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Supplementary Information for manuscript

Probing Two PESIN-Indocyanine dye-Conjugates: Significance of the Used Fluorophore

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Content

Experimental Section

General

All commercially available chemicals and solvents were at least of analytical grade and used, if not otherwise stated, without further purification. Fmoc-protected amino acids and rink amid resin (loading = 0.52 mmol/g) were purchased from NovaBiochem. Fmoc-PEG(4)-COOH (PEG = Polyethylenglycol; PEG 1820) was obtained from Iris Biotech. Dichloromethane, diethylether, dimethylformamide, HBTU ((2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) and water where purchased from Carl Roth, acetonitrile from Häberle Labortechnik, DIPEA (*N,N*-Diisopropylethylamine), TIS (Triisopropylsilane) and IR-820 (2-((*E*)-2-((*E*)-2-chloro-3-((*E*)-2-(1,1-dimethyl-3-(4-sulfobutyl)-1,3-dihydro-2*H*-benzo[*e*]indol-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-1,1-dimethyl-3-(4-sulfobutyl)-1*H*-benzo[e]indol-3-ium) from Sigma-Aldrich, 4-Carboxyphenylboronic acid and $Pd(PPh_3)_4$ (Tetrakis(triphenylphosphine)palladium(0)) from TCI.

For HPLC chromatography, an Agilent 1200 system was used together with Chromeleon Software (Version 6.80). For analytical chromatography, a Chromolith Performance (RP-18e, 100-4.6 mm, Merck, Germany) and for semipreparative analyses, a Chromolith (RP-18e, 100-10 mm, Merck, Germany) column were used, respectively. ESI (Electrospray Ionization) and MALDI (Matrix-Assisted Laser Desorption/Ionization) spectra were obtained with Finnigan MAT95Q and Bruker Daltronics Microflex spectrometers. γ-counting was performed using a 2480 Wizard gamma counter system from Perkin Elmer. A Cary 100 Bio system (Varian) was used to record the UV/Vis-Spectra. Fluorescence measurements were carried out on a Cary Eclipse spectrometer (Varian) and the procedure for determination of the relative fluorescence quantum yield was described before[1]. For all optical measurements, 4 mL PMMA cuvettes from Sigma-Aldrich were used.

The human tumor cell line PC-3 (GRPR positive) was obtained from DSMZ, [^{125}I]-Tyr^4-bombesin was purchased from Perkin Elmer (NEX258010UC, molar activity: 81.4 GBq/µmol). RPMI 1640 medium, Opti-MEM I (GlutaMAX I), L-Glutamine and PenStrep were obtained from Gibco, FCS (fetal calf serum) from BioCell and Dulbecco's phosphate buffered saline (PBS), 0.25% Trypsin and 0.02% EDTA Solution in PBS from Sigma-Aldrich.

Syntheses

The PESIN peptide sequence was synthesized by standard peptide synthesis methods, using standard solid phase peptide synthesis Fmoc-based protocols[2-4] and subsequent conjugation of the respective amino acids. The dye **LS277** (2-((*E*)-2-((*E*)-4'-carboxy-6-((*E*)-2-(1,1-dimethyl-3-(4-sulfobutyl)-1,3-dihydro-2H-benzo[*e*]indol-2-ylidene)ethylidene)-3,4,5,6-tetrahydro-[1,1'-biphenyl]-2-yl)vinyl)-1,1-dimethyl-3-(4-sulfobutyl)-1*H*-benzo[*e*]indol-3-ium) was synthesized according to literature methods[5].

The dye **CK002** (2-((*E*)-2-((*E*)-6-(2-((*E*)-5-carboxy-3,3-dimethyl-1-(4-sulfobutyl)indolin-2-ylidene)ethylidene)-3,4,5,6-tetrahydro-[1,1'-biphenyl]-2-yl)vinyl)-3,3-dimethyl-5-sulfo-1-(4-sulfobutyl)-3*H*-indol-1-ium) was prepared according to previous reports[1].

General Synthesis of peptide-dye-conjugates

25 µmol of freshly Fmoc-deprotected PESIN (H_2N-PEG_3-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2) on rink amid resin were reacted with 1.5 eq activated dye (each 34 mg) and heated (80°C) in DMF (4 mL) for 3 – 4 h. The activation of the dyes was carried out beforehand with HBTU (0.95 eq.) and DIPEA (1.0 eq.) as base, for 10 minutes in DMF (2 mL). After the reaction was finished, the resin was filtered from solvent and washed subsequently thrice with DMF, water, dichloromethane and diethylether. After drying, the conjugates were cleaved from resin by using a TFA/TIS (95% / 5%, 5 mL) mixture for one hour. The acid was removed by reduced pressure and the residue was purified by HPLC. Detailed HPLC conditions, yields and analytical data are listed below.

PCD 1 HPLC gradient (semipreparative): 35 – 85 % MeCN + 0.1 % TFA in 8 min, R_t = 4.21 min. HPLC gradient (analytical): 0 – 100 % MeCN + 0.1 % TFA in 8 min, R_t = 4.10 min, yield: 10 % (5 mg), purity: 99%, ESI-MS (*m/z*) for $[M-2H]^{2-}$ (calculated): 1039.97 (1039.98); ESI-MS (*m/z*) for $[M+2H]^{2+}$ (calculated): 1041.99 (1031.96). MALDI-MS (*m/z*) for $[M+H]^+$ (calculated): 2080.26 (2080.95); $[M+Na]^+$ (calculated): 2102.28 (2103.94); $[M+K]^+$ (calculated): 2118.25 (2119.92).

PCD 2 HPLC gradient (semipreparative): 25 – 65 % MeCN + 0.1 % TFA in 8 min, R_t = 4.38 min. HPLC gradient (analytical): 0 – 100 % MeCN + 0.1 % TFA in 8 min, R_t = 3.46 min, yield: 8 % (4 mg), purity: 99%, ESI-MS (*m/z*) for $[M+2H]^{2+}$ (calculated): 1031.95 (1031.94), ESI-MS (*m/z*) for $[M-2H]^{2-}$ (calculated): 1029.93 (1029.94), MALDI-MS (*m/z*) for $[M+H]^+$ (calculated) 2061.59 (2061.88); $[M+Na]^+$ (calculated): 2083.60 (2083.87); $[M+K]^+$ (calculated): 2099.60 (2099.84).

