Supporting Information

Collagen labelling with an azide-proline chemical reporter in live cells

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1. Supporting Results

Supporting Figure 1

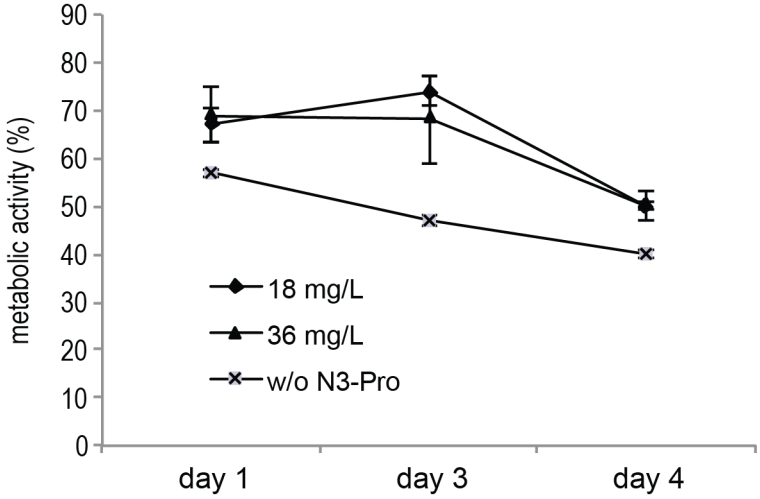

Figure S1. Alamar Blue Assay for measurement of metabolic activity of foetal ovine osteoblats upon incubation with N_3-Pro. Data was collected after 1, 3 and 4 days. Error bars represent the standard error from 3 different cell culture wells.

Supporting Figure 2

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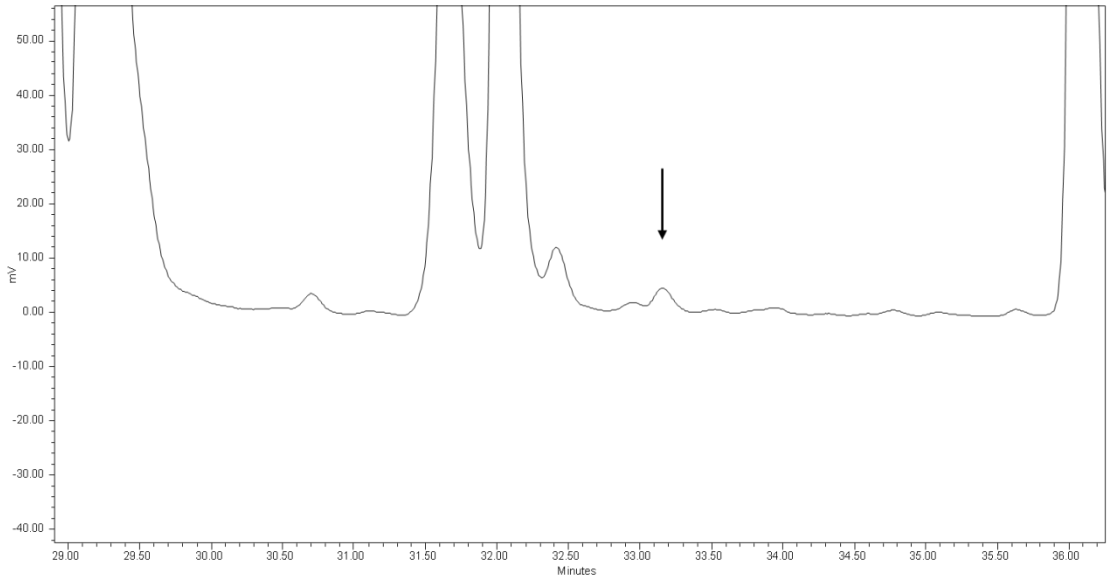

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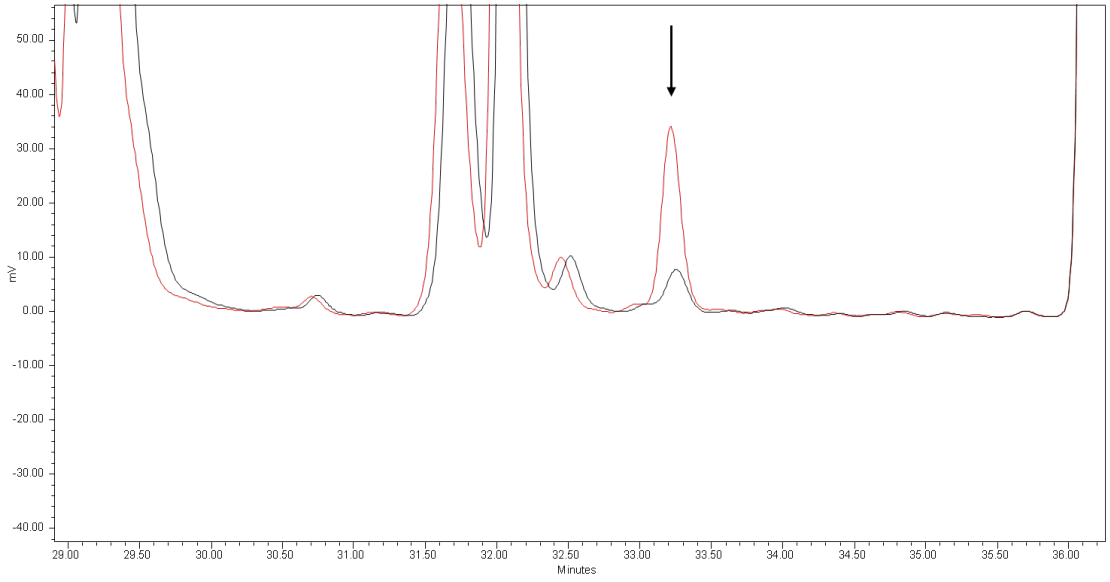

