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Review

Biotechnological applications of *Candida antarctica* lipase A: State-of-the-art

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Abstract

The yeast *Candida antarctica* produces two different lipases, lipases A and B. While lipase B (CAL-B) is probably the mostly employed hydrolase in the biocatalysis field, the use of the lipase A (CAL-A) has been rather scarce and consequently its tridimensional structure has not been elucidated yet. However, CAL-A is a useful biocatalyst with many different applications that have been described especially in the last few years. Its attractiveness results from its unique features among hydrolases: the high thermostability, allowing operation at $T > 90\,^{\circ}C$; the ability to accept tertiary and sterically hindered alcohols, which has recently been attributed to the existence of a specific aminoacidic sequence in the active site; the sn-2 recognition in hydrolysis of triglycerides; the selectivity towards *trans*-fatty acids; the stability in the acidic pH range. Furthermore, it is considered to be an excellent biocatalyst for the asymmetric synthesis of amino acids/amino esters, due to its chemoselectivity towards amine groups. Considering all these aspects, in the present review, the origin, the properties and the applications of the CAL-A are briefly described and discussed, pointing out the unique characteristics of this biocatalyst.

Keywords: *Candida antarctica* lipase A; Thermostability; *trans*-Fatty acids selectivity; Chemoselective *N*-acylation; Amino acids; Tertiary alcohols

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1. Origin and biochemical features of *Candida antarctica* lipase A (CAL-A)

Nowadays, the possibilities that lipases offer for synthetical purposes are generally well accepted and supported by a plethora of excellent examples ([1–4], and references therein). Lipases produced by different strains of genus *Candida* sp. are among the most widely used enzymes for biocatalytical purposes, especially *C. antarctica* lipase B (CAL-B), for which an enormous number of different applications have been reported [5–7].

The yeast *C. antarctica* is 1 of the 154 species of the genus *Candida* sp. [8]. Originally, it was isolated from the lake Vanda, in Antarctica, which is perennially covered with ice [6,8–9]. In fact, several aspects related to this yeast represent the majority of claims in patents reporting on discoveries in Antarctica [8]. In the late 1980s, the yeast was also isolated from Japanese natural samples [6]. Because of the many applications of lipases published, and the strong interest shown for those biocatalysts, the production of *C. antarctica* lipases was optimised [10] and two isoenzymes (called lipases A and B) were characterised [10,11] and purified [12].

Since the production of lipases from the original *C. antarctica* strains was rather scarce to allow full exploitation of their biotechnological potential, both isoenzymes were cloned and overexpressed in *Aspergillus oryzae* as host organism [13]. This has led to the production of sufficient amount of pure biocatalysts to be used at practical scale.

As stated previously, much attention has been paid to the utilisation of CAL-B, especially in the asymmetric synthesis ([5–7], and references therein). In contrast, the use of CAL-A attracted clearly less interest, despite its unique properties. An explanation for that could be that a relatively high CAL-A loading seems to be needed, especially when compared with that required for CAL-B. This may represent a limitation for its commercial use. However, in the last few years, CAL-A has found many remarkable applications, as demonstrated by the increasing number of publications (see below).

C. antarctica lipase A, a calcium dependent enzyme, has a molecular weight of 45 kDa, a pH optimum of approximately 7 and an isoelectric point (p*I*) of 7.5 [7]. It shows higher interfacial activation than CAL-B, but less than the one observed from *Humicola lanuginosa* [7,14]. The N-terminal sequence of both lipases A and B is known and there is no homology between them. In the case of CAL-A, there is also no similarity with other lipases [11,13]. While the crystallographic structure of CAL-B is available, and even some research in the development of new mutants has been done [15], the tridimensional structure of CAL-A still remains unknown and the work on mutants scarce. In this respect, as unique contribution, a new CAL-A with four-fold higher specific activity was developed by changing Phe135 and Phe139 by two Trp residues [16].

Probably, the most surprising biochemical property of CAL-A is its high thermostability. Up to date, it is considered to be the most thermostable lipase known, being able to work efficiently at >90 °C [5,7,10,11,17]. It appears to be quite strange that a microorganism which is able to grow under the cold conditions of Antarctica can produce such thermostable proteins. Perhaps, the mechanisms involved in the resistance at extreme temperatures are similar. In any case, the CAL-A thermostability represents an important advantage for its practical use.

Therefore, taking into account the recent increasing relevance of this topic, in the present review, we highlight the most important uses that have been described for CAL-A, focusing on the following aspects: (i) advantages of CAL-A thermostability; (ii) specificity of CAL-A in the hydrolysis of triglycerides, and its particular recognition of the *trans–trans*-fatty acids; (iii) CAL-A chemoselectivity towards amine groups, which makes it ideal for the asymmetric synthesis of amino acids and related compounds; (iv) asymmetric synthesis involving sterically hindered alcohols; (v) other synthetic applications/remarks.

