L-Dopa− and Dopamine−(*R*)-α-Lipoic Acid Conjugates as Multifunctional Codrugs with Antioxidant Properties

Antonio Di Stefano,*[,§] Piera Sozio,[§] Alessandra Cocco,[§] Antonio Iannitelli,[§] Eleonora Santucci,[‡] Mara Costa,[†] Laura Pecci,[†] Cinzia Nasuti,[||] Franco Cantalamessa,[||] and Francesco Pinnen[§]

Dipartimento di Scienze del Farmaco, Università "G. D'Annunzio", Via dei Vestini 31, 66100 Chieti, Italy, Dipartimento di Studi di Chimica e Tecnologia delle Sostanze Biologicamente Attive, Università "La Sapienza", P.le A.Moro, 00185 Roma, 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 multifunctional codrugs (**1**−**4**), obtained by joining L-Dopa (LD) and dopamine (DA) with (*R*)-α-lipoic acid (LA), was synthesized and evaluated as potential codrugs with antioxidant and iron-chelating properties. These multifunctional molecules were synthesized to overcome the pro-oxidant effect associated with LD therapy. The physicochemical properties, together with the chemical and enzymatic stabilities of synthesized compounds, were evaluated in order to determine both their stability in aqueous medium and their sensitivity in undergoing enzymatic cleavage by rat and human plasma to regenerate the original drugs. The new compounds were tested for their radical scavenging activities, using a test involving the Fe (II)−H_2O_2-induced degradation of deoxyribose, and to evaluate peripheral markers of oxidative stress such as plasmatic activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) in the plasma. Furthermore, we showed the central effects of compounds **1** and **2** on spontaneous locomotor activity of rats in comparison with LD-treated animals. From the results obtained, compounds **1**−**4** appeared stable at a pH of 1.3 and in 7.4 buffered solution; in 80% human plasma they were turned into DA and LD. Codrugs **1**−**4** possess good lipophilicity (log $P > 2$ for all tested compounds). Compounds **1** and **2** seem to protect partially against the oxidative stress deriving from auto-oxidation and MAO-mediated metabolism of DA. This evidence, together with the "in vivo" dopaminergic activity and a sustained release of the parent drug in human plasma, allowed us to point out the potential advantages of using **1** and **2** rather than LD in treating pathologies such as Parkinson's disease, characterized by an evident decrease of DA concentration in the brain.

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder associated primarily with loss of dopamine (DA) neurons in the nigrostriatal system.[1] Current therapy for PD is essentially symptomatic, and L-Dopa (LD), the direct precursor of DA, is the treatment of choice. Oxidative stress plays a central role in the pathogenesis of PD, and LD may paradoxically contribute to the progression of the disease because of its pro-oxidant properties, deriving from its auto-oxidative metabolism generating a variety of free radicals.[2] In particular, it was demonstrated that systemic administration of LD in the rat increases the production of free radicals in the substantia nigra;[3] in addition, cell-death, both of the necrotic and apoptotic types, was observed in neuronal and nonneuronal cell cultures treated with LD.[4] Experimental studies have also shown that LD alters cellular energy metabolism, probably by inducing oxidative damage of specific enzymes of the mitochondrial respiratory chain.[5] Analogously, it was observed that the pretreatment of mesencephalic cultures of dopaminergic neurons with rotenone, an inhibitor of mitochondrial enzyme complex I, enhances the vulnerability of these cells to LD toxicity.[6] Whether LD is toxic per se or because of its biotransformation to DA, which also causes cell death in various cell culture systems, is unclear.[7] Furthermore, Miller et al. confirmed that catecholamines, including the neurotransmitter DA and its biochemical precursor LD, are subject to autoxidation.[8] This process, which is accelerated in the presence of pro-oxidative transition metals, such as iron and copper, is enhanced by the presence of neuromelanin in dopaminergic neurons, due to its reported ability to accumulate iron and consequently acting through promotion of Fenton and Haber−Weiss reactions with production of potentially cytotoxic reactive oxygen species (ROS).[9]

These ROS have been hypothesized to play a role in the progressive and selective loss of nigrostriatal dopaminergic neurons that occurs in aging and in neurodegenerative disorders such as PD and Alzheimer disease.[10] If correct, this hypothesis suggests that inhibition of catecholamine autoxidation and the scavenging of ROS produced by such oxidation are important strategies for preventing or slowing down the progression of aging and aged-related neurodegenerative disorders. As is well-known, low molecular weight natural free radical scavengers such as glutathione, vitamin E, carnosine, and ascorbic acid have been extensively studied as useful neuroprotective agents.[11−13] However, the use of these natural antioxidants as therapeutic agents is limited, mainly due to the marginal efficiency of these scavengers to cross the blood−brain barrier. Conversely, antioxidant (e.g. ascorbate or α-tocopherol) interventions that do not affect iron accumulation have shown only limited efficacy in lowering oxidant stress in the aging brain.[14,15] Thus new,

* Corresponding author. Tel.: +39-871-3555230; fax: +39-871-3555267; e-mail: adistefano@unich.it.
[§] Università "G. D'Annunzio".
[‡] Dipartimento di Studi di Chimica e Tecnologia delle Sostanze Biologicamente Attive, Università "La Sapienza".
[†] Dipartimento di Scienze Biochimiche, Università "La Sapienza".
[||] Università di Camerino.

