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Detection of N-Acetyltranylcypromine and Glucuronide of Phenyl-Hydroxylated N-Acetyltranylcypromine from Tranylcypromine-Dosed Rat Urine: Pharmacological Implications*

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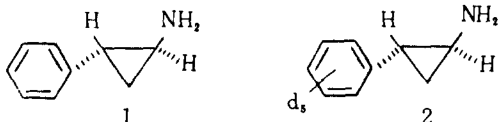
Abstract☐In order to use for metabolic studies of tranylcypromine (TCP), TCP-phenyl-d_5 was synthesized via the intermediates, 3-benzoylpropionic acid-d_5 and *trans*-2-phenylcyclopropanecarboxylic acid-d_5. TCP (0.22 mmole/kg) and its deuterated analog were administered s.c. to the rats and GC/MS analyses of the urines led to the detection of N-acetyltranylcypromine (ATCP) and glucuronide conjugate of phenyl-hydroxylated ATCP. MAO activities in rat brain were measured using serotonin as the substrate. *In vitro* IC_{50} of ATCP was determined to be 10^{-3} M. The inhibitions by ATCP were not dependent on the preincubation time and were reversed by washing sedimented mitochondrial pellets after the preincubation. *In vivo* MAO inhibitions at various times of 0.5, 1.5, 3, 6, 12, and 24 hr after the administration of 0.4 mmole/kg (i.p.) of ATCP were found to be 0, 13, 73, 90, 89, and 74%, respectively. Similarly, the inhibition percents by 0.015 mmole/kg (i.p.) of TCP were 94, 99, 95, 91, 71, and 49%. The results strongly suggest that deacetylated product of ATCP may account for its *in vivo* MAO inhibition. The relationship between the metabolism via phenylhydroxylation and the *in vivo* potency of TCP was examined by QSAR study and it was found that groupings discriminating between the compounds with *p*-substituents and those without them only ensure high correlations, suggesting that ring-hydroxylation which occurs at the para position in most of the compounds is a determining factor to the potency of TCP.

Keywords☐Tranylcypromine, $[^2H_5]$Tranylcypromine, Metabolic studies, N-Acetyltranylcypromine, Glucuronide of phenyl-hydroxylated N-acetyltranylcypromine, MAO inhibition studies, QSAR.

Tranylcypromine (*trans-dl*-2-phenylcyclopropylamine, TCP, **1**) is an antidepressant clinically available which can be considered as a rigid-structural compound derived from either amphetamine or phenylethylamine. The drug has been used as a probe to investigate a mechanism of the inhibition of monoamine oxidase (MAO, E.C. 1.4.3.4).[1]



Two recent reports have described oxidative structural modifications of TCP in connection with a suicidal inhibition of MAO. 2-Phenylcyclopropanone was isolated from MAO-inhibitor complex and Paech *et al.*[2] hypothesized that an oxidized product of TCP by MAO, of either imine or ketone structure, is responsible for the covalent binding to the MAO protein. Silverman[3] reported an identification of a cinnamaldehyde, instead of a 2-phenylcyclopropanone, on the basis of which it was proposed that one electron abstraction occurs from the amino nitrogen of TCP by MAO, followed by cyclopropyl ring opening, thus forming a free radical at the benzylic position which ultimately binds to the MAO protein. The proposed mechanism was analogous to that presented for an irreversible inhibition of MAO by N-(1-methylcyclopropyl) benzylamine.[4,5]

In addition, two *in vivo* metabolites of TCP have appeared in literatures. Youdim *et al.*[6] reported a presence of amphetamine in the plasma of a patient taking an overdose of TCP. The result, however, was found not to be reproducible, so that, according to Reynolds *et al.*,[7] the amphetamine could not be detected in both plasma and urine down to a sensitivity, in urine, of less than 0.01% of the TCP dose. Calverley *et al.*[8] demonstrated a presence of N-acetyl-TCP in rat brain as an interference in GC analysis of TCP which was extracted from the

* Mechanism of the MAO inhibition by 2-phenylcyclopropylamines VI

tissue homogenate after the N-acetylation.

Despite the significant implications of the biotransformed molecules of TCP in biochemistry and pharmacology of the drug, as described above, we found that a systematic study to determine its metabolic pathways had not been done. It was further considered that structural informations on *in vivo* metabolites would be first essential to the determination of biopharmaceutic profiles of TCP especially in relation to its interactions with either another stereoisomers or other therapeutic agents. For such studies on metabolic pathways, recent trends are to use stable isotope compounds preferably labeled at the sites which are not easily eliminated during metabolic processes, combined with a GC/MS discriminating between labeled and unlabeled molecules, thus assisting to trace and identify administered drugs and metabolites arising from them.[9]

Accordingly, in this study, we synthesized TCP-phenyl-d_5 (**2**) and administered it separately with an unlabeled TCP to the rats. GC/MS analyses of the urines led to the identification of N-acetyl-TCP (ATCP, **6**) and glucuronide conjugate of phenylhydroxy N-acetyl-TCP (*trans*-2-(4-hydroxyphenyl)-1-cyclopropylamine, **7**) (**8**). Inhibitions of the rat brain mitochondrial MAO by ATCP were determined in both *in vitro* and *in vivo* experiments using serotonin as the substrate. The mechanism of the MAO inhibition by ATCP including its potential *in vivo* hydrolysis to TCP was sought in comparison with TCP. In addition, the involvements of *in vivo* phenyl-hydroxylation in the potency of TCP and its derivatives were examined by using the method of quantitative structure-activity relationships (QSAR). A preliminary communication has been published on the *in vivo* metabolic study using an unlabeled TCP.[10]

EXPERIMENTAL METHODS

Materials

Benzene-d_6 (>99.5 atom% D) was purchased from Fluka AG, Switzerland. Serotonin creatinin sulfate complex, ß-glucuronidase (type H-3, 98,700 units/ml) and pargyline hydrochloride were obtained from Sigma Chemical Co., St. Louis, U.S.A. Tranylcypromine sulfate was a generous gift from Dr. S. Crooke of Smith Kline & French Laboratories, U.S.A. Diazomethane was generated using *p*-toluenesulfonyl-N-methyl-N-nitrosamide (Tokyo Kasei, Japan) by the method of Levitt.[11] Unlabeled synthetic intermediates of TCP and authentic TCP HCl were obtained as previously described.[12] Authentic 3-benzoylpropionic acid was purchased from Tokyo Kasei, Japan. The other reagents and solvents were of reagent grade.

