Degenerative Chemistry of Malondialdehyde. Structure, Stereochemistry, and Kinetics of Formation of Enaminals from Reaction with Amino Acids[1]

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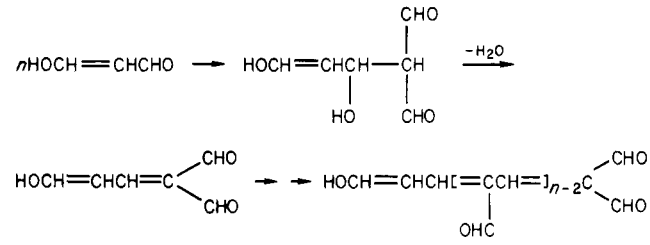
Abstract: Malondialdehyde (MDA) is a naturally occurring compound produced in biological materials as a result of polyunsaturated lipid oxidation or from irradiation of certain compounds. Malondialdehyde chemistry has long been of interest in food science and has received attention recently because of suggestions that it may play a role in cellular aging and in a host of other degenerative biological transformations associated with lipid peroxidation. The detrimental effects of MDA are believed to result from its reaction with primary amino groups of biological macromolecules. So that further insight into the specific nature of these interactions could be gained, the reaction of pure MDA with several amino acid derivatives was studied at pH 4.2. The products from the reaction were found to be enaminals, and ^1H and ^{13}C NMR spectroscopy showed evidence of both *s*-cis and *s*-trans conformations in $CDCl_3$ while only the *s*-trans form was evident in D_2O. This represents the first detailed structural analysis of these products. The rate and equilibrium constants for enaminal formation were determined with the use of UV spectroscopy. With histidine, tyrosine, arginine, and tryptophan there was no evidence for reaction of MDA at positions other than the α-amino group, and the possible implications of this finding in MDA–protein interactions are discussed. The reactions of the more stable methylmalondialdehyde were examined as well.

Malondialdehyde (MDA) (**1**) is a naturally occurring three-carbon dialdehyde produced in the oxidation of polyunsaturated lipids.[2-7] It is generated also in the irradiation of carbohydrates,[8] and certain amino acids.[9] The measurement of MDA has been used by the food industry as a measure of oxidative rancidity.[7,10-12] It has been suggested that the production of MDA in foods alters their nutritive value.[13-16] Recent reports that MDA is toxic,[17,18] carcinogenic,[17] and mutagenic[18-20] and may be involved in a number of age-related disorders[21-24] have generated wide interest in its chemistry.

The detrimental effects of MDA are likely to result from its ability to covalently bond and to cross-link a variety of biological macromolecules by reaction with functional groups such as primary amino groups. Products such as **2** and **3** have been isolated

$$\underset{\mathbf{1}}{\mathrm{OHCCH{=}CHOH}} + \mathrm{RNH_2} \rightarrow \underset{\mathbf{2}}{\mathrm{RNHCH{=}CHCHO}} \xrightarrow{\mathrm{RNH_2}} \underset{\mathbf{3}}{\mathrm{RNHCH{=}CHCH{=}NR}}$$

Scheme I



from the reaction of MDA and glycine.[25,26] The 1-amino-3-iminopropenes exhibit fluorescence with an excitation maximum at 370 nm and an emission maximum at 450 nm. The reaction of MDA with proteins has been said to result in cross-linking and the products have fluorescence spectra similar to 1-amino-3-iminopropenes. This suggests that the cross-linking of these macromolecules may occur through formation of 1-amino-3-iminopropene linkages. It should be noted, however, that MDA is unstable and undergoes an aldol-type self condensation reaction to produce a mixture of polymers (Scheme I).[27] Some of these polymers exhibit fluorescence similar to those of 1-amino-3-iminopropenes and also are capable of cross-linking proteins.[27-30] Interestingly, an age skin pigment called lipofuscin which has been suggested as being derived from the reaction of MDA with skin proteins also has a fluorescence spectrum similar to the 1-amino-3-iminopropenes.[21,26]

Although MDA is known to react with proteins, the reaction products have not been characterized adequately.[14,25,26,31-34] Valuable information on both the reactive sites and the structural nature of protein modification and cross-linking can be obtained through investigation of simple model systems that represent the

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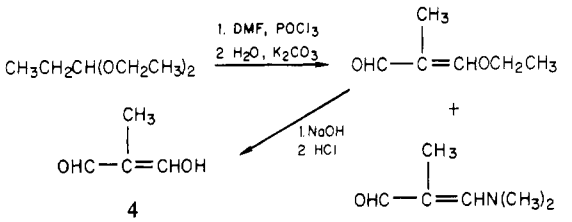
Scheme II



vulnerable protein components. We report here the first complete account of the structure, stereochemistry, and kinetics of the initial reaction of MDA with representative amino acids. Studies of MDA chemistry are complicated by its tendency to undergo self-condensation reactions. Methylmalondialdehyde (MMDA) (**4**) is a much more stable yet closely related derivative of malondialdehyde. The reactions of MMDA with amino acid derivatives were therefore studied as well to determine the feasibility of its use in model studies of MDA reactions. The focal point of this study is the initial reaction of MDA with the amino acids to form enaminals. The rates and equilibria involved in the reactions were examined by UV spectroscopy, and the stereochemical features of the products in solution were analyzed by ^1H and ^{13}C NMR spectroscopy.

Results and Discussion

The choice of amino acid derivatives for the study was based on several considerations. Initially, the reactions of glycine and alanine derivatives were examined as their structural simplicity facilitated isolation and identification of the products. Lysine, histidine, tyrosine, arginine, and tryptophan derivatives were chosen to determine if the dialdehydes react at positions other than the α-amino group. The only simple primary amino groups present in protein are the ϵ-amino groups of lysine and the N-terminal amino groups. Crawford et al. determined that MDA does, in fact, react with the ϵ-amino group of lysine and the N-terminal amino acid, asparagine, of bovine plasma albumin.[14] Subsequently, a number of enzymes,[25,26,30] food proteins,[13] and collagen[35-37] were reacted with MDA, hydrolyzed, and subjected to amino acid analysis. The results of these studies vary depending on the protein studied and the reaction conditions, but the amino acids which consistently appear to be altered to some degree are histidine, tyrosine, arginine, lysine, and methionine. Cysteine and tryptophan are destroyed in protein hydrolysis, but tryptophan residues of protein have been shown to react with triose reductone (hydroxymalondialdehyde).[38] Methionine- and cysteine-malondialdehyde interactions are currently under study in our laboratory, and these results will be reported elsewhere.

