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Analogs of Host-Specific Phytotoxin Produced by *Helminthosporium maydis*, Race T

II. Biological Activities

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Inhibition of dark CO_2 fixation by susceptible corn leaves was used to compare the relative toxicity of synthetic analogs with that of the host-specific phytotoxin produced by the fungal corn pathogen, *Helminthosporium maydis*, race T. Analogs with C_{15}, C_{25}, or C_{26} chain lengths and 1,5-dioxo-3-hydroxy functions were only slightly less toxic ($2–6 \times 10^{-7}$ *M*) than native T toxin ($C_{35}–C_{45}$ chain lengths) or its individual components (3×10^{-8} *M*). Like native toxin, analogs were host-specific in that they did not inhibit dark CO_2 fixation in leaf tissue of resistant corn at concentrations $10^2–10^3$ times greater than those effective with susceptible corn. These findings support the structures previously proposed for native T toxin.

INTRODUCTION

In a previous paper (*1*) the synthesis of some simple ($C_{15}–C_{26}$) analogs of the host-specific corn pathotoxin produced by *Helminthosporium maydis*, race T, was reported. Although synthesis of the major C_{39} and C_{41} components of native toxin is still in progress, data on the biological activities of the compounds synthesized to date (Figs. 1 and 2) are of interest for several reasons.

The biological activity of native T toxin is high ($10^{-8}–10^{-9}$ *M*) but it can be argued, as with any natural product, that toxicity is due to a chemically uncharacterized contaminant. Thus, the toxicity, and especially the biological specificity, toward Texas male sterile corn of compounds structurally related to native T toxin bears on the purity of our preparation, as well as the validity of the structures proposed for it. In addition, the availability of synthetic analogs provides an initial basis for structure-activity considerations of toxicity and/or specificity. These considerations may be useful in defining the as yet unknown biochemical site of action.

RESULTS

A number of processes (ion leakage (*2*), respiration (*3*), mitochondrial oxidation (*4*, *5*), photosynthesis (*6*)) are affected by T toxin, but we chose inhibition of dark

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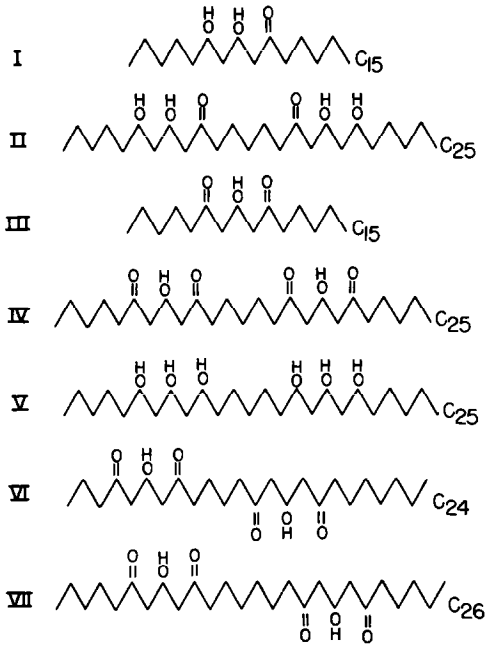

FIG. 1. Synthetic analogs of race T toxin prepared by Grignard addition of aldehyde intermediates to di(bromomagnesium) alkanes (*1*).

CO_2 fixation as a bioassay because of its sensitivity (nanograms toxin per milliliter) and the rapidity of the assay (2–3 hr). A dosage–response curve for native toxin is shown (Fig. 3) to illustrate that the response is logarithmic and that inhibition has a maximum of 60 to 70%. The sensitivity of susceptible leaf tissue to toxin is somewhat variable with leaf age but, in our experience, no more so than other processes we have examined. Variability is evidenced in two distinct ways: changes in the slope of the response curve and the maximum inhibition that can be obtained.