Instrumentation

The melting points were determined using a Sybron Thermolyne, Olympus, Tokyo and uncorrected. Infrared spectra were recorded on a Perkin-Elmer Model 710 infrared spectrophotometer. UV/VIS absorptions were measured using a Hitachi Model 200-20 UV-VIS spectrophotometer. Nuclear magnetic resonance spectra were taken on Varian EM-360L 60MHz spectrometer using tetramethylsilane as internal standard (s = singlet, d = doublet, t = triplet, M = multiplet). Hewlett Packard Model HP 5985B GC/MS System was used to collect all direct probe mass spectral data and some GC/MS data noted therein. The operating conditions were as follows: Electron ionization voltage, 70eV; SE-54 capillary column (25m x 0.2mm i.d.); column temperature, 100°C, 2 min hold and programmed to 260°C at 5°C min^{-1}; injection temperature, 230°C. Gas chromatographic data were obtained using Hitachi Model 163 gas chromatograph. A glass column (2.0m x 3mm i.d.) packed with 3% OV-17 on 80/100 Chromosorb W(HP) was used with carrier gas (N_2) at 50ml min^{-1}. The standard operating conditions were: column temperature, 100°C, programmed to 250°C at 10°C min^{-1}; injector temperature, 250°C; and detector temperature, 250°C. Finnigan 4021 gas chromatograph-mass spectrometer with BP-5 Vitreous silica capillary column (25m x 0.33 mm) was used to collect all GC/MS data. The analysis conditions were as follows; column temperature, 100°C, 1 min hold and programmed to 230°C at 10°C min^{-1}; injector temperature, 230°C; carrier gas (He) flow rate, 5ml min^{-1}; electron energy, 70 eV; scan time, 0.95 sec. A Sorvall superspeed refrigerated centrifuge, RC 2-B, Sorvall Inc., U.S.A. and a motor-driven glass homogenizer (Potter Elvehjem type) with a Teflon resin pestle were used. Elementary analyses were done by Analytical Research Section of Dong-a Pharm. Co. Ltd., Seoul.

Synthesis of tranylcypromine-phenyl-d_5 (2)

Deuterium labelling was introduced in the step of the synthesis of 3-benzoylpropionic acid.[13] Subsequent synthetic procedures were taken from Kaiser *et al.*[14] and

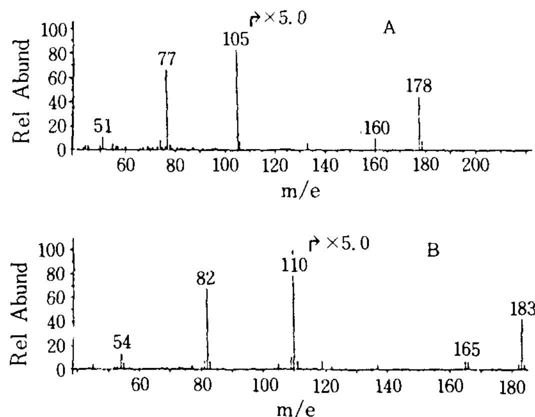


Fig. 1. Direct probe mass spectra of 3-benzoylpropionic acid (A) and [$^2H_5$] analog (B).

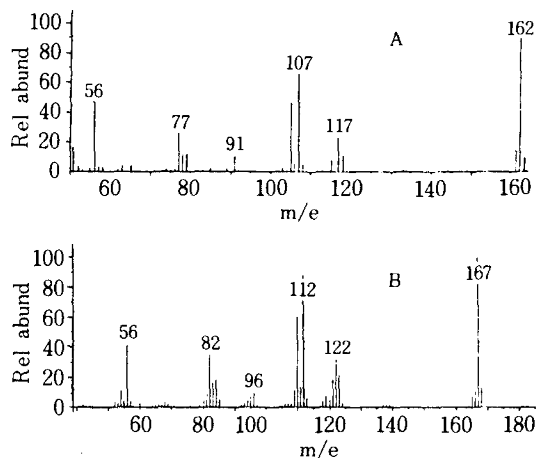


Fig. 2. Direct probe mass spectra of γ-phenyl-γ-butyrolactone (A) and [$^2H_5$]alalog (B).

from the methods previously described for TCP-d_2.[12] Succinic anhydride (3.45g, 0.034 mol) and benzene-d_6 (20g, 0.237 mol) gave 3.90g (64%) of 3-benzoylpropionic acid-phenyl-d_5 (**3**). A portion of benzene-d_6 (7ml) was recovered. mp 111-113°C (lit[13] 114-115°C). IR (KBr) 1700 cm^{-1} (C=O), aryl C-H at 700, 780 cm^{-1} (absent). NMR ($CDCl_3$) δ10.80(broad s, 1H, OH), 2.72 (t, 2H, CH_2 COOH), 3.25 (t, 2H, $C_6H_5COCH_2$), phenyl protons at 7.52, 7.90 (absent). Mass spectrum was shown in Fig. 1 with that of an unlabeled compound. GC t_R (12.1min) was consistent with that of an authentic unlabeled compound. From 3.66g (0.02mol) of **3**, 3.0g(90%) of γ-phenly-γ-butyrolactone-phenyl-d_5 (**4**) was obtained. IR(KBr) 2280 cm^{-1} (aryl C-D), aryl C-H at 690, 760 cm^{-1} (absent). NMR ($CDCl_3$) phenyl protons at δ7.35(absent). Mass spectrum (Fig. 2). From 2.8g(0.017mol) of **4**, total 0.7g (25%) of *trans*-2-phenylcyclopropanecarboxylic

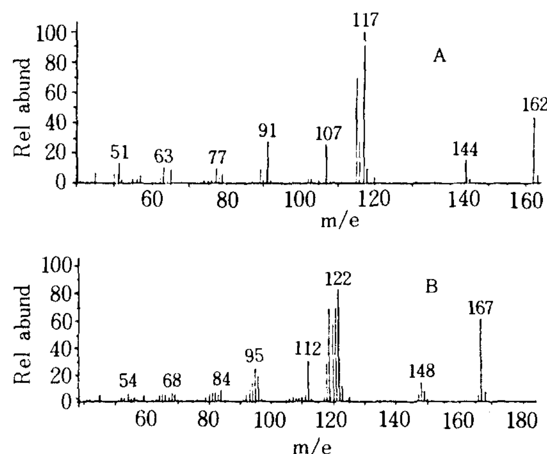


Fig. 3. Direct probe mass spectra of *trans*-2-phenylcyclopropanecarboxylic acid (A) and [$^2H_5$]analog (B).

