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Non-enzymatic Oxidation of the New Cardiotonic Agent Denopamine and Its Derivatives: Comparison with Enzymatic Oxidation

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Chemical oxidation of the positive inotropic agent denopamine, (−)-(*R*)-1-(*p*-hydroxyphenyl)-2-[(3,4-dimethoxyphenethyl)amino] ethanol, and its derivatives by Udenfriend's model system for enzymatic oxidation was studied. The oxidation products were separated and identified by gas chromatography-mass spectrometry and high-performance liquid chromatography. All the metabolites of denopamine produced by enzymatic oxidation were also formed in Udenfriend's system. However, compared to enzymatic oxidation, the chemical oxidation was less selective as to the position of demethylation: *i.e.*, although enzymatic 4′-O-demethylation took place overwhelmingly in preference to 3′-O-demethylation, comparable amounts of the two demethylated isomers were produced in Udenfriend's system. It was also found that the chemical oxidation was more powerful than the enzymatic oxidation because hydroxylation at the *ortho* or *para* position to the methoxy group took place in all the substrates tested, while such metabolites have not been detected in biological systems. As in the enzymatic system, tetrahydroisoquinoline-type compounds were formed from substrates in which the hydroxy group was attached at the *meta* position of the benzene ring. This is presumably a result of Pictet–Spengler-type condensation with formaldehyde generated in the reaction mixture.

Keywords——denopamine; oxygenation; non-enzymatic oxidation; Udenfriend's system; O-demethylation; ring hydroxylation; Pictet–Spengler condensation; monooxygenase; tetrahydroisoquinoline; formaldehyde

Denopamine, (−)-(*R*)-1-(*p*-hydroxyphenyl)-2-[(3,4-dimethoxyphenethyl)amino]ethanol (I), is a new, orally active, selectively positive inotropic agent.[1] In a previous study on its urinary metabolites excreted after oral administration of denopamine to man, we identified and quantitated five metabolites (II—VI).[2] As is evident from the structures, the initial reaction steps in denopamine metabolism involve either oxidative O-demethylation of the two adjacent methoxy groups or ring hydroxylation *ortho* to the phenolic hydroxy group by monooxygenase.

The catechol compound (VII) formed *in vivo* by ring hydroxylation is immediately methylated by catechol O-methyltransferase (COMT) to IV and V. Although VII is the obligatory metabolic intermediate to IV and V, VII was not detected in human plasma and urine samples after administration of denopamine. However, the formation of VII was confirmed by incubation of denopamine with rat liver microsomal fraction containing the reduced nicotinamide adenine dinucleotide phosphate (NADPH) generating system.[3]

As for the two adjacent methoxy groups of denopamine, both *para*- and *meta*-O-demethylated metabolites are formed by oxidative demethylation. These isomers were detected not only in the above human urine samples, but also as products of *in vitro* incubation of denopamine with rat liver preparations. However, the amount of the *para*-demethylated isomer (II) was overwhelmingly greater than that of the *meta*-demethylated compound (III) in both systems.[2,3]

Fenton's reagent ($FeCl_2$, H_2O_2)[4] and Udenfriend's system ($FeCl_2$, ascorbic acid, ethylenediamine tetraacetic acid (EDTA))[5] are both well known as chemical reaction models

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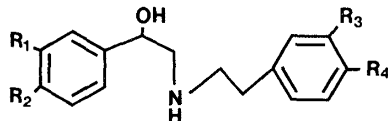

	R_1	R_2	R_3	R_4
denopamine (I)	H	OH	OCH_3	OCH_3
II	H	OH	OCH_3	OH
III	H	OH	OH	OCH_3
IV	OCH_3	OH	OCH_3	OCH_3
V	OH	OCH_3	OCH_3	OCH_3
VI	OCH_3	OH	OCH_3	OH
VII	OH	OH	OCH_3	OCH_3
VIII	OH	OH	OCH_3	OH
IX	H	H	OCH_3	OCH_3
X	H	OCH_3	OCH_3	OCH_3
XI	H	OH	OH	OH
XII	OCH_3	OH	OH	OCH_3
XIII	H	OH	H	OCH_3
XIV	H	OH	OCH_3	H

Chart 1

of the monooxygenase system. Since the chemical reaction pattern of denopamine in Udenfriend's system was similar to that of enzymatic oxidation with the rat liver microsomal fraction in our preliminary experiments, we selected Udenfriend's system to study in detail the non-enzymatic oxidation products of denopamine and its derivatives and compare them to the enzymatic oxidation products of denopamine. The oxidation products were determined by gas chromatography-mass spectrometry (GC-MS), and, when the isomers were not separated from each other by GC-MS, by a combination of high-performance liquid chromatography (HPLC) and GC-MS. The present paper describes the non-enzymatic ring hydroxylation and oxidative O-demethylation of denopamine in Udenfriend's system and the occurrence of ring condensation products in comparison with *in vitro* enzymatic oxidation.

