Journal of Medicinal Chemistry Article

*J. Med. Chem.* **2010,** *53,* 8727–8733 **8727**
DOI: 10.1021/jm101208x

# Design, Synthesis, and Pharmacological Activity of Nonallergenic Pyrazolone-Type Antipyretic Analgesics

Naoto Uramaru,[†,‡] Hidenari Shigematsu,[‡] Akihisa Toda,[§] Reiko Eyanagi,[§] Shigeyuki Kitamura,[‡] and Shigeru Ohta*[,†]

[†]*Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan,*
[‡]*Nihon Pharmaceutical University, 10281 Komuro, Ina-machi, Kitaadachi-gun, Saitama 362-0806, Japan, and*
[§]*Daiichi College of Pharmaceutical Sciences, 22-1 Tamagawa-cho, Minami-ku, Fukuoka 815-8511, Japan*

*Received September 18, 2010*

To develop novel nonallergenic pyrazolone analgesics, we synthesized a series of compounds in which position 1 of the pyrazolone ring was substituted in place of the original methyl group in order to block the formation of allergenic metabolites via *N*-dealkylation. These pyrazolone analogues were found to show as potent an antipyretic and analgesic effect as antipyrine (AT). In an examination of allergenicity, AT induced a typical skin reaction in guinea pigs, whereas the pyrazolone analogues were inactive. When AT was administered (po) to rats, norantipyrine (NORA) as an active metabolite was detected in the urine, whereas similar administration of the pyrazolone analogues did not afford NORA. We conclude that these novel pyrazolone analogues were nonallergenic because they were not converted to allergenic metabolites in vivo. Because these compounds retain the antipyretic and analgesic activities of AT, they are considered to be promising candidates for nonallergenic antipyretic analgesics.

## Introduction

The pyrazolones are the oldest synthetic pharmaceuticals, and antipyrine (AT)[a], which has a pyrazolone skeleton, was first prepared by Knorr as an alternative to quinine in 1883.[1] Their derivatives, such as AT, aminopyrine, isopropylantipyrine, and sulpyrine (Figure 1), are widely used as nonsteroidal anti-inflammatory drugs that exhibit potent antipyretic and analgesic activities.[2−4] Unfortunately, however, the pyrazolones are allergenic, and various side effects, including anaphylaxis,[5] cutaneous reactions,[5,6] and agranulocytosis,[5,7] have been reported. Consequently, the clinical use of pyrazolones is restricted in several countries.

The metabolic pathways of pyrazolones have been well clarified by many researchers (Scheme 1),[8−12] and it is considered that reactive intermediates generated by metabolic activation are converted to dimer, trimer, and conjugates.[13,14] For example, AT was converted to conjugates, dimer, and trimer derived via norantipyrine (NORA).[14] NORA itself is an unstable and active metabolite. Also, we reported that NORA has antigenicity in guinea pig skin reaction.[14] These results strongly suggested that pyrazolones become antigenic after binding to biomacromolecules following metabolic activation. We hypothesized that *N*-demethylation is the key reaction in the pathway leading to allergenic products and that blocking of *N*-demethylation at position 1 of the pyrazolone ring would be an effective approach to prevent allergenicity.

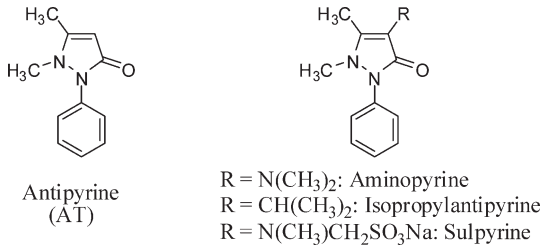



**Figure 1.** Basic structures of pyrazolone derivatives.

Therefore, we designed and synthesized several pyrazolone compounds with substituents that were expected to block *N*-elimination reaction. The antipyretic and analgesic activities of these pyrazolone analogues were examined in animal models of fever and inflammation, and skin allergenicity was examined in guinea pigs. Furthermore, metabolism of these compounds by rats was examined in vivo and in vitro.

## Results

**Chemistry.** Pyrazolone derivatives **1**, **2**, and **3** were synthesized from hydrazine derivatives and diketene in chloroform with triethylamine (Scheme 2) based on the method of Kato et al..[15] Compound **1** or **2** was synthesized from 1-phenyl-2-(2,2,2-trifloroethyl) hydrazine or 1,2-diphenyl hydrazine, respectively, in satisfactory yield. Compound **3** was synthesized from 1-(1,1-dimethylethyl)-2-phenyl hydrazine and diketene; two pyrazolone derivatives of the same molecular weight were obtained, and their structures were elucidated as 1-(1,1-dimethylethyl)-5-methyl-2-phenyl-1,2-dihydro-3*H*-pyrazol-3-one (**3a**) and 2-(1,1-dimethylethyl)-5-methyl-1-phenyl-1,2-dihydro-3*H*-pyrazol-3-one (**3b**) using a combination of

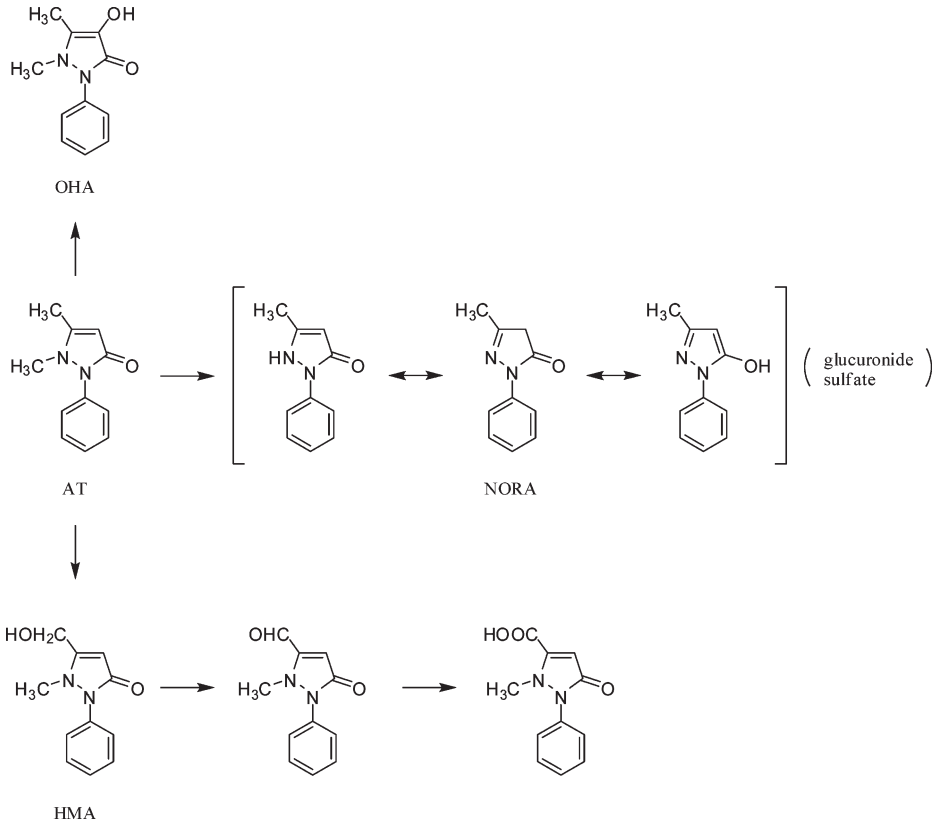
*To whom correspondence should be addressed. Phone: +81-82-257-5325. Fax: +81-82-257-5329. E-mail: sohta@hiroshima-u.ac.jp.

