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Synthesis of a Novel Cyclic Prodrug of *S*-Allyl-glutathione Able To Attenuate LPS-Induced ROS Production through the Inhibition of MAPK Pathways in U937 Cells

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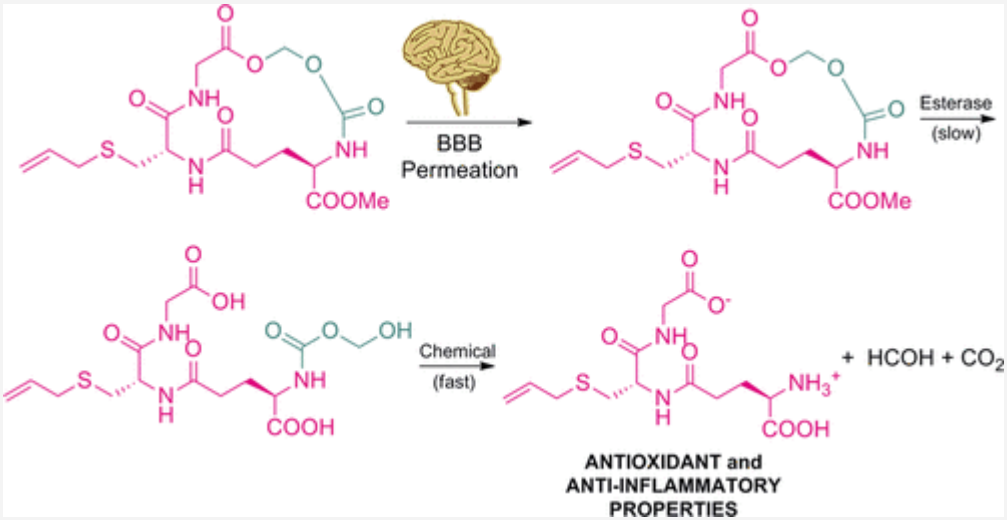
Abstract



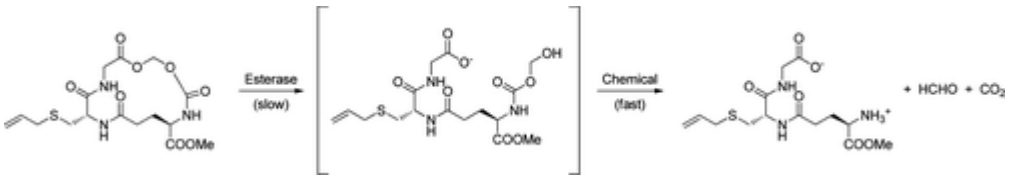
A novel cyclic prodrug of S-allyl-glutathione (CP11), obtained by using an acyloxy-alkoxy linker, was estimated for its pharmacokinetic and biological properties. The stability of CP11 was evaluated at pH 1.2, 7.4, in simulated fluids with different concentrations of enzymes, and in human plasma. The anti-inflammatory ability of CP11 was assessed in U937 cells, an immortalized human monocyte cell line. Results showed that CP11 is stable at acidic pH showing a possible advantage for oral delivery due to the longer permanence in the stomach. Having a permeability coefficient of 2.49 × 10–6 cm s–1, it was classified as discrete BBB-permeable compound. Biological studies revealed that CP11 is able to modulate inflammation mediated by LPS in U937 cells preventing the increase of ROS intracellular levels through interaction with the MAPK pathway.

Introduction

GSH is a tripeptide involved in xenobiotic metabolism and antioxidant defense playing a central role in scavenging free radicals, detoxifying electrophiles, and modulating many cellular processes and the immune system. (1, 2) To date, studies have confirmed that GSH is crucial in counteracting oxidative stress derived from both physiological and pathological conditions that characterize many diseases such as aging, neurodegenerative diseases, and other pathologies. (3)

A variety of GSH derivatives have been extensively studied to discover therapeutic molecules for the treatment of the above-reported disorders. (4-7) Unfortunately, GSH possesses some limitations in its pharmacological use due to its unfavorable pharmacokinetic properties, having a plasmatic half-life lower than 3 min. (8) These disadvantages can be overcome using the prodrug strategy, a medicinal-chemistry-based approach widely used to enhance the delivery features of drugs. (9) To date, literature data have reported several cyclic peptide prodrugs endowed with better physicochemical properties (good membrane permeation, increased hydrophobicity, and enzymatic stability) with respect to their parent peptides, but no example of cyclic GSH prodrugs has been documented, yet. (10-12) In fact, the attempt of obtaining the direct cyclization of GSH failed as reported by Sheh et al., (13) while the replacement of the glycine with β-Ala or γ-aminobutyric acid afforded the desired cyclic peptide.

Herein is described the synthesis of a novel GSH prodrug (**CP11**), sensitive to esterase hydrolysis, introducing an acyloxy-alkoxy moiety as intramolecular cleavable linker. (14, 15) Two steps are involved in the hydrolysis of **CP11**: initially a slow enzymatic hydrolysis of the ester bond and then a fast chemical hydrolysis of the carbamate moiety to release *S*-allyl-GSH, CO_2, and HCOH (Scheme 1).



