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Online NMR for monitoring biocatalysed reactions—the use of lipases in organic solvents

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Abstract

The lipase-catalysed acetylation of 2-hydroxymethylpiperidine was carried out in the NMR tube and monitored by ^{1}H NMR spectroscopy for defined periods of time. Two different acylating agents were used: ethyl acetate in C_6D_6 and vinyl acetate in C_6D_6. The lipase employed was porcine pancreatic lipase (PPL). Ethyl acetate in C_6D_6 was hydrolysed by the enzyme but no formation of acylated product could be detected in these dilute solutions. In case of vinyl acetate as the acyl donor, the reaction did not give the desired *N*-acylated compound but the corresponding oxazolidine-derivative, which was formed by the reaction of the aminoalcohol and the acetaldehyde. This compound was assigned unambiguously without isolation from the reaction medium by total correlation spectroscopy (TOCSY) and gHSQC experiments.

Keywords: Lipases in organic solvents; NMR; Biocatalysis; Aminoalcohols; Porcine pancreatic lipase

1. Introduction

The monitoring of biocatalysed reactions by NMR spectroscopy has been successfully used by several groups in recent years [1–7]. Its main advantage is that the reactions can be carried out directly in the NMR magnet without operator intervention and the data can be collected for prolonged time periods. Moreover, it is a non-invasive method, which means that no material is consumed during the analysis [8]. Biocatalysts in several stages of purity have been successfully used in these transformations [9].

For the present study we decided to look at lipases in organic solvents and to use a substrate, which has several possibilities to react, making the analysis more complicated but also showing the power of NMR to analyse these mixtures. We had already undertaken the lipase-catalysed acetylation of racemic 1-phenylethanol in deuterated benzene in the NMR tube and were able to watch the reaction as it proceeded to give acetaldehyde and the acetylated product [10]. In this study the substrate was 2-hydroxymethylpiperidine for mainly two reasons:

- Aminoalcohols of this type in optically pure form are important building blocks for a number of interesting drugs [11–13].
- The substrate has two potentially reactive centres, namely the amine nitrogen and the alcohol oxygen.

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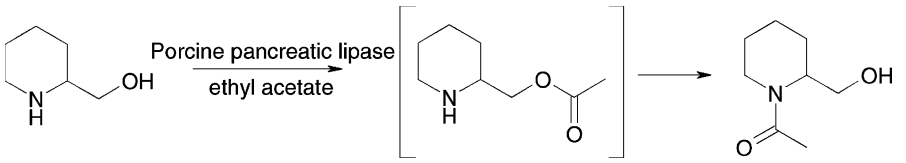


Scheme 1. PPL-catalysed acetylation of 2-hydroxymethylpiperidine in ethyl acetate as solvent via the putative *O*-acylated compound.

Previous studies using this compound in the PPL-catalysed acetylation in ethyl acetate suggested that the reaction occurred at the oxygen, but then the acetyl moiety was transferred to the nitrogen yielding the only isolated product—*N*-monoacetylated aminoalcohol—in varying e.e.s depending on the reaction conditions [13]. We hoped to get some additional evidence for this hypothesis by running the reaction in the NMR tube and maybe be able to watch the occurrence of the putative *O*-acyl intermediate. The biotransformation is shown in Scheme 1.

