Enzymatic Stereodivergent Synthesis of Azaspiro[2.y]alkanes

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ABSTRACT: Azaspiro[2.y]alkanes are increasingly valuable scaffolds in pharmaceutical drug discovery; however, an asymmetric catalytic method for their synthesis remains unknown. Here, we present a stereodivergent carbene transferase platform for the cyclopropanation of unsaturated exocyclic *N*-heterocycles to provide structurally-diverse and pharmaceutically-relevant azaspiro[2.y]alkanes in high yield (21–>99% yield) and excellent diastereo- and enantioselectivity (dr from 52.5:47.5 to >99.5:0.5; er from 51:49 to >99.5:0.5). These engineered protoglobin-based enzymes operate on gram scale, with no organic co-solvent, at substrate concentrations up to 150 mM (25 g/L) using lyophilized *E. coli* lysate as the catalyst. This platform represents a practical, scalable, and stereodivergent biocatalytic synthesis of azaspiro[2.y]alkanes using low-cost engineered protoglobins and their native iron-heme cofactors.

Azaspiro[x.y]alkanes are emerging scaffolds in pharmaceutical drug discovery, having been shown to enhance key pharmacokinetic and physicochemical properties, including potency, lipophilicity, target selectivity, and metabolic stability (**Figure 1A**).[1–5] These advantages are largely attributed to their rigid yet three-dimensional structure, featuring an expanded set of spatial vectors for chemical interactions with target proteins, compared to planar alternatives.[6–9]

Within this class of molecular frameworks, azaspiro[2.y]alkanes, characterized by a cyclopropane moiety joined at a quaternary carbon of a *N*-heterocycle, have been incorporated into numerous pharmaceutical drug candidates spanning various therapeutic areas. For example, Genentech showed that replacing an aromatic linker with an azaspiro[2.5]octane improved the binding potency of a nicotinamide phosphoribosyltransferase (NAMPT) inhibitor while minimizing off-target interactions with the CYP2C9 enzyme (**Figure 1B**).[10] AstraZeneca similarly utilized an azaspiro[2.5]octane in a histamine-3 receptor (H_3R) antagonist and found improved target selectivity and *in vivo* efficacy, while also reducing off-target effects.[11] In each of these cases, the stereochemical configuration proved to be a key determinant of compound potency. Beyond these examples, the growing utility of azaspiro[2.y]alkanes is evidenced by an increasing number of reports describing their incorporation into drug candidates, including azaspiro[2.3]hexanes,[12–22] azaspiro[2.4]heptanes,[13,15,20,23–32] and azaspiro[2.5]octanes,[22,33–47] among other scaffolds.[40]

The linchpin intermediate toward these structurally-diverse drug candidates commonly features an oxidized functionality bound to the cyclopropane—typically an ethyl ester—which, along with the amine, serves as a versatile handle for synthetic diversification. Conventional syntheses toward this intermediate involve a Horner-Wadsworth-Emmons olefination of the requisite ketone, followed by a Corey-Chaykovsky cyclopropanation.[7] Alternatively, Rh- and Cu-catalyzed cyclopropanations of the corresponding alkene with an acceptor-type diazo carbene precursor are known.[11,40] However, an asymmetric catalytic method has hitherto not been reported and chiral chromatographic separation is required to access enantioenriched intermediates, rendering these syntheses both costly and time and resource intensive.

