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Baeyer−Villiger Monooxygenase-Mediated Synthesis of Esomeprazole As an Alternative for Kagan Sulfoxidation

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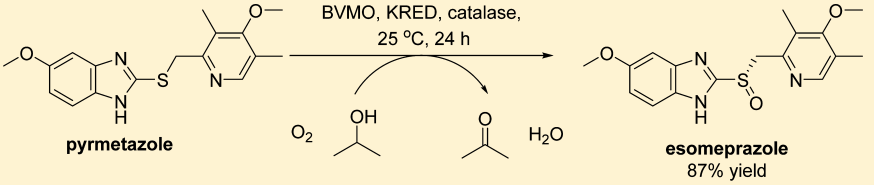
Supporting Information



ABSTRACT: A wild-type Baeyer−Villiger monooxygenase was engineered to overcome numerous liabilities in order to mediate a commercial oxidation of pyrmetazole to esomeprazole, using air as the terminal oxidant in an almost exclusively aqueous reaction matrix. The developed enzyme and process compares favorably to the incumbent Kagan inspired chemocatalytic oxidation, as esomeprazole was isolated in 87% yield, in >99% purity, with an enantiomeric excess of >99%.

INTRODUCTION

Chiral sulfoxides are gaining interest as design elements in drug discovery as well as chiral auxiliaries in chemical synthesis.[1] The "prazole" proton pump inhibitors for the treatment of gastric reflux are possibly the most significant examples of sulfoxide-containing active pharmaceutical ingredients (API). Esomeprazole, the *S*-enantiomer of omeprazole, is the API of the blockbuster drug Nexium.

Esomeprazole is produced commercially using variations of the Kagan−Sharpless−Pitchen sulfoxidation[2] of the parent sulfide, pyrmetazole (Scheme 1).[3] However, the enantioselectivity of the titanium-catalyzed asymmetric oxidation is sensitive to the amount of water present.[4] Over oxidation of the sulfoxide to sulfone also poses a problem as the downstream removal of sulfone is challenging.[5] In addition, waste treatment, process safety, and environmental impact are also important aspects to consider for the reaction.

Controlled asymmetric oxidations are uncommon in traditional syntheses, and the development of safe scalable oxidations in general has been identified as a key requirement if more oxidations are to be used in the synthesis of active pharmaceutical ingredients.[6] Asymmetric oxidations that proceed near room temperature in water using molecular oxygen as the oxidant are particularly desirable. A biocatalytic approach can be considered for asymmetric sulfoxidation which would combine these highly desirable attributes with other potential benefits in terms of milder processing conditions, minimal organic solvent waste, better chirality control, and chemo-selectivity.[7] However, several aspects of oxidative biocatalysis need to be addressed for commercial utilization. First of all, wild-type enzymes are typically less stable under the desired reaction conditions that do not necessarily reflect the native conditions the enzymes originally evolved under. Besides that, enzymes identified in the nature, generally, are limited in substrate range and do not possess sufficient activity or selectivity to the substrate of interest. Furthermore, the soluble expression of enzymes, especially oxidative enzymes, through heterologous hosts can be challenging. Nevertheless, with CodeEvolver directed evolution technology, many of the above-mentioned concerns have been addressed.[8] For esomeprazole, a whole cell biocatalytic reaction has been reported, but in low yield.[9] Therefore, a process using specifically engineered isolated sulfoxdiation enzyme as the oxidation catalyst and a suitable enzyme for cofactor recycling is attractive.

Cyclohexanone monooxygenase (CHMO) was first identified in *Acinetobacter sp. strain NCIB 9871*.[10] This flavoprotein monoxygenenase, also referred to as a Baeyer−Villiger monoxygenase (BVMO), catalyzes the Baeyer−Villiger oxidation of cyclohexanone to the ring expanded lactone.[11] BVMOs are known to catalyze other oxidative reactions such

