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Enzymatic Process for the Synthesis of *cis/trans*-(1*R*,5*R*)-Bicyclo[3.2.0]hept-6-ylidene-acetate: Solvent Effect and NMR Study

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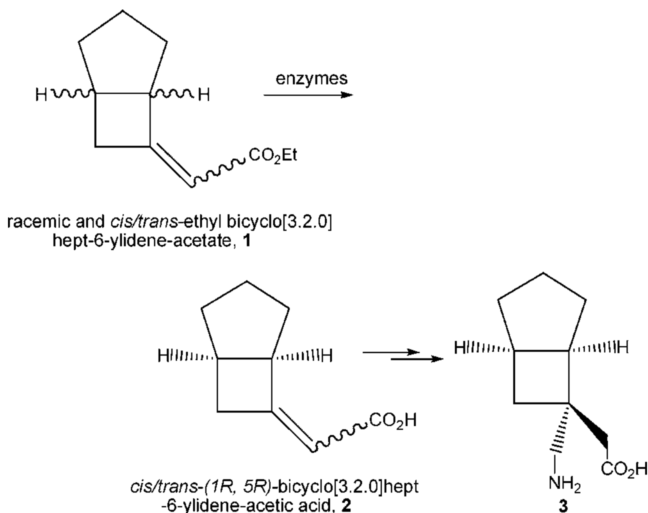
Abstract:

An efficient enzymatic process has been developed to resolve a diastereomeric mixture of racemic ethyl bicyclo[3.2.0]hept-6-ylidene-acetate (1). Using 40% acetone, not only was the enantioselectivity of *Candida antarctica* lipase B (CAL-B) significantly improved to $E > 200$ from $E = 2.7$, remarkably the enzyme is able to maintain low diastereoselectivity for the *Z* (*cis*)- and *E* (*trans*)-isomers leading to an overall high isolated yield (40−45%, vs maximum theoretical yield 50%) and excellent enantiomeric excess (>98.5% ee). Preliminary studies using 2D TROSY shows that there is probably a global conformational change in the N^{15}-labeled enzyme CAL-B when the content of organic cosolvents increases from 0% to 30%. The route was successfully scaled to 63 kg for the synthesis of GABA analogues.

Scheme 1



Introduction

(1*R*,5*R*)-Bicyclo[3.2.0]hept-6-ylidene-acetic acid (**2**) is a key intermediate toward the synthesis of GABA (γ-aminobutyric acid) analogues such as **3**, therapeutically useful agents for the treatment of epilepsy and neuropathic pain (Scheme 1).[1] To our knowledge, no asymmetric synthesis of this molecule has been reported prior to this study. We describe here the development of an efficient and cost-effective enzymatic process to prepare enantiomerically pure *E/Z*-**2** by the resolution of racemic and *E/Z*-ethyl bicyclo-[3.2.0]hept-6-ylidene-acetate (**1**). In this method, both the *E*- and *Z*-isomers of the (1*R*,5*R*)-enantiomer (**2**) are desired to be converted into **3** (Scheme 1). While enzymes have been widely used for the resolution of racemic molecules, this is a rare example where the resolution involves a mixture of four stereoisomers rendering the problem much more challenging and interesting.

Results and Discussions

Enzyme Screening. The racemic and *E/Z*-ethyl bicyclo-[3.2.0]hept-6-ylidene-acetate (**1**) was synthesized from bicyclo-[3.2.0]heptan-6-one according to a literature protocol (see Experimental Section for details).[1] Using an automated enzyme screening protocol developed in our lab,[2] *Candida antarctica* lipase B (CAL-B) was one of the few enzyme candidates identified that was able to catalyze the hydrolysis of the ethyl ester with some selectivity and high reactivity. Unfortunately, the enantioselectivity of the enzyme was quite low ($E = 2.7$, or 33% ee at a conversion of 50%) under screening conditions with a pH 7 buffer and 10% acetonitrile (ACN). Previous experience in working with CAL-B, however, gave us hope that we might be able to improve the selectivity towards this substrate via medium engineering. It should be noted that the overall rate of the reaction is dependent on the ratio of two diastereomers. CAL-B hydrolyzes the *tran*-isomer at a rate approximately 2 times faster than the *E*-isomer. The K_{cat} for the *Z*-(1*R*,5*R*)-ethyl bicyclo[3.2.0]hept-6-ylidene-acetate is about 115 min^{-1}. In most batches screened, the ratio of the *Z- to E*-diastereomers in the starting material (**1**) was about 4:1 or greater which ultimately increased the throughput of the reaction.