Experimental

Chemicals——Chemicals were obtained from the following sources: trimethylsilylimidazole (TMSI) from Tokyo Kasei Kogyo Co., Tokyo, Japan; ethyl acetate, acetonitrile, and disodium ethylenediaminetetraacetate (EDTA-2Na) from Nakarai Chemicals Co., Tokyo, Japan; ferrous chloride from Kanto Kagaku Co., Tokyo, Japan; L-ascorbic acid from the Tokyo Plant, Tanabe Seiyaku Co., Ltd.

TMSI and ethyl acetate were distilled before use. Denopamine and related compounds, II—X and XIII, were synthesized by Dr. T. Iwakuma of the Organic Chemistry Research Laboratory, Tanabe Seiyaku Co., Ltd. Compounds XI, XII, and XIV were synthesized by us as described by Iwakuma *et al.*[6] Compounds XV—XVIII were synthesized as follows. Compound III (80 mg, 0.24 mmol) in MeOH (10 ml) was treated with 37% formaldehyde (0.2 ml) for 1 h at room temperature. The reaction mixture was concentrated *in vacuo* to give a solid residue, which was recrystallized from iso-PrOH to give a mixture of XV and XVI (60 mg, 73%), mp 152—156 °C; XV and XVI were separated by preparative thin-layer chromatography (TLC) on silica gel with $CHCl_3$–MeOH = 9 : 1. Compound V (150 mg, 0.39 mmol) was treated similarly. A solid residue was recrystallized from iso-PrOH–MeOH–diethyl ether to give a mixture of XVII and XVIII (100 mg, 65%), mp 140—145 °C; Compounds XVII and XVIII were separated by preparative TLC on silica gel with $CHCl_3$–MeOH = 9 : 1. All these compounds were characterized by mass spectrometry.

Non-enzymatic Oxidation——Non-enzymatic oxidation was carried out in a modified Udenfriend's system.[5] To 10 ml of 0.1 M phosphate buffer, pH 6.6, was added 2 mg of substrate, 4 mg of ferrous chloride, 80 mg of EDTA-2Na, and 50 mg of L-ascorbic acid. The mixture was shaken for 1 h at 37 °C in air. Then the reaction mixture was made alkaline with 1 N NH_4OH, and extracted with 10 ml of ethyl acetate. After evaporation of the ethyl acetate *in vacuo*, the residue was subjected to HPLC directly or to GC-MS after derivatization.

Derivatization for GC-MS——O-TMS derivatives of the oxidation products were prepared as follows. Ethyl acetate (50 μl) and TMSI (50 μl) were added to the dried residue. The tube was allowed to stand at room temperature for 30 min.

GC-MS——A Shimadzu 6020 GC-MS instrument equipped with a DEC 11/23 minicomputer was employed. The column was a 1 meter-long and 2 mm i.d. glass coil packed with 1.5% OV-1 on 100/120 mesh Gas Chrom Q. The flow rate of carrier gas (helium) was 35 ml/min. The temperatures of the injection port and the separator were both 280 °C and that of the ionization source was 290 °C. The mass spectrometer was operated at an ionization energy of

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30 eV and an accelerating voltage of 3.5 kV in the electron impact (EI) mode. As previously reported,[2] the mass spectra of the O-TMS derivatives of denopamine and related compounds gave characteristic fragment ions resulting from the cleavage of the bond between the substituted benzyl alcohol and phenethylamine moieties. Consequently, these ions were monitored in the mass chromatograms to characterize the products.

HPLC——Dried samples were dissolved in the mobile phase and subjected to HPLC with a Waters Associates model 6000A pump system equipped with a model U6-K universal injector and a Shimadzu SPD-2A spectrophotometer. Separation was accomplished on a Waters Radial Pak C_{18} column (10 cm × 8 mm i.d.), particle size 10 μm, by isocratic elution with a solvent (water–methanol–acetonitrile, 16 : 3 : 1, v/v) containing 1% (v/v) AcOH and 2% (v/v) triethylamine. The compounds were detected by measuring ultraviolet absorption at 280 nm.

