BIOSYNTHESIS

A carbonate architect emerges

Carbonates are known to exist in natural products, but their biosynthesis has not been linked to any enzymes. An unusual fungal Baeyer-Villiger monooxygenase CcsB is now revealed to catalyze an in-line carbonate formation via a two-step mechanism of oxygen atom insertions.

Yuhui Sun

The biosynthesis of complex polyketide and nonribosomal peptide natural products routinely involves formation of a core structure by stepwise chain elongation followed by enzymatic tailoring steps such as acylation, methylation, glycosylation and redox transformation. Among the fascinating enzymes that catalyze these tailoring reactions are flavin-dependent monooxygenases[1]. These are multifunctional enzymes that catalyze a variety of different types of C-O bond formation, including Baeyer-Villiger (BV) oxidations[2,3], catalyzed by the BV monooxygenases (BVMOs). The key intermediate flavin-peroxide species (Fl-4a-OO$^-$) serves as a nucleophilic equivalent in BV transformations in the conversion of keto groups to esters in numerous natural product pathways. In contrast, oxidation to the carbonate moiety has rarely been observed in either chemical synthesis or enzymatic transformation, and its biosynthetic origins are unknown. Hu *et al.*[4] now demonstrate one route by which a carbonate moiety is generated in their biochemical characterization of a remarkable multifunctional BVMO enzyme, CcsB, in the biosynthetic pathway to the polyketide-peptide cytochalasin E in the fungus *Aspergillus clavatus*.

BV oxidation reactions have been studied over many years, and the mechanisms of BVMOs, such as OxyS from the oxytetracycline pathway[5] and GilOII from the gilvocarcin pathway[6], have been explored. However, BV oxidation reactions are typically limited to a single O insertion with no sequential O insertions demonstrated so far. Additionally, no enzymatic oxidation of a ketone or ester to the corresponding carbonate has been reported. The carbonate functionality is rarely seen in natural products; the most prominent examples are found in several members of the cytochalasin family, discovered in fungi and produced by enzymes in the *ccs* gene cluster in *A. clavatus*[7]. The construction of the carbonate moiety via oxidation of a keto or an ester group is a challenging synthetic or enzymatic transformation. The oxidation of an ester via BV reactions would require transfer of a nucleophilic oxygen atom from Fl-4a-OO$^-$ to the ester carbonyl, but the ester carbonyl group is a relatively electron-rich moiety; indeed, if it were favorable for an additional C-O bond to form, the Criegee complex (a tetrahedral intermediate formed by nucleophilic attack or addition of the peroxy acid to the carbonyl) in BV reactions would be unstable. Thus, studying the biochemical mechanism of carbonate moiety formation in cytochalasin E was expected to yield important insights into an unusual process.

In their research, Hu *et al.*[4] started by identifying the biosynthetic origin of oxygen atoms in cytochalasin E using isotope feeding experiments. The oxygen atoms in the carbonate group were found to be derived from molecular oxygen, indicating an insertion pathway catalyzed by oxygenases. By screening the *ccs* gene cluster and aligning all of the high-scoring sequence homologs, CcsB was identified as a predicted BVMO that is likely to catalyze a BV reaction on a keto group of a macrocyclic intermediate owing to moderate sequence identity to well-characterized type I BVMOs. It contains the fingerprint motif FXGXXXHXXXW for NADPH binding and a highly conserved active site Arg421 for stabilizing the peroxide-flavin species. Deletion of the *ccsB* gene led to accumulation of a new compound (**1**), which strongly implicated CcsB in catalyzing the oxidative transformation from keto to ester or carbonate (**Fig. 1**). Incubation of purified **1** with recombinant CcsB gave **3** as a major product (the carbonate-containing compound) with **2′** as a minor product. Crucially, the authors showed, with the help of deuterium labeling, that **2′** arises from the

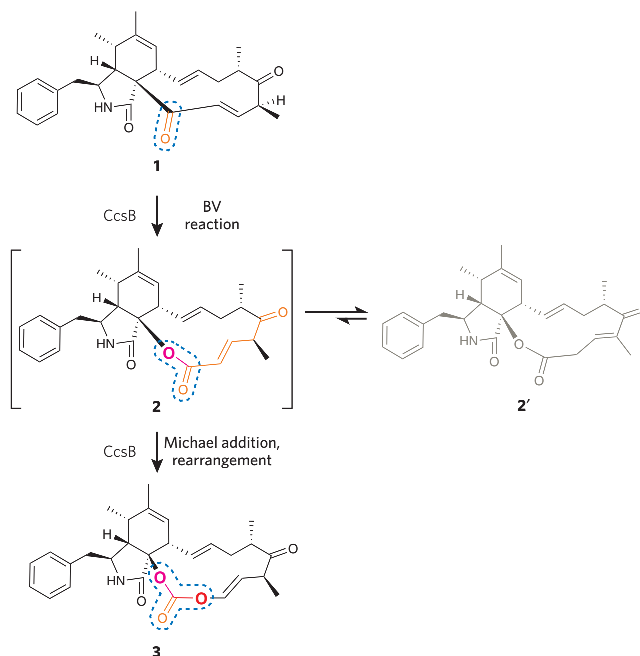

Figure 1 | The mechanism of carbonate formation catalyzed by CcsB. The first and second oxygen atoms inserted by the BVMO CcsB are highlighted in magenta and red, respectively. The vinylogous 1,5-diketo moiety required for the second oxidation is shown in orange.

