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Design and Synthesis of Highly Active Peroxisome Proliferator-Activated Receptor (PPAR) β/δ Inverse Agonists with Prolonged Cellular Activity

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Based on 3-(((4-(hexylamino)-2-methoxyphenyl)amino)sulfonyl)-2-thiophenecarboxylic acid methyl ester (ST247, compound **2**), a recently described peroxisome proliferator-activated receptor (PPAR)β/δ-selective inverse agonist, we designed and synthesized a series of structurally related ligands. The structural modifications presented herein ultimately resulted in a series of ligands that display increased cellular activity relative to **2**. Moreover, with methyl 3-(*N*-(2-(2-ethoxyethoxy)-4-(hexylamino)phenyl)sulfamoyl)thiophene-2-carboxylate (PT-S264, compound **9u**), biologically relevant plasma concentrations in mice were achieved. The compounds presented in this study will provide useful novel tools for future investigations addressing the role of PPARβ/δ in physiological and pathophysiological processes.

Introduction

The three peroxisome proliferator-activated receptor (PPAR) subtypes α (NR1C1), β/δ (NR1C2), and γ (NR1C3) are members of the nuclear receptor family, a group of transcription factors regulated by endogenous fatty acids or synthetic ligands.[1] Depending on the nature of the interacting ligand, they modulate the transcription of their target genes by recruiting either co-activators (agonists) or co-repressors (inverse agonists), thus inducing or repressing transcription.

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Although several PPAR ligands are currently in clinical use (e.g., lipid-lowering fibrates for PPARα and antidiabetic glitazones for PPARγ), no drug has been approved for PPARβ/δ. PPARβ/δ is expressed in many cell types and hence involved in diverse biological processes, such as lipid and glucose metabolism,[2] cell differentiation,[3] proliferation and apoptosis,[4,5] immune regulation,[6] and tumorigenesis,[7,8] thus representing an attractive pharmacological target. While its role in the lipid and glucose metabolism is well documented, and an agonist had entered clinical trials,[9] its role in cell differentiation, proliferation, and tumorigenesis is not yet fully understood.[5,7,8,10] We recently demonstrated that inverse PPARβ/δ agonists repress the ANGPTL4-mediated invasion of human breast cancer cells into a three-dimensional matrix in vitro, indicating that selectively repressing PPARβ/δ target genes could be advantageous under certain pathophysiological conditions.[11]

The development of subtype-selective and bioavailable ligands with a distinct profile (agonist, inverse agonist, antagonist) is a prerequisite to elucidate the complex system of PPARβ/δ-mediated transcriptional processes, especially in tumorigenesis in vivo. This may not only result in a better understanding of the underlying processes, but also clarify whether PPARβ/δ is a valid target for the development of new chemotherapeutics. However, as research has so far predominantly focused on the activation of PPARβ/δ rather than on its inhibition, the detailed exploration of PPARβ/δ functions is mainly hampered by the lack of subtype-selective, bioavailable and highly potent inverse agonistic or antagonistic ligands, whereas agonistic ligands are abundant.[1,12]

GSK0660 (**1**), disclosed in 2008, was the first PPARβ/δ-selective inverse agonist described, but suffered from rapid clearance in rodents (Figure 1).[13] Development of stilbene deriva-

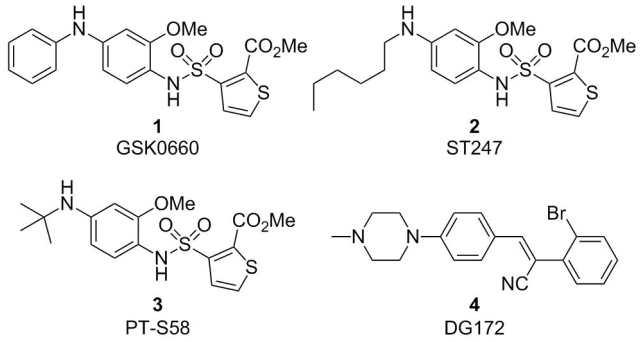


Figure 1. Structures of GSK0660 (**1**),[13] ST247 (**2**),[16,17] PT-S58 (**3**),[16,17] and DG172 (**4**).[14]

tive DG172 (**4**),[14] resulted in an orally available PPARβ/δ-selective inverse agonist, but turned out to exhibit off-target effects in myeloid cells.[15] Based on the structure of **1**, we previously reported a structure–activity relationship (SAR) study which led to the identification of ST247 (**2**), being one of the most active inverse PPARβ/δ agonists known to date. In the same study, we also described PT-S58 (**3**), representing the first pure PPARβ/δ antagonist.[11,16,17]

However, it turned out that **2** was seemingly unsuitable for long-term cellular experiments aimed to elucidate the effects of PPARβ/δ inhibition, especially in cell differentiation. Moreover, with **2** and related analogues, no relevant plasma concentrations could be reached after oral and even intravenous application. We therefore revisited our initial SAR, which had led to the identification of **2** focusing on the further improvement of this ligand class.

Results and Discussion

The large ligand binding pocket of PPARβ/δ has a volume of ~1300 Å^3, is Y-shaped and, except for a small polar section, mainly lipophilic. Flexible ligands such as the fatty acid eicosapentaenoic acid (EPA) adopt different conformations within the binding pocket, varying only in the occupation of the hydrophobic sub-pockets (Figure 2).[18]

Based on these findings we envisioned that by introducing an additional substituent into **2** we would not only be able to better mimic the Y shape of the binding cleft, but also increase hydrophobic interactions within the binding pocket, which should be advantageous for the binding affinity of the ligands. As revealed by our initial SAR studies, a significant decrease in binding affinity to the PPARβ/δ ligand binding domain (LBD) is observed if the methoxy substituent of **1** is moved from its original position adjacent to the sulfonamide or if it is completely missing. Interestingly though, replacement of the methoxy moiety by a hydrogen substituent in **2** resulted in a compound being less potent than **2** but still significantly more potent than the initial lead **1**. Further screening of various substitution patterns at the central phenyl moiety of **2** (data not shown) finally confirmed that the 2-position seems to be optimal for further structural variations (Figure 2).

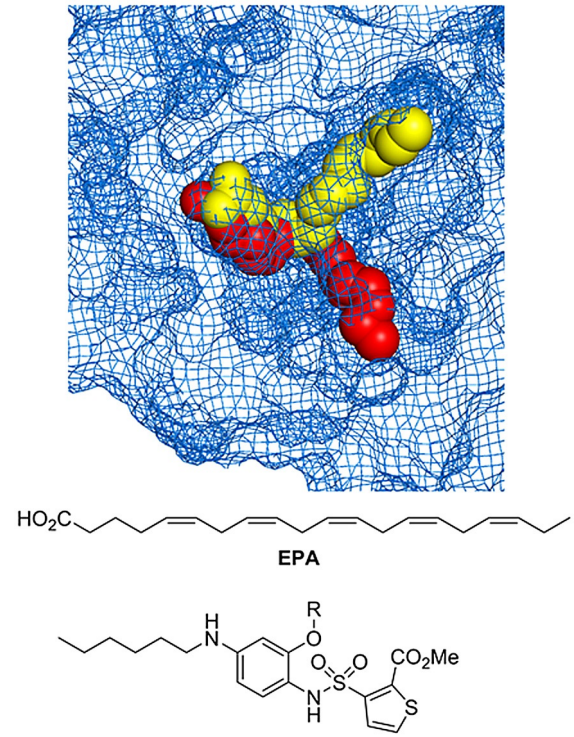
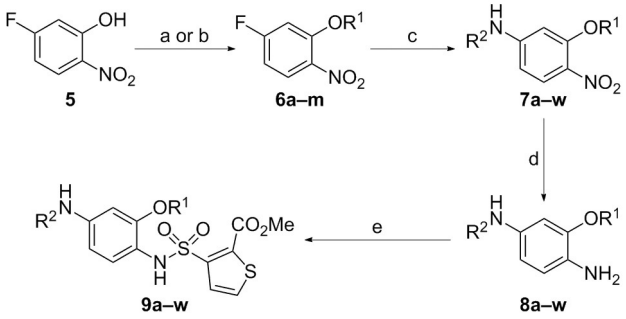


