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Efficient, Chemoenzymatic Process for Manufacture of the Boceprevir Bicyclic [3.1.0]Proline Intermediate Based on Amine Oxidase-Catalyzed Desymmetrization

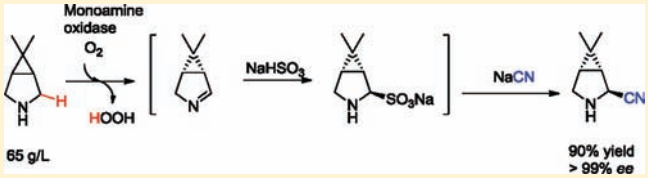
Tao Li,*[,†,§,▽] Jack Liang,[‡,▽] Alexandre Ambrogelly,[†] Tim Brennan,[†,∥] Guy Gloor,[†,⊥] Gjalt Huisman,[‡] James Lalonde,[‡] Azzeddine Lekhal,[†] Ben Mijts,[‡] Sheela Muley,[‡] Lisa Newman,[‡] Matt Tobin,[‡] George Wong,[†] Aleksey Zaks,[†,#] and Xiyun Zhang[‡]

[†]Merck & Co., Inc., 126 East Lincoln Avenue, Rahway, New Jersey 07065, United States

[‡]Codexis, Inc., 200 Penobscot Drive, Redwood City, California 94063, United States

Supporting Information

ABSTRACT: The key structural feature in Boceprevir, Merck's new drug treatment for hepatitis C, is the bicyclic [3.1.0]proline moiety "P2". During the discovery and development stages, the P2 fragment was produced by a classical resolution approach. As the drug candidate advanced through clinical trials and approached regulatory approval and commercialization, Codexis and Schering–Plough (now Merck) jointly developed a chemoenzymatic asymmetric synthesis of P2 where the net reaction was an oxidative Strecker reaction. The key part of this reaction sequence is an enzymatic oxidative desymmetrization of the prochiral amine substrate.



INTRODUCTION

Imine preparation by amine oxidation is a highly desirable synthetic tool, because it opens many strategic opportunities for C–C bond formation.[1,2] Selective, clean, and efficient amine oxidations are very important for pharmaceutical process development, because the economic and environmental impacts of a commercial manufacture process usually last as long as the patent life cycle of the drug. Among all the available methods, biocatalysis with amine oxidases[1a] is particularly attractive by virtue of its selectivity and environmental sustainability: enzymatic oxidation is highly enantioselective, it uses dioxygen as the oxidant, and the reaction is in water. Still, this reaction suffers from problems including low enzyme activity at practical substrate concentration, instability of imine product, and fire hazard at large scale. In this article, we report the development of a practical imine preparation method based on biocatalysis with amine oxidases and the application of this method in developing a commercial process to manufacture a bicyclic [3.1.0]proline intermediate for boceprevir.

Boceprevir (Victrelis, **1**, Scheme 1) is a newly approved, first in class oral medication developed for treating chronic hepatitis C infection. It is recommended as an addition to the current standard regimen of PEG–interferon and ribavirin in treating patients infected with HCV genotype 1. Phase III clinical trial results showed that the addition of boceprevir improved the sustained viral response from <30% to close to 70%.[3] This new therapy represents a major advance in treating HCV infection.

Boceprevir is a peptidomimetic protease inhibitor with four moieties, P1–P3 and a Cap, where P1 is a racemic *β*-aminoamide, P2 is a chiral dimethylcyclopropylproline analog, P3 is (*S*)-*tert*-leucine, and Cap is a *tert*-butylcarbamoyl group. The 3-azabicyclo[3.1.0]hexane structure of P2 adopts a constrained conformation, so that the *gem*-dimethyl group has a fixed angle with respect to the bicyclic ring structure. The incorporation of the 3-azabicyclo[3.1.0]hexane moiety results in a 1000-fold increase in NS3 protease binding over proline in a pentapeptide scaffold. When boceprevir binds to the NS3 protease, the P2 moiety interacts with four amino acid residues at the active site.[4,5]

