Supporting Information:

Fluoromethylated derivatives of carnitine biosynthesis intermediates – synthesis and applications

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General experimental

Enzyme production

Human BBOX was prepared as described[1]. *Pseudomonas sp* AK1 BBOX (for sequence see Fig **S7**) was cloned into *E. coli* expression vector under IPTG inducible promoter. Expression was performed in *E. coli* BL21 (DE3) cells. Further details of construct preparation (utilizing pCOLDI plasmid) and purification of the *Pseudomonas sp* AK1 BBOX will be reported elsewhere.

Cell lysate assays

E. coli BL21 (DE3) cells were transformed with pCOLD I plasmid bearing the psBBOX gene. 100 mL cultures were grown in 2TY media containing ampicillin as a selection marker for 4 h in 37°C until the OD_{600} reached 0.5 and then induced with 0.2 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) and grown overnight (15°C). Cells were pelleted by centrifuging 7000 rpm for 10 min at 4°C. The precipitate was resuspended in 5 times weight of binding buffer (50 mM HEPES pH 7.6, 0.5 M NaCl, 5 mM imidazole) and lysed by sonication. The lysates were centrifuged and the supernatant used in further experiments. SDS-PAGE molecular weight marker was from Thermo Scientific (PageRuler Prestained Protein Ladder 10-170 kDa). NMR samples were prepared by addition of 10% 50 mM Tris-d_{11} D_2O pH 7.5, 10% 50 mM Tris-d_{11} H_2O pH 7.5, 0.5 mM trifluoroacetic acid as a chemical shift reference, 1 mM fluoromethyl GBB and 3 mM 2OG.

HEK 293T cell experiments

HEK 293T cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Lonza/ BioWhittaker) with a Penicillin G/ Streptomycin antibiotic mixture (Sigma), 2% GlutaMAX (Gibco) and 10% FBS (PAA) in 10 cm sterile Petri dishes at 37°C, in presence of 5% CO_2. Cell cultures were incubated in media supplemented with TML or TMLNF at concentrations of 50 μM, or without compounds in case of a control cultures for 48 h. Media was discarded and cells were washed 3 times with PBS (5 mL), harvested and centrifuged (5 min, 1000 rpm). Cells were re-suspended in PBS, lysed in a sonication water bath and spun down (10 min, 14800 rpm). Supernatants were collected and transferred to MS vials for measurement.

Chromatography was performed on Aquity UPLC system (Waters). Column: PrimeSep 200 mixed mode, 2.1×250mm, particles 5μm (SIELC, Prospect Heights, US). Mobile phase: Solvent A – 9:1 H_2O-acetonitrile mixture, 0.05% formic acid, solvent B – 8:2 H_2O-acetonitrile mixture, 0.2% formic acid. Gradient: Linear gradient from 0% to 100% B in 25 min, column reconditioning: 25-26 min from 0% to 100% A, 26-30 min 100% A. Flow rate: 0.3 mL/min. Detection was performed using a Waters Quattro Micro instrument (triple quadrupole MS, electrospray ionisation). Single ion mode was used and scan mode was running as a control.

NMR assays

Reagents were from Sigma-Aldrich (Dorset, UK). 2OG was used as its disodium salt, ascorbate as monosodium salt, Fe(II) was in form of $(NH_4)_2Fe(SO_4)_2$ salt. GBBF was synthesized as described[2].

Tris-d_{11} was obtained from Cambridge Isotope Laboratories. NMR spectra were recorded using Bruker AVIII 700 with inverse TCI cryoprobe and Bruker AVII 500 machines.

^{1}H NMR hydroxylation/ succinate formation assays contained: GBB analogue 100 µM, 2OG 500 µM, ascorbate 500 µM, KCl 200 mM, Fe(II) 50 µM, enzyme 400 nM, buffer: Tris-d_{11} 50mM pH 7.5 in H_2O, 10% D_2O. Reactions were performed in a final volume of 160 µL, initiated by addition of enzyme, and then followed by ^{1}H NMR in real time. ^{1}H NMR K_M measurements were done using analogous conditions but with saturating concentration of 2OG (1 mM).

^{19}F NMR inhibition assays used the following conditions: GBBNF 150 µM, 2OG 100 µM, ascorbate 500 µM, KCl 80 mM, Fe(II) 40 µM, enzyme 25 nM, buffer: Tris-d_{11} 50mM pH 7.5 in H_2O. Samples were prepared in final volume of 0.4 mL. Reaction was initiated by addition of hBBOX and quenched after 10 min by addition of 0.1 mL of CD_3CN. Curves were fitted using GraphPad Prism 5.04 Software.

Figures

GBBNF (9) conversion to CARNF (14) can be observed by ^{1}H NMR

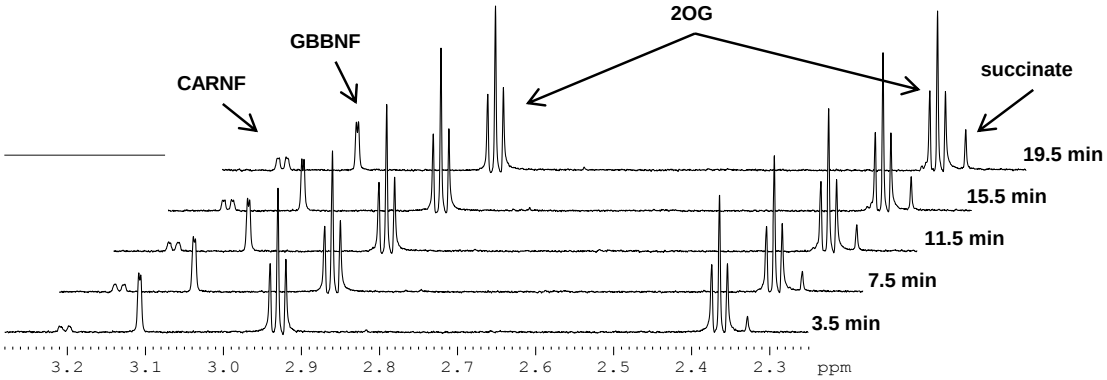

Figure S1 ^{1}H NMR spectra of GBBNF turnover to CARNF by BBOX – time course experiment. Reaction conditions: 400 nM hBBOX, 50 µM Fe(II), 100 µM GBBNF, 500 µM 2OG, 500 µM ascorbate, 200 mM KCl in 50 mM Tris-d_{11} buffer at pH 7.5.