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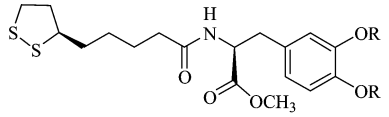
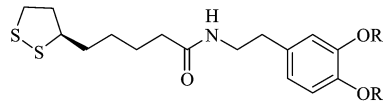


1: R=$COCH_3$
2: R=H



3: R=$COCH_3$
4: R=H

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

nontoxic, therapeutic agents that improve both alterations in iron and antioxidant status may be needed.

(*R*)-α-Lipoic acid (LA) is a dithiol compound normally bound to lysine residues of mitochondrial α-keto acid dehydrogenases. However, supplementing the diet with LA results in a transient accumulation of non-protein-bound LA. Moreover, LA readily crosses the blood−brain barrier and accumulates in all neuronal cell types.[16] There, cytosolic and mitochondrial dehydrogenases rapidly reduce it to dihydrolipoic acid (DHLA).

Previous in vitro studies showed that DHLA lowers the redox activities of non-protein-bound iron and copper.[17] In addition, other investigators showed that the metal-chelating properties of LA contribute to its $HO^{\bullet}$ radical scavenging effects.[18] More recently, Goralska and co-workers reported beneficial effects of LA treatment in preventing iron accumulation in lens epithelial cells.[19] Thus, it is plausible that LA may also be beneficial in normalizing the adverse effects of iron accumulation in the aging brain.

Starting from these data, our study was focused on providing molecular combinations obtained joining an antioxidant molecule with a therapeutic compound also able to generate a targeted antioxidant. These compounds could permit a targeted delivery of the antioxidant moiety directly to specific groups of cells, including neurons, where cellular stress is associated with pathology, including that associated with normal aging and neurodegenerative disorders.[20] With regard to PD, an analysis of the literature reveals that despite antioxidant therapy having been explored in a number of pathological conditions, the joining of an antioxidant molecule to a group capable of targeting a specific population of dying cells has not so far been considered.

In particular, we proposed the synthesis of novel molecular combinations (**1**−**4**) in which LD and DA are linked to antioxidant and iron-chelating agents such as LA (Figure 1). These multifunctional codrugs, containing antioxidant molecules whose benefits have been demonstrated in several neurodegenerative disorders, could represent useful dopaminergic agents devoid of the pro-oxidant effects associated with the presence of the catecholic moiety.[21]

Chemistry

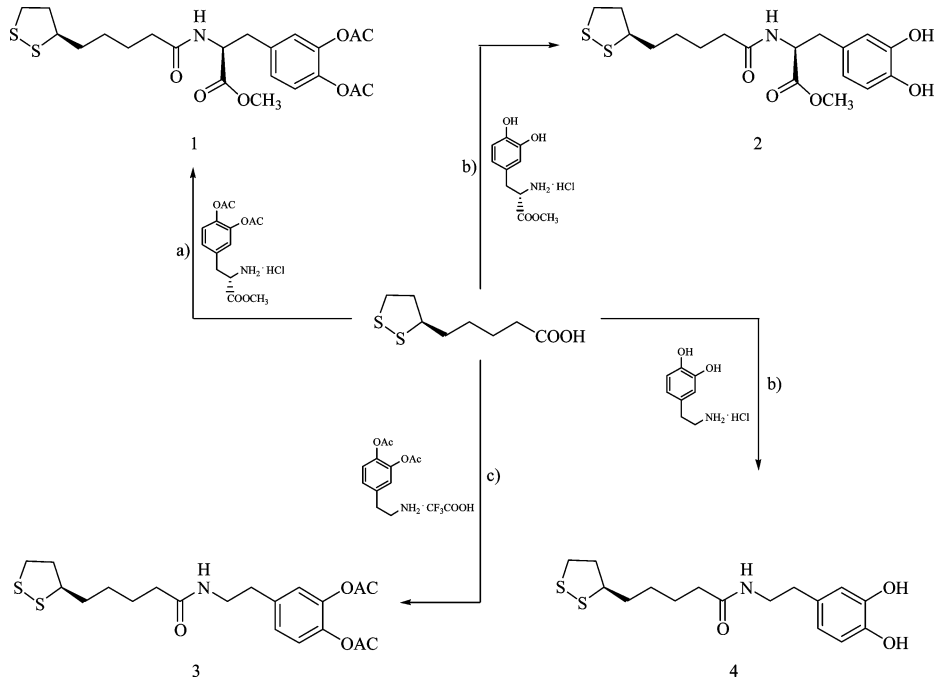
The amides **1**−**4** were synthesized by the classical methods through the interaction of (*R*)-α-lipoic acid with 3,4-diacetyloxy-L-phenylalanine methyl ester, 3,4-dihydroxy-L-phenylalanine methyl ester, 2-(3,4-diacetoxy)-phenylethylamine, and 2-(3,4-dihydroxy)-phenylethylamine, respectively.[22] The synthesis is outlined in Scheme 1.

Results and Discussion

The evaluation of solubility and apparent coefficient partition (log *P*) of codrugs **1**−**4** was performed by means of classical approach based on the saturation shake-flask method.[23−25] The concentration of codrugs was performed using HPLC with UV detection.

Lipophilicity is an important factor controlling the interaction of drugs with biological membranes. It is generally accepted that good absorption of an orally administered drug could be obtained when the log *P* value is more than 2 and the aqueous solubility is more than 10 μg/mL.[26] To assess this potential, the log *P* of the studied compounds **1**−**4** were determined in *n*-octanol/phosphate buffer of pH 7.4. The concentrations of codrugs in *n*-octanol and buffer layers were evaluated by correlating the peak areas in HPLC to a known concentration

Scheme 1[a]



[a] Reagents: (a) dioxane, Et_3N, DCC; (b) DCC, DMAP, anhydrous pyr; (c) anhydrous CH_2Cl_2, Et_3N, DCC.

