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Synthesis and characterization of carnitine nitro-derivatives

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Abstract—Nitric oxide (NO) acts as an autacoid molecule that diffuses from its endothelial production site to the neighboring muscular cells. NO-donors are often used to mimic the physiological effects of NO in biological systems. Organic nitrates are commonly used as NO-donors; the most popular, glycerol trinitrate (GTN), has been used in therapy for more than a century. Carnitine nitrates have been synthesized using an endogenous non-toxic molecule: (L)-carnitine. The biotransformation of carnitine nitro-derivatives in biological fluids (saliva and blood plasma) and in red blood cells (RBC) has been monitored by an electrochemical assay and the interaction of carnitine nitrates with the plasma membrane carnitine transporter has been investigated. Differences in the way carnitine nitro-derivatives are metabolized in biological fluids and cells and transported by OCTN2 transporter are modulated by the chemical structures and by the length of the acyl template which carries the nitro-group.

1. Introduction

Nitric oxide is a highly diffusible, lipid-soluble free radical species that plays an important physiological role in vascular regulation,[1] neuronal communication,[2] and cytotoxic action of macrophages during bacterial infections.[3] NO is a messenger both inside and outside the cells and has a very short half-life (5–15 s). It has the properties of a highly reactive radical species and, in the presence of oxygen, it is quickly transformed into nitrite and nitrate. NO synthase (NOS) catalyzes the synthesis of NO from the guanidinic nitrogen of arginine. Three isoforms of the enzyme have been well characterized: the neuronal (nNOS) and endothelial (eNOS) ones are constitutive and Ca^{2+}-calmodulin-dependent,[4] whereas the third isoform (iNOS) is inducible, does not depend on Ca^{2+}, and is generally expressed by neutrophils and macrophages as a consequence of inflammatory processes. A Ca^{+2}-dependent mitochondrial NOS (mtNOS) was reported recently.[5] Most biological effects ascribed to NO on blood vessels are mediated by the activation of a soluble guanilate cyclase that causes an increase in cGMP levels, followed by a decrease of cytosolic calcium in target cells.[6] NO acts as an autacoid molecule by diffusing from its endothelial production site to the neighboring muscular cells.[7]

NO-donors are often used to mimic the physiological effects of NO in biological systems. The degradation pathways that lead to NO formation differ noticeably among the different commercial NO-donors. The chemical diversity of NO-donors implies that some donors release NO only in the presence of suitable enzymes, while others require the presence of thiols and still others spontaneously release NO. Some compounds reduce, whereas others oxidize during the release of NO.

Organic nitrates and esters of nitric acid are commonly used as NO-donors. Among these, the most popular is glycerol trinitrate (GTN). Although it has been used in therapy for more than a century, its mechanism of action is still uncertain.[8] GTN leads to tolerance and loss of efficacy in ischemia protection.[9] The release of NO from this compound requires an enzymatic activation and/or the presence of thiols.[9]

The development of new NO-donors is an important field of research, aimed at improving the efficiency of NO-release and reducing cytotoxicity. Both these characteristics pertain to the application in diffuse and severe pathologies that respond to NO such as ischemias. Low

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cytotoxicity is also important in the study of NO signaling in the absence of side effects.

The synthesis of the carnitine nitrates has been based on the use of an endogenous non-toxic molecule: (L)-carnitine. Carnitine derivatives should improve bioavailability, since their delivery in the cell is facilitated by the transport systems for carnitine. In addition, carnitine, that plays a role in shuttling fatty acyl groups in the cell, has a protective function in ischemic tissues.[10] In the literature, there have been hints that NO is associated with carnitine effects. Carnitine, as such, prevents some neurovascular effects in diabetic rats and this phenomenon is enhanced by NO.[11] Thus the research aimed at designing alternative NO-donor-like carnitine nitrates is also very important in view of pharmacological applications.

On the basis of these factors, we have synthesized novel nitrate esters using (L)-carnitine as carrier. (L)-Carnitine hydrochloride (**1**) was chosen as the chiral building block because it allows nitrates to be synthesized that possess the following structural characteristics: (i) chiral and optical purity, and (ii) the nitrate group is bound to either a short- or long-chain ester linkage.

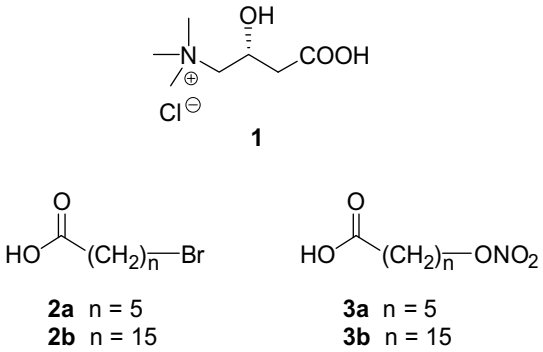
The biotransformation of carnitine nitro-derivatives in biological fluids (saliva and blood plasma) and in human red blood cells (RBC) was monitored by an electrochemical assay.[12]

The interaction of carnitine nitrates with the plasma membrane carnitine transporter OCTN2 was investigated in reconstituted artificial membranes. OCTN2 is expressed in kidney, heart, skeletal muscle, liver, and other tissues and is the transporter with the highest affinity for carnitine.[10]

2. Results and discussion

2.1. Chemistry

Commercially available (L)-carnitine hydrochloride (**1**) was used as starting material for the synthesis. 6-(Nitrooxy)hexanoic acid **3a** and 16-(nitrooxy)hexadecanoic acid **3b** were prepared from the corresponding bromoacids **2a**–**b** by reaction with $AgNO_3$ in CH_3CN at reflux (Scheme 1).



