Supporting Information

Isoprenylcysteine carboxylmethyltransferase-based therapy for Hutchinson–Gilford progeria syndrome

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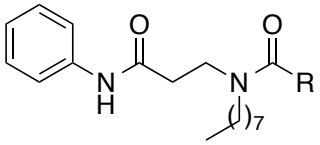
1. Supporting Table S1



2-25

Cpd	**R**	**ICMT inhibition (%)**[a]	**Viability (%)**[b]	
			Wild type mouse fibroblasts	Progeroid mouse fibroblasts
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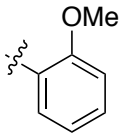
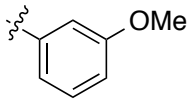
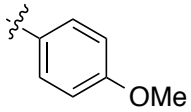
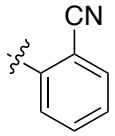
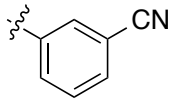
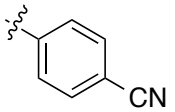
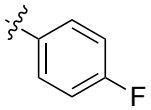
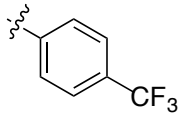
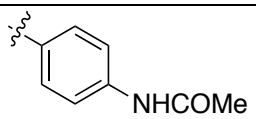
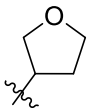
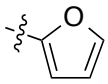
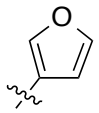
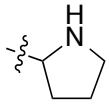
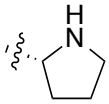
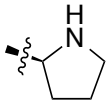
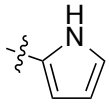
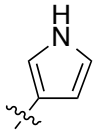
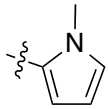
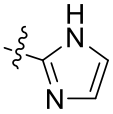
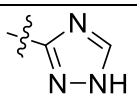
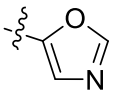
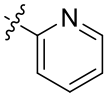
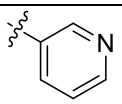
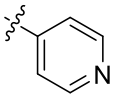
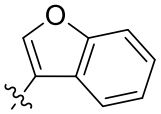
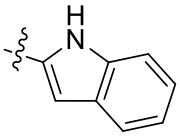
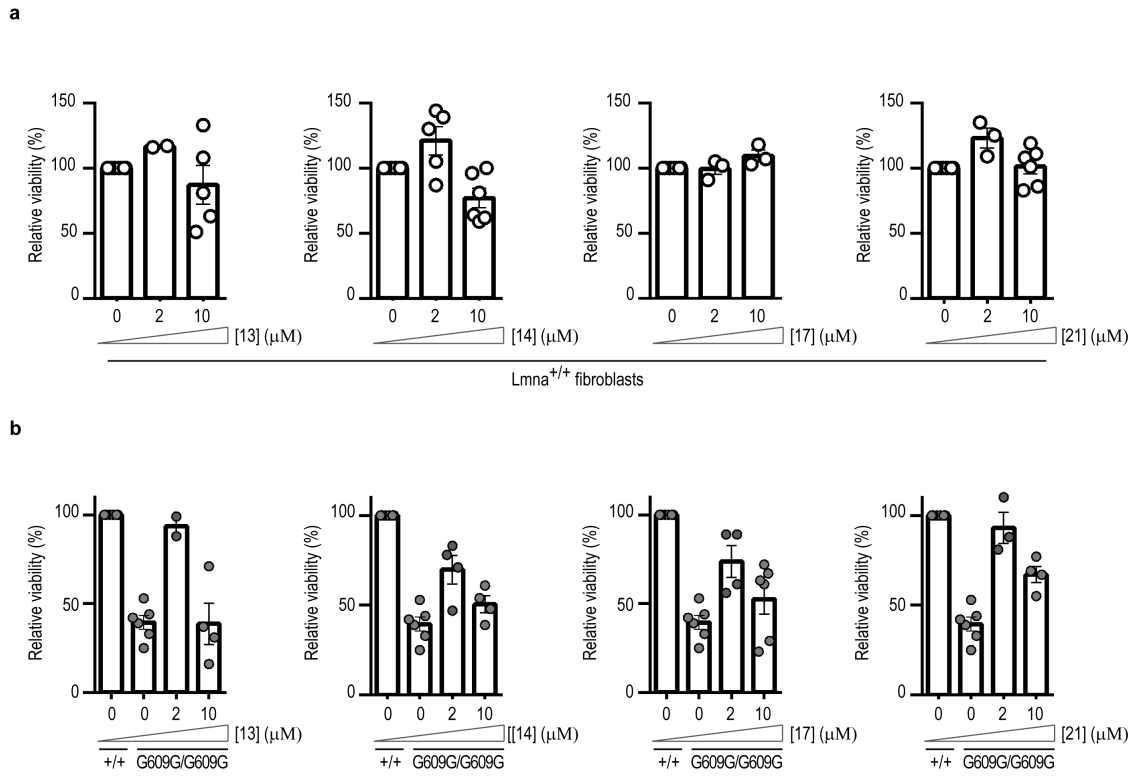
2		32	-	-
3		26	-	-
4		52	-	-
5		47	-	-
6		53	-	-
7		66	86±2	56±4
8		42	-	-
9		18	-	-

10		10	-	-
11		41	-	-
12		49	-	-
13		81	116±2	77±5
14		62	121±11	70±8
(*R*)-14		61	114±6	80±7
(*S*)-14		64	114±10	70±5
15		75	107±5	45±6
16		81	109±4	53±13
17		75	99±5	74±9
18		34	-	-
19		0	-	-
20		56	-	-
21		73	113±9	93±9

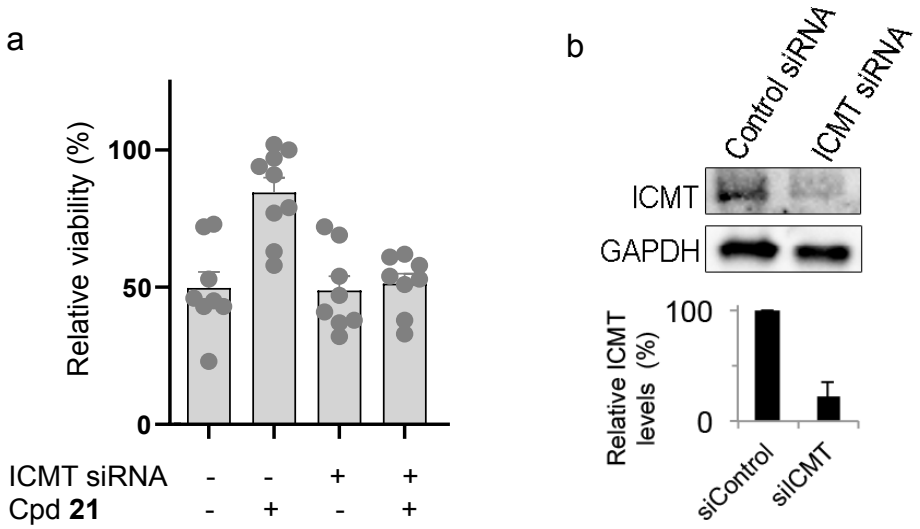
22		38	-	-
23		30	-	-
24		47	-	-
25		15	-	-

[a]ICMT inhibition values were determined at a concentration of 50 μM. Results are expressed as the mean from at least two independent experiments performed in triplicate. The sem is within a 10% of the mean value. [b]Cell viability was determined by the MTT assay. Data are expressed as the mean±sem from three independent experiments performed in triplicate at a compound concentration of 2 μM. The percentage of viability is expressed considering 100% viability in vehicle-treated wild type fibroblasts.

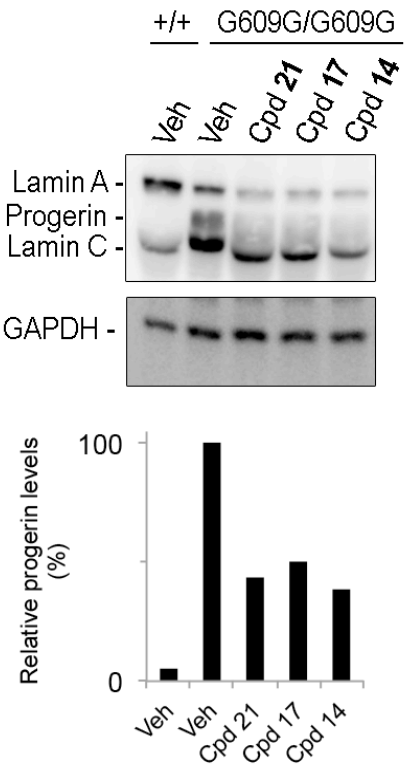
2. Supporting Figures



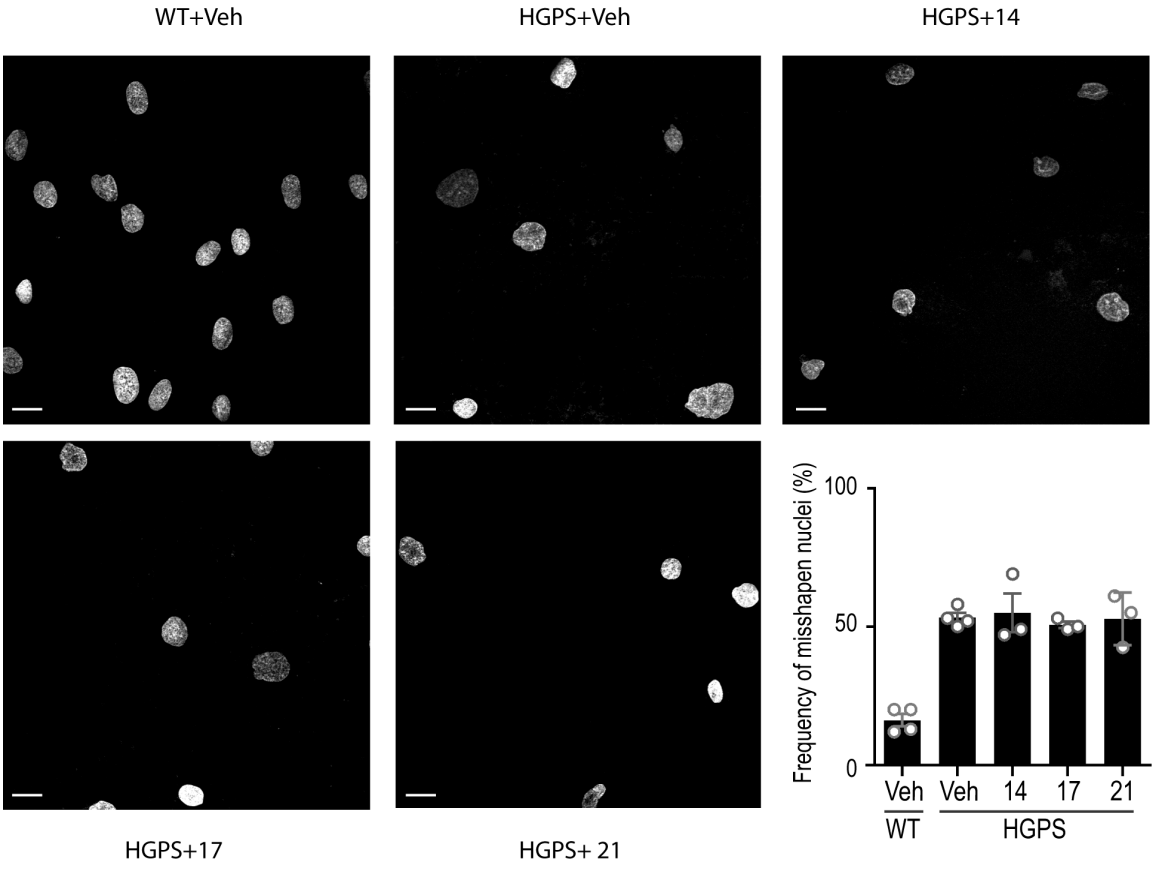
Supporting Figure S1. Effect of compounds in the viability of *Lmna*$^{+/+}$ and *Lmna*$^{G609G/G609G}$ mouse fibroblasts. a, *Lmna*$^{+/+}$ and **b,** *Lmna*$^{G609G/G609G}$ cells were incubated with 0.1% DMSO (vehicle, veh) or different compounds at 2 μM or 10 μM during 3 days. Viability was determined in MTT assay. Plots show percentage of cell viability (n≥3 independent experiments).



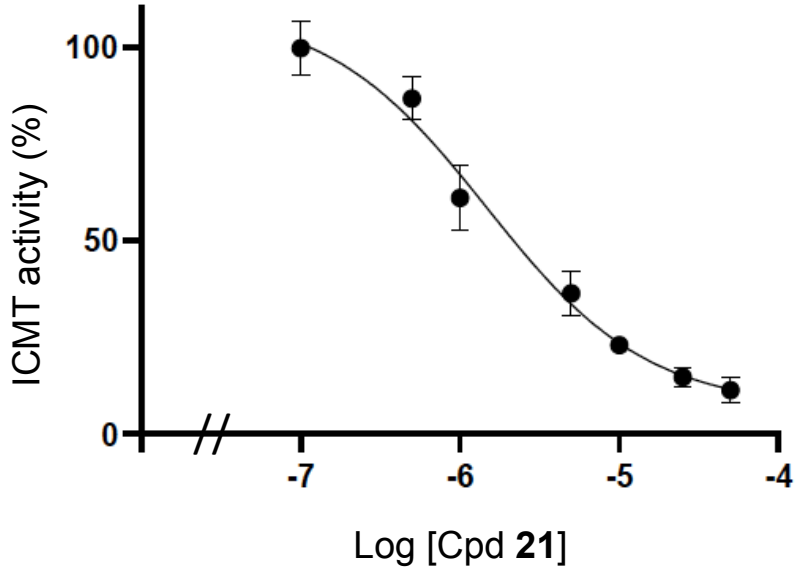
Supporting Figure S2. Compound 21 does not increase cell viability in *Lmna*$^{G609G/G609G}$ mouse fibroblasts in which ICMT has been knocked down using a specific siRNA. a, Cells were treated with a control siRNA or with the ICMT siRNA and were incubated with 0.1% DMSO (vehicle) or with 2 μM of compound **21** during 3 days. Viability was determined in MTT assay. Plot shows percentage of relative cell viability (n≥4, two independent experiments) with respect to *Lmna*$^{+/+}$ cells, which were considered as the 100% viability value. **b,** *Lmna*$^{G609G/G609G}$ mouse fibroblasts were transfected with siRNA-targeting ICMT or with a control siRNA for 72 h. Total protein homogenates from cells transfected with the siRNA targeting ICMT or with control siRNA were subjected to immunoblotting analyses with antibodies against ICMT (Novus Biologicals NBP1-69579, 1:400) or GAPDH as loading control. Results shown are a representative blot and the average±SEM of two independent experiments.



Supporting Figure S3. Immunoblot of progerin from *Lmna*$^{+/+}$ or *Lmna*$^{G609G/G609G}$ mouse fibroblasts incubated with vehicle or ICMT inhibitors (2 μM) during 14 days. To quantify relative progerin levels, protein levels were normalized against total GAPDH.

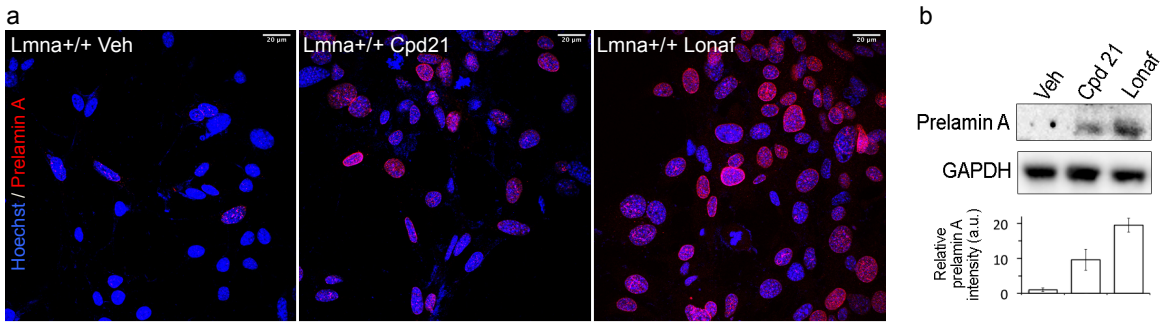


Supporting Figure S4. ICMT inhibitors do not affect misshapen nuclei in HGPS human cells. Immunofluorescence images of wild type (WT) and HPGS human fibroblasts treated with 0.1% DMSO (vehicle, veh) or different ICMT inhibitors at 2 μM during 17 days and stained with Hoechst 33342. Plot shows percentage average of misshapen nuclei in at least five images per condition from ≥3 experiments. No significant differences (one way ANOVA) were found between treated and non-treated HGPS cells. Scale bar: 20 μm.

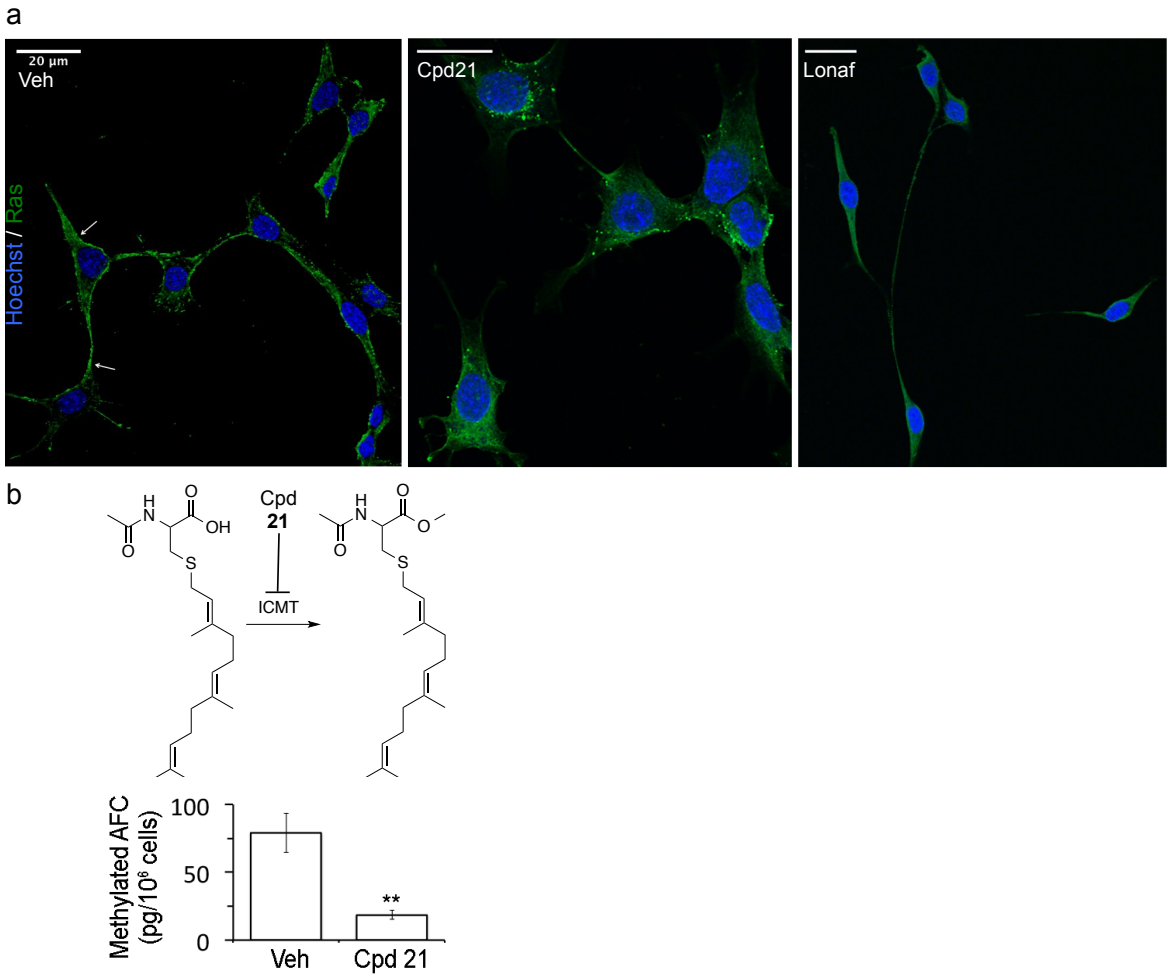


Supporting Figure S5. Concentration-response curve for compound **21**. ICMT activity is reported as the percentage of remaining activity compared to the activity in the absence of compound. Shown data correspond to one representative experiment carried out in duplicate and represent means and SEM (when not enclosed by the symbol). ICMT inhibition was determined using Sf9 membranes containing the recombinantly expressed enzyme using a mixture of biotin-farnesyl-L-cysteine and tritiated *S*-adenosylmethionine as previously described.[1]

