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Naphthalenones and isocoumarins of the fungus *Ceratocystis fimbriata* f. sp. *platani*

Nicolas Bürki, Armelle Michel and Raffaele Tabacchi

Institute of Chemistry, University of Neuchâtel, Avenue de Bellevaux 51
CH-2007 Neuchâtel, Switzerland

Summary. The chemical composition of the culture filtrates of *Ceratocystis fimbriata* f. sp. *platani* was investigated. Ten compounds potentially toxic to *Platanus acerifolia* were isolated and identified. These metabolites were mainly isocoumarins and naphthalenones. Three of these induced extensive necrosis on plane tree tissues.

Key words. *Platanus* sp., canker stain, phytotoxins, 1,4-naphthoquinones.

Introduction

Ceratocystis fimbriata is a perithecial ascomycete that infects high-value crops and trees such as hevea, sweet potato, coffee, cacao, oak, coconut palm and plane tree (Chevaugeon *et al*., 1957; Kojima, 1993). Canker stain of plane tree, caused by *C. fimbriata* f. sp. *platani,* is one of the most important and most widely reported diseases on plane (Panconesi, 1999). This infection has spread very quickly in Europe, mainly through France and Italy, and only prophylactic treatments can prevent its dispersal towards the North.

The pathogenic agent enters through wounds in the roots and branches, and causes foliar withering accompanied by trunk canker (El Modafar *et al*., 1974). These symptoms are limited to organs situated above the inoculation site, which suggests that fungal metabolites are involved in the decay process.

Previous studies carried out on susceptible species *(Platanus acerifolia, P. orientalis)* inoculated with *C. fimbriata* f. sp. *platani* showed a loss of ability to cicatrize wounds and the occurrence of large necrotic lesions in infected tissues (El Modafar *et al*., 1987; Ake *et al*., 1992). The former symptom could be assigned to fimbriatan, a protein of low molecular weight excreted by the fungal pathogen (Ake *et al*., 1992), and which completely mimics some of the observed symptoms *in vivo*. Very little work has been done on the fungal metabolites involved in the formation of necrotic lesions.

This work reports for the first time the production of biologically active secondary metabolites in the culture medium of *C. fimbriata* f. sp. *platani*. Three of these metabolites induce large necrotic lesions in plane tree tissues, as was observed *in vivo*. At a later stage, some of the moderately active metabolites were detected in infected wood. The structure and biological properties of these fungal compounds are described.

Corresponding author: R. Tabacchi
Fax: +41 32 7182511
E-mail: raphael.tabacchi@unine.ch

Materials and methods

Fungal cultures

Ceratocystis fimbriata f. sp. *platani* strains (Madonna del Piano, Ticino, Switzerland) were grown on malt agar plates and broth cultures were prepared by inoculating 250 ml of synthetic ZMA medium (Witt, 1990) with 2 ml of a 6-day-old culture of the pathogen. An equal amount of sterile vitamin B1 (aneurin hydrochloride) was added to each plate after sterilization of the contents. The 48 conical flasks containing the broth were incubated for 24 days at 25°C on an orbital shaker at 120 rpm; the mycelium was then filtered off and the aqueous phase centrifuged at 15,000 *g* (rotor diam. 15 cm) for 30 minutes and sterilized through a 1 μm membrane filter.

Extraction, purification and analysis of metabolites

The aqueous phases were mixed with NaCl (100 g l^{-1}) and two times extracted with EtOAc. The organic extract was then partitioned between methanol and hexane, and the alcoholic phase chromatographed on an RP-C18 column, which gave eight fractions of decreasing polarity. Subsequent silica gel columns (EtOAc-hexane mixtures) and final RP-HPLC made possible the purification of compounds **1–10.**

HPLC: Bischoff (Methrom AG, Wallisellen, CH) Nuc100-5μ C18 column (250×4.6 mm) on an HP 1050 quaternary pump, or an HP 1100 (Agilent Technologies), (eluent: MeOH-Water, variable gradient), detection 254 nm. Five μm RP-C18 or Merck silica gel 60 was used as the chromatographic phase for HPLC. LC/MS: Finnigan (Palo Alto, CA, USA) LCQ ion trap MS-MS^n equipped with an ESI source. ^1H and ^{13}C NMR Bruker AMX 400. FT-IR Perkin-Elmer 1720X and low-resolution mass spectrometry: NERMAG R30-10 (EI, 70eV). CD spectra were recorded on a JASCO J-500C.