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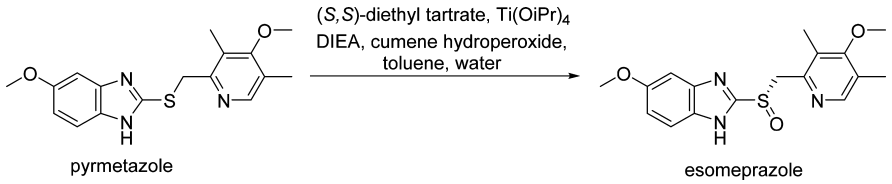
Scheme 1. Kagan–Sharpless–Pitchen-Based Oxidative Formation of Esomeprazole



Scheme 2. BVMO-FAD Catalytic Cycle and NADPH Recycle

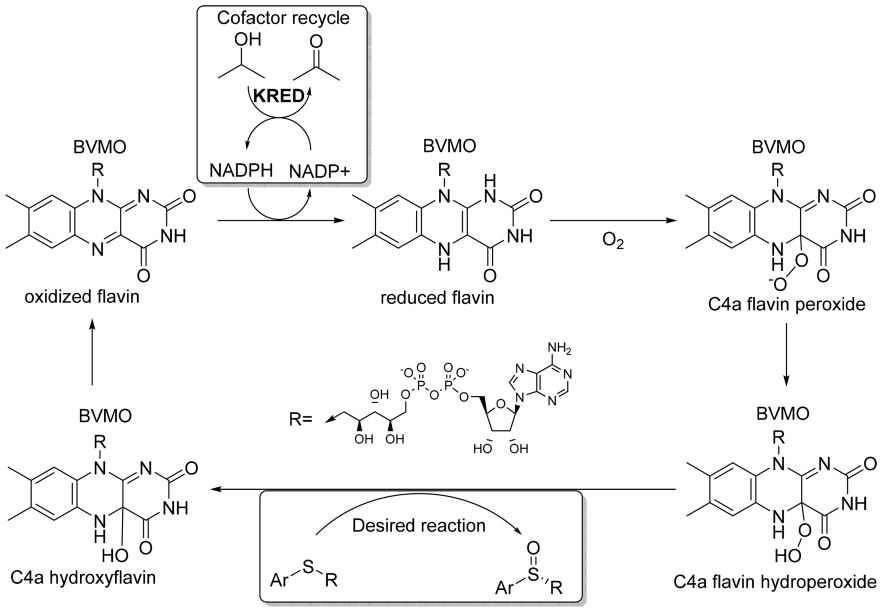


Table 1. Key BVMO and Reaction Performance Parameters over the Course of BVMO Evolution

	fold improvement in productivity[a]	ee (*S*)	sulfone (%)	$NADP^+$ (g/L)
BVMO WT	1	Nd	Nd	0 (an equivalent NADPH was used)
round 2 hit	800	−95%	Nd	2
round 3 hit	610	92%	1	2
round 7 hit	~6600	96%	0.5	0.5
round 13 hit	~85000	98%	0.5	0.5
round 19 hit	~140000	>99%	0.1	0.1

[a]Fold improvement was calculated by using the ratio of productivity (gP/(gE·hr) of the evolved variants with respect to wild-type.

as hydroxylation, epoxidation, amine oxidation, halogenations, and, of specific interest in this context, sulfoxidation.[12] BVMO requires NADPH to reduce the oxidized flavin adenosine nucleotide (FAD) cofactor generated per catalytic cycle (Scheme 2). The cost of NADPH prohibits stoichiometric use, and therefore a recycling system is used. Numerous recycling systems are available, but for this reaction a ketoreductase (KRED)-mediated propan-2-ol (IPA) oxidation was selected to efficiently enable NADPH cofactor regeneration.

Through Codexis' CodeEvolver directed evolution technology, a BVMO was improved to allow a synthesis of esomeprazole in high chemical and enantiomeric purity with a catalytic efficiency suitable for use in commercial production. The variants produced in the directed evolution program were also found to be useful for enantioselective sulfoxidaton of other prazole precursor sulfides.[13]

