Electronic supporting information for:

Renal-specific delivery of prednisolone-folate conjugates for renal ischemia/reperfusion injury

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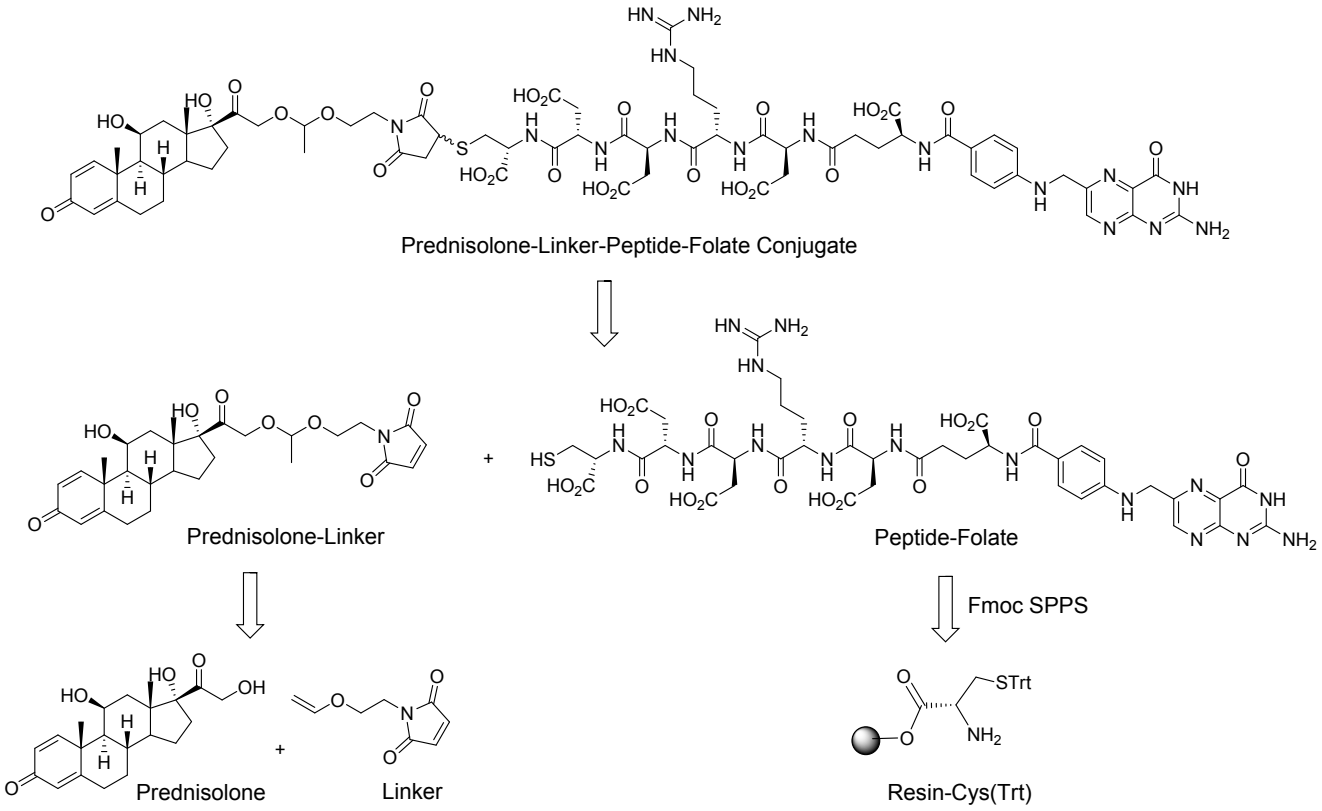
General remarks

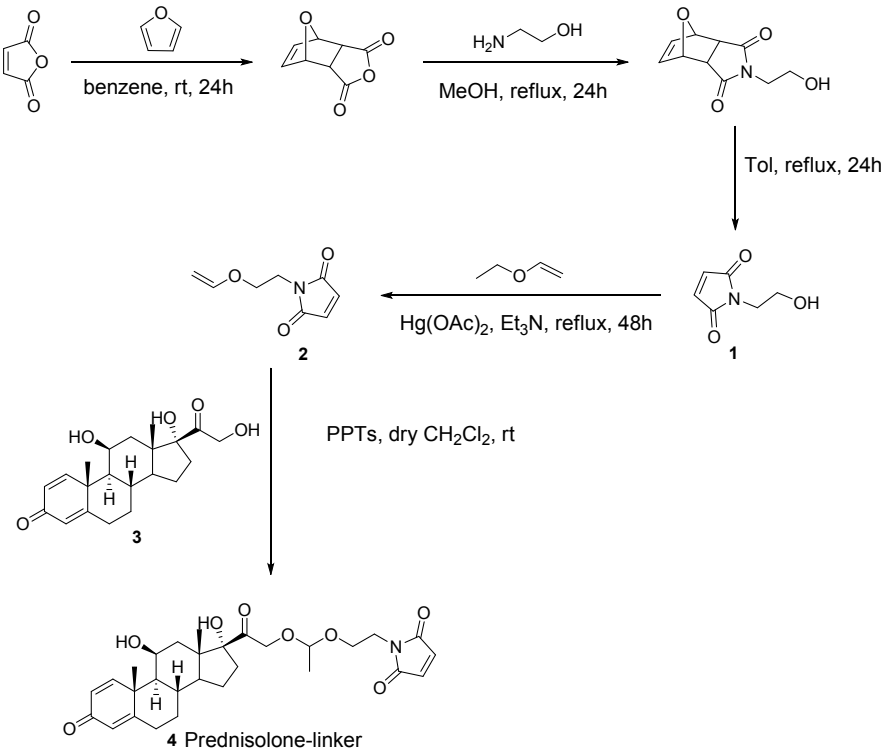
All reactions in non-aqueous media were conducted under a positive pressure of dry nitrogen in glassware that had been oven dried prior to use unless noted otherwise. Anhydrous solutions of reaction mixtures were transferred via an oven dried syringe or cannula. All solvents were dried prior to use unless noted otherwise. Prednisolone was purchased from Lihua Pharmaceutical Co. (Henan, China). Folic acid was purchased from Sinopharm Chemical Reagent Co. Ltd (China). Reagents were purchased from Sigma-Aldrich or Fisher unless otherwise noted. Thin layer chromatography was performed using precoated silica gel plates (EMD Chemical Inc. 60, F254). Infrared spectra (IR) were obtained on a Nicolet FT-IR 20SXB spectrophotometer. ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) spectra were obtained on a Varian Unity-Inova 400 MHz recorded in ppm (δ) downfield of TMS (δ = 0) in $CDCl_3$. Signal splitting patterns were described as singlet (s), doublet (d), triplet (t), quartet (q), quintet (quint), or multiplet (m), with coupling constants (*J*) in hertz. High resolution mass spectra (HRMS) were performed by Analytical Instrument Center at the West China School of Pharmacy on an Electron Spray Injection (ESI) mass spectrometer. Male Sprague-Dawley (SD) rats (by weight 200 ± 20 g), provided by the West China Experimental Animal Center of Sichuan University (China), were maintained in a germ-free environment and allowed free access to food and water for a week. All animal experiments were conducted after the approval by the Animal Ethical Experimentation Committee of Sichuan University according to the requirements of the National Act on the use of experimental animals (China).

