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Topographical Geometric Factors Governing the Biological Activities of Methomyl Derivatives on Binding Receptors of Maize Mitochondria

I. Biological Activities

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The topographical study of the binding receptors of T and N mitochondria was undertaken with methomyl derivatives acting as molecular probes. The two biological activities related to topography and geometric isomerism are distinguishable from the receptor sites of T and N mitochondria.

INTRODUCTION

Methomyl, or *S*-methyl *N*-[(methylcarbamoyl)oxy]thioacetimidate, is an insecticide with a specific action toward sterile male Texas maize. This activity is analogous to that of toxins produced by the phytopathogenic fungi *Helminthosporium* and *Phyllosticta maydis*. Even though this action is less intense than that of the toxin, methomyl has the undeniable advantage of being available in large quantities and at a satisfying degree of purity (*1*). Prior results have indicated that there are relationships between toxicity toward T and N cytoplasms and the structural variations of methomyl (*2, 3*). In addition, the biological activity of the two geometric isomers of methomyl toward T and N has been investigated and it was found that specific action is a conformational property of the molecule (*4*). Nevertheless, these results raise questions concerning receptor topography. Thus, do toxicity and specific action correspond to distinct receptors or to the same receptor whose topography can bind the two geometric isomers of methomyl? In other words, can we explain the specific action of methomyl as a bivalent phenomenon in terms of T and N cytoplasms.

In this context, we have synthesized a family of molecules which may be useful for elucidating receptor topography. These molecular probes, as well as prior results (*2–4*), should lead to the distinction of a number of topographic geometric criteria involved in the biological activity of methomyl.

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RESULTS

The different carbamates in Table 1, previously described (*2–4*) and synthesized in this work (**2**, **3**, **4**, **6**, **7**, and **8**), were tested against T and N maize mitochondria, using previously described criteria, techniques, and procedures (*3, 4*). The tests used involve (1) the oxidation of succinate, malate, and NADH by T or N mitochondria; and (2) changes in absorbance at 520 nm (A_{520}) by mitochondrial suspensions.

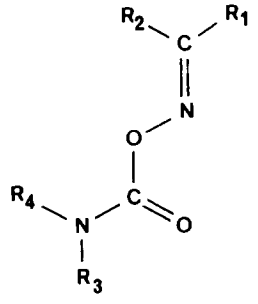
The results of this test showed that toxin HmT and geometric isomer *Z* of methomyl: (a) are inactive on N mitochondria; and (b) present the following activity (activity A) on T mitochondria: stimulation of NADH oxidation (novel and characteristic effect of these products); stimulation of succinate oxidation; inhibition of malate oxidation; and decreased A_{520} of mitochondrial suspensions.

In addition, geometric isomer *E* of methomyl and some methomyl derivatives have a nonspecific action (activity B) on both N and T mitochondria, shown by the

TABLE 1

RELATIONSHIP BETWEEN BIOLOGICAL ACTIVITY AND STRUCTURE OF INDICATED CARBAMATES

1	$R_1 = CH_3$	$R_2 = SCH_3$	$R_3 = H$	$R_4 = CH_3$ [a]
2	$R_1 = R_2 = SCH_3$		$R_3 = H$	$R_4 = CH_3$ [a]
3	$R_1 = CH_3$	$R_2 = S(CH_2)_3CH_3$	$R_3 = H$	$R_4 = CH_3$ [a]
4	$R_1 = CH_3$	$R_2 = OCH_2CH_3$	$R_3 = H$	$R_4 = CH_3$ [a]
5	$R_1 = CH_3$	$R_2 = SCH_3$	$R_3 = H$	$R_4 = CH_2CH_3$ [a]
6	$R_1 = CH_3$	$R_2 = SCH_3$	$R_3 = H$	$R_4 = (CH_2)_3CH_3$ [a]
7	$R_1 = CH_3$	$R_2 = SCH_3$	$R_3 = H$	$R_4 = CH(CH_3)_2$ [a]
8	$R_1 = R_2 = CH_2CH_3$		$R_3 = H$	$R_4 = CH_3$ [b]
9	$R_1 = CH_3$	$R_2 = (CH_2)_3CH_3$	$R_3 = H$	$R_4 = CH_3$ [b]
10	$R_1 = CH_3$	$R_2 = SCH_3$	$R_3 = R_4 = CH_3$	[c]
11	$R_1 = CH_3$	$R_2 = SOCH_3$	$R_3 = H$	$R_4 = CH_3$ [c]
12	$R_1 = CH_3$	$R_2 = CH(CH_3)COOC_2H_5$	$R_3 = H$	$R_4 = CH_3$ [c]
13	$R_1 = CH_3$	$R_2 = (CH_2)_2C(=NOCONHCH_3)CH_3$	$R_3 = H$	$R_4 = CH_3$ [c]



[a] Active and specific.
[b] Active and not specific.
[c] Nonactive.

