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Synthesis of Vibegron Enabled by a Ketoreductase Rationally Designed for High pH Dynamic Kinetic Reduction

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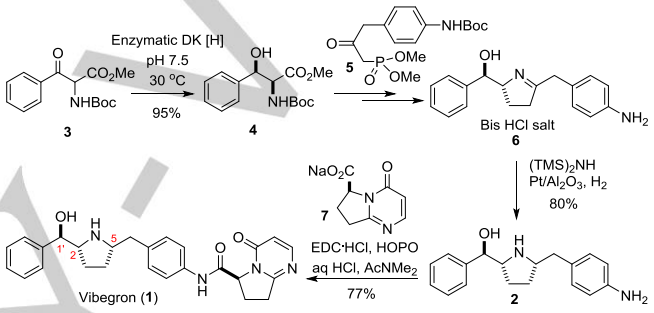
Abstract: Described here is an efficient stereoselective synthesis of vibegron, enabled through an enzymatic dynamic kinetic reduction in a high pH process environment. To overcome enzyme performance limitations under these conditions, a ketoreductase has been evolved using a computationally and structurally aided strategy to increase cofactor stability via tighter binding.

Overactive bladder syndrome (OAB) is a condition of urinary frequency and urgency that negatively affects quality of life. The prevalence of OAB is expected to increase by 18% from 2008 to 2018.[1] Before 2012, muscarinic antagonists were the standard-of-care prescribed pharmacological therapy, but these agents lack sufficient efficacy and have side effects due to blockade of M3 muscarinic receptor.[2] To address the unmet medical need, β_3-adrenergic receptor (β_3-AR) agonists have recently emerged as a promising new class of agents for the treatment of OAB.[3]

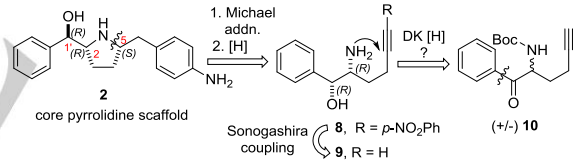
Vibegron[4] (**1**) is a potent and selective β_3-AR agonist currently in Phase 3 clinical trials. It structurally differs from acyclic β-hydroxylamine β_3-AR agonists,[3a-b,4a] and possesses a unique *cis* pyrrolidine moiety linking the C2 and C5 substituents. The discovery of the *cis* pyrrolidine backbone improved the pharmacological properties,[4a] but raised the synthetic complexity.

In 2013 we reported an asymmetric synthesis[5] of *cis*-2,5-disubstituted pyrrolidine **2**, the core scaffold of vibegron. In this approach (Scheme 1), the *R,R* (C1',C2) stereochemistry of **1** was efficiently established through an enzymatic dynamic kinetic (DK) reduction of β-keto ester **3**. The third stereogenic center, C5, was established by diastereoselective hydrogenation of imine **6**. Vibegron (**1**) was obtained after coupling with pyrimidinone acid sodium salt **7**.[6]

The first generation route provided a robust means to prepare large quantities (>100 kg) of vibegron to support early safety and clinical studies. Despite the acceptable overall yield (32%), we envisioned that the atom economy could be improved. The central problem of the first generation synthesis lies in the arduous nature of creating the pyrrolidine imine moiety in intermediate **6** with multiple transformations. The application of a Horner–Wadsworth–Emmons reaction using **5** offered convergence to the first generation route, but the low atom-economy and the phosphorus waste were of concern in the context of an environmentally responsible synthesis for large scale preparation. Furthermore, the transformation of hydroxy amino ester **4** to imine **6** introduced a reduction-oxidation-reduction sequence and protection-deprotection,[5] contributing to synthetic inefficiency.



Scheme 1. Key steps of the first generation process of vibegron (**1**).



Scheme 2. Retro-synthetic analysis of pyrrolidine **2**

We envisioned that a more concise synthesis of vibegron could arise from reducing a corresponding pyrrolidine imine formed by intramolecular Michael addition to aryl alkyne **8** (Scheme 2). The inclusion of an electron-withdrawing nitro group in precursor **8** not only promotes the Michael addition, but also allows for unveiling of the aniline functionality in **2** through a global reduction.

