Synthesis and reduction of (*S*)-(-)-nicotine-*N´*-oxide and *N,N´*-dioxides by rat liver S-9 fraction

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Dedicated to Professor Keiichiro Fukumoto on the occasion of his 70th birthday

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Abstract

cis- And *trans*-Nicotine-*N*'-oxide (**5**) and *N,N*'-dioxide (**7**) diastereomers were synthesized in good yields from nicotine (**1**) using *m*-CPBA. Asymmetric reductions of nicotine *N*-oxides with rat liver S-9 fraction were investigated.

Keywords: Nicotine, MO calculations, nicotine-*N'*-oxide, nicotine-*N,N'*-dioxides, S-9

Introduction

Nicotine (**1**) is one of the main pharmacologically active constituents of tobacco, and when nicotine (**1**) is administered it is well absorbed by digestive and respiratory organs as is metabolized to cotinine (**2**), 5'-hydroxycotinine (**3**) and 3'-hydroxycotinine (**4**) by CYP2A6. Nicotine (**1**) is also metabolized by FMO to *trans*-nicotine-*N'*-oxide (**5a**) (Scheme 1).[1,2] The *N*-oxidation of nicotine (**1**) shows an interesting stereoselectivity in the formation of *cis*- and *trans*-nicotine-*N'*-oxides (**5a** and **5b**).[3] The kinetic properties of the formation of these diastereoisomeric nicotine-*N'*-oxides (**5a** and **5b**) in rat liver microsomes were only recently reported.[4] Interestingly, in humans, the *trans*-isomer was the major excreted urinary metabolite, while *cis*-nicotine-*N'*-oxide (**5b**), nicotine-*N*-oxide (**6**), and the further oxidized product, nicotine-*N,N'*-dioxide (**7**) are not found in the urine. To understand these observations nicotine-*N*-oxides were prepared using *m*-CPBA and the transition state energies of the reaction intermediates were investigated by an *ab-initio* MO method. The metabolic reduction of nicotine *N*-oxides using the rat liver S-9 fraction was also investigated.

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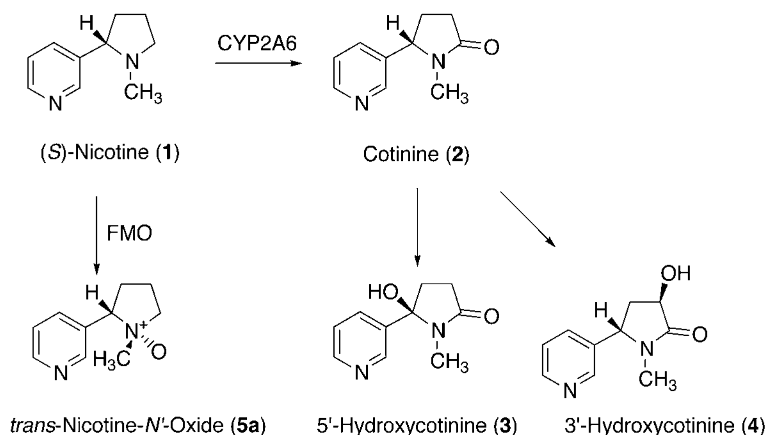


