Hydrolysis and Acyl Migration of a Catechol Monoester of L-Dopa: L-3-(3-Hydroxy-4-pivaloyloxyphenyl)alanine

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Abstract □ Hydrolysis and acyl migration studies on L-3-(3-hydroxy-4-pivaloyloxyphenyl)alanine (**1**, NB-355), which produced long-lasting plasma L-dopa levels after oral dosing, have been conducted. Compound **1** exists as pure 4-*O*-pivaloyl-L-dopa in the solid state, but it converts rapidly to a mixture of the 3- and 4-*O*-isomers in solution. The rate of acyl migration increased with increases in pH and temperature, and the content of the 4-*O*-isomer in the equilibrium state was 53–59%. The hydrolysis rate of **1** to L-dopa (**6**) also increased with increases in pH and temperature, and accelerated steeply at neutral and alkaline pH. The rapid hydrolysis at neutral pH was not observed with *O*-pivaloyl-L-tyrosine (**3**), di-*O*-pivaloyl-L-dopa (**4**), or L-dopa methyl ester (**5**). Because of this chemical lability, **1** was hydrolyzed in rat plasma far faster than the other tested catechol esters. However, in rat intestinal homogenate at pH 6.0, **1** was hydrolyzed at the slowest rate among the tested esters, predominantly by a diisofluorophosphate (DFP)-sensitive esterase. Thus, **1** showed a unique in vitro profile on hydrolysis and acyl migration due to the existence of a neighboring hydroxyl group. The stability of **1** in the intestine might be essential for the long-lasting plasma L-dopa profile after oral dosing of **1**.

L-Dopa has a short half-life in vivo resulting in wide interdose variation in drug levels. The fluctuation of plasma L-dopa levels may be responsible in part for some problems, namely dyskinesia and wearing-off phenomenon, that occur during chronic L-dopa therapy for Parkinson's disease.[1–7] Many L-dopa prodrugs have been prepared in an effort to solve these problems;[8–13] however, none of them has led to an agent clinically more useful than L-dopa itself. In a previous report[14] we described a novel type of L-dopa prodrug, 4-*O*-pivaloyl-L-dopa (**1**, NB-355), which produced a sustained L-dopa plasma level and large L-dopa bioavailability after oral dosing in rats and dogs. Slow absorption from the GI tract was proposed as the mechanism for the duration; therefore, stability of **1** in the intestine would be essential for the slow absorption. Marrel et al.[11] reported that L-dopa carboxyl esters were unstable to enzymatic hydrolysis by esterase. This lability to esterase might be the reason that these L-dopa carboxyl esters have not led to improvement in the duration of plasma L-dopa levels. In this report, we demonstrate the unique profile of **1** in terms of hydrolysis and acyl migration.

Results and Discussion

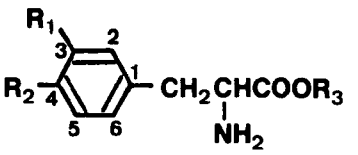
Structure Determination of 4-*O*-Pivaloyl-L-dopa (1, NB-355)—Table I shows the ^{13}C and ^{1}H NMR data of **1**, di-*O*-pivaloyl-L-dopa (**4**), and L-dopa (**6**) in their aromatic region. The assignment for ^{13}C chemical shift values of **1** in the aromatic region was made by long-range selective proton decoupling (LSPD) experiments. The signal at δ_c 150.1 was assigned to the C-3 carbon based on long-range couplings with H-2 (3.0 Hz) and H-5 (6.7 Hz), and the signal at δ_c 139.5 was assigned to the C-4 carbon based on long-range couplings with H-2 (9.0 Hz), H-5 (4.0 Hz), and H-6 (9.0 Hz). The acylated position on **1** was deduced by a comparison of the gated decoupling ^{13}C NMR data with those of **4** and L-dopa (**6**). The chemical shifts for C-4 in **1**, **4**, and L-dopa (**6**) are δ_c 139.5, 143.0, and 145.6, respectively, while those for C-3 are δ_c 150.1, 143.5, and 146.2, respectively. The upfield shift for C-4 and downfield shift for C-3 in **1** indicate that the acylated position on **1** is C-4.[15]

Table I—^{13}C and ^{1}H NMR Chemical Shift Values[a] of 1, 4, and L-Dopa (6) in the Aromatic Region

Assignment	1		4		6	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	133.9		134.3		126.3	
2	118.5	6.92d	125.3	7.20d	117.2	6.78d
3	150.1		143.5		146.2	
4	139.5		143.0		145.6	
5	124.0	6.93d	124.7	7.18d	116.7	6.79d
6	121.4	6.79dd	128.8	7.31dd	121.7	6.64dd

[a] Measured in CD_3OD containing DCl; chemical shifts are shown in ppm from TMS as internal standard.

Further confirmation of the chemical structure of **1** was achieved by preparing individual authentic specimens of 3-(**15**) and 4-*O*-pivaloyl-L-dopa (**14**) as summarized in Scheme I. *N*-Benzyloxycarbonyl-L-dopa benzyl ester (**7**)[16] reacted with 1.03 equivalents of benzyl chloride in the presence of potassium carbonate in acetone to afford a mixture of its 3- (**8**) and 4-*O*-benzyl esters (**9**), both of which were separated by medium-pressure liquid chromatography (MPLC). The chemical structures of **8** and **9** were determined by *O*-methylation with



	R_1	R_2	R_3
1	OH	OPiv	H
2	OPiv	OH	H
3	H	OPiv	H
4	OPiv	OPiv	H
5	OH	OH	CH_3
6	OH	OH	H

