APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1978, p. 412-420
0099-2240/78/0036-0412$02.00/0

Vol. 36, No. 3
Printed in U.S.A.

Mollicellins: Mutagenic and Antibacterial Mycotoxins

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Received for publication 29 June 1978

Eight mollicellins (depsidones) were assayed for mutagenicity and antibacterial activity in *Salmonella*/microsome tests involving histidine reversion and forward mutation to 8-azaguanine resistance. Two of them, mollicellins C and E, which contain a 3-methylbutenoic acid moiety, were mutagenic and bactericidal for *Salmonella typhimurium* in the absence of microsomes. Mollicellins D and F, each containing a chlorine atom, were bactericidal but not mutagenic. The mutagenic activity was completely abolished and the antibiotic activity was greatly reduced by coincubation with rat liver microsomes.

Mollicellins, the major products of the fungus *Chaetomium mollicellum* MIT M-37 (G. Büchi, K. Matsuo, B. Kobbe, A. L. Demain, G. N. Wogan, N. Eickman, and J. Clardy, submitted for publication) belong to the depsidone group of polyketide metabolites, which up to this point have been produced primarily by lichens. Recent reports indicate that the mycobiont moieties of lichens and free fungi are capable of complete synthesis of depsidones: salazinic acid is produced by the fungus isolated from the lichen *Ramalina crassa* (8), and yasimin is produced by *Aspergillus unguis* (7). The biosynthesis of depsidones involves acetate, malonate (7, 9, 11), and formate; the structures of about 20 compounds have been established (5). Because of their reported antibiotic activity, toxicity, and unique structural features (Büchi et al., submitted for publication), we felt it important to determine whether the mollicellins possess mutagenic properties. Indeed, as shown in the present paper, two mollicellins containing a 3-methylbutenoic acid moiety are mutagens. Because of their stability and their production by *Chaetomium* on rice (Büchi et al., submitted for publication), one may define them as stable environmental mutagens that may be produced on foodstuffs during storage.

MATERIALS AND METHODS

Mycotoxins. All mollicellins employed in this study were produced and purified as previously described (Büchi et al., submitted for publication).

Bacterial strains. *Salmonella typhimurium* TA100 was provided by B. N. Ames, University of California, Berkeley, Calif. *S. typhimurium* TM677 was obtained from W. G. Thilly, Massachusetts Institute of Technology, Cambridge, Mass.

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Bacterial stocks, media, and conditions for growth. All bacteriological procedures and growth media for *S. typhimurium* TA100 were according to Ames et al. (1). *S. typhimurium* TM677 was grown from very small inocula (<10^3 cells per 50 ml of culture) in nutrient broth (Difco) containing 20 mg of glucose per ml and 30 μg of ampicillin trihydrate per ml (Ayerst Laboratories) for 18 h at 37°C. Cultures were quickly chilled to 0°C and assayed for the frequency of 8-azaguanine resistant ($8AG^R$) mutants as described by Skopek et al. (10). Cultures with low spontaneous mutant frequencies were diluted 10-fold and grown up to 10^9 cells per ml at 37°C. Dimethyl sulfoxide was added to 10% (vol/vol), and the cultures were frozen and stored at −80°C.

Preparation of cell suspensions. Suspensions of *S. typhimurium* TA100 were prepared as described (1). A frozen stock of *S. typhimurium* TM677 was thawed, diluted fivefold in nutrient broth containing 20 mg of glucose per ml, shaken for 15 to 20 min at 37°C, and stored at 0°C until used.

Reaction mixtures and conditions for mutagenesis. Reaction mixtures contained, in a final volume of 540 μl: 50 μmol of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate buffer (pH 7.4) (Sigma), 16.5 μmol of KCl, 4.0 μmol of $MgCl_2$, 2.0 μmol of nicotinamide adenine dinucleotide phosphate (Sigma), 2.5 μmol of glucose-6-phosphate (Sigma), 28 μmol of glucose, and 60 μl of 9,000 × *g* supernatant of rat liver extract (S-9), prepared from livers of rats treated with sodium phenobarbital (1). Reactions conducted without S-9 did not contain nicotinamide adenine dinucleotide phosphate. The mixtures were sterilized by filtration and stored at 0°C.

