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A Major Urinary Protein of the Domestic Cat Regulates the Production of Felinine, a Putative Pheromone Precursor

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Summary

Domestic cats spray urine with species-specific odor for territorial marking. Felinine (2-amino-7-hydroxy-5,5-dimethyl-4-thiaheptanoic acid), a putative pheromone precursor, is excreted in cat urine. Here, we report that cauxin, a carboxylesterase excreted as a major urinary component, regulates felinine production. In vitro enzyme assays indicated that cauxin hydrolyzed the felinine precursor 3-methylbutanol-cysteinylglycine to felinine and glycine. Cauxin and felinine were excreted age dependently after 3 months of age. The age-dependent increases in cauxin and felinine excretion were significantly correlated. In mature cats, cauxin and felinine levels were sex-dependently correlated and were higher in males than in females. In headspace gas of cat urine, 3-mercapto-3-methyl-1-butanol, 3-mercapto-3-methylbutyl formate, 3-methyl-3-methylthio-1-butanol, and 3-methyl-3-(2-methyldisulfanyl)-1-butanol were identified as candidates for felinine derivatives. These findings demonstrate that cauxin-dependent felinine production is a cat-specific metabolic pathway, and they provide information for the biosynthetic mechanisms of species-specific molecules in mammals.

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Introduction

Most mammals have a highly developed olfactory sense, and urinary odorants and pheromones are important molecules for chemical communication used in reproduction, territoriality, and conspecific recognition. It is thought that the biosynthetic and excretion mechanisms of the molecules have evolved independently in each animal species, although evidence is limited to a small number of species, which include rodents and elephants [1].

The domestic cat (*Felis catus*) is a popular pet, and it is commonly known that cat urine has a characteristic "catty odor." Male cats frequently spray urine with the odor onto vertical objects, such as walls, in order to mark their territory. In cat urine, a sulfur-containing amino acid, 2-amino-7-hydroxy-5,5-dimethyl-4-thiaheptanoic acid, known as felinine (Figure 1), is excreted [2, 3]. Felinine excretion is regulated by testosterone [4], and male cats excrete a large amount of felinine (122 μmol/kg body weight/day) compared to castrated males and mature females (41 and 36 μmol/kg body weight/day, respectively) [5]. Chemically synthesized felinine and native felinine purified from urine develop an odor during storage that is characteristic of cat urine [6]. Based on these findings, it has been postulated that felinine is a biologically important molecule in the cat, and that it is used as a territorial marker for intraspecies communication [7, 8] and is a putative precursor of a pheromone that plays a role in attracting females [4].

A felinine precursor is present in cat blood as 3-methylbutanol-glutathione (3-MBG) and is formed via a glutathione S-conjugation reaction between glutathione and isopentenyl pyrophosphate as an intermediate of cholesterol biosynthesis [9]. In general, glutathione S-conjugates such as 3-MBG are converted to cysteinylglycine S-conjugates by γ-glutamyl transferase (Enzyme Commission [EC] numbers 2.3.2.2) in the kidney, and the cysteinylglycine S-conjugates are hydrolyzed by renal dipeptidase (EC 3.4.13.11). The resulting cysteine S-conjugates, including felinine, are *N*-acetylated by microsomal *N*-acetyltransferase (EC 2.3.1.5) and are ultimately excreted in the urine as *N*-acetyl cysteine S-conjugates, known as mercapturic acids [10, 11]. The metabolic pathway by which 3-MBG is hydrolyzed to felinine, however, remains unknown.

Here, we report that cauxin, a major urinary protein, is involved in the production of felinine in the domestic cat. We previously found that urine of normal male cats contains a high concentration of proteins (about 0.5–1.0 mg/ml), and we found that more than 90% of these proteins are a 70 kDa component named cauxin [12]. Cauxin is a member of the carboxylesterases (EC 3.1.1.1), which hydrolyze a wide variety of aromatic and aliphatic substrates containing ester, thioester, and amide bonds [13, 14]. Cauxin is expressed only in the kidney and is secreted from the kidney-proximal straight tubular cells into the urine. Recently, we found that cauxin is excreted in a species-, sex-, and age-dependent manner [15]. Although the cauxin gene is conserved in several

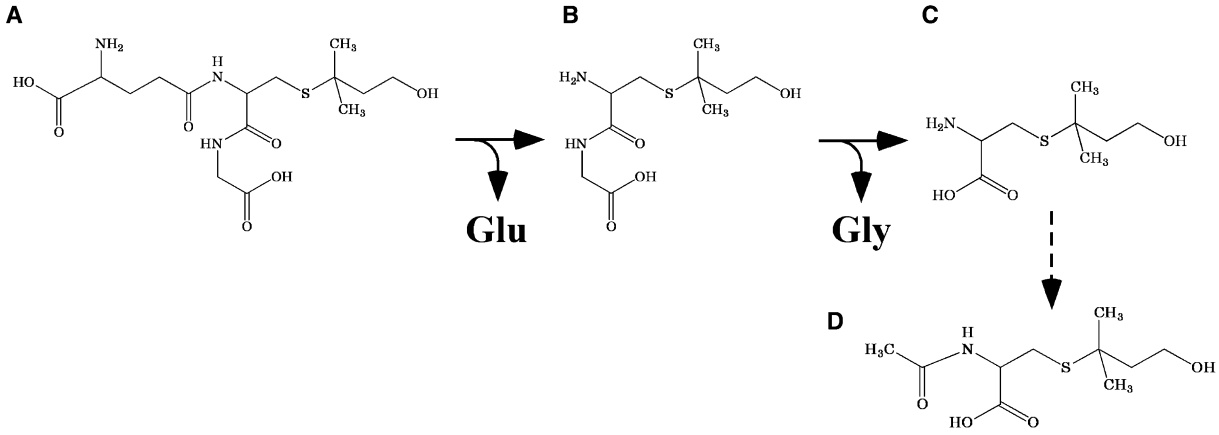

Figure 1. Chemical Structures of Felinine-Related Molecules Discussed in This Paper

(A) 3-Methylbutanol-glutathione (3-MBG).

(B) 3-Methylbutanol-cysteinylglycine (3-MBCG).

(C) 2-Amino-7-hydroxy-5,5-dimethyl-4-thiahepanoic acid (felinine).

