

Metabolism of the Food-Associated Carcinogen 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine by Human Intestinal Microbiota

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2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine is a putative human carcinogenic heterocyclic aromatic amine formed from meat and fish during cooking. Although the formation of hazardous PhIP metabolites by mammalian enzymes is well-documented, nothing is known about the PhIP transformation potency of human intestinal bacteria. In this study, the *in vitro* metabolism of PhIP by human fecal samples was investigated. Following anaerobic incubation of PhIP with stools freshly collected from six healthy volunteers, we found that PhIP was extensively transformed by the human intestinal bacteria. HPLC analysis showed that the six human fecal microbiota transformed PhIP with efficiencies from 47 to 95% after 72 h incubation, resulting in one major derivative. ESI-MS/MS, HRMS, 1D (¹H, ¹³C, DEPT) and 2D (gCOSY, gTOCSY, gHMBC, gHSQC) NMR, and IC analysis elucidated the complete chemical identity of the microbial PhIP metabolite as 7-hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydropyrido[3',2':4,5]imidazo[1,2-*a*]pyrimidin-5-ium chloride. At present, no information is available about the biological activity of this newly discovered bacterial PhIP metabolite. Our findings however suggest that bacteria derived from the human intestine play a key role in the activation or detoxification of PhIP, a digestive fate ignored so far in risk assessments. Moreover, the variation in transformation efficiency between the human microbiota indicates interindividual differences in the ability to convert PhIP. This may predict individual susceptibility to carcinogenic risk from this suspected dietary carcinogen.

KEYWORDS: heterocyclic amine; meat; cooking; intestinal bacteria; HPLC; LC-MS; NMR

INTRODUCTION

Cooked muscle meats, major components of the Western diet, contain potent genotoxic carcinogens belonging to the heterocyclic aromatic amine class of chemical compounds (**Figure 1**) (*1*). Of the 19 heterocyclic amines identified, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is frequently the most mass abundant heterocyclic amine produced during the cooking of beef, pork, and chicken (2–7). The highest levels of PhIP (**1**) can be found in grilled or fried meats. In very well-done flame-grilled chicken, up to 400 ng/g PhIP (**1**) has been

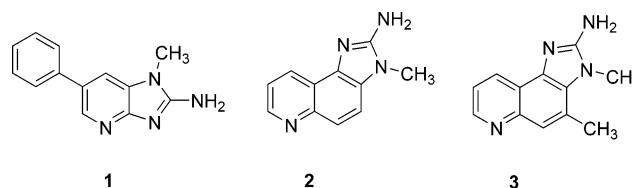


Figure 1. Chemical structures of heterocyclic aromatic amines.

measured (2). The human intake of PhIP (**1**) varies with food type and cooking conditions and is estimated to range from nanograms to tens of micrograms per day, depending on individual dietary and cooking preferences (4, 8). Assessment studies based on rodent tumor data (9–12) and the abundance of PhIP (**1**) in the diet have indicated that this heterocyclic amine may be a risk factor in human colon, breast, and prostate carcinogenesis (13, 14).

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As a means of determining the potential health risks associated with heterocyclic amines, several dietary studies have been conducted on the metabolism and disposition of these compounds in humans. So far, most investigations focused on the activation and detoxification of heterocyclic amines by mammalian enzymes. The genotoxic/carcinogenic effect of heterocyclic amines is closely related to a highly complex metabolism involving xenobiotic metabolizing enzymes generating very reactive metabolites as well as detoxified derivatives (15). On the other hand, the involvement of the intestinal microbiota in the digestive fate of heterocyclic amines remains underinvestigated (16). Recent research showed that the amount of PhIP (1) metabolites excreted in the 0–24 h urine represented $17 \pm 10\%$ of the ingested PhIP (1) in a meat matrix (17). In an earlier study with patients given PhIP (1) in a capsule, 90% of the ingested dose was recovered in the urine (18). This indicates that PhIP (1) provided in capsule form is more bioavailable than PhIP (1) ingested from meat. The nonbioavailable fraction reaches the colon intact to come there into contact with the resident microbiota. Direct binding of heterocyclic amines to the cell walls of intestinal bacteria has been reported and is currently considered as a detoxification mechanism, since it prevents absorption of heterocyclic amines through the intestinal mucosa (19, 20). On the other hand, results of 2-amino-3-methylimidazo[4,5-f]quinoline (IQ)-induced genotoxicity assays in germ-free and conventional rodents showed that the presence of intestinal microbiota is essential for the induction of DNA damage in the colon and liver cells (21, 22). These findings suggest that the intestinal microbiota play a significant role in the bioconversion of heterocyclic amines into harmful metabolites. Indications exist that hydrolysis of heterocyclic aminoglucuronides by bacterial β -glucuronidase may release mutagenic intermediates (23). Information on the bacterial metabolism of native heterocyclic amines is however still scarce. Several researchers report that incubation of the heterocyclic amine IQ (2) with mixed human feces under anaerobic conditions results in the formation of the hydroxy metabolite 7-OH-IQ (24–27). The bacterial metabolism of the heterocyclic amine PhIP (1) has to our knowledge not been investigated yet.

