Phytochemistry, Vol. 26, No. 7, pp. 1909–1913, 1987.
Printed in Great Britain.

0031–9422/87 $3.00 + 0.00

METHOMYL ANALOGUES WITH INCREASED BIOLOGICAL ACTIVITY TOWARDS F_7T MAIZE MITOCHONDRIA

GÉRARD ARANDA, PHILIPPE DURLIN* and CHRISTIAN GAUVRIT*†

Laboratoire de Synthèse Organique, Ecole Polytechnique, 91128 Palaiseau Cedex, France; *Laboratoire des Herbicides, INRA BV 1540, 21034 Dijon Cedex, France

(*Revised received* 5 *December* 1986)

Key Word Index—*Zea mays*; Graminae; maize; male sterility; *Helminthosporium maydis* race T toxin; Methomyl; structure–activity relationships; lipophily.

Abstract—Methomyl analogues were synthesized by substituting alkyl moieties (C_2–C_{21}) in the place of the carbamic methyl. They were assayed on mitochondria isolated from male sterile (F_7T) and male fertile (F_7N) maize. They had no action on F_7N mitochondria. The heptadecyl (C_{17}) and heneicosanyl (C_{21}) derivatives had no conspicuous effect on F_7T mitochondria. By contrast, the ethyl, propyl, butyl, nonyl, tridecyl (C_{13}) and pentadecyl (C_{15}) derivatives had the same type of activity as Methomyl on F_7T mitochondria, namely stimulation of NADH oxidation and inhibition of malate oxidation. Moreover, the concentration at which they were maximally effective decreased from 10 mM (Methomyl) to 3 μM (tridecyl derivative); hence, the latter compound has a biological activity which is nearly the same as that of *Helminthosporium maydis* toxin.

INTRODUCTION

Maize (*Zea mays* L.) with a mitochondrial gene for male sterility (Texas male sterile cytoplasm or T) is highly susceptible to *Helminthosporium maydis* race T (HmT). A host-specific pathotoxin produced by HmT was purified and showed the same specificity as HmT [1]. A growing body of evidence suggests that mitochondria are the site of toxin action [2]. On isolated mitochondria HmT toxin stimulates exogenous NADH oxidation and inhibits malate oxidation [2, 3]. The insecticide Methomyl (*S*-methyl-*N*-methylcarbamoyl-oxythioacetimidate, Me-NH–CO–O–N=C(Me)–S·Me) was found to mimic the action of HmT toxin, both in plants [4] and on isolated mitochondria [5]. The actions of HmT toxin and Methomyl on F_7T mitochondria are thought to involve membrane permeation to NAD (and possibly to NADH) [6, 7], as well as uncoupling [8]. According to this view, following addition of HmT toxin or Methomyl to mitochondria oxidizing exogenous NADH, this substrate can enter the matrix and be oxidized by both the external and internal NADH dehydrogenases. It results in an increase in NADH rate of oxidation. As NAD is a cofactor of malate oxidation, inhibition of this reaction would be caused by NAD depletion in the matrix [7].

Purified Methomyl shows toxic and specific action against isolated mitochondria and on sprouting of corn seeds with a Texas cytoplasm [9]. We also carried out studies on the activity of Methomyl analogues on these two systems. We showed that only the *Z*- and not the *E*-isomer of Methomyl mimics toxin activity in F_7T mitochondria [10]. We also found that the efficiency of Methomyl is governed by very stringent structural requirements and by the extent of its electron conjugated system [9, 11, 12]. For instance, substitution of the carbamic methyl moiety by a phenyl group leads to an ineffective and non-selective compound. By contrast, substitution by an ethyl moiety gives a derivative that retains the selective action of Methomyl towards F_7T mitochondria. Hence, we synthesized analogues with alkyl (C_2–C_{21}) moieties in the place of the carbamic methyl, in order to see the resulting change in activity on F_7T mitochondria.

†Author to whom correspondence should be addressed.

RESULTS

Oxidative phosphorylation

All derivatives were without any effect on F_7N maize mitochondria (data not shown). Fig. 1 shows that most of them had basically the same type of action on F_7T mitochondria as Methomyl, namely stimulation of NADH oxidation and inhibition of malate oxidation. Their action on succinate oxidation was less characteristic.

