Synthesis and Study of L-Dopa−Glutathione Codrugs as New Anti-Parkinson Agents with Free Radical Scavenging Properties

Francesco Pinnen,[†] Ivana Cacciatore,[†] Catia Cornacchia,[†] Piera Sozio,[†] Antonio Iannitelli,[†] Mara Costa,[‡] Laura Pecci,[‡] Cinzia Nasuti,[§] Franco Cantalamessa,[§] and Antonio Di Stefano*[,†]

Dipartimento di Scienze del Farmaco, Università "G. D'Annunzio", Via dei Vestini 31, 66100 Chieti, Italy, Dipartimento di Scienze Biochimiche, Università "La Sapienza", P. le A. Moro, 00185 Roma, Italy, and Dipartimento di Scienze Farmacologiche e Medicina Sperimentale, Università di Camerino, Via Scalzino, 62032 Camerino (MC), Italy

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A series of novel molecular combinations (**1**−**4**), in which L-dopa (LD) is linked covalently via an amide bond with glutathione (GSH), were synthesized and evaluated as potential anti-Parkinson agents with antioxidant properties. These conjugates were characterized by evaluating solubility, chemical and enzymatic stabilities, and apparent partition coefficient (log *P*). Derivatives **2** and **4** were tested for their radical scavenging activities, by use of a test involving the Fe(II)/H_2O_2-induced degradation of deoxyribose. In this study, the antioxidant efficacy of codrugs **1** and **3** was also assessed through the evaluation of plasmatic activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx). Furthermore, the central nervous effects and rat striatal concentration of LD and dopamine (DA) have been evaluated after oral administration of codrugs **1** and **3**. Tested compounds prolonged the plasma LD levels and were able to induce sustained delivery of DA in rat striatum with respect to an equimolar dose of LD. The results suggest that compounds **1** and **3** could represent useful new anti-Parkinson agents devoid of the pro-oxidant effects associated with LD therapy and potentially able to restore the GSH depletion evidenced in the substantia nigra pars compacta (SNpc) of PD patients.

Introduction

Parkinson's disease (PD)[a] is the most common neurodegenerative movement disorder, primarily associated with degeneration of the pigmented neurons in the substantia nigra pars compacta (SNpc), resulting in decreased dopamine (DA) nigrostriatal system availability and formation of Lewis bodies mainly composed of fibrillar α-sinuclein.[1,2] Numerous drugs are available for treating PD, but none has surpassed the clinical efficacy of L-dopa (LD). This drug, which is converted to DA in the brain, still remains the gold standard of pharmacotherapy to which all other therapies are compared, but unfortunately, it seems to contribute to the progression of the disease because of its pro-oxidant properties deriving from autoxidative metabolism; administration of LD in the rat can increase the production of free radicals in the SNpc.[3] Thus catecholamine metabolism by monoamine oxidases (MAO) leads to the formation of H_2O_2, which can be converted into the more reactive hydroxyl radicals through interaction with ferrous iron; the reaction involving H_2O_2, iron, and DA may be a source of a dopaminergic neurotoxin such as 6-hydroxydopamine.[3−6] For these reasons, the search for novel therapeutic strategies based on DA replacement therapy is still ongoing. During the last years, studies of the pathogenesis of PD have centered on the oxidative damage to the SNpc region of the midbrain. Biochemical changes occurring in PD [increased iron levels, inhibition of mitochondrial complex I activity, and decreased reduced glutathione (GSH) levels] suggest that oxidative stress and free radical species may be a key factor in nigrostriatal system degradation; increased free radical production and an inadequate antioxidant defense system have been reported, which could contribute to the biopathology of PD.[7−9] The reactive oxygen species (ROS), whose major source is represented by mitochondrial respiration, are rapidly deactivated by scavenging systems before they can cause damage.[10] In some cases, pathologic conditions lead to increased formation of ROS and decreased scavenging capacity. In particular, decreased GSH levels may be an early component of the process: the postmortem lower levels of GSH in the SNpc of PD patients are remarkably decreased if compared with those of healthy subjects.[11] Thus, it has been suggested that low levels of nigrostriatal GSH contents and consequent oxidative stress might contribute to the degeneration of dopaminergic neurons in idiopathic PD.[12] GSH plays an important role in protecting dopaminergic SN cells against oxidative stress and is also involved in many cellular process such as DNA metabolism and protein synthesis.[13] Although it is not the only antioxidant molecule reported to be altered in PD, the magnitude of its depletion is hypothesized to be the earliest indicator of nigrostriatal degeneration and an important factor in losses in mitochondrial NADH-dehydrogenase activity, which seems to play an important role in neurodegeneration associated with the ethiology of PD;[14] furthermore, striatal DA content and GSH levels are not altered in areas of the brain other than SNpc or in other diseases affecting dopaminergic neurons.[15−17]

GSH is known to protect proteins against oxidation by conjugating with oxidized thiol groups to form protein−SS−G mixed disulfides, which can then be reduced to protein and GSH by glutathione reductase (GR), thioredoxine, or protein disulfide isomerase.[18] In dopaminergic cells, GSH can also bind to quinones formed during oxidation of DA and prevent these

* Corresponding author: tel +39-871-3555230; fax +39-871-3555267; e-mail adistefano@unich.it.
[†] Università "G. D'Annunzio".
[‡] Università "La Sapienza".
[§] Università di Camerino.

[a] Abbreviations: ANOVA, analysis of variance; DA, dopamine; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; LD, L-dopa; MAO, monoamine oxidases; MDA, malondialdehyde; PD, Parkinson's disease; ROS, reactive oxygen species; SGF, simulated gastric fluid; SNpc, substantia nigra pars compacta; SOD, superoxide dismutase; TBA, thiobarbituric acid; TFA, trifluoroacetic acid.

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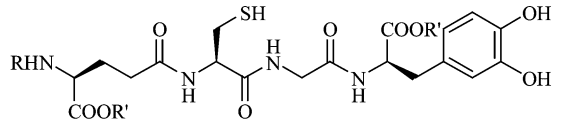
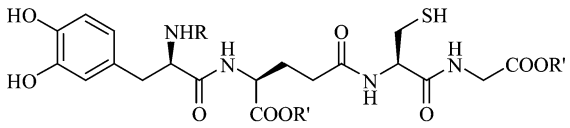


1: R=Ac; R'=Me **2**: R=H; R'=H



3: R=Ac; R'=Me **4**: R=H; R'=H

Figure 1. Chemical structures of multifunctional codrugs **1**−**4**.

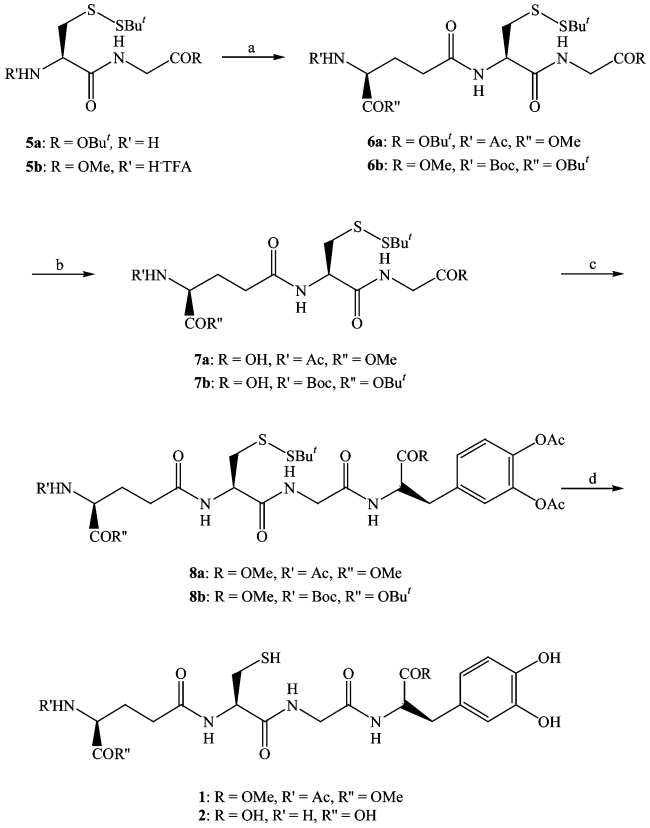
compounds from reacting with protein sulfhydryl groups.[19] Furthermore, thiol oxidation and loss of mitochondrial complex I activity precede excitatory amino acid-mediated neurodegeneration.[20] Both could be prevented by treatment with antioxidant thiol agents. It has been suggested that GSH is involved in the repair of oxidized iron-sulfur centers of mitochondrial complex I.[21,22] Furthermore, Sechi et al.[23] confirmed that when GSH was administered by iv injection to PD patients, a significant improvement in disease-related disability was observed, demonstrating that maintenance of thiol homeostasis is critical for the protection of dopaminergic SNpc neurons against neurodegeneration.

