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Supplementary Materials

Novel (*S*)-Selective Hydrolase from *Arthrobacter* sp. K5 for Kinetic Resolution of Cyclic Amines

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S.1 Screening of microorganisms degrading N-acetyl-2-MPI

Soil samples were collected from various area of Japan. A few grams of the soil sample was put in 5 mL of medium containing 1.88 g L^{-1} *N*-acetyl-2-MPI, 1 g L^{-1} NH_4Cl, 5 g L^{-1} Na_2HPO_4, 2 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 1% (v/v) metal solution and 1% (v/v) vitamin mixture in tap water, pH 7. Metal solution consists of 0.3 g L^{-1} H_3BO_3, 0.4 g L^{-1} $CaCl_2 \cdot 2H_2O$, 0.2 g L^{-1} $FeSO_4 \cdot 7H_2O$, 0.1 g L^{-1} KI, 0.4 g L^{-1} $MnCl_2 \cdot 4H_2O$, 0.04 g L^{-1} $CuSO_4 \cdot 5H_2O$, 0.22 g L^{-1} $Na_2MoO_4 \cdot 2H_2O$ and 1% (v/v) HCl (6 M) in distilled water. Vitamin mixture consists of 100 mg L^{-1} biotin, 20 mg L^{-1} pantothenate·Ca, 100 mg L^{-1} inositol, 20 mg L^{-1} nicotinic acid, 20 mg L^{-1} pyridoxine·HCl, 10 mg L^{-1} 4-aminobenzoic acid, 10 mg L^{-1} riboflavin and 0.5 mg L^{-1} folic acid in distilled water. Cultivation was aerobically carried out at 28°C with shaking (120 rpm). After 7-10 days, 0.1 mL of culture broth was inoculated into new medium and cultivated at the same conditions. This process was repeated once more. The final culture broth was spread onto 2% (w/v) agar plates of the above medium. The colonies isolated on the agar plates were cultivated in 5 mL medium with 0.2% (w/v) yeast extract at 28°C for 2-6 days. Cells were harvested by centrifugation and washed with 0.85% (w/v) NaCl. The reaction mixture (2 mL) was consisted of 10 mM *N*-acetyl-2-MPI, 100 mM potassium phosphate buffer (pH 7.0), and cells derived from 5 mL culture broth. The mixture was incubated at 30°C for 24 h with shaking (120 rpm). The reaction was quenched by centrifugation for removal of cells and the resulting supernatants were analyzed by TLC and HPLC. Microorganisms exhibiting hydrolase activity for *rac*-*N*-acetyl-2-methylpiperidine were evaluated and the strains with high stereoselectivity for other *N*-acyl-2-MPI was selected. Among the microorganisms, strain K5 with the highest stereoselectivity was identified by Techno-Suruga Laboratory Co. Ltd. based on 16S rDNA gene analysis.

S.2 Optimization of culture conditions for Arthrobacter sp. K5

To optimize the culture medium, the following compounds were added to nutrient medium containing 5 g L^{-1} polypepton, 5 g L^{-1} meat extract, 2 g L^{-1} NaCl, 0.5 g L^{-1} yeast extract in tap water (pH 7, adjusted by 6M NaOH): *N*-acetyl-2-MPI, *N*-acetyl-2-methylpyrrolidine, *N*-acetylpiperidine, *N*-acetylpyrrolidine, *N*,*N*′-diacetyl-2-methylpiperazine, acetanilide, *N*-acetylaspartic acid, 2-MPI, benzamide and nicotinamide.

S.3 Cultivation of Arthrobacter sp. K5

Arthrobacter sp. K5 was cultivated at 28°C for 24 h in 5 mL nutrient medium containing 5 g L^{-1} polypepton, 5 g L^{-1} meat extract, 2 g L^{-1} NaCl, 0.5 g L^{-1} yeast extract in tap water (pH 7). Cultivation was performed at 28°C and 120 rpm for 24 h in 40 mL scale containing nutrient medium with 0.4% (v/v) *N*-acetylpiperidine for the induction of hydrolase activity. Cells were harvested by centrifugation, washed twice with 0.85% (w/v) NaCl, and suspended in the same solution. The cell growth of *Arthrobacter* sp. K5 was estimated turbidimetrically at 610 nm.

S.4 Enzyme assay

Enzymatic reaction was assayed in the reaction mixture containing 10 mM *N*-benzoylpiperidine and 1 μL enzyme solution in 100 mM potassium phosphate buffer (pH 7.0). One unit (U) of hydrolase activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol benzoate at 30°C per min. Enzyme activity was determined with HPLC using *N*-benzoylpiperidine by detecting benzoate released by hydrolysis. HPLC analysis was performed at 254 nm at a flow rate of 1.0 ml min^{-1} using an Atlantis dC18 5μm 4.6×150 mm column (Waters) and 10 mM sodium phosphate buffer (pH 2.8) /acetonitrile (3:2, v/v). Retention time of benzoate was 4 min under the above conditions.

S.5 Enzyme purification from Arthrobacter sp. K5

All purification steps were conducted at 4°C in 100 mM potassium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol.

S.5.1 Preparation of cell-free extract

Cells of *Arthrobacter* sp. K5 were suspended in 100 mM potassium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol and disrupted by ultrasonication at 100 W for 20 min at 4°C using a cell disrupter (19 kHz, Insonator 201 M; Kubota, Japan). The cell debris was removed by centrifugation at 4°C and 8,000 rpm for 30 min and the supernatant solution was used as cell-free extract.

S.5.2 Ammonium sulfate fractionation

Solid ammonium sulfate was added to the cell-free extract to reach 30% saturation. Proteins precipitated was removed by centrifugation at 4°C and 8,000 rpm for 30 min. To the supernatant, solid ammonium sulfate was added to 60% saturation and resulting precipitate was collected by centrifugation at 4°C and 8,000 rpm for 30 min. The precipitate was dissolved in 100 mM potassium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol and dialyzed in the same dissolving solution.

S.5.3 DEAE-Sephacel column chromatography

Protein solution obtained by ammonium sulfate fractionation was applied to DEAE-Sephacel resin (GE-healthcare, Tokyo, Japan) equilibrated with 100 mM potassium phosphate buffer (pH 7.0) containing 100 mM potassium chloride. Proteins bound to the resin were eluted with stepwise concentration of 100, 150 and 200 mM potassium chloride in 100 mM potassium phosphate buffer (pH 7.0).

S.5.4 Phenyl-Sepharose column chromatography

Solid ammonium sulfate was added to the protein solution obtained by DEAE-Sephacel column chromatography to reach 20% saturation. This solution was applied to phenyl-Sepharose (GE-healthcare) column chromatography equilibrated with 100 mM potassium phosphate buffer (pH 7.0) containing 20% ammonium sulfate. Proteins bound to the resin were eluted with stepwise concentration of 20 and 15% ammonium sulfate in 100 mM potassium phosphate buffer (pH 7.0).

S.5.5 Butyl-Toyopearl column chromatography

Solid ammonium sulfate was added to the protein solution obtained by phenyl-Toyopearl (Tosoh, Tokyo, Japan) column chromatography to reach 20% saturation. This solution was applied to butyl-Sepharose column chromatography equilibrated with 100 mM potassium phosphate buffer (pH 7.0) containing 20% ammonium sulfate. Proteins bound to the resin were eluted with stepwise concentration of 20, 10, 2.5 and 0% ammonium sulfate in 100 mM potassium phosphate buffer (pH 7.0).

S.5.6 Protein analysis

Protein concentration was determined by the Bradford method [1], using a protein assay kit (Bio-Rad Laboratories, Inc., California, USA) and bovine serum albumin as a standard. In column chromatography, absorbance at 280 nm was measured to detect eluted protein. SDS-PAGE was performed on 10% polyacrylamide gel with Tris-glycine buffer system according to Laemmli [2]. Protein were stained with Coomassie Brilliant Blue R250 and gels destained in ethanol/acetate/H_2O (3:1:6, v/v/v). Native molecular mass of the enzyme was estimated by gel permeation HPLC on a TSK G-3000SW column (7.5 × 600 mm; Tosoh, Tokyo, Japan) with 100 mM sodium phosphate buffer (pH 7.0) containing 200 mM NaCl as a mobile phase at a flow rate of 0.7 mL min^{-1}. The N-terminal and internal amino acid sequences of purified enzyme were determined by APRO Science Inc.

S.6 Effect of temperature and pH on purified enzyme

Optimal temperature of the hydrolase was examined in a 100 mM potassium phosphate buffer (pH 7.0) containing an appropriate amount of the purified enzyme and 10 mM *N*-benzoylpiperidine at temperatures from 15 to 60°C. Optimal pH was examined at 30°C using various 100 mM buffers with

different pH values, including sodium citrate (pH 3.0-6.0), potassium phosphate buffer (pH 6.0-8.0), Tris-HCl (pH 7.5-9.0) and glycine-NaOH (pH 9.0-11.0). In all cases, incubation was carried out for 10 min. To determine the thermal stability of the hydrolase, the enzyme solution was pre-incubated at temperatures from 0 to 55°C for 30 min and cooled on ice. For evaluation of pH stability, the enzyme solution was pre-incubated at 30°C for 30 min using the various buffers with different pH values (3.0-11.0). The enzyme solutions incubated at various temperature or pH were used for reaction mixture containing 100 mM potassium phosphate buffer (pH 7.0) and 10 mM *N*-benzoylpiperidine at 30°C.

References

1. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3
2. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **1970**, *227*, 680–685. https://doi.org/10.1038/227680a0

Table S1. Effect of amine compound on *Arthrobacter* sp. K5 growth and hydrolase activity

Compound	Growth [1] (OD_{610}) [2]	(*S*)-2-MPI [3] (mM)
None	4.30	0.12
N-Acetyl-2-MPI	4.51	1.51
N-Acetyl-2-methylpyrrolidine	4.23	1.25
N-Acetylpiperidine	6.02	1.67
N-Acetylpyrrolidine	5.53	0.71
N,*N*′-Diacetyl-2-methylpiperazine	3.67	0.26
2-MPI	4.48	0.10
Acetanilide	5.47	0.09
Benzamide	0.56	0.18

[1] *Arthrobacter* sp. K5 was cultivated at 28°C and 120 rpm for 2 days with 0.2% (w/v) amine compound.
[2] OD_{610} = optical density at 610 nm.
[3] The reactions were performed at 30°C for 27 h in 2 mL of 100 mM potassium phosphate buffer (pH 7.0) containing 10 mM *N*-pivaloyl-2-MPI and whole cells derived from 4 mL culture broth.

Table S2. Effect of *N*-acetylpiperidine concentration on *Arthrobacter* sp. K5 growth and hydrolase activity

N-Acetylpiperidine (%, w/v)	Growth [1] (OD_{610})	(*S*)-2-MPI [2] (mM)
0	4.30	0.12
0.05	4.69	1.10
0.1	4.48	1.21
0.2	5.90	1.98
0.3	5.91	1.99
0.4	6.91	2.65
0.5	6.02	1.44

[1] *Arthrobacter* sp. K5 was cultivated at 28°C and 120 rpm for 2 days.
[2] The reactions were performed at the same condition as described in table S1.

