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5-hydroxymethylfurfural conversion by fungal aryl-alcohol oxidase and unspecific peroxygenase

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Oxidative conversion of 5-hydroxymethylfurfural (HMF) is of biotechnological interest for the production of renewable (lignocellulose-based) platform chemicals, such as 2,5-furandicarboxylic acid (FDCA). To the best of our knowledge, the ability of fungal aryl-alcohol oxidase (AAO) to oxidize HMF is reported here for the first time, resulting in almost complete conversion into 2,5-formylfurancarboxylic acid (FFCA) in a few hours. The reaction starts with alcohol oxidation, yielding 2,5-diformylfuran (DFF), which is rapidly converted into FFCA by carbonyl oxidation, most probably without leaving the enzyme active site. This agrees with the similar catalytic efficiencies of the enzyme with respect to oxidization of HMF and DFF, and its very low activity on 2,5-hydroxymethylfurancarboxylic acid (which was not detected by GC-MS). However, AAO was found to be unable to directly oxidize the carbonyl group in FFCA, and only modest amounts of FDCA are formed from HMF (most probably by chemical oxidation of FFCA by the H_2O_2 previously generated by AAO). As aldehyde oxidation by AAO proceeds via the corresponding *geminal* diols (aldehyde hydrates), the various carbonyl oxidation rates may be related to the low degree of hydration of FFCA compared with DFF. The conversion of HMF was completed by introducing a fungal unspecific heme peroxygenase that uses the H_2O_2 generated by AAO to transform FFCA into FDCA, albeit more slowly than the previous AAO reactions. By adding this peroxygenase when FFCA production by AAO has been completed, transformation of HMF into FDCA may be achieved in a reaction cascade in which O_2 is the only co-substrate required, and water is the only by-product formed.

Introduction

Over recent years, the use of renewable carbon sources instead of fossil sources has become a necessity due to the expected decrease of crude oil reserves and the concomitant rise in prices, as well as to reduce the sharp increase in associated greenhouse-effect emissions [1]. Lignocellulosic biomass may be

Abbreviations
AAO, aryl-alcohol oxidase; DFF, 2,5-diformylfuran; DMSO-d_6, deuterated dimethylsulfoxide; FDCA, 2,5-furandicarboxylic acid; FFCA 2,5-formylfurancarboxylic acid; HMF, 5-hydroxymethylfurfural; HMFCA, 2,5-hydroxymethylfurancarboxylic acid; TMSi, trimethylsilyl; UPO, unspecific peroxygenase.

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able to substitute for fossil sources due to its renewable nature and other attractive properties that make it unique among other carbon resources. Some valuable chemicals obtained from lignocelluloses are furaldehydes. These are primarily furfural and 5-hydroxymethylfurfural (HMF). The former is derived from pentoses present in the hemicelluloses, whereas the latter is derived from hexoses, which are present both in cellulose and hemicelluloses. HMF is synthesized by dehydration of monosaccharides, generally fructose [2], but direct conversion of glucose is also possible [3]. After their optimized hydrolysis, disaccharides and polysaccharides can be the starting materials for these renewable building blocks [4].

The presence of two functional groups in HMF, combined with the furan aromatic ring, makes it an appealing starting material for various chemical applications. Much attention has been paid to its oxidation and reduction, because they provide convenient synthetic pathways for the production of chemical building blocks for the polymer industry. One of these important building blocks is 2,5-furandicarboxylic acid (FDCA), which originates from oxidation of HMF. Its importance resides in the ability to co-polymerize with diols, producing poly(ethylene-2,5-furandicarboxylate) among other polyesters. These polymers are thought to be able to substitute for polyesters based on terephthalic acid, such as poly(ethylene-terephthalate), poly(propylene-terephthalate) or poly(butylene-terephthalate), which are biologically not degradable, and the precursors of which are fossil resources. Poly(ethylene-2,5-furandicarboxylate) exhibits good mechanical and barrier properties [5], and has the double advantage of being renewable and biodegradable. The oxidative pathway leading from HMF to FDCA takes place via 2,5-formylfurancarboxylic acid (FFCA) and its alternative precursors 2,5-diformylfuran (DFF) and 2,5-hydroxymethylfuran carboxylic acid (HMFCA) (Fig. 1). FFCA and other partially oxidized compounds are also of interest as intermediates for the preparation of surfactants, biofuels, resins and other compounds [6].

Aryl-alcohol oxidase (AAO, EC 1.1.3.7) is a secreted flavoenzyme that is involved in lignin degradation by several white-rot basidiomycetes [7]. Within this multienzymatic process, which enables the recycling of carbon fixed by photosynthesis in land ecosystems, AAO reduces O_2, providing the H_2O_2 required by ligninolytic peroxidases to oxidize the recalcitrant lignin polymer [8]. In this way, subsequent hydrolytic breakdown of plant polysaccharides is made possible [9]. Here we show that AAO is also able to oxidize some furanic compounds such as HMF and DFF. This is not only an important scientific finding, given the structural differences from previously known AAO substrates, but is also of biotechnological relevance due to the importance of these renewable chemicals. In the present study, AAO was combined with an unspecific peroxygenase (UPO, EC 1.11.2.1) from *Agrocybe aegerita* [10] for full oxidative conversion of HMF in an enzymatic cascade. This peroxygenase belongs to the recently described superfamily of heme-thiolate peroxidases, and is capable of incorporating peroxide-borne oxygen into diverse substrate molecules [11]. Among other interesting reactions, it catalyzes the H_2O_2-dependent stepwise hydroxylation of aliphatic and aromatic alcohols into the corresponding aldehydes (via *gem*-diol intermediates) and finally into carboxylic acids [12,13].