2. Biotechnological processes employing CAL-A at high temperature

In many industrial processes, the combination of an enzymatic method with high temperatures would lead to more effective and economic performances. Considering that most of the hydrolases presently known are not active at temperatures higher than ca. 45 °C, the thermostability shown by CAL-A is a plus in, e.g. pitch control in the paper industry, and in the pulp and wax industries, since CAL-A remains stable, whereas other hydrolases deactivate. On the basis of this advantage, some applications were recently reviewed [6], including several patents that claim possibilities like hydrolysis of triglycerides in the pulp industry [18,19], or the wax lubricants field [20] at high temperatures. In addition, a proper immobilisation of the CAL-A leads to an even higher thermostability [11,17]. Moreover, the capability to perform reactions at high temperature makes this enzyme useful for asymmetric syntheses, which will be discussed later in this review.

3. CAL-A in the hydrolysis of triglycerides: selectivity towards *trans*-fatty acids and reactivity

The hydrolysis of triglycerides and their chemical handling to enhance the proportion of certain fatty acids represent an important application within the lipases field [1]. Most of the lipases known for this application have a sn-1,3 preference (related to the position of the fatty acid in the glycerol molecule). On the contrary, CAL-A shows a sn-2 preference [5,21,22], although such preference in the structure of triglycerides is not pronounced enough to enable selective synthesis of 1,3-diglycerides or 2-monoglycerides [7]. Consequently, for practical interesterifications, CAL-A has been reported as a non-selective biocatalyst [11].

Furthermore, CAL-A displays an outstanding selectivity towards *trans*-fatty acids when compared with the corresponding *cis*-derivatives. This is not a common feature within the lipases field, where usually *cis*-selectivities towards those substrates are found. On the basis of these features, a mixture of CAL-A and CAL-B was employed successfully in the synthesis of enriched polyunsaturated fatty acids [23], by combining

the different properties that both isoenzymes display towards production of different fatty acids derivatives [7].

In addition, some authors studied the CAL-A transesterification of *n*-butanol with different *cis*- or *trans*-fatty acids. Borgdorf and Warwel [24] reported that the transesterification rate of the *trans*-9-octadecenoic acid with *n*-butanol, catalysed by CAL-A, was 15 times higher than the rate observed for the *cis*-derivative. The same effect was noticed when different *trans*-polyunsaturated acids were tested. For instance, the use of *trans*-9,12-octadecadienoic acid revealed the extraordinary selectivity of CAL-A towards such structures, combined with a 200-fold faster reactivity as compared to the use of the *cis*-derivative (linolenic acid) [25,26].

This aspect seems to be unique among the hydrolases currently known. In the cases of lipases from *Candida rugosa* and *Geotrichum candidum*, the existence of a tunnel in the substrate recognition site (acyl part) was reported [27]. Such a tunnel is not straight, but has an L shape, which made it quite useful for the acceptance of *cis*-fatty acids (i.e. oleic acid) [28], as empirically observed [26]. Since the structure of some *C. rugosa* lipase (CRL) isoenzymes are well known, in a recent paper the development of mutants of the CRL-Lip1, with specific aminoacidic changes at some places of the tunnel, led to different biocatalytic selectivities towards different fatty acids [29]. Thus, the elucidation of the tridimensional structure of the CAL-A would shed some light on this surprising *trans*-preference, and what is more, would open possibilities for selective genetic modifications, enabling the production of new CAL-A mutants, specifically suitable for applications in the triglycerides/fatty acids chemistry field. This approach, combined with the thermostability, would represent an excellent tool from a practical point of view.

4. CAL-A—chemoselective *N*-acylation

CAL-A shows high chemoselectivity for the *N*-acylation of β-amino esters, under experimental conditions in which other hydrolases would lead to a competition between *N*-acylation at the amino group and transesterification at the ester group. This capability makes the CAL-A a useful catalyst in the production of enantiopure amino acids and related molecules, as it has been widely described in the last few years with a plethora of examples (see below).

4.1. CAL-A synthesis of alicyclic amino esters

CAL-A is able to acylate cyclic structures sterically hindered (**1**) via kinetic resolution (see reaction scheme above Table 1). Thus, several alicyclic β-aminocarboxylic acids esters – building blocks for the synthesis of various pharmaceutical important 1,3-heterocycles – were acylated with different acyl donors. The alicyclic β-aminocarboxylic acids were used in the ester form in order to overcome the intermolecular formation of amides, while the use of esters as acyl donors avoids the production of water during the esterification, keeping the water activity constant during the reaction [30]. On the other hand, the choice of the solvent and the acyl donor is crucial for achieving a high enantioselectivity. Thus, as reported in different articles during the last years, the best conditions for some applications were obtained by using activated trifluoroethyl esters as acyl donors in a slight excess – in order to circumvent the non-enzymatic acylation – and several ethers as solvents [30–32]. In addition, the reaction mixture had an easy work-up, and the pure product was isolated simply by removing the unreacted β-amino carboxylate either by extraction or by crystallisation [30]. The best results obtained by using different solvents are summarized in Table 1. In those examples (**2a**–**h**), high yields in short times (up to 40 h) were achieved. In some cases, synthesis at gram-scale in the optimised conditions was also carried out [30,31]. Finally, Gyarmati et al. reported the resolution of different β-amino acids and β-lactams by using similar reaction conditions [31,32].

