Supporting Information

Supramolecular Complexation with Kinetic Stabilization:

Cucurbit[6]uril Encapsulated Doxorubicin-Based Prodrugs for pH-Responsive Controlled Release

Weipeng Mao, Shuyi Wang, Dake Mao, Yamin Liu, Libai Li, Da Ma*

School of Pharmaceutical and Materials Engineering & Institute for Advanced Studies, Taizhou University, Jiaojiang, Zhejiang 318000, China.
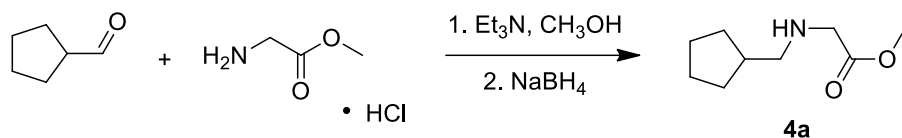Department of Chemistry, Fudan University, 220 Handan Road, Shanghai, 200433, China.
Email:dama@fudan.edu.cn

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Synthesis Procedures

Compound 4a. Cyclopentanecarboxaldehyde (1.96 g, 20 mmol) and Glycine methyl ester hydrochloride (2.51 g, 20 mmol) were dissolved in CH_3OH (40 mL). Trimethylamine (5.53 mL) was added into the solution. The solution was stirred at room temperature for 8 h. $NaBH_4$ (1.56 g, 40 mmol) was slowly added into solution. The suspension was stirred at room temperature for 3 h. The reaction was quenched by water. The product was extracted with ethyl acetate, and the solvent was removed by rotary evaporation. The residue was purified by column chromatography (CH_2Cl_2/CH_3OH) to give compound **4a** (700 mg, 20%) as colorless liquid. ^1H NMR (400 MHz, CDCl$_3$): δ = 3.73 (s, 3 H), 3.42 (s, 2 H), 2.53 (d, J = 8 Hz, 2 H), 2.01 (m, 1 H), 1.82 – 1.74 (m, 2 H), 1.64 – 1.53 (m, 4 H), 1.20- 1.15 (m, 2 H). ^{13}C NMR (100 MHz, CDCl$_3$): δ = 173.0, 55.4, 51.6, 51.0, 40.0, 30.7, 25.2. HR-MS: m/z 172.1331 ([M+H]$^+$, calcd for 172.1338).
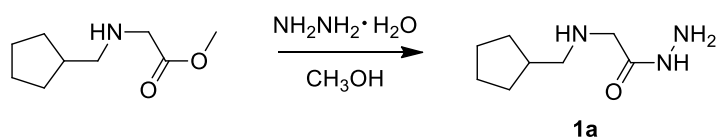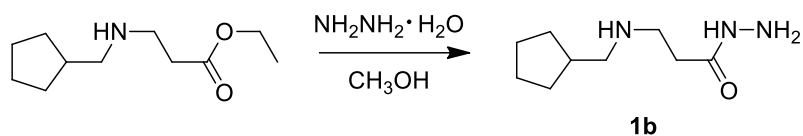

Compound 1a. Compound **4a** (511 mg, 3 mmol) and $NH_2NH_2 \cdot H_2O$ (300 mg, 6 mmol) were dissolved in CH_3OH (5 mL). The solution was heated at reflux for 12 h. The solvent was removed by rotary evaporation, and the residue was recrystallized by ethanol to yield compound **1a** (300 mg, 53%) as colorless solid. ^1H NMR (400 MHz, DMSO-d_6): δ = 8.83 (s, 1 H), 4.19 (s, 2 H), 3.04 (s, 2 H), 2.43 (d, J = 6.4 Hz, 2 H), 1.95 – 1.88 (m, 1 H), 1.71 – 1.64 (m, 2 H), 1.55 – 1.45 (m, 4 H), 1.16 – 1.11 (m, 2 H). ^{13}C NMR (100 MHz, DMSO-d_6): 170.9, 55.2, 51.6, 39.9, 30.7, 25.3. HR-MS: m/z 172.1446 ([M+H]$^+$, calcd for 172.1450).
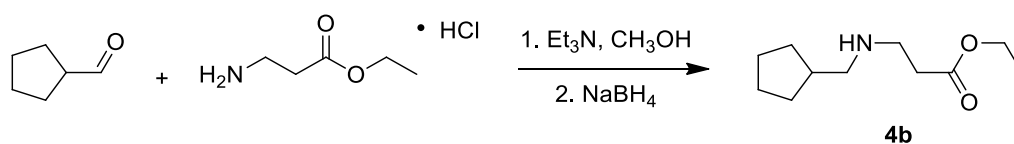

Compound 4b. Cyclopentanecarboxaldehyde (980 mg, 10 mmol) and β-alanine ethyl ester hydrochloride (1536 mg, 10 mmol) were dissolved in CH_3OH (40 mL). Trimethylamine (3.03 g, 30 mmol) was added into the solution. The solution was stirred at room temperature for 8 h. $NaBH_4$ (780 mg, 20 mmol) was slowly added. The suspension was stirred at room temperature for 3 h. The reaction was quenched by water. The product was extracted with ethyl acetate, and solvent was removed by rotary evaporation. The residue was purified by column chromatography (ethyl acetate/petroleum ether) to give compound **4b** (900 mg, 20%) as colorless liquid. ^1H NMR (400 MHz, CDCl$_3$): δ = 4.14 (q, J = 7.2 Hz, 2 H), 2.88 (t, J = 6.4 Hz, 2 H), 2.55

– 2.50 (m, 4 H), 2.04 – 1.97 (m, 1 H), 1.80 – 1.72 (m, 2 H), 1.64 – 1.49 (m, 4 H), 1.26 (t, J = 7.2 Hz, 3 H), 1.19 – 1.10 (m, 2 H). ^{13}C NMR (100 MHz, CDCl$_3$): δ = 172.8, 60.3, 55.5, 45.3, 19.9, 34.7, 30.8, 25.2, 14.2. HR-MS: m/z 200.1645 ([M+H]$^+$, calcd for 200.1651).


1b

Compound **1b**. Compound **4b** (700 mg, 3.6 mmol) and NH$_2$NH$_2$·H$_2$O (360 mg, 7.2 mmol) were dissolved in CH$_3$OH (6 mL). The solution was heated at reflux for 12 h. Solvent was removed by rotary evaporation, and the residue was purified by column chromatography (ethyl acetate/petroleum ether) to give compound **1b** (300 mg, 53%) as colorless solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 9.01 (s, 1 H), 4.14 (s, 2 H), 2.68 (t, J = 7.8 Hz, 2H), 2.97 (d, J = 7.2 Hz, 2H), 2.16 (t, J = 7.8 Hz, 2H), 1.95 – 1.87 (m, 1 H), 1.70 – 1.63 (m, 2 H), 1.56 – 1.44 (m, 4 H), 1.16 – 1.11 (m, 2 H). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 171.3, 55.2, 46.3, 39.9, 34.4, 30.8, 25.3. HR-MS: m/z 186.1603 ([M+H]$^+$, calcd for 186.1606).

2a
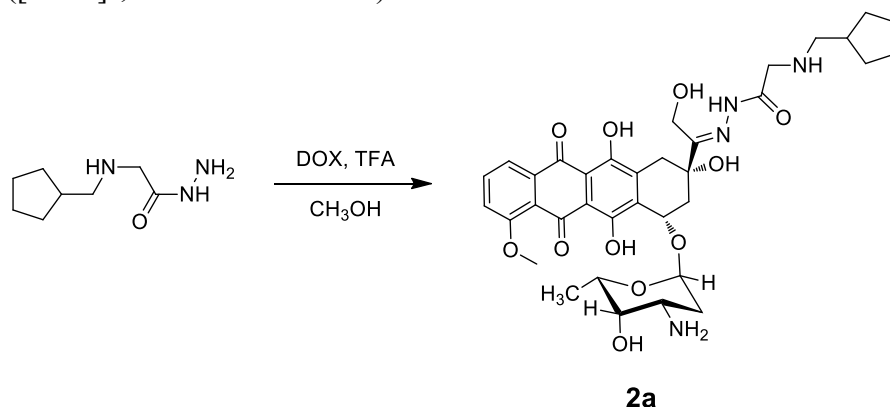
Compound **2a**. Compound **1a** (34.2 mg, 200 μmol) and doxorubicin hydrochloride (40 mg, 69 μmol) were dissolved in CH$_3$OH (16 mL). Trifluoroacetic acid (80 μL) was added into the solution and stirred at room temperature for 12 h. Solvent was removed by rotary evaporation. EtOAc (4 mL) was added to the mixture and sonicated for 15 min to afford a red precipitate. The red precipitate was collected by centrifugation and washed by EtOAc (6 mL×2) and CH$_2$Cl$_2$ (6 mL×2), and dried under high vacuum to give compound **2a** (35 mg, 69%) as a red solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 13.319(s, 1 H), 11.002(s, 1 H), 7.932 – 7.886(m, 5 H), 7.690(d, J = 6.8 Hz, 1 H), 5.840(s, 1 H), 5.626(s, 1 H), 5.496(d, J = 4.8 Hz, 1 H), 5.304(s, 1 H), 4.895(t, J = 6.4 Hz, 1H), 4.452(s, 2 H), 4.039(d, J = 6.8 Hz, 1 H), 3.993(s, 3 H), 3.831(d, J = 16.4 Hz, 1 H), 3.626 – 3.575(m, 2 H), 3.446(d, J = 16.8 Hz, 1 H), 2.910 – 2.686(m, 4 H), 2.203 – 1.863(m, 4 H), 1.746 – 1.451(m, 8 H), 1.234 – 0.988(m, 7 H). ^{13}C NMR: δ = 187.1, 187.0, 167.2, 161.3, 156.8, 156.4, 154.4, 137.0, 136.7, 136.0, 135.2, 137.0, 136.7, 136.0, 135.2, 120.5, 120.3, 119.5, 111.2, 111.1, 99.4, 72.8, 72.2, 66.8, 66.4, 60.18, 57.1, 56.1, 52.4, 47.5, 47.0, 36.4, 30.5, 30.3, 25.0, 25.0, 17.3, 14.5. HR-MS: m/z 697.3098 ([M+H]$^+$, calcd for 697.3085).