NADH oxidation. On F_7T mitochondria, heptadecyl (C_{17}) and heneicosanyl (C_{21}) derivatives were only marginally effective on NADH oxidation (Figs 1–3). They did not inhibit oxidative phosphorylation since they did not prevent establishment of state 3 (ADP phosphorylation) then state 4 (non-phosphorylative state). All other derivatives stimulated NADH oxidation in state 4. Their dose-response curves were bell-shaped (Fig. 2) and up to the tridecyl (C_{13}) derivative the longer the alkyl chain, the lower the concentration at which they were maximally effective: C_1 (Methomyl), 10 mM; C_2, 9–10 mM; C_3, 4 mM; C_4, 0.3 mM; C_9, 7 μM; C_{13}, 3 μM. Hence, the

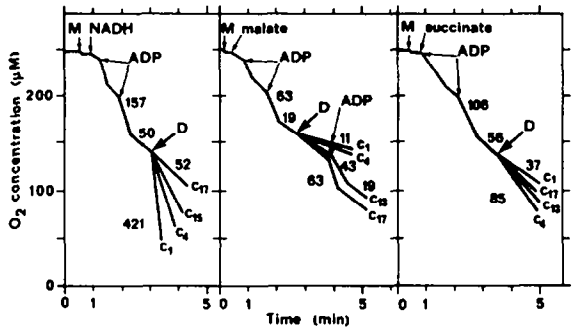

Fig. 1. Action of some Methomyl derivatives on the oxidation of NADH, succinate and malate by F_7T mitochondria. M, purified mitochondria. Substrates concentrations were: NADH, 2 mM; succinate, 12.5 mM; malate, 50 mM. ADP additions were 0.1 then 0.2 mM. Methomyl derivatives additions (D) were: C_1 (Methomyl), 10 mM; C_4, 0.3 mM; C_{13}, 3 μM; C_{15}, 50 μM; C_{17}, 0.1 mM. The numbers on the figure are O_2 consumption rates (nmol O_2/min/mg protein). These data are typical of experiments which were at least triplicated.

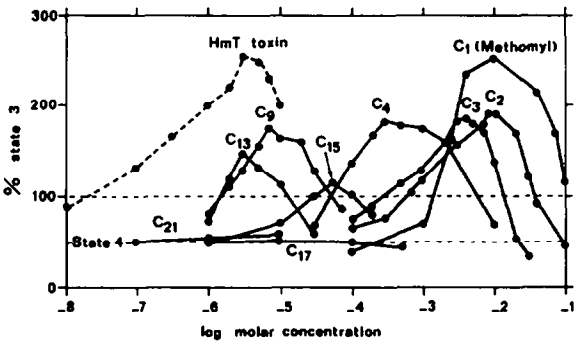

Fig. 2. Dose-response curves for stimulation of NADH oxidation in F_7T mitochondria by Methomyl derivatives. The compounds are indicated by the number of carbon atoms in their *N*-alkyl chain. Data are presented as explained in 'Experimental' and come from experiments that were at least triplicated.

tridecyl (C_{13}) derivative and HmT toxin showed their maximum activity in the same concentration range. The pentadecyl (C_{15}) derivative did not follow this trend since it was maximally effective at 50 μM.

The extent of maximal stimulation of NADH oxidation decreased with increasing chain length. It was, as expressed in percent of state 3 rate of NADH oxidation: C_1 (Methomyl), 157; C_2, 95; C_3, 89; C_4, 84; C_9, 79; C_{13}, 50; C_{15}, 18; C_{17} and C_{21}, marginal and not determined (HmT toxin, 161).

Malate oxidation. At the pH we used (7.2) malate is prominently oxidized by malate dehydrogenase [13]. Methomyl as well as its analogues up to the pentadecyl (C_{15}) derivative inhibited malate oxidation. This action is shown in Fig. 3, at concentrations corresponding to the maximal effect on NADH oxidation. The extent of state 4 inhibition regularly decreased as chain length increased (ethyl, 68%; tridecyl, 23%). The C_{17} and C_{21} derivatives were without any effect and did not prevent a further phosphorylation cycle following ADP addition.