The starting material for the P2 moiety is the HCl salt of the dimethylcyclopropylproline methyl ester **2**. For medicinal chemistry studies, it was prepared by cyclopropanation of a Δ^3-pyrroline derivative of pyroglutamic acid.[6] For large scale preparation, caronic anhydride was selected as the starting material. In the first generation process,[7] desymmetrization of the anhydride was used to set up the two chiral centers in **4**. The third chiral center was installed by a diasterespecific cyanation of **3**. Athough this approach is asymmetric, the procedure requires eight additional linear steps after desymmetrization. In the second generation method, the synthesis was simplified by maintaining molecular symmetry to the last possible step.[8] In two steps, caronic anhydride was converted to 6,6-dimethyl-3-azabicyclo[3.1.0]hexane (**6**). The amine oxidation with chlorination and elimination gave (±)-**5**. The *trans*-carboxylate was installed by diastereoselective *trans*-cyanation and then nitrile methanolysis under Pinner conditions. In the final step, the desired enantiomer was isolated by classical resolution. Although the second generation synthesis is significantly shorter, it still suffers from at least 50%

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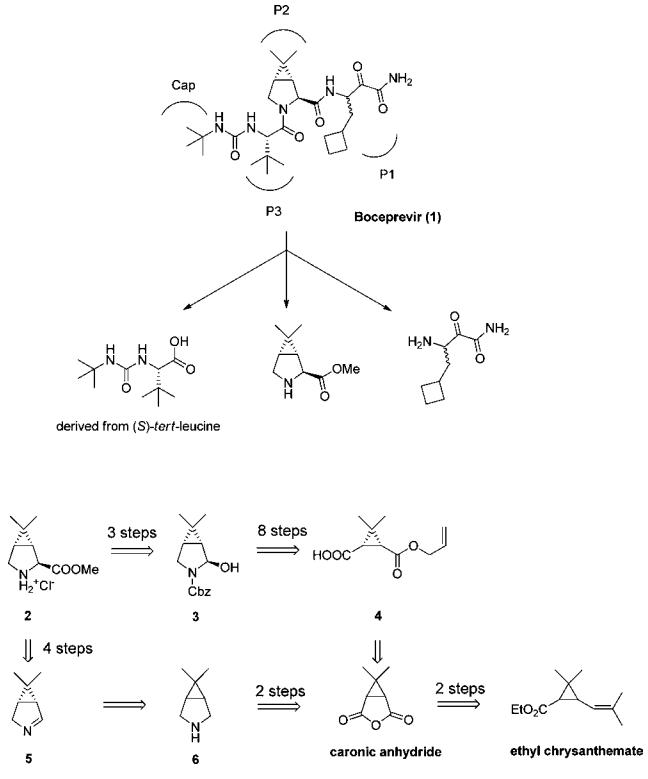
Scheme 1. Key Disconnections in Boceprevir and the Retro-Synthesis of the Central P2 Moiety



yield loss in the resolution at the last step. This is especially significant considering that the synthesis of the pyrrolidine **6** from ethyl chrysanthemate involves the use of excess of both a metal-based oxidant (e.g., $KMnO_4$) and reductant (e.g., BH_3 or $LiAlH_4$). That is, the material lost (i.e., the waste stream) upon resolution is compounded by the reagents used in the preparation of the starting material. Given the high cost and large demand for this intermediate, there were strong motivations to develop a more efficient approach.

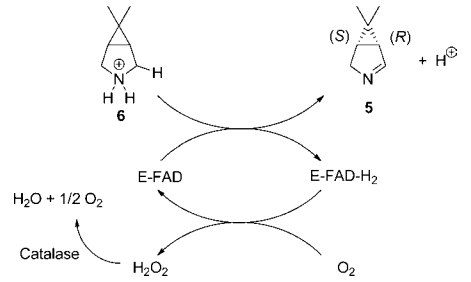
One can envision a superior process where **6** is desymmetrized directly to give the desired enantiomer of **5**. Indeed, asymmetric phase transfer catalysis has been applied to the dehydrohalogenation of the corresponding chloroamine, but the enantiomeric excess (ee) was only 20%.[8c] A biocatalytic approach was therefore explored to carry out the asymmetric amine oxidation.

Biocatalytic desymmetrization of substituted pyrrolidines is an attractive approach to access proline analogs. Recently, Turner's group[9a] demonstrated that a variety of 3,4-substituted *meso*-pyrrolidines can be enantioselectively oxidized by the MAON D5 mutant. The Δ^1-pyrroline products were converted to the amino acids through the corresponding nitriles. In another application, the chiral Δ^1-pyrrolines were directly used to prepare prolyl peptides in a Ugi reaction.[9b] This reaction has been applied to an efficient synthesis of Telaprevir.[9c] However, the amine oxidation was carried out with low substrate concentrations ($\leq$25 mM) in all the examples. Applying this reaction for practical preparation remains to be a challenge.