Figure 2. PPARβ/δ binding site (blue mesh) with EPA bound in two different conformations (yellow and red spheres), PDB ID: 3GWX;[18] image created using PyMOL 0.99.[19]

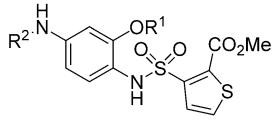
Chemistry

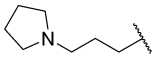
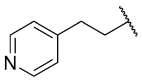
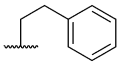
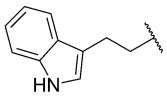
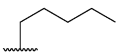
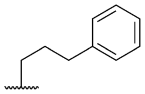
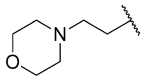
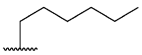
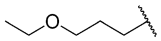
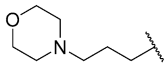
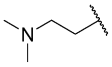
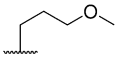
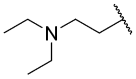
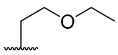
All subsequent compounds were synthesized using a four-step procedure starting from commercially available 2-nitro-5-fluorophenol (**5**), which was alkylated with various alkyl halides (Scheme 1). These reactions proceeded smoothly, giving rise to the corresponding aryl alkyl ethers, in most cases without the need for further purification by column chromatography, with yields ranging from 40 to 95%. For cases in which an alkyl chloride or bromide was used, a catalytic amount of sodium iodide was added to facilitate the reaction. The following synthetic steps of this sequence were carried out as described



Scheme 1. Synthesis of PPARβ/δ inverse agonists. *Reagents and conditions*: a) R^1I, K_2CO_3, acetone, 70 °C; b) R^1Cl/Br, NaI, acetone, 70 °C; c) R^2NH_2, MeCN, 120 °C, microwave irradiation; d) Pd/C, H_2, EtOAc, RT; e) methyl 3-(chlorosulfonyl)thiophene-2-carboxylate, DMAP, pyridine, RT.

Table 1. PPARβ/δ inverse agonists of the different series.



Compd	R^1	R^2	Compd	R^1	R^2	Compd	R^1	R^2
9a		*n*Hex	**9h**		*n*Hex	**9o**	Me	
9b		*n*Hex	**9i**		*n*Hex	**9p**	Me	
9c		*n*Hex	**9j**		*n*Hex	**9q**	Me	
9d		*n*Hex	**9k**		*n*Hex	**9r**	Me	
9e		*n*Hex	**9l**	Me		**9s**	Me	
9f		*n*Hex	**9m**	Me		**9t**		*n*Hex
9g		*n*Hex	**9n**	Me		**9u**		*n*Hex

previously,[16] rendering the sulfonamides **9a**–**w** in overall yields ranging from 8 to 53% over four steps (Scheme 1 and Table 1).

Variation of the methoxy and amino substituents

As the initial SAR had revealed that the effect of the different substituents at the 4′-amino position (R^2) and the central 1,4-diaminobenzene moiety (R^1) on the ligand's affinity toward the PPARβ/δ LBD is merely additive, we focused first on evaluating the optimal size and shape of the attached side chains R^1 and R^2 by introducing linear and branched alkyl chains as well as alkyl-chain-linked aryl moieties and several amines (Scheme 1 and Table 1). The synthesized compounds were tested in a qualitative manner for their inverse-agonistic effect by measuring the recruitment of co-repressor peptides in a time-resolved fluorescence resonance energy transfer (TR-FRET) assay in vitro in comparison with **1** and **2** (Tables S1–S3, Supporting Information).

In an initial series, we focused on evaluating the optimal size and shape of the attached side chain R^1 by introducing linear and branched alkyl chains as well as alkyl-chain-linked aryl moieties (**9a**–**9k**). Interestingly, the introduction of linear alkyl chains increases the recruitment of co-repressors significantly for some of the compounds, with **9d** (Table 1 and Table S1) having the strongest effect, albeit the underlying SAR is not linear. However, as **9d**, being equipped with an additional hydrophobic moiety, was even more lipophilic than **2**, we decided to focus next on optimizing the solubility of the compounds with respect to assay conditions. Besides the implementation of non-ionizable functionalities such as polyhydroxy or polyethylene glycol moieties, the introduction of polar, protonatable functionalities is a valid and frequently applied methodology.[20]

Consequently, we introduced an ethoxypropyl moiety as well as a variety of more polar alkyl amine residues at R^2 (**9l**–**9s**; Table 1 and Table S2). Most modifications of the *n*-hexyl moiety resulted in a significant decrease in the ability to recruit co-repressor peptides, as revealed in the TR-FRET assay.

Only the morpholinopropyl-substituted derivative **9s** clearly exceeded the inverse-agonistic effect observed for **2** (Table 1 and Table S2). Thus, in a third series the *n*-pentyl substituent at position R^1 (**9d**) was replaced by the equally sized but significantly more polar methoxypropoxy and 2-ethoxyethoxy residues, respectively (**9t**, **9u**; Table 1 and Table S3). Because the ethoxyethoxy-substituted derivative **9u** (PT-S264) was significantly more potent than **2** in the TR-FRET assay, albeit less active than **9d**, we considered further testing of **9u** (PT-S264) to be worthwhile (Figure 3 and Table S3).

In the final series, we combined the substitution patterns of the most active derivatives of both series and synthesized the corresponding ligands **9v** and **9w** bearing a morpholinopropylamino substituent as well as a pentyl or ethyloxyethyl substituent (Table 2). To elucidate their potency further, **9d** and **9u**–**w** as well as **2** as reference were tested with respect to their ability to repress the PPARβ/δ target genes *Angptl4*, *PDK4*, and *Cpt1a* in cell-based assays. As illustrated in Figure 3, the newly designed ligands showed a 2.5- to 5-fold enhanced re-

