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An Enzyme-Initiated Domino Hydroxylation-Oxidation-Carbo-Diels-Alder Reaction Cascade

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Abstract: In a domino hydroxylation-oxidation-Diels-Alder reaction sequence which is initiated by the enzyme tyrosinase simple phenols are converted in high yield into highly functionalized bicyclic 1,2-diketones. This example demonstrates that enzymatic transformations can successfully be combined with non-enzymatic reactions in domino reaction sequences. Copyright © 1996 Elsevier Science Ltd

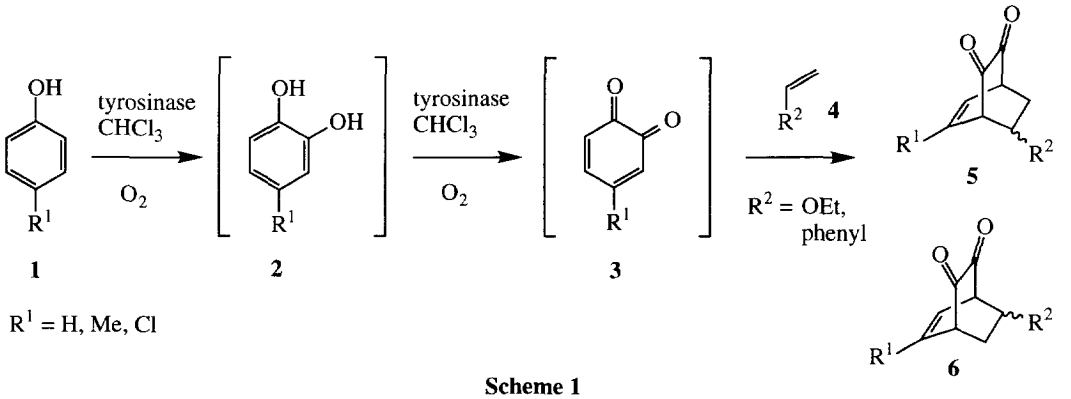
Domino reactions are sequential bond forming or breaking processes during which the subsequent transformations occur at the functionalities generated in the preceding step. The applicaton of such cascade reactions has led to the development of a variety of elegant and effective synthetic methods and thereby opened up new and promising opportunities for organic synthesis.[1] The prototype, of such domino processes are the sequential transformations catalyzed in nature by biocatalysts, resulting in series of bond formations or scissions without the accumulation of intermediates. However, although the use of biocatalysts for synthetic purposes is well established,[2] the incorporation of an enzymatic transformation in a series of sequential non-enzymatic reactions has not been described.

We now report that the biocatalyzed hydroxylation of phenols to catechols and their subsequent enzymatic oxidation to *ortho* quinones can advantageously be combined with a non-enzymatic carbo Diels-Alder reaction to give highly functionalized bicyclic 1,2-diketones in one step from simple starting materials.

Ortho quinones are reactive compounds which can be generated from phenols by oxidation with e. g. Fremy's salt. They react readily with nucleophiles, electrophiles and in cycloaddition reactions[3] and might serve as advantageous intermediates in domino sequences provided that the reagents needed for the subsequent steps are not attacked by the strong oxidant.

Nature employs *ortho* quinones during the synthesis of pigments and structural materials (e. g. the insect cuticle) and uses for instance the enzyme tyrosinase [EC 1.14.18.1][4] for their generation. This biocatalyst hydroxylates phenols (e.g. **1**) to catechols (e.g. **2**) and oxidizes these aromatic diols further to 1,2-dicarbonyl compounds, i.e. *ortho* quinones (e.g. **3**). This facile biocatalytic transformation cannot be used for preparative purposes if aqueous solutions, i.e. the natural environment of the enzyme, are employed since the quinones rapidly polymerize under these conditions.[5] However, if tyrosinase is immobilized on glass beads, a biocatalyst preparation results which functions in organic solvents and generates solutions of *ortho* quinones under mildest conditions which may serve well in further transformations.[5,6]

In the light of these findings we reasoned that the enzymatic oxygenation of phenols to catechols and their subsequent oxidation to *ortho* quinones might advantageously be combined with e. g. carbo Diels-Alder reactions in domino processes which would yield highly functionalized bicyclic compounds in one single step from simple aromatic starting materials.