2b
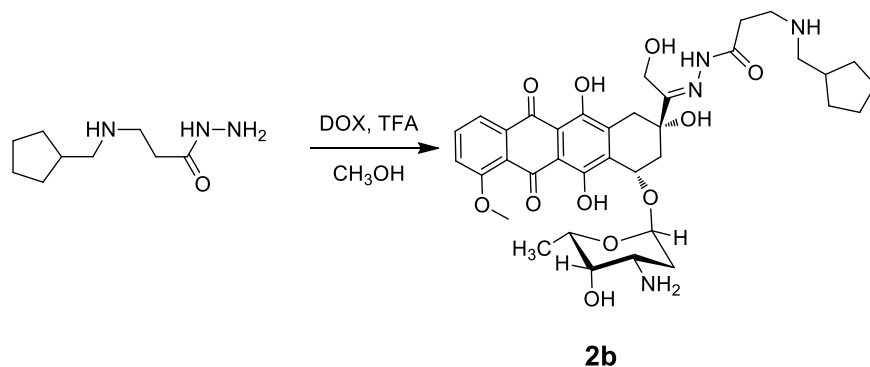
Compound **2b**. Compound **1b** (37.2 mg, 200 μmol) and doxorubicin hydrochloride (40 mg, 69 μmol) were dissolved in CH_3OH (16 mL). Trifluoroacetic acid (80 μL) was added to the solution and stirred at room temperature for 12 h. Then, the solvent was removed by rotary evaporation. EtOAc (4 mL) was added and sonicated for 15 min to afford a red precipitate. The precipitate was collected by centrifugation and washed by EtOAc (6 mL×2) and CH_2Cl_2 (6 mL×2), and dried under high vacuum to give compound **2b** (40 mg, 77%) as a red solid. ^1H NMR (400 MHz, DMSO-d_6): δ = 14.084(s, 1 H), 13.317(s, 1 H), 10.658(s,1 H), 7.931-7.869(m, 5 H), 7.868-7.664(m, 1 H), 5.888(s, 1 H), 5.484(s, 1 H), 5.380(s, 1 H), 5.320(s, 1 H), 4.985(t, J = 5.2 Hz, 1 H), 4.464(s, 2 H), 3.990(s, 3 H), 3.576(s, 1 H), 3.226(d, J = 17.6 Hz, 1 H), 3.114 – 3.104(m, 2 H), 2.930 – 2.829(m, 4 H), 2.716 (t, J = 6.8 Hz, 2 H), 2.399 – 2.349(m, 1 H), 2.269 – 2.225(m, 1 H), 2.154 – 2.077(m, 1 H), 1.894(t, J = 12.8 Hz, 1 H), 1.592 - 1.506(m, 2 H), 1.592 – 1.506(m, 4 H), 1.233 – 1.170(m, 7 H). ^{13}C NMR: δ = 187.1, 187.0, 171.7, 161.3, 156.7, 155.2, 154.7, 136.7, 136.2, 136.0, 135.3, 130.1, 120.6, 120.2, 119.5, 111.2, 99.6, 72.5, 71.9, 66.8, 66.5, 66.4, 57.1, 56.6, 52.0, 47.0, 42.9, 36.8, 33.8, 30.4, 28.5, 27.0, 25.0, 17.2. HR-MS: m/z 711.3223 ([M+H]$^+$, calcd for 711.3141)
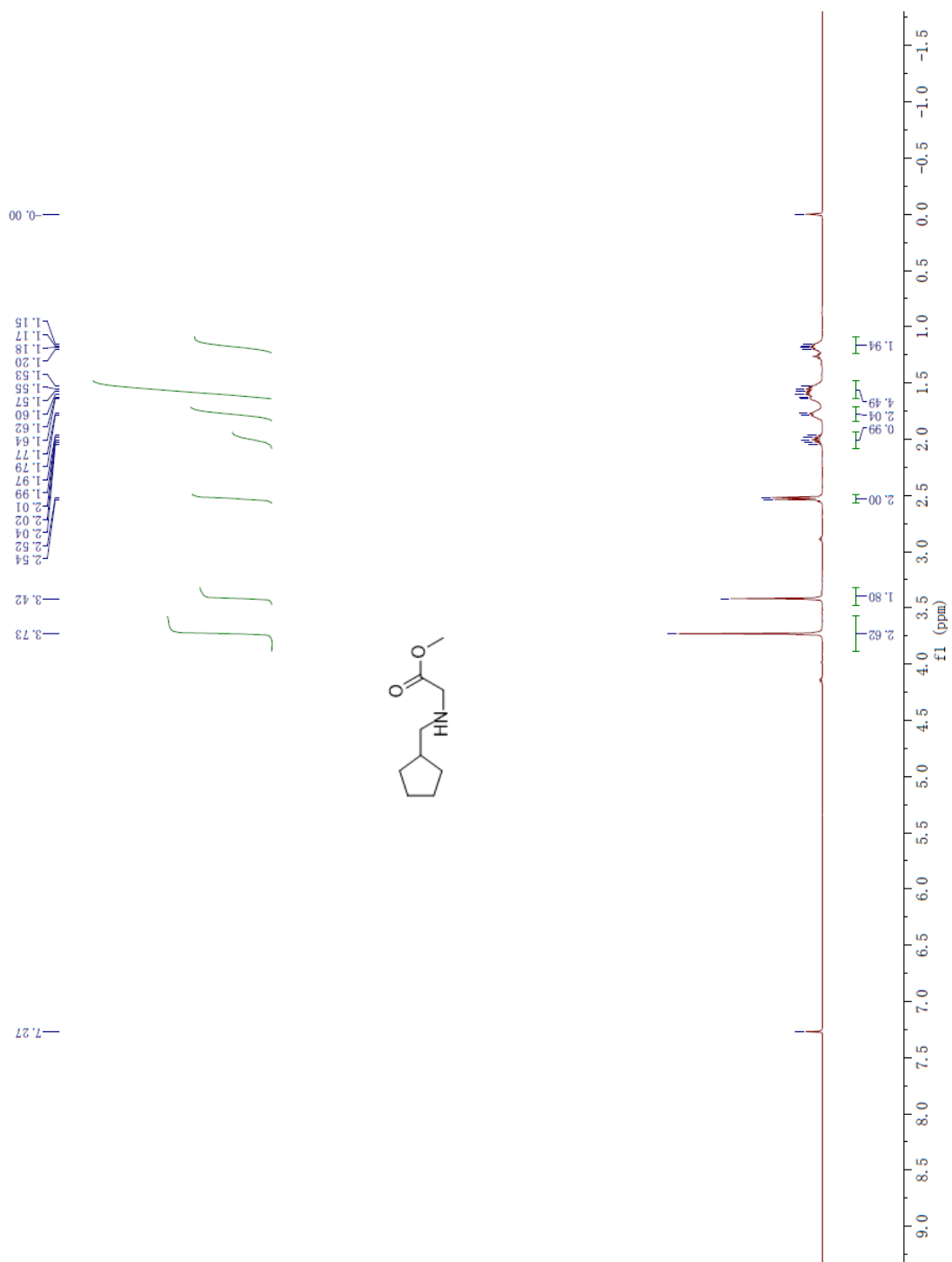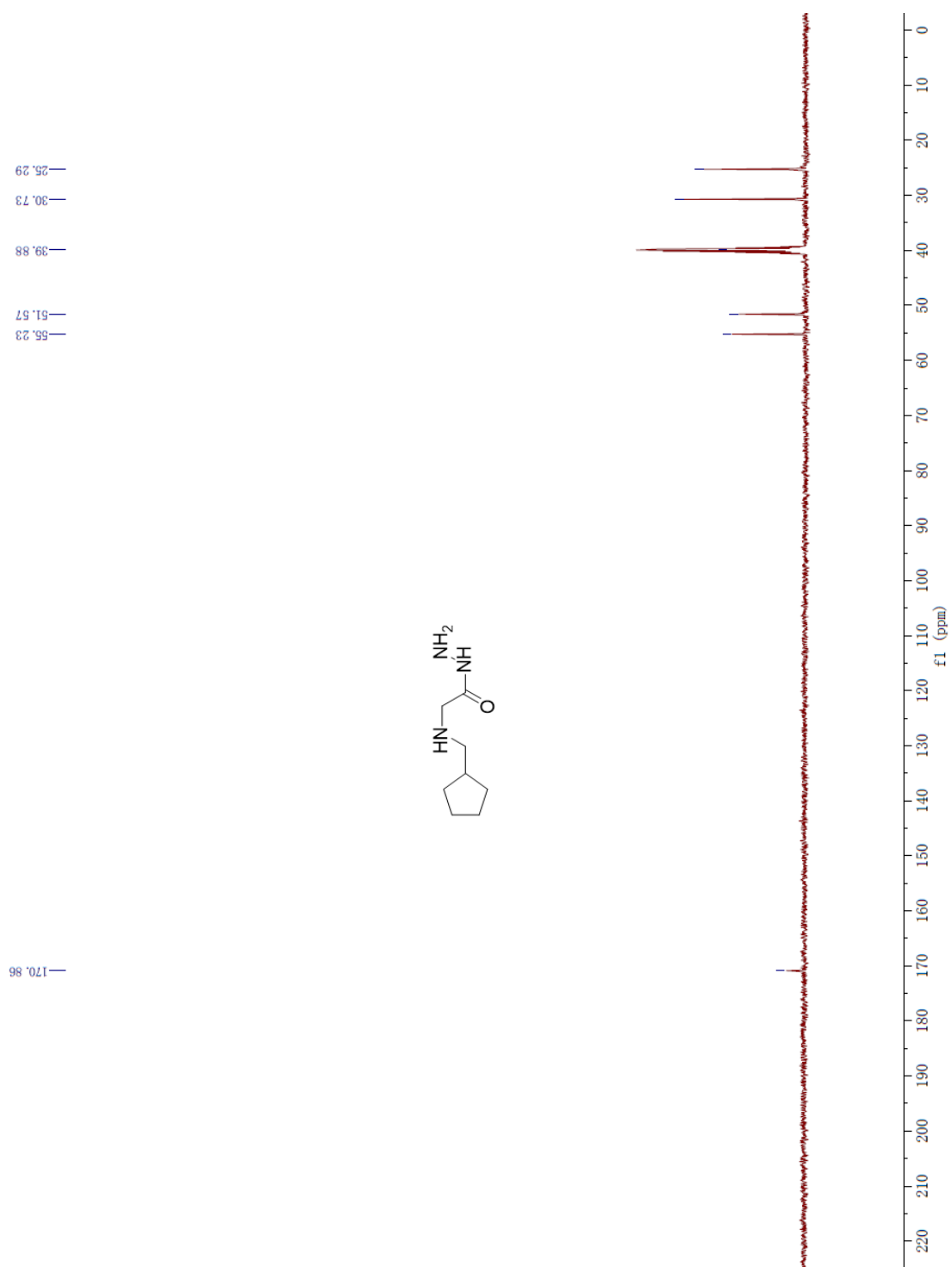
Figure S1. ¹H NMR recorded (400 MHz, CDCl₃, 25 °C) for compound **4a**
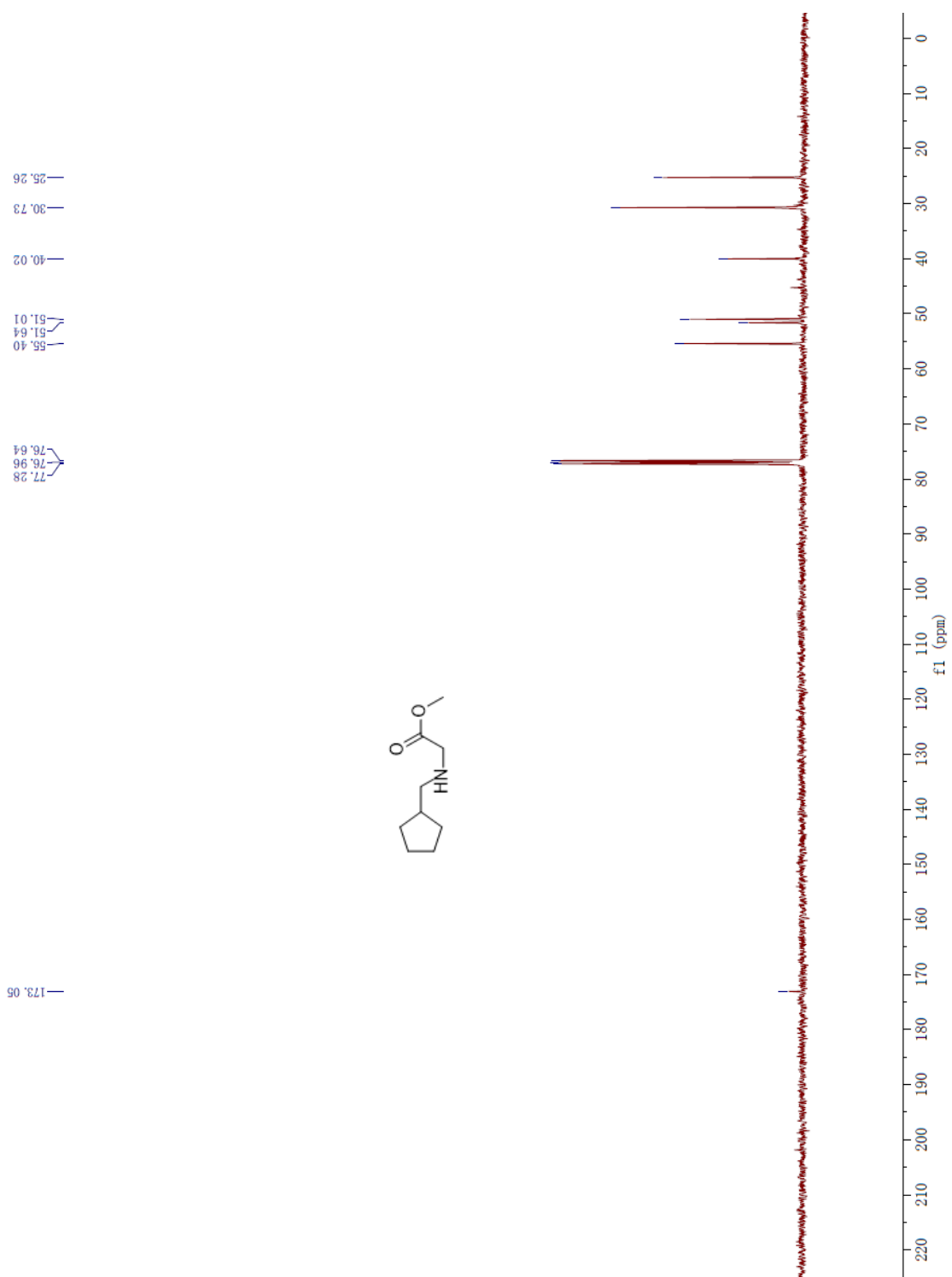
Figure S2. ^{13}C NMR recorded (100 MHz, CDCl$_3$, 25 °C) for compound **4a**
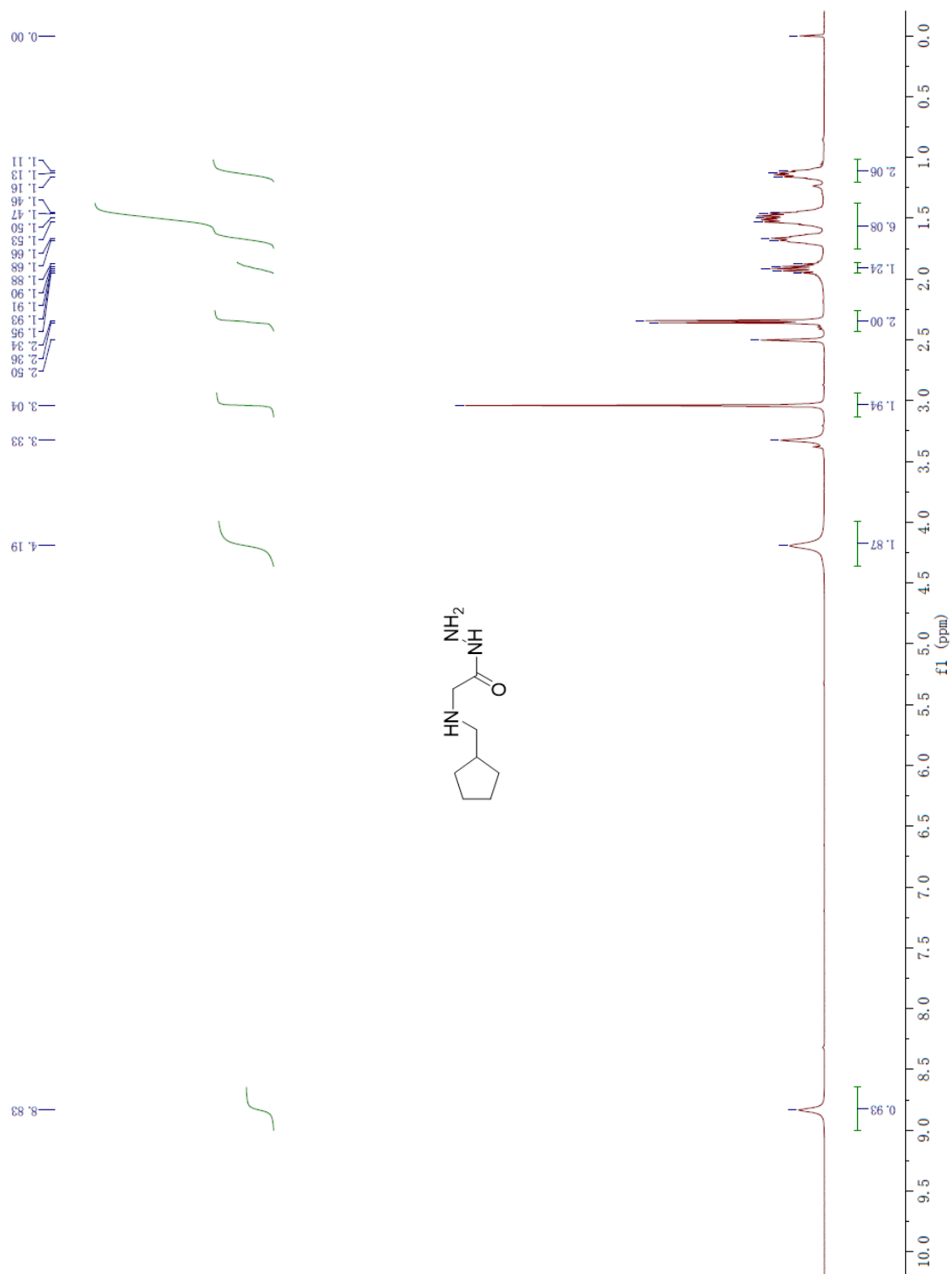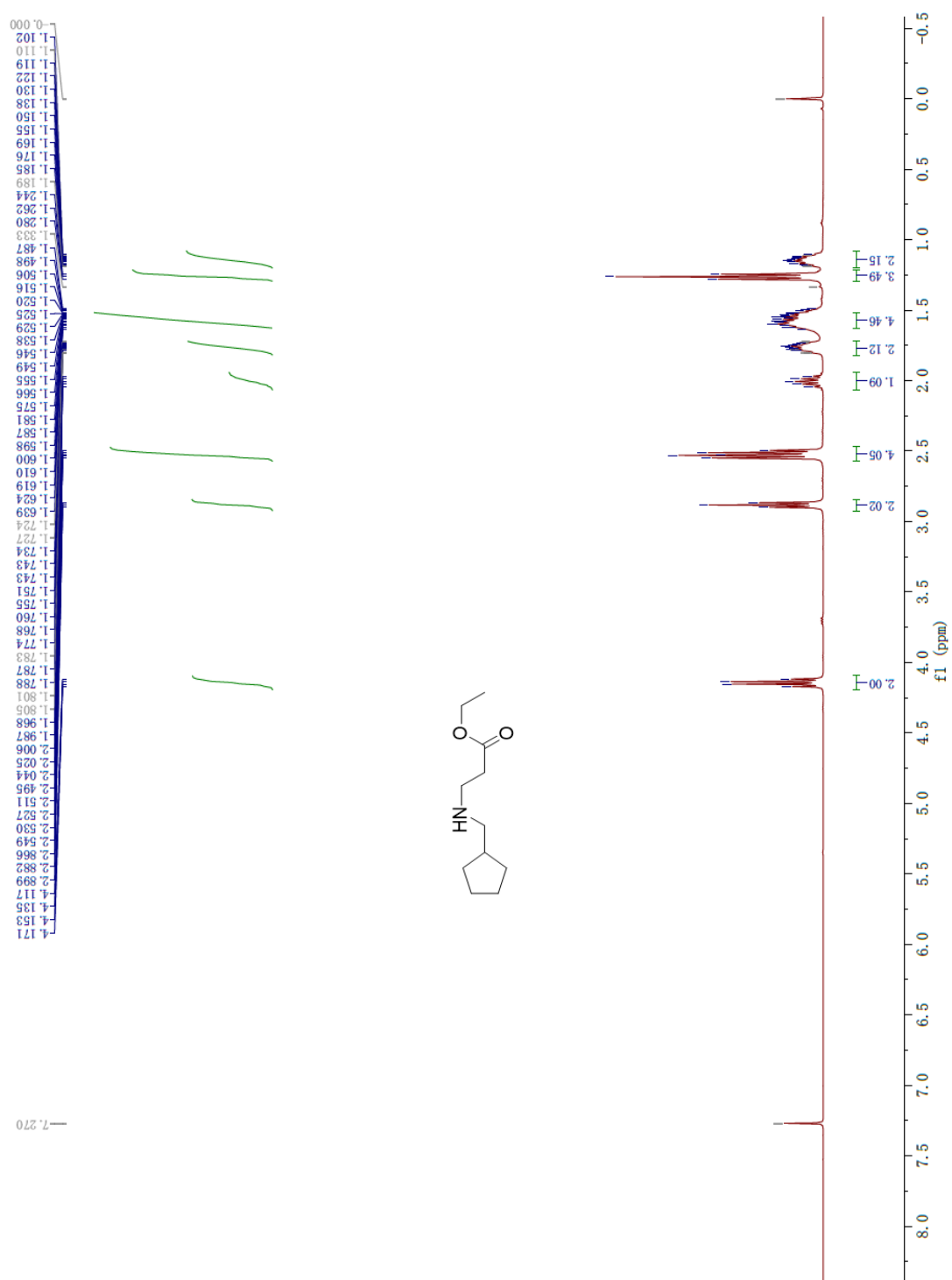
Figure S3. ^{1}H NMR recorded (400 MHz, DMSO-d_6, 25 °C) for compound **1a**

