

## RESEARCH ARTICLE

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View Journal | View IssueCite this: *Org. Chem. Front.*, 2020, 7, 3193Identification, cloning, expression and functional interrogation of the biosynthetic pathway of the polychlorinated triphenyls ambigol A–C from *Fischerella ambigua* 108b†Elke R. Duell, ‡<sup>a,f</sup> Tobias M. Milzarek, ‡<sup>a,b</sup> Mustafa El Omari,<sup>c</sup> Luis J. Linares-Otaya,<sup>d,e</sup> Till F. Schäberle, <sup>d,e</sup> Gabrielle M. König <sup>c</sup> and Tobias A. M. Gulder <sup>\*a,b</sup>

The terrestrial cyanobacterium *Fischerella ambigua* 108b produces the three polychlorinated triphenyls ambigol A–C that exhibit interesting antimicrobial, antiviral and cytotoxic activities. They are structurally related to polybrominated diphenylethers synthesized by diverse marine bacteria that are known to be highly toxic and are bioaccumulating in natural food webs. All ambigols display unusual connectivities: Ambigols A and B exhibit chlorination and ambigol C biaryl-ether bonds in the relative *meta* position at the central phenol unit, which is flanked by two 2,4-dichlorophenol units in all three compounds. Here we report on the identification of the biosynthetic gene cluster (BGC) responsible for ambigol production in *F. ambigua*. After bioinformatic discovery of a putative ambigol BGC (*ab*) containing 10 genes, we cloned and heterologously expressed this cluster in *Synechococcus elongatus* PCC 7942 using Direct Pathway Cloning (DiPaC). *In vivo* and *in vitro* characterization of the two cytochrome P450 enzymes present in the *ab* BGC revealed complementary selectivity for either biaryl-ether bond (Ab2) or biaryl formation (Ab3) and provided a biosynthetic route to the ambigols.

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## Introduction

Polyhalogenated aromatics can be found almost everywhere in Nature and are often associated with negative biological effects on human and animal life, especially as they are known to be highly persistent and bioaccumulative in natural food webs. Many toxic representatives of this class of molecules have an anthropogenic source and their incautious distribution in the environment is clearly caused by humankind. Amongst these

substances are the polychlorinated biphenyls (PCBs, **1**), formally used, *e.g.*, as industrial coolants and plasticizers, and the polybrominated diphenyl ethers (PBDEs, **2a/b**), utilized as flame retardants (Fig. 1A). These compounds are now restricted from commercial use due to their toxicity and aggregation in the environment. Polyhalogenated dibenzo-*p*-dioxines (PHDDs), *e.g.*, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, **3**), which is a side product in the synthesis of herbicides such as 2,4,5-trichlorophenoxyacetic acid (trioxone), is one of the most toxic and environmentally persistent man-made pollutants known today.<sup>1–3</sup> The structurally related substance triclosan (**4**), which was developed as disinfectant, can still be found in toothpastes, soaps and detergents. However, recent studies suggest it to be very likely toxic for animals and humans,<sup>4–6</sup> especially due to formation of PHDDs from **4** via its photodegradation.<sup>7,8</sup>

However, not all harmful polyhalogenated aromatic substances are of anthropogenic origin. A wide range of PBDEs, such as **5** and **6**, has been isolated from marine invertebrates<sup>9–11</sup> and only recently it was shown that they are produced by a variety of marine bacteria such as  $\gamma$ -proteobacteria of the genus *Pseudoalteromonas* and the cyanobacterial *Hormosira spongeliae* spp.<sup>12,13</sup> These naturally occurring PBDEs, including their hydroxylated (OH-BDEs) and

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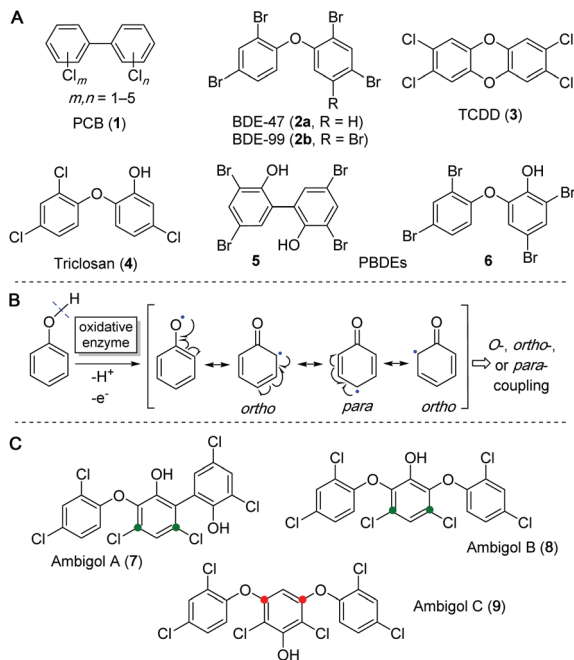
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**Fig. 1** (A) Structures of selected synthetic (1–4) and naturally occurring (5, 6) polyhalogenated aromatic compounds. (B) The radical mechanism underlying oxidative coupling of phenols allows reactions only at the phenol oxygen or in relative *ortho* and *para* position to this activating group. The identical positions are activated for nucleophilic halogenation by flavin-dependent halogenases. (C) Ambigols A (7) and B (8) show chlorination in *meta* position (green dots), ambigol C (9) contains two biaryl-ether-bonds in *meta* position (red dots) within the central phenol building block.