As the biological potency of PhIP (1)-induced carcinogenicity is strongly dependent upon its digestive fate, a comprehensive understanding of the metabolism, mammalian as well as microbial, of this putative carcinogen is essential for human risk assessment. Therefore, the focus of the present study was to investigate the role of the intestinal microbiota in the metabolism of PhIP (1). Interindividual differences occur with regard to the species composition and the metabolic activities of the human intestinal microbiota (28). Therefore, the bioconversion potential of fecal samples collected from different subjects was examined.

MATERIALS AND METHODS

Chemicals. PhIP (1) was purchased from Toronto Research Chemicals (Toronto, Canada). The constituents of the culture media, namely tryptone and yeast extract, were obtained from AppliChem (Darmstadt, Germany). All other chemicals were obtained from Sigma-Aldrich (Bornem, Belgium). The solvents for HPLC and LC-MS analysis were of HPLC grade and purchased from Acros Organics (Geel, Belgium).

Incubation Conditions. Collection and Preparation of Human Fecal Samples. Fecal samples were obtained from six healthy subjects (three males and three females) between the ages of 20 and 35. Donors were on a Western-type diet and none had a history of digestive pathology nor had received antibiotics during 3 months prior to sample delivery. Fecal slurries of 20% (w/v) fresh fecal inocula were prepared by homogenizing the feces with phosphate-buffered saline (0.1 M, pH 7),

containing 1 g/L sodium thioglycolate as reducing agent. The particulate material was removed by centrifugation for 2 min at 500g.

Incubation. All incubation experiments were performed in TY broth (30 g/L tryptone, 20 g/L yeast extract, 0.5 g/L cysteine, pH 7.0). Fecal bacteria require anaerobic conditions (low redox potential) for growth. Therefore, resazurin (2 mg/L) was added as a redox indicator. A pink color indicated a redox potential higher than -80 mV, a colorless solution showed a redox potential below this limit, i.e., anaerobic. The redox potential in the large intestine typically ranges between -150 and -280 mV (29). The medium was autoclaved at 121 °C for 15 min. Prior to addition to the autoclaved growth medium in the incubation vessels, PhIP (1) was dissolved in dimethyl sulfoxide (DMSO). The incubation volume was either 20 or 40 mL. Each batch culture consisted of 90% TY broth medium and 10% fecal inoculum in phosphate-buffered saline. The batch cultures were added with PhIP (1) dissolved in DMSO to give a final concentration of 1, 10, 100, or 1000 mg/L and less than 5% DMSO (v/v). Each batch was sealed with butylrubber tops and anaerobiosis was obtained by flushing the flasks with N_2 during 15 cycles of 2 min each at 800 mbar overpressure and 900 mbar underpressure. Cultures were incubated at 37 °C and 150 rpm for the duration of the experiment. Samples were taken at regular time intervals using syringes. All experiments were performed in triplicate.

To assess the extent of bacterial transformation, a number of control samples were included in the experimental setup. First, undosed fecal cultures were analyzed to serve as a negative control, as they presumably do not contain PhIP (1). Second, an undosed fecal culture was autoclaved for 20 min at 121 °C and added with PhIP (1) to ascertain that the disappearance of the substrate could be assigned to the metabolic activity of viable cells and not to passive adsorption on bacterial cell walls.

Chemical Analysis. Extraction Protocol. For HPLC and LC-MS analysis the PhIP (1) parent component and its metabolite were extracted from the digests (1 mL sample) by performing a solid-phase extraction using STRATA C18-U cartridges (Phenomenex, Belgium). After centrifugation for 10 min at $7000g$ at 4 °C, the resulting supernatant was loaded onto a 200-mg C18-U cartridge preconditioned with 3 mL each of acetonitrile, water, and ammonium acetate (0.1 mM, pH 3.5). A vacuum manifold and an evaporation manifold (Alltech, Lokeren, Belgium) were used for manipulations with SPE cartridges and solvent evaporation, respectively. The cartridge was washed with 3 mL of water and eluted with 3 mL ammonium acetate (0.1 mM, pH 3.5):acetonitrile (1:4) (v/v). The eluate obtained was dried under a N_2 stream, and the residue was reconstituted in 1 mL of ammonium acetate (0.1 mM, pH 3.5):acetonitrile (1:4) (v/v), transferred into HPLC vials, and stored at 4 °C until analysis. The recovery of PhIP (1) and its microbial metabolite using the latter protocol was determined in fecal digests at two concentrations, 1 and 100 mg/L, and gave recoveries of $95 \pm 1.3\%$ for PhIP (1) and its microbial metabolite. To further improve the recovery, DMSO (1.5%) was added to the ammonium acetate and acetonitrile mixture, since DMSO is a very good solvent for PhIP (1). Although the recovery was better than using the ammonium acetate and acetonitrile mixture (approximately 99%), the evaporation of DMSO was difficult and, therefore, unsuitable for larger sample volumes or greater numbers of samples.

