In the Search for New Anticancer Drugs. XXV: Role of *N*-Nitrosated Amadori Compounds Derived from Glucose–Amino Acid Conjugates in Cancer Promotion or Inhibition

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Received April 21, 1992, from the *Department of Chemistry, University of Wisconsin-Milwaukee, Milwaukee, WI 53201.* Accepted for publication November 2, 1992.

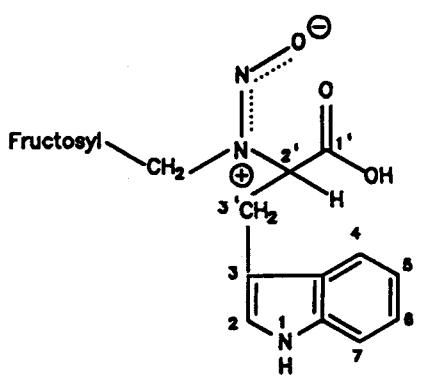
Abstract □ Earlier investigators found that some *N*-nitrosated Amadori compounds, derived from glucose and amino acid condensation reactions, exhibit mutagenic properties and theorized that these potentially carcinogenic compounds might be formed in the human digestive system. To further investigate these compounds, *N*-nitrosated Amadori compounds [i.e., *N*-(1-deoxy-D-fructos-1-yl)-L-*N*-nitroso-glycine (**5a**), -threonine (**5b**), -methionine (**5c**), -valine (**5d**), -phenylalanine (**5e**), and -tryptophan (**5f**)] were synthesized by modifications of known methods. Acute toxicity tests of **5a**, **5b**, **5c**, **5d**, **5e**, and **5f** in male Swiss mice produced the following lowest lethal limits of toxicity: 2000, 2000, 4000, 3000, 2000, and 6000 mg/kg, respectively, whereas the highest tolerated doses were 1750, 1500, 3000, 1500, and 5000 mg/kg, respectively. The 50% lethal dose (intraperitoneally) for **5b** in mice was ~1777 mg/kg. This value is at least three times higher than that for the over-the-counter drug ibuprofen (i.e., 495 mg/kg, intraperitoneally, in mice). Compounds **5b**, **5c**, **5d**, and **5f** were evaluated in vitro by the National Cancer Institute primary antitumor screen consisting of 60 cell lines. None of the four compounds caused a significant inhibition of cell growth, even at the maximum dosage of 10^{-4} M. Compounds **5a–f** were tested in vivo against the lymphocytic leukemia P388, and **5b** and **5f** were tested against the lymphoid leukemia L1210 in CDF_1 male mice following the National Cancer Institute protocol. There were no significant differences in results between the control and drug-treated mice. The percent increase in lifespan ranged from −15 to +15 (T/C = 85–115) for P388 and from −5 to +1 (T/C = 95–101) for L1210, whereas the values for the positive control 5-fluorouracil were 71 and 67 (T/C = 171 and 167), respectively. The combination of very low acute toxicity, low in vitro cytotoxicity in primary tumor screens, and no activity in vivo led to the hypothesis that none of the compounds **5a–f** are carcinogenic or cytotoxic. It is possible that these compounds are not metabolically activated by either α- or β-oxidations or by retro-aldol cleavage reactions.

Conjugates of carbohydrates and proteins (i.e. glycoproteins) are widely distributed in nature and often possess very important biological functions (e.g., they represent components of such entities as blood groups, antigens, and antibodies). The nonenzymatic condensation products of sugars, amino acids, and peptides also are frequently encountered in the biological environment.[1–22] These products have been found[1,2,5–7] in stored and heat-processed foodstuffs containing ingredients of reducing sugars and amino acids (the Maillard Browning reaction). Nonenzymatic browning products have also been detected in the so-called, long-lived human proteins, such as collagen and eye lens crystallins, membranes of arteries, nerves, and connective tissue.[23] Furthermore, it was found[23] that in vitro interactions of DNA with glucose and glucose-6-phosphate resulted in browning products that are similar to those derived from proteins and carbohydrates. Presumably, these products are formed by initial reactions of the amino groups of heterobases of DNA with carbohydrates. On the basis of these results, it was assumed that such processes could also occur in vivo, thereby causing genetic damages and, hence, contributing to the aging process.

Whereas the chemistry of biological systems involving Maillard transformations are extremely complex and result in a wide variety of products,[1–7] the reactions involving amino acids can be delineated[1,7,11,20,21] with well-defined monomeric compounds (Scheme I). The initial step in the Maillard reaction between carbohydrates and amino acids probably results in the formation of Schiff base intermediates that cyclize to give *N*-glucosyl amino acids (3) in the case of D-glucose (1).[1,14,18,20] Analogous reactions with D-fructose result in the formation of *N*-fructosyl amino acids.[1,14,18,20] Compounds 3 can undergo the Amadori rearrangement in acidic media to give *N*-(1-deoxy-D-fructos-1-yl)-L-amino acids (4). The analogous Heyns rearrangement of the fructose derivatives results in the formation of *N*-(1-deoxy-D-glucos-1-yl)-L-amino acids.[1,11,14,15,20] These compounds readily react with nitrites under conditions that exist in the environment (e.g., in cured meats, tobacco, saliva, and stomach) to give *N*-nitroso derivatives 5.[24–30] The nitrites (Scheme I) in the digestive system can be derived from various endogenous and exogenous sources. Hence, it is most probable that human and animal bodies are capable of generating *N*-nitroso compounds of type 5.

It is well documented that the biological action of *N*-nitroso compounds varies considerably with structure, ranging from nonmutagenic and/or noncarcinogenic, as in the case of nitrosoproline,[31,32] to highly mutagenic and/or carcinogenic.[33–37]

A number of very effective *N*-nitroso anticancer drugs,[38,39] such as CCNU (lomustine), MeCCNU (semustine), BCNU (carmustine), MCNU (cymerin), and streptozotocin, exhibit various degrees of toxicity, although the naturally occurring streptozotocin has a very low bone marrow toxicity. These alkylating anticancer drugs interact with the DNA and therefore can inflict genetic damage to both cancerous and healthy cells. A large number of studies[40,41] have been conducted in an



