Supporting Information

# Nematode Signaling Molecules Derived from Multimodular Assembly of Primary Metabolic Building Blocks

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## 1. Supporting Methods

**1.1. Nematode-derived modular metabolite (NDMM) nomenclature.** Ascarosides are named using Small Molecule IDentifiers ("SMIDs"), representing searchable, gene-style identifiers that consist of four lower case non-italicized letters followed by a pound sign and a number. The SMID database (www.smid-db.org) is an electronic resource maintained by Profs. Frank C. Schroeder and Lukas Mueller at the Boyce Thompson Institute/Cornell University, in collaboration with Prof. Paul Sternberg at Caltech and WormBase (www.wormbase.org). This database catalogues newly identified nematode small molecules, assigns a unique four-letter SMID (a searchable gene-style identifier), and for each compound includes a list of other names and abbreviations used in the literature.

**1.2. Analytical instrumentation.** NMR spectra were recorded on a Varian INOVA-600 (600 MHz for $^{1}$H, 151 MHz for $^{13}$C), INOVA-500 (500 MHz for $^{1}$H and 125 MHz for $^{13}$C), and INOVA-400 (400 MHz for $^{1}$H, 100 MHz for $^{13}$C) instruments. HPLC-MS and –MS/MS was performed using an Agilent 1100 Series HPLC system equipped with a diode array detector and an Agilent Eclipse XDB-C18 column (4.6 x 250 mm, 5 µm particle diameter), connected to a Quattro II spectrometer (Micromass/Waters). Flash chromatography was performed using a Teledyne ISCO CombiFlash system. Preparative HPLC separation was performed using the Agilent 1100 Series HPLC system equipped with an Agilent Eclipse XDB-C18 column (9.4 x 250 mm, 5 µm particle diameter) coupled to a Teledyne ISCO Foxy 200 fraction collector. High resolution mass spectra were acquired using a Xevo G2 QTOF mass spectrometer.

**1.3. *P. pacificus* strains and culture conditions.** The following *P. pacificus* strains were used for the study: RS2333 (novel metabolite identification, dauer formation assays), RS5134, RS5205, RS5399, RS5380, and RSB020 (dauer formation assays). Plates and liquid cultures of worms were prepared as described previously.[1]

**1.4. Preparation of exo-metabolome extracts and fractionation.** ~3 L *P. pacificus* RS2333 liquid culture supernatant (exo-metabolome) was lyophilized to a fine powder and extracted with 750 ml of a 95:5 mixture of ethanol and water for 16 h (2 times). The exo-metabolome extract was then concentrated *in vacuo*, loaded onto 12 g of ethyl acetate-washed Celite® and fractionated using a Teledyne ISCO CombiFlash system over a RediSep® Rf GOLD 30 g HP C18 reverse-phase column using a water-methanol solvent gradient, starting with 15 min of 98% water, followed by a linear increase of methanol content up to 100% at 60 min. The eluate was divided into 8 fractions, which were evaporated *in vacuo* and prepared for HPLC-MS/MS, NMR spectroscopic analyses, or further HPLC enrichment.

**1.5. 2D NMR spectroscopic metabolome analyses.** Non-gradient phase-cycled dqfCOSY spectra were acquired using the following parameters: 0.8 s acquisition time, 400-600 complex increments, 8-32 scans per increment. dqfCOSY spectra were zero-filled to 8k × 4k and a cosine bell-shaped window function was applied in both dimensions before Fourier transformation. Gradient and non-gradient HSQC and HMBC spectra were acquired using 0.25 s acquisition time and 256-500 complex increments. NMR spectra were processed using Varian VNMR, MestreLabs' MestReC, and MNova software packages.

**1.6. HPLC protocol, LC-MS/MS, and SIM-LCMS analyses.** HPLC-MS was performed using an Agilent 1100 Series HPLC system equipped with an Agilent Eclipse XDB-C18 column (4.6 x 250 mm, 5 µm particle diameter) connected to a Quattro II spectrometer (Micromass/Waters). A 0.1% acetic acid-acetonitrile solvent gradient was used at a flow rate of 1 mL/min, starting with an acetonitrile content of 5% for 5 min which was increased to 100% over a period of 40 min. Exo-metabolome fractions were analyzed by HPLC-

ESI-MS in negative and positive ion modes using a capillary voltage of 3.5 kV and a cone voltage of -35 V and +20 V respectively. HPLC-MS/MS screening for precursor ions of $m/z$ = 73.0 (negative mode) performed using argon as collision gas at 2.1 mtorr and 40 eV. Quantifications were based on intetegration of HPLC-MS signals from the corresponding ion-traces. Concentrations were calculated using response factors determined for synthetic standards.

The analytical HPLC protocol mentioned above was translated to a semi-preparative Agilent Eclipse XDB-C18 column (9.4 x 250 mm, 5 μm particle diameter) with a flow rate of 3.6 mL/min and used for MS-assisted enrichment of pasay#9, pasy#9, and npar#3 from exo-metabolome extract fractions, as well as for synthetic sample purification.

**1.7. General methods for chemical synthesis.** Thin-layer chromatography (TLC) was used to monitor progress of reactions unless stated otherwise using J. T. Baker Silica Gel IB2-F. Unless stated otherwise, reagents were purchased from Sigma-Aldrich and used without further purification. *N,N*-dimethylformamide (DMF), dichloromethane (DCM) were dried over 4 Å molecular sieves prior to use. Tetrahydrofuran (THF), 1,4-dioxane were distilled prior to use. Optical rotations were measured on a Perkin Elmer 341 polarimeter. Solvent used for taking optical rotations (methanol) was not further purified prior to use.

**1.8. Dauer formation assay.** Dauer formation assay was performed as described previously.[2]

**1.9. Statistical analysis.** Error bars represent a 95% confidence interval in Figure 3A calculated using a binomial test on the total count data. All experiments were conducted in triplicate (or in five replicates for mouth-form concentration-curve assays) for each treatment. Significant differences (*$P$<0.01 and **$P$<0.001) between each chemical treatment and the control (EtOH) treatment in Figure 3A were determined using Fisher's exact test in the program R.

## 2. Supporting Figures

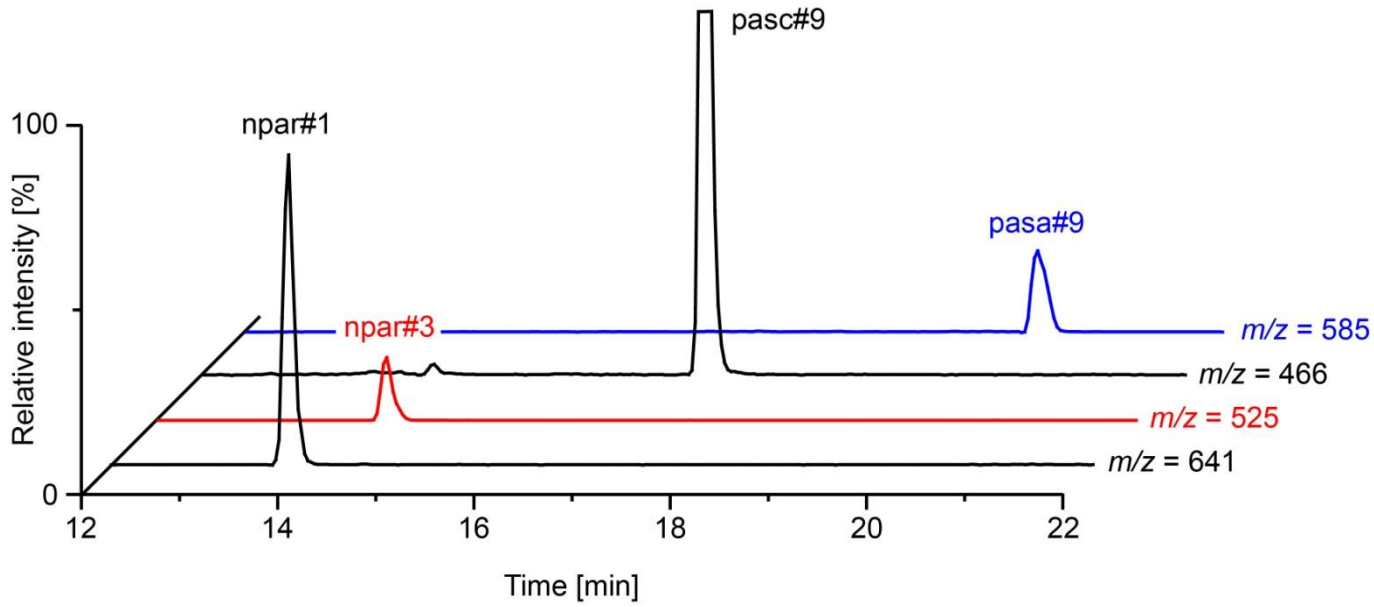



**Figure S1.** HPLC-MS analysis of exo-metabolome extract fractions from *P. pacificus* cultures showing peaks for the novel compounds npar#3 and pasa#9, reported in this work. The retention times of npar#3 and pasa#9 are compared to the structurally related and previously identified[3] compounds npar#1 and pasc#9 respectively (refer to Figure 1 for structures).

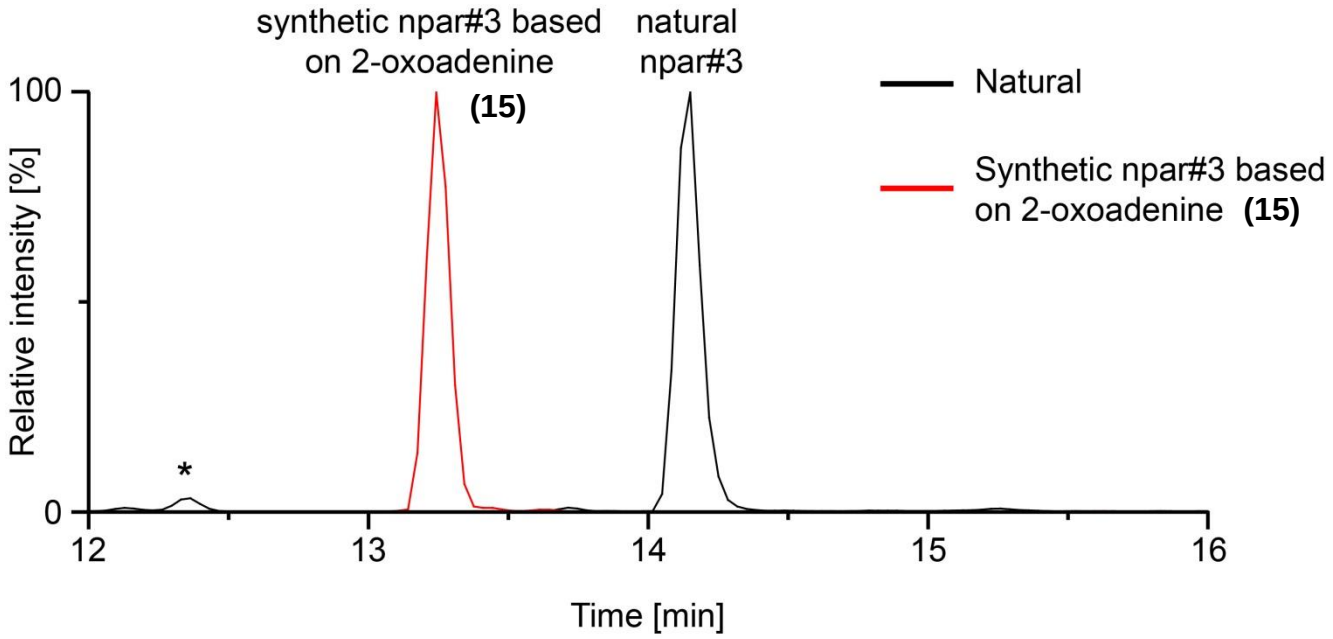



**Figure S2.** Comparison of HPLC-MS retention times ($ESI^-$, extracted ion chromatogram for $m/z$ = 525) of natural npar#3 (black) and synthetic **15** (npar#3-isomer based on 2-oxoadenine in red). Retention times do not match; indicating that natural npar#3 is not based on 2-oxoadenine.
*Represents unrelated compound(s) in the natural sample.

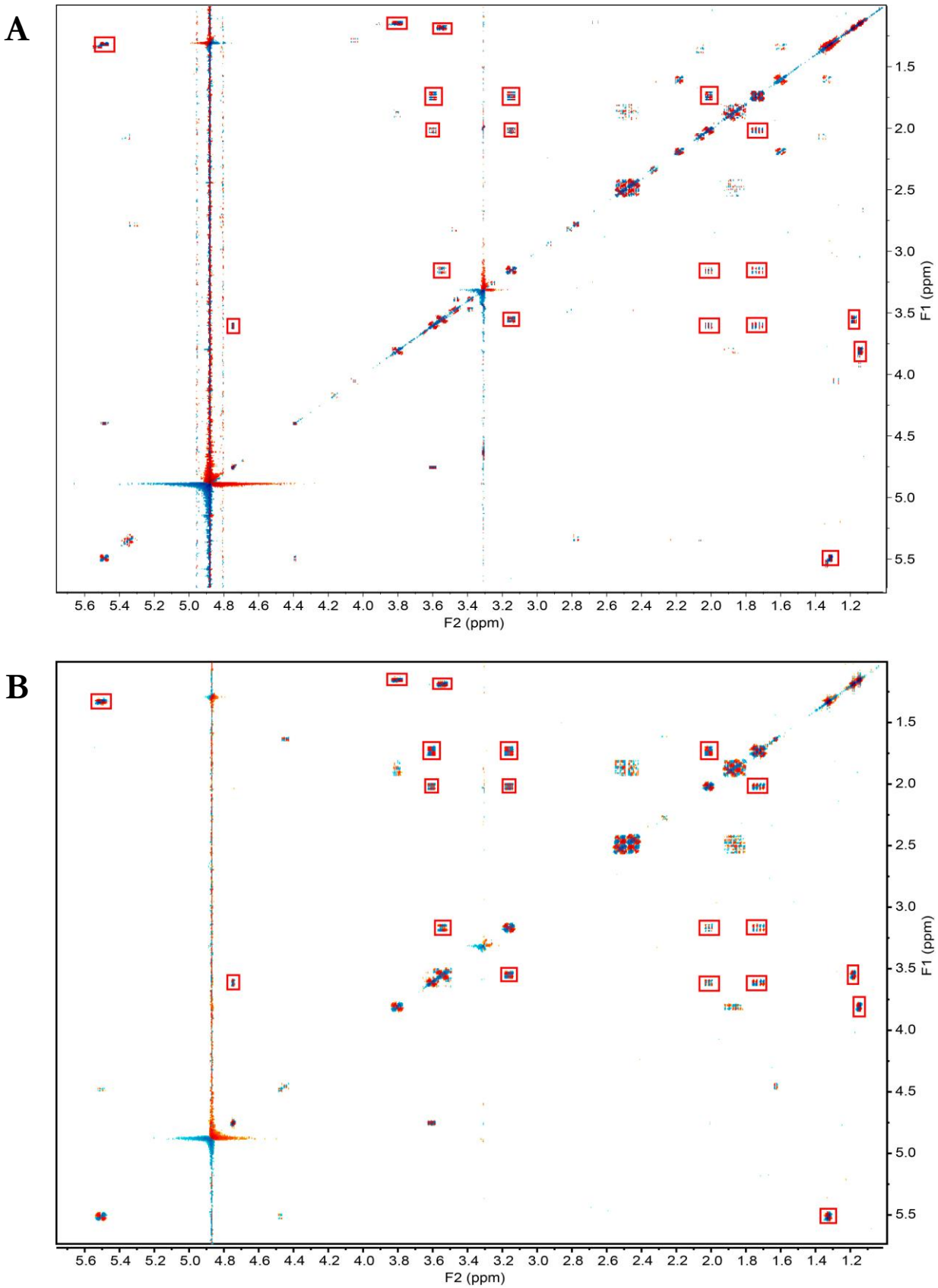



**Figure S3.** Sections of dqfCOSY spectra (600 MHz, methanol-$d_4$) confirming presence of npar#3 in *P. pacificus* exo-metabolome. A) HPLC-enriched *P. pacificus* exo-metabolome extract fraction containing npar#3. B) Synthetic sample of npar#3 based on 8-oxoadenine. Characteristic crosspeaks for npar#3 are boxed red.

