VIP Biocompatible Chemistry Very Important Paper

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Rescuing Auxotrophic Microorganisms with Nonenzymatic Chemistry**

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Chemical manipulations of small-molecule metabolites take place in all living systems and are essential for sustaining life through cellular metabolism.[1] The vast majority of these transformations are carried out by macromolecular, enzymatic catalysts. In contrast to the natural world, most chemical reactions employed in laboratory syntheses utilize nonbiological reagents and catalysts.[2] Although nonbiological catalysts and reagents have been shown to function in the presence of cells,[3] the question of whether or not their reactivity can interface with cellular metabolism and influence biological function remains underexplored. We are interested in further developing this aspect of reaction methodology, which we term biocompatible chemistry: nonenzymatic chemical transformations that can be used to manipulate the structures of small molecules in the presence of living organisms. Successful integration of biocompatible chemistry with cellular metabolism would have implications for biotechnology, because it could expand the capabilities of biotransformation in ways that are not possible using enzymes. Establishing a link between nonenzymatic catalysis and metabolism could also have potential implications for prebiotic chemistry, because it would suggest that primitive cells could have relied upon nonenzymatic transformations occurring in the surrounding environment to generate essential metabolites.[4] Here, we describe biocompatible chemical reactions that interact with microbial metabolism and rescue auxotrophy through the production of essential metabolites. This experimental approach enables us to directly connect the growth of a living organism to the success of a nonbiological chemical transformation.

Auxotrophs are organisms that lack the ability to synthesize key nutrients required for growth and survival.[5] The necessary compounds must instead be obtained from external sources, typically the diet or metabolic activities of other organisms living in close proximity. The numerous examples of auxotrophs in nature include humans, who are unable to synthesize many essential vitamins and metabolites. It is possible to rescue auxotrophy in bacteria by transformation with genes encoding enzymes that can replace or complement the missing metabolic activity. Restoration of growth in this way constitutes an extremely powerful strategy for the selection of evolved biomolecules in the context of directed evolution.[6] Recently, Hecht and co-workers demonstrated that it is possible to rescue the growth of *E. coli* auxotrophs using de novo protein scaffolds not found in natural systems.[7] Significantly, this result implies that catalysts other than native enzymes can replace key metabolic functions in living cells.

Our interest in merging nonenzymatic chemistry with metabolism led us to investigate whether reactions carried out by nonbiological catalysts could support the growth of auxotrophic microorganisms (Figure 1 A). Rescuing auxotrophy in this manner requires linking a transformation of interest to the production of an essential cellular metabolite. We decided to initially apply a ruthenium-catalyzed allyloxycarbonyl (Alloc) removal reaction developed by Streu and Meggers, a reaction that had been previously used in human cell culture.[8] We envisioned connecting this reaction to the release of *p*-aminobenzoic acid (PABA), a biosynthetic precursor to folic acid (Figure 1 B). Organisms that cannot biosynthesize PABA are unable to grow because of defects in nucleotide metabolism. By using Alloc removal to generate PABA, the success of this nonenzymatic reaction would enable the growth of PABA auxotrophs.

We accessed the rescue substrate Alloc-PABA (**1**) in a single step as reported.[9] We then evaluated whether the deprotection could take place in growth media. Using [Cp*Ru(cod)Cl] (**2**, 20 μM) as a catalyst in PABA-free M9 glycerol minimal medium at 37 °C, we were able to detect the formation of PABA from **1** (100 μM, Table 1, entry 1). As had previously been observed, the inclusion of a thiol additive boosted conversion (entries 2, 3). The production of PABA was dependent on the presence of catalyst **2** (entry 4) and was also observed at lower substrate concentrations (entry 5). Overall, these experiments confirmed that deprotection could proceed in growth media, albeit with low efficiency.