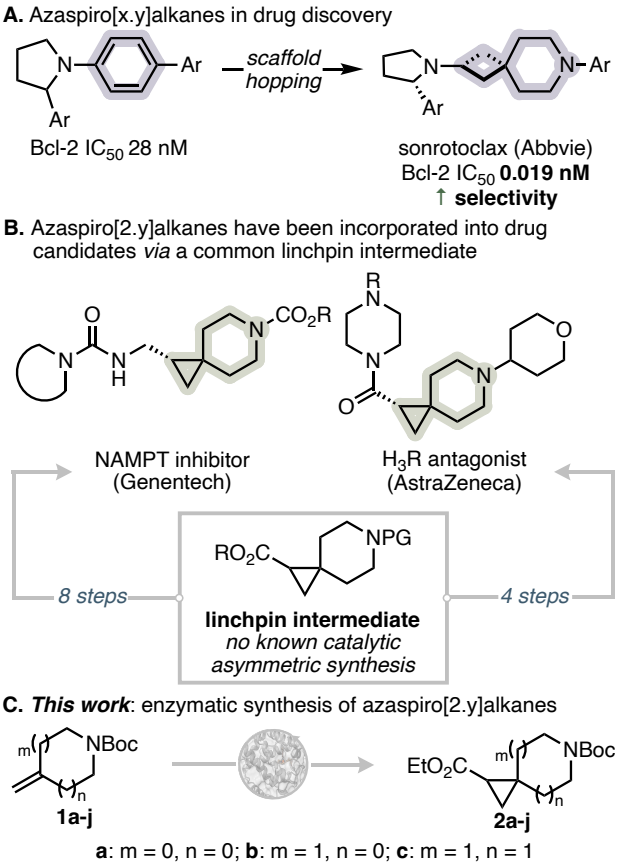


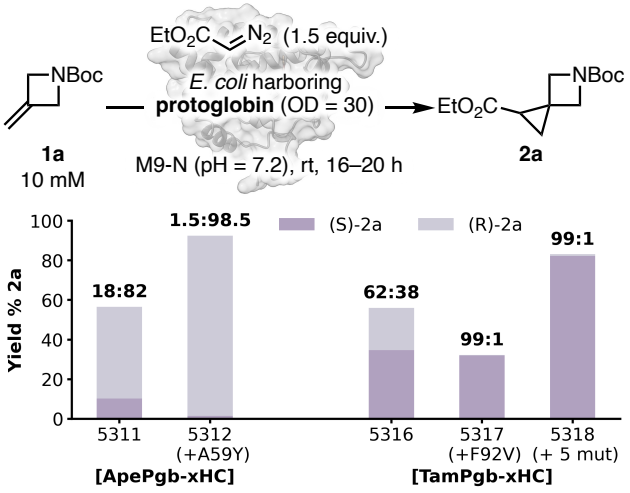
Figure 1. (A) Azaspiro[x.y]alkanes are rigid, three-dimensional alternatives to aromatic linkers. (B) Azaspiro[2.y]alkanes have been incorporated into drug candidates spanning a range of therapeutic areas. (C) This work describes a stereodivergent enzymatic synthesis of azaspiro[2.y]alkanes of varying ring size and substitution.

Biocatalysis offers a promising asymmetric catalytic solution by providing a highly-ordered stereochemical environment that

can be tuned to enhance activity, selectivity, and scalability.[48,49] Our group has previously engineered heme-dependent enzymes for *new-to-nature* carbene transfer reactions, including cyclopropanations with acceptor-type diazo compounds.[50–53] We therefore hypothesized that an engineered carbene transferase could provide an asymmetric catalytic route to this valuable linchpin intermediate and that directed evolution (DE) would enable access to stereodivergent pathways (**Figure 1C**).[54,55] Our goal was to develop a platform of engineered enzymes that exhibit stereodivergence across a broad application-driven substrate scope, establishing a general, practical route to enantioenriched azaspiro[2.y]alkanes for drug discovery and production.