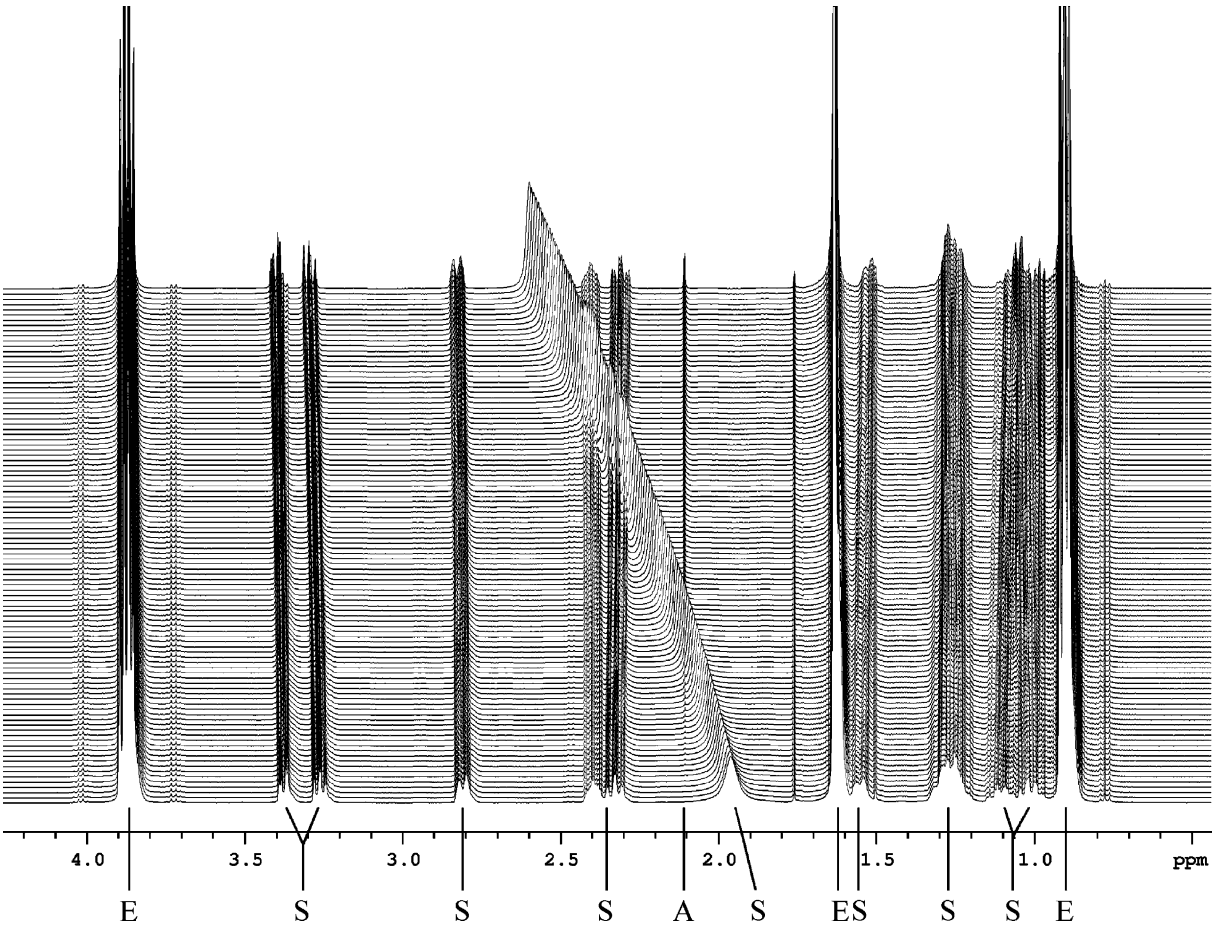


Fig. 1. ^{1}H NMR spectra of a mixture of 2-hydroxymethylpiperidine (2.8 mg), ethyl acetate (12 ml) and PPL (2 mg) in C_6D_6 (500 ml). Spectra were recorded automatically every 10 min on a Varian INOVA 500 for 14 h at 30 °C. Signals are: E for ethyl acetate (^{13}C-satellites can be seen for these large signals); S for the substrate (2-hydroxymethylpiperidine), A for acetic acid.

2. Results and discussion

2.1. PPL-catalysed acylation of 2-hydroxymethylpiperidine using ethyl acetate as acyl donor and deuterated benzene as solvent

For the NMR experiments we had to modify the originally published conditions [13]. Ethyl acetate as solvent is not suitable because it would introduce huge signals in the ^{1}H NMR spectrum and thereby make the substrate and product signals almost invisible. Solvent suppression of course would be an option, but it would also be interesting to monitor the disappearance of ethyl acetate as reagent which would not be possible when suppressing the solvent (i.e. ethyl acetate) signal. Therefore, we decided to dilute the reaction mixture with deuterated benzene, which had worked very well in our previous studies [10]. Deuterated benzene acted as a fairly cheap solvent required for deuterium lock and showed similar properties compared with the typically used organic solvents (usually hydrophobic solvents which do not displace the residual water from the enzyme are preferred).

To test if spontaneous reaction occurred between 2-hydroxymethylpiperidine and ethyl acetate, the former was dissolved in C_6D_6 in an NMR tube and ethyl acetate was added. ^{1}H NMR spectra were measured every 10 min for 5 h at 30 °C revealing that no reaction occurred and that the mixture did not change at all.

Now PPL was added and the measurement was restarted collecting ^{1}H NMR spectra every 10 min for a period of 14 h. The spectra are shown in Fig. 1.

