



Synthesis and evaluation of 4-(3-methyl-2-butenoxy)isonitrosoacetophenone, a radiation-induced stress metabolite in Citrus

Ian A. Dubery*, Anna E. Louw, Fanie R. van Heerden

Department of Chemistry and Biochemistry, Rand Afrikaans University, P.O. Box 524, Auckland Park, 2006, South Africa

Received 18 February 1998

Abstract

The time- and dose-dependent occurrence of 4-(3-methyl-2-butenoxy)isonitrosoacetophenone, a γ -irradiation-induced stress metabolite was investigated. The chemical synthesis of the compound is reported. The compound exhibits antifungal activity, as well as antioxidant activity, as indicated by its ability to scavenge reactive oxygen radicals in a chemiluminescence assay. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: *Citrus sinensis*; Rutaceae; Acetophenones; γ -irradiation; Antifungal; Chemiluminescence; Antioxidant

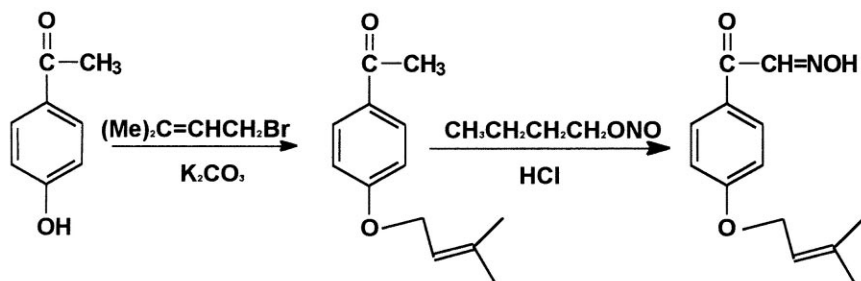
1. Introduction

The irradiation of plant tissues with γ -rays leads to the intracellular generation of active oxygen radicals and H_2O_2 , agents that are known to play a role in signalling plant defence responses [1]. The plant tissue responds to the radiation treatment and cellular protective processes are induced. Stress ethylene is produced and metabolic changes in phenylpropanoid metabolism occur with increased synthesis of phenolic compounds [2]. Receptors for different elicitors might network into the same signalling pathways, thereby leading to the similarities between radiation- and pathogen-induced hypersensitive stress conditions [3].

The radiosensitive external flavedo tissues of citrus peel frequently develops peel damage (pitting) if the fruit is unable to overcome the stress condition. Studies on the effect of γ -radiation on citrus fruits indicated accumulation of phenolic compounds in the peel tissues; this was suggested to be the cause of cell death and consequent peel necrosis [2].

Analysis of peel extracts from γ -irradiated mature oranges (*Citrus sinensis* cv. Valencia) revealed the enhanced synthesis and accumulation of several antifungal phenolic compounds, of which 7-hydroxycoumarin (umbelliferone) and 7-hydroxy-6-methoxycoumarin (scopoletin) [4] were identified. The presence of some compounds that did not occur in the extracts from non-irradiated fruits was also observed. Two of these novel stress metabolites, which possess antifungal activity against *Cladosporium cucumerinum*, were identified as scoparone (6,7-dimethoxycoumarin) [4] and 4-(3-methyl-2-butenoxy)isonitrosoacetophenone (**1**) [5]. The latter is a stress metabolite/phytoalexin of unknown metabolic origin. Although acetophenones have not often been reported as stress metabolites or phytoalexins [5, 6], previous examples include *p*-hydroxyacetophenone from gymnosperms [7], xanthoxylin (2'-hydroxy-4',6'-dimethoxyacetophenone) from *Citrus* [6], 2',6'-dihydroxy-4'-methoxyacetophenone from *Sanguisorba minor* [8], three 4'-hydroxyacetophenone-related phytoalexins from *Polymnia sonchifolia* [9], as well as 3'-hydroxyacetophenone, a phytoanticipin, in carnation [10]. Prenylated [11] and diprenylated

* Author to whom correspondence should be addressed.



Scheme 1.

acetophenones [12] have been identified as common metabolites in the genus *Ophryosporus*.

Evidence for a role for the dietary intake of phytochemicals in the maintenance of health and protection from disease is accumulating. One such protective mechanism by which such chemicals react, is antioxidant (radical-scavenging) activity and recent studies indicated the potential importance of flavonoids, phenylpropanoids and phenolic acids as antioxidants [13].