Testing the ability of this reaction to rescue PABA auxotrophy required a microorganism that was unable to synthesize this metabolite. We obtained three different *E. coli* mutants that are PABA auxotrophs from the Keio collection (Figure 1 C).[10] Two of these strains (Δ*pabA* and Δ*pabB*) have mutations in the genes encoding the two subunits of aminodeoxychorismate synthase, which catalyzes the penultimate step in PABA biosynthesis.[11] The third mutant (Δ*aroC*) is

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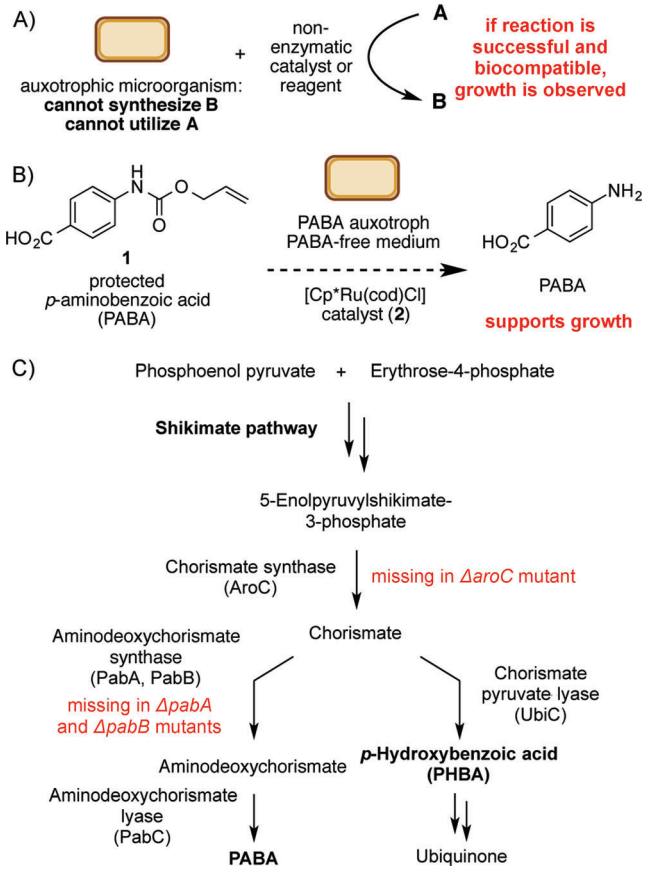
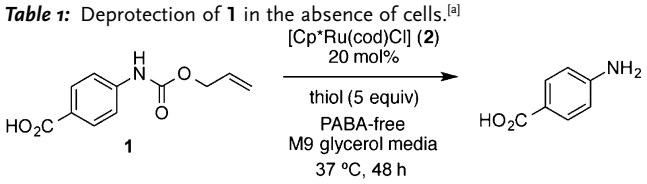


Figure 1. Rescuing auxotrophy with biocompatible chemistry. A) Overall strategy for auxotroph rescue. B) Plan for connecting the biocompatible Alloc deprotection to the generation of the essential metabolite PABA. C) Biosynthesis of aromatic metabolites in *E. coli*, and mutations leading to PABA and PHBA auxotrophies.

Table 1: Deprotection of **1** in the absence of cells.[a]



Entry	**1** [μM]	**2** [μM]	Thiol	Conversion [%][b]
1	100	20	none	8
2	100	20	PhSH	9
3	100	20	GSH	14
4	100	0	PhSH	0
5	10	2	GSH	2

[a] Reactions were run on a 5 mL scale for 48 h. [b] Conversions were determined by HPLC analysis of reaction mixtures. PhSH = thiophenol, GSH = glutathione, Cp* = pentamethylcyclopentadiene, cod = cycloocta-1,5-diene.

missing the gene encoding the key shikimate pathway enzyme chorismate synthase, and is unable to synthesize not only PABA but also the aromatic amino acids L-phenylalanine, L-tyrosine, and L-tryptophan, as well as the aromatic metabolites *para*-hydroxybenzoic acid (PHBA) and 2,3-dihydroxybenzoic acid.[12] We confirmed that all three strains were unable to grow in PABA-free M9 glycerol minimal medium, and established the inability of each strain to utilize substrate **1** in place of PABA. All media used for the *ΔaroC* mutant were supplemented with the additional required nutrients. Given that strains from the Keio collection contain a kanamycin resistance cassette, we routinely cultivated the auxotrophs in media containing this antibiotic to prevent contamination with wild-type strains.