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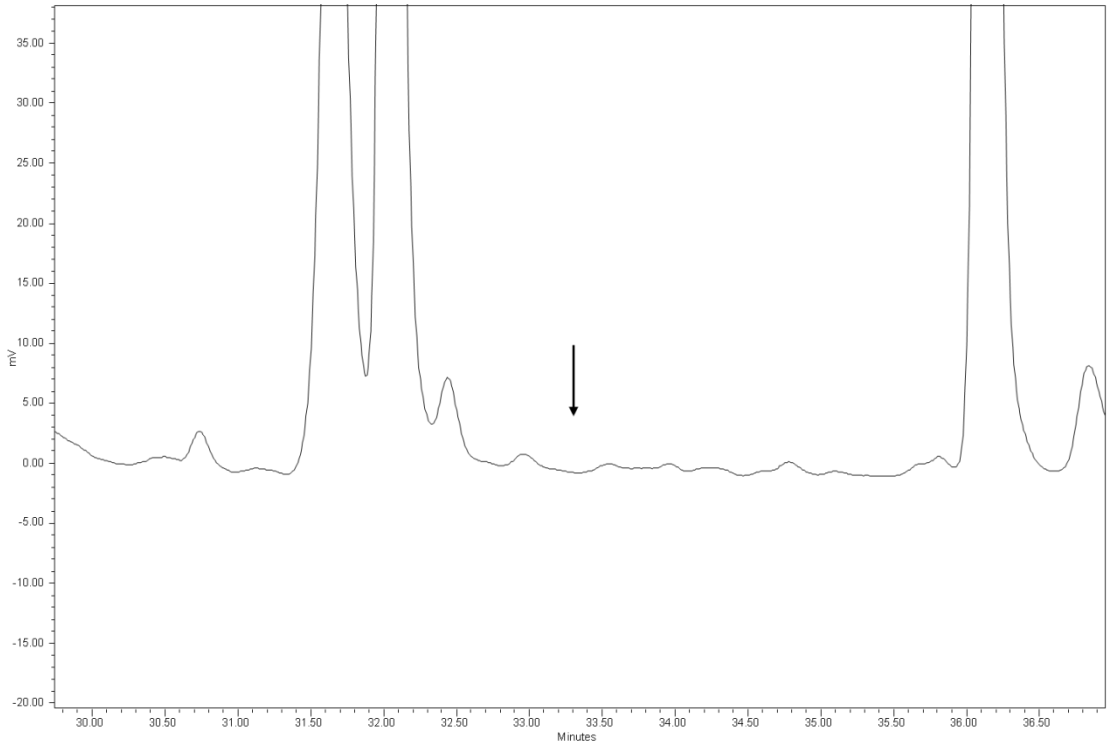
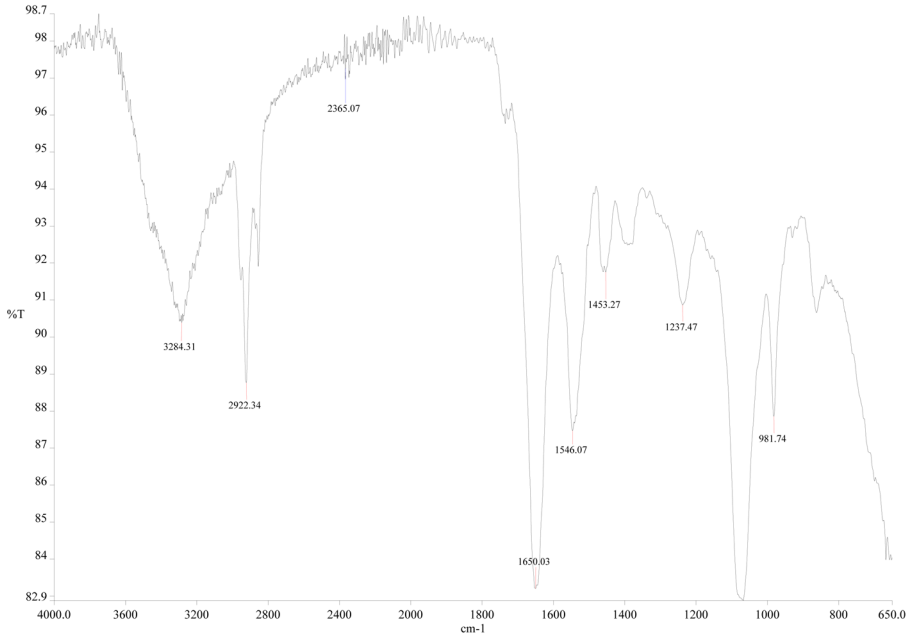


Figure S2. HPLC chromatogram of amino acid analysis on harvested ECM. HPLC chromatograms for (A) N_3-Pro-labelled ECM with (B) spiking of N_3-Pro reference substance (in red) and (C) non-labelled ECM. N_3-Pro was detected at a retention time of 33.5 min for labelled samples, whereas the chromatogram of unlabelled ECM does not exhibit the expected peak. The shift towards a lower retention time (0.5 min) in unlabelled ECM (C) must be noted.

Supporting Figure 3

A



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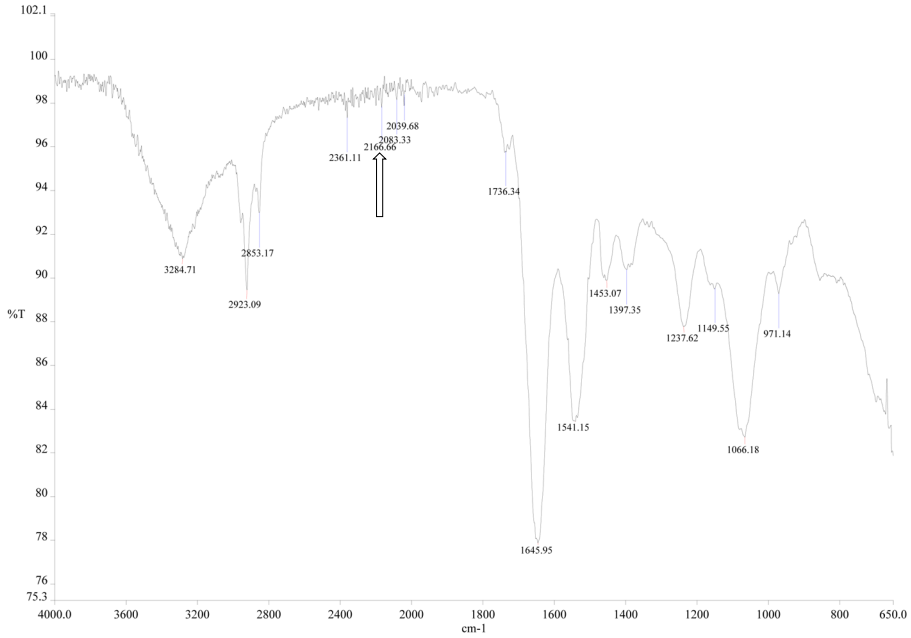

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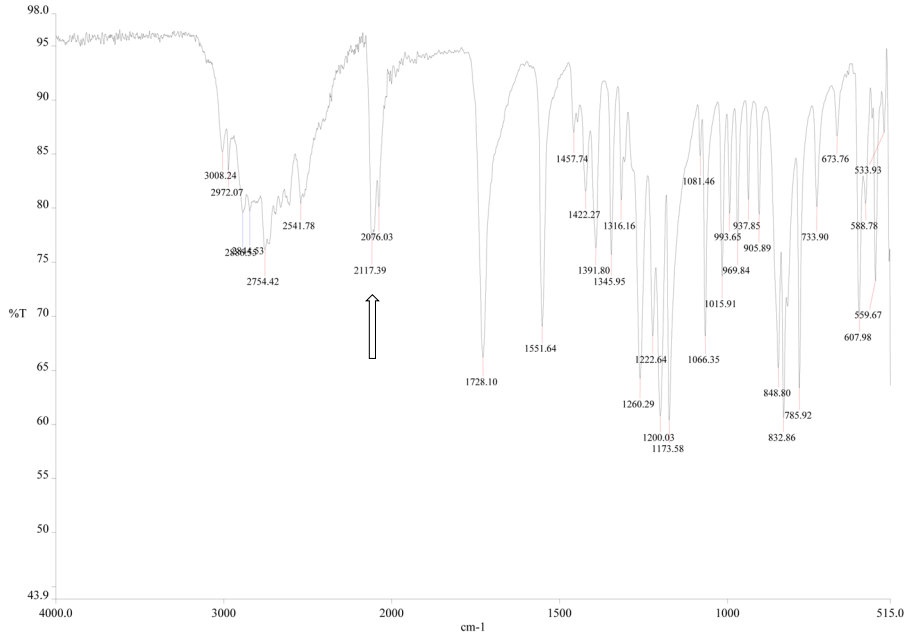


Figure S3. Infrared spectra on harvested ECM: Infrared spectra of ECM grown in the absence (A) or presence (B) of 36 mg/L N_3-Pro. (C) Reference spectrum for N_3-Pro. Data obtained with a Spectrum One FT-IR Spectrometer (Perkin Elmer) with 100 scans per sample. The arrow indicates the frequency region (2160 - 2120 cm^{-1}) corresponding to the azide stretching.