5f

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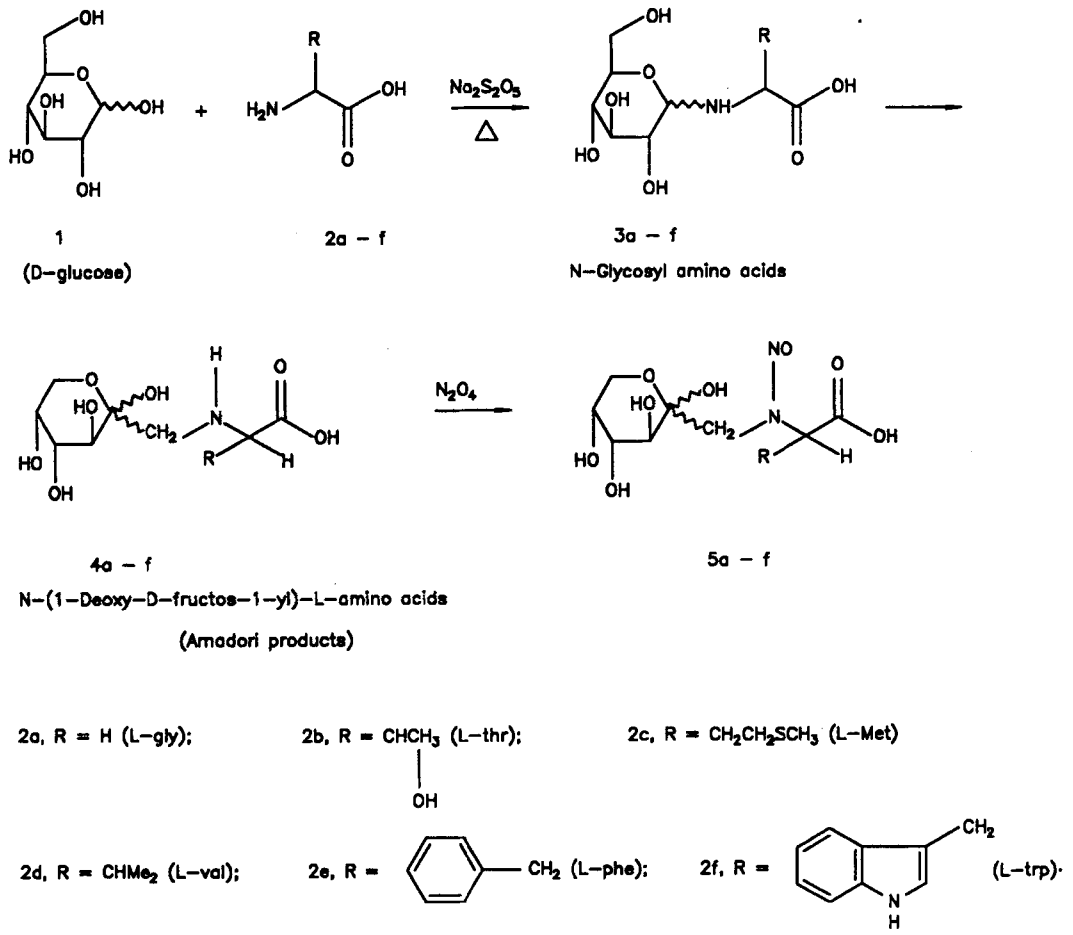


Scheme I—Syntheses of *N*-(1-deoxy-D-fructose-1-yl)-*N*-nitroso-L-amino acids.

effort to assess the possibilities of formation of chemotherapy-induced second tumors. On the basis of these experiences the questions arise[24–30] whether the nitroso derivatives of the ubiquitous Maillard–Amadori–Heyns products are involved in either cancer promotion or inhibition, or both.

Although several excellent studies dealing with the syntheses[22,26–28] and testing of mutagenicity[27–30,35–37] of these compounds have been published, to the best of our knowledge there exists no published report on the in vivo evaluation of these compounds for their toxicity and anticancer activity. The present study is restricted to *N*-nitrosated Amadori compounds of type 5 (Scheme I), representing three nonmutagenic[27] compounds (**5a**, **5c**, and **5e**), one borderline[27] compound (**5d**), and two mutagenic[27] compounds (**5b** and **5f**).

Results and Discussion

Synthesis—*Amadori Products*—The syntheses of fructose amino acid conjugates (Amadori products) and of the corresponding nitrosated derivatives were described in the literature.[11,14,15,18–22] However, in an attempt to prepare larger quantities of those materials for biological testing in vivo, it became necessary to critically evaluate all previously reported syntheses and make appropriate modifications, because the scale-up procedures often gave unexpected results. Thus, the fructose–glycine conjugate (**4a**) was prepared by a previously described procedure;[20] however, the solvent system for the recrystallization (i.e., water–methanol–*n*-butanol) was adapted from a different procedure.[15] The compound fructose–threonine (**4b**) was obtained essentially by following a previously described method.[11,15,21] The conjugate fructose-methionine (**4c**) was synthesized by a modification of the literature method[21] by replacing the 50% aqueous methanol with water, because reaction in 50% aqueous methanol resulted in very low yields of product **4c**. Analogously, the fructose–valine compound (**4d**) was obtained with water instead of the 50% aqueous methanol.[21] However, for the condensation of glucose with phenylalanine to give the fructose-phenylalanine derivative (**4e**), absolute methanol was used as the solvent[14] instead of the 50% aqueous methanol as previously described.[21]

The elution of this product (**4e**) from a Dowex 50X8 column was accomplished with 0.5 N instead of 0.1 N ammonium hydroxide solutions as reported.[21] Ion-exchange chromatography of the reaction mixture always resulted in fractions of uniform product, followed by fractions containing mixtures of product and phenylalanine. These latter fractions were combined and recycled through a Dowex 50X8 column. The product fractions and the final mixture fractions were separately recrystallized from a methanol–*n*-butanol solvent system[14] to give pure product **4e**. This purification procedure was more useful for large scale reactions than the proposed silica gel chromatography with water-saturated *n*-butanol.

Analogously, the fructose–tryptophan product (**4f**) was synthesized with absolute methanol[19] as solvent rather than the aqueous methanol system.[21] Similar to **4e**, the product **4f** was eluted from a Dowex 50X8 column with 0.5 N instead of 0.1 N ammonium hydroxide solution as previously reported.[21] The crude product from the Dowex column was further purified by crystallization from a methanol–*n*-butanol system, rather than by chromatography on silica gel with

water-saturated *n*-butanol as the solvent system.[21] Small amounts of this product (**4f**) were purified by flash chromatography on silica gel with water-saturated *n*-butanol as eluant. In general, the yields of the Amadori products were either about equal or better than those reported in the literature.[14,15,18–21]

N-Nitroso Derivatives of the Amadori Products—The nitrosated products were obtained by a published method[22] with some modifications. Thus, the order of addition was reversed (i.e., 1 N hydrochloric acid was added to an aqueous solution of the Amadori products and sodium nitrite rather than addition of the sodium nitrite solution to the fructose–amino acid in 1 N hydrochloric acid solution).[22] During attempts to desalt the nitrosated compounds according to the literature method,[22] it was found that the nitrosated products were strongly adsorbed on the Dowex 2X8 (HO^-, 50–100 mesh) resin at pH 3. With narrow-range pH papers (i.e., 1.0–2.5 and 3.0–5.5), the UV absorbance of the eluate was shown to decrease greatly between pH 2 and 3. The same result was also obtained with Amberlite IRA 400 (HO^-). This adsorption on the columns was proven by eluting the products from the resins with 1 N acetic acid. Because the pK_a values of the nitrosated products were reported[22] to be in the range 3.10–3.30, it would be expected that the carboxylate anion should exchange with the hydroxide ion of the resin at pH $\geq$ 3.

