Supplementary Information

Synthesis and structure-activity study of the antimicrobial lipopeptide brevibacillin

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MATERIAL AND EQUIPMENT

***Reagents*:** All the chemical reagents and solvents were purchased from commercial sources (Sigma-Aldrich, Fisher, Combi-Block and Oakwood Chemical) and used without further purification, unless otherwise indicated. Coupling reagents and *N*-Fmoc-protected amino acids were purchased from Matrix Innovation (Québec City, QC, Canada). Wang resin was purchased from Chem-Impex (Wood Dale, IL). Reactions on solid support were performed in filter columns (2 and 10 mL) from Roland Vetter Laborbedarf OHG (Ammerbuch, Germany).

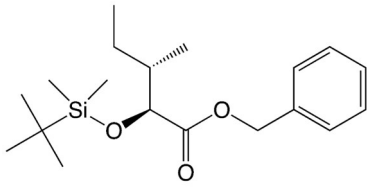
***Equipment*:** Peptide synthesis was performed on a Prelude peptide synthesizer from Gyros Protein Technologies (Tucson, AZ).

Nuclear magnetic resonance (NMR) spectra were recorded using a Bruker Ascend 300 MHz spectrometer (Billerica, MA, USA). Chemical shifts are reported in parts per million downfield from TMS. The chemical shifts (d) are expressed in ppm and referenced to acetone (2.06 ppm, ^{1}H), chloroform (7.26 ppm, ^{1}H and 77.0 ppm, ^{13}C), dimethylsulfoxide (2.50 ppm, ^{1}H) or methanol (3.33 ppm, ^{1}H). Splitting patterns are designated as s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), br (broad singlet) and m (multiplet).

HPLC and LC-MS analyses were conducted on a Shimadzu Prominence LCMS-2020 system equipped with an electro-spray ionization (ESI) probe using a Phenomenex Kinetex EVO C18 column (100 mm × 4.6 mm, 100 Å, 2.6 µm) using 0.1%HCOOH/H_2O (A) and 0.1% HCOOH/CH_3CN (B), with a linear gradient from 10 to 90% (B) for 10.5 min at a rate of 1.4 mL/min and ultraviolet detection at 220 and 254 nm.

High resolution mass spectra (HRMS) were measured with an Agilent 6210 LC Time of Flight mass spectrometer in electrospray mode with Agilent HP-0921 as internal standard. Either protonated molecular ions $[M + nH]^{n+}$, sodium adducts $[M + Na]^{+}$ or ammonium adducts $[M + NH_4]^{+}$ were used for empirical formula confirmation.

SYNTHESIS OF THE N-TERMINAL DIPEPTIDE

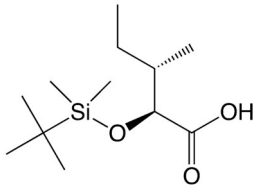


2

Benzyl (2S,3S)-2-{[tert-butyl(dimethyl)silyl]oxy}-3-methylpentanoate (2). A solution of $NaNO_2$ (3.16 g, 45.74 mmol) in 20 mL H_2O was added dropwise to a solution of L-isoleucine (3.0 g, 22.87 mmol) in 20 mL H_2O and conc. H_2SO_4 (0.755 mL, 13.72 mmol) at 0°C. Afterward, the mixture was stirred at room temperature for 16 h and 20 mL of 1N aqueous HCl solution and 3.0 g of NaCl were added to the reaction. After complete dissolution, the aqueous solution was extracted with AcOEt (5 x 30 mL). The organic layer was dried over Na_2SO_4 and concentrated in vacuo to give 2.72 g of isoleucic acid as a white waxy solid which was used in the next step without further purification.

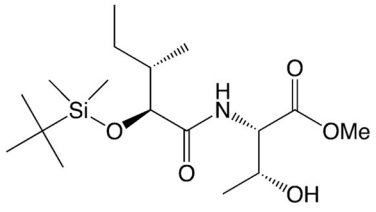
Isoleucic acid (2.72 g, 20.66 mmol) was dissolved in 60 mL DMF and 3 mL H_2O at 0°C followed by the addition of 1.1 equiv of K_2CO_3 (3.14 g, 22.73 mmol) and 1.05 equiv of BnBr (2.59 mL, 21.69 mmol). The mixture was stirred at room temperature for 16 h followed by quenching of the reaction by adding 1 g of K_2CO_3, 5 mL of MeOH and 50 mL H_2O. After stirring for 30 min, the solution was extracted with AcOEt (3 x 50 mL) and the organic phase washed with H_2O (50 mL), brine (50 mL) to be dried over Na_2SO_4 and concentrated in vacuo to give the benzyl ester as a yellow oil which was used in the next step without any further purification.

The benzyl ester was dissolved in CH_2Cl_2 (42 mL) and 2.4 equiv of imidazole (3.38 g, 49,58 mmol) and 1.2 equiv of TBSCl (3.74 g, 24.79 mmol) were added to the solution at 0°C. The mixture was stirred at room temperature for 16 h and the reaction quenched with the addition of 100 mL of sat. NH_4Cl solution. The solution was extracted with CH_2Cl_2 (3 x 50mL) followed by washing with brine (1 x 50 mL), drying over Na_2SO_4, and solvent removal under reduced pressure. The product was purified by flash column chromatography (SiO_2, 0 to 5% AcOEt/Hex; R_f = 0.50 in 5% AcOEt/Hex) to afford 6.22 g (78% over 3 steps) of **2** as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.04 (s, 3H), 0.05 (s, 3H), 0.87 (t, J = 7.4 Hz, 3H), 0.94 (d, J = 7.0 Hz, 3H overlapping s, 9H), 1.12 – 1.31 (m, 1H), 1.40 – 1.60 (m, 1H), 1.78 – 1.93 (m, 1H), 4.09 (d, J = 5.0 Hz, 1H), 5.18 (dd, J = 19.0, 12.3 Hz, 2H), 7.30 – 7.45 (m, 5H). ^{13}C NMR (76 MHz, $CDCl_3$) δ -5.4, -4.9, 11.5, 15.5, 18.3, 24.0, 25.7, 26.0, 39.5, 66.3, 76.6, 128.3, 128.5, 135.8, 173.4. HRMS (ESI+): *m/z* $[M+H]^+$ calcd for $C_{19}H_{33}O_3Si$: 337.2193, found 337.2199 The preparation and characterization of this compound has been previously reported.[1]



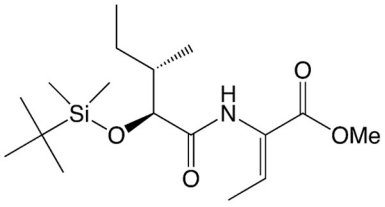
3

(2S,3S)-2-{[tert-butyl(dimethyl)silyl]oxy}-3-methylpentanoic acid (3). To a stirred solution of **2** (6.22 g, 18.48 mmol) in 37 mL of dry THF was added 0.622 g of 10% Pd/C (10 wt%). The atmosphere of the flask was purged with hydrogen and the mixture was stirred at room temperature for 4 h. The mixture was filtered on a pad of celite 545® and the solvent of the filtrate evaporated. The product was purified by flash column chromatography (SiO_2, 5% to 20% AcOEt/Hex; R_f = 0.1 in 5% AcOEt/Hex + 0.1% AcOH) to afford 4.16 g (91% yield) of acid **3** as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.10 (s, 6H), 0.89 (t, J = 7.3 Hz, 3H), 0.93 (s, 9H), 0.95 (d, J = 6.9 Hz, 3H), 1.17 – 1.38 (m, 1H), 1.41 – 1.61 (m, 1H), 1.73 – 1.91 (m, 1H), 4.13 (d, J = 4.0 Hz, 1H), 8.88 (br, 1H). ^{13}C NMR (76 MHz, $CDCl_3$) δ -5.2, -5.0, 11.7, 15.1, 18.1, 24.1, 25.7, 39.8, 76.2, 175.8. HRMS (ESI+): *m/z* $[M+H]^+$ calcd for $C_{12}H_{27}O_3Si$: 247.1724, found 247.1726. The preparation and characterization of this compound has been previously reported.[1]



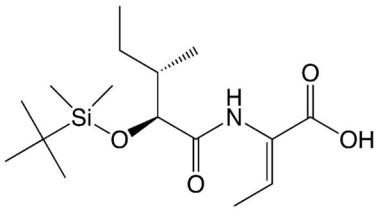
4

Methyl N-[(2S,3S)-2-{[tert-butyl(dimethyl)silyl]oxy}-3-methylpentanoyl]-L-threoninate (4). The *O*-protected isoleucic acid **3** (0.87 g, 3.54 mmol) and H-L-Thr-OMe (HCl) (0.66 g, 3.89 mmol) were dissolved in 8 mL of DMF and the solution cooled to 0°C. Then, 1.1 equiv of HATU (1.48 g, 3.89 mmol) and 3 equiv of NMM (1.42 mL, 10.62 mmol) were successively added and the resulting mixture stirred at room temperature for 16 h. The reaction was quenched by adding 50 mL of sat. $NaHCO_3$ solution and the mixture stirred for 10 min. After extraction with EtOAc (3 x 25 mL), the organic phase was washed with water (50 mL), brine (50 mL) and dried over Na_2SO_4. The solvent was removed under reduced pressure and the crude purified by flash column chromatography (SiO_2, 25% to 35% AcOEt/Hex, R_f = 0.25 in 25% AcOEt/Hex) to afford 0.99 g (78% yield) of dipeptide **4** as off-white shards. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.11 (s, 6H), 0.90 (t, J = 7.4 Hz, 3H), 0.96 (d, J = 7.0 Hz, 3H overlapping s, 9H), 1.21 (d, J = 6.5 Hz, 3H), 1.15 – 1.36 (m, 1H), 1.42 – 1.61 (m, 1H), 1.74 – 1.90 (m, 1H), 2.49 (br, 1H), 3.75 (s, 3H), 4.09 (d, J = 3.4 Hz, 1H), 4.35 (qd, J = 6.4, 2.5 Hz, 1H), 4.55 (dd, J = 9.0, 2.5 Hz, 1H), 7.31 (d, J = 8.8 Hz, 1H). ^{13}C NMR (76 MHz, $CDCl_3$) δ -5.3, -4.9, 11.9, 15.4, 18.0, 20.0, 24.0, 25.7, 39.8, 52.3, 56.7, 67.8, 77.4, 171.2, 173.7. HRMS (ESI+): *m/z* $[M+H]^+$ calcd for $C_{17}H_{36}NO_5Si$: 362.2357, found 362.2364.



5

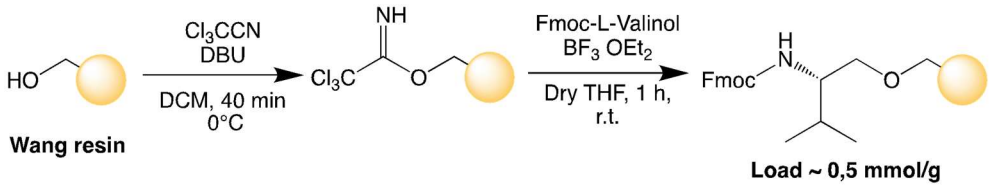
Methyl (2Z)-2-{[(2S,3S)-2-{[tert-butyl(dimethyl)silyl]oxy}-3-methylpentanoyl]amino}but-2-enoate (5). To a stirred solution of dipeptide **4** (0.97 g, 2.68 mmol) in 27 mL of CH_2Cl_2 was added 7 equiv of EDAC·HCl (3.6 g, 18.76 mmol) and 3 equiv of CuCl (0.79 g, 8.04 mmol). After stirring the mixture for 16 h at room temperature, the solvent was removed under reduced pressure and the product purified by flash column chromatography (SiO_2, 10% to 20% AcOEt/Hex, R_f = 0.7 in 25% AcOEt/Hex) to afford 0.81 g (87% yield) of **5** as a colorless oil that solidified upon freezing. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.12 (s, 6H), 0.91 (t, J = 7.4 Hz, 3H), 0.96 (s, 9H), 0.99 (d, J = 7.1 Hz, 3H), 1.16 – 1.35 (m, 1H), 1.49 – 1.65 (m, 1H), 1.77 (d, J = 7.2 Hz, 3H), 1.74 – 1.87 (m, 1H), 3.75 (s, 3H), 4.12 (d, J = 3.6 Hz, 1H), 6.80 (q, J = 7.2 Hz, 1H), 7.92 (s, 1H). ^{13}C NMR (76 MHz, $CDCl_3$) δ -5.2, -4.9, 12.0, 14.9, 15.3, 18.0, 24.0, 25.7, 40.3, 52.1, 77.6, 125.5, 133.3, 133.5, 164.7, 171.1. HRMS (ESI+): *m/z* $[M+H]^+$ calcd for $C_{17}H_{34}NO_4Si$: 344.2252, found 344.2257.



6

(2Z)-2-{[(2S,3S)-2-{[tert-butyl(dimethyl)silyl]oxy}-3-methylpentanoyl]amino}but-2-enoic acid (6). Dipeptide **5** (0.42 g, 1.23 mmol) was dissolved in THF (4 mL) and MeOH (1.4 mL) followed by the addition of 1N NaOH aqueous solution (1.36 mL, 1.36 mmol). The mixture was stirred until complete transformation of the starting material (typically 3 h). After completion, 25 mL of 1N HCl was poured in the flask and the mixture stirred for 5 min before extraction with EtOAc (3 x 20 mL). The organic phase was washed with brine, dried over Na_2SO_4 and concentrated in vacuo. The product was purified by flash column chromatography (SiO_2, 10% to 25% AcOEt/Hex, R_f = 0.3 in 25% AcOEt/Hex + 0.1% AcOH) to afford 0.29 g (72% yield) of **6** as an off-white waxy solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.13 (s, 3H), 0.14 (s, 3H), 0.92 (t, J = 7.4 Hz, 3H), 0.97 (s, 9H), 0.99 (d, J = 6.9 Hz, 3H), 1.20 – 1.38 (m, 1H), 1.49 – 1.69 (m, 1H), 1.83 (d, J = 7.3 Hz, 3H), 1.78 – 1.89 (m, 1H), 4.16 (d, J = 3.6 Hz, 1H), 6.97 (q, J = 7.2 Hz, 1H), 8.00 (s, 1H), 10.94 (s, 1H). ^{13}C NMR (76 MHz, $CDCl_3$) δ -5.2, -4.9, 12.0, 15.3, 15.3, 18.0, 24.0, 25.8, 40.3, 77.5, 125.1, 135.8, 169.2, 171.6. HRMS (ESI+): *m/z* $[M+H]^+$ calcd for $C_{16}H_{32}NO_4Si$: 330.2095, found 330.2097.