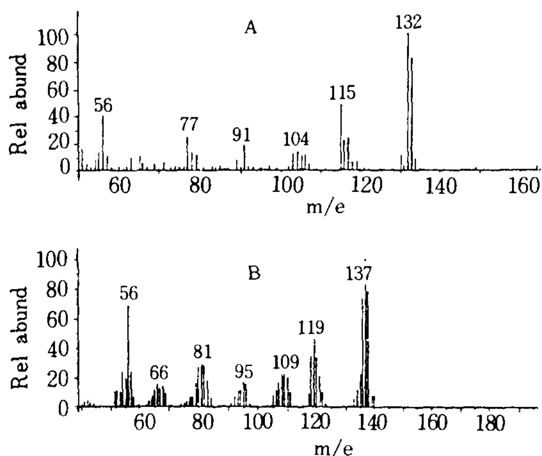


Fig. 4. Direct probe mass spectra of tranylcypromine (A) and[$^2H_5$]analog (B).

acid-phenyl-d_5 (**5**) was obtained, which was a combined yield with 0.51g recovered directly from the reaction mixture before the hydrolysis of ethyl *trans*-2-phenylcyclopropanecarboxylate. In this study 0.023 mol of potassium was used to prepare potassium t-butoxide. Thus, when the reaction mixture was made acidic and extracted with ether, two peaks were observed by GC (150-250°C, 5°C min^{-1}) at t_R 5.4 min (176°C) and 6.6 min (183°C). The peak at t_R 5.4 min was consistent with that of **5** and a peak at t_R 6.6 min was presumably from the *cis* isomer. The observation was confirmed by NMR. Treatment of the residue with hot water gave 0.51g of **5**. mp 85-86°C (lit.[14] 86-88°C) IR (KBr) 2280 cm^{-1} (aryl C-D), 1680, 1700 (C=O). NMR($CDCl_3$) δ10.7 (broad s, 1H, OH), 2.3-2.8 (m, 1H, benzylic H) 1.1-2.1 (m, 3H, cyclopropyl H). Mass spectrum (Fig. 3). GC t_R (8.6min). By this stage the labelling percents for the compounds, **3**, **4**, and **5** were over 98% by NMR. From 580mg (0.0035mol) of **5**, 270mg (46%) of **2** HCl was obtained after two recrystallizations from MeOH/EtOAc/ether. mp 153-155°C (lit[15] 151-154°C). IR(KBr) 2280cm^{-1} (aryl C-D). NMR of the base (CCl_4) δ1.4 (s, 2H, $-NH_2$), 2.15-2.80 (m, 1H, cyclopropyl H), 1.45-2.10 (m, 1H, cyclopropyl H), 0.60-1.35 (m, 2H, cyclopropyl H). The labelling percent was determined to be 95-96% by NMR. Mass spectrum (Fig. 4). GC t_R of the base (4.7min).

Synthesis of trans-N-acetyl-2-phenylcyclopropylamine (N-acetyltranylcypromine, ATCP, 6)

A 500mg (2.7 mmole) of TCP sulfate was dissolved in a small amount of H_2O and it was mixed with 5g of sodium acetate in 10ml of H_2O. Maintaining the temperature at 0-5°C, 0.33ml (3.5 mmole) of acetic anhydride was added to the mixture and stirring continued for 30min. White precipitates were collected and two recrystallizations from H_2O/EtOH (9:1) gave 300mg (60%) of **6**. mp 88-89°C. TLC (Kiesel gel 60G,

EtOAc/NH_4OH (99:1) R_f 0.50, TCP base R_f 0.27. IR(KBr) 3440cm^{-1} (NH) 1680 (amide I), 1530 (amide II). NMR ($CDCl_3$) δ 7.17 (s, 5H, aromatic H), 6.5-6.9 (broad s, 1H, NH) 2.65-3.15 (m, 1H, cyclopropyl H), 1.8-2.25 (4H, CH_3 (s) mixed with cyclopropyl H (m)), 0.8-1.5 (m, 2H, cyclopropyl H). MS, m/e(relative intensity) 175 (M^{+}, 2), 60(48), 84(50), 92(10), 105(30), 116(82), 132 (base peak). Anal. Calcd. for $C_{11}H_{13}ON$: C, 75.43; H, 7.43; N, 8.00. Found: C, 75.77; H, 7.70; N, 8.36.

In vivo metabolic studies

A 40mg/kg dose of TCP sulfate ([$^2H_5$] TCP·HCl) was administered s.c. to a male Sprague Dawley rat (150-200g). From one group of 7 rats kept in metabolic cages, 60-70 ml of urine was collected over 35hr. After centrifugation to remove solid substances, pH of the urine was adjusted to pH 2.5-3.0 with 0.5 M H_2SO_4 and the solution extracted twice with two volumes of $CHCl_3$. The $CHCl_3$ extracts were evaporated under reduced pressure, redissolved in 3ml of MeOH and a half of the MeOH solution was analyzed directly by GC/MS. (nonconjugated fraction extracted at pH 3.0) Another half was treated with diazomethane and analyzed (methylated nonconjugated fraction extracted at pH3.0). The aqueous layer was centrifuged to remove solid substances and made to pH 11.0 using 1N NaOH. Then, the solution was extracted twice with two volumes of $CHCl_3$. After the distillation of $CHCl_3$, the residue was dissolved in 3ml of MeOH. A half of them was analyzed directly (nonconjugated fraction extracted at pH 11.0) and another half treated with diazomethane to obtain a methylated nonconjugated fraction extracted at pH 11.0. The pH of the remaining aqueous layer was adjusted to pH 4.5 using dilute acetic acid. The solution (total 150ml) was transferred to 3 incubation flasks to each of which was added 1.9 ml of ß-glucuronidase. The solution was then incubated at 37°C for 24hr. After the hydrolysis, the combined solution was centrifuged to remove solid substances, followed by the adjustment to pH 2.5-3.0 using 0.5M H_2SO_4 and the acidic solution was extracted twice with two volumes of $CHCl_3$. After evaporating $CHCl_3$ under reduced pressure, the residue was dissolved in 3ml of MeOH. A half of the MeOH solution was analyzed directly (conjugated fraction extracted at pH 3.0) and another half treated with diazomethane to obtain a methylated conjugated fraction extracted at pH 3.0. The $CHCl_3$-extracted aqueous layer was made to pH 11.0 with 1N NaOH and extracted twice using two volumes of $CHCl_3$. The $CHCl_3$ was distilled under reduced pressure and residue was dissolved in 3ml of MeOH. A half of them was analyzed directly (conjugated fraction extracted at pH 11.0) and another half treated with diazomethane (methylated conjugated fraction extracted at pH 11.0).

