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ARTICLE TYPE

Laccase-catalysed biotransformation of collismycin derivatives. A novel enzymatic approach for the cleavage of oximes†

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Analogues of the natural product collismycin bearing a carboxylic acid moiety has been efficiently synthesised from several collismycin precursors through a laccase-catalysed oxidation under mild conditions with TEMPO (2,2,6,6-tetramethylpiperidin-1-oxyl) as mediator and aerial O_2 as oxidant in aqueous medium. The biotransformations proceeded with excellent yields (85-95%) and involved, depending on the precursor, the oxidation of a benzylic hydroxyl group or the bioconversion of an aldoxime group into a carboxylic acid. Since the latter is herein reported for the first time, we explored the potential of this novel oxime cleavage with several synthetic aldo- and ketoximes. Thus, the laccase/TEMPO system proved to be an efficient and green alternative for the deprotection of both aromatic and aliphatic ketoximes into the corresponding ketones. On the other hand, the reaction with aldoximes leads to dimeric species generated by coupling reactions.

Introduction

The group of the 2,2´-bipyridyl-containing natural products is constituted by compounds sharing a bipyridine ring system which is further modified with some tailoring modifications. Members of this family include SF2738A-F,[1] pyrisulfoxins,[2] caerulomycins[3] and collismycins.[4] Particularly, the latter were isolated for the first time by Gomi *et al.* from *Streptomyces* species. The biological activities displayed by this family of compounds comprise antibacterial, antifungal and cytotoxic activities[3-5] and collismycin A (**1a**, Figure 1) is also an inhibitor of dexamethasone-glucocorticoid receptor binding and therefore a potential anti-inflammatory agent.[3] Structurally, collismycin derivatives belong to the hybrid polyketides-nonribosomal peptides. Recently, the isolation and characterisation of the gene cluster of **1a** has been described,[6] enabling the expansion of the chemical space of this natural product by genetic engineering. Thus, insertional inactivation allowed the isolation of seven novel analogues modified in the second ring of collismycin[7] whereas a mutasynthesis approach led to two more analogues modified in the first pyridine ring.[8] Although the cytotoxicity of the parental **1a** was not improved, some of them exhibited enhanced neuroprotective activity against oxidative stress in a zebra fish model.[8]

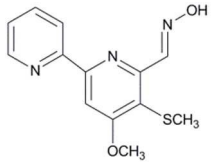

Figure 1 Chemical structure of collismycin A (**1a**).

Laccases are copper-containing enzymes belonging to the group of oxidoreductases. In nature, laccases are involved in biological processes such as lignification in plants or lignin degradation in fungi, and catalyse oxidation of substrates such as phenols, polyphenols and anilines by means of four-electron transfer processes while reducing O_2 to water.[9] Typically, these molecules are preliminary oxidised to reactive radical intermediates which can undergo coupling to provide complex mixtures of dimeric, oligomeric or polymeric derivatives, or can be further oxidised. At first, applications of laccases were aimed at the field of bioremediation and pulp/textile bleaching due to their ability to degrade many recalcitrant pollutants in soil and water. However, recent improvements in protein engineering enable today easy access to these enzymes, which have moved from mere anti-pollutant reagents to smart biocatalysts for biooxidation processes. Thus, the employment of chemical (natural or artificial) mediators has allowed to oxidise not only phenolic compounds but also benzylic, aliphatic, allylic or propargylic alcohols, and the radical species triggered in these processes can led to very complex molecules via tricky cyclisation or coupling reactions.[10] Additionally, the laccase-mediated biooxidations proceed in water, demand O_2 as oxidant and generate only water as by-product, which perfectly fits the present requirements of the chemical industry in its quest for eco-friendly processes. Taking in mind these considerations and our ongoing interest in the discovery of novel collismycins, we wish to report herein our findings regarding the laccase-mediated biooxidation of **1a** and some analogues. Moreover, a novel enzymatic protocol for the deprotection of both aromatic and aliphatic ketoximes under mild reaction conditions is also reported.

