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ANTIMUTAGENS FROM *MOMORDICA CHARANTIA* LINN.

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ABSTRACT

A mixture of antimutagens, D, were isolated from the ethanol extract of the green fruits of *Momordica charantia* Linn., locally known as ampalaya, by solvent partitioning and repeated column chromatography. The major component of the mixture, D', was separated from the minor components by preparative high pressure liquid chromatography (HPLC). HPLC-pure D' was shown to be an intractable mixture of D'-1, as the major component, and D'-2, as the minor component.

The mixtures D and D' significantly reduced the number of micronucleated polychromatic erythrocytes induced by the well-known mutagen, Mitomycin C. At a dosage range of 12.5 ug – 50 ug per gram mouse, D' reduced the mutagenicity of Mitomycin C by 80%.

The structures of D' were elucidated by high-field proton nuclear magnetic resonance spectroscopy (^{1}N NMR), carbon-13 nuclear magnetic resonance spectroscopy (^{13}C NMR), Fourier transform infrared spectroscopy (FTIR), mass spectroscopy (MS), and chemical modification (acetylation, saponification, and methanolysis) followed by chromatographic and spectral analysis.

Spectral and chromatographic data indicated that D'-1 was 3-O-[6'-O-palmitoyl-β-D-glucosyl]-stigmasta-5,25(27)-dien while D'-2 was the stearyl derivative.

Structure-activity correlation studies suggested that the antimutagenic activity may reside in the peculiar lipid-like structure of the acylglucosylsterols which may allow them to be absorbed in the plasma membrane, thereby adversely affecting the membrane permeability towards Mitomycin C.

A computer search of the Biological Abstract and the Chemical Abstract revealed that no previous work has been published on the isolation, structure elucidation, and antimutagenic activity of these acylglucosylsterols. The structure elucidation of these novel compounds will be published in a forthcoming issue of *Phytochemistry* (in press).

Introduction

Previous studies (Sylianco, 1986) showed that the expressions and/or decoctions of the leaves, fruits, and seeds of some 50 Philippine plants exhibited antimutagenic activity towards some well known mutagens like Mitomycin C, tetracycline, and dimethylnitrosoamine. This particular study on *Momordica charantia* Linn., locally known as ampalaya, was the first attempt to isolate, purify, and elucidate the structure of the active antimutagenic components from Philippine plants.

Antimutagenicity is a relatively new area of research, particularly the search for antimutagens of plant origin. The literature shows that a relatively few researchers have reported the isolation and characterization of some substances from plants found to inhibit the mutagenicity of various mutagens. These substances were found to be structurally diverse. Among the antimutagens found from plants were cinnamaldehyde from cinnamon bark oil (Ishii, 1984; Ohta, 1983), coumarin from lavender, woodruff, and sweet clover and unbeliferone from umbelliferae plants (Ohta, 1983), protoanemonin from Ranunculus and Anemone plants (Minakata, 1982), emodin from Rhubarb (Kushi, 1980), and epigallo-catechin-gallate from green tea leaves (Kada, 1985). Extracts of some common vegetables like cucumber, celery, lettuce, brocoli, spinach, cabbage, and parsely have also been shown to exhibit antimutagenic activity (Inoue, 1981; Morita, 1982; Shimoi, 1985). The active components of these vegetables await future research work.

The importance of antimutagens can not be overemphasized at this point. There are at present many mutagens in the environment and even in the common food we eat, many of which are thought to be related to cancer, genotoxicity, and aging. This has created worldwide interest in the study of antimutagenic agents and their modes of action in an effort to minimize, if not eliminate, such mutagens. Studies on antimutagens are important not only for the elucidation of the genetic mechanisms of mutagesis but also for the prevention of cancer (Ohta, 1983).

Experimental

Isolation and Purification

Fresh green fruits of *Momordica charantia* were homogenized in distilled ethanol at room temperature. The filtered extract was concentrated under reduced pressure at 40°C and subsequently partitioned between water, dichloromethane, petrol, aqueous methanol, and carbon tetrachloride using the method of Kupchan (Kupchan, 1978) as shown in Scheme I. The petrol and carbon tetrachloride extracts, which were shown by the bioassay tests to be antimutagenic, were subjected to repeated and sequential flash column chromatography using vacuum elution and pressure elution until thin layer chromatography (TLC)-pure fractions were obtained. Mixtures of hexane-ethylacetate of varying polarities were used as solvent. The elution was monitored by analytical TLC on precoated silica gel plates.

