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From Phenols to Antimicrobial Phenazines: Tyrosinase-like Catalytic Activity of a Bisguanidine Based Bis(μ-oxido) Complex

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Dedicated to Prof. Dr. Takashi Hayashi on the occasion of his 60th birthday

The catalytically active center of the enzyme tyrosinase, standing out for its unusual substrate diversity, consists of a side-on μ-η^2:η^2-peroxido complex (^{S}**P**). Several ligand systems stabilizing a ^{S}**P** and able to mimic the catalytic activity of the enzyme towards simple phenolic substrates are known. Only a few catalytically active systems based on the isoelectronic isomer structure of ^{S}**P**, a bis(μ-oxido)dicopper(III) complex (**O**), were investigated until now. Two years ago, we presented with the TMGbenza a hybrid guanidine based tyrosinase model system stabilizing an **O** species with an exceptional substrate diversity. Herein we studied the catalytic activity of another **O** species stabilized by the bisguanidine ligand TMG_2tol. The reaction conditions for the catalytic oxygenation were optimized and a broad spectrum of phenolic substrates like naphthol, quinolinols and indolols was tested. Naturally occurring phenazine derivatives like phenazine-1-carboxylamide (PCN) and phenazine-1-carboxylic acid (PCA) show antifungal as well as antibacterial activity, functioning as biological control agents against crop disease like take-all. Hence, the synthesized phenazines were evaluated for their potential antimicrobial activities against representative gram-positive and gram-negative bacteria.

Introduction

Implementing sustainability in industrial processes gained increasing interest within the last decade.[1] Based on the model of green chemistry, toxic chemicals posing a major risk for environment and humans have to be reduced.[2] Currently, industrial oxidation processes mostly use molecular oxygen, air or peroxides as an oxidant. molecular oxygen and air seem to be environmentally friendly in contrast to hazardous peroxides, but to function as an oxidizing agent toxic transition metal catalysts containing rhodium, ruthenium or palladium are needed.[3] molecular oxygen needs to be transferred from an inert triplet ground state to an activated peroxide ion in a singlet state by reduction. Therefore, metal catalysts able to reversibly bind and release molecular oxygen by changing their oxidation state are needed.[4]

In nature, activation, transfer and/or reversible binding of molecular oxygen are catalyzed by enzymes containing the transitions metals iron or copper. Well-known examples are the enzymes cytochrome C oxidase and hemocyanin controlling the oxygen transfer within the respiratory chain of aerobic living organisms.[5,6] Another important oxidase is tyrosinase which is capable of activating molecular oxygen. The catalytically active center consists of two copper centers being coordinated by six histidine ligands and a peroxide moiety, a so called side-on μ-η^2:η^2-peroxido complex (^{S}**P**).[7–9] Tyrosinase, in contrast to most enzymes, is not limited to a specific substrate molecule: it can generate quinone products from either diphenolic or monophenolic substrates.[6,10] This special characteristic is highly attractive for catalytic applications in industrial processes. Enzymes are meeting many goals of "green chemistry". They operate under mild reaction conditions in aqueous reaction media displaying a high activity and selectivity.[1] However, their catalytic activity is usually substrate specific and limited to a certain product.[11] By modeling the enzymatic function with transition metal catalysts the gap of "green" catalysts could be filled circumventing the disadvan-

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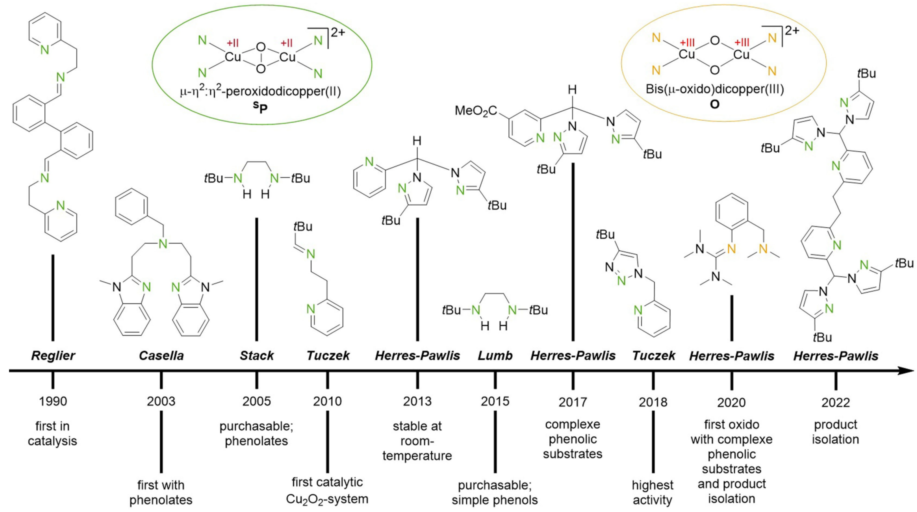


Figure 1. Catalytically active tyrosinase model systems with a side-on peroxido (^{S}**P**) or oxido (**O**) Cu_2O_2 center developed within the last 35 years.[13–16,18–21,23]

tages of enzymes. Tyrosinase activates oxygen and shows an exceptional substrate flexibility.[6,10] Thus, mimicking the active Cu_2O_2 center of tyrosinase to create a catalyst with a high substrate flexibility working under various reaction conditions is an intensively investigated target.

Already 35 years ago, first attempts to create a catalytically active tyrosinase model system were made.[12] A few years later Réglier presented the first catalytically active model system. The N-donor system based on pyridinyl and iminyl donors was able to generate 3,5-di-*tert*-butyl-*o*-benzoquinone (DTBQ) from 2,4-di-*tert*-butylphenol (DTBP–H) using triethylamine as the auxiliary base (Figure 1).[13]

Despite this breakthrough, just a few substrate-converting tyrosinase model systems were found within the following twenty years.[10] In 2003, Casella et al. demonstrated the first system with a tyrosinase-like peroxido complex which was able to transform sodium 4-carbonylmethoxyphenolate to the related quinone.[14] Only in recent years, more catalytically active ligand systems were established. Tuczek et al. presented the first catalytically active system based on a peroxido complex in 2010. It consists of a pyridinyl and an iminyl donor bridged by an ethylene spacer.[15] A few years later, the catalytic activity could be increased by replacing the iminyl donor with a triazolyl donor.[16] At the same time, the groups around Lumb and Stack worked with the purchasable ligand *N*,*N*-di-*tert*-butylethylenediamine. Stack et al. used phenolates as substrate while Lumb et al. worked with simple phenolic substrates to generate quinones.[17,18] In 2013, our working group reported the first catalytically active room temperature stable tyrosinase model system. In contrast to previously reported systems, more complex phenolic substrates like 8-quinolinol could be transformed to the corresponding quinones.[19] Extending the pyridinyl donor with an ester function led to a decrease of the reaction time to 10 seconds.[20] Last year, we presented a dinuclear bis(pyrazolyl)(pyridinyl) ligand which is able - as peroxide complex - to transform complex phenolic substrates like 2-naphthol.[21] Furthermore, reaction products could be isolated as phenazine products using a condensation reaction with 1,2-phenylendiamine.[21,22] Figure 1 summarizes the discussed tyrosinase model systems based on side-on peroxido complexes chronologically.

