tested [11]. Further exploration of this topic is limited, because of the lack of a rapid and simple procedure for analysis and monitoring of the reaction mixture. The objective of this study was to investigate the possibility of analysis of a DCC transamidation reaction in the presence of *Candida rugosa* lipase (CRL). The reaction system designed is illustrated in Scheme 1. The reaction between an amide and combinatorial mixture of six amines performed in a system containing lipase was compared with reaction of the same system but without the enzyme. It was shown that DCC transamidation of the benzyl amide of 8-hydroxyquinoline-7-carboxylic acid with a combinatorial library of several amines



Scheme 1

System designed for DCC experiment with pathways of the reaction performed with or without lipase

in water selectively furnished the least likely product, because the amine with the lowest nucleophilicity yielded the main product.

The reactants were incubated under optimum reaction conditions (ethanol solution buffered to pH 6.9 with Na₂HPO₄ and KH₂PO₄) at 40°C for 3 days. Samples were collected from the reaction mixture at 24-h intervals and monitored by HPLC and ¹³C NMR.

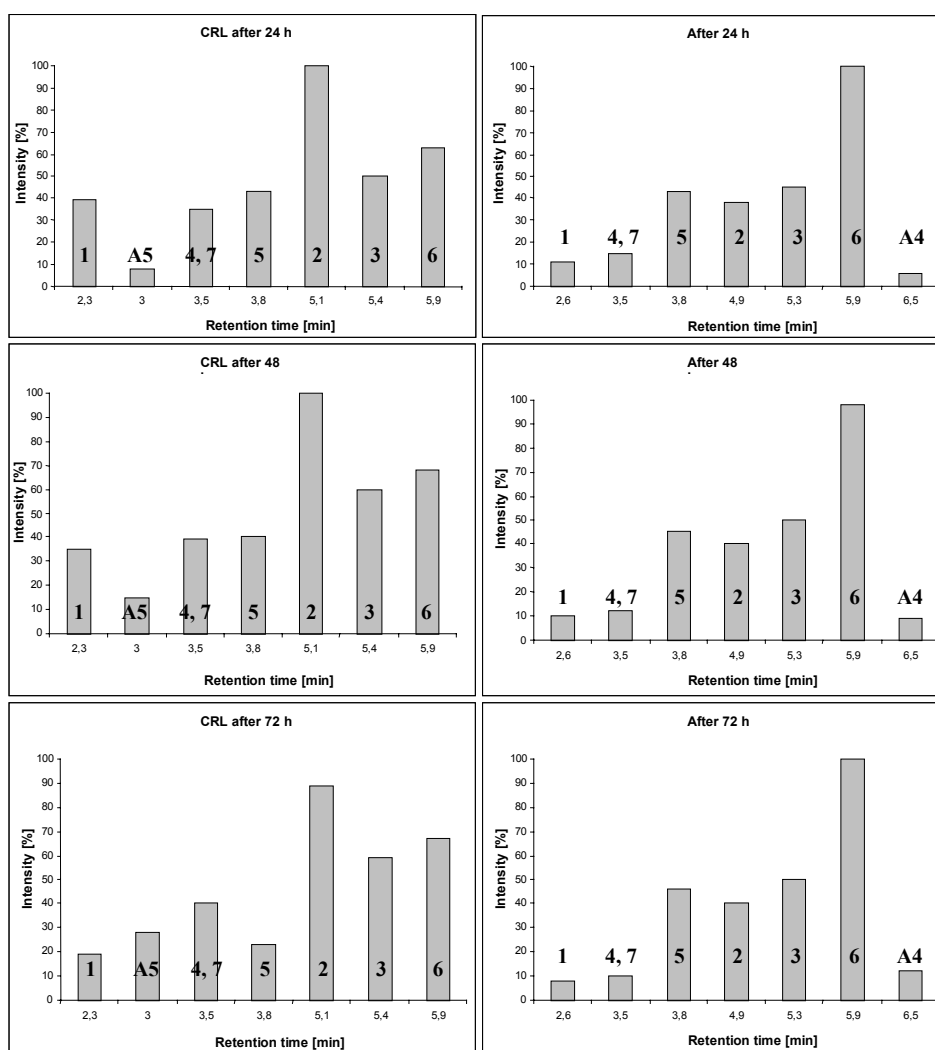


Fig. 1

Results from HPLC analysis of samples incubated with and without CRL for the specified period of time