4.2. CAL-A synthesis of acyclic amino acids

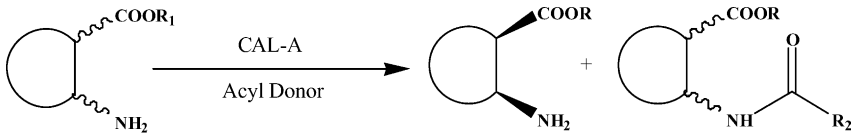
A high number of applications in the resolution of amino acids have been reported in the last few years, leading in many cases to enantiospecific reactions. The most relevant examples are reported in Table 2 (**5a**–**m**).

Once again, the choice of the best reaction media turned out to be crucial for the correct asymmetric resolutions. As reported in Table 2, in some cases, the reaction was performed using butyl butanoate as acyl donor. Since this compound is not as reactive as the 2,2,2-trifluoroethyl derivative, in those examples, the excess of the acyl donor was not a problem, and thus the reaction was carried out using solvent free procedure, adding the enzyme and the racemic substrate in butyl butanoate. In any case, better enantioselectivities and reactivities were observed in the presence of activated acyl donors [36,37].

Moreover, in many of those examples, preparative synthesis at gram-scale was successfully attempted, demonstrating the applicability that those processes could have at practical scale [35–39]. Notably, Kanerva and Sundholm reported better results in terms of enantioselectivity when CAL-A was immobilised onto Celite in the presence of sucrose [40]. Furthermore, the immobilisation strategy led to a remarkable higher stability under the reaction conditions and allowed the re-use of the enzyme. An explanation postulated for the beneficial effect was the highly hydrophilic nature of the Celite, which might lead to an optimal water level for a good performance of the enzyme in organic solvent [36]. Regarding immobilisation procedures, recently an enhancement of the CAL-A activity has been reported by immobilising it in static emulsions in silicone spheres [41].

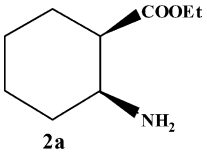
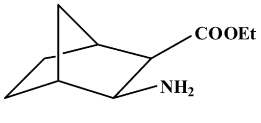
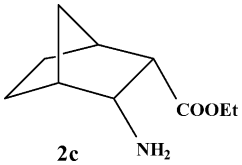
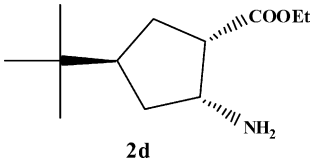
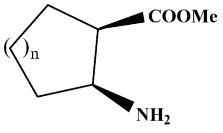
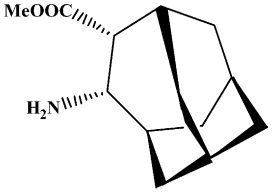
Furthermore, the chemoselective *N*-acylation of the β-tryptophan nitrile derivative (**7**) has been reported [38] (Scheme 1). Interestingly, other frequently used lipases (i.e. CAL-B) were unable to display any activity towards this substrate, which emphasises once again the potential of CAL-A, being unique for many synthetic processes [38].

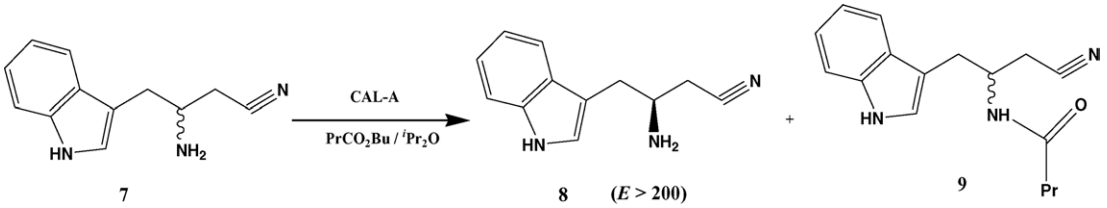
For the reaction reported in Scheme 1, the selection of acyl donor and solvent was crucial. A high enantioselectivity ((**8**) $E > 200$) was achieved when the reaction was performed in diisopropyl ether and using butyl butanoate as acyl donor [38].



1 **2** **3**

Table 1
Examples of the CAL-A asymmetric synthesis of alicyclic amino acids

Product (**2**)	Acyl donor (R_2)	Solvent	*E*	Reference
 **2a**	2,2,2-Trifluoroethyl hexanoate	Et_2O	≫100	[30]
 **2b**	2,2,2-Trifluoroethyl hexanoate	Et_2O	≫100	[30]
 **2c**	2,2,2-Trifluoroethyl hexanoate	Et_2O	≫100	[30]
 **2d**	2,2,2-Trifluoroethyl acetate	$^iPr_2O$	≫100	[30]
 **n: 3, 4, 8**	2,2,2-Trifluoroethyl butanoate	$^iPr_2O$	≫200	[31]
2e-g  **2h**	2,2,2-Trifluoroethyl butanoate	$^iPr_2O$	≫200	[32]



7 **8** (*E* > 200) **9**

Scheme 1. CAL-A asymmetric resolution of β-tryptophan nitrile derivative, recently reported [38].

