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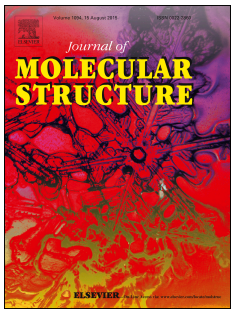

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The synthesis and characterization of tetramic acid derivatives as Mdm2-p53 inhibitors

Damian Muszak,[†] Beata Łabuzek,[†] Mateusz Z. Brela,[‡ *] Aleksandra Twarda-Clapa,[§] Miroslawa Czub,[†] Bogdan Musielak,[†] Ewa Surmiak,[†*] Tad A. Holak[†]

† Faculty of Chemistry, Department of Organic Chemistry, Jagiellonian University, Gronostajowa 2, 30-387 Krakow, Poland

‡ Faculty of Chemistry, Department of Physical Chemistry, Jagiellonian University, Gronostajowa 2, 30-387 Krakow, Poland

§ Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Gronostajowa 7, 30-387 Krakow, Poland

Corresponding Author

*E-mail: ewa.surmiak@uj.edu.pl, brela@chemia.uj.edu.pl

KEYWORDS

Mdm2-p53 inhibitors, tetramic acid, DFT, tautomerism

HIGHLIGHTS

- Synthesis and activity of the tetramic acid core inhibitors are presented.
- Binding to Mdm2 was tested by two orthogonal methods: the FP assay and the ^{1}H-^{15}N HSQC titration experiments.
- The tautomeric effect of the 3-phenylthio-substituted tetramic acids were studied by spectroscopic and DFT methods and showed that they exist in solution in a usually not-preferred enol form.

ABSTRACT

We present syntheses, prediction of tautomer forms and activities of the second generation of the Mdm2-p53 inhibitors that are based on the tetramic acid scafolld. The inhibitors do not contain 6-chloroindole. Binding of these compounds to Mdm2 was checked by two orthogonal methods: the fluorescence polarization and the ^{1}H-^{15}N HSQC NMR titration experiments. We discovered that the 3-phenylthio-substituted tetramic acid derivatives exist in solution solely in their enol forms which is in contrast to the similar 3-aliphatic substituted derivatives. The inhibitory (K_i) and dissociation (K_D) constants are in low micromolar ranges with the best binding compound **9a** having K_D = 2.9 μM. Furthermore, our data show that the compounds indeed bind to the p53-binding pocket of Mdm2 and do not cause dimerization of Mdm2. The current work provides solid base for further rational design of the Mdm2/p53 inhibitors.

1. Introduction

The p53 tumor suppressor protein is inactivated or mutated in nearly all types of human cancers [1-3]. Restoration of the p53 activity should in principle be a promising strategy against cancer. The Mdm2 protein is an E3 ubiquitin ligase and a main negative regulator of the p53 tumor suppressor protein. p53 is inactivated by mutations in over 50% of all cancers. Most of the remaining malignancies have the wild-type p53 (wt-p54) present but this p53 is deactivated by overexpression of p53-regulatory proteins, such as Mdm2 and MdmX [2-6]. In such cases, the restoration of wt-p53 activity is possible by introducing small-molecule inhibitors, which by binding to Mdm2, can liberate wt-p53 from the Mdm2/p53 complex [2,4]. Therefore, a low-molecular-weight antagonist could inhibit or reverse tumor formation and provide a non-genotoxic anticancer therapy [5,6]. The vast majority of the small-molecule Mdm2 inhibitors are designed using a three-finger-pharmacophore-model, based on the triad of p53 amino acids: Phe19, Trp23 and Leu26, which inserts deeply into the binding pocket of Mdm2 [7-9]. Nevertheless, recently several new approaches were proposed which exploit additional induced pockets [10-11], dual inhibitors [12,13] or dimerization [14-15].

In dimerization of Mdm2 by 1,5-dihydro-2*H*-pyrrol-2-ones and 1,5-dihydro-2*H*-furan-2-ones described by us, the 6-chloroindole moieties point outside the p53 binding pockets of Mdm2 [15]. In order to change the physicochemical properties and binding mode of these compounds, we have decided to obtain a second generation of substituted 1,5-dihydro-2*H*-

pyrrol-2-ones (**Fig. 1**). First and foremost, we decided to replace the 6-chloroindole moiety in the new scaffold, as there is no inhibitor in the clinical trials with this moiety [8,16]. Even though the 6-chloroindole usually interacts with the Trp23 pocket of Mdm2, in our 1,5-dihydro-2*H*-pyrrol-2-one molecules it caused protein dimerization, which now we wanted to avoid. Another highly important aspect of our study was shortening and simplifying the compound preparation. For that reason we decided to proceed with a new scaffold that comprises the tetramic acid core, which is both capable of providing suitable substituents arrangement and could be easily synthesized.

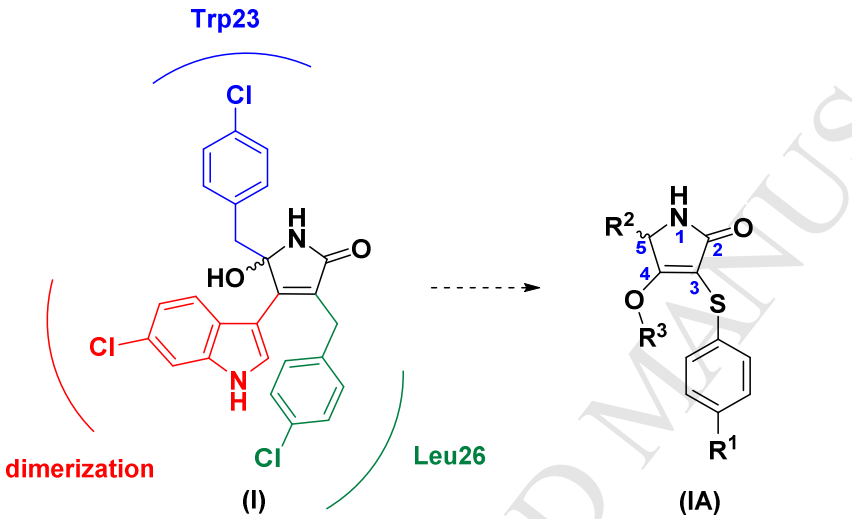


Figure 1. Structure of the scaffold based on the 1,5-dihydro-2*H*-pyrrol-2-one inhibitor (**I**) and its second generation (**IA**). (Numbering of atoms in the central ring of tetramic acid is shown in blue)

The tetramic acid core possessing compounds (2,4-pyrrolidinedione) are of interest of researchers both due to their biological functions, unusual physical properties and challenging synthesis. The tetramic acid ring can be found in many naturally occurring products with biological functions, mostly antibiotic and antiviral [17-18]. Naturally occurring tetramic acid derivatives are most commonly isolated from marine species and possess a 3-acyl moiety and 5C chirality originating from the amino acid used in their biosynthesis. Examples of such compounds are: the antibacterial Ravenic acid isolated from *Penicillium* [19], the antiviral Trichobotysin A from *Trychobotrys effuse* [20], the anticancer Cylindramid from the marine sponge *Halichondria cylindrata* [21], the cytotoxic Palau'imide from cyanobacteria *Lyngbya* [22], an anti-inflammatory lipoxygenase inhibitor Tetrapetalone A from *Streptomyces* sp. USF-4727 [23] or the antifungal dihydromaltophilin from *Lascobacter enzymogenes* [24].

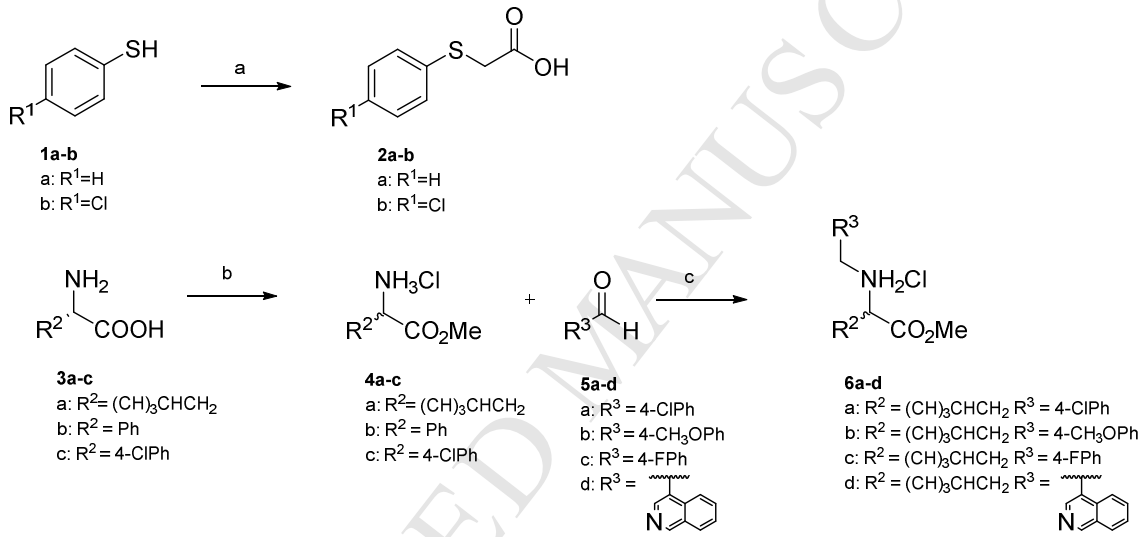
Tetramic acid can be found in both the ketone and enol states. Traditionally it is often presented as an enolic derivative, due to its structural similarity to the highly acidic oxygen analogue - tetronic acid. Although, this is the preferred form only when the compound structure contains the 3-acyl moiety – in which case four tautomers interconvert slowly (Fig. S1) [25]. In these compounds, the 4-hydroxyl group is acidic and can be deprotonated even in physiological conditions, which makes them a perfect chelator for a variety of metals [26]. Moreover, in some natural products the enol form is trapped by the 4-O alkyl ether group, like for example in the Palau'imide. Even though the enol form of tetramic acid derivatives can be stable, in most cases the ketone form is predominant which is reflected in their significantly reduced acidity ($pK_a \approx 6.4$) [27] when compared to the oxygen-based tetronic acids. The keto-enol tautomerization equilibrium depends on the various factors, like solvent properties, and therefore can be shifted toward one of possible forms. Taken together, the tetramic acid properties and structural identification can be difficult to predict due to the presence of several tautomeric forms in solution. The knowledge of the compound structure, especially in aqueous solutions is crucial to their biological activity. Herein we present synthesis, structural characterization and the Mdm2 binding of a series of 3-phenylthio substituted tetramic acid derivatives.

2. Experimental and computational details.

2. 1. Synthesis

The compounds were synthesized as depicted in **Schemes 1 and 2**. Flash chromatography was performed with a Reveleris® X2 Flash Chromatography, using Grace® Reveleris Silica flash cartridges. Monitoring of the reactions was carried out using the silica gel TLC plates silica Merck 60 F_{254}. Spots were visualized by UV light at 254 and 365 nm. All NMR spectra were recorded on a Bruker Avance 600 MHz. Chemical shifts for ^{1}H NMR were reported as δ values and coupling constants were in Hertz (Hz). The following abbreviations were used for spin multiplicity: s = singlet, br = broad, d = doublet, t = triplet, q = quartet, quin = quintet, m = multiplet, sept = septet. Chemical shifts for ^{13}C NMR were reported in δ calibrated to the solvent peak. IR absorption spectra were recorded on a Nicolet IR200 spectrometer using ATR technique. HRMS were carried out by the Laboratory for Forensic Chemistry Faculty of Chemistry, Jagiellonian University with the microOTOF-QII spectrometer using ESI ionization technique. The UPLC-MS/MS system consisted of a Waters ACQUITY® UPLC® (Waters Corporation, Milford, MA, USA) coupled to a Waters TQD mass spectrometer (electrospray ionization mode ESI-tandem quadrupole). Chromatographic separations were

carried out using the Acquity UPLC BEH (bridged ethyl hybrid) C_{18} column; 2.1 × 100 mm, and 1.7 µm particle size, equipped with Acquity UPLC BEH C18 VanGuard pre-column; 2.1 × 5 mm, and 1.7 µm particle size. The column was maintained at 40°C, and eluted under gradient conditions using from 95% to 0% of eluent A over 10 min, at a flow rate of 0.3 mL min^{-1}. Eluent A: water/formic acid (0.1%, v/v); eluent B: acetonitrile/formic acid (0.1%, v/v). The purity of all final compounds, determined using chromatographic LC-MS, was >95%. All reagents were obtained from commercial sources and used without further purification unless otherwise stated. Anhydrous solvents were purchased from Sigma-Aldrich or Alfa-Aesar, anhydrous Et_2O and THF were distilled from sodium-benzophenone and stored over molecular sieves (4Å, 3-5 mm beads).



Scheme 1. Synthesis of compounds **2a-b**, **4a-c,** and **6a-d**. Reagents and conditions: (a) **1a-b** (1 eq.), TBAB (0.01 eq), NaOH (2 eq.) bromoacetic acid (1 eq.), THF/H_2O (1/2), 0°C to RT, 16 h, yield 90-91%; (b) **3a-c** (1 eq.), $SOCl_2$ (1.5 eq), anh. MeOH, 0°C to RT, 16 h, yield 80-100%; (c) (i) **4a** (1 eq.), Et_3N (1.1 eq), anh MeOH, RT, 15 min. (ii) **5a-d** (1.1 eq), $NaBH_3CN$ (1 eq), RT, 16 h, yield 60-79%.

2.2. General synthetic procedure for preparation and analytical data of 2-(phenylthio)acetic acid derivatives (2a-b)

The corresponding thiophenol (**1a-b**) (1 eq.), tetrabutylammonium bromide (TBAB) (0.01 eq.) and THF/H_2O (1:2 mixture) were placed in a round bottom flask. The reaction mixture was cooled to 0ºC then NaOH (2 eq.) and bromoacetic acid (1 eq.) were added. The reaction was stirred at RT overnight (16 h). After this time diethyl ether (20 ml) was added and the reaction was poured into 1M HCl solution (30 ml) and then extracted with diethyl ether (3 x 20 ml). Organic layers were collected, washed with brine, dried over anhydrous $MgSO_4$ and evaporated.

2.2.1. 2-(Phenylthio)acetic acid (2a)

Thiophenol (**1a**) (2.05 ml, 20.0 mmol), TBAB (0.08 g 0.2 mmol), bromoacetic acid (2.78 g, 20.0 mmol), NaOH (1.60 g, 40.0 mmol) and THF/H_2O (10/20 ml) were used. The crude product was precipitated and washed with petroleum ether giving compound **2a** with 91% yield (3.36 g) as colorless solid. The product was recrystallized from water. The compound is reported in the literature [28].

