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Mutagenic Activity of Austocystins—Secondary Metabolites of *Aspergillus ustus*

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Mycotoxins constitute a group of toxic secondary fungal metabolites. Fungi that produce these toxins frequently contaminate food and feed, creating a potential threat to human and animal health. Biological activities of mycotoxins include, amongst others: toxicity, mutagenicity and carcinogenicity, which can be expressed with or without metabolic activation (Uraguchi and Yamazaki 1978; Ames et al. 1973). One of the best known and most extensively studied examples of mycotoxins is aflatoxin B_1, a highly toxic, mutagenic and carcinogenic metabolite of Aspergillus flavus (Butler 1974). A closely related group of mycotoxins, austocystins, is produced by another fungus, namely Aspergillus ustus. Austocystins are similar in structure to aflatoxin B_1 (Fig. 1) and are probably synthesized in a similar manner (Steyn et al. 1980).

The Ames Salmonella test, a widely accepted method employed for the detection of mutagenic activity of various chemical compounds (Ames et al. 1973, 1975), was used for testing the mutagenic activity of different mycotoxins (Nagao et al. 1976). As aflatoxin B_1 was found by the Ames test to be highly mutagenic, the same test was applied for the study of possible mutagenicity of the austocystins. The mutagenic activity of these compounds was studied with and without metabolic activation using two tester strains of S. typhimurium, one capable of detecting frame shift mutation (strain TA98) and the other capable of detecting base pair substitution (strain TA100).

MATERIALS AND METHODS

Aflatoxin B_1 and austocystins A, B, C, D and H were isolated and purified by Steyn and Vleggaar (1974, 1975). All compounds were dissolved prior to testing in dimethylsulphoxide (DMSO, Merck, West Germany).

Bacterial strains of S. typhimurium, strain TA98 and TA100, were obtained from Dr W.O.K. Grabow (NIWR, CSIR, Pretoria, South Africa). Cultures were tested for their histidine and biotin

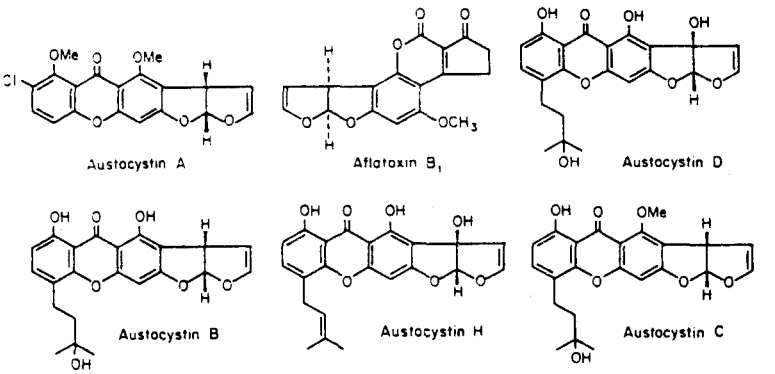

Figure 1. Molecular structure of austocystins and aflatoxin B_1

requirements, ampicillin resistance and crystal violet sensitivity. The rate of spontaneous reversion to the prototrophic state was repeatedly tested. The strains were kept frozen at -80°C. Overnight shake cultures grown in nutrient broth (Oxoid, London, England) at 37°C were used for all experiments.

The microsomal fraction of rat liver, S_9, as well as the S_9 mixture, were prepared as described by Ames et al. (1975). Male Wistar rats of about 300 g each were injected with aroclor 1254 (Analabs, Conn. USA), and after three days their livers were used for S_9 preparation. This S_9 preparation was incorporated into the S_9 mixture in a ratio of 0.05 ml per 1 ml of the mixture.

Quantitative mutagenicity tests were carried out as described by Ames et al. (1975). Positive controls, i.e. spot tests (Ames et al. 1975) were done with N-methyl-N'-nitro-N-nitrosoguanidine (Sigma, St. Louis, Mo, USA), 2-aminofluorene (Sigma, St. Louis, Mo, USA) and sodium azide (BDH Chemicals Ltd, Poole, England). Spontaneous reversion rates of both tester strains, and the influence of the solvent DMSO (in quantities not exceeding 100 µl per plate) on their spontaneous reversion rates, were examined.

