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Laccase-mediated synthesis of 2-methoxy-3-methyl-5-(alkylamino)- and 3-methyl-2,5-bis(alkylamino)-[1,4]-benzoquinones

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ABSTRACT

The synthesis of 5-alkylamino- and 2,5-bis(alkylamino)-[1,4]-benzoquinones, showing structural similarity to natural mitomycins, was performed through coupling of 2-methoxy-3-methylhydroquinone with primary amines such as *n*-octylamine, geranylamine and cyclooctylamine using laccases from *Myceliophthora thermophila* (*Mt*L) and *Pycnoporus cinnabarinus* SBUG-M 1044 (*Pc*L). Product spectra of laccase reactions differ due to reaction systems pH values (pH 7.0 for *Mt*L and pH 5.0 for *Pc*L) applied to assure enzymes optimal catalytic efficiency. The *Mt*L- and *Pc*L-mediated formation of monoaminated products was achieved at equimolar reactant concentrations with amine coupling at the *meta*-position to benzoquinones methyl group. Increased formation of diaminated products occurred in *Pc*L-mediated reactions and generally when the amine was supplied in excess. Diamination entailed elimination of the benzoquinone methoxy group (amination in *para*-position to the first amine substituent). Six products were synthesised and characterised by NMR and HR-MS analysis. The laccase-mediated amine coupling to 2-methoxy-3-methylhydroquinone confers two of the essential pharmaceutical active motifs from mitomycins: (i) a stable 1,4-benzoquinoic parent structure and (ii) a biological active alkylation function (—NH).

1. Introduction

The enzyme class of laccases (*para*-diphenol:dioxygen oxidoreductases, EC 1.10.3.2) finds increasing significance in a variety of organic synthetic methodologies and the manufacture of synthetic building blocks for fine chemistry due to a broad substrate scope, the variety of reactions catalysed, and in particular the high resistance towards harsh reaction conditions [1–4]. The versatility of laccases enables a broad range of applications from the production of polymers and food additives to the synthesis of fragrance compounds, and cosmetic and pharmaceutical precursors in the field of white biotechnology [5–7].

The family of mitomycins comprised pharmaceutical active, antineoplastic compounds. Mitomycins provide a broad spectrum of efficacy due to their antibiotic and cytotoxic characteristics and therefore selected mitomycins are used in control of infectious diseases caused by Gram positive as well as Gram negative bacteria, and also some viruses [8,9]. They are also employed as chemotherapeutics in the treatment of several types of cancer, including solid carcinomas [10,11]. The biological activity of mitomycins has been related to certain biologically active structure motifs such as a *para*-benzoquinoidic parent structure, a strong alkylating function, especially that of a secondary amino-group, and partially a urethane-function (Fig. 1A) [12].

Approaches for the chemical synthesis of mitomycins are challenging due their demanding stereochemistry, the difficult workability of quinones, and often requiring complex reaction steps [13–15]. With respect to the aforementioned difficulties, in particular quinone instability, the use of the biocatalyst laccase affords mild reaction conditions and can catalyse the transformation of dihydroxylated compounds into the corresponding stable benzoquinones. Previously, it was shown that laccases can mediate a wide range of heteromolecular reactions (e.g. C—C, C—O, C—N, C—S coupling) at which the enzyme substrate radical is linked with diverse coupling agents yielding stable hybrid molecules [16–19]. In most of the published laccase-mediated C—N coupling reactions, simple substituted *para*-hydroquinones acting as native laccase substrates due to position of hydroxyl groups were

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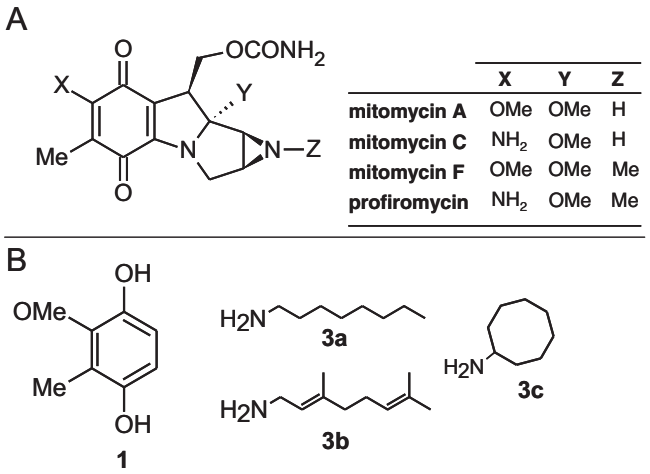

	X	Y	Z
mitomycin A	OMe	OMe	H
mitomycin C	NH_2	OMe	H
mitomycin F	OMe	OMe	Me
profiromycin	NH_2	OMe	Me

Fig. 1. Model structure for selected naturally occurring mitomycins (A). Laccase-substrate **1** and primary amines **3a–c** (coupling agents) used in reactions focused on the enzyme-mediated synthesis of mitomycin-like compounds (B).

applied in assays containing aromatic amines [20–22]. It was demonstrated that product formation in such laccase-mediated C—N coupling results in satisfying yields and is equivalent or better when compared to synthetic routes, as for example use of the coupling-mediator sodium iodate [23,24].

