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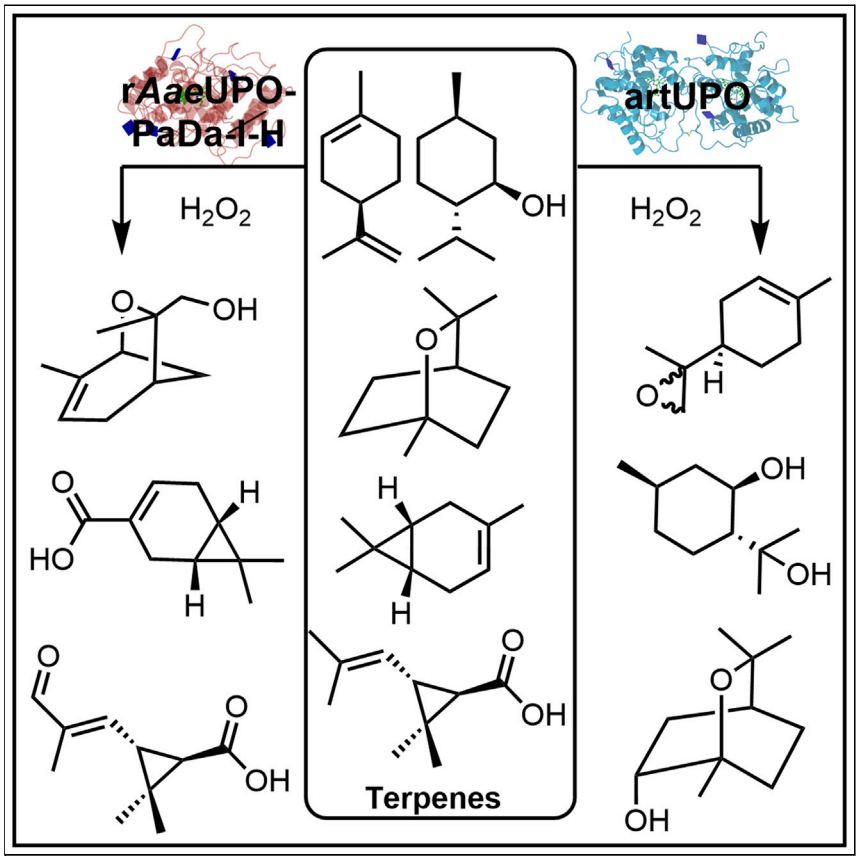
Complementary specificity of unspecific peroxygenases enables access to diverse products from terpene oxygenation



The selective oxygenation of terpenoids has been performed on a preparative scale using unspecific peroxygenase (UPO) enzymes. UPOs can be applied as easy-to-use lyophilized powders and can catalyze the oxygenation of carbon atoms at the expense of only hydrogen peroxide as the external oxidation. In some cases, UPOs are able to perform these reactions with excellent regio- and enantioselectivities. Hence, menthol was transformed exclusively to *p*-menthane-3,8-diol, and a racemic pyrethroid fragment underwent kinetic resolution through methyl hydroxylation, each reaction giving products on a preparative scale.

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Highlights

Unspecific peroxygenases (UPOs) have been applied to the transformations of terpenes

Menthol was selectively transformed to *p*-menthane-2,8-diol on a gram scale

The resolution of a racemic pyrethroid fragment was achieved on preparative scale

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Complementary specificity of unspecific peroxygenases enables access to diverse products from terpene oxygenation

Benjamin Melling,[1] Tamara Mielke,[1] Adrian C. Whitwood,[1] Timothy J.C. O'Riordan,[2] Nicholas Mulholland,[2] Jared Cartwright,[3] William P. Unsworth,[1,*] and Gideon Grogan[1,4,*]

SUMMARY

Unspecific peroxygenases (UPOs) have emerged as attractive biocatalysts for selective oxygenations because, unlike cytochromes P450, they can be employed as easy-to-use lyophilized powders and depend only upon hydrogen peroxide as the external oxidant. The application of UPOs to a range of synthetic challenges relies on the characterization of activity and specificity of complementary enzymes. Here, we show that two UPOs, artUPO and r*Aae*UPO-PaDa-I-H, which are representative members of the "short" family I and "long" family II UPOs, display complementary activity in a series of scalable, preparative biotransformations of a diverse array of terpenes. The UPOs were also applied to the biotransformation of chrysanthemic-acid-derived fragments relevant in the agrichemical industry, culminating in the highly diastereoselective and enantioselective oxidation of a racemic synthetic pyrethroid derivative via kinetic resolution.

INTRODUCTION

The selective oxygenation of unactivated carbon atoms under mild reaction conditions remains a major challenge in synthetic organic chemistry and was listed as one of the ten key green chemistry research areas by members of the ACS Green Chemistry Institute Pharmaceutical Roundtable in 2018.[1] The necessity for greener and more sustainable methods of oxidation has led many researchers to consider whole-cell or enzymatic biocatalysts for their processes,[2,3] as these offer advantages of selectivity, mild reaction conditions, and non-toxicity that align with the precepts of sustainable synthesis. By far, the most studied oxygenation biocatalysts are the heme-dependent cytochromes P450 (P450s),[4–6] which have been employed for decades within whole-cell systems for the industrial hydroxylation of steroids.[7] Despite these advances, the application of P450s is hampered by their requirements for whole microbes or, when used *in vitro*, their requirements for expensive, exogenous nicotinamide cofactors (NAD(P)H) and electron transport proteins (P450 reductase or ferredoxin reductase/ferredoxin systems). These are required for the delivery of electrons to the catalytic heme iron within the active site, where the iron reacts with molecular oxygen to form the catalytic iron (IV) oxo species (compound I) that is the active oxidant.[6] In 2004, Hofrichter and co-workers described the discovery of a new class of secreted fungal heme oxygenases, now known as "unspecific peroxygenases" (UPOs),[8] which displayed a catalytic repertoire similar to that of P450s but that were dependent upon only the addition of hydrogen peroxide to generate compound I.[9] As secreted enzymes, UPOs displayed high activities and stabilities that were amenable to their application to scalable oxygenation reactions. Major

THE BIGGER PICTURE

Selective C–H activation remains a major challenge in organic chemistry, and for years, enzymes such as cytochromes P450 have been targeted as possible solutions. However, the use of P450s presents its own challenges to the organic chemist, as either whole-cell microbes or complex enzyme systems are dependent upon multiple proteins and expensive cofactors. In this context, unspecific peroxygenases (UPOs) are a promising alternative, as they can be applied as easy-to-use lyophilized powders and are dependent only upon hydrogen peroxide to catalyze their oxygenation reactions.