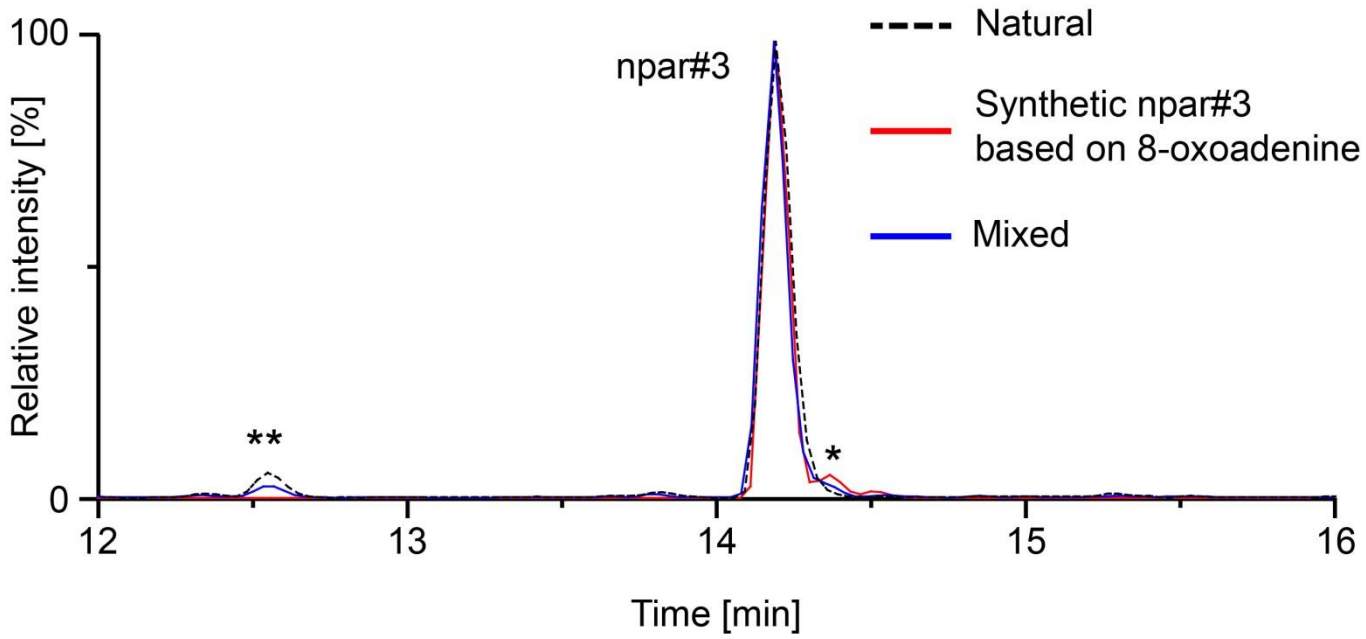


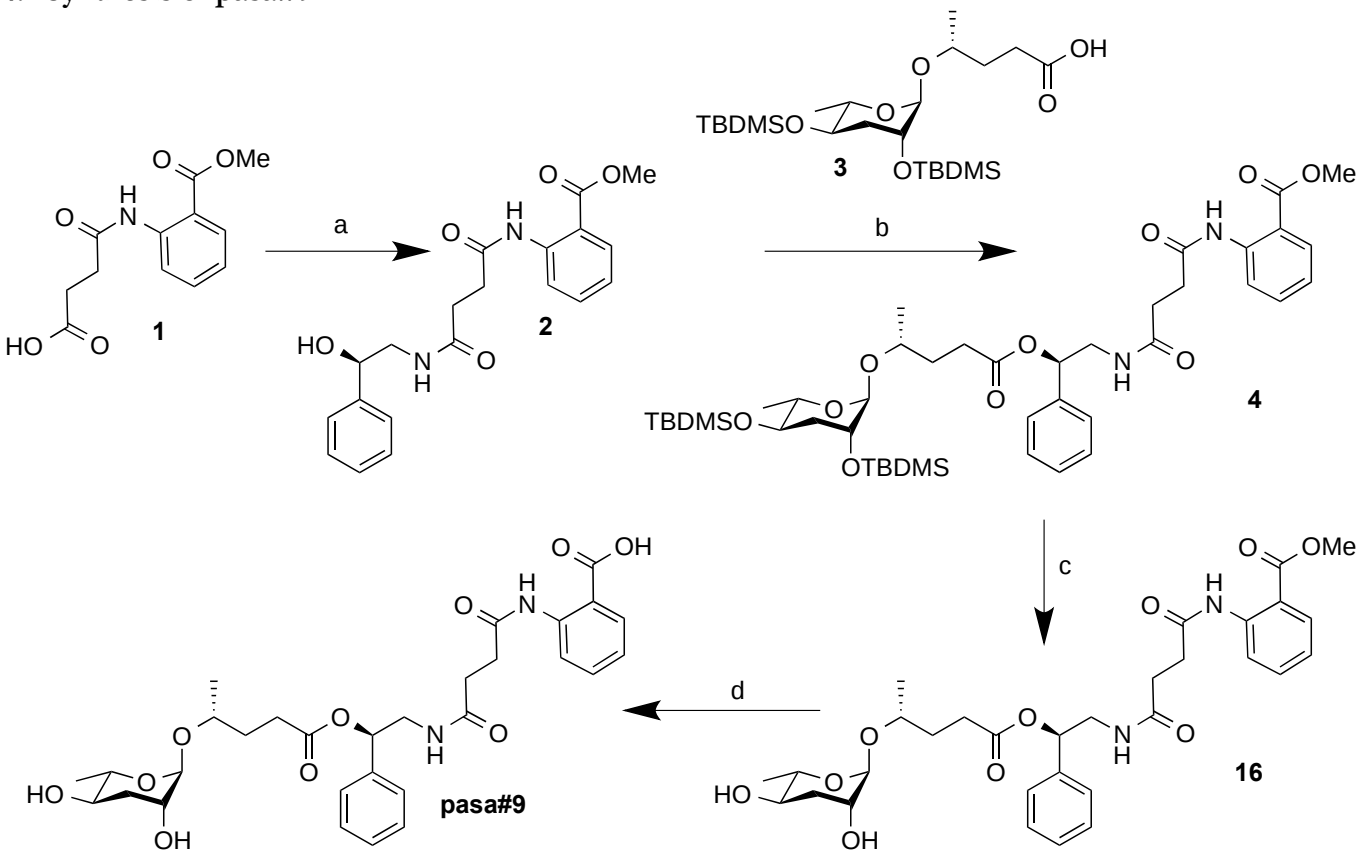

**Figure S4.** Comparison of HPLC-MS retention times ($ESI^-$, extracted ion chromatogram for $m/z$ = 525) of natural npar#3 (dotted black), synthetic npar#3 based on 8-oxoadenine (red), and a mixture of the natural and synthetic samples (blue). Retention times and NMR crosspeaks (Figure S4) precisely match, indicating that natural npar#3 is based on 8-oxoadenine.
*Represents minor sideproduct(s) in the synthetic sample.
**Represents unrelated compound(s) in the natural sample.

**3. Supporting Table S1. High-resolution MS data of new metabolites.**

| SMID | Detected molecular ion | LC retention time [Min] ±SD | *m/z* calculated | *m/z* observed |
|---|---|---|---|---|
| npar#3 | $C_{21}H_{29}N_6O_{10}^-$ | 14.19 ± 0.06 | 525.1951 | 525.1963 |
| pasa#9 | $C_{30}H_{37}N_2O_{10}^-$ | 20.14 ± 0.08 | 585.2454 | 585.2453 |
| pasy#9 | $C_{23}H_{32}NO_8^+$ | 19.34 ± 0.06 | 450.2122 | 450.2109 |

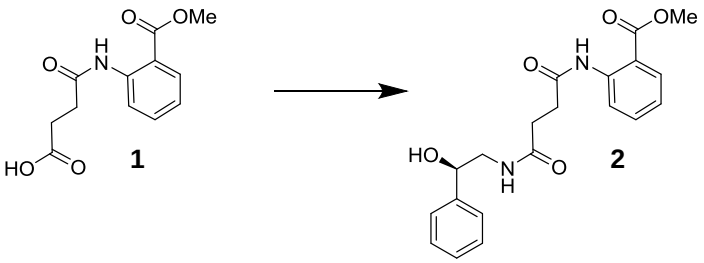
## 4. Chemical Synthesis

### 4.1 Synthesis of pasa#9




**Synthetic Scheme S1. Overview of synthesis of pasa#9.** Reagents and conditions: **(a)** EDC, DMAP, DCM; **(b)** EDC, DMAP, DCM; **(c)** 40% HF, MeCN; **(d)** LiOH, THF/dioxane/$H_2O$, 40 °C.

**Synthesis of (2)**
methyl (*R*)-2-(4-((2-hydroxy-2-phenylethyl)amino)-4-oxobutanamido)benzoate

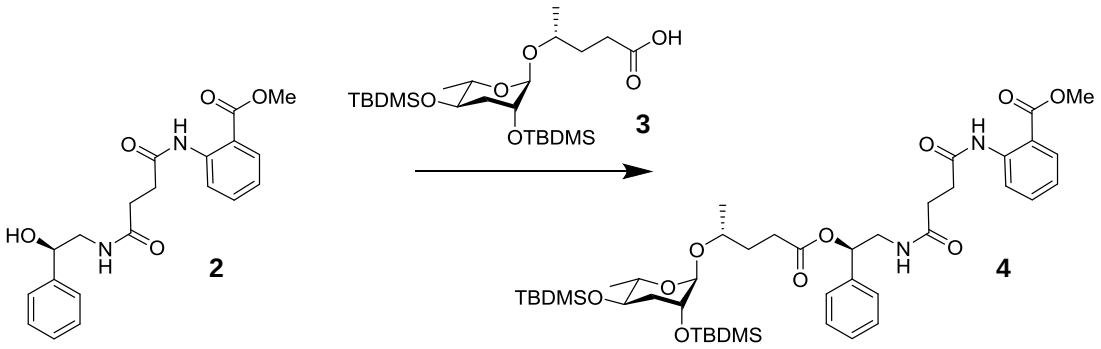



A solution of **1** (124 mg, 490 μmol) in 8 mL dry dichloromethane was treated with 4-dimethylaminopyridine (95 mg, 780 μmol) and EDC hydrochloride (150 mg, 780 μmol). After stirring for 10 minutes, **(*R*)-phenyl ethanolamine** (100 mg, 740 μmol) in 2 mL dry dichloromethane was added to the mixture. After stirring for 18 h, the reaction was concentrated *in vacuo*. Flash column chromatography on silica using a gradient of 0-10% methanol in dichloromethane with 0.25% acetic acid afforded **2** (116 mg, 310 μmol, 63%).

$^1$H NMR (600 MHz, chloroform-*d*): δ (ppm) 11.16 (s, 1H), 8.60-8.57 (m, 1H), 8.03-7.99 (m, 1H), 7.54-7.48 (m, 1H), 7.37-7.33 (m, 2H), 7.33-7.28 (m, 2H), 7.25-7.21 (m, 1H), 7.10-7.06 (m, 1H), 6.74-7.67 (m, 1H), 4.86 (dd, *J* = 8.3, 3.3 Hz, 1H), 3.92 (s, 3H), 3.69 (ddd, *J* = 14.1, 6.9, 3.3 Hz, 1H), 3.29 (ddd, *J* = 13.9, 8.3, 5.1 Hz, 1H), 2.89-2.76 (m, 2H), 2.65-2.55 (m, 2H).

**Synthesis of (4)**

Methyl 2-(4-(((*R*)-2-(((*R*)-4-(((2*R*,3*R*,5*R*,6*S*)-3,5-bis((*tert*-butyldimethylsilyl)oxy)-6-methyltetrahydro-2*H*-pyran-2-yl)oxy)pentanoyl)oxy)-2-phenylethyl)amino)-4-oxobutanamido)benzoate



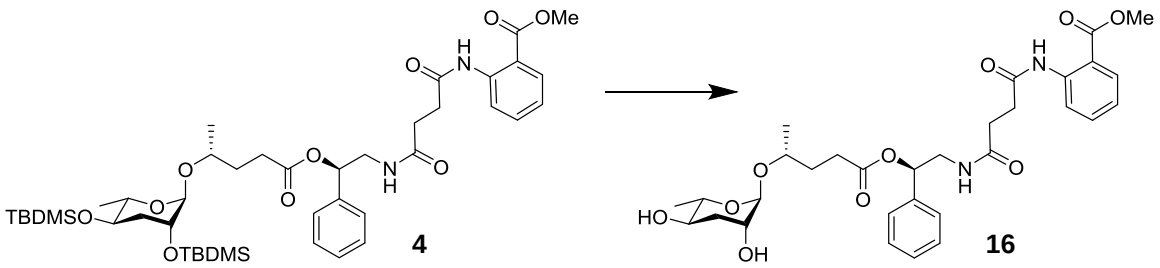

A solution of **3**[3] (13.3 mg, 28 μmol) in 500 μL dry dichloromethane was treated with 4-dimethylaminopyridine (7 mg, 56 μmol) and EDC hydrochloride (9 mg, 56 μmol). After stirring for 15 minutes, **2** (21 mg, 56 μmol) in 500 μL dry dichloromethane was added to the mixture. After stirring for 18 h, the reaction was concentrated *in vacuo*. Flash column chromatography on silica using a gradient of 20-80% ethyl acetate in hexanes afforded **4** (19 mg, 23 μmol, 82%).