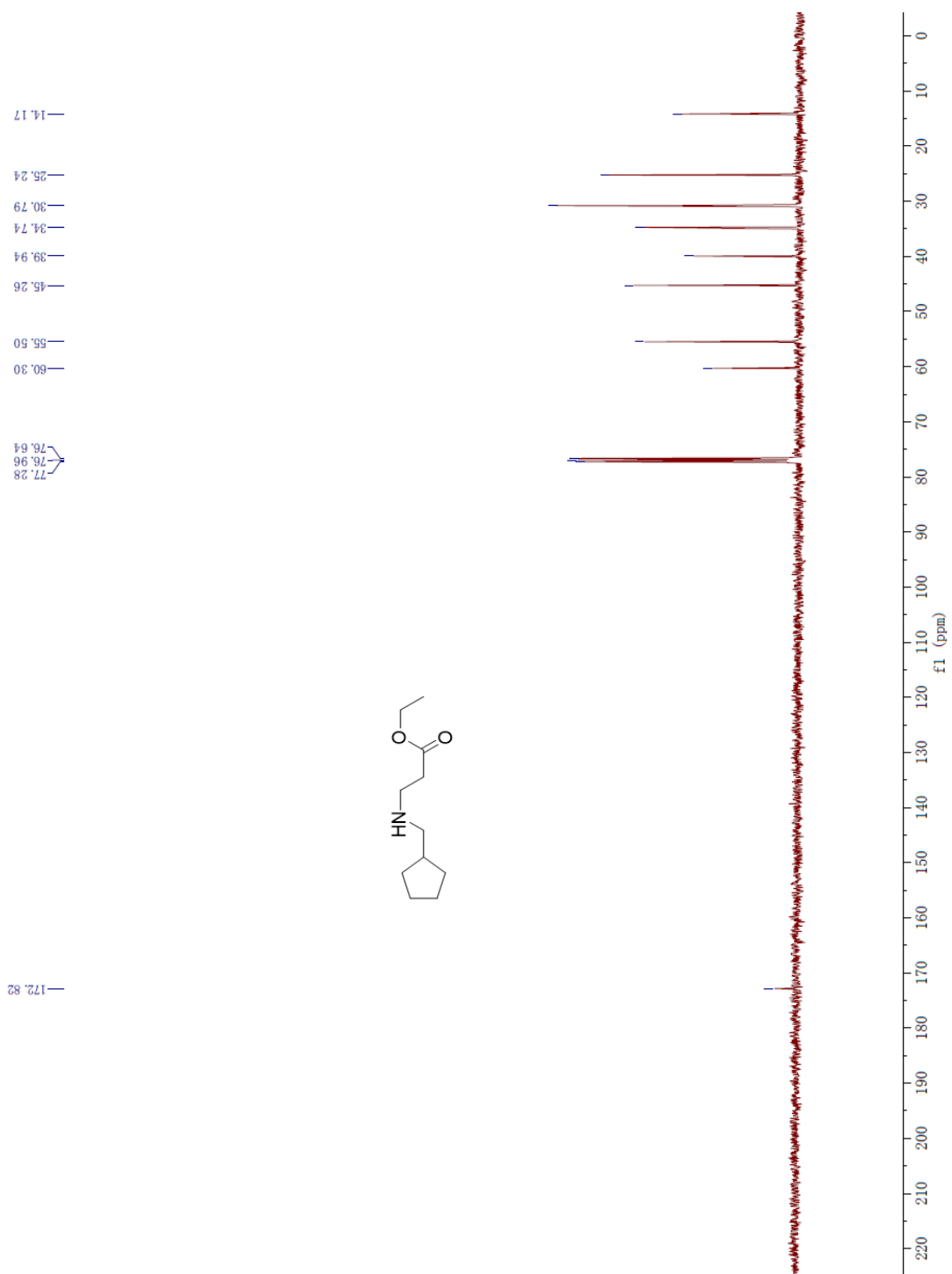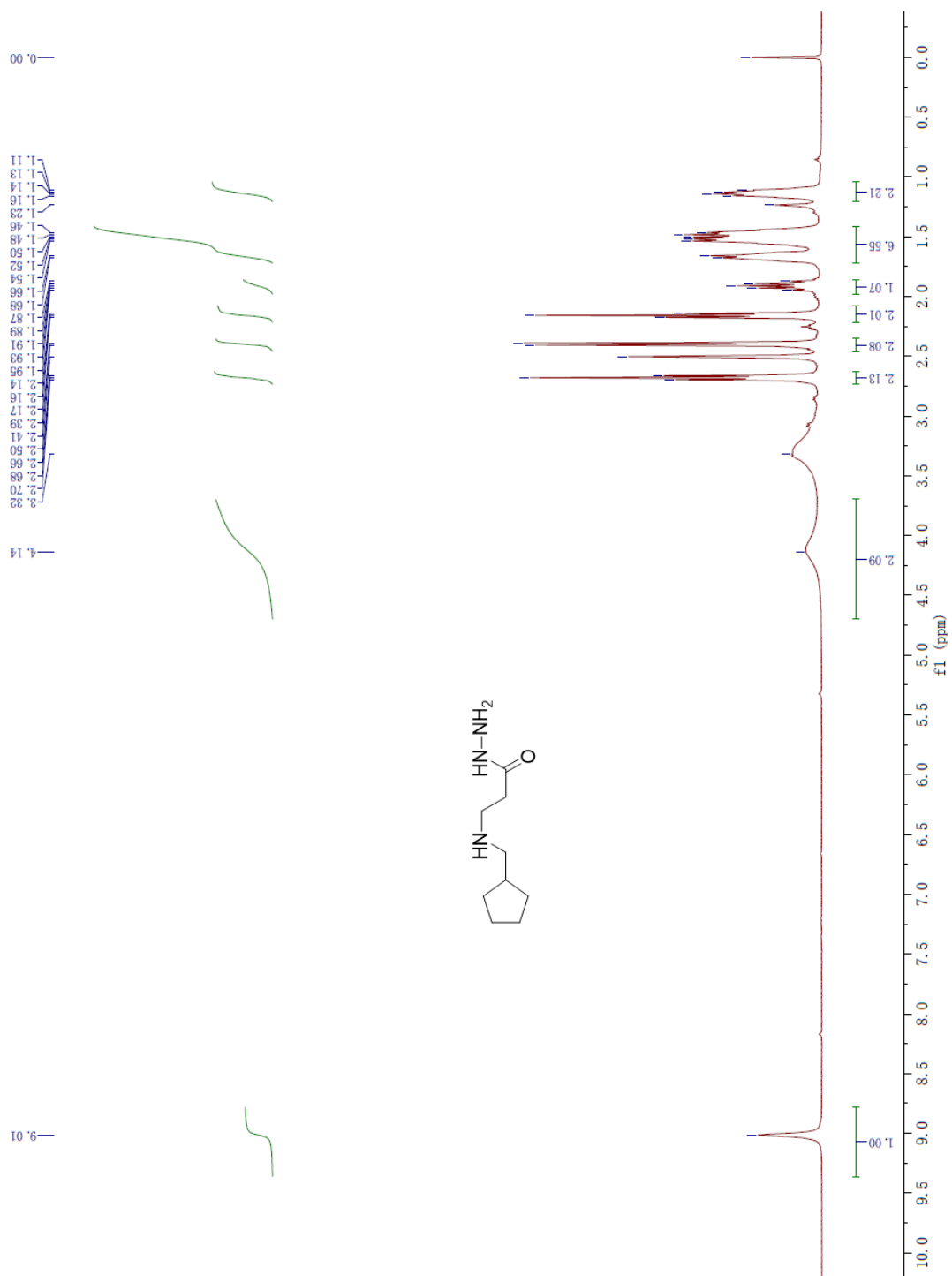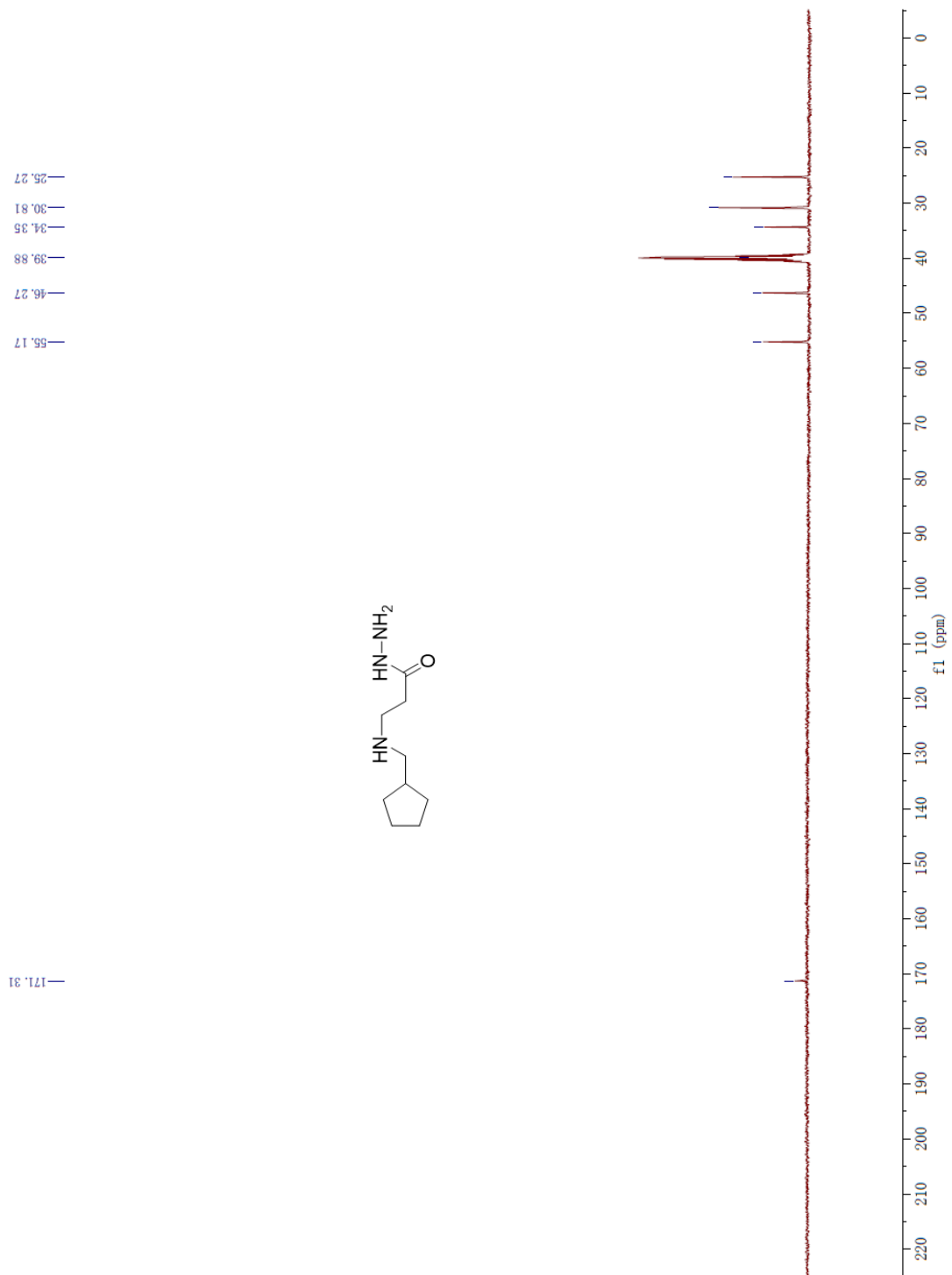**Figure S4.** ^{13}C NMR recorded (100 MHz, DMSO-d_6, 25 °C) for compound **1a**.

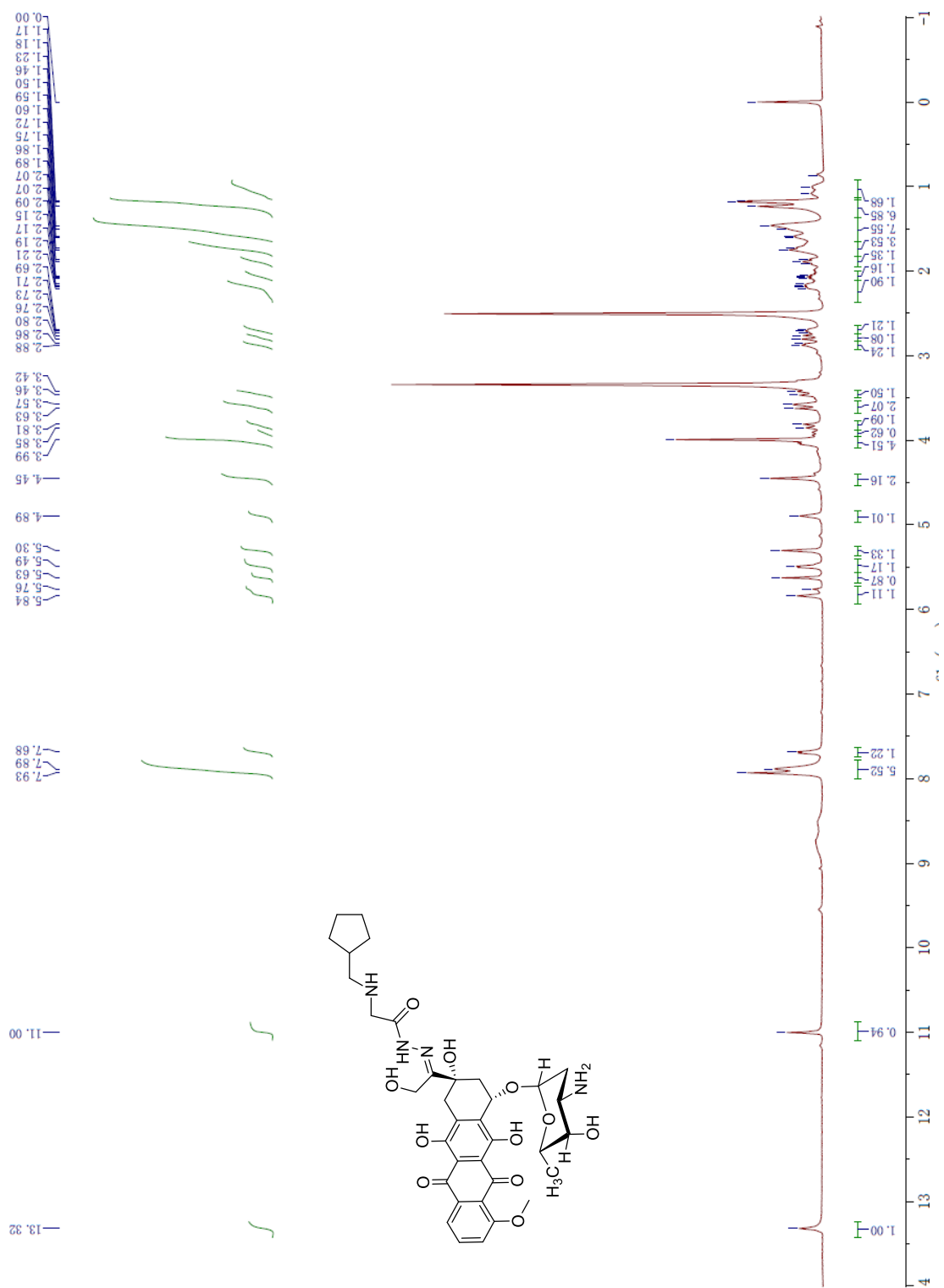**Figure S5.** ^{1}H NMR recorded (400 MHz, CDCl$_3$, 25 °C) for compound **4b**.

