

Diphenylthiourea, a Common Rubber Chemical, Is Bioactivated to Potent Skin Sensitizers

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Received July 20, 2010

Diphenylthiourea (DPTU) is a known skin sensitizer commonly used as a vulcanization accelerator in the production of synthetic rubber, for example, neoprene. The versatile usage of neoprene is due to the multifaceted properties of the material; for example, it is stretchable, waterproof, and chemical- and abrasion-resistant. The wide application of neoprene has resulted in numerous case reports of dermatitis patients allergic to DPTU. The mechanism by which DPTU works as a contact allergen has not been described; thus, the aim of the present study was to investigate if DPTU is a prohaptens that can be activated by skin metabolism. The metabolic activation and covalent binding of ¹⁴C-labeled DPTU to proteins were tested using a skinlike cytochrome P450 (P450) cocktail containing the five most abundant P450s found in human skin (CYP1A1, 1B1, 2B6, 2E1, and 3A5) and human liver microsomes. The incubations were carried out in the presence or absence of the metabolite trapping agents glutathione, methoxylamine, and benzylamine. The metabolism mixtures were analyzed by LC-radiochromatography, LC-MS, and LC-MS/MS. DPTU was mainly metabolically activated to reactive sulfoxides resulting in desulfurated adducts in both enzymatic systems used. Also, phenylisothiocyanate and phenylisocyanate were found to be metabolites of DPTU. The sensitizing capacity of the substrate (DPTU) and three metabolites was tested in the murine local lymph node assay. Two out of three metabolites tested were strong skin sensitizers, whereas DPTU itself, as previously known, was negative using this mouse model. In conclusion, DPTU forms highly reactive metabolites upon bioactivation by enzymes present in the skin. These metabolites are able to induce skin sensitization and are probable causes for DPTU allergy. To increase the possibilities of diagnosing contact allergy to DPTU-containing items, we suggest that suitable metabolites of DPTU should be used for screening testing.

Introduction

Diphenylthiourea (DPTU)¹ (Scheme 1) is a skin sensitizer included in the patch test series of shoe and rubber chemicals for diagnosis of allergic contact dermatitis (ACD) (1). DPTU is present in various rubber and plastic products due to its use as a stabilizer in the manufacturing of polyvinyl chloride (PVC) and as an accelerator in the production of neoprene. Neoprene has become a widely used material since it is waterproof, stretchable, and chemical- and abrasion-resistant. Its extensive use is mirrored in case reports of ACD to DPTU from various exposures, for example, from contact with wet suits, swim goggles, running shoes, orthopedic devices, mouse pads, and iPod holders (2–9). However, contact allergy caused by rubber products is not only due to DPTU since other derivatives of thioureas can be used in the synthesis of rubber (10).

During the years, the allergenic activity of DPTU has been studied in three different animal models: the murine local lymph node assay (LLNA) (11), the sensitive local lymph node assay (SLNA) (12), and the guinea pig maximization test (GPMT) (13). DPTU was identified as a sensitizer in the GPMT and the SLNA but was found to be nonsensitizing in the LLNA. This disparity is most probably due to differences in the administration of the compound in the LLNA versus the SLNA and the GPMT. In the two latter methods, the test compound is administered intradermally in addition to topical application. Intradermal injections seem to be important to obtain a positive response to DPTU as the penetration of DPTU probably is low when applied topically on intact skin (14).

The majority of contact allergens, known as haptens, are low molecular weight chemicals able to react with nucleophilic groups on proteins to form immunogenic hapten–protein complexes. However, compounds inherently unreactive to proteins may also induce ACD as a result of bioactivation to reactive compounds in the skin. These compounds, prohaptens, contain structural moieties susceptible to metabolic transformation into protein-reactive species able to cause contact allergy (15, 16). For many years, the skin was considered to be an inert passive structural barrier, but it is now obvious that the skin plays a key role in the penetration and metabolism of topically applied compounds (15, 17–19). Bioactivation generally involves oxidative processes that are attributed to monooxyge-

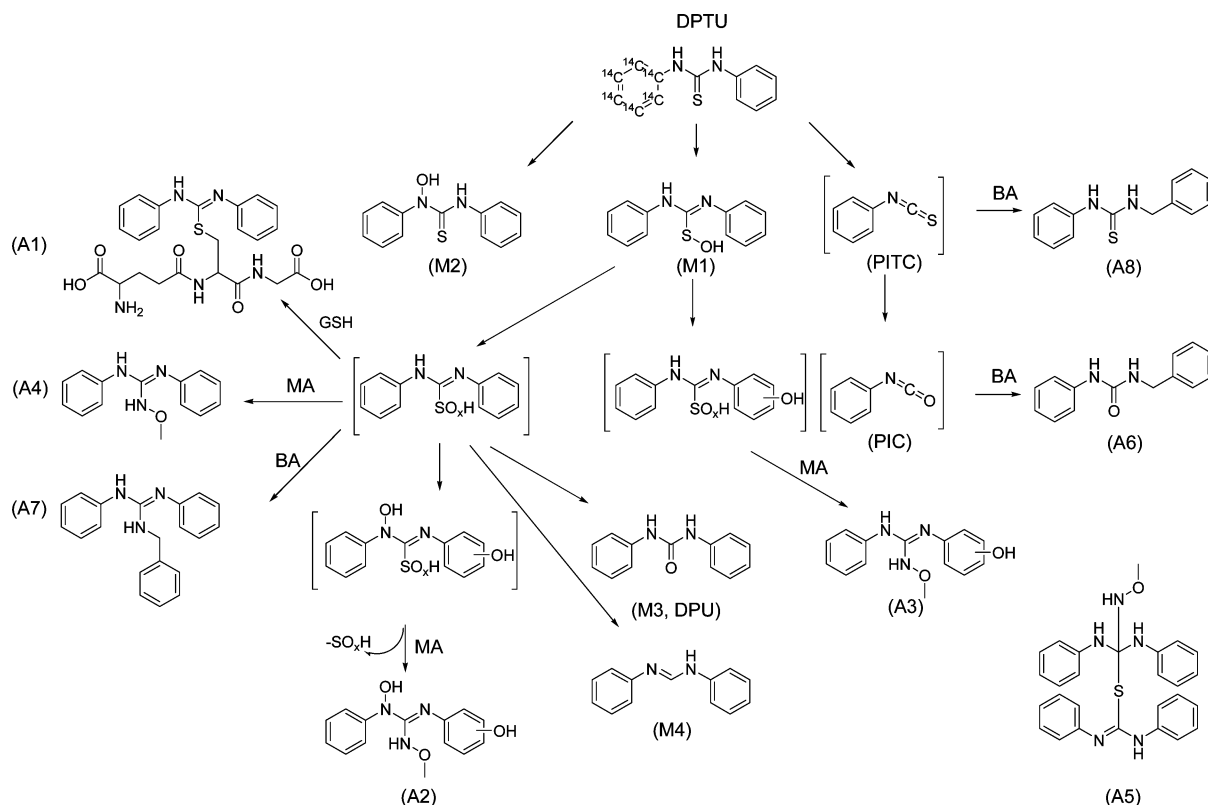
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¹ Abbreviations: ACD, allergic contact dermatitis; A:DBP, acetone: dibutylphthalate; BA, benzylamine; DPTU, diphenylthiourea; DPU, diphenylurea; ESI, electrospray interface; FMO, flavin-containing monooxygenases; GPMT, guinea pig maximization test; LLNA, local lymph node assay; MA, methoxylamine; MEST, mouse ear swelling test; PIC, phenylisocyanate; PITC, phenylisothiocyanate; PVC, polyvinyl chloride; RAD, radioactivity detection; SI, stimulation index; SLNA, sensitive local lymph node assay.

Scheme 1. Proposed Metabolic Pathways of DPTU in the Skinlike P450 Cocktail and Human Liver Microsomes^a

^a Structures within brackets are suggested intermediate compounds. Adducts A1–A8 were detected in incubations with reactive metabolite trapping agents glutathione (GSH), methoxylamine (MA), and benzylamine (BA).

nases, such as cytochrome P450 (P450) and flavin-containing monooxygenases (FMO) (20, 21). The most common phase I metabolizing enzymes present in the skin are P450s (15), and the most abundant P450s found in human skin samples are CYP1A1, 1B1, 2B6, 2E1, and 3A5 (22). A cocktail was developed to reflect the metabolism in human skin (22) as there are highly expressed cutaneous P450s that are not as pronounced in the liver (23). The ratios between the individual P450 enzymes are the same in the cocktail as in the skin. As the ability of a hapten or metabolite to form hapten–protein complexes is pivotal for the development of contact allergy, bioactivation and covalent binding to proteins can be assessed *in vitro* by incubating radiolabeled compounds with microsomal preparations and measuring nonextractable radioactivity associated with proteins. In addition to studying covalent binding, the structure of the reactive metabolites can be probed using reactive intermediate trapping experiments (24).