Malondialdehyde was prepared by hydrolysis of its bis(dimethyl acetal) and was purified and stored as its relatively stable enolic sodium salt.[39,40] The sodium salt of MDA crystallizes from ethanol/ether as white needles, mp 246 °C dec. Its UV spectrum in 0.01 M HCl showed λ_{max} 245 nm (ϵ 12 800). A bathochromic shift to λ_{max} 267 nm (ϵ 29 400) occurred in 0.01 M NaOH. Its ^1H NMR spectrum in D_2O (external Me_4Si) showed resonances at δ 9.08 (2 H, d, J = 10.1 Hz) and 5.73 (1 H, t, J = 10.1 Hz), suggesting an *s*-trans stereochemistry.[40] Interestingly, the triplet at δ 5.73 slowly disappeared, and examination of its ^{13}C NMR spectrum showed C_2 as a triplet at δ 110.1, suggesting slow exchange of the hydrogen at C_2 with deuterium.

For purposes of comparison and for extension of the scope of these studies, a substituted malondialdehyde also was utilized. The choice of a specific substituted malonadialdehyde for use in these studies was governed by the requirements not only of stability but also of inherent structural closeness to the parent compound. The substituted compound that best fulfilled these requirements was methylmalondialdehyde (MMDA). This was prepared by a modified literature procedure involving the Vilsmeier-Haack-Arnold acylation of propionaldehyde diethyl acetal as outlined in Scheme II.[41-43] We have discovered in our laboratory that phosphorus oxychloride is a better reagent to use than phosgene or oxalyl chloride. The ethyl ether of **4** is produced as a side product, and it can be easily converted to **4** by hydrolysis. Analytically pure samples of **4** can be prepared by taking advantage of its amphoteric character. Thus, purification can be achieved through conversion of **4** to its hydrochloride salt.[43] Methylmalondialdehyde is a solid mp 89 °C. It exhibits a strong UV absorption in neutral solution at 274 nm (ϵ 29 800). This provides a convenient method for monitoring some reactions of this compound. Additionally, the methyl group on this compound provides an excellent marker for following its reaction by ^1H and ^{13}C NMR spectroscopy.

In general, the reactions were carried out by combining aqueous solutions of the dialdehyde enolic sodium salt and a molar

Table I. Products from the Reaction of Malondialdehyde and Methylmalondialdehyde with Amino Acid Derivatives

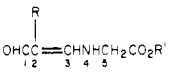
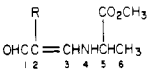
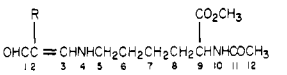
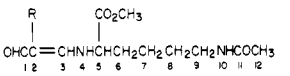
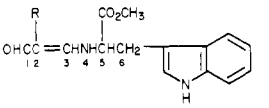
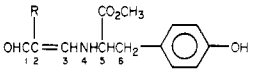
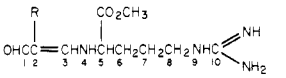
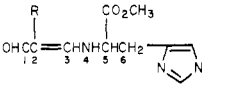
amino acid derivative	product
glycine	 **5**, R = H, R′ = Na; **6**, R = CH_3, R′ = H
glycine methyl ester	**7a**, R = H, R′ = CH_3; **7b**, R = R′ = CH_3
alanine methyl ester	 **8a**, R = H; **8b**, R = CH_3
α-acetyllysine methyl ester	 **9a**, R = H; **9b**, R = CH_3
ϵ-acetyllysine methyl ester	 **10a**, R = H; **10b**, R = CH_3
tryptophan methyl ester	 **11a**, R = H; **11b**, R = CH_3
tyrosine methyl ester	 **12a**, R = H; **12b**, R = CH_3
arginine methyl ester	 **13a**, R = H; **13b**, R = CH_3
histidine methyl ester	 **14a**, R = H; **14b**, R = CH_3
α-acetylhistidine methyl ester	no reaction
α-acetyltyrosine methyl ester	no reaction

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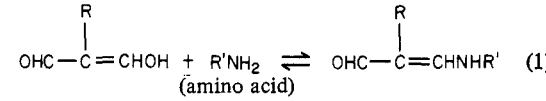
Table II. Physical and Spectral Properties of Enaminal Products[a]

compd	method[b]	R_f[b]	yield, %	mp, °C	UV_{max} (log ε)[c]
6	B[d]		56	170–171	289 (4.51)
7a	A	0.53	50	103–104	277 (4.52)
7b	A	e	63	oil[e]	288 (4.55)
8a	A	0.53	32	oil	279 (4.53)
8b	A	0.63	18	oil	288 (4.54)
9a	B	0.23	10	oil	282 (4.53)
9b	B	0.27	12	oil	289 (4.56)
10a	B	0.42	10	oil	280 (4.52)
10b	B	0.50	10	oil	289 (4.56)
11a	A	0.60	97	89–90	280 (4.46)[f]
11b	A	0.60	88	57–59	285 (4.56)[f]
12a	B	0.41	30	164–165	282 (4.53)
12b	B	0.50	25	59–61	290 (4.50)
13a	B	g	20	165 dec	283 (4.53)
13b	B	g	50	165 dec	292 (4.57)
14a	B	0.15	45	45–46	279 (4.52)
14b	B	0.30	29	42–43	288 (4.51)

[a] All compounds gave satisfactory elemental analyses. [b] Methods A and B and chromatographic techniques are described in the Experimental Section. [c] In 0.2 M, pH 4.2 acetate buffer solution. [d] Purified by recrystallization from 2-propanol. [e] Purified by distillation on a micromolecular still at 0.2 torr, 180–185 °C (bath temperature). [f] UV spectrum was taken in ethanol because of low solubility in aqueous buffer. [g] Purified by high pressure liquid chromatography.