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Results and Discussion

Laccase-catalysed oxidation of collismycins 1a-6a

From the plethora of reported analogues, a panel of six collysmicins was selected,[7,8] the parental **1a** and the compounds **2a-6a** (Figure 2), attending to criteria such as its neuroprotectant activity and the diversity of the functional groups present. In fact, laccases have provided to effectively oxidise hydroxyl groups in benzylic positions, like those contained in **4a-6a**, to aldehydes and carboxylic acids.[11] Moreover, there are some examples of laccase-catalysed biotransformations involving pyridylic compounds through oxidative or coupling reactions.[12]

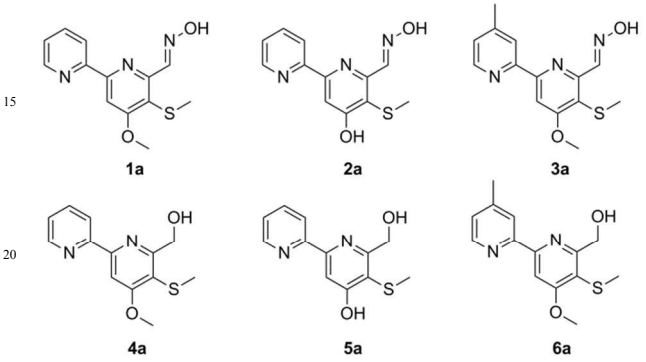
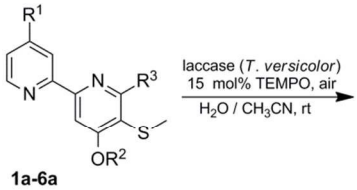


Figure 2 Collismycin derivatives selected for the laccase-catalysed biooxidation.

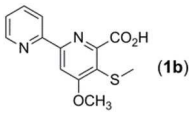
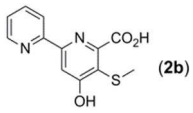
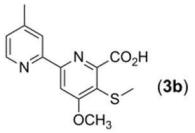
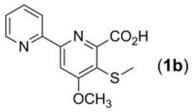
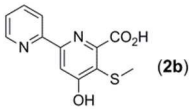
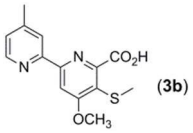
The screening biotransformation conditions were attempted with the most commonly used laccase/mediator system, that is, laccase from *Trametes versicolor* and TEMPO (Table 1). First, the reaction with **1a** was conducted at room temperature, in citrate buffer (pH 5.0), in an open vessel under vigorous stirring. Monitoring by reversed-phase HPLC showed **1a** totally converted after 24 h into a single new compound (**1b**). Additionally, control experiments performed respectively in the absence of laccase or mediator revealed that the biotransformation could not proceed without the presence of both components. MS analysis of **1b** showed a molecular weight of 276 versus 275 mass units for collismycin A. Once isolated, the structure of **1b** was unambiguously identified by NMR spectroscopy as 4-methoxy-5-(methylthio)-[2,2'-bipyridine]-6-carboxylic acid. This compound, which was previously reported by us and named collismycin C,[7] differs from **1a** in the presence of a carboxylic group instead of the aldoxime group. Similarly, the aldoxime-based derivatives **2a** and **3a** led to the corresponding carboxylic acids **2b** and **3b** in excellent yields and purity. Regarding collismycins **4a-6a** bearing a benzylic alcohol, we performed the biotransformation foreseeing in this case the oxidation of the hydroxyl group. This was indeed the result, and the corresponding carboxylic acid derivative was the only isolated product in each case. It is also noteworthy that despite monitoring the process (MS-HPLC) at short reaction time, only traces of the expected aldehyde-intermediate were detected, which suggests fast kinetics of the full bioconversion. Also interestingly, although the collysmicin derivatives **1a-6a** were very soluble in slightly acid solutions like the citrate buffer used herein (pH 5.0), the presence of acid and basic groups in **1b-3b** made challenging their isolation. In order to improve isolation, the biotransformations were performed in water to avoid salts in the reaction medium. All oxidations proceeded well and the products were isolated in excellent isolated yield without chromatographic purification (see Experimental Section).

Table 1 Laccase-catalysed oxidation of collismycins **1a-6a** in the presence of TEMPO and aerial O_2[a]



1a-6a **1b-3b**

R^1, R^2 = H, CH_3
R^3 = CH_2OH, C=N-OH

Entry	Substrate	*t* (h)	Conv./Yield[b]	Product
1	**1a**	24	100 / 85	 (**1b**)
2	**2a**	24	100 / 90	 (**2b**)
3	**3a**	24	100 / 88	 (**3b**)
4	**4a**	12	100 / 95	 (**1b**)
5	**5a**	12	100/ 90	 (**2b**)
6	**6a**	12	100/ 95	 (**3b**)

[a] Reaction conditions: **1a-6a** (0.04 mmol), laccase (1.84 U), TEMPO (15% mol), water (1 mL) and acetonitrile (100 μL), stirring in an open vessel at rt. [b] Conversion measured by HPLC. Isolated yields after lyophilisation.

