Selective Functionalization of Aliphatic Amines via Myoglobin-Catalyzed Carbene N–H Insertion

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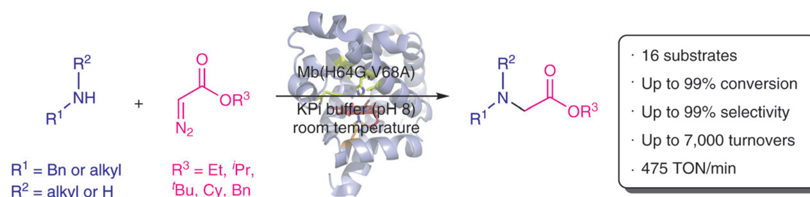
Published as part of the Cluster *Biocatalysis*

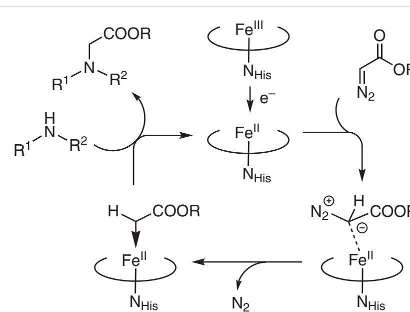


Received: 07.05.2019
Accepted after revision: 01.07.2019
Published online: 11.07.2019
DOI: 10.1055/s-0039-1690007; Art ID: st-2019-r0254-l

Abstract Engineered myoglobins have recently gained attention for their ability to catalyze a variety of abiological carbene transfer reactions including the functionalization of amines via carbene insertion into N–H bonds. However, the scope of myoglobin and other hemoprotein-based biocatalysts in the context of this transformation has been largely limited to aniline derivatives as the amine substrates and ethyl diazoacetate as the carbene donor reagent. In this report, we describe the development of an engineered myoglobin-based catalyst that is useful for promoting carbene N–H insertion reactions across a broad range of substituted benzylamines and α-diazo acetates with high efficiency (82–99% conversion), elevated catalytic turnovers (up to 7,000), and excellent chemoselectivity for the desired single insertion product (up to 99%). The scope of this transformation could be extended to cyclic aliphatic amines. These studies expand the biocatalytic toolbox available for the selective formation of C–N bonds, which are ubiquitous in many natural and synthetic bioactive compounds.

Key words biocatalysis, myoglobin, protein engineering, C–N bond formation, carbene N–H insertion

Strategies for the formation of C–N bonds are of fundamental importance in organic chemistry, due the ubiquitous presence of nitrogen-containing functional groups in synthetic building blocks and biologically active compounds. The insertion of metal-carbenoid units into Y–H groups (Y = N, O, B, Si, S) constitutes an attractive strategy for the construction of carbon–heteroatom bonds.[2] Over the past years, a number of organometallic catalysts, including Rh, Ru, Ag, Ir, Au, Pd, Cu, and Fe complexes, have been investigated for the synthesis of functionalized amines via metal-catalyzed carbenoid N–H insertions.[3] Metal-catalyzed N–H insertion reactions have also been exploited for bioconjugation purposes.[2a,4] More recently, heme-dependent proteins such as myoglobin (Mb)[5] and cytochrome P450s[6] have been identified as viable biocatalysts for promoting carbene transfer reactions,[7] including carbene N–H insertion reactions.[5,6] In previous work, our group has established that the engineered myoglobin variant Mb(H64V,V68A) is capable of catalyzing the insertion of α-diazo esters into the N–H bond of aniline derivatives with high efficiency (up to 7,000 turnovers)[5] via a process involving a reactive iron-carbenoid intermediate (Scheme 1).[8] Engineered cytochrome P450-BM3 variants[6] as well as myoglobin-based artificial metalloenzymes harboring Mn, Co, Ru, Rh, and Ir-substituted cofactors have also been employed for this reaction.[7g,9]



Scheme 1 Plausible mechanism for the myoglobin-catalyzed carbene insertion into N–H bonds

Despite this progress, the scope of biocatalytic carbene N–H insertion reactions has so far remained limited to aniline and aromatic amines. On the other hand, functionalization of alkylamines via transition-metal-catalyzed carbene transfer presents challenges because of amine-induced catalyst poisoning effects and/or limited chemoselectivity for the single N–H insertion product.[2] Here, we report the development of an efficient and sustainable biocatalytic strategy for the selective functionalization of alkylamines via hemoprotein-based carbene transfer.