GBBNF (9) reaction product assignment

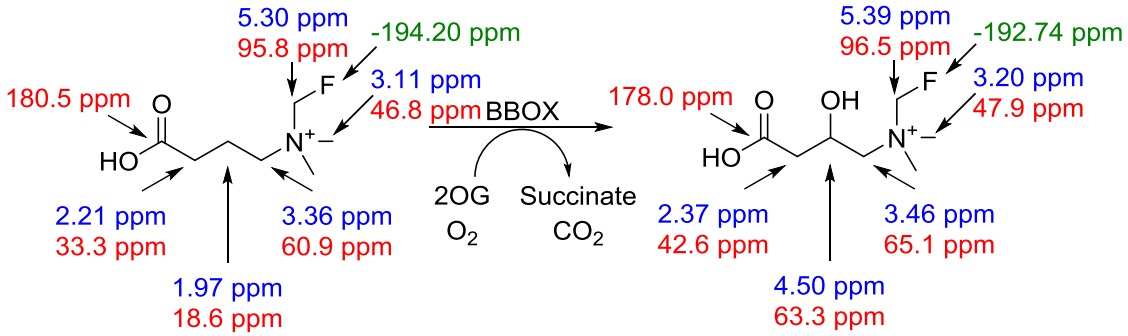

Figure S2 ^{1}H NMR (blue), ^{13}C NMR (red) and ^{19}F NMR (green) assignments of substrate (GBBNF, (**9**)) and product (CARNF, (**10**)) of BBOX catalysed reaction at 280K.

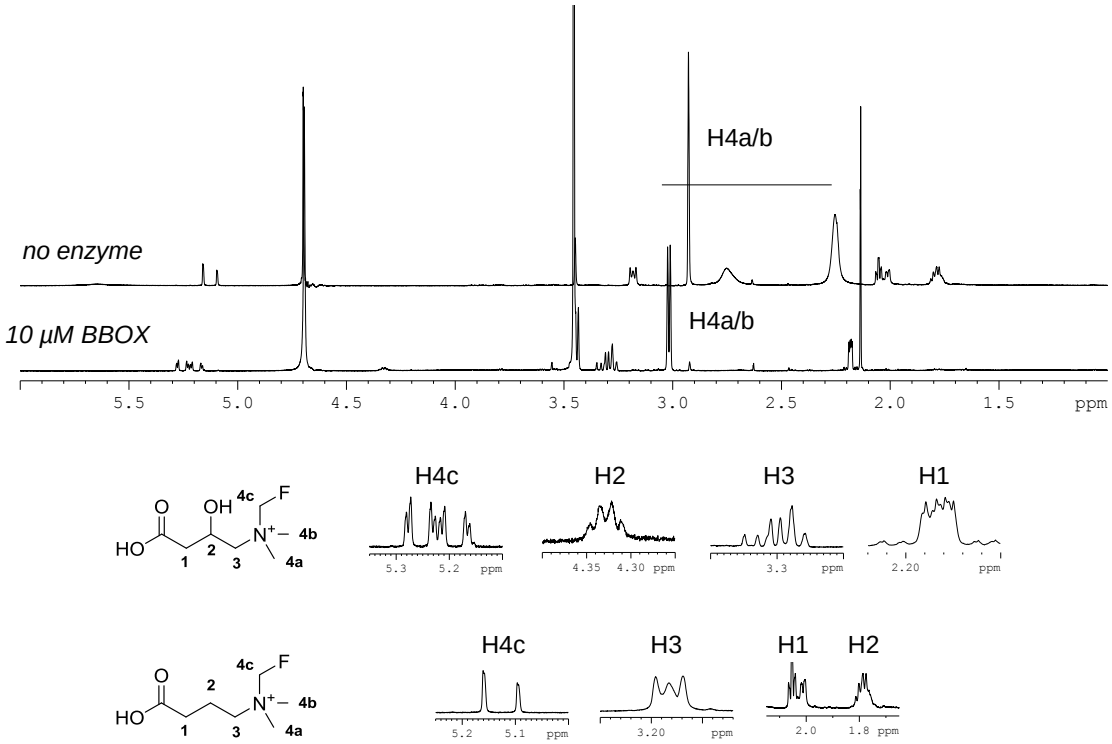

Figure S3 ^{1}H NMR spectra of BBOX catalysed GBBNF (9) hydroxylation (bottom) and control reaction with no enzyme added (top).

