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β-Diketone hydrolases

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Abstract

The hydrolytic cleavage of carbon–carbon bonds by β-diketone hydrolases (E.C. 3.7.1.X) is a biotransformation reaction that has received limited attention. However, studies of purified enzymes of this class have shown that they are simple hydrolases, with no complex cofactor requirement and as such, they may be a source of economical catalysts with potential application in organic synthesis. In this review, recent developments in the study of β-diketone hydrolases are surveyed, including our own work, which focuses on the first example of an asymmetric reaction catalysed by one of these enzymes—the cleavage of bicyclic β-diketones to yield keto acids of high optical purity.

Keywords: β-Diketone; Hydrolases; Biotransformation

1. Introduction

The use of hydrolases in organic synthesis has become widespread in recent years owing to the mild reaction conditions used in their application and the selectivities displayed by these enzymes in the transformation of a variety of organic substrates. Much of this research has focused on the use of carbon–heteroatom bond hydrolases such as esterases, lipases, proteases [1] and more recently, epoxide hydrolases [2]. By contrast, examples of the cleavage of carbon–carbon bonds by hydrolases are rare in the literature and their application to organic synthetic transformations has not been considered.

In 1997, Pokorny et al. published a review entitled ‘β-Ketolases—forgotten hydrolytic enzymes’, which focused on literature reports of the enzymatic cleavage of carbon–carbon bonds in 1,3-dioxo substituted compounds [3]. A section of this review dealt with the enzymatic process of β-diketone cleavage, catalysed by enzymes classified by the Enzyme Commission on Nomenclature in the Hydrolase Group (E.C. 3.7.1.X), which cleave a carbon–carbon bond between two carbonyl groups to yield two products; one bearing a methyl ketone terminus and the other, a carboxylic acid terminus (Fig. 1).

There are only 10 listed examples of C–C bond hydrolases acting on ketonic substances, including β-diketone hydrolases [4] (Table 1). In work leading up to the review of Pokorny et al., little was known about the mechanisms that may be responsible for these enzymatic reactions, but in recent years, structural studies on representative members of the group have revealed more detailed information. β-Diketone hydrolases may be trivially divided into two classes, based on the structure of the substrate on which they act: β-diketo acid hydrolases, for which a terminal carboxylate group in the substrate is essential for

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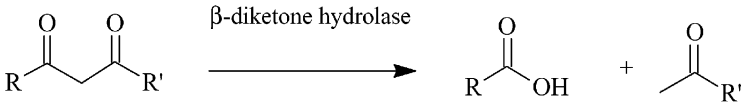


Fig. 1. General reaction catalysed by β-diketone hydrolases.

Table 1
E.C. list of 'hydrolases acting on carbon–carbon bonds in ketonic substances' (E.C. 3.7.1.X) [4]

Classification entry	Name of enzyme
3.7.1.1	Oxaloacetase
3.7.1.2	Fumarylacetoacetase
3.7.1.3	Kynureninase
3.7.1.4	Phloretin hydrolase
3.7.1.5	Acylpyruvate hydrolase
3.7.1.6	Acetylpyruvate hydrolase.
3.7.1.7	β-Diketone hydrolase.
3.7.1.8	2,6-Dioxo-6-phenylhexa-3-enoate hydrolase
3.7.1.9	2-Hydroxymuconate-semialdehyde hydrolase
3.7.1.10	Cyclohexane-1,3-dione hydrolase

molecular recognition, and β-diketone hydrolases, which hydrolyse a range of 'neutral' acyclic and cyclic β-diketones.

1.1. β-Diketo acid hydrolases

Early observations of C–C bond fission in β-diketo acids were made by Meister and Greenstein [5], who noted the ability of rat liver extracts to catalyse the hydrolytic transformation of acetylpyruvate **1** to acetate **2** and pyruvate **3** (Fig. 2). In later work, Stanier and co-workers observed the equivalent transformation to be catalysed by cell extracts of *Pseudomonas arvilla* [6].

Ribbons and co-workers subsequently purified and partially characterised an inducible enzyme from orcinol induced *Pseudomonas putida* 01 which was capable of catalysing the same reaction in vitro [7]. The enzyme, named acetylpyruvate hydrolase (APH; E.C. 3.7.1.6), was shown to have a molecular weight of 38 kDa and to require Mg^{2+} or Mn^{2+} as cofactor. These early results were recently embellished by a further publication wherein the purification protocol for the enzyme was improved and the stoichiometry of the natural reaction monitored by ^{1}H NMR in situ [8]. These studies revealed that the substrate for the enzymatic reaction in solution was likely not to be the diketone **1**, but rather the 4-keto-2-enol form **4** (Fig. 2). In addition, it was found that the enzyme was able to transform substrate analogues substituted at the 4-position, with improved rates of hydrolysis observed for the 4-isopropyl **5** (100%), 4-*tert*-butyl **6** (68%) and 4-cyclohexyl **7** (25%) compared to acetylpyruvate

