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**We report a new chemoenzymatic cascade starting with aldehyde synthesis by carboxylic acid reductase (CAR) followed by chemical *in situ* oxime formation. The final step to the nitrile is catalyzed by aldoxime dehydratase (Oxd). Full conversions of phenylacetic acid and hexanoic acid were achieved in a two-phase mode.**

The nitrile functionality is versatile.<sup>1</sup> Nitriles serve as building blocks for a range of chemical transformations *e.g.* hydrolysis to acids, hydration to amides, reduction to amines, cyclization to N-heterocyclic compounds and many others. In conventional organic synthesis, nitriles may be synthesized by addition of cyanide or substitution of leaving groups by cyanide. Other applied reactions use transition metal-catalyzed cyanation and electrophilic cyanide transfer and involve highly toxic cyanide.<sup>2</sup> The carbon chain is extended by one C atom in these reactions (Scheme 1A).<sup>3</sup> Avoiding the use of cyanide in nitrile synthesis is an important step in replacing hazardous and highly toxic chemicals. Cyanide-free procedures for nitrile syntheses from aldehydes, amines, azides, oximes, halides and arenes have therefore been established.<sup>2,4</sup>

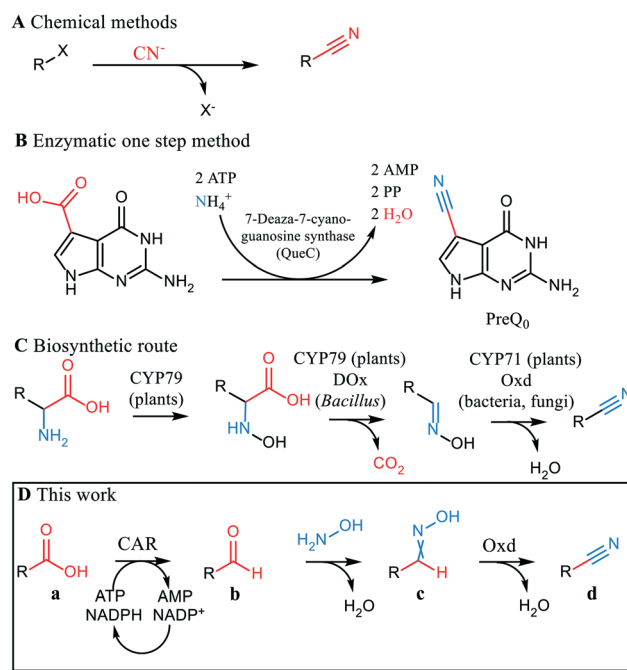
Carboxylic acids are abundant in biomass and ways to replace current methods that start from fossil precursors are sought after. A classical route from carboxylic acids to nitriles consists of acid activation followed by amide formation and chemical dehydration with phosphoric acid at high temperature. These conditions are only compatible with simple, unfunctionalized molecules, and not appropriate *e.g.*

## Chemoenzymatic one-pot reaction from carboxylic acid to nitrile *via* oxime†

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for late stage modifications. Recently, a single step reaction from acid to nitrile was reported that involves light-driven decarboxylation and cyanation of an acid in the presence of stoichiometric amounts of cyanobenziodoxolones. CO<sub>2</sub> is eliminated and cyanide introduced in this synthetic sequence.<sup>5</sup>

The direct conversion of a carboxylic acid to the respective nitrile was described in the biosynthesis pathway of deazapurine-containing compounds such as the



**Scheme 1** Routes from acid to nitrile. A: Substitution of leaving group by cyanide. Carbon chain is extended; B: acid carbon becomes nitrile carbon. Carbon chain length is retained; C: acid carbon is released as CO<sub>2</sub>. Carbon chain length is reduced; CYP79 = cytochrome P450 (CYP79 family), CYP71 = cytochrome P450 (CYP71 family), DOx = decarboxylase/oxidase, Oxd = aldoxime dehydratase; D: chemoenzymatic route. CAR = carboxylic acid reductase. Carbon chain length is retained.