The retrosynthetic pathway of Prednisolone-Linker-Peptide-Folate Conjugate (PFC)



Scheme S1 the retrosynthetic pathway of prednisolone-linker-peptide-folate conjugate (PFC)

The synthetic route of prednisolone-linker



Scheme S2 the synthetic route of prednisolone-linker

Compound **4**

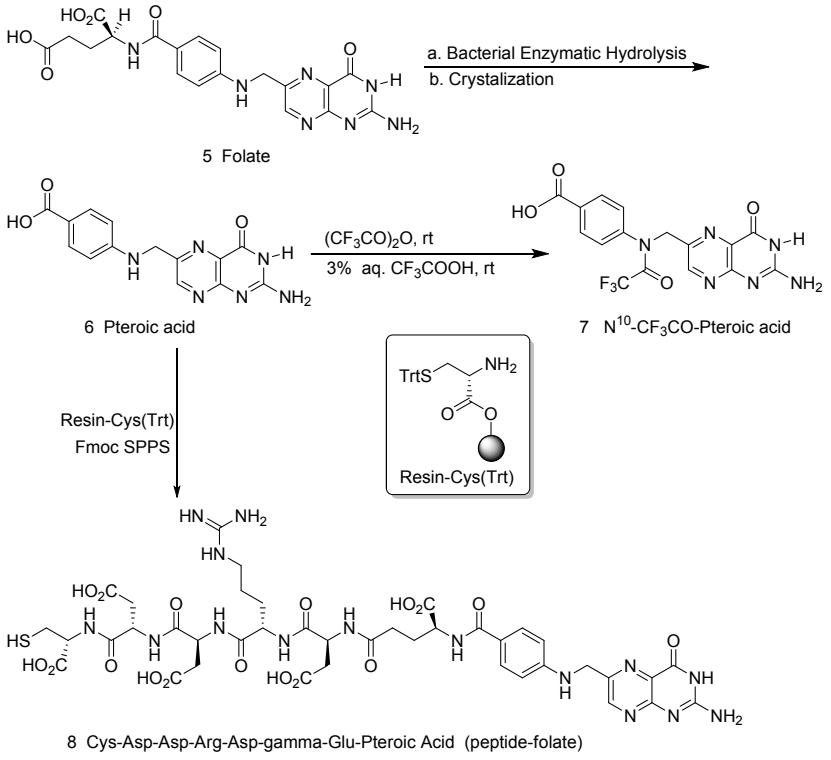
Accurately weighed 1.67 g (10 mmol) compound 2 and 1.80 g (5 mmol) prednisolone were placed in a dry round bottom flask. 125 mg (0.5 mmol) *p*-toluenesulfonic acid was added to the mixture and dissolved in about 60 ml dry dichloromethane under nitrogen protection. The reaction mixture was stirred till the white suspension gradually turned transparent. The product was then subjected to column chromatography to obtain a white solid, (1.90 g, 72.1%). Compound 4 is a mixture of diastereoisomers which was separated by chiral HPLC. NMR and HRMS were used for structural analysis.

Compound 4 isomer-1 (Prednisolone-Linker Isomer-1): ^{1}H NMR (400 MHz, DMSO-d_6-TMS) δ 0.76 (s, 3H), 0.86-0.91 (m, 1H), 0.98-1.03 (m, 1 H), 1.16 (d, *J* = 5.2 Hz, 3H), 1.23-1.29 (m, 1H), 1.39 (s, 3H), 1.39 (m, 1H), 1.49-1.53 (m, 1H), 1.57-1.64 (m, 2H), 1.84-1.87 (m, 1H), 2.01-2.04 (m, 2H), 2.28-2.30 (m, 1H), 2.50-2.54 (m, 2H), 3.52-3.61 (m, 4H), 4.10 (d, *J* = 18.4 Hz, 1H), 4.29 (s, 1H), 4.54 (d, *J* = 18.4 Hz, 1H), 4.63-4.67 (m, 2H), 5.23 (s, 1H), 5.92 (s, 1H), 6.17 (dd, *J* = 10 Hz and 1.6 Hz, 1H), 7.03 (s, 2H), 7.33 (d, *J* = 10 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$-TMS) δ 17.2, 18.8, 20.9, 23.9, 31.2, 32.0, 33.6, 34.0, 37.6, 39.5, 44.2, 47.2, 51.2, 55.2, 60.5, 68.0, 70.0, 89.7, 98.6, 122.1, 127.4, 134.2, 157.0, 170.8, 170.9, 186.8, 208.3. HRMS: Exact mass calculated for $C_{29}H_{37}NO_8$: 527.2519, observed $[M+H]^+$: 528.2594.

Compound 4 isomer-2 (prednisolone-linker Isomer-2): ^{1}H NMR (400 MHz, DMSO–d_6-TMS) δ 0.754 (s, 3H), 0.875-0.909 (m, 1H), 0.961-1.049 (m, 1 H), 1.165 (d, *J* = 5.6 Hz, 3H), 1.235-1.315 (m, 1H), 1.387 (s, 3H), 1.387 (m, 1H), 1.500-1.533 (m, 1H), 1.570-1.652 (m, 2H), 1.840-1.881 (m, 1H), 2.012-2.036 (m, 2H), 2.272-2.306 (m, 1H), 2.500-

2.536 (m, 2H), 3.515-3.627 (m, 4H), 4.133 (d, *J* = 18.4 Hz, 1H), 4.291 (s, 1H), 4.493 (d, *J* = 18.4 Hz, 1H), 4.629-4.677 (m, 2H), 5.231 (s, 1H), 5.918 (s, 1H), 6.165 (dd, *J* = 10 Hz and 1.6 Hz, 1H), 7.021 (s, 2H), 7.323 (d, *J* = 10 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$-TMS) δ 17.342, 18.966, 20.894, 23.866, 31.189, 31.961, 33.724, 33.973, 37.645, 39.506, 44.192, 47.386, 51.111, 55.255, 60.664, 68.758, 70.001, 89.491, 99.201, 122.073, 127.459, 134.172, 156.957, 170.780, 170.852, 186.778, 208.244. HRMS: calculated for $C_{29}H_{37}NO_8$: 527.2519, observed $[M+Na]^+$: 550.2418.