IR (ATR): [cm^{-1}]: 3500-2500 (br), 3055, 2903, 2696, 2588, 1704, 1482, 1441, 1427, 1392, 1314, 1198, 900, 736; **NMR: ^{1}H** (600 MHz, DMSO-d_6): δ [ppm] 12.75 (s, 1H), 7.35-7.30 (m, 4H), 7.19 (tt, *J* = 7.1, 1.0 Hz, 1H), 3.79 (s, 2H); **^{13}C** (151 MHz, DMSO-d^6): δ [ppm] 170.6, 135.7, 129.0, 127.7, 125.9, 34.9; **LC-MS (DAD/ESI):** t_r = 4.48 min, Calcd for $C_8H_8O_2$ (m/z): $[M-H]^-$ 167.02, Found: $[M-H]^-$ 166.95; **HRMS (ESI):** Calcd for $C_8H_8O_2$ (m/z): $[M-H]^-$ 167.0167, Found: $[M-H]^-$ 167.0161.

2.2.2. 2-((4-Chloro)phenylthio)acetic acid (2b)

4-chlorothiophenol (**1b**) (2.67 g, 20.0 mmol), TBAB (0.08 g 0.2 mmol), bromoacetic acid (2.78 g, 20.0 mmol), NaOH (1.60 g, 40.0 mmol) and THF/H_2O (10/20 ml) were used. The crude product was precipitated and washed with petroleum ether giving compound **2b** with 90% yield (4.10 g) as colorless solid. The product was recrystallized from water. The compound is reported in the literature [28].

IR (ATR): [cm^{-1}] 3500-2500 (br), 3001, 2921, 2581, 1694, 1478, 1427, 1390, 1305, 1203, 1097, 1010, 889, 812; **NMR: ^{1}H** (600 Mhz, DMSO-d_6) δ [ppm]:12.80 (s, 1H), 7.39-7.34 (m, 4H), 3.82 (s, 2H); **^{13}C** (151 Mhz, DMSO-d_6) δ [ppm]: 170.4, 134.9, 130.5, 129.5, 128.9, 34.9; **LC-MS (DAD/ESI):** t_r = 5.39 min, Calcd for $C_8H_7ClO_2S$ (m/z): $[M-H]^-$ 200.98 $[M+2-H]^-$ 202.98 , Found: $[M-H]^-$ 200.90 $[M+2-H]^-$ 202.90; **HRMS (ESI):** Calcd for $C_8H_7ClO_2S$ (m/z): $[M-H]^-$ 200.9777 $[M+2-H]^-$ 202.9748 , Found: $[M-H]^-$ 200.9771 $[M+2-H]^-$ 202.9742.

2.3. General synthetic procedure for preparation and analytical data of amino acid methyl ester hydrochloride derivatives (4a-c)

The corresponding amino acid (**3a-c**) (1 eq.) and anhydrous methanol were placed in round bottom flask. The reaction mixture was cooled to 0ºC then thionyl chloride (1.5 eq.) was added dropwise. The reaction was stirred at RT overnight (16 h). After this time solvent was evaporated. To the residual oil another potion of methanol was added (approx. 30 ml) and evaporated again. This procedure was repeated twice.

2.3.1 Leucine methyl ester hydrochloride (4a)

L-leucine (**3a**) (1.31 g, 10 mmol), $SOCl_2$ (1.09 ml, 15.0 mmol) and methanol (20 ml) were used. The crude product was precipitated from petroleum ether giving compound **4a** with 80% yield (1.46 g). The product was recrystallized from ethyl acetate. The optical purity of the obtained compound was not established. The compound is reported in the literature [29].

IR (ATR): [cm^{-1}] 2958, 1848, 1738, 1507, 1452, 1250, 1227, 1044; **NMR:** ^{1}**H** (600 MHz, DMSO-d_6) δ [ppm] 8.66 (s, 3H), 3.92 (t, *J* = 7.1 Hz, 1H), 3.73 (s, 3H), 1.79-1.72 (m, 1H), 1.70-1.60 (m, 2H), 0.89 (d, *J* = 6.5 Hz, 6H); ^{13}**C** (151 MHz, DMSO-d_6) δ [ppm] 170.3, 52.7, 50.5, 23.7, 22.1, 22.0 **LC-MS (DAD/ESI):** t_r = 1.06 min, Calcd for $C_7H_{15}NO_2$ (m/z): $[M+H]^+$ 146.12, Found $[M+H]^+$ 146.15; **HRMS (ESI):** Calcd for $C_7H_{15}NO_2$ (m/z): $[M+H]^+$ 146.1181, Found $[M+H]^+$ 146.1176.

2.3.2 Phenylalanine methyl ester hydrochloride (4b)

L-phenylalanine (**3b**) (6.30 g, 38.0 mmol), $SOCl_2$ (4.15 ml, 57 mmol) and methanol (80 ml) were used. The crude product was precipitated from petroleum ether giving compound **4b** with quantitative yield (8.20 g). The product was recrystallized from ethyl acetate/methanol mixture. The optical purity of the obtained compound was not established. The compound is reported in the literature [30].

IR (ATR): [cm^{-1}] 2842, 1744, 1583, 1495, 1448, 1241, 1146, 1084; **NMR:** ^{1}**H** (600 MHz, DMSO-d^6) δ [ppm] 8.75 (s, 3H), 7.33 (t, *J* = 7.3 Hz, 2H), 7.29-7.26 (m, 1H), 7.24 (d, *J* = 7.0 Hz, 2H), 4.23 (dd, *J* = 7.4, 5.8 Hz, 1H), 3.65 (s, 3H), 3.21 (dd, *J* = 14.0, 5.7 Hz, 1H), 3.10 (dd, *J* = 14.0, 7.5 Hz, 1H); ^{13}**C** (151 MHz, DMSO-d^6) δ [ppm] 169.5, 134.7, 129.4, 128.6, 127.3, 53.2, 52.5, 35.8; **LC-MS (DAD/ESI):** t_r = 2.19 min, Calcd for $C_{10}H_{13}NO_2$ (m/z): $[M+H]^+$ 180.10, Found: $[M+H]^+$ 180.10; **HRMS (ESI):** Calcd for $C_{10}H_{13}NO_2$ (m/z): $[M+H]^+$ 180.1024, Found: $[M+H]^+$ 180.1018.

2.3.3 (4-Chloro)phenylalanine methyl ester hydrochloride (4c)

4-Chloro-DL-phenylalanine (**3c**) (2.99 g, 15 mmol), $SOCl_2$ (1.63 ml, 22.5 mmol) and methanol (25 ml) were used. The crude product was precipitated from petroleum ether giving compound **4c** with quantitative yield (3.73 g). The optical purity of the obtained compound was not established. The compound is reported in the literature [31].

IR (ATR): [cm^{-1}] 2908 (br), 1741, 1550, 1489, 1239, 1148, 1096, 1018, 948, 858, 805; **NMR:** ^{1}**H** (600 MHz, DMSO-d_6) δ [ppm] 8.77 (br, 3H), 7.38 (d, *J* = 7.4 Hz, 2H), 7.29 (d, *J* = 7.3 Hz, 2H), 4.28-4.21 (m, 1H), 3.67 (s, 3H), 3.23-3.17 (m, 1H), 3.17-3.10 (m, 1H) ^{13}**C** (151 MHz, DMSO-d_6) δ [ppm] 169.2, 133.8, 132.0, 131.4, 128.5, 53.0, 52.6, 34.9; **LC-MS**

(DAD/ESI): t_r = 3.01 min, Calcd for $C_{10}H_{12}ClNO_2$ (m/z): $[M+H]^+$ 214.06 $[M+2+H]^+$ 216.06, Found: $[M+H]^+$ 214.06 $[M+2+H]^+$ 216.06; **HRMS (ESI)**: Calcd for $C_{10}H_{12}ClNO_2$ (m/z): $[M+H]^+$ 214.0635 $[M+2+H]^+$ 216.0605, Found: $[M+H]^+$ 214.0629 $[M+2+H]^+$ 216.0600.

2.4 General synthetic procedure and analytical data for reductive amination (compounds 6a-d)

Leucine methyl ester hydrochloride (**4a**) (1.eq) and anhydrous MeOH (excess) were placed in a round bottom flask equipped with $CaCl_2$ tube, then triethylamine was added (1.1 eq.) and the reaction was stirred for 15 minutes. After this time suitable aldehyde (**5a-d**) (1.1 eq.) and sodium cyanoborohydride (1 eq.) were added respectively. The reaction was stirred at RT for 3 days. After this time solvent was evaporated. To the residual slurry water was added (50 ml) and the mixture was extracted with ethyl acetate (3 x 20 ml). Organic layers were collected, washed with water and brine, dried over anhydrous $MgSO_4$ and evaporated. Crude products were purified by flash chromatography (SiO_2, petroleum ether/ethyl acetate 10:1). After column chromatography compounds were transformed into their hydrochlorides. The corresponding amine was put into a round bottom flask with excess of 4M HCl in 1,4-dioxane and stirred for 5 minutes at RT. After this time solvent was evaporated and final product was precipitated from petroleum ether.

2.4.1. Methyl N-(4-chlorobenzyl)leucinate hydrochloride (6a)

Leucine methyl ester hydrochloride (**4a**) (2.50 g, 13.8 mmol), 4-chlorobenzaldehyde (**5a**) (2.13 g, 15.1 mmol), triethylamine (2.10 ml, 15.1 mmol), $NaBH_3CN$ (0.86 g, 13.8 mmol) and anhydrous methanol (70 ml) were used. Compound **6a** was obtained as a colorless solid, with a 60% yield (2.55 g). The compound is reported in the literature [32].

IR (ATR): [cm^{-1}] 2957, 2648 (br), 1749, 1542, 1472, 1254, 1204, 1093, 1019, 843, 808; **NMR:** ^{1}**H** (600 MHz, DMSO-d_6) δ[ppm]: 10.27 (s, 1H), 9.79 (s, 1H), 7.60 (d, *J* = 8.4 Hz, 2H), 7.50 (dt, *J* = 8.5 Hz, 2.0, 2H), 4.22 (d, *J* = 13.0 Hz, 1H), 4.11 (d, *J* = 13.0 Hz, 1H), 3.94 (dd, *J* = 9.2, 4.6 Hz, 1H), 3.73 (s, 3H), 1.88-1.81 (m, 1H), 1.77-1.70 (m, 1H), 1.69, 1.62 (m, 1H), 0.88 (d, *J* = 6.1 Hz, 6H); ^{13}**C** NMR (151 MHz, DMSO-d_6) δ[ppm]: 160.4, 133.9, 132.5, 130.6, 128.6, 57.4, 53.0, 48.2, 37.9, 24.2, 23.0, 21.2; **LC-MS (DAD/ESI):** t_r = 4.33 min, Calcd for $C_{14}H_{20}NO_2$ (m/z): $[M+H]^+$ 270.13 $[M+2+H]^+$ 272.12, Found $[M+H]^+$ 270.15 $[M+2+H]^+$272.21; **HRMS (ESI):** Calcd for $C_{14}H_{20}NO_2$ (m/z): $[M+H]^+$ 270.1261 $[M+2+H]^+$ 272.1231, Found $[M+H]^+$ 270.1255 $[M+2+H]^+$272.1222.

2.4.2. Methyl N-(4-methoxybenzyl)leucinate hydrochloride (6b)

Leucine methyl ester hydrochloride (**4a**) (3 g, 16.5 mmol), 4-methoxybenzaldehyde (**5c**) (2.20 ml, 18.1 mmol), triethylamine (2.53 ml, 18.1 mmol), $NaBH_3CN$ (1,04 g, 16,5 mmol) and anhydrous methanol (70 ml) were used. Compound **6b** was obtained as a colorless solid, with a 79% yield (3.94 g). The compound is reported in the literature [33].

IR (ATR): [cm^{-1}] 3456, 2931, 1586, 1418, 1050, 1008, 923; **NMR:** ^{1}**H** (600 MHz, DMSO-d_6) δ[ppm]: 7.17 (dt, *J* = 8.7, 2.1 Hz, 2H), 6.84 (dt, *J* = 8.7, 2.1 Hz, 2H), 3.70 (s, 3H), 3.64 (d, *J* = 13.2 Hz, 1H), 3.60 (s, 3H), 3.42 (d, *J* = 13.2 Hz, 1H), 3.12 (t, *J* = 6.7 Hz, 1H), 2.23 (s, 2H), 1.67 (sept, *J*= 6.6 Hz, 1H), 1.42-1.37 (m, 1H), 1.35-1.30 (m, 1H), 0.83 (d, *J* = 6.7 Hz, 3H), 0.76 (d, *J* = 6.6 Hz, 3H); ^{13}**C** (151 MHz, DMSO-d_6) δ[ppm]: 175.0, 158.5, 132.6, 113.9, 58.6, 55.3, 51.8, 50.8, 42.4, 26.0, 24.8, 23.2, 22.4; **HRMS (ESI):** Calcd for $C_{15}H_{23}NO_3$ (m/z): $[M+H]^+$ 266.1756 Found $[M+H]^+$ 266.1750.

2.4.3. Methyl N-(4-fluorobenzyl)leucinate hydrochloride (6c)

Leucine methyl ester hydrochloride (**4a**) (1.50 g, 8.3 mmol), 4-fluorobenzaldehyde (**5b**) (0.95 ml, 9.1 mmol), triethylamine (1.27 ml, 9.1 mmol), $NaBH_3CN$ (0.52 g, 8.3 mmol) and anhydrous methanol (40 ml) were used. Compound **6c** was obtained as a colorless solid, with a 68% yield (1.64 g). The compound is reported in the literature [34].

IR (ATR): [cm^{-1}] 2960, 2727 (br), 1750, 1742, 1604, 1513, 1417, 1227, 1056, 1023, 840, 822; **NMR:** ^{1}**H** (600 MHz, DMSO-d_6) δ[ppm]: 10.42 (s, 1H), 9.83 (s, 1H), 7.66 (dd, *J*= 8.7, 5.5 Hz, 2H), 7.27 (tt, J= 8.9, 3.0 Hz, 2H), 4.21 (d, *J*= 13.0 Hz, 1H), 4.11 (d, *J*= 13.0 Hz, 1H), 3.92 (dd, *J*= 9.5, 4.7 Hz, 1H), 3.73 (s, 3H), 1.90-1.86 (m, 1H), 1.76-1.72 (m, 1H), 1.70-1.64 (m, 1H), 0.88 (d, *J*= 6.5 Hz, 6H); ; ^{13}**C** (151 MHz, DMSO-d_6) δ [ppm]: 169.8, 163.7-162.1 (d, J_{FC} = 245.6 Hz), 133.4-133.3 (d, J_{FC} = 8.4 Hz), 128.3, 115.9-115.8 (d, J_{FC} = 21.5 Hz), 57.6, 53.3, 48.5, 38.3, 24.7, 23.4, 21.6; **HRMS (ESI):** Calcd for $C_{14}H_{20}FNO_2$ (m/z): $[M+H]^+$ 254.1556 Found $[M+H]^+$ 254.1539.