Reactions were started by adding 60 μl of cell suspension containing up to 10^9 cells to the reaction mixture. For tests of mutagenesis in suspension, up to 30 μl of a mycotoxin solution was added, and the mixture was incubated in the dark for 2 h at 37°C while shaking at 120 to 140 rpm, or for 30 min in the case of aflatoxin B_1. Reactions were quenched by the addition of 4.5 ml of cold saline (0.85% NaCl) and centrifuged at 6,000 to 10,000 × *g* for 10 min in the cold; the cell pellets were suspended in 1.2 ml of saline.

For spot tests, reaction mixtures were mixed with 3.0 ml of soft (0.6%) agar and overlaid onto the appropriate plates. Sterile filter-paper disks (6.3-mm diameter, Schleicher & Schuell) were placed on the agar, and 10 to 20 μl of mycotoxin solution was applied.

The frequency of histidine prototrophic revertants of strain TA100 was assayed on minimal agar plates as described by Ames et al. (1). The appearance of $8AG^R$ mutants of strain TM677 was assayed on freshly prepared minimal agar plates containing 50 μg of 8AG per ml (Sigma), prepared as described by Skopek et al. (10).

The antibacterial activity of mycotoxins was determined by plating appropriate dilutions of cell suspensions after exposure to the mycotoxins onto minimal agar plates (strain TM677) or plates containing 100 μM L-histidine (strain TA100). All plates were incubated for 18 h at 37°C in the dark.

Preparation of mutagen solutions. Unless otherwise noted, mycotoxins were freshly dissolved in dimethyl sulfoxide to give solutions of 1 to 10 mg/ml. Solutions were stored at 0°C for no longer than 30 min prior to exposure of the cells to them. Aflatoxin B_1 (isolated and purified by the method of Asao et al. [2]) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (K&K Laboratories) were dissolved in methanol.

All organic solvents were obtained from Fisher Scientific Co. Acridine orange was from Calbiochem, and ethyl methane sulfonate (EMS) was from Pfaltz & Bauer.

RESULTS

Histidine reversion assay: spot test. Spot tests employing the reversion to histidine independence were used first for mutagenic activity of *Chaetomium* products. Table 1 presents results obtained with strain TA100 in the presence and absence of rat liver microsomes. No mutagenicity of the mollicellins was observed, and the only effect detected was inhibition of bacterial growth manifested by clear zones around the disks. In all cases, the presence of rat liver microsomes decreased the diameter of the growth inhibition zones. Known mutagens such as MNNG, EMS, or aflatoxin B_1 showed both antibacterial and mutagenic activities under similar conditions. Thus, the spot test for histidine reversion of strain TA100 failed to reveal mutagenicity of the *Chaetomium* products.

Histidine reversion assay: suspension test. Results different from those described above for the spot test were obtained when the mutagenesis assay was carried out in suspension. Mollicellins C and E increased the number of histidine-independent colonies of strain TA100, whereas none of the other mollicellins did (Table 2). The number of mutants in the surviving fraction was proportional to the concentration of mollicellin C or E. Mollicellin E was more effective than mollicellin C both as antibiotic and as mutagen (Fig. 1): at the concentration range of 20 to 50 μg per reaction, the antibacterial activity of mollicellin E was 10-fold higher than that of mollicellin C (Fig. 1A). The mutagenic activity of mollicellin E was about four times higher than that of mollicellin C (Fig. 1B). Mollicellin A was inactive under the same conditions.

It should be noted that the antibacterial activity of mollicellins C and E was determined by 30-min exposure times, terminated by serial dilution prior to plating on permissive medium, whereas the appearance of mutants was determined by long exposure times on the selective plates, resulting from the plating of the whole volume of undiluted reaction mixtures after 30 min of incubation in suspension. Short (30-min) exposure times followed by washing of the cells prior to plating did not result in significant increase in mutant frequency (unpublished results). The addition of rat liver microsomes abolished the mutagenic activity of mollicellins C and E (data not shown) and, as will be shown below, greatly reduced their antibiotic activity.