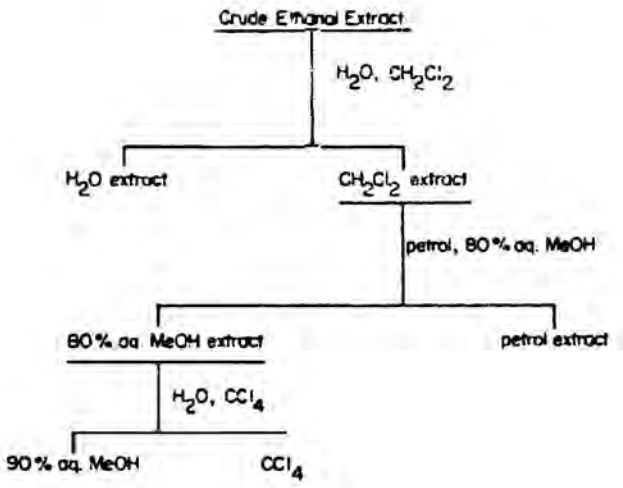
The TLC-pure fractions were finally subjected to preparative HPLC to isolate the intractable mixture D'.

Bioassay: The Micronucleus Test

The micronucleus test (Schmid, 1978) was used to monitor the antimutagenic activity of the various fractions during the isolation process.

Seven- to nine-week old Swiss mice were used as test animals. Mitomycin C was used as the mutagenic control. A uniform dosage of 3 μg per gram mouse was

FRACTIONATION SCHEME



injected intraperitoneally to the test animals. Two treatments were given – 30 and 6 hours – before the mice were sacrificed.

The test extracts were dissolved in 50% dimethylsulfoxide (DMSO) and administered twice orally at varying dosages to the test animals using a feeding gavage. Three to five mice were used for each test dosage.

Immediately after sacrificing the mice, the bone marrow from the femura bones were suspended in fetal calf serum. The suspensions were centrifuged at 1000 rpm for 5 minutes and the cells in the sediment were smeared on glass slides. Three sides were smeared for each mouse. The air-dried slides were stained successively with May Grunwald and Giemsa stains. The slides were scored under a high power microscope by counting the number of micronucleated polychromatic erythrocytes (PCE) per 1000 PCE.

The data on the number of micronucleated PCE per 1000 PCE were processed on an IMB PC using the "Microstat" software, specifically the program on the analysis of variance. The calculated F ratios were compared with the critical F values found in statistical tables at 5% and 1% probability levels. The treatment effects were considered statistically significant when the calculated ratios were higher than the corresponding critical F ratios.

Structure Elucidation

The structure of the antimutagens were elucidated using high-field (400MHz) proton and carbon-13 NMR, Fourier transform infrared spectroscopy, and high and low resolution mass spectroscopy. Confirmation of the structure was obtained from chemical modification of the structure was obtained from chemical modification studies (acetylation, saponification, and methanolysis) followed by spectral and chromatographic analysis of the chemically modified products.

Structure-Activity Correlation Studies

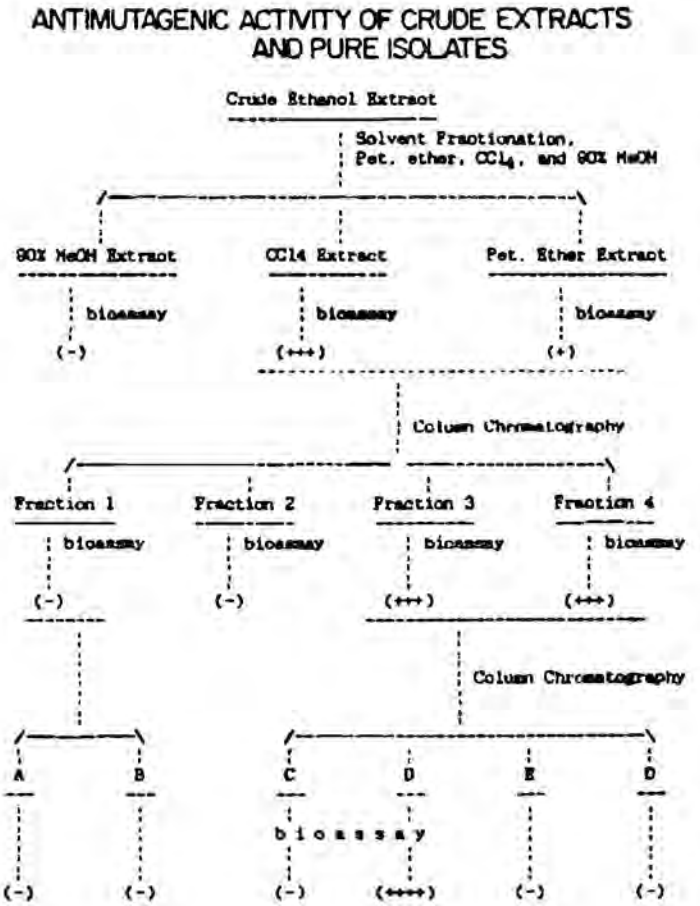
The structure-activity correlation studies were done by testing the antimutagenic activities of the native D* and the chemically modified products. The micronucleus test was used to monitor the antimutagenic activities.