[a] Abbreviations: AT, antipyrine; NORA, norantipyrine; NOESY, nuclear Overhauser effect spectroscopy; IL-1$\beta$, interleukin-1$\beta$; MF, mefenamic acid; ipl, intraplantar; IM, indometacin; id, intradermal; NADPH, $\beta$-nicotinamide-adenine dinucleotide phosphate reduced form; HPLC, high-performance liquid chromatography; HMA, hydroxymethylantipyrine; OHA, hydroxyantipyrine; TLC, thin layer chromatography; IR, infrared spectrometry; $^1$H NMR, $^1$H nuclear magnetic resonance; TMS, tetramethylsilane; HRMS, high-resolution mass spectra; icv, intracerebroventricular.

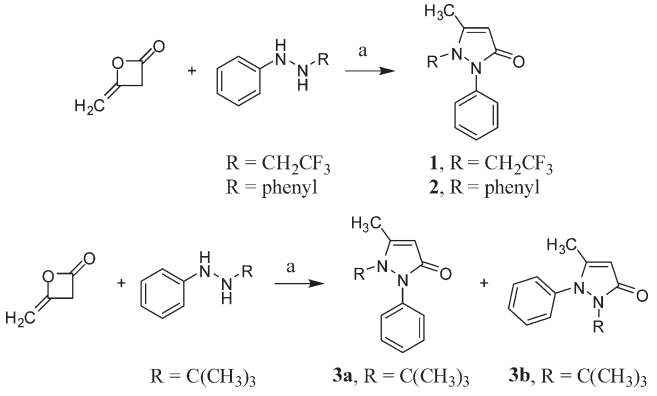


Published on Web 12/01/2010

**Scheme 1.** Metabolic Pathways of AT




**Scheme 2.** Synthesis of Pyrazolone Derivatives **1**, **2**, and **3**[a]




[a] Reagents and conditions: (a) diketene, triethylamine, $CHCl_3$, reflux.

2D NMR techniques and nuclear Overhauser effect spectroscopy (NOESY).

**Effect on Body Temperature of Rats with Interleukin-1$\beta$ (IL-1$\beta$)-Induced Fever.** The mean temperatures of the experimental animals, measured rectally, were 38.13 ± 0.06 °C at 30 min before treatment. The body temperatures of the IL-1$\beta$-treated rats significantly increased (+1.75 ± 0.18 °C) within 2 h after injection of IL-1$\beta$. Administration of mefenamic acid (MF) (100 mg/kg) or AT (100 mg/kg) significantly decreased the IL-1$\beta$-induced fever, restoring the body temperature to the level prior to IL-1$\beta$ administration from 2 h. Compounds **1**, **2**, and **3a** showed dose-dependent antipyretic effects, and at the dose of 50 or 100 mg/kg, they were as potent as MF (100 mg/kg) or AT (100 mg/kg) after 2 h (Figure 2).

**Analgesic Activity of Antipyrine and the Analogues on Inflamed Tissue.** Hyperalgesia was induced by intraplantar (ipl) injection of 0.1 mL of carrageenin (10 mg/mL) into the right hindpaw. The nociceptive threshold of the carrageenin-treated hindpaw decreased to about 30% of the basal value at 1 h, and this was maintained for at least 3 h, followed by a slow recovery. The carrageenin-induced nociception in the hyperalgesic rat was completely blocked by indometacin (IM) (2 mg/kg) and AT (100 mg/kg). Compounds **1**, **2**, and **3a** (100 mg/kg) also significantly suppressed the nociceptive threshold from 1 h after administration, being as potent as IM and AT (Figure 3).

**Guinea Pig Skin Reaction.** The antigenicity of AT and compounds **1**, **2**, and **3a** was examined using the guinea pig skin reaction test to evaluate the delayed skin reaction on day 21 after sensitization with NORA. AT (1 mg/body) elicited a significant skin reaction at 24 h after intradermal (id) injection, whereas compounds **1**, **2**, and **3a** (1 mg/body) did not. Figure 4 shows photographs of the excised abdominal skin.

***N*-Demethylation of Antipyrine and the Analogues by Rat Microsomes.** AT was incubated with liver microsomes of untreated rats in the presence of $\beta$-nicotinamide-adenine dinucleotide phosphate reduced form (NADPH) to obtain metabolites as described in the Experimental Section. Three peaks, which were not detected in the control, were found on the high-performance liquid chromatography (HPLC) of the extract of incubation mixtures. The retention times of 3.0, 5.4, and 9.4 min corresponded to those of hydroxymethylantipyrine (HMA), NORA, and hydroxyantipyrine (OHA), respectively (Figure 5a). The amounts of *N*-demethylation products increased linearly for 20 min (data not shown). When AT was incubated, HMA and OHA were produced at similar levels of about 40 nmol/20 min/mg