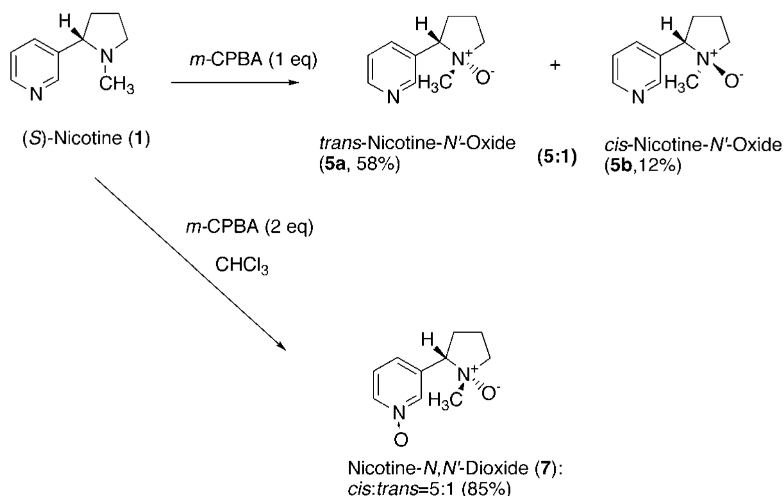
Scheme 1

Results and Discussion

Chemistry

Scheme 2 shows the synthesis of *cis*- and *trans*-nicotine-*N'*-oxides (**5a** and **5b**) and nicotine-*N,N'*-dioxide (**7**) diastereomers by treating (*S*)-(-)-nicotine (**1**) with *m*-CPBA. Treating (*S*)-(-)-nicotine (**1**) with 1 eq. of *m*-CPBA resulted in a diastereomeric mixture of *trans*- and *cis*-nicotine-*N'*-oxide (**5a**, **5b**), while the reaction of nicotine with 2 eq. of *m*-CPBA afforded a diastereomeric mixture of *trans*- and *cis*-nicotine-*N,N'*-dioxides (**7**). However, nicotine-*N*-oxide was not found in any reactions.

To explain these results, the energies associated with each step of the reactions and the activation energies (Ea) were calculated and are listed in Table 1. Fig. 1 shows the energy diagram for the reaction of (*S*)-nicotine (**1**) with *m*-CPBA to prepare *trans*-nicotine-*N'*-oxide (**5a**) and the structures in the Eq, TS, and final state.



Scheme 2

Table 1. Yields of products and E_a in the reactions of (*S*)-nicotin4e (**1**) and its *N*-oxides with *m*-CPBA, using Gaussian 98 at the RHF/6-31++G(d,p)//RHF/6-31G(d) level[7]

Compound		Energy (kcal/mol)				Yield (%)
Reactant	Product	Initial[a]	Eq	TS	Ea	
S-Nicotine (**1**)	*trans* -*N'*-Oxide (**5a**)	0.0	-13.32[b]	13.69	27.01	68
S-Nicotine (**1**)	*cis* -*N'*-Oxide (**5b**)	0.0	-13.32[b]	15.72	29.05	13
S-Nicotine (**1**)	*N*-Oxide (**6**)	0.0	-13.32[b]	24.71	38.03	0
trans –*N'*-Oxide (**5a**)	*trans* -*N,N'*-Dioxide (**7a**)	0.0	-24.29	16.50	40.79	68
cis -*N'*-Oxide (**5b**)	*cis* -*N,N'*-Dioxide (**7b**)	0.0	-20.77	15.82	36.59	14

[a] The energy of the initial state is normalized to zero.

[b] The products **5a**, **5b**, and **6** are formed from the same reactants via the same equilibrium state.

In the energy diagrams of those reactions, Ea for the formation of *cis* and *trans* nicotine-*N'*-oxides (**5a** and **5b**) were 29.05 and 27.01 kcal/mol, respectively. Our calculations suggest that the *trans* isomer is more stable than the *cis* isomer, and that the formation rate of the *cis* isomer is much smaller than that of the *trans* isomer. The highest Ea value (38.03 kcal/mol) was for forming nicotine-*N*-oxide (**6**) (Table 1), which is consistent with the experimental result that nicotine-*N*-oxide (**6**) was not obtained in the reaction of (*S*)-nicotine (**1**) with *m*-CPBA. The Ea for the oxidation of the *N* of *cis*- and *trans*- nicotine-*N'*-oxides (**5a** and **5b**) were 36.59 and 40.79 kcal/mol, respectively, and these values were much higher than those corresponding to *N'*.