We have recently demonstrated that multifunctional codrugs, obtained by linking together LD with (*R*)-α-lipoic acid, seem to protect partially against the oxidative stress deriving from autoxidation and the MAO-mediated metabolism of DA.[24] Starting from these data, our study was focused on providing molecular combinations, obtained by joining GSH with LD, able to generate a targeted antioxidant. These compounds could permit a targeted delivery of the GSH (whose lower SNpc levels may be related to PD) directly to specific groups of neurons, where cellular stress is associated with PD.[25] To modify the physicochemical properties of LD in order to obtain improved absorption after oral administration, and, in addition, to take advantage of the apparent synergistic mechanism of GSH and LD, here we proposed the synthesis of novel molecular combinations in which LD is linked covalently via an amide bond to GSH (Figure 1). The new codrugs **1**−**4**, with a low degree of chemical and enzymatic stability under physiological conditions, can afford more efficacious central nervous system (CNS) delivery than can LD and GSH alone. These conjugates containing GSH, whose benefits have been demonstrated in PD, could represent useful new anti-Parkinson agents devoid of the pro-oxidant effects associated with the presence of the catecholic moiety and able to restore the GSH depletion evidenced in the SNpc of PD patients.[26]

Chemistry

Boc-Glu-[Cys(SBu^t)-Gly-OMe]-OMe (**6c**), H-LD(Ac)$_2$-OMe·HCl, and Boc-LD(Ac)$_2$-OH were synthesized as previously reported.[27−29] The H-LD(Ac)$_2$-OH·HCl was N-acetylated as described by Chenault et al.[30] to obtain Ac-LD(Ac)$_2$-OH. Codrugs **1**−**4** were synthesized as outlined in Schemes 1 and 2; solution-phase procedures were employed by elongation of the suitably protected GSH peptide chain in the C (compounds **1** and **2**) or N (compounds **3** and **4**) direction with the LD derivative. Simultaneous deprotection of the cysteine -SH and the cathecolic hydroxy groups to give the desired codrugs **1**−**4** was obtained when the corresponding protected tetrapeptide precursor was treated for 1.5 h at room temperature with a small excess (1.2 equiv) of tri-*n*-butylphosphine in a water/*n*-propanol solution, made slightly alkaline (pH 8.5) by aqueous ammonia.

Scheme 1[a]



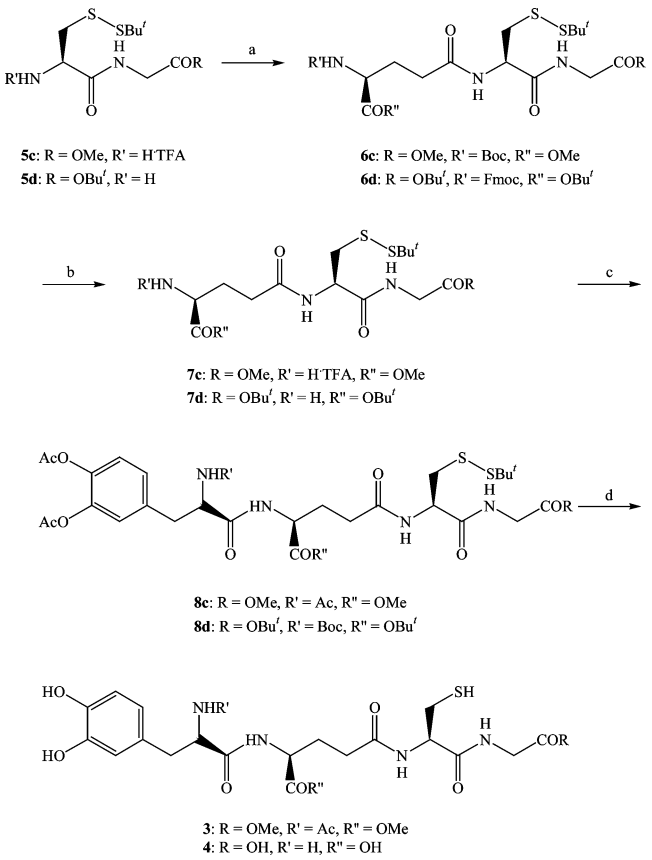
5a: R = OBu^t, R' = H
5b: R = OMe, R' = H·TFA
6a: R = OBu^t, R' = Ac, R" = OMe
6b: R = OMe, R' = Boc, R" = OBu^t
7a: R = OH, R' = Ac, R" = OMe
7b: R = OH, R' = Boc, R" = OBu^t
8a: R = OMe, R' = Ac, R" = OMe
8b: R = OMe, R' = Boc, R" = OBu^t
1: R = OMe, R' = Ac, R" = OMe
2: R = OH, R' = H, R" = OH

[a] Reagents and conditions were as follows. For **5a**−**8a** and **1**: (a) Ac-Glu-OMe, DCC, dry THF, 3 h, 0 °C, and then 16 h, 5 °C; (b) TFA, 2 h, rt; (c) H-LD(Ac)$_2$-OMe·HCl, IBCF, TEA, DMF, 3 h, 0 °C, and then 15 h, 4 °C; (d) (*n*-Bu)$_3$P, *n*-PrOH/H$_2$O (2:1), 1.5 h, rt, pH 8.5. For **5b**−**8b** and **2**: (a) Boc-Glu-OBu^t, DCC, TEA, dry THF, 3 h, 0 °C, and then 16 h, 5 °C; (b) NaOH 1 N, MeOH, 1 h, rt; (c) H-LD(Ac)$_2$-OMe·HCl, IBCF, TEA, DMF, 3 h, 0 °C, and then 15 h, 4 °C; (d) TFA, 5 h, rt, and then (*n*-Bu)$_3$P, *n*-PrOH/H$_2$O (2:1), 1.5 h, rt, pH 8.5.

Results and Discussion

Apparent coefficient partition (log *P*) measurement is a useful parameter for understanding the behavior of drug molecules; for example, it may be used to predict the distribution of a drug compound in a biological system. Furthermore, factors such as absorption, excretion and penetration of the CNS may be related to the log *P* value of a drug. In general, if passive transport through biological membranes is assumed, good intestinal absorption of an orally administered drug could be obtained with log $P = 1.35$; instead, a log *P* of 2 ± 0.7 can be suitable for optimum CNS penetration.[31] To assess this potential, the log *P* values of compounds **1**−**4** were determined in *n*-octanol/phosphate buffer, pH 7.4, by a saturation shake-flask method.[32−34] The concentrations of codrugs were determined on a C_{18} reverse-phase column, by use of an HPLC apparatus with UV detection; concentrations of compounds **1**−**4** in *n*-octanol and buffer layers were evaluated by correlating the peak areas in HPLC with known concentrations of the compounds. The same method was employed for evaluation of aqueous solubility in water and buffer solutions

Scheme 2[a]



[a] Reagents and conditions were as follows. For **5c**−**8c** and **3**: (a) Boc-Glu-OMe, DCC, TEA, dry THF, 3 h, 0 °C, and then 16 h, 5 °C; (b) TFA, 2 h, rt; (c) Ac-LD(Ac)$_2$-OH, IBCF, TEA, DMF, 3 h, 0 °C, and then 15 h, 4 °C; (d) (*n*-Bu)$_3$P, *n*-PrOH/H$_2$O (2:1), 1.5 h, rt, pH 8.5. For **5d**−**8d** and **4**: (a) Fmoc-Glu-OBu^t, DCC, dry THF, 3 h, 0 °C, and then 16 h, 5 °C; (b) DBU, CH_2Cl_2, 20 min, rt; (c) Boc-LD(Ac)$_2$-OH, IBCF, TEA, DMF, 3 h, 0 °C, and then 15 h, 4 °C; (***d***) TFA, 5 h, rt, and then (*n*-Bu)$_3$P, *n*-PrOH/H$_2$O (2:1), 1.5 h, rt, pH 8.5.

at pH 1.3, 5.0, and 7.4. The obtained log *P* and solubility values are shown in Table 1. Our results indicate that codrugs **1**−**4** showed high lipophilicity when compared to the parent drug LD (log $P = -2.39$).[35] In particular, derivatives **1** and **3** showed log $P = 0.89$ and 0.94, respectively, which is near the optimum for good intestinal absorption with good water solubility (about 27 mg/mL for compound **1**). The new codrugs were studied in vitro to evaluate their chemical and enzymatic stability. The chemical hydrolysis rates of our compounds were measured under three pH conditions (pH 1.3, 5.0, and 7.4) at 37 °C. Table 2 reports the half-life ($t_{1/2}$) values of compounds **1**−**4** obtained by regression analysis from slopes of semilogarithmic plots of the codrug concentrations versus time.[36]

The rate data show that the amide bonds allowed us to obtain codrugs with high stability in nonenzymatic simulated gastric fluid (SGF) at pH 1.3 ($t_{1/2} > 20$ h). In fact, degradation of compound **3** was not significant within 4 h ($t_{1/2} > 30$ h). This stability implies that compounds **1**−**4** pass unhydrolyzed through the stomach after oral administration. At pH 5.0 and 7.4, all the compounds are stable enough ($t_{1/2} > 7$ h) to be absorbed intact from the intestine.

In rat plasma (80% rat plasma containing 20% 0.02 M phosphate buffer, pH 7.4), catechol esters and amide bonds of the studied derivatives were cleaved and LD was formed in one step. A rapid conversion of our compounds to LD was observed. In particular, the HPLC analysis of the rat plasma samples showed complete disappearance of codrug **1** with the concomitant appearance of LD. These derivatives were identified by liquid chromatography/mass spectrometry (LC/MS) and NMR analysis.