The novel structure of **1** prompted us to investigate its time- and dose-dependent induction in irradiated citrus flavedo after γ -radiation, its chemical synthesis and an evaluation of some of its properties, including tests for antifungal activity and determination of potential antioxidant activity.

2. Results and discussion

An investigation of the occurrence of **1** as a function of radiation dose and time during a post-irradiation period of 9 days was followed using bioautography [14] which involved the spraying of TLC plates with spore suspensions of *Cladosporium cucumerinum*, followed by the detection of zones of inhibition at a R_f corresponding to that of **1**, using C_6H_6 -EtOAc (2:1) as solvent system (Fig. 1). Radial zones of no fungal growth, indicating fungitoxic activity due to the presence of **1**, were only evident in the peel extracts of citrus after γ -radiation with doses of 2 kGy and 4 kGy; no antifungal activity could be detected with doses of 0.75 kGy and 1 kGy. A small inhibition zone due to the presence of the stress metabolite was already discernable one day after exposure to a radiation dose of 2 kGy. It increased from days 3 to 6 and decreased towards day 9. In contrast, a dose of 4 kGy was sufficient to result in a more sustained response with the appearance of large inhibition zones one day after irradiation that reached a maximum between days 3 to 6 and declined towards day 9. A 4 kGy irradiation dose was also reported to result in sustained

high levels of PAL activity [2,15,16] against a background of almost complete inhibition of total protein synthesis [17]. No inhibition zones were detected on TLC plates of extracts from non-irradiated fruit.

The stress metabolite content of stressed tissues represents a balance between the synthesis and degradation of these compounds. High levels of phytoalexin accumulation do not normally persist in plants once a pathogen or stress condition has been contained and plant metabolism has returned to normal [18]. The persistent high levels of **1** seem to indicate that the tissue is unable to overcome the stress condition, which then results in the extensive pitting of peel tissue associated with the 4 kGy dose.

The yield of **1** from necrotic regions of flavedo tissue was low (48 mg kg^{-1}) and, therefore, the chemical synthesis of **1** was attempted. The synthesis of **1** consisted of two main steps. Firstly, the formation of the 4-(3-methyl-2-butenyloxy)acetophenone (**2**) from *p*-hydroxyacetophenone using standard procedures [19] and, secondly, a nitrosation reaction on the product of the first reaction (Scheme 1). Using the acid-catalyzed reaction for the introduction of a nitroso group into a ketone [20], **1** was obtained as light yellow crystals in a yield of 15%. The base-catalyzed reaction for the introduction of a nitroso group into a ketone was less satisfactory despite the use of potassium tertiary butoxide as base and more forcing reaction conditions.

The data obtained from NMR and mass spectral analyses corresponded with the data obtained for the compound isolated from irradiated citrus flavedo. The absorbance spectrum of **1** revealed two peaks in the ultraviolet region which were identified as $\lambda_{\text{max}}^{\text{MeOH}}$ nm (log ϵ): 302 (4.22) and $\lambda_{\text{max}}^{\text{MeOH}}$ nm (log ϵ): 228 (4.16).

Fig. 2 shows the antifungal activity of **1** and **2** as detected by TLC bioassay. Zones of no growth were detected for **1**, as well as for **2**, against the organisms *Cladosporium cucumerinum*, *Penicillium expansum* and *Aspergillus niger*. It has previously been reported that the introduction of allyl groups into a stilbene-like compound with no antifungal activity resulted in a