dioxine-like derivatives, can be found in all trophic levels of marine life, are accumulating in the food chain *via* marine plants<sup>14</sup> and algae,<sup>15–17</sup> and are ultimately detectable in marine animals<sup>18–20</sup> and even human tissue.<sup>21–24</sup> Similar to their man-made relatives, they exhibit toxic effects on mammalian hormone mediated signalling pathways and essential enzymatic reactions.<sup>24–28</sup> The biosynthesis of these PBDEs of natural origin has been extensively studied by pioneering work of the Moore laboratory over the last years, leading to the discovery of the encoding biosynthetic gene cluster (BGC) *bmp*, which can be found in varying genetic compositions in all bacterial strains associated with PBDE biosynthesis.<sup>12,13</sup> The biosynthesis of PBDEs utilizes the primary metabolite chorismic acid (10), which is converted to 4-hydroxybenzoic acid (4-HBA, 11) by the chorismate lyase *Bmp6* (*cf.* Fig. 3).<sup>29,30</sup> A so far unprecedented decarboxylative phenol bromination mechanism, catalyzed by the halogenase *Bmp5*, uses 11 as a substrate to form 2,4-dibromophenol (2,4-DBP).<sup>12</sup> The cytochrome P450 enzyme *Bmp7* is then able to couple two 2,4-DBP units to form a variety of different dimeric PBDEs.<sup>12,13,30</sup> These biaryl- and biaryl-ether-coupling reactions between aromatic rings follow the common principle of phenol oxidative coupling, where an initial hydroxyl radical is formed by abstraction of one electron and one proton (Fig. 1B).<sup>31</sup> Due to mesomeric delocalization of the free electron on the benzene ring in relative *ortho* and *para*

position to the oxygen functionality, a large diversity of C–C- and C–O–C-bond containing coupling products can be biosynthesized.<sup>32</sup>

Not only marine but also terrestrial cyanobacteria produce polyhalogenated phenols. Due to its abundance in soil, chlorine is generally favoured over bromine for incorporation into halogenated natural products of terrestrial origin. In 1993 and in 2005, the three polychlorinated ambigols A–C (7–9, Fig. 1C) were discovered from the soil-dwelling filamentous cyanobacterium *Fischerella ambigua* 108b. Ambigol A (7) exhibits strong antimicrobial, antifungal and virostatic activities and moderate cytotoxicity whereas ambigol C (9) shows exclusive, potent activity against Gram positive bacteria and fungi and ambigol B (8) generally displays only weak activity in biological assays.<sup>33–35</sup> At first glance, 7–9 are structurally related to the marine PBDEs as they also contain two halogen atoms per phenol and are linked either by biaryl- or biaryl-ether-bonds. Unlike the PBDEs, 7–9 incorporate a third dihalophenol unit. In addition, either the chlorine substituents (in 7 and 8) or the biaryl ether structural motif (in 9) are not located at the typical relative *ortho* or *para* position to the activating phenol function in the central aromatic portion, but rather situated in *meta* position (Fig. 1C). Owing to the interesting biological activities of the ambigols 7–9 and these structural peculiarities pointing at unusual biosynthetic transformations, we investigated ambigol biosynthesis within this work, with a focus on the installation of the biaryl structural features.

