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# First and second sphere interactions accelerate non-native *N*-alkylation catalysis by the thermostable, methanol-tolerant $B_{12}$-dependent enzyme MtaC†

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**The corrinoid protein MtaC, which is natively involved in methyl transferase catalysis, catalyzes *N*-alkylation of aniline using ethyl diazoacetate. Our results show how the native preference of $B_{12}$ scaffolds for radical *versus* polar chemistry translates to non-native catalysis, which could guide selection of $B_{12}$-dependent proteins for biocatalysis. MtaC also has high thermal stability and organic solvent tolerance, remaining folded even in pure methanol.**

Vitamin $B_{12}$ catalyzes many chemical transformations, including alkyl halide and alkene coupling reactions, that are not known for $B_{12}$-dependent enzymes in nature.[1] $B_{12}$ is poorly soluble in most organic solvents, and a pendant dimethylbenzimidazole coordinates to its cobalt center in the +2 or +3 oxidation state in solution,[2] which complicates the use of synthetic ligands to tune $B_{12}$ reactivity.[3] To address these limitations, chemists have developed organic-soluble $B_{12}$ derivatives to catalyze organic transformations.[3,4] Limited variation of tethered axial ligands on these complexes (*i.e.* the primary coordination sphere) has been achieved *via* chemical synthesis[5,6] and semisynthesis starting from microbially produced cobamides,[7–9] but axial ligand variation is more typically achieved using external ligands.[4] Controlling the second coordination sphere of synthetic $B_{12}$ derivatives (*i.e.* those atoms that surround the metal center but are not bound to it) is also difficult since their planar structure places functional groups distal to the metal center.[10]

We recently reported that a variant of the $B_{12}$-dependent transcription factor CarH, CarH*, catalyzes non-native alkylation of styrenes using ethyl diazoacetate (EDA)[11] with improved selectivity relative to $B_{12}$ itself.[12] Kinetic and solvent isotope effects, TEMPO trapping experiments, and product distribution analyses suggest that second sphere interactions between the CarH* scaffold and radical intermediates involved in the alkylation reaction are responsible for CarH* selectivity. This transformation is initiated by reaction of the cob(I)alamin form of CarH* (**i**) with ethyl diazoacetate (EDA) to form an ethyl carboxymethylcob(III)almin intermediate **ii**, which can undergo selective radical addition to styrene within CarH* (Fig. 1).

Given the importance of second sphere interactions in this system, we hypothesized that other scaffolds might catalyze other non-native transformations involving EDA either by modulating the reactivity of intermediate **ii** or by accessing other reactivity manifolds involving EDA (Fig. 1). We were particularly interested in the possibility of non-native alkylation reactions involving $S_N2$-attack on **ii** given that methylcobalamin-dependent (MeCbl) methyltransferases[13] catalyze the analogous methylation reaction, albeit using a variety of native methyl donors rather than diazoacetates. Analyzing the activity of different $B_{12}$ proteins toward this reaction could therefore establish whether the native preference of $B_{12}$ scaffolds for radical *versus* polar chemistry translates to non-native catalysis and guide selection of $B_{12}$-dependent proteins for biocatalysis.

Sequence-based searches for CarH homologues revealed many uncharacterized proteins annotated as MerR family

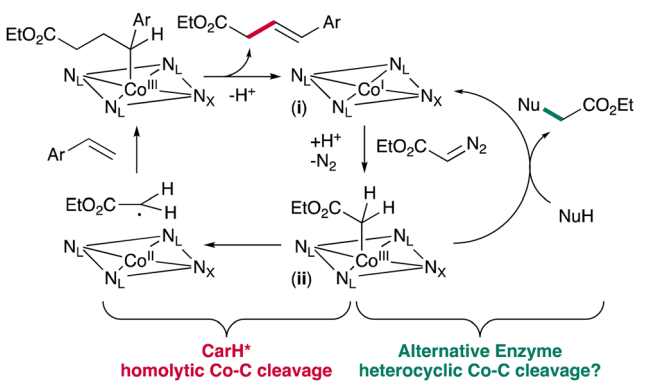


Fig. 1 Enzyme-dependent reactivity of $B_{12}$ intermediate (i) previously reported styrene alkylation *via* homolytic Co–C cleavage in CarH* and (ii) newly reported alkylation of nucleophiles *via* heterolytic Co–C cleavage using different $B_{12}$-dependent enzymes.