As can be seen the reaction started immediately after the addition of lipase giving rise to a singlet at 2.14 ppm and the continuous downfield shift of the "acidic" protons (OH and NH, 1.97–2.62 ppm). The shifts of the substrate also moved slightly upfield during the course of the reaction. The first assumption

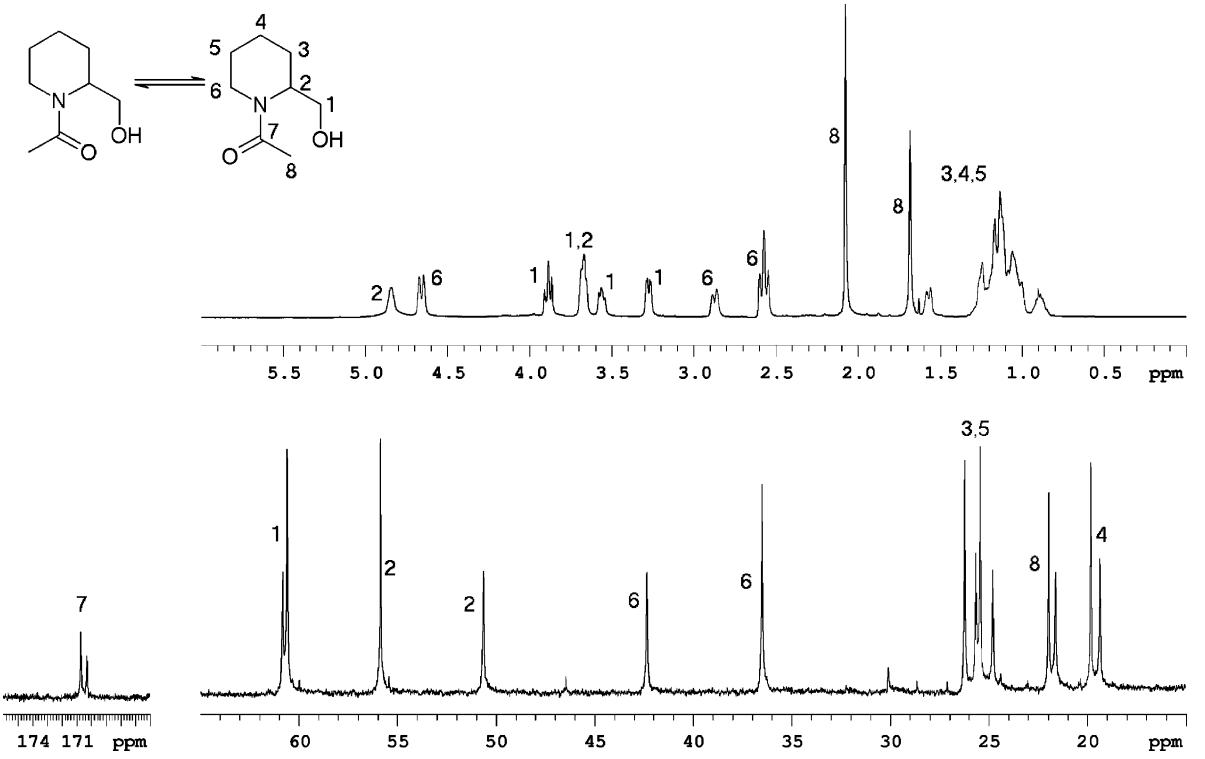


Fig. 2. ^{1}H and ^{13}C NMR spectra of 2-hydroxymethyl-*N*-acetylpiperidine in deuterated benzene. Both spectra show that two isomeric forms are present with very different chemical shifts, especially for signals 2 and 6.

was that the signal at 2.14 ppm could be the acetyl group of 2-hydroxymethyl-*N*-acetylpiperidine. However, this proved not to be the case by addition of authentic chemically synthesised reference material. Fig. 2 shows the ^{1}H and ^{13}C NMR of 2-hydroxymethyl-*N*-acetylpiperidine. At 30 °C this compound is present as a mixture of *E*/*Z* isomers (this is due to the partial double bond character of the C–N bond in amides), which have remarkable chemical shift differences. The signals for these isomers coalesced when heating the sample to 75 °C.

It was found that the singlet in Fig. 1 belonged to acetic acid being formed by enzymatic hydrolysis of ethyl acetate. Another couple of hours later the formation of ethanol could also be recognised in the spectra (not shown). Obviously, no *N*-acetyl product was produced by this biotransformation, which might be because of the more dilute reaction medium compared with the originally published conditions.

2.2. PPL-catalysed acylation of 2-hydroxymethylpiperidine using vinyl acetate as acyl donor and deuterated benzene as solvent

Vinyl acetate is a much more popular acylating agent in enzymatic transesterifications than ethyl