Another synthetic challenge of preparing vibegron is its unique *R,R* (C1',C2) stereochemistry. The methodologies of choice to establish syn stereochemistry of 1,2-amino alcohols in an open-chain system have been lacking.[7] Our previous enzymatic DK reduction approach prepared optically pure syn 1,2 amino alcohol **4** effectively.[5] However, our new strategy (Scheme 2) eliminates the activating ester group that facilitated the epimerization of keto ester **3** at pH 7.5.[5] In contrast, epimerization studies on **10**, prepared by treating amino nitrile **11**[8] with phenyl Grignard reagent (Scheme 3), confirmed that fast racemization could only be realized at elevated temperature (≥ 45°C) and high pH (≥ pH 10). These conditions are generally recognized as harsh for ketoreductases (KREDs), challenging the limits of enzyme performance.

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Table 1. Selected results of DK reduction through KRED evolution

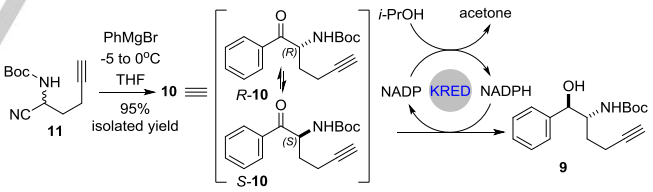
entry	KRED	evolution round	temp. (°C)	pH	substrate concentration (g/L)	enzyme loading (wt% relative to **10**)	conv. (%)[a]	ee (%)[b]	dr (syn/ anti)[b]
1	KRED-P1B2	0	30	7.0	25	35	43[c]	>99	16:1
2	KRED-P1B2	0	30	10.0	15	100	5[d]	>99	n.d.
3	KRED-p101	1	45	10.0	50	200	52	>99	28:1
4	KRED-p201	2	45	10.0	50	25	38	>99	64:1
5	KRED-p301	3	45	10.0	50	1	95	>99.4	>100:1

[a] Unless otherwise noted, reactions were carried out in 50% aqueous *i*-PrOH with 0.2 M borate buffer at pH 10.0 and 0.1 g/L $NADP^+$. [b] Determined by HPLC analysis. [c] In aqueous *i*-PrOH with 0.1 M phosphate buffer at pH 7.0 and 1.0 g/L $NADP^+$. [d] 2g/L $NADP^+$

At the inception of this work, the literature precedence for ketoreductase reactions was limited to pH <8 and ambient temperature, reflecting typical physiological conditions.[9] Examples of enzymatic DK reductions have been reported on β-keto esters or activated ketones with a pKa range of 7–12[5,10] with the exception of rare reports using native extremophile KREDs at pH 9.[10a] Alternatively, a strategy of modifying functional group(s) in precursor **10** to lower the pKa and achieve epimerization at pH <8 was unattractive and would reduce synthetic efficiency. To pursue our retro-synthetic target (Scheme 2), we first needed to identify a KRED that could produce alcohol **9** from **10**. Given the advances in protein engineering technologies,[11] we envisioned tailoring this enzyme to the required reaction conditions by employing directed evolution with computationally and structurally designed mutations to rapidly create an optimal catalyst.