To evaluate whether or not Ru-catalyzed deprotection of **1** could restore the growth of these strains, we serially diluted overnight cultures of Δ*pabA*, Δ*pabB*, and Δ*aroC* mutants into PABA-free M9 glycerol minimal medium, inoculated each strain into PABA-free M9 glycerol minimal medium containing **1** (10 μM), added catalyst **2** (2 μM), and then incubated the cultures at 37 °C. As a positive control, we inoculated the auxotrophs into M9 glycerol minimal medium containing PABA (10 μM); and as negative controls, we used M9 glycerol minimal medium lacking both PABA and **1** or PABA-free M9 glycerol minimal medium containing only **1** (10 μM). Measurement of the optical density of the cultures (OD_{600}) after 48 h revealed considerable growth of each of the mutant strains in the presence of substrate **1** and catalyst **2** (Figure 2 A,B). The final optical densities were comparable to the cultures grown in the presence of PABA. As expected, no growth was observed in the absence of PABA. We evaluated the time course of growth under each of these conditions by

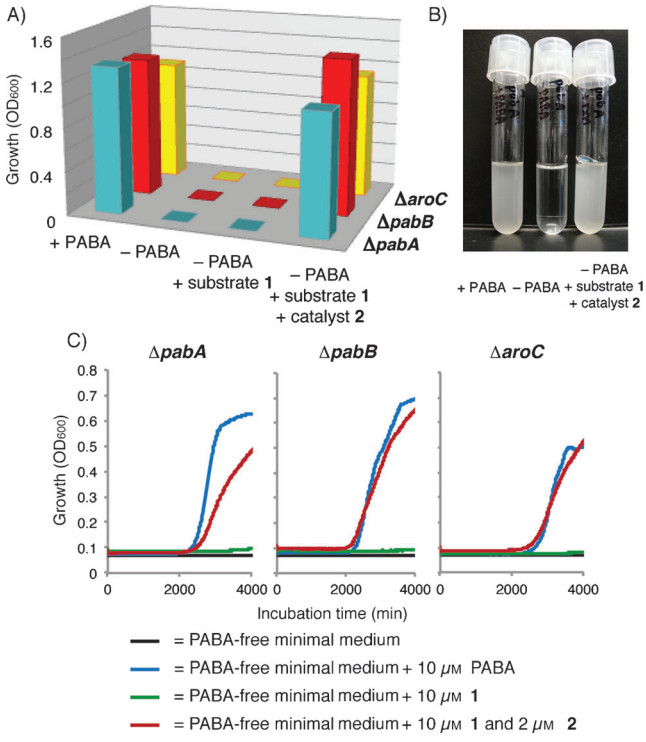


Figure 2. Biocompatible Alloc deprotection rescues the growth of *E. coli* PABA auxotrophs. A) Final optical densities of *E. coli* mutants grown in 5 mL PABA-free M9 glycerol minimal media for 48 h at 37 °C. B) Image of Δ*pabA* mutant cultures after 48 h of growth. C) Growth curves for *E. coli* mutants grown in 150 μL of PABA-free M9 glycerol minimal media at 37 °C. Growth curves shown represent the mean of three replicate cultures.

recording growth curves over 72 h. The growth curves for mutants grown in the presence of **1** and **2** and mutants grown in medium containing PABA (10 μM) were similar, with only the rescued Δ*pabA* mutant reaching a lower optical density with **1** and **2** than with directly added PABA (Figure 2C). We delineated the requirements for successful auxotroph rescue with a series of control reactions, in which we varied the components of the defined minimal medium and measured the final OD_{600} values after 48 h at 37 °C (Table S3 in the Supporting Information). We found that the presence of both substrate **1** and catalyst **2** is essential. Thiol addition had a minimal impact on growth, perhaps indicating that the improved conversion observed with these additives does not affect the efficiency of rescue. This result could also suggest that thiols generated by *E. coli* are involved in the deprotection in vivo.

