Supporting Information for:

On the Intrinsic Reactivity of Highly Potent Trypanocidal Cruzain Inhibitors

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1. Synthesis and characterization

General Consideration

Melting points were determined on a Büchi 510 oil bath apparatus and are uncorrected. Infrared spectra were obtained from FT-IR Thermo Scientific Nicolet 380. Reagents, starting materials and solvents were of commercial quality and were used without further purification unless otherwise stated. All syntheses started with enantiopure amino acids. TLC analysis was carried out on Merck 60 F_{254} silica gel plates and visualized under UV light at 254 nm and 365 nm or by using a ninhydrin staining solution.

Purity was determined with an LC-MS instrument (AmaZon SL ESI-MS, Shimadzu LC) with a cellulose-2 Phenomenex column (250 x 4.6 mm, 5 µm) or a Diacel column (IC-chiralpak, 250 x 4.6 mm, 5 µm). Isocratic elution with MeCN and water was applied as specified, stop time 60 min, flow 0.5 mL/min. NMR spectra were recorded on Bruker Avance 400 MHz and Bruker Avance DRX 500 MHz NMR spectrometers. Chemical shifts are reported in ppm relative to TMS or the residual proton peak of the re-protonated deuterated solvent, and the spectra were calibrated against the residual proton peak of the used deuterated solvent. The following symbols indicate spin multiplicities: s (singlet), s br (broad singlet), d (doublet), dd (doublet of doublet), t (triplet), tt (triplet of triplet), q (quartet), sept (septet), and m (multiplet). HRMS spectra were recorded on a Thermo Scientific LTQ Velos Orbitrap, in electrospray ionization (ESI) mode by direct injection.

Synthesis of 4-nitroisoxazole (**1**): isoxazole (15 mmol, 960 µL) was dissolved in TFAA (7.3 mL); then, NH4NO3 (22.5 mmol,1.81 g) was added in 0.3-g portions, each 15 min, keeping the reaction mixture at 25-30°C. After complete addition, the mixture was kept at room temperature for 2 h after that poured in ice water (30 mL) and this aqueous washing was extracted with $CHCl_3$ (3 x 15 mL); the combined organic extracts were dried over Na_2SO_4 and evaporated (bath at room temperature) to give an oil that was triturated with *n*-hexane to give a yellow solid (50 % yield). ^{1}H-NMR ($CDCl_3$) δ= 9.29 (s,1H), 8.83 (s,1H) ppm**[1]**.

Synthesis of 4-aminoisoxazole (**2**): to a yellow solution of 4-nitroisoxazole (**1**,160 mg, 1.4 mmol) in 6 M HCl (7 mL) $SnCl_2$ (1.327 g, 7 mmol) was added in one portion. After 1.5 h at room temperature, the resulting orange solution was treated with a saturated solution of Na_2CO_3 until pH was 9. The formed solid was removed by filtration, and the aqueous solution was extracted with ethylacetate (5 x 50 mL); the organic phase, dried over $MgSO_4$, was evaporated to give a brown oil (R_f = 0.64 ethylacetate 100% / silica) stored at 4°C and inert atmosphere (65% yield). ^{1}H-NMR (DMSO-d_6) δ = 8.16 (s,1H), 8.13 (s,1H), 4.26 (s br, 2H) ppm.

Synthesis of 2-benzamido-3-phenylpropanoic acid (**3**) or 3-(1H-indol-3-yl)-2-(phenylformamido)propanoic acid (**4**) 2.75 mmol of the corresponding amino acid was dissolved in 1M NaOH (6 mL) in an ice-bath. Benzoyl chloride (261 µL, 2.25 mmol) was added. After 5 min, the reaction mixture was allowed to stand at room temperature. After 20 min, the solution was cooled in ice and 1M $KHSO_4$ (16 mL) was added slowly. The obtained white solid was washed with 1 M $KHSO_4$ (3 x 5 mL), H_2O (10 x 3 mL), 9:1 EtOH:H_2O (3 x 3 mL) and dried under vacuum on P_2O_5

(yield 88%). For compound **3**: ^{1}H NMR (500 MHz, DMSO-d_6): 12.62 (s br, 1H, D_2O exchange), 10.75 (s, 1H, D_2O exchange), 8.56 (m, 1H, D_2O exchange), 7.28 (m, 8H), 7.13 (m, 2H), 4.54 (m, 1H), 3.29 (m, 1H), 3.19 (m, 1H). For compound **4**: ^{1}H NMR (500 MHz, DMSO-d_6): 12.69 (s br, 1H, D_2O exchange), 10.80 (s, 1H, D_2O exchange), 8.61 (d, J = 8.0 Hz, 1H, D_2O exchange), 7.81 (d, J = 8.5 Hz, 2H), 7.59 (d, J = 8 Hz, 1H), 7.51 (t, J = 7.0 Hz, 1H), 7.44 (t, J = 7.5 Hz, 2H), 7.31 (d, J = 8.0 Hz, 1H), 7.20 (s, 1H), 7.05 (t, J = 7.5 Hz, 1H), 6.98 (t, J = 7.5 Hz, 1H), 4.65 (m,1H), 3.30 (m, 1H, H_2O overlapping), 3.19 (m,1H).