Scheme 1.

(L)-Carnitine ethyl ester iodide (**4**), prepared as described in the literature[13] by treating (L)-carnitine hydrochloride (**1**) with EtOH/HI (10:1) at reflux, was reacted with **3a** or **3b** in the presence of dicyclohexylcarbodimmide (DCC), 4-(dimetylammino)pyridine (DMAP) in CH_2Cl_2 at room temperature to give, after 20 h, the acylnitrooxy carnitine ethyl ester iodides **5** and **6** in 75% and 84% yield, respectively (Scheme 2). The products were isolated in pure form by column chromatography and recrystallization with diethyl ether/methanol solution.

The iodides **5** and **6** were exchanged with chloride by anion exchange column chromatography with Amberlite IRA 400 (Cl); (L)-ethyl-3-[(6(nitrooxy)hexanoyl)oxy]-4-(trimethylammonio)butanoate chloride (**7**) and (L)-ethyl-3-[(16(nitrooxy)hexadecanoyl)oxy]-4-(trimethylammonio)butanoate chloride (**8**) were obtained in pure form.

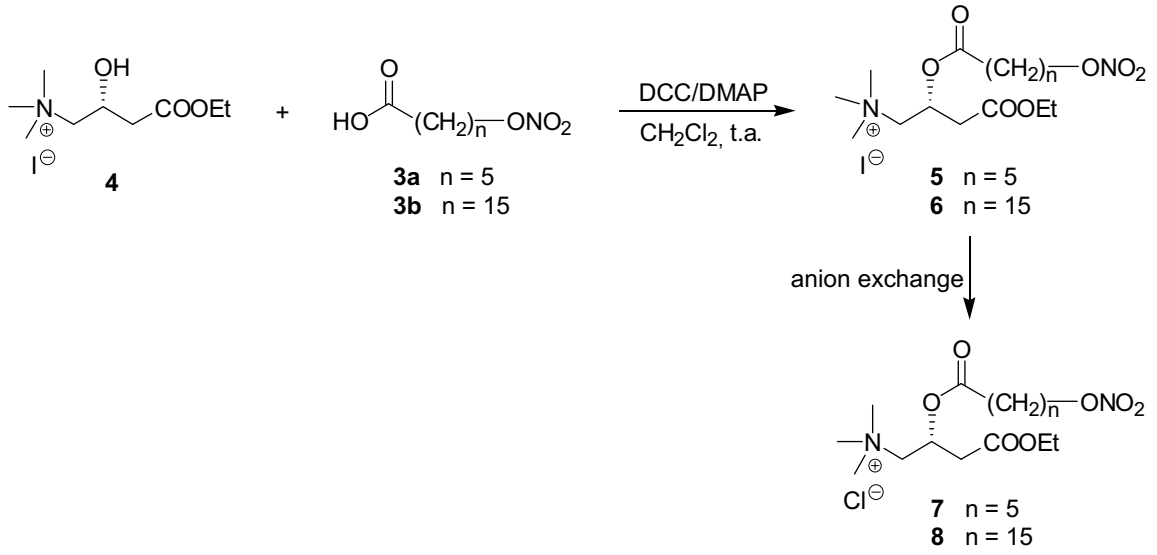
(L)-6-(Nitrooxy)hexanoyl carnitine chloride **10** and (L)-16-(nitrooxy)hexadecanoyl carnitine chloride **11** were prepared in 84% and 90% yield, respectively, first by treating **3a** and **3b** with $SOCl_2$ at 60 °C[14] and then by reaction of the corresponding acyl chloride **9a** and **9b** with (L)-carnitine hydrochloride in Cl_3C–COOH solution at 60 °C[14] (Scheme 3).

2.2. Determination of carnitine nitrates

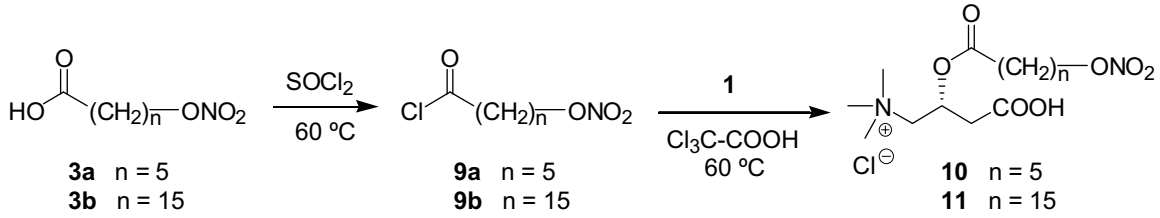
The carnitine-bound nitrate-group was determined by using an electrochemical assay after the reduction of organic nitrates with Cd in the presence of $CuSO_4$. The released nitrite was transformed into NO by reaction with $CuCl_2$ and cysteine in the sensor reaction chamber and determined as a NO gas with an amperometric sensor.[12] The amount of NO formed allowed the nitro-group of each compound to be monitored, in the various experimental conditions described in this paper. The release of NO is related to the molecular structure of the organic nitro-derivatives. The 6-nitrooxy-hexanoyl derivatives **7** and **10** showed a decrease in reactivity of about 50% with respect to the 16-nitrooxy-hexadecanoyl analogues **8** and **11**.

2.3. Biotransformation in biological fluids

The carnitine nitro-derivatives were exposed to biological fluids, phosphate buffer (PBS), human saliva, and plasma for 24 h. All the compounds were stable in phosphate buffer at pH 7.4. In human saliva the carnitine nitro-derivative **8** was degraded by about 25%, while only the carnitine derivative **10** was degraded by about 55% in plasma. The remaining nitro-derivatives were not affected by the experimental conditions (Table 1). The water phase remaining after chloroform extraction was used for the nitrite determination. The nitrite values remained at the basal level when all of the carnitine nitro-derivatives were incubated in plasma.