(D) 3-Methylbutanol-*N*-acetyl cysteine (*N*-acetyl felinine).

mammals, including humans, mice, and dogs, a large amount of cauxin is found only in the domestic cat, bobcat (*Lynx rufus*), and lynx (*Lynx lynx*), all of which are relatively close members in the Felidae lineage [16]. Urinary cauxin is detected in cats older than ~3 months of age and is significantly higher in intact males than in castrated males or mature females. Based on these findings, we reasoned that products of the cauxin enzyme reaction should vary with species, sex, and age in the same way as cauxin. We therefore focused our attention on felinine, whose excretion is species, sex, and age dependent [8], and we hypothesized that cauxin hydrolyzes the felinine precursors, 3-methylbutanol-cysteinylglycine (3-MBCG) and/or 3-MBG. In this study, we found that cauxin hydrolyzes the peptide bond of 3-MBCG and produces felinine. Furthermore, in the analysis of headspace gas of cat urine, cat-specific odorants 3-mercapto-3-methyl-1-butanol, 3-mercapto-3-methylbutyl formate, 3-methyl-3-methylthio-1-butanol, and 3-methyl-3-(2-methyldisulfanyl)-1-butanol were identified as candidates for degradative products of felinine. To our knowledge, these findings provide new information for the biosynthetic mechanisms of species-specific urinary chemicals in mammals.

Results

Cauxin Enzyme Activity toward Felinine Precursors

Using in vitro enzyme assays, we examined the peptidase activity of cauxin toward the felinine precursors, 3-MBCG and 3-MBG. The reaction products analyzed by high-performance thin-layer chromatography (HPTLC) are shown in Figure 2A. When 3-MBCG was used as the substrate (lane 3), two products, *a* and *b*, comigrated with the authentic compounds of felinine (lane 1) and glycine (lane 2), respectively. As shown in Figure 2B, the reaction products were also analyzed by an amino acid analyzer, and peaks *a* and *b* showed the same elution times as those of the authentic compounds of felinine and glycine, respectively. As shown in Figure 2C, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) indicated that product *a* purified by HPLC with an octadecylsilica (ODS) column exhibited ions at *m/z* 208.1 for $[M+H]^+$, *m/z* 230.1 for $[M+Na]^+$, and *m/z* 246.1 for $[M+K]^+$, confirming that product *a* was felinine (molecular weight: 207.29). When 3-MBG was used as the substrate, no product was detected by HPTLC (Figure 2A, lane 5). These results indicate that cauxin hydrolyzed the peptide bond of 3-MBCG and produced felinine and glycine, but did not hydrolyze 3-MBG. The K_m and V_{max} values of cauxin for hydrolysis of 3-MBCG, which were calculated from a Lineweaver-Burk plot, were 1.8 mM and 3.3 nmol/mg/min, respectively (Figure 2D). Cauxin had hydrolytic activity toward 3-MBCG in the pH range of 5.5–7.5, as shown in Figure 2E, and cat urine falls within this range (males 6.37 ± 0.07; females 5.97 ± 0.10) [17].

Temporal Changes in Excretion of Cauxin, Felinine, and 3-MBCG in Immature Cats

Figure 3 shows the excretion levels of cauxin, felinine, and 3-MBCG in male (Figure 3A) and female (Figure 3B) littermate cats from birth to 4.8 months of age. Cauxin concentrations (g/l) were normalized to urinary creatinine levels (g/l), and felinine and 3-MBCG concentrations (nmol/ml) were normalized to urinary creatinine concentrations (μmol/ml). Cauxin excretion was detected in males older than 2.8 months of age and in females older than 3.1 months of age, and levels were found to increase with age. Felinine was detected in males older than 2.5 months of age and in females older than 3 months of age, and its excretion level increased with age in a very similar manner to that of cauxin for cats older than 3 months of age. Notably, the felinine precursor 3-MBCG was present for cats younger than 3 months of age, and its concentration did not vary with age. During this time, cauxin was below the detection limit of western blotting with anti-cauxin antibody, and very little felinine was excreted in the urine.

Concentrations of 3-MBCG per milliliter urine were significantly lower ($p < 0.05$) during the first month

