Chloroperoxidase-catalyzed amino alcohol oxidation: substrate specificity and novel strategy for the synthesis of *N*-Cbz-3-aminopropanal

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Highlights

- Influence of amino alcohols structure on chloroperoxidase specificity was assessed
- Effect of peroxide and substrate on the enzyme stability was evaluated
- A chemical reaction between the amino aldehyde and peroxides was identified
- High *N*-Cbz-3-aminopropanol conversion was obtained by enzymatic oxidation

Abstract

The ability of chloroperoxidase (CPO) to catalyze amino alcohol oxidations was investigated. The oxidations of compounds with different configurations with respect to the amine position towards hydroxyl – using H_2O_2 and *tert*-butyl hydroperoxide (*t*-BuOOH) – were analyzed in terms of the initial reaction rate, substrate conversion, and CPO operational stability. It was observed that the further the amino group from the hydroxyl, the lower the initial reaction rate. The effect of the amino-protecting group and other substituents (i.e., methyl and hydroxyl) was also examined, revealing an increase in steric hindrance due to the effect of bulky substituents. The observed reaction rates were higher with *t*-BuOOH, whereas CPO was more stable with H_2O_2. Moreover, CPO stability had to be determined case by case as the enzyme activity was modulated by the substrate. The oxidation of *N*-Cbz-3-aminopropanol (Cbz, carboxybenzyl) to *N*-Cbz-3-aminopropanal was investigated. Main operational conditions such as the reaction medium, initial amino alcohol concentration, and peroxide nature were studied. The reaction kinetics was determined, and no substrate inhibition was observed. By-products from a chemical reaction between the formed amino aldehyde and the peroxide were identified, and a novel reaction mechanism was proposed. Finally, the biotransformation was achieved by reducing side reactions and identifying the key factors to be addressed to further optimize the product yield.

Keywords: chloroperoxidase (CPO); amino alcohol oxidation; substrate specificity for chloroperoxidase; *N*-Cbz-3-aminopropanal synthesis; aldehyde–peroxide reaction; D-fagomine precursor.

Chemical compounds studied in this article: *N*-Cbz-2-aminoethanol (PubChem CID: 280458), *N*-Cbz-3-aminopropanol (PubChem CID: 562256), *N*-Cbz-3-aminopropanal (PubChem CID: 10398106), *N*-Cbz-3-aminopropanoic acid (PubChem CID: 736104), 3-amino-1-propanol (PubChem CID: 9086), 4-amino-2-butanol (PubChem CID: 170254), *N*-Cbz-2-hydroxy-3-aminoethanol (PubChem CID: 3362144), *N*-Fmoc-4-aminobutanol (PubChem CID: 57365309), *N*-Cbz-5-aminopentanol (PubChem CID: 4072414), *N*-Cbz-6-aminohexanol (PubChem CID: 24890214).

1. Introduction

Chloroperoxidase (CPO, EC 1.11.1.10), isolated from the fungus *Caldariomyces fumago*, is one of the most versatile heme-containing enzymes as it catalyzes a wide variety of reactions, apart from its natural halogenation.[1–3] The halide-independent reactions catalyzed by this heme protein include oxidative dehydrogenation, H_2O_2 dismutation, and oxygen transfer reactions (e.g. alcohol oxidation).[4–9] Although CPO catalyzes alcohol oxidation without requiring cofactors, it uses peroxides, such as hydrogen peroxide (H_2O_2) or tert-butyl hydroperoxide (*t*-BuOOH), as electron acceptors. Although peroxides are required for this reaction, they rapidly deactivate CPO.[10–12] Thus, the peroxide nature and its rate of addition to the reactor are key operation parameters due to their effect on CPO stability.

Comentari [A1]: Revised sentence.

In this work, we performed CPO-catalyzed oxidation of amino alcohols to yield amino aldehydes. Amino aldehydes are used as substrates of aldolases for aldol addition of dihydroxyacetone (DHA) or dihydroxyacetone phosphate (DHAP) to yield aldol adducts.[13–17] These compounds are precursors of the well-known iminocyclitols, which bind strongly to glycosidases and glycotransferases. Accordingly, they have been extensively studied for their antiviral, anticancer, and antidiabetic effects.[18–21]

However, only few studies have been published on CPO oxidation of amino alcohols, which were restricted to α-amino alcohols.[22,23] The ability of the enzyme to oxidize amino alcohols in other positions – particularly focusing on β-amino alcohols – is worth investigating so as to discover new valuable intermediates and products.

