Monoamine oxidase (MAO-N) whole cells biocatalyzed aromatization of 1,2,5,6-tetrahydropyridines into pyridines

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ABSTRACT. A sustainable MAO-N biocatalyzed process for the synthesis of pyridines from aliphatic tetrahydropyridines (THP) has been developed. Pyridine compounds were synthesized under mild reaction conditions and with high conversion exploiting MAO-N whole cells as aromatizing biocatalysts. The kinetic profile of the whole cell biocatalytic transformation was finally investigated via in situ ^{19}F NMR.

KEYWORDS. biocatalysis, MAO-N, pyridine, aromatization, tetrahydropyridine, *in-situ* NMR

INTRODUCTION

The pyridine ring system represents the major class of heterocycles and constitute the key structural unit of many pharmaceutical, agrochemical and material compounds.[1] Natural molecules such as the vitamins niacin and pyridoxine or the ubiquitous redox system NADP/NADPH contain a pyridine nucleus as well as many drugs like the antitubercular agent isoniazid **1**, the analgesic etoricoxib **2** or the anticancer agent sorafenib **3**.[2] Pyridine derivatives also find application in flavor and fragrance industry due to their peculiar olfactory properties ranging from the unpleasant fish-like smell of pyridine itself to the fried chicken smell of 2-pentylpyridine **4** or the peppermint odor of lutidine.[3] A plethora of methodologies for the synthesis of pyridines has been reported in the literature so far.[4] The two most commonly used methods to synthesise substituted pyridines are the Hantzsch[5] and the Chichibabin syntheses.[6] Both these approaches show some limitations though, often leading to desired pyridines in low yields and with the formation of many side products. In addition, the Hantzsch synthesis of pyridines is a two steps protocol which requires an oxidation step commonly carried out with nitric acid, metal complexes or high temperatures.[7] Due to the versatility of pyridine rings and their broad use in different research fields, new approaches to access this important class of compounds are constantly developed.[8]

In an effort to find new, greener and more sustainable routes to access aromatic nitrogen heterocycles from aliphatic precursors, we recently discovered that the monoamine oxidase MAO-N, variants from *Aspergillus niger*, can act as aromatizing biocatalysts and can catalyze the synthesis of pyrrole derivatives **A** from 3-pyrrolines **B** and allylamine substrates[9] (Figure 1). In nature, MAO enzymes are known to metabolize the xenobiotic 1,2,5,6-tetrahydropyridines (THPs) into pyridine derivatives or pyridinium compounds through an aromatization biotransformation. The natural human monoamine oxidase MAO-B catalyzes

the oxidation of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) into the neurotoxin 1-methyl-4-phenylpyridinium (MPP^+), a toxic metabolite involved in Parkinson's disease, through a double oxidative process *via* the 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) intermediate.[10] Inspired by this metabolic biotransformation and as a natural evolution of our previous work, we decided to investigate the possibility to exploit MAO-N as aromatizing biocatalysts in the synthesis of pyridine derivatives and to develop a robust biocatalytic process to access this important class of compounds.

Herein, the development of an aromatizing biocatalytic protocol for the synthesis of pyridines and pyridinium derivatives **D** from aliphatic THP substrates **C** using freeze-dried MAO-N whole cell biocatalysts under mild conditions is described. In addition, an *in-situ* ^{19}F-NMR kinetic study of the MAO-N whole cell biocatalyzed aromatization reaction is presented. *In-situ* NMR allows the real time monitoring and analysis of chemical reactions including biocatalytic processes, as previously described.[11] However, to the best of our knowledge, no examples of *in-situ* NMR studies on whole cell biocatalyzed transformations have been reported yet.