Medium Study. Attempts at improving the enantioselectivity of the enzyme were made via medium engineering. After screening a number of representative solvents with log *P* values ranging from −1 to 5 and various cosolvent contents from 20% to 50%, it was found that *t*-BuOH (log *P* 0.35), acetonitrile (log *P* −0.34), and acetone (log *P* −0.24) have the desired physical properties to tune the enantioselectivity of the enzyme. Figure 1 and Figure 2 illustrate in more detail the effect of these solvents on the reactivity and enantioselectivity of CAL-B towards the substrate **1**. As the cosolvent content was increased from 20% to 50%, the ee's increased from 78% to 98.1% for *t*-BuOH, 85% to 95% for

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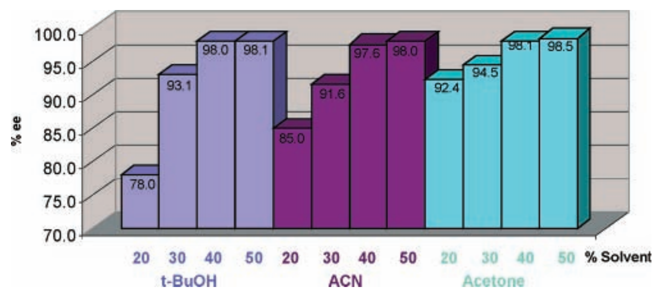

Figure 1. **Effect of solvent and solvent content on the enantioselectivity of CAL-B towards 1. Reaction conditions: 10% enzyme loading (w/w), 100 mg/mL of 1, pH 7.0, rt, 16 h.**

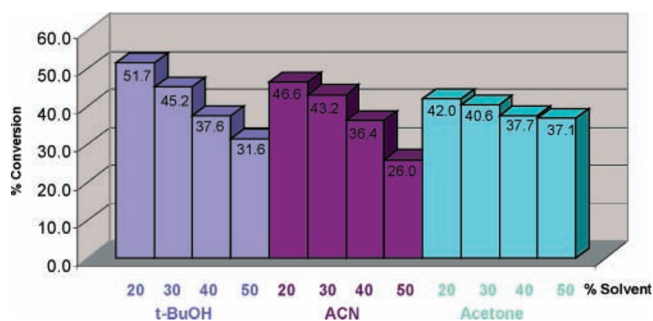

Figure 2. **Effect of solvent and solvent content on the reactivity of CAL-B towards 1. Reaction conditions: 10% enzyme loading (w/w), 100 mg/mL of 1, pH 7.0, rt, 16 h.**

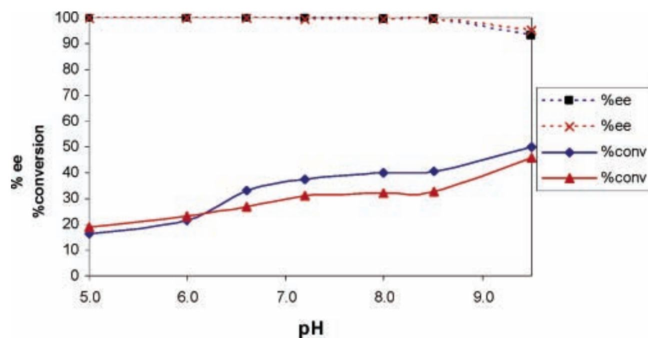

Figure 3. **Effect of pH on the reactivity and enantioselectivity of CAL-B towards 1. Reaction conditions: 40% cosolvent (red line − *t*-BuOH, blue line − acetone); potassium phosphate buffers (KPB) with varying pH, 10% enzyme loading (w/w), 100 mg/mL of 1, rt, 16 h.**

acetone, and 92.4% to 98.5% for ACN (Figure 2). At the same time, the rate of the reaction decreased for all three cosolvents (Figure 3). Therefore, a dramatic solvent effect was observed, where not only was the enantioselectivity significantly improved to $E > 200$, but remarkably the enzyme was also able to maintain low diastereoselectivity for the *E*- and *Z*-isomers as well. This was essential to maximize the overall yield of the reaction because both the *E*- and *Z*-(1*R*,5*R*)-**2** will be turned into the same intermediate for the synthesis of **3**. Acetone (40%) is ideal for further process development since it allows us to achieve the desired enantioselectivity and maintains a high reactivity (98.1% ee, 37.7% conversion at pH7 after 16 h). Acetone is also inexpensive and easier to handle at large scale for both the reaction and downstream separation.

pH Effect. As the pH changes from 5.0 to 8.0, the rate of the reaction increases without any sacrifice to enantioselectivity (Figure 3). At pH's higher than 8.5, a decrease in ee's is observed presumably due to background chemical hydrolysis. The optimal pH was chosen at 8.0.

