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Synthesis and antiproliferative activity of the salicyl-based Weinreb amides and their derivatives in cancer cell lines

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A series of 11 pseudotripeptide Weinreb amides and 8 pseudodipeptide sulfonylhydrazides were synthesized and assessed using the resazurin-based method to determine their antiproliferative activity against the following cancer cell lines: lymphoblastic leukaemia (CEM), acute myeloid leukaemia (MV4-11), multiple myeloma (U266), and breast adenocarcinoma (MCF-7). The investigated compounds have shown a mid-micromolar range of inhibition. When L-Trp was included in the centre of the tripeptide chain, the derivatives exhibited a single-digit micromolar activity against the MV4-11 and U266 cell lines. Further immunoblotting experiments and caspase-3/7 activity assays for the most active compound ***3e*** *have confirmed apoptosis as the mechanism of cell death.*

Keywords: Cytotoxicity; Peptides; Apoptosis; Weinreb amide

Introduction

Peptides belong to the widespread class of compounds whose diversity is affected by their length, distinctive integrated amino acids, and amino acid modifications. The combination of these parameters leads to a broad and diverse chemical variability comprising not only new chemical entities but also compounds

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characterized by novel chemical and biological properties. Therefore, peptides and their mimetics have been widely investigated for a variety of activities, such as anti-hypertensive, antioxidant, antimicrobial, anti-coagulant, anti-obesity, anti-diabetic, and anticancer [1]. The main efforts of further peptidomimetic investigations reflect improvements not only in their biological properties but also in biochemical stability towards proteolytic enzymes and cellular permeability. Further chemical modifications have yielded new peptidomimetics and their variants, including peptoids, azapeptides, azatides, carbamates, sulfur-containing peptides, and others [2].

The functional moiety *N,O*-dimethylhydroxylamine, most commonly known as the Weinreb amide (WA) [3], has a variety of useful synthetic applications [4]. These compounds are synthetically available from either the corresponding acid or its functional derivatives [5]. WAs are the excellent leaving groups that maintain good stability of the starting materials [6]. Due to these properties, WAs are common synthetic intermediates to obtain the carbonyl compounds.

Phenylsulfonyl hydrazides have gained attention due to their properties, such as stability, and also present interesting biological activities [7–9]. The introduction of this interesting functional moiety into a pseudopeptide molecule should produce final compounds with significant bioactivities. For example, phenylsulfonyl hydrazides were described as potent MMP-9 inhibitors [10], and these compounds could be an interesting option for cancer treatment [11].

Previously, we prepared modified dipeptides and tripeptides decorated with salicylate or by *O*-benzylsalicylate on the N-terminus and with variety modifications to the C-terminus. Based on multiple alterations, these pseudopeptides exhibited diverse biological effects, including disruption of the dynamics of the actin cytoskeleton [12], inhibition of the proteasome [13], modulation of adhesion-related processes via FAK signalling [14] or other yet unknown, accompanying antiproliferative properties in the cancer cells [15].

In this work, we modified the C-terminus of salicylate-substituted dipeptide **1**, of which some (**1a**,**1c**–**k**) had been synthesized and characterized previously [13,14,16]. For the purposes of this study, additional original dipeptidic acids were modified at the C-terminus by Weinreb amide or by phenylsulfonyl hydrazide functional groups. The compounds bearing these moieties usually serve only as simple and stable intermediates in organic synthesis, and their biological activities are often neglected. We have assumed that the implementation of WA functional groups onto salicylamide pseudopeptides brings a new option to study the biological activity of these compounds.

Experimental part

Chemistry

All reagents and solvents were purchased from commercial sources (TCI Europe, Sigma Aldrich, Acros Organics, Fluorochem, Merck, and Lach-Ner). Commercial grade reagents were used without further purification. Reactions were monitored by thin-layer chromatography plates coated with 0.2 mm silica gel 60 F_{254} (Merck, Darmstadt, Germany). The TLC spots were visualized with a 5% solution of phosphomolybdic acid in ethanol and eventually by UV-irradiation (254 nm). All melting points were determined on a Melting Point B-540 apparatus (Büchi, Flawil, Switzerland) and left uncorrected. NMR spectra were measured in $CDCl_3$, CD_2Cl_2, DMSO or eventually D_2O at ambient temperature on a Bruker Avance™ III 400 spectrometer at frequencies ^1H (400 MHz) and ^{13}C (100.26 MHz) or a Bruker Ascend™ 500 spectrometer (Bruker, Rheinstetten, Germany) at frequencies ^1H (500.13 MHz) ^{13}C $\{^1H\}$ (125.76 MHz). The chemical shifts, δ, are given in ppm relative to the residual solvent peaks: $CDCl_3$ – 7.26, CD_2Cl_2 – 5.32, DMSO – 2.50, and D_2O – 4.79. The coupling constants (*J*) are reported in Hz. Elemental analyses (C, H, N) were performed with automatic microanalyser (Flash 2000 Organic elemental analyser, Thermo Fisher Scientific, Waltham, MA, USA). High-resolution mass spectrometry was determined by the "dried droplet" method using a MALDI LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) equipped with a nitrogen UV laser (337 nm, 60 Hz). Spectra were measured in the positive ion mode and in regular mass extent with a resolution of 100 000 at m/z = 400. 2,5-Dihydrobenzoic acid (DBH) was used as the matrix.

Starting materials were synthesized according to our previously published approach [13]. Acids **1a, 1c–1k** are described and characterized in our previous publications [14–16]. The synthetic approach to the hydrochloride salts of the amino acid methyl esters starting from optically pure amino acids is generally known and described in the literature [15,19]. All salts used were prepared according to this procedure, and their characterization was in good agreement with literature data. All general synthetic procedures were recalculated to the 1 mmol scale. The amount of each compound used in the experiments is described in the characterization section.

General experimental procedure for the synthesis of starting materials **1a–k**

Starting materials were synthesized according to our previous approaches [14–16].

Characterization data of starting materials **1a**–**k**

*(S)-2-((S)-2-(2-(benzyloxy)-5-chlorobenzamido)-3-phenylpropanamido)-4-methylpentanoic acid **1a***

The procedure, characterization, and yield correspond to the literature [15].

*(S)-methyl 2-((S)-2-(2-(benzyloxy)-5-chlorobenzamido)-3-phenylpropanamido)-6-(((benzyloxy)carbonyl)amino)hexanoate **1b***

Reaction was done in 3 mmol scale. Light yellow oil; yield 87 %; R_f = 0.277 (ethylacetate/*n*-hexane 1:1, eluated 1×). **^{1}H NMR** (400 MHz, $CDCl_3$): δ 8.46–8.43 (1H, m, N*H*), 8.19 (1H, t, *J* = 3.00 Hz, N*H*), 7.50–7.33 (11H, m, 11 × C*H*(Ar)), 7.27–7.19 (3H, m, 3 × C*H*(Ar)), 7.11–7.06 (2H, m, 2 × C*H*(Ar)), 6.99–6.95 (1H, m, C*H*(Ar)), 6.86–6.82 (1H, m, C*H*(Ar)), 5.26–5.11 (4H, m, O–CH_2–Ph, Ph–CH_2–COO), 5.18–5.04 (1H, m, N*H*), 4.95–4.84 (1H, m, NH–C*H*–C=O), 4.60–4.54 (1H, m, NH–C*H*–C=O), 3.76–3.72 (3H, m, CH_3–O), 3.19–3.05 (2H, m, Ph–CH_2), 3.13–2.87 (2H, m, CH_2–NH), 1.91–1.47 (4H, m, CH_2–CH_2), 1.41–1.19 (2H, m, CH_2). **Elemental analysis:** calc. for $C_{38}H_{40}ClN_3O_7$ (686.19): C, 66.51; H, 5.88; N, 6.12. Found: C, 66.41 ± 0.19; H, 5.94 ± 0.03; N, 5.99 ± 0.10. **HRMS:** *m*/*z* calc. for $C_{38}H_{40}ClN_3O_7$: 686.26275 $[M+H]^+$, 724.21864 $[M+K]^+$; found: 686,26514 $[M+H]^+$, 724,22119 $[M+K]^+$.

*(S)-2-((S)-2-(2-(benzyloxy)-5-chlorobenzamido)-4-methylpentanamido)-4-methylpentanoic acid **1c, 1f, 1g, 1h, 1k***

The procedure, characterization, and yield correspond to the literature [15].

*(S)-2-((S)-2-(2-(benzyloxy)-5-chlorobenzamido)-4-methylpentanamido)hexanoic acid **1d***

The procedure, characterization, and yield correspond to the literature [16].

*(S)-2-((S)-2-(2-(benzyloxy)-5-chlorobenzamido)-4-methylpentanamido)-3-(1H-indol-3-yl)propanoic acid **1e***

The procedure, characterization, and yield correspond to the literature [16].

(S)-2-((S)-2-(2-(benzyloxy)-5-chlorobenzamido)-4-methylpentanamido)-3-cyclohexylpropanoic acid ***1i***

The procedure, characterization, and yield correspond to the literature [16].

(S)-1-((S)-2-(2-(benzyloxy)-5-chlorobenzamido)-4-methylpentanoyl)pyrrolidine-2-carboxylic acid ***1j***

The procedure, characterization, and yield correspond to the literature [16].

General experimental procedure for the synthesis of tripeptidic methyl esters **2a**–**k**

Liberation of the amino acid methyl ester free base: The hydrochloride salt of the amino acid methyl ester (1 mmol) was dissolved in distilled water (2.5 mL), and potassium carbonate (1 mmol) was added in one portion. The reaction mixture was stirred at RT for 10 mins. After this time, the reaction mixture was washed with DCM (3 × 2 mL), and the collected organic fractions dried over Na_2SO_4. The mixture was filtered, and the solvent evaporated under reduced pressure. The free base was used in the next step without further purification.