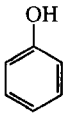
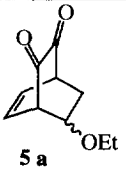
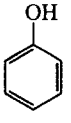
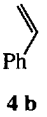
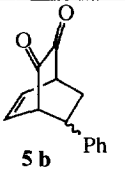
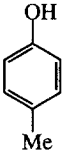
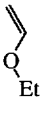
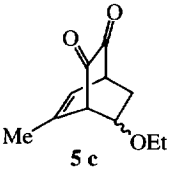
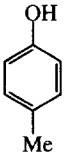
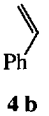
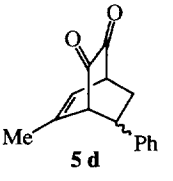
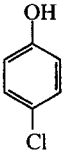
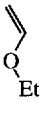
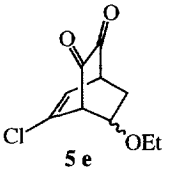
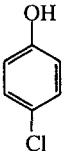
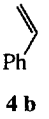
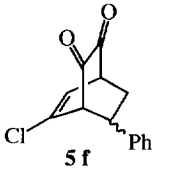
Scheme 1

To test this notion, the phenols **1** were treated with immobilized tyrosinase (vide infra) in mixtures of $CHCl_3$ and a dienophile **4** in the presence of oxygen (Scheme 1; $CHCl_3$ is known to dissolve oxygen well[5,7]). In the ensuing transformation the biocatalyst first hydroxylates the position *ortho* to the aromatic OH group to give the catechols **2** which are further oxidized by the enzyme to the quinones **3**. Indeed, these highly reactive intermediates directly undergo a carbo Diels-Alder reaction with the dienophile **4** to yield the bicyclic addition products **5** and **6** in high yields (Scheme 1, Table 1).[8]

The formation of undesired products which might arise e.g. from the participation of the 1,2-diketones as heterodienes in the cycloaddition and which would lead to dioxins[9] is not observed under these conditions. In principle, four different diastereomeric cycloadducts **5/6** can be formed in the chemoenzymatic domino process, one *endo* and one *exo* product in which R^1 and R^2 are in 1,3-orientation, i.e. **5** as well as one *endo* and one *exo* product in which the substituents are in 1,4-orientation, i.e. **6** (Scheme 1). In general, in the enzyme initiated reaction sequence one isomer was formed in large excess. Whereas we could not determine if the *endo* or the *exo* isomers predominate, ^1H NMR spectroscopic analysis clearly revealed that in the major isomers both substituents R^1 and R^2 are next to the same bridge head, i.e. the diastereomers **5** are the main products. Unfortunately, the cycloadducts **5** and **6** are racemic indicating that although tyrosinase does catalyze the incorporation of oxygen and the subsequent oxidation it does obviously not influence the final Diels-Alder step.

The success of the domino sequence described above critically depends on the purity of the protein used for the catalyst preparation. In our initial experiments a crude preparation obtained from mushrooms (*Agaricus bisporus*) according to the procedure described by S. Burton[6] was employed. In the presence of this catalyst the cycloadduct **5c** was obtained from p-cresol and ethyl vinyl ether in only 15% yield although the aromatic alcohol was completely consumed. Since we could not detect substantial amounts of a by-product in the organic phase we assume that the *ortho* quinone was formed and underwent undesired reactions with proteins still present in the crude mushroom extract. Much better results were obtained if the raw protein was purified by means of hydrophobic interaction chromatography.[10] After immobilization[10] on glass beads the enzyme preparation obtained thereby made the desired cycloadducts **5** and **6** available in yields of 55 to 82% (Table 1). In addition, this biocatalyst is remarkably stable. Thus, it is capable of working in solutions containing up to 50 vol % of ethyl vinyl ether (Table 1) and, if it is stored at -80°C, even after 150 days still ca. 80% of its activity is retained.

