Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Comparison of the substrate selectivity and biochemical properties of human and bacterial γ-butyrobetaine hydroxylase

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Contents

Pseudomonas sp. AK1 BBOX purification

A **B**

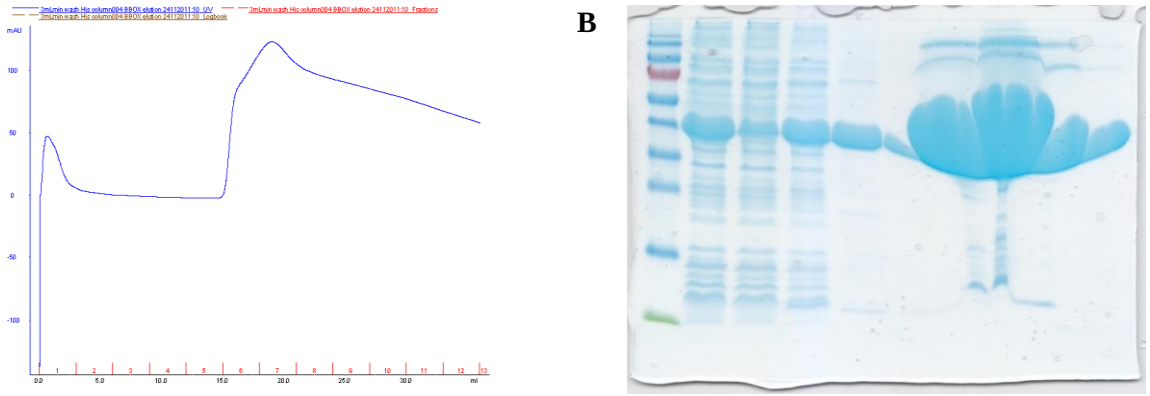


Figure S1 Ni-NTA chromatogram, B. SDS PAGE gel analysis. Lanes: 1. Molecular weight markers (Thermo Scientific PageRuler Prestained Protein Ladder 10-170 kDa, 2. Total cell lysates, 3. Cell lysate flow through, 4. Wash flow through, then fractions 6-11.

A **B**

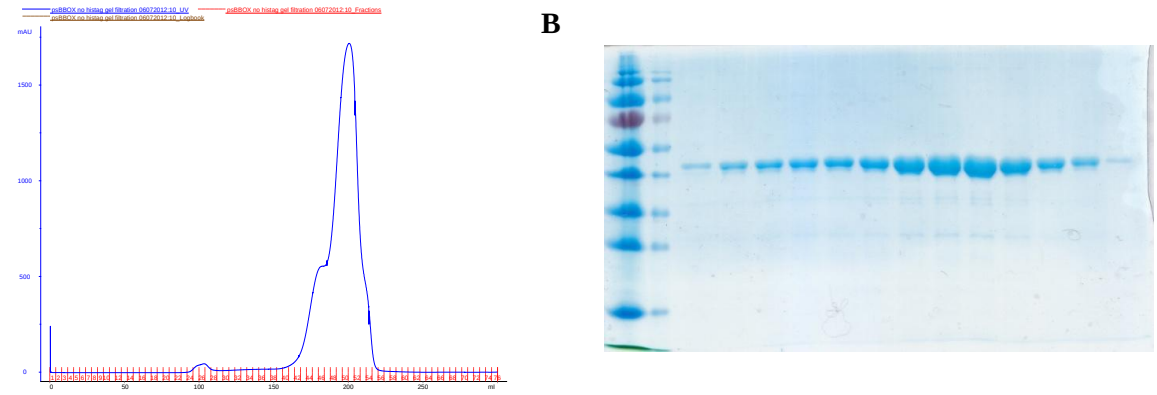


Figure S2 S200 column gel filtration chromatogram, B. SDS PAGE gel analysis. Lanes: 1-2. Molecular weight marker (Thermo Scientific PageRuler Prestained Protein Ladder 10-170 kDa), then fractions 43-55.

A **B**

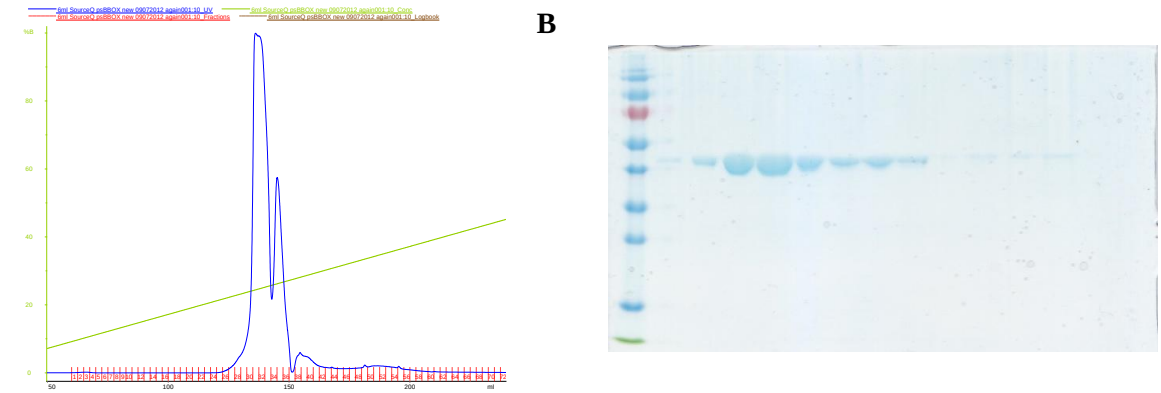


Figure S3 SourceQ column ion exchange chromatogram, B. SDS PAGE gel analysis. Lanes: 1. Molecular weight marker (Thermo Scientific PageRuler Prestained Protein Ladder 10-170 kDa), then fractions 29-40. Protein was eluted with 200-250 mM NaCl.

Sequence alignment

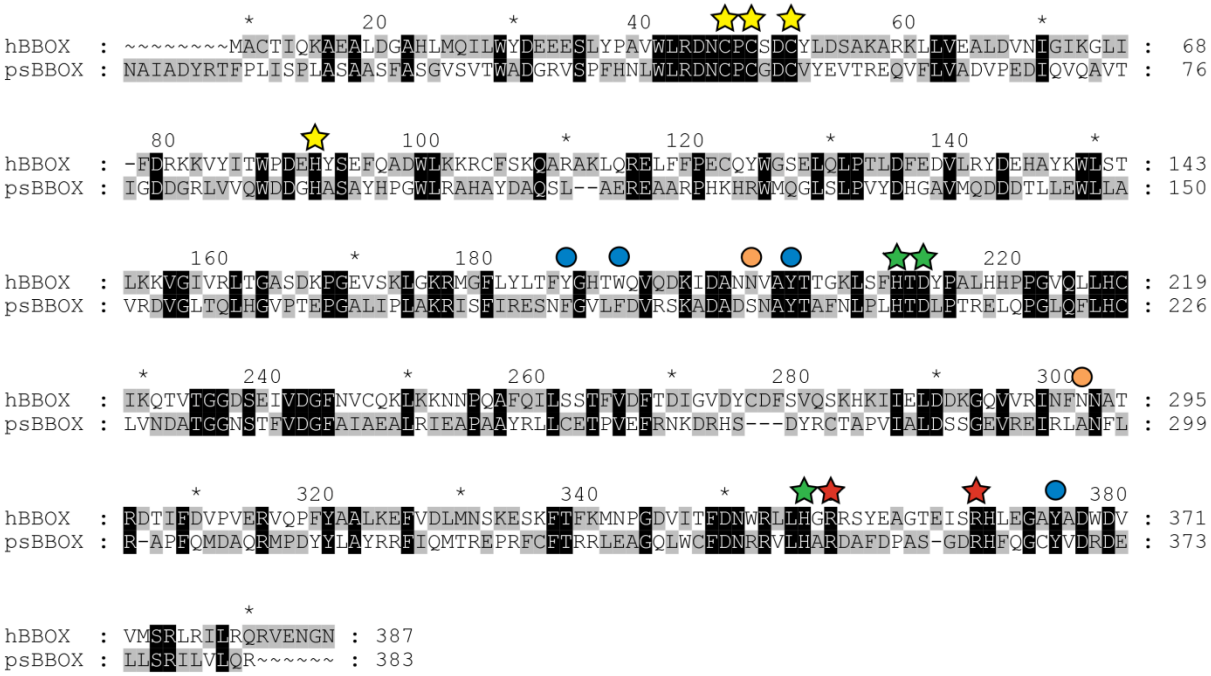


Figure S4 Sequence alignment of human (gi|158261239) (hBBOX) and *Pseudomonas* sp. AK1 (gi|231642) BBOX (psBBOX). The Zn(II)-binding domain is present in both hBBOX and psBBOX and residues responsible for Zn(II) binding (yellow stars) are highly conserved. The HxD/E…H – an iron binding motif characteristic of the 2OG oxygenases (green stars) as well as the arginines binding the C-5 carboxylate of 2OG (red stars) are conserved. Blue circles indicate residues involved in formation of the aromatic cage, which interacts with the trimethylammonium group of GBB via hydrophobic and π-cation interactions. For both hBBOX and psBBOX these residues have aromatic side chains, i.e. phenylalanine and tyrosine (psBBOX) or tyrosine and tryptophan (hBBOX). Residues involved in binding of the GBB carboxylate present in hBBOX (orange circles) are not conserved in psBBOX, suggesting why binding of GBB may be less tight in the case of the psBBOX.

Kinetic analyses

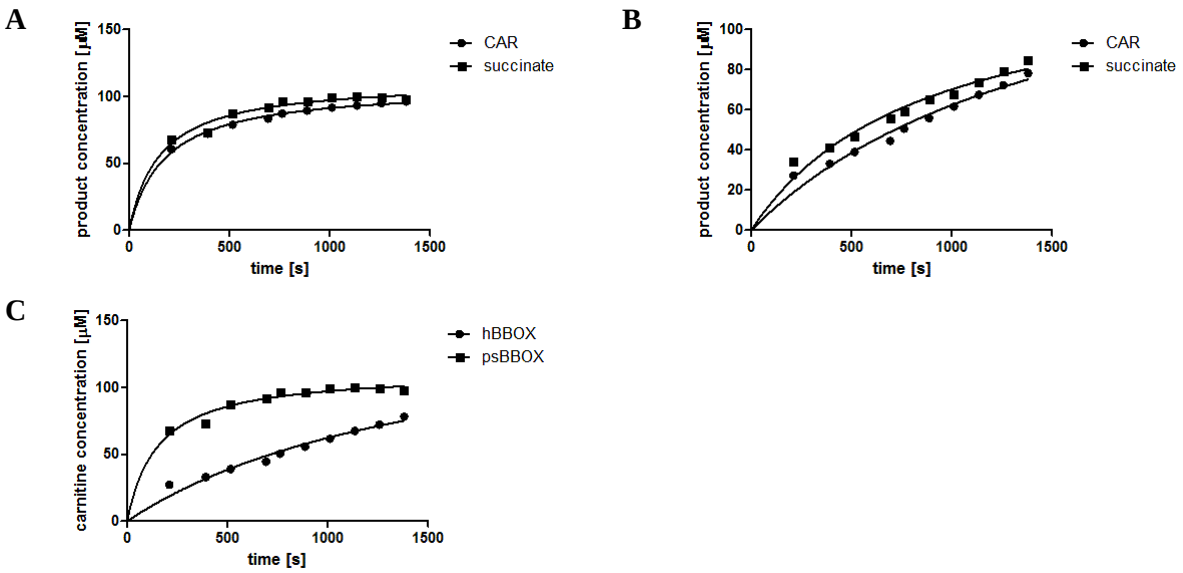


Figure S5 Time course analyses of psBBOX (A) and hBBOX (B) catalysed reactions as observed by carnitine and succinate formation using NMR. Overlay of hBBOX and psBBOX catalysed GBB hydroxylation (C).