It is possible to circumvent this problem by direct precipitation of the products from ethanolic solutions of the concentrated eluate obtained from the Dowex 50X8 columns, as in the case of fructose–*N*-nitroso-glycine (**4a**) and -threonine (**4b**), by using the weak basic anion-exchange resin Rexyn 203 (HO^-, 16–50 mesh), or by adding small amounts of Dowex 2X8 (HO^-) resin to the acidic solutions of the product to remove the anions, as in the case of fructose–*N*-nitrosophenylalanine (**5e**). Desalting of the reaction mixture of fructose–*N*-nitrosotryptophan (**5f**) was achieved by passage through an Amberlite XAD-2 column before, rather than after, the column chromatography on silica gel,[22] to remove any possible impurities from the desalting process. Flash chromatography also proved convenient for the purification of this compound (**5f**).

The yields of the nitrosated target compounds were similar in most cases to those reported in the literature,[22] except for fructose–*N*-nitrosomethionine (53 versus 97%[22]) and for fructose–*N*-nitrosotryptophan (53 versus 31%[22]). There were several unexplainable discrepancies with the literature concerning the optical rotation values of the nitrosated products. Thus, for fructose–*N*-nitroso-threonine (**5b**), -methionine (**5c**), -phenylalanine (**5e**), and -tryptophan (**5f**) the values were $[\alpha] = -153.8°$, $-119.5°$, $-178.6°$, and $-166.7°$, respectively, whereas the reported values in the literature[22] were $[\alpha] = -117.4°$, $-72.3°$, $-132.3°$ and $-79.6°$, respectively. The measurements of these compounds were repeated after 6 months of storage in a deep freeze, with a different polarimeter (Jasco), and no significant change in values was observed; for **5b**, **c**, **e**, and **f** values of $-150.1°$, $-119.9°$, $-202.5°$, and $-171.3°$, respectively, were obtained. The uniformity of all nitrosated compounds **5a–f** was ascertained by thin-layer chromatography (TLC) analyses.

Difficulties were experienced in completely removing the water of crystallization from the otherwise pure **4b**, and, in particular, from **5f** whose optical rotation was also quite different from the reported value.[22] Because no melting (decomposition) points for any of the nitrosated derivatives were available for comparison from previous work,[22] the ^1H NMR spectrum of **5f** was compared with that from the literature.[22] In general, the spectra were in agreement. The chemical shifts and shapes of the peaks were about the same. Although the complex multiplet peak assembly arising from the carbohydrate and amino acid side-chain moieties could not be resolved and individually assigned, the general pattern was in agreement with the more detailed analysis described in a previous study,[22] and the integration of protons was indicative of the correct molecular identity of **5f**.

Biological Evaluation—Acute toxicities for **5a–f** were obtained with Swiss male mice. All compounds exhibited very low toxicity in the range 2000–6000 mg/kg. Specifically, the lowest lethal toxic doses were as follows: 2000 mg/kg for **5a**, **5b**, and **5e**, 3000 mg/kg for **5d**, 4000 mg/kg for **5c**, and 6000 mg/kg for **5f**. Interestingly, the compound with the highest in vitro mutagenicity (**5f**)[22] possessed the lowest acute toxicity. The 50% lethal dose (LD_{50}) for one of the reported[22] mutagenic compounds (**5b**) was determined by the Weil method.[42] Thus, four logarithmically spaced single doses were injected intraperitoneally (ip) into four groups of four Swiss male mice. The high value of 1777 mg/kg for **5b** confirmed a comparatively low toxicity for this compound. In comparison, the LD_{50} for the over-the-counter drug ibuprofen, administered ip into mice, was only 495 mg/kg[43] (i.e., ibuprofen is at least three times as toxic as **5b**).

Compounds **5b–d** and **5f** were evaluated in vitro by the National Cancer Institute (NCI) disease-oriented primary antitumor screen.[44] The cell panel consists of 60 lines representing leukemias, non-small-cell lung cancers, small-cell lung cancers, colon cancer, central nervous system cancer, melanoma, ovarian cancer, and renal cancer. The compounds were tested at log 10 of sample concentrations ranging in values from -8 to -4 M. In none of the cell lines did **5b–d** and **5f** exhibit any significant cell growth inhibition, even at a maximum dose of 10^{-4} M. These results support the contention that the *N*-nitrosated Amadori products are neither acutely toxic nor cytotoxic.

The anticancer activity was evaluated in vivo against the murine lymphocytic leukemia P388 and lymphoid leukemia L1210 in CDF_1 male mice in accordance with the NCI Institute Protocol[45] (Tables I and II). All compounds were inactive against both tumor lines. The fact that the T/C values (the ratio of the mean survival times of treated mice to tumor-bearing control mice) for **5a–f** fluctuated only slightly at all doses against P388 and L1210 may also indicate that these compounds lack toxicity. This lack of anticancer activity combined with low toxicity is somewhat surprising, considering that conjugates **5a–f** are *N*-nitroso compounds, which, as a class, often exhibit highly toxic properties,[33,34,40,41] albeit with some exceptions.[31,32] Hence, an explanation is deemed necessary to explain the properties of these important compounds (**5a–f**) that are believed to be involved in various food consumptions by humans.