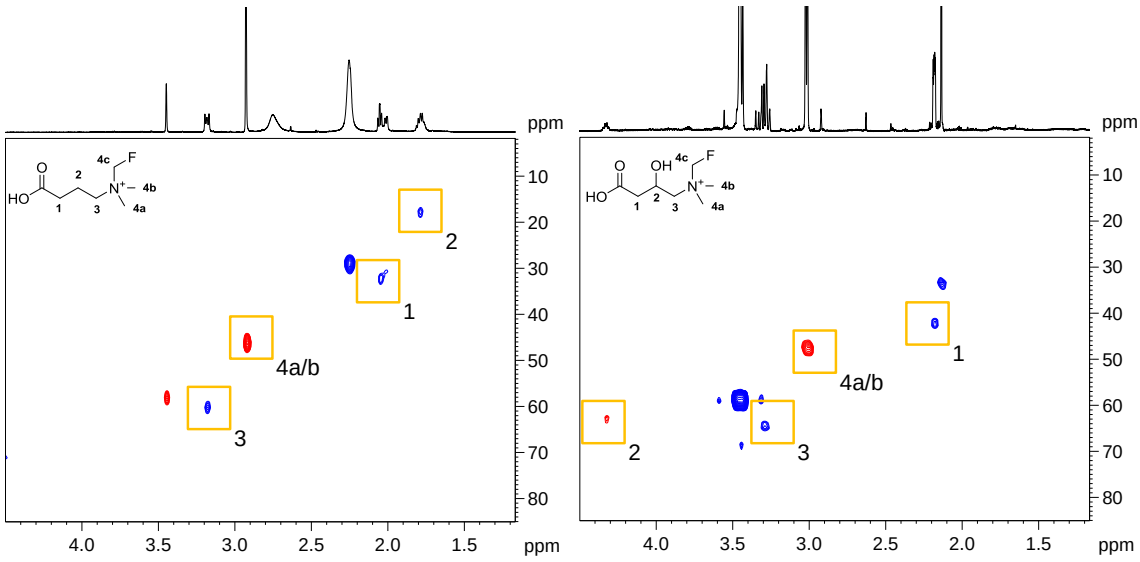

Figure S4A ^{1}H-^{13}C HSQC spectra of BBOX catalysed GBBNF (9) hydroxylation (left) and control with no BBOX added (right).

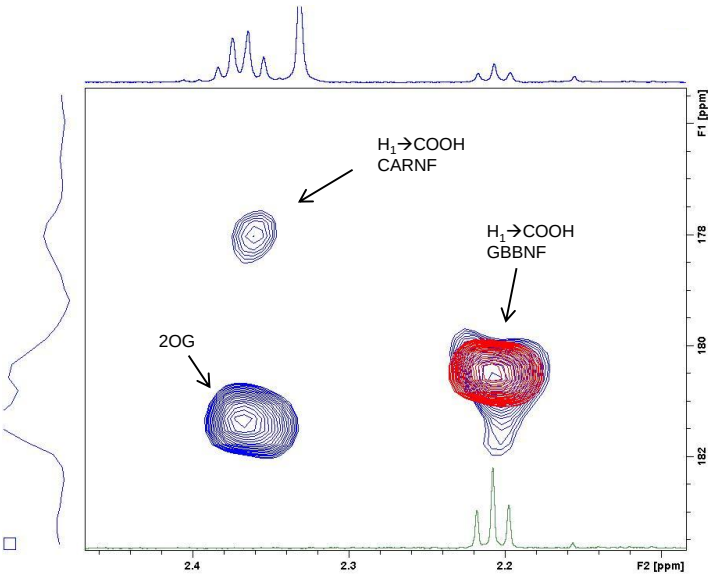

Figure S4B Overlay of HMBC spectra of GBBNF (9) (red) and a reaction mixture of BBOX catalysed GBBNF (**9**) hydroxylation (blue).

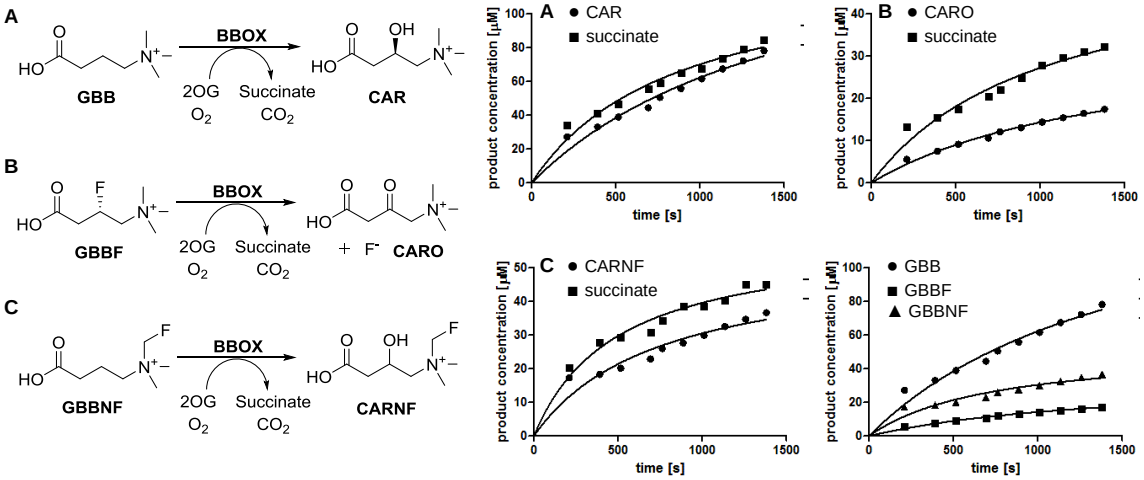

Figure S5 Time course of the hBBOX catalysed hydroxylation of GBB analogues.