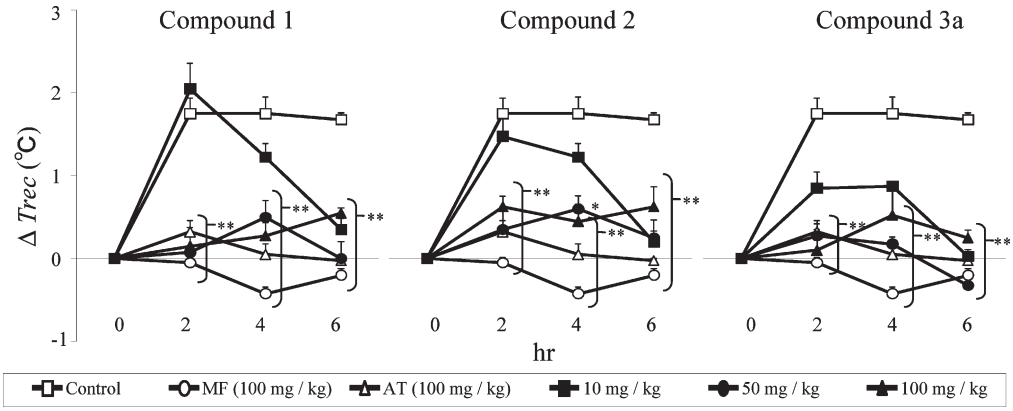



**Figure 2.** Changes in the rectal temperature of rats. Each pain threshold value represents the mean ± SEM of four rats. AT (100 mg/kg), MF (100 mg/kg), and compounds **1**, **2**, and **3a** (10−100 mg) were administered orally, immediately after the icv injection of IL-1$\beta$ (200 units). Rectal temperature was measured in the large intestine 7 cm above the anus at 2 h intervals. $\Delta T_{rec}$ was calculated as described in the Experimental Section. *$p < 0.05$, **$p < 0.01$ compared with control.

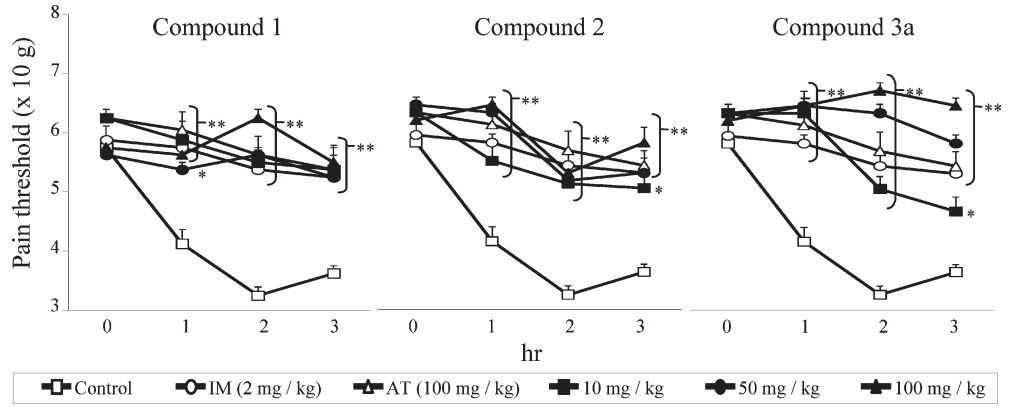



**Figure 3.** Analgesic effects in rats, evaluated by means of Randall−Selitto's method. Each pain threshold value represents the mean ± SEM of four rats. AT (100 mg/kg), IM (2 mg/kg), and compounds **1**, **2**, and **3a** (10−100 mg/kg) were administered orally, immediately after ipl injection of 0.1 mL of carrageenin (10 mg/mL) into the right hindpaw. Analgesic effect was calculated as described in the Experimental Section. *$p < 0.05$, **$p < 0.01$ compared with control.

protein and NORA was formed at a level of 27 nmol/20 min/mg protein (Figure 5b). When compounds **1**, **2**, and **3a** were incubated under the same conditions, the amount of NORA formed was below the HPLC detection limit (0.1 nmol/mL in this experiment equivalent to 0.1 nmol/20 min/mg protein) (Figure 5c).

**Metabolism of Antipyrine and the Analogues in Rats in Vivo.** When AT was administered to rats, four peaks, which were not detected in the control, were detected in an HPLC chromatogram of the extract of the urine. The retention times of 3.0, 5.4, 6.0, and 9.4 min corresponded to these of HMA, NORA, AT, and OHA, respectively (Figure 6a). NORA was detected in AT (100 mg/kg) treated rats, at a level corresponding to approximately 2% of the dose of AT, together with AT, HMA, and OHA (Figure 6b). On the other hand, NORA was not detected in the urine of rats administered compounds **1**, **2**, and **3a** (100 mg/kg), i.e., it was below the HPLC detection limit (0.2 nmol/mL in this experiment) (Figure 6c).

## Discussion and Conclusion

The newly synthesized pyrazolone analogues **1**, **2**, and **3a** (100 mg/kg) showed antipyretic activity equivalent to that of AT (100 mg/kg) in a rat fever model. Further, they showed equivalent analgesic effect to AT in a rat model of carrageenin-induced paw inflammation after single oral administration at the same dose. Therefore, the antipyretic and analgesic effects were retained irrespective of the substituent at position 1. On the other hand, compounds **1**, **2**, and **3a** were not metabolized to NORA in vivo or in vitro, as expected, and did not elicit an allergic reaction in guinea pigs, unlike AT.

Drug allergy is rare, but seriously and potentially life-threatening. There are many reports that T-lymphocytes react specifically to low-molecular-weight compounds, even though peptide structures are required for antigen presentation.[16,17] The reason for such hypersensitivity is that some low-molecular-weight drugs are metabolically activated and form complexes with proteins in vivo.[18,19] Himly et al. reported that biomacromolecules bearing norantipyrine as an antigen were formed following isopropylantipyrine administration, and this supports the idea that the allergenicity of antipyrine involves a similar mechanism.[20]

We found that the hydroxylation reaction at position 4 of the pyrazolone ring is the main metabolic pathway of AT in rats, together with oxidation at position 5 and demethylation at position 1, both in vitro and in vivo, in agreement with the findings of Shimeno et al.[21] It is thought that the *N*-demethylation is due to deprotonation of the α-carbon, leading to the formation of NORA by *N*-elimination reaction.

