H-Bonding Promotion of Peptide Solubility and Cyclization by Fluorinated Alcohols

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Abbreviations.

Abbreviations used for amino acids and the designations of peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in *J. Biol. Chem.* **1982**, *247*, 977−983. The following additional abbreviations are used: MeCN, acetonitrile; 2-ClTr, 2-chlorotrityl; 2-ClTrCl, 2-chlorotrityl chloride (Barlos); DIEA, *N,N*-diisopropylethylamine; DIC, *N,N´*-diisopropylcarbodiimide; DMF, *N,N*-dimethylformamide; HATU, 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo-[4,5-b]pyridinium hexafluorophosphate 3-oxide; HBTU, 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo-[4,5-b]pyridinium hexafluorophosphate 3-oxide; COMU, 1-[(1-(Cyano-2-ethoxy-2-oxoethylideneaminooxy)-dimethylamino-morpholinomethylene)]methanaminium Hexafluorophosphate; PyAOP, azabenzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)phosphoniumhexafluorophosphate; PyBOP, benzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate; PyOxP, 1-(Cyano-2-ethoxy-2-oxoethylideneaminooxy) tris(pyrrolidino)phosphoniumhexafluorophosphate; DPPA, diphenyl phosphorazidate; HOAt, 7-aza-1-hydroxybenzotriazole(3-hydroxy-3H-1,2,3-triazolo-[4,5-b]pyridine); HOBt, 1-hydroxybenzotriazole; Oxyma, ethyl cyanoglyoxylate-2-oxime; MeOH, methanol; EtOH, ethanol; 2-PrOH, 2-propanol; t-BuOH, tertiary-butanol (2,2-dimethyl-2-propanol); TFA, trifluoroacetic acid; t-Bu, tertiary-butyl; HPLC, high performance liquid chromatography; MS, mass spectrometry; LC-ESI/MS, liquid chromatography electrospray ionization mass spectrometry; rt, room temperature; h, hour. Amino acid symbols denote L-configuration unless indicated otherwise. All reported solvent ratios are expressed as v/v, unless otherwise stated.

General.

DIC, DIEA, HFIP, TFE, and t-BuOH were obtained from Fluka (Buchs, Switzerland). DMF (peptide synthesis grade), DCM (analysis grade), Acetonitrile (HPLC grade), MeOH (HPLC grade), EtOH (anhydrous), 2-PrOH, t-BuOH, dioxane, Et_2O were obtained from SDS (Peypin, France). All solvent were used without any additional treatment. Ratios of solvent mixture were given in v/v.
HPLC columns (XBridge C18 column, 2.5 µm x 75 mm x 4.6 mm) were obtained from Waters (Ireland). Analytical HPLC was carried out on a Waters instrument comprising a separation module (Waters 2695), automatic injector, photodiode array detector (Waters 996), and system controller (Millenium32 login). UV detection was at 220 and 254 nm, and linear gradients of MeCN (+0.036% TFA) into H_2O (+0.045% TFA), were run at 1.0 mL/min flow rate over 8 min. All peptides and byproducts were identified by HPLC-MS systems comprising a separation module (Waters 2795 or

Waters 2695), automatic injector, photodiode array detector (Waters 2487 or 2998), ESI-MS detector (Waters Micromass ZQ spectrometer) and system controller (MassLynx). HPLC columns was Symmetry® C18 reversed-phase analytical column, 5.0 μm × 4.6 mm × 150 mm for 15 min analysis, UV detection was at 220 and 254 nm, and linear gradients of MeCN (+0.080% formic acid) into H_2O (+0.100% formic acid), were run at 1.0 mL/min flow rate over 15 min. HPLC columns was Sunfire® C18 reversed-phase analytical column, 3.5 μm × 2.1 mm × 100 mm for 8 min analysis, UV detection was at 220 and 254 nm, and linear gradients of MeCN (+0.080% formic acid) into H_2O (+0.100% formic acid), were run at 0.3 mL/min flow rate over 8 min. HR-MS spectra were obtained on a JEOL JMS-T100CS (JEOL, Japan). ^{1}H NMR (500 MHz) spectra were recorded on a BRUKER ADVANCE 500 and 600 spectrometer (Bruker BioSpin Co., Germany) with D_2O as solvents. Chemical shifts are reported in δ (ppm), and coupling constants (*J*) are in Hertz (Hz).

General procedures for linear hexapeptide synthesis

Loading of the resin: 2-ClTrCl resin (1.58 mmol/g) in polyisopropylene filter tube (5, 20, 50 mL) was swollen with DCM (15 mL/g resin) for 10 minutes, and was washed with DCM (10 mL/g resin) three times. A solution of Fmoc-Xaa-OH (1.00 mmol/g resin) and DIEA (1.0 mL/g resin) in DCM (10 mL/g resin) was added to the resin. The mixture was stirred on vortex mixer at rt for 1 h. The solvent was drained and the resin was washed sequentially with DCM (3 x 10 mL/g resin) and DMF (3 x 10 mL/g resin).

Fmoc deprotection: Removal of the Fmoc group was carried out with piperidine−DMF (1:4) (12 mL/g resin, 2 × 10 min), and then the resin was washed with DMF (3 x 10 mL/g resin), DCM (3 x 10 mL/g resin), and DMF (3 x 10 mL/g resin).

Peptide coupling: Fmoc-Xaa-OH (3 equiv.) and Oxyma (3 equiv.) in DMF (3.3 mL/mmol of Fmoc-Xaa-OH) was activated by treatment with DIC (3 equiv.) for 30 seconds, before addition to the resin. After 1 h, the resin was washed with DMF (3 x 10 mL/g resin).

Cleavage from the resin without t-Bu group deprotection: A mixture of HFIP-DCM (1:4, 10 mL/g resin) was added to the resin and the mixture was stirred on vortex mixer at rt for 1h. The resin was washed with HFIP-DCM (1:1, 5 mL/g resin) and HFIP-DCM (1:4, 10 mL/g resin) twice. The filtrate was collected and concentrated *in vacuo*. The crude hexapeptide was washed with ether under sonication at rt and insoluble hexapeptide was collected by centrifugation, and this ether washing manipulation was repeated three times. The peptide was dried *in vacuo* and the residue was analyzed with HPLC to show > 90% purity. Each precipitate of hexapeptide was used for cyclization without HPLC purification to avoid formation of trifluoroacetamide on *N*-terminal in the cyclization step.

H-Thr(t-Bu)-Ala-Ala-Thr(t-Bu)-Ala-Ala-OH (3).

^{1}H NMR (D_2O, 500 MHz): δ 4.34 (Q, 1H, *J* = 7.1 Hz), 4.28 (Q, 1H, *J* = 7.1 Hz), 4.23 (m, 2H), 4.18 (Q, 1H, *J* = 7.3 Hz), 4.03 (m, 2H), 3.73 (d, 1H, *J* = 5.3 Hz), 1.29 (m, 12H), 1.18 (d, 3H, *J* = 6.3 Hz), 1.11 (s, 9H), 1.07 (s, 9H), 1.06 (d, 3H, *J* = 6.5 Hz). HRMS (ESI) *m*/*z* calced for $C_{28}H_{53}N_6O_9$ $[M+H]^+$ 617.3874, found 617.3895.

H-Ala-Thr(t-Bu)-Ala-Ala-Thr(t-Bu)-Ala-OH (4).

^{1}H NMR (D_2O, 500 MHz): δ 4.32-4.23 (m, 4H), 4.01-3.99 (m, 4H), 1.44 (d, 3H, *J* = 7.1 Hz), 1.31 (d, 3H, *J* = 7.5 Hz), 1.29 (d, 3H, *J* = 7.4 Hz), 1.23 (d, 3H, *J* = 7.1 Hz), 1.09 (s, 9H), 1.08 (s, 9H), 1.06 (m, 6H). HRMS (ESI) *m*/*z* calced for $C_{28}H_{53}N_6O_9$ $[M+H]^+$ 617.3874, found 617.3885.

H-Thr(t-Bu)-Ala-Ala-Thr(t-Bu)-Ala-Ala-OH (5).

^{1}H NMR (D_2O, 500 MHz): δ 4.35-4.21 (m, 6H), 4.03 (m, 1H), 3.99 (Q, 1H, *J* = 7.1 Hz), 1.44 (d, 3H, *J* = 7.1 Hz), 1.30 (m, 9H), 1.18 (d, 3H, *J* = 6.3 Hz), 1.11 (s, 9H), 1.08 (s, 9H), 1.06 (d, 3H, *J* = 6.5 Hz). HRMS (ESI) *m*/*z* calced for $C_{28}H_{53}N_6O_9$ $[M+H]^+$ 617.3874, found 617.3888.

H-Thr(t-Bu)-Ala-Ala-Thr(t-Bu)-Ala-d-Ala-OH (8).