The synthetic route of peptide-folate

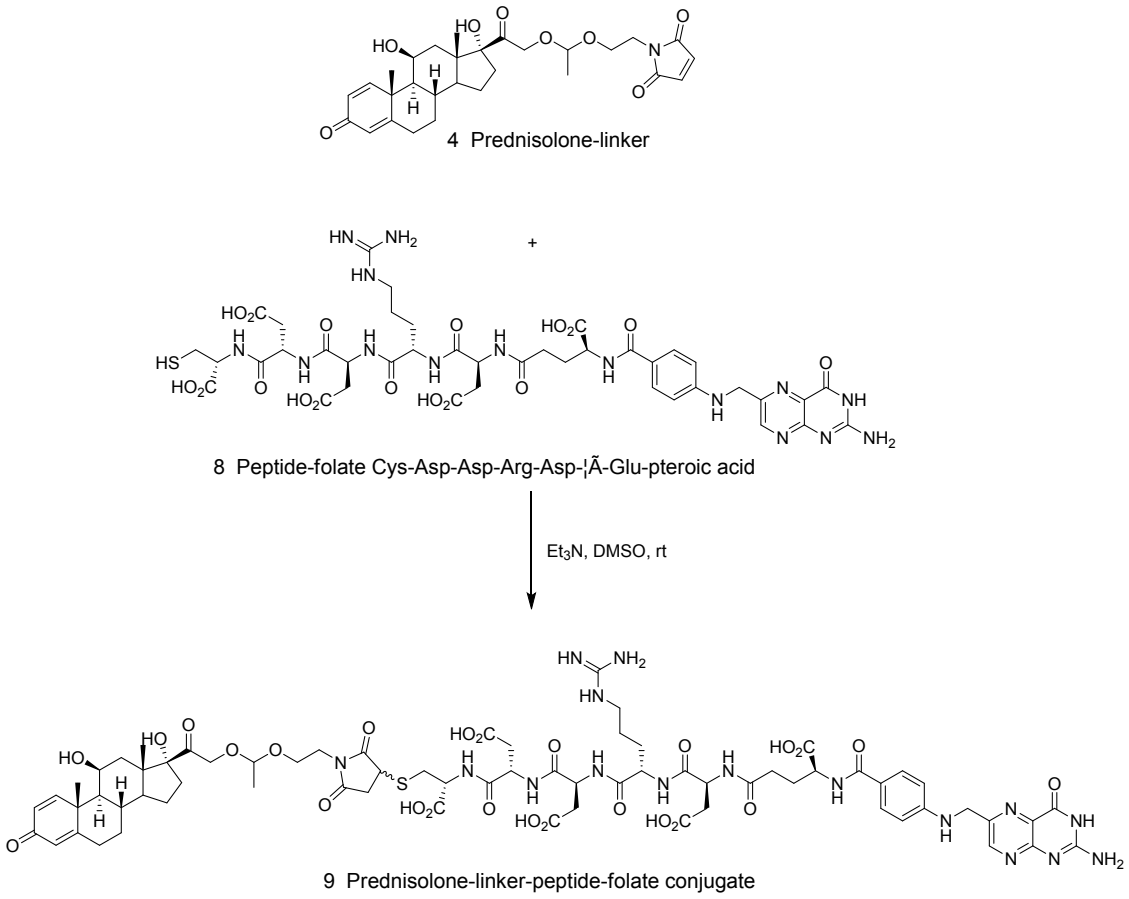


Scheme S3 the synthetic route of peptide-folate

Compound **8** (peptide-folate)

Peptide-folate (Asp-Asp-Arg-Asp-γGlu-pteroic acid) was synthesized on a Fmoc-Cys(Trt)-2-Chlorotrityl resin by the solid phase peptide synthesis methodology. Reagent I (20% piperidine, 80% DMF) was used to deprotect Fmoc. Reagent II (TFA:H_2O:EDT = 94:3:3) was used for peptide cleavage. The obtained peptide-folate conjugate was purified by HPLC and freeze-dried to obtain a fluffy yellow solid (1.25 g). Selected ^1H NMR data (400 MHz, D_2O+DMSO-*d*6-TMS) δ 8.710 (s, 1H, FA H-7), 7.666 (d, *J* = 8.4 Hz, 2H, FA H-12 & 16), 6.676 (d, *J* = 8.8 Hz, 2H, FA H-13 & 15), 4.508-4.587 (m, 5H), 4.382-4.424 (m, 1H), 4.296-4.332 (m, 1H), 4.175-4.208 (m, 1H), 3.075 (m, 2H), 2.490–2.879 (series of m, 8H), 2.220-2.256 (m, 2H), 2.048–2.065 (m, 1H), 1.886–1.939 (m, 1H), 1.474-1.698 (m, 4H). LC-MS: calculated for $C_{40}H_{51}N_{15}O_{17}S$: 1045.3, observed $[M+2H]^{2+}$: 523.7

The synthetic route of prednisolone-linker-peptide-folate conjugate



Scheme S4 the synthetic route of prednisolone-linker-peptide-folate conjugate

Compound **9** (prednisolone-linker-peptide-folate conjugate)

In a dry round flask, 0.66 g (1.25 mmol) compound 4 isomer I and 0.325 g (0.31 mmol) compound 8 (Peptide-folate) were dissolved in about 5 ml dry DMSO under an atmosphere of nitrogen. About 0.13 ml (0.93 mmol) triethylamine was added dropwise to the above solution and the reaction mixture was stirring at RT in dark for 60 min. The obtained product was purified by preparative HPLC. The preparative HPLC system consisted of a Shimadzu LC-10ATvp pump, a Shimadzu sPD-M10ATvp detector, and a Xbridge Prep C18 column (5 μm, 19mm × 150 mm). The mobile phase consisted of pH7.0 10 mM ammonium acetate (82%, v/v) and acetonitrile (18%, v/v).

Selected ^{1}H NMR data (400 MHz, D_2O+DMSO-*d*6-TMS) δ 8.605 (s, 1H, FA H-7), 7.542 (d, *J* = 8.4 Hz, 2H, FA H-12 & 16), 7.375 (d, *J* = 10.4 Hz, 1H, Pred H-1), 6.617 (d, *J* = 8.8 Hz, 2H, FA H-13 & 15), 6.165 (d, *J* = 10 Hz, 1H, Pred H-2), 5.914 (s, 1H, Pred H-4), 1.285 (s, 3H, Pred H-19), 1.099 (d, *J* = 4.4 Hz, 3H, Acetal), 0.680 (s, 3H, Pred H-18)

LC-MS: calculated for $C_{69}H_{88}N_{16}O_{25}S$: 1572.6, observed $[M+2H]^{2+}$: 787.3

Rat peritoneal macrophage isolation and culture

Male SD rats were given 5 ml thioglycollate medium (Sigma-Aldrich, USA) via an intraperitoneal injection. 4 days later, rats were sacrificed and sprayed with 70% ethanol. According to the protocol for mouse peritoneal macrophage isolation [1], all the following procedures were conducted under sterile conditions. (i) The outer skin of the peritoneum was cut with a scissors and forceps to expose the inner skin lining the peritoneal cavity. (ii) About 20 ml ice cold PBS was carefully injected into the peritoneal cavity. (iii) Using a 25 g needle, fluid in the peritoneum was gently collected and filtered through 70 μm-sieves. Filtered fluid was collected and deposited in tubes kept on ice. Step (ii) and (iii) were repeated twice and the filtered fluids were combined and centrifuged (1500 rpm for 5 min at 4 °C). The supernatant was removed and cell pellets were resuspended in 1 ml red blood cell lysis buffer and incubated on ice for 5 min. The cell suspension was centrifuged (1500 rpm for 5 min at 4 °C) and resuspended in 5 ml ice cold PBS. After centrifugation (1500 rpm for 5 min at 4 °C), cell pellets were further suspended with 5 ml RPMI-1640 containing 1% FBS and seeded at the density of 2×10^5 cell/well in the 96-well plate. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 for 2 h. After cell attachment, culture medium was removed and cells were gently rinsed with sterile PBS twice to remove nonadherent cells and then cultured with RPMI-1640 containing 10% FBS for anti-inflammatory and cytotoxicity study.