RESULTS AND DISCUSSION

Directed Evolution of BVMO. The wild-type BVMO from *Acinetobacter* (Uniprot accession number P12015) showed only a trace detectable activity on pyrmetazole, the sulfide precursor to esomeprazole. With such barely detectable activity using LC-MS, the BVMO enzyme was evolved to improve four critical factors namely, the enzyme productivity (gram of product formed per gram enzyme per hour), the enantioselectivity, the chemoselectivity (sulfoxidation versus sulfone formation), and cofactor loading. In this approach, libraries of thousands of monooxygenase variants incorporating programmed mutations were tested for activity, stereoselectivity, and chemoselectivity for the desired target in rounds of evolution. Variants were identified with improved function, and algorithms used to assign predicted coefficients of the contributions of individual mutations to the overall fitness of the catalyst. Beneficial mutations were recombined in iterative rounds of directed evolution until biocatalysts were produced that met the targeted catalytic performance. In this work, tens of thousands of variants were produced and tested,

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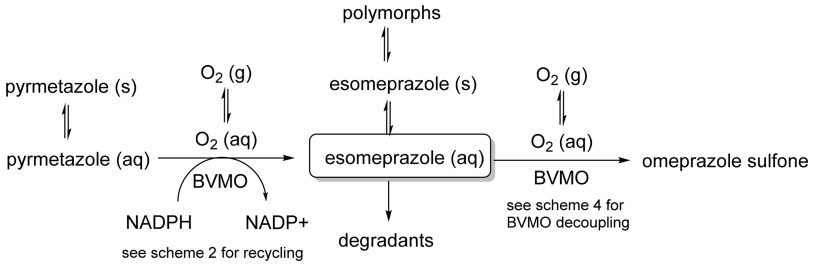
J. Org. Chem. XXXX, XXX, XXX–XXX

Scheme 3. Overall Reaction Scheme



and the final biocatalyst was improved over 140,000-fold over the initial wild-type enzyme.

Table 1 indicates the improvements made against the four key measures at various points through the engineering program. Various evolution library design strategies were employed, ranging from random mutation, recombination of diversity from related homologous enzyme, to protein-model guided design. As evolution progressed through successive rounds, the high-throughput assay was adjusted to apply the appropriate evolutionary pressure on the mutants, such as the substrate loading, cofactor loading, %IPA cosolvency, as well as reaction time, pH, and temperature.

As demonstrated in Table 1, the activity toward pyrmetazole was significantly improved by round 2. However, the variant showed undesirable *R*-enantioselectivity. The enantioselectivity was then immediately targeted, reversed in round 3. The enantioselectivity was subsequently improved to 96.5% ee (*S*), in addition a further 8-fold increase in productivity in round 7. With continued evolution, the productivity was further enhanced, but the enantioselectivity was less than ideal with significant overoxidation to the sulfone. This level of sulfone was highly undesirable as the downstream removal of sulfone was challenging. In addition, a cost analysis suggested a significant positive impact could be made with a reduction in NADP cofactor used. As a result, the subsequent evolution effort was aimed at the fine-tuning of enantioselectivity, suppression of overoxidation, and reduction in cofactor loading. The best round 19 hit was approximately 140,000-fold improved in productivity, over the starting wild-type enzyme. A review of the mutations introduced to the final variant, unsurprisingly, showed a significant number in the substrate- and cofactor-binding regions. These mutations are believed to be influential in enantioselectivity improvement, sulfone suppression, cofactor loading reduction, as well as improvement in enzyme productivity. Interestingly, most of the mutations in the substrate-binding region were guided by the protein homology model (e.g., identifying regions for saturation mutagenesis through examination of protein model), while random and homology-based mutations accounted for most of the mutations in the nonsubstrate binding region.

During the course of evolution, the BVMO's native capability of catalyzing Baeyer−Villiger oxidation was diminished, signaling a fundamental change in the underlying catalytic mechanism. The catalysis for Baeyer−Villiger oxidation is likely to proceed through *nucleophilic* attack of the substrate by the FAD-peroxide intermediate species, while the sulfoxidation is likely to involve *electrophilic* attack at sulfur by hydroperoxide intermediate.[14] The mutations around the active site have apparently changed the dominant mode of catalysis, but as yet no defining structural motifs have been identified.