The thionocarbonyl group of thioureas, such as DPTU, is known to be oxidized by FMOs and P450s to sulfenic ($S-OH$), sulfinic ($-SO_2H$), or sulfonic ($-SO_3H$) acids, which can cause alkylation of proteins (25, 26). Thioureas are metabolized *in vivo* into desulfurated products. This is thought to be a result of nucleophilic substitution or elimination reactions of the sulfenic- and sulfinic acid metabolite intermediates. The oxidized thiourea compounds are also believed to be responsible for the toxicity of thioureas. Due to the instability of the intermediate metabolites of thioureas, the formation of oxidation products and the alkylating potential in metabolic mixtures have been studied by indirect measurements in different assays. Studies have shown that thioureas need to be metabolically activated to become protein reactive (27, 28). However, thioureas can also form reactive isothiocyanates by thermal decomposition (29).

The aim of the present study was to investigate if DPTU can act as a prohapten upon skin contact and to identify possible reactive metabolites that could be formed in the skin. The potential of DPTU to covalently bind to proteins was also investigated. Furthermore, we were interested in studying the formation of the putative reactive metabolite phenylisothiocyanate (PITC), since simultaneous reactions to PITC and DPTU have been observed in patients (9). As isothiocyanates can be metabolically converted into the corresponding isocyanate, we were also interested in studying the formation of phenylisocyanate (PIC) from DPTU. The metabolism of ¹⁴C-labeled DPTU was examined *in vitro* using the skinlike P450 cocktail and human liver microsomes. The covalent binding of DPTU and metabolites to proteins was studied in the P450 cocktail and in human liver microsome incubations and has to our knowledge not been studied in this context before. To gain further insight into the nature of the reactive metabolites formed, trapping experiments using glutathione (GSH), methoxylamine (MA), and benzylamine (BA) were performed. The sensitizing capacities of DPTU and three putative metabolites were investigated using the LLNA in mice (30). In addition, elicitation studies using the mouse ear swelling test (MEST) method were performed (31).

Experimental Procedures

Chemicals and Biochemicals. ¹⁴C-DPTU was synthesized by Isotope Chemistry at AstraZeneca R&D Mölndal (Mölndal, Sweden). A mixture of ¹⁴C₆-DPTU and unlabeled DPTU with a specific activity of 4.51 kBq/nmol was used for the microsomal incubations. PITC ($\geq 99\%$), PIC (99%), and BA ($\geq 99\%$) were purchased from Fluka (Buchs, Switzerland). Dibutyl phthalate (DPB, $\geq 99\%$) and acetone ($\geq 99\%$) used in a 1:1 mixture as vehicle in the animal experiments were purchased from Merck (Darmstadt, Germany).

and Sigma-Aldrich (Schnelldorf, Germany), respectively. Human liver microsomes and P450 enzymes were purchased from BD Biosciences (San José, CA) and Tebu-Bio (Offenbach, Germany), respectively. OptiPhase Hisafe 3 from PerkinElmer (Waltham, MA) was used as a scintillation cocktail.

Synthesis. 1-Benzyl-3-phenylthiourea (A8 in Scheme 1). PITC (10 mmol, 1.53 mL) was added to a solution of BA (10 mmol, 1.05 mL) in hexane (10 mL). The mixture was stirred at room temperature for 1 h, and the product was collected by filtration and washed with hexane yielding 86% of A8. ^1H and ^{13}C NMR spectroscopy (400 and 100 MHz, respectively) were performed on a JEOL Eclipse 400 instrument using deuterated DMSO as solvent. Chemical shifts (δ) are reported using residual DMSO δ 2.5 and δ 50.0 as references for ^1H and ^{13}C shifts, respectively. ^1H NMR: δ 8.0 (s, 1H), 7.18–7.42 (m, 10H), 6.3 (s, 1H), and 4.9 (d, 2H, J = 6 Hz). ^{13}C NMR: δ 181.3, 139.7, 139.6, 129.2, 128.8, 128.0, 127.4, 124.9, 123.9, and 47.8. MS/MS 243 (M^+ 45%), 119 (9%), 94 (31%), and 91 (100%).

1-Benzyl-3-phenylurea (A6 in Scheme 1). PIC (10 mmol, 1.09 mL) was added to BA (10 mmol, 1.53 mL) in hexane (10 mL) during stirring. The mixture was stirred at room temperature for 1 h, and the product was collected by filtration and washed with hexane. The yield was 91% of A6. ^1H NMR: δ 4.3 8.5 (s, 1H), 7.3 (m, 9H), 6.9 (t, 1H, 1.1), 6.6 (t, 1H, J = 5.8), (d, 2H, J = 5.8). ^{13}C NMR: δ 155.8, 141.0, 140.9, 129.2, 128.8, 127.7, 127.3, 121.6, 118.2, and 43.3. MS/MS 227 (M^+ 45%), 119 (9%), 94 (31%), and 91 (100%).

1,3-Diphenylurea (DPU; M3 in Scheme 1). DPU was obtained in 80% yield by adding PIC (10 mmol, 1.07 mL) to a solution of aniline (10 mmol, 0.91 mL) dissolved in hexane (5 mL) and stirred at room temperature for 1 h. The product was collected by filtration and washed with portions of hexane. ^1H NMR: δ 8.6 (s, 2H), 7.4 (d, 4H, J = 7.7 Hz), 7.3 (t, 4H, J = 7.7 Hz), 6.9 (t, 2H, J = 7.3 Hz). ^{13}C NMR: δ 153.1, 140.3, 129.4, 122.4, and 118.7. MS/MS 213 (M^+ 81%), 120 (2%), and 94 (100%).

Human Liver Microsomal and Skinlike P450 Cocktail Incubations. ^{14}C -DPTU (50 μM , 4.51 kBq/nmol) was incubated with human liver microsomes (1 mg protein/mL) or the skinlike P450 cocktail in the presence of NADPH (1 mM) and MgCl_2 (5 mM) in potassium phosphate buffer (0.1 M, pH 7.4) in a total volume of 500 μL . The P450 cocktail was prepared by mixing human P450s expressed in bacterial along with cytochrome P450 reductase. The total P450 content was 56 pmol, a concentration that was 2.5 times more than that used in the original protocol according to Bergstrom et al. (22). The content of the skinlike P450 cocktail was 1A1 (9.0 pmol), 1B1 (5.0 pmol), 2B6 (0.088 pmol), 2E1 (28 pmol), and 3A5 (14 pmol). The total amount of protein in the P450 incubations was 0.5 mg/mL. Incubations run in the absence of NADPH or using heat-inactivated enzymes served as negative controls. For heat inactivation, the enzymes were preincubated at a temperature of $>75^\circ\text{C}$ for 10 min. An additional control experiment was performed without enzymes using unlabeled DPTU to probe its decomposition in the conditions used. Trapping experiments were carried out using either of the trapping agents, GSH, MA, or BA, at a final concentration of 5 mM. The reactions were initiated by the addition of NADPH after approximately 3 min of preincubation at 37°C . The incubations were terminated after 60 min, and the protein was precipitated by the addition of ice-cold acetonitrile (0.5 mL). The samples were placed on ice for 10 min and were then centrifuged at 2600g at 4°C for 15 min. The supernatants were stored at -20°C awaiting analysis. Also, the protein pellets were retained for analysis of covalent binding of radiolabeled compounds to proteins. All incubations were performed in triplicate.

