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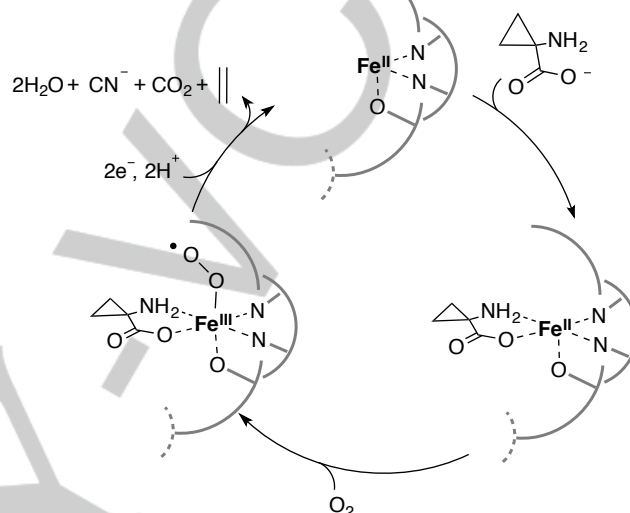
Cu(II)-containing 1-aminocyclopropane carboxylic acid oxidase is an efficient stereospecific Diels-Alderase

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Abstract: In the context of developing eco-friendly chemistry, artificial enzymes are now considered as promising tools for synthesis. They are prepared in particular with the aim to catalyze reactions that are rarely, if ever, catalyzed by natural enzymes. We discovered that 1-aminocyclopropane carboxylic acid oxidase reconstituted with Cu(II) served as an efficient artificial Diels-Alderase. The kinetic parameters of the catalysis of the cycloaddition of cyclopentadiene and 2-azachalcone were determined ($K_M = 230 \mu\text{M}$, $k_{\text{app}} = 3 \text{ h}^{-1}$), which gave access to reaction conditions that provided quantitative yield and >99 % ee of the (1*S*,2*R*,3*R*,4*R*) product isomer. This unprecedented performance was rationalized by molecular modeling as only one docking pose of 2-azachalcone was possible in the active site of the enzyme and it was the one that leads to the (1*S*,2*R*,3*R*,4*R*) product isomer.

Artificial metalloenzymes (AMEs) can be prepared by incorporating transition metals or their complexes into biomacromolecules.^[1] The use of various transition metals should grant AMEs catalytic versatility, whereas biomacromolecules should enable a stereoselective ecofriendly catalysis. Accordingly, AMEs are considered first-class catalysts, particularly in the context of ecofriendly chemistry.^[2] Their preparation strategies have evolved to produce *in vivo* examples,^[3] but the “metal substitution strategy” remains the simplest one. This strategy requires stripping a metalloenzyme from its naturally coordinated metal center, followed by the incorporation of a different metal that may provide an artificial reactivity.^[4]

The substitution of the natural Fe(II) ($K_D = 4.4 \cdot 10^{-6} \text{ M}$) of 1-aminocyclopropane carboxylic acid (ACC) oxidase (Fe(II)-ACCO) by other metal cations such as Ni(II), Co(II), Zn(II) or Cu(II) has been reported.^[5] In most cases the exogenous metal cations have been reported to occupy the active site and coordinate the same amino acids as those that originally coordinated Fe(II), namely two histidine and one aspartic acid residues.^[5] In particular, Cu(II) binding has been investigated and shown to bind at the active site of the enzyme with an affinity similar to that of the native Fe(II) ion



Scheme 1. Proposed mechanism for the catalysis of the oxidation of ACC by natural Fe(II)-ACCO leading to the formation of the phytohormone ethylene. The electrons are provided by ascorbate.

($K_D = 5.5 \cdot 10^{-6} \text{ M}$).^[5c] While the natural Fe(II)-ACCO catalyzes the oxidation of ACC by dioxygen to form the phytohormone ethylene, all its non-iron reconstituted versions have appeared to be inactive in catalyzing ethylene production.^[5b-c] The proposed mechanism of the catalysis proceeds by the coordination of the substrate ACC on Fe(II) in a bidentate fashion, *via* its amino and carboxylate groups.^[6] Subsequently, dioxygen binds to the Fe(II), which leads to the oxidation of ACC and the formation of ethylene, water, cyanide and carbon dioxide (Scheme 1).^[7]