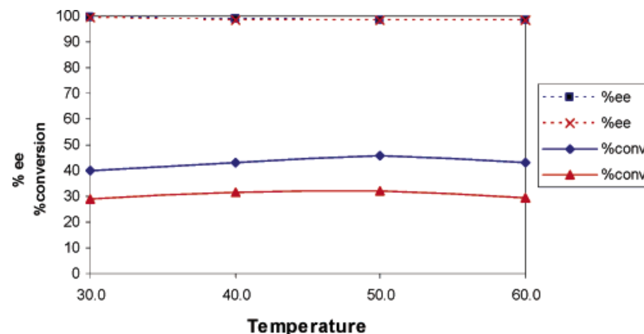

Figure 4. **Effect of temperature on the reactivity and enantioselectivity of CAL-B towards 1. Reaction conditions: 40% cosolvent (red line − *t*-BuOH, blue line − acetone); KPB pH 8.0, 10% enzyme loading (w/w), 100 mg/mL of 1, 16 h.**

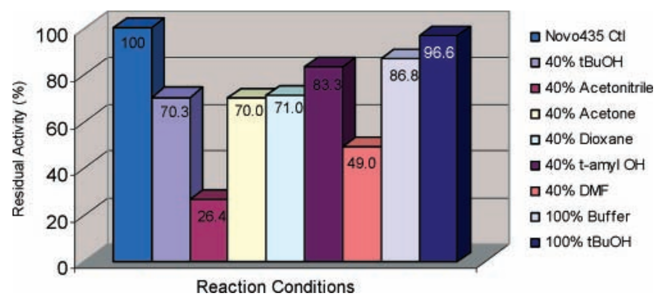

Figure 5. **Effect of cosolvents on enzyme leaching. The residual activity of recycled Novozyme 435 after a 16 h incubation in various organic solvents was measured via a subsequent reaction in KPB buffer at pH 8.0, 10% recycled enzyme (w/w), 100 mg/mL 1, 16 h. Novozyme 435 was recycled via a simple filtration of the incubated solvent/enzyme solutions.**

Temperature Effect. Temperature optimization studies were carried out from 30 to 60 °C. As seen from Figure 4, temperature has a minor effect on both the reactivity and enantioselectivity of the reaction. Therefore, all subsequent reactions were run at room temperature for higher process efficiency.

Enzyme Sources. To identify the ideal CAL-B form for process development, different forms of the enzyme from various suppliers were selected for comparison including Chirazyme L2 solution from Roche, Novozyme 435 from Novozyme, cross-linked enzyme crystal (CLEC) CAL-B from Altus, Chirazyme L2 Carrier Fixed (C.F.) from Roche, and Chirazyme L2 Lyo powder from Roche). All forms of CAL-B showed similar reactivity and price profile except for the CLEC and the Chirazyme L2 C.F., which are less reactive. In general, the lypophilized forms are substantially more expensive than the crude extracts. Ultimately, Novozyme 435 was selected, as it can be readily recycled to further enhance the process efficiency, resulting in simpler workup and reduction in the cost of goods.

Enzyme Recycling Studies. Novozyme 435 is an immobilized form of CAL-B through physical absorption to the macroporous acrylic resin. Therefore, solvents will have a strong effect on enzyme leaching and its residual activity after solvent incubation. Among the eight solvents studied, acetonitrile has the strongest negative effect on leaching (Figure 5). Over 70% of the activity was lost after incubation in 40% acetonitrile for 16 h compared to the nonincubated

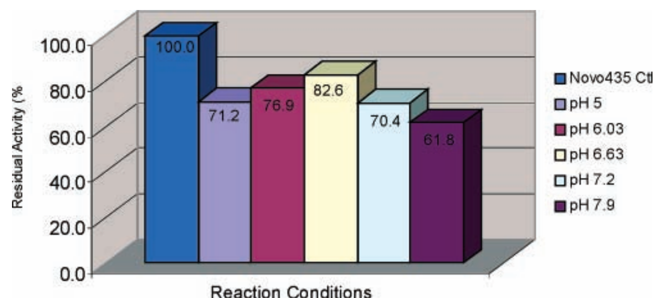

Figure 6. **Effect of pH on enzyme leaching. The residual activity of recycled Novozyme 435 after a 16 h incubation in various buffer pH's was measured via a subsequent reaction in KPB buffer at pH 8.0, 10% recycled enzyme (w/w), 100 mg/mL 1, 16 h. Novozyme 435 was recycled via a simple filtration of the incubated buffer/enzyme solutions.**