2.4.4. Methyl N-(isoquinolin-4-ylmethyl)leucinate hydrochloride (6d)

Leucine methyl ester hydrochloride (**4c**) (1.06 g, 5.9 mmol), 4-isoquinolinecarboxaldehyde (**5d**) (1.02 g, 6.5 mmol), triethylamine (0.90 ml, 6.5 mmol), $NaBH_3CN$ (0.37 g, 5.9 mmol) and anhydrous methanol (40 ml) were used. Compound **6d** was obtained as a colorless solid, with a 62% yield (1.18 g).

IR (ATR): [cm^{-1}] 3267, 3027, 2508 (br), 1569, 1394, 1310, 1219, 1150, 1060, 888; **NMR:** ^{1}**H** (600 MHz, DMSO-d_6) δ [ppm]: 10.73 (s, 1H), 10.22 (s, 1H), 9.38 (s, 1H), 9.10 (s, 1H), 8.34

(d, *J* = 8.3 Hz, 1H) 8.26 (d, *J* = 7.9 Hz, 1H), 8.08 (t, *J* = 7.3 Hz, 1H), 7.89 (t, *J* = 7.2 Hz, 1H), 4.59 (d, *J* = 13.0 Hz, 1H), 4.48 (d, *J* = 13 Hz, 1H), 4.17 (dd, *J* = 8.8, 4.2 Hz, 1H), 3.76 (s, 3H), 1.96-1.90 (m, 1H), 1.84-1.78 (m, 1H), 1.77-1.69 (m, 1H), 0.89 (d, *J* = 6.4 Hz, 6H); **^{13}C** (NMR, DMSO-d_6) δ [ppm]: 169.3, 148.7, 144.7, 140.8, 133.6, 129.1, 129.0, 127.3, 125.7, 123.6, 57.6, 53.1, 46.0, 37.8, 24.3, 23.0, 21.2; **LC-MS (DAD/ESI)**: t_r = 3.60 min, Calcd for $C_{17}H_{22}N_2O_2$ (m/z): $[M+H]^+$ 287.18 Found $[M+H]^+$ 287.23; **HRMS (ESI):** Calcd for $C_{17}H_{22}N_2O_2$ (m/z): $[M+H]^+$ 287.1760 Found $[M+H]^+$ 287.1754;

2.5. General synthetic procedure for coupling reaction and analytical data (compounds 7a-c and 8-d)

The corresponding amino acid methyl ester hydrochloride **4a-c** or **6a-d** (1 eq.) and anhydrous dichloromethane (DCM, 40 ml) were placed in round bottom flask equipped with $CaCl_2$ tube. The reaction mixture was cooled to 0ºC and triethylamine (1 eq.) was added dropwise and stirred for 15 minutes. Then suitable derivatives of 2-(phenylthio)acetic acid (**2a-b**) (1 eq.), 4-(dimethylamino)pyridine (DMAP, 0.2 eq.) and N,N'-diisopropylcarbodiimide (DIC, 1 eq.) were added respectively with approx. 5 minutes between each reagent. The reaction was stirred at 0ºC for 1 hour and then at RT overnight (16 h). After this time, water was added (30 ml) and the reaction mixture was extracted with DCM (3 x 15 ml). Organic layers were collected, washed with citric acid (10%, 2 x 15 ml), saturated $NaHCO_3$ (2 x 15 ml) and water (1 x 15 ml), then dried over anhydrous $MgSO_4$ and evaporated.

2.5.1. Methyl (2-((4-chlorophenyl)thio)acetyl)leucinate (7a)

Leucine methyl ester hydrochloride (**4a**) (0.40 g, 2.2 mmol), triethylamine (0.31 ml, 2.2 mmol), 2-((4-chloro)phenylthio)acetic acid (**2b**) (0.45 g, 2.2 mmol), DIC (0.34 ml, 2.2 mmol), DMAP (0.05 g, 0.4 mmol) were used. The crude product was purified by flash chromatography (SiO_2, petroleum ether/chloroform 1:1) giving compound **7a** as a colorless oil with a 73% yield (0.53 g).

IR (ATR): [cm^{-1}] 3275, 2963, 1732, 1669, 1653, 1540, 1480, 1397, 1289, 1256, 1097, 1009, 816; **NMR: ^{1}H** (600 MHz, $CDCl_3$) δ [ppm]: 7.29-7.25 (m, 4H), 6.99 (d, *J* = 8.2 Hz, 1H), 4.58 (td, *J* = 9.0; 5.0 Hz, 1H), 3.69 (s, 3H), 3.67 (d, *J* = 16.9 Hz, 1H), 3.60 (d, *J* = 16.9 Hz, 1H), 1.63-1.57 (m, 1H), 1.50-1.44 (m, 1H), 1.40-1.30 (m, 1H), 0.84 (d, *J* = 6.6 Hz, 6H); **^{13}C** (151 MHz, $CDCl_3$) δ [ppm]: 173.0, 167.4, 133.1, 133.1, 130.0, 129.5, 52.5, 51.0, 41.5, 37.7, 24.8, 22.9, 21.8; **LC-MS (DAD/ESI)**: t_r = 7.06 min, Calcd for $C_{15}H_{20}ClNO_3S$ (m/z): $[M+H]^+$ 330.09 $[M+2+H]^+$ 332.09, Found $[M+H]^+$ 330.16 $[M+2+H]^+$ 332.16; **HRMS (ESI)**: Calcd for

$C_{15}H_{20}ClNO_3SNa$ (m/z): $[M]^+$ 352.0750 $[M+2]^+$ 354.0721, Found $[M]^+$ 352.0743 $[M+2]^+$ 354.0712.

2.5.2. Methyl (2-(phenylthio)acetyl)phenylalaninate (7b)

Phenylalanine methyl ester hydrochloride (**4b**) (1.10 g, 5.1 mmol), triethylamine (0.71 ml, 5.1 mmol), 2-(phenylthio)acetic acid (**2a**) (0.86 g, 5.1 mmol), DIC (0.78 ml, 5.1 mmol), DMAP (0.12 g, 1.0 mmol) were used. The crude product was purified by flash chromatography (SiO_2, petroleum ether/ethyl acetate 3:1) giving compound **7b** as colorless solid with a 81% yield (1.36 g). Product was recrystallized from cyclohexane/ethyl acetate [35].

IR (ATR): [cm^{-1}] 3300, 2915, 1745, 1652, 1533, 1436, 1239, 1212, 1175, 981; **NMR: ^{1}H** (600 MHz, $CDCl_3$) δ[ppm] 7.29-7.26 (m, 2H), 7.24-7.18 (m, 6H), 6.98-6.94 (m, 2H), 4.85 (dt, *J* = 8.0, 6.0 Hz, 1H), 3.67 (s, 3H), 3.65-3.57 (m, 2H), 3.09-3.02 (m, 2H); **^{13}C** (151 MHz, $CDCl_3$) 171.6, 167.8, 135.6, 134.6, 129.4, 129.2, 128.7 128.5, 127.3, 126.9, 53.5, 52.4, 37.9, 37.6; **LC-MS (DAD/ESI):** t_r = 6.59, Calcd for $C_{18}H_{19}NO_3S$ (m/z): $[M+H]^+$ 330.12, Found $[M+H]^+$ 330.16; **HRMS (ESI):** Calcd for $C_{18}H_{19}NO_3SNa$ (m/z): $[M]^+$ 352.0983, Found $[M]^+$ 352.0979.

2.5.3. Methyl (2-((4-chlorophenyl)thio)acetyl)phenylalaninate (7c)

Phenylalanine methyl ester hydrochloride (**4b)** (1.07 g, 5.0 mmol), triethylamine (0.70 ml, 5.0 mmol), 2-((4-chloro)phenylthio)acetic acid (**2b**) (1.01 g, 5.0 mmol), DIC (0.77 ml, 5.0 mmol), DMAP (0.12 g, 1.0 mmol) were used. The crude product was purified by flash chromatography (SiO_2, petroleum ether/chloroform 1:1) giving compound **7c** as colorless solid with a 72% yield (1.32 g). Product was recrystallized from cyclohexane.

IR (ATR): [cm^{-1}] 3297, 2912, 1745, 1653, 1533, 1478, 1435, 1239, 1212, 1174, 891, 821; **NMR: ^{1}H** (600 Mhz, $CDCl_3$) δ [ppm]: 7.23-7.18 (m, 5H), 7.13 (tt, *J* = 8.7, 2.0 Hz, 2H), 7.11 (d, *J* = 7.7 Hz, 1H), 6.98 (dd, *J* = 7.4, 2.0 Hz, 2H), 4.8 (ddd, *J* = 7.8, 6.5, 5.7 Hz, 1H), 3.70 (s, 3H), 3.57 (s, 2H), 3.11 (dd, 14.0, 5.5 Hz, 1H), 3.04 (14.0, 6.6 Hz 1H); **^{13}C** (151 MHz, $CDCl_3$) δ [ppm]: 171.6, 167.5, 135.6, 133.1, 130.0, 129.5, 129.2, 128.8, 127.3, 55.5, 52.5, 37.8, 37.8; **LC-MS (DAD/ESI):** t_r = 7.17 min, Calcd for $C_{18}H_{18}ClNO_3S$ (m/z): $[M+H]^+$ 364.12 $[M+2+H]^+$ 366.12, Found $[M+H]^+$ 364.07 $[M+2+H]^+$ 366.07; **HRMS (ESI):** Calcd for $C_{18}H_{18}ClNO_3SNa$ (m/z): $[M]^+$ 386.0594 $[M+2]^+$ 388.0564, Found $[M]^+$ 386.0589 $[M+2]^+$ 388.0560.

2.5.4. Methyl 3-(4-chlorophenyl)-2-(2-((4-chlorophenyl)thio)acetamido)propanoate (7d)

4-Chlorophenylalanine methyl ester hydrochloride (**4c**) (1.25 g, 5.0 mmol), triethylamine (0.70 ml, 5.0 mmol), 2-((4-chloro)phenylthio)acetic acid (**2b**) (1.01 g, 5.0 mmol), DIC (0.77 ml, 5.0 mmol), DMAP (0.12 g, 1.0 mmol) were used. Thecrude product was purified by flash chromatography (SiO_2, petroleum ether/chloroform 1:1) giving compound **7d** as colorless solid with a 69% yield (1.38 g). Product was recrystallized from cyclohexane/acetone.

IR (ATR): [cm^{-1}] 3276, 3074, 1748, 1733, 1667, 1544, 1478, 1377, 1269, 1213, 1178, 1098, 1009, 819, 808; **NMR:** ^{1}**H** (600 MHz, $CDCl_3$) δ [ppm]: 7.72-7.23 (m, 2H), 7.18-7.13 (m, 4H), 7.10 (d, *J* = 7.7 Hz, 1H), 6.90 (d, *J* = 8.4 Hz, 2H), 4.85-4.79 (m, 1H), 3.71 (s, 3H), 3.63-3.54 (m, 2H), 3.12-3.05 (m, 1H), 3.03-2.97 (m, 1H); ^{13}**C** (151 MHz, $CDCl_3$) δ [ppm]: 171.3, 167.4, 134.0, 133.2, 133.0, 132.9, 130.4, 129.7, 129.4, 128.8, 53.2, 52.5, 37.5, 37.1; **LC-MS (DAD/ESI)**: t_r = 7.65 min, Calcd for $C_{18}H_{17}Cl_2NO_3S$ (m/z): $[M+H]^+$ 398.04 $[M+2+H]^+$ 400.04, Found $[M+H]^+$ 398.09 $[M+2+H]^+$ 400.08; **HRMS (ESI)**: Calcd for $C_{18}H_{17}Cl_2NO_3SNa$ (m/z): $[M]^+$ 420.0204 $[M+2]^+$ 422.0174, Found $[M]^+$ 420.0199 $[M+2]^+$ 422.0172.

2.5.5. Methyl N-(4-chlorobenzyl)-N-(2-((4-chlorophenyl)thio)acetyl)leucinate (8a)

Methyl N-(4-chlorobenzyl)leucinate hydrochloride (**6a**) (1.79 g, 5.8 mmol), triethylamine (0.82 ml, 5.8 mmol), 2-((4-chloro)phenylthio)acetic acid (**2b**) (1.18 g, 5.8 mmol), DIC (0.89 ml, 5.8 mmol), DMAP (0.14 g, 1.2 mmol) were used. The crude product was purified by flash chromatography (SiO_2, petroleum ether/chloroform 1:1) giving compound **8a** as a colorless oil with a 61% yield (1.60 g).

IR (ATR): [cm^{-1}] 2955, 2869, 1741, 1652, 1477, 1202, 1095, 1013, 814; **NMR:** mixture of two rotamers (approx. A/B 2:1) ^{1}**H** (600 MHz, DMSO-d_6) δ [ppm]: rotamer A 7.44-7.38 (m, 4H), 7.37-7.32 (m, 4H), 4.72 (d, *J* = 15.8 Hz, 1H), 4.65 (d, *J* = 17.1 Hz, 1H), 4.14 (s, 1H). 4.01 (d, *J* = 15.2 Hz, 1H), 3.97 (d, *J* = 15.2 Hz, 1H), 3.47 (s, 3H), 1.75-1.67 (m, 1H), 1-50-1.45 (m, 1H), 1.43-1.37 (m, 1H), 0.74 (d, *J* = 6.5 Hz, 3H), 0.69 (d, *J* = 6.6 Hz, 3H); rotamer B 7.44-7.48 (m, 4H), 7.28 (d, *J* = 8.5 Hz, 2H), 7.14 (d, *J* = 8.4 Hz, 2H), 4.82 (dd, *J* = 9.0, 5.2 Hz, 1H), 4.82 (d, *J* = 15.8 Hz, 1H), 4.39 (dd, *J* = 7.9, 6.1 Hz, 2H), 4.17 (d, *J* = 15.9 Hz, 1H), 3.56 (s, 3H), 1.75-1.67 (m, 1H), 1.61-1.55 (m, 1H), 1.36-1.30 (m, 1H), 0.87 (d, *J* = 6.5 Hz, 3H), 0.60 (d, *J* = 6.7 Hz, 3H); ^{13}**C** (151 MHz, DMSO-d_6) δ [ppm]: A and B: 171.3, 171.0, 169.5, 168.4, 137.4, 136.0, 134.6, 134.6, 132.1, 131.2, 130.8, 130.7, 130.2, 129.4, 128.8, 128.8, 128.4, 127.9, 58.7, 56.7, 56.6, 52.3, 50.2, 37.8, 35.9, 24.4, 24.2, 22.5, 22.4, 22.1, 22.0; **LC-MS (DAD/ESI):** t_r = 9.29 min, Calcd for $C_{22}H_{25}Cl_2NO_3S$ (m/z): $[M+H]^+$ 454.10 $[M+2+H]^+$ 456.10, Found $[M+H]^+$ 454.12 $[M+2+H]^+$ 456.11; **HRMS (ESI):** Calcd for

$C_{22}H_{25}Cl_2NO_3SNa$ (m/z): $[M]^+$ 476.0830 $[M+2]^+$ 448.0800, Found $[M]^+$ 476.0825 $[M+2+H]^+$ 478.0796.