Plant material

A 12-m-high plane tree *(Platanus orientalis)* from the Experimental Station "La Versiliana" (Pietrasanta, Forte dei Marmi, Italy) was artificially infected by injections of a suspension of spores of *C. fimbriata* f. sp. *platani*. Injections were repeated at 15-cm intervals all around the trunk, at a height of 1 m. After 3 months of development, the trunk was divided into discs of equal thickness and the infected discs (characterized by an extensive blackening of the wood) as well as apparently healthy wood were reduced to small chips with an electric saw. The sawdust produced (1.5 kg) was extracted with acetone for two days in a soxhlet, the extract (A, from necrotic wood, and B, from apparently healthy wood) being then filtrated through silica gel (eluent EtOAc) and Superclean® (Supelco, Bellefonte, PA, USA), (eluent MeOH) just before analysis with HPLC (gradient solvent system MeOH-water-acetic acid 1%). Sawdust of a similar but healthy plane tree was treated in the same way and used as a control (extract C). Pure fungal metabolites or commercially available compounds (*p*-aminobenzoic acid, umbelliferone and scopoletine, Fluka, Switzerland) were used as analytical standards.

Biological tests

Crude extracts, chromatographic fractions and pure metabolites were weighed and dissolved in 2% EtOH. Stems and 1–2-cm-long plantlet leaves were then immersed in these solutions and symptoms (browning or wilting) monitored over time, and compared with healthy controls in 2% EtOH. For quantification, 1 cm^2-square pieces of plane tree leaves were cut in distilled water and immersed in the test solutions. The size of necrotic black areas was again recorded over time, and results converted to percent of total leaf area.

Identification of naphthalenones

*(+)cis-3,4-Dihydro-2,4,8-trihydroxynaphthalen-1(2H)-one (**1**):*

^1H NMR (MeOD-d_4) :11.60 (*s*, OH-8), 7.57 (t, 7.7 Hz, H-6), 7.23 (*dt*, 7.4, 1.3 Hz, H-5), 6.87 (*dt*, 8.4, 1.3 Hz, H-7), 4.95 (*dd*, 11.7, 5.2 Hz, H-4), 4.43 (*dd*, 13.6, 5.2 Hz, H-2), 2.66 (*dt*, 11.7, 5.2 Hz, H-3), 2.02 (*dt*, 13.6, 11.7 Hz, H-3'); CD (MeOH, c = $1.89 \cdot 10^{-3}$ M) $[\theta]_{335}$ = -291; $[\theta]_{321}$ = 0; $[\theta]_{305}$ = +265; $[\theta]57_{280}$ = 0; $[\theta]_{263}$ = +317; $[\theta]_{230}$ = +741; $[\theta]_{226}$ = 0

(-)trans-3,4-Dihydro-2,4,8–trihydroxynaphthalen-1(2H)-one (***2***):

^1H NMR (MeOD-d_4) :11.66 (*s*, OH-8), 7.54 (*dd*, 7.4, 8.5 Hz, H-6), 6.99 (*d*, 6.8 Hz, H-5), 6.93 (*dd*, 8.5, 1.1 Hz, H-7), 5.00 (*t*, 3.5 Hz, H-4), 4.82 (*dd*, 4.8,11.1 Hz, H-2), 2.52 (*ddd*, 3.5, 4.8, 13.3, H-3), 2.28 (*ddd*, 3.5, 11.1, 13.3 Hz, H-3'); ^{13}C NMR

(MeOD-d_4) : 206.4 (C-1), 164.0 (C-8), 146.8 (C-10), 138.7(C-6), 121.6 (C-5), 118.9 (C-7), 115.2 (C-9), 70.3 (C-2), 67.9 (C-4), 39.4 (C-3) : m/z (EI,+) 194, 176, 150, 121, 102 (M-C_6H_4O) : IR (neat NaCl) : 3355, 1645, 1576, 1456 cm^{-1}; UV (MeOH): 258, 331 nm; CD (MeOH, c = $2.2 \cdot 10^{-3}$ M) $[\theta]_{337}$ = -1759; $[\theta]_{321}$ = 0; $[\theta]_{304}$ = +1296; $[\theta]_{282}$ = 0; $[\theta]_{257}$ = -14352; $[\theta]_{230}$ = -1389; $[\theta]_{227}$ = 0.

