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Improved Delivery through Biological Membranes. 4. Prodrugs of L-Dopa[1]

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Various classes of transient derivatives of L-Dopa have been synthesized, systematically protecting one or more of the main sites of metabolism in the molecule: the carboxy function, the amino, and/or the catechol system. The derivatives studied include carboxy esters, phenol esters, amides, peptides, and various combinations of these functions. A number of these derivatives effectively prevent the metabolism of L-Dopa prior to and/or during the absorption process, resulting in a significantly better bioavailability of the drug. In vivo studies using dogs showed up to 2.5-fold increase in L-Dopa blood levels. The metabolism as well as toxicity aspects of the prodrugs is also discussed.

L-Dopa (L-3,4-dihydroxyphenylalanine) is still generally accepted as the first drug of choice in the management of Parkinsonism. Long-term therapy with L-Dopa is, however, associated with a number of therapeutic problems.[2,3] The most serious limitations of L-Dopa can be summarized as follows: poor bioavailability, wide range of interpatient variations of plasma levels, unpredictable therapeutic response, and various side effects. The main factors responsible for these problems are the physical–chemical properties of the drug substance: low water solubility resulting in incomplete dissolution at and prior to the absorption site, low lipid solubility resulting in unfavorable partition, and the high susceptibility of the drug molecule to chemical and enzymatic degradation.

L-Dopa is usually administered orally and, in man as in dog, the material in solution appears to be well adsorbed, primarily in the small bowel by a special carrier transport mechanism. But, in fact, the drug is extensively metabolized in the gastrointestinal tract and/or during its first passage through the liver, so that relatively little arrives in the blood as intact L-Dopa. This metabolism of L-Dopa is unfavorable to its therapeutic intent.[4] Also, individual differences in the degree of breakdown during the passage of the drug through the gastrointestinal tract may be responsible for the highly variable blood levels observed in patients receiving similar doses.[5]

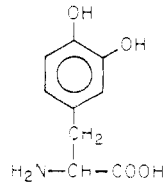
The peripheral side effects appear to be due to one or more biotransformation products rather than L-Dopa itself. Eighteen metabolites of L-Dopa were detected in the urine,[7] but the major metabolism during and prior to absorption involves primarily decarboxylation and conjugation. L-Aromatic amino acid decarboxylase has a high activity in the gastric mucosa.[8] Also, conjugation of L-Dopa and its metabolites takes place predominantly in the gastrointestinal–hepatic system.[9] L-Dopa is rapidly and continuously metabolized in blood, since only 5–8% of it is protein-bound, making it very susceptible to metabolic processes.[5] Finally, the remaining intact L-Dopa is rapidly taken up by the brain and localized in the caudate nucleus.[10,11]

Although peripheral DC inhibitors, such as L-α-methyl-Dopa hydrazine, reduced the L-Dopa requirements by up to 80%, a number of other problems related to L-Dopa therapy are still unsolved, primarily the complex problem of bioavailability of the drug, including the dissolution–absorption–metabolism processes, prior to delivery to the blood. The other still existing major problem is the side effects caused by L-Dopa. Although a close correlation between some dyskinesias and plasma L-Dopa concentration has been found,[12] other forms of involuntary movements are not associated with high plasma L-Dopa concentrations.[6]

In order to improve the therapeutic value of L-Dopa, we have carried out a systematic study of transient derivatives (prodrugs) of the molecule aimed at solving one or more of the above problems. Based on the previous evaluation of the physical–chemical properties of L-Dopa, an ideal prodrug of L-Dopa should be soluble in water and in lipids, completely adsorbed from the gastrointestinal tract without any chemical degradation or metabolism, and thus deliver L-Dopa intact in the blood stream, at a reproducible therapeutic level. A circulating prodrug which is transformed to L-Dopa might also alter the peripheral metabolism of L-Dopa. In addition, a prodrug of L-Dopa coadministered with a decarboxylase inhibitor could combine the best aspects of these two approaches. But

for resolving the dissolution–absorption–metabolism problems prior and during absorption, the prodrug approach appeared to be the most promising.

In our studies, we have protected individually and in combination the three sensitive centers in the molecule: the carboxy function, the amino group, and the catechol system. Various prodrugs were thus obtained which were studied in vivo in dogs for the extent of L-Dopa delivery and for the major metabolites [dopamine (DA), homovanillic acid (HVA), and 3,4-dihydroxyphenylacetic acid (DOPAC)].



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Simple aliphatic esters of L-Dopa have been suggested as prodrugs,[13] although no biological studies were reported. Various structural modifications of L-Dopa, including some possible prodrugs, have not reportedly[14] led to any more useful dopamimetic agent. A study on the simple di- and tripeptides of L-Dopa has also appeared.[15] Although a number of peptides were synthesized, the authors did not apparently recognize the potential value and the prodrug character of the peptides, the importance of delivery of L-Dopa, the difference in distribution, and the site of cleavage of the peptides. Thus, no wonder that "no obvious structure–activity relationships were observed", and their conclusion was that "these compounds were acting mainly by a peripheral mechanism".[15]

Results and Discussions

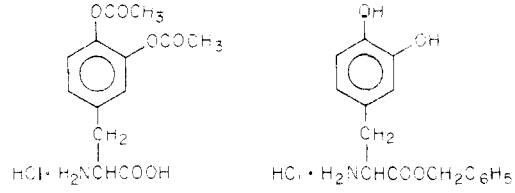
Chemistry. The selected individual protective groups used for the various reactive sites in L-Dopa were acetyl and pivalyl for the catechol, methyl and benzyl esters and N-terminal peptides for the carboxy, and formyl and C-terminal dipeptides for the amino group. The protective groups were also used in combinations for the synthesis of multiprotected prodrugs, which, as it will be shown, seems to provide the best overall result in terms of delivering intact L-Dopa to the blood stream.

The diacetyl- and dipivalyl-L-Dopa hydrochlorides were prepared by simple acid catalyzed esterification. L-Dopa was esterified to the methyl and benzyl ester, respectively, using modified versions of the thionyl chloride methods.[16] The esters were then acylated on the catechol hydroxy group under acidic conditions. N-Formylation of L-Dopa or its diacyl derivatives was performed according to the general procedure.[17] For the peptides of L-Dopa, the *N*-formyldiacetyl-L-Dopa and the diacetyl-L-Dopa esters were used, respectively. The use of the *N*-phthaloyl group for DL-Dopa peptides[18] and the carbobenzoxy group for L- and DL-Dopa peptides[19] was reported, but the formyl group is certainly the most advantageous, since we have found that it provides good yields, it is easy to handle, and it is easy to remove.

Bioavailability. The selected prodrugs were then tested for delivery of L-Dopa in vivo after oral administration to dogs. Although various physical–chemical properties (solubilities, partition coefficients, rate of hydrolysis, etc.) and in vitro tests (enzyme hydrolysis, diffusion through membranes, etc.) can generally be correlated with in vivo transport properties, in the complex case of L-Dopa, direct bioavailability measurements represent the only meaningful test for screening the L-Dopa prodrugs. It is generally accepted that the therapeutic effects of L-Dopa can be correlated with the blood L-Dopa concentration and with the blood dopamine (DA) content. Thus, for the preliminary screening of the prodrugs we determined both the L-Dopa and DA concentrations in the blood at various times after administration of equivalent doses. The results are summarized in Table I.

It is obvious that except for compound **4**, and perhaps **3**, the rest of the prodrugs result in significantly higher L-Dopa bioavailability and a much more favorable plasma L-Dopa–DA ratio. The overall results are obviously a combination of various actions: changes in water and lipid solubilities, in transport properties, and in metabolism.

Based on the bioavailability, peak level, and L-Dopa–DA ratio, we selected two simple derivatives (**2** and **7**) for further studies, along with three dipeptide derivatives of L-Dopa. The dipeptides were selected on the following basis: since both C- and N-terminal L-Dopa peptides (**9** and **11**) resulted in almost similar delivery, the more advantageous dipeptides would be composed of L-Dopa molecules. From the points of view of stability and of lipid solubility, it is also advantageous to maintain the acetylated catechol system but is unclear whether an esterified peptide (C-terminal acid ester) is better or not, although **10** does seem to give better overall results than **9** and **11**. Thus, the following structures were taken for further studies.



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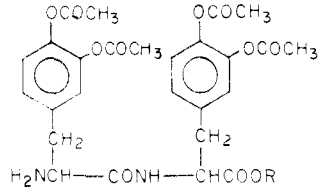
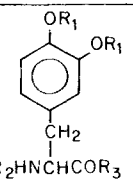


12, R = H
13, R = CH_3
14, R = $CH_2C_6H_5$

Crossover bioavailability studies on six dogs were carried out for **2**, **7**, **12**, and **13** at two dose levels. The concentrations in plasma of L-Dopa and DA, respectively, were determined. In addition, the major metabolites in the urine were also determined. Preliminary toxicity studies were also carried out (LD_{50} and acute oral toxicity in mice). Absorption characteristics of compounds **2**, **7**, **12**, and **13** are compared to that of L-Dopa in Table II. The corresponding L-Dopa and total dopamine (T-DA) blood levels vs. time are shown as Figures 1–5. The protected dipeptides containing two L-Dopa moieties do, indeed, result in a superior delivery of L-Dopa. The corresponding metabolites excreted in the urine in 24 h are summarized in Table III. The metabolic pattern of the prodrugs is remarkably close to that of L-Dopa. This is really not a surprise, since the drug is so extensively metabolized. For this kind of drug, the bioavailability as reflected by the plasma levels and AUC is more important than the physiological availability shown by the metabolites.