Scheme 2.



Scheme 3.

Table 1. Biotransformation of carnitine nitro-derivatives after incubation with the indicated fluids at 25 °C for 24 h

Compound	PBS (pH 7.4)	Saliva	Plasma
7	100 ± 3	100 ± 3	100 ± 3
8	100 ± 2	75 ± 2	100 ± 3
10	100 ± 3	100 ± 2	45 ± 4
11	100 ± 4	100 ± 3	100 ± 2

The data are expressed as percentage of unaltered compound remaining. Data are means ± SD of five different experiments.

Table 2. Biotransformation of carnitine nitro-derivatives and nitrite/nitrate produced after incubation with red blood cells (RBC) at 25 °C for 24 h

Compound	% Unchanged compound	nmol mL^{-1} NO_2^-/NO_3^-	
		t_0	t_{24}
7	100 ± 4	20 ± 4	22 ± 3
8	73 ± 3	18 ± 3	40 ± 5
10	100 ± 3	21 ± 3	23 ± 4
11	56 ± 2	19 ± 4	62 ± 5

The data are expressed as percentage of unaltered compound remaining after 24 h of incubation and as nmol of NO_2^- and NO_3^-/mL of emolysate at t_o: time zero and t_{24}: after 24 h of incubation. Data are means ± SD of five different experiments.

2.4. Biotransformation in red blood cells (RBC)

The degradation of carnitine nitro-derivatives after 24 h of incubation in RCB was different. Only the 16-nitro-oxy-acyl derivatives of carnitine **8** and **11** were degraded by 27% and 44%, respectively (Table 2). The water phase remaining after chloroform extraction was used for the nitrite and nitrate determinations. The nitrite and nitrate concentrations remained at basal levels when carnitine nitro-derivatives **7** and **10** were incubated in RBC, while the nitrite and nitrate concentrations doubled and tripled, respectively, in RBC when nitro-derivatives **8** and **11** were incubated (Table 2).

2.5. Effect of carnitine nitrates on transport

The effect of the carnitine nitro-derivatives (**7**, **8**, **10**, **11**) on transport catalyzed by the plasma membrane carnitine transporter was investigated. To achieve this objective, the carnitine transporter (OCTN2) solubilized from rat kidney brush border membranes was reconstituted into liposomes. The transport activity of OCTN2, that catalyzes a substrate antiport, was determined as [^{3}H]carnitine uptake into proteoliposomes containing internal carnitine (homologous antiport) as previously described.[10e] To evaluate the effect on transport, the carnitine nitro-derivatives were added to the proteoliposomes together with the labeled carnitine. The nitro-derivative **7** did not lead to substantial variations of the carnitine transport (Fig. 1). The effect of **8**, **10**, and **11** on the carnitine transport is shown in the dose–response

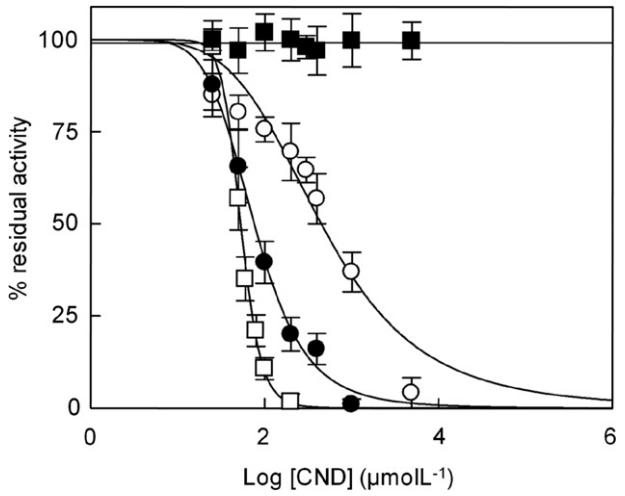


Figure 1. Semilogarithmic plots of dose–response curves for the inhibition of the reconstituted carnitine transporter. Transport was measured as [^{3}H]carnitine uptake in 10 min, that is, into proteoliposomes containing 10 mM carnitine within the initial linear range of the time course. Carnitine nitro-derivatives (CND) **7** (■), **8** (○), **10** (●), or **11** (□) were added to the proteoliposomes at the indicated concentrations together with labeled carnitine. Percent residual activity with respect to the control is reported. Data are means ± SD of three different experiments.

curves in Figure 1. The different compounds led to nearly complete transport inhibition but with variable efficiencies. The nitro-derivative **11** was the most effective inhibitor with an IC_{50} of 53 ± 5.0 μM as calculated from the dose–response curve (three experiments). The carnitine nitro-derivative **10** showed IC_{50} of 76 ± 18 μM (three experiments), slightly higher than **11**. The nitro-derivative **8** exhibited the lowest inhibition efficiency with IC_{50} of 443 ± 72 μM (three experiments). The inhibition observed indicates that the carnitine nitro-derivatives interact with the transport protein. The presence of the ethyl ester suppresses (compound **7**) or reduces (compound **8**) the interaction. Since the derivatives are structurally related to carnitine, they may be transported by the OCTN2. The transport of the carnitine nitro-derivatives mediated by the carnitine transporter has been determined as heterologous antiport, that is, carnitine uptake into proteoliposomes that contain the various carnitine derivatives. This strategy was previously adopted to study the transport of non-labeled carnitine analogues by the OCTN2.[10e] The carnitine derivatives were alternatively included in the proteoliposomes during the reconstitution procedure. The uptake of labeled carnitine was measured in these proteoliposomes and compared to the control proteoliposomes containing internal carnitine at the same concentration as each derivative (Table 3). The data show that only the nitro-derivatives **10** and **11** were transported with significant efficiencies, compared to carnitine, whereas **7** and **8** were not transported.