^{1}H NMR (D_2O, 500 MHz): δ 4.34 (Q, 1H, *J* = 7.1 Hz), 4.29-4.22 (m, 3H), 4.07-4.00 (m, 3H), 3.72 (d, 1H, *J* = 5.3 Hz), 1.30 (m, 9H), 1.22 (d, 3H, *J* = 7.2 Hz), 1.18 (d, 3H, *J* = 6.3 Hz), 1.11 (s, 9H), 1.08 (s, 9H), 1.06 (d, 3H, *J* = 6.5 Hz). HRMS (ESI) *m*/*z* calced for $C_{28}H_{53}N_6O_9$ $[M+H]^+$ 617.3874, found 617.3886.

General procedure of cyclization reactions with "stand alone" coupling reagents:

To each solutions of hexapeptide **3** (5.5 mg, 9 μmol) and coupling reagents (10 μmol, HATU, HBTU, COMU, PyAOP, PyBOP, PyOxP, or DPPA) in HFIP-DCM (1:4, 1.0 mL) was added DIEA (2.6 μL, 20 μmol) and the mixture was voltexed at rt. After 30 min, 10 μL of reaction mixture was collected and diluted with 10 μL of 1% TFA solution and 80 μL of MeCN, and the mixture was applied to HPLC analysis and LC-ESI/MS analysis. (Figure SI-1)

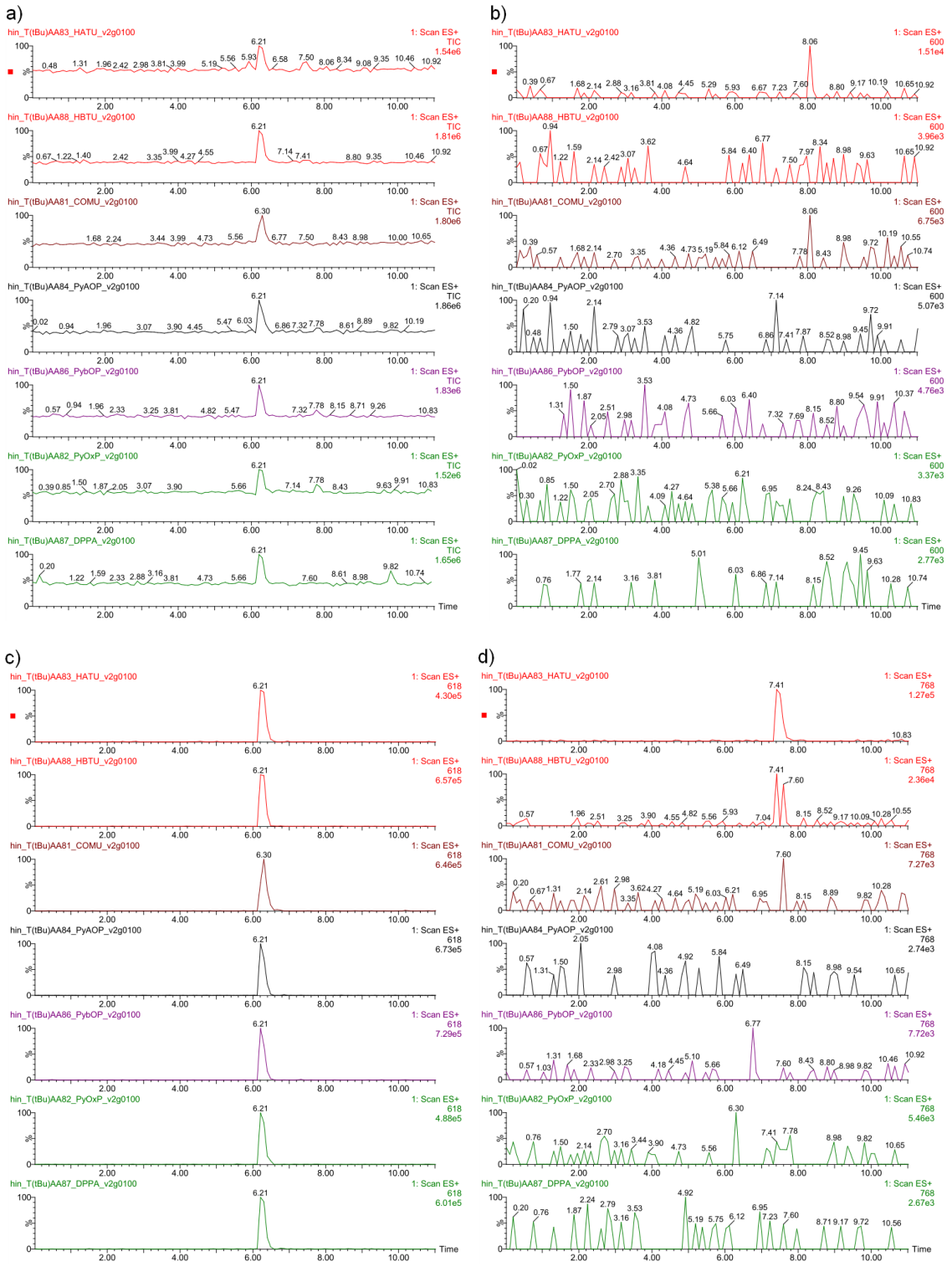


Figure SI-1. Ion chromatograms of cyclization reactions with "stand alone" reagents, a) total ion chromatogram, b) ion chromatograms of 600 Da (cyclic hexapeptide **1**), c) ion chromatograms of 618 Da (linear hexapeptide **3**), d) ion chromatograms of 768 Da (HFIP ester **7**).

General procedure of cyclization reactions with DIC-additive system:

To each solutions of hexapeptide **3** (6.2 mg, 10 μmol) and additive (10 μmol, HOAt, HOBt, or Oxyma) in 1.0 mL of solvent was added DIC (1.1 μL, 1.0 μmol) and the mixture was voltexed at rt. After 1 h, 10 μL of reaction mixture was collected and diluted with 10 μL of 1% TFA solution and 80 μL of MeCN, and the mixture was applied to HPLC analysis and LC-ESI/MS analysis to determine the ratio of the products.

Cyclo[-Thr(t-Bu)-Ala-Ala-Thr(t-Bu)-Ala-Ala-] (1).

^{1}H NMR (D_2O, 600 MHz): δ 4.28-4.25 (m, 4H), 4.22 (Q, 2H, J = 6.9 Hz), 4.07 (Q, 2H, J = 7.2 Hz), 1.40 (d, 6H, J = 7.2 Hz), 1.39 (d, 6H, J = 6.9 Hz), 1.14 (s, 18H), 1.05 (d, 6H, J = 6.2 Hz). HRMS (ESI) m/z calced for $C_{28}H_{50}N_6NaO_8$ $[M+Na]^+$ 621.3587, found 621.3577.

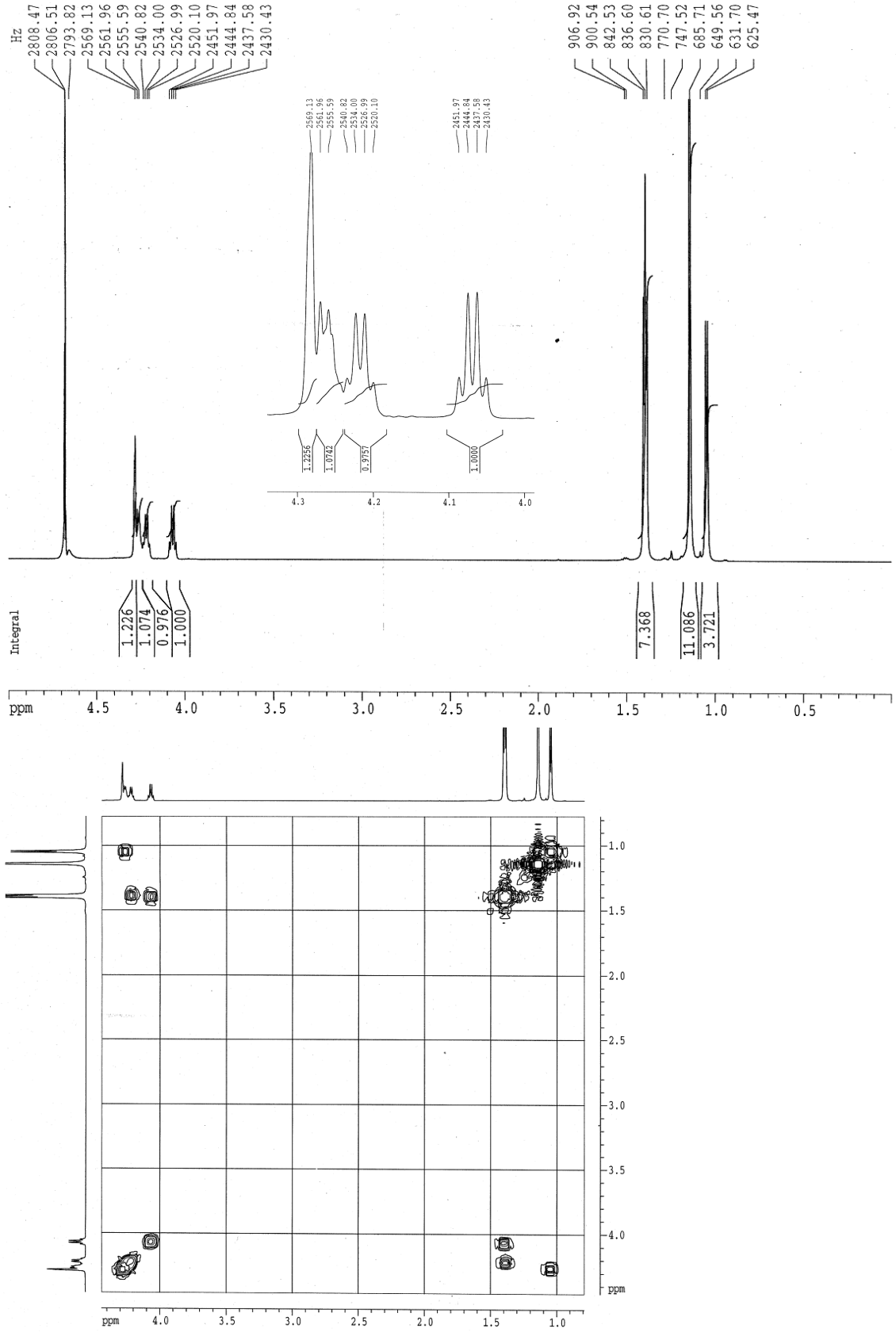
Cyclo[-Thr(t-Bu)-Ala-Ala-Thr(t-Bu)-Ala-Ala-Thr(t-Bu)-Ala-Ala-Thr(t-Bu)-Ala-Ala-] (6).

