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CHARACTERIZATION OF A γ-RADIATION-INDUCED ANTIFUNGAL STRESS METABOLITE IN CITRUS PEEL

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Key Word Index—Citrus; Rutaceae; γ-radiation; stress metabolite; antifungal activity.

Abstract—A γ-irradiation-induced stress metabolite with antifungal activity was isolated from pitted regions of γ-irradiated Citrus peel and identified as 4-(3-methyl-2-butenoxy) isonitrosoacetophenone on the basis of spectroscopic evidence and single crystal X-ray structural determination.

INTRODUCTION

The exposure of citrus fruit to γ-rays subjects it to oxidative stress [1]. The external flavedo tissues of citrus peel is much more radiosensitive than the pulp tissues and peel damage (pitting) frequently develops [2]. The triggering event might involve cellular disorganization and damage to membrane structures, leading to an increase in ethylene production [3], phenylalanine ammonia-lyase activity [2], and content of phenolic compounds, which then induce cell death and consequent peel pitting [4]. Apart from an increase in the synthesis of phenolic compounds, little is known about the biochemistry of radiation-induced stress metabolism in plant tissues.

In an analysis of peel extracts from γ-irradiated mature oranges (*Citrus sinensis* cv Valencia) and lemons (*Citrus limon* cv Eureka), we observed the presence of compounds which did not occur in the extracts from non-irradiated fruits and which exhibited antifungal activity [5]. One of these stress metabolites was identified as scoparone [6,7-dimethoxycoumarin] [5]. We now report on the isolation and structural elucidation of another stress metabolite with antifungal properties from both orange and lemon peel.

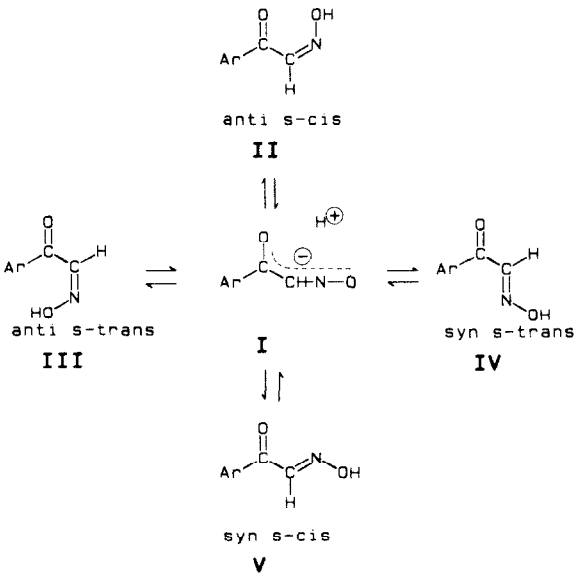
RESULTS AND DISCUSSION

Antifungal compounds in flavedo extracts were detected by TLC bioassay [6] using *Cladosporium cucumerinum* as the test organism. One of the zones inhibitory to the germination of *C. cucumerinum* spores was associated with the unknown stress metabolite. The corresponding inhibition zone was absent from the control chromatogram. This compound was purified from the flavedo tissue with yields of between 5 to 10 mg/g fresh tissue. The structure of the compound, 4-(3-Methyl-2-butenoxy) isonitrosoacetophenone (**1**), was established from its MS, ^{1}H and ^{13}C NMR spectra and an X-ray crystal structure determination.

The ^{13}C and ^{1}H NMR spectra of **1** (see Experimental for assignments) showed the presence of a 1,4-disubstituted benzene ring system. One of the substituents was identified as a 3-methyl-2-butenoxy group by comparison of the ^{1}H and ^{13}C NMR spectra with those of an authentic sample of methyl 4-(3-methyl-2-butenoxy) benzoate, leaving the other substituent of the disubstituted benzene nucleus thus to have the composition $C_2H_2NO_2$.

The molecular ion peak at *m/z* 233.1045 in the high resolution mass spectrum of **1** corresponded best with a molecular formula $C_{13}H_{15}NO_3$. Fragments at *m/z* 216.1031 and 165.0436 corresponded to the loss of a hydroxyl group and an isopentadiene molecule respectively. The ^{1}H NMR spectrum of an acetone-d_6 solution of **1** showed signals for a hydrogen-bonded hydroxyl proton and an aldoxime proton at δ 11.50 and 7.90 respectively. The chemical shifts of these protons are comparable with those of the corresponding protons in isonitrosoacetophenone (**2**) [7]. The ^{13}C NMR spectrum (proton-coupled) of a $CDCl_3$ solution of **1** showed a singlet at δ 187.5 and a broad doublet at δ 149.05 which is in agreement with the presence of a carbonyl carbon and an aldoxime carbon. The latter signal was very broad (probably due to interconversion in solution between some of the forms I to V in Scheme 1 at a rate comparable with the NMR time scale), but it was sharper in the spectrum of an acetone-d_6 solution of **1**.