The mutagenicity of a number of nitrosated D-fructose–L-amino acid conjugates (i.e., of nitrosated Amadori compounds) has been investigated[25–28,30] in vitro by the Ames test with transformations of *Salmonella typhimurium* strains, and the compounds were classified[27] into the following groups: (1) nonmutagenic (with or without metabolic activation) *N*-NO-D-fruc–gly, –L-ala, –L-phe, –L-leu, –L-ser, –L-met, –L-asp, and –L-tyr; (2) borderline (mutagenic with only some strains) –L-val; and (3) mutagenic –L-thr, –L-his, and –L-tryp. The nitrosated D-fructose–L-tryptophan (**5f**) was strongly mutagenic in all test strains,[27,28] with or without metabolic activation. It was hypothesized[27] that the mutagenic activity of *N*-NO-D-fruc–L-val, –L-thr, and –L-his analogues was caused by alkylation and base-pair substitution of the DNA, whereas the activity of the –L-tryp analogue was also the result of an intercalation of the indole ring between the base pairs of the DNA double helix.

The importance of the nitrosamine moiety for biological activity of these compounds was supported by several lines of evidence.[25–30,46] The unnitrosated D-fructose–L-amino acid conjugates and the L-amino acid precursors were shown[25–28,30]

Table I—Evaluation of *N*-(1-Deoxy-D-fructos-1-yl)-*N*-nitroso-L-amino Acids against P388 Lymphocytic Leukemia in CDF_1 Male Mice

Compound	Dose, mg/kg/day[a]	5-Day Weight Change, %[b]	10-Day Weight Change, %[b]	T/C, %[c]	Survivors on Day 30 [survivor/total]
5a	100	+5.2	+9.2	99	0/6
	200	+4.6	+23.2	89	0/6
5b	100	+8.0	+19.6	111	0/6
	200	+9.1	+20.5	112	0/6
5c	200	+9.3	+23.1	105	
	400	+4.7		85	0/6
5d	150	+6.0	+20.6	109	0/6
	300	+7.4	+29.9	97	0/6
5e	100	+3.1	+11.2	115	0/6
	200	+7.3	+21.5	111	0/6
5f	300	+7.1	+15.9	108	0/6
	600	+5.3		108	0/6
5-Fluorouracil	200[d]	−13.0	+2.6	171	0/6

[a] All compounds were administered in a 0.85% saline solution (Sigma Chemical Company). [b] The average percentage weight change on days 5 and 10 was taken as a measure of drug toxicity. [c] T/C, Ratio of mean survival times of treated mice to control mice. [d] 5-Fluorouracil in a single dose of 200 mg/kg in 0.85% saline solution (Sigma Chemical Company) was administered on day 1 in accordance with the protocol of the NCI.

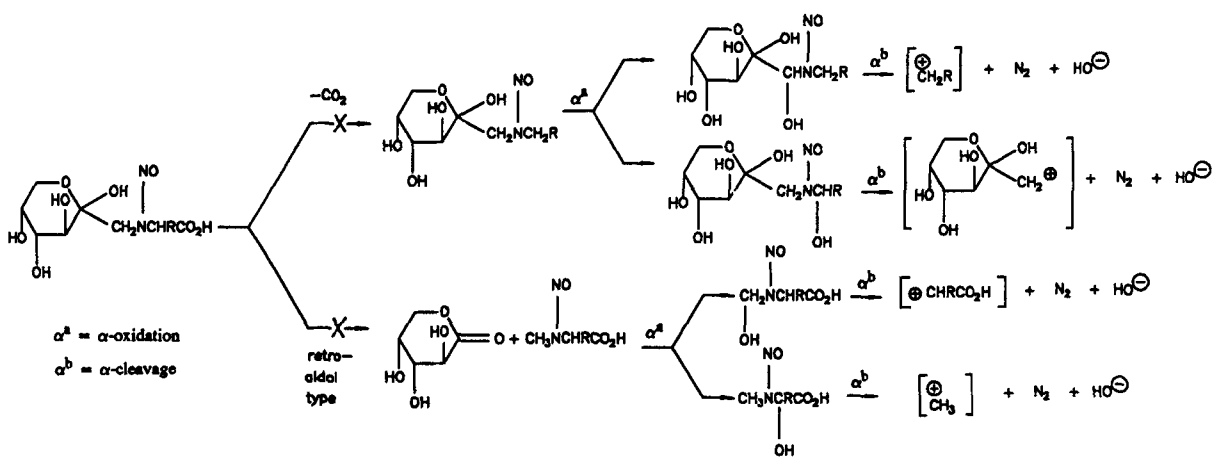
Table II—Evaluation of *N*-(1-Deoxy-D-fructos-1-yl)-*N*-nitroso-L-amino Acids against L1210 Lymphoid Leukemia in CDF_1 Male Mice

Compound	Dose, mg/kg/day[a]	5-Day Weight Change, %[b]	10-Day Weight Change, %[b]	T/C, %[c]	Survivors on Day 30 [survivor/total]
5b	50	+6.9		101	0/6
	100	+8.4		100	0/6
	200	+5.9		101	0/6
5f	150	+10.5		95	0/6
	300	+6.0		100	0/6
	600	+7.7		98	0/6
5-Fluorouracil	200[d]	−5.6	+16.4	167	0/6

[a] All compounds were administered in a 0.85% saline solution (Sigma Chemical Company). [b] The average percentage weight change on days 5 and 10 was taken as a measure of drug toxicity; the mice treated with **5b** and **5f** died before day 10. [c] T/C, Ratio of mean survival times of treated mice to control mice. [d] 5-Fluorouracil in a single dose of 200 mg/kg in 0.85% saline solution (Sigma Chemical Company) was administered on day 1 in accordance with the Protocol of the NCI.

to be nonmutagenic, whereas nitrosated tryptophan and its derivatives were mutagenic against *S. typhimurium* strains.[46] A mutagenicity study of glycosylamines (i.e., the precursors of Amadori compounds) revealed that these compounds were nonmutagenic, but were converted to mutagens by nitrosation.[30] Further evidence was presented[29] that the nitrosated D-fruc–L-tryp **5f** and the 5-OH–tryp derivative increased the DNA synthesis in HeLa 53 cells in vitro by stimulation of the DNA repair mechanisms, whereas the unnitrosated compounds had practically no effect on the DNA replication. In addition to their mutagenic potential, many nitrosamines act as carcinogens in a wide variety of animals.[33,34]

The dogma of nitrosamine carcinogenicity[47–62] requires metabolic activation by oxidative hydroxylation of the α-carbon, followed by a α-cleavage reaction forming an aldehyde or ketone and a diazohydroxide. The latter intermediate decomposes to nitrogen and an alkylating species, the so-called ultimate carcinogen, which reacts with the DNA or RNA by alkylation of the heterobases leading to a predominance of 7-alkylguanine. The nitrosated Amadori compounds **5a–f** are *bis*-β-oxidized nitrosamines and, hence, represent a special



