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# Novel platinum(IV) prodrugs with tetraacetylriboflavin axial ligand possessing enhanced light-induced toxicity

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## Materials and methods

Synthesis. The Pt(IV) prodrug "Riboplatin" **1**, with a tetraacetylriboflavin derivative as an axial ligand, was prepared in 4 successive stages described earlier[1]. Naproxen was obtained by extraction from grinded tablets. DCC (dicyclohexylcarbodiimide), DCM, EtOAc, DMF, 6-aminohexanoic acid, maleic anhydride, acetic acid, acetone, sodium sulfate, oxalic chloride, sodium azide, petroleum ether and silica gel. were from commercial sources (AKSci, Sigma Aldritch, Thermo Fisher, etc.). The solvents were purified following the literature procedures[2,3], other reagents were used without further purification.

*2-(6-Methoxynaphthalen-2-yl)propanoic anhydride (naproxen anhydride).* Naproxen (500 mg, 2.17 mmol) and 268 mg (1.3 mmol, 0.6 equiv.) of DCC were dissolved in 20 mL of anhydrous $CH_2Cl_2$ and stirred for 2 h at room temperature. The dicyclohexylurea precipitate was filtered off, the filtrate was evaporated under reduced pressure, the residue was suspended in EtOAc. The precipitate was filtered off, and the filtrate was again evaporated under reduced pressure. The product was purified by flash chromatography, eluent dichloromethane. 427 mg of 2-(6-methoxynaphthalen-2-yl)propanoic anhydride 2 were obtained as a white powder. Yield: 89%. **$^1$H NMR spectrum** (400 MHz, $CDCl_3$, δ, ppm): 7.53 (d, 1H, J=7.1 Hz, H3-naphthalene), 7.50 (d, 1H, J=7.4 Hz, H4-naphthalene), 7.43 (d, 1H, J=1.3 Hz, H1-naphthalene), 7.16 (dd, 1H, J1=8.5 Hz, J2=1.8 Hz, H8-naphthalene), 7.12 (dd, 1H, J1=8.9 Hz, J2=2.5 Hz, H7-naphthalene), 7.05 (d, 1H, J=2.5 Hz, H5-naphtalene), 3.94 (s, 3H, OCH3), 3.82 (quad, 1H, J=7.1 Hz, CH-Ph), 1.52 (d, 3H, J=7.1 Hz, $CH_3$-CH). **$^{13}$C NMR spectrum** (101 MHz, $CDCl_3$, δ ppm): 169.64, 157.35, 133.36, 133.24, 128.84, 128.40, 126.92, 125.91, 125.42, 118.65, 105.13, 54. 88, 45.88, 17.39.

Conjugate of oxoplatin, (2*S*,3*R*,4*R*)-5-(7,8-dimethyl-2,4-dioxo-3,4-dihydrobenzo[*g*]pteridin-10(2*H*)-yl)pentane-1,2,3,4-tetrayl tetraacetate and naproxen **(2)**: 15 mg (0.015 mmol, 1 equiv) of complex **1** were dissolved in 400 μl of DMF, 10.2 mg (0.023 mmol, 1.5 equiv) of naproxen anhydride were added, and the mixture was stirred at room temperature for 18 h. The solvent was evaporated under reduced pressure, the residue was suspended in 0.5 ml of methanol, and 8 ml of diethyl ether was precipitated. The precipitate was separated, dried in air, the product was purified by column chromatography, eluent $CH_2Cl_2$:MeOH 10:1. 8 mg of complex **2** were obtained as an orange powder. Yield: 43%.

**$^1$H NMR spectrum** (400 MHz, DMSO-$d_6$, δ, ppm): 7.96 (s, 1H, H9(Ar)), 7.75 (s, 1H, H6(Ar)), 7.73-7.69 (m, 3H, H1, H4, H8-naphthalene), 7.45 (d, 1H, J=8.1 Hz, H3-naphthalene), 7.25 (s, 1H, H7—naphthalene), 7.10 (dd, 1H, $J_1$=8.9 Hz, $J_2$=2.5 Hz, $H_5$-naphthalene), 6.76-6.51 (m, 7H, $NH_3$, NH(CO), 5.49-5.44 (m, 2H, 2',3'-CH(rib)), 5.31-5.28 (m, 1H, 4'–CH (rib)), 5.14-4.80 (m, 1H, 5'–$CH_2$ (rib)), 4.37 (dd, 1H, J1=12.5 Hz, J2=2.8 Hz, 1'H–CHOAc), 4.21 (dd, 1H, J1=12.3 Hz, J2=6.0 Hz, 1'H–CHOAc), 3. 87 (m, 6H, N–$CH_2$–$CH_2$, $OCH_3$, CH-Ph), 2.98-2.94 (m, 2H, $CH_2$–$CH_2$– NH–(C=O)), 2.51 (s, 3H, CH3–Ar), 2.40 (s, 3H, $CH_3$–Ar), 2.20 (s, 3H, AcO), 2.18 (s, 3H, AcO), 1.99 (s, 3H, AcO), 1.70-1.65 (m, 2H, $CH_2$–$CH_2$–$CH_2$), 1.58 (s, 3H, AcO), 1.38 (d, 3H, J=7.2 Hz, CH-$CH_3$). **$^{13}$C NMR spectrum** (101 MHz, DMSO-$d_6$, δ, ppm): 182.34, 170.54, 170.19, 170.08, 169.85, 164.23, 159.78, 157.35, 155.00, 149.65, 146.92, 137.88, 136.45, 134.43, 133.45, 131.60, 129.51, 128.80, 127.44, 126.76, 125.89, 118.76, 116.79, 106.12, 70.10, 69.19, 61.93, 55.57, 46.83, 44.20, 35.56, 31.70, 29.46, 29.25, 28.82, 22.52, 21.23, 21.19, 21.00, 20.92, 20.54, 20.27, 19.19. **$^{195}$Pt NMR spectrum** (86 MHz, DMSO-$d_6$ δ, ppm): 1252.82. **HRMS (ESI-TOF):** calculated $C_{43}H_{53}Cl_2N_7NaO_{15}Pt^+$, 1195.2522, (**3**+Na)$^+$; found $C_{43}H_{53}Cl_2N_7NaO_{15}Pt^+$, 1195.2517, (**3**+Na)$^+$. Melting point: >200ºC