In vitro MAO inhibitions

The MAO assay was carried out according to the procedures of Sjoerdsma *et al.*[16] The serotonin was extracted from the incubation mixture and measured by the U.V. method of Udenfriend *et al.*[17] Mitochondrial proteins were measured by the method of Lowry *et al.*[18] using bovine albumin (A 7906, Sigma Chemical Co.) as the standard. Optimal assay conditions suitable to the present study were examined and sought as follows:

1) Enzyme source, substrate, and incubation time

Male Sprague Dawley rats (150-200g) were decapitated, and the brain was immediatly removed and homogenized in 10 volumes of 0.32M sucrose at 0°C. All subsequent operations were also performed at 0-4°C. After centrifuging the homogenate for 20min at 2,000g, the supernatent was removed and recentrifuged for 20 min at 12,000g. The sedimented mitochondrial pellet was suspended in 0.32 M sucrose so that 1.5ml of the resulting mixture contained mitochondria equivalent to 1.0g of whole brain. One ml of the enzyme preparation contained approximately 15mg of protein. The relationship between the serotonin consumption and the enzyme concentraction (0.5, 1.0, 1.5, and 2.0 mg/ml) was examined in 3ml of the incubation mixture containing 0.2ml of 0.006 M serotonin (0.4mM) by subjecting to a 90 min incubation at 37°C. A linear relationship of the incubation time (30, 60, 90, and 120 min) with the serotonin consumption was evaluated with a fixed enzyme concentration of 1mg/ml and the 0.4mM of serotonin. All incubation mixtures were prepared in 0.1M phosphate buffer, pH 7.4.

2) Extraction, measurement, and calibration

The incubation was carried out in 12ml centrifuge tubes. The enzyme reaction was stopped by adding 0.3ml of 10% $ZnSO_4$ to the incubation mixture. Following the addition of 0.7 ml of 0.5 M NaOH, it was mixed with 3ml of n-BuOH, then vortexed for 2min and centrifuged (3,000 rpm, 15min). A 2.5ml of the supernatent n-BuOH was transferred to another centrifuge tube and the aqueous layer reextracted with 3ml of n-BuOH. The serotonin in a combined n-BuOH solution (~6ml) was then back-extracted into 4ml of 0.01 N HCl with 2 min vortexing. The absorbance of the solution was determined at 276 nm using a blank prepared with distilled water in place of the substrate. In order to construct an extraction calibration curve for serotonin, incubation mixtures containing varying concentrations of serotonin (0.1, 0.2, 0.3, and 0.4 mM) were prepared with the enzyme (1mg/ml) whose activity had been terminated by adding $ZnSO_4$ before the serotonin. Following the extraction, the absorbance corresponding to the serotonin concentration was read and plotted.

3) Optimal analytical condition and IC_{50} determination

The serotonin extraction calibration showed a linearity over the entire concentration range of

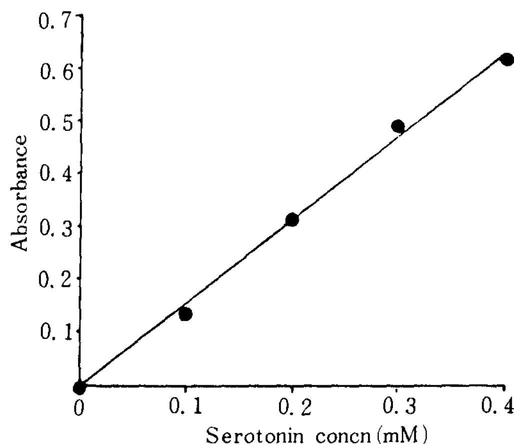


Fig. 5. Relationship betwee the amount of serotonin in a reaction mixture and the absorbance measured after the extraction. Each point represents the mean of triplicate determinations.

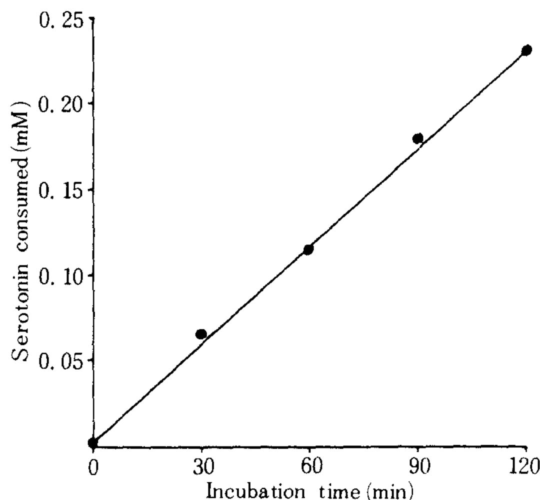


Fig. 7. Relationship between serotonin consumption and incubation time. Each point represents the mean of duplicate determinations.

0.1-0.4mM (Fig. 5). A fixed concentration of 0.4mM used for the present study gave an optimal absorbance value of ~0.6, which represents an upper limit of the serotonin absorbance for the inhibition study. A vortexing for 2 min with n-BuOH and a back-extraction into 0.01 N HCl were chosen because the prolongation of the vortexing time and the use of 0.1N HCl did not improve the extractability. When enzyme concentrations were varied over 0.5 to 2.0 mg/ml, the serotonin consumed vs. enzyme concentration curve was found to deviate a

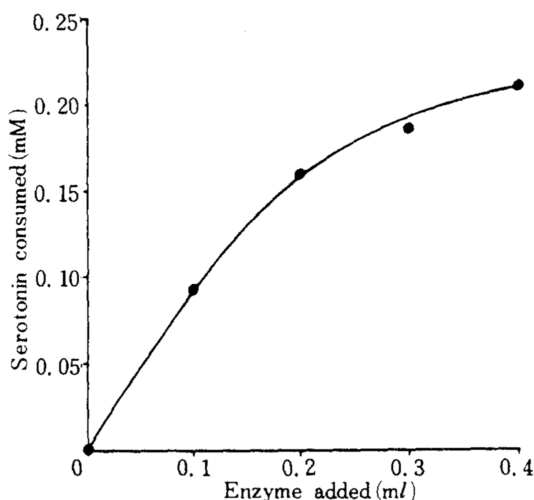


Fig. 6. Relationship between serotonin consumption and enzyme concentration. The 0.2 ml of enzyme is equivalent to 3.0 mg of protein and the resulting 0.159 mM serotonin equals to 0.477 μmole. Each point represents the mean of duplicate determinations.

little from linearity at the enzyme concentration of 1.0 mg/ml (Fig. 6) but a fixed concentration of 1.0mg/ml was used. A linearity was observed in serotonin consumptions at the varying incubation times of 30 to 120 min (Fig. 7). In the present study, 90 min of the incubation time was chosen because it gave an absorbance of ~0.3 after the enzymatic reaction. The air saturation in incubation mixtures did not change the results.

The IC_{50} was determined as follows: Ranges of the final inhibitor concentrations in 3ml of an incubation mixture were: ATCP, 2.5×10^{-3}-10^{-7} M; TCP, 10^{-3}-10^{-8} M; and pargyline, 10^{-3}-10^{-8} M. The inhibitor was preincubated with the enzyme (3mg protein/2.8 ml; final concentration, 1mg/ml) at 37°C for 30min, then 0.2ml of 0.006 M serotonin (final concentration, 0.4mM) was added and the incubation was continued for another 90 min. Following extractions, the absorbance was measured at 276 nm using a blank prepared in the absence of serotonin. The percent inhibitions calculated from the absorbance data and serotonin calibration were plotted against the inhibitor concentrations and the concentration to cause 50% inhibition was determined. In the study, the absorbance of 10^{-2} M ATCP in distilled water was found to be ~0.9. After the extraction procedures, the value decreased below 0.01 indicating that the ATCP, in the concentration range used, did not interfere in the analysis.