Scheme II

class of *N*-nitroso compounds. The β-oxidation of nitrosamine side-chains by various mechanisms has been proposed[63–75] to explain the increased biological and chemical activity of these compounds. In the case of **5a–f**, the elimination of the β-carboxyl group (Scheme II) could result in a more active compound as in, for example, the thermal[76–78] and bacterial[79] transformations of the noncarcinogenic *N*-nitrosoproline to the highly carcinogenic *N*-nitrosopyrrolidine. Furthermore, the β-hydroxy group attached to the pyranose ring could undergo a retro-aldol fragmentation reaction to give a lactone and a side-chain moiety that could be further metabolized to the ultimate carcinogen (Scheme II). There is support for such cleavage reactions involving nitrosamines (e.g., the nitrosamine group behaves like a carbonyl group in that the α hydrogens are acidic and can be readily substituted[63]). In addition, it has been demonstrated that β-hydroxynitrosamines undergo base-[68–71] and enzyme-catalyzed[72,73] retro-aldol fragmentation reactions that can convert weakly carcinogenic compounds into highly carcinogenic compounds (Scheme II).

Considering these reactions and the fact that the nitrosated Amadori conjugates **5a–f** are *bis*-β-oxidized compounds, it was somewhat surprising to find these compounds to be inactive in vivo against the P388 and L1210 cancer lines and to possess a very low overall toxicity. It is hypothesized that **5a–f** do not undergo the oxidation and fragmentation reactions shown in Scheme II and behave more like the noncarcinogenic *N*-nitrosoproline that suffers no decarboxylation in vivo to the carcinogenic *N*-nitrosopyrrolidine[31,32] and is excreted almost quantitatively by rats. Hence, the nitrosated Amadori compounds (**5a–f**) could be considered as detoxified, water-soluble products that are either excreted without degradation[80] or denitrosated by cytochrome P-450 in the liver.[81] Denitrosation reactions of a *N*-nitrosopeptide have been reported.[82] The lack of correlation between the in vitro mutagenicity tests and the in vivo results proves again that caution must be exercised when mutagenicity results are extended to in vivo animal models.[83]

Experimental Section

Mice—Male Swiss mice (for acute toxicity and LD_{50}; average weight 18–21 g) and DBA/2 mice (for tumor propagation; 6–7 weeks old) were supplied by Harlan Sprague-Dawley, Inc. (Indianapolis, IN). Male CDF_1 mice (for testing; average weight 18–21 g) were purchased from Charles River Laboratories, Wilmington, MA. The tumor-bearing mice or vials with tumor cells were obtained from the Frederick Cancer Research Facility, Frederick, MD. Mice were fed rodent Laboratory Chow 5001 (Purina Mills, Inc., St. Louis, MO) and water ad libitum.

Drugs—Compounds were administered in a 0.85% saline solution (Sigma Chemical Company).

Biological Evaluations—The acute toxicity of the compounds in Swiss mice was determined at doses of 50, 100, 150, 200, 500, 1000, 1500, 2000, 3000, 5000, and 10 000 mg/kg for **5a** and **5b**, 3000, 4000, and 5000 mg/kg for **5c**, 2000, 3000, and 5000 mg/kg for **5d** and **5e**, and 3000, 5000, and 6000 mg/kg for **5f**. The lowest doses exhibiting lethal toxicity for **5a**, **5b**, **5c**, **5d**, **5e**, and **5f** were 2000, 2000, 4000, 3000, 2000, and 6000 mg/kg, respectively, whereas the highest tolerated doses were 1750, 1500, 3000, 2000, 1500, and 5000 mg/kg, respectively.

The LD_{50} and its 95% confidence interval (1777, 1515–2086 mg/kg) was determined for **5b** by Weil's method.[42] Thus, for the calculation of the LD_{50}, four logarithmically spaced single doses (i.e., 1500, 1778, 2109, and 2500 mg/kg) were injected ip into four groups of four Swiss male mice. The observation period for the determination of the LD_{50} was 30 days. Compounds were evaluated in vivo against the lymphocytic leukemia P388 and the lymphoid leukemia L1210 in mice following the NCI protocol.[45] The CDF_1 male mice (18–21 g weight), in groups of six, were inoculated ip either with 10^6 cells of P388 or with 10^5 cells of L1210 tumor at day zero of the experiment. Compounds **5a–5f** were injected ip at doses listed in Table I for 9 successive days from day one. Then, the animals were observed according to the protocol, keeping a record of deaths and survivors.[45] The anticancer activity was evaluated by comparing the mean survival time of treated with that of the control animals (e.g., by the T/C method where T represents the mean survival time of the treated group and C the mean survival time of the tumor-bearing control group). The percent increase in life span (%ILS) parameter was calculated by the formula $[(T - C)/C] \times 100$.

Materials—All chemicals were of the best quality available commercially. The L-amino acids and α-D-glucose were purchased from United States Biochemical Corp., Cleveland, OH. The Amberlite XAD-2 and Dowex 2X8 (50–100 mesh) resins were obtained from Rohm and Haas Company, Philadelphia, PA, and Dow Chemical Company, Midland, MI, respectively. The Dowex 50X8 (H^+, 100–200 mesh) resin was obtained from Aldrich Chemical Company, Milwaukee, WI. The Rexyn 203 (HO^-) resin and sodium metabisulfite ($Na_2S_2O_5$) were purchased from Fisher Scientific Company, Fairlawn, NJ. Silica gel 60 (230–400 mesh) for flash chromatography was purchased from EM Science, Gibbstown, NJ.