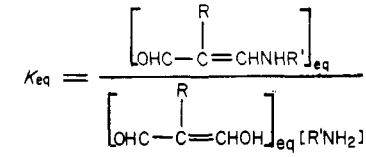
equivalent of the amino acid methyl ester hydrochloride. One equiv of HCl was added to the solutions for the reactions of the dialdehydes with the α-acetyl amino acid methyl esters. So that MDA polymerization, could be minimized, reactions involving it were carried out in solutions low in MDA concentration. The results of the reaction between the dialdehydes and the amino acid derivatives are summarized in Table I. The use of the methyl ester derivatives of the amino acids simplified isolation, purification, and structural analysis of the products. The procedures used and the yields, physical properties, and UV data for the products are shown in Table II. In every case, only the 1:1 adduct of amino acid and MDA or MMDA was observed. This is in contrast to the work of Chio and Tappel[25] who observed both 1:1 and 1:2 adducts in strongly acidic aqueous solutions. Chio and Tappel prepared MDA by the acidic hydrolysis of its bis(acetals). Malondialdehyde prepared by this method is generally contaminated with its partial hydrolysis products (3,3-dialkoxypropionaldehyde and β-alkoxyacrolein) and its polymers. These side products also are reactive toward amino acids. We have used highly purified MDA (as its sodium salt) in our studies. Thus, we believe that the reasons for this difference must be associated with the purity of the MDA and the mildly acidic conditions used in our work. Unambiguous evidence for the formation of the 1:1 adducts came from spectral data and elemental analysis. The mass spectra provided particularly convincing data. In all cases, parent ion currents were present in the mass spectra of these 1:1 adducts.

The UV absorption maximum and absorptivity of the dialdehydes vary with pH changes.[43-46] At low pH values, the UV absorption maximum is 30–40 nm lower than that of the enaminal products. Ultraviolet spectroscopy, therefore, provides a convenient means of examining the rate and equilibria involved in the dialdehyde–amino acid reaction. The reaction of MDA with glycine has been shown to be second order, first order in both amino acid and MDA, and the rate varied greatly with changes of pH having a maximum rate at pH 4.2.[33] This is also the optimum pH for the reaction of MDA with protein[30,47] and is just below the malondialdehyde acid dissociation constant of 4.5.[48] The *s*-trans protonated enol form of MDA predominates at pH 4.2. Methylmalondialdehyde with an acid dissociation constant of 4.7 shows similar behavior.[43-46] Rate studies were carried out at pH 4.2 under pseudo-first-order conditions with a large excess of amino acid derivative. The disappearance of MDA and the appearance of enaminal product were monitored at their absorption maxima of approximately 249 and 280 nm, respectively. An isosbestic point was present at 258–261 nm, and this behavior was typical of all the MDA–amino acid reactions studied here. Methylmalondialdehyde reactions were monitored at 252 and 288 nm. The MMDA reactions exhibit an isosbestic point at 265–269 nm. The observed pseudo-first-order rate constants (k_{obsd}) for MDA disappearance and for enaminal formation agreed within experimental error. Where applicable, absorbance values of A_∞ at these wavelengths were corrected for absorbance due to amino acid derivative. These values along with the molar absorptivity of the dialdehydes (MDA 249 nm, log ε 4.10; MMDA 252 nm, log ε 4.27) and the products (280 nm, log ε 3.53; 288 nm, log ε 3.35) were substituted into the appropriate Beer's law relationships and the two equations solved for the concentrations of product and dialdehyde at equilibrium. From this information, the equilibrium constant (K_{eq}), the second-order rate constant (k_f) for enaminal formation, and k_r, the pseudo-first-order rate constant for the hydrolysis were obtained by using eq 1–4.[49] The results are summarized in Table III.



$$OHC-C(R)=CHOH + R'NH_2 \rightleftharpoons OHC-C(R)=CHNHR' \quad (1)$$

(amino acid)

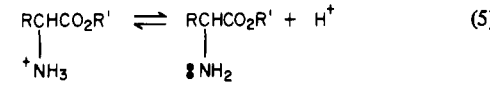


$$K_{eq} = \frac{[OHC-C(R)=CHNHR']_{eq}}{[OHC-C(R)=CHOH]_{eq}[R'NH_2]} \quad (2)$$

$$k_{obsd} = k_f[R'NH_2] + k_r \quad (3)$$

$$k_f/k_r = K_{eq} \quad (4)$$

Several classes of amino acids are represented in the study, and there appear to be several features which affect the reaction rate and equilibrium constant. For the reaction of MDA with the α-amino group of glycine, alanine, and ε-*N*-acetyllysine, there appears to be a decrease in K_{eq} and k_f with increase in the size of the alkyl substituent. However, arginine and tyrosine react as fast as glycine despite the presence of large groups so that the position of the equilibrium (eq 5) at pH 4.2 must also be taken



$$RCHCO_2R' \rightleftharpoons RCHCO_2R' + H^+ \quad (5)$$

($^+NH_3$ and NH_2)

into account. The observation that ε-*N*-acetyllysine shows a higher K_{eq} and k_f than α-*N*-acetyllysine is consistent with this (pK_2 of α-N^+H_3 is 8.95 and pK_3 of ε-N^+H_3 is 10.53).[50] The reaction of MDA with the α-amino group of histidine is exceptionally favorable. The exact reason for this effect is unclear. The possibility of participation of the imidazole ring of histidine in the rate acceleration observed at the α-amino group of this amino acid was examined. However, addition of α-*N*-acetylhistidine to solutions of other amino acids does not accelerate their reaction with MDA. It is conceivable that the facile reaction with histidine is the result of an intramolecular rate acceleration process where initial binding of MDA to the imidazole nitrogen is followed by transfer of the MDA from this nitrogen to the α-amino group.[51] The behavior of MMDA parallels that of MDA except that the