For preparative separation and subsequent spectroscopic analysis, the PhIP (1) metabolites were extracted from the digests (40 mL sample) using a liquid/liquid extraction procedure. Prior to extraction, the pH of the samples was adjusted to 9–10 with 10 mL of 1 M Na_2CO_3 . After extraction into ethyl acetate (3×25 mL), the samples were centrifuged, and the combined organic phases were extracted with 3×25 mL 0.1 M HCl. PhIP (1) and metabolites were recovered from the acidic solution by addition of 12.5 mL of 1 M Na_2CO_3 and extraction with ethyl acetate (3×50 mL). After centrifugation and separation over a funnel to remove any remaining aqueous phases, the samples were taken to dryness at 50 °C by rotary evaporation.

Analytical HPLC. Samples were analyzed on a Dionex HPLC system (Sunnyvale, CA) comprising an autosampler ASI-100, a pump series P580, and a STH585 column oven, coupled to a UVD340S UV/VIS detector and a RF-2000 fluorescence detector. A $10 \mu L$ volume of the sample was injected and separated over a 150×4.6 mm i.d., $4 \mu m$

Genesis C18 column (Jones Chromatography). The temperature was set at 25 °C and the flow rate was maintained at 1 mL/min. Solvents were 0.01% formic acid (A) and acetonitrile (B). Solvent programming was isocratic 2% B during 2 min followed by a linear gradient to 40% B in 20 min. Absorbance was monitored at 315 nm; fluorescence was monitored at 316 nm (excitation) and 370 nm (emission). Data were collected and peaks integrated using the Chromeleon chromatography manager software (Dionex). Identification of PhIP (**1**) was based on the identity of the retention time and the absorption spectrum with those of an authentic standard (Research Chemicals Inc.), and quantification was achieved using a standard curve from 1 ng/mL to 100 µg/mL. The detection limit for quantification of PhIP (**1**) was 1 ng/mL for fluorescence and 1 µg/mL for absorbance detection, based on the criterion that the signal-to-noise ratio should be >3 for quantification purposes. Relative productions of the microbial PhIP (**1**) metabolite over time and between samples could be compared by integrating the peak areas. Quantification of the PhIP (**1**) metabolite was achieved using a standard curve obtained after preparative separation and purification of the metabolite.

LC-MSⁿ. The HPLC apparatus comprised a P4000 quaternary pump and an AS3000 autosampler (Thermo Finnigan, San Jose, CA). Chromatographic separation was achieved using a 150 × 3 mm i.d., 5 µm Zorbax SB-C3 column obtained from Agilent Technologies (Diegem, Belgium). The mobile phase consisted of a mixture of acetonitrile (A) and water with 0.01% formic acid (B). A linear gradient was run from 2% A for 2 min, increasing to 40% A over 20 min, maintaining 40% A for 8 min, and finally increasing to 100% A in the minute at a flow rate of 0.3 mL/min. The analysis was performed using a LCQ Deca ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with an electrospray ionization (ESI) interface. Both positive and negative ion modes were used, but only the positive ion mode allowed observing PhIP and metabolite peaks. To perform MS² and MS³, the precursor isolation width was set to 2 Da, the activation Q to 0.35, and the collision energy to 45%.

Preparative HPLC. Preparative separation was performed on a Gilson preparative HPLC system (Gilson International B. V., Middleton, WI) comprising a H322 pump system and a 206 fraction collector, coupled to a model 156 UV/vis detector. Chromatographic separation was achieved using an Omnisphere 250 × 21.4 mm i.d., 10 µm C18 column obtained from Varian (St.-Katelijne-Waver, Belgium). Compounds were eluted by an isocratic solvent mixture containing 85% water with 0.05% formic acid and 15% acetonitrile with 0.05% formic acid, the flow rate was 20 mL/min. Absorbance was monitored at 307 nm.

HRMS. High-resolution mass spectra (HRMS) were recorded on a Finnigan MAT 95 XP-API-GC-trap tandem mass spectrometer (Thermo Finnigan, Bremen, Germany). ESI-MS was performed in the positive mode under the following operating parameters: probe voltage, 3 kV; capillary temperature, 250 °C. The mobile phase consisted of a mixture of acetonitrile and water with 0.1% formic acid (50:50) (v/v) at a flow rate of 50 µL/min. PEG 200/300 (2.5 ng/µL sample) was used as internal standard.