cis -3,4 -Dihydro-3,4,6,8 tetrahydroxynaphthalen-1(2H)-one (***3***):

^{1}H NMR (MeOD-d_4) :12.76 (*s*1036, OH), 6.53 (*dd*, 2.0, 0.4 Hz), 6.15 (*d*, 2.0 Hz), 4.70 (broad *d*, 2.8 Hz), 4.21 (*ddd*, 2.8, 4.2, 6.8 Hz), 2.88 (*dd*, 6.8,13.0 Hz), 2.81 (*dd*, 4.2, 13.0 Hz); CD (MeOH, c = $1.9 \cdot 10^{-3}$M) $[\theta]_{305}$ = +3579; $[\theta]_{294}$ = 0; $[\theta]_{279}$ = -6684; $[\theta]_{237}$ = -3737; $[\theta]_{225}$ = 0.

trans-3,4-Dihydro-3,4,6,8- tetrahydroxynaphthalen-1(2H)-one (***4***):

^{1}H NMR (MeOD-d_4) :12.80 (*s*, OH), 6.60 (*dd*, 2.2,1.0 Hz), 6.18 (*d*, 2.2 Hz), 4.48 (broad *d*, 7.4 Hz), 3.96 (*ddd*, 8.6, 4.2, 7.4 Hz), 2.96 (*dd*,17.0, 4.2 Hz), 2.60 (*dd*, 17.0, 8.6 Hz); CD (MeOH, c = $1.43 \cdot 10^{-3}$ M) $[\theta]_{329}$ = -699; $[\theta]_{317}$ = -1119; $[\theta g]_{305}$ = -629; $[\theta]_{279}$ = -9441; $[\theta]_{239}$ = -4545; $[\theta]_{228}$ = 0.

Results and discussion

From *C. fimbriata* f. sp. *platani* culture filtrate (Witt, 1990) we obtained an organic extract that induced a strong darkening of plane tree leaves even at moderate concentration (1 mg ml^{-1}). As regards necrosis of the leaves, the aqueous phases of the medium exhibited a much weaker activity even at relatively high concentrations (solution of 10 mg ml^{-1} of freeze-dried aqueous extract). RP-C18 chromatography of this extract yielded eight fractions, only three of which showed consistent toxicity to plantlet leaves. Subsequent chromatographic separations and purifications yielded nine active compounds, whose structures were confirmed by comparing their spectroscopic data with those in the literature (Fig. 1). These compounds consisted mainly of naphthalenones (compounds **1–5**) involved in the branched pathway of fungal DHN-melanine biosynthesis, and of isocoumarins (compounds **6–9**). All these compounds are known metabolites that have been isolated from various fungal strains (Iwasaki *et al.*, 1973; Bell *et al*, 1976; McGraw and Hemingway, 1977). The last of these compounds, p-aminobenzoic acid (PABA, **10**), which is produced mainly during the first two weeks of growth, is reported to be a fungal metabolite and biosynthetic intermediate (Turner, 1983) but is not considered a phytotoxin.

It should be noted that both diastereomers (*cis / trans* **1**, **2** and **3**, **4**) were found in the *C. fimbriata* f. sp. *platani* culture medium, as was confirmed by their ^{1}H-^{1}H correlations and the CD spectra (experimental part). Each isomer could be purified by RP-HPLC using the solvent system MeOH-water-THF (15:80:5) for **1** and **2**, and (10:85:5) for **3** and **4**.

Together with compounds **1** and **2**, PABA (**10**) showed necrotic activity against plane tree tissues, causing large lesions throughout the sample. *Cis* and *trans* naphthalenone **1** and **2** were previously reported as phytotoxins of *Mycosphaerella fijiensis* (Ichahara *et al.*, 1989) the causative agent of Black Sigatoka disease of bananas.