Effects of the preincubation time and the washings upon the inhibition of MAO activity.

1) Effect of the preincubation time

The concentrations of TCP and ATCP used were 10^{-7} and 10^{-3}M, respectively. The inhibitors were preincubated with the enzyme for up to 60 min at an interval of 10 min prior to adding the substrate to initiate an en-

zymatic reaction. The MAO activity was measured as described and plotted as a semilogarithmic curve against the preincubation time.

2) Effect of washing the mitochondrial pellet

Following the preincubation of inhibitors (TCP, 10^{-4} and 10^{-6} M; ATCP, 2.5 x 10^{-3} and 10^{-3} M) with the enzyme for 30 min, the mixture was centrifuged for 20 min at 20,000g and the sedimented mitochondria washed twice with 1ml of phosphate buffer. The washed pellet was assayed for the MAO activity by resuspending it in 2.8 ml of the same buffer, followed by adding 0.2ml of serotonin and incubating for 90min. Lower limit of the serotonin absorbance (0% inhibition) was obtained by following the same procedures in the absence of the inhibitor. In order to obtain an upper limit (100% inhibition), the suspended enzyme was kept in boiling water bath for 15 min prior to adding the serotonin and the absorbance value obtained as described. For a comparative study designated as "before washing" in Table I, enzymes were not sedimented and the assay procedure was essentially the same as that described for IC_{50} determination at the inhibitor concentrations.

In vivo MAO inhibitions

TCP sulfate was prepared in saline. ATCP was dissolved in a few drops of EtOH and the solution was mixed with an olive oil. A 70mg/kg (0.4mmol/kg) dose of ATCP and a 2.73 mg/kg (0.015 mmole/kg) of TCP sulfate were administered respectively to the rats in a volume of ~0.5ml by intraperitoneal injection. After various times of 0.5, 1.5, 3, 6, 12, and 24 hr, rat brains were removed and the MAO activities were measured as previously described for *in vitro* inhibitions. Controls to obtain the lower and upper limits of the serotonin absorbance were prepared using the enzyme from the rats administered vehicles only.

QSAR study

The purpose of the study was to correlate an aromatic hydroxylation which occurs at the para position to the *in vivo* potency of TCP and its derivatives and to prove it by QSAR. Therefore, in complying with the assumption, 2-(substituted-phenyl)cyclopropylamines were grouped into two, one with *p*-substituents and another without them. For the study, structure-activity data for 2-(substituted-phenyl) cyclopropylamines were taken from Zirkle *et al.*[19] In addition, substituent effect on the *in vivo* potency were analyzed only with respect to both hydrophobic (π) and electronic effects (σ). Hence, the basic equation is $\log 1/C = -k\pi^2 + k'\pi\pi_0 - k''\pi_0^2 + \rho\sigma + K'''$ as derived by Hansch *et al.*[20] and the simplest form is $\log 1/C = a\pi + \rho\sigma + c$. Multiple regression analysis was done using HP 85B micro computer with a general statistics pack MLR.

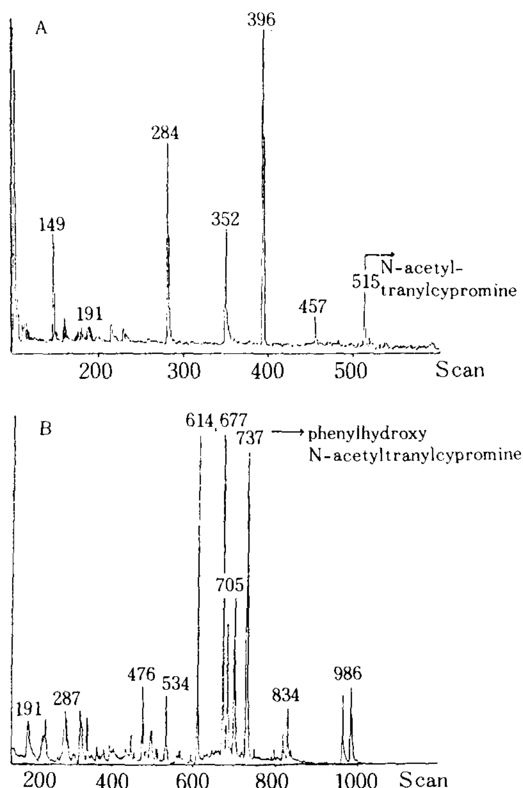


Fig. 8. TIC profiles of pH 3.0-extracted nonconjugated (A) and conjugated (B) fractions from urines of tranylcypromine-dosed rats.

RESULTS AND DISCUSSION

In vivo metabolic studies

Fig. 8A represents a TIC profile of the nonconjugated fraction extracted at pH 3.0. A peak at scan 515 was identified to be N-acetyltranylcypromine by mass spec-

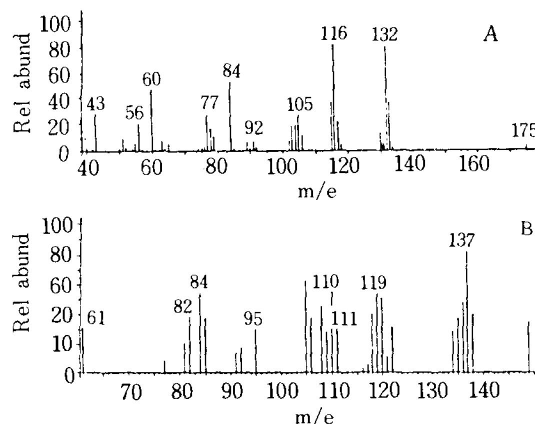


Fig. 9. Mass spectra of N-acetyltranylcypromine (A) and [$^2H_5$]N-acetyltranylcypromine (B) obtained from pH 3.0-extracted [$^2H_5$] analog-dosed rat urines.