NMR Analysis. NMR spectra were recorded at 298.1 K using a Varian Mercury 300 spectrometer equipped with a 5 mm PFG-probe, observing ¹H at 300.0 and ¹³C at 75.4 MHz. The compound was dissolved in 1 mL of DMSO-*d*₆ and transferred to a 5-mm NMR tube. All chemical shifts are expressed in ppm relative to TMS for ¹H spectra (δ 0 ppm) and DMSO-*d*₆ for ¹³C spectra (δ 39.52 ppm). The ¹H NMR spectra were acquired using 128 transients, with spectral widths of 4803.1 Hz and digitized with 32 K data points. For ¹³C NMR spectra 12 000 transients were recorded, and a spectral width of 18 867.9 Hz digitized with 128 K points was used. Relaxation delays were set to 1 s, and a 45° excitation pulse was used. DEPT-45°, DEPT-135°, DEPT-90° experiments were performed to distinguish methyl, methylene, methine, and quaternary carbon resonances. For ¹³C NMR spectra a line broadening of 1 Hz was applied during processing. Gradient-enhanced ¹H-¹H COSY and TOCSY correlation experiments were performed through standard pulse sequences, as suggested by the manufacturer. The gCOSY was performed using a spectral width of 4.8 kHz and 2K data points with eight transients for each of the 200 *t*₁ increments. The gTOCSY was performed using a spectral width of

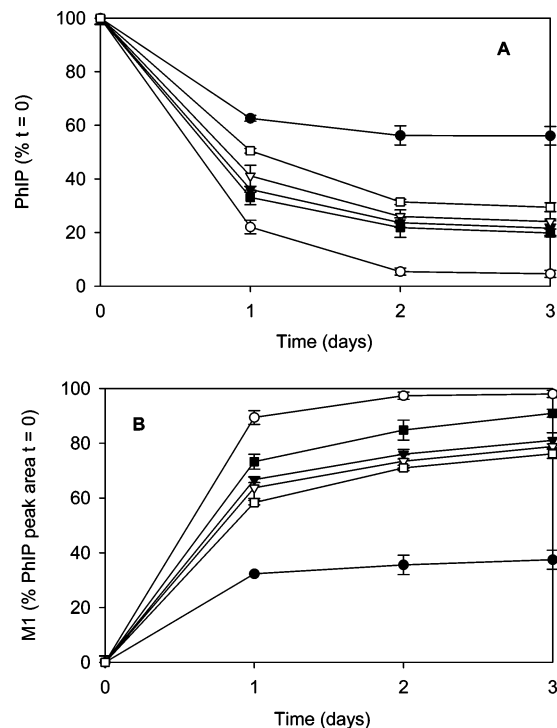


Figure 2. PhIP (**1**) degradation (**A**) and formation of its microbial metabolite **M1** (**B**) in cell suspensions derived from six human stools (▼, □, ▽, ●, ○, ■). PhIP (**1**) initial concentration was 1 mg/L. PhIP (**1**) and metabolite concentrations were determined by HPLC analysis and presented as average (±SD) percentage of the PhIP (**1**) peak area at day 0 (*n* = 3).

4.8 kHz, 2K data points, and a mixing time of 80 ms with 32 transients for each of the 256 *t*₁ increments. Data were multiplied by a sine bell function in both dimensions and transformed into the frequency domain as a 2048 × 2048 data matrix. The one-bond ¹H, ¹³C correlation experiments were acquired using the manufactures gradient HSQC pulse program with spectral width of 4.8 kHz in *f*₂ and 12.8 kHz in *f*₁ (32 transients, 2K data points, and 512 *t*₁ increments). Data were multiplied by a Gaussian function in both dimensions and transformed into the frequency domain as an 8192 × 2048 data matrix. The long-range ¹H-¹³C correlation experiments were recorded using the manufactures gradient HMBG pulse sequence with spectral width of 4.8 kHz in *f*₂ and 18.1 kHz in *f*₁ (32 transients, 2K data points, and 512 *t*₁ increments), and an evolution delay of 62.5 ms (*J*(C,H) = 8 Hz). Data were multiplied by a sine bell function in both dimensions and transformed into the frequency domain as a 2048 × 2048 data matrix.

IC Analysis. The anionic counterpart of the microbial PhIP (**1**) metabolite was determined using a Metrohm 761 compact ion chromatograph (Herisau, Switzerland) equipped with a conductivity detector. The operational parameters were as follows: column, Metrosep A supp 5; eluent, 1.06 g/L Na₂CO₃; flow, 0.7 mL/min; sample loop, 20 µL.