Covalent Binding to Proteins. The precipitated protein pellets from the microsomal incubations were washed with methanol ($2 \times 1\text{ mL}$), acetonitrile ($2 \times 1\text{ mL}$), and acetonitrile:water (4:1) ($2 \times 1\text{ mL}$). The pellets were disintegrated by vortex mixing, and the washing solvent was removed to waste after centrifugation at 2600g and 4°C for 10 min. After the sixth wash, aliquots (200 μL) of the washing liquid were analyzed using liquid scintillation counting

(Wallac 1409, Wallac Oy, Kuopio, Finland). An activity of <500 dpm was considered satisfactory to ensure that the pellets had been adequately washed. Each pellet was solubilized by heating and shaking in aqueous sodium hydroxide (1 mL, 0.1 M) containing sodium dodecyl sulfate (5%) at 40°C overnight in a water bath. The resulting solutions were analyzed for total radioactivity using static liquid scintillation counting, and the protein content was determined using the Markwell-modified Lowry assay with bovine serum albumin as standard (32). The levels of nonextractable radioactivity were expressed as pmol equivalents per milligram protein (pmol equiv/mg protein) using the specific activity of the test compound and the protein levels for each sample, according to the following equation:

$$\text{amount of binding (pmol equiv/mg protein)} = \frac{\text{measured radioactive concentration (Bq/mL)}}{\text{specific activity (Bq/pmol)} \times \text{protein concentration (mg/mL)}}$$

LC/MS/RAD Analysis of Incubation–Mixture Supernatants.

The supernatants were analyzed using LC combined with radioactivity detection (RAD) for quantification and MS for structural elucidation of parent compound and metabolites. The LC system (Agilent Series 1100, Palo Alto, CA) consisted of an autosampler, a solvent degassing unit, and a gradient pump. The column oven was set to 25°C , and the sample tray temperature was set to 8°C . The mobile phases used were (A) H_2O with 20 mM ammonium acetate and (B) acetonitrile. Chromatographic separations were performed on a Hypurity C18 LC column (4.6 mm i.d. $\times$ 150 mm; particle size 5 μm) protected by an Optiguard CN column (1 mm i.d.). A flow rate of 1 mL/min was used. All samples were diluted with water to a concentration of 20% acetonitrile in water before analysis, and the injection volume was 40 μL . Used was the following LC gradient: The gradient program started with a 30 min linear gradient from 10 to 30% B, followed by a 15 min gradient from 30 to 60% B. An increase from 60 to 90% B during 25 min was followed by 5 min of isocratic elution at 90% B, after which the eluent composition returned to the initial conditions (10% B) for 10 min. The total run time was 115 min.

RAD was performed with an online radioactivity detector (Radiomatic Flo One model TR-525, Packard, Meriden, CT) with a 100 μL flow cell and Ultima Flo AP scintillation fluid. The counting results were analyzed using Laura (version 3, LabLogic).

For MS detection, ~ 0.1 mL/min of the LC eluent was directed to the mass spectrometer. The mass spectrometer used was a hybrid quadrupole time-of-flight mass spectrometer (Micromass Q-ToF 2) with an electrospray interface (ESI) and a LockSpray probe. Specific mass spectrometric source conditions were as follows: cone voltage, 40 V; capillary voltage, 3.20 kV; MCP, 2000 V; TOF, 9.10 kV; source temperature, 120°C ; and desolvation temperature, 320°C . MassLynx (version 4, Waters) was used for controlling the mass spectrometer and for data evaluation. The mass range was 80–1000 Da, and MS spectra were acquired in the positive ionization mode. MS/MS spectra were acquired using collision energies ranging between 15 and 25 V. The MS/MS mass range was m/z 80–650.

A second LC-MS/MS instrument was used with the LC system (Agilent Series 1200) consisting of an autosampler, a solvent degassing unit, and a gradient pump. The column oven was set to 25°C , and the sample tray temperature was set to 8°C . The same column and elution system was used as to the Agilent Series 1100. For MS detection, about 0.4 mL/min of the LC eluent was directed to the 6410B Triple Quad (Agilent) with an ESI interface. The data were analyzed using MassHunter. This instrument was only used in the product ion scan mode with the collision energies ranging from 10 to 30 V. The fragmentor voltage was set to 50 V. The mass range was from m/z 80 to parent ion plus 50 Da.

Experimental Sensitization Studies (LLNA). The LLNA (33) was used to determine the sensitizing capacities of DPTU, DPU, PITC, and PIC. All animal procedures were approved by the local ethics committee. Female CBA/Ca mice, 8–12 weeks, in groups of three animals, were exposed to a test compound dissolved in A:DPB (1:1) in five different concentrations (Table 1). A sham-

Table 1. Cell Proliferation Induced by DPTU, DPU, PITC, and PIC Using the LLNA^a

compound concentration (% w/v)	DPM/lymph node	SI ^b	EC3 ^c	
			%	mM
DPTU			—	—
0.10	710	0.86		
1.0	981	1.2		
2.5	994	1.2		
5.0	824	1.0		
10	1564	1.9		
vehicle ^d	828			
DPU			—	—
0.10	1029	1.1		
1.0	1096	1.1		
2.5	915	0.95		
5.0	1320	1.4		
10	1779	1.2		
vehicle	966			
PITC			0.40	30
0.0010	704	0.59		
0.010	763	0.64		
0.10	1758	1.5		
1.0	7204	6.0		
2.0	10533	8.8		
vehicle	1196			
PIC			0.020	1.7
0.0010	1380	1.5		
0.10	7077	7.9		
1.0	7212	8.0		
5.0	12224	14		
10	15009	17		
vehicle	898			

^a The local lymph node experiments were performed as described in the Experimental Procedures. ^b An increase in [³H]-thymidine incorporation relative to vehicle-treated controls was derived for each experimental group and recorded as a SI. ^c EC3 is the estimated concentration required to induce an SI of 3 and is calculated using linear interpolation. ^d Acetone:dibutylphthalate (A:DBP) (1:1).

treated control group was exposed to the vehicle alone. The highest test concentrations used for DPTU and DPU were saturated solutions at room temperature. The results of LLNA were obtained as mean dpm/pooled lymph nodes of each animal group from which the stimulation index (SI) was calculated, that is, the test group dpm/control group dpm ratio. An EC3 value, that is, the estimated concentration of the test material required to induce an SI of 3 (the limit to be classified as a sensitizer), was calculated by linear interpolation (34).

Irritation Study. Prior to the MEST (see below), a control experiment was performed to determine what test concentrations should be used to avoid irritation. Under isoflurane narcosis, the ear thickness of naïve male CBA/Ca mice, 8–12 weeks old, was measured with a caliper before and 24 h after topical application of vehicle, PITC, or PIC on the dorsum of the ears. Used concentrations of the compounds are listed in Table S1 in the Supporting Information. To reduce the number of animals, each mouse was exposed to two different compounds, or one compound

and the vehicle (A:DBP, 1:1), one on each ear. For each treatment, the percent relative ear thickness was calculated from the sum of the quotient of ear thickness before and 24 h after exposure divided by the number of ears treated. For the vehicle-treated mice, the mean of four ears was obtained, while the mean of two ears was calculated for the sensitizers. Neither of the compounds increased the ear thickness, indicating no irritation at the concentrations tested (Table S1 in the Supporting Information).

Cross Reactivity Studies Using the MEST. The MEST experiments were performed as described in literature (35) with some modifications: change of mouse strain, vehicle, and omission of adjuvant or other enhancement techniques to perform the experiment using the same animal strain and vehicle as in the LLNA. Male CBA/Ca mice 8–12 weeks old were used in groups of eight or four animals (Table 2). Under isoflurane narcosis, induction was carried out on days 0, 1, and 2 by topical applications on the shaven backs (approximately 2 cm²) of the mice with 100 μ L of the test compounds in A:DBP (1:1) (Table 2). The ear thickness was measured under isoflurane narcosis at day 7, after which the mice were challenged topically on the dorsum of both ears with 25 μ L of test compound in the vehicle as shown in Table 2. Ear thickness measurements were performed again after 24 and 48 h. The relative ear thickness at these two time intervals after challenge was calculated for each mouse using the following formula: relative ear thickness (%) = 100[(A_x/B_x) + (A_y/B_y)]/2 (A = the ear thickness after 24 h, B = the ear thickness prior to exposure, X = ear 1, and Y = ear 2). The mean relative ear thickness for each group of mice is presented in Table 2.

Statistical Analysis. The results from the MEST were statistically evaluated using an exact permutation variant of Wilcoxon's rank sum test. The individual increase in relative ear thickness observed of each mouse within the groups was used when groups 2–5 were compared with control group 1.