RESULTS AND DISCUSSION

The MAO-N mediated aromatization of the THP **5a** into the pyridine **6a** was first investigated. Freeze-dried whole cells containing MAO-N variants D5, D9, and D11 were selected on the basis on their known activity and selectivity toward structurally related piperidines.[12] The THP **5a** was suspended in a buffer solution (pH = 7.8) in the presence of minimum amount of cosolvent (DMSO or isooctane[8]) and treated with different MAO-N biocatalysts at 37 ºC for 18-24h. The conversion (%) of the biotransformation reaction was determined by ^1H-NMR integration of the crude mixture or by HPLC. Results are reported in Table 1. The treatment of **5a** with MAO-N variants D5, D9 and D11 in a 1:66 buffer/DMSO

mixture lead to the desired pyridine **6a** with good conversions (*entries 1-3*) with the best result obtained with enzyme D9 (76%). Interestingly, when a DMSO/DMF 1:4 mixture was used as cosolvent (1.6% of the whole reaction mixture) an increase in the yield of **6a** was observed (*entry 4*). On the other hand, excellent and greater conversions (>90%, *entries 5-7*) were obtained when isooctane was employed. In the presence of isooctane, the aromatization reaction was completed in 18h with all the MAO-N variants and, again, the variant D9 proved to be the best biocatalyst. The role of the cosolvent seems to be crucial to some extent for the outcome of the reaction as shown in our previous work.[9] Isooctane is a water non-miscible solvent, which, together with the buffer solution, generates a biphasic system with low mass transfer.[13] We hypothesised that, even if in small concentration, a water miscible solvent like DMSO may have, in the long term, a detrimental effect on the MAO-N biocatalyst leading to its progressive inactivation. On the other hand, in the presence of a non-water miscible solvent like isooctane this inactivation could be prevented, thus leading, as final result, to higher substrate conversion. Although substrate/product phase transfer mechanisms are not fully clear for this biotransformation, it is reasonable to envisage that the aromatization of **5a** into **6a** could occur at or in proximity of the water-solvent interphase. It is also possible that different cosolvents affect the permeability of cell membranes of the whole cell biocatalysts, in turn aiding the substrate uptake and improving the reaction outcome. Interestingly, as further corroboration of the key role of the cosolvent, when the biotransformation was carried out in buffer only, the pyridine **6a** was obtained with only 80% conversion (*entry 8*). The aromatization of **5a** with the nicotine oxidase biocatalyst 6-HDNO E350L/E352D[14] was also explored, but no conversion was observed and the THP was fully recovered from the reaction mixture (*entry 9*). Finally, as confirmation that the aromatization of **5a** is catalyzed by the MAO-N, rather than spontaneously promoted by the oxygen in the air or other chemicals and side products present in the reaction mixture, a series of blank experiments were carried out.

When no biocatalyst was added to the reaction mixture (*entry 10*), no traces of the pyridine **6a** were detected after 24h, confirming that the MAO-N is responsible for the aromatization of **5a**. Similarly, the treatment of **5a** with *E. coli* BL21(DE3) cells no harboring MAO-N enzymes under optimized conditions did not lead to any pyridine **6a** (*entry 11*). The MAO-N catalyzed biotransformations requires oxygen as co-substrate for the oxidation reaction, thereby producing H_2O_2 as a side-product. In order to confirm if the H_2O_2 formed during the biotransformation affects the aromatization of the THP, the oxidation of **5a** in the presence of the enzyme catalase was investigated. In fact, catalase acts as H_2O_2 scavenger, converting the hydrogen peroxide formed during the MAO-N biocatalyzed aromatization into H_2O. When the substrate **5a** was treated with MAO-N D9 in the presence of catalase, the desired pyridine **6a** was formed with 72% conversion after 18h (*entry 12*), thus confirming that the H_2O_2 has no effect (or only minor effect) on the biocatalytic aromatization of THPs into pyridines. As further confirmation that H_2O_2 was not responsible for the aromatization of the THP, the substrate **5a** was suspended in buffer solution (pH 7.8) at 37 °C and treated with H_2O_2 30% w/w. Only traces (<4%) of the pyridine **6a** were detected from the reaction mixture after 24h.

According to previous studies on the MAO metabolism,[10] we assume that the biocatalytic aromatization of THP substrates occurs in two steps as shown in Scheme 1. The enzyme MAO-N catalyzes first the C-N oxidation of **5a** leading to the 5,6-dihydropyridinium intermediate **7a.** This latter can spontaneously aromatize into **6a** in the presence of O_2 from the air or alternatively tautomerize into the intermediate **7b** and, eventually, undergoes a second MAO-N catalyzed C-N oxidation leading to **6a**.