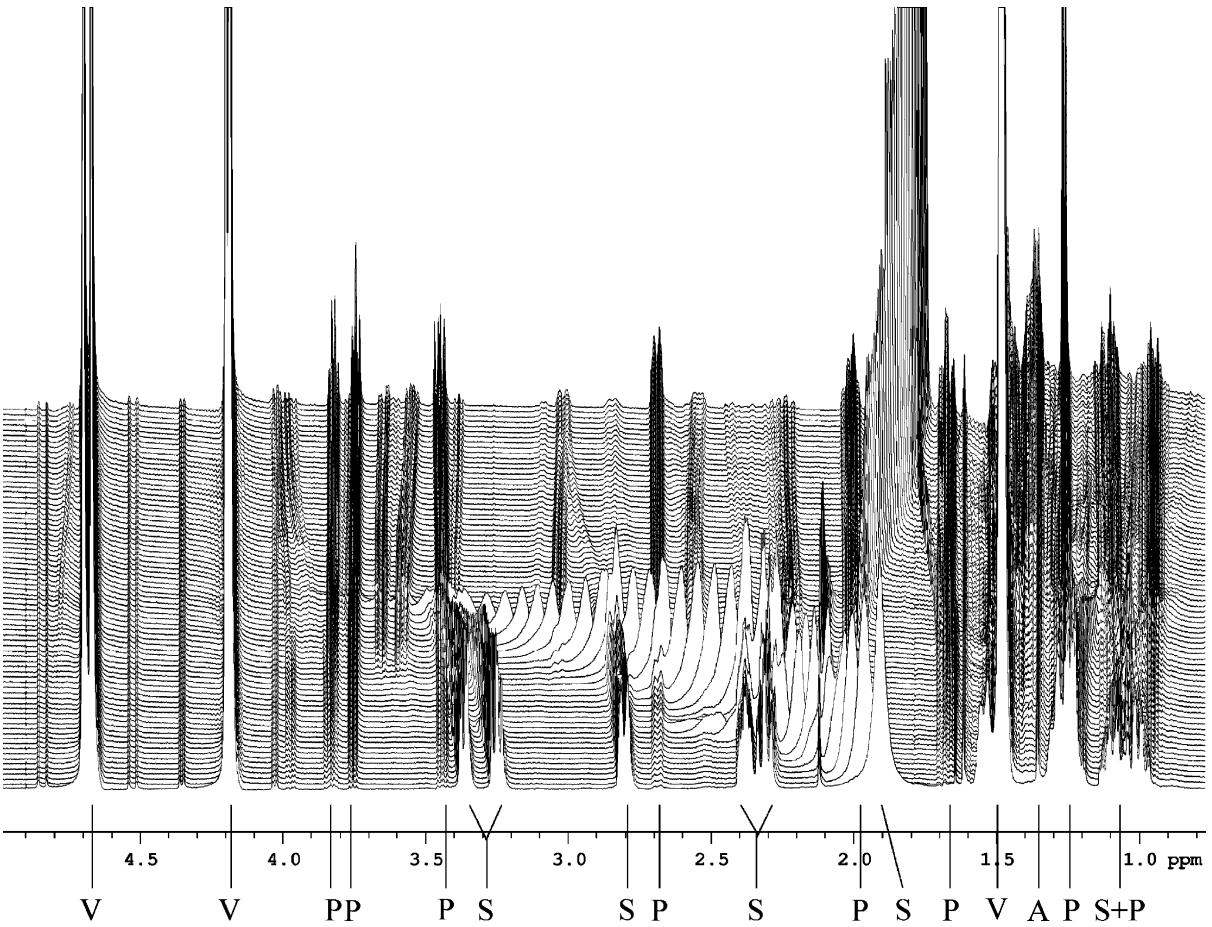


Fig. 3. ^{1}H NMR spectra of a mixture of 2-hydroxymethylpiperidine (2.1 mg), vinyl acetate (12 ml) and PPL (2 mg) in C_6D_6 (500 ml). Spectra were recorded automatically every 10 min on a Varian INOVA 500 for 24 h at 30 °C. The time difference between the spectra shown in the figure is 40 min. Signals are: V for vinyl acetate (^{13}C-satellites can be seen for these large signals); S for the substrate (2-hydroxymethylpiperidine), P for the product, A for acetaldehyde.