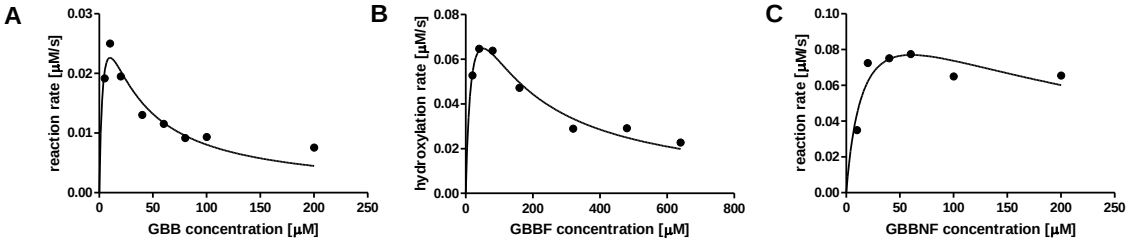

Figure S6 Dependence of initial rate of hydroxylation on GBB analogue concentration. A – GBB, B – GBBF, C – GBBNF. Reaction conditions: hBBOX 50 nM (A)/ 400 nM (B)/ 800 nM (C), 50 µM Fe(II), 1 mM 2OG, 500 µM ascorbate, 200 mM KCl, 10% D_2O in 50 mM Tris-d_{11} buffer at pH 7.5. Experiments performed using NMR based assay.

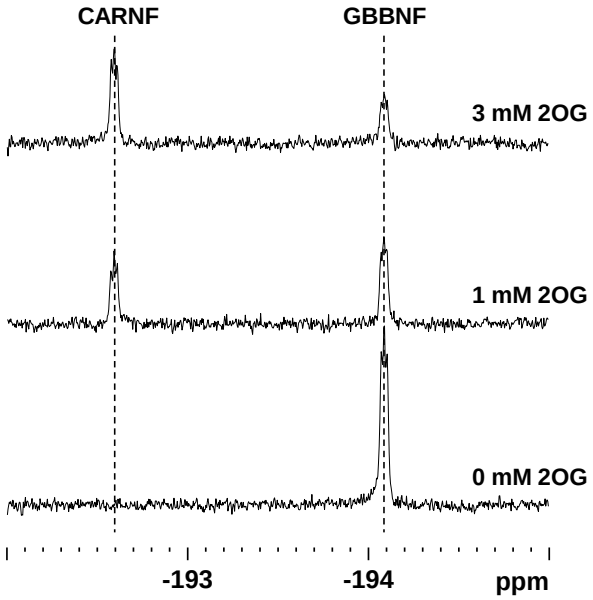

Figure S7 Turnover of GBBNF in cell lysates is dependent on the amount of 2OG.

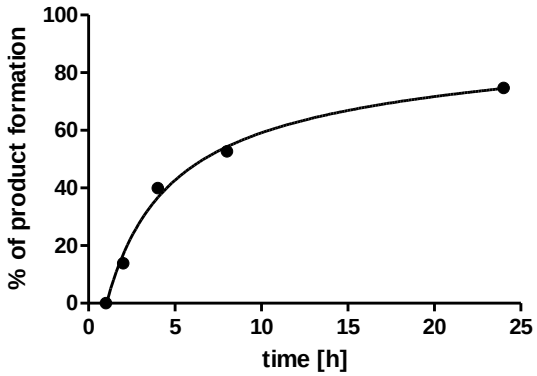

Figure S8 Dependence of GBBNF turnover in cell lysate on time after induction by IPTG. GBBNF turnover in cell lysate plotted against time elapsed from induction by IPTG was fitted with equation: y=a-bln(x+c), where a=7.8, b=-21.6, c=-0.34 and R^2=0.97 (OriginPro 8.5.1 software).

NAIADYRTFPLISPLASAASFASGVSVTWADGRVSPFHNLWLRDNCPCGDCVYEVTREQVFL
VADVPEDIQVQAVTIGDDGRLVVQWDDGHASAYHPGWLRAHAYDAQSLAEREAARPHKH
RWMQGLSLPVYDHGAVMQDDDTLLEWLLAVRDVGLTQLHGVPTEPGALIPLAKRISFIRES
NFGVLFDVRSKADADSNAYTAFNLPLHTDLPTRELQPGLQFLHCLVNDATGGNSTFVDGFAI
AEALRIEAPAAYRLLCETPVEFRNKDRHSDYRCTAPVIALDSSGEVREIRLANFLRAPFQMDA
QRMPDYYLAYRRFIQMTREPRFCFTRRLEAGQLWCFDNRRVLHARDAFDPASGDRHFQGCY
VDRDELLSRILVLQR

Figure S9 Sequence of the *Pseudomonas* sp. AK1 BBOX used in this study. Organism: *Pseudomonas* sp. AK1, EC 1.14.11.1, gi 385463.