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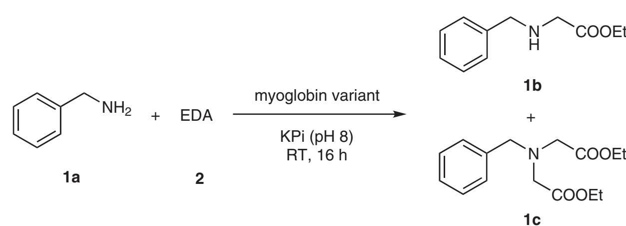
Owing to the versatility of myoglobin-based biocatalysts in a variety of carbene-mediated Y–H insertions (Y = N, S, Si),[5,7d,g,10] we selected this scaffold as a promising starting point for the development of a catalyst useful for the functionalization of alkylamines. Toward this goal, we tested the catalytic activity of wild-type sperm whale myoglobin (Mb) and selected engineered Mb variants in the model reaction with benzylamine (**1a**) and ethyl α-diazo acetate (EDA; **2**) as the carbene donor (Table 1). This initial panel of carbene transferase catalysts included Mb(H64V,V68A), which was previously identified as a highly active catalyst for N–H insertion with arylamines (1,000–6,900 TON) as well as Mb(L29A) and Mb(L29A,H64V), which were previously found to possess enhanced reactivity, relative to Mb(H64V,V68A), toward less reactive arylamine substrates such as *N*-methyl-aniline (up to 3,600 TON).[5] While wild-type Mb showed only modest activity in this reaction

Table 1 Catalytic Activity and Selectivity of Selected Engineered Myoglobin Variants in the Carbene N–H Insertion Reaction with Benzylamine and EDA[a]



Entry	Catalyst	Conversion (%) (**1b**+**1c**)	TTN	**1b** Yield (%)[b]	**1b** Select. (%)[c]
1	WT	14	70	1	7
2	Mb(H64V,V68A)	>99	>500	12	12
3	Mb(L29A)	58	290	2	3
4	Mb(H64V)	>99	>500	2	2
5	Mb(V68A)	86	430	6	7
6	Mb(L29A,H64V)	>99	>500	4	4
7	Mb(L29F,H64V)	>99	>500	1	1
8	Mb(H64V,V68F)	72	360	1	14
9	Mb(H64V,I107S)	>99	>500	16	16
10	Mb(H64V,I107Y)	>99	>500	1	1
11	Mb(F43V,V68A)	73	365	1	14
12	Mb(H64G,V68A)	>99	>500	32	32
13[d]	Mb(H64G,V68A)	>99	>125	99	99
14[e]	Mb(H64G,V68A)	75	7,500	70	93

[a] Reaction conditions: **1a** (10 mM), EDA (10 mM), enzyme (20 μM), $Na_2S_2O_4$ (10 mM), phosphate buffer (50 mM; pH 8.0).
[b] Based on GC conversion using calibration curve with isolated **1a**.
[c] Based on **1b**/(**1b**+**1c**).
[d] Using 5 mM **1a** and 2.5 mM EDA.
[e] Using 20 mM **1a**, 10 mM EDA, 1 μM enzyme.

(14% conversion, 70 TON; Table 1, entry 1), the engineered variants Mb(H64V), Mb(H64V,V68A), and Mb(L29A,H64V) led to quantitative conversion of the benzylamine substrate at a catalyst loading of 0.2 mol% (>500 TON; Table 1, entries 2, 4, and 6). In all cases, however, the desired single N–H insertion product **1b** was produced in minor amounts (2–12%), with the double insertion adduct **1c** representing the major product. This result mirrors those reported for iron-porphyrin and other transition-metal catalysts[3j,n,11] and stands in contrast to the high chemoselectivity observed for Mb-catalyzed functionalization of arylamines, highlighting the challenges associated with the carbene-mediated functionalization of alkylamines compared with arylamines under biocatalytic conditions in water.