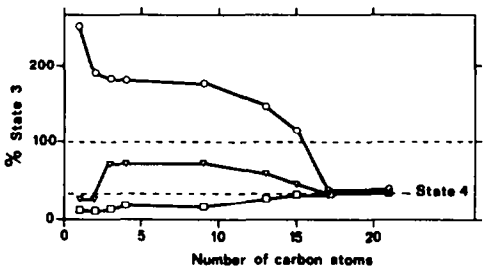

Fig. 3. Action of Methomyl derivatives on NADH, malate and succinate oxidation in F_7T mitochondria.

Methomyl derivatives were administered in state 4 and the resulting rate of substrate oxidation was expressed in % state 3. Results were normalized to a state 3/state 4 ratio of 2.85.

Effects of Methomyl derivatives on malate and succinate oxidation were measured at the concentration which gave maximal stimulation on NADH oxidation. NADH, ○; malate, □; succinate, ▽.

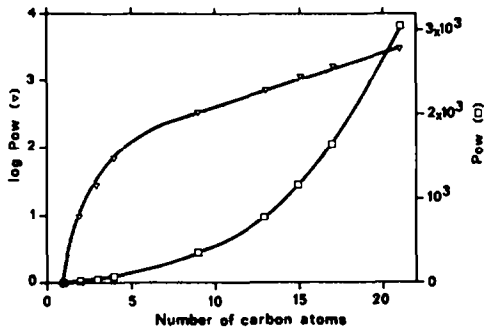

Fig. 4. *n*-Octanol/water partition coefficients (Pow) of the Methomyl analogues as a function of the number of carbon atoms in their alkyl chain.

Succinate oxidation. Figure 3 shows that the ethyl and propyl analogues slightly inhibited succinate oxidation in state 4. The butyl, nonyl, tridecyl and pentadecyl analogues stimulated it (by 73% in the case of the butyl and nonyl derivatives). The mitochondria were uncoupled in the presence of the ethyl to nonyl derivatives, and remained only partially coupled in the presence of the tridecyl and pentadecyl derivatives (data not shown). The C_{17} and C_{21} analogues were without any conspicuous action.

In our experiments, Methomyl behaved as a poor uncoupler, as was also observed by Koeppe *et al.* [5]. This seems to contradict Klein and Koeppe's results [8], which showed that Methomyl permeabilizes the inner mitochondrial membrane to H^+ ions. The discrepancy could be due to an inhibition of the succinate-driven electron flow, the inhibitory action concealing the stimulation brought about by the uncoupling. Indeed, Methomyl inhibited state 3 succinate oxidation (data not shown).

n-*Octanol/water partition coefficients*

Figure 4 shows that the *n*-octanol/water partition coefficients of the Methomyl analogues regularly in-

creased with the alkyl chain length. They were: C_1 (Methomyl), 0.9; C_2, 9; C_3, 28; C_4, 68; C_9, 355; C_{13}, 768; C_{15}, 1162; C_{17}, 1653; C_{21}, 3058.

DISCUSSION

The most striking feature of our results is given by the ethyl–tridecyl Methomyl analogues. First, the longer their *N*-alkyl chain, the lower the concentration at which the analogues were maximally active. In the case of the tridecyl derivative, this concentration was about the same as for HmT toxin. Secondly, these compounds retain the selective action of Methomyl (and HmT toxin) towards F_7T maize mitochondria. In other words, they are without any action against F_7N (male fertile) mitochondria, while they stimulate NADH oxidation and inhibit malate oxidation in F_7T mitochondria.

The increase in the *N*-alkyl chain length results in a tremendous increase in lipophilicity since the *n*-octanol/water partition coefficient of Methomyl (C_1) is 0.9 whereas that of the tridecyl derivative is 768. The target of Methomyl is probably located in the inner mitochondrial membrane [7]; hence, these compounds equilibrate with their target in a lipophilic environment. The higher their lipophilicity, the higher their partition into the mitochondrial membrane, the higher their concentration in the vicinity of the target and the greater their occupancy of the active site. This would explain why from the ethyl to the tridecyl derivatives we observed a decrease in the active concentrations.