Results

Oxidation of Denopamine and Its Metabolites

Figure 1 shows a total ion intensity trace and mass chromatograms of an extract obtained after non-enzymatic oxidation of denopamine (I) with $FeCl_2$, ascorbic acid and EDTA under the conditions of Udenfriend's system. The mass numbers selected represent the characteristic fragment ion structures shown in Chart 2. The two peaks at about 5.5 min with characteristic ions at m/z 252 and 267 gave identical mass spectra. These mass spectra were

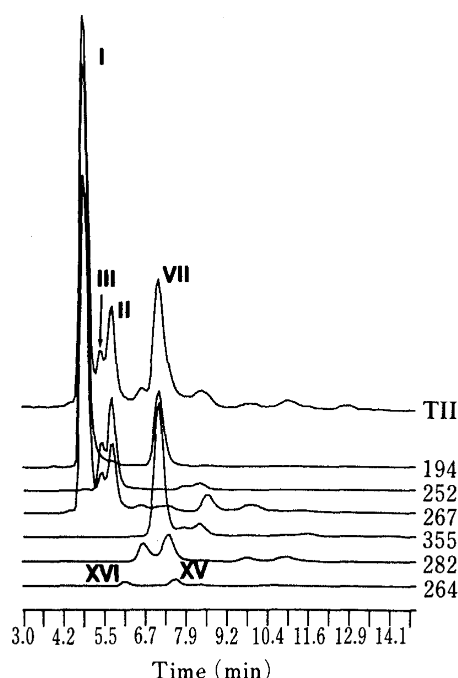

Fig. 1. Mass Chromatogram of O-TMS Derivatives of Oxidation Products of Denopamine

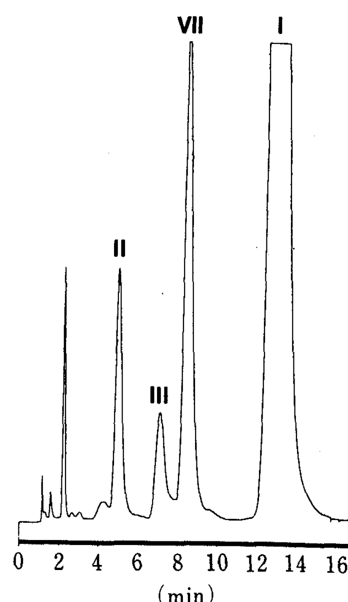

Fig. 2. High-Performance Liquid Chromatogram of Oxidation Products of Denopamine

R_1	R_2	m/z
H	OTMS	267
OCH_3	OTMS	297
OTMS	OCH_3	297
OTMS	OTMS	355
H	H	179
H	OCH_3	209

R_3	R_4	m/z
OCH_3	OCH_3	194
OCH_3	OTMS	252
OTMS	OCH_3	252
OTMS	OTMS	310
H	OCH_3	164
OCH_3	H	164

Chart 2

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indicative of O-demethylated denopamine.

The retention time of synthetic 4′-O-demthylated denopamine (II) was slightly longer than that of synthetic 3′-O-demethylated denopamine (III). Consequently, the larger peak was identified as II. When the extract residue was subjected to HPLC as described in the previous paper,[2)] the peaks of II and III were completely separated from each other as shown in Fig. 2. The ratio of II to III was 1.6 : 1. The peak at 7.0 min in the mass chromatograms of the characteristic ions at m/z 194 and 355 gave a mass spectrum identical to that of synthetic hydroxylated denopamine, *i.e.*, the catechol compound VII. In addition, two peaks were