Anti-inflammatory effect and cytotoxicity of PFC *in vitro*

Nitrite is the stable product of NO, indicating the production of NO [2]. Nitrite concentration was determined by the Griess reagent in the study. Rat peritoneal macrophages were cultured in a 96-well plate at a density of 2×10^5 cell/well. The cells were cultured for 24 h and treated with LPS (final concentration 5 μg/ml) alone or in combination with different concentrations of prednisolone, PFC and free folate ranging from 0.012 to 400 μM. The control and LPS treated groups received 100 μl RPMI-1640 containing 10% FBS and no folic acid. Briefly, 100 μl supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% N-1-naphylethylenediamine dihydrochloride in water) at room temperature for 10 min. The absorbance was measured at 540 nm. Nitrite concentration in the supernatants was determined from a standard curve using known concentrations of sodium nitrite. Also, The concentration-nitrite production curve was established for prednisolone, PFC and folate, respectively and the IC_{50} values were determined by the concentration for inhibiting 50% nitrite production as stimulated by LPS.

The cytotoxicity of prednisolone, PFC and folate was evaluated using MTT assay as described before [2]. Rat peritoneal macrophages (2×10^5 cell/well) were seeded in a 96-well plate and incubated at 37 °C for 24 h. Cells were treated with LPS (final concentration 5 μg/ml) alone or with prednisolone, PFC and folate at various concentrations. After 24 h of incubation at 37 °C, 15 μl MTT reagent (5 mg/ml) was added to each well and incubated for 3 h. After removal of supernatant, cells were lysed with 150 μl DMSO. The plate was shaken for 10 min and the optical densities (OD_{490}) were determined within 30 min and compared with the OD values of the control or LPS-stimulated wells to assess the cell viability. For the cytotoxicity study, prednisolone, PFC and folate displayed minimum cytotoxicity with over 85% of rat peritoneal macrophages remaining viable at the maximum treatment concentrations of 50, 400, and 400 μM, respectively.

Table S1. Inhibitory activity of prednisolone, PFC, and folate on LPS-induced NO production in rat peritoneal macrophages at 24h. Data represent mean ± SD, (n=3).

Compound	IC_{50}
Prednisolone	171.2 ± 38.6 nM
PFC	12.3 ± 1.2 μM
Folate	54.1 ± 19.8 μM

Sample preparation and quantitative analysis by LC-MS

Plasma or tissue homogenates (0.3 ml) were first mixed with 0.1 ml 10% methanol and subjected to hydrolysis with 10 µl 6N HCl solution at 37 °C for 1 h. 10 µl 6N NH_4OH was then added to neutralize the mixed solution followed by the addition of 800 µl methanol. Sample mixture was vortexed for 5 min, centrifuged at 14000 rpm for 20 min, and the supernatant was filtered through 0.22 µm membrane filter. 1 µl filtrate was used for LC-MS/MS analysis.

The LC-MS/MS system consisted of an Agilent 1200 series RRLC system, including an SL autosampler, degasser, and SL binary pump and an Agilent triple-quadrupole MS. The system was controlled with B01.03 software for qualitative analysis and B01.04 software for quantification. Separation was performed on a Zorbax XDB-C18 column (50 × 2.1 mm, ODS, 1.8 µm) with the corresponding guard column (ODS, 5 µm). The column was maintained at 30 °C and a 1 µl aliquot was injected into the LC-MS/MS system. The mobile phase consisted of water (A) and methanol (B) and a gradient elution was performed: 0 – 4 min, 20% – 80% B; 4 – 6 min, 80% B; 6 – 6.5 min, 80% B – 20% B; 6.5 – 10 min, 20% B. Flow rate was maintained at 0.3 ml/min. The mass spectrometer was operated using an electrospray source and configured to positive ion mode, and quantification was performed using multiple reaction monitoring (MRM). Selected reaction monitoring mode with transitions of *m*/*z* 405.2 → 329.1 was adopted to quantify the prednisolone derivate. Instrumental parameters were as follows: gas temperature, 350 °C; gas flow, 8 ml/min; nebulizer, 30 psi; capillary, positive 4000 v, negative 4000 v. The calibration curves for prednisolone derivative were measured over a range of (i) 100 – 1000 ng/ml (Linearity equation Y = 0.5919X + 10.6281, R^2 = 0.9999) and (ii) 1000 – 20000 ng/mL (Y = 0.5525X + 135.4997, R^2 = 0.9987) in plasma and tissue homogenates (20 – 4000 ng/ml and 4000 – 40000 ng/ml for the kidney homogenate) and were proven to be linear with a correlation coefficient (R^2) of 0.99 of all the calibration curves.

Pharmacokinetics and biodistribution of PFC in rats

Male SD rats were randomly divided into two groups and given prednisolone and PFC through tail vein injection. For each preparation and sampling time point, 5 rats were treated with a single dose of prednisolone and PFC equivalent to 3 mg/kg bodyweight of prednisolone. The rats were sacrificed at 4, 15, 30, 60, and 120 min post injection. The blood plasma and tissue homogenates (hearts, livers, spleens, lungs, kidneys) were collected and immediately stored at -40 °C before use.

Pharmacodynamic study using rat renal IRI model

Male SD rats (250 ± 20 g) were divided into the following four groups: (i) sham operated group, rats were subjected to the same surgical procedure besides renal I/R (n = 5); (ii) I/R group, rats were injected with normal saline via caudal vein 3 days before renal ischemia (n = 5); (iii) I/R + prednisolone group: rats received prednisolone (3 mg/kg/day) via caudal vein 3 days before renal ischemia (n = 5); (iv) I/R + PFC group: rats received PFC (equivalent to 3 mg/kg/day of prednisolone) via caudal vein 3 days before renal ischemia (n = 5). Renal function, and histological morphology were evaluated in all animals.

Renal ischemia-reperfusion (I/R) injury model in rats was established according to previous studies with some modifications [3, 4]. 1% pentobarbital sodium (3 ml/kg) was given via intraperitoneal injection. Rats were maintained in the ischemia 24 h post the surgery, both kidneys were removed from each rat, fixed with 10% buffered formalin and embedded in paraffin. 5 µm sections were placed on glass slides and counterstained with hematoxylin and eosin (H&E). The stained slides were observed under upright Metallurgical microscope (Nikon Eclipse 80i, Nikon Instech Co. Ltd., Japan) at 200× magnification.