Scheme 1. Release of *S*-Allyl-GSH Following **CP11** Ring-Opening

The GSH thiol group has been alkylated with an allyl chain for the following reasons: (a) different papers report that the sulfhydril group of cysteine exerts a pro-oxidative effect that, together with autoxidation, can generate very reactive singlet oxygen ($^1O_2$); (16) (b) *S*-allyl-cysteine has got antioxidant properties scavenging ROS produced in neuronal cells, and exhibiting chelating properties on Fe^{2+} and Cu^{2+} ions, thus avoiding the well-known Fenton reaction. (17)

In this study, we gauged the chemical stability of **CP11** at different values of pH and the enzymatic stability in simulated fluids and human plasma. The cell membrane permeation characteristics of **CP11** were measured using PAMPA-BBB, as model for CNS permeability, while the anti-inflammatory activity was assayed *in vitro* on LPS-activated U937 cells.

Materials and Methods

Chemistry

H-Cys(Allyl)-OH and Boc-Glu-OMe were purchased from Bachem. The synthesis of Boc-Gly(OCH_2O)-*p*-nitro-phenyl-carbonate was performed as described in the literature. (14) All the organic layers were dried on Na_2SO_4. Chromatography was executed on silica gel (Merck 60, 70–230 mesh). ^1H and ^{13}C NMR spectra were registered in ppm (δ) by Varian VXR-300 spectrometer with Me_4Si as internal standard. Mass analysis was carried out using an LCQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an ESI source. The injector temperature was 300 °C, and the spray voltage was 4.25 kV. N_2 was used as sheath and auxiliary gas.

H-Cys(Allyl)-OBu^t (6)

A suspension of H-Cys-(Allyl)–OH (4.16 g, 25.8 mmol) in AcOBu^t (375 mL) was slowly added to $HClO_4$ (7.74 mL) at 20 °C. The resulting solution was maintained under stirring for 4 h, then neutralized with solid $NaHCO_3$, and extracted with AcOEt. After evaporation under vacuum, the crude was chromatographed using $CHCl_3$:MeOH (97:3) to afford the *tert*-butyl ester **6** (4.5 g). Yield: 80%. R_f = 0.65, $CHCl_3$:MeOH (97:3). ^{1}H NMR ($CDCl_3$) δ: 1.46 (9H, s, OBu^t), 2.36 (2H, s, Cys NH_2), 2.64–2.88 (2H, m, Cys β-CH_2), 3.14–3.16 (2H, m, Cys S-C*H*$_2$-CHCH$_2$), 3.53 (1H, m, Cys α-CH), 5.09–5.15 (2H, m, Cys S-CH_2-CH-C*H*$_2$), 5.75 (1H, m, Cys S-CH_2-C*H*-CH_2). ^{13}C NMR ($CDCl_3$) δ: 28.24 (OBu^t), 35.35 (Cys β-CH_2), 35.82 (S-CH_2), 54.71 (Cys α-CH), 82.03 (OBu^t), 117.80 (S-CH_2-CH-*C*H_2), 134.19 (S-CH_2-*C*H-CH_2).

Boc-Glu[Cys(Allyl)-OBu^t]-OMe (7)

Boc-Glu-OMe (2.55 g, 9.77 mmol) was treated with dry DMF (147 mL), isobutyl chloroformate (1.28 mL, 9.77 mmol), and TEA (1.36 mL, 9.77 mmol) under stirring for 20 min at −20 °C. H-Cys(Allyl)-OBu^t (4.26 g, 19.54 mmol) was added to the reaction flask and left for 3 h at 0 °C, then 19 h at 20 °C. After filtration, the solution was extracted with $CHCl_3$/$KHSO_4$ 1 N, $NaHCO_{3ss}$, $NaCl_{ss}$, and then evaporated. The crude was purified with $CHCl_3$ providing dipeptide **7** (4.5 g). Yield: 99%. R_f = 0.22, $CHCl_3$. ^{1}H NMR ($CDCl_3$) δ: 1.41 (9H, s, OBu^t), 1.45 (9H, s, Boc), 1.88–2.19 (2H, m, Glu β-CH_2), 2.21–2.35 (2H, m, Glu γ-CH_2), 2.75–2.97 (2H, m, Cys β-CH_2), 3.11–3.14 (2H, m, Cys S-C*H*$_2$-CH-CH_2), 3.72 (3H, s, OMe), 4.32–4.36 (1H, m, Cys α-CH), 4.61–4.68 (1H, m, Glu α-CH), 5.08–5.14 (2H, m, Cys S-CH_2-CH-C*H*$_2$), 5.31 (1H, d, Cys NH), 5.67–5.81 (1H, m, Cys S-CH_2-C*H*-CH_2), 6.53 (1H, d, Glu NH). ^{13}C NMR ($CDCl_3$) δ: 28.20 (OBu^t), 28.53 (Boc), 32.39 (Glu β-CH_2), 33.11 (Cys β-CH_2), 35.36 (S-CH_2), 52.51 (Glu γ-CH_2), 52.71 (Cys α-CH), 53.09 (Glu α-CH), 76.46 (OMe), 82.04 (OBu^t), 85.10 (Boc), 118.05 (S-CH_2-CH-*C*H_2), 133.89 (S-CH_2-*C*H-CH_2), 170, 173, 174, 186, and 197 (5 × CO).

Boc-Gly(OCH$_2$O)-Glu[Cys(Allyl)-OBu^t]-OMe (9)

The dipeptide **7** (4.37 g, 9.48 mmol) was dissolved with TFA (9.43 mL, 123.24 mmol) and dry CH_2Cl_2 (19.55 mL) for 4 h at 0 °C. After evaporation, the residue was treated with Et_2O to afford the corresponding pure dipeptide **8** (4.5 g, quantitative yield).