We screened a Codexis® KRED panel under standard aqueous conditions at pH 7–8. The results not only showed excellent reactivity, but also gave us a hit on stereoselectivity. Although epimerization of **10** did not occur at pH 7 and 30°C, kinetic resolution with KRED-P1B2 gave desired alcohol **9** with high dr (16:1) and excellent ee (>99%) (Table 1, entry 1) while the undesired ketone enantiomer *S*-**10** remained intact. As expected, the reaction underperformed with increasing pH, producing only minimal amounts of **9** at pH 10 (Table 1, entry 2). Clearly, variant P1B2 required improvements in both diastereoselectivity and productivity at higher temperature and pH. To address stereoselectivity, we focused on seven positions[8] in and around the substrate binding pocket. These positions underwent saturation mutagenesis in an attempt to identify improved amino acids. Screening of the libraries revealed seven unique mutations at five of the targeted positions that were 1.5 to 13.8-fold improved in activity over P1B2, and a mutation to phenylalanine at position 206 (M206F) which improved the enzyme selectivity. The phenylalanine was structurally predicted to have favorable interaction with the phenyl group of the substrate when bound by the enzyme in a conformation that yields the desired (*R,R*) alcohol **9** (Figure 1A). In contrast, the substrate is disfavored from binding in the opposite conformation that would result in the (*S,R*) or (*S,S*) alcohol **9** due to steric interactions with the Boc or alkyne group (Figure 1B).

In parallel, we targeted to recover overall conversion at the desired conditions. The degradation rate of $NADP^+$ increases significantly above pH 8, in particular at elevated temperature.[12] We concluded that poor performance of the reduction in the basic environment was the result of cofactor rather than enzyme instability. Since the catalytic nicotinamide cofactor cycles between its oxidized and reduced form throughout the reaction (Scheme 3), decomposition of $NADP^+$ eventually exhausts all available cofactor and shuts down the reaction. We hypothesized that improved binding of the cofactor to the KRED's active site would provide a protective environment for the cofactor and increase its stability.



Scheme 3. Enzymatic reduction of ketone **10**.

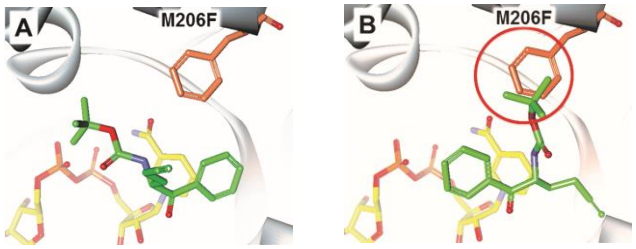


Figure 1. M206F beneficial mutation. The NADPH cofactor, substrate **10**, and M206F mutation are depicted in yellow, green, and salmon, respectively. The docked **10** is shown in a conformation that results in the desired (*R,R*) alcohol **9** at the targeted carbon center in panel A and in the corresponding undesired (*R,S*)-**9** in panel B. The predicted steric clash between the Boc group and the phenylalanine group at position 206 is circled in red.

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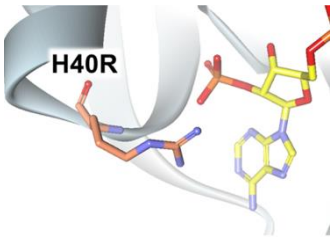


Figure 2. H40R beneficial mutation. The NADPH cofactor and the H40R mutation are depicted in yellow and salmon respectively. The mutation to arginine at position 40 is shown interacting with the phosphate of the cofactor.

We applied a rationally guided strategy, analyzing the KRED cofactor binding pocket for improvements. Position 40 was quickly identified as a good candidate for mutation based on computational structural analysis, and showed benefits from mutating it to an arginine. The H40R mutation displayed favorable electrostatic interactions with the phosphate of NADPH (Figure 2) and likely stabilized binding of the cofactor. Engineering of the H40R mutation into the selectivity hit M206F produced the first round hit KRED-p101. Experimental characterization of this double mutant showed KRED-p101 to be successfully improved in cofactor binding, validating the structural predictions. In the absence of the H40R mutation, variant M206F converts over 40% of 5 g/L **10** in 24 hours at 0.5 g/L cofactor (Figure 3A). However, at 0.1 g/L cofactor, the activity drops by an order of magnitude while the variant with the H40R mutation retained its activity and showed close to 70% conversion (Figure 3B). The result is consistent with the hypothesis that tighter binding serves to protect the cofactor from prolonged exposure to the higher pH environment and thus supresses its breakdown.

Combining directed evolution approaches and rational mutagenesis revealed key mutations improving both stereospecific control in the reduction of **10** and stabilization of the cofactor in the enzyme, effectively increasing the enzyme's operating window to the target conditions in a single round of evolution.