In this study, we have expanded the application of UPO reactivity to a range of terpene substrates and showed that, with representative enzymes from two UPO families, we can effect complementary transformations of terpenoids to give different valuable products on preparative scales. The results provide further evidence that UPOs are credible catalysts for selective and scalable oxygenation reactions.

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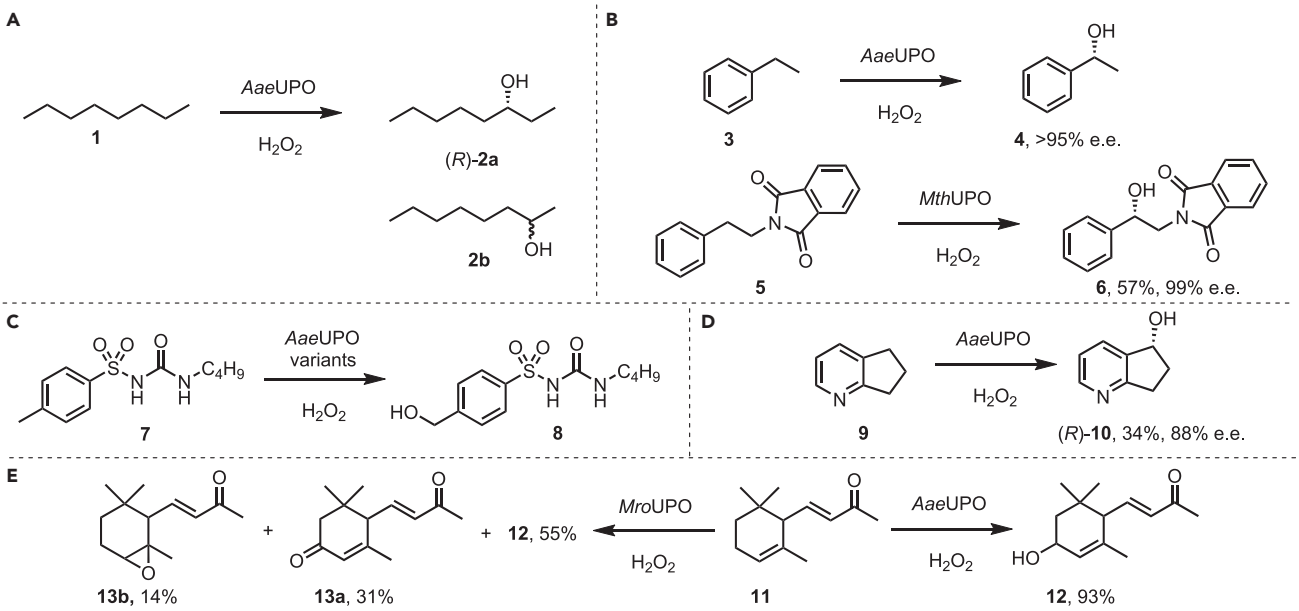

Scheme 1. Oxygenations by unspecific peroxygenases

*Aae*UPO, *Mth*UPO, and *Mro*UPO are the enzymes from *Agrocybe aegerita*, *Myceliophthora thermophilla*, and *Marasmius rotula*, respectively.

(A) UPO oxygenation of octane.

(B) Enantioselective UPO benzylic oxygenation.

(C) UPO oxygenation of pharmaceutical tolbutamide.

(D) Enantioselective UPO transformation of *N*-heterocycles.

(E) UPO transformations of α-ionone **11**.

advances in the heterologous expression of first the model enzyme from *Agrocybe aegerita* (*Aae*UPO), in *Pichia pastoris*,[10] meant that UPOs could also be produced in bulk in tractable microbial systems. In a recent paper, Hollmann and co-workers describe the production of 735 g of a variant of *Aae*UPO, PaDa-I, in a 2,500 L fermentor and the application of the enzyme to the scalable oxidation of ethylbenzene to give 5 g L-1 of the (*R*)-phenyl-1-ethanol product.[11] In addition, the 115-mol-scale oxidation of gaseous butane to butanol and butanone by *Aae*UPO has been reported.[12] Since the discovery of UPOs, they have now been applied to the oxygenation of a range of organic substrates, including alkanes such as octane **1** (Scheme 1A),[13] aromatics such as **3**[14] and **5**[15] (Scheme 1B), and pharmaceuticals such as tolbutamide **7** (Scheme 1C).[16] We have also recently applied a lyophilized preparation of the PaDa-I variant of *Aae*UPO to the selective oxygenation of a range of pharmaceutically relevant *N*-heterocycles, including **9**, in 100-mg-scale reactions (Scheme 1D).[17] Summaries of these applications can be found in recent reviews.[18–21]

Since the discovery of *Aae*UPO, many more putative UPO sequences have been identified in sequence databases, and two large distinct families have been identified.[22] The first of these, family I UPOs, typified by the enzyme from *Marasmius rotula*,[23] are comparatively short in length (molecular weight [MW] of approximately 25 kDa) and also include the examples *Cvi*UPO from *Collariella virescens*[24] and *Hsp*UPO from *Hypoxolon* sp. EC38.[25] Family II UPOs, typified by *Aae*UPO, are longer, by virtue of a long C-terminal helical segment that covers the entrance to the active site, and also include *Pab*UPO from *Psathyrella aberdarensis*.[15,26] Data on the comparative catalytic properties of these families are only just emerging, although on the whole, the family II enzymes appear to display greater peroxygenase activity.[25] Intriguingly, some of the family I enzymes can be expressed in

