Electronic Supplementary Information

Affinity resins as new tools for identifying target proteins of ascorbic acid

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I. Supplementary methods

Specificity of AA-affinity resins for ascorbate oxidase. All subsequent manipulations of affinity chromatography were done at a temperature of about 4˚C. AA-affinity resins **4** and **8** and the control resin (1.0 ml) were each transferred to a poly-prep column and equilibrated with 3 bed volumes of binding buffer. Solutions of ascorbate oxidase (AO) from *Cucurbita* sp. (Wako Pure Chemical Industries, Osaka, Japan) in binding buffers (total protein of 30 μg) without or with 0.1 mM and 1.0 mM AA derivative **5** were each applied onto the affinity resins. The resins were washed with 3 bed volumes of binding buffer to remove unbound proteins. The bound proteins were each eluted with 2 bed volumes of 0.1 mM AA derivative **5** solution in 10 mM Tris-HCl buffer (pH 7.4) and then with 2 bed volumes of 1.0 mM AA derivative **5** solution in the same buffer. The protein elution fractions were each concentrated by ultrafiltration with a Vivaspin 500. Both the concentrated protein elution fractions and the wash fractions with binding buffer were diluted 2-fold with SDS-PAGE sample buffer and heated at 100˚C for 5 min. The heated samples were separated on SDS-PAGE (12.5% e-PAGEL) and the proteins were visualized by using a Sil-best stain one silver staining kit.

Recyclability of AA-affinity resins by using ascorbate oxidase (AO). All subsequent manipulations of affinity chromatography were done at a temperature of about 4˚C. AA-affinity resins **4** and **8** and the control resin (1.0 ml) were each transferred to a poly-prep column and equilibrated with 3 bed volumes of binding buffer. A solution of AO in binding buffer (total protein of 30 μg) was applied onto the affinity resins. The resins were washed with 3 bed volumes of binding buffer to remove unbound proteins. The bound proteins were each eluted with 2 bed volumes of 0.1 mM AA derivative **5** solution in 10 mM Tris-HCl buffer (pH 7.4) and then with 2 bed volumes of 1.0 mM AA derivative **5** solution in the same buffer. The protein elution fractions were each concentrated by ultrafiltration with a Vivaspin 500. Both the concentrated protein elution fractions and the wash fractions with binding buffer were diluted 2-fold with SDS-PAGE sample buffer and heated at 100˚C for 5 min. The heated samples were separated on SDS-PAGE (12.5% e-PAGEL) and the proteins were visualized by using a Sil-best stain one silver staining kit. The affinity resins already used were re-equilibrated with more than 3 bed volumes of binding buffer and reused in the same manner.

Competitive binding assay of oxd-cyt c with AA-affinity resins. All subsequent manipulations of affinity chromatography were done at a temperature of about 4˚C. AA-affinity resins **4** and **8** and the control resin (1.0 ml) were each transferred to a poly-prep column and equilibrated with 3 bed volumes of binding buffer. Solutions of oxd-cyt c in binding buffers (total protein of 20 μg) without or with 0.1 mM AA and 0.1 mM derivative **5** were each applied onto the affinity resins. The resins were washed with 3 bed volumes of binding buffer to remove unbound proteins, and the wash fractions were collected by 1.0-ml fractions. The bound proteins were each eluted with 2 bed volumes of 0.1 mM AA derivative **5** solution in 10 mM Tris-HCl buffer (pH 7.4) and then with 2 bed volumes of 1.0 mM AA derivative **5** solution in the same buffer. The protein elution fractions were each concentrated by ultrafiltration with a Vivaspin 500. Both the concentrated protein elution fractions and the No.1 and No.3 of the three wash fractions with binding buffer were diluted 2-fold with SDS-PAGE sample buffer and heated at 100˚C for 5 min. The heated samples were separated on SDS-PAGE (12.5% e-PAGEL) and the proteins were visualized by using a Sil-best stain one silver staining kit.