A suspension of Boc-Gly(OCH$_2$O)-*p*-nitro-phenyl-carbonate **4** (3.33 g, 9.01 mmol), HOBt (1.22 g, 9.01 mmol), and NMM (0.99 mL, 9.01 mmol) in dry DMF (210 mL) was added to a solution of **8** (4.27 g, 9.01 mmol) and NMM (0.99 mL, 9.01 mmol) in DMF (210 mL); the flask was left for 2 h at 0 °C under nitrogen atmosphere and 19 h at 20 °C. After evaporation, the residue was washed with CH_2Cl_2/5% citric acid (×2), H_2O (×1), $NaHCO_3$ (×2), H_2O (×1), and NaCl (×1) and then evaporated. Compound **9** (2.4 g) was obtained after chromatographic purification using $CHCl_3$:MeOH (97:3). Yield: 45%. R_f = 0.40, $CHCl_3$:MeOH (97:3). ^{1}H NMR ($CDCl_3$) δ: 1.42 (9H, s, OBu^t), 1.46 (9H, s, Boc), 1.96–2.24 (2H, m, Glu β-CH_2), 2.29–2.38 (2H, m, Glu γ-CH_2), 2.75–2.98 (2H, m, Cys β-CH_2), 3.11–3.15 (2H, m, Cys S-C*H*$_2$-CH-CH_2), 3.73 (3H, s, OMe), 3.89 (2H, d, Gly α-CH_2), 4.36–4.41 (1H, m, Glu α-CH), 4.60–4.65 (1H, m, Cys α-CH), 5.00 (1H, d, Gly NH), 5.09–5.12 (2H, m, Cys S-CH_2-CH-C*H*$_2$), 5.64–5.76 (1H, m, Cys S-CH_2-C*H*-CH_2), 5.79 (2H, s, OCH_2O), 6.02 (1H, d, Glu NH), 6.41 (1H, d, Cys NH). ^{13}C NMR ($CDCl_3$) δ: 28.04 (Glu β-CH_2), 28.19 (OBu^t), 28.52 (Boc), 32.21 (Glu γ-CH_2), 33.13 (Cys β-CH_2), 35.33 (S-CH_2), 42.53 (Gly α-CH_2), 52.39 (Cys α-CH), 52.92 (Glu α-CH), 53.71 (OMe), 76.83 (OCH_2O), 80.57 (OBu^t), 83.09 (Boc), 118.12 (S-CH_2-CH-*C*H_2), 133.88 (S-CH_2-CH-CH_2), 154.41, 169.34, 169.88, 170.11, 171.66, and 172.12 (6 × CO).

Cyclo-{Gly(OCH$_2$O)-Glu[Cys(Allyl)]-OMe} (CP11)

The tripeptide **9** (2.26 g, 3.82 mmol) was treated with TFA (63 mL, 821.3 mmol) for 5 h at 20 °C. After evaporation, the treatment with ether provided the fully deprotected tripeptide **10** (2.1 g) (quantitative yield).

Compound **10** (1.8 g, 3.37 mmol) and NMM (3.92 mL, 35.7 mmol) in CH_2Cl_2 (800 mL) were added over 2 h to an ice-cold mixture of bis(2-oxo-3-oxazolidinyl)phosphonic chloride (4.29 g, 16.85 mmol) and 4-(dimethylamino)pyridine (410 mg, 3.37 mmol) in CH_2Cl_2 (600 mL). The reaction mixture was left for 72 h at 20 °C. After evaporation, the residue was treated with CH_2Cl_2 and washed with 5% citric acid (×2), H_2O (×1), $NaHCO_{3ss}$ (×2), H_2O (×1), and $NaCl_{ss}$ (×1), and the organic layers were evaporated. After RP-chromatographic purification on silica gel with gradient scale of CH_3CN:H_2O (from 5:95 to 40:60) as eluant, the final product **CP11** (300 mg) was achieved. Yield: 20%. R_f = 0.7, $CHCl_3$:MeOH (9:1). ^{1}H NMR ($CDCl_3$) δ: 1.93–2.21 (2H, m, Glu β-CH_2), 2.27–2.35 (2H, m, Glu γ-CH_2), 2.61–2.99 (2H, m, Cys β-CH_2), 3.16–3.19 (2H, m, Cys S-CH_2-CH-CH_2), 3.70 (3H, s, OMe), 3.92 (2H, d, Gly α-CH_2), 4.40–4.48 (1H, m, Glu α-CH), 4.51–4.62 (1H, m, Cys α-CH), 5.07–5.19 (2H, m, Cys S-CH_2-CH-CH_2), 5.71–5.83 (1H, m, Cys S-CH_2-C*H*-CH_2), 5.84 (2H, s, OCH_2O), 8.03–8.09 (1H, d, Gly NH), 8.15–8.17 (1H, d, Glu NH), 8.22–8.25 (1H, d, Cys NH). ^{13}C NMR ($CDCl_3$) δ: 27.15 (Glu β-CH_2), 32.03 (Cys β-CH_2), 33.67 (Glu γ-CH2), 34.63 (S-CH_2), 47.23 (Gly α-CH_2), 51.95 (OMe), 55.69 (Glu α-CH), 61.54 (Cys α-CH), 86.52 (OCH_2O), 118.31 (S-CH_2-CH-*C*H_2), 133.12 (S-CH_2-*C*H-CH_2), 157.5, 171.3, 171.9, 172.9, and 175.8 (5 × CO). MS (ESI): 418.2 *m*/*z* $(M + H)^+$.

Pharmacokinetic Studies

Determination of cLogP was performed by ACD LogP software package, version 4.55 (Advanced Chemistry Development Inc., Toronto, Canada). LogP analysis was performed as previously reported. (18) The purity of **CP11** was detected using the experimental HPLC conditions reported in the Supporting Information.