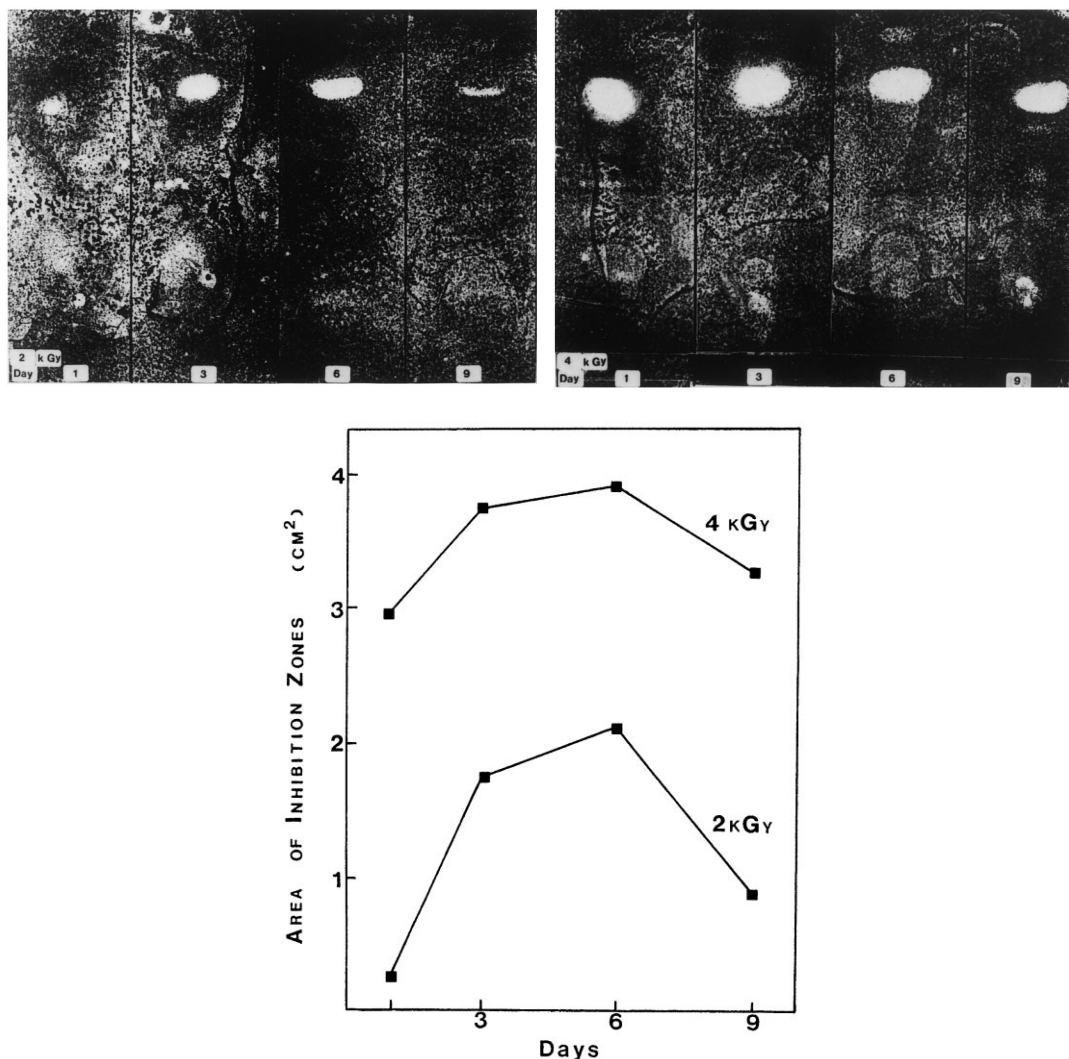


Fig. 1. 1(a). Bio-autography of fungitoxic compounds in extracts from 2 kGy and 4 kGy γ -irradiated citrus peel at intervals of 1, 3, 6 and 9 days after irradiation. Experimental conditions are described in the text. Fig. 1(b). Inhibition zones due to the presence of 4-(3-methyl-2-butenoxy)is-nitrosoacetophenone (**1**) as determined by bio-autography. No inhibition zones were found on TLC plates of extracts of 0.75- and 1.0 kGy-treated fruits.

compound with high antifungal activity against a wide spectrum of organisms [21]. It is interesting that the presence of a prenyl grouping, which could be regarded as an allyl group where the terminal hydrogen atoms have been substituted by methyl groups, also resulted in a compound with antifungal activity against more than one organism.

An evaluation of the antioxidant capacity of **1** was made by using a chemiluminescence (CL) assay [22] in which the chemiluminescence probe, MCLA, was used. MCLA [2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3-one] is a sensitive probe for the detection of superoxide radicals, as well as singlet oxygen, probably *via* the dioxetanone analogue of

MCLA [23], and has widely been used in biological [23] and chemical systems [24]. A hydrogen peroxide (H_2O_2)-horseradish peroxidase (HRP) system in association with MCLA was used in the assay for antioxidant activity in our study. Peroxidase catalysis involves the combination of the enzyme with H_2O_2 which oxidises its haem group to form Compound I which is reduced in sequential, one-electron transfer steps in the presence of one-electron donors, indicating a peroxidative mechanism for catalysis in the H_2O_2 -HRP system [25]. However, Takahashi *et al.* [26] suggested that Compound I of ovoperoxidase can abstract an electron from H_2O_2 to yield superoxide, a proposal supported by Sugioka and Nakano (unpub-