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

compd	log P^a	solubility in water[a] (μg/mL)	solubility at pH 1.3[a] (μg/mL)	solubility at pH 7.4[a] (μg/mL)	solubility at pH 5.0[a] (μg/mL)
1	2.77 (±0.12)	233 (±12)	39 (±2)	38 (±2)	24 (±1)
2	2.16 (±0.10)	12 (±1)	insoluble	13 (±2)	11 (±1)
3	3.65 (±0.16)	49 (±4)	0.47 (±0.02)	52 (±3)	47 (±3)
4	3.40 (±0.17)	0.36 (±0.2)	0.87 (±0.04)	1.1 (±0.05)	insoluble

[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

	pH 1.3[a]		pH 5.0[a]		pH 7.4[a]	
compd	$t_{1/2}$ (h)	K_{obs} (h^{-1})	$t_{1/2}$ (h)	K_{obs} (h^{-1})	$t_{1/2}$ (h)	K_{obs} (h^{-1})
1	51.64 (±1.81)	0.013 (±0.9 × 10^{-3})	50.73 (±2.03)	0.014 (±0.5 × 10^{-3})	12.83 (±0.59)	0.054 (±2.4 × 10^{-3})
2	68.27 (±3.41)	0.010 (±0.5 × 10^{-3})	> 200		66.04 (±3.30)	0.010 (±5.0 × 10^{-3})
3	52.29 (±2.41)	0.013 (±0.6 × 10^{-3})	200.39 (±8.42)	0.003 (±0.1 × 10^{-3})	19.11 (±0.75)	0.036 (±1.4 × 10^{-3})
4	85.17 (±2.56)	0.008 (±0.2 × 10^{-3})	97.08 (±2.43)	0.007 (±0.2 × 10^{-3})	57.70 (±2.65)	0.012 (±0.5 × 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

	rat plasma[a]		human plasma[a]	
compd	$t_{1/2}$ (min)	K_{obs} (min^{-1})	$t_{1/2}$ (min)	K_{obs} (min^{-1})
1	immediate hydrolysis		52.8 (±4.2)	0.012 (±2 × 10^{-3})
2	immediate hydrolysis		97.8 (±4.2)	0.007 (±1 × 10^{-3})
3	immediate hydrolysis		10.3 (±0.5)	0.067 (±2 × 10^{-3})
4	144.6 (±7.2)	0.005 (±1 × 10^{-3})	81.0 (±0.6)	0.009 (±2 × 10^{-3})

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

of the compounds. The obtained values of log *P* and solubility are listed in Table 1. Tested compounds showed log *P* more than 2, indicating that codrugs **1**−**4** meet the requirements for gi absorption. In particular codrugs **1**−**3** possess the requirements for good oral absorption.

All new compounds were also assayed "in vitro" to evaluate their chemical and enzymatic stability. The kinetics of chemical hydrolysis was studied at 37 °C in buffer solutions of pH 1.3, 5.0, and 7.4. (Table 2).[27] The reactivities to chemical hydrolysis were evaluated by pseudo-first-order rate constants, obtained from slopes of semilogarithmic plots of the codrug concentrations against time. The decomposition product has not yet been identified. The rate data show that all the codrugs are stable under buffer solutions; in particular, a considerable chemical stability of all codrugs was observed at pH 1.3 ($t_{1/2}$ > 52 h) nonenzymatic simulated gastric fluid (SGF). This stability implies that compounds **1**−**4** pass unhydrolyzed through the stomach after oral administration. At pH 7.4, all the compounds are stable enough ($t_{1/2}$ > 12 h) to be absorbed intact from the intestine.

In 80% rat plasma (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. These derivatives were identified by LC/MS and NMR analysis. The data are listed in Table 3. Hydrolysis in 80% human plasma (human plasma containing 20% 0.02 M phosphate buffer, pH 7.4) generally proceeds more slowly with formation of LD. The faster hydrolysis in rat than in human plasma may be ascribed to the reportedly different enzyme systems that are highly efficient in rat plasma.[28] The degradation process was found correlated with first-order kinetics, and the LD was released in quantitative amounts. The rate constants (K_{obs}) and the corresponding half-life times are shown in Table 3. Figure 2 shows LD plasma concentration trends obtained in rats over time following administration of codrugs **1** and **2** and of LD. The values of LD plasma concentration at 3 h postdose were ca. 120 μg/mL for compound **1**, 90 μg/mL for compound **2**, and 75 μg/mL for LD, with rapid decrease of concentration levels 90 min after LD administration. Human and animal biochemical investigations clearly confirm that wearing-off phenomenon or end-of-dose deterioration is directly related to LD plasma levels fluctuation after long-term PD with chronic LD therapy.[29,30] For these reasons, codrugs **1** and **2** prolong plasma LD levels and could be beneficial in the treatment of motor fluctuation.

