Very-Long-Chain Fatty Acid Biosynthesis is Inhibited by Cafenstrole, *N,N*-Diethyl-3-mesitylsulfonyl-1*H*-1,2,4-triazole-1-carboxamide and Its Analogs

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Cafenstrole, Rice Herbicide, Very-Long-Chain Fatty Acids

The rice herbicide cafenstrole and its analogs inhibited the incorporation of [1-^{14}C]-oleate and [2-^{14}C]-malonate into very-long-chain fatty acids (VLCFAs), using *Scenedesmus* cells and leek microsomes from *Allium porrum*. Although the precise mode of interaction of cafenstrole at the molecular level is not completely clarified by the present study, it is concluded that cafenstrole acts as a specific inhibitor of the microsomal elongase enzyme involved in the biosynthesis of fatty acids with alkyl chains longer than C_{18}. For a strong VLCFA biosynthesis inhibition an -SO_2- linkage of the 1,2,4-triazole-1-carboxamides was required. Furthermore, *N,N*-dialkyl substitution of the carbamoyl nitrogen and electron-donating groups such as methyl at the benzene ring of 1,2,4-triazole-1-carboxamides produced a strong inhibition of VLCFA formation. A correlation was found between the phytotoxic effect against barnyardgrass (*Echinochloa oryzicola*) and impaired VLCFA formation.

Introduction

Cafenstrole, *N,N*-diethyl-3-mesitylsulfonyl-1*H*-1,2,4-triazole-1-carboxamide, is a new herbicide for rice cultivation which especially inhibits the germination of grass weeds, e.g. *Echinochloa oryzicola* or *Cyperus difformis* and some dicotyledonous weeds like *Elatine triandra* or *Lindernia pyxidaria* (Kanzaki *et al.*, 1999 ; 2000). Its phytotoxic symptoms, namely impaired seedling emergence and stunting have been reported as very similar to those of chloroacetamide herbicides, such as alachlor or butachlor (Fukazawa *et al.*, 1995). Recently, a strong inhibition of very-long-chain fatty acid (VLCFA) biosynthesis by cafenstrole has been found in unicellular *Scenedesmus* cells (Couderchet *et al.*, 1998), in cucumber cotyledons (Matthes *et al.*, 1998), and with a cell-free microsomal preparation from leek seedlings (*Allium porrum*; Böger *et al.*, 2000). Incorporation of exogenously applied [^{14}C]-oleic acid into an insoluble non-lipid fraction of *Scenedesmus acutus* cells was drastically decreased by the herbicide. This is attributed to a marked inhibition of oleic acid elongation to monounsaturated VLCFAs. The inhibition of oleic acid elongation correlated with growth inhibition of the algal cells, indicating that the reduced formation of VLCFAs was responsible for the phytotoxicity of the herbicide (Couderchet *et al.*, 1998).

The *in-vitro* biosynthesis of VLCFAs and localization of enzymes related to elongation, as well as their purification, have been examined for seed elongases of various plants and particularly for the extraplastidic elongase of leek cells (Cassagne *et al.*, 1994; Matthes *et al.*, 1998; Böger *et al.*, 2000). This information on VLCFA biosynthesis in leek prompted us to use this plant as a model to study inhibitors affecting the biosynthesis pathway.

In this paper, incorporation of [^{14}C]-oleate and [2-^{14}C]-malonate into VLCFAs was compared both in cell and cell-free assays with cafenstrole and its analogs present, using *Scenedesmus* cells and leek microsome preparations, respectively. Furthermore, the relationship of structure of cafenstrole and its analogs to their inhibition of

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VLCFA biosynthesis will be discussed by data obtained with leek microsomal assays.