According to the same bioassays, the other naphthalenones **3** and **4** were less active (comparison after 48 hours). Scytalone (**5**), generally considered phytotoxic, showed significant necrosis only after 7 days. The isocoumarins (**6–9**) were only slightly active, causing slight browning at the base of the leaves. We again observed (Gremaud and Tabacchi, 1994) the toxic activity of these isocoumarins, produced by *C. fimbriata coffea,* the causal agent of coffee canker tree disease, on the leaves. These results prompted us to synthesize the most toxic metabolites, **1**, **2** and **10**, in amounts sufficient to improve the biological tests. Compounds **1** and **2** were synthesised in racemic form with *Fujimoto's* procedure (Fujimoto and Satoh, 1986) and both diastereomers were separated by HPLC. The asymmetric synthesis of **1** and **2** confirmed the stereochemistry of the natural naphathalenones **1** (cis): (-)-(2S,4S) isomer and **2** (trans): (+)-(2S,4R)isomer(+) (Couché *et al.*, 2003).

We then quantified their biological activity merely by measuring the necrotic areas occurring on square pieces of plane tree leaves immersed in test solutions. The resulting curves are presented below (Fig. 2). PABA (**10**) at a concentration of 1 mg ml^{-1} induced visible symptoms on

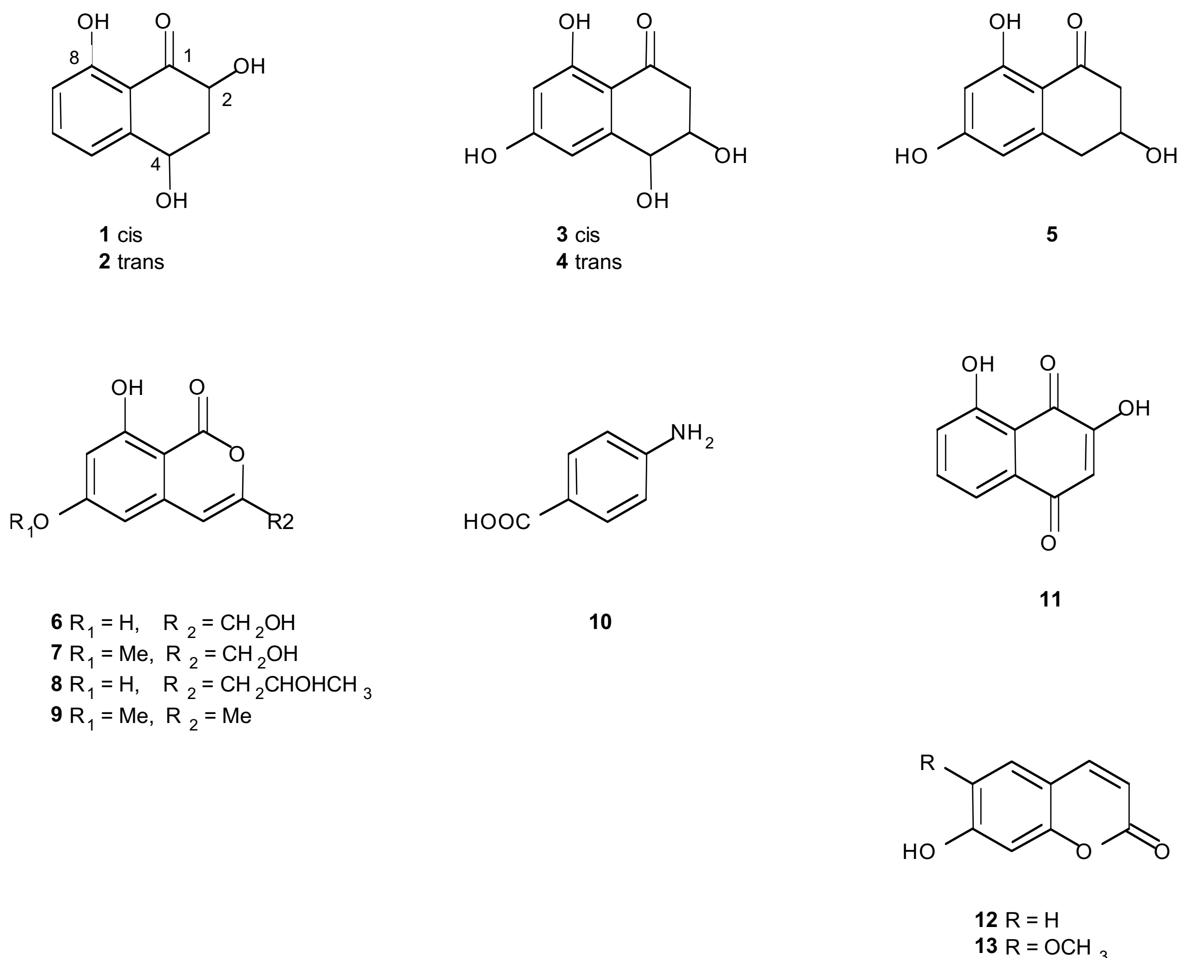