Table S3. Effect of culture time on *Arthrobacter* sp. K5 growth and hydrolase activity

Preculture (day)	Culture (day)	Growth [1] (OD_{610})	(*S*)-2-MPI [2] (mM)
1	1	8.24	3.27
1	2	6.16	2.28
2	1	9.91	3.91
2	2	6.65	2.19
2	3	4.13	1.38
3	1	9.95	3.80
3	2	7.01	2.84
3	3	4.24	1.45

[1] *Arthrobacter* sp. K5 was cultivated at 28°C and 120 rpm in 5 mL nutrient medium containing 5 g L^{-1} polypepton, 5 g L^{-1} meat extract, 2 g L^{-1} NaCl, 0.5 g L^{-1} yeast extract in tap water (pH 7), then 5 mL preculture was inoculated into 40 mL the nutrient medium and further cultivated at 28°C.
[2] The reactions were performed at the same condition as described in table S1.

Table S4. Purification of *Arthrobacter* sp. K5 hydrolase.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification (-fold)
Cell-free extract [1]	3340	11000	3.30	100	1
$(NH_4)_2SO_4$ (30-60%)	1440	5170	3.60	47	1.09
DEAE-Sephacel	364	4980	13.7	45	4.14
Phenyl-Sepharose	133	4110	30.9	37	9.36
Butyl-Toyopearl	68	2420	35.5	22	10.8

[1] The cell-free extract was prepared by disruption of cells obtained from 4.84 L of culture broth.

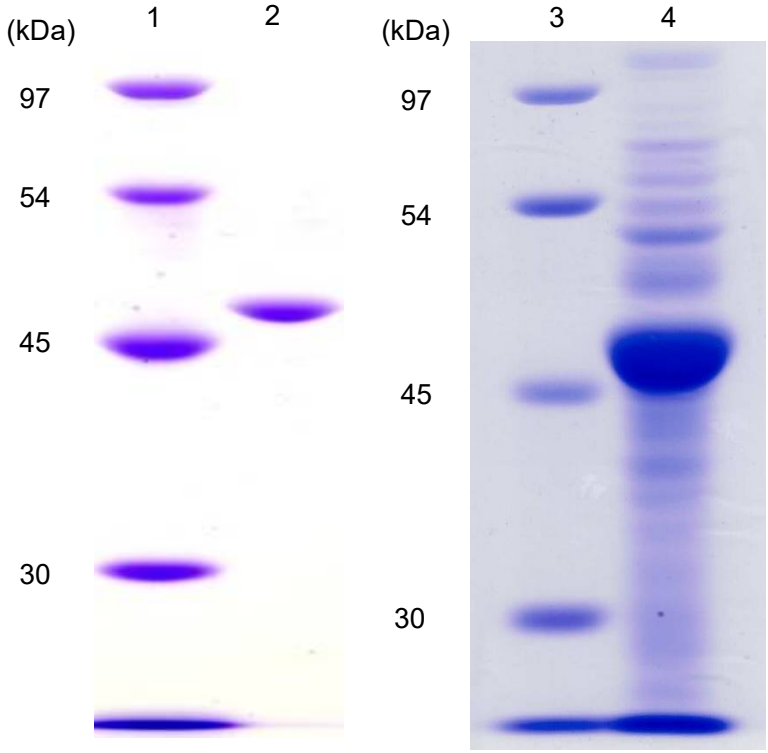

Figure S1. SDS-PAGE analysis of *Arthrobacter* sp. K5 hydrolase. Lanes 1, 3: molecular weight markers, 2: purified hydrolase from *Arthrobacter* sp. K5, 4: cell-free extract of recombinant *R. erythropolis* overexpressing (*S*)-selective hydrolase (SHA) gene.

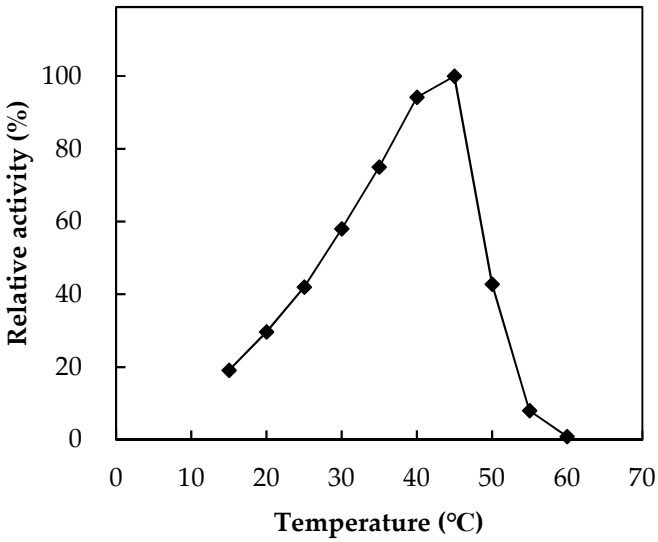

Figure S2. Optimum temperature of the purified hydrolase. 100% = 141 μmol min^{-1} mg^{-1}.

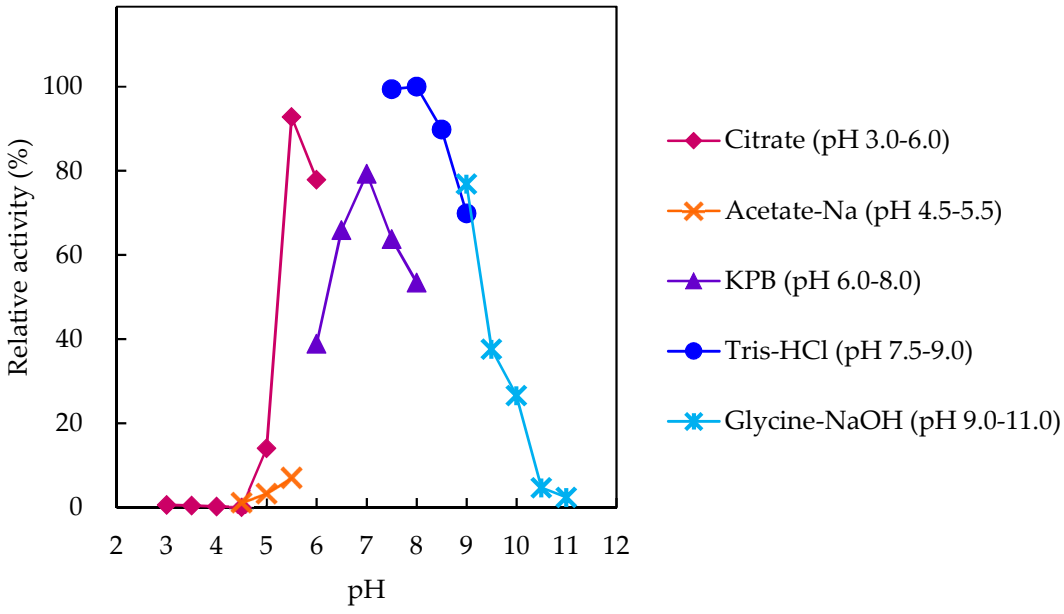

Figure S3. Optimum pH of the purified hydrolase. 100% = 114 μmol min^{-1} mg^{-1}.

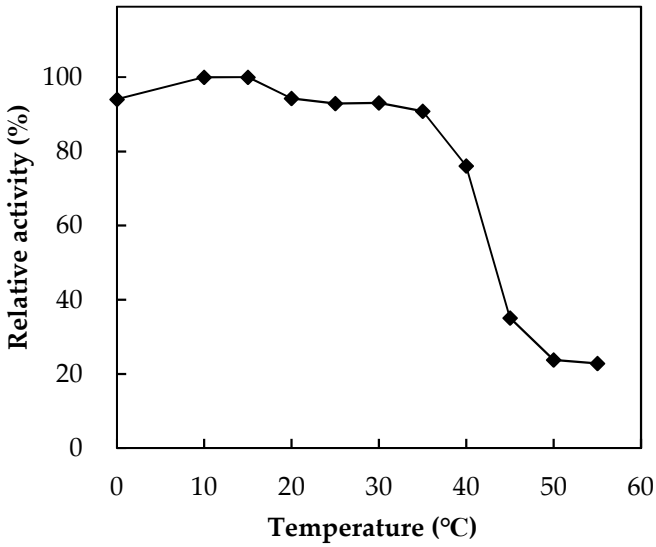

Figure S4. Thermal stability of the purified hydrolase. 100% = 89.1 μmol min^{-1} mg^{-1}.

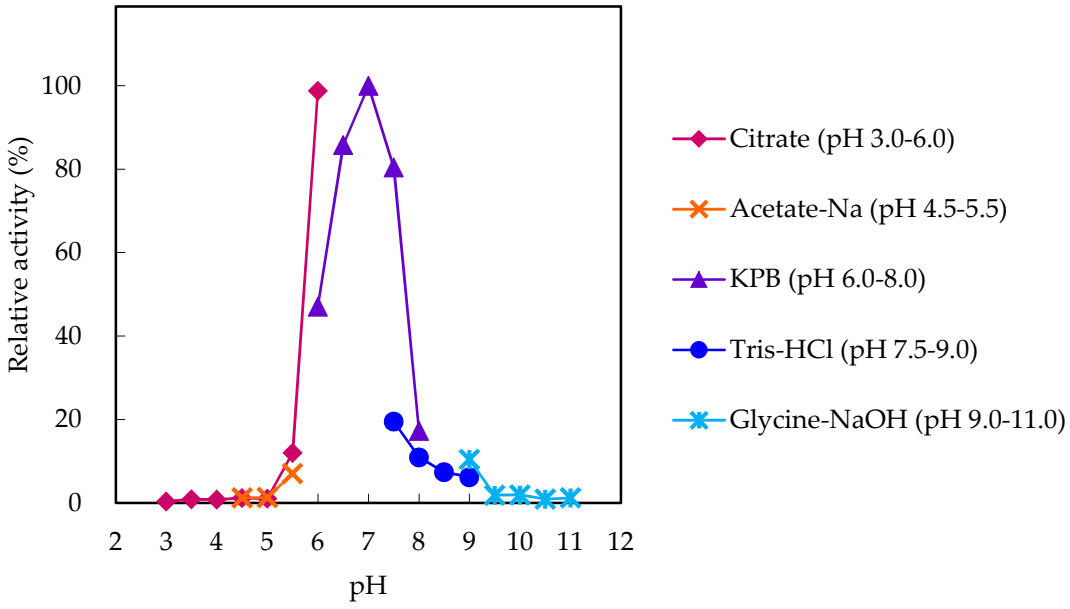

Figure S5. pH stability of the purified hydrolase. 100% =76.3 μmol min^{-1} mg^{-1}.

Table S5. HPLC analysis conditions for detection of benzoate and cyclic amines.

Compound [1]	Column	Solvent	Retention time (min)
Benzoate	Atlantis dC18 5μm 4.6×150 mm	sodium phosphate buffer (10 mM, pH 2.8):MeCN = 3:2	4.0
2-MPI	Atlantis dC18 5μm 4.6×150 mm	sodium phosphate buffer (10 mM, pH 2.5):MeOH = 55:45	36.8 (*R*) [3] 38.5 (*S*)
N-Benzoyl-3-MPI [2]	CHIRALPAK AD-H 4.6×250 mm	*n*-hexane:*i*-PrOH = 95:5	9.1 10.4
2-Methylpyrrolidine	Atlantis dC18 5μm 4.6×150 mm	sodium phosphate buffer (10 mM, pH 2.5):MeOH = 55:45	20.8 (*S*) [3] 21.9 (*R*)
2-Methylindoline	Atlantis dC18 5μm 4.6×150 mm	60% (v/v) MeOH	17.3 19.4
1,2,3,4-tetrahydroquinaldine	Atlantis dC18 5μm 4.6×150 mm	60% (v/v) MeOH	21.0 22.3

[1] Cyclic amines except for 3-MPI were derivatized with GITC to determine the yield and optical purity.

