In vitro Inhibition Studies of Phytoene Desaturase by Bleaching Ketomorpholine Derivatives

Gerhard Sandmann*,† and Glynn Mitchell‡

Botanisches Institut, Goethe Universität, P.O. Box 111932, D-60054 Frankfurt, Germany, and ZENECA Agrochemicals, Jealott's Hill International Research Centre, Bracknell, Berkshire RG42 6ET, U.K.

The herbicidal activities of homochiral steroisomeric 5-methyl-2-(3-trifluoromethylbenzyl)-3-ketomorpholine derivatives were investigated in vitro as inhibitors of phytoene desaturase, a key enzyme in carotenoid biosynthesis. It was demonstrated that ketomorpholines are classical bleaching compounds which directly inhibit phytoene desaturase, accumulating phytoene at the expense of colored carotenoids. Ketomorpholines interact with phytoene desaturase in a noncompetitive manner with respect to phytoene. A structure−activity investigation for in vitro inhibition of phytoene desaturase activity revealed that the relative and absolute stereochemistry is important for optimum inhibition for the 5-methyl derivatives, and that the distance of the phenyl group from the ketomorpholine ring is critical for the inhibitory potential. The average herbicidal score on 7 weeds and the in vitro I_{50} values related very well with the exception of two compounds. It was postulated that the discrepancies may possibly occur through modification in plants to compounds that are either more or less active herbicides.

Keywords: *Phytoene desaturase inhibition; bleaching; herbicidal activity; ketomorpholine; carotenoid biosynthesis*

INTRODUCTION

Phytoene desaturase is the target enzyme for a range of structurally diverse classes of bleaching herbicides (*1*). Inhibition of phytoene desaturase prevents the formation of colored carotenoids, which are essential for the photoprotection of chlorophylls: when carotenoids are depleted, photooxidative destruction of chlorophylls is a consequence. All of the herbicidal phytoene desaturase inhibitors studied to date interact in a noncompetitive manner with respect to the substrate, phytoene (*1*). Several lines of investigation have been pursued in order to gain further information about the herbicidal binding site; studies with mutants of the cyanobacteria *Synechococcus* (*2*, *3*) and *Synechocystis* (*4*) led to a model of overlapping binding sites for the phytoene desaturase inhibitors norflurazon, fluridone, fluorochloridone, and flurtamone.

Structure activity investigations provided the most conclusive results on the relationship between herbicidal activity and the nature of various ligands (*5*). However, in many cases an overall herbicidal effect is compared to the structural modifications of a lead compound. Because this whole plant parameter may comprise herbicide uptake, translocation, target interaction, and chemical modification, including degradation, it is difficult to explain the significance of mechanistic contribute. For different chemical classes of bleaching herbicides, structure activity investigations were carried out at the enzyme level by measuring the inhibiton of phytoene desaturase activity. These data exclusively describe the inhibitor target interaction. They provided the first direct information on the very strict spatial arrangement of certain structural elements that are required for potent phytoene desaturase inhibition. This approach may lead to a better understanding of the essential structural elements which determine the potency of a phytoene desaturase inhibitor.

In a certain group of phytoene desaturase inhibitors, the 3-ketomorpholines (*6*), chiral stereoisomers are possible (*7*). Thus, they represent a useful class of phytoene desaturase inhibitors to study the steric requirement for their interaction with the target enzyme. In the present work, the inhibition properties of homochiral stereoisomers of 5-methyl-2-(3-trifluoromethylbenzyl)-3-ketomorpholine on the target enzyme, phytoene desaturase, were determined by enzyme kinetic studies. In addition, the structure−activity investigations for a range of 3-ketomorpholines were carried out by determination of I_{50} values for in vitro inhibition of enzyme activity, to provide more detailed information on the optimized substitution pattern of this series of herbicides for inhibition of the target enzyme.

MATERIALS AND METHODS

Growth Conditions. For supply of phytoene and phytoene desaturase, *E. coli* JM 101 carrying either plasmid pACCRT-BE (*8*) or plasmid pG-pds was used (*9*), respectively. *E. coli* transformants were grown in LB medium (*10*) with either chloramphenicol (35 μg/mL) or ampicillin (0.1 mg/mL), overnight at 37 °C. The cyanobacterium, *Synechococcus* PCC 7942, was grown for 2 days as previously described (*11*) in the presence of inhibitors as indicated.