## Results and discussion

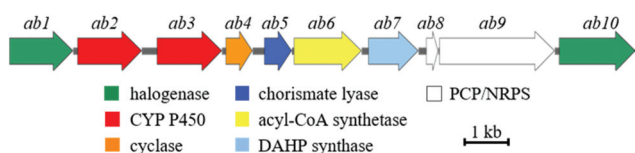
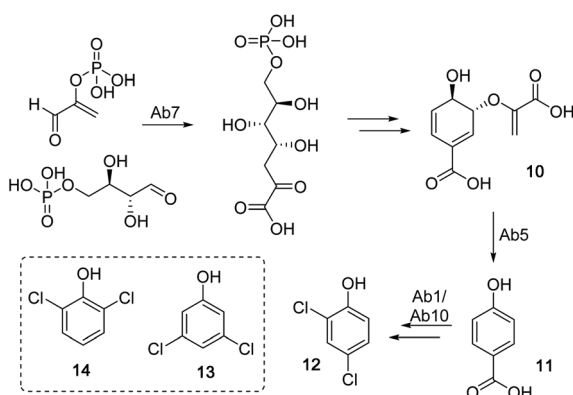
### Bioinformatic analysis of the *ab* BGC

To identify the ambigol BGC, the natural producer *Fischerella ambigua* 108b was submitted to 454 genome sequencing (see ESI†). Bioinformatic screening for the presence of halogenases allowed for the identification of a putative ambigol BGC *ab* (14.3 kb) containing 10 genes (Table 1), among them a number of genes predicted to be required for ambigol biosynthesis (Fig. 2). All 10 putative *ab* genes appear to be located on a single transcript. Upstream of *ab1* a non-coding DNA region is followed by a gene encoding a protein kinase while *ab10* is neighbored by genes encoding two small hypothetical proteins and two transposases, thus indicating the likely borders of the *ab* BGC. Besides genes encoding halogenating enzymes, additional similarities to the gene clusters encoding the structurally related PBDEs were expected. Indeed, *ab* contains a gene encoding a chorismate lyase *ab5* that is highly homologous to *bmp6* found in *H. spongelliae*.<sup>13</sup> In analogy to the *bmp* pathway, *ab5* thus likely recruits building blocks from the pool of primary metabolites for ambigol biosynthesis by formation of 4-HBA (11) from chorismic acid (10). Furthermore, *ab* contains a gene encoding a 3-deoxy-7-phosphoheptulonate (DAHP) synthase (*ab7*), an enzyme catalyzing the first dedicated step in the shikimic acid biosynthetic pathway towards 10 (see Fig. 3).



**Table 1** Genetic composition of the *ab* BGC

| Gene        | Size (bp) | Proposed function                     | Closest xBLAST hit   | Identity (protein) | GenBank acc. number |
|-------------|-----------|---------------------------------------|--|--------------------|---------------------|
| <i>ab1</i>  | 1452      | Precursor chlorination                | NAD(P)/FAD-dependent oxidoreductase [ <i>Scytonema hofmanii</i> ]      | 281/486 (58%)      | WP_017744817.1      |
| <i>ab2</i>  | 1464      | Biaryl coupling                       | Cytochrome P450 [ <i>Hormosilla spongelliae</i> GUM020]                | 337/485 (69%)      | AQU14168.1          |
| <i>ab3</i>  | 1476      | Biaryl coupling                       | Cytochrome P450 [ <i>Hormosilla spongelliae</i> GUM020]                | 301/474 (64%)      | AQU14168.1          |
| <i>ab4</i>  | 615       | Unknown                               | Cyclase [ <i>Nostoc punctiforme</i> NIES-2108]                         | 123/180 (68%)      | RCJ31428.1          |
| <i>ab5</i>  | 624       | Synthesis of <b>11</b> from <b>10</b> | Chorismate lyase [ <i>Hormosilla spongelliae</i> GUM096]               | 106/177 (60%)      | AQU14179.1          |
| <i>ab6</i>  | 1536      | Acyl activation                       | Acyl-CoA synthetase [filamentous cyanobacterium CCP3]                  | 410/511 (80%)      | WP_106922238.1      |
| <i>ab7</i>  | 1131      | Synthesis of DAHP                     | 3-Deoxy-7-phosphoheptulonate synthase [ <i>Nostoc minutum</i> NIES-26] | 296/374 (79%)      | RCJ32495.1          |
| <i>ab8</i>  | 273       | PKS (ACP)                             | Acyl carrier protein [ <i>Chroogloeocystis siderophila</i> ]           | 49/81 (60%)        | WP_073551326.1      |
| <i>ab9</i>  | 2631      | NRPS (C-PCP-TE)                       | non-ribosomal peptide synthetase [ <i>Moorea producens</i> ]           | 519/870 (60%)      | WP_008188544.1      |
| <i>ab10</i> | 1725      | Precursor chlorination                | FAD-dependent oxidoreductase [ <i>Scytonema</i> sp. HK-05]             | 430/573 (75%)      | WP_073630058.1      |

**Fig. 2** Schematic illustration of the genetic organization within the *ab* BGC from *F. ambigua* containing ten genes.**Fig. 3** Biosynthetic route to the ambigol precursor **12** from chorismic acid (**10**) via 4-HBA (**11**). Box: alternative chlorinated precursors **13** and **14**.

