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STRUCTURE–ACTIVITY RELATIONSHIPS OF NITROSAMINES AND NITRAMINES WHICH STIMULATE UDP-GLUCURONOSYLTRANSFERASE ACTIVITIES *IN VITRO*

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Abstract—Examination of twelve nitrosamines and seven nitramines revealed that nitramines modify UDP-glucuronosyltransferase activity in a manner similar to that of nitrosamines. Only *N*,*N*-diethyl-substituted nitrosamine and nitramine significantly stimulated transferase activity toward 2-aminophenol and 4-nitrophenol but not toward phenolphthalein and androsterone. Elongation of the alkyl chains or introduction of carboxy, hydroxy, or oxo groups into the alkyl chains did not result in stimulatory ability, and some of these compounds inhibited the transferase activity.

Conjugation of endogenous and exogenous compounds with glucuronic acid is catalyzed by hepatic microsomal UDP-glucuronosyltransferase (EC 2.4.1.17). This enzyme is latent in freshly prepared microsomes and can be activated by physical, chemical or enzymatic perturbation of the microsomal membrane [1, 2].

The hereditary deficiency of UDP-glucuronosyltransferase activity toward bilirubin and several substrates is well known in Gunn rats, a mutant strain of Wistar rats [1, 2]. When *N*,*N*-diethylnitrosamine is added to Gunn rat liver enzymes, the deficiency in transferase activity toward 2-aminophenol disappears and in addition, its activity in preparations from both Gunn and Wistar rats is stimulated to equally high levels [3, 4]. *N*,*N*-Diethylnitrosamine was subsequently found to increase the transferase activities toward certain substrates such as paracetamol, 2-aminobenzoic acid, 4-nitrophenol, and 4-methylumbelliferone [1]. However, this nitrosamine does not stimulate the activities toward menthol, phenolphthalein, 2-aminothiophenol, 4-nitrothiophenol, and bilirubin [1]. Since the discovery of the carcinogenicity of *N*,*N*-dimethylnitrosamine in the rat [5], a large number of nitrosamines have been synthesized and found to be carcinogenic in various experimental animals [6–8]. Though activation of UDP-glucuronosyltransferase toward 2-aminophenol and 4-nitrophenol by *N*,*N*-diethylnitrosamine has been studied extensively [1, 2], the potential of other nitrosamines as activators of the transferase has not been reported. It was mentioned briefly by Dutton [1] that *N*,*N*-dimethylnitrosamine did not activate markedly the transferase. Recently, alkyl ketones such as butan-2-one, pentan-2-one, and pentan-3-one were shown to activate the transferase toward 2-aminophenol in a manner similar to *N*,*N*-diethylnitrosamine [9, 10]. Another example of genetic deficiency of UDP-glucuronosyltransferase activity was reported recently [11, 12], and its deficient activity toward androsterone was not reversed by addition of *N*,*N*-diethylnitrosamine and pentan-2-one [12].

To extend the structure–activity relationships of *in vitro* activators, we examined twelve nitrosamines and seven nitramines for the ability to modify UDP-glucuronosyltransferase activities toward 2-aminophenol, 4-nitrophenol, phenolphthalein, and androsterone.

MATERIALS AND METHODS

Materials. UDP-glucuronic acid disodium salt and UDP-*N*-acetylglucosamine were purchased from Boehringer, Mannheim, Germany. [1,2-^{3}H]Androsterone (40.8 Ci/mmole) was obtained from the New England Nuclear Corp., Boston, MA, U.S.A. Androsterone and 2-aminophenyl-β-D-glucuronide were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. Other acceptor substrates were commercial samples of the purest grade, and 2-aminophenol was purified by resublimation. Triton X-100, *N*,*N*-dimethyl-, *N*,*N*-diethyl-, *N*,*N*-dipropyl-, and *N*,*N*-dibutylnitrosamines were obtained from the Tokyo Kasei Kōgyo Co., Tokyo, Japan. *N*-Methyl-*N*-butylnitrosamine was purchased from the Izumi Chemical Laboratory, Yokohama, Japan. These nitrosamines were purified by distillation under reduced pressure. *N*,*N*-Dipentylnitrosamine, *N*-methyl-*N*-benzylnitrosamine, and *N*-ethyl-*N*-(2-hydroxyethyl)nitrosamine were prepared by the procedure reported previously [6]. *N*-Ethyl-*N*-(4-hydroxybutyl)nitrosamine [13], *N*-butyl-*N*-(2-oxopropyl)nitrosamine [13], *N*-ethyl-*N*-(3-carboxypropyl)-nitrosamine [14], and *N*-methyl-*N*-dodecylnitrosamine [15] were prepared as previously described. *N*,*N*-Diethyl- and *N*,*N*-dibutylnitramines were prepared by the procedure reported previously [16], and *N*,*N*-dimethyl-, *N*,*N*-dipropyl-, and *N*,*N*-dipentylnitramines were prepared by a similar pro-

