0014-4754/94/020137-05$1.50 + 0.20 © Birkhäuser Verlag Basel, 1994

Identification of mucondialdehyde as a novel stress metabolite

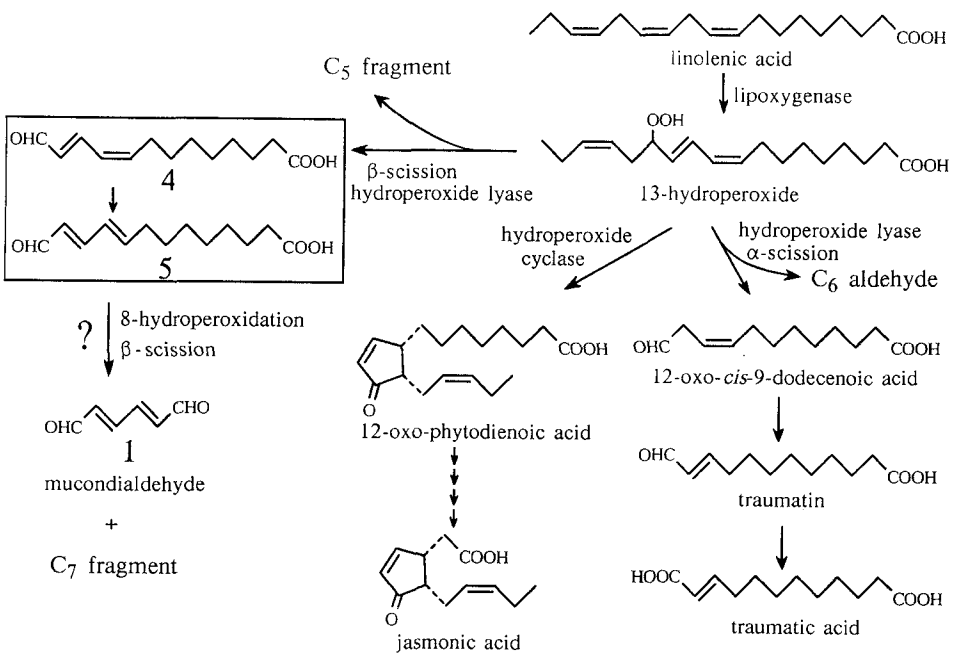
S. Tahara, S. Kasai, M. Inoue, J. Kawabata[a] and J. Mizutani

Department of Applied Bioscience and [a]Department of Bioscience and Chemistry, Faculty of Agriculture, Hokkaido University, Kita-ku, Sapporo 060 (Japan)
Received 4 October 1993; accepted 18 November 1993

Abstract. In a survey of antifungal stress compounds induced by cupric chloride we found that leaves of *Chenopodium album* exuded a highly fungitoxic metabolite mucondialdehyde (*trans*-2,*trans*-4-hexadienedial), which was associated with 13-oxo-9,11-tridecadienoic acids (*cis*-9,*trans*-11 and *trans*-9,*trans*-11 isomers) presumably resulting from β-scission of 13-hydroperoxy-octadecadi(tri)enoic acid. The biogenesis and role as a general defensive agent in plants are briefly discussed.
Key words. Chenopodium album; stress compound; fungitoxin; oxylipin; mucondialdehyde (*trans*-2,*trans*-4-hexadienedial); 13-oxo-9,11-tridecadienoic acids.

Antimicrobial oxygenated fatty acids or their degradation products (oxylipins)[1] are quite common in plants, whether healthy[2], pathogen infected[3] or wounded[4]. These physiologically active aliphatic compounds are produced by metabolism of unsaturated fatty acids, especially the lipoxygenase pathway for linoleic and linolenic acids[5,6]. The lipoxigenase pathway consists of two significant branches, 1) to regulatory substances such as jasmonic acid and its derivatives, and 2) to protective substances, for example, antifungal and antifeeding substances and wound-healing agents. The former is derived from linolenic acid by 13-lipoxygenase, hydroperoxide dehydrase and allene oxide cyclase followed by successive β-oxidation, and the latter is mediated by 13-lipoxygenase and hydroperoxide lyase which cleaves the bond between C-12 and C-13 (α-scission: scission of α-bond from the *trans*-11 double bond) to give C_6 aldehyde (hexanal from linoleic acid, and *cis*-3-hexenal from linolenic acid which is variously isomerized into *trans*-3 or *trans*-2-hexenal) and 12-oxo-trans-9-dodecenoic acid which is finally transformed into traumatin and traumatic acid which have a wound-healing effect[4,7].