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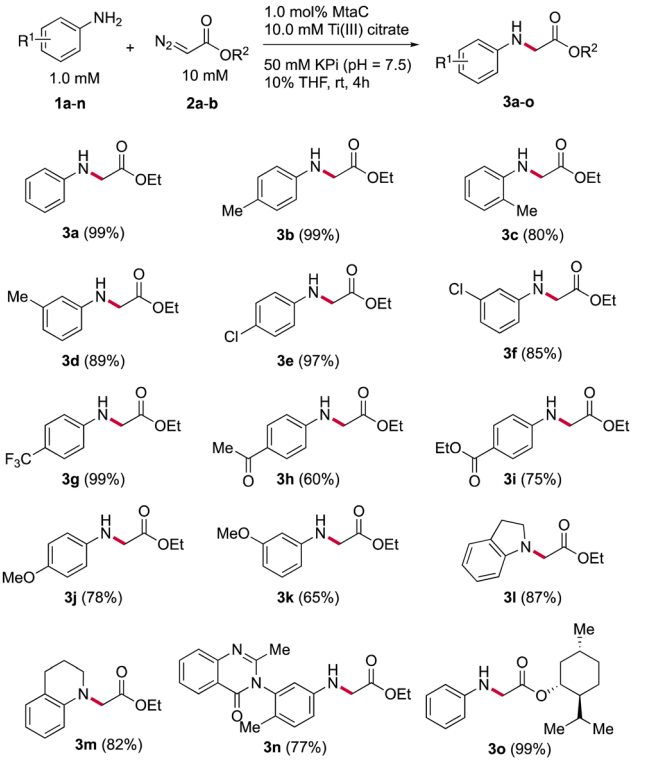
transcriptional regulators,[14] and multiple-sequence alignment indicated that their putative active site residues are conserved. We therefore used the DALI protein structure comparison server[15] to find scaffolds with low sequence identity but high structural homology to CarH. This approach led to the identification of MtaC from the methanol-coenzyme M methyltransferase complex MtaABC from *M. barkeri*,[16] the monomethylamine corrinoid protein MMCP from *M. barkeri*,[17] and MetH from *E. coli*,[18] which we previously examined[12] and excluded from this study due to its low expression yield. Because MtaC and MMCP are both involved in methyl transferase processes, we examined DhaF4611[19] and TCP,[17] which are also methyl transferase components. The native activity of these enzymes suggested that if they could form alkylcob(III)almin intermediates like **ii** in Fig. 1 upon reduction and reaction with alkyl diazoacetates, they might catalyze non-native alkylation reactions.[19,20]

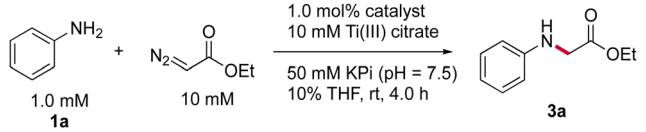
Genes encoding MtaC, MMCP, DhaF4611, and TCP were expressed in *E. coli* (Fig. S1, ESI†), and the apo proteins were purified and reconstituted with hydroxycob(III)alamin in 84-98% yield based on ICP-MS analysis (see supporting information). The alkylation of aniline (**1a**) using EDA was selected as a model reaction since aniline possesses a sufficiently low $pK_a$ to ensure that it would be deprotonated and therefore serve as a good nucleophile at the pH values required for biocatalysis. Similar levels of alkylation were provided by CarH*, Dhfa4611 and TCP, but MtaC provided quantitative conversion and negligible dialkylation under the standard reaction conditions (Table 1). $B_{12}$ itself is essentially unreactive under these conditions, indicating that the different scaffolds, and MtaC in particular, activate the $B_{12}$ toward *N*-alkylation catalysis. This contrasts with our styrene alkylation reaction, for which CarH* provided only moderate acceleration relative to $B_{12}$.[12] Moreover, MtaC provided modest yields as a catalyst for our previously reported styrene alkylation (35% vs 51% for CarH*, Fig. S2, ESI†), showing that these enzymes exhibit complementary non-native activity.