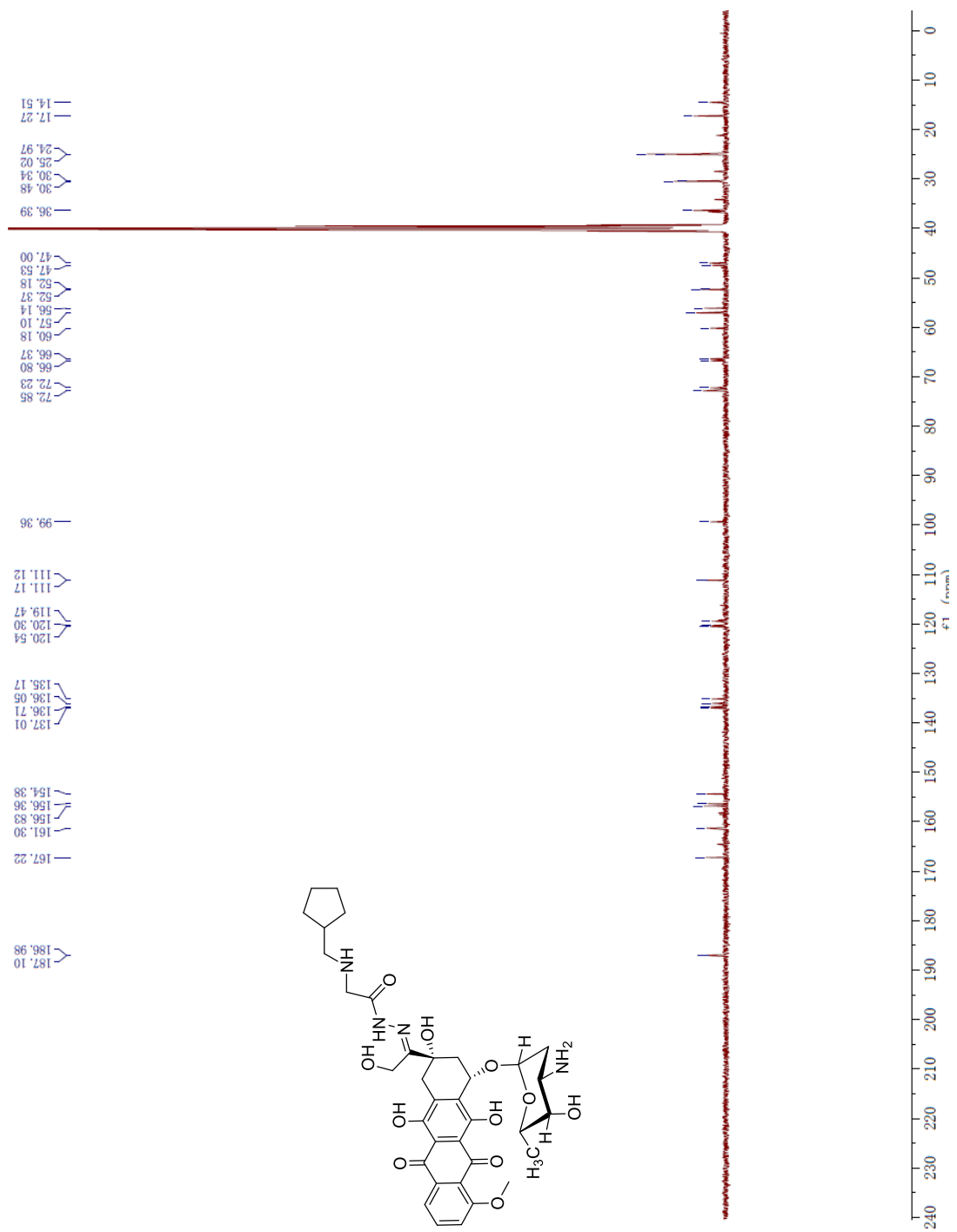**Figure S6.** ^{13}C NMR recorded (100 MHz, CDCl$_3$, 25 °C) for compound **4b**.

Figure S7. ^{1}H NMR recorded (400 MHz, DMSO-d_6, 25 °C) for compound **1b**.

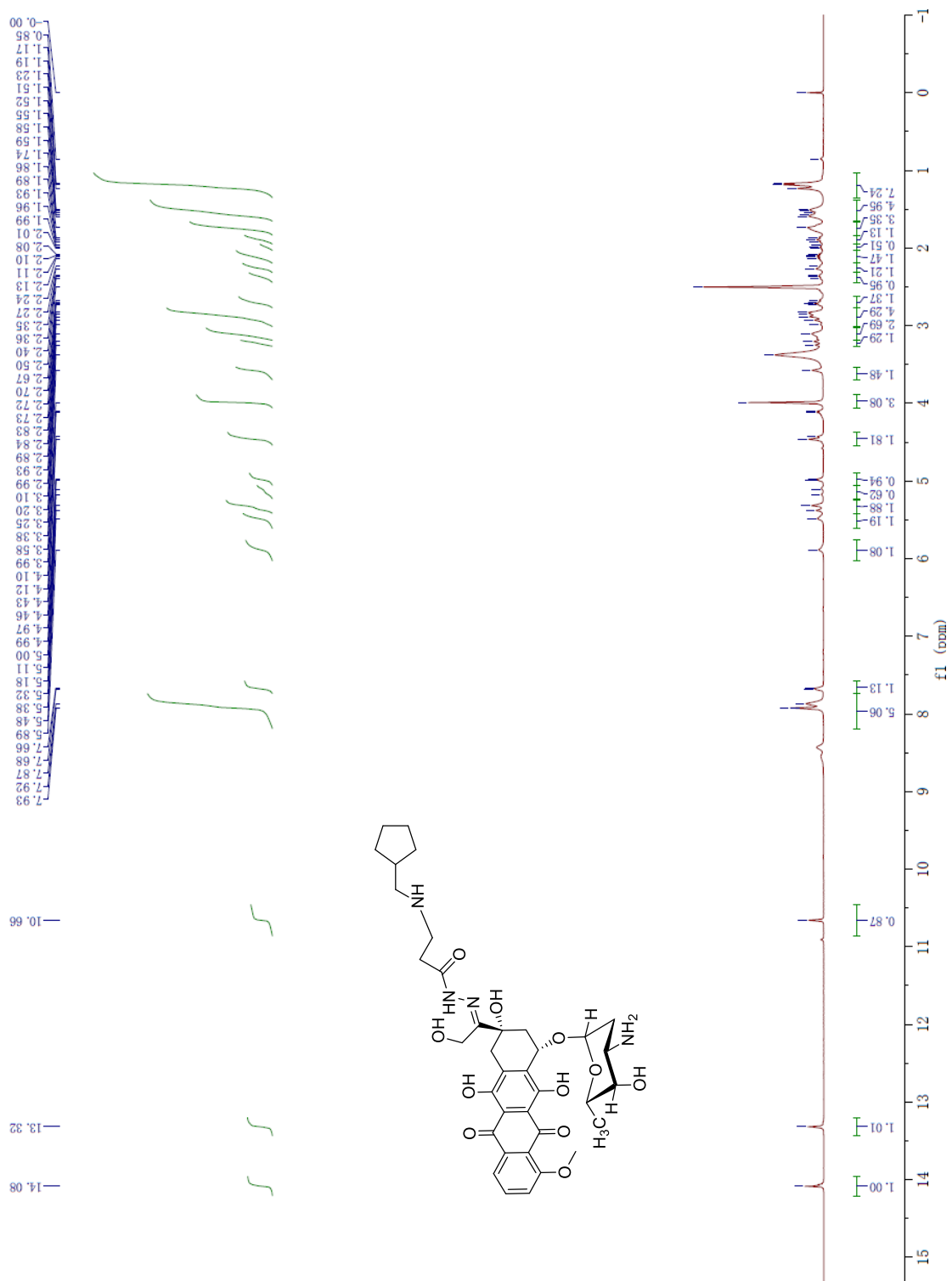**Figure S8.** ^{13}C NMR recorded (100 MHz, DMSO-d_6, 25 °C) for compound **1b**.
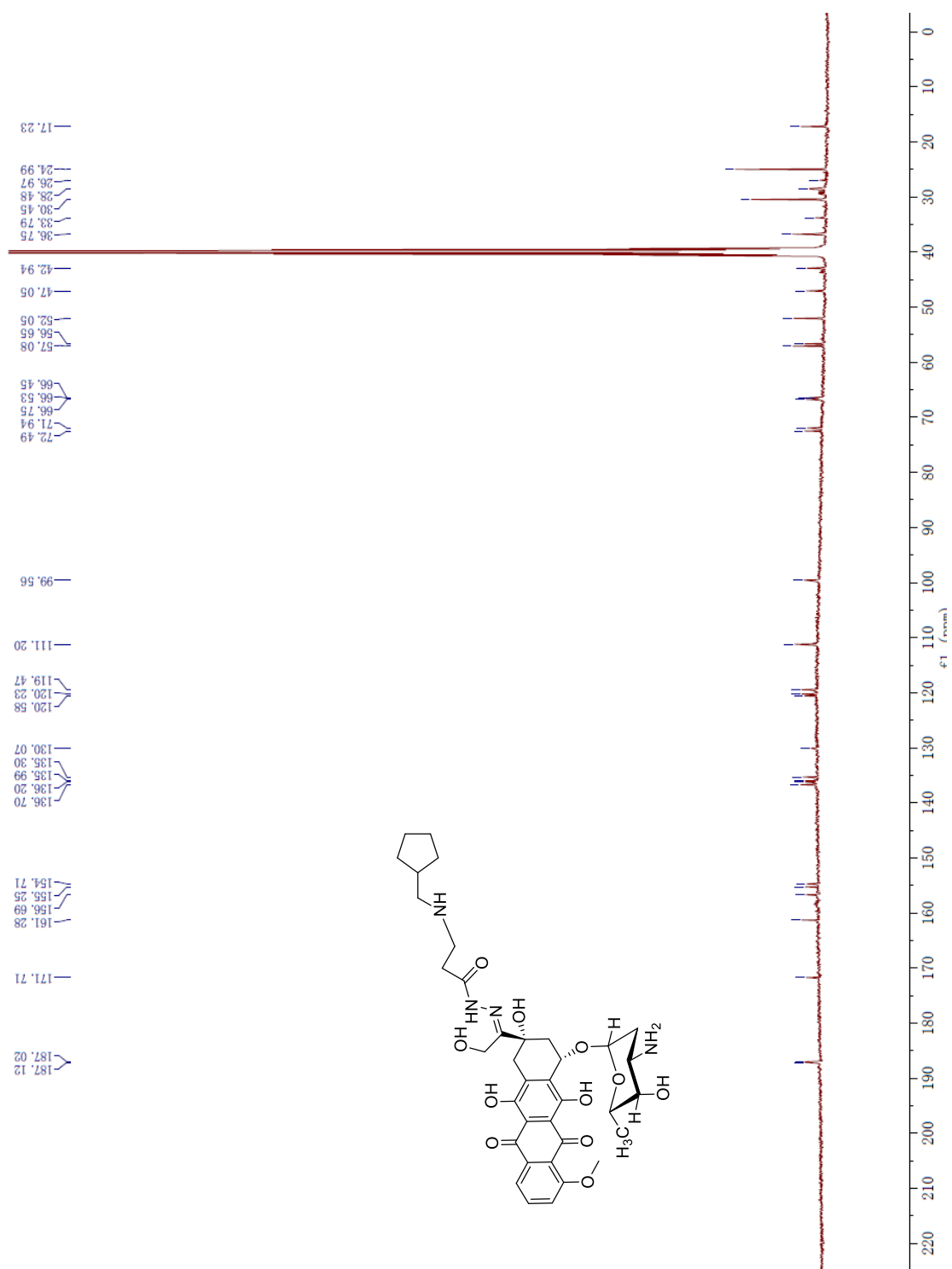
Figure S9. ^{1}H NMR recorded (400 MHz, DMSO-d_6, 25 °C) for compound **2a**.

Figure S10. ^{13}C NMR recorded (100 MHz, DMSO-d_6, 25 °C) for compound **2a**.

Figure S11. ^{1}H NMR recorded (400 MHz, DMSO-d_6, 25 °C) for compound **2b**.

17.23
24.99
26.97
28.48
30.45
33.79
36.75
42.94
47.05
52.05
56.65
57.08
66.45
66.53
66.75
71.94
72.49
99.56
111.20
119.47
120.23
120.58
130.07
135.30
135.99
136.20
136.70
154.71
155.25
156.69
161.28
171.71
187.02
187.12