Hydrolysis in human plasma (80% human plasma containing 20% 0.02 M phosphate buffer, pH 7.4) proceeds more slowly with respect to rat plasma with formation of LD. The process was correlated with first-order kinetics, and the LD was released. The faster hydrolysis in rat than in human plasma may be ascribed to the different enzyme systems that are highly efficient in rat plasma.[37] The rate constants (K_{obs}) and the corresponding half-life times are shown in Table 3. Our data indicate that the enzymatic hydrolysis of compounds **2** and **4** proceeds more slowly than that of **1** and **3**, although inverse results could be expected. Generally, enzymes having broad specificity are most active against one particular substrate. In our case we can hypothesize that the initial interaction between enzyme and codrugs **2** and **4** is relatively weak when compared to more lipophilic codrugs **1** and **3**. Figure 2 shows the LD plasma concentration profile obtained in rats over time following oral administration of codrugs **1** and **3** and of LD. The values of LD plasma concentration at 2 h postdose were about 105 μg/mL for compound **1**, 85 μg/mL for compound **3**, and 83 μg/mL for LD, with rapid decrease of concentration levels 100 min after LD administration. The reduction of LD plasma levels was slower after administration of compounds **1** and **3**; in this case the plasma concentration was still elevated after 8 h (82 μg/mL for compound **1**). The new compounds were also evaluated by comparison of neostriatal LD and DA levels after administration of codrugs **1**, **3**, and LD. A previously reported high-performance liquid chromatography (HPLC) method with electrochemical detection (EC) was utilized.[38] The changes in striatal levels of LD and DA are shown in Figure 3. The studied compounds are of particular interest because they were able to induce sustained delivery of both LD and DA in rat striatum with respect to an equimolar dose of LD; the striatal levels of DA was still elevated after 12 h (about 40 pmol/mg for compounds **1** and **3**). To minimize the conversion to DA outside the CNS, LD is usually given in combination with peripheral inhibitors of amino acid decarboxylase (carbidopa and benserazide). During chronic treatment with LD, a variety of problems may emerge. Patients experience a decrease in the duration of drug effect ('wearing-off' phenomenon) and, as the number of functioning DA neurons decreases in the SNpc, the patient becomes more sensitive to LD plasma level fluctuations (on/off effects). Our data showed that codrugs **1** and **3** are able to prolong rat plasma LD levels and striatal DA concentration and could be beneficial in the treatment of PD.[39,40]

In this study, we also assessed the antioxidant efficacy of selected codrugs **1** and **3** that could be used also in therapy as potential neuroprotective agents through the evaluation of peripheral markers of oxidative stress. This biochemical evaluation allowed us to compare rats treated with compounds **1** and **3** containing an antioxidant portion and those treated with LD for the activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx) in the plasma. Antioxidant enzymes such as SOD and GPx may play an important role in the protective mechanisms against oxidative stress, and then their activities might be critical for a protective effect against the oxidative stress produced by LD.[41,42]

Table 4 shows the activities of antioxidant enzymes that form the primary defense system against ROS in the plasma, 1.5 h after drug administration. GPx activity (1.5 h after treatment) was significantly ($P < 0.05$) increased in plasma of **1**-treated

Table 1. Physicochemical Properties of Prodrugs **1**−**4**

compd	log P^a	solubility[a] (mg/mL) in water	at pH 1.3	at pH 7.4	at pH 5.0
1	0.89 (±0.04)	27.03 (±1.35)	35.10 (±1.76)	25.64 (±1.28)	25.51 (±1.27)
2	0.75 (±0.02)	1.80 (±0.07)	2.15 (±0.07)	1.93 (±0.04)	1.67 (±0.06)
3	0.94 (±0.05)	7.89 (±0.40)	2.74 (±0.14)	3.99 (±0.20)	3.84 (±0.19)
4	0.80 (±0.04)	0.32 (±0.01)	0.55 (±0.02)	0.46 (±0.01)	0.69 (±0.03)

[a] Values are means of three experiments; standard deviation is given in parentheses.

Table 2. Kinetic Data for Chemical Hydrolysis of Codrugs **1**−**4** at 37 °C

compd	pH 1.3[a] $t_{1/2}$ (h)	pH 1.3[a] K_{obs} (h^{-1})	pH 5.0[a] $t_{1/2}$ (h)	pH 5.0[a] K_{obs} (h^{-1})	pH 7.4[a] $t_{1/2}$ (h)	pH 7.4[a] K_{obs} (h^{-1})
1	20.14 (±0.73)	0.034 (±1.2 × 10^{-3})	40.76 (±0.94)	0.017 (±0.4 × 10^{-3})	7.22 (±0.31)	0.096 (±4.1 × 10^{-3})
2	28.12 (±1.21)	0.025 (±1.1 × 10^{-3})	57.26 (± 1.83)	0.012 (±0.4 × 10^{-3})	12.23 (±0.49)	0.057 (±2.3 × 10^{-3})
3	31.02 (±0.46)	0.022 (±0.4 × 10^{-3})	69.30 (±2.56)	0.010 (±0.4 × 10^{-3})	15.07 (±0.66)	0.046 (±2.0 × 10^{-3})
4	40.37 (±1.05)	0.017 (±0.4 × 10^{-3})	80.08 (±1.52)	0.009 (±0.2 × 10^{-3})	23.16 (±0.86)	0.030 (±1.1 × 10^{-3})

[a] Values are means of three experiments; standard deviation is given in parentheses.

Table 3. Rate Constants for the Hydrolysis of Codrugs **1**−**4** in 80% Rat Plasma and 80% Human Plasma at 37 °C

compd	rat plasma[a] $t_{1/2}$ (min)	rat plasma[a] K_{obs} (min^{-1})	human plasma[a] $t_{1/2}$ (min)	human plasma[a] K_{obs} (min^{-1})
1	*b*		3.2 (±0.1)	0.217 (±6 × 10^{-3})
2	2.7 (±0.1)	0.257 (±8 × 10^{-3})	15.1 (±0.4)	0.046 (±1 × 10^{-3})
3	1.9 (±0.1)	0.365 (±13 × 10^{-3})	4.7 (±0.1)	0.148 (±4 × 10^{-3})
4	4.1 (±0.1)	0.169 (±4 × 10^{-3})	13.3 (±0.4)	0.052 (±2 × 10^{-3})

[a] Values are means of three experiments; standard deviation is given in parentheses. [b] Immediate hydrolysis.

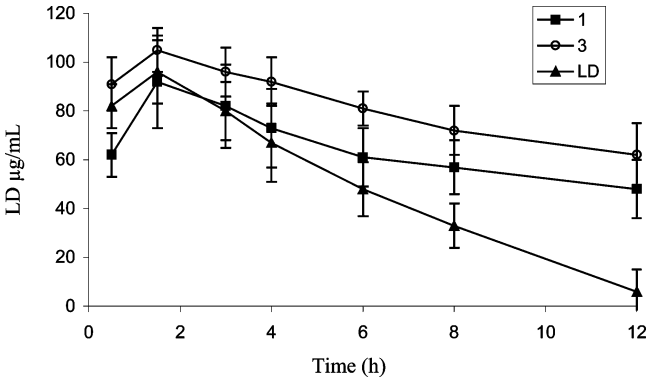


Figure 2. Plasma concentration profile of LD after administration of LD, **1**, and **3** in rats. Data are expressed as mean ± SE. Each experiment was performed in triplicate.

rats [847.78 ± 3.55 units/(g·Hb)] compared with the LD-treated ones [795.60 ± 15.76 units/(g·Hb)], whereas those of the **3**-treated group remained at similar values [821.00 ± 25.23 units/(g·Hb)] ($P > 0.05$) with respect to those of the LD-treated group. The higher enzymatic activity of the **1**-treated group could indicate a decreased production of free radicals, a hypothesis supported by a previous study where a line of PC12 cells overexpressing GPx protected against the cell damage induced by exposure to LD.[43,44]

Similar behavior can be observed with regard to the plasma SOD activity. One and a half hours after treatment with **1**, a significant reduction of the plasma SOD activity [174.86 ± 2.39 units/(g·Hb)] was measured compared to LD-treated groups [203.40 ± 5.98 units/(g·Hb)] ($P < 0.05$). This behavior could be a consequence of the lesser production of superoxide anion ($O_2^{\bullet -}$) generated during the autoxidation of DA, following treatment with **1**. As reported in our previous studies, xenobiotics induced oxidative stress in rats by elevated levels of $O_2^{\bullet -}$ followed by increased activity of SOD.[45,46]

On the whole, one of the potential advantages of using **1** rather than LD in treating PD is that, via its antioxidant properties, it seems to protect in part against the oxidative stress deriving from autoxidation and the MAO-mediated metabolism of DA.

For in vitro evaluation of pro-oxidant activity, compounds **2** and **4** were chosen, which correspond to the N-deacetyl derivatives of codrugs **1** and **3**, respectively, with hydrolyzed methyl ester groups. We selected codrugs **2** and **4** because the incubation medium [deoxyribose, phosphate buffer, ethylenediaminetetraacetic acid (EDTA), and H_2O_2] lacks enzymatic activity and was not able to hydrolyze the *N*-acetyl and methyl ester groups of compounds **1** and **3** as observed from in vivo conditions. To better evaluate whether LD pro-oxidant ability was affected by the chemical modification present in the synthetic derivatives **2** and **4**, we compared their effect on the Fe(II)/H_2O_2-induced degradation of deoxyribose (Figure 4). As previously reported, LD (from 1 to 50 μM) caused a concentration-dependent increase of deoxyribose degradation that leveled off at about 50 μM LD.[24] Compound **4**, over the same concentration range, exerted pro-oxidant properties significantly lower than LD, whereas codrug **2** did not stimulate deoxyribose damage. These results clearly indicate that modification of the amino group of LD through amide bonds with GSH produces molecules with a decreased susceptibility to iron-induced autoxidation; furthermore, the different linkage of GSH to LD may play an important role by modulating LD pro-oxidant activity.