OPiv = $OCOC(CH_3)_3$

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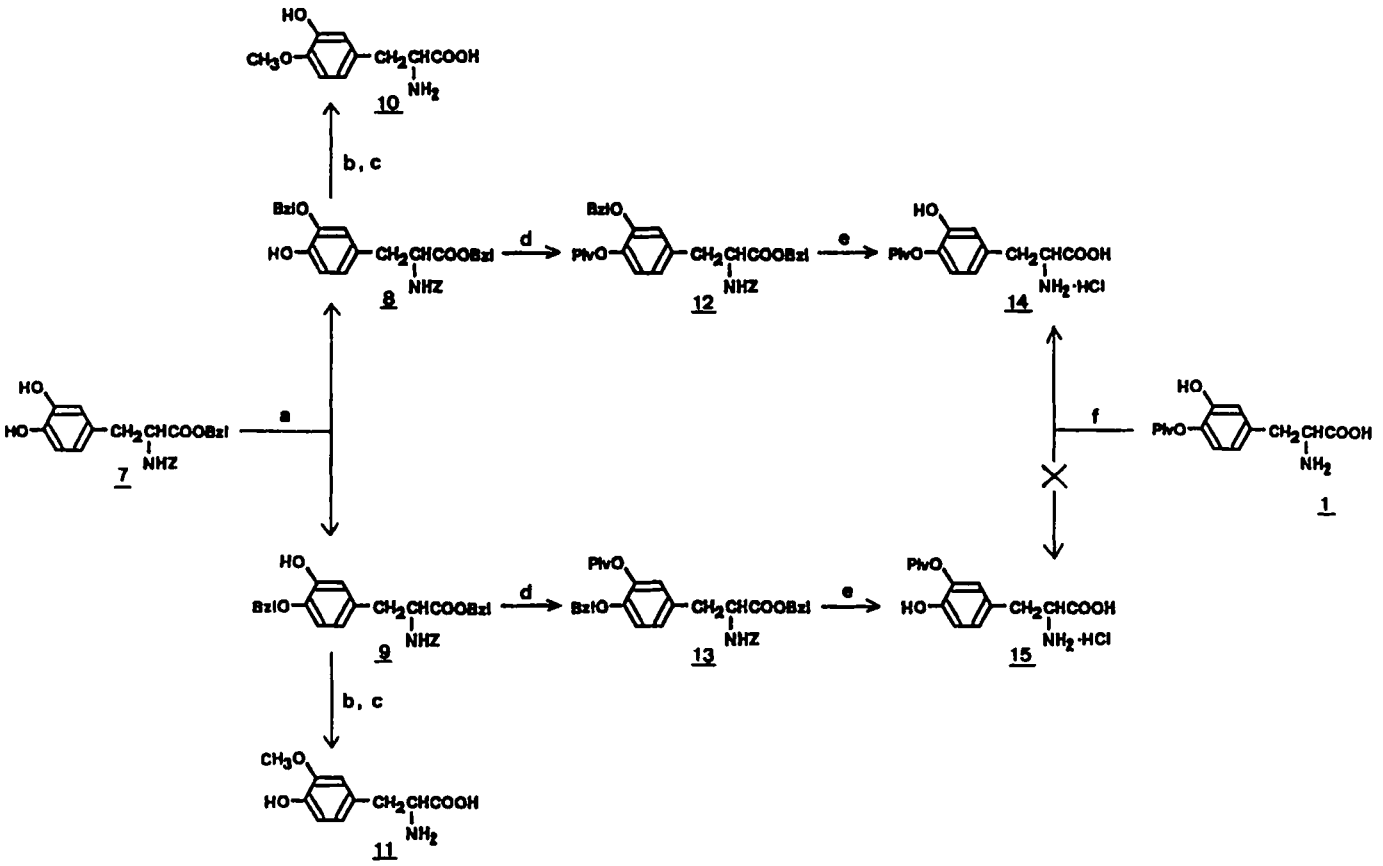

Scheme I—Structural determination of **1** by chemical approach (Z = benzyloxycarbonyl, Bzl = benzyl, Piv = pivaloyl). Method: (a) 1.03 eq. $PhCH_2Cl$:K_2CO_3:NaI in acetone, reflux; (b) CH_2N_2 in Et_2O; (c) H_2:Pd-C in MeOH; (d) $(CH_3)_3CCOCl$:TEA:DMAP in DMF, 90 °C; (e) H_2:Pd-C in HCl:MeOH; (f) HCl:MeOH:isopropyl ether.

diazomethane in ethyl ether, followed by hydrogenolysis to give the corresponding 4-*O*-methyldopa (**10**) and 3-*O*-methyldopa (**11**).[17] Both **8** and **9** were acylated with pivaloyl chloride in the presence of triethylamine and 4-dimethylaminopyridine in *N*,*N*-dimethylformamide to afford pivalates **12** and **13**, respectively. Then **8** and **9** were hydrogenated in methanol containing hydrogen chloride to give 4-(**14**) and 3-*O*-pivaloyl-L-dopa hydrochlorides (**15**), respectively. The NMR and IR data indicate that the hydrochloride of **1** is not 3-*O*-pivaloyl-L-dopa (**15**) derived from the 4-*O*-benzyl ether (**9**), but 4-*O*-pivaloyl-L-dopa (**14**) derived from the 3-*O*-benzyl ether (**8**). Furthermore, the HPLC chromatograms (method A) of **1**, the 4-*O*-isomer (**14**), and the 3-*O*-isomer (**15**) indicate that all of them are stable in 0.1 M HCl:methanol and that the peak of **1** does not coincide with that of the 3-*O*-isomer hydrochloride (**15**; retention time: 12.9 min), but with that of the 4-*O*-isomer hydrochloride (**14**; retention time: 10.9 min). Thus, the structure of **1** was concluded to be 4-*O*-pivaloyl-L-dopa.

***O*→ *O* Acyl Migration of 1 in Solution**—Compound **1**(4-*O*-isomer) and the 3-*O*-isomer (**2**) in 0.1 M HCl:methanol gave separate peaks on the HPLC chromatograms (method A) as described above, while 15 min after dissolving in 50 mM phosphate buffer (pH 5) at 4 °C, both isomers gave the same composition mixture of their isomers on the chromatograms. These data indicate that a rapid and reversible *O*→ *O* acyl migration, moving the pivaloyl function, occurs in solution.

