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Development of a Practical, Biocatalytic Reduction for the Manufacture of (*S*)-Licarbazepine Using an Evolved Ketoreductase

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Supporting Information

ABSTRACT: This contribution describes the development of a ketoreductase enzyme over four rounds of directed evolution and the associated process development that enabled a practical, scalable process to (*S*)-licarbazepine (eslicarbazepine).

INTRODUCTION

Eslicarbazepine acetate (Aptiom in U.S.A.; Exalief in EU) (**3**) is approved as a voltage-gated sodium channel inhibitor for the treatment of epileptic seizures in adults.[1] It is an acetate ester prodrug of (*S*)-licarbazepine (**2**), which itself is an active metabolite of oxcarbazepine (Trileptal) (**1**) (Figure 1).[2]

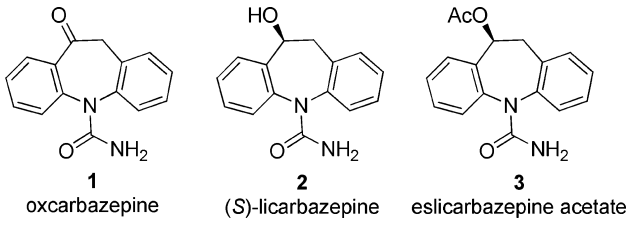

Figure 1. Structural relationships of carbazepine APIs.

Reported syntheses of eslicarbazepine are summarized in Table 1 and include classical resolution of diastereomeric intermediates,[1b,3] asymmetric reduction of the ketone in oxcarbazepine (**1**) to (*S*)-licarbazepine (**2**) via asymmetric hydrogenation[4] and Corey–Bakshi–Shibata catalyzed hydroboration followed by esterification.[5] Enzymatic processes involving hydrolytic resolution of racemic eslicarbazepine esters have also been reported.[6] The chemical and biocatalytic resolution approaches are naturally limited to a maximum yield of 50% and in general are two-step processes with modest yields in the recovery of eslicarbazepine in the second step. Asymmetric transfer hydrogenation is attractive as it can be high yielding and highly selective, but this has to be weighed against the dependence on complex ligands that are potentially air sensitive and the use of expensive rare metals, the prices of which are market driven and difficult to predict in the long term.

Biocatalytic asymmetric reductions of oxcarbazepine (**1**) have been described using either *Saccharomyces cerevisiae*[7] or *Pichia methanolica*[8] whole cell systems, and although these processes provide the desired product in high enantiomeric purity, their overall poor volumetric productivities and high catalyst loadings render them unsuitable for practical, large-scale use. However, biocatalytic reduction does offer the possibility of high yield and high selectivity similar to the hydrogenation approaches, but by using sustainably produced enzymes under inherently safer and greener conditions.

The continuing advances in modern directed-evolution technologies, such as Codexis' Code Evolver platform, have provided rapid and efficient access to improved enzyme variants such that substandard enzyme performance no longer needs to be tolerated.[9] Enzymes can now be engineered to operate under a much wider range of nonphysiological, process relevant conditions and can provide greener processes than traditional chemistries when the catalytic activity is high and the reaction workup is straightforward.[10] A number of high-volume API intermediates and APIs can be produced with such evolved proteins as catalysts which often provide economic advantages over traditional chemical methods.[11]

Herein we report the biocatalytic reduction of oxcarbazepine (**1**) using an isolated ketoreductase (KRED) specifically evolved for the efficient manufacture of (*S*)-licarbazepine (**2**) in >99% conversion with >95% isolated yield, >98% chemical purity and >99% ee with a substrate concentration of 100 g/L (0.39 M) and beyond and enzyme loading of 1% w/w with respect to substrate (Scheme 1).[12] The enzymatic step was conducted in aqueous triethanolamine buffer using isopropanol (IPA) as both cosolvent and terminal reductant, and was successfully demonstrated at a pilot 500 mL scale.