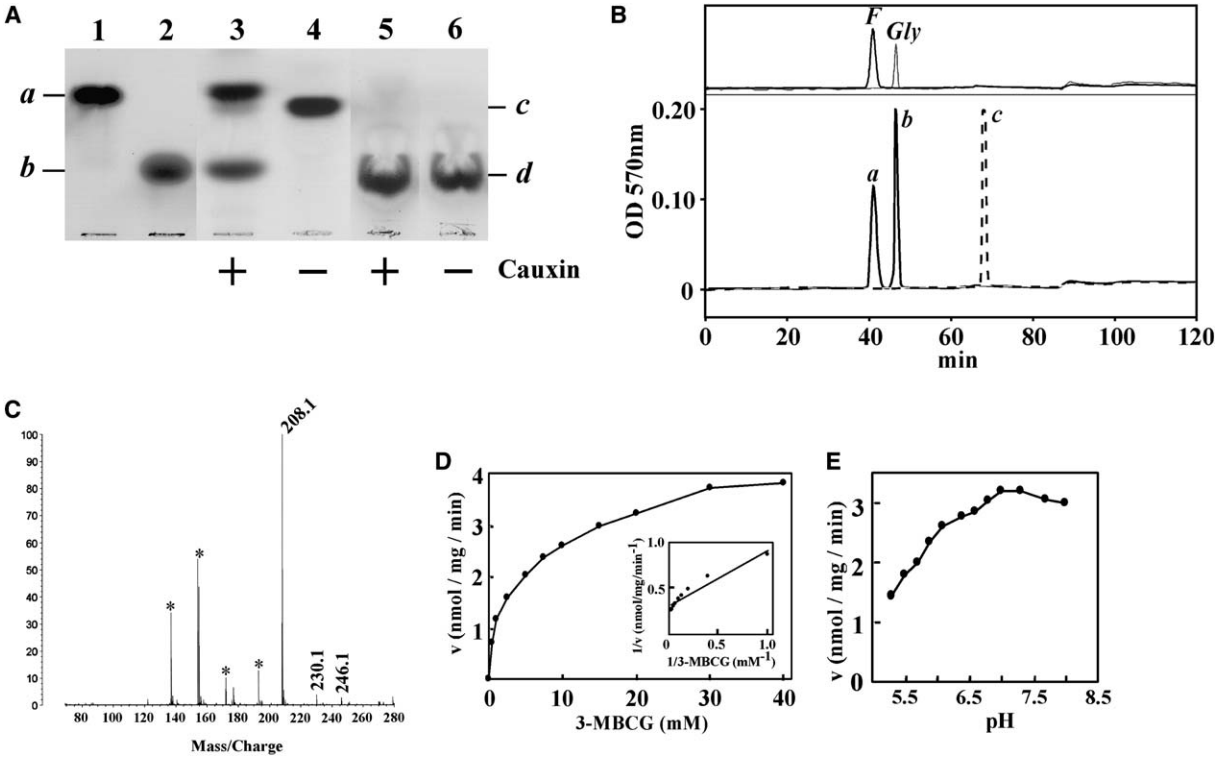

Figure 2. Hydrolytic Activity of Cauxin toward Felinine Precursors

(A) 3-MBCG (lanes 3 and 4) or 3-MBG (lanes 5 and 6), 20 mM each, was incubated with or without cauxin (1.5 mg/ml) as described in the Experimental Procedures, and the reaction mixtures were analyzed by HPTLC with ninhydrin staining. Lanes 1 and 2 show the authentic compounds of felinine and glycine, respectively. Lane 3 shows that 3-MBCG (*c*) was hydrolyzed to felinine (*a*) and glycine (*b*). Lane 5 shows that 3-MBG (*d*) was not hydrolyzed by cauxin.

(B) In the lower chromatogram, the reaction products (solid line) and 3-MBCG (dotted line) were analyzed by an amino acid analyzer employing the ninhydrin colorimetric method. Peaks were detected by a UV detector at 570 nm. Peaks *a* and *b* had the same elution times as authentic compounds of felinine (F) and glycine (Gly), respectively, shown in the upper chromatogram.

(C) A mass spectrum of peak *a* analyzed by MALDI-TOF MS. Reaction product *a* was recovered by HPLC with an ODS column. An asterisk indicates ions derived from matrix, 2,5-dihydroxy benzoic acid (DHB).

(D) Kinetic analysis of the hydrolytic activity of cauxin toward 3-MBCG. The graph shows the effects of 3-MBCG concentration (mM) on the initial velocity of the hydrolytic reaction by cauxin. The inset shows a Lineweaver-Burk plot used for the determination of K_m and V_{max} values.

(E) The effects of pH on hydrolytic activity of cauxin toward 3-MBCG.

(male, 137.6 ± 39.9 nmol/ml; female, 96.3 ± 44.2 nmol/ml) than during the subsequent months (male, 395.0 ± 56.6 nmol/ml; female, 268.2 ± 25.4 nmol/ml). However, as shown in Figures 3A and 3B, corrected concentrations of 3-MBCG were higher during the first 2 months than subsequent months because urinary creatinine was significantly lower ($p < 0.05$) during the first month (2.25 ± 1.24 μmol/ml) or second month (11.89 ± 1.86 μmol/ml) than during the period from 2 to 4.8 months (17.89 ± 3.49 μmol/ml).

Excretion Levels of Cauxin, Felinine, and 3-MBCG in Mature Cats

Figure 4B shows a correlation diagram of urinary cauxin (g/l) and felinine concentrations (μmol/ml) in eight intact males, seven castrated males, and seven intact females. This diagram shows that cauxin concentration was positively correlated ($R^2 = 0.95$) with felinine concentration in a sex-dependent manner. Figure 4C presents the urinary concentrations of felinine, 3-MBCG, and glycine, which were normalized to urinary creatinine concentrations (μmol/ml). In contrast to sex-dependent felinine excretion, concentrations of 3-MBCG in intact males and intact females did not differ significantly ($p > 0.05$). Glycine concentrations were markedly lower than felinine concentrations.

Cauxin and Renal Dipeptidase Expression in Kidney

Real-time quantitative reverse transcriptase-PCR demonstrated that cauxin mRNA is expressed in an age- and sex-dependent manner in the kidney (Figure 5A). Although cauxin protein was not detected by western blotting with anti-cauxin antibody for cats up to 3 months old (Figures 3A and 3B), cauxin mRNA was detected at a very low level at 0.5 months, and its concentration increased with age. The amount of cauxin mRNA was much higher in mature cats than in immature cats. In addition, the cauxin mRNA content was higher in a mature, intact male than in a castrated male and a mature female. In contrast, cat renal dipeptidase expression varied slightly with age during the first 3 months, but no difference was observed between 3-month-old and mature cats (Figure 5B). In mature cats, renal dipeptidase was expressed in a sex-independent manner, and its levels did not differ between males and females.

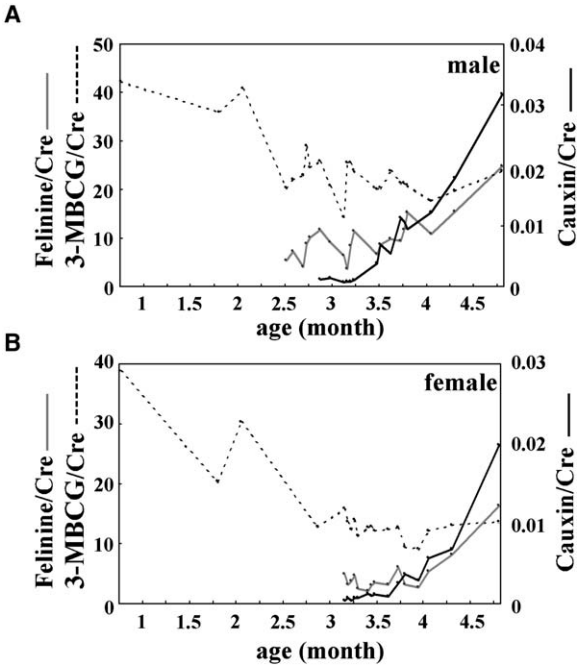

Figure 3. Changes in Excretion Levels of Cauxin, Felinine, and 3-MBCG in Immature Cats

(A and B) Levels of excreted cauxin, felinine, and 3-MBCG in a (A) male and a (B) female cat are shown up to 4.8 months after birth. The urinary cauxin to creatinine ratio was calculated by dividing the urinary cauxin concentration (g/l) by the urinary creatinine concentration (g/l). Urinary felinine and 3-MBCG to creatinine ratios were calculated by dividing urinary felinine and 3-MBCG concentrations (nmol/ml), respectively, by the urinary creatinine concentration (μmol/ml).