RESULTS

Microbial Conversion of PhIP by Human Feces. Incubation of PhIP with Human Fecal Samples. The capacity of the microbial cultures obtained from six human stool samples to transform the food carcinogen PhIP was tested by incubating the cultures with 1 mg/L PhIP (**1**) for a period of 3 days (**Figure 2**). All six human feces transformed PhIP (**1**), though with different efficiencies. Indeed, the fraction of PhIP (**1**) degraded over 72 h ranged from 47 to 95% of the initial quantity for the low- and high-degrading microbiota, respectively. The formation of one metabolite (further referred to as **M1**) accompanied PhIP (**1**) degradation in each fecal incubation experiment (**Figure 3**). This metabolite peak was not observed upon incubation of

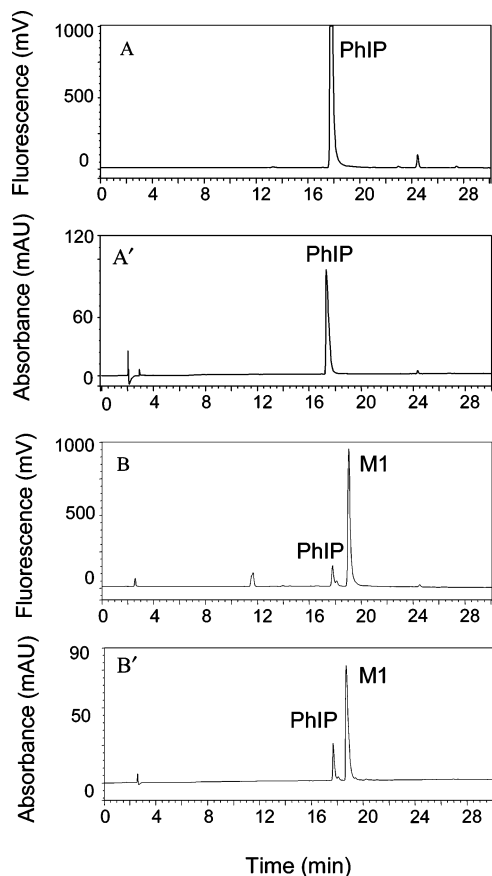


Figure 3. HPLC chromatograms with fluorescence (A, B) and absorbance (A', B') detection of PhIP (1) and its metabolite M1 produced by human intestinal microbiota. (A, A') Standard: 10 and 500 ng of PhIP (1). (B, B') Metabolic products of PhIP (1) incubated with human intestinal microbiota for 3 days. Initial incubation concentration was 10 mg/L PhIP (1).

undosed fecal cultures, confirming its PhIP (1) origin. Interindividual differences between the kinetics of metabolite formation paralleled those between the kinetics of PhIP (1) transformation. This resulted in a time-dependent increase of 55–98% of the metabolite peak area relative to the initial PhIP (1) peak area at day 0. Upon incubation of PhIP (1) with fecal material that was inactivated prior to incubation, no decrease in PhIP (1) concentration or metabolite formation was observed.

Characterization of the PhIP Metabolism by Human Fecal Cultures. The data obtained from Figure 2 showed that the capacity of the human microbiota to transform PhIP (1) varied with the origin of the fecal sample. Yet the majority of the fecal microbiota belonged to the intermediate-degrading category. Therefore, further investigation of the PhIP (1) transformation was performed with an intermediate degrading fecal culture. To thoroughly screen for microbial PhIP (1) metabolite production, a 12-h experiment was performed during which unprocessed incubation medium was sampled every hour and analyzed by HPLC with fluorescence and UV detection (Figure 4). This approach allowed the formation of solely one transformation product to be observed. The increase in concentration of this metabolite paralleled the decrease in PhIP (1) concentration in a time-dependent manner. Subsequent experiments were conducted using five different incubation concentrations of PhIP (1) ranging from 1 to 1000 mg/L for a period of 3 days. Again only one PhIP (1) metabolite could be observed, and the transformation occurred with a conversion efficiency of $80 \pm 2\%$, regardless of the initial concentration of PhIP (1).

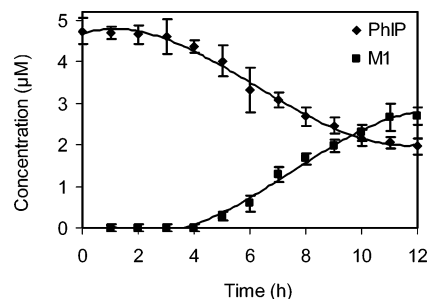


Figure 4. Kinetics of PhIP (1) transformation and metabolite formation in cell suspensions derived from human feces. Results are presented as average (\pm SD) concentrations of PhIP and the microbial PhIP metabolite ($n = 3$).

Chemical Identification of Microbial PhIP Metabolite.