Supporting Figure 4

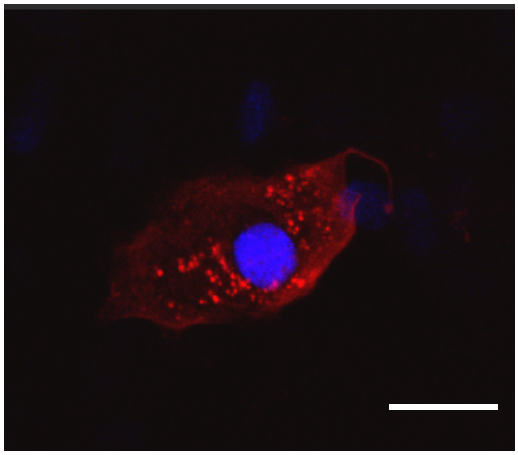


Figure S4. Confocal microscopy of labelled foetal ovine osteoblats. Staining with 5 μM DIBO-Alexa Fluor 555 and counterstaining with Hoechst 33342. The staining shows a dotted structure which is likely to be non-secreted pro-collagen. Scale bar represents 20 μm.

Supporting Figure 5

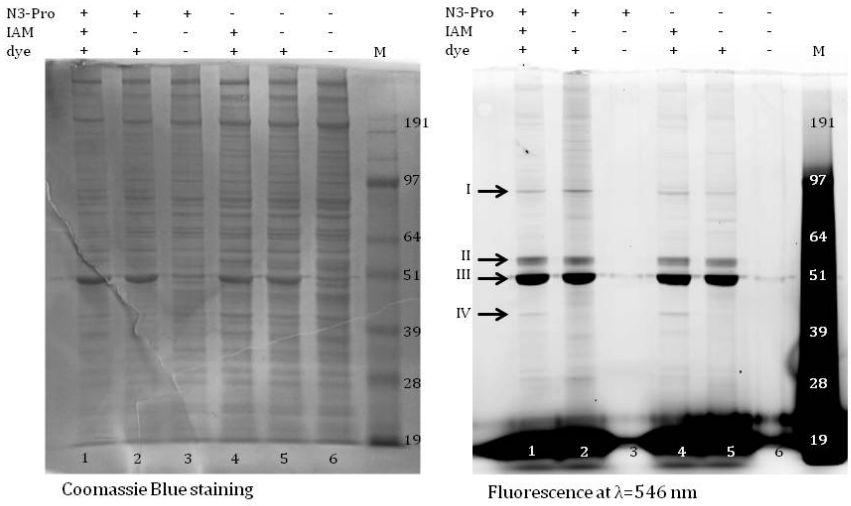
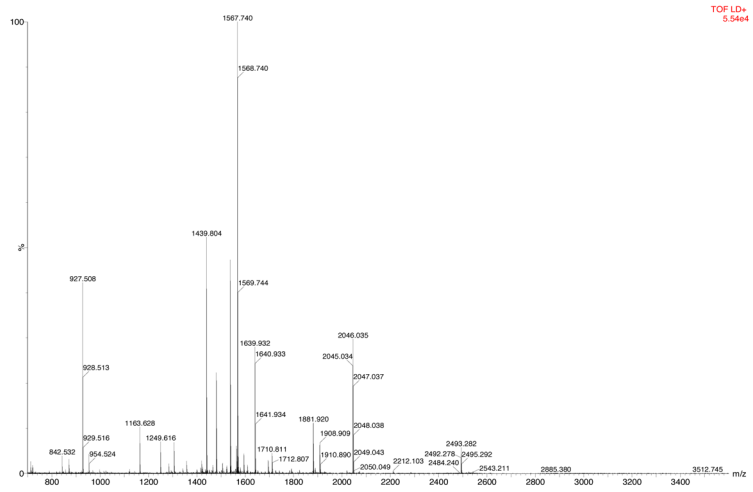


Figure S5. In-gel fluorescence: Sodium dodecyl sulphate- Polyacrylamide gel electrophoresis of foetal ovine osteoblast cell lysates grown in presence of 36 mg/L N_3-Pro (lanes 1-3) or without (lanes 4-6). Samples were pre-incubated with 5 mM IAM in lane 1 and 4. Left: Coomassie Blue staining. Right: Fluorescence imaging with a Typhoon Trio imager (GE Healthcare) at wavelength 546 nm. Protein identification by mass spectrometry of bands III and IV showed staining of bovine serum albumin (BSA) and actin, respectively. The other two bands (I and II) are likely collagen IV and tubulin.

Supporting Figure 6

A



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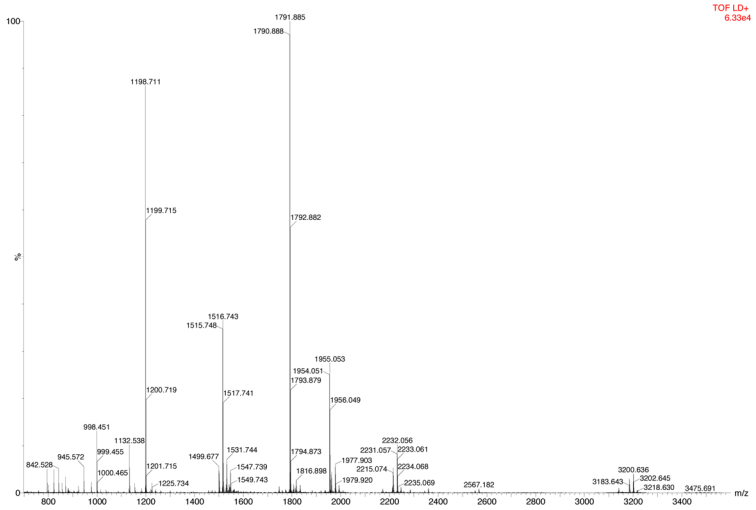


Figure S6. Protein identification using mass-spectrometry: (A) Mass spectrum of protein III. The gel band was excised and digested and mass spectrometry was carried out with MALDI ionisation by a micro MX mass spectrometer (Waters). Spectrum was afterwards edited: lockmass-calibrated and deisotoped. Subsequent Mascot search gave the highest score for ALB protein [bos taurus], also known as bovine serum albumin (BSA). (B) Mass spectra of protein IV. Protein identification procedure as described previously. The mascot search gave the highest score for actin of many species.

