Total Synthesis, NMR Solution Structure and Binding Model of the Potent Histone Deacetylase Inhibitor FR235222

Manuela Rodriguez, Stefania Terracciano, Elena Cini, Giulia Settembrini, Ines Bruno, Giuseppe Bifulco, Maurizio Taddei and Luigi Gomez-Paloma

a Professors L. Gomez-Paloma, I. Bruno, G. Bifulco, Doctors M. Rodriguez and S. Terracciano, Dipartimento di Scienze Farmaceutiche, Università di Salerno, via Ponte don Melillo, 84084 Fisciano (Salerno), Italy. Fax: (+39)-089-962828. E-mail: gomez@unisa.it

b Professor Maurizio Taddei, E. Cini and G. Settembrini, Dipartimento Farmaco Chimico Tecnologico, Università di Siena, via A. Moro 2, 53100, Siena, Italy. Fax: +39-0577-234333. Email: taddei.m@unisi.it

Abbreviations:
AA: amino acid
Bn: benzyl;
Boc: tert-butyloxycarbonyl;
CTrt-Cl: 2-chlorotrityl chloride-resin;
DCC: N,N’-dicyclohexylcarbodiimide;
DMF: N,N-dimethylformamide;
DIEA: N,N-diisopropylethylamine;
Fmoc: 9-fluorenylmethyloxycarbonyl;
HATU: O-(7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate;
HBTU: O-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate;
HOBt: N-hydroxybenzotriazole;
TBDMS: tert-butyldimethylsilyl;
TFA: trifluoroacetic acid;
TFE: 2,2,2-trifluoroethanol;
TIS: trisopropylsilane.
General Experimental Data

Proton detected (1H, HMBC, HSQC, TOCSY, ROESY) and carbon detected NMR spectra were recorded on Bruker instruments of Avance series operating at 300, 400, 600 MHz and 75, 100 and 150 MHz, respectively. Chemical shifts are expressed in parts per million (ppm) on the delta (δ) scale. The solvent peak was used as internal reference: for 1H NMR CDCl3 = 7.25 ppm, DMSO-d6 = 2.49 ppm; for 13C NMR: CDCl3 = 77.0 ppm, DMSO-d6: 39.5 ppm. Multiplicities are reported as follows: (s = singlet; d = doublet; t = triplet; q = quartet; dd = doublet of doublets; dt = doublet of triplets; b = broad). Mass spectra were recorded on a LCQ DECA TermoQuest (San José, California, USA) mass spectrometer using an electrospray ion source (ES-MS).

Analytical and semipreparative reverse phase HPLC purifications were performed on a Jupiter C-18 column (250 × 10.00 mm, 10 µ, 300 Å, respectively). For estimation of Fmoc amino acids loading on the resin, absorbance at 301 nm was reading using a Shimadzu UV 2101 PC spectrophotometer.

All the reaction solvents were previously dried following standard procedures. Analytical TLC was performed on silica gel 60 F254 (Merck) plates. Visualization was done under UV (λ = 254 nm). Flash chromatography was done using a Sepacore® system or 60/230-400 mesh silica gel.

(S)-2-Dibenzylamino-6-oxo-hexanoic acid benzyl ester (4).