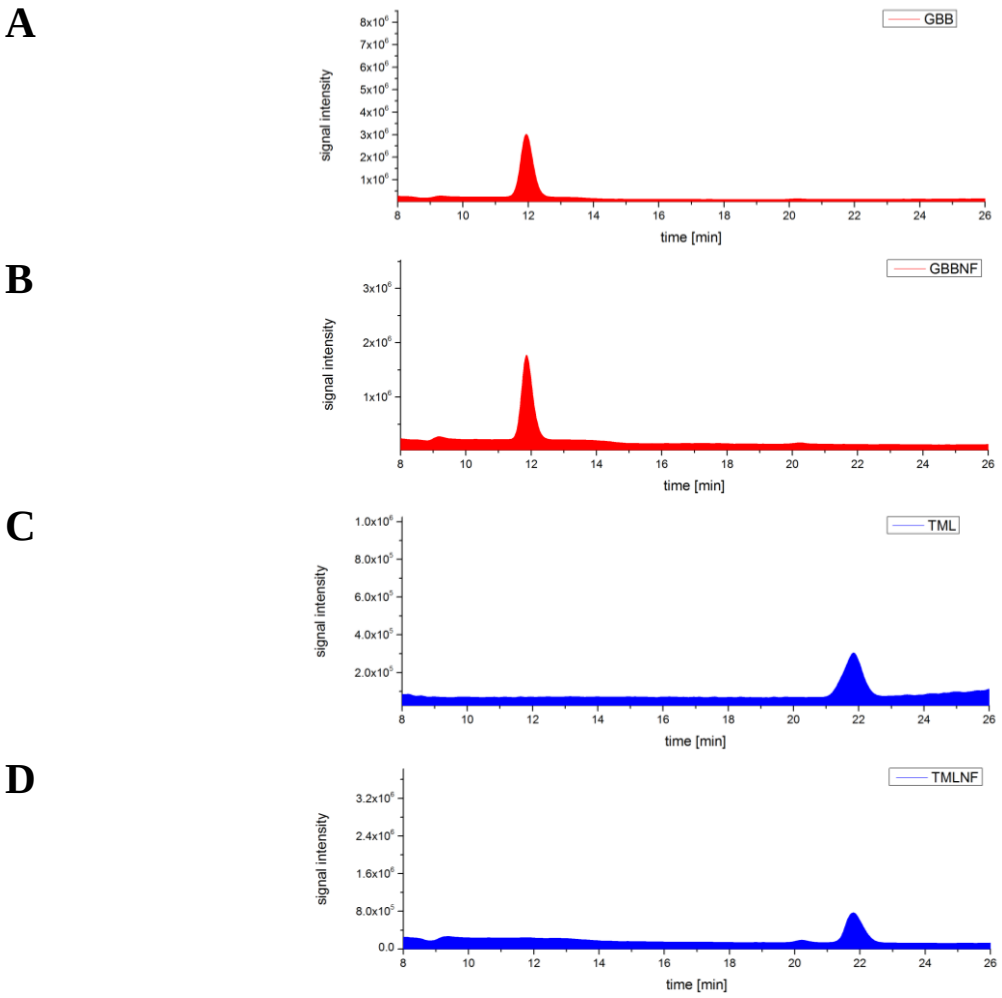

Figure S10 LC-MS chromatograms of carnitine biosynthesis metabolites standards. A – GBB, B – GBBNF, C – TML, D – TMLNF. For details on chromatographic separation see Experimental section.