$^{1}$H NMR (500 MHz, chloroform-*d*): δ (ppm) 11.10 (broad s, 1H), 8.67-8.63 (m, 1H), 8.04-7.99 (m, 1H), 7.55-7.49 (m, 1H), 7.36-7.24 (m, 5H), 7.10-7.04 (m, 1H), 6.58-6.49 (m, 1H), 5.85 (dd, *J* = 8.6, 4.0 Hz, 1H), 4.55 (broad s, 1H), 3.92, (s, 3H), 3.84-3.70 (m, 3H), 3.69-3.58 (m, 2H), 3.48 (ddd, *J* = 14.0, 8.6, 5.0 Hz, 1H), 2.84-2.72 (m, 2H), 2.59-2.53 (m, 2H), 2.50-2.44 (m, 2H), 1.85-1.71 (m, 4H), 1.22 (d, *J* = 5.8 Hz, 3H), 1.10 (d, *J* = 6.1 Hz, 3H), 0.89 (s, 9H), 0.88 (s, 9H), 0.063 (s, 3H), 0.056 (s, 3H), 0.05 (s, 3H), 0.03 (s, 3H).

$^{13}$C NMR (125 MHz, chloroform-*d*) δ (ppm) 172.9, 172.1, 171.0, 168.7, 141.5, 138.0, 134.7, 130.9, 128.7 (2C), 128.4, 126.5 (2C), 122.6, 120.5, 115.1, 97.5, 74.8, 71.4, 70.7, 70.1, 68.8, 52.5, 44.4, 37.1, 33.2, 32.4, 31.1, 31.0, 26.0 (3C), 25.9 (3C), 19.6, 18.3, 18.16, 18.15, 4.1, -4.5, -4.78, -4.84.

**Synthesis of (16)**

methyl 2-(4-(((*R*)-2-(((*R*)-4-(((2*R*,3*R*,5*R*,6*S*)-3,5-dihydroxy-6-methyltetrahydro-2*H*-pyran-2-yl)oxy)pentanoyl)oxy)-2-phenylethyl)amino)-4-oxobutanamido)benzoate



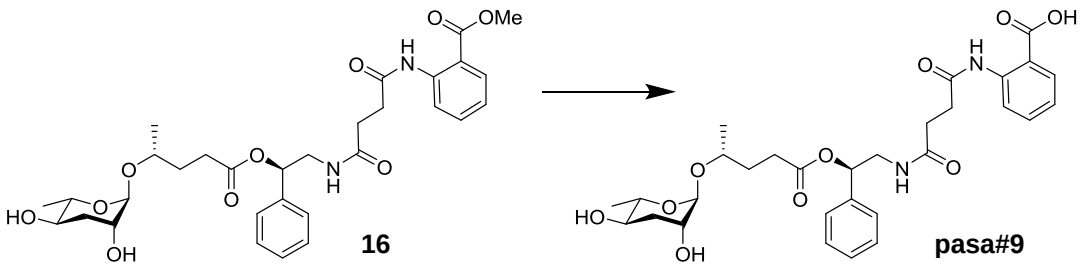

A solution of **4** (19 mg, 23 μmol) in acetonitrile (1 mL) was treated with 40% HF (3 drops) at 0 °C and allowed to stir. After 2 h, the reaction was taken to r.t. and allowed to stir for 1 h. After stirring, the reaction was neutralized with sodium bicarbonate and quickly acidified with acetic acid and concentrated *in vacuo*. Flash column chromatography on silica using a gradient of 0-20% methanol in dichloromethane afforded **16** (12.5 mg, 21 μmol, 91%).

$^{1}$H NMR (500 MHz, methanol-$d_4$): δ (ppm) 8.53-8.46 (m, 1H), 8.07-8.01 (m, 1H), 7.59-7.51 (m, 1H), 7.40-7.23 (m, 5H), 7.18-7.12 (m, 1H), 5.84 (dd, $J$ = 8.3, 4.4 Hz, 1H), 4.63 (s, 1H), 3.94 (s, 3H), 3.81-3.73 (m, 1H), 3.72-3.68 (m, 1H), 3.62-3.43 (m, 4H), 2.76-2.68 (m, 2H), 2.62-2.44 (m, 4H), 1.98-1.91 (m, 1H), 1.85-1.72 (m, 3H), 1.19 (d, $J$ = 6.0 Hz, 3H), 1.11 (d, $J$ = 6.1 Hz, 3H).

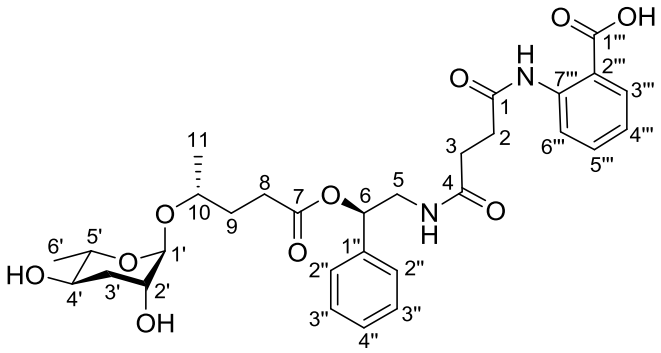
$^{13}$C NMR (125 MHz, methanol-$d_4$) δ 174.7, 174.3, 172.7, 169.7, 141.9, 139.7, 135.3, 132.0, 129.6 (2C), 129.3, 127.5 (2C), 124.1, 121.9, 117.5, 97.4, 75.7, 71.6, 71.3, 69.9, 68.3, 53.0, 45.4, 36.0, 33.9, 33.3, 31.60, 31.59, 19.1, 18.1.

**Synthesis of pasa#9**

2-(4-(((*R*)-2-(((*R*)-4-(((2*R*,3*R*,5*R*,6*S*)-3,5-dihydroxy-6-methyltetrahydro-2*H*-pyran-2-yl)oxy)pentanoyl)oxy)-2-phenylethyl)amino)-4-oxobutanamido)benzoic acid




A solution of **16** (12.5 mg, 21 μmol) in THF (300 μL) was treated with LiOH (400 μg, 17 μmol) in $H_2O$:dioxane (3:7, 600μL) at 40 °C and allowed stir. After stirring for 1 h, the reaction was treated with an additional LiOH (200 μg, 8 μmol) in $H_2O$:dioxane (3:7, 300μL), and the reaction was stirred for another 1 h. After stirring, the reaction was quenched with acetic acid and concentrated *in vacuo*. HPLC afforded **pasa#9** (3.2 mg, 5.5 μmol, 26%). $\alpha_D^{20}$ = -66.7 (c, 0.23, methanol). For NMR spectroscopic data, see next page.

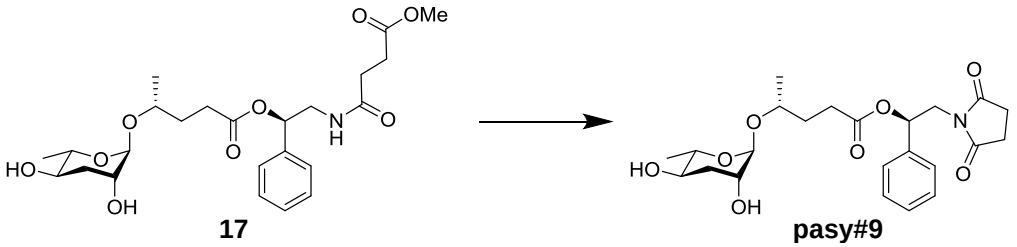


NMR Spectroscopic data for **pasa#9**. $^{1}$H (600 MHz), $^{13}$C (151 MHz), and HMBC NMR spectroscopic data for **pasa#9** in methanol-$d_4$. Chemical shifts were referenced to ($CD_2\underline{H}OD$) = 3.31 ppm and ($\underline{C}D_3OD$) = 49.00 ppm.

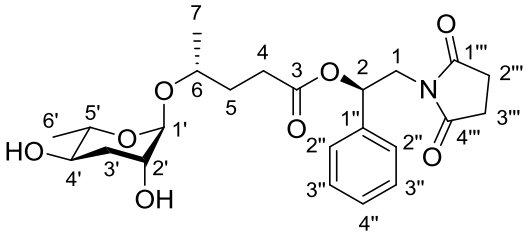
| Position | $^{13}$C [ppm] | $^{1}$H [ppm] | $^{1}$H-$^{1}$H coupling constants (Hz) | Key HMBC correlations |
|---|---|---|---|---|
| 1 | 172.3 | --- | | |
| 2 | 33.7 | 2.72 | $J_{2,3}$ = 7.0 | C-1 |
| 3 | 31.4 | 2.57 | | C-4 |
| 4 | 174.5 | --- | | |
| 5 | 45.1 | 5a = 3.51<br>5b = 3.59 | $J_{5a,5b}$ = 14.2, $J_{5a,6}$ = 8.5,<br>$J_{5b,6}$ = 4.4 | C-4 |
| 6 | 75.5 | 5.84 | | C-7, C-1” |
| 7 | 174.0 | --- | | |
| 8 | 31.3 | 2.52 | | C-7 |
| 9 | 33.0 | 1.79 | | |
| 10 | 71.3 | 3.77 | $J_{10,11}$ = 6.0 | C-1’ |
| 11 | 18.9 | 1.11 | | |
| 1’ | 97.1 | 4.64 | $J_{1',2'}$ = 2.3 | C-2’, C-3’, C-5’ |
| 2’ | 69.6 | 3.71 | $J_{2',3'\,(ax)}$ = 6.5, $J_{2',3'\,(eq)}$ = 6.0 | |
| 3’ | 35.6 | 1.76(ax)<br>1.96(eq) | $J_{3'\,(ax),3'\,(eq)}$ = 13.0, $J_{3'\,(ax),4'}$ = 12.2<br>$J_{3'\,(eq),4'}$ = 4.6 | |
| 4’ | 68.0 | 3.51 | $J_{4',5'}$ = 12.1 | |
| 5’ | 71.0 | 3.58 | $J_{5',6'}$ = 6.3 | |
| 6’ | 17.9 | 1.19 | | |
| 1” | 139.4 | --- | | |
| 2” | 127.3 | 7.36 | | C-6, C-4” |
| 3” | 129.3 | 7.34 | | |
| 4” | 129.0 | 7.28 | | |
| 1”’ | 171.4 | --- | | |
| 2”’ | 117.8 | --- | | |
| 3”’ | 132.3 | 8.08 | $J_{3''',4'''}$ = 7.9 | C-1”’, C-2”’, C-7”’ |
| 4”’ | 123.5 | 7.12 | $J_{4''',5'''}$ = 6.9 | C-1”’ |
| 5”’ | 134.5 | 7.53 | $J_{5''',6'''}$ = 8.5 | |
| 6”’ | 121.0 | 8.56 | | C-1”’, C-2”’, C-7”’ |
| 7”’ | 142.1 | --- | | |

**4.2 Synthesis of pasy#9**

(*R*)-2-(2,5-dioxopyrrolidin-1-yl)-1-phenylethyl (*R*)-4-(((2*R*,3*R*,5*R*,6*S*)-3,5-dihydroxy-6-methyltetrahydro-2*H*-pyran-2-yl)oxy)pentanoate



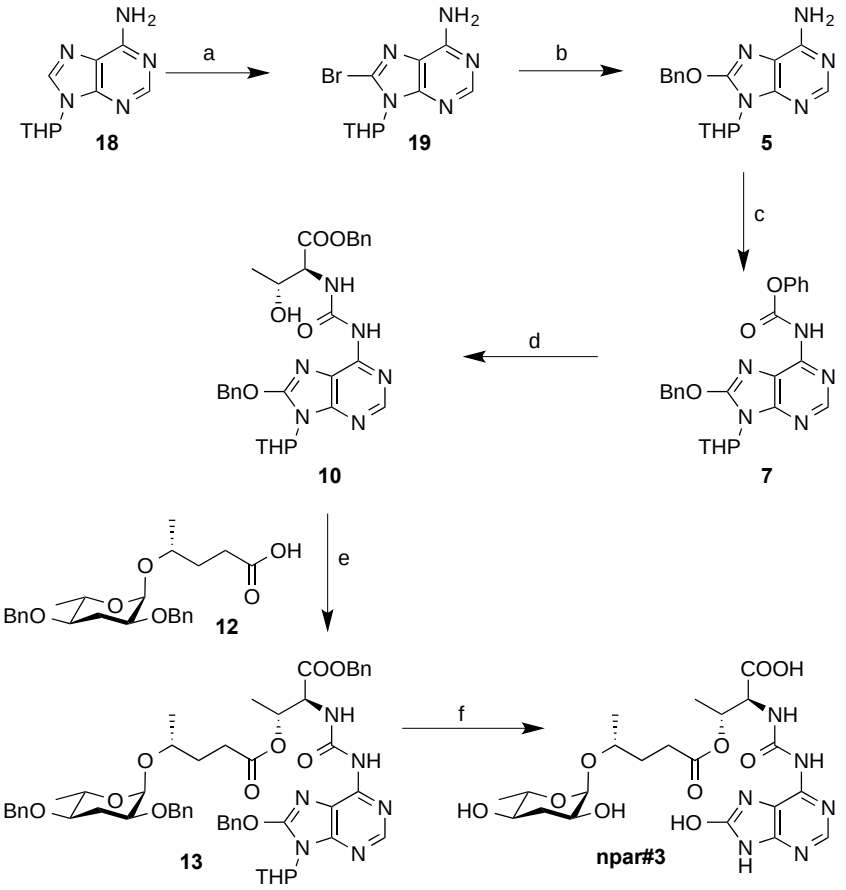

A solution of **17** (8.5 mg, 18 μmol) in THF (250 μL) was treated with LiOH (340 μg, 14 μmol) in $H_2O$:dioxane (3:7, 500μL) at 40 °C and allowed stir. After stirring for 4 min, the reaction was quenched with acetic acid and concentrated *in vacuo*. HPLC afforded **pasy#9** (1.7 mg, 3.8 μmol, 21%) and **pasc#9** (3.0 mg, 6.4 μmol, 36%). $\alpha_D^{20}$ = -86.0 (*c*. 0.1, methanol). For NMR spectroscopic data, see next page.