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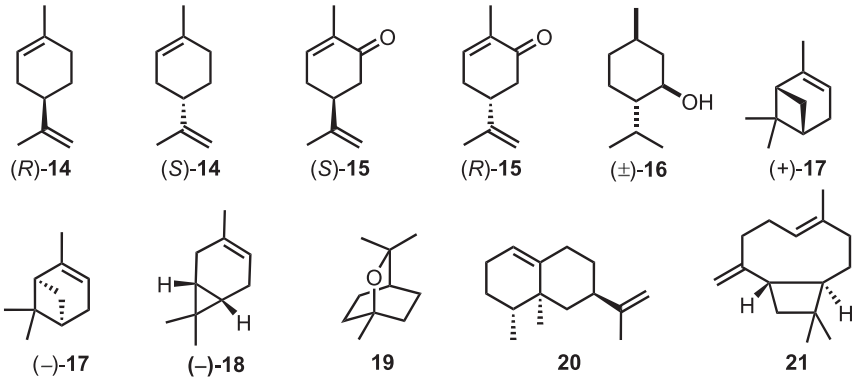

Figure 1. Terpene substrates selected from an initial screen

E. coli,[27,28] increasing their accessibility and also the speed at which expression and mutation can be enabled. The diversity of UPOs presents an extensive reservoir of possible activities that might be mined for selective oxygenation reactions.

In this article, we describe the complementary activity of a family I and family II UPO to the biotransformation of terpenes, an important class of hydrocarbons. The oxygenation products of terpenes include value-added chemicals in the flavor, fragrance, and also pesticide industries. In previous work, Gutiérrez and co-workers had shown that family I and family II UPOs displayed complementary activity toward the terpene compounds α- and β-damascone and α- and β-ionone.[29] For example, α-ionone **11** was mostly converted into the 3-hydroxy metabolite **12** by the family II *Aae*UPO but also into significant amounts of ketone **13a** and epoxide **13b** products by the family I enzyme from *Marasmius rotula* (*Mro*UPO) (Scheme 1E), although in that case, products were only characterized by gas chromatography-mass spectrometry (GC-MS) analysis. Larger-scale transformations of β-ionone were carried out during studies by Weissenborn and co-workers in which the UPO from *Myceliophthora thermophila* was evolved to give variants of both (*R*) and (*S*) selectivities for a 4-hydroxy ionone product.[30] These results prompted us to make a wider study of terpene biotransformations by family I and II UPOs, in 100-mg-scale reactions that permit the full spectroscopic analysis of products. The results reveal a stark difference in substrate specificity between UPO families that enables access to new products inaccessible using the more widely used *Aae*UPO and without the need for additional mutations.

RESULTS AND DISCUSSION

The work described in this article follows our study on the family I enzyme "artUPO," which is a 73% sequence homolog with *Mro*UPO and was described in a patent in 2016.[31] Our studies[32] showed that artUPO displayed complementary selectivity to the PaDa-I variant of *Aae*UPO, particularly with a model series of thioether substrates, giving the (*S*) series of sulfoxide products, compared to the (*R*) given by r*Aae*UPO-PaDa-I-H. We also showed that while artUPO could be expressed and purified from the bacterial host *E. coli* in an unglycosylated form, this enzyme was generally less robust in biotransformations than its glycosylated form expressed in *Pichia pastoris*. In this comparative study of terpene biotransformations, we have therefore employed the *Pichia*-expressed artUPO as a family I representative and compared this with the catalytic performance of the PaDa-I variant, expressed previously by us in an alternate construct[33] and named r*Aae*UPO-PaDa-I-H.

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The terpene substrates explored in this study are shown in Figure 1. This selection was made following an initial screen (10 mM substrate concentration, see supplemental information section 3 for full details) of a wider range of terpenes, with those exhibiting the most promising conversions (analyzed by GC) chosen for further study. The selection consisted of the separate enantiomers of limonene **14** and carvone **15**, racemic menthol **16**, and the separate enantiomers of α-pinene **17**, 3-carene **18**, eucalyptol (1,8-cineole) **19**, valencene **20**, and β-caryophyllene **21**. In addition to these preliminary screens, additional optimization reactions were performed on substrates **14–21** to probe the effect of different H_2O_2 loadings on the reaction outcomes; these results are also included in the supplemental information (section 4), with the general trend that increased H_2O_2 led to the formation of higher-oxidation-state products as expected, but tempered by reduced conversion at higher H_2O_2 loadings, likely as a result of oxidative deactivation of the enzyme. The data from these screens informed the synthetic-scale biotransformations in this study, using both *rAae*UPO-PaDa-I-H and artUPO in each instance, described in detail below for substrates **14–21** in turn.

Importantly, all of the preparative-scale biotransformations that follow were performed using >100 mg terpene, using conditions previously determined to be optimal for UPO-catalyzed conversions in our laboratory.[17] This means that even in cases in which the reaction produced complex mixtures of products, we were generally able to isolate analytically pure synthetic samples to enable the products to be identified and assigned reliably, using GC, nuclear magnetic resonance (NMR), and MS methods. Synthetic samples were also prepared by standard chemical synthetic methods to further support characterization of the products formed, with full synthetic procedures, spectroscopic data, and details of their structural assignments included in the supplemental information.

Limonene, carvone, and menthol

The first substrate examined was the *p*-menthane monoterpene limonene, which has been the subject of many previous biotransformation studies.[34] (+)-(*R*)-Limonene (***R*)-14**, for example, can be transformed into (+)-*trans*-piperitenol, a direct precursor of menthol, by a limonene-3-hydroxylase from *Aureobasidium pullulans*.[35] (−)-(*S*)-Limonene (***S*)-14** has also been converted into (−)-(*R*)-carvone by a two-step enzymatic system of a limonene-6-hydroxylase and a carveol dehydrogenase (CDH) expressed in *E. coli*.[36] In this study, preliminary screens indicated that both UPOs can transform limonene into various oxidized products, and this was well illustrated by the preparative-scale reactions (Scheme 2A). Both limonene enantiomers (***R*)-14** and (***S*)-14** were transformed efficiently by artUPO (97% and 80% conversion, respectively) via epoxidation of both alkene groups, each giving a mixture of mono- and bis-epoxides **22a/b** and **23a/b**. Products **22a/b** and **23a/b** were all formed as mixtures of diastereoisomers, indicating that the enzyme imparts little stereocontrol in this case. The product mixtures obtained using r*Aae*UPO-PaDa-I-H were more complex in contrast; epoxidation was again observed, with epoxide products **22**, **23**, and **26** all identified, alongside hydroxylated products (**24a/b**) and (−)-bottrospicatol **25**, which presumably formed via the rearrangement of epoxide **24a**. Another key difference in the reaction outcome using r*Aae*UPO-PaDa-I-H was in the stereoselectivity; in contrast to the artUPO reactions, which showed very little stereoselectivity, all of the products identified using r*Aae*UPO-PaDa-I-H were formed either as single diastereoisomers (e.g., **24a**, **25**) or were highly enriched as one diastereoisomer (e.g., **23a**). Interestingly, the same absolute stereoinduction was seen when using both (***R*)-14** and (***S*)-14** as substrates, suggesting that the stereoselectivity is governed purely by the enzyme and not by limonene's existing stereogenic center.