EDC·HCl mediated coupling reaction: Dipeptidic acids **1a–k** (1 mmol) were dissolved in DCM (10 mL), HOBt (1.1 mmol) and EDC·HCl (1.2 mmol) was added in one portion. The reaction mixture was stirred for 1 h, and then a solution of the free base methylester amino acid in DCM (3 mL) was added via syringe over 1 min. The reaction mixture stirred for 18 h at RT. DCM was removed by rotary evaporation, and the residue dissolved in EtOAc (10 mL). The solution was washed with a saturated solution of $NaHCO_3$ (3 × 3 mL), 5% citric acid (3 × 3 mL) and saturated NaCl (2 × 3 mL) to obtain tripeptidic methyl esters **2a–k**. The esters were used in the next step without further purification or characterization.

General experimental procedure for the synthesis of the investigated Weinreb amides **3a**–**k**

N,O-Dimethylhydroxylamine hydrochloride (2.5 mmol) was slowly added to a solution of tripeptidic methylesters **2a–k** (1 mmol) in dry THF (16 mL) under a N_2 atmosphere. The reaction mixture was cooled to −20 °C, and then LiHMDS (7 mmol) was added dropwise over 45 mins. After complete conversion of the starting material (by TLC control, usually 30 min), the solution was warmed to room temperature, and the reaction quenched by the addition of a saturated aqueous solution of NH_4Cl (10 mL). The solution was washed three times with Et_2O

(3 × 7 mL). The combined organic phases were washed with water (2 × 7 mL) and dried over sodium sulfate. The crude product was purified using flash chromatography. The final Weinreb amides **3a–k** were obtained with 53 % to 93 % yield.

Characterization data of investigated Weinreb amides **3a**–**k**

*2-(benzyloxy)-5-chloro-N-((5S,8R,11S)-5,8-diisobutyl-3-methyl-4,7,10-trioxo-12-phenyl-2-oxa-3,6,9-triazadodecan-11-yl)benzamide **3a***

Reaction was done in 0,8 mmol scale. White amorph; yield 93 %; R_f = 0.17 (ethylacetate/*n*-hexane 1:1). **^{1}H NMR** (400 MHz, $CDCl_3$): δ 8.32 (1H, d, *J* = 7.0 Hz, N*H*), 8.12 (1H, d, *J* = 2.8 Hz, N*H*), 7.38 (6H, m, 6 × C*H*(Ar)), 7.17 (3H, m, 3 × C*H*(Ar)), 6.99 (2H, m, 2 × C*H*(Ar)), 6.90 (1H, d, *J* = 8.9 Hz, C*H*(Ar)), 6.65 (1H, d, *J* = 8 Hz, C*H*(Ar)), 6.56 (1H, d, *J* = 8.7 Hz, N*H*), 5.12 (2H, dd, *J* = 11.6; 30.5 Hz, CH_2–O–Ph), 5.0 (1H, m, NH–C*H*–C=O), 4.81 (1H, m, NH–C*H*–C=O), 4.41 (1H, m, NH–C*H*–C=O), 3.78 (3H, s, CH_3–O), 3.19 (3H, s, CH_3–O), 3.10 (1H, dd, *J* = 5.6; 14.1 Hz, C*H*H–Ph), 2.84 (1H, m, CH*H*–Ph), 1.62 (2H, m, 2 × C*H*), 1.52 (4H, m, 2 × CH_2), 0.93 (6H, dd, *J* = 6.4; 9.9 Hz, (CH_3)$_2$), 0.84 (6H, dd, *J* = 2.88; 6.3 Hz, (CH_3)$_2$). **^{13}C NMR** (100.6 MHz, $CDCl_3$): δ 171.63, 171.04, 164.54, 155.48, 136.85, 136.30, 135.23, 132.95, 132.28, 129.26, 129.13, 128.73, 127.89, 127.14, 127.00, 122.46, 114.53, 71.74, 55.32, 52.13, 47.92, 41.87, 41.19, 37.08, 30.51, 24.97, 24.84, 23.50, 23.16, 22.04, 21.87, 1.53, 0.18. **Elemental analysis:** calc. for $C_{37}H_{47}ClN_4O_6$ (679,32): C, 65,42; H, 6,97; N, 8,25. Found: C, 66,10 ± 0,26; H, 7,29 ± 0,02; N, 6,92 ± 0,01. **HRMS:** *m*/*z* calc. for $C_{37}H_{47}ClN_4O_6$: 701.30763 $[M+Na]^+$; found: 701.30811 $[M+Na]^+$.

*Benzyl ((R)-5-((S)-2-(2-(benzyloxy)-5-chlorobenzamido)-3-phenylpropanamido)-6-(((S)-1-(methoxy(methyl)amino)-4-methyl-1-oxopentan-2-yl)amino)-6-oxohexyl) carbamate **3b***

Reaction was done in 1.2 mmol scale. White amorph; yield 54 %; R_f = 0.33 (ethylacetate/*n*-hexane 1:1). **^{1}H NMR** (400 MHz, $CDCl_3$): δ 8.53–8.4 (1H, m, N*H*), 8.19–8.11 (1H, m, N*H*), 7.52–6.94 (18H, m, 18 × C*H*(Ar)), 5.52 (1H, d, *J* = 30.3 Hz, N*H*), 5.28–5.12 (4H, m, CH_2–O–Ph, O–CH_2–Ph), 4.99–4.86 (2H, m, 2 × C*H*), 4.46 (1H, s, C*H*), 3.88–3.79 (3H, m, CH_3–O), 3.22–3.11 (3H, m, CH_3–N), 3.22–2.78 (4H, m, 2 × CH_2), 1.91–1.31 (9H, m, CH_2, CH_2–CH_2–CH_2, C*H*), 1.17 (1H, s, N*H*), 1.00–0.92 (6H, m, (CH_3)$_2$–CH). **^{13}C NMR** (100.6 MHz, $CDCl_3$): δ 171.31, 171.05, 170.82, 164.46, 156.81, 155.54, 136.97, 136.74, 135.30, 132.94, 132.21, 129.29, 129.23, 129.08, 128.76, 128.70, 128.62, 128.29, 128.17, 127.94, 127.09, 127.02, 122.51, 114.60, 71.78, 66.68, 61.67, 55.97, 55.52, 53.21, 48.24, 41.34, 40.43, 37.46, 31.93, 29.21, 25.01, 23.49, 22.08, 21.71. **Elemental**

analysis: calc. for $C_{45}H_{54}ClN_5O_8$ (828,39): C, 64,10; H, 6,83; N, 10,10. Found: C, 65,35 ± 0,05; H, 6,56 ± 0,02; N, 8,07 ± 0,02. **HRMS:** *m*/*z* calc. for $C_{45}H_{54}ClN_5O_6$:850,35531 $[M+Na]^+$; found: 850,35785 $[M+Na]^+$.

N-((5S,8R,11S)-5-benzyl-8-isobutyl-3,13-dimethyl-4,7,10-trioxo-2-oxa-3,6,9-triazatetradecan-11-yl)-2-(benzyloxy)-5-chlorobenzamide ***3c***

The procedure, characterization, and yield correspond to the literature [15].

2-(benzyloxy)-N-((5S,8R,11S)-8-butyl-5-isobutyl-3,13-dimethyl-4,7,10-trioxo-2-oxa-3,6,9-triazatetradecan-11-yl)-5-chlorobenzamide ***3d***

Reaction was done in 5 mmol scale. White amorph; yield 73 %; R_f = 0.272 (ethylacetate/*n*-hexane 1:1.2). **^{1}H NMR** (400 MHz, $CDCl_3$): δ 8.25 (1H, m, N*H*), 8.15 (1H, m, N*H*), 7.50–7.38 (7H, m, 7 × C*H*$_{Ar}$), 7.05 (1H, m, C*H*$_{Ar}$), 6.75 (1H, m, N*H*), 5.15 (2H, m, Ar–C*H*$_2$–O), 4.95 (1H, m, C*H*), 4.5–4.31 (2H, m, 2 × C*H*), 3.75 (3H, s, C*H*$_3$–O), 3.13 (3H, s, C*H*$_3$–N), 1.87 (1H, m, C*H*), 1.67–1.14 (11H, m, 5 × C*H*$_2$, C*H*), 0.90–0.84 (9H, m, 3 × C*H*$_3$), 0.79 (3H, d, *J* = 6.4 Hz, C*H*$_3$), 0.70 (3H, d, *J* = 6.8 Hz, C*H*$_3$). **^{13}C NMR** (100.6 MHz, $CDCl_3$): δ 171.90, 171.59, 164.60, 155.82, 136.85, 135.01, 132.95, 132.37, 129.28, 128.72, 128.57, 127.07, 122.44, 114.19, 72.12, 61.70, 53.28, 52.78, 47.88, 41.59, 40.02, 32.31, 31.90, 27.73, 24.88, 24.83, 23.40, 22.94, 22.51, 22.02, 21.79, 14.09. **CHN analysis**: calc. for $C_{34}H_{49}ClN_4O_6$ (645.23): C, 63.29; H, 7.65; N, 8.68. Found: C, 63.51 ± 0.36; H, 7.77 ± 0.02; N, 8.32 ± 0.09. **HRMS:** *m*/*z* calc. for $C_{34}H_{49}ClN_4O_6$: 667.32328 $[M+Na]^+$; found: 667.32501 $[M+Na]^+$.