To evaluate the dopaminergic activity of equimolar doses of compounds, we studied the effects of **1** and **3** on spontaneous locomotor activity of rats in comparison with LD-treated animals. As can be observed in Figure 5, drug administration 1.5 h after gavage led to a pattern of behavioral depression in the treated groups compared with the control group, which received vehicle only, or with the GSH group that received glutathione. The results obtained after treatment with **3** showed less behavioral depression on locomotion (162.6 ± 25) compared to both the **1**- (61 ± 27.47) and LD-treated groups (82 ± 23.44) ($P < 0.05$). Besides, grooming episodes of **3**-treated animals (209.35 ± 27.55) were significantly ($P < 0.05$) decreased compared to the **1**-treated group (358.20 ± 32.98) ($P < 0.05$). As shown in the literature, grooming behaviors depend on DA neurotransmission in basal ganglia circuits, as the pattern is disrupted by dopaminergic nigrostriatal lesions, while grooming is increased by systemic administration of D1 agonists.[47−49] In our case, it seems that, compared to both LD

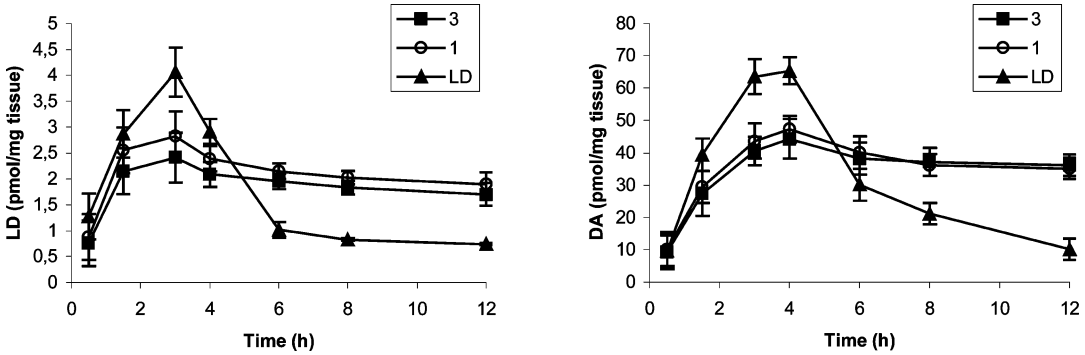


Figure 3. Rat striatal levels of LD and DA after administration of LD, **1**, and **3**. Data are expressed as mean ± SE. Each experiment was performed in triplicate.

Table 4. Values of Superoxide Dismutase and Glutathione Peroxidase Activities[a]

	SOD activity, units/(g·Hb)	GPx activity, units/(g·Hb)
L-dopa	203.40 (±5.98)	795.60 (±15.76)
1	174.86 (±2.39)[b]	847.78 (±3.55)[b]
3	200.59 (±24.79)	821.00 (±25.23)

[a] Activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) were measured 1.5 h after administration of LD, **1**, and **3** in rats. Data are expressed as mean ± SE of five experiments. [b] $P < 0.05$ compared to LD-treated group.

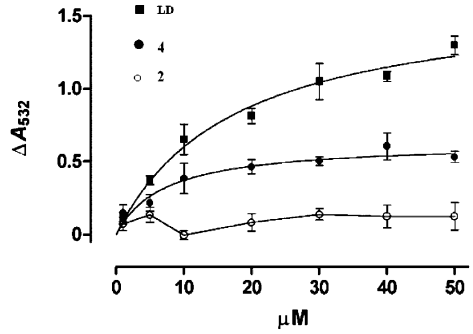


Figure 4. Pro-oxidant effect of LD and compounds **2** and **4** on Fe(II)/EDTA/H_2O_2-induced deoxyribose degradation. The data are presented as increases of absorbance at 532 nm with respect to a control containing deoxyribose alone ($A_{532} = 0.404$). Results are the mean ± SEM of three separate experiments performed in duplicate.

and codrug **1**, treatment with **3** induced less dopaminergic activity: decreased locomotion and impaired grooming behavior. This may be related to the slower biodistribution in the CNS of codrug **3** used at the same molar doses as both LD and codrug **1**. The data obtained allow us to distinguish the different pharmacokinetic properties of compounds **3** and **1**: the higher values of plasma concentration of LD after administration of **3** could be attributed to the slower DA delivery to the CNS [analysis of variance (ANOVA) one-way test $P < 0.05$].

The responses to anxiety-like behavior 1.5 h after drug administration are reported in Figure 6. As shown, treatment with LD and **1** and **3** codrugs induced a significant ($P < 0.05$) decrease of entries and time spent in the center of open field compared with both control and GSH groups. As observed for locomotor activity, also in anxiety-like behavior, treatment with LD, **1**, and **3** led to a pattern of behavioral depression. No significant modifications were revealed in anxiety-like activities among the three groups treated with LD, **1**, and **3**.

In short, this report describes a series of LD and GSH conjugates as LD prodrugs with antioxidant properties. The present findings show that the new codrugs possessed good stability toward gastrointestinal simulated fluids and released LD in rat and human plasma after enzymatic hydrolysis.

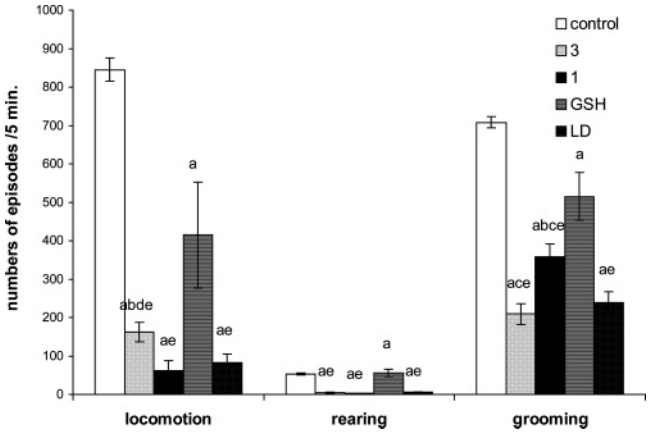


Figure 5. Locomotion, rearing, and grooming, 1.5 h after administration of LD, GSH, **1**, and **3** in rats. Data are expressed as mean ± SE of 10 experiments. (a) $P < 0.05$ compared to control group; (b) $P < 0.05$ compared to LD-treated group; (c) $P < 0.05$ compared to **3**-treated group; (d) $P < 0.05$ compared to **1**-treated group; (e) $P < 0.05$ compared to **1**-treated group.

Codrugs **2** and **4** displayed an antioxidant effect with a decreased susceptibility to iron-induced autoxidation compared to LD. Among the studied products, codrugs **1** and **3** were of particular interest because they are able to induce sustained release of DA in rat striatum with respect to equimolar dose of LD. Taken together, these results demonstrated the possible therapeutic application of new derivatives in PD; in particular, conjugate **1** seems to protect partially against the oxidative stress deriving from autoxidation and the MAO-mediated metabolism of DA.

Experimental Section

Microanalyses were performed on a 1106 Carlo Erba CHN analyzer, and the results were within 0.4% of the calculated values. Optical rotations were taken at 25 °C on a Perkin-Elmer 241 polarimeter. ^{1}H and ^{13}C NMR spectra were recorded on a Varian VXR 300-MHz spectrometer. Chemical shifts are reported in parts per million (δ) downfield from the internal standard tetramethylsilane (Me_4Si). The LC-MS/MS system used consisted of an LCQ (Thermo Finnigan) ion trap mass spectrometer (San Jose, CA) equipped with an electrospray ionization (ESI) source. The capillary temperature was set at 300 °C and the spray voltage at 4.25 kV. The fluid was nebulized by use of nitrogen (N_2) as both the sheath gas and the auxiliary gas. The identity of all new compounds was confirmed by elemental analysis, NMR data, and LC-MS/MS system; homogeneity was confirmed by thin-layer chromatography (TLC) on Merck 60 F_{254} silica gel. Solutions were routinely dried over anhydrous sodium sulfate prior to evaporation. Chromatographic purifications were performed on a Merck 60 70−230 mesh ASTM silica gel column.