An alternate explanation for auxotroph rescue could be the accumulation of mutations during the course of the experiment that either alleviate PABA auxotrophy or enable the strains to generate PABA from substrate **1**. We investigated these possibilities by re-inoculating rescued cultures back into minimal medium lacking PABA (Figure S3 and Table S4 in the Supporting Information). None of the rescued cultures grew under these conditions, which confirms that they were still PABA auxotrophs. The rescued strains also remained unable to grow in PABA-free medium containing substrate **1** (10 μM), a result that indicates that they had not acquired the ability to deprotect Alloc-PABA. All of the rescued cultures retained the ability to grow in medium containing PABA and could be rescued a second time in PABA-free medium containing substrate **1** and catalyst **2**. Finally, the fact that three auxotrophic strains, each containing a distinct mutation, could be rescued using this deprotection reaction adds to the weight of evidence against genetic changes leading to bacterial growth in these experiments.

A final piece of data supporting the link between Alloc removal and growth was the detection of PABA in rescued cultures. We determined the concentration of PABA necessary to achieve rescue by inoculating Δ*pabA* and Δ*aroC* mutants into M9 glycerol minimal media containing varying amounts of PABA (10 μM–10 pM). We found that at least 10 nM of PABA was required to rescue either mutant strain (Table S5 in the Supporting Information). We then used liquid chromatography–mass spectrometry (LC–MS) to quantify the PABA produced during a rescue experiment (Table S6 in the Supporting Information). Analysis of the culture medium after rescue of the Δ*pabA* mutant revealed a PABA concentration of 60 nM; therefore, the concentration of PABA generated from the Ru-catalyzed deprotection of **1** is sufficient to allow growth of these auxotrophs. Although we detected PABA in the medium, we do not know to what extent deprotection occurs intracellularly versus extracellularly; gaining a deeper understanding of where biocompatible reactions take place is a future area of interest.

This initial demonstration of auxotroph rescue relied upon a reaction that had previously been applied in living cells. We decided to explore the generality of this phenomenon by rescuing a different type of auxotrophy using iron(II)-catalyzed hydroxylation, a chemically challenging transformation that had not previously been utilized in the presence of living organisms. The hydroxylation of aromatic substrates in water using low-valent transition metals and molecular oxygen has been extensively investigated, because these systems mimic the reactivity of monooxygenase enzymes.[13] In particular, oxygenation using iron(II) complexes, molecular oxygen, and reducing agents (ascorbic and citric acids) has been previously carried out in aqueous solvent at ambient temperatures, conditions that could potentially be compatible with living organisms.[14] These hydroxylations are thought to proceed through formation of reactive oxygen species, including hydroxyl radicals, making it unclear whether this type of transformation could be biocompatible.[15] However, this reaction has biological relevance, because it resembles chemistry that may have occurred during the oxygenation of the Earth's atmosphere, as molecular oxygen reacted with iron present in the environment.[16]

We envisioned rescuing the Δ*aroC* mutant, a *p*-hydroxybenzoic acid (PHBA) auxotroph, through an iron-catalyzed *para* hydroxylation of benzoic acid (**3**, Figure 3A). We first confirmed that the Δ*aroC* mutant could not grow in PHBA-free M9 glycerol minimal medium, and that medium containing benzoic acid (100 μM) did not rescue growth (Figure 3B). In a manner analagous to that of the earlier rescue of PABA auxotrophy, the media contained all nutrients required for growth except PHBA. We then inoculated the *ΔaroC* mutant into PHBA-free minimal media containing **3** (10, 100, or 200 μM), $FeSO_4$ (10, 100 or 200 μM), and citric acid (0.2, 2, or 4 mM).[14e] The cultures containing higher concentrations of substrates and catalysts were rescued. Control experiments revealed that all reaction components were required for growth (Table S7 in the Supporting Information). Comparison of the growth curves for cultures grown in the presence of PHBA with those for rescued cultures revealed a more pronounced delay of entry into the exponential growth phase during rescue than was observed for the PABA auxotrophs (Figure 3C). We hypothesize that this difference may be due to cell death arising from reactive oxygen species generated during the hydroxylation reaction, an effect that would reduce the inoculum. Similarly to the rescued PABA auxotrophs, the rescued PHBA auxotrophs remained unable to grow in the presence of **3**, thus indicating a key role for catalysis in the restoration of growth (Table S8 and Figure S4 in the Supporting Information). Culturing the Δ*aroC* mutant in media containing varying amounts of PHBA revealed that 10–100 nM PHBA is sufficient for growth (Table S9 in the Supporting Information). We confirmed that PHBA was produced in rescued cultures by using LC–MS, and detected 70 nM of PHBA in the medium after rescue (Table S10 in the Supporting Information).

