9 Gutierrez, G., The rate of oxygen release and its effect on capillary O_2 tension: a mathematical analysis. Respir. Physiol. *63* (1986) 79–96.
10 Haab, P. E., Hogan, M. C., Bedout, D. E., Gray, A., Wagner, P. D., and West, J. B., Limitation of O_2 uptake in working muscle due to the presence of carbon monoxide in blood. Faseb J. *2* (1988) A760.
11 Haldane, J. B. S., The dissociation of oxyhemoglobin in human blood during partial CO-poisoning. J. Physiol., Lond. *45* (1912) XXII.
12 Hirsch, G. L., Sue, D. Y., Wasserman, K., Robinson, T. E., and Hansen, J. E., Immediate effects of cigarette smoking on cardio-respiratory responses to exercise. J. appl. Physiol. *58* (1985) 1975–1981.
13 Hlastala, M. P., McKenna, H. P., Franada, R. L., and Detter, J. C., Influence of carbon monoxide on hemoglobin-oxygen binding. J. appl. Physiol. *41* (1976) 893–899.
14 Hogan, M. C., Roca, J., Wagner, P. D., and West, J. B., Limitation of maximal O_2 uptake and performance by acute hypoxia in dog muscle in situ. J. appl. Physiol. *65* (1988) 815–821.
15 Hogan, M. C., Roca, J., Wagner, P. D., and West, J. B., Dissociation of maximal O_2 uptake from O_2 delivery in canine gastrocnemius in situ. J. appl. Physiol. *66* (1989) 1219–1226.
16 Holland, R. A. B., Shibata, H., Scheid, P., and Piiper, J., Kinetics of O_2 uptake and release by red cells in stopped-flow apparatus: effects of unstirred layer. Respir. Physiol. *59* (1985) 71–95.
17 Honig, C. R., Frierson, J. L., and Gayeski, T. E. J., Anatomical determinants of O_2 flux density at coronory capillaries. Am. J. Physiol. *256* (1989) H375–H382.
18 Honig, C. R., Gayeski, E. J., Federspiel, W., Clark, A. Jr, and Clark, P., Muscle O_2 gradients from hemoglobin to cytochrome: new concepts, new complexities. Adv. exp. Med. Biol. *169* (1984) 23–38.
19 Joels, N., and Pugh, L. G. C. E., The carbon monoxide dissociation curve of human blood. J. Physiol., Lond. *142* (1958) 63–77.
20 King, C. E., Dodd, S. L., and Cain, S. M., O_2 delivery to contracting muscle during hypoxic or CO hypoxia. J. appl. Physiol. *63* (1987) 726–732.
21 Moray, R., A relation of persons killed with subterraneous damps. Phil. Trans. R. Soc., Lond. *1* (1665) 44–45.
22 Nag, A. C., Chen, K. C., and Cheng, M., Effects of carbon monoxide on cardiac muscle cells in culture. Am. J. Physiol. *255* (1988) C291–C296.
23 Okada, Y., Tyuma, I., Ueda, Y., and Sugimoto, T., Effect of carbon monoxide on equilibrium between oxygen and hemoglobin. Am. J. Physiol. *230* (1976) 471–475.
24 Roca, J., Hogan, M. C., Story, D., Bebout, D. E., Haab, P., Gonzalez, R., Ueno, O., and Wagner, P. D., Evidence for tissue diffusion limitation of $\dot{V}_{O_{2max}}$ in normal humans. J. appl. Physiol. *67* (1989) 291–299.
25 Rose, C. P., and Goresky, C. A., Limitations of tracer oxygen uptake in the canine coronary circulation. Circ. Res. *56* (1985) 57–70.
26 Roughton, F. J. W., and Forster, R. E., Relative importance of diffusion and chemical reaction rates in determining rate of exchange of gases in the human lung, with special reference to true diffusing capacity of pulmonary membrane and volume of blood in the lung capillaries. J. appl. Physiol. *11* (1957) 290–302.
27 Roughton, F. J. W., Forster, R. E., and Candor, L., Rate at which carbon monoxide replaces oxygen from combination with human hemoglobin in solution and in the red cell. J. appl. Physiol. *11* (1957) 269–276.
28 Savoy, J., Michoud, M.-C., Robert, M., Geiser, J., Haab, P., and Piiper, J., Comparison of steady state pulmonary diffusing capacity estimates for O_2 and CO in dogs. Respir. Physiol. *42* (1980) 43–59.
29 Stainsby, W. N., and Otis, A. B., Blood flow, blood oxygen tension, oxygen uptake, and oxygen transport in skeletal muscle. Am. J. Physiol. *206* (1964) 858–866.
30 Wittenberg, B. A., and Wittenberg, J. B., Transport of oxygen in muscle. A. Rev. Physiol. *51* (1989) 857–878.
31 Wittenberg, B. A., and Wittenberg, J. B., Myoglobin-mediated oxygen delivery to mitochondria of isolated cardiac myocytes. Proc. natl Acad. Sci. USA *84* (1987) 7503–7507.
32 Zwart, A., Kwant, G., Oeseburg, B., and Zijlstra, W. G., Human whole-blood oxygen affinity: Effect of carbon monoxide. J. appl. Physiol. (Respir. envir. Exercise Physiol.) *57* (1984) 14.