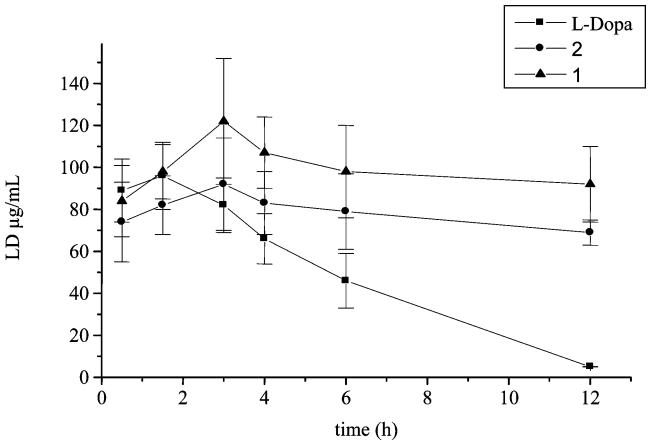


Figure 2. Plasma concentration profile of LD after administration of LD, **1**, and **2** in rats. Data are expressed as mean ± SE. Each experiment was performed in triplicate.

In the present study, we assessed the antioxidant efficacy of codrugs that could be used also in therapy as potential neuroprotective agents. The evaluation of peripheral markers of oxidative stress allowed us to compare rats treated with compounds **1** and **2** containing an antioxidant part and those treated with LD on the total antioxidant status (TAS), as well as 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 oxidative stress produced by LD.[31,32]

Table 4. Values of Total Antioxidant Status (TAS), Superoxide Dismutase (SOD) and Glutathione Peroxidase (GPx) Activities, 1 and 3 h after the Administration of LD, **1**, and **2** in Rats[a]

	1 h			3 h		
	TAS (mmol/L)	SOD (U/g·Hb)	GPx (U/g·Hb)	TAS (mmol/L)	SOD (U/g·Hb)	GPx (U/g·Hb)
L-Dopa	1.56 (±0.03)	545.50 (±16.03)	1003.40 (±19.88)	1.70 (±0.06)	523.00 (±36.47)	1034.25 (±40.91)
1	1.55 (±0.02)	444.60 (±63.03)	1073.25 (±24.49)[b]	1.48 (±0.06)	318.33 (±24.92)[b]	1107.00 (±37.29)
2	1.55 (±0.08)	308.33 (±3.67)[b,c]	1097.25 (±6.99)[a]	1.48 (±0.05)	462.50 (±73.73)	1167.67 (±24.17)[b]

[a] Data are expressed as mean ± SE of five experiments. [b] $P < 0.05$ compared to LD-treated group. [c] $P < 0.05$ compared to **1**-treated group.

Both **1** and **2** were observed to induce an increase of plasmatic GPx activity 1 h after drug administration. Besides, this effect was still noted 3 h after administration of **2**. However, the higher enzymatic activity of treated groups could indicate a decreased production of free radicals, a hypothesis supported by a previous study where a line of PC12 cells overexpressing GPx protected the cell damage induced by LD exposure.[33,34] With regard to the SOD activity, the data obtained indicate that compounds **1** and **2** induced a decrease of SOD activity 1 and 3 h, respectively, after drug administration. This behavior could be a consequence of the lesser production of superoxide anion ($O_2^{\cdot -}$) generated during the autoxidation of DA, following treatment with **1** and **2**. As reported in our previous studies, xenobiotics induced oxidative stress in rats by elevated levels of $O_2^{\cdot -}$ followed by increased activity of SOD.[35,36]

It has been suggested that the TAS of plasma is due to the relative concentration in endogenous plasma antioxidants, albumin, and uric acid, which constitute most of it (about 70%), and in molecules maintaining antioxidant substances in the reduced form.[37] However, despite the observed increase of enzymatic protection against oxidative stress produced by dopaminergic activity, the administration of **1** or **2** had no significant effect on TAS. One can speculate that the magnitude of the changes in plasma antioxidant concentrations might have been too small to significantly increase the TAS.

On the whole, one of the potential advantages of using **1** and **2** rather than LD in treating PD is that, via their antioxidant properties, they seem to protect in part against the oxidative stress deriving from autoxidation and the MAO-mediated metabolism of DA.

Table 4 shows the activities of those antioxidant enzymes that form the primary defense system against ROS in the plasma, 1 and 3 h after drug administration: no significant modifications were revealed in the TAS of plasma among the three groups (LD, **1**, and **2**).

GPx activity (1 h after treatment) was significantly ($P < 0.05$) increased in plasma of **1**- (1073.25 ± 24.49 U/g Hb) and **2**-treated rats (1097.25 ± 6.99 U/g Hb) compared with the LD-treated ones (1003.4 ± 19.88 U/g Hb). Three hours after treatment, the rise of GPx activity caused by treatment with **1** (1107 ± 37.29 U/g Hb) fell to the levels of the LD-treated group (1034.25 ± 40.91 U/g Hb), whereas those of the **2**-treated group remained at higher values (1167.67 ± 24.17 U/g Hb) ($P < 0.05$) with respect to those of the LD-treated group.

One hour after treatment with **2**, a significant reduction of the plasmatic SOD activity (308.33 ± 3.67 U/g Hb) was measured compared to both the **1**- (444.6 ± 63.03 U/g Hb) and LD- (545.5 ± 16.03 U/g Hb) treated groups ($P < 0.05$). After 3 h, the rats treated with **1** showed a significant decrease of plasmatic SOD activity compared with the **2**- and LD-treated rats (Table 4) ($P < 0.05$).