Unlike most enzymatic transformations that functionalize aromatic scaffolds, the iron-catalyzed hydroxylation used to generate PHBA is not expected to be regioselective. To establish the selectivity of this reaction, we performed the hydroxylation in culture medium using **3** (1 mM). At this higher substrate concentration, we were able to separate and quantify all three monohydroxylation products by using HPLC (Figure S5 and Table S11 in the Supporting Informa-

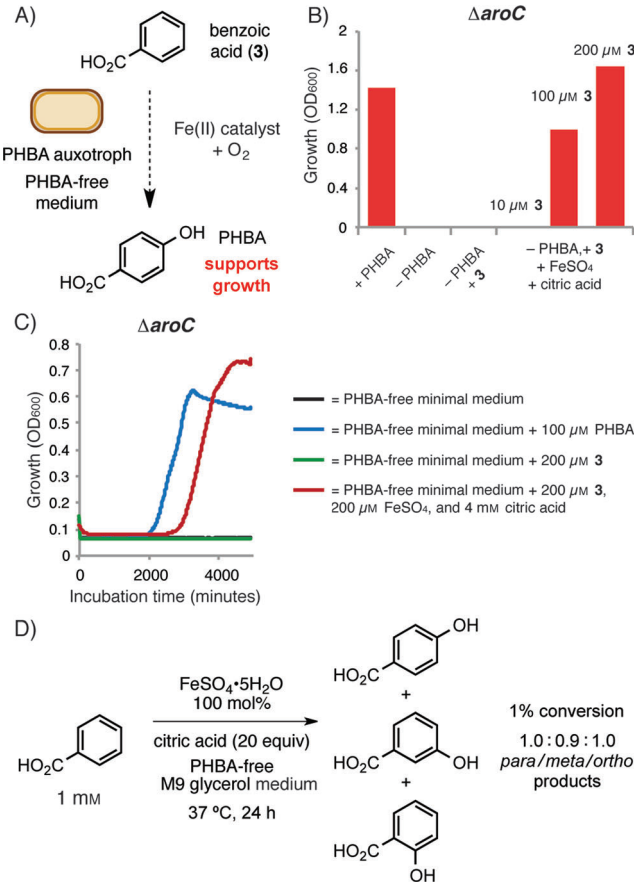


Figure 3. Rescuing PHBA auxotrophs using iron-catalyzed hydroxylation. A) Plan for generating PHBA through the hydroxylation of benzoic acid. B) Final optical densities for the *E. coli* Δ*aroC* mutant grown in 5 mL PHBA-free M9 glycerol minimal media for 48 h at 37 °C. C) Growth curves for the Δ*aroC* mutant grown in 150 μL of PHBA-free M9 glycerol minimal media at 37 °C. Growth curves shown represent the mean of three replicate cultures. D) Selectivity of the $FeSO_4$-catalyzed hydroxylation of benzoic acid. Product ratio determined by HPLC analysis.

tion). As anticipated, the reaction provided a mixture of *para*, *meta*, and *ortho* hydroxylation products (Figure 3D). This result indicates that a nonenzymatic transformation does not have to proceed with the same degree of selectivity as an enzymatic reaction to support growth, an observation that may have significance for prebiotic chemistry and the evolution of new enzymatic function. It also raises the question of whether there may be residual promiscuity in native metabolic reactions and pathways that could provide insights into their origins.

In summary, we have demonstrated for the first time that biocompatible, nonenzymatic transformations can rescue the growth of auxotrophic microorganisms by generating essential metabolites. More broadly, this approach enables us to link the growth of a living organism to the success of a nonbiological reaction. We anticipate that this phenomenon will constitute a powerful screening strategy for the discovery of new biocompatible reactions. Important future challenges include establishing methods to discover more efficient transformations, as well as identifying reactions that can directly replace missing cellular enzymes, for use within metabolic engineering and medicine. Overall, this work represents an important step toward interfacing biocompatible transformations with metabolism, and constitutes a new way to apply synthetic chemistry in the context of biological systems.

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