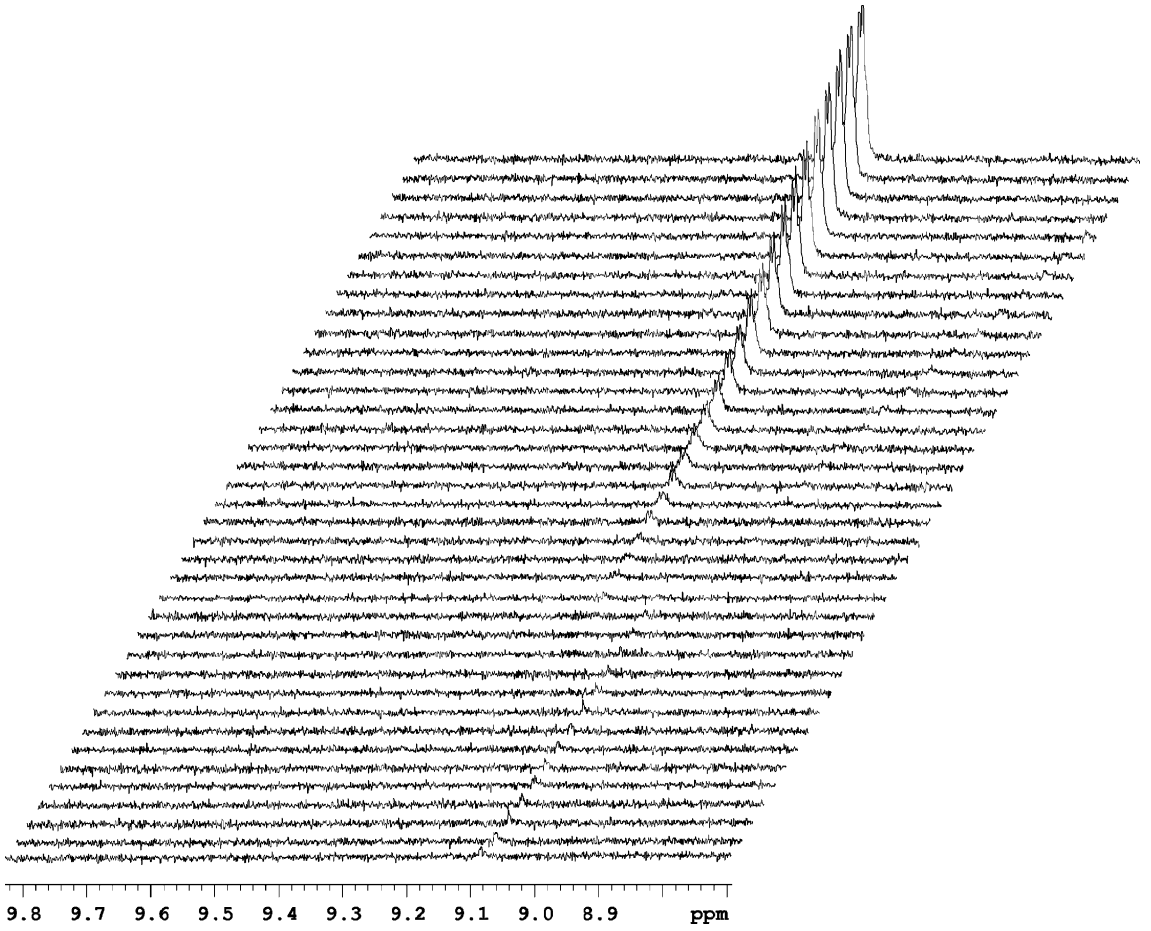


Fig. 4. ^{1}H NMR spectra of a mixture of 2-hydroxymethylpiperidine (2.1 mg), vinyl acetate (12 ml) and PPL (2 mg) in C_6D_6 (500 ml). The acetaldehyde quartet is shown. Spectra were recorded automatically every 10 min on a Varian INOVA 500 for 24 h at 30 °C. The time difference between the spectra shown in the figure is 40 min.

acetate. The reason of course is that acetaldehyde is produced immediately from the vinylalcohol by enol to keto tautomerization thus shifting the equilibrium almost completely to the product side. We have previously performed the CAL-B-catalysed acetylation of 2-hydroxymethylpiperidine with vinyl acetate and found that a number of products are formed, whose structures could not be assigned at that time [14]. Therefore, we wanted to reinvestigate this conversion, now using PPL as biocatalyst.

Mixing substrate and vinyl acetate in C_6D_6 and monitoring this mixture by ^{1}H NMR for several hours at 30 °C showed that also in this case no reaction occurred without the addition of enzyme. After adding PPL the reaction started immediately as can be seen from Figs. 3 and 4.

It is evident that in this case the reaction mixture is much more complex compared with the ethyl acetate conversion. The substrate is consumed after about 8 h, the “acidic” protons move over a much larger shift range, the acetaldehyde formation starts after about 8 h and one main product is formed, which is different from the expected 2-hydroxymethyl-*N*-acetylpiperidine.

We took up the challenge to determine the structure of this major product in the reaction mixture without isolation.

Two selective total correlation spectroscopy (TOCSY) experiments were very helpful in this case. Excitation of one of the two putative CH_2O proton signals using a shaped pulse (iBURP2) and an array of spin lock times from 0 to 150 ms revealed the whole cyclic spin system of the product as shown in Fig. 5.

The magnetisation is transferred from the hydroxymethylene-protons first to the CHN, then along the CH_2 ring atoms to the CH_2N as shown in Fig. 5. The quartet at 3.83 ppm seemed to belong to this compound as well because of the integration and the same characteristic curve of formation over time. Excitation of this quadruplet transferred magnetisation to the methyl dublet at 1.27 ppm (see Fig. 6).