0014-4754/90/11-12/1202-05$1.50 + 0.20/0

Research Articles

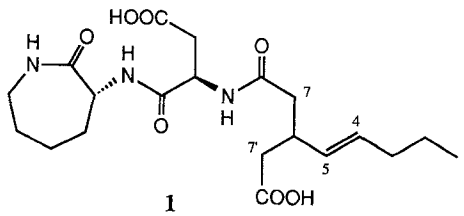
The structure of circinatin, a non-toxic metabolite from the plant pathogenic fungus *Periconia circinata*

V. Macko[a,1], M. B. Stimmel[a], H. Peeters[a], T. J. Wolpert[a,2], L. D. Dunkle[b], W. Acklin[c], R. Bänteli[c], B. Jaun[c] and D. Arigoni[c,1]

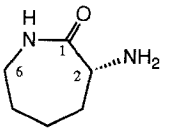
[a]*Boyce Thompson Institute, Cornell University, Ithaca (New York 14853, USA),* [b]*USDA-ARS, Department of Botany and Plant Pathology, Purdue University, W. Lafayette (Indiana 47907, USA), and* [c]*Laboratorium für Organische Chemie, ETH-Zentrum, CH-8092 Zürich (Switzerland)*
Received 29 June 1990; accepted 1 August 1990

Summary. A new non-toxic metabolite, circinatin, has been isolated from culture filtrates of the fungus *Periconia circinata* grown under modified conditions which suppress the normal production of host-specific toxins. The structure of the new compound has been established as in **1** by combination of instrumental analysis and chemical degradation.
Key words. Milo disease of sorghum; *Periconia circinata*; circinatin; D-cyclolysine; D-aspartic acid; 3-(E-pent-1'-enyl)-glutaric acid.

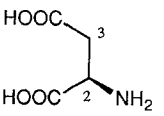
The fungus *Periconia circinata* (Mangin) Sacc. produces host-specific toxins that are important pathogenicity factors causing disease symptoms on cultivars of sorghum susceptible to the fungus[3]. Abundant toxin production is observed when the pathogenic isolate is grown in 1-liter Roux bottles containing 200-ml or in 400-ml prescription bottles containing 100 ml of modified Fries' medium supplemented with 0.1% yeast extract[4,5]. From such cultures Wolpert and Dunkle[5] purified two PC-toxins which were characterized as peptides (MW < 2000) resistant to proteases and having aspartic acid as one of their constituents. We have now observed that when the fun-