Biocatalytic Process Development. Despite having a productive enzyme, the process development for esomeprazole synthesis was challenging. As demonstrated in Scheme 3, the reaction is a three-phase three-enzyme system with numerous potential side reactions, and the product isolation is further complicated by the presence of esomeprazole polymorphs.[15]

Molecular oxygen is the oxygen donor in the reaction. However, the aqueous solubility of oxygen is low (~8 mg/L, ~0.2 mM at 25 °C).[16] The mass transfer rate of oxygen between the gaseous and aqueous phases was frequently found to be reaction rate determining during process development. The use of oxygen blanket was found to deliver superior reaction rate as compared to air. On the other hand, the low aqueous solubility of substrate pyrmetazole (<1 g/L/) in the reaction matrix creates another phase transfer process in the reaction. The low concentration of the reactants, oxygen and pyrmetazole, suggests that the enzyme possesses a very high catalytic efficiency, enabling the reaction to proceed at a practically useful rate.

A third phase transfer process is related to the poorly soluble product esomeprazole in the reaction system. At pH 9, the esomeprazole is mainly in the free acid form. The esomeprazole continuously precipitates out from the reaction solution as the reaction progresses, giving rise to a "slurry to slurry" process. Numerous esomeprazole polymorphs have been reported. In this reaction, the presence of different polymorphs was suspected to result in a viscous reaction medium. The viscosity posed a challenge to the reactor design, as efficient mixing is crucial to ensure the reaction is not limited by oxygen mass transfer rate. Additionally, the solid particles in the reaction medium tended to clump together at high solid loadings but in an unpredictable manner, and product polymorphism was suspected to be the culprit. A seeding strategy was implemented that controlled the variation in reaction slurry viscosity.

Stability of the products and safety of the reaction are key concerns. Esomeprazole is unstable at neutral or low pH which sets a window of operation for the reaction pH and temperature.[17] The reaction is kept above pH 8 to ensure product stability, and the process temperature is kept at 25 °C. The reaction is also protected from excessive ambient light as light exposure is known to accelerate the rate of esomeprazole impurity formation.[18] One of the significant side reactions is the overoxidation of the product to give omeprazole sulfone. The esomeprazole USP specification for sulfone impurity is stringent (<0.2%),[19] but the sulfone is difficult to remove. As a result, it is desirable to minimize the sulfone formation at source in the oxidative reaction. The biocatalytic chemoselectivity can be effectively controlled though directed enzyme

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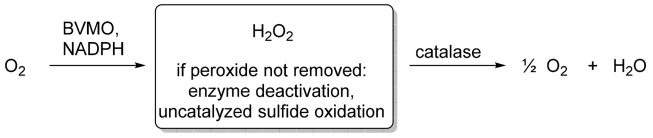
J. Org. Chem. XXXX, XXX, XXX−XXX

evolution, although a tendency for more sulfone formation at high pH set a new boundary for the reaction pH. The uncoupled reaction, where inefficient binding of substrate in the active site results in enzyme turnover with release of hydrogen peroxide, is a known side reaction for BVMO.[20] Hydrogen peroxide is highly detrimental to the reaction as it oxidatively inactivates enzymes and can also oxidize pyrmetazole in an uncatalyzed fashion to the achiral sulfoxide and further to sulfone (Scheme 4).

Scheme 4. Uncoupled Reaction, Formation of Hydrogen Peroxide

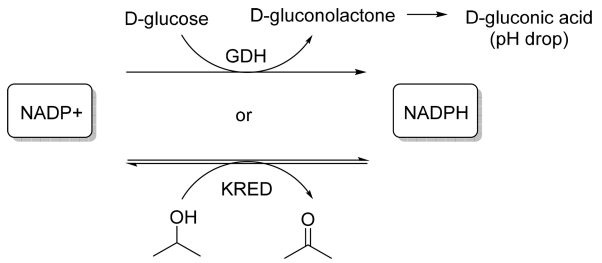


In order to minimize decoupling, a BVMO with high binding affinity for pyrmetazole is needed, which was addressed through directed evolution. Additionally, operating the reaction where substrate mass transfer from solid to solution phase is not rate limiting and will also minimize the effects of decoupling. Lastly, the detrimental oxidative harm by hydrogen peroxide is removed by introduction of catalase, which degrades the hydrogen peroxide into water and oxygen. In terms of safety, the use of volatile and flammable cosolvents in the process should be kept to a minimum and at a concentration such that the flash point is well in excess of the desired reaction temperature. In the final process, 4% IPA (3.4 mol equiv) was used in tandem with an additional ketoreductase (KRED) to effect necessary regeneration of the expensive NADPH cofactor. The flash point of 5%v/v IPA in water is reported to be 50 °C, which was considered a suitable excess over the reaction temperature of 25 °C.[21]