II. Supplementary figures

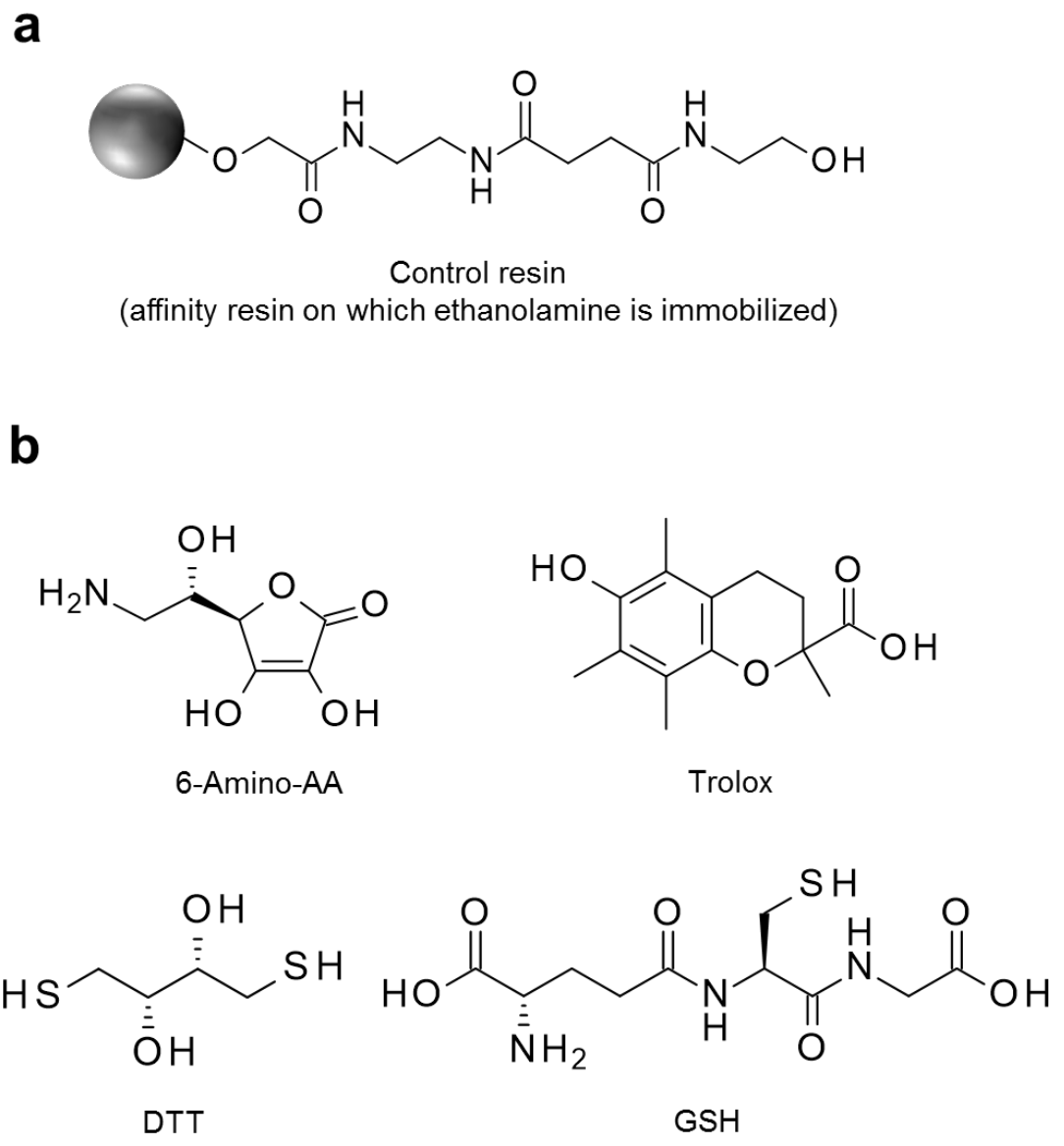


Figure S1. Structures of control resin and reducing agents. (a) Control resin. (b) Reducing agents used in the oxd-cyt c reduction assay and FRAP assay.