In a study of defensive agents in wild plants, we have now found an unstable and potent antifungal substance which accumulates in the exudates from the leaves of *Chenopodium album* L. var. *album* (pigweed) treated with a cupric chloride solution. The fungitoxin, which is presumably related to unsaturated fatty acids, was identified as mucondialdehyde (*trans*-2,*trans*-4-hexa-



dienedial). Mucondialdehyde was found in association with weakly fungitoxic 13-oxo-9,11-tridecadienoic acids (*cis*-9,*trans*-11 and *trans*-9,*trans*-11 isomers), in addition to aromatic antifungal substances *p*-hydroxybenzaldehyde and cinnamic acid.

Results

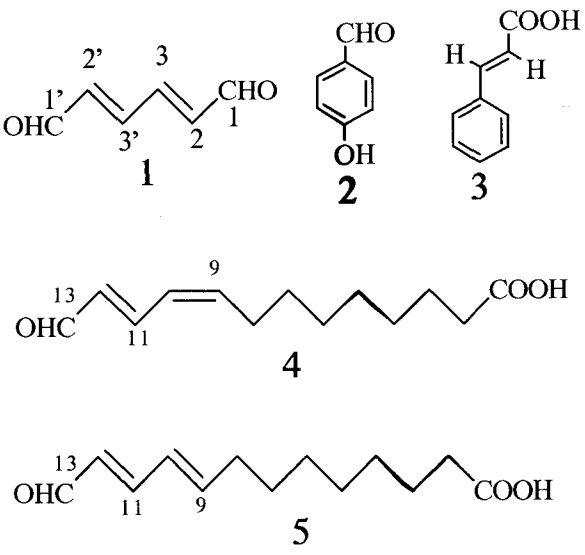
When the cupric chloride-induced antifungal acitivities of the leaf exudates were compared, the exudates from pigweed (*Chenopodium album* L. var *album*) a wild species of Chenopodiaceae, were far more potent than those from sugar beet (*Beta vulgaris* L. subsp. *vulgaris*) and from leaf beet (*B. vulgaris* L. subsp. *cicla*), both cultivated species of the same family. Sugar beets are prone to *Cercospora* leaf spots, whereas pigweeds are resistant to the pathogen. We supposed that the incompatibility between pigweed and *Cercospora beticola*, a causal pathogen of the beet leaf spot, is due to the chemical barrier of this wild plant. In fact, TLC bioautography[8] using pigweed exudates equivalent to 300 mg of fresh leaves and *Cladosporium herbarum* as the test fungus revealed the presence of some antifungal substances, which were not clearly detected in the exudates of sugar beet or leaf beet. The antifungal principles in the exudates released into the cupric chloride solution for 24 h were extracted with ethyl acetate and isolated by silica gel column, preparative thin-layer and high performance liquid chromatography. The most potent fungitoxin (compound **1**) in the neutral/phenolic fraction was shown to have a molecular formula of $C_6H_6O_2$ by high resolution mass spectroscopy, and to be a carbonyl compound because of the positive response to 2,4-dinitrophenylhydrazine (2,4-DNPH, reddish orange). The ^{1}H NMR spectrum showed two aldehydic protons at δ 9.11 as a doublet and four olefinic protons giving two multiplets (ddd). These results are indicative of a hexadienedial structure for compound **1**. Direct comparison of compound **1** with synthetic *trans*-2,*trans*-4-hexadienedial[9] (mucondialdehyde) by ^{1}H NMR spectroscopy at 500 MHz and mass spectroscopy revealed that the compounds were indeed identical.