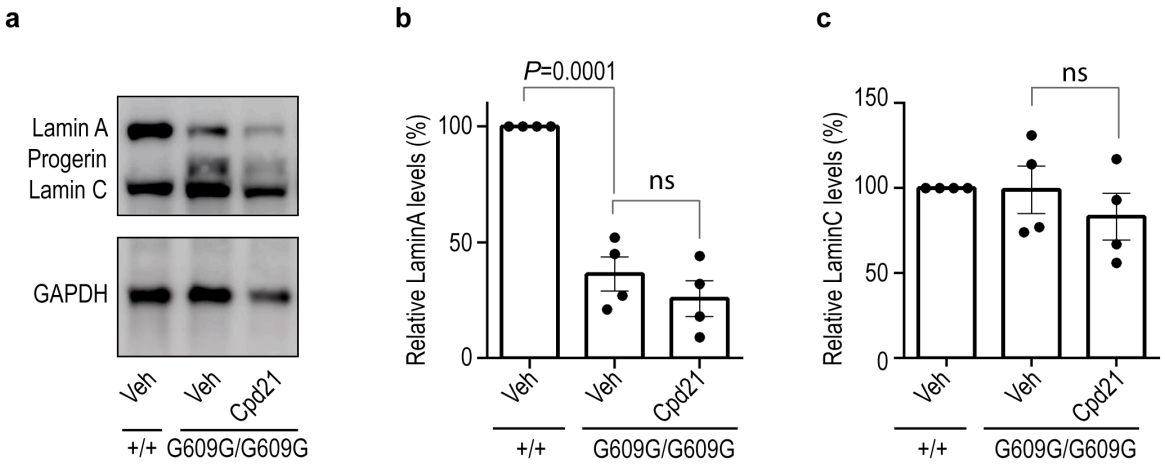
[1] Marín-Ramos, N.I.; Balabasquer, M.; Ortega-Nogales, F.J.; Torrecillas, I.R.; Gil-Ordóñez, A.; Marcos-Ramiro, B.; Aguilar-Garrido, P.; Cushman, I.; Romero, A.; Medrano, F.J.; Gajate, C.; Mollinedo, F.; Philips, M.R.; Campillo, M.; Gallardo, M.; Martín-Fontecha, M.; López-Rodríguez, M.L.; Ortega-Gutiérrez, S. *J. Med. Chem.* **2019**, *11*, 6035-6046.



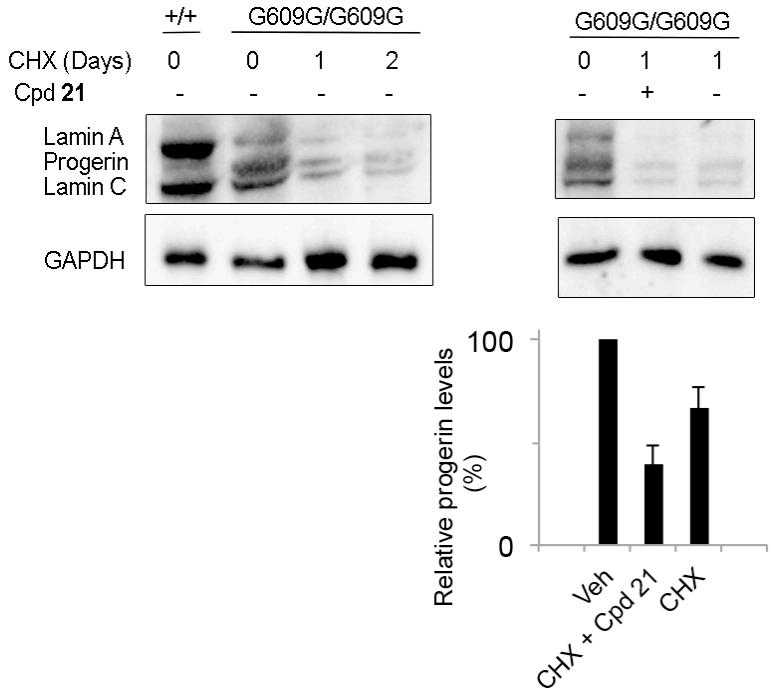
Supporting Figure S6. Compound 21 induces prelamin accumulation in *Lmna*$^{+/+}$ cells. a, Immunofluorescence images of *Lmna*$^{+/+}$ mouse fibroblasts treated with 0.1% DMSO (vehicle, veh), compound **21** at 2 µM during 5 days, or lonafarnib at 10 µM during 3 days and stained with anti-prelamin A antibody (1:1000, Millipore; visualized with the corresponding secondary antibody in red) and with Hoechst 33342 (blue). Images are representative from ≥2 independent experiments. Scale bar: 20 µm. **b,** Representative blot showing the levels of prelamin A and GAPDH. Bar graph shows quantification of prelamin A levels normalized against total GAPDH (n=2 independent experiments, plot shows mean±sem).



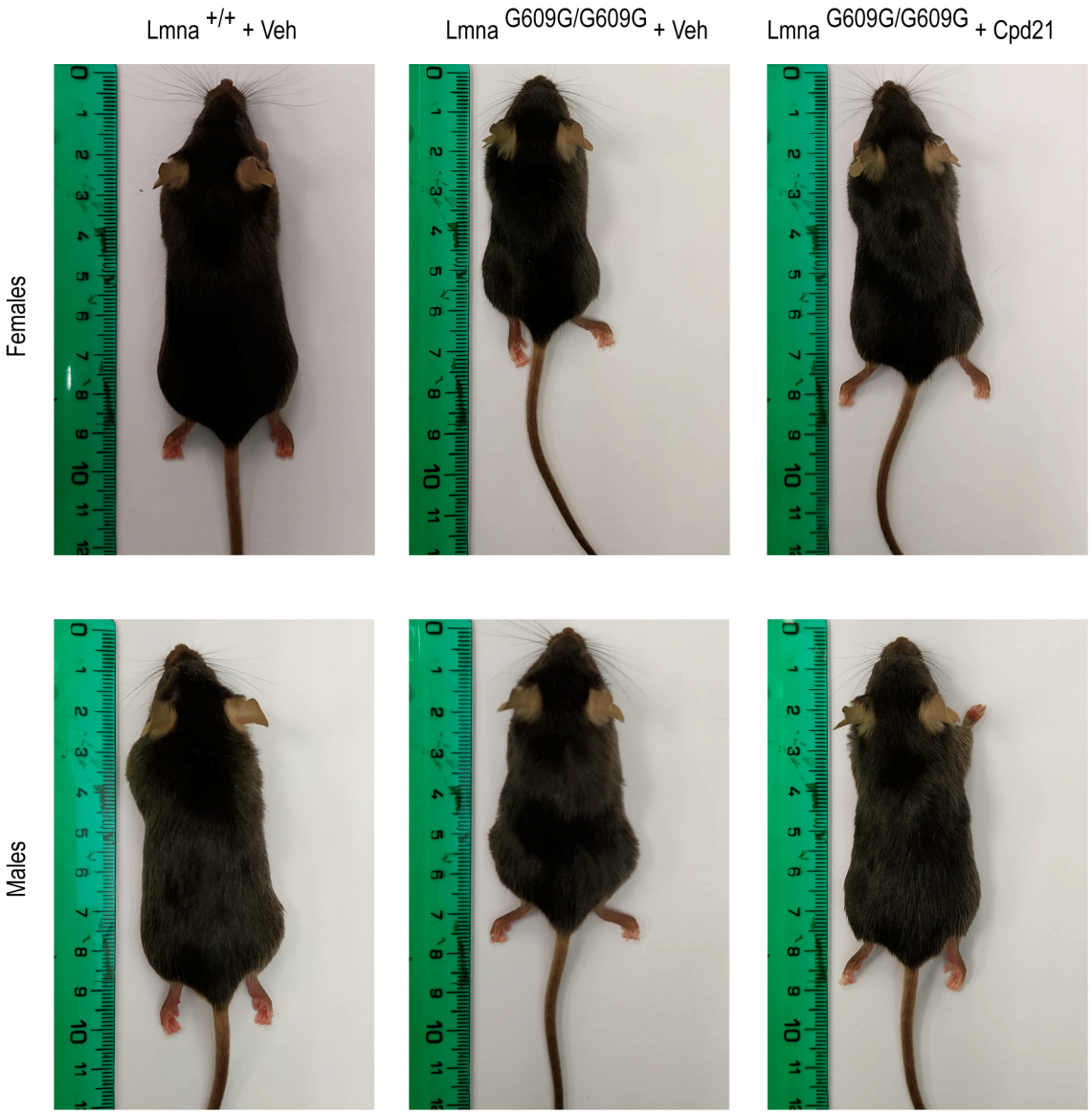
Supporting Figure S7. a, Compound 21 induces Ras mislocalization in *Lmna*$^{G609G/G609G}$ cells. Immunofluorescence images of *Lmna*$^{G609G/G609G}$ mouse fibroblasts treated with 0.1% DMSO (vehicle, veh), compound **21** at 2 µM during 5 days, or lonafarnib at 10 µM during 3 days and stained with an anti-Ras antibody (1:200, Thermo Scientific; visualized with the corresponding secondary antibody in green) and with Hoechst 33342 (blue). Arrows indicate membrane localization of Ras. Images are representative from ≥2 independent experiments. Scale bar: 20 µm. **b, Compound 21 decreases the level of methylated acetylfarnesylcysteine (AFC) in *Lmna*$^{G609G/G609G}$ cells.** Cells (2.5 millions per point) were incubated in the presence of vehicle or compound **21** (2 µM) for 24 h. Then, AFC (100 µM) was added and cells were incubated for additional 72 h. After that, cells were washed and lysed in 0.5 mL PBS. Whole cell homogenates were extracted with a mixture of chloroform (1 mL) and methanol (0.5 mL). Organic phase was evaporated and resuspended in methanol (50 µL) and the levels of methylated AFC was measured by LC MS/MS. Results shown are the average±SEM of two independent experiments carried out in duplicates (** $p<0.01$, two-tailed Student´s t-test).



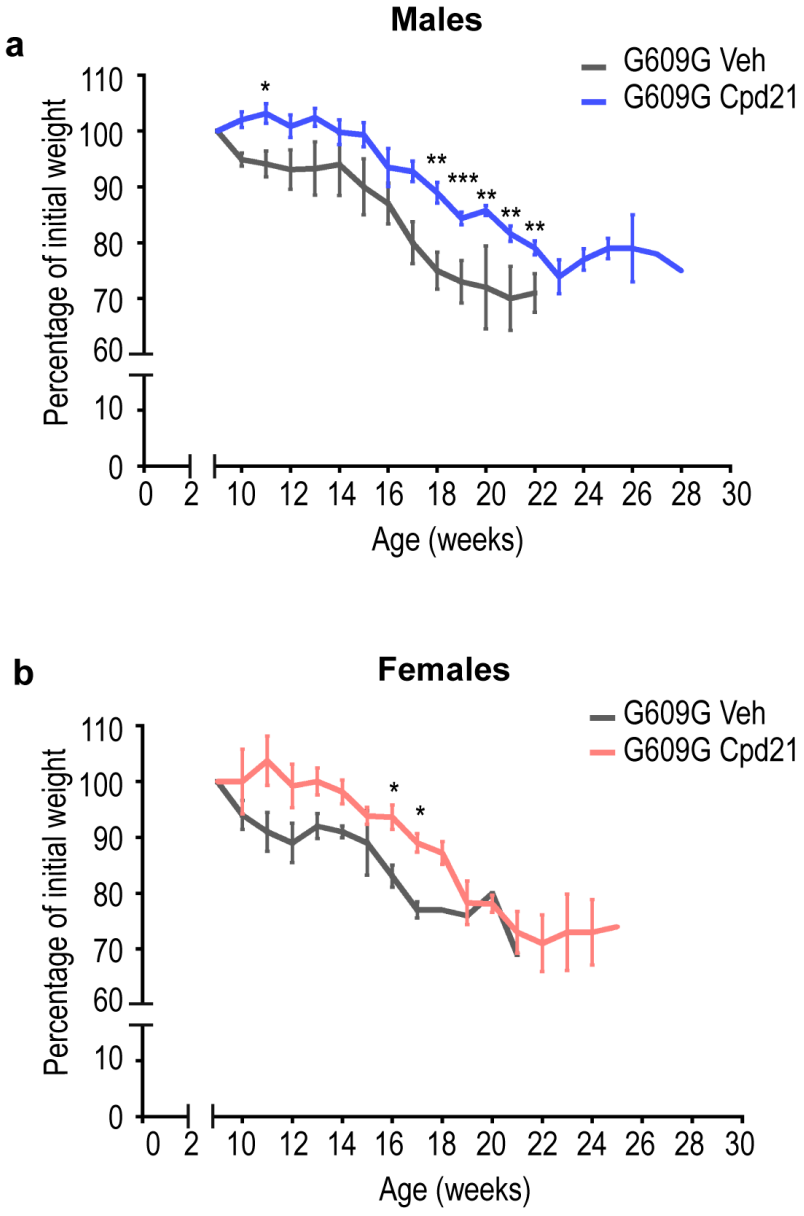
Supporting Figure S8. ICMT inhibitors do not affect lamin A and lamin C levels. *Lmna*$^{+/+}$ and *Lmna*$^{G609G/G609G}$ fibroblasts were incubated with 0.1% DMSO (vehicle, veh) or compound **21** at 2 μM during 14 days and lamin A and lamin C levels were analysed by western blot. **a,** Representative blot showing the levels of lamins A and C, progerin, and GAPDH. **b,** Quantification of lamin A levels. **c,** Quantification of lamin C levels (n=4 independent experiments; plots show protein level±sem normalized against GAPDH levels, two-tailed Student´s t-test).



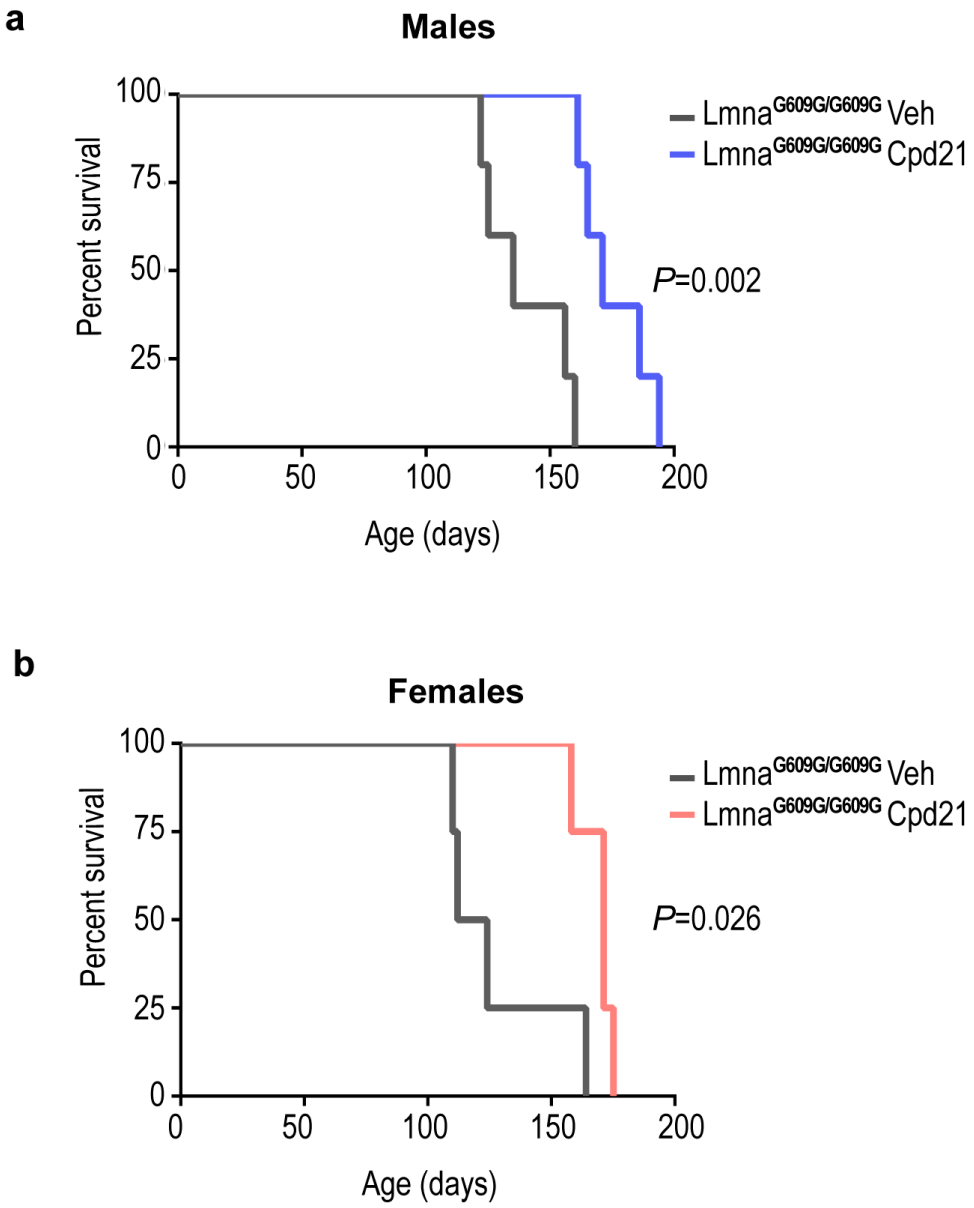
Supporting Figure S9. Immunoblot showing the progerin remaining in progeroid cells treated with cycloheximide. *Lmna*$^{+/+}$ and *Lmna*$^{G609G/G609G}$ fibroblasts were incubated with 0.1% DMSO (vehicle) or compound **21** at 2 μM during 14 days and then with cycloheximide (20 μg/mL, C4859, Sigma-Aldrich) to stop protein synthesis and progerin levels were analysed by western blot. Results shown are a representative blot and the average±SEM of two independent experiments.



Supporting Figure S10. Compound 21 improved body size in male and female *Lmna*$^{G609G/G609G}$ mice. Photographs of three months old sex-matched mice *Lmna*$^{+/+}$ or *Lmna*$^{G609G/G609G}$ treated with vehicle compared to *Lmna*$^{G609G/G609G}$ mice treated with compound **21**.