^{1}H NMR (D_2O, 600 MHz): δ 4.28 (Q, 4H, J = 7.0 Hz), 4.22-4.19 (m, 8H), 4.12 (m, 4H), 1.35 (d, 12H, J = 7.2 Hz), 1.33 (d, 12H, J = 7.2 Hz), 1.10 (s, 36H), 1.07 (d, 6H, J = 6.3 Hz). HRMS (ESI) m/z calced for $C_{56}H_{100}N_{12}Na_2O_{16}$ $[M+2Na]^{2+}$ 621.3587, found 621.3592; for $C_{56}H_{100}N_{12}NaO_{16}$ $[M+Na]^+$ 1219.7278, found 1219.7341.

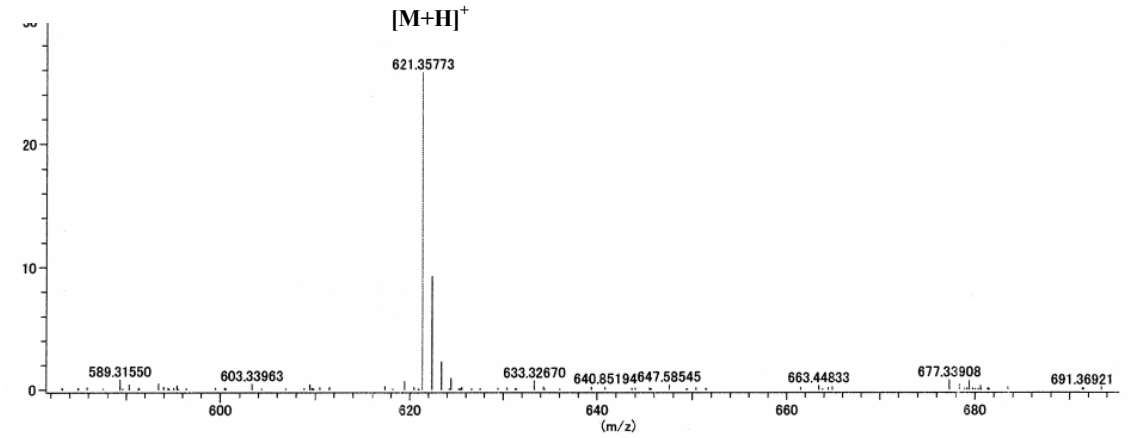
Cyclo[-Thr(t-Bu)-Ala-Ala-Thr(t-Bu)-Ala-d-Ala-] (9).

^{1}H NMR (D_2O, 600 MHz): δ 4.41 (m, 1H), 4.28-4.16 (m, 7H), 4.05 (Q, 1H, J = 7.2 Hz), 1.50 (d, 3H, J = 7.3 Hz), 1.36 (d, 3H, J = 7.3 Hz), 1.32 (d, 3H, J = 6.6 Hz), 1.32 (d, 3H, J = 6.8 Hz), 1.12 (s, 18H), 1.08 (d, 3H, J = 6.3 Hz), 1.01 (d, 3H, J = 5.8 Hz). HRMS (ESI) m/z calced for $C_{28}H_{50}N_6NaO_8$ $[M+Na]^+$ 621.3587, found 621.3571.

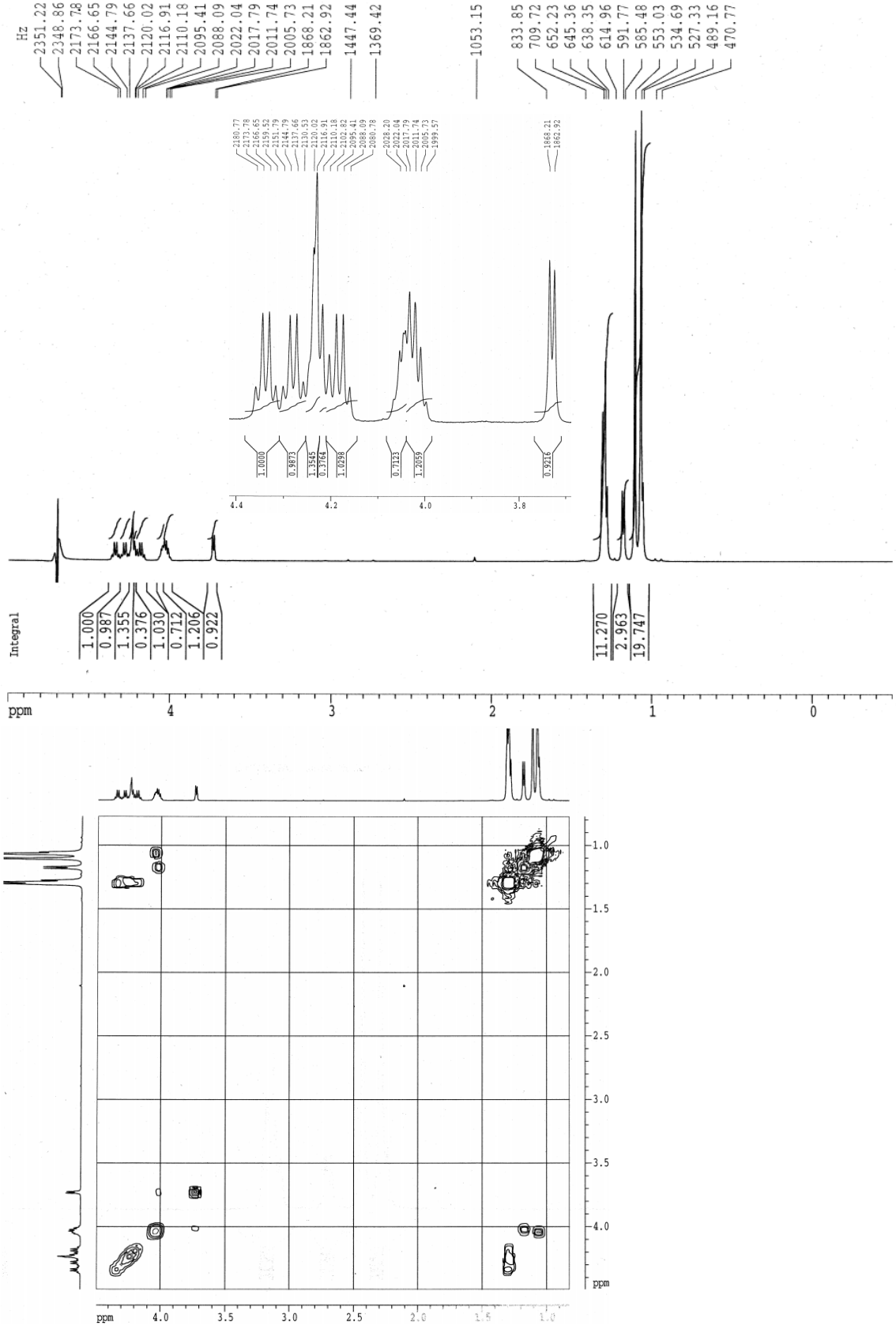
Cyclo[-Thr(t-Bu)-Ala-Ala-Thr(t-Bu)-Ala-Ala-] (1).