One reaction of particular interest is the CPO-catalyzed oxidation of *N*-Cbz-3-aminopropanol (Cbz, carboxybenzyl) in order to yield *N*-Cbz-3-aminopropanal, an intermediate for the aldol addition of DHA catalyzed by D-fructose-6-phosphate aldolase (FSA, EC 4.1.2.). The Cbz protection of the amino group is necessary to avoid

Schiff base formation between the amine and aldehyde groups of the formed amino aldehyde. The above coupled reaction produces the Cbz-aldol adduct precursor of D-fagomine, an iminocyclitol with significant therapeutic potential. This nutraceutical product reduces the health risks associated with excessive intake of fast-digestible carbohydrates, or an excess of potentially pathogenic bacteria.[24–27]

In recent studies, some authors have chemically synthesized protected amino aldehydes.[28–30] Nevertheless, oxidative biocatalysis may have certain advantages over the chemical process: higher selectivity and hence more control and predictability of the obtained product structure, as well as fewer chemical reagents used. In a recent study, *N*-Cbz-3-aminopropanol oxidation was reported to be catalyzed by horse liver alcohol dehydrogenase; however, in this case, cofactor NADH regeneration was required.[31]

In the present study, the substrate specificity in amino alcohol oxidation catalyzed by CPO is analyzed. For this purpose, several amino alcohol compounds are intended to be oxidized by targeting their molecular substituents. Different configurations according to the hydroxyl position towards amine (α to ε) are considered in terms of the substrate conversion rate and enzyme stability. The effect of peroxide nature is also examined. In addition, a novel enzymatic procedure for the synthesis of *N*-Cbz-3-aminopropanal is presented, using CPO as the biocatalyst and H_2O_2 or t-BuOOH as the peroxide. The main operational conditions (reaction medium, substrate concentration, and peroxide nature) are determined.

Comentari [A2]: Revised sentence.

2. Materials and methods

2.1. Materials

CPO from *C. fumago* (chloride–hydrogen peroxide oxidoreductase, EC 1.11.1.10) was supplied by Chirazyme Labs (Greenville, NC, USA) as a solution of partially purified enzyme (9.54 mg protein ml^{-1}) with a specific activity of 1400 U mg^{-1} protein.

Monochlorodimedone (1,1-dimethyl-4-chloro-3,5-cyclohexadione) and 1-fluoro-2,4-dinitrobenzene (DNFB) were obtained from Fluka (Milwaukee, WI, USA). *N*-Cbz-2-aminoethanol (benzyl *N*-(2-hydroxyethyl)carbamate) **(1,** see Table 1), *N*-Cbz-3-aminopropanol (benzyl *N*-(3-hydroxypropyl)carbamate) **(2.a)**, 3-amino-1-propanol **(2.b)**, *N*-Fmoc-4-aminobutanol **(3)**, *N*-Cbz-5-aminopentanol **(4)**, *N*-Cbz-6-aminohexanol **(5)**, *N*-Cbz-3-aminopropanal (3-[(Benzyloxycarbonyl)amino] propionaldehyde), *N*-Cbz-3-aminopropanoic acid (Z-β-Ala-OH), *tert*-butyl hydroperoxide (*t*-BuOOH, 70 wt. % in H_2O), hydrogen peroxide (H_2O_2, 30 wt. % in H_2O), and trifluoroacetic acid (TFA) were purchased from Sigma Aldrich (St. Louis, MO, USA). 4-Amino-2-butanol **(2.c)** was procured from Acros Organics (Geel, Belgium). *N*-Cbz-2-hydroxy-3-aminoethanol **(2.d)** was supplied by Dr. Clapés from the Biotransformation and Bioactive Molecules Research Group (CSIC, Barcelona, Spain). High-performance liquid chromatography (HPLC)-grade acetonitrile was obtained from Carlo Erba (Milan, Italy). Milli-Q-grade water was used for analytical HPLC. All deuterated solvents for nuclear magnetic resonance (NMR) analysis were purchased from Cortecnet (Voisins-le-Bretonneux, France). All other reagents (analytical grade) were commercially available products.

2.2. CPO stability

The CPO stability was determined by measuring its activity along time. For simplicity, the chlorination activity test was performed. The CPO half-life time ($t_{1/2}$) was experimentally estimated from activity versus time data.