Supporting Figure 7

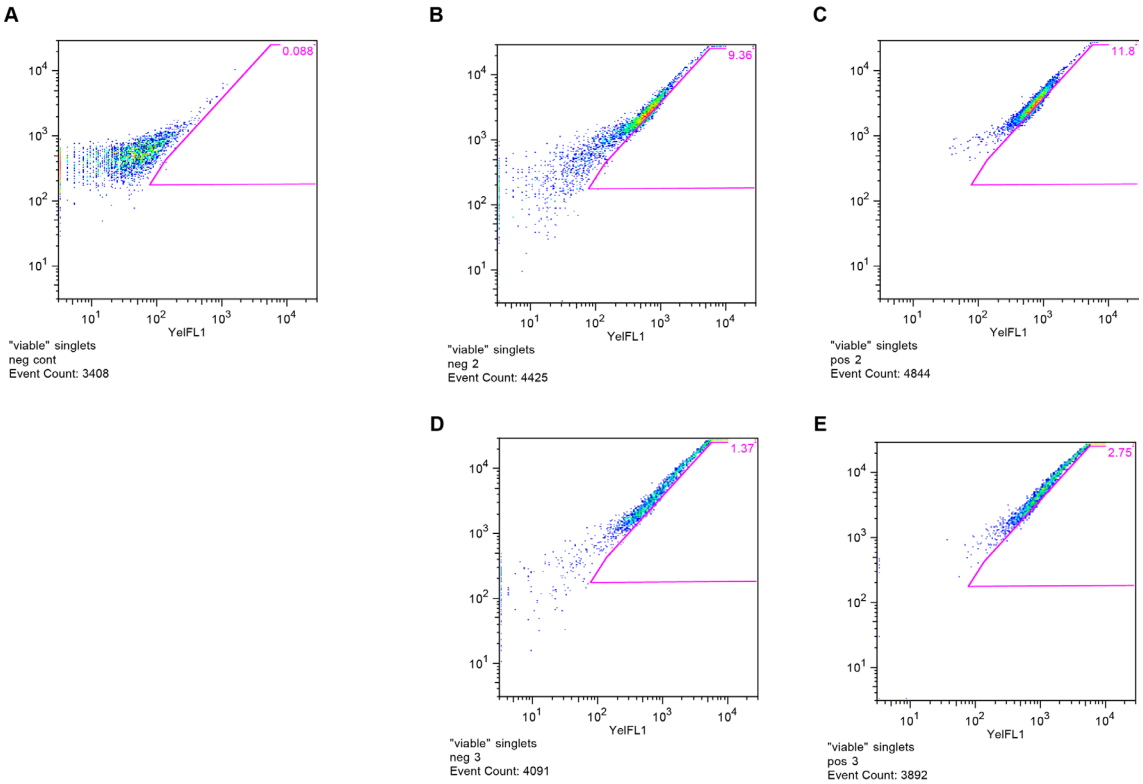
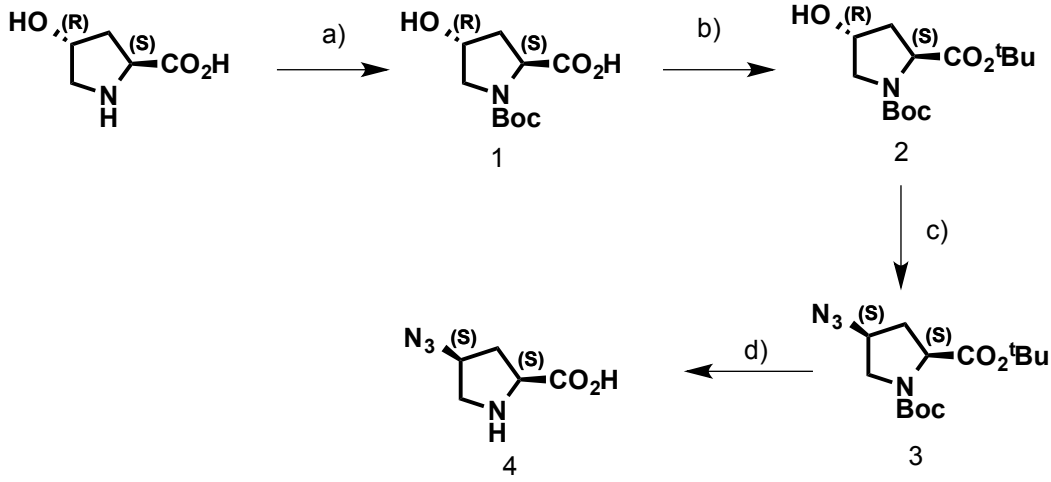


Figure S7. Assessment of the effect of pre-incubation of cells with iodoacetamide by flow cytometry analysis: (A) N_3-Pro negative cells; (B) Stained N_3-Pro negative cells incubated with 5 mM IAM; (C) Stained N_3-Pro positive cells incubated with 5 mM IAM; (D) Stained N_3-Pro negative cells; (E) Stained N_3-Pro positive cells.

2. Synthesis of *cis*-3-azido-L-proline[1]



a) Boc_2O, TEA, dioxane/H_2O/MeOH(1:2:3). b) DCC, ^tBuOH, CuCl, DCM. c) DPPA, DEAD, Ph_3P, THF. d) TFA/DCM (1:1).

N-Boc-*cis*-3-hydroxy-L-proline (1). Boc_2O (2.65 g, 12.13 mmol) was added in one portion to a stirred solution of *cis*-3-hydroxy-L-proline (1.59 g, 12.13 mmol) and TEA (2.54 mL, 18.19 mmol) in H_2O (15 mL), MeOH (23 mL), and dioxane (7.5 mL) at room temperature. The solvent was removed after 4 h on a rotary evaporator, and the mixture was resuspended in 25 mL of water and washed 3 times with toluene. The water layer was acidified with citric acid (10%) in water and extracted with ethyl acetate. The organic layer was dried and the solvent removed on the rotary evaporator. The compound was purified by flash chromatography (ethyl acetate:MeOH:AcOH, 10:1:0 to 10:1:0.05), giving 2.30 g (86%) of a white solid. This compound appears in the ^1H NMR spectrum as a distinct pair of rotomers. ^1H NMR (400 MHz, MeOD) δ: 1.45 and 1.48 (2s, 9H, $C(CH_3)_3$), 2.08 (m, 1H, $1H_\beta$), 2.29 (m, 1H, $1H_\beta$), 3.45 (m, 2H, $2H_\delta$), 4.33 (m, 1H, $1H_\alpha$), 4.41 (m, 1H, $1H_\gamma$). ^{13}C NMR (100 MHz, MeOD) δ: 27.1 ($C(\underline{C}H_3)_3$), 38.7 (C_β), 54.1 (C_δ), 58.0 (C_α), 68.6 (C_γ), 80.3 ($\underline{C}(CH_3)_3$), 154.6 (CO), 175.4 (CO). **HRMS (ESI+) m/z:** calcd. for $C_{10}H_{17}NO_5$ $[M+Na]^+$: 254.0999 found: 254.0992.

N-Boc-*trans*-3-hydroxy-L-proline *tert*-butyl ester (2). *N,N′*-Dicyclohexylcarbodiimide (6.63 g, 31.83 mmol), *tert*-butyl alcohol (3.02 mL, 40.78 mmol) and CuCl (98.4 mg, 0.99 mmol) were stirred for 5 days at room temperature. DCM (60 mL) and N-Boc-*cis*-3-hydroxy-L-proline (1) (2.30 g, 9.95 mmol) were added followed by continued stirring for 4 h. The solution was first filtered through a pad of

Celite and then washed with H_2O, dried over Na_2SO_4, filtered and concentrated. The residue was purified by flash column chromatography (hexane:ethyl acetate, 1:1) to afford N-Boc-*trans*-3-hydroxy-L-proline *tert*-butyl ester (2), (2.1 g, 70%) as a white solid. This compound appears in the ^{1}H NMR spectrum as a distinct pair of rotomers.^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.43-1.50 (m, 18H, $C(CH_3)_3$), 1.99-1.12 (m, 1H, $1H_\beta$), 1.20-1.38 (m, 1H, $1H_\beta$), 3.39-3.66 (m, 2H, $2H_\delta$), 4.23-4.36 (m, 1H, $1H_\alpha$), 4.46-4.53 (m, 1H, $1H_\gamma$). ^{13}C NMR (100 MHz, $CDCl_3$)) δ: 28.0 ($C(\underline{C}H_3)_3$), 28.3 ($C(\underline{C}H_3)_3$), 39.1 (C_β), 54.6 (C_δ), 58.5 (C_α), 69.3 (C_γ), 80.1 ($\underline{C}(CH_3)_3$),81.2 ($\underline{C}(CH_3)_3$), 154.2 (CO), 172.1 (CO). **HRMS (ESI+) m/z:** calcd. for $C_{14}H_{25}NO_5$ $[M+H]^+$: 288.1794 found: 288.1805.

N-Boc-*cis*-3-azido-L-proline *tert*-butyl ester (3). DEAD (1,38 mL, 9.00 mmol) was slowly added dropwise via syringe to an ice-cooled stirred solution of **2** (2.10 g, 6.92 mmol) and Ph_3P (2.18 g, 8.31 mmol) in dry THF (30 mL) under nitrogen. DPPA (1.6 mL, 8.31 mmol) was added dropwise via syringe to the solution, and the temperature was allowed to rise to room temperature. After 24 h the solvent was removed by rotary evaporation. The compound was purified by flash chromatography (ethyl acetate:hexane, 1:5) to obtain 1.20 g (52%) of a white solid. This compound appears in the ^{1}H NMR spectrum as a distinct pair of rotomers. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.47 (m, 18H, $C(CH_3)3$), 2.04-2.23 (m, 1H, $1H_\beta$), 2.33-2.55 (m, 1H, $1H_\beta$), 3.37-3.53 (m, 1H, $1H_\delta$), 3.63-3.77 (m, 1H, $1H_\delta$), 4.08-4.19 (m, 1H, $1H_\gamma$), 4.19-4.34 (m, 1H, $1H_\alpha$). ^{13}C NMR (100 MH, D_2O) δ: 27.9 ($C(\underline{C}H_3)_3$), 28.3 ($C(\underline{C}H_3)_3$), 36.1 (C_β), 50.8 (C_δ), 58.1, 58.3 (C_α, C_γ), 80.2 ($\underline{C}(CH_3)_3$), 81.5 ($\underline{C}(CH_3)_3$), 153.6 (CO), 170.7 (CO). **HRMS (ESI+) m/z:** calcd. for $C_{14}H_{24}N_4O_4$ $[M+H]^+$: 313.1861 found: 313.1870.