SOLID-PHASE PEPTIDE SYNTHESIS



Preparation of the Fmoc-valinol-Wang resin:[2] Wang resin (1.09 mmol/g) was first swelled in CH_2Cl_2 for 15 min by mechanical stirring followed by addition of trichloroacetonitrile (15 equiv). After cooling to 0°C, DBU (0.7 equiv) was added over a period of 5 min and the mixture stirred for 40 min at 0°C. The resin was then filtered in a peptide synthesis glass vessel and washed successively with DCM (3 × 30 s), DMSO (3 × 30 s), THF (3 × 30 s) and DCM (3 × 30 s). The resulting trichloroacetimidate resin (TCA-Wang resin) can be dried and stored at this point.

The TCA-Wang resin (1.09 mmol/g) was swelled in CH_2Cl_2 for 15 min followed by washing with dry THF (5 × 30 s). In a separate flask, Fmoc-L-Valinol (2.5 equiv) was dissolved in dry THF and the solution transferred to the resin. Afterward, $BF_3{\cdot}OEt_2$ (0.5 equiv) was added to the resin and the mixture stirred at room temperature for 60 min. MeOH (10% the volume of THF) was then added to the resin and stirring continued for another 15 min. The resin was filtered and washed successively with THF (3 × 30 s), DCM (3 × 30 s) and MeOH (3 × 30 s). The resin was dried in vacuo before the loading capacity determined by the Fmoc dosage assay described by Gude et *al*. and evaluated to 0.49 mmol/g.[2]

General solid-phase peptide synthesis procedure: Peptides were prepared by standard solid-phase peptide synthesis (SPPS) using the Fmoc/tBu strategy on preloaded Fmoc-Valinol-Wang resin. Briefly, the Fmoc protecting group was removed from the resin by treatments with 20 % piperidine in DMF (v/v) (2 x 7 min) and amino acid couplings were performed with Fmoc-Xaa-OH (3 equiv), HATU (3 equiv) and NMM (6 equiv) in DMF (2 × 20 min).

Dipeptide 6 conjugation: The peptides were synthesized up to the L-leucine (position 2) residue on the automatic peptide synthesizer and the terminal Fmoc was removed by treating the resin with a solution of 20% piperidine/DMF (2 x 7min). The resin (~100 mg, 0.049 mmol) was then washed with DMF (5 x 30 s) and transferred in a 5 mL glass vial equipped with a pressure relief septum. In a separate flask, 5 equiv of dipeptide **6** (0.081 g, 0.245 mmol) and 5 equiv of HOAt (0.033 g, 0.245 mmol) were dissolved in 2 mL of DMF. After addition of DIC (0.038 mL, 0.245 mmol, 5 equiv), the solution was added to the resin and the mixture was left without stirring for 16 h at 40 °C. After completion, the resin was filtered and washed with DMF (5 x 30 s).

Peptide cleavage and side chain deprotection: The resin (0.049 mmol) was washed with DCM (5 x 30 s) and treated with 6 mL of a deprotection solution of TFA/TIS/H_2O (95:2.5:2.5) for 90 min. After filtration of the resin, the filtrate was evaporated under reduced pressure and the peptide precipitated with cold Et_2O (45 mL). The precipitate was washed twice with diethyl ether and dried under vacuum.

The peptides were purified by semi-preparative RP-HPLC with a Shimadzu Prominence system on a Phenomenex Kinetex EVO C18 column (250 mm × 21.2 mm, 300 Å, 5µm) using 0.1%TFA/H_2O (A) and 0.1% TFA/CH_3CN (B), with a linear gradient from 10 to 60% (B) for 20 min at a rate of 12 mL/min and ultraviolet detection at 220 and 254 nm. The collected fractions containing the peptide with >95% purity were lyophilized to afford the desired peptide as a white powder.

Brevibacillin (**1**): White powder, 14% isolated yield (13.8 mg). RP-HPLC t_R = 8.93 min. HRMS (ESI-TOF) *m/z* calcd for $C_{80}H_{142}N_{16}O_{16}$: $[M+H]^+$ 1584.0862, $[M+2H]^{2+}$ 792.5468; found 1584.0914, 792.5457

Brevibacillin Val5 (**7**): White powder, 20% isolated yield (19.6 mg). RP-HPLC t_R = 8.89 min. HRMS (ESI-TOF) *m/z* calcd for $C_{79}H_{140}N_{16}O_{16}$: $[M+H]^+$ 1570.0706, $[M+2H]^{2+}$ 785.5389; found 1570.0749, 785.5363.

Brevibacillin Val4,5 (**8**): White powder, 21% isolated yield (21.2 mg). RP-HPLC t_R = 8.66 min. HRMS (ESI-TOF) *m/z* calcd for $C_{78}H_{138}N_{16}O_{16}$: $[M+H]^+$ 1556.0549, $[M+2H]^{2+}$ 778.5311; found 1556.0604, 778.5302

Brevibacillin Ile8 (**9**): White powder, 16% isolated yield (18.4 mg). RP-HPLC t_R = 9.28 min. HRMS (ESI-TOF) *m/z* calcd for $C_{81}H_{144}N_{16}O_{16}$: $[M+H]^+$ 1598.1019, $[M+2H]^{2+}$ 799.5546; found 1598.1082, 799.5537.

Brevibacillin COOH (**10**): White powder, 28% isolated yield (28 mg). RP-HPLC t_R = 8.60 min. HRMS (ESI-TOF) *m/z* calcd for $C_{80}H_{140}N_{16}O_{17}$: $[M+H]^+$ 1598.0655, $[M+2H]^{2+}$ 799.5364; found 1598.0715, 799.5351.

Brevibacillin CONH$_2$ (**11**): White powder, 7% isolated yield (7.6 mg). RP-HPLC t_R = 8.74 min. HRMS (ESI-TOF) *m/z* calcd for $C_{80}H_{141}N_{17}O_{16}$: $[M+H]^+$ 1597.0815, $[M+2H]^{2+}$ 799.5364; found 1597.0882, 799.5351.

Brevibacillin Thr1 (**12**): White powder, 18% isolated yield (17.8 mg). RP-HPLC t_R = 8.68 min. HRMS (ESI-TOF) *m/z* calcd for $C_{80}H_{144}N_{16}O_{17}$: $[M+H]^+$ 1602.0968, $[M+2H]^{2+}$ 801.5520; found 1602.1031, 801.5512.

Brevibacillin Ser1 (**13**): White powder, 15% isolated yield (14.9 mg). RP-HPLC t_R = 8.56 min. HRMS (ESI-TOF) *m/z* calcd for $C_{79}H_{142}N_{16}O_{17}$: $[M+H]^+$ 1588.0812, $[M+2H]^{2+}$ 794.5442; found 1588.0869, 794.544.

Brevibacillin Ala1 (**14**): White powder, 14% isolated yield (12.7 mg). RP-HPLC t_R = 9.00 min. HRMS (ESI-TOF) *m/z* calcd for $C_{79}H_{142}N_{16}O_{16}$: $[M+H]^+$ 1572.0862, $[M+2H]^{2+}$ 786.5468; found 1572.0924, 794.5459.

Brevibacillin Abu1 (**15**): White powder, 20% isolated yield (20 mg). RP-HPLC t_R = 9.02 min. HRMS (ESI-TOF) *m/z* calcd for $C_{80}H_{144}N_{16}O_{16}$: $[M+H]^+$ 1586.1019, $[M+2H]^{2+}$ 793.5546; found 1586.1079, 793.5533.

Brevibacillin Val1 (**16**): White powder, 6% isolated yield (7.9 mg). RP-HPLC t_R = 9.23 min. HRMS (ESI-TOF) *m/z* calcd for $C_{81}H_{146}N_{16}O_{16}$: $[M+H]^+$ 1600.1175, $[M+2H]^{2+}$ 800.5624; found 1600.1237, 800.5606.

Brevibacillin Abu1/Val4,5 (**17**): White powder, 10% isolated yield (12.2 mg). RP-HPLC t_R = 8.52 min. HRMS (ESI-TOF) *m/z* calcd for $C_{78}H_{140}N_{16}O_{16}$: $[M+H]^+$ 1558.0706, $[M+2H]^{2+}$ 779.5389; found 1558.0763, 779.5378.

Brevibacillin Abu1/CONH$_2$ (**18**): White powder, 9% isolated yield (8.7 mg). RP-HPLC t_R = 8.83 min. HRMS (ESI-TOF) *m/z* calcd for $C_{80}H_{143}N_{17}O_{16}$: $[M+H]^+$ 1599.0971, $[M+2H]^{2+}$ 800.0522; found 1599.1037, 800.0502.

Brevibacillin Ac-Ile/Abu1 (**19**): White powder, 12% isolated yield (15.8 mg). RP-HPLC t_R = 9.18 min. HRMS (ESI-TOF) *m/z* calcd for $C_{82}H_{147}N_{17}O_{16}$: $[M+H]^+$ 1627.1284, $[M+2H]^{2+}$ 814.0679; found 1627.1354, 814.0663.

Brevibacillin Ac-Ile/Abu1/*CONH$_2$* (**20**): White powder, 7% isolated yield (8 mg). RP-HPLC t_R = 8.83 min. HRMS (ESI-TOF) *m/z* calcd for $C_{82}H_{146}N_{18}O_{16}$: $[M+H]^+$ 1640.1237, $[M+2H]^{2+}$ 820.5655; found 1640.1295, 820.5643.

Figure S1. ^{1}H NMR spectra of compounds **2**-**6**.

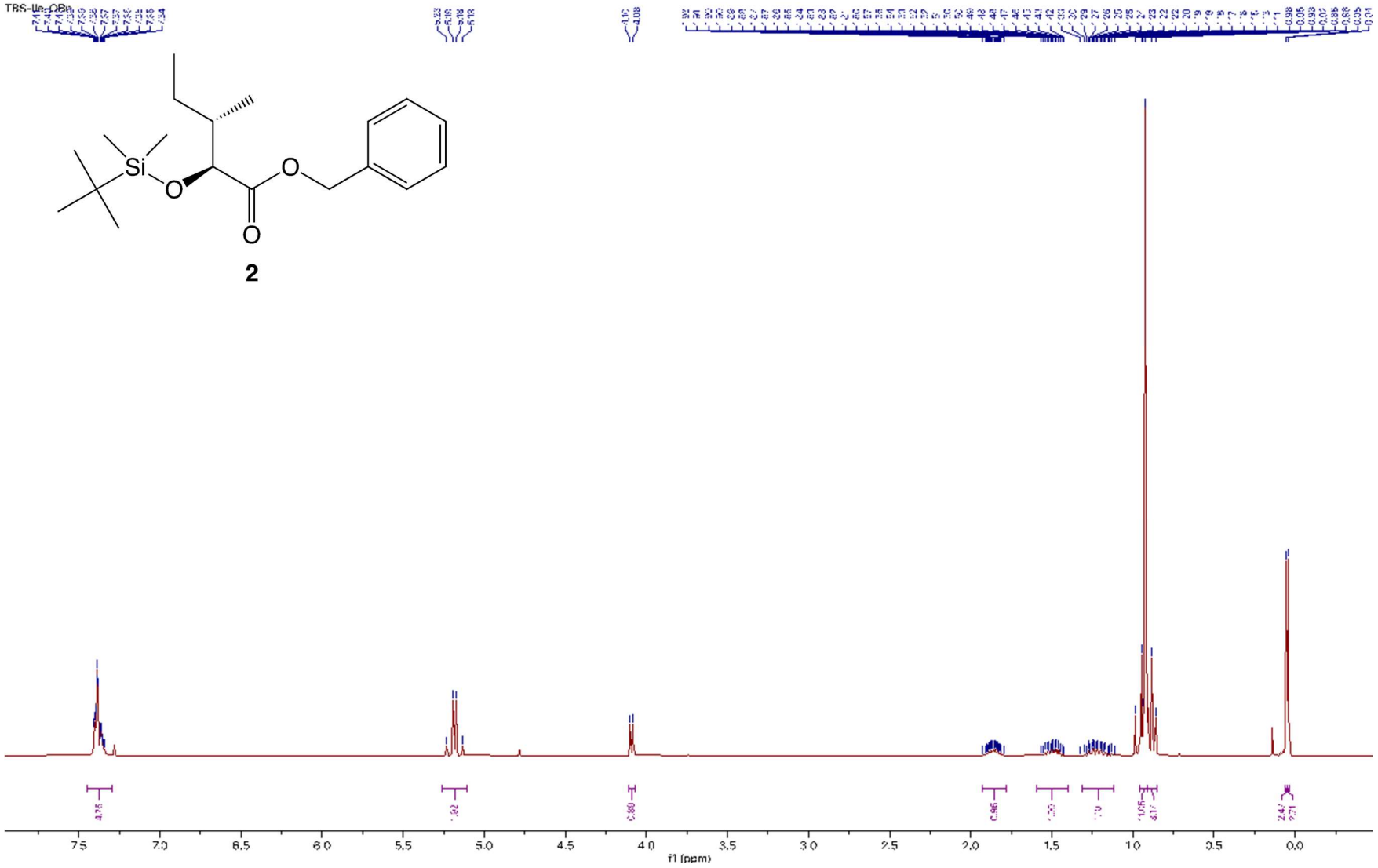


Figure S1. (Continued)