Mechanism insights

Conventional chemical transformations of oximes (aldoximes and ketoximes) comprise hydrolyses catalysed by inorganic salts to yield aldehydes or ketones,[13] reductions with metals or hydrides to produce amines[14] and the Beckmann rearrangement into amides,[15] which can be subsequently transformed into acids by hydrolysis under harsh conditions. Regarding enzymatic processes, oximes are often found in several metabolic pathways, being converted by the action of enzymes. Thus, there have been

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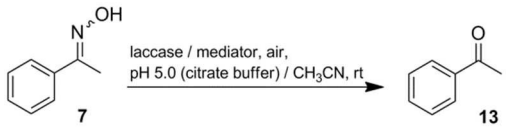
characterised aldoxime dehydratases able to produce nitriles from the corresponding aldoximes.[16] Moreover, Kroutil and co-workers[17] reported a promiscuous activity of alcohol dehydrogenases which reduces aldoximes to alcohols through an initial reduction to the unstable imine (which hydrolyses spontaneously) and a second reduction of the resulting aldehyde.

The unexpected biocatalytic activity ─namely the conversion of an aldoxime into a carboxylic acid occurred in entries 1-3 (Table 1)─ would imply two steps: i) oxime disproportionation into the corresponding aldehyde and hydroxylamine; ii) oxidation of the triggered aldehyde to carboxylic acid (Scheme 1). The initial aldoxime hydrolysis has been previously assumed in biotransformations involving baker's yeast and whole cell biocatalysts[18] and was supported by the detection of the aldehyde by HPLC/MS in selected experiments.[19] So far in the literature there are four main pathways for the deoximation, including hydrolytic, reductive, oxidative pathways as well as transoximation. In general, the oxidative and reductive pathways only need weak acid conditions, since oximes are reduced or oxidised to form unstable intermediates which are much more susceptible to hydrolysis than oximes themselves. Herein, our enzymatic system gives an oxidative environment under mild acid conditions which could transform the oxime into a more readily hydrolysable species. Likewise, deconvolution experiments demonstrated that both enzyme and mediator must be present for the reaction to happen. Although the role of TEMPO cannot be ensured at this point, we postulate that it transforms the oxime into a radical compound which, after some evolution, can be easily hydrolysed.[20] In Scheme 1 is formally depicted the loss of hydroxylamine although another species could be released. Then, the oxidation of the resulting aldehyde to render the carboxylic acid would be easily accomplished by the oxidizing system as in literature reports.[10,11,21] In the case of derivatives **4a-6a**, the benzylic hydroxyl group is particularly activated and prone to undergo oxidation to acid through the reactive aldehyde intermediate. Accordingly, the oxidative bio-deoximation presented herein to convert oximes into acids could be a green alternative to the conventional chemical processes and also expands the biocatalysis toolbox for chemists.

Laccase-catalysed cleavage of aldoximes and ketoximes

The previous findings with collismycins bearing an oxime moiety (**1a-3a**) prompted us to study the scope of this unprecedented enzymatic transformation. First, the laccase-catalysed biotransformation of acetophenone oxime (**7**) was taken as a benchmark reaction and the catalytic activity of several laccases explored in the presence of TEMPO as mediator (Table 2, entries 1-4). Thus, it was found that the bio-deoximation happened exclusively with laccases from *Trametes versicolor* and *Coriolus versicolor* (entries 1 and 4, respectively), the reaction being slightly faster in the first case. Specifically, complete conversion was reached after 14 h with *Trametes versicolor* laccase in citrate buffer 50 mM at pH 5.0 and rt. Once identified the optimal laccase, the reaction was also tested in presence of mediators such as *N*-hydroxyphthalimide (NHPI), hydroxybenzotriazole (HOBT), 2-azaadamantane *N*-oxyl (AZADO) or 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic (ABTS). Although the first three showed incomplete reaction after 24 h, ABTS turned out to be particular effective and reached complete conversion in just 2 h. However, the process was not as clean as the TEMPO-mediated biotransformation and several by-products proved detrimental for the yield. Next, we prepared a set of aldoximes and ketoximes starting from the corresponding carbonyl compounds. While in the first case the oxidative bio-deoximation would led to acid derivatives, in the second a ketone would be obtained.