N-((5S,8R,11S)-8-((1H-indol-3-yl)methyl)-5-isobutyl-3,13-dimethyl-4,7,10-trioxo-2-oxa-3,6,9-triazatetradecan-11-yl)-2-(benzyloxy)-5-chlorobenzamide ***3e***

Reaction was done in 4 mmol scale. White amorph; yield 53 %; R_f = 0.40 (ethylacetate/*n*-hexane 1:1).**^{1}H NMR** (500 MHz, $CDCl_3$): δ 8.06 (1H, d, *J* = 3 Hz, N*H*), 8.01 (1H, d, *J* = 6.5 Hz, N*H*), 7.64 (1H, d, *J* = 8 Hz, N*H*), 7.41 (7H, m, 7 × C*H*$_{Ar}$), 7.20 (1H, d, *J* = 8 Hz, C*H*), 7.05 (5H, m, 5 × C*H*$_{Ar}$), 6.67 (1H, d, *J* = 8 Hz, N*H*), 5.07 (2H, m, Ar–C*H*$_2$–O), 4.95 (1H, m, C*H*), 4.72 (1H, m, C*H*), 4.47 (1H, m, C*H*), 3.73 (3H, s, C*H*$_3$–O), 3.27 (2H, m, C*H*$_2$–Trp), 3.16 (3H, s, C*H*$_3$–N), 1.52–1.12 (6H, m, 2 × C*H*$_2$, 2 × C*H*), 0.88 (6H, dd, *J* = 6, 14.5 Hz, (C*H*$_3$)$_2$), 0.75 (3H, d, *J* = 6.5 Hz, C*H*$_3$–CH–CH$_3$), 0.67 (3H, d, *J* = 6.5 Hz, CH$_3$–CH–C*H*$_3$). **^{13}C NMR** (100.6 MHz, $CDCl_3$): δ 171.66, 170.93, 164.19, 155.48, 136.85, 136.08, 134.65, 132.70, 132.07, 129.17, 129.09, 129.05, 128.56, 128.42, 127.41, 126.78, 123.53, 122.09, 121.86, 119.41, 118.74, 113.94, 111.02, 110.22, 71.81, 61.54, 53.68,

52.89, 52.44, 47.75, 41.48, 39.72, 27.51, 24.63, 23.27, 22.90, 21.58. **CHN analysis:** calc. for $C_{39}H_{48}ClN_5O_6$ (718,28): C, 65.21; H, 6.74; N, 9.75. Found: C, 64.80 ± 0.06; H, 6.70 ± 0.01; N, 9.21 ± 0.05. **HRMS:** *m*/*z* calc. for $C_{39}H_{48}ClN_5O_6$: 740.31853 $[M+Na]^+$; found: 740.32031 $[M+Na]^+$.

N-((5S,8R,11S)-5-((1H-indol-3-yl)methyl)-8-isobutyl-3,13-dimethyl-4,7,10-trioxo-2-oxa-3,6,9-triazatetradecan-11-yl)-2-(benzyloxy)-5-chlorobenzamide ***3f***

Reaction was done in 3 mmol scale. White amorph; yield 90 %; R_f = 0.288 (ethylacetate/*n*-hexane 1:1, eluated 3×). **^{1}H NMR** (500 MHz, $CDCl_3$): δ 8.24–8.20 (1H, m, N*H*), 8.16–8.10 (1H, m, N*H*), 7.58–7.51 (1H, m, C*H*(Ar)), 7.47–7.36 (7H, m, 7 × C*H*(Ar)), 7.31–7.30 (1H, m, C*H*(Ar))m 7.14–7.10 (1H, m, C*H*(Ar)), 7.08–7.05 (1H, m, C*H*(Ar)), 7.02–6.96 (2H, m, 2 × C*H*(Ar)), 6.69–6.59 (1H, m, N*H*), 5.19–5.06 (3H, m, N*H*, CH_2–O–Ph), 4.56–4.37 (2H, m, CH_2–Trp), 3.67 (3H, s, CH_3–O), 3.25–3.12 (3H, m, CH_3–N), 1.56–1.47 (3H, m, C*H*–CH_2), 1.44–1.35 (3H, m, C*H*–CH_2), 1.32–1.12 (3H, m, 3 × C*H*), 0.84 (3H, d, *J* = 6.3 Hz, CH_3), 0.79 (6H, t, *J* = 6.6 Hz, 2 × CH_3), 0.70 (3H, d, *J* = 6.5 Hz, CH_3). **^{13}C NMR** (100.6 MHz, $CDCl_3$): δ 172.13, 171.49, 164.65, 155.68, 136.27, 134.92, 133.11, 132.30, 129.30, 128.57, 127.87, 127.22, 123.84, 123.56, 122.32, 121.94, 119.37, 118.50, 114.20, 111.51, 110.15, 72.05, 61.74, 52.66, 51.97, 50.17, 41.00, 40.85, 40.35, 34.72, 32.37, 28.01, 24.98, 23.15, 23.10, 22.02, 21.74. **Elemental analysis:** calc. for $C_{39}H_{48}ClN_5O_6$ (717.28): C, 65.21; H, 6.74; N, 9.75. Found: C, 65.16 ± 0.08; H, 6.93 ± 0.02; N, 9.66 ± 0.06. **HRMS:** *m*/*z* calc. for $C_{39}H_{48}ClN_5O_6$: 740.31853 $[M+Na]^+$; found: 740.32098 $[M+Na]^+$.

2-(benzyloxy)-5-chloro-N-((5S,8R,11S)-5-(cyclohexylmethyl)-8-isobutyl-3,13-dimethyl-4,7,10-trioxo-2-oxa-3,6,9-triazatetradecan-11-yl)benzamide ***3g***

Reaction was done in 3 mmol scale. White amorph; yield 57 %; R_f = 0.4 (ethylacetate/*n*-hexane 1:1). **^{1}H NMR** (500 MHz, $CDCl_3$): δ 8.21–8.12 (2H, m, 2 × N*H*), 7.49–7.39 (6H, m, 6 × C*H*(Ar)), 7.00 (1H, d, *J* = 8.7 Hz, C*H*(Ar)), 6.76–6.59 (1H, m, C*H*(Ar)), 5.19–5.09 (2H, m, CH_2–O–Ph), 5.01–4.97 (1H, m, N*H*), 4.53–4.29 (2H, m, CH_2–CHA), 3.79–3.73 (3H, m, CH_3–O), 3.17–3.11 (3H, m , CH_3–N), 1.84–1.32 (12H, m, C*H*–CH_2–C*H*–(CH_2)$_5$), 1.27–1.04 (5H, m, C*H*–CH_2–C*H*, C*H*–CH_2–CH), 0.92–0.69 (15H, m, CH–CH_2–C*H*, 4 × CH_3). **^{13}C NMR** (100.6 MHz, $CDCl_3$): δ 172.00, 164.45, 155.61, 134.92, 132.96, 132.32, 129.27, 128.76, 128.53, 127.16, 122.49, 114.17, 72.05, 61.76, 53.77, 53.06, 52.42, 51.98, 47.18, 41.02, 40.29, 40.04, 34.12, 34.07, 32.41, 26.61, 26.36, 26.16, 24.84, 23.18, 23.07, 22.06, 21.96. **Elemental analysis:** calc. for

$C_{37}H_{53}ClN_4O_6$ (685.29): C, 64.85; H, 7.80; N, 8.18. Found: C, 65.01 ± 0.10; H, 7.80 ± 0.06; N, 8.50 ± 0.02. **HRMS:** *m*/*z* calc. for $C_{37}H_{53}ClN_4O_6$: 707.35458 $[M+Na]^+$; found: 707.35645 $[M+Na]^+$.

2-(benzyloxy)-N-((5S,8R,11S)-5-butyl-8-isobutyl-3,13-dimethyl-4,7,10-trioxo-2-oxa-3,6,9-triazatetradecan-11-yl)-5-chlorobenzamide ***3h***

Reaction was done in the 4 mmol scale. White amorph; yield 44 %; R_f = 0.377 (ethylacetate/*n*-hexane 1:1). **^{1}H NMR** (500 MHz, $CDCl_3$): δ 8.26–8.11 (2H, m, 2 × N*H*), 7.50–7.40 (6H, m, 6 × C*H*(Ar)), 7.02 (1H, d, *J* = 8.8 Hz, C*H*(Ar)), 6.90–6.76 (1H, m, C*H*(Ar)), 5.21–5.11 (2H, m, CH_2–O–Ph), 4.90 (1H, d, *J* = 33.3 Hz, N*H*), 4.55–4.26 (2H, m, CH–CH_2–CH_2), 3.81–3.74 (3H, m, CH_3–O), 3.20–3.14 (3H, m, CH_3–N), 1.84–1.19 (13H, m, C*H*–CH_2–C*H*, C*H*–CH_2–C*H*, C*H*–CH_2–CH_2–CH_2–CH_3), 0.93–0.70 (15H, m, 5 × CH_3). **^{13}C NMR** (100.6 MHz, $CDCl_3$): δ 172.05, 164.43, 155.61, 134.93, 132.94, 132.29, 129.26, 128.53, 127.13, 122.51, 114.17, 72.04, 61.75, 53.80, 53.07, 52.75, 52.41, 52.00, 51.65, 49.87, 49.63, 49.27, 40.98, 40.08, 32.33, 27.53, 24.83, 23.18, 23.02, 22.51, 22.01, 14.04. **Elemental analysis:** calc. for $C_{34}H_{49}ClN_4O_6$ (685.29): C, 63.29; H, 7.65; N, 8.68. Found: C, 63.59 ± 0.28; H, 7.74 ± 0.05; N, 8.48 ± 0.05. **HRMS:** *m*/*z* calc. for $C_{37}H_{53}ClN_4O_6$: 667.32328 $[M+Na]^+$; found: 667.32538 $[M+Na]^+$.