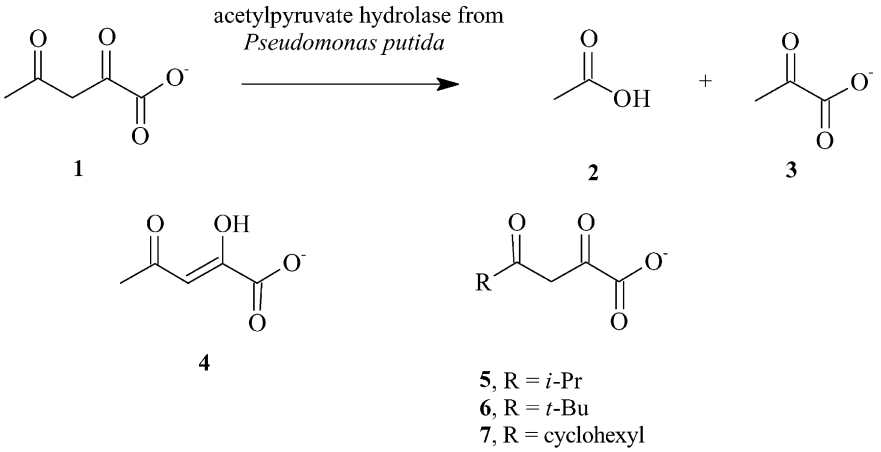


Fig. 2. Hydrolysis of acetyl pyruvate by acetylpyruvate hydrolase and further substrates for APH.

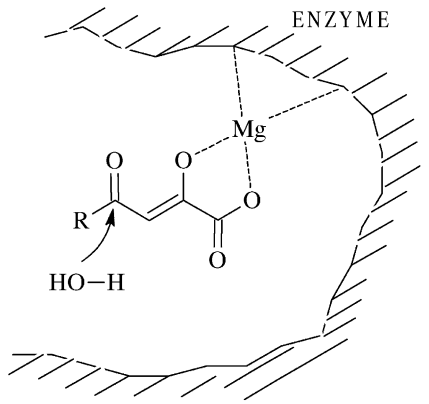


Fig. 3. Proposed mechanism of substrate binding by acetylpyruvate hydrolase from *Pseudomonas putida* showing chelation of carboxylate/enolate substrate by divalent Mg^{2+} ions.

1 itself (4%). The possibilities of effecting regio- or enantioselective transformations of chiral or prochiral substrates by APH were not investigated. A role for Mg^{2+} ions in the mechanism of APH catalysed C–C bond hydrolysis of substrate **4** was proposed, whereby co-ordination of the divalent metal ion to the enolate dianion shown would facilitate substrate binding for subsequent hydrolytic cleavage (Fig. 3).

Whilst no conclusive structure-based mechanistic data are available for APH, the mechanism for another β-diketo acid hydrolase enzyme, fumarylacetoacetate hydrolase (FAH; E.C. 3.7.1.2), has been elucidated with the aid of an X-ray crystallographic structure. FAH was also reported to accept acetylpyruvate as a substrate for the reaction shown in Fig. 2. FAH catalyses the final step in tyrosine metabolism in mammals, transforming fumarylacetoacetate **8** to fumarate **9** and acetoacetate **10** (Fig. 4). A deficiency of FAH activity causes the metabolic condition hereditary tyrosinaemia Type 1. Schmidt and co-workers described the purification of FAH from beef liver using acetylpyruvate as the assay substrate [9]. Subsequent work by the same group revealed the enzyme to be a homodimer with a subunit molecular weight of 46 kDa [10].

The structure of FAH has recently been solved by Timm et al. [11]. In common with APH, FAH also requires divalent metal ions, in this case Ca^{2+}, as cofactor. Structural evidence suggests that cleavage of the carbon–carbon bond is catalysed by a water molecule activated as part of a glutamate/histidine/water triad, wherein the histidine serves as a general base (Fig. 5). The Ca^{2+} ion has three roles in catalysis: Binding of the carboxylate of the substrate **8**, which was proposed in this case to bind in its diketo form; indirect positioning of the water nucleophile, by co-ordinating the glutamate which activates the water molecule; and stabilisation of the carbanion leaving group which will become the product acetoacetate. The authors suggested that the requirement for a metal ion in the hydrolytic mechanism, unusual for catalytic triad type hydrolases, could be due to the relative inactivity of the carbon–carbon bond toward hydrolysis. Although the enzyme was also reported to accept acetylpyruvate **1** as a substrate, the structure of FAH suggests that **1** would not be ideally accommodated in the active site. Any extrapolations of the mechanism of FAH-catalysed hydrolysis to acetylpyruvate hydrolase may therefore be premature at this stage.