NADPH is a significant cost contributor in the process. Hence, a NADPH recycling system is needed to enable practical synthesis of esomeprazole. Both glucose dehydrogenase (GDH)/glucose-dependent and KRED/IPA-dependent cofactor recycling systems were evaluated. Both systems have pros and cons in various applications, but in this instance the KRED/IPA system was selected as it offered a more atom economical and simpler process without the need for constant pH adjustment that the GDH system required due to the formation of gluconic acid (Scheme 5).

The envisioned three enzyme (BVMO, KRED, and catalase) process that also had several phase transitions required careful optimization. The KRED and catalase loadings were selected, so that these enzymes were not the limiting enzyme for the process. At the same time, the overall enzyme loading needed to be kept to the minimum to reduce the overall process cost. A search through Codexis' in-house KRED and catalase collections identified the suitable enzymes for the process.

Scheme 5. Comparison of KRED and GDH-Mediated NADPH Recycling Systems



At the end of the enzyme and process developmental efforts, the optimal biocatalytic process involved the use of 50 g/L pyrmetazole, 1 g/L BVMO variant, 0.5 g/L KRED CDX-019, 0.1 g/L $NADP^+$, 0.2% v/v catalase CAT-101, 2.5 g/L esomeprazole seed, and 4% v/v IPA, in 50 mM phosphate buffer pH 9.0, under oxygen blanket, at 25 °C overnight. Downstream reaction workup through extraction into isobutyl methyl ketone (MiBK) and filtration afforded esomeprazole in 87% isolated yield, 99% purity (by HPLC), 99.9% ee, and <0.1% sulfone. As compared to Kagan–Sharpless–Pitchen oxidation process, this biocatalytic process serves as an alternative practical process with good productivity at attractive cost. The reaction is mainly aqueous based, with only minimal organic solvent, IPA. The downstream process workup is high yielding due to the high enantiopurity of the product with minimal sulfone impurity.

Enantioselective Sulfoxidation of Other Sterically Demanding Sulfides. The BVMO variants produced in the evolution program described were found to be active on other sterically demanding sulfides, such as the other proton pump inhibitors, "prazole" sulfides and the modafinil sulfide.[22] This demonstrated the broader applicability of the evolved BVMO as catalyst for enantioselective sulfoxidation. Although the enantioselectivity and productivity might not be ready for commercial manufacturing of those materials, further directed evolution could be employed to improve the biocatalytic process. This further exemplifies the usefulness of BVMO-based biocatalytic approaches as a practical alternative to Kagan–Sharpless–Pitchen oxidation.

CONCLUSION

Kagan–Sharpless–Pitchen oxidation remains the method of choice for many chemists when considering enantioselecitve sulfoxidation. However, this report offers a practical alternative process using an evolved BVMO for enantioselective sulfoxidation which gives high catalytic efficiency with much superior chemo- and enantioselectivity. Through both directed evolution and process development, high enantioselectivity is demonstrated with minimal side products formation, on top of good productivity, cost effectiveness, and environmental sustainability. The enzyme variants produced in the course of this evolution effort were also useful for the enantioselective sulfoxidation of other bulky–bulky compounds, such as armodafinil and rabeprazole.

For esomeprazole, specific physical challenges were encountered, such as mass transfer challenges, reaction medium viscosity, and product instability. Within the boundaries set by these physical limitations, directed evolution technology was employed to improve the enzyme performance and characteristics. The biocatalytic sulfoxidation process parameters can be first adjusted to address the physical mass transfer limitations, and then directed evolution technology can be used to adapt the enzyme to the new process conditions, instead of having to further restrict the reaction parameters to accommodate enzyme limitations.