Cyclo[-Thr(t-Bu)-Ala-Ala-Thr(t-Bu)-Ala-Ala-] (1).



H-Thr(t-Bu)-Ala-Ala-Thr(t-Bu)-Ala-Ala-OH (3).



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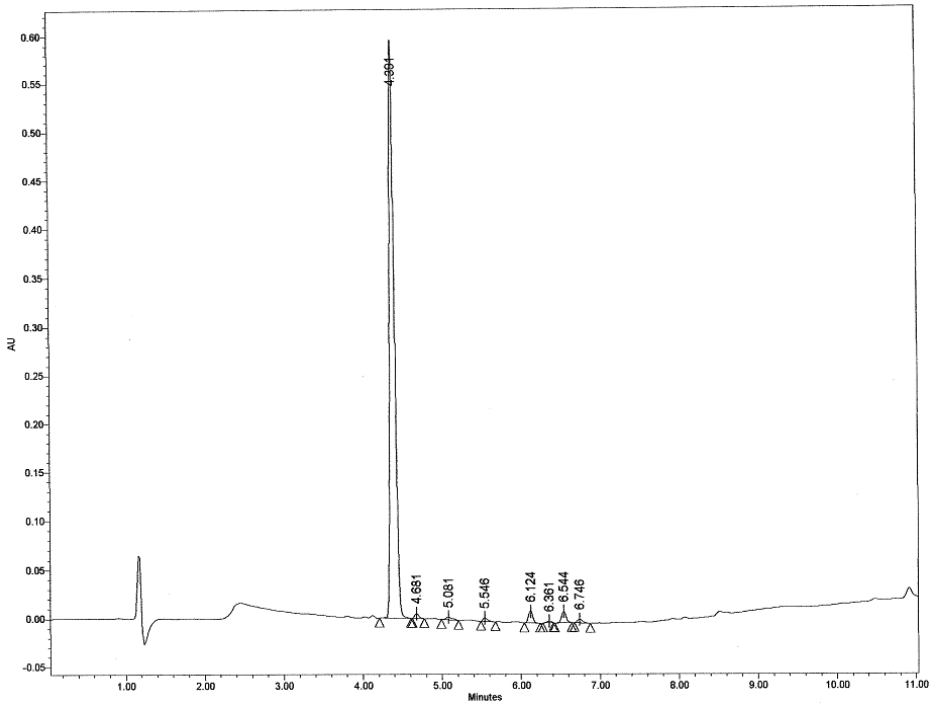
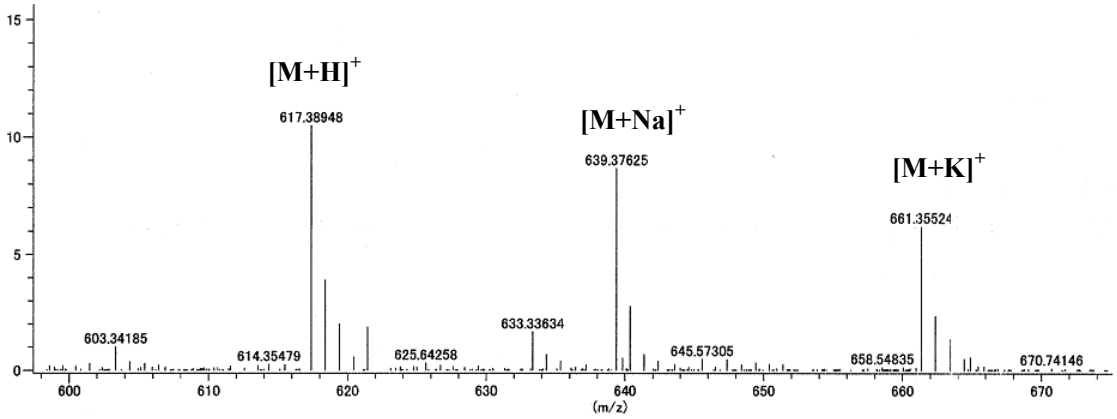
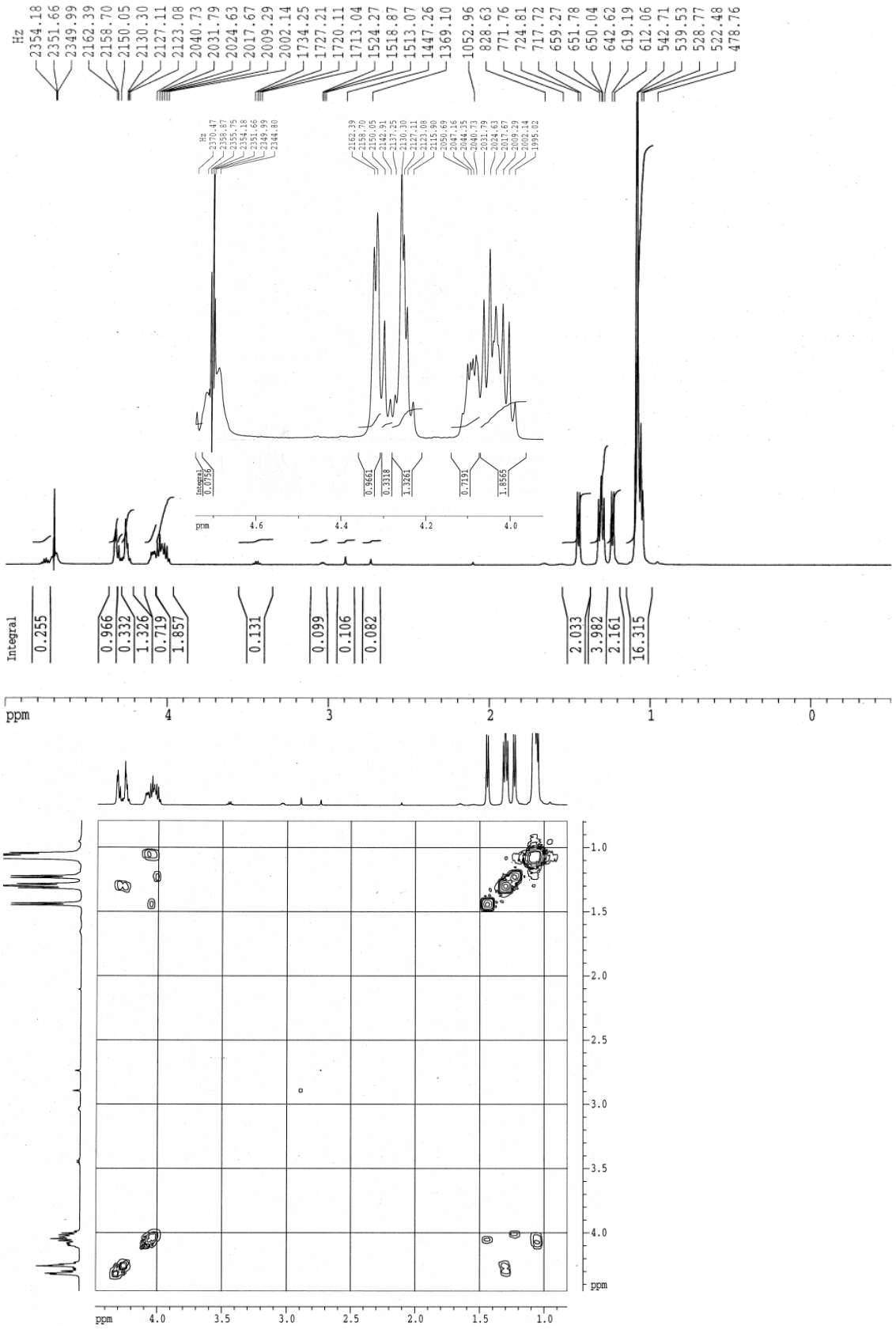
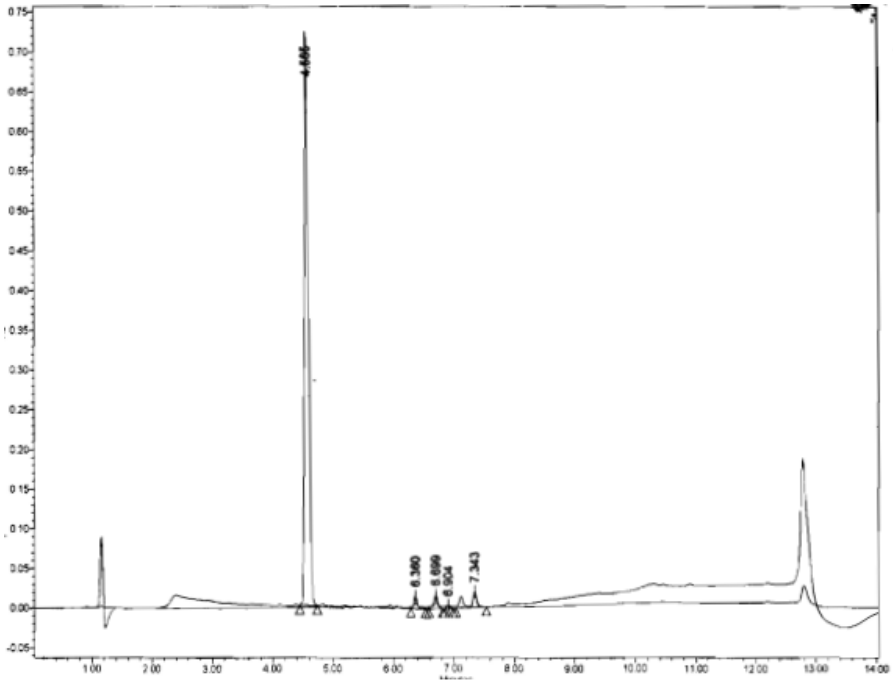


