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Formation of the food associated carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in model systems

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Abstract

Food-related mutagens play a major role in human carcinogenesis. Heterocyclic aromatic amines which are formed in meat or fish during cooking contribute to food-related carcinogenesis, at least in rodents. The mechanism of formation of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) was clarified by using ^{13}C-labelled phenylalanine as a reaction partner in a model system containing additionally creatinine. Isolation of the labelled reaction product (PhIP) and ^{13}C-NMR experiments showed that the carbon atoms of phenylalanine form a part of the pyridine moiety. Carbon atoms C-5, C-6 and C-7 in PhIP originated quantitatively from phenylalanine, leading to the conclusion that PhIP is formed by a defined mechanism and that two phenylalanine molecules are needed to form PhIP in this model system. In the proposed mechanism phenylacetaldehyde plays a key role which undergoes an aldol condensation with creatinine. The six-membered pyridine ring is completed by formation of a Schiff's base and cyclisation. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: PhIP; Model system; Food associated mutagens; Heterocyclic amines

1. Introduction

2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) (Fig. 3) is a heterocyclic aromatic amine belonging to a class of mutagens found in foods. So far about 20 different heterocyclic aromatic amines have been isolated from cooked meat and fish products. Among these heterocyclic amines, PhIP often occurs in the highest concentration (Becher, Knize, Nes, & Felton, 1988; Felton & Knize, 1991; Skog, 1993; Wakabayashi, Nagao, Esumi, & Sugimura, 1992). PhIP was first isolated in 1984 by the group of Sugimura in a model system with creatinine, glucose and glycine (Negishi et al., 1984) and identified by the group of Felton in 1986 (Felton et al., 1986) in fried ground beef. Later studies have shown that PhIP may also be present in beer, wine, and cigarette smoke condensate (Manabe, Suzuki, Wada, & Ueki, 1993; Manabe, Tohyama, Wada, & Aramaki, 1991). It was designated a colon and mammary carcinogen and has been shown to be carcinogenic to experimental animals (Esumi, Ohgaki, Kohzen, Takayama, & Sugimura, 1989; Ito et al., 1991).

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The formation of the food carcinogens was shown in model systems using creatine/ine carbohydrates and different amino acids as reactants (Jägerstad, Skog, Grivas, & Olsson, 1991). In such heated model systems, using a mixture of creatine/creatinine and phenylalanine with or without sugar, the formation of PhIP can be observed (Manabe, Kurihara, Wada, Tohyama, & Aramaki, 1992; Shioya, Wakabayashi, Sato, Nagao, & Sugimura, 1987). The model systems used for studying this reaction are normally a mixture of phenylalanine with creatinine and occasionally carbohydrates. These substances are heated to 150 to 200°C as solutions in ethylene glycol or as dry substances (Felton & Knize, 1990; Manabe et al.; Övervik, Kleman, Berg, & Gustafsson, 1989; Shioya et al.; Skog & Jägerstad, 1991). Addition of glucose increases the yield of PhIP (Skog & Jägerstad, 1991) but also results in a substantially more complex mixture of products with many polymeric substances from the Maillard reaction.

In this study the investigation of the mechanism of the formation of PhIP was the primary aim. In a first step the model system was optimized since about 1 mg of PhIP was needed for NMR analysis. The optimization was

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done only with respect to reaction temperature and molar ratio of phenylalanine to creatinine. The incorporation of the carbon atoms during the formation of PhIP was traced by using ^{13}C-labelled phenylalanine. Three different reactions were carried out using phenylalanine labelled at positions 1, 2 and 3.

2. Materials and methods

All chemicals used were of analytical quality purchased from Merck (Darmstadt, Germany). PhIP was purchased from Toronto Research Chemicals (Toronto, Canada). The solvents used for HPLC were of gradient grade and purchased from Promochem (Wesel, Germany). Blue cotton was self-made with Remazol Turquoise Blue G 133 (EE AF 505), a gift from Hoechst (Vienna, Austria).