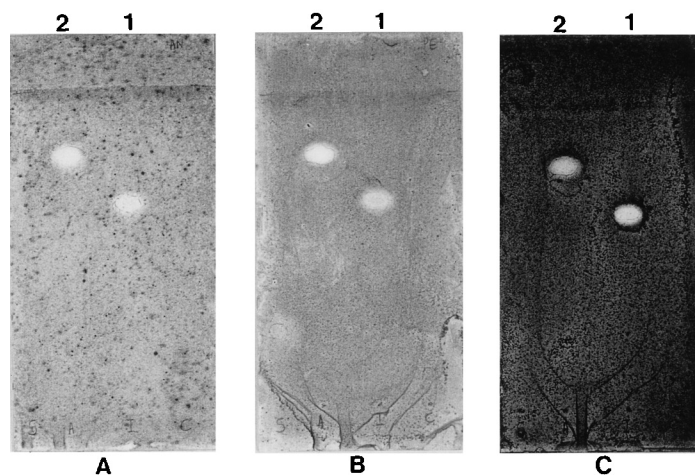


Fig. 2. Antifungal activity of 4-(3-methyl-2-butenoxy)isonitrosoacetophenone (**1**) and 4-(3-methyl-2-butenoxy)acetophenone (**2**) against *Aspergillus niger* (Plate A), *Penicillium expansum* (Plate B) and *Cladosporium cucumerinum* (Plate C), as indicated by zones of no fungal growth. Experimental conditions are described in the text.

lished in [26]) whom gave evidence of the production of superoxide radicals in a system containing H_2O_2 , HRP and MCLA with inhibition of the CL signal by superoxide dismutase. The necessity for the presence of Compound I of HRP in our system was illustrated when almost no chemiluminescence (≈ 20 mV) was observed in reaction mixtures consisting of MCLA and either HRP or MCLA. The addition of HRP to a

mixture of MCLA and H_2O_2 led to a CL signal detectable in a concentration-dependent manner for H_2O_2 with the constant production of radicals. It could be that MCLA reacted with compound I analogous to the reaction sequence proposed for the H_2O_2 -HRP-catalyzed chemiluminescent oxidation of luminol [27]. Alternatively, H_2O_2 probably acted as a hydrogen acceptor for the specific formation of Compound I of

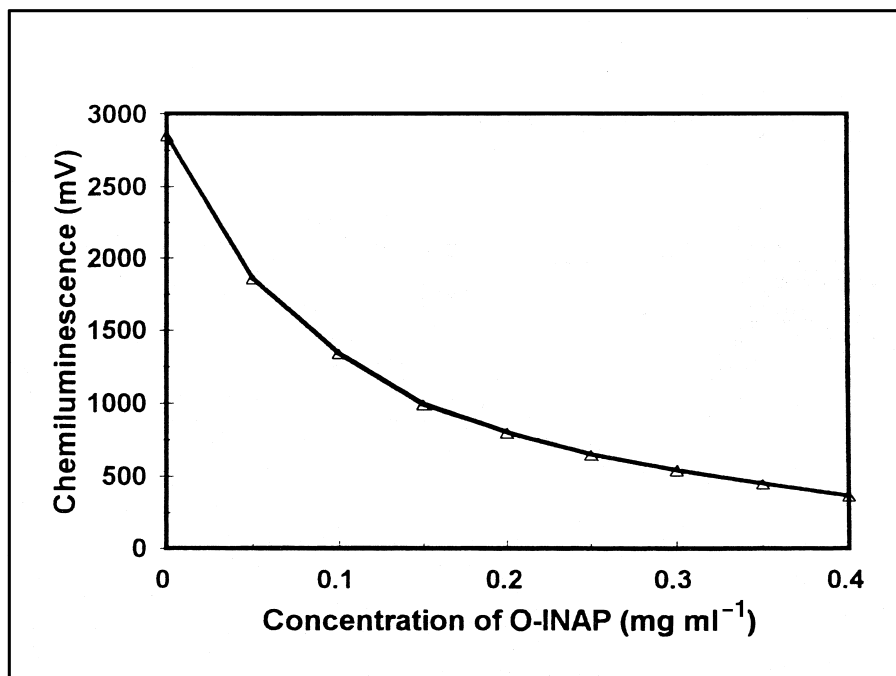


Fig. 3. Chemiluminescence output against concentration of 4-(3-methyl-2-butenoxy)isonitrosoacetophenone (**1**) indicating the anti-oxidant ability of **1** as shown by the decrease in the chemiluminescence signal. Experimental conditions are described in the text.