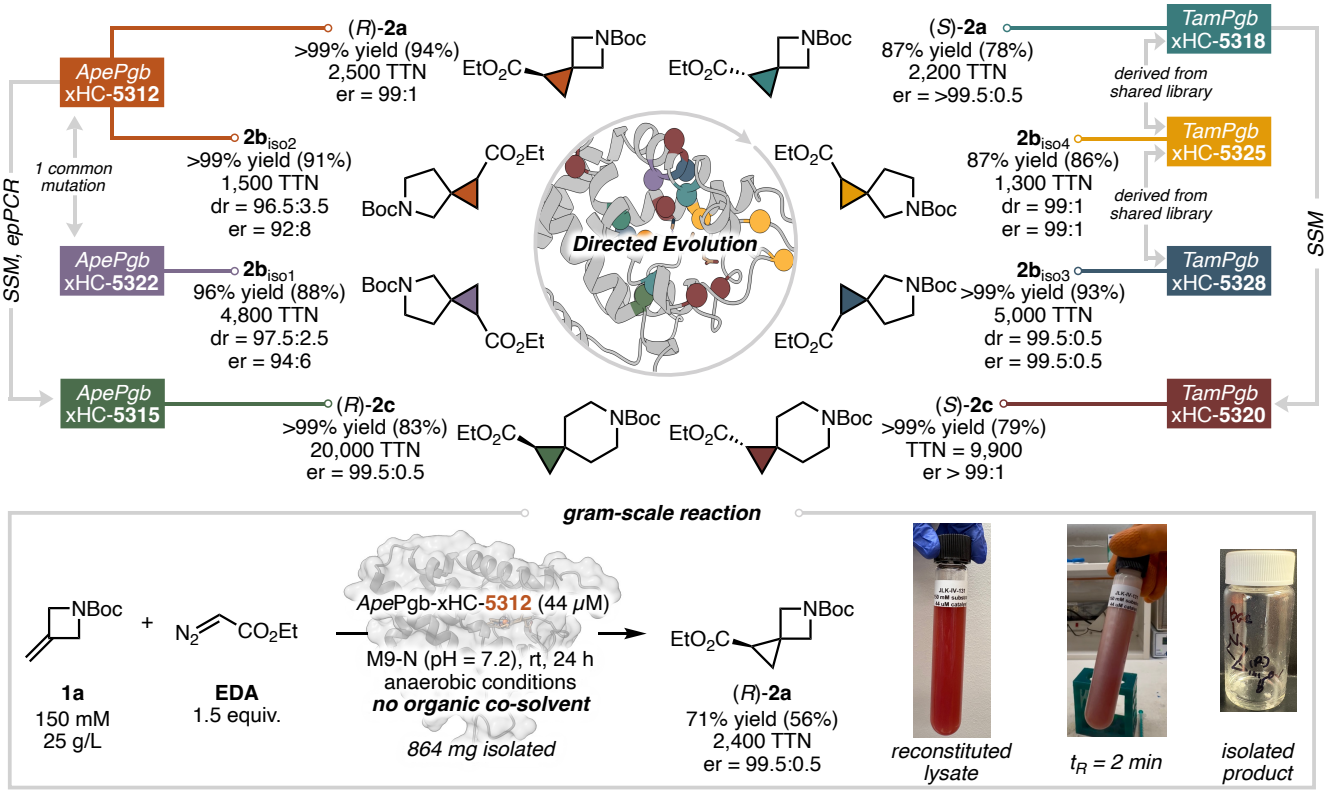
At the outset, we screened an in-house library of 60 protoglobins previously engineered to effect carbene transformations. Protoglobins are small (~200 amino acids), highly thermostable hemoproteins that are easily synthesized by host microbes like *Escherichia coli*.[56,57] Excitingly, we discovered an *Aeropyrum pernix* protoglobin (*Ape*Pgb) containing four mutations from wild type (W59A Y60G V63P F145W) (denoted *Ape*Pgb-xHC-**5311**; where xHC = eXo-cyclic Heterocycle Cyclopropanation) that catalyzes the cyclopropanation of *tert*-butyl 3-methyleneazetidine-1-carboxylate (**1a**) with ethyl diazoacetate (EDA) to yield (*R*)-**2a** in 56% yield with good enantioselectivity (er = 82:18) in a whole-cell context (**Scheme 1**). Fortuitously, within the same enzyme library, a protoglobin from *Thermus amyloliquefaciens* (*Tam*Pgb, 60% pairwise amino acid (AA) sequence identity to *Ape*Pgb) containing two mutations from wild type (W59L Y60Q) (denoted *Tam*Pgb-xHC-**5316**) favored (*S*)-**2a** in 56% yield with an er = 62:38.