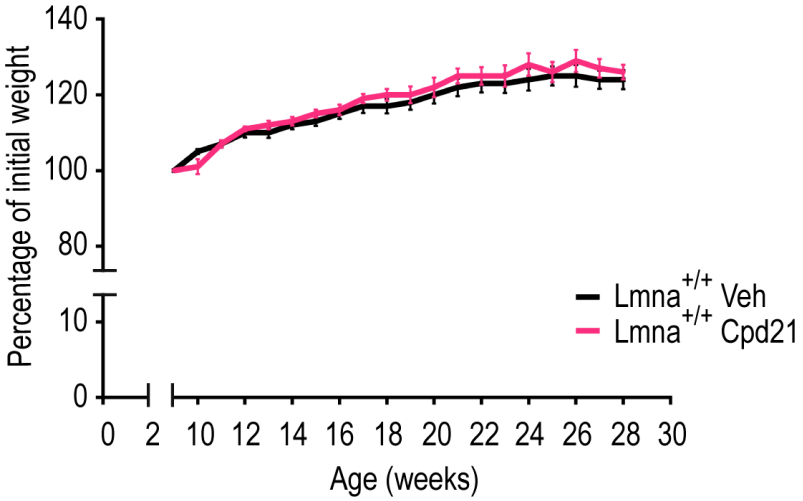


Supporting Figure S11. Progression of body weight of male and female *Lmna*$^{G609G/G609G}$ mice treated with compound 21 or with vehicle. a, Body weight versus age plot of untreated (n=4) and treated (n=6) male *Lmna*$^{G609G/G609G}$ mice (plot shows mean±sem, two-tailed Student´s t-test, p-value *<0.05, **<0.005, ***<0.0005). **b,** Body weight versus age plot of untreated (n=2) and treated (n=4) female *Lmna*$^{G609G/G609G}$ mice (plot shows mean±sem, two-tailed Student´s t-test, p-value *<0.05, **<0.005, ***<0.0005).

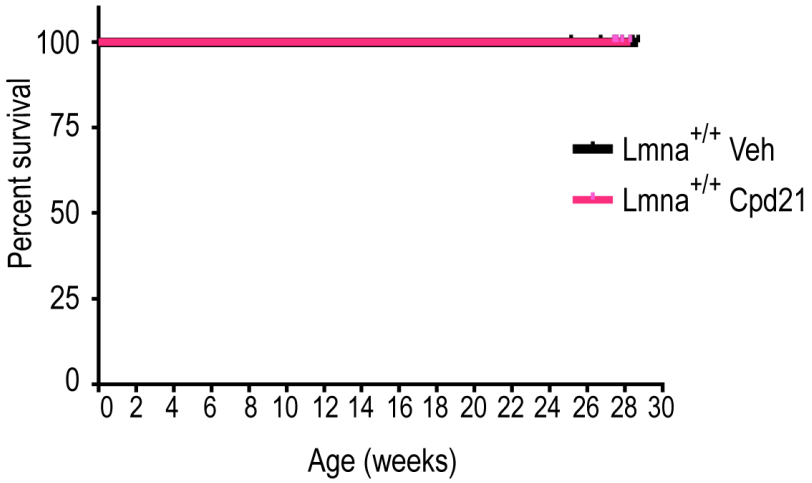


Supporting Figure S12. Compound 21 increases the survival of male and female *Lmna*$^{G609G/G609G}$ mice. a, Kaplan-Meier survival plot of *Lmna*$^{G609G/G609G}$ male mice treated with compound **21** (n=6) or vehicle (n=5), (P=0.002, log-rank/Mantel-Cox test). **b,** Kaplan-Meier survival plot of *Lmna*$^{G609G/G609G}$ female mice treated with compound **21** (n=4) or vehicle (n=4), (P=0.026, log-rank/Mantel-Cox test).

a

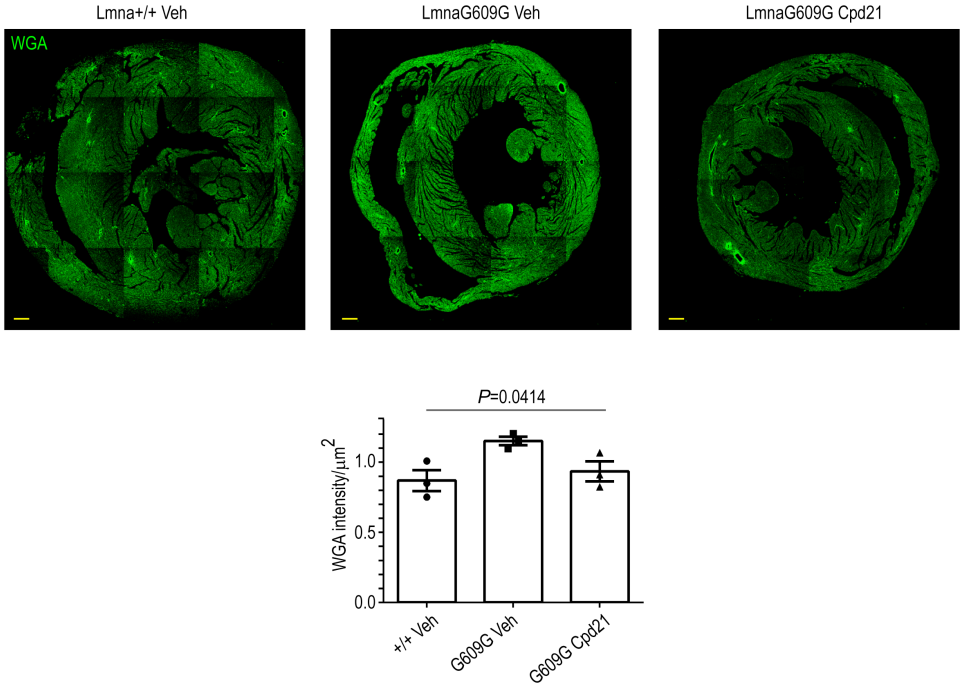


b

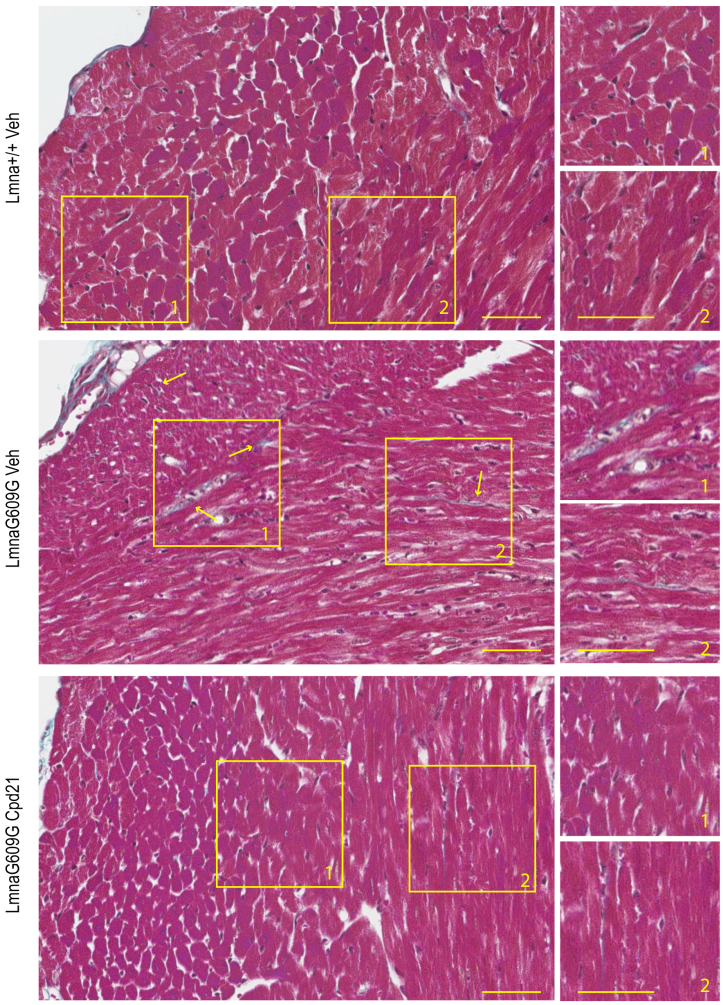


Supporting Figure S13. Compound 21 does not affect the progression of body weight and survival of *Lmna*$^{+/+}$ mice. a, Plot of body weight versus age of *Lmna*$^{+/+}$ mice treated with compound **21** (n=7) or with vehicle (n=10) (plot shows mean±sem). **b**, Kaplan-Meier survival plot of *Lmna*$^{+/+}$ mice treated with compound **21** (n=7) or vehicle (n=10).

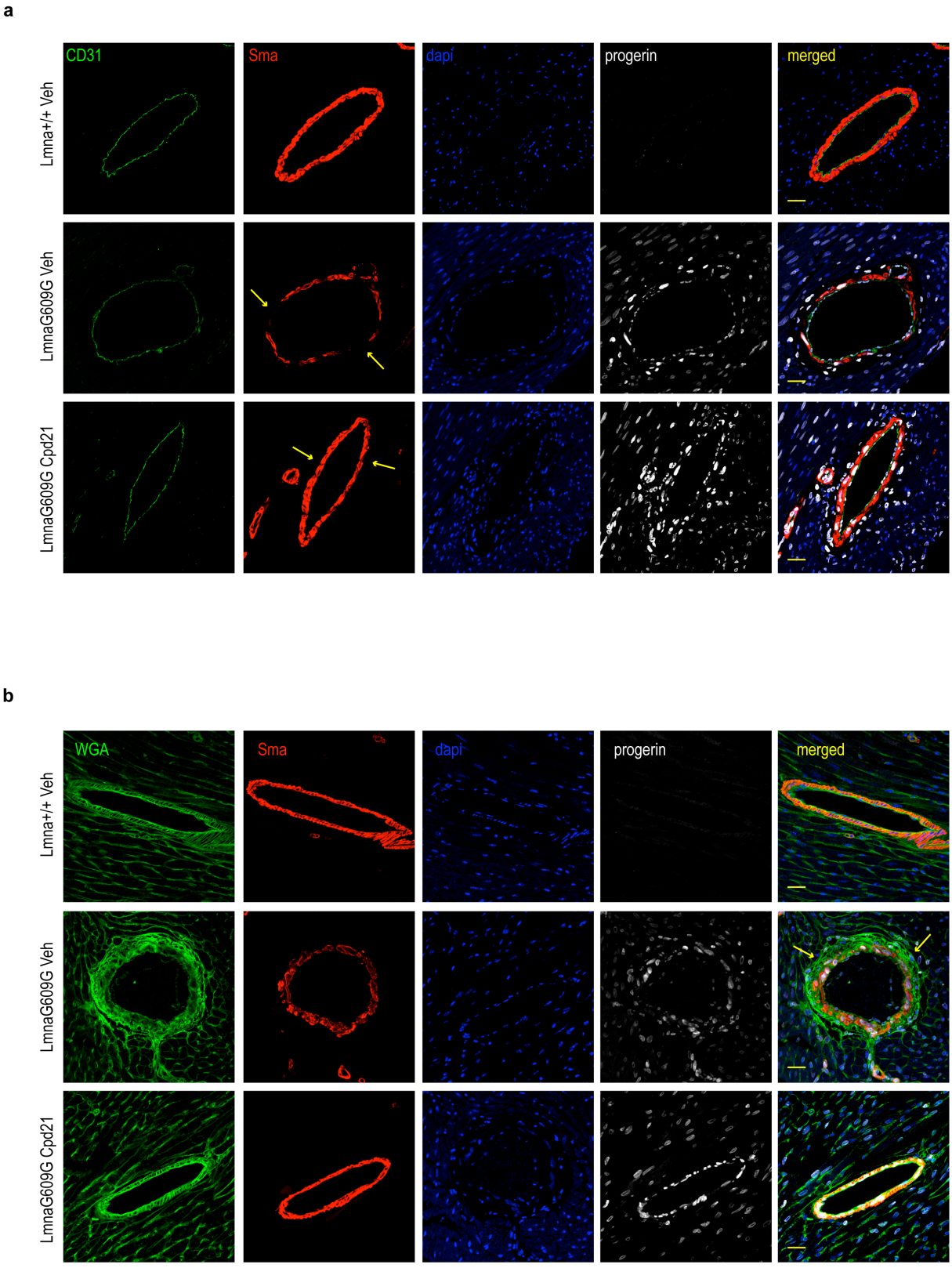
a


Lmna+/+ Veh
LmnaG609G Veh
LmnaG609G Cpd21
WGA
P=0.0414
WGA intensity/µm2
1.0
0.5
0.0
+/+ Veh
G609G Veh
G609G Cpd21

b

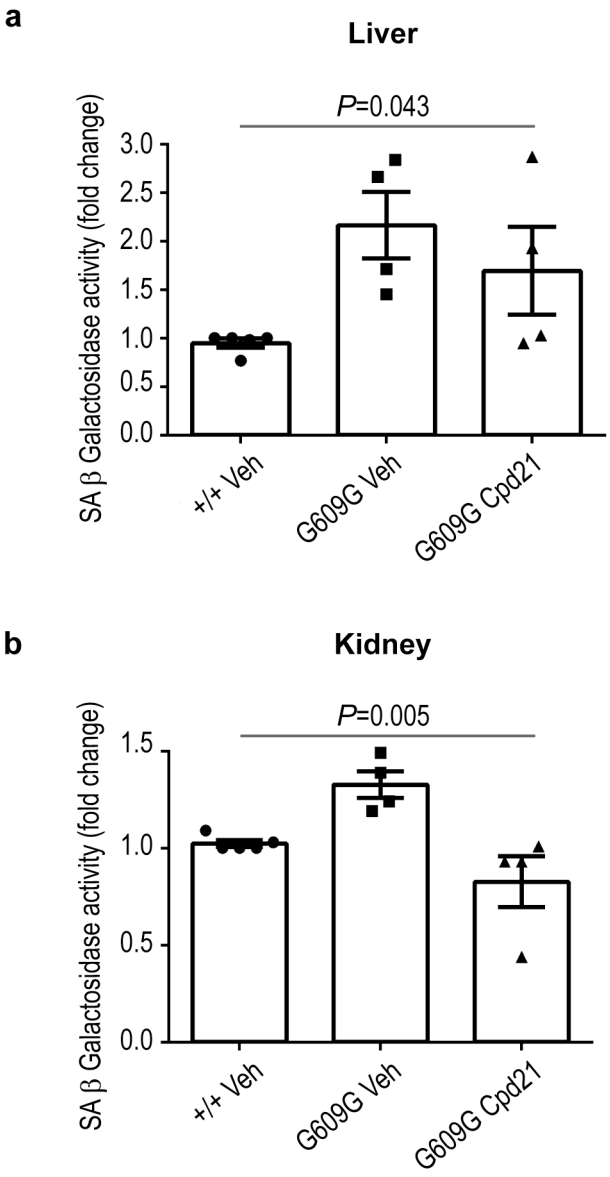

Lmna+/+ Veh
LmnaG609G Veh
LmnaG609G Cpd21
1
2

Supporting Figure S14. Reduced heart fibrosis in progeric *Lmna*$^{G609G/G609G}$ mice treated with compound 21. a, Septum heart fibrosis was revealed by wheat germ agglutinin (WGA) staining. Representative images are shown. Bottom plot shows mean±sem and individual values (n=3 animals per condition, one-way ANOVA) of WGA intensity per μm^2. Scale bars=250 μm. **b,** Heart fibrosis partial recovery in *Lmna*$^{G609G/G609G}$ mice treated with compound **21** was confirmed through Masson's trichrome staining. Representative images are shown. Yellow arrows point at interstitial fibrosis (blue signal) detected in *Lmna*$^{G609G/G609G}$ mice treated with vehicle (see zoom images in right panels 1 and 2). Note that, in addition, tissue in *Lmna*$^{G609G/G609G}$ mice treated with compound **21** appears more compacted and homogeneous, similar to WT tissue, when compared to vehicle treated *Lmna*$^{G609G/G609G}$ mice. Scale bars=50 μm.



Supporting Figure S15. Reduced microvascular cell loss and fibrosis in progeric *Lmna*$^{G609G/G609G}$ mice treated with compound 21. a, Vascular smooth muscle cell (VSMC) loss in the medial layer of apex arterioles in *Lmna*$^{G609G/G609G}$ mice was partially recover after treatment with compound **21**. Representative immunofluorescence images show staining for a-smooth muscle actin (SMA) in red, CD31 endothelial cell marker in green, progerin in

white and nuclei in blue. Yellow arrows indicate interruption in arterial wall, which correspond to VSMC loss and is evident in the case of vehicle treated *Lmna*$^{G609G/G609G}$ mice but almost unnoticeable in the case of *Lmna*$^{G609G/G609G}$ mice treated with compound **21**. Scale bars=25 mm. From the analyzed animals, no wild type mice showed vascular loss (n=3), 75% of *Lmna*$^{G609G/G609G}$ mice treated with vehicle showed vascular loss (n=4), and only in the 25% of *Lmna*$^{G609G/G609G}$ mice treated with compound **21** vascular loss was detected (n=4). **b,** Wheat germ agglutinin (WGA) perivascular deposition (indicative of fibrosis in arterial wall) was partially recovered in apex arterioles of *Lmna*$^{G609G/G609G}$ mice treated with compound **21**. Representative immunofluorescence images show staining for WGA in green, a-smooth muscle actin (SMA) in red, progerin in white and nuclei in blue. Yellow arrows point at WGA perivascular deposition in vehicle treated *Lmna*$^{G609G/G609G}$ mice. Scale bars=25 mm. From the animals analyzed, no WT mice showed WGA perivascular deposition (n=3), 100% of the vehicle treated *Lmna*$^{G609G/G609G}$ mice showed WGA perivascular deposition (n=4), and WGA perivascular deposition was detected only in the 25% of *Lmna*$^{G609G/G609G}$ mice treated with compound **21** (n=4).



Supporting Figure S16. Compound 21 reduces senescence-associated (SA) β-galactosidase activity in liver and kidney of progeric *Lmna*$^{G609G/G609G}$ mice. The activity of SA β-galactosidase was determined by incubating tissue lysates with Pierce β-galactosidase Assay Reagent. The reaction was incubated for 30 min and the absorbance was measured at 405 nm. Values were normalized to total protein levels. (n≥4 animals; plot shows mean±sem, one-way ANOVA).

3. Compound syntheses

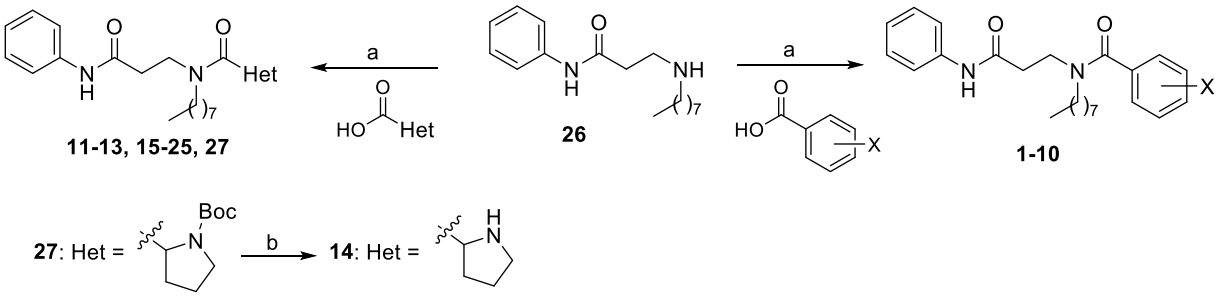
3.1. General

Unless stated otherwise, starting materials, reagents and solvents were purchased as high-grade commercial products from Sigma-Aldrich, Acros, Abcr, Fluorochem, Scharlab or Honeywell, and were used without further purification. Dichloromethane (DCM) was dried using a Pure Solv™ Micro 100 Liter solvent purification system. All reactions in organic solvents were performed under an argon atmosphere in dry glassware. Analytical thin-layer chromatography (TLC) was run on Merck silica gel plates (Kieselgel 60 F-254) with detection by UV light (254 nm), ninhydrin solution, or 10% phosphomolybdic acid solution in ethanol. Flash chromatography was performed on a Varian 971-FP flash purification system using silica gel cartridges (Varian, particle size 50 μm) or on glass columns using silica gel type 60 (Merck, particle size 230 μm, 400 mesh). All compounds were obtained as oils, except for those whose melting points (mp) are indicated, which were solids. Melting points (mp, uncorrected) were determined on a Stuart Scientific electrothermal apparatus. Infrared (IR) spectra were measured on a Bruker Tensor 27 instrument equipped with a Specac ATR accessory of 5200-650 cm^{-1} transmission range; frequencies (ν) are expressed in cm^{-1}. Optical rotation [α] was measured on an Anton Paar MCP 100 modular circular polarimeter using a sodium lamp (λ = 589 nm) with a 1 dm path length; concentrations (c) are given as g/100 mL. NMR spectra were recorded at room temperature on a Bruker DPX 300 (^{1}H, 300 MHz; ^{13}C, 75 MHz) spectrometer at the Universidad Complutense de Madrid (UCM) NMR facilities. Chemical shifts (δ) are expressed in parts per million relative to internal tetramethylsilane; coupling constants (*J*) are in hertz (Hz). The following abbreviations were used to describe peak patterns when appropriate: s (singlet), d (doublet), t (triplet), q (quartet), qt (quintet), m (multiplet), and br (broad). 2D NMR experiments (HMQC and HMBC) of representative compounds were carried out to assign protons and carbons of the new structures. High resolution mass spectrometry (HRMS) was carried out on a FTMS Bruker APEX Q IV spectrometer in electrospray ionization (ESI) mode at UCM's spectrometry facilities. Elemental analyses of solid compounds were obtained on a LECO CHNS-932 apparatus at the UCM's analysis services and were within 0.4% of the theoretical values.