Log_D determination

The water/octanol partition coefficient (log_D) was determined by semipreparative HPLC. For this purpose, 10 µL DMSO solution (c = 5 x 10^{-4} mol/L) of the respective substance was added to a mixture of 1 mL 1-octanol and 990 µL phosphate buffered solution (pH 7.4) and vigorously shaken for 5 minutes. After centrifugation, the phases were separated and both phases were analyzed by semipreparative HPLC as described before. For each compound, the log_D was determined by three separate measurements, each experiment performed in triplicate.

Competitive receptor binding assay

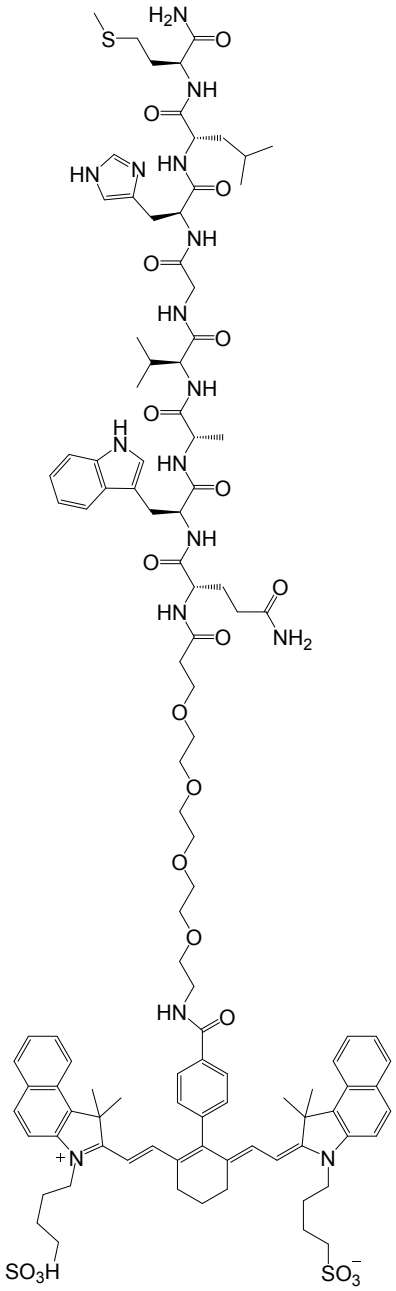
The human tumor cell line PC-3 was cultured at 37°C in RPMI 1640 medium supplemented with 10% FCS, 1% L-Glutamine and 1% PenStrep in a humidified atmosphere containing 5% CO_2. The medium was exchanged every two or three days and cells were split at >75% confluence. In vitro binding affinities were determined via competitive displacement experiments which were performed at least trice, each experiment performed in triplicate. A Millipore Multiscreen punch kit and Millipore 96 well filter plates were used. The plates were incubated with PBS/BSA (1%) solution (each well 200 µL) for one hour before use. PC-3 cells were harvested and suspended carefully in Opti-MEM I (GlutaMAX I) medium. 50 µL of a cell suspension containing 10^5 cells

were seeded in each well. To this, a total volume of 50 µL was added to each well, containing 25 µL (0.012 kBq/µL) of the GRPR-specific radioligand [^{125}I]-Tyr^4-bombesin (81.4 GBq/µmol) and 25 µL of the respective competitor **PDC 1**, **PDC 2** or endogenous bombesin (BBN, used as reference compound). The competitor was added in 11 increasing concentrations ranging from 0.5 – 1000 nM for **PDC 1** and **PDC 2** or 0.1 – 250 nM for BBN, whereat the twelfth well contained no competitor to ensure 100% binding of the radioligand. After one hour of incubation at ambient temperature, the solution was filtrated and the filters were washed with cold PBS (3 times). The filters were collected and measured by γ-counting. The 50% inhibitory concentration (IC_{50}) values of **PDC 1**, **PDC 2** and bombesin were calculated by fitting the obtained data via a nonlinear regression analysis using GraphPad Prism Software (version 5.04).

References:

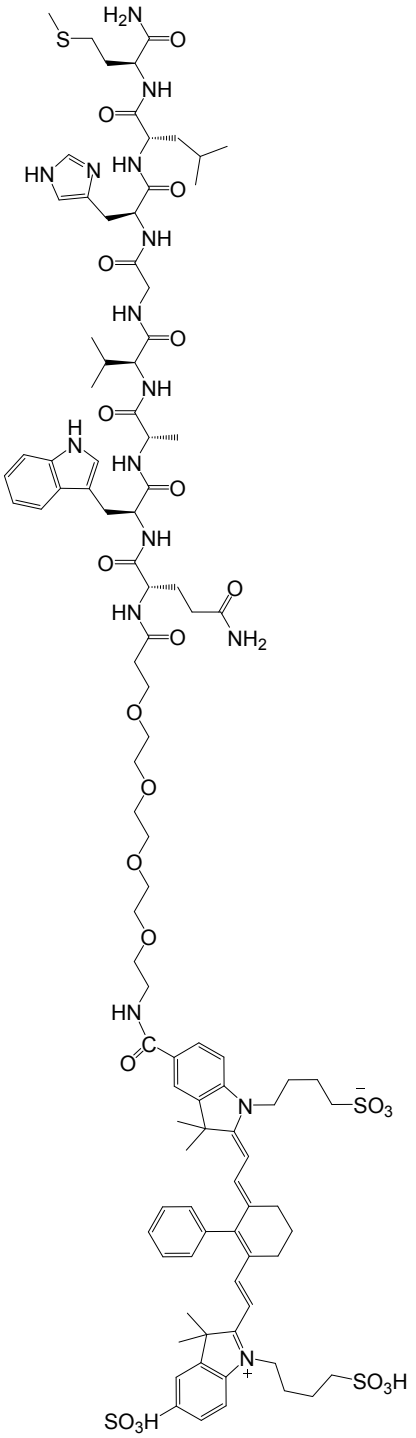
1. S. G. König and R. Kramer, *Chemistry*, 2017, **23**, 9306-9312.
2. D. A. Wellings and E. Atherton, *Methods in Enzymology*, 1997, **289**, 44 - 67.
3. C. Wängler, S. Maschauer, O. Prante, M. Schäfer, R. Schirrmacher, P. Bartenstein, M. Eisenhut and B. Wängler, *ChemBioChem*, 2010, **11**, 2168 - 2181.
4. C. Wängler, B. Waser, A. Alke, L. Iovkova, H.-G. Buchholz, S. Niedermoser, K. Jurkschat, C. Fottner, P. Bartenstein, R. Schirrmacher, J.-C. Reubi, H.-J. Wester and B. Wängler*, *2010*, 2010, **21**, 2289 - 2296.
5. J. C. M. Hyeran Lee, and Samual Achilefu, *J. Org. Chem.*, 2006, **71**, 7862.