In a separate set of experiments, the dipeptide benzyl ester **14** was compared to the methyl ester **13** and L-Dopa. The absorption characteristics are shown in Table IV. The blood level curves of **13** and **14** are compared indi-

Table I. Bioavailability of L-Dopa Delivered Orally as Its Prodrugs to Beagle Dogs[a]



No.	R_1	R_2	R_3	L-Dopa in blood[b] AUC, (μg h)/mL	L-Dopa in blood[b] t_{max}, h	L-Dopa in blood[b] C_{max}, μg/mL	DA in blood[b] AUC, (μg h)/mL	DA in blood[b] t_{max}, h	DA in blood[b] C_{max}, μg/mL	R^c (ratio)
1	H	H	OH	2.22 ± 0.09	1.31 ± 0.09	1.21 ± 0.05	3.24 ± 0.33	1.57 ± 0.17	1.02 ± 0.11	0.69
2	CH_3CO	H·HCl	OH	4.85 ± 0.15	0.83 ± 0.17	3.42 ± 0.40	3.50 ± 0.50	1.70 ± 0.20	0.98 ± 0.30	1.38
3	$(CH_3)_3CCO$	H·HCl	OH	3.17 ± 0.06	1.17 ± 0.17	1.33 ± 0.21				
4	$(CH_3)_3CCO$	HCO	OK	0.77 ± 0.07	1.17 ± 0.17	0.57 ± 0.12	0.37 ± 0.04	1.67 ± 0.17	0.2 ± 0.01	1.35
5	H	H·HCl	OCH_3	3.43 ± 0.66	0.67 ± 0.17	2.08 ± 0.37	2.78 ± 0.12	2.17 ± 0.44	0.85 ± 0.07	1.23
6	CH_3CO	H·HCl	OCH_3	3.68 ± 0.40	1.0 ± 0.0	2.19 ± 0.14	2.09 ± 0.60	1.67 ± 0.17	0.79 ± 0.23	1.68
7	H	H·HCl	$OCH_2C_6H_5$	3.96 ± 0.73	0.5 ± 0.0	3.95 ± 0.48	2.12 ± 0.39	1.00 ± 0.29	0.83 ± 0.14	1.87
8	CH_3CO	H·HCl	$OCH_2C_6H_5$	4.06 ± 0.18	0.83 ± 0.17	3.18 ± 0.40	3.23 ± 0.41	1.83 ± 0.17	1.06 ± 0.05	1.28
9	CH_3CO	HCl·NH_2CH_2CO-	OH	3.28 ± 0.27	0.75 ± 0.25	2.36 ± 0.12	1.83	1.50	0.82	1.39
10	CH_3CO	HCl·NH_2CH_2CO-	OCH_3	3.66 ± 0.05	0.83 ± 0.17	2.29 ± 0.08	2.57 ± 0.48	1.67 ± 0.17	0.89 ± 0.13	1.42
11	CH_3CO	H·HCl	$NHCH_2COOH$	3.51 ± 0.44	1.0 ± 0.0	2.67 ± 0.36	3.14 ± 0.20	2.0 ± 0	1.06 ± 0.03	1.31

[a] L-Dopa equivalents (100 mg) were given orally. The values obtained represent the average of three dogs [except L-Dopa (1) where the values are the average of 16 dogs]. Standard errors are given. [b] AUC = area under the curve. t_{max} = time to reach peak L-Dopa or DA levels, respectively. C_{max} = peak concentrations. [c] R = (L-Dopa area under the curve)/(DA area under the curve).

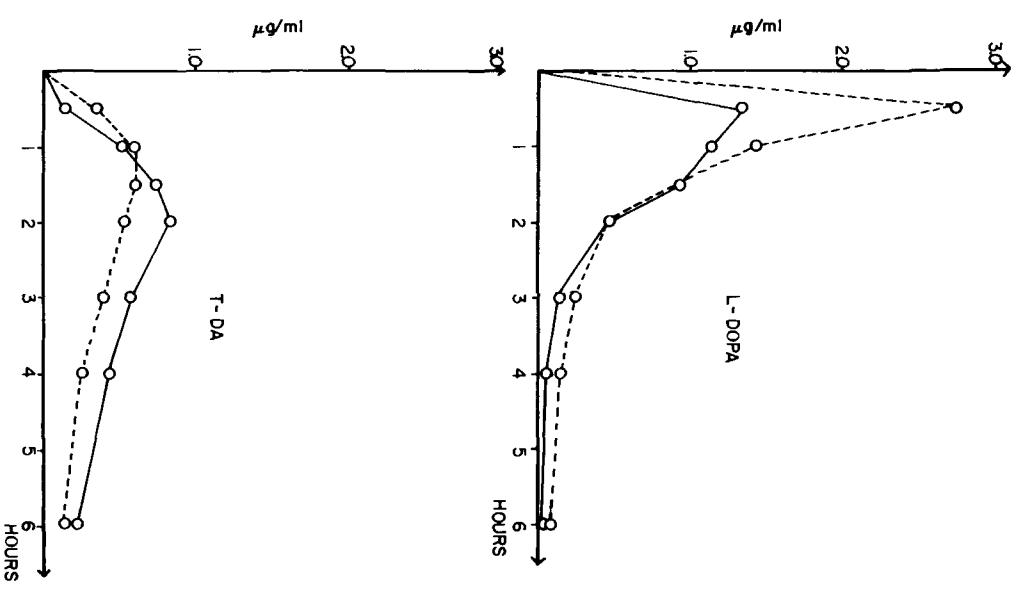


Figure 1. L-Dopa and T-DA blood levels after administration of 100 mg equiv of L-Dopa (1, O—O) and the benzyl ester 7 (O---O). Crossover test on six dogs.

vidually to L-Dopa on Figures 6 and 7. Besides resulting in a significantly higher L-Dopa delivery, both dipeptides are characterized by a highly favorable L-Dopa–T-DA ratio, which is the true measure of the L-Dopa delivery vs. peripheral metabolism.

Interestingly, the metabolic products excreted in the urine during 48 h differ to some extent from the previous studies in a higher T-DA and lower DOPAC (3,4-dihydroxyphenylacetic acid) concentration, resembling more an iv administration, as shown in Table V. In this case, again, the set of experiments is self-consistent in that the metabolism of the L-Dopa and that of the prodrugs is essentially identical. The metabolic pattern also supports our observations that no racemization during dipeptide formation occurs. Any D-Dopa present would significantly alter the excreted Dopa and DA amounts.[7]

The oral LD_{50} values in mice of the selected prodrugs were also determined. The results are given in Table VI. The benzyl ester **7** is the only derivative which appears to be more toxic than L-Dopa itself. It also caused cataract-like changes in the eyes of the animals. On the other hand, the diacetyl derivative **2** showed an unusually steep dose–response curve (Figure 8). The dose–response curves of the dipeptides **13** and **14**, however, are very close to that of L-Dopa (Figure 9). The acute intoxication symptoms were also different from that of L-Dopa. Some of the locomotion (hyperactivity) and excitation (convulsions, tremors, biting, jumping) scores were significantly smaller with the prodrugs as compared to L-Dopa, when all

Table II. Absorption Characteristics of L-Dopa and Its Selected Prodrugs (Crossover Studies Using Six Beagle Dogs)[a]

Compd (dose)[b]	Maximum plasma levels		Area under the curve, (μg h)/mL		
	L-Dopa, μg/mL	t_{max}, h	L-Dopa	T-DA	L-Dopa/T-DA
L-Dopa (1) (100 mg)	1.98 ± 0.28	0.88 ± 0.15	2.53 ± 0.24	2.80 ± 0.32	0.90 ± 0.12
L-Dopa (1) (50 mg)	0.85 ± 0.12	0.75 ± 0.16	0.95 ± 0.10	1.42 ± 0.10	0.67 ± 0.10
Diacetyl 2 (100 mg)	3.11 ± 0.30	0.92 ± 0.08	4.32 ± 0.52	3.43 ± 0.44	1.33 ± 0.21
Diacetyl 2 (50 mg)	1.75 ± 0.21	0.67 ± 0.11	1.30 ± 0.16	1.14 ± 0.07	1.16 ± 0.15
Benzyl ester 7 (100 mg)	2.84 ± 0.48	0.58 ± 0.10	3.33 ± 0.41	1.95 ± 0.16	1.72 ± 0.18
Benzyl ester 7 (50 mg)	1.57 ± 0.32	0.75 ± 0.17	1.70 ± 0.13	1.53 ± 0.13	1.15 ± 0.15
Dipeptide 12 (100 mg)	2.94 ± 0.38	0.75 ± 0.17	3.09 ± 0.22	2.50 ± 0.09	1.25 ± 0.10
Dipeptide 13 (100 mg)	2.99 ± 0.18	0.75 ± 0.17	3.64 ± 0.21	2.67 ± 0.14	1.42 ± 0.25

[a] The dogs were fasted for 16 h. The compounds were administered with 10 mL of tap water. Each point is the average of six dogs, except L-Dopa, which was administered to 12 dogs. [b] In L-Dopa equivalent.

Table III. Major Metabolites Excreted in 24 h in the Urine of Dogs Administered Orally L-Dopa and Its Prodrugs[a]

Compd (dose)	Percent of dose and SE				
	L-Dopa	T-DA	DOPAC	HVA	Total
1 (100 mg)	0.42 ± 0.04	10.86 ± 1.01	12.34 ± 1.18	36.20 ± 1.76	59.82 ± 1.68
1 (50 mg)	0.29 ± 0.04	15.07 ± 1.39	10.13 ± 0.84	36.25 ± 1.28	59.74 ± 1.72
2 (100 mg)	0.67 ± 0.10	10.20 ± 1.41	11.57 ± 0.83	34.57 ± 2.12	57.00 ± 2.00
2 (50 mg)	0.35 ± 0.04	11.28 ± 1.01	11.20 ± 1.01	38.00 ± 1.80	60.80 ± 2.20
7 (100 mg)	0.58 ± 0.07	10.43 ± 0.58	11.65 ± 0.94	34.25 ± 2.45	56.91 ± 2.89
7 (50 mg)	0.38 ± 0.03	11.17 ± 0.57	10.78 ± 0.58	35.75 ± 2.00	58.06 ± 1.92
12 (100 mg)	0.52 ± 0.09	12.83 ± 1.22	10.97 ± 1.10	33.70 ± 2.25	58.02 ± 2.75
13 (100 mg)	0.61 ± 0.13	11.65 ± 0.89	12.20 ± 1.86	34.27 ± 2.07	58.90 ± 2.60

[a] See footnote a, Table II.

Table IV. Absorption Characteristics of L-Dopa and Its Catechol- and Carboxy-Protected Dipeptides (Crossover Studies on Six Beagle Dogs)[a]

Compd (dose)[b]	Peak values			Areas under the curve		
	L-Dopa, μg/mL	T-DA, μg/mL	Ratio	L-Dopa, (μg h)/mL	T-DA, (μg h)/mL	Ratio
L-Dopa (1)	1.40 ± 0.24	0.89 ± 0.08	1.57 ± 0.10	1.14 ± 0.10	2.36 ± 0.32	0.48 ± 0.05
Dipeptide 13 (50 mg)	1.60 ± 0.22	0.75 ± 0.10	2.37 ± 0.42	1.56 ± 0.09	2.25 ± 0.25	0.72 ± 0.05
Dipeptide 14 (50 mg)	1.59 ± 0.10	0.66 ± 0.12	2.75 ± 0.44	1.51 ± 0.05	2.09 ± 0.32	0.81 ± 0.12

[a] The dogs were fasted for 18 h. The compounds were administered in capsules with 10 mL of tap water. The L-Dopa values represent an average of 12 experiments. [b] In L-Dopa equivalent.