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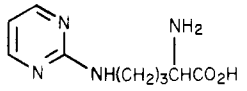
Table III. Rate and Equilibrium Constants[a] and Related Ultraviolet Spectral Data[b] for the Reaction of Dialdehyde and Amino Acid

compd	log ε (249 nm)	log ε (280 nm)	log K_{eq}	k_f, M^{-1} min^{-1}	k_r, 10^3 min^{-1}	compd	log ε (252 nm)	log ε (288 nm)	log K_{eq}	k_f, 10^2 M^{-1} min^{-1}	k_r, 10^4 min^{-1}
7a	3.65	4.51	2.87	2.51	3.35	7b	3.38	4.55	2.20	2.76	1.76
8a	3.55	4.53	2.38	0.82	3.39	8b	3.30	4.54	1.90	1.24	1.55
9a	3.57	4.53	2.09	0.14	1.12	9b	3.65	4.55	1.29	0.16	0.82
10a	3.48	4.54	2.55	1.09	3.07	10b	3.18	4.56	2.10	1.21	0.97
12a	3.43	4.50	2.78	2.23	3.73	12b	3.34	4.49	2.72	2.64	0.50
13a	3.48	4.53	2.82	2.26	3.46	13b	3.25	4.57	2.47	2.68	0.91
14a	3.58	4.51	3.81	9.27	1.45	14b	3.39	4.51	3.48	13.4	0.44

[a] The rate constant k_f is the second-order rate constant for the formation of the enaminals; k_r is the first-order rate constant for the reverse reaction, i.e., enaminal hydrolysis; K_{eq} is the equilibrium constant for enaminal formation. [b] The molar absorptivity (ε) for the enaminals is given at wavelengths near the absorption maxima and the molar absorptivity (ε) for the dialdehydes is given at the absorption maxima, in 0.2 M pH 4.2 buffer. These values along with the absorptivity of the dialdehydes were used to calculate the concentrations of dialdehyde and enaminal at equilibrium.

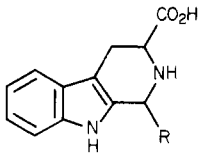
reactions are generally about 100 times slower and the values of K_{eq} are somewhat smaller.

The kinetic studies also show that, under the conditions employed, MDA reacts with the amino acid methyl esters exclusively at the α-amino group. The observation of an isosbestic point and the fact that identical values of k_{obsd} were calculated from MDA disappearance and enaminal appearance precludes any significant amount of side reactions. It is notable that there is no evidence for reaction of the guanidino group of arginine. In strongly acidic solution, arginine has been reported to react with MDA to give δ-*N*-(2-pyrimidinyl)-L-ornithine (**15**).[34] Tryptophan was not



15

included in the kinetic studies because of the low solubility of the product in water. However, the enaminal, **11a**, was isolated in nearly quantitative yield from the reaction of tryptophan methyl ester with MDA in acetate buffer at pH 4.2. Interestingly, under somewhat more forcing conditions (1 N H_2SO_4, 50–60 °C, 1 h), tryptophan has been reported to give the cyclic product **16** on

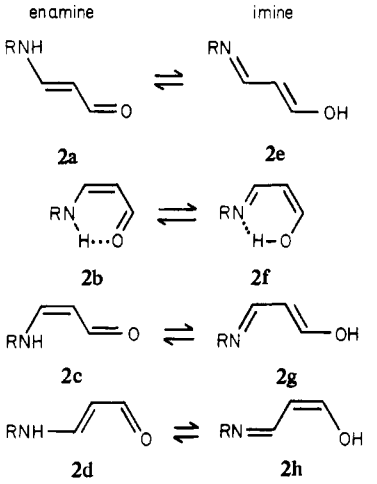


16

reaction with simple aldehydes.[52,53] However, there was no evidence of a product such as **16** from the MDA reaction.

The α-*N*-acetyl derivatives of histidine, tyrosine, and lysine methyl esters were also exposed to MDA in acetate buffer, under the same conditions as in the rate studies. Except in the case of lysine, there was no evidence for a decrease in MDA concentration even after several days at 25 °C. Even at higher temperatures (pH 4.2, 60 °C, 7 days) and higher pH values (pH 7.0, 25 °C, 4 days), there was no apparent reaction between MDA (or MMDA) and α-*N*-acetyltyrosine or α-*N*-acetylhistidine methyl esters. Several studies have suggested that MDA reacts with histidine and tyrosine residues of proteins, but the nature of this interaction remains unclear. It has also been suggested that the product from the reaction of MDA with the ε-amino group of lysine residues in proteins is quite resistant to hydrolysis.[35] However, the relatively low values of k_f and K_{eq} obtained for the reaction of MDA with the ε-amino group of α-*N*-acetyllysine indicate that this product is fairly susceptible to hydrolysis even under conditions chosen to favor enaminal formation rather than hydrolysis. Apparently, protein provides an environment more

Scheme III



favorable to some of these interactions, or the complexities involved in studying MDA protein interactions hinder one from obtaining a clear picture of the nature of the reactions on the basis of the simple model systems utilized in this study.

The ^1H and ^{13}C NMR spectra of most of the products were obtained in $CDCl_3$ and provided insight into the stereochemical features of the products in that solvent. The NMR spectra of the glycine methyl ester enaminals (**7**) were determined in both $CDCl_3$ and D_2O for purposes of comparison. Due to their insolubility in nonhydroxylic solvents, the NMR spectra of **5**, **6**, **13**, and **14** were run in D_2O. The products shown in Table I in the enaminal form are capable of enamine–imine tautomerism and may assume any of the four tautomeric pairs of isomers illustrated in Scheme III. These isomers are all interconvertable by the two processes of tautomerism and hindered rotation about a carbon–carbon single bond. By analogy to related compounds, it was expected that the trans-enamine form **2a** would be the most preferred conformation in hydrogen-bonding solvents and that **2a** and the cis-chelated form **2b** would both be important in non-hydrogen-bonding solvents. The NMR spectra of the products supported these assumptions. Our subsequent use of the terms trans and cis isomers will refer to forms **2a** and **2b**.