Synthesis of N-(1-(isoxazol-4-ylamino)-1-oxo-3-phenylpropan-2-yl)benzamide (**Neq0646**): to a suspension of (±)-2-benzamido-3-phenylpropanoic acid (3, 216 mg, 0.70 mmol), HOBt (123 mg, 0.91 mmol) and EDC (175 mg, 0.91 mmol) in CH_2Cl_2 (8 mL) were added under argon at 0°C. After stirring of 1 hour at room temperature, the mixture was kept on ice-bath, and a solution of 4-aminoisoxazole (235 mg, 2.80 mmol) in dry CH_2Cl_2 (2 mL) was added. The resulting mixture was kept overnight at room temperature, then the solvent was evaporated, and the residue treated with AcOEt (30 mL) and washed H_2O (2 x 20 mL) and brine (2 x 20 mL). The organic phase was dried over $MgSO_4$ and evaporated to give a crude residue that was purified by chromatographic column on silica using as mobile phase $CHCl_3$/AcOEt (1:1), to give with solid (R_f = 0.4) crystallized from ACOEt (36% yield).

Secondary purification was carried out on cellulose-2 Phenomenex column, in isocratic elution with a flow rate of 2.36 mL min^{-1}, at 32°C; the mobile phase composition was *n*-hexane/ethanol (70:30) (v/v) to give Neq0646. ^{1}H-NMR (500

MHz, DMSO-d_6) δ = 10.80 (s br, 1H), 10.51 (s, 1H), 9.13 (s,1H), 8.72 (d, *J* = 8.0 Hz, 1H), 8.63 (s, 1H), 7.84 (m, 2H), 7.69 (d, *J* = 8.0 Hz, 1H), 7.52 (tt, *J* = 7.5 Hz, *J* = 1.5 Hz, 1H), 7.44 (m, 2H), 7.31 (dt, *J* = 8.0 Hz, *J* = 1.0 Hz, 1H), 7.23 (d, *J* = 2.0 Hz, 1H), 7.05 (m, 1H), 6.98 (m, 1H), 4.83 (qd, *J* = 9.5 Hz, *J* = 8.0 Hz, *J* = 5.0 Hz, 1H), 3.30 (dd, *J* = 14.5 Hz, *J* = 5.0 Hz, 1H), 3.22 (dd, *J* = 14.5 Hz, *J* = 9.5 Hz, 1H) ppm. ^{13}C NMR (DMSO-d_6): 170.72, 166.90, 147.56, 144.85, 136.52, 134.23, 131.86, 128.63, 127.97, 127.59, 124.23, 121.41, 120.17, 118.89, 118.73, 111.83, 110.55, 54.89, 27.74 ppm. HRMS (+) Calc. for $[C_{19}H_{17}N_3O_3]^+$ 335.12699, found: 336.12663 $[M+H]^+$.

Synthesis of N-(3-(1H-indol-3-yl)-1-(isoxazol-4-ylamino)-1-oxopropan-2-yl)benzamide (**Neq0673**): to a suspension of (±)-2-benzamido-3-phenylpropanoic acid (216 mg, 0.70 mmol), HOBt (124 mg, 0.91 mmol) and EDC (175 mg, 0.91 mmol) in CH_2Cl_2 (8 mL) were added under argon at 0°C. After stirring of 1 hour at room temperature, the mixture was kept on ice-bath and a solution of 4-aminoisoxazole (235 mg, 2.80 mmol) in dry CH_2Cl_2 (2 mL) was added. The resulting mixture was kept overnight at RT, then the solvent was evaporated, and the residue treated with AcOEt (30 mL) and washed H_2O (2 x 20 mL) and brine (2 x 20 mL). The organic phase was dried over $MgSO_4$ and evaporated to give a crude residue that was purified by chromatographic column on silica using as mobile phase $CHCl_3$/AcOEt (1:1), to give with solid (R_f = 0.4) crystallized from ACOEt (36% yield).

Secondary purification was carried out on cellulose-2 Phenomenex column, in isocratic elution with a flow rate of 2.36 mL min^{-1}, at 32°C; the mobile phase

composition was *n*-hexane/ethanol (70:30) (v/v) to give Neq0673. ^{1}H-NMR (500 MHz, DMSO-d_6) δ = 10.80 (s br, 1H), 10.51 (s, 1H), 9.13 (s,1H), 8.72 (d, *J* = 8.0 Hz, 1H), 8.63 (s, 1H), 7.84 (m, 2H), 7.69 (d, *J* = 8.0 Hz, 1H), 7.52 (tt, *J* = 7.5 Hz, *J* = 1.5 Hz, 1H), 7.44 (m, 2H), 7.31 (dt, *J* = 8.0 Hz, *J* = 1.0 Hz, 1H), 7.23 (d, *J* = 2.0 Hz, 1H), 7.05 (m, 1H), 6.98 (m, 1H), 4.83 (qd, *J* = 9.5 Hz, *J* = 8.0 Hz, *J* = 5.0 Hz, 1H), 3.30 (dd, *J* = 14.5 Hz, *J* = 5.0 Hz, 1H), 3.22 (dd, *J* = 14.5 Hz, *J* = 9.5 Hz, 1H) ppm. ^{13}C NMR (DMSO-d_6): 170.72, 166.90, 147.56, 144.85, 136.52, 134.23, 131.86, 128.63, 127.97, 127.59, 124.23, 121.41, 120.17, 118.89, 118.73, 111.83, 110.55, 54.89, 27.74 ppm. HRMS (+) Calc. for $[C_{21}H_{18}N_4O_3]^+$ 374.13789, found: 375.13895 $[M+H]^+$.