Synthesis of Compounds. Homochiral ketomorpholines A, B, and C (structures in Figure 1) were derived from optically pure (*S*)- and (*R*)-alaninol. 5(*S*)-Methyl-3-ketomorpholine was prepared from (*S*)-alaninol and chloroacetyl chloride by a two-step process, comprising an initial *N*-acylation followed by a sodium hydride-mediated cyclization. Generation of the dian-

* To whom correspondence should be addressed. Fax: +49 69 79824822. E-mail: sandmann@em.uni-frankfurt.de.

† Goethe Universität.

‡ ZENECA Agrochemicals.

10.1021/jf0010432 CCC: $20.00
Published on Web 12/09/2000

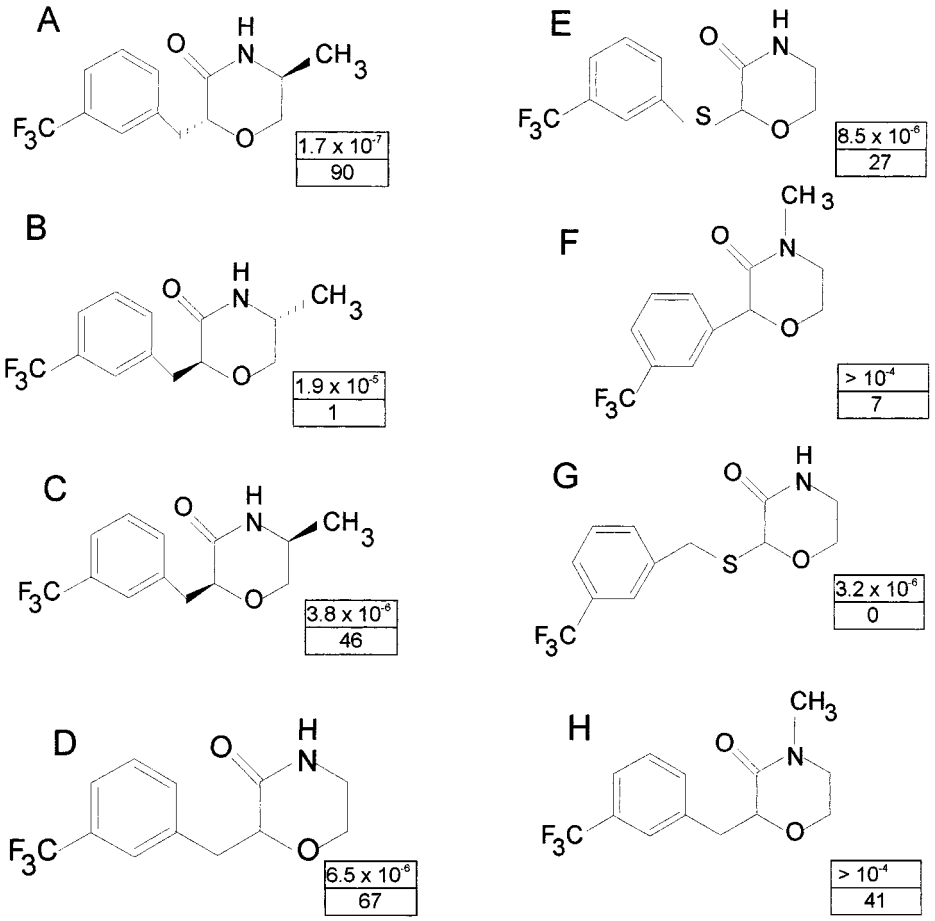

Figure 1. Structures of ketomorpholine derivatives, their I_{50} values (M) for inhibition of phytoene desaturase (top value) and averaged herbicidal activity at 1000 g ha^{-1} (bottom value).

ion of this ketomorpholine using two equivalents of *n*-butyllithium followed by reaction with (3-trifluoromethyl)benzyl chloride afforded the diastereoisomeric compounds A and C, which were readily separated by column chromatography. By similar methods, compound B (the enantiomer of compound A) was prepared from 5(*R*)-methyl-3-ketomorpholine, which was itself obtained from (*R*)-alaninol.