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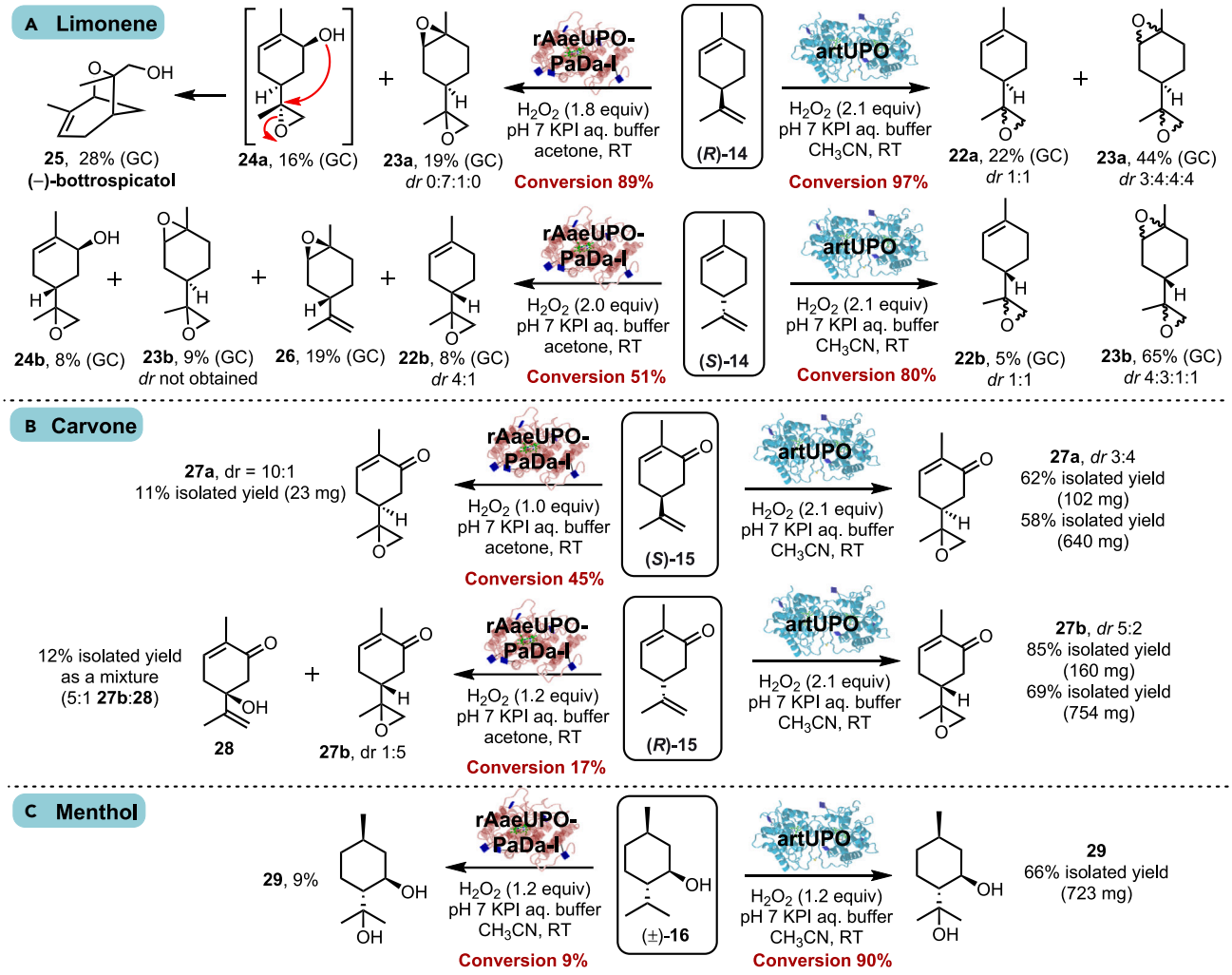

Scheme 2. Biotransformations of *p*-menthane monoterpenes limonene, carvone, and menthol

These results contrast with recent observations made by Li and co-workers in which the PaDa-I mutant of *Aae*UPO was reported to largely produce carveol and carvone in a cascade system with choline oxidase for the production of peroxide.[37]

Carvone **15** has also been well studied as a whole-cell biotransformation substrate, with the presence of C=C and C=O bonds meaning that reduction products have predominated,[38] although both hydroxylation[39] and epoxidation followed by epoxide hydrolysis[40] have been observed on the propene side chain. In this study, we found that r*Aae*UPO-PaDa-I-H is able to partially convert both carvone enantiomers (*S*)-**15** and (*R*)-**15**, predominantly via epoxidation of the more electron-rich alkene to form **27a/b**, albeit in low yield (Scheme 2B). Pleasingly, artUPO was a much more effective catalyst both in terms of conversion and selectivity, allowing analytically pure samples of **27a/b** to be synthesized in good yields (58% and 69%) on a gram scale. As for limonene, both enantiomers of starting material were converted to similar extents.