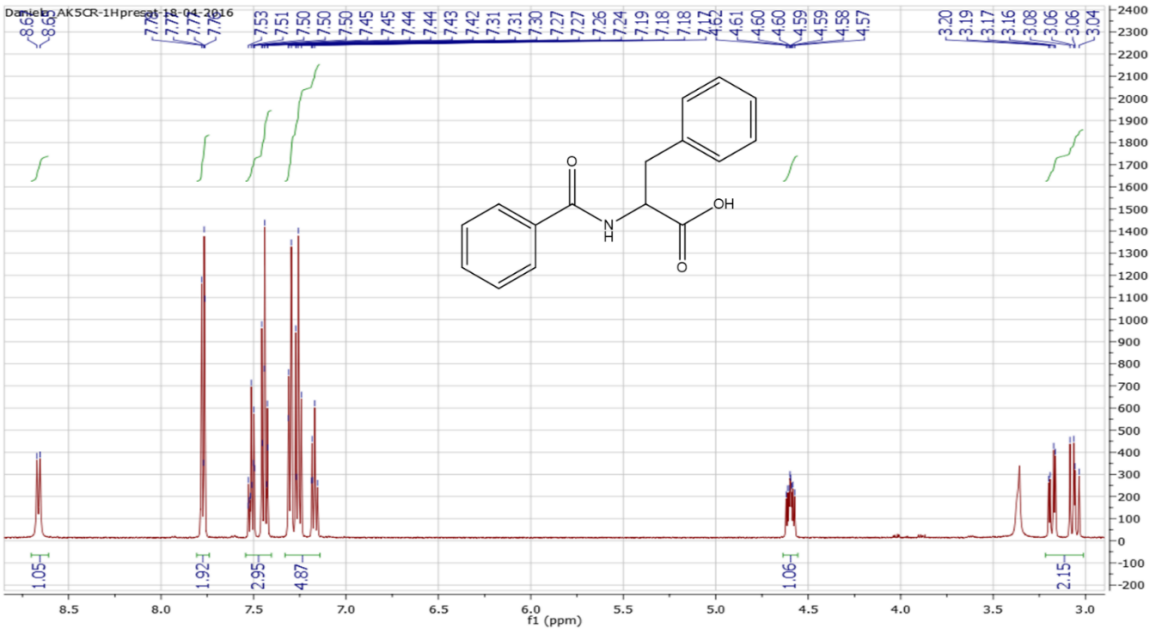

Figure S1. ^{1}H-NMR (400 MHz, $CDCl_3$) of compound **4**

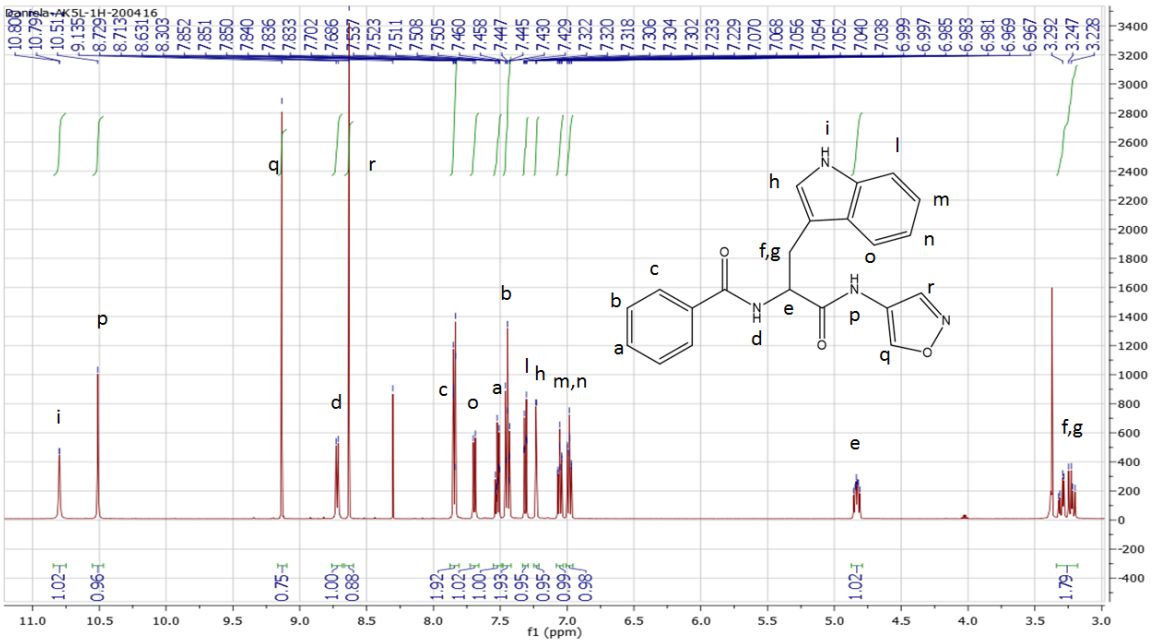

Figure S2. ^{1}H-NMR (400 MHz, DMSO-*d*$_6$) of compound **Neq0673**

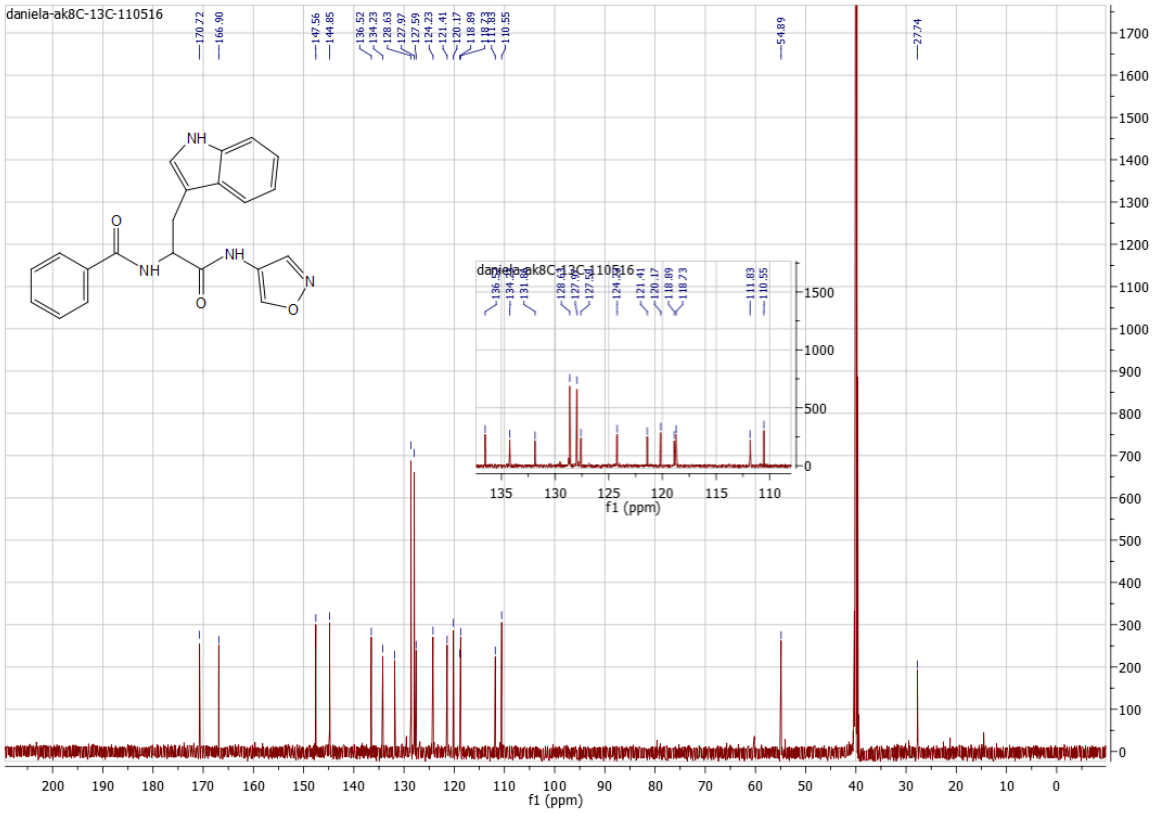

Figure S3. ^{13}C-NMR (100 MHz, DMSO-*d*$_6$) of compound **Neq0673**

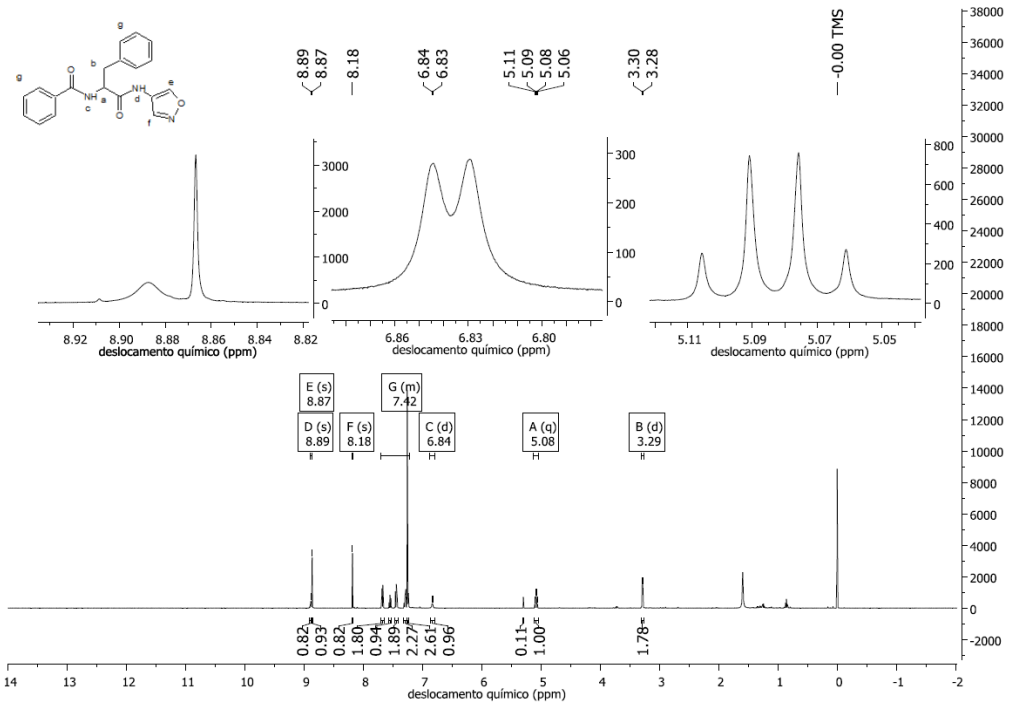

Figure S4. ^{1}H-NMR (400 MHz, DMSO-d_6) of compound **Neq0646**

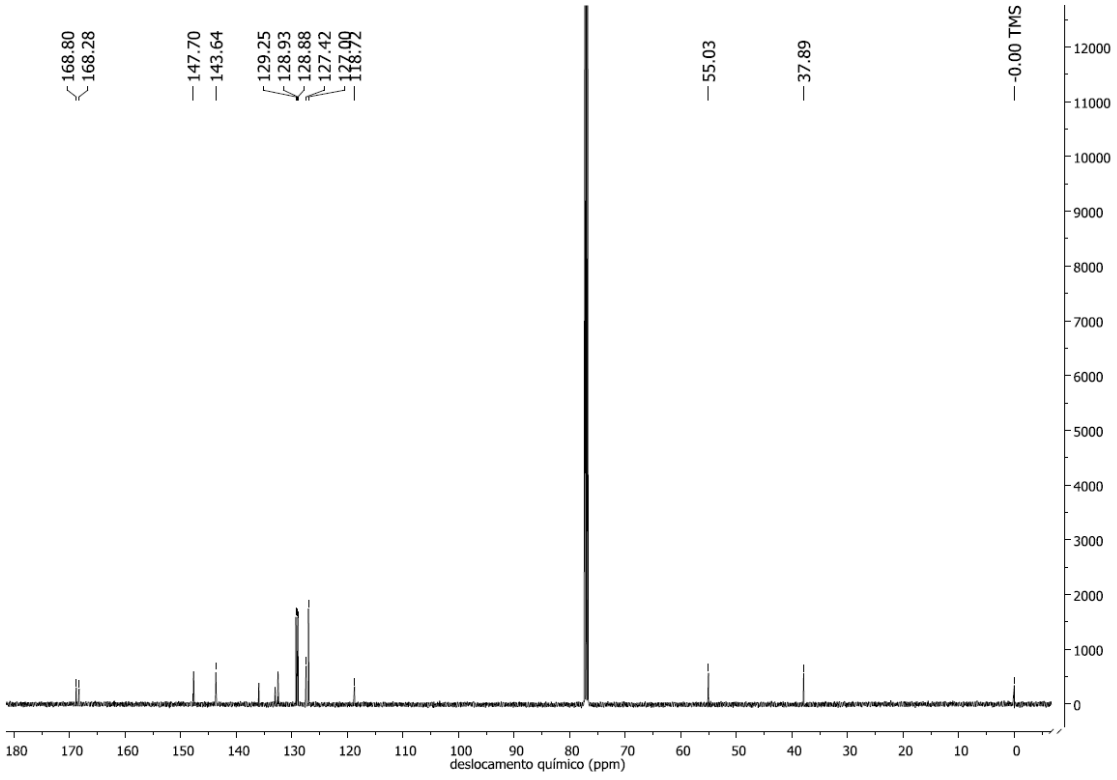

Figure S5. ^{13}C-NMR (100 MHz, DMSO-d_6) of compound **Neq0646**