RESULTS AND DISCUSSION

Amine oxidation is a common pathway in biogenic amine metabolism. Typically, an amine is oxidized by molecular oxygen to give the corresponding imine and hydrogen peroxide as products. The reaction is catalyzed by amine oxidases that are either copper-containing or FAD-dependent.[10] In an earlier report, Turner's group described that directed evolution of a FAD-containing monoamine oxidase (MAO) from *Aspergillus niger* (MAOs)[11] provided a N336S variant with a broadened substrate range as well as high enantioselectivity for several α-methyl amines.[12a] In further enzyme evolution, an improved variant was identified (N336S/I246M) for secondary amines. For synthetic application, a secondary amine deracemization protocol was developed by using the N336S/I246M variant.[12b] Intriguingly, both variants exhibited exceptionally high activity for isoindoline, a 3,4-fused bicyclic pyrrolidine similar to compound **6**. MAON is a FAD-dependent enzyme that can oxidize a wide range of amines.[11] It is also related to MAOA and MAOB, two of the most important mammalian MAOs known for their promiscuity in oxidizing xenobiotic amines.[13] Inspired by these results, we embarked on the development of a MAO-based desymmetrization process (Scheme 2).

Scheme 2. Mechanism for Monoamine Oxidase-Catalyzed Oxidation of 6,6-Dimethyl-3-azabicyclo[3.1.0]hexane



The catalytic cycle of the desired reaction as shown in Scheme 2 has significant implications for both process chemistry and enzyme optimization. From a process development standpoint, the key aspects of the reaction are the use of molecular oxygen and the release of a proton per turnover. For safety reasons, the use of molecular oxygen precludes the use of most organic solvents and makes the process potentially subjective to gas–liquid mass transfer limitations. The hydrogen peroxide byproduct is to be decomposed to water and oxygen by catalase, a highly efficient enzyme available in bulk quantity. The release of protons provides an opportunity to maintain the pH of the reaction by titration with substrate free base and to monitor the progress of the reaction based on the amount of added substrate. From an enzyme optimization perspective, the high throughput screening of MAO libraries could be performed by measuring the hydrogen peroxide byproduct in an amplex red-based assay.[14]

A collection of amine oxidases was tested for oxidation of **6**. MAON from *Aspergillus niger* and *Aspergillus oryzae* showed activity on **6** in the amplex red-based assay. Larger scale reactions generated sufficient quantity of **5** for absolute configuration verification. The product from MAON catalysis was converted to compound **2** and found to be the desired enantiomer with an ee >99%. The undesired enantiomer was never observed throughout the course of the enzyme optimization. Of the two active wild type MAOs, the *A. niger* enzyme was more stable but less active compared to the *A. oryzae* enzyme.

Enzyme optimization was initiated using the *A. niger* enzyme (MAON) as the parent (see the Supporting Information for sequence). Initial libraries were generated via random muta-

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genesis using error-prone PCR as well as via a homology model-inspired approach since the crystal structure of MAON was not available. Screening of these round 1 libraries yielded a number of variants with improved activity on **6**. The best variant, MAON156, contained one targeted mutation K348Q and one random mutation A289V. Its activity was 2.4-fold higher than that of the wild type enzyme. MAON156 was selected as the backbone for the next round of evolution, where mutations from other round 1 hits provided new diversity for recombination into this backbone. In addition, a parallel strategy was performed by family shuffling of MAON156 with the homolog from *A. oryzae*. Two improved variants were identified from the second round evolution including MAON274, a hybrid *A. niger*/*A. oryzae* variant from the family shuffling approach, and MAON291 from a hit shuffling library. MAON274 and MAON 291 provided a ~3-fold and a ~6-fold improvement over MAON156, respectively. The MAON291 variant contained an S-to-G mutation at position 465 that resulted in a significant change in enzyme solubility as observed by SDS-PAGE analysis. Retrospective modeling studies (Figure 1) performed after the enzyme evolution had been completed