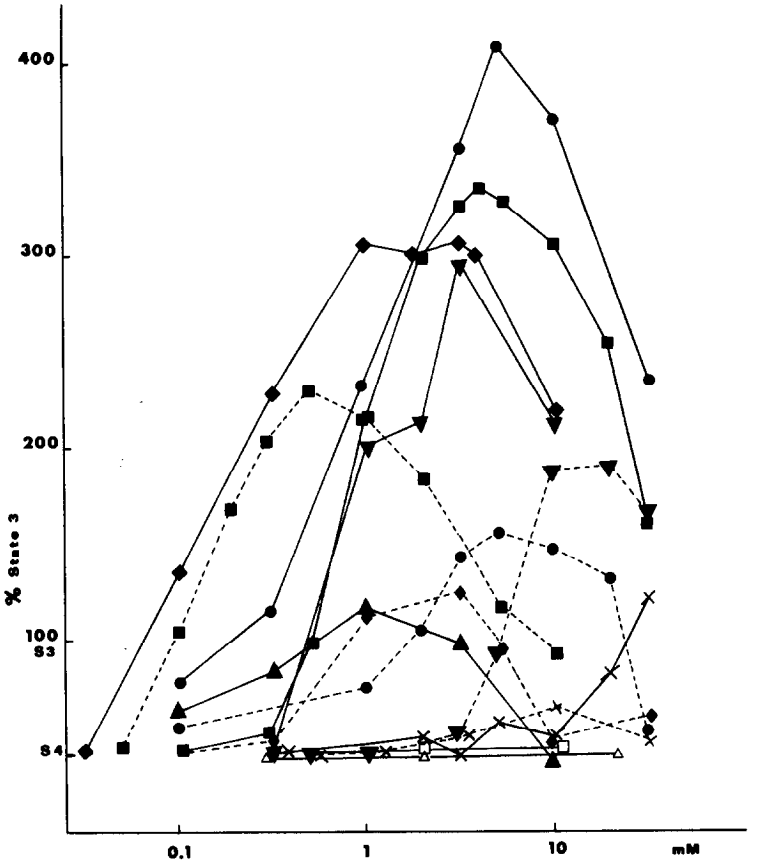


FIG. 1. Dose–response curves of methomyl analogs. Results were normalized as explained in the text. Symbols: (■—■) methomyl, (●—●) *N*-ethyl, (◆—◆) *N*-propyl, (▼—▼) *N*-ipropyl, (▲—▲) "9," (■---■) S-butyl, (●---●) 2(S-CH_3), (◆---◆) OC_2H_5, ▼---▼) 2(C_2H_5), (×---×) $N(CH_3)_2$, (×—×) sulfoxide, (□—□) "12," (△—△) "13," (S3) state 3, (S4) state 4.

inhibition of oxidation of *all substrates* (succinate, malate, NADH) and increased A_{520} absorption of mitochondrial suspensions.

Figure 1 shows that 4 mM methomyl is maximally active against NADH oxidation. Its efficiency regularly increases from 0.5 to 4 mM and subsequently decreases. Figure 1 also shows that the chemical modifications of the methomyl molecule resulted in distinct types of changes in the dose–response curve exhibited by the resulting derivatives as far as NADH oxidation was concerned:

(1) The *N*-ethyl, the *N*-isopropyl, and the $S(CH_3)_2$ derivatives had, like methomyl, a maximum activity in the range 3–5 mM. The $S(CH_3)_2$ derivative was about twice less as effective as methomyl in stimulating NADH oxygen consumption.

(2) The 2(C_2H_5), $N(CH_3)_2$, and sulfoxide derivative had a maximum activity at concentrations higher than 5 mM. At 10 mM, the two latter compounds had only a marginal action. Maximum activity for sulfoxide could not be determined because of the very high concentrations implied.