NMR Spectroscopic data for **pasy#9**. $^{1}$H (600 MHz), $^{13}$C (151 MHz), and HMBC NMR spectroscopic data for **pasy#9** in methanol-$d_4$. Chemical shifts were referenced to ($CD_2\underline{H}OD$) = 3.31 ppm and ($\underline{C}D_3OD$) = 49.00 ppm.

| Position | $^{13}$C [ppm] | $^{1}$H [ppm] | $^{1}$H-$^{1}$H coupling constants (Hz) | Key HMBC correlations |
|---|---|---|---|---|
| 1 | 44.3 | 5a = 3.98<br>5b = 3.70 | $J_{5a,5b}$ = 14.0, $J_{5a,6}$ = 9.2,<br>$J_{5b,6}$ = 3.8 | C-1''' |
| 2 | 73.8 | 6.02 | | C-3, C-1" |
| 3 | 174.2 | --- | | |
| 4 | 31.3 | 2.43<br>2.50 | | C-3 |
| 5 | 33.0 | 1.78~1.79 | | |
| 6 | 71.2 | 3.79 | $J_{6,7}$ = 6.0 | C-1' |
| 7 | 18.7 | 1.13 | | |
| 1' | 97.1 | 4.63 | $J_{1',2'}$ = 2.5 | C-2', C-3', C-5' |
| 2' | 69.6 | 3.70 | $J_{2',3'\,(eq)}$ = 6.5, $J_{2',3'\,(ax)}$ = 6.1 | |
| 3' | 35.7 | 1.75(ax)<br>1.94(eq) | $J_{3'\,(ax),3'\,(eq)}$ = 13.0, $J_{3'\,(ax),4'}$ = 11.5<br>$J_{3'\,(eq),4'}$ = 4.5 | |
| 4' | 68.0 | 3.50 | $J_{4',5'}$ = 9.5 | |
| 5' | 71.1 | 3.55 | $J_{5',6'}$ = 6.1 | |
| 6' | 17.9 | 1.19 | | |
| 1" | 138.4 | --- | | |
| 2" | 127.3 | 7.41 | | C-2, C-1", C-4" |
| 3" | 129.6 | 7.39 | | |
| 4" | 129.5 | 7.33 | | |
| 1''' | 179.3 | --- | | |
| 2''' | 28.7 | 2.68~2.66 | | |
| 3''' | 28.7 | 2.68~2.66 | | |
| 4''' | 179.3 | --- | | |

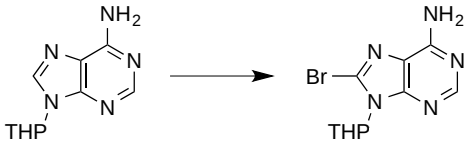
### 4.3 Synthesis of natural npar#3 (8-oxo)




**Synthetic Scheme S2. Overview of synthesis of npar#3.** Reagents and conditions: **(a)** $Br_2$, acetate buffer, THF, MeOH, r.t.; **(b)** BnOH, NaH, DMF, 55 °C; **(c)** phenyl chloroformate, N-methyl imadazole, DCM, toluene, 0 °C to r.t.;[4] **(d)** L-threonine benzyl ester, pyridine, 35 °C;[5] **(e)** EDC, DMAP, DCM; **(f)** TFA, anisole and 10% Pd/C, $H_2$ (*g*) 10% formic acid in MeOH.

**Synthesis of (19)**

8-bromo-9-(tetrahydro-2*H*-pyran-2-yl)-9*H*-purin-6-amine



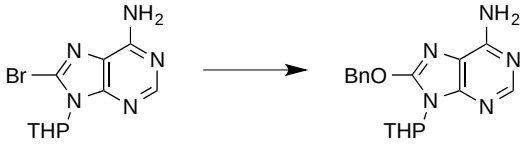

A solution of **18** (86 mg, 390 μmol) in NaOAc buffer (pH =5.2, 2 mL), methanol (2 mL), and THF (2 mL) was treated with $Br_2$ (42 μL, 790 μmol) and stirred at r.t. After stirring for 3 h, the reaction was concentrated to 1/3 its initial volume. The reaction was extracted with ethyl acetate, the organic layer was washed with water and subsequently washed with brine. The organic layer was dried over $Na_2SO_4$ and concentrated *in vacuo*. Flash column chromatography on silica using a gradient of 0-15% methanol in dichloromethane afforded **19** (103 mg, 344 μmol, 88%).

$^1$H NMR (600 MHz, methanol-$d_4$): δ (ppm) 8.15 (s, 1H), 5.69 (dd, $J$ = 11.3 Hz, $J$ = 2.5 Hz, 1H), 4.15-4.08 (m, 1H), 3.75-3.68 (m, 1H), 3.00-2.90 (m, 1H), 2.09-2.02 (m, 1H), 1.88-1.68 (m, 3H), 1.61-1.53 (m, 1H).

$^{13}$C NMR (151 MHz, methanol-$d_4$): δ (ppm) 156.2, 153.7, 151.9, 127.6, 120.8, 86.3, 70.1, 29.9, 25.8, 24.2.

**Synthesis of (5)**

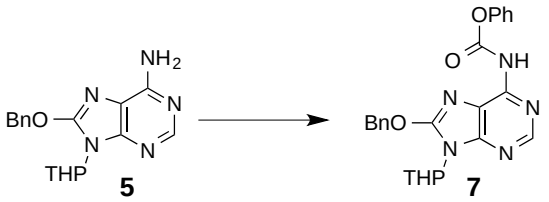
8-(benzyloxy)-9-(tetrahydro-2*H*-pyran-2-yl)-9*H*-purin-6-amine




A solution of **19** (376 mg, 1.26 mmol) in DMF (3 mL) was added to a stirring solution of benzyl alcohol (2.74 mL, 26.5 mmol) and NaH (110 mg, 4.5 mmol) in DMF (9 mL). The mixture was then heated to 55 °C and allowed to stir for 2.5 h. After stirring, the reaction was quenched with acetic acid and concentrated *in vacuo*. Flash column chromatography on silica using a gradient of 0-10% methanol in dichloromethane afforded **5** (295 mg, 910 μmol, 72%).

$^1$H NMR (400 MHz, methanol-$d_4$): δ (ppm) 8.09 (s, 1H), 7.55-7.48 (m, 2H), 7.45-7.31 (m, 3H), 5.63-5.52 (m, 3H), 4.12-4.03 (m, 1H), 3.75-3.62 (m, 1H), 2.80-2.65 (m, 1H), 2.08-1.94 (m, 1H), 1.83-1.49 (m, 4H).

**Synthesis of (7)**
phenyl (8-(benzyloxy)-9-(tetrahydro-2*H*-pyran-2-yl)-9*H*-purin-6-yl)carbamate



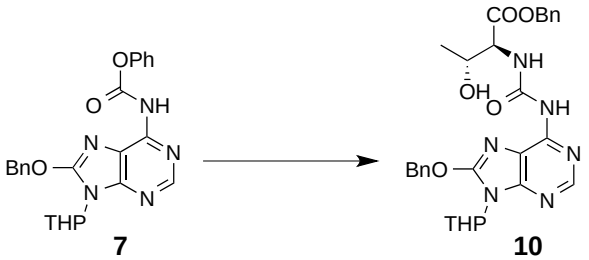

A solution of **5** (150 mg, 460 μmol) in dichloromethane : toluene (9:1, v/v, 10 mL) was treated with phenyl chloroformate (230 μL, 1.84 mmol) and N-methyl imidazole (300 μL, 3.76 mmol) at 0 °C and allowed to gradually warm up to r.t. After stirring for 20 h, the reaction was concentrated *in vacuo*. Flash column chromatography on silica using a gradient of 10-80% acetone in dichloromethane afforded **7** (36 mg, 81 μmol, 17%).

$^{1}$H NMR (600 MHz, chloroform-*d*): δ (ppm) 8.69 (s, 1H), 8.27 (s, 1H), 7.53-7.47 (m, 2H), 7.46-7.37 (m, 5H), 7.29-7.23 (m, 3H), 5.65-5.58 (m, 3H), 4.19-4.12 (m, 1H), 3.74-3.66 (m,1H), 2.91-2.80 (m, 1H), 2.10-2.02 (m, 1H), 1.85-1.55 (m, 4H).

$^{13}$C NMR (125 MHz, chloroform-*d*): δ (ppm) 156.2, 151.9, 150.9, 150.5, 149.4, 145.8, 134.9, 129.6, 129.5, 128.9, 128.8, 128.3, 125.9, 121.6, 81.9, 72.5, 69.2, 28.8, 24.9, 23.4.

**Synthesis of (10)**

benzyl ((8-(benzyloxy)-9-(tetrahydro-2*H*-pyran-2-yl)-9*H*-purin-6-yl)carbamoyl)-*L*-threoninate



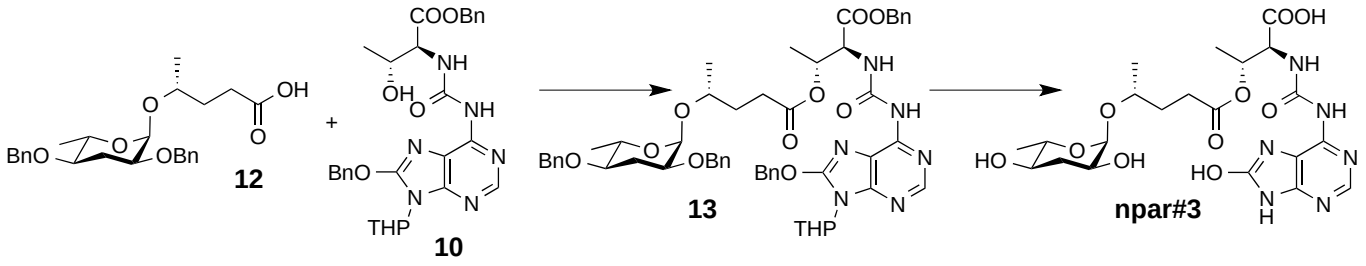

A solution of **7** (17.5 mg, 39 μmol) in pyridine (700 μL) was treated with L-threonine benzyl ester **9** (25 mg, 120 μmol) and stirred at 35 °C. After stirring for 9 h, the reaction was coevaporated *in vacuo* with toluene 5 times to ensure all pyridine was evaporated. Flash column chromatography on silica using a gradient of 0-40% acetone in dichloromethane afforded **10** (10 mg, 17 μmol, 44%).

$^{1}$H NMR (500 MHz, chloroform-*d*): δ (ppm) 10.14 (d, *J* = 8.5 Hz, 1H), 8.44-8.41 (m, 1H), 7.59 (s, 1H), 7.53-7.47 (m, 2H), 7.45-7.29 (m, 8H), 5.61-5.54 (m, 3H), 5.28-5.22 (m, 2H), 4.73 (dd, *J* = 8.5 Hz, *J* = 2.9 Hz, 1H), 4.48-4.41 (m, 1H), 4.19-4.12 (m, 1H), 3.73-3.66 (m, 1H), 2.87-2.76 (m, 1H), 2.27-2.21 (m, 1H), 2.10-2.02 (m, 1H), 1.83-1.58 (m, 3H), 1.33-1.29 (m, 3H).

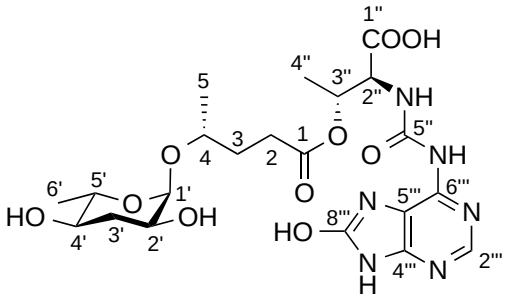
$^{13}$C NMR (125 MHz, chloroform-*d*): δ (ppm) 171.0, 155.9, 154.7, 150.8, 149.6, 147.2, 135.6, 135.2, 128.9, 128.85 (2C), 128.77 (2C), 128.53, 128.49, 128.48, 128.26, 128.25, 117.3, 82.0, 72.6, 69.2, 68.63 (0.5C), 68.62 (0.5C), 67.4, 59.08 (0.5C), 59.06 (0.5C), 28.95, 24.99, 23.5, 20.17 (0.5C), 20.16 (0.5C).

**Synthesis of npar#3**

*O*-((*R*)-4-(((2*R*,3*S*,5*R*,6*S*)-3,5-dihydroxy-6-methyltetrahydro-2*H*-pyran-2-yl)oxy)pentanoyl)-*N*-((8-hydroxy-9*H*-purin-6-yl)carbamoyl)-*L*-threonine



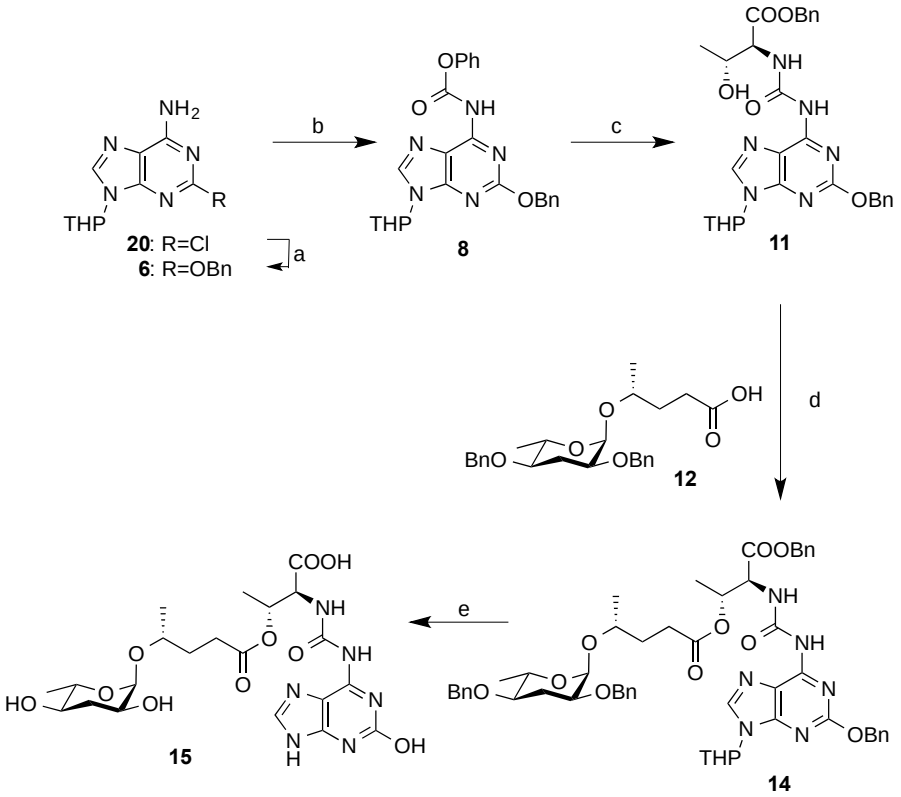

A solution of **12**[3] (2.6 mg, 6.1 μmol) in 500 μL dry dichloromethane was treated with 4-dimethylaminopyridine (1.9 mg, 15.2 μmol) and EDC hydrochloride (2.9 mg, 15.2 μmol). After stirring for 20 minutes, **10** (3.75 mg, 6.7 μmol) in 500 μL dry dichloromethane was added to the mixture. After stirring for 15 h, the reaction was concentrated *in vacuo*. Flash column chromatography on silica using a gradient of 20-100% ethyl acetate in hexanes afforded mixture of undesired side products and product **13** (4.6 mg). This mixture was used without further purification in the next step. To a solution of TFA (150 μL) in anisole (200 μL), 1010 was added and stirred for 4 h, monitoring by ESI-MS (positive mode) showed THP had fallen off. This reaction mixture was then concentrated in vacuo. A solution of Pd/C (3 mg, 10%, *w*/*w*) in 500 μL of methanol containing 10% formic acid was first flushed with argon for 5 minutes and subsequently with a moderate flow of $H_2$ gas. To this stirring solution was added a solution of the crude mixture from the THP deprotection in 500 μL methanol containing 10% formic acid. After 1 h, the reaction mixture was filtered over a pad of silica to remove Pd/C catalyst. This mixture was then concentrated *in vacuo* and subjected to HPLC purification (see Methods), affording **npar#3** (200 μg, 0.4 μmol, 6.5%, over two steps). $\alpha_D^{20}$ = -2.2 (*c*. 0.09, methanol). For NMR spectroscopic data, see next page.