Statistics

The area under the curve ($AUC_{0\text{-}t}$) and the maximal concentration (C_{max}) were calculated by Data and Statistics (DAS, Shanghai, China). The statistical analysis was performed using Student's *t*-test. *p*-values < 0.05 were considered statistically different. The relative uptake efficiency (Re_{kidney}) and concentration efficiency (Ce_{kidney}) were calculated to evaluate the kidney targetability of PFC. The values of Re_{kidney} and Ce_{kidney} are defined as follows:

$Re_{kidney} = (AUC_{0\text{-}t,\ kidney})_{PFC}/(AUC_{0\text{-}t,\ kidney})_{P}$

$Ce_{kidney} = (C_{max,\ kidney})_{PFC}/(C_{max,\ kidney})_{P}$

P represents prednisolone.

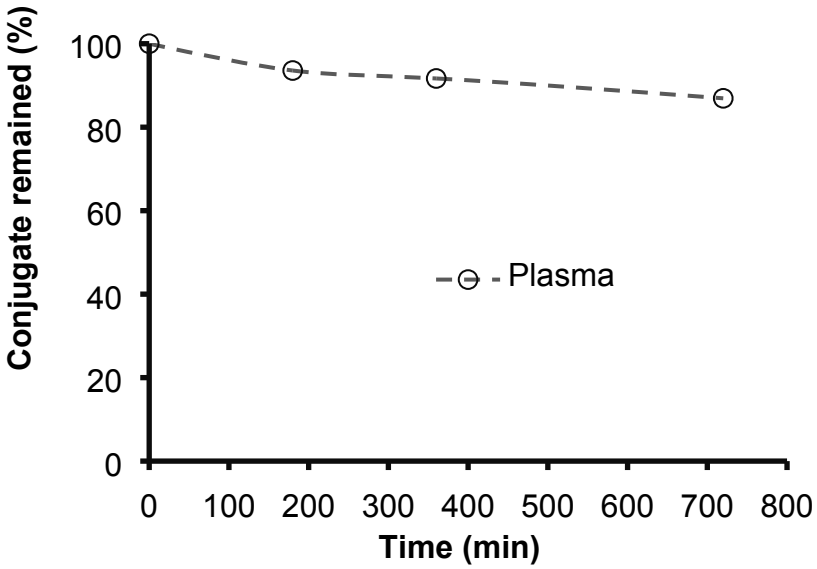


Figure S1. Degradation profile of PFC in the rat plasma (37°C).

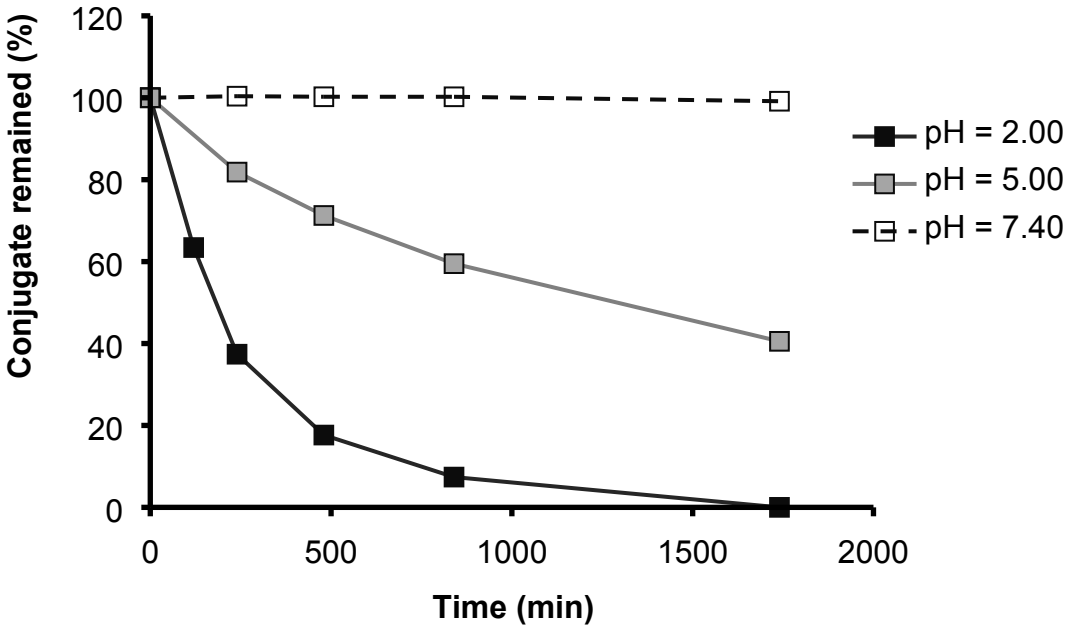


Figure S2. Degradation profile of PFC at pH 2.0, 5.0, and 7.4 (37°C).

Table S2. The pharmacokinetic parameters of prednisolone and PFC in plasma (equivalent to 3 mg·kg^{-1} prednisolone) Data represent mean ± SD, (n = 5).

Parameter	$AUC_{(0-t)}$ (μg/L·min)	$MRT_{(0-t)}$ (min)	$t_{1/2}$ (min)	CL (L/min/kg)	V (L/kg)	C_{max} (μg/L)
Prednisolone	42887.124 ± 4728.906	13.968 ± 1.551	16.283 ± 1.845	0.066 ± 0.009	1.541 ± 0.221	2485.384 ± 481.913
PFC	330786.344 ± 17024.061*	26.584 ± 1.946*	30.033 ± 7.621*	0.009 ± 0.001*	0.392 ± 0.117*	15017.664 ± 1670.896*

*$p < 0.05$ vs. prednisolone

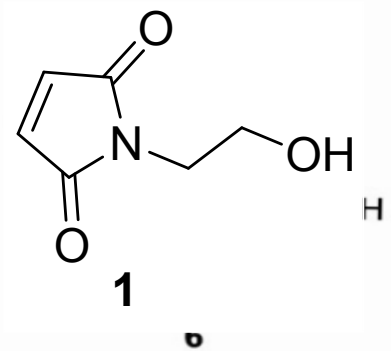
Table S3. Pharmacokinetic parameters of prednisolone and PFC in kidney after *i.v.* injection in rats. Data represent mean ± SD, (n = 5).

Parameters	**$AUC_{(0-t)}$ (μg/L·min)**	**$MRT_{(0-t)}$ (min)**	**C_{max} (μg/L)**	**Re**	**Ce**
Prednisolone	12219.0 ± 1567.0	13.4 ± 2.3	724.7 ± 163.4	-	-
PFC	1427639.4 ± 84931.9	40.1 ± 1.5	29436.7 ± 2775.6	116.8	40.6

Table S4. Blood urea nitrogen (BUN) and serum creatine (CRER) after 3 days of treatment and I/R operation or sham operation. Data represent mean ± SD (n = 5).