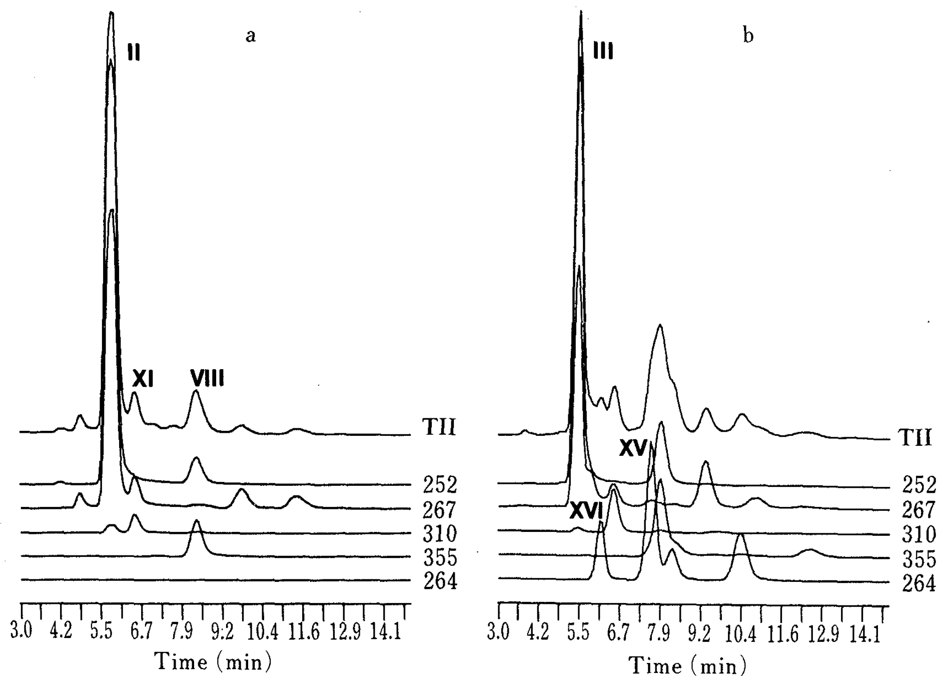

Fig. 3. Mass Chromatograms of O-TMS Derivatives of Oxidation Products of (a) II and (b) III

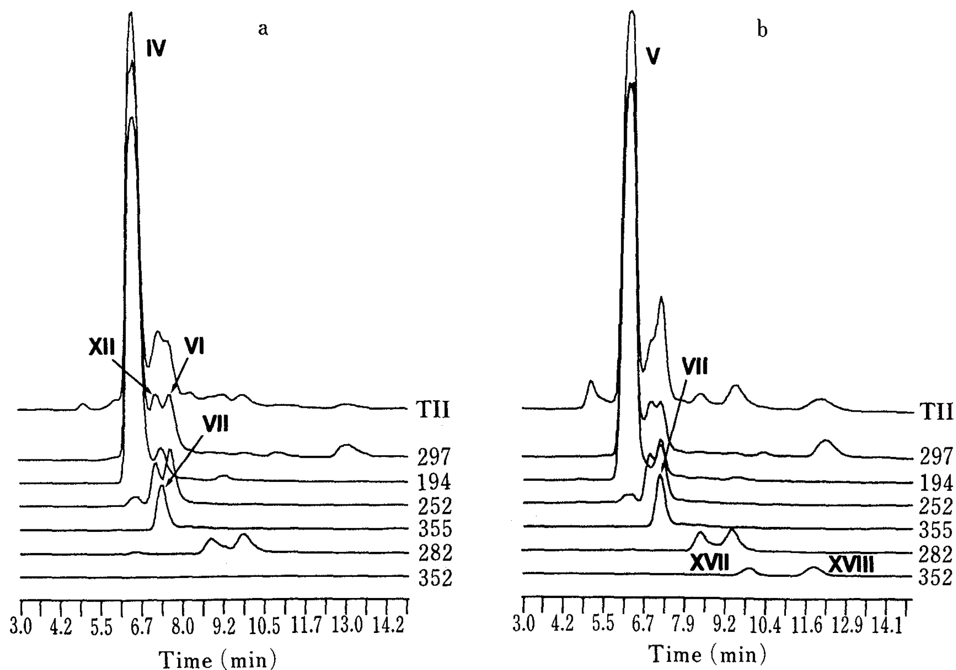

Fig. 4. Mass Chromatograms of O-TMS Derivatives of Oxidation Products of (a) IV and (b) V

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detected at 6.7 and 7.2 min on the mass chromatogram trace of m/z 282. The ion at m/z 282 indicated the presence of compounds with a hydroxylated dimethoxyphenethylamine moiety, suggesting that ring hydroxylation had also taken place on the dimethoxybenzene ring.

Figure 3-a shows mass chromatograms of an extract obtained after oxidation of II. In this case, two catecholic compounds were the main products. The peak at 7.8 min having the ions at m/z 252 and 355 was found to be due to VIII resulting from ring hydroxylation of the benzyl alcohol moiety, and the peak at 6.5 min having the ions at m/z 267 and 310 was found to be due to XI resulting from demethylation of the hydroxy-methoxyphenethylamine moiety.

Mass chromatograms of an extract obtained after oxidation of III, an isomer of II, looked more complex as shown in Fig. 3-b. The peaks of the mass chromatogram of m/z 264 were characteristic of the oxidation products of III. The assignment of these peaks will be described later.

Figure 4-a shows mass chromatograms of an extract obtained after oxidation of IV. Both 4′- and 3′-O-demethylation took place with IV, as shown by the peaks of VI and XII having the ions at m/z 252 and 297. Moreover, demethylation also took place at the methoxy group of the benzyl alcohol moiety, as shown by the peak of VII having the ions at m/z 194 and 355.