2-(benzyloxy)-5-chloro-N-((5S,8R,11S)-8-(cyclohexylmethyl)-5-isobutyl-3,13-dimethyl-4,7,10-trioxo-2-oxa-3,6,9-triazatetradecan-11-yl)benzamide ***3i***

Reaction was done in 4 mmol scale. Light yellow amorph; yield 24 %; R_f = 0.377 (ethylacetate/*n*-hexane 2:3, eluated 4×). **^{1}H NMR** (500 MHz, $CDCl_3$): δ 8.31–8.24 (1H, m, N*H*), 8.21–8.15 (1H, m, N*H*), 7.52–7.41 (6H, m, 6 × C*H*(Ar)), 7.06–7.02 (1H, m, C*H*(Ar)), 6.81–6.79 (1H, m, C*H*(Ar)), 5.22–5.12 (2H, m, CH_2–O–Ph), 4.97–4.96 (1H, m, N*H*), 4.55–4.44 (2H, m, 2 × NH–C*H*–C=O), 3.88–3.77 (3H, m, CH_3–O), 3.20–2.99 (3H, m, CH_3–N), 1.82–1.50 (10H, m, CH_2–CH_2–CH_2–CH_2–CH_2), 1.46–1.37 (2H, m, CH–CH_2–CH), 1.29–1.26 (1H, m, C*H*–CH_2–CH), 1.23–1.12 (4H, m, 2 × CH–CH_2–CH), 0.99–0.90 (3H, m, C*H*–CH_2–CH, C*H*–CH_2–C*H*), 0.89–0.74 (12H, m, 4 × CH_3). **^{13}C NMR** (100.6 MHz, $CDCl_3$): δ 171.92, 164.64, 155.86, 135.00, 133.01, 132.43, 129.29, 128.75, 128.59, 127.08, 122.36, 114.18, 72.17, 61.72, 52.93, 52.25, 51.20, 47.87, 41.85, 41.51, 39.97, 39.32, 34.31, 34.11, 34.02, 32.39, 26.58, 26.23, 24.88, 23.41, 22.82, 22.20, 21.81. **Elemental analysis:** calc. for $C_{37}H_{53}ClN_4O_6$ (685.29): C, 64.85; H, 7.80; N, 8.18. Found: C, 64.44 ± 0.32; H, 7.82 ± 0.10; N, 8.33 ± 0.14. **HRMS:** *m*/*z* calc. for $C_{37}H_{53}ClN_4O_6$: 707.35513 $[M+Na]^+$; found: 707.35458 $[M+Na]^+$.

(R)-1-((S)-2-(2-(benzyloxy)-5-chlorobenzamido)-4-methylpentanoyl)-N-((R)-1-(methoxy(methyl)amino)-4-methyl-1-oxopentan-2-yl)pyrrolidine-2-carboxamide ***3j***

Reaction was done in 3 mmol scale. Yellow amorph; yield 83 %; R_f = 0.39 (ethylacetate/*n*-hexane 3:1). **^{1}H NMR** (400 MHz, $CDCl_3$): δ 8.31 (1H, d, *J* = 7.6 Hz, N*H*), 8.14 (1H, d, *J* = 2.7 Hz, N*H*), 7.51 (2H, d, *J* = 7.1 Hz, C*H*(Ar)), 7.45–7.36 (4H, m, 4 × C*H*(Ar)), 7.1 (1H, d, *J* = 8.3 Hz, C*H*(Ar)), 7.0 (1H, d, *J* = 8.8 Hz, C*H*(Ar)), 5.23–5.08 (2H, m, CH_2–O–Ph), 4.97–4.96 (1H, m, NH–C*H*–C=O), 4.9–4.84 (1H, m, NH–C*H*–C=O), 4.59–4.57 (1H, m, N–C*H*–C=O), 3.91–3.55 (2H, m, CH_2–N), 3.79 (3H, s, CH_3–O), 3.19 (3H, s, CH_3–N), 2.31–2.27 (1H, m), 2.21–2.11 (1H, m), 2.04–2.02 (1H, m), 1.95–1.86 (1H, m), 1.64–1.54 (1H, m), 1.51–1.42 (3H, m), 1.38–1.32 (2H, m), 0.96–0.82 (9H, m, 2 × (CH_3)$_2$–CH), 0.74 (3H, d, *J* = 6.5 Hz, CH_3–CH). **^{13}C NMR** (100.62 MHz, $CDCl_3$): δ 172.64, 171.18, 164.03, 155.68, 135.19, 132.68, 132.54, 132.21, 129.15, 129.05, 128.91, 128.62, 128.57, 126.99, 122.90, 114.27, 71.97, 61.73, 59.88, 49.87, 47.92, 47.46, 41.66, 41.51, 27.34, 25.39, 24.82, 23.62, 23.46, 21.81, 21.74. **CNH Analysis:** calc. for $C_{33}H_{45}ClN_4O_6$ (629,19): C, 62,99; H, 7,21; N, 8,90. Found: C, 63,50 ± 0.36; H, 7,57 ± 0.09; N, 8.29 ± 0.01. **HRMS**: *m*/*z* calc. for $C_{33}H_{45}ClN_4O_6$: 146.11756 $[M+H]^+$; found: 146.11762 $[M+H]^+$.

1-((R)-2-((S)-2-(2-(benzyloxy)-5-chlorobenzamido)-4-methylpentanamido)-4-methylpentanoyl)-N-methoxy-N-methylpyrrolidine-2-carboxamide ***3k***

Reaction was done in 1.2 mmol scale. White amorph; yiled 40 %; R_f = 0.288 (ethylacetate/*n*-hexane 2:1). **^{1}H NMR** (500 MHz, $CDCl_3$): δ 8.14–8.03 (2H, m, 2 × N*H*), 7.47–7.34 (6H, m, 6 × C*H*(Ar)), 7.15–6.86 (2H, m, 2 × C*H*(Ar)), 5.21–5.09 (2H, m, CH_2–O–Ph), 4.86–4.74 (2H, m, 2 × NH–C*H*–C=O), 4.58–4.50 (1H, m, N–C*H*–C=O), 3.82–3.80 (1H, m, N–C*H*H), 3.78–3.75 (3H, m, CH_3–O), 3.63–3.50 (1H, m, N–CH*H*), 3.16–3.10 (3H, m, CH_3–N), 2.22–2.06 (2H, m, N–CH_2–CH_2–CH_2), 1.89–1.84 (1H, m, N–CH_2–CH_2–C*H*H), 1.71–1.44 (4H, m, 2 × CH–CH_2–CH), 1.40–1.35 (1H, m, N–CH_2–CH_2–CH*H*), 1.23–1.16 (1H, m, C*H*(CH_3)$_2$), 0.97–0.65 (13H, m, C*H*(CH_3)$_2$, 4 × CH_3). **^{13}C NMR** (100.6 MHz, $CDCl_3$): δ 172.03, 171.12, 164.22, 155.59, 135.07, 135.04, 132.77, 132.36, 129.22, 128.57, 128.52, 127.08, 122.74, 114.12, 71.95, 61.44, 56.87, 52.52, 49.20, 47.39, 41.75, 41.23, 40.61, 32.35, 28.88, 24.94, 24.84, 23.56, 23.21, 22.02, 21.72. **Elemental analysis:** calc. for $C_{33}H_{45}ClN_4O_6$ (629,18): C, 62.99; H, 7.21; N, 8.90. Found: C, 62.32 ± 0.09; H, 7.25 ± 0.01; N, 8.40 ± 0.03. **HRMS:** *m*/*z* calc. for $C_{33}H_{45}ClN_4O_6$: 667.26592 $[M+K]^+$; found: 667.26794 $[M+K]^+$.

General experimental procedure for benzyl protected dipeptidic phenyl sulfonyl hydrazides **4a–d**

Dipeptidic acid **1a**, **1c**, **1h** or **1i** (1 mmol) was dissolved in dry acetonitrile (ACN, 18 mL), HOAt (1.16 mmol) and EDC·HCl (1.25 mmol) was added in one portion under inert atmosphere. After activation of starting acid (TLC control, approx. 30 min) phenylsulfonyl hydrazide (1.25 mmol) was added as solution in acetonitrile (9 mL). Reaction mixture was heated up to 40 °C and stirred overnight. After, ACN was evaporated under reduced pressure and residue dissolved in ethyl acetate (18 mL) and extracted with water (9 mL). Separated water phase was extracted with ethyl acetate (2 × 9 mL). Combined organic phases were extracted with 1% water solution of sodium hydrogen carbonate (3 × 9 mL) and with water (3 × 9 mL). Organic phase was dried over sodium sulfate. Benzyl protected dipeptidic phenylsulfonyl hydrazides was purified by column chromatography in ethyl acetate – *n*-hexane mixture.

Characterization data of prepared benzyl protected dipeptidic phenylsulfonyl hydrazides **4a–d**

*2-(benzyloxy)-5-chloro-N-((S)-4-methyl-1-(((S)-4-methyl-1-oxo-1-(2-(phenylsulfonyl) hydrazinyl)pentan-2-yl)amino)-1-oxopentan-2-yl)benzamide **4a***

Reaction was done in 2 mmol scale. White crystals; yield 82 %; *mp* = 183.2–186.5 °C. R_f = 0.20 (ethylacetate/*n*-hexane 1:1). **^{1}H NMR** (500 MHz, DMSO): δ 10.29 (1H, d, *J* = 4.8 Hz, N*H*), 9.96 (1H, d, *J* = 4.8 Hz, N*H*), 8.22 (1H, d, *J* = 8.2 Hz, N*H*), 8.02 (1H, d, *J* = 8.1 Hz, N*H*), 7.77 (2H, d, *J* = 7.8 Hz, 2 × C*H*(Ar)), 7.70 (1H, d, *J* = 2.7 Hz, C*H*(Ar)), 7.62–7.48 (6H, m, 6 × C*H*(Ar)), 7.40–7.32 (4H, m, 4 × C*H*(Ar)), 5.24 (2H, s, CH_2–O–Ph), 4.41–4.45 (1H, m, NH–C*H*), 4.23–4.27 (1H, m, NH–C*H*), 1.33–1.51 (2H, m,CH_2–CH–$(CH_3)_2$), 1.22–1.27 (2H, m, CH_2–CH–$(CH_3)_2$), 1.15–1.19 (2H, m, 2 × C*H*), 0.84 (3H, d, *J* = 6.6 Hz, CH_3), 0.77–0.74 (6H, m, 2 × CH_3), 0.66 (3H, d, *J* = 6.4 Hz, CH_3). **^{13}C NMR** (400 MHz, $CDCl_3$): δ 172.03, 171.51, 163.70, 155.59, 139.59, 136.41, 133.52, 132.51, 130.55, 129.31, 129.17, 128.90, 128.81, 128.39, 125.40, 125,01, 116.23, 71.44, 51.93, 49.69, 41.93, 41.62, 24.79, 24.61, 23.70, 23.48, 22.18, 21.95. **CHN analysis:** calc. for $C_{32}H_{39}ClN_4O_6S$ (643.19): C, 59.76; H, 6.11; N, 8.71; S, 4.99. Found: C, 59.88 ± 0.18; H, 6.15 ± 0.01; N, 8.32 ± 0.22; S, 4.48 ± 0.05. **HRMS:** *m*/*z* calc. for $C_{32}H_{39}ClN_4O_6S$: 665.21710 $[M+Na]^+$; found: 665.21881 $[M+Na]^+$.