Results

AAO oxidation of HMF and its partially oxidized derivatives

The ability of fungal AAO to oxidize HMF and its partially oxidized derivatives (HMFCA, DFF and FFCA) was tested by incubating them with the enzyme, usually in 24 h experiments. All the compounds present in the reaction mixture at various

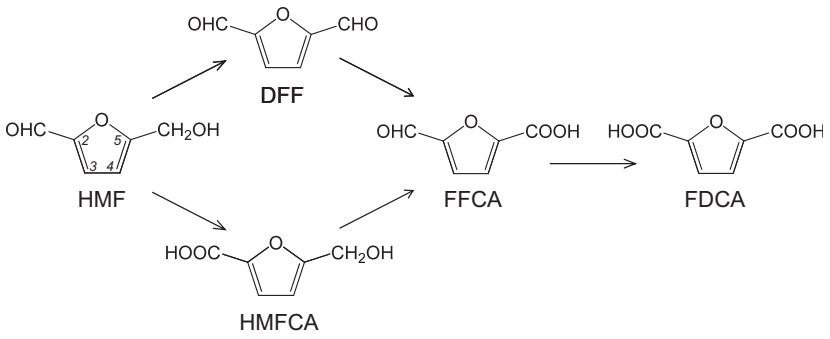


Fig. 1. Pathway for 5-hydroxymethylfurfural (HMF) conversion into 2,5-furandicarboxylic acid (FDCA), via 2,5-formylfurancarboxylic acid (FFCA), including 2,5-diformylfuran (DFF) or 2,5-hydroxymethylfurancarboxylic acid (HMFCA) intermediates.

times (i.e. the remaining substrate and products) were analyzed by GC-MS after trimethylsilyl (TMSi) derivatization of the alcohol and carboxylic groups, and the corresponding molar percentages were estimated. MS identification of the various compounds present in these and other enzymatic reactions is described in Doc. S1.

When AAO was incubated with HMF at pH 6 (the optimal pH for the enzyme), almost all HMF (98% molar percentage) had been converted into FFCA by 4 h, and very little FDCA was formed (Fig. 2A). The proportion of FFCA slowly decreased over time due to formation of some FDCA (6% after 24 h). Interestingly, neither DFF nor HMFCA were detected in the analyses. These results show that AAO is able to oxidize HMF, as well as DFF and/or HMFCA, since these two compounds are obligate intermediates in the pathway from HMF to FFCA.

To reveal which pathway is more likely to be followed (Fig. 1), DFF was mixed with AAO to test the ability of the enzyme to oxidize it, and the reaction compounds were analyzed (Fig. 2B). Similarly to what was seen with HMF, almost 90% FFCA and only 3% FDCA were detected after 2 h of incubation, and these percentages changed slightly during the subsequent incubation (approximately 10% FDCA after 24 h). However, when HMFCA was incubated with AAO, no product formation was observed.

The above reactions took place during long-duration incubations (24 h) at room temperature. In order to test whether the enzyme was active throughout the complete reaction time, its residual activity was determined. The main activity decrease during HMF conversion by AAO (Fig. 2A) occurred during the first 2 h, decreasing from 100% to 84%. Between 2 and 24 h, the AAO activity slowly decreased further, but retained 71% of its activity by the end of the 24 h period. These results demonstrate that the enzyme was catalytically active over the whole experiment, despite loss of some activity.

AAO kinetic parameters for HMF and its partially oxidized derivatives

Having observed that HMF was converted into several products by AAO, we estimated the kinetic parameters of the enzyme for the various potential substrates (HMF, DFF, HMFCA, and FFCA) in 3 min reactions on the basis of H_2O_2 release measured using a peroxidase-coupled assay (Table 1).

HMF was the best AAO substrate in terms of catalytic efficiency, although the k_{cat} for DFF was higher. In contrast, HMFCA was a poor substrate for AAO. Although some AAO activity was revealed on the basis of H_2O_2 formation, it was impossible to assess the kinetic parameters as no enzyme saturation by HMFCA was observed, and only a k_{obs}/concentration value (referring the observed turnover to substrate concentration) was calculated. Thus, it is likely that the reaction of HMF to FFCA proceeds via the formation of DFF from HMF (upon oxidation of the alcohol group), and then one aldehyde group is further oxidized to a carboxylic group, in agreement with the GC-MS results (see below). With regard to FFCA, no activity was detected in the kinetic study.

In order to confirm which products were formed in the above kinetic studies, the reaction mixtures were analyzed by GC-MS, showing that, during 3 min of

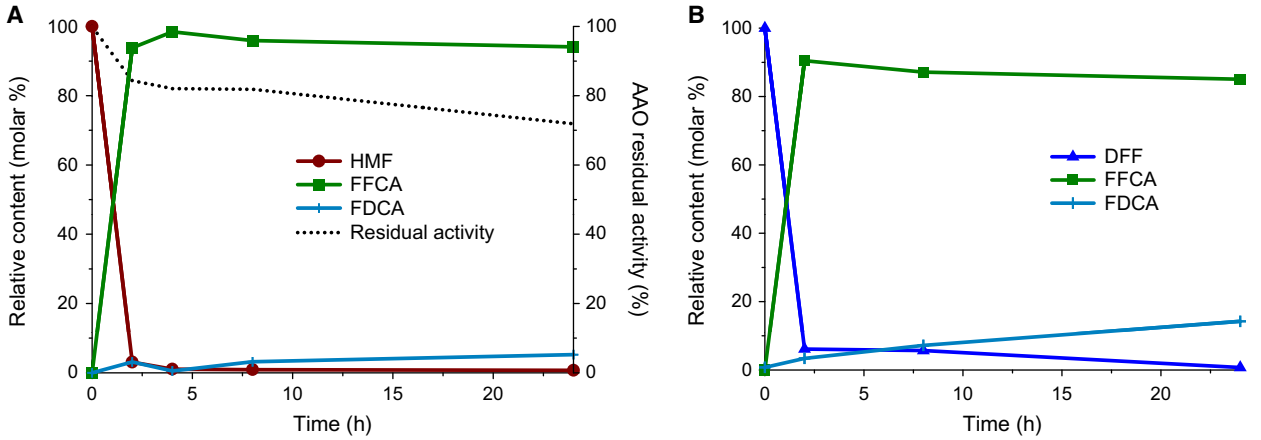
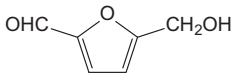
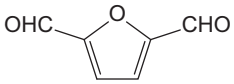
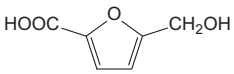
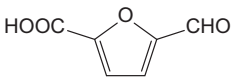


Fig. 2. HMF and DFF transformation by *P. eryngii* AAO. Treatment of 3 mM HMF (A) or DFF (B) in a 5 mL reaction volume with AAO (5 μM, corresponding to 54 units measured with veratryl alcohol as substrate), yielding 90–98% FFCA after 4 h, which was then slowly oxidized to FDCA (6–10% after 24 h). The AAO residual activity is also shown in (A).

Table 1. Kinetic parameters for HMF, DFF, FFCA and HMFCA oxidation by AAO (estimated on the basis of H_2O_2 formed).