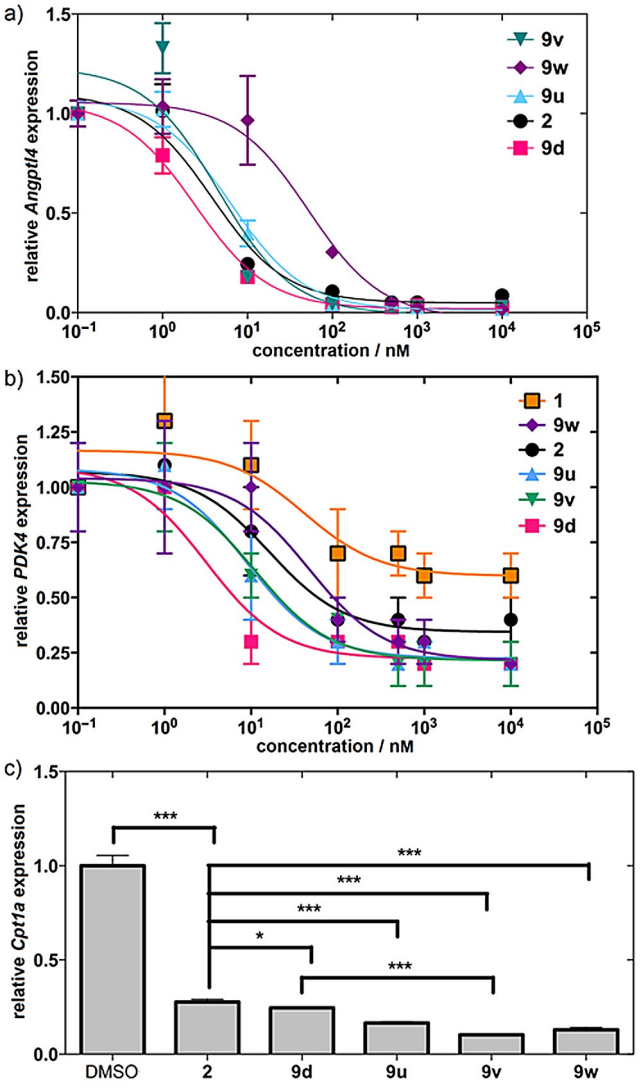


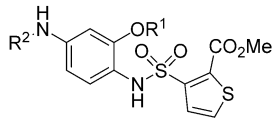
Figure 3. Concentration-dependent repression of the a) *Angptl4* gene in C2C12 cells and the b) *PDK4* gene in MDA-MB-231 cells by derivatives of parent compound **2**. Cells were treated with the indicated concentrations for 24 h and analyzed by RT-qPCR. c) Repression of the *Cpt1a* gene in C2C12 cells by derivatives of the parent compound **2**. Cells were treated with the indicated compounds at a concentration of 1 μM for 24 h and analyzed by RT-qPCR. DMSO: solvent control. Values are the average ± SD of triplicates; significant difference from DMSO-treated sample: ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ (*t*-test).

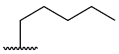
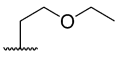
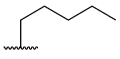
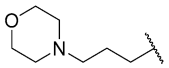
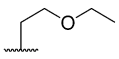
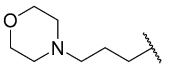
pression of the respective genes relative to the hitherto most active compound **2**.

Besides IC_{50} and subtype specificity (Figure S1) determination for the two derivatives **9d** and **9u**, their impact on *PDK4* expression in primary human monocyte-derived macrophages was evaluated over a time period of six days (Figure 4). The experimental data clearly reveal the superior effect of both compounds relative to **2**. There is no discernible loss of transcriptional repression within the observation period in cells treated with **9d** or **9u** (PT-S264), while *PDK4* transcription rises steadily in **2**-treated cells after its initial repression.

Finally, **9u** was administered i.v. to mice, and plasma concentrations were determined at various time points (Figure 5).

Table 2. Inhibitory activity of selected PPARβ/δ inverse agonists.



Compd	R^1	R^2	IC_{50} [nM][a] *Angptl4*	*PDK4*
2	Me	*n*Hex	3.84	16.17
9d		*n*Hex	2.42	3.03
9u		*n*Hex	6.43	9.08
9v			4.56	11.12
9w			52.22	46.96

[a] Relative expression.

In contrast to **2** and **1**, for **9u**, biologically relevant plasma concentrations could be determined 120 min post-application. Thus **9u** represents the first member of this ligand class that might prove useful for further in vivo experiments.

Conclusions

Systematic structural optimization of the PPARβ/δ-inverse agonist **2** resulted in a series of ligands displaying significantly increased potency relative to **2**. Compounds **9d** and **9u** (PT-S264) exhibited prolonged activity in cellular assays. Moreover, one of the ligands (**9u**) gave rise to biologically relevant plasma concentrations in mice. Compound **9u** (PT-S264) will therefore not only be a useful tool to address the role of PPARβ/δ in physiological and pathophysiological processes in vitro but also in vivo. The extended half-life of the compound in cell-based assays will enable studies which require several days of cultivation, such as differentiation processes.

Experimental Section

Chemistry

Materials and methods. Unless stated otherwise, all commercially available starting materials and solvents were purchased and used without further purification. Thin-layer chromatography (TLC) was performed on pre-coated TLC plates (silica gel 60 F_{254} Merck). Flash column chromatography was performed on prepacked flash chromatography columns (PF 30-SIHP-JP/12G) purchased from Interchim using a Büchi separation system. Cyclohexane p.a. (from Grüssing), EtOAc p.a. (Sigma–Aldrich or Acros), were distilled prior to use. HPLC-quality acetonitrile was obtained from VWR. Pyridine was kept over KOH for 48 h, distilled, and stored over molecular sieves (4 Å) under an argon atmosphere. ^1H and ^{13}C NMR spectra were recorded on Jeol ECX-400 or Jeol ECA-500 spectrometers. Chemical shifts (δ) are given in ppm with the residual solvent signal used as reference ($CDCl_3$: s, 7.26 ppm (^1H) and t, 77.1 ppm (^{13}C); [D_6]DMSO: quint, 2.50 ppm (^1H) and quint, 40.1 ppm (^{13}C)). Spectra with $CDCl_3$ as solvent were recorded at ambient tempera-

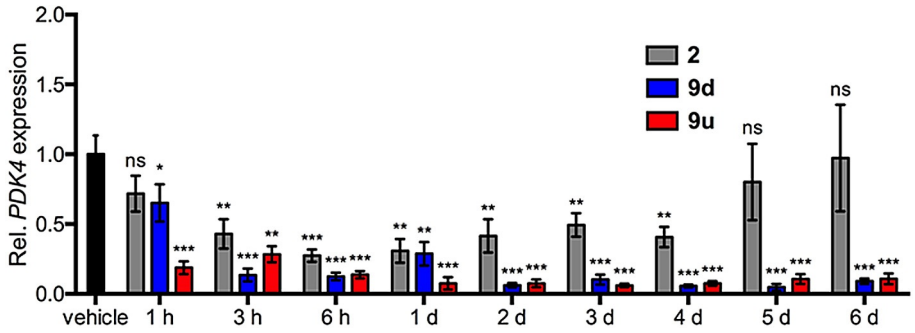


Figure 4. Kinetics of *PDK4* repression by inverse agonists in primary human monocyte-derived macrophages. After six days of differentiation, cells were treated with the compounds (300 nM) and harvested at the indicated time points. RT-qPCR was performed. Expression values were calculated relative to solvent control (DMSO-treated cells). Error bars represent standard deviations derived from technical triplicates. A representative experiment is shown. Significant difference from DMSO-treated sample: ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ (*t*-test).

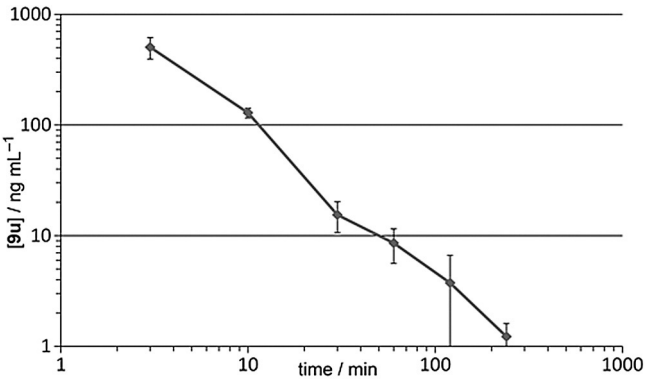


Figure 5. Plasma concentration of **9u** after administration (1 mg kg^{-1} i.v.) in mice. Values are the average ± SD of $n = 3$ biological replicates.

ture while spectra with [D_6]DMSO as solvent were recorded at 30.0 °C. Coupling constants (*J*) are reported in Hz. Mass spectra were recorded on a double-focusing sector field spectrometer type 70/70 H (Vacuum Generators), on a double-focusing sector field spectrometer type AutoSpec (Micromass), on a triple quadrupole spectrometer type EP 10+ (MS Vision) or on a triple quadrupole spectrometer type Q-Trap 2000 (Applied Biosystems). Elemental combustion analyses were performed on a Vario MICRO cube (ElementarAnalysensysteme GmbH). Microwave irradiation was applied with a Discover BenchMate Plus (CEM GmbH). Melting points were determined using a melting point meter KSP1N (A. KrüssOptronic GmbH) and are uncorrected. Unless stated otherwise, all tested compounds were at least 95% pure as determined by combustion analysis. All compounds were synthesized following a synthetic procedure as described previously.[16] However, for the synthesis of branched ligands an additional synthetic step for the generation of 2-alkoxy-4-fluoro-1-nitrobenzenes was introduced.