The entire *ab* BGC is framed by two genes encoding FAD-dependent halogenases, *ab1* and *ab10*. As in PBDE biosynthesis, **11** has to be chlorinated twice to give 2,4-dichlorophenol (**12**) as monomeric building block found in all ambigol structures. Formation of monomeric precursors in case of PDBE biosynthesis was shown to be catalyzed by a single halogenase, Bmp5, which not only performs an *ortho* bromination, but also a highly unusual second decarboxylative halogenation, thus replacing the acid function in *para* position to the phenol in **11** by a bromine substituent.<sup>12</sup> In contrast, the *ab* BGC contains genes encoding two FAD-dependent halogenases *ab1* and *ab10*. The underlying mechanistically different halogenation reactions towards **12** therefore might require two separate halogenases in the case of ambigol biosynthesis. Interestingly, neither *ab1* nor *ab10* show a particularly strong

homology to *bmp5*. Alternatively, one of the halogenases might be involved in the assembly of 3,5-dichlorophenol (**13**) or 2,6-dichlorophenol (**14**), as potential further required central ambigol biosynthetic building blocks (Fig. 3). Three additional genes located in *ab*, i.e., *ab6*, *ab8*, and *ab9*, resemble elements of PKS/NRPS biosynthesis for precursor activation (*ab6*) and covalent tethering (*ab8*, *ab9*). The corresponding proteins might thus likewise be involved in enzymatic halogenation, as PCP-tethering is known to be required by a number of FAD-dependent halogenases.<sup>36–39</sup>

However, despite considerable attempts to obtain soluble halogenases Ab1 and Ab10 (data not shown), we did not yet succeed in recombinant production of these enzymes in a soluble form and thus could not further probe their functions. In case of PDBEs, cross-coupling of the phenolic building blocks typically occurs in the mechanistically permitted *ortho* and *para* positions relative to an activating phenol function (cf. Fig. 1B). The *bmp* BGC in  $\gamma$ -proteobacteria only contains a single cytochrome P450 encoding gene, *bmp7*. The corresponding protein catalyzes the required biaryl bond formations between two units of 2,4-dibromophenol.<sup>13</sup> In contrast, the *ab* cluster harbours two genes encoding the closely related CYP P450 homologs Ab2 and Ab3. It was thus tempting to speculate that one of these enzymes catalyzes biaryl bond formation, while the other homolog is involved in biaryl ether assembly. Such a complementary set of C–C- and C–O–C- coupling enzymes would be a valuable tool for the biocatalytic synthesis of biaryl structures. We thus set out to firmly link the putative ambigol BGC to ambigol production and to characterize the function of the cytochrome P450 enzymes Ab2 and Ab3.

### Cloning and expression of the *ab* BGC in *E. coli* and *S. elongatus*

To unambiguously link the putative ambigol BGC *ab* to ambigol biosynthesis, we first aimed at recombinant production of the target molecules. Mutation free cloning of the whole *ab* cluster into pET-28b-SUMO as well as subsequent addition of a *gfp* gene downstream of the *ab* BGC as reporter for successful transcription of the entire pathway<sup>40</sup> was achieved using our DiPaC strategy.<sup>40–42</sup> Initial expression tests with the resulting construct in *E. coli* BAP1 harbouring pET-28b-SUMO::*ab-gfp* did not show production of any new