[2] 3-MPI obtained by enzymatic hydrolysis was analyzed by chiral HPLC after acylation with benzoyl chloride.

[3] Absolute configurations of chiral cyclic amines were assigned using commercially available chiral reagents.

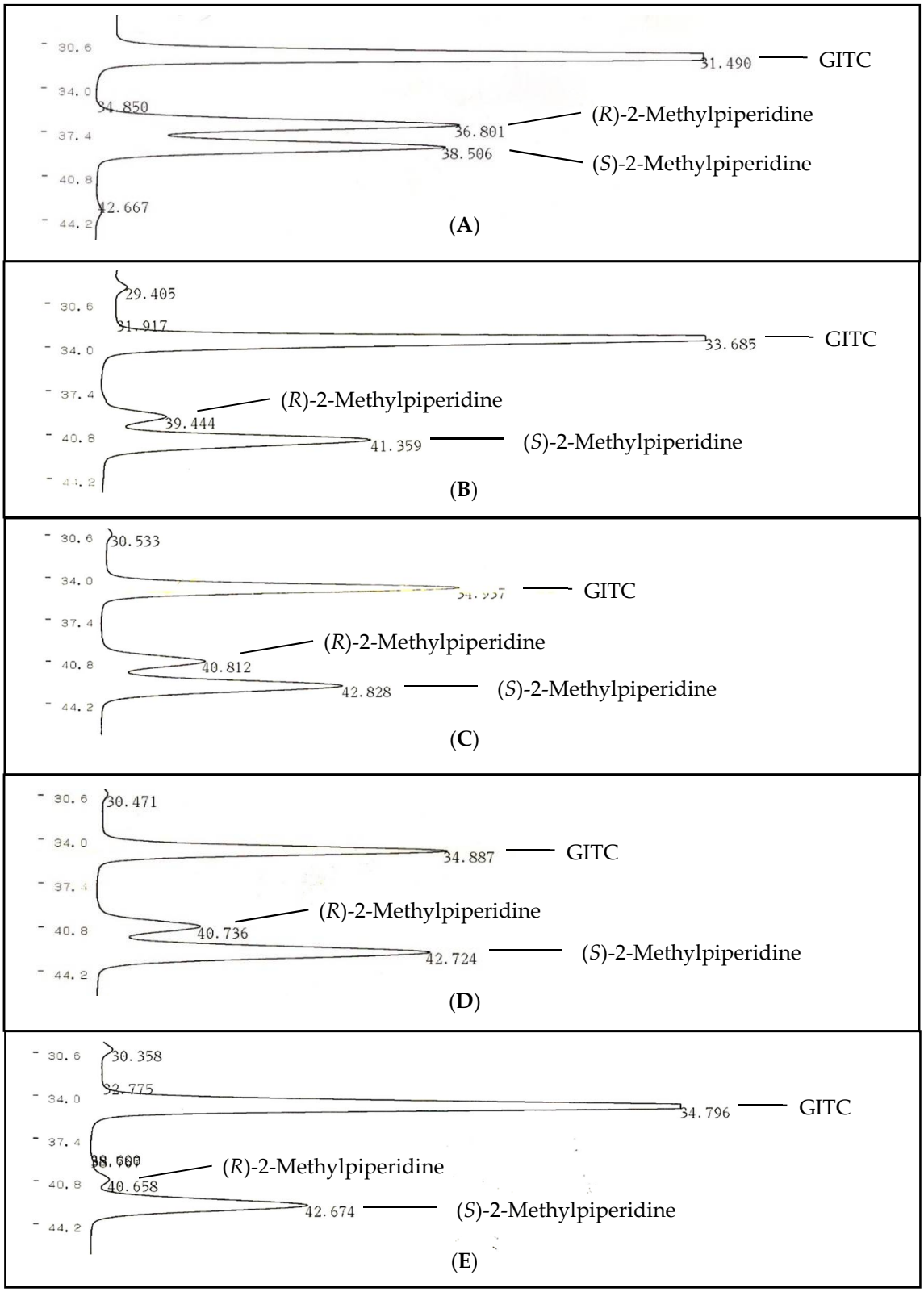

Figure S6. HPLC chromatograms of 2-MPI-GITC after *rac-N*-acyl-2-MPI hydrolysis using purified *Arthrobacter* sp. K5 hydrolase. (**A**) Racemic 2-MPI-GITC as a standard. (**B**) Hydrolysis of *N*-benzoyl-2-MPI. (**C**) Hydrolysis of *N*-acetyl-2-MPI. (**D**) Hydrolysis of *N*-crotonoyl-2-MPI. (E) Hydrolysis of *N*-pivaloyl-2-MPI.

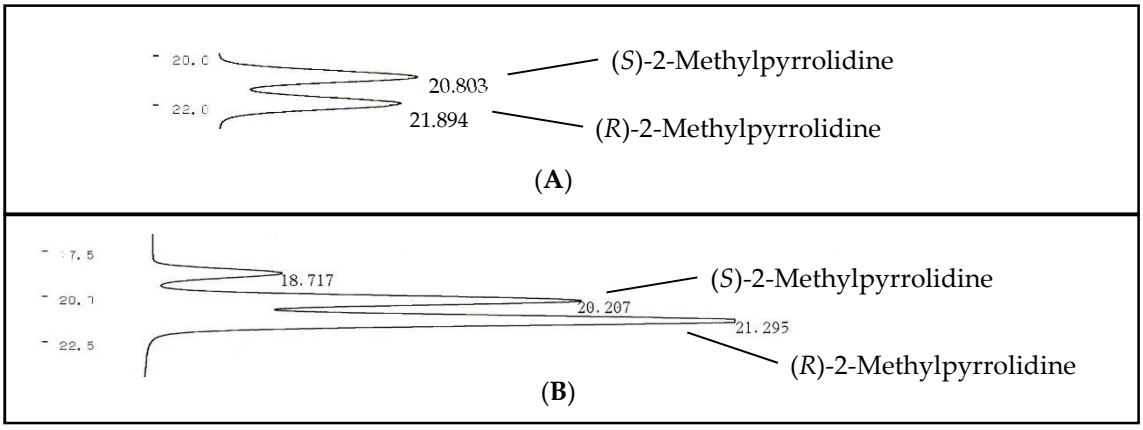

Figure S7. HPLC chromatograms after *N*-benzoyl-2-methylpyrrolidine hydrolysis using purified *Arthrobacter* sp. K5 hydrolase. (A) Racemic 2-methylpyrrolidine as a standard. (B) Hydrolysis of *N*-benzoyl-2-methylpyrrolidine.

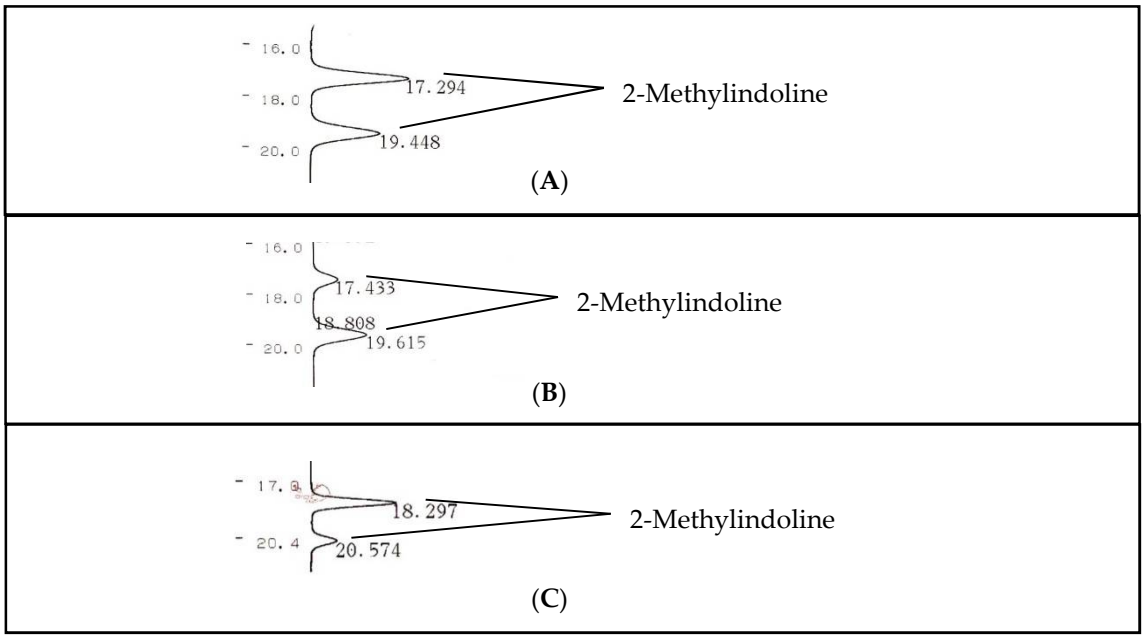

Figure S8. HPLC chromatograms after *N*-acyl-2-methylindoline hydrolysis using purified *Arthrobacter* sp. K5 hydrolase. (A) Racemic 2-methylindoline as a standard. (B) Hydrolysis of *N*-benzoyl-2-methylindoline. (C) Hydrolysis of *N*-acetyl-2-methylindoline.

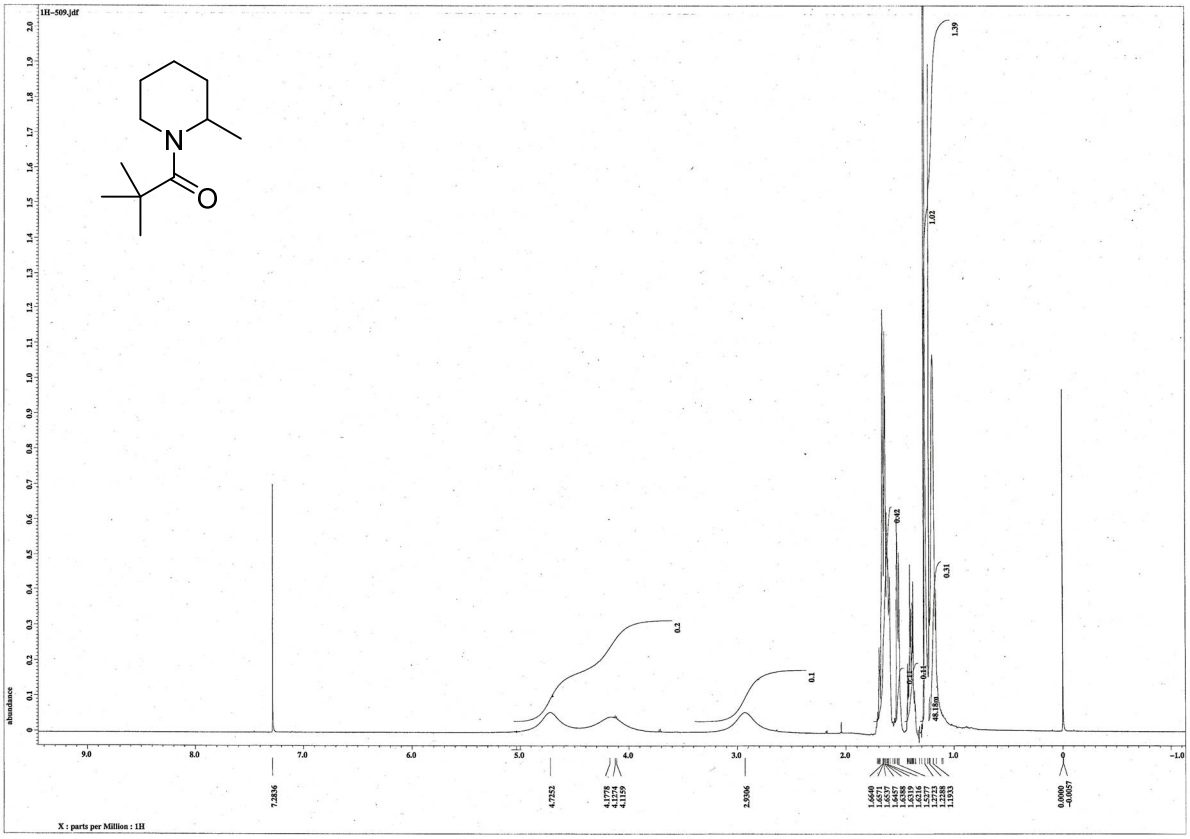

Figure S9. ^{1}H-NMR and ^{13}C-NMR spectrum of *N*-pivaloyl-2-methylpiperidine.