2.5.6. Methyl N-(2-((4-chlorophenyl)thio)acetyl)-N-(4-methoxybenzyl)leucinate (8b)

Methyl N-(4-methoxybenzyl)leucinate hydrochloride (**6b**) (2.71 g, 9.0 mmol), triethylamine (1.26 ml, 9.0 mmol), 2-((4-chloro)phenylthio)acetic acid (**2b**) (1.82 g, 9.0 mmol), DIC (1.38 ml, 9.0 mmol), DMAP (0.22 g, 1.8 mmol) were used. The crude product was purified by flash chromatography (SiO_2, petroleum ether/chloroform 1:1) giving compound **8b** as a colorless oil with a 70% yield (2.83 g).

IR (ATR): [cm^{-1}] 2964, 2934, 1741, 1645, 1513, 1467, 1338, 1250, 1158, 1094, 1023, 815; **NMR:** mixture of two rotamers (aprox. A/B 3:1) **^{1}H** (600 MHz, DMSO-d_6) δ [ppm]: rotamer A; 7.33 (s, 4H), 7,26 (d, *J* = 8.7 Hz, 2H), 6.90 (dt, *J* = 8.7, 3.0 Hz, 2H), 4.62 (d, *J* = 16.6 Hz, 1H), 4.57 d, *J* = 16.6 Hz, 1H), 4.34 (dd, *J* = 7.9, 6.1 Hz, 1H), 4.03 (d, *J* = 15.2 Hz, 1H), 3.97 (d, *J* = 15.1 Hz, 1H), 3.72 (s, 3H), 3.46 (s, 3H), 1.73-1.68 (m, 1H), 1.48-1.44 (m, 1H), 1.43-1.36 (m, 1H), 0.72 (d, *J* = 6.5 Hz, 3H), 0.67 (d, *J* = 6.6 Hz, 3H); rotamer B; 7.40 (dt, *J* = 8.8, 2.4 Hz, 2H), 7.38 (dt, *J* = 8.8, 2.4 Hz, 2H), 7.04 (d, *J* = 8.6 Hz, 2H), 6.77 (d, *J* = 8.7 Hz, 2H), 4.78 (dd, *J* = 8.9, 5.3 Hz, 1H), 4.60-4.57 (m, 1H), 4.14-4.11 (m, 3H), 3.69 (s, 3H), 3.51 (s, 3H), 1.69-1.65 (m, 1H), 1.63-1.59 (m, 1H), 1.35-1.29 (m, 1H), 0.85 (d, *J* = 6.6 Hz, 3H), 0.59 (d, *J*= 6.7 Hz, 3H); **^{13}C** (151 MHz, DMSO-d_6) δ [ppm]: rotamer A; 171.5, 168.6, 159.1, 135.2, 131.2, 130.6, 129.4, 129.3, 129.2, 114.3, 56.9, 55.6, 52.2, 50.9, 38.3, 36.5, 24.9, 22.9, 22.5; rotamer B; 171.8, 169.7, 158.5, 135.5, 132.3, 130.3, 130.2, 129.3, 129.0, 113.8, 58.9, 55.8, 52.7, 46.4, 40.5, 38.2, 24.6, 22.9, 22.5; **LC-MS (DAD/ESI):** t_r = 8.72 min, Calcd for $C_{23}H_{28}ClNO_4SNa$ (m/z): $[M]^+$ 472.13, Found $[M]^+$ 471.99; **HRMS (ESI):** Calcd for $C_{23}H_{28}ClNO_4SNa$ (m/z): $[M]^+$ 472.1325, Found $[M]^+$ 472.1318.

2.5.7. Methyl N-(2-((4-chlorophenyl)thio)acetyl)-N-(4-fluorobenzyl)leucinate (8c)

Methyl N-(4-fluorobenzyl)leucinate hydrochloride (**6c**) (1.30 g, 4.5 mmol), triethylamine (0.63 ml, 4.5 mmol), 2-((4-chloro)phenylthio)acetic acid (**2b**) (0,91 g, 4.5 mmol), DIC (0.69 ml, 4.5 mmol), DMAP (0.11 g, 0.9 mmol) were used. The crude product was purified by flash chromatography (SiO_2, petroleum ether/chloroform 1:1) giving compound **8c** as an colorless oil with a 67% yield (1.32 g).

IR (ATR): [cm^{-1}] 2956, 2870, 1741, 1652, 1510, 1223, 1096, 820; **NMR:** mixture of two rotamers (aprox. A/B 2:1) **^{1}H** (600 MHz, DMSO-d_6) δ [ppm]: rotamer A 7.39-7.37 (m, 2H), 7.33 (s, 4H), 7.17 (tt, *J* = 8.8, 1.9 Hz, 2H), 4.70-4.63 (m, 2H), 4.35 (dd, *J*= 7.8, 6.1 Hz 1H).

4.04 (d, *J* = 15.2 Hz, 1H), 3.98 (d, *J* = 15.2 Hz, 1H), 3.46 (s, 3H), 1.74-1.69 (m, 1H), 1.48-1.44 (m, 1H), 1.42-1.35 (m, 1H), 0.72 (d, *J* = 6.5 Hz, 3H), 0.67 (d, *J* = 6.6 Hz, 3H); rotamer B 7.41-7.37 (m, 4H), 7.19-7.15 (m, 2H), 7.03 (t, *J* = 8.8 Hz, 2H), 4.81-4.79 (dd, *J* = 9.1, 5.2 Hz, 1H), 4.70-4.63 (m, 1H), 4.18 (d, *J* = 15.7 Hz, 1H), 4.12 (s, 2H), 3.54 (s, 3H), 1.69-1.67 (m, 1H), 1.61-1.57 (m, 1H), 1.34-1.28 (m, 1H), 0.86 (d, *J* = 6.5 Hz, 3H), 0.58 (d, *J* = 6.7 Hz, 3H); **^{13}C** (151 MHz, DMSO-d_6) δ [ppm]: rotamer A; 171.4, 168.73, 162.8-161.2 (d, J_{FC} = 243.5 Hz), 135.15, 133,5 (d, J_{FC} = 2 Hz), 131.2, 130.6, 130.2 (d, J_{FC} = 8.1 Hz), 129.20, 115.7 (d, J_{FC} = 21.3 Hz), 57.1, 52.2, 50.7, 38.3, 36.5, 24.9, 22.9, 22.4; rotamer B; 171.7, 169.9, 161.5 (d, J_{FC} = 242.5 Hz), 135.1, 135.9 (d, J_{FC} = 1.6 Hz), 131.2, 130.6, 129.9 (d, J_{FC} = 7.9 Hz), 129.3, 115.1 (d, J_{FC} = 21.1 Hz), 59.1, 52.8, 46.4, 50.5, 24.7, 22.9, 22.5; **LC-MS (DAD/ESI):** t_r = 8.86 min, Calcd for $C_{22}H_{25}ClFNO_3S$ (m/z): $[M-H]^-$ 436.11 $[M+2-Cl]^-$ 438.11, Found $[M-H]^-$ 435.97 $[M+2-Cl]^-$ 438.03; **HRMS (ESI):** Calcd for $C_{22}H_{25}ClFNO_3SNa$ (m/z): $[M]^+$ 460.1125 $[M+2]^+$ 462.1096, Found $[M+H]^+$ 460.1133 $[M+2]^+$ 462.1115.

2.5.8. Methyl N-(2-((4-chlorophenyl)thio)acetyl)-N-(isoquinolin-4-ylmethyl)leucinate (8d)

Methyl N-(isoquinolin-4-ylmethyl)leucinate hydrochloride (**6d**) (0.60 g, 1.8 mmol), triethylamine (0.26 ml, 1.8 mmol), 2-((4-chloro)phenylthio)acetic acid (**2b**) (0.38 g, 1.8 mmol), DIC (0.28 ml, 1.8 mmol), DMAP (0.05 g, 0.4 mmol) were used. The crude product was purified by flash chromatography (SiO_2, petroleum ether/chloroform 1:1) giving compound **8d** as a colorless oil with a 80% yield (0.70 g).

IR (ATR): [cm^{-1}] 2955, 2915, 1737, 1651, 1497, 1477, 1201, 1095, 1048, 826; **NMR**: mixture of two rotamers (approx. A/B 3:1) **^{1}H** (600 MHz, DMSO-d6) δ [ppm]: rotamer A; 8.91 (d, *J* = 2.2 Hz, 1H), 8.29 (d, *J* = 1.3 Hz, 1H), 8.02-7.94 (m, 2H), 7.55-7.50 (m, 1H), 7.62-7.57 (m, 1H), 7.37-7.30 (m, 4H), 4.96 (d, *J* = 17.2 Hz, 1H), 4.89 (d, *J* = 17.2 Hz, 1H), 4.42 (dd, *J* = 8.2, 5.9 Hz, 1H), 4.17 (d, *J* = 15.2 Hz, 1H), 4.11 (d, *J* = 15.2 Hz, 1H), 3.40 (s, 3H), 1.72-1.70 (m, 1H), 1.56-1.51 (m, 1H), 1.45-1.38 (m, 1H), 0.70 (d, *J* = 6.5 Hz, 3H), 0.64 (d, *J* = 6.6 Hz, 3H); rotamer B; 8.75 (d, *J* = 2.1 Hz, 1H), 8.21 (s, 1H), 8.02-7.94 (m, 2H), 7.55-7.50 (m, 1H), 7.62-7.57 (m, 1H), 7.41-7.40 (dt, *J* = 8.7, 2.5 Hz, 2H), 7.33-7.31 (m, 2H), 4.81 (d, *J* = 16.0 Hz, 1H), 4.47 (d, *J* = 16.0 Hz, 1H), 4.18 (m, 1H), 3.61 (s, 2H), 3.50 (s, 3H), 1.79-1.73 (m, 1H), 1.56-1.51 (m, 1H), 1.45-1.36 (m, 1H), 0.99 (d, *J* = 6.5 Hz, 3H), 0.87 (d, *J* = 6.5 Hz, 3H) ; **^{13}C** (151 MHz, DMSO-d6) δ [ppm]: rotamer A; 171.5, 168.9, 151.3, 147.4, 135.1, 134.7, 131.2, 130.7, 130.0, 129.2, 129.1, 128.4, 128.1, 127.7, 127.4, 57.1, 52.2, 49.5, 38.2, 36.3, 24.9, 23.0, 22.4; rotamer B; 171.8, 170.2, 152.1, 147.0, 135.2, 134.3, 131.8, 130.5, 130.4, 129.6, 129.3, 128.3, 127.9, 127.7, 127.2, 59.1, 58.9, 52.8, 38.3, 36.2, 23.8, 22.9, 22.5

2.6. General synthetic procedure for cyclisation reaction and analytical data (compounds 9a-d and 10a-d)

The corresponding amide **7a-d** or **8a-d** (1 eq.) and anhydrous 1,4-dioxane (30 ml) were placed in a round bottom flask equipped with $CaCl_2$ tube and reflux condenser. Then fresh potassium *tert*-butoxide (1.5 eq.) was added in one portion and reaction was heated at 80ºC for 1.5 hours. After this time reaction was cooled down, poured into 1 M HCl solution (15 ml) and extracted with ethyl acetate (3 x 20 ml). Organic layers were collected, washed with water and brine, dried over anhydrous $MgSO_4$ and evaporated.

2.6.1. 3-((4-Chlorophenyl)thio)-4-hydroxy-5-isobutyl-1,5-dihydro-2*H*-pyrrol-2-one (9a)

Compound **7a** (0.23 g 0.7 mmol) and ^{t}BuOK (0.12 g, 1.1 mmol) were used. The crude product was purified by flash chromatography (SiO_2, ethyl acetate/methanol 1:1) as a result giving compound **9d** as colorless solid with a 37% yield (0.07 g). The product was recrystallized from cyclohexane/acetone.

IR (ATR): [cm^{-1}] 3179, 2956, 2932, 2907, 2869, 2617, 1655, 1588, 1476, 1390, 1090, 823; **NMR:** ^{1}**H** (600 MHz, DMSO-d_6) δ [ppm]: 12.24 (s, 1H), 7.94 (s, 1H), 7.34-7.30 (m, 2H), 7.12-7.08 (m, 2H), 4.11 (dd, *J* = 9.8; 2.6 Hz, 1H), 1.86-1.78 (m, 1H), 1.66-1.57 (m, 1H), 1.33-1.27 (m, 1H), 0.91 (dd, *J* = 6.5; 4.9 Hz, 6H); ^{13}**C** (151 MHz, DMSO-d_6) δ [ppm]: 181.6, 171.2, 136.6, 129.4, 128.7, 127.2, 92.2, 55.1, 41.7, 24.4, 23.7, 21.5 **LC-MS (DAD/ESI):** t_r = 5.54 min, Calcd for $C_{14}H_{16}ClNO_2S$ (m/z): $[M-H]^-$ 296.05 $[M+2-H]^-$ 298.05, Found $[M-H]^-$ 296.00 $[M+2-H]^-$ 298.00; **HRMS (ESI)**: Calcd for $C_{14}H_{16}ClNO_2SNa$ (m/z): $[M]^+$ 320.0488 $[M+2]^+$ 322.0458, Found $[M]^+$ 320.0483 $[M+2]^+$ 322.0453.