Various concentrations of the different austocystins (Tables 1 and 2) with and without the addition of the S_9 fraction of liver microsomes were assayed with the same set of experiments, i.e. the same cultures ofthe tester strains, the same batch of minimal plates, and identical incubation conditions were used. All tests were carried out at least in triplicate and always included positive, negative (spontaneous reversion) and sterility controls. Plates were incubated at 37°C for 48 h before bacterial colonies were counted.

The results obtained are expressed as mutagenicity ratios (MR), which represent a ratio of the number of revertant colonies on test plates (spontaneous plus induced revertants) to the number

of colonies on negative control plates (spontaneous revertants). In this study an MR value of 1.0 obtained for a particular compound at a certain concentration was considered to indicate a lack of mutagenic activity.

RESULTS AND DISCUSSION

Cultures of the tester strains TA98 and TA100 were found to maintain their auxotrophic requirement for L-histidine and biotin and their ampicillin resistance, and showed sensitivity to crystal violet. It was therefore concluded that the strains used in this study retained their original phenotypes. The average numbers of spontaneous revertant colonies per plate were 19 for strain TA98 and 131 for strain TA100 (Tables 1 and 2). Control plates, on which spontaneous revertant colonies were tested in the presence of the S_9 liver fraction, showed an increase in the number of these colonies to 31 per plate for strain TA98 and 153 for strain TA100 (Tables 1 and 2). These numbers are in agreement with the numbers of spontaneous revertants previously observed for these two tester strains (Ames et al. 1975). Both tester strains reverted to the prototrophic state in the presence of known mutagens which were previously found to mutate them, namely sodium azide and N-methyl-N'-nitro-N-nitrosoguanidine, which acted directly, and 2-aminofluorene and aflatoxin B_1, both of which required metabolic activation.

As is apparent from the results obtained with tester strain TA100 (Table 1), austocystins A, C and D showed a lack of or very mild direct mutagenic activity within the range of concentrations tested - a concentration of 50 µg/plate resulted in an MR value of 1.0 for austocystin D, 1.7 for austocystin C and 2.2 for austocystin A. Austocystins H and B showed some mutagenic activity. Austocystin B showed an MR value of 1.6 at a concentration of 0.1 µg/plate and 4.6 at a concentration of 50 µg/plate. A concentration of 5 µg/plate of austocystin H resulted in an MR value of 1.6, which increased to 3.7 when a 50 µg/plate was used. The MR values obtained for aflatoxin B_1 followed the trend observed in austocystin B and reached 6.6 for a concentration of 50 µg/plate. Neither austocystin A, B, C, D or H nor aflatoxin B_1, when tested without metabolic activation, showed any toxic effect within the range of concentrations examined.

Metabolic activation greatly enhanced the mutagenicity of all five austocystins tested (Table 1). The increase in mutagenicity was clearly evident in the lower range of concentrations, as at higher concentrations metabolic activation converted austocystins to highly toxic compounds. Due to the toxic effect of the compounds, a decrease in the size of revertant colonies was observed and in many cases colonies were not found at all. A concentration of 1 µg/plate of austocystin B caused a five-fold reduction in the MR value in comparison to the MR value observed for the concentration of 0.1 µg/plate. Austocystin D showed a two-fold reduction at the same concentration range.

Toxic activity was observed at different concentrations for the different austocystins. For austocystin H, a 5 μg/plate was toxic, whereas austocystin A only showed its toxic effect at a concentration of 50 μg/plate (Table 1). The known mutagen, aflatoxin B_1, when tested using strain TA100 at the same concentration range, showed a high MR value at 0.1 μg/plate. At any higher concentration tested the toxic effect led to the absence of revertant colonies (Table 1).

According to the results discussed above, the trend of mutagenic activity of all the austocystins tested was similar to that observed for aflatoxin B_1. The MR values obtained for austocystins A and D are in agreement with the results reported by Wehner et al. (1978). The discrepancies between the results obtained in this study with metabolically activated austocystins A and D and the results obtained by Wehner et al. (1978) may be attributed to the differences in the spontaneous reversion rates and the use of different S_9 preparations (Table 1).

