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Letter

Selective P450$_{BM3}$ Hydroxylation of the Spiro[3.3]heptane Core as a Route to Potential Drug Fragment Molecules

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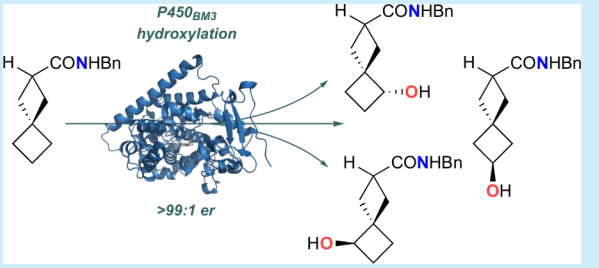
ABSTRACT: Engineered P450$_{BM3}$ enzyme variants, developed from an initial screening panel of 42 enzymes, convert *N*-benzyl spiro[3.3]-heptane-2-carboxamide into three distally monohydroxylated regioisomers with essentially complete enantioselectivity. Two α-hydroxyamide derivatives are also produced. Elaboration of the metabolites by tethered C−H amination leads to spiro[3.3]heptane motifs substituted with three different functional groups ready for further derivatization.



The spiro[3.3]heptane ring system, first described in 1907,[1] is gaining in prominence within early stage drug discovery efforts, including as a template for fragment development[2] and as a bioisosteric replacement for benzene (Figure 1).[3] This interest has developed sharply over the past

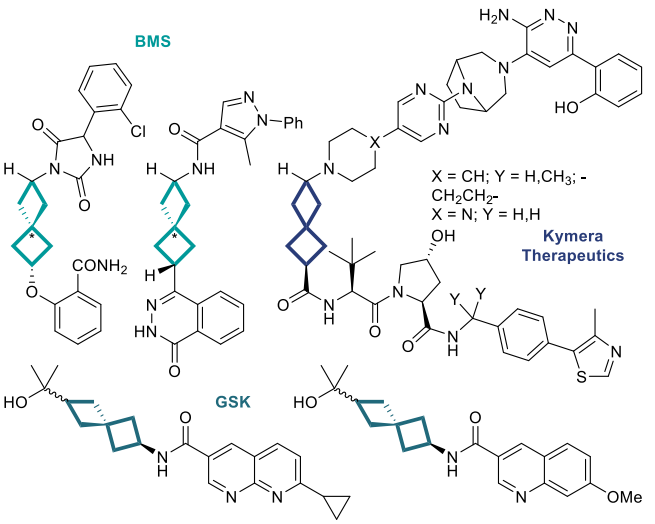


Figure 1. Spiro[3.3]bicyclohepta-2,6-diyl linking motifs in ROCK inhibitors (BMS),[10] anticancer SMARCA degraders (Kymera),[11] and H-PGDS inhibitors (GSK).[12]

decade, no doubt stimulated by widely promoted alternatives to 'traditional' medicinal discovery chemistry strategies as exemplified by Lovering's influential paper correlating the fraction of sp^3 carbons (Fsp^3) and number of stereogenic carbons with success in progressing from hit through lead to clinical drug.[4] Taking benzene as a representative "flatland" template (Fsp^3 = 0), for which there are only three possible isomers of a given disubstituted derivative, in contrast there are 18 ways to arrange two different substituents around separate rings of the three-dimensional spiro[3.3]heptane core (Fsp^3 = 1.0) and all 18 isomers are chiral.[5] Gaining access to all these isomers would allow a thorough exploration of the relationship between the disposition of functionality around the core and the nature and strength of binding with a given biological target; however, to do so using current methods would be onerous because each isomer would require its own tailored synthesis.

In this communication we illustrate a synthetic route to representative 2,6- and 1,6- disubstituted spiro[3.3]heptanes, substitution patterns which represent around 90% and 10%, respectively, of the almost 9,000 reported spiro[3.3]heptanes bearing a single substituent in both rings.[6] Our strategy of diversifying from a single starting point by P450$_{BM3}$-biocatalytic hydroxylation,[7] broadly follows the workflow summarized in our recent publications,[7a,8] here extending the range of cyclobutane-containing hydroxylation substrates to incorporate spirocycles.[9]

In this project, spiro[3.3]heptane-2-carboxamide derivative **1** (Scheme 1) was selected for screening based on its ease of preparation by sequential Perkin ring synthesis,[13] lack of chirality to avoid complications arising from the differential reactivity of enantiomers, incorporation of polar functionality to bias productive conformations within the enzyme active site, and inclusion of a UV chromophore to aid analytical detection.