**Figure S12.** ^{13}C NMR recorded (100 MHz, DMSO-d_6, 25 °C) for compound **2b**.

Measurement of K_a and dissociation kinetics in buffered solution

Determination of K_a value. The value of K_a was determined according to literature method.[1,2] Based on the slow exchange of free and bound guests on the NMR timescale, the K_a was measured by comparing ^{1}H NMR integral of free and bound guests. The following equation was used to calculate the value of K_a as an average of three experiments with different concentrations.
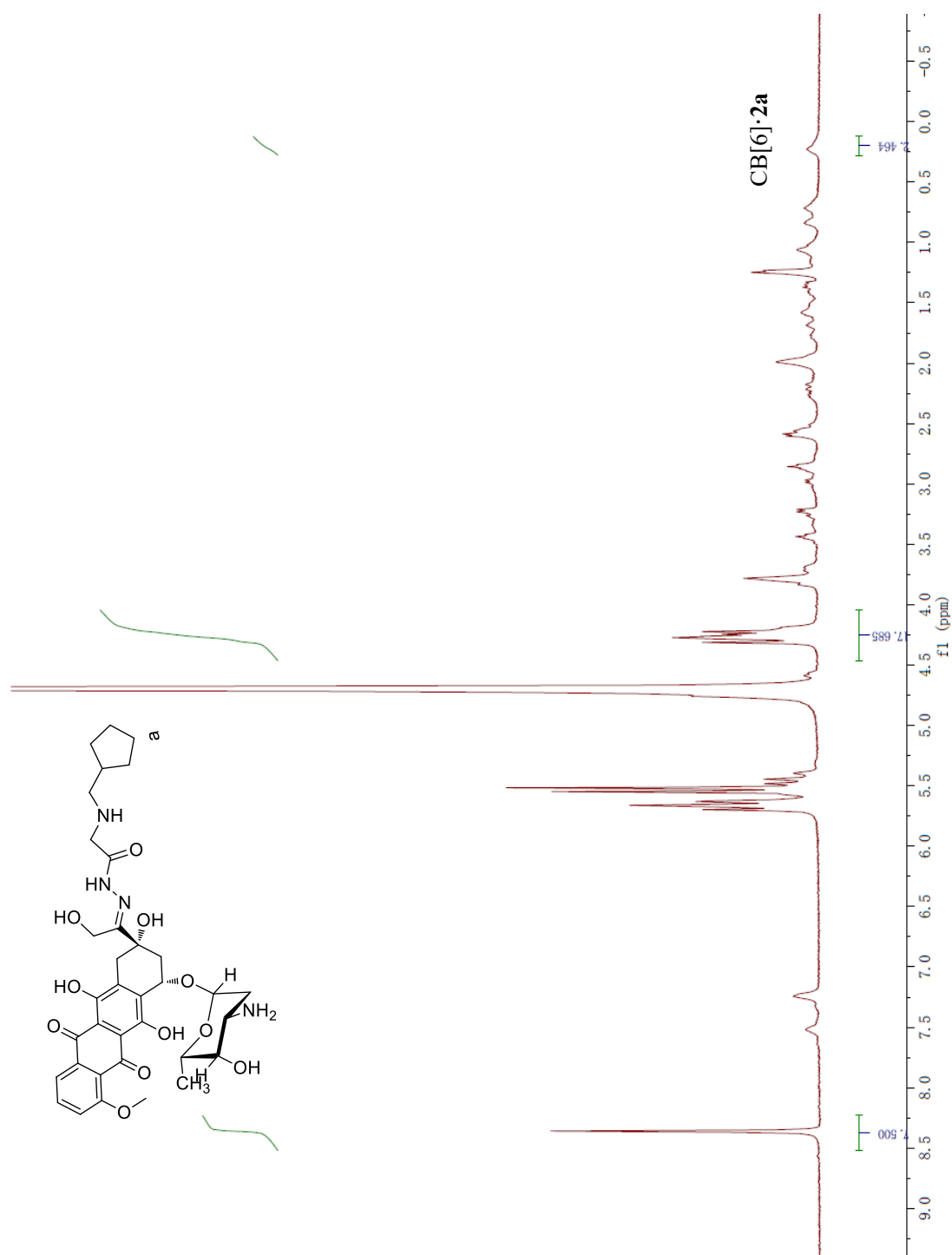
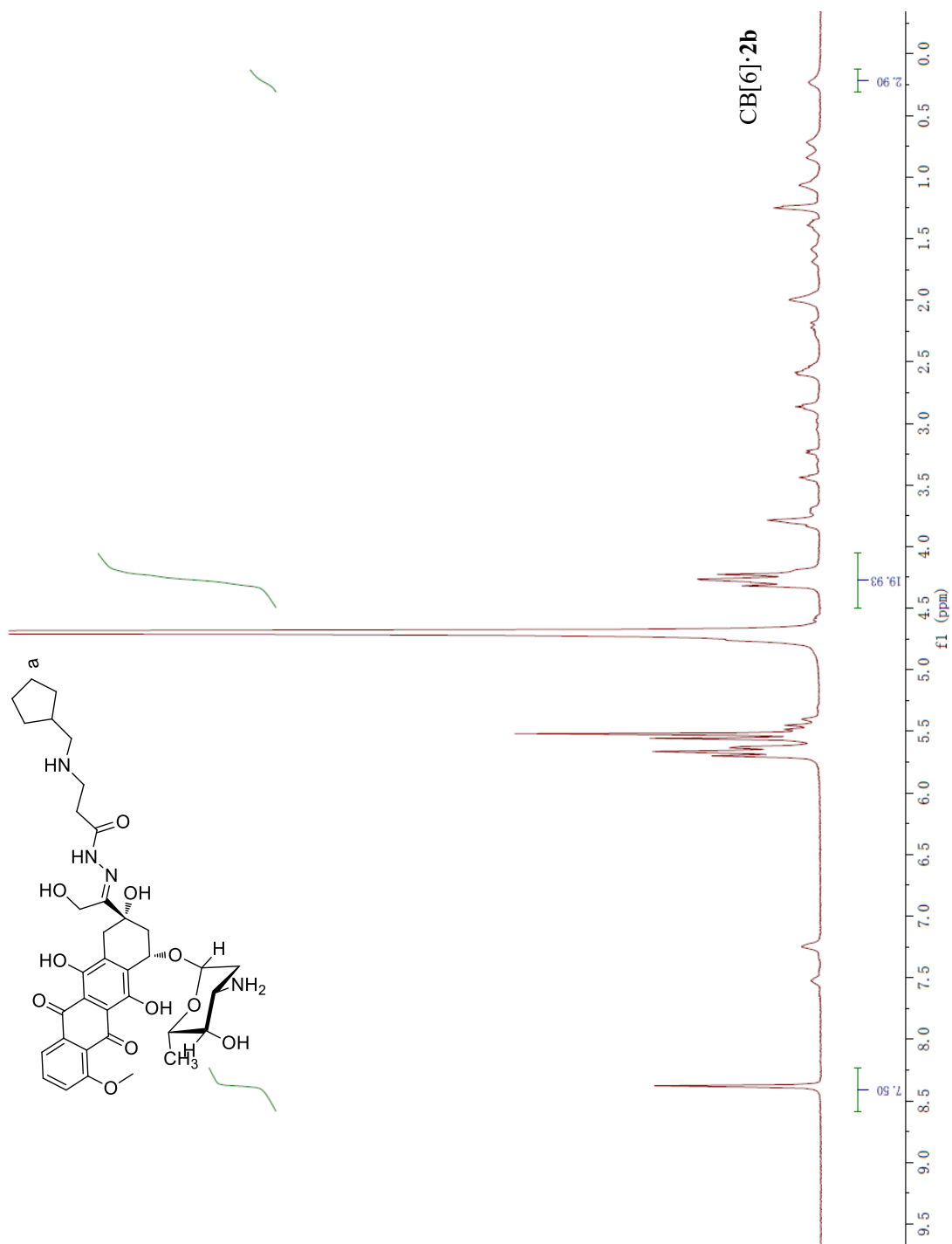K_a = [bound guest]/([host] × [free guest])
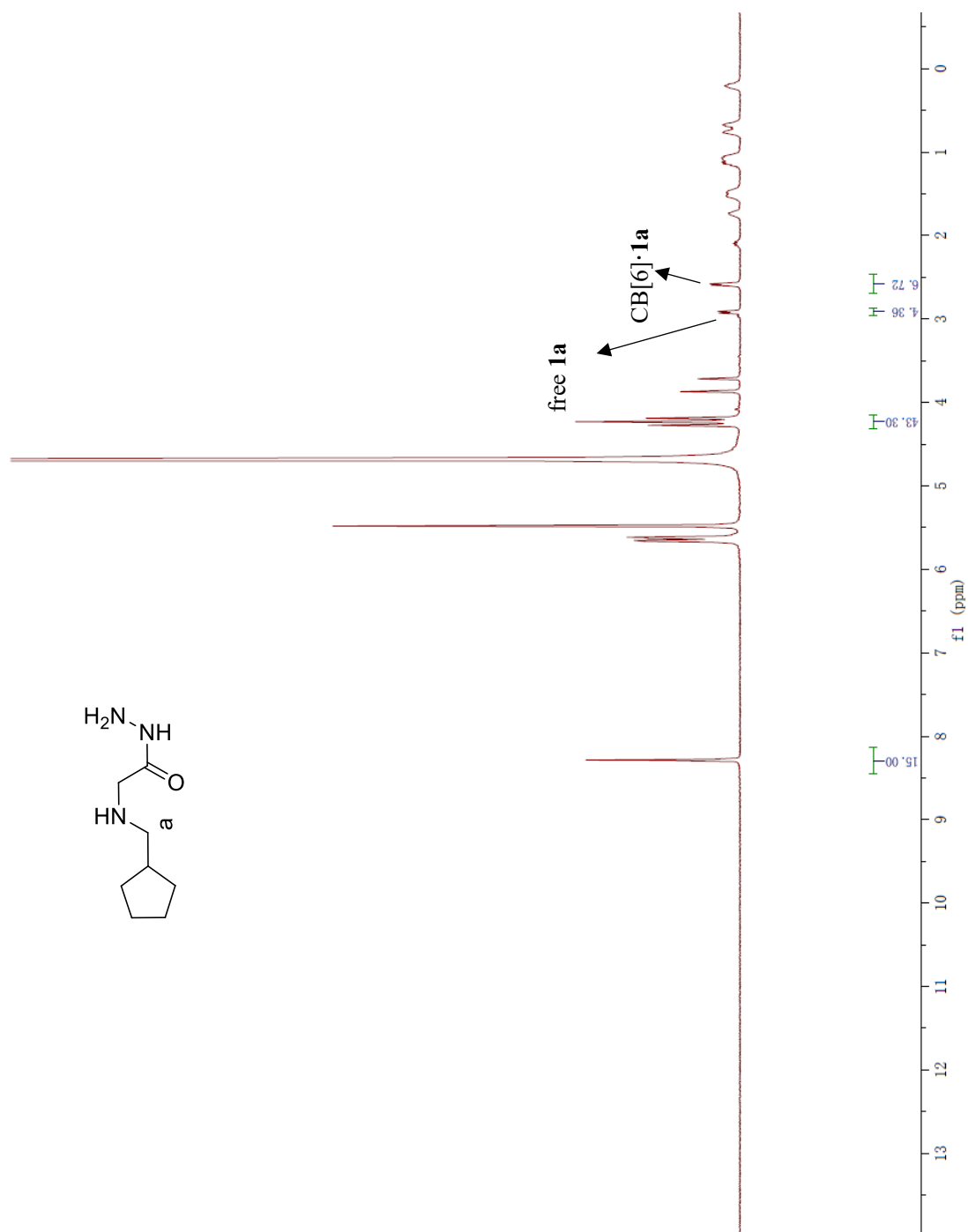
Figure S13. Representative ^1H NMR spectrum recorded for CB[6]·**1a** in NaD$_2$PO$_4$ (pD = 7.4) to determine the K_a value (K_a = (7.5 ± 1.4) × 10^3 M^{-1}). The integral of proton a was compared to internal reference (benzene-1,3,5-tricarboxylic acid) to calculate the concentration of complexed and free guest.
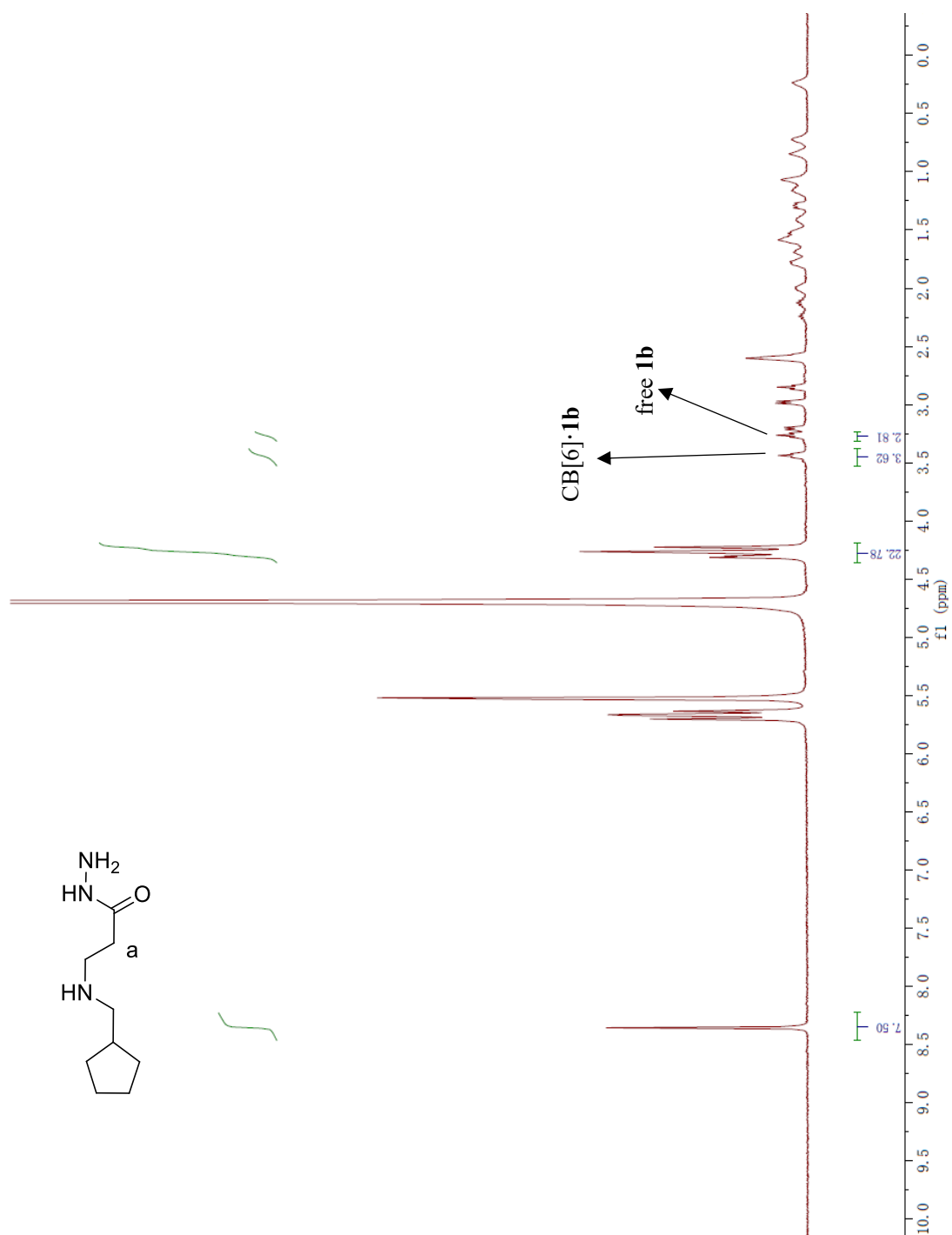
Figure S14. Representative ^{1}H NMR spectrum recorded for CB[6]·**1b** in NaD$_2$PO$_4$ (pD = 7.4) to determine the K_a value (K_a = (1.4 ± 0.1) × 10^4 M^{-1}). The integral of proton a was compared to internal reference (benzene-1,3,5-tricarboxylic acid) to calculate the concentration of complexed and free guest.

Figure S15. Representative ^{1}H NMR spectrum recorded for CB[6]·**2a** in NaD$_2$PO$_4$ (pD = 7.4) to determine the K_a value (K_a = (7.9 ± 0.5) × 10^3 M^{-1}). The integral of proton a was compared to internal reference (benzene-1,3,5-tricarboxylic acid) to calculate the concentration of complexed guest.