Methoxymethyltriphenylphosphonium chloride (0.77 g, 2.25 mmol) was dissolved in dry THF (8 mL) and to this solution, cooled to 0 °C, LiN(SiMe3)2 (2.4 mL of a 1 M solution in THF) was slowly added. After 1 h at 0 °C, aldehyde (2.06 g, 15 mmol) was added in dry THF (8 mL). The mixture was stirred overnight at room temperature. Water was added and the two phases separated. The organic layer was washed several times with EtOAc, the collected organic fractions, were dried and evaporated. The crude product was purified using a Sepacore® system (silica gel column, petroleum ether 60-80/ EtOAc 3:1) to give compound 3 (0.48 g, 75% yield). This product was dissolved in EtOAc (8 mL) and then a solution of HCl 6N (4 mL) was added. The mixture was stirred at room temperature for 20 min, and then a saturated solution of Na2CO3 was added. The organic phase was separated and the aqueous layer washed several times with EtOAc. The organic fractions were collected and dried, the solvent evaporated to give crude compound 4 (0.436 g, 94% yield) as an oil. 1H NMR (400 MHz, CDCl3) δ: 1.60–1.80 (m, 4H), 2.05 (m, 2H), 2.23 (t-like, 1H), 3.41 (d-like, 2H), 3.80 (d-like, 2H), 5.12 (AB system, 2H), 7.15–7.40 (m, 15 H), 9.57 (s-like, 1H). 13C NMR (100 MHz, CDCl3) δ: 16.8, 28.9, 54.2, 54.4, 60.2, 64.0, 74.3, 124.2, 126.9, 127.0, 128.2 (2C), 128.3 (2C), 128.4 (2C), 128.5 (2C), 128.8 (2C), 129.0 (2C), 134.2 (2C), 136.2, 174.3, 202.1. ES-MS m/z 416 [M+H]+.

(2S,9R)-2-Dibenzylamino-9-((tert-butylimidemethylsilyloxy)-8-oxo-decanoic acid benzyl ester (5).

To a solution of [(R)-dimethyl-(2-[tert-butylimidemethylsilyloxy]-1-oxo-propyl] phosphonate (0.293 g, 0.94 mmol) in dry MeCN (5 mL), dry LiCl (42.7 mg, 0.94 mmol) and then freshly distilled DIEA (98.0 mg, 0.78 mmol) were added. After stirring for 2 h at rt, aldehyde (0.30 g, 0.72 mmol) in MeCN was added and the mixture was stirred at room temperature for 72 h. A saturated solution of NaCl was added and the organic layer separated, dried and purified using a Sepacore® system (silica gel column, petroleum ether 60-80/ EtOAc 6:1) to give compound 5 (0.37 g, 87% yield). 1H NMR (400 MHz, CDCl3) δ: 0.12 (s, 3H) and 0.15 (s, 3H), 0.89 (s, 9H), 1.28 (d, J = 7 Hz, 3H), 1.40 (m, 1H), 1.59 (m, 1H), 1.70 (m, 1H), 1.77 (m, 1H), 2.01 (m, 2H), 3.24 (X part of an ABX system, 2H), 7.15–7.40 (m, 15 H), 9.57 (s-like, 1H). 13C NMR (100 MHz, CDCl3) δ: -4.9, -4.8, 18.1, 21.1, 24.4 (3C), 25.7, 28.9, 32.1, 54.2, 54.4, 60.2, 64.0, 74.3, 124.2, 126.9, 127.0, 128.2 (2C), 128.3 (2C), 128.4 (2C), 128.5 (2C), 128.8 (2C), 129.0 (2C), 132.7, 134.6, 135.1, 139.4, 148.2, 172.3, 201.7. ES-MS m/z 430 [M+H]+.

(2S,9R)-2-((tert-Butoxy-carbonylamino)-9-((tert-butyldimethylsilyloxy)-8-oxodecanoic acid (6).

Pd(OH)2 on C, (20 mg) was dissolved in dry MeOH (4 mL) and put in a pressure bottle connected with a Parr apparatus. Two cycles of vacuum-nitrogen were performed and compound 5 (0.200 g, 0.334 mmol) dissolved in dry MeOH (2 mL) was added followed by addition of Boc2O (0.145 g, 0.66 mmol). The bottle was filled with H2 at 6 atm and shaken at room temperature for 12 h. The bottle was degassed; the catalyst filtered (attention: the residue Pd may be pyrophoric) and washed several times with MeOH. The solvent was evaporated and the crude product was purified using the Sepacore® system (silica gel column, CHCl3: MeOH 98:2) giving 6 (0.108 g, 75% yield). 1H NMR (400 MHz, CDCl3) δ: 0.10 (s, 6H), 0.87 (s, 9H), 1.21 (d, J = 7 Hz, 3H), 1.45 (s, 9H), 1.4-1.9 (m, 8H), 2.55 (m, 2H), 4.12 (q, J = 7Hz, 1H), 5.20 (m, 1H), 5.90 (bs, 1H), 11.2 (bs, 1H). 13C NMR (100 MHz, CDCl3) δ -3.7, -3.5, 18.1, 21.1 (3C), 24.3 (3C), 25.0, 25.7, 28.9, 29.2, 32.1, 33.4, 54.7, 75.3, 80.6, 158.2, 172.5, 211.3. ES-MS m/z 430 [M+H]+. Anal Calcd. for C23H26NO5Si: C, 58.43; H, 9.57; N, 3.25. Found C, 58.33, H, 9.52, N, 3.23.