For all final compounds, purity was determined by high-performance liquid chromatography coupled to mass spectrometry (HPLC-MS) using an Agilent 1200LC-MSD VL instrument, and satisfactory chromatograms confirmed a purity of at least 95% for all tested compounds. LC separation was achieved with an Eclipse XDB-C18 column (5 μm, 4.6 mm x 150 mm), together with a guard column (5 μm, 4.6 mm x 12.5 mm) with a flow rate of 0.5 mL/min. The gradient mobile phase consisted of A (95:5 water/acetonitrile) and B (5:95 water/acetonitrile) with 0.1% formic acid as solvent modifier. The gradient started at 0% B (for 5 min), increased linearly to 90% B over the course of 10 min, and up to 100% B for 7 min more, before being kept isocratic at 100% B for 4 min and decreased to 0% B for the final 4 min (total run time = 30 min). Spectra were acquired in positive or negative ionization mode from 100 to 1000 m/z and in UV-mode at four different wavelengths (210, 230, 254, and 280 nm). MS analysis was performed with an ESI source. The capillary voltage was set to 3.0 kV and the fragmentor voltage was set at 72 eV. The drying gas temperature was 350 °C, the drying gas flow was 10 L/min, and the nebulizer pressure was 20 psi. The enantiomeric excess (ee) was determined by HPLC using a chiral column in reversed-phase chromatography. Chiralpak® IA (5 μm, 4.6 mm x 15.0 mm) was used as the stationary phase, equipped with an Eclipse XDC-C18 precolumn (5 μm, 4.6 mm x 12.5 mm) using a flow of 0.5 mL/min. The mobile phase consisted of A (20 mM NH_4HCO_3, pH 9) and B (acetonitrile) using an isocratic method of 80% B for 10 (chiral HPLC method A) or 30 min (chiral HPLC method B). HPLC traces were

compared to racemic samples obtained by mixing equal amounts of the enantiopure compounds independently obtained.

3.2. Synthesis of compounds 1-25

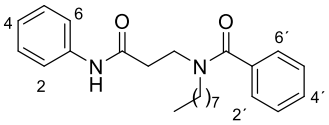


Scheme S1. Reagents and conditions: a) EDC, HOBT, DCM, rt, 16 h, 36-98%; b) TFA, DCM, rt, 70-90%.

N^3-Octyl-N^1-phenyl-β-alaninamide (**26**) was synthesized as previously described and its spectroscopic data are in agreement with those reported.[2]

General procedure for the synthesis of compounds 1-13, 15-25 and 27. To a solution of the corresponding carboxylic acid (1-2 equiv) in anhydrous DCM (4 mL/mmol), EDC (1-2 equiv) and HOBt (1-2 equiv) were added. The reaction mixture was stirred at rt for 1 h. Then, a solution of the secondary amine **26** (1 equiv) in anhydrous DCM (2 mL/mmol) was added and the reaction mixture was stirred at rt for 16 h. The reaction crude was washed with saturated aqueous solutions of $NaHCO_3$ and NaCl, consecutively. The organic extracts were dried over Na_2SO_4, filtered, and the solvent removed under reduced pressure. The residue was purified by column chromatography obtaining the desired amides.

***N*²-Benzoyl-*N*²-octyl-*N*¹-phenyl-β-alaninamide (1)**. Obtained from amine **26** (150 mg, 0.54 mmol), benzoic acid (132 mg, 1.1 mmol), EDC (207 mg, 1.1 mmol) and HOBt (146 mg, 1.1 mmol) in 91% yield (164 mg). Chromatography: hexane/EtOAc, 9:1.

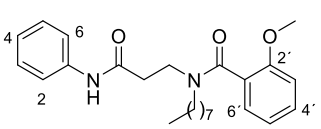


R_f (hexane/EtOAc, 7:3): 0.28. Mp: 65-67 °C. IR (ATR, ν): 3307 (NH), 1686 (CON), 1607, 1546, 1498, 1439 (Ar). ^{1}H-NMR ($CDCl_3$, δ): 0.85 (t, J = 6.9 Hz, 3H, CH_3), 1.09-1.15 (m, 10H, $(CH_2)_5CH_3$), 1.50 (m, 2H, $CH_2(CH_2)_5CH_3$), 2.75 (m, 2H, CH_2CO), 3.25 (m, 2H, $(CH_2)_6CH_2N$), 3.81 (m, 2H, $COCH_2CH_2N$), 7.04 (t, J = 7.4 Hz, 1H, H_4), 7.21-7.27 (m, 2H, $H_{3'}$, $H_{5'}$), 7.32-7.40 (m, 5H, H_3, H_5, $H_{2'}$, $H_{4'}$, $H_{6'}$), 7.51 (d, J = 7.7 Hz, 2H, H_2, H_6), 9.35 (br s, 1H, NH). ^{13}C-NMR ($CDCl_3$, δ): 14.0 (CH_3), 22.6, 26.4, 28.8, 29.0 (2C), 31.7 ($(CH_2)_6CH_3$), 36.2 (CH_2CO), 42.6 (CH_2N), 50.6 ($(CH_2)_6CH_2N$), 119.9 (C_2, C_6), 123.9 (C_4), 126.4 ($C_{2'}$, $C_{6'}$), 128.5 ($C_{3'}$, $C_{5'}$), 128.8 (C_3, C_5), 129.5 ($C_{4'}$), 136.4 ($C_{1'}$), 138.6 (C_1), 169.8,

[2] See reference in footnote 1.

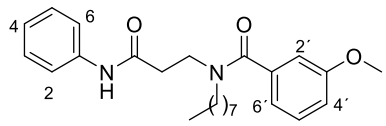
172.7 (2CO). MS (ESI, m/z): 381.2 $[M+H]^+$. Elemental analysis calculated for $C_{24}H_{32}N_2O_2$: C, 75.75; H, 8.48; N, 7.36; found: C, 75.45; H, 8.21; N, 7.41.

***N*²-(2-Methoxyphenylcarbonyl)-*N*²-octyl-*N*¹-phenyl-β-alaninamide (2).** Obtained from amine **26** (100 mg, 0.36 mmol), 2-methoxybenzoic acid (66 mg, 0.43 mmol), EDC (67 mg, 0.43 mmol) and HOBt (58 mg, 0.43 mmol) in 74% yield (110 mg). Chromatography: hexane/EtOAc, 1:1.



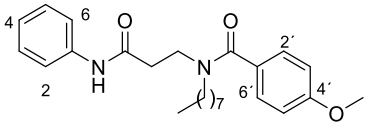
R_f (hexane/EtOAc, 1:1): 0.42. IR (ATR, ν): 3270 (NH), 1686, 1547 (CON), 1599, 1498, 1441 (Ar). ^{1}H-NMR ($CDCl_3$, δ): amide rotamers A:B, 9:1; 0.86 (t, J = 7.0 Hz, 3H, CH_3CH$_2$), 1.08-1.31 (m, 10H, (CH_2)$_5$CH$_3$), 1.44-1.51 (m, 2H, CH_2(CH$_2$)$_5$CH$_3$ rotamer A), 1.52-1.73 (m, 2H, CH_2(CH$_2$)$_5$CH$_3$ rotamer B), 2.17 (m, 2H, CH$_2$CO rotamer B), 2.39-2.48 (m, 2H, (CH$_2$)$_6$CH_2N rotamer B), 2.80-2.85 (m, 2H, CH$_2$CO rotamer A), 3.13-3.20 (m, 2H, (CH$_2$)$_6$CH_2N rotamer A), 3.42-3.57 (m, 2H, COCH$_2$CH_2N rotamer B), 3.67 (s, 3H, CH$_3$O rotamer A), 3.72-4.06 (m, 5H, COCH$_2$CH_2N rotamer A, CH$_3$O rotamer B), 6.86 (d, J = 8.3 Hz, 1H, H$_{3'}$), 6.95 (t, J = 7.4 Hz, 1H, H$_{5'}$), 7.04 (t, J = 7.4 Hz, 1H, H$_4$), 7.19 (dd, J = 7.4, 1.7 Hz, 1H, H$_{6'}$), 7.24 (t, J = 8.1 Hz, 2H, H$_3$, H$_5$), 7.32 (td, J = 7.1, 1.7 Hz, 1H, H$_{4'}$), 7.53 (d, J = 7.7 Hz, 2H, H$_2$, H$_6$), 9.47 (br s, 1H, NH). ^{13}C-NMR ($CDCl_3$, δ): 14.2 (*C*H$_3$CH$_2$), 22.7, 26.5, 28.6, 29.1 (2C), 31.8 ((*C*H$_2$)$_6$CH$_3$), 36.3 (*C*H$_2$CO), 42.0 (CH$_2$N), 49.9 ((CH$_2$)$_6$*C*H$_2$N), 55.4 (CH$_3$O), 110.9 (C$_{3'}$), 120.0 (C$_2$, C$_6$), 120.8 (C$_{5'}$), 123.7 (C$_4$), 125.9 (C$_{1'}$), 127.6 (C$_{6'}$), 128.7 (C$_3$, C$_5$), 130.5 (C$_{4'}$), 138.8 (C$_1$), 155.2 (C$_{2'}$), 170.0, 170.5 (2CO). HRMS (ESI, m/z): Calculated for $C_{25}H_{34}N_2O_3Na$ $[M+Na]^+$: 433.2467; found: 433.2466.

***N*²-(3-Methoxyphenylcarbonyl)-*N*²-octyl-*N*¹-phenyl-β-alaninamide (3).** Obtained from amine **26** (100 mg, 0.36 mmol), 3-methoxybenzoic acid (66 mg, 0.43 mmol), EDC (67 mg, 0.43 mmol) and HOBt (58 mg, 0.43 mmol) in 78% yield (115 mg). Chromatography: hexane/EtOAc, 1:1.



R_f (hexane/EtOAc, 1:1): 0.48. IR (ATR, ν): 3273 (NH), 1687, 1547 (CON), 1601, 1498, 1442 (Ar). ^{1}H-NMR ($CDCl_3$, δ): amide rotamers A:B, 9:1; 0.86 (t, J = 6.9 Hz, 3H, CH_3CH$_2$), 1.12-1.27 (m, 10H, (CH_2)$_5$CH$_3$), 1.52 (m, 2H, CH_2(CH$_2$)$_5$CH$_3$), 2.48 (m, 2H, CH$_2$CO rotamer B), 2.77 (t, J = 6.0 Hz, 2H, CH$_2$CO rotamer A), 3.27 (t, J = 7.5 Hz, 2H, (CH$_2$)$_6$CH_2N rotamer A), 3.45 (m, 2H, (CH$_2$)$_6$CH_2N rotamer B), 3.69 (s, 3H, CH$_3$O), 3.81-3.92 (m, 2H, COCH$_2$CH_2N), 6.83 (s, 1H, H$_{2'}$), 6.90 (t, J = 7.4 Hz, 1H, H$_{5'}$), 6.91 (d, J = 8.6 Hz, 1H, H$_{4'}$), 7.06 (t, J = 7.4 Hz, 1H, H$_4$), 7.23-7.29 (m, 3H, H$_3$, H$_5$, H$_{6'}$), 7.54 (d, J = 7.9 Hz, 2H, H$_2$, H$_6$), 9.39 (br s, 1H, NH). ^{13}C-NMR ($CDCl_3$, δ): 14.2 (*C*H$_3$CH$_2$), 22.7, 26.5, 28.9, 29.1, 29.2, 31.8 ((*C*H$_2$)$_6$CH$_3$), 36.2 (*C*H$_2$CO), 42.7 (CH$_2$N), 50.7 ((CH$_2$)$_6$*C*H$_2$N), 55.3 (CH$_3$O), 111.7 (C$_{2'}$), 115.6 (C$_{6'}$), 118.6 (C$_{5'}$), 120.0 (C$_2$, C$_6$), 124.0 (C$_4$), 128.9 (C$_3$, C$_5$), 129.8 (C$_{4'}$), 137.6 (C$_{1'}$), 138.6 (C$_1$), 159.7 (C$_{3'}$), 169.9, 172.6 (2CO). HRMS (ESI, m/z): Calculated for $C_{25}H_{33}N_2O_3$ $[M-H]^-$: 409.2496; found: 409.2491.

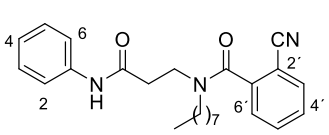
***N*²-(4-Methoxyphenylcarbonyl)-*N*²-octyl-*N*¹-phenyl-β-alaninamide (4).** Obtained from amine **26** (50 mg, 0.18 mmol), 4-methoxybenzoic acid (33 mg, 0.21 mmol), EDC (34 mg, 0.21 mmol) and HOBt (29 mg, 0.21 mmol) in 73% yield (52 mg). Chromatography: hexane/EtOAc, 7:3 to 3:7.



R_f (hexane/EtOAc, 7:3): 0.31. IR (ATR, ν): 3309 (NH), 1688, 1547 (CON), 1607, 1504, 1441 (Ar). ^{1}H-NMR ($CDCl_3$, δ): 0.86 (t, J = 6.9 Hz, 3H, CH_3CH$_2$), 1.14-1.26 (m, 10H, (CH_2)$_5$CH$_3$), 1.53 (m, 2H, CH_2(CH$_2$)$_5$CH$_3$), 2.77 (m, 2H, CH$_2$CO), 3.32 (m,

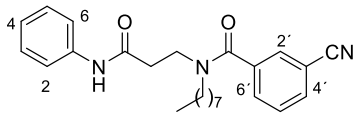
2H, $(CH_2)_6CH_2N$), 3.81-3.86 (m, 5H, $COCH_2CH_2N$, CH_3O), 6.86 (d, J = 8.8 Hz, 2H, $H_{3'}$, $H_{5'}$), 7.07 (t, J = 7.4 Hz, 1H, H_4), 7.29 (t, J = 9.2 Hz, 2H, H_3, H_5), 7.30 (m, 2H, $H_{2'}$, $H_{6'}$), 7.56 (d, J = 7.7 Hz, 2H, H_2, H_6), 9.31 (br s, 1H, NH). ^{13}C-NMR ($CDCl_3$, δ): 14.2 (CH_3CH_2), 22.7, 26.6, 28.9, 29.2 (2C), 31.8 ($(CH_2)_6CH_3$), 36.4 (CH_2CO), 42.8 (CH_2N), 50.9 ($(CH_2)_6CH_2N$), 55.4 (CH_3O), 113.8 ($C_{3'}$, $C_{5'}$), 120.0 (C_2, C_6), 124.0 (C_4), 128.5 (C_3, C_5), 128.9 ($C_{2'}$, $C_{6'}$), 138.6 (C_1), 143.0 ($C_{1'}$), 160.7 ($C_{4'}$), 169.9, 172.9 (2CO). HRMS (ESI): Calculated for $C_{25}H_{34}N_2O_3Na$ $[M+Na]^+$: 433.2467; found: 433.2450.

***N*²-(2-Cyanophenylcarbonyl)-*N*²-octyl-*N*¹-phenyl-β-alaninamide (5).** Obtained from amine **26** (100 mg, 0.36 mmol), 2-cyanobenzoic acid (63 mg, 0.43 mmol), EDC (67 mg, 0.43 mmol) and HOBt (58 mg, 0.43 mmol) in 65% yield (96 mg). Chromatography: hexane/EtOAc, 1:1.



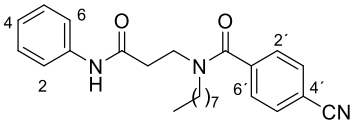
R_f (hexane/EtOAc, 1:1): 0.50. IR (ATR, ν): 3308 (NH), 2229 (CN), 1729, 1687, 1546 (CON), 1601, 1498, 1442 (Ar). ^{1}H-NMR ($CDCl_3$, δ): amide rotamers A:B 85:15; 0.85 (t, J = 7.0 Hz, 3H, CH_3), 1.08-1.29 (m, 10H, $(CH_2)_5CH_3$), 1.52 (m, 2H, $CH_2(CH_2)_5CH_3$ rotamer A), 1.60-1.73 (m, 2H, $CH_2(CH_2)_5CH_3$ rotamer B), 2.56 (t, J = 7.0 Hz, 2H, CH_2CO rotamer B), 2.83 (t, J = 6.4 Hz, 2H, CH_2CO rotamer A), 3.15 (t, J = 7.6 Hz, 2H, $(CH_2)_6CH_2N$ rotamer A), 3.47-3.62 (m, 4H, $(CH_2)_6CH_2N$, $COCH_2CH_2N$ rotamer B), 3.94 (t, J = 6.3 Hz, 2H, $COCH_2CH_2N$ rotamer A), 7.08 (t, J = 7.4 Hz, 1H, H_4), 7.30 (t, J = 7.9 Hz, 2H, H_3, H_5), 7.43 (d, J = 7.6 Hz, 1H, $H_{6'}$), 7.50 (td, J = 7.7, 1.1 Hz, 1H, $H_{4'}$), 7.56 (d, J = 7.8 Hz, 2H, H_2, H_6), 7.64 (td, J = 7.7, 1.2 Hz, 1H, $H_{5'}$), 7.67 (d, J = 7.8 Hz, 1H, $H_{3'}$), 8.50 (br s, 1H, NH). ^{13}C-NMR ($CDCl_3$, δ): 14.2 (CH_3), 22.6, 26.5, 28.8, 29.0 (2C), 31.8 ($(CH_2)_6CH_3$), 36.3 (CH_2CO), 42.3 (CH_2N), 50.1 ($(CH_2)_6CH_2N$), 109.9 ($C_{2'}$), 117.1 (CN), 120.0 (C_2, C_6), 124.2 (C_4), 126.9 ($C_{6'}$), 128.9 (C_3, C_5), 129.5 ($C_{5'}$), 132.8 ($C_{3'}$), 133.2 ($C_{4'}$), 138.3 (C_1), 140.7 ($C_{1'}$), 168.3, 169.8 (2CO). HRMS (ESI): Calculated for $C_{25}H_{30}N_3O_2$ $[(M-H)^-]$: 404.23435; found: 404.23578.

***N*²-(3-Cyanophenylcarbonyl)-*N*²-octyl-*N*¹-phenyl-β-alaninamide (6).** Obtained from amine **26** (50 mg, 0.18 mmol), 3-cyanobenzoic acid (32 mg, 0.21 mmol), EDC (10 mg, 0.21 mmol) and HOBt (12 mg, 0.21 mmol) in 66% yield (51 mg). Chromatography: hexane/EtOAc, 1:1.