Menthol provides a different test for the UPOs, as it lacks alkene groups to undergo epoxidation. The biotransformation of (–)-menthol (–)-**16** by whole cells of

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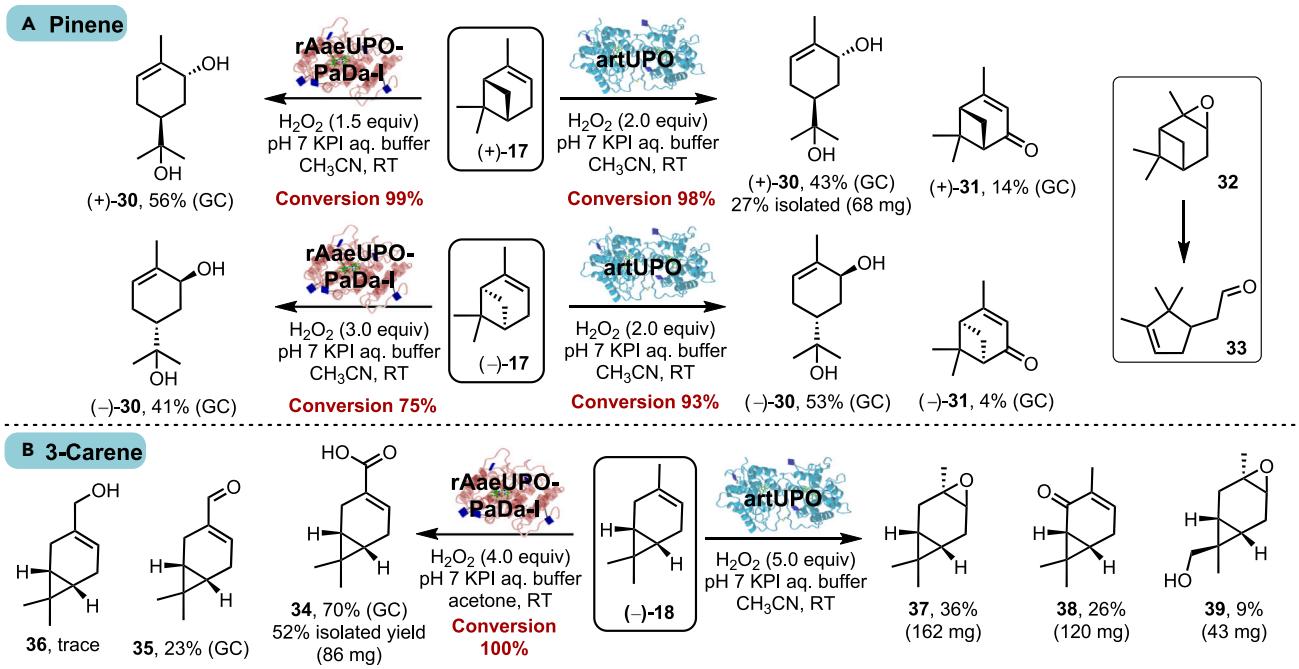

Scheme 3. Biotransformations of pinene and 3-carene

Rhizoctonia solani has been reported, with this biocatalyst leading to the formation of a mixture of products hydroxylated in the 1, 8, and 6 positions.[41] In this study we tested UPO reactivity on racemic menthol (±)-**16**, which was converted poorly by r*Aae*UPO-PaDa-I-H, giving only 9% conversion to the tertiary alcohol product **29** (Scheme 2C). In contrast, artUPO converted the same substrate far more effectively, allowing **29** to be obtained in 66% isolated yield on a gram scale, with complete regioselectivity for hydroxylation of the menthol 8 position. *p*-Menthane-3,8-diol **29** is a high-value compound, as it is an active constituent of insect repellents[42] and has been chemically synthesized from citronellal using sulfuric acid.[43]

Pinene and 3-carene

Pinene **17** and (*S*)-3-carene **18** are the major constituents of turpentine from pine resin.[44] The biotransformation of pinene to useful hydroxylated metabolites has been of interest since as early as 1960, when Bhattachyra and co-workers showed that this substrate could be converted to products including *cis*-verbenol and verbenone using whole-cell preparations of fungi.[45] More recently, Siddhardha and co-workers showed that the enantiomers of α-pinene were converted to α-terpineol and isoterpineol by cells of *Absidia corulea*.[46] In this study, both UPOs were challenged with the separate enantiomers of pinene (+)-**17** and (–)-**17** (Scheme 3A). In this series, the major product was the same for both UPOs, with *trans*-sobrerol **30** formed as the major product (product identity confirmed by X-ray crystallography, CCDC: 2283917). Broadly, the same outcome was observed when using either enantiomer of pinene, with the same selectivity for the *trans*-diastereoisomer in each enantiomeric series. These reactions were complicated by the formation of additional side products also; for example, a small amount of ketone **31** was observed in the artUPO reactions. The formation of *trans*-sobrerol **30** presumably proceeds via the initial formation of α-pinene oxide **32**, followed by a known rearrangement.[47] Quantifying the amount of α-pinene oxide **32** formed in the biotransformation was not easy due to its proclivity to rearrange (both in the reaction and during purification) into **30** and other rearranged products such as campholenic

aldehyde **33**, which was also observed during chromatographic purification (see supplemental information).

(−)-3-Carene (−)-**18** has also been the subject of a number of biotransformation studies, including those of Vanek and co-workers, who identified (1*S*,3*S*,4*R*)-3,4-epoxycarane and (1*R*)-*p*-mentha-1(7),2-dien-8-ol as the most rapidly produced metabolites using whole cells of *Picea abies*.[48] In this study, transformation of (−)-**18** with r*Aae*UPO-PaDa-I-H resulted in the exclusive oxidation of the carene 10 position, with chaminic acid **34**, caren-10-al **35**, and caren-10-al **36** all formed (Scheme 3B). The ratio of these products is, unsurprisingly, influenced by the number of equiv H_2O_2 used in the biotransformation (see supplemental information), with our best preparative synthetic example illustrated in Scheme 3A; in this case, analytically pure chaminic acid **34** was obtained in 52% isolated yield. Notably, chaminic acid **34** was the exclusive product of microbial biotransformation of **20** by *Mycobacterium smegmatis* DSM 43061.[49] In contrast, artUPO gave a completely different reaction outcome; no products arising from the oxidation of the 10 position of carene were observed. Rather, a mixture of products arising from diastereoselective epoxidation to form *trans* **37**, oxidation of the carene 5-position to form enone **38**, and 8-methyl oxygenation to form epoxy alcohol **39** was obtained. This artUPO reaction worked well on a preparative scale, with all three products **37**–**39** isolated in reasonable yield from a single biotransformation.