Enzymatic activity was determined by the decrease in absorbance at 278 nm due to the conversion of monochlorodimedone (MCD, ε_{278} =12.2 mM^{-1} cm^{-1})[32] to dichlorodimedone (DCD), according to the method of Hager and Morris.[1] The enzymatic assay contained 100 mM potassium phosphate buffer of pH 2.75, 0.16 mM MCD, 20 mM KCl, 2 mM hydrogen peroxide, and 50 µL of the sample with CPO, in a

total volume of 1 mL. The absorbance was measured at 25 °C, using a Cary 50 Bio UV–Visible Spectrophotometer (Varian, Palo Alto, CA, USA). One activity unit of CPO is defined as the amount of enzyme required for the conversion of 1 μmol of MCD per minute at pH 2.75 and 25 °C. The standard deviation of the CPO activity test was calculated from measurements performed by duplicate.

2.3. Amino alcohol oxidation: monitoring the concentration of substrates and products

The concentrations of amino alcohols and *N*-Cbz-3-aminopropanol-oxidized products (*N*-Cbz-3-aminopropanal and *N*-Cbz-3-aminopropanoic acid) were measured by HPLC analysis in a HPLC Dionex UltiMate 3000 with UltiMate 3000 Variable Wavelength Detector. For chromatographic separation, a reversed-phase column (XBridge BEH C18, 130Å, 5 μm, 4.6 × 250 mm) from Waters (Milford, MA, USA) was used. The reaction samples were dissolved in acetonitrile (MeCN), which deactivates the enzyme and arrests the reaction. All separations were performed by injecting 30 μL of the sample at a flow rate of 1 mL min^{-1} at 30 °C. For all substrates, the solvent system consisted of solvent A (0.1% (v/v) TFA in H_2O) and solvent B (0.095% (v/v) TFA in MeCN/H_2O 4:1 (v/v)). Samples were eluted using a specific multistep gradient for each substrate molecule: (**1**) gradient from 20% to 36% in 24 min, λ = 200 nm; (**2.a**, *N*-Cbz-3-aminopropanal, *N*-Cbz-3-aminopropanoic acid) gradient from 5% to 31% B in 0.35 min and isocratic elution at 30% B over 14.65 min, λ = 254 nm; (**2.d**) gradient from 5% to 28% B in 0.35 min and isocratic elution at 30% B over 9.65 min, λ = 254 nm; (**3**) gradient from 20% to 50% B in 1.5 min and isocratic elution at 30% B over 18.5 min, λ = 200 nm; (**4**) gradient from 20% to 60% B in 1.5 min and isocratic elution at 30% B over 7.5 min, λ = 200 nm; and (**5**) gradient from 20% to 65% B in 1.5 min and isocratic elution at 30% B over 8.5 min, λ = 200 nm.

157 **2.b** and **2.c** were analyzed via a well-established derivatization method with DNFB.[33] In
158 both cases, 50 µL of the sample was mixed with 80 µL of DNFB (37.6 mM in acetone)
159 and 20 µL of 1 M $NaHCO_3$. The mixture was incubated for 1.5 h at 40 °C. The reaction
160 was terminated by the addition of 40 µL of 1 M HCl and 200 µL of MeCN. Samples
161 were eluted with a gradient from 31.3% to 48.8% in 25 min, λ = 360 nm.
162 A quantitative analysis of all compounds was performed by prior calibration with
163 standards of known concentration. The concentration values were converted into molar
164 amount (µmol); that is, the volume of the peroxide added in pulses via a microburette
165 and the volume of the reaction medium withdrawn for each analysis (<10% of the initial
166 volume) were considered. The standard deviation of molar quantities was calculated
167 from HPLC measurements performed by duplicate.
168 *2.4. CPO-catalyzed oxidation of selected amino alcohols*
169 The reaction conditions were adapted from a previous study of our research group.[22]
170 The reaction medium was prepared by dissolving the amino alcohol in 100 mM sodium
171 acetate buffer (pH 5.0) and ethyl acetate (90:10 v/v) in a final volume of 5 mL. The
172 substrate concentration range was 5 mM for α/β-amino alcohols or 0.5 mM for γ/δ/ε-
173 amino alcohols. This difference in the order of magnitude was due to solubility
174 restrictions. All reactions were performed at 25 °C and 1000 rpm of orbital stirring
175 (MultiTherm™, Benchmark Scientific, Edison, NJ, USA), and 450 U mL^{-1} of CPO was
176 used. The reaction was initiated by a pulse of 0.8 mM peroxide and carried out by
177 continuous addition of 3 mM h^{-1} peroxide using a single-syringe automatic
178 microburette (Crison Instruments, Barcelona, Spain). A linear fitting of substrate
179 concentration versus time, at a substrate conversion <10%, was used to estimate initial
180 reaction rates.