Table 3. Dependence of carnitine transport on counter substrate in reconstituted liposomes

Compound	Transport (% of control)
7	1.5 ± 1.2
8	3.1 ± 1.6
10	20.0 ± 3.5
11	16.3 ± 3.3

Transport was measured as [^{3}H]carnitine uptake in 30 min into proteoliposomes that contain compounds **7**, **8**, **10**, or **11**, in the internal compartment, at the concentration of 2 mmol L^{-1}. In the control samples, the transport was measured in proteoliposomes containing 2 mmol L^{-1} internal carnitine. The percentage of carnitine transport was calculated for each sample, containing the carnitine derivative, with respect to its control sample (referred to as 100%). Data are means ± SD of three different experiments.

2.6. Discussion

The ideal NO-releasing compound should release NO in solution with definite kinetics; it should not interfere with the systems modulated by NO or with other cellular pathways. If the NO-donor has a very low toxicity, its use could be extended to a pharmacological application.

A new class of NO-releasing compounds (carnitine nitrates) has been synthesized and their biotransformation in biological fluids and human erythrocytes has been investigated. Carnitine has its own way of cellular uptake and subcellular distribution via transport systems localized on the plasma membrane or on membranes of other cellular compartments.[10] As a native molecule it has a protective function in the ischemic tissues;[10c] as an organic nitrate, its NO effects may combine with those of carnitine.

The biotransformation of nitro-derivatives in biological fluids was different from erythrocytes. In human saliva (Table 1), the carnitine nitro-derivative **8** was degraded by 25%. The presence of bacterial flora in this biological fluid may play a role in this process. It is known that the inorganic nitrates present in vegetables are transformed into nitrites through the activity of bacterial mouth flora.[15] It is probable that these enzymatic activities are responsible for the selective degradation of the carnitine nitro-derivatives.

In human plasma (Table 1), only carnitine derivative **10** was degraded by almost 45%, but the nitrite level remained at the basal level after incubation with all the carnitine nitro-derivatives. If nitrite is a product, it is probably converted to NO by enzymatic reduction and/or non-enzymatic transformation.[16,17]

In RBC, the nitro-derivatives **8** and **11** were metabolized by almost 27% and 44% and the nitrate and nitrite levels, recovered in the water phase, increased two and three times, with respect to the basal levels (Table 2). Nitrite forms from NO through auto-oxidation and nitrate forms from NO reaction with oxy-hemoglobin.[18]

These results suggest that the degradation of carnitine derivatives, in biological fluid and in red blood cells, was selective and was probably carried out by several enzymatic activities.

An important feature influencing the bioavailability of the nitro-derivatives is their ability to enter into the cell. The OCTN2 protein is one of the most widely expressed transport systems for carnitine[10e] and is probably the way that the carnitine-derivatives enter inside the cells of various tissues. The effect of the synthesized compounds on the OCTN2 transporter extracted from rat kidney has been investigated. The data obtained may also pertain to other tissues like muscle and liver in which OCTN2 is expressed.[10b] The interaction study was conducted on proteoliposomes, an experimental model that has the advantage of reducing interference due to other transporters or enzymes, and allows the experimental conditions in both the external and internal compartments to be controlled. Under the conditions used, only the OCTN2 transport activity is detected.[10e] This study highlights the importance of maintaining the chemical structure of the native carnitine. In fact, when the –COOH group was substituted for an ethyl ester, the derivatives (**7** and **8**) were not transported. In the case of product **8**, the ethyl group did not completely prevent the interaction with the transporter as demonstrated by the inhibition. Derivatives **10** and **11** that lacked the ethyl group were transported; this indicated that the greater inhibition of free carnitine transport found in these products is related to their transport. Furthermore, the micromolar IC_{50} values on the external face of the transporter indicate a relatively high affinity for these compounds. Another important design feature is the length of the acyl template and its effect on the chemical coupling of the nitro-group to carnitine. The long-chain ester linkage (C_{16}) increased the degradation in RBC and reduced the OCTN2 transporter efficiency. The short-chain ester linkage (C_6) reduced the degradation and gave compounds which were easier to transport.

The results of this study provide information that is very relevant for the design of carnitine nitro-derivatives. The best compromise between optimal bioavailability and biotransformation properties was found in the carnitine nitro-derivative **11**. It was transported into the cell, not degraded in blood plasma and biotransformed in RBC after 24 h of incubation.

3. Conclusion

The carnitine nitrates are newly developed NO-releasing molecules with optimal features in terms of efficiency of NO release and transport into the cells.

This strategy for synthesizing organic nitrates shows the importance of specific functional groups of the carnitine derivatives, that provide a better perspective than the existing NO-releasing compounds.

4. Experimental

4.1. Chemistry

4.1.1. General. All of the compounds prepared were characterized by ^{1}H NMR, ^{13}C NMR, and IR spectra. NMR spectra were recorded at 400 and 200 MHz for proton and 100.6 and 50.3 MHz for carbon nuclei in CD_3OD and $CDCl_3$. IR spectra were recorded with a FT-IR instrument, in KBr pellets or in $CHCl_3$ solution. The products were purified by column chromatography carried out on silica gel (230–400 mesh). The Cl-anion exchange columns were prepared with Amberlite IRA 400 (Cl). (L)-Carnitine chloride (**1**) was purchased from Sigma. (L)-Carnitine ethyl ester iodide (**4**)[13] was prepared as described in the literature.