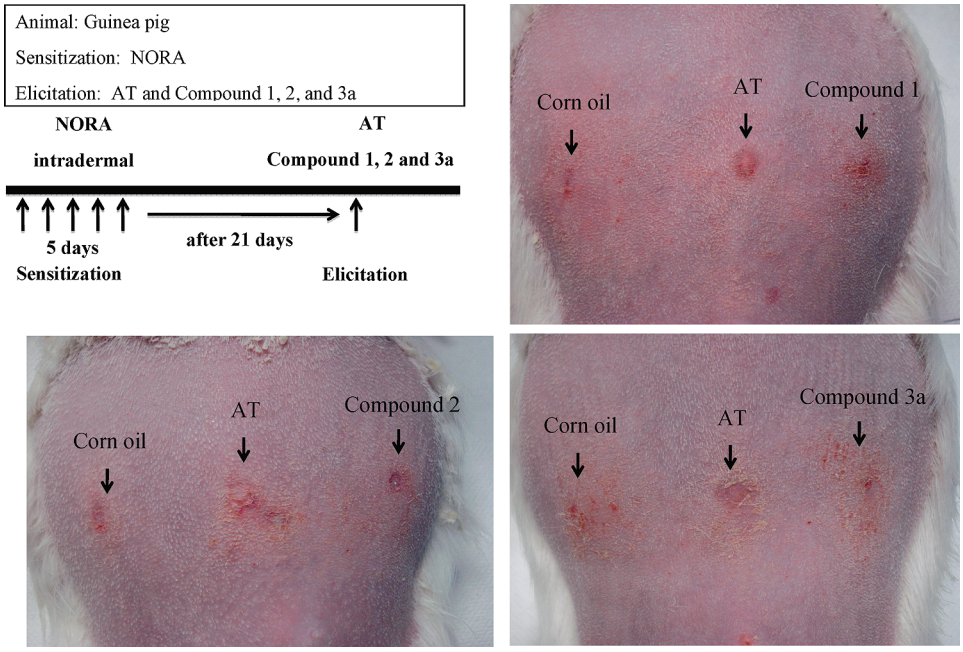



**Figure 4.** Elicitation of skin reaction in guinea pigs sensitized with AT. Sensitization and elicitation were performed as described in the Experimental Section. The photograph shows erythema at 24 h after id injection of 0.1 mL of corn oil, or a corn oil solution of AT (1 mg/body) or compounds **1**, **2**, and **3a** (1 mg/body).

However NORA was not detected as a metabolite of the newly synthesized pyrazolone analogues, suggesting that these compounds are metabolically stable in this respect, as anticipated. As NORA is considered to mediate the allergenicity of AT, compounds **1**, **2**, and **3a** should not show NORA-mediated allergenicity, and indeed, they did not elicit any allergic reaction in the guinea pig skin test. Thus, we have obtained pyrazolone derivatives which retain the antipyretic and analgesic effects of AT but lack the allergenicity of AT, as summarized in Figure 7.

In conclusion, our results confirm the validity of our strategy of blocking metabolic activation of pyrazolones by means of structural modification to obtain compounds that can not be metabolically activated to bind to biomacromolecules and so do not show allergenicity but retain potent antipyretic and analgesic activities. These new pyrazolones are considered to be candidate nonallergenic analgesic agents.

## Experimental Section

**General Methods.** All commercial chemicals and solvents are reagent grade and were used without further purification. The residue was purified by silica gel column (63−200 $\mu$m) chromatography or recrystallized from an appropriate solvent. The progress of reactions was monitored by thin layer chromatography (TLC) using commercially prepared silica gel 60 $F_{254}$ glass-backed plates. Compounds were visualized under ultraviolet light. Male Sprague−Dawley rats (weighing 210−240 g) and male Hertly guinea pigs (weighing 350−400 g) were purchased from Japan SLC Inc.. Urine was stored at −80 °C until analyzed by HPLC. Melting points were determined with a Yanagimoto hot-stage melting point apparatus and are uncorrected. Infrared spectrometry (IR) spectra were recorded on a Shimadzu FTIR-8400S (KBr). The $^1$H nuclear magnetic resonance ($^1$H NMR) spectra and NOESY data were obtained on a JEOL JMN-ECA500 spectrometer. Proton chemical shifts were referenced to the tetramethylsilane (TMS) internal standard. *J* values are given in Hz. High-resolution mass spectra (HRMS) were obtained with a JEOL JMS-AX 505 W double-focusing spectrometer interfaced to DA win data system. Elemental analysis was carried out with a PerkinElmer 2400II CHNS/O recorder, and results were within ±0.4% of the theoretical values. The analytical HPLC system consisted of SPD-20A Shimadzu chromatograph and equipped with a CAPCELLPAK C18 UG120 column (Shiseido Co., Ltd., Japan), 5 $\mu$m (4.6 mm × 250 mm). Elution was performed with 0.1 M sodium acetate aqueous solution, adjusted with acetic acid to pH 6.6, and acetonitrile (85:15, v/v). The flow rate was 2 mL/min, and the column temperature was set to 30 °C.[22] Column effluents were monitored at a wavelength of 254 nm. The determination of the substrate and metabolites, AT, HMA, NORA, and OHA were used authentic samples. The limits of detection (*S*/*N* value of 3) of NORA in the in vivo and in vitro experiments were 0.2 nmol/mL and 0.1 nmol/mL, respectively.

**General Procedure for Synthesis of Pyrazolone Derivatives.**[15] A mixture of hydrazine derivative (0.01 mol) and diketene (0.01 mol) in chloroform (30 mL) was stirred at room temperature. Triethylamine (0.01 mol) was added, and the reaction mixture was refluxed for 3 h. After evaporation of the solvent, the residue was dissolved in ethyl acetate (100 mL), and the resulting solution was extracted with 10% hydrochloric acid. The extract was basified with 5 M sodium hydroxide in an ice bath and extracted with ethyl acetate. The organic layer was washed with $H_2O$ and brine, dried over $MgSO_4$, and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel (*n*-hexane/ethyl acetate) or recrystallized from *n*-hexane/ethyl acetate to give the corresponding pyrazolone derivative.

**5-Methyl-2-phenyl-1-(2,2,2-trifluoroethyl)-1,2-dihydro-3*H*-pyrazol-3-one (1).** According to the general procedure, compound **1** was obtained in 68% yield as white needles after recrystallization from *n*-hexane/ethyl acetate. mp 91−92 °C. IR (KBr) cm$^{-1}$: 1676 (CO). $^1$H NMR (500 MHz, $CDCl_3$-*d*) $\delta$: 7.48 (t, 2H, *J* = 10.0 Hz, Ar*H*), 7.36−7.32 (m, 3H, Ar*H*), 5.53 (s, C*H*), 4.09 (q, *J* = 5.0 Hz, $CF_3CH_2$), 2.31(s, $CH_3$). HRMS (EI) *m*/*z* calcd for $C_{12}H_{11}F_3N_2O$ 256.0823; found 256.0814 [M$^+$]. Anal. Calcd for $C_{12}H_{11}F_3N_2O$: C, 56.25; H, 4.33; N, 10.93. Found: C, 56.20; H, 3.99; N, 10.92.