(3) The *N*-propyl, *N*-butyl, *S*-butyl, and OC_2H_5 derivatives as well as compound **9** had a maximum activity at concentrations lower than 2 mM. The two latter ones had only a limited action.

(4) Compounds **12** and **13** had no effects.

DISCUSSION

Let us consider the conformation of methomyl **1** and its various derivatives **2–12**. The preferential conformation of these carbamates, deduced from X-ray diffraction studies of **1**, is the geometric isomer *Z*, which is unique for derivatives **1**, **2**, **5–8**, and **10**. ^{13}C NMR had confirmed the attribution of this conformation. In the other cases, an $E \rightleftarrows Z$ equilibrium is noted. It is in fact of utmost importance to take geometric isomerism into account when estimating specific action. Carbamates **1–9** are active against Texas cytoplasm and some of them are specific to varying degrees, according to the position of the above equilibrium. Prior results (*3, 4*) have indicated the relationship between biological activity and electron delocalization of methomyl, which changes according to the substituents R_1 and R_2. We will not deal with the intensity of this activity here.

Let us consider the planar representation of the different carbamates (Fig. 2) (*5*). Consider the upper part of the molecule, characterized by linear substituents R_1 and R_2, with or without heteroatoms (active carbamates **1–9**, Table 1). A topography which could accept all these substituents is shown in Fig. 2, with one lack of precision corresponding to the hybridization direction given by atom *X*. It is important to note that the hybridation of carbon, hydrogen, oxygen, and sulfur leads to a valence angle very close to 110° in the three cases. Consequently, carbamates **10–13** which present substitutions on the hydrocarbonated chain are totally inactive. On the basis of the preceding representation (Fig. 2) we may conclude that they can no longer adapt by their ramification to the receptor.

Geometric isomerism is defined by a 180° rotation of the carbamate function around the C=N double bond. Biological activities A and B against T and N mitochondria have been characterized and we may envision that they depend on the *cis* or *trans* conformation of the four atoms *X*C=NO, respectively. The activity of the *Z* conformers of methomyl and its derivatives **3**, **4**, and **9** are similar to that of *Helminthosporium maydis* toxins, specific for T mitochondria, while the activity of *E* conformers is different and nonspecific. It is thus very probable that the receptor contains an *X* cavity perfectly adapted to sulfur, although this atom is not indispensable to biological activity (*X* = S, O, CH_2—carbamates **1–4**, **8**, and **9**). The van der Waals radius of sulfur is greater than those of oxygen and carbon and so the interaction of these two atoms with the receptor is considerably attenuated. The corresponding carbamates are consistently less active than methomyl, since oxygen and methylene are not isosteric with sulfur.

Now let us consider the lower part of the molecule and the axes of rotation I, II, and III, centered on the carbamate function. The corresponding rotational isomerisms are compatible with the geometric isomerism. The preferential conformation adopted around axis I (Fig. 2) accounts for the structure determined by X-ray

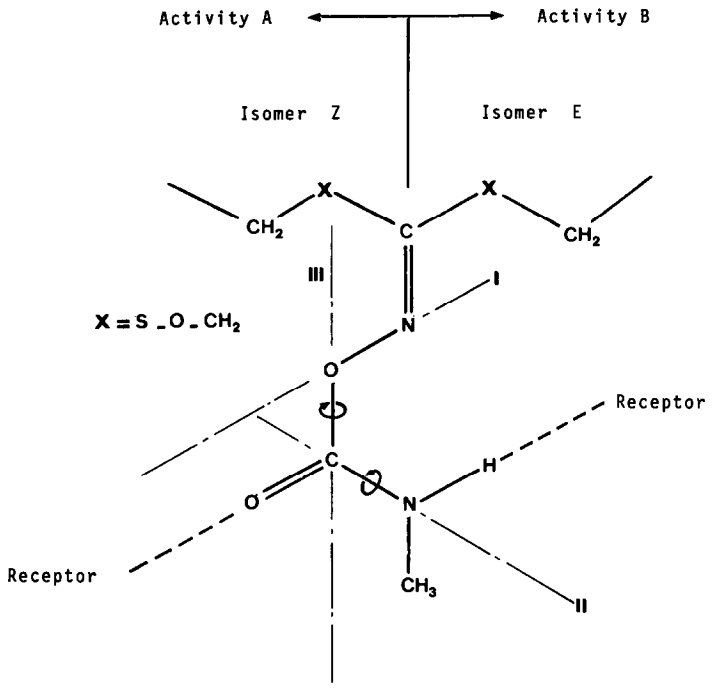


FIG. 2. Limit positioning of methomyl derivatives on the site binding of mitochondria. The arrow indicates the conformation of the carbamate function with respect to the plane of geometric isomerism. The dotted lines represent hydrogen bonds with the receptor (*6*).