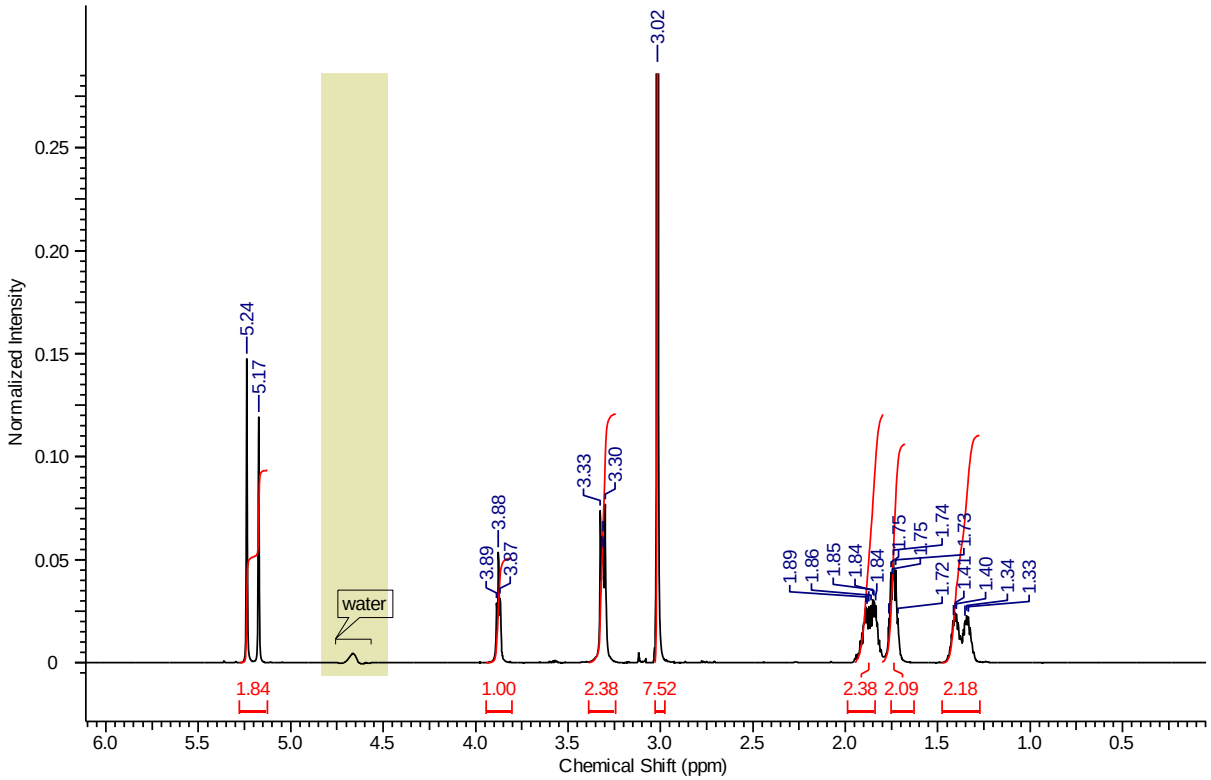

Figure S11 ^{1}H NMR spectrum of TMLNF

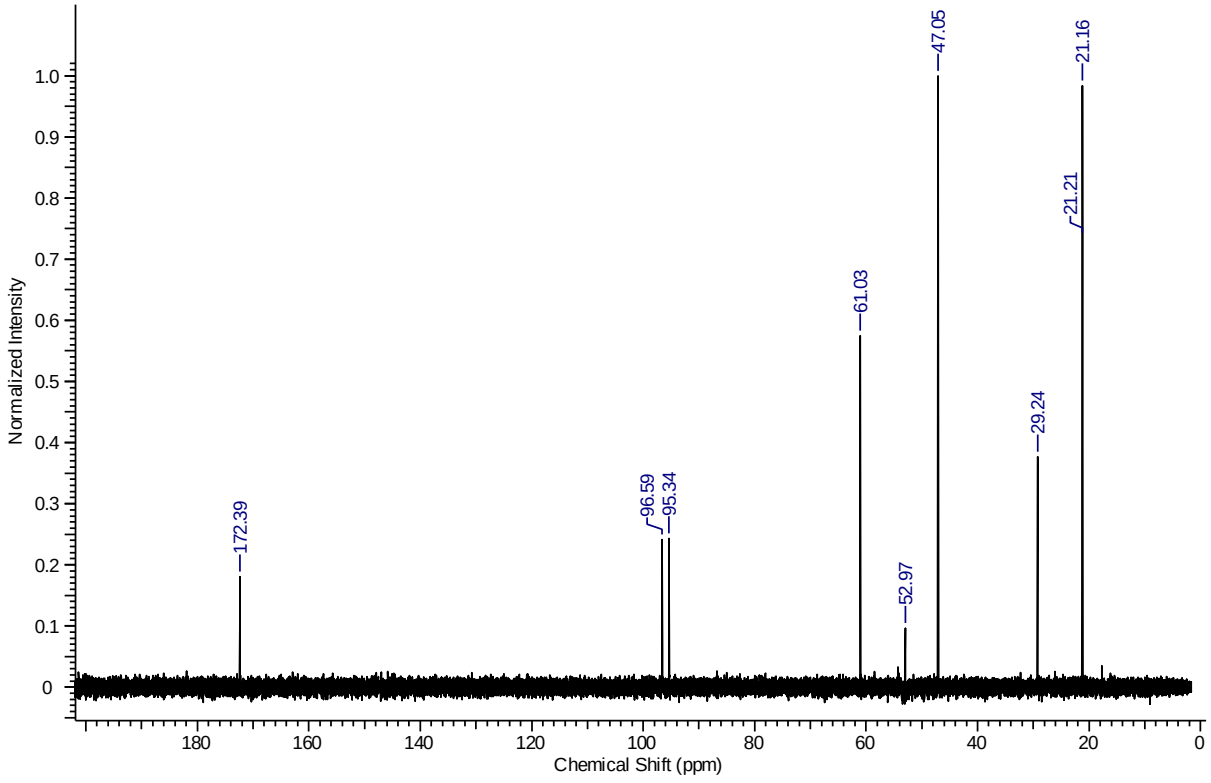

Figure S12 ^{13}C NMR spectrum of TMLNF

Synthesis

Chemicals were from Sigma-Aldrich (Dorset, UK) and used without further purification. Solvents for chemical transformations, work-up and chromatography were from Aldrich at HPLC grade, and used without further purification. Silica gel 60 F254 analytical thin layer chromatography (TLC) plates were from Merck (Darmstadt, Germany) and visualized under UV light, or with potassium permanganate. Chromatographic purifications were performed using prepacked SNAP columns on a Biotage SP1 Purification system (Uppsala, Sweden). Deuterated solvents were from Sigma and Apollo Scientific Ltd. ^{1}H NMR spectra were recorded using Bruker AVANCE AV400 (400 MHz), Bruker AV 500 MHz with ^{13}C cryoprobe and variable temperature setup, Bruker AVIII 700 with inverse TCI cryoprobe or Bruker AVII 500 machines. Signal positions were recorded in δ ppm with the abbreviations s, d, t, q, and m denoting singlet, doublet, triplet, quartet and multiplet respectively. NMR chemical shifts were referenced to residual solvent peaks. Coupling constants, *J*, are registered in Hz to a resolution of 0.5 Hz. High Resolution (HR) mass spectrometry data (m/z) were obtained from a Bruker MicroTOF instrument using an ESI source and Time of Flight (TOF) analyzer. Values are reported as ratio of mass to charge in Daltons. Melting points were obtained using a Leica VMTG heated-stage microscope or Stuart SMP-40 automatic melting point apparatus. Fourier transform Infrared (FT-IR) spectra were recorded on a Bruker Tensor 27 instrument. Optical rotations were recorded using a Perkin Elmer 241 Polarimeter.

Fluoromethylsulphinyl benzene (6)

The preparation of (**6**) employed a modification of the reported procedure[3] for the synthesis of the unstable fluoromethyl phenyl sulphide that was directly oxidized to (**6**).

A stirred suspension of chloromethyl phenyl sulphide ((**5)**, 5.00 g, 31.5 mmol, 1 eq.) and CsF (10.0 g, 66.2 mmol, 2 eq.) in PEG200/CH_3CN (20 mL, 1:2 v/v, dried over molecular sieves) was heated to 80 °C for 1.5 h. The reaction mixture was then cooled to room temperature and partitioned between water and CH_2Cl_2. The aqueous layer was then extracted with CH_2Cl_2. The combined organic layers were dried (Na_2SO_4), then concentrated *in vacuo* (water bath at room temperature). The resultant yellow oil was immediately taken up in a mixture of MeOH (50 mL) and water (5 mL) and cooled to 0 °C in an ice-water bath. *N*-Bromosuccinimide (11.5 g, 64.6 mmol, 2.05 equiv.) was added in small portions such that the internal temperature remained below 10 °C, and stirred 1 h at 0 °C. The mixture was diluted with CH_2Cl_2 (100 mL) and washed with 10% $Na_2S_2O_3$ (100 mL), 5% H_2SO_4 (100 mL), satd. $NaHCO_3$ (100 mL) and brine (100 mL). The organic layer was dried (Na_2SO_4) and concentrated *in vacuo*. The residue was purified by automated flash column chromatography (Biotage KP-SIL SNAP 100 g cartridge, eluting with hexane–ethyl acetate) to afford the desired compound (2.64 g, 59%) as a pale yellow oil.