**5-Methyl-1,2-diphenyl-1,2-dihydro-3*H*-pyrazol-3-one (2).** According to the general procedure, compound **2** was obtained in 80% yield as white needles after recrystallization from *n*-hexane/

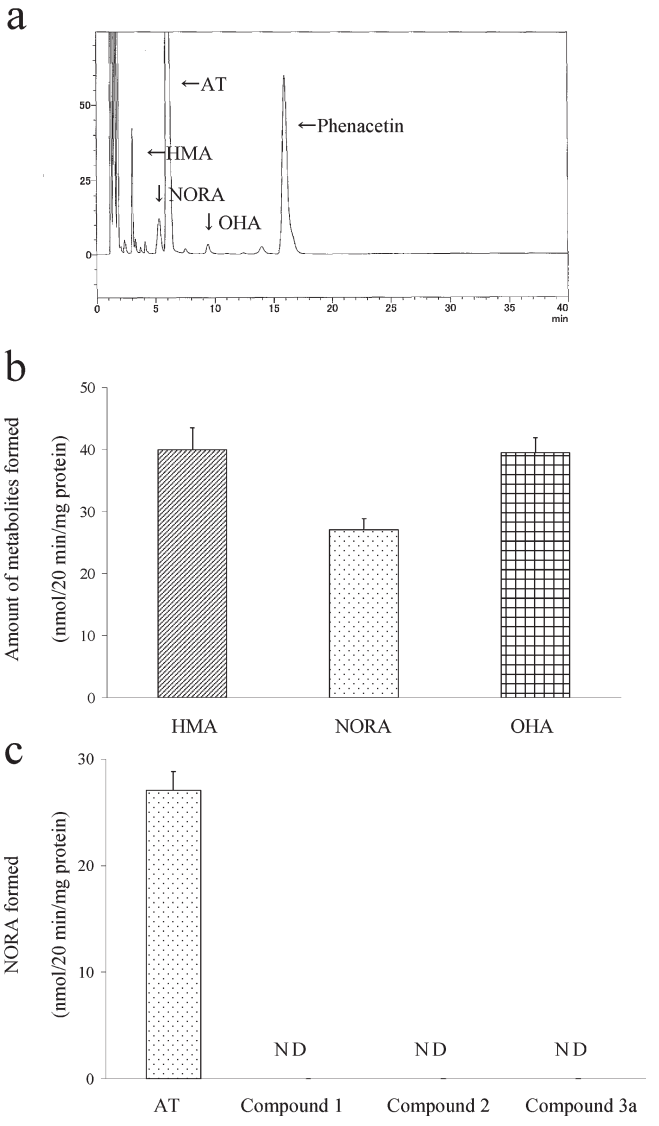



**Figure 5.** In vitro metabolism of AT by rat liver microsomes. (a) HPLC chromatogram of HMA, NORA, AT, and OHA after incubation of AT with rat microsomes; for details, see the Experimental Section. (b) Quantitative analysis of metabolites. Each bar represents the mean ± SEM of four rats. (c) Comparison of formation of NORA from AT and compounds **1**, **2**, and **3a**. ND: not detected.

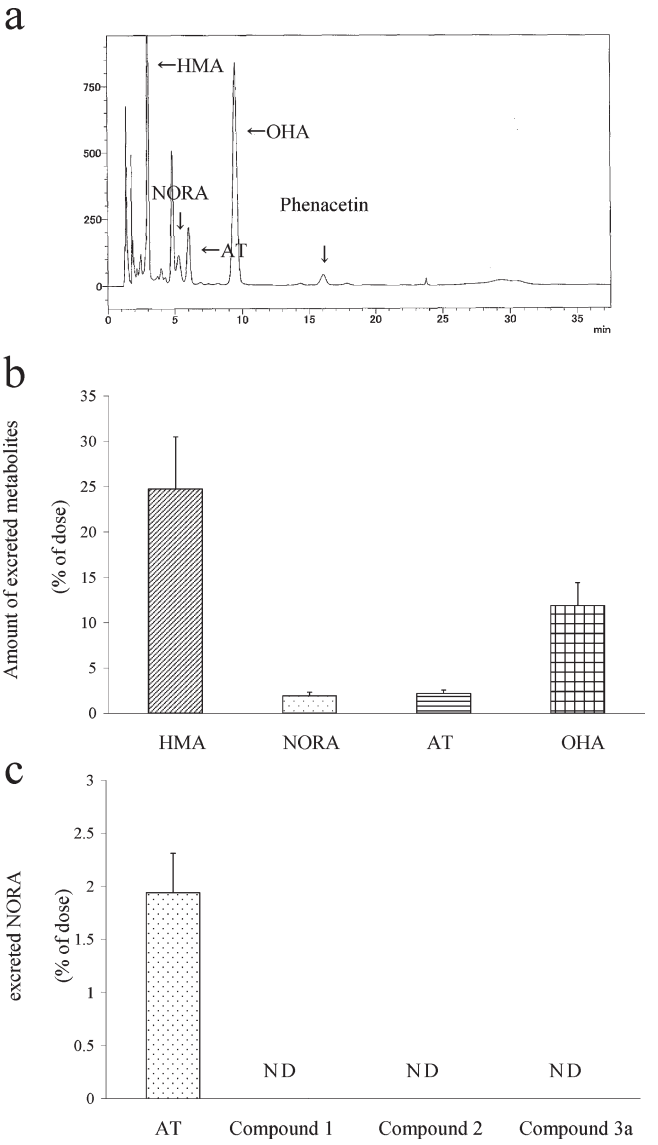



**Figure 6.** In vivo metabolism of AT by rats. (a) HPLC chromatogram of extract of rat urine after administration of AT; for details, see the Experimental Section. (b) Quantitative analysis of metabolites. Each bar represents the mean ± SEM of four rats. (c) Comparison of formation of NORA from AT and compounds **1**, **2**, and **3a**. ND: not detected.

ethyl acetate. mp 126−127 °C. IR (KBr) $cm^{-1}$: 1664 (CO). $^1H$ NMR (500 MHz, $CDCl_3$-*d*) $\delta$: 7.37−7.09 (m, 10H, Ar*H*), 5.56 (s, C*H*), 2.08 (s, $CH_3$). HRMS (EI) *m*/*z* calcd for $C_{16}H_{14}N_2O$ 250.1106; found 250.1099 [$M^+$]. Anal. Calcd for $C_{16}H_{14}N_2O \cdot {}^1/_2H_2O$: C, 74.11; H, 5.83; N, 10.80. Found: C, 74.13; H, 5.76; N, 10.79.