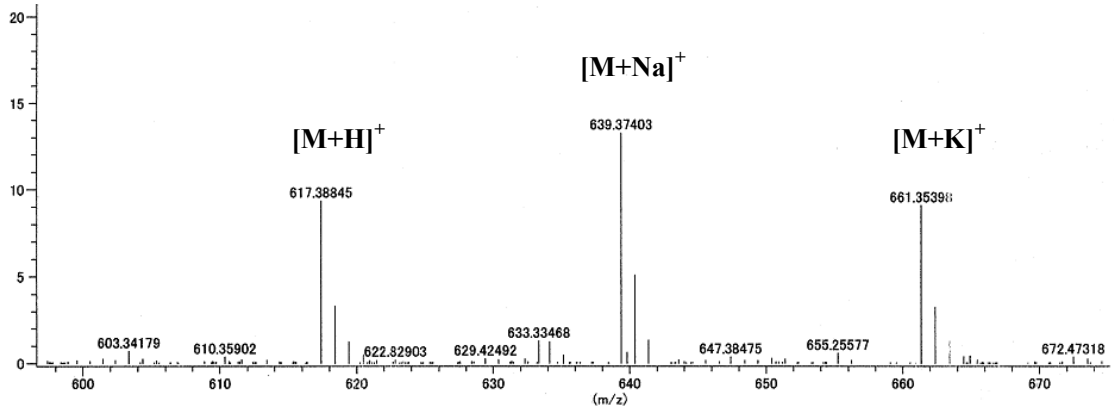


H-Ala-Thr(t-Bu)-Ala-Ala- Thr(t-Bu)-Ala-OH (4).

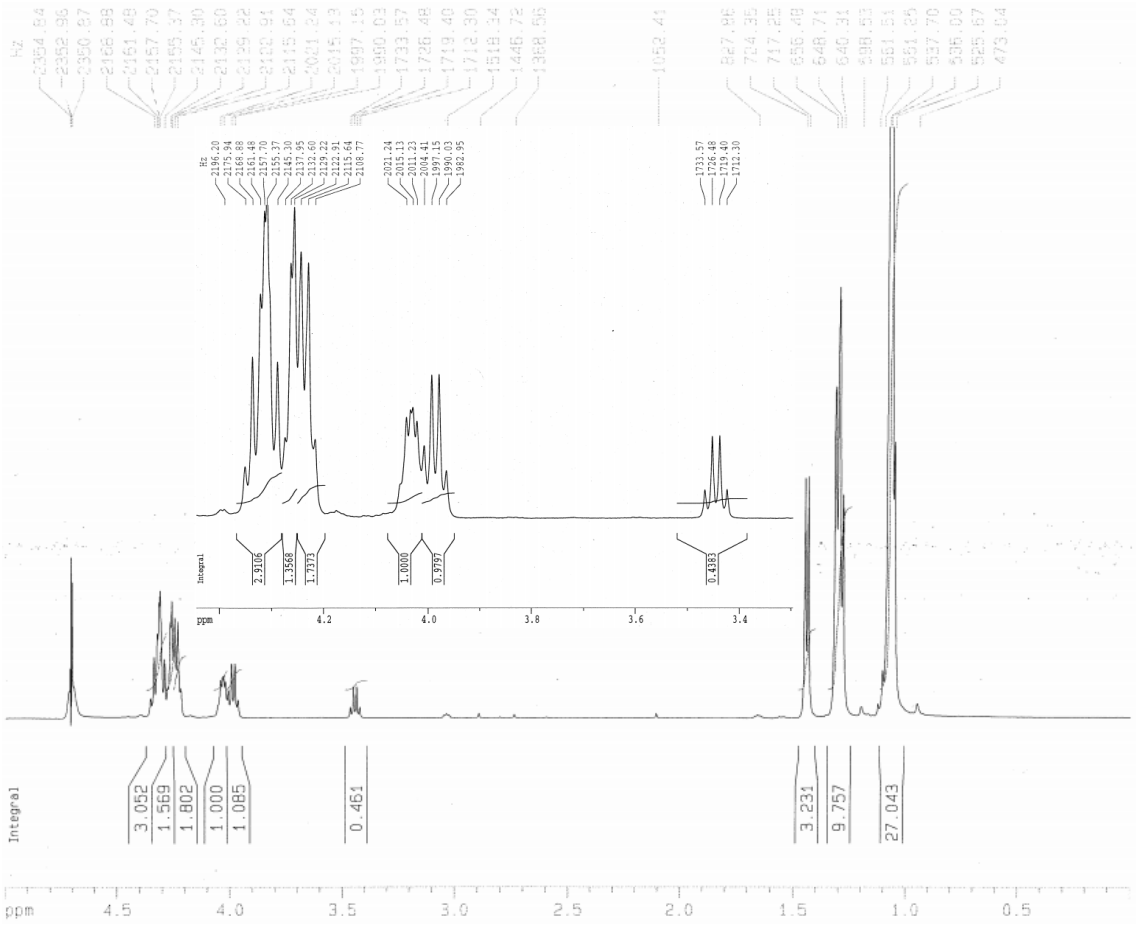


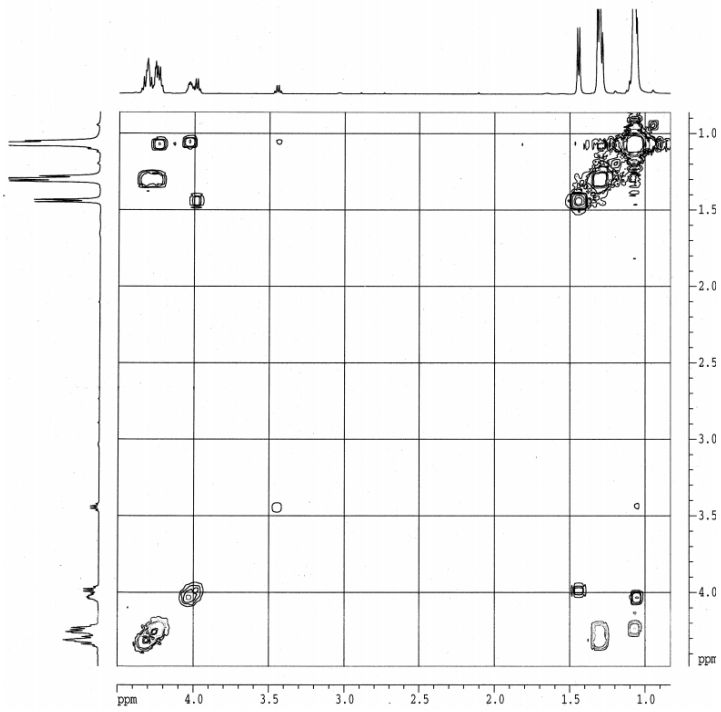
H-Ala-Thr(t-Bu)-Ala-Ala- Thr(t-Bu)-Ala-OH (4).