This hypothesis is in line with the work of Susuki *et al.* [14, 15] who studied the biological activity of synthetic HmT toxin analogues. They found that the selective action of the derivatives was governed by strict requirements about the relative position of the β-ketol moieties: they were more active if they were spaced by $-(CH_2)_3-$ or $-(CH_2)_5-$ bridges. Moreover, as in our study, there was a clear relationship between the length of the alkyl chain in the analogues and the concentration at which they were active. Hence, lipophilicity plays a great part in the activity of both HmT toxin and Methomyl derivatives.

However, this view cannot account for two of our results. Firstly, the pentadecyl derivative is far less active than the tridecyl derivative in spite of a higher partition coefficient, and the even more lipophilic heptadecyl (C_{17}) and heneicosanyl (C_{21}) analogues were virtually inactive (Fig. 2). Secondly, although the ethyl derivative is about 10 times more lipophilic than Methomyl (C_1), it is nevertheless maximally active at the same concentration.

As far as the ethyl derivative and Methomyl are concerned, one can notice that the first compound is 40% less active than Methomyl in stimulating NADH oxidation (Figs 2, 3 and 5). The ethyl moiety has a slightly higher electron-donating effect than the methyl moiety as shown by the pK_a values of the alkyl organic acids [16]. Hence the replacement of methyl by ethyl in Methomyl can be expected to perturb the conjugated electron system which, as we have shown [9–12], takes a part in the ineraction with the target. As this perturbation is limited, it results in a decrease in activity, not a complete loss. This could explain why the higher lipophilicity of the ethyl analogue, as compared to Methomyl, is not paralleled by a higher biological activity.

In order to explain the low to zero activity of long-chain Methomyl derivatives, it is helpful to have a look at Fig. 3 which summarizes the actions of the whole series on the

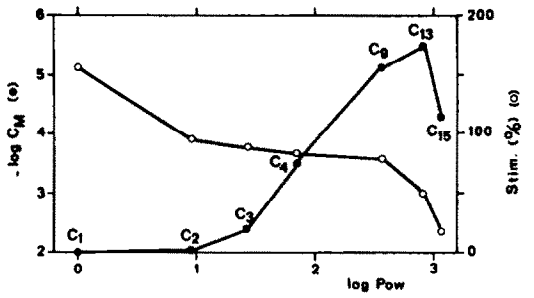

Fig. 5. Relationship between the lipophilicity of Methomyl derivatives and their action on NADH oxidation in F_7T mitochondria.

Pow, *n*-octanol/water partition coefficient. C_M, molar concentration at which a derivative is maximally active. Stim., stimulation of NADH oxidation in percent of the previous state 3 rate.

oxidation of NADH, malate and succinate by F_7T maize mitochondria. Figures 3 and 5 show that as the chain length of Methomyl analogues increases, the extent to which they stimulate NADH oxidation decreases. The same pattern is observed with the inhibition of malate oxidation by the analogues. The decrease is slight from ethyl to nonyl derivatives, then becomes sharper with the tridecyl derivative. Hence, the longer the alkyl chain, the less effective the interaction with the target; a critical length is observed for 13–15 carbon atoms. As the electron-donating properties in the alkyl series are very close for the moieties longer than ethyl [16], they cannot account for the observed decrease in biological activity. Steric hindrances might be responsible for it.

Another explanation takes into account the location of the putative Methomyl target in the mitochondrion. As it very probably is a protein in the inner membrane [7], Methomyl analogues have first to go through the mitochondrial outer membrane to reach their target. They undergo successive partitions between the suspension medium, the outer membrane, the inter-membrane space, and finally the inner membrane. It allows the analogues with an average lipophilicity to reach the target in sufficient amounts to be active. By contrast, one can expect that the more lipophilic compounds are extensively accumulated in the outer membrane, but not readily released into the intermembrane space. Hence, they would tend to be blocked in the outer membrane and reach the inner membrane only in limited amounts.

As a conclusion, we can draw the following hypothesis. The increase in the alkyl chain length of Methomyl analogues could affect their biochemical activity in three ways: the resulting increase in lipophilicity favours their accumulation in the vicinity of their site of action in the mitochondrial inner membrane; for the most lipophilic derivatives, the increase in lipophilicity can also have an adverse effect since they could be blocked in the mitochondrial outer membrane; the increased chain length might decrease the effectiveness of their interaction with the target.