**1-(1,1-Dimethylethyl)-5-methyl-2-phenyl-1,2-dihydro-3*H*-pyrazol-3-one (3a).** According to the general procedure, compound **3a** was obtained in 36% yield as white needles after column chromatography (eluent: *n*-hexane/ethyl acetate 6/1); mp 129−130 °C. IR (KBr) $cm^{-1}$: 1678 (CO). $^1H$ NMR (500 MHz, $CDCl_3$-*d*) $\delta$: 7.38−7.34 (m, 4H, Ar*H*), 7.20 (t, 1H, $J$ = 5.0 Hz, Ar*H*), 5.41 (s, C*H*), 2.40(s, $CH_3$), 1.24 (s, $C(CH_3)_3$). HRMS (EI) *m*/*z* calcd for $C_{14}H_{18}N_2O$ 230.1419; found 230.1418 [$M^+$]. Anal. Calcd for $C_{14}H_{18}N_2O \cdot {}^1/_4H_2O$: C, 71.61; H, 7.94; N, 11.93. Found: C, 71.60; H, 8.14; N, 11.55. $R_f$ (*n*-hexane/ethyl acetate 1/1) = 0.17.

**2-(1,1-Dimethylethyl)-5-methyl-1-phenyl-1,2-dihydro-3*H*-pyrazol-3-one (3b).** According to the general procedure, compound **3b** was obtained in 14% yield as yellow needles after column chromatography (eluent: *n*-hexane/ethyl acetate 6/1); mp 127−128 °C. IR (KBr) $cm^{-1}$: 1663 (CO). $^1H$ NMR (500 MHz, $CDCl_3$-*d*) $\delta$: 7.38 (t, 2H, Ar*H*, $J$ = 10.0 Hz), 7.29 (t, 1H, Ar*H*, $J$ = 5.0 Hz), 7.21 (d, 2H, Ar*H*, $J$ = 5.0 Hz), 5.41 (s, C*H*), 1.83 (s, $CH_3$), 1.40 (s, $C(CH_3)_3$). HRMS (EI) *m*/*z* calcd for $C_{14}H_{18}N_2O$ 230.1419; found 230.1416 [$M^+$]. Anal. Calcd for $C_{14}H_{18}N_2O \cdot {}^1/_4H_2O$: C, 71.61; H, 7.94; N, 11.93. Found: C, 71.92; H, 8.06; N, 11.68. $R_f$ (*n*-hexane/ethyl acetate 1/1) = 0.29.

**Cannula Implantation.**[23] The animals were anesthetized with diethylether and positioned in a stereotaxic apparatus (SR-5R, Narishige Scientific Instrument Lab., Japan). The overlying skin and connective tissue were cleared from the skull. A hole was drilled in the skull and a 22-G stainless steel guide cannula was inserted unilaterally into the lateral ventricle. The stereotaxic coordinates used for cannula placement were 0.8 mm posterior to bregma, 1.5 mm lateral from midline, and 4.2 mm below the surface of the skull. The cannula was secured using dental cement (Quick resin, Shofu Inc., Japan) and anchored to the skull with two stainless steel screws. A wire dummy cannula was used to seal the guide cannula. The animals were provided with

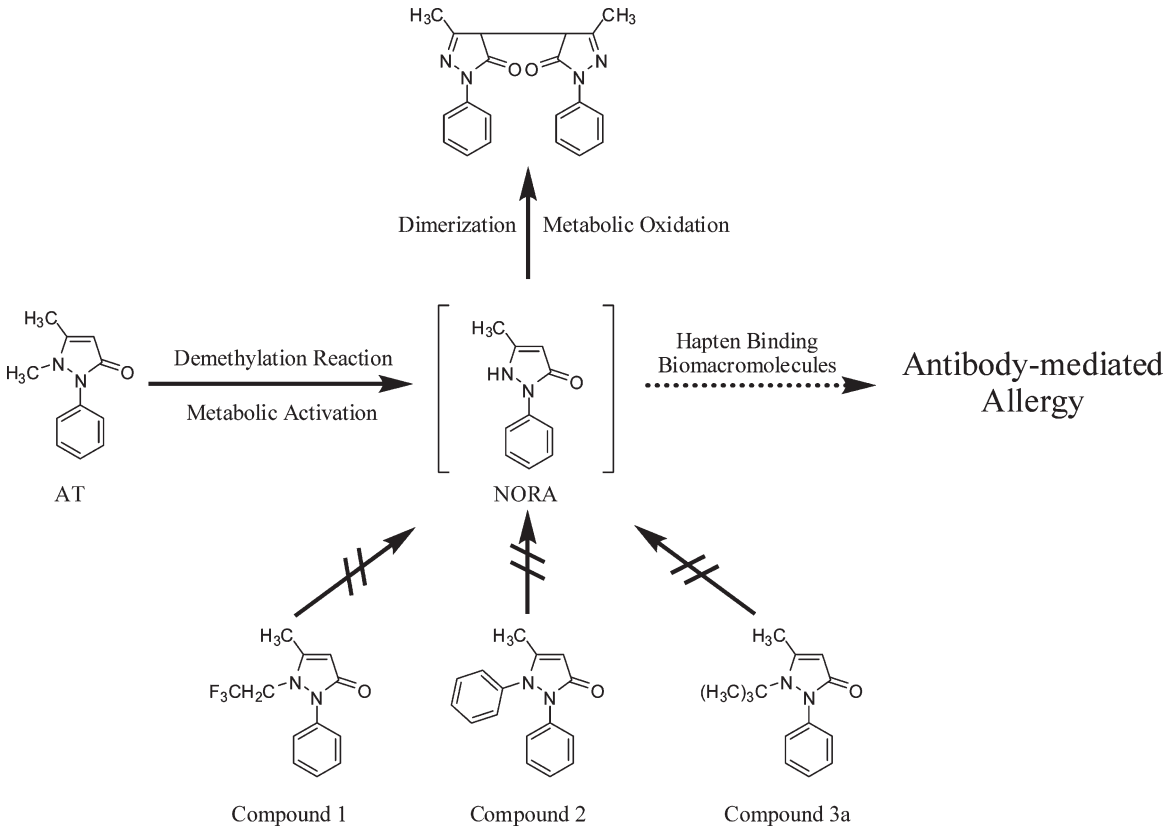



**Figure 7.** Summary of the effect of various substituents at position 1 of pyrazolone analgesics on the allergenicity of the compounds.

food and water ad libitum and were allowed to recover in individual cages for 7 days. Proper positioning of the cannula was verified at the end of experiment by intracerebroventricular (icv) injection of methylene blue.