PDC 1



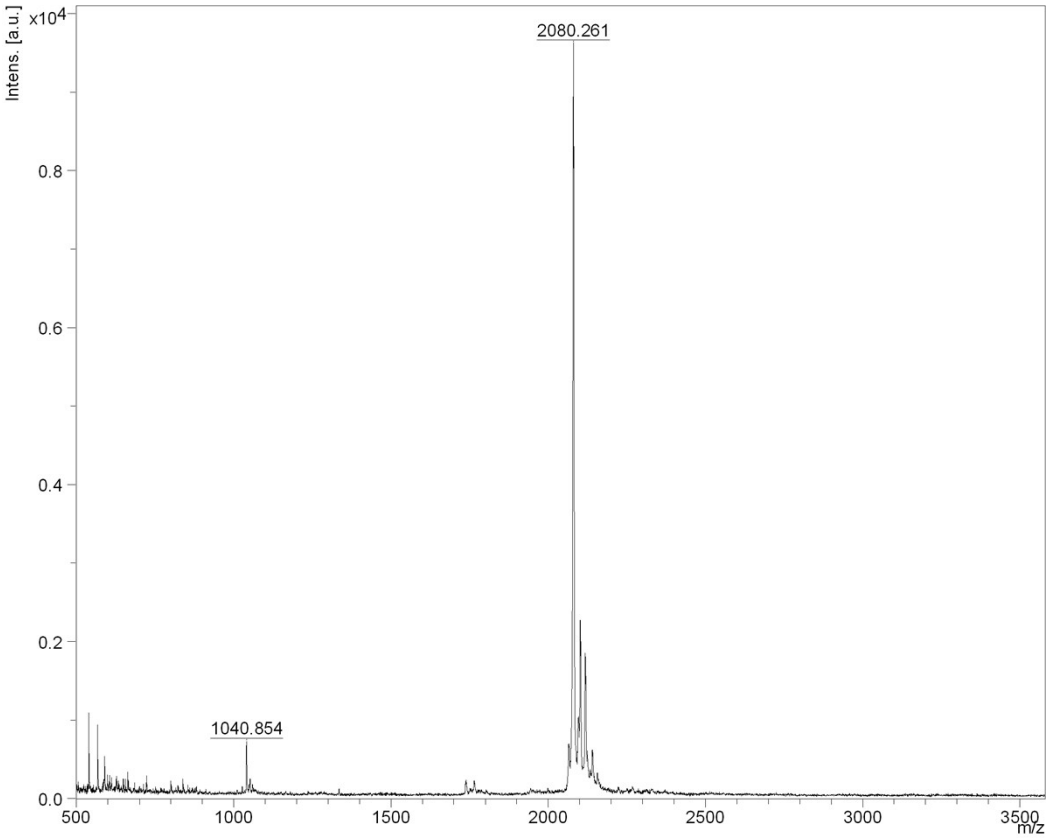
Chemical Formula: $C_{107}H_{140}N_{16}O_{21}S_3$
Exact Mass: 2080,954

PDC 2



Chemical Formula: $C_{99}H_{136}N_{16}O_{24}S_4$
Exact Mass: 2060,880

Fig. S1: Structures and mass data for both conjugates **PDC 1** and **PDC 2**.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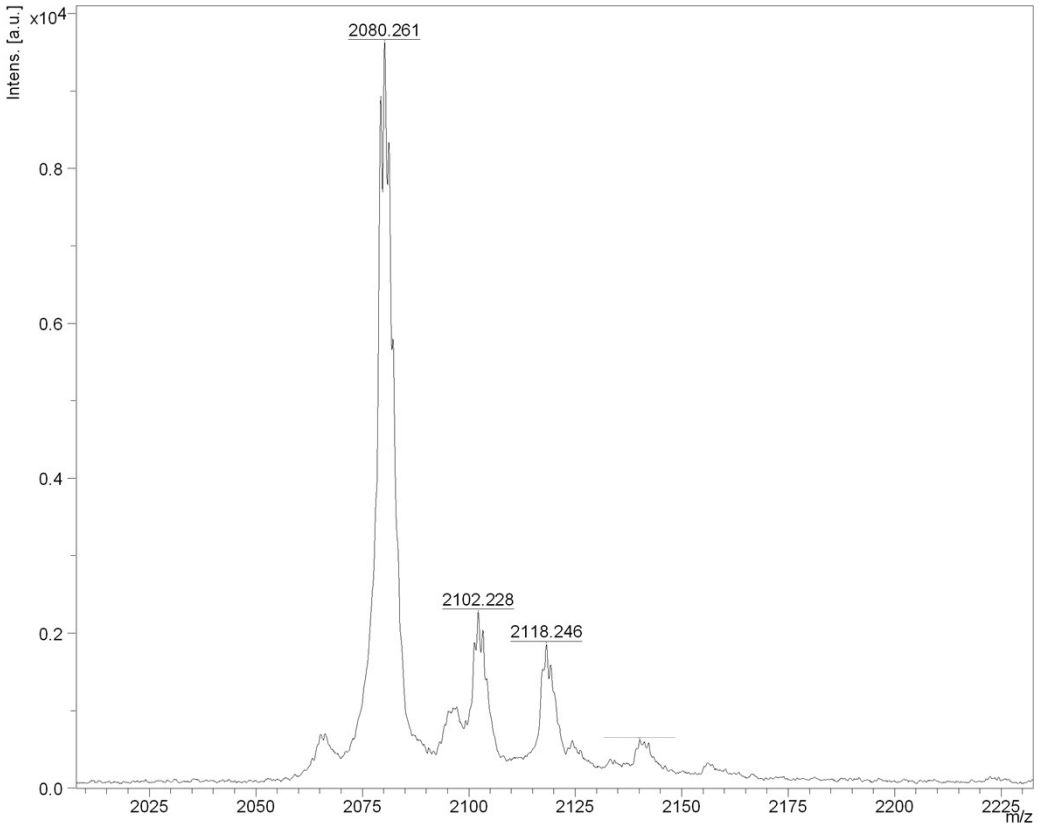
1500
2000
2500
3000
3500
m/z