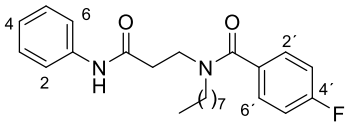
R_f (hexane/EtOAc, 1:1): 0.35. IR (ATR, ν): 3308 (NH), 2232 (CN), 1687, 1619, 1545 (CON), 1601, 1579, 1499, 1442 (Ar). ^{1}H-NMR ($CDCl_3$, δ): amide rotamers A:B 9:1; 0.89 (t, J = 6.9 Hz, 3H, CH_3), 1.14-1.29 (m, 10H, $(CH_2)_5CH_3$), 1.54 (m, 2H, $CH_2(CH_2)_5CH_3$ rotamer A), 1.69 (m, 2H, $CH_2(CH_2)_5CH_3$ rotamer B), 2.54 (m, 2H, CH_2CO rotamer B), 2.83 (t, J = 6.4 Hz, 2H, CH_2CO rotamer A), 3.26 (t, J = 7.5 Hz, 2H, $(CH_2)_6CH_2N$ rotamer A), 3.53 (m, 2H, $(CH_2)_6CH_2N$ rotamer B), 3.65 (m, 2H, $COCH_2CH_2N$ rotamer B), 3.87 (t, J = 6.4 Hz, 2H, $COCH_2CH_2N$ rotamer A), 7.13 (t, J = 7.4 Hz, 1H, H_4), 7.32 (t, J = 7.5 Hz, 2H, H_3, H_5), 7.50-7.60 (m, 4H, H_2, H_6, $H_{5'}$, $H_{6'}$), 7.65 (s, 1H, $H_{2'}$), 7.71 (d, J = 7.4 Hz, 1H, $H_{4'}$), 8.76 (br s, 1H, NH). ^{13}C-NMR ($CDCl_3$, δ): 14.2 (CH_3), 22.7, 26.5, 28.9, 29.1, 31.8 ($(CH_2)_6CH_3$), 36.1 (CH_2CO), 42.8 (CH_2N), 50.7 ($(CH_2)_6CH_2N$), 113.1 ($C_{3'}$), 117.9 (CN), 119.9 (C_2, C_6), 124.4 (C_4), 129.0 (C_3, C_5), 129.7 ($C_{5'}$/$C_{6'}$), 130.2 ($C_{2'}$), 130.8 ($C_{5'}$/$C_{6'}$), 133.1 ($C_{4'}$), 137.8, 138.1 (C_1, $C_{1'}$), 169.3, 170.2 (2CO). HRMS (ESI): Calculated for $C_{25}H_{31}N_3O_2Na$ $[(M+Na)^+]$: 428.23085; found: 428.23095.

***N*²-(4-Cyanophenylcarbonyl)-*N*²-octyl-*N*¹-phenyl-β-alaninamide (7).** Obtained from amine **26** (50 mg, 0.18 mmol), 4-cyanobenzoic acid (32 mg, 0.21 mmol), EDC (10 mg, 0.21 mmol) and HOBt (12 mg, 0.21 mmol) in 68% yield (55 mg). Chromatography: hexane/EtOAc, 1:1.



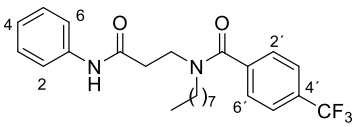
R_f (hexane/EtOAc, 1:1): 0.30. Mp: 90-91 °C. IR (ATR, ν): 3311 (NH), 2232 (CN), 1686, 1611, 1545 (CON), 1498, 1439 (Ar). ^{1}H-NMR ($CDCl_3$, δ): amide rotamers A:B, 9:1; 0.87 (t, J = 6.9 Hz, 3H, CH_3), 1.10-1.27 (m, 10H, $(CH_2)_5CH_3$), 1.50 (m, 2H, $CH_2(CH_2)_5CH_3$ rotamer A), 1.66 (m, 2H, $CH_2(CH_2)_5CH_3$ rotamer B), 2.49 (m, 2H, CH_2CO rotamer B), 2.78 (t, J = 6.4 Hz, 2H, CH_2CO rotamer A), 3.22 (t, J = 7.8 Hz, 2H, $(CH_2)_6CH_2N$ rotamer A), 3.50 (m, 2H, $(CH_2)_6CH_2N$ rotamer B), 3.60 (m, 2H, $COCH_2CH_2N$ rotamer B), 3.84 (t, J = 6.4 Hz, 2H, $COCH_2CH_2N$ rotamer A), 7.01 (t, J = 7.3 Hz, 1H, H_4), 7.28 (t, J = 7.8 Hz, 2H, H_3, H_5), 7.42 (d, J = 8.1 Hz, 2H, $H_{3'}$, $H_{5'}$), 7.49 (d, J = 8.0 Hz, 2H, H_2, H_6), 7.65 (d, J = 8.1 Hz, 2H, $H_{2'}$, $H_{6'}$), 8.13 (br s, 1H, NH rotamer B), 9.02 (br s, 1H, NH rotamer A). ^{13}C-NMR ($CDCl_3$, δ): 14.1 (CH_3), 22.6, 26.4, 28.8, 29.0 (2C), 31.7 ($(CH_2)_6CH_3$), 35.8 (CH_2CO), 42.6 (CH_2N), 50.6 ($(CH_2)_6CH_2N$), 113.4 ($C_{4'}$), 118.0 (CN), 119.9 (C_2, C_6), 124.2 (C_4), 127.2 ($C_{3'}$, $C_{5'}$), 128.9 (C_3, C_5), 132.5 ($C_{2'}$, $C_{6'}$), 138.2 (C_1), 140.7 ($C_{1'}$), 169.3, 170.6 (2CO). HRMS (ESI): Calculated for $C_{25}H_{31}N_3O_2Na$ [$(M+Na)^+$]: 428.23085; found: 428.23099. Elemental analysis calculated for $C_{25}H_{31}N_3O_2$: C, 74.04; H, 7.70; N, 10.36; found: C, 73.81; H, 7.54; N, 10.19.

***N*^{2}-(4-Fluorophenylcarbonyl)-*N*^{2}-octyl-*N*^{1}-phenyl-β-alaninamide (8).** Obtained from amine **26** (100 mg, 0.36 mmol), 4-fluorobenzoic acid (59 mg, 0.42 mmol), EDC (67 mg, 0.43 mmol) and HOBt (58 mg, 0.43 mmol) in 75% yield (108 mg). Chromatography: hexane/EtOAc, 7:3 to 3:7.



R_f (hexane/EtOAc, 1:1): 0.42. Mp: 76-77 °C. IR (ATR, ν): 3306, 3136 (NH), 1688, 1666, 1546 (CON), 1602, 1499, 1442 (Ar). ^{1}H-NMR ($CDCl_3$, δ): 0.86 (t, J = 6.9 Hz, 3H, CH_3), 1.11-1.26 (m, 10H, $(CH_2)_5CH_3$), 1.51 (m, 2H, $CH_2(CH_2)_5CH_3$), 2.75 (t, J = 5.9 Hz, 2H, CH_2CO), 3.26 (t, J = 7.2 Hz, 2H, $(CH_2)_6CH_2N$), 3.82 (t, J = 6.0 Hz, 2H, $COCH_2CH_2N$), 7.03 (t, J = 8.6 Hz, 2H, $H_{2'}$, $H_{6'}$), 7.07 (t, J = 7.4 Hz, 1H, H_4), 7.27 (t, J = 7.9 Hz, 2H, H_3, H_5), 7.32 (dd, J = 8.4, 5.5 Hz, 2H, $H_{3'}$, $H_{5'}$), 7.52 (d, J = 7.9 Hz, 2H, H_2, H_6), 9.29 (br s, 1H, NH). ^{13}C-NMR ($CDCl_3$, δ): 14.2 (CH_3), 22.7, 26.5, 28.9, 29.1 (2C), 31.8 ($(CH_2)_6CH_3$), 36.1 (CH_2CO), 42.8 (CH_2N), 50.8 ($(CH_2)_6CH_2N$), 115.7 ($C_{3'}$, $C_{5'}$), 119.9 (C_2, C_6), 124.1 (C_4), 128.7 (d, J = 8.4 Hz, $C_{2'}$, $C_{6'}$), 128.9 (C_3, C_5), 132.5 ($C_{1'}$), 138.2 (C_1), 163.3 ($C_{4'}$), 169.7, 171.8 (2CO). MS (ESI): 399.1 [$(M+H)^+$]. Elemental analysis calculated for $C_{26}H_{36}FN_3O_2$: C, 72.33; H, 7.84; N, 7.03; found: C, 72.41; H, 7.78; N, 6.98.

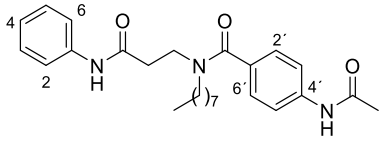
***N*^{2}-[4-(Trifluoromethyl)phenylcarbonyl]-*N*^{2}-octyl-*N*^{1}-phenyl-β-alaninamide (9).** Obtained from amine **26** (100 mg, 0.36 mmol), 4-(trifluoromethyl)benzoic acid (82 mg, 0.43 mmol), EDC (66 mg, 0.43 mmol) and HOBt (49 mg, 0.43 mmol) in 78% yield (126 mg). Chromatography: hexane/EtOAc, 7:3 to 1:1.



R_f (hexane/EtOAc, 1:1): 0.53. Mp: 80-81 °C. IR (ATR, ν): 3307 (NH), 1688, 1666, 1615, 1546 (CON), 1499, 1443 (Ar). ^{1}H-NMR ($CDCl_3$, δ): amide rotamers A:B, 9:1; 0.88 (t, J = 6.9 Hz, 3H, CH_3), 1.12-1.28 (m, 10H, $(CH_2)_5CH_3$), 1.53 (m, 2H, $CH_2(CH_2)_5CH_3$ rotamer A), 1.69 (m, 2H, $CH_2(CH_2)_5CH_3$ rotamer B), 2.51 (m, 2H, CH_2CO rotamer B), 2.83 (t, J = 6.4 Hz, 2H, CH_2CO rotamer A), 3.26 (t, J = 7.5 Hz, 2H, $(CH_2)_6CH_2N$ rotamer A), 3.53 (m, 2H, $(CH_2)_6CH_2N$ rotamer B), 3.64 (m, 2H, $COCH_2CH_2N$ rotamer B), 3.88 (t, J = 6.4 Hz, 2H, $COCH_2CH_2N$ rotamer A), 7.10 (t, J = 7.4 Hz, 1H, H_4), 7.28 (t, J = 7.8 Hz, 2H, H_3, H_5), 7.45 (d, J = 8.0 Hz, 2H, $H_{2'}$, $H_{6'}$ rotamer A), 7.50 (d, J = 8.0 Hz, 2H, H_2, H_6), 7.64 (d, J = 8.1 Hz, 2H, $H_{3'}$, $H_{5'}$ rotamer A), 7.72 (d, J = 8.2 Hz, 2H, $H_{2'}$, $H_{6'}$ rotamer B), 8.17 (d, J = 8.1 Hz, 2H, $H_{3'}$, $H_{5'}$ rotamer B), 9.25 (br s, 1H, NH). ^{13}C-NMR ($CDCl_3$, δ): 14.1 (CH_3), 22.6, 26.4, 28.8, 29.0, 29.1, 31.7 ($(CH_2)_6CH_3$), 35.9

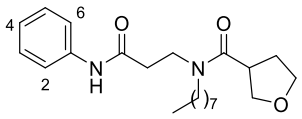
(CH_2CO), 42.8 (CH_2N), 50.7 ($(CH_2)_6CH_2N$), 120.0 (C_2, C_6), 124.2 (C_4), 125.7 (q, J = 3.6 Hz, $C_{3'}$, $C_{5'}$), 126.9 ($C_{2'}$, $C_{6'}$), 127.3 (CF_3), 128.9 (C_3, C_5), 131.7 ($C_{4'}$), 138.3 (C_1), 139.9 ($C_{1'}$), 169.6, 171.3 (2CO). MS (ESI): 449.2 [$(M+H)^+$]. Elemental analysis calculated for $C_{25}H_{31}F_3N_2O_2$: C, 66.95; H, 6.97; N, 6.25; found: C, 66.23; H, 6.73; N, 6.01.

***N*²-(4-Acetamidophenylcarbonyl)-*N*²-octyl-*N*¹-phenyl-β-alaninamide (10).** Obtained from amine **26** (85 mg, 0.31 mmol), 4-acetamidobenzoic acid (66 mg, 0.37 mmol), EDC (57 mg, 0.37 mmol) and HOBt (50 mg, 0.37 mmol) in 80% yield (108 mg). Chromatography: hexane/EtOAc, 2:8.



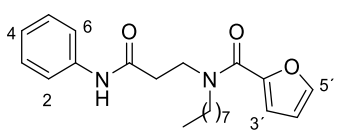
R_f (hexane/EtOAc, 2:8): 0.19. IR (ATR, ν): 3300 (NH), 1669, 1535 (CON), 1602, 1468, 1438 (Ar). ^{1}H-NMR ($CDCl_3$, δ): amide rotamers A:B, 8:2; 0.85 (t, J = 6.8 Hz, 3H, CH_3CH_2), 1.16-1.29 (m, 10H, $(CH_2)_5CH_3$), 1.49-1.62 (m, 2H, $CH_2(CH_2)_5CH_3$ rotamers A and B), 1.97 (s, 3H, CH_3CO rotamer B), 2.15 (s, 3H, CH_3CO rotamer A), 2.22 (m, 2H, CH_2CO rotamer B), 2.76 (m, 2H, CH_2CO rotamer A), 3.26 (m, 2H, $(CH_2)_6CH_2N$ rotamer A), 3.45 (m, 2H, $(CH_2)_6CH_2N$ rotamer B), 3.60 (m, 2H, $COCH_2CH_2N$ rotamer B), 3.83 (m, 2H, $COCH_2CH_2N$ rotamer A), 7.07 (t, J = 7.4 Hz, 1H, H_4), 7.20 (d, J = 7.6 Hz, 2H, $H_{2'}$, $H_{6'}$), 7.27 (t, J = 7.8 Hz, 2H, H_3, H_5), 7.44 (d, J = 7.4 Hz, 2H, $H_{3'}$, $H_{5'}$), 7.53 (d, J = 7.7 Hz, 2H, H_2, H_6), 8.15 (br s, 1H, NH), 9.19 (br s, 1H, NH). ^{13}C-NMR ($CDCl_3$, δ): 14.2 (CH_3CH_2), 22.7 (CH_2), 24.6 (CH_3CO), 26.6, 26.7, 29.0, 29.2, 31.8 ($5CH_2$), 36.3 (CH_2CO), 42.6 (CH_2N), 50.8 ($(CH_2)_6CH_2N$), 119.7 ($C_{3'}$, $C_{5'}$), 120.1 (C_2, C_6), 124.2 (C_4), 127.6 ($C_{2'}$, $C_{6'}$), 129.0 (C_3, C_5), 131.8 ($C_{1'}$), 138.4 (C_1), 139.5 ($C_{4'}$), 169.0, 169.1, 169.9 (3CO). HRMS (ESI): Calculated for $C_{26}H_{35}N_3O_3Na$ [$(M+Na)^+$]: 460.2570; found: 460.2583.

***N*²-Octyl-*N*¹-phenyl-*N*²-(tetrahydrofuran-3-ylcarbonyl)-β-alaninamide (11).** Obtained from amine **26** (100 mg, 0.36 mmol), tetrahydrofuran-3-carboxylic acid (84 mg, 0.72 mmol), EDC (138 mg, 0.72 mmol) and HOBt (97 mg, 0.72 mmol) in 84% yield (113 mg). Chromatography: hexane/EtOAc, 7:3.



R_f (hexane/EtOAc, 7:3): 0.13. Mp: 68-70 °C. IR (ATR, ν): 3309 (NH), 1684, 1545, (CON), 1624, 1494, 1442 (Ar). ^{1}H-NMR ($CDCl_3$, δ): 0.89 (t, J = 6.7 Hz, 3H, CH_3), 1.28-1.30 (m, 10H, $(CH_2)_5CH_3$), 1.56-1.61 (m, 2H, $CH_2(CH_2)_5CH_3$), 2.01-2.23 (m, 2H, CH_2CH_2O), 2.69 (t, J = 6.6 Hz, 2H, CH_2CO), 3.22 (qt, J = 7.6 Hz, 1H, CH), 3.34 (t, J = 6.8 Hz, 2H, $NCH_2(CH_2)_6$), 3.70 (t, J = 6.6 Hz, 2H, NCH_2CH_2CO), 3.81-4.02 (m, 4H, $2CH_2O$), 7.09 (t, J = 7.4 Hz, 1H, H_4), 7.31 (t, J = 7.9 Hz, 2H, H_3,H_5), 7.54 (d, J = 7.9 Hz, 2H, H_2, H_6), 8.47 (br s, 1H, NH). ^{13}C-NMR ($CDCl_3$, δ): 14.0 (CH_3), 22.6, 26.8, 29.2, 29.3, 29.5 ($(CH_2)_5CH_3$), 30.8 (CH_2CH_2O), 31.7 ($CH_2(CH_2)_5$), 36.8 (CH_2CO), 41.2 (CH), 43.2 (NCH_2CH_2CO), 49.0 ($NCH_2(CH_2)_6$), 68.6, 71.2 ($2CH_2O$), 119.8 (C_2, C_6), 124.1 (C_4), 128.9 (C_3, C_5), 138.2 (C_1), 168.4, 174.1 (2CO). MS (ESI): 375.2 [$(M+H)^+$]. Elemental analysis calculated for $C_{22}H_{34}N_2O_3$: C, 70.55; H, 9.15; N, 7.48; found: C, 70.83; H, 8.77; N, 7.50.

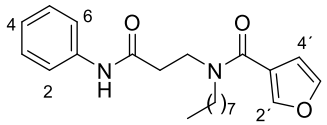
***N*²-(2-Furoyl)-*N*²-octyl-*N*¹-phenyl-β-alaninamide (12).** Obtained from amine **26** (85 mg, 0.31 mmol), 2-furoic acid (76 mg, 0.62 mmol), EDC (96 mg, 0.62 mmol) and HOBt (84 mg, 0.62 mmol) in 95% yield (109 mg). Chromatography: hexane/EtOAc, 1:1.



R_f (hexane/EtOAc, 1:1): 0.40. Mp: 91-93 °C. IR (ATR, ν): 3315 (NH), 1668 (CON), 1605 (Ar). ^{1}H-NMR ($CDCl_3$, δ): 0.87 (t, J = 6.6 Hz, 3H, CH_3), 1.25 (m, 10H, $(CH_2)_5CH_3$), 1.65 (m, 2H, $CH_2(CH_2)_5CH_3$), 2.75 (t, J = 6.3 Hz, 2H, CH_2CO), 3.61 (m, 2H, $(CH_2)_6CH_2N$), 3.83 (m, 2H, $COCH_2CH_2N$), 6.43 (m, 1H, $H_{4'}$),

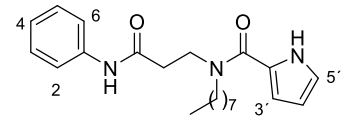
6.95 (m, 1H, $H_{3'}$), 7.06 (t, J = 7.4 Hz, 1H, H_4), 7.27 (t, J = 7.8 Hz, 2H, H_3, H_5), 7.41 (m, 1H, $H_{5'}$), 7.49-7.60 (m, 2H, H_2, H_6), 9.29 (br s, 1H, NH). ^{13}C-NMR ($CDCl_3$, δ): 14.1 (CH_3), 22.6, 26.8, 29.2, 29.3, 29.7, 31.8 ((CH_2)$_6CH_3$), 36.2 (CH_2CO), 44.3 (CH_2N), 49.6 ((CH_2)$_6CH_2N$), 111.4, 116.5 ($C_{3'}$, $C_{4'}$), 119.9 (C_2, C_6), 123.9 (C_4), 128.8 (C_3, C_5), 138.5 (C_1), 144.0 ($C_{5'}$), 147.9 ($C_{2'}$), 160.5 (CON), 169.6 (CONH). MS (ESI): 371.2 [$(M+H)^+$]. Elemental analysis calculated for $C_{22}H_{30}N_2O_3$: C, 71.32; H, 8.16; N, 7.56; found: C, 71.35; H, 8.03; N, 7.30.