Results and Discussion

Antimutagenic Activity

Scheme 2 summarizes the results of the bioassay tracing the antimutagenic activity during the separation scheme.

Bioassay of the crude extracts showed that the activity was in the carbon tetrachloride and petroleum ether extracts (Figure 1). Further fractionation of these active extracts were done until TLC-pure isolaes, A, B, C, D, E, and F, were obtained. Bioassay data graphically presented in Figure 2 showed that only D was antimutagenic.



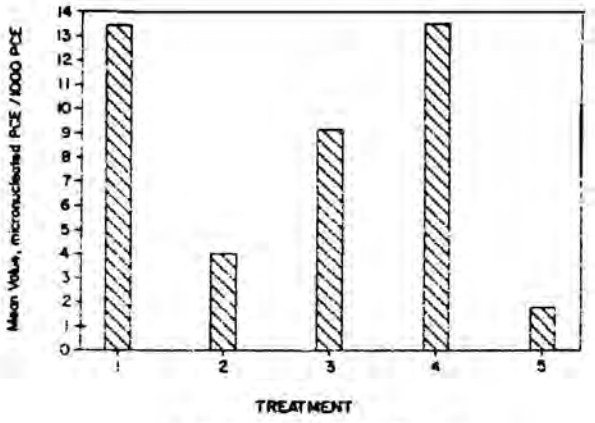


Figure 1. BIOASSAY OF CRUDE FRACTIONS.

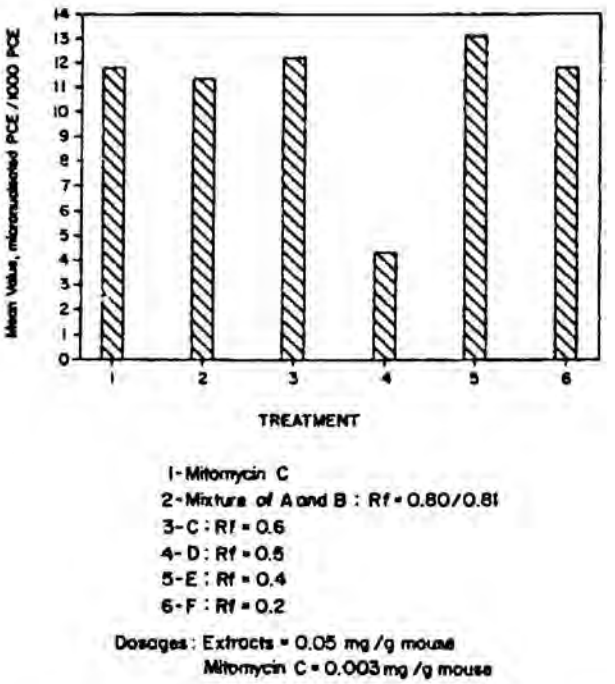


Figure 2. BIOASSAY OF PURE ISOLATES.

TLC-pure D was further purified by preparative HPLC to separate the major component, D'. Data shown in Figure 3 confirmed the antimutagenic activity of D' at a dosage range of 12.5 $\mu g - 50\ \mu g$ D' per gram mouse. At this dosage range, the mutagenicity of Mitomycin C was reduced by 80%. These data indicated that the antimutagenic principle of *Momordica charantia* is D'.