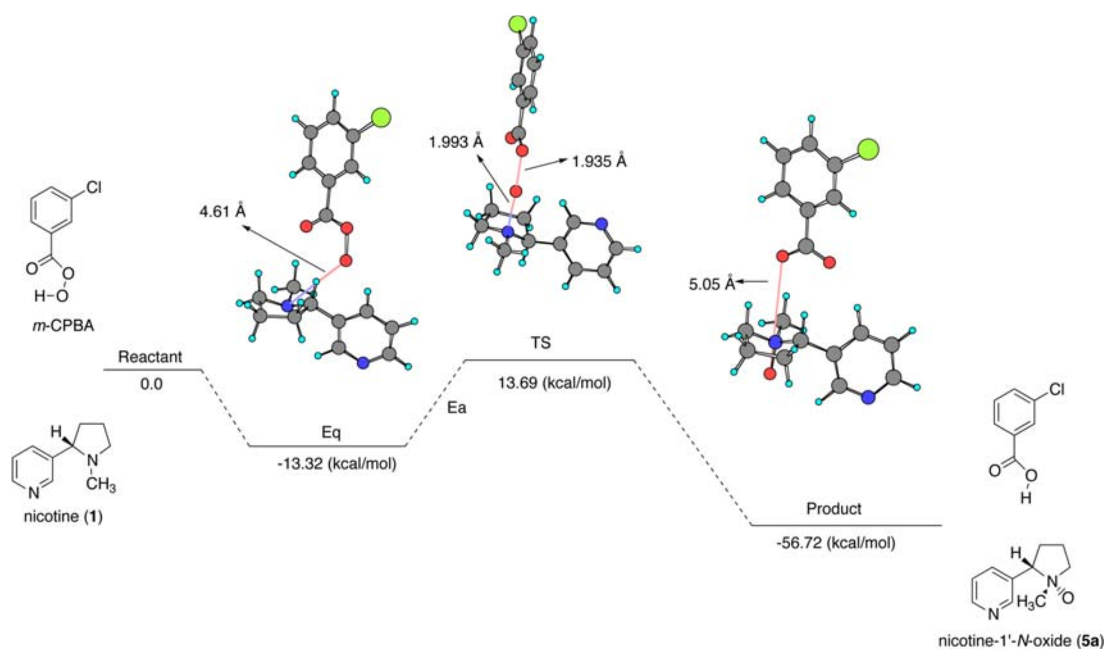
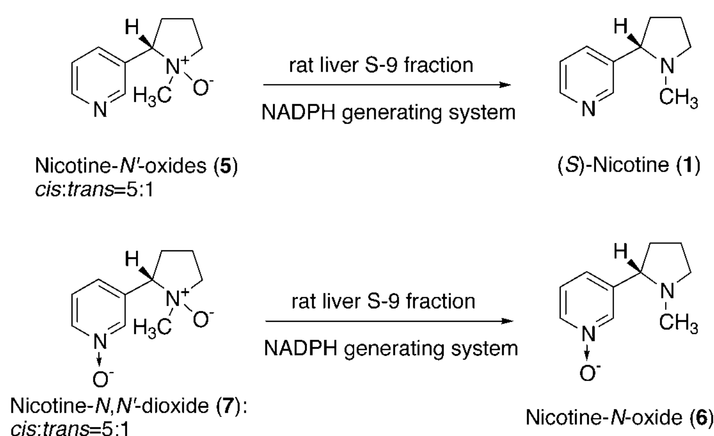


Figure 1. The energy diagram for the reaction of (*S*)-nicotine with *m*-CPBA to yield *trans*-nicotine-*N'*-oxide (**5a**). The energies are calculated using Gaussian 98 at the RHF/6-31++G (d,p)//RHF/6-31G (d) level. The sum of the energies of (*S*)-nicotine (**1**) and *m*-CPBA is normalized to zero.