x10^4
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2225
m/z

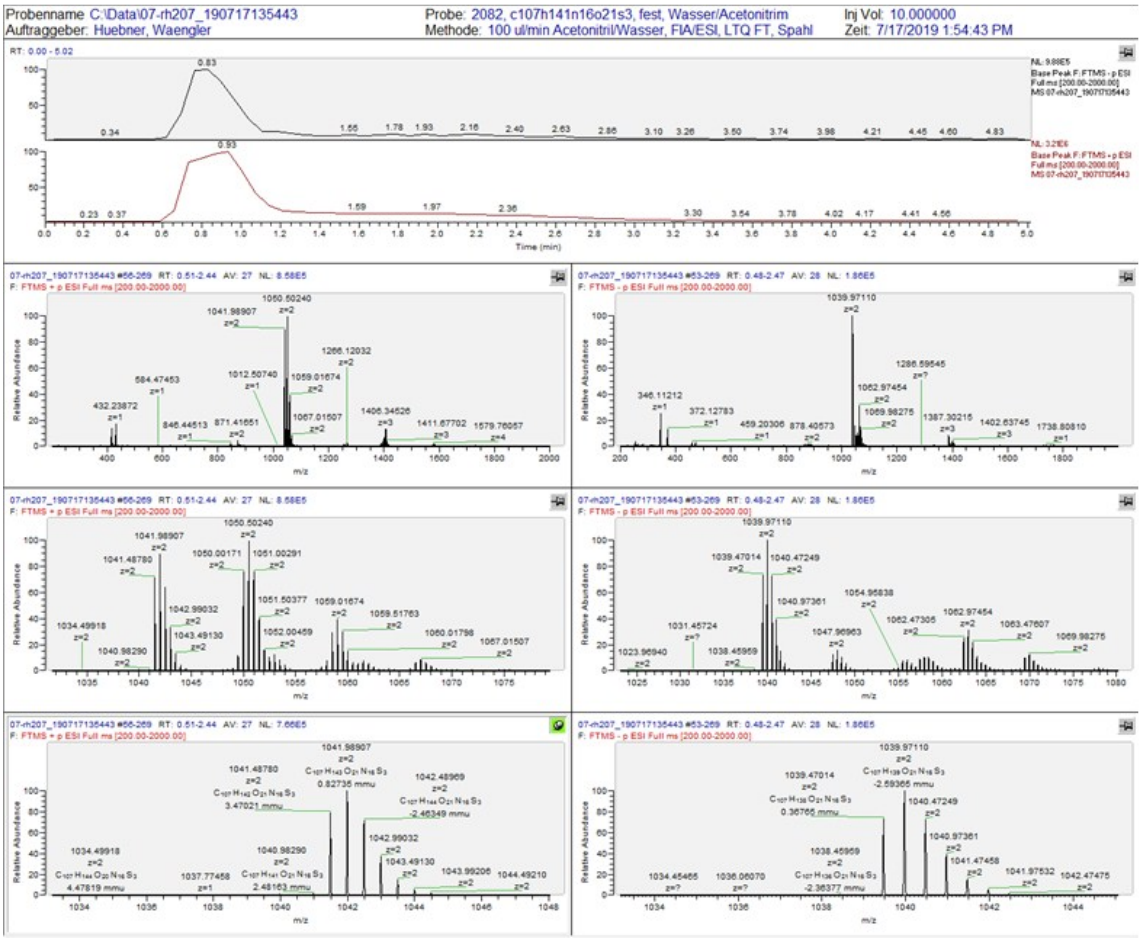
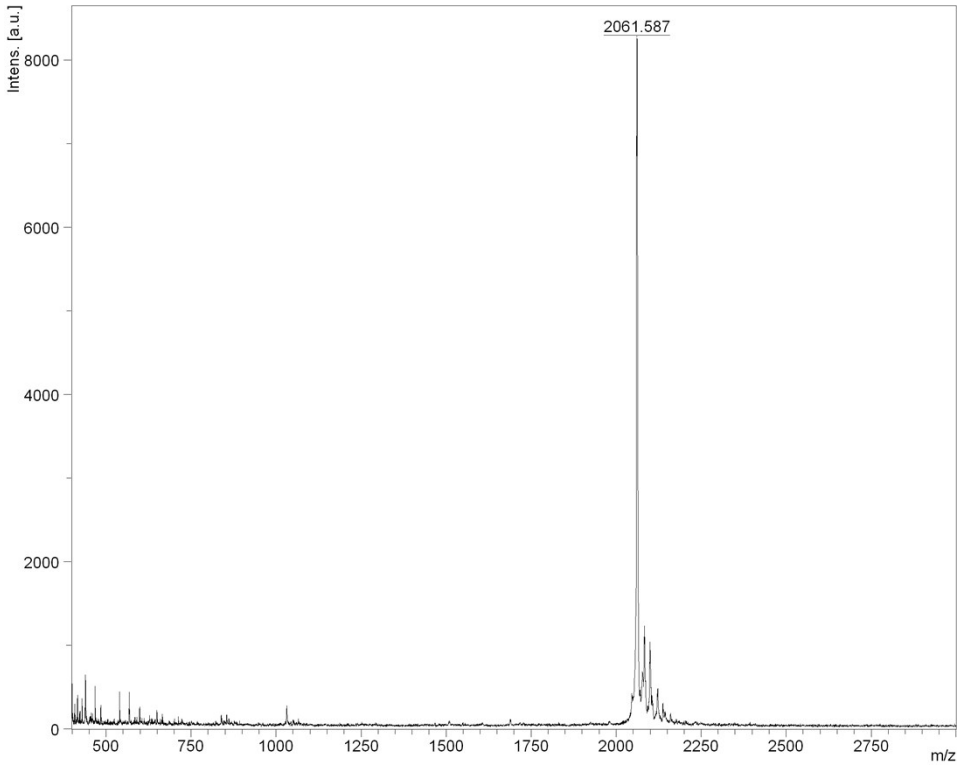