Once the best reaction conditions were identified the scope of the aromatizing biotransformation was investigated. A series of THPs **5b-f** and **14a-b** were synthesised as reported in Scheme 2.[15] The commercial *N*-Boc-piperidone was treated with different aryl-

Grignard reagents leading to tertiary alcohol derivatives **8b-e**, which were in turn treated with TFA leading to **5b-e** through dehydratation and concomitant Boc-cleavage (Scheme 2a). The synthesis of THP **14a-b** was carried out according to the protocol described in Scheme 2b. Benzaldehyde or hexanal and allylamine were reacted to form the imines **11a-b**, which were in turn treated with allylmagnesium bromide in the presence of $ZnEt_2$ and $(Boc)_2O$ to afford the dienes **12a-b**.[16] Ring-closing metathesis of **12a-b** led to the THPs **13a-b** which were finally deprotected with HCl/AcOEt to give desired **14a-b** as HCl salts. The THPs **5b-f** and **14a-b**, together with the *N*-methyl-THP **15a-c**[17] were then treated with the MAO-N whole cell biocatalysts. The results are reported in Tables 2 and 3. The THP **5b** was fully converted (99%, *entry 1*) into pyridine **6b** by MAO-N D9 when the reaction was carried out in buffer/isooctane mixture. Comparable conversions were observed with MAO-N D5 and D11, whilst slightly lower conversions were obtained when DMSO was used as cosolvent. The pyridines **6c-d** were also obtained with excellent conversions (*entries 2-3*), in particular when additional loading of the MAO-N biocatalysts was used in the course of the reaction. The chloro-derivative **6e** was obtained in slightly lower amount (57%, *entry 4*),[18] while the bromo-derivative **6f** was formed with only 34% conversion (*entry 5*) probably due to the steric hindrance of the halogen substituent on the phenyl ring. The *N*-methyl derivatives **15a-b** were fully converted (99-87%) into the desired pyridinium ions **16a-b** by the MAO-N D9 biocatalyst. On the other hand, arecoline **15c**, a THP bearing an electron withdrawing substituent, was not converted into the desired pyridinium **16c** even when higher biocatalyst loading and longer reaction times were employed. The THPs **14a-b** bearing a stereocentre at C6 were only partially converted by MAO-N D9 and D11 into the corresponding pyridines **17** and the flavoring agent **4** (Table 3). These results were not unexpected, since it is well documented that MAO-N biocatalysts can stereoselectively oxidize one enantiomer over the other in chiral amines. In fact, MAO-N enzyme selectively aromatized the enantiomers (*S*)-**14a-b** into pyridines **17** and **4** (*entries 1-*

2), whilst the opposite enantiomers (*R*)-**14a-b** were recovered from the reaction mixture as major products with approximately 20% ee.[19] It is also plausible that factors (i.e. steric factors) may contribute to the low conversion observed for **14a-b**. Attempts to increase the conversion of **14a-b** by increasing the catalyst loading or the reaction time were unsuccessful.

The MAO-N whole cell biocatalyzed aromatization of THP **5d** was finally investigated by *in situ* ^{19}F NMR spectroscopy. The experiment was carried out under standard conditions (buffer pH 7.8, isooctane cosolvent, 37 ºC) using the whole cell biocatalyst MAO-N D9. Attempts to monitor the biocatalytic aromatization of THP substrates *via in situ* ^{1}H NMR proved to be tricky due to the interference caused by the cells in the reaction mixtures, as well as the cosolvent.[20] The THP **5d**, bearing a fluorine atom, was thus chosen as candidate for the *in situ* NMR experiments, as it would allow monitoring of the ^{19}F nucleus with less interferences arising from the whole cell biocatalyst. The substrate **5d** was suspended in buffer solution containing 1.6% isooctane and treated with MAO D9 at 37 ºC. The reaction was monitored for 24h during which time ^{19}F NMR experiments were recorded in sequence every 15 minutes (number of scans: 128) using the proton-decoupled ^{19}F sequence. A selected number of ^{19}F NMR spectra is reported in Figure 2a. After approximately 90 min from the addition of the biocatalysts to the reaction mixture, a signal at -113.61 pm belonging to the pyridine **6d** appeared in the NMR spectrum and it became clearer after 120 min. The intensity of the signal at -115.56 ppm belonging to the substrate **5d** start to decrease already after 30 min and the signal disappear completely after 10h with concomitant increase of the **6d** fluorine peak. According to the *in-situ* NMR experiment the reaction was completed within 12h. As the integration of the peaks is proportional to the concentration of the species involved, the kinetic profiles of each species were reported by plotting the integration of each peak against the time (Figure 2b). The less sharp and defined signals of **6d**, compared to **5d**, are most likely due to residues of the biocatalyst (cells and their organelles present in the reaction mixture) or affinity