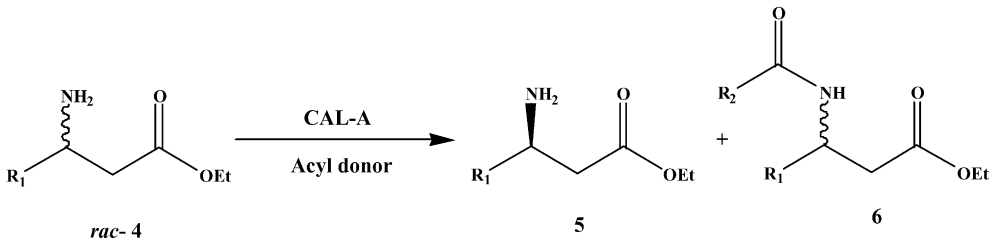
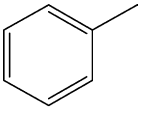
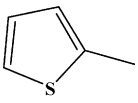
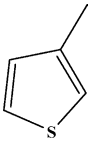
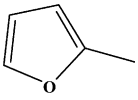
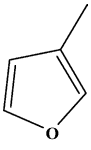
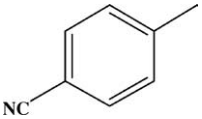
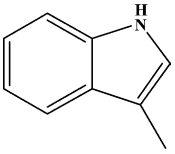
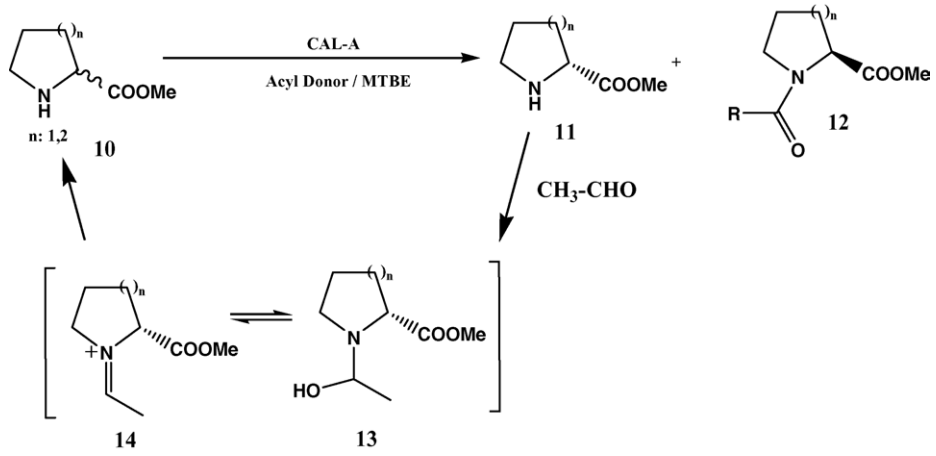


Table 2
Selection of examples of the CAL-A asymmetric synthesis of acyclic β-amino acids

R_1 (**5**)	Acyl donor	Solvent	*E*	Reference
$-CH_3$ (**5a**)	2,2,2-Trifluoroethyl butanoate	$^iPr_2O$	100	[33]
−COOMe (**5b**)	2,2,2-Trifluoroethyl butanoate	AcCN	20	[34]
$-CH_2-CH_3$ (**5c**)	Butyl butanoate	$^iPr_2O$	>200	[35]
$-CH-(CH_3)_2$ (**5d**)	2,2,2-Trifluoroethyl butanoate	$^iPr_2O$	106	[35]
$-CH_2-CH_2-CH_3$ (**5e**)	Butyl butanoate	$^iPr_2O$	>100	[35]
 (**5f**)	2,2,2-Trifluoroethyl butanoate	$^iPr_2O$	>100	[35]
 (**5g**)	2,2,2-Trifluoroethyl butanoate	$^iPr_2O$	75	[35]
 (**5h**)	Butyl butanoate	Butyl butanoate (neat)	580	[36]
 (**5i**)	2,2,2-Trifluoroethyl butanoate	$^iPr_2O$	245	[36]
 (**5j**)	Butyl butanoate	Butyl butanoate (neat)	220	[36]
 (**5k**)	2,2,2-Trifluoroethyl butanoate	$^iPr_2O$	510	[36]
 (**5l**)	Butyl butanoate	Butyl butanoate (neat)	143	[37]
 (**5m**)	Butyl butanoate	$^iPr_2O$	>200	[38]



Scheme 2. Dynamic kinetic resolution performed recently by Kanerva and co-workers [43].

4.3. Chemoselective N-acylation of N-heterocyclic α-amino esters

Considering the capability of CAL-A to acylate the NH group, its application was extended to secondary cyclic amines, such as proline and pipecolinate derivatives (**10**). As reported by Liljeblad et al. [42,43], the reaction proceeded at competitive time and with high chemoselectivity even at gram-scale. Moreover, the dynamic kinetic resolution approach was applied successfully, by racemising the remanent *N*-heterocyclic enantiomer with acetaldehyde, via several intermediates (**13**–**14**) (Scheme 2).