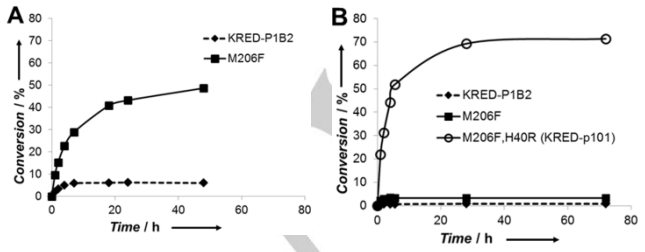
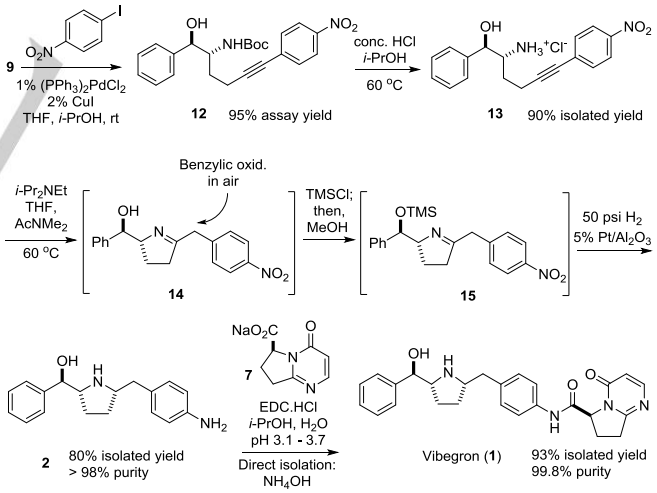


Figure 3. Impact of the H40R mutation on productivity (5 g/L of **10**) at decreased cofactor loading and pH 10 at 35°C. a) The performance of the parent P1B2 vs. the M206F mutant at 0.5 g/L cofactor. b) The performance of P1B2, mutant M206F and the double mutant M206F, H40R (KRED-p101) at 0.1 g/L cofactor.

The subsequent protein engineering focused on further improving selectivity and specific activity to achieve higher conversion at decreased enzyme loading. In the second round of evolution, 20 positions[8] around the substrate binding pocket were targeted for saturation mutagenesis. Screening of the library identified ten unique mutations at eight positions that were beneficial over the parent. The best mutations G94P, I144V, and L196M were improved in activity by 1.7-, 2.1-, and 2.1-fold, respectively. The three mutations were combined in KRED-p201, resulting in a round 2 variant more active and selective than KRED-p101 (Table 1, entry 4). The final round of evolution recombined all beneficial diversity identified in the previous two rounds in one library to fully optimize the enzyme. The final variant,[8] KRED-p301, contained six additional mutations relative to KRED-p201 and was shown to be at least 50-fold improved overall, resulting in an efficient and selective high pH DK reduction biocatalyst (Table 1, entry 5). In practice, treatment of **10** with 1wt% KRED-p301 and only 0.1g/L NADP in pH 10 aqueous borate buffer containing 50% *i*-PrOH at 45°C afforded **9** in 95% assay yield with >99% ee and >100:1 dr. Sonogashira coupling of crude **9** with *p*-iodonitrobenzene in the presence of <1 mol% $(PPh_3)_2PdCl_2$ and 0.5–2 mol% CuI in THF and *i*-PrOH at ambient temperature gave the desired coupling product **12** in 95% yield.

Carrying out the Sonogashira reaction in alcohol solvents allowed significant reduction in catalyst loading, presumably because the reduction capability of alcohol solvents maintained the Pd(0) catalyst reactivity.[13] Without isolation, intermediate **12** was treated with conc. HCl in *i*-PrOH to afford HCl salt **13**.