The pH dependency of the acyl migration profile is shown in Figure 1. Even immediately after dissolution at pH 2, ~5% of the 3-*O*-isomer existed on the HPLC chromatogram. A similar composition of the 3-*O*-isomer existed on the HPLC chromatogram of **1** in DCl:methanol-d_4, although the 3-

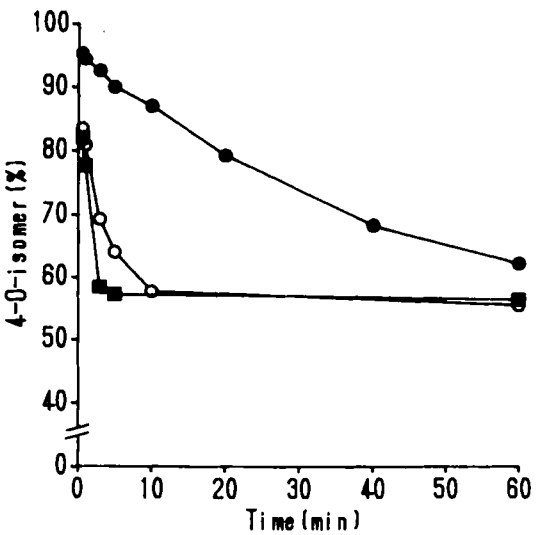

Figure 1—Effect of pH values on the time course of migration of **1** in 50 mM phosphate buffer at 4 °C. Key: (●) pH 2; (○) pH 3; (■) pH 4.

O-isomer did not exist on the NMR spectrum of the same solution. These results indicate that ~5% of the 3-*O*-isomer detected immediately after dissolution at pH 2 is due to conversion to the 3-*O*-isomer in the injection port and the HPLC line before the separation column. Compound **1** took >60 min to reach equilibrium with the 3-*O*-isomer at pH 2 and 4 °C, while the acyl migration rate accelerated steeply with a rise in the pH value. The calculated rate constants of

acyl migration at 4 °C are 0.011 min^{-1} at pH 2, 0.124 min^{-1} at pH 3, and 0.511 min^{-1} at pH 4. At pH values >5, the rate of acyl migration to the equilibrium state was too rapid to estimate exactly. In addition, the migration rate was also accelerated with a rise in the temperature at pH 2, as shown in Figure 2. The rate constants of acyl migration at pH 2 are 0.011 min^{-1} at 4 °C, 0.071 min^{-1} at 20 °C, and 0.264 min^{-1} at 37 °C. The ratio of the 4-*O*-isomer in the equilibrium state was 53 to 59% in the pH range 2–7.4; the ratio increased slightly with increases in pH. In addition, the equilibrium ratio at pH 2 was constant at any temperature observed (4, 20, and 37 °C). These data suggest that after oral dosing, **1** momentarily converts to an equilibrium mixture with an almost constant ratio between the 4- (1) and 3-*O*-isomers (2) at the physiological conditions (~37 °C) of the GI tract.

Hydrolysis of 1 and Related Compounds in Phosphate Buffer—The hydrolysis rates of **1** over the pH range 2–7.4 at 37 °C in 50 mM phosphate buffer were studied (Figure 3). Under all the experimental conditions used, **1** was hydrolyzed to yield L-dopa (**6**) quantitatively as estimated by HPLC method B. At constant pH and temperature, the hydrolysis displayed strict first-order kinetics. Although acid-catalyzed hydrolysis was not detected at pH 2 to 5, the rapid hydrolysis was observed at pH values >6.5. The rate constant of the hydrolysis at pH 7.4 and 37 °C was 0.0311 ± 0.0008 min^{-1} ($t_{1/2}$: 22.3 min). In addition, the hydrolysis rate at pH 7.0 was not influenced by the concentration of **1** and phosphate buffer (data not shown), indicating that the base-catalyzed rapid hydrolysis of **1** was not due to intermolecular interaction and not likely to be influenced by the ionic strength.

The rapid base-catalyzed hydrolysis of **1** was not seen with the other phenolic pivaloyl esters (**3** and **4**; Table II). Therefore, the rapid base-catalyzed hydrolysis of **1** would be responsible for the existence of a neighboring hydroxyl group, which is characteristic of the L-dopa catechol monoester.

Hydrolysis of 1 and Related Compounds in Rat Intestinal and Pancreatic Homogenate—The surface of the rat intestinal wall is weakly acidic (~ pH 6.2) due to the microclimate, which keeps the surface at pH 6.2 and protects against pH change by the various conditions of the lumen.[18] Therefore, the studies of the hydrolysis of **1** and the related compounds **3**, **4**, and **5** in the GI tract were conducted at pH 6.0 in rat intestinal and pancreatic homogenates (in rat, enzymes are secreted into the intestine).

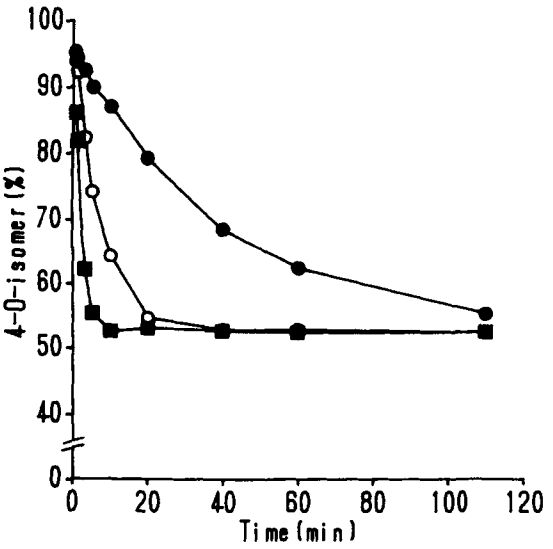

Figure 2—Effect of temperature on the time course of migration of **1** in 50 mM phosphate buffer at pH 2. Key: (●) 4 °C; (○) 20 °C; (■) 37 °C.