Results

Identification of DPTU Metabolites. The metabolism of ¹⁴C₆-DPTU was assessed, and metabolites were detected after incubations with the skinlike P450 cocktail or human liver microsomes. The metabolites and trapped adducts were separated using reversed-phase HPLC and online RAD for quantification. MS/MS was used for structure elucidation. The total conversion of DPTU after 60 min of incubation in human liver microsomes and the P450 cocktail was 29 and 12%, respectively. A loss of approximately 10% of parent was observed in the control experiments without NADPH and is considered to be due to decomposition of DPTU (data not shown). This was confirmed in an additional control experiment run in the absence of metabolizing enzymes.

Radiochromatograms from the human liver microsome incubations with and without NADPH are shown in Figure 1. In total, six metabolites were detected in the human liver microsome incubations without trapping agents. Four of the metabolites were detected by MS and are denoted M1–M4 in

Table 2. Results from the MEST^a

group	no. of animals	induction compound (%, w/v)	challenge compound (%, w/v)	mean relative ear thickness ^b ($\pm$ SD)		observed reactions ^c at 24 and 48 h
				24 h	48 h	
1	8	PITC (2.0)	vehicle ^d	104 (2.7)	104 (3.3)	—
2	8	PITC (2.0)	DPTU (5.0)	101 (1.5)	101 (1.6)	—
3	8	PITC (2.0)	PIC (0.50)	113 (7.0)	113 (5.7)	+
4 ^e	4	PITC (2.0)	PITC (2.0)	112 (3.6)	111 (4.8)	+
5 ^e	4	PIC (0.50)	PIC (0.50)	121 (10.5)	140 (5.0)	+

^a The experiment was performed as described in the Experimental Procedures. ^b Relative ear thickness at 24 and 48 h after challenge as compared to 0 h was calculated for each mouse as follows: (%) = 100(A_x/B_x + A_y/B_y)/2, where A = the ear thickness after challenge (24 or 48 h), B = the ear thickness prior to challenge (0 h), X = right ear, and Y = left ear. ^c Groups 2–5 were compared to group 1 using an exact permutation variant of Wilcoxon's rank sum test. Groups 3–5 were all statistically distinguished from the control group 1, a positive (+) observed reaction. The *p* values were <0.01 in all cases except for the reaction of group 4 at 48 h, which had a *p* value of <0.05. No increased ear thickness was observed in the DPTU exposed mice as compared to the vehicle. ^d A:DBP (1:1). ^e Positive controls.

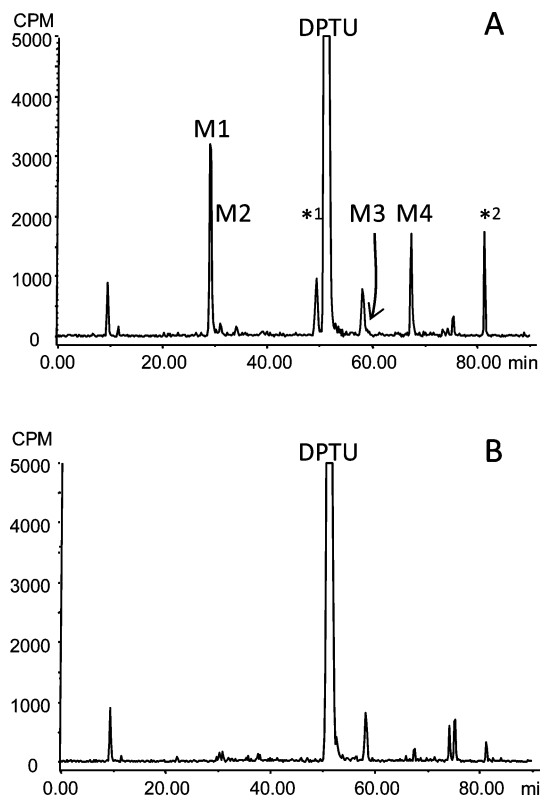


Figure 1. LC radiochromatograms obtained from metabolism of ^{14}C -labeled DPTU in human liver microsomes. Panels A and B show experiments with and without NADPH, respectively. The incubations were run in triplicate and pooled before LC analysis. Each incubation contained a substrate concentration of $50\ \mu\text{M}$ at $0.5\ \text{mg protein/mL}$ and was incubated for 60 min at $37\ ^\circ\text{C}$. The metabolites (M1–M4) are numbered in order of elution and were further identified with LC-MS/MS. Peaks marked with * could not be detected by LC/MS. The arrow denotes the metabolite M3, seen as a shoulder of a peak from a NADPH-independent coeluting unknown compound.

order of elution time. No mass information, neither in positive nor in negative ionization mode, could be obtained for the remaining two DPTU metabolites, which are denoted *1 and *2 (Figure 1A). In the P450 cocktail incubations, M1–M4 were detected, however, the unidentified peaks from the human liver microsome incubations were not seen (Figure 2A). Amounts of metabolites formed and masses of metabolites are presented in Table 3. M1, a monooxygenated metabolite, was the major metabolite formed in incubations with both the P450 cocktail and the human liver microsomes. The minor metabolites M2–M4 were formed via monooxygenation (M2), desulfuration (M4), and combined oxygenation and desulfuration (M3). Proposed metabolic pathways of DPTU are shown in Scheme 1. The compositions of DPTU and metabolites thereof are based on LC-MS/MS data (Table 3), and all metabolites detected by MS/MS have the same isotope pattern as DPTU. The m/z given for the compounds is the unlabeled MH^+ . The MS/MS spectrum of DPTU (MH^+ at m/z 229) contains product ions at m/z 195, 136, and 94. Proposed structures of the DPTU product ions are shown in the Supporting Information (Figure S1). M1 and M2 have pseudomolecular ions of m/z 245 corresponding to a net mass gain of +16 Da and are thus monooxygenated metabolites. Both M1 and M2 produced product ions at m/z 227, which indicates that both compounds lose the mass of one water molecule upon fragmentation of the parent at m/z 245. Hydroxylation on aromatic rings is a common metabolic pathway, but loss of water is unlikely to occur for phenols when subjected to collision-induced dissociation. Thus, on the basis of MS/MS interpretation, M1 and M2 are postulated to be the sulfenic acid