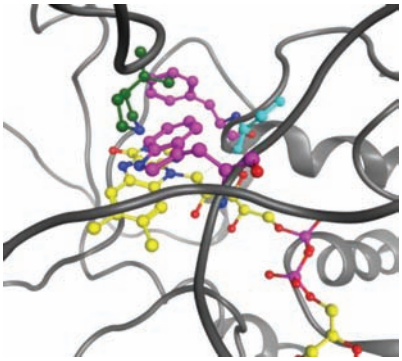

Figure 1. MAON active site contour with substrate (green), aromatic cavity (purple; W430, F466), G465 (turquoise), and FAD (yellow) indicated.

and when the crystal structure of the enzyme became available[15] suggest that this mutation in the active site loop may lead to increased flexibility in the substrate binding site. As a consequence, the "aromatic cage" might be reoriented for binding the non-natural substrate and enhance the nucleophilicity of the amine moiety.

The optimization of MAON continued through two more rounds of evolution. Screening strategies were adjusted to identify variants with tolerance to substrate and product inhibition as well as thermostability improvement. Thermostability is a desirable characteristic in a biocatalyst as it enables reactions at higher temperatures, typically at a higher reaction rate. It also correlates with storage stability, a necessary feature for industrial application (see below). The thermostability of the most active round 2 variants, MAON291 and MAON274, was evaluated over a range of temperatures. MAON291 lost ~80% activity when incubated at 40 °C overnight, whereas MAON274 retained 100% activity at 40 °C and lost only ~30−35% activity at 50 °C. In round 3, family shuffling of MAON274 and MAON291 produced improved hybrid variants MAON301, MAON304, and MAON308 that were more stable than their parents at 50 °C. In round 4, 12 positions in the active site of backbone MAON304 were targeted for further mutation. The final variant, MAON401, was identified that met the activity and stability objectives. This final variant was a hybrid enzyme with one additional F382L mutation and was 2.8-fold more active than MAON304. MAON401 was completely stable upon storage under air at −20 °C (standard storage) for 33 days followed by 10 days at room temperature. When incubated under air at 40 °C for three days, lyophilized MAON401 retained more than 85% of its initial activity. The evolution of the natural MAOs is summarized in Table 1 (Sequences of the variants are described in the Supporting Information).[16]

Table 1. Summary of MAON Improvement over the Four Rounds of Evolution

round	MAON variant	mutation	diversity source	improvement
	wild type	*A. niger* wild type		
1	156	*A. niger* + A289V, K348Q	active site targeted library	activity
2	274	many (see the Supporting Information)	*A. niger* and *A. oryzae*	thermal stability at 40 °C
2	291	150 + S465G	diversity from round 1	activity and solubility
3	301/304/308	many (see the Supporting Information)	MAON274 and MAON291	thermal stability at 50 °C
4	401	304 + F382L	active site library	8-fold increase in activity

As the enzyme optimization proceeded, we evaluated process development-related challenges. In the beginning, MAON291 was used in the development of a laboratory protocol for preparative amine oxidation. When MAON401 became available later, we characterized the enzyme comprehensively and modified the reaction condition for large scale production based on the underlying principles in reaction design.

The first issue we addressed was limitation of oxygen availability in aqueous medium. Under 1 atm air at 25 °C the solubility of oxygen in pure water is ~250 μM and is expected to decrease as the salinity in the reaction medium increases. For practical synthesis, oxygen supply needs to be facilitated. In our set up oxygen was introduced to the aqueous solution by passing air through a gas dispersion tube to maximize mass transfer. The off gas was led through a condenser (−10 °C) to minimize loss of volatile product **5** and water. The flow rate was adjusted to meet the expected reaction rate while minimizing material loss (solubility of **5** is approximately 6 g/L). For easy scale adjustment the flow rate is expressed as relative volume (with respect to reaction volume) per unit of time.

Initial experiments were run at 22 g/L (200 mM) of **6** at an air flow rate of 0.1 v/v per minute. Catalase was added to decompose hydrogen peroxide byproduct. The reaction was initiated by adding 5 wt % (1.1 g/L) of MAON291. The pH was maintained at 7.4 by NaOH titration (instead of using free-base of **6**) with a pH stat. After 11 h, the reaction came to a complete stop at about 50% conversion (estimated concentration for compound **5** = 92 mM). Adding more MAON291 marginally increased conversion. Enzyme assays showed that MAON291 was completely deactivated at this point. Further analysis revealed that **5** was a strong inhibitor to MAON and the inhibition was irreversible (see the Supporting Information).