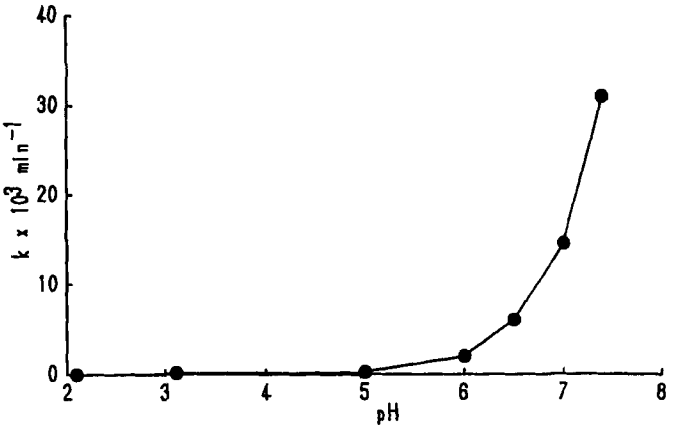

Figure 3—The pH–rate constant profile for hydrolysis of **1** in 50 mM phosphate buffer at 37 °C.

Table II—Rate Constants (*k*) of Hydrolysis[a]

pH	Compound			
	1	**3**	**4**	**5**
6.0	1.94 ± 0.07[b]	N.D.[c]	N.D.[c]	N.D.[c]
7.4	31.1 ± 0.8[b]	N.D.[c]	N.D.[c]	1.77 ± 0.08[b]

[a] Measured in 50 mM phosphate buffer at 37 °C. [b] Expressed as mean k ± S.D. × 10^3 (min^{-1}). [c] Not detected.

In intestinal homogenate at pH 6.0, **1** was hydrolyzed at the rate of 2.6 × 10^{-3} min^{-1} · mg $protein^{-1}$ · mL; this rate is 1.8 and 4.0 times slower than that of the other phenolic esters **3** and **4**, respectively (Table III). These hydrolyses were inhibited by the esterase inhibitor diisofluorophosphate (DFP). The observation that the hydrolysis of **1** in intestinal homogenate proceeded at the slowest rate of the compounds tested suggests that the neighboring hydroxyl group on **1** is responsible for resistance to the hydrolysis by esterase.

The pH dependency of the hydrolysis rate of **1** in intestinal homogenate is shown in Figure 4. The total hydrolysis rate increased with increases in pH. When DFP was added to the homogenate, the hydrolysis rate decreased to the same level as that without homogenate. These data indicate that **1** is hydrolyzed enzymatically in intestinal homogenate by a DFP-sensitive esterase, with the rate shown as the total hydrolysis minus DFP-treated hydrolysis. The rate of enzymatic hydrolysis was almost constant between pH 6.5 and 7.4.

In pancreatic homogenate at pH 6.0, phenolic esters including **1** were resistant to enzymatic hydrolysis, but the L-dopa methyl ester (**5**) was quickly hydrolyzed to L-dopa (**6**; Table III). The lability of the L-dopa methyl ester (**5**) to esterase in the GI tract may account for the reported short half-life of plasma L-dopa (**6**) after oral dosing.[10] Therefore, the stability of **1** in the intestine may be essential for the long-lasting

Table III—Rate Constants (*k'*) of Hydrolysis in Rat Tissue Homogenate[a]

Homogenate	Compound			
	1	**3**	**4**	**5**
Intestine	2.6 ± 0.09[b]	4.6 ± 1.1[b]	10.5 ± 0.4[b]	39.9 ± 1.0[b]
+ DFP	0.29 ± 0.07[b]	N.D.[c]	N.D.[c]	24.2 ± 0.1[b]
Pancreas	0.16 ± 0.008[b]	N.D.[c]	N.D.[c]	176.8 ± 9.4[b]
+ DFP	0.11 ± 0.01[b]	N.D.[c]	N.D.[c]	64.7 ± 1.3[b]

[a] Measured at pH 6.0 and 37 °C. [b] Expressed as mean k' ± S.D. × 10^3 (min^{-1} · mg $protein^{-1}$ · mL). [c] Not detected.

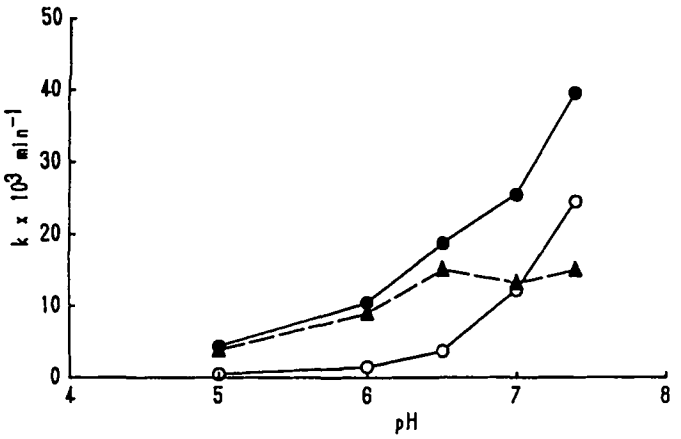

Figure 4—The pH–rate profiles for hydrolysis of **1** in 10% (w/v) rat intestinal homogenate at 37 °C. Key: (●) total hydrolysis (A); (○) hydrolysis in the presence of 100 μm DFP (B); (▲) DFP-sensitive hydrolysis estimated as A − B.

profile of plasma L-dopa (**6**) levels that is due to the slow absorption from the intestine after oral dosing of **1**.[14]

Hydrolysis of 1 and Related Compounds in Rat Plasma—The rate constants of hydrolysis of **1** and the related compounds **3**, **4**, and **5** in 50% rat plasma at pH 7.4 are shown in Table IV. The rate constant of **1** (catechol mono-*O*-pivaloylester) is 0.0513 ± 0.0012 min^{-1}, a value which is 11.9 and 4.5 times as rapid as those for **3** (phenol *O*-pivaloylester) and **4** (catechol di-*O*-pivaloylester), respectively. Since the hydrolysis of **1** was not inhibited by DFP, **1** will be chemically hydrolyzed to L-dopa (**6**) primarily in the plasma. However, the rate of hydrolysis in plasma was 1.65 times as fast as the rate of chemical hydrolysis in 50 mM phosphate buffer at pH 7.4. Therefore, **1** might also be hydrolyzed by DFP-insensitive enzymes that are different from the DFP-sensitive esterase observed in the intestinal homogenate. This lability of **1** in plasma would lead to rapid generation of L-dopa (**6**) in the systemic circulation after oral dosing of **1**.