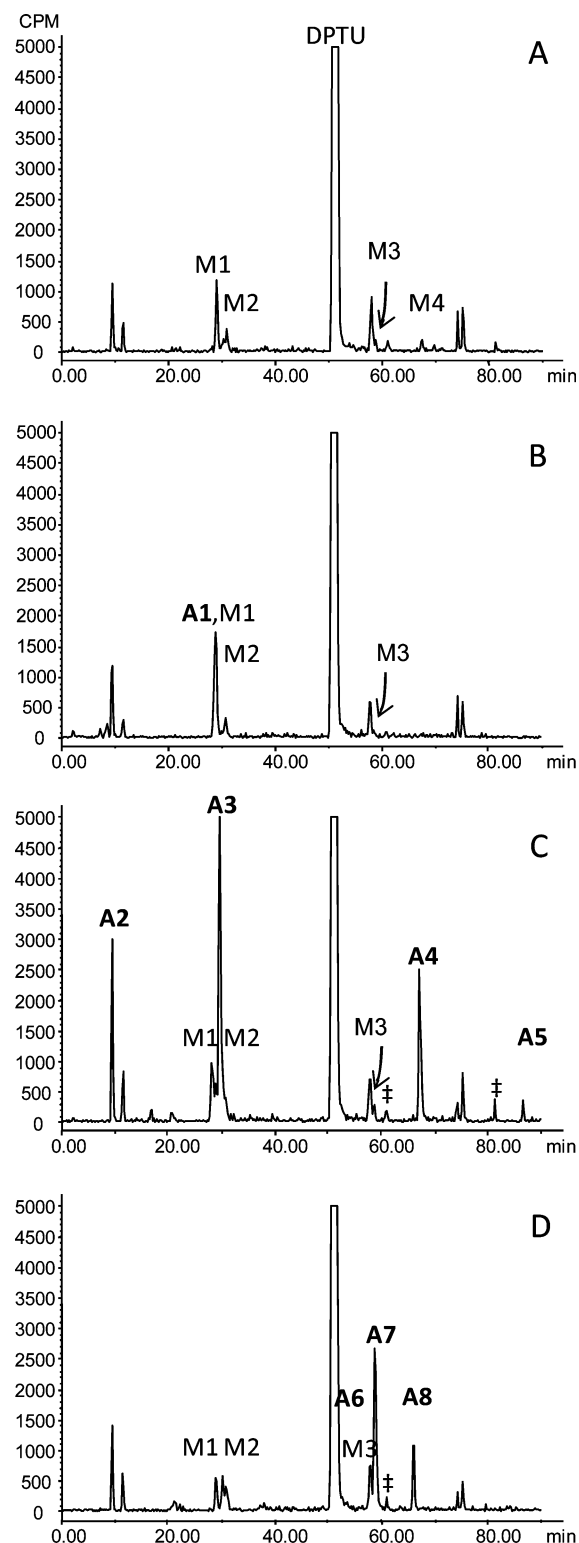


Figure 2. LC radiochromatograms obtained from ^{14}C -labeled DPTU in the skinlike P450 cocktail. Panel A is without trapping agent, and panels B–D show profiles after incubation with GSH, MA, and BA, respectively. The chromatograms are the results of three pooled reactions, each performed with a substrate concentration of $50\ \mu\text{M}$ at $0.25\ \text{mg protein/mL}$ for 60 min at $37\ ^\circ\text{C}$. The designated peaks of metabolites (M1–M4) and adducts (A1–A8) are in order of elution and were further identified with LC-MS/MS. Peaks marked with ‡ could not be detected by MS. The arrow denotes the metabolite M3, seen as a shoulder of a peak from a NADPH-independent coeluting unknown compound.

(S–OH) and hydroxylamine (N–OH) of DPTU, respectively. S- and N-oxidations are catalyzed both by microsomal P450 and FMO (25). S-oxidation produces sulfenic (S–OH), sulfinic

Table 3. Percentages^a, Masses, and MS/MS Data of DPTU and Metabolites Formed in Human Liver Microsome and P450 Incubations

metabolites (<i>t_R</i> , min)	MH ⁺ (<i>m/z</i>)	product ions (<i>m/z</i>)	human liver microsomes + NADPH (%)	P450 + NADPH (%)
DPTU (50.3)	229	195, 136, 94		
M1 (29.1)	245	227, 195, 169, 152, 92	47	64
M2 (30.8)	245	227, 152, 136, 110, 94	1.5	20
M3 ^b (58.1)	213	120, 94	1.1	7.7
M4 (67.3)	197	104, 94	20	9.6
*1 (49.2)			15	
*2 (81.2)			15	

^a Areas in the radiochromatograms were used to calculate the percent of formed metabolites. ^b The NADPH-independent compound from the control experiment coeluting with M3 (Figures 1 and 2) was subtracted from the incubations with NADPH.

(SO₂H), and sulfonic acids (SO₃H), summarized as sulfoxides (SO_xH) in Scheme 1. Thiourea sulfoxides are unstable and difficult to isolate and identify. For this reason, only the sulfenic acid of DPTU (M1) was identified in the metabolic mixture. The MS/MS spectra of M1 and M2 are shown in the Supporting Information (Figure S2). Using a synthetic standard, metabolite M3 at *m/z* 213 was identified as diphenyl urea (DPU, Scheme 1). M3 produced the same MS/MS spectrum and had the same retention time as the reference compound DPU (data not shown). Metabolite M4 was the most nonpolar metabolite identified. This compound was found in relatively low amounts and only in incubates without trapping agents. The protonated molecular ion had a *m/z* of 197 and is proposed to be diphenyl formamidine (Scheme 1). Its MS/MS product ions at *m/z* 104 and 94 correspond to C₇H₆N and protonated aniline.

Formation and Identification of Trapped Adducts. The formation of reactive intermediate metabolites from DPTU in incubations with the skinlike P450 cocktail and human liver microsomes was investigated using GSH, MA, and BA as reactive metabolite trapping agents. The incubates were analyzed using LC-MS/RAD, and radiochromatograms from the P450 incubations are shown in Figure 2. The results from the P450 incubations were qualitatively similar to those of the human liver microsome incubations except for the two compounds not detected with MS. Peaks marked with ‡ could not be identified by MS detection. In the trapping experiments, one GSH adduct (A1, Figure 2B), four MA adducts (A2–A5, Figure 2C), and three BA adducts (A6–A8, Figure 2D) were found. All adducts had the same isotope pattern as DPTU and were subjected to MS/MS analysis. The proposal of structures for adducts A1, A4, and A7 (Scheme 1) is based on MS/MS data, and the fact that thioureas are known to be bioactivated into reactive sulfenic, sulfinic, and sulfonic acid metabolites. These acids will readily react with amino and thiol compounds to form guanidines and isothioureases, respectively (36, 37). The relative amounts of adducts formed in the trapping experiments of the P450 incubations are shown in Table 4. The guanidine and isothiourease adducts are the major adduct type formed in the various incubations. The MS/MS spectra and proposed fragment structures for the GSH adduct A1, the MA adduct A4, and the BA adduct A7 are shown in Figure 3. Similar to DPTU and M1, A4 and A7 produced a fragment at *m/z* 195, which is suggested to be a nitrilium ion (Figure 3). The product ion of A4 at *m/z* 210 is loss of the methoxy group from the MA moiety, and *m/z* 119 is subsequent loss of one aniline group. The loss of C₆H₅–N from A4 gave the product ion of *m/z* 134, whereas *m/z* 93 corresponds to C₆H₅NH₂⁺. Upon collision of adduct A7, the product ions were observed as follows: 209, 195, 119, 108, and 91. The *m/z* 195 and 119 product ions formed from A7 are

Table 4. Relative Amounts of Adducts Formed in Reactive Metabolite Trapping Experiments in Human Liver Microsomes^a

adduct (retention time, min)	GSH (%) ^b	MA (%)	BA (%)
A1 (28.9)	41.0	ND	ND
A2 (9.4)	ND	11	ND
A3 (29.5)	ND	41	ND
A4 (67.0)	ND	17	ND
A5 (86.5)	ND	1.5	ND
A6 (54.1) ^c	ND	ND	NQ
A7 (58.7)	ND	ND	42
A8 (65.9)	ND	ND	12

^a ND, not detected. The limit of detection was set to 1000 cpm. NQ, not quantified. ^b The relative amount (%) is calculated from the total radioactivity of all metabolites and adducts detected in the incubations. ^c A6 coeluted with DPTU and could therefore not be quantified.

the same as those found for A4. The ion at *m/z* 209 is analogous to the product of A4 at *m/z* 134 but contains BA as trapping agent instead of MA. From A7, protonated BA is formed and detected as *m/z* 108. Collision-induced dissociation of BA products identified in this study did all produce the mass of 91, which likely is the tropylium ion C₇H₇⁺ (38). Proposed structures of adducts A2, A3, and A5 found in the incubates of MA and NADPH are shown in Scheme 1. A2 is the result of a dihydroxylated product of a DPTU-sulfoxide, while A3 results from the corresponding monohydroxylated product. A5 was identified as a DPTU dimer trapped by MA. The MS/MS spectra of A2, A3, and A5 as well as proposed structures of product ions are shown in Figure S3 in the Supporting Information.

Adducts A6 and A8 (*m/z* at 227 and 243, respectively) were detected in incubations with BA (Figure 2). Their MS/MS spectra and proposed fragment structures are shown in Figure S4 in the Supporting Information. Retention times and MS/MS spectra of A6 and A8 are identical to the synthetic standards, and their structures are shown in Scheme 1. A6 and A8 are formed from the reactive metabolites PIC and PTC (Scheme 1), respectively. PTC may also be formed as a thermal decomposition product of DPTU (38), and small amounts of A8 were also found in the controls (data not shown). Isothiocyanates may be metabolically converted into the corresponding, and even more reactive, isocyanate PIC (39, 40), and thus, the adduct A6 was formed as a result of further metabolism of PTC to PIC. The product ions obtained from A6 and A8 are *m/z* 136, 119, 94, 91, and 119, 94, and 91, respectively. *m/z* 136 corresponds to PTC and *m/z* 119 to C₆H₅–NCNH₂; the latter was also present as a product ion of A4 and A7. The observed fragment ion at *m/z* 94 is suggested to be anilinium ion, and the ion at *m/z* 91 is (C₇H₇)⁺ as previously reported (38).