2. Electronic parameters

Table S1. The energy (E) of neutral, anionic and cationic form, ionization potential (IP), electron affinity (EA), chemical potential (μ) and hardness (η) for compounds.

Compound (Neq)	$E_{Neutral}$ (E_h)	E_{Anion} (E_h)	E_{Cation} (E_h)	IP (eV)	EA (eV)	μ (eV)	η (eV)
500	-1122.56	-1122.54	-1122.20	9.799	-0.473	-5.136	10.272
539	-1235.59	-1235.58	-1235.27	8.844	-0.462	-4.653	9.306
615	-1352.25	-1352.22	-1351.89	9.722	-0.836	-5.279	10.558
646	-1121.32	-1121.30	-1120.99	8.809	-0.353	-4.581	9.161
652	-1509.98	-1509.96	-1509.66	8.738	-0.459	-4.599	9.198
653	-1274.83	-1274.81	-1274.52	8.653	-0.636	-4.645	9.289
654	-1451.45	-1451.43	-1451.12	9.030	-0.534	-4.782	9.564
655	-1199.53	-1199.52	-1199.24	7.731	-0.302	-4.016	8.032
656	-1220.78	-1220.76	-1220.43	9.669	-0.494	-5.082	10.163
657	-1392.40	-1392.38	-1392.06	9.228	-0.684	-4.956	9.912
673	-1252.56	-1252.55	-1252.24	8.712	-0.397	-4.555	9.109
675	-1198.78	-1198.74	-1198.39	10.383	-0.830	-5.606	11.213
677	-1216.92	-1216.90	-1216.57	9.683	-0.593	-5.138	10.276
690	-989.82	-989.80	-989.46	9.701	-0.689	-5.195	10.389