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hypermodified tRNA bases queuosine<sup>6</sup> and archaeosine<sup>7</sup> as well as the nucleoside antibiotics toyocamycin<sup>8</sup> and sangivamycin.<sup>9</sup> The reaction is catalyzed by 7-cyano-7-deazaguanine synthase (EC 6.3.4.20, QueC), which activates the carboxylate at the expense of ATP. Ammonia acts as a nucleophile,<sup>9</sup> and an intermediate amide reacts with a second ATP to give the nitrile.<sup>10</sup> The conceptual elegance of this enzymatic reaction cannot be exploited for synthesis, because QueC synthases are strictly specific for their natural substrate<sup>11</sup> (Scheme 1B). Of biogenic nitriles other than the deazanucleoside preQ<sub>0</sub>, phenylacetonitrile is derived from amino acids in the bacterium *Bacillus* sp. OxB-1 through a decarboxylase/oxidase (DOx) – aldoxime dehydratase (Oxd) pathway (Scheme 1C).<sup>12</sup> In this case, CO<sub>2</sub> is released, and the carbon chain of the substrate shortened. The Oxd (EC 4.99.1.5-7) in this reaction sequence is a heme-protein that catalyzes oxime dehydration. Oxd's were found in the bacteria *Rhodococcus*, *Pseudomonas*, *Bradyrhizobium* and in fungi (*Fusarium*, *Sclerotinia*)<sup>13–15</sup> and have been studied as catalysts for mild and sustainable nitrile synthesis.<sup>16,17</sup> Their biocatalytic potential lies in their ability to dehydrate oximes in an aqueous medium and thus produce nitriles in a cyanide-free fashion. Oxd's were shown to react with aliphatic and (chiral) aryl aliphatic oximes,<sup>18,19</sup> oximes attached to alicycles,<sup>20</sup> dioximes<sup>17</sup> and benzisoxazoles<sup>21</sup> (*de facto* circular oximes) in a Kemp-type elimination.<sup>22</sup> However, little or no activity of Oxd's were found for substrates with the oxime moiety directly attached to a (hetero)aromatic ring.<sup>13</sup>

We report here a new artificial pathway from acid to nitrile (Scheme 1D), in which the carbon atom of the carboxylic acid eventually becomes the nitrile carbon and thus, the carbon chain length is retained. The proposed cascade includes two enzymatic steps and one chemical reaction. In the first step, a carboxylic acid reductase (EC 1.2.1.30, CAR) reduces the carboxylate to the respective aldehyde. In the intermediate chemical step, the aldehyde is trapped by oximation with hydroxylamine. The resultant aldoxime finally undergoes enzymatic dehydration mediated by Oxd (Scheme 1D).

The first step in the envisaged cascade reaction was a CAR-mediated acid reduction to the respective aldehyde in a whole cell system. In this setup, oxygen is required for cell viability and constant ATP formation, the cofactor needed for carboxylate activation. The presence of hydroxylamine in the reaction medium should lead to chemical scavenging of the aldehyde as an aldoxime, the substrate for the second enzymatic (dehydration) step. The majority of Oxd's were highly active only under anaerobic conditions, which maintained the catalytically essential ferrous state of the heme. The enzyme OxdBr1 from *Bradyrhizobium* sp. LTSPM299 is an exception, since it does not require strictly anaerobic conditions.<sup>15</sup> This fact was important in light of the oxygen requirement of the CAR-containing living cell biocatalyst and rendered OxdBr1 a promising candidate for the cascade.

In contrast to CARs with their exceptionally broad substrate tolerance,<sup>25</sup> little was known about OxdBr1