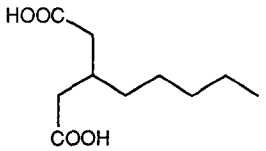
1



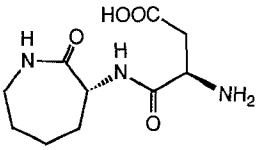
2



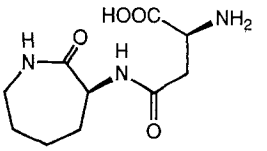
3



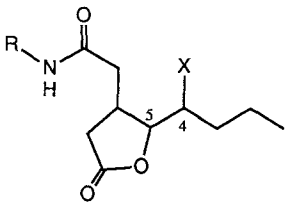
4



6

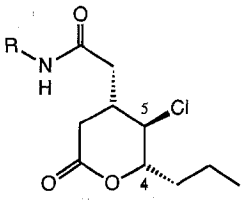


7

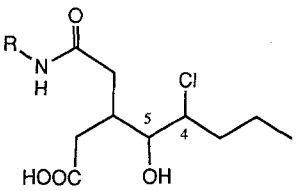


5, X = Br

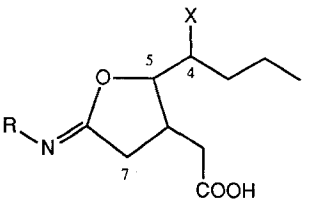
8, X = Cl



9



10



11

gus is grown in 500-ml Erlenmeyer flasks containing 100 ml of medium toxin production is suppressed, while a non-toxic metabolite is produced in relatively large quantities. The availability of substantial quantities of the new compound, which we have named circinatin, and its suspected biogenetic relationship to the PC-toxins made it interesting to tackle its structure in the hope that this might serve as a good prelude for later studies of the less abundant PC-toxins. In this paper we provide evidence which establishes **1** as the structure of circinatin. Circinatin, [white crystals from H_2O, soluble in methanol and DMSO, m.p. 165 °C, $[\alpha]_D = +29°$ (c = 0.9, H_2O)] has the elemental composition $C_{20}H_{31}O_7N_3$, as indicated by its FAB mass spectrum ($[M + H^+] = 426$ in thioglyc matrix) and evaluation of the ^1H- and ^{13}C-NMR spectra (cf. tables 1 and 2). The compound fails to display a ninhydrin reaction, titrates as a dibasic acid ($pK^*_{MCS} = 6.68$[6], equivalent weight 212.0), and can be converted by treatment with diazomethane into a dimethylester. Hydrolysis of **1** with 6 N HCl under forced conditions (110 °C/16 h) provided a complex mixture of products, two of which could be identified as cyclic D-lysine (**2**) and partially racemized D-aspartic acid (**3**) by comparison with authentic samples[7]. Hydrogenation of **1** in aqueous solution in the presence of Adams catalyst yielded a saturated dihydroderivative, hydrolysis of which gave, in addition to **2** and **3**, a C_{10}-component, identified as β-pentylglutaric acid (**4**) by comparison with an authentic specimen[8]. The presence of a trans disubstituted double bond in the corresponding C_{10}-moiety of circinatin is clearly revealed by the NMR data (cf. tables

Table 1. 400 MHz ^1H-NMR data[a] of circinatin (**1**)

Subunit	Position	in D_2O	in DMSO-d_6
C_{10}-diacid	C-1	0.84 (3H, t; 7.3)	0.82 (3H, t; 7.3)
	2	1.34 (2H, sext; 7.3)	1.28 (2H, sext; 7.3)
	3	1.96 (2H, q; 7.2)	1.88 (2H, q; 6.9)
	4	5.57 (dt; 15.3, 7.2)	5.40 (dt; 15.5, 7)
	5	5.33 (ddt; 15.3, 8.3, 1.3)	5.31 (dd; 15.5, 6.5)
	6	2.85 (m)	2.82 (sext, 7)
	7	2.51 (dd; 14.4, 5.5)	2.18 (2H, m)
		2.38 (dd; 14.4, 9.2)	
	7'	2.46 (dd; 13.7, 5.5)	2.34 (dd; 15.2, 5.3)
		2.33 (dd; 13.7, 9.6)	2.14 (dd; 15.2, 8.7)
asp	C-2	4.76 (dd; 7.7, 5.5)	4.56 (ddd; 8.0, 7.7, 6.0)
	3	2.91 (dd; 16.7, 5.5)	2.71 (dd; 16.5, 6.0)
		2.78 (dd; 16.7, 7.8)	2.40 (dd; 16.5, 7.7)
	N-2		8.25 (d; 8.0)
cyclolys	C-2	4.58 (dd; 11.2, 1.6)	4.31 (dd; 10.8, 6.4)
	3a	1.63 (dddd; 13.6, 12.0, 2, 3)	1.28 (q; 13)
	3e	1.86 (m)	1.85–1.70 (m)
	4a	1.76 (ddddd; 13.6, 12.0, 12.0, 3, 3)	1.62 (q; 13)
	4e	2.01 (ddd; 13.6, 4, 3)	1.85–1.70 (m)
	5a	1.40 (ddddd; 13, 12, 12, 4, 4)	1.17 (q, 13)
	5e	1.86 (m)	1.85–1.70 (m)
	6a		3.16 (ddd; 15, 11, 5)
		3.30 (2H, m)	
	6e		3.04 (m)
	N-2		7.65 (d; 6.4)
	N-6		7.85 (dd; 6, 5)

[a] δ-values relative to external DSS = 0 ppm; in parenthesis: number of hydrogens (if more than one), multiplicities, and J-values rounded to the next significant number. Connectivities were verified by COSY-2D measurements.