In addition to MAON inhibition effects, compound **5** also has low solubility in aqueous medium and tends to vaporize. Its

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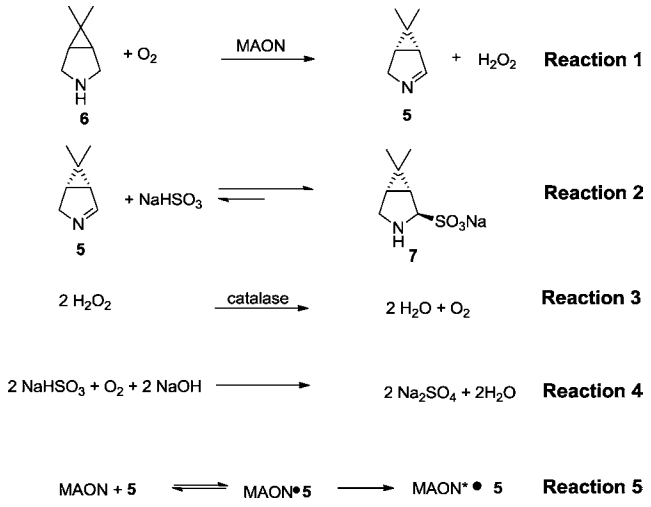
volatility posed a safety challenge as the vapor constitutes a fire hazard when mixing with oxygen. Therefore, we explored conversion of **5** into a water-soluble intermediate during the enzymatic reaction. This would increase the volumetric yield in the aqueous phase and also minimize the fire hazard by reducing the concentration of **5** in the gas phase. We considered a one step oxidative Strecker reaction involving imine capture with a cyanation cascade but dropped the idea for two reasons: cyanide is a strong inhibitor of catalase, and aminonitrile **8** racemizes in aqueous medium. Bisulfite adducts of **5**[8b] and a related des-methyl pyrroline[17] were known to be highly soluble in water. We hypothesized that the formation of amino sulfonate would minimize the concentration of **5** under oxidative conditions and product **7** (Scheme 3) would be stable enough to accumulate as the reaction proceeds.

Scheme 3. List of Reactions in Amine Oxidation and Imine Sulfonations Cascade



We tested the formation of the amino sulfonate in the enzymatic reaction stream. Sodium bisulfite was added to the reaction mixture after the concentration of **5** (~100 mM) reached the point where enzyme had been completely inactivated. ^{1}H NMR analysis showed that the formation of the bisulfite adduct **7** was instantaneous and the reaction resumed as soon as additional enzyme was added. In a reaction with 22 g/L of **6**, the conversion reached 100% after 3.3 g of MAON291 was added in three equal portions (with additional sodium bisulfite) over 29 h. The material balance was 94%, suggesting very little loss of **5** in the gas phase. Based on this result we also conclude that the amino sulfonate **7** does not inhibit or inactivate MAON.

For preparative amine oxidation of **6** we designed a streamlined process to maximize both volumetric yield of sulfonate **7** and turnover of MAON. In this plan, bisulfite is added to the enzyme solution as a mixture with **6** so that imine **5** is instantaneously converted to sulfonate **7** in a cascade of reactions 1 and 2 (Scheme 3). This would maximize the volumetric yield and also minimize the inhibition of MAON (reaction 5). Unfortunately, under oxidation conditions, the thermodynamically favored bisulfite oxidation (reaction 4) competes with sulfonation (reaction 2). When reaction 4 is a major pathway, the concentration of imine **5** is expected to increase and cause MAON inactivation. To suppress this scenario, the reaction conditions have to be such that reactions 1 and 2 predominate over reaction 4. Our process design is equipped with two variables to accommodate the need: the ratio of bisulfite to **6** can be adjusted to compensate for bisulfite oxidation, and the addition rate of the substrate can be fine-tuned to match the rate of reaction 1. The understanding of MAON kinetics, formation, and decomposition of amino sulfonate **7** allowed us to identify the reaction condition space.

For laboratory protocol development, we characterized the impact of substrate **6** and product **5** on the activity of MAON291 under air (see the Supporting Information).