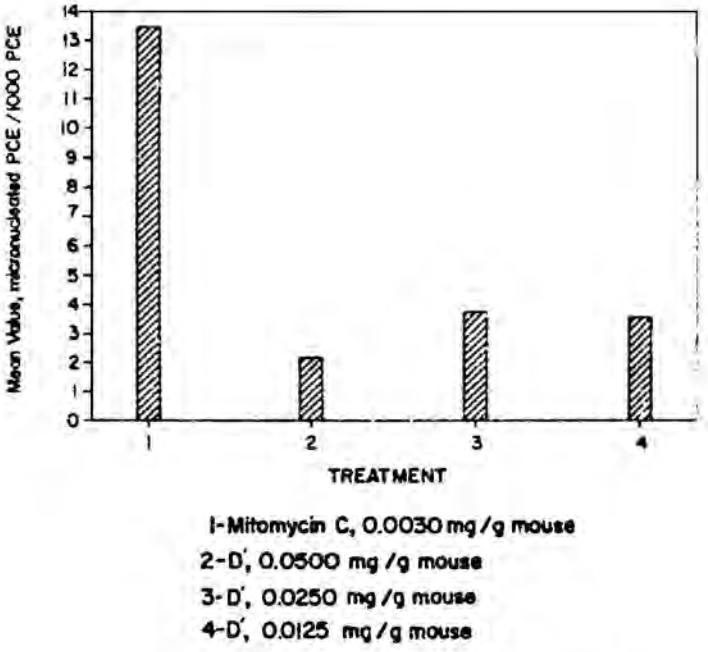


Figure 3. BIOASSAY OF HPLC-PURE D'.

Structure Elucidation

The ^1H NMR spectra of the native and acetylated (Figure 4) D' indicated D' was an acylglucosylsterol. The ^1H NMR characteristic signals attributed to the sterol moiety were consistent with published data for stigmasta-5,25(27)-dien-3β-ol (Sucrow, 1965; Akihisa, 1986; Garg, 1984). The identify of the sterol moiety was further confirmed by the electron impact mass spectrum of the free sterol, D'-S, obtained from the methanolysis reaction of D'. The spectrum showed a $[M^+]$ at m/z 412 corresponding to $C_{29}H_{48}O$. The mass fragmentation pattern (m/z 397, 394, 314, 299, 273, 255) was also consistent with the structure of stigmata-5,25(27-dien-3β-ol (Garg, 1984; Itoh, 1980). Final confirmation of the identity of the sterol moiety was obtained from the ^{13}C NMR spectum of D'. Comparison with available data from literature (Table 1) confirmed the presence of Δ^5 – nucleus and a double bond at the 25(27) positions.

The very intense broad ^1H NMR singlet at 1.26 ppm (ascribed to a long methylene chain) and the triplet at 2.36 ppm (ascribed to methylene attached to a carbonyl group), the ^{13}C NMR signal at 174.6 ppm (ascribed to carbonyl carbon) and the intense signal at 29 ppm (ascribed to many methylene carbons)