Table IV lists the ^1H NMR data for the products **5–14**. So that our interpretation and correlation of this data with structure (enamine vs. imine) and stereochemistry (cis and trans) could be exemplified, a discussion of the 90-MHz ^1H NMR spectrum in $CDCl_3$ of **7a**, the product from the reaction of MDA with glycine methyl ester, is presented. The spectrum (Figure 1) shows two complete sets of resonances which can be assigned to the trans and cis forms. The chemical shift of the NH and its splitting of H_3 and H_5 provide evidence for the enamine rather than the imine tautomer. The predominant isomer is the trans form with $J_{1,2}$ = 8.0 Hz and $J_{2,3}$ = 13.2 Hz. The cis isomer shows values of $J_{1,2}$ = 2.1 Hz and $J_{2,3}$ = 7.4 Hz. Because of intramolecular hydrogen bonding in the cis form, the NH resonance appears at δ 10.0, much further downfield than that of the trans isomer at δ 5.8.[54] These

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Table IV. ^{1}H NMR Data for Enaminals[a]

compd	solvent[b]	isomer[c] (%)	H_1	H_2	H_3	H_4	H_5	OCH_3	$J_{1,3}$	$J_{2,3}$	$J_{3,4}$	$J_{1,3}$[d]	$J_{4,5}$	H_{others}
5	D_2O	trans (100)	9.30	5.76	8.13		4.30		9.0	13.0				
6	D_2O	trans (100)	8.95		7.77		4.63							2.10 (s, 3 H, R = CH_3)
7a	$CDCl_3$	trans (78)	9.16	5.23	7.24	5.80	3.88	3.82	8.0	13.2	7.4		5.1	
7a	$CDCl_3$	cis (22)	9.20	5.12	6.70	10.0	3.97	3.78	2.1	7.3	12.5	3.2	6.3	
7a	D_2O	trans (100)	9.25	5.67	8.05		4.57	4.22	9.0	13.0				
7b	$CDCl_3$	trans (100)	8.97		6.78	5.45	4.07	3.80			13.2		4.8	1.67 (s, 3 H, R = CH_3)
7b	D_2O	trans (100)	9.02		7.62		4.63	4.20						2.04 (s, 3 H, R = CH_3)
8a	$CDCl_3$	trans (70)	9.08	5.26	7.22	6.7	4.1	3.78	8.3	13.2	8.3			1.49 (d, J = 7.1, 3 H, H_6)
8a	$CDCl_3$	cis (30)	9.20	5.09	*e*	10.0	4.1	3.78	2.2	7.1	*e*	*e*		1.52 (d, J = 7.3, 3 H, H_6)
8b	$CDCl_3$	trans (100)	9.01		6.79	5.3	4.0	3.80			13.7			1.54 (d, J = 7.4, 3 H, H_6), 1.68 (s, 3 H, R = CH_3)
9a	$CDCl_3$	trans (51)	9.06	5.26	7.1	5.9	3.2	3.75	8.5	13.0	*f*			1.2–1.9 (m, 6 H, H_6, H_7, H_8), 2.03 (s, 3 H, H_{12}), 4.6 (m, 1 H, H_9), 6.4 (m, 1 H, H_{10})
9a	$CDCl_3$	cis (49)	9.08	4.98	6.76	10.0	3.2	3.75	2.3	6.9	13.4	3.0		1.2–1.9 (m, 6 H, H_6, H_7, H_8), 2.03 (s, 3 H, H_{12}), 4.6 (m, 1 H, H_9), 6.4 (m, 1 H, H_{10})
9b	$CDCl_3$	trans (100)	8.80		6.93	6.2	3.4	3.74			13.3			1.2–1.9 (m, 6 H, H_6, H_7, H_8), 1.67 (s, 3 H, R = CH_3), 2.03 (s, 3 H, H_{12}), 4.7 (m, 1 H, H_9), 6.74 (d, $J_{9,10}$ = 7.9, 1 H, H_{10})
10a	$CDCl_3$	trans (57)	9.2	5.29	7.19	6.1	3.90	3.77	5.1	13.2	8.5			1.2–1.9 (m, 6 H, H_6, H_7, H_8), 1.98 (s, 3 H, H_{12}), 3.2 (m, 2 H, H_9), 6.40 (t, $J_{9,10}$ = 7.3, H_{10})
10a	$CDCl_3$	cis (43)	9.2	5.10	6.74	10.0	3.90	3.77	2.1	7.6	12.4	2.9		1.2–1.9 (m, 6 H, H_6, H_7, H_8), 1.98 (s, 3 H, H_{12}), 3.2 (m, 2 H, H_9), 6.40 (t, $J_{9,10}$ = 7.3, H_{10})
10b	$CDCl_3$	trans (100)	8.98		6.75	5.3	3.90	3.79			13.5			1.2–1.9 (m, 6 H, H_6, H_7, H_8), 1.70 (s, 3 H, R = CH_3), 1.97 (s, 3 H, H_{12}), 3.2 (m, 2 H, H_9), 5.8 (br m, 1 H, H_{10})
11a	$CDCl_3$	trans (63)	9.0	5.29	*e*	5.7	4.30	3.73	8.2	13.1	*e*			3.3 (m, 2 H, H_6), 6.7–7.7 (m, 5 H, indole), 8.4 (br m, 1 H, indole NH)
11a	$CDCl_3$	cis (27)	9.1	4.92	6.42	10.0	4.30	3.73	2.0	7.1	8.8	2.9		3.3 (m, 2 H, H_6), 6.7–7.7 (m, 5 H, indole), 8.4 (br m, 1 H, indole NH)
11b	$CDCl_3$	trans (100)	8.78		6.48	5.3	4.30	3.78			13.7			1.56 (s, 3 H, R = CH_3), 3.32 (t, $J_{5,6}$ = 6.0, 2 H, H_6), 6.9–7.6 (m, 5 H, indole), 8.7 (br m, 1 H, indole NH)
12a	$(CD_3)_2CO$	trans (100)	8.96	5.31	7.31		4.33	3.69	8.4	12.4				3.13 (m, 2 H, H_6), 6.75 (d, J = 9.0, 2 H, aromatic), 7.05 (d, J = 9.0, 2 H, aromatic)
12b	$CDCl_3$	trans (100)	8.80		6.41	5.5	4.30	3.80			14.0			1.61 (s, 3 H, R = CH_3), 3.11 (d, 2 H, H_6), 6.8 (m, 5 H, phenol)
13a	D_2O	trans (100)	9.24	5.80	8.00		4.8	4.23	8.5	13.2				2.0–2.5 (m, 4 H, H_6, H_7), 3.66 (t, J = 6.4, H_8)
13b	D_2O	trans (100)	8.60		7.10		4.2	3.80						1.52 (s, 3 H, R = CH_3), 1.7 (m, 4 H, H_6, H_7), 3.2 (m, 2 H, H_8)
14a	D_2O	trans (100)	9.15	5.71	7.90		4.95	4.21	7.8	13.0				3.6 (m, 2 H, H_6), 7.48 (s, 1 H, imidazole), 8.33 (s, 1 H, imidazole)
14b	$CDCl_3$	trans (100)	8.75		7.25	6.6	4.35	3.80						1.55 (s, 3 H, R = CH_3), 3.20 (d, J = 7.0, 2 H, H_6), 6.80 (s, 1 H, imidazole), 7.6 (s, 1 H, imidazole), 7.25 (s, 1 H, imidazole NH)