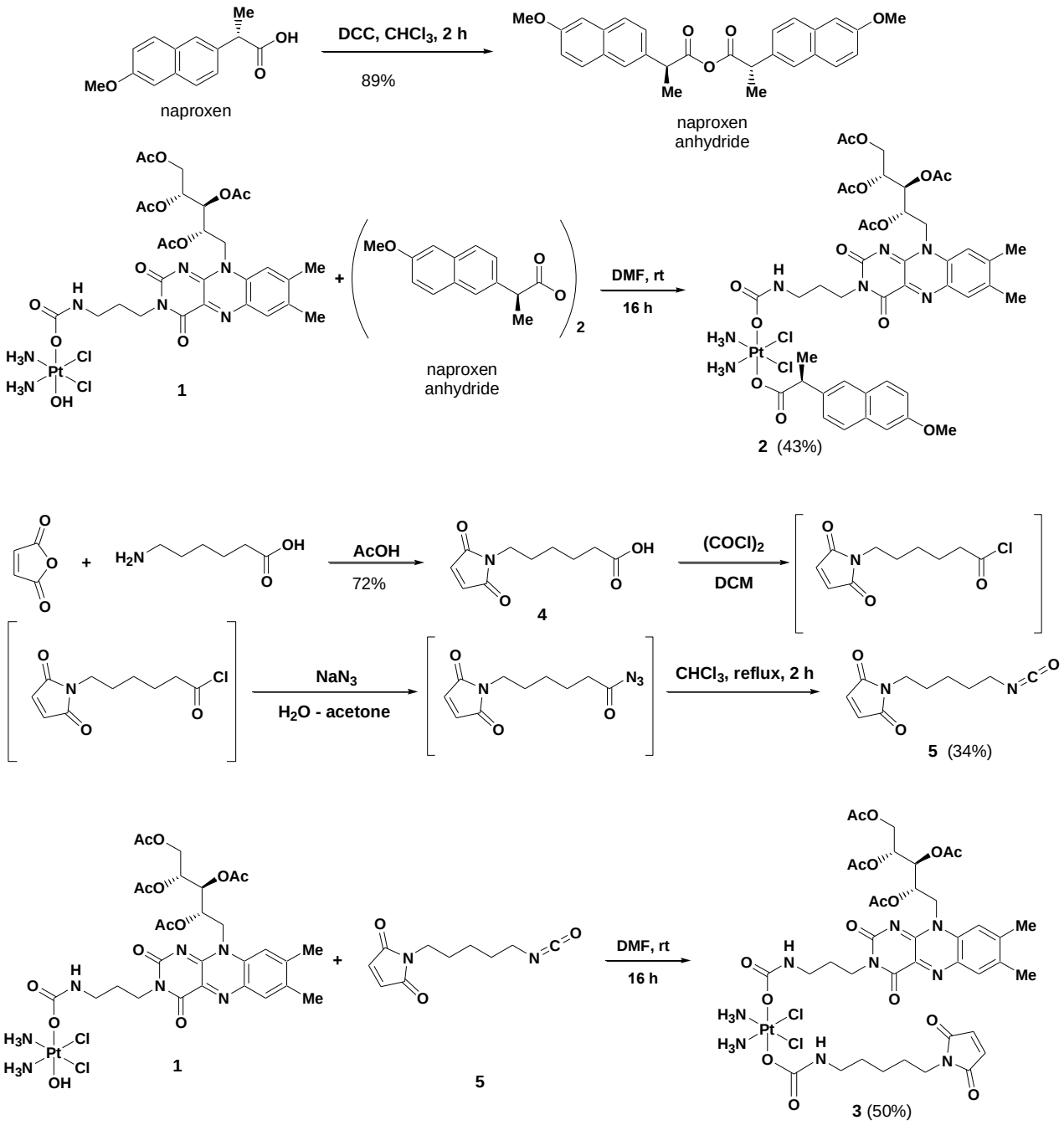
*6-(2,5-Dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoic acid* **(4)**: 390 mg (2.67 mmol, 1 equiv) of 6-aminohexanoic acid and 350 mg (3.57 mmol, 1.2 equiv) of maleic anhydride were suspended in 20 ml of acetic acid, the reaction mixture was refluxed for 6 h, then cooled to room temperature and poured into 50 ml of water. The mixture was extracted with EtOAc (3x80 ml), the organic fraction was dried over anhydrous sodium sulfate, the solvent was evaporated under reduced pressure, the product was purified by column chromatography, eluent EtOAc/acetone 50:1. 450 mg of 6-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)hexanoic acid **4** was obtained as a white powder. Yield: 72%. **$^{1}$H NMR spectrum** (400 MHz, DMSO-$d_6$, δ, ppm): 12.10 (br.s., 1H, COOH), 6.99 (s, 2H, CH=CH), 3.36 (t, 2H, J=7.1 Hz, $CH_2$-N), 2.16 (t, 2H, J=7.3 Hz, α-$CH_2$), 1.50-1.43 (m, 4H, β,δ-$CH_2$), 1.23-1.19 (m, 2H, γ-$CH_2$). **$^{13}$C NMR spectrum** (101 MHz, DMSO-$d_6$, δ, ppm): 175.35, 172.09, 135.46, 37.95, 34.47, 28.70, 26.66, 25.00.

*1-(5-Isocyanatopentyl)-1H-pyrrole-2,5-dione* **(5)**: 450 mg (2.13 mmol, 1 equiv) of 6-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)hexanoic acid **4** were dissolved in dichloromethane, 206 μl (2.34 mmol, 1.1 equiv) of oxalyl chloride were added over 30 min, and the reaction mixture was stirred for 5 h. The solvent was evaporated under reduced pressure, and the residue was dried overnight in a vacuum desiccator. The resulting yellow oil was dissolved in 1 ml of acetone and added dropwise to a solution of 204 mg (3.14 mmol, 1.5 equiv) of sodium azide in 4 ml of water, cooled to 0°C. The reaction mixture was stirred at 0°C for 1 h, after which the aqueous layer was washed with chloroform (3 x 50 ml). The organic fractions were dried over sodium sulfate, the solvent was evaporated under reduced pressure to a solution volume of 6-8 ml and dried over pre-calcined 3Å molecular sieves. The resulting solution was refluxed for 2 h. The reaction mixture was cooled to room temperature, after which the solvent was evaporated under reduced pressure, the product was purified by flash chromatography, eluent petroleum ether:EtOAc 2:1. 1-(5-isocyanatopentyl)-1H-pyrrole-2,5-dione 4 (150 μl) was obtained as a pale yellow liquid. Yield: 34%. **$^{1}$H NMR spectrum** (400 MHz, $CDCl_3$, δ, ppm): 6.63 (s, 2H, CH=CH), 3.43 (t, 2H, J=7.1 Hz, $CH_2$-N), 3.21 (t, 2H, J=6.6 Hz, α-$CH_2$), 1.56 -1.50 (m, 4H, β,δ-$CH_2$), 1.31-1.24 (m, 2H, γ-$CH_2$). **$^{13}$C NMR spectrum** (101 MHz, $CDCl_3$, δ, ppm): 170.38, 133.62, 121.37, 42.24, 36.92, 30.10, 27.35, 2314.