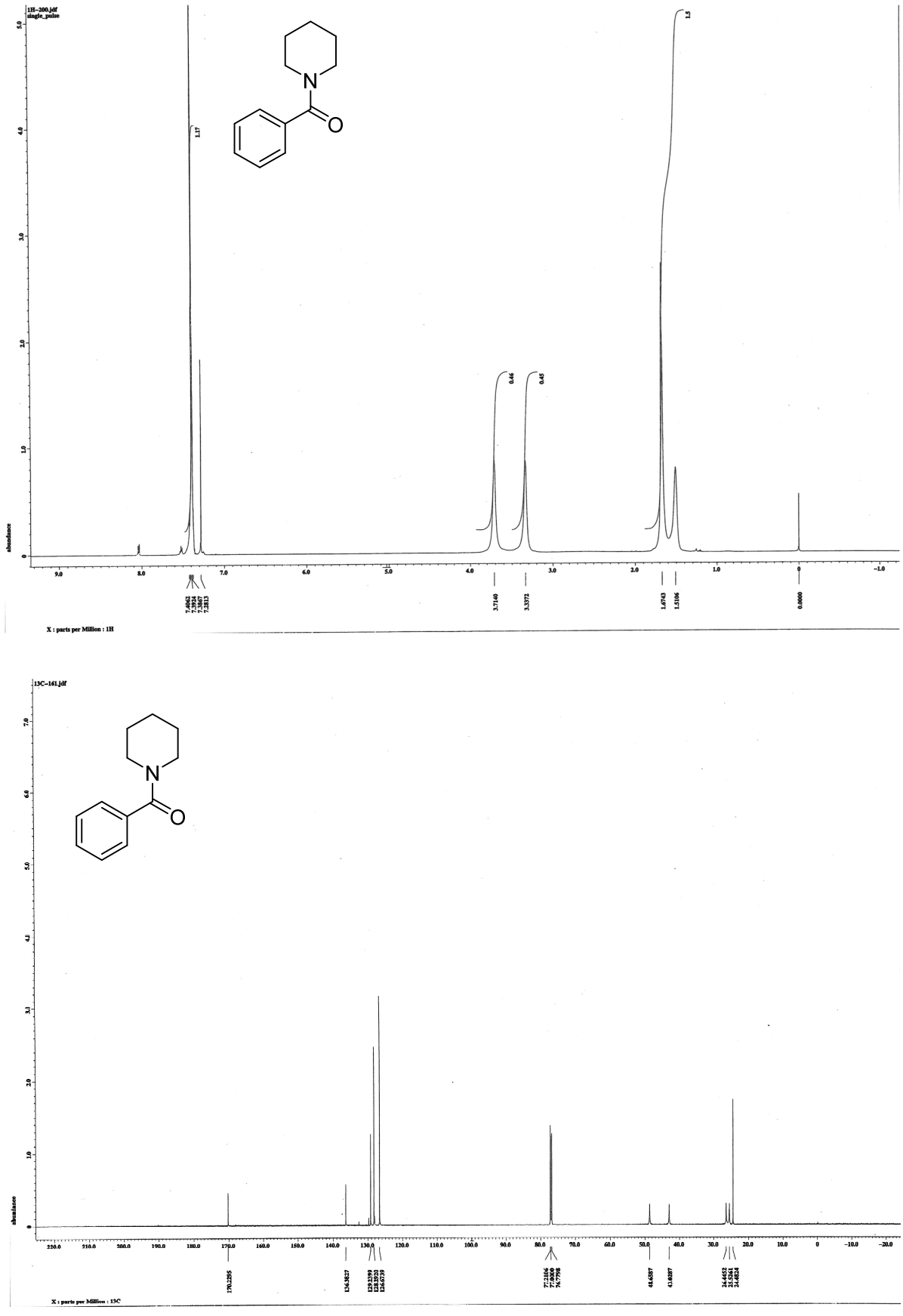

Figure S10. ^{1}H-NMR and ^{13}C-NMR spectrum of *N*-benzoylpiperidine.

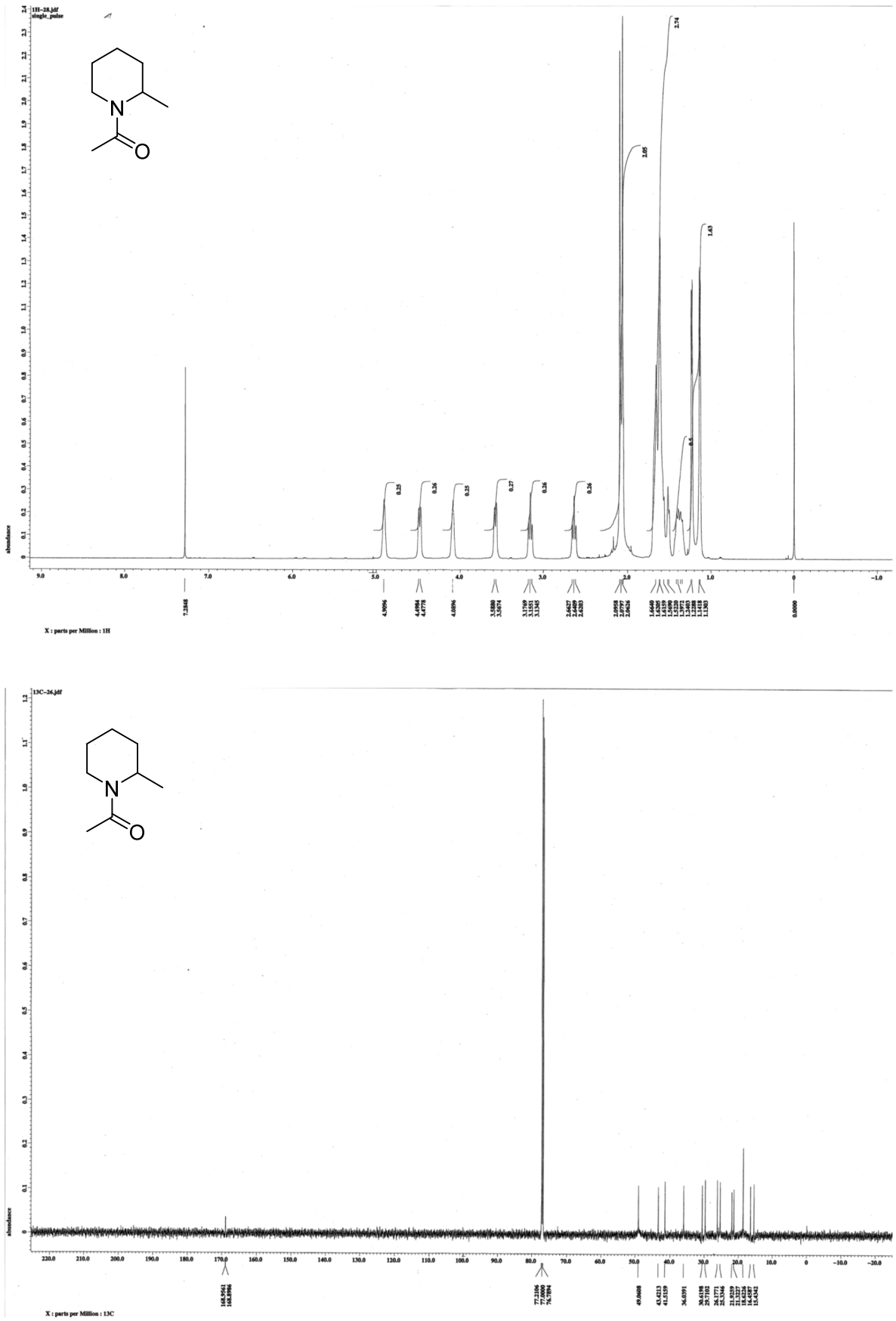

Figure S11. ^{1}H-NMR and ^{13}C-NMR spectrum of *N*-acetyl-2-methylpiperidine.