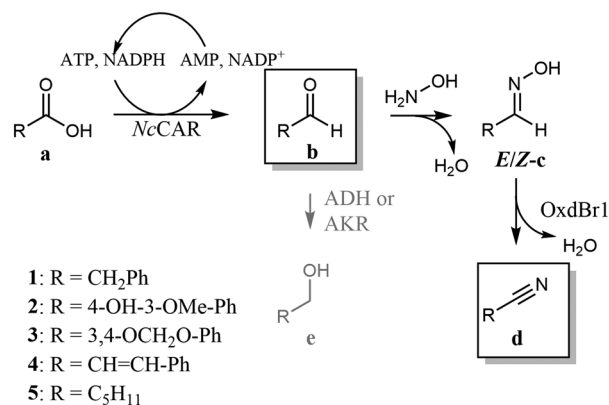
activities for substrates other than (aryl)aliphatic aldoximes. Therefore, we began the development of the reaction setup by searching for compounds compatible with both biocatalysts. Whole-cell reactions mediated by OxdBr1 with structurally diverse *E/Z*-aldoximes achieved the highest conversions for phenylacetaldehyde oxime **1c** and hexanal oxime **5c**, followed by medium conversions of cinnamaldehyde oxime **4c** and vanillin oxime **2c** as analogues of typical CAR products (Table 1).<sup>25</sup> A low conversion was found for piperonal oxime **3c**. The same trend was observed when OxdBr1 was applied as partly purified enzyme (ESI,† Fig. S1A and B). Whereas oximes **1c** and **5c** are usually well accepted by other Oxd's,<sup>13</sup> oximes **2c**, **3c**, **4c** have not yet been explored, which broadens the range of potential Oxd's uses. In this work, the best substrates **1a** and **5a** were selected to examine the proposed pathway. The CAR from *Neurospora crassa* is one of the most active CARs reported for **5a** reduction (Scheme 2),<sup>26,27</sup> and was selected for the whole cell-mediated aldehyde synthesis step of the cascade.

The metabolic activity of CAR-expressing *E. coli* cells needs to be high to ensure efficient ATP and NADPH supply. Therefore, the cytotoxic aldehyde should be eliminated using, *e.g.*, a follow-up reaction<sup>28–30</sup> or *in situ* product removal (ISPR).<sup>26,31</sup> In this work, the aldehyde is removed by follow-up reaction as an oxime. However, in solely aqueous systems, aliphatic and highly volatile compounds were particularly challenging to handle in terms of mass balance (see Table 1, entry **5c**). Therefore, we took advantage of ISPR as additional

**Table 1** Substrate scope of aldoxime dehydratase OxdBr1

|                       | Substrate                | Conv. <sup>a</sup> [%] | Anal. yield of nitrile <b>d</b> [mM] |
|-----------------------|--------------------------|------------------------|--------------------------------------|
| <b>1c<sup>b</sup></b> | Phenylacetaldehyde oxime | 93.2 ± 1.5             | 9.40 ± 0.16                          |
| <b>2c</b>             | Vanillin oxime           | 25.7 ± 1.5             | 1.28 ± 0.08                          |
| <b>3c<sup>b</sup></b> | Piperonal oxime          | 1.9 ± 0.3              | 0.11 ± 0.02                          |
| <b>4c<sup>b</sup></b> | Cinnamaldehyde oxime     | 32.3 ± 4.9             | 2.66 ± 0.41                          |
| <b>5c<sup>b</sup></b> | Hexanal oxime            | 100.0 ± 2.7            | 5.89 ± 0.17                          |

<sup>a</sup> Single-phase whole cell reaction, OD<sub>10</sub>, HEPES pH 7.5, 1 h, 28 °C, 10 mM of **c**. <sup>b</sup> Synthesized according to literature as described in ESI.†<sup>23,24</sup>



**Scheme 2** Chemoenzymatic cascade from carboxylic acid to nitrile. Compounds in frames are predominantly present in the organic phase when the reaction is performed in two-phase mode.