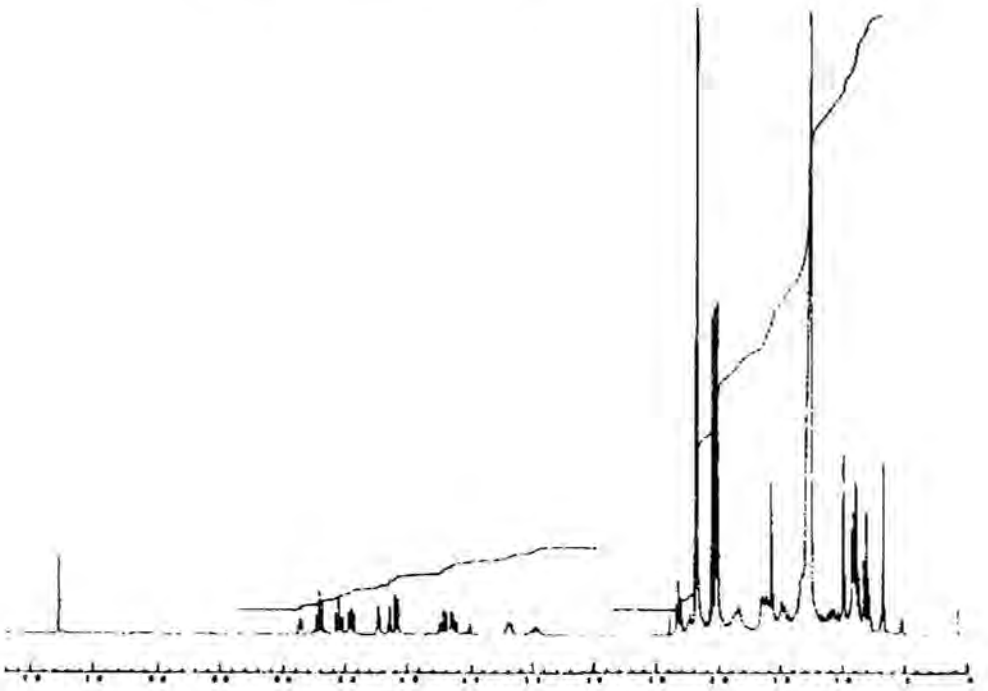


Figure 4. ^{1}H NMR SPECTRUM OF ACETYLATED D' IN $CDCl^3$.

Table 1. ^{13}C NMR Chemical Shifts

		Sitosterol		*Stigmastadiene-3 β-OAc*	
Carbon	*D'*	*Δ^5-3β-OH*	*Δ^5-3β-OAc*	*Y $\Delta^{7,25(27)}$*	*$\Delta^{8,25(27)}$*
C-1	37.3	37.3	37.0	36.8	34.8
2	29.3	31.8	27.8	27.5	27.2
3	74.0	71.8	74.0	73.4	78.8
4	38.9	42.3	38.1	33.8	36.0
5	140.3	140.8	139.1	40.1	47.1
6	122.2	121.7	122.6	29.5	20.7
7	32.0	32.0	31.9	117.3	28.1
8	31.9	32.0	31.9	139.5	133.3
9	50.2	50.2	50.0	49.3	134.8
10	36.7	36.6	36.6	34.2	36.2
11	21.1	21.3	21.0	21.4	21.8
12	39.8	39.8	39.7	39.5	25.4
13	42.8	42.3	42.3	43.3	44.5
14	56.8	58.9	56.7	55.0	49.8
15	24.3	24.4	24.3	23.0	31.0
16	28.2	28.9	28.2	27.9	30.7
17	56.1	56.1	56.0	56.0	50.4
18	11.8	12.2	11.9	12.1	15.7
19	19.4	19.4	19.3	13.0	18.8
20	36.3	40.4	36.3	36.0	36.2

Table 1. ^{13}C NMR Chemicals Shifts (Continued)

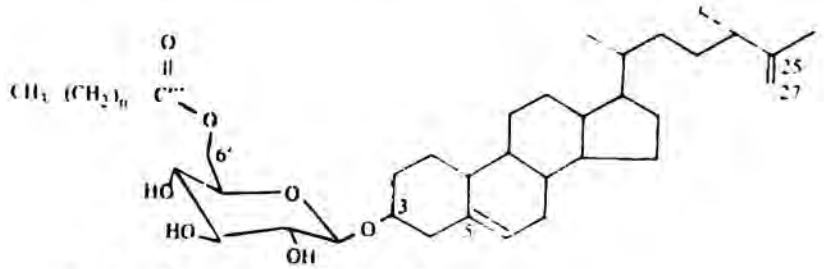
		Sitosterol		*Stigmastadiene-3 βOAc*	
Carbon	*D'*	*Δ^5-3β-OH*	*Δ^5-3β-OAc*	*$\Delta^{7,25(27)}$*	*$\Delta^{8,25(27)}$*
21	18.7	21.1	18.8	18.8	18.6
22	33.7	33.8	33.9	33.6	33.9
23	29.2	29.4	26.4	29.5	29.7
24	49.5	51.3	46.1	49.5	49.5
25	147.5	32.0	29.0	147.4	147.6
26	17.8	21.3	19.0	17.7	17.8
27	111.4	19.0	19.0	111.4	111.4
28	26.5	25.5	23.0	26.5	26.6
29	11.9	12.1	12.3	11.8	12.0
1'	101.3				
2'	73.9				
3'	76.3				
4'	70.5				
5'	76.3				
6'	63.4				
1"	174.6				
2"	32.0				
3"-14"	29.7/29.3				
14"	22.7				
15"	14.1				

were indicative of the presence of a long chain fatty acid. The gas liquid chromatogram-mass spectra of the fatty acid methyl esters obtained from the saponification reaction (followed by methylation) of D' confirmed the presence of palmitate and stearate at a ratio of 2.3: 1.