These considerations, plus constraints in the number of samples (30 or less) that could be handled in a single experiment, required two protocols for establishing

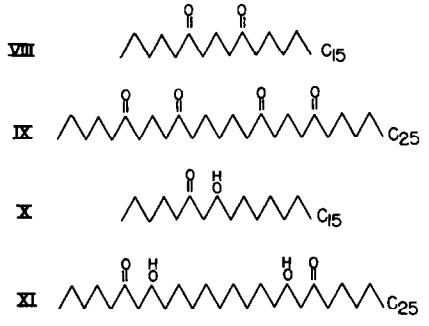

FIG. 2. Compounds with partial functional groups of race T toxin.

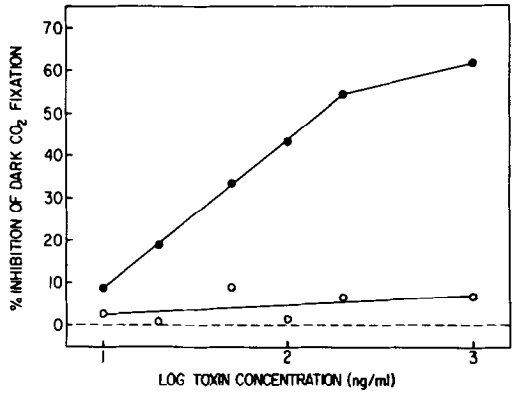

FIG. 3. Dosage response to native toxin as measured by inhibition of dark CO_2 fixation in leaves of susceptible (●) and resistant (○) corn.

the relative activity of synthetic compounds. For the data of Table 1, a single concentration of each compound was tested on the same set of susceptible leaf slices. These were ordered with least significant difference for several single concentrations in separate experiments (Table 1). In order to confirm the ranking, a second protocol directly compared two of the compounds at five concentrations. Over 60 such comparisons resulted in 3 to 4 separate tests for each active compound. Compounds IX and XI were not soluble enough in the assay solution to provide reliable data. Typical comparisons are given in Table 2. Because of possi-

TABLE 1

RELATIVE TOXICITIES OF NATIVE TOXIN AND SYNTHETIC COMPOUNDS ON DARK CO_2 FIXATION IN SUSCEPTIBLE W64A Tms CORN LEAF SLICES[a]

	Inhibition (%)				
	25 ng/ml	50 ng/ml	200 ng/ml	1 μg/ml	10 μg/ml
Native toxin	35 a	43 a	47 a	57 a	56 ab
Band 3	30 a	41 a	45 a	57 a	59 a
Reduced native toxin[b]	32 a	43 a	49 a	56 a	61 a
IV	14 b	24 b	34 b	40 b	50 bcd
III	7 bc	14 c	26 bc	40 b	54 abc
V	5 c	8 c	19 c	29 bc	45 de
VI	5 c	5 c	7 d	26 cd	46 cde
II			+1[c] de	15 de	38 e
I			+6 e	7 ef	8 f
X				2 f	14 f
VIII				+3 f	16 f
LSD	7	9	9	11	8

[a] Letters in common indicate no significant difference in activity at that concentration.

[b] A mixture of C_{35} to C_{45} of polyalcohols prepared by borohydride reduction of native toxin.

[c] Plus indicates rates above control.

TABLE 2

TYPICAL DATA OBTAINED IN DIRECT COMPARISONS OF PAIRS OF EXPERIMENTAL COMPOUNDS ON SUSCEPTIBLE W64A Tms LEAF SLICES

	Percent inhibition of dark CO_2 fixation							
	Concentration (ng/ml)							
Compound	12.5	25	50	10^2	10^3	10^4	2×10^4	4×10^4
Native toxin	8	15	27	36	50			
Band 3 toxin	16	21	26	38	58			
Native toxin	13	19	31	37	59			
Reduced toxin	11	—	17	25	48			
IV	20	22	29	32	62	74		
III	9	6	13	17	49	64		
II				+3[a]	+3	43	48	53
X				0	0	13	21	33
I				10	—	13	21	32
II				+2	10	34	41	—
VIII				+3	+5	3	+4	+4
X				+2	+2	19	27	42

[a] Plus indicates rates above controls.

ble chemical instability of compounds with β hydroxyls in the cis configuration (*1*), only the trans versions of compounds I and II were tested extensively, although a comparison of freshly made solutions of cis and trans forms of I indicated equal activity.