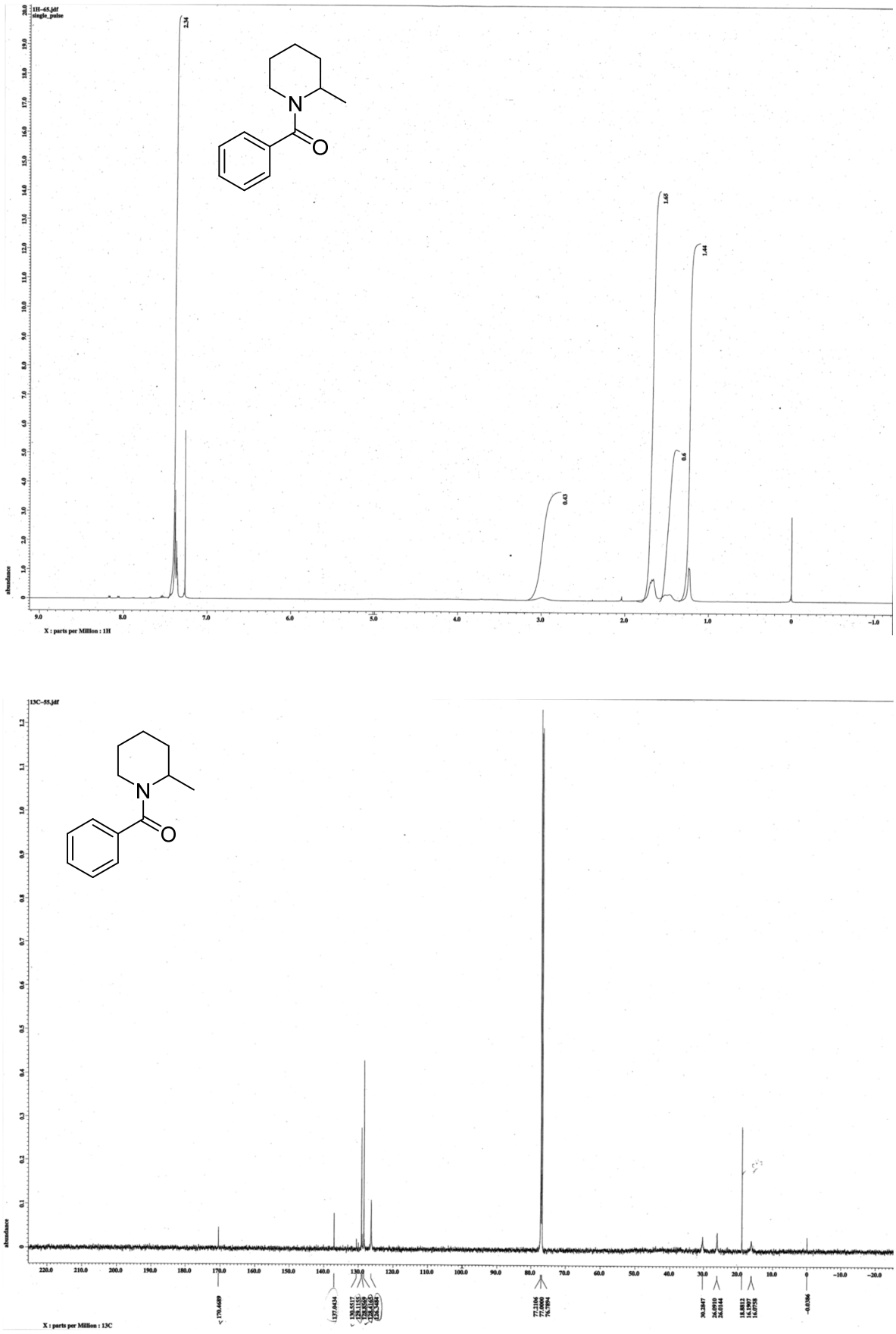

Figure S12. ^{1}H-NMR and ^{13}C-NMR spectrum of *N*-benzoyl-2-methylpiperidine.

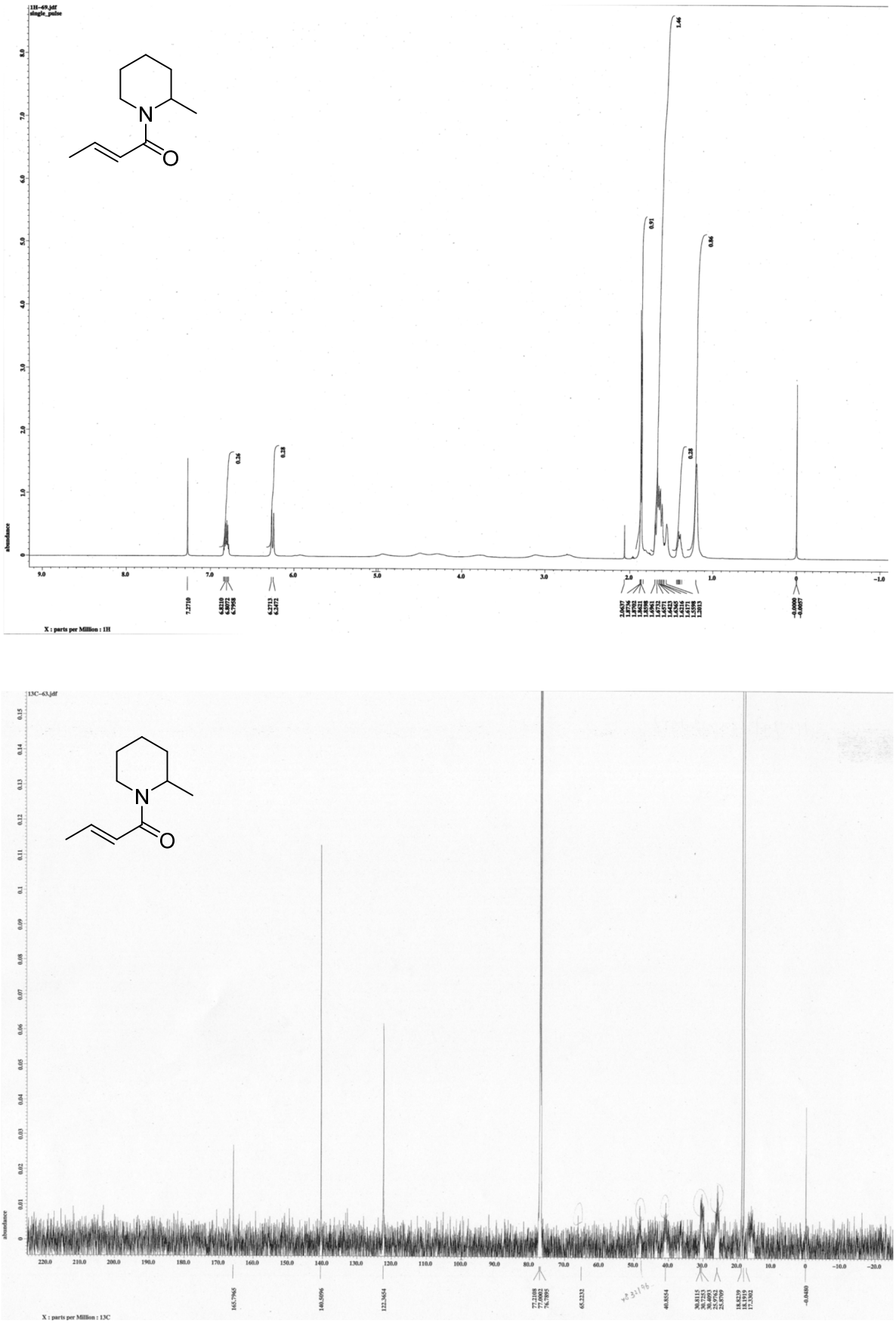

Figure S13. ^{1}H-NMR and ^{13}C-NMR spectrum of *N*-crotonoyl-2-methylpiperidine.

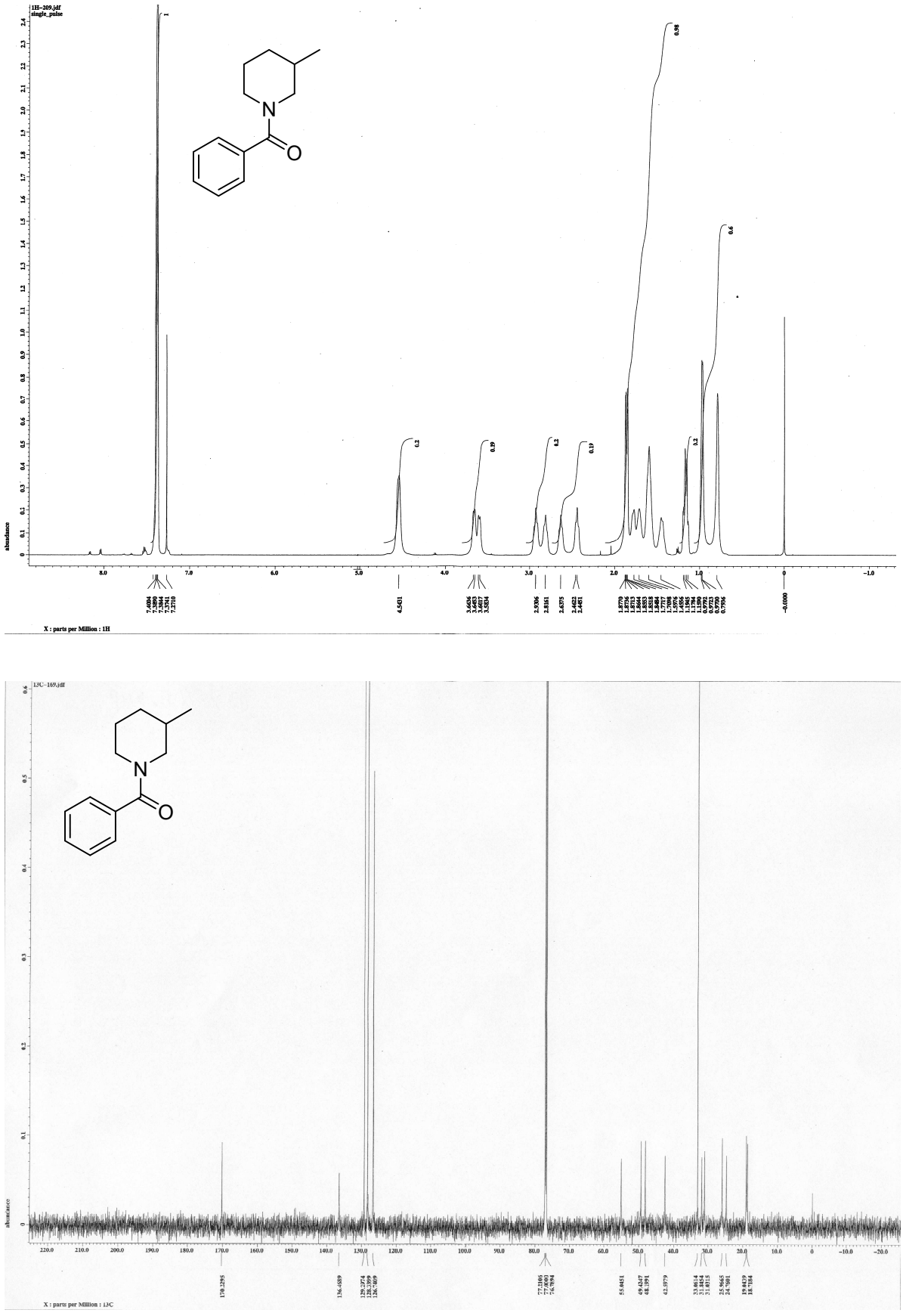

Figure S14. ^{1}H-NMR and ^{13}C-NMR spectrum of *N*-benzoyl-3-methylpiperidine.