Two reaction controls were performed for each substrate oxidation to prevent false-positive results: (a) the substrate was incubated in a reaction buffer, without enzyme or peroxide. This control checked the stability of the substrate itself under reaction conditions. (b) The substrate was incubated in a reaction buffer with peroxide (H_2O_2/*t*-BuOOH) addition at 3 mM h^{-1} and in the absence of enzyme. This second control accounted for possible chemical reactions between the substrate and peroxide, in the absence of enzyme.[34–38]

2.5. CPO-catalyzed oxidation of N-Cbz-3-aminopropanol

The reaction medium contained *N*-Cbz-3-aminopropanol dissolved in 100 mM sodium acetate buffer (pH 5.0) in a final volume of 5 mL. The initial substrate concentration range was 5–38 mM. For reactions performed with an organic solvent – ethyl or butyl acetate – in monophasic or biphasic systems, the initial *N*-Cbz-β-aminopropanol concentration was set at maximum solubility in each medium: 38 mM in an aqueous buffer, 53 mM in an acetate buffer saturated with ethyl acetate, 69 mM in an acetate buffer saturated with butyl acetate, and 85 mM in a biphasic medium of aqueous buffer and ethyl acetate (90:10 v/v). In addition, 450 U ml^{-1} of CPO was used. The reaction performance (peroxide addition, temperature, and agitation) was the same as previously mentioned.

Oxidation reactions at high enzyme load and high peroxide–substrate ratio were carried out at an initial substrate concentration of 15 mM. *N*-Cbz-3-aminopropanol was dissolved in 100 mM sodium acetate buffer (pH 5.0) in a final volume of 0.3 mL, containing 350 mM *t*-BuOOH. For this purpose, 15,000 U mL^{-1} of CPO was used. The reaction was performed in duplicate at 25 °C and 1000 rpm of orbital stirring.

2.6. Amino aldehyde–peroxide chemical reaction

205 *N*-Cbz-3-aminopropanol (β-OH), *N*-Cbz-3-aminopropanal (β-CHO), and *N*-Cbz-3-
206 aminopropanoic acid (β-COOH) were incubated at maximum concentration (38, 17, and
207 11 mM, respectively) in 5 mL of 100 mM sodium acetate buffer (pH 5.0). The reaction
208 was allowed to take place under orbital agitation in a MultiTherm™ device overnight
209 for 19 h. Peroxide was continuously added to the reactor at 3 mM h^{-1}.

210 *2.7. Identification of side-reaction products (compounds **6**–**8**)*

211 Products **6**–**8** were identified either by HPLC–mass spectrometry (MS)–MS or by MS.
212 NMR analyses of the three molecules confirmed the proposed structure. Further
213 information is presented in the related data article.[39]

214 **3. Results and discussion**

215 *3.1. Substrate specificity in CPO-catalyzed amino alcohol oxidations*

216 The amino alcohol oxidative capacity of CPO was investigated by the oxidation of
217 compounds **1**–**5** to amino aldehydes (see *Table 1*). These compounds are amino alcohols
218 with similar hydrocarbon chains selected due to (i) the distinct amine position toward
219 hydroxyl, (ii) presence or absence of an amino-protecting group, and (iii) other specific
220 carbon substitutions. Each substrate oxidation was performed by continuous addition of
221 oxidants such as hydrogen peroxide or t-BuOOH, as explained in the section "Materials
222 and Methods."

223 As seen in Table 2, CPO could accept nearly all compounds **1**–**5** as substrates to
224 catalyze their oxidation, with **2.d** and **3** being the only non-converted molecules. For all
225 substrates, the initial reaction rates (r_0) and conversion at the reaction end point (time at
226 which all substrate was converted or no remaining CPO activity was detected) are
227 described in relation to compound structural issues. The operational stability of CPO
228 toward peroxide nature was also compared.