It is clear that the mechanism of carbon–carbon bond cleavage by the β-diketone hydrolases is dependent on the co-ordination of the terminal carboxylate group that is a feature of these substrates. It is likely that different mechanisms may account for the cleavage of β-diketones wherein there is no carboxylate moiety.

1.2. β-Diketone hydrolases

There are a few examples of β-diketone hydrolases which act on non-carboxylic acid substrates in the

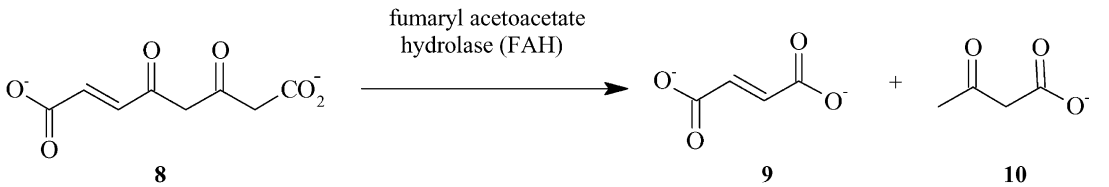


Fig. 4. Hydrolysis of fumarylacetoacetate by FAH from beef liver.

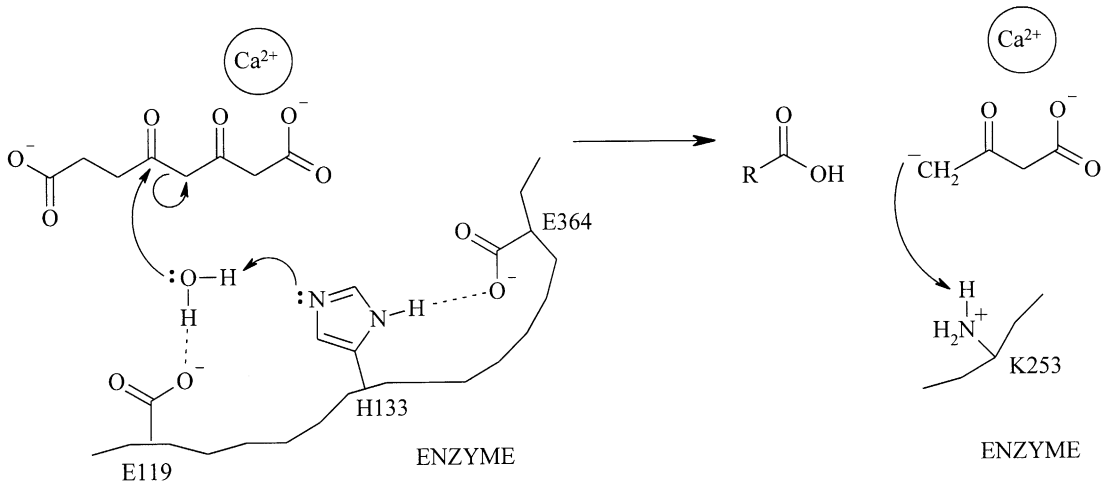


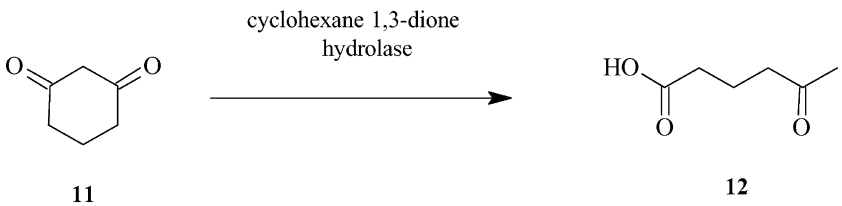
Fig. 5. Mechanism of FAH hydrolysis of fumarylacetoacetate highlighting catalytic hydrolytic triad and protonation of the carbanion product by lysine 253.

microbiology literature, with some limited characterisation of cell-free extract activities and purified enzymes.

1,3-Cyclohexanedione hydrolase (CHDH; E.C. 3.7.1.10) is active in the anaerobic metabolism of cyclohexanol by a strain of *Pseudomonas* sp. [12]. The pathway involves sequential enzymatic steps including cyclohexanol oxidation followed by desaturation, hydration and oxidation reactions to yield the symmetrical cyclohexane-1,3-dione **11**. The authors showed that **11** was subsequently cleaved by cell extracts to yield 5-oxocaproic acid **12** (Fig. 6). Cyclopentane-1,3-dione and cycloheptane-1,3-dione were not substrates for the hydrolytic activity. Cyclohexane-1,2-dione was reported to be inhibitory, and the addition of Coenzyme A to reaction mixtures had no effect. No further purification of the enzymatic activity was reported.