Racemic ketomorpholines D, E, and F were synthesized from 3-ketomorpholine, which was itself prepared from 2-aminoethanol and chloroacetyl chloride. The dianion of 3-ketomorpholine, generated using two equivalents of *n*-butyllithium, reacted with (3-trifluoromethyl)benzyl chloride to afford compound D. Similarly, treatment of the 3-ketomorpholine dianion with bis-((3-trifluoromethyl)phenyl)disulfide and bis-((3-trifluoromethyl)benzyl)disulfide afforded compounds E and F, respectively. Racemic ketomorpholine G was obtained by *N*-methylation of compound D using sodium hydride and methyl iodide. Racemic ketomorpholine H was prepared from α-chloro-3-(trifluoromethyl)phenylacetyl chloride and 2-methylaminoethanol. All of the ketomorpholines were obtained pure, and were fully characterized by proton nuclear magnetic resonance (NMR) and mass spectrometry (MS).

Biological Testing. The preemergence activity of each of compounds A−H was determined in the glasshouse. The plant species included in the tests were *Abutillon theophrasti*, *Amaranthus retroflexus*, *Cassia obtusifolia*, *Chenopodium album*, *Galium aparine*, *Ipomea hederacea*, and *Xanthium strumarium*. Seeds were sown in a loamy sand at a depth of 1 cm and were watered 2 h before spraying and lightly watered afterward. Each compound was formulated in 4% JF4400 (21.8 g $liter^{-1}$ Span 80, 78.2 g $liter^{-1}$ Tween 20, made up to 1 liter with methyl cyclohexanone), and the test plants were sprayed at a spray volume equivalent to 400 liter ha^{-1} at a range of application rates. The treated plants were assessed against untreated plants after 20 days, and herbicidal activity was assigned on a 0 to 100 scale, where 0 is 0% damage and 100% is complete kill. For overall herbicidal potency of the test compounds, the average score was determined for each compound across all the plant species at a single concentration of 1000 g ha^{-1}.

In vitro Phytoene Desaturase Assay. An active desaturase was prepared from the *E. coli* transformant carrying plasmid pG-pds, and the nonradioactive cell-free assay for phytoene desaturase was carried out according to the previously described details (*9*). The substrate phytoene for the enzymatic reaction was extracted from the transformant JM101/pACCRT-EB with hot methanol containing 6% KOH (65 °C, 20 min) and partitioning into 10% ether in petrol. Then, the absorbance at 445 nm was measured, and the carotenoids were quantitated by HPLC (*9*) using a 25-cm Spherisorb ODS 1 column and acetonitrile/methanol/2-propanol (85:10:5, v/v).

RESULTS

Interaction of compound A with phytoene desaturase could be demonstrated after expression of the *Synechococcus* enzyme in *E. coli* (*9*). Inhibition of phytoene desaturase activity by the lead compound A, (2R),(5S) 5-methyl-2-(3-trifluoromethylbenzyl)-3-ketomorpholine (structure in Figure 1), was measured by determination of the in vitro activity of phytoene desaturase from *Synechococcus*, which, on a sequence level, represents a typical plant enzyme (*8*). With increasing concentrations of compound A, the enzymatic formation of ζ-carotene was gradually decreased and phytoene accumulated instead (data not shown). This in vitro assay was used to analyze the nature of the inhibition by compound A by enzyme kinetic studies. To obtain a

Table 1. ED_{50} Values (g ha^{-1}) for Ketomorpholine Derivatives A to H Determined for Different Weeds. Chemical Structures of the Ketomorpholines Are Shown in Figure 1

compound	*Abutillon theophrasti*	*Amaranthus retroflexus*	*Cassia obtusifolia*	*Chenopodium album*	*Galium aparine*	*Ipomea hederacea*	*Xanthium strumarium*
A	50	50	200	25	130	130	340
B	>100000	>10000	>10000	>10000	4000	4000	>10000
C	500	600	1550	500	1500	1500	2300
D	150	80	350	140	380	380	800
E	1000	780	2500	850	4800	4800	>10000
F	5500	>10000	>10000	10000	>10000	>10000	9500
G	>10000	>10000	>10000	>10000	>10000	>10000	>10000
H	1400	1250	500	450	500	500	8900