Figure 1 shows the molecular structure of **1** as established from X-rays and Fig. 2 the lamellar crystal packing. The pseudo-monoclinic crystals contain four independent molecules per asymmetric unit, connected in an extended hydrogen-bonding network, part of which is shown in Fig. 3. Since not all hydrogen atoms could be located from difference Fourier maps, the identity of all the atoms in the α-oxo aldoxime side-chain could not be unequivocally established from the X-ray data only. It was however possible to derive the configuration shown by combining the information from the NMR data with the interatomic distances observed in the crystal. The bond lengths observed are, within experimental error, the same as the corresponding bond lengths reported for **2** [8] and pyruvaldehyde-1-oxime (**3**) [9] (See Table 1). A search of the Cambridge crystallographic database [10] showed that the small C–N–O angle of *ca* 112° is a common feature of *syn* aldoximes (i.e. the *E*-stereoisomer in the case of aldoximes). In the crystal structure of **1** the



Scheme 1

α-oxo aldoxime moiety has a *syn* s-*trans* conformation and hydrogen bonding occurs in infinite chains between the hydroxyl hydrogen atom and the carbonyl oxygen atom of a neighbouring molecule (see Fig. 3), while in **2** it has a *syn* s-*cis* conformation and dimeric hydrogen bonding occurs between the hydroxyl hydrogen atoms and the nitrogen atoms of two neighbouring molecules. Because of the planarity of, and the nature of the H-bonding pattern, the crystal packing exhibited is of alternating layers of molecules almost perpendicular to the long axis, each layer consisting of parallel 'ribbons' of the H-bonded molecules extending along one of the short axis directions. The molecular structure of **3** as determined by gas phase electron diffraction has the α-oxo aldoxime in the *syn* s-*trans* conformation [9].

Table 1. Bonding parameters (Å, degrees) for the α-oxo aldoxime moiety of **1** compared with the corresponding parameters of **2** and **3**

	1*	2	3
O (1)–C (7)	1.24 (1)	1.220 (3)	1.212 (3)
C (1)–C (7)	1.46 (1)	1.480 (4)	—
C (7)–C (8)	1.48 (1)	1.478 (5)	1.479 (4)
C (8)–N (1)	1.25 (1)	1.263 (4)	1.260 (4)
N (1)–O (2)	1.38 (1)	1.375 (4)	1.382 (3)
O (2)–N (1)–C (8)	112.0 (8)	112.0 (2)	112.3 (4)

*Mean values for the four independent molecules in the asymmetric unit.

Compound **1** appears to be biosynthetically derived from the glucosinolate pathway [11] and might be related to the aldoxime intermediate resulting from tyrosine as amino acid precursor. A marked increase in the

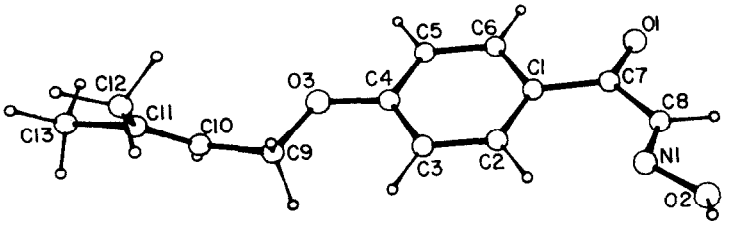


Fig. 1. Molecular structure of compound **1**.

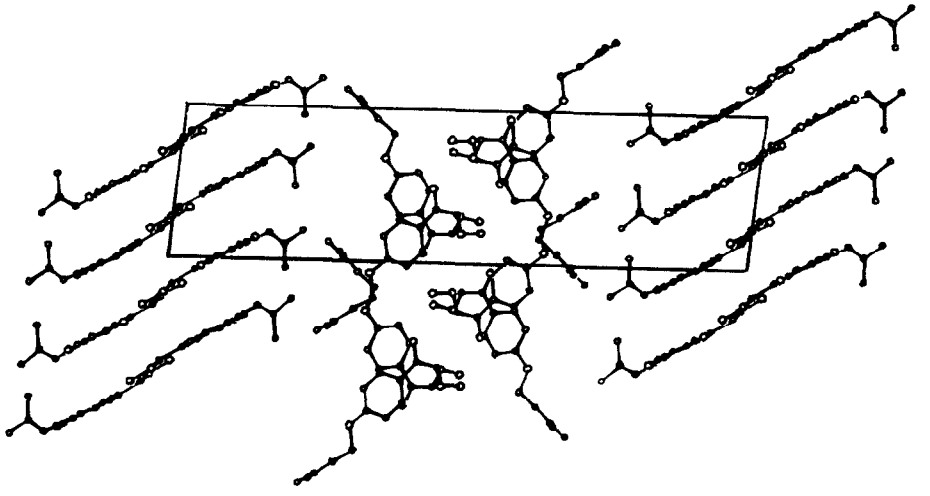


Fig. 2. The layered packing in crystals of **1**. The view direction of this parallel projection is along the crystallographic *b*-axis.