2-(benzyloxy)-5-chloro-N-((S)-1-oxo-1-(((S)-1-oxo-3-phenyl-1-(2-(phenylsulfonyl) hydrazinyl)propan-2-yl)amino)-3-phenylpropan-2-yl)benzamide ***4b***

Reaction was done in 1.8 mmol scale. White crystals; yield 95 %; *mp* = 141.4–144.0 °C. R_f = 0.23 (ethylacetate/*n*-hexane 1:1). **^{1}H NMR** (500 MHz, DMSO): δ 10.4 (1H, s, N*H*), 10.02 (1H, d, *J* = 4.5 Hz, N*H*), 8.35 (1H, d, *J* = 8.5 Hz, N*H*), 8.25 (1H, d, *J* = 8.1 Hz, N*H*), 7.8 (2H, d, *J* = 7.9 Hz, 2 × C*H*(Ar)),7.61–7.58 (2H, m, 2 × C*H*(Ar)), 7.52–7.45 (3H, m, 3 × C*H*(Ar)), 7.37–7.28 (5H, m, 5 × C*H*(Ar)). 7.21–7.08 (9H, m, 9 × C*H*(Ar)), 6.91 (2H, d, *J* = 6.7 Hz, 2 × C*H*(Ar)), 5.19 (2H, s, CH_2–O–Ph), 4.67–4.63 (1H, m, NH–C*H*–C=O), 4.56–4.51 (1H, m, NH–C*H*–C=O), 2.91–2.8 (2H, m, CH_2–Ph), 2.62–2.48 (2H, m, CH_2–Ph). **^{13}C NMR** (400 MHz, $CDCl_3$): δ 171.00, 170.55, 163.18, 155.22, 139.39, 139.30, 137.66, 137.64, 136.28, 133.43, 132.41, 130.48, 129.62, 129.42, 129.27, 129.02, 128.71, 128.51, 128.30, 128.20, 128.17, 126.85, 126.75, 125.13, 124.20, 116.18, 70.83, 54.39, 52.58, 38.18. **CHN analysis:** calc. for $C_{38}H_{35}ClN_4O_6S$ (711.23): C, 64.17; H, 4.96; N, 7.88; S, 4.51. Found: C, 64.76 ± 0.12; H, 4.95 ± 0.03; N, 7.94 ± 0.12; S, 4.17 ± 0.09. **HRMS:** *m*/*z* calc. for $C_{38}H_{35}ClN_4O_6S$: 733.18580 $[M+Na]^+$; found: 733.18604 $[M+Na]^+$.

2-(benzyloxy)-5-chloro-N-((S)-1-(((S)-4-methyl-1-oxo-1-(2-(phenylsulfonyl) hydrazinyl)pentan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)benzamide ***4c***

Reaction was done in 1,5 mmol scale. White crystals; yield 48 %; *mp* = 165.0–170.6 °C. R_f = 0.5 (ethylacetate/*n*-hexane 1:1). **^{1}H NMR** (400 MHz, $CDCl_3$): δ 8.89 (1H, s, N*H*), 8.40 (1H, s, N*H*), 8.10–8.08 (1H, m, C*H*(Ar)), 7.86–7.85 (2H, m, 2 × N*H*), 7.56–7.51 (2H, m, 2 × C*H*(Ar)), 7.45–7.36 (9H, m, 9 × C*H*(Ar)), 7.20–7.19 (3H, m, 3 × C*H*(Ar)), 7.02–6.92 (3H, m, 3 × C*H*(Ar)), 5.18–5.07 (2H, m, CH_2–O–Ph), 4.75 (1H, s, NH–C*H*–C=O), 4.21 (1H, m, NH–C*H*–C=O), 2.99–2.85 (2H, m, CH_2–Ph), 1.28–1.15 (3H, m, CH–CH_2–C*H*–$(CH_3)_2$), 0.73–0.59 (6H, m, 2 × CH_3). **^{13}C NMR** (400 MHz, $CDCl_3$): δ 171.75, 170.67, 165.14, 155.66, 136.58, 136.29, 135.10, 133.83, 133.33, 132.31, 129.30, 129.22, 129.02, 128.97, 128.95, 128.43, 128.11, 127.38, 127.23, 122.04, 114.56, 71.99, 55.76, 50.69, 39.77, 37.21, 24.50, 22.77, 21.89. **CHN analysis:** calc. for $C_{35}H_{37}ClN_4O_6S$ (677,21): C 62.07; H 5.51; N 8.27; S 4.73. Found: C 61.94 ± 0.26; H 5.48 ± 0.03; N 8.22 ± 0.03; S 4.51 ± 0.18. **HRMS:** *m*/*z* calc. for $C_{35}H_{37}ClN_4O_6S$: 699.20145 $[M+Na]^+$; found: 699.20337 $[M+Na]^+$.

2-(benzyloxy)-5-chloro-N-((S)-4-methyl-1-oxo-1-(((S)-1-oxo-3-phenyl-1-(2-(phenylsulfonyl)hydrazinyl)propan-2-yl)amino)pentan-2-yl)benzamide ***4d***

Reaction was done in 9,5 mmol scale. White crystals; yield 94 %; *mp* = 169.6–174.6 °C. R_f = 0.43 (ethylacetate/*n*-hexane 3:2). **^{1}H NMR** (400 MHz, DMSO): δ 10.42 (1H, d, *J* = 7.9 Hz, N*H*), 10.03 (1H, d, *J* = 3.8 Hz, N*H*), 8.15 (1H, t, *J* = 8.6 Hz, C*H*(Ar)), 7.87–7.84 (1H, m, C*H*(Ar)), 7.83 (1H, s, N*H*), 7.67 (1H, dt, *J* = 2.4; 4.6 Hz, C*H*(Ar)), 7.62–7.52 (5H, m, 5 × C*H*(Ar)), 7.43–7.35 (5H, m, 5 × C*H*(Ar)), 7.28–7.22 (5H, m, 5 × C*H*(Ar)), 5.30 (1H, d, *J* = 4.1 Hz, N*H*–CH), 5.27 (1H, s, N*H*), 4.56–4.40 (2H, m, CH_2–O–Ph), 2.88–2.82 (1H, m, N*H*–CH), 2.67–2.61 (1H, m, C*H*–$(CH_3)_2$), 1.32–1.17 (2H, m, CH_2), 1.07–0.95 (2H, m, CH_2), 0.75 (3H, d, *J* = 8.1 Hz, CH_3), 0,68 (3H, d, *J* = 4.2 Hz, CH_3). **^{13}C NMR** (400 MHz, DMSO): δ 172.06, 170.69, 163.64, 155.57, 139.58, 137.89, 136.37, 133.57, 132.51, 130.60, 129.78, 129.42. 129.17, 128.99, 128.89, 128.78, 128.63, 128.33, 126.94, 125.38, 125.00, 116.16, 71.46, 52.58, 52.00, 38.26, 24.70, 23.57, 21.98. **CHN analysis:** calc. for $C_{35}H_{37}ClN_4O_6S$ (677,21): C, 62.07; H, 5.51; N, 8.27; S, 4.73. Found: C, 61.47 ± 0.02; H, 5.47 ± 0.04; N, 8.79 ± 0.13; S, 5.33 ± 0.05. **HRMS:** *m*/*z* calc. for $C_{35}H_{37}ClN_4O_6S$: 699.20146 $[M+Na]^+$; found: 699.20135 $[M+Na]^+$.

General experimental procedure for the synthesis of dipeptidic phenylsulfonyl hydrazides **5a–d**

Benzyl-protected dipeptidic phenylsulfonyl hydrazides **4a–d** (1 mmol) were dissolved in tetrahydrofurane, and a catalytic amount of 10% palladium on carbon was added. In a hydrogen atmosphere, the reaction mixture was stirred to complete conversion of the starting materials (under TLC control, approx. 3 h). The catalyst was removed, and the ethyl acetate was evaporated under reduced pressure. The obtained crude dipeptidic phenylsulfonyl hydrazides were crystalized from an ethyl acetate – *n*-hexane mixture.

Characterization data of the prepared dipeptidic phenylsulfonyl hydrazides **5a–d**

5-chloro-2-hydroxy-N-((S)-4-methyl-1-(((S)-4-methyl-1-oxo-1-(2-(phenylsulfonyl)hydrazinyl)pentan-2-yl)amino)-1-oxopentan-2-yl)benzamide ***5a***

Reaction was done in 1,5 mmol scale. White crystals; yield 98 %; *mp* = 108.7–114.4 °C. R_f = 0.53 (ethylacetate/*n*-hexane 1:1). **^{1}H NMR** (500 MHz, DMSO): δ 12.25–12.14 (1H, m, O*H*), 10.26 (1H, s, N*H*), 9.92 (1H, s, N*H*), 8.86–8.69 (1H, m, N*H*), 8.09 (1H, d, *J* = 8 Hz, C*H*(Ar)), 8.00 (1H, s, C*H*(Ar)), 7.79–7.75 (2H, m, 2 × C*H*(Ar)), 7.61 (1H, t, *J* = 7.3 Hz, C*H*(Ar)), 7.51 (2H, t, *J* = 7.5 Hz, 2 × C*H*(Ar)), 7.47–7.37 (1H, m, C*H*(Ar)), 6.96–6.87 (1H, m, N*H*), 4.53–4.51 (1H, m, NH–C*H*–C=O),

4.29–4.24 (1H, m, NH–C*H*–C=O), 1.61–1.40 (4H, m, 2 × CH–CH_2–CH), 1.29–1.16 (2H, m, 2 × CH_2–C*H*–$(CH_3)_2$), 0.90–0.77 (12H, m, 4 × CH_3). **^{13}C NMR** (100.6 MHz, DMSO): δ 171.96, 171.51, 167.12, 158.47, 139.60, 133.83, 133.52, 129.31, 128.79, 128.39, 123.11, 119.80, 118.03, 52.01, 49.79, 41.60, 41.27, 24.99, 24.61, 23.85, 23.46, 22.17, 22.03. **CHN analysis:** calc. for $C_{25}H_{33}ClN_4O_6S$ (553,07): C, 55.29; H, 6.01; N,10.13; S, 5.80. Found: C, 55,57 ± 0.05; H, 6.15 ± 0.04; N, 9.80 ± 0.06; S, 4.56 ± 0.18. **HRMS:** *m*/*z* calc. for $C_{25}H_{33}ClN_4O_6S$: 575.17015 $[M+Na]^+$; found: 575.17200 $[M+Na]^+$.