2.6.2. 5-Benzyl-4-hydroxy-3-(phenylthio)-1,5-dihydro-2*H*-pyrrol-2-one (9b)

Compound **7b** (0.59 g 1.3 mmol) and ^{t}BuOK (0.22 g, 2.0 mmol) were used. The crude product was purified by flash chromatography (SiO_2, ethyl acetate/methanol 1:1) as a result giving compound **9b** as colorless solid with a 83% yield (0.33 g). The product was recrystallized from cyclohexane/ethyl acetate.

IR (ATR): [cm^{-1}] 3434, 3395, 3216, 3028, 1691, 1626, 1579, 1496, 1241, 1133, 1087, 1024, 904

NMR: ^{1}**H** (600 MHz, DMSO-d_6) δ [ppm]: 12.21 (s, 1H), 7.82 (s, 1H), 7.32-7.26 (m, 3H), 7.25-7.21 (m, 2H), 7.07-7.03 (m, 2H), 7.02-6.96 (m, 1H), 6.49-6.42 (m, 2H), 4.43 (dd, *J* = 4.3, 3.5 Hz, 1H), 3.06 (qd, *J* = 13.8, 4.3 Hz, 2H); ^{13}**C** (151 MHz, DMSO-d_6) δ [ppm]: 179.1, 170.9, 137.0, 135.3, 130.0, 128.6, 127.9, 126.6, 124.8, 124.3, 93.6, 56.9, 36.0; **LC-MS**

(DAD/ESI): t_r = 4.77 min, Calcd for $C_{17}H_{15}NO_2S$ (m/z): $[M+H]^+$ 298.09 Found $[M+H]^+$ 297.93; **HRMS (ESI):** Calcd for $C_{17}H_{15}NO_2SNa$ (m/z): $[M]^+$ 320.0721 Found $[M]^+$ 320.0719.

2.6.3. 5-Benzyl-3-((4-chlorophenyl)thio)-4-hydroxy-1,5-dihydro-2*H*-pyrrol-2-one (9c)

Compound **7c** (0.45 g 1.2 mmol) and ^{t}BuOK (0.21 g, 1.9 mmol) were used. The crude product was purified by flash chromatography (SiO_2, ethyl acetate/methanol 1:1) as a result giving compound **9c** as colorless solid with a 64% yield (0.26 g). The product was recrystallized from cyclohexane/acetone [36].

IR (ATR): [cm^{-1}] 3362, 3030, 2577 (br), 1661, 1595, 1472, 1387, 1359, 1224, 1089, 101, 822, 698; **NMR: ^{1}H** (600 MHz, DMSO-d_6) δ [ppm]:12.32 (s, 1H), 7.85 (s, 1H). 7.34-7.27 (m, 3H), 7.24-7.21 (m, 2H), 7.07 (dt, *J* = 8.6, 2.0, 2H), 4.43 (t, *J* = 3.9 Hz, 1H), 3.11-2.99 (m, 2H); **^{13}C** (151 MHz, DMSO-d_6) δ [ppm]: 179.2, 170.6, 136.2, 135.3, 130.0, 128.8, 128.4, 127.9, 126.6, 126.5, 56.9, 35.9; **LC-MS (DAD/ESI):** t_r = 5.42 min, Calcd for $C_{17}H_{14}ClNO_2S$ (m/z): $[M-H]^-$ 330.03 $[M+2-H]^-$ 332.02, Found $[M-H]^-$ 330.04 $[M+2-H]^-$ 332.03; **HRMS (ESI):** Calcd for $C_{17}H_{14}ClNO_2SNa$ (m/z): $[M]^+$ 354.0331 $[M+2]^+$ 356.0302, Found $[M]^+$ 354.0327 $[M+2]^+$ 356.0300.

2.6.4. 5-(4-Chlorobenzyl)-3-((4-chlorophenyl)thio)-4-hydroxy-1,5-dihydro-2*H*-pyrrol-2-one (9d)

Compound **7d** (1.12 g 2.8 mmol) and ^{t}BuOK (0.47 g, 4.2 mmol) were used. The crude product was purified by flash chromatography (SiO_2, ethyl acetate/methanol 1:1) as a result giving compound **9d** as colorless solid with a 57% yield (0.55 g). The product was recrystallized from cyclohexane/acetone.

IR (ATR): [cm^{-1}] 3352, 2927, 2584 (br), 1683, 1661, 1595, 1492, 1475, 1387, 1229, 1091, 1012, 854, 817, 758; **NMR: ^{1}H** (600 MHz, DMSO-d_6) δ [ppm]: 12.39 (s, 1H), 7.88 (s, 1H), 7.40-7.33 (m, 2H), 7.25-7.19 (m, 2H), 7.11 (dt, *J* = 8.7, 2.1 Hz, 2H), 6.43 (dt, *J* = 8.8, 2.1 Hz, 2H), 4.45 (t, *J* = 3.9 Hz, 1H), 3.09 (dd, *J* = 13.8, 4.1 Hz, 1H), 3.01 (dd, *J* = 13.8, 4.2 Hz, 1H); **^{13}C** (151 MHz, DMSO-d_6) δ [ppm]: 179.0, 170.5, 136.2, 134.3, 131.9, 131.4, 129.0, 128.3, 128.0, 126.5, 93.5, 56.6, 35.1, 30.7; **LC-MS (DAD/ESI)**: t_r = 5.88 min, Calcd for $C_{17}H_{13}Cl_2NO_2S$ (m/z): $[M-H]^-$ 364.00 $[M+2-H]^-$ 366.00, Found $[M-H]^-$ 363.99 $[M+2-H]^-$ 365.92; **HRMS (ESI)**: Calcd for $C_{17}H_{13}Cl_2NO_2SNa$ (m/z): $[M]^+$ 387.9942 $[M+2]^+$ 389.9912, Found $[M]^+$ 387.9935 $[M+2]^+$ 389.9909.

2.6.5. 1-(4-Chlorobenzyl)-3-((4-chlorophenyl)thio)-4-hydroxy-5-isobutyl-1,5-dihydro-2*H*-pyrrol-2-one (10a)

Compound **8a** (1.11 g 2.5 mmol) and ^{t}BuOK (0.42 g, 3.7 mmol) were used. The crude product was purified by flash chromatography (SiO_2, ethyl acetate/methanol 1:1) as a result giving compound **10a** as colorless solid with a 57% yield (0.60 g). The product was recrystallized from cyclohexane/acetone.

IR (ATR): [cm^{-1}] 2951, 2925, 2868, 2602, 1640, 1475, 1405, 1382, 1090, 818; **NMR: ^{1}H** (600 MHz, DMSO-d_6) δ [ppm]: 12.57 (s, 1H), 7.41 (dt, *J* = 8.4, 1.9 Hz, 2H), 7.35 (dt, *J* = 8.7, 2.1 Hz, 2H), 7.25 (d, *J* = 8.5 Hz, 2H), 7.14 (dt, *J* = 8.7, 2.0 Hz, 2H), 4.79 (d, *J* = 15.8 Hz, 1H), 4.24 (d, *J* = 15.8 Hz, 1H), 3.99 (t, *J* = 4.8 Hz, 1H), 1.71-1.61 (m, 2H), 1.57 (sept, *J* = 6.6 Hz, 1H), 0.77 (dd, *J* = 6.6, 1.5 Hz, 6H); **^{13}C NMR** (151, MHz, DMSO-d_6) δ [ppm]: 180.1, 169.9, 137.2, 136.3, 131.8, 129.7, 129.5, 128.9, 128.6, 127.5, 92.6, 58.6, 42.9, 23.4, 23.1; **LC-MS (DAD/ESI):** t_r = 7.98 min, Calcd for $C_{21}H_{21}Cl_2NO_2S$ (m/z): $[M-H]^-$ 419.05 $[M+2-H]^-$ 421.05, Found $[M-H]^-$ 419.95 $[M+2-H]^-$ 422.01; **HRMS (ESI):** Calcd for $C_{21}H_{21}Cl_2NO_2SNa$ (m/z): $[M]^+$ 444.0568 $[M+2]^+$ 446.0538, Found $[M]^+$ 444.0565 $[M+2]^+$ 446.0542.

2.6.6. 3-((4-Chlorophenyl)thio)-4-hydroxy-5-isobutyl-1-(4-metoxybenzyl)-1,5-dihydro-2*H*-pyrrol-2-one (10b)

Compound **8b** (2.00 g 4.0 mmol) and ^{t}BuOK (0.67 g, 6.0 mmol) were used. The crude product was purified by flash chromatography (SiO_2, ethyl acetate/methanol 1:1) as a result giving compound **10b** as colorless solid with a 79% yield (1.32 g). The product was recrystallized from cyclohexane/acetone.

IR (ATR): [cm^{-1}] 2952, 2867, 2837, 2584, 1611, 1513, 1379, 1247, 818; **NMR: ^{1}H** (600 MHz, DMSO-d_6) δ [ppm]: 12.45 (s, 1H), 7.35 (dt, *J* = 8.8, 2.8 Hz, 2H), 7.16-7.10 (m, 4H), 6.90 (dt, *J* = 8.7, 2.9 Hz, 2H), 4.82 (d, *J* = 15.3 Hz, 1H), 4.08 (d, *J* = 15.3 Hz, 1H), 3.89 (t, *J* = 4.4 Hz, 1H), 3.73 (s, 3H), 1.68-1.54 (m, 3H), 0.79-0.76 (dd, *J* = 6.2, 2.7 Hz, 6H); **^{13}C** (151, MHz, DMSO-d_6) δ [ppm]: 181.0, 170.8, 159.5, 137.4, 130.9, 130.8, 130.0, 129.9, 128.5, 115.1, 93.9, 59.1, 56.2, 44.0, 38.3, 24.5, 24.2; **LC-MS (DAD/ESI):** t_r = 7.52 min, Calcd for $C_{22}H_{24}ClNO_3S$ (m/z): $[M-H]^-$ 416.11 $[M+2-H]^-$ 418.11, Found $[M-H]^-$ 416.03 $[M+2-H]^-$ 417.89; **HRMS (ESI):** Calcd for $C_{22}H_{24}ClNO_3SNa$ (m/z): $[M]^+$ 440.1063 $[M+2]^+$ 442.1034, Found $[M]^+$ 440.1058 $[M+2]^+$ 442.1038.

2.6.7. 3-((4-Chlorophenyl)thio)-1-(4-fluorobenzyl)- 4-hydroxy-5-isobutyl-1,5-dihydro-2*H*-pyrrol-2-one (10c)

Compound **8c** (1.25 g 2.9 mmol) and ^{t}BuOK (0.49 g, 4.4 mmol) were used. The crude product was purified by flash chromatography (SiO_2, ethyl acetate/methanol 1:1) as a result giving compound **10c** as colorless solid with a 81% yield (0.94 g). The product was recrystallized from cyclohexane/acetone.

IR (ATR): [cm^{-1}]2956, 2868, 2562 (br), 1603, 1508, 1475, 1379, 1225, 1088, 1011, 810; **NMR: ^{1}H** (600 MHz, DMSO-d_6) δ [ppm]: 12.52 (s, 1H), 7.34 (dt, *J* = 8.7, 2.8 Hz, 2H), 7.26 (dd, *J* = 8.6, 5.6 Hz, 2H), 7.17-7.12 (m, 4H), 4.78 (d, *J* = 15.6 Hz, 1H), 4.21 (d, *J* = 15.6 Hz, 1H), 3.96 (t, *J* = 4.7 Hz, 1H), 1.70-1.63 (m, 2H), 1.57 (m, *J* = 6.6 Hz, 1H), 0.75 (dd, *J* = 6.5, 2.6 Hz, 6H); **^{13}C** (151, MHz, DMSO-d_6) δ [ppm]: 180.5, 170.3, 162.6-161.0 (d, J_{FC} = 242.8 Hz), 136.7, 134.7 (d, J_{FC} = 2.1 Hz), 130.2, 130.1, 130.0, 129.4 (d, J_{FC} = 15.2 Hz), 127.9, 115.8 (d, J_{FC} = 21.4 Hz), 93.2, 58.9, 43.3, 24.0, 37.7, 23.5; **LC-MS (DAD/ESI):** t_r = 7.52 min, Calcd for $C_{21}H_{21}ClFNO_2S$ (m/z): $[M-H]^-$ 404.09 $[M+2-H]^-$ 406.09, Found $[M-H]^-$ 404.07 $[M+2-H]^-$ 405.93; **HRMS (ESI):** Calcd for $C_{21}H_{21}ClFNO_2SNa$ (m/z): $[M]^+$ 428.0863 $[M+2]^+$ 430.0834, Found $[M]^+$ 428.0860 $[M+2]^+$ 430.0836.

2.6.8. 3-((4-Chlorophenyl)thio)-4-hydroxy-5-isobutyl-1-(isoquinolin-4-ylmethyl)-1,5-dihydro-2*H*-pyrrol-2-one (10d)

Compound **8d** (0.18 g 0.4 mmol) and ^{t}BuOK (0.07 g, 0.6 mmol) were used. The crude product was purified by flash chromatography (SiO_2, ethyl acetate/methanol 1:1) as a result giving compound **10d** as yellowish solid with a 19% yield (0.03 g).

IR (ATR): [cm^{-1}] 2956, 2927, 2616 (br), 1674, 1599, 1474, 1385, 1243, 1089, 1010, 812, 753; **NMR: ^{1}H** (600 MHz, DMSO-d_6) δ [ppm]: 12.59 (s, 1H), 8.83 (d, *J* = 2.1 Hz, 1H), 8.20 (d, *J* = 1.4 Hz, 1H), 8.03-7.96 (m, 2H), 7.75 (dt, *J* = 8.4, 1.4 Hz, 1H), 7.62 (dt, *J* = 6.9, 1.1 Hz, 1H), 7.33 (dt, *J* = 8.6, 2.7 Hz, 2H), 7.17 (dt, *J* = 8.6, 2.6 Hz, 2H), 4.98 (d, *J* = 15.9 Hz, 1H), 4.50 (d, *J* = 15.9 Hz, 1H), 4.16 (t, *J* = 4.4 Hz, 1H), 1.77-1.71 (m, 2H), 1.59 (sept. *J*= 6.5 Hz, 1H), 0.73 (t, *J* = 6.1 Hz, 6H); **^{13}C** NMR (151, MHz, DMSO-d_6) δ [ppm]: 181.3, 171.0, 151.8, 147.7, 137.3, 135.5, 132.4, 130.8, 130.6, 129.9, 129.6, 129.1, 128.6, 128.1, 93.8, 59.8, 42.6, 38.2, 24.5, 24.2; **LC-MS (DAD/ESI):** t_r = 6.05 min, Calcd for $C_{24}H_{23}ClN_2O_2S$ (m/z): $[M-H]^-$ 437.11 $[M+2-H]^-$ 439.1061, Found $[M-H]^-$ 436.83 $[M+2-H]^-$ 438.03; **HRMS (ESI):** Calcd for $C_{24}H_{23}ClN_2O_2SNa$ (m/z): $[M]^+$ 461.1066 $[M+2]^+$ 463.1037, Found $[M]^+$ 461.1041 $[M+2]^+$ 463.1017.