^{1}H NMR (400 MHz; $CDCl_3$) δ = 7.63–7.75 (2 H, m, Ar*H*), 7.52–7.63 (3 H, m, Ar*H*), 5.08 (2 H, d, *J*=47.5 Hz, C*H*$_2$F) ppm. ^{13}C NMR (101 MHz; $CDCl_3$) δ = 138.5, 132.0, 129.5, 124.6, 98.2 (d, J_{CF}=221

Hz) ppm. ^{19}F NMR (377 MHz; $CDCl_3$) δ = 211.8 ppm. FT-IR ν_{max} (film) 3059, 3001, 2932, 1478, 1445, 1093, 1056, 1025 cm^{-1}. Spectroscopic data are consistent with those reported[4].

_S_-Monofluoromethyl-_S_-phenyl-2,3,4,5-tetramethylphenylsulphonium tetrafluoroborate (7)[4]

To a stirred solution of fluoromethylsulphinyl benzene (**6**) (2.65 g, 18.6 mmol, 1 eq.) and 1,2,3,4-tetramethylbenzene (2.76 mL, 18.6 mmol, 1 eq.) in dry Et_2O (25 mL) was added triflic anhydride (3.13 mL, 18.6 mmol, 1 eq.) dropwise over a period of 30 min at 0 °C. After stirring for 1 h, the precipitated solid was filtered, washed with cold Et_2O (5 × 10 mL) dissolved in 60 mL DCM and washed with an aqueous $NaBF_4$ solution (1M, 5 × 80 mL). The organic layer was dried over $MgSO_4$, volatiles were removed by rotary evaporation and the product was dried *in vacuo* to afford (**7**) (3.96 g, 59%) as an off-white solid.

^{1}H NMR (400 MHz; $CDCl_3$) δ = 7.78 (2 H, d, *J*=8.0 Hz, Ar*H*), 7.72 (1 H, d, *J*=7.5 Hz, Ar*H*), 7.61–7.70 (2 H, m, Ar*H*), 7.43 (1 H, s, Ar*H*), 6.50–6.61 (1 H, dd, *J*=9.5 Hz, CH_2F), 6.44 (1 H, dd, *J*=9.0 Hz, CH_2F), 2.48 (3 H, s, CH_3), 2.37 (3 H, s, CH_3), 2.30 (3 H, s, CH_3), 2.28 (3 H, s, CH_3) ppm. ^{13}C NMR (101 MHz; $CDCl_3$) δ = 143.96, 139.4, 138.3, 137.4, 134.4, 131.4 (2 × Ar*C*H), 130.9 (2 × Ar*C*H), 128.3 (d, *J*=5.0 Hz), 121.2 (d, J_{CF}=2.0 Hz), 116.1, 89.4 (d, J_{CF}=241 Hz), 21.1, 17.7, 16.9, 16.8 ppm. ^{19}F NMR (377 MHz; $CDCl_3$) δ = −151.3, −151.4, −207.1 ppm. Mp 127–130 ºC. Spectroscopic data are consistent with those reported[4].

Sec-butyl 4-(dimethylamino)butanoate hydrochloride (8)

4-(Dimethylamino)butanoic acid (1 g, 5.97 mmol) was dissolved in 2-butanol (7 mL) and treated with 3.25 mL of 2 M solution of HCl in Et_2O. The mixture was refluxed overnight. The resultant solution was then cooled, concentrated *in vacuo* and then coevaporated several times with acetone to afford (**8**) (1.24 g, 5.55 mmol, 93%) as a colourless oil.

^{1}H NMR (400 MHz; $CDCl_3$) δ =4.71 - 4.88 (1 H, m), 3.00 - 3.14 (2 H, m), 2.82 (3 H, s, CH_3), 2.81 (3 H, s, CH_3), 2.43 (2 H, t, *J*=7.0 Hz), 2.04 - 2.22 (2 H, m), 1.40 - 1.66 (2 H, m), 1.17 (3 H, d, *J*=6.5 Hz), 0.85 (3 H, t, *J*=7.5 Hz) ppm. ^{13}C NMR (101 MHz; $CDCl_3$) δ = 171.6, 73.1, 69.5, 56.9, 42.8, 31.9, 30.9, 28.7, 19.4, 9.7 ppm. FT-IR ν_{max} (film) 3407, 2974, 2698, 1724, 1469, 1380, 1204 cm^{-1}. HRMS (ESI-TOF) calcd for $C_{10}H_{22}NO_2$ [M+H^+]: 188.1645, found: 188.1645.

3-Carboxy-_N_-(fluoromethyl)-_N_, _N_-dimethylpropan-1-aminium) chloride (9)

Cs_2CO_3 (655 mg, 2.01 mmol, 1.5 eq) was added to a solution of (**8**) (300 mg, 1.34 mmol) in dry MeCN (20 mL) and the resulting mixture was stirred vigorously. (**7**) (970 mg, 2.68 mmol, 2 eq) was added and the mixture was stirred overnight at 50°C. The reaction mixture was then filtered and the filtrate was extracted with cyclohexane and coevaporated several times with methanol and CH_2Cl_2 to afford 4-sec-butoxy-*N*-(fluoromethyl)-*N*,*N*-dimethyl-4-oxobutan-1-aminium) tetrafluoroborate (**8a**) (320 mg, 1.04 mmol, 78%) as a yellow oil.