HPLC Analysis of Human Feces Incubated with PhIP. When PhIP (1) was incubated with microbial cultures derived from human feces, one microbial PhIP (1) metabolite could be observed by HPLC with fluorescence (Figure 3B) and absorbance (Figure 3B') detection. The elution profile of the metabolic products included PhIP (1) at 17.77 min and the PhIP (1) metabolite M1 at 19.06. These products showed distinct absorbance maxima [PhIP (1) (204, 227, and 316 nm), M1 (205, 228, and 307 nm)] and fluorescence excitation maxima [PhIP (1) (316 nm), M1 (312 nm)].

MSⁿ Analysis of Human Feces Incubated with PhIP. In evaluating the chemical structure of the microbial PhIP (1) metabolite, the LC–MSⁿ mass spectra in ESI positive ion mode of a 3-day incubation extract were recorded. In MS-full scan, the pseudomolecular ions with m/z 225 and 281 appeared at the respective retention times 17.77 and 19.06 min. MS²-full scan of the pseudomolecular ion m/z 225 showed the product ion with m/z 210. Fragmentation of this product ion gave rise to fragments at m/z 183 and 168. MS²-full scan of the pseudomolecular ion m/z 281 showed the product ions with m/z 263 and 225. Fragmentation of the most mass abundant product ion m/z 263 derived from M1 showed fragments at m/z 248, 236, 222, and 210.

HRMS Analysis. The exact molecular formula of the PhIP (1) metabolite M1 was determined by recording the high-resolution mass spectrum of a sample containing $5 \mu\text{g}/\mu\text{L}$ of M1, purified by preparative HPLC. A mass was measured of 281.1398 corresponding with the theoretical mass of 281.139 69 and molecular formula of $\text{C}_{16}\text{H}_{17}\text{N}_4\text{O}$.

NMR Analysis. Sufficient quantities of the PhIP (1) metabolite M1 for NMR analysis were obtained by incubating 20 mg of PhIP (1) in 40-mL batch culture for 5 days. Purification of the PhIP (1) metabolite extract was achieved by preparative HPLC. Approximately 8.9 mg of the major PhIP (1) metabolite (99.2% purity by LC–MS/MS) was obtained by this approach.

For the complete and unambiguous assignment of all ^1H and ^{13}C chemical shifts and coupling constants of the PhIP (1) metabolite M1, a combination of two-dimensional gCOSY, gHSQC, and gHMBC experiments were acquired in $\text{DMSO}-d_6$. These data are summarized in Table 1. DEPT analysis showed one methyl group, two methylene, and eight methine groups; the ^{13}C NMR spectrum revealed five quaternary carbons. These groups accounted for 15 of the 17 protons seen in the ^1H spectrum. The missing hydrogens, bound to heteroatoms, were identified as a hydroxyl group and a secondary amine, thus being in agreement with the molecular formula of $\text{C}_{16}\text{H}_{17}\text{N}_4\text{O}$. The odd mass and the presence of four nitrogens showed that the molecule was protonated. The additional unsaturation in the PhIP

Table 1. ^1H and ^{13}C NMR Chemical Shifts, δ (ppm); Multiplicities and Coupling Constants, J (^1H , ^1H) (Hz); ^1H – ^1H Correlations in gCOSY; and ^1H – ^{13}C Correlations in gHMBC for the Microbial PhIP (**1**) Metabolite **M1** in $\text{DMSO}-d_6$

Position	δ (^{13}C)	H	δ (^1H)	Multiplicity ^a	J (Hz)	gCOSY	gHMBC
2	147.7	—	—	—	—	—	—
13-NH	—	NH	10.81	br s	—	H-12	—
5	141.3	5	8.63	d	1.9	H-7	C-7,9,1
7	116.6	7	8.38	d	2.0	H-5	C-5,6,8,9
6	136.9	—	—	—	—	—	—
8	124.6	—	—	—	—	—	—
9	141.9	—	—	—	—	—	—
N-CH ₃	29.7	CH ₃	3.78	s	—	—	C-2,8
1	132.1	—	—	—	—	—	—
2	127.1	2	7.81	d	7.3	H-3	C-1,3,4,5,6
3	129.2	3	7.54	t	7.3	H-2,4	C-6,5
4	128.1	4	7.45	t	7.3	H-,3,5	C-2,6
5	129.2	5	7.54	t	7.3	H-4,6	C-6,3
6	127.1	6	7.81	d	7.3	H-,5	C-1,2,3,4,5
10	34.8	10a	4.43	ddd	12.6, 5.2, 2.6	H-10b,11b	—
		10b	4.04	td	12.3, 4.4	H-10a,11b	—
11	26.2	11a	2.18–2.27	m	—	H-11b,12	—
		11b	1.99–2.12	m	—	H-11a,10a,10b	—
12	71.2	12	5.37	dd	5.3,2.6	OH, NH, H-11a	—
12-OH	—	OH	7.00	d	5.3	H-12	—

^a br s, broad singlet; d, doublet; t, triplet; m, multiplet.