***N*^{2}-(3-Furoyl)-*N*^{2}-octyl-*N*^{1}-phenyl-β-alaninamide (13).** Obtained from amine **26** (100 mg, 0.36 mmol), 3-furoic acid (81 mg, 0.72 mmol), EDC (138 mg, 0.72 mmol) and HOBt (97 mg, 0.72 mmol) in 98% yield (128 mg). Chromatography: hexane/EtOAc, 7:3.



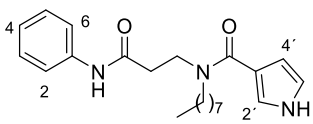
R_f (hexane/EtOAc, 7:3): 0.14. IR (ATR, ν): 3281 (NH), 1685 (CON), 1605, 1547, 1501, 1441 (Ar). ^{1}H-NMR ($CDCl_3$, δ): 0.88 (t, J = 6.7 Hz, 3H, CH_3), 1.25 (m, 10H, (CH_2)$_5CH_3$), 1.61 (m, 2H, CH_2(CH_2)$_5CH_3$), 2.76 (m, 2H, CH_2CO), 3.44 (t, J = 7.9 Hz, 2H, (CH_2)$_6CH_2N$), 3.82 (t, J = 6.6 Hz, 2H, $COCH_2CH_2N$), 6.55 (m, 1H, $H_{4'}$), 7.08 (t, J = 7.4 Hz, 1H, H_4), 7.29 (t, J = 8.3 Hz, 2H, H_3, H_5), 7.41 (t, J = 1.7 Hz, 1H, $H_{5'}$), 7.55 (d, J = 7.8 Hz, 2H, H_2, H_6), 7.68 (m, 1H, $H_{2'}$). ^{13}C-NMR ($CDCl_3$, δ): 14.1 (CH_3), 22.6, 26.6, 29.2 (3C), 31.7 ((CH_2)$_6CH_3$), 36.5 (CH_2CO), 43.2 (CH_2N), 50.1 ((CH_2)$_6CH_2N$), 110.1 ($C_{4'}$), 119.8 (C_2, C_6), 121.3 ($C_{3'}$), 124.1 (C_4), 128.9 (C_3, C_5), 138.3 (C_1), 143.1, 143.2 ($C_{2'}$, $C_{5'}$), 165.6 (CON), 169.4 (CONH). MS (ESI): 371.2 [$(M+H)^+$]. Elemental analysis calculated for $C_{22}H_{30}N_2O_3$: C, 71.32; H, 8.16; N, 7.56; found: C, 71.15; H, 7.91; N, 7.61.

***N*^{2}-Octyl-*N*^{1}-phenyl-*N*^{2}-(1*H*-pyrrol-2-ylcarbonyl)-β-alaninamide (15).** Obtained from amine **26** (85 mg, 0.31 mmol), 1*H*-2-pyrrolcarboxylic acid (70 mg, 0.62 mmol), EDC (95 mg, 0.62 mmol) and HOBt (84 mg, 0.62 mmol) in 51% yield (58 mg). In this case the carboxylic acid, the coupling reagents and the amine **26** were all dissolved in DCM at the same time to avoid the acylation of the pyrrole ring. Chromatography: hexane/EtOAc, 7:3.



R_f (hexane/EtOAc, 1:1): 0.40. Mp: 105-107 °C. IR (ATR, ν): 3264 (NH), 1666 (CON), 1599, 1548, 183, 1445 (Ar). ^{1}H-NMR ($CDCl_3$, δ): 0.88 (t, J = 6.7 Hz, 3H, CH_3), 1.28-1.31 (m, 10H, (CH_2)$_5CH_3$), 1.71 (m, 2H, CH_2(CH_2)$_5CH_3$), 2.74 (t, J = 6.7 Hz, 2H, CH_2CO), 3.58 (m, 2H, (CH_2)$_6CH_2N$), 3.87 (m, 2H, $COCH_2CH_2N$), 6.25-6.28 (m, 1H, $H_{4'}$), 6.54 (m, 1H, $H_{3'}$), 6.90 (m, 1H, $H_{5'}$), 7.07 (t, J = 7.3 Hz, 1H, H_4), 7.28 (t, J = 7.8 Hz, 2H, H_3, H_5), 7.55 (d, J = 7.8 Hz, 2H, H_2, H_6), 8.84 (br s, 1H, NH), 9.84 (br s, 1H, NH). ^{13}C-NMR ($CDCl_3$, δ): 14.1 (CH_3), 22.6, 26.8, 28.6, 29.2, 29.4, 31.8 ((CH_2)$_6CH_3$), 36.7 (CH_2CO), 44.3 (CH_2N), 49.3 ((CH_2)$_6CH_2N$), 110.3, 112.2 ($C_{3'}$, $C_{4'}$), 119.9 (C_2, C_6), 121.3 ($C_{5'}$), 124.1 (C_4), 124.4 ($C_{2'}$), 128.9 (C_3, C_5), 138.3 (C_1), 162.5 (CON), 169.4 (CONH). MS (ESI): 370.2 $[M+H]^+$. Elemental analysis calculated for $C_{22}H_{30}N_2O_3$: C, 71.51; H, 8.46; N, 11.37; found: C, 71.29; H, 8.11; N, 11.35.

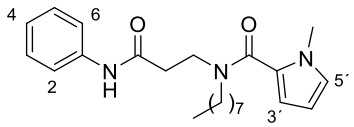
***N*^{2}-Octyl-*N*^{1}-phenyl-*N*^{2}-(1*H*-pyrrol-3-ylcarbonyl)-β-alaninamide (16).** Obtained from amine **26** (80 mg, 0.29 mmol), 1*H*-3-pyrrolcarboxilic acid (65 mg, 0.58 mmol), EDC (90 mg, 0.58 mmol) and HOBt (78 mg, 0.58 mmol) in 54% yield (58 mg). In this case the carboxylic acid, the coupling reagents and the amine **26** were all dissolved in DCM at the same time to avoid the acylation of the pyrrole ring. Chromatography: hexane/EtOAc, 3:7.



R_f (EtOAc): 0.25. Mp: 99-101 °C. IR (ATR, ν): 3250 (NH), 1598 (CON), 1548 (Ar). ^{1}H-NMR ($CDCl_3$, δ): 0.79 (t, J = 6.7 Hz, 3H, CH_3), 1.16 (m, 10H, (CH_2)$_5CH_3$), 1.54 (m, 2H, CH_2(CH_2)$_5CH_3$), 2.61 (m, 2H, CH_2CO), 3.41 (t, J = 7.2 Hz, 2H, (CH_2)$_6CH_2N$), 3.74 (t, J = 6.6 Hz, 2H, $COCH_2CH_2N$), 6.26 (m, 1H, $H_{4'}$), 6.56 (m, 1H, $H_{5'}$), 6.92 (m, 1H, $H_{2'}$),

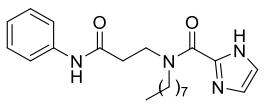
6.97 (t, J = 7.4 Hz, 1H, H_4), 7.18 (t, J = 7.8 Hz, 2H, H_3, H_5), 7.49 (d, J = 7.9 Hz, 2H, H_2, H_6), 9.37 (br s, 2H, 2NH). ^{13}C-NMR ($CDCl_3$, δ): 14.1 (CH_3), 22.6, 26.7, 28.9, 29.2, 29.3, 31.8 ((CH_2)$_6CH_3$), 36.6 (CH_2CO), 43.4 (CH_2N), 49.8 ((CH_2)$_6CH_2N$), 108.6 ($C_{4'}$), 118.3 ($C_{5'}$), 118.4 ($C_{3'}$), 120.0 (C_2, C_6), 121.1 ($C_{2'}$), 123.9 (C_4), 128.8 (C_3, C_5), 138.5 (C_1), 168.4 (CON), 170.0 (CONH). MS (ESI): 370.2 $[M+H]^+$. Elemental analysis calculated for $C_{22}H_{30}N_2O_3$: C, 71.51; H, 8.46; N, 11.37; found: C, 71.40; H, 8.22; N, 11.18.

N^2-[(1-Methyl-1*H*-pyrrol-2-yl)carbonyl]-N^2-octyl-N^1-phenyl-β-alaninamide (17). Obtained from amine **26** (163 mg, 0.59 mmol), 1-methyl-1*H*-pyrrol-2-carboxylic acid (150 mg, 1.2 mmol), EDC (230 mg, 1.2 mmol) and HOBt (162 mg, 1.2 mmol) in 49% yield (111 mg). Chromatography: hexane/EtOAc, 7:3.



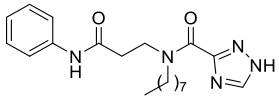
R_f (hexane/EtOAc, 7:3): 0.44. IR (ATR, ν): 3277 (NH), 1684 (CO), 1602, 1543, 1473, 1439 (Ar). ^{1}H-NMR ($CDCl_3$, δ): 0.87 (t, J = 6.7 Hz, 3H, CH_3CH_2), 1.23-1.30 (m, 10H, (CH_2)$_5CH_3$), 1.58-1.65 (m, 2H, CH_2(CH_2)$_5CH_3$), 2.73 (t, J = 6.8 Hz, 2H, CH_2CO), 3.54 (t, J = 7.6 Hz, 2H, NCH_2(CH_2)$_6$), 3.67 (s, 3H, CH_3N), 3.80 (t, J = 6.8 Hz, 2H, NCH_2CH_2CO), 6.06 (dd, J = 3.7, 2.7 Hz, 1H, $H_{4'}$), 6.30 (dd, J = 3.7, 1.4 Hz, 1H, $H_{3'}$), 6.66 (t, J = 2.9 Hz, 1H, $H_{5'}$), 7.07 (t, J = 7.4 Hz, 1H, H_4), 7.28 (t, J = 7.8 Hz, 2H, H_3, H_5), 7.55 (d, J = 8.1 Hz, 2H, H_2, H_6), 9.11 (br s, 1H, NH). ^{13}C-NMR ($CDCl_3$, δ): 14.5 (CH_3CH_2), 23.0, 26.9, 29.2, 29.6 (2C), 32.1 ((CH_2)$_6CH_3$), 35.9 (CH_3N), 36.8 (CH_2CO), 43.5 (NCH_2CH_2CO), 50.6 (NCH_2(CH_2)$_6$), 107.5, 112.5 ($C_{3'}$, $C_{4'}$), 120.2 (C_2, C_6), 124.4 (C_4), 125.7 ($C_{2'}$), 126.6 ($C_{5'}$), 129.2 (C_3, C_5), 138.8 (C_1), 165.6 (CON), 170.1 (CONH). HRMS (ESI, m/z): Calculated for $C_{23}H_{34}N_3O_2$ $[M+H]^+$: 384.2646; found: 384.2655.

N^2-(1*H*-Imidazol-2-ylcarbonyl)-N^2-octyl-N^1-phenyl-β-alaninamide (18). Obtained from amine **26** (95 mg, 0.33 mmol), 1*H*-imidazol-2-carboxylic acid (150 mg, 0.66 mmol), EDC (118 mg, 0.66 mmol) and HOBt (64 mg, 0.66 mmol) in 60% yield (71 mg). Chromatography: hexane/EtOAc, 7:3.



R_f (hexane/EtOAc, 7:3): 0.44. Mp: 96-97 ºC. IR (ATR, ν): 3201 (NH), 1668 (CO), 1604, 1546, 1483, 1443 (Ar). ^{1}H-NMR ($CDCl_3$, δ): amide romaters A:B 6:1, 0.81 (t, J = 6.7 Hz, 3H, CH_3), 1.20-1.26 (m, 10H, (CH_2)$_5CH_3$), 1.60 (m, 2H, CH_2(CH_2)$_5CH_3$), 2.71 (m, 2H, CH_2CO, rotamer B), 2.82 (t, J = 8.1 Hz, 2H, CH_2CO, rotamer A), 3.47 (t, J = 7.5 Hz, 2H, NCH_2(CH_2)$_6$, rotamer A), 3.81 (m, 2H, NCH_2CH_2CO, rotamer B), 4.21 (m, 2H, NCH_2(CH_2)$_6$, rotamer B), 4.30 (t, J = 8.0 Hz, 2H, NCH_2CH_2CO, rotamer A), 7.03 (t, J = 7.4 Hz, 1H, H_4), 7.16 (m, 1H, CH_{HetAr}), 7.19 (s, 1H, CH_{HetAr}), 7.25-7.30 (m, 2H, H_3, H_5), 7.45-7.55 (m, 2H, H_2, H_6), 8.29 (br s, 1H, NH, rotamer B), 9.98 (br s, 1H, NH, rotamer A), 10.94 (br s, 1H, NH, rotamer B), 11.52 (br s, 1H, NH, rotamer A). ^{13}C-NMR ($CDCl_3$, δ): 14.1 (CH_3), 22.6, 27.1, 27.8, 29.2, 29.4, 31.8 ((CH_2)$_6CH_3$), 39.7 (CH_2CO), 46.8 (NCH_2CH_2CO), 48.3 (NCH_2(CH_2)$_6$), 118.8 (CH_{HetAr}), 119.6 (C_2, C_6), 123.9 (C_4), 129.1 (C_3, C_5), 129.8 (CH_{HetAr}), 138.6 (C_1), 141.5 ($C_{1'}$), 158.3 (CON), 169.1 (CONH). MS (ESI): 371.2 $[(M+H)^+]$. Elemental analysis calculated for $C_{21}H_{30}N_4O_2$ C, 68.08; H, 8.16; N, 15.12; found: C, 68.42; H, 8.49; N, 15.18.

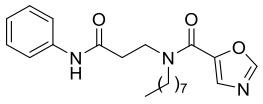
N^2-Octyl-N^1-phenyl-N^2-(1*H*-1,2,4-triazol-3-ylcarbonyl)-β-alaninamide (19). Obtained from amine **26** (150 mg, 0.54 mmol), 1*H*-1,2,4-triazol-3-carboxylic acid (124 mg, 1.1 mmol), EDC (171 mg, 1.1 mmol) and HOBt (149 mg, 1.1 mmol) in 45% yield (90 mg). Chromatography: EtOAc.



R_f (EtOAc): 0.25. Mp: 110-111 ºC. IR (ATR, ν): 3281 (NH), 1623 (CO), 1547 (Ar). ^{1}H-NMR ($CDCl_3$, δ): amide rotamers A:B 3:2, 0.84-0.89 (m, 3H, CH_3), 1.25-1.29 (m, 10H, (CH_2)$_5CH_3$), 1.61-1.78 (m, 2H,

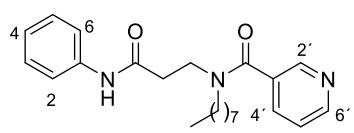
C*H*$_2$(CH$_2$)$_5$CH$_3$), 2.81-2.87 (m, 2H, CH$_2$CO), 3.55 (t, *J* = 7.6 Hz, 2H, NC*H*$_2$(CH$_2$)$_6$, rotamer B), 3.92 (t, *J* = 7.3 Hz, 2H, NC*H*$_2$CH$_2$CO, rotamer A), 4.08-4.15 (m, 2H, NC*H*$_2$(CH$_2$)$_6$, rotamer A), 4.27 (t, *J* = 7.7 Hz, 2H, NC*H*$_2$CH$_2$CO, rotamer B), 7.05-7.12 (m, 1H, H$_4$), 7.31 (t, *J* = 8.1 Hz, 2H, H$_3$, H$_5$), 7.54-7.58 (m, 2H, H$_2$, H$_6$), 8.13 (s, 1H, H$_{5'}$, rotamer A), 8.22 (m, 1H, CH$_{HetAr}$, rotamer B), 8.96 (br s, 1H, NH), 13.94 (br s, 1H, NH). ^{13}C-NMR (CDCl$_3$, δ): 14.1 (CH$_3$), 22.6, 26.6, 27.0, 27.6, 29.2 (2C), 29.3, 31.8 ((*C*H$_2$)$_6$CH$_3$, rotamers A and B), 35.7, 38.9 (*C*H$_2$CO, rotamers A and B), 45.6, 46.1 (N*C*H$_2$CH$_2$CO, rotamers A and B), 48.1, 50.2 (N*C*H$_2$(CH$_2$)$_6$, rotamers A and B), 119.7, 120.0 (C$_2$, C$_6$, rotamers A and B), 124.3 (C$_4$), 128.9, 129.0 (C$_3$, C$_5$, rotamers A and B), 138.1, 138.2 (C$_1$, rotamers A and B), 150.8 (C$_{5'}$), 158.8 (C$_{3'}$), 168.7, 169.0 (CO, rotamers A and B), 171.2 (CO). MS (ESI): 372.2 [(M+H)$^+$]. Elemental analysis calculated for $C_{20}H_{29}N_5O_2$ C, 64.66; H, 7.87; N, 18.85; found: C, 64.49; H, 7.66; N, 18.39.

***N*^{2}-Octyl-*N*^{1}-phenyl-*N*^{2}-(1,3-oxazol-5-ylcarbonyl)-β-alaninamide (20).** Obtained from amine **26** (120 mg, 0.43 mmol), 1,3-oxazol-5-carboxylic acid (97 mg, 0.86 mmol), EDC (133 mg, 0.86 mmol) and HOBt (116 mg, 0.86 mmol) in 95% yield (150 mg). Chromatography: hexane/EtOAc, 1:1.



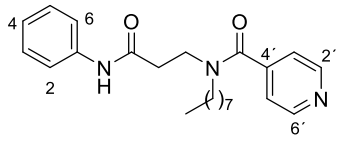
R$_f$ (hexane/EtOAc, 1:1): 0.21. IR (ATR, ν): 3312 (NH), 1684 (C=O), 1623, 1546, 1495, 1442 (Ar). ^{1}H-NMR (CDCl$_3$, δ): 0.88 (t, *J* = 6.7 Hz, 3H, CH$_3$), 1.27 (m, 10H, (C*H*$_2$)$_5$CH$_3$), 1.66 (m, 2H, C*H*$_2$(CH$_2$)$_5$CH$_3$), 2.79 (m, 2H, CH$_2$CO), 3.59 (m, 2H, NC*H*$_2$(CH$_2$)$_6$), 3.85 (m, 2H, NC*H*$_2$CH$_2$CO), 7.08 (t, *J* = 7.3 Hz, 1H, H$_4$), 7.28 (t, *J* = 7.8 Hz, 2H, H$_3$, H$_5$), 7.54-7.56 (m, 3H, H$_2$, H$_6$, CH$_{4'}$), 7.91 (m, 1H, CH$_{2'}$), 8.52 y 8.94 (br s, 1H, NH). ^{13}C-NMR (CDCl$_3$, δ): 13.0 (CH$_3$), 21.6, 25.6, 28.1, 28.2, 28.4, 30.7 ((*C*H$_2$)$_6$CH$_3$), 34.8 (*C*H$_2$CO), 43.3 (N*C*H$_2$CH$_2$CO), 48.7 (N*C*H$_2$(CH$_2$)$_6$), 118.9 (C$_2$, C$_6$), 123.2 (C$_4$), 127.9 (C$_3$, C$_4$), 130.4 (C$_{4'}$), 137.2 (C$_1$), 144.2 (C$_{5'}$), 150.7 (C$_{2'}$), 157.6 (CON), 168.3 (CONH). HRMS (ESI): Calculated for $C_{21}H_{29}N_3O_3Na$ [(M+Na)$^+$]: 394.2101; found: 394.2908.

***N*^{2}-Octyl-*N*^{1}-phenyl-*N*^{2}-(pyridin-3-ylcarbonyl)-β-alaninamide (22).** Obtained from amine **26** (100 mg, 0.36 mmol), nicotinic acid (87 mg, 0.72 mmol), EDC (138 mg, 0.72 mmol) and HOBt (97 mg, 0.72 mmol) in 36% yield (50 mg). Chromatography: hexane/EtOAc, 2:8.