^{1}H NMR (500 MHz; $CDCl_3$) δ = 5.38 (2 H, d, *J*=45.0 Hz, CH_2F), 4.82 (1 H, m), 3.40 - 3.55 (2 H, m), 3.21 (6 H, s, 2×CH_3), 2.45 (2 H, t, *J*=6.5 Hz), 2.02 - 2.11 (2 H, m), 1.44 - 1.68 (2 H, m), 1.20 (3 H, d,

J=6.5 Hz), 0.88 (3 H, t, *J*=7.5 Hz) ppm. ^{13}C NMR (126 MHz; $CDCl_3$) δ = 171.7, 97.0, 95.2, 73.2, 60.8, 47.1, 30.1, 28.7, 19.3, 17.5, 9.7 ppm. FT-IR ν_{max} (film) 2976, 2360, 2341, 1724, 1382, 1056 cm^{-1}. HRMS (ESI-TOF) calcd for $C_{11}H_{23}FNO_2^+$ [M^+]: 220.1707, found: 220.1708.

To a solution of (**8a**) (320 mg, 1.04 mmol) in H_2O (5 mL) $LiOH{\cdot}H_2O$ (218 mg, 5.20 mmol, 5 eq) was added and the mixture was stirred overnight at room temperature. The solvent was co-evaporated several times with methanol and CH_2Cl_2, the residue was suspended in MeOH and filtered. The filtrate was concentrated *in vacuo*, redissolved in water and brought to pH 7 by 1M aqueous solution of HCl. The aqueous solution was subjected to purification by HPLC (C18 column, solvent system: A – H_2O, 0.05% CF_3COOH, B – MeCN, 0.1% CF_3COOH; gradient 50% of B in 15 min, retention time 10.5 min, ELSD detection). Fractions containing the product were concentrated *in vacuo* to give (**9**) (50 mg, 24%) as a white hygroscopic solid.

^{1}H NMR (250 MHz; MeOH) δ = 8.39 (1H, s, OH), 5.44 (2H, d, *J* = 45.0 Hz, C*H*$_2$F), 3.43 - 3.54 (2H, m), 3.21 (3H, s, CH_3), 3.20 (3H, s, CH_3), 2.40 (2H, t, *J* = 7.0 Hz), 1.99 - 2.16 (2H, m) ppm. ^{13}C NMR (126 MHz; D_2O) δ = 180.5, 95.8, 60.9, 46.8, 33.3, 18.6. ^{19}F NMR (377 MHz; $CDCl_3$) δ = -193.7 ppm. HRMS (ESI-TOF) calcd for $C_7H_{15}FNO_2^+$ [M^+]: 164.1081, found: 164.1081.

***N*$^{\epsilon}$,*N*$^{\epsilon}$-Dimethyl-*N*$^{\epsilon}$-fluoromethyl-L-lysine (11)**

A 50-mL round-bottom flask was charged with *N*-Boc dimethyllysine (Bachem, Weil am Rhein, Germany; 100 mg, 0.36 mmol, 1 equiv.) and Cs_2CO_3 (180 mg, 0.55 mmol, 1.5 equiv.) in CH_3CN (10 mL). The reaction mixture was stirred vigorously at room temperature, then monofluoromethyl sulphonium salt (**7**) (400 mg, 1.09 mmol, 3 equiv.) was added in one portion. The resultant suspension was stirred at 60°C overnight and filtered. The filtrate was extracted with cyclohexane (3 × 10 mL) and co-evaporated with MeOH and CH_2Cl_2. The residue was dissolved in CF_3COOH /MeOH (1:1 v/v, 10 mL) and a solution of LiOH (50 mg) in water (10 mL) was added. The reaction mixture was stirred at room temperature (4 h), then co-evaporated with MeOH and CH_2Cl_2. The residue was suspended in MeOH, filtered and the filtrate was concentrated *in vacuo*. The resultant residue was purified by reverse phase HPLC (C18 column, solvent system: A – H_2O, 0.05% CF_3COOH, B – MeCN, 0.1% CF_3COOH; gradient 50% of B in 15 min, retention time 14 min, ELSD detection). Fractions containing product were freeze-dried to yield *N*-Boc-*N*$^{\epsilon}$,*N*$^{\epsilon}$-dimethyl-*N*$^{\epsilon}$-fluoromethyl-L-lysine (**10a**) (36 mg, 0.09 mmol, 24%) as a yellow hygroscopic solid.

^{1}H NMR (700 MHz; D_2O) δ = 5.27 (2H, d, *J*=45.0 Hz, C*H*$_2$F), 4.04 (1H, m, C*H*NH), 3.36 (2H, t, *J*=10.0 Hz, C*H*$_2$N$^+$), 3.08 (3H, s, CH_3), 3.08 (3H, s, CH_3), 1.75 (4H, m), 1.40 (2H, m), 1.36 (9H, s, $C(CH_3)_3$) ppm.

The obtained solid (**10a**) was treated with 5 mL of 2M solution of HCl in Et_2O and stirred at room temperature (2 h). Solvent were evaporated to give (**11**) as white hygroscopic solid (HCl salt, 22 mg, 0.08 mmol, 90%).

^{1}H NMR (700 MHz; D_2O) δ = 5.26 (2H, d, *J*=45.0 Hz, C*H*$_2$F), 3.87 (1H, t, *J*=6.5, C*H*NH), 3.36 (2H, m, C*H*$_2$N$^+$), 3.07 (6H, s, 2×CH_3), 1.90 (2H, m), 1.79 (2H, m), 1.42 (2H, m) ppm. ^{13}C NMR (136 MHz; D_2O) δ = 172.4, 96.0 (d, *J*=45.0 Hz), 61.0, 53.0, 47.1, 29.2, 21.2, 21.1 ppm. ^{19}F NMR (377 MHz; $CDCl_3$) δ = -193.7 ppm. $[\alpha]_D^{20} = +5.1$ (c = 0.5 in MeOH). HRMS (ESI-TOF) calcd for $C_9H_{20}FN_2O_2^+$ [M^+]: 207.1503, found: 207.1504.

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