2.1. Model system

The reaction was done by refluxing the reaction mixture in a round-bottom flask (0.2 g phenylalanine, 0.13 g creatinine, 25 ml diethylene glycol) with controlled temperature for 2 h. For HPLC analysis, the clean-up was done by dilution of the diethyleneglycol phase with 0.1 M HCl to 150 ml and extraction with dichloromethane. The pH was set to below 2 by addition of 5 M HCl. After removal of apolar reaction products, the pH was set to 9 with 5 M NaOH and PhIP was extracted from the aqueous phase three times with dichloromethane. After evaporation, the residue was dissolved in 2 ml of methanol and diluted with water. For further purification of the sample, a solid phase extraction was done with 'blue cotton' which is a copper phthalocyanin complex covalently bound to cotton. The column was loaded with the aqueous phase, washed with water, and the PhIP eluted with $MeOH/NH_3$ (50/1). The eluent was evaporated and the residue dissolved in methanol for HPLC analysis.

2.2. HPLC analysis

For HPLC analyses of the extracts, 10 μl of the sample were injected onto a reversed phase column. The HPLC equipment used was a Merck/Hitachi (LaChrom) liquid chromatograph with a diode array detector (L 7450) and a fluorescence detector (L 7480), equipped with a LiChrospher RP Select B (5 μm, 250 × 4 mm) and a precolumn LiChroCART 4-4 (LiChrospher RP18 ec, 5 μm). The column was eluted with a mobile phase of methanol/acetonitrile/acetic acid/water (15/25/2/58) at pH 5.1. The flow rate was set to 1.0 ml/min and the effluent was monitored at $\lambda = 320$ nm and the fluorescence at an excitation wavelength, λ_{ex}, of 316 nm, and an emission wavelength, λ_{em}, of 370 nm.

2.3. Isolation of ^{13}C-labelled reaction products

The isolation procedure used for preparative isolation of the labelled PhIP was the same as for quantitative analysis. After solid phase extraction with blue cotton a preparative HPLC separation was done. For preparative HPLC a LiChrospher RP Select B (5 μm, 250 × 8 mm) was used with an injection volume of 700 μl. The UV-absorption was monitored at 320 nm and the peak, at a retention time according to the reference material, was isolated. The collected peak was diluted with 0.1 M NaOH and extracted three times with dichloromethane. After evaporation of the solvent, the residue was used for NMR analysis.

2.4. Identification and quantification

PhIP was identified by comparing the retention time, UV spectra, and fluorescence signal with that of an authentic standard (Murkovic, Friedrich, & Pfannhauser, in press). The quantification was based on an external standard method where the calibration curves were determined daily. All standard solutions were prepared daily from a stock solution which was stored in the dark at −20°C.

2.5. NMR analysis

2.5.1. ^{13}C-Labelled products

^{13}C-NMR spectra were measured on a Bruker MSL 300 at 75.47 MHz. The products obtained after HPLC purification (about 1 mg) were dissolved in d_6-DMSO. 20000 scans were accumulated for each experiment to obtain a sufficient S/N-ratio.

2.5.2. Assignment of ^{13}C-signals

For these experiments a commercially obtained PhIP without ^{13}C-enrichment was used. INEPT (insensitive nuclei enhanced polarization transfer) with a selective excitation soft pulse (Sarkar & Bax, 1985) was done on a Gemini 200 at 50.289 MHz. ^{13}C-satellites of signals of 8.30, 7.74, 7.45 and 7.34 ppm in the ^1H-spectrum were irradiated with a DANTE (delays alternating with nutation for tailored excitation) generated soft pulse (Width 10 Hz, duration 50 ms), JNXH 8.0 Hz, Waltz-Decoupling and accumulation of 5000 scans.

