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Synthesis of some heterocyclic amino-imidazoazarenes

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The reaction mechanism of the synthesis of 2-amino-3,4-dimethylimidazo(4,5-f)-quinoline and 2-amino-3,4,8-trimethylimidazo(4,5-f)quinoxaline as compounds of the new class of mutagenic heterocyclic amines, amino-imidazoazarenes, in the reaction between aldoses, amino acids and creatinine, has been studied. The reaction involves two reaction steps: (a) the initial step and (b) the steady-state reaction step. The initial step depends on the existence and kinetics of the Maillard and Strecker reactions and pyridine and pyrazine free radical formations. Stabilization of the formed free radicals occurs in the steady-state step, giving pyridine and pyrazine derivatives. These compounds reacted with creatinine and formed amino-imidazoazarenes.

The proposed reaction mechanism of the formation of the amino-imidazoazarenes has been studied by Electron Spin Resonance (ESR), ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) spectroscopy and Mass spectrometry (MS).

INTRODUCTION

The elucidation of the mechanism of the reaction between aldoses and amino acids or proteins and the formation of flavour and off-flavour constituents and other organic compounds, such as heterocyclic amines, in cooked and fried protein foods, is one of the most important problems in food chemistry.

Namiki & Hayashi (1975) reported the first observation of free radicals formed by the reaction between carbonyl and amine compounds. Complete data about the formation (Milić & Piletić, 1984) and kinetics (Milić & Piletić, 1984; Milić, 1987) of pyrazine and pyridine free radicals were published recently.

Suyama & Adachi (1980) and Milić & Piletić (1984) reported that many pyrroles, pyrazines and pyridines were formed in the reactions of aldehydes, ketones, unsaturated carbonyl and dicarbonyl compounds with ammonia, amines and amino acids. A large and very important group of flavour constituents of foods consists of pyrazine and pyridine derivatives, but there is still no evidence that pyrazine and pyridine derivatives are precursors in the formation of amino-imidazoazarenes which are very potent mutagenic substances.

In an effort to clarify the mechanism of the formation of amino-imidazoazarenes in animal protein-rich foods under temperature treatment, the present paper examines the formation of pyrazine and pyridine free radicals as intermediates in the initial step, and pyrazine and pyridine derivatives in the steady-state reaction step between D(+)-glucose and 2-3,- and 4-aminobutyric acids, respectively. These final Maillard reaction products reacted with creatinine, which was present in the reaction mixtures, and gave different heterocyclic amines—the new class of mutagenic substances—amino-imidazoazarenes (Jagerstad *et al.*, 1986).

MATERIALS AND METHODS

All chemicals used in these experiments were of analytical purity.

Model systems for ESR determination on pyrazine cation free radicals were obtained by mixing equimolar amounts of D(+)-glucose and 2-, 3- and 4-aminobutyric acids separately in the water solution of 0·01 M NaOH at pH 9·0. The final concentrations of the reactants were 1·0 M.

The model systems for ESR determination of pyridine free radicals were obtained from D(+)-glucose and 2-, 3-, and 4-aminobutyric acids and 2,3-diamino-1,4-naphthohydroquinone at pH 6·5. The final concentrations of glucose and amino acids were 1·0 M, and the concentration of diaminonaphthohydroquinone was 0·02 M.

All reaction mixtures for the free radical determination were heated to 95°C in the quartz cell of a Bruker 4121 VT-RS high temperature control system.

All the ESR spectra were recorded on the Bruker 300E electron spin resonance spectrometer set as follows: field sweep width, 100 g; receiver gain, 6·30 × 10^4; time constant 1·28 ms; modulation amplitude,

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1·021 g; microwave power, 6·26 mW; centre-field, 3440 g scan time 1·8 × 10 s; modulation frequency, 100 kHz; microwave frequency, 9·540 GHz; and temperature, 25°C, for free radical determination. The g-values were determined by means of the Bruker double cavity TE 104 with PPADS (potassium peroxylamine disulfonate: g = 2·0036 ± 0·0002) as a standard. The isotropic hyperfine splitting constants of ESR spectra were determined with the Bruker Data System ES 1600 in relation to the isotropic splitting constant of the Mn^{2+} ion (I = 5/2), with a_{Mn} = 51·23 g, which were brought by ZnS as an impurity.

The reaction mixtures of the creatinine, 2,5-dimethylpyrazine or 2-methylpyridine with acetaldehyde (1:2:2) were heated in diethylene glycol containing 11% water for 3 h at pH 6·0 and temperature 130°C. The obtained reaction mixtures were extracted with 1-butanol and the extracts were purified on a column of Dowex 50 H^+-form by passing 0·1 M ethanolic ammonia. The eluates from the resin were evaporated to dryness and then dissolved in a small amount of methanol and rechromatographed on a YMC A-300 and Sep-Pak columns by HPLC. High-performance liquid chromatography was performed using a Waters Associates instrument under the following conditions: first column, YMC A-300 (240 × 5·2 mm i.d.); second column, Sep-Pak (120 × 4·8 mm i.d.) 5 μm particle size, mobile phase, methanol/H_3PO_4 0·01 M, NaOH 0·01 M pH 7·4 (1 : 1·2); flow rate, 0·8 ml/min at 25°C. The eluates were evaporated to dryness at 50°C and each fraction was analysed.