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cedure. *N*-Butylnitramine was prepared according to the procedure published previously [17], and *N*-ethylnitramine was prepared in a similar way. High purity of all these compounds was shown by high-pressure liquid chromatography or gas chromatography and by nuclear magnetic resonance spectroscopy.

Animals and preparation of microsomal fractions. Male Wistar rats (400–520 g) with high UDP-glucuronosyltransferase activity toward androsterone [11, 12] were decapitated, and a 20% (w/v) liver homogenate was prepared in ice-cold 0.25 M sucrose in 0.1 M Tris–HCl buffer, pH 7.4, with a Teflon–glass homogenizer. Microsomal fractions were collected by differential centrifugation (2,000 *g* for 10 min, 16,000 *g* for 45 min, and 105,000 *g* for 60 min). The microsomal pellets were resuspended in 0.25 M sucrose in 0.1 M Tris–HCl buffer, pH 7.4. All procedures for the isolation of microsomal fractions were done at 0–4°. The microsomal protein was determined by the method of Lowry *et al.* [18], using bovine serum albumin as protein standard.

Enzyme assays. UDP-glucuronosyltransferase activities toward 4-nitrophenol, phenolphthalein, and androsterone were assayed by the slight modification of the method described previously [19, 20]. The standard incubation medium contained microsomal fraction (1.0 to 1.7 mg of protein), 0.1 M Tris–HCl buffer, pH 7.4, 10 mM $MgCl_2$, 2 mM UDP-glucuronic acid, and 0.17 mM [^{3}H]androsterone (0.023 μCi), 0.36 mM 4-nitrophenol, or 0.12 mM phenolphthalein in a total volume of 1.0 ml. The incubation was carried out for 20 min at 37° and was terminated by heating in a boiling-water bath for 1 min, followed by cooling in ice-water. For the assay of the modified UDP-glucuronosyltransferase activity, 2 mM UDP-*N*-acetylglucosamine [21], 0.05% (w/v) Triton X-100 [21], 10 mM nitrosamine [22], or 10 mM nitramine was added to the incubation medium in which UDP-glucuronic acid was omitted. The mixture was then preincubated for 30 min at 4°, the reaction was started by addition of UDP-glucuronic acid, and the incubation was allowed to proceed for 20 min at 37°. The blank values were obtained from control incubations in which UDP-glucuronic acid was omitted. The transferase activities toward 4-nitrophenol and phenolphthalein were assayed colorimetrically from the disappearance of the substrates [19, 20]. The activity toward androsterone was measured radiometrically from the appearance of androsterone glucuronide [20]. The transferase activity toward 2-aminophenol was assayed essentially as described previously [1], except that the incubation was done for 20 min at 37° in 0.1 M Tris–HCl buffer, pH 7.4. With these incubation conditions, glucuronide formation was linear with time and protein concentration.

