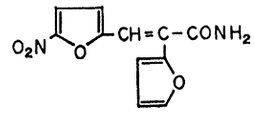
Mutagenic and Recombinogenic Activities of the Food Additive Furylfuramide in Eukaryotes

Abstract. *The mutagenic and recombinogenic activities of furylfuramide, an antimicrobial food additive, were tested in a two-component heterokaryon of* Neurospora crassa *and a diploid strain of* Saccharomyces cerevisiae, *respectively. The results show that furylfuramide is genetically active in both eukaryotic organisms; it induces mutations in* Neurospora crassa *and mitotic crossing over in* Saccharomyces cerevisiae.

Furylfuramide (AF-2), 2-(2-furyl)-3-(5-nitro-2-furyl)acrylic acid amide, is commonly used in Japan as an antimicrobial food additive. Since little is known with regard to the potential mutagenic, carcinogenic, and teratogenic effects of this agent in humans, investigations related to these properties are urgently needed. Studies in our laboratory were carried out to determine whether AF-2 causes genetic alterations in *Neurospora* and yeast.



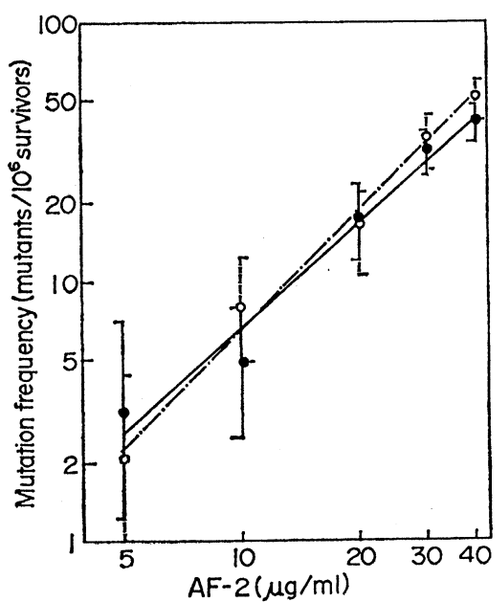
AF-2

In *N. crassa*, the mutagenic activity of AF-2 was tested at the *ad-3* region. In this region, recessive-lethal mutations resulting from chromosomal deletions or intragenic alterations can be recovered, and the type of mutagenic event can be determined (*1*). Conidia (6×10^6 per milliliter) from a genetically marked two-component heterokaryon of *N. crassa* (*2*), prepared as described by Ong and de Serres (*3*), were treated with different concentrations of AF-2 for 2 hours at 30°C in the dark in a water bath shaker. AF-2 was dissolved in dimethyl sulfoxide (DMSO) before being added to the treatment flasks containing specified amounts of 0.067*M* KH_2PO_4–Na_2HPO_4 (*p*H 7) buffer and conidia. The DMSO concentration (2 percent) in the control flasks was similar to that in the treatment flasks. DMSO was used as a solvent for AF-2 in these experiments because the mutagenic activities of some other nitrofurans that are insoluble in water but can be dissolved in DMSO were also studied in *N. crassa*. At the end of the treatment period, the solutions were centrifuged and the supernatants were decanted. The conidia were washed twice and resuspended in Fries basal medium (*p*H 8). Samples of conidia were then analyzed for the presence of *ad-3* mutants by the direct method (*4*).

In yeast, mitotic recombination was detected with the use of the diploid strain D5 which has recently been developed by Zimmermann (*5*). Cells were taken from stock cultures, inoculated into liquid YPG medium [0.15 per cent KH_2PO_4, 0.05 percent $MgSO_4$, 0.45 percent $(NH_4)_2SO_4$, 0.35 percent protease peptone (Difco), 0.5 percent yeast extract (Difco), and 2 percent glucose] and incubated at 30°C in a shaking water bath until growth reached the stationary phase. The cultures were stored at 4°C until required. Immediately before they were used, the cells were harvested by centrifugation and washed twice with phosphate buffer (*p*H 7.2). The AF-2 was dissolved in the same buffer and added to the cell suspension (1.2×10^8 cell/ml) to give a final concentration of 50 or 100 μg/ml. The control and treated cell suspensions, in Pyrex "low actinic" erlenmeyer flasks, were then incubated at 30°C in a shaking water bath. At appropriate intervals cell samples were withdrawn, centrifuged, and washed twice with phosphate buffer. The cell samples were then diluted, spread on YPG plates, and incubated for 5 days at 30°C. Colony-forming ability was the criterion of survival, and mosaic, red and pink (twin spot) colonies were scored as mitotic recombinants. Other phenotypically altered colonies (such as pink or red complete colonies) were indicative of genetic alterations of an unspecified nature.