Substrate	Structure	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
HMF		1.6 ± 0.2	20.1 ± 0.6[a]	12.9 ± 1.2[a]
DFF		3.3 ± 0.2	31.4 ± 0.7	9.4 ± 0.5
HMFCA		–	–	1.0 ± 0.1[b]
FFCA		0	0	–

[a]Values were corrected taking into account that two H_2O_2 equivalents are formed in two successive oxidations to convert HMF into FFCA (as shown by GC-MS).
[b]AAO was not saturated at increasing HMFCA concentrations, and only a k_{obs}/concentration value was obtained.

HMF incubation with AAO, FFCA is formed, with a concomitant decrease in HMF. As in the case of the 24 h reactions, no DFF and only traces of HMFCA were detectable (after 30 s, 1 min and 3 min). Therefore, the amount of H_2O_2 estimated in the HMF reactions corresponds to two subsequent oxidation steps performed by AAO (from an alcohol group to a carboxylic group), and the corresponding correction was introduced for k_{cat} estimation in Table 1. In the case of DFF, the product formed during the 3 min incubation was FFCA, as expected, accompanied by a decrease in DFF.

In the light of the above results, we propose that the oxidative pathway leading from HMF to FFCA proceeds via formation of DFF that is rapidly oxidized to FFCA. AAO shows very low activity towards HMFCA, which, if formed, should have accumulated and then been detectable by GC-MS.

Oxidation of the aldehyde group of FFCA by H_2O_2

As no activity of AAO on FFCA was observed, and low but significant amounts of FDCA were detected in the reaction mixtures with HMF and DFF, its origin remained unclear. H_2O_2 is a strong oxidant, and hence its possible involvement in (chemical) FFCA oxidation was taken into consideration (H_2O_2 is formed by AAO in stoichiometric amounts via O_2 reduction). To assess this possibility, FFCA (3 mM) was incubated for 24 h with 6 mM H_2O_2, the maximal concentration produced by AAO during 3 mM HMF oxidation to FFCA, and the reaction products were analyzed by GC-MS. The amount of FDCA formed (11%) was in a similar range to that obtained in the reactions of HMF and DFF with AAO (6–10%), and this strongly supports the assumption of chemical oxidation of FFCA into FDCA by AAO-derived H_2O_2. This was confirmed by the finding that a higher H_2O_2 concentration (200 mM) resulted in a higher amount of FDCA formed (84%).

Use of fungal peroxygenase for HMF conversion

Because AAO was apparently unable to oxidize FFCA into FDCA, we tested the ability of a second fungal enzyme, *A. aegerita* UPO, to catalyze the reaction. UPO requires H_2O_2 as co-substrate to perform the desired

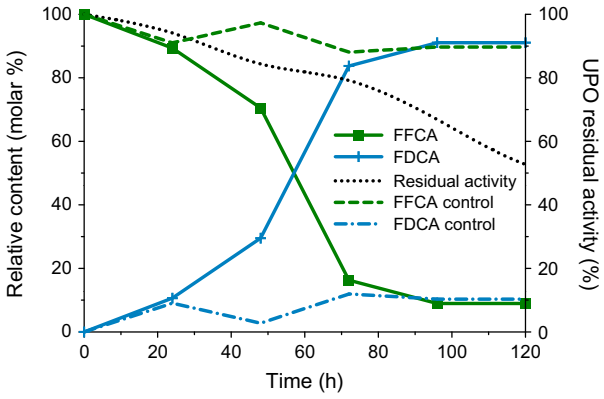


Fig. 3. FFCA transformation by *A. aegerita* UPO. FFCA (3 mM in a 5 mL reaction volume) was slowly oxidized by UPO (0.65 μM, corresponding to 20 units measured with veratryl alcohol as substrate) and H_2O_2 (4 mM) to FDCA (approximately 90% after 120 h). The UPO residual activity is also shown, as well as comparisons with control reactions without enzyme.

oxidation, and AAO produces it by reducing O_2, so the former enzyme will benefit from the activity of the latter.

First we used GC-MS to follow the oxidation of HMF by UPO, in the presence of exogenous H_2O_2, to determine how the conversion proceeds with this enzyme. In contrast to AAO, the UPO reaction was found to start with oxidation of the HMF carbonyl group, yielding HMFCA (72% after 8 h, giving a turnover rate of 7 min^{-1}, and 97% after 24 h). Then, HMFCA was converted into FFCA (up to 50%) and some FDCA (up to 10%). The latter conversion was confirmed by FFCA treatment with UPO, forming FDCA (Fig. 3), although the reaction was much slower than observed with HMF, and 96 h were required to achieve 90% conversion (approximately 80% conversion in 72 h, giving a turnover rate of 0.9 min^{-1}). The oxidation of FFCA was catalyzed by UPO, and was not the result of the H_2O_2 added, as only 10% FDCA was obtained in controls without enzyme (in agreement with previous results). As in the case of AAO, UPO retained the majority of its activity (over 90%) during the 24 h reaction, and more than 50% activity after 120 h.

Complete HMF conversion by an AAO–UPO reaction cascade

Given the above results, we designed a 'one-pot' reaction system using HMF as substrate and AAO and UPO as biocatalysts. In the first experiment, both enzymes were added together from the start of the reaction, and left to react for 24 h. UPO catalyzed oxidation at the expense of H_2O_2 released by AAO. After 24 h, no HMF was left and almost equal amounts of FFCA and HMFCA (46–47%) had been produced, together with some FDCA. This result suggests that, while AAO catalyzes oxidation of the hydroxyl group of HMF, UPO performs oxidation of the carbonyl group (as shown in the short-duration incubations), at the expense of the H_2O_2 simultaneously produced by AAO. Then, the reaction stops due to the inability of AAO to further oxidize the HMFCA produced by UPO.