Forward mutation assay: spot test. The various mollicellins were also tested for their ability to induce the $8AG^R$ mutation in *S. typhimurium* TM677. $8AG^R$ mutant zones formed around disks immersed in solutions of mollicellins C or E (Fig. 2 and 3). Mollicellins D and F, on the other hand, were antibacterial but not mutagenic. Little or no antibacterial and no mutagenic activity was observed with mollicellin H (Fig. 2), and mollicellin G was inactive (data not shown). Both growth inhibition zones and mutant zone diameters were proportional to the logarithm of the concentration of all active mycotoxins. There was a significant decrease in the activity of mollicellin E upon storage, manifested by smaller growth inhibition and mutation zone radii of dimethyl sulfoxide solutions stored for 30 days at 4°C (Fig. 2 and 3). Mollicellin C was quite stable under the same storage conditions. Similar to mollicellins C and E, known base pair mutagens such as MNNG and EMS and a frameshift mutagen such as acridine orange yielded distinct $8AG^R$ mutant zones (Fig. 3). The presence of microsomes in the assay mixture resulted in confluent growth of the tester strain even on those plates containing 8AG, thus allowing the measurement of growth inhibition but not of mutagenesis in the spot test. This phenomenon will be discussed below.

Forward mutation assay: suspension test. When the forward mutation test employing the $8AG^R$ mutation in strain TM677 was carried out in suspension culture, it was found that mollicellins A, B, and G were neither antibacte-

TABLE 1. *Antibacterial and mutagenic activities of mollicellins, aflatoxin B_1, MNNG, and EMS in the reversion assay (spot test with S. typhimurium TA100)*[a]

Compound	μg/disk	Zone diam (mm)			
		Growth inhibition		Mutation	
		No microsomes	+ Microsomes	No microsomes	+ Microsomes
Mollicellin					
A	10	9.0	8.0	NZ[b]	NZ
	40	10.0	NT[c]	NZ	NZ
	200	10.6	NT	NZ	NZ
B	10	8.0	7.9	NZ	NZ
	40	8.0	NT	NZ	NZ
	200	8.0	NT	NZ	NZ
C	10	9.4	7.7	NZ	NZ
	40	12.0	9.6	NZ	NZ
D	10	17.0	14.9	NZ	NZ
	50	23.0	16.0	NZ	NZ
E	10	13.3	8.9	NZ	NZ
	50	19.0	10.0	NZ	NZ
F	10	9.9	8.9	NZ	NZ
	50	17.0	10.0	NZ	NZ
G	10	8.1	NZ	NZ	NZ
	50	8.6	NZ	NZ	NZ
	200	9.0	NT	NZ	NZ
H	50	9.0	NZ	NZ	NZ
	200	10.0	NZ	NZ	NZ
Aflatoxin B_1	0.4	NZ	16.0	NZ	23
	0.5	NZ	18.0	NZ	26
	1.0	NZ	23.0	NZ	32
MNNG	2.5	7.0	NZ	36	32
	5.0	11.0	10.0	43	37
EMS	25	16.0	18.0	52	62
	50	23.0	23.0	60	67

[a] Reaction conditions and procedures are described in the text.
[b] NZ, No zone of inhibition of growth or zone of mutagenesis observed.
[c] NT, Not tested.

rial nor mutagenic (Table 3). Mollicellins D and F showed considerable antibiotic activity and little, if any, mutagenic activity. Mollicellin H showed some bactericidal activity and very little mutagenic activity. Mollicellin C was antibacterial only in the absence of S-9 and had no mutagenic activity. Mollicellin E was inhibitory and mutagenic, and both activities were eliminated or markedly reduced in the presence of rat liver microsomes.

DISCUSSION

Methodology. The differences in mutagenicity that we have observed by using different genetic loci on the one hand and different assay conditions on the other indicate that a single mutagenesis test is not sufficient to estimate the mutagenic potential of a particular antibacterial compound. We have found the plate assay (i.e., spot test) involving the *his* reversion to be less sensitive than a similar test using the $8AG^R$ forward mutation. For example, mollicellins C and E were inactive as mutagens in the his reversion spot test but strongly active in the spot test using the $8AG^R$ mutation. However, the presence of microsomes in the latter test led to confluent growth of the whole bacterial population in the presence of 8AG, thus rendering this

plate assay ineffective for the detection of metabolically activated mutagens.

The ability of rat liver microsomes to enable 8AG-sensitive cells to grow in the presence of the drug is probably due to the fact that rat liver microsomal fractions contain the enzymes guanase and xanthine oxidase (6). 8AG can be deaminated by guanase (4) to 8-azaxanthine and possibly further converted to the corresponding analog of uric acid. Moreover, the optimum pH for guanase action on 8AG is approximately the same as that of the 8AG-containing medium (6, 10). 8AG is probably converted into a noninhibitory compound by microsomes, thus enabling the entire bacterial population to grow in its presence.