Thus, **1** showed a unique hydrolysis and acyl migration profile in vitro due to the existence of a neighboring hydroxyl group. After oral dosing of **1**, acyl migration to equilibrium of the 4- (**1**) and 3-*O*-isomer (**2**) occurs momentarily; slow hydrolysis and/or slow absorption[14] might follow it, because the rate of acyl migration is faster than the rate of hydrolysis at physiological temperature (37 °C). Furthermore, the resistance to DFP-sensitive esterase in the intestine might be essential for the long-lasting plasma levels of L-dopa that are due to slow absorption from the GI tract after oral dosing of **1**.

Experimental Section

Elemental analyses were done by Sumika Chemical Analysis Service, Ltd. Melting points were uncorrected. The ^1H and ^{13}C NMR spectra were obtained on a Varian VXR-300 spectrometer, and shift values are reported in δ ppm downfield from tetramethysilane as the internal standard. The NMR abbreviations used are as follows: s, singlet; d, doublet; t, triplet; dd, doublet of doublets; dt, doublet of triplets; m, multiplet. Low- and high-resolution mass spectra were recorded on a Jeol JMS-DX300 spectrometer. The IR spectra were recorded on a Hitachi 270-30 spectrometer. Optical rotation was obtained with a Horiba SEPA-200 polarimeter. Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. 3-*O*-Methyldopa was purchased from Sigma Chemical Company, and 4-*O*-methyldopa was prepared by the method of Fennoy.[17] L-3-(3,4-Dipivaloyloxyphenyl)alanine (**4**) and L-3(3,4-dihydroxyphenyl)alanine methyl ester hydrochloride (**5**) were prepared according to Bodor et al.[9]

Table IV—Rate Constants (*k*) of Hydrolysis in 50% Rat Plasma[a]

	Compound			
	1	**3**	**4**	**5**
Plasma	51.3 ± 1.2[b]	4.3 ± 0.5[b]	11.5 ± 2.4[b]	214 ± 93[b]
+DFP	49.4 ± 1.9[b]	N.D.[c]	N.D.[c]	5.9 ± 0.7[b]

[a] Measured at pH 7.4 and 37 °C. [b] Expressed as mean *k* ± S.D. × 10^3 (min^{-1}). [c] Not detected.

Synthesis of L-3-(3-Hydroxy-4-pivaloyloxyphenyl)alanine Hemihydrate (1; NB-355)—To a solution of L-dopa (60 g, 0.30 mol) in 300 mL of trifluoroacetic acid, pivaloyl chloride (39 mL, 0.32 mol) was added in a dropwise manner over a period of 5 h, with stirring and while holding the temperature below 0 °C. After 6 h, the mixture was evaporated under reduced pressure. Then the residue was dissolved in 1050 mL of water. After 3 h, a precipitate of the di-*O*-pivaloyl ester was removed by filtration and the filtrate, which was diluted with 250 mL of methanol, was passed through a column of 200 mL of Diaion HP-20; this was followed by washing the column with 500 mL of 20% aqueous methanol. The effluent and washings were combined and concentrated to a volume of 1000 mL under reduced pressure and then adjusted to pH 3.5 by the addition of 1 M NaOH aqueous solution. The precipitate was collected by filtration and washed with 300 mL of water and 200 mL of isopropyl alcohol to yield 50.7 g of **1** (57.4%). An analytical sample was recrystallized from 25% aqueous isopropyl alcohol, mp 223 °C (dec.); $[\alpha]_D^{20}$: −12.6° (C = 1.0, 1 M HCl); MS(FAB): *m/z* 282[M + H]$^+$; ^1H NMR (DCl/CD_3OD): δ 1.35 (9H, s, $(CH_3)_3C$), 3.09 (1H, dd, *J* = 7.9, 14.7 Hz, CH_2Ar), 3.29 (1H, dd, *J* = 5.2, 14.7 Hz, CH_2Ar), 4.23 (1H, dd, *J* = 5.2, 7.9 Hz, CHN), 6.79 (1H, dd, *J* = 2.0, 8.2 Hz, H6-Ar), 6.92 (1H, d, *J* = 2.0 Hz, H2-Ar), and 6.93 (1H, d, *J* = 8.2 Hz, H5-Ar); IR (KBr): 3400, 2980, 2400–3100(broad), 1743, 1635, 1593, 1420, 1134, and 763 cm^{-1}.

Anal.—Calc. for $C_{14}H_{19}NO_5 \cdot 1/2H_2O$: C, 57.92; H, 6.94; N, 4.82. Found: C, 58.08; H, 6.86; N, 4.79.

Synthesis of L-3-(4-Pivaloyloxyphenyl)alanine (3)—To a solution of L-tyrosine (1.8 g; 0.01 mol) in trifluoroacetic acid (15 mL) was added pivaloyl chloride (2.0 mL; 0.016 mol) in a dropwise manner, with stirring at 0–5 °C. After the mixture was stirred for 5 h at 0–5 °C, the solvent was removed under reduced pressure. The residue was treated with isopropyl ether (50 mL) and was filtered to give the trifluoroacetic acid salt of the desired product (3.3 g). The salt was dissolved in water (50 mL) and the solution was adjusted to pH 3.6 with 1 M NaOH aqueous solution. The precipitate was collected to give 2.0 g of **3** (75.5%). An analytical sample was recrystallized from 20% aqueous methanol, mp 218–220 °C (dec.); $[\alpha]_D^{20}$: −11.0° (C = 1.0, 1 M HCl); MS (FAB): *m/z* 266[M + 1]; ^1H NMR (CD_3OD): δ 1.35 (9H, s, $((CH_3)_3C)$, 3.01 (1H, dd, *J* = 9.0, 14.4 Hz, CH_2Ar), 3.35 (1H, dd, *J* = 4.2, 14.4 Hz, CH_2Ar), 3.77 (1H, dd, *J* = 4.2, 9.0 Hz, CHN), 7.03 (2H, d, *J* = 8.7 Hz, Ar), and 7.35 (2H, d, *J* = 8.7 Hz, Ar); IR (KBr): 2974, 1752, 1512, 1485, 1404, 1200, and 1167 cm^{-1}.