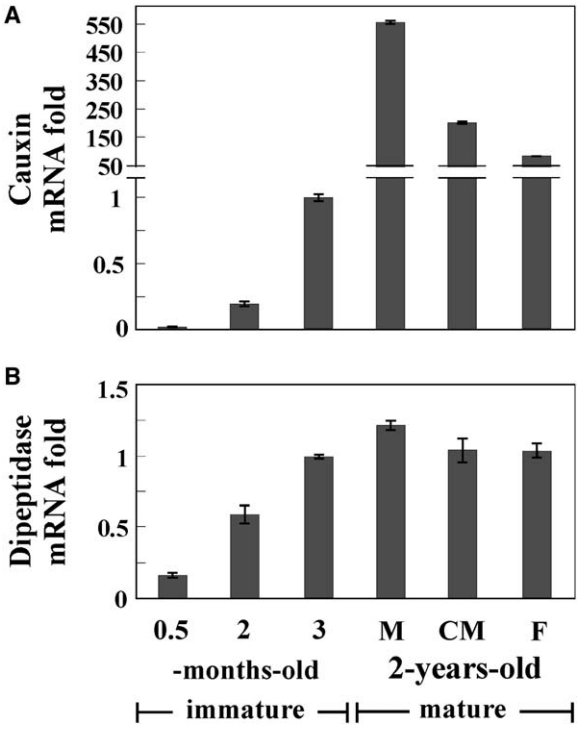

Figure 5. Expression of Cauxin and Renal Dipeptidase mRNA in the Cat Kidney

(A and B) Real-time reverse transcriptase-PCR was performed with kidney mRNA. Amounts of (A) cauxin and (B) renal dipeptidase mRNA were normalized to the GAPDH mRNA. Data shown represent the mean ± SE of three independent experiments. M, intact male; CM, castrated male; and F, spayed female.

Identification of Volatile Components as Candidates of Felinine Degradation Products in the Headspace of Cat Urine

The headspace gas of cat urine was individually collected from three intact males, one castrated male, three intact females, and one immature, 2-month-old male by heating the urine samples at 200°C for 15 min, prior to analysis with gas chromatograph (GC)-MS. Figure 6A shows a representative total ion chromatogram of mature, intact male urine.

3-Mercapto-3-methyl-1-butanol (Figure 6B, b) was detected as the major peak at 16.3 min. Additionally, 3-mercapto-3-methylbutyl formate (Figure 6B, a), 3-methyl-3-methylthio-1-butanol (Figure 6B, c), and 3-methyl-3-(2-methyldisulfanyl)-1-butanol (Figure 6B, d) were identified as minor peaks at 14.4, 17.7, and 22.7 min, respectively. In the analysis of castrated male (Figure S1A; see the Supplemental Data available with this article online) and mature female (Figure S1B) urine, 3-mercapto-3-methyl-1-butanol was clearly detected,

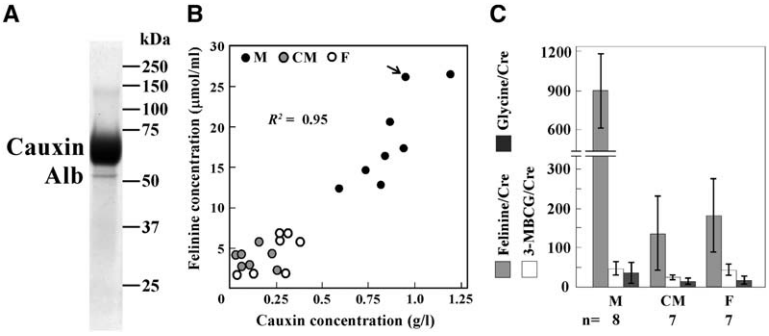

Figure 4. Concentrations of Cauxin, Felinine, and 3-MBCG in Cat Urine

(A) An example of SDS-PAGE analysis with Coomassie blue staining. Cat urine (10 μl) was applied. A single and heavily stained band of cauxin was detected at 70 kDa. The amounts of cauxin were determined by image analysis of SDS-PAGE results.

(B) Correlation between urinary cauxin and felinine concentrations in eight intact males (M, 2.7 ± 1.5 years old), seven castrated males (CM, 3.3 ± 2.0 years old), and seven intact females (F, 3.6 ± 2.0 years old). The individual giving the urine analyzed in (A) is indicated by the arrow. R^2 indicates the correlation coefficiency.

(C) The relative amounts (mean ± SD) of excreted felinine, 3-MBCG, and glycine (nmol/ml) to urinary creatinine (μmol/ml) in the same samples shown in (B).