Table 2. 100 MHz ^{13}C-NMR data[a] of circinatin (**1**)

Subunit	Atom No.	in D_2O[b]	in DMSO-d_6[c]
C_{10}-diacid	1	15.4 q	13.2 q
	2	24.4 t	21.8 t
	3	36.5 t	33.8 t
	4	136.0 d	129.5 d
	5	132.3 d	131.9 d
	6	39.6 d	35.2 d
	7	43.5 t	40.4 t
	7'	42.3–42.8 t[e]	38.8 t
	8	177.2 s	170.7 s
	8'	179.4–179.7 s[e]	173.0 s
asp	1	173.9 s	169.3 s
	2	52.7 d	49.2 d
	3	38.2–39.6 t[e]	35.7 t
	4	176.7–177.0 s[e]	171.7 s
cyclolys	1	179.4 s	173.7 s
	2	55.0 d	51.4 d
	3	32.5 t	30.8 t
	4	30.0 t[d]	27.5 t
	5	30.2 t[d]	28.7 t
	6	39.6 t	35.2 t

[a] δ-values relative to external TSP = 0 ppm; multipicities derived from DEPT spectrum. [b] Assignments corroborated by the results of incorporation experiments with $CH_3{}^{13}COOH$ and $^{13}CH_3{}^{13}COOH$. [c] δ-value is pH-dependent, signal occasionally diffuse or missing. [d] May be interchanged. [e] Assignments based on HMBC and HMQC experiments (cf. text).

1 and 2) which also locate its position as indicated in **1**. A linear arrangement of the three subunits of circinatin was first suggested by the appearance in the FAB-MS spectrum of peaks at m/z 244 and 183 which were attributed to a dipeptide encompassing the subunits **2** and **3** and to an anhydride of the unsaturated C_{10}-component, respectively; formation of the latter requires that the corresponding subunit of **1** displays one free carboxyl group.

Independent evidence for the suggested mode of linkage of the three subunits in circinatin came from additional NMR investigations of **1** in dimethyl-d_6 sulfoxide (DMSO-d_6) including ^{1}H-detected $^1H-{}^{13}C$ one bond correlations (HMQC)[9] and long range correlations (HMBC)[10]. Specifically, the three NH signals in the downfield region of the DMSO-d_6 spectrum were assigned as in table 1 from the COSY spectrum of **1**. In the HMBC spectrum, cross peaks to both lys-6-NH and lys-2-NH identify the ^{13}C-signal at 173.7 as lys-1. The asp-C_α-H shows long range couplings to the three carbonyl carbons at 171.7, 170.7 and 169.3, whereas both asp-C_β-protons have cross peaks to only two of these (169.3 and 171.7). A strong cross peak to the asp-NH corroborates the assignment of the signal at 170.7 to the amide carbonyl group of the C_{10}-acid component. A set of two cross peaks from C-6-H to the CO-resonances at 170.7 and 173.0 confirms this and assigns the signal at 173.0 to the free carboxylate of the C_{10}-unit. Long range couplings between the CO-carbons of the C_{10}-unit and their respective α-methylene protons show that the signals at 2.34 and 2.14 correspond to the protons next to the free carboxylate, whereas the methylene group α to the amide-group resonates as a higher order multiplet at 2.18. A strong cross peak between lys-2-NH and the asp-CO signal at 169.3 confirms the sequence cyclolys-asp. However, within the aspartate moiety each of the three protons displays couplings to both CO-carbons and a decision concerning the position of the amide bond cannot be met on this basis. Evidence for a specific amidation of the α-carboxyl group was obtained by showing that irradiation of the lys-2-NH signal causes a strong NOE on the asp-α-H signal without affecting the two β-H signals.