RESULTS AND DISCUSSION

Enzyme Screening and Evolution. Initial screening of the KRED Codex panel plates showed that the parent wild-type KRED from *Lactobacillus kefir* was inactive, but gratifyingly identified several variants capable of the conversion of oxcarbazepine to (*S*)-licarbazepine with high enantioselectivity (>98% ee) albeit with low substrate loading (~2 g/L). The top

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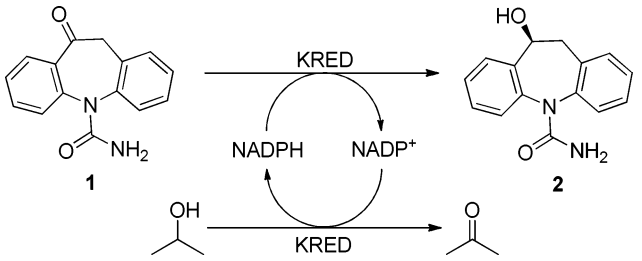
Table 1. Synthetic approaches to eslicarbazepine

ref	synthetic approach	yield (%)	HPLC purity (%)	selectivity (% ee)
3a	diacetyl tartrate half ester formation and crystallization followed by base hydrolysis	49 (ester) 84 (eslicarbazepine)	not given	96
3b	(*S*)-ibuprofen half ester formation; purification, followed by base hydrolysis	23 (ester) 87 (eslicarbazepine)		99.7 (ester)
3c	(*S*)-ibuprofen half ester formation; purification, followed by base hydrolysis	93 (ester) no yield given (eslicarbazepine)	99.8	92 (ester) 98
3d	acetyl (*R*)-mandelic half ester formation followed by base hydrolysis	no yield given (ester) 88 (eslicarbazepine)	not given	97.6 (ester)
4a	transfer hydrogenation of oxacarbazepine	no yield given	not given	>99
4b	transfer hydrogenation of oxacarbazepine	95	99.6	97.8
4c	transfer hydrogenation of oxacarbazepine	74	99.4	98.8
4d	transfer hydrogenation of oxacarbazepine	86	not given	99.4
4e	transfer hydrogenation of enol acetate	no yield given	not given	28−94 (of acetate)
5a, b	*R*-MeCBS catalyzed borane reduction	55	96.6	93.6
5a	naproxen ester followed by base hydrolysis	25 (ester) 50 (eslicarbazepine)	91.8 (ester) 96.7	93.9 (ester) 92.3
5c	D-tartaric acid and phenylboronic acid moderated $NaBH_4$ reduction of oxacarbazepine	70	99.5	96.4
6a	enzymatic hydrolysis of methoxyacetate ester, removal of undesired alcohol by hemisuccinate formation; sodium hydroxide hydrolysis of isolated ester	40 (methoxyacetate) no yield given (eslicarbazepine)	99.4	99.99

Scheme 1. Enzymatic synthesis of (*S*)-licarbazepine



performing variants were then tested at small scale under more challenging conditions (100 g/L oxcarbazepine and 5 g/L enzyme) and Variant 1 was found to give ∼5−10% conversion. This activity was insufficient for a practical process for the large-scale manufacture of (*S*)-licarbazepine, and Variant 1 was improved via directed evolution towards a practical and economic process.

Libraries of variant KREDs were generated and in total approximately 15,000 KRED variants were screened under process-like conditions over four rounds of evolution. It is critical to reflect the desired end process conditions in the screening assay used for enzyme evolution so that the correct evolutionary pressure is maintained. The screening therefore targeted mutations (and variants) that enabled low cofactor loading (to minimize cost), high IPA concentrations (to maximize solubility and drive the equilibrium), and high temperature (to increase reaction rate and improve solubility). The screening of the enzyme libraries was performed in 96-well high throughput (HTP) format (HTP assay >600 variants/day) under conditions mimicking the final process conditions, *vide infra*. The DNA of variants that showed improved product formation compared to control variants was sequenced to identify the mutations beneficial for the desired process. The mutations found in the improved variants were collected, analyzed, and combined in subsequent rounds of evolution.