Conjugate of oxoplatin, (2*R*,3*S*,4*S*)-5-(7,8 -dimethyl-2,4-dioxo-3,4-dihydrobenzo[*g*]pteridin-10(2*H*)-yl)pentane-1,2,3,4-tetrayl tetraacetate and 1-(5-isocyanatopentyl)-1*H*-pyrrole -2,5-dione **(3)**: From 15 mg (0.015 mmol, 1 equiv.) of complex **1** and 20 mg (0.09 mmol, 6 equiv.) 1-(5-isocyanatopentyl)-1*H*-pyrrole-2,5-dione **5** following the protocol given for complex 3, 9 mg of complex **3** was obtained as an orange powder. Yield 50%. **$^{1}$H NMR spectrum (400 MHz, DMSO-$d_6$, δ, ppm):** 7.96 (s, 1H, H9(Ar)), 7.75 (s, 1H, H6(Ar)), 6.99 (s, 2H, CH =CH), 6.77-6.50 (m, 7H, NH3, 5'H(rib)), 5.49-5.44 (m, 2H, 2',3'–CH (rib)), 5.31-5.28 (m, 1H, 4'–CH (rib)), 5.14-4.80 (m, 1H, 5'–CH2 (rib)), 4.37 (dd, 1H, J1=12.5 Hz, J2=2.8 Hz, 1' H–CHOAc), 4.23-4.18 (dd, 1H, J1=12.3 Hz, J2=6.0 Hz, 1' H–CHOAc), 3.89-3.85 (m, 2H, N–CH2–CH2), 2.99-2.84 (m, 6H, $CH_2$– $CH_2$– NH–(CO), CH2-N, α-CH2), 2.51 (s, 3H, CH3–Ar), 2.40 (s, 3H, CH3–Ar), 2.20 (s, 3H, AcO), 2.18 ( s, 3H, AcO), 1.99 (s, 3H, AcO), 1.71-1.27 (m, 2H, $CH_2$–$CH_2$–$CH_2$, AcO, β,γ,δ-CH2), 1.58 (s, 3H, AcO). **HRMS (ESI-TOF):** calculated $C_{39}H_{53}Cl_2N_9NaO_{16}Pt^+$, 1191.2533, (**6**+Na)+; found $C_{39}H_{53}Cl_2N_9NaO_{16}Pt$, 1191.2523, (6+Na)$^+$. Melting point: >200ºC

**Cytotoxicity.** Immortalized human fibroblasts WI-26 were seeded in a 96-well plate ($5\times10^4$ cells/well) in complete RPMI-1640 medium and incubated overnight. Then working solutions of the studied compounds (0.05-100 μM) were added to the cells (100 μl) for 2 h in a $CO_2$ incubator. Then, to determine dark toxicity, the cells were left in the dark for another 72 h. Light toxicity was assessed using two approaches. In the first case, after 2 h of incubation, the medium was replaced with fresh one and then irradiated with a dose of 1 J/cm$^2$ (450 nm, 80 s). In the second case, irradiation (450 nm, 1 J/cm$^2$, 80 s) was carried out in a medium containing the tested compounds. Cytotoxicity was measured after 72 h using the MTT assay, the viability of control cells was taken as 100%.

**Confocal microscopy.** MCF-7 human breast adenocarcinoma cells were seeded in 8-well plates and incubated overnight. Then, 50 μM of each compound for 1 h in a $CO_2$ incubator. The cells were then washed with PBS (pH 7.4), fixed in 4% PFA after which the nuclei were additionally stained with LumiTracker® Mito Red CMXRos or LumiTracker® Lyso Red and analyzed using a Leica TCS SPE confocal system (ex. 488 nm, em. 500-600 nm).




**Scheme S1**

*Comments on spectral data*. In $^{1}$H NMR spectra for **2** multiplets at 7.71, 7.47, 7.25 and 7,12 correspond to the naproxen moiety, while singlets at 7.95 and 7.75 belong to the protons 9 and 6 of the isoalloxazine moiety of the TARF axial ligand. The characteristic broad singlet of $NH_3$ ligands of $Pt^{IV}$ core is at 6.63, a 0.5 ppm shift compared to the peak at 6.10 ppm in $^{1}$H NMR spectrum of Riboplatin prodrug, which indicate the presence of two carboxylate axial ligands in **3**, namely TARF derivative and naproxen. The presence of platinum(IV) in the complex was also proven by the $^{195}$Pt NMR peak at 1254.4 ppm (Figure S5), which is typical of dicarboxylated $Pt^{IV}$ prodrugs. In $^{13}$C spectra for complex **2** a presence of naproxen moiety is indicated by the appearance of carboxylic atom peak at 181.87 ppm, as well as eight new signals in 137.42-105.65 ppm region, compared to the spectra of TARF and is consistent with NMR spectra for naproxen derivatives. For complex **3**, 9- and 6-positioned of isoalloxazine moiety at 7.96 and 7.75 ppm are observed as well, together with singlet at 6.99 ppm for the maleimide double bond. The broad singlet of $NH_3$ protons is observed for **3** at 6.67 ppm, similarly as for **2**. The broad singlet at 6.55 of two carbamate protons proves the formation of dicarbamate complex **3.**