D-γ,N-dibenzylyglycine acid dimethyl ester (8).

A solution of (D)-N,N-dibenzyglycine acid dimethyl ester (7, 1.10 g, 3.08 mmol) in dry THF (15 mL) was cooled to –78 °C under nitrogen and magnetic stirring. Iodomethane (1.32 g, 9.29 mmol) was added followed by a slow addition of a solution of KHDSMS (12.39 mL of a 0.5 M solution in THF, 6.19 mmol). After the addition, the mixture was stirred at –78 °C for 30 min. A saturated solution of NH4Cl (5 mL) was added with a syringe and the mixture was slowly warmed
to room temperature. The organic layer was separated and the aqueous layer extracted several times with AcOEt. When the solvent had been removed, the residue was dried (MgSO₄) and washed (2x3 mL) with acetone to room temperature. The organic layer was separated and the aqueous layer extracted several times with AcOEt. The silica gel chromatography (eluent hexane: AcOEt 9:1) gave product 3 as a mixture of diastereoisomers approximatively 1:4 (1.06 g, 92% yield). The spectroscopic data of the major isomer were following reported. \[^1H\] NMR (300 MHz, CDCl₃) \(\delta\): 1.15 (d, \(J = 7\) Hz, 3H), 1.75 (m, 1H), 2.01 (m, 1H), 2.75 (m, 1H), 3.48 (m, 1H), 3.55 (d-like, 2H), 3.60 (s, 3H), 3.75 (s, 3H), 3.95 (d-like, 2H), 7.30 (m, 10 H). \[^13C\] NMR (75 MHz, CDCl₃) \(\delta\): 16.6, 33.6, 36.8, 50.2, 51.6, 57.5, 58.9, 63.9, 127.5 (2C), 128.9 (4C), 129.3 (4C), 134.9 (2C), 172.3, 174.5. ES-MS m/z 370 [M+H]⁺.

(5R, 3S)-1-tert-Butyloxycarbonyl-5-carboxyethyl, 3-methyl-2-pyrrolidinone (9). Compound 8 (0.410 g, 1.11 mmol) was dissolved in dry MeOH (8 mL) and the solution poured in the pressure bottle of a Parr hydrogenation apparatus. Pd(OH)₂ (20% on C was added (50 mg) and the mixture shaken under 6 atm of H₂ for 12 h. The bottle was degassed, the catalyst filtered (attention: the residue Pd may be pyrophoric) and washed several times with MeOH. The solvent was evaporated and the crude product was dissolved in dry MeCN (15 mL). Boc₂O (0.186, 0.85 mmol) was added followed by addition of Et₃N (0.9 g, 0.9 mmol) and DMAP (64 mg, 0.07 mmol). The mixture was stirred at rt for 48 h. The solvent was evaporated under vacuum and the residue purified by column chromatography (eluent hexane: AcOEt 1:1) to give trans isomer 9 as major compound (rf = 0.35, 110 mg), together with a small amount of cis isomer (rf = 0.30, 32 mg). \[^1H\] NMR data of 9 (300 MHz, CDCl₃) \(\delta\): 1.15 (d, \(J = 7\) Hz, 3H), 1.45 (s, 9H), 1.85 (m, 1H), 2.20 (m, 1H), 2.60 (m, 1H), 3.71 (s, 3H), 4.50 (d-like, 1H). \[^13C\] NMR (75 MHz, CDCl₃) \(\delta\): 14.6, 27.6, 29.1, 35.3, 52.4, 56.8, 82.1, 150.3, 171.4, 176.3. ES-MS m/z 244 [M+H]⁺.