Materials and Methods

Chemicals

[2-^{14}C]-Malonyl-CoA (1.8 GBq/mmol) and [1-^{14}C]oleic acid (1.85 GBq/mmol) were purchased from Moravek Biochemicals, Inc., Brea CA, USA, and Amersham, Braunschweig, Germany, respectively. Other analytical grade and fine chemicals such as buffers were purchased from Sigma (Steinheim, Germany). Cafenstrole and its analogs were prepared according to Kanzaki *et al.* (2000). Their melting points are shown in Tables I, III to V. Reference herbicides were alachlor (2-chloro-2', 6'-diethyl-*N*-methoxymethylacetanilide), metazachlor (2-chloro-*N*-(1*H*-pyrazol-1-ylmethyl)-2',6'-diethylacetanilide), bensulfuron-methyl (methyl 2-[3-(4,6-dimethoxypyrimidin-2-yl)ureidosulfonylmethyl]-benzoate) and oxyfluorfen (2-chloro-4-trifluoromethylphenyl 3'-ethoxy-4'-nitrophenyl ether).

Microsome preparation from leek seedlings

Leek (*Allium porrum*) seeds obtained from Hild (Marbach, Germany) were germinated in plastic pots containing filter paper and tap water for 14 days in the dark at 20 °C. Following operations were carried out at 4 °C. Etiolated leek seedlings (4 g) were ground in a mortar in 40 ml HEPES (*N*-[2-hydroxyethyl]piperazine-*N*'- [2-ethanesulfonic acid])/KOH buffer (80 mM, pH 7.2), containing 320 mM sucrose, 10 mM DTT (dithiothreitol), 2 mM EDTA and 0.3 mM phenylmethanesulfonyl fluoride. The homogenate was filtered through eight layers of nylon gauze and the filtrate centrifuged at 10,000 × *g* for 20 min. The supernatant was centrifuged at 100,000 × *g* for 60 min using a Hitachi ultracentrifuge CP-56. The microsomal pellet obtained was resuspended in 800 µl HEPES (80 mM, pH 7.2), containing 0.1% Triton X-100, 1 mM DTT, and 1 mM $MgCl_2$. The protein content (1000~1300 µg/ml) of the suspension was determined according to Bradford (1976). For elongase inhibition assays, the microsomal protein solution thus obtained was used as the enzyme preparation (600 µg/ml).

In-vitro assay for elongase inhibition

In a final volume of 51 µl the assay included: 25 µl microsomal protein preparation, 10 µl cofactor mixture (5 µl of 2 mM NADPH + 5 µl of 2 mM NADH), 5 µl of 250 µM C18:0-CoA and 10 µl of 100 µM [2-^{14}C]-malonyl-CoA. This mixture was added to the reaction vials together with 1 µl of either 0.1 µM, 1 µM or 10 µM solution of the compounds dissolved in ethanol. One µl ethanol was used as control. The assay was incubated for 20 min at 30 °C, and the reaction terminated by 50 µl of 60% KOH.

The elongation products of the *in-vitro* assay were saponified for 30 min at 80 °C and acidified with 175 µl 6% HCl. Fatty acids were extracted with 700 µl of a mixture acetone/*n*-hexane (4:3, v/v). To remove non-reacted [^{14}C]-malonyl-CoA from the organic phase the layer was washed with 100 µl 6% HCl and then the radioactivity of an aliquot of the organic layer was measured by liquid-scintillation counting. Thus the percent inhibition of malonyl-CoA incorporation into arachidic acid was determined (Schmalfuß *et al.*, 2000).

Each assay set was run three times; the same holds for the following *in-vivo* test with *Scenedesmus.*

Determination of inhibition of oleic acid incorporation into a sporopollenin fraction of Scenedesmus acutus (Couderchet *et al.*, 1998)

One-day old cultures of *Scenedesmus acutus* (no. 276–3a, Algae Collection, Göttingen, Germany) were concentrated to 50 µg chlorophyll (Chl) ml^{-1}. The algal suspensions (1.8 ml per sample) were preincubated in capped 12-ml test tubes for 30 min after adding 10 µl 1 M $NaHCO_3$ and 2 µl of the ethanolic test compound. The test tubes were placed almost horizontally on an orbital shaker under illumination of about 55 µmol PAR (photosynthetically active radiation) $m^{-2} \times sec^{-1}$ at 25 °C. Each assay set contained the test compound with three concentrations (0.01, 0.1, or 1 µM) and a control without test compound.