In some cases, suspension assays yielded results superior to those obtained in plate tests. For example, a short (30-min) incubation of strain TA100 cells in high concentrations of mollicellins C or E, followed by a long (48-h) exposure to a 30-fold-lower concentration (achieved by plating the reaction mixture without washing out the mutagen [Fig. 1]) resulted in significant increases in the his^+ revertant frequency; the same amounts of the mollicellins applied to paper disks and incubated for the same period of time failed to do so.

Almost invariably, rat liver microsomes decreased the antibiotic activity and abolished the mutagenic activity of the mollicellins. This demonstrates the in vitro detoxification of these compounds by liver enzymes. Although many mutagens/carcinogens require metabolizing systems for their activation, the fact that both mutagenic and antibiotic activities of mollicellins are independent of microsomes and were diminished by the latter prompts us to recommend the inclusion of a test where S-9 is omitted as a component of any mutagenicity screening method.

The suspension test combined with a washing step, as suggested by Skopek et al. (10), has the advantage of accurately defining the time of exposure. However, the exposure time periods routinely employed (2 h) may not be sufficient for the detection of certain mutagens. For example, mollicellin C was active in assays involving long exposure periods, whereas exposure for only 2 h did not result in a significant increase in mutant frequency. Also, the mutagenic activity of mollicellin E in the $8AG^R$ forward mutation assay on plates using strain TM677 was much more pronounced than it was in suspension. This phenomenon suggests that mollicellins C and E are slow-acting mutagens. Interestingly enough, the strong antibacterial activity of these compounds could be detected after short (30-min to 2-h) periods of exposure. This difference in the rates of antibacterial and mutagenic actions suggests that those activities reside in different moieties of the mollicellin molecule. This possibility will be discussed below.

TABLE 2. *Mutagenicity of mollicellins in the reversion assay (suspension test with S. typhimurium TA100)*[a]

Compound	μg/reaction	Microsomes	Mutants/plate[b]
Mollicellin			
A	20–50	–	0
	20–50	+	0
B	20–50	–	0
	20–50	+	0
C	20	–	142
	20–50	+	0
D	20–50	–	0
	20–50	+	0
E	20	–	100
	20–50	+	0
F	20–50	–	0
	20–50	+	0
G	20–50	–	0
	20–50	+	0
H	20–50	–	0
	20–50	+	0
Aflatoxin B_1	0.05	–	0
	0.10	–	0
	0.05	+	2,200
	0.10	+	2,600

[a] Experimental details were as described in the text except that reaction mixtures contained 1.7 μmol of nicotinamide adenine dinucleotide phosphate and 15 nmol of L-histidine. The mixtures were shaken at 37°C for 30 min after the addition of 2 to 5 μl of a 10-mg/ml mollicellin solution (aflatoxin B_1 was at 2 μg/ml in methanol and 25 to 50 μl was added). Soft agar (3 ml) was then added to each reaction tube, and the suspension was added as an overlayer to the minimal agar plates.

[b] Net increase over background.

Relationship between structure and activity of mollicellins. The structures of the eight depsidones (mollicellins A through H) have recently been elucidated (Büchi et al., submitted for publication) and are shown in Fig. 4. Of the eight compounds tested, mollicellins C, D, E, and F were active against *S. typhimurium*. This pattern of antibiotic activity is very similar to that observed against the gram-positive *Bacillus megaterium* (B. Kobbe, unpublished re-