Scheme 1. Enantiodivergent directed evolution of protoglobins toward (*R*)- and (*S*)-**2a** from **1a**



We considered *Ape*Pgb-xHC-**5311** and *Tam*Pgb-xHC-**5316** excellent starting points for the development of an enzyme-controlled enantiodivergent pathway to **2a** and conducted DE to improve both activity and selectivity in each stereochemical direction. Site-saturation mutagenesis (SSM) on active site residues revealed ApePgb-xHC-**5312**, containing a mutation at site 59 (A59Y), which provides (*R*)-**2a** in 93% yield and excellent enantioselectivity (er = 98.5:1.5). Toward (*S*)-**2a**, a critical mutation at site 92 (F92V) provided *Tam*Pgb-xHC-**5317**, which

Scheme 2. Engineered carbene transferase platform for synthesis of azaspiro[2.y]alkanes[a]



[a]Reactions were performed using lyophilized lysate powder on 0.4 mmol scale (unless otherwise specified). Analytical yields were determined by comparison to an internal standard using GC-FID and a corresponding calibration curve. Isolated yields are designated in parentheses. TTNs are based off analytical yield. Stereoselectivities (er and dr) were determined on crude reaction mixtures using GC-FID equipped with a chiral stationary phase. The absolute configuration of (*R*)-**2a** was determined by x-ray crystallography and was assigned by analogy to **2c**.

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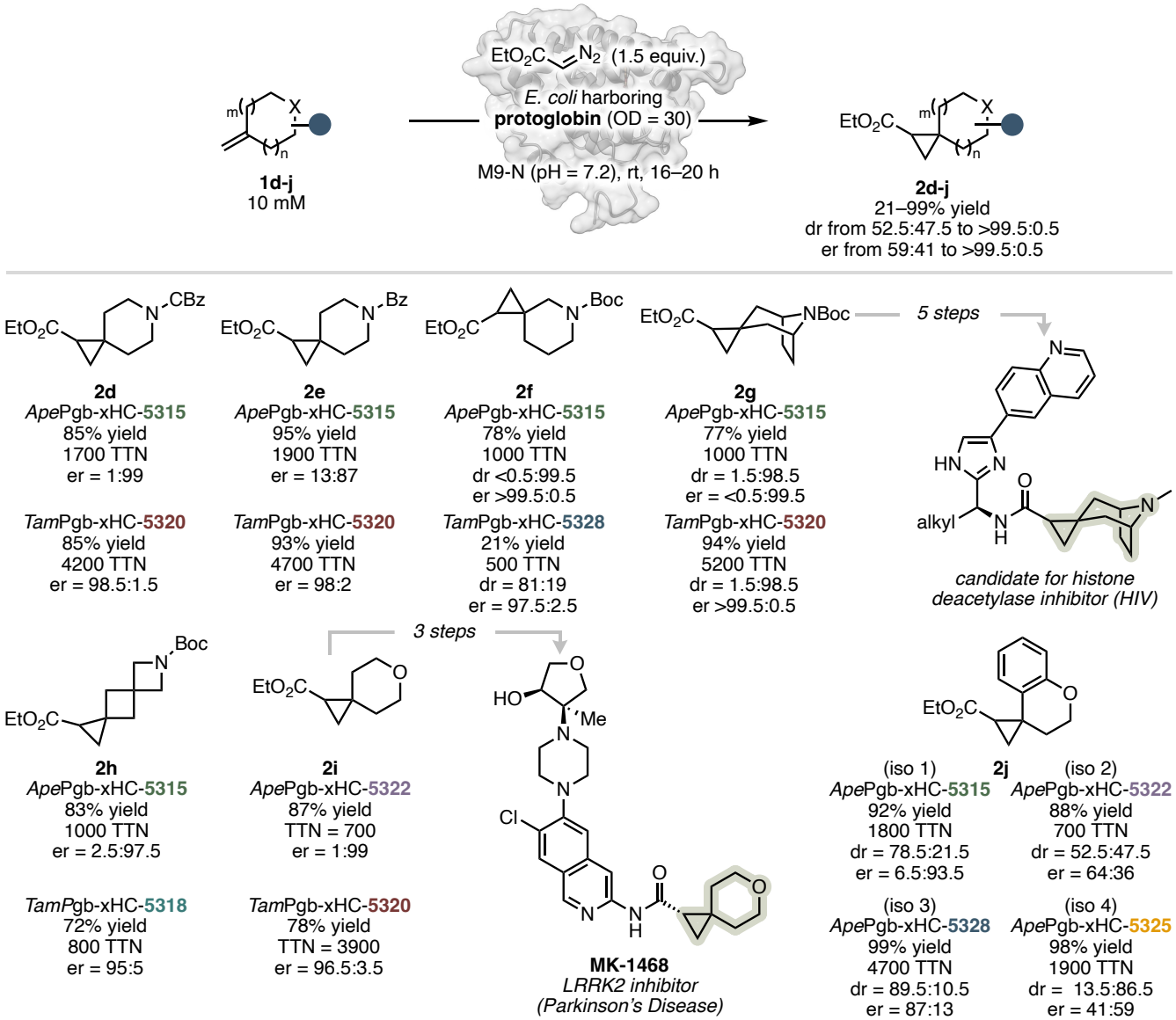
Scheme 3. Engineered carbene transferase platform for synthesis of azaspiro[2.y]alkanes[a]