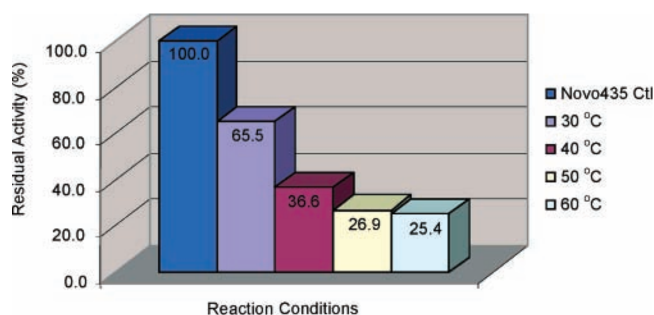

Figure 7. **The effect of temperature on enzyme leaching. The residual activity of recycled Novozyme 435 after a 16 h incubation in KPB buffer at various temperatures was measured via a subsequent reaction in KPB buffer at pH 8.0, 10% recycled enzyme (w/w), 100 mg/mL 1, 16 h. Novozyme 435 was recycled via a simple filtration of the incubated buffer/enzyme solutions.**

control (Novo 435 Ctl, Figure 5). In contrast, in 40% acetone, not only does CAL-B retain most of its activity (70%) after one recycle run, it is also more active than other solvents under the same cosolvent content (see Medium Study section). Essentially no loss in activity was observed in 100% *t*-BuOH for this form of CAL-B. It should also be noted that the same enzyme retains almost 87% of the activity after incubation and recycling from 100% aqueous phosphate buffer. On the other hand, pH does not have a significant effect on the recycling of Novozyme 435, and pH 8.0 was chosen due to the high activity of CAL-B under the condition (Figure 6). Temperature has a strong effect on the leaching and residual activity of Novozyme 435, which lost almost 75% of the activity after incubation in a buffer at 50−60 °C for 16 h (Figure 7). Therefore, the reaction should be conducted at room temperature.

Process Description. Under the optimal reaction conditions (Novozyme 435, 40% acetone, pH 8.0, rt), the reaction was initially demonstrated at 100-g scale. With a substrate loading of 100 g/L and an enzyme loading of 25 g/L, the reaction reached 45−50% conversion in a batch reactor controlled by an auto pH titrator within 24 h. After filtration of the immobilized enzyme, the desired mixture of *E/Z*-(2*R*,5*R*)-bicyclo[3.2.0]hept-6-ylidene-acetate (**2**) was isolated with a yield of 40−45% and 98.5% ee ($E > 200$ at a conversion of 49%) after removal of the leftover *E/Z*-enantiomer **4** (Scheme 2) (for details see Experimental Section). Both the *E*/*Z*-**2** was carried on to the next step for the synthesis of **3** without further purification. The immobilized enzyme was recycled for the next batch of hydrolytic resolution, leading to lower material cost. Subsequently, the enzymatic process was successfully scaled up to 62.8 kg scale with a reaction volume of 630 L in a 1450-L glass-lined reactor.

NMR Study of the Solvent Effect. Due to the dramatic solvent effect observed in this case, and the dynamic nature of enzymatic catalysis, we decided to prepare the N^{15}-labeled CAL-B and use NMR to monitor the change of the protein backbone as the content of solvent is increasing. While it is known that solvents have strong effects on both reactivity and enantioselectivity, the fundamental mechanism is still not clear,[3,4] and no NMR studies have been reported to study the solvent effects of N^{15}-CAL-B.[5] The 2D TROSY experiment affords a fingerprint of the protein amide region that is highly sensitive to changes in the protein environment. It is, therefore, a many-parameter NMR probe for studying intermolecular interactions of the protein. Structural and functional changes within a protein created by a chemical or physical event, such as solvation or binding to another molecule, can be probed by monitoring the chemical shift changes in a TROSY spectrum.[6,7] To prepare N^{15}-CAL-B, the CAL-B gene was cloned from the genomic DNA isolated from *Candida antarctica* (ATCC 32657) and amplified by PCR.[8] Subsequently, the N^{15}-CAL-B was expressed in *Pichia pastoris* using a pPIC9 secretion vector (Invitrogen, CA) under a minimal medium supplemented with $^{15}NH_4Cl$. The expressed recombinant protein was purified by Sepharose column.[9,10] Figure 8a shows the 2D, $^1H/^{15}N$ TROSY spectra for CAL-B in the absence (black) and presence (red) of acetonitrile (30 vol %) (for details see Experimental Section). It can be seen that many of the CAL-B amide resonances are shifted upon the addition of acetonitrile. A possible interpretation of these data is that CAL-B adopts a very different conformation in the presence of acetonitrile as compared to the normal H_2O/buffer state. It should be noted that the TROSY spectrum from the normal state was observed again when the 30% acetonitrile was removed from the mixture (Figure 8b). We are currently preparing an inactive mutant of CAL-B (Ser105Ala in the catalytic triad) which eventually shall allow an in situ monitoring of the backbone change during reaction when solvent contents change. The inactive form may also help to understand the substrate solvation using microcalorimetry. Solvation studies of CAL-B crystals in various solvent contents may also