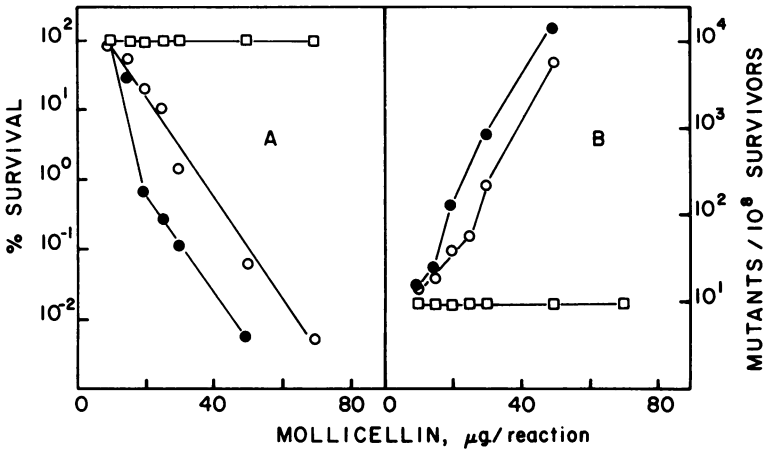

FIG. 1. *Antibacterial and mutagenic activities of mollicellins A, C, and E in a quantitative histidine reversion assay (in suspension using S. typhimurium TA100). Reaction mixtures were as described in the text except that S-9 and nicotinamide adenine dinucleotide phosphate were omitted. Incubation was for 30 min at 37°C; 50-μl samples were withdrawn for serial dilution and plating on histidine-containing plates. The remaining 550 μl was plated on minimal medium with 3 ml of soft agar. Colonies were counted after 48 h of incubation at 37°C. (A) Relative survival; (B) his⁺ revertants per 10^8 survivor cells. Symbols: ○, mollicellin C; ●, mollicellin E; □, mollicellin A.*

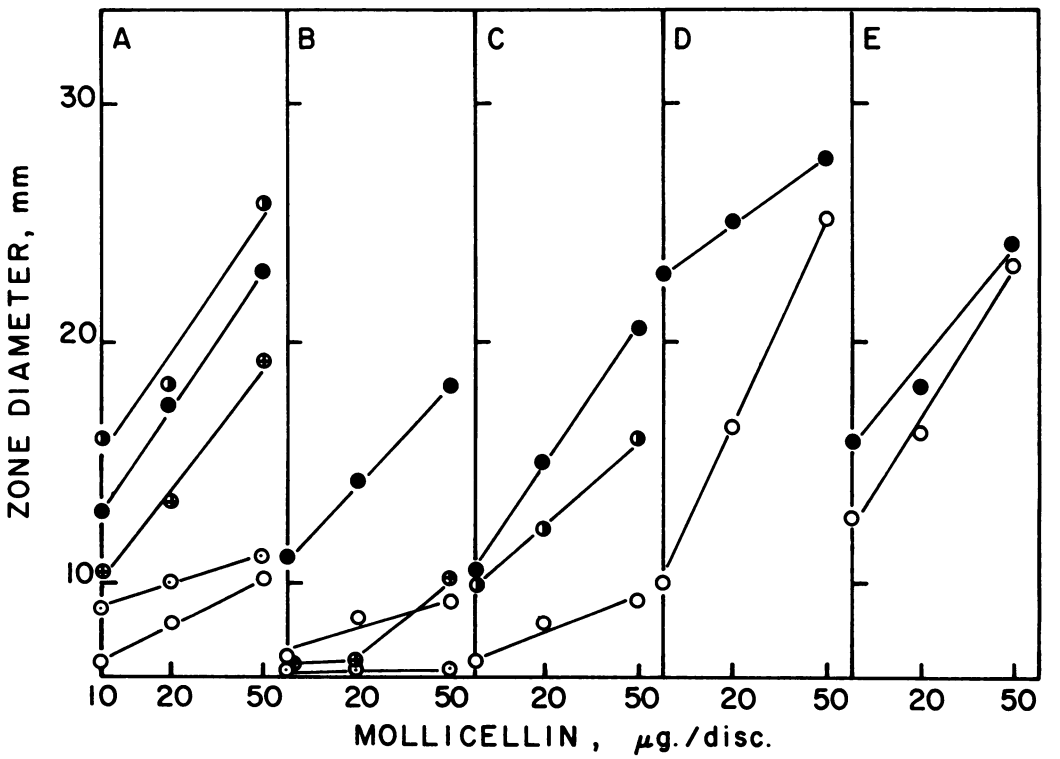

FIG. 2. *Antibacterial and mutagenic activities of mollicellins C, D, E, F, and H in the forward mutation assay (spot testing using S. typhimurium TM677). Mollicellin concentration is plotted on a logarithmic scale. Experimental conditions are as in the text except that plates were assayed after 4 days of incubation at 37°C. (A) antibacterial activity of fresh solutions in the absence of S-9; (B) antibacterial activity of fresh solutions in the presence of S-9; (C) antibacterial activity of 30-day-old solution in the absence of S-9; (D) mutagenic activity of fresh solutions in the absence of S-9; (E) mutagenic activity of 30-day-old solutions in the absence of S-9. Symbols: ○, mollicellin C; ◑, mollicellin D; ●, mollicellin E; ⊕, mollicellin F; ⊙ mollicellin H.*