4.1.2. 6-(Nitrooxy)hexanoic acid (3a) and 16-(nitrooxy)hexadecanoic acid (3b). In a 25 mL flask fitted with reflux condenser, bromo acid **2a** or **2b** (5.0 mmol), silver nitrate (5.9 mmol, 1 g), and 10 mL of acetonitrile were warmed at 85 °C under magnetic stirring for 4 h. After cooling to room temperature, the reaction mixture was filtered off to eliminate the AgBr precipitate. The solution was evaporated at reduced pressure and the residue was dissolved in diethyl ether. The solution was again filtered off and concentrated at reduced pressure. The 6-(nitrooxy)hexanoic acid (**3a**) was obtained as oil (710 mg, 86% yield) and the 16-(nitrooxy)hexadecanoic acid (**3b**) was obtained as white solid (1.27 g, 80% yield).

4.1.2.1. 6-(Nitrooxy)hexanoic acid (3a). ^{1}H NMR (200 MHz, $CDCl_3$) δ: 4.46 (t, 2H, CH_2–ONO_2, J = 6.5 Hz), 2.39 (t, 2H, CH_2–COOH, J = 7.1 Hz), 1.73 (m, 4H, –CH_2–CH_2–CH_2–), 1.49 (m, 2H, CH_2–CH_2–CH_2–). IR ($CHCl_3$) ν (cm^{-1}): 3080 (O–H); 1710 (C=O); 1631, 1280 (O–NO_2). Anal. Calcd for $C_6H_{11}NO_5$: C, 40.68; H, 6.26; N, 7.91. Found: C, 40.63; H, 6.24; N, 7.90.

4.1.2.2. 16-(Nitrooxy)hexadecanoic acid (3b). Pf: 55–56 °C. ^{1}H NMR (200 MHz, $CDCl_3$) δ: 4.44 (t, 2H, CH_2–ONO_2, J = 6.6 Hz), 2.35 (t, 2H, CH_2–COOH, J = 7.5 Hz), 1.70 (m, 4H, –CH_2–$(CH_2)_{11}$–CH_2–), 1.26 (m, 22H, –CH_2–$(CH_2)_{11}$–CH_2–). IR ($CHCl_3$) ν (cm^{-1}): 3086 (O–H); 1711 (C=O); 1629, 1279 (O–NO_2). Anal. Calcd for $C_{16}H_{31}NO_5$: C, 60.54; H, 9.84; N, 4.41. Found: C, 60.58; H, 9.82; N, 4.43.

4.1.3. (L)-Ethyl-3-[(6(nitrooxy)hexanoyl)oxy]-4-(trimethylammonium)butanoate chloride (7). In a 50 mL flask, a solution of (L)-carnitine ethyl ester iodide (**4**)[13] (2.2 mmol, 700 mg), dicyclohexylcarbodimmide (DCC) (3.3 mmol, 680 mg), 6-(nitrooxy)hexanoic acid (**3a**) (2.3 mmol, 410 mg), and 4-(dimethylammino)pyridine (DMAP) (0.11 mmol, 13.4 mg) in CH_2Cl_2 (18 mL) were stirred at room temperature for 20 h. The reaction mixture was filtered off to eliminate the DCU. The solution was evaporated at reduced pressure and the residue dissolved in $CHCl_3$. The solution was again filtered off and concentrated at reduced pressure. The residue, now free of most DCU, was chromatographed (silica gel, $CHCl_3$/MeOH = 75:25) and then recrystallized from diethyl ether/MeOH 9:1 to provide **5** as a white solid (790 mg, 75% yield). The iodide was exchanged with chloride by anion exchange column chromatography to give 600 mg of **7** as an oil.

4.1.3.1. (L)-Ethyl-3-[(6(nitrooxy)hexanoyl)oxy]-4-(trimethylammonium)butanoate iodide (5). Pf: 118–120 °C (Et_2O/MeOH). $[\alpha]_D$ −10.8 (*c* 1.0, MeOH). ^1H NMR (200 MHz, CD_3OD) δ: 5.62 (m, 1H, CH–O–CO), 4.45 (t, 2H, CH_2–ONO_2, J = 6.4 Hz), 4.12 (q, 2H, O–CH_2–CH_3, J = 7.1 Hz), 3.89 (dd, 1H, C*H*H–N^+Me_3, J = 14.4, 8.1 Hz), 3.75 (dd, 1H, CH*H*–N^+Me_3, J = 14.4, 1.6 Hz), 3.21 (s, 9H, $(CH_3)_3N^+$), 2.77 (d, 2H, CH_2–COOEt, J = 6.2 Hz), 2.39 (t, 2H, O–CO–CH_2, J = 7.3 Hz), 1.66 (m, 4H, –CH_2–CH_2–CH_2–), 1.41 (m, 2H, –CH_2–CH_2–CH_2–), 1.21 (t, 3H, O–CH_2–CH_3, J = 7.1 Hz). ^{13}C NMR (50.3 MHz, CD_3OD) δ: 173.84, 170.77, 74.52, 69.31, 66.14, 62.34, 54.82 (3C), 38.18, 34.81, 27.46, 26.16, 25.11, 14.48. IR ($CHCl_3$) ν (cm^{-1}): 1738 (C=O); 1631, 1280 (O–NO_2). Anal. Calcd for $C_{15}H_{29}IN_2O_7$: C, 38.82; H, 6.14; N, 5.88. Found: C, 37.78; H, 6.13; N, 5.91.