Another fungitoxin (compound **2**) in the neutral fraction was identified as *p*-hydroxybenzaldehyde by comparison with the commercially available authentic material.

The formula for both compounds **4** and **5** in the acidic fraction from the pigweed exudate extracts was shown to be $C_{13}H_{20}O_3$ (EI-HR-MS) and their ^{1}H NMR spectra were very similar. Therefore, they were suggested to be geometrical isomers of each other. The ^{1}H NMR spectra of **4** revealed the presence of an α, β; γ, δ-unsaturated aldehydic terminal affording a coupling sequence from the aldehydic proton (Hal, d, $J = 8.0$ Hz at δ 9.61) to Hδ coupled with the allyl methylene protons ($CH_2 - 8$, $J = 7.8$ Hz) via Hα, Hβ and Hγ i.e., J(Hal-Hα) = 8.0 Hz, J(Hα-Hβ) = 15.2 Hz (*trans*), J(Hβ-Hγ) = 11.6 *Hz* and J(Hγ-Hδ) = 10.8 Hz (*cis*). Together with this terminal structure, the ^{13}C NMR (DEPT) detection of seven methylene groups and a carboxyl group suggested the whole structure 13-oxo-*cis*-9,*trans*-11-tridecadienoic acid for compound **4**. The configuration of compound **5** was determined by the comparison of spectroscopic data with those of **4**. Although the coupling constant of H-9 with H-10 could not be read, that of H-11 with H-12 (15.3 Hz) was evidently indicative of *trans*-11 geometry. Therefore, the conjugation of *cis*-9,*cis*-11, *cis*-9,*trans*-11 (=compound **4**), and *trans*-9,*cis*-11 were easily ruled out to give the remaining structure 13-oxo-*trans*-9,*trans*-11-tridecadienoic acid to compound **5**. The ^{13}C NMR data for the olefinic carbons of **5**, δ_c in acetone-d_6: 147.6 (C-9), 129.8 (C-10). 153.5 (C-11) and 131 (C-12) were also compatible with the estimated geometry. Artificial isomerization of **4** and **5** under ambient conditions has been reported[10].

Another antifungal acid compound (**3**) was identified to be cinnamic acid.

As shown in the table, mucondialdehyde (**1**) exhibited a potent growth inhibitory activity against *Cercospora beticola*, a pathogen of *Beta vulgaris* (Chenopodiaceae). Although other compounds (**2**–**5**) have been detected by TLC bioautography using *C. herbarum* as the test fungus, none of them inhibited the growth of *C. beticola* at the dose level of 50 µg/disk. When treated with a biotic elicitor prepared from mycerial walls of *C. herbarum*[11], **1** was also induced as with the abiotic elicitor cupric chloride. The latter elicitor revealed to induce both **1** and *p*-hydroxybenzaldehyde in the cultured cells of *C. album*[12]. Judging from the inducibility and the antifungal activity of these isolates, it was supposed that the key compound in the chemical defence of *C. album* is **1** and other compounds (**2**–**5**) might play minor roles.



1 2 3

4

5

Antifungal activity of mucondialdehyde (**1**) against *Cercospora beticoal*[a]

Compound	Inhibitory zone (mm)[b]			
Dosage μg/disk	1	2	5	10
Mucondialdehyde	±	14	15	19
PCP[c]	±	±	13	15

[a]Strain KSM3-1 grown on PSA medium for 48 h.
[b]Width from the edge of the paper disk to that of the inhibitory zone; duplicated results were averaged; ±, inhibitory zone less than 2 mm. Compounds **2**–**5** were inactive at the level of 50 μg/disk.
[c]Pentachlorophenol as the standard fungicide.