All tyrosinase model systems mentioned so far are based on side-on peroxido complexes stabilized by basic N-donor ligands like the catalytically active center in the enzyme tyrosinase. The μ-η^2:η^2-peroxido complex refers to the Cu_2O_2 systems and exists in an equilibrium with another isoelectronic isomer structure, a so-called bis(μ-oxido) complex (**O**).[9] The oxido structure is similar to the one of side-on peroxido complexes ^{S}**P** but due to the missing O–O bridge the two copper atoms are in the higher oxidation state +III (Figure 2). The equilibrium between both isomers can be controlled by the choice of the ligand system, corresponding anion or solvent.[8,24–27]

Numerous ligand systems stabilizing bis(μ-oxido) complexes are known,[25–27,28,29–31] but only a few of them convert simple phenolic derivatives like DTBP–H or 4-OMe–Ph stoichiometrically to C–C coupling products.[29–31] Three years ago, we presented with the TMGbenza ligand the first catalytically active

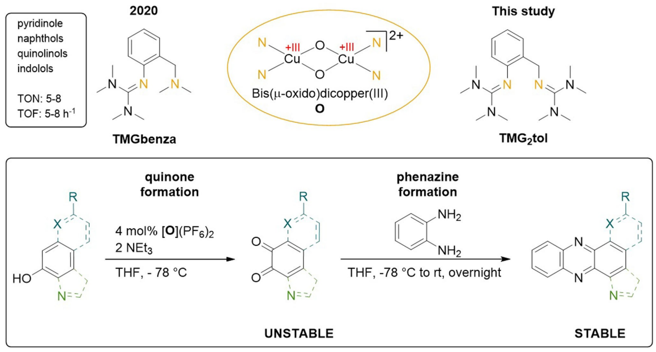


Figure 2. Oxygenation of phenolic substrates with a bis(μ-oxido) complex (**O**) based on a guanidine ligand as catalyst. First, phenolic substrates are converted in an unstable quinone product. A subsequent condensation with o-phenylendiamine (PDA) results in a stable phenazine product.[22]

tyrosinase model system based on a bis(μ-oxido) complex capable to catalyze a broad substrate scope of phenol derivatives to the corresponding *o*-quinones and subsequently to phenazines (TON 5–8) (see Figure 1 and 2). The method established by Réglier[13] and Bulkowski[32] later optimized by Tuczek et al.[15,16,33] with 25 equivalents substrates and 50 equivalents of the auxiliar base triethylamine was extended by a condensation reaction with 1,2-phenylendiamine (PDA).[22] Already 75 years ago, PDA was used to investigate catalytic activity of tyrosinase in potatoes by forming fluorescent-active phenazines.[34] The method was later applied on various *o*-quinones to detect their presence via fluorescence spectroscopy.[35] That way, the unstable, highly reactive and fluorescent-inactive quinone products[36] are converted to fluorescent-active phenazines, which are stable at ambient conditions (Figure 2).

Although the TMGbenza system appeared promising as a tyrosinase-like catalyst for industrial applications, an increase to an industrial reaction scale would be complicated. The synthesis of TMGbenza is time- and cost-intensive, needing at least three days as well as hazardous chemicals like hydrazine.[22] To fill this gap, a ligand system similar to TMGbenza is needed, capable of catalyzing the oxygenation of various phenolic substrates, whose synthesis is time- and cost-efficient.

The ligand TMG_2tol is structurally based on the same backbone as TMGbenza, an *ortho* tolyl group. TMGbenza, which is a hybrid guanidine ligand, has a dimethylamine group in *ortho* position to a tetramethylguanidine group. In contrast, TMG_2tol is a bisguanidine ligand containing two identical tetramethylguanidine groups (Figure 2). In previous studies, TMG_2tol showed the ability to stabilize a Cu_2O_2 center at $-78\,°C$. The formed bis(μ-oxido) complex ($[Cu_2O_2(TMG_2tol)_2](PF_6)_2$; [**O1**]$(PF_6)_2$) was stable at $-78\,°C$ for several hours.[37] Due to its structural analogy with the Cu_2O_2 system based on TMGbenza we propose a similar oxygenation reactivity.

Phenazines cannot only be synthesized by the condensation with PDA. They are also produced naturally by bacteria starting from the carboxylic acid chorismic acid.[38] The phenazine derivatives phenazine-1-carboxylamide (PCN) and phenazine-1-carboxylic acid (PCA) are synthesized in the rhizosphere of crop plants like wheat by soil bacteria.[39] In this plant-root-interphase[40] they are functioning as antibiotic containing the spread of fungal diseases like take-all, which results in root death.[41] Experiments using PCA as antibacterial agent against various types of bacteria showed a high toxicity of PCA against gram-positive as well as gram-negative bacteria at low pH values. At pH values near the neutral range (>5) gram-negative bacteria were found to be resistant against the antimicrobial function of PCA while gram-positive bacteria could still be destroyed.[42] As the phenazine derivatives synthesized in this study are structural modifications of PCA their antibacterial function will be tested using the bacteria *Bacillus subtilis* and *Escherichia coli.*

Results and Discussion

Catalytic Oxygenation of Phenolic Substrates

The proposed catalytic activity of the TMG_2tol based complex [**O1**]$(PF_6)_2$ towards various phenolic substrates was investigated using a catalyst concentration of 4 mol%, analogous to the former study with the TMGbenza based system, at a reaction temperature of $-78\,°C$ and with a reaction time of one hour. Subsequently, PDA was added and the mixture was stirred at room temperature overnight. Generated phenazine products as well as regained unconverted substrate were isolated via column chromatography and verified with NMR spectroscopy.