To better evaluate whether the LD pro-oxidant ability was affected by the chemical modification present in the selected synthetic derivative **2**, we compared the effect of codrug **2** on the Fe(II)−H_2O_2-induced degradation of deoxyribose (Figure 3) using as referred compound the *N*-acetyl derivative of LD. As expected, LD (from 10 to 100 μM) caused a concentration-dependent increase of deoxyribose degradation which leveled off at about 100 μM LD. Compound **2** (an *N*-lipoyl derivative of LD) and *N*-acetyl derivative of LD, over the same concentration range, exert pro-oxidant properties significantly lower than LD itself. Since the two derivatives have been synthesized as methyl esters, LD methyl ester was also tested. As shown in Figure 3, this chemical modification does not affect the pro-oxidant activity of LD. These findings clearly indicate that modification of the amino group of LD produces molecules with a decreased susceptibility to iron-induced autoxidation and, thus, may play an important role in modulating its pro-oxidant activity.

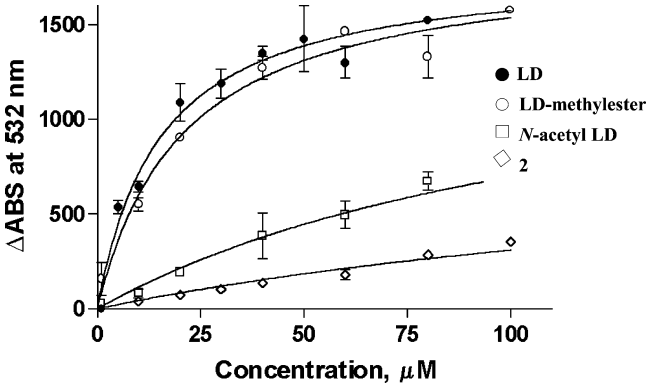


Figure 3. Pro-oxidant effect of LD and its derivatives on Fe(II)/EDTA/H_2O_2-induced deoxyribose degradation. The data are presented as increases of absorbance at 532 nm with respect to a control containing deoxyribose alone ($A_{532} = 0.404$). Results are the mean ± SEM of three separate experiments performed in duplicate.

To evaluate the dopaminergic activity of equimolar doses of compounds, we studied the effects of **1** and **2** on spontaneous locomotor activity of rats in comparison with LD-treated animals.

As can be observed, drug administration, 1, 2, and 3 h after gavage led to a pattern of behavioral depression in the treated groups compared to the control group, which received vehicle only. Figure 4 shows the responses obtained 1 h after the administration of compounds. The results obtained after treatment with **2** on locomotion (256.6 ± 120.7), rearing (7.6 ± 2.56), and grooming (310.4 ± 63.64) are similar to those with LD (283.67 ± 101.96, 16 ± 5.57, 442 ± 56.48, respectively). With regard to animals treated with **1**, grooming episodes were significantly ($P < 0.05$) decreased (231 ± 64.75) compared to the LD-treated group (442 ± 56.48). The behavioral responses 2 h after drug administration are reported in Figure 5. As shown, only treatment with **2** induced a significant decrease of grooming (342.4 ± 79.45) compared to the group treated with LD (577± 66.02) ($P < 0.05$). Three hours after treatment, no significant modifications were revealed in behavioral activities among the three treated groups (Figure 6). With the behavioral activities test, the active and recreative behavioral phenomena could be evaluated by measuring locomotion and rearing, on one hand,

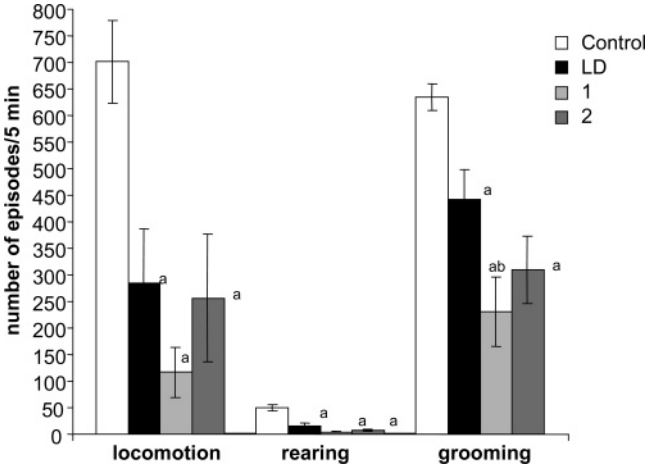


Figure 4. Locomotion, rearing, and grooming, 1 h after administration of LD, **1**, and **2** in rats. Data are expressed as mean ± S. E. of five experiments. [a]$P < 0.05$ compared to control group. [b]$P < 0.05$ compared to LD-treated group.

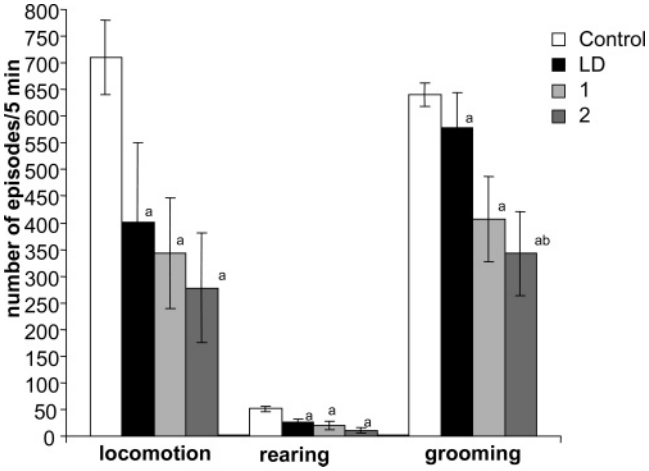


Figure 5. Locomotion, rearing, and grooming, 2 h after administration of LD, **1**, and **2** in rats. Data are expressed as mean ± SE of five experiments. [a]$P < 0.05$ compared to control group. [b]$P < 0.05$ compared to LD-treated group.