Reduction of the nicotine *N*-oxides by rat liver S-9 fraction

Scheme 3 shows the results from the metabolic reductions of nicotine *N*-oxides. Diastereomeric mixture of nicotine-*N'*-oxide (**5**) was transformed to nicotine (**1**), and nicotine-*N,N'*-dioxide (**7**) was converted into nicotine-*N*-oxide (**6**). In these reactions, the aliphatic *N*-oxide was reduced faster than the aromatic *N*-oxide.

Experiments performed to determine the apparent K_m and V_{max} for the reduction of the diastereo mixture of nicotine-*N'*-oxides (**5**) and nicotine-*N,N'*-dioxides (**7**) were conducted



Scheme 3

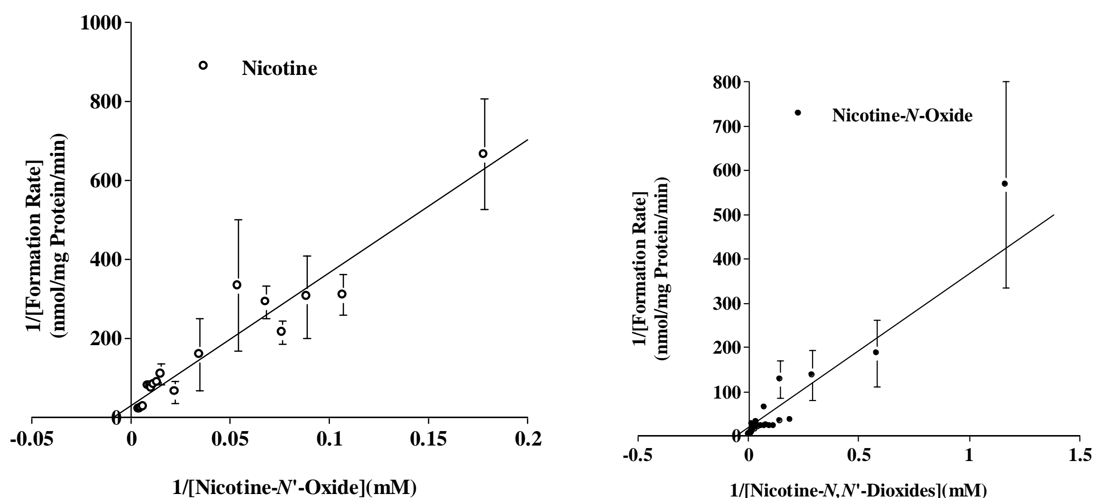


Figure 2. Lineweaver–Burk plots for the *N´*-deoxygenation of nicotine-*N'*-oxides (**5**) and nicotine *N,N'*-dioxides (**7**) by rat liver S-9 fraction. Each point represents the mean from three trials.

at a final protein concentration of 6.0 mg/mL (30 min incubation) and in the presence of increasing concentrations of nicotine-*N'*-oxide (5.6–262.2 mM) or nicotine-*N,N'*-dioxide (0.86–240.55 mM). Under these conditions, the metabolite formation was linear with respect to the S-9 concentration and time of incubation. Kinetic analyses of the enantioselective reductive metabolism of nicotine-*N*-oxides with rat liver S-9 fraction were conducted using Lineweaver–Burk plots. The reductions of the diastereomeric mixture of nicotine-*N'*-oxides (**5**) and nicotine-*N,N'*-dioxides (**7**) were characterized by V_{max} of 0.037 and 0.271 nmol/mg protein/min, respectively, whereas the estimated apparent K_m for these reactions were 114.07 and 81.73 mM, respectively (Fig. 2). Kinetic analyses of the enantioselectivity of the reductive metabolism of nicotine-*N*-oxides with rat liver S-9 fraction showed that V_{max}/K_m values were 0.3 nL/mg protein/min for reducing of nicotine-*N'*-oxide (**5**) to nicotine (**1**) and 3.3 nL/mg protein/min for the reduction of nicotine-*N,N'*-dioxide (**7**) to nicotine *N*-oxide. These results suggest that the reduction of nicotine-*N,N'*-dioxide (**7**) is much faster than that of nicotine-*N'*-oxide (**5**) and that reduction of aromatic *N*-oxide does not proceed under these experimental conditions.