In the second experiment, a sequential reaction system (cascade) comprising AAO with UPO added later was established. The aim was to enable AAO to oxidize HMF to FFCA, releasing two H_2O_2 equivalents, and then add UPO, which uses the H_2O_2 to convert FFCA into FDCA. While all previous reactions were performed at pH 6, this reaction was performed at pH 7, which is the optimal pH for UPO reactions, and still a good pH for AAO. After a 4 h incubation of HMF with AAO, UPO was added, and the reaction was allowed to proceed until HMF to FDCA conversion was completed (Fig. 4). During the first 4 h, almost all HMF was converted by AAO to FFCA (98%) and a small amount of FDCA, and atmospheric O_2 was reduced to H_2O_2. After adding UPO, the levels of FFCA progressively decreased, accompanied by increasing amounts of FDCA. After 120 h, only a small proportion of FFCA remained (9%), and the rest was almost completely converted into FDCA (91%). Three sample chromatographic profiles of the above reaction (after 0, 4 and 120 h) are presented in Fig. 5A–C, showing the HMF, FFCA and DFCA peaks. The identification of these compounds was based on the corresponding mass spectra (Fig. 5D–F), as well as the retention times compared with authentic standards (Doc. S1 and Fig. S1).

A final comparison of the results of the various conversions described above is provided in Table 2, including (a) the oxidation of HMF by AAO, UPO and AAO + UPO added simultaneously or successively, (b) the oxidation of DFF by AAO, and (c) the oxidation of FFCA by 6 mM and 200 mM H_2O_2.

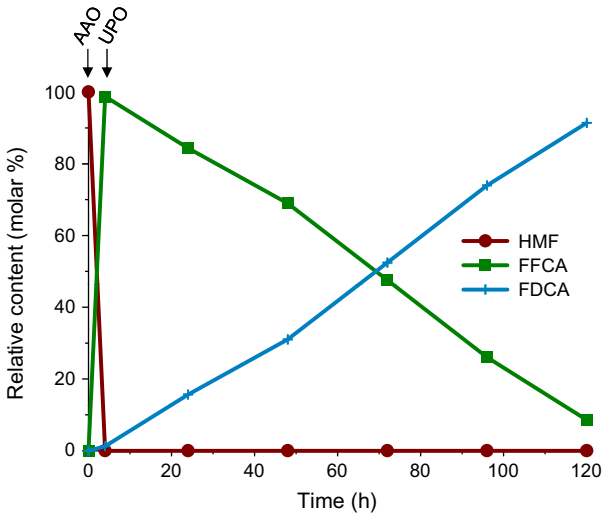


Fig. 4. HMF transformation by *P. eryngii* AAO plus *A. aegerita* UPO (successive addition). After 4 h treatment of 3 mM HMF with 5 μM AAO (corresponding to 54 units measured with veratryl alcohol as substrate, in 5 mL reaction volume), almost all HMF was converted into FFCA, and then 0.65 μM UPO (corresponding to 20 units measured with veratryl alcohol as substrate) was added to complete the HMF transformation into FDCA (91% after 120 h).

NMR analysis of carbonyl hydration in HMF-derived furanaldehydes

The ^{1}H-NMR spectrum of HMF in deuterated dimethylsulfoxide (DMSO-d_6) shows five signals corresponding to the aldehyde proton (H_1, 9.5 ppm), the

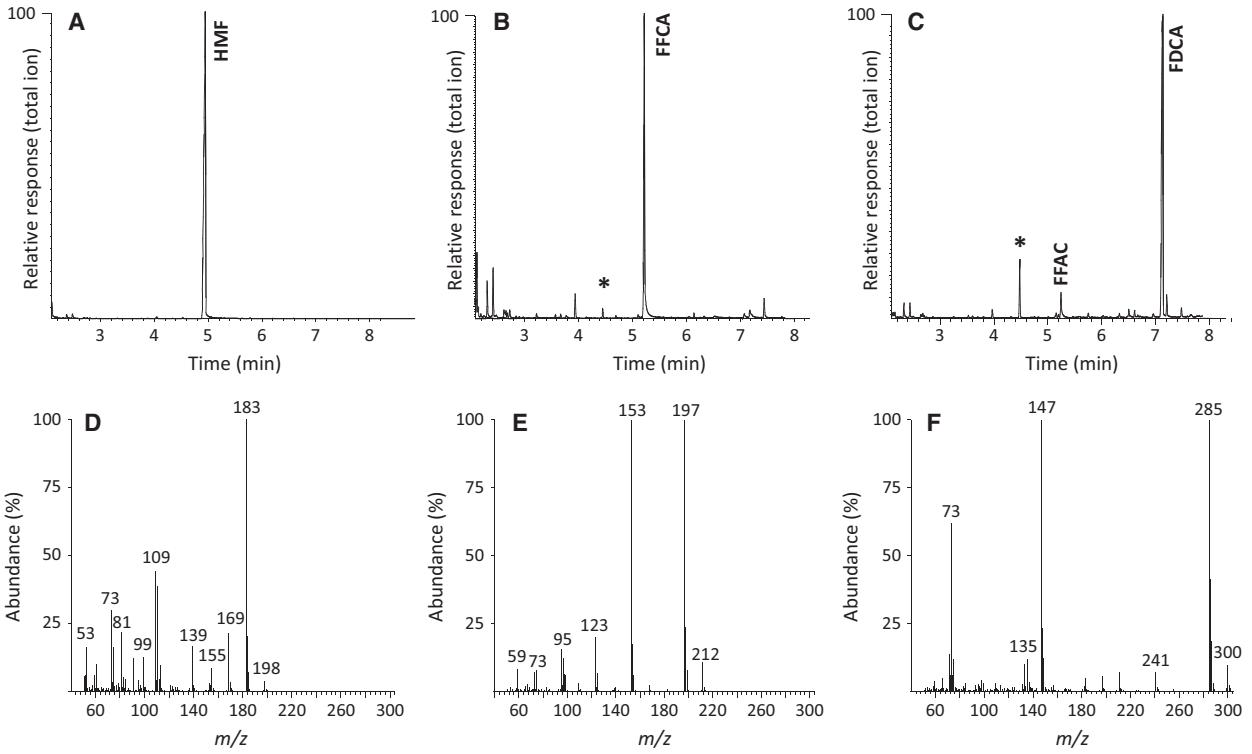


Fig. 5. GC-MS analyses (TMSi derivatization) during HMF transformation by *P. eryngii* AAO (added at time 0) and *A. aegerita* UPO (added after 4 h reaction with AAO). (A–C) Initial chromatogram showing the TMSi-HMF peak (A), 4 h reaction showing the main peak of TMSi-FFCA (and a small peak of TMSi-FDCA) (B), and 48 h reaction showing the main peak of TMSi-FDCA (and small peak of TMSi-FFCA) (C). The asterisks indicate a peak of the TMSi ester of the phosphoric acid used to buffer the enzymatic reactions. (D–F) Mass spectra of silylated HMF, FFCA and FDCA, respectively.