229 The chemical structures of compounds **1**, **2.a**, **4**, and **5** differ only in the *n* value of CH_2
230 groups and therefore in the amine position toward the hydroxyl. According to the initial
231 reaction rate analysis, the highest r_0 was observed for substrate **1** and the lowest for **5**
232 using either H_2O_2 or *t*-BuOOH as the oxidant. Thus, these results revealed that the
233 further the amino group from the hydroxyl in the substrate molecule, the lower the r_0.
234 Conversely, the presence of amino-protecting groups altered the reaction course in
235 terms of the initial rate and maximum substrate conversion. Thus, the Cbz group in
236 substrate **2.a** reduced r_0 compared with the non-protected substrate **2.b**; due to non-
237 amino protection, r_0 increased 1.4-fold with H_2O_2 as the oxidant and 3.1-fold with *t*-
238 BuOOH. Moreover, for compound **3**, which was protected by
239 fluorenylmethyloxycarbonyl (Fmoc) instead of Cbz, no oxidation was observed,
240 presumably because the protecting group was bulky.
241 The extra methyl substituent in substrate **2.c** R^1 compared with **2.b** had a different
242 impact on the initial rate depending on the oxidant used. The lower initial rate with *t*-
243 BuOOH can be attributed to the steric hindrance produced by the methyl group. Finally,
244 no conversion was observed for substrate **2.d** with any peroxide, probably due to steric
245 hindrance or changes in electron density caused by the presence of the CH_2OH group.
246 Oxidant selection affected the operational stability of CPO and consequently the
247 reaction time course, as reflected by enzyme half-life values obtained in each case. In
248 many substrate reactions, $t_{1/2}$ of CPO was higher with H_2O_2 than with *t*-BuOOH, thus
249 achieving longer reaction periods; in other cases, $t_{1/2}$ was practically the same for both
250 peroxides. For Cbz-protected substrates, conversion of similar order of magnitude was
251 achieved for both oxidants, due to lower reaction rates and higher enzyme stability with
252 H_2O_2 as the peroxide.

253 Substrate species were also found to have a modulatory effect on enzyme stability.
254 Under the same reaction conditions, the specific substrate determined the CPO
255 deactivation degree. As an example, $t_{1/2}$ of CPO was 7.8-fold higher for compound **2.b**
256 than **4** with H_2O_2 as the oxidant. This stabilizing effect of the substrate toward the
257 enzyme is well established.[40,41]

258 *3.2. N-Cbz-3-aminopropanol oxidation catalyzed by CPO*

259 As mentioned previously, our target reaction in the present work is the enzymatic
260 oxidation of *N*-Cbz-3-aminopropanol (β-OH, also compound **2.a**) to yield *N*-Cbz-3-
261 aminopropanal (β-CHO) (see *Scheme 1*). The possible CPO-catalyzed oxidation of the
262 amino aldehyde can yield *N*-Cbz-3-aminopropanoic acid (*N*-Cbz-β-alanine, β-COOH),
263 an undesirable compound if the target is the aldehyde product or an eventual coupling
264 with the FSA-catalyzed aldol addition. Several assays were performed to determine the
265 main operational conditions for β-OH oxidation. According to above CPO-catalyzed
266 oxidation of amino alcohols, *t*-BuOOH was preliminarily chosen as the peroxide for the
267 main reaction as higher r_0 values were obtained with this oxidant.

268 The solubility of β-OH in an aqueous buffer is moderate (maximum 38 mM in 100 mM
269 sodium acetate buffer of pH 5.0). To overcome this limitation, three other media with
270 selected eco-friendly solvents were used: (i) acetate buffer saturated with ethyl acetate,
271 (ii) acetate buffer saturated with butyl acetate, and (iii) biphasic medium of aqueous
272 buffer and ethyl acetate (90:10 v/v). All four media were tested for the CPO operational
273 stability and substrate oxidation. The reactions were performed at an initial β-OH
274 concentration corresponding to maximum solubility in each medium.