Protein Binding. As the formation of hapten–protein complexes is a key event in contact allergy, the covalent binding of radiolabeled products to proteins in ¹⁴C₆-DPTU-containing microsomal incubates was investigated. To determine the protein binding propensity of DPTU before and after metabolic activation, the retained protein pellets from the skinlike P450 cocktail and human liver microsome incubations were washed and analyzed. Incubations run with NADPH were used to investigate the protein reactivity of DPTU following metabolic activation. Incubations run in the absence of NADPH, or heat-inactivated microsomes were used to determine the inherent reactivity of DPTU. Incubations run in the presence of the reactive metabolite trappers GSH, MA, and BA were used to probe the nature of the protein-reactive species. The covalent binding results are presented in Figure 4. Protein binding was observed in both human liver microsome and in skinlike P450 incubations and in both the presence and the absence of NADPH. The human

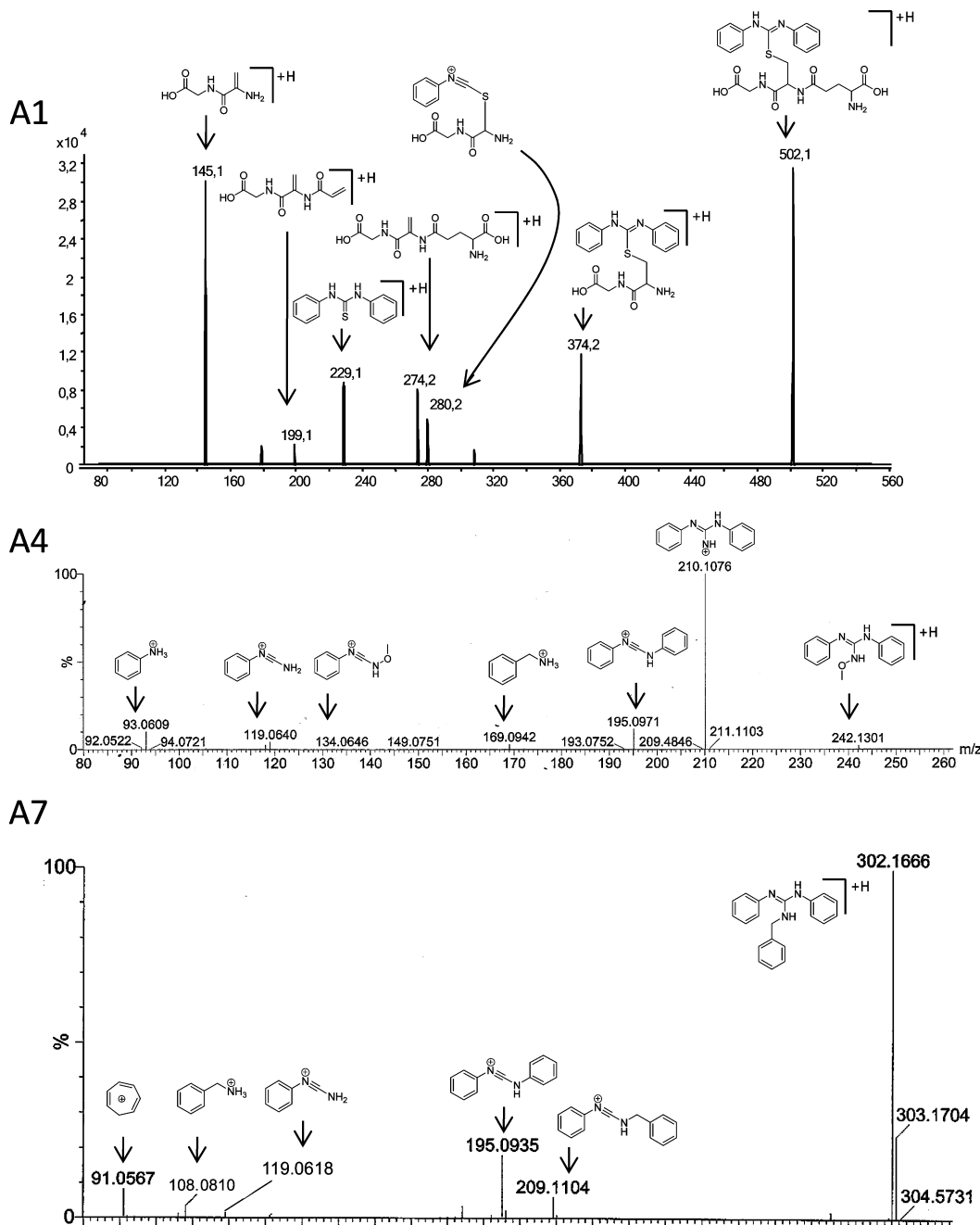


Figure 3. MS/MS spectra of the three major adducts produced (A1, A4, and A7) from both human liver microsomes and skinlike P450 cocktail.

liver microsome incubations in the presence of NADPH revealed that 22% of the total radioactivity added to the incubations was protein bound. This is approximately two times more than that of the NADPH-free or heat-deactivated incubations (Figure 4), thus showing that DPTU has undergone metabolic activation. For the incubations with the skinlike P450s, the difference in binding between the NADPH supplemented and the NADPH-free incubations is not as pronounced, 10 and 7%, respectively. A decrease in metabolism-dependent covalent binding to proteins was observed in all human liver microsome experiments containing trapping agents when compared to NADPH without trapper (Figure 4A). This was also observed in the skinlike P450 incubations but was not as pronounced when using MA as trapping agent (Figure 4B). Interestingly, decreased levels of binding were also observed in the NADPH-free experiments using the P450 cocktail incubations with GSH and MA and for all NADPH-free trapping experiments using the human liver

microsomes, which could be due to trapping of reactive degradation products (Figure 4).

Sensitizing Capacity in Mice. The LLNA was used to determine the sensitizing capacities of DPTU and the three metabolites DPU (M3), PITC, and PIC. As various thiourea sulfoxides previously have been reported to be unstable and difficult to isolate (41, 42), the oxidized products of DPTU were neither synthesized nor tested. The murine LLNA is the method of choice according to the OECD Guidelines for predictive testing of the sensitizing capacity of compounds (43). However, it has been shown to give false negative results when testing thioureas (12, 13). Despite in previous attempts to provide a more sensitive LLNA, we tried an alternative vehicle and used saturated solutions as the highest test concentrations for DPTU and DPU. Saturated solutions are found to increase the degree of penetration of a chemical into the epidermis, which is an important factor in the sensitization process in the LLNA (44).

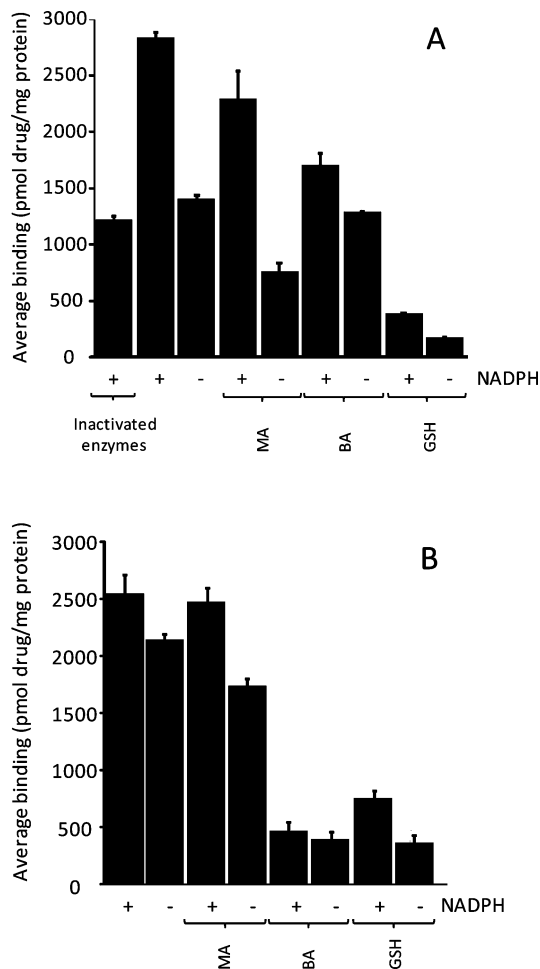


Figure 4. Covalent binding of ¹⁴C-DPTU in human liver microsomes (A) and the skinlike P450 cocktail (B). Error bars represent the standard deviation of the mean of the replicates. The first bar in panel A is the result from heat-inactivated enzymes. The other bars in both A and B show the results from active enzymes but with or without NADPH (+ or -) and incubations with the three reactive metabolite trapping agents, MA, BA, and GSH.