3. Results

The optimization of the reaction was necessary to obtain enough of the food carcinogen PhIP which is a product of a side reaction in this model system and thus is obtained in only very low concentrations. As can be seen in Fig. 1, the highest amount of PhIP is formed at 200°C. Fig. 2 shows that the molar ratio of the two

compounds has to be around 1 for optimal product formation. The normal reaction time was 2 h. Immediate clean up was necessary since PhIP disappeared completely when the reaction mixture was left overnight at room temperature. The disappearance of PhIP in the reaction solution could be due to its binding to melanoidins that are formed during the reaction. On the other hand, in this reaction mixture a lot of radicals and other reactive substances are present that could react with PhIP.

In order to find the positions of incorporation of the phenylalanine framework into PhIP, studies, using specifically labelled phenylalanine with ^{13}C in positions C-1, C-2, C-3, were undertaken. The products of these reactions were isolated and their ^{13}C-spectra measured, showing a largely increased carbon-signal for those carbons with specific labeling. The results are collated in Table 1.

Comparing the results with published data, it appeared that the results were highly improbable. Regarding this we undertook a thorough investigation using INEPT and selective excitation. This showed that the published ^{13}C-NMR data were incorrectly assigned. The result of our interpretation is as follows:

$$\text{NMR} - \text{spectrum of PhIP}: 28.45\ (\text{Me}-\text{C}), 112.06\ (\text{C}-7), 129.19\ (1\text{s}, \text{C}-2', \text{C}-6'), 128.23\ (1\text{s}, \text{C}-4'), 126.6\ (1\text{s}, \text{C}-6), 126.94\ (1\text{s}, \text{C}-3', \text{C}-5'), 139.68\ (1\text{s}, \text{C}-5), 156.7\ (1\text{s}, \text{C}-2), 158.36\ (1\text{s}, \text{C}-9), 139.44\ (1\text{s}, \text{C}-1', \text{C}-8)\ (\text{Fig.3}).$$

Since phenylacetaldehyde was found to be a thermal degradation product of phenylalanine (data not shown) the model system was modified. Phenylacetaldehyde was mixed with creatinine and heated for 15 min. After removal of unreacted creatinine from the reaction mixture, an amino acid was added and the reaction continued for 45 min. The results are shown in Fig. 4.

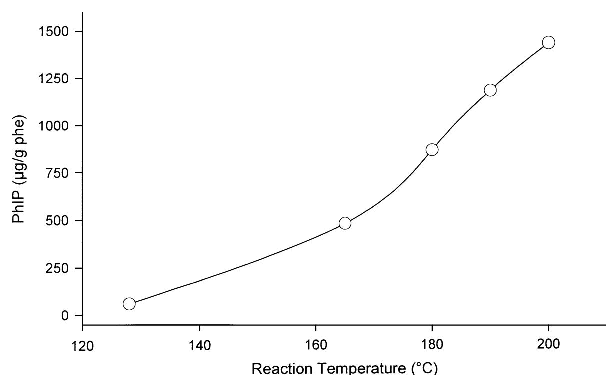

Fig. 1. Formation of PhIP in relation to reaction temperature. The reaction time was 2 h.

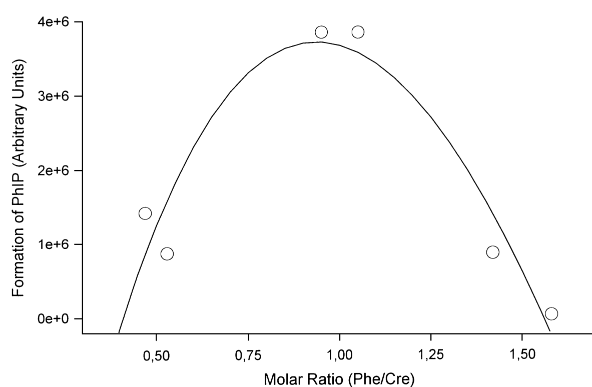

Fig. 2. Formation of PhIP in relation to molar ratio of phenylalanine to creatinine. The reaction time was 2 h.

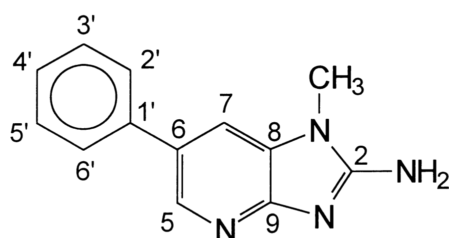

Fig. 3. Molecular structure and used carbon numbering of PhIP.