To develop a more selective catalyst for this reaction, the steric bulk at the active site positions Leu29, Val68, and Ile107 (see Figure S1) was varied by substituting each of these residues with an amino acid carrying a larger or smaller side-chain group (for example, Leu29→Ala or Phe). All these substitutions were tested in the presence of the H64V substitution at the level of the distal histidine, as this mutation was previously established to help increase the accessibility of the heme pocket to solvent/reagents as determined by X-ray crystallography.[8b] Also in the context of the present reaction, this mutation alone significantly enhances N–H insertion activity on benzylamine when compared to wild-type Mb (Table 1, entry 4 vs. 1). Surprisingly, increasing the steric bulk at position 29 (L29F), 68 (V68F), and 107 (I107Y) showed little to no beneficial effect toward improving the selectivity of the reaction toward formation of the desired single insertion product **1b** (Table 1, entries 7, 8, and 10, respectively, vs. 4). However, analysis of the structure–activity data gathered with the different Mb variants suggested a positive effect of the V68A mutation, either alone or in combination with other mutations, toward increasing the selectivity for **1b**. Promisingly, the combination of this mutation with H64G yielded a double-site variant, Mb(H64G,V68A), which exhibits high catalytic activity (>99% conversion) along with significantly improved chemoselectivity for the desired single carbene N–H insertion product (32%; Table 1, entry 12). This transformation could further be optimized to yield the functionalized amine **1b** in quantitative yield and excellent selectivity (99%; Table 1, entry 13). Furthermore, the same transformation could be efficiently performed at a catalyst loading of as little as 0.005 mol%, while maintaining high yield (70%) and selectivity (93%) (Table 1, entry 14). Under these conditions, the hemoprotein catalyst supports over 7,000 catalytic turnovers for the formation of the desired product with an initial turnover rate of 475 turnovers min^{-1}. The latter is comparable, albeit slightly lower, to the Mb(H64V,V68A)-catalyzed N–H insertion with aniline and EDA (740 turnovers min^{-1}).[5] Only trace amounts of the product **1b** were formed in the absence of the catalyst or in the absence of

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the reductant $Na_2S_2O_4$, the latter indicating that ferrous form of the hemoprotein is responsible for the observed catalytic activity.

Encouraged by these findings, we investigated the scope of the Mb(H64G,V68A)-catalyzed reaction with benzylamine **1a** in the presence of different diazo acetate reagents carrying varying alkyl ester groups, including ^{i}Pr, ^{t}Bu, Cy, or Bn moieties (**3a**–**6a**; Figure S2). Notably, Mb(H64G,V68A) was found to exhibit a remarkable tolerance toward variation of the carbene donor reagent, showing good selectivity for formation of the single N–H insertion product across all the tested diazo compounds. This result is in contrast to those obtained for the other Mb variants, which show improved chemoselectivity only in the presence of a subset (typically, 1–2) of these carbene donor reagents (Figure S2), confirming the choice of Mb(H64G,V68A) for further studies. After optimization of the reaction conditions (Table S1), the desired single insertion products **3b**–**6b** could be obtained in moderate to good yields (23–89%) and high catalytic efficiencies (970–4,660 total turnovers, TTN; Table 2). Across different diazoesters, a pH of 8 was found to be optimal for formation of the single insertion product, whereas lower chemoselectivity was observed at more acidic or basic pH values (see representative data with **5a** in Table S1). From these experiments, it also became apparent that the nature of the ester substituent has an impact on the activity of the biocatalyst, as reflected by the decrease in total turnover numbers (TTN) with an increase in the steric bulk of the alkyl substituent (i.e., Et < ^{i}Pr < ^{t}Bu < Bn < Cy).

Next, we investigated the substrate scope of Mb(H64G,V68A) with respect to the amine substrate by using a panel of variously substituted benzylamine derivatives. As summarized in Table 3, all of the substrates **7a**–**13a** could be converted by the Mb(H64G,V68A) variant into the desired N–H insertion products in good to high yields (60–89%), with the catalyst exhibiting a number of total turnovers ranging from 1,170 to 4,890. The results with **7a**–**10a** indicate that electron-withdrawing (e.g, CF_3, Cl) as well as electron-donating (e.g., OMe, Me) substituents are well tolerated by the catalyst (Table 3, entries 1–4). Furthermore, benzylamine derivatives bearing substituents in *ortho*-, *meta*-, and *para*-position of the benzene ring (**10a**–**12a**) were efficiently processed to the corresponding secondary amine (Table 3, entries 4–6). Lastly, more sterically hindered benzylamine derivatives such as **13a** are also well accepted by the biocatalyst (Table 3, entry 7). In all cases, little to none of the related double insertion byproducts **7c**–**13c** were observed (<5%), confirming the high chemoselectivity of this biocatalyst across benzylamine substrates.