Inspired by the active site of Fe(II)-ACCO and by the way ACC binds to Fe(II), we decided to investigate Cu(II)-ACCO as an artificial Diels-Alderase. Diels-Alder cycloadditions are important reactions for chemical synthesis.^[8] They often take place in organic solvents and require the use of polluting catalysts. Natural enzymes that catalyze these reactions are rare,^[9a-f] which has often incited scientists to prepare artificial alternatives that can function under ecofriendly conditions.^[10]

Known natural Diels-Alderases are metal-free proteins that catalyze the reaction by positioning substrates into the corresponding transition state conformation in what is known as the template effect.^[9a-f] Aided by computational design, artificial metal-free Diels-Alderases relying on the template effect have been prepared and genetically evolved to become highly efficient.^[9g] Catalytic antibodies that rely on this approach were also developed to catalyze Diels-Alder cycloadditions.^[9h-k] By contrast, Mg(II)-containing macrophomate synthase was suspected to be a metallo-Diels-Alderase that coordinates its

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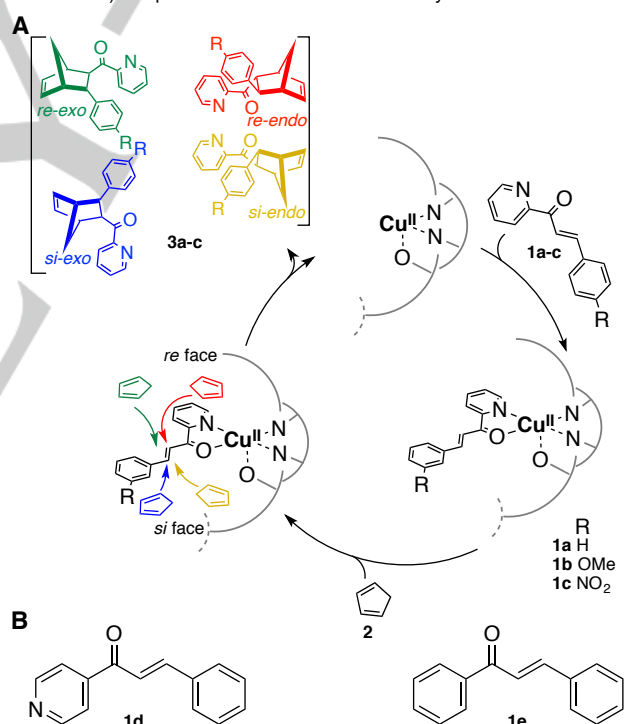
dienophile substrate making it electron-deficient and thus more prone to react with the diene substrate.^[9l-m] It was further demonstrated, that macrophomate synthase functioned instead by catalyzing a Michael addition and a subsequent aldol addition.^[9m-n] Nonetheless, artificial metallo-Diels-Alderase catalyzing Diels-Alder cycloadditions are developed based on the coordination of dienophiles on transition metals using benchmark substrates 2-azachalcon (1a) and cyclopentadiene (2) that provide up to four product isomers (3). It has been shown that Cu(II) is the most efficient water compatible transition metal catalyst for this cycloaddition^[11] and therefore most artificial metallo-Diels-Alderase have been prepared by incorporating Cu(II) complexes into biomacromolecules.^[12] The mechanism of the reaction has been described to proceed by the bidentate coordination of dienophile 1a on Cu(II) *via* the nitrogen and the oxygen of its amino and carbonyl groups, respectively. Diene 2 has been shown to be more prone to react with coordinated, hence electron-deficient 1a, as opposed to non-coordinated 1a.^[11a-b, 13] Thus, the bidentate binding mode of 1a on copper centers is similar to that of ACC on the active-site metal ion in ACCO.^[6a-b, 7] Cu(II)-reconstituted ACCO thus contains the desired transition metal catalyst for the Diels-Alder cycloaddition of 1a and 2 and the required coordination sites to accommodate bidentate 1a are available, along with an easily accessible active site as demonstrated *via* the binding of diverse substrate analogues and inhibitors (Scheme 2A).^[5d] Accordingly, the mechanistic requirements for the catalysis of this Diels-Alder cycloaddition are seemingly fulfilled by Cu(II)-ACCO.