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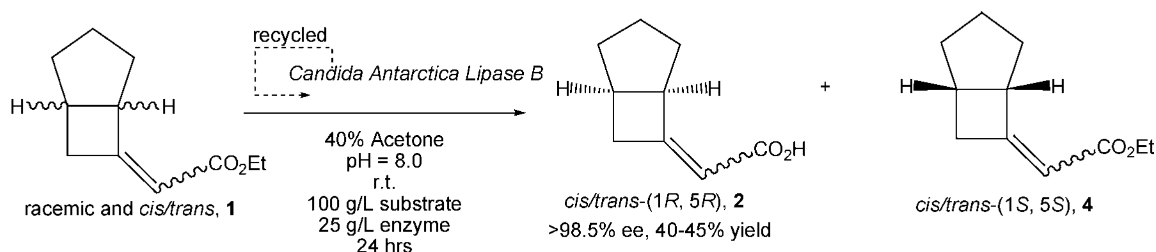
Scheme 2



provide useful information on the solvent effect at the molecular level.[3]

Conclusions

The challenge in this process was to identify an enzyme that is selective towards both the *E*- and *Z*- diastereomers of one enantiomer leaving behind the *E/Z* isomers of the other enantiomer. The only enzyme identified upon screening was *Candida antarctica* lipase B, but it exhibited an *E* value of only 2.7 under screening conditions with 10% organic cosolvent. Comprehensive medium engineering yielded a dramatic effect, improving the *E* value of CAL-B to >200 towards this substrate using 40% acetone. This process was further optimized using an immobilized CAL-B to reduce the cost of goods by enzyme recycling and to enhance process efficiency through simpler workup. Finally the enzymatic transformation was successfully scaled up to 62.8 kg scale in a batch reactor. This process represents the first synthesis of enantiomerically pure *E/Z*-(2*R*,5*R*)-bicyclo[3.2.0]-hept-6-ylidene-acetic acid, a key intermediate needed for the preparation of GABA analogues. This is a rare example where enzymatic resolution involved four stereoisomers and the success resulted from a strong solvent effect. A preliminary 2D NMR study using N^{15}-CAL-B indicates the organic solvent probably has a global effect on the change of protein conformation.

Experimental Section

General Procedure. For a general procedure of enzyme screening in a 96-well plate please see ref 2. The majority of enzymes utilized in the preparation of screening kits were obtained from various enzyme suppliers including Amano (Nagoya, Japan), Roche (Basel, Switzerland), Novozymes A/S (Bagsvaerd, Denmark), Altus Biologics Inc. (Cambridge, MA), Biocatalytics (Pasadena, CA), Toyobo (Osaka, Japan), Sigma-Aldrich (St. Louis, MO). Reactions were performed in an Eppendorf thermomixer-R (VWR), and HPLC analysis was carried out by an Agilent 220 HPLC autosampler. Solvents were obtained from EM Science (Gibbstown, NJ) and were of the highest purity available. All reactions involving air-sensitive reagents were performed under dry nitrogen. All materials obtained from commercial suppliers were used without further purification. ^{1}H NMR spectra were recorded at 400 MHz on a Varian Mercury spectrometer with the solvent as an internal standard (δ H: $CDCl_3$ 7.26 ppm). ^{13}C NMR spectra were recorded at 75 MHz on a Varian Inova spectrometer with the solvent as the internal standard (δ C: $CDCl_3$ 77.03 ppm). Chiral purity was measured by HPLC employing a Chiralpak AS-RH (4.6 mm × 150 mm) at a column temperature of 20 °C and UV wavelength of 225 nm. The gradient elution is tabulated below, and the flow rate was 0.7 mL/min. Sample concentration was 1 mg/mL, and the retention times were observed as follows; (1*R*,5*R*,*E*)-bicyclo[3.2.0]hept-6-ylideneacetic acid 12.1 min; (1*R*,5*R*,*Z*)-bicyclo[3.2.0]hept-6-ylideneacetic acid 12.7 min; (1*S*,5*S*,*E*)-bicyclo[3.2.0]hept-6-ylideneacetic acid 13.5 min; (1*S*,5*S*,*Z*)-bicyclo[3.2.0]hept-6-ylideneacetic acid 18.0 min.