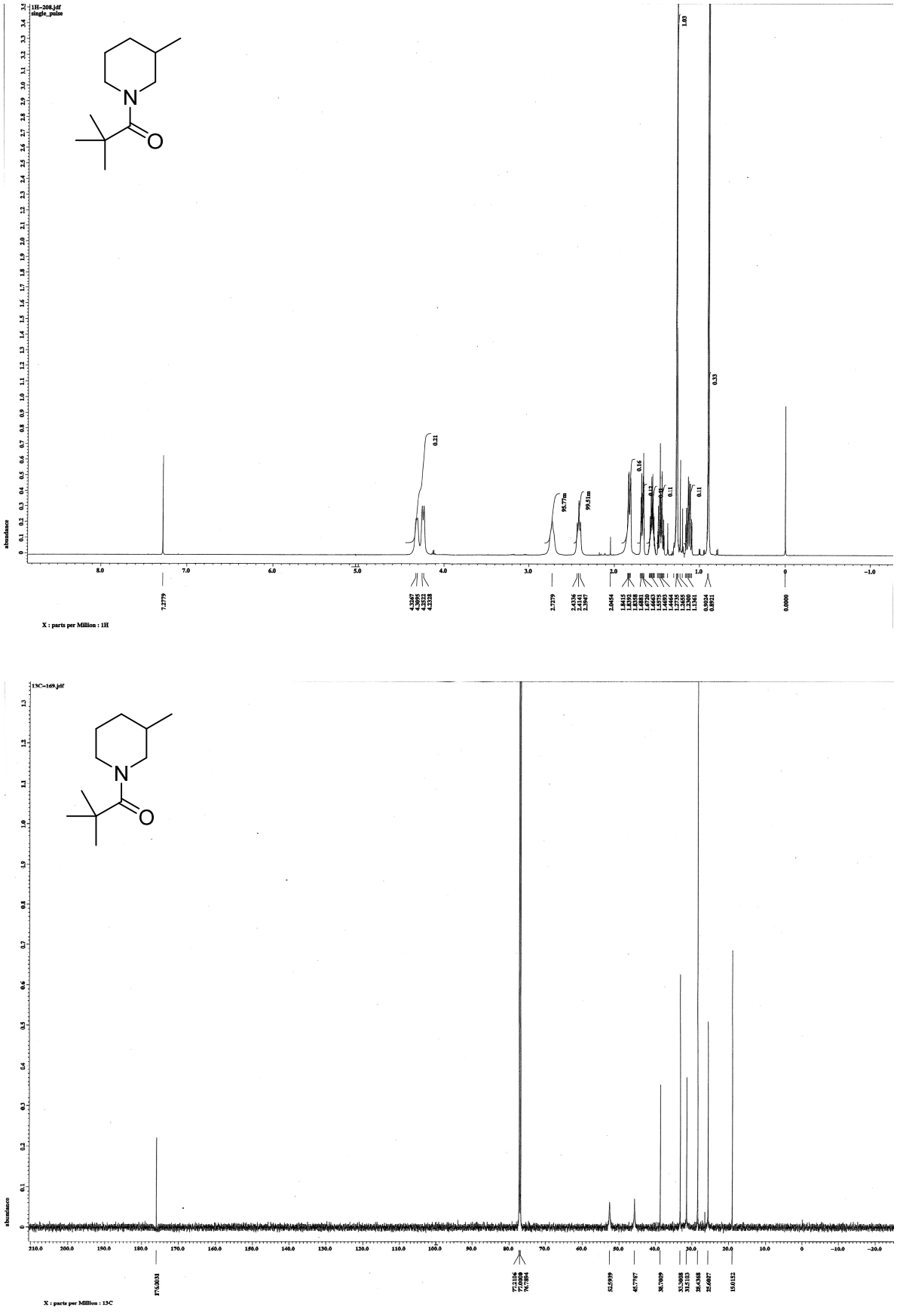

Figure S15. ^{1}H-NMR and ^{13}C-NMR spectrum of *N*-pivaloyl-3-methylpiperidine.

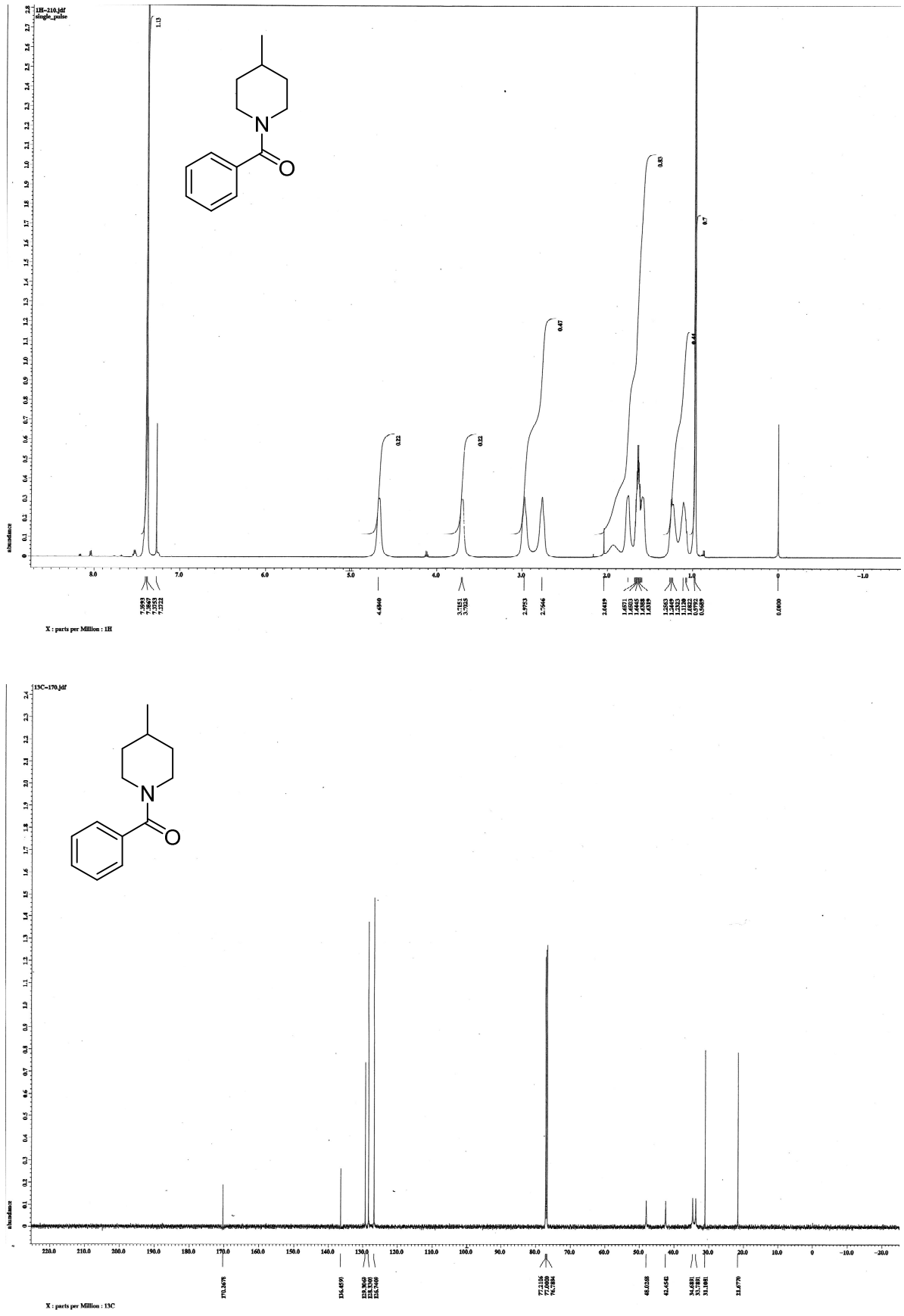

Figure S16. ^{1}H-NMR and ^{13}C-NMR spectrum of *N*-benzoyl-4-methylpiperidine.

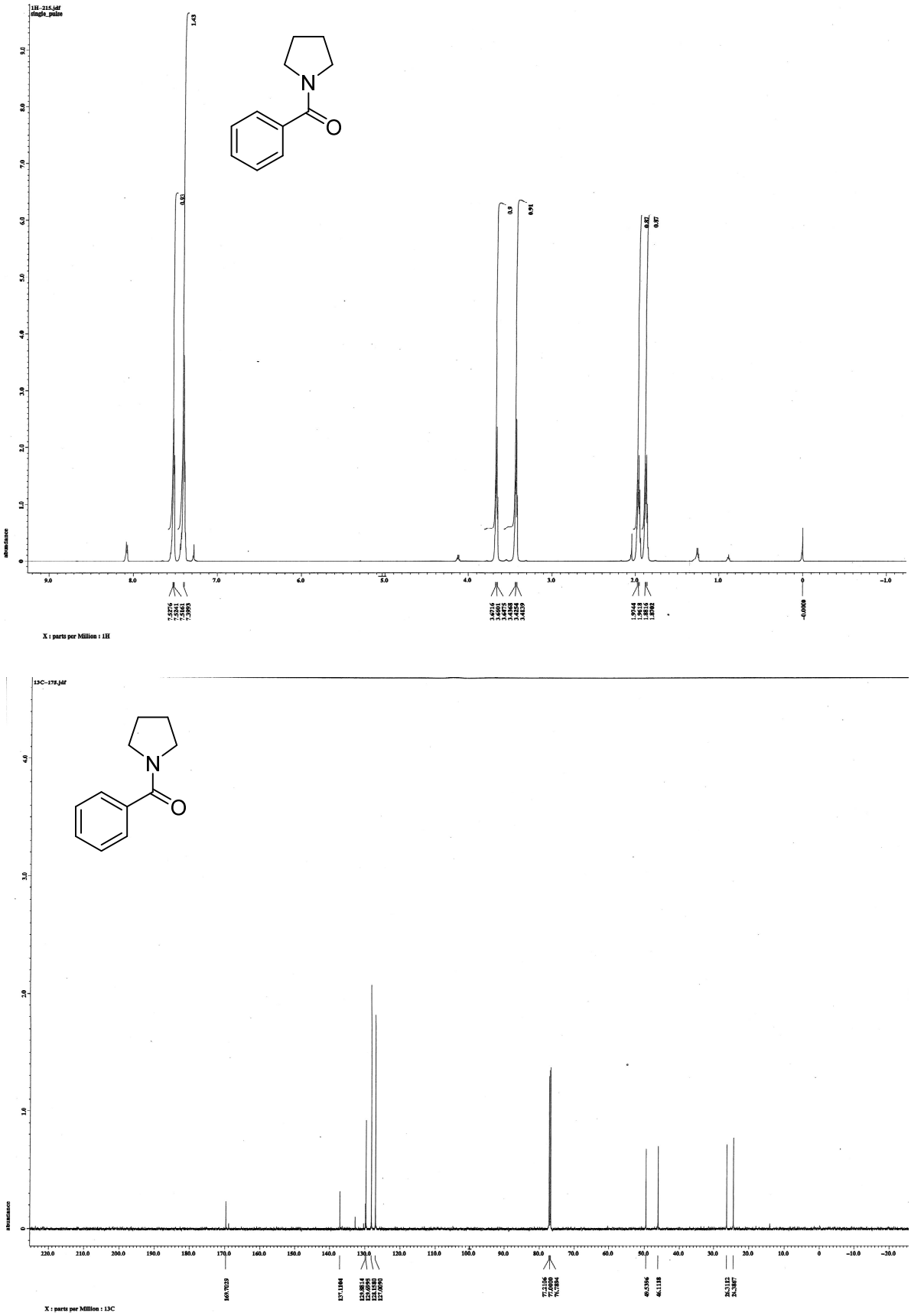

Figure S17. ^{1}H-NMR and ^{13}C-NMR spectrum of *N*-benzoylpyrrolidine.