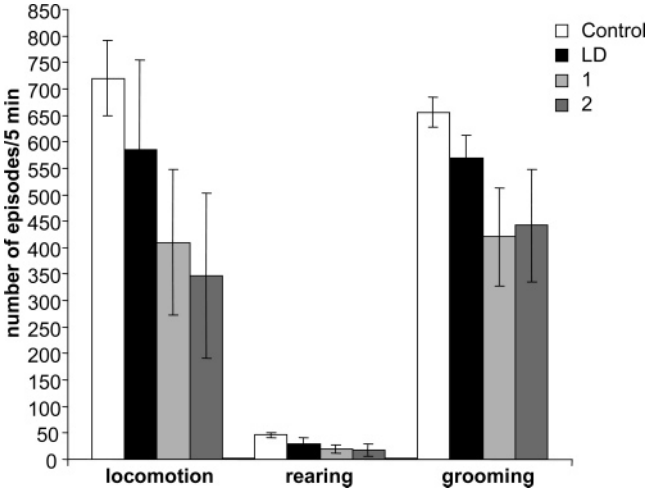


Figure 6. Locomotion, rearing, and grooming, 3 h after administration of LD, **1**, and **2** in rats. Data are expressed as mean ± SE of five experiments. $P > 0.05$.

and grooming, on the other. As reported in the literature, administration of LD and derivates led to changes in the behavioral phenomena in rat since LD influences the dopaminergic transmission.[37,39] The behavioral analysis of the open field test allowed the screening of new dopaminergic compounds and their comparison with LD as reference substance.

Our data showed some behavioral differences between the LD-treated group and **1**- and **2**-treated rats: 1 h after administration, codrug **1** induced a decrease of grooming; instead, 2 h after treatment, a similar trend was created by codrug **2**. As shown in the literature, grooming plays an important role in behavioral adaptation to stress. It has been reported that mild stress, such as exposure to a novel environment, has been known to induce grooming in rats.[40] In our case, it seems that, compared to LD treatment, both new codrugs induce sedative effects impairing the grooming behavior. This may be related to the increased potency of codrugs used at the same molar doses as with the LD treatment. Besides, the data obtained allow us to distinguish the different pharmacokinetic properties of compounds **1** and **2**: it could be that the maximum values of plasma concentration of DA are reached 1 and 3 h after the administration of **1** and **2**, respectively.

Conclusion

In conclusion, we designed and synthesized a series of LD/DA−LA conjugates such as LD and DA prodrugs with radical scavenging properties. The present findings indicate that codrugs show good stability toward g.i. hydrolysis and release LD and DA in human plasma after enzymatic hydrolysis. Codrugs **1** and **2** displayed an antioxidant effect when compared to LD. In particular, a sustained release of LD for compounds **1** and **2** was observed after oral administration of codrugs. Taken together, these results are of significance for possible therapeutic application of codrugs **1** and **2** in pathological events in which free radical damage and decreasing DA concentration in the brain are involved.

Experimental Section

Melting points (mp) were determined on a Buchi B-540 apparatus and are uncorrected. Microanalyses were performed on a 1106 Carlo Erba CHN analyzer, and the results were within (0.4%) of the calculated values. ^{1}H 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 using 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 TLC on silica gel Merck 60 F_{254}. Solutions were routinely dried over anhydrous sodium sulfate prior to evaporation. Chromatographic purifications were performed by Merck 60 70−230 mesh ASTM silica gel column.

Methyl *O*-Acetyl-3-(acetyloxy)-*N*-{5-[(3*R*)-1,2-dithiolan-3-yl]-pentanoyl}-L-tyrosinate (1). To *O*,*O*-diacetyl-L-Dopa methyl ester hydrochloride (1.9 g, 5.8 mmol) in dioxane (25 mL) was added Et_3N (1.3 g, 12.8 mmol). The suspension was filtered and was added to a solution of (*R*)-α-lipoic acid (1.0 g, 4.8 mmol) in dioxane (25 mL). Then dicyclohexylcarbodiimide (DCC, 1.2 g, 5.8 mmol) was added over a period of 30 min at 25 °C. The mixture was stirred at room temperature for an additional 3 h. The precipitated dicyclohexylurea was filtered, and the solvent was evaporated. The product was purified by column chromatography with cyclohexane/AcOEt 4:6 as eluent (R_f: 0.37); yield 61%; ^{1}H NMR ($CDCl_3$): δ 7.04 (m, 3H, Ar), 5.94 (d, 1H, J = 7.8 Hz, NH), 4.87 (m, 1H, αCH), 3.76 (s, 3H, $COOCH_3$), 3.56 (m, 1H, CH), 3.09 (m, 2H, CH_2), 2.46 (m, 1H, CH_2) 2.30 (s, 6H, 2 $OCOCH_3$), 2.24 (m, 6H, 3 CH_2), 1.89 (m, 1H, CH_2), 1.62 (m, 4H, 2 CH_2); ^{13}C NMR

($CDCl_3$): δ 172.67 (s, 1C, CONH), 172.02 (s, 1C, COO), 168.52 (s, 1C, CO), 168.41 (s, 1C, CO), 168.41 (s, 1C, CO), 142.14 (s, 1C, Ar), 141.30 (s, 1C, Ar), 134.91 (s, 1C, Ar), 127.47 (s, 1C, Ar), 124.73 (s, 1C, Ar), 123.68 (s, 1C, Ar), 56.58 (s, 1C, CH), 52.93 (s, 1C, CH), 52.78 (s, 1C, OCH_3), 40.45 (s, 1C, CH_2), 38.70 (s, 1C, CH_2), 36.36 (s, 1C, CH_2), 34.83 (s, 1C, CH_2), 29.03 (s, 1C, CH_2), 25.39 (s, 1C, CH_2), 20.95 (s, 1C, OCH_3), 20.89 (s, 1C, OCH_3); MS (ESI) *m/z* 506 $(M - Na)^+$. Anal. ($C_{22}H_{29}NO_7S_2$) C, H, N, S.