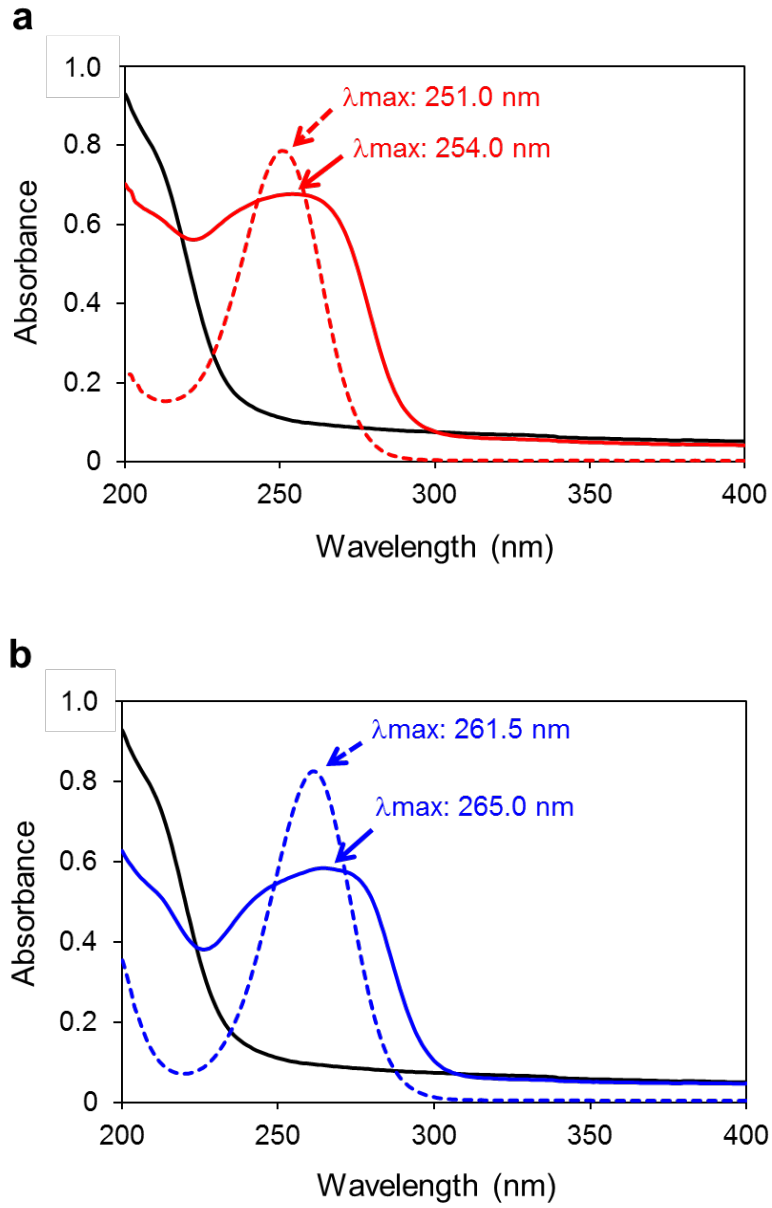


Figure S2. Comparison of UV spectra of AA-affinity resin **4** with AA derivative **9** and control resin or AA-affinity resin **8** with AA derivative **10** and control resin in binding buffer/glycerol (1:1, v/v). (a) AA-affinity resin **4** (red solid line), 50 μM AA derivative **9** (red dotted line) and control resin (black solid line). (b) AA-affinity resin **8** (blue solid line), 50 μM AA derivative **10** (blue dotted line) and control resin (black solid line).

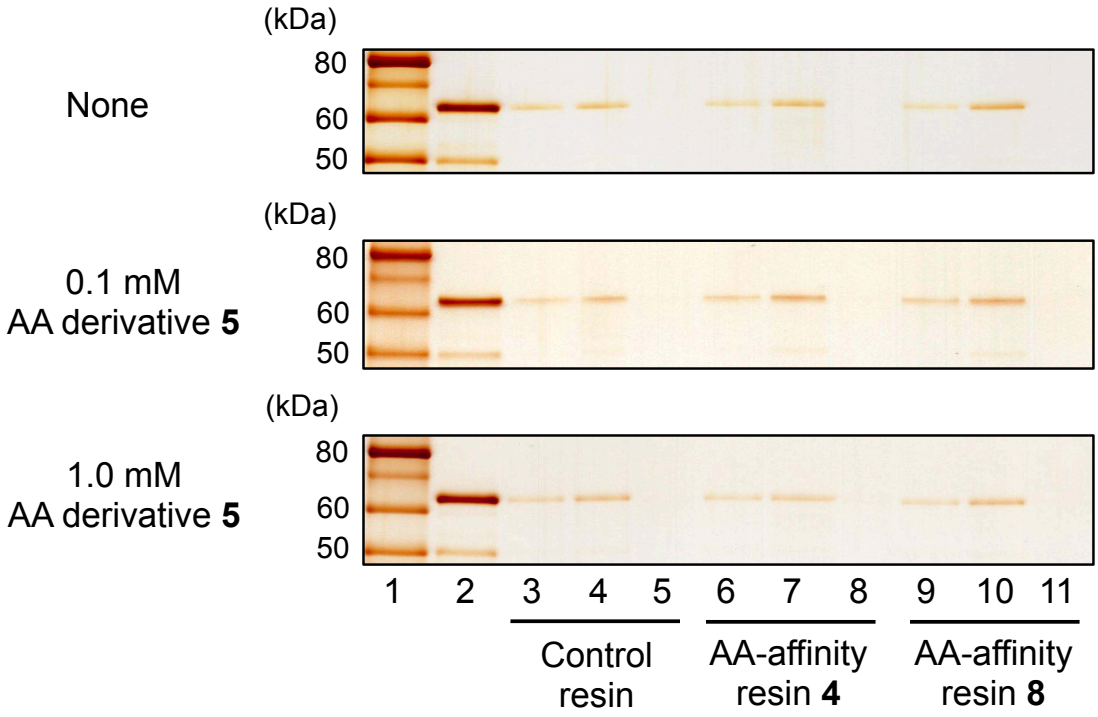


Figure S3. Specificity of AA-affinity resins for ascorbate oxidase (AO). Affinity chromatography of AO for AA-affinity resins **4** and **8** and control resin were carried out without or with 0.1 mM and 1.0 mM AA derivative **5** as a competitor. Lane 1, molecular weight marker; lane 2, AO standard; lanes 3, 6, and 9, wash fractions; lanes 4, 7 and 10, 0.1 mM AA derivative **5** elution fractions; lanes 5, 8 and 11, 1.0 mM AA derivative **5** elution fractions.