Figure 4-b shows mass chromatograms of an extract obtained after oxidation of V. Reactions common to both IV and V took place, *i.e.*, demethylation in both the benzyl alcohol and dimethoxyphenethylamine moieties. However, in this case, two peaks having the ion at m/z 352 were detected. The assignment of these peaks will be described later.

Oxidation of Denopamine Analogs

Figure 5 shows mass chromatograms of an extract obtained after oxidation of IX, which possesses no substituent on the benzene ring of its benzyl alcohol moiety. Both 4′- and 3′-O-demethylated IX having the ion of m/z 252 were detected at about 6 min. The prominent peak at 13 min in the mass chromatograms of m/z 194 and 355 gave a mass spectrum identical to that of synthetic dihydroxylated IX, *i.e.*, catechol compound VIII, but the amount of the peak corresponding to *p*-monohydroxylated IX, *i.e.*, denopamine (I), was small.

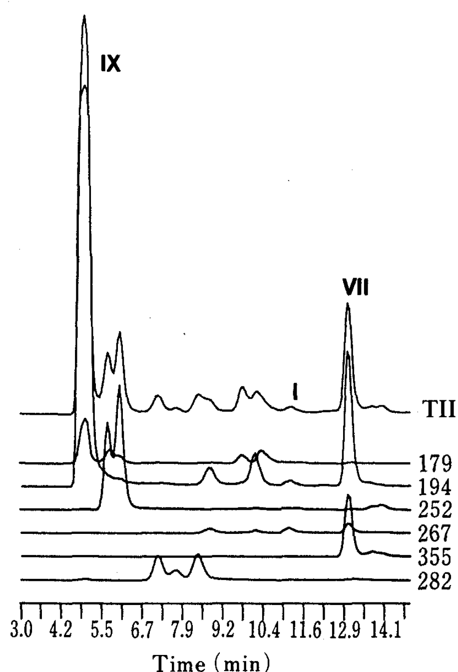

Fig. 5. Mass Chromatogram of O-TMS Derivatives of Oxidation Products of IX

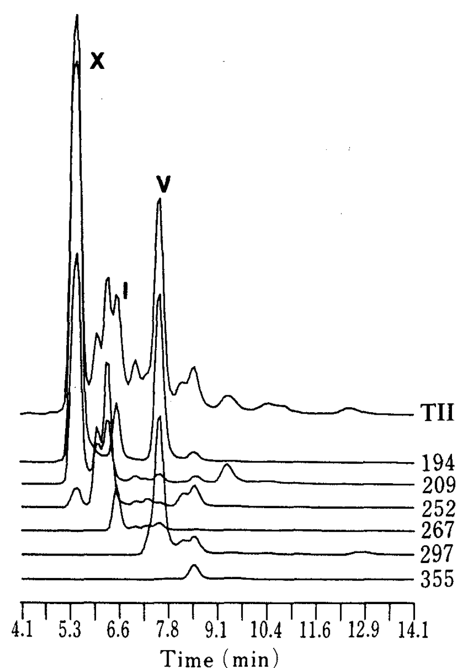

Fig. 6. Mass Chromatogram of O-TMS Derivatives of Oxidation Products of X

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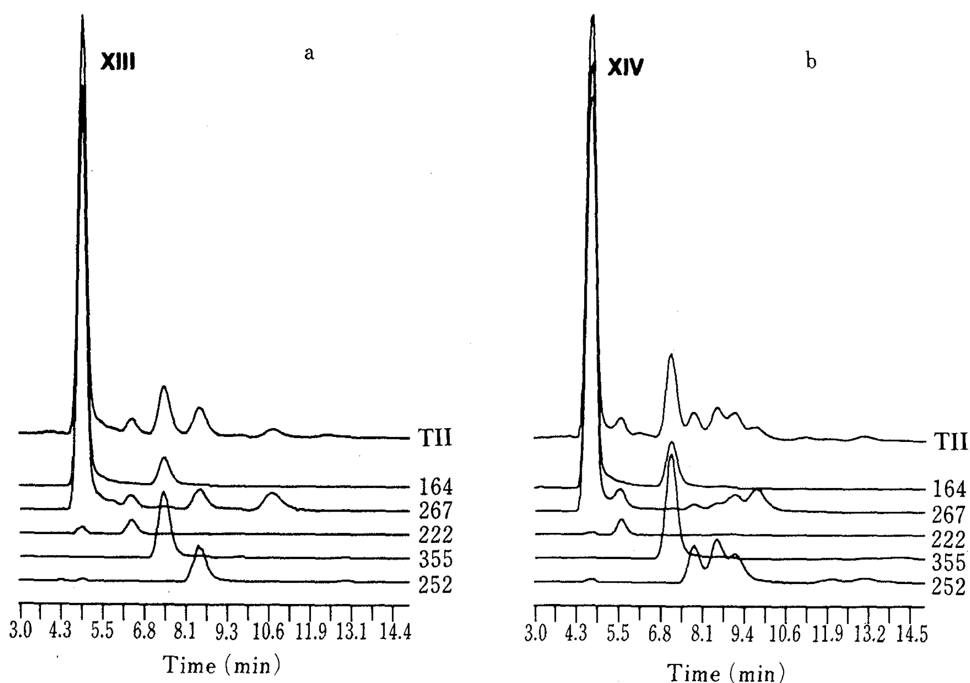