Ethyl Bicyclo[3.2.0]hept-6-ylideneacetate (1). Bicyclo-[3.2.0]hept-2-en-6-one (42 kg, 388 mol) was dissolved in *n*-heptane (82 L), and 5% Pd/C (50% wet, 0.12 kg) was added. The reaction mixture was hydrogenated at 30 psi (1551 Torr) of hydrogen and 30 °C for 8 h. The reaction mixture was filtered, and the vessel and catalyst residue were washed with *n*-heptane (20 L). The filtrate was then used directly in the next step without further purification. Separately, to a suspension of sodium ethoxide (27.2 kg, 400 mol) in *n*-heptane (107 L) was added triethylphosphonoacetate (95.9 kg, 428 mol) over 1 h, maintaining the temperature at ←45 °C. The reaction mixture was then stirred at −45 °C for 75 min. The solution of bicyclo[3.2.0]heptan-6-one in *n*-heptane (388 mol) from the previous step described above was added over 2.5 h, maintaining the temperature at −48 to −38 °C. The reaction mixture was then stirred at − 45 °C for 2 h and quenched with a 25% aqueous solution of hydrochloric acid (80 L). The phases were allowed to warm to room temperature and then separated. To the *n*-heptane phase was added a 17.6% Na_2CO_3 solution in water (375 kg) prepared by dissolving Na_2CO_3 (66 kg, 623 mol) in water (309 L). The mixture was stirred for 10 min, and the phases were separated. To the *n*-heptane phase was added water (202 L), the mixture was stirred for 10 min, and the phases were separated. The organic phase was concentrated in vacuo to give the product (60.8 kg, 337 mol) in 87% yield as a slightly yellowish oil (*Z*:*E* ratio typically >4:1): ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.58−5.55 (m, 1H, *Z* isomer), 5.54−5.52 (m, 1H, *E* isomer), 4.20 (q, 2H, *E* isomer), 4.14 (q, 2H, *Z* isomer), 3.76−3.70 (m, 1H, *E* isomer), 3.41−3.35 (m, 1H, *Z* isomer), 3.25−3.15 (m, 1H, *Z* isomer), 2.90−2.82 (m, 1H, *E* + *Z* isomers), 2.60−2.50 (m, 1H, *Z* isomer), 2.30−2.20 (m, 1H, *E* isomer), 2.05−1.97 (m, 1H, *E* isomer), 1.80−1.50 (m, 6H, *E* + *Z* isomers), 1.28 (t, 3H, *E* + *Z* isomers) in agreement with literature data.[1]

K_{cat} Measurement. The *Z*-(1*R*,5*R*)-ethyl bicyclo[3.2.0]-hept-6-ylidene-acetate was prepared by prep-HPLC, and its concentrations were varied from 8 to 5000 μM in the presence of 5 μL of CAL-B (20.6 μM). After 3 min, the reaction was quenched by the addition of a 10% 3.2%-TFA solution. K_{cat} and K_m were calculated using the Kaleidagraph 3.5 program. In the presence of 10% acetonitrile at room temperature, V_{max} = 59.4 μM/min or K_m = 303 μM and K_{cat} = 115 min^{-1}.