means to improve product recovery. We previously reported that CAR biocatalysts retain their activity, when exposed to water-immiscible organic solvents like *n*-heptane or biocompatible *n*-hexadecane for ISPR,<sup>26,27</sup> but little was known about co-solvent effects of these particular solvents on Oxd. Previous studies with different Oxds reported on (di) nitrile products as second layer to achieve exceptional product titers in oxime dehydration reactions,<sup>32</sup> which hints towards organic solvent tolerance of Oxds.<sup>32</sup> To verify this assumption, immiscible organic solvents previously selected for aldehyde production<sup>26</sup> were examined as media in the oxime dehydration step. At the same time, the robustness of OxdBr1 towards oxygen was assessed because the reactions were carried out in tubes with a large headspace of air. Full conversion of **5c** was observed under these conditions, indicating no significant inhibition by oxygen. Small amounts of nitrile in control reactions were the result of thermal dehydration of the oxime in the GC injector. These did not occur when reactions were analyzed by HPLC. Mass balance was greatly enhanced when *n*-heptane or *n*-hexadecane were applied for the formation of highly volatile **5d** (Fig. 1). The use of nitrile products as second phase would constitute the most elegant solvent. While this is feasible for Oxd reactions, it is expected to be problematic for the carboxylic acid reduction step because CARs are inhibited by **1d** (ESI,† Fig. S3).

Scheme 2 summarizes the chemoenzymatic cascade reaction from acid to nitrile. Having established an overlapping substrate profile and explored the presence of oxygen and organic solvents, we considered further critical reaction parameters, like pH value and hydroxylamine concentration. The rate of oxime formation is particularly high at very acidic or basic conditions and hydroxylamine in

excess (50-fold and more).<sup>33</sup> However, these conditions might be critical 1) in view of cell viability and 2) in view of inhibition phenomena.

The pH of *in situ* oxime formation is predetermined by the living cell catalyst's preference. Aldehyde formation by CAR enzymes has been described in a broad pH range. We opted for pH 6.5, because living *E. coli* cells can cope with a pH of 6.5<sup>27</sup> and hydroxylamine was reported to react with aldehydes and ketones under these conditions to form aldoximes and ketoximes, respectively.<sup>34</sup>

High hydroxylamine concentrations to push aldehyde conversion to oximes were expected to be problematic.<sup>35–39</sup> We used a moderate molar excess (1.5-fold) of hydroxylamine compared to carboxylic acid substrate.

The cascade reactions (Scheme 2) were studied in two different reaction setups. In the first approach, a one-pot cascade was performed with a mix of *E. coli* whole cells expressing NcCAR and others expressing OxdBr1 in the presence of **a** (10 mM), and hydroxylamine (1.5 eq.) (one-pot, Fig. 2). All constituents were incubated in a two-phase system (aqueous/*n*-heptane 1/1) for 24 h at 28 °C. Fig. 2 shows nitrile formation. Phenylacetic acid **1a** was fully reduced to aldehyde **1b**. The intermediate oxime formation was incomplete, as judged by remaining aldehyde. The enzymatic dehydration step, on the other hand, was highly efficient, as oxime was only detected in trace amounts. Due to incomplete oxime formation, aldehyde was partly over-reduced to alcohol **1e**. The resulting composition in case of the aliphatic acid **5a** was less on the product side. In this case, neither acid

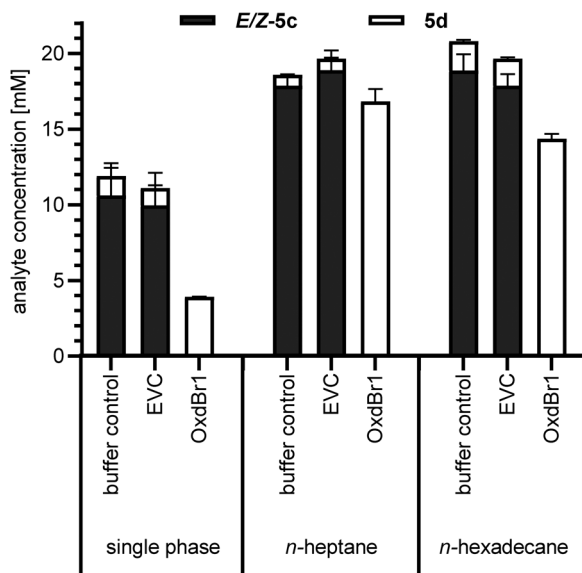


Fig. 1 Comparison of OxdBr1 catalyzed dehydration of hexanal oxime (**5c**, 20 mM) in single-phase (aqueous) and two-phase (*n*-heptane/aqueous or *n*-hexadecane/aqueous) mode. Whole cells, OD10; 4 h, 28 °C. EVC = empty vector control. Error bars are shown for technical triplicates.