A β-diketone hydrolase was also implicated in the anaerobic metabolism of atropine by another *Pseudomonas* sp. [13]. In this case, a number of enzymatic catabolic steps led to the production of cyclohepta-1,3,5-trione **13**, a substrate which may conceivably be cleaved at either of two site to yield 4,6-dioxoheptanoate **14** or 3,6-dioxoheptanoate **15** (Fig. 7). Interestingly, regioselectivity was displayed in this hydrolysis as only product **14** is observed. **14** was then cleaved by another β-diketone hydrolase catalysed reaction to yield succinate **16** and acetone **17**. Only limited biochemical characterisation of either carbon–carbon bond cleavage enzyme was conducted and no purification of the relevant proteins was carried out.

The biodegradation of polyvinyl alcohol **18** by *Pseudomonas vesicularis* var. polyvaloyiticus involves two enzymatic steps (Fig. 8). Initially, the secondary



11 **12**

Fig. 6. Hydrolysis of cyclohexane 1,3-dione by cyclohexane 1,3-dione hydrolase from *Pseudomonas* sp.

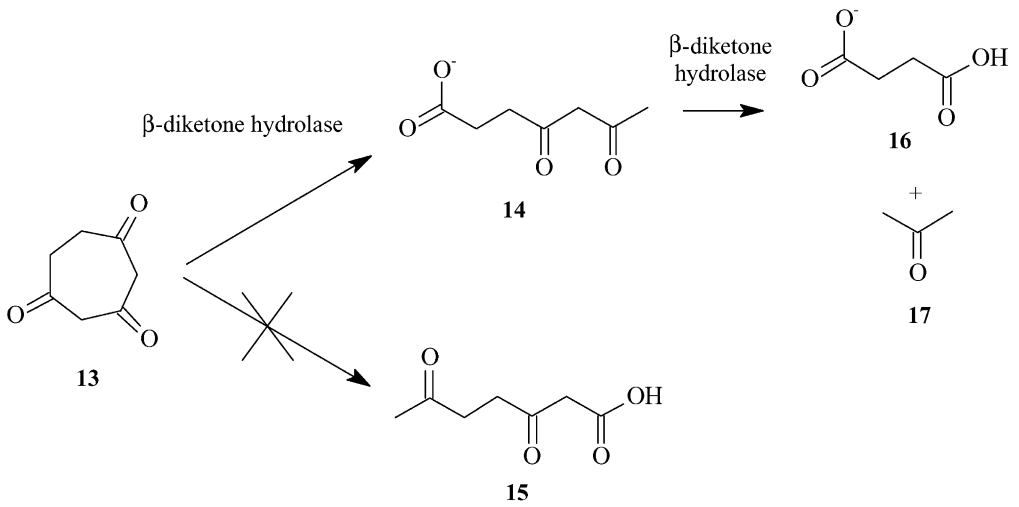


Fig. 7. Regioselective hydrolysis of cyclohepta-1,3,5-trione by a β-diketone hydrolase from *Pseudomonas* sp.

hydroxyls are oxidised to carbonyl groups by the action of polyvinyl alcohol oxidase. In the second step, the polyvinylketone **19** is cleaved at sites between the carbonyl groups by an enzymatic β-diketone hydrolase reaction [14]. The enzyme which catalyses the second step was purified by using an assay which exploited the ability of the enzyme to hydrolyse pentane-2,4-dione **20** to acetate **2** and acetone **17** [15]. The enzyme was thus termed pentane-2,4-dione hydrolase (PDH; E.C. 3.7.1.7). Purified PDH was estimated to have a molecular weight between 65 kDa and 75 kDa. The activity was inhibited by Hg^{2+} or Mg^{2+} and also by relatively low concentrations of chloride and bromide ions. No amino acid sequence data for PDH were reported.

It is notable that although the enzymes that catalyse the cleavage of β-diketones appear to be simple hydrolases, with little or no complex cofactor specificity,

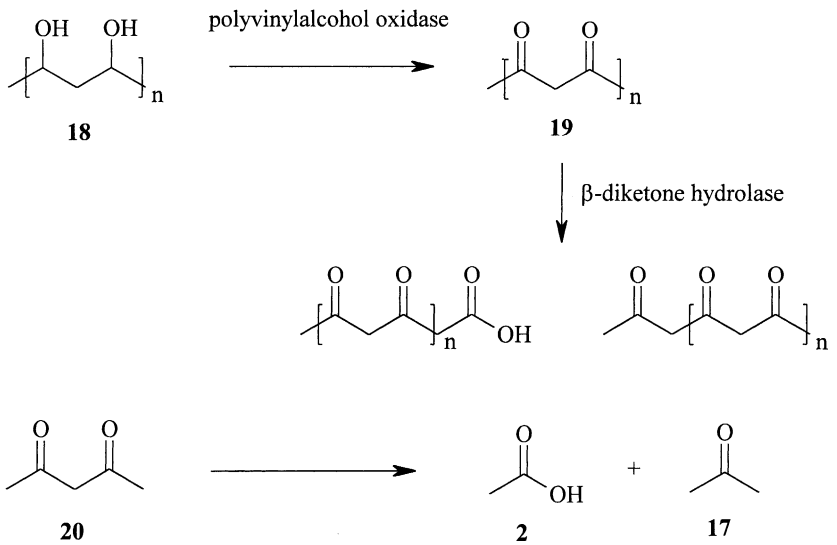


Fig. 8. Hydrolysis of polyvinylketone and pentane-2,4-dione by PDH from *Pseudomonas vesicularis* var. *povaloyliticus* PH.