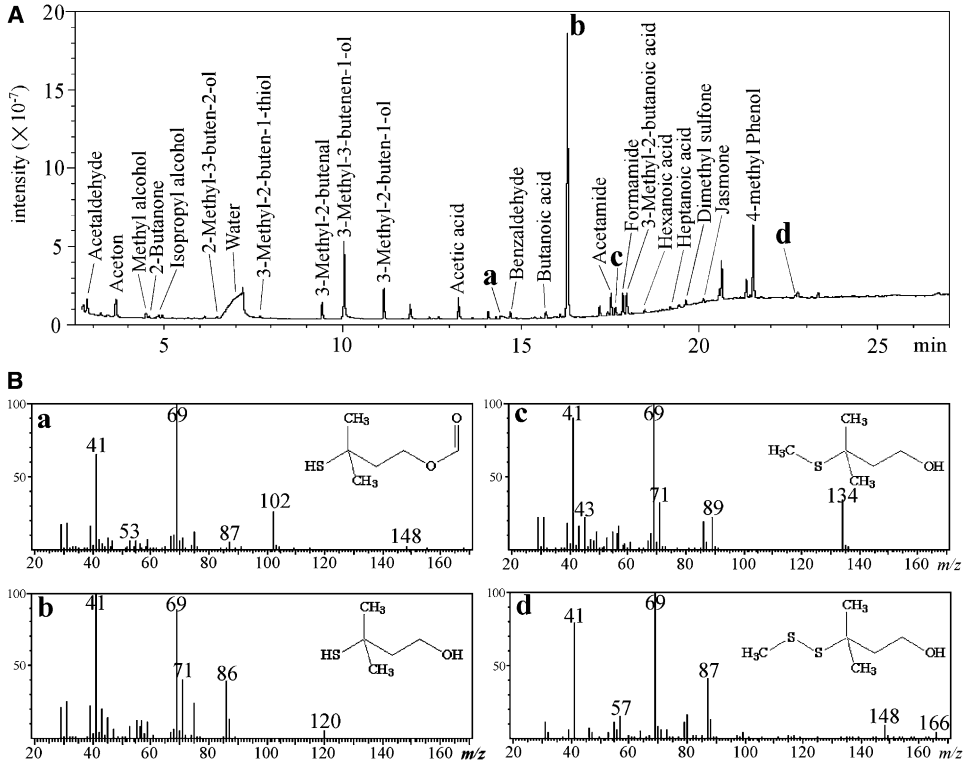

Figure 6. Analysis of Volatile Compounds Present in Male Cat Urine

(A) A GC-MS total ion chromatogram of the headspace gas of intact male cat urine. A total of 25 volatile compounds detected in the headspace gas are indicated.

(B) (a)–(d) show the GC-mass spectra and chemical structures of 3-mercapto-3-methylbutyl formate, 3-mercapto-3-methyl-1-butanol, 3-methyl-3-methylthio-1-butanol, and 3-methyl-3-(2-methyldisulfanyl)-1-butanol, respectively.

but the peak height was significantly lower than that of the intact males (Figure S2A). While 3-methyl-3-methylthio-1-butanol levels were about half the levels of the mature males (Figure S2B), 3-mercapto-3-methylbutyl formate and 3-methyl-3-(2-methyldisulfanyl)-1-butanol were not detectable. In the analysis of immature male urine (Figure S1C), 3-mercapto-3-methyl-1-butanol and 3-methyl-3-methylthio-1-butanol were detected as very minor peaks.

The headspace gas of male cat urine was trapped by a solid-phase adsorbent of a porous polymer resin at room temperature and was analyzed by GC-MS. Peaks representing 3-mercapto-3-methyl-1-butanol, 3-mercapto-3-methylbutyl formate, and 3-methyl-3-methylthio-1-butanol are seen in Figure S3, which indicates that the sulfur-containing volatile compounds collected by heating urine at 200°C for 15 min were not artificial degradation products.

Discussion

A carboxylesterase termed cauxin is a major component of cat urine [12]. In this study, we found that cauxin hydrolyzes the peptide bond of 3-MBCG to produce felinine and glycine (Figure 2). Based on the daily excretion of cauxin in mature male cats (about 25 mg/kg body weight) [15] and the V_{max} value of cauxin for 3-MBCG (3.3 nmol/mg/min), we calculated that ~119 μmol/kg body weight of 3-MBCG is hydrolyzed by cauxin daily. Since the daily excretion of felinine in male cats is ~122 μmol/kg body weight [5], the amount of cauxin excreted daily is reasonably sufficient for the production of felinine at the level observed in male cats. We therefore suggest that cauxin hydrolyzes 3-MBCG to produce felinine in vivo.

We speculated that a positive correlation existed between species-, age-, and sex-dependent cauxin and felinine excretion, if cauxin is involved in the production of felinine in vivo. Only the domestic cat, bobcat, and lynx, which are close members in the Felidae lineage [16], excrete both cauxin and felinine as major components of their urine [8, 15]. Age-dependent increases in cauxin and felinine excretions are significantly correlated in immature cats older than 3 months of age (Figure 3). In mature cats, cauxin and felinine excretions are sex-dependently correlated and are higher in intact males than in castrated males and mature females (Figure 4B). These results support our hypothesis that cauxin is an important enzyme for the production of felinine in the domestic cat.

It is widely believed that glutathione S-conjugates, such as 3-MBG, are ultimately metabolized to *N*-acetyl cysteine S-conjugates via a mercapturic acid pathway, and that *N*-acetyl cysteine S-conjugates, but not cysteine S-conjugates such as felinine, are excreted in cat urine [10, 11]. In this pathway, cysteinylglycine S-conjugates are hydrolyzed by renal dipeptidase in the brush border of proximal tubular cells. Therefore, we examined the contribution of cat renal dipeptidase to the production of felinine excreted in urine. We found that