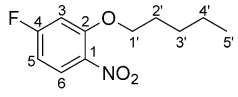
General procedure 1 for the synthesis of 2-alkoxy-4-fluoro-1-nitrobenzenes (6a–m): 5-fluoro-2-nitrophenol (**5**, 1.0 equiv) and the respective alkyl halide (1.5 equiv) were dissolved in dry acetone (10 mL). Potassium carbonate (2.0 equiv) and, if a chloride or bromide was used, potassium iodide (0.5 equiv) were added. The reaction mixture was then heated at reflux until complete conversion of the starting material occurred (TLC). The solvent was subsequently removed under reduced pressure and the resulting residue was suspended in dichloromethane. After the addition of sodium sulfate, the mixture was filtered and the solvent was removed under reduced pressure to give the pure products without further purification.

General procedure 2 for the synthesis of *N*-alkyl-4-nitro-3-alkoxyanilines (7a–w): **Procedure A**: The corresponding 2-alkoxy-4-fluoro-1-nitrobenzene (**6a–m**, 1.0 equiv) and the respective amine (4.0 equiv) were dissolved in acetonitrile (2–4 mL) while stirring. The reaction mixture was heated at 120 °C by microwave irradiation in a sealed 10 mL reaction vessel for 60 min. Subsequent removal of the solvent and purification by flash column chromatography (cyclohexane/EtOAc or dichloromethane/MeOH) yielded the target compounds. **Procedure B**: The corresponding 2-alkoxy-4-fluoro-1-nitrobenzene (**6a–m**, 1.0 equiv), the respective amine (1.0–2.0 equiv) and triethylamine (3.0–5.0 equiv) were dissolved in acetonitrile (2–4 mL) while stirring. The reaction mixture was heated at 120 °C by microwave irradiation in a sealed reaction vessel for 60 min. Subsequent removal of solvent and purification by flash column chromatography (cyclohexane/EtOAc or dichloromethane/MeOH) yielded the target compounds.

General procedure 3 for the synthesis of 1,4-phenylenediamines (8q–w): The corresponding *N*-alkyl-4-nitro-3-alkoxyanilines (**7a–w**, 1.0 equiv) were dissolved in EtOAc (~10 mL mmol^{-1}) and Pd/C (10% *w/w*) was added (26 mg mmol^{-1}). After air removal and flushing with hydrogen the reaction mixture was stirred at RT under light protection for 24 h. Filtration through a pad of Celite and concentration in vacuo gave the light-sensitive amines, which were directly used in the next step without further purification.

General procedure 4 for the synthesis of sulfonamides (9a–w): The corresponding 1,4-phenylenediamines (**8a–w**, 1.0 equiv), DMAP (0.5 equiv) and methyl 3-(chlorosulfonyl)thiophene-2-carboxylate (1.1 equiv) were dissolved in anhydrous pyridine (10 mL mmol^{-1}) while stirring under an argon atmosphere. The reaction mixture was stirred for 48 h protected from light. Subsequent removal of the solvent and purification by flash column chromatography (cyclohexane/EtOAc or dichloromethane/MeOH) yielded the target compounds.

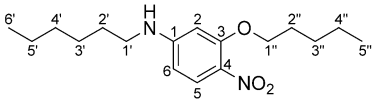
4-Fluoro-1-nitro-2-(pentyloxy)benzene (6d): Compound **6d** was prepared according to general procedure 1 using **5** (323 mg, 2.06 mmol) and 1-iodopentane (593 mg, 3.00 mmol, 1.5 equiv). The title compound was obtained as a yellow liquid (408 mg, 87%): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.93$ (dd, $^3J_{H,H} = 9.0$ Hz, $^4J_{H,F} = 6.1$ Hz, 1H, 6-H), 6.76 (dd, $^3J_{H,F} = 10.5$ Hz, $^4J_{H,H} = 2.5$ Hz, 1H, 3-H), 6.70 (ddd, $^3J_{H,F} =$



9.1 Hz, $^3J_{H,H}$ = 7.4 Hz, $^4J_{H,H}$ = 2.5 Hz, 1H, 5-H), 4.07 (t, $^3J_{H,H}$ = 6.4 Hz, 2H, 1′-H_2), 1.89–1.82 (m, 2H, 2′-H_2), 1.51–1.44 (m, 2H, 3′-H_2), 1.43–1.34 (m, 2H, 4′-H_2), 0.94 ppm (t, $^3J_{H,H}$ = 7.2 Hz, 3H, 5′-H_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 165.8 (d, $^1J_{C,F}$ = 255.3 Hz, 1C, C-4), 154.9 (d, $^3J_{C,F}$ = 11.6 Hz, 1C, C-2), 136.2 (d, $^4J_{C,F}$ = 2.9 Hz, 1C, C-1), 128.1 (d, $^3J_{C,F}$ = 11.6 Hz, 1C, C-6), 107.1 (d, $^2J_{C,F}$ = 23.1 Hz, 1C, C-5), 102.1 (d, $^2J_{C,F}$ = 26.0 Hz, 1C, C-3), 70.2 (1C, C-1′), 28.5 (1C, C-2′), 28.0 (1C, C-3′), 22.4 (1C, C-4′), 14.0 ppm (1C, C-5′); MS (EI): *m/z* (%) = 141 (57) $[M-C_5H_{10}O]^+$, 157 (93) $[M-C_5H_{10}]^+$, 227 (100) $[M]^+$; HRMS (EI) *m/z* $[M]^+$: calcd for $C_{11}H_{14}FNO_3$: 227.095772, found: 227.097703: Anal. calcd for $C_{11}H_{14}FNO_3$: C 58.14%, H 6.21%, N 6.16%; found: C 58.59%, H 6.30%, N 6.34%.

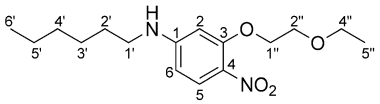
2-(2-Ethoxyethoxy)-4-fluoro-1-nitrobenzene (6m): Compound **6m** was prepared following a slightly altered general procedure 1 with a higher amount of potassium iodide (1.0 equiv instead of 0.5 equiv) using **5** (471 mg, 3.00 mmol) and 1-chloro-2-ethoxyethane (651 mg, 6.00 mmol, 2.0 equiv). The title compound was obtained as a yellow solid (636 mg, 92%): mp: 37.2–39.7 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.94 (dd, $^3J_{H,H}$ = 9.2 Hz, $^4J_{H,F}$ = 6.0 Hz, 1H, 6-H), 6.84 (dd, $^3J_{H,F}$ = 10.3 Hz, $^4J_{H,H}$ = 2.5 Hz, 1H, 3-H), 6.73 (ddd, $^3J_{H,F}$ = 9.2 Hz, $^3J_{H,H}$ = 7.1 Hz, $^4J_{H,H}$ = 2.5 Hz, 1H, 5-H), 4.24 (t, $^3J_{H,H}$ = 4.7 Hz, 2H, 1′-H_2), 3.85 (t, $^3J_{H,H}$ = 4.7 Hz, 2H, 2′-H_2), 3.85 (q, $^3J_{H,H}$ = 7.0 Hz, 2H, 4′-H_2), 1.22 ppm (t, $^3J_{H,H}$ = 7.0 Hz, 3H, 5′-H_3); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 165.8 (d, $^1J_{C,F}$ = 256.7 Hz, 1C, C-4), 154.8 (d, $^3J_{C,F}$ = 12.0 Hz, 1C, C-2), 136.1 (1C, C-1), 128.1 ($^3J_{C,F}$ = 12.0 Hz, 1C, C-6), 107.2 ($^2J_{C,F}$ = 22.8 Hz, 1C, C-5), 102.3 ($^2J_{C,F}$ = 26.4 Hz, 1C, C-3), 68.4 (1C, C-4′), 66.8 (1C, C-1′), 58.8 (1C, C-5′), 29.2 ppm (1C, C-2′); MS (ESI): *m/z* (%) = 171 (10) $[M-C_3H_6O]^+$, 230 (100) $[M+H]^+$, 247 (39) $[M+NH_4]^+$, 252 (21) $[M+Na]^+$; HRMS (EI) *m/z* $[M]^+$ calcd for $C_{10}H_{12}FNO_4$: 229.075036, found: 229.073714; Anal. calcd for $C_{10}H_{12}FNO_4$: C 52.40%, H 5.28%, N 6.11%, found: C 52.66%, H 5.47%, N 6.23%.