2.7. General synthetic procedure for Williamson ether synthesis and analytical data (compounds 11a-c and 12a-d)

The corresponding derivatives of tetramic acid **9a** or **10a-b** (1 eq.), anhydrous potassium carbonate (2 eq.) and anhydrous acetone (15 ml) were placed in a round bottom flask equipped with $CaCl_2$ tube and reflux condenser and heated at 60°C for 15 minutes. Then suitable alkyl bromide (1 eq.) in 5 ml of anhydrous acetone was added. The reaction was refluxed for 3 hours. After this time the reaction was cooled down, poured into saturated ammonium chloride solution (20 ml) and extracted with ethyl acetate (3 x 15 ml). Organic layers were collected, washed with water and brine, dried over anhydrous $MgSO_4$ and evaporated.

2.7.1. 3-((4-Chlorophenyl)thio)-5-isobutyl-4-(isopentyloxy)-1,5-dihydro-2*H*-pyrrol-2-one (11a)

Compound **9a** (0.15 g 0.5 mmol), isoamyl bromide (0.06 ml, 0.5 mmol) and potassium carbonate (0.14 g, 1.0 mmol) were used. The crude product was purified by flash chromatography (SiO_2, hexane/ethyl acetate 1:1) as a result giving compound **11a** as colorless solid with a 8% yield (0.015 g).

IR (ATR) [cm^{-1}]: 3193, 3077, 2958, 1683, 1596, 1473, 1320, 1089, 1055, 1007, 815; **NMR: ^{1}H** (600 MHz, DMSO-d_6) δ [ppm]: 8.20 (s, 1H), 7.35 (dt, *J* = 8.7, 2.1 Hz, 2H), 7.14 (dt, *J* = 8.7, 2.1 Hz, 2H), 4.56 (t, *J* = 6.7 Hz, 2H), 4.20-4.16 (m, 1H), 1.84-1.76 (m, 1H), 1.67-1.59 (m, 1H), 1.58-1.45 (m, 3H), 1.34 (ddd, *J* = 13.7, 9.4, 4.5 Hz, 1H), 0.91 (dd, *J* = 6.6, 2.5 Hz, 6H), 0.81 (t, *J* = 6.8 Hz, 3H); **^{13}C** (151 MHz, DMSO-d_6) δ [ppm]: 179.1, 170.5, 136.7, 129.8, 129.0, 127.2, 94.0, 69.9, 55.3, 55.3, 41.9, 37.5, 24.4, 24.3, 23.6, 22.3, 22.1, 21.6; **LC-MS (DAD/ESI):** t_r = 9.10 min, Calcd for $C_{19}H_{26}ClNO_2S$ (m/z): $[M+H]^+$ 368.14 $[M+2+H]^+$ 370.14, Found $[M+H]^+$ 368.14 $[M+2+H]^+$ 370.14; **HRMS (ESI):** Calcd for $C_{19}H_{26}ClNO_2SNa$ (m/z): $[M]^+$ 390.1270 $[M+2]^+$ 392.1241, Found $[M]^+$ 390.1265 $[M+2]^+$ 392.1238.

2.7.2.Methyl 2-((4-((4-chlorophenyl)thio)-2-isobutyl-5-oxo-2,5-dihydro-1*H*-pyrrol-3-yl)oxy)acetate (11b)

Compound **9a** (0.15 g 0.5 mmol), methyl bromoacetate (0.05 ml, 0.5 mmol) and potassium carbonate (0.14 g, 1.0 mmol) were used. The crude product was purified by flash chromatography (SiO_2, hexane/ethyl acetate 1:1) as a result giving compound **11b** as colorless solid with a 21% yield (0.04 g).

IR (ATR): [cm^{-1}] 3187, 3080, 2953, 1761, 1682, 1605, 1477, 1338, 1224, 1206, 1090, 1006, 820; **NMR: ^{1}H** (600 MHz, DMSO-d_6) δ [ppm]: 8.32 (s, 1H), 7.35 (dt, *J* = 8.6, 2.0 Hz, 2H), 7.11 (dt, *J* = 8.7, 2.0 Hz, 2H), 5.26 (d, *J* = 16.5 Hz, 1H), 5.18 (d, *J* = 16.5 Hz, 1H), 4.22 (ddd,

J = 9.7, 3.4, 1.2 Hz, 1H), 3.49 (s, 3H), 1.89-1.79 (m, 1H), 1.62 (ddd, *J* = 13.5, 9.9, 3.5 Hz, 1H), 1.38 (ddd, *J* = 13.8, 9.7, 4.3 Hz, 1H), 0.92 (dd, *J* = 6.6, 2.5 Hz, 3H); **^{13}C** (151 MHz, DMSO-d_6) δ [ppm]: 177.7, 169.9, 167.6, 135.8, 130.0, 128.8, 127.5, 95.2, 66.9, 55.3, 52.0, 42.0, 24.4, 23.6, 21.5; **LC-MS (DAD/ESI):** t_r = 6.70 min, Calcd for $C_{17}H_{20}ClNO_4S$ (m/z): $[M+H]^+$ 370.09 $[M+2+H]^+$ 372.08, Found $[M+H]^+$ 370.11 $[M+2+H]^+$ 372.10; **HRMS (ESI)**: Calcd for $C_{17}H_{20}ClNO_4SNa$ (m/z): $[M]^+$ 392.0699 $[M+2]^+$ 394.0670, Found $[M]^+$ 392.0691 $[M+2]^+$ 394.0664.

2.7.3.4-((4-Chlorobenzyl)oxy)-3-((4-chlorophenyl)thio)-5-isobutyl-1,5-dihydro-2*H*-pyrrol-2-one (11c)

Compound **9a** (0.15 g 0.5 mmol), 4-chlorobenzyl bromide (0.10 g, 0.5 mmol) and potassium carbonate (0.14 g, 1.0 mmol) were used. The crude product was purified by flash chromatography (SiO_2, hexane/ethyl acetate 1:1) as a result giving compound as colorless solid **16c** with a14% yield (0.03 g).

IR (ATR): [cm^{-1}] 3203, 3078, 2959, 1683, 1604, 1324, 1089, 807; **NMR: ^{1}H** (600 MHz, DMSO-d_6) δ [ppm]: 8.26 (s, 1H), 7.38-7.34 (m, 4H), 7.29 (dt, *J* = 8.7, 2.8 Hz, 2H), 7.08 (dt, *J* = 8.7, 2.8 Hz, 2H), 5.60 (d, *J* = 12.2 Hz, 1H), 5.57 (d, *J* = 12.2 Hz, 1H), 4,25 (ddd, *J* = 9.6, 3.4, 1.2 Hz, 1H), 1.83-1.76 (m, 1H), 1.61-1.57 (m, 1H), 1.38-1.33 (m, 1H), 0.90 (dd, *J* = 6.7, 1.4 Hz, 6H); **^{13}C** NMR (151 MHz, DMSO-d_6) δ [ppm]: 178.9, 170.6, 136.6, 135.3, 133.4, 130.3, 129.9, 129.3, 129.0, 128.0, 95.8, 72.1, 55.8, 42.3, 24.9, 24.0, 21.0; **LC-MS (DAD/ESI)**: t_r = 8.79 min, Calcd for $C_{21}H_{21}Cl_2NO_2S$ (m/z): $[M+H]^+$ 422.07 $[M+2+H]^+$ 424.07, Found $[M+H]^+$ 421.88 $[M+2+H]^+$ 423.81; **HRMS (ESI)**: Calcd for $C_{21}H_{21}Cl_2NO_2SNa$ (m/z): $[M]^+$ 444.0568 $[M+2]^+$ 446.0538, Found $[M]^+$ 444.0562 $[M+2]^+$ 446.0537.

2.7.4. Methyl 2-((1-(4-chlorobenzyl)-4-((4-chlorophenyl)thio)-2-isobutyl-5-oxo-2,5-dihydro-1*H*-pyrrol-3-yl)oxy)acetate (12a)

Compound **10a** (0.2 g 0.47 mmol), methyl bromoacetate (0.045 ml, 0.47 mmol) and potassium carbonate (0.13 g, 0.94 mmol) were used. The crude product was purified by flash chromatography (SiO_2, hexane/ethyl acetate 3:1) as a result giving compound **12a** as colorless solid with a 21% yield (0.05 g).

IR (ATR): [cm^{-1}] 2963, 1754, 1682, 1614, 1222, 1090, 813; **NMR: ^{1}H** (600 MHz, DMSO-d_6) δ [ppm]: 7.43 (dt, *J* = 8.5, 1.9 Hz, 2H), 7.38 (dt, *J* = 8.7, 2.0 Hz, 2H), 7.24 (d, *J* = 8.5 Hz, 2H), 7.14 (dt, *J* = 8.8, 2.1 Hz, 2H), 5.27 (d, *J* = 16.4 Hz, 1H), 5.20 (d, *J* = 16.4 Hz, 1H), 4.79

(d, *J* = 15.8 Hz, 1H), 4.26 (d, *J* = 15.8 Hz, 1H), 4.10 (t, *J* = 4.8 Hz, 1H), 3.49 (s, 3H), 1.78-1.71 (m, 1H), 1.68-1.61 (m, 1H), 0.81 (dd, *J* = 6.3, 3.0 Hz, 6H); **^{13}C NMR** (151 MHz, DMSO-d_6) δ [ppm]: 176.9, 169.0, 168.1, 137.1, 135.9, 132.4, 130.8, 129.9, 129.4, 129.1, 128.2, 95.8, 67.8, 58.9, 52.4, 43.4, 38.1, 24.2, 23.4; **LC-MS (DAD/ESI):** t_r = 8.91 min, Calcd for $C_{24}H_{25}Cl_2NO_4S$ (m/z): $[M+H]^+$ 494.09 $[M+2+H]^+$ 496.09, Found $[M+H]^+$ 494.13 $[M+2+H]^+$ 496.12; **HRMS (ESI)(:** Calcd for $C_{24}H_{25}Cl_2NO_4SNa$ (m/z): $[M]^+$ 516.0779 $[M+2]^+$ 518.0750, Found $[M]^+$ 516.0775 $[M+2]^+$ 518.0760.

2.7.5. 1-(4-Chlorobenzyl)-4-((4-chlorobenzyl)oxy)-3-((4-chlorophenyl)thio)-5-isobutyl-1,5-dihydro-2*H*-pyrrol-2-one (12b)

Compound **10a** (0.18 g 0.43 mmol), 4-chlorobenzyl bromide (0.09 g, 0.43 mmol) and potassium carbonate (0.12 g, 0.86 mmol) were used. The crude product was purified by flash chromatography (SiO_2, hexane/ethyl acetate 3:1) as a result giving compound **12b** as colorless solid with a 35% yield (0.034 g).

IR (ATR): [cm^{-1}] 2960, 2932, 1695, 1608, 1319, 1087, 806; **NMR: ^{1}H** (600 MHz, DMSO-d_6) δ [ppm]: 7.42-7.38 (m, 4H), 7.37-7.34 (m, 4H), 7.24 (d, *J* = 8.4 Hz, 2H), 7.16 (dt, *J* = 8.6, 1.9 Hz, 2H), 5.61 (q, *J* = 12.1 Hz, 2H), 4.78 (d, *J* = 15.8 Hz, 1H), 4.25 (d, *J* = 15.8 Hz, 1H), 4.16 (dd, *J* = 5.4, 4.0 Hz, 1H), 1.75-1.69 (m, 1H), 1.65-1.60 (m, 1H), 1.60-1.53 (m, 1H), 0.76 (dd, *J* = 6.5, 2.9 Hz, 6H); **^{13}C** (151 MHz, DMSO-d_6) δ [ppm]: 177.6, 169.4, 137.2, 136.3, 135.0, 133.5, 132.3, 130.7, 130.1, 130.0, 129.5, 129.1, 129.0, 128.3, 96.0, 72.6, 59.1, 43.4, 37.8, 24.0, 23.4; **LC-MS (DAD/ESI):** t_r = 10.40 min, Calcd for $C_{28}H_{26}Cl_3NO_2S$ (m/z): $[M+H]^+$ 546.08 $[M+2+H]^+$ 548.08, Found $[M+H]^+$ 546.11$[M+2+H]^+$ 548.10; **HRMS (ESI):** Calcd for $C_{28}H_{26}Cl_3NO_2SNa$ (m/z): $[M]^+$ 568.0648 $[M+2]^+$ 570.0618, Found $[M]^+$ 568.0643$[M+2]^+$ 570.0619.

2.7.6.4-((4-Chlorobenzyl)oxy)-3-((4-chlorophenyl)thio)-5-isobutyl-1-(4-methoxybenzyl)-1,5-dihydro-2*H*-pyrrol-2-one (12c)

Compound **10b** (0.2 g 0.48 mmol), 4-chlorobenzyl bromide (0.1 g, 0.48 mmol) and potassium carbonate (0.13 g, 0.96 mmol) were used. The crude product was purified by flash chromatography (SiO_2, hexane/ethyl acetate 3:1) as a result giving compound **12c** as colorless solid with a 58% yield (0.15 g).

IR (ATR): [cm^{-1}] 3000, 2964, 2921, 1691, 1615, 1510, 1318, 1247, 818, 805; **NMR: ^{1}H** (600 MHz, DMSO-d_6) δ [ppm]: 7.38-7.33 (m, 6H), 7.16-7.11 (m, 4H), 6.90 (dt, *J*= 8.7, 2.9 Hz, 2H), 5.61 (d, *J*= 12.0 Hz, 1H), 5.57 (d, *J*= 12.0 Hz, 1H), 4.79 (d, *J*= 15.3 Hz, 1H), 4.11 (d, *J*=

15.3 Hz, 1H), 4.06-4.04 (m, 1H), 3.72 (s, 3H), 1.73-1.69 (m, 1H), 1.64-1.55 (m, 2H), 0.76 (dd, *J*= 8.9, 6.3 Hz, 6H); **^{13}C** (151 MHz, DMSO-d_6) δ [ppm]: 177.4, 169.3, 159.0, 136.4, 135.1, 133.5, 130.7, 130.1, 129.9, 129.5, 129.4, 128.9, 128.4, 114.5, 96.0, 72.6, 58.8, 55.6, 43.4, 37.8, 24.1, 23.6, 23.4; **LC-MS (DAD/ESI):** t_r = 10.40 min, Calcd for $C_{29}H_{29}Cl_2NO_3S$ (m/z): $[M+H]^+$ 542.13$[M+2+H]^+$ 544.13, Found $[M+H]^+$ 542.05$[M+2+H]^+$ 543.91; **HRMS (ESI):** Calcd for $C_{29}H_{29}Cl_2NO_3SNa$ (m/z): $[M]^+$ 564.1143 $[M+2]^+$ 566.1113, Found $[M]^+$ 564.1137$[M+2]^+$ 566.1118.