The stock solution of the test compound in EtOH was used to prepare the sample solutions whose EtOH concentrations were equal to or less than 1 ml l^{-1}. [l-^{14}C]-oleic acid (13 µl, *ca.* 20 nmol; 2.04 TBq mol^{-1}) in toluene was placed in a 12-ml silanized glass tube and dried under nitrogen. To

the glass tube was added algal growth medium (5 ml) and dispersed by ultrasonication for 5 min. After the culture was preincubated for 30 min with the test compound, 200 µl of the algal growth medium containing labeled oleate was added to the 1.8-ml culture and further incubated in the light as mentioned above for 3 h. The total radioactivity per tube was approx. 1.7 kBq.

At the end of the 3-h incubation, incorporation of oleic acid into the algae was terminated by addition of 10% KOH in MeOH (7 ml, w/v) and the algal suspension was saponified at 65 °C for 30 min. The mixture was cooled, centrifuged (*ca.* 1,000 *g*) for 5 min and the supernatant discarded. The pellet which contained the non-lipid fraction (NLF, see Couderchet *et al.,* 1998), was washed once with a mixture of $CHCl_3$ and MeOH (4 ml; 2:1 v/v), suspended in MeOH (1 ml) and radioactivity determined by liquid scintillation counting to quantify the oleic acid incorporated into the NLF. Inhibition of oleic acid incorporation into sporopollenin of *Scenedesmus acutus* was thus determined with 0.01, 0.1 and 1 µM concentrations of compounds tested. The cellular assay with *Scenedesmus* and the cell-free leek test exhibited different sensitivity against the herbicides tested; see Böger and Matthes, (2002) for explanation. The comparative inhibition response of both assays against the compounds was practically the same.

Results and Discussion

Phytotoxic mode of action of cafenstrole and its analogs

Herbicidal mode of action of cafenstrole, a new triazolecarboxamide herbicide developed by Chugai Pharmaceutical Co. Ltd., was investigated together with some analogs. Phytotoxicitiy exhibited by cafenstrole was assayed using *Scenedesmus acutus* and compared with that of two chloroacetamides (alachlor and metazachlor). Inhibition of bensulfuron-methyl on acetolactate synthase (ALS) from corn and peroxidizing activity of oxyfluorfen was included. Results are shown in Table I.

Oxyfluorfen caused a strong inhibition of a chlorophyll biosynthesis step (pI_{50} protoporphyrinogen-IX oxidase, (Protox) = 8.10), induced photooxidative destruction of thylakoid membranes observed as ethane formation, (pI_{50} (ethane) = 7.38), and decreased the chlorophyll content, (pI_{50} (chlorophyll) = 7.42), as shown in Table I. These inhibitions are typical characteristics for peroxidizing herbicides (Wakabayashi and Böger, 1999). For cafenstrole, such inhibitory effect was not observed at all even at higher concentrations, e.g. the pI_{50} (chlorophyll) was found at 6.02 for *Scenedesmus.* No ethane formation was detected at 10^{-4} M as well as a very small inhibition of Protox at 10^{-6} M only, indicating that cafenstrole neither di-

Table I. General phytotoxicities by selected herbicides.