5-chloro-2-hydroxy-N-((S)-1-oxo-1-(((S)-1-oxo-3-phenyl-1-(2-(phenylsulfonyl) hydrazinyl)propan-2-yl)amino)-3-phenylpropan-2-yl)benzamide ***5b***

Reaction was done in 0,6 mmol scale. White crystals; yield 94 %; *mp* = 220.5–223.4 °C. R_f = 0.37 (ethylacetate/*n*-hexane 1:1). **^{1}H NMR** (400 MHz, DMSO): δ 12.09–12.01 (1H, m, O*H*), 10.46–10.45 (1H, m, N*H*), 10.07–10.06 (1H, m, N*H*), 8.87–8.78 (1H, m, N*H*), 8.46–8.39 (1H, m, N*H*), 7.93–7.83 (3H, m, 3 × C*H*(Ar)), 7.71–7.66 (1H, m, C*H*(Ar)), 7.61–7.57 (2H, m, 2 × C*H*(Ar)), 7.47–7.39 (1H, m, C*H*(Ar)), 7.29–7.25 (7H, m, 7 × C*H*(Ar)), 7.22–7.19 (2H, m, 2 × C*H*(Ar)), 6.96–6.90 (2H, m, 2 × C*H*(Ar)), 4.78–4.73 (1H, m, NH–C*H*–C=O), 4.61–4.55 (1H, m, NH–C*H*–C=O), 3.05–2.99 (1H, m, C*H*H), 2.94–2.86 (2H, m, CH_2), 2.71–2.65 (1H, m, CH*H*). **^{13}C NMR** (400 MHz, DMSO): δ 171.10, 170.72, 166.67, 158.23, 139.59, 138.21, 137.85, 134.28, 133.81, 133.62, 129.86, 129.74, 129.46, 128.74, 128.68, 128.37, 127.00, 125.57, 123.57, 119.73, 117.94, 117.75, 54.59, 52.85, 37.81, 35.03, 31.06. **CHN analysis:** calc. for $C_{31}H_{29}ClN_4O_6S$ (621.10): C, 59.95; H, 4.71; N, 9.02; S, 5.16. Found: C, 61.32 ± 0.42; H, 5.35 ± 0.13; N,8.02 ± 0.04; S, 4.12 ± 0.13. **HRMS:** *m*/*z* calc. for $C_{31}H_{29}ClN_4O_6S$: 643.13885 $[M+Na]^+$; found: 643.13898 $[M+Na]^+$.

5-chloro-2-hydroxy-N-((S)-1-(((S)-4-methyl-1-oxo-1-(2-(phenylsulfonyl) hydrazinyl)pentan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)benzamide ***5c***

Reaction was done in 0.6 mmol scale. White amorph; yield 93 %; *mp* = 154.5–161.0 °C. R_f = 0.58 (ethylacetate/*n*-hexane 6:1). **^{1}H NMR** (400 MHz, DMSO): δ 12.03 (1H, s, O*H*), 10.30–10.27 (1H, m, N*H*), 9.94–9.88 (1H, m, N*H*), 8.97–8.86 (1H, m, N*H*), 8.39–8.26 (1H, m, N*H*), 7.97–7.90 (1H, m, C*H*(Ar)), 7.89–7.75 (2H, m, 2 × C*H*(Ar)), 7.62–7.59 (1H, m, C*H*(Ar)), 7.54–7.38 (3H, m, 3 × C*H*(Ar)), 7.27–7.14 (5H, m, 5 × C*H*(Ar)), 6.99–6.89 (1H, m, C*H*(Ar)), 4.77–4.72 (1H, m, NH–C*H*–C=O), 4.33–4.20 (1H, m, NH–C*H*–C=O), 3.05–2.85 (2H, m, CH_2–Ph), 1.52–1.47 (1H, m, C*H*), 1.29–1.22 (2H, m, CH_2), 0.86–0.73 (6H, m, 2 × CH_3). **^{13}C NMR** (100.6 MHz, DMSO): δ 171.52, 171.06, 166.78, 158.18, 139.64, 138.29, 133.76, 133.54, 129.77, 129.33, 128.75, 128.68, 128.41, 126.99, 123.10, 119.75, 118.13, 54.76,

49.94, 41.78, 37.92, 24.65, 23.45, 22.25. **CHN analysis:** calc. for $C_{28}H_{31}ClN_4O_6S$ (587.09): C, 57.28; H. 5.32; N, 9.54; S, 5.46. Found: C, 55.46 ± 0.24; H, 5.92 ± 0.07; N, 8.13 ± 0.04; S, 4.79 ± 0.04. **HRMS:** *m*/*z* calc. for $C_{28}H_{31}ClN_4O_6S$: 609.15450 $[M+Na]^+$; found: 609.15649 $[M+Na]^+$.

5-chloro-2-hydroxy-N-((S)-4-methyl-1-oxo-1-(((S)-1-oxo-3-phenyl-1-(2-(phenylsulfonyl)hydrazinyl)propan-2-yl)amino)pentan-2-yl)benzamide ***5d***

Reaction was done in 0.7 mmol scale. White crystals; yield 97 %; *mp* = 162.6–170.3 °C. R_f = 0.29 (ethylacetate/*n*-hexane 1:1). **^{1}H NMR** (400 MHz, DMSO): δ 12.25 (1H, s, O*H*), 10.36 (1H, s, N*H*), 9.98 (1H, s, N*H*), 8.72 (1H, d, *J* = 8 Hz, N*H*), 8.21 (1H, d, *J* = 8.4 Hz, C*H*(Ar)), 7.97–7.96 (1H, m, C*H*(Ar)), 7.79 (2H, d, *J* = 7.7 Hz, 2 × C*H*(Ar)), 7.63 (1H, t, *J* = 7.3 Hz, C*H*(Ar)), 7.54 (2H, t, *J* = 7.6 Hz, 2 × C*H*(Ar)), 7.46–7.42 (1H, m, C*H*(Ar)), 7.20–7.13 (5H, m, 5 × C*H*(Ar)), 6.94 (1H, d, *J* = 8.8 Hz, N*H*), 4.51–4.45 (2H, m, 2 × NH–C*H*–C=O), 2.82–2.77 (1H, m, C*H*H), 2.63–2.59 (1H, m, CH*H*), 1.55–1.48 (2H, m, CH_2), 1.39–1.33 (1H, m, CH_2–C*H*–$(CH_3)_2$), 0.87–0.83 (6H, m, 2 × CH_3). **^{13}C NMR** (100.6 MHz, DMSO): δ 171.93, 170.70, 167.01, 158.50, 139.58, 137.93, 133.84, 133.58, 129.78, 129.42, 128.76, 128.60, 128.35, 126.93, 123.07, 119.78, 117.93, 52.67, 51.92, 41.15, 38.20, 24.90, 23.77, 22.09. **CHN analysis:** calc. for $C_{28}H_{31}ClN_4O_6S$ (587.09): C, 57.28; H, 5.32; N, 9.54; S, 5.46. Found: C, 56.89 ± 0.06; H, 5.42 ± 0.02; N, 9.58 ± 0.01; S, 5.06 ± 0.01. **HRMS:** *m*/*z* calc. for $C_{28}H_{31}ClN_4O_6S$: 609.15450 $[M+Na]^+$; found: 609.15625 $[M+Na]^+$.

Cancer cell lines and cytotoxicity assay

Human cancer cell lines were obtained from the European Collection of Authenticated Cell Cultures (MCF-7, CEM, U266) or Cell Lines Service (MV4-11) and cultivated according to the provider's instructions. In brief, cell lines were maintained in DMEM or RPMI8226 medium supplemented with foetal bovine serum, penicillin (100 U/mL), and streptomycin (100 mg/mL) at 37 °C in 5% CO_2. The cells were assayed with compounds in triplicate. After treatment, resazurin solution (Sigma Aldrich, final concentration 10.5 μg/ml) was added for 4 h, and the fluorescence of resorufin, corresponding to the number of live cells, measured at 544 nm/590 nm (excitation/emission) using a Fluoroskan Ascent microplate reader (Labsystems, Helsinki, Finland). The GI_{50} value, the drug concentration lethal to 50 % of the tumour cells, was calculated from the obtained dose-response curves.

Caspase-3/7 assay

The harvested cells were homogenized in extraction buffer (10 mM HEPES, 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.5 % NP-40, pH 7.4) on ice for 20 min. The homogenates were separated by centrifugation at 10 000 × *g* for 30 min at 4 °C, and then the proteins quantified and diluted to an equal concentration. Next, the lysates were incubated for 4 h with 100 mM Ac-DEVD-AMC, the substrate of caspases 3 and 7, in assay buffer (25 mM PIPES, 2 mM EGTA, 2 mM $MgCl_2$, 5 mM DTT, pH 7.3). The fluorescence of the product was measured using a Fluoroskan Ascent microplate reader (Labsystems) at 355/460 nm (excitation/emission).