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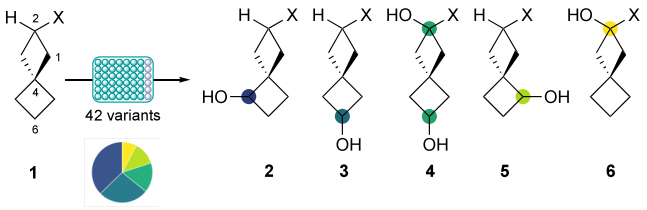
Scheme 1. Products from the Initial Screen of Amide 1[a]



[a]X = CONHBn; the products are presented in decreasing overall order of abundance, and the pie chart depicts this as the fraction (GC) of each product summed across all variants.

From a preliminary screening of 95 $P450_{BM3}$ variants, 42 converted the substrate into hydroxylation products **2**–**6**[14] along with several minor, unidentified products (Supporting Information (SI), Table S2.2).

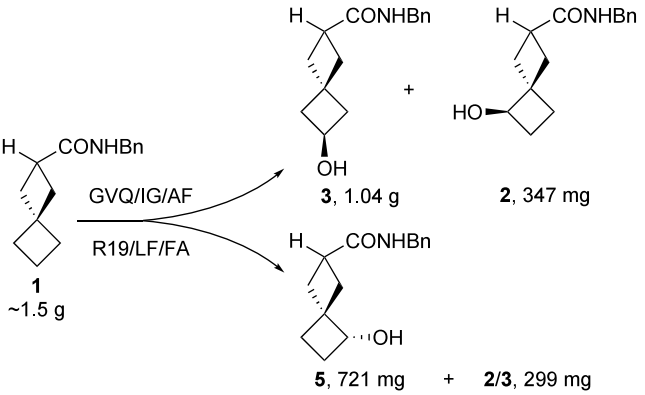
Taken together, the enzyme variants effected hydroxylation at the *trans*-5 (**2**)- and 6 (**3**, **4**)-positions in roughly equal ratio, these three products comprising about 80% of the total. From this first screening, of the variants producing the most monohydroxylation product **2**, K19/F87V/I263G, rather than GQ/I263G/A330L, was selected for optimization via iterative docking-guided mutagenesis (IDGM)[8] because of its stability and reliable expression. These docking studies (SI, S7.1–S7.4) suggested that both substrate conversion and selectivity for **2** would be improved by introducing bulkier residues at S72 and L75, smaller residues at F87, and residues at A330 to encourage binding of the benzyl group. Nine single-point variants were screened (SI, Table S2.3a), six of which showed either improved substrate conversion or improved selectivity for **2**. All gave the same major enantiomer, later assigned as (2*S*,4*r*,5*R*)-**2** by Mosher's ester analysis (SI, S5.1). Of these, the A330F variant delivered complete substrate conversion and the highest ratio of **2** with respect to the next most abundant product (**2**/**3** = 81:19) and so this was used for a second IDGM round focused on improving both regio- and stereoselectivity while retaining conversion. From this, 23 new single- and double-residue variants were generated aiming primarily to block nonproductive docking poses (SI, Table S2.3b); five of these were more regioselective and six were more enantioselective than K19/F87V/I263G. The F87L variant gave 100% conversion, a **2**/**3** ratio of 90:10, and the major product (2*S*,4*r*,5*R*)-**2** in 90% ee; figures for the next best **2**-producing variant (F87I/A330I) were 95% conversion, 84:16 **2**/**3** ratio, and >99% ee.

From the first screening, maximum production of the 6-hydroxylated metabolite **3** was achieved by variant GVQ/I263G and so the IDGM approach was applied from this starting point with single-point variants at four residues (SI, S7.5). Bulkier residues at F87 (V87 in GVQ) and T260 were intended to block nonproductive poses. The F and W variants of A330 were intended to encourage phenyl group binding, with L and I variants employed for comparison purposes. Three F87/A328 double variants were also screened. Primers for four S332 variants became available from a separate project and were also introduced (SI, Table S2.4). Many of the 17 new variants generated (2*R*,4*r*,6*R*)-**3** (assigned by X-ray crystallography, SI, S6.1) with essentially complete enantiopurity, and it was notable that all the variants retaining F87 gave either a lower ee or resulted in production of the opposite enantiomer (up to 70% ee). Five variants showed higher selectivity for **3** relative to **2** although there were no clear correlations in this aspect with residue changes. Of the four variants giving an increased production of this metabolite at higher ee, the A330F variant was the most regioselective (**3**/**2** = 75:25); the F87I variant was marginally less reactive than this but hydroxylation gave both **3** and **2** in >99% ee and with a higher ratio (70:30) than GVQ/I263G itself (**3**/**2** = 65:35; ee 95% and 90%, respectively).