Herbicides*[1]	mp [°C]	Target site inhibited	pI_{50}*[4]			Protox (1 µM) % inhibition*[5]
			Growth	Chlorophyll	Ethane	
Cafenstrole	114~116	VLCFA elongase	5.31	6.02	n.d.	14.0
Alachlor	40~41	VLCFA elongase	4.67	5.23	n.d.	8.3
Metazachlor	84~85	VLCFA elongase	5.45	5.70	n.d.	1.8
Bensulfuron-methyl	186~188	Acetolactate synthase*[2]	6.03	5.90	n.d.	0.0
Oxyfluorfen	84~86	Protoporphyrinogen-IX oxidase*[3]	7.14	7.42	7.38	100.0

n.d.: not detected.
*[1] Cafenstrole: *N,N*-diethyl-3-mesitylsulfonyl-1*H*-1,2,4-triazole-1-carboxamide; alachlor: 2-chloro-2',6'- diethyl-*N*-methoxymethylacetanilide; metazachlor: 2-chloro-*N*-(1*H*-pyrazol-1-ylmethyl)-2',6'-diethylacetanilide; bensulfuron-methyl: methyl 2-[3-(4,6-dimethoxy- pyrimidin-2-yl)ureidosulfonylmethyl]benzoate; oxyfluorfen: 2-chloro-4-trifluoromethylphenyl 3'-ethoxy-4'- nitrophenyl ether.
*[2] pI_{50} (ALS : corn) = 7.20.
*[3] pI_{50} (Protox : corn) = 8.10.
*[4] pI_{50} (growth), pI_{50} (chlorophyll) and ethane formation at 10^{-4} M were assayed using *Scenedesmus acutus.*
*[5] % inhibition of corn Protox was tested with 1 µM of compounds. For assays see "Target Assays for Modern Herbicides and Related Phytotoxic Compounds" (P. Böger, G. Sandmann, eds.) Lewis Publ., Boca Raton, FL (1993).

rectly inhibits chlorophyll biosynthesis nor causes photooxidative ethane formation, and hence is not classified as a peroxidizing herbicide. Bensulfuron-methyl strongly inhibited acetolactate synthase but cafenstrole did not even at 10^{-4} M, indicating that the latter is not an inhibitor for this enzyme (data not shown). Phytotoxic parameters (pI_{50}-(growth), pI_{50}(chlorophyll), lack of ethane formation and protox inhibition documented for cafenstrole are quite similar to those of alachlor and metazachlor.

Elongation inhibition of stearate and oleate by cafenstrole was assayed with cucumber cotyledons (Matthes *et al.*, 1998) and *Scenedesmus acutus* cells (Couderchet *et al.*, 1998), respectively. The results in Table II confirm previous reports that cafenstrole strongly and specifically inhibits VLCFA formation in both cucumber cotyledons and *Scenedesmus* cells, indicating over 80% inhibition of C18:0 elongation at 10^{-6}M in cucumber and an approximate I_{50} of 10^{-7}M for C18:1 elongation in *Scenedesmus* cells. This inhibition pattern is quite similar to that of alachlor and thenylchlor. The inhibition of VLCFA formation by cafenstrole, alachlor or metazachlor is assumed as inhibition of oleic acid incorporation into sporopollenin which is part of the non-lipid fraction (NLF) from *Scenedesmus acutus* (Böger, 1997). The inhibition of oleic acid incorporation into the NLF is a reliable indicator for inhibition of VLCFA biosynthesis (Couderchet *et al.*, 1998; Böger and Matthes, 2002).

To confirm this finding also at the cell-free level, inhibition of C18:0-CoA elongation by cafenstrole was assayed using a leek microsome preparation. Cafenstrole and the two chloroacetamides assayed strongly inhibited C18:0-CoA elongation, all exhibiting an I_{50} value of about 10^{-6}M and less (Table II). Although the precise mode of interaction of cafenstrole with its enzyme target is not completely clarified by the present study, it is concluded that cafenstrole acts as an inhibitor of the enzyme(s) involved in biosynthesis of fatty acids with an alkyl chain longer than C18.