(**1**) metabolite must be due to the formation of an extra ring. All proton and carbon resonances of the PhIP (**1**) template could be unambiguously assigned using gCOSY, gHSQC, and gHMBC and were in agreement with data reported on PhIP (**1**) (8, 30). In **M1**, the carbons at positions 2 and 9 were significantly shifted upfield from δ 158.7 and 157.0 to δ 147.7 and 141.9, respectively, suggesting that the new ring was fused to the imidazole. The alcohol (12-OH) appeared as a doublet at δ 7.00 ppm and the secondary amine (NH-13) as a broad singlet at δ 10.81 ppm. Analysis of the gCOSY spectrum showed correlation of these two signals with a methine signal at δ 5.37 (H-12), which led to the identification of a hemiaminal. In addition, three new carbon resonances were present in **M1** at δ 71.2 (C-12), 26.2 (C-11), and 34.8 (C-10), correlating with signals at δ 5.37 (H-12), 1.99–2.12 (H-11b), 2.18–2.27 (H-11a), 4.04 (H-10b), and 4.43 (H-10a). The gCOSY and gTOCSY spectra confirmed that these three groups were adjacent in the nonaromatic heterocyclic ring. This spin system terminates at one end as a hemiaminal group and ends at the other edge at a nitrogen atom. The hemiaminal is derived from the primary amine in PhIP (**1**) and the other end of the new moiety is necessarily attached to N-3; otherwise, the imidazole would be deconjugated and aromaticity would be lost. The anionic part of **M1** was determined using ion chromatography. IC analysis of 1.6 mmol/L of the purified **M1** metabolite corresponded with an equivalent concentration of chloride. Consequently, the metabolite **M1** was assigned as 7-hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydropyrido[3',2':4,5]imidazo[1,2-*a*]pyrimidin-5-ium chloride as depicted in **Figure 5**.

DISCUSSION

In the present study, we have shown that intestinal microorganisms derived from human feces actively transform the food carcinogen PhIP (**1**), resulting in the formation of one major metabolite. We elucidated the chemical structure of the microbial PhIP (**1**) metabolite by a combination of mass spectrometric and NMR spectroscopic evidence. Moreover, we investigated the interindividual variation in PhIP (**1**) metabolism between six human microbiota and the kinetics at different PhIP (**1**) incubation concentrations.

Like many other environmental carcinogens, PhIP (**1**) requires metabolic activation to exert toxic effects. Previous studies

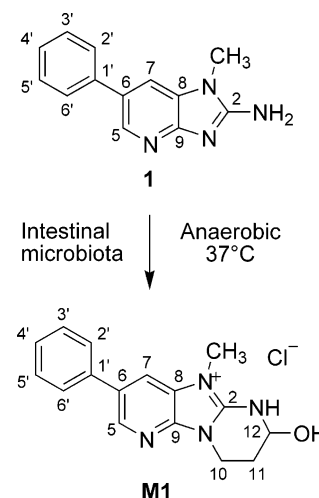


Figure 5. Molecular structure of PhIP (**1**) and its microbial metabolite 7-hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydropyrido[3',2':4,5]imidazo[1,2-*a*]pyrimidin-5-ium chloride. C-atom numbering for **M1** refers to the respective numbering of the PhIP (**1**) parent compound.

indicate that PhIP (**1**) is converted into two primary products: 2-hydroxyamino-PhIP ($\text{N}^2\text{-OH-PhIP}$) and 4'-hydroxyamino-PhIP (4'-OH-PhIP), the former being highly mutagenic and the latter being nonmutagenic (31, 32). These metabolites may subsequently be conjugated with acetyl, glucuronide, glutathione, or sulfate to form secondary phase II metabolites. According to literature, the biotransformation of PhIP (**1**) is highly dependent upon the cytochrome P4501A2 isozyme, mainly expressed in the liver (33). However, the liver is not the only transformation site inside the human body. The human colon contains $\sim 10^{12}$ microorganisms/ cm^3 , with an enormous metabolic potential. Bacterial enzymes catalyze many reactions, including hydrolysis, dehydroxylation, demethylation, ring cleavage, and carboxylation (34). Numerous findings show that intestinal microorganisms and lactobacilli contained in dairy products play a key role in the activation and detoxification of various classes of DNA-reactive carcinogens such as nitrosamines, aflatoxins, polycyclic aromatic hydrocarbons, azo compounds, nitroarenes, and glycosides (35–38). Our results confirm a similar microbial activity

toward the food carcinogen PhIP (**1**), since it can be converted by the intestinal microbiota as well.