KRED-mediated reductions are equilibrium reactions that need to be driven, in this case with IPA as the reductant for cofactor ($NADP^+$) recycling. A low level of $NADP^+$, 0.05 g/L, was used in screening from the outset to minimize the use of expensive cofactor in the process. Initial process development scoping studies performed using Variant 1 confirmed the importance of high IPA concentration in shifting the reaction equilibrium to the desired product and increasing the solubility of substrate in order to achieve product titers of ∼100 g/L. Therefore, round 1 of evolution targeted increased IPA tolerance. In the subsequent rounds of evolution the reaction parameters were gradually changed so that sufficient activity

Table 2. Round-to-round HTP screening conditions and performance improvements

evolution round	variant selected from round	improvement compared to predecessor (improvement from Variant 1)	total # of mutations vs wild type (∼# of variants screened)	screening conditions
wild type	*L. kefir*	no activity for wild type	–	–
initial screen	Variant 1	N/A	9 (400)	5 g/L oxcarbazepine, 5% (v/v) DMSO,[a] 20% IPA, 70% (v/v) crude lysate, 25 °C
round 1	Variant 2	1.5 (1.5)	10 (5800)	5 g/L oxcarbazepine, 5% (v/v) DMSO,[a] 70% IPA, 20% (v/v) crude lysate, 40 °C
round 2	Variant 3	10 (15)	13 (1200)	5 g/L oxcarbazepine, 5% (v/v) DMSO,[a] 70% IPA, 10% (v/v) crude lysate, 40 °C
round 3	Variant 4	1.3 (20)	19 (6900)	75 g/L oxcarbazepine, 70% IPA, 10% (v/v) crude lysate, 50 and 57 °C
round 4	Variant 5 *aka* CDX-021	1.3 (26)	30(1300)	75 g/L oxcarbazepine, 70% IPA, 1% (v/v) acetone, 10% (v/v) crude lysate, 57 and 62 °C

[a]Small amount of DMSO was used to prepare a solution of oxcarbazepine to aid the dispensing of the oxcarbazepine into 96 well plates. Not required in final process.

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Table 3. Round-to-round process parameters and enzyme performance

parameter	Variant 1[a] (screening hit)	Variant 2[a]	Variant 3[b]	Variant 4[c]	Variant 5[c]
substrate (g/L)	100	100	100	100	100
enzyme (g/L)	5	5	3	1	1
$NADP^+$ (g/L)	0.5	0.1	0.1	0.1	0.1
IPA (% v/v)	20	70	70	70	70
temp (°C)	25	45	45	55	55
pH	7.0	9.0	9.0	9.0	9.0
ee (% *S*)	99	>99	>99	>99	>99
time (h)	24	24	24	28	<24
conversion (%)	7	15	99	99	99
n-fold improvement over Variant 1	1	2	18	60	71

[a]Lyophilized enzyme powders on 10 mL scale. [b]Lyophilized enzyme powders on 10 mL scale with nitrogen sweep (acetone removal). [c]Lyophilized enzyme powders on 500 mL scale with nitrogen sweep (acetone removal).