Results obtained from HPLC analysis of the reaction mixtures compared in Fig. 1. In the presence of the enzyme transamidation starts after incubation for 24 h. From this point the concentration of *p*-nitroaniline (**5**) decreases regularly in favour of the amide (**A5**) up to 72 h. Comparison with the HPLC chromatograms obtained from the reaction mixture without the enzyme reveals the processes occurring are not similar. Under both sets of conditions, i.e. in the presence and absence of the lipase, a minute amount of amide **A4** is formed. To prove conclusively the structures of amides formed we recorded ^{13}C NMR spectra of the products. Schematic diagrams of the ^{13}C NMR spectra are shown in Fig. 2. These enabled identification of **A5** and **A4** as the transamidation products of *p*-nitroaniline and *p*-aminophenol, respectively.

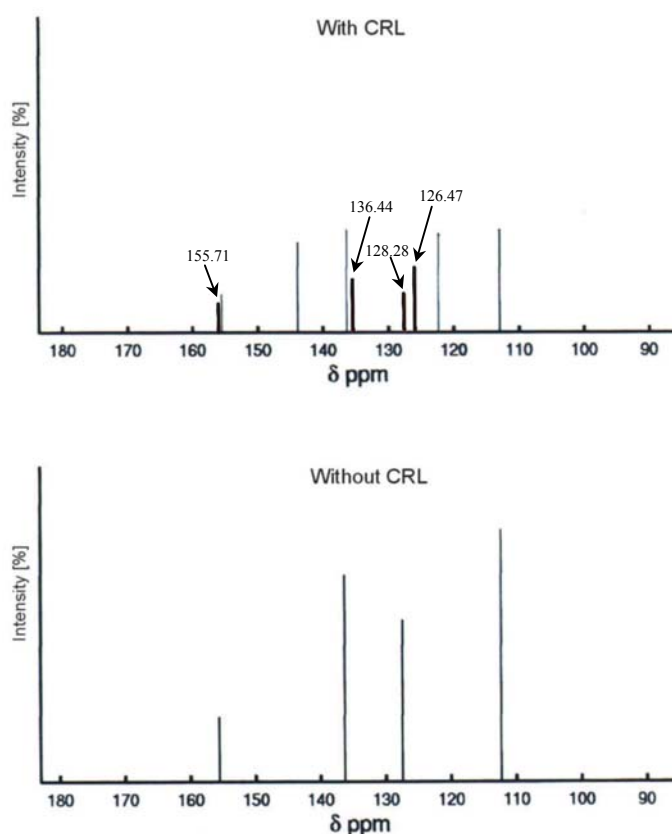


Fig. 2

Schematic diagram of ^{13}C NMR spectra after 48 h. Arrows indicate peaks larger than those detected in previous samples, or detected for the first time

Thus, CRL controls the DCC system, favouring reaction with *p*-nitroaniline, which would be the least reactive amine (*p*-NO₂ results in low nucleophilicity) in the non-catalytic system. In the reaction without *Candida rugosa* lipase only a minute amount of transamidation with *p*-aminophenol occurs (*p*-OH results in high nucleophilicity).

CONCLUSION

In conclusion, we found that use of HPLC supported by ¹³C NMR spectroscopy enabled effective monitoring and analysis of dynamically controlled reactions. This elegant and reliable procedure could be successfully used in conjunction with DCC technology.

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