RESULTS

Effects of nitrosamines and nitramines on UDP-glucuronosyltransferase activities. Ten nitrosamines and a structurally related candidate, *N, N*-diethylnitramine, were tested for the ability to stimulate transferase activity towards 2-aminophenol, 4-nitrophenol, phenolphthalein, and androsterone (Table 1). When 2-aminophenol was used as a substrate, the highest activation was obtained by addition of *N, N*-diethylnitrosamine and *N, N*-diethylnitramine, followed by *N*-methyl-*N*-butylnitrosamine. Elongation of the alkyl chains and introduction of carboxy, hydroxy, or oxo groups into the alkyl chains did not affect the stimulatory activity. *N, N*-Dipentylnitrosamine, *N*-methyl-*N*-benzylnitrosamine, and *N*-butyl-*N*-(2-oxopropyl)nitrosamine inhibited transferase activity. With 4-nitrophenol as substrate, transferase activity was increased slightly by addition of *N, N*-dibutylnitrosamine, *N, N*-dipentylnitrosamine, and *N*-methyl-*N*-butylnitrosamine, as compared with *N, N*-diethylnitrosamine and *N, N*-diethylnitramine. The activity toward phenolphthalein was not changed significantly by addition of these compounds, except that the activity was increased slightly by *N, N*-dipentylnitrosamine. The activity toward androsterone was not increased significantly by these compounds, but it was inhibited by *N*-methyl-*N*-benzylnitrosamine and *N*-butyl-*N*-(2-oxopropyl)nitrosamine.

Table 1. Effects of ten nitrosamines and a nitramine on UDP-glucuronosyltransferase activity*

Compound (10 mM)	UDP-glucuronosyltransferase activity [nmoles · min^{-1} (mg protein)$^{-1}$]			
	2-Aminophenol	4-Nitrophenol	Phenolphthalein	Androsterone
No addition	0.25 ± 0.05	0.76 ± 0.35	0.37 ± 0.08	0.76 ± 0.13
N, N-Diethylnitrosamine	0.83 ± 0.13‡	2.09 ± 0.50‡	0.40 ± 0.11	0.74 ± 0.15
N, N-Dibutylnitrosamine	0.24 ± 0.18	1.36 ± 0.38†	0.33 ± 0.06	0.50 ± 0.27
N, N-Dipentylnitrosamine	0.15 ± 0.06†	1.59 ± 0.39†	0.57 ± 0.11†	0.68 ± 0.16
N-Methyl-*N*-butylnitrosamine	0.44 ± 0.09‡	1.64 ± 0.61†	0.45 ± 0.07	0.61 ± 0.07
N-Methyl-*N*-dodecylnitrosamine	0.22 ± 0.03	1.01 ± 0.34	0.41 ± 0.12	0.62 ± 0.09
N-Methyl-*N*-benzylnitrosamine	0.15 ± 0.02‡	1.35 ± 0.69	0.37 ± 0.08	0.33 ± 0.03‡
N-Ethyl-*N*-(2-hydroxyethyl)nitrosamine	0.31 ± 0.06	1.11 ± 0.44	0.45 ± 0.05	0.68 ± 0.12
N-Ethyl-*N*-(4-hydroxybutyl)nitrosamine	0.20 ± 0.09	0.95 ± 0.40	0.36 ± 0.12	0.65 ± 0.09
N-Ethyl-*N*-(3-carboxypropyl)nitrosamine	0.22 ± 0.06	0.94 ± 0.30	0.38 ± 0.05	0.66 ± 0.09
N-Butyl-*N*-(2-oxopropyl)nitrosamine	0.16 ± 0.04†	0.95 ± 0.54	0.37 ± 0.05	0.49 ± 0.11‡
N, N-Diethylnitramine	0.88 ± 0.25‡	2.04 ± 0.59‡	0.39 ± 0.14	0.64 ± 0.13

* Each value is the mean ± S.D. for five animals. Experimental details for the enzyme assay are described in Materials and Methods.

†,‡ Significantly different from control level (Student's *t*-test): † $P < 0.05$ and ‡ $P < 0.01$.

Table 2. Effects of three nitrosamines and six nitramines on UDP-glucuronosyltransferase activity*