Table V. Average Urinary Excretion of L-Dopa and Its Metabolites after Oral Administration of L-Dopa and Dipeptides 13 and 14

Compd (50-mg dose)	Percent of dose and SE				
	L-Dopa	T-DA	DOPAC	HVA	Total
L-Dopa (1)	0.63 ± 0.10	22.0 ± 1.85	1.85 ± 0.3	38.6 ± 3.30	63.1 ± 4.30
Dipeptide 13	1.4 ± 0.10	20.0 ± 0.8	2.6 ± 0.7	40.5 ± 2.6	64.1 ± 2.6
Dipeptide 14	1.2 ± 0.15	19.2 ± 1.7	1.14 ± 0.3	40.8 ± 4.99	62.4 ± 5.79

Table VI. LD_{50} Values of L-Dopa and Its Prodrugs in Male Mice

Compd	LD_{50} values, mg/kg	
	Derivatives	Conversion values as L-Dopa
Dipeptide 13	5300 (4600-6100)[a]	3600 (3100-4100)[a]
Dipeptide 14	5500 (5000-6100)	3200 (2900-3600)
Diacetyl 2	7800 (7200-8400)	4800 (4500-5200)
Benzyl ester 7	4300 (3300-5700)	2600 (2000-3500)
L-Dopa (1)		3300 (2600-4100)

[a] The values in parentheses indicate 95% confidence limits.

compounds were administered at the level of their corresponding LD_{50} values.

Very interesting information about the value of the prodrugs was obtained when the dipeptide 13 was compared to L-Dopa in an effervescent enteric coated formulation,[20] which protects L-Dopa from degradation in the stomach, while quickly releasing it in the small intestine. As shown in Table VII and Figure 8, the prodrug is superior even to the "superenteric" L-Dopa formulation, which suggests that besides protection in the gastrointestinal tract, the prodrug provides protection against metabolism during absorption and/or enhances the ab-

Table VII. Comparison of the Absorption Characteristics of L-Dopa and Its Dipeptide 13 in Effervescent Enteric Coated Formulation[a]

Compd[b]	Peak level			Area under the curve		
	L-Dopa, μg/mL	T-DA, μg/mL	Ratio	L-Dopa, (μg h)/mL	T-DA, (μg h)/mL	Ratio
L-Dopa (1)	1.82 ± 0.24	0.74 ± 0.06	2.58 ± 0.44	1.69 ± 0.33	2.13 ± 0.16	0.94 ± 0.16
Dipeptide 13	2.25 ± 0.15	0.65 ± 0.03	3.43 ± 0.31	2.43 ± 0.22[c]	1.92 ± 0.11	1.29 ± 0.16[c]

[a] Each value is the average of five dogs. [b] Dose, 50 mg of L-Dopa equivalent. [c] Significant at $p < 0.05$.

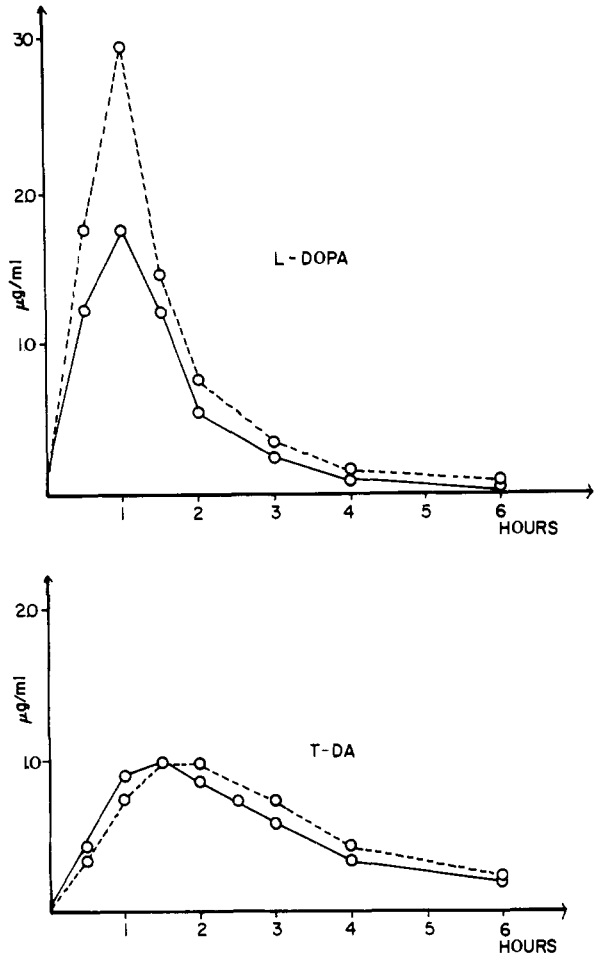


Figure 2. L-Dopa and T-DA blood levels after administration of 100 mg equiv of L-Dopa (1, O—O) and the diacetyl derivative 2 (O---O). Crossover test on six dogs.

sorption of L-Dopa possibly by increasing the passive transport contribution. The corresponding plasma levels with the expected delayed peaking are shown in Figure 10.

In conclusion, the prodrug approach provides an extremely useful way for improving delivery of L-Dopa. The protected dipeptides seem to promise significant improvements in the therapeutic value of L-Dopa.

Experimental Section

Synthesis. All melting points were uncorrected. Microanalyses were performed by Midwest Microlab, Ltd., Indianapolis, Ind., and all analyses were within ±0.4%. TLC were run on Brinkmann polygram sil G/uv$_{254}$. NMR spectra were run on a Varian T-60 spectrometer using $(CH_3)_4Si$ or the sodium salt of trimethylsilylpropanesulfonic acid as internal standard. Optical rotations were obtained using a Carl Zeiss instrument. Commercially available Sankyo L-Dopa was used for all the syntheses. Unless otherwise specified, all other chemicals were obtained from Aldrich Chemical Co., Inc.

Preparation of 3,4-Diacetyloxy-L-phenylalanine Hydrochloride (2). 3,4-Dihydroxy-L-phenylalanine (1, 50 g, 0.025 mol) was dissolved in 1.5 L of glacial acetic acid at 100 °C while gaseous HCl was introduced through the mixture. The resulting clear solution was cooled to 45 °C and 130 mL (143 g, 1.82 mol) of acetyl chloride was slowly added. After 16 h, the reaction mixture was added to 3 L of anhydrous ether and the precipitate formed was filtered and dried in vacuo to give 59.4 g (mp 184–184.5 °C, 75% yield) of 2: NMR (D_2O) δ 7.35–7.1 (m, 3, aromatic H), 4.45–4.15 (m, 1, C*H*NH_3^+), 3.4–3.0 (m, 2, $CH_2C_6H_3$–), and 2.36 (s, 6, CH_3C=O); $[\alpha]^{24}_D$ −8.9° (*c* 9.5, CH_3OH). Anal. Calcd for $C_{13}H_{16}ClNO_6$: C, 49.14; H, 5.08; N, 4.41. Found: C, 48.80; H, 5.22; N, 4.31.

Preparation of 3,4-Dipivaloxy-L-phenylalanine Perchlorate (3). 1 (20 g, 0.01 mol) was suspended in 240 mL of ethyl acetate. The suspension was heated to 70 °C and then 10 mL of 70% $HClO_4$ was added slowly, resulting in a clear solution.

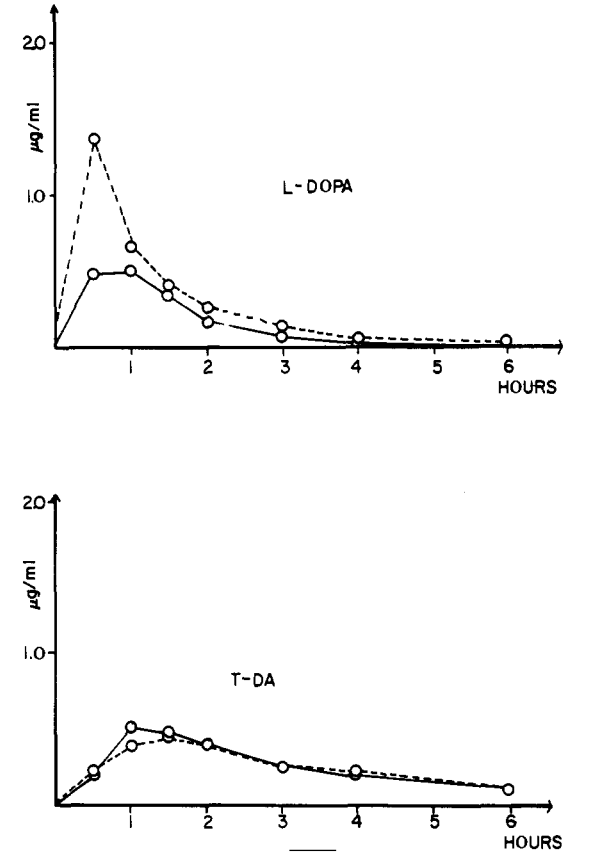


Figure 3. L-Dopa and T-DA blood levels after administration of 50 mg equiv of L-Dopa (1, O—O) and the benzyl ester 7 (O---O). Crossover test on six dogs.