**Induction of Fever and Drug Administration.**[23] Fever was induced by repeated injection of IL-1$\beta$ (200 units/rat) dissolved in sterile pyrogen-free 10 mM sodium phosphate buffer containing 0.15 M sodium chloride (PBS, pH 7.5) into the lateral ventricle of the brain. The degree of fever was assessed by measuring rectal temperature with a thermoelectric monitor (CTM-303, TERUMO, Japan) via a thermal probe inserted to a depth of 7 cm. To adapt animals to the technique and to minimize stress-induced hyperthermia, the rectal temperature was measured twice a day for 3 days before the IL-1$\beta$ injection. Changes in the rectal temperature ($\Delta T_{rec}$) were calculated from the following formula: $\Delta T_{rec} = T - T_0$, where $T_0$ and $T$ represent the rectal temperatures before and after IL-1$\beta$ injection, respectively. All experiments were started at 8:00 to minimize the influence of possible circadian variations.

**Nociceptive Assay.**[24,25] The paw pressure test described by Randall and Selitto was applied to rats.[24] Carrageenin is a natural polysaccharide (carbohydrate) extracted from red seaweed. It is commonly used to induce acute inflammation in experimental animal models.[25] An analgesy meter (37215, Ugo Basile Srl, Italy) with a pencil-shaped wooden paw-presser with a dull tip was used; pressure was gradually applied to the hind paw at an increasing linear rate of 16 g/s. The pressure required to elicit nociceptive responses, such as squeaking and struggling, was determined as the mechanical nociceptive threshold. A cutoff value of 250 g was used to prevent damage to the paw. The nociceptive threshold of each rat was measured 6−8 times, and only rats with stable thresholds were used in experiments. The control threshold for each rat was defined as the mean of the values of the last four stable thresholds because the initial 2−4 values were, in general, high and unstable. Results are expressed as a percentage of the control threshold.

**Skin Reaction in Guinea Pigs.**[26] Sensitization: a corn oil solution of NORA (10 mg/mL) was administered subcutaneously to the back of three guinea pigs at a volume of 0.1 mL of 10 mg/mL solution per site (total volume of 1 mL). Elicitation: 21 days after sensitization, a corn oil solution of AT (1 mg/body) or compound **1**, **2**, or **3a** (1 mg/body), or corn oil alone as a control, at a volume of 0.1 mL was intradermally injected into the side of the abdomen of guinea pigs shaved with an electric clipper and hair remover. Erythema at the injected site was observed at 24 h after id injection.

**Preparation of Microsomal Fraction from Rat Liver.**[27] Livers were excised and homogenized in four volumes of 1.15% KCl with a Potter−Elvehjem homogenizer. The homogenate was centrifuged for 20 min at 9000$g$, and for 60 min at 105000$g$ successively to prepare the microsomal and cytosolic fractions. The microsomal fraction was washed by resuspension in the KCl solution and resedimented. The microsomal pellet was resuspended in the solution to make 1 mL equivalent to 1 g of liver. Protein content was determined by the method of Lowry et al. with bovine serum albumin as a standard protein.[28]

**Assay of Liver Microsomal Oxidase Activity.**[29] The incubation mixture consisted of 2 $\mu$mol of AT or an analogue, 10 $\mu$mol of NADPH, 4 mg of $Na_2S_2O_5$, and 0.2 mL of liver microsomes equivalent to 200 mg of liver wet weight (2−2.5 mg protein) in a final volume of 2 mL of 0.1 M K/Na phosphate buffer (pH 7.4). Incubation was performed at 37 °C for 20 min. After addition of 670 $\mu$L of 20% trichloroacetic acid, the samples were centrifuged for 10 min at 4000 rpm. After addition of 80 mg of $Na_2S_2O_5$ and 0.4 mL of 4 M NaOH to the supernatant, unchanged AT was extracted 3 times with 5 mL of toluene. The pH of the aqueous phase was then adjusted to 7.0 by addition of 4 mL of 1 M potassium phosphate buffer (pH 6.0). After addition of 1.3 g of NaCl, the samples were vortexed for at least 30 s. Then 50 nmol of phenacetin was added as an internal standard, and the metabolites were extracted with 5 mL of chloroform/methanol 9:1 (v/v). This extraction step was repeated once more with

5 mL of chloroform/methanol 9:1 (v/v). The organic phases from the two extractions were combined and evaporated to dryness, the residue was dissolved in 0.2 mL of equal parts of methanol and distilled water, and an aliquot (20 $\mu$L) was analyzed by HPLC.

**Assay of Urinary Metabolites.**[30] AT or an analogue was given orally to rats at a single dose of 100 mg/kg, and urine was collected for 24 h. Then, a mixture of 1 mL of urine, 4 mg of $Na_2S_2O_5$, and 40 $\mu$L of $\beta$-glucuronidase/sulphatase in a final volume of 4 mL of 0.1 M acetate buffer (pH 4.5) was incubated at 37 °C for 24 h. Then 50 nmol of phenacetin was added as an internal standard. The incubation mixture was extracted with 4 mL of chloroform/methanol 9:1 (v/v). The extract was evaporated to dryness, the residue was dissolved in 0.2 mL of equal parts of methanol and distilled water, and an aliquot (20 $\mu$L) was analyzed by HPLC.

**Statistical Analysis.** All data were expressed as the mean ± SEM. The statistical significance of differences was evaluated by means of two-way repeated-measures analysis of variance (ANOVA) followed by contrasts for mean values comparison. A $p$ value $< 0.05$ was considered significant.

**Acknowledgment.** We are grateful to the SC-NMR Laboratory of Nihon Pharmaceutical University for conducting the NMR experiment.