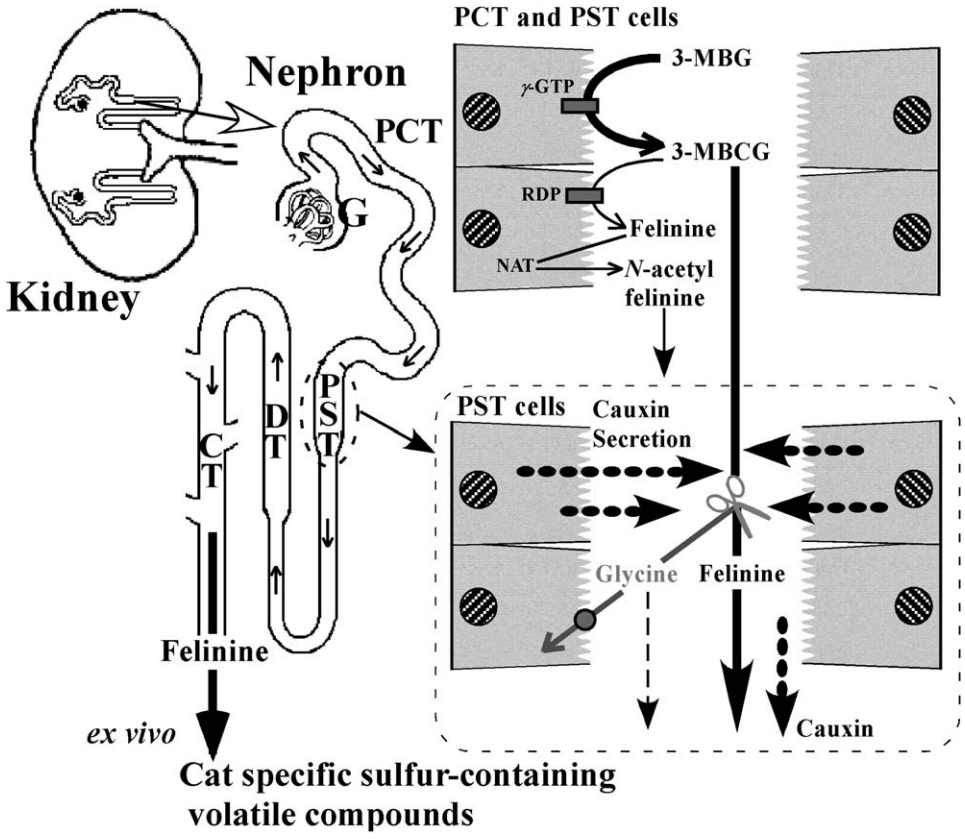

Figure 7. Proposed Metabolic Pathways for Conversion of 3-MBCG to Felinine in the Cat Kidney

Schematic diagrams of a kidney and a nephron are shown on the left. A nephron is the basic structural and functional unit of the kidney, and a normal kidney contains roughly one million nephrons. The felinine precursor 3-MBG is filtered from blood to urine through the renal glomerulus (G) and is converted to 3-MBCG by γ-glutamyl transferase (γ-GTP) localized at the brush border of proximal convoluted tubular (PCT) and proximal straight tubular (PST) cells. A small portion of 3-MBCG is hydrolyzed to felinine and glycine by renal dipeptidase (RDP) in the brush border of PCT and PST cells. Felinine is then absorbed by the PCT and PST cells, where it is converted to *N*-acetyl felinine by *N*-acetyltransferase (NAT). Most of the remaining 3-MBCG is hydrolyzed to felinine and glycine by cauxin. Glycine is reabsorbed by glycine transporters, and felinine is excreted into the urine and derivatized, sulfur-containing volatile compounds. DT, distal tubules; CT, collecting tubules.

cat renal dipeptidase expressed in COS-7 mammalian cells hydrolyzes 3-MBCG to felinine and glycine (Figure S4). Based on the expression level of cat renal dipeptidase (Figure 5B), we initially hypothesized that if renal dipeptidase mainly hydrolyzes 3-MBCG in proximal tubules, two correlates should hold true. First, felinine should be detected in the urine of cats younger than 3 months of age, because their urine contains the substrate 3-MBCG. Second, 3-MBCG should be excreted at different levels in the urine of mature males, castrated males, and females, because a large amount of 3-MBCG is hydrolyzed to felinine in males. However, this hypothesis was discarded by the results shown in Figures 3 and 4C, respectively: felinine is not detected in immature cats younger than 2.5 months of age, and no significant difference was observed in 3-MBCG levels between intact male and female cat urine. Recent studies indicate that cat urine contains 3-methylbutanol-*N*-acetyl cysteine (*N*-acetyl felinine) at a concentration significantly lower than felinine and is not sex dependent, suggesting that *N*-acetyl felinine is produced by the mercapturic acid pathway [18, 19]. Taken together, this evidence indicates that cat renal dipeptidase is not involved in the production of felinine excreted in urine, but is instead responsible for the production of *N*-acetyl felinine (Figure 7).

Cauxin secreted from the proximal straight tubular cells hydrolyzes a large amount of 3-MBCG to felinine and glycine (Figure 7). Glycine concentrations were markedly lower than felinine concentrations (Figure 4C), indicating that the hydrolysis of 3-MBCG to felinine and glycine by cauxin occurs predominantly in the renal tubules, and that only glycine is reabsorbed into the renal tubular cells. In addition, we suggest that the hydrolytic reaction of 3-MBCG by cauxin occurs in the bladder and/or ex vivo based on the report that felinine content in intact male cat urine increased over the first 24 hr when stored at 5°C [20] and on our finding that urinary 3-MBCG is hydrolyzed to felinine and glycine by incubating intact male cat urine obtained from the bladder in a test tube at 38°C (data not shown).

It is generally known that normal mammals excrete only small amounts of protein into the urine to prevent loss of proteins from the body, and that proteinuria is an indicator of renal abnormality [21]. Several studies, however, have proposed that urinary proteins excreted under physiological conditions are involved in chemical communication in mammals, although evidence is currently limited to a small number of species. In mice (*Mus musculus*), lipocalin superfamily proteins known as major urinary proteins (MUPs) are excreted at high

concentrations (1–5 mg/ml) and act as carriers of volatile pheromones, such as 2-*sec*-butyl-4,5-dihydrothiazole and 3,4-dehydro-*exo*-brevicomin [22, 23]. MUPs are also used to communicate individuality within a mouse population [24]. In Asian elephants (*Elephas maximus*), urinary albumin binds the pheromone (*Z*)-7-dodecenyl acetate and releases it via a pH change [25]. To our knowledge, our finding in cats reveals a new function of proteinuria, in which a major urinary protein is excreted to act as an enzyme in the synthesis of a putative pheromone precursor, rather than reversibly binding pheromones.

In the headspace gas of cat urine, we identified sulfur-containing volatile compounds, 3-mercapto-3-methyl-1-butanol, 3-mercapto-3-methylbutyl formate, 3-methyl-3-methylthio-1-butanol, and 3-methyl-3-(2-methyldisulfanyl)-1-butanol. The levels of these compounds were shown to be both sex and age dependent (Figures S1 and S2). Based on the presence of sulfur and the isopentyl backbone in the chemical structure, as well as the report that these compounds, excluding 3-mercapto-3-methylbutyl formate, were detected in bobcat urine that contains felinine [26], we suggest that these compounds are felinine derivatives and are capable of imparting the species-specific odor to cat urine. Although it remains unknown how felinine breaks down into these compounds in cat urine, it is suggested that enzymatic and nonenzymatic reactions are involved. It is known that β-lyase (EC 4.4.1.13) degrades the carbon-sulfur bond of cysteine S-conjugates to release mercaptan, ammonium, and pyruvic acid [27]. Similar to β-lyase, enzymes may degrade felinine to release 3-mercapto-3-methyl-1-butanol, which could then be derivatized to 3-mercapto-3-methylbutyl formate, 3-methyl-3-methylthio-1-butanol, and 3-methyl-3-(2-methyldisulfanyl)-1-butanol by enzymatic or chemical reactions in cat urine. Alternatively, Hendriks et al. [6] reported that odorless synthetic felinine can develop an odor similar to that of cat urine during storage at room temperature. Therefore, it is also possible that felinine is degraded to volatile compounds such as 3-mercapto-3-methyl-1-butanol by a nonenzymatic reaction in cat urine.