However, neither DPTU nor DPU provoked sensitization (stimulation indices of more than three) at the concentrations tested. PITC and PIC were, as expected, both sensitizers with EC₃ values of 0.40 (30 mM) and 0.020% (1.7 mM), respectively. PITC, previously assessed as an intermediate sensitizer (11) in the GPMT, was found to be a strong sensitizer according to LLNA. The more electrophilic isocyanate PIC, previously identified as a sensitizer in the MEST method (45), was found to be an extreme sensitizer using the LLNA. The SI values and the test concentrations used are presented in Table 1.

Cross-Reactivity Study in Mice (MEST). The MEST method was chosen as an *in vivo* method to elucidate a possible cross-reactivity between the DPTU and the metabolite PITC, as well as between PITC and PIC. Because of the low ability of thioureas to induce sensitization in mice after topical application, the mice were induced with the strongly sensitizing metabolite PITC and challenged with the prohaptens DPTU, in an attempt to increase the sensitivity of the method. In PITC-sensitized mice, PITC itself was able to induce an ear swelling challenge response (Table 2). However, no increased ear thickness was obtained after DPTU challenge in the PITC-sensitized mice; thus, no cross-reactivity was observed. This is probably due to the low penetration of DPTU as discussed above. The cross-reactivity between PITC and PIC was probed by challenging PITC-sensitized mice with PIC. An increased

ear thickness was detected after PIC challenge; that is, cross reactivity was demonstrated (Table 2). As positive controls, mice were sensitized and challenged with either PITC (group 4) or PIC (group 5), showing that these are contact sensitizers able to induce a challenge response. The results are presented as mean relative ear thickness after 24 and 48 h in Table 2.

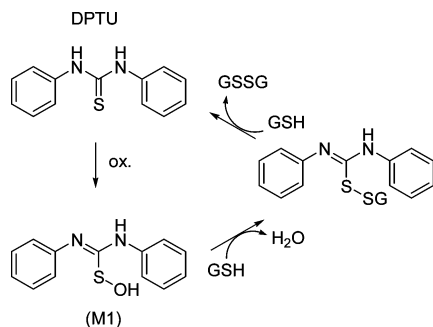
Discussion

We have, for the first time, shown that DPTU can act as a prohaptens. DPTU was bioactivated to reactive metabolites *in vitro* by the major enzymes found in the skin as well as by human liver microsomes. Four metabolites and eight trapped adducts were identified from incubations with an enriched skinlike P450 cocktail and human liver microsomes, respectively (Scheme 1). Metabolites were also shown to undergo covalent binding to proteins following NADPH-dependent bioactivation of DPTU, an experiment previously not used for studying prohaptens in contact allergy. The observed metabolism in the skinlike P450 cocktail indicates that DPTU can be metabolized to potent sensitizers in human skin. In addition to metabolic activation, DPTU was also found to undergo nonenzymatic activation to reactive species.

As contact allergens enter the body via the skin, the metabolic activity in this tissue is of major importance for topically applied prohaptens. To study skin metabolism, we used an earlier developed skinlike P450 cocktail (22). Also, human liver microsomes, commonly used to study *in vitro* metabolism, were used as a positive control for metabolic activation of DPTU. There are several hepatic P450s expressed in the skin, and four of the five most common P450s present in the cocktail are not highly expressed in the liver (2E1 is the exception) (23, 46). Two of the metabolites (*1 and *2 in Figure 1) were found in the human liver microsome incubates but not in the skinlike P450 incubates. This variation is likely to be due to the difference in enzyme composition between the two oxidative systems used. Unfortunately, the structures of these metabolites could not be revealed as they were not detected with MS. The metabolites formed in the human liver microsome incubates might be produced in human skin *in vivo* by other enzymes except for the five P450s present in the cocktail.

The major metabolite formed in both the P450 cocktail and the human liver microsome experiments was the sulfenic acid of DPTU (M1 in Table 3). M1 can either be further oxidized to reactive sulfinic and sulfonic acids or react directly with nucleophiles, making it an interesting compound in mechanistic investigations of contact allergy. Urea derivatives are often formed by metabolism from the corresponding thioureas, and this was also observed in the current study as DPU (M3) was identified as one of the metabolites. DPU was found in low amounts and coeluted with a degradation product. The MS subtraction of DPU at *m/z* 213 was only found in incubates with NADPH. Ureas are not considered to be toxic and DPU was found to be a nonsensitizer in the LLNA. The diphenyl-formamidinium metabolite (M4) was only found in the incubations without trapping agents, indicating that it is a reactive metabolite and probably involved in the formation of the sulfamidinium adducts A1 and A4 and the guanidine adduct A7. These identified sulfamidinium and guanidine adducts are mainly thought to be formed by substitution reactions of sulfoxides from oxidized DPTU. They were the major adducts formed in both oxidative systems and constituted more than 40% of the metabolites and adducts detected in the GSH-, MA-, and BA-containing incubations, respectively (Table 4). These adducts are formed via oxidation of the thiono group, suggesting this

Scheme 2. Suggested Regeneration Mechanism of DPTU from M1 via GSH Conjugation



initial transformation to be of significant importance in the development of contact allergy to DPTU. The identification of adducts A6 and A8 (formed via BA trapping of PIC and PITC, respectively) constitutes indirect evidence that the isocyanate and the isothiocyanate were formed metabolically from DPTU. The PITC adduct (A8) was also found in the NADPH-free controls but in lower amounts as compared to the NADPH-supplemented incubation mixtures. This is probably due to decomposition of DPTU to PITC under the conditions used and shows that DPTU forms reactive species also nonenzymatically.

The formation of protein complexes with haptens is a key event in contact allergy. Covalent binding of prohaptens in metabolism experiments *in vitro* has to our knowledge not been studied in the field of contact allergy. The NADPH-dependent adduct formation and covalent binding to proteins after incubation with DPTU found in this study make the activation of DPTU into sulfoxides, PITC, and PIC *in vivo* likely. Several thiourea-containing compounds have previously been shown to form reactive sulfoxide intermediates with alkylating potential of the metabolites (37, 47). The radioactivity observed in the pellet from the NADPH-free control experiments is thought to correspond to binding of PITC, formed from degradation of DPTU (38). Although previous studies (37, 47) support the opinion that thioureas need to be oxidized into unstable intermediary metabolites to achieve an alkylating potential, it cannot be excluded that DPTU also is inherently reactive toward nucleophiles. Lower levels of protein binding were found in the trapping experiments, since the trapping agents compete with the nucleophilic amino acids in the proteins. GSH was found to be the most efficient inhibitor of covalent binding. The fact that only one GSH adduct was identified supports that a NADPH-dependent recycling mechanism of DPTU takes place (Scheme 2) (25); however, no disulfide GSH adduct of DPTU was detected in the incubates. Overall, when studying prohaptens in contact allergy, *in vitro* covalent binding assays could be of future interest as a good complement to reactive metabolite trapping studies with prohaptens.

Our interest in studying PITC as a metabolite of DPTU stemmed from a previous study showing simultaneous patch test reactions to PITC and DPTU in patients with suspected rubber allergy (9). The results from the current metabolism experiments showed that PITC is formed following bioactivation of DPTU. Isothiocyanates are known sensitizers (2), and accordingly, PITC was also found to be a strong sensitizer in our study. Furthermore, it is known that isothiocyanates (PITC) can be enzymatically converted to isocyanates (48–50), as was indicated in the present investigation where PIC was identified as an adduct in the oxidative systems. Experimental studies in mice showed cross-reactivity between PITC and PIC, corroborating the *in vitro* results. Because of the thermal instability of DPTU (38), PITC could also be formed as a degradation

product and thus be present in, for example, neoprene and PVC products, as these are produced at high temperatures. Thus, it is likely that people using DPTU-containing items are simultaneously exposed to DPTU and PITC.