The kinetic features for MAON401 have been comprehensively characterized. This enzyme was purified to homogeneity by a protocol involving ammonium sulfate fractionation followed by ion-exchange chromatography on a Q-Sepharose fast flow column (GE Healthcare). The specific activity of the purified enzyme for **6** was 17.7 μmol min^{-1} mg^{-1}, representing an 8.4-fold increase over wild type MAON. It exhibited an optimal pH between 7.2 and 7.5. The reaction rate increased significantly as the concentration of oxygen rose above 0.26 mM ([O_2] in water saturated with air at 1 atm). Fitting the data to kinetic models indicated the reaction followed a Ping Pong mechanism[18] with kinetic parameters listed in Table 2. The k_{cat} was estimated to be 48 s^{-1} assuming a molecular weight for MAON of 55.6 kDa. Although MAON401 is a more active variant with better thermal stability, it is still inhibited by **6** as well as **5** just like its progenitor. At 0.8 mM, imine **5** reduces the activity of MAON401 by 40%. The inhibition is irreversible (see the Supporting Information).

Table 2. Summary of Kinetic Parameters for MAON401

parameter	value	standard error
V_{max}	51.8 μmol min^{-1} mg^{-1}	1.14
K_m (**6**)	0.29 mM	0.019
K_{oxygen}	0.21 mM	0.14

Since sulfonation is reversible and bisulfite is oxidized via reaction 4, we evaluated the formation and stability of **7** under oxygen. Although sulfonate **7** is known to form from the reaction of **5** with bisulfite under nitrogen, it had never been isolated and characterized.[8b] We prepared sulfonate **7** by reacting (1*S*,5*R*)-**5** with bisulfite in nitrogen saturated water. The isolated product was a crystalline solid with very high water solubility (200−250 g/L, 25 °C). The oxidation of **7** was carried out by adding three equivalents of sodium bisulfite to a solution of **5** at 25 °C with pH maintained at 7.4. The concentration of **5** was measured over the course of 180 min. This reaction was carried out under nitrogen or under pure oxygen with 70 and 130 mmHg gauge pressure (1.4 and 2.5 Psig). The result showed fast sulfonate formation followed by slow degradation (Figure 2) through the equilibrium shift due to bisulfite oxidation. The degradation accelerated as the pressure of oxygen increased. Under 130 mmHg oxygen more than 99% of sulfonate degraded in 180 min. Since the formation of the sulfonate is much faster than its degradation, sulfonate **7** is expected to accumulate as long as adequate bisulfite concentration is maintained. In preparative method development we optimized the reaction condition by maximizing the kinetic advantage of the cascade involving reactions 1 and 2 over reactions 4 and 5 (Scheme 3).

In the laboratory protocol we used MAON291 as the catalyst and air as the oxidant. Typically the reaction was carried out by adding a mixture of bisulfite and **6** to a solution of MAON291

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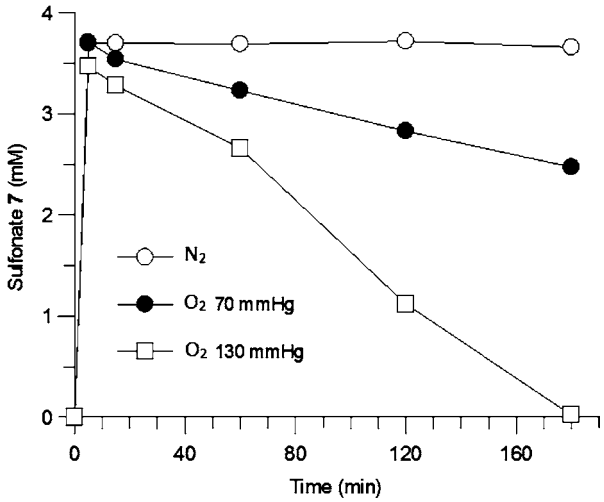


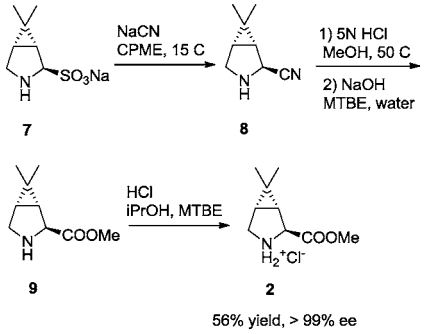
Figure 2. Formation and decomposition of sulfonate **7** under nitrogen or under oxygen with 70 and 130 mmHg gauge pressure.

and catalase at a constant rate over 24 h. At the end of addition (65 g/L of **6**) the product was mostly sulfonate **7** and a small amount of imine **5** (<10%). The combined concentration of the products was equivalent to approximately 63 g/L of **5**. The solution yield was >95%, and the product enantiopurity was >99% ee.