When a diastereomeric mixture of nicotine-*N'*-oxides (**5**) was treated with rat liver S-9 fraction, *cis*-nicotine-*N'*-oxide (**5b**) was reduced faster than *trans*-nicotine-*N'*-oxide (**5a**). (Fig. 3).

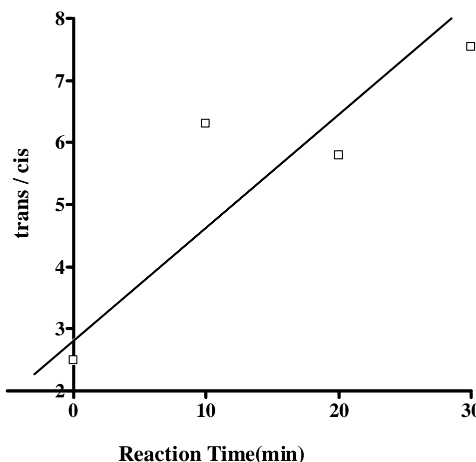


Figure 3. *trans*- and *cis*- Ratios in the reduction of a diastereomeric mixture of nicotine *trans*- and *cis*-*N'*-oxides (**5**) in rat liver S-9 fraction.

Acute toxicity

The acute toxicities (LD_{50}) of nicotine-*N*-oxides (diastereomeric mixtures of **5** and **7**, and **6**) in mice was determined by the Litchfield–Wilcoxon method.[10] The results demonstrated that,

unlike nicotine (LD_{50} = 9.5 mg/kg), these nicotine *N*-oxides did not show an acute toxicity even at 200 mg/kg after intraperitoneal injection (Table 2). The acute toxicities of the nicotine-*N*-oxides clearly show that when the N atoms are oxidized, the toxicity decreases.

Table 2. Acute toxicity of the nicotine *N*-oxides

	Nicotine-*N*-oxide (**6**)	Nicotine-*N*´-oxide (**5**)	Nicotine-*N,N*´-dioxide (**7**)
LD_{50} mg/kg	245	615	>2200
(mmol/kg)	(13.8)	(34.6)	(>113.4)

Since the oxidation of the aliphatic amine was more effective in reducing the toxicity than that of the pyridine nitrogen, in humans, metabolization of nicotine (**1**) to nicotine-*N'*-oxide (**5a**) accomplishes two goals. First nicotine (**1**) is transformed into a non-toxic metabolite. Second, excretion is facilitated.

Experimental Section

General Procedures. All reactions were conducted in oven-dried glassware under a nitrogen atmosphere. Wako gel C-200 (70–150 µm, Wako pure chemicals) was used for column chromatography. Precoated Kieselgel 60F-254 plates (0.25 mm, Merck) were used for TLC analysis and the spots were detected by absorption of UV light at 254 nm and by spraying with Dragendorff's reagent. Optical rotations were measured on JASCO DIP-360 polarimeters. 1H NMR spectra were recorded on JEOL JNM EX270 (270 MHz), JEOL JNM 400 (400 MHz), and JEOL JNM 600 (600 MHz) spectrometers. Mass spectra were recorded on JEOL JMS DX-303/JMA-DA 5000 spectrometers.

Chemicals

(*S*)-(-)-Nicotine (**1**) was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). $NADP^+$ and glucose 6-phosphate were from Nacalai Tesque, Inc. (Kyoto, Japan). Glucose 6-phosphate dehydrogenase was from Sigma Chemical Co. (St Louis, MO, USA). *m*-CPBA was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals and solvents were of the highest commercially available grade.