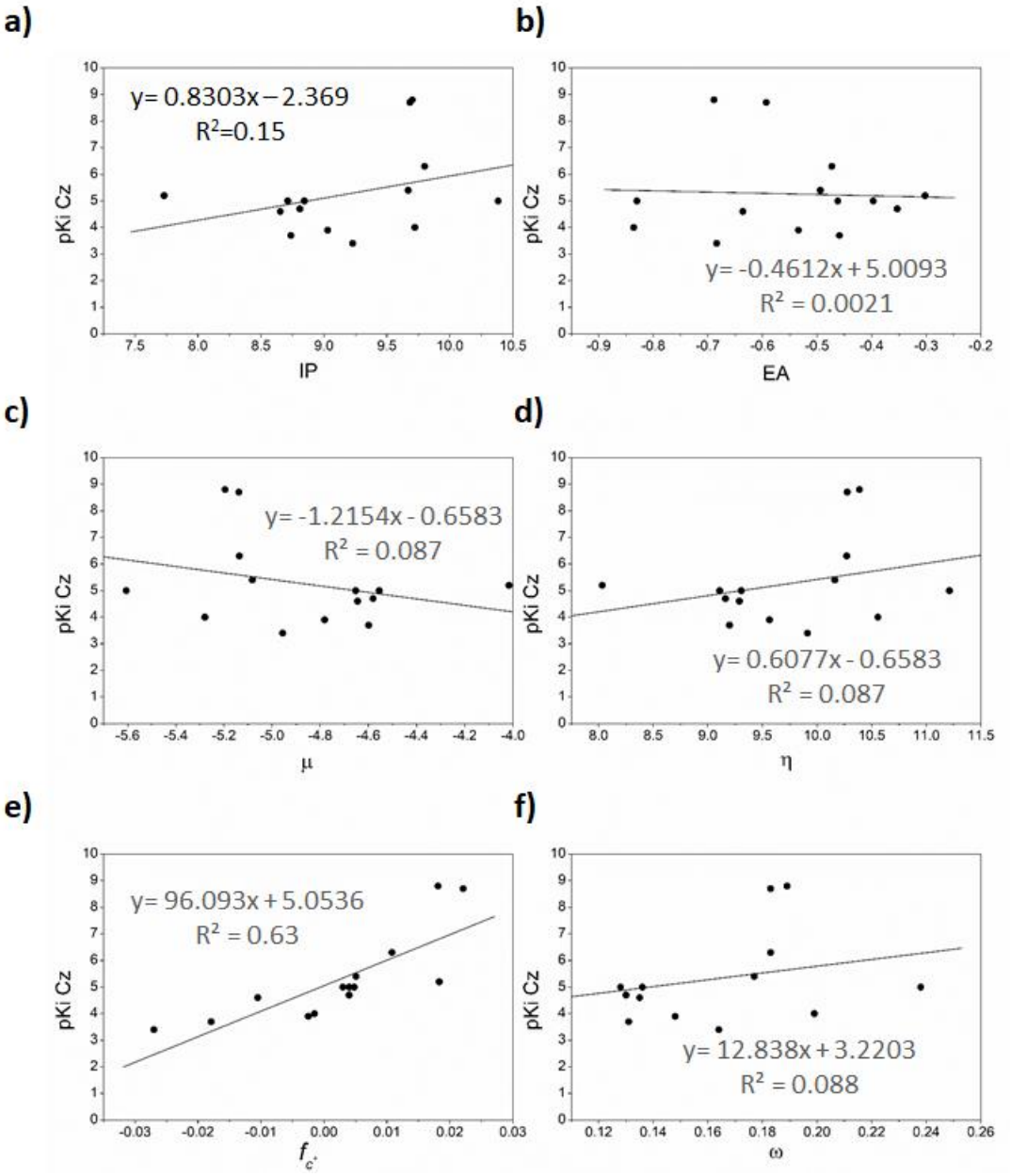

Figure S6. The putative linear equation and the coefficient of determination obtained through the linear correlation between the calculated parameter and pKi against Cz. **a)** Ionization potential **b)** electron affinity **c)** chemical potential **d)** hardness **e)** electrophilic Fukui function and **f)** global electrophilicity, all parameters values are in eV but electrophilic Fukui function.

3. Half-life measurement and decay constant by HPLC

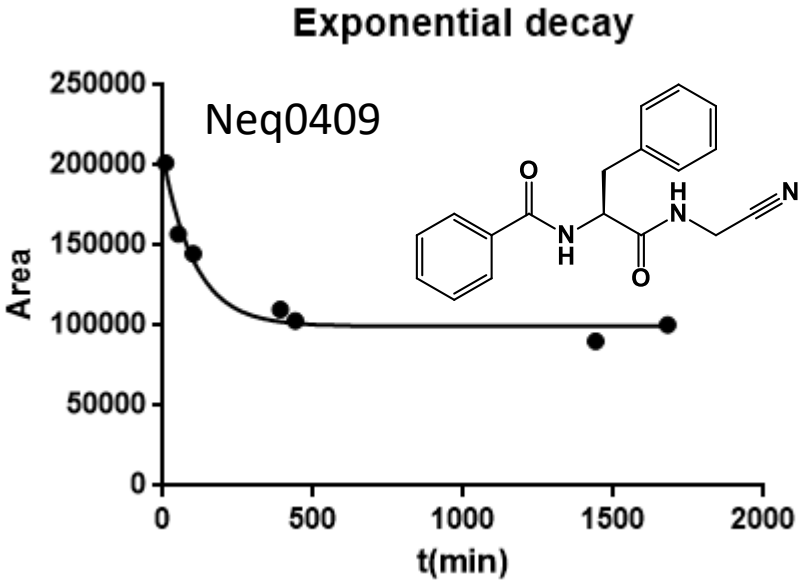

Figure S7. Decay curve of **Neq0490** with cysteine.

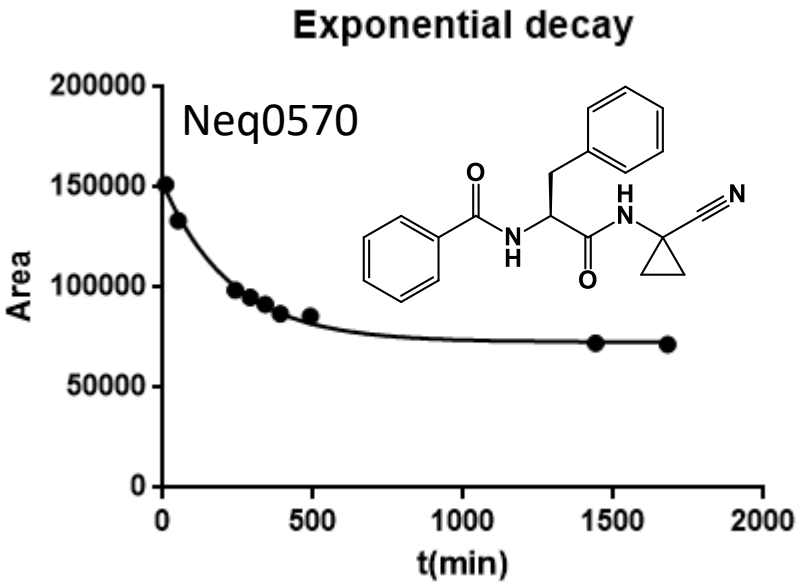

Figure S8. Decay curve of **Neq0570** with cysteine.