of the substrate to the cell membranes, which, limiting the mobility of the compounds, may be responsible for the broadening of the fluorine peak. To confirm the full conversion of the biotransformation, the reaction was stopped after 24h, extracted and the crude mixture was purified affording THP **6d** in 87% isolated yield.[21] Interestingly, the ^{19}F signal of **6d** starts to appear after a consistent time (more than 90 min) while the signal of **5d** start to decrease after few minutes. It is likely that during the first phase of the biotransformation, the THP **5d** is converted into the partially oxidized intermediates **7** (Scheme 1) before being fully aromatized into **6d**. A weak signal at around -108.75 ppm was detected by ^{19}F-NMR spectra after 30 min. This peak disappears completely after 4h and it might belong to intermediates **7**.[22] It is plausible that the low concentration and short half-life of intermediates **7** over the reaction time, as well as the interference caused by the whole cell biocatalyst, made their detection not clearly evident.

CONCLUSIONS

In conclusion, an efficient biocatalytic protocol for the synthesis of pyridines from aliphatic tetrahydropyridine precursors exploiting MAO-N whole cell biocatalysts has been described. The methodology represents a mild, alternative and more sustainable approach to access 6-membered nitrogen heterocycles through the aromatization of aliphatic substrates. In particular, the biocatalyst MAO-N D9 was found able to catalyze the biotransformation of a variety of aryl-THPs into aryl pyridines **6** and pyridinium ions **15** with excellent conversions by mimicking the natural metabolic activity of human MAO enzymes. Also the challenging chiral THP substrates **14a-b** were partially converted into the pyridine **17** and the flavouring agent **4**. Finally, the first example of *in situ* ^{19}F NMR study of a whole cell biocatalyzed reaction was reported.

FIGURES.