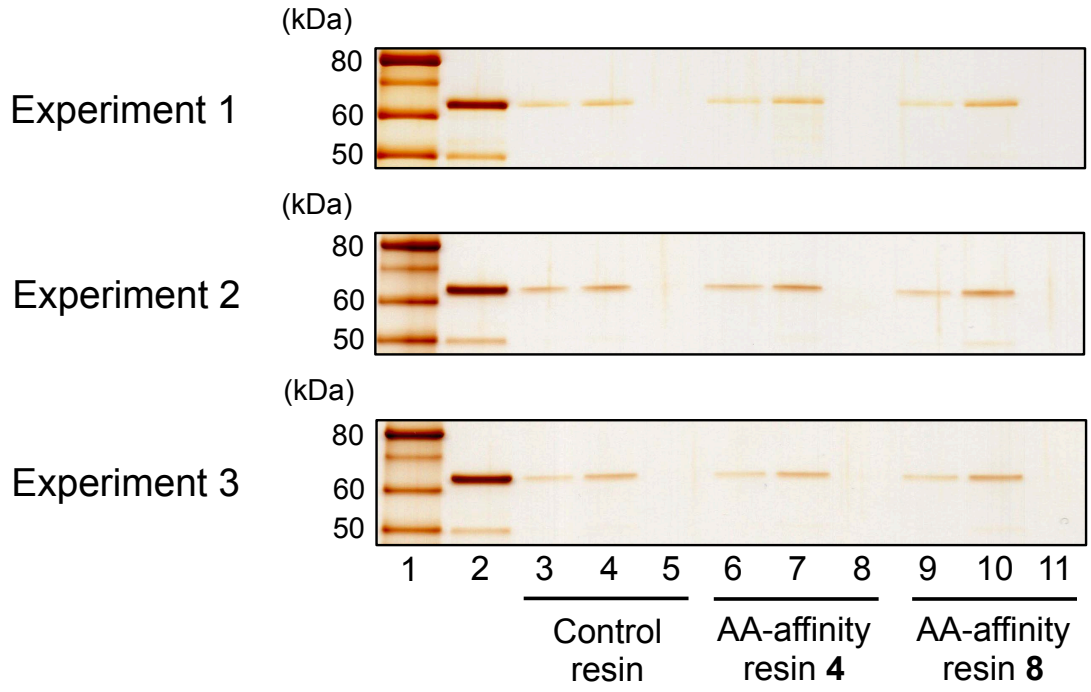


Figure S4. Recyclability of AA-affinity resins by using ascorbate oxidase (AO). Affinity chromatography of AO for AA-affinity resins **4** and **8** and control resin were carried out by three times repetitive experiments using the same respective resins. Lane 1, molecular weight marker; lane 2, AO standard; lanes 3, 6, and 9, wash fractions; lanes 4, 7 and 10, 0.1 mM AA derivative **5** elution fractions; lanes 5, 8 and 11, 1.0 mM AA derivative **5** elution fractions.

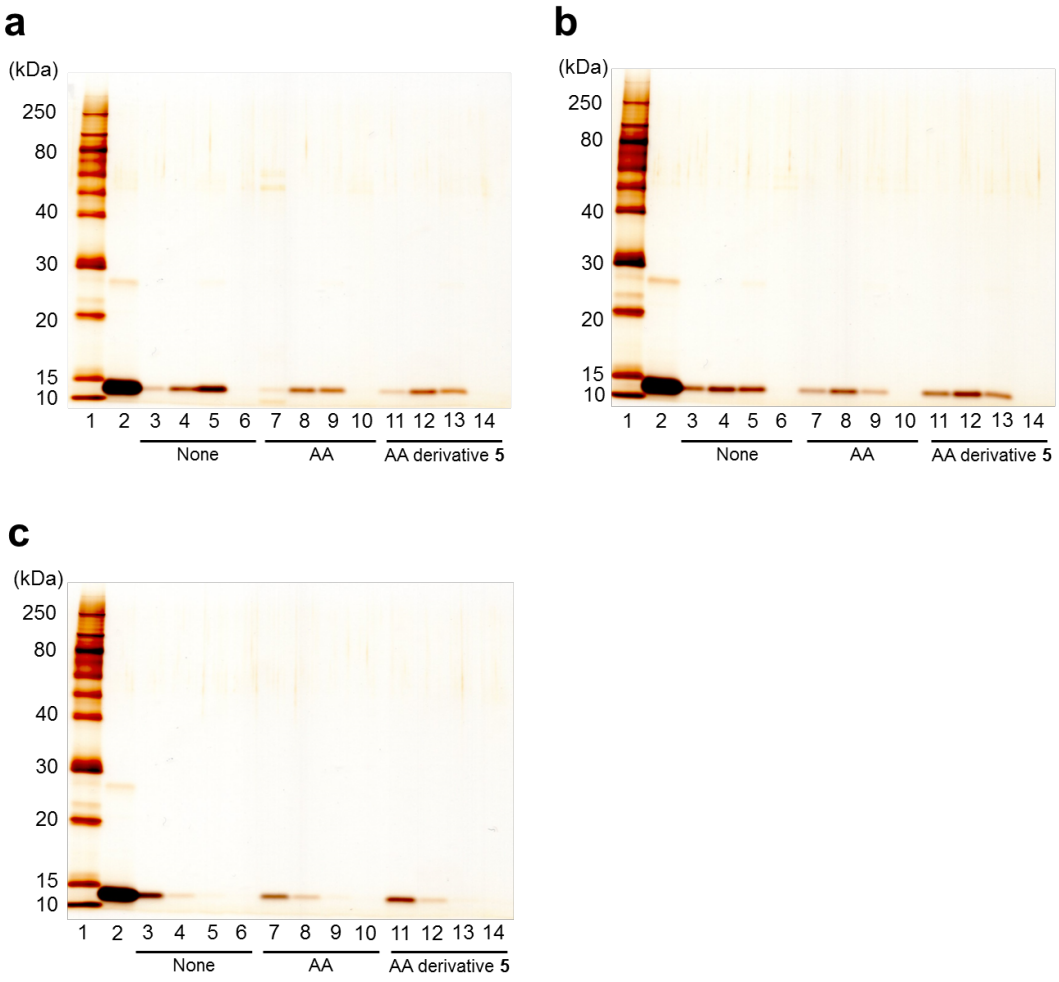


Figure S5. Competitive inhibition assay of oxd-cyt c without or with AA and AA derivative **5** for AA-affnity resin **4** (a), AA-affnity resin **8** (b) and control resin (c). Lane 1, molecular weight marker; lane 2, oxd-cyt c standard; lanes 3, 7, and 11, wash fractions (No. 1); lanes 4, 8 and 12, wash fractions (No. 3); lanes 5, 9 and 13, 0.1 mM AA derivative **5** elution fractions; lanes 6, 10 and 14, 1.0 mM AA derivative **5** elution fractions.