TABLE 3

SPECIFICITY OF INHIBITION OF DARK CO_2 FIXATION BY NATIVE TOXIN AND SYNTHETIC COMPOUNDS

Compound	Concentration (μg/ml)	Inhibition (%)	
		Tms Cytoplasm	N Cytoplasm
Native toxin	20	55	2
IV	40	53	7
III	40	72	10
V	40	64	+1[a]
VI	40	53	0
II	40	49	+2
I	60	41	9
X	40	28	4
VIII	40	+3	2

[a] Plus indicates rates above control.

TABLE 1

VARIOUS MODEL STRUCTURES

Model	Molecules[a]	
I[b]	^{195}Me–^{57}Im–^{102}Fo(−)	Side chain of Ser 195, His 57, and Asp 102
II-1	^{195}Me–^{57}Im–^{102*}Fo(−)	Correction of the remaining moiety of Ser 195, His 57, and Asp 102
II-2	^{195}Me–^{57}Imp–^{102*}Fo(−)	
II-3	^{195}Mep–^{57}Im–^{102*}Fo(−)	
II-4	^{195*}Me(−)–^{57}Im(+)–^{102}Fo(−)	
II-5	^{195*}Me(−)–^{57}Imp(+)–^{102}Fo(−)	
II-6	^{195*}Me(−)–^{57}Im(+)–^{102}Fop(−)	
III-1	I, $^{214}CH_3OH$	Side chain of Ser 214 and —NH— of Ala 56
III-2	I, $^{56}NH_3$	
IV-1	II-1,$^{42}CH_3S$—$^{58}SCH_3$	Disulfide bridge of Cys 42–Cys 58 and peptide moieties of Ser 213–Trp 214 and Val 214–Ser 215
IV-2	II-1, ^{213}HCO—$^{214}NH_2$	
IV-3	II-1, ^{214}HCO—$^{215}NH_2$	
IV-4	II-4, $^{42}CH_3S$—$^{58}SCH_3$	
IV-5	II-4, ^{213}HCO—$^{214}NH_2$	
IV-6	II-4, ^{214}HCO—$^{215}NH_2$	
V-1	III-1, #IAA, $^{15(I)}$#Lys	Ionic amino acid residues (IAA), Lys 15 (I), and main chain (MCR)
V-2	II-1, *MCR	
V-3	II-4, *MCR	
VI	I, $^{15(I)}$HCO—$^{16(I)}NH_2$	Peptide moiety of Lys 15 (I)–Ala 16 (I)
VII[c]	CH_3OH—$C_3N_2H_4$—HCO_2(−)	Geometry optimized system

[a] The fragments labeled by * and # are approximated by point fractional charges and integral charges in the calculation, respectively.

[b] The distances between Ser 195 O^{γ} and His 57 $N^{\epsilon 2}$ (R_1) and between His 57$^{\delta 1}$ and Asp 102 $O^{\delta 2}$ (R_2) are 2.68 and 2.72 Å, respectively.

[c] R_1 in A, B, and D states are 2.82, 2.49, and 2.56 Å, respectively. R_2 in A, B, and D states are 2.73, 2.53, and 2.72 Å, respectively.

The effects of the hydrogen bond between $O^{\delta 1}$ of Asp 102 and —NH— group of His 57 and the van der Waals' contacts of the backbone moieties are included in $dE_{BA(II)}$ and $dE_{DB(II)}$. In model III the effects of the hydrogen bonds between O^{γ} of Ser 214 and $O^{\delta 2}$ of Asp 102 and between —NH— group of Ala 56 and $O^{\delta 1}$ of Asp 102 are estimated (*12*). The relative energies in which the effects of the hydrogen bonds are included are estimated in the assumption of additivity as

$$dE_{BA(III)} = dE_{BA(II)} + dE_{BA(III-1)} + dE_{BA(III-2)} - 2dE_{BA(I)}$$

and

$$dE_{DB(III)} = dE_{DB(II)} + dE_{DB(III-1)} + dE_{DB(III-2)} - 2dE_{DB(I)}.$$

In model IV the effects of the disulfide bridge of Cys 42–Cys 58, the peptide moiety of Ser 214–Trp 215, and the peptide moiety of Val 213–Ser 214 which

cant effects by the most active compounds (particularly III–V and VII) on susceptible corn (Tables 1 and 2).