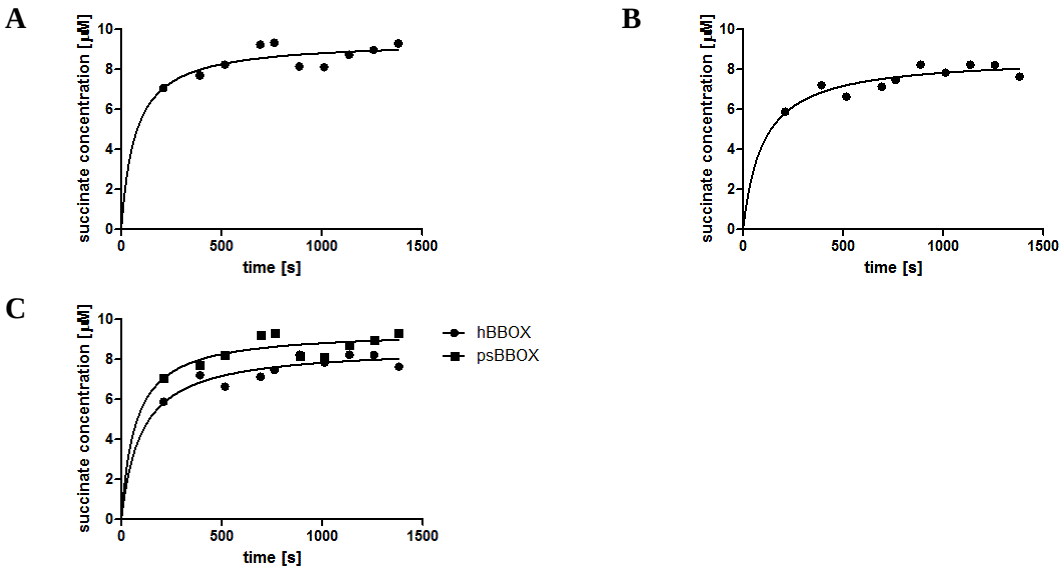


Figure S6 Uncoupled turnover in the absence of substrate for psBBOX (A) and hBBOX (B) catalysed reactions as observed by succinate formation (i.e. in the absence of GBB). Overlay of hBBOX and psBBOX catalysed 2OG turnover in the absence of GBB (C).

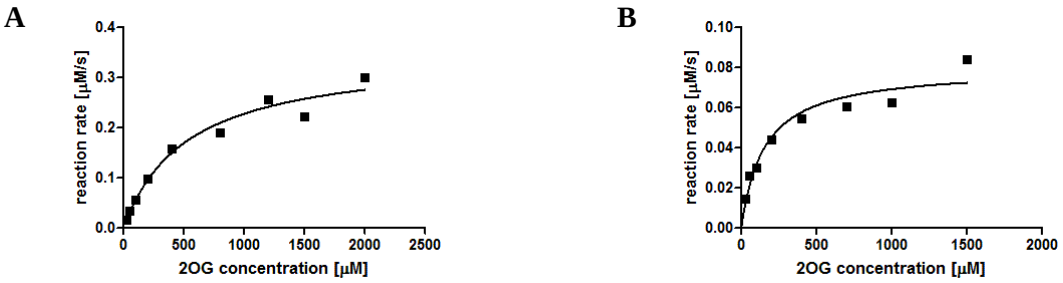


Figure S7 Dependence of initial reaction rates on 2OG concentration for psBBOX (A) and hBBOX (B) as observed by succinate formation.

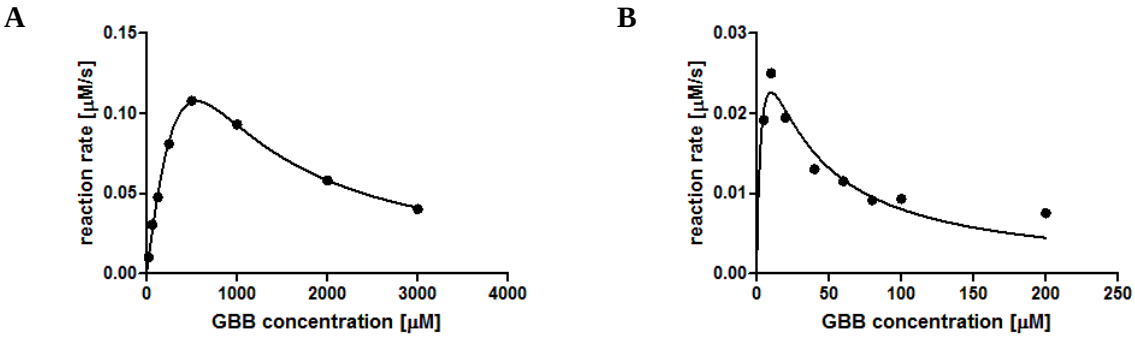


Figure S8 Dependence of initial reaction rates on the GBB concentration for psBBOX (A) and hBBOX (B).

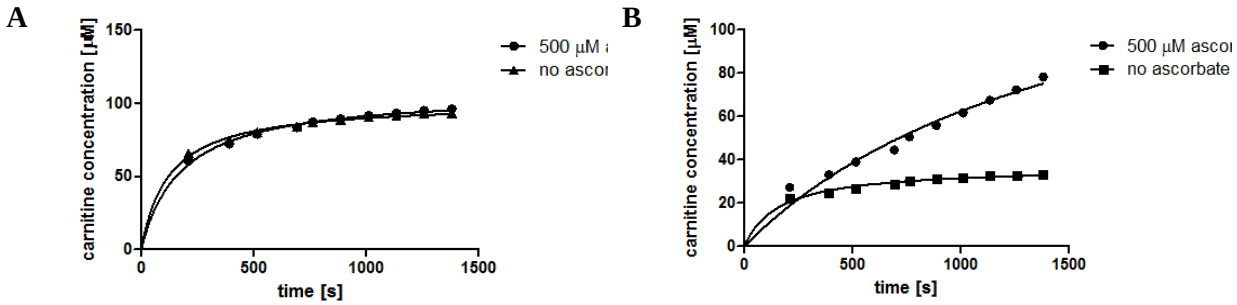


Figure S9 Comparison of time courses of psBBOX (A) and hBBOX (B) catalysed oxidations of GBB, in the presence and absence of ascorbate as monitored by product formation by ^{1}H NMR.

Reactions with D- and L-carnitine

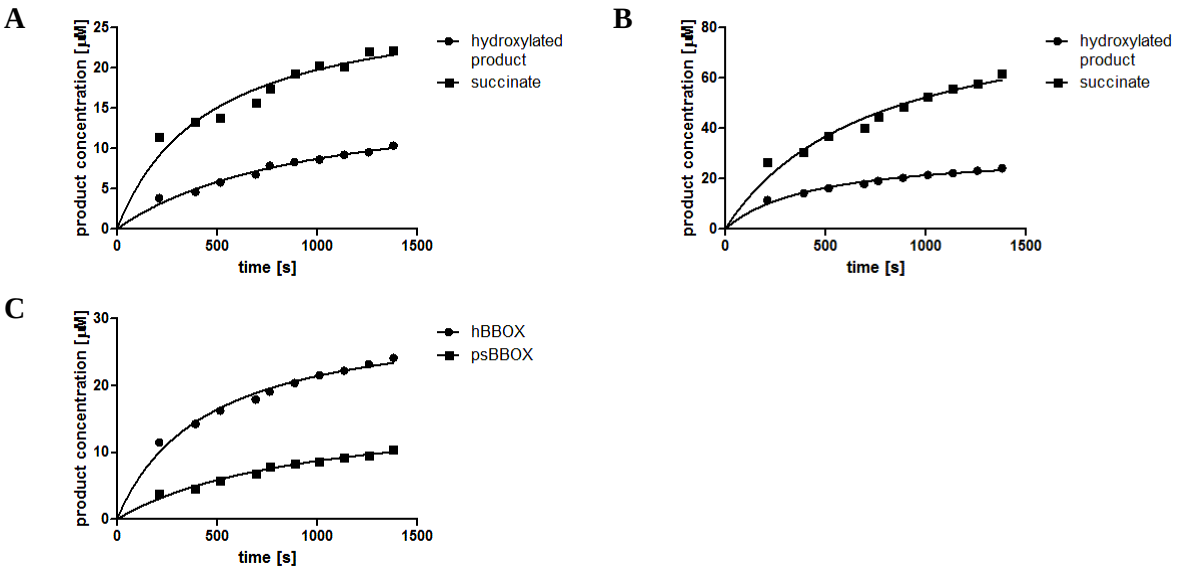


Figure S10 Time course analyses of psBBOX (A) and hBBOX (B) catalysed reaction with D-carnitine as observed by oxo-GBB and succinate formation. Overlay of hBBOX and psBBOX catalysed GBB hydroxylation (C).

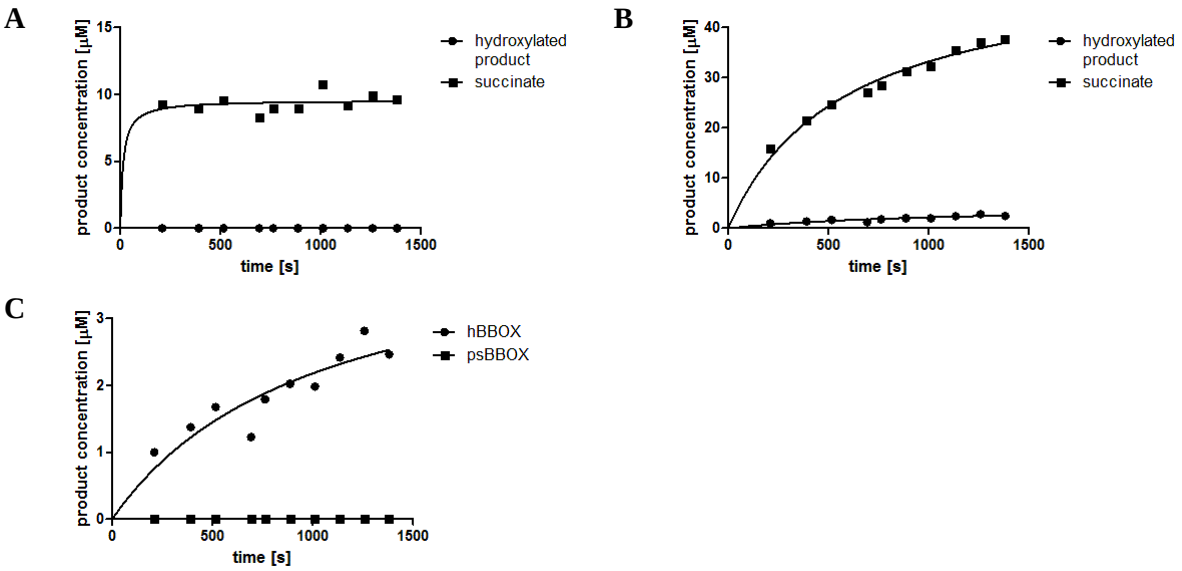


Figure S11 Time course analyses of psBBOX (A) and hBBOX (B) catalysed reaction with L-carnitine as observed by oxo-GBB and succinate formation. Overlay of hBBOX and psBBOX catalysed GBB hydroxylation (C).