Eventually, independent chemical proof for the correctness of formula **1** was obtained as follows. Treatment of circinatin with one equivalent of bromine in aqueous solution gave a complex mixture from which two pure compounds could be isolated by HPLC on a RP-C_{18} column. The less polar material, isolated in low yield, was identified by its spectroscopic data as the bromo-γ-lactone with partial formula **5** {FAB in NOBA [M + H$^+$] = 504, 1 Br; IR(KBr): 1780, ^{1}H-NMR: new signals at 4.42 (ddd; 10, 4.1, 3.3): C-4-H and at 4.48 (t; 4.1): C-5-H}. The more polar compound, eluted with the void volume, was further purified on a Bio-Gel P-2 column (Biorad) and identified as the dipeptide **6** by comparison of its properties (FAB-MS, HPLC, ^{1}H-NMR, CD: $\Delta\varepsilon_{218} = -2.47\,l \cdot mol^{-1} \cdot cm^{-1}$) with those of authentic synthetic specimens of **6** (CD: $\Delta\varepsilon_{218} = -2.56$) and **7** (CD: $\Delta\varepsilon_{221} = 1.73$, $\Delta\varepsilon_{205} = 0$)[11]. Similar results were obtained by treatment of circinatin (**1**) with chloroperoxidase (EC 1.11.1.10, Sigma C 0887) in the presence of KCl in H_2O. Four compounds were isolated and characterized: (a) the dipeptide **6**, (b) a chloro-γ-lactone **8** {FAB-MS in glyc-thioglyc: [M + H$^+$] = 460, 1 Cl; IR(KBr): 1780; ^{1}H-NMR: new signals at 4.30 (ddd; 10, 4.1, 3.2): C-4-H and 4.46 (t; 4.1): C-5-H}, (c) a chloro-δ-lactone **9** {FAB-MS in glyc-thioglyc: [M + H$^+$] = 460, 1 Cl; IR(KBr): 1730; ^{1}H-NMR: new signals at 4.39 (ddd; 10, 6, 2.5): C-4-H and at 4.01 (t; 10): C-5-H}, (d) a chlorohydrine **10** {FAB-MS in glyc-thioglyc: weak signal at m/z 478, 1 Cl: [M + H$^+$], strong signals at m/z 129 (cyclolys), 244 (cyclolys-asp) and 460 (1 Cl): [M + H$^+$]$-H_2O$; IR(KBr): 1682, 1210, 1180, 1140; ^{1}H-NMR: new signals at 4.50 (td; 7, 2.2): C-4-H and at 4.60 (d; 2.2); C-5-H}. From the ^{1}H-NMR data, a trans-trans-triequatorial arrangement of the substituents at C-4, C-5 and C-6 can be inferred for the δ-lactone **9**, whereas no conclusion can be drawn about the relative stereochemistry of the newly formed chiral centers in **5**, **8** and **10**. Formation of the lactones **5**, **8**, and **9** vindicates the presence of a free carboxylate group and the position of the double bond in the C_{10}-component of circinatin (**1**), while the release of the dipeptide **6** is suggestive of a process in which the amidated side chain participates in the halogenation step to yield an iminoester of type **11** as a labile intermediate.

These results provide a firm basis for assigning to circinatin structure **1**, in which only the absolute configuration of the chiral center in the C_{10}-subunit remains undefined.

Acknowledgments. Work at BTI was supported by grants from U.S. Department of Agriculture (88-37234-3805) and from U.S. National Science Foundation (INT-8721608). The ETH group thanks Prof. J. Seibl and Dr W. Amrein for the FAB mass spectra. Financial support from Sandoz AG (to D.A.) is gratefully acknowledged.

Abbreviations used: FAB, fast atom bombardment; NOBA, 3-nitrobenzylalcohol; glyc, glycerol; thioglyc, thioglycerol; DMSO, dimethylsulfoxide.