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isomerization of the true (more unstable) intermediate **2** (**Fig. 1**). On the basis of their experimental results, the authors propose a mechanism for carbonate formation in which CcsB inserts the first oxygen into the ketone-containing substrate **1** via a canonical BV mechanism and **2** is then further oxidized by Michael addition of Fl-4a-OO$^-$ to the α,β-unsaturated ketone of **2**. The resulting rearrangement leads to **3**.

This work highlights an unprecedented conversion of a keto group to a carbonate in a natural product catalyzed by a remarkable multifunctional BVMO CcsB. Sequence analysis of CcsB shows that it is closely related to well-characterized BVMOs[2] catalyzing single O-insertion. Therefore, it is an intriguing question whether other BVMOs might also catalyze the successive oxidation of ketone intermediates to carbonates if provided the structural context of the neighboring vinylogous 1,5-diketone. A crystal structure of CcsB would help to address this question. This additional understanding of the CcsB catalytic mechanism could also pave the way to synthetic strategies that exploit a similar arrangement of functional groups to install the carbonate moiety. Finally, *ccsB* is a worthwhile probe for screening genomes to uncover further carbonate-containing natural products and may be useful in combinatorial approaches to form diverse cytochalasin structures. ■

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Competing financial interests

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DNA METABOLISM

Bases of DNA repair and regulation

Recent studies have identified the existence of modified cytosine bases in DNA that result from ten eleven translocation (Tet)-mediated oxidation of 5-methylcytosine. The demonstration that Tet oxidizes thymine to 5-hydroxymethyluracil has implications for our current view of DNA metabolism.

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Methylation of cytosine residues in DNA to generate 5-methylcytosine (5mC) has been known since 1948 and has a fundamental impact on cellular processes including genome stability, transcription, differentiation and development. The methyltransferases that catalyze the formation of 5mC are well characterized, but the mechanistic pathways by which DNA is actively demethylated are not well understood. More recently, enzymes that can catalyze the removal of the methyl group have been identified. The identification of the Tet enzymes, which oxidize 5mC to 5-hydroxymethylcytosine (5hmC) and other bases, indicates that DNA repair pathways could be used for dynamic DNA demethylation[1]. Pfaffender *et al.*[2] now show that 5-hydroxymethyluracil (5hmU) can be produced by direct Tet-mediated oxidation of thymine bases in stem cell DNA; this suggests that Tet-catalyzed oxidation is not limited to modified bases derived from cytosine and that 5hmU in genomic DNA may have regulatory roles.

In addition to oxidizing 5mC, the Tet proteins can progressively oxidize 5hmC, yielding 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC)[3,4]. The excision of these base modifications is catalyzed by thymine DNA glycosylase[3]. Therefore, a complete enzymatic DNA demethylation pathway can be composed of the following steps: Tet-catalyzed oxidation of 5mC to either 5fC or 5caC followed by thymine DNA glycosylase–initiated base excision repair. Although all of the aforementioned base modifications are produced from cytosine, 5hmU and 5-formyluracil (5fU) are also found at reasonable amounts in genomic DNA. Some of these modifications are oxidative DNA lesions produced by reactive oxygen species (ROS) reacting with thymine in DNA. As Tet1 overexpression results in a notable increase in the amount of 5hmU[5], it was suggested that 5hmC deamination by activation-induced cytosine deaminase (AID) would produce 5hmU[5,6]. Thus, 5hmU in genomic DNA could be the result of ROS acting on thymine or deamination of 5hmC. However, AID is an unlikely candidate for active DNA demethylation as it has low deamination activity on 5mC and no detectable deamination activity on 5hmC, and overexpression of AID does not increase the quantity of 5hmU[7].

Examining the origins of 5hmU in cells in more detail, Pfaffender *et al.*[2] provide solid evidence that a majority of 5hmU in mouse embryonic stem cell (mESC) genomic DNA results from Tet-catalyzed thymine oxidation (**Fig. 1**)[2]. Relative to other cell types, 5hmU levels are elevated in mESCs and are found in amounts that are comparable to 5caC. As 8-oxoguanine is a major ROS product, the measurement of 8-oxoguanine in control experiments excludes ROS oxidation of thymine as the sole source of elevated 5hmU. Although 5hmU levels were unchanged by depletion of DNA methyltransferases, Tet knockdown resulted in a substantial reduction in 5hmU. Isotope tracing experiments in cells firmly established that the precursor of 5hmU is not 5hmC. Therefore, in mESCs, 5hmU modifications result from both Tet oxidation and exposure to ROS, the majority of which are introduced by Tet oxidation of thymine.

5hmU modifications resulting from thymine oxidation appear in the 5hmU:A base pair context. Boorstein *et al.*[8] previously identified SMUG1 as the DNA glycosylase that excises 5hmU paired with adenosine. In line with this