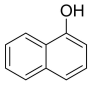
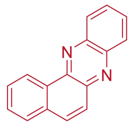
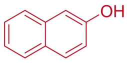
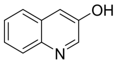
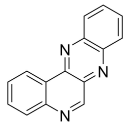
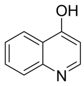
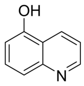
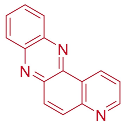
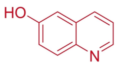
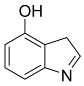
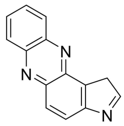
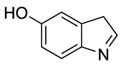
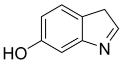
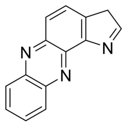
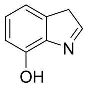
The study based on TMGbenza took place at $-90\,°C$ as the catalytically active bis(μ-oxido) complex showed a fast decay at higher temperatures.[22] In this study, the reaction temperature was slightly increased to $-78\,°C$, as the bis(μ-oxido) complex [**O1**]$(PF_6)_2$ displayed a high stability at this temperature for several hours in a former study.[37]

Yields and turn over number (TON) in the following experiments are calculated using the mass of the isolated fractions after purification via column chromatography and the concentration of the catalyst [**O1**]$(PF_6)_2$ (4 mol%). Yield loss due to side reactions forming C–C and/or C–O coupling products, which could not be isolated but were identified via mass spectrometry, are discussed at the end of this section.

First test experiments using 6-indolol (**S9**) as substrate resulted in a low yield (4%) of the desired product pyrrolo[2,3-*a*]phenazine (**P5**) while 50% of the substrate, **S9**, remained unconverted. A decrease of regained substrate **S9** to 26% could be observed with increasing reaction time (3 h), but the yield of the desired product **P5** did not increase (see Table S3 in the supporting information). Even a longer reaction time of five as well as eight hours did not lead to higher product yields. However, the amount of unconverted substrate was minimized to 1%. With a yield of 3–4% and a catalyst concentration of 4 mol% **S9** was only converted with a TON of 1 to the desired phenazine product by the TMG_2tol system (Table 1). Thus, no significant catalytic activity could be determined. To evaluate whether the TMG_2tol system is unable to convert a specific substrate group or if it is catalytically inactive, additional phenolic substrates were investigated. In the following experiments, 2-naphthol (**S2**) and 6-quinolinol (**S6**) were converted with [**O1**]$(PF_6)_2$ as catalyst over a reaction time of five and eight hours. Both substrates were successfully transformed to the desired phenazine products. In the case of **S2**, the yield of the desired product benzo[*a*]phenazine (**P1**) considerably increased with extending reaction time (14% after 5 h, 31% after 8 h). However, a large amount of substrate **S2** was still regained unconverted (37% after 8 h). In contrast, the conversion of **S6** revealed only a slight increase of the desired product's yield with longer reaction times (11% **P3** after 5 h, 16% **P3** after 8 h), while no unconverted substrate **S6** was isolated after eight hours reaction time (Table 1).

[**O1**]$(PF_6)_2$ demonstrates a contradictory reactivity in the catalytic oxygenation of phenolic substrates. While **S2** and **S6** were converted successfully (TON 8and 4; see Table 1 marked in

Table 1. Phenolic substrate **S** investigated in the catalytic oxygenation with [**O1**]$(PF_6)_2$ and subsequent condensation with PDA as well as averaged, isolated yields and TON of desired phenazine products **P**. Substrates showing catalytic activity (TON > 1) are highlighted in red.

entry	substratee		Y[a] [%]	TON[b]	phenazine product	
1		**S1**	1	< 1		**P1**
2		**S2**	31	8		
3		**S3**	5	1		**P2**
4		**S4**	0	0		
5		**S5**	0	0		**P3**
6		**S6**	16	4		
7		**S7**	0	0		**P4**
8		**S8**	4	1		
9		**S9**	4	1		**P5**
10		**S10**	0	0		

[a] Isolated yield after purification via column chromatography; Reaction conditions: catalytic oxygenation at −78 °C, 8 h; PDA condensation at room temperature (20 °C), overnight. [b] TON calculated using the isolated product yield and the concentration of [**O1**]$(PF_6)_2$ (4 mol %).

red), the activity towards **S9** was low (TON < 1). For **S2** and **S6**, an increase in the reaction time resulted in higher yields of the desired products. In addition, after 8 hours reaction time, no unconverted substrate was regained when converting **S6**. Thus, further investigations were performed with a reaction time of 8 hours. To verify the presumed inactivity of the TMG_2tol based Cu_2O_2 system towards specific substrate groups, the study was enlarged by various naphthols, quinolinols and indolols already successfully converted in a former study by the TMGbenza based Cu_2O_2 system.[22] Contrary to the TMGbenza based Cu_2O_2 system,[22] no phenazine products were observed in the catalytic oxygenation of 4-quinolinol (**S4**), 5-quinolinol (**S5**), 4-indolol (**S7**) and 7-indolol (**S10**) with [**O1**]$(PF_6)_2$. However, the TMG_2tol based Cu_2O_2 system did show a slight activity towards 1-naphthol (**S1**), 3-quinolinol (**S3**), 5-indolol (**S8**) and 6-indolol (**S9**) with yields between 3 and 5 % (Table 1).

The contradictory reactivity of [**O1**]$(PF_6)_2$ towards phenolic substrates indicates the inhibition of the desired catalytic oxygenation to *o*-quinones and subsequently to phenazines by side reactions or decay of the unstable *o*-quinone intermediate. The used copper salt, $[Cu(MeCN)_4]PF_6$, is known to catalyze the conversion of DTBP–H to the corresponding *ortho*-quinone without the need for a supporting ligand system.[43] Hence, test experiments using the pure copper salt in the catalytic conversion were performed to exclude $[Cu(MeCN)_4]PF_6$ as a possible catalyst. Therefore, the most reactive substrate out of each substrate group was selected for catalytic investigations using pure copper salt under the given reaction conditions (see Table 1). All three substrates, **S2**, **S6** and **S8**, were successfully converted to the desired phenazines but only with small yields of 4 % to 5 % (see Table S5 in the supporting information). The obtained yields with pure copper salt are in the same range as the ones of the catalytic conversion of **S1**, **S3**, **S8** and **S9** with [**O1**]$(PF_6)_2$. Consequently, a clear distinction between the catalytic activity of [**O1**]$(PF_6)_2$ and the catalytic activity of the pure copper salt towards those substrates is impossible. As a result, an actual catalytic activity of [**O1**]$(PF_6)_2$ in the oxygenation of phenolic substrates could only be proven for **S2** (31 %, TON 8) and **S6** (16 %, TON 4) (see Table 1 marked in red).