A gHSQC of the mixture (Fig. 7) confirmed the structure of this major product as being the oxazolidine shown later (Scheme 2) which is formed by reaction of 2-hydroxymethylpiperidine with acetaldehyde formed after hydrolysis of vinyl acetate by the lipase.

This finding explains why acetaldehyde formation only starts after all substrate is consumed. The formation of unexpected acetyl-derivatives in enzymatic resolutions has been described recently by Högberg

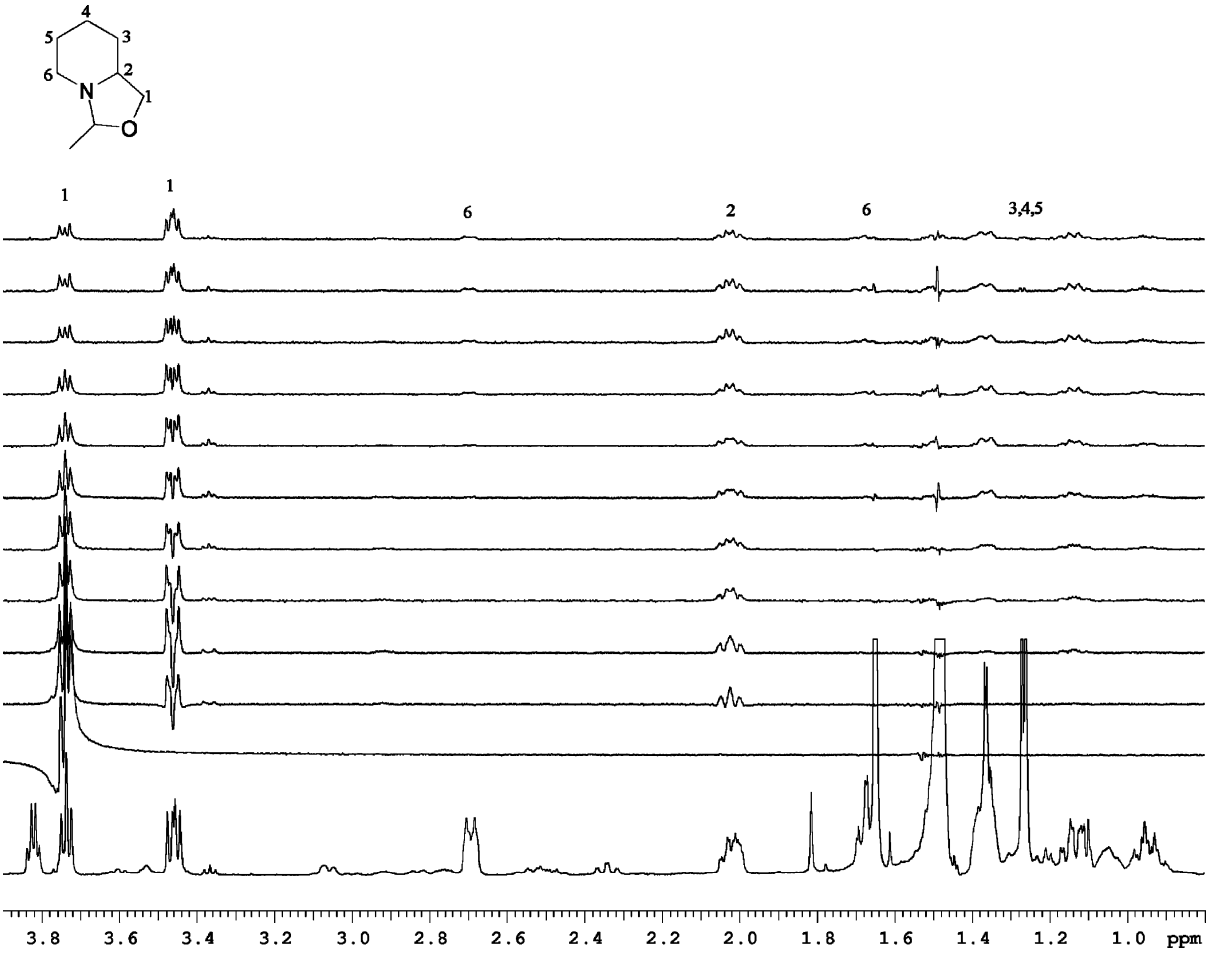


Fig. 5. Selective TOCSY1D spectra obtained by excitation of the signal at 3.72 ppm using an iBURP2 shaped pulse and its evolution at different spin lock times (0–150 ms).