2.7.7. 3-((4-Chlorophenyl)thio)-5-isobutyl-1-(4-methoxybenzyl)-4-phenethoxy-1,5-dihydro-2*H*-pyrrol-2-one (12d)

Compound **10b** (0.2 g 0.48 mmol), 2-phenylethyl bromide (0.07 ml, 0.48 mmol) and potassium carbonate (0.13 g, 0.96 mmol) were used. The crude product was purified by flash chromatography (SiO_2, hexane/ethyl acetate 3:1) as a result giving compound **12d** as colorless solid with a 63% yield (0.158 g).

IR (ATR): [cm^{-1}] 2956, 1684, 1596, 1512, 1473, 1406, 1316, 1247, 1173, 1087, 1039, 1007, 812; **NMR: ^{1}H** (600 MHz, DMSO-d_6) δ [ppm]: 7.36 (dt, *J* = 5.7, 2.8 Hz, 2H), 7.23 (m, 2H), 7.18-7.14 (m, 5H), 7.10 (dt, *J* = 8.6, 2.8 Hz, 2H), 6.88 (dt, *J* = 8.7, 2.9 Hz, 2H), 4.80-4.78 (m, 1H), 4.76 (d, *J* = 15.3 Hz, 1H), 4.73-4.69 (m, 1H), 4.06 (d, *J* = 15.3 Hz, 1H), 3.87 (m, 1H), 3.71 (s, 3H), 2.95-2.89 (m, 2H), 1.58-1.53 (m, 1H), 1.51-1.43 (m, 2H), 0.69 (d, *J* = 6.4 Hz, 3H), 0.59 (d, *J* = 6.3 Hz, 3H); **^{13}C** (151 MHz, DMSO-d_6) δ [ppm]: 177.9, 169.4, 159.0, 137.9, 136.8, 130.6, 129.9, 129.5, 129.4, 129.4, 128.8, 128.0, 126.9, 114.5, 94.6, 72.8, 58.7, 55.6, 43.4, 40.5, 37.8, 35.6, 24.0, 23.5, 23.0; **LC-MS (DAD/ESI):** t_r = 9.87 min, Calcd for $C_{30}H_{32}ClNO_3S$ (m/z): $[M+H]^+$ 522.19$[M+2+H]^+$ 524.18, Found $[M+H]^+$ 522.11b $[M+2+H]^+$ 524.04; **HRMS (ESI):** Calcd for $C_{30}H_{32}ClNO_3SNa$ (m/z): $[M]^+$ 544.1689 $[M+2]^+$ 546.1660, Found $[M]^+$ 544.1681$[M+2]^+$ 546.1659.

2.8. DFT calculations

DFT calculations based on the Becke88 exchange [37] and Perdew86 correlation [38,39] functional (BP86) were performed using the Amsterdam Density Functional (ADF) program, versions 2014 [40-42]. A standard triple-ζ STO basis containing one set of polarization functions (TZP) as well as standard triple-ζ STO basis containing a double set of polarization functions (TZP), was adopted for all elements. The scalar relativistic effects by employing the Zero Order Regular Approximation (ZORA) were considered [43]. In the geometry optimization, the following convergence criteria were applied: -0.001 au and 0.0003 au/A° for

changes in the energy gradients. The integration accuracy was set to 5. Geometry optimization was performed for the three depicted in **Fig. 2** tautomers of tested compound **9a**.

Vibrational frequencies were obtained through numerical differentiation of the analytical gradients [40-42]. Free energy (ΔG) at 298.15K were calculated from electronic bond energies (ΔE) and vibrational frequencies using standard thermochemistry relations for ideal gas [44]. **Table 3** shows the relative energy and free energy of each tautomers as well as the relative population p_i.

2.9. Protein Expression and Purification.

Two variants of the N-terminal domain of human Mdm2 (1-118 and 18-125) were cloned into pET-20 vector (Novagen) and expressed in the *Escherichia coli* BL21(DE3) as described previously [44]. In brief, cells were grown at 37°C and induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at OD_{600} of 0.6-0.8 and grown for an additional 5 h at 37°C. Cells were harvested by centrifugation (4000*g* for 15 min). Then cells were resuspended in phosphate-buffered saline (PBS) supplemented with protease inhibitor cocktail and lysed by sonication until the lysate was homogeneous. Centrifugation of the lysate at 20000*g* for 15 min yielded the inclusion body pellet. The pellet was washed in ice-cold PBS containing 0.05% Triton-X100 for three times and then in PBS for the next three times. Each wash was followed by centrifugation (20 000*g*, 15 min). After the final centrifugation the inclusion body pellet was solubilized in 10-20 ml of a solution comprising 6 M guanidine hydrochloride, 100 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 mM 2-mercaptoethanol and slowly rotated at 4^{o}C for at least 2h. The protein was centrifuged at 20 000*g* for 20 min to remove insoluble material and then dialyzed against 4 M guanidine hydrochloride (pH 3.5), 10 mM 2-mercaptoethanol overnight. The protein was refolded by dropwise addition into 10 mM Tris-HCl (pH 7.0), 1 mM EDTA, 10 mM 2-mercaptoethanol and slowly stirred at 4°C for 16 h. Then ammonium sulphate was added to the final concentration of 1.5 M and the solution was stirred for additional 2h at 4^{o}C and centrifuged at 20 000*g* for 10 min. The refolded protein was recovered on Butyl Sepharose 4 Fast Flow (GE Healthcare). The resign was washed with 10 mM Tris-HCl (pH 7.0), 1.5M ammonium sulphate, 1 mM EDTA, 10 mM 2-mercaptoethanol, and the protein was eluted using 100 mM Tris-HCl (pH 7.2), 5 mM 2-mercaptoethanol and further purified by gel filtration using HiLoad 16/600 Superdex75 (GE Healthcare).

2.10. FP measurements

Fluorescence Polarization (FP) assay was used to monitor interactions between Mdm2 and its antagonists. For each assay, fresh protein stocks of Mdm2 (1-118) were thawed and the protein concentration was determined using the Bradford method. Assay buffer contained 50 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA and 5 % DMSO.

The binding affinity of P2 peptide (sequence: LTFEHYWAQLTS labeled with carboxyfluorescein) towards Mdm2 was first determined. For this purpose, 10 nM of the fluorescent P2 peptide was contacted with serial dilutions of tested protein (range from 750 to 0.012 nM) in a final volume of 100 µl and fluorescence polarization was determined. K_d was established by fitting the curve described by the below equation to experimental data:

$$FP = FPmin + \frac{(FPmax - FPmin) \cdot c}{K_d + c}$$

where FP is the determined value of fluorescence polarization, FP_{min}- fluorescence polarization for ligand only, FP_{max}- fluorescence polarization at protein concentration saturating the ligand, and c – protein concentration. Competition binding assay was performed using 10 nM fluorescent P2 peptide and optimal protein concentration for the measurement calculated based on determined K_d according to Huang, 2003 (f_0 = 0.8) [45]. Tested compounds were dissolved in DMSO at 50 µM. Serial dilutions (50 µM to 0.05 µM) were prepared in DMSO. Nutlin-3a was used as positive controls for Mdm2 (**Table S2**). All the experiments were prepared in duplicates.

Fluorescence polarization was determined using Tecan InfinitePro F200 plate reader with the 485 nm excitation and 535 nm emission filters. The fluorescence intensities, parallel and perpendicular to the plane of excitation, were determined in Corning black 96-well NBS assay plates at room temperature. Fluorescence polarization values were expressed in millipolarization units (mP). Inhibition curves were fitted to obtain K_i values.

2.11. ^{1}H-^{15}N HSQC Measurements for K_D determination.

Uniform ^{15}N isotope labeling was achieved by expression of the protein in the M9 minimal media containing $^{15}NH_4Cl$ as the sole nitrogen source. The final step of purification of Mdm2 (residues 1-118) [46] consisted of gel filtration into the NMR buffer (50 mM phosphate buffer pH 7.4 containing 150 mM NaCl, 5 mM DTT). 10% (v/v) of D_2O was added to the samples to provide lock signal. Water suppression was carried out using the WATERGATE sequence [47]. All the spectra were recorded at 300 K using a Bruker Avance 600 MHz spectrometer. ^{1}H-^{15}N heteronuclear correlations were obtained using the fast HSQC pulse sequence [48]. Assignment of the amide groups of Mdm2 was obtained after Stoll *et al*. [49].

For each compound, the two-dimensional ^{1}H-^{15}N correlated NMR spectrum was recorded at 7-9 different ligand/protein ratios, ranging from 0:1 to 5:1, respectively. The samples were prepared by adding small amounts of a 50 mM ligand stock solution in DMSO to the protein solution (0.12 ml) containing the ^{15}N labeled Mdm2 fragment at a concentration of 0.16 – 0.30 mM. The acquisition parameters for each HSQC spectrum was as follows: the size of the FID F2: 2048, F1: 100, and a number of scans: 10. Spectra were visualized using *TopSpin 4.0.2* (**Fig. 3, Fig. S7, Fig. S8**). For the determination of dissociation constants (K_D), nonlinear fits of chemical shifts of single residue versus ligand ratio were performed using the program *OriginPro* (version 9.1) according to the following equation [50,51]:

$$\Delta_{obs} = \Delta_M \frac{([P]_0 + [L]_0 + K_D) - \sqrt{([P]_0 + [L]_0 + K_D)^2 - 4[P]_0[L]_0}}{2[P]_0}$$

Where

$[L]_0$ – ligand concentration

$[P]_0$ – protein concentration

Δ_{obs} – observed chemical shift perturbation normalized according to the Pythagoras formula with ^{15}N weighting factor of 0.2.

Δ_M –chemical shift for single residue obtained with maximum ligand concentration

K_D – dissociation constants

The final value of K_D was calculated as a weighted mean value of K_D's obtained for at least four most perturbed residues which undergo fast chemical exchange: Gly58, Tyr60, Lys94, Leu57, His73, Tyr93, Thr63, Val108, Thr23.

Reference ^{1}H-^{15}N HSQC spectra of MDM2 titrated with Nutlin-3a can be found in Krajewski *et al*. [52]

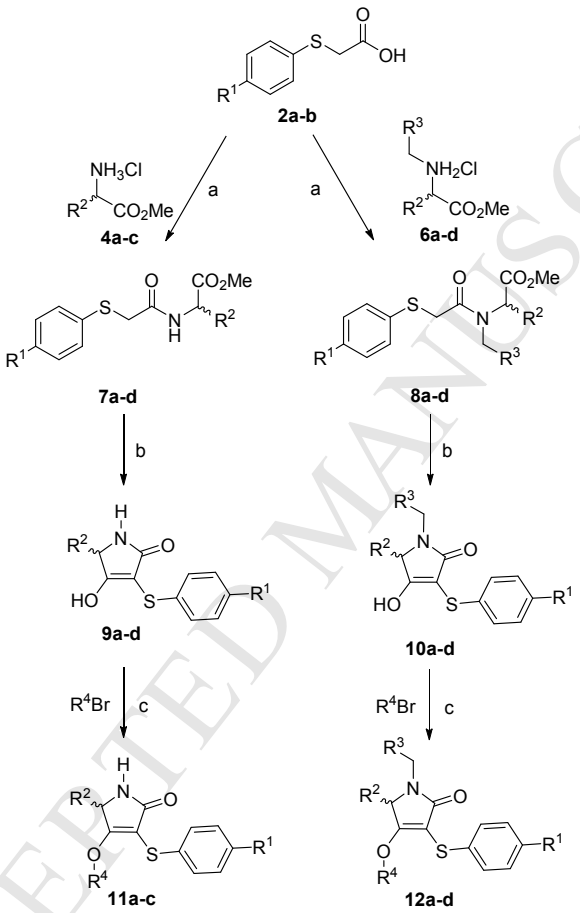
3. Results and discussion

3.1. Synthesis of tetramic acid derivatives

We first focused on the synthesis of the derivatives of tetramic acid (**IA**). There are numerous methods of the syntheses of the tetramic acid derivatives, out of which, the ones based on the Dieckmann condensation, the Lacey-Dieckmann condensation or the Meldrum's acid ring opening are among the most often used [18,53].

For the synthesis of the compounds, we chose to use the Dieckmann condensation of the N-alkoxycarbonylacety derivatives [54]. We obtained the desired compounds starting from the amino acids methyl esters (**4a-c**, **6a-d**) and substituted 2-(phenylthio)acetic acids (**2a-b**)

(Scheme 1, Scheme 2). Further, these reactants were condensed with the appropriate amides (**7a-d**, **8a-d**), using the carbodiimide coupling agent: diisopropylcarbodiimide (DIC), supported by a catalytic amount of DMAP (4-dimethylaminopyridine). Although the N-substituted tetramic acid is usually obtained using the Meldrum's acid, we decided to proceed with the modification made in reactants that were used in the Dieckmann condensation. We introduced a suitable substituent before the coupling step, which was done by reductive amination of appropriate amino acid esters.

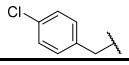
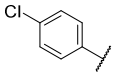
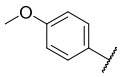
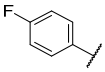
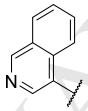


Scheme 2. Synthesis of tetramic acid derivatives. Reagents and conditions: (a) (i) **4a-c** or **6a-d** (1 eq.), Et_3N (1 eq.), anh. DCM, 0°C, 15 min (ii) **2a-b** (1 eq.), DMAP (0.2 eq.), DIC (1 eq), 0°C to RT, 16 h; (b) **7a-d** or **8a-d** (1 eq.), ^{t}BuOK (1.5 eq.), anh. 1,4-dioxane, 80°C, 1.5 h; (c) (i) **9a** or **10a-b** (1 eq.), anh. K_2CO_3 (2 eq.), anh. acetone, 60°C, 15 min (ii) alkyl bromide (1 eq.), reflux, 3h.