In the catalytic conversion of phenolic substrates different side reactions are known to occur. Converting DTBP–H, Tuczek et al.[43] as well as Lumb et al.[44] observed a C–C coupling reaction generating a tetra-*tert*-butyl-biphenol. Using 3-*tert*-butylphenol or 4-OMe–Ph a C–O coupling reaction between

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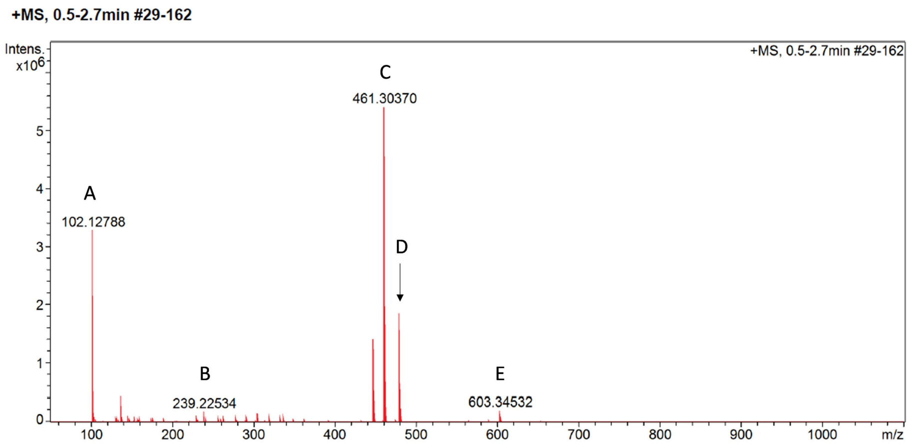
two substrate molecules with subsequent *o*-quinone formation took place.[16] Also with the TMGbenza based Cu_2O_2 system C–O coupling reactions could be observed for simple phenolic substrates like 4-OMe–Ph or *para*-*tert*-butylphenol.[22] To investigate whether these side reactions are inhibiting the conversion of phenolic substrates with [**O1**]$(PF_6)_2$ to *o*-quinones and subsequently to phenazines ESI mass spectrometry was performed. Therefore, one substrate out of each substrate group showing no or only slight phenazine formation was selected. ESI-MS measurements were performed at two points of the reaction using substrates **S1**, **S4** and **S7**: (i) directly after substrate addition at low temperature (−78 °C) and (ii) after 8 h reaction time, condensation with PDA and work up at room temperature (20 °C). Starting with **S1**, ESI-MS spectra at low temperature indicate C–O as well as C–C coupling products of the substrate and desired quinones. Measurements after condensation with PDA showed the formation of a condensation product resulting from two phenazine molecules as well as the C–O coupling products already detected before (see Figure 3 and Figure S1 in the supporting information). Neither unconverted **S1** nor the desired phenazine product benzo[a]phenazine (**P1**) could be detected via CSI-MS or ESI-MS. Moreover, small amounts of 2,3-dinitrosophenazine were detected. PDA is known to build condensation products with itself under the presence of an oxidizing agent like molecular oxygen or a catalyst like Cu^{2+}.[45] In the presence of an oxido-peroxido molybdenum catalyst, aromatic amines are converted into nitroso products.[46] Thus, with the oxido catalyst [**O1**]$(PF_6)_2$ and dissolved oxygen present in the reaction mixture, two PDA molecules could be linked towards each other forming 2,3-diamino-phenazine which was oxidized to 2,3-dinitrosophenazine afterward (Entry B in Figure 3).

CSI and ESI mass spectra of the catalytic conversion with **S4** as well as **S7** did not show any C–C or C–O coupling products. However, nitroso products resulting from PDA oxidation (1,2-dinitrosobenzene) (Entry B in Table S8 and S9 in the supporting information) or PDA condensation and subsequent oxidation (2,3-dinitrosophenazine (Entry H in Table S8, Entry F in S9 in the supporting information) could be found in both reaction mixtures (see also Figure S2 and S4 in the supporting information). While mass spectra of the conversions with **S4** contained traces of substrate and the desired product quinolino[3,4-b]quinoxaline (**P2**) (Entry C andG in Table S8, Figure S3 in the supporting information), spectra of the conversion with **S7** did not show any substrate or product.

As a result, [**O1**]$(PF_6)_2$ is able to convert several types of phenolic derivatives like naphthols, quinolinols or indolols. However, the *o*-quinone formation is either inhibited by the



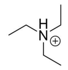
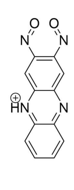
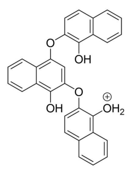
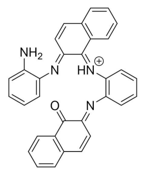
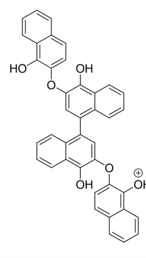
	A	B	C	D	E
m/z detected	102.1279	239.2253	461.3037	479.1875	603.3453
m/z calculated	102.1278	239.0564	461.1384	479.1867	603.1803
molecular formula	$C_6H_{16}N^+$	$C_{12}H_7N_4O_2^+$	$C_{30}H_{21}O_5^+$	$C_{32}H_{23}N_4O^+$	$C_{30}H_{21}O_6^+$
molecule structure					

Figure 3. ESI-MS spectrum and assigned m/z values of the catalytic conversion of 1-naphthol with [**O1**]$(PF_6)_2$ and subsequent condensation with PDA after extraction of the crude reaction mixture.

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formation of C–O and C–C coupling products or a contrary reaction speed within the two consecutive reactions where the slow phenol conversion to *o*-quinones vs. fast *o*-quinone conversion to phenazines is diminishing the yield of phenazines.