Anal.—Calc. for $C_{14}H_{19}NO_4$: C, 63.38; H, 7.22; N, 5.28. Found: C, 63.61; H, 7.29; N, 5.53.

L-*N*-Benzyloxycarbonyl-3-(3-benzyloxy-4-hydroxyphenyl)alanine Benzyl Ester (8) and L-*N*-Benzyloxycarbonyl-3-(4-benzyloxy-3-hydroxyphenyl)alanine Benzyl Ester (9)—To a solution of *N*-benzyloxycarbonyl-L-dopa benzyl ester (**7**; 9.0 g, 21.4 mmol) in 150 mL of acetone were added sodium iodide (0.48 g, 3.2 mmol), benzyl chloride (2.56 mL, 22.3 mmol), and potassium carbonate (9.0 g, 65 mmol). The mixture was refluxed with stirring for 7.5 h in an atmosphere of argon. After the reaction was over, the inorganic salt was removed by filtration and the filtrate was evaporated under reduced pressure. Then, the residue was purified by MPLC (Lobar column Lichroprep Si60; elution solvent: hexane:ethyl acetate = 10:1–4:1). The two isomeric mono-*O*-benzyl ethers **8** and **9** were obtained.

The chemical identities of **8** and **9** were established by methylation with diazomethane in ethyl ether, followed by hydrogenation in methanol with 10% palladium-carbon catalyst, to give 4-*O*-methyldopa (**10**) and 3-*O*-methyldopa (**11**), respectively. Both **10** and **11** were shown to be identical with authentic samples[17] by TLC, HPLC, IR, and NMR comparison.

3-O-Benzyl Isomer (8)—Compound **8** was isolated in a yield of 1.49 g (13.6%) as a colorless oily substance; ^1H NMR ($CDCl_3$): δ 3.04 (2H, d, *J* = 6.1 Hz, CH_2Ar), 4.65–4.68 (1H, m, CHN), 4.89 (2H, s, CH_2O), 5.05–5.15 (4H, m, CH_2Ox2), 5.22 (1H, d, *J* = 8.1 Hz, NH), 5.56 (1H,

s, OH), 6.52 (1H, dd, J = 8.1 Hz, 1.7 Hz, H6-Ar), 6.63 (1H, d, J = 1.7 Hz, H2-Ar), 6.77 (1H, d, J = 8.1 Hz, H5-Ar), and 7.32–7.40 (15H, m, Phx3); IR (KBr): 3376, 2926, 1722, 1518, 1275, 1236, 1197, and 699 cm^{-1}; MS (FAB): m/z 512[M + H]$^+$, 378 (base peak); HRMS (EI): m/z 511.1979 ($C_{36}H_{29}NO_6$ requires 511.1995).

4-O-Benzyl Isomer (9)—Compound 9 was isolated in a yield of 1.1 g (10.0%) as a colorless oily substance; ^1H NMR ($CDCl_3$): δ 3.01 (2H, d, J = 5.6 Hz, CH_2Ar), 4.65 (1H, m, CHN), 5.04 (2H, s, CH_2O), 5.09 (2H, s, CH_2O), 5.14 (2H, s, CH_2O), 5.22 (1H, d, J = 8.6 Hz, NH), 5.59 (1H, s, OH), 6.46 (1H, dd, J = 8.1 Hz, 1.9 Hz, H6-Ar), 6.66 (1H, d, J = 1.9 Hz, H2-Ar), 6.73 (1H, d, J = 8.1 Hz, H5-Ar), and 7.25–7.40 ppm (15H, m, Phx3); IR (KBr): 3412, 3070, 2944, 1728, 1515, 1341, 1275, 1026, 738, and 699 cm^{-1}; MS (FAB): m/z 512[M + H]$^+$, 167 (base peak); HRMS (EI): m/z 511.2013 ($C_{36}H_{29}NO_6$ requires 511.1995).

L-*N*-Benzyloxycarbonyl-3-(3-benzyloxy-4-pivaloyloxyphenyl)alanine Benzyl Ester (12)—To a solution of 8 (1.0 g, 1.96 mmol) in 15 mL of *N,N*-dimethylformamide were added triethylamine (0.81 mL, 5.81 mmol), 4-dimethylaminopyridine (597 mg, 4.89 mmol), and pivaloyl chloride (0.72 mL, 5.85 mmol), and the mixture was heated for 25 min at 90 °C. After the reaction was over, ethyl acetate and water were added to the reaction mixture. The organic layer that separated was washed with a saturated aqueous NaCl solution and then dried over anhydrous magnesium sulfate. The extract was evaporated under reduced pressure, and the residue was purified by chromatography on silica gel (Wakogel C-200; eluant: hexane:ethyl acetate = 5:1–3:1). This was followed by recrystallizing from a mixture of ethyl ether and hexane to afford 0.85 g of 12 (73%), mp 71–72 °C; ^1H NMR ($CDCl_3$): δ 1.24 (9H, s, $(CH_3)_3C$), 3.08 (2H, d, J = 5.3 Hz, CH_2Ar), 4.68–4.71 (1H, m, CHN), 4.84 (2H, s, CH_2O), 5.05–5.14 (4H, m, CH_2Ox2), 5.29 (1H, d, J = 7.9 Hz, NH), 6.60 (1H, dd, J = 8.2 Hz, 2.0 Hz, H6-Ar), 6.71 (1H, d, J = 2.0 Hz, H2-Ar), 6.86 (1H, d, J = 8.2 Hz, H5-Ar), and 7.23–7.39 ppm (15H, m, Phx3); IR (KBr): 1755, 1716, 1509, 1350, 1188, and 1122 cm^{-1}; HRMS (EI): m/z 595.2568 ($C_{36}H_{37}NO_7$ requires 595.2570).