275 The enzyme half-life time increased from 3.4 h in an aqueous buffer to 5.1 h in a
276 biphasic medium, as presented in Fig. 1.A. The saturated media stabilities were similar
277 to that measured for the acetate buffer. However, the oxidation curves of the reactions

278 catalyzed in ethyl or butyl acetate (saturated or biphasic) were not as expected with low
279 β-OH conversion (see Fig. 1.B). Maximum initial rate and final β-OH conversion were
280 noted for the aqueous buffer. Conversion in acetate buffer was 21%, but <10% in other
281 cases. This result can be attributed to two possible factors: (a) a protective effect of the
282 substrate toward the enzyme (maximum initial substrate concentration in each case) or
283 (b) a likely *t*-BuOOH partition between the two phases leading to lower peroxide
284 concentration in the aqueous phase. In turn, this would explain the lower deactivation of
285 CPO observed as well as the low oxidation rate in the presence of an organic phase.
286 To establish the appropriate initial β-OH concentration for the biotransformation,
287 several reactions in the aqueous buffer were carried out at different initial substrate
288 concentrations: 10, 16, 19, 26, and 36 mM. The half-life time of CPO increased with the
289 initial substrate concentration in the range studied (see Fig. 2). It was 1.6-fold higher at
290 38 mM than at 11 mM. These results were in concordance with the low $t_{1/2}$ values
291 obtained for the oxidation of compounds 1–5 (initial substrate concentration was 0.5–5
292 mM). Again, the data revealed the protective effect of the substrate on the operational
293 stability of the enzyme.
294 However, substrate inhibition in CPO-catalyzed reactions has been reported
295 previously.[42,43] Nevertheless, it was not significant in the solubility range of β-OH in
296 acetate buffer. The initial reaction rates were calculated and adjusted to a Michaelis–
297 Menten model (see Fig. 3). The estimated K_M and r_{max} values were 34.0 mM and 5.4
298 mM h^{-1}, respectively.
299 Two reactions were carried out in 100 mM acetate buffer at the same substrate
300 concentration (38 mM β-OH) and peroxide addition rate (3 mM h^{-1}), each one with a
301 different oxidant (*t*-BuOOH or H_2O_2). The evolution of enzymatic activity over time

(see Fig. 4.A), β-OH conversion, and formation of β-CHO and β-COOH (see Fig. 4.B) were evaluated.

The CPO half-life time increased 2.4-fold when hydrogen peroxide was used instead of *t*-BuOOH; however, as expected, the initial β-OH oxidation rate was lower with H_2O_2. Nevertheless, both oxidants led to similar degrees of final β-OH conversion degree (CPO remained active for a longer time with H_2O_2). β-CHO production was faster and higher with *t*-BuOOH, but it conducted to high amino acid yield.

Comentari [A3]: Revised sentence.

3.3. Identification of side reactions in CPO-catalyzed oxidation of N-Cbz-3-aminopropanol

On carefully evaluating the mole number of converted substrate and products obtained with reaction time (see Fig. 4.B), we found a mass imbalance: (a) using *t*-BuOOH at t = 7h, 45.5 µmol of oxidized β-OH yielded only 18.7 µmol of products (16.1 µmol β-CHO and 2.6 µmol β-COOH). (b) Using H_2O_2 at t = 24h, 41.3 µmol of oxidized β-OH yielded only 13 µmol of products (11.5 µmol β-CHO and 1.5 µmol β-COOH).

Therefore, in addition to the reactions presented in Scheme 1, secondary reactions conducting to by-products were assumed to take place. This was hypothesized to be a chemical reaction between any of the three compounds and the peroxide. To confirm this, each molecule was incubated separately in acetate buffer for 19 h with the addition of 3 mM h^{-1} H_2O_2 or t-BuOOH. Both β-OH and β-COOH were not oxidized by any peroxygen species, but the amino aldehyde did react with the two peroxides: (i) 13.5% of initial β-CHO reacted with *t*-BuOOH after 19-h incubation and (ii) 87.1% of β-CHO reacted with H_2O_2. A white precipitate was observed in the reactor when hydrogen peroxide was used. This chemical reaction between the amino aldehyde and the peroxide could explain the mass balance non-closure. This type of reaction has been described elsewhere.[44,45]

Thus, a novel reaction scheme was proposed (see *Scheme 2*). It includes one by-product when the selected oxidant is *t*-BuOOH and two with H_2O_2. Then, for *t*-BuOOH, the putative product is benzyl (3-(*tert*-butylperoxy)-3-hydroxypropyl)carbamate ($C_{15}H_{23}NO_5$, compound **6**). For H_2O_2 reactions, the proposed hydroxy alkyl peroxide – benzyl (3-hydroperoxy-3-hydroxypropyl)carbamate ($C_{11}H_{15}NO_5$, compound **7**) – can further react with another amino aldehyde molecule and form a dialkyl peroxide (dibenzyl (peroxybis(3-hydroxypropane-3,1-diyl))dicarbamate; $C_{22}H_{28}N_2O_8$, compound **8**). The dialkyl peroxide is only formed with H_2O_2, as the hydroxy peroxy intermediate formed by *t*-BuOOH is not sufficiently chemically reactive to react with another aldehyde. To characterize these molecules, all three compounds were obtained in the absence of salts, which might interfere in the identification analysis. The concentration of each target compound was maximized for the identification according to the mole ratio of peroxide/β-CHO. Compounds **6–7** were soluble under reaction conditions, whereas **8** was highly insoluble and formed a solid precipitate.