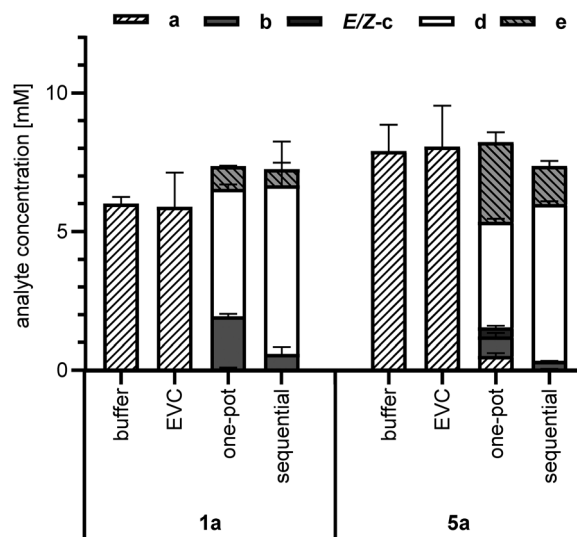
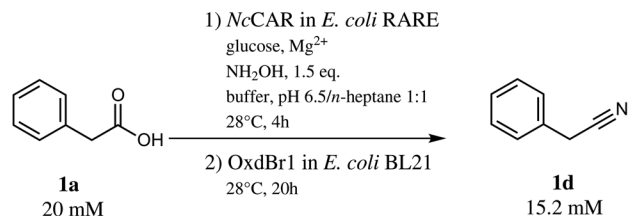


Fig. 2 Chemoenzymatic cascade from carboxylic acid (**a**) to nitrile (**d**) in biphasic system (aqueous/*n*-heptane 1/1). Whole cell-mediated reduction of **1a** or **5a** using NcCAR, *in situ* oximation by hydroxylamine (1.5 eq.) and enzymatic dehydration to **1d** or **5d** by OxdBr1 in whole cells. One-pot: mixed culture of cells harbouring either NcCAR or OxdBr1. Time: 24 h. Sequential: first, NcCAR harbouring cells were incubated with hydroxylamine for 4 h. Second, OxdBr1 harbouring cells were added and reactions were stopped after 24 h. 10 mM substrate was dissolved in *n*-heptane; temp.: 28 °C. Error bars are shown for technical triplicates. EVC = empty vector control.





**Scheme 3** One-pot two-step transformation of phenylacetic acid (**1a**) to phenylacetonitrile (**1d**).

reduction, oxime formation nor dehydration went to completion. Not surprisingly, also over-reduction was more prominent in case of this short chain fatty acid.<sup>26</sup> The one-pot reactions resulted in 4.6 mM of nitrile **1d** or 3.8 mM of nitrile **5d**, respectively.

In the second approach (sequential, Fig. 2), the cascade was divided into two subsequent steps. *In vivo* bioreduction and *in situ* oxime formation were performed during the first 4 h. Without any work-up, the resulting mixture was supplemented with *OxdBr1* biocatalyst. Full consumption of both **1a** and **5a** was detected, but approximately 20% of the added material was not recovered. Of the recovered product mixture, 83% was the desired aryl-aliphatic product **1d** in case of **1a**. In case of **5a**, 76% was nitrile **5d** and the major side product was **5e**. In total, nitrile titers were higher in these sequential two-step/one-pot reactions (6.1 mM **1d** and 5.7 mM **5d**, respectively) as compared to the simultaneous one-pot reactions (Fig. 2). One of the reasons might be that the *NcCAR* was inhibited by **d**, which only emerges in the final reaction step.

With **1a** in a concentration of 20 mM, full substrate consumption was again achieved in the one-pot two-step mode. After 24 h, 15.2 mM (76% yield) of nitrile **1d** were detected, in addition to 0.4 mM of **1b**, 2.3 mM of **E** and **Z-1c** and 1.4 mM of **1e** (Scheme 3).