^{1}H- and ^{13}C-NMR spectra of main AA derivatives

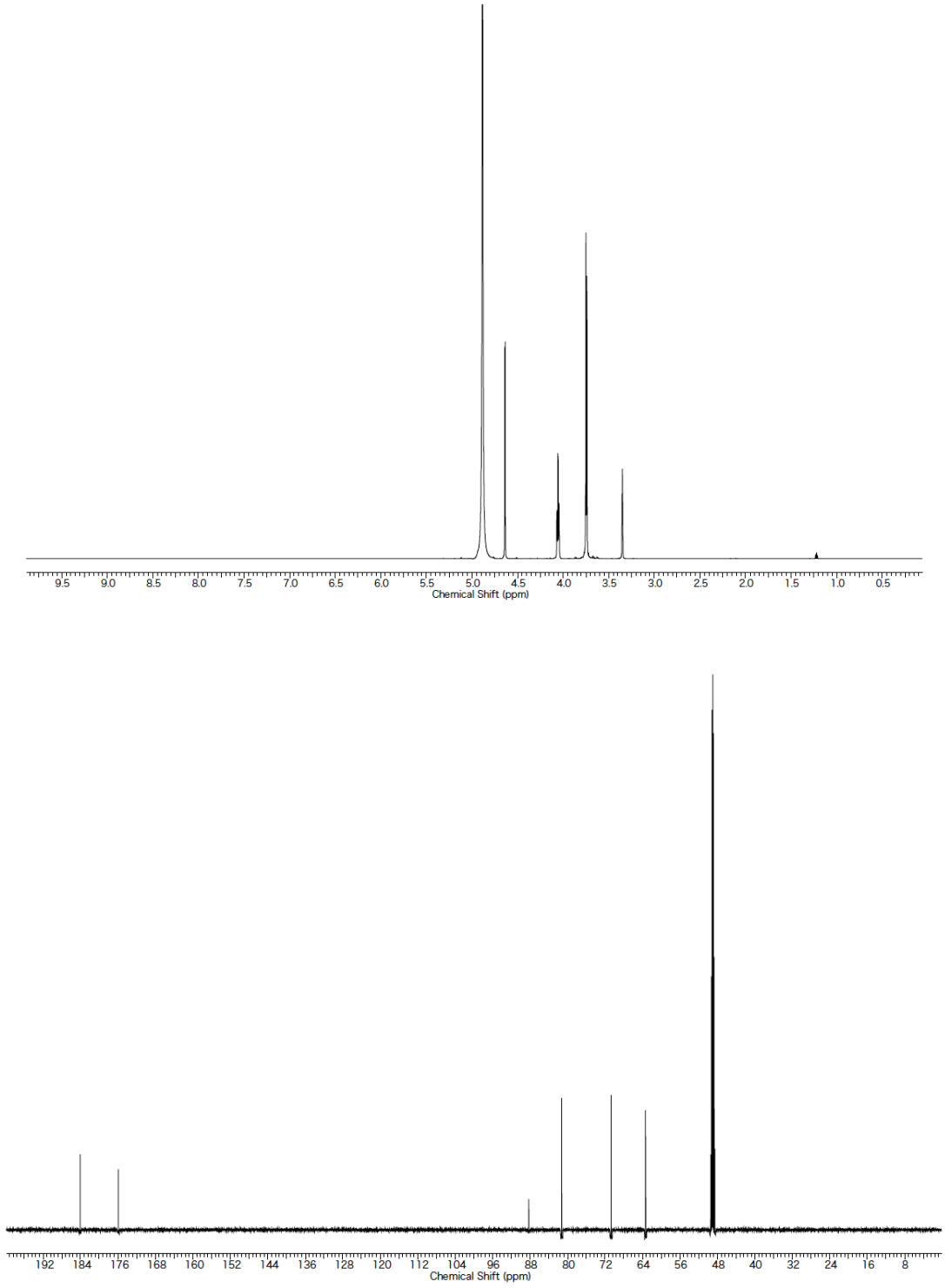


Figure S6. ^{1}H- and ^{13}C-NMR of 2-deoxy-2-amino-L-ascorbic acid (3).