The results of mutation induction experiments in *N. crassa* are summarized in Fig. 1. The *ad-3* mutation frequency increased with increasing concentration of AF-2. After conidia were treated with AF-2 (40 μg/ml), mutation frequency increased to 45 per 10^6 survivors, which is a 120-fold increase over the average spontaneous mutation frequency (0.38×10^{-6}) for this strain. The *ad-3* mutation frequencies in the control experiments are not higher than the average spontaneous mutation frequency. At concentrations of AF-2 higher than 5 μg/ml, survival of conidia decreases linearly with concentration. Survival is only 30 percent after treatment with 40 μg of AF-2 per milliliter.

The induction of aberrant colonies by AF-2 in yeast strain D5 is shown in Fig. 2. This compound increases the



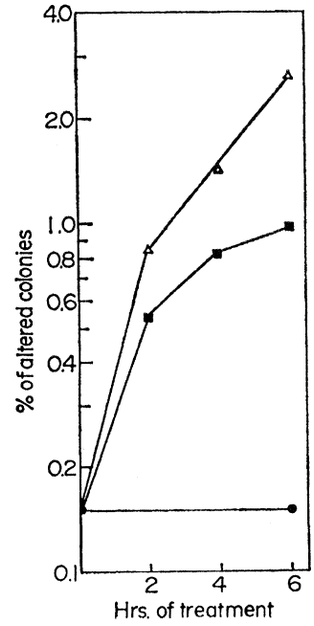


Fig. 1 (left). Frequencies of *ad-3* mutants after conidia of *Neurospora crassa* were treated with various concentrations of AF-2. The results from two independent experiments are plotted with their 95 percent confidence limits; (○) experiment 1; (●) experiment 2. Regression lines are indicated by broken line for experiment 1 and solid line for experiment 2. Fig. 2 (right). Percentage of altered colonies of *Saccharomyces cerevisiae* strain D5 induced by 50 μg (■) and 100 μg (△) of AF-2 per milliliter; (●) controls.

number of aberrant colonies as a function of incubation time. The frequency of altered colonies increased from 0.15 percent (control) to 2.63 percent after D5 cells were treated with 100 μg of AF-2 per milliliter for 6 hours. This is about an 18-fold increase over the spontaneous frequency. If the increase in aberrant colonies is based only on mitotic crossing-over events which generate twin-spotted colonies, however, the frequency is 65 times higher than that of the controls. It is interesting to note that under our experimental conditions no cell inactivation was detectable at either of the two concentrations of AF-2 used.

Our results indicate that AF-2 causes genetic alterations in eukaryotes and that this food additive is a potent mutagen. On a per mole basis, the mutagenic activity of this compound in *N. crassa* is higher than that of many well-known chemical mutagens, such as nitrous acid, ethyl methanesulfonate, methyl methanesulfonate, hydroxylamine, ethylenimine and hycanthone. In yeast AF-2 had a higher genetic activity than hycanthone or SQ18,506 (*6*). Studies by others have shown that AF-2 induces mutations in bacteria (*7*) and causes chromosome aberrations in human lymphocytes (*8*). Although Miyaji (*9*) reported that AF-2 failed to increase tumor incidence in mice fed with this compound, he cautiously indicated that his study was not adequate to draw general conclusions on tumorigenicity of AF-2 and stated that more suitable studies need to be carried out. Present knowledge of the genetic activity of AF-2 emphasizes the need for information relating to the possible mutagenic, carcinogenic, and teratogenic hazards of this compound in man and the need for reevaluation of the use of this compound as a food additive.

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References and Notes

1. F. J. de Serres, *Genetics* **50**, 21 (1964); *J. Cell. Comp. Physiol.* **64** (Suppl. 1), 33 (1964).
2. ——— and B. B. Webber, *Neurospora Newslett.* **3**, 3 (1963).
3. T. Ong and F. J. de Serres, *Mutation Res.* **13**, 276 (1971).
4. F. J. de Serres and H. G. Komalk, *Nature (Lond.)* **182**, 1249 (1958).
5. F. K. Zimmermann, *Mutation Res.* **21**, 263 (1973).
6. M. M. Shahin, in preparation.
7. T. Kada, *Jap. J. Genet.* **48**, 301 (1973); S. Kondo and H. Ichikawa-Ryo, *ibid.*, p. 283.
8. A. Tonomura and M. S. Sasaki, *ibid.*, p. 291.
9. T. Miyaji, *Tohoku J. Exp. Med.* **103**, 331 (1971).
10. AF-2 was supplied by Dr. T. Matsushima, Institute of Medical Science, University of Tokyo, Tokyo, Japan, through the courtesy of Dr. T. Sugimura, National Cancer Center Research Institute, Tokyo, Japan.