CP11 water solubility was established using HPLC methods as reported before. (19) The adopted procedures for the evaluation of chemical and enzymatic hydrolysis were previously developed by Estour et al. (20) To determine the stability of **CP11**, human plasma was treated as reported. (21) To assess the hydrolytic stability of **CP11** to pepsin and pancreatin, the SGF and SIF were produced as described elsewhere. (22, 23)

For SPE of **CP11** from human plasma we used 3 mL AFFINIMIP GSH cartridges following the manufacturer's instructions. After SPE, the residue was dissolved in D_2O for NMR analysis.

The **CP11** permeability was determined using PAMPA-BBB and PAMPA-GI protocols already developed. (24-27)

Cytotoxicity Assay

MTT assay was used to evaluate U937 cells' sensitivity to **CP11**. Cells (2×10^5), cultured as previously described, (28) were seeded in a 96-well plate and treated with **CP11** and GSH (as control) at concentrations of 1, 10, 100, and 1000 µM for 24 h. The MTT solution was added to each well and incubated at 37 °C for 4 h. The resulting formazan crystals were solubilized with DMSO (200 µL/well) at 37 °C for 30 min. Microplate Reader was adopted to read the OD_{570}, and the percentage of cell vitality was calculated. The experiments were performed in duplicate.

Measurement of ROS and Antioxidant Enzyme

The production of O_2^- was determined by NBT assay. (29) The amount of NBT-formazan was quantified spectrophotometrically (SpectraMax 190, Molecular Devices) at 630 nm. The SOD activity was detected spectrophotometrically as described elsewhere. (30)

Determination of TNF-α

TNF-α was assayed using specific ELISA development systems (Pierce, Rockford, IL, USA) on the supernatants removed from monocyte cultures.

Western Blot Analysis

The U937 cell lysates were analyzed by Western blot analysis. (31, 32) The primary antibodies used were the following: anti-p-ERK (Thr202/Tyr204, sc-101760), anti-p-p38 (Thyr180/Tyr182, sc-101759), and mouse monoclonal anti-p-JNK (Thr183/Tyr 185, sc-81502) (all from Santa Cruz Biotechnology, CA, USA).

Measurement of Apoptotic Cells by Annexin-V/PI Detection

Apoptosis was detected using Annexin-V-FITC/PI Kit (Bender Med System, Vienna, Austria). The data were examined by CXP analysis software (Beckman Coulter, Miami, FL, USA). (33)

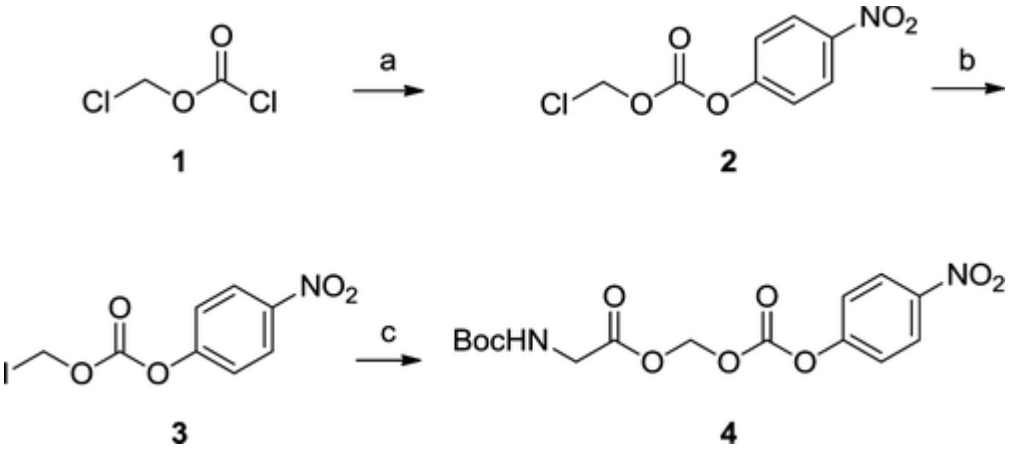
Statistics

Analysis is reported as the mean ± SD. Statistical significance was calculated by one-way analysis of variance (ANOVA), and $p < 0.05$ values were significative.