L-*N*-Benzyloxycarbonyl-3-(4-benzyloxy-3-pivaloyloxyphenyl)alanine Benzyl Ester (13)—Using the 4-*O*-benzyl isomer (9; 0.80 g, 1.56 mmol) as a starting material, the same procedure as described above was performed to give a colorless oily product; that is, the 3-*O*-pivaloyl derivative (13; 0.61 g, 65%); ^1H NMR ($CDCl_3$): δ 1.24 (9H, s, $(CH_3)_3C$), 3.03 (2H, d, J = 5.7 Hz, CH_2Ar), 4.65–4.67 (1H, m, CHN), 4.97 (2H, s, CH_2O), 5.09 (2H, s, CH_2O), 5.11 (2H, s, CH_2O), 5.32 (1H, d, J = 8.0 Hz, NH), 6.79–6.80 (3H, m, Ar), and 7.23–7.39 (15H, m, Phx3); IR (KBr): 1752, 1515, 1266, 1215, and 1122 cm^{-1}; HRMS (EI): m/z 595.2577 ($C_{36}H_{37}NO_7$ requires 595.2570).

L-3-(3-Hydroxy-4-pivaloyloxyphenyl)alanine Hydrochloride (14)—Compound 12 (100 mg, 0.168 mmol) was dissolved in 6.1 mL of 0.57% hydrogen chloride:methanol solution and reduced at a hydrogen pressure of 4 kg/cm^2 for 3.5 h in the presence of 10% palladium-carbon catalyst (250 mg). After the reduction was completed, the catalyst was removed by filtration and the filtrate was evaporated under reduced pressure. The resulting residue was dissolved in 0.2 mL of 3.3% hydrogen chloride:methanol solution and the precipitate was removed by filtration. Isopropyl ether was added to the filtrate to afford a crystalline mass of 14 (34 mg, 64%). A pure sample was obtained by recrystallization from a mixture of methanol and isopropyl ether, mp 190 °C (dec.); ^1H NMR (DCl/CD_3OD): δ 1.35 (9H, s, $(CH_3)_3C$), 3.06 (1H, dd, J = 14.5 Hz, 7.9 Hz, CH_2Ar), 3.28 (1H, dd, J = 14.5 Hz, 5.2 Hz, CH_2Ar), 4.23 (1H, dd, J = 7.9 Hz, 5.2 Hz, CHN), 6.78 (1H, dd, J = 8.2 Hz, 2.2 Hz, H6-Ar), 6.90 (1H, d, J = 2.2 Hz, H2-Ar), and 6.92 (1H, d, J = 8.2 Hz, H5-Ar); IR (KBr): 3346, 2974, 1746, 1728, 1302, 1239, 1143, and 1116 cm^{-1}.

L-3-(4-Hydroxy-3-pivaloyloxyphenyl)alanine Hydrochloride (15)—Using the 3-*O*-pivaloyl isomer (13; 100 mg, 0.168 mmol) as a starting material, catalytic hydrogenation was performed as described above to afford the 3-*O*-pivaloyl isomer (15; 45 mg, 72%), mp 195 °C (dec.); ^1H NMR (DCl/CD_3OD): δ 1.36 (9H, s, $(CH_3)_3C$), 3.14 (1H, dd, J = 14.7 Hz, 7.3 Hz, CH_2Ar), 3.26 (1H, dd, J = 14.7 Hz, 5.5 Hz, $ArCH_2$), 4.24 (1H, dd, J = 7.3 Hz, 5.5 Hz, CHN), 6.94 (1H, d, J = 2.1 Hz, H2-Ar), 6.98 (1H, d, J = 8.3 Hz, H5-Ar), and 7.07 (1H, dd, J = 8.3 Hz, 2.1 Hz, H6-Ar); IR (KBr): 3418, 2980, 1737, 1521, 1293, 1251, and 1137 cm^{-1}.

Compound 1 (NB-355) Hydrochloride—Compound 1 (0.40 g, 1.38 mmol) was dissolved in 1.5 mL of 1.5% hydrogen chloride:methanol solution. The resulting solution was diluted with 30 mL of isopropyl ether and allowed to stand for 2 h at 0–5 °C. The crystalline precipitate was collected by filtration, washed with isopropyl ether, and then dried to afford 0.30 g of 1 hydrochloride (68.6%), mp 190 °C (dec.); ^1H NMR (DCl/CD_3OD): δ 1.35 (9H, s, $(CH_3)_3C$), 3.10 (1H, dd, J = 14.3 Hz, 7.9 Hz, CH_2Ar), 3.30 (1H, dd, J = 14.3 Hz, 5.4 Hz, CH_2Ar), 4.24 (1H, dd, J = 8.0 Hz, 5.4 Hz, CHN), 6.80 (1H, dd, J = 8.0 Hz, 1.9 Hz, H6-Ar), 6.92 (1H, d, J = 1.9 Hz, H2-Ar), and 6.93 (1H, d, J = 8.0 Hz, H5-Ar); IR (KBr): 3346, 2974, 1746, 1728, 1302, 1239, 1143, and 1116 cm^{-1}.

High-Performance Liquid Chromatography Analysis—*Method A*—4-(1) and 3-*O*-Monopivalates (2) of L-dopa were measured by an HPLC system (JASCO TRIROTOR VI) equipped with a variable wavelength detector (JASCO UVIDEC 100 VI) under the following analytical conditions: precolumn: Newguard RP-18 7 μm, 15 × 3.2 mm i.d. (Brownlee Labs); column: Nucleosil C_{18} 5 μm, 250 × 4.6 mm i.d. (Macherey-Nagel); column temperature: 4 °C; mobile phase: 0.06 M $NaClO_4$ [adjusted to pH 2.5 with $HClO_4$:CH_3CN (68:32)]; flow rate: 0.7 mL/min; detection: UV 273 nm.

Method B—L-Dopa concentrations generated from 1 and 5 were measured by an HPLC apparatus (Waters 600E) equipped with an electrochemical detector (Waters 460) under the following analytical conditions: precolumn: Newguard RP-18 7 μm, 15 × 3.2 mm i.d.; column: Nucleosil C_{18} 5 μm, 250 × 4.6 mm i.d.; column temperature: 40 °C; mobile phase: 0.1 M citric acid:0.1 M sodium citrate (3:6) containing 0.1 mM EDTA · 2Na; flow rate: 0.8 mL/min; applied voltage: 600 mV.