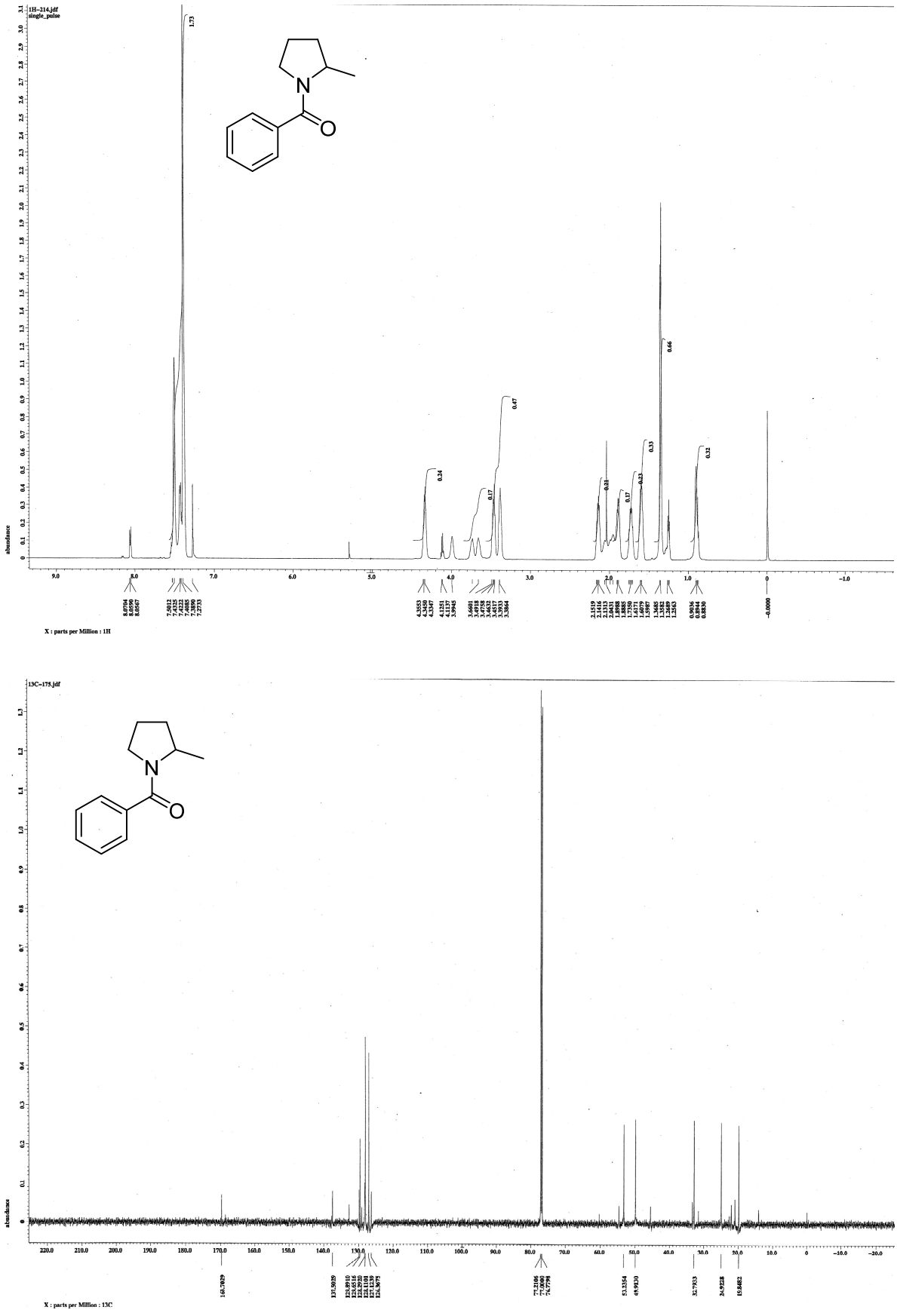

Figure S18. ^{1}H-NMR and ^{13}C-NMR spectrum of *N*-benzoyl-2-methylpyrrolidine.

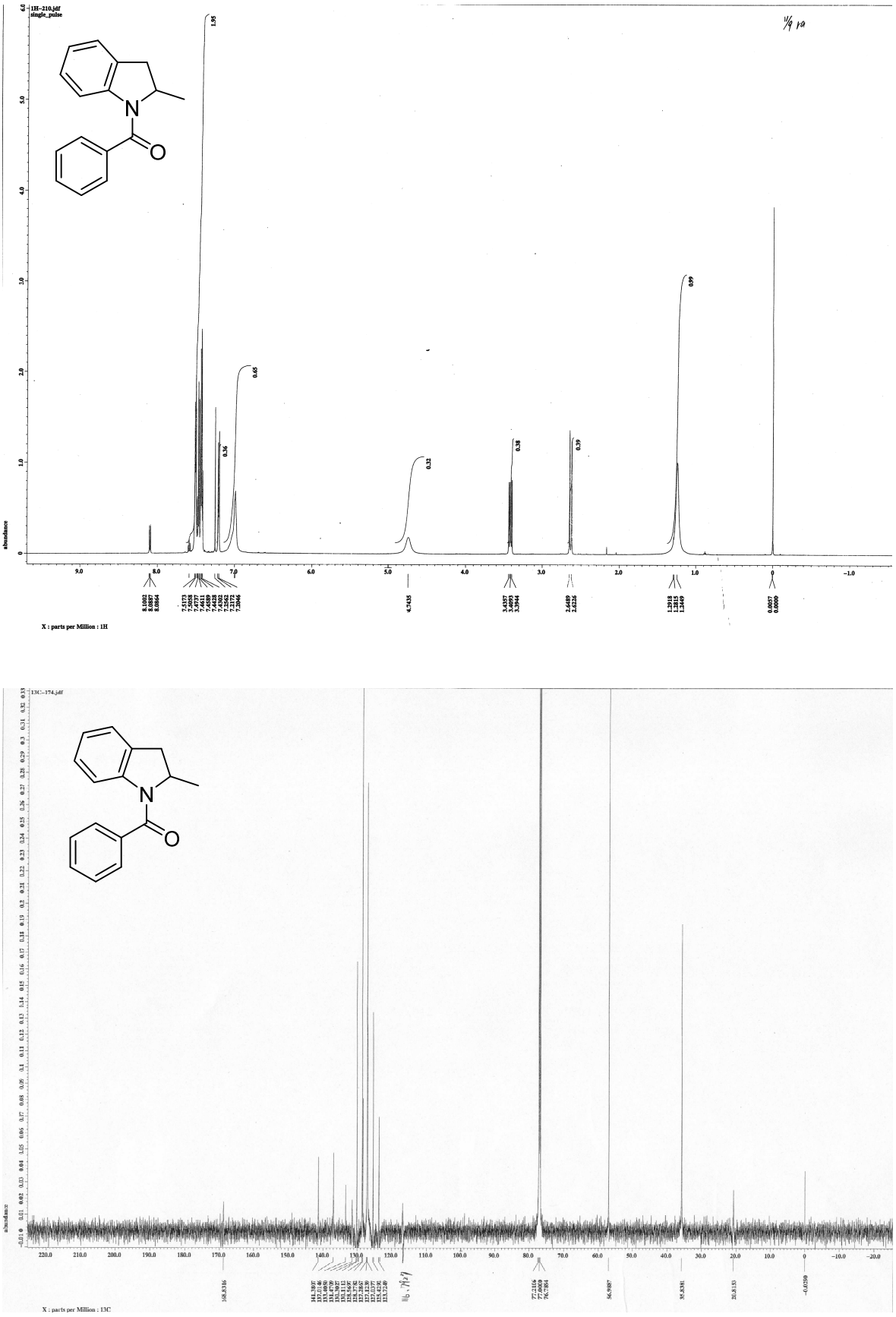

Figure S19. ^{1}H-NMR and ^{13}C-NMR spectrum of *N*-benzoyl-2-methylindoline.

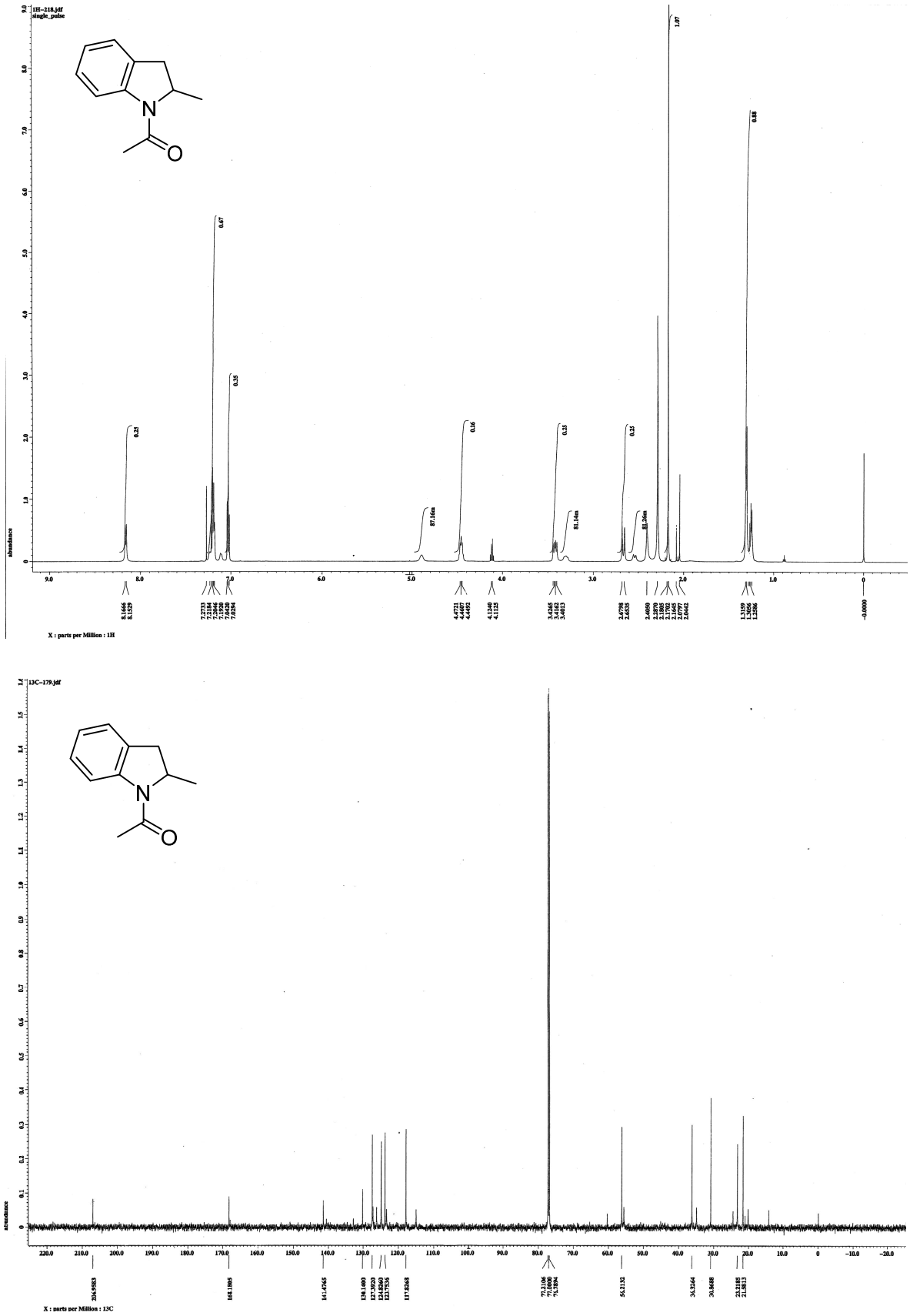

Figure S20. ^{1}H-NMR and ^{13}C-NMR spectrum of *N*-acetyl-2-methylindoline.