The ^1H NMR spectra of synthesized amino-imidazoazarenes in $CDCl_3$ were determined on the Bruker WP-200 SY spectrometer with tetramethylsilane, as an internal standard.

The MS spectra of synthesized compounds were recorded on the Varian mass spectrometer which was set as follows: ionization voltage, 70 eV; ion source temperature, 200°C; ion acceleration voltage, 3100 V; and emission current, 80 μA.

RESULTS AND DISCUSSION

The ESR spectra, shown in Figures 1(a) & (b) for the D(+)-glucose and 2- and 3-aminobutyric acids with the splitting constants: a^N = 8·73, g, for the quintet due to two equivalent N-atoms (I = 1); a^H = 2·81 g, for the quintet of the isotropic splitting constant for four equivalent H-atoms (I = 1/2); and for D(+)-glucose and 4-aminobutyric acid (Fig. 1(c)), with a^N = 8·23 g, a^H = 2·81 g and a^H = 5·44 g, indicated the presence of 1,4-dialkylpyrazine cation radicals in the reaction mixtures if the reactions were done at 95°C and pH 9·0. The splitting constant a^H = 5·44 g for the radicals, obtained from the D(+)-glucose-4-aminobutyric acid model system, indicated that the two α-H atoms of the alkyl group, linked to the N-atom of the pyrazine ring, possessed a stereo arrangement which enabled magnetic interaction with the π-orbital of an unpaired electron.

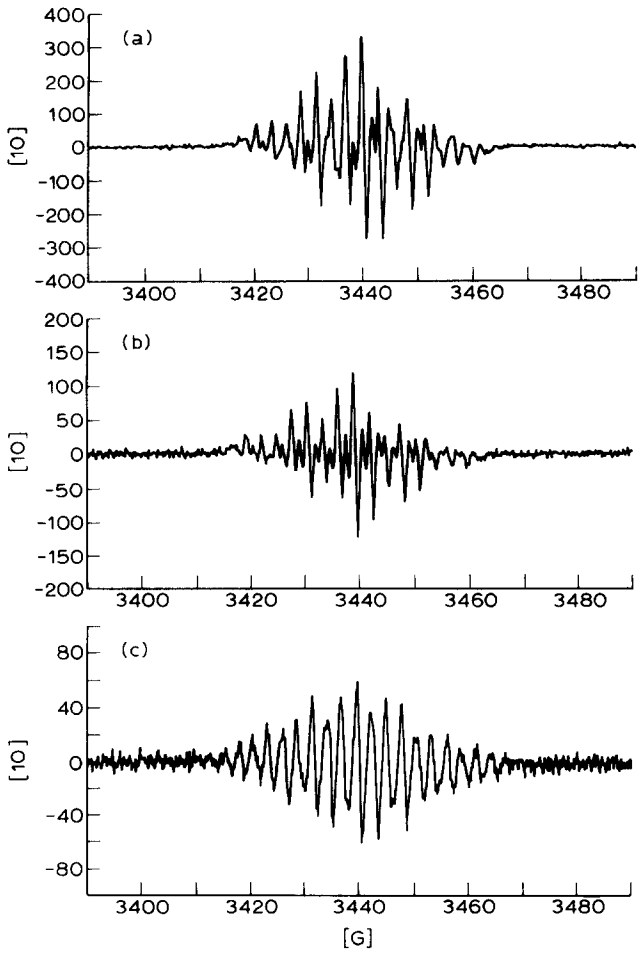


Fig. 1. ESR spectra of pyrazine cation radicals obtained by heating the reaction mixtures of D(+)-glucose and 2-,3- and 4-aminobutyric acids, (a), (b) & (c), at 95°C and pH 9·0.

If the reaction was performed under the same conditions, but in the presence of 2,3-diamino-1,4-naphthohydroquinone in a slightly acidic solution (pH 6·5), the ESR spectra obtained with splitting constants (a^N = 5·44 g and a^H = 0·34 g) indicated the presence of the 2,6-dialkylpyridine cation radical (Fig. 2), and these were similar in all examined cases.