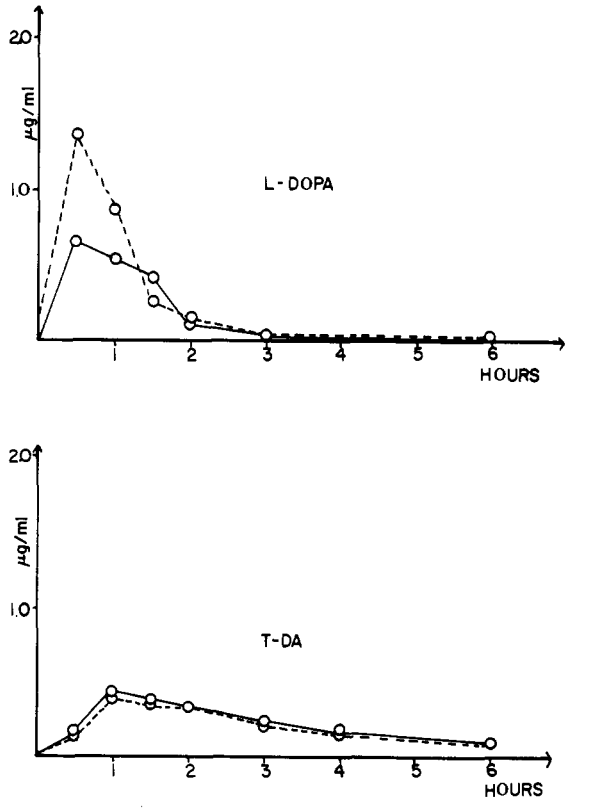


Figure 4. L-Dopa and T-DA blood levels after administration of 50 mg equiv of L-Dopa (1, O—O) and the diacetyl derivative 2 (O---O). Crossover test on six dogs.

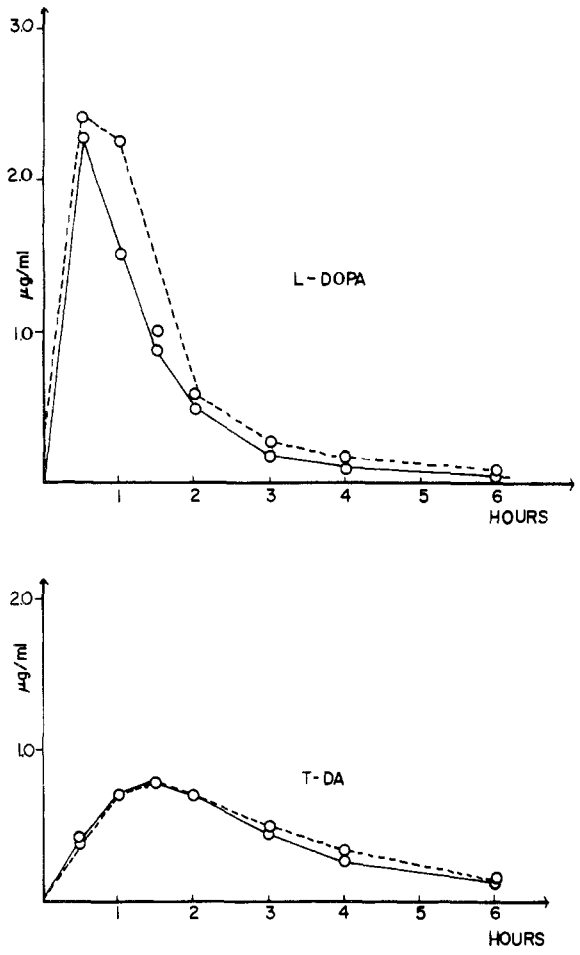


Figure 5. L-Dopa and T-DA blood levels after administration of 100 mg equiv of the dipeptide **12** (O—O) and the dipeptide **13** (O- - -O). Crossover test on six dogs.

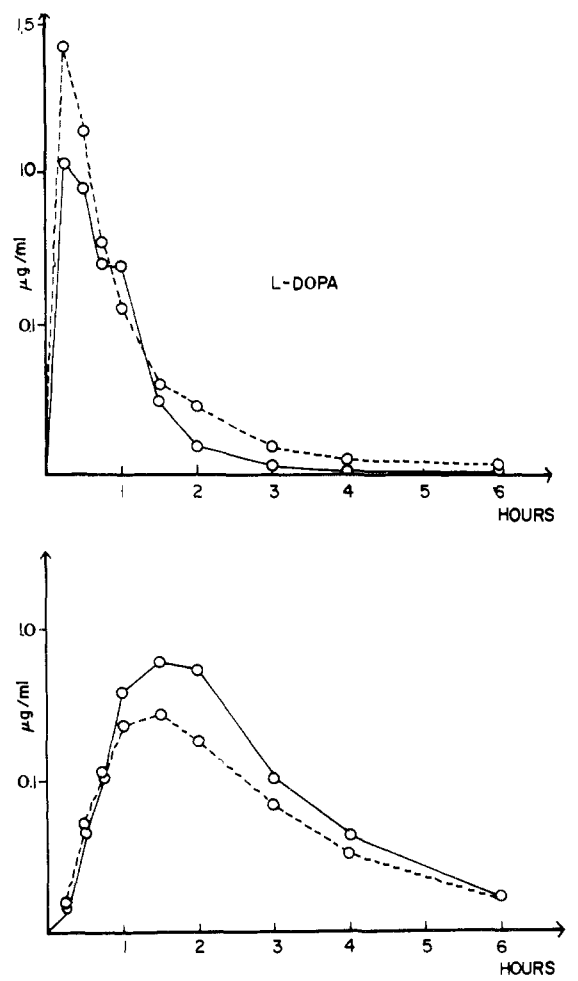


Figure 6. L-Dopa and T-DA blood levels after administration of 50 mg equiv of L-Dopa (**1**, O—O) and the dipeptide **13** (O- - -O). Crossover test on six dogs.

Pivalyl chloride (200 mL) was added to the solution which was heated for an additional 0.5 h at 70 °C and then allowed to cool to room temperature. The reaction mixture was then concentrated in vacuo and the residue was triturated with ether (600 mL) three times. There was thus obtained 32 g (mp 176–178 °C dec, 67% yield) of **3** as a white powder: NMR (Me_2SO-d_6) δ 7.3–7.15 (m, 3, aromatic H), 4.4–4.1 (m, 1, $CHNH_3^+$), 3.3–3.0 (m, 2, $CH_2C_6H_3$–), and 1.33 [m, 18, $(CH_3)_3CC{=}O$]; $[\alpha]^{25}_D$ –7.5° (*c* 13.1, CH_3OH). Anal. Calcd for $C_{19}H_{28}ClNO_{10}$: C, 48.98; H, 6.06; N, 3.01. Found: C, 48.80; H, 5.74; N, 2.79.

Preparation of *N*-Formyl-3,4-diacetyloxy-L-phenylalanine Potassium Salt (4). **2** (41.4 g, 0.13 mol) was dissolved in a mixture of CH_3OH and CH_2Cl_2 (90:1200) and allowed to react with 13.5 g (0.13 mol) of $(C_2H_5)_3N$ dissolved in 100 mL of CH_2Cl_2. The resulting gelatinous mass was stirred for 1 h and filtered. The residue was resuspended in 1.2 L of dichloromethane for 1 h and filtered again. The residue was dried in vacuo for 3 h at 60 °C, then crushed to a powder, and resuspended in 1.2 L of CH_2Cl_2. The suspension was filtered and the residue was dried in vacuo to give 34 g (mp 179–183 °C, 93% yield) of 3,4-diacetyloxy-L-phenylalanine (**15**) as a white powder. **15** (68.4 g, 0.24 mol) was added to a cold (0 °C) solution of CH_3CO_2OCH prepared from 32.5 mL of acetic anhydride and 16.2 mL of formic acid. The reaction mixture, which had solidified after about 10 min, was suspended in 300 mL of acetone at room temperature for 3 h and then the suspension was filtered to give, after drying in vacuo, 7.2 g (mp 133–136 °C) of *N*-formyl-3,4-diacetyloxy-L-phenylalanine (**16**) as a white powder. The filtrate was stirred vigorously and diluted with 300 mL of hexane which resulted in the precipitation of another 57.8 g (mp 132–135 °C) of **16** for a total yield of 86%: TLC (silica gel, acetone) R_f 0.54; NMR (Me_2SO-d_6) δ 8.5–8.2 (m, 1, NH), 7.97 (s, 1, NCH=O), 7.25–7.0 (m, 3, aromatic H), 3.15–2.8 (m, 2, $CH_2C_6H_3$–), and 2.23 (s, 6, $CH_3C{=}O$); $[\alpha]^{24}_D$ +54.2° (*c* 9.3, CH_3OH). Anal. Calcd for $C_{14}H_{14}NO_7$: C, 54.37; H, 4.89; N, 4.53. Found: C, 54.49; H, 4.97; N, 4.37.

16 (2 g, 0.005 mol) was converted to its potassium salt **4** by allowing it to react with 5 mL of 1 N KOH in CH_3OH and then evaporating the solution in vacuo to give **4** as a white powder. Anal. Calcd for $C_{14}H_{14}NO_7K$: C, 48.41; H, 4.06; N, 4.03. Found: C, 48.83; H, 4.58; N, 4.08.

Preparation of 3,4-Dihydroxy-L-phenylalanine Methyl Ester Hydrochloride (5). To 500 mL of CH_3OH at –5 °C was added, with stirring and cooling, 50 mL of $SOCl_2$ at such a rate that the temperature of the reaction mixture did not reach 10 °C. The clear, colorless solution was cooled to –5 °C and 101 g (0.51 mol) of **1** was added. After 1 min, the resulting clear solution was refluxed gently overnight, protected from atmospheric moisture. The solution was concentrated in vacuo at an oil bath temperature not exceeding 75 °C until the entire reaction residue was a foamy white solid. Absolute ethanol (450 mL) was added and the temperature of the oil bath was raised to 80 °C at which point all of the solid was dissolved. The ethanol solution was cooled to room temperature and titrated with 55 mL of ether, and the clear solution was cooled in the refrigerator overnight. More ether was added to the ethanol solution in portions followed by cooling in an ice bath until another 845 mL of ether had been added. The resulting precipitate was filtered and dried in a vacuum oven to give 115.3 g (mp 170.5–171.5 °C, 91% yield) of the methyl ester hydrochloride **5** as a white solid: TLC [silica gel, ethanol–diethyl ether (1:3)] R_f 0.41; IR (KBr) 1740 cm^{-1} (s, C=O); NMR (D_2O) δ 7.1–6.6 (m, 3, aromatic H), 4.5–4.3 (m, 1, $CHNH_3^+$), 3.56 (s, 3, CH_3O), and 3.35–3.0 (m, 2, $CH_2C_6H_3$–); $[\alpha]^{22}_D$ +14.7° (*c* 12.5, CH_3OH). Anal. Calcd for $C_{10}H_{14}ClNO_4$: C, 48.48; H, 5.70; N, 5.66. Found: C, 48.77; H, 5.75; N, 5.38.