diffraction. It is consistent to admit that this conformation minimizes intramolecular interactions, since a 180° rotation of the carbamate function around axis I causes considerable steric hindrance with substituent R_1. It is for this reason that after synthesis, carbamates are present in the form of uniquely the *Z* isomer or as a mixture of *E* and *Z* isomers, the latter predominant.

If we admit free rotation around axis II, the hydrogen and methyl on the sp_3 nitrogen are interchangeable. As a result of this, the receptor can no longer distinguish between the $NHCH_3$ and $N(CH_3)_2$ derivatives. Determinations of activities against T and N mitochondria, however, indicate exactly the opposite, i.e., the nitrogen dimethyl derivative **10** is no longer active. It should be noted that the —NHC=O group is included in a large number of molecules with biological properties with broad diversity. It is admitted that this structure leads to a considerable variety of complexes via hydrogen bonds (*6*). That utilized (Fig. 2) has no particular significance although it is the most frequently depicted because his favored energetic interaction. One may obviously imagine other complexes by appropriate rotations around the axis II and III. As a result, it is thus highly probable that the final conformation depends on hydrogen bonds with the receptor. These bonds would lead to the conformational fixation of the carbamate function and its intimate interaction with the active biological site. Esters of noncarbamate acids which do not have this structural feature are inactive (*2*, *3*).

Variations of substituent R_4 (carbamates **5**, **6**, and **7**) maintain specific action which decreases considerably when passing to the thioamide function (*3*, *4*). The sulfur atom is more polarizable than oxygen and so has a larger van der Waals radius. Its contribution to the pseudocycle formed by hydrogen bonding with the receptor is thus considerably disturbed, probably by a conformational change induced by steric interaction. Furthermore, carbamates **5–7** show that there is not topographical restriction for the size of the first nitrogen substituent.

Finally, we note that axis III situated in the plan of *X*C=NO atoms, corresponds to the privileged axis of specific action, to the extent that the two intrinsic interactions of specificity are located on this axis (activity A). The nonspecific *E* isomer of methomyl is not characterized by this axis (activity B). Carbamate **2** was synthesized and this molecular probe corresponds somewhat to the superimposition of methomyl conformers *E* and *Z*. Activity tests of these molecules toward T and N cytoplasms have shown the following:

Molecules	T	N
methomyl *Z*	active (A)	inactive
methomyl *E*	active (B)	active (B)
2	active (A)	inactive
3	active (A)	inactive
4 (*E* + *Z*)	active (A)	active (B)
4 (*Z*)	active (A)	inactive

It can be seen that activity toward N mitochondria is a dual phenomenon by conformers *E* and *Z*, which is not the case for T mitochondria. Carbamate **2** is specific and so we may admit that it adapts on the T receptor according to a topographical model similar to that described previously. All these results indicate that the biological activity of some carbamates toward T mitochondria may be reflected by special and specific interactions with these mitochondria. It thus corresponds to a structural differentiation between T and N mitochondria, which would lead to an additional interaction with T mitochondria. The latter thus have the possibility of interacting with *Z* and *E* sulfur atoms, while N mitochondria can interact only with *E* sulfur. In other words, T mitochondria possess a receptor site able to interact separately with the *E* and *Z* isomers and the carbamate **2** superposition of the two formers. For this reason, the partial occupation of the receptor site by *E* and *Z* isomers corresponds to two biological answers A and B. Topographically speaking, these interactions are probably represented by two cavities adapted to the van der Waals radius of sulfur and by a specific binding obtained with specific hydrogen bonds between the receptor site and the carbamate function.