NMR Spectroscopic data for **npar#3**. $^{1}$H (600 MHz), $^{13}$C (151 MHz), and HMBC NMR spectroscopic data for **npar#3** in methanol-$d_4$. Chemical shifts were referenced to ($CD_2\underline{H}OD$) = 3.31 ppm and ($\underline{C}D_3OD$) = 49.00 ppm.

| Position | $^{13}$C [ppm] | $^{1}$H [ppm] | $^{1}$H-$^{1}$H coupling constants (Hz) | Key HMBC correlations |
|---|---|---|---|---|
| 1 | 174.1 | --- | | |
| 2 | 31.7 | 2.53, 2.45 | | C-1 |
| 3 | 33.3 | 1.80-1.93 | | |
| 4 | 72.6 | 3.81 | $J_{4, 5}$ = 6.2 | C-1’ |
| 5 | 19.0 | 1.15 | | |
| 1’ | 96.3 | 4.75 | $J_{1', 2'}$ = 3.9 | C-3’, C-5’ |
| 2’ | 68.5 | 3.61 | $J_{2', 3' (ax)}$ = 12.1, $J_{2', 3' (eq)}$ = 6.3 | |
| 3’ | 37.0 | 1.73 (ax)<br>2.02 (eq) | $J_{3' (ax), 3' (eq)}$ = 12.3, $J_{3' (ax), 4'}$ = 11.6<br>$J_{3' (eq), 4'}$ = 4.6 | C-2’ |
| 4’ | 71.8 | 3.16 | $J_{4', 5'}$ = 9.7 | |
| 5’ | 70.1 | 3.55 | $J_{5', 6'}$ = 6.3 | |
| 6’ | 17.6 | 1.19 | | C-5’ |
| 1” | 176.3 | --- | | |
| 2” | 59.6 | 4.48 | $J_{2", 3"}$ = 6.6 | |
| 3” | 72.7 | 5.51 | $J_{3", 4"}$ = 6.3 | |
| 4” | 17.6 | 1.33 | | C-2”, C-3” |
| 5” | 156.1 or 156.2 | --- | | |
| 2”’ | 151.4 | 8.30 | | C-6”’, C-4”’ or C-5”’ |
| 4”’ | 141.2 or 141.5 | --- | | |
| 5”’ | 141.2 or 141.5 | --- | | |
| 6”’ | 151.4 | --- | | |
| 8”’ | 156.1 or 156.2 | --- | | |

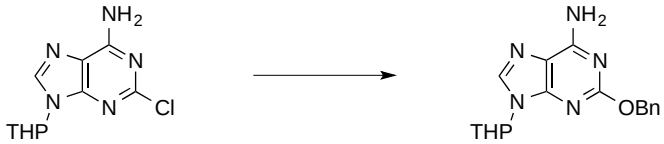
### 4.4 Synthesis of npar#3-isomer (2-oxo) (15)




**Synthetic Scheme S3. Overview of synthesis of npar#3-isomer (2-oxo) (15).** Reagents and conditions: **(a)** BnOH, NaOH, r.t.; **(b)** phenyl chloroformate, N-methyl imadazole, DCM, toluene, 0 °C to r.t.;[4] **(c)** L-threonine benzyl ester, pyridine, 35 °C;[5] **(d)** EDC, DMAP, DCM; **(e)** 10% Pd/C, $H_2$ (*g*) 10% formic acid in MeOH, 2M HCl.

**Synthesis of (6)**

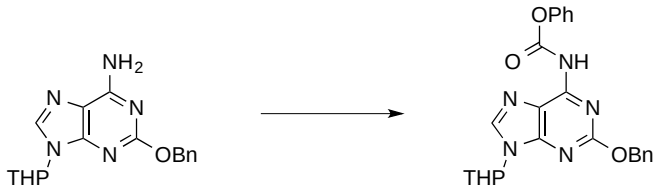
2-(benzyloxy)-9-(tetrahydro-2*H*-pyran-2-yl)-9*H*-purin-6-amine




A solution of **20** (120 mg, 0.47 mmol) in BnOH (2 mL) was stirred under argon atmosphere. To this reaction, NaOH (184mg, 4.6 mmol) was added and the reaction was left to stir for 10 h. Upon stirring for 10 h, reaction was heated to 80 °C and allowed to stir overnight under argon. After stirring, the reaction was concentrated *in vacuo*. Flash column chromatography on silica using a gradient of 0-10% methanol in dichloromethane afforded **6** (107 mg, 0.33 mmol, 70%).

$^1$H NMR (600 MHz, DMSO-$d_6$): δ (ppm) 8.13 (s, 1H), 7.48-7.45 (m, 2H), 7.39-7.35 (m, 2H), 7.34-7.29 (m, 3H), 5.50 (dd, $J$ = 11.0, 2.3 Hz, 1H), 5.35–5.27 (m, 2H), 4.02-3.97 (m, 1H), 3.70-3.62 (m, 1H), 2.26-2.19 (m, 1H), 1.98-1.92 (m, 1H), 1.91-1.86 (m, 1H), 1.76-1.66 (m, 1H), 1.60-1.53 (m, 2H).

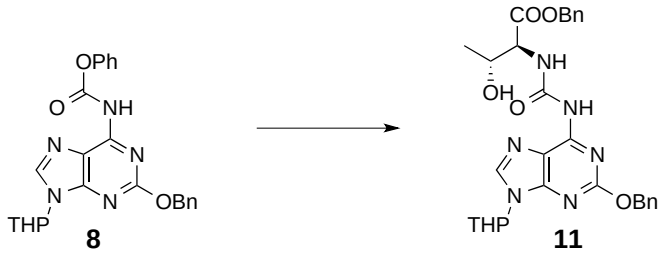
**Synthesis of (8)**
phenyl (2-(benzyloxy)-9-(tetrahydro-2*H*-pyran-2-yl)-9*H*-purin-6-yl)carbamate




A solution of **6** (22 mg, 67 μmol) in dichloromethane (1 mL) was treated with phenyl chloroformate (34 μL, 270 μmol) and N-methyl imidazole (44 μL, 550 μmol) at 0 °C under argon and allowed to gradually warm up to r.t. After stirring for 17 h, the reaction was concentrated *in vacuo*. Flash column chromatography on silica using a gradient of 10-40% ethyl acetate in hexanes afforded **8** (4.6 mg, 10 μmol, 15%) and recovered **6** (11 mg, 34 μmol, 51%).

$^1$H NMR (500 MHz, chloroform-*d*): δ (ppm) 9.05 (broad s, 1H), 8.05 (s, 1H), 7.54-7.49 (m, 2H), 7.44, 7.38 (m, 2H), 7.35-7.22 (m, 6H), 5.70 (dd, *J* = 10.8, 2.4 Hz, 1H), 5.51, (s, 2H), 4.19-4.12 (m, 1H), 3.79-3.70 (m, 1H), 2.08-2.02 (m, 1H), 1.98-1.87 (m, 1H), 1.81-1.60 (m, 4H).

**Synthesis of (11)**
benzyl ((2-(benzyloxy)-9-(tetrahydro-2*H*-pyran-2-yl)-9*H*-purin-6-yl)carbamoyl)-*L*-threoninate



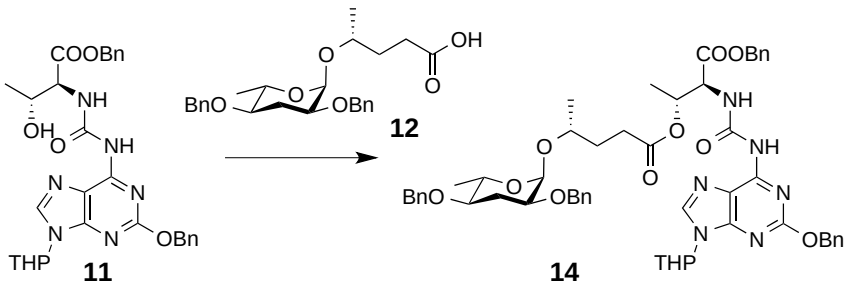

A solution of **8** (4.6 mg, 10 μmol) in pyridine (500 μL) was treated with L-threonine benzyl ester **9** (6.5 mg, 31 μmol) and heated to 35 °C with stirring. After stirring for 4 days, the reaction was concentrated *in vacuo* and the residue was co-evaporated with toluene (3 × 1 mL). Flash column chromatography on silica using a gradient of 0-10% methanol in dichloromethane afforded **11** (3.1 mg, 6 μmol, 54%).

$^{1}$H NMR (500 MHz, methanol-$d_4$): δ (ppm) 8.28 (s, 1H), 7.50-7.45 (m, 2H), 7.40-7.23 (m, 8H), 5.66 (dd, $J$ = 10.8, 2.2 Hz, 1H), 5.52-5.44 (m, 2H), 5.21 (s, 2H), 4.66-4.62 (m, 1H), 4.48-4.41 (m, 1H), 4.14-4.08 (m, 1H), 3.84-3.76 (m, 1H), 2.23-2.13 (m, 1H), 2.10-2.03 (m, 2H), 1.87-1.68 (m, 2H), 1.67-1.60 (m, 1H), 1.28 (dd, $J$ = 6.4, 1.5 Hz, 3H).

$^{13}$C NMR (125 MHz, methanol-$d_4$): δ (ppm) 172.33 (0.5C), 172.31 (0.5C), 161.8, 156.30 (0.5C), 156.29 (0.5C), 153.4, 152.1, 141.44 (0.5C), 141.42 (0.5C), 137.9, 137.20 (0.5C), 137.18 (0.5C), 129.5 (2C), 129.4 (2C), 129.20 (0.5C), 129.19 (0.5C), 129.1 (2C), 129.0 (2C), 128.9, 117.2, 83.22 (0.5C), 83.18 (0.5C), 71.7, 69.7, 68.55 (0.5C), 68.53 (0.5C), 68.14, 60.5, 32.0, 26.0, 23.9, 20.8

**Synthesis of (14)**

(2*R*,3*S*)-4-(benzyloxy)-3-(3-(2-(benzyloxy)-9-(tetrahydro-2*H*-pyran-2-yl)-9*H*-purin-6-yl)ureido)-4-oxobutan-2-yl (4*R*)-4-(((2*R*,3*S*,5*R*,6*S*)-3,5-bis(benzyloxy)-6-methyltetrahydro-2*H*-pyran-2-yl)oxy)pentanoate




A solution of **12**[3] (1.5 mg, 3.6 μmol) in 300 μL dry dichloromethane was treated with 4-dimethylaminopyridine (1.3 mg, 11 μmol) and EDC hydrochloride (1.9 mg, 10 μmol). After stirring for 10 minutes, **11** (2.0 mg, 3.6 μmol) in 500 μL dry dichloromethane was added to the mixture. After stirring for 24 h, the reaction was concentrated *in vacuo*. Flash column chromatography on silica using a gradient of 0-15% isopropanol in dichloromethane afforded **14** (2.5 mg, 2.6 μmol, 72%).

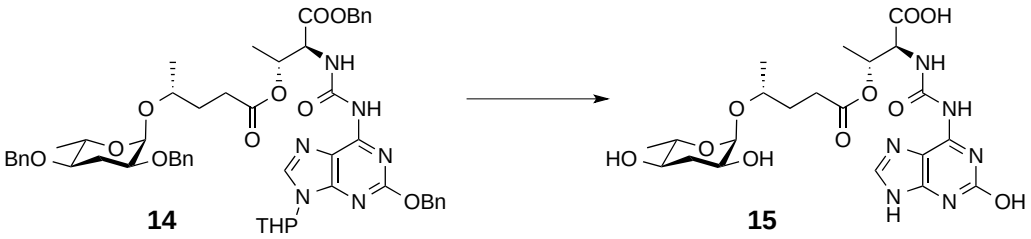
$^{1}$H NMR (600 MHz, methanol-$d_4$): δ (ppm) 8.25 (m, 1H), 7.47 (m, 2H), 7.38-7.21 (m, 18H), 5.66 (m, 1H), 5.53 (m, 1H), 5.52 (m, 1H), 5.43 (d, $J$ = 11.9Hz, 1H), 5.24 (dd, $J$ = 4.1, 12.2 Hz, 1H), 5.10 (d, $J$ = 12.2 Hz, 1H), 4.83, (m, 1H), 4.75 (m, 1H), 4.56 (dd, $J$ = 2.2, 11.6 Hz, 1H), 4.52 (s, 2H), 4.40 (dd, $J$ = 2.2, 11.6 Hz, 1H), 4.10 (m, 1H), 3.78 (m, 1H), 3.57 (m, 1H), 3.49 (m, 1H), 3.43 (m, 1H), 2.99, (m, 1H), 2.25 (m, 1H), 2.18 (m, 1H), 2.11 (m, 1H), 2.05 (m, 2H), 2.00 (m, 1H), 1.76 (m, 2H), 1.66 (m, 1H), 1.63 (m, 2H), 1.52 (m, 1H), 1.33 (d, $J$ = 6.5 Hz, 3H), 1.07 (t, $J$ = 6.2 Hz, 3H), 0.96 (d, $J$ = 6.4 Hz, 3H).

$^{13}$C NMR (151 MHz, methanol-$d_4$): δ 173.5, 170.8, 161.3, 155.90, 152.9, 141.0, 139.5, 139.4, 137.6, 136.7, 129.3-128.5 (20 C), 116.9, 94.3, 83.0, 78.8, 75.4, 72.9, 71.6, 71.5, 71.4, 70.9, 69.3, 68.4, 68.2, 57.9, 32.5, 31.0, 30.9, 30.8, 25.8, 23.4, 19.2, 17.8, 17.2.

C-6 adenine carbon shift not observed.