Compounds **6–8** were identified by MS and NMR spectroscopy. The exact mass of compound **6** was obtained by MS: 320.1469 (ESI+, positive electrospray ionization). The chemical structure of molecule **6** was elucidated by ^{1}H NMR spectroscopy, via one-dimensional (1D)-selective nuclear Overhauser effect spectroscopy (NOESY) experiments. Reaction intermediate **7** was detected, and its mass was identified by HPLC-MS (264.0842). By HPLC–MS/MS, the main ion fragment was identified (201.0769), which confirmed the presence of the Cbz substituent. The concerted analysis of 1D and 2D NMR experiments allowed full ^{1}H and ^{13}C NMR characterization of **7**, confirming its molecular structure. Finally, compound **8** was analyzed by MS–ESI+ (471.1744). The chemical structure of **8** was obtained by 1D, 2D, and diffusion-ordered spectroscopy (DOSY) NMR experiments.[39]

Comentari [A4]: Revised sentence.

352 An experimental verification of the proposed reaction mechanism was required to
353 ensure its consistency. The test was performed using hydrogen peroxide, because the
354 secondary reactions in this case are more significant. β-OH (380 μmol) in 10 mL of
355 acetate buffer was oxidized by CPO with the addition of 3 mM h^{-1} H_2O_2. After 24 h,
356 82.1 μmol of β-OH underwent conversion. Only 63.6 μmol of aldehyde and acid
357 products were obtained; 18.5 μmol was missing. The 18.5 μmol represented compounds
358 **7** and **8**. Under the reaction conditions, the concentration of by-product **7** was negligible
359 (HPLC analysis quantification). Applying the proposed mechanism scheme and
360 molecular weight of by-product **8**, 4.15 mg was missing (equivalent to 9.25 μmol of by-
361 product **8**). The reaction medium was carefully filtered, the filter and the empty reactor
362 vessel were dried at 35 °C overnight, and the weight increase was considered to be the
363 weight of compound **8** (4.0 ± 0.6 mg). Hence, the proposed mechanism was confirmed.
364 *3.4. Intensification of the CPO-catalyzed oxidation of N-Cbz-3-aminopropanol*
365 The abovementioned results of β-OH oxidation indicated a necessary compromise
366 between CPO stability, rates of substrate oxidation and key product formation, and the
367 presence of side reactions. High substrate conversion was achieved by increasing the
368 enzyme load and the peroxide/substrate ratio. Although hydrogen peroxide is an eco-
369 friendly oxidant with a weaker enzyme-deactivating effect, *t*-BuOOH was the chosen
370 peroxide because it leads to higher initial oxidation rate and a lower degree of side
371 reactions.
372 The reaction was performed under conditions indicated in the section "Materials and
373 Methods." The results are shown in Fig. 5. In this case, 87% β-OH conversion was
374 achieved after 60 min of reaction. In terms of the target product formation, yields of
375 16% β-CHO and 47% β-COOH were obtained. As can be seen in Fig. 5, the product
376 selectivity was in favor of the acid product. It could be driven toward the amino

aldehyde by coupling the CPO-catalyzed reaction with a consecutive one, thus preventing the formation of compound **6**.

4. Conclusions

The amino alcohol oxidative capacity of CPO regarding substrate structure was studied. It is worth noting that CPO accepts α to ε configurations as substrates for the reaction. The effect of this configuration on enzyme catalysis was investigated. It was found that the further the amino group from the hydroxyl in the substrate molecule, the lower the initial reaction rate. The influence of substituents on the amino alcohol molecule was also discussed, especially with respect to the amino-protecting group. The presence of this substituent was significant in the reaction progression, which prevented the chemical reaction between the amino alcohol and the peroxide. However, the reaction rates and final conversions were lower when the amino group was protected.

The deactivation of CPO by peroxides was also examined. Higher stability was observed with hydrogen peroxide than *t*-BuOOH in many reactions. Nevertheless, the substrate species influenced enzyme operational stability: the substrate was found to have a modulatory effect. Therefore, the exact deactivation of CPO should be determined case by case, depending on the substrate.