The participation and importance of the hydrogen bond has been shown in the case of *Helminthosporium maydis* and *Phyllosticta maydis* toxins (*7–9*), which are polyketols and polyols with long linear chains, capable of forming this type of donor–receptor bonds. The biological sites responsible for the activity of these molecules are not yet known and we cannot yet say if they are identical to the

active biological sites of phytotoxins and methomyl. A number of hypotheses have been put forth to explain the mechanism of action. One of them may be reasonably rejected, since ethers specific to the cations Li, K, and Na, as well as calcimycin, are inactive toward T and N mitochondria and toward the germination of T and N maize seeds. Nor is it possible to categorically reject the hypothesis of polyketols and polyols being potential ionophores, especially for Ca^{2+} (*10, 11*). The topographic description of the biological site active toward methomyl appears to be more appropriate to the possibility of a specific interaction with a polypeptide specific to T mitochondria. The receptor site of E conformers would be present in both N and T mitochondria.

Results obtained by Forde *et al.* (*12*) are compatible with the above conclusions. Thus, specific action reflects a molecular structural differentiation between T and N mitochondria, clearly shown by the methomyl derivatives. We are currently attempting to identify this polypeptide by using an appropriate labeled carbamate.

EXPERIMENTAL

Synthesis of carbamates ***1–13.*** The synthesis and spectroscopic data of methomyl derivatives will be described elsewhere.

Preparation of mitochondria. They were isolated from epicotyls of F_7T (male sterile) and F_7N (male fertile) maize (*Zea mays* L.) lines. The plants were grown on eight layers of wet germination paper for 7 days at 25°C in the dark. Epicotyls (about 10 g fresh wt) were ground in a mortar and pestle in 30 ml of a medium containing 0.4 M mannitol, 1 mM EGTA, 1 mg · ml^{-1} bovine serum albumin (BSA), 4 mM cysteine, and 0.1 M morpholinopropanesulfonate buffer (Mops), pH 7.6. After straining through four layers of cheesecloth, the homogenate was centrifuged at 2000*g* for 10 min, and the resulting supernatant fraction was centrifuged at 10,000*g* for 10 min. The pellet from the second centrifugation ws resuspended in 2 ml of 0.3 M mannitol, 1 mg · ml^{-1} BSA, and 10 mM Mops, pH 7.2, and was layered on a discontinuous Percoll gradient (15, 22.5, and 45% (v/v) Percoll in the same mannitol–MOPS–BSA solution as before). After centrifuging for 15 min at 40,000*g*, the mitochondria were collected at the 22.5–45% Percoll interface and centrifuged at 10,000*g* for 10 min to remove most of the Percoll from the fraction. All operations were done at 0–4°C.

Oxidative studies were performed conventionally (see Aranda *et al.* (*1*) for details). The reaction medium contained 300 mM mannitol, 10 mM KCl, 5 mM $MgCl_2$, 10 mM KP_i, pH 7.2. Respiratory control ratios were consistently above 2.5 with either succinate (12.5 mM) or NADH (2 mM) as substrates, indicating that the mitochondria were coupled. QO_2 was 650–850 nmol O_2 · min^{-1} · mg $protein^{-1}$ for NADH in state 3 (excess ADP). Purified maize mitochondria were used throughout this study, since they showed no decreased sensitivity toward methomyl with time, as first described with washed mitochondria by Pham and Gregory (*5*) and experienced by ourselves (*3, 4*). In order to study the stimulation of NADH oxidation by methomyl analogs, about 0.25 mg · ml^{-1} mitochondrial protein was suspended in the respiratory medium, the reaction was started by adding

2 mM NADH, a first phosphorylation cycle was initiated by 0.1 mM ADP, and the compound under study was added 1 min after the return to state 4 conditions (ADP limiting). Most methomyl analogs induced a nonlinear enhancement of NADH oxidation and so their effect was measured 3 min after their addition to the reaction medium. The resulting stimulation was expressed as per cent of the previous state 3 rate of oxygen consumption.

There were some variations in respiratory control ratios between different mitochondrial preparations as well as in the extent of the stimulation of NADH oxidation. Although they were limited (σ were lower than 20% of the mean), in order to present in Fig. 1 a comprehensive view of the activity of methomyl analogs, we had to normalize both respiratory control ratios and the stimulation of NADH oxidation. As reference numbers, we choose, respectively, 2.5 and 335%, for respiratory control ratio and stimulation of NADH oxidation by 4 mM methomyl (a 4 mM methomyl control was done for each preparation).

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