Method C—The concentrations of 3 and 4 were measured by an HPLC apparatus equipped with a fluorometric detector (Jasco 820 FP) after derivatization with *o*-phthalaldehyde (OPA). Samples were reacted at a ratio of 1:1 (v/v) with an OPA reagent [4% borate buffer:methanol (4:1) solution containing 60 mM OPA and 50 mM *N*-acetylcysteine), then vortexed for a few minutes at room temperature, and stored at 2 °C. The HPLC conditions were as follows: precolumn: Newguard RP-8 7 μm, 15 × 3.2 mm i.d., column: μBondasphere C_8 5 μm, 150 × 3.9 mm i.d.; column temperature: 50 °C; mobile phase: 50 mM phosphate buffer (pH 7.0):methanol (50:50 for 3 assay, 45:55 for 4 assay); flow rate: 0.8 mL/min; excitation wavelength: 340 nm; emission wavelength: 450 nm.

Migration Study—The kinetic studies of migration were undertaken using 50 mM phosphate buffer containing 50 μg/mL of 1 over the range of pH from 2 to 7.4 at 4, 20, and 37 °C. The samples taken at various time intervals were injected directly into the HPLC system (method A) in triplicate. The ratio of the 4-*O*-isomer was calculated from the peak heights of the 3- (2) and 4-*O*-isomers (1) in the following method. The integration values of the peaks on the ^1H NMR spectrum at 6.99 [H-6 of 3-*O*-isomer (2)] and 6.73 [H-6 of 4-*O*-isomer (1)] ppm were in the ratio 44.9:55.1 in a 38 mM sample of 1 (2 h after 1 was dissolved in methanol-d_4, while the peak heights of the 3- and 4-*O*-isomers measured by HPLC method A were in the ratio 41.3:58.7 in the same sample. Since this discrepancy is due to the different UV absorbance at 273 nm in the HPLC analysis, the corrected ratio of the 4-*O*-isomer was calculated according to the following equation using a coefficient parameter (1.16) calculated from the HPLC and NMR data described above:

$$[C] = \frac{[P4]}{1.16\,[P3] + [P4]} \quad (1)$$

where [C], [P3], and [P4] represent the corrected ratio of the 4-*O*-isomer in the isomeric mixture, and the peak heights of the 3-*O*-isomer and the 4-*O*-isomer on the chromatogram obtained by the HPLC method A, respectively.

Since the migration is reversible, the rate constants k_1 and k_{-1} can be described by the following equation:[19]

$$\log \frac{[I]_o - [I]_e}{[I]_t - [I]_e} = (k_1 + k_{-1})t/2.303 \quad (2)$$

where $[I]_o$, $[I]_t$, and $[I]_e$ represent the concentrations of 4-*O*-isomer at initiation, time t, and equilibrium, respectively. In addition, the rate constants can be described by the equilibrium constant (K_e) as follows:

$$K_e = k_1 / k_{-1} \quad (3)$$

From eqs 2 and 3, the following relationship can be determined:

$$\log \frac{[I]_o - [I]_e}{[I]_t - [I]_e} = \frac{K_e + 1}{K_e} k_1 t/2.303 \quad (4)$$

Since the K_e values were calculated by the ratio of the 4-*O*-isomer in the equilibrium state, the first-order rate constants (k_1) were estimated from the slope of the linear line [i.e., $(K_e + 1)/(K_e \cdot k_1)$].

Hydrolysis Study in Phosphate Buffer—Compound **1** (50 μM) was incubated in 50 mM phosphate buffer over the pH range 2.1–7.4 at 37 °C. Samples were collected at various time intervals. The L-dopa concentrations generated were measured by HPLC method B. Furthermore, the related compounds **3**, **4**, and **5** (50 μM) were incubated at 37 °C and pH 6.0 and 7.4. L-Dopa concentrations were measured by HPLC method B. The concentrations of **3** and **4** were measured by HPLC method C after OPA derivatization.

Hydrolysis Study in Rat Pancreas and Intestinal Homogenate—Intestine and pancreas tissues were obtained from male Sprague-Dawley rats (8–11 weeks). Intestine and pancreas tissues were washed with 50 mM phosphate buffer and homogenized with nine volumes (w/v) of 50 mM phosphate buffer (pH 6.0) by a polytron (setting 6, 30 s × 5). Compound **1** and the related compounds **3**, **4**, and **5** (50 μm) were added to the tissue homogenate in the presence of 100 μM carbidopa and incubated at 37 °C. Samples were collected at various time intervals. To measure the kinetics of **1** and **5**, the samples were treated with four volumes of 0.5 M perchloric acid and centrifuged at 10 000 rpm for 10 min at 4 °C. L-dopa concentrations in the supernatants were measured by HPLC method B. To measure the kinetics of esters **3** and **4**, the samples were treated with four volumes of ethanol and centrifuged at 10 000 rpm for 10 min at 4 °C. The concentrations of the esters **3** and **4** were measured by HPLC method C. Similar experiments were also performed with the addition of 100 μM diisofluorophosphate (DFP).

Hydrolysis study in rat plasma—Rat plasma was obtained from EDTA-treated fresh blood of male Sprague-Dawley rats (8–11 weeks). Compound **1** and the analogues (50 μM) were incubated in 0.2 M phosphate buffer (pH 7.4) containing 50% rat plasma in the presence of 100 μM carbidopa at 37 °C, and samples were withdrawn at various time intervals. The kinetics of hydrolysis were measured with procedures similar to those described above.

Calculations for Hydrolysis Study—For the calculation of the rate constants (k) of hydrolysis, the concentrations measured were entered into the following equation of a first-order reaction:

$$\log C_i = \log C_o - k/2.303 \cdot t \quad (5)$$

where C_i and C_o are the concentrations of the esters at $t = 0$ and $t =$ i, respectively. The concentrations of **1** and **5** were calculated from the L-dopa concentrations generated. The rate constants (k') of hydrolysis in intestinal and pancreatic homogenate were calculated by the following equation:

$$k' = k/C_p \quad (6)$$

where C_p is the concentration of protein in the homogenate determined by the method of Lowry et al. using bovine serum albumin as the standard.[20] The rate constants were determined in triplicate.

References and Notes

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