For this reaction, vinyl butanoate was used as acyl donor. Thus, the aldehyde formed (as by-product) racemised in situ the remanent substrate. By optimising the reaction conditions, the product was obtained with high enantioselectivity (ee 97%) and a yield of approximately 90% [43].

Finally, the application of the CAL-A in the asymmetric synthesis of different hydroxymethylpiperidines was evaluated. The purpose of this evaluation was to study the behaviour of CAL-A in the presence of a molecule with two nucleophilic groups [44]. The enzymatic reaction proceeded selectively towards the *O*-acylation, but competition with the chemical $O \rightarrow N$ migration was observed. Consequently, mixtures of products (*N*- and *O*-acylated, *N*-acylated and/or *O*-acylated) were obtained. Due to the competition between the enzymatic reaction and chemical $O \rightarrow N$ migration, a careful control of the reaction conditions was crucial to achieve the desired selectivity in the synthesis of the product [44].

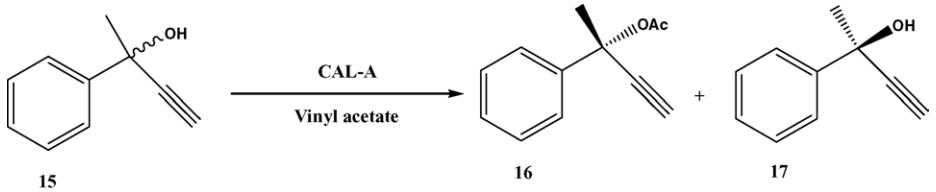
5. Applications of the CAL-A acceptance of sterically hindered alcohols

Another important feature of CAL-A is its capability to accept highly sterically hindered alcohols [45]. This is not very common among hydrolases, and thus represents a promising research line, since such bulky structures are a useful group of building blocks.

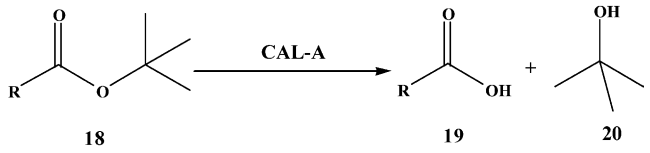
5.1. Enzymatic CAL-A activity towards tertiary alcohols

Recently, it has been discovered that such capability is due to an aminoacidic motif within the oxyanion binding pocket of various lipases and esterases [46,47]. Therefore, only those enzymes which have this amino acid motif – actually the GGGX sequence (G: Glycine, X: any amino acid) – are able to accept such hindered structures [48]. Together with CAL-A, *C. rugosa* lipases, pig liver esterase and esterase from *Bacillus subtilis* among others, also show the mentioned motif and conclusively are active in such reactions [46,47]. The discovery of the mentioned motif is noteworthy, since now the classic screening for finding new hydrolases able to accept such structures is not necessary anymore [49].

Addressing this fact, several examples have been reported for the CAL-A catalysis of those bulky structures [45,48,50]. The first example of highly enantioselective enzyme-catalysed resolution of a tertiary alcohol (**15**) was published by Hari Krishna et al. [48] (Scheme 3). The reaction proceeds with high enantioselectivity ($E > 87$) in different reaction condi-

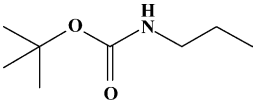
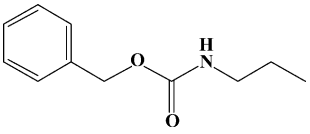
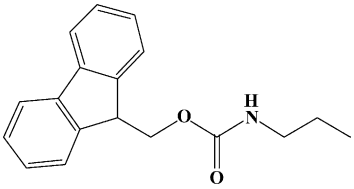
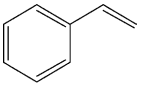
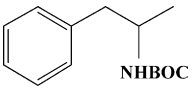
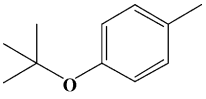


Scheme 3. CAL-A-catalysed kinetic resolution of 2-phenylbut-3-yn-2-ol in organic solvents, recently reported [48].



18 19 20

Table 3
CAL-A utilisation in the removal of *tert*-butyl protecting group, as reported recently [49]

R (**18**)	Reaction media	Reaction time (h)	Yield (isolated) (%)
 (**18a**)	Buffer	48	50
 (**18b**)	Buffer	24	80
 (**18c**)	Buffer	48	40
 (**18d**)	Buffer	48	21
 (**18e**)	Buffer/hexane	48	68
 (**18f**)	Buffer/hexane	48	12
 (**18g**)	Buffer/hexane	60	–

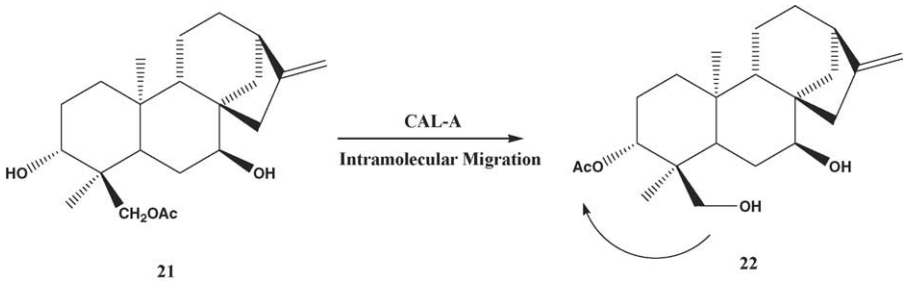
Temperature: 37–50 °C; 20 mM substrate.

tions, although moderate yields were obtained (up to 25%), which presumably were related to obvious steric hindrance. In relation to this, the directed evolution strategies would represent an excellent alternative to improve the preliminary results reported.