Chemicals. Ac-Glu-OMe, Boc-Glu-OBu^t, and Fmoc-Glu-OBu^t were obtained from Bachem. Glutathione reductase, NADPH,

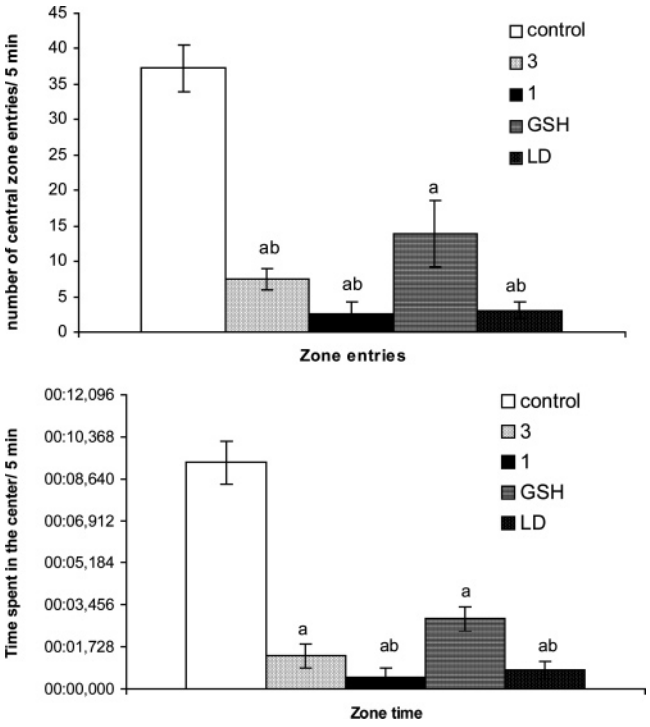


Figure 6. Anxiety test 1.5 h after administration of LD, GSH, **1**, and **3** in rats. Data are expressed as mean ± SE of five experiments. (a) $P < 0.05$ compared to control group; (b) $P < 0.05$ compared to GSH-treated group.

benserazide, 2-deoxy-D-ribose, and LD were purchased from Sigma (St. Louis, MO). All other chemicals used were of the highest purity commercially available.

General Method for Tripeptide Synthesis. To a stirred solution of dipeptide Cys-Gly (8.38 mmol) in dry tetrahydrofuran (THF, 15 mL) was added Glu (8.38 mmol) in dry THF (10 mL) at 0 °C, followed by portionwise addition of a solution of DCC (8.38 mmol) in dry THF (10 mL). After 3 h at 0 °C and 16 h at 5 °C, the reaction mixture was filtered and the resulting solution was evaporated under vacuum. The residue was taken up in EtOAc and the organic layer was washed with 1 N $KHSO_4$, saturated aqueous $NaHCO_3$, and brine. The residue obtained after drying and evaporation was chromatographed on silica gel with $CHCl_3$/MeOH as eluant to give the corresponding pure tripeptide.

Ac-Glu[-Cys(SBu^t)-Gly-OBu^t]-OMe (6a). Yield 92%; R_f = 0.54, $CHCl_3$/MeOH (95:5); $[\alpha]_D^{25}$ −43.6° (*c* 1, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.30 (9H, s, SBu^t), 1.44 (9H, s, OBu^t), 1.95−2.03 (1H, m, Glu β-CH_A), 2.00 (3H, s, Glu Ac), 2.16−2.20 (1H, m, Glu β-C_B), 2.33−2.38 (2H, m, Glu γ-CH_2), 3.06−3.09 (2H, m, Cys β-CH_2), 3.72 (3H, s, OMe), 3.80−3.99 (2H, m, Gly α-CH_2), 4.60 (1H, m, Glu α-CH), 4.75 (1H, m, Cys α-CH), 6.84 (1H, d, J = 7.48 Hz, Glu NH), 7.07 (1H, d, J = 7.92 Hz, Cys NH), 7.14 (1H, t, J = 5.27 Hz, Gly NH); ^{13}C NMR ($CDCl_3$) δ 23.26 (Glu Ac), 28.16 (Glu β-CH_2), 28.25 (OBu^t), 30.05 (SBu^t), 32.47 (Glu γ-CH_2), 42.02 (Cys β-CH_2), 42.34 (Gly α-CH_2), 48.51 (SBu^t), 52.02 (Glu α-CH), 52.79 (OCH_3), 53.01 (Cys α-CH), 82.55 (OBu^t), and 168.88, 170.43, 170.93, 172.75, and 172.86 (5× CO).

Boc-Glu-[Cys(SBu^t)-Gly-OMe]-OBu^t (6b). Yield 75%; R_f = 0.42, $CHCl_3$/MeOH (97:3); $[\alpha]_D^{25}$ −61.4° (*c* 1, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.32 (9H, s, SBu^t), 1.35 and 1.45 (18H, 2× s, OBu^t), 1.82−1.94 (1H, m, Glu β-CH_A), 2.14−2.18 (1H, m, Glu β-CH_B), 2.33−2.38 (2H, m, Glu γ-CH_2), 3.11−3.14 (2H, m, Cys β-CH_2), 3.74 (3H, s, OMe), 3.90−4.10 (2H, m, Gly α-CH_2), 4.26 (1H, m, Glu α-CH), 4.76 (1H, m, Cys α-CH), 5.27 (1H, d, J = 8.1 Hz, Glu NH), 7.0 (1H, d, J = 7.04 Hz, Cys NH), 7.09 (1H, t, J = 5.27 Hz, Gly NH); ^{13}C NMR ($CDCl_3$) δ 28.19 and 28.52 (2× OBu^t) 29.44 (Glu β-CH_2), 30.05 (SBu^t), 32.62 (Glu γ-CH_2), 41.49 (Gly α-CH_2), 41.64 (Cys β-CH_2), 48.48 (SBu^t), 52.57 (OMe), 53.01 (Cys α-CH), 53.47 (Glu α-CH), 80.21 and 82.50 (2× OBu^t), 156.16 (OCONH), and 170.19, 170.75, 171.69, and 172.88 (4× CO).

Fmoc-Glu-[Cys(SBu^t)-Gly-OBu^t]-OBu^t (6d). Yield 94%; R_f = 0.61, $CHCl_3$/MeOH (97:3); $[\alpha]_D^{25}$ −36.9° (*c* 1, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.25 (9H, s, SBu^t), 1.41 and 1.43 (18H, 2× s, OBu^t), 1.62−1.70 (1H, m, Glu β-CH_A), 1.80−1.95 (1H, m, Glu β-CH_B), 2.25−2.35 (2H, m, Glu γ-CH_2), 3.05−3.10 (2H, m, Cys β-CH_2), 3.85−3.96 (2H, m, Gly α-CH_2), 4.01 (1H, m, Fmoc α-CH), 4.30−4.41 (3H, m, Glu α-CH and Fmoc CH_2), 4.70 (1H, m, Cys α-CH), 5.63 (1H, d, J = 8.0 Hz, Glu NH), 6.80−6.92 (2H, m, Cys NH and Gly NH), 7.20−7.80 (8H, m, ArH); ^{13}C NMR ($CDCl_3$) δ 28.22 and 28.25 (2× OBu^t), 29.37 (Glu β-CH_2), 30.07 (SBu^t), 32.62 (Glu γ-CH_2), 41.72 (Cys β-CH_2), 42.38 (Gly α-CH_2), 47.40 (Fmoc α-CH), 48.56 (SBu^t), 52.99 (Glu α-CH), 53.94 (Cys α-CH), 67.29 (Fmoc β-CH_2), 82.54 and 82.80 (2× OBu^t), 120.19−144.10 (Ar), 156.71 (OCONH), and 168.69, 170.28, 171.37, and 172.67 (4× CO).

Ac-Glu-[Cys(SBu^t)-Gly-OH]-OMe (7a). The above-reported tripeptide **6a** (3.80 g, 7.48 mmol) was dissolved in TFA (9.5 mL). After 2 h at room temperature, the solution was evaporated to dryness and the residue was repeatedly evaporated with ether to give 7**a** in quantitative yield. This product was used without further purification (3.28 g, 97%).

Boc-Glu-[Cys(SBu^t)-Gly-OH]-OBu^t (7b). To a solution of the above-reported tripeptide **6b** (5.20 g, 9.19 mmol) in MeOH (21 mL) was added 1 N NaOH under stirring at room temperature. After 1 h, the pH was adjusted to 6 by 1 N $KHSO_4$ and the aqueous solution was concentrated to afford the corresponding tripeptide **7b** (4.36 g, 86%). This product was used without further purification.

H-Glu-[Cys(SBu^t)-Gly-OMe]-OMe TFA (7c). Deprotection of **6c** (4.0 g, 7.39 mmol) was performed as described for tripeptide **7a** to give **7c** (3.97 g, 99%). This product was used without further purification.

H-Glu-[Cys(SBu^t)-Gly-OBu^t]-OBu^t (7d). To a solution of the above-reported di-*t*-butyl ester **6d** (4.50 g, 6.17 mmol) in CH_2Cl_2 (44 mL) was added DBU (1.03 g, 6.79 mmol) at room temperature. After 20 min, the solution was evaporated to dryness and the residue was chromatographed on silica gel with $CHCl_3$/MeOH (97:3) as eluant to yield pure N-deprotected tripeptide di-*t*-butyl ester **7d** (2.50 g, 80%).

General Method for Coupling with L-Dopa. To an ice-cold solution of deprotected tripeptide (4.63 mmol) in dry DMF (25 mL) were added TEA (0.65 mL, 4.63 mmol) and isobutyl chloroformate (IBCF) (0.61 mL, 4.63 mmol) under stirring. After 15 min at −15 °C, L-dopa (4.63 mmol) in TEA (0.65 mL, 4.63 mmol) and dry DMF (30 mL) were added to the mixture at −15 °C with stirring. After 3 h at 0 °C and 16 h at 5 °C, the reaction mixture was filtered and the resulting solution was evaporated under vacuum. The residue was taken up in $CHCl_3$ and the organic layer was washed with 1 N $KHSO_4$, saturated aqueous $NaHCO_3$, and brine. The residue obtained after drying and evaporation was chromatographed on silica gel with $CHCl_3$/MeOH as eluant.