their application to transformations of synthetic interest has not been studied. This may be because the substrates for such enzymes have been insufficiently interesting to merit further study, or perhaps because in each case, no transformations of chiral or prochiral substrates by these enzymes have been reported. However, at least one report in the literature had suggested the existence of an enzyme catalysed desymmetric carbon–carbon bond hydrolysis [16].

In 1966, the group of Gunsalus determined, by metabolite extraction, the catabolic pathway for the degradation of (1*R*)-(+)-camphor **21** by a strain of *Corynebacterium* (now deposited as *Rhodococcus* sp. NCIMB 9784) [16]. Hydroxylation in the 6-*endo*-position to give **22** is followed by an oxidative dehydrogenase catalysed reaction to yield the symmetrical bicyclic β-diketone, 6-oxo camphor **23** (Fig. 9). **23** is then cleaved by an enzymatic *retro*-Claisen reaction to yield the chiral α-campholinic acid **24**, which was reported to have a negative optical rotation. This transformation, which was postulated by the authors to be catalysed by a 'β-ketolase' therefore constituted not only a hydrolytic carbon–carbon bond cleavage but also a desymmetrisation reaction.

Because of the ongoing interest in enzymatic desymmetrisation processes in synthesis [17], we purified the enzyme responsible for the cleavage of **23** and characterised the stereoselectivity of its natural reaction in vitro. The enzyme was named oxo-camphor hydrolase (OCH) based on its apparent relationship to hydrolases, such as cyclohexanedione hydrolase, which had been previously classified in the E.C. 3.7.1.X group. The enzyme was challenged with a series of commercially available β-diketones and found to have no activity toward, for example, pentane-2,4-dione, 2-acetylcyclohexanone or 2-acetylcyclopentanone, cyclohexane-1,3-dione 2-methyl cyclohexane-1,3-dione, or 2,2-dimethyl cyclohexane-1,3-dione. However, some activity was observed towards 2,2-dialkylated cyclohexane-1,3-diones in which one substituent was methyl and the other ethyl, **25** *n*-propyl **26** or *n*-butyl **27**. In addition, the enzyme was shown to be active toward the demethyl analogue **28** of its natural substrate and the similar [2.2.2] bicyclic compound **29** [18] (Fig. 10). It was apparent that in order for a compound to be a substrate for the enzyme, the substrate must be non-enolisable, either due to disubstitution at the 2-position or in the case

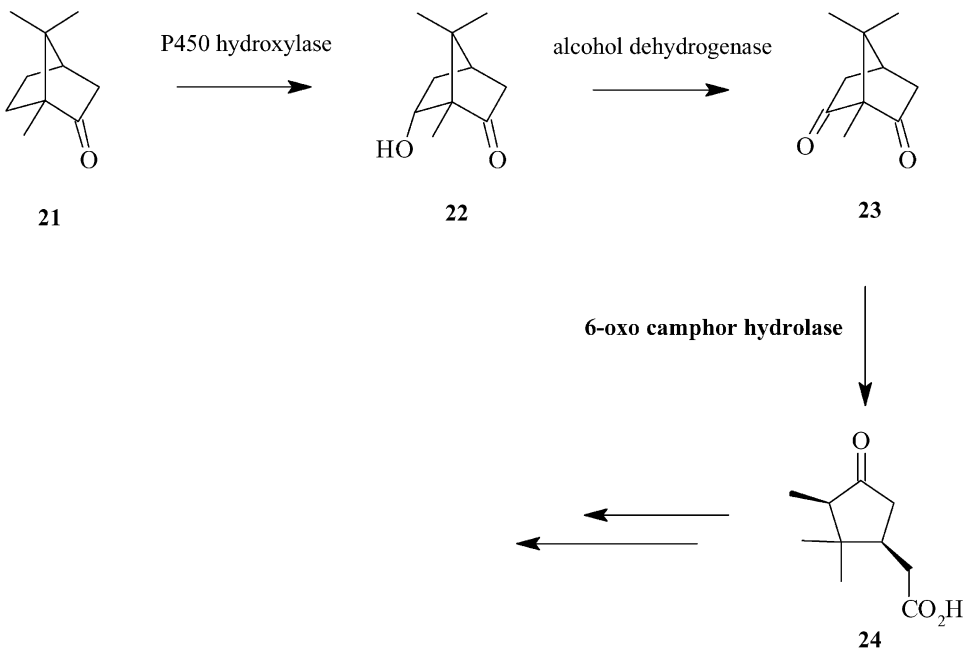
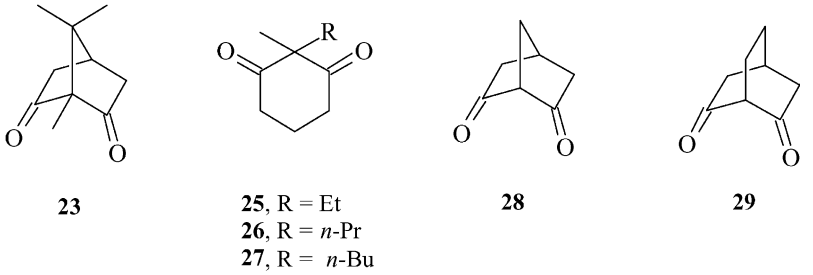