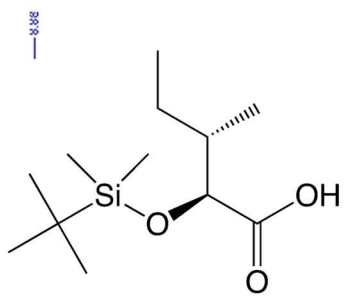


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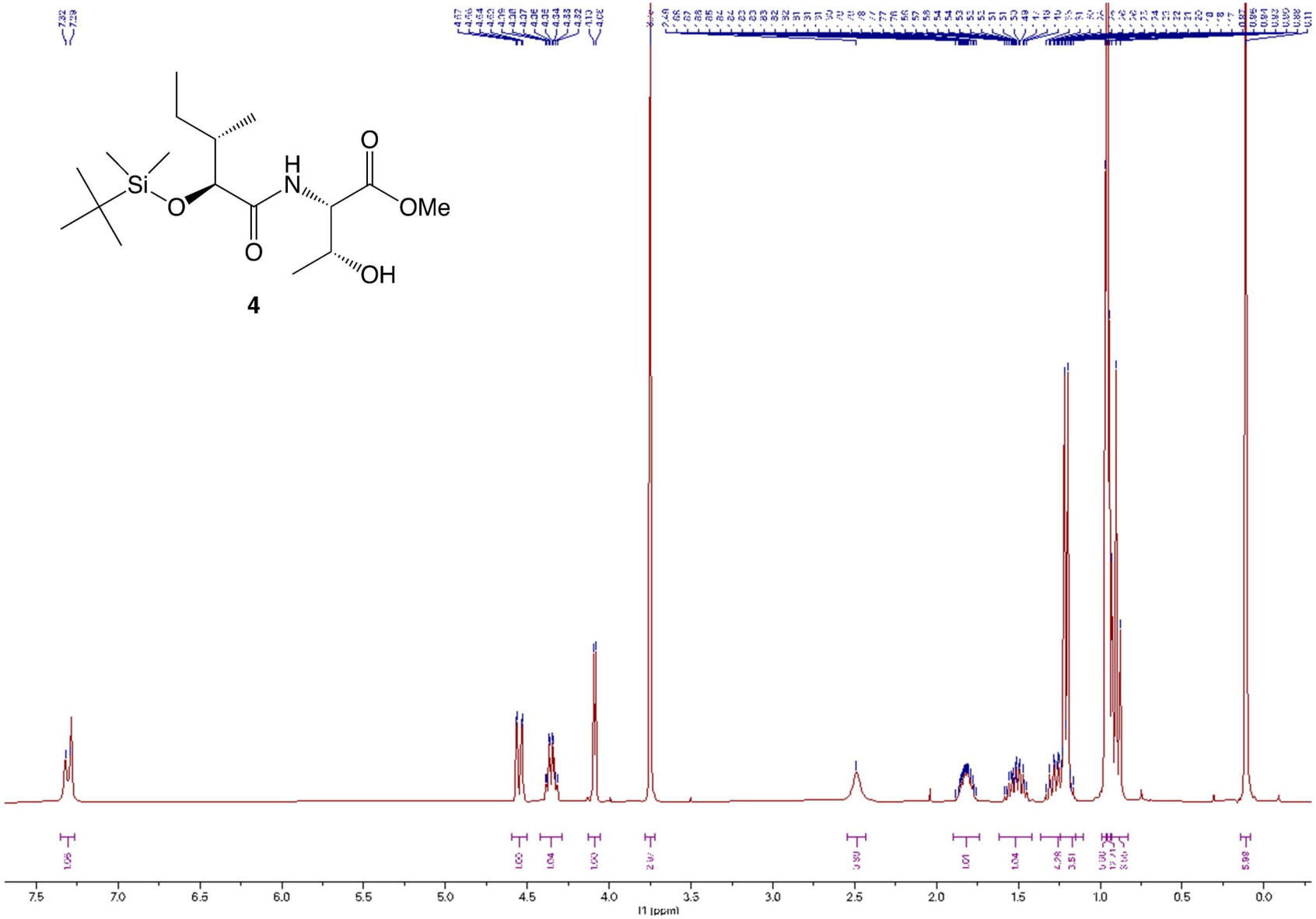
Figure S1. (Continued)



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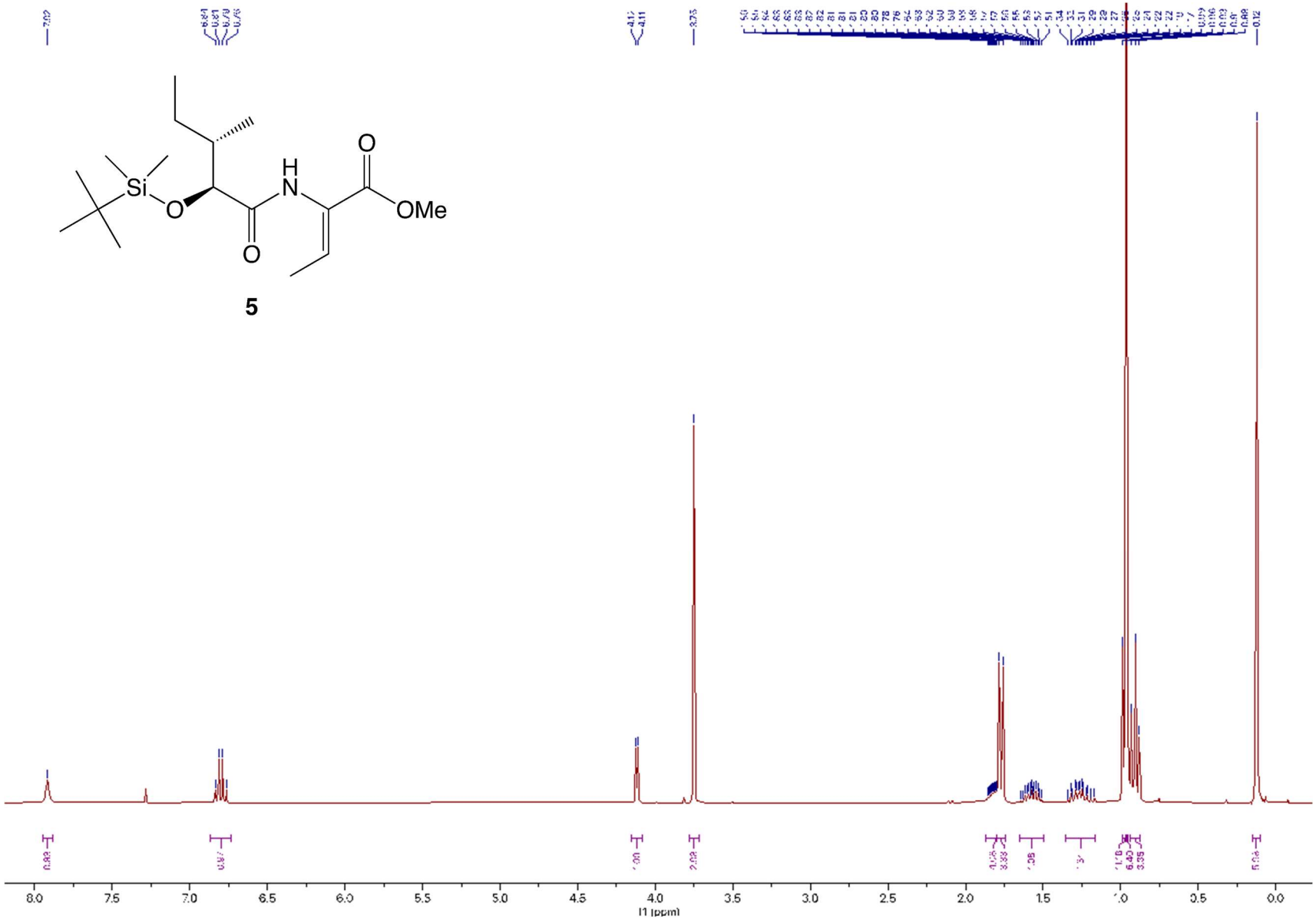

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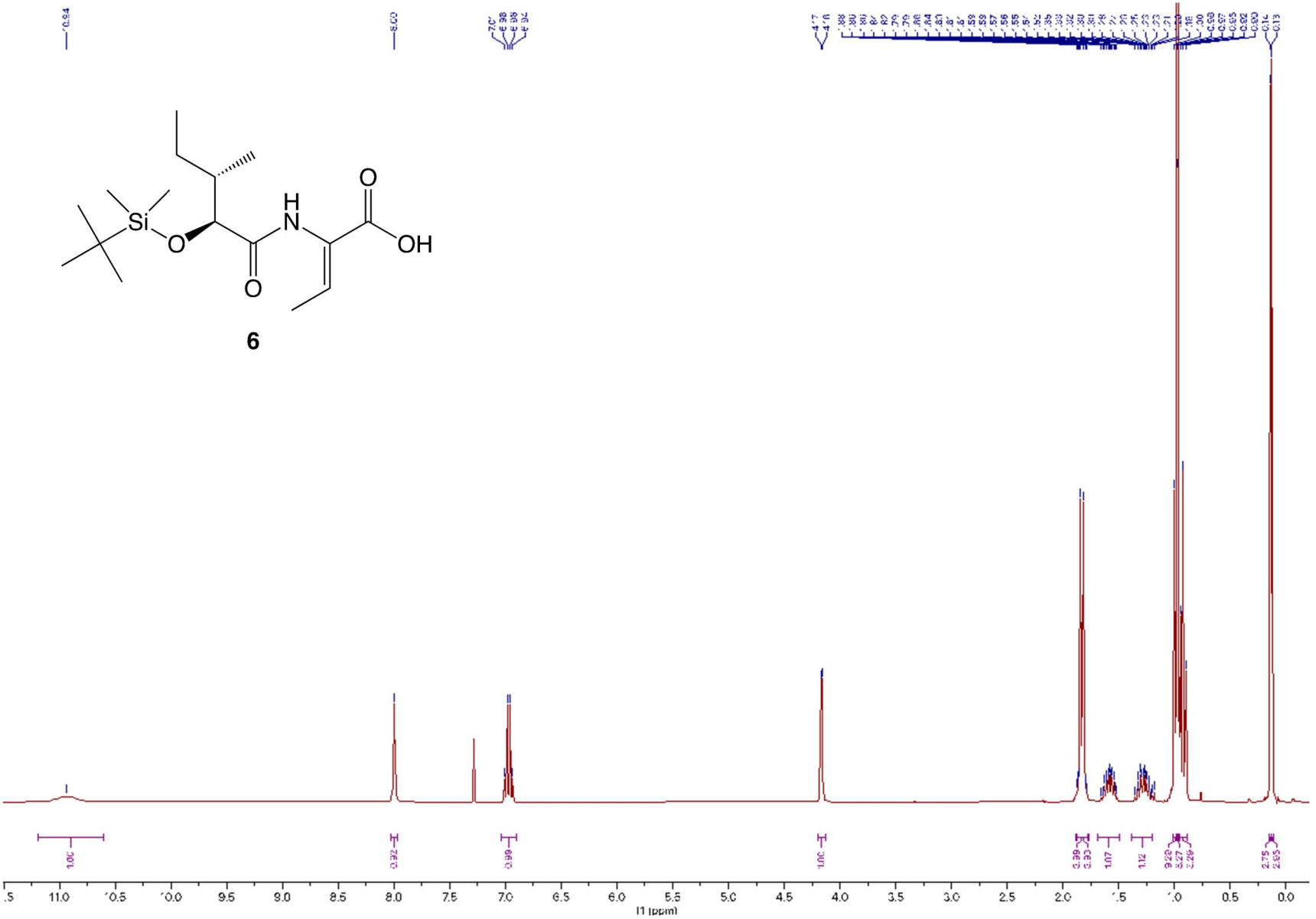


6

Figure S2. ^{13}C NMR spectra of compounds **2**-**6**.