Additionally, the TMGbenza Cu_2O_2 system, successfully converting various phenolic substrates, consists of the same backbone as TMG_2tol but contains only one bulky guanidine donor combined with a smaller amine donor. Thus, the two bulky guanidine donors in TMG_2tol might hinder the substrate access to the copper centers which would explain the amounts of unconverted substrate regained in the conversion of **S9**. Consequently, PDA undergoes oxidation and condensation reactions with itself instead of condensation reactions with the *o*-quinones to the desired phenazines.

Antimicrobial Activity of the Phenazine Products

The antimicrobial activities of the new phenazines (**P1**, **P2**, **P3**, **P4**, and **P5**) were evaluated against different bacterial strains using the disk-diffusion assay. *Bacillus subtilis* was inhibited by all the phenazines at 20 μg, with **P2** demonstrating the highest zone of inhibition at 22.2 ± 1.13 mm ($p < 0.01$) (Table S10 in the supporting information). *Pseudomonas putida* PG5 had a lower degree of susceptibility, with only phenazines **P3** and **P5** showing zones of inhibition at 10.9 ± 0.42 mm and 10.8 ± 0.00 mm, respectively. None of the tested phenazines exhibited inhibitory effects on the other tested bacteria (Table S10 in the supporting information). Additionally, we investigated the impact of four phenazines (**P1**, **P2**, **P3**, and **P4**) at higher amount (10 mg) on liquid cultures of *B. subtilis and Escherichia coli* over a 4-hour incubation period at 37 °C. While there was no significant difference in the reduction of the colony forming unit (CFU) for both organisms after incubation with the control dimethyl sulfoxide (DMSO), *B. subtilis* displayed significant decrease in CFU for all phenazines (Figure 4). In contrast, *E. coli* exhibited no significant reduction of CFU for phenazine **P2** but showed significant decrease for **P1** and **P4** (supporting information, Figure S5).

The bioactivity of the new phenazines against gram-positive bacterium *B. subtilis* was clearly demonstrated. This has been observed in earlier studies where phenazines, such as pyocyanin and phenazine-1-carboxylic acid (PCA), are inhibitory to gram-positive but not to gram-negative bacteria,[42,47] attributed to a lack of repulsion of the phenazines against gram-positive cell wall at low pH, resulting to a permissive accumulation in the cell.[42] However, the new phenazines showed no inhibitory effect against the other gram-positive bacteria tested. It is likely that they have resistance mechanisms against these phenazines like the use of efflux pumps for detoxification.[48] Similarly, majority of the tested gram-negative bacteria displayed resistance to most of the new phenazines. This resistance is often attributed to the additional protective barrier formed by the outer membrane, which prevents phenazines from entering and accumulating within the bacterial cell.[49]

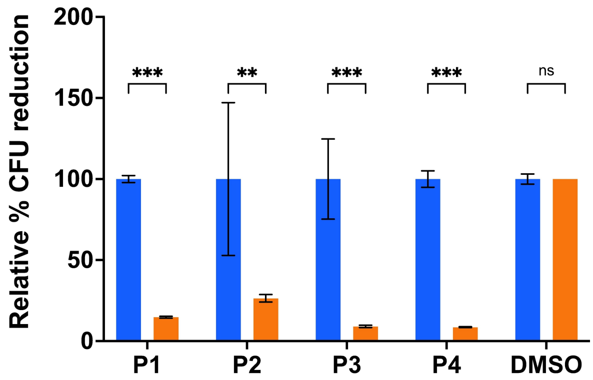

Figure 4. Assessment of the antimicrobial activity of the new phenazines in *Bacillus subtilis.* Liquid cultures (10 mL) of *B. subtilis* were amended with 10 mg of phenazine **P1**, **P2**, **P3**, or **P4** and incubated at 37 °C. DMSO was added at the same amount as negative control. At both the initial time of incubation (T0, blue bars) and 4 hours after (T4, orange bars), aliquots of 100 μL from a 1:1000 serial dilution were plated. The percent reduction of colony forming unit (CFU), relative to the control, were compared between time points. Significant difference is indicated by asterisks (2-way ANOVA: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; $n = 2$); ns, not significant.

However, the susceptibility of *P. putida* to **P3** and **P5**, and *E. coli* to **P1** and **P4** at higher amount, suggests the possibility of distinct inhibition mechanisms associated with the diverse structures of the newly synthesized phenazines. Furthermore, the role of phenazines extends beyond their synthetic origin, as the ability of *Pseudomonas aeruginosa* to produce these compounds has been linked to antagonistic effects on bioactive metabolites, including antimicrobials.[50] This antagonism not only promotes antibiotic resistance but also contributes to virulence, highlighting the intricate interplay between bacterial phenazines and their potential consequences in microbial communities and infections.

While phenazines are commonly recognized to induce the production of reactive oxygen species, rendering them effective against a broad range of bacteria,[51] our phenazines possess unique structures that likely modify their antimicrobial mechanisms. For instance **P2** contains moieties of quinolinones, which are known to inhibit gyrases and topoisomerases,[52] and quinoxalines, which inhibit nucleotide synthesis and are reported to be highly active against gram-positive bacteria.[53] These characteristics may explain the heightened susceptibility of *B. subtilis* to **P2** compared to other phenazines. On the other hand, phenazine **P3** contains a pyridine ring, while **P4** and **P5** feature pyrrole moieties. Antimicrobials containing these *N*-heterocyclic rings have diverse modes of action, including the inhibition of DNA gyrase during replication, as exemplified by the broad-spectrum pyridine-containing nalidixic acid and pyrrole-containing aminocoumarins.[54] This diversity may account for the extended antimicrobial spectrum observed with **P3** and **P5** against *P. putida*, though why it is resistant to **P4** remains unclear. Targeted studies are warranted to precisely elucidate how these phenazines work as antibacterial agents. It is also crucial to note that while the new phenazines have exhibited potential antimicrobial properties, a comprehensive assessment should involve comparing their activities with

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known antimicrobials that are structurally related or with clinically established antibiotics to fully gauge their efficacy. Expanding the range of tested microorganisms will give a comprehensive overview of the spectrum of activity of the phenazines.