Fig. 9. Metabolic pathway for degradation of (1*R*)-(+)-camphor **21** by *Rhodococcus* sp. NCIMB 9784.

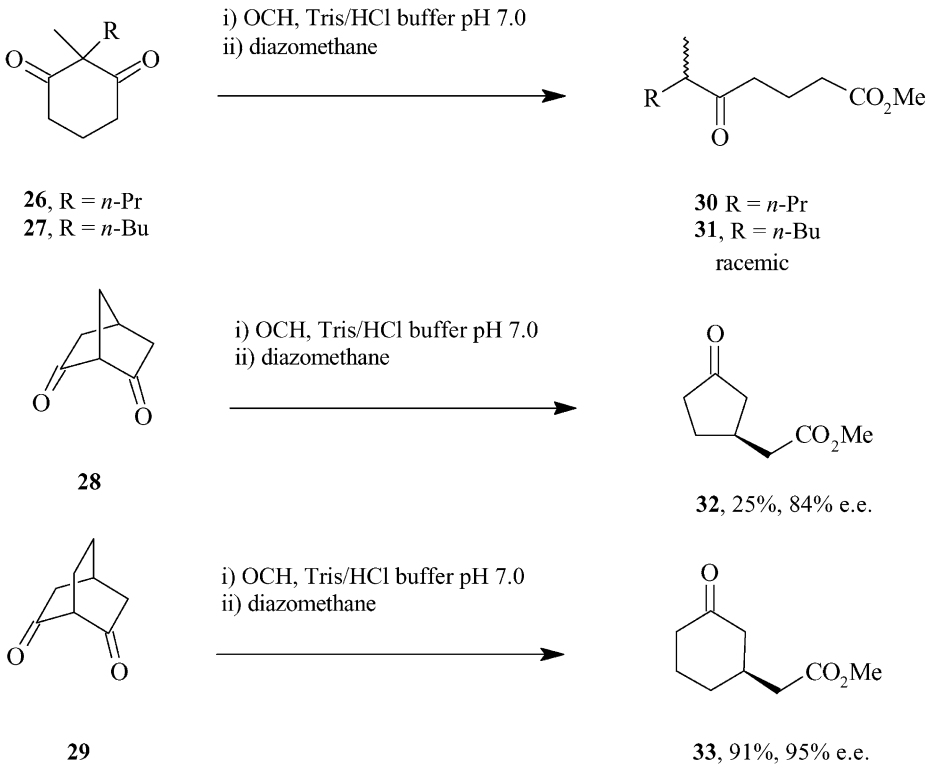


23 **25**, R = Et; **26**, R = *n*-Pr; **27**, R = *n*-Bu **28** **29**

Fig. 10. β-Diketone substrates of oxo camphor hydrolase.

of bicyclic compounds, due to restrictions imposed according to Bredt's rule.

The transformation of these symmetrical compounds raised the possibility of chiral hydrolytic reactions. The enantioselectivity of the transformations of compounds **23**, and **26** to **29** was assessed. The transformation of the cyclohexanediones **26** and **27** to cyclic keto acid products (converted to their methyl esters **30** and **31** for ease of analysis) were found to proceed with no enantioselectivity (Fig. 11), however, the transformation of the [2.2.1] analogue **28** of the natural substrate gave a cyclic keto acid product (converted to methyl ester **32**) of 84% e.e. The poor yield of this product may be due to the poor aqueous stability of the compound, which was shown to degrade in the reaction buffer within 2 h. However,



26, R = *n*-Pr; **27**, R = *n*-Bu

30 R = *n*-Pr; **31**, R = *n*-Bu racemic

28

32, 25%, 84% e.e.

29

33, 91%, 95% e.e.

Fig. 11. Hydrolysis of non-enolisable diketones by oxo camphor hydrolase.

transformation of the more stable [2.2.2] compound **29** yielded product **33**, after methylation, in high yield with an enantiomeric excess of 95% e.e. [18].