Assays were performed to catalyze the cycloaddition of 1a-e and 2, using Cu(II)-ACCO at 2.5 mol% (Scheme 2 A and B, see SI). The obtained results (Table 1) revealed that with substrate 1a, the reaction was quantitative, the *endo/exo* ratio of 3a isomers was 99/1, and the *ee* was 95 % in favor of the major *endo* product. Similar results were obtained with 1b though with a slightly lower yield (95 %), most probably due to its electron donating methoxy substituent as previously observed.^[11a-b, 14] Nitro substituted 1c on the other hand, provided the lowest yield (85 %) and *ee* (54 %) and an *endo/exo* ratio of 83/17, but was fully converted which indicated that side reactions took place as previously noted with this reactive substrate.^[11a-b, 14] It is noteworthy that 1d-e, which are only capable of monodentate coordination on Cu(II) instead of the bidentate coordination that is allowed with reactive 1a-c, did not react. Consequently, it can be assumed that here also the Diels-Alder reaction requires the bidentate coordination of the dienophiles 1a-c on Cu(II). Thereafter, diene 2 reacted with the bound and electron deficient dienophiles.

To our knowledge, no other artificial Diels-Alderase was ever prepared by following the “metal substitution strategy”, instead, only the “covalent anchoring strategy” and supramolecular insertion of metal cofactors into proteins using either the “host-guest strategy” or the “Trojan-horse strategy” were followed.^[3b, 15]

anchoring of 1,10-phenanthroline-Cu(II) complex into the mutant M89C of the lactococcal multidrug resistance regulator.^[15d] It provided 3a in up to 93 % yield and 97 % *ee* with an *endo/exo* ratio of 95/5. In light of the herein reported results, Cu(II)-ACCO stands out as an artificial Diels-Alderase that is simple to prepare and provides similarly highly stereoselective catalysis but with quantitative yield associated with an *endo/exo* ratio of 99/1. The crystal structure of natural Fe(II)-ACCO,^[5b] revealed a tetrameric form with the C-terminal part of one enzyme monomer interlocking with the C-terminal part of an adjacent monomer. This C-terminal part is thought to be involved in the catalysis as it has been found essential to the natural activity,^[16] thus the crystallographic tetrameric form is probably not representing an active conformation of the enzyme. In order to investigate the importance of the C-terminal part in the Diels-Alder activity, we produced an enzyme variant truncated from residue 290, so called ACCOt^[16b] and prepared the corresponding Cu(II)-ACCOt AME. We found that unlike Fe(II)-ACCOt that was incapable of ethylene production, both Cu(II)-ACCO and Cu(II)-ACCOt AMEs showed similar catalytic properties (Table 1). These results suggest that the C-terminal loop is neither involved in the coordination of Cu(II) nor in that of substrates 1a-c.

Scheme 2. A) Proposed mechanism for the catalysis of the Diels-Alder



cycloaddition reaction of substrates 1a-c to 2 by Cu(II)-ACCO leading to the formation of products 3a-c. B) Monodentate substrate analogues.

Table 1. Results of the catalysis of the Diels-Alder cycloaddition of the corresponding substrates 1a-e 2 mM and 2 20 mM after 72 h at 4 °C.

Catalyst ^[a]	Yield (%) ±3 ^[b]					<i>endo/exo</i> ^[b]					<i>ee</i> (%) ±1 ^[b]				
	3a	3b	3c	3d	3e	3a	3b	3c	3d	3e	3a	3b	3c	3d	3e
Buffer ^[c]	5	<3	<3	<3	<3	82/18	83/17	65/35	n.d.	n.d.	0	0	0	n.d.	n.d.
Cu(NO ₃) ₂	50	32	21	<3	<3	82/18	83/17	65/35	n.d.	n.d.	0	0	0	n.d.	n.d.
apo ACCO	4	4	10	<3	<3	86/14	n.d.	68/32	n.d.	n.d.	0	0	0	n.d.	n.d.
apo ACCOt	5	3	11	<3	<3	86/14	n.d.	68/32	n.d.	n.d.	0	0	0	n.d.	n.d.
Cu(II)-ACCO	100	95	85	<3	<3	99/1	98/2	83/17	n.d.	n.d.	95	93	54	n.d.	n.d.
Cu(II)-ACCOt	100	95	86	<3	<3	99/1	98/2	83/17	n.d.	n.d.	95	93	53	n.d.	n.d.

[a] 2.5 mol% loading [b] All measurements were performed in triplicates (see SI). [c] 20 mM MOPS buffer, pH 7.4 and 50 mM NaCl. n.d.: not determined

The most efficient of these was prepared by the covalent

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ACCOT^[16b] and prepared the corresponding Cu(II)-ACCOT AME. We found that unlike Fe(II)-ACCOT that was incapable of ethylene production, both Cu(II)-ACCO and Cu(II)-ACCOT AMEs showed similar catalytic properties (Table 1). These results suggest that the C-terminal loop is neither involved in the coordination of Cu(II) nor in that of substrates **1a-c**.