Preparation of 3,4-Diacetyloxy-L-phenylalanine Methyl Ester Hydrochloride (6). **5** (90 g, 0.36 mol) was dissolved in glacial acetic acid (500 mL) heated at 100 °C. The solution was

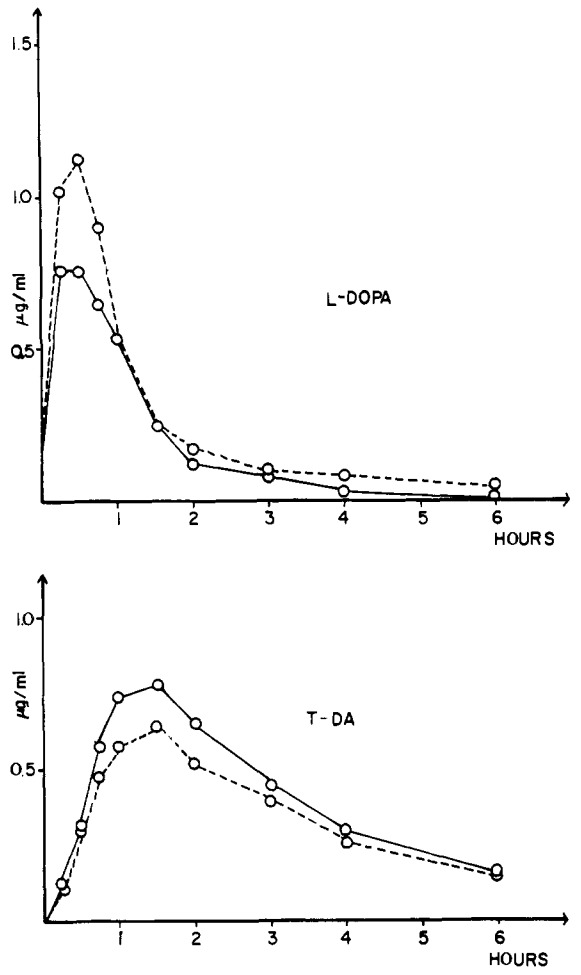


Figure 7. L-Dopa and T-DA blood levels after administration of 50 mg equiv of L-Dopa (1, O—O) and the dipeptide 14 (O---O). Crossover test on six dogs.

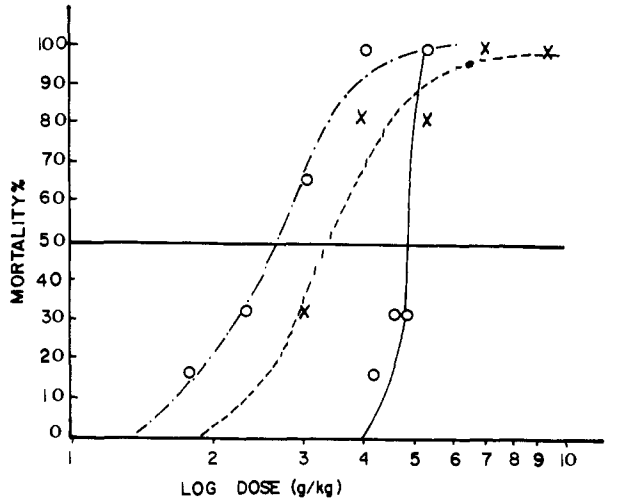


Figure 8. Dose–response curves of acute oral toxicity in male mice: (—O—) diacetyl derivative **2**; (-·O·-) benzyl ester **7**; (---X---) L-Dopa (1).

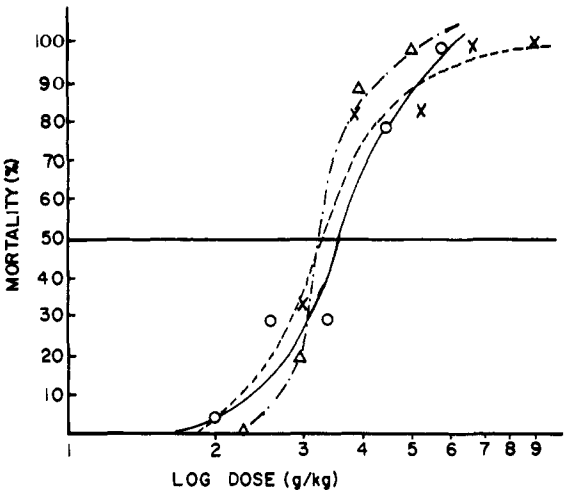


Figure 9. Dose–response curves of acute oral toxicity in male mice: (—O—) dipeptide 13; (-·Δ·-) dipeptide 14; (-·X·-) L-Dopa (1).

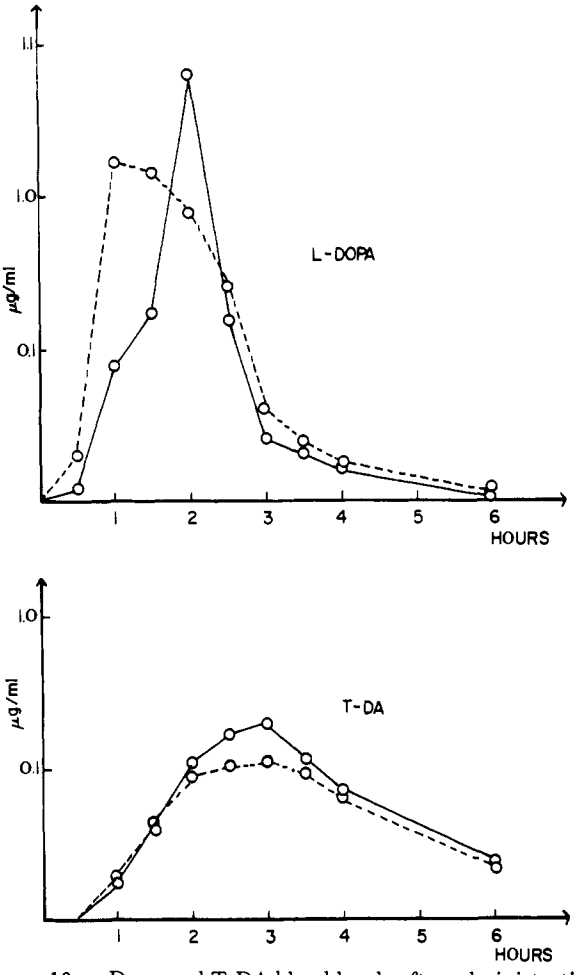


Figure 10. L-Dopa and T-DA blood levels after administration of 50 mg equiv of L-Dopa (1, O—O) and the dipeptide 13 (O---O), both formulated as effervescent enteric coated tablets. Crossover test on five dogs.

cooled to 40 °C while HCl was bubbled through the solution; then 330 mL (363 g, 4.66 mol) of acetyl chloride was added slowly to the solution. The resulting mixture was allowed to cool to room temperature overnight. Ether (1 and 2 L) was added in two portions to the reaction mixture and the precipitate that resulted was filtered, washed with ether, and dried in vacuo at 85 °C for 5 h to give 112.4 g (mp 182–182.5 °C, 94% yield) of **6** as a white solid: NMR (D_2O) δ 7.4–7.15 (m, 3, aromatic H), 4.6–4.4 (m, 1, C*H*NH_3^+), 3.56 (s, 3, CH_3O), 3.55–3.2 (m, 2, $CH_2C_6H_5$–), and 2.43 (s, 6, $CH_3C{=}O$); $[\alpha]^{25}_D$ +7.5° (*c* 14.2, CH_3OH). Anal. Calcd for $C_{14}H_{18}ClNO_6$: C, 50.68; H, 5.47; N, 4.22. Found: C, 50.45; H, 5.67; N, 4.09.

Preparation of 3,4-Dihydroxy-L-phenylalanine Benzyl Ester Hydrochloride (7). 1 (50 g, 0.25 mol) was dissolved in 150 mL of 6 N HCl. Benzyl alcohol (300 mL) was added and the biphasic mixture was heated at 50 °C until a homogeneous solution was obtained. The solution was distilled to remove the water; then the solution was cooled to room temperature and HCl was bubbled through the solution for 15 min. The above procedure

was repeated twice more. After the solution had cooled overnight, it was triturated with ether (600 mL) and filtered. The residue was washed thoroughly with ether; then it was suspended in boiling acetone (300 mL) and methanol was added until a solution was obtained. Isopropyl ether was added until the solution was turbid. The precipitate was filtered to give 57.6 g (mp 190.5–191 °C, 71% yield) of **7** as white crystals: TLC (silica gel, THF) R_f 0.73; NMR (Me_2SO-d_6) δ 7.6–7.1 (m, 5, C_6H_5), 6.85–6.35 (m, 3, C_6H_3), 5.16 (s, 2, O=COC$H_2C_6H_5$), 4.35–4.0 (m, 1, CHNH_3^+), and 3.25–2.90 (m, 2, C$H_2C_6H_5$); $[\alpha]^{24}_D$ −9.5° (*c* 1.4, CH_3OH). Anal. Calcd for $C_{16}H_{18}ClNO_4$: C, 59.35; H, 5.60; N, 4.33. Found: C, 59.47; H, 5.32; N, 4.76.

Preparation of 3,4-Diacetyloxy-L-phenylalanine Benzyl Ester Hydrochloride (8). **7** (20 g, 0.06 mol) was dissolved in 400 mL of glacial acetic acid by heating the mixture to 110 °C and bubbling HCl through the solution. After solution was achieved, the solution was cooled to 40 °C and 90 mL of acetyl chloride was added. The next day, the solution was triturated with 2 L of ether and the precipitate was filtered to give 24 g (mp 190–191 °C, 95% yield) of **8** as white crystals: NMR (Me_2SO-d_6) δ 7.55–7.1 (m, 8, aromatic H), 5.16 (s, 2, O=COC$H_2C_6H_5$), 4.5–4.0 (m, 1, CHNH_3^+), 3.45–3.0 (m, 2, C$H_2C_6H_3$), and 2.33 (s, 6, CH_3C=O); $[\alpha]^{25}_D$ −13.9° (*c* 8.1, CH_3OH). Anal. Calcd for $C_{20}H_{22}ClNO_6$: C, 58.89; H, 5.44; N, 3.43. Found: C, 58.87; H, 5.60; N, 3.30.