(2R, 4S)-1-tert-Butyloxycarbonyl-2-carboxyethyl, 4-methyl-pyrrolidin-2-carboxylic acid (12). To a solution of compound 10 (0.14 g, 0.58 mmol) in THF (5 mL) LiOH (0.049 g, 1.16 mmol in 2 mL of H₂O) was added and the mixture was stirred in vacuo and the crude residue was dissolved in water. HCl 0.5 N was added dropwise until precipitation of a white solid (I) that was filtered off. The acid (I) was dissolved in dry CH₂Cl₂ (2.5 mL) under argon atmosphere and at rt, followed by addition of TIS (0.39 g, 2.47 mmol) and TFA (2.45 mL 32 mmol). After 3 h the solvent was evaporated in vacuo and the crude product was triturated with Et₂O to obtain a powder. The amino acid was dissolved in H₂O (8 mL) followed by addition of TEA (0.22 mL, 1.6 mmol) and FmocOSu in acetonitrile (0.26 g, 0.77 mmol). Finally TEA was progressively added over 45 min to obtain a stable basic solution (pH 8.5-9). After addition of HCl 1N, ethyl acetate was used to extract the desired product 12 (144 mg, 70% yield). \[^1H\] NMR (300 MHz, DMSO-d₆, 353 K) \(\delta\): 1.03 (d, \(J = 6.5\) Hz, 3H), 1.87 (m, 1H), 2.08 (m, 1H), 2.34 (m, 1H), 2.95 (bt, \(J = 9.4\) Hz, 1H), 3.59 (dd, \(^6J = 9.4\) Hz, \(^3J = 7.7\) Hz, 1H), 4.29 (m, 4H), 7.35 (t, \(J = 7.9\) Hz, 2H), 7.43, (t, \(J = 7.9\) Hz, 2H), 7.67 (d, \(J = 7.2\) Hz, 2H), 7.88 (d, \(J = 7.2\) Hz, 2H). \[^13C\] NMR (75 MHz, CDCl₃, 353 K) \(\delta\): 18.3, 32.7, 38.1, 47.9, 54.0, 59.8, 67.9, 121.2 (2C), 126.3 (2C), 128.3 (2C), 128.8 (2C), 141.9 (2C), 145.0 (2C), 154.9, 174.9. ES-MS m/z 351 [M+H]⁺.

Fmoc-L-Iva

The Fmoc-group was introduced by stirring L-Iva (0.50 g, 3.7 mmol), 9-fluorenylmethylchloroformate (1.053 g, 4.07 mmol), and Na₂CO₃ (1.176 g, 11.1 mmol) in 50% acetone/water (75 mL) overnight; after evaporation of acetone, the aqueous solution was washed with ether and acidified with 10% citric acid. The precipitated acid, washed with water, was dissolved in ethyl acetate, dried (MgSO₄), and evaporated. Hexane was added to precipitate the pure acid. After being dried in vacuo, Fmoc-L-Iva-OH was obtained in 79% yield. ES-MS m/z 362 [M+Na]⁺. \[^1H\] NMR (600 MHz, CDCl₃) \(\delta\): 0.80 (CH₃, 3H, m), 1.50 (CH₃, 3H, s) 1.80-2.10 (CH₂, 2H, m), 4.2-4.4 (CH, CH₂, 3H, m), 7.25-7.76 (aromatic protons, 8H, m)

Final stages of the FR235222 synthesis.

Solid Phase Synthesis of the linear precursor and cyclization of cyclo-(2S,9R)-Ahoda-L-Iva-L-Phe-(2R,4S)-4-MePro-]

a) Loading of the 2-CITrt-Cl resin:

The 2-CITrt-Cl resin was placed in a 25 mL polypropylene ISOSOLUTE syringe on a VAC MASTER system, swollen in DMF (3 mL) for 1 h, and then washed with 2x3 mL of DCM.