Kinetic studies with fluorinated GBB analogues

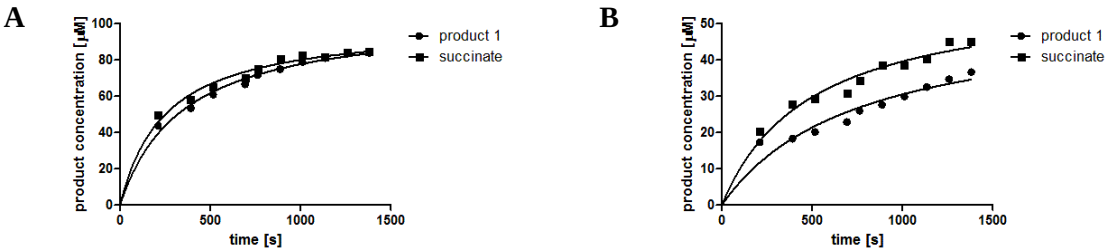


Figure S12 Comparison of time course analyses of psBBOX (A) and hBBOX (B) catalysed oxidations of GBBNF, as observed by carnitine and succinate formation.

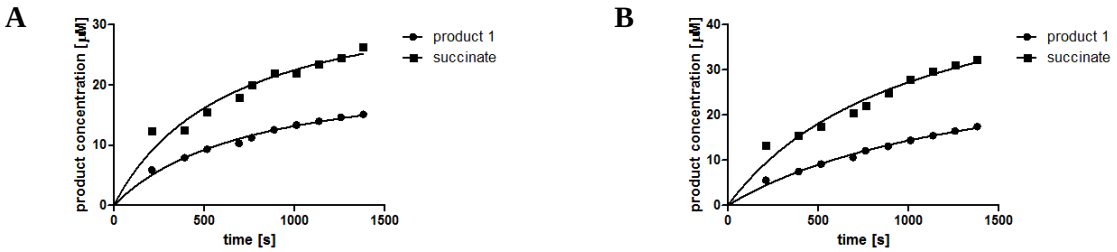


Figure S13. Comparison of time course analyses of psBBOX (A) and hBBOX (B) catalysed oxidations of GBBF, as observed by carnitine and succinate formation.

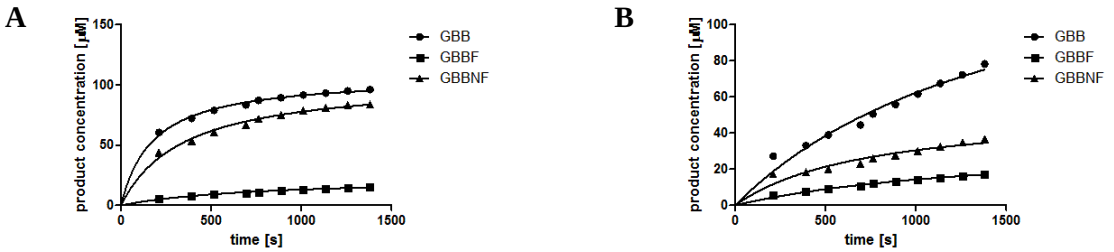


Figure S14 Comparison of time course analyses of psBBOX (left) and hBBOX (right) catalysed oxidations of GBB, GBBF and GBBNF.

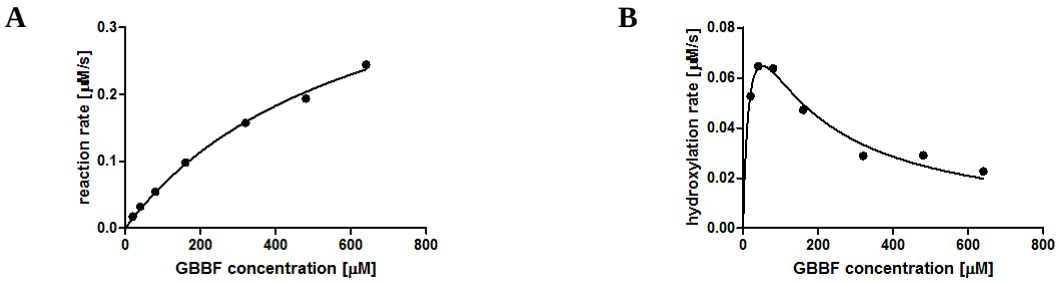


Figure S15 Dependence of initial reaction rates on the GBBF concentration for psBBOX (A) and hBBOX (B).

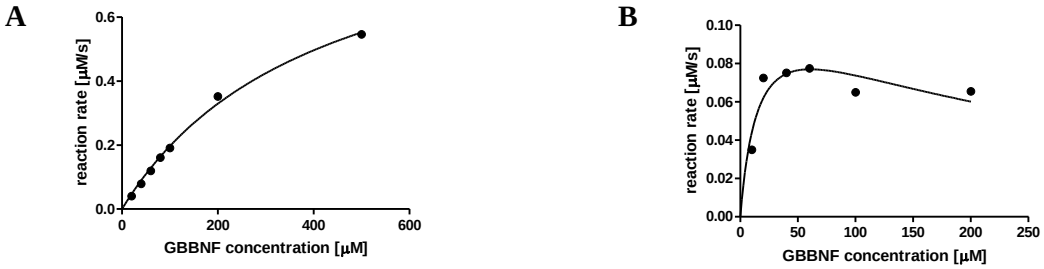
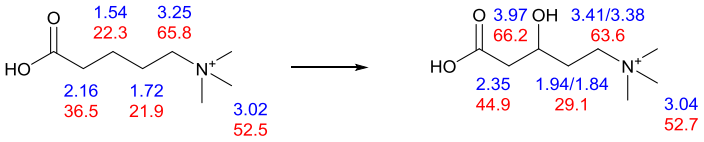


Figure S16 Dependence of initial reaction rates on the GBBNF concentration for psBBOX (A) and hBBOX (B).

Reactions of BBOX with GBB analogues with variable chain length

A



B

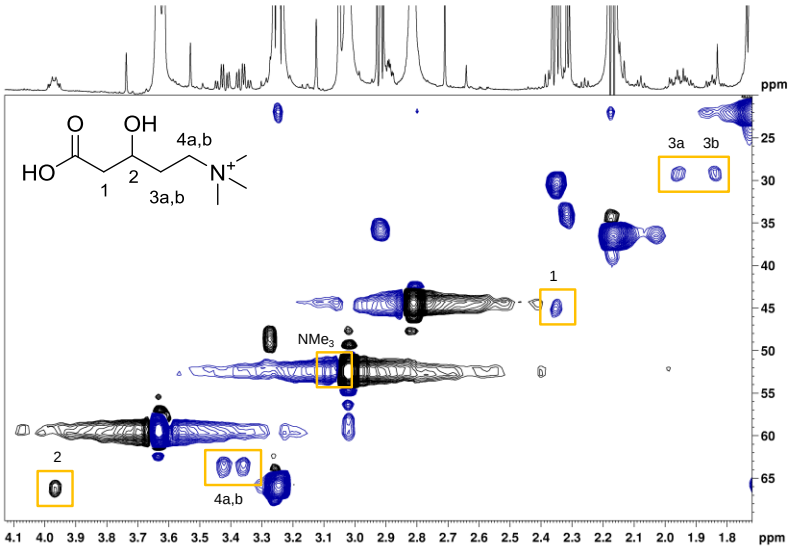
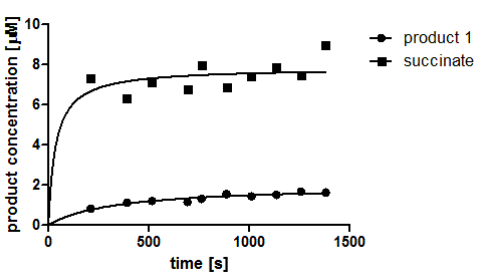


Figure S17 ^{1}H and ^{13}C NMR assignments of GBB-5 and its hydroxylation product (A). GBB-5 hydroxylation by psBBOX – ^{1}H-^{13}C HSQC NMR spectra of reaction mixture (B).

A



B

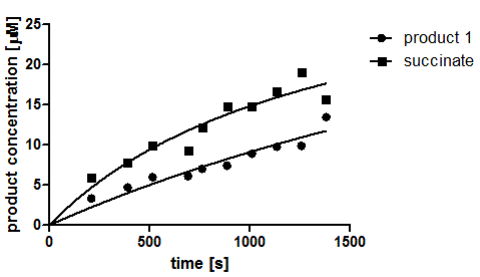
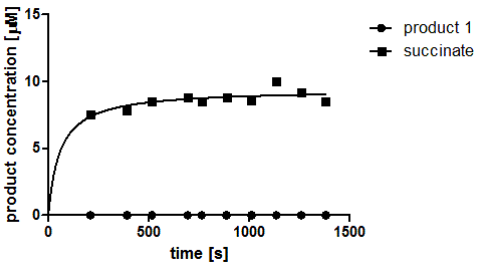


Figure S18 Time course analyses of psBBOX (A) and hBBOX (B) catalysed reaction with GBB-5 as observed by hydroxylated product and succinate formation.

A



B

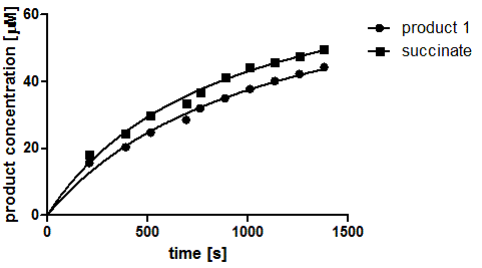
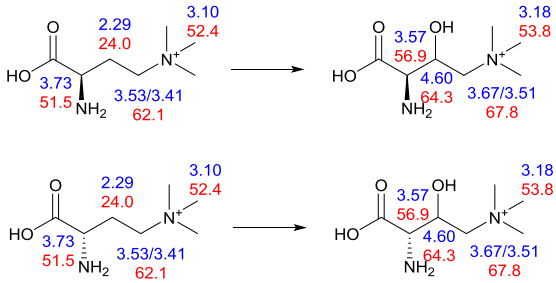


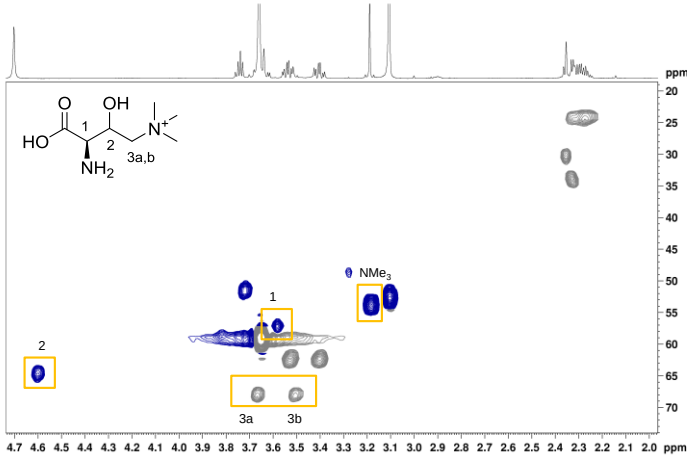
Figure S19 Time course analyses of psBBOX (A) and hBBOX (B) catalysed reaction with GBB-3 as observed by hydroxylated product and succinate formation.