Cat urine odor is a problem for many people who perceive the odor as unpleasant. Removing the odor from subjects is therefore a matter of commercial importance. The odor is distinctly stronger and more pungent in the urine of intact males than in castrated males and in mature females, suggesting that a cause of the odor is the cat-specific excretion of felinine that breaks down into mercaptans, such as 3-mercapto-3-methyl-1-butanol. In this study, we found that cauxin is a key enzyme for producing felinine from the precursor 3-MBCG. The possibility therefore exists that developing cauxin-specific inhibitors and including them as supplements in cat food could eliminate the odor problem by blocking felinine production.

Domestic cats spray urine with a characteristic odor on their territory, suggesting that odorants in cat urine are used as species-specific chemical mediators associated with species, subspecies, sex, age, social status, individuality, and reproductive conditions. It has been shown that sulfur-containing volatile compounds, such as 2-*sec*-butyl-4,5-dihydrothiazole from mice and dimethyl disulfide from hamsters, can function as species-specific pheromones [28–30]. Therefore, it is possible that the cat-specific, sulfur-containing volatile compounds 3-mercapto-3-methyl-1-butanol, 3-mercapto-3-methylbutyl formate, 3-methyl-3-methylthio-1-butanol, and 3-methyl-3-(2-methyldisulfanyl)-1-butanol are pheromones used for conspecific recognition and reproductive purposes in mature cats. Based on our preliminary behavior bioassays, which indicate that male and female cats showed interest in 3-mercapto-3-methyl-1-butanol, but not in purified felinine, we hypothesize that felinine itself could be a precursor of these compounds, not a cat pheromone. We are now designing a study to elucidate the biological roles of these sulfur-containing volatile compounds. Further studies would clarify the biological significances of felinine and cauxin excretion as a species-specific mechanism.

Significance

To our knowledge, we present new evidence for the concept that species-specific odorants and pheromones are produced by species-specific biosynthetic mechanisms in mammals. Felinine, 2-amino-7-hydroxy-5,5-dimethyl-4-thiaheptanoic acid, is a putative pheromone precursor in the domestic cat. It was established that the felinine precursor is biosynthesized by a glutathione S-conjugation reaction between glutathione and isopentenyl pyrophosphate; however, the hydrolytic pathway from 3-MBG to felinine is unknown. In this study, we found that cauxin, a major component of cat urine, regulates the production of felinine in a species-, sex-, and age-dependent manner. Our findings indicate that felinine is produced by a cat-specific hydrolytic pathway using cauxin, but not by a mercapturic acid pathway that is the general metabolic mechanism that converts glutathione S-conjugates to *N*-acetyl cysteine S-conjugates. We suggest that cauxin-dependent felinine production is an important aspect of metabolism in the cat because species-specific, sulfur-containing volatile compounds, 3-mercapto-3-methyl-1-butanol, 3-mercapto-3-methylbutyl formate, 3-methyl-3-methylthio-1-butanol, and 3-methyl-3-(2-methyldisulfanyl)-1-butanol, were identified as candidates of felinine derivatives in cat urine. These volatile compounds give a species-specific odor to cat urine and may be putative pheromones.

Experimental Procedures

Urine Sample Collection

Urine samples were collected from 22 adult cats (8 intact males, 7 castrated males, and 7 intact females; 1–6 years old) and from immature male and female littermate cats up to 4.8 months after birth. For cats up to 1 month old, samples were taken by direct manual pressure on the bladder. For all other samples, cats were housed individually in metabolism cages to collect urine samples. Protocols for urine sample collection and preparation of kidney mRNA were approved by the Animal Research Committee of the Faculty of Agriculture, Iwate University (Japan). Experiments were conducted according to the Iwate University Guidelines for Animal Experimentation.

Cauxin Purification and Analysis

To purify cauxin, male cat urine was applied to a Sephadex G-200 column (2.5 × 60 cm) equilibrated with 10 mM Tris-HCl (pH 7.2) at a flow rate of 0.33 ml/min. Fractions, 3.3 ml each, were collected

and analyzed by SDS-PAGE with Coomassie brilliant blue R-250 staining. Fractions that contained cauxin were pooled and concentrated by using a Centriprep with a 30 kDa cutoff membrane (Millipore, Billerica, MA).

Aliquots (10 μl) of urine samples of mature cats were resolved by SDS-PAGE (12% polyacrylamide gel) under nonreducing conditions with Coomassie staining. The cauxin band was quantified by using a LAS 2000 image analyzer coupled with Fujix Multigage Bioimaging software (Fuji Film, Tokyo, Japan), and the urinary cauxin concentration was determined using purified cauxin as the standard. Aliquots (10 μl) of urine samples from immature cats were analyzed by western blotting with an anti-cauxin peptide antibody [12]. The cauxin band was quantified using a LAS 2000 image analyzer, and the urinary cauxin concentration was determined using the cauxin standard.

Preparation of Felinine, 3-MBCG, and 3-MBG

Felinine and 3-MBCG from cat urine were purified by two chromatographies. Male cat urine was deproteinized by using a Centriprep with a 3 kDa cutoff membrane (Millipore) and was then applied to a 2.5 × 50 cm Dowex AG50W-X8 column (H^+ form; Bio-Rad, Hercules, CA). The resin was washed with water, and the urinary amino acids and peptides were subsequently eluted with 3 M ammonia. The eluted fractions were dried by a rotary evaporator at 38°C, dissolved in solvent I (0.1% trifluoroacetic acid and 0.2% acetonitrile), and separated by HPLC (CL10A; Shimadzu Corp., Kyoto, Japan) with a 4.6 × 250 mm PEGASIL ODS column (Senshu Corp., Tokyo, Japan) and a linear gradient from 0% to 20% solvent II (0.1% trifluoroacetic acid and 80% acetonitrile) in solvent I for 30 min, starting 15 min after sample injection.