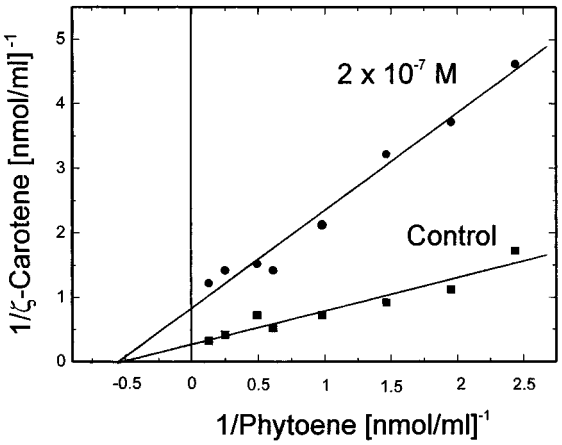

Figure 2. Lineweaver–Burk plot of phytoene desaturase activity versus the concentration of phytoene as a substrate to determine the type of inhibition by ketomorpholine A.

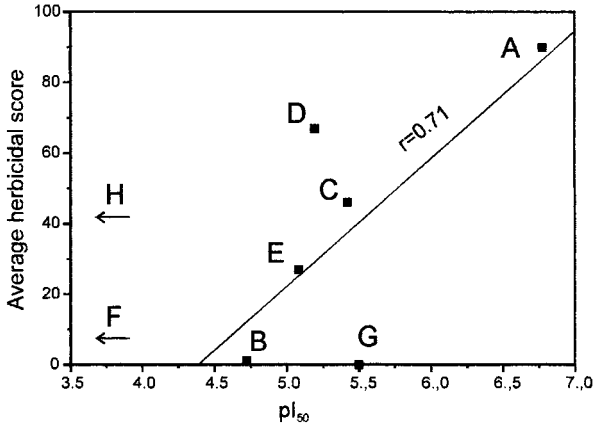

Figure 3. Plot of pI_{50} values for inhibition of phytoene desaturase vs. the average score for each compound across all tested plant species.

Lineweaver–Burk plot, the substrate concentration was varied and the formation of the reaction product was determined in two sets of experiments (Figure 2): one set was a herbicide-free control, and 0.2 μM of compound A was applied in the other set. For both sets of results, straight lines were obtained in a double-reciprocal plot, with a common intersection at the abscissa indicating a noncompetitive interaction between compound A and phytoene desaturase.

A structure–activity investigation for direct inhibitor interaction with the target enzyme phytoene desaturase was carried out with the eight ketomorpholine derivatives shown in Figure 1 (values on top). The I_{50} values for in vitro inhibition of phytoene desaturase by compounds A–H were determined. For the chiral stereoisomers, A – C, compound A [(2R), (5S)-form] is the most potent inhibitor of phytoene desaturase in vitro; compound C [(2S), (5S)-form] was less active, and compound B [(2S), (5R)-form] exhibited very low activity. Racemic ketomorpholine D, which lacks the 5-methyl substituent, is half as inhibitory as compound C. The connection of the 3-CF_3 phenyl ring to position 2 of an equally substituted ketomorpholine moiety is varied in compounds D, E, and G. Highest inhibition was observed with a thiomethylene group (compound G). Replacement of the methylene group of compound D by a sulfur bridge in the isosteric compound E increased the I_{50} value about 3-fold. The *N*-methyl derivatives, H and F, showed no in vitro inhibition of phytoene desaturase.

An influence of different substituents of the ketomorpholine molecule on transport and processes and possible chemical modification before the target enzyme is reached may be revealed by comparison of in vitro parameters for enzyme inhibition with the herbicidal effects on whole plants. Therefore, the herbicidal activity against a range of broad-leaved weeds was obtained for each compound (Table 1). From the data collected for Table 1 the average herbicidal activities at 1000 g ha^{-1} were determined (Figure 1, bottom values) and plotted against the pI_{50} values for in vitro inhibition of phytoene as shown in Figure 3. With the exception of compounds F and H with in vitro I_{50} values $>10^{-4}$, all other ketomorpholines were considered in the plot. A relationship between the pI_{50} values for inhibition of phytoene desaturase and herbicidal activity is evident for most of the compounds. The biggest discrepancy was observed for compounds G and also for compound H. Compound G is moderately active at the enzyme level but completely inactive on whole plants, whereas ketomorpholine H is inactive at the enzyme level but is moderately active as a herbicide.