Analytical Procedures—Melting points were determined on a Thomas-Hoover melting point apparatus (model 6406-K) with a calibrated thermometer. Optical rotations were determined first on a model SR-6 Polarimeter, PolyScience Group, Niles, IL, and later on a Jasco model DIP-370 Digital Polarimeter, Japan Spectroscopic Company, Ltd., Tokyo, Japan. The ^1H NMR spectra were recorded on either a Bruker 250 (250 MHz) or GE QE-500 (500 MHz) NMR spectrometers. The ^1H NMR spectrum of **5f** was recorded at 500 MHz in D_2O with sodium 3-(trimethylsilyl)-1-propanesulfonate (δ 0.015 ppm) as an internal standard. Microanalyses were performed on a Perkin-Elmer elemental analyzer (model 240C). All reactions, chromatographic procedures, and purity of products were monitored by TLC. TLC analyses were performed on silica gel 60 F_{254} precoated sheets (EM Science) with a layer thickness of 0.2 mm. The elution solvents for TLC were as follows: (A) chloroform:methanol:ammonium hydroxide (3:3:1, v/v/v) mixture for fructose–threonine, fructose–valine, fructose–methionine, and all *N*-nitrosated products; (B) chloroform:methanol:ammonium hydroxide (3:3:0.5, v/v/v) for fructose–phenylalanine and fructose–tryptophan; and (C) 1-butanol:acetic acid:water (2:2:1, v/v/v) for fructose–glycine. Visualization of thin-layer chromatograms was accomplished for the fructose–amino acids by spraying with a 1% ethanolic ninhydrin solution, or the Lemieux von-Rudloff reagent ($NaIO_4$-$KMnO_4$),[84] or a 50% methanolic H_2SO_4 solution, and for the N-nitrosated products either by UV light (254 nm) or spraying with Griess reagent[85,86] (sulfanilic acid-1-naphthylamine in acetic acid) followed by UV light (254 nm) irradiation. Flash chromatography[87] was carried out with silica gel 60 (230–400 mesh, EM Science). The separation of the fructose–amino acids was accomplished by ion-exchange chromatography on columns of Dowex 50X8 (H^+, 100–200 mesh), with at least threefold excess of the resin. The unreacted glucose and colored reaction byproducts were eluted with 10 column volumes of water:ethanol (1:1, v/v) and three column volumes of water. The products were eluted either with 0.1–0.5 N ammonium hydroxide or with water as specified. The ion-exchange column chromatography was monitored by TLC as well as by testing the aliquots with either boiling Benedict reagent[88] or warm 0.5% methanolic *o*-dinitrobenzene reagent.[18] Recrystallizations from dual solvent systems were carried out by first dissolving the products in hot solvent and then adding the second solvent until faint turbidity, and cooling. Water was removed from fructose–amino acid solutions on a rotating evaporator at 35–40 °C and 20 Torr and from fructose–*N*-nitrosoamino acids at 20–25 °C and 20 Torr and then at 0.1 Torr.

Preparation of *N*-(1-Deoxy-D-fructos-1-yl)-L-Amino Acids—*N-(1-Deoxy-D-fructos-1-yl)-L-glycine (4a)*—D-Glucose (86.48 g, 480 mmol) was dissolved in water (18 mL) on a steam bath. To this solution were added L-glycine (9.01 g, 120 mmol) and sodium metabisulfite (11.41 g, 60 mmol). The reaction mixture was heated on a steam bath for 1 h with occasional shaking. The resulting orange solution was cooled to 25 °C, diluted with water:ethanol mixture (1:1, v/v, 200 mL) and applied to a column of Dowex 50X8 (H^+, 350 meq) column. Elution of the column with 10 column volumes of water:ethanol (1:1, v/v), three column volumes of water, and then with 0.1 N ammonium hydroxide solution yielded **4a**, followed by unreacted glycine. The appropriate fractions were combined and the solvent was removed under reduced pressure leaving a thick syrup. Remaining water was removed from the syrup by flash distillation

with absolute ethanol (5 × 5 mL) to give **4a** as a white solid. Recrystallization of the solid from water:methanol:1-butanol (3:3:1, v/v/v)[15] afforded 9.77 g (34%) of **4a** as white prisms; mp 153–155 °C (dec.); $[\alpha]_D^{20}$ −68 ° (*c* 1.0, H_2O) [lit. mp 145–146 °C (dec.)[20] or 157 °C (dec.);[15] $[\alpha]_D^{20}$ −69 °, *c* 1.2, H_2O)[20] or $[\alpha]_D^{20}$ −65.8 *c* 2.0, H_2O)[15]].

Anal.—Calcd for $C_8H_9NO_7$: C, 40.51; H, 6.37; N, 5.90. Found: C, 40.55; H, 6.50; N, 5.85.

N-(1-Deoxy-D-fructos-1-yl)-L-threonine (4b)—D-Glucose (72 g, 400 mmol) was dissolved in water (60 mL) on a steam bath. To this solution were added L-threonine (12 g, 100.7 mmol) and sodium metabisulfite (9.58 g, 50.4 mmol). The reaction mixture was heated on a steam bath for 2 h with occasional shaking. The product was separated as described for **4a**, except that the product was eluted with water (~18 column volumes). Removal of the solvent from appropriately combined fractions resulted in a white solid. Recrystallization of this solid from methanol-1-butanol (1:1, v/v) afforded 7.30 g (26%) of **4b**; mp 108–114 °C (dec.); $[\alpha]_D^{20}$ −48.8 ° (*c* 2.0, H_2O) [lit. mp 108 °C (dec)[21]; $[\alpha]_D^{20}$ −49.8 ° (*c* 2.0, H_2O)[15] or $[\alpha]_D^{20}$ −47 ° (*c* 0.4, H_2O)[21]].

Anal.—Calcd for $C_{10}H_{19}NO_8 \cdot 1/2H_2O$: C, 41.38; H, 6.95; N, 4.83. Found: C, 41.52; H, 6.82; N, 4.73.

N-(1-Deoxy-D-fructos-1-yl)-L-methionine (4c)—D-Glucose (64.84 g, 360 mmol) was dissolved in water (30 mL) on a steam bath. To this solution were added L-methionine (13.43 g, 90 mmol) and sodium metabisulfite (8.56 g, 45 mmol). The reaction mixture was heated on a steam bath for 2 h with occasional shaking. The product was separated as described for **4a**, except that the product was eluted with water (~30 column volumes). The removal of solvent from appropriately combined fractions resulted in 12.44 g (44%) of **4c** as an amorphous white solid; mp 98–100 °C (dec.); $[\alpha]_D^{20}$ −38.1 ° (*c* 1.1, H_2O) [lit. 95 °C (dec)[21]; $[\alpha]_D^{20}$ −34 ° (*c*, 0.35, H_2O)[21]].

Anal.—Calcd for $C_{11}H_{21}NO_7S$: C, 42.44; H, 6.80; N, 4.50. Found: C, 42.39; H, 6.86; N, 4.29.

N-(1-Deoxy-D-fructos-1-yl)-L-valine (4d)—D-Glucose (57.65 g, 320 mmol) was dissolved in water (50 mL) on a steam bath. To this solution were added L-valine (9.37 g, 80 mmol) and sodium metabisulfite (7.60 g, 40 mmol). The reaction mixture was heated on a steam bath for 4 h with occasional shaking. The product was separated as described for **4a**, except that the product was eluted with water (~50 column volumes). Removal of the solvent from appropriately combined fractions resulted in crude **4d** as an amorphous white solid. Recrystallization of this solid from water:ethanol:1-butanol (1:1:1, v/v/v) afforded 7.1 g (31%) of **4d**; mp 155–156 °C (dec.); $[\alpha]_D^{20}$ −45 ° (*c* 2.0, H_2O) [lit. 156 °C (dec.)[15]; $[\alpha]_D^{20}$ −42.9 ° (*c* 2.0, H_2O)[15] 155–160 °C (dec)[14]; $[\alpha]_D^{20}$ −47 ° (*c* 2.0, H_2O)[14]].