[a] Chemical shift values are in δ from internal Me_4Si except for D_2O solutions where external Me_4Si (capillary) was used as reference. Because of broadening and overlap, chemical shifts in some cases could be determined only to one decimal place. Coupling constants are given in Hz to one decimal place. See Table I for numbering. [b] The concentration of enaminal is 0.1 M. [c] Trans and cis refer to forms **2a** and **2b**. The percent of isomers present in solution was calculated from integrated intensities of peaks. [d] The magnitude of the coupling constants is given but the signs were not determined. [e] Obscured by overlapping peaks. [f] Unresolved multiplet.

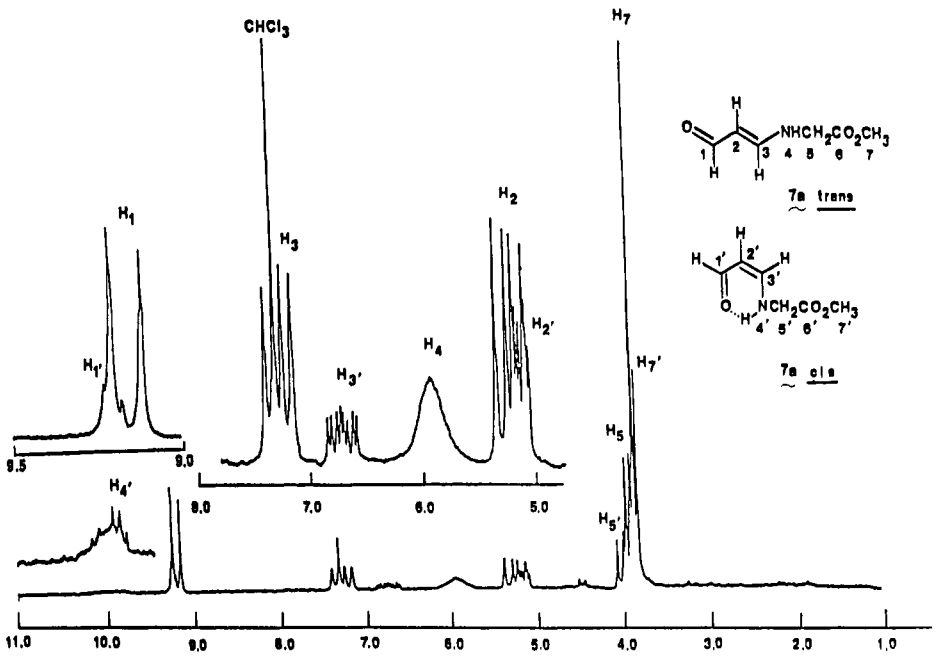

Figure 1. ^{1}H NMR Spectrum of **7a** in $CDCl_3$. Chemical shift values are in δ from internal Me_4Si.

broad peaks disappear on exchange with D_2O, and the splittings between H_3 and H_4 and between H_4 and H_5 are no longer present. An interesting feature of the spectrum of the cis isomer was the presence of a four-bond coupling $J_{1,3}$ = 3.2 Hz while no such coupling was observed for the trans isomer. This difference can be attributed to a favorable (*W*) stereochemical relationship for long-range coupling of H_1 and H_3 in the cis form which was not present in the trans isomer.[55] On the basis of integrated intensities, the trans isomer was 78% of the isomeric mixture. The ^{13}C NMR data of **7a** in $CDCl_3$ (Table V; see paragraph concerning supplementary material) also show a mixture of isomers, and assignment of resonances to the carbons of cis and trans forms was based partly on the relative intensities of the pairs of peaks. The NMR spectra of the other products were interpreted with the use of similar reasoning. The percent of cis isomer present in $CDCl_3$ solutions showed an increase with increasing size of the nonpolar amino substituent. This trend has also been observed in related compounds.[54] The MMDA products gave much simpler ^{1}H NMR spectra with only one isomer in evidence. In the $CDCl_3$ spectra, the splitting of H_5 by the NH again confirms the presence of the enamine tautomer, and H_4 chemical shifts of δ 5–6 are consistent with the expected trans form. The ^{13}C NMR spectra of these enaminals in $CDCl_3$ also show only one isomer. For both MDA and MMDA adducts **5–14**, the spectra in D_2O provide evidence for a single isomer. For the MDA products, the trans structure can be assigned based on the magnitude of splittings between H_1, H_2, and H_3. Although unequivocal assignments cannot be made for the MMDA products in D_2O, it is reasonable to assume that the trans form predominates for these compounds as well.