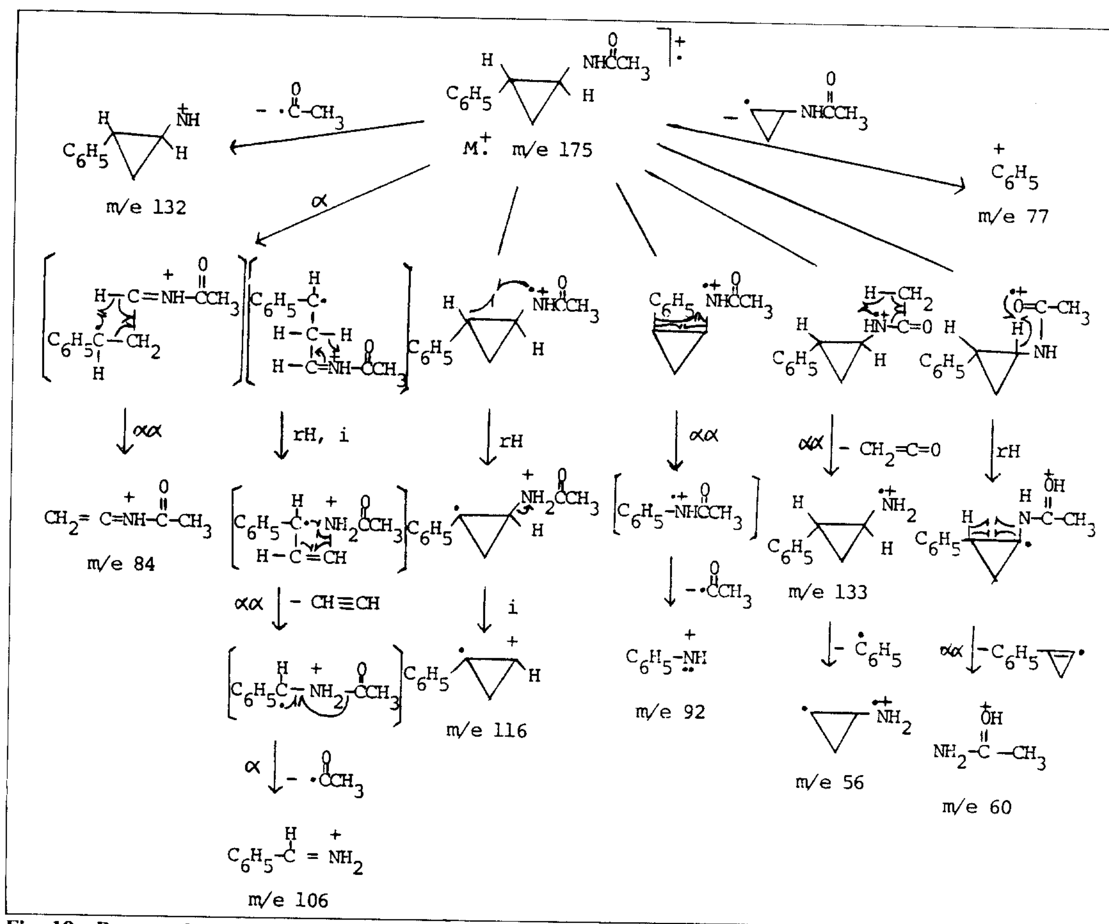


Fig. 10. Proposed mass fragmentation processes for N-acetyltranylcypromine.

tra (Fig. 9) and using an authentic compound. Five a.m.u. shifts were observed in the fragment ions such as m/e 132, 105, and 77. The ions, m/e 56, 60, and 84 did not contain phenyl portions. Mass fragmentation processes for N-acetyltranylcypromine (Fig. 10) were proposed using a spectrum from the high concentrated authentic compound. Minor discrepancies between the proposed fragment ions and the observed mass shifts in Fig. 9 might result from ion impurities in the spectra of metabolites and from the differing background subtraction.

It was assumed that the nonconjugated fraction extracted at pH 3.0 would contain acidic and neutral molecules such as cinnamaldehyde and cinnamic acid; which normally appear at scan 415 and 571, respectively by HP GC/MS. In this study, neither of them was detected. We also screened for phenylhydroxy TCP in a nonconjugated fraction extracted at pH 11.0 and in its methylated fraction in an attempt to find an evidence for the formation of phenylhydroxy ATCP from the phenylhydroxy TCP. However, in the fraction, TCP at scan 245 was the only component arising from the drug. Amphetamine was not detected either.

A TIC profile of the conjugated fraction extracted at pH 3.0 was shown in Fig. 8B, in which a peak at scan 737 was assigned a phenylhydroxy ATCP by the mass spectra originating from the administration of TCP and its deuterated analog (Fig. 11). A presence of phenylhydroxyl group in this metabolite was evident by 4 a.m.u. shifts and the methoxy fragments of its methylated compound shown in the previous communication.[10] Determination of the exact site of hydroxylation on the basis of mass spectral data was not possible. It has been known that para-hydroxylation usually predominates for the monosubstituted benzene compounds.[21] The phenylhydroxy ATCP was only detected after the hydrolysis using ß-glucuronidase. Therefore, it was certain that ATCP undergoes a ring hydroxylation followed by glucuronide conjugation to **8.** A glucuronide of phenylhydroxy TCP was not detected in the conjugated fraction extracted at pH 11.0. Mass fragmentation processes for phenylhydroxy ATCP were proposed in Fig. 12 with the comparisons among the unlabeled, labeled, and methylated metabolites.

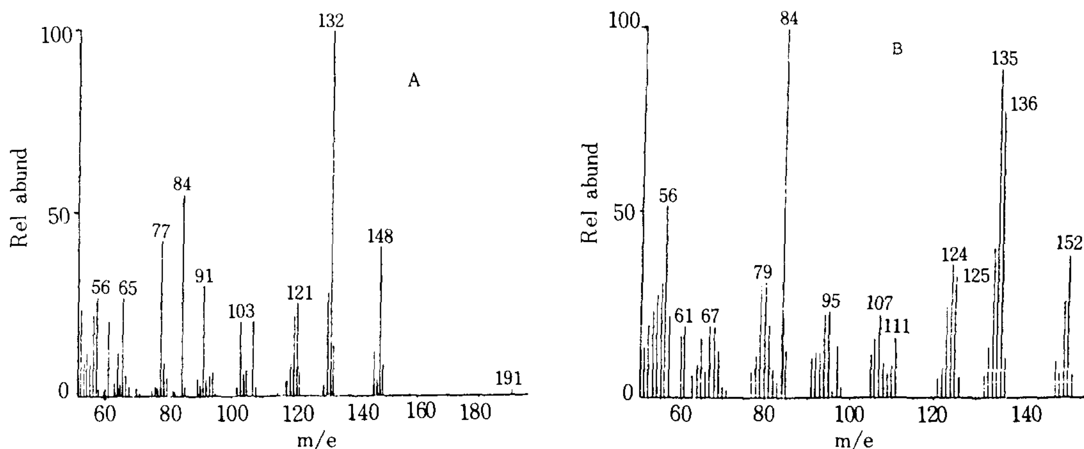


Fig. 11. Mass spectra of phenylhydroxy N-acetyltranylcypromine (A) and [$^2H_4$] metabolite (B) isolated from urines of tranylcypromine and its [$^2H_5$] analog-dosed rats.

Fig. 12. Proposed mass fragmentation processes for phenyl;hydroxy N-acetyltranylcypromine.