compounds when compared to empty cells as controls, nor of Gfp-mediated fluorescence. The T7 promoter was therefore exchanged by ptetO, as this promoter is known to efficiently upregulate larger transcripts.<sup>43</sup> While the resulting system pET28b-ptetO::ab-gfp facilitated the expression of Gfp, as indicated by significant fluorescence of the recombinant *E. coli* BAP1 host cells (see ESI Fig. S5†) thus clearly also confirming complete transcription of all ab genes, again no production of ambigols or potential intermediates was observable. The reason for this is likely the inability of *E. coli* to translate and/or fold one or more enzymes of the ab BGC into their catalytically active forms. Related problems were observed by Moore *et al.* when expressing selected genes of the bmp BGC of cyanobacterial origin,<sup>13</sup> while the bmp genes from  $\gamma$ -proteobacteria, by contrast, were expressible in *E. coli*.<sup>12,30,39,44</sup> We thus focused on the recombinant production of ambigols in a phylogenetically more closely related species when compared to *F. ambigua*, i.e., the cyanobacterial heterologous host *Synechococcus elongatus* PCC 7942<sup>45,46</sup> previously utilized for the production of PBDEs<sup>13</sup> and industrially important compounds such as butyrate,<sup>47</sup> 2-deoxy-scylo-inose<sup>48</sup> and propanediol.<sup>48,49</sup> Due to the size of the ab BGC and the known difficulties in introducing large DNA fragments into *S. elongatus* by natural transformation, we decided to split the ab BGC in two halves of approx. 7–8 kb and to sequentially integrate these parts into two independent genomic sites of *S. elongatus* PCC 7942. Mutation free integration of ab1–5 into pAM5051<sup>46</sup> and ab6–10 into pCV0094<sup>13</sup> as well as the integration of ab6–10 into the chromosomal site NSII of *S. elongatus* by natural transformation was successful (see ESI Fig. S3†). Generation of double transformants additionally carrying ab1–5 in all copies of NSI was subsequently verified by PCR. To the best of our knowledge, this is the first introduction of a >10 kb BGC into the *S. elongatus* host system. More importantly, heterologous production of the encoded metabolites was observable using this system (Fig. 4): production of a dichlorophenol-dimer with  $m/z$  323.1 [M – H]<sup>–</sup>, later identified to be 2,4-dichloro-6-(2,4-dichlorophenoxy)-phenol (36, see Fig. 5 and ESI Fig. S7†), and one trimeric molecule with  $m/z$  482.7 [M – H]<sup>–</sup> corresponding to the ambigol molecular mass was observed at the retention time of ambigol A (7, cf. Fig. 7D). In addition, two dichlorophenol-trimers each lacking one Cl-atom with  $m/z$  449.1 [M – H]<sup>–</sup> were produced. As no dichlorophenol monomer was detectable, we assume that this intermediate is rapidly consumed by the downstream enzymes to give the observed dimeric and trimeric products. Each of the observed products displayed the expected isotopic pattern of multi-chlorinated substances (Fig. 4). The corresponding signals were not present in wildtype *S. elongatus* control experiments, thus firmly linking ambigol production to the identified ab BGC. Unfortunately, the recombinant production system proved to be unstable, likely due to the metabolic stress exerted on the recombinant *S. elongatus* host system resulting from integration of two genetic elements requiring cultivation of the highly sensitive host system in the presence of multiple antibiotics. In general, the double mutant *S. elongatus* cells

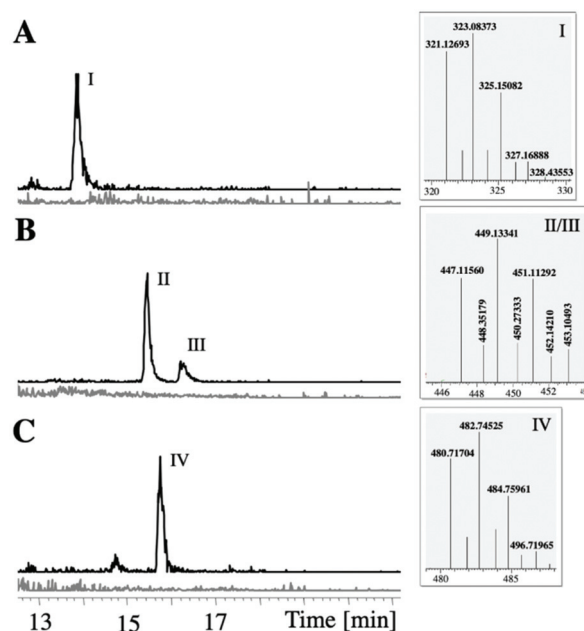


Fig. 4 LC-MS analysis of pellet extracts of *S. elongatus* WT (grey) and *S. elongatus* simultaneously carrying ab1–5 in NSI and ab6–10 in NSII (black). The production of a DCP-dimer I (A, detected at  $m/z$  = 322.5–323.5), two trimers-Cl II and III (B, detected at  $m/z$  = 448.0–450.0) and one ambigol trimer IV (C, detected at  $m/z$  = 482.0–483.0, retention time corresponding to ambigol A (7), cf. Fig. 7) showing the characteristic Cl-isotopic pattern (see boxes) can only be observed in the presence of the ab cluster.

took significantly longer to grow when compared to wild type or single mutant cells and were visibly less vital.