Amino acid hydroxylations

A



B



C

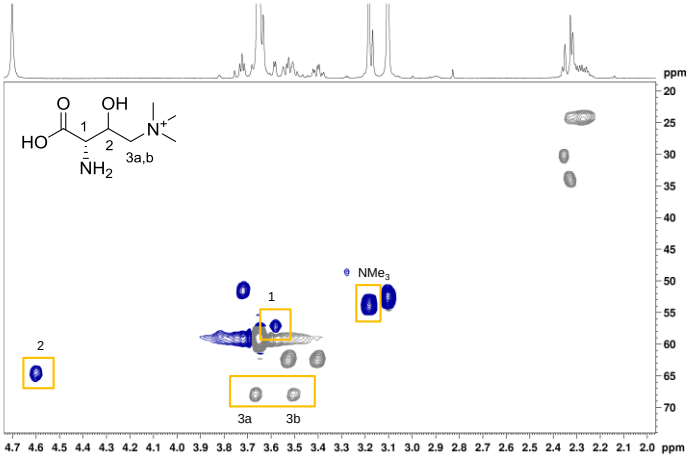
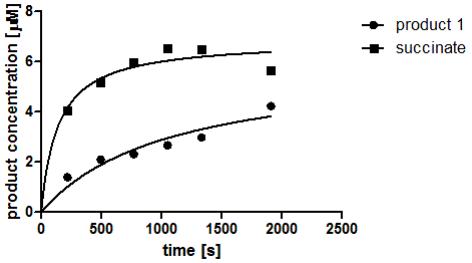


Figure S20 A – ^{1}H (blue) and ^{13}C (red) NMR assignments of GBB-NH(R), GBB-NH(S) substrates and their hydroxylation product at 280K.. B, C – NMR assignments of GBB-NH(R) and GBB-NH(S) hydroxylation products. ^{1}H-^{13}C HSQC assignment of enzymatic reaction mixture containing hydroxylated (B) – GBB-NH(R), (C) – GBB-NH(S).

A



B

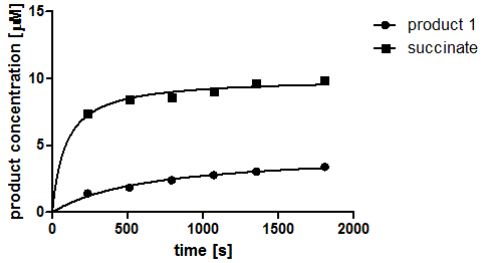
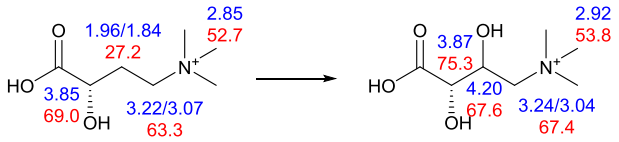


Figure S21 Time course analyses of psBBOX catalysed hydroxylation of GBB-NH(S) (A) and GBB-NH(R) (B) as observed by hydroxylated product and succinate formation.

Hydroxylation of 2-hydroxy GBB

A



B

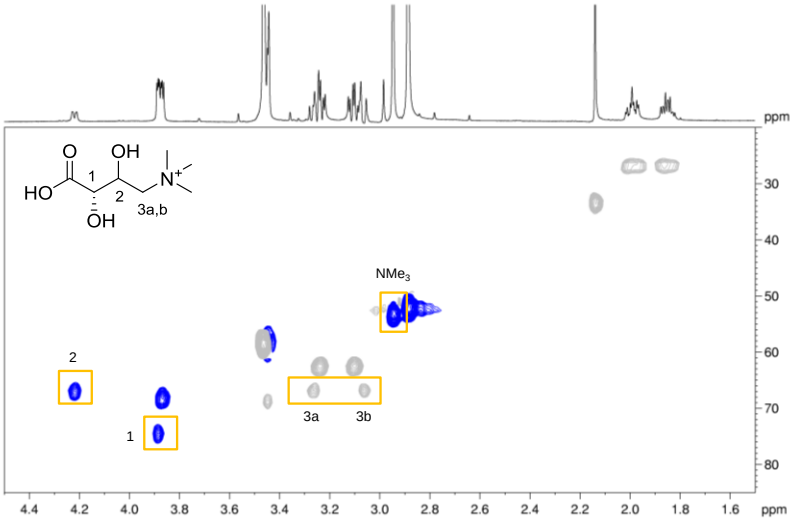


Figure S22 (A) – ^{1}H (blue) and ^{13}C (red) NMR assignments of GBB-OH and its hydroxylation product at 280K. (B) – ^{1}H-^{13}C HSQC assignments of GBB-OH hydroxylation products.

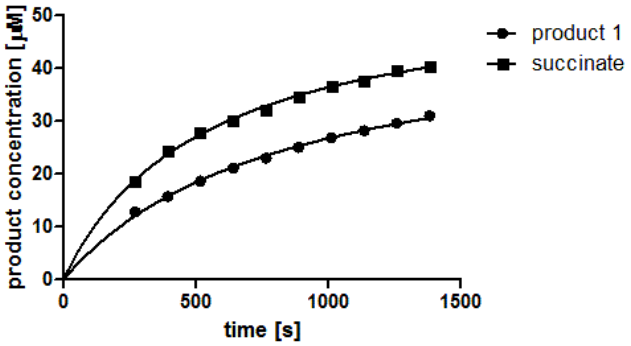


Figure S23 Time course analyses of psBBOX catalysed hydroxylation of GBB-OH as observed by hydroxylated product and succinate formation.

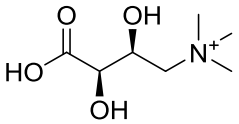


Figure S24 Structure of Anthopleurine (stereochemistry as assigned by Musich et al[1])

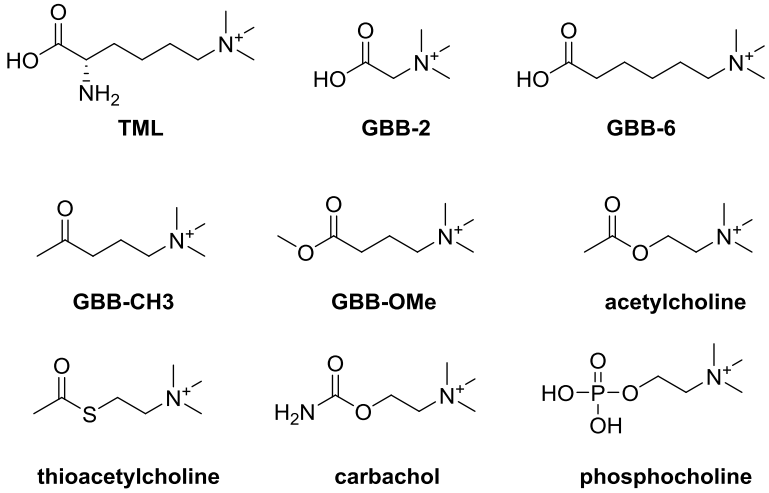


Figure S25 GBB analogues, which were found not to be substrates for BBOXs, revealing optimal carbon chain length of GBB analogue is 2<n<6 and that GBB carboxylate is required for binding.

Experimental section

Materials

γ-Butyrobetaine, L-carnitine, D-carnitine, glycine betaine (GBB-2), acetylcholine, thioacetylcholine, carbachol, phosphocholine, 2-oxoglutarate and synthetic precursors were from Sigma-Aldrich (Dorset, UK) and used without further purification. GBBF and GBBNF were synthesized as described[2, 3]. Other GBB analogues were synthesized according to General Procedure I or II. Tris-d_{11} was obtained from Cambridge Isotope Laboratories. Reagents for assays were from Sigma-Aldrich (Dorste, 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.

Cloning

The codon optimized (lower GC content than wt gene) *Pseudomonas* sp. AK1 (gi 385463). gene encoding psBBOX, flanked by NdeI and BamHI restriction sites, was obtained from GeneArt Life Sciences gene synthesis services, supplied in pMA-T plasmid. For expression purposes the BBOX gene was sucloned into pCOLDI (Takara) vector. Shortly, both vectors were amplified and purified using GeneJet DNA extraction kit. Consequently after amplification both vectors were digested with NdeI and BamHI HF restriction enzymes (New England Biolabs) (3 h at 37°C). DNA fragments were separated in 1% agarose gel, bands were cut out of the gel and the DNA was purified using Gel Extraction Kit (Quiagen). The insert containing psBBOX was ligated with the digested pCOLDI vector, using T4 DNA ligase. 2 μL of the ligation mixture was then transformed into XL10 Gold competent cells and grown on agar plates containing ampicillin. Obtained colonies were inoculated into fresh media supplemented with antibiotic and grown overnight. Plasmid DNA was purified using a DNA purification kit (Quiagen) and the identity of constructs was confirmed by sequencing.

Bacterial growth

2TY media (600 mL) supplemented with ampicillin was inoculated with 6 mL of overnight starter culture of BL21 (DE3) E. coli containing desired plasmidand grown at 37°C with shaking (200 rpm) until $O.D._{600}$ reached 0.7; After bacterial culture reached required density the temperature was reduced to 15°C and after 30min of incubation without shaking (during this time cultures cooled down) cultures were supplemented with IPTG (isopropyl β-galactopyranoside) to final concentration of 0.2 mM. The culture was then incubated overnight at 15°C with shaking Next day, bacteria were pelleted by centrifugation (10 min, 7000 rpm). The cell pellet was frozen at -80°C overnight.

Protein purification

Cells were lysed by sonication and lysate were clarified by centrifugation before loading on Ni(II)-affinity column (5ml His/Trap column Novagen). Column was preequilibrated with 10 CV (column volumes) of binding buffer (50mM HEPES pH 7.6, 0.5M NaCl, 5 mM imidazole), then cell lysate was loaded on the column and the column was washed with 5 CV of binding buffer, followed by washing with washing buffer (50mM HEPES pH 7.6, 0.5M NaCl, 30 mM imidazole) until absorbance

reached base line (usually 5-10 CV). Flow rate was 1 mL/min. Protein was eluted with elution buffer (50mM HEPES pH 7.6, 0.5M NaCl, 500 mM imidazole) (5 CV) in 3 mL fractions). After analysing of protein containing fractions by SDS-PAGE, the chosen fractions were combined and concentrated. Buffer in protein containing fractions was exchange on PD10 desalting column for 50 mM Tris-HCl buffer pH 7.5 containing 200 mM NaCl. Further protein was purified by size exclusion chromatography on Superdex S200 (300 mL) column, equilibrated with the same buffer at flow rate of 2 mL/min. Fractions containing protein were analysed by SDS-PAGE Fractions with the highest content of protein were combined and concentrated. Ion exchange chromatography was performed using Source-Q exchange column using 25 mM Tris-HCl buffer pH 7.5 and gradient of 0-200 mM NaCl. Purified protein was stored at concentration of 20 mg/mL in 50 mM Tris-HCl buffer containing 200 mM NaCl at -80°C. Produced protein contained extra *N*-terminal amino acids from the vector sequence: MNHKVHHHHHHIEGRHM, which gives protein product of 399 amino acids, MW = 45.35 kDa (as determined by MS analyses). Expression yielded around 80 mg of protein per 1 L of bacterial culture. Human BBOX was produced and purified as described[4].