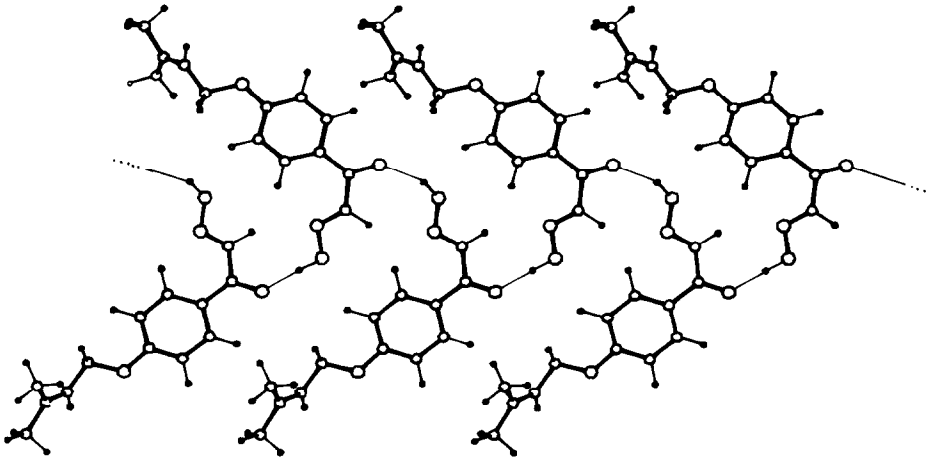
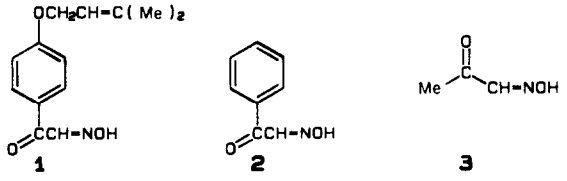


Fig. 3. A segment of one of the infinite and flat 'ribbons' of hydrogen-bonded molecules of **1**, shown end-on in Fig. 2. H-bonds are indicated by the thin lines.



concentration of the aromatic amino acids, phenylalanine (205%) and tyrosine (456%) was reported to occur in the flavedo of γ-irradiated oranges [2]. Phenylalanine in irradiated fruits is utilized for phenolic biosynthesis by phenylalanine ammonia-lyase [2]. As the enzyme from citrus does not catalyse the deamination of tyrosine [12], the accumulated tyrosine is probably metabolized by other pathways, e.g. glucosinolate biosynthesis [11].

The introduction of the α-oxo group of **1** might prevent the further metabolism of its aldoxime intermediate into the glucosinolate. Prenylation of an aromatic hydroxyl group is a very common biosynthetic event [13] and may be the last step in the biosynthesis of **1**. The amount in which this stress metabolite is synthesized would seem to suggest that it is not merely an end-product of an aberrant biosynthetic process, but that it might have a specific function in the oxidative stress metabolism of the plant tissue.

EXPERIMENTAL

MP: uncorr.

Irradiation procedure. Irradiation of mature fruit was performed as previously described [5]. Doses of 2.0 and 4.0 kGy were applied.

Extraction and purification of stress metabolite. Necrotic (pitted) regions of flavedo tissue were sampled from the fruit 7 to 9 days after irradiation. The tissue (1 kg) was homogenized in hexane (2 l) for 3 min. The homogenate was filtered and the extraction repeated. The extracts were combined and concd by vacuum evapn. Chromatography of the residue on silica gel 60 TLC plates with C_6H_6–EtOAc (2:1) revealed the presence of a compound ($R_f \simeq 0.72$), absent from the extracts of control fruit, which showed antifungal activity as shown by bio-autography performed by spraying TLC plates with a spore suspension of *Cladosporium cucumerinum* in a sterilized glucose-mineral salts medium [6]. After spraying, the TLC plates were incubated in a moist atmosphere for 2 to 3 days at 22°. The presence of fungitoxic compounds was indicated by zones of inhibition where fungal growth did not occur.