The MDA-induced cross-linking of proteins has been proposed to occur through formation of 1-amino-3-iminopropene linkages.[25,26] The failure to observe the formation of this type of product from the reaction of MDA with these amino acid derivatives under mild conditions was somewhat surprising. After completion of the rate studies, the reaction solutions (which contained a large excess of amino acid) were allowed to stand at room temperature for 30 days. After this time, the UV spectrum showed only a slight decrease in enaminal absorbance (less than 10%) and there was no significant change in the absorption maximum or in the shape of the UV spectrum. Interestingly, significant protein cross-linking by MDA has been reported to occur within 24 h.[26,30] It is possible that protein molecules provide a more favorable environment for MDA reaction than is present with simple amino acid derivatives. In relation to this, it should be mentioned that in studies in our laboratory using a "less hydrophilic" solvent (e.g., methanol), 2:1 or cross-linked adducts from MDA and amino acids have been isolated. Alternatively, MDA polymers may play a role in the observed inter- and intra-molecular cross-linking of proteins. The polymers have free aldehyde groups,[27–29] and it is possible that to some extent the observed cross-linking and the fluorescence of MDA–protein products result from reaction of the polymers with proteins.[30]

In summary, with the use of carefully controlled conditions of pH, temperature, and concentration, detailed information on the reaction of MDA with amino acid derivatives was obtained. These results serve as simple models for MDA–protein interactions, and the physical data obtained for the products should be useful in examining MDA interactions in more complex systems. Studies of MMDA show that although it reacts much slower than MDA, its behavior parallels that of MDA in other respects. The results suggest that MMDA may be useful in defining some aspects of the biological chemistry of MDA where the instability of the latter precludes acquisition of useful experimental data. Further studies are in progress regarding the formation, structure, and chemistry of the 1-amino-3-iminopropenes (**3**) and on the interaction of malondialdehyde with sulfur-containing amino acids.

Experimental Section

Melting points are uncorrected and were determined on a Thomas-Hoover melting point apparatus fitted with a microscope. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker HX-90E or a Joel FX-90Q pulse Fourier Transform NMR spectrometer. Mass spectra were determined on a Hitachi RMU-6-E mass spectrometer at an ionizing energy of 70 eV. Elemental analyses were performed by the University of Iowa microanalytical service. Ultraviolet spectra were recorded on a Cary model 219 spectrophotometer. Molar extinction coefficients were determined from the slope of plots of absorbance vs. concentration, and absorbance values were obtained from freshly made solutions of the analytically pure products (10^{-5}–10^{-6} M) in 0.2 M, pH 4.2 acetic acid–sodium acetate buffer.

Materials. Amino acid derivatives and malondialdehyde bis(dimethyl acetal) were purchased from Aldrich Chemical Co., Milwaukee, WI, or from Sigma Chemical Co., St. Louis, MO. α-*N*-Acetylhistidine,[51,56] α-*N*-acetyltyrosine,[57] α-*N*-acetyllysine,[58–60] and ϵ-*N*-acetyllysine[61] methyl

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esters were prepared by literature methods. *N*-Prop-2-enal amino acetic acid (**5**) was prepared by the method of Crawford.[33] Acetic acid–sodium acetate buffer (0.2 M, pH 4.2) was made from 19.2 g (0.32 mole) of glacial acetic acid and 6.56 g (0.08 mol) of anhydrous sodium acetate which was diluted to 2 L with deionized, degassed, distilled water. Merk silica gel PF_{254} was used for preparative layer chromatography.

Sodium malondialdehyde was prepared by a modified literature procedure.[39,40] Malondialdehyde bis(dimethylacetal) (5 mL, 30 mmol) was added to 32 mL of 1 N HCl. The flask was stoppered tightly and the suspension stirred at room temperature for several minutes until a homogeneous solution was obtained. The solution was then kept at 0 °C for 24 h. The resulting clear pale yellow solution was neutralized (pH 7–8) with 5 N NaOH and the solvent removed under reduced pressure (bath temperature <40 °C). The residue was slurried with 75 mL of CH_3OH and treated with Norit decolorizing carbon. The Norit and NaCl were filtered and the filtrate evaporated under reduced pressure. The residue was recrystallized twice from ethanol–ether and dried for 24 h at room temperature at 0.1 torr over P_2O_5 to give 1.87 g (47%) of MDA sodium salt hydrate as white needles: mp 245 °C dec; ^1H NMR-(D_2O) δ 9.08 (d, J = 10.1 Hz, 2 H, CHO), 5.73 (t, J = 10.1 Hz, 1 H); ^{13}C NMR(D_2O) δ 193.6, 110.3. After 24 h H_2 exchanges with D_2O: δ 193.6, 110.1 (t, J_{CD} = 24.9 Hz).

Anal. Calcd for the sodium salt of $C_3H_3O_2 \cdot H_2O$: C, 32.13; H, 4.50. Found: C, 32,36; H, 4.43.

Methylmalondialdehyde (4) was prepared by a modification of literature procedures.[41-43] Dimethylformamide (187 mL, 2.4 mol) was added dropwise to 322 g (2.2 mol) of phosphoryl chloride with vigorous stirring and cooling in ice. During the addition the temperature was kept below 30 °C. After the addition was complete, 132 g (1.0 mol) of propionaldehyde diethylacetal was added slowly with vigorous stirring and slight warming. When the exothermic reaction began, the heat was removed and the reaction temperature was maintained at 60–70 °C by the slow dropwise addition of the acetal. The solution changed from light orange to dark brown as the reaction progressed. When addition of the acetal was complete, the solution was warmed to 70 °C for 2 h, cooled to room temperature, poured into 1500 g of ice, and allowed to stand overnight. Anhydrous potassium carbonate was added until the solution was basic, and 1000 mL of water was added to dissolve the precipitated salts. The aqueous solution was then extracted with CH_2Cl_2 (5 × 200 mL). The combined organic extracts were dried over anhydrous K_2CO_3 and evaporated under reduced pressure. The liquid residue was fractionally distilled. After DMF was removed, a yellow liquid was collected at 75–80 °C (0.3–0.5 torr). The liquid was redistilled at 40 °C (0.26 torr), giving 29.6 g (26%) of 2-methyl-3-ethoxyprop-2-enal as a colorless liquid: ^1H NMR($CDCl_3$) δ 1.34 (t, J = 7.2 Hz, 3 H), 1.60 (s, 3 H), 4.25 (q, J = 7.2 Hz, 2 H), 7.37 (s, 1 H), 9.25 (s, 1 H); ^{13}C NMR($CDCl_3$) δ 6.3, 15.3, 71.0, 119.6, 168.6, 191.5. A second fraction was obtained at 110–115 °C (0.3 torr) which was identified as 2-methyl-3-(dimethylamino)prop-2-enal[41] (30.7 g, 27%): ^1H NMR($CDCl_3$) δ 1.93 (s, 3 H), 3.15 (s, 6 H), 6.58, (s, 1 H), 8.88 (s, 1 H). The 2-methyl-3-(dimethylamino)prop-2-enal (23.7 g, 0.21 mol) and 9.2 g (0.23 mol) of sodium hydroxide in 30 mL of water were stirred vigorously and warmed to 70 °C until a homogeneous solution was obtained from the original two layers (5 min). The solution was concentrated almost to dryness under reduced pressure (bath temperature 50 °C) and the sodium salt of MMDA precipitated by means of a 1:1 ethanol–acetone mixture. The product was filtered and the mother liquor evaporated almost to dryness. In the same manner a further portion of the sodium salt was isolated and the previous operation repeated once more. The residue was recrystallized from ethanol–ether and dried overnight over $CaSO_4$ at room temperature (0.2 torr), giving 14.0 g (62% from 2-methyl-3-(dimethylamino)prop-2-enal) of the sodium salt. The salt was also prepared from the 2-methyl-3-ethoxyprop-2-enal in the same manner. The free acid of MMDA was prepared and purified by the method of Moschel and Leonard.[43] The sodium salt was a fine white powder: mp 360 °C dec; ^1H NMR(D_2O) δ 8.90 (s, 2 H, CHO), 2.00 (s, 3 H, CH_3).

