Interconversion of Prenyl Pyrophosphates and Subsequent Reactions in the Presence of FMC 57020

Gerhard Sandmann and Peter Böger

Lehrstuhl für Physiologie und Biochemie der Pflanzen, Universität Konstanz, Postfach 5560, D-7750 Konstanz, Bundesrepublik Deutschland

Z. Naturforsch. **42c,** 803–807 (1987); received December 1, 1986

FMC 57020 Inhibition, Prenyl Pyrophosphates, Carotenes, Kaurene, Squalene

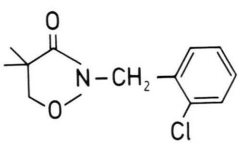
In vitro studies of the influence of FMC 57020 on terpenoid biosynthesis have shown that this herbicide acts at an early stage. It affects the conversion of isopentenyl pyrophosphate to geranylgeranyl pyrophosphate catalyzed by isopentenyl pyrophosphate isomerase and prenyl transferase. An inhibition of the carotenogenic enzymes phytoene desaturase, ζ-carotene desaturase, and lycopene cyclase could be excluded.

Comparison of I_{50} values for *in vivo* chlorophyll, carotenoid, ergosterol and gibberellin biosynthesis as well as *in vitro* formation of phytoene, phytol and kaurene *in various* autotrophic and heterotrophic organisms have shown that terpenoid biosynthesis in the chloroplast is much stronger affected than extraplastidic terpenoid formation.

Introduction

FMC 57020 is 2-(2-chlorophenyl)methyl-4,4-dimethyl-3-isoxazolidinone (Fig. 1), which was introduced as a new herbicide [1] in 1984. Its registered trade mark is Command. The proposed common name, dimethazone, was not approved and may not be used any more.

FMC 57020 is a pre-emergence herbicide for weed control in soybeans [2]. Susceptible plants treated with this compound show decreased levels of chlorophylls and carotenoids – the leaf colors range from light green and yellow to white [3]. This bleaching effect looks different to all the others previously observed with other herbicides [4, 5]. Inhibition of carotene interconversion with simultaneous accumulation of phytoene, the precursor of α- and β-carotene, which is typical for the action of norflurazon and similar herbicides [6], was not observed [7, 8]. Peroxidative properties could be excluded by comparing FMC 57020 with the *p*-nitrodiphenyl ether oxyfluorfen, and indications for a specific inhibition of the chlorophyll pathway, as observed by inhibition of chlorophyll formation in heterotrophically (dark) grown algae, were not obtained [8]. Ultrastructural effects of FMC 57020 [9] were also different to those observed after treatment with the nitrodiphenyl ether acifluorfen methyl [10]. In the presence of the latter compound, disruption of chloroplast membranes was evident within 1 h in the light. When FMC 57020 was applied to etiolated plants, the main short-term effect in the light is a retardation of chloroplast development. Disruption of chloroplast membranes occurred only after a treatment of 12 to 24 h.



FMC 57020

Fig. 1. FMC 57020 (Command).

Furthermore, FMC 57020 is effective on the phytylation of chlorophyllide after having been formed by photoconversion of photochlorophyllide in illuminated etiolated seedlings of cowpea or morningglory [3, 7]. With the green alga *Scenedesmus* it could be demonstrated that the phytol pool is much lower in cells treated with FMC 57020 [8]. As the degree of inhibition of chlorophyll formation (in the dark) was the same as for phytol formation, it was concluded that inhibition of phytol biosynthesis by FMC 57020 is the reason for a decreased chlorophyll content [8].

Other terpenoids or compounds having a terpenoid-derived moiety in the molecule are affected. These are gibberellins [7, 8] and α-tocopherol [8]. Formation of the latter is decreased in *Scenedesmus* cells in the presence of FMC 57020. This result might point to plastoquinone (having a similar prenyl side chain to α-tocopherol) as the missing component

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/87/0600–0803 $ 01.30/0

of the photosynthetic electron transport chain when leaves develop under the influence of FMC 57020 that could not yet be specified [7].