Having established the broad substrate scope of Mb(H64G,V68A) across both diazo ester and benzylamine substrates, we extended these studies to other types of alkylamines. To this end, we tested different aliphatic amines (**14a**–**18a**) in the Mb(H64G,V68A)-catalyzed reaction with EDA (Table 4). To our delight, the catalyst proved effective in the conversion of morpholine (**14a**) and thiomorpholine (**15a**) into the desired single insertion products **14b** and **15b** in good yields (82 and 37%, respectively).

Table 2 Substrate Scope for Mb(H64G,V68A)-Catalyzed Carbene N–H Insertion Reaction with Benzylamine and Different Diazo Compounds[a]

1a ($PhCH_2NH_2$) + 3a–6a ($N_2CHCOOR$) → [Mb(H64G,V68A), KPi (pH 8), RT, 16 h] → 3b–6b ($PhCH_2NHCH_2COOR$)

Entry	Product	Conv. (%) [(**3b**–**6b**)+**3c**+**6c**]	**3b**–**6b** Yield (%)[b]	Select. (%)	TON[c]
1	**3b** ($PhCH_2NHCH_2COO^{i}Pr$)	>99	89	89	4,660
2	**4b** ($PhCH_2NHCH_2COO^{t}Bu$)	59	45	76	2,020
3	**5b** ($PhCH_2NHCH_2COOCy$)	>99	49	45	970
4	**6b** ($PhCH_2NHCH_2COOBn$)	91[d]	23	25	1,220

[a] Reaction conditions: **1a** (10 mM), diazo compound (5 mM), enzyme (20 μM), $Na_2S_2O_4$ (10 mM), phosphate buffer (50 mM; pH 8.0).
[b] For single N–H insertion products. Based on HPLC conversion using calibration curves with isolated **3b**–**6b**.
[c] Reaction conditions: **1a** (20 mM), diazo compound (10 mM), enzyme (1 μM), $Na_2S_2O_4$ (10 mM), phosphate buffer (50 mM; pH 8.0).

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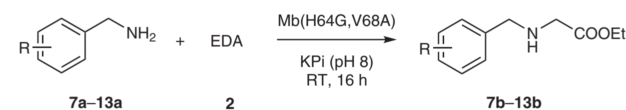
Table 3 Substrate Scope for Mb(H64G,V68A)-Catalyzed Carbene N–H Insertion Reaction with Benzylamines and EDA[a]

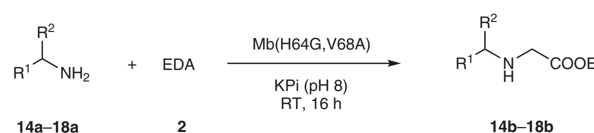


Entry	Product	Yield (%)[b]	TON[c]
1	**7b**	73	4,230
2	**8b**	77[d]	4,450
3	**9b**	89	4,890
4	**10b**	60	3,120
5	**11b**	72	1,170
6	**12b**	82	3,960
7	**13b**	72	4,240

[a] Reaction conditions: amine (10 mM), EDA (5 mM), enzyme (20 μM), $Na_2S_2O_4$ (10 mM), phosphate buffer (50 mM; pH 8.0).
[b] Based on GC conversion using calibration curves with isolated **7b–13b**.
[c] Reaction conditions: amine (20 mM), EDA (10 mM), enzyme (1 μM), $Na_2S_2O_4$ (10 mM), phosphate buffer (50 mM; pH 8.0).
[d] Using 20 mM amine and 10 mM EDA.

To evaluate the reactivity of primary versus secondary amines in this reaction, we further tested Mb(H64G,V68A) in the presence of cyclohexylmethanamine (**16a**) and hexylamine (**17a**). In this case, however, although full consumption of the starting material was observed, the desired single insertion products **16b** and **17b** were obtained only in 3% and 12% yield, respectively, with the remainder accounting for the double insertion products **16c** and **17c**. Similar results were observed for other primary alkylamines tested in this reaction, including isopropyl-, cyclohexyl-, and octylamine (data not shown). This result is not entirely surprising in light of the high propensity of primary aliphatic amines to undergo double carbenoid insertion in metal-catalyzed carbene transfer reactions.[3p] This limitation notwithstanding, Mb(H64G,V68A) was found able to accept highly sterically encumbered secondary amines such as *N*,*N*-dioctylamine **18a**, albeit with reduced activity compared to cyclic secondary amines (Table 2, entry 5 vs. 1–2). Furthermore, the differential reactivity of benzylamine **1a** vs. cyclohexylmethanamine **16a** indicate a favorable effect of the aromatic ring toward favoring the single insertion reaction as opposed to the double insertion reaction, in spite of the similar nucleophilicity (N = 13.4 versus ca. 12.9)[12] and basicity (pK_{aH}: 9.5 vs. 10.5) of these amines in water.