The reaction works best with a 10-fold excess of EDA and a 20-fold excess of Ti(III), but 10-fold of the latter was used in practice. A buffer pH of 7.5 was again used with 10% v/v THF as a co-solvent, and the reaction is slightly accelerated in the presence of blue light (470 nm). Minimal dialkylation was observed in the standard 4 h reaction time for most substrates, but longer reaction times led to dialkylation (Fig. S2, ESI†). A variety of anilines could be alkylated with good-excellent yield using these conditions (Scheme 1). Substituents at the *ortho*-, *meta*-, and *para*-positions of the aniline are tolerated (**3a–d**), as are both electron withdrawing (**3e–i**) and donating substituents (**3j**, **3k**). Cyclic anilines like indoline and tetrahydroindoline are also good substrates (**3l**, **3m**), and fragment-based coupling is possible as evidenced by reaction of quinazolinone-substituted aniline **3n** and menthol-derived diazoacetate **3o**.




Scheme 1 Substrate scope of MtaC-catalyzed aniline alkylation.[a] [a]Reactions conducted under anaerobic conditions. [b]Yields were determined by GC-MS relative to 1,3,5-trimethoxybenzene (single data point).

Table 1 *N*-alkylation of aniline catalyzed by different $B_{12}$ catalysts

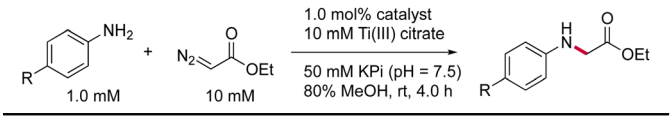



| Entry | Catalyst | **1a**/TMB[a] | **3a**/TMB[a] | Yield[b] (%) |
|---|---|---|---|---|
| 1 | MtaC | 0.0 | 3.4 | >99 |
| 2 | DhaF4611 | 0.5 | 1.3 | 38 (±4) |
| 3 | TCP | 0.5 | 1.2 | 36 (±2) |
| 4 | CarH* | 0.7 | 1.1 | 33 (±3) |
| 5 | $B_{12}$ | 1.0 | < 0.1 | < 0.1 |

[a] Ratios of peak integrals for **1a** or **3a** relative to 1,3,5-trimethoxybenzene (TMB) internal standard. [b] Yields and standard deviations determined by GC-MS relative to TMB for independent reactions conducted in triplicate.

While THF was used as a co-solvent in these reactions, up to 80% v/v methanol in phosphate buffer could also be used with only modest reductions in yield (Table 2, entries 1, 3, and 5), and the reaction scale could be increased up to 0.46 mmol (Table 2, entries 2 and 4). $B_{12}$ alone again provided negligible conversion under these conditions. MtaC is fully soluble in methanol, and its UV-vis spectrum acquired in methanol displays characteristic bands for cob(III)almine at 357, 507, and 537 nm, similar to the values obtained in phosphate buffer and for $B_{12}$ itself (Fig. 2A). CD Spectra for MtaC are nearly identical above a wavelength of 200 nm in phosphate buffer up to 90 °C or in pure MeOH indicating that at least the secondary structure of the enzyme remains intact under typically denaturing conditions (Fig. 2B). Similar CD spectra were obtained for Dhaf4611 and TCP in pure MeOH, indicating that these proteins also have high MeOH tolerance (Fig. S3, ESI†).