Compound (10 mM)	UDP-glucuronosyltransferase activity [nmoles · min^{-1} (mg protein)$^{-1}$]			
	2-Aminophenol	4-Nitrophenol	Phenolphthalein	Androsterone
No addition	0.17 ± 0.03	0.93 ± 0.41	0.27 ± 0.05	0.56 ± 0.06
N, N-Dimethylnitrosamine	0.25 ± 0.04†	1.39 ± 0.55	0.24 ± 0.02	0.62 ± 0.11
N, N-Diethylnitrosamine	0.52 ± 0.07‡	1.76 ± 0.54†	0.24 ± 0.06	0.56 ± 0.13
N, N-Dipropylnitrosamine	0.17 ± 0.03	1.00 ± 0.49	0.26 ± 0.08	0.54 ± 0.07
N, N-Dimethylnitramine	0.32 ± 0.04‡	1.22 ± 0.55	0.21 ± 0.04	0.54 ± 0.08
N, N-Dipropylnitramine	0.16 ± 0.03	1.30 ± 0.62	0.26 ± 0.12	0.26 ± 0.04‡
N, N-Dibutylnitramine	0.08 ± 0.01‡	1.06 ± 0.34	0.26 ± 0.11	0.38 ± 0.07‡
N, N-Dipentylnitramine	0.09 ± 0.01‡	1.17 ± 0.22	0.23 ± 0.07	0.35 ± 0.07‡
N-Ethylnitramine	0.16 ± 0.05	1.04 ± 0.61	0.31 ± 0.04	0.54 ± 0.06
N-Butylnitramine	0.15 ± 0.01	1.06 ± 0.38	0.22 ± 0.04	0.46 ± 0.04†

* Each value is the mean ± S.D. for four animals. Experimental details for the enzyme assay are described in Materials and Methods.

†,‡ Significantly different from control level (Student's *t*-test): † $P < 0.05$ and ‡ $P < 0.01$.

Table 2 shows the effects of another two nitrosamines and six nitramines on the transferase activities. Only *N, N*-dimethylnitrosamine and *N, N*-dimethylnitramine significantly stimulated the activity toward 2-aminophenol, though these compounds were less potent than *N, N*-diethylnitrosamine. *N*-Monosubstituted nitramines such as *N*-ethyl- and *N*-butylnitramines did not stimulate the activity, probably indicating that the presence of the *N, N*-disubstituted structure, as well as special alkyl (ethyl or methyl) groups, might be required for enzyme activation. *N, N*-Dibutyl- and *N, N*-dipentylnitramines inhibited the activity toward 2-aminophenol and androsterone. In addition, the activity toward androsterone was inhibited by *N, N*-dipropylnitramine and *N*-butylnitramine. Thus, some nitramines seem to inhibit the transferase activities toward 2-aminophenol and androsterone, as compared with the corresponding nitrosamines.

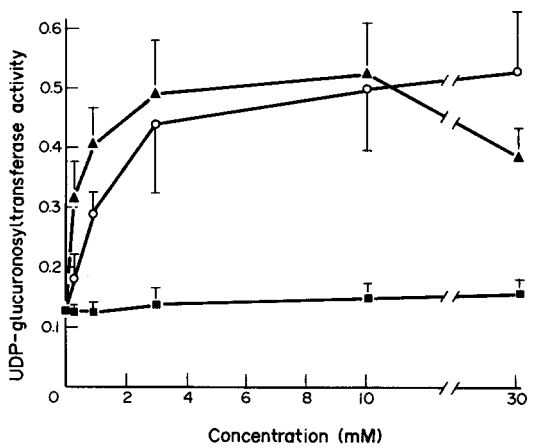

Fig. 1. Effects of various concentrations of *N, N*-diethylnitrosamine, *N*-ethyl-*N*-(2-hydroxyethyl)nitrosamine, and *N, N*-diethylnitramine on UDP-glucuronosyltransferase activity toward 2-aminophenol. The transferase activity is expressed as nmoles of glucuronide per min per mg of protein. Data are the means of three animals. The vertical lines represent one S.D. Key: (○——○) *N, N*-diethylnitrosamine; (■——■) *N*-ethyl-*N*-(2-hydroxyethyl)nitrosamine; and (▲——▲) *N, N*-diethylnitramine.

Effects of various concentrations of N,N-*diethylnitrosamine,* N-*ethyl*-N-*(2-hydroxyethyl)-nitrosamine, and* N,N-*diethylnitramine on UDP-glucuronosyltransferase activities toward 2-aminophenol.* Figure 1 shows the effects of 0.3 to 30 mM concentrations of *N, N*-diethylnitrosamine, *N*-ethyl-*N*-(2-hydroxyethyl)nitrosamine, and *N, N*-diethylnitramine on transferase activity toward 2-aminophenol. *N, N*-Diethylnitramine was slightly more effective than *N, N*-diethylnitrosamine at concentrations below 3 mM. Maximum enhancement was attained at about a 10 mM concentration of either compounds; it was somewhat inhibited by 30 mM *N, N*-diethylnitramine, but not by 30 mM *N, N*-diethylnitrosamine. It is reasonable to say that *N, N*-diethylnitramine stimulated the transferase activity in a way similar to *N, N*-diethylnitrosamine. When a hydroxy group was introduced into the ethyl group of *N, N*-diethylnitrosamine, the stimulatory property disappeared, and the transferase activity was slightly increased only at high concentrations of *N*-ethyl-*N*-(2-hydroxyethyl)nitrosamine. These results suggest that the non-stimulatory property of *N*-ethyl-*N*-(2-hydroxyethyl)nitrosamine was not due to its glucuronide formation, which might have competitively inhibited 2-aminophenyl glucuronide synthesis, but to the hydrophilic nature of the 2-hydroxyethyl moiety, which might have resulted in poor interaction with the enzyme.