Ac-Glu-[Cys(SBu^t)-Gly-LD(Ac)$_2$-OMe]-OMe (8a). Yield 81%; R_f = 0.58, $CHCl_3$/MeOH (95:5); $[\alpha]_D^{25}$ −17.8° (*c* 1, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.31 (9H, s, SBu^t), 1.85−1.90 (2H, m, Glu β-CH_2), 2.13 (3H, s, Glu Ac), 2.21 (2H, m, Glu γ-CH_2), 2.27 and 2.28 (6H, 2× s, LD Ac), 2.99−3.16 (4H, m, Cys β-CH_2 and LD β-CH_2), 3.68 and 3.70 (6H, 2× s, OMe), 3.77−3.79 (1H, m, Gly α-CH_A), 4.04−4.10 (1H, m, Gly α-CH_B), 4.53−4.60 (2H, m, Glu α-CH and LD α-CH), 4.77 (1H, m, Cys α-CH), 6.77 (1H, d, J = 7.91 Hz, Glu NH), 6.95−7.08 (5H, m, 3× ArH, Cys NH, and LD NH), 7.52 (1H, t, J = 5.72 Hz, Gly NH); ^{13}C NMR ($CDCl_3$) δ 20.93 and 21.06 (2× LD Ac) 23.22 (Glu Ac), 27.91 (Glu β-CH_2), 30.08 (SBu^t), 31.82 (Glu γ-CH_2), 36.82 (LD β-CH_2), 40.76 (Cys β-CH_2), 43.20 (Gly α-CH_2), 48.57 (SBu^t), 51.87 (Glu α-CH), 52.71 and 53.01 (2× OMe), 53.23 (Cys α-CH), 53.35 (LD α-CH), 123.58−141.84 (LD Ar), and 168.91, 169.01, 170.65, 170.69, 170.77, 171.67, 172.84, and 173.21 (8× CO).

Boc-Glu-[Cys(SBu^t)-Gly-LD(Ac)$_2$-OMe]-OBu^t (8b). Yield 26%; R_f = 0.30, $CHCl_3$/MeOH (97:3); $[\alpha]_D^{25}$ = −15.5° (*c* 1, $CHCl_3$);

^{1}H NMR ($CDCl_3$) δ 1.26 (9H, s, SBu^t), 1.31 and 1.39 (18H, 2× s, OBu^t), 1.49−1.79 (2H, m, Glu β-CH_2), 2.04−2.10 (2H, m, Glu γ-CH_2), 2.17 and 2.23 (6H, 2× s, LD Ac), 2.93−3.13 (4H, m, Cys β-CH_2 and LD β-CH_2), 3.70 (3H, s, OMe), 3.64−3.66 (1H, m, Gly α-CH_A), 4.03−4.09 (2H, m, Gly α-CH_B and Glu α-CH), 4.48 (1H, m, LD α-CH), 4.79 (1H, m, Cys α-CH), 5.26 (1H, d, J = 8.1 Hz, Glu NH), 6.61 (1H, d, J = 7.05, Cys NH), 6.77 (1H, d, J = 6.3, LD NH), 6.87−7.02 (3H, m, ArH), 7.30 (1H, t, J = 5.7 Hz, Gly NH); ^{13}C NMR ($CDCl_3$) δ 20.97 and 21.07 (2× LD Ac), 25.07 (Glu β-CH_2), 28.20 and 28.63 (2× OBu^t) 30.10 (SBu^t), 31.88 (Glu γ-CH_2), 36.96 (LD β-CH_2), 40.13 (Cys β-CH_2), 43.12 (Gly α-CH_2), 48.51 (SBu^t), 52.71 (OMe), 52.95 (Cys α-CH), 53.19 (Glu α-CH), 53.63 (LD α-CH), 80.13 and 82.18 (OBu^t), 123.54−141.85 (LD Ar), 156.14 (OCONH), and 168.87, 168.92, 169.17, 170.67, 171.01, 171.89, and 173.58 (7× CO).

Ac-LD(Ac)$_2$-Glu-[Cys(SBu^t)-Gly-OMe]-OMe (8c). Yield 74%; R_f = 0.29, $CHCl_3$/MeOH (95:5); $[\alpha]_D^{25}$ = −29.8° (*c* 1, $CHCl_3$); ^{1}H NMR (DMSO-d_6) δ 1.26 (9H, s, SBu^t), 1.74 (3H, s, LD Ac), 1.78−1.82 (1H, m, Glu β-CH_A), 1.93−1.98 (1H, m, Glu β-CH_B), 2.14−2.19 (2H, m, Glu γ-CH_2), 2.22 and 2.24 (6H, 2× s, LD Ac), 2.66−3.07 (4H, m, Cys β-CH_2 and LD β-CH_2), 3.59 and 3.61 (6H, 2× s, OMe), 3.78−3.80 (2H, m, Gly α-CH_2), 4.23 (1H, m, Glu α-CH), 4.48−4.56 (2H, m, LD α-CH and Cys α-CH), 7.13−7.15 (3H, m, ArH), 8.11 (1H, d, J = 8.41 Hz, Cys NH), 8.21 (1H, d, J = 7.81 Hz, LD NH), 8.43 (1H, t, J = 5.71 Hz, Gly NH), 8.52 (1H, d, J = 7.51 Hz, Glu NH); ^{13}C NMR (DMSO-d_6) δ 20.98, 21.03, and 23.11 (3× LD Ac), 27.59 (Glu β-CH_2), 30.21 (SBu^t), 32.15 (Glu γ-CH_2), 37.91 (LD β-CH_2), 41.36 (Gly α-CH_2), 43.07 (Cys β-CH_2), 48.38 (SBu^t), 52.20 (Glu α-CH), 52.39 and 52.59 (2× OMe), 52.93 (Cys α-CH), 54.10 (LD α-CH), 123.72−142.15 (LD Ar), and 168.86, 168.93, 170.02, 170.68, 171.16, 172.04, 172.12, and 172.86 (8× CO).

Boc-LD(Ac)$_2$-Glu-[Cys(SBu^t)-Gly-OBu^t]-OBu^t (8d). Yield 63%; R_f = 0.41, $CHCl_3$/MeOH (97:3); $[\alpha]_D^{25}$ = −39.6° (*c* 1, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.30 (9H, s, SBu^t), 1.32, 135, and 1.36 (27H, 3× s, OBu^t), 1.88−1.95 (2H, m, Glu β-CH_2), 2.05−2.10 (2H, m, Glu γ-CH_2), 2.19 and 2.21 (6H, 2× s, LD Ac), 2.89−3.11 (4H, m, Cys β-CH_2 and LD β-CH_2), 3.71−3.78 (1H, m, Gly α- CH_A), 3.98−4.06 (1H, m, Gly α-CH_B), 4.35−4.46 (2H, m, Glu α-CH and LD α-CH), 4.68 (1H, m, Cys α-CH), 5.21 (1H, d, J = 6.1 Hz, LD NH), 7.01−7.20 (5H, m, Glu NH, Gly NH, and ArH), 7.47 (1H, t, J = 7.5 Hz, Cys NH); ^{13}C NMR ($CDCl_3$) δ 20.86 and 20.91 (2× LD Ac), 27.39 (Glu β-CH_2), 28.18, 28.26, and 28.51 (3× OBu^t), 30.10 (SBu^t), 32.04 (Glu γ-CH_2), 37.62 (LD β-CH_2), 41.40 (Cys β-CH_2), 42.26 (Gly α-CH_2), 48.32 (SBu^t), 52.53 (Cys α-CH), 52.88 (Glu α-CH), 55.28 (LD α-CH), 80.13, 82.18, and 82.48 (3× OBu^t), 123.65−142.14 (LD Ar), 156.10 (OCONH), and 168.43, 168.58, 168.92, 169.52, 170.96, 172.12, and 173.26 (7× CO).

Ac-Glu-[Cys-Gly-LD-OMe]-OMe (1). A solution of the foregoing tetrapeptide dimethyl ester disulfide **8a** (2.0 g, 2.74 mmol) in a mixture of *n*-PrOH/H_2O (2:1) (45 mL) was brought to pH 8.5 with 25% aqueous NH_3 and flushed with nitrogen. After 30 min, tri-*n*-butylphosphine (0.66 g, 3.29 mmol) was added and the stoppered flask was stirred at room temperature. After 1 h the reaction mixture was concentrated and subjected to column chromatography on silica gel with $CHCl_3$/MeOH (9:1) as eluant to afford the corresponding reduced tetrapeptide dimethyl ester **1** (0.91 g, 60%). R_f = 0.20, $CHCl_3$/MeOH (9:1); $[\alpha]_D^{25}$ −26.4° (*c* 1, MeOH); ^{1}H NMR (DMSO-d_6) δ: 1.72−1.77 (1H, m, Glu β-CH_A), 1.82 (3H, s, Glu Ac), 1.88−1.96 (1H, m, Glu β-CH_B), 2.18−2.20 (2H, m, Glu γ-CH_2), 2.22 (1H, s, Cys SH), 2.60−2.81 (4H, m, Cys β-CH_2 and LD β-CH_2), 3.33 and 3.58 (6H, 2× s, OMe), 3.67−3.70 (2H, m, Gly α-CH_2), 4.19 (1H, m, Glu α-CH), 4.29−4.35 (2H, m, LD α-CH and Cys α-CH), 6.38−6.60 (3H, m, ArH), 8.10 (1H, d, J = 7.5 Hz, Glu NH), 8.19−8.29 (2H, m, LD NH and Cys NH), 8.75 (1H, d, J = 3.6 Hz, Gly NH); ^{13}C NMR (DMSO-d_6) δ 22.93 (Glu Ac), 26.75 (Glu β-CH_2), 27.38 (Cys β-CH_2), 31.96 (Glu γ-CH_2), 37.10 (LD β-CH_2), 42.27 (Gly α-CH_2), 52.04 (Glu α-CH), 52.47 and 52.50 (2× OMe), 54.70 (Cys α-CH), 55.79 (LD α-CH), 116.10−145.66 (LD Ar), and 169.22, 169.30, 170.18, 170.81, 172.65, and 173.21(6× CO); MS (ESI) *m*/*z* 555 (M − H)$^-$. Anal. ($C_{23}H_{32}N_4O_{10}S$) C, H, N, S.