Fig. 1. Structure of the compounds isolated from the culture medium of *Ceratocystis fimbriata* f. sp. *platani* and (or) from wood infected by the pathogen.

the samples after 10 hours and caused complete browning after less than four days (Fig. 2A). Naphthalenones **1** and **2** both induced the same effects, suggesting an activity independent of the stereochemistry of the molecule, but unlike what occurred with (**10**), the symptoms with **1** and **2** did not appear until three days after immersion of the pieces of leaves, after a long initial stationary phase (Fig. 2B).

HPLC analysis of these solutions revealed a new compound which was further identified as 2-hydroxyjuglone (2-HJ, **11**), a 1,4-naphthoquinone derived from **1** or **2** (2,4,8 THN) by an oxydative process. We investigated the experimental conditions and the kinetics of this reaction *in vitro* after oxidation, by HPLC (Bürki, 1996). We suggest that there is a radical induced mechanism. The oxidation process could be enhanced either by increasing the pH or by adding small amounts of an appropriate proton acceptor (proline or any non-aromatic amines), or *in vivo* by an oxidase-enzyme. 2-Hydroxyjuglone (**11**) was never detected in our crude fungal extracts, using HPLC and HPLC-MS. Consequently, naphthoquinone **11** was a secondary, toxic metabolite produced by the naphthalenones **1** or **2** (2,4,8-THN) *in vivo*. The oxidation product 2-hydroxyjuglone (2-HJ, **11**) seemed to be strongly active