Eucalyptol, valencene, and β-caryophyllene

Eucalyptol (also known as 1,8-cineole) **19** is one of the major constituents of eucalyptus oil and has also been targeted by other groups for selective biooxygenation. In recent examples, Omarini and co-workers identified both 1,3,3-trimethyl-2-oxabicyclo [2.2.2] octan-6-ol and 1,3,3-trimethyl-2-oxabicyclo [2.2.2] octan-6-one as the major products from incubation with whole cells of the fungal species *Pleurotus ostreatus* and *Favolus tenuiculus*.[50] Additionally, strains of bacteria such as *Rhodococcus* sp. C1 have been shown to metabolize the substrate via 6-*endo*-hydroxycineole and 6-oxocineole.[51] Interestingly, incubation of **19** with r*Aae*UPO-PaDa-I-H gave no substantial oxidation products. However, incubation with artUPO afforded a ≈2.5:1 mixture of regioisomeric hydroxylated products *endo*-2-hydroxycineole (−)-**40** and 5-*endo*-hydroxycineole **41** in 67% overall isolated yield on a preparative scale (Scheme 4A). As eucalyptol is a *meso* compound, its hydroxylation offers an interesting selectivity challenge, with regio-, diastereo-, and enantioselectivities all being considerations. In this case, the regioselectivity was moderate—this is reflected by the 2.5:1 ratio of (−)-**40**:**41**. Diastereoselectivity was high, evident in the fact that both products were formed exclusively as *endo*-isomers, while enantioselectivity was also high, with (−)-**40** obtained in 90% *ee*, with the absolute stereochemistry of the major isomer depicted in Scheme 4A. The structural assignments of products (−)-**40** and **41** were confirmed by comparison to literature data,[52] and the identity of (−)-**40** was further supported by X-ray crystallographic data (CCDC: 2283913).

The sesquiterpene valencene (+)-**20** has long been a target for biooxidation, as its allylic hydroxylation product is the direct precursor to the grapefruit flavor compound nootkatone.[53] Indeed, numerous biocatalytic routes to nootkatone are reported, many of which involve a P450-catalyzed hydroxylation step. For example, the F87A/Y96F mutant of P450cam gave a 70% mix of *trans*-nootkatol and 12% nootkatone.[54] As with eucalyptol, r*Aae*UPO-PaDa-I-H did not transform valencene (+)-**20** effectively; epoxide **42** was the only product isolated from a preparative-scale biotransformation, in just 5% yield. However, more promisingly, artUPO was able

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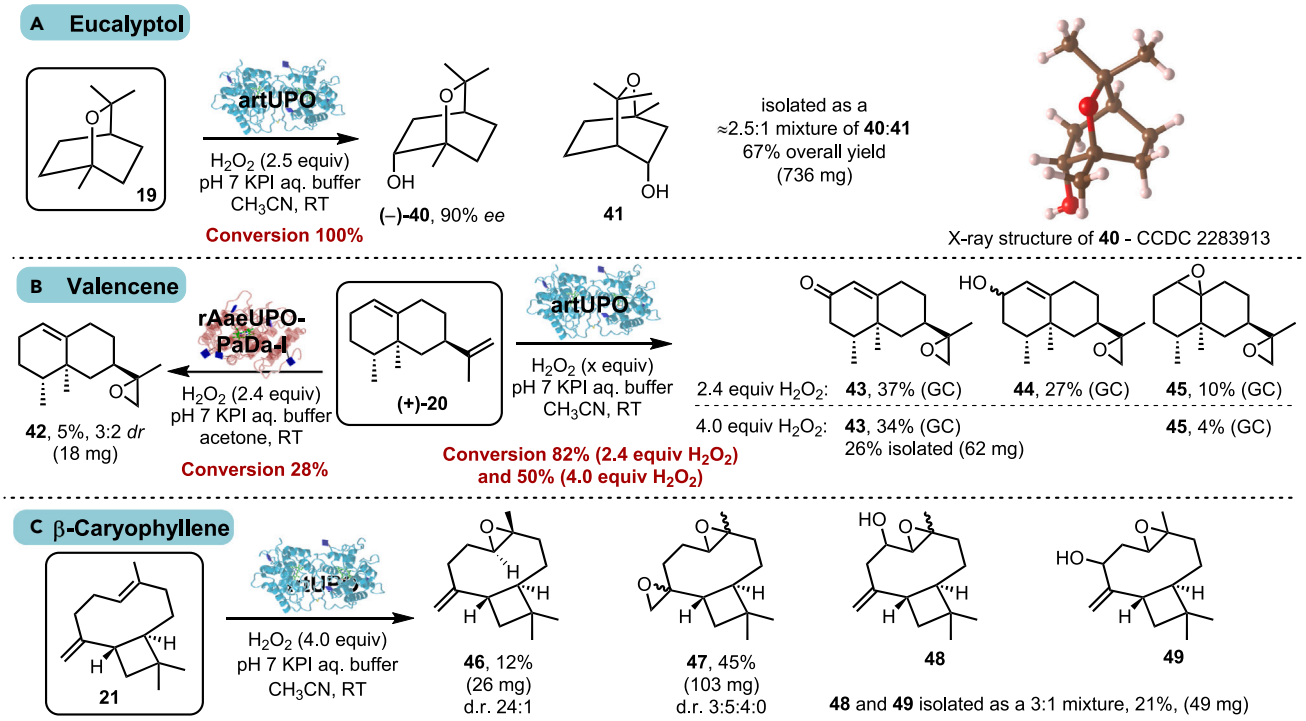

Scheme 4. Biotransformations of eucalyptol, valencene, and β-caryophyllene

to effect 82% conversion of valencene (+)-**20** when using 2.4 equiv H_2O_2, with nootkatone 13,14-epoxide **43** being the major product, alongside nootkatol 13,14-epoxide **44** and bis-epoxide **45**. The major product **43** is a metabolite that was also produced by certain mutants of P450 BM3 in experiments by Wong and coworkers.[54] In an attempt to improve conversion into **43**, the same reaction was tried using 4 equiv H_2O_2, but while this preparative-scale reaction permitted the isolation of **43**, the increased H_2O_2 loading led to an overall drop in conversion.