H-Glu-(Cys-Gly-LD-OH)-OH (2). The above-reported tetrapeptide **8b** (1.35 g, 1.63 mmol) was dissolved in TFA (26.5 mL). After 5 h at room temperature, the solution was evaporated to dryness and the residue was repeatedly evaporated with ether to give TFA•H-Glu-[Cys(SBu^t)-Gly-LD(Ac)$_2$-OMe]-OH (1.11 g, 86%). This compound, obtained in good yield, was used without further purification.

A solution of the foregoing trifluoracetate (1.11 g, 1.40 mmol) in a mixture of *n*-PrOH/H_2O (2:1) (30 mL) was brought to pH 8.5 with 25% aqueous NH_3 and flushed with nitrogen. After 30 min, tri-*n*-butylphosphine (0.34 g, 1.68 mmol) was added and the stoppered flask was stirred at room temperature. After 1 h, the reaction mixture was repeatedly washed with $CHCl_3$ and the pH of the aqueous solution was adjusted to 6.0 by use of 1 N $KHSO_4$. The solution was concentrated and subjected to column chromatography on Sephadex LH-20 with H_2O/MeOH (2:1) as eluant to afford the corresponding reduced compound **2** (0.33 g, 49%). R_f = 0.62, *n*-BuOH/AcOH/H_2O (4:5:1); $[\alpha]_D^{25}$ −37.4° (*c* 1, MeOH); ^{1}H NMR (D_2O) δ 1.90−2.01 (2H, m, Glu β-CH_2), 2.26−2.37 (2H, m, Glu γ-CH_2), 2.67−2.99 (4H, m, Cys β-CH_2 and LD β-CH_2), 3.61 (1H, m, Glu α-CH), 3.70−3.73 (2H, m, Gly α-CH_2), 4.31 (1H, m, LD α-CH), 4.51 (1H, m, Cys α-CH), 6.41−6.66 (3H, m, ArH); ^{13}C NMR (D_2O) δ 25.25 (Cys β-CH_2), 26.07 (Glu β-CH_2), 31.15 (Glu γ-CH_2), 35.99 (LD β-CH_2), 42.44 (Gly α-CH_2), 54.09 (LD α-CH), 54.18 (Glu α-CH), 55.98 (Cys α-CH), 116.32−144.03 (LD Ar), and 170.87, 172.70, 173.39, 173.95, and 175.03 (5× CO); MS (ESI) *m*/*z* 485 (M − H)$^-$. Anal. ($C_{19}H_{26}N_4O_9S$) C, H, N, S.

Ac-LD-Glu-[Cys-Gly-OMe]-OMe (3). The reaction was performed as described for tetrapeptide **1** with Ac-LD(Ac)$_2$-Glu-[Cys-(SBu^t)-Gly-OMe]-OMe (**8c,** 3.65 g, 5 mmol) in a mixture of *n*-PrOH/H_2O (2:1) (90 mL) and tri-*n*-butylphosphine (1.48 mL, 6 mmol) at pH 8.5 with 25% aqueous NH_3 to give the reduced tetrapeptide **3** in good yield (2.0 g, 72%). R_f = 0.34, $CHCl_3$/MeOH (9:1); $[\alpha]_D^{25}$ −17.9° (*c* 1, MeOH); ^{1}H NMR (DMSO-d_6) δ 1.73 (3H, s, LD Ac), 1.77−1.80 (1H, m, Glu β-CH_A), 1.96−1.99 (1H, m, Glu β-CH_B), 2.15−2.32 (2H, m, Glu γ-CH_2), 2.47 (1H, s, Cys SH), 2.62−2.80 (4H, m, Cys β-CH_2 and LD β-CH_2), 3.59 and 3.61 (6H, 2× s, OMe), 3.81−3.84 (2H, m, Gly α-CH_2), 4.21 (1H, m, Glu α-CH), 4.36−4.43 (2H, m, LD α-CH and Cys α-CH), 6.46−6.63 (3H, m, ArH), 8.01 (1H, d, J = 7.42 Hz, Glu NH), 8.41−8.47 (2H, m, LD NH and Cys NH), 8.66 (1H, br s, Gly NH); ^{13}C NMR (DMSO-d_6) δ 23.18 (LD Ac), 26.83 (Glu β-CH_2), 27.39 (Cys β-CH_2), 31.96 (Glu γ-CH_2), 37.59 (LD β-CH_2), 41.32 (Gly CH_2), 52.08 (Glu α-CH), 52.43 and 52.58 (2× OMe), 54.89 (Cys α-CH), 55.47 (LD α-CH), 115.79−145.44 (LD Ar), and 169.96, 170.82, 171.12, 172.13, 172.73, and 172.89 (6× CO); MS (ESI) *m*/*z* 555 (M − H)$^-$. Anal. ($C_{23}H_{32}N_4O_{10}S$) C, H, N, S.

H-LD-Glu-(Cys-Gly-OH)-OH (4). Removal of Boc and Bu^t groups from compound **8d** was obtained as described for **2**. Reduction of the resulting TFA•LD-Glu-[Cys(SBu^t)-Gly]-OH (0.45 g, 0.65 mmol) with tri-*n*-butylphosphine (0.16 g, 0.78 mmol) was performed as described for tetrapeptide **2** to afford H-LD-Glu-(Cys-Gly)-OH (**4**, 0.3 g, 93%). R_f = 0.29, *n*-BuOH/AcOH/H_2O (4:5:1); $[\alpha]_D^{25}$ −29.4° (*c* 1, MeOH); ^{1}H NMR (D_2O) δ 1.74−1.83 (1H, m, Glu β-CH_A), 1.90−1.94 (1H, m, Glu β-CH_B), 2.13−2.19 (2H, m, Glu γ-CH_2), 2.71−275 (2H, m, Cys β-CH_2), 2.83−2.98 (2H, m, LD β-CH_2), 3.54−3.57 (2H, m, Gly α-CH_2), 3.93 (1H, m, Glu α-CH), 4.02 (1H, m, LD α-CH), 4.35 (1H, m, Cys α-CH), 6.49−6.69 (3H, m, ArH); ^{13}C NMR (D_2O) δ 25.64 (Cys β-CH_2), 27.54 (Glu β-*C*H$_2$), 31.93 (Glu γ-*C*H$_2$), 36.10 (LD β-*C*H$_2$), 43.13 (Gly α-CH_2), 54.47 (LD α-CH), 54.87 (Glu α-CH), 55.70 (Cys α-*C*H), 116.67−144.41 (LD Ar), and 168.61, 171.93, 175.85, 176.01, and 177.01 (5× *C*O); MS (ESI) *m*/*z* 485 (M − H)$^-$. Anal. ($C_{19}H_{26}N_4O_9S$) C, H, N, S.

Animals. Male Wistar rats (n = 100) (Harlan, UD, Italy) weighing 250−300 g were employed. Twenty rats were assigned to each treatment group. The animals were housed in plastic (Makrolon) cages (five rats/cage) in a temperature-controlled room

(21 ± 5 °C) and maintained on a laboratory diet and water ad libitum. The light/dark cycle was from 7 a.m. to 7 p.m.

Drug Administration. Benserazide hydrochloride, a peripheral dopa-decarboxylase inhibitor, was dissolved in water, whereas LD and codrugs **1** and **3** were dissolved in dimethyl sulfoxide. All animals received a dose of benserazide (16.36 mg/kg) combined with LD (65.46 mg/kg) or **1** or **3** (184.79 mg/kg) in equimolar doses (0.332 mmol/kg). The drugs were given at a volume of 5 mL/kg in a single oral administration by intragastric tube. A control group ($n = 20$), receiving only water (5 mL/kg), was included in the experiment for behavioral study. This experiment was carried out in accordance with the Italian government's guidelines for the care and use of laboratory animals (D.L. n. 116 of January 27, 1992).

Pharmacokinetic Analysis. After slight anaesthesia with carbon monoxide, the blood of rats was collected for the determination of LD metabolites by cardiac puncture from five rats of each group and then collected in vials containing heparin (250 I.U.). The blood sampling schedules were 30 min and 1, 2, 3, 4, 5, 6, and 12 h after treatment with drugs. All samples were centrifuged at 2000*g* for 10 min and the plasma samples were kept at −80 °C until analysis. Aliquots (400 μL) were taken at various times and deproteinized by mixing with 40 μL of 4 M perchloric acid. After centrifugation for 5 min at 5000*g* and filtration (Millipore 0.45 μm), 10 μL of the layer supernatant was chromatographed as described below. The amounts of LD were plotted as a function of incubation time.