***N*-Hexyl-4-nitro-3-(pentyloxy)aniline (7d)**: Compound **7d** was prepared according to general procedure 2A using **6d** (227 mg, 1.00 mmol) and hexan-1-amine (405 mg, 4.00 mmol, 4.0 equiv). The title compound was obtained as a yellow solid (130 mg, 42%):



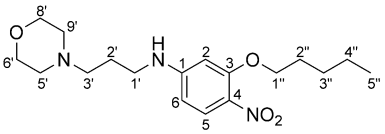
^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.95 (d, $^3J_{H,H}$ = 9.2 Hz, 1H, 5-H), 6.10 (dd, $^3J_{H,H}$ = 9.2 Hz, $^4J_{H,H}$ = 2.3 Hz, 1H, 6-H), 6.02 (d, $^4J_{H,H}$ = 2.3 Hz, 1H, 2-H), 4.36 (brt, $^3J_{H,H}$ = 5.0 Hz, 1H, N*H*), 4.04 (t, $^3J_{H,H}$ = 6.5 Hz, 2H, 1″-H_2), 3.17 (m_c, 2H, 1′-H_2), 1.90–1.83 (m, 2H, 2″-H_2), 1.64 (tt, $^3J_{H,H}$ = 7.3 Hz, $^3J_{H,H}$ = 7.3 Hz, 2H, 2′-H_2), 1.53–1.31 (m, 10H, 3′-H_2, 4′-H_2, 5′-H_2, 3″-H_2, 4″-H_2), 0.93 (t, $^3J_{H,H}$ = 7.2 Hz, 3H, 5″-H_3), 0.90 ppm (brt, $^3J_{H,H}$ = 6.9 Hz, 3H, 6′-H_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 156.6 (1C, C-3), 154.1 (1C, C-1), 129.3 (1C, C-5), 104.0 (1C, C-6), 95.7 (1C, C-2), 69.4 (1C, C-1″), 43.5 (1C, C-1′), 31.6 (1C, C-2′), 29.2 (1C, C-4′), 28.8 (1C, C-2″), 28.2 (1C, C-3″), 26.8 (1C, C-3′), 22.7 (1C, C-5′), 22.5 (1C, C-4″), 14.1 ppm (2C, C-6′, C-5″); MS (ESI): *m/z* (%) = 221 (18) $[M-C_5H_{11}O]^+$, 309 (100) $[M+H]^+$, 331 (26) $[M+Na]^+$, 617 (13) $[2M+H]^+$, 639 (15) $[2M+Na]^+$, 925 (6) $[3M+H]^+$; HRMS (ESI) *m/z* $[M+Na]^+$ calcd for $C_{17}H_{28}N_2O_3Na$: 331.199763, found: 331.199488.

3-(2-Ethoxyethoxy)-*N*-hexyl-4-nitroaniline (7u): Compound **7u** was prepared according to general procedure 2B using **6m** (229 mg, 1.00 mmol) and hexan-1-amine (202 mg, 2.00 mmol,



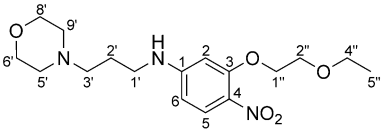
2.0 equiv) with triethylamine (202 mg, 2.00 mmol, 2.0 equiv) as additional base. The title compound was obtained as a yellow solid (297 mg, 96%): mp: 63.8–65.5 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.95 (d, $^3J_{H,H}$ = 8.9 Hz, 1H, 5-H), 6.13 (dd, $^3J_{H,H}$ = 9.2 Hz, $^4J_{H,H}$ = 2.1 Hz, 1H, 6-H), 6.10 (d, $^4J_{H,H}$ = 2.1 Hz, 1H, 2-H), 4.39 (brs, 1H, N*H*), 4.20 (t, $^3J_{H,H}$ = 5.0 Hz, 2H, 1″-H_2), 3.87 (t, $^3J_{H,H}$ = 5.0 Hz, 2H, 2″-H_2), 3.65 (q, $^3J_{H,H}$ = 7.0 Hz, 2H, 4″-H_2), 3.16 (t, $^3J_{H,H}$ = 7.2 Hz, 2H, 1′-H_2), 1.64 (tt, $^3J_{H,H}$ = 7.3 Hz, $^3J_{H,H}$ = 7.3 Hz, 2H, 2′-H_2), 1.44–1.29 (m, 6H, 3′-H_2, 4′-H_2, 5′-H_2), 1.23 (t, $^3J_{H,H}$ = 7.0 Hz, 3H, 5″-H_3), 0.90 ppm (brt, $^3J_{H,H}$ = 6.8 Hz, 3H, 6′-H_3); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 156.4 (1C, C-3), 154.1 (1C, C-1), 129.2 (1C, C-5), 129.1 (1C, C-4), 104.6 (1C, C-6), 96.2 (1C, C-2), 69.4 (1C, C-2″), 68.6 (1C, C-1″), 67.2 (1C, C-4″), 43.5 (1C, C-1′), 31.5 (1C, C-2′), 29.1 (1C, C-4′), 26.7 (1C, C-3′), 22.6 (1C, C-5′), 15.3 (1C, C-5″), 14.1 ppm (1C, C-6′); MS (ESI): *m/z* (%) = 193 (15), 265 (100) $[M-C_2H_5O]^+$, 311 (93) $[M+H]^+$, 333 (61) $[M+Na]^+$; HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{16}H_{27}N_2O_4$: 311.197083, found: 311.194174; Anal. calcd for $C_{16}H_{26}N_2O_4$: C 61.91%, H 8.44%, N 9.03%, found: C 61.68%, H 8.37%, N 8.93%.

***N*-(3-Morpholinopropyl)-4-nitro-3-(pentyloxy)aniline (7v)**: Compound **7v** was prepared according to general procedure 2B using **6d** (238 mg, 1.05 mmol) and 3-morpholinopropan-1-amine (303 mg, 2.10 mmol, 2.0 equiv) with triethylamine (425 mg,