The ^1H NMR of the acetylated D' attributed to the sugar moiety indicated the presence of a β-D-glucose moiety. Confirmation of the presence of D-glucose was obtained from the gas liquid chromatogram of the trimethylsilylated glucose obtained from the trimethylsilylation of the unknown sugar obtained from the methanolysis reaction of D' .

A comparison of the glucosidic ^1H NMR and ^{13}C NMR signals of native D' and the glucosylsterol obtained from the saponification reaction of D' showed that only the signals due to the H'-6 and C'-6, respectively, were significantly shifted uplifted. These data indicated that the fatty acids were ester linked to the glucosyl moiety at the hydroxyl group at the 6' postion. The ^1H NMR spectrum of the acetylated glucosylsterol indicated that only one molecule of fatty acid is ester linked per molecule of glucosylsterol.

In the light of these data, it is suggested that the antimutagens present in *Momordica charantia*, which is the HPLC-pure D', is an intractable mixture of acylglucosylsterols. The major component of the mixture is 3-O-[6'-O-palmitoyl-β-D-glucosyl]-stigmasta-5,25(27)-diene (D'-1) and the minor component is the stearyl derivative (D'-2).



D'-1 : n = 14 (palmitate)
D'-2 : n = 16 (stearate)

Structure-Activity Correlation

A comparison of the bioassay data for the native D' and the chemically modified products obtained from the acetylation, methanolysis, and saponification reactions are graphically shown in Figure 5.

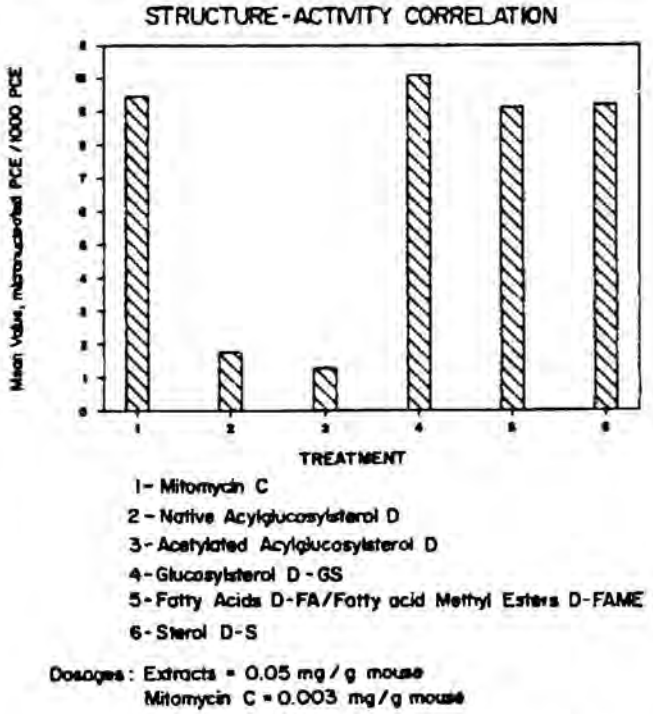


Figure 5. STRUCTURE-ACTIVITY CORRELATION

The acetylated D' also exhibited antimutagenic activity which suggested that the hydroxyl groups of the glucosyl moiety were not necessary for antimutagenic activity. It is, however, possible that the acetylated compound may be deacetylated *in vivo* to give back the active native antimutagen. On the other hand, the hydrophobic nature of the fatty acid and sterol moieties may be necessary for the activity.

The lack of antimutagenic activity of the free fatty acids, the free sterol and the glucosylsterol suggested that the activity may reside in the native arrangement of the three components – the fatty acid, the glucosyl moiety, and the sterol

moiety. It is, thus, possible that the antimutagenic activity may reside in the peculiar lipid-like nature of D'. As a lipid-like compound, ingestion of D' may result in the absorption in the membrane lipid bilayer, which could adversely affect the membrane permeability towards Mitomycin C and disrupt the cellular activity of the latter.

Acknowledgment

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