Table 2 Reaction tolerance of MeOH and scalability[a]




| Entry | R | scale (mmol) | yield (%) |
|---|---|---|---|
| 1 | Me | 0.001 | > 99 |
| 2 | Me | 0.46 | 93[b] |
| 3 | Cl | 0.001 | 95 |
| 4 | Cl | 0.35 | 90[b] |
| 5 | H | 0.001 | 80 |

[a] Reactions were conducted under the anaerobic conditions; yields determined by GC-MS relative to 1,3,5-trimethoxybenzene (single data point). [b] Catalyst loading is reduced to 0.5 mol%, and yields are reported as isolated yields (single data point).

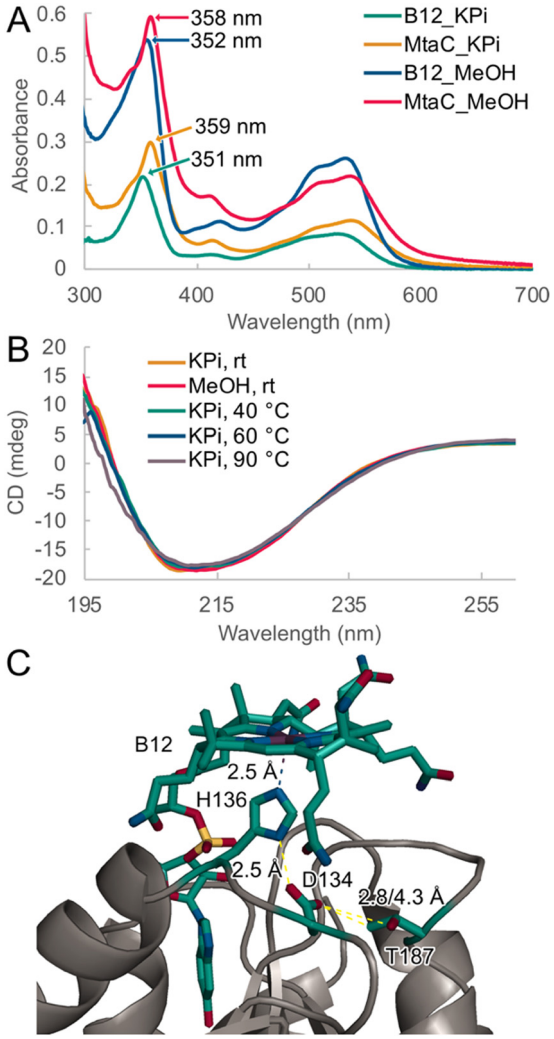



Fig. 2 (A) UV-vis and (B) CD spectra of 1 μM solutions of hydroxycob(III)alamin ($B_{12}$) and MtaC reconstituted with $B_{12}$ in phosphate buffer (50 mM, pH 7.5) and MeOH. (C) $B_{12}$-binding domain of MtaC (PDB ID 2I2X; residues 1–125 and structure of MtaB omitted)[16] showing primary (H136) and secondary (D134 and T187) residues that affect catalysis.

The methanol tolerance of MtaC and its ability to accelerate aniline *N*-alkylation relative to free $B_{12}$ indicate that it binds $B_{12}$ in a manner that activates it for non-native catalysis. While MtaC obtained from *M. barkeri* possesses a 5-hydroxybenzimidazoylcobamide cofactor,[21] it maintains its native activity after heterologous expression in *E. coli* and reconstitution with $B_{12}$[22] as was done in the current study. MtaC natively undergoes methylation by MtaB using methanol as a methyl donor to generate its methylcob(III)alamin form, which serves as a methyl donor for the MtaA-catalyzed methylation of 2-mercaptoethanesulfonate.[21]