peroxidase, as well as a hydrogen donor for the regeneration of the native enzyme, leading to the production of superoxide radicals [26] that will react with MCLA to emit light [23]. Fig. 3 shows the potential of **1** to inhibit the CL signal produced in the reaction between MCLA, HRP and H_2O_2 ; 0.12 mg ml^{-1} of **1** was needed to decrease the CL output from 2764 mV to 1382 mV in a system containing 4 nmol ml^{-1} H_2O_2 . These results are indicative of the anti-oxidative nature of **1** and the possibility exists that it acts as a scavenger of superoxide radicals present in the assay. Also, low background CL levels ($\approx 25\text{--}35 \text{ mV}$) were observed in reaction mixtures consisting of MCLA and **1**, MCLA, **1** and H_2O_2 , as well as MCLA, **1** and HRP, demonstrating the absence of other interfering radical species.

Interception of unstable free radicals by nitroso or nitron compounds leads to the production of relatively stable nitroxide radicals which possess superoxide dismutase mimetic activity and which cannot react with H_2O_2 directly [28]. It is possible that **1** represents the nitroso precursor of a nitroxide radical that possesses the ability to scavenge superoxide radicals and, hence, superoxide dismutase mimetic activity. An evaluation of the radical scavenging ability of **2**, when used at the same concentrations as **1**, indicated that this compound does not have any superoxide dismutase mimetic properties, because it had a stimulatory effect on the chemiluminescence assay mixture in the presence of the enzyme. The findings that **1** possesses antifungal, as well as antioxidative activities, were not unexpected, since acetophenones with phytoalexin activity are known to occur [5–10] and prenylation as a last step in the biosynthesis might increase their lipophilicity and consequently their antimicrobial activity [29]. The rapid induction of *O*-prenylases by elicitors has been reported and it appears to be activated predominantly to cope with stress conditions [30].

It seems that the stress condition due to γ -irradiation leads to the activation of latent enzyme systems resulting in increased concentrations of precursors [3] or to possible activation of rate-limiting steps, bringing pre-formed precursors from various pathways together [17]. The development of necrotic lesions in the flavedo layers of 2 kGy-irradiated fruit was preceded by an increase in tyrosine and phenylalanine content, the transient induction of phenylalanine ammonia-lyase (PAL), as well as the accumulation of phenols and the deposition of lignin [3]. Since PAL in citrus cannot deaminate tyrosine [2], it is possible that accumulated tyrosine is metabolized through the glucosinolate pathway [31] and **1** could be related to the aldoxime intermediate derived from tyrosine as amino acid pre-

cursor in this pathway. It is also possible that **1** is a C-nitrosated compound. Nitric oxide (NO), peroxyxynitrite as well as other products from the reaction between NO and oxygen are strong nitrosating agents, resulting in *N*-nitrosated, *S*-nitrosated and *O*-nitrosated, as well as C-nitrosated, compounds [32]. Evidence for the presence as well as for potential biological functions for NO in plants exists. An inducible nitric oxide synthase-like enzyme is present in the roots of *Lupinus alba* upon elicitation with *Rhizobium* lipopolysaccharides [33]. In pea foliage a concentration-dependent activity for NO was reported and at low concentrations of NO a stress-coping effect on leaf growth was reported, while high concentrations of NO had an inhibitory effect [34]. In potato tubers, NO-releasing agents also induced the synthesis of the phytoalexin, rishitin [35].

2-Isonitrosoacetophenone can be used for the detection of ferrous ions [36] and, therefore, **1** could bind Fe^{2+} , implying that during oxidative stress **1** could prevent hydroxyl radical formation in superoxide-driven Fenton reactions [37]. A dual role for **1** as an antioxidant is proposed, not only superoxide dismutase mimetic activity, but also chelating abilities for iron (II). It is interesting that metal-binding and antimicrobial activity were shown for the 1-hydroxypyrid-2-ones which resemble aromatic hydroxamates [38], as well as for the novel synthetic antibacterial molecule, L-573,655, which also possesses a hydroxamic acid function [39]. The biosynthesis and possible roles of **1** in oxidative stress metabolism in plants, as well as in general plant defence mechanisms, warrants further investigation.