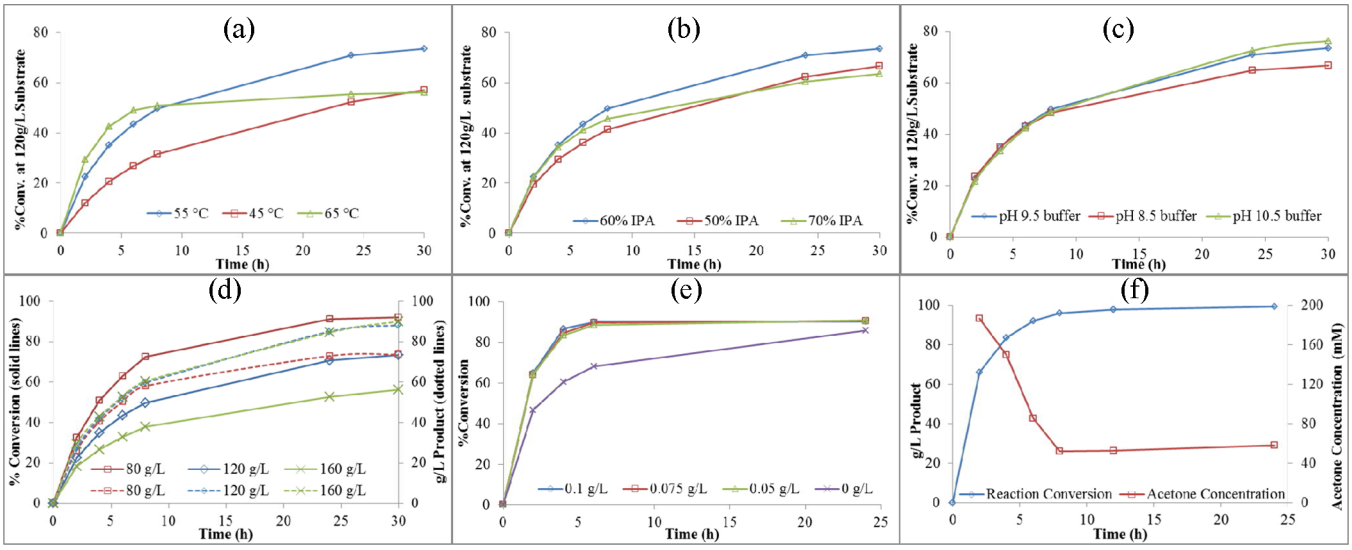

Figure 2. (a) Effect of temperature on reaction kinetics. (b) Effect of % v/v IPA on reaction kinetics. (c) Effect of buffer pH on reaction kinetics. (d) Effect of substrate loading on % conversion and g/L product produced. (e) Effect of $NADP^+$ loading on reaction kinetics. (f) 500 mL scale reaction under optimized conditions.

could be detected in the HTP screening as shown in Table 2. In the final round of evolution, as acetone is produced as a stoichiometric byproduct during the reaction, a small amount of acetone was introduced to the assay to ensure that the enzyme's acetone tolerance was not lost. The temperature was also raised further to instill greater thermostability to the enzyme. Although only a small 1.3-fold improvement was seen in the HTP performance in the final round from Variant 4 to Variant 5 (Table 2) and a similar 1.2-fold improvement in the 10-mL-scale reactions (Table 3), the thermostability of Variant 5 was significantly improved. Variant 5 retained 83% of its activity after 16 h incubation challenge at 60 °C compared to 53% retained activity for Variant 4. This improved thermostability gives a more robust catalyst that can tolerate short temperature excursions and localized hot spots during scale up to manufacturing scale.

After each round of screening selected top variants were produced at gram scale and isolated as a lyophilized powder. These enzyme powders were then used in a small scale preparative reactions to confirm enzyme performance (Table 3).

Process Optimization and Robustness Analysis via DoE. Critical process parameters such as temperature, % v/v IPA, reaction pH, and substrate loading were stressed using factorial design in a DoE study in order to analyze the effects on reaction rate, process robustness and to define any process limitations. Thirty-two experiments were conducted including eight center points, on 10 mL scale. These reactions were monitored over 30 h and quenched, and the results were used for DoE analysis. The center points for the study were set at 55 °C, 60% v/v IPA, pH 9.5, and 120 g/L substrate loading, and the parameters ranged across 45−65 °C, 50−70% v/v IPA and pH 8.5−10.5. Enzyme and $NADP^+$ loading were kept constant at 0.25 g/L and 0.1 g/L, respectively.

Effect of Temperature. As expected the reaction initial rate increased with increasing temperature (Figure 2a). However, at 65 °C the reaction slowed down after 10 h, indicating poor stability of either the enzyme or the cofactor $NADP^+$ (or both) at this temperature. At all pHs in the design space (with fixed 120 g/L substrate loading and 60% v/v IPA, Figure 3a) the maximum of the response curve shown in green indicated that a temperature range of 53−57 °C was operationally optimal. A temperature of 55 °C was chosen as the optimum process temperature as the enzyme demonstrated considerable stability and activity at that temperature.

Effect of isopropanol/buffer ratio. Comparable initial rates were observed for reactions conducted with 50−70% v/v IPA (Figure 2b). A decrease in final conversion was observed