Immunoblotting and antibodies

Briefly, the treated cells were harvested and then lysed in extraction buffer (10 mM HEPES, 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.5 % NP-40, pH 7.4). Proteins were separated on SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes. After blocking, the membranes were incubated overnight with specific primary antibodies, washed, and incubated with peroxidase-conjugated secondary antibodies. Finally, the peroxidase activity was detected with SuperSignal West Pico reagents (Thermo Scientific) using an LAS-4000 CCD camera (Fujifilm). Specific antibodies were purchased from Cell Signalling Technology (anti-PARP, clone 46D11; anti-XIAP; anti-Mcl-1, clone D35A5; anti-caspase-7; anti-cleaved caspase-9, clone E5Z7N; anti-Bcl-xl, clone 54H6; anti-Hsp70; peroxidase-labelled secondary antibodies) and Merck (anti-Bcl-2; anti-α-tubulin, clone DM1A).

Results and discussion

Chemistry

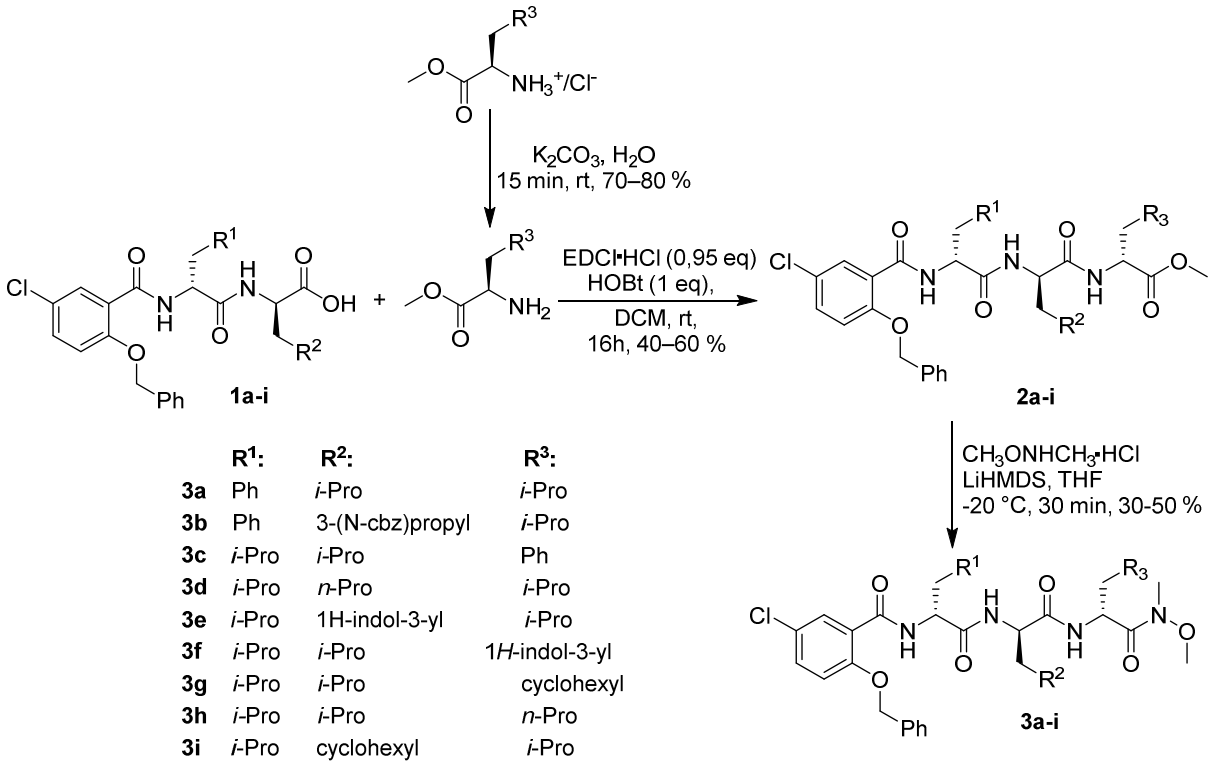
Based on our previous experience [13,14], we decided to synthesize the targeted WAs **3a–i** from the methyl esters of tripeptide of substituted salicylic acid amides **2** as outlined in **Scheme 1**. WAs derived from L-proline (**3j** and **3k**) were synthesized using a similar approach. Due to the structural differences, it is not possible to include these structures in general **Scheme 1**; therefore, the synthesis of the L-proline derivatives **3j** and **3k** is gathered into **Scheme 2**. The synthesis of the starting compounds has been previously described [13–15], producing optically pure dipeptidic acids or their analogues that contain various amino acids **1** prepared by the cascade mediated reactions involving 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl) and ester hydrolysis. The optical purity of

these starting intermediates is readily verifiable using ^{1}H NMR spectroscopy. Possible purification by crystallization is straightforward [14]. The dipeptidic moiety of acid **1** was constructed from L-Phe, L-Leu or their combination, or additionally L-Trp, L-norLeu, ε-cbz-L-Lys, or L-Pro was also added to the dipeptide moiety.

The EDC·HCl-mediated reactions of acid **1** with the free base of an amino acid methyl ester liberated from its hydrochloride salts and used immediately in the reaction, were performed in dry dichloromethane. The presence of hydroxybenzotriazole (HOBt) is also essential to minimize unsuitable reactions during amidation (e.g., racemization). The formation of tripeptidic esters **2** proceeded to the next final step without further purification and characterization.

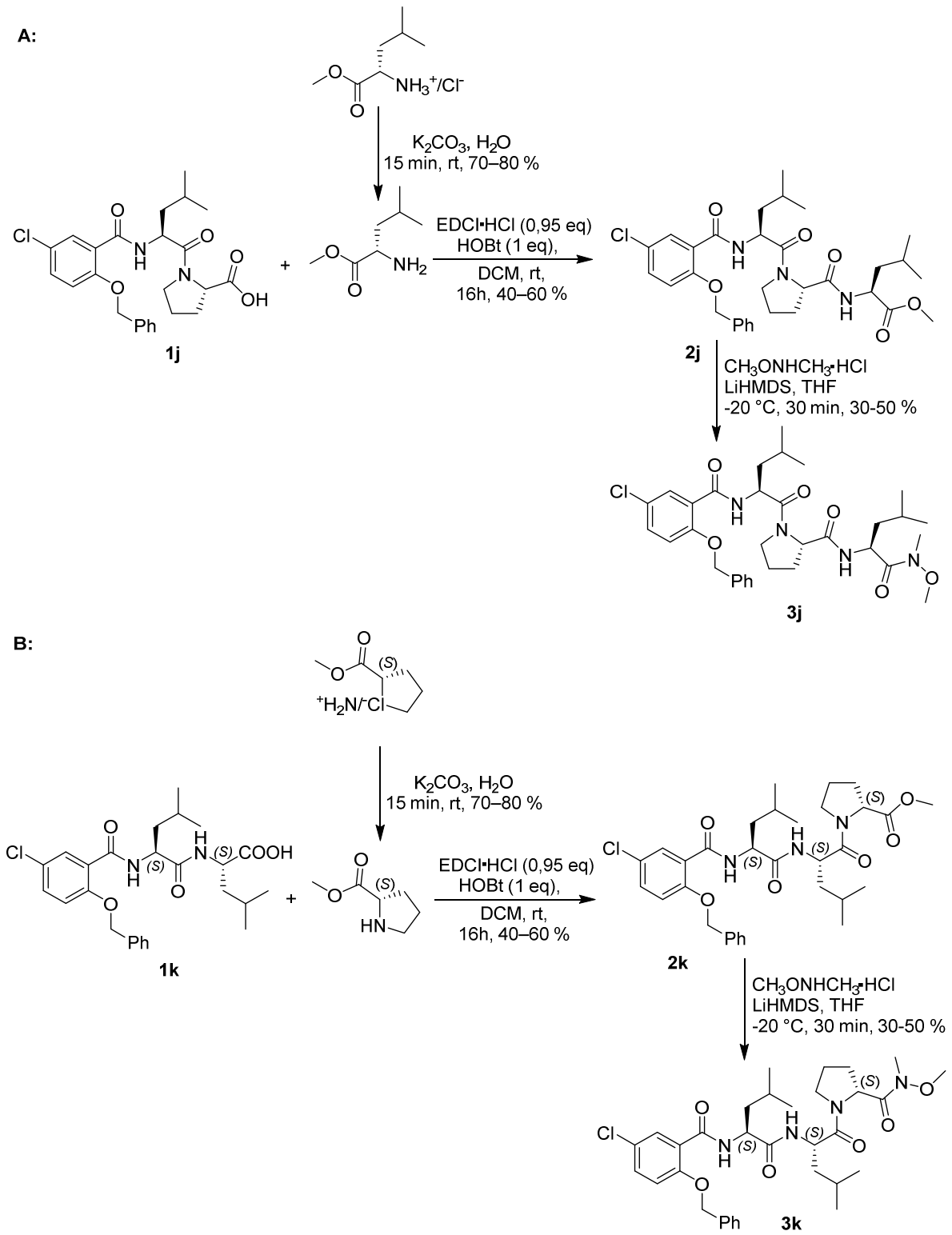
Targeted WAs **3a–k** were obtained by the conversion of methylesters **2** with *N,O*-dimethylhydroxylamine hydrochloride in the presence of lithium bis(trimethylsilyl)amide (LiHMDS) in satisfactory yields. All 11 WAs are original compounds except **3c** which was prepared and characterized in our previous study [15]. Alternatively, in literature, there is an approach involving reaction with *i*-Pro-MgCl, but this synthetic way is not suitable for our purpose due to the low selectivity that goes hand in hand with the low degree of conversion of the starting material [17].

Scheme 1 is the general synthetic pathway that describes the preparation of compounds **3a–i**. Compounds **3j** and **3k** contain L-proline in the peptide moiety at different tripeptide positions. The synthetic approach for these compounds is identical to that for compounds **3a–i** and is shown in **Scheme 2**.