Methyl *N*-{5-[(3*R*)-1,2-Dithiolan-3-yl]pentanoyl}-3-hydroxy-L-tyrosinate (2). Dicyclohexylcarbodiimide (DCC, 720 mg, 3.48 mmol) was added to a solution consisting of L-Dopa methyl ester hydrochloride (860 mg, 3.48 mmol), DMAP (21 mg, 0.17 mmol), and (*R*)-α-lipoic acid (717 mg, 3.48 mmol) in dry Pyr. The reaction mixture was stirred for about 12 h at room temperature, under anhydrous conditions. After evaporation under vacuum, the crude reaction product was partitioned between AcOEt and water. The organic phase was dried on Na_2SO_4, filtered, and then evaporated in a vacuum to give a residue which was purified by column chromatography with $CHCl_3/CH_3OH$ 95:5 as eluent (R_f: 0.13); yield 40%; ^1H NMR ($CDCl_3$): δ 6.49 (m, 3H, Ar), 6.46 (d, 1H, $J = 7.5$ Hz, NH), 4.82 (m, 1H, αCH), 3.73 (s, 3H, $COOCH_3$), 3.49 (m, 1H, CH), 3.16 (m, 2H, CH_2), 2.92 (m, 1H, CH_2), 2.43 (m, 6H, 3 CH_2), 2.44 (m, 1H, CH_2), 1.89 (m, 4H, 2 CH_2); ^{13}C NMR ($CDCl_3$): δ 173.91 (s, 1C, CONH), 172.54 (s, 1C, $COOCH_3$), 144.46 (s, 1C, Ar), 143.75 (s, 1C, Ar), 127.93 (s, 1C, Ar), 121.37 (s, 1C, Ar), 116.24 (s, 1C, Ar), 115.56 (s, 1C, Ar), 56.60 (s, 1C, CH), 53.59 (s, 1C, CH), 52.82 (s, 1C, OCH_3), 40.47 (s, 1C, CH_2), 38.68 (s, 1C, CH_2), 37.61 (s, 1C, CH_2), 36.42 (s, 1C, CH_2), 34.71 (s, 1C, CH_2), 28.91 (s, 1C, CH_2), 25.50 (s, 1C, CH_2); MS (ESI) *m/z* 398 $(M-H)^-$. Anal. ($C_{18}H_{25}NO_5S_2$) C, H, N, S.

2-(Acetyloxy)-4-[2-({5-[(3*R*)-1,2-dithiolan-3-yl]pentanoyl}-amino)ethyl]phenyl Acetate (3). To dopamine trifluroacetate (2.0 g, 5.8 mmol) in CH_2Cl_2 (25 mL) was added Et_3N (1.3 g, 12.8 mmol). The suspension was filtered and was added to a solution of (*R*)-α-lipoic acid (1.0 g, 4.8 mmol) in CH_2Cl_2 (25 mL). Then dicyclohexylcarbodiimide (DCC, 1.2 g, 5.8 mmol) was added over a period of 30 min at 25 °C. The mixture was stirred at room temperature for an additional 3 h. The precipitated dicyclohexylurea was filtered, and the solvent was evaporated. The product was purified by column chromatography with AcOEt as eluent (R_f: 0.56); yield 34%; ^1H NMR ($CDCl_3$): δ 7.00 (m, 3H, Ar), 5.47 (s, 1H, $J = 6.2$ Hz, NH), 3,08 (m, 1H, CH), 2.74 (m, 2H, CH_2), 2.39 (m, 1H, CH_2), 2.22 (s, 6H, 2 $OCOCH_3$), 2.08 (m, 2H, CH_2), 1.84 (m, 1H, CH_2), 1.59 (m, 6H, 3 CH_2), 1.38 (m, 4H, 2 CH_2); ^{13}C NMR ($CDCl_3$): δ 173.23 (s, 1C, CONH), 168.74 (s, 1C, CO), 168.62 (s, 1C, CO), 142.17 (s, 1C, Ar), 140.78 (s, 1C, Ar), 138.13 (s, 1C, Ar), 127.11 (s, 1C, Ar), 124.01 (s, 1C, Ar), 123.70 (s, 1C, Ar), 56.64 (s, 1C, CH), 40.47 (s, 1C, CH_2NH), 38.69 (s, 1C, CH_2), 36.58 (s, 1C, CH_2), 35.24 (s, 1C, CH_2), 34.83 (s, 1C, CH_2), 34.12 (s, 1C, CH_2), 29.10 (s, 1C, CH_2), 25.60 (s, 1C, CH_2), 20.92 (s, 1C, CH_3), 20.90 (s, 1C, CH_3); MS (ESI) *m/z* 426 $(M - H)^+$. Anal. ($C_{20}H_{27}NO_5S_2$) C, H, N, S.