Group	BUN (mmol/L)	CRER (mmol/L)
Sham	5.5 ± 1.2	23 ± 4.7
I/R	39.8 ± 8.4	255.4 ± 59.5
I/R + prednisolone	22.2 ± 9.4[(a)]	100.6 ± 43.8[(a)]
I/R + PFC	8.9 ± 3.1[(a)(b)]	30.6 ± 14.0[(a)(b)]

[(a)] $p < 0.05$, as compared with I/R group; [(b)] $p < 0.05$, as compared with I/R + prednisolone group



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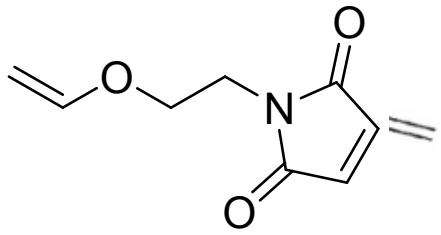

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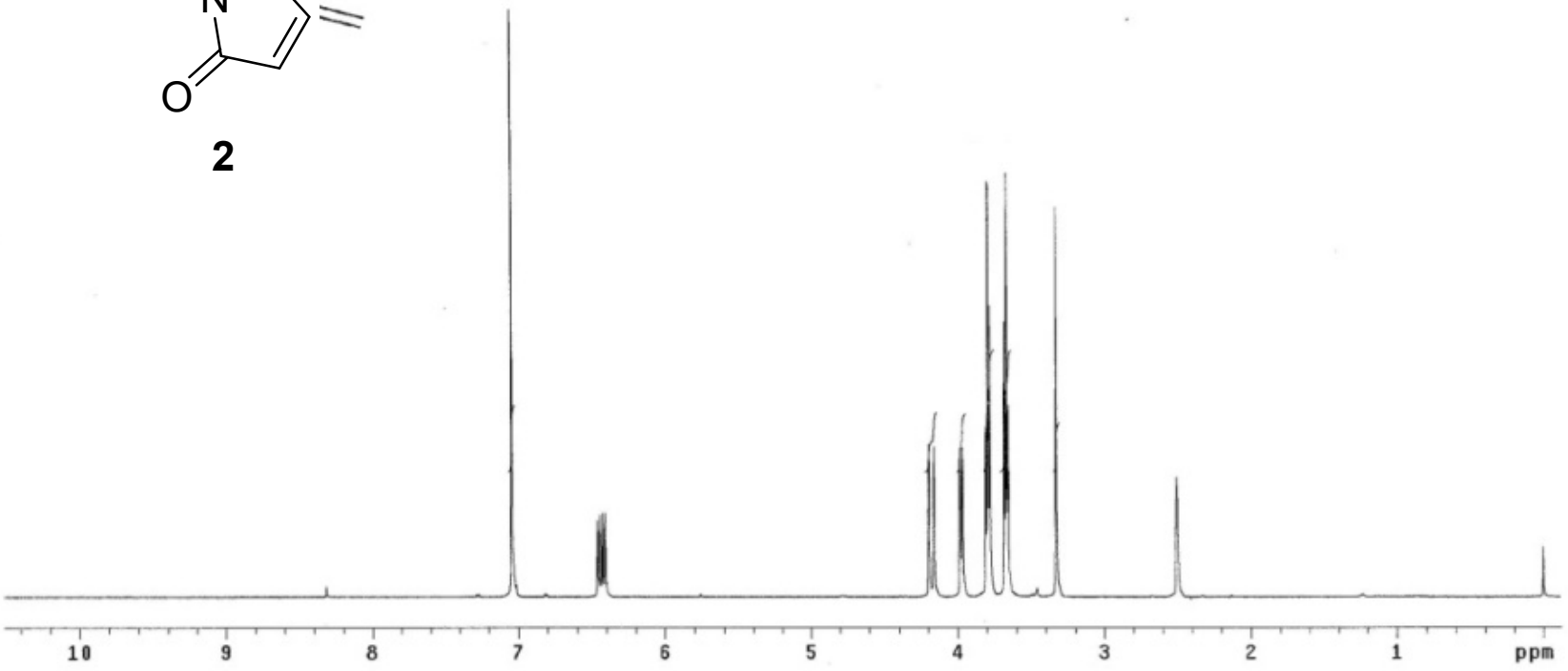
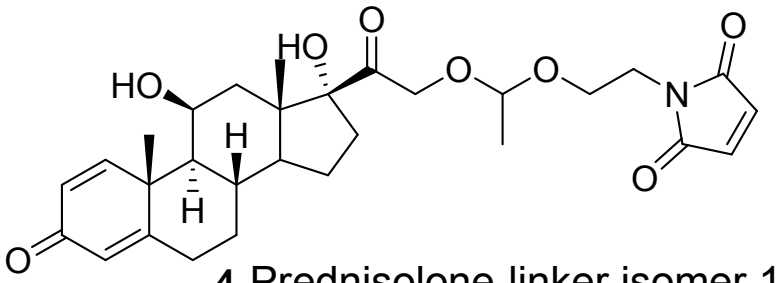
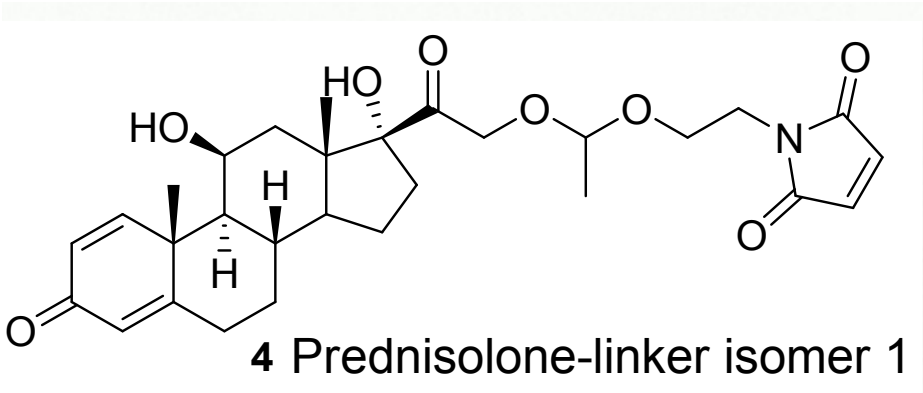
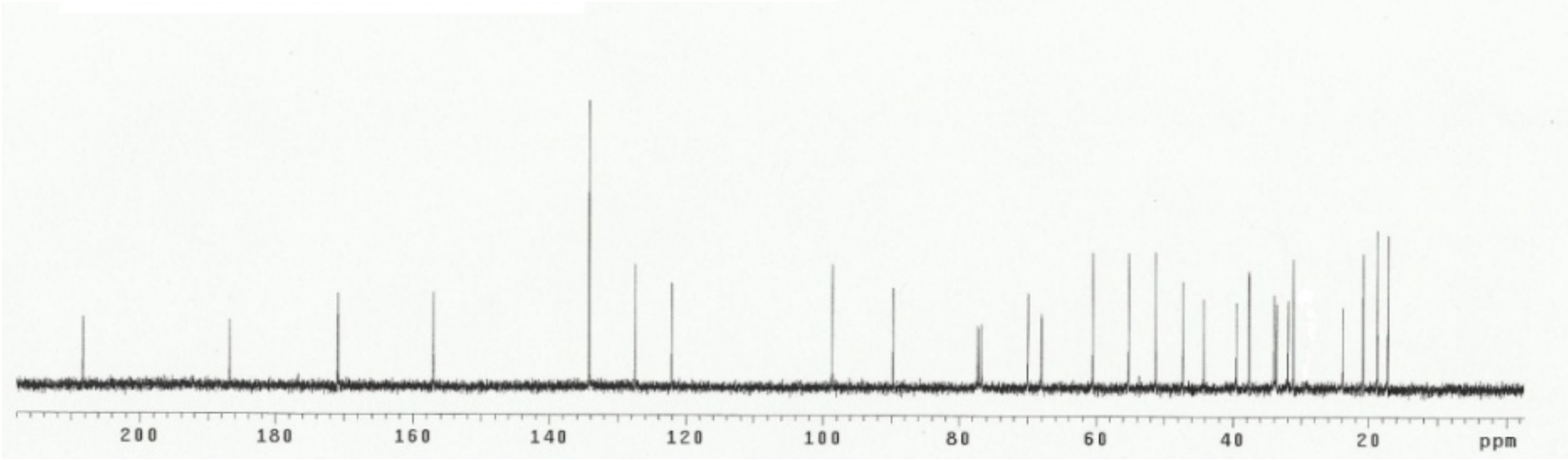