[a]Reactions were performed on 0.4 μmol scale using whole *E. coli* cells. Analytical yields were determined by comparison to an internal standard using GC-FID and a corresponding calibration curve. Stereoselectivities (er and dr) were determined on crude reaction mixtures using either GC-FID, HPLC, or SFC-MS equipped with a chiral stationary phase.

yields (*S*)-**2a** with an er = 98:2, albeit with reduced yield (32%). From here, error-prone mutagenesis (epPCR) provided *Tam*Pgb-xHC-**5318**, containing five additional AA substitutions (D57G G61D K134R P137L K153R) that reinstated high activity (83% yield) while maintaining excellent enantioselectivity (er = 99:1).

Given the pharmaceutical relevance of azaspiro[2.y]alkanes of varying ring size and substitution, we sought to build upon our initial engineering campaign to provide a suite of engineered enzymes capable of achieving stereodivergence across multiple scaffolds. To do so, we tested variants *Tam*Pgb-xHC-**5316**–**5318** and *Ape*Pgb-xHC-**5311**–**5312**, as well as select mutant libraries, on pyrrolidine- and piperidine-based substrates tert-butyl 3-methylenepyrrolidine-1-carboxylate (**1b**) and tert-butyl 4-methylenepiperidine-1-carboxylate (**1c**), respectively. We reasoned that **1b**, a non-symmetric scaffold, would expand the platform's structural diversity, while **1c**, a common motif in drug discovery, would bolster its utility.

Encouragingly, we observed promising activity and selectivity toward all six possible stereoisomers of **2b** and **2c**, with *Ape*Pgb-xHC-**5312** providing isomer 2 of **2b** in excellent yield and high diastereo- and enantioselectivity. We next conducted parallel DE campaigns, optimizing each enzyme lineage for selectivity and activity toward a given stereoisomer. We identified evolved enzymes capable of forming all possible stereoisomers of **2b** and **2c** with excellent stereoselectivity and exceptional activity (see SI section 4 for evolution details). These engineering efforts culminated in a carbene transferase platform for the stereoselective synthesis of azaspiro[2.y]alkanes (**Scheme 2**). Site 92 remained a critical mutation for stereodivergence with both **1b** and **1c**, suggesting a conserved mechanistic role toward varying azaspiro[2.y]alkane frameworks. Altogether, the sequences represented in this platform are highly conserved, with *Ape* and *Tam* variants averaging Hamming distances of 4.3 and 8.5 amino acids, respectively, underscoring the functional diversity accessible through minimal rounds of DE.

Notably, all seven final variants are active catalysts as lyophilized lysate powders at 0.4 mmol scale and can be distributed similarly to any standard chemical reagent. Under these conditions, each variant exhibits TTNs between 1,000–20,000 and isolated yields between 78–94%. These protoglobin catalysts also retain activity at high substrate concentrations. Using a 1-L expression culture of ApePgb-xHC-**5312**, subjected to lysis and lyophilization, we observed 88% conversion of 150 mM (25 g/L) of **1a** to yield **2a** in 71% yield (56% isolated yield) (2400 TTN) with an er = 99:1. This reaction is performed in a fully aqueous environment without an organic co-solvent. This is amongst the highest reported substrate loading for a carbene transferase[50], without compromising stereoselectivity.[58] These results highlight the practicality of our enzyme platform and its potential for large-scale applications without further protein engineering.