Preparation of Carbobenzyloxyglycyl-3,4-diacetyloxy-L-phenylalanine Benzyl Ester (17). A CH_2Cl_2 (100 mL) solution containing 0.95 g (9.4 mmol) of triethylamine was allowed to react with 3.64 g (8.92 mmol) of **8** for 0.3 h at room temperature; then the solution was concentrated in vacuo at room temperature to dryness. The residue was suspended in ether, filtered, and washed with ether to give 1.54 g of triethylamine hydrochloride contaminated with 3,4-diacetyloxy-L-phenylalanine benzyl ester (**18**) in various stages of deprotection; for instance, 3,4-dihydroxy-L-phenylalanine benzyl ester was obtained quantitatively if a large excess of triethylamine was used to neutralize the hydrochloride. The ether filtrate was concentrated in vacuo at room temperature and the residue was shown by NMR to be the desired **18**: NMR ($CDCl_3$) δ 7.5–6.7 (m, 2, NH_2), 7.33 (s, 5, $CH_2C_6H_5$), 7.0 [s, 3, $CH_2C_6H_3(OAc)_2$], 5.13 (s, 2, $CO_2CH_2C_6H_5$), 3.9–3.65 (m, 1, CHNH_2), 3.1–2.8 (m, 2, $C_6H_3CH_2$CH), and 2.26 (s, 6, CH_3CO_2). The residue was estimated to contain 8.15 mmol of **18**. The residue was dissolved in CH_2Cl_2 (50 mL) and carbobenzyloxyglycine (1.70 g, 8.15 mmol) and dicyclohexylcarbodiimide (DCC, 1.68 g, 8.15 mmol) were added to the solution in that order; a white precipitate of dicyclohexylurea (DCU) began to form immediately. The next day the suspension was filtered and the residue was washed with CH_2Cl_2 (50 mL) to give 1.55 g (85%) of DCU. The combined CH_2Cl_2 filtrates were washed with 10 mL each of 1 N HCl and water and then concentrated in vacuo to room temperature to give 4.6 g of an oily residue. The oily residue was chromatographed on silica gel (Mallinckrodt Silicar CC7) using ether as the eluent to give 2.00 g (44% yield) of a yellow oil (TLC, silica gel, acetone, R_f 0.63) whose NMR spectrum was consistent with that of **17**: NMR ($CDCl_3$) δ 7.33 (br s, 10, $C_6H_5CH_2O$), 7.4–6.65 [m, 4, $C_6H_3(OAc)_2$ and NHCO], 5.76 (m, 1, NHCO), 5.11 and 5.15 (2 s, 4, $C_6H_5CH_2O$), 5.0–4.7 (m, 1, NHCHCO$_2$), 3.9–3.65 (m, 2, COCH_2NH), 3.2–2.8 (m, 2, $C_6H_3CH_2$CH), and 2.23 (s, 6, CH_3CO_2). The oil would not crystallize in our hands and was used without further purification in the next step.

Preparation of Glycyl-3,4-diacetyloxy-L-phenylalanine Hydrochloride (9). **17** (2.48 g, 4.4 mmol) was dissolved in 120 mL of CH_3OH with 5 mL of glacial acetic acid. Then 10% Pd/C (0.5 g) was wetted with water (5 mL) and washed into the solution with water (~5 mL). The resulting suspension was shaken under H_2 (30 lb) for 24 h. The suspension was filtered and washed with CH_3OH (100 mL). The combined filtrates were concentrated in vacuo at room temperature. The residue was dissolved in 10 mL of glacial acetic acid saturated with HCl and treated with 10 mL of acetyl chloride in a tightly stoppered flask overnight. The solution was diluted to 125 mL with ether. After 2 h, the ether was decanted and the gummy residue was suspended in dry ether and left to stir overnight. The suspension was quickly filtered and the residue dried in vacuo at 60 °C to give 0.87 g (mp 95–118 °C) of **9**. Anal. Calcd for $C_{15}H_{19}ClN_2O_7$: C, 48.06; H, 5.11; N, 7.48. Found: C, 48.42; H, 5.38; N, 7.30.

Preparation of Glycyl-3,4-diacetyloxy-L-phenylalanine Methyl Ester Hydrochloride (10). A CH_2Cl_2 (150 mL) solution of **6** (4.3 g, 13 mmol) was allowed to react with 1.35 g (13 mmol) of $(C_2H_5)N$. After 10 min, 2.7 g (13 mmol) of carbobenzyloxyglycine was added to the CH_2Cl_2 solution and then 2.80 g (13 mmol) of DCC was added. After 2 h, the solution was filtered to give 2.62 g (90%) of DCU. The filtrate was extracted with 10 mL each of 1 N HCl and water. The CH_2Cl_2 layer was dried over Na_2SO_4 and concentrated in vacuo at room temperature to give 6.5 g of white solid: TLC (silica gel, acetone) R_f 0.42. The white solid was dissolved in CH_2Cl_2 (60 mL) and the solution was filtered and diluted to 220 mL with hexane. The next day, the solution was filtered and the residue was dried to give 5.5 g (mp 130–131 °C, 87% yield) of carbobenzyloxyglycyl-3,4-diacetyloxy-L-phenylalanine methyl ester (**19**). The mother liquor was concentrated (45 mL) to give an additional 233 mg (mp 129–131 °C, 3% yield) of **19**: NMR ($CDCl_3$) δ 7.28 (br s, 5, $CH_2C_6H_5$), 7.1–6.45 [m, 3, $CH_2C_6H_3(OAc)_2$], 5.7–5.4 (m, 1, NHCO), 5.10 (s, 2, $CH_2C_6H_5$), 5.0–4.65 (m, 1, NHCHCO$_2$), 3.9–3.7 (m, 2, COCH_2NH), 3.66 (s, 3, OCH_3), 3.2–3.0 (m, 2, $C_6H_3CH_2$CH), and 2.23 (s, 6, O_2CCH_3). Anal. Calcd for $C_{24}H_{26}N_2O_9$: C, 59.25; H, 5.39; N, 5.76. Found: C, 59.54; H, 5.39; N, 5.74.

19 (3.00 g, 6.17 mmol) was dissolved in CH_3OH (100 mL) and glacial acetic acid (5 mL). The solution was then shaken in a Parr apparatus for 16 h over 10% Pd/C (0.4 g) under H_2 (35 lb). The solution was filtered and concentrated in vacuo at room temperature. The residue was dissolved in 10 mL of glacial acetic acid saturated with HCl and treated with 10 mL of acetyl chloride. The solution was stirred at room temperature overnight in a tightly sealed reaction flask and then diluted to 125 mL with ether to give a light brown gum. The ether was decanted and the residue was suspended in anhydrous ether (100 mL). The suspension was broken up with a spatula and then allowed to stir until it became homogeneous. The suspension was filtered and the residue was dried in a vacuum oven at 60 °C to give 0.58 g (mp 80–100 °C, 24% yield from **19**) of **10**: NMR (D_2O) δ 7.25–6.9 (m, 3, C_6H_3), 3.66 (br s, 5, OCH_3 and O=CCH_2NH_2), 3.15–2.85 (m, 2, $C_6H_3CH_2$–), and 2.26 (s, 6, O_2CCH_3). Anal. Calcd for $C_{16}H_{21}ClN_2O_7$: C, 49.42; H, 5.44; N, 7.21. Found: C, 49.65; H, 5.50; N, 7.47.

Preparation of *N*-Formyl-3,4-diacetyloxy-L-phenylalanylglycine Benzyl Ester (20). **16** (4.0 g, 13 mmol) was added to a CH_2Cl_2 (200 mL) solution containing triethylamine (1.35 g, 13 mmol) and glycine benzyl ester *p*-toluenesulfonate (4.40 g, 13 mmol). The resulting suspension was vigorously shaken to get all of **16** into solution; then 2.80 g (13 mmol) of DCC was added. After 4.5 h, the reaction was filtered. The residue (2.41 g, 83% yield of DCU) was discarded and the filtrate was extracted with 10 mL each of water and 1 N HCl. The CH_2Cl_2 solution was then dried over Na_2SO_4 and concentrated in vacuo at room temperature. The residue was chromatographed on silica gel (Silicar CC-7) using acetone–ether (20:80–40:60) as eluent to give 2.33 (39% yield) of **20**: mp 120.5–121 °C; TLC [silica gel, ether–acetone (1:1)] R_f 0.46; IR (KBr) 3320 and 3280 (s, NH) and 1760 and 1730 cm^{-1} (s, C=O); NMR (acetone-d_6) δ 8.1 (s, 1, NCHO), 7.35 (s, 5, $C_6H_5CH_2O_2C$), 7.13 [s, 4, $C_6H_3(OAc)_2$], 8.0–6.8 (m, 2, NH), 5.13 (s, 2, $CH_2C_6H_5$), 5.0–4.6 (m, 1, O_2CCHNHCHO), 4.1–3.9 (m, 2, NHCH_2CO_2), 3.2–2.8 (m, 2, CH_2CHCO$_2$), and 2.23 (s, 6, CH_3CO_2). Anal. Calcd for $C_{23}H_{24}N_2O_8$: C, 60.52; H, 5.29; N, 6.14. Found: C, 60.25; H, 5.10; N, 6.01.

Preparation of 3,4-Diacetyloxy-L-phenylalanylglycine Hydrochloride (11). **20** (0.90 g, 2.0 mmol) was allowed to react with 2.2 mL of 1 N methanolic HCl (prepared by diluting 2 mL of concentrated HCl to 24 mL with CH_3OH overnight). Initially, a suspension formed and an additional 5 mL of CH_3OH was added to the suspension; after about 0.5 h, all of the solid was in solution. The solution was concentrated in vacuo at room temperature. The residue showed the absence of CH_3CO_2 and CHON upon analysis by NMR. The residue was dissolved in 60 mL of anhydrous CH_3OH to which 0.5 mL of glacial acetic acid had been added. The solution was shaken in a Parr shaker under an H_2 atmosphere over 0.1 g of 10% Pd/C overnight. The suspension was filtered to remove the Pd/C and concentrated in vacuo at room temperature. The residue was twice diluted with acetone (100 mL) and concentrated; then it was dissolved in glacial acetic acid

saturated with HCl (10 mL) and allowed to react with acetyl chloride (10 mL); a solid formed immediately but soon went back into solution. After 8 h at room temperature, the acetic acid solution was diluted to 125 mL with ether. The ether was decanted and the gummy dark residue that was left behind was suspended in ether (100 mL). The suspension was stirred overnight to give a fine white solid in suspension which was filtered quickly and dried in a vacuum desiccator to give 0.59 g (mp 120–217 °C, 78% yield) of 11: NMR (D_2O) δ 7.13 [s, 3, $C_6H_3(OAc)_2$], 3.25–2.95 (m, 2, $C_6H_3CH_2CH$), and 2.23 (s, 6, CH_3CO_2). An analytical sample was obtained by recrystallization from methanol–ether. Anal. Calcd for $C_{15}H_{19}ClN_2O_7$: C, 48.06; H, 5.11; N, 7.48. Found: C, 48.22; H, 5.34; N, 7.72.