Although the expression of FMC 57020 on plant metabolism looks as being caused by multiple effects, there is evidence that the essential mode of action of this herbicide is on a common target [8]. If a single enzyme is responsible, inhibition should occur at the level of prenyl pyrophosphate synthesis and conversion, the early steps of the terpenoid pathway. Therefore, we have concentrated our studies on the effect of FMC 57020 on *in vitro* formation of C_5- to C_{20}-prenyl pyrophosphates and compared the inhibition of *in vitro* and *in vivo* biosynthesis of certain terpenoids in chloroplasts and other cell compartments. Furthermore, a re-investigation of a possible additional inhibitory effect of FMC 57020 on the desaturation and cyclization enzymes of the carotenogenic pathway was carried out in a cell-free system.

Materials and Methods

Organisms

Spinach (*Spinacia oleracea*, var. Atlanta) was cultivated in fertilized soil in the open. Growth of *Phycomyces blakesleeanus* C5, a phytoene-accumulating mutant, was described in ref. [11]. *Fusarium moniliforme*, strain Lilly M 45,399, was grown in the same way as *Phycomyces*. The nutrient used was a 3% potato-glucose medium (Difco Laboratories). FMC 57020 was added from a 10 mM stock solution in methanol. All harvested plants and fungal material were freeze-dried and stored in a deep-freeze. Prior to use, the leaves and the mycelia were rubbed through a sieve with a mesh size of 0.4 mm in order to obtain a fine powder.

Aphanocapsa PCC 6714 was grown under conditions as previously described [12].

Terpenoid determinations

The medium of the *Fusarium* cultures was brought to pH 8 with KOH and extracted with petroleum ether. The aqueous phase was then adjusted to pH 2.5 with acetic acid and extracted three times with ethyl acetate. The combined ethyl-acetate extracts were pooled, evaporated to dryness, and resuspended in methanol. Aliquots were heated in 70% sulfuric acid at 90 °C for 10 min and their fluorescence emission at 470 nm during exitation at 280 nm determined. The dry weight related to relative fluorescence yield was used to calculate the I_{50} values.

The powdered *Fusarium* mycelium was extracted with methanol containing 6% (w/v) KOH at 65 °C for 15 min. The methanol extract was diluted with water and partitioned against ether. The ether phase was dried over anhydrous Na_2SO_4 and applied on a silica gel 60 F_{254} plate (Merck). Ergosterol was purified by developing the plate in chloroform. It can be visualized by fluorescence quenching, *i.e.* fluorescence of the plate under UV light is quenched where ergosterol is located (R_f value of about 0.2). The ergosterol band was scraped off and eluted with methanol. Ergosterol was quantitated by its absorbance at 282 nm.

In vitro formation of terpenoids

Extracts for all assays, except for *Aphanocapsa*, were prepared by mixing 0.2 g of powdered material with 1.6 ml of a 0.4 M Tris [tris-(hydroxymethyl)-aminomethane]-HCl buffer, pH 8.0, containing 5 mM dithiothreitol and 10 mM KF. The paste was centrifuged at $10{,}000 \times g$ for 15 min and the supernatant used in the assays. *Aphanocapsa* membranes were prepared by lysozyme digestion of the cell wall and osmotic shock of the spheroplasts obtained by this treatment [10]. The reaction mixture for kaurene formation contained R-[2-^{14}C]mevalonic acid (0.25 μCi or 9.25 kBq equivalent to 5 nmol), 5 μmol ATP, 3 μmol $MnCl_2$, 2 μmol $MgCl_2$, 100 μl fungal extract and the Tris-HCl buffer mentioned above in a final volume of 0.5 ml. For squalene or phytol biosynthesis from mevalonic acid, the assays were essentially the same, with 1 μmol NADPH added.

In the reaction mixtures for biosynthesis of phytoene with *Phycomyces*, or spinach as well as of subsequently formed carotenes with *Aphanocapsa* membranes, NADPH was replaced by 1 μmol of NADP. The latter assay was performed with 0.5 μCi of [^{14}C]geranylgeranyl pyrophosphate (specific activity 4.2 mCi/mmol or 0.16 GBq/mmol) as substrate and *Aphanocapsa* membranes equivalent to 150 μg of chlorophyll. All the incubations were carried out at 35 °C for 2 h and terminated by addition of 3 ml of methanol. The terpenoids were extracted with petroleum ether and purified by thin-layer chromatography (TLC). Kaurene was separated on activated sili-

ca gel plates in hexane (R_f 0.5) and carotenes in two subsequent TLC systems. The details are given in ref. [14]. Purification of phytol is described in the following paragraph. The bands are scraped off and transferred into scintillation vials. Incorporated radioactivity was determined by liquid scintillation counting.