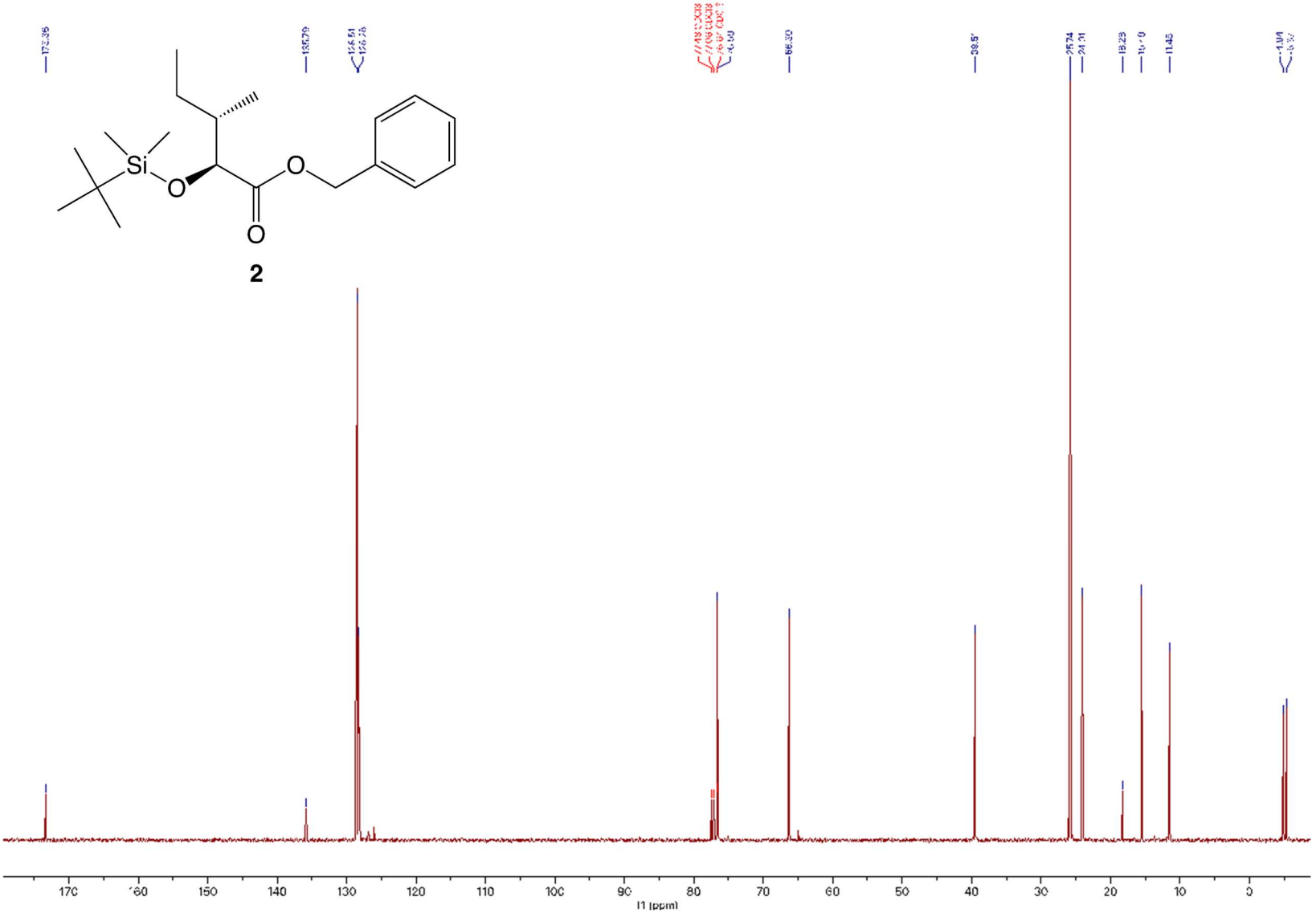


Figure S2. (Continued)

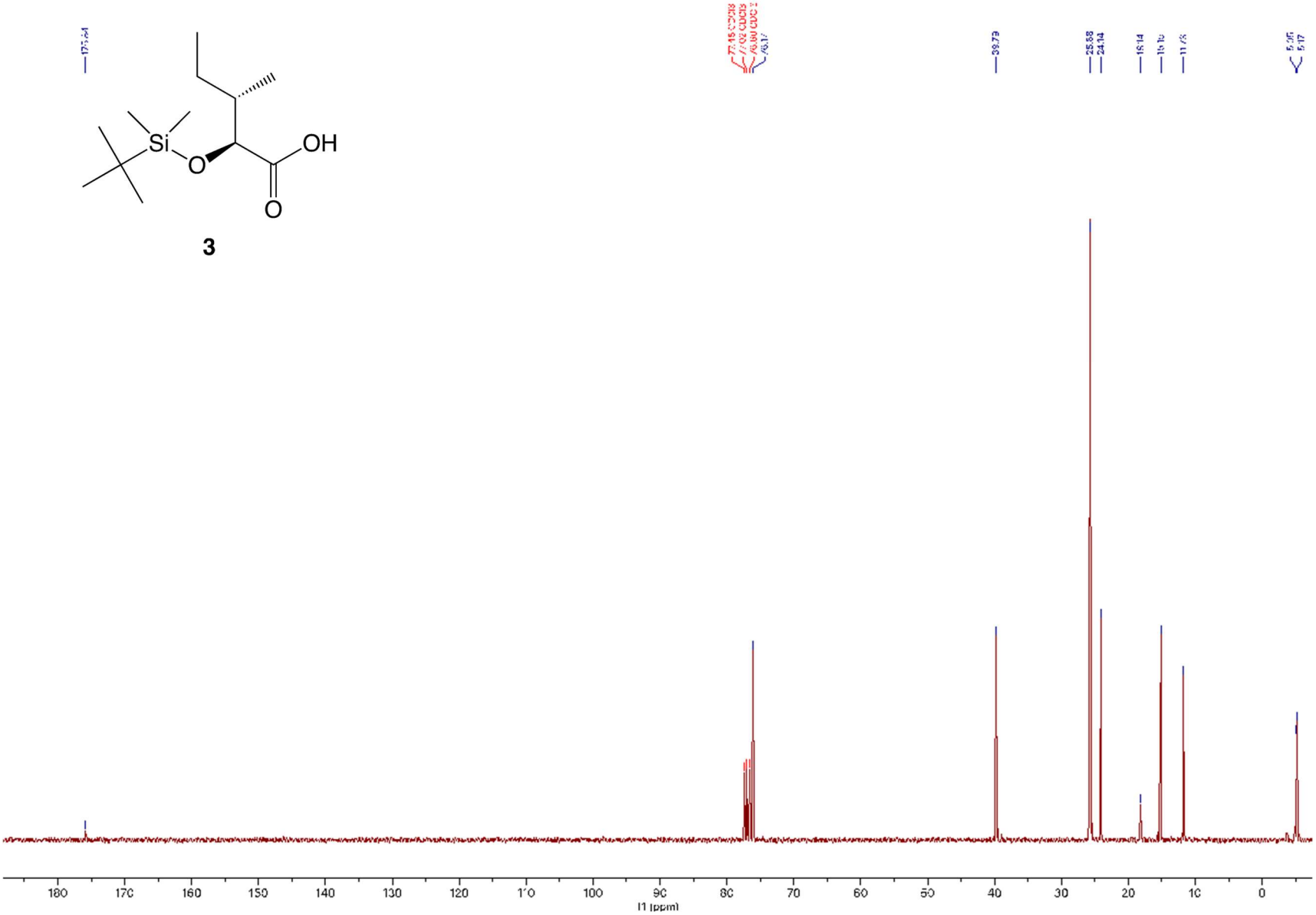


Figure S2. (Continued)

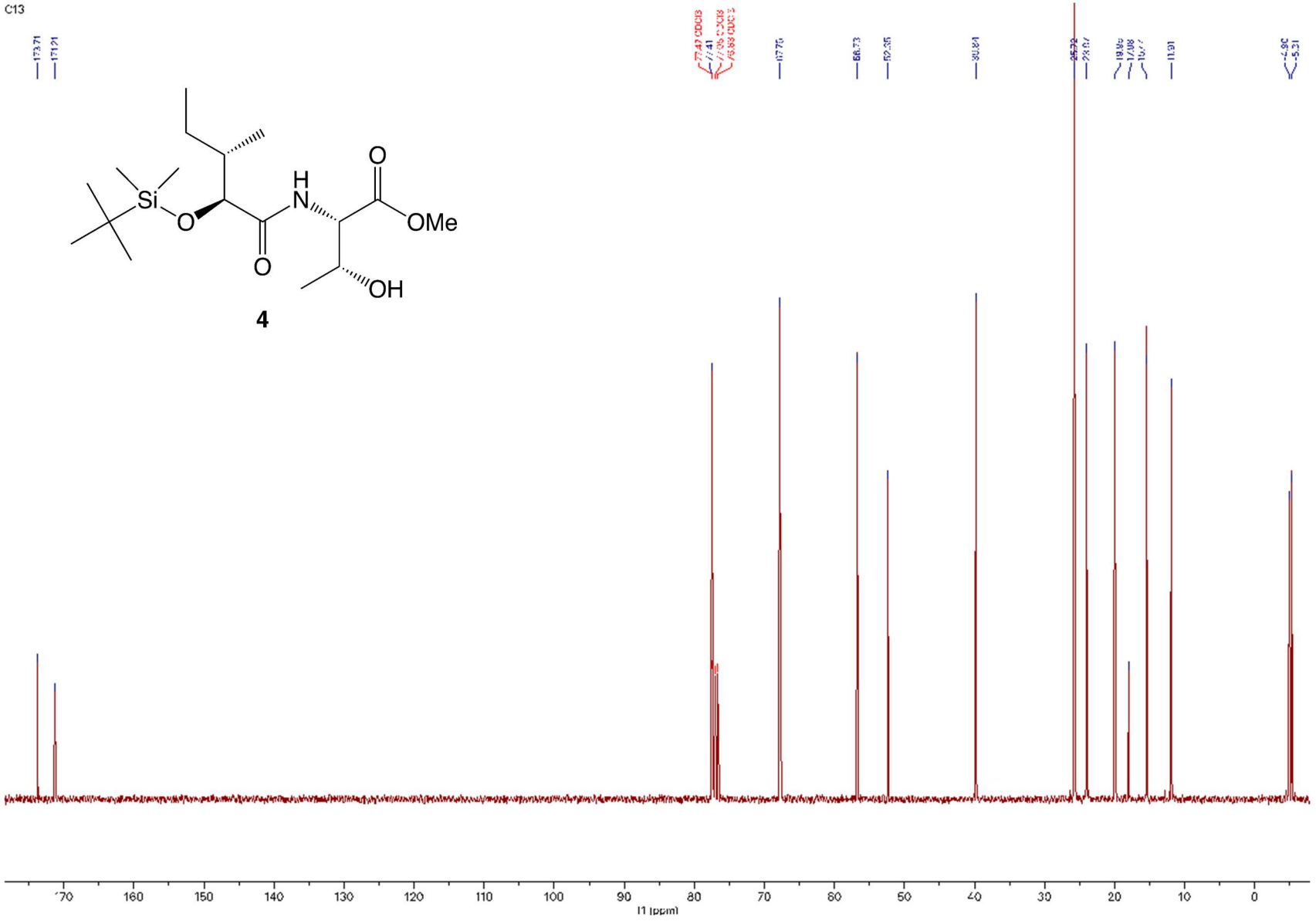


Figure S2. (Continued)

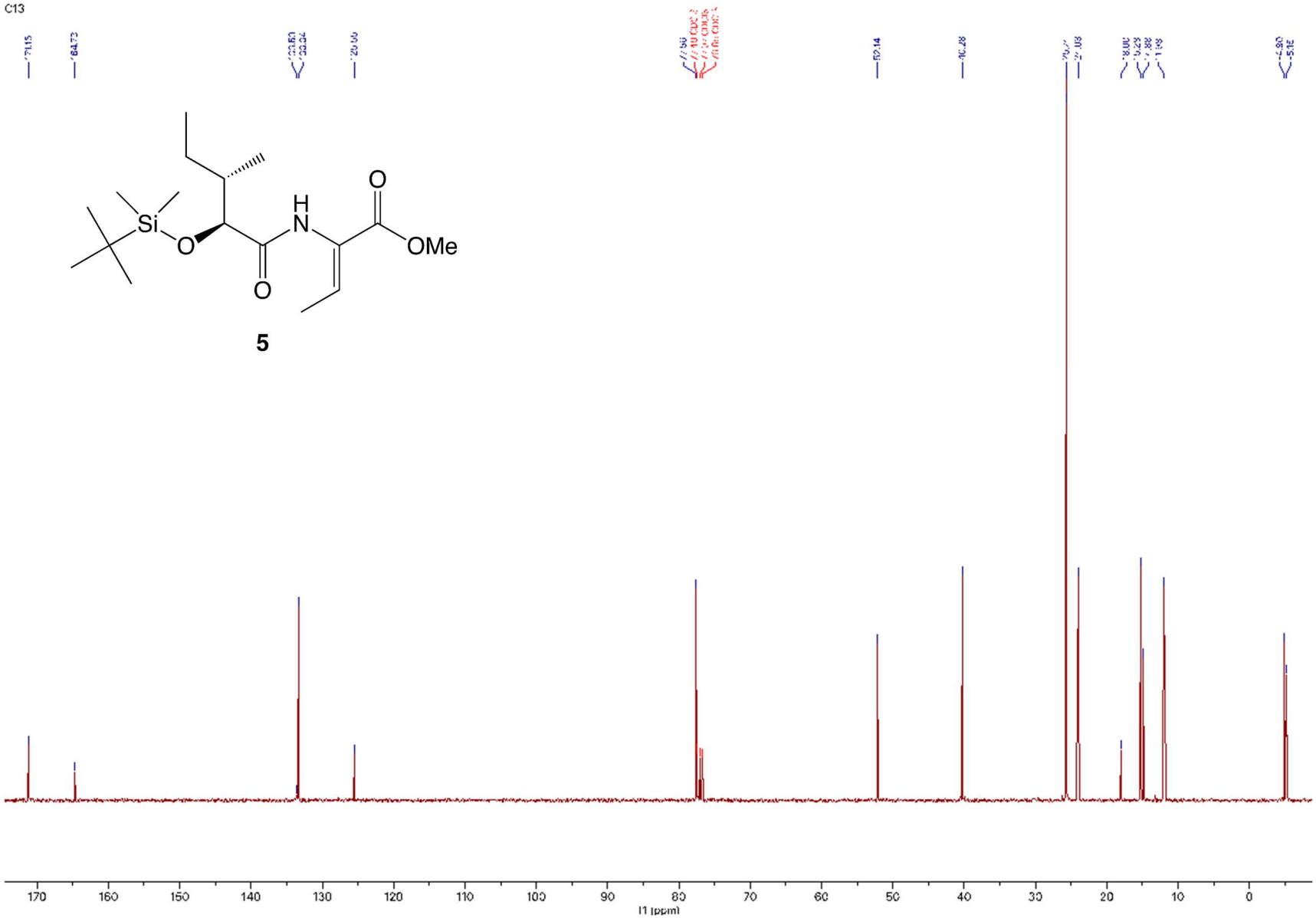


Figure S2. (Continued)