Fig. S2 Analytical data for **PDC 1** (ESI and MALDI mass spectroscopy).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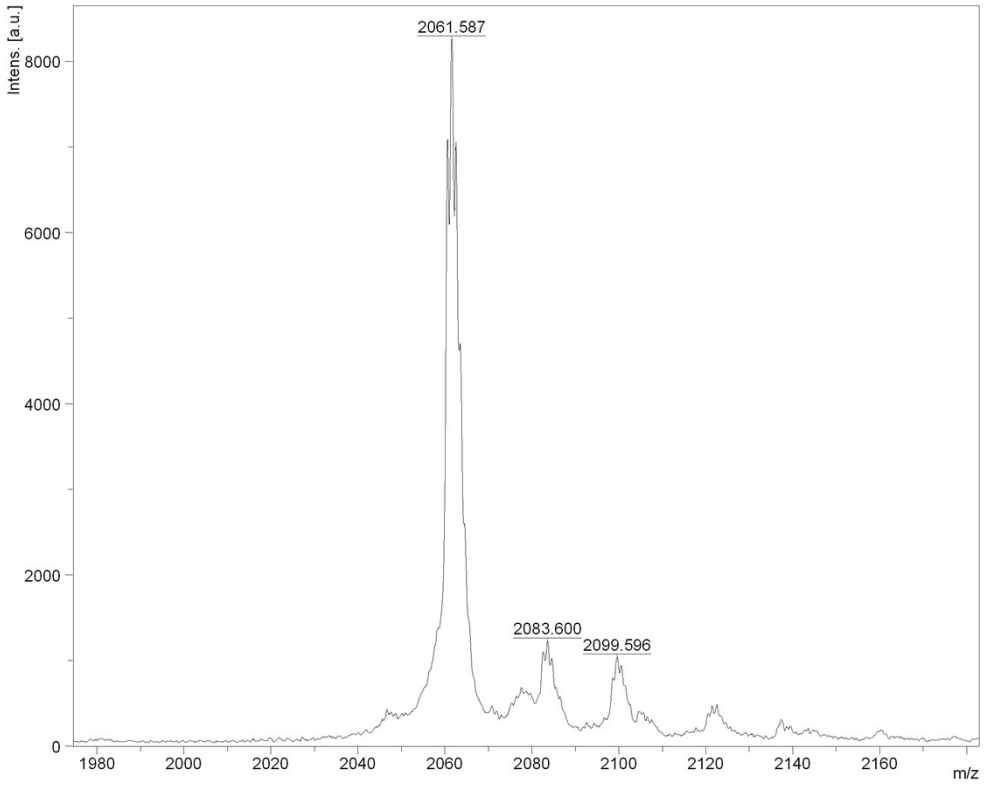
m/z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2080
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2120
2140
2160
m/z