Synthesis of nicotine-*N*´-oxides (5a and 5b). *m*-CPBA (70% solid with H_2O, 5.9 g, 33.9 mmol) was added, in portions, to a stirred solution of (*S*)-(-)-nicotine (**1**, 5 g, 30.8 mmol) in $CHCl_3$, at room temperature. After 1 h, K_2CO_3 (18 g) and H_2O (3 mL) were added and the mixture was stirred for 5 min. The resulting solution was filtered and the filtrate was concentrated *in vacuo*. Purification by column chromatography on silica gel (250 g) gave a diastereomeric mixture (5:1) of *cis*- and *trans*- nicotine-*N'*-oxides (**5**). Subsequently, the mixture was separated by HPLC using a Nanospace SI-1 (Shiseido, Tokyo, Japan) chromatograph equipped with a Chiral CD-Ph column (10.0 × 250 mm; Shiseido, Tokyo, Japan) using MeOH:H_2O (4:1, *v*/*v*) as the mobile

phase. The flow rate was 3.0 mL/min and column temperature was 40°C. The eluent was monitored at 254 nm. In this way, *trans*-nicotine-*N'*-oxide (**5a**, 3.2 g, 58%) and *cis*-nicotine-*N'*-oxide (**5b**, 659 mg, 12%) were isolated. **5a**: $[\alpha]_D$ +27.1° (*c* = 3, $CHCl_3$). **5b**: $[\alpha]_D$ –69° (*c* = 2, $CHCl_3$). All spectral data is consistent with previously reported results.[5]

Synthesis of nicotine-*N,N´*-dioxide (7). *m*-CPBA (70% solid with H_2O, 11.8 g, 67.8 mmol) was added in portions to a stirred solution of (*S*)-(-)-nicotine (**1**, 5 g, 30.8 mmol) in $CHCl_3$, was added at room temperature. After 5 h, K_2CO_3 (36 g) and H_2O (6 mL) were added and the mixture was stirred for 5 min. The resulting solution was filtered and the filtrate was concentrated *in vacuo*. Purification by column chromatography on silica gel (250 g) yielded a diastereomeric mixture (5:1) of *cis*- and *trans*-nicotine-*N,N'*-oxides (**7**). All spectral data are consistent with that previously reported.[6]

Calculation of activation energies

The oxidation reactions of nicotine and its *N*-oxides with *m*-CPBA are considered to proceed as follows: The reactant molecules interact, forming an equilibrium complex (Eq). Then, the final compound is produced through a transition state (TS). The structures of Eq and TS were optimized using Gaussian 98 at the RHF/6-31++G (d,p)//RHF/6-31G (d) level.[7] The solvent effect was not considered. The activation energy was calculated as the energy difference between the TS and the Eq. After optimizing the TS structure, the vibrational frequencies were calculated and it was confirmed that the TS had a single imaginary vibrational frequency.

Enzymatic Experiments

1. Animals

Male Wistar rats (150–180 g) were supplied by Japan SLC (Hamamatsu, Japan) and housed in an air-conditioned room (23 ± 1 °C and 55.0 ± 5% relative humidity) with a 12 h light cycle, and allowed to feed (CE-2 obtained from Japan Crea Co., Japan) and drink water *ad libitum.*

2. Preparation of rat liver 9000 *g* supernatant fraction

Rat liver S-9 fraction was prepared from untreated rats as previously described.[8] Briefly, the liver was removed and perfused with saline, and the liver sample was homogenized in an ice-cold 0.1 M K, Na-phosphate buffer. The homogenates were centrifuged (9000 *g*, 4°C) for 20 min, and the supernatant (S-9) was used in the experiments. The protein concentration was determined using the method of Bradford *et al.*[9] with bovine serum albumin as a standard.

3. *in vitro* Reduction of diastereomeric nicotine-*N'*-oxide (5) and nicotine-*N,N'*-dioxide (7).