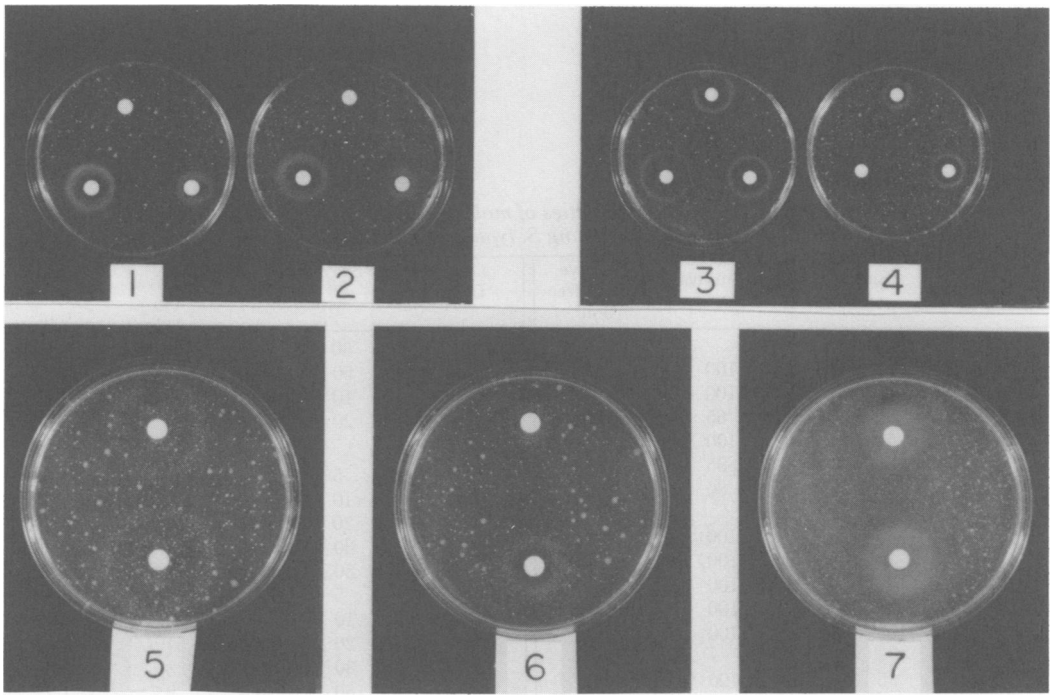

FIG. 3. *Mutagenicity of mollicellins C and E and some other known mutagens in the forward mutation assay (spot test using S. typhimurium TM677). Disks containing 10, 25, and 50 μg of mollicellins C and E in dimethyl sulfoxide (plates 1 through 4, starting at top disk, clockwise). Minimal agar medium was at pH 6.50 and contained 50 μg of 8AG per ml. Reaction mixtures were as described in the text except that S-9 and nicotinamide adenine dinucleotide phosphate were omitted. Incubation was at 37°C for 48 h. Pictures were taken after 4 days of incubation to allow maximum development of mutant rings. (Plate 1) Mollicellin C, fresh solution. (Plate 2) Mollicellin C, 30-day-old solution. (Plate 3) Mollicellin E, fresh solution. (Plate 4) Mollicellin E, 30-day-old solution. (Plate 5) MNNG: 1 μg (top); 2 μg (bottom). (Plate 6) EMS: 2 μl (top); 4 μl (bottom). (Plate 7) Acridine orange: 10 μg (top); 20 μg (bottom).*

sults). The latter is often used to test the antibacterial activity of mycotoxins and is more sensitive than the *Salmonella* strains used here.

Mollicellins C and E were active as mutagens in *S. typhimurium* at two independent genetic loci. This excludes the possibility that mollicellins protect 8AG-sensitive cells from inhibition by the drug via mechanisms other than mutation to the $8AG^R$ trait. Among the eight compounds, mollicellins C and E are the only ones that contain a 3-methylbutenoic acid moiety in position 1′. The absence of the carbonyl group from this moiety correlates with inactivity as a mutagen. Thus, the presence of O-methyl in the position ortho (2′) to the 3-methylbutenoic moiety may play a role in imparting mutagenic properties to mollicellins C and E. This possibility is currently being investigated.