Attention then turned to β-caryophyllene **21**, which is a major constituent of the essential oil of many plant products, including those of cloves and cannabis.[55] Several biotransformation products of this sesquiterpene have been reported including a number of oxygenated products using the fungus *Diplodia gossypina*,[56] many of which feature the epoxidation of the C4-C5 double bond to give caryophyllene oxide. Again, r*Aae*UPO-PaDa-I-H did not transform **21** to a significant degree, indicative of an emerging pattern in which this enzyme does not work well with larger substrates. However, artUPO performed much better; bis-epoxide **47** was the major product isolated (45% yield), alongside the known epoxide **46**, which was in 12% yield, as well as the hydroxylated epoxide isomers **48** and **49**, which were obtained in 21% combined yield (Scheme 4).

The contrasting substrate specificities of artUPO and r*Aae*UPO-PaDa-I-H for terpene substrates **14**–**21** can be summarized and contrasted. Both enzymes are effective catalysts for the transformation of terpene compounds, but with the *p*-menthane substrates limonene and carvone, the transformations are not so selective when compared to some P450 catalysts, which have been evolved to impart absolute regio- and enantioselectivities with specific substrates. The transformations of racemic menthol (±)-**16**, however, are revealing in that both UPOs furnish the production of

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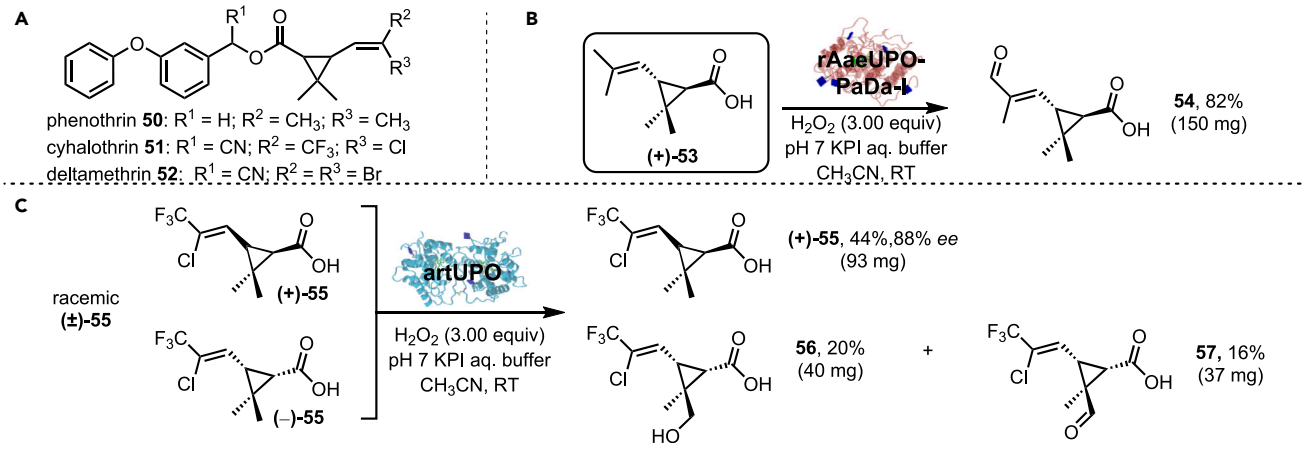

Scheme 5. Biotransformations of pyrethroids

the tertiary alcohol product **29**. A key difference in selectivity between the enzymes is illustrated by their transformation of (−)-3-carene **18**, as r*Aae*UPO-PaDa-I-H is regioselective in giving only products of the oxidation at the allylic methyl group, whereas artUPO attacks multiple carbon centers. Using our structures of r*Aae*UPO-PaDa-I-H (PDB: 8AV5) and artUPO (PDB: 7ZNM),[32] we have docked (−)-**18** into their active sites using Autodock Vina (see supplemental information and Figures S26A–S26C).[57] Three out of five of the lowest-energy poses for the first enzyme clearly show the ligand presenting the experimentally oxygenated methyl group to the heme, with the conformation of the ligand constrained by the narrower active site of r*Aae*UPO-PaDa-I-H, especially by the side chains of F69, F76, F121, and F199 (Figure S26A). Contrastingly, a mixture of poses was obtained for artUPO with (−)-**18**, presenting, for example, either the *trans*-methyl of the dimethyl group that gives product **39** (Figure S26B) or the other side of the molecule (Figure S26C), giving products such as **37** and **38** at similar energies. This lack of regioselectivity is congruent with a less-restricted active site with smaller active-site residues, such as L65, I91, and I160 substituting for some of the Phe residues in r*Aae*UPO-PaDa-I-H. The more constrained active site of r*Aae*UPO-PaDa-I-H is also presumably the reason for its failure to effectively transform the larger terpene substrates such as **19** and **21**. These differences have already been highlighted in an explanation of the superior enantioselectivity of r*Aae*UPO-PaDa-I-H over artUPO with respect to sulfoxidation reactions.[31]

Biotransformation of pyrethroids

To further showcase the potential of the enzymes to impact industrially relevant molecules, we set out to examine their efficacy at transforming pyrethroid targets, terpene-derived molecules that are important in the agrochemical industry. Synthetic pyrethroids that feature the carbon skeleton of the monoterpene acid chrysanthemic acid **53**, including phenothrin **50**, cyhalothrin **51**, and deltamethrin **52** (Scheme 5A), have assumed a central role in agriculture with applications such as pesticides, and as such, their metabolism by model systems has been the subject of much interest.[58] In terms of microbial biotransformation, strains of *Bacillus*, *Pseudomonas*, and others have previously been shown to metabolize this class of pyrethroid through the hydrolysis of the ester bond,[59] although enzymatic mechanisms for the subsequent degradation of the monoterpene acid moiety are less well described. We were therefore interested to assess the oxygenation capabilities of UPOs with respect to the chrysanthemic acid moieties of these molecules.