For large scale manufacturing it is preferred to run the reaction in a closed reactor with pure oxygen in the head space since MAON401 activity increases by up to 3-fold under pressurized pure oxygen. We determined the solubility of oxygen as a function of pressure in the head space under reaction condition and characterized K_La as the function of oxygen pressure and agitation rate. The pressure and agitation rate was set just enough to allow MAON401 to work at the optimal rate. On scale-up we chose an agitation speed that gave us similar calculated K_La.

We then developed the process for amine oxidation and adapted the downstream chemistry to the chiral sulfonate (Scheme 4). The procedure began with oxidation of **6** under

Scheme 4. Preparation of the P2 Intermediate from 7



pure oxygen at 4.0 psi (207 mmHg) gauge pressure using an induction impeller for mixing. Under optimal condition, the substrate/bisulfite mixture was added to a solution of 6 wt % (3.9 g/L) MAON401 and catalase over 20 h. All of substrate **6** (final concentration 65 g/L) was converted to sulfonate **7** with a small amount of **5** (<10%). The enzyme reaction stream was telescoped for cyanation in a mixture with cyclopentylmethyl ether (CPME). The reaction gave only *trans*-nitrile **8** in the organic phase in about 90% yield from **6**. In the next step the nitrile was transformed to the methyl ester under Pinner conditions. The product was extracted into methyl *t*-butyl ether (MTBE) after being converted to the free base **9**. In the final step the free base was crystallized as the HCl salt. The overall yield of the process was 56%, giving **2** with an ee of >99%. The conditions of the procedure were successfully applied to pilot plant scale preparation with minor modifications.

The biocatalytic step significantly improved the performance and sustainability of the manufacturing process of **9**. Compared with the resolution method the product yield was increased by 150%, raw material use was reduced by 59.8%, consumption of water was reduced by 60.7%, and the overall process waste (E-factor) was reduced by 63.1% (Table 3).[19] The product from

Table 3. Improvement in Green Chemistry Metrics

parameter	resolution process (kg/kg product)	enzyme process (kg/kg product)	reduction (%)
raw material used	2.44	0.98	59.8
water used	83.5	32.8	60.7
E-factor	191	70.3	63.1

this process meets the stringent specifications and is suitable for API manufacture. This process has been used for commercial production.

Our laboratory protocol should be useful for making the sulfonates corresponding to all imines from oxidative desymmetrization by MAON.[9] The sulfonate product is useful in all applications from the imines: it can be converted to the amino acid through cyanation, or it can also be converted back to Δ^1-pyrroline by treating with a mixture of MTBE and aqueous solution of NaOH.

CONCLUSION

We have developed a practical, highly efficient method for asymmetric oxidation of 6,6-dimethyl-3-azabicyclo[3.1.0]-hexane. This reaction involves an amine oxidase-mediated desymmetrization and a concurrent bisulfite adduct formation cascade to give the corresponding amino sulfonate. Based on this reaction, a process has been developed to prepare the P2 intermediate for boceprevir. The procedure features great improvement in efficiency as well as green chemistry performance.

ASSOCIATED CONTENT

Supporting Information

Descriptions of experimental protocols, characterization of new compounds, amine oxidase cloning and expression, enzyme evolution by protein engineering, and enzyme characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

li.tao@epa.gov

Present Addresses

[§]Sustainable Technology Division, ORD, U.S. EPA, 26 Martin L. King Dr. West, Cincinnati, OH 45268-0001

[∥]Hospira, 4876 Sterling Dr., Boulder, CO 80301

[⊥]Chesilton LLC, 3 Sunrise Ct., Colts Neck, NJ 07722

[#]Akermin, Inc. 1005 N. Warson Road, Suite 101, St. Louis, MO 63132

Author Contributions

[▽]These authors contributed equally to this work.

Notes

The authors declare no competing financial interest.

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