3. Experimental

3.1. Irradiation procedure

Freshly harvested, ripe oranges, *C. sinensis* cv. Valencia, were irradiated in the air and at room temp. in a γ beam 650 (AEC) irradiator equipped with a ^{60}Co source at a dose rate of 22 kGy h^{-1} . Radiation doses of 0.75, 1, 2 and 4 kGy were applied.

3.2. Storage and sampling

After irradiation, fruits were kept at 22° in an air-conditioned room. Sampling was conducted over a 9 day period at intervals of 3 days. To ensure representative sampling, flavedo layers from 20 fruits were removed with a grater, mixed thoroughly and frozen

in liquid N₂. Frozen tissue was stored at –20° until analysis.

3.3. Extraction of stress metabolites

Flavedo tissue (100 g) was homogenized in 200 ml hexane for 3 min. Extraction with hexane minimized the co-extraction of coumarin-derived stress metabolites. Homogenates were filtered and the extraction procedure repeated twice. Extracts were combined and concn by vacuum evapn. The final vol. of extracts was made up to 50 ml.

3.4. Bio-autography

Various extracts (100 µl) were applied to silica gel 60 TLC plates with fluorescent indicator and chromatographed with C₆H₆-EtOAc (2:1) or hexane-EtOAc (4:1). The chromatograms were then dried, viewed under UV light to detect the presence of **1** and **2** and subjected to direct bio-autography by spraying the plates with a spore suspension of *Cladosporium cucumerinum* in a glucose-mineral salt medium. After spraying, plates were incubated in a moist atmosphere for 2 to 3 days at 22°. Inhibition zones where fungal growth did not occur indicated the presence of fungitoxic compounds. Areas of inhibition zones were taken as an approximation of the concn of stress metabolite [13]. Fungitoxic activity was also tested using spore suspensions of *Aspergillus niger* and *Penicillium expansum* in glucose-mineral salt medium.

3.5. Synthesis of 4-(3-methyl-2-butenoxy)acetophenone (2)

A mixt. of *p*-hydroxyacetophenone (1.36 g, 0.01 mol), 4-bromo-2-methyl-2-butene (1.2 ml, 0.01 mol) and dry K₂CO₃ (1.38 g, 0.01 mol) in 25 ml Me₂CO was refluxed for 8 hr. The soln was dild with Et₂O (50 ml), washed with 2 M NaOH (3×50 ml), dried (Na₂SO₄) and evapd *in vacuo*. CC (hexane-EtOAc, 4:1) afforded **2** (1.73 g, 85%) as a white crystalline compound. ¹H NMR (300 MHz, CDCl₃): δ 1.73 (3H, *s*, H-4''), 1.78 (3H, *s*, H-5''), 2.53 (3H, *s*, H-2), 4.55 (2H, *d*, *J* = 6.7 Hz, H-1''), 5.45 (1H, *tm*, *J* = 8.2 Hz, H-2''), 6.91 (2H, *d*, *J* = 6.8 Hz, H-3',H-5'), 7.91 (2H, *d*, *J* = 6.8 Hz, H-2',H-6'). ¹³C NMR (300 MHz, CDCl₃): δ 18.20 (*q*, C-4''), 25.76 (*q*, C-5''), 26.25 (*q*, C-2), 65.02 (*t*, C-1''), 114.33 (*d*, C-3',C-5'), 119.0 (*d*, C-2''), 130.33 (*s*, C-1'), 130.52 (*d*, C-2',C-6'), 138.79 (*s*, C-3''), 162.83 (*s*, C-4'), 196.68 (*s*, C-1). EIMS (probe) 70 eV, *m/z* (rel. int): 204 [M]⁺ (7), 136

[M-C₅H₈]⁺ (68), 121 [M-C₅H₈-Me]⁺ (94), 69 [C₅H₉]⁺ (100).

3.6. Synthesis of *n*-butyl nitrite

To a soln of NaNO₂ (47.6 g, 0.69 mol) in H₂O (187 ml, 10 mol) at 0° an ice-cold mixture of H₂O (12.5 ml, 0.69 mol), conc. H₂SO₄ (17 ml, 0.32 mol) and *n*-BuOH (57 ml, 0.63 mol) was added. The reaction mixt. was stirred for 1 hr and then filtered. The organic phase was washed with a soln containing 2% NaHCO₃ and 25% NaCl (3×20 ml) and dried (MgSO₄). Distillation at atmospheric pressure (bp 75–77°) afforded the product in a yield of 80%. ¹H NMR (300 MHz, CDCl₃): δ 0.93 (3H, *t*, *J* = 7.2 Hz, H-4), 1.39 (2H, *tq*, *J* = 7.3 and 8.2 Hz, H-3), 1.69 (2H, *tt*, *J* = 8.2 and 6.5 Hz, H-2), 4.66 (2H, *t*, *J* = 6.5 Hz, H-1).