Preparation of *N*-Formyl-3,4-diacetyloxy-L-phenylalanyl-3,4-diacetyloxy-L-phenylalanine Benzyl Ester (21). 16 (30.0 g, 0.097 mol) in dry THF (200 mL) was cooled with stirring in a dry ice–chloroform bath while 14.5 g (0.106 mol) of isobutyl chloroformate in THF (10 mL), which had been cooled with a dry ice–chloroform bath, was added dropwise. The cooled solution was stirred for about 2 min; then 9.79 g (0.097 mol) of *N*-methylmorpholine in THF (20 mL), which had also been cooled with the dry ice–chloroform bath, was added to the solution. Immediately, a precipitate formed. After 1 min, a THF (150 mL) solution of 8 (39.6 g, 0.097 mol), which had been previously neutralized by the addition of 9.79 g (0.097 mol) of *N*-methylmorpholine and which had been cooled with a dry ice–chloroform bath, was added to the first solution. The reaction mixture was then allowed to warm to room temperature (1 h), at which time it was filtered and the filtrate was evaporated in vacuo. The residue from the evaporation was dissolved in CH_2Cl_2 (500 mL) and extracted twice with water (50 mL). The CH_2Cl_2 layer was then dried over Na_2SO_4 and concentrated to dryness in vacuo. The residue was dissolved in 75 mL of hot acetone, ether was added until the solution became turbid, and the solution was cooled at room temperature for 24 h. The solution was filtered to give 43.1 g [mp 138.5–142.5 °C (67% yield); $[\alpha]^{25}_D$ +3.0° (*c* 5.6, CH_3OH)] of 21. Anal. Calcd for $C_{34}H_{34}N_2O_{12}$: C, 61.62; H, 5.17; N, 4.22. Found: C, 61.55; H, 5.20; N, 4.25.

Preparation of 3,4-Dihydroxy-L-phenylalanyl-3,4-dihydroxy-L-phenylalanine Hydrochloride (12). 21 (3.3 g, 5.0 mmol) was dissolved in 20 mL of CH_3OH and allowed to react with 5.4 mL of 1 N HCl in CH_3OH overnight. The solution was evaporated and the residue was dissolved in 60 mL of CH_3OH and 0.5 mL of glacial acetic acid. That solution was then hydrogenated over 0.2 g of 10% Pd/C in a Parr shaker overnight. The resulting solution was concentrated in vacuo and the residue was triturated with ether (50 mL). The suspension was filtered and the residue was recrystallized from methanol–ether to give 12 as light beige crystals. Anal. Calcd for $C_{18}H_{21}ClN_2O_7$: C, 52.37; H, 5.13; N, 6.78. Found: C, 52.19; H, 5.39; N, 6.68.

Preparation of *N*-Formyl-3,4-diacetyloxy-L-phenylalanyl-3,4-diacetyloxy-L-phenylalanine Methyl Ester (22). 6 (3.4 g, 10.3 mmol) was added to a CH_2Cl_2 (150 mL) solution containing 1.1 g (10.9 mmol) of triethylamine; all of the hydrochloride went into solution. After 10 min, 3.1 g (10 mmol) of 16 followed by 2.2 g (10.7 mmol) of DCC was added to the CH_2Cl_2 solution. The solution was stirred at room temperature for 2 h; DCU began precipitating immediately. The resulting suspension was filtered and the residue (1.85 g, 77% yield of DCU) was washed with CH_2Cl_2 and then discarded. The filtrate was extracted with 10 mL of 1 N HCl and then with 50 mL of water and was dried over Na_2SO_4 and concentrated in vacuo to give 6.9 g of a waxy solid. The solid was suspended in CH_2Cl_2 (30 mL) and filtered again to remove some additional DCU. The filtrate was diluted to turbidity with hexane (30 mL) and then with CH_2Cl_2 until just barely clear; a white precipitate formed immediately. The precipitate was separated by filtration to give 4.53 g (mp 153–156 °C, 78% yield) of the methyl ester 22. Anal. Calcd for $C_{28}H_{30}N_2O_{12}$: C, 57.33; H, 5.15; N, 4.78. Found: C, 57.63; H, 5.15; N, 4.77.

Preparation of 3,4-Diacetyloxy-L-phenylalanyl-3,4-diacetyloxy-L-phenylalanine Methyl Ester Hydrochloride (13). First, the completely protected methyl ester dipeptide 22 was deacylated. 22 (3.16 g, 0.0054 mol) was suspended in 10 mL of CH_3OH and allowed to react with 6 mL (0.006 mol) of 1 N methanolic HCl. After a few hours, the suspension dissolved to give a clear, yellow-colored solution. The solution was allowed to stir at room temperature for 48 h; then it was concentrated in vacuo at 60 °C to give 2.45 g of a hygroscopic orange solid, 3,4-dihydroxy-L-phenylalanyl-3,4-dihydroxy-L-phenylalanine methyl ester hydrochloride. Part of the intermediate (1.95 g) was dissolved in 15 mL of glacial acetic acid saturated with HCl and allowed to react with 10 mL of acetyl chloride in a tightly sealed flask for 24 h. The solution was diluted to 200 mL with dry ether and the resulting suspension was stirred for 24 h at room temperature. The ether was decanted from the gummy residue which was resuspended in 200 mL of dry ether and stirred at room temperature for 24 h. The suspension was filtered and the residue was washed with ether (50 mL) and dried in a vacuum oven at 50 °C for 2 h to give 2.10 g of 13 as a light yellow solid. Anal. Calcd for $C_{27}H_{31}ClN_2O_{11}$: C, 54.50; H, 5.25; N, 4.71. Found: C, 54.51; H, 5.41; N, 4.73.

Preparation of 3,4-Diacetyloxy-L-phenylalanyl-3,4-diacetyloxy-L-phenylalanine Benzyl Ester Hydrochloride (14). First, the benzyl ester dipeptide 21 was deacylated. 21 (60 g, 0.091 mol) was suspended in 500 mL of CH_3OH and 8.37 mL (0.1 mol) of concentrated HCl dissolved in 50 mL of CH_3OH was added to the well-stirred suspension. The suspension became homogeneous after 1.5 h. The resulting solution was stirred overnight (19 h) at room temperature; then it was concentrated to dryness in vacuo to give 3,4-dihydroxy-L-phenylalanyl-3,4-dihydroxy-L-phenylalanine benzyl ester hydrochloride as an orange-yellow solid. This solid was then partially reacylated by dissolving it in 120 mL of glacial acetic acid saturated with HCl and allowing it to react with 200 mL of acetyl chloride (which was added slowly with cooling) overnight in a tightly closed flask at room temperature. The solution was diluted to 3 L with ether and the resulting suspension was stirred for 4 h. The suspension was filtered to give 36.5 g (mp 167–168 °C; 60% yield; $[\alpha]^{25}_D$ −4.1°) of 14 as a very light yellow solid. Anal. Calcd for $C_{33}H_{35}ClN_2O_{11}$: C, 59.06; H, 5.26; N, 4.18. Found: C, 58.70; H, 5.50; N, 4.11.

The yellow solid can be recrystallized from acetone–methanol–ether to give 28 g [mp 173–173.5 °C; $[\alpha]^{27}_D$ −7.1° (*c* 7.0, CH_3OH)] of 14 as a white solid.

Biological Tests. Bioavailability and metabolism studies were performed on male Beagle dogs of 10–12 kg. The compounds were administered orally in capsules with 10 mL of water to the 16–18-h fasted dogs. Blood samples were withdrawn at various time intervals and analyzed for L-Dopa and T-DA and the urine samples were analyzed for L-Dopa and metabolites, as follows.

(A) Determination of L-Dopa and Total Dopamine in the Plasma. (1) Preparation of Plasma Samples. The blood (5 mL) was taken with a heparinized syringe from the front paw vein at the scheduled time (0.5, 1.0, 1.5, 2.0, 3.0, 4.0, and 6.0 h and at time of emesis) after oral administration of the drug. Sodium metabisulfite (0.1 mL of 10% physiological saline solution) and disodium ethylenediaminetetraacetate (Na_2EDTA) (0.1 mL of 1% physiological saline solution) were added to the freshly drawn heparinized blood. After gentle mixing, the plasma was immediately separated by centrifugation at 4 °C. Plasma (1 mL) was transferred into a glass test tube containing 1 mL of 0.01 N HCl and deproteinized by the addition of 1 mL of 7% perchloric acid.

(2) Plasma L-Dopa. The method of Tyce[21] was used with a few modifications. An aliquot of the perchloric acid extracts was adjusted to pH 3.0–4.0 with 2 N KOH and was kept in the refrigerator overnight. The fine precipitate of potassium perchlorate was then removed by centrifugation. The protein-free extract was adjusted to pH 2 by addition of 1 N HCl and introduced into the 0.7-cm diameter column containing 500 mg of Bio-Rad AG 50W X4 (200–400 mesh, H^+ type). After the column was washed with 20 mL of distilled water, amino acids including L-Dopa were eluted with 8 mL of 0.5 M potassium acetate buffer (pH 6.5).

L-Dopa content of the eluate was determined by the method outlined by Laverty and Taylor.[22] The eluate (1 mL) was added to a test tube containing 1 mL of 0.5 M citrate–phosphate buffer at pH 5.4 and was adjusted to pH 5.4 with 0.5 N NaOH or 0.5 N HCl if necessary. The catechols were oxidized by the addition of 0.1 mL of iodine solution (0.02 N I_2 in 5% NaI w/v). The oxidation was stopped after 5 min by addition of 0.5 mL of alkaline sulfite solution (2.5% $Na_2SO_3 \cdot 7H_2$ w/v, 1% Na_2EDTA w/v in 2.5 N NaOH). After 5 min, 0.06 mL of glacial acetic acid was added

to the alkaline reaction mixture and the pH was adjusted to 5.7 with glacial acetic acid.

The L-Dopa concentration was measured using a fluorophotometer at the activation wavelength of 330 nm and emission wavelength 380 nm after the sample was heated at 100 °C for 30 min. A standard curve was made by analyzing a series of known amounts of L-Dopa in plasma. Unknown L-Dopa plasma levels were calculated from the standard curve. The recovery of L-Dopa added to the plasma was 81.5 ± 4.2%.