These promising results prompted us to clone the gene encoding OCH, in order to investigate more fully the nature of the enzymatic process. The gene *camK* encoding a polypeptide of 257 amino acids was isolated by using classical reverse genetics based on hybridisation studies utilising degenerate oligonucleotide primers designed using the N-terminal amino acid sequence of OCH [19]. Comparison of the amino acid sequence with the Swissprot database revealed significant homology to the superfamily of enzymes known as crotonases. The best alignments were obtained with crotonase (enoyl-CoA hydratase) from *Clostridium acetobutilicum* (45% homology) and *Escherichia coli* (42% homology). Close homology was also observed with crotonase superfamily members of different reaction specificity, 2-ketocyclohexanecarboxyl Coenzyme A hydrolase from *Rhodopseudomonas palustris* and 4-chlorobenzoyl dehalogenase from *Pseudomonas* sp. [19].

The crotonase superfamily of enzymes is a group of low sequence homology proteins, members of which catalyse distinct chemical reactions based on a similar mechanism of transition state stabilisation [20]. Some examples of the activity of crotonases are shown in Fig. 12.

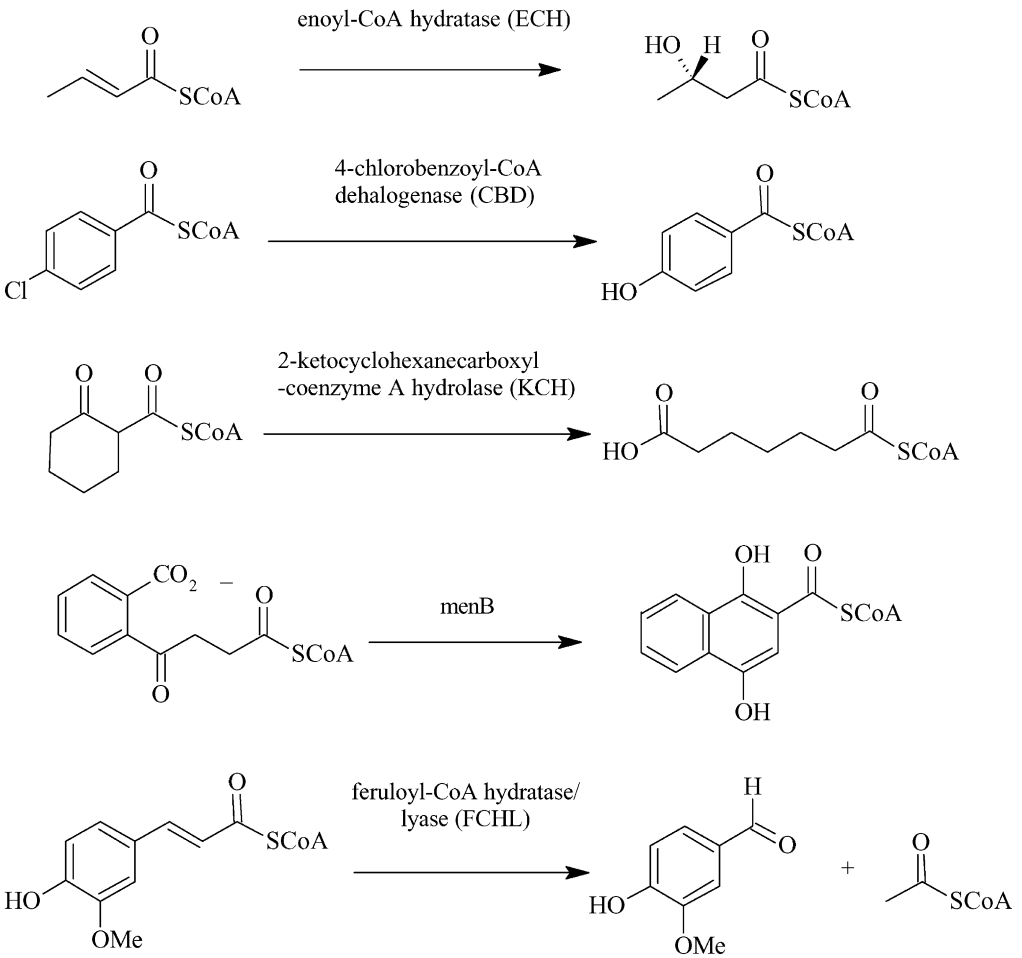


Fig. 12. Representative reactions catalysed by members of the crotonase superfamily.