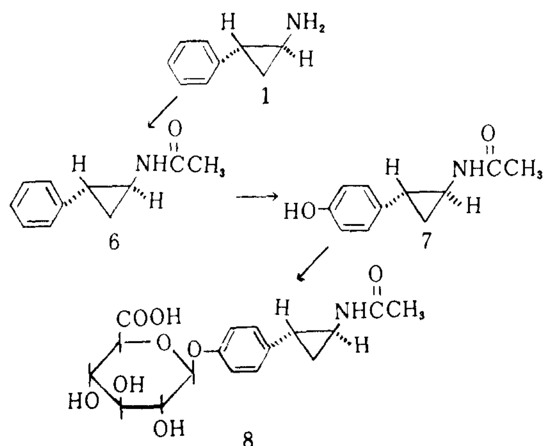


Fig. 13. Major metabolic pathways of tranylcypromine in rats.

Major biotransformation pathways of TCP are summarized in Fig. 13. The detection of N-acetyltranylcypromine in rat urines was considered new in the present study with the labeled compound. Taking into account the fact that only 12% of the administered TCP was excreted as an intact drug in rat urine,[22] it seems highly likely that metabolism via N-acetylation will play a significant role in the pharmacology and toxicology of TCP. It has been known that the ability to acetylate foreign compounds is genetically controlled.[23] Thus, for a therapeutic effect of the drug which is inactivated primarily by N-acetylation, rapid acetylators require a larger dose and slow acetylators will be more sensitive to the adverse effect. In analogy, the finding that TCP is mainly excreted via initial N-acetylation might provide a clue to the explanation why large doses of TCP have proven to be clinically ineffective to some patients.[24] It has been also reported that exaggerated effects observed in some patients given phenelzine may be due to the slow N-acetylation.[25] Moreover, a potential implication of the N-acetylated metabolite in a part of the activity resulting from TCP may be able to be made in relation to the fact that N-acetylphenelzine which is a metabolite of phenelzine is active both *in vivo* and *in vitro*.[26] There are also sulfa drugs such as N_4-acetylsulfapyridine[27] and N_4-acetylsulfamerazine,[28] which are deacetylated *in vivo* by the reversible action of the polymorphic N-acetyl transferase enzyme. In this respect, we intended to determine MAO-inhibitory activities of N-acetyltranylcypromine at both *in vitro* and *in vivo* levels.

***In vitro* MAO inhibitions**

Fig. 14 shows *in vitro* dose-inhibition curves of ATCP, TCP, and pargyline. *In vitro* inhibition by ATCP was very low in its degree (IC_{50}, 10^{-3}M) compared to those by TCP (5×10^{-7}M) and pargyline (7×10^{-6}M).

As shown in Fig. 15, MAO inhibitions by ATCP were not dependent on the preincubation time whereas the extent of the inhibition by TCP increased with the increasing preincubation time, which is a characteristic of an irreversible inhibition.[29] It was found that TCP at a concentration of 10^{-7} M did not exhibit a significant inhibition without being preincubated with an enzyme.

In order to further examine MAO-inhibitory properties of ATCP in comparison with TCP, the mitochondrial

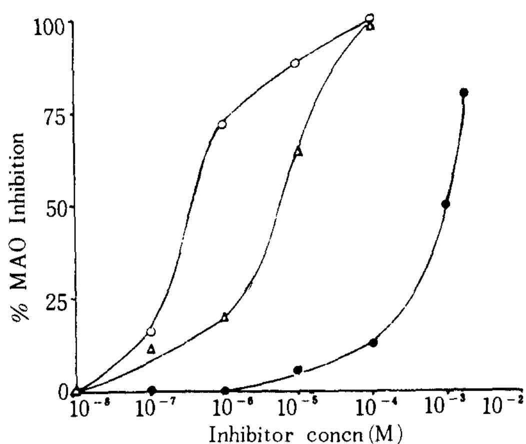


Fig. 14. *In vitro* inhibition of rat brain MAO by N-acetyltranylcypromine (-•-), tranylcypromine (-o-), and pargyline (-Δ-). Inhibitors were preincubated with enzyme for 30 min prior to substrate (serotonin) addition. Each point is the average of duplicate determinations.

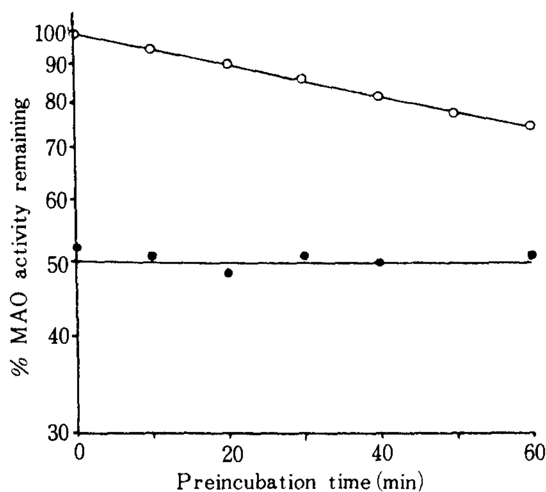


Fig. 15. Plot of percent enzyme activity remaining (logarithmic scale) against time of preincubation of rat brain MAO and inhibitor with serotonin as substrate. The concentration of N-acetyltranylcypromine (-•-) was 10^{-3} M and that of tranylcypromine (-o-) was 10^{-7} M. Each point represents the mean of duplicate determinations.

Table I. Effect of washing rat brain mitochondria upon MAO activity.

Pretreatment[a]	Serotonin consumed (μg)[b]		% Inhibition	
	Before washing	After washing	Before washing	After washing
None	206.8	171.4[c]		
TCP (1.0×10^{-4}M)	0	2.4	100	98.6
TCP (1.0×10^{-6}M)	53.8	43.2	74.0	74.8
ATCP (2.5×10^{-3}M)	34.2	157.2	83.4	8.2
ATCP (1.0×10^{-3}M)	92.2	164.3	55.4	4.1

a) TCP, tranylcypromine; ATCP, N-acetyltranylcypromine. b) The value represents the average of three determinations. c) 17% loss of activity was observed on washing.

MAO was preincubated with TCP and ATCP for 30 min at 37°C and the MAO activities were determined both without the treatment of washing and after the washings of sedimented mitochondrial pellets with a phosphate buffer. As shown in Table I, washings restored the MAO activity which had been initially inhibited by ATCP near to normal, while the inhibition by TCP could not be reversed by washing. The result indicated reversibility of the MAO inhibition by ATCP and was consistent with the aforedescribed finding that the inhibition by ATCP was independent of the preincubation time.