DISCUSSION

The results described above indicate that the ketomorpholines are classical bleaching herbicides: upon their application phytoene is accumulated at the expense of colored carotenoids. Furthermore, direct inhibition of the target enzyme phytoene desaturase could be demonstrated and this interaction was noncompetitive with respect to the substrate phytoene (Figure 2), as reported for several other bleaching herbicides (*5*). A tobacco transformant, expressing a foreign bacterial phytoene desaturase gene which is structurally completely unrelated to the plant-type enzyme and which thus lacks the common binding site for many bleaching herbicides (*12*), was totally resistant to ketomorpholines (unpublished data). This indicates that the herbicidal activity of the ketomorpholines is exclusively due to inhibition of phytoene desaturase. The I_{50} value of 1.7 $\times 10^{-7}$ M for in vitro inhibition of phytoene desaturase by compound A (Figure 1) ranks the active ketomorpholines with norflurazon and fluorochloridone (*3*). In

contrast, the most potent phytoene desaturase inhibitors, diflufenican, fluridone, and flurtamone, exhibit I_{50} values between 10^{-8} and 10^{-9} M.

For the homochiral derivatives A, B, and C, inhibition of phytoene desaturase appears to be rather more dependent on the stereochemistry of the ketomorpholine 5-methyl substituent than that of the 2-(3-trifluoromethylbenzyl) substituent; compound A [(2R), (5S)-isomer] is the most active, followed by compound C [(2S), (5S)-isomer], with compound B [(2S), (5R)-isomer] being the least active. The enhanced activity of the *trans*-isomer (compound A) over the *cis*-isomer (compound C) was predicted by our earlier model for the herbicide binding site, which was constructed on the basis of whole plant herbicidal activity (*7*) and was now established for interaction of the ketomorpholines with the target enzyme.

The in vitro inhibition of the racemic *N*-methyl derivative (compound H) is *significantly* reduced relative to the corresponding N−H derivative (compound D). By comparison of the in vitro activities of compounds D, E, and G, it is clear that the distance of the substituted phenyl group from the ketomorpholine ring is an important factor for their interaction with phytoene desaturase. The influence of either $-CH_2-$ or $-S-$ as a linking group is very similar, but $-CH_2S-$ as a linking group significantly increased phytoene desaturase inhibition of compound G. This derivative is isosteric with the CH_2-CH_2-linked compound which was predicted in our earlier modeling work (based on overall herbicidal activity) to be a less-active herbicide than compound D (*7*). The difference may be explained by a better interaction of compound G via the sulfur group in addition to the negative steric effect of the extended bridge.

Recently, a good correlation between inhibition of phytoene desaturase and preemergence herbicidal activity for a series of bleaching 3-trifluoromethyl-1,1′-biphenyl derivatives assayed with two plant species was reported (*13*). This is also the case for our ketomorpholine derivatives (Figure 3) when considering the average herbicidal score obtained with 7 different weed species. However, compounds G and H did not fit into the correlation. In whole plants, compound G is less herbicidal than expected from its phytoene desaturase I_{50} value. One explanation may be in vivo oxidation of this compound to a less potent sulfoxide or sulfone derivative: we know that the corresponding sulfides (mixture of diastereoisomers) and sulfone of compound E are inactive as herbicides (Mitchell et al., unpublished). In contrast, compound H shows rather better activity in whole plants than might be expected from its poor in vitro phytoene desaturase activity. Here again a modification in plants may occur, most likely *N*-demethylation to compound D which is a much more active herbicide; similar activation by *N*-demethylation has been reported for the bleaching herbicide metflurazon (*14*). As judged from the highly variable pattern of weed susceptibility towards compound H as compared to that of the other ketomorpholines (e.g., *Xanthium strumatum* showed a 15 to 20-fold higher ED_{50} value than *Cassia obtusifolia*, *Chenopodium album*, or *Galium aparine*; Table 1), the potential to modify compound H may be different in various plants.

ACKNOWLEDGMENT

The authors thank Dr. D. J. Collins and Mr. C. V. Coles for the herbicidal activity determinations.