Enzyme assays

Reagents were from Sigma-Aldrich. Tris-d_{11} was from Cambridge Isotopes. The standard solution of Tris-d_{11} buffer was 50 mM in H_2O pH 7.5 and contained 0.1% NaN_3 to prevent microbial growth. 2OG was used as its sodium salt, ascorbate as its potassium salt, and GBB as its hydrochloride salt. Fe(II) stock solution was prepared by dissolving $Fe(NH_4)_2(SO_4)_2$ in 20 mM HCl to final concentration of 100 mM. Reaction was performed in volume of 160ul.. NMR assays were run on Bruker AVIII 700 with inverse TCI cryoprobe using 3 mm MATCH tubes. Pulses were calibrated using single-pulse nutation method (Bruker pulsecal routine). Water suppression was achieved using the excitation sculpting method. Spectra were recorded 240 s after enzyme addition and the typical experiment length was 16 scans.

2OG turnover kinetic parameters were assigned by ^{1}H NMR using the following procedure. To a mixture containing 0.5 mM ascorbate, 0.2 M KCl, 0.1 mM GBB (in case of psBBOX)/ 0.04 mM GBB (hBBOX assays), 10% D_2O and varying concentration of 2OG in 50 mM TRIS-d_{11} pH 7.5 freshly prepared of solution of Fe(II) salt was added to final concentration of 0.05 mM. The reaction was initiated by the addition of enzyme to the reaction mixture to final concentration of 100 nM (psBBOX) or 50 nM (hBBOX).

GBB kinetic parameters were assigned by ^{1}H NMR using the following procedure. To a mixture containing 0.5 mM ascorbate, 0.2 M KCl, 1 mM 2OG, 10% D_2O and the varying concentration of GBB in 50 mM TRIS-d_{11} pH 7.5 a freshly prepared of solution of Fe(II) salt was added to final concentration of 0.05 mM. The reaction was initiated by addition of enzyme to the reaction mixture to final concentration of 50 nM.

Substrate (GBB) uncoupled turnover assays were conducted in solution containing: 0.5 mM 2OG, 0.5 mM ascorbate, 0.2 M KCl, 50 μM Fe(II) and 10% D_2O in 50 mM TRIS-d_{11} pH 7.5. The reaction was initiated by the addition of enzyme to final concentration of 400 nM. A control reaction with no enzyme added was performed to ensure there is no measurable non-enzymatic 2OG turnover in the assay conditions.

Ascorbate dependence assays performed in solutions containing: 0.5 mM 2OG, 0.1 mM GBB, 0.2 M KCl, 50 μM Fe(II), 0.5 mM or 0 mM ascorbate, 10% D_2O in 50 mM TRIS-d_{11} pH 7.5. Reactions were initiated by addition of enzyme to final concentration of 400 nM.

GBBF/GBBNF kinetic parameters were assigned by ^{1}H NMR using the following procedure. To a mixture containing 0.5 mM ascorbate, 0.2 M KCl, 1 mM 2OG, 10% D_2O and the varying concentration of GBBF/GBBNF in 50 mM TRIS-d_{11} pH 7.5, was added a freshly prepared solution of Fe(II) salt (final concentration of 0.05 mM). The reaction was initiated by addition of enzyme to the reaction mixture to give a final concentration of 800 nM (for GBBF assays)/200 nM (psBBOX, GBBNF assays, 400 nM (hBBOX, GBBNF assays).

Time-courses of GBB and its analogues (GBBF, GBBNF, GBB-5, GBB-3, GBB-NH(*R*), GBB-NH(*S*)) as well as D- and L-carnitine hydroxylations were performed in conditions containing 0.1 mM GBB analogue, 0.5 mM 2OG, 0.5 mM ascorbate, 0.2 M KCl, 50 μM Fe(II), 10% D_2O in 50 mM TRIS-d_{11} pH 7.5. The reaction was initiated by addition of enzyme to final concentration of 400 nM.

Assignments for the GBB-5, GBB-NH(R), GBB-NH(S) hydroxylation products were carried out at 280K in a solution containing 0.2 mM GBB-5, 0.5 mM 2OG, 0.2 M KCl, 50 μM Fe(II) in 50 mM TRIS-d_{11} pH 7.5 in D_2O, using 10 μM final concentration of psBBOX added in two portions with 2 h break in between additions.

MS screening

Initial screens for BBOX substrates were performed using a Waters LCT Premier Instrument, employing Electron impact Chemical Ionisation, fitted with time of flight (ToF) analyser. Samples were measured using direct injection (no column attached) and analysed for the presence of a +16 peak.

Enzymatic assays were run in a final volume of 200ul in conditions containing: 100 μM substrate, 1 mM 2OG, 100 μM Fe(II) 200 mM KCl, 0.5 mM ascorbate, and 1 μM enzyme in 50 mM phosphate pH 7.0. Each assay contained samples run with hBBOX, psBBOX and control with no enzyme added.

Homology modelling

Homology modelling was carried out using the SWISS-MODEL[5, 6] server (available from the EXPASY website: http://swissmodel.expasy.org/), using an automatic alignment mode and the human BBOX structure with PDB id: 3O2G as a template. Template for homology modeling was chosen by user, sequence alignment was performed in the automatic mode, theoretical model was obtained with

a QMEAN score of 0.62 (QMEAN score estimates model quality on the scale 0-1). Protein structures were visualised using PyMOL software.

Sequence alignments

Sequence alignments were carried out using the Clustal Omega program for multiple sequence alignment, accessible from EMBL-EBI servers (http://www.ebi.ac.uk/Tools/msa/clustalo/). Alignment was visualised using GeneDoc software.

Data processing

NMR data were processed using TopSpin 3.1 software (Bruker). Kinetic analyses were carried out using GraphPad Prism software. Standard Michaelis-Menten model was used for most fits. Substrate inhibition was fitted with built-in equation from GraphPad Prism: $Y=V_{max}*X/(K_M + X*(1+X/K_i))$, where V_{max} is the maximum enzyme velocity, if the substrate didn't also inhibit enzyme activity, expressed in the same units as Y. K_M is the Michaelis-Menten constant, presented in the same units as X. The equation is based on equation 5.44, in RA Copeland, *Enzymes*, 2nd edition, Wiley, 2000.

Synthesis

General experimental

Chemicals were from Sigma-Aldrich and used without further purification. Solvents for chemical transformations were from Aldrich at HPLC grade, and used without further purification. 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 br s., s, d, t, q, and m denoting broad singlet, singlet, doublet, triplet, quartet and multiplet respectively. All NMR chemical shifts were referenced to residual solvent peaks. Coupling constants, *J*, are registered in Hz to a resolution of 0.5 Hz. All compounds used in screening were more that 90% pure by ^{1}H NMR. 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. Low Resolution (LR) mass spectrometry data (m/z) were obtained from a Waters LCT Premier 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.

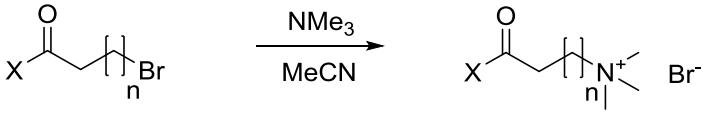
General procedure I

A solution of the corresponding bromide derivative in anhydrous acetonitrile (1 equiv.) was mixed with 4.2 M solution of trimethylamine in ethanol (1.5 equiv), stirred for 3 h, upon which a white precipitate formed. Solvents and trimethylamine were then evaporated *in vacuo*. The residue was collected in a small volume of cold acetonitrile; the resultant solid was collected by filtration and dried under vacuum to yield the required product as its bromide salt.

General procedure II

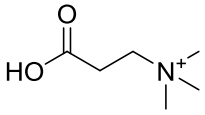
The appropriate *N*-Boc amino derivative (1 equiv.) was dissolved in methanol (5 mL) along with K_2CO_3 (4 equiv.), then treated with an excess of iodomethane (5 equiv.) and stirred at room temperature for 24 h. The methanol was then evaporated, and resultant residue dissolved in water (1 mL) and acidified with concentrated HCl. The mixture was stirred for 1 h and afterwards washed with diethyl ether. Water was evaporated *in vacuo* and resultant residue was purified by HPLC (preparative C-18 reverse phase column; gradient: 50% B in 15 min, where A – water, 0.05% formic acid, B – acetonitrile, 0.1% formic acid; fractions containing product identified using Evaporative Light Scattering Detection (ELSD). The fractions containing product were combined and freeze-dried to yield the product as a highly hygroscopic solid. Due to low level of compound recovery after HPLC purification, some of the assays used crude products if pure by ^{1}H NMR (which contained compound and KCl mixture, concentration of organic sample was determined by ^{1}H NMR).