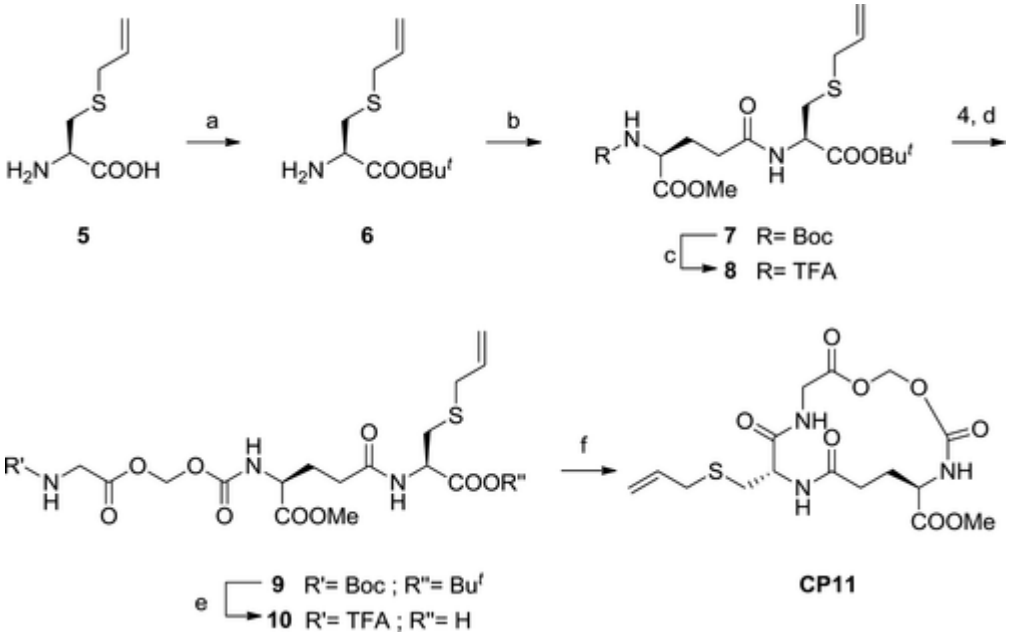
Results and Discussion

Compound Design and Synthesis of CP11

The synthesis of **CP11** was performed as reported in Schemes 2 and 3. Following a convergent approach, synthetic fragments **4** and **8** were separately synthesized and then conjugated. The synthesis of **3** was carried out as reported in the literature (Scheme 2). (14) Then, compound **3** was coupled with Boc-Gly-OCs in DMF to afford Boc-(glycyloxy)methyl *p*-nitrophenyl carbonate (**4**) and Boc-Gly-O*p*NP; this mixture was used for the subsequent reaction since it resulted to be difficult to purify.



Scheme 2. Reagents and conditions: (a) *p*-nitro-phenol, NMM, $CHCl_3$, 1 h, 0 °C and 15 h, rt; (b) NaI, acetone, 24 h, 50 °C; (c) Boc-Gly-O^-Cs^+, dry DMF, 2 h, 0 °C and 15 h, rt.



Scheme 3. Reagents and conditions: (a) $HClO_4$, $AcOBu^t$, 4 h, rt; (b) Boc-Glu-OMe, IBCF, TEA, dry DMF, 20 min at −20 °C, 3 h, 0 °C then 15 h, rt; (c) TFA, CH_2Cl_2, 3 h and 30 min, 0 °C; (d) Boc-Gly(OCH_2O)-*p*-nitro-phenyl-carbonate (**4**), NMM, HOBt, dry DMF, 2 h, 0 °C and 15, rt; (e) TFA, 5 h, rt; f) bis(2-oxo-3-oxazolidinyl)phosphonic chloride, 4-(dimethylamino)pyridine, NMM, dry CH_2Cl_2, 2 h, 0 °C and 72 h, rt.

Linear tripeptide **10** was obtained via Boc-amino acid strategy. The temporary protection of the cysteine carboxyl group **5** was achieved using $HClO_4$ in $AcOBu^t$ in 80% yield. (34) Coupling between Boc-Glu-OMe, commercially available, and *t*-butyl ester **6** in the presence of isobutyl chloroformate (IBCF) and TEA in DMF gave the dipeptide Boc-Glu-[Cys(allyl)-OBu^t]-OMe (**7**) in 99% yield. Compound **7**, following the treatment with TFA in CH_2Cl_2, gave **8** (yield 100%). The assembly of fragments **4** and **8** gave tripeptide **9** in 45% yield. The removal of Boc and OBu^t groups on **9** was fulfilled after treatment with TFA for 5 h affording the corresponding trifluoracetate **10** in quantitative yield. (35) After different attempts of cyclization, the best experimental conditions were reached employing bis(2-oxo-3-oxazolidinyl)phosphonic chloride, (36) 4-(dimethylamino)pyridine, and NMM in CH_2Cl_2. The cyclic prodrug **CP11** was obtained in low yield (20%) after RP-chromatography. The purity of **CP11** resulted as higher than 98% after HPLC purification (see Supporting Information).

Pharmacokinetic Properties of CP11

Table 1 shows the water solubility of **CP11** and its lipophilicity (LogP and cLogP). Our data revealed that **CP11** has a high water solubility (2.82 mg/mL), suggesting its favorable GI absorption. (37)

Table 1. Physicochemical Properties of CP11 (*n* = 3)[a]

lipophilicity		
LogP	**cLogP**	**water solubility (mg/mL)**
0.345 (±0.001)	–0.161 (±0.001)	2.82 (±0.07)

[a]Values ± SD.

The stability of **CP11** was evaluated in two buffers (pH 1.2 and 7.4), in simulated fluids (SGF and SIF) with different concentrations of enzymes (10 mg/mL and 40 mg/mL), and in human plasma. As reported in Table 2, **CP11** is stable at acidic pH, suggesting that it is able to pass unhydrolyzed through the gastrointestinal tract. The presence of selective esterases in human plasma resulted as responsible for the faster enzymatic degradation rate compared to the chemical one, as observed by data reported in Table 2. To confirm the real hydrolysis of **CP11** into the linear peptide (*S*-allyl-GSH) we extracted by SPE procedure the mixture obtained after 24 h incubation of **CP11** in human plasma and then analyzed it using a NMR spectrophotometer. NMR spectra showed that the hydrolysis product can be identified with the desired linear peptide (see Supporting Information), as similarly proposed for cyclic prodrugs of opioid peptides. (14)

Table 2. Pharmacokinetic Data of CP11 at 37 °C (*n* = 3)[a]

		$t_{1/2}$ (h)	k_{obs} (h^{-1} x 10^{-3})
Chemical Hydrolysis			
	pH 1.2	110 (±2)	(6.3 ± 0.1)
	pH 7.4	19.58 (±0.54)	(35.4 ± 1.0)
SGF	pepsin 10 mg/mL	108 (±3)	(6.4 ± 0.2)
	pepsin 40 mg/mL	105 (±2)	(6.6 ± 0.1)
Enzymatic Hydrolysis			
SIF	pancreatin 10 mg/mL	17.05 (±0.77)	(40.6 ± 1.8)
	pancreatin 40 mg/mL	14.23 (±0.48)	(48.7 ± 1.6)
human plasma		19.83 (±0.69)	(34.9 ± 1.2)

[a]Values ± SD.