EXPERIMENTAL SECTION

Laboratory Scale Sulfoxidation. Pyrmetazole (30 g, 0.091 mol, ex Sinojie (HK) Ltd.) and esomeprazole (1.5 g) were charged to a

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J. Org. Chem. XXXX, XXX, XXX–XXX

jacketed reactor vessel, equipped with baffle and an anchor-shaped agitator, containing potassium phosphate buffer (0.05 M, pH 9.0, 517 mL). This reaction mixture was agitated at 150 rpm at 25 °C for 10 min in order to obtain a well-suspended slurry, and the vessel was filled with oxygen via three cycles of evacuation and oxygen refill. The reaction vessel was held under positive oxygen pressure with an oxygen filled balloon. Propan-2-ol (HPLC grade, 24 mL, 0.31 mol, 3.4 mol equiv), NADP solution (60 mg in 4 mL 0.05 M phosphate pH 9.0 buffer), ketoreductase CDX-019 solution (300 mg in 15 mL 0.05 M phosphate pH 9.0 buffer), BVMO round 19 hit solution (600 mg in 40 mL 0.05 M phosphate pH 9.0 buffer), and 1.2 mL of catalase (1.2 mL, from Sigma-Aldrich) were added to the mixture which was stirred at 25 °C for 48 h. The initial stir rate of 300 rpm was gradually increased stepwise to 450 rpm throughout the reaction. The course of the reaction was followed by taking periodic samples from the reaction mixture which were diluted 100-fold with MeOH and analyzed using HPLC method 1.

Upon reaction completion, methyl isobutyl ketone (165 mL) was added to the vessel with stirring at 300 rpm, and the jacket temperature was increased to 48 °C. After complete dissolution (approximately 25 min), the stirring was stopped, and the phases allowed to separate (approximately 20 min). The aqueous turbid layer was drained and collected. The brown organic layer was subsequently drained and submitted to a warm filtration over Celite applying vacuum (the temperature of the jacket filter was adjusted to 45 °C). The Celite cake was retained for later use, the aqueous phase was transferred back to the heated vessel, and further methyl isobutyl ketone (45 mL) was added and stirred at 300 rpm for 30 min. Approximately 20 min after the agitation was stopped, the yellow turbid aqueous layer was drained and discarded. The brown organic layer was drained and filtered over the retained Celite filter cake used previously. The organic phases when combined released a small amount of water which was separated. The organic phase was transferred back to the vessel, the temperature was adjusted to 15 °C, and the agitator was set to 150 rpm for 1 h. The product precipitated from the solution as a dense slurry. The temperature was further reduced to 10 °C, and the slurry stirred at 150 rpm for 30 min. Finally the temperature was adjusted to 5 °C, and the mixture stirred at 150 rpm for 30 min. In order to increase the mobility of the slurry, *n*-heptane (240 mL) was added slowly in 30 mL portions (over 5 min). The slurry was drained and filtered off, and the filter cake was dried in a vacuum oven (3−10 mmHg) at 25 °C for 72 h. A total of 28.7 g of esomeprazole was isolated (87% isolated yield) with a chemical purity of 99% by HPLC (method 4, see Supporting Information) and >99% ee (method 5, see Supporting Information).

^{1}H NMR (400 MHz, MeOH-d_4): δ 8.09 (s,1H), 7.51 (d, J = 8.8 Hz, 1H), 7.08 (d, J = 2.4 Hz, 1H), 6.96 (dd, J = 8.8, 2.4 Hz, 1H), 4.73 (m, 2H), 3.83 (s, 3H), 3.68 (s, 3H), 2.22 (s, 3H), 2.15 (s, 3H). ^{13}C NMR (100 MHz, MeOH-d_4): δ 166.2 (s), 159.3 (s), 150.5 (s), 149.8 (s), 129.1 (s), 128.2 (s), 115.8 (s), 61.4 (s), 60.6 (s), 56.3 (s), 13.4 (s), 11.7 (s). LCMS (ESI+) m/z 346.4.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.8b00468.

Selected NMR data, analytical methods, HPLC data, and HPLC/LCMS chromatograms (PDF)

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Notes

The authors declare no competing financial interest.

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J. Org. Chem. XXXX, XXX, XXX−XXX