GBB analogues synthesized according to general procedure I

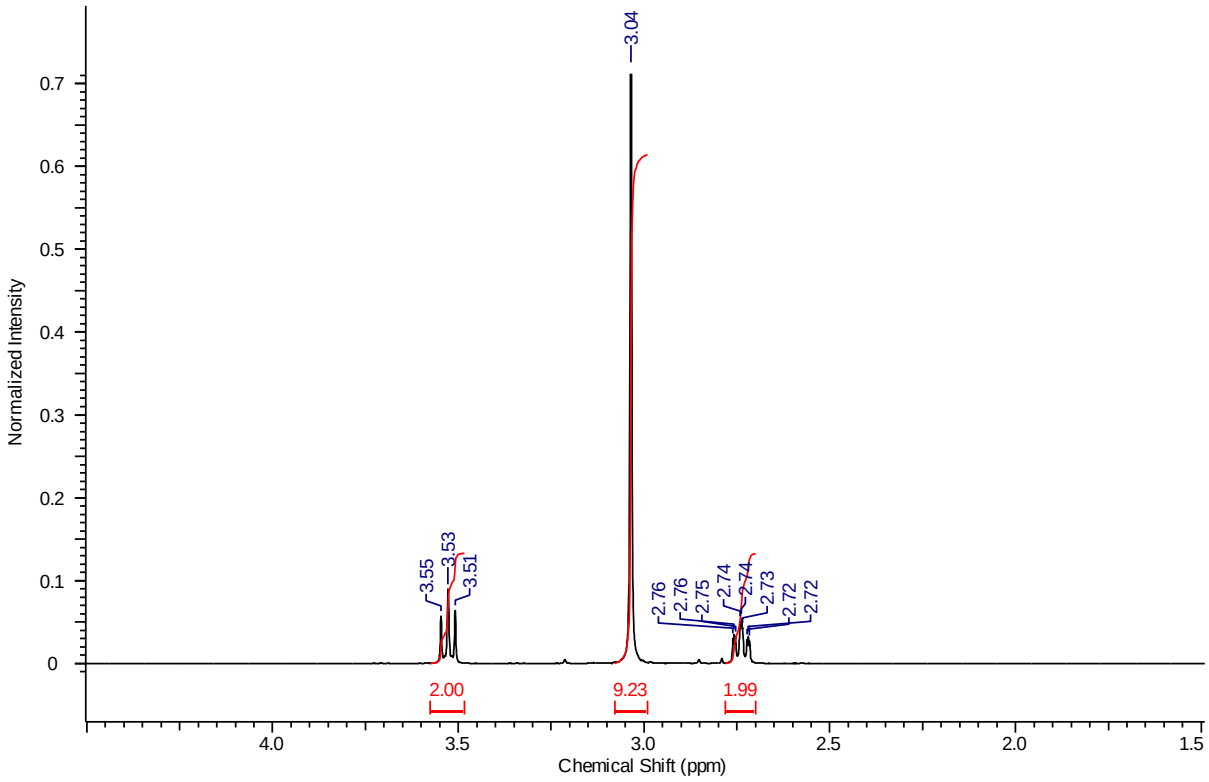


compound	n	X
GBB-3	1	H
GBB-5	3	H
GBB-6	4	H
GBB-OMe	2	OCH_3
GBB-CH3	2	CH_3

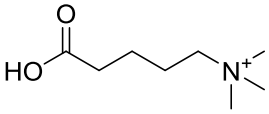
N$^{\beta}$-Trimethyl-3-aminopropionic acid bromide (GBB-3)



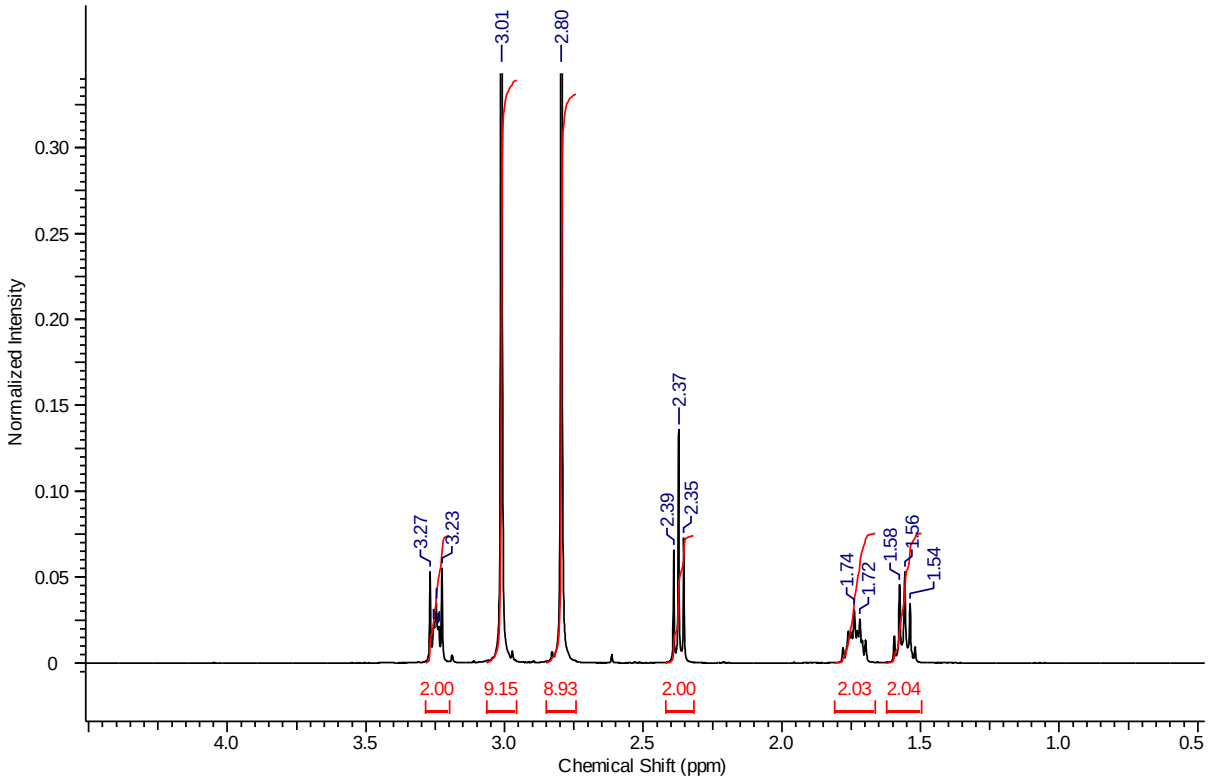
GBB-3 (125 mg, 0.6 mmol, 80%) was obtained as a white solid starting from 3-bromopropionic acid (100 mg, 0.66 mmol) and following the general procedure I. ^{1}H NMR (400 MHz, D_2O) δ = 3.53 (t, *J*=7.5 Hz, 2 H), 3.04 (s, 9 H), 2.74 (tt, *J*=7.5, 1.5 Hz, 2 H) ppm, ^{13}C NMR (101 MHz, D_2O) δ = 174.9, 62.5, 52.9, 29.5 ppm. Mp = 170-172°C. HRMS (ESI-TOF) calcd for $C_6H_{14}NO_2^+$ [M^+]: 132.1019, found: 132.1023. FT-IR νmax (neat): 2950, 1704, 1489, 1386, 1256 cm^{-1}.



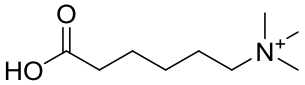
***N*$^{\delta}$-Trimethyl-5-aminopentanoic acid bromide (GBB-5)**



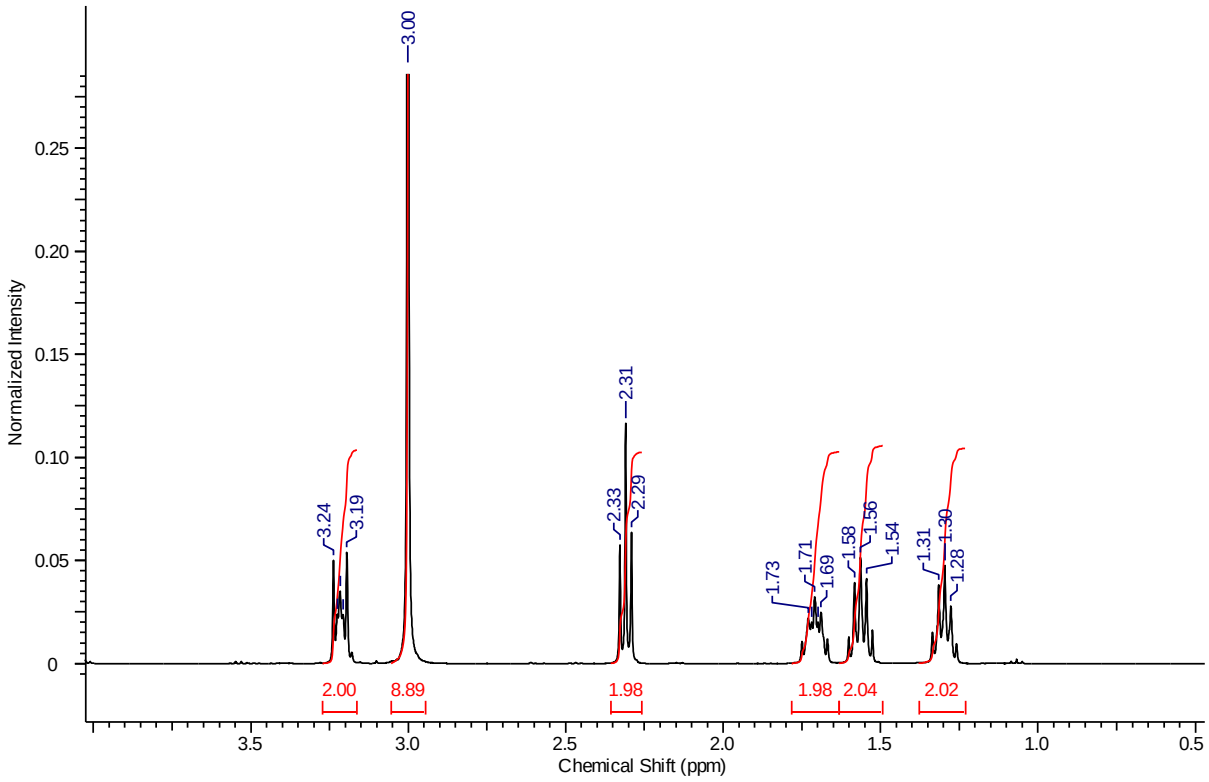
GBB-5 (105 mg, 0.4 mmol, 88%) was obtained as a white solid starting from 5-bromopentanoic acid (100 mg, 0.5 mmol) and following the general procedure I. ^{1}H NMR (400 MHz, D_2O) δ = 3.20 - 3.29 (m, 2 H), 3.01 (s, 10 H), 2.37 (t, *J*=7.0 Hz, 2 H), 1.73 (d, *J*=8.0 Hz, 2 H), 1.49 - 1.63 (m, 2 H) ppm, ^{13}C NMR (101 MHz, D_2O) δ = 178.0, 66.1, 52.8, 44.7, 33.0, 21.8, 21.0 ppm. Mp = 160-162°C. HRMS (ESI-TOF) calcd for $C_8H_{18}NO_2^+$ [M^+]: 160.1332, found: 160.1336. FT-IR νmax (neat): 2955, 2472, 2160, 1709, 1401, 1167 cm^{-1}.