These results are a promising proof of the cascade concept and the starting point for further in-depth investigation, including diversification of the product range by tailored combinations of various CARs and Oxd.

## Conclusions

In summary, we showed a chemoenzymatic route from carboxylic acid to nitrile in which the carbon-chain length of the starting material is retained. For this approach, a three-step cascade was used. In the first step, *E. coli* whole cell catalysts co-expressing CAR and PPTase reduced the carboxylic acid to the respective aldehyde. Hydroxylamine trapped this cytotoxic compound to give aldoxime in a chemical step. Thirdly, the intermediate aldoxime was enzymatically dehydrated to the desired nitrile by *Oxd*. Up to 76% of target product was obtained in a biphasic system. The organic layer was beneficial for the recovery rate, because it retained the lipophilic volatiles which would otherwise escape to the gas phase. The organic phase did not affect

*OxdBr1* performance. The proposed route is environmentally more benign since it does not require cyanide salts, toxic metals, or undesired oxidants in contrast to entirely chemical procedures. Future in depth-studies will extend this proof of concept by examination of various CAR and *Oxd* enzymes to target a wider variety of nitriles.

## Author contributions

MP, LM and MW: conceptualization; MH, VW, RR, SH, AS, BW: investigation and methodology; MH, MW, SH, BW: formal analysis; MH, MW: visualization, data curation; FR, NK, MP, LM, MW: supervision; MP, LM, MW: funding acquisition; MH, MW: writing – original draft. FR, BW, MP, LM: writing – review & editing.

## Conflicts of interest

There are no conflicts to declare.

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## References

- M. Winkler, A. Glieder and K. Steiner, in *Comprehensive Chirality*, ed. E. M. Carreira and H. Yamamoto, 2012, vol. 7, pp. 350–371.
- J. Vilím, T. Knaus and F. G. Mutti, *Angew. Chem., Int. Ed.*, 2018, **57**, 14240–14244.
- G. Yan, Y. Zhang and J. Wang, *Adv. Synth. Catal.*, 2017, **359**, 4068–4105.
- H. Gröger and Y. Asano, *J. Org. Chem.*, 2020, **85**, 6243–6251.
- F. Le Vaillant, M. D. Wodrich and J. Waser, *Chem. Sci.*, 2017, **8**, 1790–1800.
- J. S. Reader, D. Metzgar, P. Schimmel and V. de Crécy-Lagard, *J. Biol. Chem.*, 2004, **279**, 6280–6285.
- G. Phillips, M. A. Swairjo, K. W. Gaston, M. Bailly, P. A. Limbach, D. Iwata-Reuyl and V. de Crécy-Lagard, *ACS Chem. Biol.*, 2012, **7**, 300–305.
- U. Battaglia, J. E. Long, M. S. Searle and C. J. Moody, *Org. Biomol. Chem.*, 2011, **9**, 2227–2232.
- R. M. McCarty, A. Somogyi, G. Lin, N. E. Jacobsen and V. Bandarian, *Biochemistry*, 2009, **48**, 3847–3852.
- M. T. Nelp and V. Bandarian, *Angew. Chem., Int. Ed.*, 2015, **54**, 10627–10629.
- M. Winkler, K. Dokulil, H. Weber, T. Pavkov-Keller and B. Wilding, *ChemBioChem*, 2015, **16**, 2373–2378.
- Y. Kato, T. Tsuda and Y. Asano, *Biochim. Biophys. Acta, Proteins Proteomics*, 2007, **1774**, 856–865.



