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Identification of Difenylenedioxide-2,3-quinones Obtained from an Alternative Pathway for Catechol Metabolism in Tobacco

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Derivados de difenilenodióxido-2,3-quinona produzidos pela incubação de proteínas, parcialmente purificadas, obtidas das folhas de *Nicotiana tabacum* var. Xanthi, *Campsis radicans*, *Tecoma stans* e *Populus deltoides* com catecóis foram identificados com base em seus dados de RMN ^{1}H, ^{13}C e EM. Os produtos de oxidação dos catecóis foram similares àqueles previamente isolados pela incubação de catecóis com extratos de folhas de espinafre.

Diphenylenedioxide-2,3-quinone derivatives produced by incubating partially purified proteins from the leaves of *Nicotiana tabacum* var. Xanthi, *Campsis radicans*, *Tecoma stans* and *Populus deltoides* with catechols were identified based on their ^{1}H- and ^{13}C-NMR and MS data. The catechol oxidation products were similar to those previously isolated from the incubation of catechols with spinach leaf extracts.

Keywords: Nicotiana tabacum *var. Xanthi*, Campsis radicans, Tecoma stans, Populus deltoides, *catechol oxidation*

Introduction

Catechol oxidase is an intracellular enzyme widely distributed in the plant kingdom. It contains copper, with a molecular weight between 116 and 128 kDa[1,2,3], and is frequently classified as polyphenol oxidase. In general it catalyses two distinct reactions: the insertion of oxygen in an *ortho* position into an existing hydroxyl group, followed by the oxidation of the diphenol to the corresponding quinone, often referred to as cresolase activity. It can also catalyze only the oxidation step referred to above, and in this case its activity is referred to as catecholase. Since it is only one of several phenol oxidizing enzymes known to occur in plant tissues, cases of incorrect identification cannot be ruled out[4], particularly when crude preparations have been used. Furthermore, catechol oxidase is considered a difficult enzyme to purify, especially from plant tissues. Tanning reactions and binding to cell membranes in many tissues occur during the isolation of the enzyme, resulting in changes in its properties as well as in its oligomerization state. Such reactions can be partially prevented by isolation under N_2 or by the presence of reducing agents[1].

This paper presents two derivative products of the incubation of catechol and 4-chlorocatechol with cellular leaf extracts from *Nicotiana tabacum* var. Xanthi. Diphenylenedioxide-2,3-quinone derivatives were produced in small amounts and were identified on the basis of their ^{1}H- and ^{13}C-NMR and MS data. Cellular leaf extracts from *Campsis radicans, Tecoma stans* and *Populus deltoides* also presented similar products through incubation with catechol and 4-chlorocatechol.

The oxidation of catechol has been reported to occur in several plant species, leading different authors to classify them as catechol oxidizing plants[4,5,6].

Experimental

Leaves from *Nicotiana tabacum* var. Xanthi (25 g fresh weight) were frozen in liquid nitrogen, crushed with a mortar and pestle, and suspended in water (25 mL). The

mixture was filtered through cheese cloth and centrifuged (5,000 rpm) at 4 °C. The supernatant was treated with 3 volumes of cold acetone (-20 °C), and the precipitate was collected and thoroughly dried *in vacuo*.

Either catechol, or 4-chlorocatechol or 4-methyl-catechol (250 mg) in water (125 mL) was added to 250 mL of 0.1 M phosphate buffer, pH 7.4. Tobacco leaf acetone powder (0.5 g) dispersed in water (20 mL) was stirred in, and the mixture was incubated for 5 min at 25 °C. Since the product was unstable in an alkaline medium, the pH was immediately adjusted to 5.0 with HCl 0.1 M. The reaction mixture was extracted 3 times with chloroform (150 mL). After drying this mixture over anhydrous sodium sulfate, it was evaporated to a small volume *in vacuo*, and the concentrated solution was applied to a column (1.8 x 12 cm) of silica gel. The main orange-red zone was eluted rapidly with chloroform. It was concentrated *in vacuo*, and the product was precipitated by adding an excess of petroleum ether. After recrystallization from acetone, the product obtained as red prisms was used in spectroscopic analysis.