Biochemical Assays. The pro-oxidant activity of LD and its synthetic derivatives was analyzed by the $Fe(II)/H_2O_2$-induced degradation of deoxyribose assay conducted in the presence of EDTA essentially as previously described.[24,50] Briefly, the reaction mixtures contained, in a final volume of 1.0 mL, the following reagents at the final concentrations stated: deoxyribose (2.8 mM), potassium phosphate buffer, pH 7.4 (100 mM), increasing concentrations of LD or LD derivatives (1−50 μM), $(NH_4)_2Fe(SO_4)_2$ (50 μM), EDTA (0.1 mM), and H_2O_2 (1 mM). Fresh solution of ferrous ammonium sulfate was prepared in deaerated water immediately before each experiment and was used to start the reactions, which were carried out at 37 °C for 30 min. The extent of deoxyribose degradation was monitored by the formation of malondialdehyde (MDA) determined by the addition of 1 mL of 1% (w/v) thiobarbituric acid (TBA) in 50 mM NaOH and 1 mL of 2.8% (w/v) trichloroacetic acid. After heating at 100 °C for 30 min, the reaction solutions were cooled, and the resulting absorbance was read at 532 nm against appropriate blanks. LD and LD derivatives were prepared fresh in 0.01 M HCl; control experiments showed that the volumes of stock solutions added did not lead to any appreciable change in the pH of the reaction mixtures. Other control experiments showed that none of the compounds tested interfered with the assay (no effect when added at the end of the incubation, just before addition of TBA reagents), nor did they generate TBA-reactive material (controls with deoxyribose omitted).

For in vivo assays, blood from five rats of each group was collected at 1.5 h after drug administration in vials containing heparin (250 I.U.) and washed three times with physiological solution before the experiments. The blood of each subject was aliquoted and centrifuged at 2000*g* for 10 min.

GPx and SOD activities were measured in the remaining aliquot of whole blood. The former was determined by the method of Paglia and Valentine,[51] while the latter was measured by use of Bioxytech SOD-525 (Oxis International Health Products, Inc., Portland, OR). This method is based on the SOD-mediated increase in the rate of autoxidation of 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo[*c*]-fluorene in aqueous alkaline solution to yield a chromophore with maximum absorbance at 525 nm.[52]

Open-Field Studies. Automated locomotor activity boxes (MedAssociates, VT 05478) were used to quantify behavioral activity. Each animal was placed in the activity box, a square plastic box measuring 43 cm × 43 cm × 30 cm, and spontaneous locomotor activity parameters were monitored during the dark phase (6:00−7:00 a.m.). Activity was recorded for 5 min, starting 2 min after the animal was placed in the test cage. Each rat was automatically recorded by interruptions of two orthogonal light beams (3.5 and 13.0 cm above the activity box floor), which were connected to automatic software (Activity Monitor, MedAssociates). Each rat was used only once, and a total of 10 rats was used for each treatment. The behavioral tests were performed 1.5 h after treatment with drugs and before blood collection. The behavioral parameters observed were locomotion (number of ambulatory episodes), rearings (number of rears), stereotype counts (number of grooming movements), and number of entries and time spent in the central square of the open field. Locomotion counts were recorded when the low row of photocells was interrupted, while rearing counts were recorded by interruptions in the higher row of photocells. The open field is divided into two squares, and we recorded the number of entries into the central area (25 cm × 25 cm), which can be considered an unprotected area for rats. Entries into the central squares of the arena provided a measure of anxiety-like behavior.[53] Rats that spend less time in the center are regarded as more ''anxious". Between each test session, the apparatus was cleaned with alcohol (10%) and dried with a cloth.

HPLC UV Assays. All analyses were carried out on a Waters 1525 binary HPLC pump, equipped with a Waters 2996 photodiode array detector, a 20-μL Rheodyne injector, and a computer integrating apparatus. The column was a Waters X-Terra RP_{18} (5 μm, 3.0 × 15 mm); the mobile phase was a water/methanol mixture. The flow rate was 0.5 mL/min.

HPLC-EC Assays. For measurements of LD and DA, striatum from each animal (treated as described above) was individually homogenized for 2 min with a Dyna-Mix homogenizer (Fisher Scientific) in 500 μL of 0.05 N perchloric acid solution containing (w/v) 0.064% 1-octanesulfonic acid sodium salt, 0.060% heptanesulfonic acid sodium salt, 0.004% sodium EDTA, 0.010% sodium metabisulfite, and 25 ng/mL 3,4-dihydroxybenzylamine (DHBA) as an internal standard. The whole procedure was carried out on ice.[38] The resulting homogenate was then centrifuged at 4500*g* for 10 min, and the supernatant was filtered through a 0.45 μm Millipore filter. The filtrate was set in a low volume insert vial, and a portion was injected directly into the liquid chromatography equipment (10 μL). The HPLC system consisted of a PU-2080 Plus pump (Jasco), a Rheodyne 7295 injector with a 10 μL loop, and an ESA Coulochem III detector. Separation was achieved on a Waters Symmetry RP-C18 column (4.6 mm × 150 mm, 5 μm). The mobile phase consisted of 0.045 M monobasic sodium phosphate, 0.001 M 1-octanesulfonic acid sodium salt, 0.006% triethylamine, 0.015% 100 μM sodium EDTA, and 6% acetonitrile. The pH of the mobile phase was adjusted to 3.0 with *o*-phosphoric acid. The mobile phase was filtered and degassed by vacuum. A flow rate of 1 mL/min was used in all experiments. The electrochemical detection system included a high-sensitivity dual detector analytical cell: detector 1 set at +350 mV and detector 2 set at −180 mV. The signal was recorded by use of the response from detector 1.

Monoamine stock solutions were prepared at a concentration of 1 mg/mL (as a free base) in 0.05 N perchloric acid containing 0.064% 1-octanesulfonic acid sodium salt, 0.060% heptanesulfonic acid sodium salt, 0.004% sodium EDTA, and 0.010% sodium metabisulfite. These standard solutions were freshly prepared every week and stored at 4 °C for use right away.

The monoamines and their metabolites were identified on the basis of retention time. The concentration of each compound was established from the peak area ratio with DHBA as internal standard. Final values were expressed in terms of picomoles per gram of tissue. Measurements were performed in triplicate for each original sample.

Aqueous Solubility. The aqueous solubilities of codrugs **1**−**4** were determined in deionized water, in 0.02 M phosphate buffer, pH 7.4; in 0.02 M sodium acetate buffer, pH 5.0; and in 0.02 M hydrochloric acid buffer, pH 1.3. An excess of compound was added to buffer solutions and the suspensions were shaken for 15 min and filtered (Millipore 0.45 μm). The filtered solutions were analyzed by HPLC.

Octanol/Water Partition Coefficients (Log P). Octanol/water partition coefficients were determined by placing approximately 5 mg of compounds **1**−**4** in 1 mL of anhydrous *n*-octanol, shaking

vigorously for about 2 min, and filtering. An equal volume of phosphate buffer, pH 7.4, was added, and the mixture was equilibrated by repeated inversions of up to 200 times for 5 min and then allowed to stand for 30 min for the phases to fully separate. Thereafter, the respective phases were analyzed by HPLC.

Kinetics of Hydrolysis in Aqueous Solutions. A 0.02 M hydrochloric acid buffer, pH 1.3, as nonenzymatic simulated gastric fluid (SGF); a 0.02 M phosphate buffer, pH 7.4; and a 0.02 M sodium acetate buffer, pH 5.0, were used in this study. Reactions were initiated by adding 1 mL of 10^{-4} M stock solution (in acetonitrile) of the respective codrug to 10 mL of the appropriate thermostated (37 ± 0.5 °C) aqueous buffer solution, containing 20% acetonitrile. At appropriate time intervals, samples of 20 μL were withdrawn and analyzed by HPLC. Pseudo-first-order rate constants (K_{obs}) for the hydrolysis of the codrugs were then calculated from the slopes of the linear plots of log (% residual codrugs) against time. The experiments were run in triplicate for each codrug, and the mean values of the rate constants were calculated.

Kinetics of Hydrolysis in Plasma. Plasma from rats and humans was obtained by centrifugation of blood samples containing 0.3% citric acid at 3000*g* for 15−20 min. Plasma fractions (4 mL) were diluted with 0.02 M phosphate buffer (pH 7.4) to give a final volume of 5 mL (80% plasma). Incubations were performed at 37 ± 0.5 °C in a shaking water bath. The reactions were initiated by adding 100 μL of a stock solution of drug (1 mg/mL in acetonitrile) to 5 mL of preheated plasma. Aliquots (100 μL) were taken at various times and deproteinized by mixing with 200 μL of 0.01 M HCl in methanol. After centrifugation for 5 min at 5000*g*, 10 μL of the layer supernatant was chromatographed as described above. The amounts of remaining intact codrug were plotted as a function of incubation time.

Statistical Analysis. The experimental data are expressed as mean values ± SE of five rats used. The significance of differences among different treatment groups was calculated by ANOVA followed by the Newman-Keuls test. P values < 0.05 were considered statistically significant.

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Supporting Information Available: Elemental analyses of codrugs **1**−**4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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