***N*-[2-(3,4-Dihydroxyphenyl)ethyl]-5-[(3*R*)-1,2-dithiolan-3-yl]-pentanamide (4).** Dicyclohexylcarbodiimide (DCC, 720 mg, 3.48 mmol) was added to a solution consisting of dopamine hydrochloride (657 mg, 3.48 mmol), DMAP (21 mg, 0.17 mmol), and (*R*)-α-lipoic acid (717 mg, 3.48 mmol) in dry Pyr. The reaction mixture was stirred for about 12 h at room temperature, under anhydrous conditions. After evaporation under vacuum, the crude reaction product was partitioned between AcOEt and water. The organic phase was dried on Na_2SO_4, filtered, and then evaporated in a vacuum to give a residue which was purified by column chromatography with $CHCl_3/CH_3OH$ 95:5 as eluent (R_f: 0.10); yield 30%; ^1H NMR ($CDCl_3$): δ 6.68 (m, 3H, Ar), 5.82 (d, 1H, $J = 5.1$ Hz, NH), 3,48 (m, 3H, CH and CH_2CO), 3.12 (m, 2H, CH_2), 2.68 (m, 2H, CH_2), 2.41 (m, 1H, CH_2), 2.16 (m, 2H, CH_2), 1.86 (m, 1H, CH_2), 1.61 (m, 4H, 2 CH_2), 1.37 (m, 2H, CH_2); ^{13}C NMR ($CDCl_3$): δ 174.40 (s, 1C, CONH), 144.54 (s, 1C, Ar), 143.37 (s, 1C, Ar), 130.71 (s, 1C, Ar), 120.73 (s, 1C, Ar), 115.75 (s, 1C, Ar), 115.50 (s, 1C, Ar), 56.64 (s, 1C, CH), 41.24 (s, 1C, CH_2NH), 40.51 (s, 1C, CH_2), 38.70 (s, 1C, CH_2), 36.71 (s, 1C, CH_2), 35.07 (s, 1C, CH_2), 34.70 (s, 1C, CH_2), 28.97 (s, 1C, CH_2), 25.63 (s, 1C, CH_2); MS (ESI) *m/z* 340 $(M - H)^-$. Anal. ($C_{16}H_{23}NO_3S_2$) C, H, N, S.

Chemicals. Glutathione reductase, NADPH, and benserazide were obtained from Sigma Chemical Co, St. Louis, MO. Compounds **1** and **2** under study were synthesized in our laboratories. 2-Deoxy-D-ribose, LD were purchased from Sigma (St. Louis, MO). All other chemicals used were of the highest purity commercially available.

Animals. Male Wistar rats ($n = 80$) (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 the LD, **1**, and **2** codrugs were dissolved in dimethyl sulfoxide. All animals received a dose of benserazide (16.36 mg/kg) combined with LD (65.46 mg/kg), **1** (160.54 mg/kg), or **2** (132.64 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 study 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, 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 synthetic LD derivatives showed low water solubility. Stock solutions of the compounds were prepared in 0.01 M HCl and the concentration was determined by absorbance at 280 nm using the molar absorption coefficient of LD determined in the same solvent (26.2 M^{-1} cm^{-1}).

The pro-oxidant activity of LD and its synthetic derivatives was analyzed using the Fe (II)-H_2O_2-induced degradation of deoxyribose assay conducted in the presence of EDTA, essentially as previously described.[41] 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), K-phosphate buffer, pH 7.4 (100 mM), increasing concentrations of LD or LD derivatives (10−100 μM), $(NH_4)_2Fe(SO_4)_2$ (50μM), 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 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 80 °C for 20 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, and 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 and 3 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 transferred to two separate tubes; one aliquot was centrifuged at 2000*g* for 10 min, and the measurements of TAS were carried out in fresh plasma. TAS was evaluated using a commercially available kit (Randox Laboratories, BT29 4QY, UK). This method is based on the plasma antioxidant-mediated decrease in the stable blue-green color of the radical cation 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate] which is measured at 600 nm.[37]

The GPx and SOD activities were measured in the remaining aliquot of whole blood. The GPx activity was determined by Paglia and Valentine's method.[42] The SOD activity was measured using the 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.[43]

Measurement of Locomotor Activity. Automated locomotor activity boxes (MedAssociates, VT 05478) were used to quantify the 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. Activity was recorded for 5 min, starting 2 min after placing the animal 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 softwares (Activity Monitor, MedAssociates). Each rat was used only once and a total of five rats was used for each treatment. The behavioral tests were performed 1, 2, and 3 h after treatment with drugs and before blood collection. The behavioral parameters observed were locomotion (number of ambulatory episodes), rearings (number of rears), and stereotype counts (number of grooming movements). Locomotion counts were recorded when the low row of photocells was interrupted, while rearing counts were recorded by taking the higher row of photocells. Between each test session, the apparatus was cleaned with alcohol (10%) and dried with a cloth.

HPLC 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 mixture of water/methanol. The flow rate was 0.5 mL/min.

Aqueous Solubility. The aqueous solubilities of codrugs **1**−**4** were determined in deionized water, in 0.02 M phosphate buffer of pH 7.4, in 0.02 M sodium acetate buffer of pH 5.0, and in 0.02 M hydrochloridric acid buffer of 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** and **3** and 30 mg of compounds **2** and **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 hydrochloridric acid buffer of pH 1.3 as nonenzymatic simulated gastric fluid (SGF), 0.02 M phosphate buffer of pH 7.4 and 0.02 M sodium acetate buffer of 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 human 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 using 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 were 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 using the analysis of variance (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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