Figure S16. Representative ¹H NMR spectrum recorded for CB[6]·**2b** in NaD$_2$PO$_4$ (pD = 7.4) to determine the K_a value (K_a = (1.3 ± 0.3) × 10^4 M^{-1}). The integral of proton a was compared to internal reference (benzene-1,3,5-tricarboxylic acid) to calculate the concentration of complexed guest.
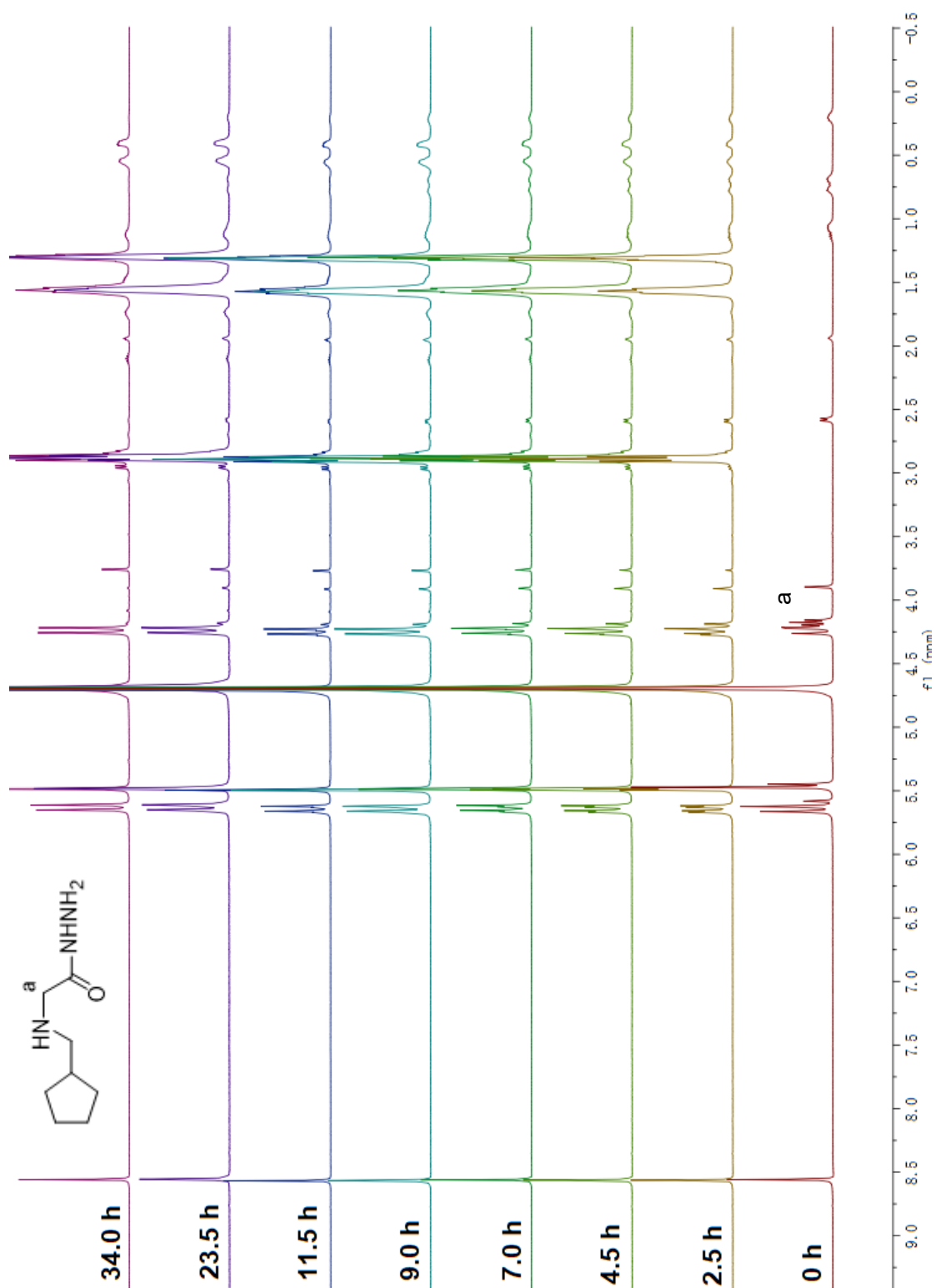
Dissociation kinetics.
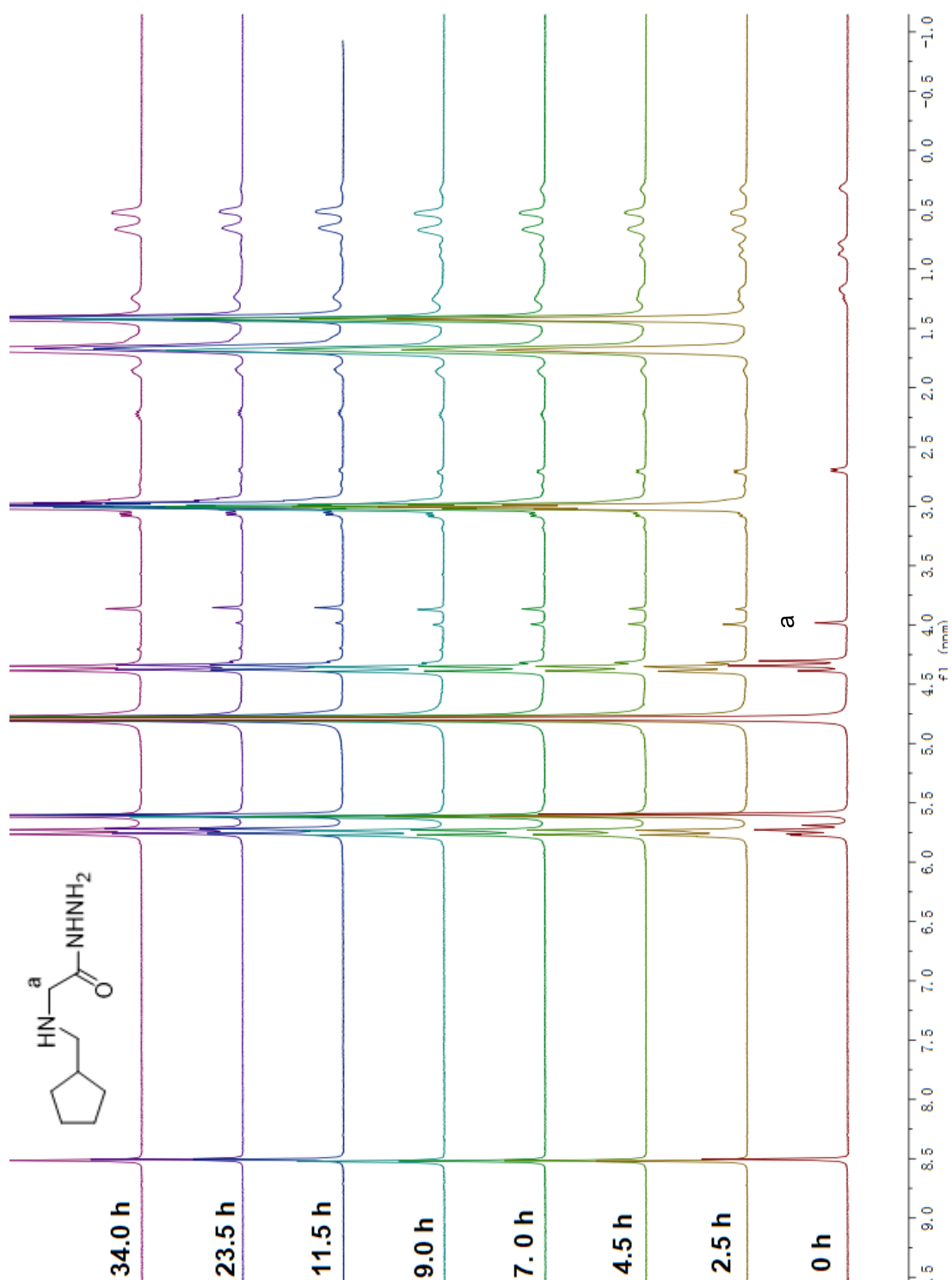
Figure S17. ^{1}H NMR recorded (400 MHz, NaD$_2$PO$_4$, pD 7.4, 37 °C) for guest **1a** (2 mM), CB[6] (3 mM) and guest **3** (20 mM) at different time. The integral of proton a was compared to internal reference (benzene-1,3,5-tricarboxylic acid) to calculate the concentration of complexed guest (k_{out} = 5.67 × 10^{-2} h^{-1}).
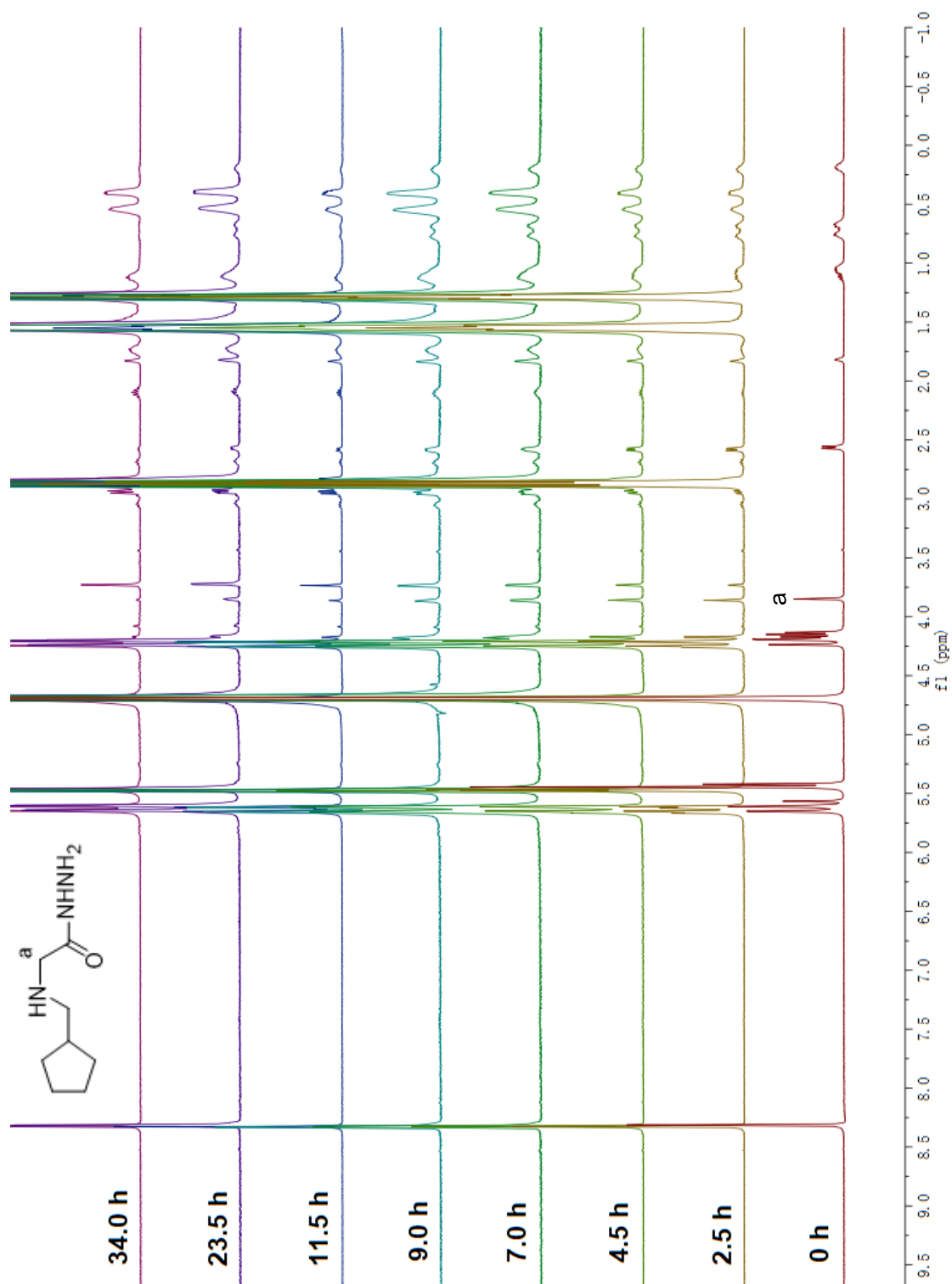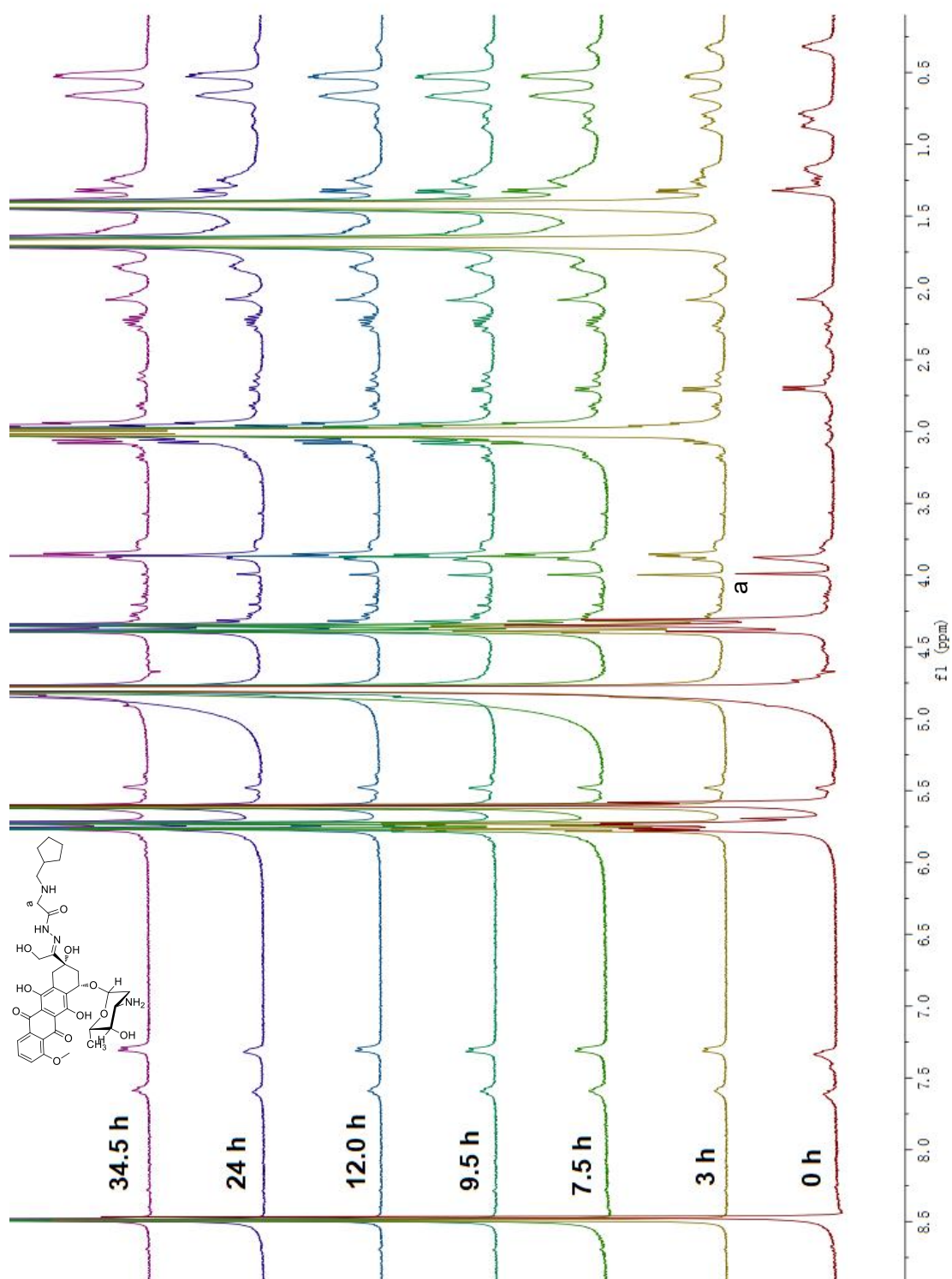
Figure S18. ^{1}H NMR recorded (400 MHz, CD$_3$COOD/CD$_3$COONa, pD 5.5, 37 °C) for guest **1a** (2 mM), CB[6] (3 mM) and guest **3** (20 mM) at different time. The integral of proton a was compared to internal reference (benzene-1,3,5-tricarboxylic acid) to calculate the concentration of complexed guest (k_{out} = 4.89 × 10^{-2} h^{-1}).
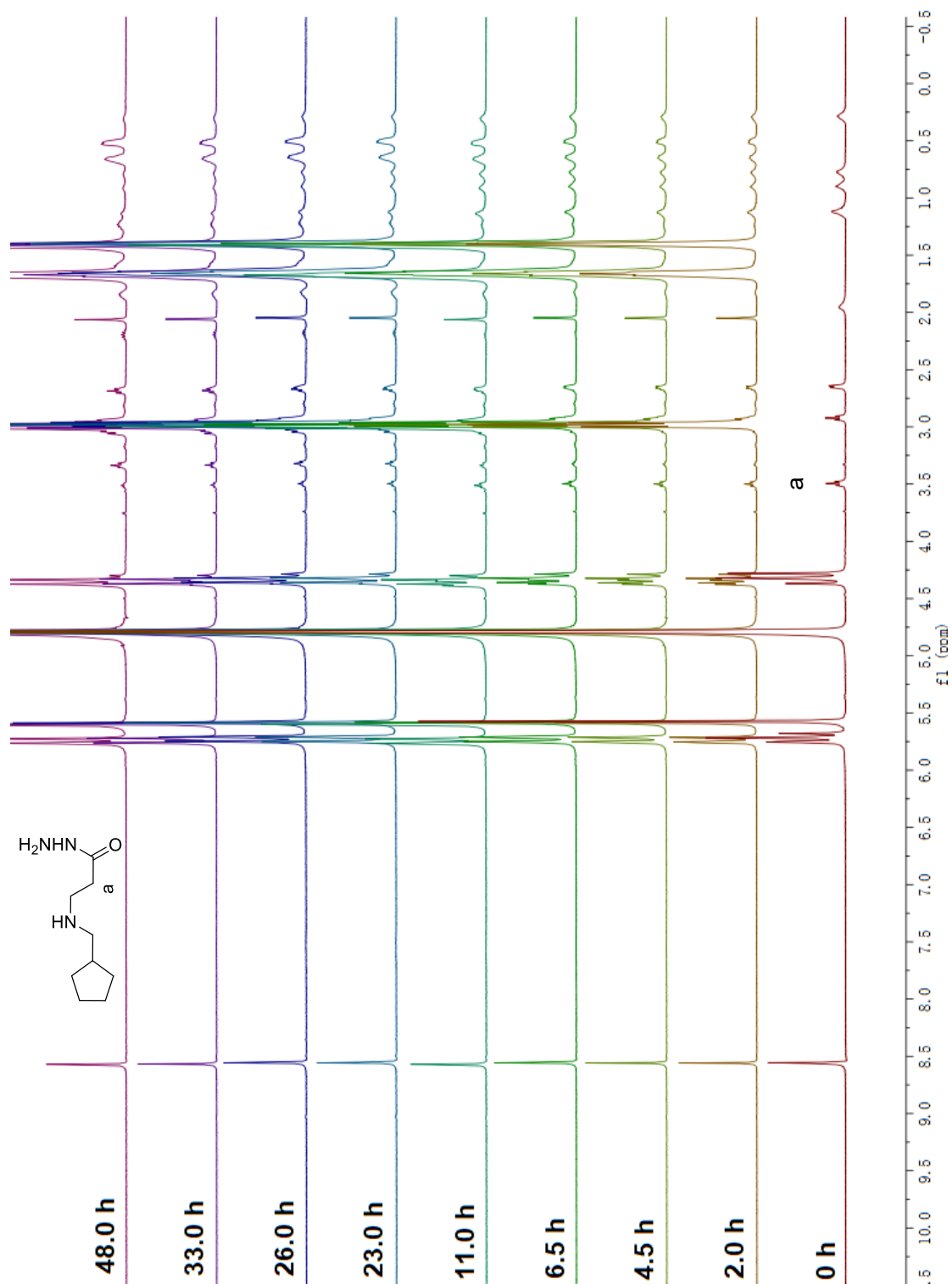
Figure S19. ^{1}H NMR recorded (400 MHz, CD$_3$COOD/CD$_3$COONa, pD 4.0, 37 °C) for guest **1a** (2 mM), CB[6] (3 mM) and guest **3** (20 mM) at different time. The integral of proton a was compared to internal reference (benzene-1,3,5-tricarboxylic acid) to calculate the concentration of complexed guest. (k_{out} = 5.30 × 10^{-2} h^{-1})
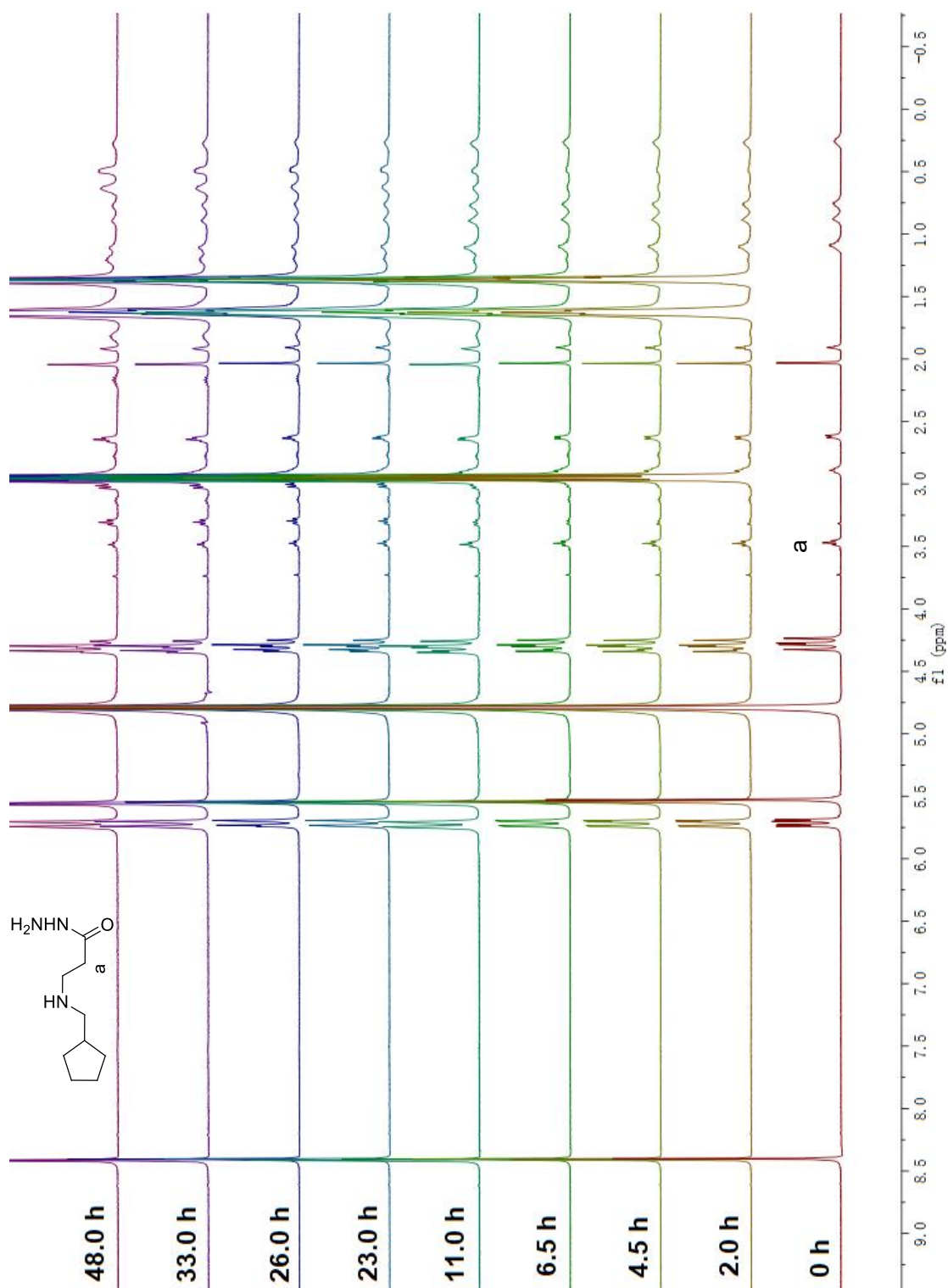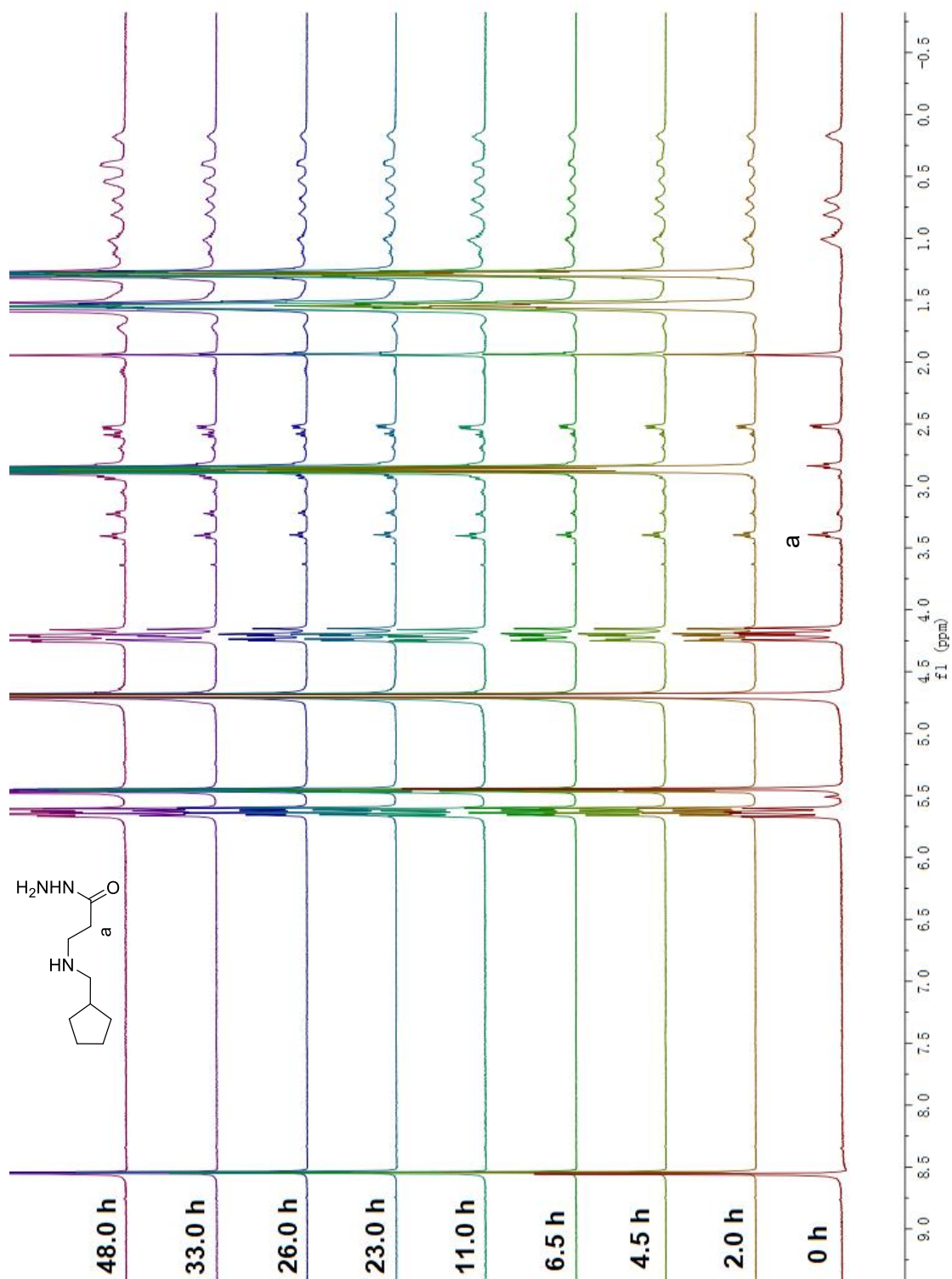
Figure S20. ^{1}H NMR recorded (400 MHz, NaD$_2$PO$_4$, pD 7.4, 37 °C) for guest **2a** (2 mM), CB[6] (3 mM) and guest **3** (20 mM) at different time. The integral of proton a was compared to internal reference (benzene-1,3,5-tricarboxylic acid) to calculate the concentration of complexed guest. (k_{out} = 5.13 × 10^{-2} h^{-1})