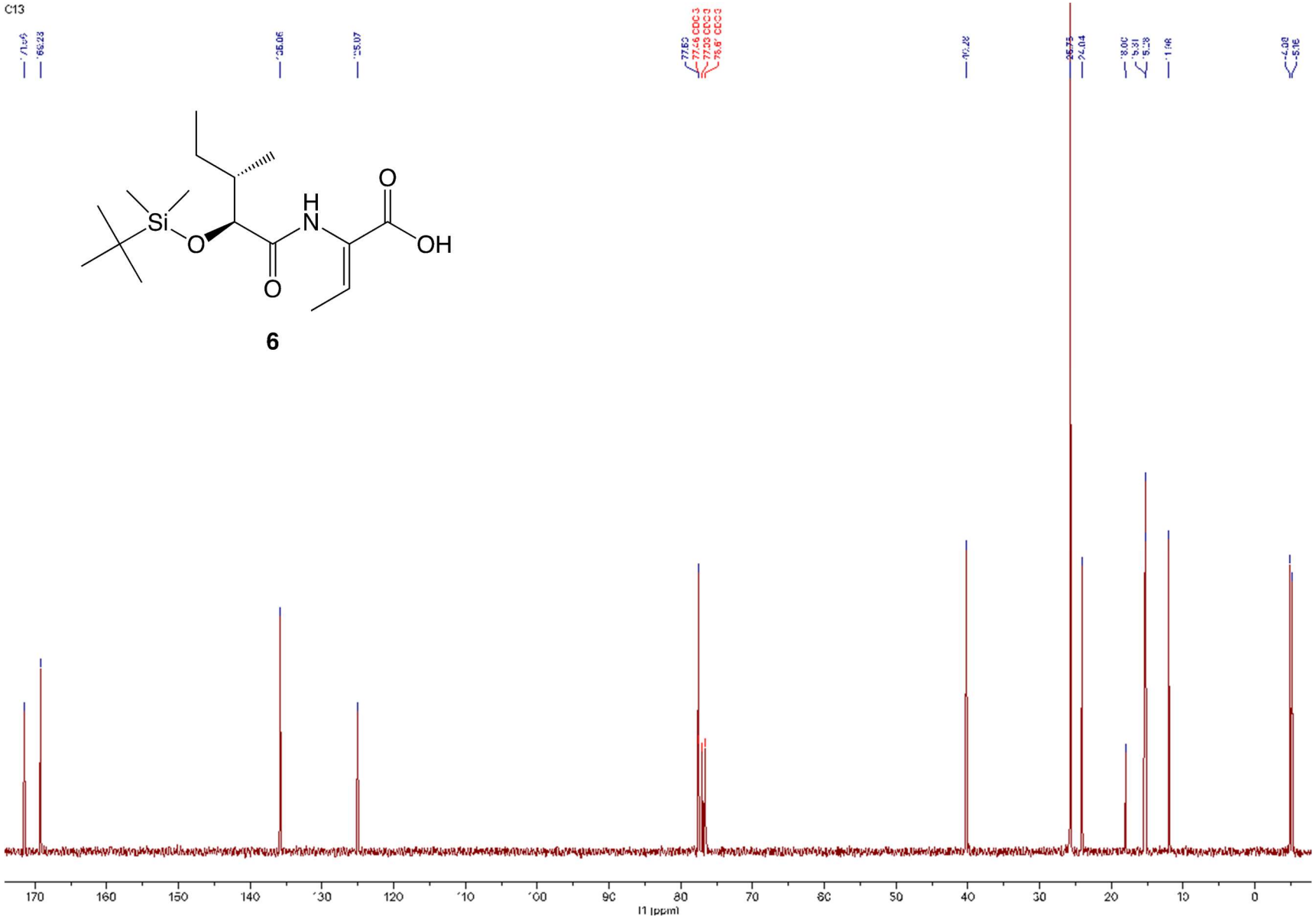
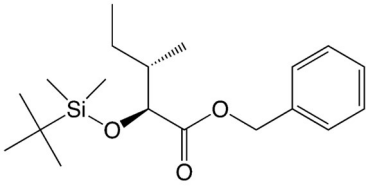


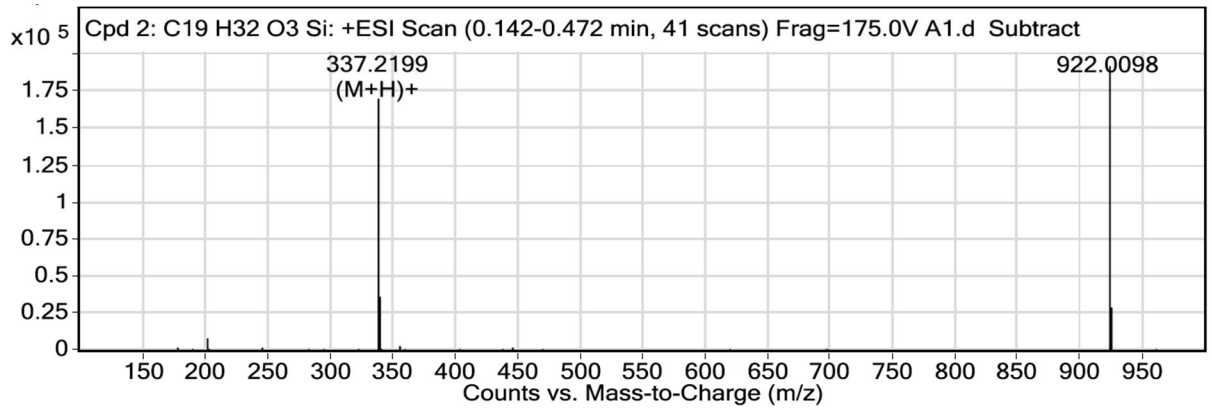
Figure S3. HRMS (ESI-TOF) spectra of compounds **2**-**6**.

Benzyl (2S,3S)-2-{[tert-butyl(dimethyl)silyl]oxy}-3-methylpentanoate (**2**)

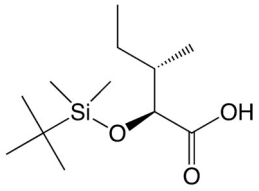


2

$[M+H]^+$ calculated for $C_{19}H_{32}O_3Si$: 337.2193; found 337.2199



(2S,3S)-2-{[tert-butyl(dimethyl)silyl]oxy}-3-methylpentanoic (**3**)



3

$[M+H]^+$ calculated for $C_{12}H_{26}O_3Si$: 247.1724; found 247.1726

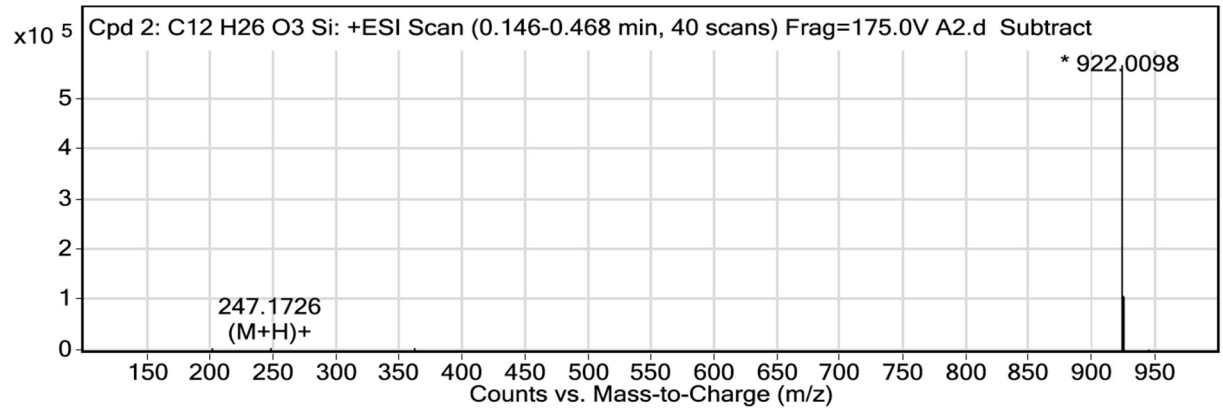
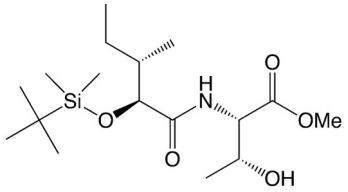


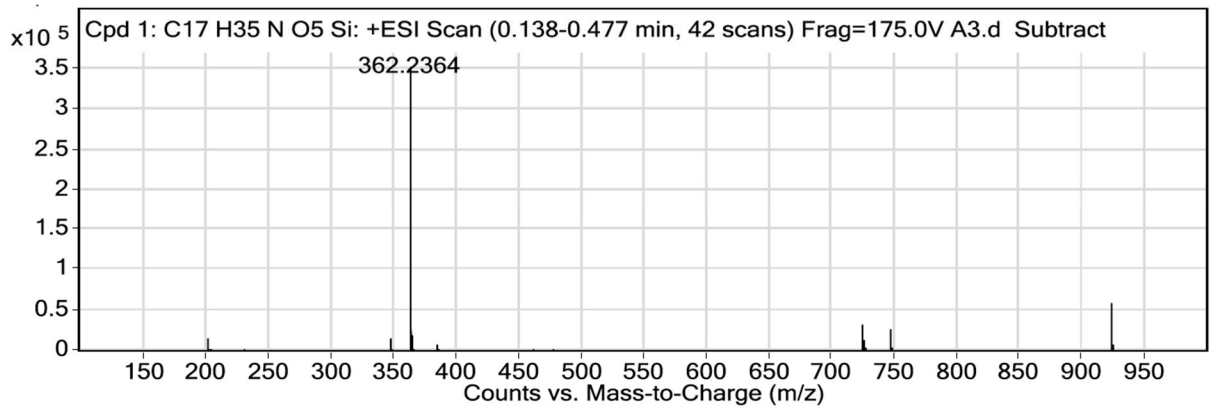
Figure S3. (Continued)

Methyl N-[(2S,3S)-2-{[tert-butyl(dimethyl)silyl]oxy}-3-methylpentanoyl]-L-threoninate (**4**)

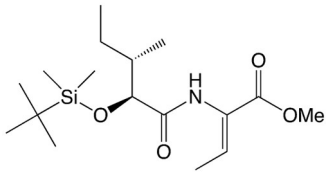


4

$[M+H]^+$ calculated for $C_{17}H_{36}NO_5Si$: 362.2357; found 362.2364



Methyl (2Z)-2-{[(2S,3S)-2-{[tert-butyl(dimethyl)silyl]oxy}-3-methylpentanoyl]amino}but-2-enoate (**5**)



5

$[M+H]^+$ calculated for $C_{17}H_{34}NO_4Si$: 344.2252; found 344.2257

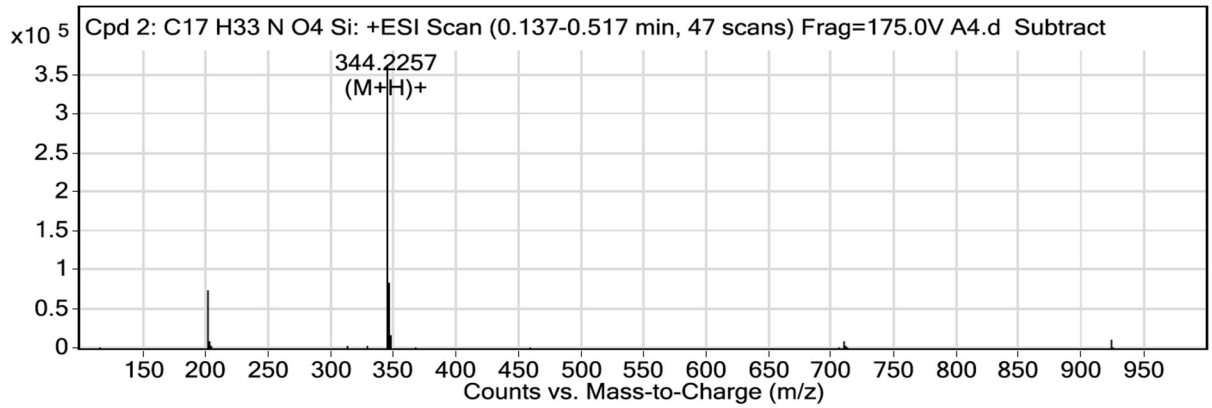
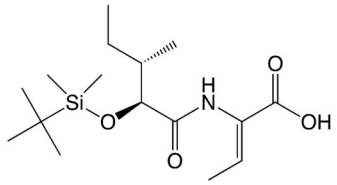


Figure S3. (Continued)

(2Z)-2-{[(2S,3S)-2-{[tert-butyl(dimethyl)silyl]oxy}-3-methylpentanoyl]amino}but-2-enoic acid (**6**)