Table 2. Molar percentages (from 5 mL reactions) after 24 h treatment of HMF (3 mM) with AAO alone (5 μM, corresponding to 54 units measured with veratryl alcohol as substrate), UPO alone (0.65 μM, corresponding to 20 units measured with veratryl alcohol as substrate), or with AAO (5 μM, 54 units) and UPO (0.65 μM, 20 units) added simultaneously or successively, after 24 h treatment of DFF (3 mM) with AAO (5 μM, 54 units), and after 24 h treatment of or FFCA (3 mM) with 6 and 200 mM H_2O_2.

	HMF					DFF	FFCA	
	AAO (24 h)	UPO (24 h)	AAO + UPO simultaneously (24 h)	AAO + UPO successively (24 h)	AAO + UPO successively (120 h)	AAO (24 h)	6 mM H_2O_2 (24 h)	0.2 M H_2O_2 (24 h)
HMF	0	3	0	0	0	–	–	–
HMFCA	0	97	46	0	0	0	–	–
DFF	0	0	0	0	0	0	–	–
FFCA	94	0	47	84	9	86	89	16
FDCA	6	0	7	16	91	14	11	84

two protons of the furanic ring (H_2 and H_3, 7.6 and 6.7 ppm, respectively), the two benzylic protons (4.5 ppm) and the hydroxyl proton (5.6 ppm) (Fig. S2A). Because the proton from the hydroxyl group interchanges with ^2H_2O, the spectrum of HMF in ^2H_2O buffer (pH 6) showed four signals that were the same as found in DMSO-d_6: H_2 (7.6 ppm), H_3 (6.7 ppm), H_1 (9.5 ppm) and H_4 (4.5 ppm) (Fig. 6A). In light of these results, it appears that HMF does not produce any *geminal* diol (*gem*-diol) form. This reinforces the hypothesis that AAO oxidation of HMF to FFCA occurs via DFF, as the absence of hydration rules out oxidation of the aldehyde group yielding HMFCA.

The DFF spectrum in DMSO-d_6 (Fig. S2B) shows only two signals due to the symmetry of the molecule.

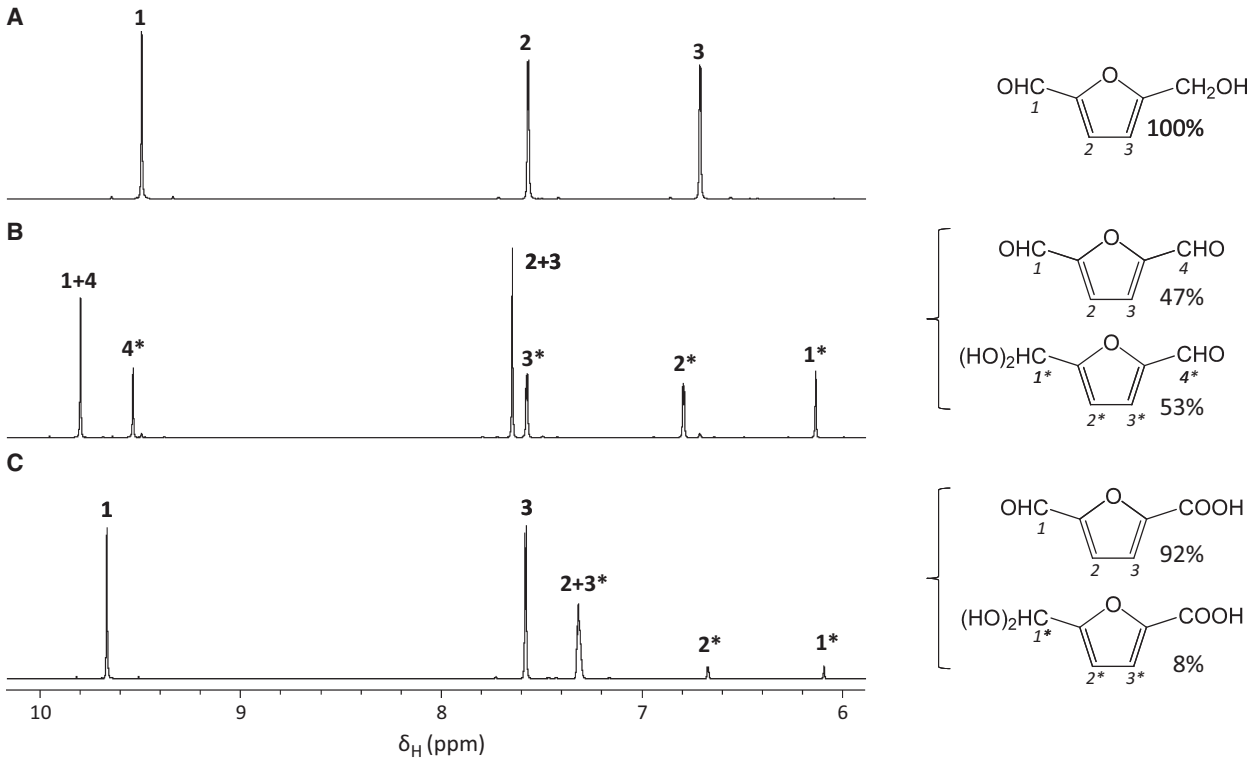


Fig. 6. ^{1}H-NMR analyses revealing various degrees of hydration. The ^{1}H-NMR spectra (5.8–10.2 ppm) of 10 mM HMF (A), DFF (B) and FFCA (C) in $^{2}H_2O$ show the H_1–H_4 signals in the aldehyde forms and in the *gem*-diol forms (asterisks), enabling calculation of their hydration degrees.

These correspond to the protons of the aldehyde (H_{1+4}) and those from the furanic ring (H_{2+3}). However, the spectrum in $^{2}H_2O$ (Fig. 6B) is more complex, displaying six signals: H_{1+4} (9.8 ppm), H_{2+3} (7.7 ppm), coinciding with those of DFF in DMSO, and H_{1*} (6.1 ppm), H_{2*}, (6.8 ppm), H_{3*} (7.6 ppm) and H_{4*} (9.5 ppm), corresponding to the hydrated forms. The similar intensities of the H_{1*}, H_{2*}, H_{3*} and H_{4*} signals indicate that only one of the DFF carbonyl groups is (partially) hydrated. The DFF hydration degree, estimated from the integration of the H_{1+4}, H_{4*} and H_{1*} signals, is 53%, and corresponds to *gem*-diol formation at one of the carbonyl groups.