Discussion

Interested in the wild plants equipped with an efficient chemical defence mechanism, we found mucondialdehyde (**1**), two 13-oxo-tridecadienoic acid isomers (**4** and **5**), *p*-hydroxybenzaldehyde (**2**) and cinnamic acid (**3**) in the exudates from *C. album* soaked in a cupric chloride solution. Compounds **2** and **3**, related to the shikimic acid pathway, are well known as stress compounds in higher plants[13,14].

13-Oxotridecadienoic acid (*cis*-9,*trans*-11 isomer, **4**) has been known as a degradation product of linoleic or linolenic acid catalyzed by lipoxygenase under oxygen deficient conditions[15–17], or lipoxygenase and hydroperoxide lyase in plants under aerobic conditions[10,18–20], whilst mucondialdehyde (**1**) has no precedent as a natural product. As shown in the scheme, 13-oxotridecadienoic acid is produced from 13-hydroperoxyoctadecadi (tri)enoic acid by β-scission affording C_5 and C_{13} products. Both α-and β-scissions of the 13-hydroperoxide seem to occur in the homogenates of higher plants for example, corn[19] and pea[20]. β-Scission products or β-scission of hydroperoxides are found both in animals[21,22] and plants[10,18–20,23]. At present we have no evidence for the direct precursor of **1**. However, we suppose that the putative precursor would be derived not from the path from C_{18} to C_6 and C_{12} which is known as a defence system responding rapidly to physical and biological injury[4] or as signal production for elicitation of phytoalexins[24], but from the C_5 and C_{13} pathway. It is possible that hydroperoxidation of 13-oxo-9,11-tridecadienoic acid (**4** or **5**) at the allylic methylene (C-8), followed by β-scission of the resulting 8-hydroperoxide could yield mucondialdehyde (**1**). Since 13-oxotridecadienoic acid formation by the combination of constituent 13-lipoxygenase and hydroperoxide lyase cleaving the β-bond from the *trans* double bond has been reported in plant homogenates[19,20], the reaction from C_{13} to C_6 and C_7 must be mediated by an enzyme(s) induced under the biotically or abiotically stressed condition.

It is not know yet if the path of mucondialdehyde formation is a shunt of the lipoxygenase pathway or not. However, this compound seems to be produced in the physiologically stressed leaves of many plants, for example Araceae, Cercidiphyllaceae, Chenopodiaceae, Compositae, Leguminosae, Ranunculaceae, Saururaceae, Ulmaceae and Umbelliferae of the species so far examined[25]. Therefore, mucondialdehyde (**1**) seems to be a promising candidate for one of general defensive agents in higher plants. The reason why **1**, which has a simple structure and such a remarkable biological activity, has not previously been reported as a stress metabolite might be due to its physiological and chemical instability. When **1** is biosynthetically correlated to the metabolism of unsaturated fatty acid(s), it may be called one of natural oxylipins[1].

Materials and methods

Antifungal test. TLC bioautography[8] was employed to detect antifungal constituents, using *Cladosporium herbarum* as the test fungus. Crude extracts of the exudates and remaining plants obtained after treatment with a cupric chloride solution (3 mM) or water for 24 h were dissolved in EtOAc (the crude extracts from 15 g of plant materials per 1 ml). The EtOAc solution (50 μl) of EtOAc solution was applied to a thin-layer plate and developed in $CHCl_3$-MeOH (25:1) followed by spraying the spore suspension. The antifungal activities of **1**–**5** against *C. beticola* were evaluated by the paper disk method using 8 mm i.d. disk (Toyo Roshi Kaisya, Ltd.), which were charged with test compounds as 25 μl of acetone solution and placed on the potato sucrose agar plates (sucrose, 2%; agar, 0.8%; $NaHPO_4 \cdot 12H_2O$, 0.2%; and $Ca(NO_3)_2 \cdot 4H_2O$, 0.05%) containing mycelia of *C. beticola*. Pentachlorophenol (PCP) was used as the standard fungitoxin.