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Plant Species Intermediate for C_3, C_4 Photosynthesis

Abstract. Mollugo verticillata *is the first plant species reported which has characteristics of both C_3 (Calvin-Benson pathway) and C_4 (Hatch-Slack pathway) plants. This plant species is intermediate between C_3 and C_4 plants in at least four features generally used to separate those two plant groups: leaf anatomy, cell ultrastructure, photorespiration, and primary photosynthetic products.*

Prior to 1965, all green plants were thought to assimilate atmospheric CO_2 via the Calvin-Benson or reductive pentose phosphate pathway (*1*). The first stable product of those reactions is the 3-carbon compound, phosphoglyceric acid (PGA). A series of reports by Kortschak and co-workers beginning in 1954 indicated that PGA was not a primary product of photosynthesis in sugar cane (*2*). A complete report on this alternative pathway of CO_2 fixation was described by Kortschak *et al.* in 1965 in which the 4-carbon acids malic and aspartic are the primary products of photosynthesis (*3*). Similar results were independently reported by Karpilov in 1960 (*4*). As a result of this discovery, flowering plants have been divided into C_3 plants (those with PGA as the first stable intermediate) and C_4 plants (those with 4-carbon acids as the first stable intermediates). The C_4 plants are also distinguished from C_3 plants by several other specific physiological and antomical features such as leaf anatomy, organelle structure, low photorespiration rates, high photosynthetic efficiency, and reduced discrimination of ^{13}C (*5*).

Within a taxonomic category, plants with C_3 photosynthesis are considered to be ancestral to those with C_4 photosynthesis; the latter having a diverse taxonomic and geographic distribution. In spite of the multiple evolution of C_4 photosynthesis, no plant species has been reported which possesses features intermediate between the syndrome of features characteristic of either C_3 or C_4 plants. All species investigated so far have been entirely C_3 or C_4. This is strange, because the distribution of C_4 plants among flowering plant families does not follow strict taxonomic lines. Even within a genus, not all species need be C_3 or C_4 (*6*). The genus *Atriplex*, for example, has a C_4 species (*A. rosea*) and a C_3 species (*A. patula*). An intermediate hybrid of these two species has been created, but the hybrid, while intermediate in leaf anatomy and many biochemical characteristics, was not intermediate in "photosynthetic performance" (*7*).

The Aizoaceae is known to contain C_3 plants, C_4 plants, and crassulacean acid metabolism (CAM) plants. Previous work with the genus *Mollugo* (Aizoaceae) indicated that it might contain a species which was intermediate between C_3 and C_4 plants (*8*). The cytology of one member of this genus, *M. cerviana*, was wholly C_4, whereas another member, *M. verticillata*, appeared to have characteristics of both C_3 and C_4 plants. This report shows that *Mollugo verticillata* possesses features intermediate between C_3 and C_4 plants in leaf anatomy, cell ultrastructure, photorespiration, and primary photosynthetic products.

Fully expanded leaves of *Mollugo verticillata* L. and *M. cerviana* L. (Aizoaceae) were obtained from plants grown in environmental chambers. A 16-hour photoperiod was maintained with temperatures of 27°C day and 18°C night. Both plants continually flowered under a light energy of 1.4×10^4 erg cm^{-2} sec^{-1} supplied by fluorescent and incandescent lamps. All $^{14}CO_2$ feeding experiments, extraction, separation, and identification of the products of photosynthesis were conducted as described (*9*). Leaves were exposed to $^{14}CO_2$ for 5 seconds. Preparation of leaf material for light microscopy has been reported (*10*).

Photorespiratory rates were determined with a method adapted from that of Zelitch (*11*). Stomata were opened prior to the assimilation period by passing CO_2-free air over the illuminated leaves for 45 minutes. Air flow was then stopped, and the leaves were allowed to completely assimilate 2 μmole of $^{14}CO_2$ for 45 minutes. The $^{14}CO_2$ evolved in the light was compared to the $^{14}CO_2$ evolved in the dark. The $^{14}CO_2$ trapping agent consisted of 50 ml 1.0*N* KOH with 0.6 percent isoamyl alcohol.