- 13 T. Betke, J. Higuchi, P. Rommelmann, K. Oike, T. Nomura, Y. Kato, Y. Asano and H. Gröger, *ChemBioChem*, 2018, **19**, 768–779.
- 14 M. S. C. Pedras, Z. Minic, P. D. Thongbam, V. Bhaskar and S. Montaut, *Phytochemistry*, 2010, **71**, 1952–1962.
- 15 R. Rädisch, M. Chmátal, L. Rucká, P. Novotný, L. Petrásková, P. Halada, M. Kotik, M. Pátek and L. Martínková, *Int. J. Biol. Macromol.*, 2018, **115**, 746–753.
- 16 P. Domínguez de María, *Molecules*, 2021, **26**, 4466.
- 17 A. Hinzmann, T. Betke, Y. Asano and H. Gröger, *Chem. – Eur. J.*, 2021, **27**, 5313–5321.
- 18 M. Gruber-Khadjawi, M. Fechter and H. Griengl, in *Enzyme Catalysis in Organic Synthesis*, ed. K. Drauz, H. Gröger and H. Griengl, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, 2012, ch. 23, pp. 917–990.
- 19 H. Yavuzer, Y. Asano and H. Gröger, *Angew. Chem., Int. Ed.*, 2021, **60**, 19162–19168.
- 20 T. Betke, P. Rommelmann, K. Oike, Y. Asano and H. Gröger, *Angew. Chem., Int. Ed.*, 2017, **56**, 12361–12366.
- 21 Y. Miao, R. Metzner and Y. Asano, *ChemBioChem*, 2017, **18**, 451–454.
- 22 S. Martí, I. Tuñón, V. Moliner and J. Bertran, *ACS Catal.*, 2020, **10**, 11110–11119.
- 23 A. Hinzmann, S. Glinski, M. Worm and H. Gröger, *J. Org. Chem.*, 2019, **84**, 4867–4872.
- 24 T. V. Hansen, P. Wu and V. V. Fokin, *J. Org. Chem.*, 2005, **70**, 7761–7764.
- 25 M. Winkler, *Curr. Opin. Chem. Biol.*, 2018, **43**, 23–29.
- 26 M. Horvat and M. Winkler, *ChemCatChem*, 2020, **12**, 5076–5090.
- 27 D. Schwendenwein, G. Fiume, H. Weber, F. Rudroff and M. Winkler, *Adv. Synth. Catal.*, 2016, **358**, 3414–3421.
- 28 S. P. France, S. Hussain, A. M. Hill, L. J. Hepworth, R. M. Howard, K. R. Mulholland, S. L. Flitsch and N. J. Turner, *ACS Catal.*, 2016, **6**, 3753–3759.
- 29 E. Klumbys, Z. Zebec, N. J. Weise, N. J. Turner and N. S. Scrutton, *Green Chem.*, 2018, **20**, 658–663.
- 30 M. Ahsan, S. Sung, H. Jeon, M. Patil, T. Chung and H. Yun, *Catalysts*, 2017, **8**, 4.
- 31 J. Citoler, S. R. Derrington, J. L. Galman, H. Bevinakatti and N. J. Turner, *Green Chem.*, 2019, **21**, 4932–4935.
- 32 A. Hinzmann, M. Stricker and H. Gröger, *ACS Sustainable Chem. Eng.*, 2020, **8**, 17088–17096.
- 33 S. Rosenberg, S. M. Silver, J. M. Sayer and W. P. Jencks, *J. Am. Chem. Soc.*, 1974, **96**, 7986–7998.
- 34 W. P. Jencks, *Phys. Org. Chem.*, 1959, **81**, 475–481.
- 35 Y. Nishigaya, Z. Fujimoto and T. Yamazaki, *Biochem. Biophys. Res. Commun.*, 2016, **476**, 127–133.
- 36 R. Jiménez, R. Pequerul, A. Amor, J. Lorenzo, K. Metwally, F. X. Avilés, X. Parés and J. Farrés, *Chem.-Biol. Interact.*, 2019, **306**, 123–130.
- 37 N. O. Kaplan and M. M. Ciotti, *J. Biol. Chem.*, 1953, **201**, 785–794.
- 38 M. Wada, T. Matsumoto, S. Nakamori, M. Sakamoto, M. Kataoka, J.-Q. Liu, N. Itoh, H. Yamada and S. Shimizu, *FEMS Microbiol. Lett.*, 1999, **179**, 147–151.
- 39 A. Putrament, H. Baranowska and J. Pachecka, *Mol. Gen. Genet.*, 1973, **122**, 61–72.