Plant materials and induction of stress metabolites. The aerial parts of *C. album* (9.2 kg, fresh wt.) were collected in the campus of Hokkaido University in August. They were patted with a nylon brush to produce slight mechanical injuries before soaking in 1 litre of 3 mM $CuCl_2$ solution for 24 h (each 250 g of plant materials in a plastic vat, W 40 × L 70 × H 20 cm). The exudates in the solution were extracted with EtOAc. The plant materials after being stressed were also extracted with 50% aq. acetone for 30 min (ca. 4 l/kg) and the extracts were concentrated. The constituents in the concentrate were partitioned between EtOAc and water. Each EtOAc extract was washed with 5% aq. $NaHCO_3$ to separate the acidic constituents. The neutral/phenolic and acidic fractions dissolved in EtOAc were subjected to TLC bioautography. Growth inhibitory zones were clearly detected in both plates containing neutral/phenolic and acidic fractions obtained from the exudates rather than from the residual plant materials, and furthermore only from plants treated with an elicitor-containing solution ($CuCl_2$, 3 mM; H_2O_2, 0.5%; or *C. herbarum* cell wall material, 1 mg/ml).

Isolation of antifungal compounds

Neutral/phenolic compounds: The neutral/phenolic crude extract (ca. 2 g) was subjected to silica gel column chromatography. After washing the column with 600 ml of benzene, antifungal substances (major 2) were eluted with benzene-EtOAc = 19:1 into 4 fractions (150 ml each). The first two fractions containing antifungal and 2,4-DNPH-positive constituents were combined and rechromatographed over silica gel. The first antifungal compound **2** (*Rf* 0.38 in $CHCl_3$-MeOH = 25:1) was eluted with hexane-EtOAc = 17:3 and purified by preparative TLC in $CHCl_3$-MeOH = 25:1, 4 mg yield). Successive elution of the column with hexane-EtOAc = 4:1 yielded the second antifungal compound **1** which was further purified by preparative TLC (*Rf* 0.68 in $CHCl_3$-MeOH = 25:1, 2 mg yield).

Acidic constituents: The acidic fraction was subjected to Sephadex LH-20 column chromatography and constituents were eluted with $CHCl_3$-MeOH = 1:1 to give 12 fractions (17 ml each) after washing the column with 270 ml of the same solvent. The 4th and 5th fractions gave an antifungal spot on silica gel thin-layer plates (*Rf* 0.73 in ether-MeOH = 50:1). Two isomeric components (**4** and **5**) corresponding to this spot were eventually isolated by preparative HPLC using an Inertsil ODS column (250 × 20 mm i.d.) and a mixture of THF-H_2O = 3:2 as eluting solvent (flow rate 8 ml/min and recycled three times), and eluates were monitored by a UV detector at 254 nm. Yields of slowly moving **4** and fast moving **5** were 6 and 5 mg, respectively. The 9th and 10th fractions were subjected to preparative TLC successively in diethyl ether-MeOH (50:1, *Rf* ca. 0.7) and in $CHCl_3$-MeOH (9:1, *Rf* 0.35) to give pure **3** (ca. 1 mg).

Physicochemical properties of Chenopodium fungitoxins. The isolated compounds **2** and **3** were confirmed to be chromatographically and spectroscopically identical to commercially available *p*-hydroxybenzaldehyde and cinnamic acid respectively.

Mucondialdehyde (**1**). Yellow gum. EI-HR-MS: *m/z* 110.0355 ($C_6H_6O_2$, calcd. 110.0368). EI-MS *m/z* (%): 110 (M^+, 91), 82 (20), 81 (M^+-CHO, 97), 54 (19), 53 (100), 52 (14), 51 (30), 50 (22), 39 (14). UV λ max, MeOH nm (log ε): 270 (4.56). ^{1}H NMR δ_{TMS} (benzene-d_6, 500 MHz, *J* = Hz): 5.75 (2H, ddd, *J* = 15.3, 7.8, 0.6, H-2 and H-2′), 5.93 (2H, ddd, *J* = 15.3, 10.7, 0.6, H-3 and H-3′), 9.11 (2H, d, *J* = 7.8, H-1 and H-1′). ^{13}C NMR δ_{TMS} (benzene-d_6, 125 MHz): 137.4 (C-2 and C-2′), 145.7 (C-3 and C-3′), 191.6 (C-1 and C-1′).