Structure-activity considerations

(a) Sulfur linkage: Modification of the sulfur linkage of *N,N*-diethyl-3-mesityl- sulfonyl-1*H*-1,2,4-triazole-1-carboxamide (**1**) and C18:1, C18:0 elongation inhibition activity together with herbicidal activity (greenhouse test) was examined (Table III). Compound **1** (cafenstrole with an -SO_2-linkage) was the strongest inhibitor. In the *Scenedesmus* assay it inhibited elongation of C18:1 with an I_{50} value of approx. 10^{-7}M; for the cell-free elongation of C18:0 it was found about 10^{-6}M. For compound **1** an ED_{90} of 5 g a.i./ha was found for *E. oryzicola*. Compounds **2** and **3** having an -SO-, or -S-linkage, respectively, showed an ED_{90} of 60 and 300 g a.i./ha. Accordingly, aryl and triazole rings should be connected by sulfoxide to achieve maximum inhibition.

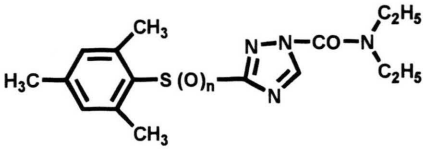
(b) Benzene substituents: Table IV shows the benzene substituents of compound **1** and phytotoxitities. Although the approximate I_{50} value for oleic acid elongation in *Scenedesmus* for compounds **4, 5** and **6** were about 10^{-6}M, the I_{50} value

Table II. VLCFA biosynthesis inhibition by herbicides in cell and cell-free assay.

Herbicides	Inhibition of VLCFA elongation (% of control)						
	C18:0-CoA	C18:1			C18:0		
	Cucumber*[1] cotyledons	*Scenedesmus acutus*[*2] cells			Leek (*Allium porrum*) cell-free		
	1 µM	0.01 µM	0.1 µM	1 µM	0.1 µM	1 µM	10 µM
Cafenstrole	84 (± 4)	30 (± 5)	53 (± 2)	73 (± 3)	10 (± 9)	33 (± 8)	80 (± 4)
Alachlor	–	29 (± 7)	49 (± 7)	62 (± 5)	48 (± 6)	62 (± 7)	82 (± 5)
Metazachlor	89 (± 1)	17 (± 13)	51 (± 12)	64 (± 3)	25 (± 5)	45 (± 5)	61 (± 10)
Bensulfuron-methyl	–	0 (± 13)	1 (± 3)	2 (± 3)	–	–	–
Oxyfluorfen	–	–3 (± 9)	–34 (± 1)	–52 (± 12)	–	–	–

*[1] For the assay with cucumber cotyledons see Matthes *et al.* (1998).
*[2] for the assay with *Scenedesmus* cells see Couderchet *et al.* (1998).

Table III. Cafenstrole and its analogs: sulfur linkage and elongation inhibition assayed with *Scenedesmus* and microsomes.

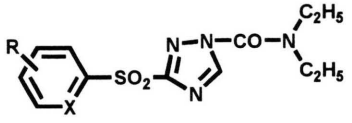


Compounds Nos.	n	mp [°C]	(ED_{90}, g/ha)*2 ECHOR*1	Inhibition of VLCFA elongation (% of control)					
				C18:1 Scenedesmus acutus cells			C18:0 Leek (*Allium porrum*) cell-free		
				0.01 µM	0.1 µM	1 µM	0.1 µM	1 µM	10 µM
1	2	114–116	5	30 (± 14)	53 (± 2)	73 (± 4)	10 (± 6)	33 (± 14)	80 (± 1)
2	1	74–76	60	12 (± 6)	35 (± 4)	67 (± 3)	5 (± 2)	6 (± 3)	32 (± 4)
3	0	70–71	300	–	–	–	2 (± 5)	9 (± 5)	11 (± 6)

*1 ECHOR: *Echinochloa oryzicola.*

*2 The compounds were applied in water three days after transplanting of rice. Herbicidal activity was estimated 21 days after application. The degree of inhibition was determined by the fresh weight of the shoot. These data were used to calculate the ED_{90}.

Table IV. Oleate elongation inhibition by 3-arylsulfonyl-*N,N*-diethyl-1*H*-1,2,4– triazole-1-carboxamide.