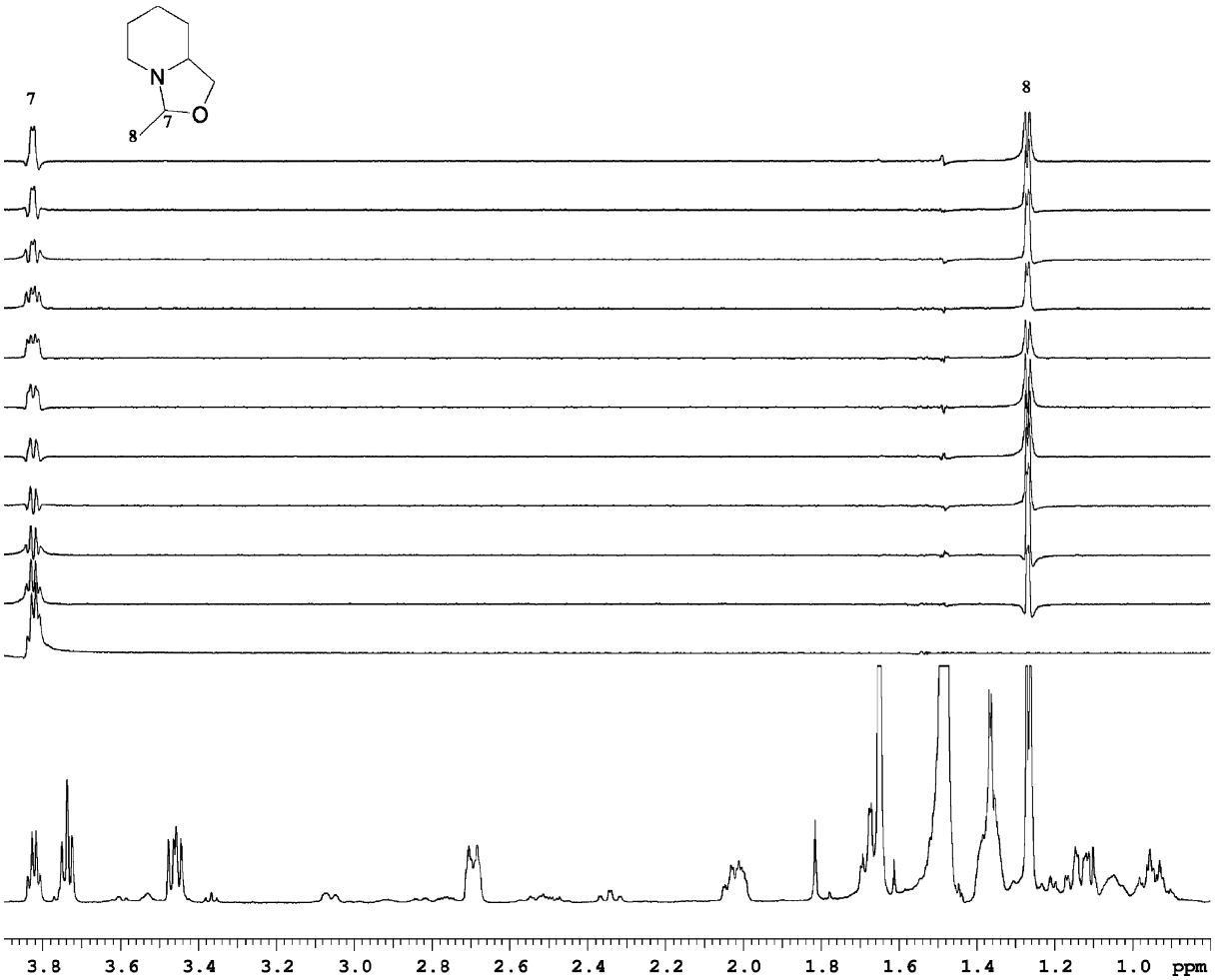
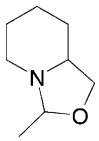


Fig. 6. Selective TOCSY1D spectra obtained by excitation of the signal at 3.83 ppm using an iBURP2 shaped pulse and its evolution at different spin lock times (0–150 ms).

et al. [15]. We plan to perform this reaction on a larger scale in order to obtain larger amounts of this oxazolidine in order to determine whether some optical activity can be found, which might indicate that the enzyme assists in the formation of this product.



Scheme 2. Structure of the major product formed in the vinyl acetate resolution.