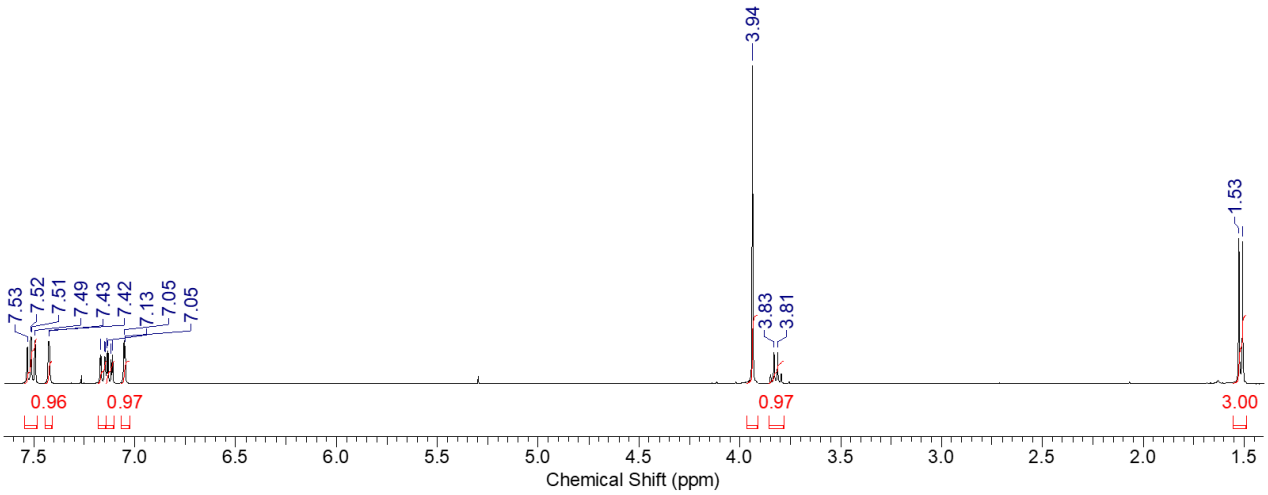



**Figure S1**. [1]H NMR spectrum of naproxen anhydride.

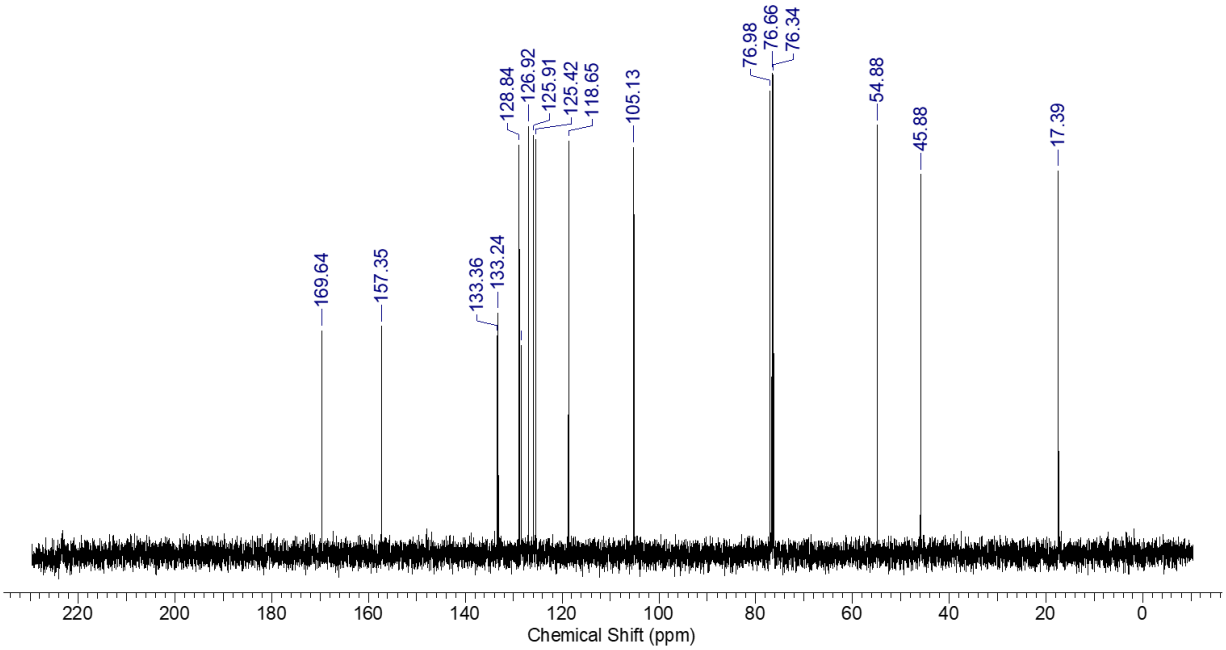



**Figure S2.** [13]C NMR spectrum of naproxen anhydride.

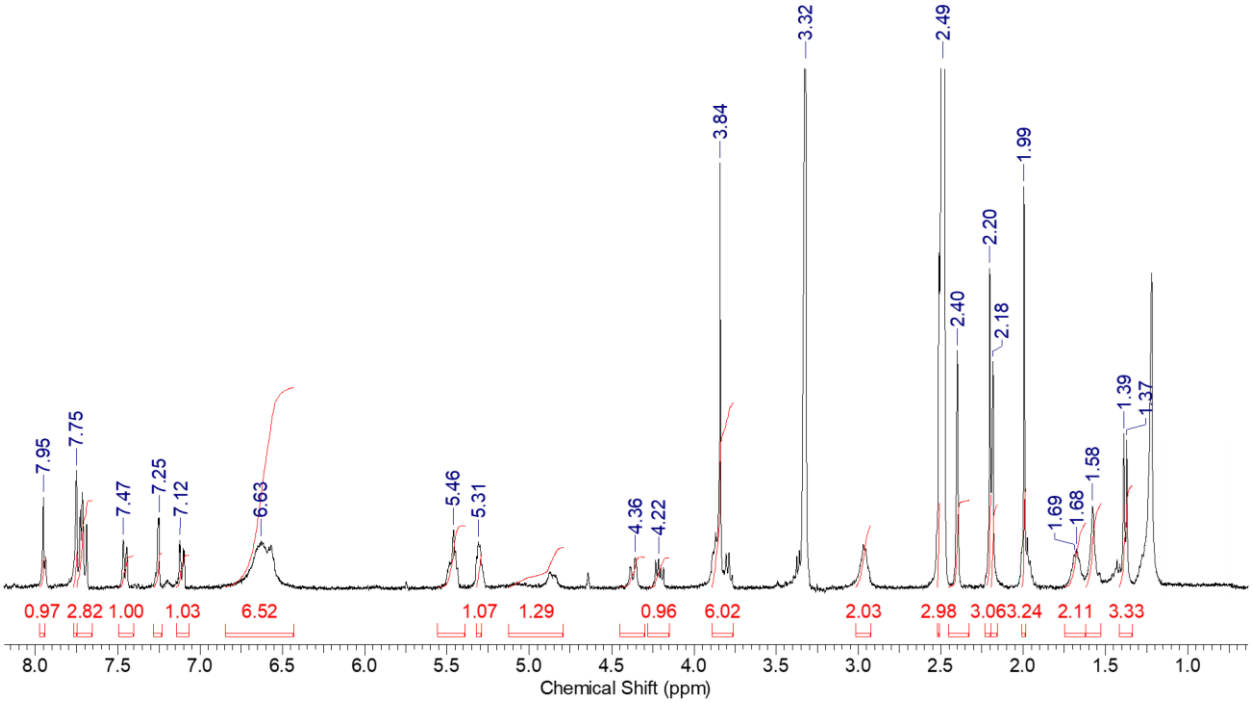



**Figure S3.** $^{1}$H NMR spectrum of **2.**

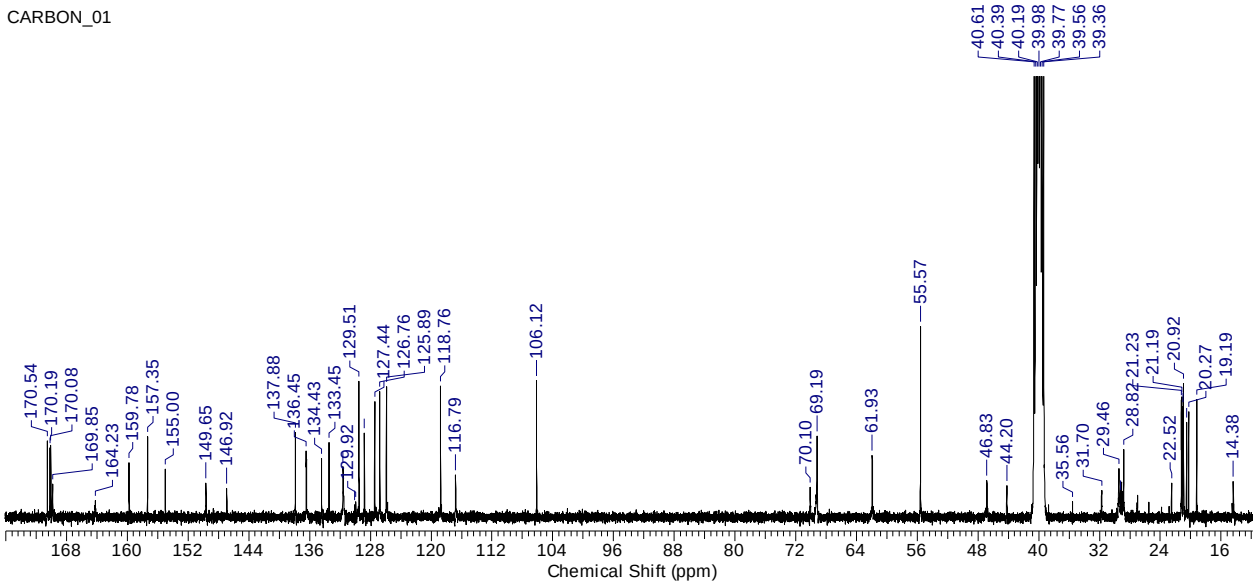



**Figure S4.** $^{13}$C NMR spectrum of **2.**

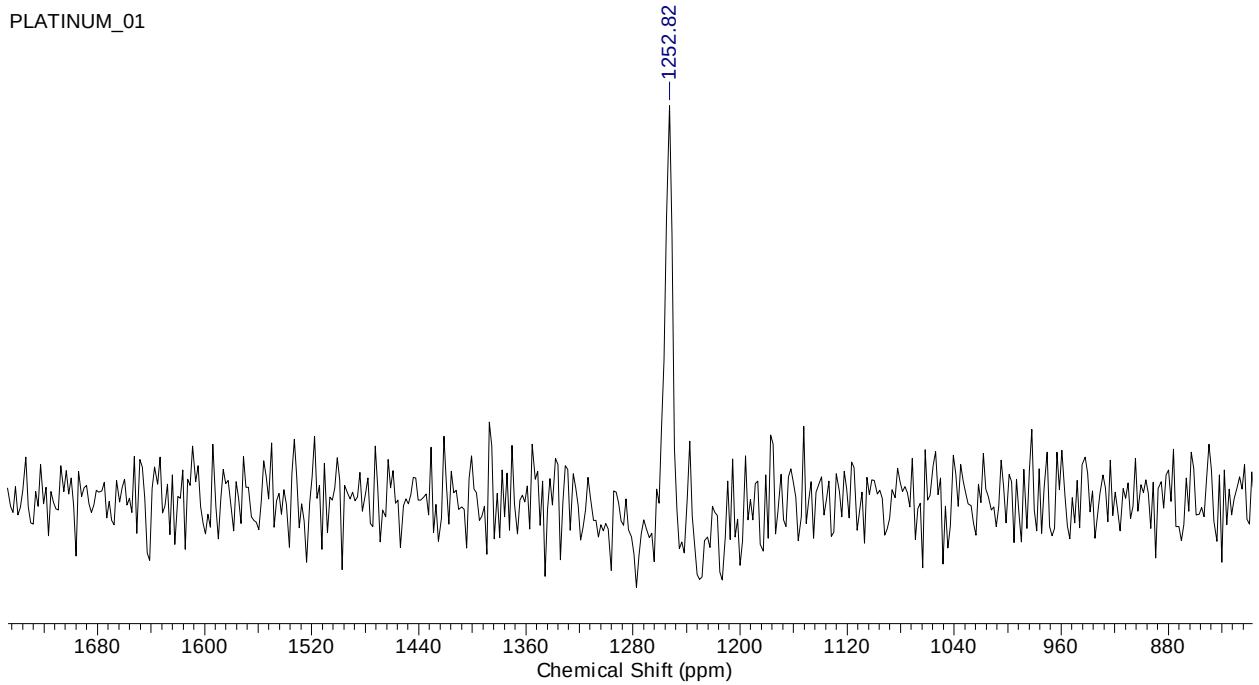



**Figure S5.** $^{195}$Pt NMR spectrum of **2.**

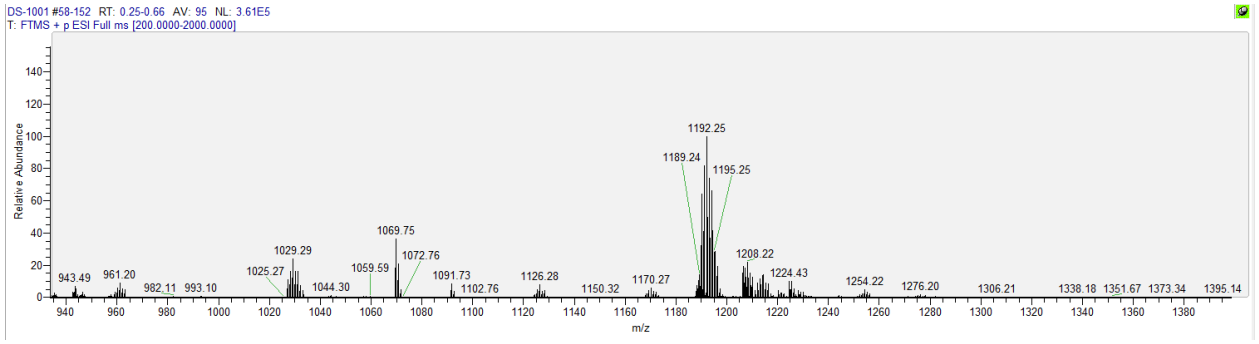