Conclusions

The reaction conditions for the catalytic conversion of phenolic substrates with the TMG_2tol based oxido complex [**O1**]$(PF_6)_2$ could be optimized successfully. After 8 hours reaction time at −78 °C and subsequent condensation with PDA, 2-naphthol (**S2**) as well as 6-quinolinol (**S6**) could be converted to the desired phenazines benzo[a]phenazine (**P1**; 31 %, TON 8) and pyrido[3,2-a]phenazine (**P3**; 16 %, TON 4). Other naphthols, quinolinols or indolols could not be converted to the desired phenazines or obtained yields in the same range as tests experiments performed with pure $[Cu(MeCN)_4]PF_6$ copper salt as catalyst (4–5 %). CSI and ESI mass spectra revealed C–C and C–O coupling reactions are taking place as well as condensation and oxidation reactions of PDA molecules with themself. Against our expectations, the bisguanidine ligand TMG_2tol stabilized Cu_2O_2 system did not show a similar activity towards a broad substrate spectrum as the hybrid guanidine stabilized TMGbenza Cu_2O_2 system. However, two substrates could be converted successfully, proving the catalytic activity of [**O1**]$(PF_6)_2$ and creating opportunities for synthesizing new bioactive compounds. Our study demonstrated the potential antimicrobial properties of the synthesized novel phenazines, notably against the gram-positive bacterium *B. subtilis*, with **P2** displaying the highest potency. While most tested gram-negative bacteria were resistant to the new phenazines, *P. putida* was susceptible to **P3** and **P5**, and *E. coli* showed sensitivity to higher concentrations of **P1** and **P4**. Further research is needed to elucidate the modes of action of the new phenazines and their spectrum of activity as potential antimicrobial agents.

Experimental Section

General Remarks: The precursor species [**C1**]PF_6 was prepared in a glove box of the company M.BRAUN under a nitrogen atmosphere. All other experiments were carried out using Schlenk technique with nitrogen (99.99 %, dried over phosphorus pentoxide) as inert gas if not stated otherwise. Solvents and triethylamine used were distilled under nitrogen atmosphere over either sodium with benzophenone as indicator (tetrahydrofuran), calcium hydride (acetonitrile, triethylamine) or magnesium (methanol). molecular sieve (3 Å) was dried with a heat gun (600 °C) under vacuum for two hours and stored in a drying oven (150 °C) afterward. 2-Aminobenzylamine was destilled under reduced pressure (p= 0.05 mbar) right before usage. Chloro-*N,N,N′,N′*-tetramethylformamidinium chloride (TMG Vilsmeier salt)[55] and tetrakis(acetonitrile)copper(I) hexafluorophosphate ($[Cu(MeCN)_4]PF_6$)[56] were synthesized according to literature. All other chemicals were used as purchased (Table S1 in the supporting information). Product purification was performed under ambient conditions using purchased solvents (Table S1 in the supporting information), Geduran Si 60 (40-63 μm; Merck), a glass column (diameter: 2.5 cm, length 40 cm) with glass filter frit (3 Å) and thin layer chromatography sheets (silicon dioxide, 0,2 mm, MACHEREY-NAGEL) for column chromatography.

Elemental analysis (CHN): Elemental analysis was conducted with an elementar vario EL and an elementar vario EL cube.

Infrared (IR) spectroscopy: FT-IR spectra were recorded on a Shimadzu IRTracer 100 using a CsI beam splitter in combination with an ATR unit (Quest model from Specac utilising a robust monolithic crystalline diamond) in a resolution of 2 cm^{-1}. For data acquisition the software LabSolution IR (Version2.15) from Shimadzu was used.

Nuclear Magnetic Resonance (NMR) Spectroscopy: ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker Avance II 400 and Bruker Avance III HD 400 spectrometer at 25 °C in NMR tubes. Resonances were referenced to the residual solvent signal (^{1}H-NMR: δ_H(DMSO-d_6) = 2.50 ppm, $\delta_H(CDCl_3)$ = 7.26 ppm; ^{13}C-NMR: $\delta_H(CDCl_3)$ = 77.2 ppm), relative to tetramethylsilane (0 ppm). Resonances in ppm are listed descending with their related multiplicity, coupling constants *J* in Hz and integration. Multiplicities are abbreviated with: s = singlet, d = doublet, t = triplet, m = multiplets. For the Bruker Avance III HD 400 the software Topspin (Version 3.5 pl 7) from Bruker and for the Bruker Avance II 400 the software TopSpin (Version 2.1) from Bruker were used for data acquisition. For visualization and examination of the NMR spectra the software MestReNova (Version 12.0.1-20560) from Mestrelab Research was used.

Mass Spectrometry: For cryospray-ionization mass spectrometry (CSI-MS) and electron spray ionization mass spectrometry (ESI-MS) measurements an ESI-quadrupole time-of-flight (qToF) mass spectrometer (UHR-TOF Bruker Daltonik maXis II) in combination with a Bruker Daltonik Cryospray unit was used. The spectrometer has a resolution of at least 80.000 FWHM. Spectra were detected in positive ion mode with a source voltage of 3.5 kV. The flow rate was 3.0 μL min^{-1}. The drying gas (N_2), to achieve solvent removal, and the spray gas were both held at −80 °C for low-temperature measurements. The mass spectrometer was calibrated prior to every experiment via direct infusion of Agilent ESI-TOF low concentration tuning mixture, which provided a *m/z* range of singly charged peaks up to 3000 Da in both ion modes.

Single Crystal X-Ray Diffraction: The single crystal diffraction data for **L1** are presented in Table 2. The data were collected with a Stadivari diffractometer of STOE with an Eulerian cradle using an Dectris Pilatus3 R 200 K hybrid-pixel detector with GeniX 3D high flux Cu radiation (λ = 1.54186 Å) at 100 K. The temperature was controlled by an Oxford Cryostream 800. Data were collected with X-Area Pilatus and integrated with X-Area Integrate and X-Area Recipe. The absorption correction was performed by Gaussian integration with Stoe X-Red32, afterwards scaling of reflections with X-Area LANA.[57] The structure were solved by direct method and all non-hydrogen atoms were refined anisotropically with full-matrix least-squares based on F^2 (XPREP,[58] ShelXT,[59] ShelXL[60] and SHELXLe[61]). All hydrogen atoms were localized at idealized positions and refined with isotropic displacement parameters $U_{iso}(H) = 1.2\ U_{eq}(C)$ and $1.5\ U_{eq}$(C methyl). All methyl groups were allowed to rotate but not to tip. Full crystallographic data have been deposited with the Cambridge Crystallographic Data Centre as supplementary no. CCDC – 2304964 for **L1**. Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: (+44)1223-336-033; e-mail: deposit@ccdc.cam.ac.uk).