## References

(1) Knorr, L. Einwirk von acetessigester auf phenylhydrazin. *Ber. Dtsch. Chem. Ges.* **1883**, *16*, 2597–2599.
(2) Beaver, W. T. Mild analgesics. A review of their clinical pharmacology. *Am. J. Med. Sci.* **1965**, *250*, 577–604.
(3) Brogden, R. N. Pyrazolone derivatives. *Drugs* **1986**, *32*, 60–70.
(4) Volz, M.; Kellner, H. M. Kinetics and metabolism of pyrazolones (propylphenazone, aminopyrine and dipyrone). *Br. J. Clin. Pharmacol.* **1980**, *10*, 299S–308S.
(5) Levy, M. Hypersensitivity to pyrazolones. *Thorax* **2000**, *55*, S72–S74.
(6) Voigtlander, V. Adverse dermatological reactions to pyrazolone. *Agents Acti. Suppl.* **1986**, *19*, 303–311.
(7) Pisciotta, A. V. Drug-induced agranulocytosis. Peripheral destruction of polymorphonuclear leucocytes and their marrow precursors. *Blood Rev.* **1990**, *4*, 226–237.
(8) Agúndez, J. A.; Martínez, C.; Benítez, J. Metabolism of aminopyrine and derivatives in man: in vivo study of monomorphic and polymorphic metabolic pathways. *Xenobiotica* **1995**, *25*, 417–427.
(9) Bottcher, J.; Bassmann, H.; Schuppel, R. Quantitation of norantipyrine sulphate and norantipyrine glucuronide as major metabolites of antipyrine in man and rat. *J. Pharm. Pharmacol.* **1984**, *36*, 391–398.
(10) Bottcher, J.; Bassmann, H.; Schuppel, R. Quantitation and urinary pattern of 4,4′-dihydroxyantipyrine, 4-hydroxyantipyrine and 3-hydroxymethylantipyrine, as main metabolites of antipyrine in man and rat. *J. Pharm. Pharmacol.* **1982**, *34*, 168–175.
(11) Yoshimura, H.; Shimeno, H.; Tsukamoto, H. Metabolism of drugs-LIX. A new metabolite of antipyrine. *Biochem. Pharmacol.* **1968**, *17*, 1511–1516.
(12) Yoshimura, H.; Shimeno, H.; Tsukamoto, H. Metabolism of Drugs. LXX. Further study on antipyrine metabolism. *Chem. Pharm. Bull.* **1971**, *19*, 41–45.
(13) Shigematsu, H.; Hisanari, Y.; Yoshida, K.; Shimeno, H. Delayed allergic reactions with aminopyrine metabolites. *Skin Res.* **1988**, *30*, 95–100.
(14) Shigematsu, H.; Hisanari, Y.; Eyanagi, R.; Toshitani, S. Sensitization and elicitation of delayed allergic skin reaction with antipyrine metabolite. *Jpn. J .Dermatoallergol.* **1994**, *1*, 278–287.
(15) Kato, T.; Sato, M.; Tabei, K.; Kawashima, E. Studies on ketene and its derivatives. LXIX. Reaction of diketene with hydrazobenzenes. *Chem. Pharm. Bull.* **1975**, *23*, 456–459.
(16) Hertl, M.; Merk, H. F. Lymphocyte activation in cutaneous drug reactions. *J. Invest. Dermatol.* **1995**, *105*, 95S–98S.
(17) Weltzien, H. U.; Padovan, E. Molecular features of penicillin allergy. *J. Invest. Dermatol.* **1998**, *110*, 203–206.
(18) Park, B. K.; Kitteringham, N. R.; Powell, H.; Pirmohamed, M. Advances in molecular toxicology—towards understanding idiosyncratic drug toxicity. *Toxicology* **2000**, *153*, 39–60.
(19) Park, B. K.; Pirmohamed, M.; Kitteringham, N. R. The role of cytochrome P450 enzymes in hepatic, extrahepatic human drug toxicity. *Pharmacol. Ther.* **1995**, *68*, 385–424.
(20) Himly, M.; Jahn-Schmid, B.; Pittertschatscher, K.; Bohle, B.; Grubmayr, K.; Ferreira, F.; Ebner, H.; Ebner, C. IgE-mediated immediate-type hypersensitivity to the pyrazolone drug propylphenazone. *J. Allergy Clin. Immunol.* **2003**, *111*, 882–888.
(21) Shimeno, H.; Yoshimura, H. Metabolism of drugs. LXXVII. In vitro metabolism of antipyrine. *Yakugaku Zassi* **1972**, *92*, 1376–1379.
(22) Mikati, M. A.; Szabo, G. K.; Pylilo, R. J.; LeDuc, B. W.; Browne, T. R.; Greenblatt, D. J. Improved high-performance liquid chromatographic assay of antipyrine, hydroxymethylantipyrine, 4-hydroxyantipyrine and norantipyrine in urine. *J. Chromatogr.* **1988**, *433*, 305–311.
(23) Kihara, T.; Toda, A.; Umesue, I.; Ono, N.; Shigematsu, H.; Soeda, S.; Shimeno, H. Effect of interleukin 1$\beta$-induced fever on hepatic drug metabolism in rat. *Xenobiotica* **1998**, *28*, 559–569.
(24) Randall, L. O.; Selltto, J. J. A method for measurement of analgesic activity on inflamed tissue. *Arch. Int. Pharmacodyn. Ther.* **1957**, *111*, 409–419.
(25) Oh-ishi, S.; Hayashi, I.; Utsunomiya, I.; Hayashi, M.; Yamaki, K.; Yamasu, A.; Nakano, T. Roles of kallikrein-kinin system in acute inflammation: studies on high- and low-molecular-weight kininogens-deficient rats (B/N-Katholiek strain). *Agents Act.* **1987**, *21*, 384–386.
(26) Eyanagi, R.; Toda, A.; Ishii, Y.; Saito, H.; Soeda, S.; Shimeno, H.; Shigematsu, H. Antigenicity of sulfanilamide and its metabolites using fluorescent-labelled compounds. *Xenobiotica* **2005**, *35*, 911–925.
(27) Kohno, Y.; Kitamura, S.; Sanoh, S; Sugihara, K.; Fujimoto, N.; Ohta, S. Metabolism of the $\alpha,\beta$-unsaturated ketones, chalcone and *trans*-4-phenyl-3-buten-2-one, by rat liver microsomes and estrogenic activity of the metabolites. *Drug Metab. Dispos.* **2005**, *33*, 1115–1123.
(28) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.
(29) Szakács, T.; Veres, Z.; Vereczkey, L. Effect of phenobarbital and spironolactone treatment on the oxidative metabolism of antipyrine by rat liver microsomes. *Pol. J. Pharmacol.* **2001**, *53*, 11–19.
(30) Danhof, M.; de Boer, A. G.; de Groot-van der Vis, E.; Breimer, D. D. Assay of antipyrine and its primary metabolites in plasma, saliva and urine by high-performance liquid chromatography and some preliminary results in man. *Pharmacology* **1979**, *19*, 215–220.