Table 4 Substrate Scope for Mb(H64G,V68A)-Catalyzed Carbene N–H Insertion Reaction with Alkylamines and EDA[a]



Entry	Product	Yield (%)[b]	TON[c]
1	**14b**	82	7,810
2	**15b**	37	3,730
3	**16b**	3 (82 for **16c**)[d]	122
4	**17b**	12 (87 for **17c**)	650
5	**18b**	10	210

[a] Reaction conditions: amine (10 mM), EDA (5 mM), enzyme (20 μM), $Na_2S_2O_4$ (10 mM), phosphate buffer (50 mM; pH 8.0).
[b] Based on GC conversion using calibration curves with isolated **14b–18b**.
[c] Reaction conditions: amine (20 mM), EDA (10 mM), enzyme (1 μM), $Na_2S_2O_4$ (10 mM), phosphate buffer (50 mM; pH 8.0).
[d] Using 20 mM amine and 10 mM EDA.

In summary, this work describes the first example of a hemoprotein-catalyzed carbene insertion into the N–H bonds of benzyl- and alkylamine substrates.[13] Mb(H64G,V68A) was found to provide a particularly promising biocatalyst for this reaction, offering excellent activity as well as high chemoselectivity in the transformation of a broad range of benzylamine substrates (up to 99% yield, 99% selectivity, 7,000 TON) and cyclic aliphatic amines, also in the presence of different α-diazo acetate reagents. It is expected that further engineering of this biocatalyst could provide a means to enhance its selectivity also for the functionalization with primary alkylamines. This work expands the repertoire of biocatalytic reactions useful for the selective construction of new C–N bonds under mild reaction conditions and in aqueous solvents.

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Funding Information

This work was supported in part by the U.S. National Institutes of Health grant GM098628 and in part by the U.S. National Science Foundation grant CHE-1609550. Mass spectrometry instrumentation is supported by the U.S. National Science Foundation grant CHE-0946653.

Supporting Information

Supporting information for this article is available online at https://doi.org/10.1055/s-0039-1690007.

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(13) **Experimental Procedures.**
Reagents and Analytical Methods: All chemicals and reagents were purchased from commercial suppliers (Sigma–Aldrich, Acros Organics, Alfa Aesar, TCI Chemicals) and used without further purification, unless otherwise stated. The diazo compounds isopropyl diazoacetate (**3a**) and cyclohexyl diazoacetate (**5a**) were prepared according to a reported procedure (see ref. 8b). All moisture- or oxygen-sensitive reactions were carried out under argon atmosphere in oven-dried glassware with magnetic stirring using standard gas-tight syringes, cannulae and septa. ^{1}H and ^{13}C NMR spectra were measured with a Bruker DPX-400 instrument (operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C) or a Bruker DPX-500 instrument (operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C). Tetramethylsilane (TMS) served as the internal standard (0 ppm) for ^{1}H NMR, $CDCl_3$ was used as the internal standard (77.0 ppm) for ^{13}C NMR. Silica gel chromatography purifications were carried out using AMD Silica Gel 60 Å 230–400 mesh. Thin-layer chromatography (TLC) was carried out using Merck Millipore TLC silica gel 60 F254 glass plates. UV/Vis measurements were performed with a Shimadzu UV-2401PC UV/Vis spectrometer. Gas chromatography (GC) analyses were carried out with a Shimadzu GC-2010 gas chromatograph equipped with a FID detector and a Chiral Cyclosil-B column (30 m × 0.25 mm × 0.25 mm film). Separation method: 1 mL injection, injector temp.: 250 °C, detector temp.: 300 °C. Gradient: column temperature set at 140 °C for 3 min, then to 160 °C at 1.8 °C/min, then to 165 °C at 1 °C/min, then to 245 °C at 25 °C/min. Total run time was 28.31 min. HPLC analyses were performed with a Shimadzu LC-2010A-HT equipped with a VisionHT C18 column and a UV/Vis detector. Injection volume: 20 µL. Flow rate: 1 mL/min. Gradient: 40% acetonitrile (0.1% TFA) in water (0.1% TFA) for 3 min, then increased to 90% over 15 min.