(3) Plasma Total Dopamine. Dopamine has been identified in free form in adrenal medula,[23] brain, spleen, splenic nerves, pancreas, lung, liver, and intestine[24] and also found both in the free and conjugated form in urine.[25] In the present studies, dopamine was found mostly in conjugated form in the plasma at levels of micrograms per milliliter in the dog after administration of L-Dopa, which was determined in free form after acid hydrolysis and resulting in the total dopamine (T-DA) values. An aliquot of the perchloric acid extracts of the plasma was taken into a siliconized glass tube and the air was replaced by bubbling N_2 stream into the extract while the tube was capped with a glass stopper. The heated extracts of the plasma hydrolysate were then adjusted to pH 3.0–4.0 with 2.0 N KOH and left in the refrigerator overnight. A Bio-Rad AG 50W column described in the determination of plasma L-Dopa was used to isolate dopamine in the protein-free extracts of the plasma. It was adjusted to pH 6.5 by adding 0.5 M potassium acetate buffer and introduced on the column. The column was washed with 8 mL of 0.5 M potassium acetate buffer of pH 6.5, followed by 20 mL of distilled water. Dopamine adsorbed on the column was then eluted with 10 mL of 2 N HCl.

Dopamine in the eluate was determined by the method outlined by Laverty and Taylor.[22] The eluate (0.5 mL) was taken into a test tube containing 1 mL of 0.5 M phosphate buffer of pH 7.0 and, while kept in an ice bath, the pH was carefully adjusted to 7.0 by dropwise addition of 2.5 N NaOH. Dopamine was oxidized by the addition of 0.1 mL of iodine solution. After 5 min, the oxidation was stopped by addition of 0.5 mL of alkaline sulfite solution. Five minutes later, 0.25 mL of glacial acetic acid was added to the alkaline reaction mixture and the pH was adjusted to 4.4 with glacial acetic acid.

The sample was measured by a fluorophotometer at the activation wavelength of 320 nm and emission wavelength of 370 nm after being heated at 100 °C for 30 min. Plasma samples containing a known amount of dopamine were treated in the same way and a standard curve was obtained, which was used for the determination of the concentration in the samples. The recovery of dopamine added to the plasma was 82.3 ± 5.6%.

(B) Determination of L-Dopa and Its Metabolites in the Urine. (1) Preparation of Urine Samples. After oral administration of L-Dopa, 0–24- or 0–48-h urine samples were collected into plastic bottles containing 5 mL of 6 N HCl and 5 mL of 0.1 N Na_2EDTA. In order to determine the total amounts of the metabolites, the acid hydrolysis of their conjugates was first performed by heating the urine samples at 100 °C for 25 min.[26]

(2) L-Dopa in the Urine. EDTA (1 mL, 0.1 M) was added to the urine sample and the pH was adjusted to about 6 with 2 N ammonium hydroxide. Acid-washed alumina (550 mg, 100–150 mesh) was then added to the solution and the pH was adjusted to 8.5 with 2 N NH_4OH. The solution was then shaken mechanically for 15 min. After sedimentation, the alumina was washed with 10 mL of distilled water. The adsorbed L-Dopa was extracted with 10 mL of 0.05 N HCl by shaking the alumina mechanically for 15 min. The HCl phase was used for the determination of L-Dopa in urine. The separation and determination of L-Dopa from the obtained solution was carried out using the same methods as for the plasma L-Dopa except that the blank sample was prepared by adding alkaline solution without oxidation with iodine.

(3) Total Dopamine in Urine. The prepared urine sample was used for the separation and determination of dopamine by the same method as for the plasma total dopamine, except that the blank sample was prepared by adding alkaline sulfite solution without oxidation with iodine.

(4) Total Phenolic Acids in Urine[27] [Homovanillic Acid (HVA) and 3,4-Dihydroxyphenylacetic Acid (DOPAC)]. An aliquot of urine was placed in a 60-mL ground glass centrifuge tube and the pH was adjusted to 1 with 6 N HCl. After bubbling nitrogen through the sample, the tube was closed. The sample was then heated to 100 °C for 25 min and cooled to room temperature, saturated with NaCl, and extracted three times with 10 mL of ethyl acetate. The ethyl acetate extracts were combined and evaporated to dryness under vacuum at 40 °C. The residue was dissolved in 0.5 mL of ethyl acetate which contained 0.2% of triphenylmethane as the internal standard. The solution was trimethylsilylated with 0.3 mL of *N,Q*-bis(trimethylsilyl)acetamide and 0.1 mL of trimethylchlorosilane overnight at room temperature, and then 1 μL of the mixture was injected into a gas chromatograph, using a 3.6-in. long, 2-mm internal diameter U-shaped glass tube column packed with 2% OV-1 on Chromosorb W (HP grade) of 80–100 mesh.

The oven temperature was maintained at 155 °C, the detector temperature was 200 °C, the inlet and outlet were 190 °C, and the flow rate of the prepurified He was 40 mL/min. A flame ionization detector was used. DOPAC and HVA authentic standards were dissolved into distilled water and carried through the entire procedure, and standard curves for DOPAC and HVA were thus obtained.

The working curves for DOPAC and HVA were obtained by analyzing a series of urine samples containing known amounts of DOPAC or HVA. The recoveries of DOPAC and HVA added to the urine were 95.5 ± 4.2 and 96.3 ± 2.5%, respectively.

Toxicity Studies (LD_{50} Values and Intoxication Symptoms). Conventional male DDY strain male mice weighing 20–23 g were used. The number of the animals used for a given dose varied from 10 to 20 (at levels closer to LD_{50}). All drugs were dissolved or suspended in 0.5% tragacanth solution, at 150 mg/mL concentration. All test solutions were administered orally in a volume of 0.15–0.74 mL. The animals were observed for 5 days following the administration. The LD_{50} and related confidence limits were calculated by the method of Litchfield and Wilcoxon. The dose response of acute oral toxicity is summarized on Figures 8 and 9.

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Antibacterial Halogenoacetyl Derivatives of Amino Acids and Simple Peptides

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The vital role of D-alanine and L-lysine in the peptidoglycan crosslinking process in the bacterial cell wall prompted preparation of various small peptides incorporating these amino acids. *N*-Iodoacetyl or -bromoacetyl derivatives of the peptides were then prepared in the hope that they would serve as active-site-directed irreversible inhibitors of cell wall transpeptidases. Certain of the halogenoacetyl dipeptide esters, but not the corresponding free acids, showed slight antistaphylococcal activity. Subsequent structural variation showed that inclusion of D-alanine or L-lysine was not necessary, since antibacterial activity was at least as good when the dipeptide unit was replaced by glycylglycine or by an ω-aminoalkanoic acid. It was concluded that the observed antibacterial activity was probably not due to specific inhibition of a cell wall transpeptidase.

A vital process in the growth and division of bacterial cells is the construction of new peptidoglycan, a macromolecular framework which determines the integrity and shape of the cell wall.[1] The mucopeptide units which comprise the peptidoglycan are elaborated in the interior of the cells, but their assembly involves a peptide cross-linking reaction in the exterior situation. Penicillins and other β-lactam antibiotics are believed to exert their antibacterial action by inhibiting the membrane-bound transpeptidase enzyme or enzymes responsible for this reaction, probably by acylation at or near the active site.[2] In all bacteria so far studied cross-linking involves a C-terminal D-alanyl-D-alanine component of one peptide chain, the terminal D-alanine being displaced by the amino group of a second peptide chain, the following reaction in staphylococci being typical.

$$\text{R-L-Lys-D-Ala-D-Ala} + \text{R}^1\text{NH}_2 \xrightarrow{\text{enzyme}} \text{R-L-Lys-D-Ala-NHR}^1 + \text{D-Ala}$$

Certain small peptides structurally related to the terminal sequences involved in the cross-linking reaction act as competitive inhibitors of the DD-carboxypeptidase/transpeptidase of certain bacteria, but none of these peptides proved toxic to intact bacteria.[3] It occurred to us that such peptides might be converted into effective antibacterial agents by the attachment of a chemically reactive group, which could facilitate irreversible combination with the target enzyme according to Baker's concept[4] of active-site-directed irreversible enzyme inhibition. Other enzymes which have been irreversibly inhibited by chemically reactive peptide derivatives include elastase,[5] neurohypophyseal hormone-stimulated adenylate cyclase,[6] trypsin, and chymotrypsin.[4] The reactive groups which have proved valuable in designing effective inhibitors include *N*-bromoacetyl or -iodoacetyl groups. In this paper we describe the incorporation of such groups in certain amino acids or di- and tripeptides. In constructing the initial range of peptides, either D-alanine or L-lysine or both were included, since these two amino acids appear to play a central role in the cross-linking process in the staphylococcal cell wall. Later use was also made of D-glutamic acid, which had proved a useful C-terminus in the previously mentioned[3] reversible inhibitors, and of glycylglycine and various ω-aminoalkanoic acids as possible substitutes for a dipeptide unit.

Chemistry. Abbreviations used are those recommended by the IUPAC–IUB Commission on Biochemical Nomenclature Symbols for Amino Acid and Peptides: DCC, dicyclohexylcarbodiimide; Cbz, benzyloxycarbonyl; Bzl, benzyl; Boc, *tert*-butyloxycarbonyl; Bu, *tert*-butyl; Phth, phthalimidyl; Suc, succinyl; Ac, acetyl; TosOH, *p*-toluenesulfonic acid.

The protected peptide esters listed in Table I were synthesized by a standard coupling technique using DCC in methylene chloride. They were then converted into the corresponding compounds containing a single free amino group (Table II). For this purpose Cbz groups were removed by catalytic hydrogenation over 10% palladium on charcoal. Boc groups were removed by means of 1 equiv of *p*-toluenesulfonic acid, a procedure[7] which does not affect *tert*-butyl or benzyl ester functions, and in these cases (**18, 22–26, 30–32, 34, 35**) the product was frequently isolated as the TosOH salt.

The free amines were then treated with mixed anhydrides prepared from iodoacetic acid or bromoacetic acid to yield the *N*-halogenoacetyl derivatives listed in Table III. Finally the free acids (Table IV) were liberated by treating the *tert*-butyl esters with trifluoroacetic acid. In the case of certain lysine-containing peptides the TFA treatment also served to remove a Boc group and gave the peptides as TFA salts (**63–66** and **72**).