**Figure S6.** HRMS spectrum of **2**.

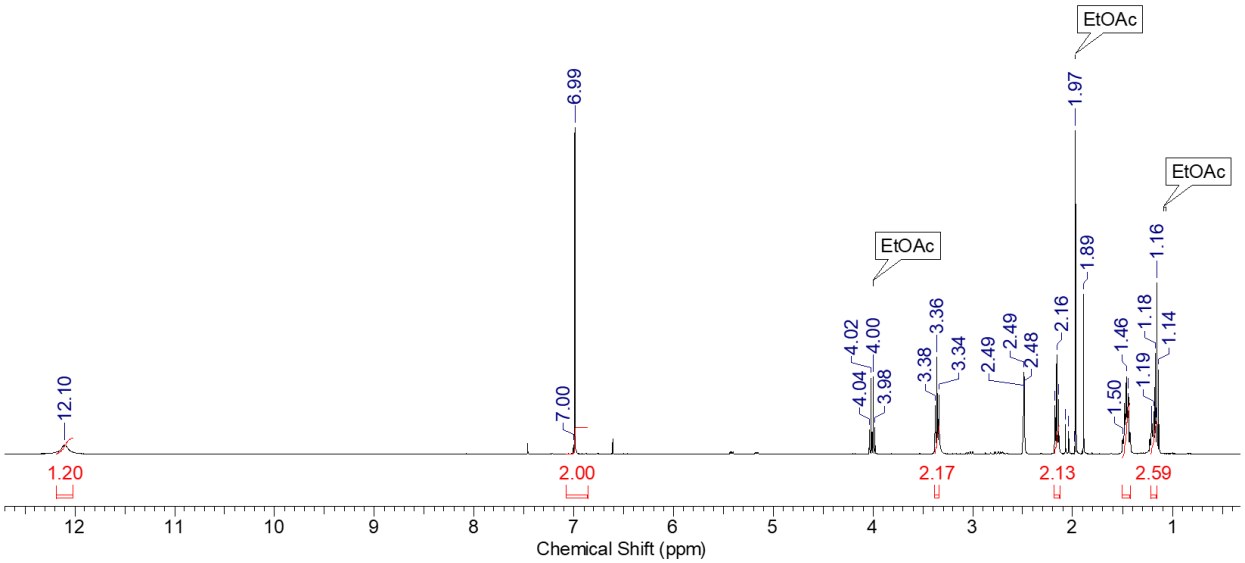



**Figure S7.** [1]H NNR spectrum of **4**.

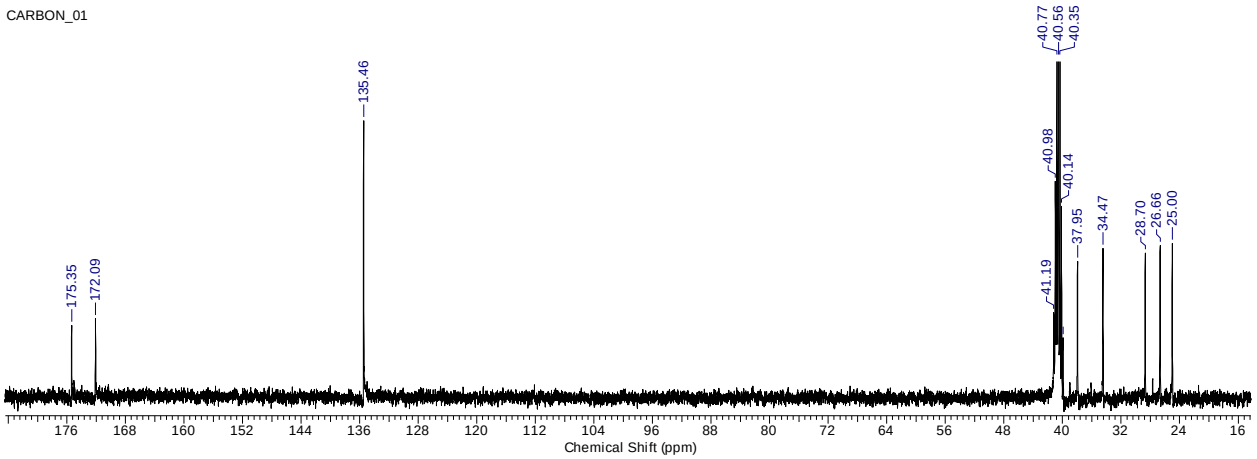



**Figure S8.** [13]C NNR spectrum of **4**.

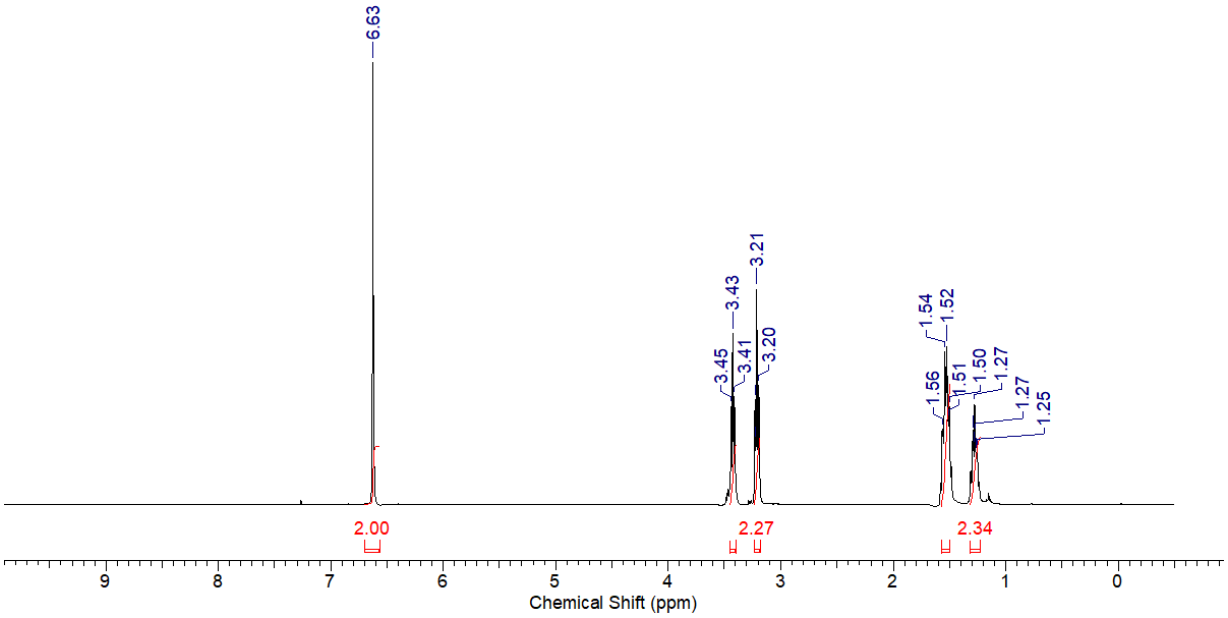



**Figure S9.** [1]H NMR spectrum of **5**.

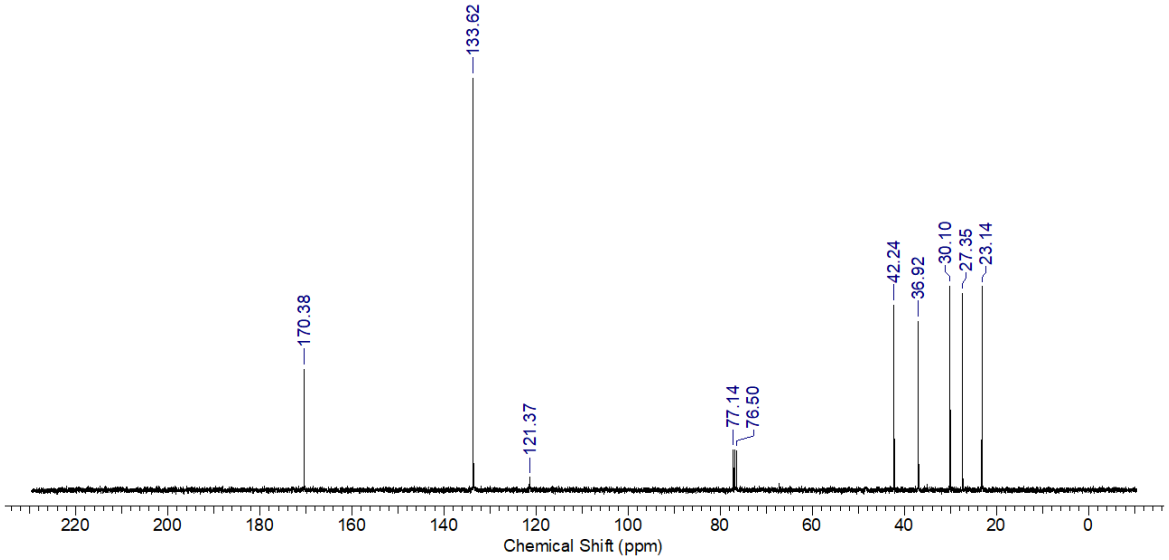



**Figure S10.** $^{13}$C NMR spectrum of **5**.

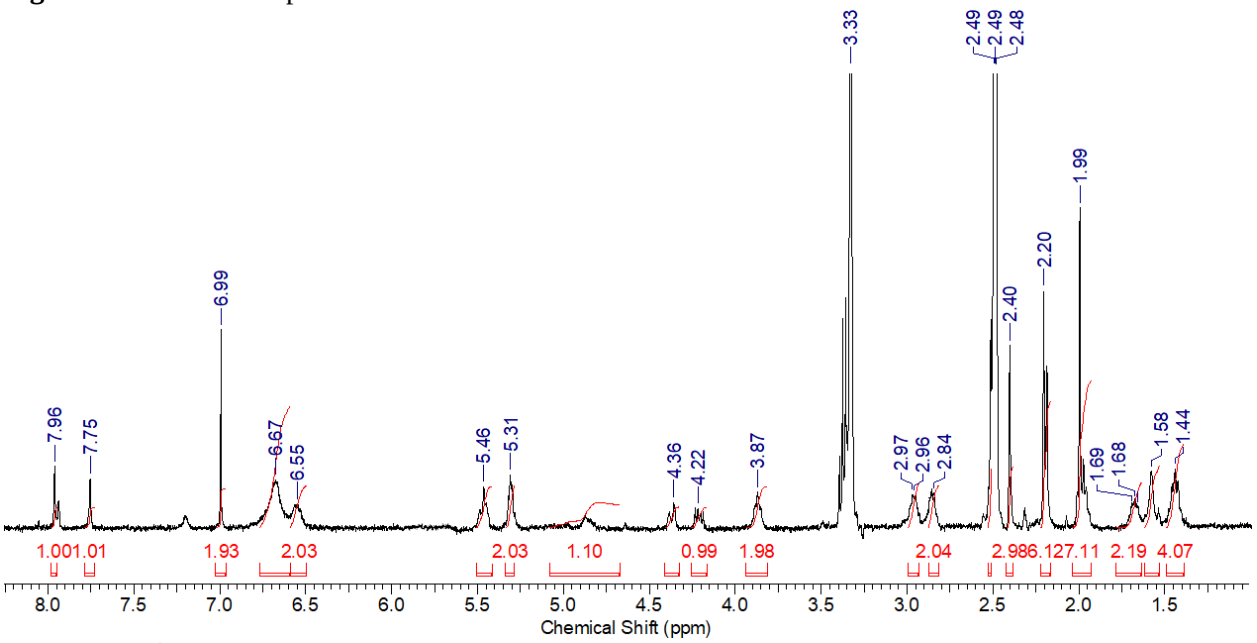