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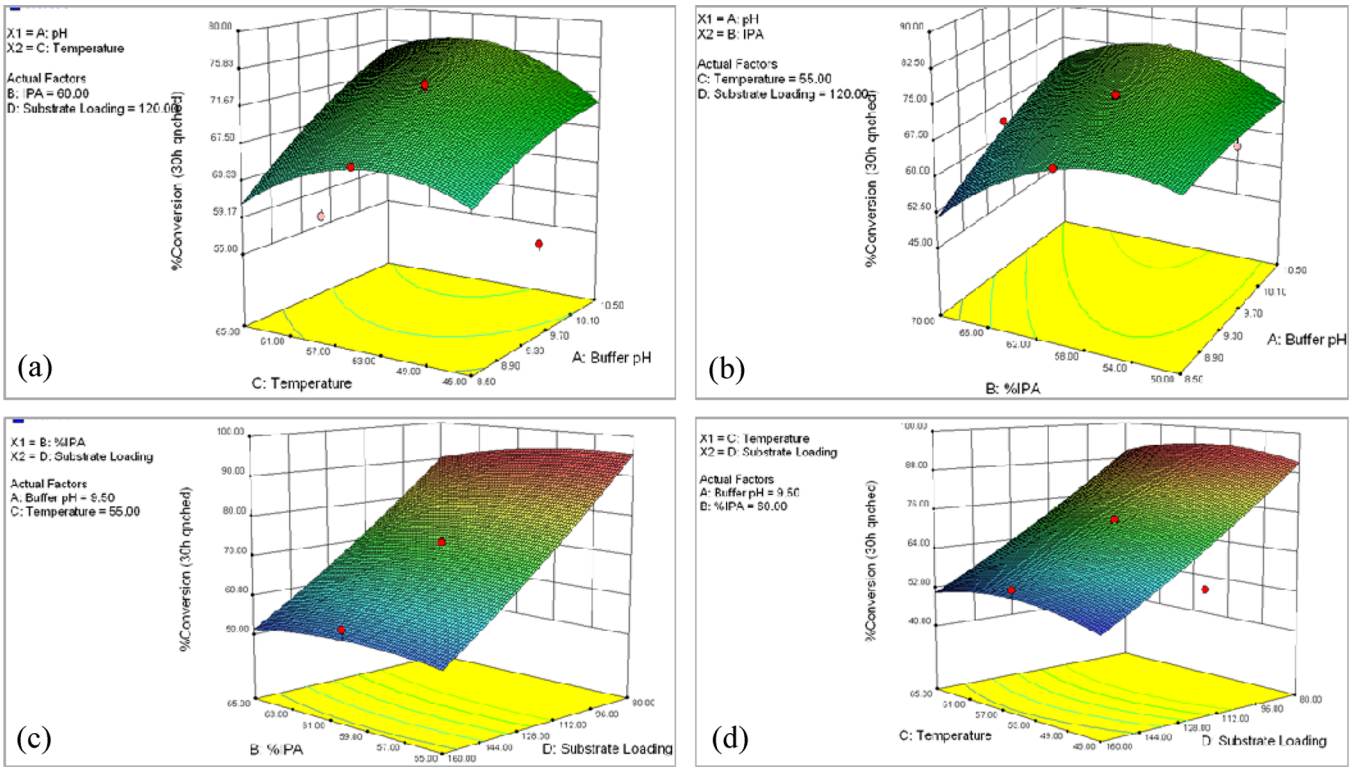

Figure 3. (a) Response surface at 120 g/L substrate and 60% IPA. (b) Response surface at 120 g/L substrate and 55 °C. (c) Response surface at 55 °C and pH 9.5. (d) Response surface at 60% v/v IPA and pH 9.5.

with greater than 60% v/v IPA and was most likely due to decreased enzyme stability. Analysis across a range of 50−70% v/v IPA suggested an operationally optimal range between 55−65% v/v IPA (Figure 3b). The optimum process parameter for the enzymatic reaction chosen was 60% v/v IPA.

Effect of Buffer pH. No significant differences in reaction rate were observed in the pH range of 8.5−10.5 (Figure 2c). 0.1 M Triethanolamine buffer at pH 10.0 was prepared at room temperature and used for the enzymatic reaction without any adjustments upon either addition of substrate or heating.

Effect of Substrate Loading. Although a higher percent conversion was seen with lower substrate loading (Figure 2d, solid lines), the absolute amount of product formed remained the same, (Figure 2d, dotted lines) which indicated little or no substrate inhibition of the enzyme. It was anticipated that with efficient acetone removal from the reaction mixture combined with replenishment of reaction volume, it would be possible to fully convert at least 100 g/L substrate with 1 g/L enzyme. Analyses of substrate loading against both the optimum temperature and optimum IPA loading (55 °C and 60% v/v IPA, Figure 3c,d) suggested an optimal substrate loading of 110−130 g/L in order to maximize product titer in g/L rather than % conversion. Eventually a slightly lower substrate loading of 100 g/L was chosen for the technical transfer in order to minimize potential mass transfer effects on scale up and ensure robust and reproducible performance.