**Synthesis of npar#3-isomer (2-oxo) (15)**

*O*-((4*R*)-4-(((2*R*,3*S*,5*R*,6*S*)-3,5-dihydroxy-6-methyltetrahydro-2*H*-pyran-2-yl)oxy)pentanoyl)-*N*-((2-hydroxy-9*H*-purin-6-yl)carbamoyl)-*L*-threonine

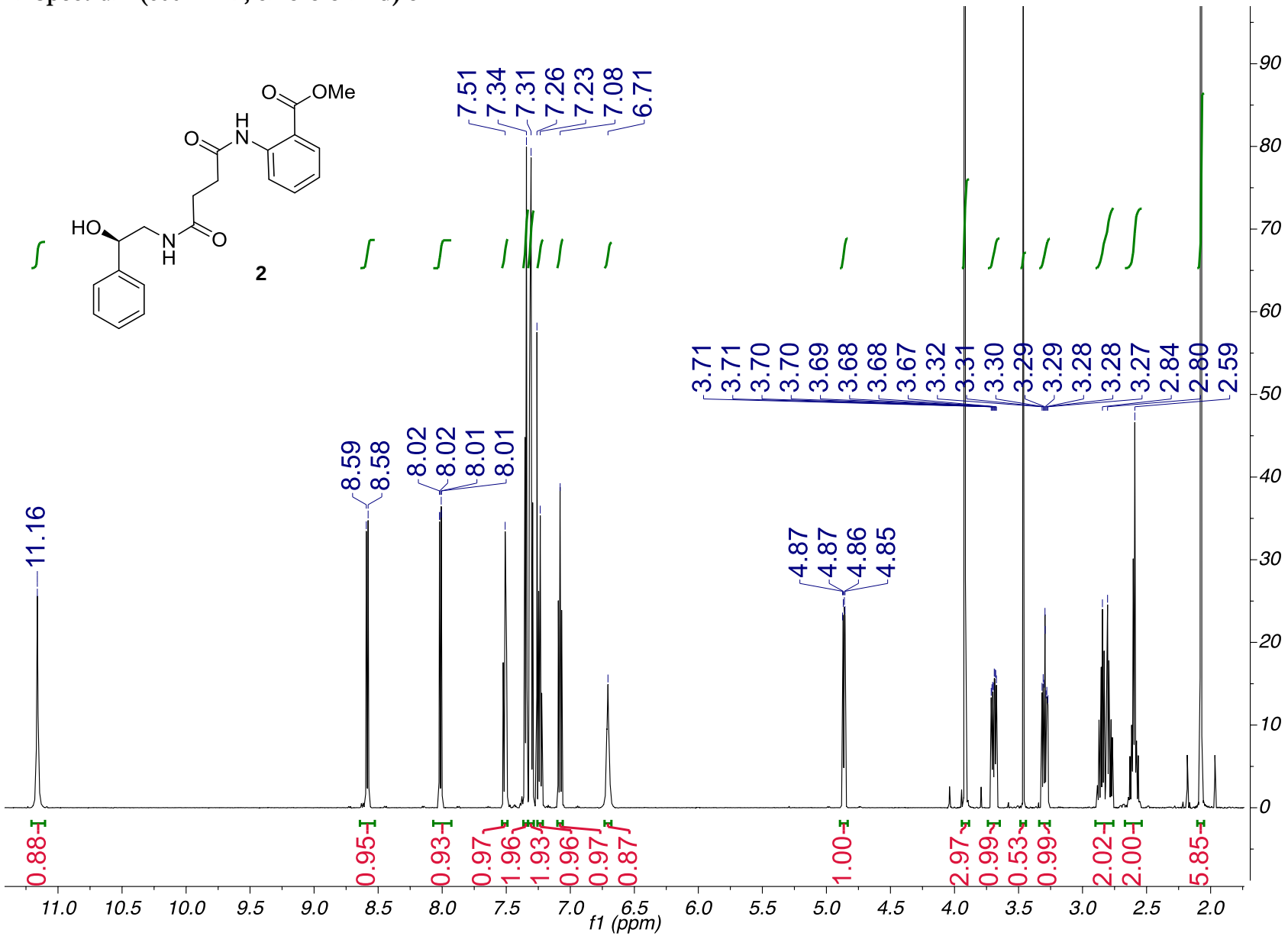



A solution of Pd/C (2.7 mg, 10%, *w*/*w*) in 500 μL of methanol containing 10% formic acid was first flushed with argon for 5 minutes and subsequently with a moderate flow of $H_2$ gas. To this stirring solution was added a solution of **14** (2.5 mg, 2.6 μmol) in 500 μL methanol containing 10% formic acid. After 3 h, 200 μL of 2 M HCl was added to the reaction. After stirring for an additional 17 h, the reaction mixture was filtered over a pad of silica to remove Pd/C catalyst and quenched with sodium formate (27 mg, 400 μmol) in 200 μL water. This mixture was then concentrated *in vacuo* and re-suspended in dichloromethane : methanol (3:1) and filtered over a pad of silica to remove excess salts. Concentration *in vacuo* and HPLC purification (see Methods) afforded **npar#3-isomer (2-oxo) (15)** (300 ug, 0.6 μmol, 22%).

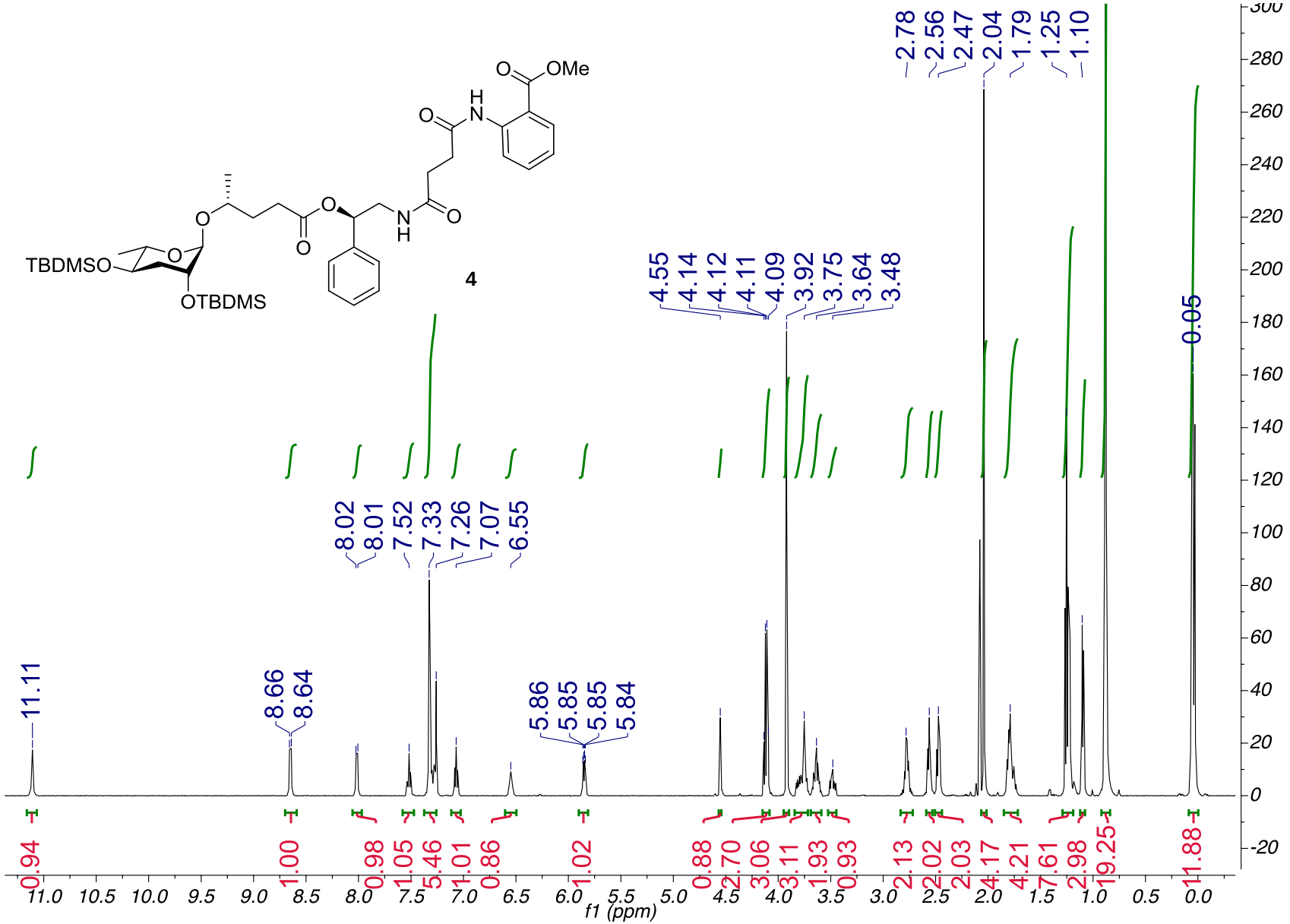
$^1$H NMR (600 MHz, methanol-$d_4$): δ (ppm) 7.94 (broad s, 1H), 5.50-5.45 (m, 1H), 4.75-4.70 (m, 1H), 4.44-4.39 (m, 1H), 3.82-3.78 (m, 1H), 3.60-3.52 (m, 2H), 3.17-3.11 (m, 1H), 2.54-2.41 (m, 2H), 2.03-1.99 (m, 1H), 1.91-1.78 (m, 2H), 1.76-1.69 (m, 1H), 1.32 (d, $J$ = 6.4 Hz, 3H), 1.18 (d, $J$ = 6.3 Hz, 3H), 1.14 (d, $J$ = 6.1 Hz, 3H).

**5. NMR Spectra of Synthetic Compounds**

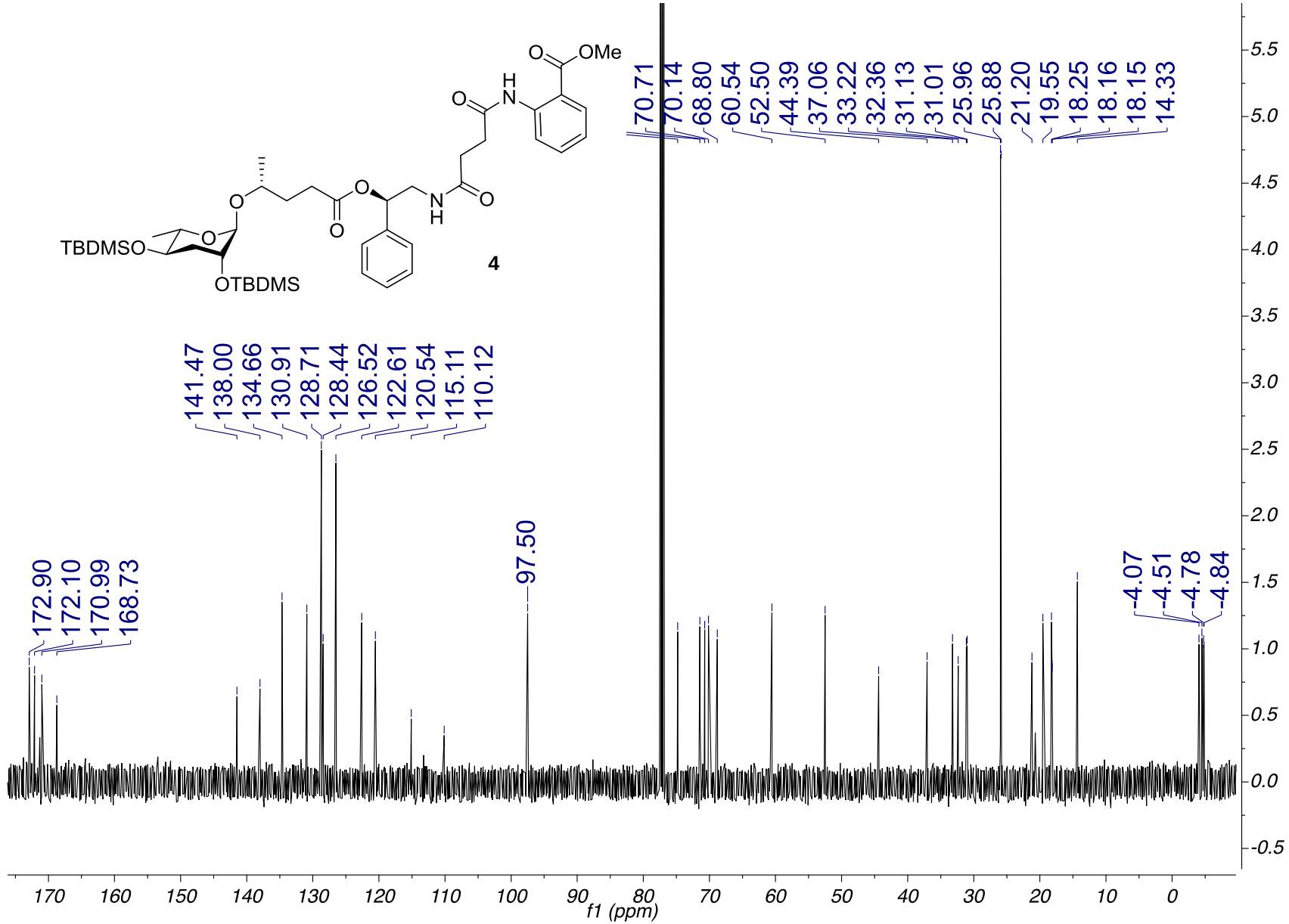
**$^1$H NMR Spectrum (600 MHz, chloroform-*d*) of 2**