As indicated above, T toxin affects a number of physiological processes in susceptible corn, and it is theoretically possible that it has several independent cellular targets (*8*). Because the molecular site(s) is not known, it is possible that a different biochemical site for dark CO_2 fixation is being affected by the analogs.

Inhibition of dark CO_2 fixation by native toxin occurs whether leaf tissue is preincubated in light or darkness, while inhibition of photosynthesis requires a minimum of about 30 min preincubation with toxin in light (*6*). Compounds II–IV, like native toxin, do not affect photosynthesis significantly even at very high concentrations when preincubated in the dark for 1 hr; but photosynthesis is inhibited if leaf slices are incubated in the light (Table 4). Similarly, ion leakage was induced by IV in leaf slices from Tms (Texas male sterile), but not N, corn at 50 ng/ml; in contrast, only 10 ng/ml of native toxin was required for equivalent leakage (unpublished data). These observations suggest that the synthetic analogs affect the same sites as native toxin.

DISCUSSION

The specificity for Tms corn of synthetic compounds containing functional groups previously assigned to native T toxin (*6, 9*) supports the structures proposed for the components of native toxin (*1*). It also indicates that the effects of our preparations obtained from cultures of *H. maydis,* race T cannot be ascribed to an unknown contaminant.

The lack of activity of VIII indicates that the 1,5-dioxo function alone is not sufficient for high toxicity (cf. VIII to III), while 1,3-dioxyoxo functions are present in all active compounds with activities greater than that of VIII. For a direct comparison consider VIII and X. For compounds of equivalent lengths,

TABLE 4

EFFECT OF PREINCUBATION CONDITIONS ON INHIBITION OF PHOTOSYNTHETIC AND DARK CO_2 FIXATION IN LEAF SLICES

Preincubation conditions	Native toxin[a]	II	III	IV
Inhibition of dark CO_2 fixation (%)				
Light	42	42	37	50
Dark	35	24	22	59
Inhibition of photosynthetic CO_2 fixation (%)				
Light	32	46	36	51
Dark	0	7	0	+5[b]

[a] Native toxin, 90 ng/ml; synthetic compounds, 40 μg/ml.

[b] Plus indicates rates above controls.

however, a 1,5-dioxo-3-hydroxy moiety compared to a 1-oxo-3,5-dihydroxy or 1,3,5-trihydroxy function increases toxicity by about an order of magnitude in all cases (cf. I to III, II to IV, and V to IV). Thus the 1,5-dioxo-3-hydroxy function appears to be quite significant in toxin activity and/or specificity. Changing the length of the central diketo bridge in the C_{24}, C_{25}, and C_{26} compounds has but a slight effect on toxicity, as would be expected from the activity of III. Nevertheless, IV, with the 1,7-diketo bridge characteristic of native toxin, is more effective than either VI or VII.

Increasing the chain length has the most marked effect on toxicity, as shown by the activities of I and II, as well as V and reduced native toxin. On a molar basis, this observation holds also for III (6×10^{-7} *M*), IV (2×10^{-7} *M*), and band 3 toxin (3×10^{-8} *M*).

The structures of the individual components of native T toxin might suggest biological activities as ionophores (*5*). Although III is much shorter in chain length than the native toxin components, it should be noted that on a weight (≅100 ng/ml), rather than on a molar basis, its activity is almost equivalent to IV (≅90 ng/ml). Compound III is not markedly less active than band 3 toxin or native toxin (≅40 ng/ml). This behavior might be the result of cooperativity in forming ion channels or pores, as is known for gramicidin (*10*). Cooperativity also could be envisioned for the weakly toxic compound X. Synthesis of additional analogs and their modification (e.g., by acetylation) will provide experimental approaches to such problems.