Fig. 7. Mass Chromatograms of O-TMS Derivatives of Oxidation Products of (a) XIII and (b) XIV

Figure 6 shows mass chromatograms of an extract obtained after oxidation of X, which possesses a *p*-methoxy group in the benzyl alcohol moiety. In this case, two prominent peaks were detected at 6.5 and 7.5 min besides the usual two peaks resulting from demethylation in the dimethoxyphenethylamine moiety. One of these at 6.5 min having the ions of m/z 194 and 267 was identified as denopamine (I) resulting from demethylation in the *p*-methoxybenzyl alcohol moiety, and the other at 7.5 min having the ions of m/z 194 and 297 resulted from *ortho*-hydroxylation in the *p*-methoxybenzyl alcohol moiety of X.

Figure 7 shows mass chromatograms of extracts obtained after oxidation of *para*-monomethoxy (XIII) and *meta*-monomethoxy (XIV) analogs of I. In both cases, the peaks of the catechol compounds at about 7.5 min having the ions of m/z 164 and 355 were conspicuous, and they resulted from ring hydroxylation of the benzyl alcohol moiety. There was no difference in the amount of the demethylation products which appeared at about 6 min and gave the ions of m/z 222 and 267. In the case of XIII, one peak having the ions of m/z 252 and 267 was observed at 8.5 min while, in the case of XIV, three peaks were observed on the traces of the above ions. These peaks were presumed to represent compounds which were ring-hydroxylated in the monomethoxyphenethylamine moiety. The difference in the pattern of the products probably arises from the fact that XIII possesses only one position which could be *ortho*-hydroxylated on the methoxybenzene ring, while XIV possesses three (two *ortho* and one *para*) such positions.

Formation of Tetrahydroisoquinolines

Two peaks were observed at 6.1 and 7.7 min in the mass chromatogram trace at m/z 264 of the extract obtained after oxidation of I (Fig. 1). These peaks were also observed in the mass chromatograms of the extract obtained after oxidation of III, but, in this case, their amounts were much greater (Fig. 3-b). Mass spectrometric analysis showed that these compounds are two isomeric tetrahydroisoquinoline-type compounds (Fig. 8), presumably formed by Pictet–Spengler-type condensation of III with formaldehyde generated by demethylation in the reaction mixture (Chart 3). Their chemical structures were confirmed as XV and XVI by comparing the retention times and mass spectra of the individual peaks with

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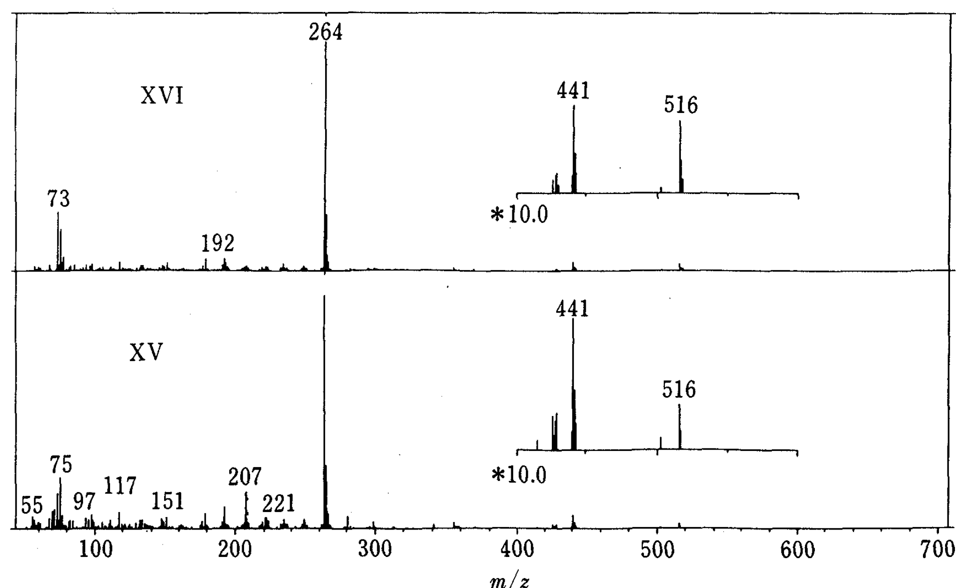