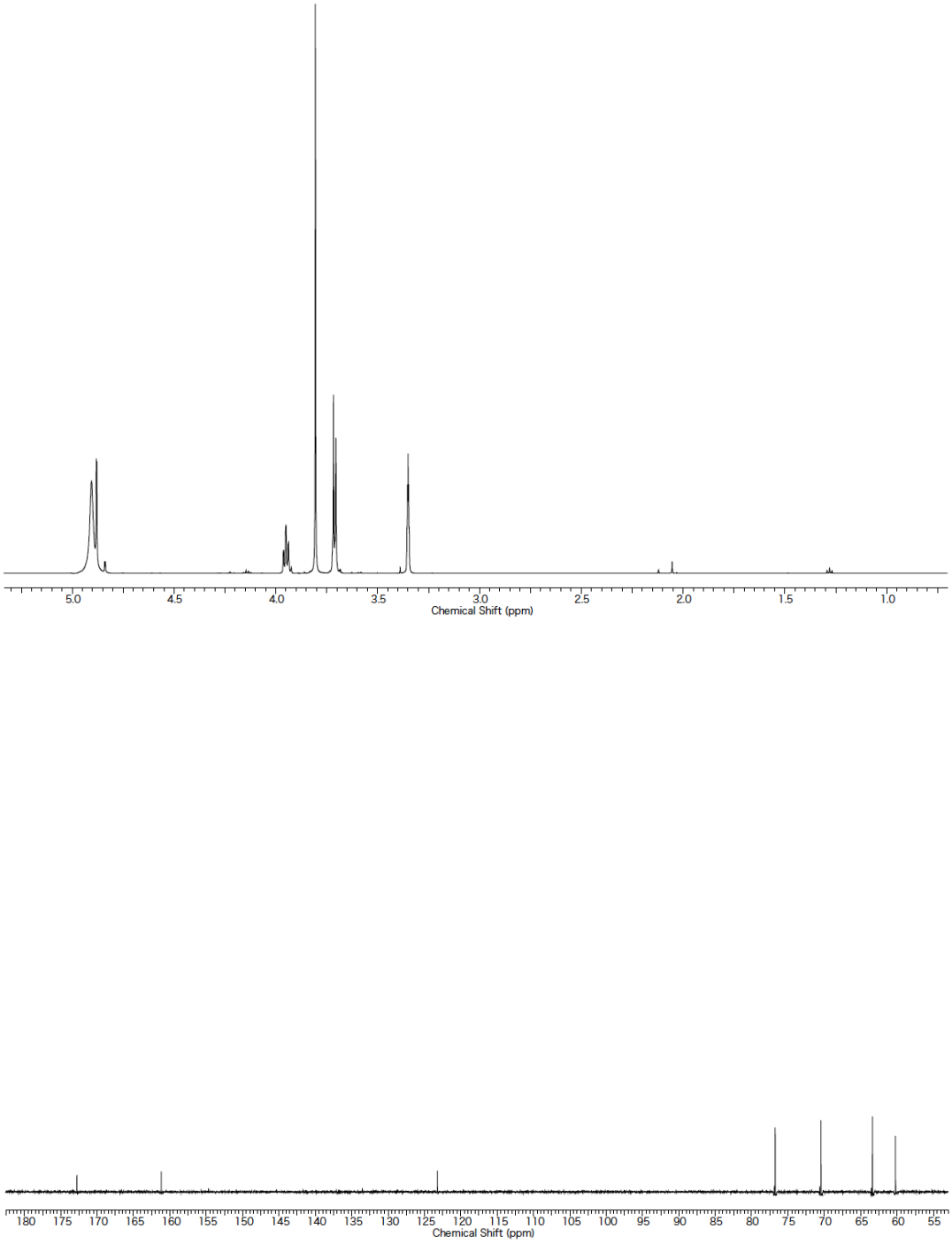


Figure S7. ^{1}H- and ^{13}C-NMR of 2-*O*-methyl-L-ascorbic acid (5).