	R1:	R2:	R3:
3a	Ph	*i*-Pro	*i*-Pro
3b	Ph	3-(N-cbz)propyl	*i*-Pro
3c	*i*-Pro	*i*-Pro	Ph
3d	*i*-Pro	*n*-Pro	*i*-Pro
3e	*i*-Pro	1H-indol-3-yl	*i*-Pro
3f	*i*-Pro	*i*-Pro	1*H*-indol-3-yl
3g	*i*-Pro	*i*-Pro	cyclohexyl
3h	*i*-Pro	*i*-Pro	*n*-Pro
3i	*i*-Pro	cyclohexyl	*i*-Pro

Scheme 1 General synthetic scheme for Weinreb amides **3a–i**



Scheme 2 Synthetic route for Weinreb amides **3j** and **3k** containing L-proline in different parts of the tripeptide moiety

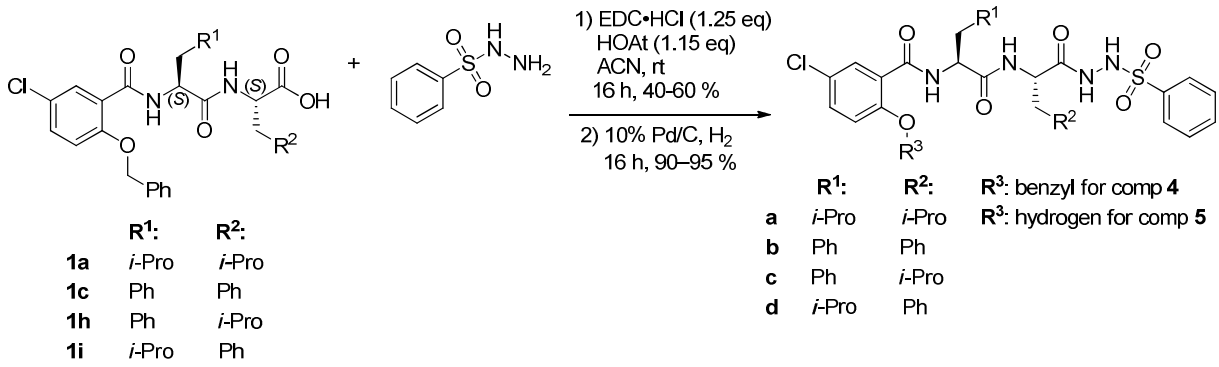
A: L-proline is in the middle of the tripeptide surrounded by L-leucine (**3j**)
B: L-proline terminates the amino acid tripeptide moiety (**3k**)

Table 1 Antiproliferative activities of novel Weinreb amides **3a–3k.**

	GI_{50} [μmol]*			
Compound	CEM	MV4-11	U266	MCF-7
3a	>25	>25	>25	>25
3b	>25	>25	>25	>25
3c	>25	>25	18.5 ± 8.0	18.4 ± 2.9
3d	>25	>25	>25	>25
3e	17.4 ± 2.7	9.7 ± 0.1	8.2 ± 2.6	>12.5
3f	>25	>25	8.5 ± 0.3	>25
3g	>25	>25	>25	>25
3h	>12.5	>12.5	>12.5	>12.5
3i	>12.5	>12.5	11.4 ± 1.1	>12.5
3j	>12.5	>12.5	>12.5	>12.5
3k	>12.5	>12.5	>12.5	>12.5
bortezomib	0.004	0.002	0.001	0.011

* tested at least in duplicates

A library of phenylsulfonyl hydrazides was prepared using similar approach to that for the methyl esters **2**. Optically pure starting acids **1** (constructed from the combination of the protected amides of 5-chloro salicylic acid, and L-phenylalanine and L-leucine) were coupled with phenylsulfonyl hydrazide by means of standard EDC·HCl reaction in the presence of HOAt and acetonitrile as the reaction solvent [18]. Hydrazides **4a–d** were obtained in very high yields, except for **4c**, which was obtained in a 48 % yield only (**Scheme 3**). Such a low value was probably caused by repeated purification of the product required to obtain a material of the adequate quality.



Scheme 3 Synthesis of protected phenylsulfonyl hydrazides **4a–d** and their debenzylated analogues **5a–d**

Antiproliferative activity of novel derivatives

Weinreb amides and sulfonyl hydrazides were tested for their antiproliferative properties against four cancer cell lines derived from acute lymphoblastic leukaemia (CEM), acute myeloid leukemia (MV4-11), multiple myeloma (U266), and breast adenocarcinoma (MCF-7). The respective data are presented in **Table 1**. Resazurin-based method was used to determine the GI_{50} value corresponding to the concentration of compound able of reducing the number of live cells to 50%.

The data show that some compounds from the series of Weinreb amides and sulphonamides resulted in GI_{50} values in the mid-micromolar range (**Table 1**). On the other hand, all sulfonyl hydrazides did not compromise cytotoxicity. Activity of Weinreb amides is affected by amino acid side chains in the peptide part of the molecules. We found that amide **3e** containing *1H*-indol-3-yl moiety (incorporation of tryptophan) in the middle part of peptide chain was able to inhibit proliferation of all cell lines derived from haematological malignancies and its GI_{50} values reached the single-digit micromolar ranges (9.7 μM and 8.2 μM for MV4-11 and U266 cell lines).

Effects of the Weinreb amides on cell death

The mechanism of the cell death after treatment with the most active compound **3e** was then analysed by immunoblotting experiments. As shown in **Figure 1A**, the dose-dependent cleavage of poly(ADP-ribose) polymerase (PARP) in all treated cells was obvious, indicating that the cell lines undergo apoptosis. This observation was further supported by the presence of activated caspases 7 and 9 that were clearly detected in CEM and MV4-11 cells, respectively. In addition, we monitored the expression of the anti-apoptotic proteins XIAP, Bcl-xl, Mcl-1 and Bcl-2 involved in the mitochondrial apoptotic pathway. Except for gradual decreases in XIAP and Mcl-1 expression in the treated MV4-11 cells, no significant changes were observed (**Figure 1A**). The trends registered from the immune-blotting results corresponded well to the biochemical caspase assays with lysates prepared from the cells treated with different concentrations of **3e** for 24 h (**Figure 1B**). Compound **3e** increased caspase activity more than five times and nine times compared to the untreated CEM and MV4-11 cells, respectively. CEM and MV4-11 cells were more sensitive than U266 cells, which confirmed apoptosis as the mechanism of cell death.

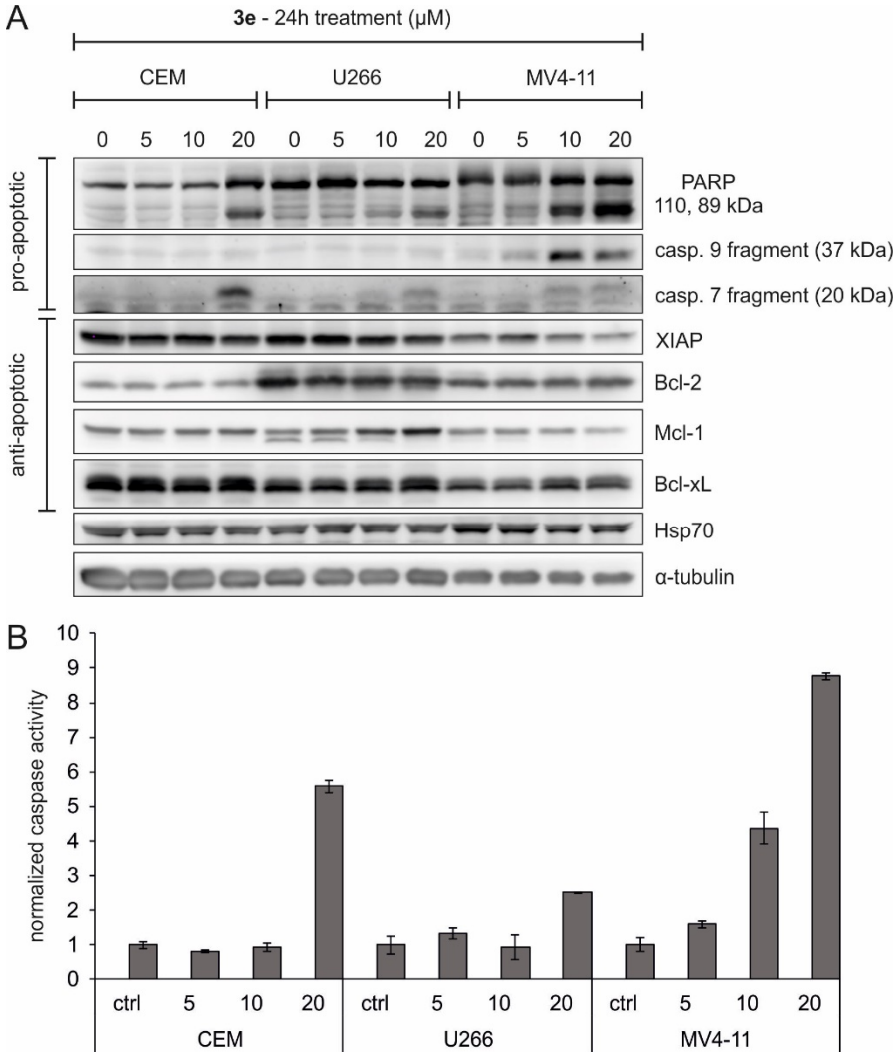


Fig. 1 **(A)** Effect of **3e** on the expression of proteins involved in programmed cell death in different cell lines

Lysates for immunoblotting analysis were prepared from cells treated with different concentrations of **3e** for 24 h
Hsp70 and α-tubulin were included as a control for protein loading

(B) The activity of caspase-3 was measured in cell lysates using the fluorogenic substrate Ac-DEVD-AMC and normalized to an untreated control

Conclusion

A series of novel pseudopeptide salicylates was synthesized and characterized using ^{1}H and ^{13}C NMR spectroscopy, HRMS, and elemental analysis. All 19 final compounds were tested for their antiproliferative activity against four cancer cell lines. The most potent Weinreb amide **3e**, with L-tryptophan incorporated, displayed the antiproliferative properties with single-digit micromolar GI_{50} values and was shown to induce apoptosis in CEM and MV4-11 cells.

Our results have demonstrated that Weinreb amides are useful not only for synthetic applications due their superior chemical properties but also they could be relevant as a part of biologically active compounds in relation to several disorders, including cancer.

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