While PhIP (**1**) is biotransformed into a large number of derivatives in the liver, the human intestinal microbiota selectively converted PhIP (**1**) into one major metabolite. By analyzing crude incubation media by HPLC with fluorescence detection, we can assert that the PhIP (**1**) derivative observed is unambiguously the only metabolite produced by bacterial conversion and rule out the possibility that other derivatives have been released yet not recovered in the extract. HPLC with fluorescence detection is a highly sensitive and powerful analytical tool for providing quantitative information on fluorescent compounds in complex biological media (39, 40). Synchronous absorbance and fluorescence spectroscopic analysis of PhIP (**1**) and its microbial metabolite **M1** revealed a decrease in wavelength of both absorbance and fluorescence excitation maxima for the PhIP (**1**) derivative compared to its precursor, indicating an alteration at the primary amine function or imidazo moiety. Crofts et al. (31) measured the fluorescence intensity for PhIP (**1**) and the phase I liver metabolites and observed a decrease in fluorescence excitation maxima upon hydroxylation of the primary amine, whereas hydroxylation of the phenyl substituent caused an increase in fluorescence maxima. Mass spectrometry gave a molecular ion at m/z 281 $[M + H]^+$, indicating that a fragment of 56 mass units had been added to PhIP (**1**) (m/z 225 $[M + H]^+$). Loss of water from the molecule ion referred to the presence of a hydroxyl group. High-resolution mass spectrometry revealed the exact molecular mass 281.1398 and molecular formula $C_{16}H_{17}N_4O$. Further elucidation of the chemical identity of the microbial PhIP (**1**) metabolite was achieved by careful analysis and interpretation of the 1D and 2D NMR and IC data, assigning the metabolite as 7-hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydropyrido[3',2':4,5]imidazo[1,2-*a*]pyrimidin-5-ium chloride (**Figure 5**).

Up to now, data regarding the microbial transformation of heterocyclic amines are scarce. Only for the quinolines IQ (**2**) and MeIQ (2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline) has it been reported that incubation with human fecal microbiota resulted in the formation of stable hydroxy metabolites (24–25, 41). The microbial metabolism of PhIP (**1**) shows, however, no resemblance to that of IQ (**2**) and MeIQ (**3**). One possible explanation for this discrepancy is the protective effect of the phenyl substituent of PhIP (**1**), thereby impairing hydroxylation on the imidazo moiety. Several reports, however, emphasize the crucial role of the intestinal bacteria in the genotoxicity of heterocyclic amines (16, 22), implying cleavage of glucuronide conjugates as the most important mechanism by which intestinal bacteria activate heterocyclic amines. In contrast, bacteria in fermented foods and dairy products are known to detoxify these heterocyclic amines by direct binding to the cell walls (16, 42). Moreover, overall health effects may result from a combination of microbial interactions with multiple and perhaps additive or interfering activities. The impact of microbial transformations on the carcinogenicity of heterocyclic amines, entering the colon in their native form, remains underinvestigated. Our results indicate that microbial transformation of PhIP (**1**) causes an increase in hydrophobicity for the metabolite, thereby facilitating its absorption from the colon to exert potential biological activity inside the human body. Research has shown that the human colonic mucosa generally has a higher permeability to hydrophobic compounds than the small intestinal mucosa (43, 44). Further in vivo studies are warranted to acquire insight into the bioavailability and biological activity of this newly discovered PhIP (**1**) metabolite throughout the intestine. However, as the

efficiencies of the fecal samples to degrade PhIP (**1**) ranged from 47 to 95%, interindividual variability in the microbial community and activity could strongly influence the individual exposure to this dietary carcinogen. Interindividual differences in microbial metabolic activities are not uncommon. A striking example is the microbial conversion of the dietary phytoestrogen daidzein (45, 46). Intensive research has shown that only approximately one-third of humans harbor an intestinal microbiota capable of transforming daidzein into equol (47). A similar interindividual variability in microbial transformation has been shown for the group of the prenylflavonoids as well (48).

In conclusion, by converting PhIP (**1**) into 7-hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydropyrido[3',2':4,5]imidazo[1,2-*a*]pyrimidin-5-ium chloride, human intestinal microbiota would contribute to the bioactivation or detoxification of a putative food-borne carcinogen. As a significant fraction of the daily exposure of PhIP (**1**) is suggested to reach the colon in its native form, this biotransformation potency has to be considered when estimating the risks related to fried meat ingestion. Moreover, we showed interindividual differences in the microbial PhIP (**1**) transformation, which may predict individual differences in susceptibility to the risks associated with this suspected dietary carcinogen.

ABBREVIATIONS USED

IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; MeIQ, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; PFG, pulsed field gradient; gCOSY, gradient enhanced correlation spectroscopy; gTOCSY, gradient enhanced total correlation spectroscopy; gHSQC, gradient enhanced heteronuclear single quantum correlation; gHMBC, gradient enhanced heteronuclear multiple bond correlation; DEPT, distortionless enhancement by polarization transfer.

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