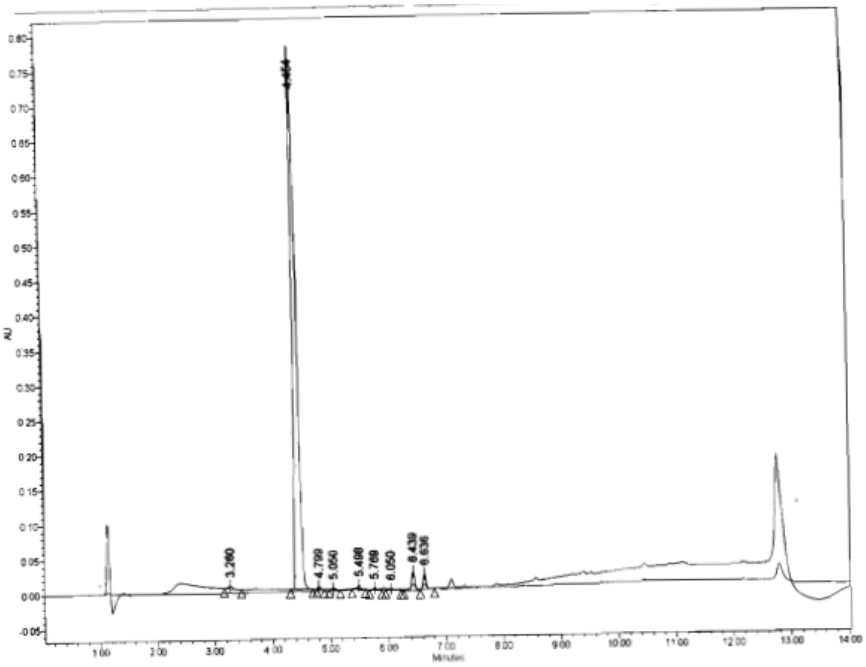


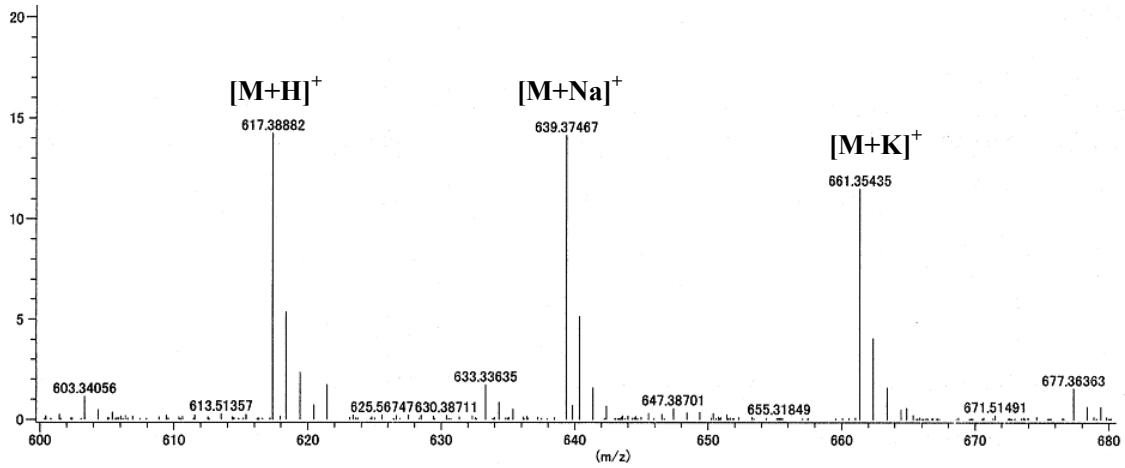
H-Ala-Ala-Thr(t-Bu)-Ala-Ala- Thr(t-Bu)-OH (5).



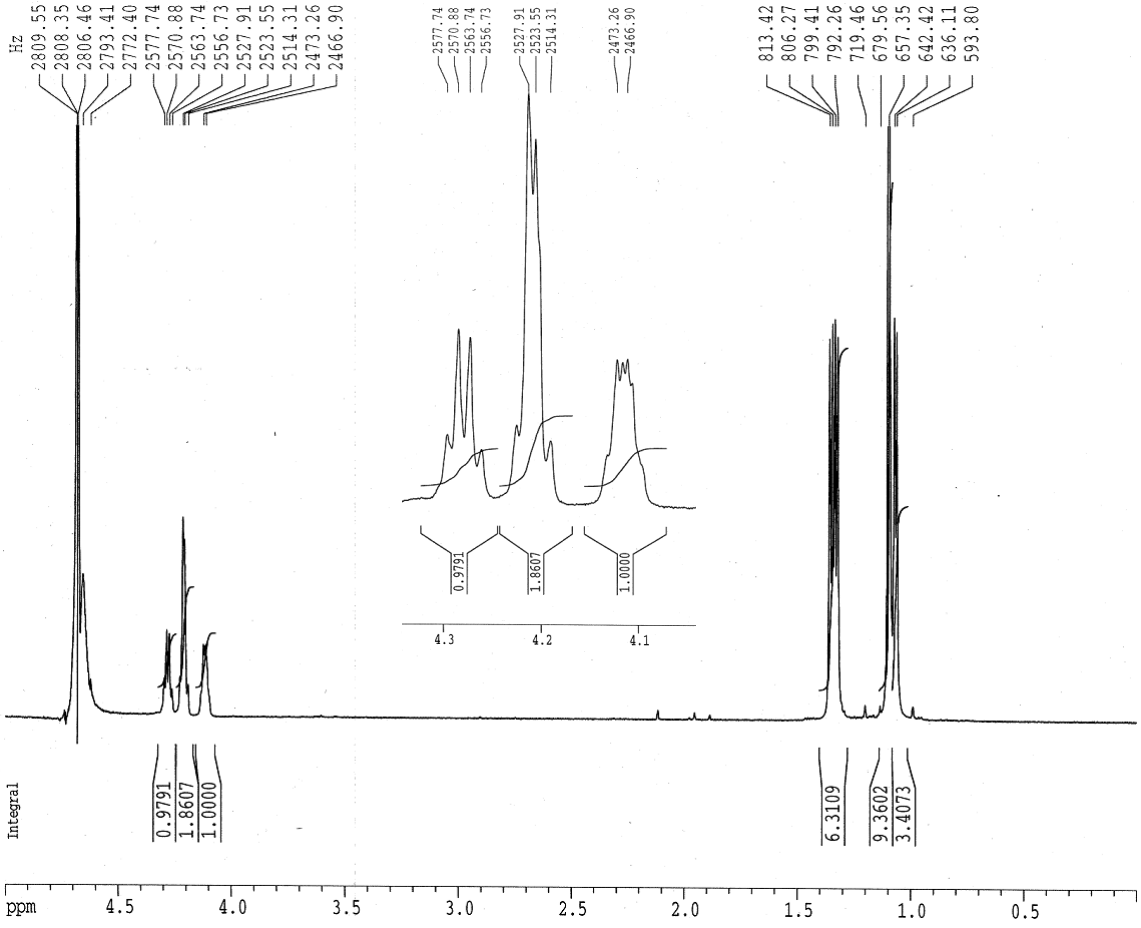


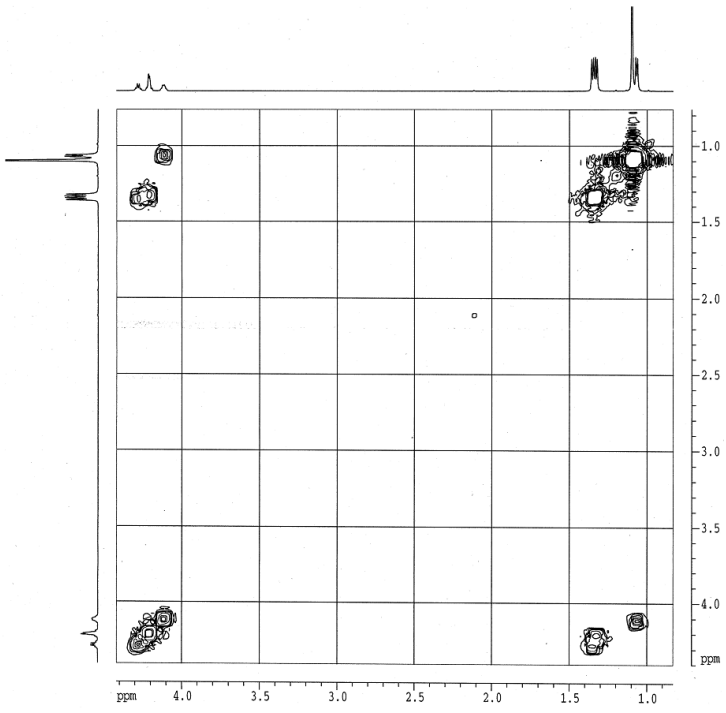
H-Ala-Ala-Thr(t-Bu)-Ala-Ala- Thr(t-Bu)-OH (5).