The biological activity of V is of particular interest. It is important to determine if toxin has a specific binding site in susceptible corn; for example, on mitochondria (*4*). Because of the high potency of toxin, however, the minimally useful specific radioactivity required would be approximately 3 mCi/μmol (1000 dpm/ng). Biosynthesis of native toxin of this activity from ^{14}C substrates or $^{3}H_2O$ has not been possible because of dilution by nonlabeled substrates or water in the culture medium. Synthesis of 100 mg of compound IV with 1000 dpm/ng by the methods (*1*) used for these studies would cost $500,000 at current prices of available radioactive reactants. The reasonable activity of V makes reduction of IV with $NaB^{3}H_4$ with specific activity of over 10,000 dpm/ng a feasible route for production of useful radioactive markers.

EXPERIMENTAL

Compounds. Native T toxin was prepared by methods described previously (*9*). Reduction of carbonyl groups to hydroxyl groups ("reduced toxin") was accomplished using $NaBH_4$ in MeOH. The product, a mixture of C_{35} to C_{45} linear polyalcohols, was purified by precipation from MeOH—CH_3COCH_3 followed by thin-layer chromatography (TLC). Complete reduction was indicated by the absence of C=O absorption at 1740 cm^{-1}. One component of native T toxin (band 3 toxin) was isolated by TLC on Merck EM silica gel with $CHCl_3$—MeOH (85 : 15) as solvent (*9*). Procedures for the preparation of synthetic compounds were described (*1*).

Because native toxin binds easily to glass, the general precautions for handling toxin discussed by Payne *et al.* (*5*) were observed. Synthesis, isolation, and storage of the compounds shown in Figs. 1 and 2 was carried out with glassware that had never been used to prepare native toxin.

CO_2 Fixation bioassay. Susceptible (Tms) and resistant (N) lines of W64A corn were grown as described previously (*11*). The fourth leaf was collected when mature (17–20 days from sowing) and cut into 1 × 8-mm thin slices by the procedures of Daly and Barna (*6*). MOPS[1] buffer (12.5 m*M*) supplemented with 20 m*M* KH_2PO_4 and 0.1 m*M* sodium pyruvate was the assay solution (pH 6.4).

Twelve or 15 leaf slices were placed in 475 μl of assay solution in 7-ml scintillation counting vials. Compounds were added in 1 or 2 μl of dimethyl sulfoxide (DMSO) and the vials sealed with serum stoppers. Controls received an equivalent amount of DMSO. In most experiments, vials were incubated for 1 hr in light at an intensity of 2×10^3 μeq m^{-2} hr^{-1} at 29°C. For dark CO_2 fixation, the slices were placed in darkness for 5 min before addition of 25 μl of 60 m*M* Na_2H $^{14}CO_3$ (specific activity 0.1 to 0.5 μCi/μmol). Vials were kept in light when assaying photosynthetic CO_2 fixation.

After 10 to 15 min exposure to $^{14}CO_2$, 0.25 ml of 50% (w/v) trichloracetic acid was added and the vials placed overnight in a hood to allow diffusion of unfixed $^{14}CO_2$. Five milliliters of Handiflour scintillation fluid was added and radioactivity determined with a Packard Model 240 CL/D spectrometer at an efficiency of 55 to 65%. Rates of dark CO_2 fixation were linear for a least 30 min and are expressed as nanomoles CO_2 fixed per leaf slice per hour. Control rates ranged from 16 to 24 nmol/slice hr^{-1}. All data are the average of either three replicates with 12 slices/vial or duplicate vials with 15 leaf slices. Standard deviations were no more than 10% of fixation rates for the data presented.

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[1] MOPS, (3-[morpholino]propanesulfonic acid.

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