Effect of $NADP^+$ (Cofactor) Loading. $NADP^+$ is a cofactor essential for KRED enzyme activity which was not included as a variable in the DoE. However, isolated robustness tests showed no detrimental effect on reaction rate at a $NADP^+$ loading as low as 0.05 g/L (Figure 2e, tested at 100 g/L substrate loading, 60% IPA, pH 10.0, 55 °C). The manufactured enzyme powder may contain $NADP^+$ from the enzyme manufacturing process, enabling the reaction to progress without additional $NADP^+$. However, for a more robust process, it is desirable to add $NADP^+$ (between 0.05 and 0.1 g/L), as the amount of intrinsic cofactor within the manufactured enzyme powder may vary from batch to batch.

Reaction Scale Up. Upon identification of optimal and robust process conditions (100 g/L substrate, 1 g/L CDX-021, 0.1 g/L $NADP^+$, 60% v/v IPA, pH 10, 55 °C), a scaled up reaction was performed at 500 mL scale. The reaction reached equilibrium at approximately 90% conversion and was driven to completion by the removal of acetone by employing a nitrogen sweep and the simultaneous addition of a pre-mixed solution of IPA and buffer to maintain the reaction volume. Upon completion of the enzymatic reaction, IPA and any remaining acetone were removed by distillation under reduced pressure. Solvent replacement of IPA with water triggered crystallization which upon subsequent filtration afforded the crude desired product **2** in high yield, purity, and enantiomeric excess (96% yield, 98.7 area %, >99% ee). A further recrystallization from methanol improved the purity to 99.6 area % in 90% recovery and removed low level traces of residual enzyme present in the crude product.[13] A typical reaction kinetic profile is shown in Figure 2f.

The ACS Green Chemistry Round Table Process Mass Intensity (PMI) Tool is an open source tool freely available for the standardization of PMI calculations.[14] By using the PMI tool, the biotransformation and the purification were calculated as 15.0 and 19.7, respectively. As PMIs for existing reductions of oxcarbazepine have not been published, direct comparison has not been possible. However, given the catalytic reaction mechanism, low molecular mass of the terminal reductant, and the direct isolation of product from the reaction by distillation, the reaction PMI can only be significantly lowered further if

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Table 4. Isolated CDX-021 KRED vs whole cell performance

biotransformation system	substrate (g/L)	substrate:catalyst ratio (w/w)	conversion (isolated yield) (%)	ee (%)
CDX-021 (60%v/v aq IPA, 0.1 g/L $NADP^+$, 55 °C)[9]	100	100:1 (KRED)	>99 (96)	>99
S. cerevisiae CGMC 226[7] (5%v/v EtOH, 70 g/L glucose, 30 °C)	73	14:1 (dry cells)	99.2 (not reported)	10
P. methanolica 103660[8] (1:1 aq:hexane, 30 °C)	1.5	0.01:1 (wet cells)	>98 (>85)	>98

IPA recycling is implemented and the heptane wash of the product cake is optimized or eliminated.[15] The PMI of the current downstream purification method is higher than that of the biotransformation and isolation due to the relatively high volume of solvents required for dissolution. Future development would be aimed towards optimization of the downstream processing, including solvent recycling where practical, to further reduce the overall PMI.

In summary we have developed a practical, scalable isolated enzyme-mediated process for the production of (*S*)-licarbazepine, that provides important benefits over previously described whole cell processes (Table 4) and chemocatalytic processes (Table 1). The process is currently being assessed for commercial manufacture of eslicarbazepine.

■ CONCLUSIONS

A ketoreductase was evolved to enable a commercially attractive process for a highly efficient biocatalytic reduction of oxcarbazepine, a first for an isolated enzyme to the best of our knowledge. The resulting enzymatic process outperforms the whole cell processes previously reported in terms of volumetric productivity and downstream processing. This process affords a key chiral intermediate of eslicarbazepine acetate in high purity and yield. The enzyme and the process have been successfully transferred to an API manufacturer and proven to achieve results similar to those observed in the laboratory setting.