The FFCA spectrum in DMSO-d_6 shows four signals: H_1 bound to the carbonyl carbon, H_2 and H_3 in the furanic ring, and that of the proton of the carboxylic group (Fig. S2C). The spectrum in $^{2}H_2O$ (Fig. 6C) shows five signals: H_1 (9.7 ppm) and H_3 (7.6 ppm), corresponding to those found in DMSO-d_6, plus H_{1*} (6.1 ppm) and H_{2*} (6.7 ppm), corresponding to the hydrated form, and H_{2+3*} (7.3 ppm), which results from overlapping of the above aldehyde H_2 signal and the *gem*-diol H_{3*} signal (the carboxylic proton is exchanged in $^{2}H_2O$). By integrating the H_1 and H_{1*} signals, an 8% hydration degree was calculated at pH 6. FFCA hydration was also estimated in $^{2}H_2O$ buffer at pH 3 and pH 9, and degrees of hydration of approximately 3% and 6%, respectively, were obtained. In agreement with this low degree of hydration, no significant FFCA oxidation by AAO was found in the pH 3–9 range.

Discussion

Substrate specificity of a fungal model flavo-oxidase (AAO)

AAO belongs to the superfamily of GMC oxidoreductases, whose name derives from three of its first and best-characterized members: glucose oxidase (EC 1.1.3.4), methanol oxidase (EC 1.1.3.13) and choline oxidase (EC 1.1.3.17) [14]. Recently, AAO structure–function relationships and mechanistic aspects have been studied in-depth [15–20], and its ability to oxidize aromatic (and some aliphatic polyunsaturated) primary alcohols, as well as related aldehydes, has been

demonstrated [21,22]. Comparison of the AAO activity for these substrates reveals much lower catalytic efficiency for oxidizing aldehydes than alcohols, due to both lower k_{cat} and higher K_m values (Table S1). Moreover, the effects of the same ring substituents on AAO activity were opposite for substituted benzyl alcohols and aldehydes, as already noted by Guillén *et al.* [23] when the AAO aldehyde oxidase activity was first observed. This is due to the required aldehyde hydration discussed below.

HMF and DFF: two new AAO substrates

The enzyme kinetics and product GC-MS analyses showed that AAO is able to perform two successive oxidations using HMF and O_2 as substrates, yielding FFCA and two molecules of H_2O_2. In the first step, AAO oxidizes the hydroxyl group of HMF to a carbonyl, giving DFF. In the second step, it oxidizes one of the aldehyde groups of DFF to the corresponding carboxylic acid to produce FFCA. The fact that DFF was not detected as a reaction product is in agreement with the higher turnover number (kcat) for this compound than for HMF, ensuring that all the DFF formed is rapidly turned into FFCA.

In the light of the above results, the possibility that four-electron oxidation of HMF to FFCA by AAO takes place, with DFF acting as a transient intermediate that remains at the active site during the whole reaction, must be considered. Simultaneous alcohol and aldehyde oxidase activities have already been reported for the reaction of AAO with other substrates (such as 3-chloro-*p*-methoxybenzyl and *m*- and *p*-fluorobenzyl alcohols) [21], and oxidation of the aldehyde intermediate without leaving the enzyme active site is supported by the ternary-complex mechanism described for AAO [19]. This means that the aldehyde product is still present at the active site when the enzyme is re-oxidized by O_2, and, if it is an AAO substrate as DFF is, will be hydrated and immediately oxidized without leaving the active site. In this scenario, the active-site histidine (His502) that acts as a catalytic base, accepting the proton from the C5 hydroxyl of HMF for subsequent hydride transfer to the flavin, accepts the proton from the C5 hydroxyl of the DFF *gem*-diol that remains at the same position at the active site. The rapid HMF conversion into FFCA by AAO contrasts with results reported for some galactose oxidase (EC 1.1.3.9) variants [24] that predominantly yielded DFF from HMF oxidation. Oxidation of aryl-alcohols by galactose oxidase variants had been previously reported [25], and the different HMF products compared with AAO are most likely the result of the different catalytic mechanisms of the enzymes, as galactose oxidase belongs to the group of copper radical oxidases [26] while AAO is a flavoenzyme.

In most cases, AAO has much lower activities on aldehydes than their alcohol counterparts (Table 2), but this is not the case for HMF and DFF. Ferreira *et al.* [21] reported that hydration of aldehydes forming the *gem*-diol forms is required for their reactivity with AAO (which always acts as an alcohol oxidase). The results from ^{1}H-NMR estimation of the hydration degree in HMF derivatives agree with this reactivity. DFF showed a 53% degree of hydration, which means that the *gem*-diol form is more abundant than the aldehyde one. Hydration of one of the carbonyls is promoted by the electron-withdrawing effect of the second one, and therefore no double hydration of DFF was detected. This means that a dynamic equilibrium exists between the two aldehydes and the aldehyde plus *gem*-diol forms, the latter being constantly formed while transformed into FFCA by AAO. On the other hand, the carbonyl substituent in HMF acts as electron withdrawer, lowering the reactivity of the alcohol group for hydride transfer to the AAO cofactor, as shown for aryl alcohols with other electron-withdrawing substituents (such as F and Cl atoms; Table S1). The opposite consequences of these electron-withdrawing effects of (a) reducing oxidation of the HMF hydroxyl group due to the simultaneous carbonyl presence, and (b) promoting hydration of one of the DFF carbonyls due to the presence of the second carbonyl group, resulted in the unexpected similar activity of AAO on the alcohol and aldehyde groups of these two furanic compounds.

Nevertheless, the enzyme is apparently unable to catalyze the next step, which is oxidation of the remaining aldehyde group present in FFCA. The carboxyl group in FFCA may also act as an electron withdrawer, promoting the carbonyl reaction with water. However, GC-MS analyses did not show products of the reaction of FFCA with AAO, in agreement with the lack of H_2O_2 release when the reaction was followed using the peroxidase-coupled assay. Both the comparatively low hydration degree (8% as estimated by ^{1}H-NMR) and the deactivating effect of the carboxyl group on hydride transfer to flavin may be responsible for the lack of AAO activity on FFCA. In the case of DFF, oxidation to FFCA may take place at the C5 carbonyl formed during AAO oxidation of HMF without substrate re-accommodation or exit from the active site, as discussed above. However, FFCA oxidation to FDCA must occur at the C2 position, and most probably requires exit and new entry of the substrate so that it may adopt the catalytically rel-

evant position, a process that is complicated by the bulky nature of the carbonyl group, making the third oxidation step by AAO more difficult.