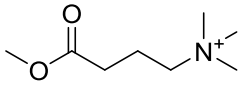
N$^{\varepsilon}$-Trimethyl-6-aminohexanoic acid bromide (GBB-6)



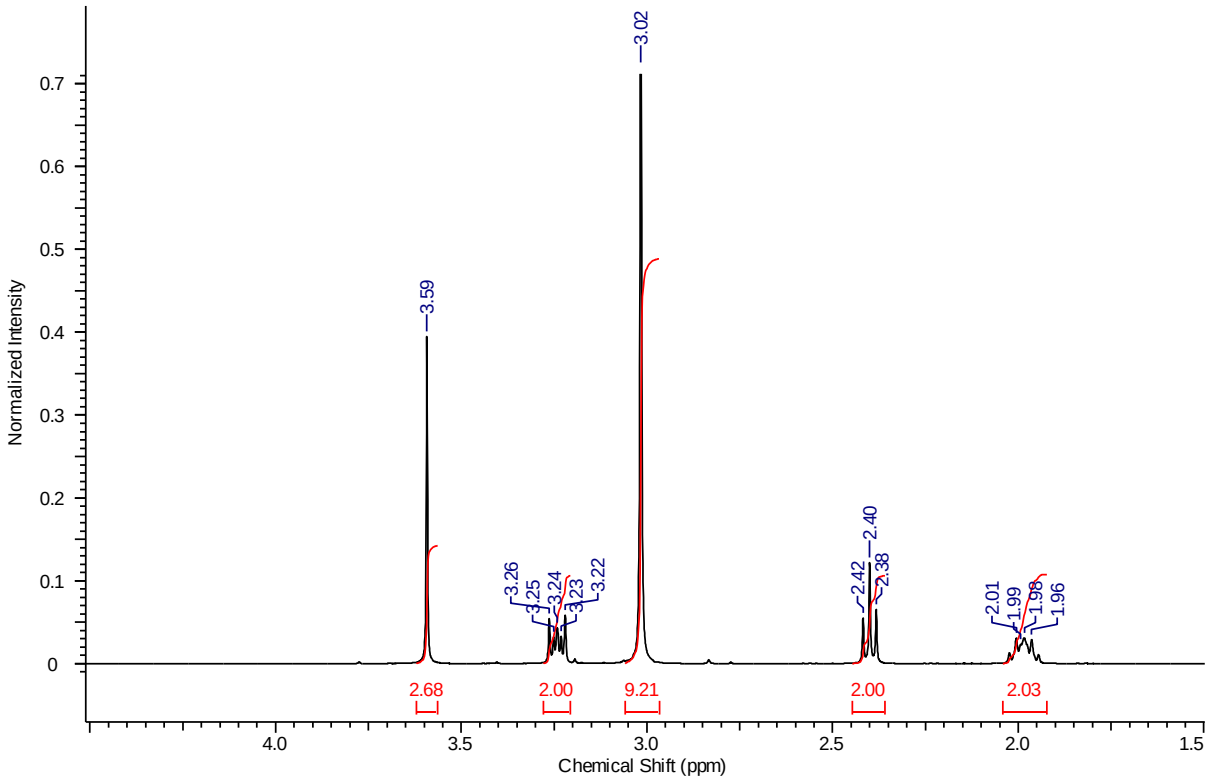
GBB-6 (105 mg, 0.4 mmol, 78%) was obtained as a white solid starting from 5-bromohexanoic acid (100 mg, 0.5 mmol) and following the general procedure I. ^{1}H NMR (400 MHz, D_2O) δ = 3.16 - 3.26 (m, 2 H), 3.00 (s, 9 H), 2.31 (t, *J*=7.5 Hz, 2 H), 1.71 (dt, *J*=8.0, 4.0 Hz, 2 H), 1.49 - 1.62 (m, 2 H), 1.24 - 1.36 (m, 2 H) ppm, ^{13}C NMR (101 MHz, D_2O) δ = 178.8, 66.4, 52.8, 33.5, 24.9, 23.7, 22.0 ppm. Mp = 177-179°C. HRMS (ESI-TOF) calcd for $C_9H_{20}NO_2^+$ [M^+]: 174.1489, found: 174.1492. FT-IR νmax (neat): 2910, 1720, 1483, 1396, 1174 cm^{-1}.



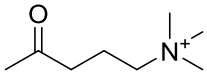
N$^{\epsilon}$-Trimethyl-4-aminobutanoic acid methyl ester bromide (GBB-OMe)



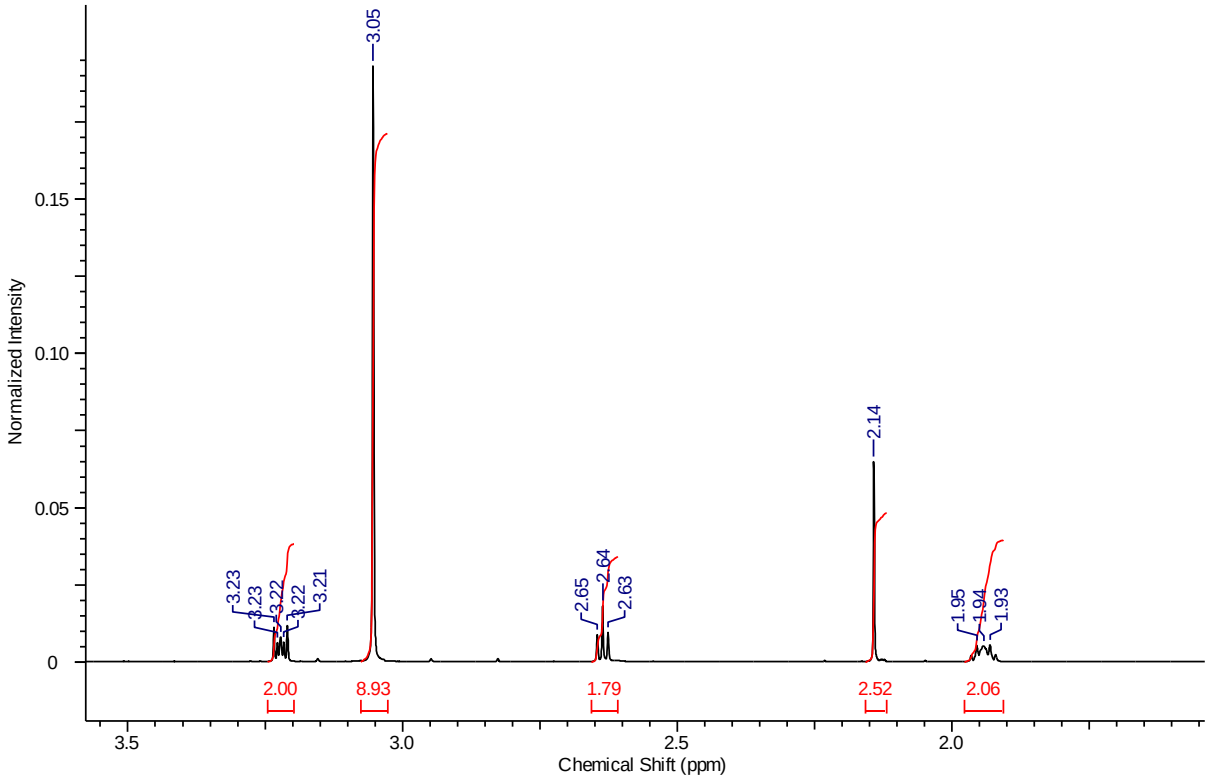
GBB-OMe (60 mg, 0.4 mmol, 75%) was obtained as a white solid starting from 4-bromobutanoic acid methyl ester (50 mg, 0.5 mmol) and following the general procedure I. ^{1}H NMR (400 MHz, D_2O) δ = 3.59 (s, 3 H), 3.19 - 3.30 (m, 2 H), 3.02 (s, 9 H), 2.40 (t, *J*=7.0 Hz, 2 H), 1.91 - 2.06 (m, 2 H) ppm, ^{13}C NMR (101 MHz, D_2O) δ = 175.0, 65.3, 52.8, 52.3, 29.9, 17.8 ppm. Mp = 75-77°C. HRMS (ESI-TOF) calcd for $C_8H_{18}NO_2^+$ [M^+]: 160.1332, found: 160.1330. FT-IR νmax (neat): 1736, 1480, 1380, 1193 cm^{-1}.



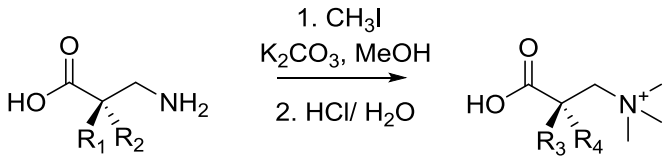
N$^{\epsilon}$-Trimethyl-4-amino-2-oxopentane bromide (GBB-CH3)



GBB-OMe (54 mg, 0.2 mmol, 80%) was obtained as a white hygroscopic solid following the general procedure I, starting from 4-bromo-2-oxopentane (50 mg, 0.3 mmol), which was prepared from α-acetylbytyrolactone according to literature procedure. ^{1}H NMR (400 MHz, D_2O) δ = 3.19 - 3.27 (m, 2 H), 3.05 (s, 9 H), 2.64 (t, *J*=7.0 Hz, 2 H), 2.14 (s, 3 H), 1.88 - 1.99 (m, 2 H) ppm, ^{13}C NMR (101 MHz, D_2O) δ = 213.2, 65.4, 65.4, 52.8, 38.9, 29.2, 16.6 ppm. Mp = 97-99°C. HRMS (ESI-TOF) calcd for $C_8H_{18}NO^+$ [M^+]: 144.1383, found: 144.1382. FT-IR νmax (neat): 1708, 1484, 1362, 1174 cm^{-1}.

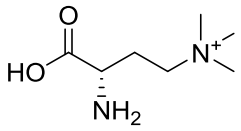


GBB analogues synthesized according to general procedure II

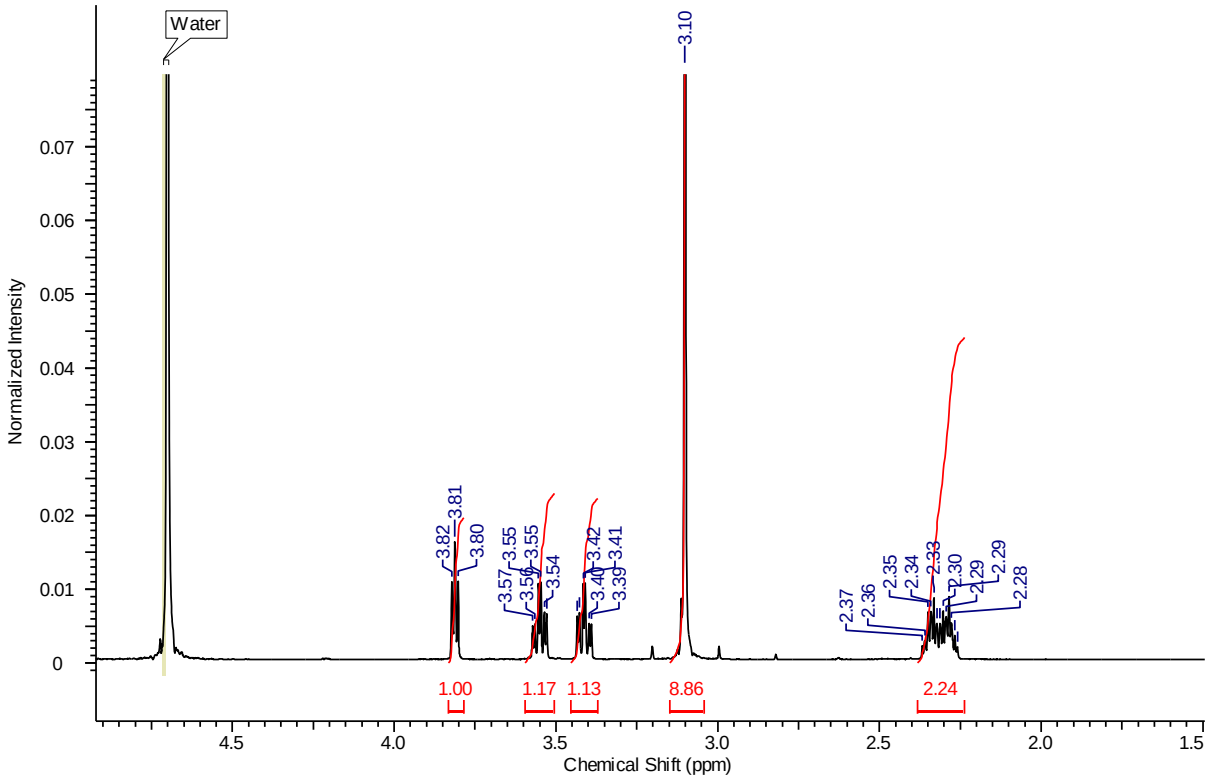


compound	R_1	R_2	R_3	R_4
GBB-NH(S)	H	NHBoc	H	NH_2
GBB-NH(R)	NHBoc	H	NH_2	H
GBB-OH	H	OH	H	OH