Figure S21. ^{1}H NMR recorded (400 MHz, NaD$_2$PO$_4$, pD 7.4, 37 °C) for guest **1b** (2 mM), CB[6] (3 mM) and guest **3** (20 mM) at different time. The integral of proton a was compared to internal reference (benzene-1,3,5-tricarboxylic acid) to calculate the concentration of complexed guest. ($k_{out} = 2.09 \times 10^{-2}$ h^{-1})
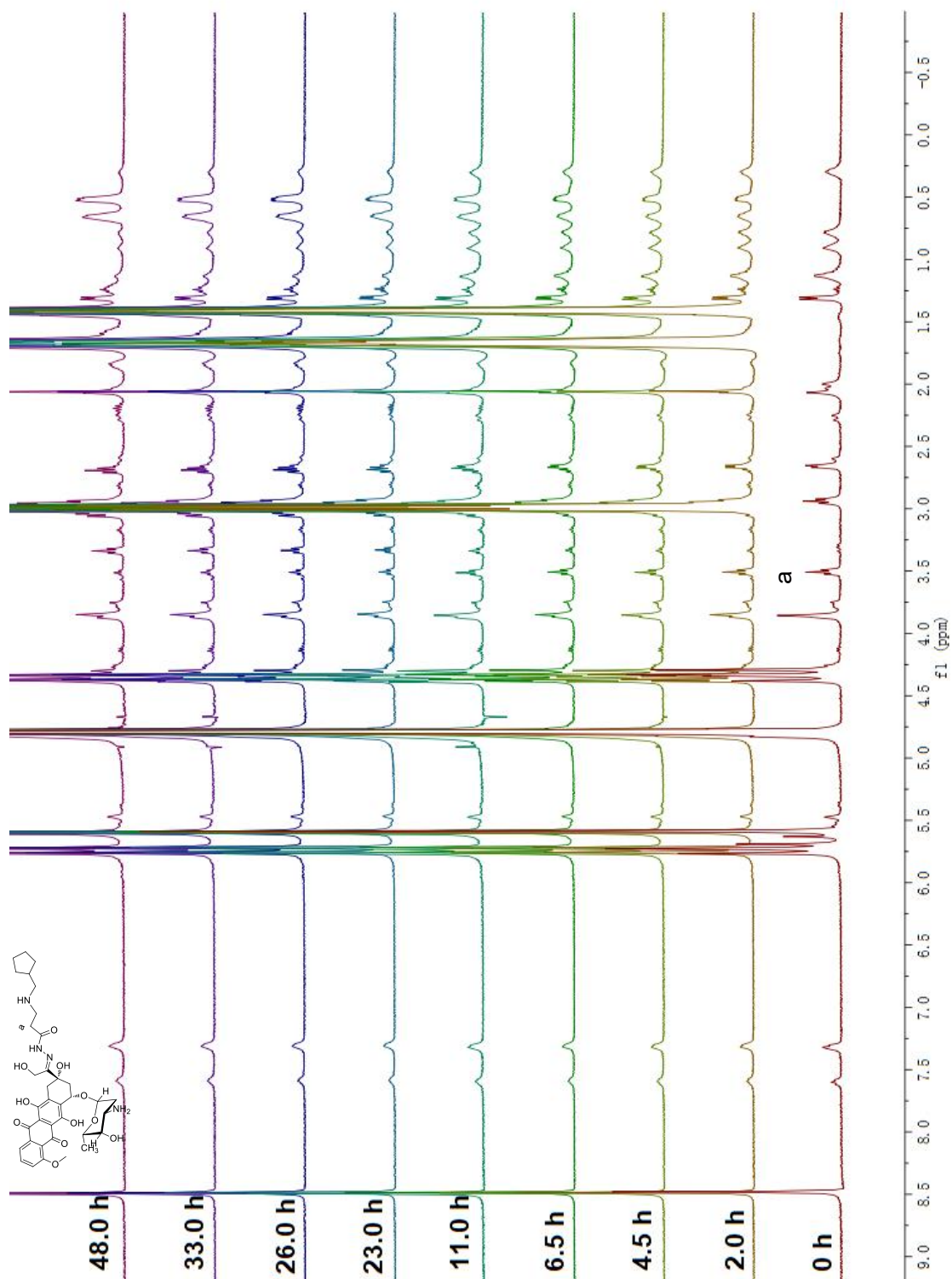
Figure S22. ^{1}H NMR recorded (400 MHz, CD_3COOD/CD_3COONa, pD 5.5, 37 °C) for guest **1b** (2 mM), CB[6] (3 mM) and guest **3** (20 mM) at different time. The integral of proton a was compared to internal reference (benzene-1,3,5-tricarboxylic acid) to calculate the concentration of complexed guest. ($k_{out} = 1.52 \times 10^{-2}$ h^{-1})

Figure S23. ^{1}H NMR recorded (400 MHz, CD₃COOD/CD₃COONa, pD 4.0, 37 °C) for guest **1b** (2 mM), CB[6] (3 mM) and guest **3** (20 mM) at different time. The integral of proton a was compared to internal reference (benzene-1,3,5-tricarboxylic acid) to calculate the concentration of complexed guest. (k_{out} = 6.81 × 10^{-3} h^{-1})