6

$[M+H]^+$ calculated for $C_{16}H_{32}NO_4Si$: 330.2095; found 330.2097

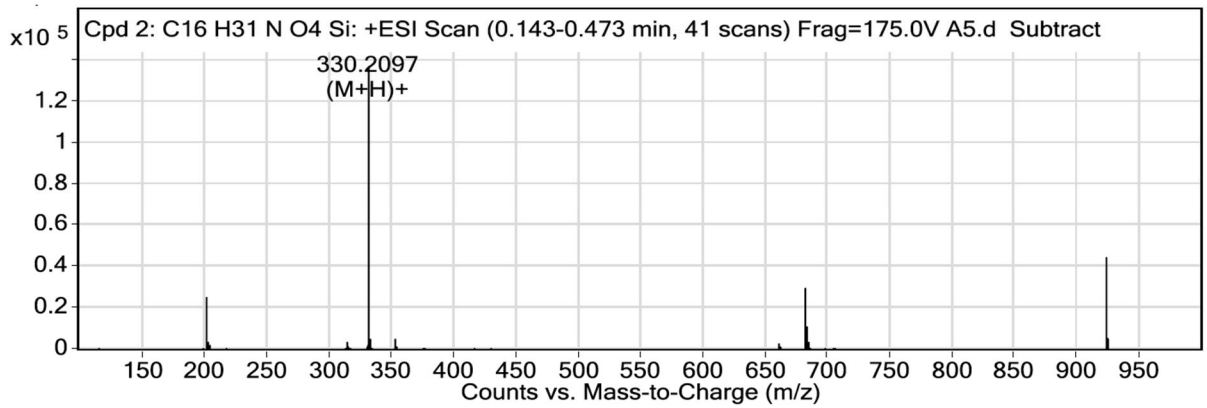
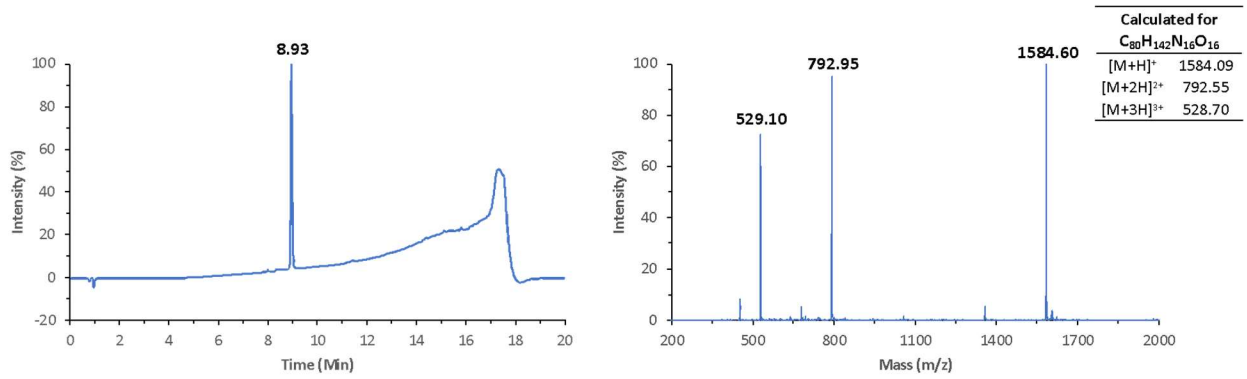
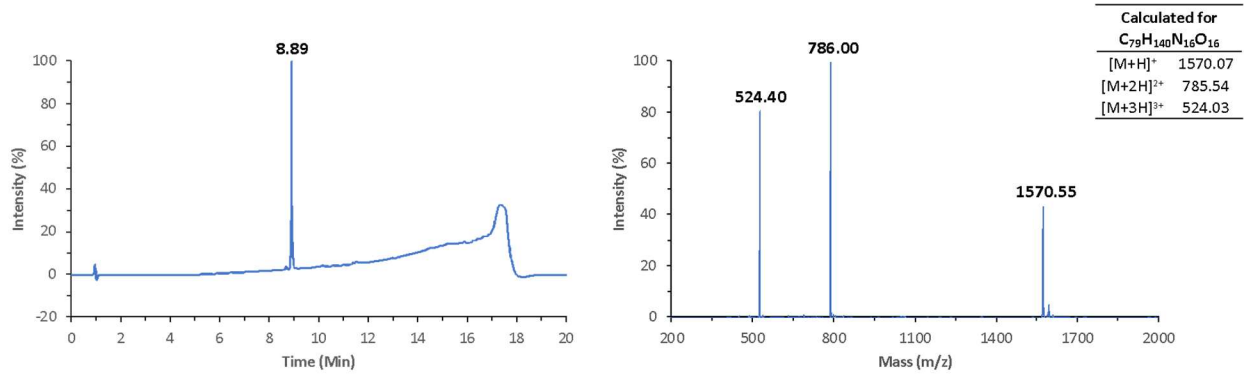


Figure S4. HPLC profiles (λ = 220 nm) and ESI-MS spectra of synthesized peptides **1**, **7**-**20**.

Brevibacillin (**1**)



Brevibacillin Val5 (**7**)



Brevibacillin Val4,5 (**8**)

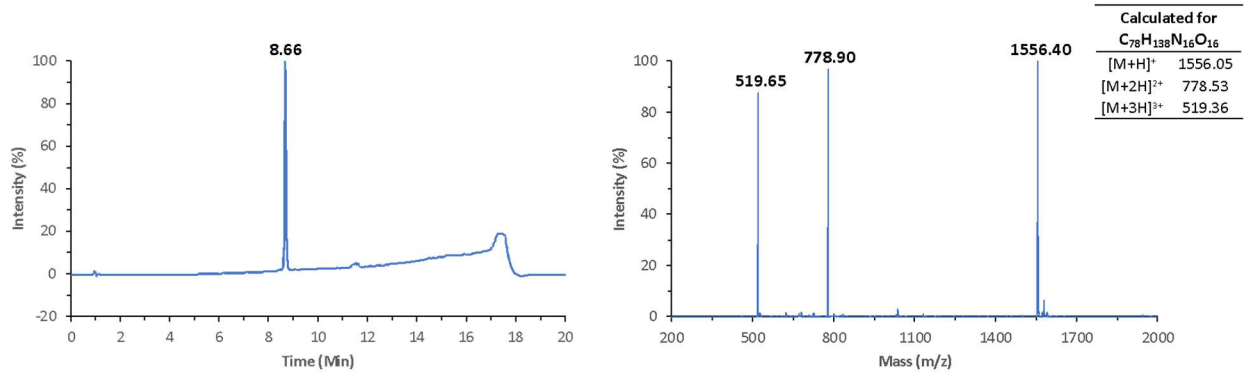
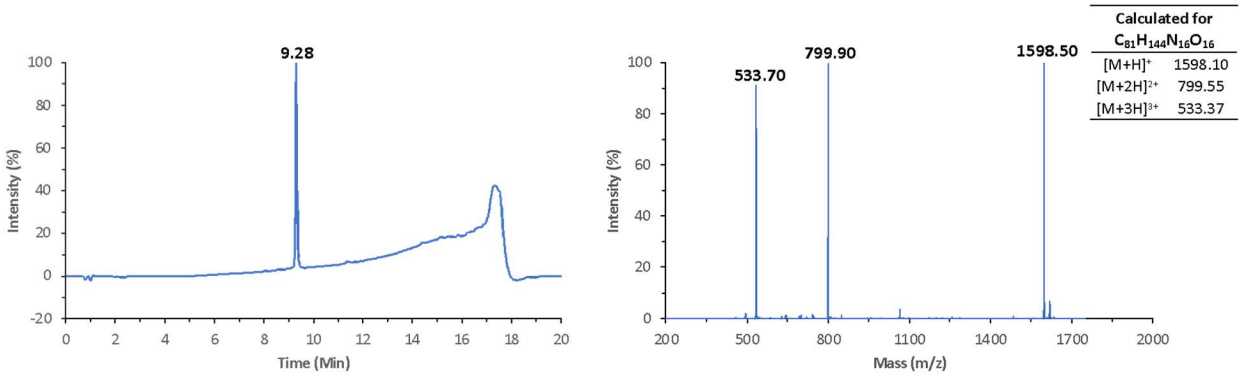
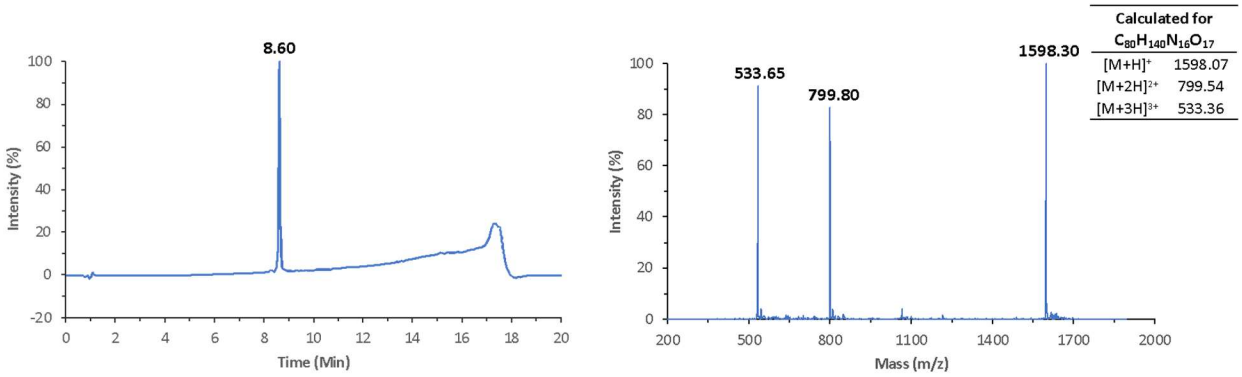


Figure S4. (Continued)

Brevibacillin Ile8 (**9**)



Brevibacillin COOH (**10**)



Brevibacillin $CONH_2$ (**11**)

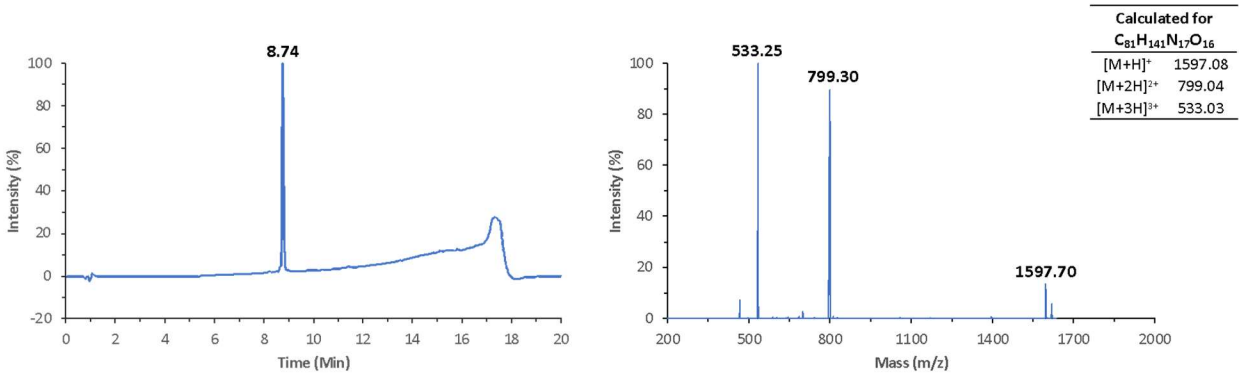
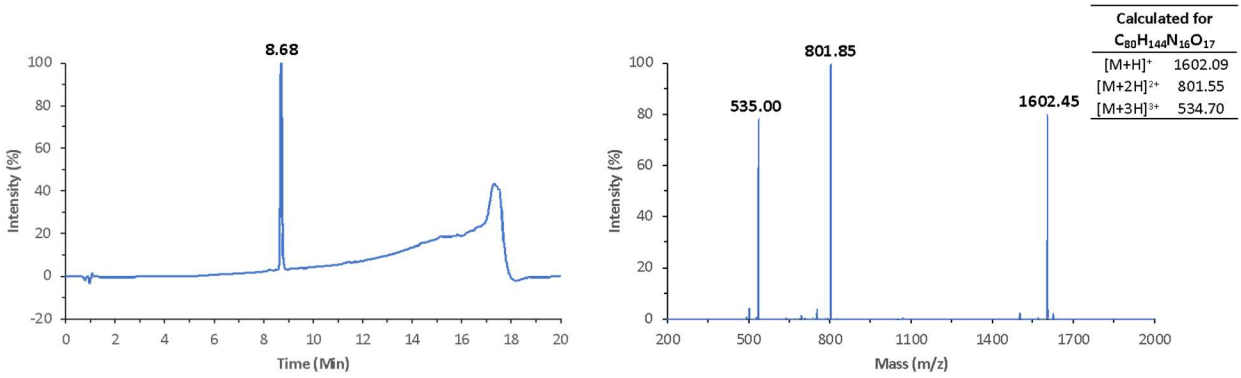
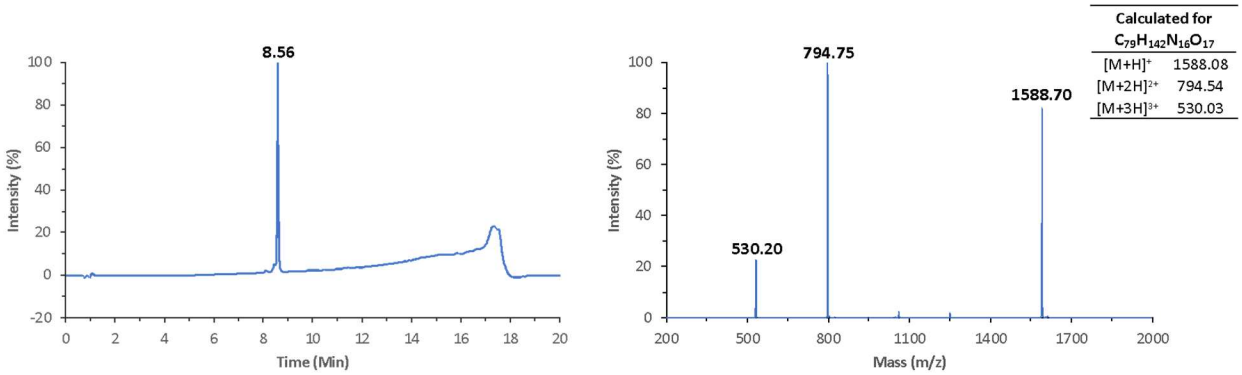


Figure S4. (Continued)

Brevibacillin Thr1 (**12**)



Brevibacillin Ser1 (**13**)



Brevibacillin Ala1 (**14**)