Fig. 8. Mass Spectra of O-TMS Derivatives of XV and XVI

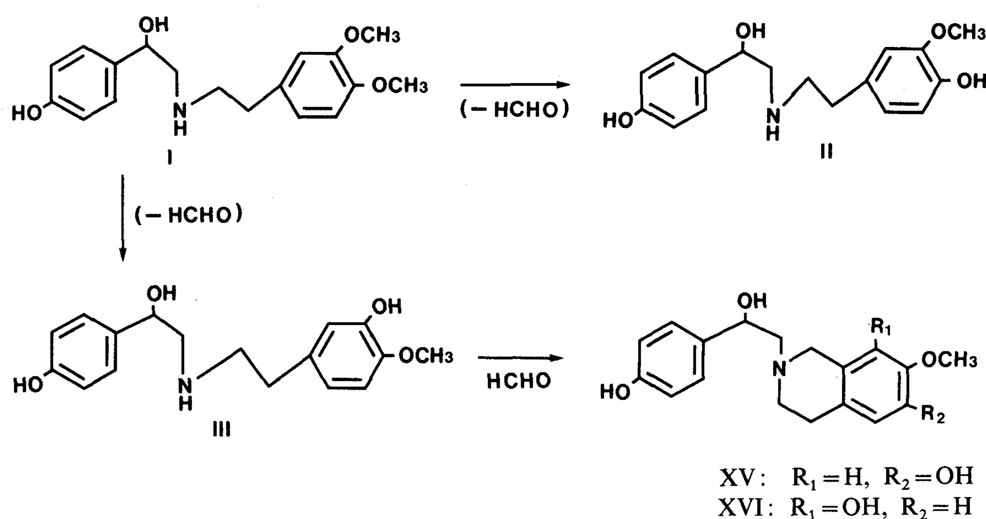

Chart 3

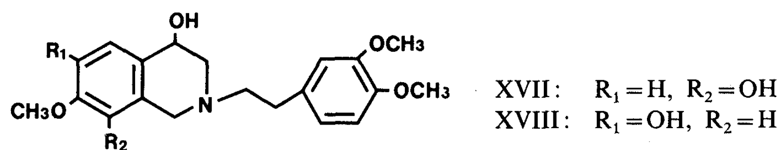

Chart 4

those of synthetic samples obtained by the reaction of III with formaldehyde. Two further peaks appeared on the mass chromatogram trace of m/z 264 at 8.3 min and 10.5 min (Fig. 3-b). From mass spectral analysis, these peaks are probably catechol-like tetrahydroisoquinolines. Similar reactions were observed in the oxidation of V, and in this case, two peaks were detected on the trace of m/z 352 at 9.8 min and 12.0 min (Fig. 4-b). These products were identified as XVII and XVIII by comparison of the retention times and mass spectra with those of synthetic samples (Chart 4). None of these tetrahydroisoquinoline-type compounds was detected in the mass chromatograms of the extracts obtained after oxidation of II and IV, which are isomers of III and V, respectively.

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Discussion

Previous studies on the metabolism of denopamine (I) *in vivo* and *in vitro* showed that enzymatic 4′-O-demethylation took place overwhelmingly in preference to 3′-O-demethylation.[2,3] However, the prsent study demonstrated that the amounts of the two isomers of demethylated denopamine (II and III) formed by non-enzymatic oxidation of I were almost equal. Thus, the high positional selectivity characteristic of enzymatic O-demethylation is not observed in the non-enzymatic oxidation in Udenfriend's system, which is a well-known chemical model of P-450-dependent monooxygenase. There was no difference in the amount of chemical demethylation products between mono-*para*-methoxy compound (XIII) and mono-*meta*-methoxy compound (XIV). On the other hand, demethylation of XIII took place in preference to that of XIV in the *in vitro* enzymatic oxidation of coexisting XIII and XIV to give a *para*-to-*meta* ratio of about 2, which is much smaller than the ratio (33) obtained in the enzymatic demethylation of 3,4-dimethoxy compounds.[3]