1 To whom inquiries concerning this paper may be addressed.
2 Current address: Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR 97330.
3 Dunkle, L. D., in: Methods for Research on Soilborne Phytopathogenic Fungi. Eds L. Singleton, C. Rush and J. D. Mihail. American Phytopathological Society Press, St. Paul, MN (1990) in press.
4 Pringle, R. B., and Scheffer, R. P., Phytopathology *57* (1967) 530 and *53* (1963) 785.
5 Wolpert, T. J., and Dunkle, L. D., Phytopathology *70* (1980) 872.
6 Simon, W., Helv. chim. Acta *41* (1958) 1835.
7 A control experiment with optically pure aspartic acid verified that under the conditions of hydrolysis racemization occurs to an extent of ca 60% within 12 h and is essentially complete after 24 h.
8 Farmer, E. H., and Marti, S. R. W., J. chem. Soc. (1933) 960.
9 Bax, A., and Subramanian, S., J. magn. Res. *67* (1986) 565.
10 Bax, A., and Summers, M. F., J. Am. chem. Soc. *108* (1986) 2093.
11 Samples of **6** and **7** were prepared by standard methods from the commercially available optically active components cyclolysine and the required Z-protected aspartic acid monobenzylesters.

0014-4754/90/11-12/1206-04$1.50 + 0.20/0

E-myrcenol in *Ips duplicatus*: An aggregation pheromone component new for bark beetles

J. A. Byers[a], F. Schlyter[a], G. Birgersson[b] and W. Francke[c]

[a] *Department of Ecology, Lund University, S-223 62 Lund (Sweden),* [b] *Department of Chemical Ecology, Göteborg University, S-400 33 Göteborg (Sweden), and* [c] *Department of Organic Chemistry, University of Hamburg, Martin-Luther-King Platz 6, D-2000 Hamburg 13 (Germany)*
Received 20 March 1990; accepted 29 June 1990

Summary. Males of the Eurasian bark beetle *Ips duplicatus*, when feeding in host Norway spruce (*Picea abies* (L.) Karst.), produced and released ipsdienol and *E*-myrcenol, which we show to be aggregation pheromone components. Bioassays using walking beetles indicated that *E*-myrcenol in synergistic combination with ipsdienol is essential for attraction. Synergism of *E*-myrcenol and ipsdienol released at natural rates in the forest was also demonstrated with a new technique using mechanical slow-rotation of sticky traps.
Key words. Pheromone; *E*-myrcenol; ipsdienol; *Ips duplicatus*; Coleoptera; Scolytidae; *Picea abies*.

The genera *Ips* and *Dendroctonus* include most of the 'aggressive' tree-killing bark beetles that account for the major losses of coniferous trees in the northern hemisphere[1,2]. These species release pheromones, leading to the aggregation of the beetles on a tree and the overpowering of its resinous defenses[1,2]. In the genus *Ips*, no aggregation pheromone components with a monoterpene structure have been discovered since ipsenol, ipsdienol and *cis*-verbenol were identified in 1966 in the American bark beetle *I. paraconfusus*[3]. Most *Ips* species use these semiochemicals alone or in mixtures as pheromone components[1-4]. A few additional compounds have been suggested as aggregation pheromone components, among which only 2-methyl-3-buten-2-ol (methylbutenol) in European *I. typographus* has been confirmed as significantly active[2,5,7].

Ipsdienol is produced by males of *I. duplicatus* feeding in spruce logs and is attractive alone[6]. The ipsdienol found in males consists of an equal ratio of (+)- and (−)-enantiomers (Birgersson, unpublished). Commercial baits for *I. typographus* consisting of ipsdienol, *cis*-verbenol and methylbutenol are also attractive to *I. duplicatus*[7], but it is not known whether the latter two compounds are essential. Therefore, in order to determine whether ipsdienol alone is responsible for aggregation, the attractiveness of a range of release rates of racemic ipsdienol was compared in a laboratory bioassay to that of volatiles from males feeding in a host log. Females were tested for their upwind attraction to an odor source as they walked in a 42-cm diameter arena[8]. In the bioassay, release rates spanning five orders of magnitude, from 0.02 to 2000 ng ipsdienol per min., were of low attractiveness (< 23% response) with the 20 ng/min. rate being most attractive (table). The attraction of females to the infested log was much higher (75%), indicating that additional components participate in eliciting the natural attraction (table).

To identify potential pheromone components in *I. duplicatus*, males were collected from nuptial chambers in a tree during the first days of attack (Torsby, Värmland, Sweden, in May 1982). Males were stored in liquid nitrogen until extraction of their hindguts in pentane with an internal standard of heptyl acetate, as described earlier[9]. Volatiles in the extracts were identified and quantified by gas chromatography and mass spectrometry (GC-MS) (fig. 1). Besides ipsdienol, other formerly discovered