4.20 mmol, 4.0 equiv) as additional base. The title compound was obtained as a yellow liquid (338 mg, 92%): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.96 (d, $^3J_{H,H}$ = 9.2 Hz, 1H, 5-H), 6.10 (dd, $^3J_{H,H}$ = 9.0 Hz, $^3J_{H,H}$ = 2.4 Hz, 1H, 6-H), 6.00 (d, $^2J_{H,H}$ = 2.3 Hz, 1H, 2-H), 4.04 (t, $^3J_{H,H}$ = 6.5 Hz, 2H, 1″-H_2), 3.77 (t, $^3J_{H,H}$ = 4.6 Hz, 4H, 6′-H_2, 8′-H_2), 3.30 (q, $^3J_{H,H}$ = 5.8 Hz, 2H, 1′-H_2), 2.57 (t, $^3J_{H,H}$ = 6.2 Hz, 2H, 3′-H_2), 2.54 (brs, 4H, 5′-H_2, 9′-H_2), 1.90–1.82 (m, 4H, 2′-H_2, 2″-H_2), 1.54–1.46 (m, 2H, 3″-H_2), 1.39 (tq, $^3J_{H,H}$ = 7.2 Hz, $^3J_{H,H}$ = 7.2 Hz, 2H, 4″-H_2), 0.94 ppm (t, $^3J_{H,H}$ = 7.2 Hz, 3H, 5″-H3); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 156.7 (1C, C-3), 154.4 (1C, C-1), 129.3 (1C, C-5), 128.8 (1C, C-4), 104.1 (1C, C-6), 95.3 (1C, C-2), 69.4 (1C, C-1″), 67.0 (2C, C-6′, C-8′), 57.6 (1C, C-3′), 53.7 (2C, C-5′, C-9′), 43.1 (1C, C-1′), 28.8 (1C, C-2″), 28.1 (1C, C-3″), 24.4 (1C, C-2′), 22.4 (1C, C4″), 14.1 ppm (1C, C-5″); MS (ESI): *m/z* (%) = 352 (100) $[M+H]^+$, 374 (4) $[M+Na]^+$; HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{18}H_{30}N_3O_4$: 352.2236, found: 352.2231; Anal. calcd for $C_{18}H_{29}N_3O_4$: C 61.52%, H 8.32%, N 11.96%, found: C 61.38%, H 8.32%, N 11.77%.

3-(2-Ethoxyethoxy)-*N*-(3-morpholinopropyl)-4-nitroaniline (7w): Compound **7w** was prepared according to general procedure 2B using **6m** (229 mg, 1.00 mmol) and 3-morpholinopropan-1-amine (288 mg, 2.00 mmol, 2.0 equiv) with triethylamine (405 mg,



4.00 mmol, 4.0 equiv) as additional base. The title compound was obtained as a yellow liquid (343 mg, 97%): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.94 (d, $^3J_{H,H}$ = 9.2 Hz, 1H, 5-H), 6.11 (dd, $^3J_{H,H}$ = 9.0 Hz, $^3J_{H,H}$ = 2.4 Hz, 1H, 6-H), 6.05 (d, $^2J_{H,H}$ = 2.3 Hz, 1H, 2-H), 4.19 (t, $^3J_{H,H}$ = 5.0 Hz, 2H, 1″-H_2), 3.86 (t, $^3J_{H,H}$ = 5.0 Hz, 2H, 2″-H_2), 3.76 (t, $^3J_{H,H}$ = 4.6 Hz, 4H, 6′-H_2, 8′-H_2), 3.65 (q, $^3J_{H,H}$ = 7.0 Hz, 2H, 4″-H_2), 3.27 (q, $^3J_{H,H}$ = 5.8 Hz, 2H, 1″-H_2), 2.56 (t, $^3J_{H,H}$ = 6.3 Hz, 2H, 3′-H_2), 2.53 (br s, 4H, 5′-H_2, 9′-H_2), 1.84 (tt, $^3J_{H,H}$ = 6.2 Hz, $^3J_{H,H}$ = 6.2 Hz, 2H, 2′-H_2), 1.22 ppm (t, $^3J_{H,H}$ = 7.0 Hz, 3H, 5″-H_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 156.5 (1C, C-3), 154.4 (1C, C-1), 129.2 (1C, C-5), 128.9 (1C, C-4), 104.7 (1C, C-6), 95.9 (1C, C-2), 69.4 (1C, C-2″), 68.6 (1C, C-1″), 67.2 (1C, C-4″), 67.0 (2C, C-6′, C-8′), 57.6 (1C, C-3′), 53.7 (2C, C-5′, C-9′), 43.1 (1C, C-1′), 24.3 (1C, C-2′), 15.3 ppm (1C, C-5″); MS (ESI): *m/z* (%) = 354 (100) $[M+H]^+$, 376 (24) $[M+Na]^+$; HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{17}H_{28}N_3O_5$: 354.2023, found: 354.2029; Anal. calcd for $C_{17}H_{27}N_3O_5 \cdot 0.25\,H_2O$: C 57.05%, H 7.74%, N 11.74%, found: C 57.21%, H 7.62%, N 11.54%.

N^1-Hexyl-3-(pentyloxy)benzene-1,4-diamine (8d): **8d** was prepared following a slightly altered general procedure 3 using **7d** (105 mg, 0.340 mmol) and PtO_2 (10 mg) as catalyst and a reaction temperature of 40 °C. The title compound was obtained as a purple solid which was directly used in the next step without further purification.

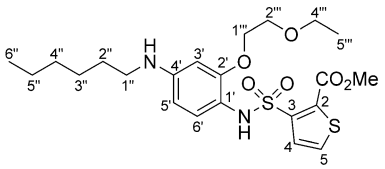
3-(2-Ethoxyethoxy)-N^1-hexylbenzene-1,4-diamine (8u): **8u** was prepared according to general procedure 3 using **7u** (155 mg, 0.500 mmol). The title compound was obtained as a purple solid which was directly used in the next step without further purification.

N^1-(3-Morpholinopropyl)-3-(pentyloxy)benzene-1,4-diamine (8v): **8v** was prepared according to general procedure 3 using **7v** (176 mg, 0.500 mmol). The title compound was obtained as a purple liquid which was directly used in the next step without further purification.

3-(2-Ethoxyethoxy)-N^1-(3-morpholinopropyl)benzene-1,4-diamine (8w): **8w** was prepared according to general procedure 3 using **7w** (177 mg, 0.500 mmol). The title compound was obtained as a purple liquid which was directly used in the next step without further purification.

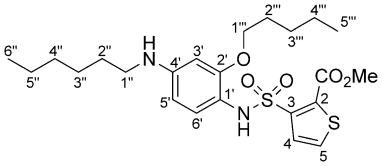
Methyl 3-(*N*-(4-(hexylamino)-2-(pentyloxy)phenyl)sulfamoyl)thiophene-2-carboxylate (9d): Compound **9d** was prepared according to general procedure 4 using **8d** (0.340 mmol). The title compound was obtained as a yellow solid (84 mg, 51% over two steps): mp: 80.4–82.9 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.18 (br s, 1H, SO_2NH), 7.38 (d, $^3J_{H,H}$ = 5.3 Hz, 1H, 5-H), 7.36 (d, $^3J_{H,H}$ = 5.3 Hz, 1H, 4-H), 7.32 (d, $^3J_{H,H}$ = 8.7 Hz, 1H, 6′-H), 6.12 (dd, $^3J_{H,H}$ = 8.6 Hz, $^4J_{H,H}$ = 2.4 Hz, 1H, 5′-H), 5.94 (d, $^4J_{H,H}$ = 2.5 Hz, 1H, 3′-H), 3.97 (s, 3H, CO_2CH_3), 3.68 (t, $^3J_{H,H}$ = 7.0 Hz, 2H, 1‴-H_2), 3.54 (br s, 1H, N*H*), 3.02 (t, $^3J_{H,H}$ = 7.2 Hz, 2H, 1″-H_2), 1.56 (m_c, 4H, 2″-H_2, 2‴-H_2), 1.40–1.28 (m, 10H, 3′-H_2, 4″-H_2, 5″-H_2, 3‴-H_2, 4‴-H_2), 0.92 (t, $^3J_{H,H}$ = 7.1 Hz, 3H, 5‴-H_3), 0.89 ppm (br t, $^3J_{H,H}$ = 7.1 Hz, 3H, 6″-H_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 60.7 (1C, CO_2CH_3), 152.5 (1C, C-2′), 147.9 (1C, C-4′), 145.9 (1C, C-3), 131.5 (1C, C-2), 131.4 (1C, C-5), 129.3 (1C, C-4), 127.4 (1C, C-6′), 114.8 (1C, C-1′), 104.4 (1C, C-5′), 96.5 (1C, C-3′), 68.2 (1C, C-1‴), 53.0 (CO_2CH_3), 44.2 (1C, C-1″), 31.7 (1C, C-4″), 29.6 (1C, C-2″), 28.7 (1C, C-2‴), 28.0 (1C, C-3‴), 26.9 (1C, C-3″), 22.7 (1C, C-5″), 22.5 (1C, C-4‴), 14.1 (1C, C-6″), 14.1 ppm (1C, C-5‴); MS (ESI): *m/z* (%) = 278 (100) $[M-C_6H_4O_4S_2]^+$, 483 (60) $[M+H]^+$, 505 (58) $[M+Na]^+$; HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{23}H_{35}N_2O_5S_2$: 483.198741, found: 483.201640; Anal. calcd for $C_{23}H_{34}N_2O_5S_2$: C 57.24%, H 7.10%, N 5.80%, found: C 57.01%, H 7.10%, N 5.79%.