3.7. Synthesis of 4-(3-methyl-2-butenoxy)isonitrosoacetophenone (1)

Dry HCl gas was bubbled through a soln of **2** (0.5 g, 2.45 mmol) in 20 ml dry Et₂O at a rate of 2–3 bubbles per sec for 1 hr. Freshly prepared *n*-butyl nitrite (0.573 ml, 4.9 mmol) was then added slowly. The soln changed colour from orange-brown to light yellow. The soln was refluxed for 2 hr until it assumed a clear, pale yellow colour, whereupon it was acidified with ice-cold 0.5 M HCl to pH 4–5. Solvent was then removed *in vacuo* and CC (hexane-EtOAc, 4:1) afforded **1** as light yellow crystals in a yield of 15% (86 mg). ¹H NMR (300 MHz, CDCl₃): δ 1.76 and 1.77 (2×3H, 2×*d*, *J* = 1 Hz, H-4'', H-5''), 4.66 (2H, *d*, *J* = 6.6 Hz, H-1''), 5.47 (1H, *m*, H-2''), 7.02 (2H, *d*, *J* = 7.0 Hz, H-3',H-5'), 7.90 (1H, *s*, H-2), 8.08 (2H, *d*, *J* = 7.0 Hz, H-2',H-6'), 11.5 (1H, *br s*, OH). ¹³C NMR (300 MHz, CDCl₃): δ 18.1 (*q*, C-4''), 25.7 (*q*, C-5''), 65.1 (*t*, C-1''), 114.5 (*d*, C-3',C-5'), 118.7 (*d*, C-2''), 129.1 (C-1'), 132.27 (C-2',C-6'), 139.12 (*s*, C-3''), 149.05 (*s*, C-2), 163.45 (*s*, C-4'), 187.5 (*s*, C-1). EIMS (probe) 70 eV, *m/z* (rel. int): 233 [M]⁺ (4), 216 [M-OH]⁺ (68), 165 [M-C₅H₈]⁺ (30), 121 [M-C₅H₈-CHNOH]⁺ (42), 69 [C₅H₉]⁺ (100)

3.8. Antioxidative activity

Radical scavenging activity of **1** was monitored as a decrease in the emitted light (λ = 465 nm) at a fixed time interval of 2 min in a luminometer from a reaction mixt. consisting of 0.1 M sodium citrate buffer pH 5.8, 100 µl horseradish peroxidase (1.1 mg ml⁻¹), 100 µl H₂O₂ (8 nmol ml⁻¹), 100 µl

MCLA (0.3 mg ml^{-1}) and increasing amounts ($10\text{--}120 \mu\text{l}$ from a soln of 5 mg ml^{-1} in Me_2CO) of **1** in a total vol. of 1 ml. Incubations were carried out at 25° . All the necessary controls were included and all gave very low background CL.