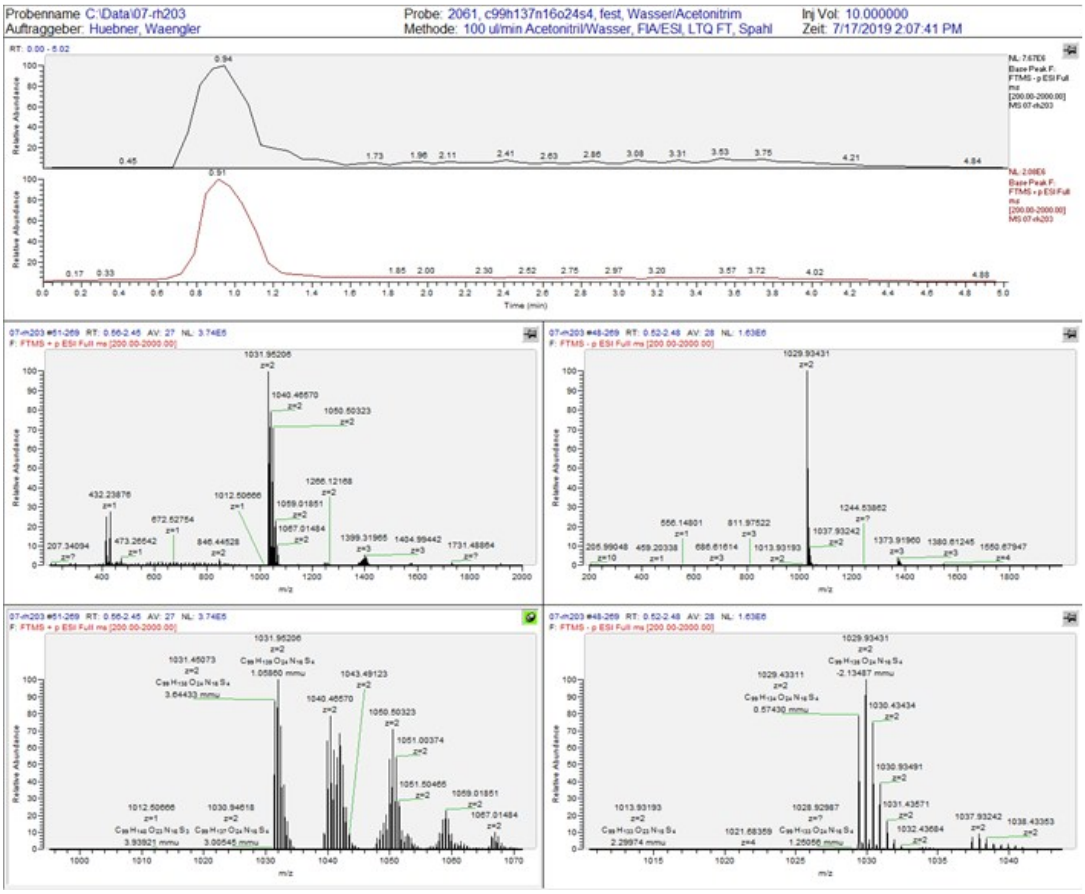
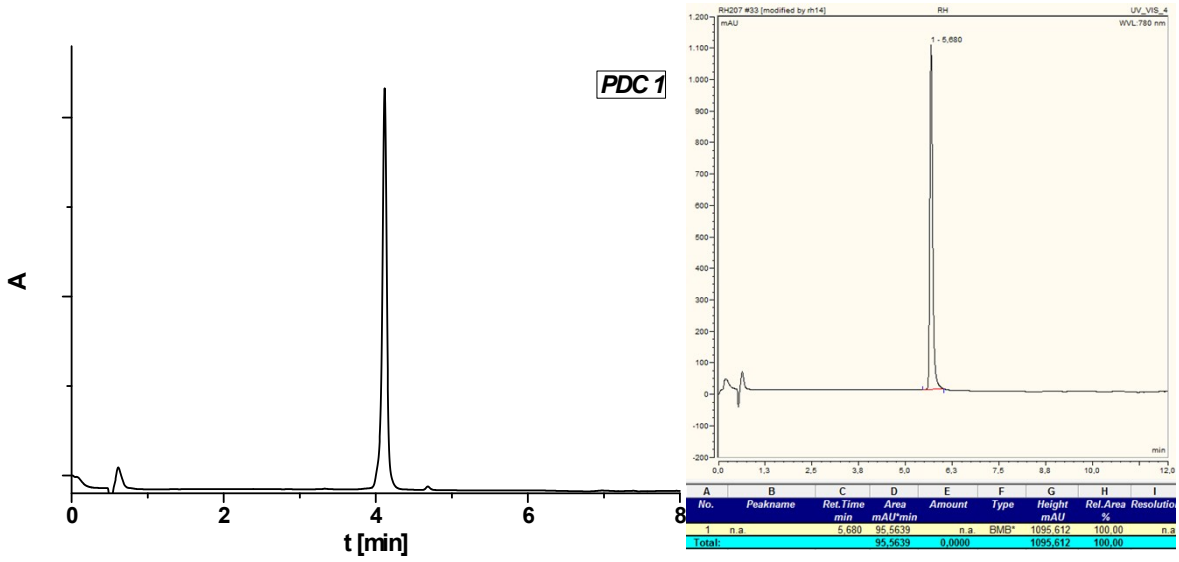
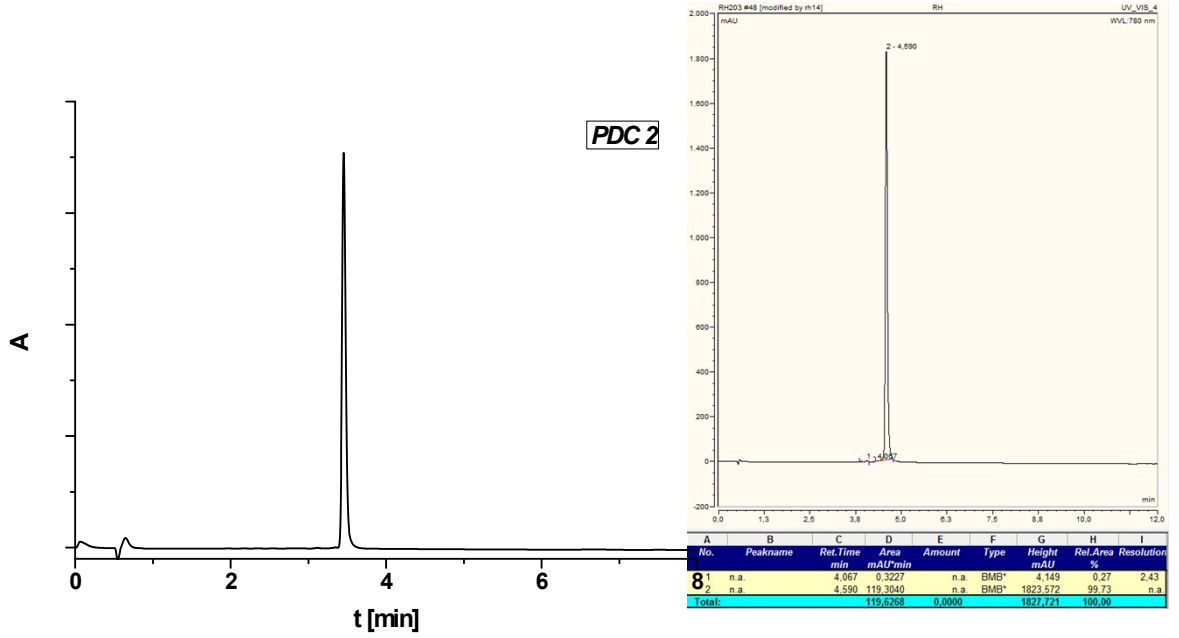


Fig. S3: Analytical data for **PDC 2** (ESI and MALDI mass spectroscopy).



No.	Peakname	Ret.Time min	Area mAU*min	Amount	Type	Height mAU	Rel.Area %	Resolution
1	n.a.	5,680	95,5639	n.a.	BMB*	1095,612	100,00	n.a.
Total:			95,5639	0,0000		1095,612	100,00	

Fig. S4: Analytical HPLC chromatogram for **PDC 1** (8 min, 0 → 100%); R_t = 4.11 min; (12 min, 0 → 100%); R_t = 5.68 min).



No.	Peakname	Ret.Time min	Area mAU*min	Amount	Type	Height mAU	Rel.Area %	Resolution
1	n.a.	4,067	0,3227	n.a.	BMB*	4,149	0,27	2,43
2	n.a.	4,590	119,3040	n.a.	BMB*	1823,572	99,73	n.a.
Total:			119,6268	0,0000		1827,721	100,00	

Fig. S5: Analytical HPLC chromatogram for **PDC 2** (8 min, 0 → 100%); R_t = 3.46 min); (12 min, 0 → 100%); R_t = 4.59 min).