Just three of the 42 variants provided reasonable conversion to the final (*cis*-5-) distally monohydroxylated product (**5**). Of these, variant R19/F87A was chosen for optimization based on its demonstrated high enantioselectivity for this product as well as its relatively clean product profile in which the only minor component observed by GC was the *trans*-5- isomer **2** (**5**/**2** = 76:24). Based on IDGM (SI, S7.6), aiming to disfavor nonproductive poses, single-point replacements of L75 and T260 were made (aromatic F or W; bulkier L or I; SI, Table S2.5). The L75F variant gave slightly improved conversion while maintaining complete enantioselectivity for (2*R*,4*s*,5*R*)-**5** assigned by Mosher's ester analysis (SI, S5.2) and confirmed by X-ray crystallography (SI, S6.2). Against the IDGM guidance, the T260 variants resulted in poor activity or much lower selectivity for **5**.

Reactions conducted on an ∼1 mmol scale for product characterization afforded moderate isolated yields of the individual monohydroxylated regioisomers **2** (38% from K19/F87I/I263G/A330I), **3** (36% from GVQ/I263G/A330F), and **5** (30% from R19/L75F/F87A). For efficient gram-scale production (≥4.5 mmol) it was necessary to lower the substrate/enzyme ratio, maintain a slightly alkaline reaction pH (7.9), ensure efficient agitation (overhead stirrer) and oxygenation (air bubbling); and include cyclodextrin to aid substrate solubilization. With these parameters established, each isomer was obtained reliably in synthetically useful quantities (Scheme 2) from reaction of ∼1.5–1.6 g of amide **1**.

Scheme 2. Gram-Scale Preparation of Monohydroxylated Products 2, 3, and 5



The products are able to be elaborated trivially by acylation, or by substitution without rearrangement under conditions which favor an S_N2 mechanism.[15,16] The corresponding ketones **7**–**9** were prepared without complication for the production of racemic standards (Scheme 3). Such transformations maintain the 2,5- and 2,6-substitution pattern in the derivatives but this spirocyclic system afforded an opportunity to develop trisubstituted motifs by engaging the newly introduced hydroxyl group to facilitate a second C–H bond functionalization. In earlier work we had discovered that Rh(II) nitrenoid insertions at nonactivated methylene sites

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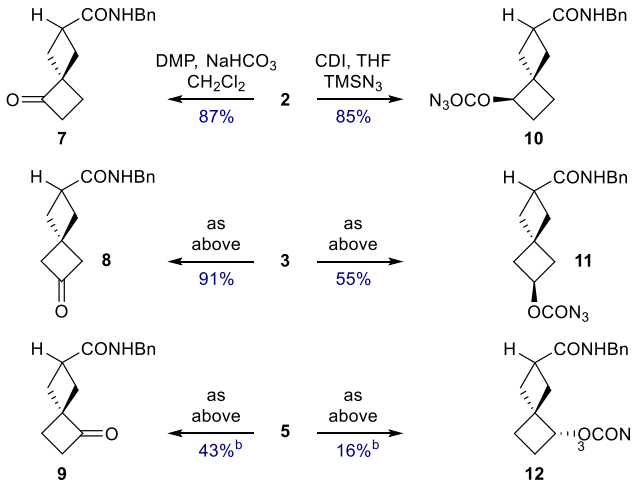
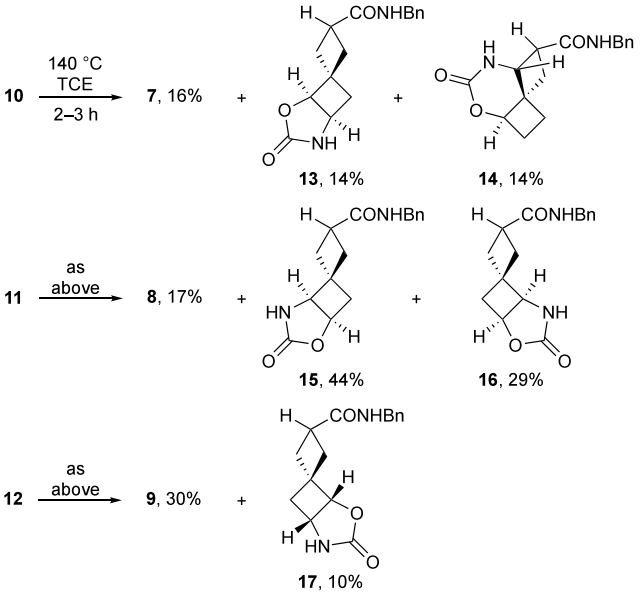
Scheme 3. Derivatives of the Monohydroxylation Products[a]