LITERATURE CITED

(1) Böger, P.; Sandmann, G. Carotenoid biosynthesis inhibitor herbicides − mode of action and resistance mechanisms. *Pestic. Outlook* **1998**, *9*, 29−35.

(2) Chamovitz, D.; Sandmann, G.; Hirschberg, J. Molecular and biochemical characterization of herbicide-resistant mutants of cyanobacteria reveals that phytoene desaturation is a rate-limiting step in carotenoid biosynthesis. *J. Biol. Chem*. **1993**, *268*, 17348−17353.

(3) Sandmann, G.; Fraser, P. D. Differential inhibition of phytoene desaturase from diverse origins and analysis of resistant cyanobacterial mutants. *Z. Naturforsch., C: J. Biosci*. **1993**, *48c*, 307−311.

(4) Martinez-Ferez, I.; Vioque, A.; Sandmann, G. Mutagenesis of an amino acid responsible in phytoene desaturase from *Synechocystis* for binding of the bleaching herbicide norflurazon. *Pestic. Biochem. Physiol*. **1994**, *48*, 185−190.

(5) Sandmann, G.; Böger, P. Chemical structure and activity of herbicidal inhibitors of phytoene desaturase. In *Rational Approaches to Structure, Activity, and Ecotoxicology of Agrochemicals*; Draber, W., Fujita, T., Eds.; CRC Press: Boca Ration, FL, 1992; pp 357−371.

(6) Mitchell, G.; Bartlett, D. L. 2-(3-Trifluoromethylbenzyl)-3-ketomorpholine − a new phytoene desaturase inhibitor: optimisation of *in vitro* activity using computer modelling. In *7th Int. Congr. Pestic. Chem., Book of Abstracts Vol I.*; Frehse, H., Kesseler-Schmitz, E., Conway, S., Eds.; Poster 01D-04, Hamburg, Germany, 1990; p 170.

(7) Mitchell, G. Phytoene desaturase. A model for the optimization of inhibitors. In *Synthesis and Chemistry of Agrochemicals IV*, Baker, D. A., Fenyes, J. G., Moberg, W. K., Cross, B., Eds.; ACS Symposium Series, Vol. 584, American Chemical Society: Washington, DC, 1995; pp 161−170.

(8) Pecker, I.; Chamovitz, D.; Linden, H.; Sandmann, G.; Hirschberg, J. A single polypeptide catalyzing the conversion of phytoene to ζ-carotene is transcriptionally regulated during tomato fruit ripening. *Proc. Natl. Acad. Sci., U.S.A.* **1992**, *89*, 4962−4966.

(9) Sandmann, G.; Schneider, C.; Böger, P. A new nonradiactive assay of phytoene desaturase to evaluate bleaching herbicides. *Z. Naturforsch., C: J. Biosci.* **1996**, *51c*, 534−538.

(10) Maniatis, T.; Fritsch, E. F.; Sambrook, J. *Molecular Cloning*. A Laboratory Manual, 2nd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1982.

(11) Sandmann, G. *In vitro* assay system for phytoene desaturase inhibitors with isolated thylakoids, In *Target Assays for Modern Herbicides and Related Phytotoxic Compounds*; Böger, P., Sandmann, G., Eds.; Lewis Publishers, Boca Raton, FL, 1993; pp 15−20.

(12) Misawa, N.; Masamoto, K.; Hori, T.; Ohtani, T.; Böger, P.; Sandmann, G. Expression of an *Erwinia* phytoene desaturase gene not only confers multiple resistance to herbicides interfering with carotenoid biosynthesis but also alters xanthophyll metabolism in transgenic plants. *Plant J*. **1994**, *6*, 481−489.

(13) Laber, G.; Usunow, E.; Wiecko, W.; Franke, H.; Köhn, A. Inhibition of *Narcissus pseudonarcissus* phytoene desaturase by herbicidal 3-trifluoromethyl-1,1′-biphenyl derivatives. *Pest. Biochem. Physiol*. **1999**, *63*, 173−184.

(14) Motooka, P. S.; Corbin, F. T.; Worsham, A. D. Metabolism of Sandoz 6706 in soybean and sicklepod. *Weed Sci*. **1977**, *25*, 9.

Received for review August 24, 2000. Revised manuscript received October 25, 2000. Accepted October 25, 2000.

JF0010432