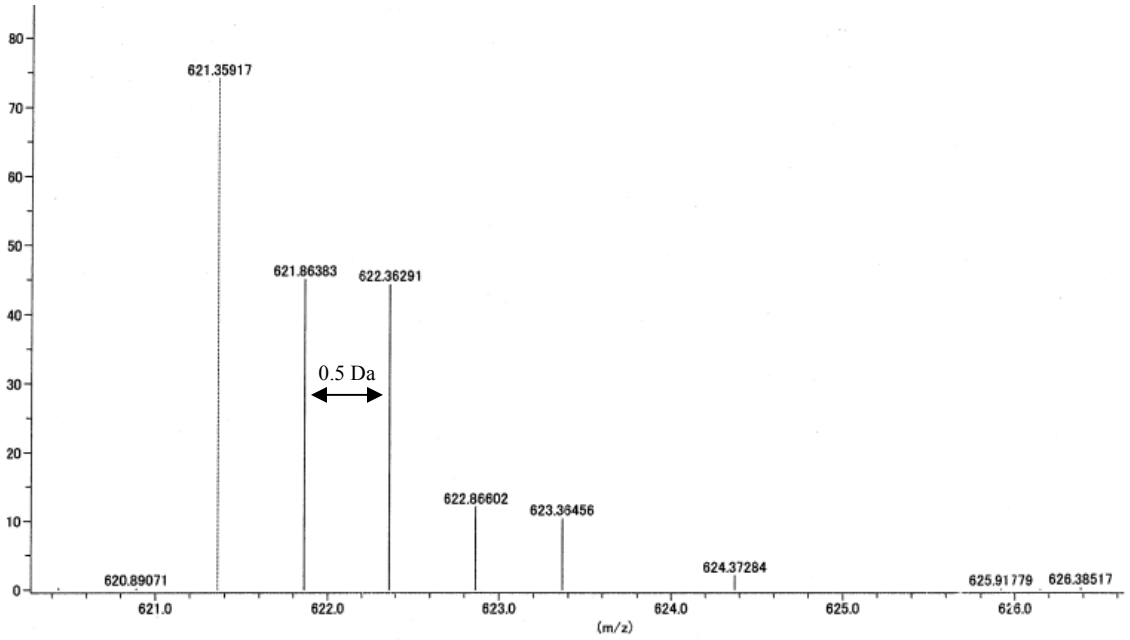
Cyclo[-Thr(t-Bu)-Ala-Ala-Thr(t-Bu)-Ala-Ala-Thr(t-Bu)-Ala-Ala-Thr(t-Bu)-Ala-Ala-] (6).



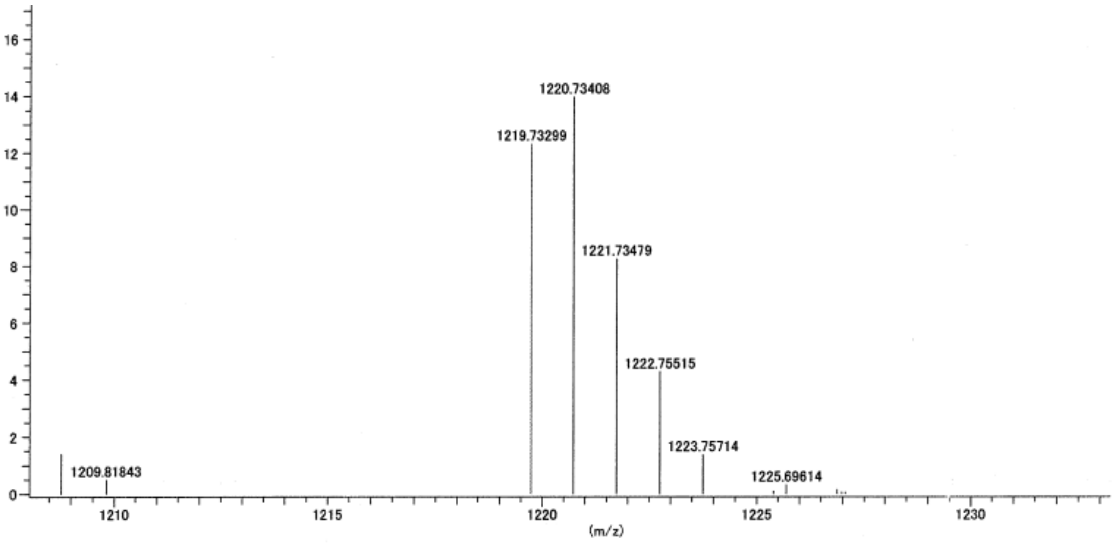


Cyclo[-Thr(t-Bu)-Ala-Ala-Thr(t-Bu)-Ala-Ala-Thr(t-Bu)-Ala-Ala-Thr(t-Bu)-Ala-Ala-] (6).