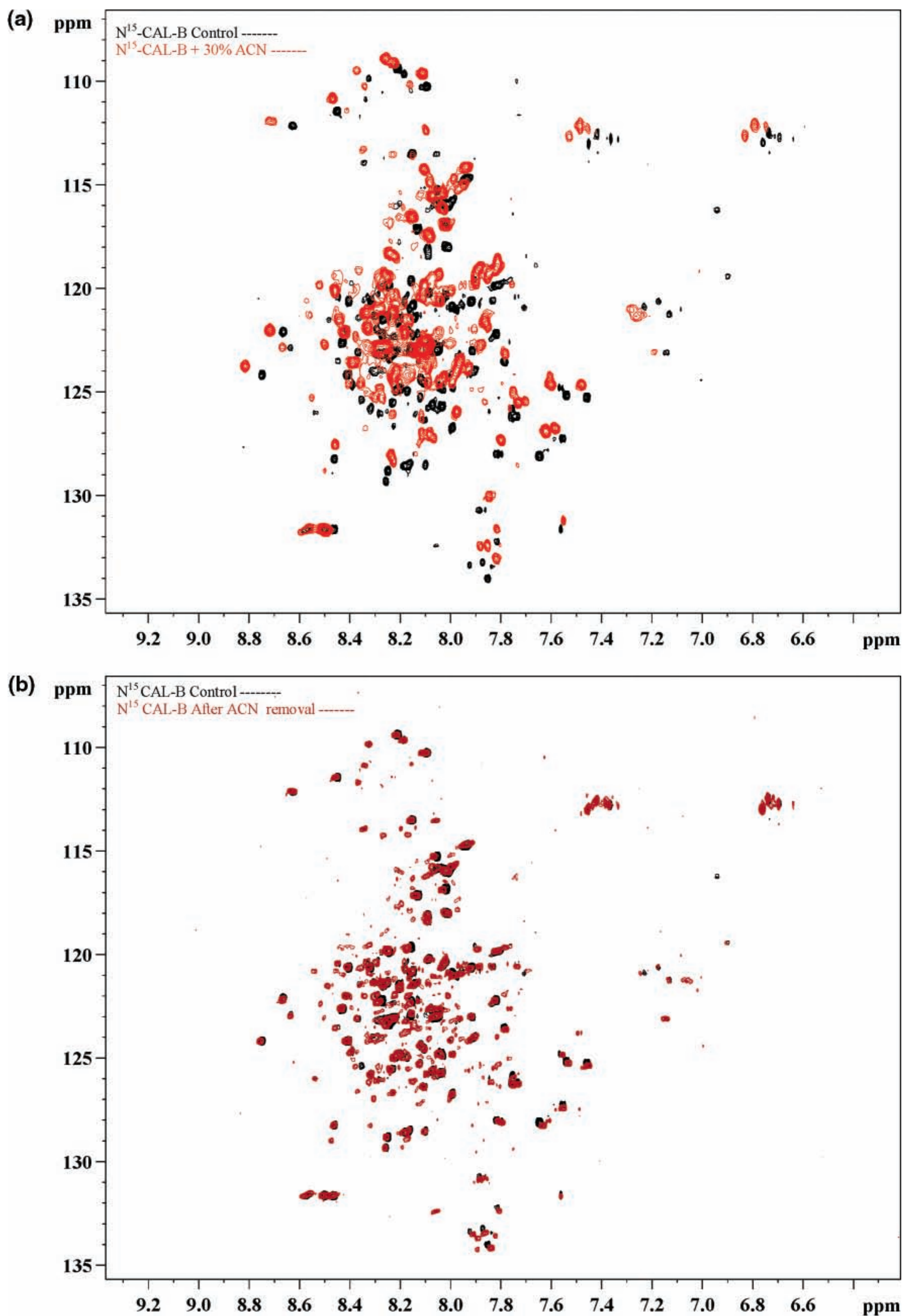


Figure 8. **(a) 2D $^{1}H/^{15}N$ TROSY spectra. The N^{15}-CAL-B control spectrum is displayed in black, and the N^{15}-CAL-B containing 30% acetonitrile is shown in red. (b) 2D $^{1}H/^{15}N$ TROSY spectra. The N^{15}-CAL-B control spectrum is displayed in black, and the N^{15}-CAL-B after acetonitrile removal is shown in red.**

(1*R*,5*R,E*)-Bicyclo[3.2.0]hept-6-ylideneacetic Acid (*Z*-2). To a reaction flask equipped with a pH electrode, an overhead stirrer, and a base addition line controlled by a 718 Stat titrino-metrohm pH titrator was added acetone (400 mL) and potassium phosphate buffer (pH = 7.2, 600 mL). The pH of the resulting mixture was adjusted to pH 8.0, and 25

Table 1

time (min)	0.1% TFA/acetonitrile (%)	0.1% TFA/water (%)
0.00	30	70
15.00	30	70
22.00	10	90
23.00	30	70
33.00	30	70

g of Novozyme 435 was added. The suspension was then stirred at room temperature for 1 min, and the racemic ethyl ester **1** (100 g) was added to the mixture. The reaction was monitored by RP-HPLC looking at both the conversion and ee's of the product, and was stopped after about 45−50% of the starting material had been consumed (approximately 24 h). After the reaction was complete, the heterogeneous mixture was filtered to recover the immobilized enzyme. The reaction mixture was concentrated under vacuum to remove the 400 mL of acetone. The aqueous solution was then acidified to pH 4.0 with 1 N HCl, and extracted three times with MTBE. The acid and ester fractions were pooled, dried with sodium sulfate, and concentrated by rotary evaporation. A vacuum distillation was then carried out to separate the acid product **2** as a *E/trans* mixture (>98.5% ee by chiral HPLC with 40−45% yields) from the residual ester: ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.58−5.55 (m, 1H, *Z* isomer), 5.54−5.52 (m, 1H, *E* isomer), 3.77−3.70 (m, 1H, *E* isomer), 3.43−3.35 (m, 1H, *Z* isomer), 3.26−3.16 (m, 1H, *Z* isomer), 2.91−2.82 (m, 1H, *Z* isomer), 2.61−2.51 (m, 1H, *Z* isomer), 2.32−2.22 (m, 1H, *E* isomer), 1.83−1.51 (m, 6H, *E* + *Z* isomers). ^{13}C NMR (75 MHz, $CDCl_3$) δ 175.2, 172.5, 112.5, 48.9, 37.5, 35.7, 33.1, 32.5, 24.6.