In vitro formation of prenyl pyrophosphates

The enzyme extract was prepared as described in the previous paragraph and the reaction mixtures were essentially the same as for kaurene biosynthesis. However, a 0.04 M Tris-HCl buffer, pH 8.0, was used instead in both cases. Incubation was carried out at 35 °C for 2 h. Then the reaction was stopped by addition of 1 ml methanol and heated at 80–100 °C for 5 min. After centrifugation at 2500 × *g* for 10 min the pellet was washed with 0.5 ml of the same buffer +3 ml H_2O and centrifuged again. The combined supernatants were treated with 750 µl of 25% HCl at 80–100 °C for 10 min to hydrolyze all prenyl pyrophosphates to the corresponding alcohols. Then 750 µl of 25% NH_4OH, 750 µl of a 1% Na-EDTA solution (w/v), and 10 µl of 1% methanol solutions (v/v) of geraniol (G–OH) and farnesol (F–OH), respectively, as well as 50 µl dimethylallyl alcohol (DMA–OH) were added as markers.

Determination of I_{50} values

I_{50} values for *in vivo* and *in vitro* inhibition of terpenoid formation were determined by plotting the inhibitor concentration *versus* the inverse metabolite concentrations or inverse reaction rates. Then the I_{50} value is obtained from the intercept of the resulting straight lines ($r > 0.9$) with the abscissa [13].

Results and Discussion

In order to prove or disprove a direct interference of FMC 57020 on the enzymes that are specific for the formation of carotenes, namely phytoene desaturase, ζ-carotene desaturase, and lycopene cyclase, the inhibitory effect on these enzymes was investigated with isolated membranes from *Aphanocapsa,* which are highly carotenogenic [14]. Norflurazon [4-chloro-5-methylamino-2-(3-trifluoromethylphenyl)-pyridazin-3(2H)one], LS 80,717 [ethyl, *trans*-5-methyl-6-ethyl-2-phenyl-5,6-dihydropyran-4-one-3 carboxylate] and CPTA [2-(4-chlorophenylthio)-triethylammonium chloride] were included for comparison (Table I). Norflurazon increased the accumulation of radioactivity in phytoene by inhibition of phytoene desaturase. LS 80,717 exhibited a similar inhibitory effect on phytoene desaturase but a stronger one on ζ-carotene desaturase resulting in an accumulation of ζ-carotene. The alkylamine CPTA prevents the cyclization of lycopene to β-carotene. FMC 57020 does not show any of these well-known effects of the compounds included in Table I [15]. Radioactivity incorporation into phytoene, ζ-carotene, lycopene as well as into β-carotene, was not significantly different to the control values. Consequently, using this *in vitro* carotenogenic system, we can definitely exclude inhibition of carotene interconversion by FMC 57020.

The early step of carotenoid biosynthesis leading also to other terpenoids is the formation and interconversion of prenyl pyrophosphates. In a newly developed *in vitro* system from spinach we were able to convert mevalonic acid (MVA) into prenyl pyrophosphates ranging from C_5 to C_{20}. After acid hydrolysis, the free alcohols were separated on a paraffin-impregnated reverse phase silica gel plate. As indicated by Fig. 2A the TLC plate shows the separation

Table I. *In vitro* carotenoid biosynthesis by *Aphanocapsa* membranes using [^{14}C]geranylgeranyl pyrophosphate as substrate.

Additions	Incorporation of radioactivity [dpm] into			
	Phytoene	ζ-Carotene	Lycopene	β-Carotene
Control	799	378	381	2672
FMC 57020, 50 µM	774	352	377	2533
Norflurazon, 0.5 µM	4835	197	208	208
LS 80717, 5 µM	1278	678	212	1270
CPTA, 40 µM	757	399	2111	775