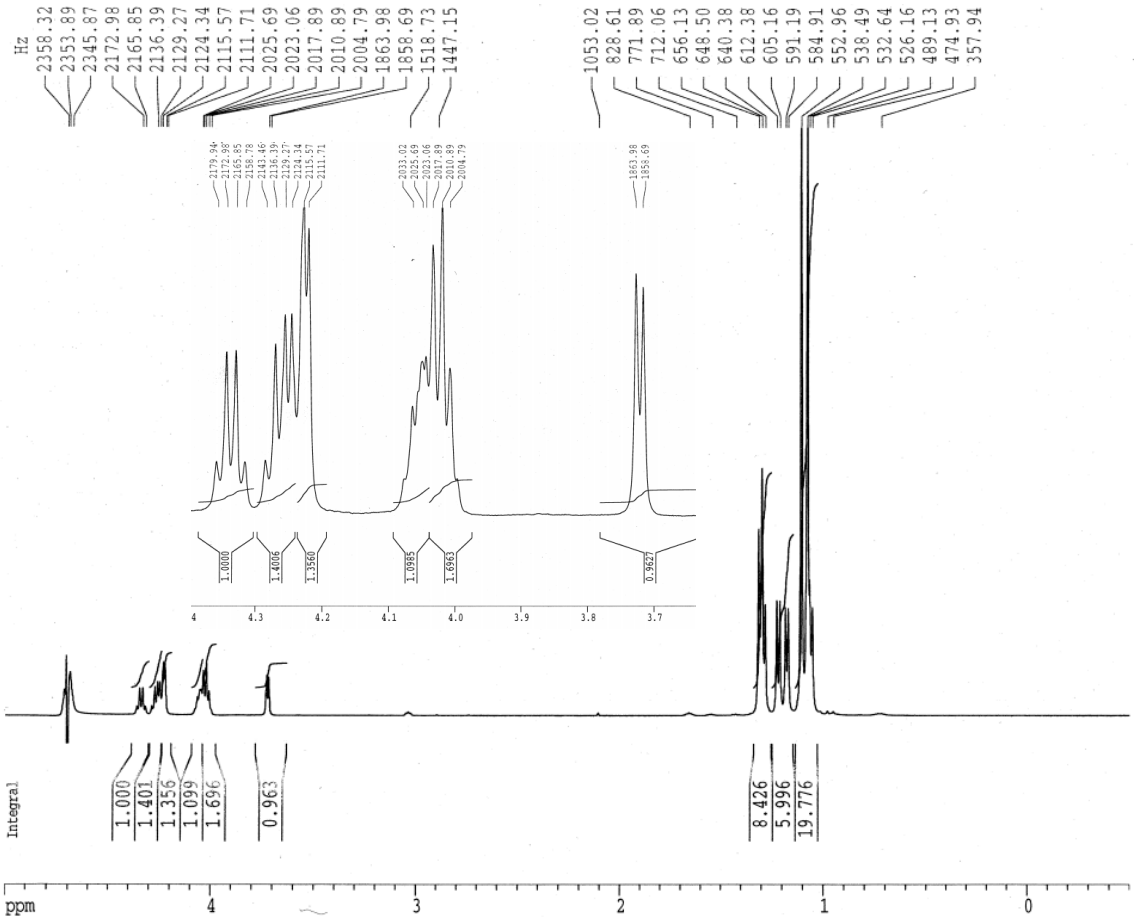
$[M + 2Na]^{2+}$

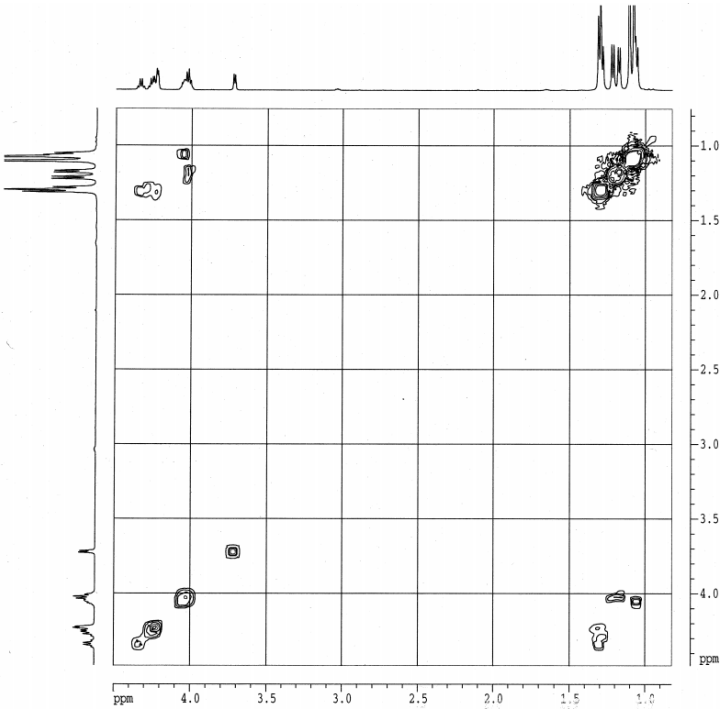


$[M + Na]^{+}$

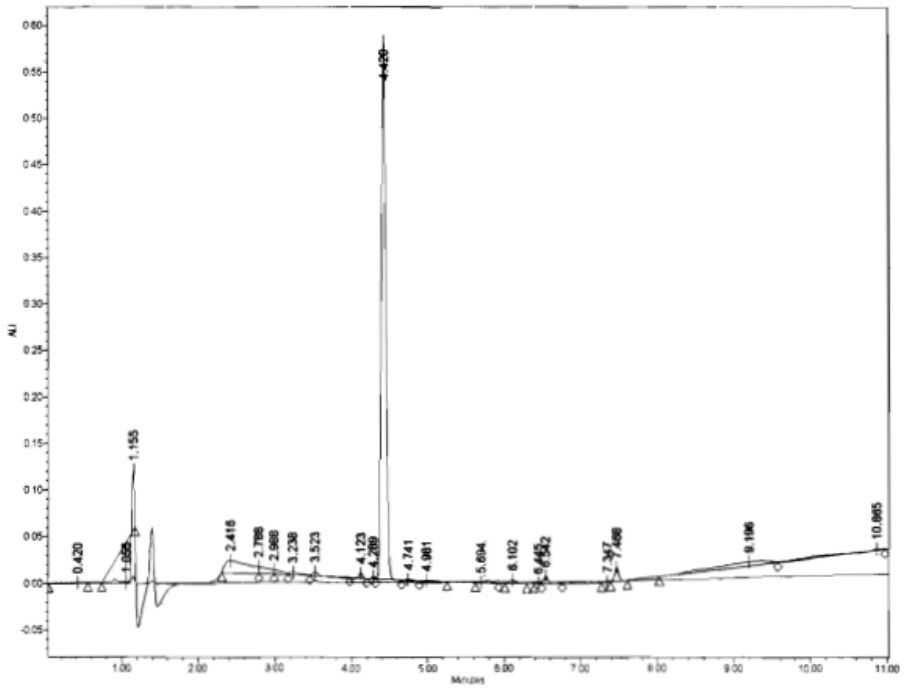


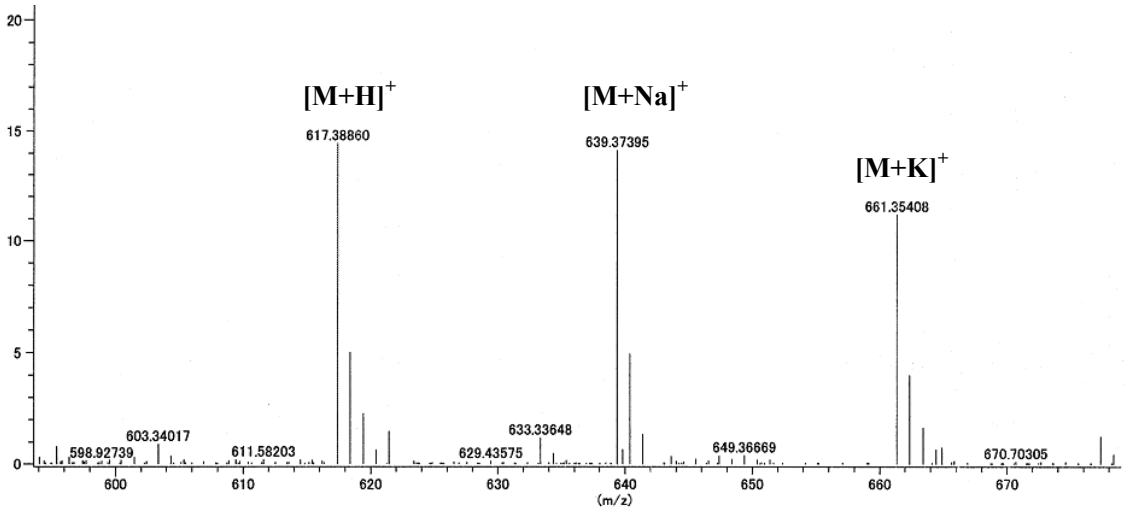
H-Thr(t-Bu)-Ala-Ala-Thr(t-Bu)-Ala-d-Ala-OH (8).



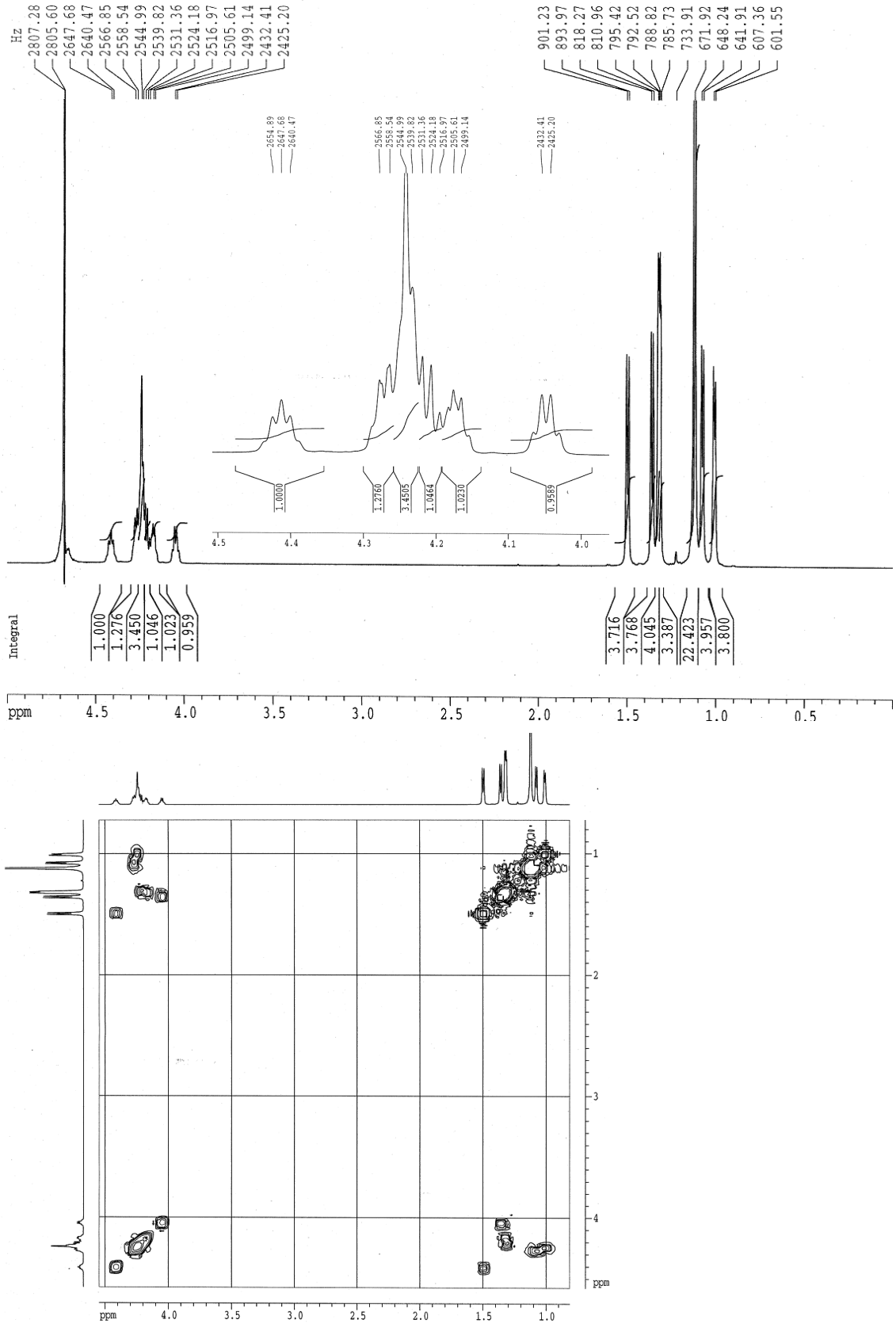


H-Thr(t-Bu)-Ala-Ala-Thr(t-Bu)-Ala-d-Ala-OH (8).





Cyclo[-Thr(t-Bu)-Ala-Ala-Thr(t-Bu)-Ala-d-Ala-] (9).



Cyclo[-Thr(t-Bu)-Ala-Ala-Thr(t-Bu)-Ala-d-Ala-] (9).