A solution of Fmoc-D-4-MePro-OH (48 mg, 0.14mmol) and DIEA (18 µL, 0.4 mmol) in 2.5 mL of dry DCM was added and the mixture was stirred for 2 h with a N₂ stream. The mixture was then removed, and the resin was washed with 3x
DCM/MeOH/DIEA (17:2:1) and successively with: DCM 3 x 3 mL, DMF 2 x 3 mL, DCM 2 x 3 mL (1.5 min each). The loading of the resin was determined by UV quantification of the Fmoc-piperidine adduct.

The assay was performed on a duplicate samples: 0.4 mL of piperidine and 0.4 mL of DCM were added to two dried samples of Fmoc-amino acid-resin in two volumetric flasks of 25 mL. The reaction was allowed to proceed for 30 min at rt and than 1.6 mL of MeOH were added and the solutions were diluted to 25 mL volume with DCM. A reference solution was prepared in a 25 mL volumetric flask using 0.4 mL of piperidine, 1.6 mL of MeOH and DCM to volume. The solutions were shaken and the absorbance of the samples versus the reference solution was measured at 301 nm. The substitution level (expressed in mmol of amino acid/g of resin) was calculated from the equation: mmol g⁻¹ = (Aₜ₀₀/7800) x (25 mL g⁻¹ of resin).

b) Fmoc deprotection conditions:
After Fmoc-D-4-MePro-O-2CITrt-resin swelling (1 h with 3 mL of DMF), removal of the Fmoc protecting group was carried out using 20% piperidine in DMF (3 mL, 1 x 1.5 min), 20% piperidine in DMF (3 mL, 1 x 10 min or 1 x 5 min); washings in DMF 2 x 3 mL, DCM 2 x 3 mL, DMF 2 x 3 mL (1.5 min each). The loading of the resin was determined by UV quantification of the Fmoc-piperidine adduct.

The solutions were shaken and the absorbance of the samples versus the reference solution was measured at 301 nm.

The crude linear peptide (N-terminus and side-chain exchangeable protons, including amide signals, could not be unambiguously assigned. For the same reason, many broad resonances at rt, preventing the observation of clear signal multiplicities. For the same reason, exchangeable protons, including amide signals, could not be unambiguously assigned.

The HPLC analysis showed one main peak at RT = 19.20 min that was identified as the linear deprotected peptide (60% yield) on the basis of ES-MS and 1H-NMR data. The 1H-NMR spectrum contained many broad resonances at rt, preventing the observation of clear signal multiplicities. For the same reason, exchangeable protons, including amide signals, could not be unambiguously assigned.

f) Cyclization conditions:
The cyclization was performed in solution at a concentration of 7.7 x 10⁻⁵ M with HATU (18.8 mg, 0.05 mmol) and DIEA (11 µl, 0.062 mmol) in DCM. The solution was stirred at 74 °C for 1h and then allowed to warm to room temperature for 1 h. The solvent was removed under reduced pressure.

The crude cyclopeptide (36.9 mg) was purified by semipreparative RP HPLC on a Jupiter C-18 column using a 40% gradient from 27:85 to 50:50 of CH₃CN/CH₂O (each containing 0.1% TFA) at a flow rate of 4 mL/min and UV detection at 240 nm. The HPLC analysis showed a main peak at t_R = 27.80 min (25% B) to 100% B over 30 min at a flow rate of 4 mL/min. The binary solvent system (A/B) was as follows: 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B). The absorbance was detected at 240 nm.

The HPLC analysis showed one main peak at t_R = 19.20 min that was identified as pure FR235222 (I) (68 % isolated yield) on the basis of ES-MS and NMR data.
$^{13}$C NMR (150 MHz, CDCl$_3$) $\delta$: **Ahoda** 19.7, 23.2, 25.4, 28.8 (2C), 37.3, 54.5, 72.5, 174.2, 212.4; **4-MePro** 18.2, 32.9, 33.1, 53.8, 58.1, 171.9; **Phe** 35.8, 53.2, 126.7, 128.6 (2C), 129.1 (2C), 137.0, 173.2; **Iva** 8.4, 22.5, 27.7, 63.0, 175.5. HRES-MS $m/z$ 557.3343 [M+H]$^+$, C$_{30}$H$_{44}$N$_4$O$_6$. 