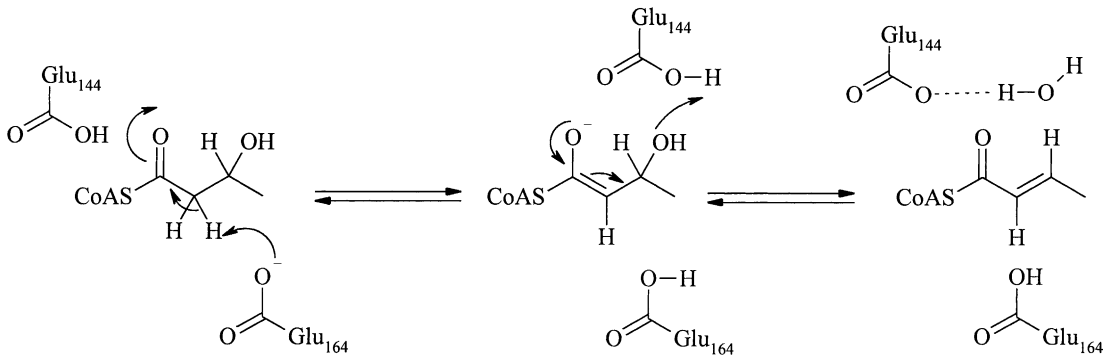


Fig. 13. Mechanism of reversible hydration of enoyl-CoA by enoyl-CoA hydratase (crotonase). Glu 144 and Glu 164 form the catalytic acid–base couple in the active site.

For each reaction catalysed by a crotonase enzyme, the substrate is an acyl CoA thioester. During the course of each reaction an intermediate enolate is evolved that must be stabilised by an oxyanion hole, which is in the case of crotonase enzymes, formed by the N–H groups of conserved glycine residues in the peptide backbone. In crotonase superfamily members studied so far, catalysis is accomplished by an acid–base couple in the active site. The residues which constitute the acid–base couple are not conserved throughout the superfamily, however. In the mechanism of ECH catalysed hydration of enoyl-CoA, two glutamate residues, E144 and E164 are the catalytic residues [21]—E144 activates a molecule of water for attack at C2 of the substrate and E164 acts as an acid to protonate the reaction intermediate (Fig. 13). Oxo camphor hydrolase (OCH) is the first member of the crotonase superfamily to be identified whose substrate, diketone **23**, is not an acyl-CoA thioester. However, we propose that the phenomenon of enolate stabilisation and acid–base catalysis is likely to persist, via the mechanism illustrated in Fig. 14. The identity of the catalytic residues is unknown at present and we are currently performing both structural studies and mutagenesis experiments in an effort to establish the mechanism of β-diketone cleavage. Four crystal structures of crotonase enzymes are available in the database for comparison: enoyl-CoA hydratase (ECH) [22], 4-chlorobenzoyl-CoA dehalogenase (CBD) [23], methylmalonyl decarboxylase (MMD) [24], and dienoyl CoA isomerase [25]. To our knowledge, OCH is the first crotonase superfamily member that Coenzyme A binding is not necessary for catalysis. A comparative structural study of OCH and other crotonase superfamily members may therefore aid in the rational design of crotonase-catalysed processes that are independent of the involvement of the Coenzyme A cofactor.

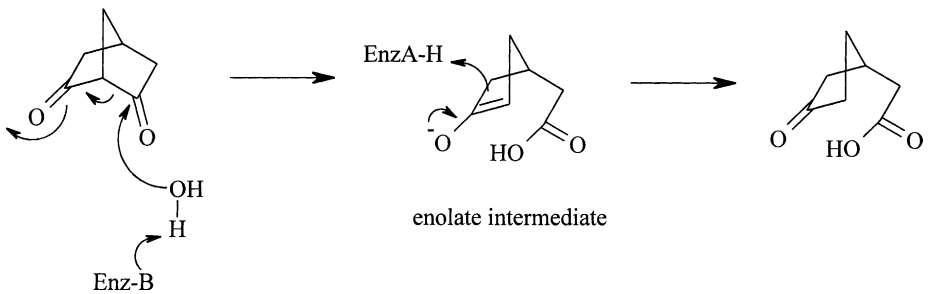


Fig. 14. Proposed ‘crotonase-type’ mechanism for the hydrolysis of **28** by 6-oxo camphor hydrolase.

2. Conclusions

It is clear from the limited data available that nature has evolved different mechanisms for carbon–carbon bond hydrolysis in β-diketones, which are heavily dependent on substrate structure. The classification of such activities is not straightforward, as the reaction based E.C. classification system fails to illustrate the evolutionary distance and mechanistic diversity among proteins that catalyse carbon–carbon bond hydrolyses in β-diketone substrates, such as the hydrolytic triad of FAH and the crotonase-type mechanism which may operate in the reaction catalysed by OCH. The mechanistic bases for the activity of pentane-2,4-dione hydrolase and cyclohexane-1,3-dione hydrolase are thus a matter for conjecture at this point, due to the absence of the relevant primary structural data and more detailed experimental studies.

The application of β-diketo acid or β-diketone hydrolases in synthesis will depend, as with other biocatalytic agents, on the demonstration of their competitive advantages over abiotic reagents for reasons of selectivity. It is envisaged that the discovery of an enantioselective β-diketone hydrolase, oxocamphor hydrolase, and the further investigation of the reaction specificity of other enzymes of related activity will stimulate further study into the potential of these simple hydrolases as applied biocatalysts.

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