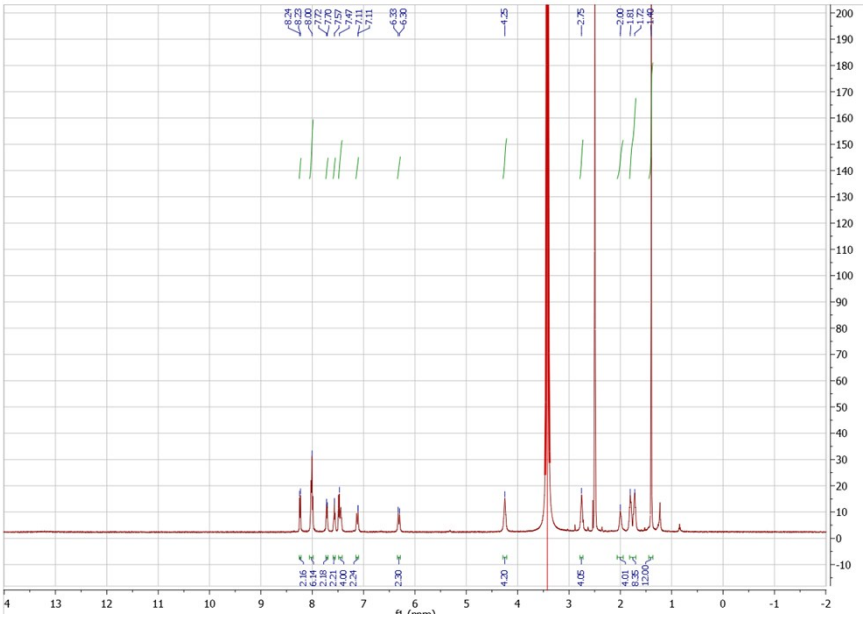


Fig. S6: ^{1}H-NMR-Data of LS277: (^{1}**H NMR** (500 MHz, DMSO-d$_6$): δ = 1.40 (s, 12H), 1.76 (m, 8H), 2.00 (m, 4H), 2.76 (m, 4H), 4.25 (m, 4H), 6.31 (d, ^{3}J=14.3 Hz, 2H), 7.13 (d, ^{3}J=13.2 Hz, 2H), 7.46 (m, 4H), 7.57 (m, 2H), 7.72 (d, ^{3}J=8.5 Hz, 2H), 8.01 (m, 6H), 8.24 (d, ^{3}J=8.2 Hz, 2H))

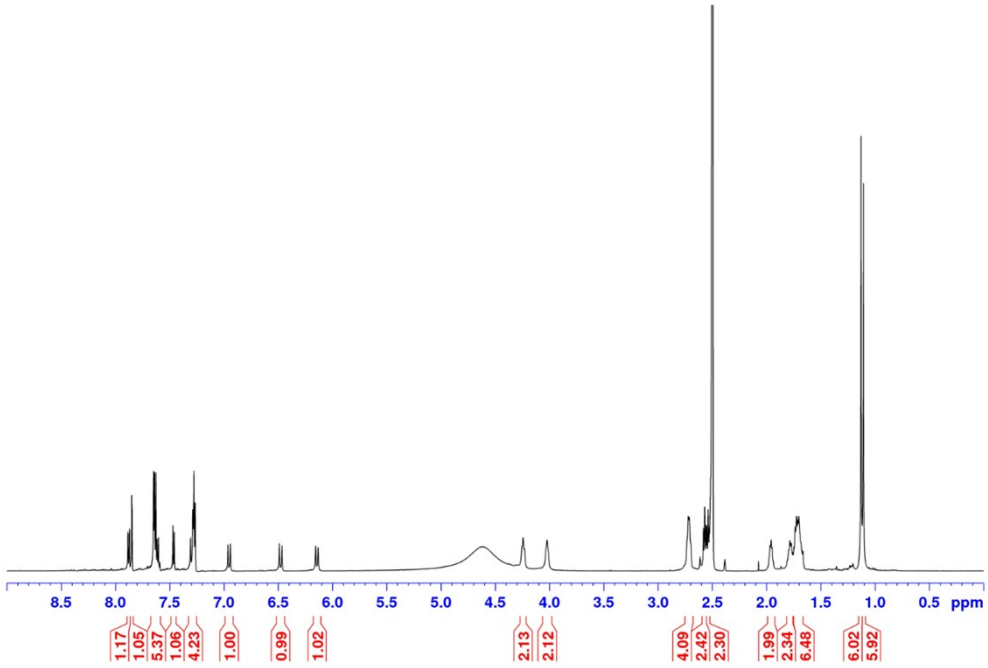


Fig. S7: ^{1}H-NMR-Data of CK002: (^{1}**H NMR** (600 MHz, DMSO-d$_6$): δ = 1.11 (s, 6H,), 1.12 (s, 6H,), 1.66 - 1.81 (m, 8H,), 1.92 - 1.99 (m, 2H,), 2.53 - 2.59 (m, 4H,), 2.68 - 2.75 (m, 2H,), 4.00 - 4.04 (m, 2H,), 4.22 - 4.47 (m, 2H,), 6.15 (d, ^{3}J =13.6 Hz, 1H,), 6.47 (d, ^{3}J =14.7 Hz, 1H,), 6.93 (d, ^{3}J = 13.6 Hz, 1H,), 7.25 - 7.31 (m, 4H,), 7.46 (d, $^{3}J_{H\text{-}H}$ = 8.2 Hz, 1H,), 7.58 - 7.68 (m, 5H,), 7.84 (d, ^{3}J = 1.4 Hz, 1H,), 7.87 (dd, ^{3}J = 8.3 Hz, ^{3}J = 1.5 Hz, 2H,))