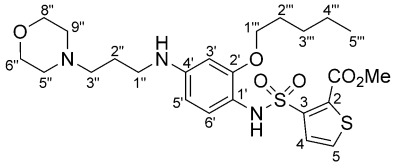
Methyl 3-(*N*-(2-(2-ethoxyethoxy)-4-(hexylamino)phenyl)sulfamoyl)thiophene-2-carboxylate (9u): Compound **9u** was prepared according to general procedure 4 using **8u** (0.500 mmol). The title compound was obtained as an orange solid (79 mg, 33% over two steps): mp: 80.8–81.5 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.20 (br s, 1H, SO_2NH), 7.36 (d, $^3J_{H,H}$ = 5.3 Hz, 1H, 5-H), 7.35 (d, $^3J_{H,H}$ = 5.0 Hz,



1H, 4-H), 7.30 (d, $^3J_{H,H}$ = 8.7 Hz, 1H, 6′-H), 6.14 (dd, $^3J_{H,H}$ = 8.6 Hz, $^4J_{H,H}$ = 2.4 Hz, 1H, 5′-H), 6.01 (d, $^4J_{H,H}$ = 2.3 Hz, 1H, 3′-H), 3.98 (s, 3H, CO_2CH_3), 3.88 (t, $^3J_{H,H}$ = 5.4 Hz, 2H, 1‴-H_2), 3.55 (t, $^3J_{H,H}$ = 5.5 Hz, 2H, 2‴-H_2), 3.51 (q, $^3J_{H,H}$ = 7.1 Hz, 2H, 4‴-H_2), 3.00 (t, $^3J_{H,H}$ = 7.1 Hz, 2H, 1″-H_2), 1.56 (tt, $^3J_{H,H}$ = 7.2 Hz, $^3J_{H,H}$ = 7.2 Hz, 2H, 2″-H_2), 1.39–1.26 (m, 6H, 3″-H_2, 4″-H_2, 5″-H_2), 1.22 (t, $^3J_{H,H}$ = 7.0 Hz, 3H, 5‴-H_3), 0.88 ppm (br t, $^3J_{H,H}$ = 6.9 Hz, 3H, 6″-H_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 160.7 (1C, CO_2CH_3), 152.2 (1C, C-2′), 147.6 (1C, C-4′), 145.3 (1C, C-3), 131.8 (1C, C-2), 131.3 (1C, C-5), 129.3 (1C, C-4), 127.1 (1C, C-6′), 115.2 (1C, C-1′), 105.3 (1C, C-5′), 97.4 (1C, C-3′), 68.4 (1C, C-2‴), 67.9 (1C, C-1‴), 66.8 (1C, C-4‴), 53.0 (1C, CO_2CH_3), 44.2 (1C, C-1″), 31.6 (1C, C-4″), 29.4 (1C, C-2″), 26.8 (1C, C-3″), 22.6 (1C, C-5″), 15.2 (1C, C-5‴), 14.1 ppm (1C, C-6″); MS (ESI): *m/z* (%) = 280 (100) $[M-C_{11}H_{26}NO_2]^+$, 301 (10), 485 (34) $[M+H]^+$, 507 (22) $[M+Na]^+$; HRMS (ESI) *m/z* $[M+Na]^+$ calcd for $C_{22}H_{32}N_2O_6S_2Na$: 507.159950, found: 507.157691; Anal. calcd for $C_{22}H_{32}N_2O_6S_2$: C 54.52%, H 6.66%, N 5.78%, found: C 54.79%, H 6.70%, N 5.68%.

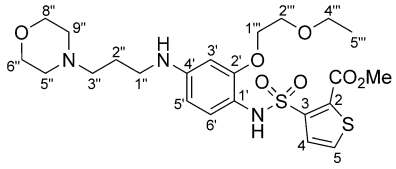
Methyl 3-(*N*-(4-((3-morpholinopropyl)amino)-2-(pentyloxy)phenyl)sulfamoyl)thiophene-2-carboxylate (9v): Compound **9v** was prepared according to general procedure 4 using **8v** (0.500 mmol). The title compound was obtained as a yellow solid (134 mg, 51% over two steps): mp: 93.9–96.7 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.19 (s, 1H, SO_2NH), 7.38 (d, $^3J_{H,H}$ = 5.4 Hz, 1H, 5-H), 7.36 (d, $^3J_{H,H}$ = 5.2 Hz, 1H, 4-H), 7.31 (d, $^3J_{H,H}$ = 8.6 Hz, 1H, 6′-H), 6.11 (dd, $^3J_{H,H}$ = 8.6 Hz, $^2J_{H,H}$ = 2.3 Hz, 1H, 5′-H), 5.95 (d, $^2J_{H,H}$ = 1.4 Hz, 1H, 3′-H), 4.63 (br s, 1H, N*H*), 3.97 (s, 3H, CO_2CH_3), 3.77 (br s, 4H, 6″-H_2, 8″-H_2), 3.67 (t, $^3J_{H,H}$ = 7.0 Hz, 2H, 1‴-H_2), 3.15 (t, $^3J_{H,H}$ = 5.9 Hz, 2H, 1″-H_2), 2.55 (br s, 6H, 3″-H_2, 5″-H_2, 9″-H_2), 1.82 (br s, 2H, 2‴-H_2), 1.56 (tt, $^3J_{H,H}$ = 7.2 Hz, $^3J_{H,H}$ = 7.2 Hz, 2H, 2″-H_2), 1.36–1.27 (m, 4H, 3‴-H_2, 4‴-H_2), 0.92 ppm (t, $^3J_{H,H}$ = 7.0 Hz, 3H, 5‴-H_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 160.6 (1C, CO_2CH_3), 152.5 (1C, C-2′), 147.9 (1C, C-4′), 145.8 (1C, C-3), 131.5 (1C, C-2), 131.3 (1C, C-5), 129.4 (1C, C-4), 127.4 (1C, C-6′),





114.7 (1C, C-1′), 104.3 (1C, C-5′), 96.5 (1C, C-3′), 68.2 (2C, C-6″, C-8″), 66.9 (1C, C-1‴), 57.5 (1C, C3″), 53.7 (2C, C-5″, C-9″), 53.0 (1C, CO_2CH_3), 43.2 (1C, C-1″), 28.7 (1C, C-2‴), 27.9 (1C, C-3‴), 25.2 (1C, C-2″), 22.5 (1C, C-4‴), 14.1 ppm (1C, C-5‴); MS (ESI): *m/z* (%) = 321 (21) $[M-C_6H_6O_4S_2]^+$, 526 (100) $[M+H]^+$; Anal. calcd for $C_{24}H_{35}N_3O_6S_2$: C 54.84%, H 6.71%, N 7.99%, found: C 54.93%, H 6.65%, N 8.08%.