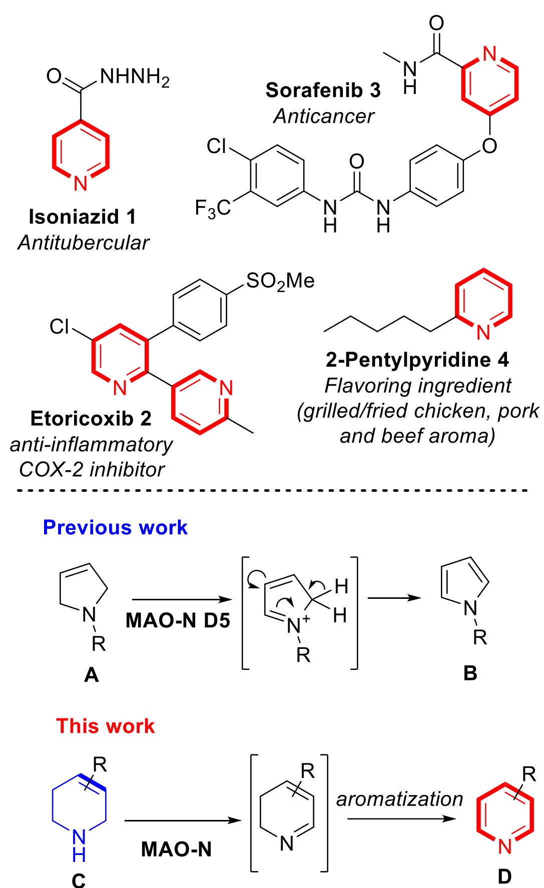


Figure 1. Pyridine nucleus containing drugs and flavoring agents

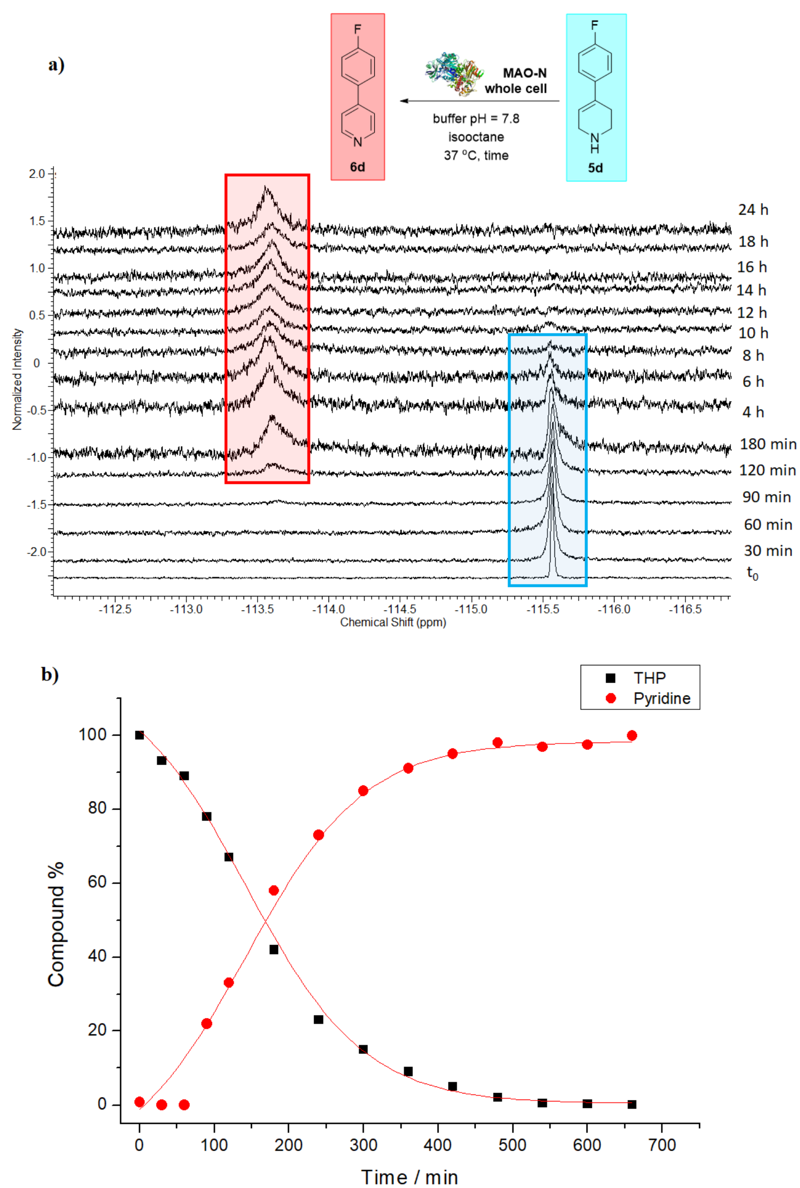
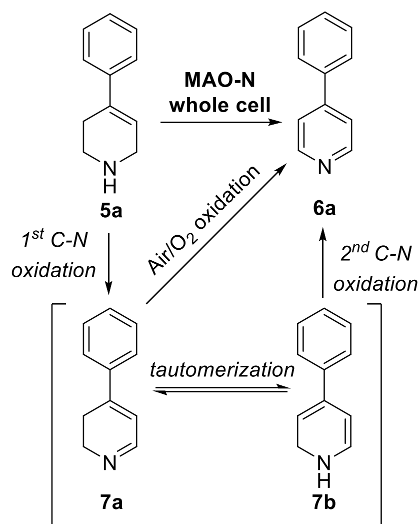
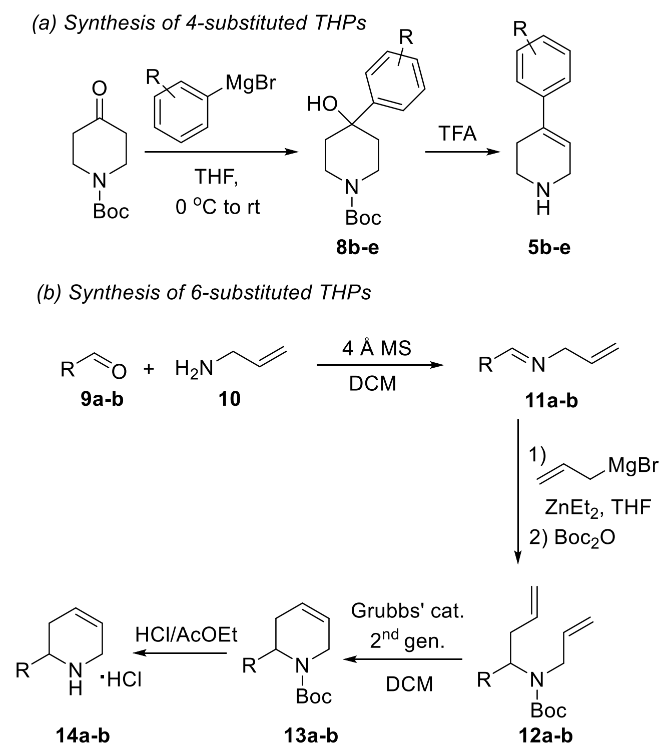