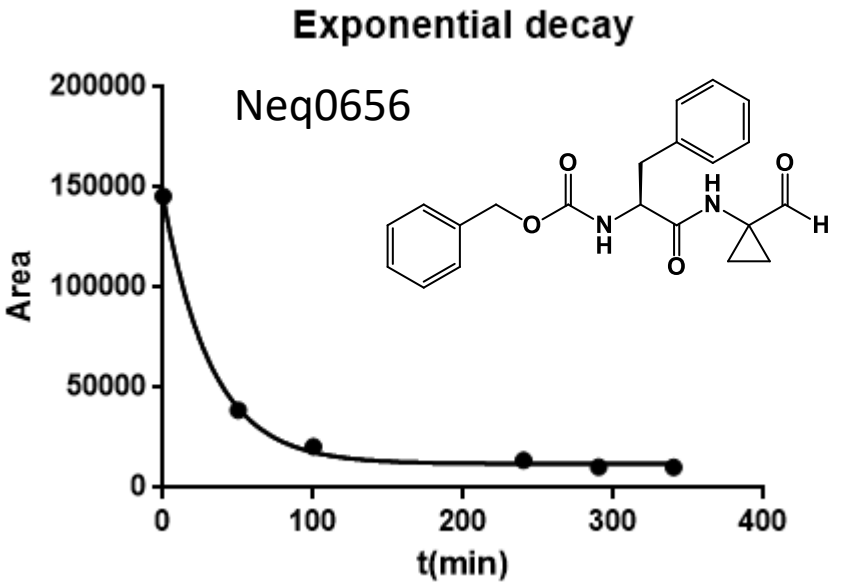

Figure S9. Decay curve of **Neq0656** with cysteine.

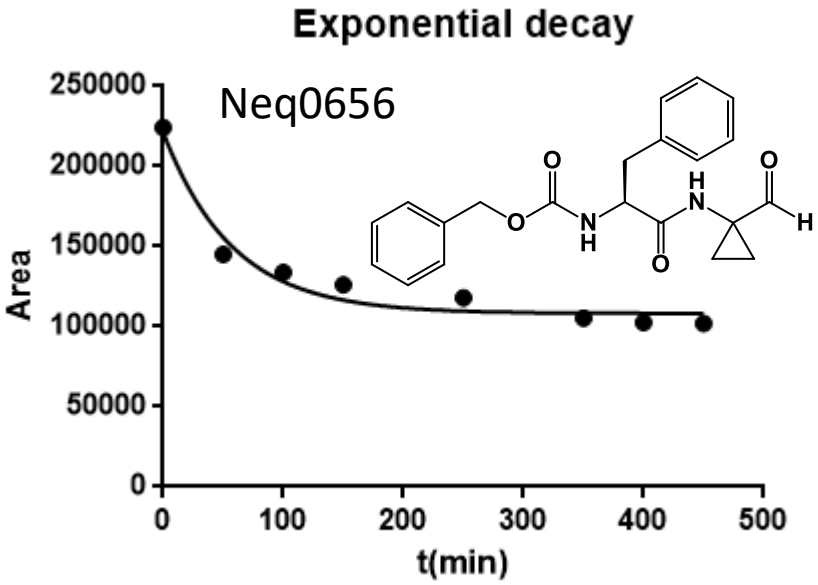

Figure S10. Decay curve of **Neq0656** with gluthatione.

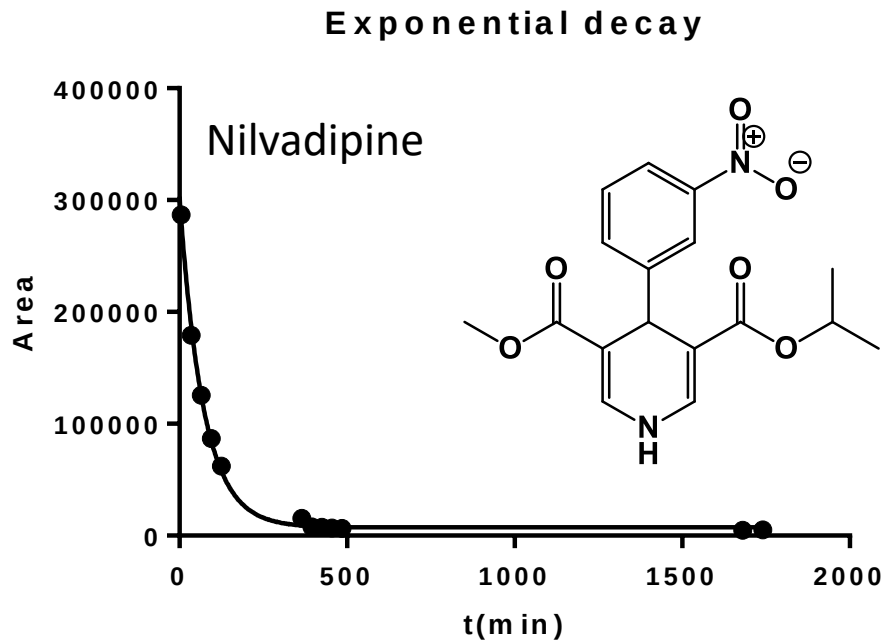

Figure S11. Decay curve of Nilvadipine with cysteine.

We also performed a blank HPLC run in order to show the stability of compounds. The decays are caused by reaction with the thiol.