The relative importance of each of these three factors is expected to vary according to the alkyl chain length. In this view, for the ethyl to nonyl derivatives the interaction with the target is partly decreased; their concentration in the inner mitochondrial membrane compensates for the loss in affinity and allows them to act at low concentra-

tions in the suspension medium. For the tridecyl derivative, the interaction is further decreased but its high lipophilicity still favours its activity. For the pentadecyl analogue, the interaction with the target is drastically lowered and its high lipophilicity only partially compensates for that, since it makes the chemical be retained in the outer mitochondrial membrane. For the heptadecyl and heneicosanyl derivatives the loss of affinity for the target is complete and they are anyway accumulated in the outer mitochondrial membrane.

EXPERIMENTAL

Methomyl analogues. These were obtained by reaction of alkyl isocyanates with the corresponding oximes. The oximes were obtained from the appropriate ketones [17]. All compounds synthesized had analytical and spectroscopic data (IR, ^1H and ^{13}C NMR) in agreement with the structure.

n-Octanol/H_2O partition coefficients. The partition coefficient (Pow) of Methomyl was determined spectrophotometrically at 234 nm. The partition coefficients (Pow) of Methomyl analogues were determined from their R_ts on reversed-phase HPLC [18]. The relationship between Pow and the R_t is: $\log(\text{Pow}) = a \log(k') + b$, where a and b are parameters depending on the chromatographic system and are obtained from compounds of known Pow and $k' = (t_r - t_0)/t_0$ where t_r is the R_t of the compound under study and t_0 the R_t of a compound which does not interact with the stationary phase.

The coefficients were determined by using an Ultrasphere ODS C_{18} column (Alteck) operated at 2000 psi in a Waters 6000 A chromatograph. One % formic acid was introduced in samples to determine t_0.

Due to their wide range of lipophilicity, Methomyl analogues had to be studied with two different mobile phases, namely 67% EtOH for the C_2–C_9 analogues and 96% EtOH for the C_9–C_{21} analogues. With 67% EtOH the reference compounds were Methomyl and the herbicides Fenuron (*N,N*-dimethyl-*N'*-phenylurea), Methoxuron [*N'*-(3-chloro-4-methoxyphenyl)-*N,N*-dimethylurea] and Monuron [*N'*-(4-chlorophenyl)-*N,N*-dimethylurea]. Their log(Pow) were respectively: −0.03, 0.72, 1.52 and 1.66. In this system, the relationship between log(Pow) and k' was: $\log(\text{Pow}) = 1.35\,k' + 2.71$, ($r^2 = 0.98$). With 96% EtOH the reference compounds were C_6H_6 and the herbicides Monuron, Diuron [*N'*-(3,4-dichlorophenyl)-*N*-*N*-dimethylurea] and Terbutryne [*N*-(1,1-dimethylethyl)-*N'*-ethyl-6-(methylthio)-1,3,5-triazine-2,4-diamine). Their log(Pow) were respectively: 2.13, 1.66, 2.60 and 3.74. In this system the relationship between log(Pow) and k' was: $\log(\text{Pow}) = 0.89\,k' + 3.43$, ($r^2 = 0.85$).

Preparation and assay of mitochondria. They were prepared by a conventional method [10] and further purified on a Percoll gradient [19]. They were assayed in the same conditions as in [10] except that we used an assay medium devoid of bovine serum albumine (BSA). It is known that lipophilic compounds and in particular pesticides can extensively adsorb on BSA [20, 21]; this phenomenon could have biased the results with the present Methomyl analogues which greatly differ in their lipophilicity. The absence of BSA in the reaction medium lowered the respiratory control ratio; however, it was consistently above 2.5 with NADH as substrate. The reaction medium was 300 mM mannitol, 10 mM KCl, 5 mM $MgCl_2$, 10 mM KPi, pH 7.2, Q_{O_2} was 150–180 nmol O_2 min/mg protein for state 3 NADH oxidation. Purified maize mitochondria were used throughout this study since their susceptibility towards Methomyl did not decrease with time. By contrast, washed mitochondria rapidly lose their susceptibility as first described by Pham and Gregory [22] and experienced by ourselves [10, 11].