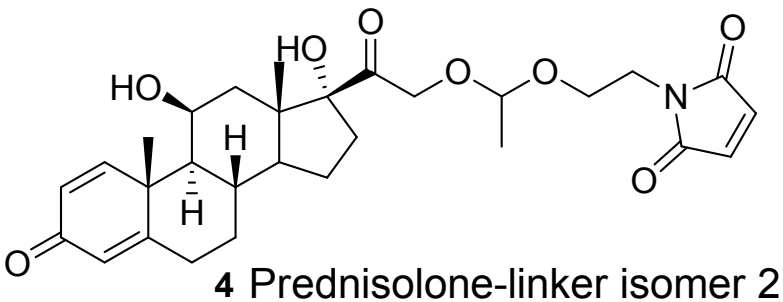


4 Prednisolone-linker isomer 1



4 Prednisolone-linker isomer 1





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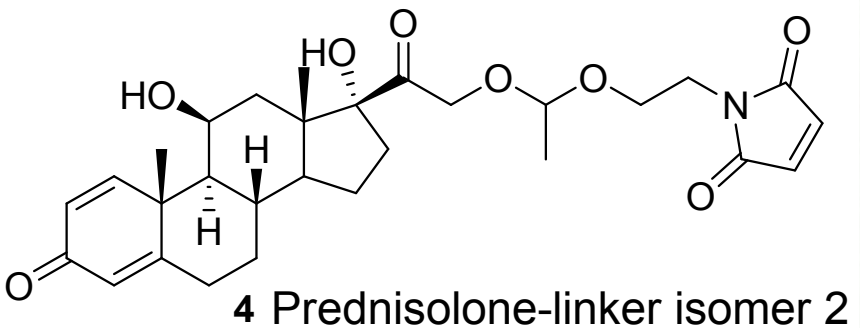
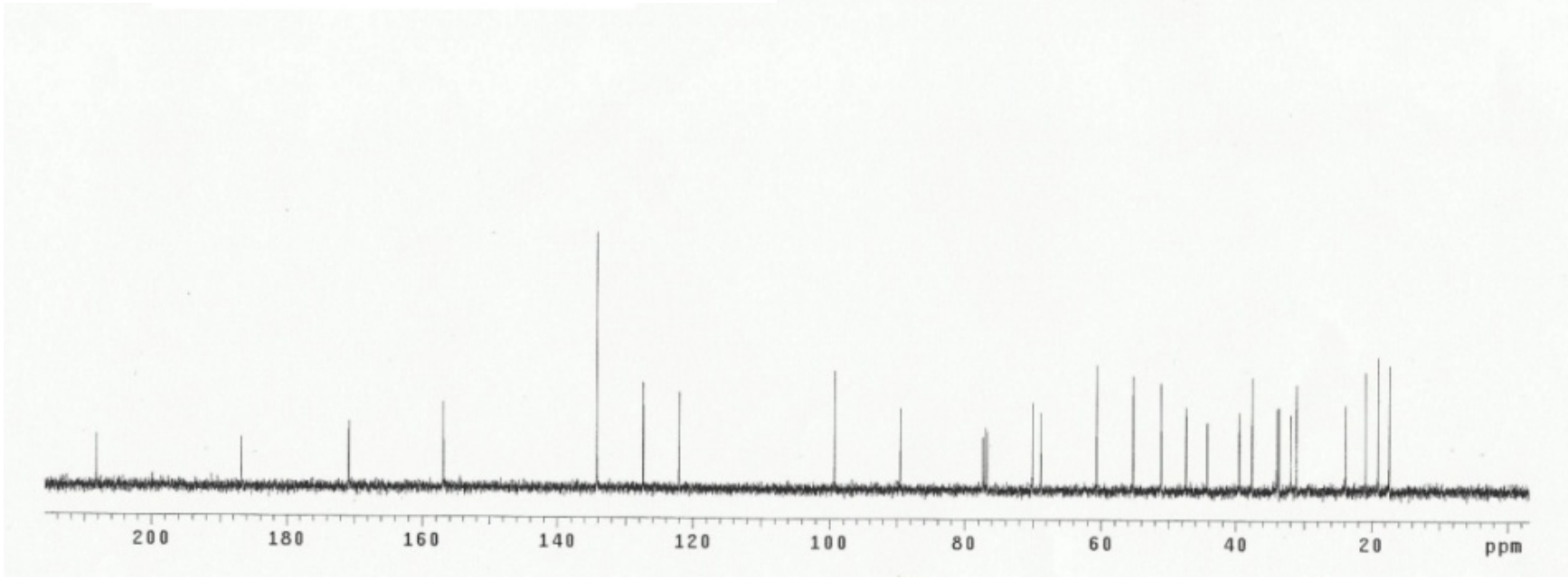
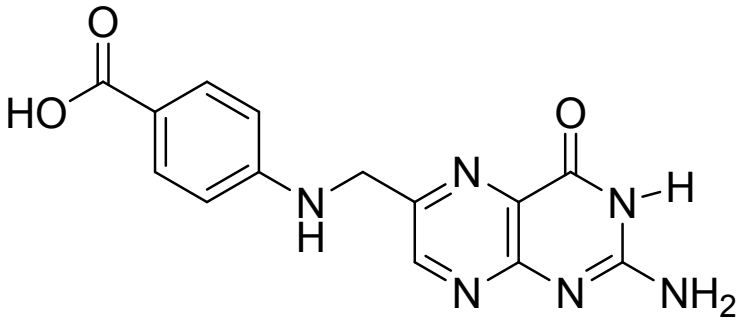
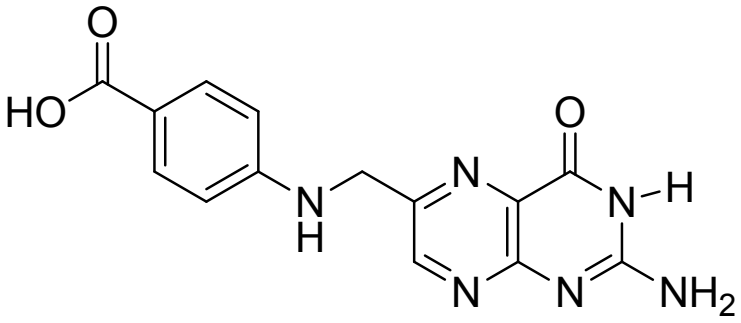