For predicting the capacity of **CP11** to cross the biological barriers, PAMPA assays were performed. To evaluate the GI permeation, the studies were implemented at pH 5.0, 6.5, and 7.4 with an 18 h incubation time; the pH of acceptor and donor compartments was always the same. (38) The PAMPA-BBB assays were conducted at pH 7.4, and porcine polar brain lipid was used as phospholipid membrane; also in this case acceptor and donor compartments had the same pH (Table 3). After 18 h of incubation, **CP11** showed low permeability through PAMPA-GI membrane, whereas it was more permeable to PAMPA-BBB and it was classified as discrete BBB permeable compound (CNS+/−). The reduced permeability of **CP11** could be attributed to its high water solubility and attitude to form H-bonds with the solvent.

Table 3. CP11 Permeability through GI and BBB Artificial Membranes (18 h of Incubation, *n* = 3)

	permeability P_e (10^{-6} cm/s)	
pH[a]	PAMPA-GI	PAMPA-BBB[b]
7.4	0.99	2.49
6.5	0.71	
5.0	0.70	

[a]pH of donor and acceptor compartments.

[b]Classification CNS+/–.

Biological Studies

ROS are responsible for the onset of several disorders. In pathological conditions, the physiological balance between oxidants/antioxidants observed in healthy individuals could be affected by a condition of increased levels of ROS and/or a decrease of antioxidant activities. This concept points out the use of antioxidants as therapeutic strategy to enhance the immune cell functions and restore the balance of oxidants/antioxidants, thus preventing the following tissue damage. Reports showed that GSH, as universal antioxidant, is implicated in the protection against ROS produced during inflammatory processes through different mechanisms. (39-41) Consequently, the knowledge of the regulatory mechanisms of GSH is vital to develop new therapies to solve the inflammatory condition.

Therefore, our purpose was to establish the anti-inflammatory characteristics of **CP11** in U937 cells, an immortalized human monocyte cell line, that represents a valid model for studying the inflammatory response. (42) In fact, monocytes possess the ability to intervene in the initial inflammatory response to LPS endotoxin through TLR4, (43) which activates several intracellular signaling proteins, thus triggering proinflammatory mediators. (44) Initially, we analyzed the effects on cell survival and cytotoxicity in U937 monocytes by MTT assay. We found that the treatment of cells for 24 h in the presence of 1, 10, 100, and 1000 μM of **CP11** and GSH (as reference compound), respectively, did not cause any toxic effect on cell viability (Figure 1). The addition of 1000 μM for either tested compound resulted in a weak decrease of cell viability (80% and 75%, respectively). Next, we conducted an Annexin-V/PI assay by flow cytometry analysis to evidence a dose-dependent modulation in apoptotic cell percentage in LPS-stimulated U937 cells preincubated with **CP11** and GSH (1, 10, 100, and 1000 μM). We observed that such variations were not significant, as confirmed by no change in viable (unlabeled) cell percentage when compared to LPS-induced U937 cells in alignment with MTT results (Figure 2). Subsequently, we focused our investigation on antioxidant ability of **CP11** in LPS-stimulated monocytes. As reported in Figure 3a, the treatment of cells with LPS produced a significant enhancement of the intracellular concentration of $O_2^{\bullet -}$ with respect to control. ROS generation was significantly reduced in LPS-stimulated U937 cells pretreated with GSH, confirming its typical antioxidant capacity. Similar antioxidant effects were obtained with **CP11** treatment, showing a higher remission with respect to GSH, at a concentration of 10 μM. In addition, to verify if the cells had maintained the delicate balance between ROS generation and antioxidants, SOD activity was evaluated (Figure 3b).