■ EXPERIMENTAL SECTION

For analytical Methods 1−5 please refer to the Supporting Information.

Preparation of Crude (*S*)-Licarbazepine (2). Triethanolamine buffer (TEoA) solution (0.1 M, pH 10.0) was prepared in a separate vessel by dissolving triethanolamine (13.3 mL) in deionized water (1 L) at room temperature. $MgSO_4 \cdot 7H_2O$ (0.25 g) was added to obtain a final magnesium concentration of 1 mM. The pH of the resulting clear solution was 10.0 (±0.1) at room temperature and was used for reaction without any pH adjustments. A 1 L jacketed reactor was charged sequentially with IPA (300 mL), TEoA buffer (190 mL), and solid oxcarbazepine (50 g). The mixture was stirred at 200 rpm and heated to reach an internal temperature of 55 °C whereupon a pale-yellow slurry was obtained with a pH of 8.7. A stock solution of enzyme (0.5 g) and $NADP^+$ (50 mg) was prepared separately in buffer (10 mL, final pH 8.6), and was charged to the reaction mixture whereupon the reaction mixture was stirred at 55 °C and 200 rpm under a nitrogen atmosphere with a sweep flow rate of 0.8 L min^{-1}. The reaction volume was maintained by the intermittent addition of a premixed solution of 60% IPA and 40% buffer (0.1 M TEoA with 1 mM $MgSO_4$, pH 10.0). The reaction course was followed by periodically taking samples from the reaction mixture, quenching, and analyzing as described in Method 1. Samples were also frequently monitored for acetone content using the procedure described in Method 4. After in-process analyses indicated >99% conversion in 24 h, the pale-yellow turbid reaction mixture was drained from the reactor into a 1 L round-bottom flask. IPA was distilled by rotary evaporation (75 Torr, 50 °C bath). Upon partial concentration of the reaction by distillation, water (100 mL) was added to the white slurry and the distillation continued to completely remove IPA. The crude product was collected by filtration on a Buchner funnel, washed with water (100 mL) and heptane (200 mL),[15] and dried for 24 h in a vacuum oven (2 mbar) at 30 °C. Crude (*S*)-licarbazepine (**2**) (48.0 g, 96% yield) was obtained as an off white solid with a chemical purity of 98.7% (HPLC Method 5) with >99.9% *e.e.* (HPLC Method 3) and a residual protein content of 80 ppm.[13]

Purification of crude (*S*)-licarbazepine (2). A suspension of crude **2** from above (10.0 g) in methanol (100 mL) was heated to 40 °C (internal temperature) to allow maximum dissolution of product. Celite (2.0 g) was added to the slightly turbid solution and the mixture was stirred at 40 °C for 15−20 min. The Celite was removed by filtration through a sintered funnel and the residue was washed with preheated methanol (20 mL, ∼40 °C). The clear filtrate was then distilled under reduced pressure to approximately 30 mL volume. The thick mixture was cooled to 5 °C, cold water (50 mL, 5 °C) was added over 30 min to the white precipitate, and the resulting slurry was stirred at 5 °C for a further 30 min. The precipitated product was filtered through a sintered funnel, rinsed, and washed with 20 mL water before being dried in a vacuum oven for 16 h (30 °C, 2 mbar). Purified product was isolated in a single crop (9.0 g, 90% recovery) as a white solid with 99.6% chemical purity (HPLC Method 5) and a residual protein content of <10 ppm.[13]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Spectra and analytical methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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(13) Protein levels of 80 ppm and <10 ppm were present in the crude and purified products, respectively. The level of residual protein was measured using a commercial UV absorbance-based protein quantification assay (SPN - Assay commercially available from GBiosciences). All materials used were those provided with the commercial kit, and the experimental protocol provided was followed without deviation. The amount of residual protein was calculated on the basis of absorbance measurements from a UV spectrophotometer.
(14) (a) For the freely available PMI tool see www.acs.org/content/dam/acsorg/greenchemistry/industriainnovation/roundtable/process-mass-intensity-calculation-tool.xls. (b) For other green chemistry tools and information see ACS Chemistry Institute Pharmaceutical Roundtable website at www.acs.org/gcipharmaroundtable.
(15) The heptane cake wash was used on this scale to assist more rapid drying and should be readily removed in future scale-up development.