References

- Apostol, I., Heinstein, P. F., & Low, P. S. (1989). *Plant Physiology*, 90, 109.
- Riov, J., Monselise, S. P., & Kahan, R. S. (1972). *Radiation Research Reviews*, 3, 417.
- Dubery, I. A. (1992). *Phytochemistry*, 31, 2659.
- Dubery, I. A., & Schabort, J. C. (1987). *South African Journal of Science*, 83, 440.
- Dubery, I. A., Holzapfel, C. W., Kruger, G. J., Schabort, J. C., & Van Dyk, M. (1988). *Phytochemistry*, 27, 2769.
- Hartman, G., & Nienhaus, F. (1974). *Phytopathologische Zeitschrift*, 81, 97.
- Osswald, W. F., Ziebold, S., Schutz, W., Firl, J., & Elstner, E. F. (1987). *Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz*, 94, 572.
- Kokubun, T., Harborne, J. B., & Eagles, J. (1994). *Phytochemistry*, 35, 331.
- Takasugi, M., & Masuda, T. (1996). *Phytochemistry*, 43, 1019.
- Curir, P., Marchesini, A., Daniel, B., & Mariani, F. (1996). *Phytochemistry*, 41, 447.
- Bohlmann, F., Wallmeyer, M., King, R. M., & Robinson, H. (1984). *Phytochemistry*, 23, 1513.
- Sigstad, E., Catalán, C. A. N., Díaz, J. G., & Herz, W. (1993). *Phytochemistry*, 33, 165.
- Rice-Evans, C. A., Miller, N. J., & Paganga, G. (1997). *Trends in Plant Science*, 2, 152.
- Homans, A. L., & Fuchs, A. (1970). *Journal of Chromatography*, 51, 327.
- Riov, J., Monselise, S. P., & Kahan, R. S. (1968). *Radiation Botany*, 8, 463.
- Riov, J., Monselise, S. P., & Kahan, R. S. (1970). *Radiation Botany*, 10, 281.
- Riov, J., & Goren, R. (1970). *Radiation Botany*, 10, 155.
- Kuc, J., & Rush, J. S. (1985). *Archives of Biochemistry and Biophysics*, 236, 455–472.
- Furniss, B. S., Hannaford, A. J., Smith, P. W. G. and Tatchell, A. R., *Vogel's textbook of Practical Organic Chemistry*. The Bath Press, Great Britain, 1989, pp. 984–989.
- Touster, O., in *Organic Reactions*, Vol. VII, eds. R. Adams, A.H. Blatt, A. C. Cope, F. C. McGraw, C. Niemann and H. R. Snyder. John Wiley and Son, Inc., USA, 1967, pp. 327–377.
- Arnoldi, A., Carughi, M., Farina, G., Merlini, L., & Parrino, M. G. (1989). *Journal of Agricultural and Food Chemistry*, 37, 508.
- Bors, W., Saran, M. and Elstner, E. F., in *Plant Toxin Analysis*, Vol. 13, eds. H. F. Linskens and J. F. Jackson Springer-Verlag, Berlin, Germany, 1992, pp. 277–295.
- Nakano, M. (1990). *Methods in Enzymology*, 186, 585.
- Suzuki, N., Suetsuna, K., Mashiko, S., Yoda, B., Nomoto, T., Toya, Y., Inaba, H., & Goto, T. (1991). *Agricultural and Biological Chemistry*, 55, 157.
- Ryan, O., Smyth, M. R., & Fágáin, C. O. (1994). *Essays in Biochemistry*, 28, 129.
- Takahashi, A., Totsune-Nakano, H., Nakano, M., Mashiko, S., Suzuki, N., Ohma, C., & Inaba, H. (1989). *FEBS Letters*, 246, 117.
- Thorpe, G. H. G., & Kricka, L. J. (1986). *Methods in Enzymology*, 133, 331.
- Krishna, M. C., & Samuni, A. (1994). *Methods in Enzymology*, 234, 580.
- Barron, D., & Ibrahim, R. K. (1996). *Phytochemistry*, 43, 921.
- Hamerski, D., Schmitt, D., & Matern, U. (1990). *Phytochemistry*, 29, 1135.
- Gross, G. G., in *Secondary Plant Products*, Vol. 7, eds. E. E. Conn. Academic Press, New York, 1981, pp. 301–316.
- Keefer, L. K. and Williams, D. L. H., in *Methods in Nitric Oxide Research*, eds. M. Feelisch and J. S. Stamler. John Wiley, Chichester, 1996, pp. 509–520.
- Cueto, M., Hernández-Perera, O., Martin, R., Bentura, M. L., Rodrigo, J., Lamas, S., & Golvano, M. P. (1996). *FEBS Letters*, 398, 159.
- Leshem, Y. Y., & Haramaty, E. (1996). *Plant Physiology*, 148, 258.
- Noritake, T., Kawakita, K., & Doke, N. (1996). *Plant and Cell Physiology*, 37, 113.
- Budavari, S., O'Neil, M. J., Smith, A., Heckelman, P. E. and Kenneary, J. F., *The Merck index*, Merck & Co., Inc., Whitehouse Station, New Jersey, USA, 1996.
- Halliwell, B., & Gutteridge, J. M. C. (1990). *Methods in Enzymology*, 186, 1.
- Lott, W. A., & Shaw, E. (1949). *Journal of the American Chemical Society*, 71, 70.
- Onishi, H. R., Pelak, B. A., Gerckens, L. S., Silver, L. L., Kahan, F. M., Chen, M.-H., Patchet, A. A., Galloway, S. M., Hyland, S. A., Anderson, M. S., & Raetz, C. R. H. (1996). *Science*, 274, 980.