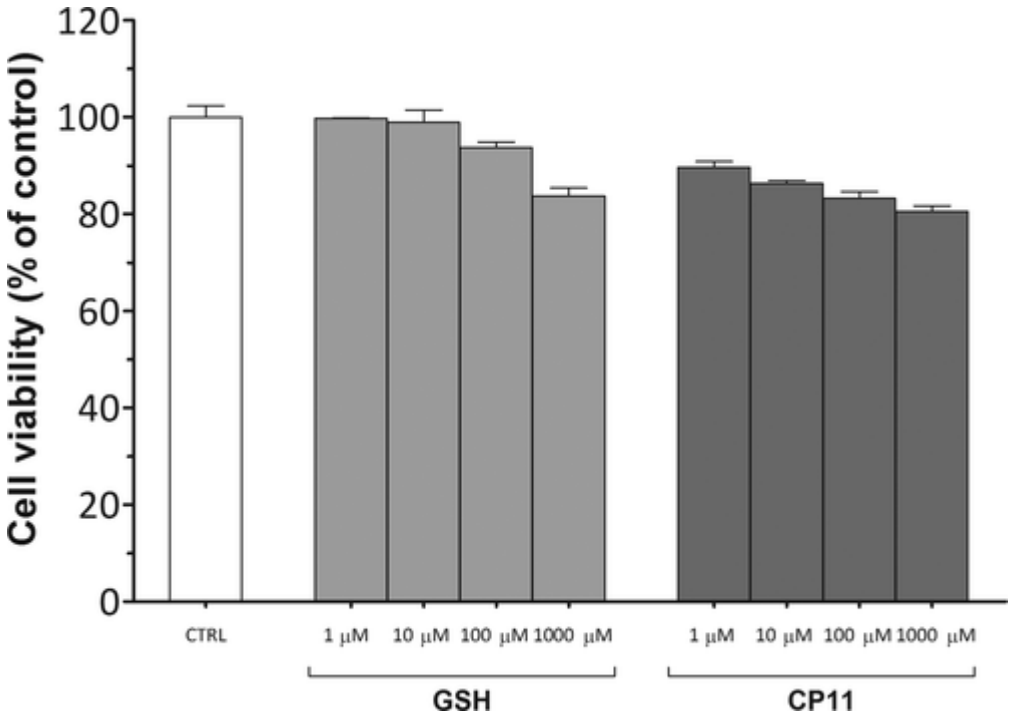


Figure 1. MTT measurements after **CP11** and GSH treatments (1–1000 μM) on U937 cells for 24 h. Results (*n* = 3) are expressed as mean ± SD.

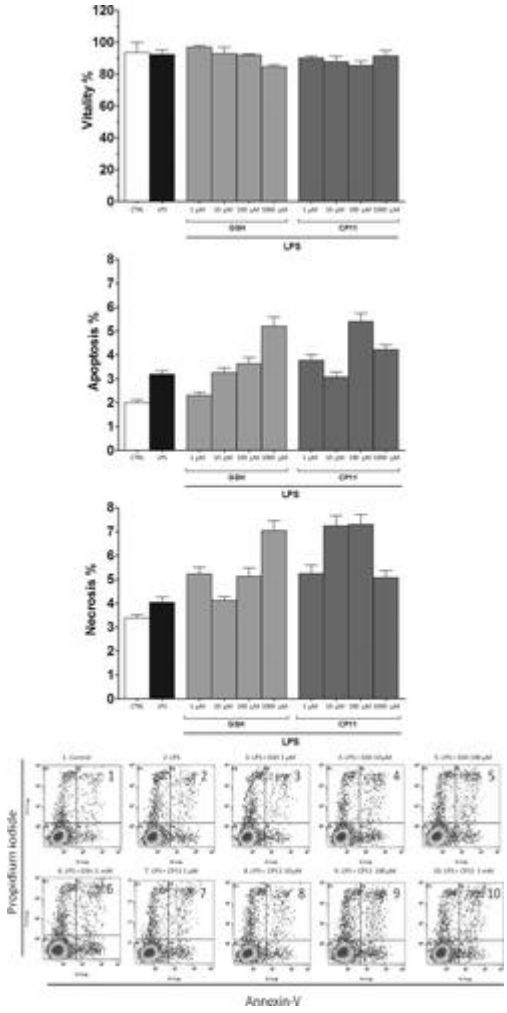


Figure 2. Effect of different concentrations of **CP11** and GSH on apoptosis in LPS-treated U937 monocytes. Apoptosis was determined by Annexin-V/propidium iodide (PI) assay. Histograms for vitality, apoptosis, and necrosis were obtained from different fluorescence emission. Results (n = 3) are expressed as percentage ± SD.

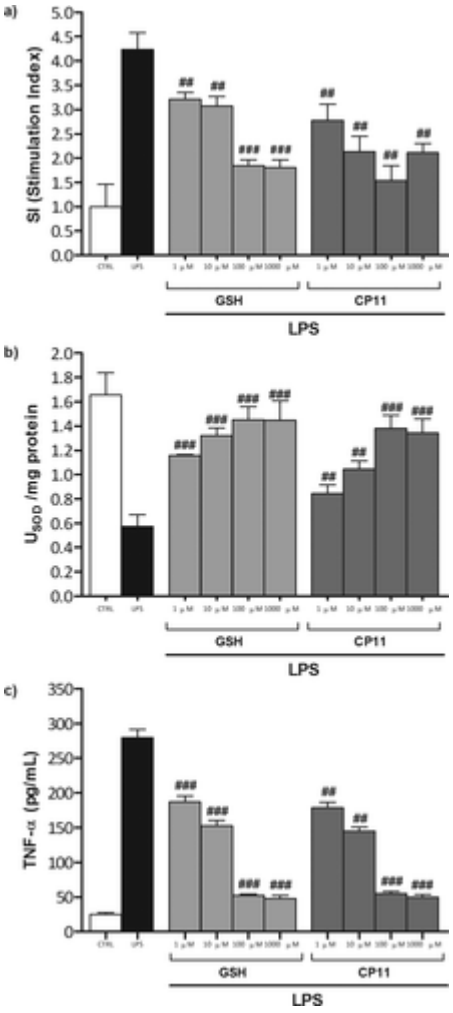


Figure 3. Effects of **CP11** and GSH on preventing LPS-induced ROS production and proinflammatory mediator in U937 cells. (a) Antioxidant activity of **CP11** and GSH against oxidative stress measured by NBT test. Results were registered as stimulation index (SI). SI value of 1 was assigned to control cells. (b) SOD activity was indicated as unit mg^{-1} of proteins. (c) Secreted TNF-α was revealed by ELISA kit. Results (n = 3) were reported as means ± SD. ## $p < 0.05$ and ### $p < 0.01$, significance with respect to LPS-stimulated cells.