General Procedure for the Preparation of the Enaminals 6–14. Method A. The amino acid methyl ester hydrochloride was dissolved in pH 4.2 acetate buffer, and 1 equiv of MDA or MMDA sodium salt was added. The solution were diluted with buffer to give reactant concentrations of 0.02–0.20 M. The solutions were allowed to stand at room temperature, and samples were withdrawn periodically and diluted with pH 4.2 buffer for analysis of the UV spectrum. When the absorbance of product reached a maximum, the solutions were treated with a slight excess of 1 N $NaHCO_3$ and the products extracted from the aqueous solution with CH_2Cl_2 ((3–6) × 15 mL). The organic extracts were dried over anhydrous Na_2SO_4, and the solvent was evaporated under reduced pressure to give the crude enaminal. The product was purified by preparative layer chromatography on silica gel. The plates were developed with 10% methanolic CH_2Cl_2 and, the product was eluted from the gel with 25% methanolic CH_2Cl_2. The pure enaminal was obtained by evaporation of the solvent under reduced pressure and was dried over P_2O_5 overnight at room temperature (0.1 torr).

Method B. The solutions were made up and monitored by their UV spectra as in method A. When the reaction was complete, the solution was neutralized with $NaHCO_3$ and solvent evaporated under reduced pressure. The dry residue was taken up in a few milliliters of methanol, and sodium chloride was filtered. The filtrate was applied to silica gel preparative layer plates and the plates developed with 2–3 immersions in 10% methanolic CH_2Cl_2. The products were eluted from the silica gel, isolated, and dried as in method A.

The products **13a** and **13b** were purified by high pressure liquid chromatography by using an Altex model 100 pump with preparative heads, and Altex injector model numbers 905-19 with a 10.2-mL loop, and Altex model 153 analytical UV (280 nm) detector with an 8-μL analytical cell, and a Texas Instruments (servo-riter) recorder. For **13a** a column 20.5-mm i.d. × 32 cm packed with Amberlite XAD-4 (75–105-μm particle size) was used.[62] A 2-mL sample of 0.1 M neutralized reaction solution was injected with water used as eluting agent at a flow rate of 16.8 mL/min (350–500 psi). Product fractions were collected at 12–22 min, pooled, and lyophilized, giving **13a** as a white solid. A 1-mL sample of 0.1 M neutralized solution of product **13b** was injected onto a column of Amberlite XAD-4 (37–44-μm particle size 6.6-mm i.d. × 25 cm. Water was used as elutant at a flow rate of 4.2 mL/min (1500 psi), and the product fraction was collected at 8–16 min, pooled, and lyophilized, giving **13b** as a white solid.

Kinetics. The amino acid derivatives were dissolved in 0.2 M pH 4.2 acetate buffer solution at concentrations of 0.001–0.06 M and the ionic strength adjusted to 0.10 M with NaCl. The solutions were placed in a cuvette and allowed to equilibrate at 25° ± 0.5 °C in the thermostated cell holder of the spectrometer. Constant temperature was maintained by a Forma Scientific model 2095 water bath and circulator. The reaction was initiated by adding 20 μL of 5 × 10^{-3} M MDA sodium salt solution. Alternatively, solutions of the amino acid derivatives were made up in volumetric flasks, equilibrated at 25 °C in the water bath, and an aqueous solution of MDA sodium salt added to give an MDA concentration of 0.001 M. Aliquots (100 μL) were withdrawn periodically and diluted to 10 mL with acetate buffer for observing the UV spectrum. The ultraviolet spectra were recorded with buffer solution in the reference beam. The disappearance of MDA was monitored at 249 nm and of MMDA at 252 nm; the appearance of their enaminal products was followed at 279 and 288 nm, respectively. The reactions were generally followed for at least three half-lives and infinity readings taken after ten half-lives. The observed first-order rate constants, k_{obsd}, were calculated from the slopes of linear plots of ln $(A_\infty - A_t)$ vs. time. The plots had correlation coefficients of >0.995. During the first 3–4, min there is a rapid decrease in the absorbance at 249 nm due to equilibration of the isomeric forms of MDA which occurs with protonation of the enolate anion. Points taken before 4 min were therefore not included in the calculations of the slopes. This behavior could be eliminated by using solutions of MDA equilibrated with buffer. However, because of its greater stability, it was generally more convenient to use aqueous solutions of the sodium salt. The rate studies were repeated at three or more different concentrations of amino acid derivative, maintaining at least a 20-fold excess of amino acid derivative over dialdehyde. Absorbance values from solutions at A_∞ were used to calculate the equilibrium constants K_{eq} in Table V. The values have an average deviation of ±10%. The rate constants k_f and k_r for the forward and reverse reaction (eq 1) were calculated from k_{obsd} values and K_{eq} and have a deviation of less than 5%.

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Supplementary Material Available: Listing of ^{13}C NMR data for enaminals (Table V) (2 pages). Ordering information is given on any current masthead page.

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