The presence of chlorine in mollicellins appears to impart antibacterial properties to the molecule: the antibiotic activity of all chlorine-containing mollicellins (D, E, and F) is 10- to 40-fold higher than that of the others. Apparently, this chlorine-dependent antibacterial activity is independent of the structure at the 1′–2′ site. The presence of chlorine in mollicellin E appears to enhance both mutagenicity and antibiotic activity as compared with mollicellin C (which lacks chlorine). Mollicellin E is also less stable (more reactive) than mollicellin C: a dimethyl sulfoxide solution of mollicellin E stored for 30 days was less active as antibiotic or as mutagen than a fresh solution, whereas almost no decrease in either activity was observed with mollicellin C. Note also the decrease in antibiotic activity of chlorine-containing mollicellin D upon storage (Fig. 2A and C). The hydroxyl moiety in mollicellin C, which contains this 3-methylbutenoic group but lacks chlorine, has much less antibacterial activity than mollicellin E, which contains both hydroxyl and chlorine, and which shows antibacterial activity close to that of the chlorine-containing mollicellins D and F. It should be noted that the 3-hydroxy-

TABLE 3. *Mutagenic and antibacterial activities of mollicellins in the 8AG^R forward mutation assay (suspension test using S. typhimurium TM667)*[a]

Compound	μg/reaction	S-9	Survival (%)	Relative mutant fraction[b]
Mollicellin				
A	10	+	100	1.21
	20	+	100	1.05
	50	+	65	2.40
	10	−	100	0.70
	20	−	95	0.80
	50	−	80	0.65
B	5	+	100	1.32
	10	+	100	1.46
	50	+	100	0.90
	20	−	100	1.18
	50	−	100	1.01
C	5	+	100	0.97
	10	+	100	1.30
	20	+	100	0.90
	50	+	100	1.06
	70	+	100	0.88
	10	−	100	1.26
	20	−	80	0.88
	30	−	40	0.63
D	10	+	100	1.05
	20	+	85	1.21
	50	+	67	1.87
	70	+	35	0.83
	10	−	40	1.62
	20	−	27	1.70
	50	−	10	1.68
E	10	+	90	1.25
	20	+	82	1.49
	30	+	66	0.97
	50	+	57	1.80
	10	−	23	2.00
	20	−	20	3.80
F	5	+	100	1.09
	10	+	100	1.57
	20	+	97	1.59
	30	+	95	0.83
	50	+	88	0.90
	5	−	100	0.98
	10	−	92	0.94
	20	−	51	0.69
	30	−	23	0.68
	50	−	9.7	1.22
G	10	+	100	1.04
	20	+	100	1.07
	30	+	100	1.04
	10	−	100	0.80
	20	−	78	1.05
	30	−	81	1.09
H	18	+	87	1.74
	30	+	100	0.97
	45	+	86	1.57
	15	−	55	1.52
	30	−	66	1.34
	45	−	50	1.56
Aflatoxin B_1 (control)	0.075	+	90	18.70

[a] Reaction mixtures and procedure were as described in the text. Cells were washed off mycotoxins prior to plating. Control reaction mixtures did not contain mollicellins and were assayed in the presence and absence of microsomes. Spontaneous 8AG^R mutant frequency in the absence of microsomes was 2.95×10^{-5}; in the presence of microsomes it was 5.58×10^{-5}.

[b] Ratio between observed mutant frequency in the presence and absence of mycotoxins.