The reactivity of CAL-A towards tertiary alcohols has recently led to another interesting application for the removal of the *tert*-butyl protecting group, even at high temperature (up to 50 °C) [49]. In Table 3, several examples of this application are summarized (**18a**–**g**).

5.2. Applications of CAL-A towards secondary sterically hindered alcohols

The capability of CAL-A to accept sterically hindered alcohols has also been exploited for selective acylation of different sterols [51]. On the other hand, a recent article describes the CAL-A acylation of the –OH group in the ring A of *ent*-Kaurane (**21**) diterpenoid via intramolecular migration/acylation of the acetate group from position 18 to 3 [52] (Scheme 4). For such purpose, the presence of the enzyme was crucial, as the migration



Scheme 4. CAL-A intramolecular acylation in the ring A of the *ent*-Kaurane diterpenoid [52].

activity was not found when reactions were conducted without lipase. Also, CAL-B, *C. rugosa* lipase, *Pseudomonas* sp. lipase and porcine pancreatic lipase showed activity in this reaction.

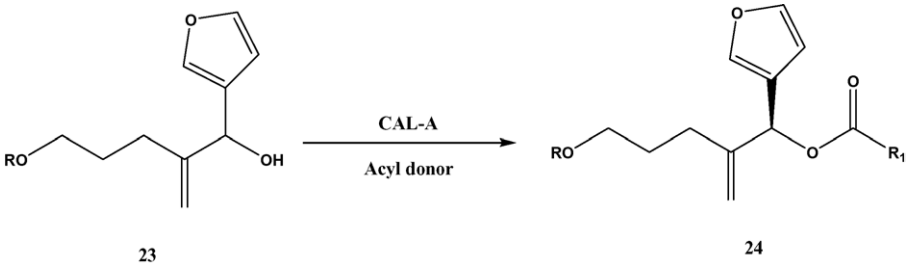
CAL-A has also been employed successfully in the kinetic resolution of a furyl substituted allyl alcohol intermediate (**23**) in different solvents with high enantioselectivity ($E > 300$) (Scheme 5) [53].

Finally, some other articles describing the applicability of CAL-A with sterically hindered alcohols have been published: the enzymatic lactonisation to produce homologues of the mosquito *Culex* sp. oviposition pheromone [54,55], the asymmetric synthesis in the resolution of piperidine hydroxy esters [56] and a chemoenzymatic synthesis of the (−)-paroxetine, a selective serotonine reuptake inhibitor [57].

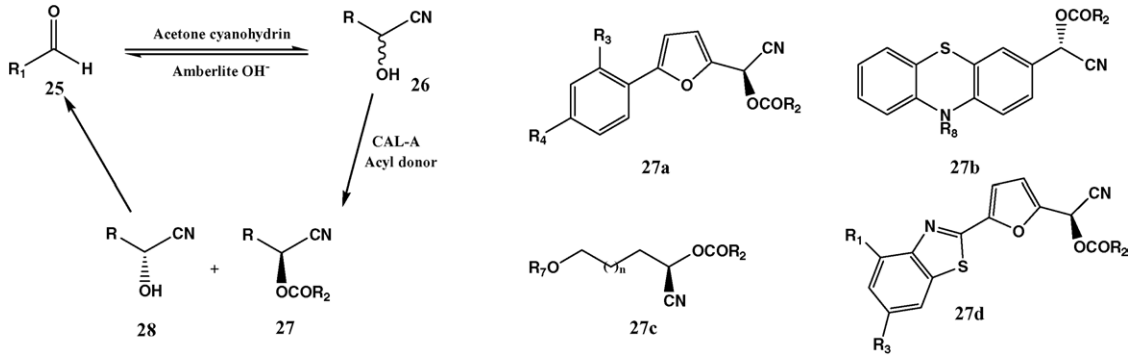
5.3. (Dynamic) kinetic resolution of cyanohydrins

Another interesting application of CAL-A is the synthesis of chiral cyanohydrins (**27a**–**d**), both in kinetic and dynamic kinetic resolutions [58–60]. The strategy is based on the equilibrium between the formation and decomposition of cyanohydrins (**25**–**26**) in the presence of Amberlite IRA-904 basic resin in organic solvents. For such purpose, the stability of the acylated cyanohydrin (**27**) in the reaction conditions, as well as the capability of CAL-A to carry out such a process providing sufficient yield and enantioselection, turns out crucial. In Scheme 6, the process, as well as the main important product groups described in recent literature (**27a**–**d**), is depicted.