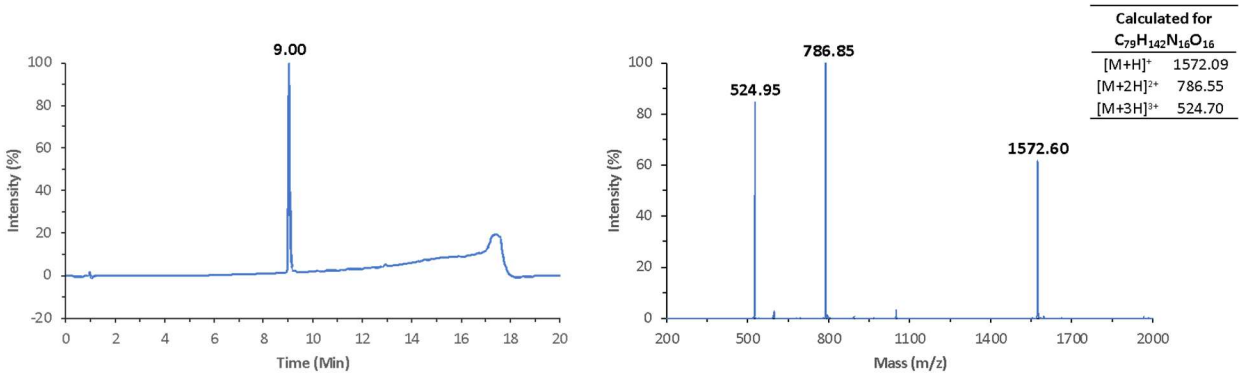
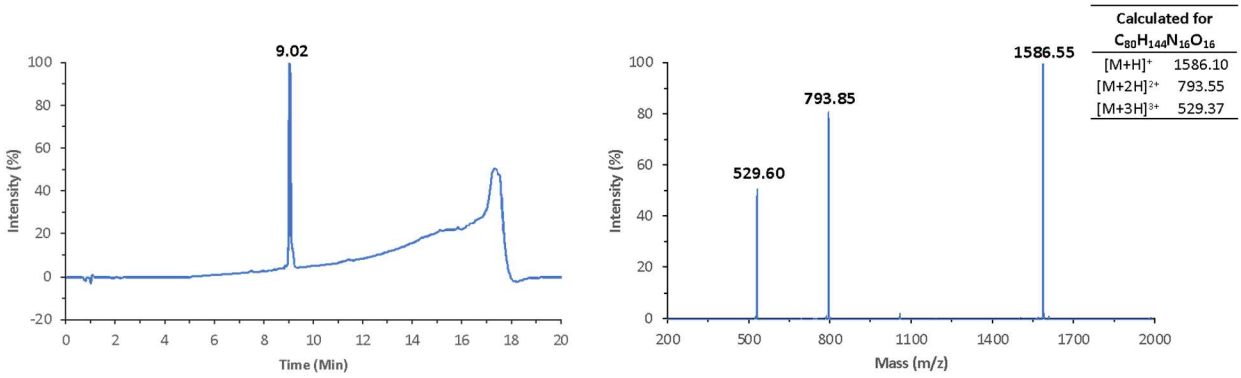
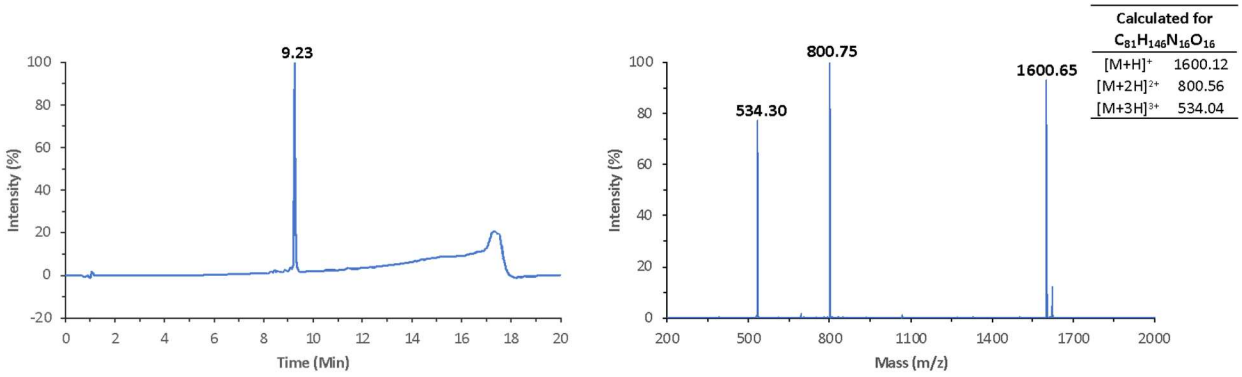


Figure S4. (Continued)

Brevibacillin Abu1 (**15**)



Brevibacillin Val1 (**16**)



Brevibacillin Abu1/Val4,5 (**17**)

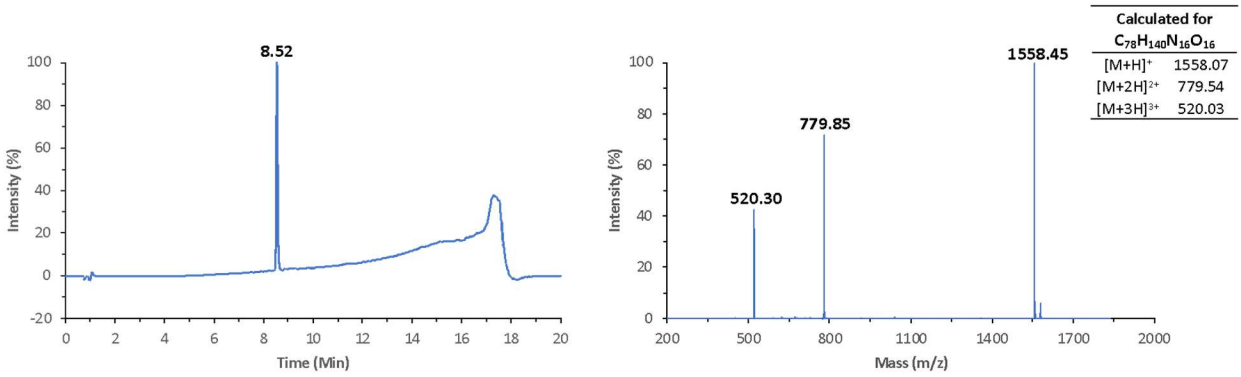
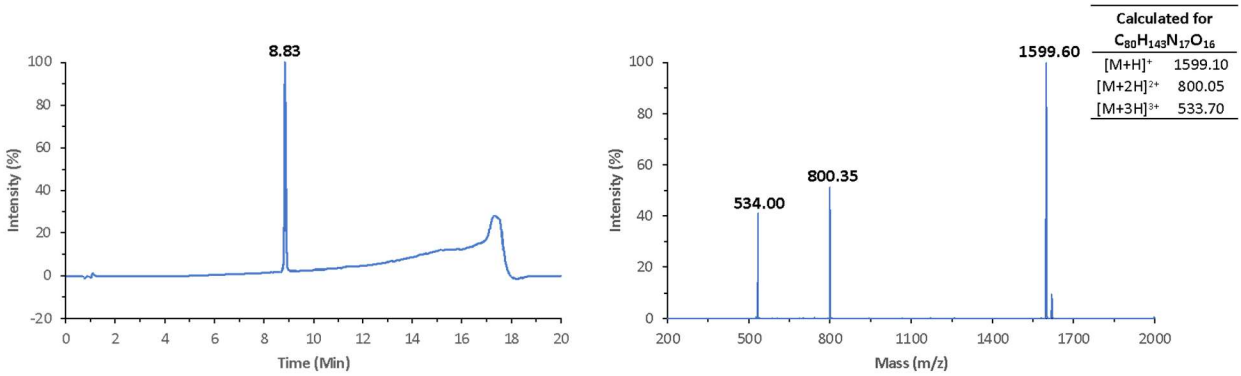
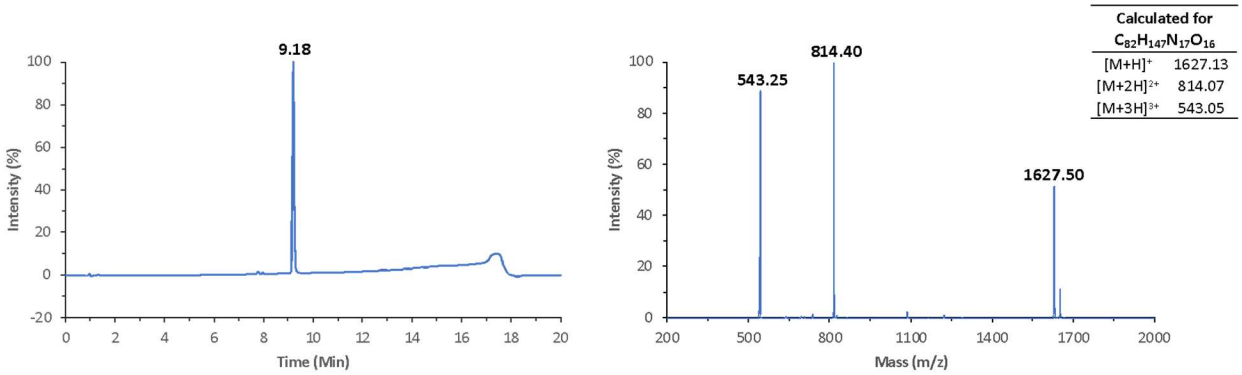


Figure S4. (Continued)

Brevibacillin Abu1/$CONH_2$ (**18**)



Brevibacillin Ac-Ile/Abu1 (**19**)



Brevibacillin Ac-Ile/Abu1/$CONH_2$ (**20**)

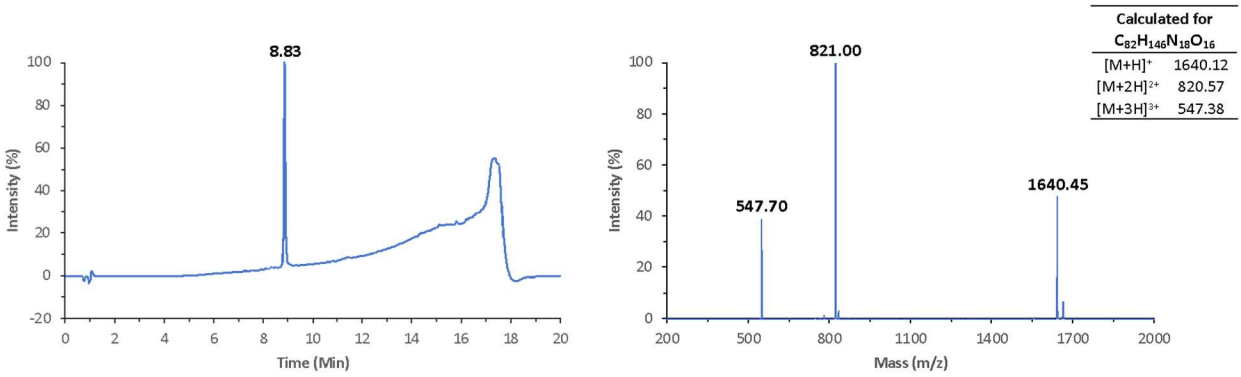


Figure S5. Hemolytic activity of compounds **1**, **7-20** at concentrations of 2, 4, 8, 16, 32, 64, 128, and 256 μg/mL.

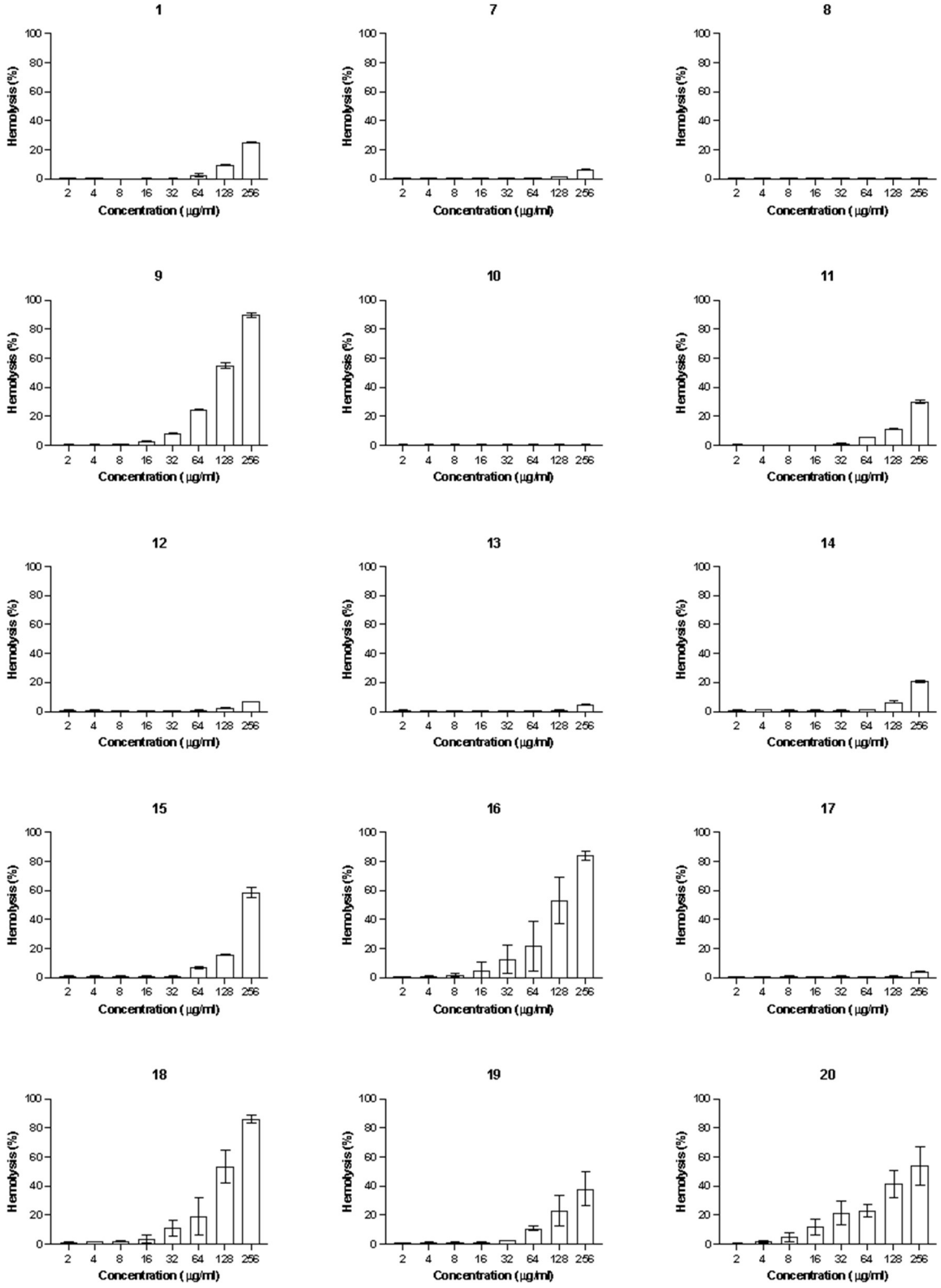


Figure S6. Dose-response of compounds **1**, **7-20** at concentrations of 2, 4, 8, 16, 32, 64, 128, and 256 µg/mL on the viability of Caco-2 cells.