Table 2 summarizes the MR values obtained when strain TA98 was tested using various concentrations of the five austocystins. Without metabolic activation austocystin B induced a very high number of revertants, with an MR value of 15.2 when a concentration of 50 μg/plate was used. Austocystins H and C showed a similar trend within the range of concentrations tested, reaching lower MR values for the highest concentration of 9.8 and 4.2 respectively. Very mild mutagenic activity was observed for austocystins A and D at the same concentration with MR values of 1.5 and 1.9 respectively. In comparison to the MR values obtained for austocystin B, aflatoxin B_1, when tested at the same range of concentrations, showed stronger mutagenic activity at a concentration of 50 μg/plate while other concentrations gave lower MR values. All austocystins tested, as well as the positive control of aflatoxin B_1, showed no toxic effect at the range of concentrations studied.

As shown in Table 2, metabolically activated austocystins showed a marked increase in mutagenicity and toxicity. Both austocystins B and D showed a very strong mutagenic activity as 1 μg/plate resulted in MR values of 89.6 and 74.9 respectively. At higher concentrations both austocystins were toxic to the bacteria.

Austocystin H showed strong mutagenic activity with an MR value of 56.5 obtained at a concentration of 5 μg/plate, whereas higher concentrations showed toxic activity. Austocystins A and C showed milder mutagenic activity, with austocystin A showing the lowest activity. At a concentration of 1 μg/plate, austocystin C gave its highest MR value of 19.0. The highest mutagenic effect of austocystin A was shown at 10 μg/plate with an MR of 5.0. For the two austocystins, one concentration above the ones mentioned was too toxic to permit any mutagenic activity. Aflatoxin B_1 gave MR values similar to those obtained for austocystins B and D

Table 1. Mutagenicity ratios (MR*) for various concentrations of austocystins A, B, C, D and H and aflatoxin B_1 with a tester strain TA100

Concentration µg/plate \ Mycotoxin	Austocystin A	Austocystin B	Austocystin C	Austocystin D	Austocystin H	Aflatoxin B_1
			Without metabolic activation			
0.1	1.0	1.6	1.0	1.0	1.1	1.0
1.0	1.0	1.6	1.0	1.0	1.2	1.2
5.0	1.0	3.2	1.0	1.0	1.6	2.1
10.0	1.1	4.1	1.0	1.0	1.4	3.8
50.0	2.2	4.6	1.7	1.0	3.7	6.6
	Average number of revertant colonies on control plates: 131					
			With metabolic activation			
0.1	1.2	10.4	1.5	4.8	2.1	
1.0	4.9	2.4	5.2	2.0	8.4	Toxic
5.0	4.1	1.2	1.8	Toxic	2.6	Toxic
10.0	4.6	1.1	2.0	Toxic	1.7	Toxic
50.0	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic
	Average number of revertant colonies on control plates: 153					

* $MR = \frac{\text{Average number of revertant colonies on treated plates}}{\text{Average number of revertant colonies on control plates}}$

Table 2. Mutagenicity ratios (MR*) for various concentrations of austocystins A, B, C, D and H and aflatoxin B_1 obtained with a tester strain TA98

Concentration µg/plate \ Mycotoxin	Austocystin A	Austocystin B	Austocystin C	Austocystin D	Austocystin H	Aflatoxin B_1
			Without metabolic activation			
0.1	1.0	1.0	1.0	1.0	1.0	1.0
1.0	1.0	1.7	1.0	1.1	1.0	1.0
5.0	1.0	6.2	1.0	1.2	1.4	3.5
10.0	1.0	9.7	1.2	1.4	3.2	4.5
50.0	1.5	15.2	4.2	1.9	9.8	25.4
	Average number of revertant colonies on control plates: 19					
			With metabolic activation			
0.1	1.8	41.3	4.8	40.3	2.3	35.5
1.0	2.1	89.6	19.0	74.9	27.0	52.0
5.0	4.1	Toxic	16.0	Toxic	56.5	Toxic
10.0	5.0	Toxic	14.0	Toxic	26.6	Toxic
50.0	3.8 (Toxic)	Toxic	Toxic	Toxic	Toxic	
	Average number of revertant colonies on control plates: 37					

$$* \text{ MR} = \frac{\text{Average number of revertant colonies on treated plates}}{\text{Average number of revertant colonies on control plates}}$$

at a concentration of 0.1 μg/plate, and higher concentrations were highly toxic.