Anal.—Calcd for $C_{11}H_{21}NO_7$: C, 47.30; H, 7.58; N, 5.02. Found: C, 47.31; H, 7.61; N, 4.88.

N-(1-Deoxy-D-fructos-1-yl)-L-phenylalanine (4e)—A mixture of D-glucose (47.25 g, 240 mmol), L-phenylalanine (10 g, 60 mmol), and sodium metabisulfite (5.70 g, 30 mmol) in methanol (500 mL) was heated on a steam bath under reflux for 4 h with occasional shaking. The color of the reaction mixture changed to yellow, then to dark orange as the reaction progressed. The reaction mixture was cooled to 25 °C and filtered, and the solvent was removed. The residue was dissolved in water:ethanol (1:1, 250 mL), and the solution was added to a Dowex 50X8 ion-exchange column (377 meq). After elution of the column with 10 column volumes of water:ethanol (1:1) and three column volumes of water, the product was eluted with aqueous 0.5 N ammonium hydroxide (~5 column volumes, 50 mL fractions). Earlier fractions containing only pure product were combined. Removal of the solvent gave 2.45 g of **4e**. Later fractions that were contaminated with unreacted phenylalanine were combined and recycled through a Dowex 50X8 column. Again, the workup gave 1.08 g of **4e**, whereas the later fractions were contaminated with phenylalanine. This final crude product contaminated with phenylalanine, as well as pure product obtained from the earlier fractions, were further purified by recrystallization from methanol:1-butanol (2:1, v/v). The combined crops amounted to 8.67 g (44%) of **4e**; mp 136–141 °C (dec.); $[\alpha]_D^{20}$ −40.9 ° (*c* 1.71, H_2O) [lit. 170 °C (dec.)[14] or 143 °C (dec)[21]; $[\alpha]_D^{20}$ −34 ° (*c* 2.2, H_2O)[21] or −33 ° (*c* 2.0, H_2O)[14]].

Anal.—Calcd for $C_{15}H_{21}NO_7$: C, 55.04; H, 6.47; N, 4.28. Found: C, 54.84; H, 6.61; N, 4.06.

N-(1-Deoxy-D-fructos-1-yl)-L-tryptophan (4f)—D-Glucose (43.24 g, 240 mmol) was dissolved in water (60 mL) on a steam bath. To this solution were added L-tryptophan (12.25 g, 60 mmol) and sodium metabisulfite (5.70 g, 30 mmol). The reaction mixture was heated on a steam bath for 4 h with occasional shaking. The color of the reaction mixture changed to yellow, then to orange as the reaction progressed. The resulting orange solution was cooled to ~25 °C, diluted with a water:ethanol mixture (1:1, v/v, 200 mL), and applied to a column of Dowex 50X8 (H^+, 400 meq) column. After eluting the column with 15 column volumes of water:ethanol (1:1, v/v) and five column volumes of water, the product (**4f**) was eluted along with unreacted tryptophan with 0.5 N ammonium hydroxide solution. Appropriate fractions were combined and solvent was removed leaving an orange solid (15.0 g). Repeated recrystallization from methanol:1-butanol (1:1, v/v) gave 7.0 g (32%) of **4f** as a white solid; mp 135–136 °C (dec.); $[\alpha]_D^{20}$ −37.4 ° (*c* 1.1, H_2O) [lit. 143 ° (dec.)[21]; $[\alpha]_D^{20}$ −7.2 ° (*c* 2.6, 2M HCl for anhydrous)[19]; $[\alpha]_D^{20}$ −40 ° (*c* 0.3, H_2O for dihydrate)[21]].

Anal.—Calcd for $C_{17}H_{22}N_2O_7 \cdot H_2O$: C, 53.12; H, 6.29; N, 7.29. Found: C, 52.92; H, 6.78; N, 6.84.

N-(1-Deoxy-D-fructos-1-yl)-L-N-nitrosoglycine (5a)—Aqueous 1 N hydrochloric acid was added in a dropwise manner at 5 °C with magnetic stirring to a solution of **4a** (1.238 g, 5.21 mmol) and sodium nitrite (0.540 g, 7.82 mmol) in water (4 mL) until pH 3 was attained. After stirring for 30 min at 5 °C, the reaction mixture was diluted with water (25 mL), and the solution was passed through a Dowex 50X8 column (25 meq) to remove Na^+. The resulting salt-free solution was concentrated at 25 °C and 20 Torr to give a thick syrup. The remaining water was removed by flash distillation with absolute ethanol (5 × 5 mL). Finally, the ethanolic solution was concentrated to turbidity and chilled in a freezer overnight. The white powder was collected by filtration, washed with cold ethanol (5 mL), and dried under reduced pressure at 25 °C and 0.5 Torr over P_2O_5 to give 1.12 g of **5a**. More product was collected from the mother liquor by concentration and chilling. The combined crops amounted to 1.35 g (97%) of **5a**; mp ´ `.5–130 °C (dec.); $[\alpha]_D^{20}$ −76.0 ° (*c* 0.92, H_2O) [lit. $[\alpha]_D^{20}$ −78.1 °, (*c* 0.-ó, H_2O)[22]].

Anal.—Calcd for $C_8H_{14}N_2O_8$: C, 36.10; H, 5.30; N, 10.52. Found: C, 35.87; H, 5.20; N, 10.21.

N-(1-Deoxy-D-fructos-1-yl)-L-N-nitrosothreonine (5b)—Aqueous 1 N hydrochloric acid was added in a dropwise manner at 5 °C with magnetic stirring to a solution of **4b** (0.697 g, 2.40 mmol) and sodium nitrite (0.249 g, 3.60 mmol) in water (5 mL) until pH 3 was attained. After stirring for 30 min at 5 °C, the product was isolated and purified as described for **5a**. Thus, 0.70 g (95%) of **5b** was obtained as a white crystalline powder; mp 144.5–146 °C (dec.); $[\alpha]_D^{20}$ −153.8 ° (*c* 0.11, H_2O) [lit. $[\alpha]_D^{20}$ −117.4 °, (*c* 0.48, H_2O)[22]].