Ligand 2-(2((bis(dimethylamino)methylene)amino)benzyl)-1,1,3,3-tetramethylguanidine (TMG_2tol, L1): TMG_2tol was synthe-

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Table 2. Crystallographic data and parameters of TMG_2tol (**L1**).

Empirical formula	$C_{17}H_{30}N_6$
Formular mass [g mol^{-1}]	318.47 g mol^{-1}
Crystal size [mm]	0.12×0.10×0.09
Temperature [K]	100
λ [Å]	1.54186
Crystal system	triclinic
Space group	$P\bar{1}$
a [Å]	8.2187(16)
b [Å]	9.823(2)
c [Å]	12.617(3)
α [°]	75.24(3)
β [°]	77.18(3)
γ [°]	70.53(3)
V [Å^3]	918.1(4)
Z	2
$\rho_{calcd.}$ [g cm^{-3}]	1.152
μ [mm^{-1}]	0.563
F(000)	348
hkl range	± 9, $-11 \leq k \leq 8$, $-14 \leq l \leq 10$
Reflections collected	5659
Independent reflections	2973
$R_{int.}$	0.0245
Number of parameters	216
$R_1[I \geq 2\sigma(I)]$	0.0346
wR_2 (all data)	0.0792
Goodness-of-fit	0.913
Largest diff. peak, hole [eÅ^{-3}]	0.125, −0.199

sized based on literature.[37] Extension of the reflux time and a subsequent fractional distillation resulted in a nearly two times higher yield and single-crystals suitable for X-ray diffraction. 2-Aminobenzylamine (13.90 g, 113.8 mmol, 1 eq.) and triethylamine (31.70 ml, 23.01 g, 227.4 mmol, 2 eq.) were added to acetonitrile (100 ml) and stirred in an ice bath (0 °C). TMG Vilsmeier salt (38.92 g, 227.5 mmol, 2 eq.) dissolved in acetonitrile (250 ml) was added dropwise. Subsequently, the colorless suspension was refluxed over the course of five hours. After cooling to room temperature (20 °C), an aqueous solution of sodium hydroxide (9.10 g, 227.5 mmol, 2 eq. in 80 ml water) was added and acetonitrile, water and triethylamine were removed under reduced pressure (p = 0.05 mbar). The following extraction and filtration were performed at ambient conditions (air atmosphere, no Schlenk technique used). The oily, yellow residue was dissolved in an aqueous solution of potassium hydroxide (50 wt %, 40.00 g in 40.00 ml water) and extracted with acetonitrile (4×100 ml). The combined organic phases were dried over magnesium sulfate and filtered over celite before the solvent was removed under reduced pressure (p = 0.05 mbar). Fractional distillation under nitrogen atmosphere (p = 0.05 mbar, $T_{oilbath}$ = 240 °C, T_{head} = 140 °C) resulted in a light yellow oil (27.30 g, 85.7 mmol, 75 %). Single crystals suitable for X-ray diffraction were grown from a concentrated pentane solution. The solution was heated up until it became turbid and was placed in a freezer (−30 °C) for five days afterwards. The molecular structure of TMG_2tol in the solid state is depicted in Figure 6. Referring molecular structure data are given in Table 2. An allocation of the atom numbers used in the ^{1}H and ^{13}C NMR spectra to the molecular structure of ligand TMG_2tol is given in Figure 5.

^{1}H NMR (400 MHz, $CDCl_3$ [7.26 ppm], ppm) δ = 7.51 (d, *J* = 7.5 Hz, 1H, H-5), 7.03 (t, *J* = 7.5 Hz, 1H, H-5), 6.85 (t, *J* = 7.4 Hz, 1H, H-3), 6.52 (d, *J* = 7.8 Hz, 1H, H-2), 4.24 (s, 2H, H-7), 2.75 (s, 6H, H-9), 2.72 (s, 6H, H-9), 2.66 (s, 12H, H-11). **^{13}C NMR** (100 MHz, $CDCl_3$ [77.2 ppm], ppm) δ = 160.6 (1 C, C-8), 158.6 (1 C, C-10), 149.2 (1 C, C-6), 134.1 (1 C, C-1), 127.4 (1 C, C-5), 126.0 (1 C, C-4), 121.3 (1 C, C-2), 120.2 (1 C, C-3), 49.4 (1 C, C-7), 39.8 (2 C, C-9), 39.7 (4 C, C-11), 39.1 (2 C, C-9). **IR**: ν [cm^{-1}] = 3057 (w, ν(C-$H_{arom.}$)), 2996 (w, ν(C-$H_{arom.}$)), 2919 (m, ν(C-$H_{arom.}$)), 2869 (m, ν(C-$H_{aliph.}$)), 2844 (m, ν(C-$H_{aliph.}$)), 2799 (w, ν(C-$H_{aliph.}$)), 1607 (vs, ν(C=$N_{gua.}$)), 1581 (vs), 1559 (vs), 1496 (m), 1477 (m), 1448 (s), 1424 (m), 1404 (w), 1366 (vs), 1313 (w), 1281 (w), 1233 (m), 1206 (w), 1131 (vs), 1108 (w), 1059 (m), 1040 (w), 1016 (vs), 977 (w), 913 (w), 855 (w), 780 (m), 743 (s), 722 (m), 695 (w), 628 (w), 604 (w), 580 (w), 548 (w), 532 (w), 447 (w), 419 (w). **HR-MS**: m/z (%) = [$C_{17}H_{31}N_6^+$, M + H] calculated: 319.2610, detected: 319.2597 (100), [$C_{12}H_{18}N_3^+$, M - $NC(N(CH_3)_2)_2$] calculated: 204.501, detected: 204.1493 (100), [$C_{13}H_{10}N_2^+$, M - $NC(N(CH_3)_2)_2$, - $N(CH_3)_2$, + H] calculated: 161.1079 detected: 161.1071 (60). **CHN**: (M = 318.25 g·mol^{-1}) $C_{17}H_{30}N_6$, calculated: C: 64.12, H: 9.50, N: 26.39; detected: C: 63.72, H: 9.07, N: 26.90.