In order to study the stimulation of NADH oxidation by Methomyl analogues, about 0.25 mg/ml mitochondrial protein was suspended in the respiratory medium, the reaction was started by addition of 2 mM NADH, a first phosphorylation cycle was initiated by 0.1 mM ADP and the compound under study was added 1 min after return to state 4 conditions (ADP depleted). As some Methomyl analogues induced a non-linear enhancement of NADH oxidation, their effect was measured in linear conditions 2 min after their addition to the reaction medium. The stimulation they brought about was expressed as a percent of the previous state 3 rate of oxygen consumption to avoid the interference that an uncoupling action [8] would have introduced in determinations based on state 4 measurements. The action of HmT toxin was determined in the same way.

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 (s.d.s were lower than 20% of the mean), in order to present in Fig. 2a comprehensive view of the activity of Methomyl analogues, we had to normalize both respiratory control ratios and the stimulations of NADH oxidation. As reference values we choose 2.85 and 157%, respectively for respiratory control ratio and stimulation of NADH oxidation by 10 mM Methomyl (a 10 mM Methomyl control was done for each preparation).

Acknowledgements—We are grateful to Dr J. Negrel for his guidance in the HPLC experiments, to Drs R. Cassini and E. Vuillaume for the supply of F_7T and F_7N maize seeds, and to Dr J. M. Daly for the gift of HmT toxin.

REFERENCES

1. Payne, G. A., Knoche, H. W., Kono, Y. and Daly, J. M. (1980) *Physiol. Plant Pathol.* **16**, 227.
2. Gregory, P., Earle, E. D. and Gracen, V. E. (1977) in *Host Plant Resistance to Pests* (Hedin P. A. ed.) ACS Symposium Series No 62, p. 90, American Chemical Society, Washington.
3. Miller, R. J. and Koeppe, D. E. (1971) *Science* **173**, 67.
4. Humaydan, H. S. and Scott, E. W. (1977) *Hort. Sci.* **12**, 312.
5. Koeppe, D. E., Kox, J. K. and Malone, C. P. (1978) *Science* **201**, 1127.
6. Matthews, D. E., Gregory, P. and Gracen, V. E. (1979) *Plant Physiol.* **63**, 1149.
7. Bervillé, A., Ghazi, A., Charbonnier, M. and Bonavent, J. F. (1984) *Plant Physiol.* **76**, 508.
8. Klein, R. R. and Koeppe, D. E. (1985) *Plant Physiol.* **77**, 912.
9. Aranda, G., Fetizon, M. and Poiret, B. (1983) *Experientia* **39**, 396.
10. Aranda, G., Gauvrit, C., Cesario, M., Guilhem, J., Pascard, C. and Tran Huu Dau, M. E. (1983) *Phytochemistry* **22**, 2431.
11. Gauvrit, C. and Aranda, G. (1983) *Phytochemistry* **22**, 33.
12. Aranda, G. and Gauvrit, C. (1986) *Bioorganic Chem.* **11**, 313.
13. Douce, R. (1985) *Mitochondria in Higher Plants* (Am. Soc. Plant Physiol. eds) p. 177. Academic Press, New York.
14. Susuki, Y., Tegtmeier, K. J., Daly, J. M. and Knoche, H. W. (1982) *Bioorg. Chem.* **11**, 313.
15. Susuki, Y., Danko, S. J., Daly, J. M., Kono, Y., Knoche, H. W. and Takeuchi, S. (1983) *Plant Physiol.* **73**, 440.

16. Dawson, R. M. C., Eliott, D. C., Eliott, W. H. and Jones, K. M. (1986) *Data for Biochemical Research* 3rd edn., pp. 33 and 167. Oxford Science Publication, Oxford.
17. Vogel, A. I. (1956) *Practical Organic Chemistry* 3rd edn. Longman, London.
18. Hellgehausen, H., d'Hondt, C. and Fuerer, R. (1981) *Pestic. Sci.* **12**, 219.
19. Gauvrit, C. (1983) *Weed Research* **23**, 85.
20. Brown, N. A., Wilson, A. G. E. and Bridges, J. W. (1982) *Biochem. Pharmacol.* **24**, 4019.
21. Camper, N. D. and Moreland, D. E. (1971) *Weed Sci.* **19**, 269.
22. Pham, H. N. and Gregory, P. (1980) *Plant Physiol.* **65**, 1173.