4.1.3.2. (L)-Ethyl-3-[(6(nitrooxy)hexanoyl)oxy]-4-(trimethylammonium)butanoate chloride (7). $[\alpha]_D$ −15.9 (*c* 0.92, MeOH). ^1H NMR (200 MHz, CD_3OD) δ: 5.59 (m, 1H, CH–O–CO), 4.44 (t, 2H, CH_2–ONO_2, J = 6.4 Hz), 4.11 (q, 2H, O–CH_2–CH_3, J = 7.1 Hz), 3.82 (dd, 1H, C*H*H–N^+Me_3, J = 14.4, 8.2 Hz), 3.65 (dd, 1H, CH*H*–N^+Me_3, J = 14.4, 1.5 Hz), 3.15 (s, 9H, $(CH_3)_3N^+$), 2.73 (d, 2H, CH_2–COOEt, J = 6.2 Hz), 2.37 (t, 2H, O–CO–CH_2, J = 7.4 Hz), 1.66 (m, 4H, –CH_2–CH_2–CH_2–), 1.41 (m, 2H, –CH_2–CH_2–CH_2–), 1.21 (t, 3H, O–CH_2–CH_3, J = 7.1 Hz). IR (KBr) ν (cm^{-1}): 1738 (C=O); 1631, 1279 (O–NO_2). Anal. Calcd for $C_{15}H_{29}ClN_2O_7$: C, 46.81; H, 7.60; N, 7.28. Found: C, 46.77; H, 7.59; N, 7.30.

4.1.4. (L)-Ethyl-3-[(16(nitrooxy)hexadecanoyl)oxy]-4-(trimethylammonium)butanoate chloride (8). In a 50 mL flask, a solution of (L)-carnitine ethyl ester iodide (**4**)[13] (2.2 mmol, 700 mg), dicyclohexycarbodimmide (DCC) (3.3 mmol, 680 mg), 16-(nitrooxy)hexadecanoic acid (**3b**) (2.3 mmol, 730 mg), and 4-(dimethylammino)pyridine (DMAP) (0.11 mmol, 13.4 mg) in CH_2Cl_2 (18 mL) were stirred at room temperature for 20 h. The reaction mixture was filtered off to eliminate the DCU. The solution was evaporated at reduced pressure and the residue dissolved in $CHCl_3$. The solution was again filtered off and concentrated at reduced pressure. The residue, now free of most DCU, was chromatographed (silica gel, $CHCl_3$/MeOH = 75:25) and then recrystallized from diethyl ether/MeOH 95:5 to provide **6** as a white solid (1.14 g, 84% yield). The iodide was exchanged with chloride by an anion exchange column chromatography to give 920 mg of **8** as a white solid.

4.1.4.1. (L)-Ethyl-3-[(16(nitrooxy)hexadecanoyl)oxy]-4-(trimethylammonium)butanoate iodide (6). Pf: 103–105 °C (Et_2O). $[\alpha]_D$ −12.5 (*c* 1.05, MeOH). ^1H NMR (200 MHz, CD_3OD) δ: 5.59 (m, 1H, CH–O–CO), 4.42 (t, 2H, CH_2–ONO_2, J = 6.6 Hz), 4.11 (q, 2H, O–CH_2–CH_3, J = 7.1 Hz), 3.83 (dd, 1H, C*H*H–N^+Me_3, J = 14.4, 8.2 Hz), 3.66 (dd, 1H, CH*H*–N^+Me_3, J = 14.4, 1.6 Hz), 3.16 (s, 9H, $(CH_3)_3N^+$), 2.73 (d, 2H, CH_2–COOEt, J = 6.2 Hz), 2.33 (t, 2H, O–CO–CH_2, J = 7.4 Hz), 1.60 (m, 4H, –CH_2–$(CH_2)_{11}$–CH_2–), 1.25 (m, 22H, –CH_2–$(CH_2)_{11}$–CH_2–), 1.21 (t, 3H, O–CH_2–CH_3, J = 7.1 Hz). ^{13}C NMR (50.3 MHz, CD_3OD) δ: 174.06, 170.75, 74.75, 69.29, 66.04, 62.30, 54.70 (3C), 38.13, 35.08, 34.71, 30.71 (3C), 30.60 (2C), 30.38, 30.25, 30.13, 27.80, 26.72, 26.04, 25.68, 14.49. IR ($CHCl_3$) ν (cm^{-1}): 1737 (C=O); 1630, 1280 (O–NO_2). Anal. Calcd for $C_{25}H_{49}IN_2O_7$: C, 48.70; H, 8.01; N, 4.54. Found: C, 48.73; H, 8.02; N, 4.57.

4.1.4.2. (L)-Ethyl-3-[(16(nitrooxy)hexadecanoyl)oxy]-4-(trimethylammonium)butanoate chloride (8). Pf: 59–60 °C (Et_2O). $[\alpha]_D$ −12.7 (*c* 0.94, MeOH). ^1H NMR (200 MHz, CD_3OD) δ: 5.59 (m, 1H, CH–O–CO), 4.42 (t, 2H, CH_2–ONO_2, J = 6.6 Hz), 4.11 (q, 2H, O–CH_2–CH_3, J = 7.1 Hz), 3.82 (dd, 1H, C*H*H–N^+Me_3, J = 14.4, 8.3 Hz), 3.65 (dd, 1H, CH*H*–N^+Me_3, J = 14.4, 1.5 Hz), 3.15 (s, 9H, $(CH_3)_3N^+$), 2.73 (d, 2H, CH_2–COOEt, J = 6.2 Hz), 2.33 (t, 2H, O–CO–CH_2, J = 7.4 Hz), 1.59 (m, 4H, –CH_2–$(CH_2)_{11}$–CH_2–), 1.24 (m, 22H, –CH_2–$(CH_2)_{11}$–CH_2–) 1.21 (t, 3H, O–CH_2–CH_3, J = 7.1 Hz). IR (KBr) ν (cm^{-1}):1740 (C=O); 1630, 1279 (O–NO_2). Anal. Calcd for $C_{25}H_{49}ClN_2O_7$: C, 57.18; H, 9.41; N, 5.33. Found: C, 57.23; H, 9.40; N, 5.35.