Table 1
Results of ^{13}C-NMR-measurements

	Measured peaks	
Labelling at C-1	–	No incorporation of ^{13}C was detected, probably due to very fast decarboxylation of the intermediate product and loss of $^{13}CO_2$.
Labelling at C-2	139.68 113.06	Increased ^{13}C-signals could be found for C-5 and C-7 in PhIP, suggesting two different possibilities for the formation of the six-membered pyridine ring.
Labelling at C-3	126.60	Increased ^{13}C-signals could be found for C-6 in PhIP.

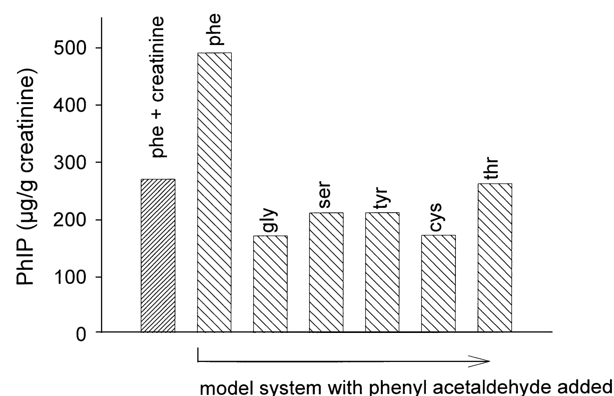

Fig. 4. Modified model system with phenyl acetaldehyde as additional reaction partner.

4. Discussion

The optimization of the reaction was necessary for producing enough PhIP to be used for the ^{13}C-NMR experiments. The optimal conditions (2 h reaction time, 200°C, molar ratio of phenylalanine to creatinine of 1) yielded about 500 μg PhIP which was sufficient for ^{13}C-NMR.

The results of the experiments suggest that PhIP is formed with a defined reaction mechanism. In contrast to results of other groups (Felton et al., 1989; Eriksson, Lov, & Olsson, 1989) it is shown in this work that C-7 is formed from a defined carbon atom having its origin in phenylalanine (Fig. 5). At first glance the result from this experiment was quite puzzling, since the ^{13}C, according to the interpretation of the published ^{13}C-spectrum, would have ended up in position C-4′ being a part of the phenyl ring (Knize & Felton, 1986).

In contrast to the results of Felton and coworkers (Felton et al., 1989) our experiments showed that no ^{13}C-labelled PhIP is found when the carboxyl carbon of phenylalanine is labelled. This suggests that the carbon dioxide resulting from decarboxylation of phenylalanine does not further contribute to the formation of PhIP.

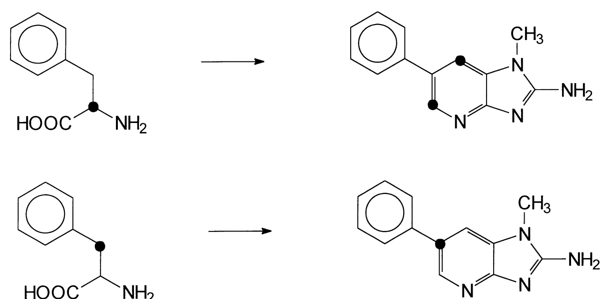

Fig. 5. Reaction scheme obtained from NMR experiments using ^{13}C-labelled phenylalanine as reaction partner (black circles indicate labeled carbon atoms).