4 Prednisolone-linker isomer 2





6 Pteroic acid



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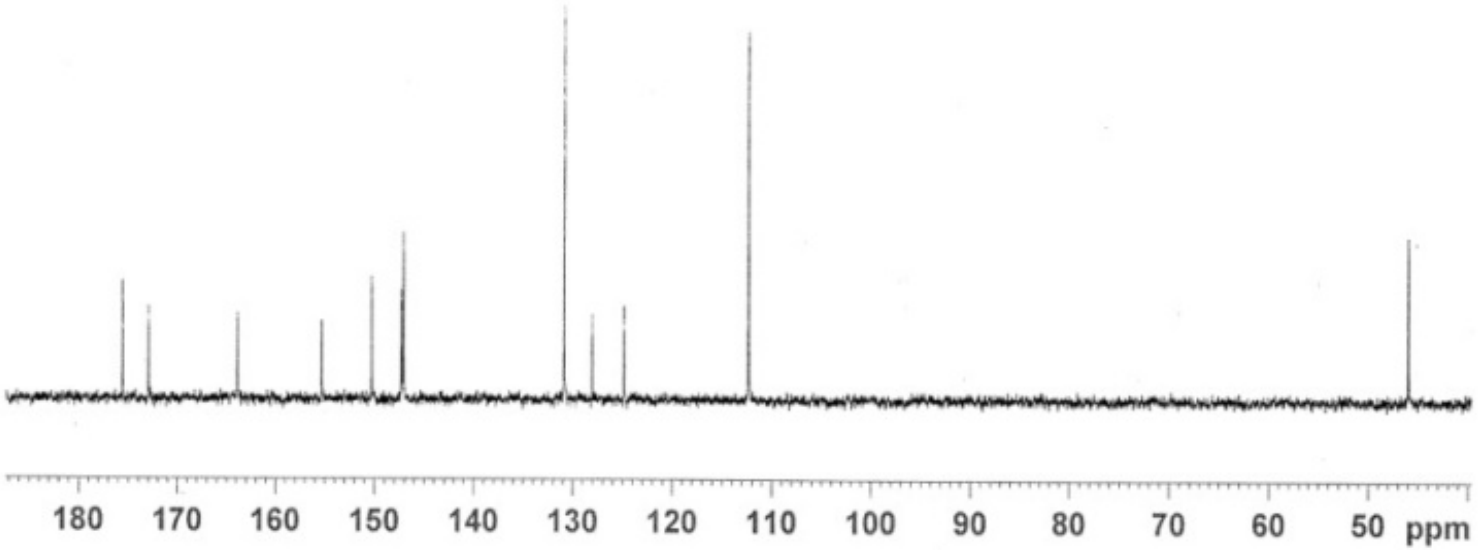
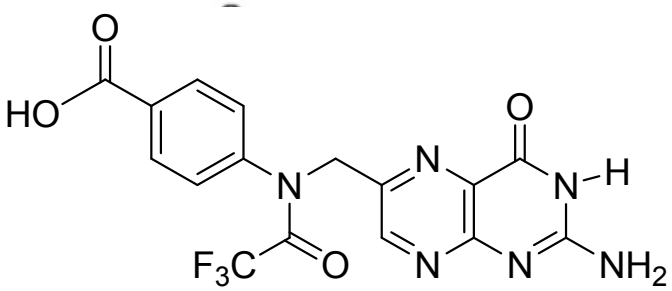
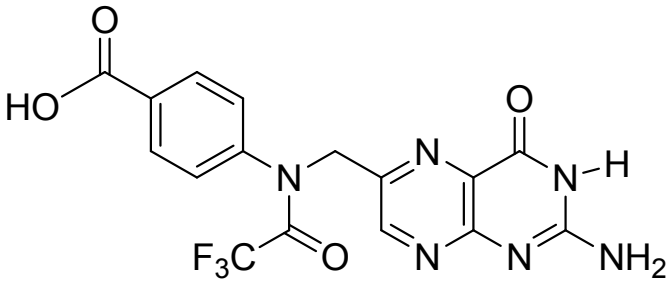
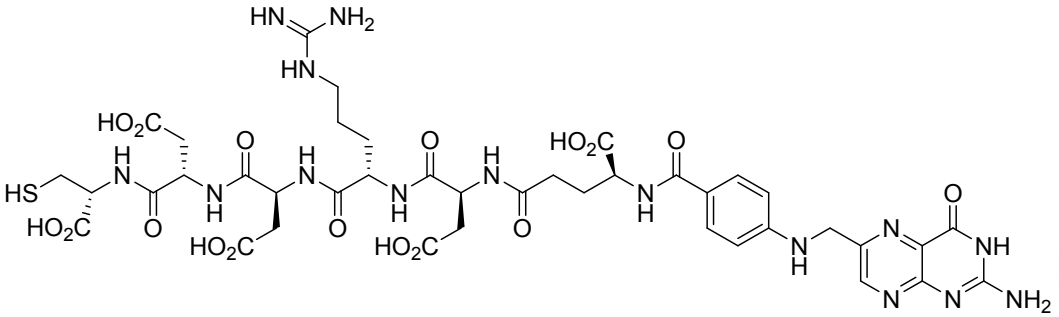




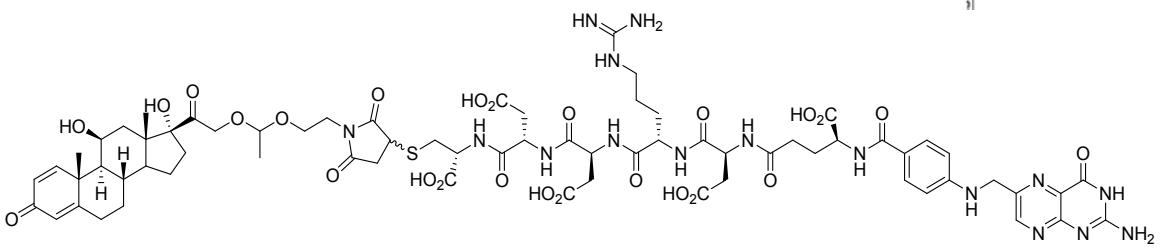
7 N^{10}-CF_3CO-Pteroic acid



7 N^{10}-CF_3CO-Pteroic acid



8 Cys-Asp-Asp-Arg-Asp-gamma-Glu-Pteroic Acid (peptide-folate)



9 Prednisolone-linker-peptide-folate conjugate

Reference

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