Figure 2. a) Stacked *in situ* ^{19}F NMR spectra of the aromatization of **5d** using MAO-N D9 whole cells. b) The kinetic profile of the reaction shows the disappearance of **5d** and the formation of the pyridine product **6d**.

SCHEMES.



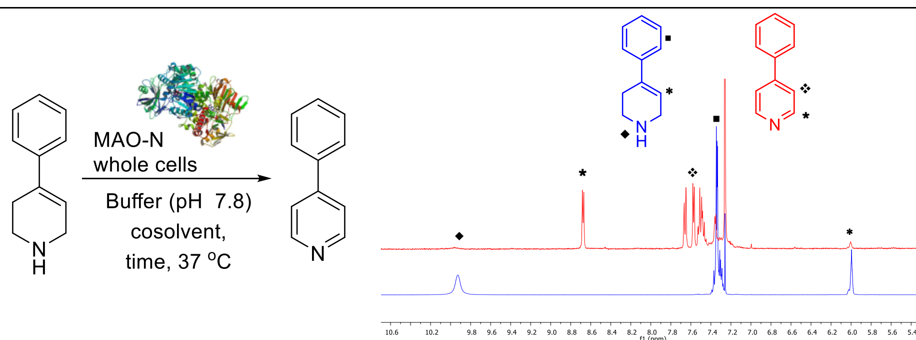
Scheme 1. Plausible mechanism of MAO-N biocatalytic aromatization.



Scheme 2. a) Synthesis of aryl THPs **5b-e**. b) Synthesis of chiral THPs **14a-b**

TABLES.

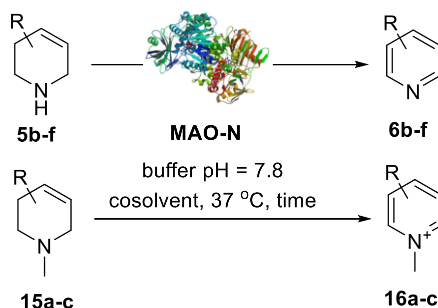
Table 1. Biocatalytic aromatization of THP **5a**



Entry	MAO-N[a]	Cosolvent (1.4%)	Time (h)	Conv. (%)[b]
1	D5	DMSO	24	70
2	D9	DMSO	24	76
3	D11	DMSO	24	67
4	D9	DMSO/DMF 1:4	24	95
5	D5	isooctane	18	92
6	D9	isooctane	18	98[f]
7	D11	isooctane	18	95
8	D9	-	15	80
9	6-HDNO	DMSO	24	0
10	- [c]	DMSO	24	0
11	- [d]	DMSO	24	0
12	D9[e]	isooctane	24	72

[a]Freeze-dried whole cells (140mg / 0.1mmol of **5a**) were used. Conditions are reported in the Supporting Information. [b]Conversion was determined by ^{1}H-NMR integration of the crude mixture or by HPLC analysis using a Chiralpak IG column. [c]No biocatalyst was added to the reaction mixture. [d]*E. coli* BL21(DE3) cells no harboring MAO-N enzymes were used. [e]Catalase was added to the reaction mixture. [f]Large scale reaction afforded **6a** with 99% conv. and 75% isolated yield