Moreover, the present study indicates that non-enzymatic oxidation shows greater reactivity than enzymatic oxidation. For example, demethylation of the methoxy group with an adjacent hydroxy group to form catechol compounds always took place in the non-enzymatic oxidation of II—V. However, none of the catechol compounds of this type was formed by incubation of II—V with rat liver microsomal fraction containing the NADPH generating system. The adjacent hydroxy group may prevent further transformation into more hydrophilic metabolites in the enzymatic system. Udenfriend *et al.* have reported non-enzymatic hydroxylation of tyramine, which possesses a pre-existing hydroxy group like denopamine, to hydroxytyramine.[7] The present study showed that denopamine was hydroxylated to form the catechol compound (VII) by non-enzymatic oxidation in Udenfriend's system. It was also found that the non-enzymatic oxidation was usually more powerful than the enzymatic oxidation: *i.e.*, hydroxylation at the *ortho* or *para* position to the methoxy group also took place in the non-enzymatic oxidation of all substrates tested. None of these hydroxylation products was observed in the enzymatic oxidation.

Udenfriend *et al.* have also reported non-enzymatic hydroxylation of phenylalanine, which possesses no pre-existing hydroxy group on its benzene ring, but they have not mentioned the formation of catechol-type products. In our present study, the catechol compound (VII) was formed by the non-enzymatic oxidation of IX. The peak of mono-hydroxylated IX, *i.e.*, denopamine (I), was only small (Fig. 5). The mechanism of the formation of VII may involve two-step hydroxylation of IX *via* I as in the enzymatic oxidation.[8] However, this two-step mechanism seems unlikely because (1) the yield of VII from I was not quantitative (Fig. 1), (2) the amount of demethylated VII having the ions of m/z 252 and 355 was negligibly small compared to the amount of VII formed (Fig. 5), and (3) the amount of I was small even in the early stage of the reaction (after 10 min). Therefore, a direct dioxygenation pathway may be more likely in the oxidation of IX to form the catechol compound VII. When IX was incubated with rat liver microsomal fraction containing the NADPH generating system, the peak of VII was not detected, although the peak of I was detected (unpublished).

All the differences described above between the enzymatic and Udenfriend's systems in the pattern of denopamine oxidation are probably related to differences in the mechanism of oxidation between the two systems. It is known that Udenfriend's system does not cause the NIH shift, a special criterion for aryl hydroxylation catalyzed by monooxygenases.[9]

The tetrahydroisoquinoline-type compounds were formed from III and V probably by Pictet–Spengler-type condensation with formaldehyde generated in the reaction mixture (Fig. 3-b and 4-b). As none of the corresponding tetrahydroisoquinoline-type compounds was formed in the oxidation of II and IV, which are isomers of III and V, respectively, it is

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suggested that the ring condensation can take place in the oxidation of hydroxylated substrates in which the hydroxy group is attached at the *meta* (3) position of the benzene ring. This is presumably a result of activation by the electron-donating hydroxy group at the *ortho* and *para* positions to the hydroxyl group. The methoxy group does not seem to contribute to the condensation reaction under the mild conditions of Udenfriend's system. Consequently, the ratio of *para*- to *meta*-demethylation may be only an apparent value, because the *meta*-demethylated compound could further react with formaldehyde.

Although the generation of formaldehyde was not confirmed, the formaldehyde taking part in the condensation reaction seems to have been derived from oxidative demethylation of the methoxy group. In our recent study, these tetrahydroisoquinoline-type compounds were also detected in human urine after oral administration of denopamine. In this case, demethylated denopamine seemed to react with endogenous formaldehyde. There are many reports dealing with the Pictet-Spengler-type condensation of catecholamines, such as dopamine,[10] with enzymatically formed formaldehyde, but few deal with such condensation products in drug metabolism.[11]

In summary, all the metabolites of denopamine produced by microsomal oxidation are also produced by chemical oxidation of denopamine under the conditions of Udenfriend's system. Compared to enzymatic oxidation, the chemical oxidation is more extensive in the sense that both methoxy groups can be demethylated, and less selective as to the position of demethylation when one of the two methoxy groups is demethylated. With the *meta*-hydroxylated metabolites of denopamine, demethylation reactions were always accompanied by ring condensation reactions with formaldehyde, presumably derived from the methyl groups, to give tetrahydroisoquinoline-type compounds, which have also been detected in human urine after denopamine administration.

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