Furthermore, *N*-Cbz-3-aminopropanal was successfully synthesized by a novel strategy using *N*-Cbz-3-aminopropanol as the starting material and CPO as the biocatalyst. Main operational conditions, such as reaction medium and initial substrate concentration, were studied. The Michaelis–Menten kinetics parameters were estimated. A secondary chemical reaction between the formed amino aldehyde and peroxides was identified, and a new reaction scheme was proposed and validated.

Finally, the extent of the target reaction was determined: high amino alcohol conversion was achieved, but with an amino aldehyde selectivity of only 18 %. Hence, further

research must focus on driving the oxidation in the desired synthetic direction, avoiding the mentioned undesirable reaction. One-pot coupling of the former oxidation and the FSA-catalyzed aldol addition of DHA to yield the D-fagomine precursor is currently being investigated.

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547 **Figure legends**

548 **Figure 1.** Oxidation of *N*-Cbz-3-aminopropanol by CPO and addition of *t*-BuOOH at
549 3mM h^{-1} in 5 mL of the four assayed reaction media: 100 mM sodium acetate buffer of
550 pH 5.0 (⬥), acetate buffer saturated with ethyl acetate (■), acetate buffer saturated with
551 butyl acetate (▲), and biphasic medium buffer/ethyl acetate 90:10 v/v (●). Initial
552 substrate concentrations corresponded to maximum solubility in each medium: 38, 53,
553 68, and 85 mM. 450 U mL^{-1} of CPO was used. **(A)** Operational stability of soluble
554 CPO. **(B)** *N*-Cbz-3-aminopropanol consumption.

555 **Figure 2.** Operational stability of CPO in relation to initial *N*-Cbz-3-aminopropanol
556 concentration: 11 mM (⬥), 21 mM (■), 34 mM (●), and 38 mM (▲). All reactions were
557 catalyzed in 5 mL of 100 mM sodium acetate buffer (pH 5.0) with the addition of 3 mM
558 h^{-1} *t*-BuOOH. 450 U mL^{-1} of CPO was used.

559 **Figure 3.** Lineweaver–Burk plot from the Michaelis–Menten adjustment of CPO-
560 catalyzed oxidation of β-OH. Reactions were performed at initial substrate
561 concentrations of 10, 16, 19, 26, and 36 mM β-OH in 5 mL of 100 mM acetate buffer
562 (pH 5.0) with the addition of 3 mM h^{-1} *t*-BuOOH. 450 U mL^{-1} of CPO was used.

563 **Figure 4.** Oxidation of *N*-Cbz-3-aminopropanol performed in 5 mL of 100 mM sodium
564 acetate buffer (pH 5.0) with initial substrate concentration set at 38 mM by addition of 3
565 mM h^{-1} *t*-BuOOH/H_2O_2. 450 U mL^{-1} of CPO was used. **(A)** Operational stability of
566 CPO. *t*-BuOOH (○); H_2O_2 (●). (**B**) Substrate consumption and oxidized product
567 formation. *t*-BuOOH: β-OH (○), β-CHO (△), β-COOH (□); H_2O_2: β-OH (●), β-CHO (▲),
568 β-COOH (■)

569 **Figure 5.** Extent of the *N*-Cbz-3-aminopropanol **o**xidation: reaction carried out in 0.3
570 mL of 100 mM sodium acetate buffer (pH 5.0) with 15 mM β-OH and 350 mM *t*-
571 BuOOH as initial concentrations. 15,000 U mL^{-1} of CPO was used. β-OH (●), β-CHO

572 (▲), β-COOH (■), operational stability of CPO (○). Inset: Measured selectivity for Cbz-
573 amino aldehyde (β-CHO) and Cbz-amino acid (β-COOH) products. Selectivity for
574 compound 6 (scheme 2) was calculated by difference.
575
576 **Scheme legends**
577 **Scheme 1**. CPO-catalyzed oxidation of *N*-Cbz-3-aminopropanol to *N*-Cbz-3-
578 aminopropanal and *N*-Cbz-3-aminopropanoic acid.
579 **Scheme 2.** Novel proposed reaction scheme for *N*-Cbz-3-aminopropanol oxidation
580 catalyzed by CPO with the addition of peroxide (H_2O_2/*t*-BuOOH)
581