Table 2. Biocatalytic aromatization of THPs **5b-f** and **15a-c**



Entry	THP	R	MAO-N[a]	Cosolvent (1.4%)	Time	Pyridine/ pyridinium	Conv. (%)[b]
1	**5b**	4-(4-Me-Ph)	D5	DMSO	20h	**6b**	65
			D9	DMSO	20h		85
			D11	DMSO	20h		72
			D5	isooctane	20h		93
			D9	isooctane	20h		99
			D11	isooctane	20h		95
2	**5c**	4-(4-MeO-Ph)	D9	DMSO	24h	**6c**	63
			D9	DMSO	48h		73
			D9[c]	DMSO	24h		98
			D9	isooctane	24h		70
			D11	DMSO	24h		58
3	**5d**	4-(4-F-Ph)	D9	DMSO	24h	**6d**	50
			D9	DMSO	48h		55
			D9[c]	DMSO	24h		98
			D9	isooctane	24h		54
			D11	isooctane	24h		28
4	**5e**	4-(4-Cl-Ph)	D9	DMSO	24	**6e**	55

			D9[c]	DMSO	24h		57
			D9	isooctane	24 h		20
			D9	DMSO	24 h		34
5	**5f**	4-(4-Br-Ph)	D9[c]	isooctane	24 h	**6f**	30
			D11	isooctane	24 h		16
6	**15a**	4-Ph	D9	isooctane	24h	**16a**	99
7	**15b**	3-Ph	D9	isooctane	24h	**16b**	87
8	**15c**	3-COOMe	D9	isooctane	24h	**16c**	0

[a]Freeze-dried whole cells were used. [b]Conversion was determined by ^{1}H-NMR integration of the crude mixture or by HPLC analysis using a C18 reverse phase column (for compounds **16a-c**). [c]Additional amount of MAO-N D9 (140 mg / 0.1 mmol substrate) was added after 9 h.

Table 3. Biocatalytic aromatization of THPs **14a-b**

Reaction scheme: **14a** R = Ph, **14b** R = Pentyl → MAO-N, buffer pH = 7.8/ isooctane 66:1, 37 °C, 24h → **17** R = Ph, **4** R = Pentyl + **(*R*)-14a** R = Ph, **(*R*)-14b** R = Pentyl

Entry	THP	MAO-N	Pyridine/THP ratio. (%)[a]	(*R*)-14a-b ee (%)[c]
1	**14a**	D9	25:75	21
		D9[b]	26:74	-
		D11	24:76	20
2	**14b**	D9	18:82	-
		D9[b]	26:74	-
		D11	24:76	-

[a]The ratio between the pyridine product (**17** or **4**) and the unreacted THP (**14a-b**) was determined by HPLC using a Chiralpak IG column. [b]Additional amount of MAO-N D9 (140 mg / 0.1 mmol substrate) was added after 9h. [c]The ee of unreacted THP was determined by GC using a Beta-DEX 325 Capillary Column.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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ASSOCIATED CONTENT

Supporting Information. Experimental procedures and full characterization for new compounds and substrates are reported in the Supporting Information. Copies of ^{1}H-NMR and ^{13}C-NMR spectra for new compounds are reported. This information is available free of charge on the ACS Publications website.

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15. See Supporting Information for synthetic procedures. The THPs **5a** and **5f** are commercially available and were purchased.
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17. The THPs **15c** is commercially available and was purchased. The THP **15a-b** was synthesised according to literature as described in the Supporting Information

18. Contrary to what observed with substrate **5a**, the conversion of THPs **5c-d** into **6c-d** was not affected by the nature of the cosolvent. In fact, comparable results were obtained when both DMSO or isooctane were used.

19. The selective oxidation of the (*S*)-enantiomer of **14a-b** was established according to the known enantioselctivity of MAO-N biocatalysts (see reference 11). The ee of (*R*)-**14a** was confirmed by GC analysis using a Beta-DEX 325 Capillary Column. Attempts to separate the enantiomers of **14b** via HPLC or GC were unsuccessful

20. An example of *in situ* ^{1}H NMR of the whole cell biocatalyzed aromatization reaction is reported in the Supporting Information together with further details on the experiment.

21. Both a ^{1}H NMR and a ^{19}F NMR spectrum in $CDCl_3$ of the crude reaction mixture after *in situ* ^{19}F NMR showing full conversion of the biotransformation are reported in the Supporting Information (Figures S4 and S5).

22. A full ^{19}F-NMR spectrum showing the peak at -108.75 is reported in the Supporting Information.

SYNOPSIS.