The enhancement of mutagenicity of the austocystins by the S_9 fraction is probably of the same nature as that described for aflatoxin B_1. The formation of a highly active intermediate of aflatoxin B_1 is associated with the change of the vinyl ether moiety (Engelbrecht and Altenkirk, 1972) to an epoxide during the exposure of the mycotoxin to the mixed function oxygenase (Carner et al., 1972 Garner, 1973). In view of the structural similarity between the austocystins and aflatoxin B_1 (Fig. 1) the mechanism of their activation should be the same or similar.

The differences in the molecular structure of one or two functional groups of the five austocystins tested appeared to significantly affect their mutagenic activity. Even though all austocystins have the similar vinyl ether moiety, which has been shown to be associated with the activation of aflatoxin B_1, the other functional groups might influence the strength of binding of the activated forms of austocystins to the nucleic acid material.

Metabolically activated austocystins B, C and H were found to be potent mutagens with strong frame-shifting activity. Austocystins B and H were also found to be moderately mutagenic to both tester strains without metabolic activation i.e. it is possible that these mutagens might be interacting with nucleic acids not exclusively via the activated vinyl ether moiety.

There appeared to be a direct correlation between the mutagenic efficiency and the degree of toxicity exhibited by the five austocystins. Metabolic activation enhanced the toxicity of the five austocystins studied.

REFERENCES

Ames BN, Lee FD, Durston WE (1973) An improved bacterial test system for the detection and classification of mutagens and carcinogens. Proc Natl Acad Sci 70:782-786.

Ames BN, McCann J, Yamasaki E (1975) Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutation Res 31:347-363.

Butler WH (1974) Aflatoxin. In: Purchase IFH (ed) Mycotoxins. Elsevier Science Publishers, Amsterdam, Oxford, New York, pp. 1-28.

Engelbrecht JC, Altenkirk B (1972) Comparison of some biological effects of sterigmatocystin and aflatoxin analogues on primary cell cultures. J Nat Cancer Inst 48:1647-1655.

Garner RC (1973) Chemical evidence for the formation of a reactive aflatoxin B_1 metabolite, by hamster liver microsomes. FEBS Letters 36:261-264.

Garner RC, Miller EC, Miller JA (1972) Liver microsomal metabolism of aflatoxin B_1 to a reactive derivative toxic to Salmonella typhimurium TA 1530. Cancer Res. 32:2058-2066.

Nagao M, Honda M, Hamasaki T, Natori S, Ueno Y, Yamasaki M, Seino Y, Yahagi T, Sugimura T (1976) Mutagenicity of mycotoxins on Salmonella. Proc Japan Assoc Mycotoxicol 3/4: 41-43.

Steyn PS, Vleggaar R (1974) Austocystins. Six novel dihydrofuro[3',2':4,5]furo[3,2-b]xanthenones from Aspergillus ustus. J Chem Soc Perkin Trans 1:2250-2256.

Steyn PS, Vleggaar R (1975) Dihydrofuro[3',2':4,5]furo[3,2-b]-xanthenones: the structures of austocystins G, H and I. J SA Chem Inst XXVIII:375-377.

Steyn PS, Vleggaar R, Wessels PL (1980) The biosynthesis of aflatoxin and its congeners. In: Steyn PS (ed) The Biosynthesis of Mycotoxins, Academic Press, New York, pp. 105-155.

Uraguchi K, Yamazaki M (1978) Toxicology, biochemistry and pathology of mycotoxins. Kodansha Scientific Books, Tokyo, Japan. pp. 107-188.

Wehner FC, Thiel PG, Van Rensburg SJ, Demasius IPC (1978) Mutagenicity to Salmonella typhimurium of some Aspergillus and Penicillium mycotoxins. Mutation Res 58:193-203.

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