When part of the phenylalanine in the model system was replaced by phenylacetaldehyde, PhIP was still found in high amounts. Phenylacetaldehyde is a Strecker degradation product of phenylalanine and is thus a common intermediate of Maillard browning reactions in foods. The isolation of the postulated intermediate of the aldol condensation was necessary to exclude the possibility that phenylalanine could react with creatinine and form PhIP by another reaction. Phenylacetaldehyde was shown to be a thermal degradation product of phenylalanine by GC/MS and HPLC with pre-column derivatisation with dinitro phenylhydrazine (DNPH). In this reaction, using phenylacetaldehyde in the first step, phenylalanine could be replaced by any other amino acid (Fig. 4). In this case the formation of PhIP was doubled in comparison to the reaction of phenylalanine with creatinine. Using other amino acids the yield of PhIP was about the same as in the reaction of phenylalanine without phenylacetaldehyde. Even when using ammonia instead of amino acids, a significant amount of PhIP was formed.

The mechanism suggested is as follows (Fig. 6): the C-5 of creatinine reacts with phenyl acetaldehyde (2) in a nucleophilic addition and subsequent dehydration to form (6) as a possible intermediate. Phenyl acetaldehyde (2) itself is formed by pyrolytic degradation of phenylalanine (1). Dimerisation to (3) is a common reaction of phenylacetaldehyde. The intermediate (6) could also explain, why C-6 in PhIP is labelled when ^{13}C-3-phenylalanine is used

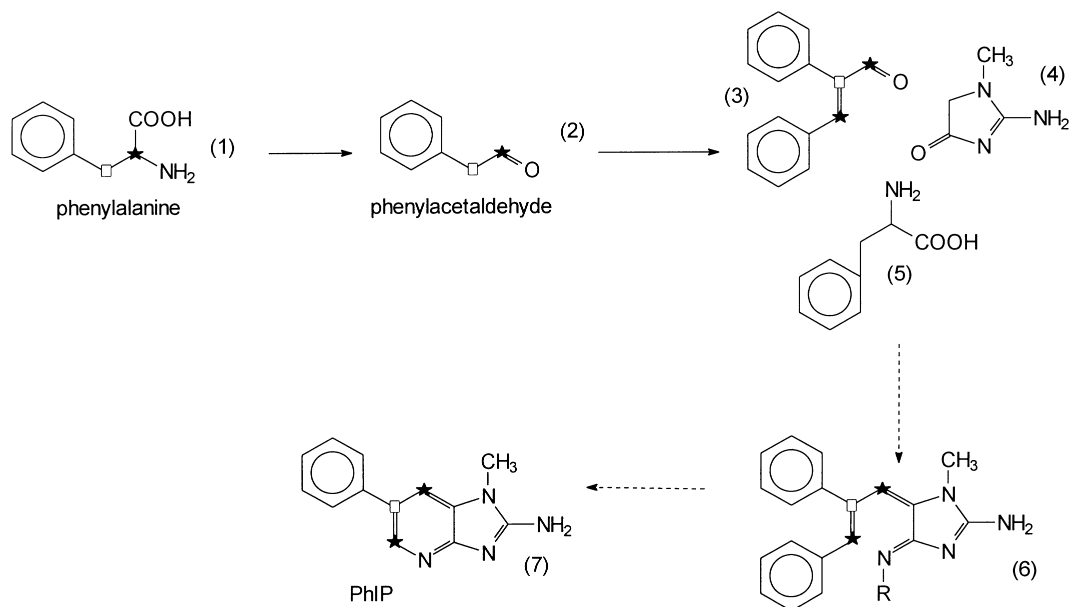

Fig. 6. Proposed mechanism of formation of PhIP in the model system. The marked carbon atoms correspond to the labelled positions in the experiments.

in the reaction. This would give (^{13}C-2)-2-phenyl-acetaldehyde and the corresponding dimer during thermal degradation of phenylalanine, resulting in the respective labelled PhIP. Phenyl-acetaldehyde (2) was shown to be a thermal degradation product of phenylalanine under these experimental conditions by headspace GC/MS (data not shown). The second reaction which would take place, is the formation of a Schiff's base by condensation of the amino group from phenylalanine with the oxygen from creatinine. This reaction would explain why position 5 is labelled when C-2-labelled phenylalanine is used as reactant.

Further work is currently being done to influence the reaction that leads to the formation of PhIP. Optimization that means reduction of the formation of heterocyclic amines in model systems and in meat is the primary aim of the continuing work.

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