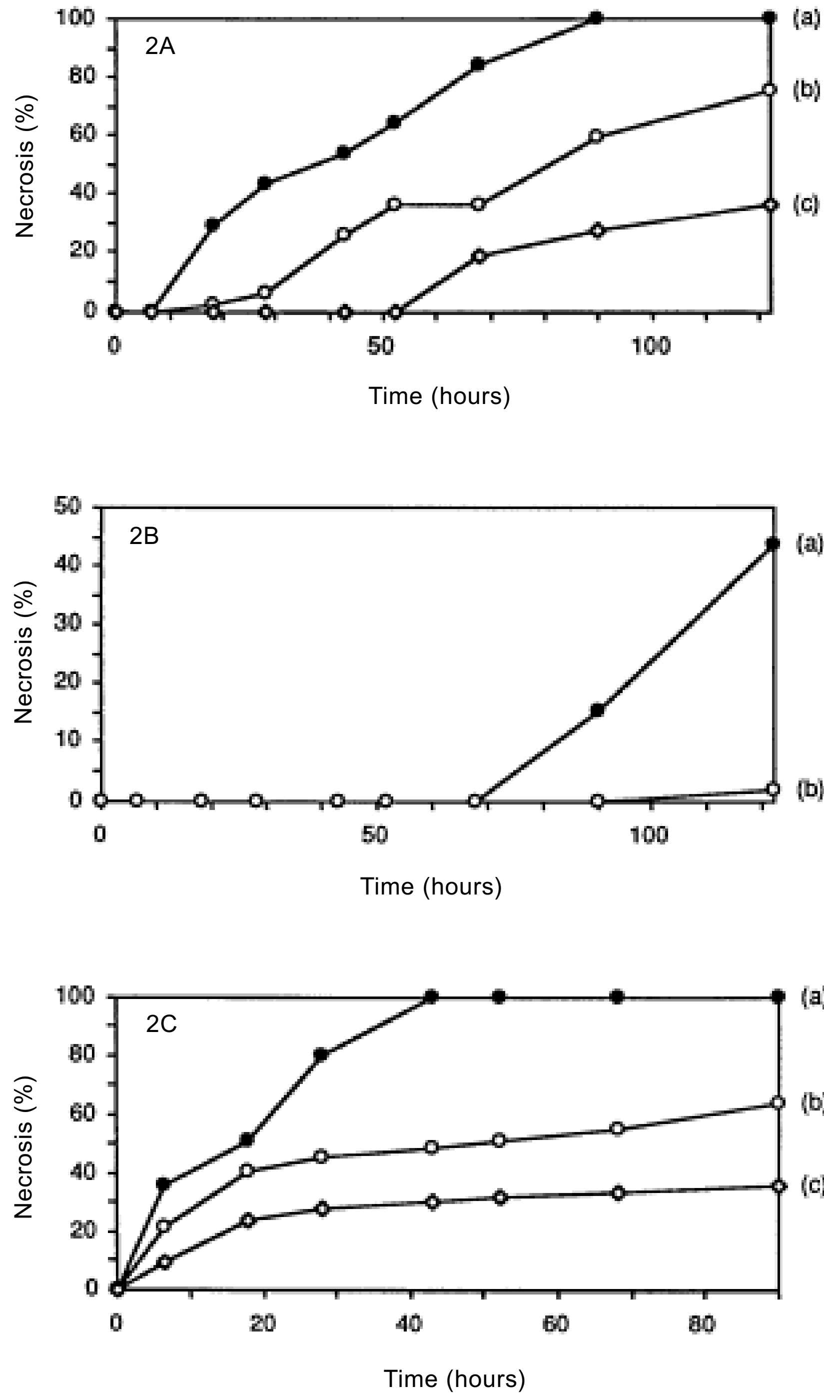

Fig. 2. Observed black areas (as % of total area) are shown as a function of time; biological tests were performed on 1 cm^2 pieces of plane tree leaves and controls were kept in 2% aqueous solutions of ethanol. 2A, PAB (10) solution at: (a)1000 ppm (7.2 μM), (b) 500 ppm (3.6 μM), and (c) 100 ppm (0.72 μM); 2B, solution of a racemic mixture of 2,4,8-THN (1 and 2) at: (a) 1000 ppm (5.15 μM), (b) 500 ppm (2.57 μM); 2C, 2-HJ (11) solution at: (a) 500 ppm (2.63 μM), (b) 100 ppm (0.52 μM), and (c) 50 ppm (0.26 μM).

against plane tree, as is shown on Fig. 2C. With a 2.6 μg ml^{-1} (500 ppm) solution of haphtoquinone **11** it took only 5 h to produce necrosis on 40% of leaves and 40 h for complete necrosis to occur.

The cytotoxicity of related naphthoquinones has often been reported (Soderquist, 1973; Oberth *et al*., 1992) and is attributed to the chemical modification of the thiol or amine protein groups. These functional groups form covalent adducts with quinones by a Michael 1,4-addition; once formed, protein-hydroquinone adducts can oxidize back to the quinone form, and react with another nucleophilic group resulting in protein cross-linking (Fig. 3) Oberth *et al*. (1992) and Oberth and Jones (1994) demonstrated the existence of such addition compounds with one or two amino acid residues by ESI-MS-MS analysis

The strong biological activity of naphthoquinone **11** explains the apparent inconsistent effects of naphthalenones **1** and **2**, a preliminary oxidation step being necessary before the appearance of phytotoxic symptoms.

In addition to fimbriatan (Ake *et al*., 1992), Pazzagli *et al*. (1999) described a new phytotoxic protein (cerato-platanin) of about 12,4 kDa, identified and isolated from the culture filtrate of *C. fimbriata* f. sp. *platani*. The N-terminus of this protein was homologous with that of cerato-ulmin, a phytotoxic protein belonging to the hydrophobin family and produced by *Ophiostoma novo-ulmi,* the fungus responsible for Dutch elm disease. Cerato-platanin consists of 120 amino acid residues and contains four cysteins, a lysine and a histidine residue that react with the 1,4-naphoquinones by means of the Michael 1,4 addition (Fig. 3).