Compounds Nos.	R	X	mp [°C]	(ED_{90}, g/ha)* ECHOR*	Inhibition of C18:1 elongation (% of control) Scenedesmus acutus		
					0.01 µM	0.1 µM	1 µM
1	2,4,6-$(CH_3)_3$	CH	114–116	5	30 (± 14)	53 (± 2)	73 (± 4)
4	H	CH	88–89	100	4 (± 7)	25 (± 2)	73 (± 4)
5	3-CH_3	CH	135–136	40	–5 (± 14)	37 (± 12)	64 (± 4)
6	2-OCH_2CF_3	CH	134–135	10	26 (± 11)	37 (± 8)	68 (± 4)
7	2-NO_2	CH	101–103	300	–	–	–
8	3-OCH_2CF_3	N	137–138	200	0 (± 11)	30 (± 3)	51 (± 5)

* For ED_{90} and ECHOR see Table III

of compound **1** (cafenstrole) was below 10^{-7}M, reflecting the strong herbicidal activity against *Echinochloa oryzicola.* Therefore, an electron-donating group such as methyl or -OCH_2CF_3 at the benzene ring (compounds **1, 5** and **6**) results in strong herbicidal activity and VLCFA biosynthesis inhibition.

The approx. I_{50} values for elongation inhibition of compounds **6** (benzene type) and **8** (pyridine type) were 10^{-6} M. The herbicidal activity against *E. oryzicola* exhibited an ED_{90} of 10 g a.i./ha for compound **6** and ED_{90} of 200 g a.i./ha for compound **8**. Introduction of an electron-withdrawing NO_2 group at the benzene ring, however, produced the herbicidally less active compound **7**.

(c) Dialkyl-carbamoyl moiety: Synthesis of 3-mesitylsulfonyl-1*H*-1,2,4-triazole-1- carboxamide failed, but mono- or dialkyl substitution of the carbamoyl nitrogen was possible (Table V). The approx. I_{50} value for elongation inhibition for compounds **1** (cafenstrole), **10** and **11,** all dialkyl substituted, was about 10^{-7} M. Compounds **9** and **12** showed a remarkable decrease of elongation inhibition (a little more than 10% with 10^{-6}M inhibitor applied), since these are *monoalkyl* substituted at the carbamoyl nitrogen. In addition, com-

pounds **9** and **12** were found also as poor inhibitors against *E. oryzicola*. Conclusively, a *dialkyl* substituted carbamoyl nitrogen is important for both herbicidal activity and elongation inhibition. This confirms previous findings with chloroacetamides (Böger *et al.*, 2000).

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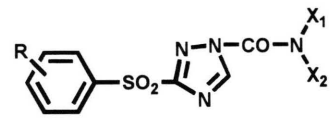
Table V. Cafenstrole and its analogs : Importance of *N,N*-dialkylcarbamoyl group for inhibition of oleate elongation.



Com pounds No.	R	X_1	X_2	mp [°C]	(ED_{90}, g/ha)* ECHOR*	Inhibition of C18:1 elongation (% of control) Scenedesmus acutus		
						0.01 µM	0.1 µM	1 µM
1	2,4,6-$(CH_3)_3$	C_2H_5	C_2H_5	114–116	5	30 (± 14)	53 (± 2)	73 (± 4)
9	2,4,6-$(CH_3)_3$	H	C_2H_5	107–110	10,000	8 (± 4)	9 (± 6)	16 (± 10)
10	2-NO_2, 4-Cl	CH_3	C_4H_9-*n*	solid	750	18 (± 8)	49 (± 6)	69 (± 3)
11	2-NO_2, 4-Cl	CH_3	$CH_2CH{=}CH_2$	solid	300	13 (± 10)	53 (± 4)	72 (± 4)
12	2-NO_2, 4-Cl	H	$CH_2CH{=}CH_2$	solid	10,000	2 (± 9)	9 (± 2)	13 (± 3)

* For ED_{90} and ECHOR see Table III

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