The observed selectivities of AMEs have been investigated with the help of molecular modelling by different groups, including ours.^[15a-c] Schrödinger Biologics Suite was used to perform the molecular docking of the substrate **1a** in Cu(II)-ACCO (see SI). The results showed that only one pose of **1a** in the protein was possible (Figure 1). The Cu(II)-bound carbonyl group and pyridine of substrate **1a** occupied a small cavity inside the catalytic pocket. The phenyl group on the other hand, dangled in a larger cavity. The double bond of **1a** that is involved in the Diels-Alder reaction was sandwiched between isoleucine 184 from the *si*-face and lysine 158 from the *re*-face. Additionally, phenylalanine 250 covered the double bond from the non-coordinated side of **1a**. Thus, the *si*-face of the double bond of **1a** stacked on the protein at the inter cavity and was therefore protected from both the *si*-endo and the *si*-exo attack of **2**. The *re*-face of the double bond was however only protected from the *re*-exo attack of **2** as it remained accessible from the coordinated side of **1a**. According to these results, the **3a** *si*-endo, *si*-exo and *re*-exo isomers were all disfavored and the AME should produce selectively the **3a** *re*-endo isomer *i.e.* the (1*S*,2*R*,3*R*,4*R*) isomer. This is in good agreement with the experimental results as the major product (95 % *ee*) was indeed in the *re*-endo configuration as determined by circular dichroism, following an already described procedure (see SI).^[15c] The minor formation of *si*-face attack products could possibly be assigned to the reaction taking place in solution *i.e.* outside the protein catalytic pocket, due to the high concentration of both substrates (5 % yield in buffer in the absence of ACCO and Cu(II)). To verify this hypothesis and in prospect of improving the reaction conditions, we performed kinetic studies.

The kinetics of the catalysis by the AME was explored using **1a** and **2** as substrates (See SI). To our knowledge, kinetic parameters have not been determined for other protein-based artificial metallo-Diels-Alderses, most probably because of the

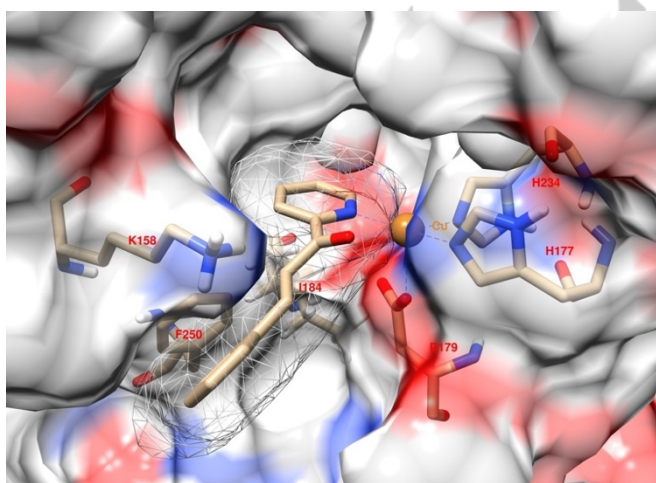


Figure 1. substrate **1a** is shown as sticks docked in the active site of Cu(II)-ACCO which is shown as a surface colored by heteroatoms. The Cu(II) center is shown as an orange sphere and the relevant amino acid residues as sticks.

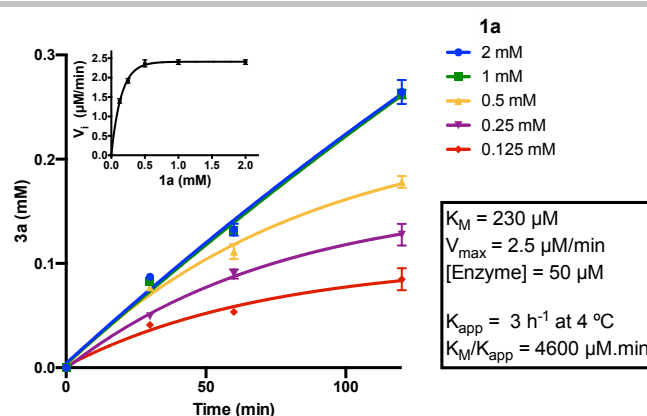


Figure 2. Kinetic of the formation of **3a**. The concentration of **2** was 400 times that of the enzyme. Insert, the extracted initial velocities (V_i) as function of substrate concentration. All measurements were repeated at least three times (see SI).