#### Cloning, expression and purification of the CYP P450 enzymes Ab2 and Ab3

We next turned our attention to the *in vivo* and *in vitro* characterization of the enzymes Ab2 and Ab3 putatively involved in biaryl formation. For the *in vivo* experiments, we again utilized *S. elongatus* as well as *E. coli* BL21 as host organisms. Mutation free integration of the native sequences of ab2 and/or ab3 into pCV0092 was confirmed by sequencing. Successful natural transformation of *S. elongatus* with these constructs was verified by PCR screening (see ESI Fig. S4†). As this heterologous system is not capable of producing high amounts of proteins after induction, the transformed cells were only used for *in vivo* feeding experiments. For *in vitro* work, *E. coli* codon optimized versions of ab2 and ab3 were successfully integrated into the pMal expression plasmid containing an N-terminal MBP-tag. Heterologous overexpression in *E. coli* BL21 and purification *via* MBP-tag based affinity chromatography led to soluble protein, resulting in a slightly yellow enzyme solution (see ESI Fig. S6†). Before characterizing the enzymatic transformation catalyzed by these CYPs, we set out to synthesize all feasible C–C and C–O–C coupling dimers as analytical standards to allow for an unambiguous structural assignment, as very similar chromatographic and spectroscopic properties were to be expected for these structurally highly related compounds.



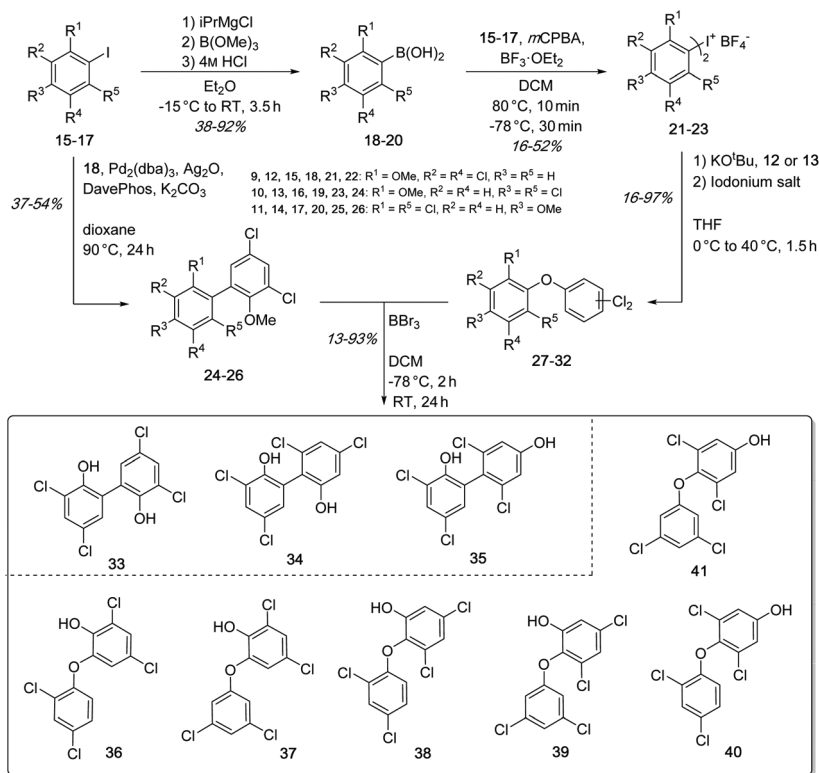


Fig. 5 Developed synthetic routes for the formation of C–C-coupled dimers 33–35 and of C–O–C-coupled dimers 36–41.

### Chemical synthesis of standards and functional characterization of the CYP P450 enzymes *in vitro* and *in vivo*

The synthesis of the biaryls 33–35 and biaryl-ethers 36–41 was performed by an improved palladium-catalyzed Suzuki cross-coupling reaction as well as a nucleophilic aromatic substitution ( $\text{S}_{\text{N}}\text{Ar}$ ) using symmetric hypervalent iodine(III) salts (Fig. 5). The basic building blocks, 2,4-dichlorophenol (12) and 3,5-dichlorophenol (13) were transformed into methoxy-protected aryl iodides 15–17 (see ESI<sup>†</sup>), which were used to synthesize the respective boronic acids 18–20 through metal-halogen exchange in 38–92% yield. By Suzuki coupling, the aryl iodides 15–17 and (3,5-dichloro-2-methoxy-phenyl)boronic acid (18) were coupled to give the protected biaryls 24–26 in 37–54% yield. The symmetric iodonium salts were synthesized using the same aryl iodides 15–17 and the corresponding boronic acids 18–20 under oxidative conditions.<sup>50</sup> The subsequent conversion in the  $\text{S}_{\text{N}}\text{Ar}$  reaction led to the methoxy-protected biaryl-ethers 27–32 in yields of 16–97%.<sup>51</sup> In order to obtain the desired dimers 33–41, the methoxy protecting groups in 24–32 were cleaved off using boron tribromide. The deprotection gave the expected products 33–41 in 13–93% yield. It should be noted that the total yield of all executed reactions is highly dependent on the steric hindrance of the building blocks. In summary, we have developed a short and fast synthetic route for the formation of all required C–C and C–O–C dimers, using functionalized aromatic building blocks.