Based on the hyperfine splitting constants of the ESR spectra, it is possible to conclude that the free

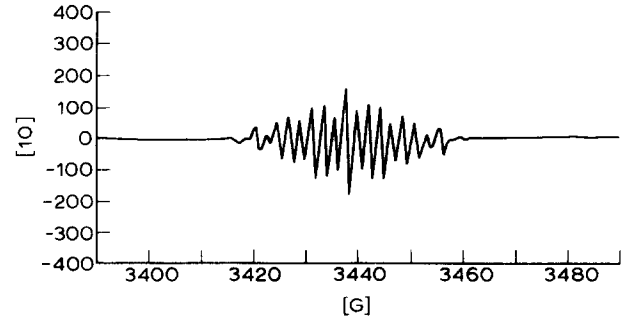
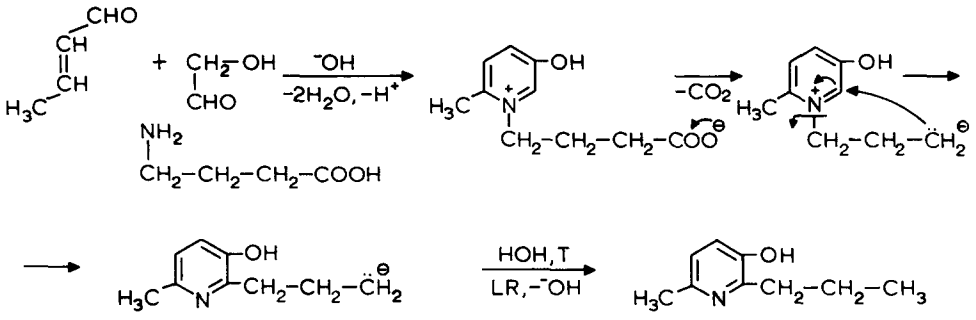


Fig. 2. ESR spectrum of pyridine cation radical obtained by heating the reaction mixtures of D(+)-glucose and 2-, 3- and 4-aminobutyric acids at 95°C and pH 6·5 in the presence of the 2,3-diamino-1,4-naphthohydroquinone.



Scheme I. The formation of the 2-methyl-5-hydroxy-6-propylpyridine.

pyrazine and pyridine radicals were formed in the initial step ($0{\cdot}6 \times 10$ s) of the reaction between D(+)-glucose and amino acids. At the beginning, this stage included the nucleophilic attack of the amino group of the amino acid on the electrophilic C-atom of the carbonyl group of the aldose, and then the Maillard reaction, Amadori rearrangement and Strecker degradation of the aldose and amino acid moiety, when ketosylamines were formed. The ketosylamines are assumed to be very important precursors for the further cyclisation and formation of the pyrazine cation radicals and pyrazine derivatives, respectively (Milić & Piletić, 1984; Milić & Djilas, 1989).

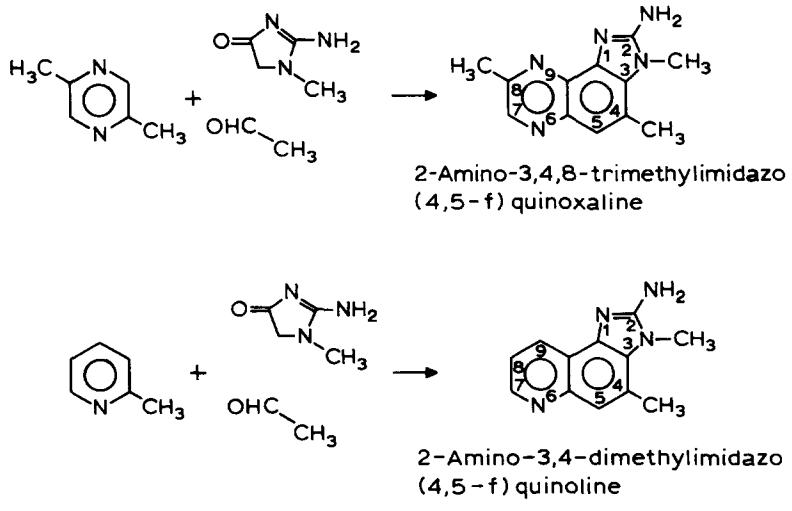
On the other hand (Milić & Piletić, 1984; Milić & Djilas, 1989), the sugar in the reaction mixture at pH 9·0 underwent alkaline dehydration to provide 1,2-enolic products, glycolaldehyde, glyceraldehyde, lactic acid and other diketo- and glucosone derivatives. The dicarbonyl compounds, formed from sugar, react readily with amino acids, and at the same time undergo Strecker degradation yielding aminoketo derivatives. These intermediates lose one molecule of water and react with another molecule of the same structure by cyclisation reaction to give dialyl- or tetraalyl-5,6-dihydropyrazines.