A mixture (final volume, 6 mL) consisting of 10 mM Na,K- phosphate buffer (pH 7.4), NADPH-generating system (1.3 mM NADP, 10 mM glucose 6-phosphate, 5 mM $MgCl_2$, 0.4 U/mL glucose 6-phosphate dehydrogenase), 6.0 mg/mL S-9 fraction and nicotine-*N*-oxides (**5** or **7**) was incubated at 37°C for 30 min. Adding a 2.0 M solution of aqueous $HClO_4$ (3 mL) followed by

caffeine as an internal standard terminated the reaction. After centrifugation (800 *g*, 15 min) to remove the protein, a 1*N* solution of aqueous NaOH (2 mL) and $CHCl_3$ (15 mL) was added to the supernatant, which was subsequently shaken for 15 min. After the phases separated, 10 mL of the organic fraction was concentrated *in vacuo* and the residue was redissolved in 200 µL of MeOH and a 20 µL portion of the sample was subjected to HPLC.

HPLC analyses were performed using a Shodex DEGAS KT-27 degasser (Showa Denko, Tokyo, Japan), and a Waters 510 HPLC Pump (Waters, Milford MA, USA) equipped with a CAPCELL PAK SCX column (4.6 × 150 mm, 5 µm; Shiseido, Tokyo, Japan). The eluent was monitored at 220 nm using a Waters 486 Tunable Absorbance Detector (Waters). The mobile phase was 100 mM K, Na-phosphate buffer and acetonitrile (1:1, *v*/*v*). The flow rate was 1.0 mL/min and the column temperature was 40°C. The metabolites were quantified by comparing the HPLC peak areas with those of authentic standards with an internal standard reference.

4. Kinetic analysis

The kinetic studies were performed using rat liver S-9 fraction incubated with nicotine-*N'*-oxide and nicotine-*N,N'*-dioxide concentrations ranging from 5.6 to 262 and 0.86 to 240 mM, respectively. The maximum velocity (V_{max}) and Michaelis–Menten constant (K_m) were evaluated by non-linear regression analysis of the untransformed kinetic data.

Acute toxicity

Male mice of the ddy strain (25–26 g) were purchased from Nihon SLC Co. (Hamamatsu, Japan). The mice were housed in groups of 10 per cage (30 × 30 × 16 cm), and kept in an air-conditioned room (22 ± 2 °C and 55.5 ± 5% relative humidity) with 12 h light cycle, and allowed to feed (F-2 obtained from Funabashi Farm Co., Funabashi, Japan) and drink water *ad libitum*. Samples were suspended in physiological saline containing 0.5% Tween 80 and intraperitoneally administered in geometrically increasing doses from 0 to 100% lethal dose (10 mL/kg body weight) 5–10 mice were used for each dose. The LD_{50} values were estimated according to the Litchfield–Wilcoxon method.[10]

Conclusions

Nicotine-*N*-oxides were successfully synthesized using *m*-CPBA and the transition state energies of the reaction intermediates were investigated by an *ab-initio* MO method. Consequently, it was found that oxidation occurred first at the *N'* position, followed by the *N* position both in theory and practice. The asymmetric reduction of nicotine-*N*-oxides with the rat liver S-9 fraction showed that the aliphatic *N*-oxide was reduced faster than the aromatic one, and *cis*-nicotine-*N'*-oxide (**5b**) was easier to reduce than *trans*-nicotine-*N'*-oxide (**5a**). These results suggest that even if *cis*-nicotine-*N'*-oxide (**5b**) is produced *in vivo*, the reduction–oxidation process should

exclusively result in the urinary excretion of *trans*-nicotine-*N'*-oxide (**5a**). The acute toxicities of the nicotine-*N*-oxides were also investigated and suggest a reason of oxidation of nicotine (**1**).

Although the mechanism of the oxidation of the nicotine in mammalian metabolic system has been previous reported, little is known about the reduction of nicotine-*N*-oxides. The present study sheds light on the reductive properties of the mammalian reductive system. Further studies are currently underway in our laboratory, including a detailed mechanistic study of the metabolism of these compounds.

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