***cis*-3-azido-L-proline (4)**. The compound **3** (1.00 g, 3.05 mmol) was dissolved in a mixture of trifluoroacetic acid (TFA) and DCM (1:1). After stirring the solution at 25 ºC for 2 h, the solvent was evaporated and the crude peptide was purified using a C18 reverse-phase cartridge to give 0.49 g (93%) of a white solid compound. ^{1}H NMR (400 MHz, D_2O) δ: 2.37-2.47 (m, 1H, $1H_\beta$), 2.50-2.61 (m, 1H, $1H_\beta$), 3.40-3.51 (m, 2H, $2H_\delta$), 4.42 (dd, J = 10.1, 3.4 Hz, 1H, $1H_\alpha$), 4.19-4.34 (m, 1H, $1H_\gamma$). ^{13}C NMR (100 MHz, D_2O) δ: 34.1 (C_β), 50.8 (C_δ), 58.8, 59.3 (C_α, C_γ), 172.1 (CO). **HRMS (ESI+) m/z:** calcd. for $C_5H_8N_4O_2$ $[M+H]^+$: 157.0719 found: 157.0720.

3. Methods

3.1 Cell isolation and maintenance

Foetal ovine osteoblats were isolated as described previously.[2] Foetal ovine osteoblasts were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% (v/v) foetal calf serum, 30 μg/mL L-ascorbic acid 2-phosphate, 0.29 mg/mL glutamine and antibiotics (100 IU/mL penicillin, 0.1 mg/mL streptomycin). Cells were maintained in a 37 ºC, 5% CO_2 humidified incubator. Media was changed every 2-3 days. Near-confluent cells were passaged with 0.05% trypsin.

3.2 Cell cytotoxicity assay

Cells were cultured in a 24-well plate with a seeding density of 7500 cells/well until confluency. Further growth was either in presence or absence of N_3-Pro (18 and 36 mg/L). Cell viability was determined using alamarBlue® assay (Life Technologies) according to the manufacturer's instructions.

3.3 Amino acid analysis on harvested ECM

The extracellular structure of cells grown in the presence and absence of azidoproline (36 mg/L) was harvested according to a protocol described previously.[2] Amino acid analysis followed the protocol developed by Spackman and co-workers.[3]

3.4 Infrared on harvested and dried ECM

The extracellular structure of cells grown in the presence and absence of N_3-Pro (36 mg/L) was harvested according to a protocol described previously.[2] After removal of the supernatant, samples were then frozen in liquid nitrogen and dried over night. Next day, infrared spectroscopy was carried out on the dry structure with a Spectrum One FT-IR Spectrometer (Perkin Elmer).

3.5 Fluorescence imaging of fixed cells

Cells were grown on glass coverslips in a 24 well plate until half-confluency. Further growth was in presence or absence of N_3-Pro (36 mg/L). After 3 days of N_3-Pro incubation, cells were washed two times with D-PBS (Life Technologies) containing 1% FCS. For staining, cell cultures were first fixed with 4 % formaldehyde in PBS (Life Technologies) for 15 min and then labelled for 60 min at room temperature with 5 μL of

DIBO-Alexa Fluor 555 in D-PBS containing 1 % FCS. They were washed 4 times (5 min each wash) with D-PBS containing 1 % FCS. Counterstaining with Hoechst 33342 (Life Technologies) in D-PBS was applied for 15 min, followed by 2 washing steps with D-PBS. DIBO-Alexa Fluor 555 has a peak excitation at 555 nm and a peak emission at 565 nm. Hoechst 33342 has a peak excitation at 350 nm and a peak emission at 461 nm. Imaging was carried out with an Olympus IX-71 inverted microscope, with the appropriate filters.

3.6 Flow cytometry

Cells were grown in a T75 flask (Greiner) for 3 days in the presence or absence of N_3-Pro (36 mg/L) and suspended by trypsinisation. Cell number was adjusted to 10^6 cells/mL per sample. Cells were pre-incubated for 60 min with IAM and then stained with 5 μM DIBO-Alexa Fluor 555 in PBS containing 1% FCS for 60 min. Cells were then washed two times with PBS. Fluorescent intensity was measured with a Cytek DxP8 cell analyser at excitation wavelenght 568 nm and detection at 613 nm.

3.7 In-gel fluorescence

Cells were cultured in a T-75 flask (Greiner) to 80 % confluency. They were harvested by trypsinisation and spinned down at 1200 rpm for 5 min. Resuspension in 500 μL lysis buffer (150 mM NaCl, 1 % Triton-X 100, 50 mM Tris HCl pH 8.0; containing 2 μg/mL Aprotinin, 5 μg/mL Leupeuptin, 1 mM PMSF, 5 mM EDTA for protease inhibition) and subsequent agitation for 30 min resulted in cell lysis. Cell debris was spinned down at 12'000 rpm for 20 min and the supernatant was collected and stored in 5 fractions of 100 μL at -20 °C. Fractions were either treated with or without 5 mM IAM prior to 5 μM DIBO-Alexa Fluor 555 for 60 min each. A negative control without Alexa Fluor was included. SDS-PAGE was carried out a with 4-12 % Tris-Bis gels (Life Technologies) in a MOPS running buffer (Life Technologies) (200 V for 55 min). Before staining with Coomassie Blue, the gel was imaged with a Typhoon Trio imager (GE Healthcare) at an excitation wavelength of 546 nm.