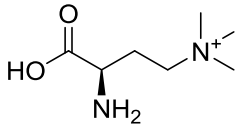
N$^{\gamma}$-Trimethyl-(S)-2,4-diaminobutanoic acid (GBB-NH(S))



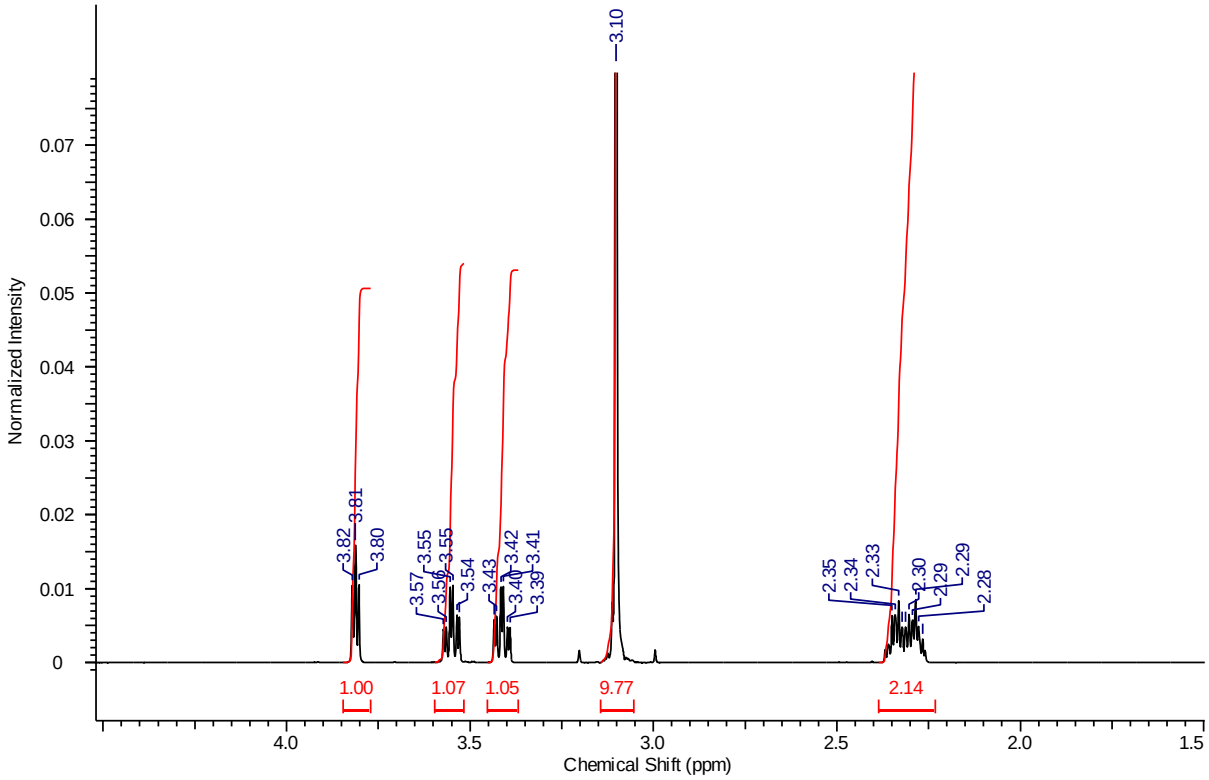
The GBB-NH(S) trifluoroacetic acid (TFA) salt (6 mg, 0.022 mmol, 10%) was obtained starting from (*S*)-4-amino-2-((tert-butoxycarbonyl)amino)butanoic acid (50 mg, 0.23 mmol) and following the general procedure 2. ^{1}H NMR (700 MHz, D_2O) δ = 3.81 (t, *J*=6.5 Hz, 1 H), 3.55 (td, *J* = 12.5, 4.5 Hz, 1 H), 3.41 (td, *J* = 12.5, 4.5 Hz, 1 H), 3.10 (s, 9 H), 2.25 - 2.38 (m, 2 H) ppm, ^{13}C NMR (126 MHz, D_2O) δ = 172.1, 62.6, 53.0, 51.5, 24.1 ppm. HRMS (ESI-TOF) calcd for $C_7H_{17}N_2O_2^+$ [M^+]: 161.1285, found: 161.1283. Due to the small amount of purified product available, IR spectra and optical rotation values are not reported for this and subsequent compounds. Melting points are not given due to highly hygroscopic nature of obtained material.



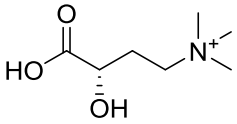
N$^{\gamma}$-Trimethyl-(R)-2,4-diaminobutanoic acid (GBB-NH(R))



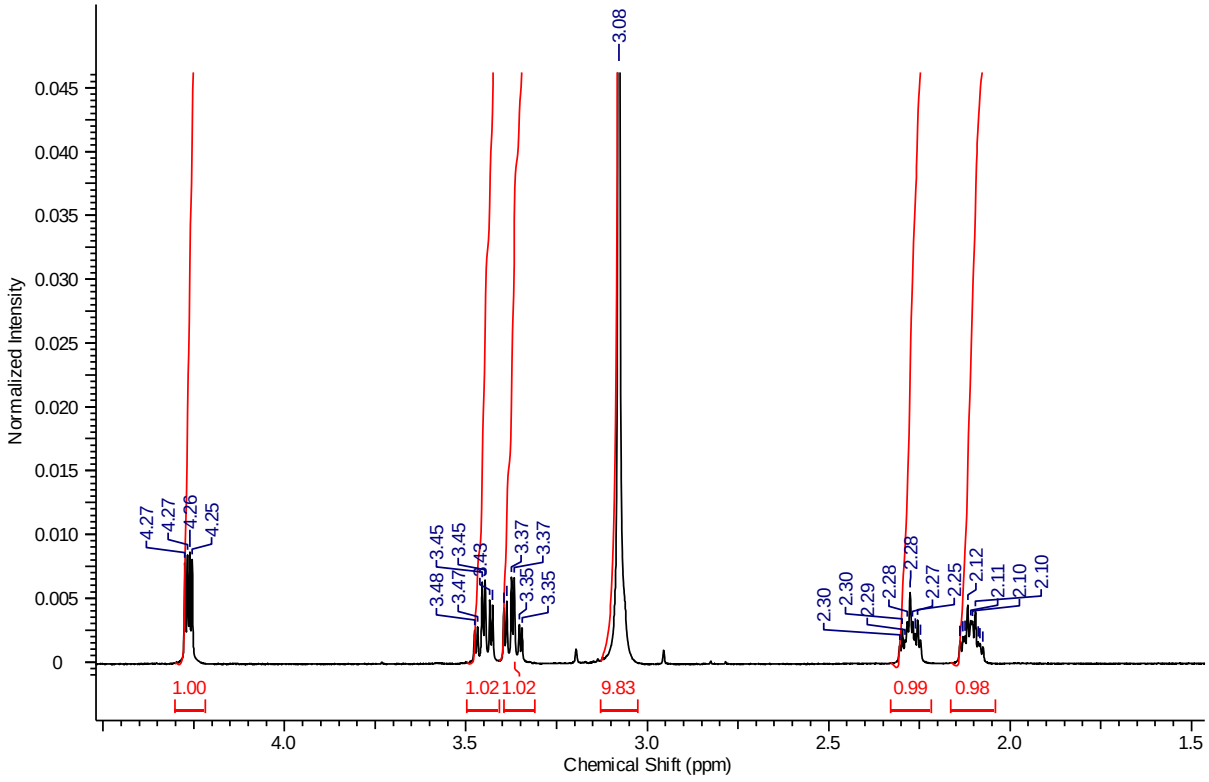
The GBB-NH(R) TFA salt (5mg, 0.018 mmol, 8%) was obtained starting from (*R*)-4-amino-2-((tert-butoxycarbonyl)amino)butanoic acid (50 mg, 0.23 mmol) and following the general procedure 2. ^{1}H NMR (700 MHz, D_2O) δ = 3.81 (t, *J*=6.5 Hz, 1 H), 3.55 (td, *J* = 12.5, 4.5 Hz, 1 H), 3.41 (td, *J* = 12.5, 4.5 Hz, 1 H), 3.10 (s, 9 H), 2.25 - 2.38 (m, 2 H) ppm, ^{13}C NMR (126 MHz, D_2O) δ = 172.1, 62.6, 53.0, 51.5, 24.1 ppm. HRMS (ESI-TOF) calcd for $C_7H_{17}N_2O_2^+$ [M^+]: 161.1285, found: 161.1283.



***N*$^{\gamma}$-Trimethyl-(S)-4-amino-2-hydroxybutanoic acid (GBB-OH(S))**



The GBB-OH(S) was obtained starting from 4-amino-(*2S*)-hydroxybutanoic acid hydrochloride and following the general procedure 2. ^{1}H NMR (400 MHz, D_2O) δ = 4.09 (dd, *J* = 7.3, 4.2 Hz, 1 H), 3.40 - 3.52 (m, 1 H), 3.27 - 3.38 (m, 1 H), 3.11 (s, 10 H), 2.21 (tt, *J* = 12.6, 4.4 Hz, 1 H), 2.06 (tdd, *J* = 12.5, 12.5, 7.2, 5.1 Hz, 1 H) ppm, ^{13}C NMR (101 MHz, D_2O) δ = 179.5, 69.3, 63.6, 53.2, 27.8 ppm. HRMS (ESI-TOF) calcd for $C_8H_{18}NO_2^+$ [M^+]: 162.1125, found: 162.1125.



References

1. J. A. Musich and H. Rapoport, *Journal of the American Chemical Society*, 1978, **100**, 4865-4872.
2. A. M. Rydzik, I. K. H. Leung, A. Thalhammer, G. T. Kochan, T. D. W. Claridge and C. J. Schofield, *Chemical Communications*, 2014, **50**, 1175-1177.
3. A. M. Rydzik, I. K. Leung, G. T. Kochan, A. Thalhammer, U. Oppermann, T. D. Claridge and C. J. Schofield, *Chembiochem : a European journal of chemical biology*, 2012, **13**, 1559-1563.
4. I. K. Leung, T. J. Krojer, G. T. Kochan, L. Henry, F. von Delft, T. D. Claridge, U. Oppermann, M. A. McDonough and C. J. Schofield, *Chemistry & biology*, 2010, **17**, 1316-1324.
5. K. Arnold, L. Bordoli, J. Kopp and T. Schwede, *Bioinformatics*, 2006, **22**, 195-201.
6. F. Kiefer, K. Arnold, M. Kunzli, L. Bordoli and T. Schwede, *Nucleic acids research*, 2009, **37**, D387-392.