13-Oxo-cis-9,trans-11-tridecadienoic acid (**4**). Colourless gum. FD-MS: *m/z* 224 (100%). EI-HR-MS: *m/z* 224.1397 ($C_{13}H_{20}O_3$, calcd. 223.1412). EI-MS *m/z* (%): 224 (M^+, 4), 95 (30), 95 (30), 83 (42), 81 (100), 67 (40), 55 (43), 41 (65). ^{1}H NMR δ_{TMS} (500 MHz, in $CDCl_3$, *J* = Hz): 1.35 (6H, br s, H_2-4, H_2-5 and H_2-6), 1.45 (2H, br t-like, *J* = ca. 7), 1.63 (2H, for t-like, J = ca. 7, H_2-3), 2.35 (2H, q, *j* = 7.8, H_2-8), 2.35 (2H, t, *J* = 7.4, H_2-2), 6.00 (1H, dt, *J* = 10.8, 7.8, H-9), 6.15 (1H, dd, *J* = 15.2, 8.0, H-12), 6.27 (1H, t-like, *J* = 11.6, 10.8, H-10), 7.44 (1H, dd, *J* = 15.2, 11.6, H-11), 9.61 (1H, d, *J* = 8.0, H-13). ^{13}C NMR δ_{TMS} (125 MHz, in $CDCl_3$): 24.6 (C-3), 28.4 (C-2), 28.9 (C-6), 29.0 (C-5), 29.0 (C-4), 29.1 (C-7), 33.7 (C-8), 126.8 (C-10), 131.8 (C-12), 143.9 (C-9), 146.8 (C-11), 178.1 (C-1), 194.1 (C-13).

13 - Oxo - trans - 9,trans - 11 - tridecadienoic acid (**5**). Colourless gum. FD-MS: *m/z* 224 (100%). EI-HR-MS: *m/z* 224.1410 ($C_{13}H_{20}O_3$, calcd. 224.1412). EI-MS *m/z* (%): 224 (M^+, 6), 95 (28), 83 (43), 81 (100), 67 (35), 55 (45), 41 (62). ^{1}H NMR δ_{TMS} (500 MHz, in $CDCl_3$, *J* = Hz): 1.33 (6H, br s, H_2-4, H_2-5 and H_2-6), 1.46 (2H, br t-like, *J* = ca. 7, H_2-7), 1.64 (2H, m, H_2-3), 2.22 (2H, q, *J* = 7.0, H_2-8), 2.36 (2H, t, *J* = 7.5, H_2-2), 6.08 (1H, dd, *J* = 15.3, 8.1, H-12), 6.29 (2H, m, H-9 and H-10), 7.08 (1H, dd, *J* = 15.3, 9.9, H-11), 9.57 (1H, d, *J* = 8.1, H-13). ^{13}C NMR δ_{TMS} (125 MHz, in acetone-d_6): 129.8 (C-10), 131.0 (C-12), 147.6 (C-9), 153.5 (C-11), 194.1 (C-13).

Acknowledgments. We should like to express our thanks to Dr. K. Saito and Dr. M. Kozaki, Nagoya Institute of Technology for the gift of synthetic mucondialdehyde, and to Dr. T. Shimanuki and Dr. Y. Homma, Hokkaido National Agricultural Experiment Station for kind supply of *Cercospora beticola*. Financial support (to S. Tahara) from the Hokkaido Agriculture Frontier Movement (President: Dr. H. Okajima) is gratefully acknowledged.