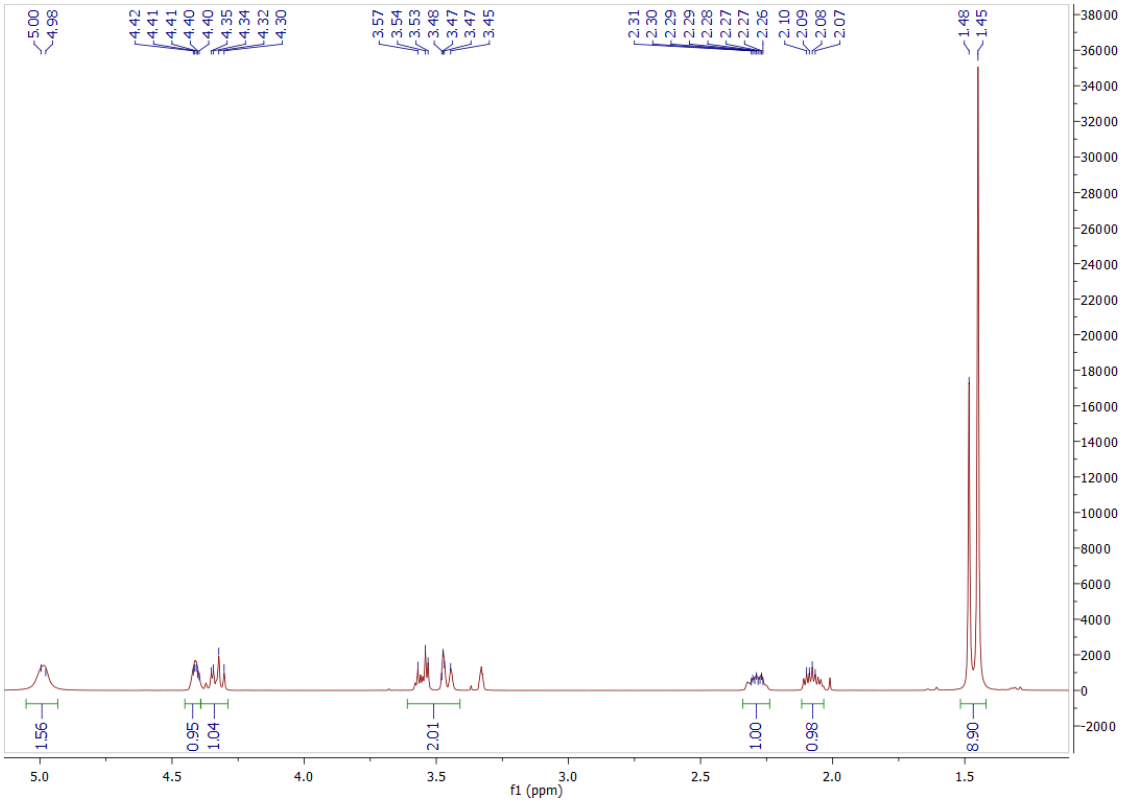
3.8 Electron microscopy

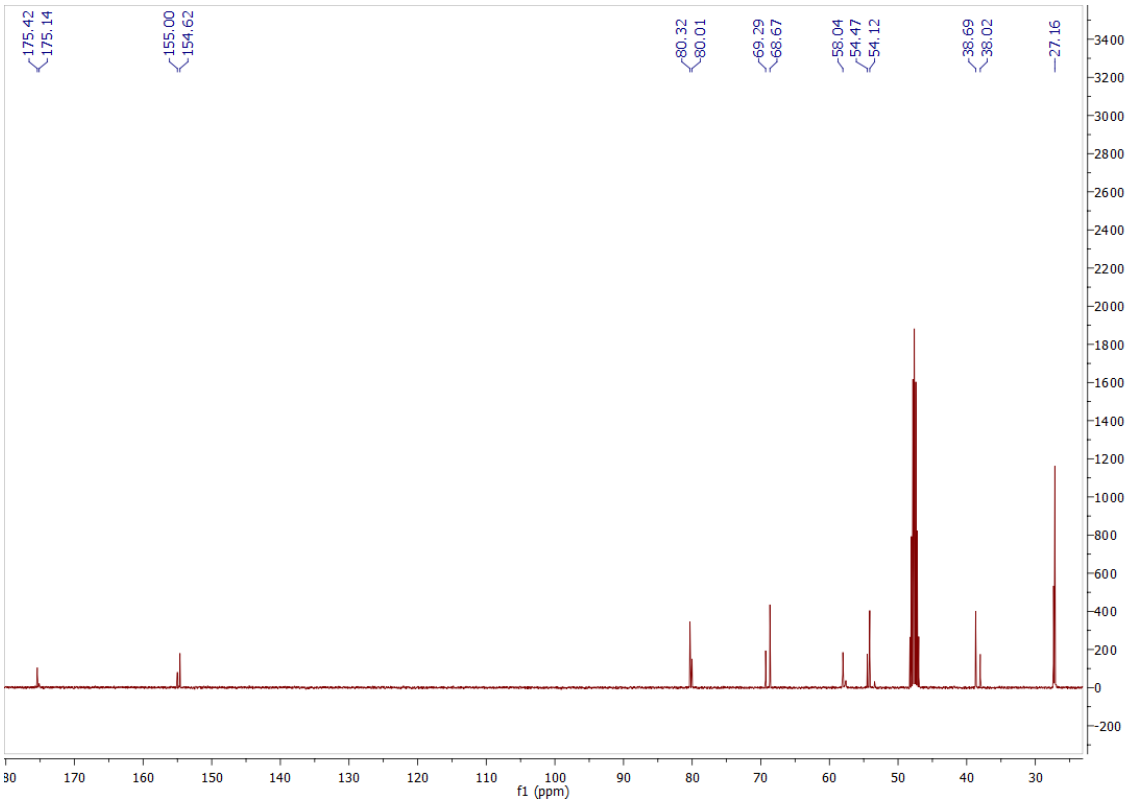
Adherent cells on 10mm diameter cover-slips were fixed in 2% glutaraldehyde in 0.1 M piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES) buffer at pH 7.4 for 18 hours at 4 °C. They were rinsed in deionized water (DIW) and treated with 1% osmium

ferricyanide at 4 °C for 18 hours. They were rinsed in DIW and treated with 2% uranyl acetate in 0.05M maleate buffer at pH 5.5 for 18 hours at 4 °C. They were again rinsed in DIW and dehydrated in an ascending series of ethanol solutions from 70% to 100%. The coverslips were placed in a Polaron critical point dryer (Quorum/Emitech, UK) where the ethanol was replaced with liquid CO_2 which was heated to 37 °C where it changed state to its gaseous phase. The CO_2 was vented and the coverslips were glued to SEM stubs with colloidal silver. The stubs were coated with 10 nm of gold in a Quorum/Emitech K575X sputter coater. They were viewed in an FEL-Philips XL30 FEGSEM at 5 kV.