Figure S24. ¹H NMR recorded (400 MHz, NaD$_2$PO$_4$, pD 7.4, 37 °C) for guest **2b** (2 mM), CB[6] (3 mM) and guest **3** (20 mM) at different time. The integral of proton a was compared to internal reference (benzene-1,3,5-tricarboxylic acid) to calculate the concentration of complexed guest. (k_{out} = 2.18 × 10^{-2} h^{-1})
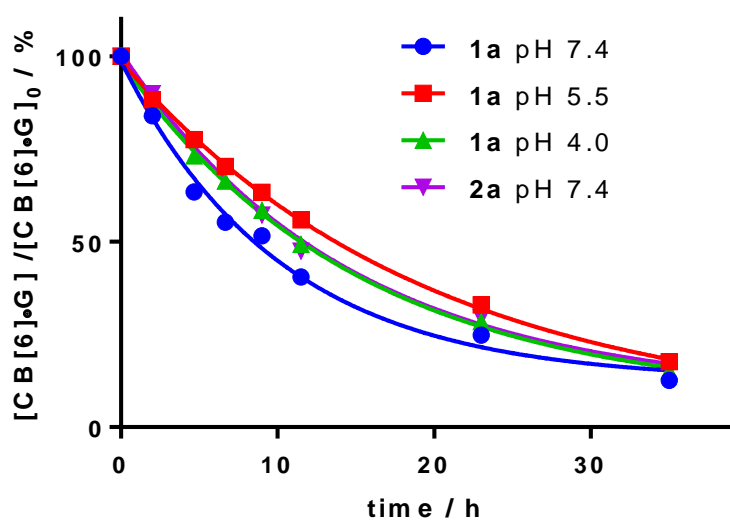
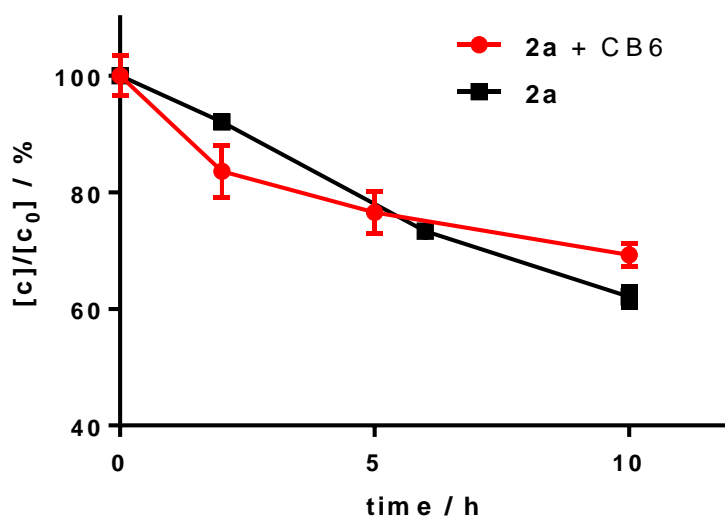**Figure S25.** Time-dependent guest displacement assay of complexes CB[6]·**1a** and CB[6]·**2a** at different pH.
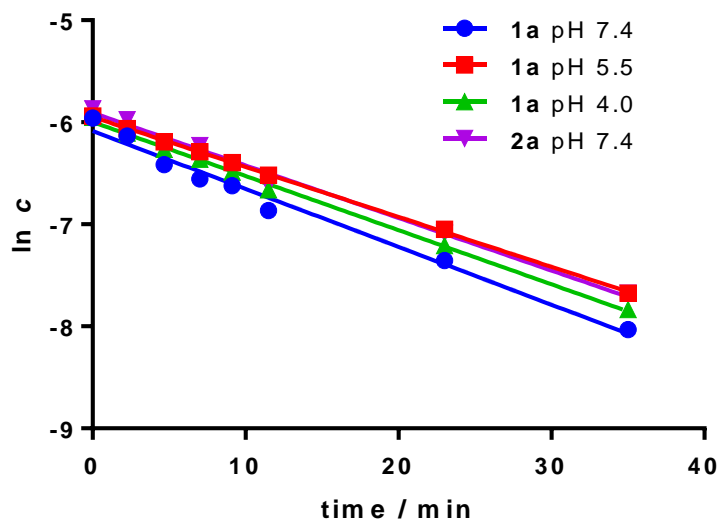
Figure S26. Linear fitting of lnC vs t to calculate dissociation rate constant k_{out}.

Complex stability study in blood serum.

Figure S27. Time-dependent concentration of complexed guest **2a** (CB[6]+**2a**, red circle) or guest **2a** (guest **2a** only, black square) when incubated in blood serum (FBS) at 37 °C; data are displayed as mean ± SD ($n = 3$). [CB[6]] = 60 μM, [**2a**] = 40 μM.
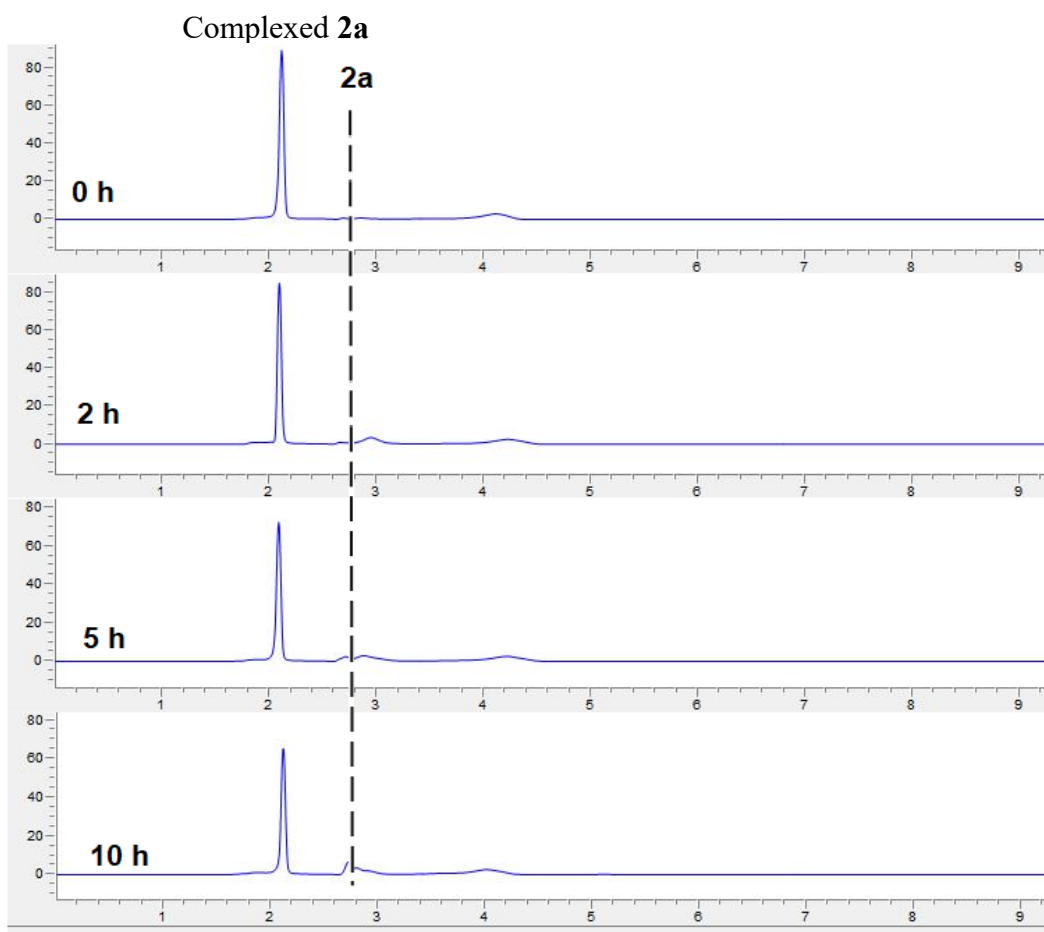
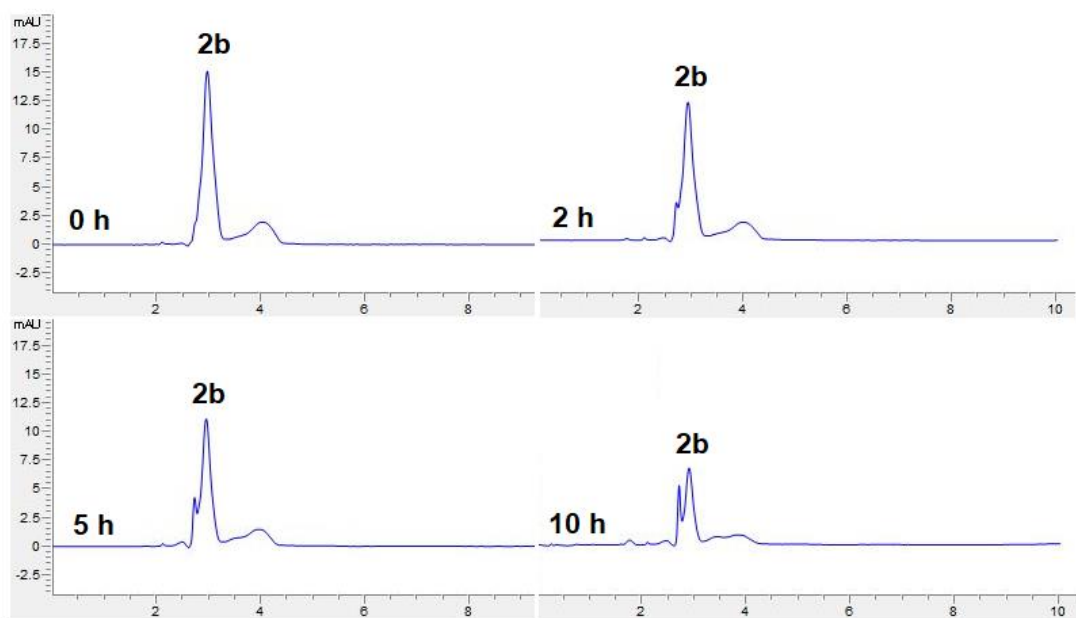**Figure S28.** Representative time-dependent RP-HPLC traces of CB[6]·**2a** when incubated in blood serum (FBS) at 37 °C. [CB[6]] = 60 µM, [**2a**] = 40 µM.
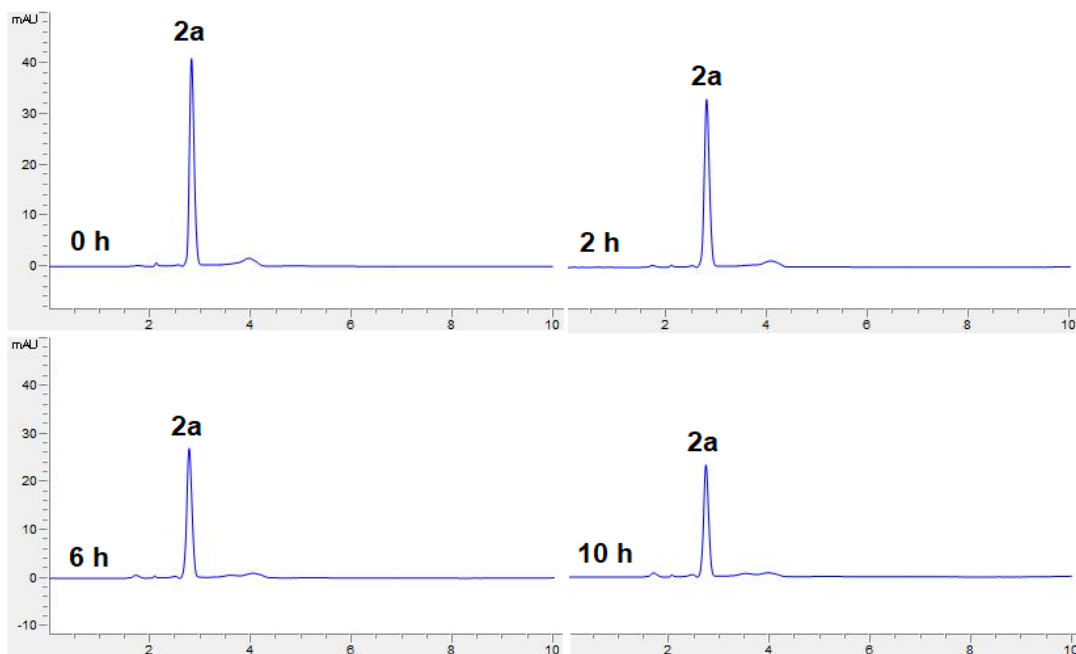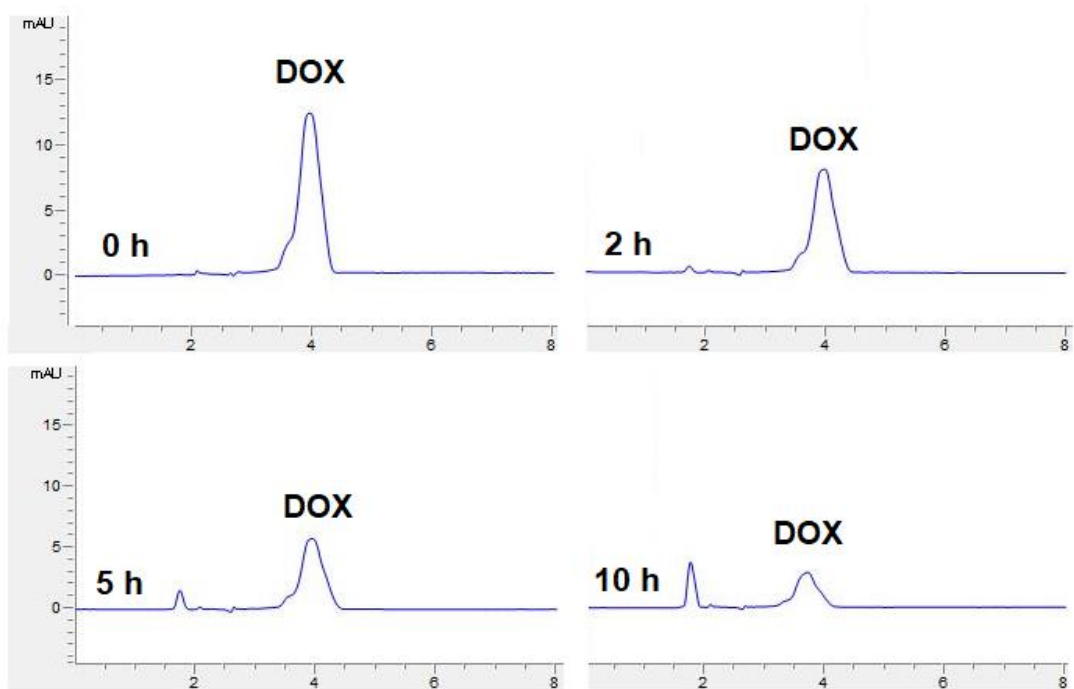
Figure S29. Representative time-dependent RP-HPLC traces of **2a** when it was incubated alone in blood serum (FBS) at 37 °C. [**2a**] = 40 µM.


Figure S30. Representative time-dependent RP-HPLC traces of **2b** when it was incubated alone in blood serum (FBS) at 37 °C. [**2b**] = 40 µM.

Figure S31. Representative time-dependent RP-HPLC traces of doxorubicin hydrochloride (DOX) when it was incubated alone in blood serum (FBS) at 37 °C. [**DOX**] = 40 μM.

Cell study

Minimum essential medium eagle (MEM), Hank's balanced salt solution, L - Glutamine solution and penicillin-streptomycin was purchased from Sigma. Trypsin was purchased from Gibco. Fetal bovine serum was purchased from Zhejiang Tianhang Biotechnology co., Ltd.
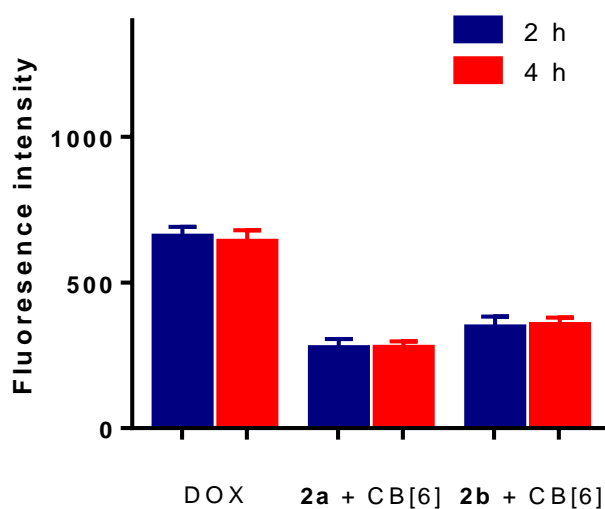
Figure S32. Time-dependent cell uptake assay of complexes CB[6]·**2a** and CB[6]·**2b**. HeLa cell medium pH = 7.4. [DOX] = [**2a**] = [**2b**] = 10 μM, [CB[6]] = 15 μM. Stock solution concentration in PBS: [**2a**] = [**2b**] = 2 mM, [CB[6]] = 3 mM. Incubation time: 2 h (blue) and 4 h (red). Fluorescent intensity is displayed as mean ± SD (*n* = 9).

References

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