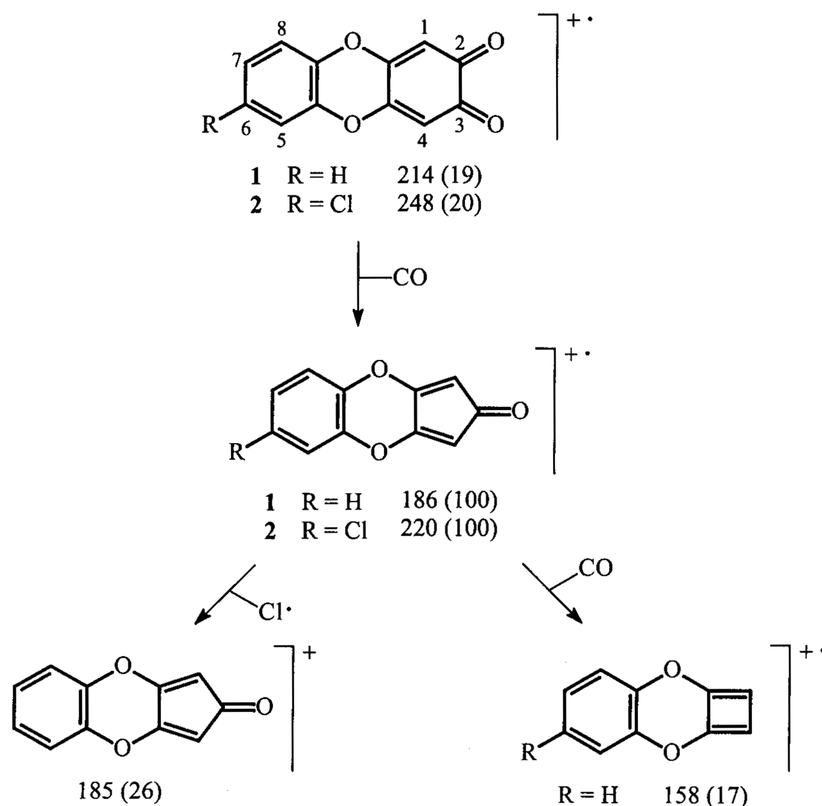
The same procedure was used with *Campsis radicans*, *Tecoma stans* and *Populus deltoides* leaf extracts, and the oxidation products presented similar characteristics.

Diphenylenedioxide-2,3-quinone (**1**). Red solid; m.p. 260-261 °C dec., UV λ max ($CHCl_3$): 240, 275, 390 nm (log ε 4.30, 4.26, 3.87); IR ν max (KBr): 3060, 2350, 1653, 1583, 1490, 1388, 1258, 1197, 1098, 859, 677 cm^{-1}, MS m/z (rel.int.): Scheme 1. ^{1}H-NMR (200 MHz, $CDCl_3$): δ 6.45 (s, H-1 and 4), 6.97 (d, J = 8HZ, H-6 and 7), 7.24 (d, J = 8Hz, H-5 and 8).

6-Chlorodiphenylenedioxide-2,3-quinone (**2**). Red solid, m.p. undet.. UV λ max ($CHCl_3$): 240, 283, 390 nm (log ε 4.32, 4.28, 3.88); IR ν max (KBr): 3060, 2350, 1653, 1583, 1490, 1388, 1258, 1197, 1098, 859, 781, 677 cm^{-1}, MS m/z (rel.int.): Scheme 1. ^{1}H-NMR (200 MHz, $CDCl_3$): δ 6.32 (s, H-4 or 1), 6.33 (s, H-4 or 1), 7.29 (dd, J = 8.8, 2.3, H-7), 7.42 (d, J = 8.8, H-8), 7.58 (d, J = 2.3, H-5). ^{13}C-NMR (50.32 MHz, $CDCl_3$): δ 108.6 (C-1 or C-4), 108.8 (C-4 or C-1), 117.5 (C-5), 118.1 (C-8), 128.8 (C-7).

Results and Discussion

The dried acetone extract, prepared from frozen *Nicotiana tabacum* var. Xanthi leaves[2,7], contains enzymes (polyphenol oxidase) which oxidize catechol and chlorocatechol to the quinone (diphenylenedioxide-2,3-quinone, **1**) and Cl-quinone (6-chlorodiphenilenedioxide-2,3-quinone, **2**), respectively. These substances were characterized by their spectroscopic data. The UV absorptions of **1** and **2** are characteristic of ortho-quinone (240, 289 ± 4 and 390 nm), and the IR data for both are similar, with the exception of compound **2** which has a characteristic chloride absorption at 781 cm^{-1}. The fragmentation of com-



Scheme 1. M S fragmentation of **1** and **2**.

pounds **1** and **2** in the MS (Scheme 1) confirmed the proposed structures. Structure **1** was confirmed by the ^{1}H-NMR data and Structure **2** by the ^{1}H- and partial ^{13}C-NMR data. As compound **1** is a symmetrical molecule, its ^{1}H-NMR spectrum only contains three signals, the attributions of which were determined on the basis of their coupling constants. In 6-chlorodiphenylenedioxide-2,3-quinone (**2**) the signals attributed to H-4 and H-1 are not equivalent, appearing at δ 6.32 and 6.33. The multiplicity of the signals of H-5 (7.58 δ) and H-8 (7.42 δ) appear as doublets, while H-7 (7.29 δ) appears as a double doublet, as expected.

The enzyme (polyphenol oxidase) presents activity with catechol, 4-chlorocatechol and 4-methylcatechol in the pH range of 7 to 8. Its temperature optimum was 30 °C. The enzyme is insensitive to DIECA, but its activity is increased by the addition of $CuSO_4$. It is inhibited by cyanide, benzoic acid and ascorbic acid.

We have checked the ability of other plants to oxidize catechol and 4-chlorocatechol to quinones (*Campsis, Tecoma, Nicotiana* and *Populus*). In these studies the same products (**1** and **2**) were obtained as when using *N. tabacum* extracts. This property could be a general feature observed in several plants which could be exploited in the recovery of waste sites. Additionally, it is first necessary to establish whether these plants have endogenous hydroxylases with phenol oxidase activity which can convert chlorinated phenols to catechol, and then to carry out the subsequent oxidation.

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