Amides **7a-d**, **8a-d** were subjected to the Dieckmann condensation carried out with strong bases, leading to the tetramic acid derivatives **9a-d** and **10a-d**. The best yields were obtained using the freshly distilled anhydrous 1,4-dioxane as a solvent and the potassium *tert*-butoxide as a base. This is consistent with the literature findings, which reported that addition of water promotes dimerization of tetramic acid [55]. The yields vary from moderate to high (Table 1). Significantly lower yields were observed for cyclisation with the leucine amino acid derivative leading to compound **9a** - as compared to the aromatic amino acid derivatives

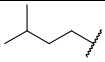
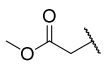
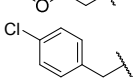
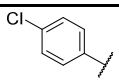
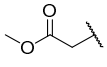
(leading to **9b-d**). With the exception of the isoquinoline derivative **10d,** the obtained yields were better when the N-alkylated compounds were used (**10a-c**).

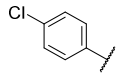
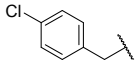
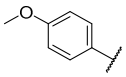
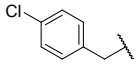
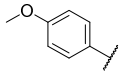
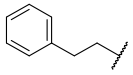
Table 1. Structures, Dieckmann condensation yields and activities measured by fluorescence polarization (FP) assay (K_i) of compounds **9a-d** and **10a-d**.

No	R^1	R^2	-	Yield [%]	K_i FP [µM]
9a	Cl			37	2.9
9b	H			83	>100
9c	Cl			55	9.0
9d	Cl			57	5.8
No	R^1	R^2	R^3	**Yield [%]**	**K_i FP [µM]**
10a	Cl			57	35.7
10b	Cl			79	26.3
10c	Cl			81	>100
10d	Cl			19	>100

To introduce another substituent to our scaffold, we decided to proceed with the Williamson ether synthesis [56] which gave the O-substituted products (**11a-c**,**12a-d**) with a rather low yield, probably due to the modest acidity of the 4-hydroxyl group (Table 2). The formation of the O-alkylated product rather than C-alkylated is confirmed by analysis of the chemical shift of the methylene protons of R^4 substituent. For example in the compound **11c** and **12b,** these proton signals appear at 5.60 ppm and 5.61 ppm respectively. In this case such kind of downfield presence of aliphatic signals can be caused only by the deshielding effect of the neighboring oxygen. Surprisingly the N-alkylated compounds (**10a** and **10c**), seem to be more prone to alkylation than those without substituents on the nitrogen atom (**9a**).

Table 2. Structures, alkylation yield, and activity of compounds **11a-c** and **12a-d**.[a]

No	R^1	R^2	R^4	-	Yield [%]
11a	Cl			-	8
11b	Cl			-	21
11c	Cl			-	14
No	R^1	R^2	R^3	R^4	**Yield [%]**
12a	Cl				21

12b	Cl				35
12c	Cl				58
12d	Cl				63

[a] K_i (FP) obtained for these compounds were above 100 μM

3.2. Tautomerization of 3-phenylthio- tetramic acid derivative 9a

Having in mind a possible tautomerization of the tetramic acid derivatives and as a consequence its chemical and biochemical properties, we decided to determine whether the ketone or enol forms are predominant in our compounds (Fig. 2). To achieve this goal, we both analyzed spectral data and carried out theoretical calculations for compound **9a**.

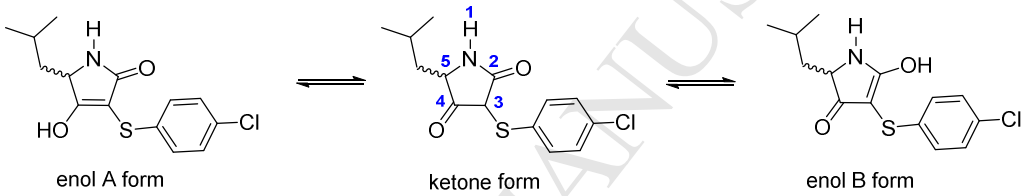


Figure 2. Possible tautomeric forms of **9a**. (Numbering of atoms in the central ring of tetramic acid is shown in blue)

^{1}H NMR spectra for **9a** show a proton signal at 12.24 ppm which is a region characteristic for enol forms of tetronic and tetramic acids. This suggested that the main form present in solution (DMSO) is the enolic one. To further verify this assumption, we decided to measure the DEPT-135 ^{13}C NMR (**Fig. S2**). The signals originating from carbons C(2) – 171.2 ppm, C(3) – 92.2 ppm and C(4) – 181.6 ppm (taken from ^{13}C spectrum) are not present at DEPT-135 spectrum. The absence of signal originating from C(3) is due to lack of hydrogen attached to C(3) – situation present only in ketone form (the signal assignment was done using 2D NMR: COSY, HSQC and HMBC experiments; **Table S1**, **Fig. S3-5**). Further, the chemical shift value of C(4) more likely originates from an enolic carbon atom (enol A form) than the ketone one (enol B form). Moreover, an infrared spectrum of **9a** shows the C=O stretching vibration at 1655 cm^{-1} which is characteristic for an amide carbonyl group, in contrast to ketone forms for which the values 1750-1700 cm^{-1} are expected (**Fig. S6**). A similar situation is present in all compounds, which suggest the main tautomer in all cases is the enol A form.

Furthermore, to verify this hypothesis we performed DFT calculations of energy for all possible tautomeric forms of **9a**. The energy, as well as free energy, are collected in **Table 3**. The COSMO model has been applied for the consideration of the solvent effects. The various

solvent with the different character has been used. These calculations clearly show that the enol A form is preferred over both the ketone and enol B form. The energy of the ketone is around 2 kcal higher and the energy in the gas phase of enol B is around 1 kcal higher than the one for the enol A. The calculation with taking into account the solvation effect shows even bigger preference for the enol form. The observed probability, based on the thermodynamics, for the enol A form is bigger than for the ketone and for the enol B forms. It should be pointed out that in cyclohexane the ketone is preferred over enol B form. However the enol A is still the most probable form.

Table 3. The energy difference, the free energy difference as well as population (p_i) of the alternative tautomers of the compound **9a**.

			Gas phase		*Chloroform*		*cycohexane*		*DMSO*		*Ethanol*	
forms:	*Δ E* [kcal/mol]	p_i	*Δ G* [kcal/mol]	p_i	*Δ G* [kcal/mol]	p_i	*Δ G* [kcal/mol]	p_i	*Δ G* [kcal/mol]	p_i	*Δ G* [kcal/mol]	p_i
enol A	0.00	0.950	0.00	0.811	0.00	0.944	0.00	0.994	0.00	0.944	0.00	0.940
ketone	2.18	0.024	1.92	0.032	2.31	0.019	3.18	0.005	2.74	0.009	2.24	0.020
enol B	2.13	0.026	0.97	0.157	1.93	0.038	3.82	0.001	2.07	0.029	1.90	0.040

3.3. Biological evaluation

To evaluate the potency of obtained inhibitors, we checked their activity in the fluorescence polarization (FP) and ^{1}H-^{15}N HSQC NMR titration assays (**Table 1**).

The FP method show moderate activities of our compounds. The series of the di-substituted tetramic acid (**9a-d**) shows the best binding to Mdm2. These results show that it is beneficial to introduce a chlorine atom to the molecule structure of designed inhibitor. This trend is illustrated by increasing binding affinity: from the not binding **9b** (without the chlorine atoms) to the good affinity of **9d** that has two chlorine atoms. From the other series, only the N-p-chlorobenzylated tetramic acid **10a** and **10b** show binding towards Mdm2. None of the O-alkylated compounds (**11a-c**, **12a-d**) was found active. Such situation can be caused by either the importance of the 4-OH substituent for the binding, relative positions of substituents in the central core or the decreasing compound solubility.

To confirm the activities of compounds found in the FP assay, we proceeded with the ^{1}H-^{15}N HSQC NMR titration experiments for selected compounds: **9d** and **10a**. The method used is based on monitoring of chemical shift changes in protein amide backbone resonances upon titration with an increasing amount of the tested compound [50,51,57]. Moreover, since all cross peaks in the Mdm2 spectrum were assigned to particular amino acid residues [49], it

was also possible to analyze the interface interaction between inhibitors and the Mdm2 protein. In general, the perturbation of the protein spectra due to the formation of the complex can be divided in ^{1}H-^{15}N HSQC NMR to three cases: fast chemical exchange presented as the cross-peak movement, intermediate chemical exchange presented as the signal broadening and disappearing and slow chemical exchange presented as signal doubling [58,59]. As expected, the ligand-induced perturbations in the Mdm2 spectra were indeed observed for the reported active compounds. We decided to verify the binding interaction of our inhibitors with Mdm2 protein and for this purpose, we chose compounds **9d** and **10a** which share the biggest similarity to the first generation inhibitors based on 1,5-dihydro-2*H*-pyrrol-2-one (by the presence of two 4-chlorophenyl substituents) [15]. It allowed us not only to qualitatively evaluate the interaction, (if the interaction occurs) but also to semi-quantitatively estimate the binding affinity. Obtained values of their K_D's are 5.8±1.1 μM for **9d** (**Fig. 3**) and 11.4±2.0 μM for **10a** (**Fig. S7**).

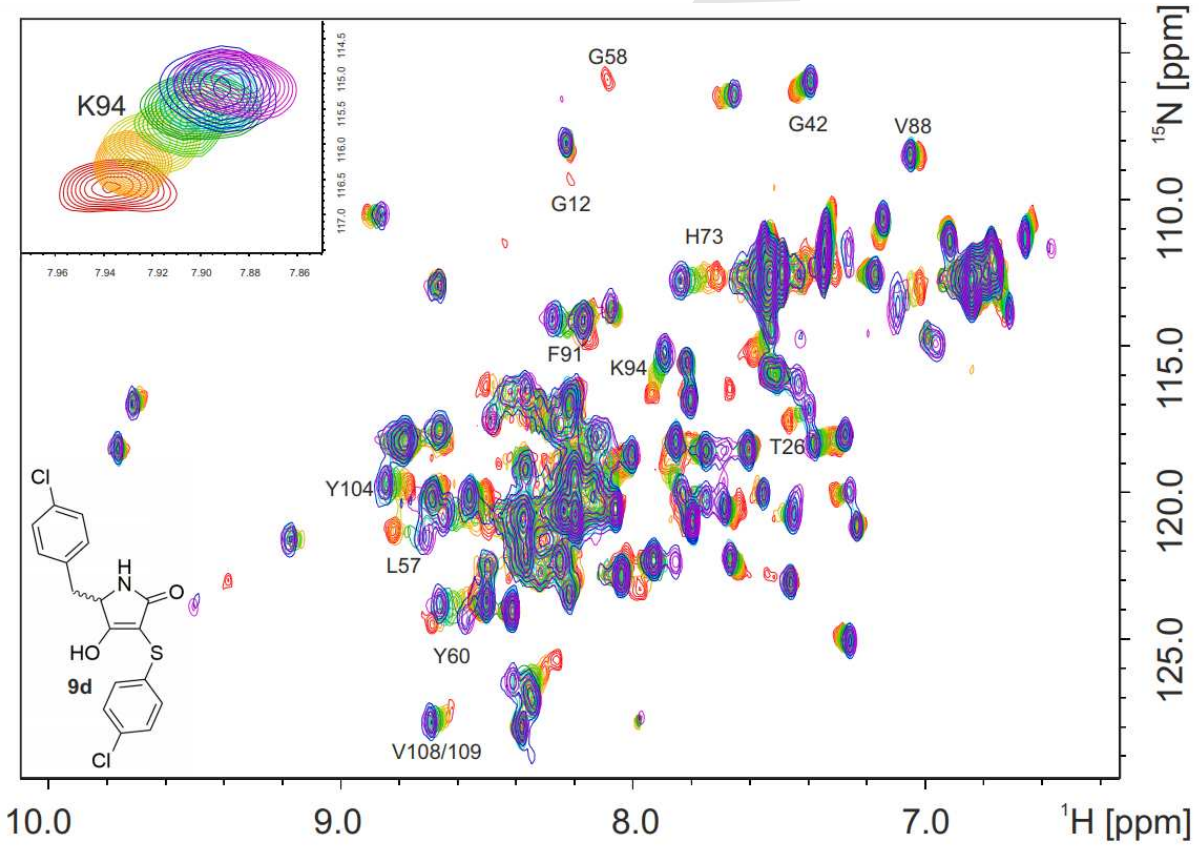


Figure 3. ^{1}H-^{15}N HSQC titration experiment for the compound **9d**. Key residues changes were assigned on spectra. Exemplary cross peak movement (K94) is shown enlarged. Red-reference Mdm2; orange – Mdm2:**9d** 8:1; yellow Mdm2:**9d** 8:2; light green – Mdm2:**9d** 8:3; green – Mdm2:**9d** 8:4; light blue – Mdm2:**9d** 8:6; blue – Mdm2:**9d** 1:1; purple – Mdm2:**9d** 1:2.

The binding patterns observed for both the tetramic acid derivatives are similar and indicate the interaction with the p53 binding pocket of Mdm2. Particularly, the induced changes in NMR signals are seen for the residues which are located inside the p53 binding pocket; these are, for example, Gly58 (disappearing), Met62, Leu57, Phe91 and Leu82 (movement). Additionally, several more cross-peaks placed in the vicinity of the p53 binding pocket of Mdm2 are changed due to interaction with the inhibitors (e.g. Lys94, His73, Thr63, Tyr60).

Furthermore, we verified the lack of the possible dimerization of Mdm2 in the presence of our compounds [15]. Because of the lack of 6-chloroindole moiety in new compounds, we suspected that they would not cause protein dimerization. Indeed, analysis of the ^{1}H signal linewidths of titration experiments for **9d** showed no broadening of signals, which would have been characteristic for the dimer formation (**Fig. S8**).

4. Conclusions

In this work we have described the synthesis of the second generation derivatives of Mdm2-p53 inhibitors based on the tetramic acid scaffold. Specifically, our focus has been on the tetramic acid derivatives, investigating their tautomerization and activity. Binding towards Mdm2 was tested by two orthogonal assays: the FP assay and the ^{1}H-^{15}N HSQC titration experiments. Our results demonstrated the binding of the inhibitors into the p53-binding pocket of Mdm2. Moreover, we have shown that eliminating the 6-chloroindole group from the current compounds prevents dimerization of Mdm2. Our work provides a solid base for the further rational design of potent Mdm2/p53 inhibitors without the 6-chloroindole group and demonstrates the strategy of synthetic pathways for the design of new compounds. Furthermore, we investigated tautomerization of the 3-phenylthio-substituted tetramic acids and showed that they exist in solution in a usually not-preferred enol form.

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Conflicts of interest

The authors declare no competing financial interest.

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