Methyl 3-(*N*-(2-(2-ethoxyethoxy)-4-((3-morpholinopropyl)amino)-phenyl)sulfamoyl)thiophene-2-carboxylate (9w): Compound **9w** was prepared according to general procedure 4 using **8w** (0.500 mmol). The title compound was obtained as a yellow solid (104 mg, 39% over two steps): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.20 (s, 1H, SO_2NH), 7.37 (d, $^3J_{H,H}$ = 5.2 Hz, 1H, 5-H), 7.36 (d, $^3J_{H,H}$ = 5.2 Hz,



1H, 4-H), 7.30 (d, $^3J_{H,H}$ = 8.6 Hz, 1H, 6′-H), 6.13 (dd, $^3J_{H,H}$ = 8.6 Hz, $^2J_{H,H}$ = 2.6 Hz, 1H, 5′-H), 6.00 (d, $^2J_{H,H}$ = 2.3 Hz, 1H, 3′-H), 4.62 (br s, 1H, N*H*), 3.99 (s, 3H, CO_2CH_3), 3.88 (t, $^3J_{H,H}$ = 5.3 Hz, 2H, 1‴-H_2), 3.77 (br s, 4H, 6″-H_2, 8″-H_2), 3.56 (t, $^3J_{H,H}$ = 5.3 Hz, 2H, 2‴-H_2), 3.52 (q, $^3J_{H,H}$ = 7.0 Hz, 2H, 4‴-H_2), 3.13 (t, $^3J_{H,H}$ = 6.3 Hz, 2H, 1″-H_2), 2.54 (br s, 6H, 3″-H_2, 5″-H_2, 9″-H_2), 1.81 (tt, $^3J_{H,H}$ = 6.3 Hz, $^3J_{H,H}$ = 6.3 Hz, 2H, 2″-H_2), 1.22 ppm (t, $^3J_{H,H}$ = 7.0 Hz, 3H, 5‴-H_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 160.7 (1C, CO_2CH_3), 152.3 (1C, C-2′), 147.9 (1C, C-4′), 145.4 (1C, C-3), 131.8 (1C, C-2), 131.3 (1C, C-5), 129.3 (1C, C-4), 127.2 (1C, C-6′), 115.3 (1C, C-1′), 105.1 (1C, C-5′), 97.4 (1C, C-3′), 68.5 (1C, C-2‴), 68.0 (1C, C-1‴), 66.8 (1C, C-4‴), 66.7 (2C, C-6″, C-8″), 57.4 (1C, C-3″), 53.6 (2C, C-5″, C-9″), 53.1 (1C, CO_2CH_3), 43.1 (1C, C-1″), 25.1 (1C, C-2″), 15.2 ppm (1C, C-5‴); MS (ESI): *m/z* (%) = 323 (37) $[M-C_6H_6O_4S_2]^+$, 528 (100) $[M+H]^+$; HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{23}H_{34}N_3O_7S_2$: 528.1838, found: 528.1833; Anal. calcd for $C_{23}H_{33}N_3O_7S_2 \cdot 0.25H_2O$: C 51.91%, H 6.35%, N 7.90%, found: C 51.79%, H 6.21%, N 7.97%.

Biological data

Mice: C57 BL6 mice were purchased from Charles River Laboratories (Wilmington, MA, USA). Mouse experiments were approved by the local authorities (Regierungspräsidium Giessen).

Cell culture: C2C12 murine myoblasts (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM), complemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin. MDA-MB-231-luc2 (Caliper Life Science, Mainz, Germany) human breast cancer cells were cultured in McCoy's 5A (Modified) medium complemented with 10% FCS, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin. Cells were maintained in a humidified incubator at 37 °C and 5% CO_2. Cells were exposed to ligands (1 μM or indicated concentrations) for 24 h. Mononuclear cells were isolated from peripheral blood of healthy volunteers by Lymphocyte Separation Medium 1077 (PromoCell GmbH, Heidelberg, Germany) density gradient centrifugation followed by adherent cell positive selection. Cells were cultured in RPMI 1640 supplemented with 10% FCS for six days. Ligand treatment was performed as indicated at 300 nM final concentrations.

TR-FRET: Interaction of the PPARβ/δ LBD with co-repressor peptides was determined using the Lanthascreen TR-FRET PPARβ/δ co-regulator assay with a fluorescein-labeled co-repressor peptide derived from the SMRT-interaction domain 2.[17] Measurements were performed on a VICTOR3V Multilabel Counter (WALLAC 1420; PerkinElmer, Waltham, MA, USA).

IC_{50} calculations: IC_{50} values were determined by nonlinear regression analysis using Prism version 6.0 software (GraphPad Software, San Diego, CA, USA).

Quantitative RT-PCR: RNA isolation was carried out according to the manufacturer's instructions using the NucleoSpin RNA Isolation Kit (Macherey–Nagel GmbH & Co. KG, Düren, Germany). cDNA was synthesized from 500 ng of RNA according to the manufacturer's protocol with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories GmbH, Munich, Germany). Real-time quantitative PCR was performed in triplicate reactions in an Mx3000P RT-qPCR system (Stratagene, La Jolla, CA, USA) using ABsolute QPCR SYBR Green Mix (Thermo Fisher Scientific, Waltham, MA, USA) at a primer concentration of 0.25 μM. *RPL27*/*Rpl27* primers were used for normalization.

Human primers: RPL27 forward: AAA GCT GTC ATC GTG AAG AAC, RPL27 reverse: GCT GTC ACT TTG CGG GGG TAG, PDK4 forward: TTA TAC ATA CTC CAC TGC ACC A, PDK4 reverse: ATA GAC TCA GAA GAC AAA GCC T.

Mouse primers: Rpl27 forward: AAA GCC GTC ATC GTC AAC AAC, Rpl27 reverse: GCT GTC ACT TTC CCG GGG ATA G, Angptl4 forward: CTC TGG GGT CTC CAC CAT TT, Angptl4 reverse: TTG GGG ATC TCC GAA GCC AT, Cpt1a forward: AGT GTG TGA GGC CAC TGA TG, Cpt1a reverse: TCG CTG CCT GAA TAT GGG TT.

Pharmacokinetics in mice: Compounds were administered intravenously (i.v.) at 1 mg kg^{-1} in 200 μL PBS/Solutol 5% to male mice (six months of age, weight: ~25 g, three animals per data point), and blood samples were taken at the indicated time. Plasma samples (50 μL) were diluted with acetonitrile (200 μL) and centrifuged for 15 min at 4 °C (6000*g*); 180 μL of the supernatant were diluted with water (100 μL) and again centrifuged (15 min, 3900*g*); 200 μL of the resulting supernatant were analyzed further by HPLC–MS/MS. The measurements were carried out on a Waters Alliance separation system (Model 2795, Milford, MA, USA). Samples were separated on a Phenomenex Kinetex column (C_{18}, length: 50 mm, inner diameter: 2.1 mm, particle size: 2.6 μm, pore size: 100 Å) using isocratic conditions (80% MeCN, 20% 10 mM NH_4OAc, flow rate: 0.2 mL min^{-1}, injection volume: 10 μL, run time: 8 min). The separation system was coupled to a nano-electrospray interface of a Micromass Quattro Micro mass spectrometric detector (Beverly, MA, USA). The compounds were detected in multiple reaction monitoring mode. For quantification, a ten-point calibration curve was used with the corresponding transitions (**9u**: *m/z* = 485 → *m/z* = 280). Data analysis was performed using Masslynx software Version 4.1 (Waters, Milford, MA, USA). The coefficient of determination (R^2) was better than 0.99. Limit of quantification (LOQ) for **9u**: 0.005 ng μL^{-1}; limit of detection (LOD) for **9u**: 0.0015 ng μL^{-1}.

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Keywords: inverse agonists · nuclear receptors · PPARβ/δ · ST247 · structure–activity relationships

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