[a]DMP, Dess–Martin periodinane; CDI, carbonyl diimidazole. [b]The yields reflect the relatively low reactivity of the alcohol in **5** which is both neopentylic and *cis*-disposed to the bulky CONHBn group.

were unacceptably inefficient.[17] Thermolytic reactions of analogous azidoformates[18,19] were more successful but side reactions dominate with flexible substrates such as medium ring cyclic amines. The relative rigidity of the spiro[3.3]-heptane framework was, however, expected to promote more predictable reactivity. Thermolysis of azidoformate **10** (Scheme 4) was complete within 3 h at 140 °C to afford

Scheme 4. C–H Insertion Products from Thermolysis of Azidoformates 10–12



ketone **7**, along with the 1,2- and 1,3-C–H amination products **13** and **14**, respectively, in roughly equal proportions. Similarly, azidoformate **11** produced ketone **8** and the 1,2-C–H amination regioisomers **15** and **16** in ~20:50:30 ratio, respectively. Thermolysis of azidoformate **12** was the least efficient, generating only 1,2-C–H amination product **17** in low yield, with ketone **9** being the major product from this isomer.

In conclusion, this work demonstrates that each site of the unfunctionalized cyclobutane ring in amide **1** can be accessed by biocatalytic hydroxylation to produce the *trans* (**2**)- and *cis* (**5**)-5-ol, and 6-ol (**3**) isomers, essentially enantiomerically pure at a synthetically useful scale (>multihundred mg). Combined with a secondary C–H insertion event, made possible by the initial hydroxylation,[20] trisubstituted spiro[3.3]heptane derivatives are accessible in just three steps from a common substrate. The so-formed templates bear spatially distinct combinations of carboxyl, hydroxyl, and amino functionality (Figure 2), each of which could be elaborated independently to yield a wide range of derivatives for drug development applications.[21]

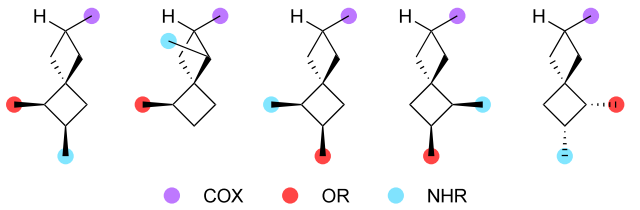


Figure 2. Trisubstituted spiro[3.3]heptane motifs available by sequential C–H functionalization from amide **1**.

This general approach is equally applicable to other monosubstituted spiro[3.3]heptane derivatives. For example, in a preliminary screening of *N*-Boc-spiro[3.3]heptyl-2-amine (**1**, X = NHBoc), nine variants of the enzyme library (SI, Table S2.1) converted the substrate into three major products in variable ratio: the *trans*-1-, *cis*-5-, and *trans*-5-hydroxylated derivatives.[22] Validation and optimization of these hydroxylations, and analysis of their absolute stereochemical course will be the subject of further work in this area.

■ ASSOCIATED CONTENT

Data Availability Statement

The data underlying this study are available in the published article and its Supporting Information.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.5c01265.

Enzyme variants, experimental procedures, characterization data, brief computational details, copies of ^{1}H and ^{13}C NMR spectra for all compounds, and crystallographic details (PDF)

Accession Codes

Deposition Numbers 2424349 and 2424351 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge via the joint Cambridge Crystallographic Data Centre (CCDC) and Fachinformationszentrum Karlsruhe Access Structures service.

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Author Contributions

The manuscript was written through contributions of all authors.

Notes

The authors declare no competing financial interest.

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(15) Examples of hydroxyl activation and azide displacement are provided in ref 14.

(16) Reactions which promote ionization result in ring contraction. For example, treatment of alcohols **2** or **5** with DAST in DMF resulted in *N*-benzyl 3-cyclopropyl-3-fluorocyclobutane-1-carboxamide; similar reactivity was displayed during tosylation to give the 3-cyclopropyl-3-hydroxy analogue.

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(21) Transformations of the carboxamide group also widen access to diverse functional group combinations; for example Hofmann rearrangement[14] connects to the 2,5- and 2,6-aminoalcohol series, of interest as potential aminophenol isosteres.

(22) Quantities of the mono-hydroxylation products sufficient for spectroscopic characterization were obtained by preparative-scale reactions with RT2/S72G/A330W and RT2/V78A/I263G/A330W.

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