These findings encouraged us to study the laccase-mediated synthesis of mitomycin-like compounds. The 2-methoxy-3-methylbenzoquinone represents in its protonated form a suitable laccase substrate (**1**) and was hence investigated in the coupling with aliphatic (*n*-octylamine **3a** and geranylamine **3b**) as well as cyclic (cyclooctylamine **3c**) primary amines using laccases from *Myceliophthora thermophila* (*Mt*L) and *Pycnoporus cinnabarinus* SBUG-M 1044 (*Pc*L) (Fig. 1B). The influence of pH values of buffered reaction systems, as well as reactant ratios, on product yields and formation of mono- or diaminated products was studied.

2. Experimental

2.1. Chemicals

All chemicals were of reagent grade or better and purchased from Sigma–Aldrich (Steinheim, Germany) and Merck (Darmstadt, Germany).

2.2. Enzymes

M. thermophila laccase (*Mt*L) was commercially obtained from Novozymes® (Bagsvaerd, Denmark). Laccase from the white-rot fungus *P. cinnabarinus* SBUG-M 1044 was produced as described by Herter et al. [25] according to methods of Kordon et al. [26]. Laccase activity was determined by monitoring the oxidation of 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium (ABTS) spectrophotometrically at 420 nm ($\varepsilon = 3.6 \times 10^4\ M^{-1}\ cm^{-1}$) in sodium acetate buffer (100 mM, pH 5) at 25 °C as described earlier. One unit (1 U) of activity is defined as the amount of enzyme, which catalyzes the conversion of 1 μmoL mL^{-1} min^{-1} substrate at 25 °C.

2.3. Laccase-mediated heteromolecular coupling of 2-methoxy-3-methylhydroquinone with primary amines

Analytical assays were conducted in sealed brown 6-mL-glass flasks with a total reaction volume of 5 mL at room temperature and shaking at 200 rpm. Reactions were performed either in phosphate-citrate buffer (pH 7.0) with *M. thermophila* laccase (1 μmoL mL^{-1}) or in sodium acetate buffer (20 mM, pH 5.0) with *P. cinnabarinus* SBUG-M 1044 laccase (1 μmoL mL^{-1}). Educts were prepared as 50 mM methanolic stock solutions and biocatalysis conducted either in equimolar concentration or with an excess of the primary amines. Product formation was followed by analytical HPLC at regular intervals. Preparative scale reactions were performed in 100-mL-Erlenmeyer flasks protected against light, containing 50 mL of the reaction mixture using the same conditions described above. Monoaminated products were obtained using a 2:2 mM ratio between 2-methoxy-3-methylhdroquinone **1** and the primary amines **3a–c** from *Mt*L-catalysed reactions. Diaminated products were enriched using a 2:5 mM ratio under the same conditions as described for enrichment of monoaminated products.

2.4. Analytical HPLC

Reaction batches were sampled at regular intervals over 24 h and analyzed via HPLC (Shimadzu, Duisburg, Germany) consisting of a SCL-10A VP control unit, a LC-A FCV-10AL VP fluid chromatograph and a SPD-10A VP photodiode array detector. Grading of analytes was accomplished with a LiChroCart 125-4 RP-18 end-capped column of 5-μm particle size (Merck, Darmstadt, Germany). The mobile phase consisted of an aqueous component A (0.1% phosphoric acid) and a methanol component B at an initial ratio of 30% B to 70% A, simultaneously raised up to 100% B within 14 min. The flow rate was always adjusted to 1 mL min^{-1} with an injection volume of 40 μL.

2.5. Isolation of heteromolecular coupling products

Products were isolated by solid reverse phase extraction with a Strata C18-E silicagel column (60 mL Giga Tubes, 10 g absorbent material, Merck Darmstadt, Germany), progressively charged with 50 mL of the reaction mixture. Residual starting material and low-molecular by-reaction products were eluted with 60 mL of a solution composed of 60% methanol and 40% bidistilled water. The monoaminated products were obtained with 60 mL of a mixture consistent of 85% methanol and 15% acetic acid (0.1%, v/v in bidistilled water), followed by elution of the corresponding diaminated products due to addition of pure methanol (40 mL). The methanol concentration of samples was initially reduced via rotary evaporation at 30 °C. Afterwards, samples were diluted with bidistilled water to reach a final methanol concentration of 5% and subsequently frozen at −20 °C for 24 h, followed by freezing at -70 °C for further 4 h. Products were obtained as solids via lyophilisation (20 °C).

2.6. Structural characterisation

HR-MS measurements were conducted on an ESI-TOF/MS (Agilent Technologies, Böblingen, Germany) in positive ion mode ($[M+H]^+$, $[M+Na]^+$) by direct injection of methanolic samples lacking a previous chromatographic column separation using a MeOH/0.1% acetic acid mobile phase in a 9:1 ratio (v/v). ^1H, ^{13}C, DEPT and two-dimensional NMR spectra (^1H,^1H COSY, ^1H,^1H NOESY, ^{13}C,^1H HMBC, ^{13}C, ^1H HSQC) were recorded on Bruker Avance spectrometers AV 600, AV 500, and AV 250, Karlsruhe, Germany, in deuterated methanol (MeOH-d_4, $\delta(^1H) = 3.31$, $\delta(^{13}C) = 49.1$).

2.6.1. 2-Methoxy-3-methyl-5-(octylamino)-[1,4]-benzoquinone (5a)

Red solid. $HPLC_{Rt}$ 14.9 min, UV-vis (MeOH) λ_{max} 212, 309, 498 nm. HR-MS (ESI): calcd for $C_{16}H_{26}NO_3$ $[M+H]^+$ 280.19072;