4.1.5. (L)-3-[(6(Nitrooxy)hexanoyl)oxy]-4-(trimethylammonium)butanoic acid chloride (10). In a 25 mL flask fitted with reflux condenser and $CaCl_2$ valve, 6-(nitrooxy)hexanoic acid (**3a**) (2 mmol, 360 mg) and $SOCl_2$ (190 μL, 2.6 mmol) were first warmed at 60 °C under magnetic stirring for 30 min. ^1H NMR spectra analysis showed the complete conversion into the corresponding acyl chloride **9a**. (L)-Carnitine chloride **1** (198 mg, 1 mmol) and trichloroacetic acid (1 g) were then added at 40 °C and the mixture stirred at 60 °C for 3 h. Dry diethyl ether (6 mL) was added to the cooled mixture under stirring and the oleose solid product was separated by decantation and washed again with dry diethyl ether (10 mL under stirring). The residue was dissolved in MeOH and the solution filtered to eliminate the insoluble impurities. The solvent was evaporated at reduced pressure to give 300 mg of **10** as a transparent, highly viscous oil (yield 84%).

$[\alpha]_D$ −15.4 (*c* 1.22, MeOH). ^1H NMR (400 MHz, CD_3OD) δ: 5.57 (m, 1H, CH–O–CO), 4.44 (t, 2H, CH_2–ONO_2, J = 6.4 Hz), 3.80 (dd, 1H, C*H*H–N^+Me_3, J = 14.4, 8.5 Hz), 3.75 (dd, 1H, CH*H*–N^+Me_3, J = 14.4, 1.3 Hz), 3.14 (s, 9H, $(CH_3)_3N^+$), 2.69 (m, 2H, CH_2–COOH), 2.37 (t, 2H, O–CO–CH_2, J = 7.3 Hz), 1.66 (m, 4H, –CH_2–CH_2–CH_2–), 1.39 (m, 2H, –CH_2–CH_2–CH_2–). ^{13}C NMR (100.6 Hz, CD_3OD) δ: 173.92, 172.6, 74.49, 69.40, 66.41, 54.59 (3C), 38.03, 34.76, 27.44, 26.15, 25.12. IR ($CHCl_3$) ν (cm^{-1}): 3367 (OH), 1732 (C=O); 1624, 1280 (O–NO_2). Anal. Calcd for $C_{13}H_{25}ClN_2O_7$: C, 43.76; H, 7.06; N, 7.85. Found: C, 43.71; H, 7.04; N, 7.82.

4.1.6. (L)-3-[(16(Nitrooxy)hexadecanoyl)oxy]-4-(trimethylammonium)butanoic acid chloride (11). In a 25 mL flask fitted with reflux condenser and $CaCl_2$ valve, 6-(nitrooxy)hexadecanoic acid (**3b**) (2 mmol, 634 mg) and $SOCl_2$ (190 μL, 2.6 mmol) were first warmed at 60 °C under magnetic stirring for 30 min. ^1H NMR

spectra analysis showed the complete conversion into the corresponding acyl chloride **9b**. (L)-Carnitine chloride **1** (198 mg, 1 mmol) and trichloroacetic acid (1 g) were then added at 40 °C and the mixture stirred at 60 °C for 3 h. Dry diethyl ether (6 mL) was added to the cooled mixture under stirring and the white solid product was separated by filtration under nitrogen pressure and washed again with dry diethyl ether (10 mL under stirring). The residue was solved in MeOH and the solution filtered to eliminate the insoluble impurities. The solvent was evaporated at reduced pressure to give 450 mg of **11** as a white solid (yield 90%).

Pf: 70–72 °C. $[\alpha]_D$ −12.7 (*c* 0.94, MeOH). ^{1}H–NMR (400 MHz, CD_3OD) δ: 5.56 (m, 1H, CH–O–CO), 4.42 (t, 2H, CH_2–ONO_2, J = 6.6 Hz), 4.11 (q, 2H, O–CH_2–CH_3, J = 7.1 Hz), 3.83 (dd, 1H, C*H*H–N^+Me_3, J = 14.4, 8.5 Hz), 3.64 (dd, 1H, CH*H*–N^+Me_3, J = 14.4, 1.1 Hz), 3.14 (s, 9H, $(CH_3)_3N^+$), 2.67 (m, 2H, CH_2COOH), 2.33 (t, 2H, O–CO–CH_2, J = 7.2 Hz), 1.50–1.70 (m, 4H, –CH_2–$(CH_2)_{11}$–CH_2–), 1.24 (m, 22H, –CH_2–$(CH_2)_{11}$–CH_2–). ^{13}C NMR (100.6 Hz, CD_3OD) δ: 174.13, 172.76, 74.75, 69.45, 66.38, 54.57 (3C), 38.15, 35.09, 30.72 (2C), 30.69 (2C), 30.60, 30.56, 30.51, 30.38, 30.24, 30.16, 27.81, 26.72, 25.69. IR ($CHCl_3$) ν (cm^{-1}): 3408 (OH), 1730 (C=O); 1633, 1283 (O–NO_2). Anal. Calcd for $C_{23}H_{45}ClN_2O_7$: C, 55.58; H, 9. 31; N, 5.64. Found: C, 55.52; H, 9.29; N, 5.67.