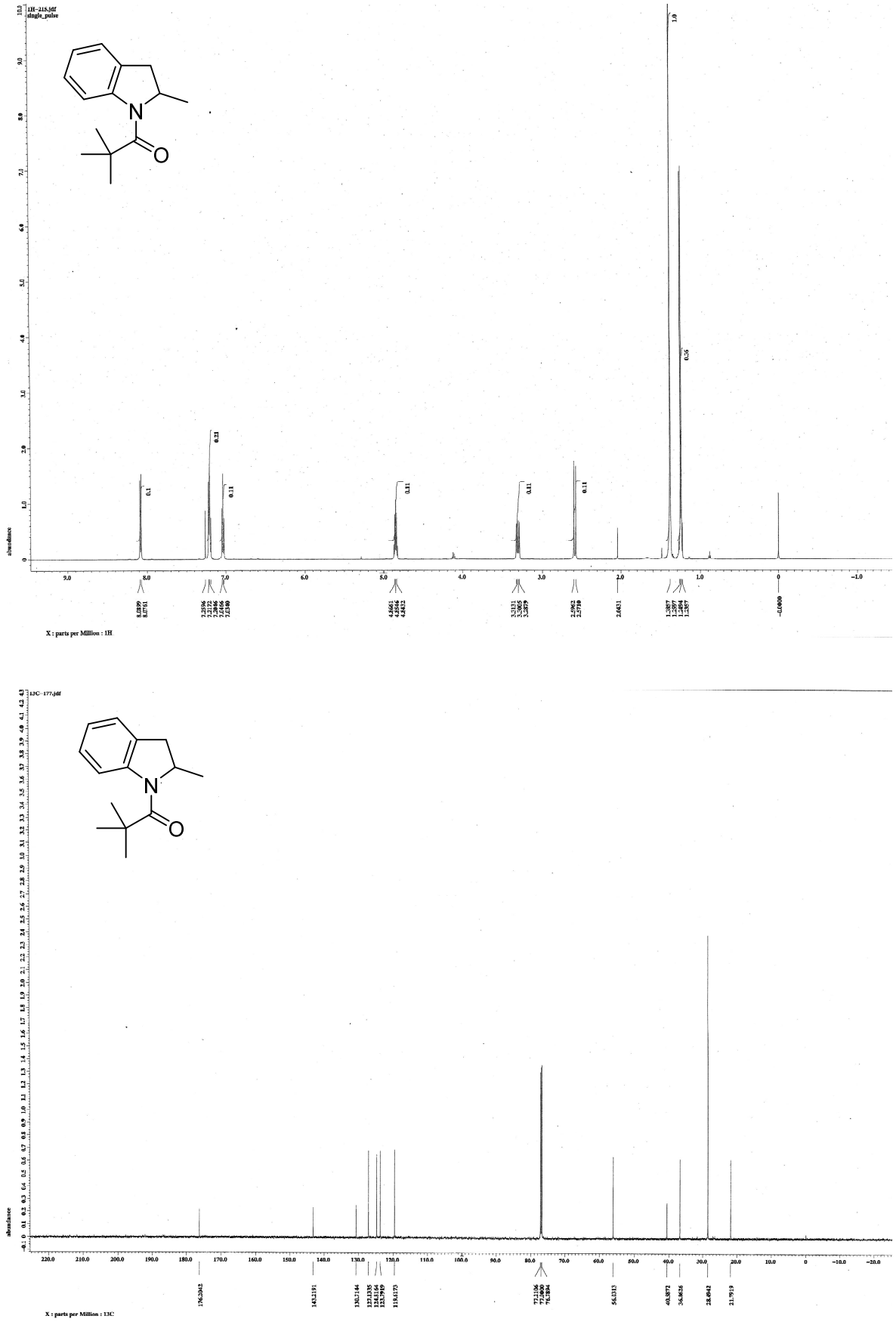

Figure S21. ^{1}H-NMR and ^{13}C-NMR spectrum of *N*-pivaloyl-2-methylindoline.

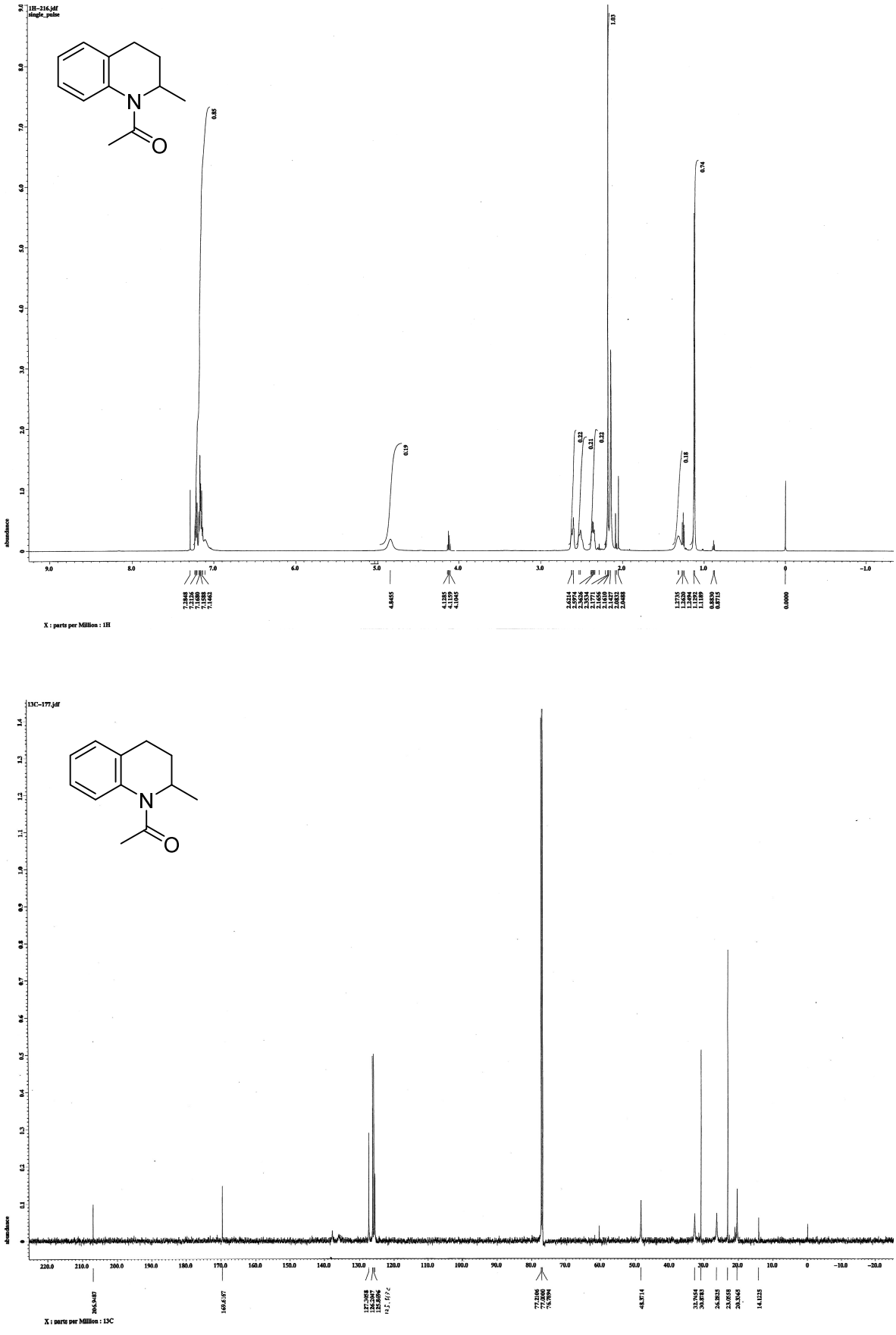

Figure S22. ^{1}H-NMR and ^{13}C-NMR spectrum of *N*-acetyl-1,2,3,4-tetrahydroquinaldine.

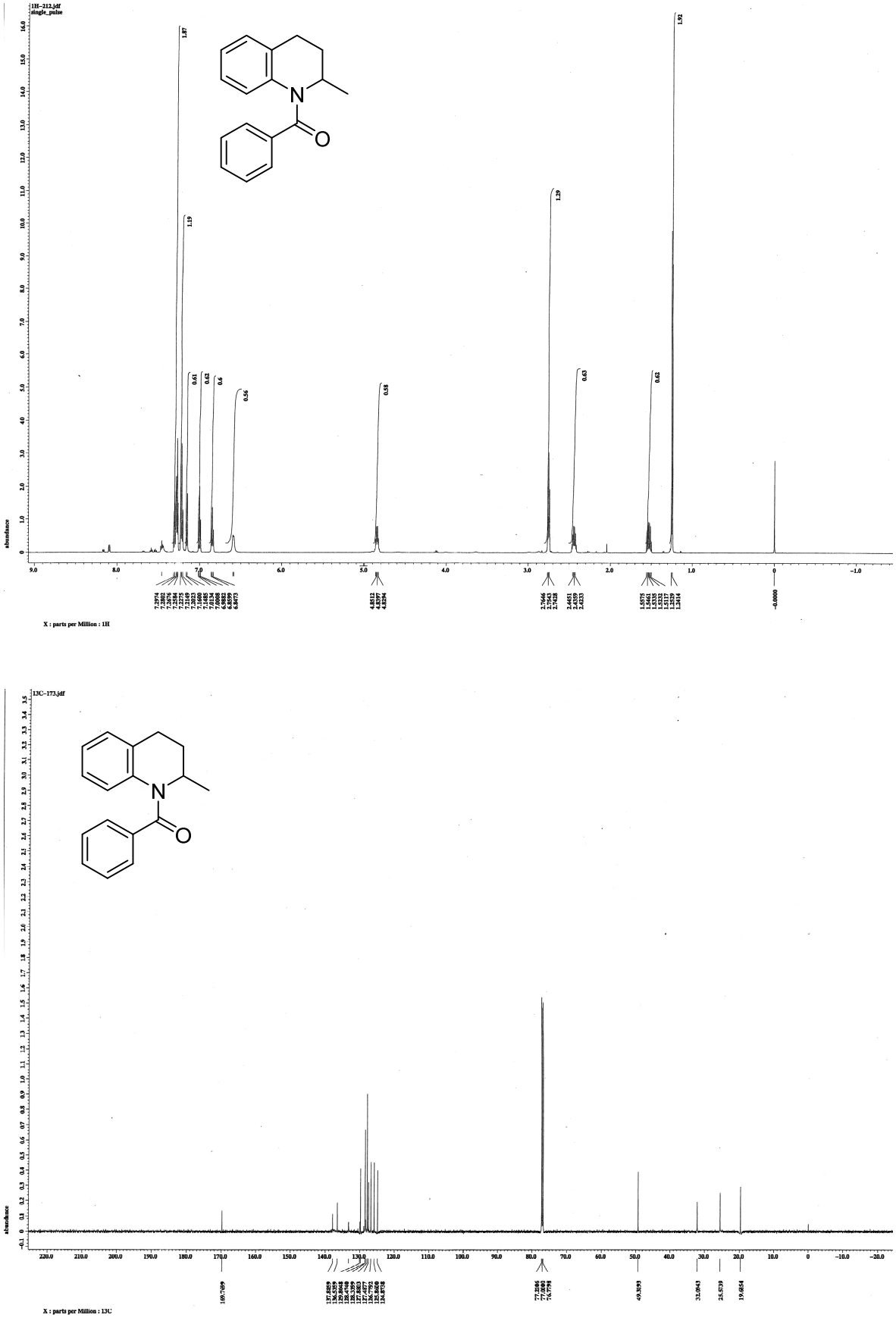

Figure S23. ^{1}H-NMR and ^{13}C-NMR spectrum of *N*-benzoyl-1,2,3,4-tetrahydroquinaldine.