The NADPH-dependent formation of metabolites and adducts together with LLNA experiments show that DPTU can be bioactivated into reactive intermediates able to cause skin sensitization. However, which route of activation of DPTU (metabolism or thermal instability) that is the most important in developing contact allergy depends on the situation of exposure. In real life, neoprene products are used for extended periods of time, in close contact with the skin, and often under occlusion, which enhances the skin absorption and risk of sensitization. Diagnostic patch testing using DPTU in dermatitis patients can cause false negative results, as the dose of the metabolites becomes very small as the skin penetration of DPTU is limited. To increase the possibilities of diagnosing contact allergy to DPTU-containing items, we suggest that suitable metabolites of DPTU should be used for screening testing. This is an objective for future studies.

Acknowledgment. We thank Petri Karhunen for skillful technical assistance. At Astra Zeneca R&D, we thank Claire Milne for the opportunity of collaboration and Cecilia Ericsson, Isotope Chemistry team, for providing the radiolabeled DPTU. We also thank Lars Weidolf at Astra Zeneca R&D and Lars G. Nilsson for valuable discussions and for reading the manuscript and Martin Gillstedt for statistical calculations. In addition, we thank Astra Zeneca R&D for instrumentation. The work was performed within the Centre for Skin Research, University of Gothenburg.

Supporting Information Available: Figures of MS/MS spectra for DPTU, M1, and M2 and adducts A2, A3, A5, A6, and A8 including a table of the relative ear thickness results from the irritation study. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Table S1. Relative ear thickness results from an irritation study.

Test Material	Relative ear thickness ^a	Number of ears
Vehicle	98%	4
PITC 1.0%	100%	2
PITC 2.0%	99%	2
PITC 4.0%	100%	2
PIC 0.10%	95%	2
PIC 0.50%	98%	2
PIC 1.5%	101%	2

^a The ear thickness was measured before and 24 h after exposure. The percentage relative ear thickness was calculated from the sum of the quotients of the ear thicknesses at these two time intervals for each treatment divided by the number of ears exposed. For the vehicle treated mice, the mean of 4 ears was calculated while the mean of 2 ears was calculated for the sensitizers.

Figure S1 . MS/MS spectra of DPTU.

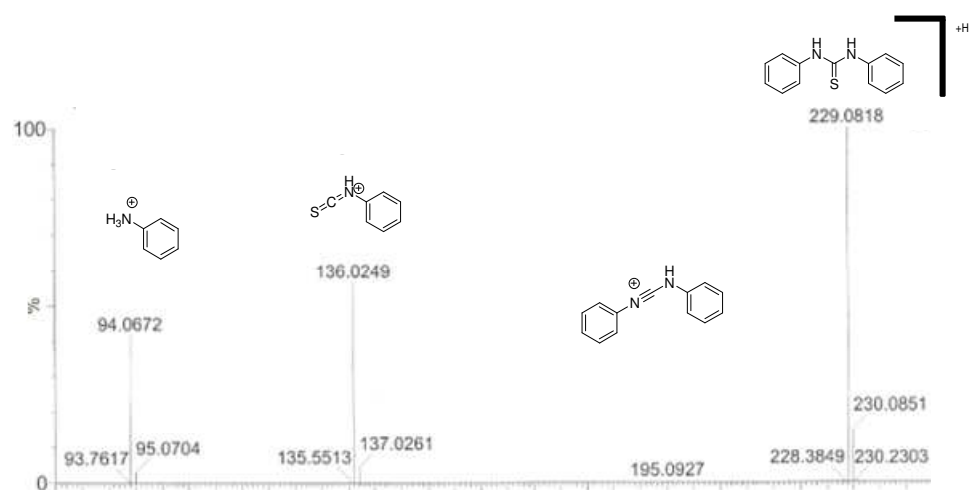
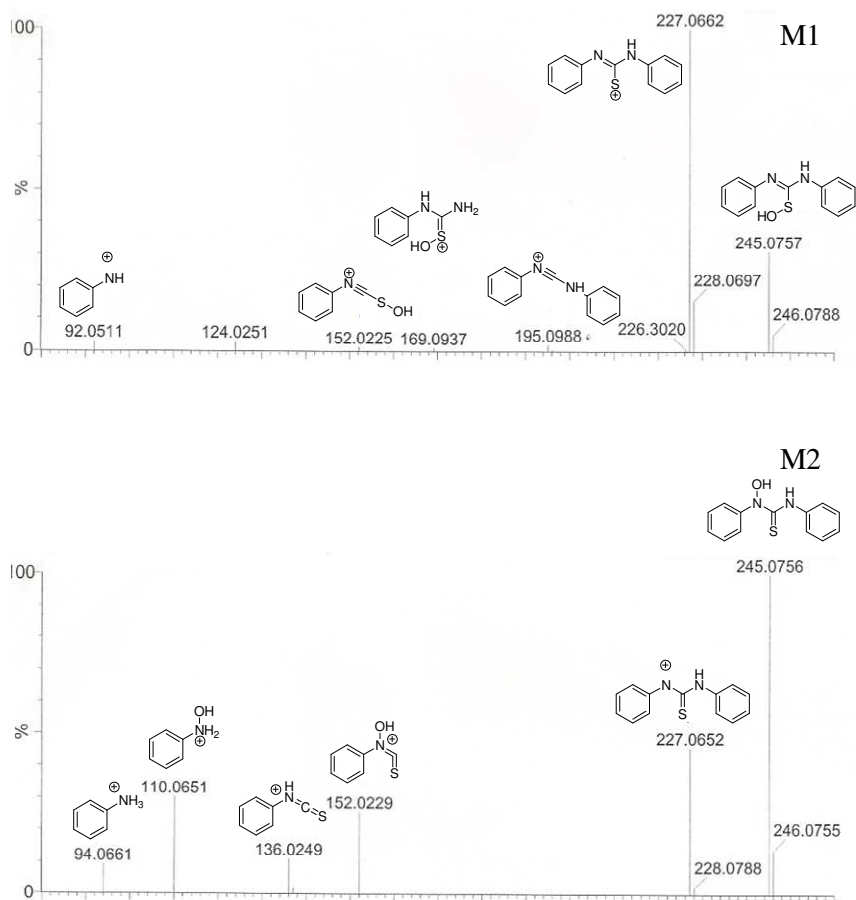


Figure S2. MS/MS spectra of M1 and M2.



microsome incubates with MA as trapping agent.

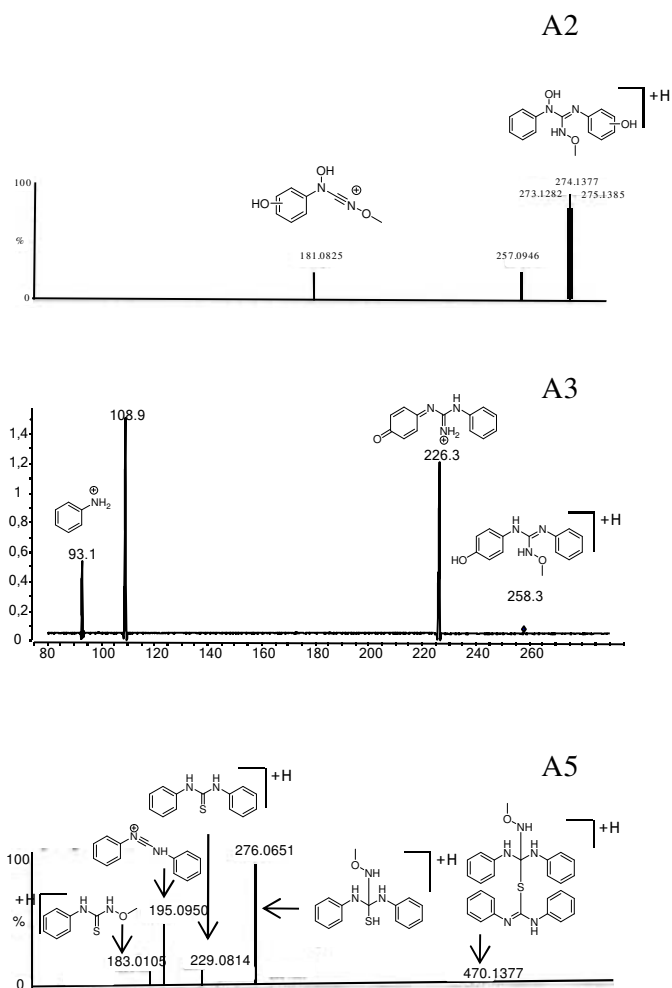


Figure S4. MS/MS spectra of A6 and A8 formed in the P450 and human liver microsme incubates with BA as trapping agent.