4.2. Biological studies

4.2.1. Materials. $NaNO_2$, HCl, $CuCl_2$, $HClO_4$, and $KMnO_4$ were purchased from Aldrich (Buchs, Switzerland). Metallic cadmium was purchased from Sigma Chemical (St. Louis, MO, USA). Pure nitrogen (5.5 IP) and gaseous NO (1000 ppm in N_2) were supplied by Rivoira s.p.a., Gas Tecnici (Foligno, Italy). Gases were delivered through a flow controller (Bronkhorst, Ruurlo, The Netherlands). Amberlite XAD-4, egg yolk phospholipids (3-*sn*-phosphatidylcoline from egg yolk) and $C_{12}E_8$ were purchased from Fluka; L-[^{3}H]carnitine from Amersham; Sephadex G-75, Triton X-100 from Sigma. All other reagents were of analytical grade.

4.2.2. Determination of carnitine nitrates. Carnitine nitro-derivatives (**7**, **8**, **10**, **11**) were dissolved in methanol (5 mg/mL) and were diluted 1:20 (v/v) in 10 mmol L^{-1} phosphate buffer (pH 7.4). This solution (100 μL) was mixed with 100 μL of 0.1 mol L^{-1} $CuSO_4$ and 50 mg of metallic Cd to reduce organic nitrate to nitrite. After 2 min of incubation at room temperature, the suspension was centrifuged at 10,000 rpm for 2 min. The supernatant (20 μL) was injected into the reaction chamber of the amperometric sensor. The chamber contained 0.05 mol L^{-1} cysteine and 0.05 mol L^{-1} $CuCl_2$, in a final volume of 2 mL. The NO released by each sample was determined with an amperometric sensor, as described elsewhere.[12]

4.2.3. Biotransformation in biological fluids. The amount of carnitine derivatives was determined after incubation with 10 mmol L^{-1} phosphate buffer (pH 7.4), blood plasma, and saliva either at zero time or after 24 h at room temperature.

Carnitine nitro-derivatives were dissolved in methanol (5 mg mL^{-1}) and 25 μL of this solution was added to 1 mL of the above reported fluids. Samples were analyzed at time 0 and after 24 h. Nitro-derivatives were extracted twice by adding 2 mL of chloroform, dried under a gentle nitrogen flux, and suspended in 1 mL of methanol. Aliquots were used to determine nitro-groups.

In addition, nitrites were determined in the water phase after chloroform extraction. The samples were centrifuged at $5000g \times 5$ min and the supernatant (200 μL) was used to determine the nitrite levels, as reported above.

4.2.4. Biotransformation in the presence of red blood cells (RBC). Human red blood cells (RBC) were obtained by centrifugation of 5 mL heparinated blood at $800g \times$ 10 min. The pellet was suspended in 5 mL of 10 mmol L^{-1} phosphate buffer (pH 7.4) and divided into two aliquots. One aliquot was used as such and incubated at room temperature with 25 μL of carnitine nitro-derivatives (dissolved in methanol; 5 mg mL^{-1}). The samples were extracted with chloroform and analyzed as reported above at the beginning of the incubation and after 24 h.

In addition, nitrates and nitrites were determined in the water phase after chloroform extraction. To this purpose, the water phase was boiled at 100 °C for 1 min. The samples were centrifuged at $5000g \times 5$ min to remove hemoglobin. The supernatant (100 μL) was mixed with 100 μL of 0.1 mol L^{-1} $CuSO_4$ and 50 mg Cd, as reported above.

4.2.5. Reconstitution of the carnitine transporter. The carnitine transporter was solubilized from rat kidney brush border membranes as previously described[10e] and reconstituted into liposomes by the cyclic detergent removal procedure.[19,20] In this procedure the mixed micelles-containing detergent, protein, and phospholipids were repeatedly passed through the same Amberlite XAD-4 column. The composition of the initial mixture used for reconstitution was: 25 μL of the solubilized protein (15–25 μg protein in 2% $C_{12}E_8$), 85 μL of 10% $C_{12}E_8$, 100 μL of 10% egg yolk phospholipids in the form of sonicated liposomes, L-carnitine or the carnitine derivatives at the concentrations indicated in the legends of Tables and Figures, and 20 mmol L^{-1} Hepes/Tris (pH 6.0) in a final volume of 700 μL. After vortexing, this mixture was passed through the same Amberlite column (0.5×3 cm) 15 times after it had been preequilibrated with a buffer containing 20 mmol L^{-1} Hepes/Tris (pH 6.0) and 10 mmol L^{-1} L-carnitine. All the operations were performed at 4 °C, except the passages through Amberlite, which were carried out at room temperature.

4.2.6. Transport measurements. To remove the external substrate, 550 μL of proteoliposomes was passed through a Sephadex G-75 column (0.7×15 cm) preequilibrated with 10 mmol L^{-1} Hepes/Tris (pH 6.0). Transport was started by adding 10 μmol L^{-1} [^{3}H]carni-

tine and 50 mmol L^{-1} NaCl to the proteoliposomes that contained as internal substrates carnitine or carnitine nitro-derivatives added to the initial reconstitution mixture (see above); transport was stopped by adding 50 μmol L^{-1} mersalyl at the desired time interval. In the control samples, 50 μmol L^{-1} mersalyl was added at time zero according to the inhibitor stop method.[17] The assay temperature was 25 °C. The assay pH was 6.0, that represents the optimal condition of transport.[10e] Finally, each sample of proteoliposomes (100 μL) was passed through a Sephadex G-75 column (0.6 × 8 cm) in order to separate the external from the internal radioactivity. Liposomes were eluted with 1 mL of 50 mmol L^{-1} NaCl and collected in 4 mL of scintillation mixture, vortexed, and counted. The experimental values were corrected by subtracting the respective control value.

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