**Figure S11.** $^{1}$H NMR spectrum of **3**

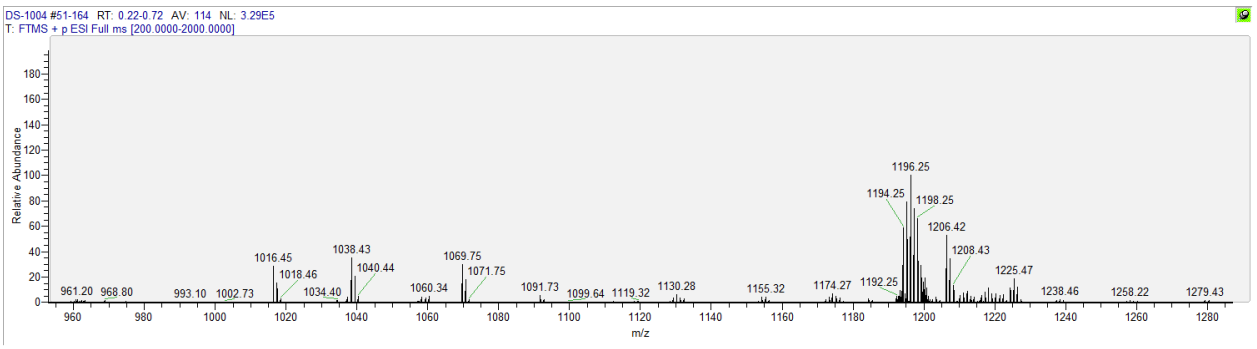



**Figure S12.** HRMS spectrum of **3.**

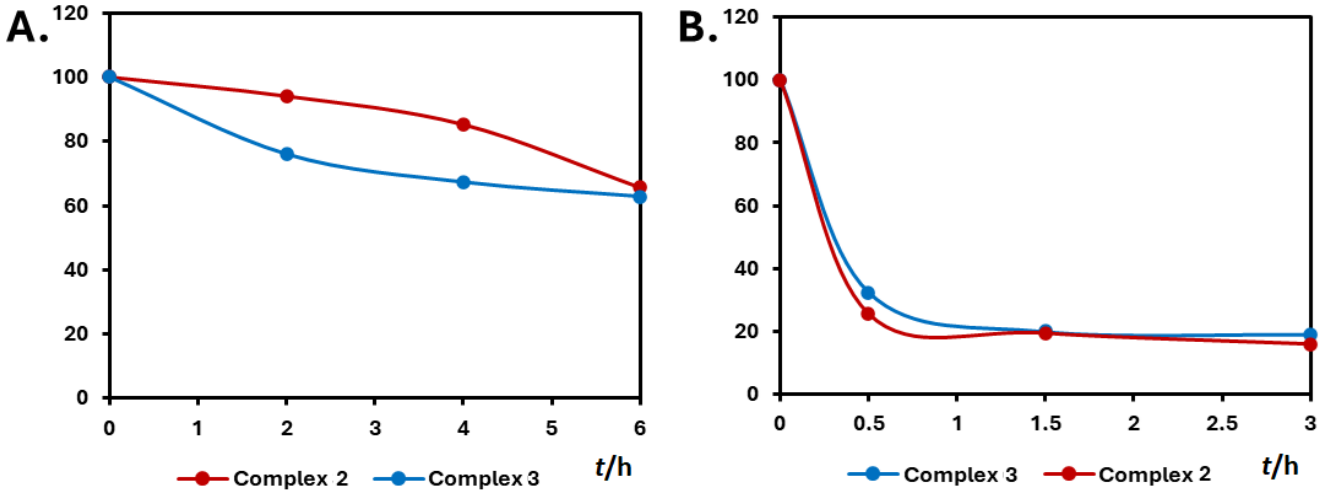



**Figure S13.** (*a*) Stability of $Pt^{IV}$ prodrugs **2** and **3** in DMSO:MeOH:$H_2O$ (60:30:10) in the absence of light. (*b*) Reduction of $Pt^{IV}$ prodrugs **2** and **3** in DMSO/MeOH/$H_2O$ (60:30:10) in the absence of light.

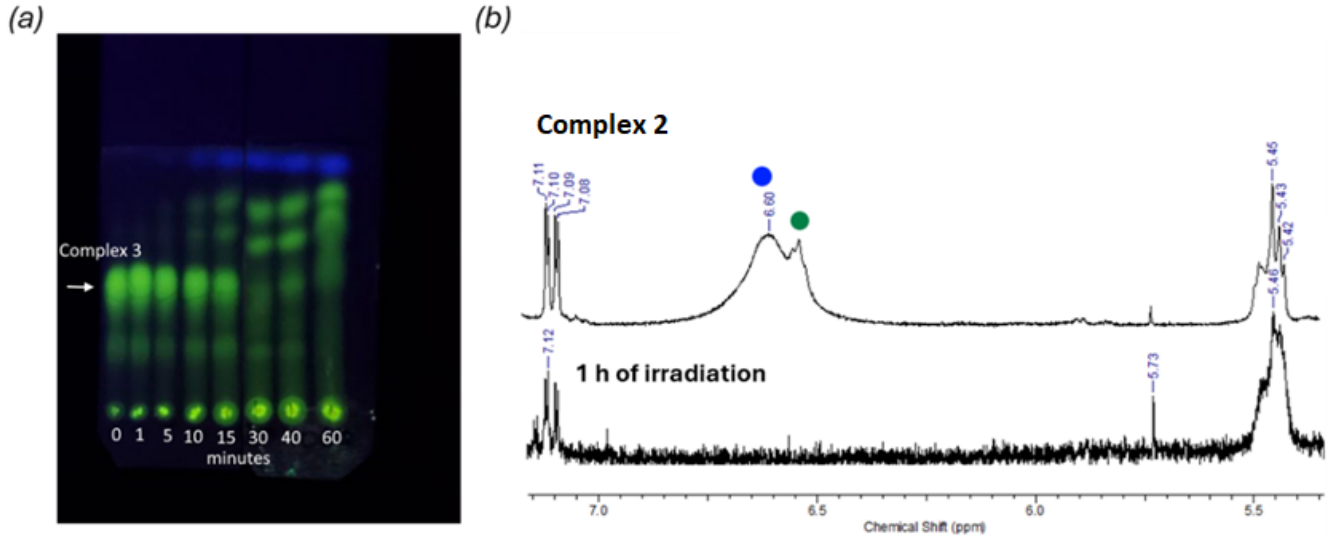



**Figure S14** (*a*) TLC plate of $Pt^{IV}$ prodrug **2** solution in methanol (2 mg $ml^{-1}$) under blue light irradiation (450 nm, 10.4 mW $cm^{-2}$). Each point was placed at fixed time point (before irradiation/0 min, 1, 5, 10, 15, 30, 40 or 60 minutes). (*b*) $^1H$ NMR spectra of complex **2** before and after irradiation with blue light (450 nm, 10.4 mW/$cm^2$, 60 minutes). Blue circle indicates the $NH_3$ ligands peak, green circle indicates the peak corresponding to the NH carbamate group**.**