However, some FDCA was always detected by GC-MS when HMF, or even DFF, was incubated with AAO. As AAO releases H_2O_2, we hypothesized that it may chemically oxidize the aldehyde group of FFCA to some extent [27], and this was in fact demonstrated by an appropriate experiment with FFCA and a higher amount of H_2O_2. In conclusion, it is possible to state that AAO directly oxidizes HMF to FFCA, and may indirectly oxidize some FFCA (via the previously formed H_2O_2) yielding FDCA.

Oxidase/peroxygenase cascade for HMF conversion into FDCA

Even though the oxidative biotransformation of HMF into FFCA by AAO (via DFF) was a good achievement, we were also interested in biocatalytic synthesis of FDCA, because of its potential as precursor of renewable polyesters [28]. That is why we introduced a second enzyme, the UPO from the basidiomycete *A. aegerita* [10], to complete the enzymatic conversion of HMF. Due to the wide range of reactions catalyzed by UPO, we started by elucidating the oxidative pathway that the enzyme uses for HMF oxidation. We observed that it preferentially catalyzed oxidation of the aldehyde group of HMF, producing HMFCA, which is the opposite of what AAO does when oxidizing the hydroxyl group to yield DFF. Then the reaction proceeded to FDCA, although the conversion was rather inefficient, as UPO activity towards FFCA is much lower compared to HMF. Although oxidation of HMF and related compounds has not previously been described for a basidiomycete UPO, similar reactions have been reported for another member of the heme-thiolate peroxidase superfamily, chloroperoxidase (CPO, EC 1.11.1.10) from the ascomycete *Caldariomyces fumago*, albeit in the presence of high enzyme concentrations [29].

The simultaneous action of AAO and UPO on HMF did not result in its substantial conversion into FDCA, most probably because the H_2O_2 generated by the AAO oxidation of HMF to DFF was immediately used by UPO to oxidize the same compound (HMF) to HMFCA. According to the reactions described above, this proceeded in such a way that a mixture of HMFCA and FFCA was finally obtained. However, addition of UPO when most of the HMF had already been transformed into FFCA resulted in almost complete conversion of HMF into FDCA due to FFCA oxidation by UPO (in a long-term reaction) using the

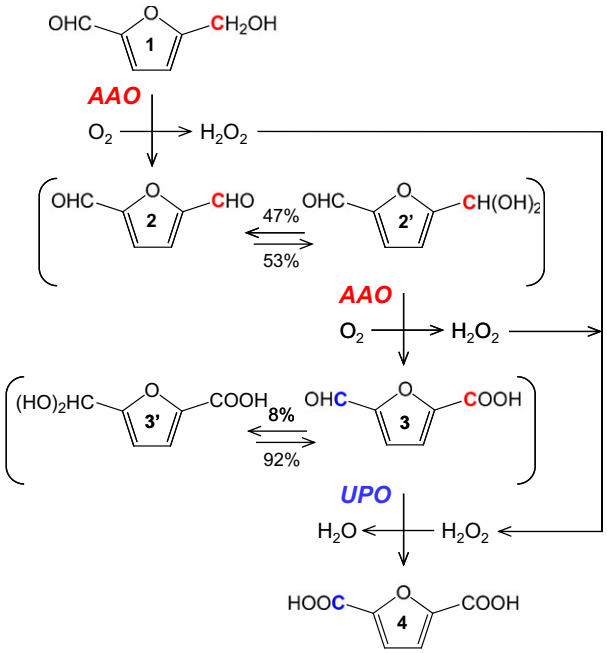


Fig. 7. Scheme for enzymatic conversion of HMF into FDCA in a cosubstrate-free reaction cascade involving *P. eryngii* AAO and *A. aegerita* UPO. HMF (**1**) is oxidized by AAO to DFF (**2**), whose hydrated (*gem*-diol) form (**2′**) undergoes a second AAO oxidation yielding FFCA (**3**). The H_2O_2 formed during AAO reduction of O_2 enables UPO to convert unhydrated FFCA (**3**) into FDCA (**4**), with H_2O as the only by-product. The low amount of hydrated FFCA (**3′**) results in the inability of AAO to catalyze the last step in the reaction cascade, which is successfully catalyzed by UPO.

H_2O_2 excess provided by AAO. This enzymatic cascade, whose two initial steps are the AAO-catalyzed oxidation of HMF and DFF (*gem*-diol form) with the concomitant reduction of two O_2 to two H_2O_2, followed by a third step catalyzed by UPO, which results in oxidation of predominantly unhydrated FFCA at the expense of the previously formed H_2O_2, is illustrated in Fig. 7.

Conclusions

The present findings describing AAO-catalyzed oxidation of the hydroxymethyl and carbonyl substituents of the HMF furan ring expand our knowledge of the range of alcohols and aldehydes that this flavoenzyme is able to oxidize. Thus, it attacks the benzylic position of substituted aromatic heterocycles (such as HMF and DFF) in the same way as the respective position in the substituted aromatic carbocycles previously considered to be typical AAO substrates [7]. The newly identified activities of fungal AAO are similar to those of a bacterial HMF oxidase recently described in a *Methylovorus* species [30,31] that appears to be

involved in a HMF degradation pathway [32]. More importantly, although the use of AAO for HMF conversion into FDCA is covered by one patent, which also describes use of chloroperoxidase, albeit with very modest FDCA yields in all cases [27], this is the first time that full enzymatic conversion of HMF into FDCA has been reported using a reaction cascade in which the H_2O_2 generated by AAO during oxidative transformation of HMF into FFCA is used by a peroxygenase to catalyze conversion of the latter compound into FDCA.

Experimental procedures

Chemicals

HMF, DFF, HMFCA, FDCA, methyl-*t*-butyl ether and ^2H_2O were purchased from Sigma-Aldrich (St. Louis, MO, USA). FFCA was purchased from TCI America (Portland, OR, USA). AmplexRed® and horseradish peroxidase were obtained from Invitrogen (Walthem, MA, USA). DMSO-d_6 was obtained from Merck (Darmstadt, Hessen, Germany).