1 Gerwick, W. H., Moghaddam, M. F., and Hamberg, M., Archs Biochem. Biophys. *290* (1991) 436: Oxylipin is introduced as an encompassing term for oxygenated compounds which are formed from fatty acids by reaction(s) involving at least one step of mono or dioxygenase-catalyzed oxygenation. As such, this term includes the eicosanoids as well as biosynthetically related compounds of longer and shorter chain length.
2 Kato, T., Yamaguchi, Y., Namai, T., and Hirukawa, T., Biosci. Biotech. Biochem. *57* (1993) 283.
3 Vernenghi, A., and Ravisé, A., Can. J. Bot. *64* (1986) 973.
4 Croft, K. P. C., Jüttner, F., and Slusarenko, A. J., Pl. Physiol. *101* (1993) 13.
5 Vick, B. A., and Zimmerman, D. C., in: The Biochemistry of Plants Vol. 9, p. 53. P. K. Stumpf and E. E. Conn. Academic Press, Orlando 1987.
6 Siedow, J. N., A. Rev. Pl. Physiol. Pl. Molec. Biol. *42* (1991) 145.
7 Hatanaka, A., Kajiwara, T., and Sekiya, J., Chem. Phys. Lipids *44* (1987) 341.
8 Homans, A. L., and Fuchs, A., J. Chromat. *51* (1970) 327.
9 Saito, K., Kozaki, M., and Takahashi, K., Heterocycles *31* (1990) 1491.
10 Vick, B. A., and Zimmerman, D. C., Pl. Physiol. *90* (1989) 125.
11 Ayers, A. R., Ebel, J., Valent, B. S., and Albersheim, P., Pl. Physiol. *57* (1976) 760.
12 Murashige, T., and Skoog, F., Pl. Physiol. *15* (1962) 473. The suspension culture of *C. albus* was raised from a callus initially dedifferentiated from the seedlings, and grown at 27 °C on a shaker in 500-ml shaking flasks containing 250 ml of MS medium; (sucrose, 3%; kinetin, 0.1 ppm; and 2,4-D, 2 ppm). The cells cultured for 2 weeks were stressed by the addition of $CuCl_2$ (1.5 mM) and another two days incubation, at which point the filtered medium was extracted to give stress metabolites.
13 Bonner, J., and Galston, A. W., Bot. Gaz. *106* (1944) 185.

14 Abdul-Wahab, A. S., and Rice, E. L., Bull. Torrey Bot. Club *94* (1967) 486.
15 Garssen, G. J., Vliegenthart, J. F. G., and Boldingh, J., Biochem. J. *122* (1971) 327.
16 Chamulitrat, W., and Mason, R. P., Archs Biochem. Biophys. *282* (1990) 65.
17 Salzmann, U., Künn, H., Schewe, T., and Rapoport, S. M., Biochim. biophys. Acta *795* (1984) 535.
18 Andrianarison, R.-H., Beneytout, J.-L., and Tixier, M., Pl. Physiol. *91* (1989) 1280.
19 Hamberg, M., Adv. Prostaglandin, Thromboxane, Leucotriene Res. *21A* (1990) 117.
20 Gafarova, T., Grechkin, A., and Tarchevskii, I., in: Proc. 9th Int. Symp. Plant Lipids, p. 301. Eds P. J. Quinn, and J. L. Harwood. Portland Press, London 1990.
21 Lam, B. K., Lin, Y.-L., Ho, H.-Y., and Wong, P. Y.-K., Biochem. biophys. Res. Commun. *149* (1987) 1111.
22 Vaz, A. D. N., Roberts, E. S., and Coon, M. J., Proc. natl Acad. Sci. USA *87* (1990) 5499.
23 Bernart, M. W., Whatley, G. G., and Gerwick, W. H., J. Nat. Prod. *56* (1993) 245.
24 Zeringue, H. J. Jr, Phytochemistry *31* (1992) 2305.
25 The presence of mucondialdehyde was confirmed in the exudates from many plants stressed with a cupric chloride solution by TLC, TLC bioautography and/or GC-MS in comparison with an authentic compound. A further survey of stress compounds revealed that 6-hydroxy-*trans*-2,*trans*-4-hexadienal was produced by *Hypochoeris radicata* L. (Compositae) in addition to mucondialdehyde. The results (Y. Maruta et al.) will be published elsewhere.