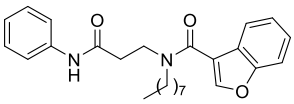
R$_f$ (EtOAc): 0.28. IR (ATR, ν): 3312 (NH), 1685 (CON), 1612, 1546, 1495, 1440 (Ar). ^{1}H-NMR (CDCl$_3$, δ): amide rotamers A:B, 6:1; 0.87 (t, *J* = 6.8 Hz, 3H, CH$_3$), 1.13-1.18 (m, 10H, (C*H*$_2$)$_5$CH$_3$), 1.53 (m, 2H, C*H*$_2$(CH$_2$)$_5$CH$_3$), 2.50 (m, 2H, CH$_2$CO, rotamer B), 2.79 (t, *J* = 5.7 Hz, 2H, CH$_2$CO, rotamer A), 3.28 (t, *J* = 7.0 Hz, 2H, (CH$_2$)$_6$C*H*$_2$N, rotamer A), 3.49 (m, 2H, (CH$_2$)$_6$C*H*$_2$N, rotamer B), 3.64 (m, 2H, COCH$_2$C*H*$_2$N, rotamer B), 3.86 (m, 2H, COCH$_2$C*H*$_2$N, rotamer A), 7.07 (t, *J* = 7.3 Hz, 1H, H$_4$), 7.24-7.32 (m, 3H, H$_3$, H$_5$, H$_{5'}$), 7.53 (d, *J* = 7.6 Hz, 2H, H$_2$, H$_6$), 7.67 (d, *J* = 7.7 Hz, 1H, H$_{4'}$), 8.64 (d, *J* = 1.4 Hz, 2H, H$_{2'}$, H$_{6'}$), 9.27 (br s, 1H, NH). ^{13}C-NMR (CDCl$_3$, δ): 14.0 (CH$_3$), 22.5, 26.4, 28.9, 29.0 (2C), 31.6 ((*C*H$_2$)$_6$CH$_3$), 35.8 (*C*H$_2$CO), 42.7 (CH$_2$N), 50.7 ((CH$_2$)$_6$*C*H$_2$N), 119.9 (C$_2$, C$_6$), 123.4 (C$_{5'}$), 124.1 (C$_4$), 128.8 (C$_3$, C$_5$), 132.4 (C$_{3'}$), 134.2 (C$_{4'}$), 138.3 (C$_1$), 147.3, 150.6 (C$_{2'}$, C$_{6'}$), 169.5, 169.8 (CONH, CON). HRMS (ESI, m/z): Calculated for $C_{23}H_{31}N_3O_2Na$ [M+Na]$^+$: 404.2308; found: 404.2302.

***N*^{2}-Octyl-*N*^{1}-phenyl-*N*^{2}-(pyridin-4-ylcarbonyl)-β-alaninamide (23).** Obtained from amine **26** (85 mg, 0.31 mmol), isonicotinic acid (53 mg, 0.43 mmol), EDC (67 mg, 0.43 mmol) and HOBt (58 mg, 0.43 mmol) in 43% yield (51 mg). Chromatography: hexane/EtOAc, 2:8.



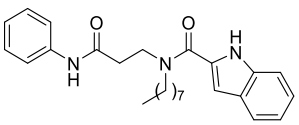
R_f (EtOAc): 0.19. IR (ATR, ν): 3310 (NH), 1624 (CON), 1547 (Ar). ^{1}H-NMR ($CDCl_3$, δ): amide rotamers A:B, 4:1: 0.76 (t, J = 6.9 Hz, 3H, CH_3), 1.02-1.07 (m, 10H, $(CH_2)_5CH_3$), 1.41 (qt, J = 6.9 Hz, 2H, $CH_2(CH_2)_5CH_3$), 2.40 (m, 2H, CH_2CO, rotamer B), 2.70 (t, J = 6.5 Hz, 2H, CH_2CO, rotamer A), 3.12 (t, J = 7.6 Hz, 2H, $(CH_2)_6CH_2N$, rotamer A), 3.41 (m, 2H, $(CH_2)_6CH_2N$, rotamer B), 3.50 (m, 2H, $COCH_2CH_2N$, rotamer B), 3.74 (t, J = 6.5 Hz, 2H, $COCH_2CH_2N$, rotamer A), 7.00 (t, J = 7.3 Hz, 1H, H_4), 7.11 (d, J = 5.8 Hz, 2H, $H_{3'}$, $H_{5'}$), 7.16-7.21 (m, 2H, H_3, H_5), 7.41 (d, J = 7.9 Hz, 2H, H_2, H_6), 8.55 (d, J = 5.7 Hz, 2H, $H_{2'}$, $H_{6'}$), 8.78 (br s, 1H, NH). ^{13}C-NMR ($CDCl_3$, δ): 14.0 (CH_3), 22.5, 26.4, 28.8, 29.0 (2C), 31.6 ($(CH_2)_6CH_3$), 35.9 (CH_2CO), 42.4 (CH_2N), 50.4 ($(CH_2)_6CH_2N$), 119.8 (C_2, C_6), 120.8 ($C_{3'}$, $C_{5'}$), 124.2 (C_4), 128.9 (C_3, C_5), 138.2 (C_1), 144.1 ($C_{4'}$), 150.3 ($C_{2'}$, $C_{6'}$), 169.2, 169.9 (CON, rotamers A and B), 171.1 (CONH). HRMS (ESI, m/z): Calculated for $C_{23}H_{31}N_3O_2Na$ $[M+Na]^+$: 404.2308; found: 404.2311.

***N*^{2}-(1-Benzofuran-3-ylcarbonyl)-*N*^{2}-octyl-*N*^{1}-phenyl-β-alaninamide (24).** Obtained from amine **26** (100 mg, 0.36 mmol), benzofuran-3-carboxylic acid (117 mg, 0.72 mmol), EDC (138 mg, 0.72 mmol), and HOBt (97 mg, 0.72 mmol), in 57% yield (86 mg). Chromatography: DCM/MeOH, 95:5.



R_f (DCM/ethanol 95:5): 0.38. IR (ATR, ν): 3313 (NH), 1683 (CO), 1609, 1548, 1492, 1443 (Ar). ^{1}H-NMR ($CDCl_3$, δ): 0.84 (t, J = 6.9, 3H, CH_3), 1.14-1.26 (m, 10H, $(CH_2)_5CH_3$), 1.54 (m, 2H, $CH_2(CH_2)_5CH_3$), 2.76 (m, 2H, CH_2CO), 3.45 (t, J = 7.5, 2H, $NCH_2(CH_2)_6$), 3.85 (t, J = 6.5, 2H, NCH_2CH_2CO), 7.06 (t, J = 7.4, 1H, H_4), 7.15 (t, J = 7.4, 1H, CH_{benzof}), 7.22-7.33 (m, 3H, H_3, H_5, CH_{benzof}), 7.46-7.61 (m, 4H, H_2, H_6, $2CH_{benzof}$), 7.74 (s, 1H, H_3, H_{benzof}), 9.15 (br s, 1H, NH). ^{13}C-NMR ($CDCl_3$, δ): 14.0 (CH_3), 22.5, 26.5, 29.1 (3C), 31.7 ($(CH_2)_6CH_3$), 36.2 (CH_2CO), 43.0 (NCH_2CH_2CO), 50.4 ($NCH_2(CH_2)_6$), 111.7 (CH_{benzof}), 117.2 (C_{benzof}), 119.9 (C_2, C_6), 120.9, 123.7 ($2CH_{benzof}$), 124.1 (C_4), 125.3 (CH_{benzof}), 125.4 (C_{benzof}), 128.8 (C_3, C_5), 138.4 (C_1), 143.9 (CH_{benzof}), 154.6 (C_{benzof}), 165.4 (CON), 169.6 (CONH). HRMS (ESI, m/z): Calculated for $C_{26}H_{32}N_2O_3Na$ $[M+Na]^+$: 443.2305; found: 443.2319.

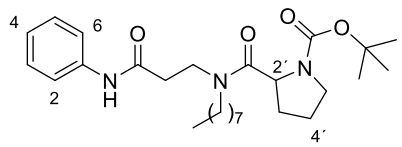
***N*^{3}-(1*H*-Indol-2-ylcarbonyl)-*N*^{3}-octyl-*N*^{1}-Phenyl-β-alaninamide (25).** Obtained from amine **26** (387 mg, 1.4 mmol), 1*H*-indol-2-carboxylic acid (451 mg, 2.8 mmol), EDC (435 mg, 2.8 mmol), and HOBt (378 mg, 2.8 mmol), in 73% yield (420 mg). Chromatography: hexane/EtOAc, 7:3.



R_f (hexane/EtOAc, 1:1): 0.47. mp: 142-144 ºC. IR (ATR, ν): 3284 (NH), 1666 (CO), 1600, 1533, 1442 (Ar). ^{1}H-NMR ($CDCl_3$, δ): 0.79 (t, J = 6.6, 3H, CH_3), 1.16 (m, 10H, $(CH_2)_5CH_3$), 1.59 (m, 2H, $CH_2(CH_2)_5CH_3$), 2.62 (t, J = 6.8, 2H, CH_2CO), 3.50 (m, 2H, $NCH_2(CH_2)_6$), 3.80 (m, 2H, NCH_2CH_2CO), 6.63 (m, 1H, CH_{indol}), 6.92-7.03 (m, 2H, H_4, CH_{indol}), 7.09-7.16 (m, 3H, H_3, H_5, CH_{indol}), 7.26 (d, J = 8.2 Hz, 1H, CH_{indol}), 7.40 (d, J = 7.9 Hz, 2H, H_2, H_6), 7.51 (d, J = 7.8 Hz, 1H, CH_{indol}), 8.53 (br s, 1H, NH), 9.82 (br s, 1H, NH). ^{13}C-NMR ($CDCl_3$, δ): 14.1 (CH_3), 22.7, 26.8, 28.8, 29.2, 29.3, 31.8 ($(CH_2)_6CH_3$), 36.3 (CH_2CO), 44.5 (NCH_2CH_2CO), 49.9 ($NCH_2(CH_2)_6$), 105.2, 111.8 ($2CH_{indol}$), 120.0 (C_2, C_6), 120.6, 122.1 ($2CH_{indol}$), 124.3 (C_4), 124.6 (CH_{indol}), 127.9 (C_{indol}), 128.9 (C_3, C_5), 129.2, 135.7 ($2C_{indol}$), 138.1 (C_1), 163.4 (CON), 169.5 (CONH). MS (ESI): 420.4 $[(M+H)^+]$. Elemental analysis calculated for $C_{26}H_{33}N_3O_2$: C, 74.43; H, 7.93; N, 10.02; found: C, 74.19; H, 7.83; N, 10.00.

***N*^{2}-Octyl-*N*^{1}-phenyl-*N*^{2}-(*N*-Boc-pyrrolidin-2-ylcarbonyl)-β-alaninamide (27).** Obtained from amine **26** (71 mg, 0.26 mmol), *N*-(tert-butoxycarbonyl)-proline (110 mg, 0.51 mmol),

EDC (98 mg, 0.51 mmol), and HOBt (69 mg, 0.51 mmol), in 64% yield (79 mg). Chromatography: hexane/EtOAc, 1:1.

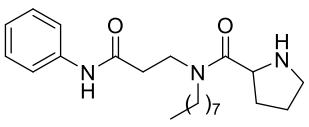


R_f (hexane/EtOAc, 1:1): 0.27. IR (ATR, ν): 3312 (NH), 1685, 1667 (CO), 1604, 1546, 1441 (Ar). ^{1}H-NMR ($CDCl_3$, δ): amide rotamers A:B, 2:1: 0.80-0.88 (m, 3H, CH_3CH$_2$), 1.17-1.28 (m, 10H, (CH_2)$_5$CH$_3$), 1.44-1.67 (m, 11H, CH_2(CH$_2$)$_5$CH$_3$, C(CH_3)$_3$), 1.74-1.95 (m, 2H, CH_2CH$_2$CH), 2.06-2.19 (m, 2H, CH_2CH), 2.31-2.51 (m, 2H, CH$_2$CO), 2.60-2.79 (m, 2H, CH$_2$NBoc, rotamer A), 3.00-3.32 (m, 2H, NCH_2(CH$_2$)$_6$), 3.52-3.65 (m, 2H, NCH_2CH$_2$CO), 3.67-3.87 (m, 2H, CH$_2$NBoc, rotamer B), 4.41-4.57 (m, 1H, CH), 7.01 (t, J = 7.3 Hz, 1H, H$_4$), 7.26 (d, J = 7.9 Hz, 2H, H$_3$, H$_5$), 7.55 (d, J = 7.7 Hz, 2H, H$_2$, H$_6$, rotamer A), 7.74 (d, J = 7.7 Hz, 2H, H$_2$, H$_6$, rotamer B), 9.13 (br s, 1H, NH, rotamer A), 9.34 (br s, 1H, NH, rotamer B). ^{13}C-NMR ($CDCl_3$, δ): 14.0 (CH$_3$CH$_2$), 22.6 (2C), 24.4, 24.7, 26.7, 27.7 (4CH$_2$, rotamers A and B), 28.5, 28.6 (C(CH_3)$_3$, rotamers A and B), 29.1, 29.3, 30.0, 30.1, 30.2, 31.7 (2C) (4CH$_2$, rotamers A and B), 37.1, 37.9 (CH_2CO, rotamers A and B), 44.9, 46.0, 47.2, 47.3, 47.9, 50.0 (3CH$_2$N, rotamers A and B), 56.1, 56.5 (CH, rotamers A and B), 80.0, 80.1 (C(CH$_3$)$_3$, rotamers A and B), 120.1, 120.2 (C$_2$, C$_6$, rotamers A and B), 123.8, 124.0 (C$_4$, rotamers A and B), 128.7 (C$_3$, C$_5$), 138.5, 139.0 (C$_1$, rotamers A and B), 154.7, 154.9 (NCOO, rotamers A and B), 169.6, 171.2, 172.5, 173.1 (2CO, rotamers A and B). MS (ESI): 474.3 $[M+H]^+$, 374.0 $[M\text{-Boc}+H]^+$.

N^2-Octyl-N^1-phenyl-N^2-(*N*-Boc-*R*-pyrrolidin-2-ylcarbonyl)-β-alaninamide (*R*-27). Obtained from amine **26** (100 mg, 0.36 mmol), *N*-(tert-butoxycarbonyl)-D-proline (156 mg, 0.72 mmol), EDC (139 mg, 0.72 mmol), and HOBt (98 mg, 0.72 mmol), was obtained in 74% yield (105 mg). Spectroscopic data were in agreement with those described for its racemic counterpart. Chiral HPLC-MS, r_t (min): 6.66 (Chiral HPLC method A). ee> 99%

N^2-Octyl-N^1-phenyl-N^2-(*N*-Boc-*S*-pyrrolidin-2-ylcarbonyl)-β-alaninamide (*S*-27). Obtained from amine **26** (72 mg, 0.26 mmol), *N*-(tert-butoxycarbonyl)-L-proline (109 mg, 0.50 mmol), EDC (97 mg, 0.50 mmol), and HOBt (67 mg, 0.50 mmol), was obtained in 78% yield (97 mg). Spectroscopic data were in agreement with those described for its racemic counterpart. Chiral HPLC-MS, r_t (min): 7.45 (Chiral HPLC method A). ee> 99%

• **N^2-Octyl-N^1-phenyl-N^2-(pyrrolidin-2-ylcarbonyl)-β-alaninamide (14).** To a solution of *N*-Boc pyrrolidine **27** (63 mg, 0.13 mmol) in anhydrous DCM (0.5 mL), TFA (200 μL, 2.7 mmol) was added. The reaction mixture was stirred at rt for 1 h, basified with $NaHCO_3$ until pH 8 and extracted with DCM. The organic extract was washed with a saturated solution of NaCl, dried over Na_2SO_4, filtered, and the solvent was removed under reduced pressure, obtaining the desired final compound **14** in 70% yield (34 mg).



R_f (EtOAc/methanol, 7/3): 0.19. IR (ATR, ν): 3269 (NH), 1644 (CO), 1605, 1548, 1494, 1444 (Ar). ^{1}H-NMR ($CDCl_3$, δ): amide rotamers A:B, 2:1: 0.83-0.88 (m, 3H, CH$_3$), 1.21-1.24 (m, 10H, (CH_2)$_5$CH$_3$), 1.45-2.30 (m, 6H, CH_2(CH$_2$)$_5$CH$_3$, (CH_2)$_2$CH), 2.56-3.05 (m, 4H, CH$_2$CO, CH_2NH), 3.15-3.34 (m, 2H, NCH_2(CH$_2$)$_6$), 3.55-3.76 (m, 2H, NCH_2CH$_2$CO), 3.91 (t, J = 7.5 Hz, 1H, CH, rotamer A), 4.37 (t, J = 7.5 Hz, 1H, CH, rotamer B), 5.10 (br s, 1H, NH), 7.05 (t, J = 7.3 Hz, 1H, H$_4$), 7.23-7.30 (m, 2H, H$_3$, H$_5$), 7.56 (d, J = 7.7 Hz, 2H, H$_2$, H$_6$, rotamer A), 7.61 (d, J = 7.7 Hz, 2H, H$_2$, H$_6$, rotamer B), 8.97 (br s, 1H, NH, rotamer A), 9.58 (br s, 1H, NH, rotamer B). ^{13}C-NMR ($CDCl_3$, δ): 14.1 (CH$_3$), 22.6, 25.8, 26.3, 26.8, 27.6, 29.1, 29.2 (2C), 29.3 (2C), 29.7, 30.6, 31.1, 31.7 ((CH_2)$_6$CH$_3$, (CH_2)$_2$CH, rotamers A and B), 36.3, 37.2 (CH_2CO, rotamers A and B), 43.2, 44.0

(N*C*H$_2$CH$_2$CO, rotamers A and B), 46.8, 46.9, 47.4, 48.3 (N*C*H$_2$(CH$_2$)$_6$, CH$_2$NH, rotamers A and B), 57.8, 58.2 (CH, rotamers A and B), 119.9 (C$_2$, C$_6$), 124.1 (C$_4$), 128.8, 128.9 (C$_3$, C$_5$), 138.4 (C$_1$), 168.7, 169.5, 171.4, 173.9 (2CO, rotamers A and B). HRMS (ESI, m/z): Calculated for $C_{22}H_{36}N_3O_2$ $[M+H]^+$: 374.2796; found: 374.2802.

***N*^{2}-Octyl-*N*^{1}-phenyl-*N*^{2}-(*R*-pyrrolidin-2-ylcarbonyl)-β-alaninamide (*R*-14).** Obtained from (*R*)-**27** (75 mg, 0.16 mmol) and TFA (244 μL, 3.2 mmol) in 90% yield (59 mg). Spectroscopic data were in agreement with those described for racemic compound **14**. Chiral HPLC-MS, r_t (min): 5.99 (Chiral HPLC method B). ee> 99%. $[\alpha]^D_{20}$: -103.2º (c = 0.4, methanol).

***N*^{2}-Octyl-*N*^{1}-phenyl-*N*^{2}-(*S*-pyrrolidin-2-ylcarbonyl)-β-alaninamide (*S*-14).** Obtained from (*S*)-**27** (90 mg, 0.19 mmol) and TFA (293 μL, 3.8 mmol) in 70% yield (50 mg). Spectroscopic data were in agreement with those described for racemic compound **14**. Chiral HPLC-MS, r_t (min): 10.18 (Chiral HPLC method B). ee> 99%. $[\alpha]^D_{20}$: + 100.8º (c = 0.4, methanol).

4. Cell viability and proliferation assays.

Cells were seeded in 96-well plates at a density of 5 x 10^2 cells per well in the corresponding medium with 10% FBS for 24 h prior to treatments. The medium was then replaced by fresh medium containing different concentrations of compounds tested or by medium containing the equivalent volume of dimethylsulfoxide (DMSO, vehicle control). Cells were treated for 72 h, and then medium was replaced by fresh medium with 2 mg/mL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich) and cells were incubated for 4 h at 37 ºC in the dark. Once supernatants were removed, formazan crystals previously formed by viable cells were dissolved in DMSO (100 μL/ well) and absorbance was measured at 570 nm (OD570-630) using an Asys UVM 340 (Biochrom Ltd., Cambridge, UK) microplate reader. Background absorbance from blank wells containing only media with compound or vehicle were substracted from each test well.