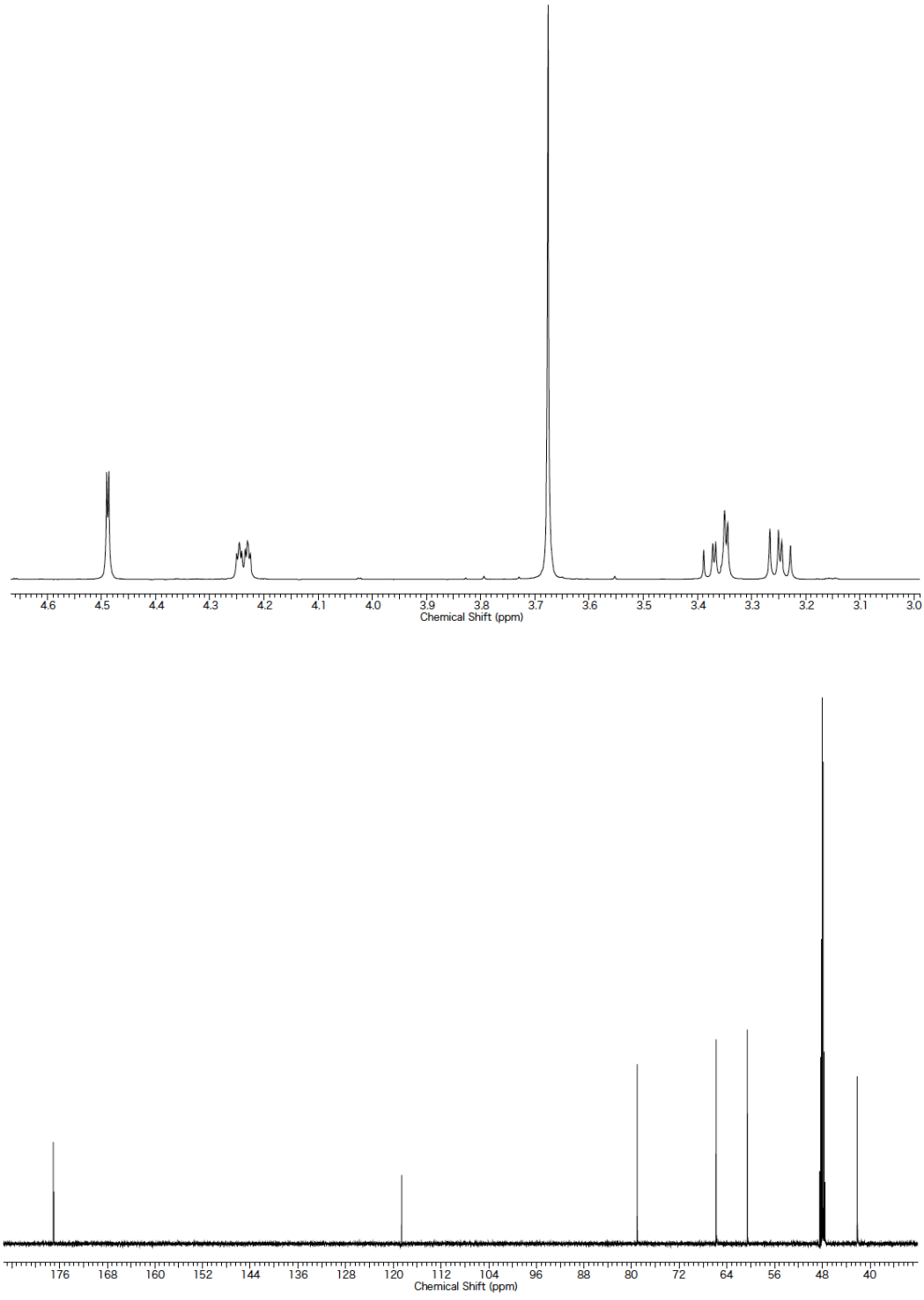
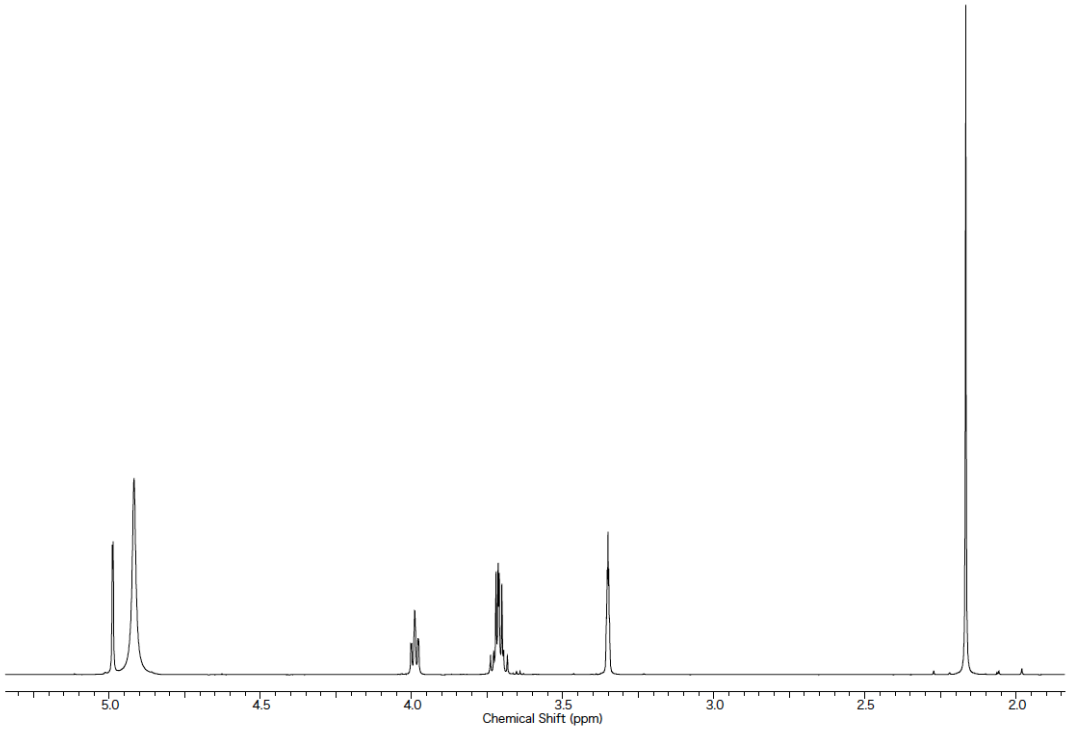


Figure S8. ^{1}H- and ^{13}C-NMR of 6-amino-6-deoxy-2-*O*-methyl-L-ascorbic acid (7).