Alternatively, the product can be obtained by an acid/base workup (62.8-kg scale): the enzyme was removed by filtration using a centrifuge after the reaction was finished from an initial set up with 62.8 kg of racemic *E/trans*-ethyl bicyclo[3.2.0]hept-6-ylideneacetate in 248 L of acetone and 512 L of buffer. The enzyme was washed with *n*-heptane (313 L), and the pH of the filtrate was adjusted to pH 2 using concentrated hydrochloric acid (15.5 L). The aqueous layer was separated and extracted with MTBE (150 L). The combined organic layers were extracted with 1 N NaOH (121 L) and the layers separated. The organic phase was extracted a second time with 1 N NaOH (50 L), and the layers were separated. The combined basic aqueous layers were washed twice with *n*-heptane (2 × 54 L) to remove the starting material. The pH of the aqueous phase was then adjusted to pH 1−2 using 36% commercial grade concentrated hydrochloric acid (30 L) and extracted twice with MTBE (2 × 155 L). The combined MTBE extracts were then washed with water (100 L) and concentrated in vacuo to give the crude product (17.2 kg) raw as a pale-yellow oil, which can be further purified as a white powder by crystallization in ethanol−water (2.2:3.4 v/v) with a melting point of 68 °C by DSC.

N^{15}-CAL-B Cloning, Expression, and Purification. The genomic DNA was isolated from *Candida antarctica* (ATCC 32657) and the CAL-B gene amplified by PCR (polymerase chain reaction) using primers CTTCGAATTCCTACCTTC-CGGTTCGGACC and GCGGATACAGCGGCCGCGGG-GGTGACGATGCCGGAG (see ref 8). The protein was cultured and expressed in *Pichia pastoris* using a pPIC9 vector. The CAL-B construct was transformed into GS115 strain. Transformants were screened for mut+ phenotype following manufacturer's recommended protocol (Invitrogen product manual, CA). In general, a single colony was inoculated into 25 mL of culture media and grown until OD 2−6 (18−24 h). The cells were then spun down, and the pellet was resuspended to an OD of 1.0 in minimal media to induce expression. Methanol was then added every 24 h (1%), and the culture was induced for 72 h (for details, see the Invitrogen manual). The media supernatant was tested for the activity using the ethyl ester **1**. Once the activity of the CLA-B was confirmed, steps were taken to minimize degradation by changing growth media to BMG media (phosphate buffer, pH6.0). One set was grown in the absence of exgenous isotopes, and the other was grown with $^{15}NH_4$-Cl (3 g/L) (see ref 10). Isotope labeled cultures demonstrated comparable expression (data not shown). The protein content was determined using a Bradford assay and was estimated to have an expression of 15 mg/L of cultures. Using a 1-mL butyl sepharose column, 500 mL of the filtered protein-containing media was purified. For purification, ammonium acetate was added to the supernatant to reach final concentration of 0.8 M. Onto a 1-mL butyl sepharose column, was loaded 500 mL of the sample without concentration. The supernatant was applied to the butyl sepharose column and eluted with a gradient of 0.8 M ammonium acetate in phosphate buffer (50 mM) and water. The eluted fractions were tested for activity, and fractions containing the desired purity (B10-A11) were pooled to get a total of 2 mg of N^{15}-CAL-B.

NMR Analysis. NMR spectra were recorded at 30 °C using a Bruker-Biospin AV700 spectrometer operating at 700 MHz for ^{1}H and equipped with a 5 mm TCI *z*-gradient Cryoprobe. TROSY (transverse relaxation optimized spectroscopy) data were acquired using the Bruker pulse sequence trosyf3gpphsi19 (see refs 6−7); 256 t_1 increments were collected using 128 acquisitions per increment, and 4096 t_2 data points. Prior to Fourier transformation, each dimension was apodized by a 90 degree shifted sine-bell function and zero-filled in t_1 to form a final matrix size of 2048 × 512. Data processing and acquisition were performed using the TOPSPIN 1.3 software package (Bruker-Biospin). Proton chemical shifts are referenced to internal 3-(methylsilyl) propane-1,1,2,2,3,3,-d_6-sulfonic acid sodium salt (DSS). The ^{15}N chemical shifts are referenced indirectly to DSS using the absolute frequency ratios.

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