Enzyme production

Recombinant AAO from *Pleurotus eryngii* was obtained by expressing the mature AAO cDNA (GenBank accession number AF064069) in *Escherichia coli*, and activating and purifying the recombinant enzyme *in vitro*, as previously described [33]. AAO concentrations were determined using a molar absorption coefficient (ε_{463}) of 11 050 $M^{-1}\cdot cm^{-1}$ [33], and activity was estimated using veratryl alcohol (on the basis of an ε_{310} value for veratraldehyde of 9300 $M^{-1}\cdot cm^{-1}$) in 50 mM sodium phosphate, pH 6, at 25 °C. One AAO unit was defined as the amount of enzyme oxidizing 1 μmol of substrate per min.

The UPO-producing *A. aegerita* strain is deposited at the Deutsche Stammsammlung für Mikroorganismen und Zellkulturen (Braunschweig, Germany) under accession number DSM-22459. The extracellular UPO preparation (*Aae*UPO isoform II, 44 kDa) was produced and purified as described previously [10]. The enzyme preparation was homogeneous, as shown by SDS/PAGE, and exhibited an A_{418}/A_{280} ratio of 1.75. Its specific activity was 117 units·mg^{-1}, where 1 unit represents the oxidation of 1 μmol of veratryl alcohol to veratraldehyde in 1 min at 23 °C and pH 7, in the presence of 2.5 mM H_2O_2. The turnover rate (kcat) of the purified enzyme for veratryl alcohol was estimated as 85 s^{-1} (with a Michaelis–Menten K_m constant of approximately 2.4 mM).

Kinetic studies

Steady-state kinetic parameters for AAO oxidation of HMF, DFF, HMFCA and FFCA were calculated by monitoring AAO-catalyzed production of H_2O_2 during oxidation of different substrates concentrations (0.125–32 mM) using a horseradish peroxidase-coupled assay at 25 °C in air-saturated 50 mM sodium phosphate, pH 6. The reactions were initiated by adding the enzyme (0.11 μM). In the presence of the H_2O_2 generated by AAO, horseradish peroxidase (6 units/mL) oxidized AmplexRed® (60 μM), forming resorufin (ε_{563} of 52 000 $M^{-1}\cdot cm^{-1}$) with a 1:1 H_2O_2/resorfurin stoichiometry. Kinetic parameters were obtained by fitting the data to the Michaelis–Menten equation using SigmaPlot software (Systat Softwarwe Inc., San Jose, CA, USA).

AAO and UPO reactions

The time course of 24 h conversions of HMF by AAO was followed at 25 °C in 5 mL sodium phosphate (pH 6) containing 5 μM enzyme (corresponding to 54 units measured with veratryl alcohol as substrate) and 3 mM substrate. Samples were taken after 2, 4, 8 and 24 h. Similar reactions were performed using the partially oxidized HMF derivatives DFF, HMFCA and FFCA.

UPO oxidation of the same substrates (3 mM in 5 mL volume reactions) was followed for up to 120 h using 0.65 μM enzyme (corresponding to 20 units measured with veratryl alcohol) in 50 mM sodium phosphate (pH 7) containing 10 mM H_2O_2.

The effect of UPO on the HMF conversion by AAO was evaluated by simultaneous addition of 0.65 μM UPO (from the start of the AAO reaction) or successive addition of 0.65 μM UPO (after 4 h of AAO reaction under the conditions described above, in 50 mM sodium phosphate (pH 7).

In addition to the above 24 h reactions, the time courses of initial transformation of HMF (32 mM) by AAO (0.3 μM, 3.4 units) and UPO (86 nM, 2.6 units) were followed (after 30, 60 and 180 s) using 50 mM sodium phosphate at pH 6 and 7, respectively.

AAO and UPO residual activities during incubation with HMF and FFCA were tested by taking samples at various reaction times, and measuring the activity as described above (activity immediately after adding the enzyme was taken as 100%).

GC-MS analyses

After various incubation times during 24 h and 3 min reactions of HMF (and its partially oxidized derivatives) with AAO and/or UPO, samples were taken and reactions were stopped by adding 6 M HCl to pH 2–3. The products were liquid/liquid-extracted using methyl-*t*-butyl ether. The extracts were dried with Na_2SO_4 to remove water traces, evaporated in a rotary evaporator, and derivatized using bis (trimethylsilyl)trifluoroacetamide, at 25 °C for 15 min [34]. A gas chromatograph equipped with an HP-5MS column (Agilent, Santa Clara, CA, USA; 30 m × 0.25 mm internal

diameter; 0.25 μm film thickness) coupled to a quadrupole mass detector was used. The oven program started at 110 °C for 2 min, increasing at 20 °C min^{-1} until 310 °C. Helium was used as the carrier gas at a flow rate of 1.2 mL min^{-1}. The compounds involved in the HMF oxidative pathway were identified by comparing their mass spectra with those of standards. Response factors were obtained using various concentrations of these standard compounds to confirm a linear response in the concentration ranges analyzed. These response factors were used to estimate the molar percentage of each of the compounds in the reactions.

NMR studies

The hydration rates of aldehyde solutions (approximately 10 mM) in 50 mM sodium phosphate, pH 6, and 50 mM sodium borate-acetate-phosphate Briton-Robinson buffer, pH 3 and pH 9, prepared using 99.9% isotopic purity ^2H_2O were estimated by ^{1}H-NMR using a Bruker (Billenica, MA, USA) Avance 600 MHz instrument. The internal reference for chemical shifts was the signal from the residual water proton (δ_H, 4.9 ppm). The signal of the H-C-$(OH)_2$ proton in the *gem*-diol form was integrated, and referred to the signal of the H-C=O proton of the aldehyde species for estimation of the hydration degree. Spectra in DMSO-d_6 (isotopic purity ≥ 99.8%) were run as a reference, since they only showed the non-hydrated species.

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Author contributions

ATM and PF planned the experiments; JC, LR, AP, AA, AS, BB, AG, RU and MH performed the experiments and contributed essential material; JC, PF, LR, AP, AA, JJ-B analyzed data; ATM and JC wrote the paper.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Fig. S1. GC-MS analysis of standard compounds.
Fig. S2. ^{1}H-NMR spectra for HMF, DFF and FFCA in DMSO-d_6.
Table S1. Kinetic constants for a variety of AAO substrates.
Doc. S1. MS identification of products from HMF conversion into FDCA.