In order to find evidence of the occurence of any or all of the potential phytotoxins isolated in the plant, 1.5 kg of infected and necrotic *Platanus orientalis* wood was extracted with acetone (extract A).

Using HPLC (gradient solvent system MeOH-water-acetic acid 1%) coupled to UV and MS detectors, the LC-ESI-MS-MS^n technique detected four of the active compounds previously isolated from the culture medium of the fungus: **1**, **2**, **7** and **9**. Other signals corresponding to m/z 193 (M^- - 1), were observed, but the corresponding MS-MS^n spectrum could not confirm the occurrence of **3** and **4**. By contrast, the highly phytotoxic oxidation products, 2-hydroxyjuglone (**11**), and 3-hydoxyjuglone, arising from hydroxynaphthalenones **1**, **2**, and **3**, **4** respectively, were detected in SIM-negative mode. PABA (**10**) seems not to occur in

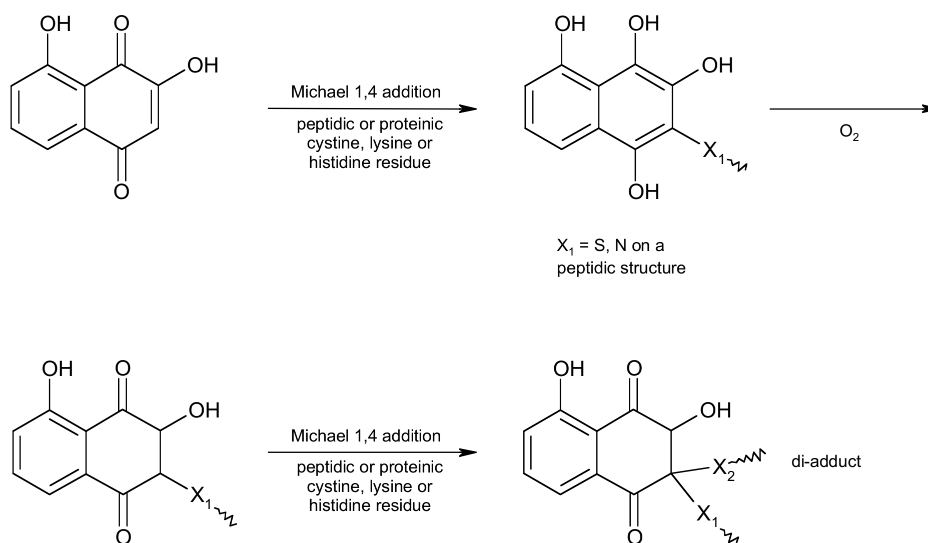

Fig. 3. Proposed mechanism of protein cross-linking induced by 1,4 naphthoquinones.

the plant material.

We also confirmed the occurrence of large amounts of umbelliferone (**12**) and scopoletine (**13**), reported as phytoalexins of *Platanus acerifolia* (El Modafar *et al.,* 1993; Ibitssam *et al*., 1998.). Extract A was compared, using the same analytical conditions, with extract B, obtained from apparently healthy wood of the same infected tree cut 1 m above the last black necrosis, and with extract C, obtained from another, non-infected plane tree. Except for trans-1(2*H*)3,4-dihydro-2,4,8-trihydroxynaphthalenone (2,4,8-THN, **2**) which occurred in extract B, none of the metabolites of C. *fimbriata* f. sp. *platani* were detected in extract B or C. The phytoalexins unmbelliferone (**12**) and scopoletine (**13**) occurred in these two extracts, but the quantity decreased from the necrosed (A), to the infected (B) and the healthy wood (C).

As Stierle *et al*. (1991) suggested, the absence of the most phytotoxic compounds in infected materials is not surprising, considering their high chemical reactivity and their strong tendency to undergo further oxidation, reduction or other enzymatic reactions *in vivo*. According to Oberth *et al*. (1992), and Oberth and Jones, 1994), a good option would now be to investigate and look for biological derivatives of such molecules in infected plane tree wood.

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