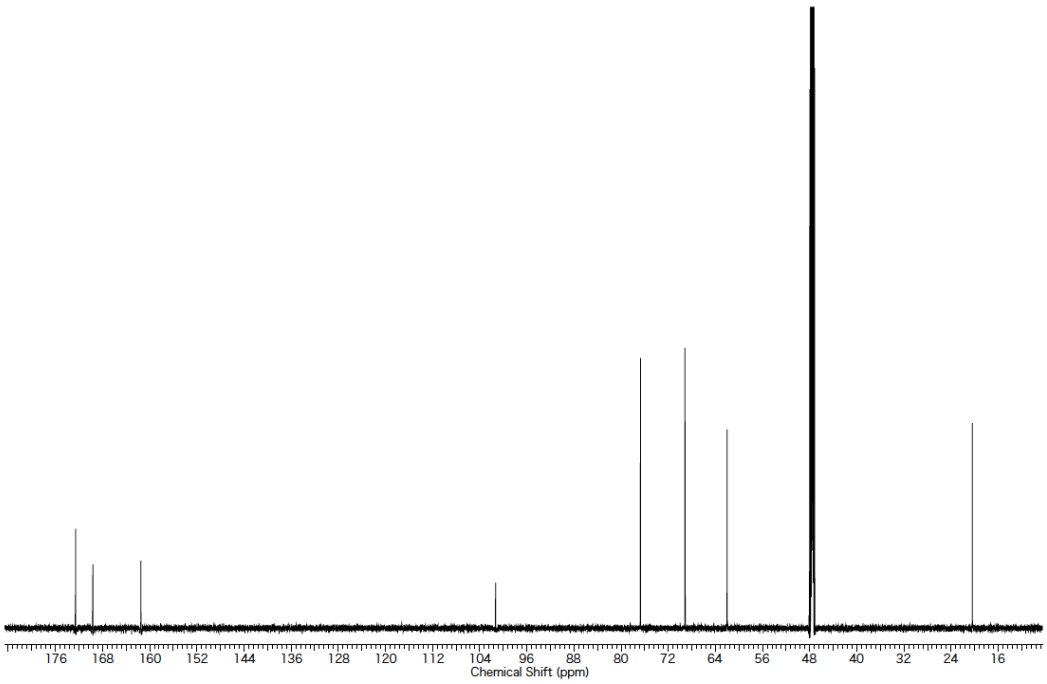


Figure S9. ^{1}H- and ^{13}C-NMR of 2-acetylamino-2-deoxy-L-ascorbic acid (9).

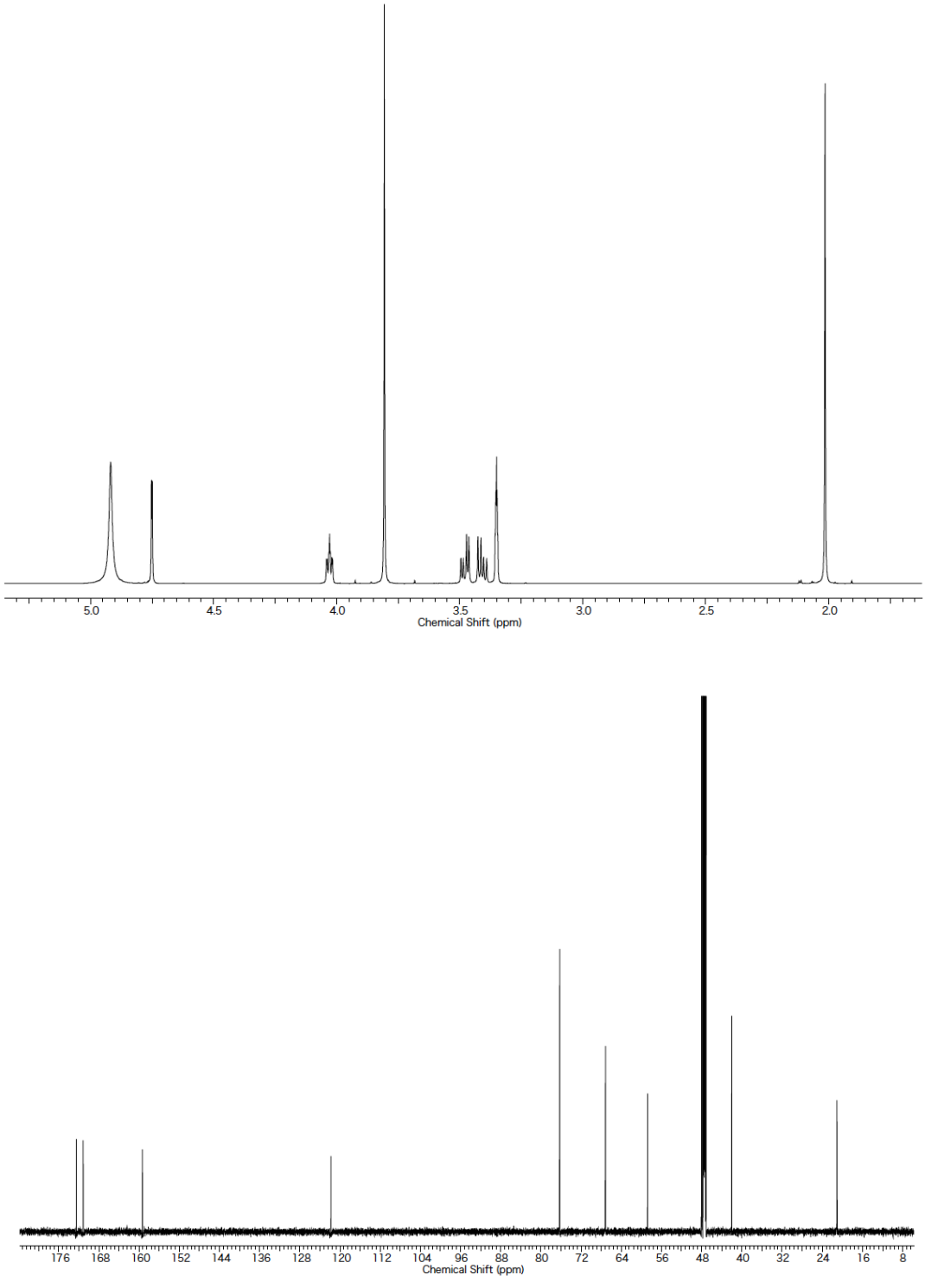


Figure S10. ^{1}H- and ^{13}C-NMR of 6-acetylamino-6-deoxy-2-*O*-methyl-L-ascorbic acid (10).