***In vivo* MAO inhibitions**

The results are shown in Table II. An inhibition over 90% reached 0.5hr after the administration of TCP, stayed at the similar level to 6 hr and declined to 50% by 24 hr. On the other hand, with ATCP as an inhibitor, 90% inhibition level was obtained at 6hr after the administration and the inhibition declined to 73.8% by 24hr. The rapid approach to a 90% inhibition at 0.5 hr with TCP appears to be due to the fast absorption and thus high concentration of TCP in the brain at 0.5hr. It has been reported that TCP reached a peak concentration in about 15 min after the i.p. injection.[30] Whereas, slow onset of the inhibition by ATCP may be explained in relation to both the slow absorption of ATCP in an olive oil suspension and the delay in the hydrolysis to the active TCP *in vivo*.

As judged by the area under the inhibition curve, the degree of inhibition by 0.4mmole/kg of ATCP was similar to that by 0.015mmole/kg of TCP over 24hr. Therefore, it can be assumed that a conversion of 3-4% of the administered ATCP to TCP might occur *in vivo*. A work is in progress to determine a quatitative yield of *in vivo* hydrolysis by analyzing urine samples obtained following the administration of ATCP.

Table II. *In vivo* effects of tranylcypromine and N-acetyltranylcypromine on MAO activity in rat brain.

Drug[a]	Dose (mmole/kg i.p.)	% Inhibition[b] at various times (hr) following the injection					
		0.5	1.5	3	6	12	24
TCP	0.015	93.6	98.5	94.6	90.6	70.6	49.0
ATCP	0.4	0	12.7	73.0	89.7	88.2	73.8

a) TCP, tranylcypromine; ATCP, N-acetyltranylcypromine. b) The value represents the average of three rats.

QSAR study

As shown in the metabolic pathways (Fig. 13), the potency of TCP will be eventually terminated by its transformation to ATCP although it was proposed that part of the activity of TCP might be ascribed to the ATCP which is in equilibrium with TCP *in vivo*. However, the effect of ring-hydroxylation to the potency appears not clear, although it can be assumed that the aromatic hydroxylation may play a critical role in the shift of an overall metabolism to the side terminating the activity. In order to prove if the metabolic *p*-hydroxylation of the phenyl group is a contributing factor to the potency of TCP, it was assumed in this QSAR study that the compounds with *p*-substituents would behave all in a similar way in terms of the transport to the brain because of similar metabolic extractions differing from a series of compounds without *p*-substituents in which the ring-

Table III. Parameters used to derive equations for the inhibition of MAO by 2-phenylcyclopropylamines containing substituents on phenyl ring.

No.	x	ED_{50} (p.o.)[a] Mmoles/kg $\times 10^{3}$	log 1/C obsd.	log 1/C calcd.[b]	log 1/C Δlog 1/C	σ[c]	π[c]
1	4-Cl	1.7	5.77	5.80	-0.03	0.23	0.80
2	3,4-Cl_2	4.4	5.36	5.34	0.02	0.60	1.61
3	4-CF_3	1.1	5.96	5.94	0.02	0.55	1.09
4	4-CH_3	3.0	5.52	5.52	0.00	-0.17	0.52
5	4-OCH_3	1.2	5.92	5.90	0.02	-0.27	0.11
6	H	1.1	5.96	6.20	-0.24	0	0
7	3-Cl	6.4	5.19	5.06	0.13	0.37	0.81
8	2,5-Cl_2	360	3.48	3.78	-0.30	0.60	1.61
9	3-CF_3	16	4.80	4.59	0.21	0.43	1.09
10	2-Cl	7.4	5.13	4.93	0.20	0.23	0.80

a) Taken from the work of Zirkle et al.[19] b) Calculated either by eq. 1 (No. 1-5) or by eq. 2 (No. 6-10). c) Taken from the report of Hansch et al.[20]

hydroxylation can occur.

The results of QSAR study are shown in Table III. Equations derived by multiple regression analysis are as follows;

For compounds, 1-5 (*p*-substituent compounds);

$\log 1/C = 1.62\sigma - 1.31\pi + 6.48$ (r=0.992) (Eq. 1)

For compounds, 6-10 (compounds without *p*-substituents);

$\log 1/C = 1.10\sigma - 1.91\pi + 6.20$ (r=0.922) (Eq. 2)

When the compounds, 1-10 are considered;

$\log 1/C = 0.81\sigma - 1.33\pi + 6.22$ (r=0.447)

The equations indicated that groupings discriminating between the compounds with *p*-substituents and those without them only ensure high correlations in structure-activity relationships. When compounds, 1-10 are arranged in one group and analyzed, a complete lack in the correlation was observed. In this study such correlations were achieved only by using two constants, σ and π, and a simple equation, $\log 1/C = a\pi + \rho\sigma + c$. Therefore, it can be concluded that the results of the QSAR study were in accordance with our primary assumption that ring-hydroxylation which occurs at the para-position in most of the compounds is a determining factor to the potency of TCP.

It was also found by equations that electron-withdrawing groups in both series of compounds contributed positively to the potency. On the other hand, increasing π values resulted in the decrease in the potency. Since σ values used in the study were those derived from equilibrium constants,[20] the σ contributions in the compounds analyzed for this study were not restricted to the phenyl ring but reached to all molecules including cyclopropyl ring and the amino group attached to it. Negative contributions by π values can be explained in consideration of two factors; one, difficulty in the transport to the brain due to the high lipid solubility as reflected on its high π value and another, in relation to metabolic factors; that is, increasing π values will enhance the metabolic extraction rate, leading to the decrease in potency.

According to the similar QSAR study for the same series of compounds done by Fujita,[31] a steric constant, E_s as well as π and σ_2 constants was introduced in the equation to achieve high correlations and the steric factor was that considered only meta substituents as shown in the following equation:

$\log 1/C = -0.746\pi + 1.858\sigma_2 + 0.502\ E_s^3 + 5.180$ (r=0.939)

The only explanation made with respect to E_s^3 for the equation was that steric influence at the meta position might be involved in the drug-enzyme interaction. Fujita's equation also differs from ours in that high correlations could be achieved only by using σ_2 instead of σ. They attempted to explain this by proposing interactions such as a charge-transfer complex between benzene-ring and MAO active site.

In this study, the use of σ value was to take into account electronic effects affecting a whole molecule, paying a special attention to our finding that in tranylcypromine, electrons are highly delocalized over the molecule as seen by its low pKa value of 8.2.[32] Electron-withdrawing groups lower pKa values and thus the concentration of the unionized molecules at physiological pH will be increased, which might be an active form interacting with a MAO. Similar applications of σ value to the QSAR of MAO inhibitors can be found in propynylamine derivatives by Martin *et al.*[33] and N-isopropylhydrazides by Johnson.[34]

In addition, it was found in the equations that the magnitudes of σ and π effects contributing to the overall potency are different between the two series of compounds. The higher slope value of π compared to that of σ shown in the compounds without *p*-substituents, which is opposite in the *p*-substituent compounds, strongly suggest that the effects governing the metabolism are more important in determining the potency of the compounds which can undergo a para-hydroxylation.

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