HPLC fractions corresponding to absorbance peaks at 210 nm were collected and analyzed by MALDI-TOF MS by using an AXIMA CFR spectrometer (Shimadzu Corp.). A peak eluted at 9.0% solvent II had an $[M+H]^+$ molecular ion at *m/z* 207.9, which closely matched the calculated molecular weight of felinine (207.29). A peak eluted at 13.2% solvent II had strong molecular ions at *m/z* 265.0 and 287.0, corresponding to the $[M+H]^+$ and $[M+Na]^+$ ions, respectively; these ions were consistent with the calculated molecular weight of 3-MBCG (264.35). Each fraction containing felinine or 3-MBCG was dried by a rotary evaporator and then dissolved in distilled water.

The reverse reaction of γ-glutamyl transferase was used for enzymatic synthesis of 3-MBG from 3-MBCG and γ-glutamyl-alanine (Wako, Osaka, Japan). The synthetic 3-MBG was purified by HPLC as described above. MALDI-TOF MS analysis indicated that a peak eluted at 16.6% solvent II had strong molecular ions at *m/z* 393.8 and 415.8, corresponding to the $[M+H]^+$ and $[M+Na]^+$ ions, respectively. These ions were consistent with the calculated molecular weight of 3-MBG (393.46).

Urinary Concentrations of Felinine, 3-MBCG, and Glycine

Urinary concentrations of felinine, 3-MBCG, and glycine were analyzed by using a JEOL JLC-500 amino acid analyzer (JEOL Ltd., Tokyo, Japan) equipped with a JEOL LC30-DK20 data-analyzing system. The retention time and concentration of felinine, 3-MBCG, and glycine were determined by using purified standards. These concentrations vary with urine volume and were therefore normalized to the urinary creatinine concentrations measured with a creatinine test (Wako). Creatinine is produced from creatine in muscle fibers by a nonenzymatic process and is excreted exclusively in the urine. For statistical analysis, data were analyzed by using Mann-Whitney U tests. Significance was defined as $p < 0.05$.

Cauxin Enzyme Activity Assay

Cauxin purified from urine was mixed with 20 mM 3-MBG or 20 mM 3-MBCG in 100 mM Tris-HCl (pH 7.2) at a concentration of 1.5 mg/ml. The reaction mixture (40 μl final volume) was incubated at 38°C for 10 hr. Aliquots (2 μl) of each reaction mixture were spotted on a silica gel HPTLC plate, which was developed with a solvent system of *n*-butanol:acetic acid:water (4:1:1). Spots were detected by spraying 0.7% ninhydrin in ethanol and heating at 120°C for 3 min. The amount of glycine released was determined by a LAS 2000 image analyzer and was used for the calculation of the hydrolytic activity of cauxin toward 3-MBCG. K_m and maximal velocity (V_{max}) values were determined by using a Lineweaver-Burk plot. For product identification, a large-scale incubation (200 μl) was performed under the same incubation conditions as described above, and the products were separated by HPLC with an ODS column and the gradient elution described in the preparation of felinine, 3-MBCG, and 3-MBG. To estimate the optimal pH for the hydrolytic activity of cauxin toward 3-MBCG, 50 mM Tris-maleate buffer (at various pHs from 5.3 to 8.2) containing 20 mM 3-MBCG was used.

Real-Time Quantitative Reverse Transcriptase-PCR

Kidney mRNA was prepared from six cats: 0.5- and 2-month-old intact male cats; a 3-month-old intact female cat; and 2-year-old intact male, castrated male, and female cats. The cDNA was reverse transcribed from 1 μg mRNA by using a SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Real-time PCR was performed with an ABI PRISM 7900HT Sequence Detection System (TaqMan; Applied Biosystems, Foster City, CA). Primers for cauxin (Accession number: AB045377) were 5′-CCCTATGCTGCACCTCCT CTA-3′ (forward), 5′-TGTGGCATTTCGGAAGTCATT-3′ (reverse), and 5′-CCCTGCGATTTAAG-3′ (TaqMan probe). Primers for cat renal dipeptidase (Accession number: AB208053) were 5′-CAGAACAAGG ATGCCGTGAAG-3′ (forward), 5′-TCCGGATACATCTGGCACATG-3′ (reverse), and 5′-CCCTAGAGCAGATCGA-3′ (TaqMan probe). Primers for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene were used as an internal control to normalize the amount of mRNA in each sample.

GC-MS Analysis of Headspace Gas of Cat Urine

The headspace gas of urine samples taken from three intact males (4 years old), one castrated male (6 years old), three intact females (4 years old), and one immature cat (2 months old) were individually analyzed by a QP-2010 GC-MS (Shimadzu Corp.) with an automated headspace sampler (TurboMatrixHS40, PerkinElmer Life Sciences, Wellesley, MA) and equipped with a 60 m Stabiliwax capillary column (length, 60 m; internal diameter, 0.32 mm; layer thickness, 0.5 μm; Restek, Bellefonte, PA). In the headspace autosampler, 10 μl urine was heated at 200°C for 15 min to allow endogenous volatiles to enter the gas phase. Samples were injected at 210°C (split 1:20), and the oven temperature was held at 40°C for 1 min, then increased to 230°C by 10°C/min and held at 230°C for 10 min. Helium was used as the carrier gas. The mass spectrometer was operated in electron impact (EI) mode with the electron energy set at 70 eV and the source temperature set at 200°C. Spectra were acquired in full scan operation from *m/z* 29 to *m/z* 400. Volatile compounds were identified by using fragmentation patterns of mass spectra compared to those from the NIST mass spectral library and the report by Mattina et al. [26].

Supplemental Data

Supplemental Data include GC-MS analyses (Figures S1–S3) and hydrolytic activity of cat renal dipeptidase toward 3-MBCG (Figure S4) and are available at http://www.chembiol.com/cgi/content/full/13/10/1071/DC1/.

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Accession Numbers

The DNA sequence for cat renal dipeptidase has been deposited in GenBank under accession number AB208053.