Table 2 Laccase-catalysed bio-deoximation of **7** in the presence of TEMPO and aerial O_2[a]



7 13

Entry	Laccase	Mediator	*t* (h)	*c* (%)[b]
1	*T. versicolor*	TEMPO	14	100
2	*R. vernicifera*	TEMPO	24	---
3	*A. bisporus*	TEMPO	24	---
4	*C. versicolor*	TEMPO	24	100
5	*T. versicolor*	ABTS	2	100
6	*T. versicolor*	HOBT	24	60
7	*T. versicolor*	NHPI	24	25
8	*T. versicolor*	AZADO	24	80

[a] Reaction conditions: **7** (0.04 mmol), laccase (2 mg; 1.84 U for *T. versicolor*, 100 U for *R. vermicifera*, 8 U for *A. bisporus*, 0.6 U for *C. versicolor*), mediator (15% mol), citrate buffer 50 mM pH 5.0 (1 mL) and acetonitrile (100 µL), stirring in an open vessel at rt. [b] Conversion measured by HPLC.

First, ketoximes from acetophenone (**7**), 1-phenylbutan-2-one (**8**) and benzophenone (**9**) were chosen to cover examples with the oxime moiety in both benzylic and non-benzylic positions as well as a sterically hindered substrate. As can be seen in Table 3, deoximation of ketoximes with the laccase-TEMPO system (entries 1-3) took place efficiently and the corresponding ketones were isolated in excellent yields (>90%). Furthermore, the reaction rate decreased in the following order: **9** > **7** > **8**, presumably caused by the activation of the benzylic positions. In contrast to the previous biotransformations with **1a-6a**, reactions exhibited herein certain turbidity despite using 10% of acetonitrile. However, this percentage of cosolvent enabled a partial solution of the starting products, without inactivation of the laccase, and led to excellent conversions. Regarding aldoximes, we selected those coming from benzaldehyde (**10**), pyridine-2-carboxaldehyde (**11**) and 2-phenylpropanal (**12**). However, in this occasion the expected acids were not formed as in the case of the collismycin derivatives. Instead, mainly we detected by HPLC/MS far more apolar compounds, which are attributable to dimeric species. Actually, this kind of reaction products is very common since laccase-mediated systems typically generate radicals, which are subsequently involved in either oxidative coupling (i.e., bond formation) or bond cleavage. Further efforts to promote the oxidative deoximation against the coupling reaction consisted of experiments performed with a 10- and 100-fold dilution, but were unsuccessful in both cases. Finally, by changing the mediator to ABTS (Table 3, entries 4-6), the target acids **16-18** were obtained although in very low yields

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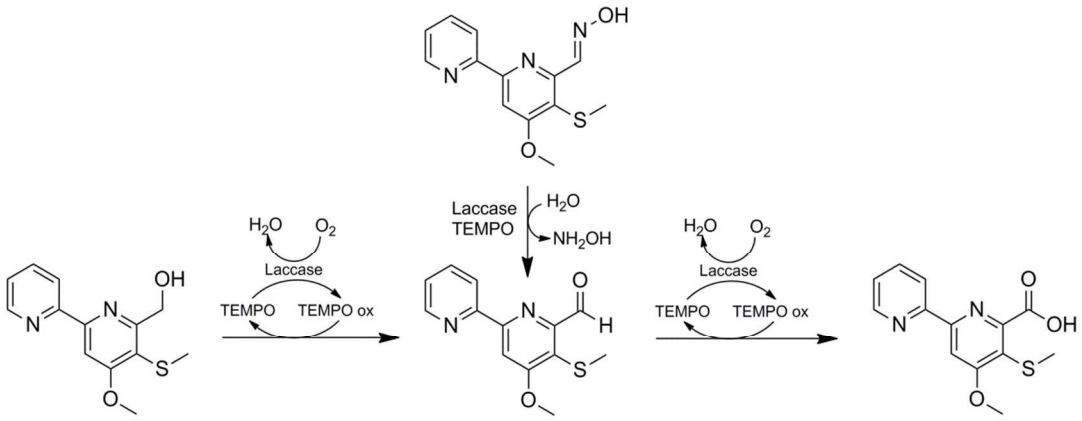
due to by-products. This evidence, taken together with the reaction described above for collismycin, supports the hypothesis that the 2,2'-bipyridyl moiety in collismycin stabilizes the intermediate radical, avoiding thus the coupling reaction and enabling the attack of a water molecule to render, after re-oxidation, a carboxylic acid.

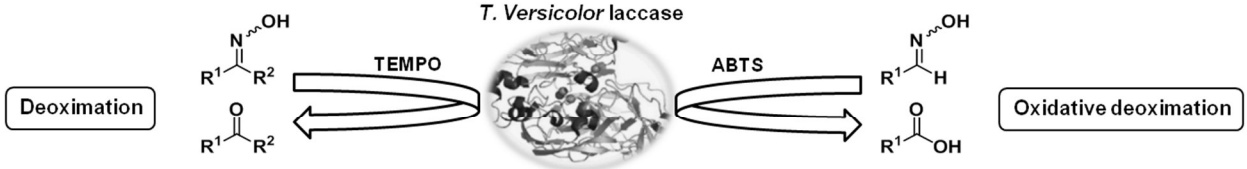
Although the chemical scope of laccases for synthetic purposes has been significantly broaden in recent years, it is worth noting that most applications are aimed to oxidative processes. In fact, there are only two examples of laccases involved in the cleavage of protecting groups, namely the *p*-methoxyphenyl and the benzyl group, both in amines.[22,23] Hence, the enzymatic cleavage of ketoximes disclosed herein represents a new contribution to biocatalytic deprotecting strategies.