relatively high quantities of AMEs that are required for such studies. The products formation was monitored at 4°C as a function of time at different concentrations of **1a** while the concentration of the AME was maintained at 50 µM and that of **2** at 20 mM (Figure 2), and initial velocities were extracted (Figure 2 inset). The results revealed that Cu(II)-ACCO follows a pseudo first order Michaelis-Menten kinetics with apparent parameters $K_M = 230 \mu\text{M}$ and $k_{app} = 3 \text{ h}^{-1}$. At V_{max} , the reaction catalyzed by the AME was about 100 times faster than that catalyzed by Cu(II) in the absence of protein.^[3b, 15b] In comparison, Fe(II)-ACCO was reported to catalyze the oxidation of ACC at 29°C with $K_M = 150 \mu\text{M}$ and $k_{cat} = 11 \text{ min}^{-1}$.^[16a] Note that under the initially used conditions, **1a** and **2** resulted in 5 % yield of all four **3a** isomers in the absence of ACCO and Cu(II).

With the kinetic parameters in hand, we attempted to further increase the selectivity of the catalysis by avoiding the formation of products outside the catalytic pocket of the AME. To limit this non-selective reaction of substrates in excess with respect to the AME (2.5 % AME), the addition of both **1a** and **2** was fractionated in time. With 1 equiv. AME, the reaction was performed by addition of 5 equiv. **1a** and 50 equiv. **2** every 8 h, for up to 40 times followed by an additional 48-50 h then extraction and products analysis. By following this procedure, the excess of substrates was limited. These conditions were very effective as adding up to 90 equiv. of **1a** formed exclusively the **3a** *re*-endo product isomer in a quantitative yield (TON = 90, *ee* > 99 %). Furthermore, the full conversion of up to 185 equiv. of **1a** was possible but **3a** *re*-

Table 2. Results of the catalysis using 50 mM AME upon fractionated addition of substrates.

equiv. of 1a ^[a]	Reaction time for full conversion (h) ^[b]	endo/exo ^[c]	ee (%) ± 1 ^[c]
20	80	100/-	>99
60	144	100/-	>99
90	192	100/-	>99
95	200	100/-	99
120	240	100/-	99
160	304	99/1	98
180	336	99/1	97
185	345	98/2	95
200	>400 ^[d]	97/3	92

[a] Fractionated in 5 equiv. and added every 8h with 50 equiv. of **2**. [b] Time necessary to achieve full conversion of **1a**. [c] All measurements were performed in triplicates (see SI). [d] Full conversion not reached, 96 % yield after 400 h.

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endo was obtained with 95 % ee. Upon further addition of substrate, full conversion could not be reached most probably because the enzyme was denaturing after more than 16 days at 4 °C (Table 2, see SI).

In summary, ACCO reconstituted with Cu(II) following the metal substitution strategy was discovered to be an efficient and stereoselective Diels-Alderase. Computational studies were performed and revealed the origin of the high selectivity of the reaction. Additionally, thanks to the simplicity of the preparation of the artificial enzyme, kinetic parameters could be measured, and reaction conditions adapted to perform quantitative and stereospecific Diels-Alder cycloadditions of up to 90 equiv. substrate.

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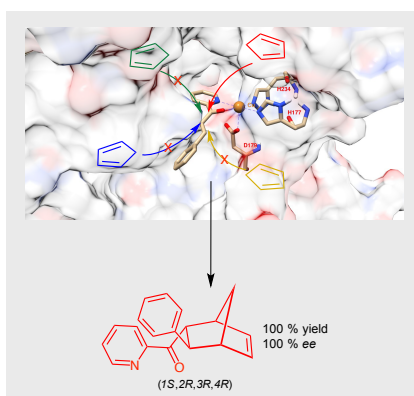
COMMUNICATION

Entry for the Table of Contents (Please choose one layout)

Layout 1:

COMMUNICATION

Artificial enzymes catalyze reactions that are rarely, if ever, performed by natural enzymes. We discovered and that 1-aminocyclopropane carboxylic acid oxidase reconstituted to contain Cu(II) served as a Diels-Alderase ($K_M = 230 \mu\text{M}$, $k_{app} = 3 \text{ h}^{-1}$) which provided up to a quantitative yield and >99 % ee of products. This unprecedented performance was rationalized by molecular modeling.



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