In LPS-treated monocytes, we observed a significant diminution of SOD activity with respect to control cells. The effect was completely reverted after 1 h of preincubation with **CP11** as a function of the concentration; a similar trend was observed with GSH pretreatment. Lastly, we explored the effect of **CP11** on the release of TNF-α, an important endogenous inflammatory mediator secreted by LPS-stimulated monocytes. (45) As shown in Figure 3c, LPS stimulates U937 cells to secrete a TNF-α amount 11-fold superior to GSH. On the contrary, pretreatment of cells with **CP11** showed a significant dose-dependent inhibitory effect on TNF-α release from monocytes treated with LPS, comparable to GSH. To analyze the molecular mechanism underlying the anti-inflammatory effect of **CP11**, we further examined its effect on phosphorylation of MAPKs as JNK, ERK 1/2, and p38 (Figure 4). Literature data evidenced that ROS, produced during oxidative stress, manage the MAPK signal transduction pathway which is responsible for the regulation of inflammatory genes and inflammatory cytokines. (46, 47) Studying this pathway, we found that LPS

treatment produced a dramatic induction of phospho-p38 (1.98-fold vs control), phospho-JNK (3.31-fold vs control), and phospho-p42/p44-ERK (1.73-fold vs control) compared to control cells. Such inductions were all attenuated by pretreatment with **CP11** and GSH at concentrations of 10, 100, and 1000 µM in LPS-stimulated cells, while no significant modulation was revealed at concentrations of 1 µM (data not shown). Overall, these results proved that both peptides have a similar trend of ability to decrease MAPK expression/activity in LPS-stimulated cells, with a significantly greater effect for **CP11** compared to GSH: in fact, **CP11** resulted in a broad spectrum of inhibition of signal transduction pathway.

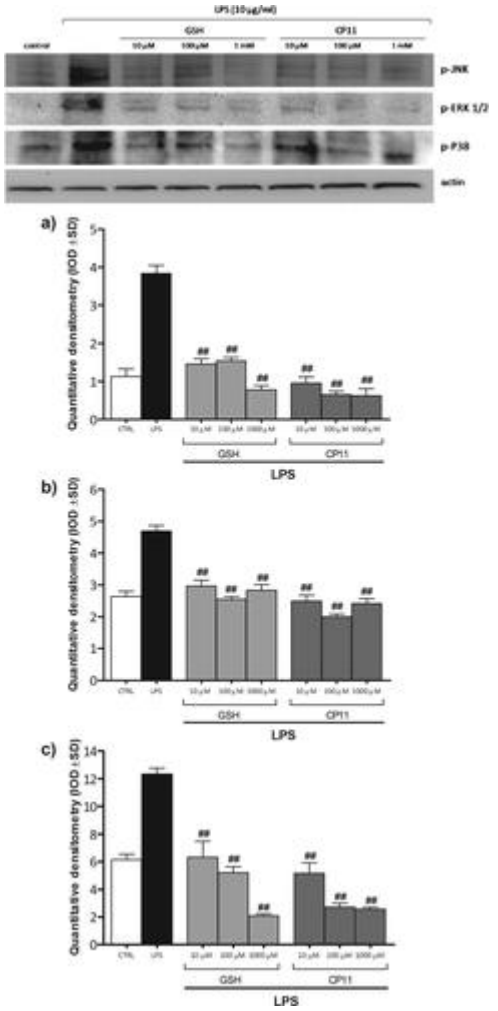


Figure 4. Effect of **CP11** and GSH at concentrations of 10, 100, and 1000 µM on LPS-induced signal transductions in U937 cells. Representative image of Western blot analysis for p-JNK (a), p-ERK (b), p-p38 (c), and β-actin (top). At the bottom, in the densitometric analysis (n = 3), each bar is reported as the intensity of optical density (IOD) ± SD. ## $p < 0.05$, significance with respect to LPS-stimulated cells.

Conclusion

Over the years the prodrug strategy has been successfully employed in the design of novel compounds with improved biopharmaceutical properties. We applied this medicinal chemistry approach to synthesize a novel cyclic GSH prodrug, bearing an acyloxy-alkoxy linker, with the aim of ameliorating the pharmacokinetic profile of the native tripeptide. *In vitro* pharmacokinetic and biological results showed that **CP11** could be considered an interesting therapeutic candidate since (a) it is stable in acidic conditions; (b) it releases allyl-GSH more quickly in the enzymatic environment than in the chemical one, due to the presence of plasma esterases; (c) it is classified as discrete BBB-permeable compound; (d) it is able to modulate the LPS-induced inflammation in U937 cells, thus preventing the increase of ROS intracellular levels via the MAPK pathway. Consequently, our encouraging outcomes indicate that **CP11** could be a new

chemical tool potentially valuable for the treatment of neurological pathologies characterized by a tight correlation between oxidative stress and neuroinflammation. This prodrug might constitute the progenitor of a novel class of compounds able to permeate the BBB and act inside the CNS, even though further studies will be required to investigate the biological behavior of **CP11** in *in vivo* experimental models.

Supporting Information

Additional experimental data of **CP11** including HPLC chromatogram and ^{1}H NMR spectrum and effects of **CP11** and GSH on U937 cell viability. This material is available free of charge via the Internet at http://pubs.acs.org.

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- **Notes**

The authors declare no competing financial interest.

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Abbreviations Used

BBB	blood brain barrier
GI	gastrointestinal tract

GSH	glutathione
LPS	lipopolysaccharides
MAPKs	mitogen-activated protein kinases
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PAMPA	parallel artificial membrane permeability assay
ROS	reactive oxygen species
SGF	simulated gastric fluid
SIF	simulated intestinal fluid
SPE	solid phase extraction
SOD	superoxide dismutase
TLR4	Toll-like receptor 4
TNF-α	tumor necrosis factor α

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