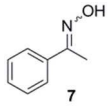
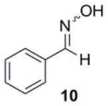
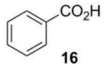
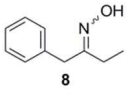
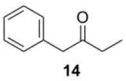
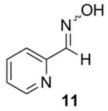
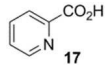
The number of reported methodologies to regenerate carbonyl compounds from the corresponding oximes is impressive. Although most of these methods work well, they suffer from several drawbacks: for example, use of toxic and hazardous transition metal oxidants such as Mn,[24] Cr,[25] Ti,[26] Th,[27] Hg,[28] and problems associated with waste disposal, use of strong Lewis[29] and Bronsted acids,[30] moderate yields and difficulties in the downstream and isolation of products.[31] Additionally, other strategies involve microwave irradiation and photosensitization[25] or drastic conditions of pressure and temperature.[32] Our methodology fits some of the key requirements to be considered as a catalytic green chemical process.[33,34] Thus, the absence of tedious intermediate downstream and purification steps improves the ecological footprint and the efficiency of the process.[35,36] Additionally, to deliver truly *greener* processes the use of a safe, non-toxic, biorenewable and cheap solvent such as water is highly desirable.[37]



Scheme 1. Pathways for the laccase-catalysed biotransformation of collismycins.

Table 3 Laccase-catalysed biotransformation of ketoximes and aldoximes in the presence of TEMPO or ABTS and aerial O_2[a]



Entry	Substrate[b]	*t* (h)	Conv./Yield	Product	Entry	Substrate[b]	*t* (h)	Conv./Yield	Product
1	 **7**	14	100 / 95	 **13**	4	 **10**	12	100 / 20[c]	 **16**
2	 **8**	30	100 / 92	 **14**	5	 **11**	12	100 / 25[c]	 **17**

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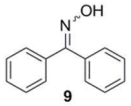
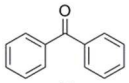
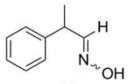
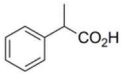
3	 9	10	100 / >95	 15	6	 12	12	100 / 20[c]	 18

[a] Reaction conditions: **7-12** (0.5 mmol), laccase (16.6 U), TEMPO or ABTS (10% mol), citrate buffer 50 mM pH 5.0 (10 mL) and acetonitrile (1 mL), stirring in an open vessel at rt. [b] Z/E mixtures of different ratio depending on the substrate. [c] Yield-drop due to the formation of dimeric species and by-products.

Conclusion

To summarise, an unprecedented and eco-friendly method for the deprotection of ketoximes with laccase as catalyst, TEMPO as mediator and aerial O_2 as the oxidant has been developed. Their simplicity, mildness and efficiency turn this enzymatic approach into a valuable alternative to the existing methods. On the other hand, although the oxidative system suffered from low yields when applied to aldoximes, the presence of an intramolecular stabilising motiff, in this case the 2,2´-bipiridyl unit found in collismycin, makes possible the efficient conversion into the corresponding carboxylic acid.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Experimental procedures, substrate characterization, *E*-factor calculation, analytical data and NMR spectra.

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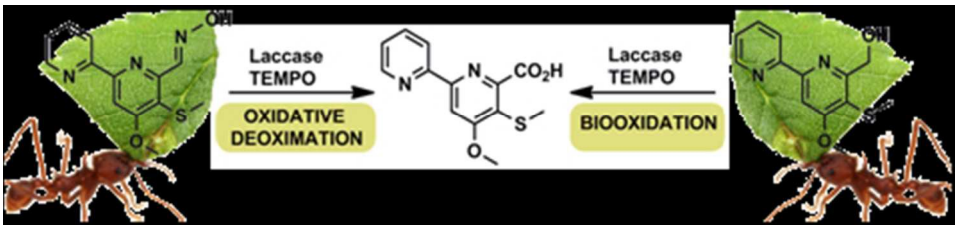

An unprecedented and eco-friendly method for the deprotection of oximes with a laccase/TEMPO system was developed
80x18mm (150 x 150 DPI)