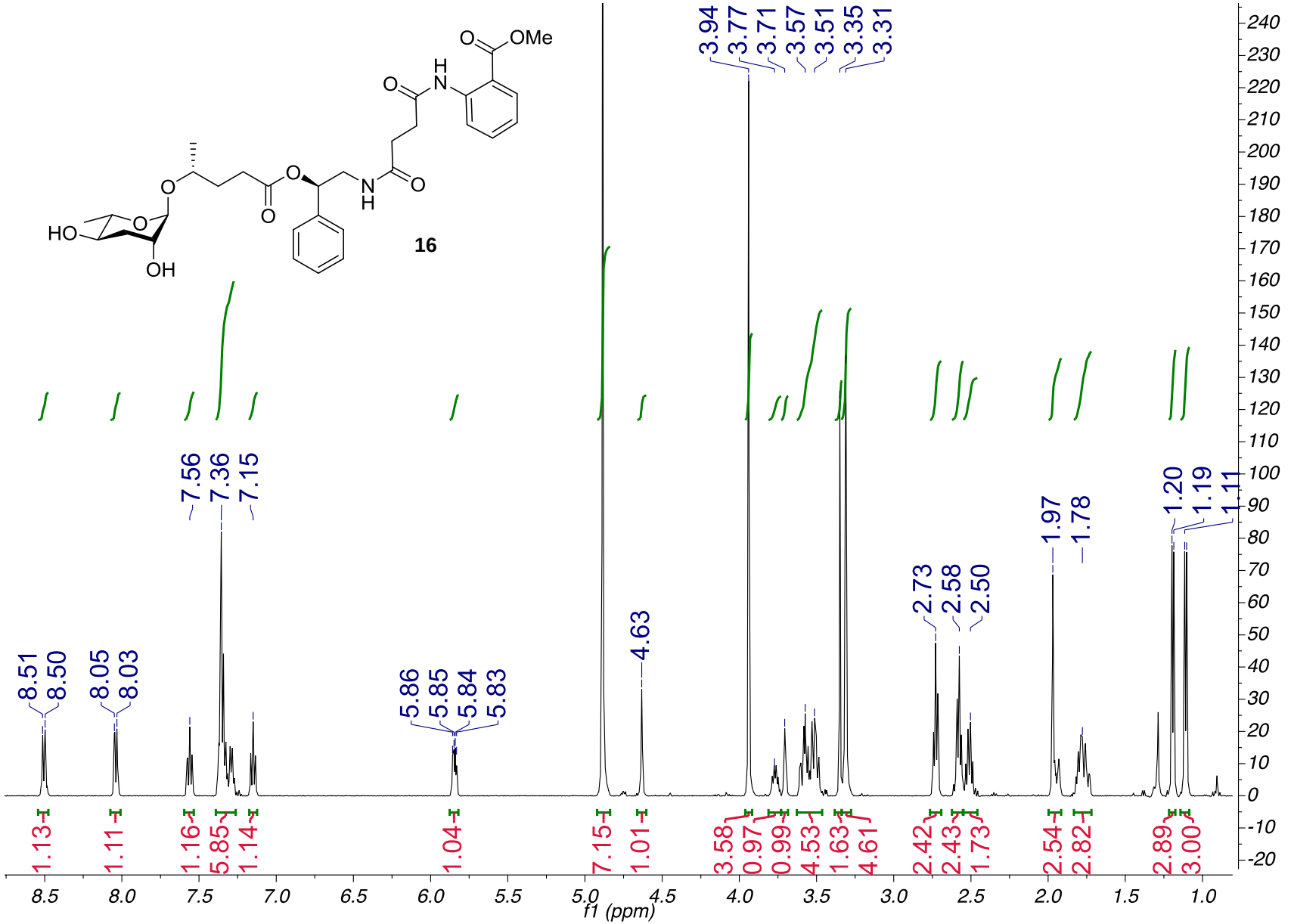
**$^{1}$H NMR Spectrum (500 MHz, chloroform-*d*) of 4**



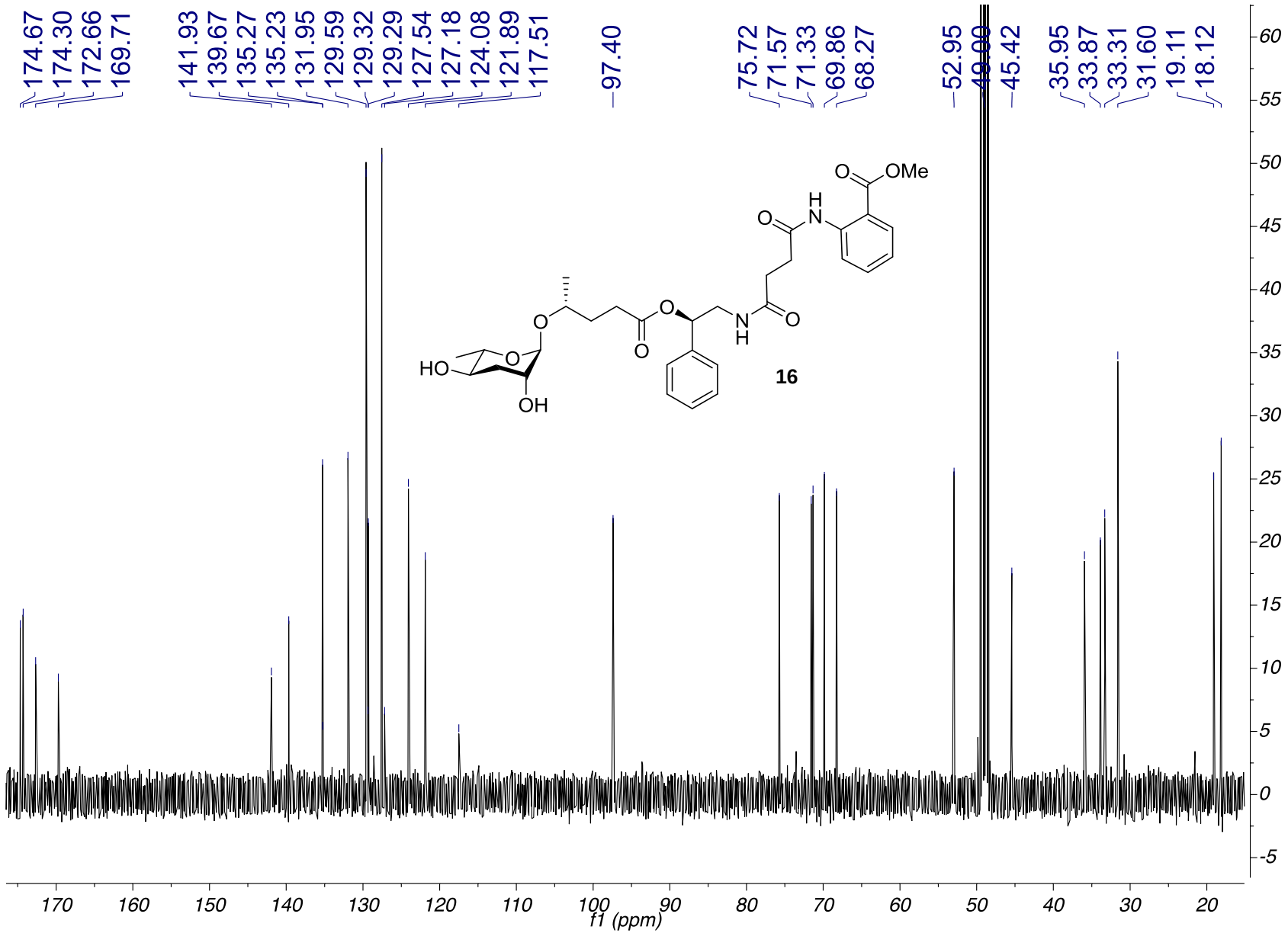
**$^{13}$C NMR Spectrum (125 MHz, chloroform-*d*) of 4**



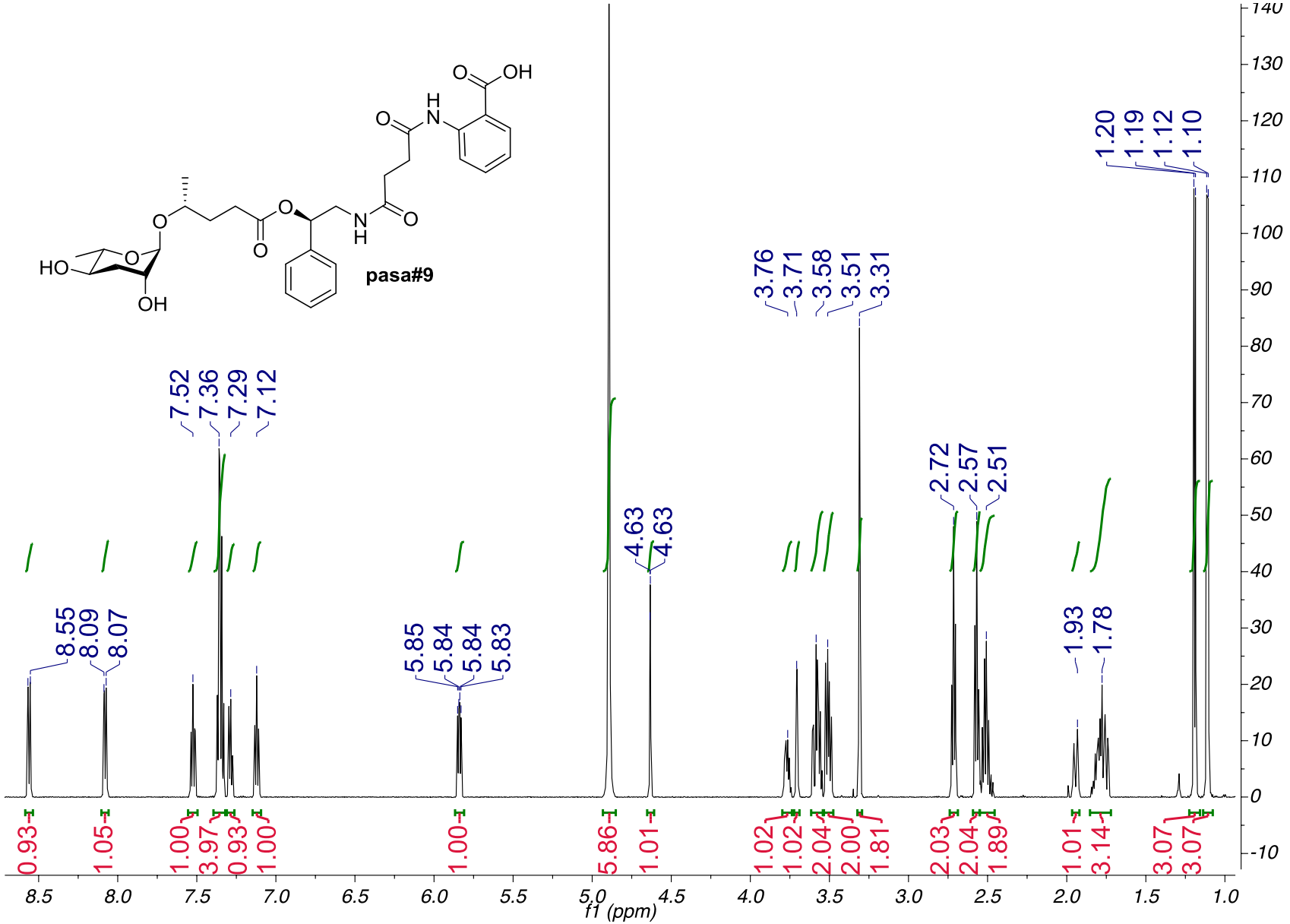
**$^1$H NMR Spectrum (500 MHz, methanol-$d_4$) of 16**



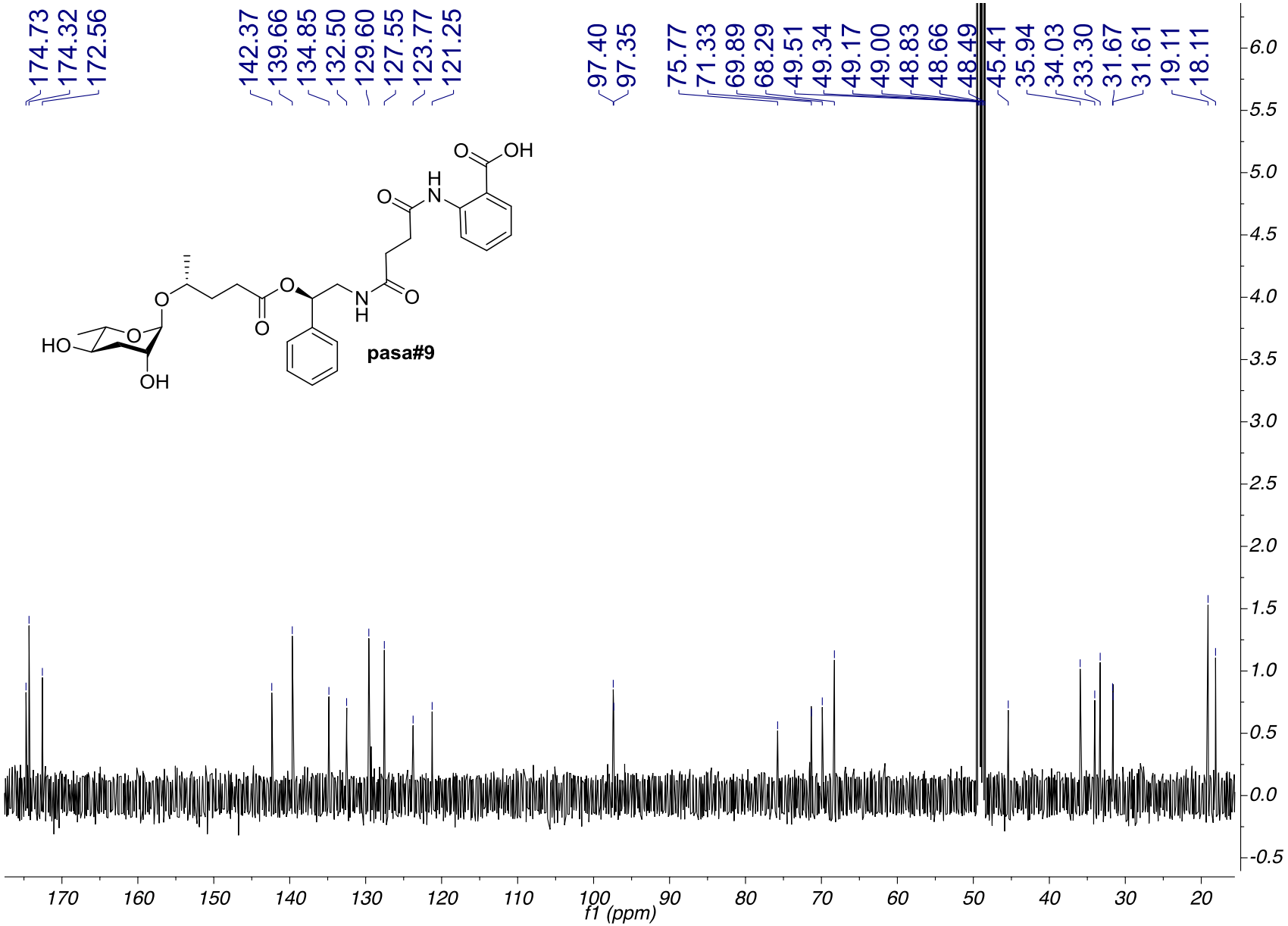
**$^{13}$C NMR Spectrum (125 MHz, methanol-$d_4$) of 16**