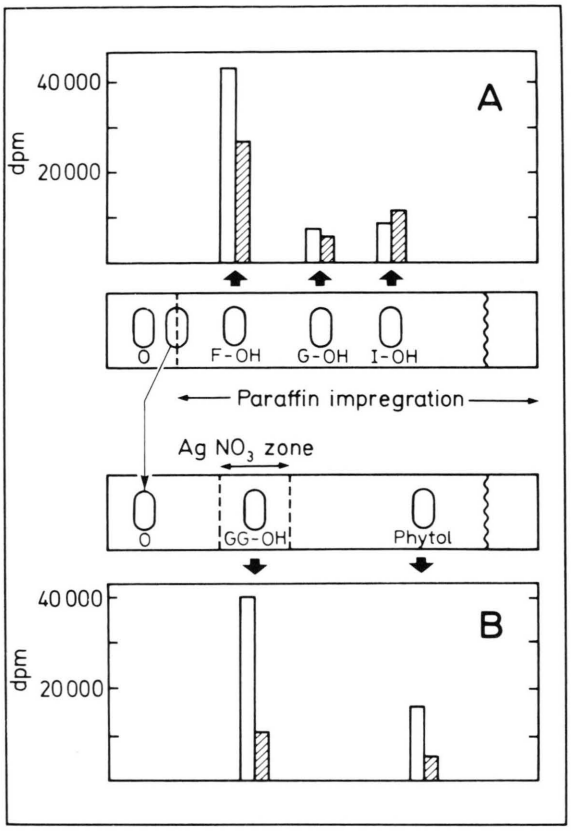


Fig. 2. Biosynthetic incorporation of radioactivity from mevalonic acid (MVA) into terpenoids by spinach extracts and separation of prenyl pyrophosphates and corresponding alcohols as well as phytol in the presence of FMC 57020 (20 μM).
The left bars represent the control value, the right ones the incorporated radioactivity from MVA with FMC 57020 present. The solvent for system A was 70% (v/v) methanol in water and for system B 15% (v/v) ethylacetate in petroleum ether (b.p. 100–140 °C).
F–OH, farnesol; G–OH, geraniol; I–OH, isopentenol; GG–OH, geranylgeraniol; O, origin.

of geraniol (G–OH), farnesol (F–OH) and isopentenol (I–OH). The spot on the impregnation border (see the dotted line) was eluted and the containing alcohols re-chromatographed on a silica gel plate with an $AgNO_3$ strip across it, as indicated in Fig. 2B. This TLC step separates phytol from geranylgeraniol (GG–OH) the latter being absorbed in the $AgNO_3$ zone. All corresponding prenyl pyrophosphates were formed from MVA. Radioactivity is preferentially accumulated in the longer chain prenyl pyrophosphates. In the presence of 20 μM FMC 57020, a slight increase of isopentenyl pyrophosphate (IPP) compared to the control was observed. But formation of all the subsequent prenyl pyrophosphates was inhibited by FMC 57020. This inhibition was less pronounced in geranyl pyrophosphate (GPP), the direct reaction product of IPP and dimethylallyl pyrophosphate (DMAPP) condensation than in the subsequent products farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). Their labeling was much stronger inhibited. Realizing the reaction sequence of Fig. 3, the inhibitory site of FMC 57020 can be assumed between IPP and GGPP. The enzymes catalyzing this part of the pathway are IPP isomerase and prenyl transferase.

According to the results of Fig. 2, biosynthesis of kaurene, phytol and phytoene as well is supposed to be inhibited by FMC 57020. Table II shows I_{50} values for these three pathways branching off from GGPP and from FPP in case of squalene (Fig. 3). Inhibition of several terpenoid pathways by FMC 57020 was investigated in two fungal species, *Phycomyces* and *Fusarium,* as well as in the photosynthetic green alga *Scenedesmus* and spinach. In the latter two autotrophic species, biosynthesis of the terpenoid moiety, phytol, of the chlorophylls as well as of carotenoids take place in the chloroplast [16],

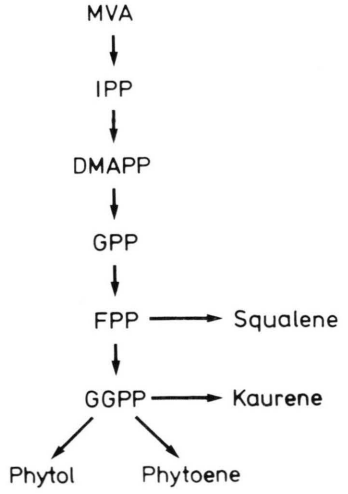


Fig. 3. Pathway of prenyl pyrophosphate formation and interconversion into terpenoids.
MVA, mevalonic acid; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate.

Table II. I_{50} values to inhibit the *in vivo* and *in vitro* terpenoid biosynthesis by FMC 57020 in various species.