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We began by examining transformations of (+)-chrysanthemic acid (+)-**53** and found that this substrate was converted very well by r*Aae*UPO-PaDa-I-H, with aldehyde **54** isolated in high yield on a preparative scale. Notably, with four methyl groups present in (+)-**53**, only one was oxygenated to an appreciable extent, leading to the selective formation of the (*E*)-enal product **54**. In contrast, artUPO did not transform (+)-**53** effectively, affording a mixture of products in low conversion (data not shown; see supplemental information). Even more remarkable were the results obtained using (±)-**55**, a fragment of the commercial pesticide cyhalothrin **51**. In this case, racemic (±)-**55** was used as the substrate with the intention of exploring whether kinetic resolution of the enantiomers might be possible alongside selective oxygenation. r*Aae*UPO-PaDa-I-H was tested first but did not transform (±)-**55**; this result was somewhat expected given the previously observed trend that this enzyme does not generally accept larger substrates. In contrast, artUPO was much more effective; despite the reaction being performed using sufficient oxidant to transform all of the substrate (3 equiv H_2O_2 was used), a very efficient kinetic resolution was observed, with ≈50% (±)-**55** being consumed and highly enantioenriched (88% *ee*) unreacted (+)-**55** being isolated from the reaction in 44% isolated yield out of a theoretical maximum of 50%. Thus, the enzyme oxygenates the (−)-**55** enantiomer with high selectivity. Furthermore, the oxygenation is also highly diastereoselective, with two oxidized products formed (alcohol **56** and aldehyde **57**), both arising from selective oxygenation of the *trans*-methyl group. The *ee*s of **56** and **57** were not measured directly, as racemic standards were not available, but given that both must derive predominantly from (−)-**55**, their *ee*s presumably will be high and broadly mirror those of the recovered (+)-**55**.

Conclusion

The selective oxidations of terpenes can yield valuable oxygenated products that have complementary or improved bioactivities compared to the starting materials. These oxidations can also provide clues and analytical standards for studies of the metabolic disposition of the molecules in the environment. We have shown that the application of even only two UPOs can offer a range of complementary selectivities in these oxygenation reactions, with family I and II UPOs displaying different substrate specificities and regio- and enantioselective chemistries. Moreover, these reactions can be performed at significant scale in an organic synthesis laboratory, without the use of microbes, cofactors, or the electron transport proteins that are often required for P450-mediated biotransformations. The study culminates in the diastereoselective and enantioselective kinetic resolution of racemic pyrethroid (±)-**55**, an important compound in agrochemistry, well demonstrating the power of these UPOs to transform industrially relevant targets.

EXPERIMENTAL PROCEDURES

Resource availability

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Gideon Grogan (gideon.grogan@york.ac.uk).

Materials availability

All materials generated in this study are available from the lead contact without restriction upon reasonable request.

Data and code availability

All data supporting this study are available in the manuscript or supplemental information. CCDC: 2283917 (**30**) and 2283913 (**40**) contain the crystallographic data (see www.ccdc.cam.ac.uk/data_request/cif).

Experimental procedures are included in the supplemental information and include details of materials and general methods, details on production of UPOs, details of small-scale screening of UPO reactivity with terpenes, details of the H_2O_2 loading study, synthetic procedures and characterization data for substrate standards and biotransformations, ^{1}H and ^{13}C NMR spectra images, and details on modeling studies comparing UPO active sites.

Representative synthetic procedures for biocatalysis

Representative biotransformation using artUPO

Liquid artUPO expression secretate (5.4 mg mL^{-1}, 10.00 mL) was added to a reaction mixture consisting of KPi buffer (100 mM, 125 mL, pH = 7.0) and deionized water (16 mL) in a 500 mL round-bottom flask. (±)-Menthol (±)-**16** (6.40 mmol, 1.00 g) was dissolved in acetonitrile (25 mL) and added to the reaction. The reaction was initiated by the slow, continuous addition of an aqueous H_2O_2 solution (7.50 mmol, 3% w/v, 10 mL) added over 5 h via a syringe pump at room temperature (RT). The reaction was extracted with ethyl acetate (2 × 250 mL, 1 × 200 mL), and the combined organic extracts were washed with saturated brine (2 × 250 mL), dried over $MgSO_4$, and filtered. The solvent was removed *in vacuo*, and the crude material was purified via column chromatography (eluent: hexane/ethyl acetate = 3:7) to afford pure (±)-*trans*-*para*-menthane-3-8-diol **29** (723 mg, 66%) as a white solid.

Representative biotransformation using rAaeUPO-PaDa-I-H

Lyophilized r*Aae*UPO-PaDa-I-H expression secretate (0.01 U mg^{-1}, 3.87 g) was dissolved in a reaction mixture consisting of KPi buffer (100 mM, 50 mL, pH = 7.0) and deionized water (30 mL) in a 250 mL round-bottom flask. (+)-*trans*-Chrysanthemic acid (+)-**53** (1.00 mmol, 0.153 mL) was dissolved in acetonitrile (20 mL) and then added to the reaction. The reaction was initiated by the continuous addition of an aqueous H_2O_2 solution (3.00 mmol, 1.7% w/v, 5 mL) added over 9.8 h via a syringe pump at RT; after the H_2O_2 addition, the reaction was left to stir for 12 h. The reaction was acidified to pH ca 1.0 via dropwise addition of HCl (aq). The reaction was extracted with diethyl ether (3 × 130 mL), and the combined organic extracts were washed with saturated brine (200 mL), dried over $MgSO_4$, and filtered, and the solvent was removed *in vacuo*. The crude material was purified via column chromatography (eluent: diethyl ether/n-hexane = 7:3 with 1% acetic acid) to afford carboxylic acid **54** (150 mg, 82% yield) as a white solid.

Full details of all synthetic and biosynthetic transformations in this manuscript are included in the supplemental information.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.checat.2023.100889.

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AUTHOR CONTRIBUTIONS

G.G., W.P.U., N.M., T.J.C.O'R., and J.C designed experiments. B.M. and T.M. performed experiments. A.C.W. collected, processed and analyzed the X-ray crystallographic data. All authors contributed to the writing of the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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