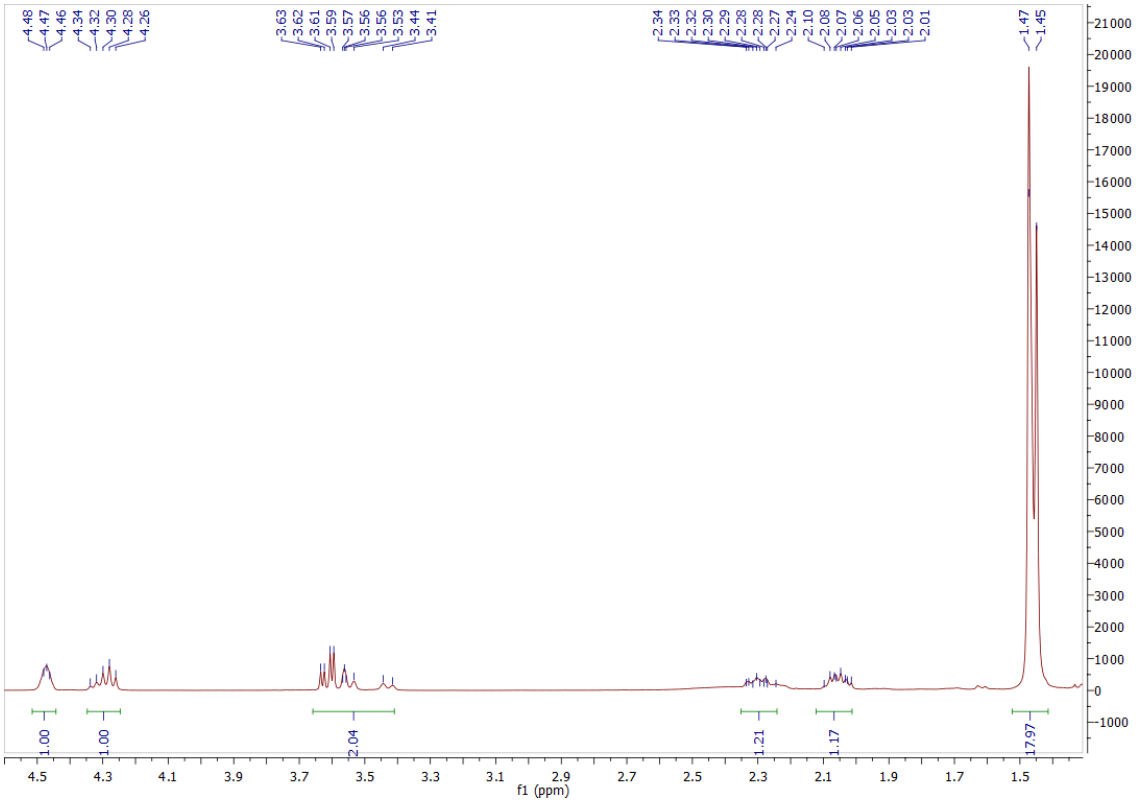
4. ^{1}H- and ^{13}C-NMR spectra

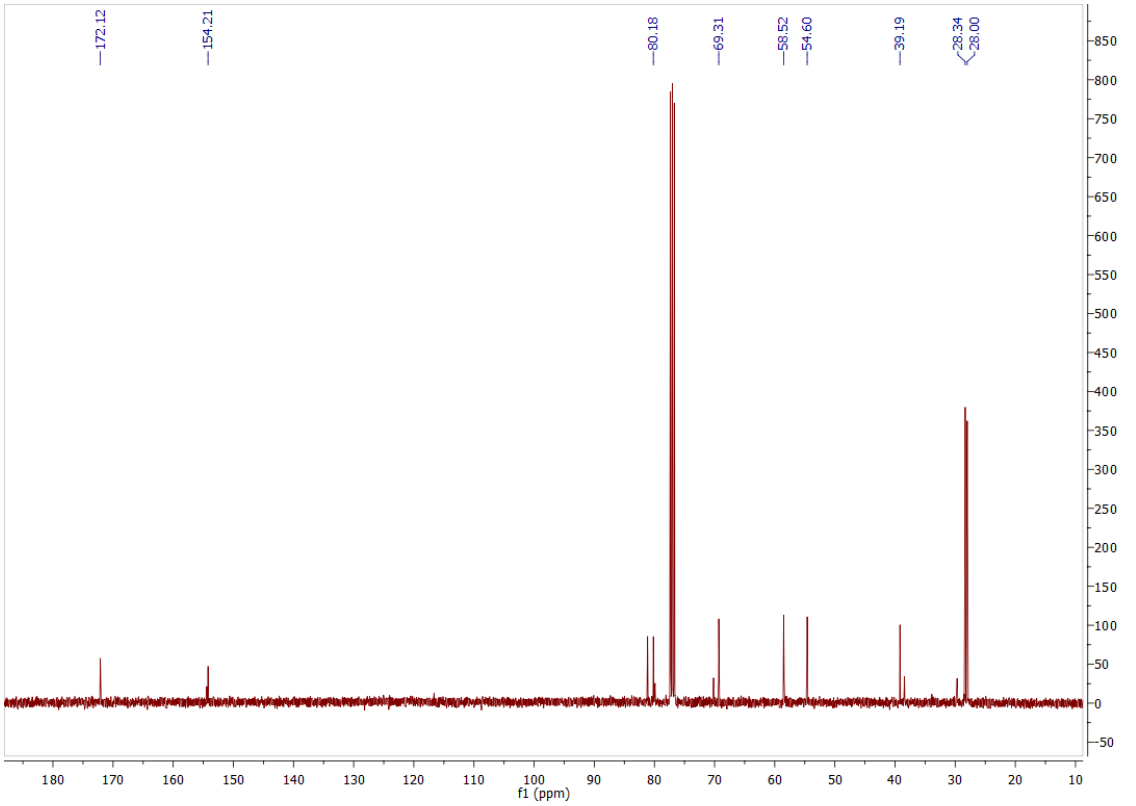
N-Boc-*cis*-3-hydroxy-L-proline (1).