3. Experimental

^{1}H NMR spectra were measured on a Varian INOVA 500 at 499.98 MHz. ^{13}C NMR were measured at 125.69 MHz. The probe was a gradient indirect detection probe (S/N 850:1 on 0.01% ethylbenzene). Total correlation spectroscopy spectra are typically 2D spectra. However, they can be made selective and one-dimensional by the inclusion of shaped pulses and then are called TOCSY1D [16]. Shaped pulses (iBURP2; inversion band-selective, uniform response, pure phase pulses) were created using PBox [17]. Heteronuclear single quantum coherence (HSQC)

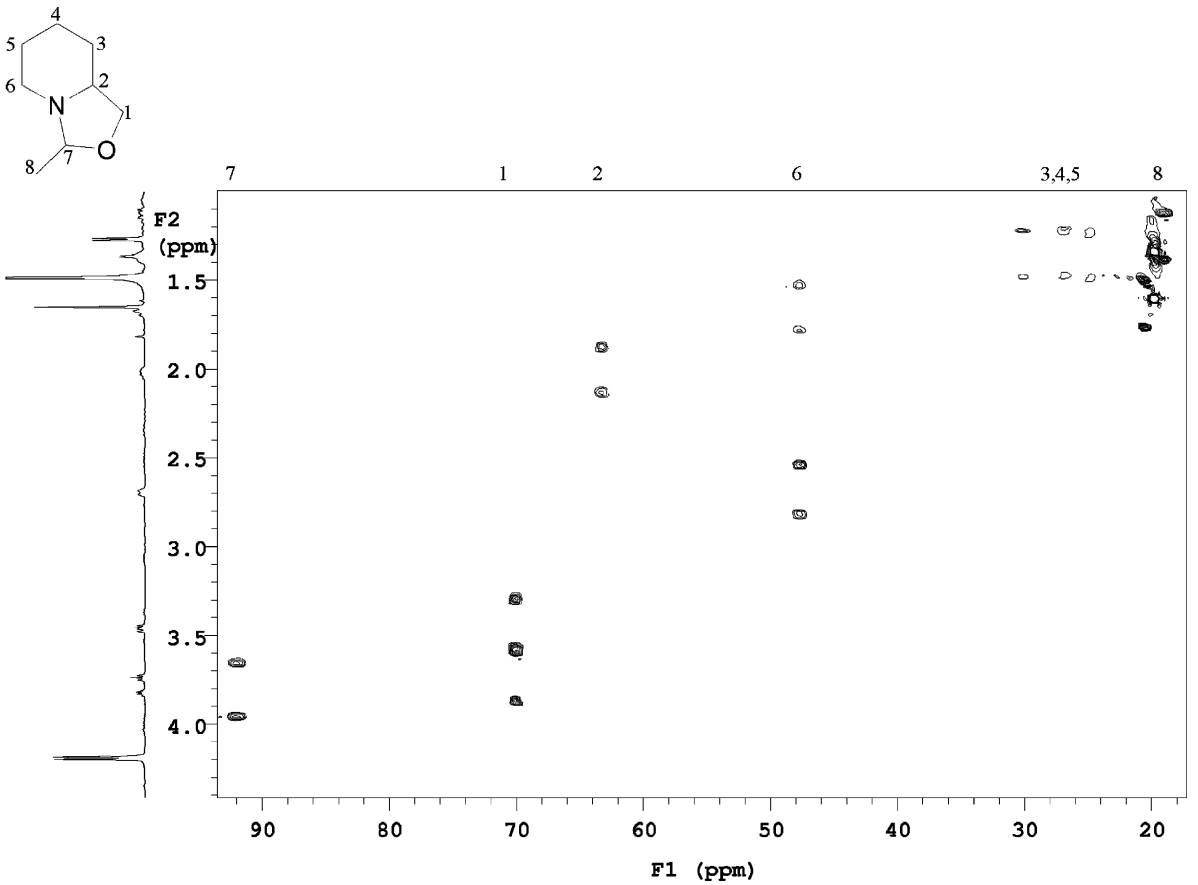


Fig. 7. The gHSQC spectrum of the aliphatic part of the reaction mixture after 40 h.

spectra are indirectly detected 2D-spectra correlating protons with ^{13}C to which they are attached [18].

Porcine pancreatic lipase was a gift from Biocatalysts, UK (batch no. 2078995). CAL-B was *Candida antarctica* Lipase B from Novo (Novo SP 525).

4. Conclusions

It could be shown that NMR spectroscopy is very useful to analyse complex mixtures formed by unexpected biotransformation reactions in this case. It was possible to assign the structure of an unknown product without isolation directly in the reaction medium. The complete time course of the formation of products and intermediates is very useful for the understanding of transformations if they do not give the desired products. In this case it would have been difficult to monitor the occurrence of acetaldehyde by other analytical techniques.[1]

Acknowledgements

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[1] A referee suggested to use dried solvents and products to minimise the formation of the by-product. However, as we have already found in [10], hydrolysis of vinyl acetate without formation of product occurs even under “dry” conditions.

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