	Species	Terpenoids	I_{50} value [μM]
A. *In vivo*			
	Scenedesmus	Carotenoids	10*
		Chlorophylls	11*
	Fusarium	Ergosterol	52
		Gibberellins	60
B. *In vitro*			
	Spinach	Phytoene	14
		Phytol	12
		Squalene	42
	Phycomyces	Phytoene	44
		Squalene	48
	Fusarium	Kaurene	45

* These values were taken from ref. [8].

whereas sterols (*i.e.* squalene and subsequent sterols) are formed in an extraplastidic pathway. Of course, in fungi squalene, kaurene, and phytoene are synthesized by cytoplasmic enzymes [17].

I_{50} values for the effect of FMC 57020 on *in vivo* and *in vitro* formation of phytoene/carotenoids and phytol/chlorophylls are very close together, when *Scenedesmus* and spinach is compared; they range from 10 to 14 μM (Table II). The I_{50} value for spinach extraplastidic squalene biosynthesis *in vitro* was found about 3.5-fold higher. In *Phycomyces* a similar high sensitivity for squalene formation as well as for phytoene biosynthesis was measured. In addition, inhibition of kaurene synthesis shows a similar I_{50} value between 44 and 48 μM FMC 57020.

From the results of Table II we conclude that the target enzyme exhibits a different sensitivity against FMC 57020 depending on its intracellular location. The extraplastidic isoenzyme, either from a higher plant or from fungi, needs about a 4-fold higher concentration of FMC 57020 to exhibit the same degree of inhibition as the enzyme in the chloroplast. As possible reactions affected by FMC 57020 either remain the conversion of IPP to DMAPP catalyzed by IPP isomerase or the subsequent condensation reactions in which additional IPP molecules are stepwise added by prenyl transferase(s). To answer the question, whether either IPP isomerase or prenyl transferase are inhibited by FMC 57020, kinetic studies with both enzymes are necessary. This work is in progress in our laboratory.

Acknowledgements

This study was supported by the Stiftung "Umwelt und Wohnen" and the Deutsche Forschungsgemeinschaft which is gratefully acknowledged. Thanks are expressed to Mrs. S. Kuhn for expert technical assistance.

[1] J. H. Chang and M. J. Konz, Amer. Chem. Soc., Abstracts, 187th Natl. Meeting, Pestic. Chem. Div., No. 22 (1984).
[2] T. R. Warfield, D. B. Carlson, S. K. Bellman, and H. L. Guscar, Weed Sci. Soc. Amer. (WSSA), Abstr. **25,** 105 (1985).
[3] S. O. Duke and W. H. Kenyon, Pestic. Biochem. Physiol. **25,** 11–18 (1986).
[4] S. O. Duke and W. H. Kenyon, Weed Sci. Soc. Amer., Abstr. **25,** 69 (1985).
[5] G. E. Darke and J. W. Bolcsak, Weed Sci. Soc. Amer., Abstr. **25,** 69 (1985).
[6] G. Sandmann and P. Böger, in: Biochemical Responses induced by Herbicides (D. E. Moreland, J. B. St. John, and F. D. Hess, eds.), Amer. Chem. Soc. Symposium Series 181, pp. 111–130, Washington D.C., 1982.
[7] S. O. Duke, W. H. Kenyon, and R. N. Paul, Weed Sci. **33,** 786–794 (1985).
[8] G. Sandmann and P. Böger, Z. Naturforsch. **41,** 729–732 (1986).
[9] S. O. Duke and N. Paul, Pestic. Biochem. Physiol. **25,** 1–10 (1986).
[10] G. L. Orr and F. D. Hess, Plant Physiol. **69,** 502–507 (1982).
[11] A. Than, P. M. Bramley, B. H. Davies, and A. F. Rees, Phytochemistry **11,** 3187–3192 (1972).
[12] G. Sandmann, Photosynth. Res. **6,** 261–271 (1985).
[13] G. Sandmann, K.-J. Kunert, and P. Böger, Z. Naturforsch. **34c,** 1044–1046 (1979).
[14] G. Sandmann and P. M. Bramley, Planta **164,** 259–263 (1985).
[15] G. Sandmann and P. Böger, in: Wirkstoffe im Zellgeschehen (P. Böger, ed.), pp. 139–169, Universitätsverlag Konstanz 1985.
[16] G. Schultz, J. Soll, E. Fiedler, and D. Schultz-Siebert, Physiol. Plant. **64,** 123–129 (1985).
[17] P. M. Bramley, Adv. Lipid Research **21,** 243–279 (1985).