Compound **1** was purified by chromatographing the extract on silica 60 TLC plates with $CHCl_3$ as solvent. The zone corresponding to the unknown compound ($R_f \simeq 0.20$), was detected by UV light, the silica was scraped off and the compound was extracted within Et_2O. The above process was then repeated with C_6H_6–EtOAc (2:1) as solvent, and finally with C_6H_6–MeOH (9:1) as solvent ($R_f \simeq 0.60$). The compound was then extracted from the silica with Et_2O, concd to dryness and used for structural analysis. The pure compound 4-(3-methyl-2-butenoxy)isonitrosoacetophenone (**1**), (48 mg) was obtained as a colourless crystalline solid, mp 104–105° (from CH_2Cl_2–hexane), UV λ_{max}^{MeOH} nm: 227, 302; IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3240, 1683, 1600, 980; ^1H NMR (200 MHz, acetone-d_6): δ 1.76 and 1.77 (2 × 3H, 2 × *d*, $J = 1$ Hz, $C(CH_3)_2$), 4.66 (2H, *d*, $J = 6.6$ Hz, OCH_2), 5.47 (1H, *m*, CH = C), 7.02, (2H, *d*, $J = 7.0$ Hz, arom.), 7.90 (1H, *br s*, CH=N), 8.08 (2H, *d*, $J = 7.0$ Hz, arom.), 11.50 (1H, *br s*, OH); ^{13}C NMR (50.3 MHz, $CDCl_3$): δ 18.32 and 25.88 (2 × *q*, $C(Me)_2$, 65.14 (*t*, OCH_2), 114.49 (*d*, arom. CH), 118.73 (*d*, CH=C), 129.10 (*s*, arom. C), 132.27 (*d*, arom CH), 139.12 (*s*, $(Me)_2$ C=), 149.05 (*d*, CH=N), 163.45 (*s*, arom. C), 187.50 (*s*, C=O); MS *m/z* (rel. int.): 233 $[M]^+$ (13), 216 (38), 165 (48), 121 (77), 69 (100); High resolution MS *m/z*: M^+ 233.1045 (calc. for $C_{13}H_{15}NO_3$ 233.1052), 216.1031 (calc. for $C_{13}H_{14}No_2$ 216.1025), 165.0436 (calc. for $C_8H_7NO_3$ 165.0426).

Crystal structure determination of **1**. Crystal data: $C_{13}H_{15}NO_3$, $M_r = 233.27$, triclinic, space group $P\bar{1}$, $a = 8.9869$ (12), $b = 8.9902$ (8), $c = 31.491(7)$Å, $\alpha = 95.881$ (12), $\beta = 95.326$ (14), $\gamma = 90.438$ (9)°, $V = 2519.63$ Å^3, $Z = 8$, $d_c = 1.23$ g cm^{-3}, μ (CuK_α) $= 0.47$ mm^{-1}, $T = 298$ K.

Crystals barely suitable for X-ray analysis could be obtained on one occasion only by crystallisation from CH_2Cl_2–hexane. A pseudo-monoclinic cell was discarded in favour of the triclinic one because of unacceptable deviations from right angles for α and γ, and large differences in intensity of reflections that were supposed to be equivalent. A crystal of dimensions 0.45 × 0.35 × 0.10 mm was used for data collection on an Enraf–Nonius CAD4 diffractometer with graphite monochromated CuK_α radiation ($\lambda = 1.5418$ Å). Data were collected by ω-2θ scans of variable speed (max 4.12°/min). Reflections with $5 \leq \theta \leq 78°$ were measured and yielded 10638 reflections of which 4934 with

$I > 5\sigma(I)$ were used in the refinement. Three standard reflections showed a 3.5% loss in intensity over the exposure time of 120 hr. Data were corrected for crystal decay and Lorentz and polarisation effects.

The structure was solved by direct methods and refined by least-squares procedures minimizing the function $\Sigma w (|F_o| - |F_c|)^2$ with $w = [\sigma(F)]^2 + .005F^{2-1}$ using the SHELX76 [14] computer programme. Computer memory constraints necessitated refinement in two blocks (each block containing two molecuels each). Non-H atoms were treated anisotropically and H atoms isotropically with a common isotropic temperature factor for each of the four molecules in the asymmetric unit. H atoms on C atoms were included in calculated positions for an ideal Dreiding model (all C–H = 1.08 Å, methyl groups refined as rigid groups free to rotate). H atoms of hydroxyl groups were fixed in positions required by the hydrogen bonds between adjacent molecules (O–H = 1.0 Å) [15]. Discrepancy indices converged to $R = 0.010$ and $R_w = 0.013$. These high values were expected because of the poor quality of the crystals. Equivalent bond lengths in the four independent molecules all agree within experimental error. Refined coordinates and other relevant data have been deposited at the Cambridge Crystallographic Data Centre.

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