Activation of UDP-glucuronosyltransferase by combination of N,N-*diethylnitrosamine or* N,N-*diethylnitramine with UDP*-N-*acetylglucosamine or Triton X*-100. To see whether transferase activity treated with *N, N*-diethylnitrosamine or *N, N*-diethylnitramine could be further increased upon addition of other types of stimulators, UDP-*N*-acetylglucosamine, an endogenous activator, and Triton X-100, a detergent, were added to the incubation media, and their stimulatory effects were measured (Table 3). With 2-aminophenol as substrate, UDP-*N*-acetylglucosamine alone exhibited a degree of stimulation (3.3-fold) similar to that of the nitrosamine and the nitramine. The nitrosamine- and the nitramine-stimulated activities were further

Table 3. Activation of UDP-glucuronosyltransferase by *N, N*-diethylnitrosamine, *N, N*-diethylnitramine, UDP-*N*-acetylglucosamine, and Triton X-100*

Compound	UDP-glucuronosyltransferase activity [nmoles · min^{-1} (mg protein)$^{-1}$]		
	2-Aminophenol	4-Nitrophenol	Phenolphthalein
No addition	0.22 ± 0.03	1.02 ± 0.18	0.35 ± 0.06
10 mM *N, N*-Diethylnitrosamine	0.73 ± 0.02	1.64 ± 0.35	0.41 ± 0.04
10 mM *N, N*-Diethylnitramine	0.73 ± 0.06	1.67 ± 0.22	0.28 ± 0.03
2 mM UDP-*N*-acetylglucosamine	0.73 ± 0.07	3.99 ± 0.26	1.09 ± 0.16
2 mM UDP-*N*-acetylglucosamine + 10 mM *N, N*-diethylnitrosamine	2.31 ± 0.16	5.27 ± 0.40	1.10 ± 0.08
2 mM UDP-*N*-acetylglucosamine + 10 mM *N, N*-diethylnitramine	2.22 ± 0.12	4.83 ± 0.40	0.94 ± 0.14
0.05% Triton X-100	0.29 ± 0.03	7.14 ± 0.34	2.33 ± 0.21
0.05% Triton X-100 + 10 mM *N, N*-diethylnitrosamine	2.84 ± 0.11	10.10 ± 0.46	2.08 ± 0.25
0.05% Triton X-100 + 10 mM *N, N*-diethylnitramine	3.90 ± 0.13	10.70 ± 0.80	2.05 ± 0.24

* Each value is the mean ± S.D. for three animals. Experimental details for the enzyme assay are described in Materials and Methods.

increased by addition of UDP-*N*-acetyglucosamine, resulting in an overall 10-fold activation. Though Triton X-100 alone slightly increased the transferase activity toward 2-aminophenol, the combined addition of Triton X-100 and the nitrosamine or the nitramine yielded the maximal activation (13- to 18-fold), the nitramine stimulating the activity more than the nitrosamine. With 4-nitrophenol as substrate, UDP-*N*-acetylglucosamine or Triton X-100 alone stimulated the activity by 4- or 7-fold, respectively; however, further addition of the nitrosamine and the nitramine resulted in 5- or 10-fold activation respectively. The activation toward 4-nitrophenol was not as great as that toward 2-aminophenol. When phenolphthalein was used as substrate, the transferase was activated only by UDP-*N*-acetylglucosamine and Triton X-100, no further activation being obtained by addition of the nitrosamine or the nitramine to the assay medium. These results strongly support the idea that the nitramine activated the transferase in a way similar to that of the nitrosamine.