Precursor Species [Cu(TMG_2tol)]PF_6 ([C1]PF_6): [Cu(MeCN)$_4$]PF_6 (74.5 mg, 0.2 mmol, 1 eq.) was diluted in acetonitrile (4.0 ml). A solution of TMG_2tol (**L1**) (63.6 mg, 0.2 mmol, 1 eq.) in acetonitrile (1.0 ml) was added dropwise. The resulting colorless precursor solution was drawn in a gastight Hamilton syringe and stored in a Schlenk tube under nitrogen atmosphere until further usage (max. 8 hours).

Catalyst Species Bis(μ-oxido)dicopper(III) complex [Cu_2(μ-O)$_2$(TMG_2tol)$_2$](PF_6)$_2$, ([O1](PF_6)$_2$): [**O1**](PF_6)$_2$ was synthesized in-situ during the catalytic oxygenation reaction. Tetrahydrofuran (20 ml) and molecular sieve (300–400 mg) were stirred in an ethanol/dry ice cooling bath (−78 °C). molecular oxygen was bubbled through the solution for 5 minutes. The previously synthesized precursor solution was added to form the catalyst species [**O1**](PF_6)$_2$

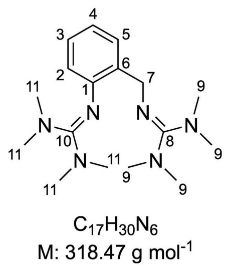

Figure 5. Molecular structure of TMG2tol, numbers refer to the labels given in the ^{1}H and ^{13}C NMR spectra.

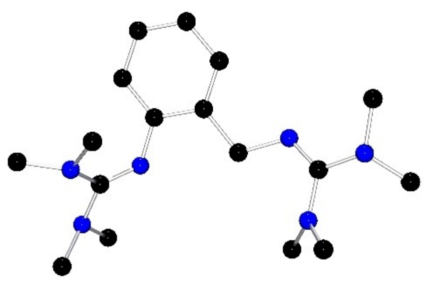

Figure 6. Molecular structure of ligand TMG_2tol in the solid state. (Hydrogen atoms are omitted for clarity).

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(0.1 mmol, 1 eq.) in-situ. The reaction mixture stirred for 15 minutes at −78 °C to insure a complete formation of the catalyst species.

Catalytic oxygenation reactions with [O1](PF_6)$_2$: Catalytic oxygenation of phenolic substrates with [**O1**](PF_6)$_2$ as catalyst was performed as described in literature.[22] Test experiments were performed with reaction time varying between one and eight hours. Catalytic oxygenation resulting in products **P1** – **P5** were performed with a reaction time of eight hours. The purification process was slightly adapted using different solvent mixtures for column chromatography. Blank experiments were performed diluting the given amount of [Cu(MeCN)$_4$]PF_6 without any ligand species.

After the formation of the catalyst species [**O1**](PF_6)$_2$ the oxygenation reaction was started by adding the substrate solution (substrate 2.5 mmol, 25 eq.; triethylamine 0.7 ml, 5.0 mmol, 50 eq.; see Table S2 in the supporting information) to the stirring reaction mixture at −78 °C. After 8 hours 1,2-phenylendiamine (540.7 mg, 5.0 mmol, 50 eq.) dissolved in tetrahydrofuran (5 ml) was added and the cooling bath was removed. The reaction mixture was allowed to warm up to room temperature and stirred overnight. After adding an aqueous solution of hydrochloride solution (0.5 M, 30 ml) and 3 tablespoons of ethylenediaminetetraacetic acid (EDTA) the solvents were removed under reduced pressure (p = 50 mbar). The residue was extracted with dichloromethane (3×40 ml). The combined organic phases were dried over magnesium sulfate. After filtration of the suspension, the solvents were removed under reduce pressure (p = 50 mbar). The crude product was further purified by column chromatography. All catalyses were performed two or three times at least to provide reproducibility. Yield and TON are given as averaged in Table 1 over all experiments performed.

A list of all test experiments, blank experiments and catalytic oxygenation reactions performed as well as details on the purification process via column chromatography including NMR spectroscopic data of **P1** – **P5** are given in the supporting information section 2.

CSI-MS and ESI-MS measurements of the catalytic conversion of phenolic substrates with [O1](PF_6)$_2$: The catalytic conversions of 1-naphthol, 4-quinolinol and 4-indolol were investigated using mass spectrometry. Experiments were performed as noted in the section catalytic oxygenation reactions with [O1](PF_6)$_2$ before. CSI-MS spectra were recorded directly after addition of the substrate solution. A few milliliter of the solution were removed with a gas-tight, pre-cooled Hamilton syringe and injected in the spectrometer. ESI-MS spectra were recorded after 8 hours reaction time, addition of PDA and extraction of the crude reaction mixture.

Supporting Information

Detailed information about the chemicals purchased, a list of all catalytic experiments performed including purification details and NMR spectroscopic data of **P1** - **P5** as well as further mass spectra and data for antimicrobial experiments are given in the supporting information.

Original data files of the NMR and IR spectroscopic data are openly available via the Chemotion Repository: https://www.doi.org/10.14272/reaction/SA-FUHFF-UHFFFADPSC-PJLMQMBEXP-UHFFFADPSC-NUHFF-NUHFF-NUHFF-ZZZ

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Conflict of Interests

The authors declare no conflict of interest.

Keywords: copper · oxygenation · guanidines · tyrosinase model system · phenazines

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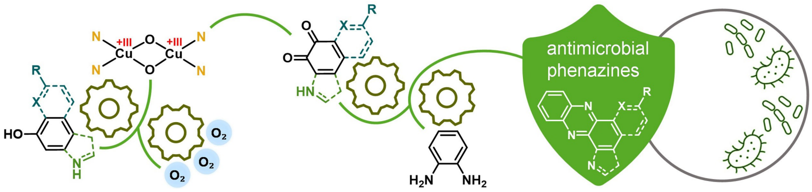
RESEARCH ARTICLE



Complex phenolic derivatives like naphthols, quinolinols and indolols are converted via a catalytic oxygenation reaction using a bisguanidine stabilized oxido complex as catalyst. Subsequent condensation with 1,2-phenylendiamine results in phenazines products which function as biological antimicrobial agents against bacteria.

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From Phenols to Antimicrobial Phenazines: Tyrosinase-like Catalytic Activity of a Bisguanidine Based Bis(μ-oxido) Complex