We next evaluated all seven final variants across a range of substrates, focusing on the synthesis of azaspiro[2.y]alkanes with established pharmaceutical relevance. First, we tested various *N*-protecting groups, finding that the platform is amenable to *N*-Cbz and *N*-Bz protecting groups, forming **2d** and **2e** in high yield and with excellent enantioselectivity, with distinct variants selectively producing either the (*R*)- or (*S*)-enantiomer in each case (**Scheme 3**).

We next tested a non-symmetric piperidine substrate (**1f**) to form **2f**, a motif featured in numerous drug candidates, including anti-bacterial agents[36,59], MDM2 modulators[45], and DGAT1 inhibitors[44]. Excitingly, *Ape*Pgb-xHC-**5315** furnished one isomer of this compound in high yield and with excellent enantio- and diastereoselectivity. In contrast, *Tam*Pgb-xHC-**5328** yields another isomer in moderate yield and diastereoselectivity, with excellent enantioselectivity. Further, *Ape*Pgb-xHC-**5322** and *Tam*Pgb-xHC-**5325** provide excellent starting points to engineer variants selective for the remaining two possible stereoiomers (see SI section 8 for details).

The platform similarly provides excellent starting points to selectively access multiple stereoisomers of **2g**, a tropane derivative that has been incorporated into drug candidates targeting histone deacetylase[40] and DGAT1[44]. *Tam*Pgb-xHC-**5320** and *Ape*Pgb-xHC-**5315** provide two of the four possible stereoisomers in high yields and with excellent levels of diastereo- and enantioselectivity. Further, *Ape*Pgb-xHC-**5312** is a promising starting point for the formation of a diastereomer highly disfavored in the Rh-catalyzed cyclopropanation of **1g** (see SI section 8 for details).

Additionally, **2h** has shown promising potency as a muscarinic acetylcholine receptor (mAChR) antagonist.[60] Excitingly, *Ape*Pgb-xHC-**5315** furnishes **2h** in 83% yield with excellent enantioselectivity (er = 2.5:97.5). In contrast, *Tam*Pgb-xHC-**5318** delivers the antipode in 72% yield with an er of 95:5.

We additionally probed oxygen heterocycles and found that the platform is amenable to the synthesis of **2i**, a pyran derivative, which has been incorporated into LRRK2 inhibitor MK-1468 by Merck[61] and studied by many others.[40,62–65] We observed excellent activity and importantly, stereodivergence: *Ape*Pgb-xHC-**5322** provides the **2i** with an er = 1:99 and *Tam*Pgb-xHC-**5320** provides the antipode with an er = 96.5:3.5.

We next evaluated chromane derivative **1j**, a scaffold that is markedly distinct from those used during platform evolution and has also been featured in EP_4 receptor antagonists.[66,67] Despite the substrate's structural divergence, the platform enables access to all four diastereomers of product **2j** in high yield, with different variants selectively favoring each isomer. This result highlights the platform's substrate promiscuity, suggesting broad applicability to structurally diverse scaffolds beyond those explicitly represented in the evolution substrate set.

We describe a carbene transferase platform for the cyclopropanation of unsaturated exocyclic *N*-heterocycles to yield azaspiro[2.y]alkanes in high yields and with excellent levels of enantio- and diastereoselectivity. This stereodivergent platform shows excellent substrate generality, obviates the need for post-synthetic resolution steps, and is amenable to gram-scale synthesis; thus, improving applicability for both drug discovery and production efforts. The observation of stereodivergence on a substrate that is structurally distinct from those evolved on suggests that this enzyme platform contains biocatalysts capable of accessing a wide range of desired azaspiro[2.y]alkane stereoisomers. We envision this platform as a starting point for the selective synthesis of any desired stereoisomer of azaspiro[2.y]alkanes and anticipate its integration into drug discovery pipelines.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

General procedures, discovery of initial activity, control experiments, directed evolution lineage trajectories and plots, DNA sequences of evolved enzymes, gram-scale enzymatic reaction at high substrate concentration, yields and enantioselectivities of enzymatic substrate scope reactions, synthesis of starting materials, synthesis and characterization of authentic standards, calibration curves for authentic standards, analytical traces for stereoselectivity determination, x-ray crystallography data, spectroscopic data.

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Notes

The authors declare no competing financial interest.

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Table 1. Example of a Double-Column Table

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