Early studies indicated that His136 in MtaC binds to the Co(III) center of $B_{12}$,[22] and the crystal structure of the enzyme later showed that this residue, along with Asp134 and Thr187, form a second-sphere H-bonding network (Fig. 2C) that is essential to the activity of other $B_{12}$-dependent methyl transferases.[16] In the $B_{12}$ binding domain of MetH from *E. coli*, for example (35% similarity to MtaC),[21] this triad is believed to stabilize the His-on methylcob(III)alamin and His-off cob(I)alamin forms of the enzyme during catalysis *via* alternate H-bonding arrangements governed by the protonation state of the Asp residue.[18,23] The effects of triad mutations on MtaC activity have not been established because the H136A variant could not be reconstituted with the cob(II)alamin or methylcob(III)alamin cofactors used to study its native activity.[22] We found that H136A, D134A, and T187A variants of MtaC (Fig. 2C) could be reconstituted with hydroxycob(III)alamin in 66, 52, and 76% yield, respectively, based on ICP-MS analysis (Fig. S8, ESI†) and that the native secondary structures of these variants remained intact based on CD spectroscopy (Fig. S9, ESI†). Aniline alkylation yields for these variants were reduced (Fig. S5, ESI†), and their initial rates were 6.6-, 1.3-, and 1.9-fold lower than MtaC after accounting for differential metalation levels (Fig. S6, ESI†). These results suggest that H136 plays a significant role in activating $B_{12}$ toward non-native aniline alkylation and that second sphere residues D134 and T187 also have some effect in this regard. We hypothesize that these residues could be acting to stabilize different states of the $B_{12}$ cofactor during non-native catalysis just as they do in native MetH methyl transfer catalysis.[23]

In this study, we established that reconstituting the corrinoid protein MtaC with hydroxycob(III)alamin gives an active catalyst for aniline *N*-alkylation using alkyl diazoacetates while $B_{12}$ itself is essentially unreactive toward this reaction. MtaC exhibited good substrate scope, selectivity for monoalkylation, and high thermal stability and methanol tolerance. The complementary specificity of MtaC and CarH* for non-native polar and radical reactions, respectively, mirrors their native reactivity and suggests that such preferences should be considered when exploring different scaffolds as catalysts for non-native reactions.

Cytochrome P450 and myoglobin variants catalyze the same reaction with similar selectivity,[24,25] and these enzymes have been engineered to give high activity and enantioselectivity.[26,27] Other systems, including *de novo* designed heme proteins[28] can also achieve good activity and selectivity for monoalkylation of anilines. On the other hand, the heme cofactor in these enzymes is itself an active catalyst[24,25] while $B_{12}$ is not, so MtaC

appears to play a greater role in activating $B_{12}$ toward this reaction than P450s and myoglobin do for their heme cofactor. This finding constitutes a notable example of protein accelerated catalysis,[29] which was first described in the context of artificial metalloenzymes[30] and has important implications for engineering systems whose cofactors are not directly incorporated *in vivo*. If libraries of a protein scaffold must be reconstituted following expression, some means of mitigating background cofactor reactivity must be employed,[31,32] which complicates directed evolution. If the scaffold activates the cofactor, scaffold libraries can be screened following cofactor addition,[33] and efforts to engineer MtaC variants using this approach are currently underway.

A His-Asp-Thr triad was presumed important for the native methyl transferase activity of MtaC,[16] but the effects of mutations to these residues were not evaluated.[22] We found that mutating each of these residues to Ala reduces the rate of aniline *N*-alkylation catalysis, suggesting that they play a similar role in this reaction as the related triad in MetH.[23] This finding adds to a growing body of research showing how first and second sphere interactions that give rise to different native reactivity in homologous proteins can affect the utility of those proteins for non-native catalysis.[34] Further modulation of second sphere interactions could be possible by including MtaB and MtaA in biocatalytic reactions since these are known to complex MtaC, changing the microenvironment surrounding the $B_{12}$ cofactor.[16] Combined with our report of styrene alkylation using CarH*[12] and studies exploring the substrate scope of $B_{12}$-dependent methyl transferases,[19,20] this study highlights the utility of corrinoid proteins for biocatalysis.

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## Conflicts of interest

There are no conflicts to declare.

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