Scheme 4. Preparation of vibegron

Upon treatment of HCl salt **13** with *i*-Pr_2NEt in THF-$AcNMe_2$ at 60°C, the desired intramolecular Michael addition proceeded smoothly under an atmosphere of nitrogen to afford imine **14**. The cyclization was highly selective, and Michael addition of the benzylic OH to the electron deficient carbon-carbon triple bond was not observed. Isolation of **14** was undesirable since the free base was prone to benzylic oxidation in air. However,

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maintaining an inert atmosphere to prevent oxidation was straightforward at plant scale. Therefore, the crude stream **14** was carried forward to the hydrogenation step and treated *in situ* with TMSCl to form the corresponding silyated intermediate **15**, which was required to enhance the diastereoselectivity of the hydrogenation.[5] Methanol was added to consume excess TMSCl before the reaction stream **15** was subjected to global hydrogenation conditions. In the presence of Pt/Al_2O_3, pyrrolidine **2** was obtained in 95:5 dr. Surprisingly, preliminary results showed that the initial hydrogenation lacked reproducibility in terms of conversion and reaction rate. In addition, the high catalyst loading (>3 mol%) was not cost effective, even though the heterogeneous catalyst could be recovered easily on large scale. After several experiments, we found that residual palladium carried through from the Sonogashira coupling was responsible for catalyst poisoning, which was easily rejected to <150 ppm by a slurry wash of the wet cake of **13** during isolation. The resulting robust hydrogenation was then achieved with low catalyst loading (<1 mol%). Upon aqueous workup, pyrrolidine **2** was isolated in 80% yield and the undesired diastereomer was rejected to <1%.

The initial coupling of **2** with pyrimidione acid **9** was carried out in the presence of EDC·HCl in aqueous $AcNMe_2$ (Scheme 1).[6] However, an undesired solvate form of **1** was isolated in 77% yield, which required a recrystallization to obtain the desired, more bioavailable crystal form. To improve the volumetric productivity and eliminate the recrystallization for crystal form correction, a process to carry out the EDC coupling in pH controlled aqueous *i*-PrOH was developed. The pyrrolidine nitrogen in **2** was selectively protonated with HCl, leaving the less basic aniline nitrogen available to effectively couple with the free acid of **7**. Worthy of note is the operational ease of this endgame process at large scale. After adjusting the pH of a solution of **2** and sodium salt **7** in aqueous *i*-PrOH with HCl to pH 3.1–3.7, EDC·HCl was charged at 5–10°C to cleanly afford desired product **1**. Direct addition of aqueous NH_4OH to the reaction stream followed by filtration completed the synthesis of vibegron, which was obtained in its desired crystal form in 93% yield with 99.8% purity and nearly perfect ee.

In summary, a highly efficient, asymmetric synthesis of vibegron (**1**), which has been implemented on a manufacturing scale, has been described. The synthesis features an enzymatic DK reduction to establish the challenging C'1,C2 stereochemistry in excellent enantio- and diastereo-selectivity. Incorporating structurally designed mutations into directed enzyme evolution produced a ketoreductase suitable for high pH and elevated temperature conditions in only three evolution rounds. Subsequent intramolecular Michael addition followed by diastereoselective hydrogenation concisely constructed the backbone of vibegron. The second generation route to vibegron is realized by integration of bioengineering technology into organic synthesis and is atom efficient with minimal transformations of functional groups, requiring only one protecting group in the early intermediates.

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Keywords: enzyme • evolution • ketoreductase • synthesis

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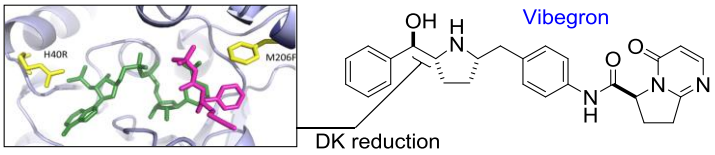

Evolution for synthetic efficiency: Target-driven evolution optimizes enzymes for synthetically desired transformations under conditions that challenge and surpass known limitations of enzyme performance. A biocatalyst, engineered for stereoselective, dynamic kinetic reduction in a high pH environment, has enabled an efficient synthesis of vibegron, a potent β_3-adrenergic agonist for the treatment of overactive bladder syndrome.

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