LC-MS analysis of both *in vivo* (feeding experiments) and *in vitro* assays of Ab2 with substrate 12 showed the predominant

formation of one coupling product with  $m/z$  323.0  $[\text{M} - \text{H}]^-$  (Fig. 6). NMR analysis of the isolated product suggested the production of 2,4-dichloro-6-(2,4-dichlorophenoxy)phenol (36). Comparison of HPLC retention times and  $^1\text{H}$ -NMR spectra of biochemically produced dimer with the synthetic standards indeed confirmed the identity of 36 (see ESI Fig. S7, S12 and 13<sup>†</sup>). A formation of trimeric products was not observed.

Ab3 catalyzed the formation of a major product with identical mass but different retention time when compared to the

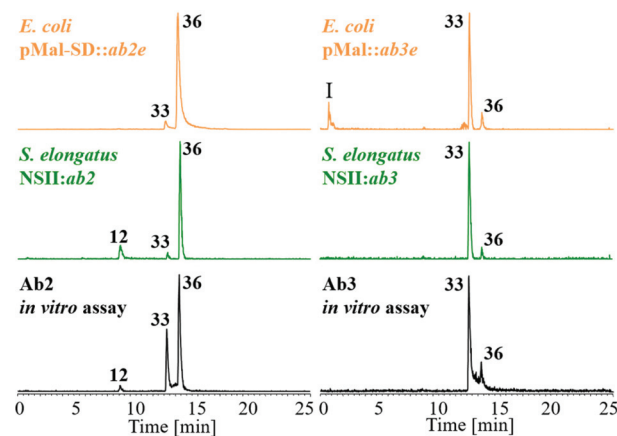


Fig. 6 *In vivo* and *in vitro* catalytic activity of Ab2 and Ab3 when incubated with 12. Ab2 predominantly catalyzed the formation of the C–O–C coupled dimer 36 whereas Ab3 selectively produced the C–C coupled dimer 33. EIC (ESI-) at  $m/z$  = 322.5–323.5. I = injection peak.



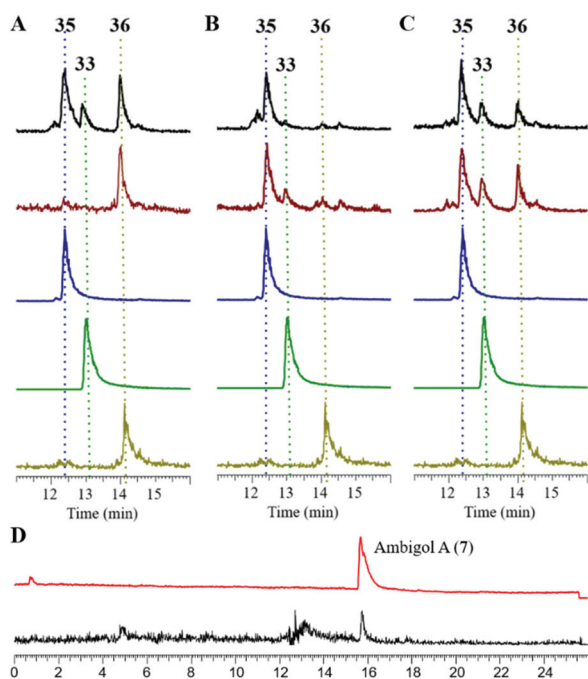
Ab2 product in both *in vitro* and *in vivo* experiments (Fig. 6). Comparison to the synthetic standards revealed the dimer to be 3,3',5,5'-tetrachloro-[1,1'-biphenyl]-2,2'-diol (**33**) resulting from a biaryl-coupling of two units of **12** in relative *ortho* position to the phenol functions (see ESI Fig. S9 and 14†). Particularly in *in vitro* assays, Ab2 and Ab3 were found to also catalyze the formation of small amounts of the dimer characteristic of the respective other CYP P450 enzyme. In addition to these products, small amounts of two DCP trimers at  $m/z$  449.1  $[M - H]^-$  and  $m/z$  449.0  $[M - H]^-$ , thus lacking one chlorine substituent, were likewise produced both *in vitro* and *in vivo* in the presence of Ab3 (see ESI Fig. S11†), in agreement with the observed product spectrum in the *S. elongatus* recombinant *ab* expression host (see above). The combination of both Ab2 and Ab3 in one assay did not lead to the formation of any additional, new products. *In vivo* and *in vitro* assays with 2,4-DBP instead of **12** showed that both Ab2 and Ab3 can accept and couple this substrate as well, following the same site selectivity. On the basis of these experimental results, we identified two cytochrome P450 enzymes that complementarily perform either biaryl- or biaryl-ether bond formation with **12** as substrate.