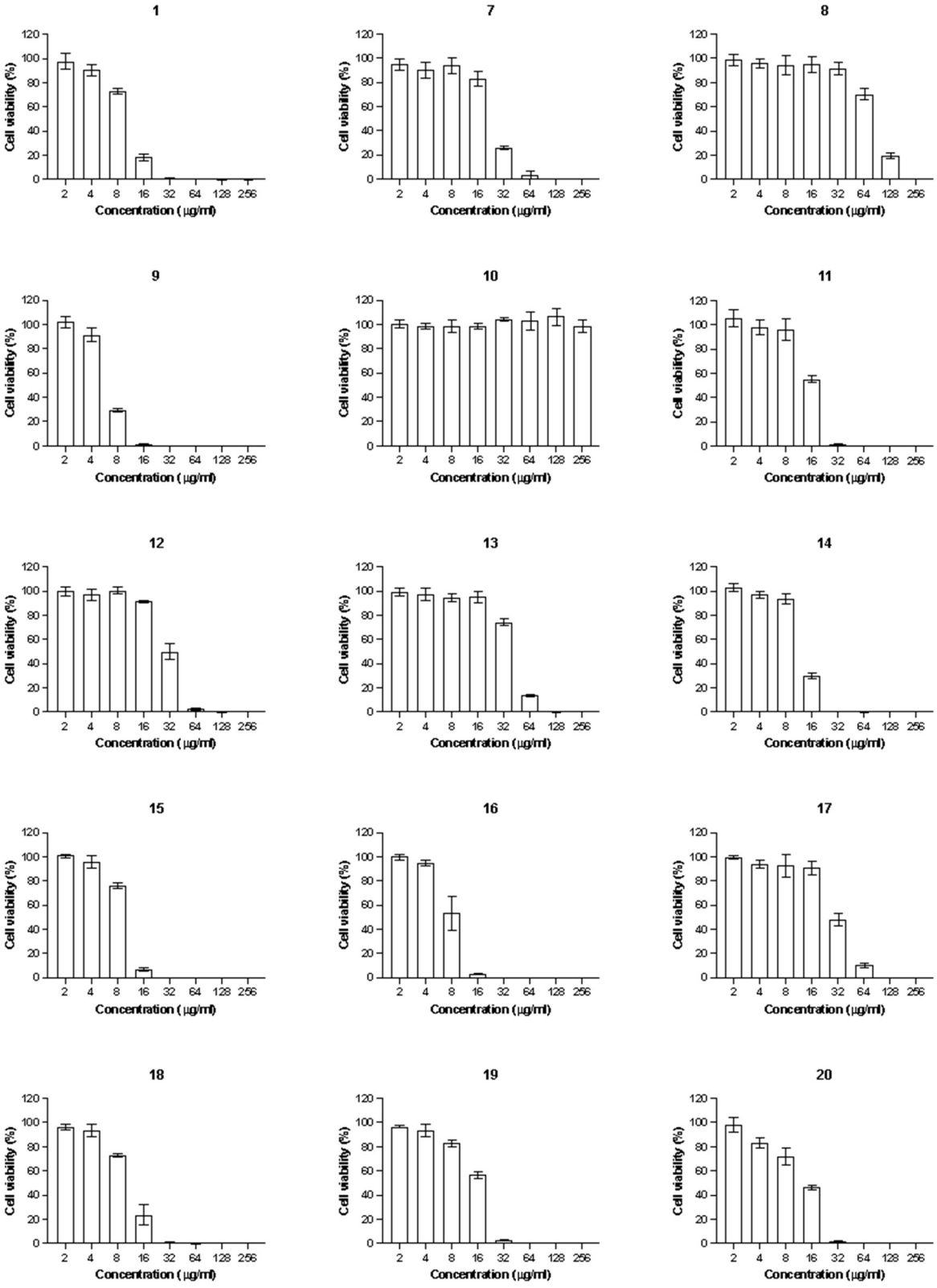


Table S1. Chemical characterization of synthesized peptides.

#	Peptide	Chemical formula	Yield (%)	HPLC rt (min)	Purity (%)[a]	**HRMS** (ESI-TOF) **(da)**	
						Calculated	Observed
1	Brevibacillin (Brevi)	$C_{80}H_{142}N_{16}O_{16}$	14	8.93	>98	$[M+H]^+$ 1584.0620 $[M+2H]^{2+}$ 792.5468	1584.0914 792.5457
7	Brevi Val5	$C_{79}H_{140}N_{16}O_{16}$	20	8.89	95	$[M+H]^+$ 1570.0706 $[M+2H]^{2+}$ 785.5389	1570.0749 785.5363
8	Brevi Val4,5	$C_{78}H_{138}N_{16}O_{16}$	21	8.66	95	$[M+H]^+$ 1556.0549 $[M+2H]^{2+}$ 778.5311	1556.1082 778.5302
9	Brevi Ile8	$C_{81}H_{144}N_{16}O_{16}$	16	9.28	>98	$[M+H]^+$ 1598.1019 $[M+2H]^{2+}$ 799.5546	1598.1082 799.5537
10	Brevi COOH	$C_{80}H_{140}N_{16}O_{17}$	28	8.60	97	$[M+H]^+$ 1598.0655 $[M+2H]^{2+}$ 799.5364	1598.0715 799.5351
11	Brevi $CONH_2$	$C_{80}H_{141}N_{17}O_{16}$	7	8.74	96	$[M+H]^+$ 1597.0815 $[M+2H]^{2+}$ 799.0444	1597.0882 799.0435
12	BreviThr1	$C_{80}H_{144}N_{16}O_{17}$	18	8.68	96	$[M+H]^+$ 1602.0968 $[M+2H]^{2+}$ 801.5520	1602.1031 801.5512
13	Brevi Ser1	$C_{79}H_{142}N_{16}O_{17}$	15	8.56	95	$[M+H]^+$ 1588.0812 $[M+2H]^{2+}$ 794.5442	1588.0869 794.5440
14	Brevi Ala1	$C_{79}H_{142}N_{16}O_{16}$	14	9.00	>98	$[M+H]^+$ 1572.0862 $[M+2H]^{2+}$ 786.5468	1572.0924 794.5459
15	Brevi Abu1	$C_{80}H_{144}N_{16}O_{16}$	20	9.02	95	$[M+H]^+$ 1586.1019 $[M+2H]^{2+}$ 793.5546	1586.1079 793.5533
16	Brevi Val1	$C_{81}H_{146}N_{16}O_{16}$	6	9.23	95	$[M+H]^+$ 1600.1175 $[M+2H]^{2+}$ 800.5624	1600.1237 800.5606
17	Brevi Abu1/Val4,5	$C_{78}H_{140}N_{16}O_{16}$	10	8.52	96	$[M+H]^+$ 1558.0706 $[M+2H]^{2+}$ 779.5389	1558.0763 779.5378
18	Brevi Abu1/$CONH_2$	$C_{80}H_{143}N_{17}O_{16}$	9	8.83	>98	$[M+H]^+$ 1599.0971 $[M+2H]^{2+}$ 800.0522	1599.1037 800.0502
19	Brevi Ac-Ile/Abu1	$C_{82}H_{147}N_{17}O_{16}$	12	9.18	97	$[M+H]^+$ 1627.1284 $[M+2H]^{2+}$ 814.0679	1627.1354 814.0663
20	Brevi Ac-Ile/Abu1/$CONH_2$	$C_{82}H_{146}N_{18}O_{16}$	7	8.83	97	$[M+H]^+$ 1640.1237 $[M+2H]^{2+}$ 820.5655	1640.1295 820.5643

[a]Peptide purities were determined by HPLC with UV absorption at 220 nm

Table S2. Minimum bactericidal activity of the synthesized peptides **1**, **7**-**20**.

#	Peptide	Minimum Bactericidal Concentration (µg/mL)								
		Gram-positive					Gram-negative			
		S.a[a]	***L.m***[a]	***B.s***[a]	***E.f***[a]	***C.p***[b]	***P.a***[c]	***S.e***[c]	***E.c***[c]	***C.c***[d]
1	Brevibacillin (Brevi)	4	4	2	8	4	4	64	32	8
7	Brevi Val4	8	4	2	32	8	2	NA	64	16
8	Brevi Val4,5	32	16	8	64	16	16	NA	128	64
9	Brevi Ile8	2	2	0.5	2	2	2	128	32	8
10	Brevi COOH	NA	256	NA	NA	128	NA	NA	NA	64
11	Brevi $CONH_2$	8	4	4	32	16	4	NA	NA	NA
12	Brevi Thr1	4	8	1	32	4	2	NA	32	16
13	Brevi Ser1	16	4	4	NA	8	4	NA	32	32
14	Brevi Ala1	2	4	2	4	2	1	NA	64	32
15	Brevi Abu1	1	0.5	1	2	2	1	NA	NA	32
16	Brevi Val1	4	2	1	8	8	4	NA	NA	16
17	Brevi Abu1/Val4,5	4	8	4	NA	8	4	NA	128	32
18	Brevi Abu1/$CONH_2$	8	4	2	128	8	8	NA	NA	64
19	Brevi *N*-Ac-Ile/Abu1	4	4	4	32	2	4	NA	NA	64
20	Brevi *N*-Ac-Ile/Abu1/$CONH_2$	NA	NA	NA	NA	NA	NA	NA	NA	NA

Abbreviations and selected strains: ***S.a*** (*Staphylococcus aureus* ATCC 6538), ***L.m*** (*Listeria monocytogenes* ATCC 19111), ***B.s*** (*Bacillus subtilis* ATCC 23957), ***E.f*** (*Enterococcus faecalis* ATCC 29212), ***C.p*** (*Clostridium perfringens* ATCC 3628), ***P.a*** (*Pseudomonas aeruginosa* ATCC 25853), ***S.e*** (*Salmonella enterica serovar Newport* ATCC 6962) and ***E.c*** (*Escherichia coli* ATCC 25922), ***C.c*** (*Campylobacter coli* ATCC 33559).

Used medium and growth conditions: [a]Incubated in TSB (Tryptic Soy Broth) for 20 h at 37°C, [b] Incubated in RCM (Reinforced Clostridial Medium) for 20 h at 37°C, [c] Incubated in LB (Luria-Bertani) for 20 h at 37°C, [d]Incubated in Brucella for 48 h at 37°C

Table S3. MBC/MIC ratio of the synthesized peptides **1**, **7**-**20**.

#	Peptide	MBC/MIC ratio								
		Gram-positive					Gram-negative			
		S.a[a]	***L.m***[a]	***B.s***[a]	***E.f***[a]	***C.p***[b]	***P.a***[c]	***S.e***[c]	***E.c***[c]	***C.c***[d]
1	Brevibacillin (Brevi)	2	2	2	2	1	4	1	1	1
7	Brevi Val4	2	4	1	2	2	1	NA	NA	1
8	Brevi Val4,5	8	4	1	2	1	4	NA	NA	2
9	Brevi Ile8	1	2	1	1	1	2	2	2	1
10	Brevi COOH	NA	2	NA	NA	1	NA	NA	NA	2
11	Brevi $CONH_2$	2	2	1	2	1	1	NA	NA	NA
12	Brevi Thr1	2	2	1	2	1	1	NA	NA	1
13	Brevi Ser1	2	4	2	NA	1	1	NA	NA	1
14	Brevi Ala1	1	4	1	1	1	1	NA	NA	4
15	Brevi Abu1	1	1	1	1	1	1	NA	NA	8
16	Brevi Val1	4	2	1	2	2	4	NA	NA	2
17	Brevi Abu1/Val4,5	2	2	1	NA	1	2	NA	NA	2
18	Brevi Abu1/$CONH_2$	4	2	1	2	1	4	NA	NA	4
19	Brevi *N*-Ac-Ile/Abu1	2	2	1	1	1	2	NA	NA	4
20	Brevi *N*-Ac-Ile/Abu1/$CONH_2$	NA	NA	NA	NA	NA	NA	NA	NA	NA

Abbreviations and selected strains: ***S.a*** (*Staphylococcus aureus* ATCC 6538), ***L.m*** (*Listeria monocytogenes* ATCC 19111), ***B.s*** (*Bacillus subtilis* ATCC 23957), ***E.f*** (*Enterococcus faecalis* ATCC 29212), ***C.p*** (*Clostridium perfringens* ATCC 3628), ***P.a*** (*Pseudomonas aeruginosa* ATCC 25853), ***S.e*** (*Salmonella enterica serovar Newport* ATCC 6962) and ***E.c*** (*Escherichia coli* ATCC 25922), ***C.c*** (*Campylobacter coli* ATCC 33559).

Used medium and growth conditions: [a]Incubated in TSB (Tryptic Soy Broth) for 20 h at 37°C, [b] Incubated in RCM (Reinforced Clostridial Medium) for 20 h at 37°C, [c] Incubated in LB (Luria-Bertani) for 20 h at 37°C, [d]Incubated in Brucella for 48 h at 37°C

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