The assumed mechanism of pyridine cation radical formation is based mainly on the fact that, in the reaction mixtures of aldoses and amino acids at pH 6·5, in the presence of 2,3-diamino-1,4-naphthohydroquinone, D(+)-glucose gives rise to carbonyl compounds by the transformation and fragmentation of the open aldehyde form. Crotonal and glyoxal, which are easily formed (Suyama & Adachi, 1980), react with amino acid molecules by condensation reaction to give 1-(3'-carboxypropyl)-2-methyl-5-hydroxypyridine cation radicals. The obtained cation radicals are stabilized by decarboxylation, and by Ledenburg rearrangement to give 2-methyl-5-hydroxy-6-propylpyridine, according to the proposed mechanism presented in Scheme I.

The proposed mechanisms of pyrazine and pyridine derivative formations are not the only ones, and are still speculative because of the complexity of the reaction, even in very simple model systems.

These compounds, pyrazine and pyridine derivatives, are the precursors, and in the reaction with creatinine they form different amino-imidazoazarenes.

In establishing the reaction pathway, which would correspond to the real reaction, the syntheses of amino-imidazoazarenes were performed by direct reaction between pyrazine and pyridine derivatives, e.g., 2,5-dimethylpyrazine or 2-methyl-pyridine with creatinine and acetaldehyde. These changes, which really started from aldose, amino acid and creatinine, were unavoidable because of the reaction complexity. Judging from the HPLC retention times and peak areas of the chromatographed compounds from the reaction mixtures, the first fraction contained almost pure 2-amino-3,4,8-trimethylimidazo(4,5-f)quinoxaline (yield 77·6%) while



Scheme II. Proposed reaction pathway of imidazoquinoline and imidazoquinoxaline formations.

Table 1. ^{1}H NMR and MS spectra characteristics of synthesized and commercial (A) 2-amino-3,4-dimethyl-imidazo(4,5-f)quinoline, and (B) 2-amino-3,4,8-trimethylimidazo(4,5-f)quinoxaline

Compounds	Spectra data	
	^{1}H NMR (δ)	MS (ppm)
A. Synthesized	1·63 (H_2O), 2·83(d—Me), 3·91 (e—Me) 4·80 (f—NH_2), 7·42(c—H), 8·65 (b—H) and 9·30 (a—H)	211—M^+, 198, 183, 170, 156 142, 129, 114, 99, 86, 72, 58, 54 and 44.
A. Commercial	1·60 (H_2O), 2·82(d—Me), 3·87(e—Me) 4·82(f—NH_2), 7·40(c—H), 8·66(b—H) and 9·35 (a—H)	211—M^+, 198, 180, 170, 155, 142, 130, 115, 100, 86, 74, 59, 54 and 45.
B. Synthesized	1·60 (H_2O), 2·75(a—Me), 2·82(d—Me) 3·94(e—Me), 4·80(f—NH_2), 7·40 (c—H) and 8·68(b—H)	226—M^+, 212, 199, 185, 173 159, 144, 131, 117, 104, 90, 85, 76, 69, 63 and 52.
B. Commercial	1·60 (H_2O), 2·77 (a—Me), 2·80 (d—Me), 3·92(e—Me), 4·81(f—NH_2), 7·44(c—H) and 9·00(b—H)	226—M^+, 212, 199, 185, 173, 160, 147, 130, 116, 104, 91, 86, 76, 69, 65 and 53.

NB: Commercial compounds of analyzed imidazoquinoline and imidazoquinoxaline were obtained from Department of Nutrition and Food Sciences, Ochanomizu University Tokyo, Japan.

the second one contained 2-amino-3,4-dimethylimidazo-(4,5-f)quinoline (yield 81·5%) with minor impurity. The proposed reaction pathway (Scheme II) was confirmed on the basis of the results of ^{1}H NMR and MS spectra of the synthesized and commercial compounds which are listed in Table 1.

The ^{1}H NMR spectra of synthesized compounds were in good agreement with the results obtained from analyses of both commercial compounds. The ^{1}H NMR spectrum of 2-amino-3,4-dimethyl-imidazo-(4,5-f)quinoline showed the singlets at 2·83, 3·91 and 4·80 ppm which corresponded to d–CH_3, e–CH_3, and f–NH_2 protons. The quartet corresponding to signals c–H, b–H and a–H at 7·42, 8·65 and 9·30 ppm was also detected, although with minor intensity. The ^{1}H NMR spectrum of 2-amino-3,4,8-trimethylimidazo (4,5-f)quinoxaline showed similarity to the above results, but with the signals at 2·75 ppm, which corresponded to a–CH_3 protons. Nonexistence of the signal at 9·30 ppm, which corresponded to an a–H proton, suggested that the methyl group was bound at this position (Nyhammer *et al.*, 1986).

The mass spectra confirmed the results of the NMR spectra. The masses of 212 and 227 corresponded to the proposed isolated compounds.

The validity of the proposed mechanisms of the amino-imidazoazarene formations should be checked by isotopic labelling experiments.

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