DISCUSSION

UDP-glucuronosyltransferase is strongly bound to hepatic endoplasmic reticulum and exhibits latency in freshly prepared microsomes. Its activity can be increased by several membrane-perturbing procedures [1, 2]. The latency of the transferase activity may be due to restricted access of substrates to the enzyme and/or to poorly active conformation of the membrane-bound enzyme. Therefore, activation processes should remove these accessibility barriers and/or should convert the enzyme into a more active conformation. There is much evidence that supports either compartmentational or conformational theories [1, 2].

When *N, N*-diethylnitrosamine is added to liver homogenates or purified enzymes from Gunn rats, transferase activity toward 2-aminophenol is restored to high levels [3, 4]. Interaction of *N, N*-diethylnitrosamine with the enzyme is reversible [23]; removal of the nitrosamine prevents activation of the enzyme, but this is recovered by addition of the nitrosamine. Low 4-nitrophenol transferase activity in Gunn rats has been ascribed to decreased affinity of the transferase for UDP-glucuronic acid, which would be enhanced by addition of *N, N*-diethylnitrosamine [24]. These results favor its interaction with the enzyme molecule. From studies on structure–activity relationships of alkyl ketones, Burchell and his coworkers [10] described the minimum structural requirement for activation of the transferase as being the presence of both an electron-attracting (oxo) and a hydrophobic (ethyl chain) group.

To clarify the structural requirements for the activation of the enzyme in more detail, and to extend this activation to other substrates, we examined twelve nitrosamines and seven nitramines for their abilities to stimulate transferase activity, using 2-aminophenol, 4-nitrophenol, phenolphthalein, and androsterone as substrates. Nitramines, in which the *N*-nitroso group of nitrosamines is substituted for the *N*-nitro group, are, in general, less toxic and more stable than nitrosamines and seem to have structural characteristics similar to nitrosamines. *N*-Monosubstituted nitrosamines are very unstable and readily undergo spontaneous decomposition, while the corresponding nitramines are comparatively stable. Therefore, nitramines could be suitable compounds for elucidation of structure–activity relationships of the activators.

From studies of modification of the transferase activities by nitrosamines and nitramines, it was apparent that the nitramines modified the transferase activities in a manner similar to nitrosamines, and that the compounds with an *N, N*-diethyl structure exhibit maximum activation toward 2-aminophenol and 4-nitrophenol. Stimulation of the activity toward 4-nitrophenol was not as great as that toward 2-aminophenol. Cooperative activation of the transferase by the combined addition of UDP-*N*-acetylglucosamine or Triton X-100 suggests that the

activation mechanisms of *N, N*-diethylnitrosamine and *N, N*-diethylnitramine differ from those of UDP-*N*-acetylglucosamine and Triton X-100. These results are consistent with those reported by Winsnes [21]. The maximum transferase activities toward 2-aminophenol and 4-nitrophenol were obtained by the combined addition of *N, N*-diethylnitramine and Triton X-100. Only the *N, N*-diethyl-substituted nitrosamine and nitramine highly stimulated the transferase activity, and this stimulation was found only with certain substrates. This suggests precise structural requirements for optimal activation of a specific transferase. In fact, some of the nitrosamines and nitramines may interact in the wrong way with the enzyme molecule and inhibit the activity. Inhibition of activity by several nitrosamines and nitramines was pronounced when 2-aminophenol and androsterone were used as substrates. The activity of the transferase is divided roughly into two groups either on the basis of late-foetal and neonatal development of activity [25] or on the basis of the inducibility by 3-methylcholanthrene (GT_1) and phenobarbital (GT_2) [26]. There is a remarkable similarity between late-foetal and GT_1 substrates (planar compounds such as 2-aminophenol, 4-nitrophenol, and 4-methylumbelliferone) and between neonatal and GT_2 substrates (nonplanar compounds such as morphine, phenolphthalein, and 4-hydroxybiphenyl) [1]. Moreover, some of the late-foetal and GT_1 clusters are activated by *N, N*-diethylnitrosamine and *N, N*-diethylnitramine. These functional heterogeneities of the transferase may be a reflection of sequential or conformational heterogeneities of the enzymes. However, further study is required to obtain insight into the stimulatory mechanism of UDP-glucuronosyltransferase activity.

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