Table 1. Results of the domino hydroxylation - oxidation - Diels - Alder reaction sequence initiated by tyrosinase

entry	phenol	dieno-phile	main product	$CHCl_3$/ dienophile (v/v)[8]	reaction time [d]	isomer ratio **5 : 6**	enzyme / substrate [U/mmol]	yield[d] [%]
1	 **1 a**	 **4 a**	 **5 a**	1 : 1	3	-	520	70
2	 **1 a**	 **4 b**	 **5 b**	4 : 1	6	-	775	69
3	 **1 b**	 **4 a**	 **5 c**	1 : 1	2.5	33 : 1[a]	345	77
4	 **1 b**	 **4 b**	 **5 d**	4 : 1	1.6	17 : 4[b]	520	75
5	 **1 c**	 **4 a**	 **5 e**	1 : 1	4	51 : 1[a]	690	55
6	 **1 c**	 **4 b**	 **5 f**	4 : 1	6	7 : 2[c]	1035	82

[a] Only two isomers have been found; [b] Three isomers are found in the ratio 17 : 4 : 1; [c] Three isomers are found in the ratio 7 : 2 : 1; [d] Based on chromatographically purified compounds. All compounds were identified by ^1H NMR spectroscopy (250- or 400 MHz; $CDCl_3$).

In conclusion we have shown that enzymatic transformations can successfully be combined with non-enzymatic processes in domino reactions. In the sequence described above simple phenols are converted in high yields in a one-pot but three step reaction cascade consisting of a hydroxylation, an oxidation and a subsequent Diels-Alder reaction into bicyclic 1,2-diketones which carry further substituents and open up opportunities for additional transformations. The incorporation of different reactions and the addition of further reaction steps to this enzyme-initiated domino sequence are actively pursued in our laboratory.

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8. Experimental Procedure: To a solution of 1 mmol of the phenol **1** in 100 ml of a mixture of $CHCl_3$ and the dienophile **4** (see Table 1) 345-1035 units[11] of the immobilized biocatalyst and 0.5 ml of phosphate buffer (pH 7, 0.05 M) is added. The mixture is gently shaken at ambient temperature until tlc control indicates the complete disappearance of the phenol (1.5 to 6 d). The solution is decanted, the solvent is evaporated in vacuo and the remaining residue is purified by flash chromatography using hexane/ethyl acetate mixtures as eluents.
9. C. Grundmann, in *Methoden der organischen Chemie (Houben Weyl) Band VII/3b* (Ed.:E. Müller), Georg Thieme Verlag, Stuttgart 1979, pp 160 - 164.
10. Isolation and immobilization of tyrosinase: 3 kg of fresh mushrooms are frozen with liquid nitrogen, homogenized in acetone (5.5 l) using a Waring blender and centrifuged at 5000 rpm for 20 min. The pellet is treated with liquid nitrogen and then suspended in 2 l of phosphate buffer (pH 7, 0.1 M). The mixture is kept overnight at 4°C while nitrogen is bubbled gently through to remove remaining acetone. The suspension is centrifuged for 20 min at 5000 rpm and the pellet is discarded. After addition of 176 g of $(NH_4)_2SO_4$ per l solution (30% saturation) the mixture is centrifuged (10000 rpm, 40 min) and the pellet is discarded. After addition of 1.66 l phosphate buffer (pH 7, 0.1 M) to adjust the $(NH_4)_2SO_4$ concentration to 0.7 M the solution is filtered (filter G4) and the filtrate is passed over a column loaded with phenyl sepharose (Pharmacia: Phenylssepharose HP, column volume 210 ml). After washing with 2 l phosphate buffer (0.1 M, pH 7) containing 0.65 M $(NH_4)_2SO_4$ and elution with phosphate buffer (0.1 M, pH 7) containing 0.2 M $(NH_4)_2SO_4$ the fractions containing the enzymatic activity are pooled, concentrated to 100 ml with an ultrafiltration system (30 kDa membrane), dialyzed against distilled water and lyophilized to yield 306 mg of protein. To immobilize the tyrosinase 44 g of glass beads (0.1 to 0.25 mm diameter) are placed in a flat polyethylene dish and a solution of 306 mg of the protein in 17 ml of 0.05 M phosphate buffer (pH 7) is slowly added. The water is evaporated by slowly passing an airstream over the dish and the remaining dried glass beads are stored at - 80 °C in Eppendorf tubes. By this procedure 17250 units[11] of enzymatic activity are obtained, which can be stored at -60°C for several months with only little loss of activity (after 50 days 90% and after 150 days 80% of the activity are retained).
11. The activity was assayed using an adapted "dopachrome" method as described[6]. 1 unit of enzymatic activity is defined as the amount of enzyme which produces 1 µmol of dopachrome/min in a solution of L-dopa (10 mM in phosphate buffer (pH 6.0, 50 mM)). The reaction is followed with a spectrophotometer monitoring the increase in absorbance due to dopachrome formation at 475 nm (ε = 3600 / M cm).

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