Anal.—Calcd for $C_{10}H_{18}N_2O_9$: C, 38.71; H, 5.85; N, 9.03. Found: C, 38.99; H, 5.94; N, 8.83.

N-(1-Deoxy-D-fructos-1-yl)-L-N-nitrosomethionine (5c)—Aqueous 1 N hydrochloric acid was added in a dropwise manner at 5 °C with magnetic stirring to a solution of **4c** (0.571 g, 1.83 mmol) and sodium nitrite (0.190 g, 2.75 mmol) in water (3 mL) until pH 3 was attained. After stirring for 30 min at 5 °C, the reaction mixture was worked up to remove Na^+ as described for **5a**. The resulting acidic solution was passed through a short column of Rexyn 203 (HO^-, 16–50 mesh, 1 mL), and the solvent was removed from the eluate. Drying of the residue at 25 °C and 0.5 Torr over P_2O_5 gave 0.33 g (53%) of **5c** as a hygroscopic light yellow, glassy solid; mp softens at 45 °C, dec. 59–70 °; $[\alpha]_D^{20}$ −119.5 ° (*c* 4.5, H_2O) [lit. $[\alpha]_D^{20}$ −72.3 °, (*c* 0.23, H_2O)[22]].

Anal.—Calcd for $C_{11}H_{20}N_2O_8S$: C, 38.82; H, 5.92; N, 8.23. Found: C, 39.17; H, 6.20; N, 7.61.

N-(1-Deoxy-D-fructos-1-yl)-L-N-nitrosovaline (5d)—Aqueous 1 N hydrochloric acid was added in a dropwise manner at 5 °C with magnetic stirring to a solution of **4d** (3.32 g, 11.9 mmol) and sodium nitrite (0.779 g, 11.3 mmol) in water (12 mL) until pH 3 was attained. After stirring for 30 min at 5 °C, the reaction mixture was worked up to remove Na^+ as described for **5a**. The resulting acidic solution was passed through a short column of Dowex 2X8 (HO^-, 50–100 mesh, 1 mL). Removal of the solvent from the resulting solution gave a thick syrup. This syrup was dissolved in absolute ethanol (5 mL), triturated with methylene chloride (2 mL), and cooled in a freezer overnight. The resulting white solid was filtered and washed with ethyl acetate:ether (1:1, v/v, 10 mL). Drying of the solid at 25 °C and 0.5 Torr over P_2O_5 gave 3.23 g (93%) of **5d**; mp 72–73 °C (dec.); $[\alpha]_D^{20}$ −108 ° (*c* 0.28, H_2O) [lit. $[\alpha]_D^{20}$ −115.9 °, (*c* 0.26, H_2O)[22]].

Anal.—Calcd for $C_{11}H_{20}N_2O_8$: C, 42.86; H, 6.54; N, 9.09. Found: C, 43.07; H, 6.64; N, 8.95.

N-(1-Deoxy-D-fructos-1-yl)-L-N-nitrosophenylalanine (5e)—Concentrated formic acid was added in a dropwise manner at 0 °C with magnetic stirring to a solution of **4e** (0.245 g, 0.75 mmol) and sodium nitrite (0.049 g, 0.71 mmol) in water (3 mL) until pH 3 was

attained. After stirring for 30 min at 0 °C, the reaction mixture was worked up to remove Na^+ as described for **5a**. The volume of the resulting solution was adjusted to 10 mL, then Dowex 2X8 (HO^-, 1 mL) was added to this solution. The mixture was stirred for a few minutes and then filtered. Removal of the solvent from the filtrate gave a thick syrup. The remaining water was azeotropically removed from the syrup with absolute ethanol (5 × 5 mL) by rapid distillation. Drying of the residue at 25 °C and 0.5 Torr over P_2O_5 gave 0.22 g (87%) of **5e** as an off-white powder; $[\alpha]_D^{20}$ −178.6 ° (*c* 0.21, H_2O) [lit. $[\alpha]_D^{20}$ −132.3 °, (*c* 0.56, H_2O)[22]].

Anal.—Calcd for $C_{15}H_{20}N_2O_8$: C, 50.56; H, 5.66; N, 7.86. Found: C, 50.26; H, 6.08; N, 7.08.

N-(1-Deoxy-D-fructos-1-yl)-L-N- nitrosotryptophan (5f)—Aqueous 1 N hydrochloric acid was added in a dropwise manner at 5 °C with magnetic stirring to a solution of **4f** (1.464 g, 4.0 mmol) and sodium nitrite (0.552 g, 8.0 mmol) in water (43 mL) until pH 3 was attained. The color changed to chartreuse, then to orange during the reaction. After stirring for 1 h at 2–5 °C, the reaction mixture was concentrated to 20 mL and added to a column of XAD-2 adsorption resin (2.4 × 27 cm). The column was eluted with water until no Cl^- was detected in the eluant. Then, the product mixture adsorbed on the resin was eluted with methanol. Removal of the solvent from the combined fractions gave a dark orange solid. This solid was flash chromatographed on a silica column (2.7 × 40.5 cm) with water-saturated 1-butanol as eluant. Removal of the solvent from the appropriately combined fractions gave 0.84 g (53%) of **5f** as light yellow powder; mp 155–158 °C (dec); $[\alpha]_D^{20}$ −166.7 ° (*c* 0.42, H_2O) [lit. $[\alpha]_D^{20}$ −79.6 °, (*c* 0.40, H_2O)[22]]; ^1H NMR (D_2O)[22]: δ 7,74 (d, H-7E), 7.61 (d, H-7Z), 7.51 (d, H-4E), 7.49 (d, H-4Z), 7.25 (fused m, H-5), 7.18 (fused m, H-6), 7.15 (s, H-2E), 7.10 (s, H-2Z), 5.11 (dd, H-2'aE), 4.94 (dd, H-2'aZ), remaining protons appear as a complex multiplet between 4.12 and 3.32 ppm (for comparison see ref 22).

Anal.—Calcd for $C_{17}H_{21}N_3O_8 \cdot H_2O$: C, 47.32; H, 5.84; N, 9.74. Found: C, 46.67; H, 4.85; N, 10.01.

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Acknowledgments

The authors thank the Elsa U. Pardee Foundation, Midland, MI, for generous financial support. The authors are also indebted to Dr. Margaret L. Kornguth, Starks C.P., Rockville, MD, for arranging the primary antitumor screens, to the NCI for the actual test results, to the Rohm and Haas Company, Philadelphia, PA, and Dow Chemical Company, Midland, MI, for samples of ion-exchange resins, and to the Frederick Cancer Facility, NCI, Frederick, MD, for the P388 and L1210 tumor lines.