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Protein Expression and Purification: Wild-type Mb and engineered Mb variants were cloned and expressed in *E. coli* BL21(DE3) or *E. coli* C41(DE3) cells as described previously (see ref 7b). Briefly, cells were grown in TB medium (ampicillin, 100 mg L^{-1}) at 37 °C (180 rpm) until OD_{600} reached 0.6. Cells were then induced with 0.25 mM β-D-1-thiogalactopyranoside (IPTG) and 0.3 mM δ-aminolevulinic acid (ALA). After induction, cultures were shaken at 27 °C (180 rpm), harvested after 20 h by centrifugation (4,000 rpm, 20 min, 4 °C) and resuspended in Ni-NTA Lysis Buffer (50 mM KPi, 250 mM NaCl, 10 mM histidine, pH 8.0). Resuspended cells were frozen and stored at – 80 °C. Cell suspensions were thawed at room temperature, lysed by sonication, and clarified by centrifugation (14,000 rpm, 50 min, 4 °C). The clarified lysate was transferred to a Ni-NTA column equilibrated with Ni-NTA Lysis Buffer. The protein was washed with Ni-NTA Wash Buffer (50 mM KPi, 250 mM NaCl, 20 mM histidine, pH 8.0). Proteins were eluted with Ni-NTA Elution Buffer (50 mM KPi, 250 mM NaCl, 250 mM histidine, pH 7.0). After buffer exchange (50 mM Kpi, pH 7.0), the proteins were stored at +4 °C. Myoglobin concentration was determined by UV/Vis spectroscopy using an extinction coefficient of ε_{410} = 157 mM^{-1} cm^{-1}.

N–H Insertion Reactions: Under standard reaction conditions, reactions were carried out at a 400 μL scale using 1 or 20 μM myoglobin, 5–20 mM amine, 2.5–10 mM diazo compound, and 10 mM sodium dithionite. In a typical procedure, in an anaerobic chamber, a solution containing the desired myoglobin variant was mixed with a solution of sodium dithionite in argon-purged potassium phosphate buffer (50 mM, pH 8.0). Reactions were initiated by addition of amine (400 mM stock solution in EtOH) followed by the addition of diazo compound (200 or 400 mM stock solution in EtOH), and the reaction mixtures were stirred in the chamber for 12 h at room temperature.

Product analysis: The reactions were analyzed by adding 8 μL of internal standard (fluorenone, 50 mM in DMSO) to the reaction mixture, followed by extraction with 400 μL of dichloromethane and analysis by GC-FID for reactions with substrates **1a**, **7a**–**18a** (see the Supporting Information Reagents and Analytical Methods section for details on GC analyses). For reactions with substrates **3a**–**6a**, the organic layer was removed via evaporation and the residue was dissolved in 300 μL methanol, filtered through 0.22 μm syringe filters, and analyzed by HPLC (see the Supporting Information Reagents and Analytical Methods section for details on HPLC analyses). Calibration curves for quantification of the different N–H insertion products were constructed using authentic standards prepared as described in the Supporting Information Synthetic Procedures. All measurements were performed at least in duplicate. For each experiment, negative control samples containing either no enzyme or no reductant were included.

Synthetic Procedures: Detailed procedures for the synthesis of **3b**–**18b**, relevant double insertion products as well as the precursors **19**–**20** are provided in the Supporting Information.

Synthesis of Ethyl Benzylglycinate (1b): To a flame-dried round-bottom flask under argon, equipped with a stir bar, was added benzylamine **1a** (1 equiv) and $Rh_2(OAc)_4$ (1 mol%) in toluene (2–3 mL). To this solution was added a solution of ethyl diazoacetate (1 equiv) in toluene (1–2 mL) over 30 minutes at 0 °C. The resulting mixture was heated at 80 °C for another 15–18 h. The solvent was removed under vacuum and the crude mixture was purified by flash column chromatography (20% ethyl acetate/hexanes) to provide the title compound **1b** (82% yield) as a pale-yellow oil. R_f = 0.18 (20% EtOAc/hexanes). GC-MS: *m*/*z* (%) = 193 (1.4), 120 (66.3), 106 (31.2), 91 (100), 77 (2.0), 65 (15.0). ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 7.21–7.18 (m, 2 H), 6.76 (t, *J* = 7.5 Hz, 1 H), 6.62 (d, *J* = 8.0 Hz, 2 H), 3.80 (s, 2 H), 1.50 (s, 9 H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 170.3, 147.2, 129.2, 118.0, 113.0, 81.9, 45.5, 28.1

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