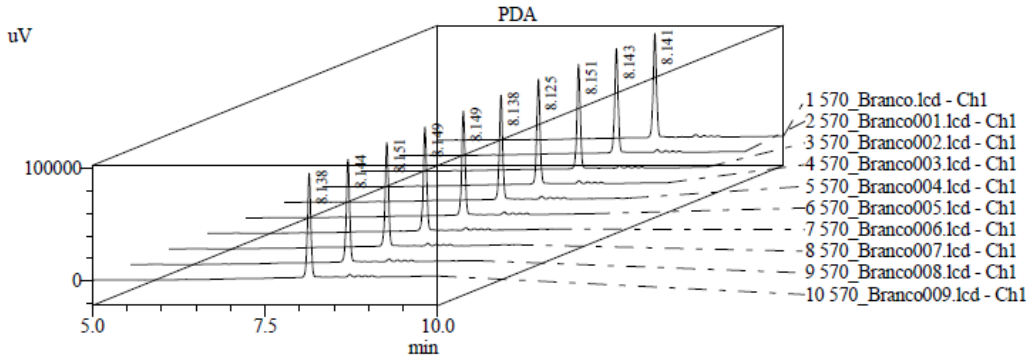

Figure S12. Blank of **Neq0570**.

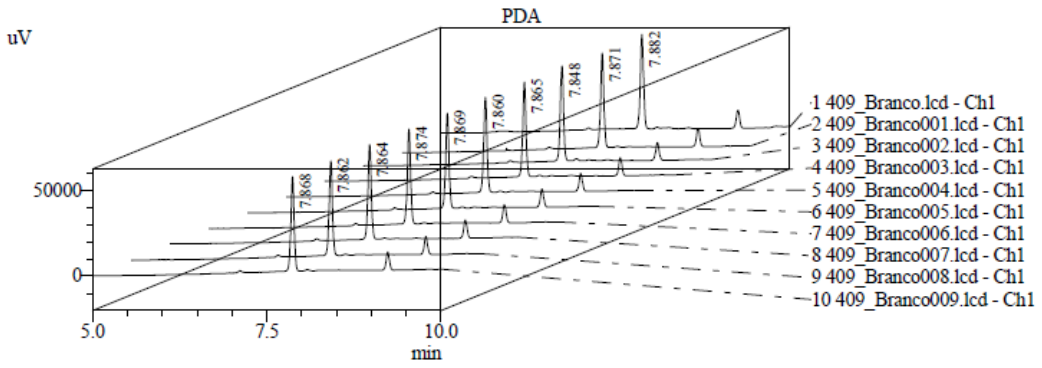

Figure S13. Blank of **Neq0409**.

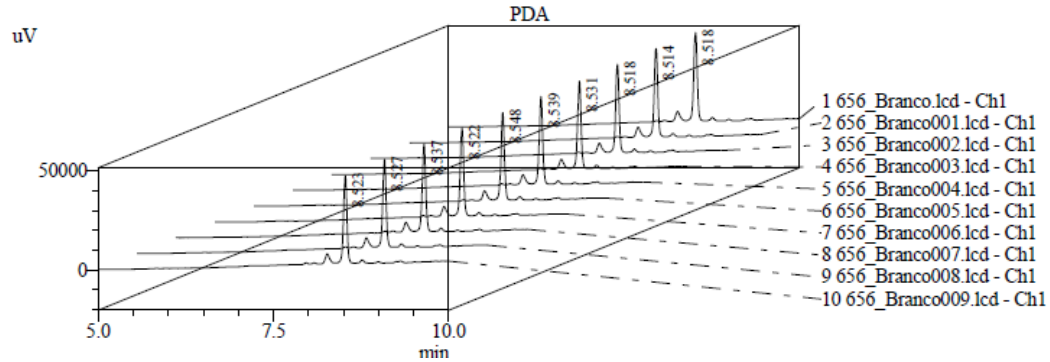

Figure S14. Blank of **Neq0656**.

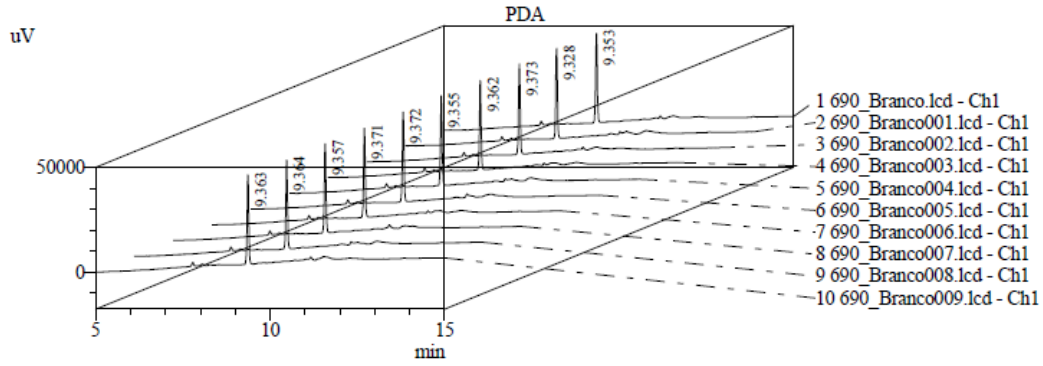

Figure S15. Blank of **Neq0690**.

4. Reference

(1) Wriede, U.; Fernandez, M.; West, K. F.; Harcour, D.; Moore, H. W. Synthesis of Halodimethoxy-1,2-Benzoquinones. *J. Org. Chem.* **1987**, *52* (20), 4485–4489. https://doi.org/10.1021/jo00229a011.