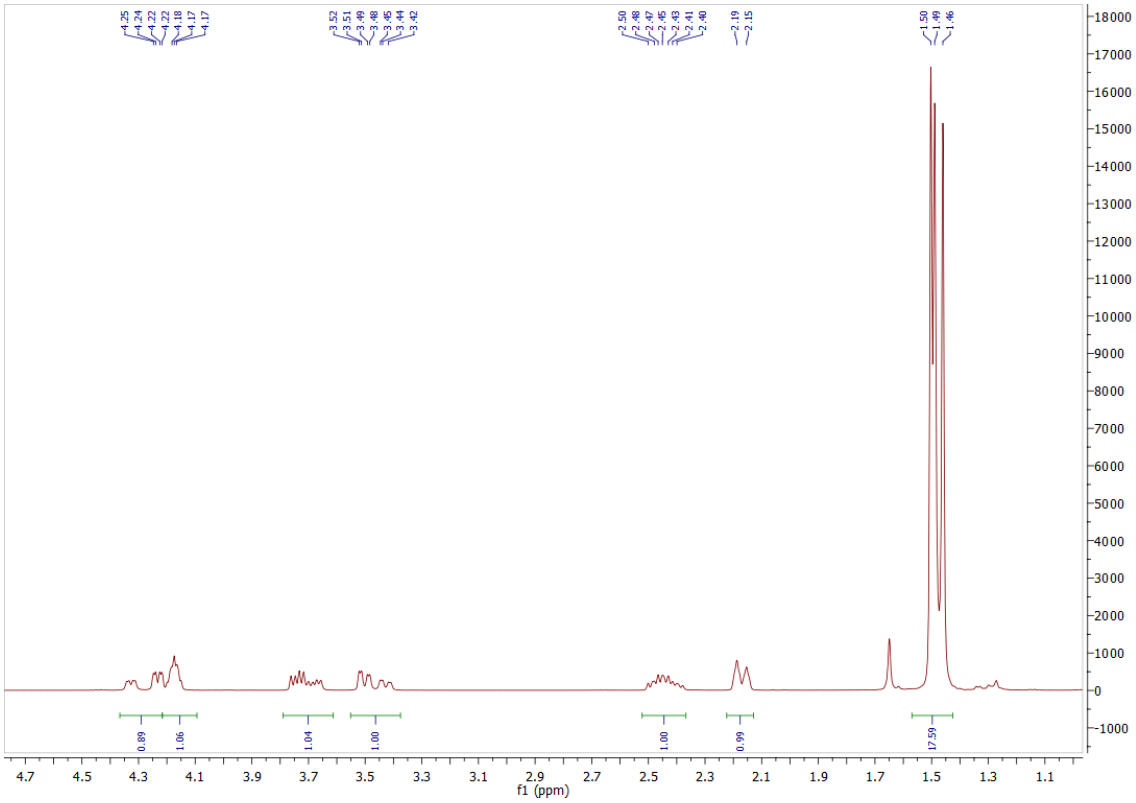


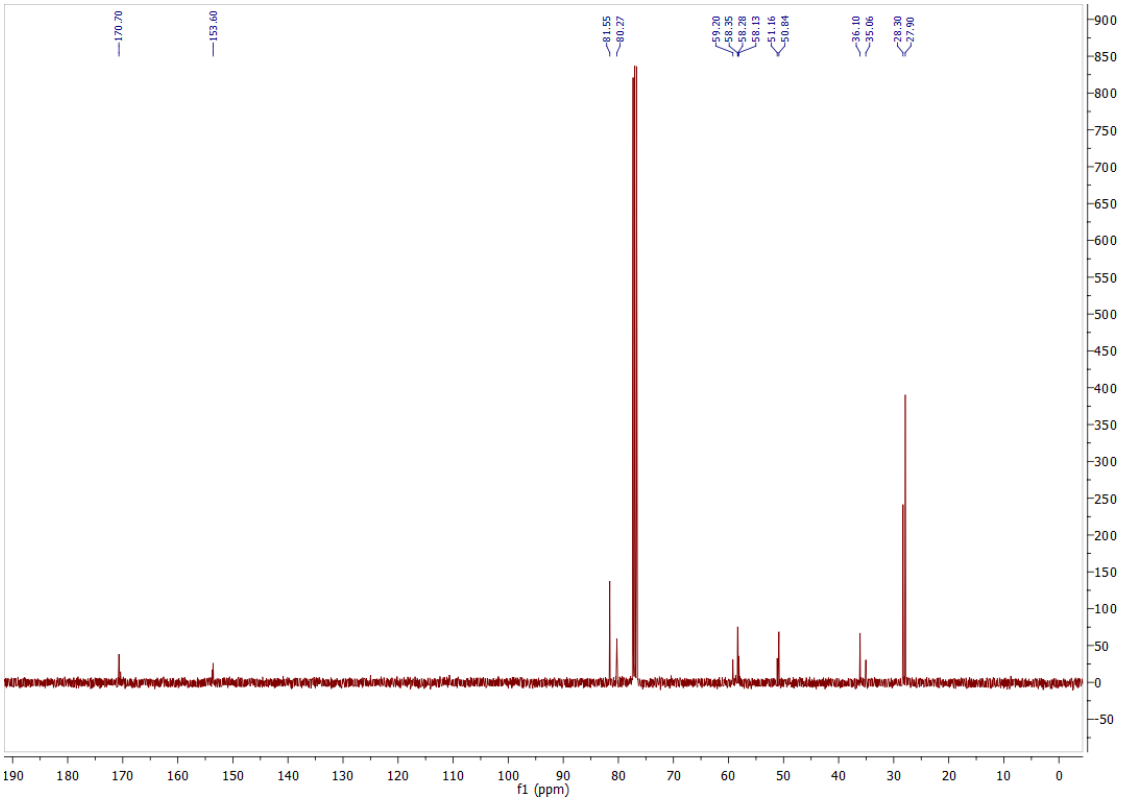
N-Boc-*trans*-3-hydroxy-L-proline tert-butyl ester (2).



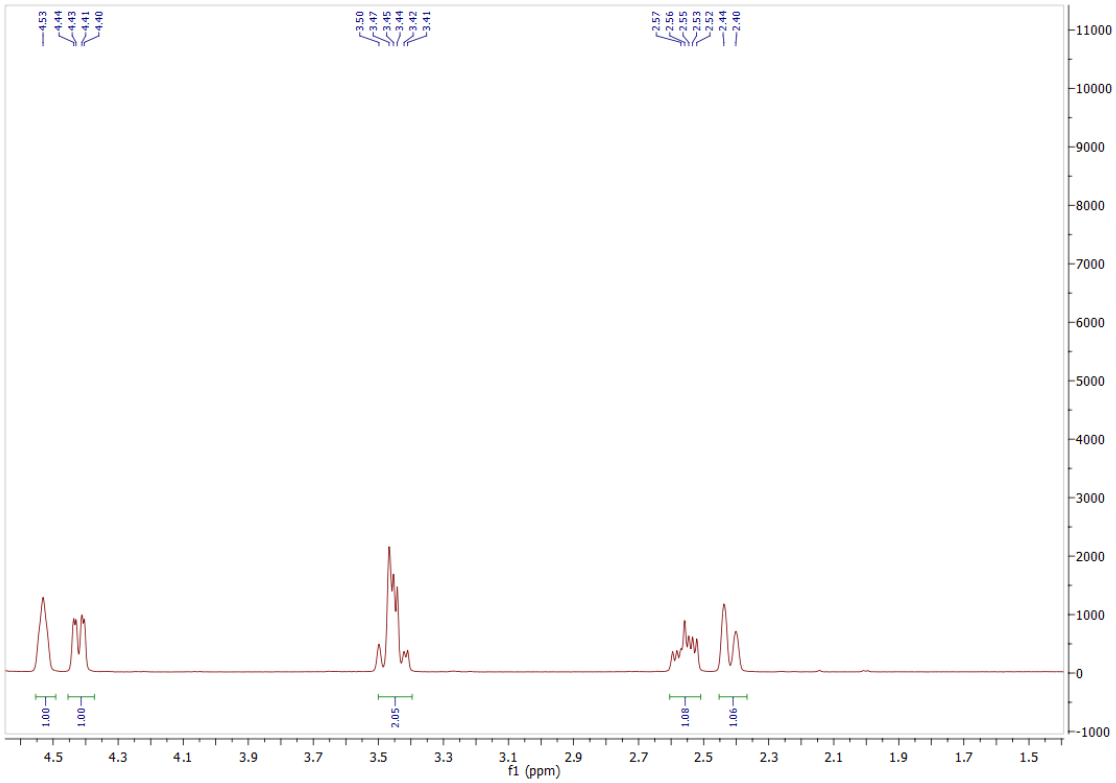


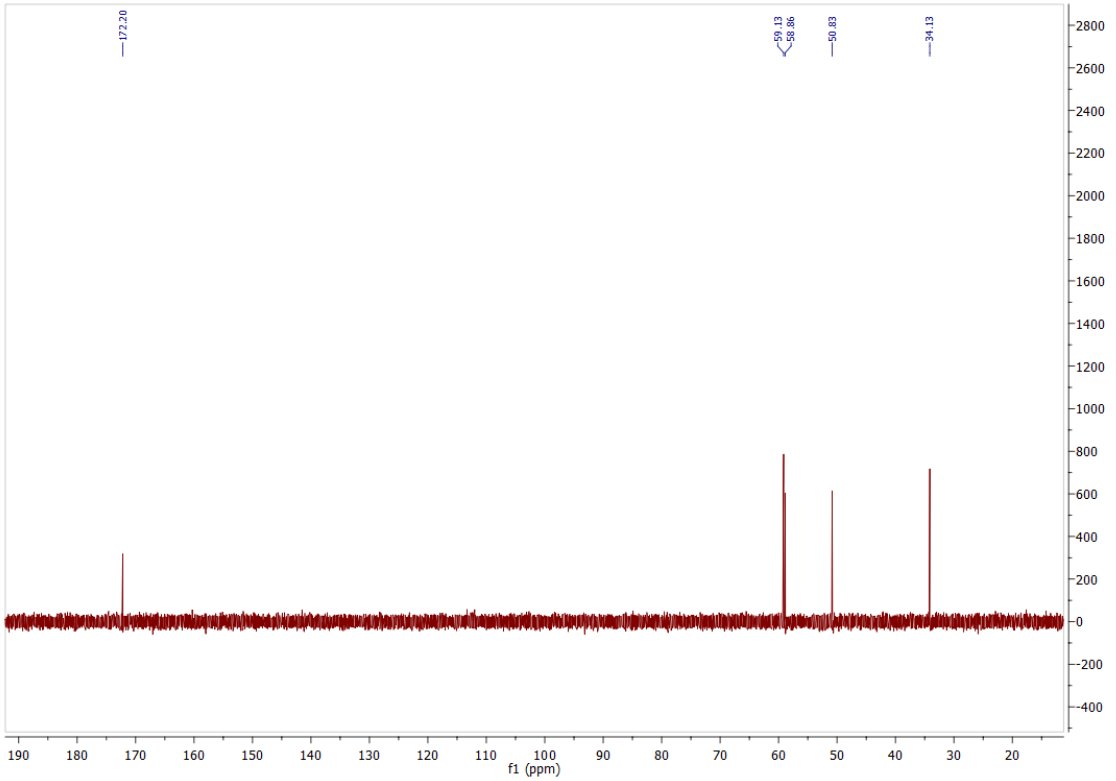
N-Boc-*cis*-3-azido-L-proline tert-butyl ester (3).





***cis*-3-azido-L-proline (4)**.





5. References

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2. W. Y. Chow, R. Rajan, K. H. Muller, D. G. Reid, J. N. Skepper, W. C. Wong, R. A. Brooks, M. Green, D. Bihan, R. W. Farndale, D. A. Slatter, C. M. Shanahan and M. J. Duer, *Science*, 2014, **344**, 742-746.
3. D. H. Spackman, W. H. Stein and S. Moore, *Anal. Chem.*, 1958, **30**, 1190.