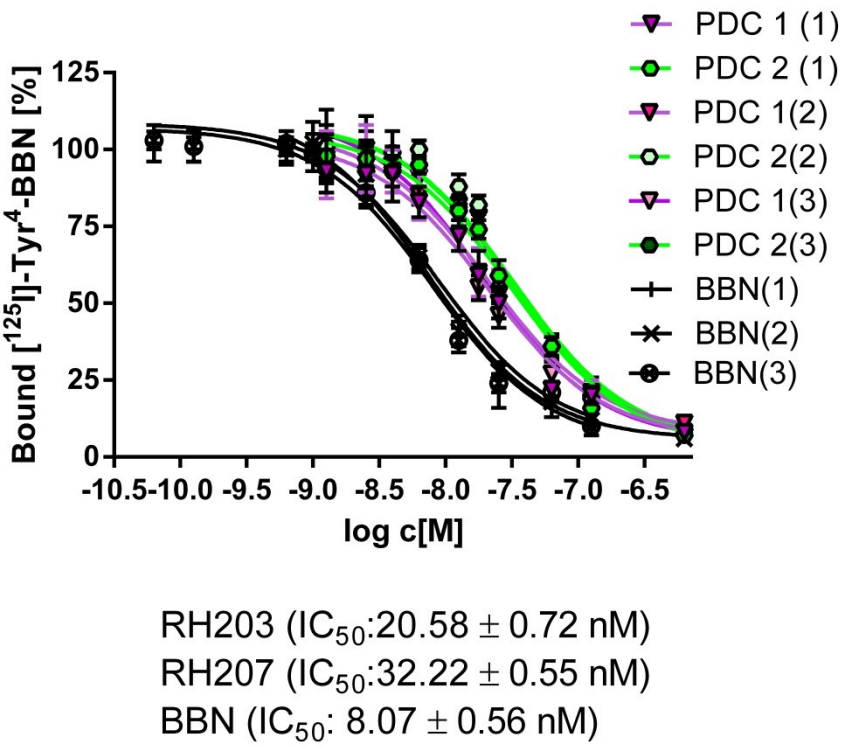


Fig. S8: Summarized results of competitive bindings experiments of BBN, **PDC 1** and **PDC 2** determined on PC-3 cells.

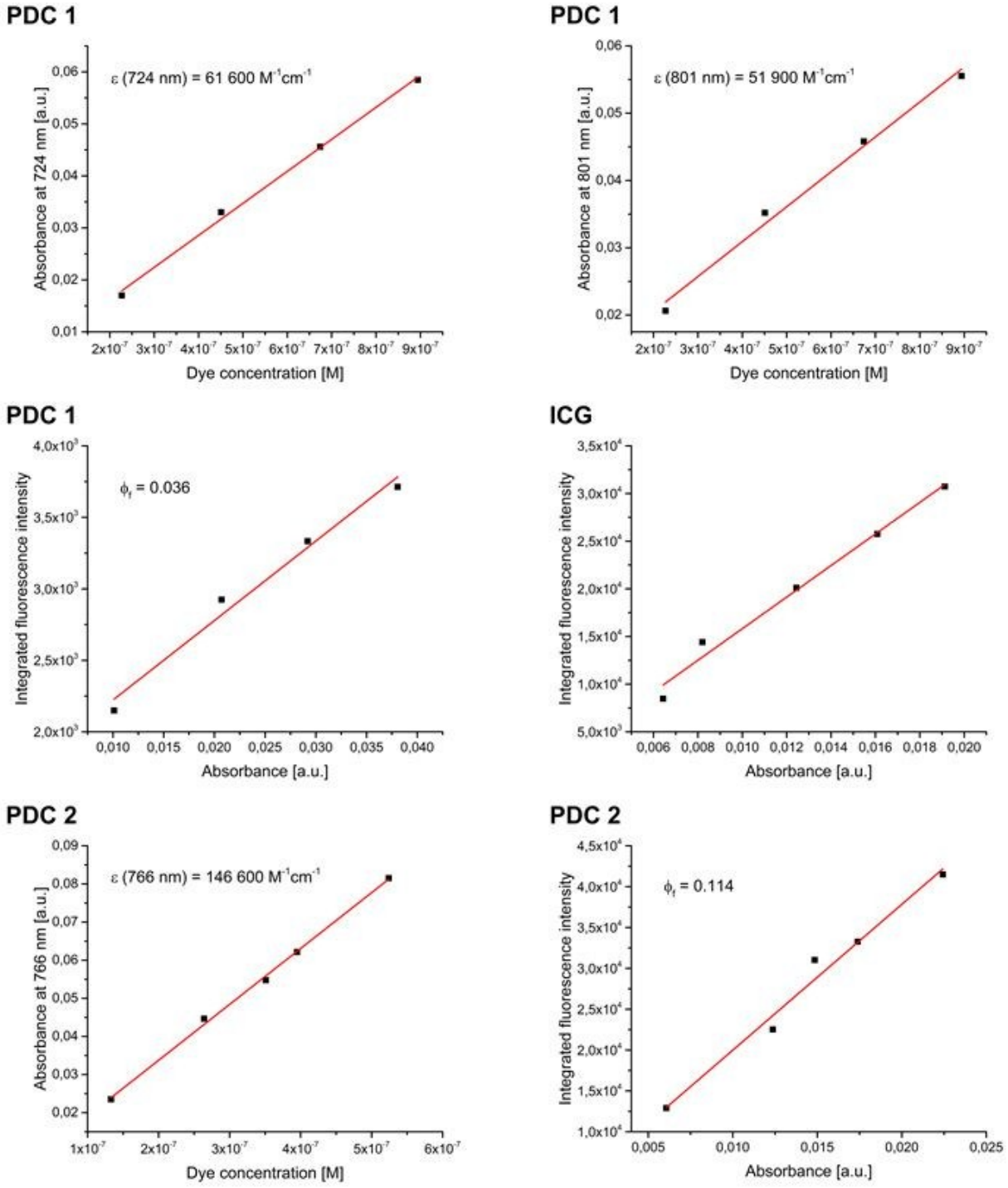


Fig. S9: Results of photophysical measurements of **PDC 1** and **PDC 2** (absorption coefficients and quantum yield). All measurements were conducted in PBS (pH=7.4). Quantum yields are referenced to ICG (ϕ_f = 0.13 in DMSO) and were determined using an excitation wavelength of λ_{ex}= 705 nm (here RH205 = PDC 1 and RH203 PDC 2).