Measurements were performed in triplicate, and each experiment was repeated at least three times. The viability values were calculated from the percentage of viable cells with respect to 100% viability of the vehicle-treated cells.

For proliferation assays, AF3 mouse fibroblasts (15 x 10^3 cells) or human progeroid fibroblasts (25 x 10^3 cells) were seeded on 24-well plates in the corresponding medium. After 24 h, the medium was replaced by fresh medium containing different concentration of compounds or the equivalent volume of DMSO. Every three (mouse cells) or seven (human cells) days, cells were treated with trypsin, counted, and reseeded with fresh medium and compound in increasing plate sizes. Population doublings (PD) were calculated using the formula PD=Log (harvested/seeded)/Log(2).

5. Immunoblot analysis.

Mouse or human progeroid cells previously incubated with compound of interest or DMSO were seeded at density of 50 x 10^3 cells on 24-well plates and incubated for 24 h in the appropriate medium with fresh compound. Then, cells were lysed with cold laemmly buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Igepal) with a mixture of protease and phosphatase inhibitors (1 mM PMSF, 10 mM NaF, 0.3 mg/mL CalyculinA, 1 mM sodium

orthovanadate, 10 mM β-glycerol-phosphate; all from Sigma-Aldrich and complete protease inhibitor cocktail, from Roche). Proteins were denatured at 95 ºC for 5 min and the lysates were immediately used or stored at -20 ºC until their use. Samples were analyzed by electrophoresis in polyacrylamide gels (4-20% SDS-PAGE, BioRad) and the proteins transferred to nitrocellulose membranes (GE Healthcare, Amersham). After 1 h of incubation in blocking buffer [100 mM Tris-HCl pH 8.0, 150 mM NaCl and 0.05% Tween-20 (TBS-T) with 3% of bovine serum albumin (BSA)], blots were incubated overnight at 4 ºC with the corresponding primary antibody: anti-phospho-histone 2AX (Ser139) (EMD Millipore, 05-636, 1:1000), anti-phospho-Akt (Cell Signaling, 4060S, 1:1000), anti-Akt (Cell Signaling, 9272, 1:1000), anti-progerin (Nourshargh S. lab, 1:1000), anti-lamin A/C (Santa Cruz Biotechnology, sc-376248, 1:500) and anti-GAPDH (Cell Signaling, 2118S, 1:10000) as antibody for loading control. Then, membranes were washed (3x10 min) with TBS-T and incubated with the corresponding secondary antibody conjugated with peroxidase (Sigma-Aldrich) for 1 h at rt. Membranes were washed (6x5 min) with TBS-T and proteins were visualized by quimioluminiscence (GE Healthcare Amersham™ ECL™ Western Blotting Detection Reagents, Fisher Scientific) in a Fujifilm LAS-3000 imager. The bands were quantified by densitometry using the program ImageJ (NIH).

For progerin degradation studies, mouse progeroid cells previously incubated with compound of interest or DMSO during 14 days were seeded at density of 50 x 10^3 cells on 24-well plates in the appropriate medium with fresh compound and bafilomicyn A (24 h at 25 nM) or MG-132 (5 h at 5 μM) were added. Then, cells were lysed and subjected to immunoblot analysis for progerin as described above.

6. Immunocytofluorescence analysis

Immunocytofluorescence. Human progeroid cells previously incubated with compound of interest or DMSO were seeded at a density of 2 x 10^4 cells/well over 12 mm diameter slide covers and were incubated for 24 h at 37 ºC with compound of interest. Cells were fixed with cold methanol (Sigma-Aldrich) during 15 min and permeabilized with PBS containing 0.1% Triton X-100 (PBS-T, Sigma-Aldrich). Then, cells were blocked with TBS 3% BSA during 20 min at rt, followed by incubation with an anti-progerin antibody (Santa Cruz Biotechnology, SC-81611, 1:500) in TBS with 3% BSA at 37 °C for 3 h. After that, cells were washed with TBS (1x) and incubated in absence of light for 1 h with fluorescent goat anti-mouse (1:500, Alexa Fluor 488, Life Technologies) diluted in TBS with 3% BSA. Later, cells were additionally washed with TBS (1x) and incubated with Hoechst 33258 (5 μg/mL, Sigma-Aldrich) for 10 min at rt in order to visualize cellular nucleus. Finally, cells were washed with TBS (2x) and the slide cover was carefully mounted with Immumount (Thermo Scientific). The visualization was carried out using confocal microscopy (Olympus IX83) equipped with a 40X oil immersion lens and the appropriate excitation and emission filters at the microscopy UCM facilities.

Quantification of progerin intensity or internalization was performed using ImageJ (NIH). For quantification of progerin intensity, images stained for progerin in green and Hoescht in blue were taken under the same conditions. Then, the tool "threshold" was applied to blue Hoescht channel to select each nucleus. Then, regions containing each cell nucleus were created with the tool "Analyze particles". These regions were extrapolated to progerin channel and green fluorescence mean intensity was measured for each nucleus. For progerin internalization quantification, the same protocol was followed, but once the nuclei regions were determined for progerin positive cells, they were made 2 pixels smaller and subtracted from the original

region using the tool "enlarge". To generate a new region comprising the fluorescence intensity at nucleus periphery, the tool "make band" was applied to the new smaller region to create a 2 pixels band around it. The mean pixel intensity of progerin included in this peripheral band region was quantified and compared with fluorescence intensity from the intranuclear region area.

7. Senescence-associated (SA) β-galactosidase activity.

Human progeroid cells previously incubated with compound of interest or vehicle were seeded at a density of 5 x 10^3 cells per well in triplicates in a 96-well plate overnight for attachment, washed, and then fixed for 5 min in 2% formaldehyde and 0.2% glutaraldehyde buffered with PBS. After washing thrice in PBS, the fixed cells were incubated in the staining solution [0.2 mM fluorescein di- β -D-galactopyranoside (FDG, Sigma-Aldrich F2756), 0.2 M citric acid, 0.4 M Na_2HPO_4 (citrate–phosphate buffer), 100 mM potassium ferricyanide, 5 M NaCl, 0.2 M $MgCl_2$, (all from Sigma Aldrich) in deionized water] in the dark in a humidified incubator at 37 ºC for 24 h without CO_2 supply. Then, an equal volume of the supernatant of each well was transferred to a new 96-well plate for fluorescence measurement. The resultant production of fluorescein was measured using a FluoStar Optima instrument (BMG Labtech) with an excitation of 485 nm and emission at 535 nm. One well containing the reaction mixture without the cells was used as blank for subtracting the background fluorescence.

For mouse tissue, senescence-associated β-galactosidase quantitative assay was performed according to Desdín-Micó et al.[3] Briefly, indicated tissues were lysed with T-PER Tissue Protein 5 Extraction Reagent (Thermo Scientific, 78510). Lysates were centrifuged at 10,000*g* for 5 min and the supernatant was collected. Fifty microliters of protein lysates were mixed with 50 µL of Pierce β-galactosidase Assay Reagent (Thermo Scientific, 75705) for the assay. The reaction was incubated for 30 min and the absorbance was measured at 405 nm. Values were normalized to total protein levels.

8. Stability assays.

Stability in cell culture medium and in mouse and human serum was assayed by adding 625 µL of a 2 mM solution of test compound in PBS (pH 7.4) to 1875 µL of mouse or human serum (Europa Bioproducts, EQSM-0100) pre-warmed at 37 ºC or to 1875 µL of cell culture medium. Next, solutions were incubated at 37 ºC for 24 h, taking aliquots of 125 µL at different time points (0, 1, 4, 8 and 24 h). Each aliquot was quenched with 500 µL of cold acetonitrile, vortexed, incubated for 10 min in ice and centrifuged at 39000*g* for 10 min. Supernatants were then analyzed by HPLC-MS using SIM mode. Concentrations were quantified by measuring the area under the peak ($[M+H]^+$) normalized with an internal standard and converted to the percentage of compound remaining, using the time zero peak area value as 100%.

For measuring the stability in mouse and human liver microsomes, compounds were incubated at 37 °C at a final concentration of 1 or 5 µM in PBS, respectively, together with a solution of nicotinamide adenine dinucleotide phosphate (NADPH) in PBS (final concentration of 2 mM) and a solution of $MgCl_2$ in PBS (final concentration of 5 mM).

[3] Desdín-Mico, G.; Soto-Heredero, G.; Aranda, J.F.; Oller, J.; Carrasco, E.; Gabandé-Rodríguez, E.; Blanco, E.M.; Alfranca, A.; Cussó, L.; Desco, M.; Ibañez, B.; Gortazar, A.R.; Fernández-Marcos, P.; Navarro, M.N.; Hernaez, B.; Alcamí, A.; Baixauli, F.; Mittelbrunn, M. *Science* **2020**, *368*, 1371-1376.

Reactions were initiated by the addition of a suspension of mouse liver microsomes (MLMs) (male CD-1 mice pooled, Sigma-Aldrich) or human liver microsomes (HLMs) (male human pooled, Sigma-Aldrich), respectively, at a final protein concentration of 1 mg/mL. The solutions were vortexed and incubated at 37 °C. Aliquots of 100 μL were quenched at time zero and at seven points ranging to 1 h (MLM) or 2 h (HLM) by pouring into 100 μL of ice-cold acetonitrile. Quenched samples were centrifuged at 10000*g* for 10 min, and the supernatants were filtered through a polytetrafluoroethylene (PTFE) membrane syringe filter (pore size of 0.2 μm, 13 mm of diameter, GE Healthcare Life Sciences). The relative disappearance of the compound under study over the course of the incubation was monitored by HPLC-MS using SIM mode. Concentrations were quantified by measuring the area under the peak ($[M+H]^+$) normalized with an internal standard and converted to the percentage of compound remaining, using the time zero peak area value as 100%. The natural logarithm of the remaining percentage versus time data for each compound was fit to a linear regression, and the slope was used to calculate the degradation half-life ($t_{1/2}$). The intrinsic clearance (CL_{int}) was calculated by the equation $V \cdot \ln 2/t_{1/2}$ where V is the incubation volume (in μL) / protein (mg) for microsomal stability.

9. Human serum albumin (HSA) binding assay.

An eight-well unit of the TRANSIL assay plate was used for each compound; six wells contain increasing concentrations of HSA immobilized on silica beads suspended in PBS at pH 7.4, and two wells contain buffer only and serve as references to account for non-specific binding. The TRANSIL assay plate was thawed for 3 h at rt and centrifuged at 750*g* for 5 s. Then, 15 μL of an 80 μM stock solution of the compound in PBS (for a final concentration of 5 μM) was added to each well of the eight-well unit, and the plate was incubated on a plate shaker at 1000 rpm for 12 min at rt. After this time, the plate was centrifuged at 750*g* for 10 min, and 50 μL of the supernatants were transferred for analytical quantification by HPLC-MS using selected ion monitoring (SIM) and quantification was estimated by measuring the area under the peak ($[M+H]^+$) normalized with an internal standard. The binding percentage was calculated from the remaining free compound concentration in the supernatant of each well, using the spreadsheet and algorithms supplied with the kit.

10. Animal experiments

For pharmacokinetic studies, compound **21** was administered intraperitoneally and blood was collected at the selected time points post-dose (n=3 per time point) by cardiac puncture. Blood was allowed to clot at room temperature for 30 min and centrifuged at 4 ºC for 10 min at 16000*g*. The supernatant was transferred to a clean polypropylene tube and stored at -80 ºC until analysis. For analysis, a volume of cold acetonitrile was added to the serum. The sample was incubated in an ice bath for 10 min and centrifuged at 4 ºC for 10 min at 16000*g*. The resulting organic layer was filtered through a polytetrafluoroethylene filter (0.2 μm, 13 mm diameter, Fisher Scientific) and 20 μL of the sample analysed by LC-MS/MS at the UCM's Mass Spectrometry CAI. Separation was performed using a Phenomenex Gemini 5 μm C18 110A 150x2 mm column (run time 10 min; flow 0.5 mL/min; gradient: 5 min 5% B – 3 min 100% B – 2 min 5% B; Phase A: water with formic acid 0.1%; Phase B: acetonitrile). The entire LC eluent was directly introduced to an electrospray ionization (ESI) source operating in the positive ion mode for LC MS/MS analysis on a Shimadzu LCMS8030 triple

quadrupole mass spectrometer coupled to UHPLC with an oven temperature of 31.5 °C. The mass spectrometer ion optics were set in the multiple reaction monitoring mode and the transition selected for quantification was 382.10 > 217.15 (CE: -19 V).

For in vivo treatment, compound **21** was administrated at a concentration of 40 mg/kg diluted in sterile corn oil (Sigma-Aldrich) intraperitoneally five times per week starting at the age of 6 weeks. The same quantity of corn oil was injected to control $Lmna^{G609G/G609G}$ and $Lmna^{+/+}$ mice. Treatments did not produce any apparent damage or stress in mice. The weight of each mouse was registered once a week during the duration of the treatment.

To analyse blood glucose, animals were starved for 7 h and glucose levels were measured with a FreeStyle Optium Neo glucometer (Abbott) using blood from the tail vein.

Forepaw strength of in the three groups of mice was measured as previously described.[4] Briefly, seven weights of 20, 33, 46, 59, 72, 85 and 98 grams were used. The ability of each mouse to hold each weight from lighter to heavier was determined. A final total score is calculated as the product of the number of links in the heaviest chain held for the full 3 seconds, multiplied by the seconds it is held. If the heaviest weight is dropped before 3 seconds, an intermediate value is calculated.

In vivo magnetic resonance imaging (MRI) was performed at the Preclinical MRI Unit of the Complutense University using a 1-Tesla benchtop MRI scanner (Icon 1T-MRI; Bruker BioSpin GmbH, Ettlingen, Germany). MRI system consists of a 1 T permanent magnet (without extra cooling required for the magnet) with a gradient coil that provides a gradient strength of 450 mT/m. Animal monitoring systems and solenoid radiofrecuency coil (oval shape, inner diameter 59 x 50 mm^2 and length 90 mm) are integrated into the magnet bed. They allowed both the animals accurate positioning and the correct monitoring of vital signs during acquisition. Animals were anesthetized using 2% isofluorane (IsoFlo, Zoetis, NJ, USA) and they were positioned unrestrained in order to visualize the natural curvature of their spinal corn. The main MRI experiment consisted of three dimensional images (0.3 x 0.3 x 1.0 mm^3). It was acquired using a gradient echo sequence with a repetition time of 160 ms, an echo time of 3.5 ms, and a flip angle of 45°. The total acquisition time was less than 6 min. All MRI images were analysed using ImageJ software.

11. Pathological analysis and immunofluorescence.

After 6 weeks of treatment, 12-week-old mice were euthanized by CO_2 inhalation. Immediately after sacrifice, the thoracic and abdominal cavities were opened. Mice were perfused with cold PBS and spleen, thymus, heart, and aorta were excised and used for different protocols. For immunohistofluorescence, heart and aorta segments were fixed overnight in 4% paraformaldehyde and dehydrated in an ascending ethanol series. Samples were embedded in paraffin, cut into 4-μM sections, and stained with Masson's trichrome. These samples were imaged with OPT scanner 3001 (OPT, Bioptonics Microscopy). Alternatively, heart and aorta (thoracic arch) sections were used for immunohistofluorescence experiments. Antigen retrieval was performed with 10 mM sodium citrate buffer (pH 6). Samples were blocked and permeabilized for 1 h at rt in PBS containing 0.3% TritonX-100 (9002-93-1, Sigma), 5% normal goat serum (005-000-001, JacksonImmunoResearch), and 5% bovine serum albumin (BSA, A7906, Sigma). Sections were incubated overnight at 4 °C with

[4] Deacon, R.M.J. *J. Vis. Exp.* **2013**, *76*, 2610. Doi: 10.3791/2610

rabbit anti-progerin polyclonal antibody (1:300) generated using peptide immunogens and standard immunization procedures (S. Nourshargh et al., manuscript in preparation) diluted in PBS containing 2.5% normal goat serum, with anti-CD31 (Dianova DIA-310, 1:50) antibody, with wheat germ agglutinin-Alexa Fluor 488 (W11261, ThermoFisher), or for 2 h at rt with anti-SMA-Cy3 antibody (C6198, Sigma, 1:200). Next, samples were incubated in 2.5% normal goat serum in PBS containing the corresponding secondary antibody [anti-rabbit Alexa Fluor-647 (111-607-008, Jackson ImmunoResearch) or anti-rat Alexa Fluor-488 (A-11006, Molecular Probes)] and Hoechst 33342 (B2261, Sigma) for 2 h at rt. Samples were mounted using Fluoromount G imaging medium (00-4958-02, Affymetrix eBioscience).

For fibrosis determination, heart sections processed as indicated above were incubated overnight at 4 °C with anti-CD31 (Dianova DIA-310 1:50) antibody, or Wheat Germ Agglutinin-Alexa fluor 488 (W11261, ThermoFisher) and for 2 hours at rt with anti-SMA-Cy3 antibody (C6198, Sigma, 1:200). Next, samples were incubated with anti-rabbit Alexa Fluor-647 secondary antibody (111-607-008, Jackson ImmunoResearch) and anti-rat Alexa Fluor-488 secondary antibody (A-11006 Molecular probes) and Hoechst 33342 for 2 h at rt, and mounted in Fluoromount-G imaging medium.

Fluorescence images were acquired under the same conditions with a Zeiss LSM 700 confocal microscope. Image analysis was performed using ImageJ Fiji software by a researcher blinded to genotype.

Vascular smooth muscle cell (VSMC) number was determined using Fiji. Briefly, medial area of the aortic arch was selected using wand tracing tool. Then, nucleus in DAPI channel included in this area were selected and counted using threshold and analyze particles tools.

Quantification of progerin intensity was performed in Fiji. First, medial layer was selected using wand tracing tool. Then, nucleuses positive for progerin in this area were selected using threshold and analyze particle tools. Finally, mean intensity of these nucleuses was measured and represented.

Quantification of heart WGA intensity (as a measure of fibrosis) was performed in Fiji. Whole apex cut images were taken under the same conditions and stained area was selected using wand tracing tool. Mean WGA intensity was determined, background signal was subtracted and result normalized against total area to determine mean signal per micrometer. Quantification of smooth muscle cell loss in the medial layer of septum arterioles was determined through α-smooth muscle actin (SMA) I stain. Heart apex arteriole images were taken under the same conditions and stained area was selected using wand tracing tool.

12. Determination of the levels of methylated AFC in cells.

Lmna$^{G609G/G609G}$ cells (2.5 millions per point) were incubated in the presence of vehicle or compound **21** (2 μM) for 24 h. Then, acetylfarnesylcysteine (AFC, 100 μM) was added and cells were incubated for additional 72 h. After that, cells were washed and lysed in 0.5 mL PBS. Whole cell homogenates were extracted with a mixture of chloroform (1 mL) and methanol (0.5 mL). Organic phase was evaporated and resuspended in methanol (50 μL) and analysed by LC-MS/MS at the UCM's Mass Spectrometry CAI. Separation was performed using a Phenomenex Gemini 5 μm C18 110A 150x2 mm column (run time 8 min; flow 0.5 mL/min; gradient: 0.5 min 10% Phase B – 2 min 60% Phase B – 4.5 min 100% Phase B – 6 min 100% Phase B– 7min-8min 10% Phase B; Phase A: water with formic acid 0.1%; Phase B: methanol). The entire LC eluent was directly introduced to an electrospray ionization (ESI)

source operating in the positive ion mode for LC MS/MS analysis on a Shimadzu LCMS8030 triple quadrupole mass spectrometer coupled to HPLC with an oven temperature of 45 °C. The mass spectrometer ion optics were set in the multiple reaction monitoring mode and the transition selected for quantification was 382.30 > 143.90 (CE: -17.0 v).