To obtain further insights into the biosynthesis of ambigol precursor molecules, Ab2 and Ab3 were simultaneously probed with **12** and **13**, both *in vivo* in *E. coli* (Fig. 7; brown chromatogram) and *in vitro* (Fig. 7; black). Compared to incubation with

**12** alone, Ab2 retained identical selectivity for production of **36** *in vivo* (cf. Fig. 6) and also showed similar product distribution of **33** versus **36** *in vitro* (Fig. 7A). However, the *in vitro* experiment additionally revealed the formation of **35**, the central building block of ambigol A (**7**), the major ambigol congener in *F. ambigua*.

For Ab3, by contrast, a significantly altered product spectrum was observed. While only small amounts of dimer **33** were still detectable, Ab3 predominantly produced biaryl **35** both *in vivo* and *in vitro* (Fig. 7B), thus showing pronounced selectivity for the production of the mixed cross-coupling product of **12** and **13**. Combination of Ab2 and Ab3 in a single assay delivered the result expected for combining the reaction outcomes of the individual enzymatic transformations (Fig. 7C).

Given the selectivity of Ab3 to form the C–C-coupled precursor **35** of ambigol A (**7**) and the previously observed selectivity of Ab2 to perform C–O–C-bond formation (cf. Fig. 6), we next incubated Ab2 with **35** and **12**, the missing building block to conclude ambigol A (**7**) assembly *via* C–O–C-coupling. This indeed led to the selective formation of **7**, as evidenced by HPLC-MS analysis and comparison with an authentic standard of **7** isolated from *F. ambigua* (Fig. 7D). Traces of **7** were also obtained upon simultaneous incubation of Ab2 and Ab3 with both **12** and **13**. These experiments thus unambiguously proof that Ab2 and Ab3 perform step-wise phenol-oxidative cross-coupling of **12** and **13** to yield ambigol A (**7**).



**Fig. 7** (A)–(C) HPLC-MS analysis at  $m/z = 322.5$ – $323.5$  of the product spectrum of Ab2 (A), Ab3 (B) and a combination of Ab2 and Ab3. (C) *In vitro* (black trace) and *in vivo* (brown trace) when simultaneously incubated with **12** and **13** as substrates compared to synthetic standards of **33** (green), **35** (blue) and **36** (olive). (D) *In vitro* assembly of ambigol A (**7**) from **12** and **35** catalyzed by Ab2 (black) compared to an authentic standard of **7** (red).

## Conclusions

Within this work, we set out to identify the biosynthetic machinery for the production of the polyhalogenated ambigols. A putative ambigol BGC *ab* was identified by sequencing of the natural producer *F. ambigua* 108b. Using the model cyanobacterial heterologous expression system *S. elongatus*, we firmly linked ambigol A (**7**) production to the identified *ab* BGC containing ten genes. To our knowledge, this study is the first example for a successful construction of *S. elongatus* double mutants by integrating in total more than 14 kb foreign DNA into the two neutral sites NSI and NSII.

To shed light on the biaryl coupling biochemistry involved in ambigol biosynthesis, the function of the two cytochrome P450 enzymes, Ab2 and Ab3, encoded in *ab* were probed both *in vivo* (in *E. coli* and *S. elongatus*) and *in vitro* using purified recombinant protein produced in *E. coli*. Using **12** as a substrate, all these experiments consistently revealed Ab2 to selectively form the *O,ortho*-coupled biaryl ether **36**, whereas Ab3 produces the *ortho,ortho*-linked, C–C-coupled biaryl **33**. The identity of these products was unambiguously established by comparison with all feasible coupling products **33**–**41** that were prepared by chemical synthesis. Given the structural simplicity of the joint precursor molecule 2,4-dichlorophenol (**12**), the exceptional site selectivity of Ab2 and Ab3 is highly remarkable and warrants future studies on the underlying structural basis of this selectivity in these biaryl coupling enzymes.



Key to fully uncover the biosynthetic assembly of **7** were *in vitro* experiments using the two bis-halogenated monomeric phenols **12** and **13** as substrates for Ab2 and Ab3. These experiments revealed the production of the mixed C–C-coupled **35**, a central precursor to **7**, particularly by Ab3. Incubation of **35** and **12** with Ab2 selectively delivered ambigol A (**7**), revealing the stepwise assembly of this polyhalogenated polyphenol by an interplay of the two cytochrome P450 enzymes Ab2 and Ab3 encoded in the ambigol BGC *ab*.

## Conflicts of interest

There are no conflicts to declare.

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