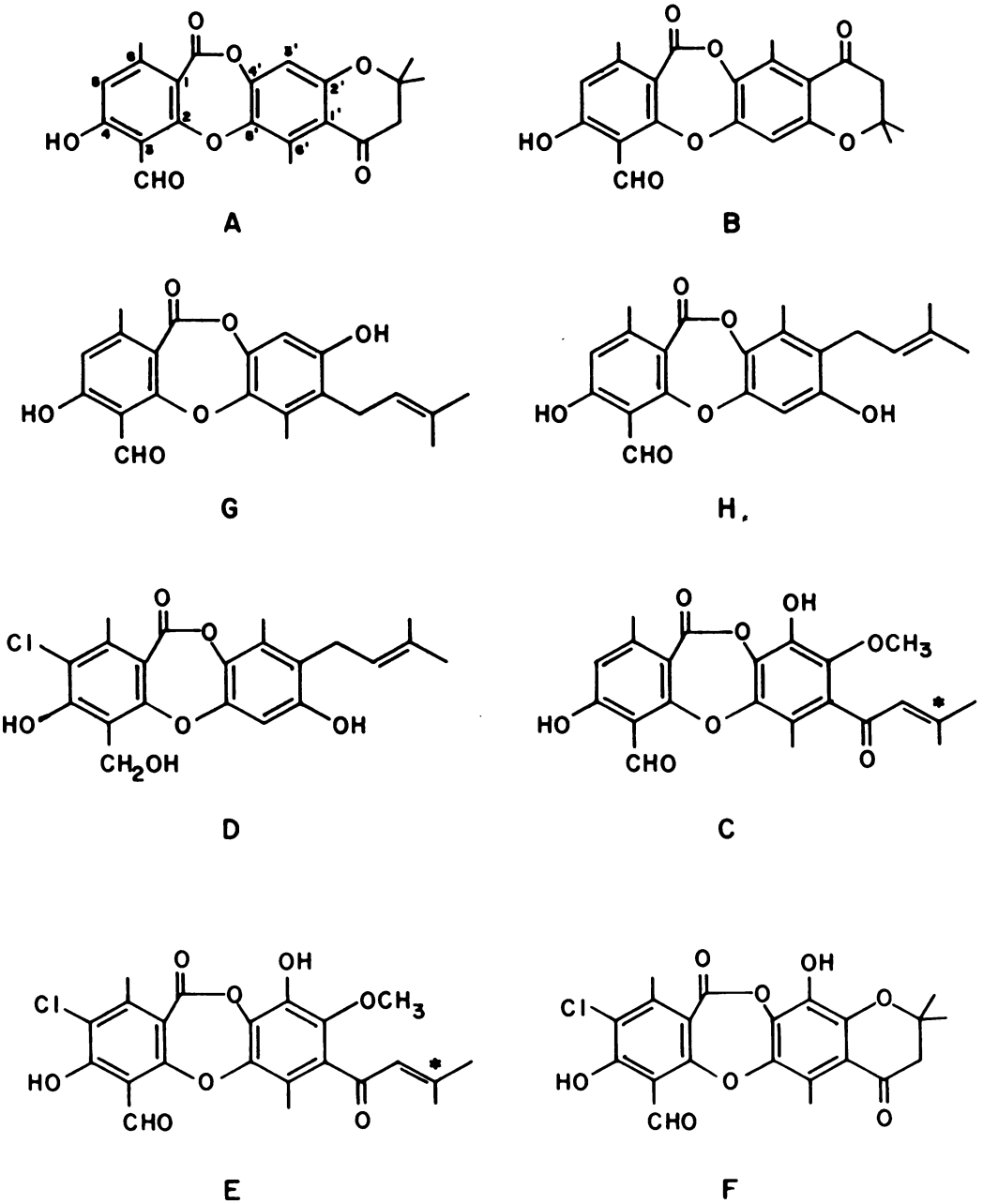

FIG. 4. *Structures of mollicellins (according to Büchi et al., submitted for publication) (nomenclature of carbon atoms according to Culberson [3]).*

methyl group in mollicellin D may also play a role in imparting antibiotic properties to mollicellin D. However, it is difficult to draw any such conclusion, since no mollicellin possessing a 3-hydroxymethyl group but lacking chlorine has been isolated. It thus appears that both mutagenic and antibiotic properties reside in the 1'-3-methylbutenoic acid moiety, whereas the presence of chlorine increases only antibiotic activity of the mollicellin series.

It is possible that the mollicellins containing four rings, i.e., A, B, and F, are formed from

open structures resembling mollicellins C and E by cyclization at the carbon atom marked with an asterisk in Fig. 4. The reactivity of this carbon and, in fact, its electrophilicity, could be responsible for mutagenic and antibacterial activity. The correlation of the 1'-3-methylbutenoic moiety (including its position relative to the 2'-O-methyl group) with mutagenicity is, to our knowledge, a unique finding.

It is tempting to hypothesize that the microsomal conversion of mollicellins C and E into nonmutagenic species involves the cyclization of the 3-methylbutenoic group. This question is currently under investigation.

ACKNOWLEDGMENTS

We thank W. G. Thilly for *S. typhimurium* TM677 and for many helpful discussions concerning the forward mutation assay. We also thank B. N. Ames for *S. typhimurium* TA100.

Support for this work was provided by Public Health Service contract 1CP33217 from the National Cancer Institute.

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