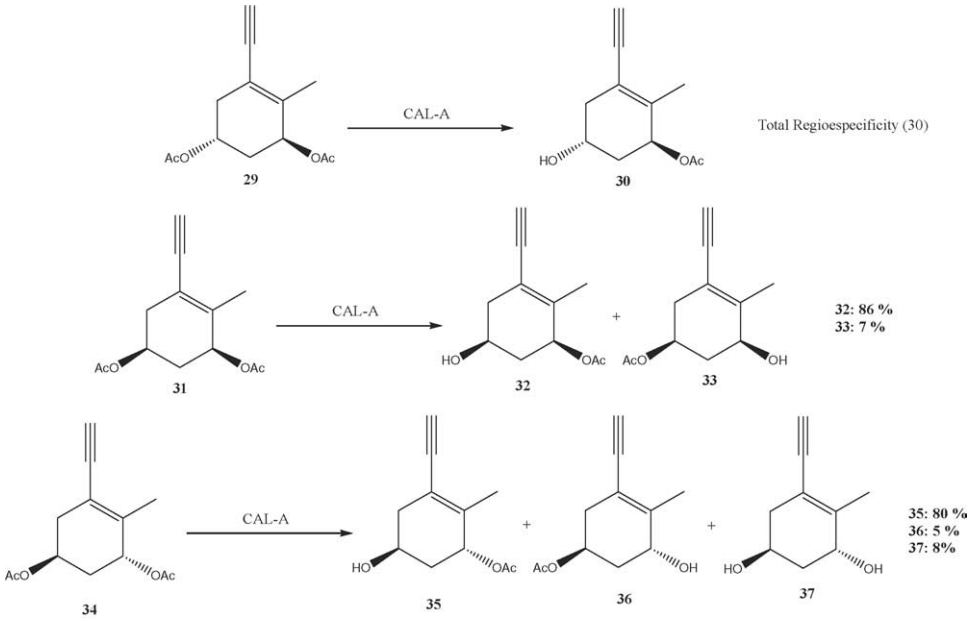
A high number of successful examples have been reported in this field. Acetonitrile turned out to be the best solvent, providing acceptable solubility for the cyanohydrin. In addition, this solvent was not detrimental to the enzymatic activity. As acyl donor, the use of vinyl acetate resulted in good yields, even though the exclusive use of isoprenyl acetate had been reported for *Pseudomonas* lipase PS for such processes [61]. Interestingly, the lipase PS did not display any activity towards furylbenzotiazol cyanohydrins derivatives (**27d**), for which the CAL-A turned out to be an excellent catalyst (conversion > 99%; ee > 96%) [58–60]. For other cyanohydrin structures (**27b** and **c**), a similar CAL-A performance was found. For these reactions,



Scheme 5. CAL-A acylation of a hindered furyl substituted allyl alcohol, reported recently [53].



Scheme 6. Strategy for the dynamic kinetic resolution of in situ formed cyanohydrins. Structures resolved by CAL-A reported in literature.



Scheme 7. CAL-A regioselectivity hydrolysis of cyclic diacetates [63].

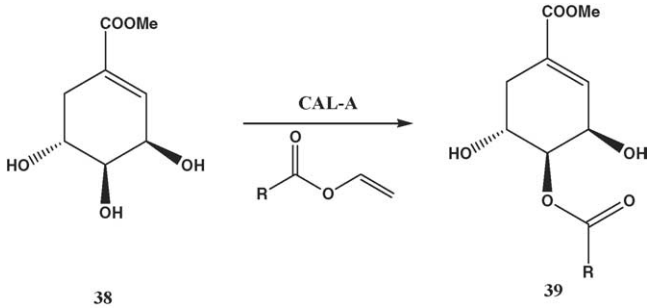
the amount of enzyme used was crucial for achieving a high enantioselectivity [58]. The synthetic platform was also useful for the production of racemates in mild conditions. Thus, when the CAL-A acylation of phenylfuran-based cyanohydrin (**27a**) was tested in toluene as solvent, racemate esters were obtained. These molecules were subsequently used as substrates for the enzymatic lipase PS racemic resolution [62]. This two-enzymes-based approach is an attractive alternative since the chemical synthesis usually leads to different mixtures of products.

Furthermore, the influence of the protecting group R_7 (**27c**) was studied by molecular modelling techniques, in the case of the lipase PS, since the tridimensional structure of this lipase is available [60]. Once again, the elucidation of the structure of the CAL-A will probably bring more applications in the field by starting from the theoretical data reported.