*Comments on Figure S14*. To evaluate the light-responsive properties of novel $Pt^{IV}$ prodrugs, complex **2** was selected. Its 2 mg $ml^{-1}$ solution **2** in MeOH was irradiated with low-dose blue light (450 nm, 10 mW $cm^{-2}$, 1 h). The emergence of new bands could be clearly seen on the TLC plate under UV light (365 nm), while the starting band of the complex **3** gradually disappeared within 1 hour of irradiation (Figure S14, part *a*), To confirm the light-induced decay of prodrug, after 1 hour of irradiation the solution was analyzed by $^1H$ NMR. The characteristic broad singlet of $NH_3$ protons at 6.65 ppm has disappeared, which indicated that the $Pt^{IV}$ complex has decayed (part *b*). The disappearance of the NH carbamate peak at 6.53 ppm indicates the cleavage of carbamate group and the release of the TARF amine derivative.

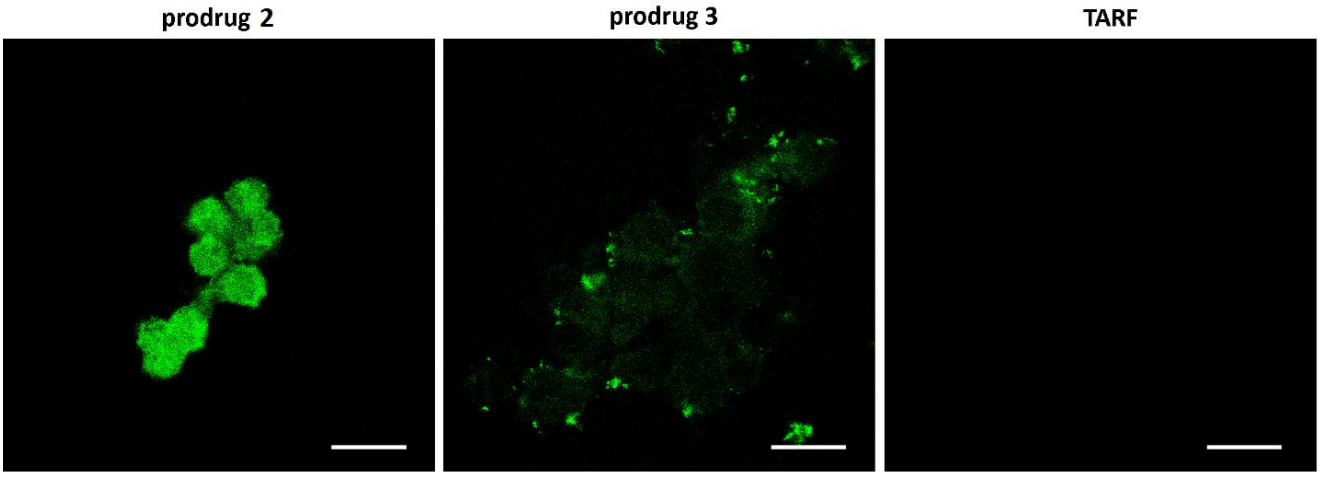



**Figure S15.** Intracellular distribution of prodrug **2** and TARF (green) in human breast adenocarcinoma cells, 50 μM, 1 h incubation. Scale-bar 20 μm.

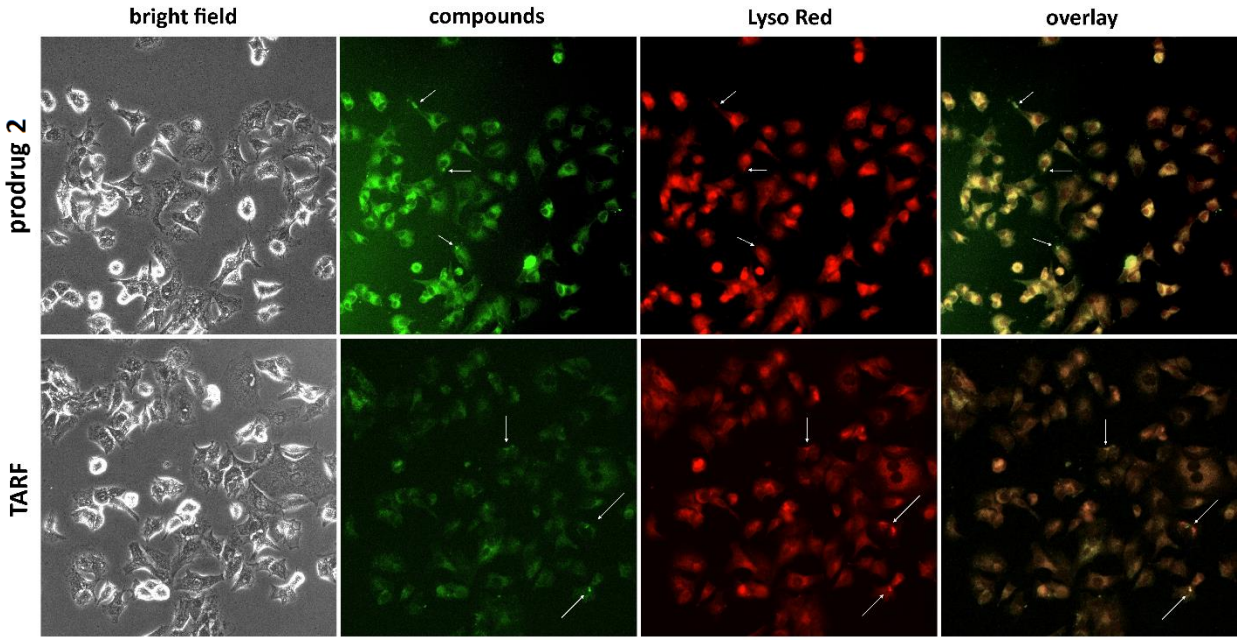



**Figure S16.** Intracellular distribution of prodrug **2** and TARF (green) in human breast adenocarcinoma cells, 50 μM, 1 h incubation. Cells were additionally stained with LumiTracker® Lyso Red probes. The arrows indicate the most representative regions (separate bright spots with high compound accumulation).

**References**

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S2 L. F. Tietze and T. Eicher, *Reaktionen und Synthesen im organisch-chemischen Praktikum und Forschungslaboratorium*, Wiley, 1991; https://doi.org/10.1002/3527601716.

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