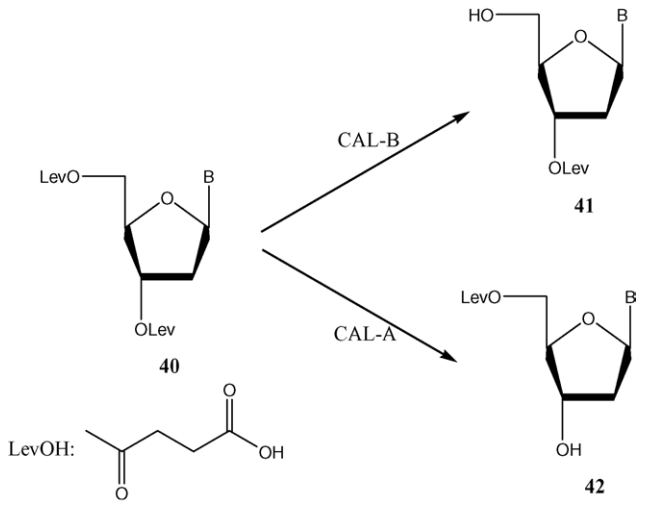
5.4. CAL-A regioselectivity towards cyclic alcohols

Some CAL-A hydrolytic and synthetic reactions have been described in the field. Thus, different CAL-A selectivities towards the hydrolysis of several 1-(2-yn-ethyl)-2-methyl-3,5-diacetate-1-cyclohexene (**29**, **31** and **34**) have been published [63] (Scheme 7). These molecules are useful building blocks in the synthesis of Vitamin D_3 derivatives. As observed, CAL-A shows regioselectivity towards the C-5, being strongly dependent on the absolute configuration of the diacetates formed. Depending on that, either a total regiospecific synthesis is observed or mixtures of compounds are achieved. In some cases, also the diol was formed [63] (Scheme 7).

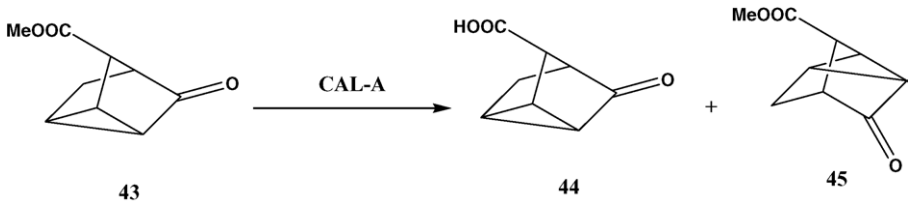
The behaviour of CAL-A changes if analogous molecules are tested. Thus, when the regioselective acylation of methyl shikimate (**38**) is performed, a regioselection towards the –OH located in the C-4 position is observed. A complete study of the parameters that control such regioselection was reported [64,65] (Scheme 8).



Scheme 8. CAL-A regioselective acylation of methyl shikimate [64,65].



Scheme 9. CAL-A and CAL-B regioselective hydrolysis of different levulinyl-nucleosides [75].



Scheme 10. Hydrolysis of sterically hindered acids catalysed by CAL-A in aqueous media [77].

6. Other biotechnological applications and remarks

The asymmetric alkoxycarbonylation of different vinyl carbonates with (*R*,*S*)-1-phenyl-ethyl-amine catalysed by CAL-A has been reported with high yields and enantioselectivities [66], as well as the asymmetric amidation, which also proceeded with excellent results when CAL-A was used as the biocatalyst [67].

Some syntheses with diols have been reported [68], as well as solvent free alcoholysis at preparative scale [69]. Besides, the role of a contaminant esterase present in the CAL-A crude samples (Novozymes 868) has been investigated by purifying both enzymes (CAL-A as such, and esterase) and studying them separately [70]. On the other hand, synthetic applications of the CAL-A in ionic liquids were carried out, with different effects depending on the reaction/substrates tested [48,71,72]. This topic represents a promising task for the development of more fundamental knowledge concerning CAL-A, as well as for biocatalysis in general, as reported for recent reviews in the field [73,74].

The use of CAL-A for biotechnological purposes has also found applications in the synthesis of nucleosides. In this area, it has been reported that CAL-A and CAL-B show opposite regioselection in the enzymatic regioselective hydrolysis of 3′,5′-di-*O*-levulinylnucleosides (**40**) (Scheme 9) [75]. This fact is remarkable, since two different structures can be achieved by using both lipases, with almost complete regioselection. The process was also feasible at relative high substrate concentrations (0.25 M).

Finally, the hydrolysis of different sterically hindered carboxylic acids (**43**) catalysed by CAL-A has been reported at gram-scale [76,77]. In these processes, conducted in buffer, CAL-A was the best biocatalyst tested (Scheme 10).

7. Conclusions and outlook

The present review aims to highlight the most important and promising biotechnological applications of CAL-A, derived from its almost unique properties within the hydrolases. Although this enzyme did not receive special attention in the past, recently the number of its applications widely increased. There are good reasons to believe that in the forthcoming years, even more applications will be reported. In relation to this, one of the most promising lines will be the enhancement of the CAL-A substrate spectra in the asymmetric synthesis. On the other hand, once that the tridimensional structure of the CAL-A will be elucidated, it will be possible to combine this with the molecular biology techniques in order to develop CAL-A mutants for certain specific uses. Hence, the combination of the cloning technologies with the impressive thermostability of CAL-A will give many possibilities in a wide range of synthetic applications. Furthermore, the *trans*-fatty acid preference will also represent an important tool for future processes.

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