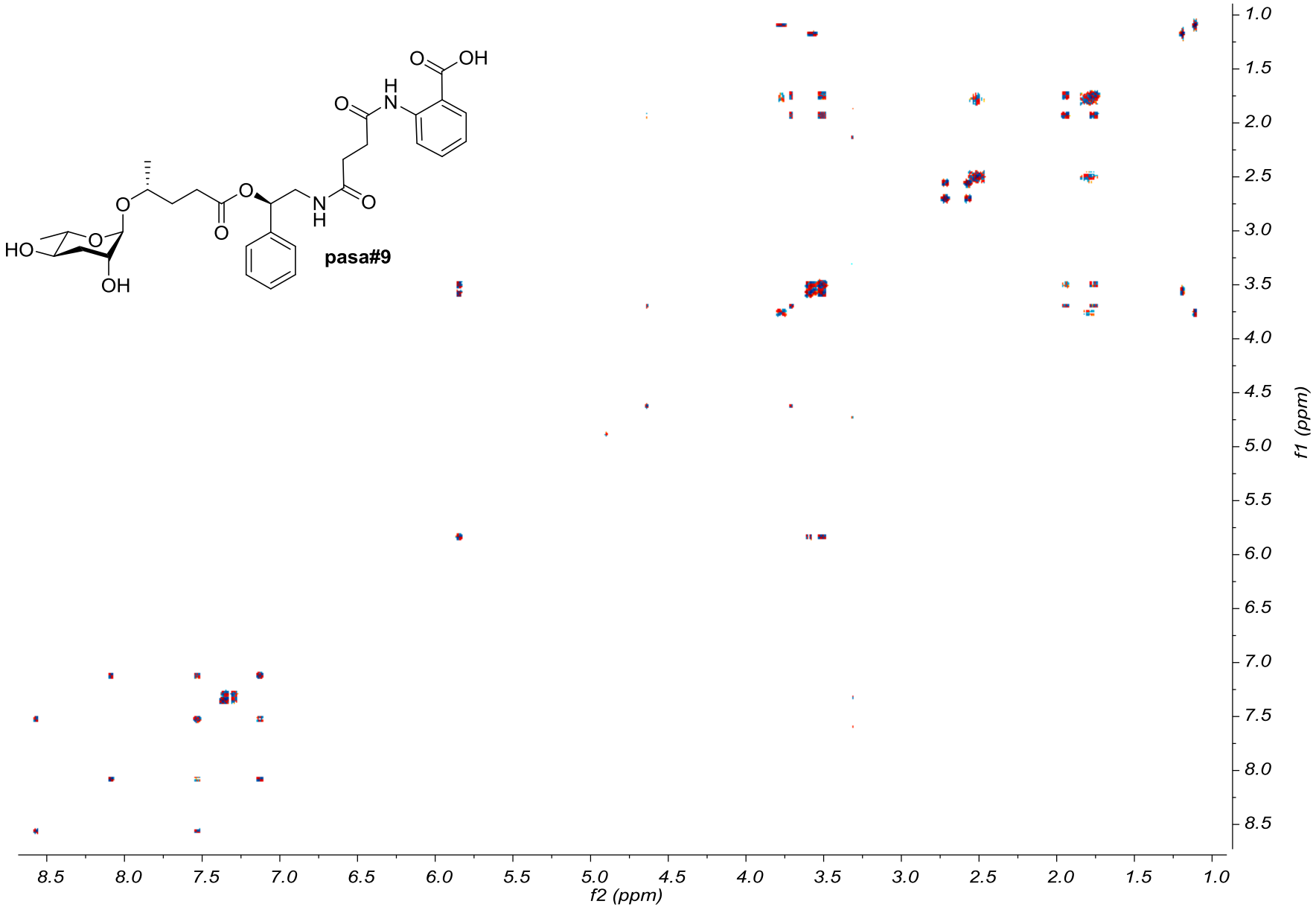
**$^1$H NMR Spectrum (600 MHz, methanol-$d_4$) of pasa#9**



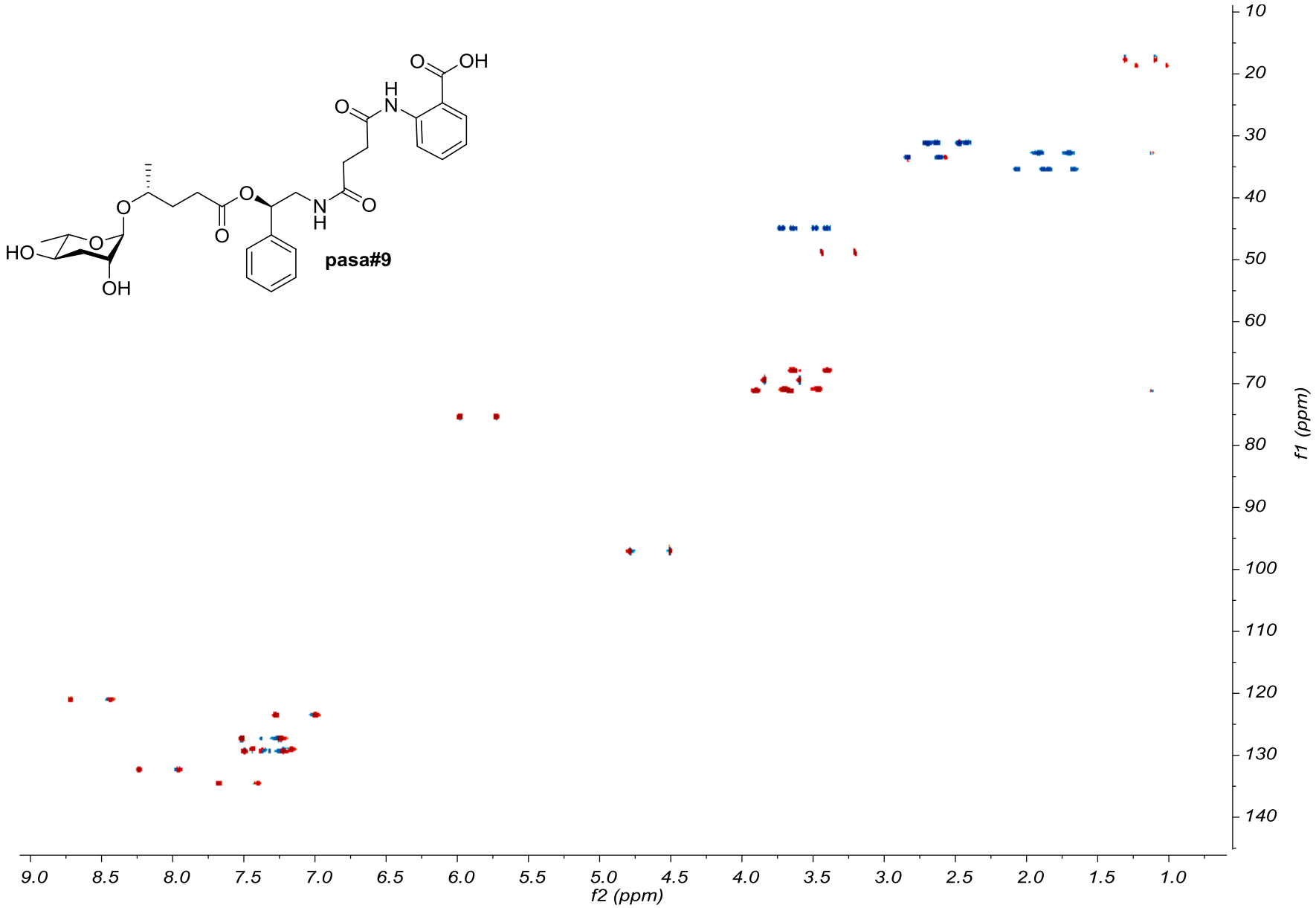
**$^{13}$C NMR Spectrum (125 MHz, methanol-$d_4$) of pasa#9**



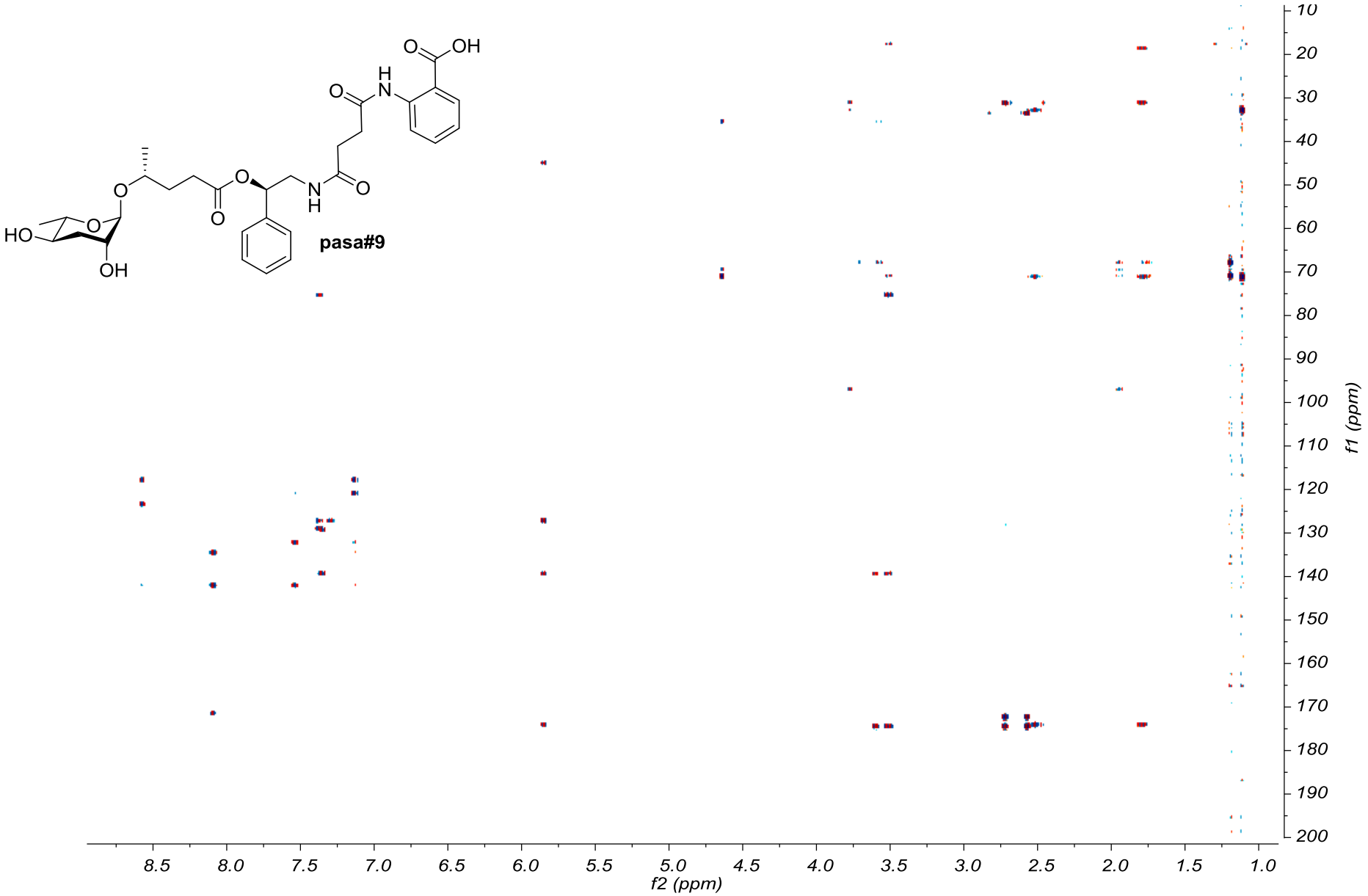
**dqfCOSY Spectrum (600 MHz, methanol-$d_4$) of pasa#9**



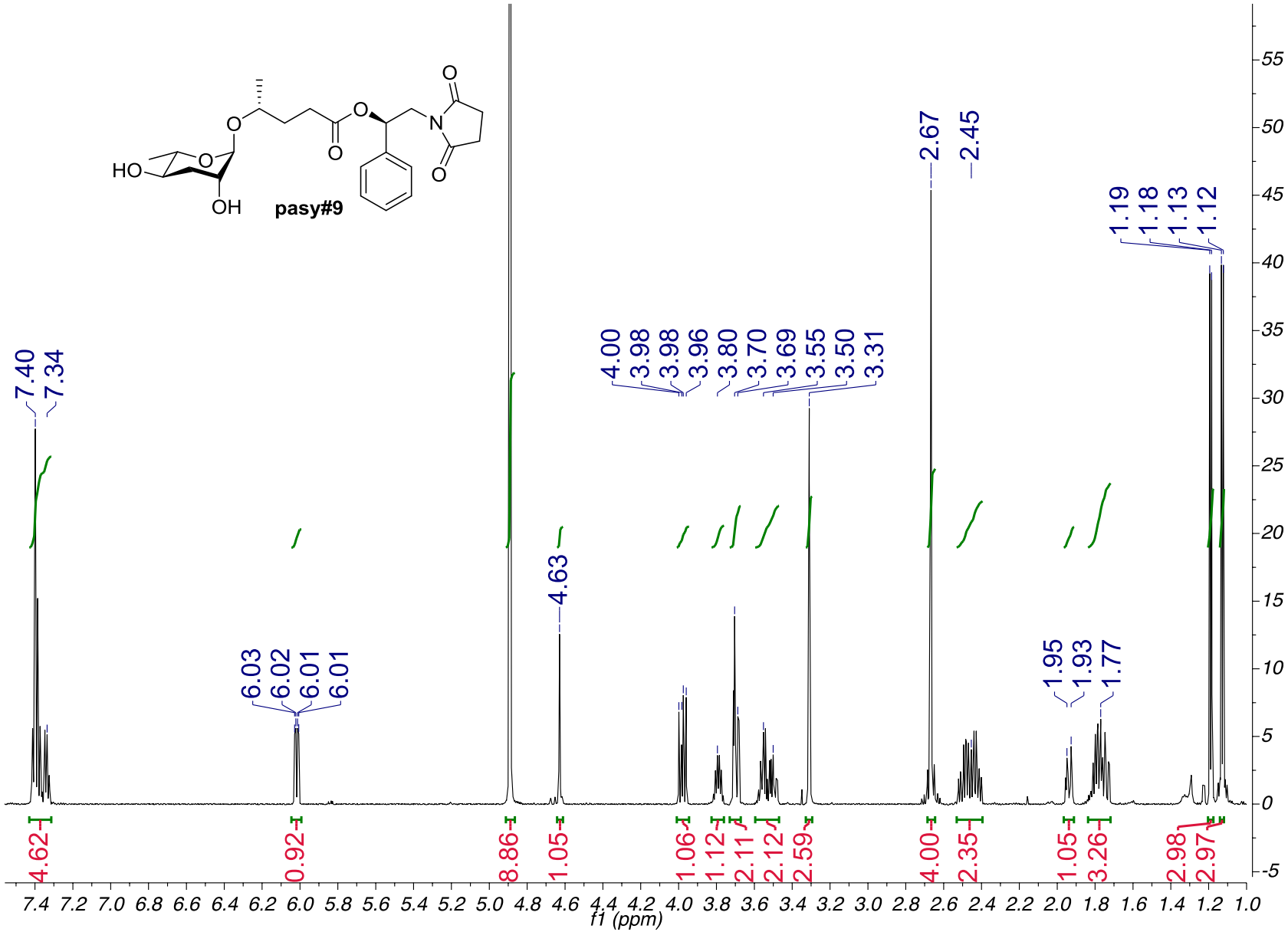
**HSQC Spectrum (600 MHz for $^{1}H$, 151 MHz for $^{13}C$, methanol-$d_4$) of pasa#9**



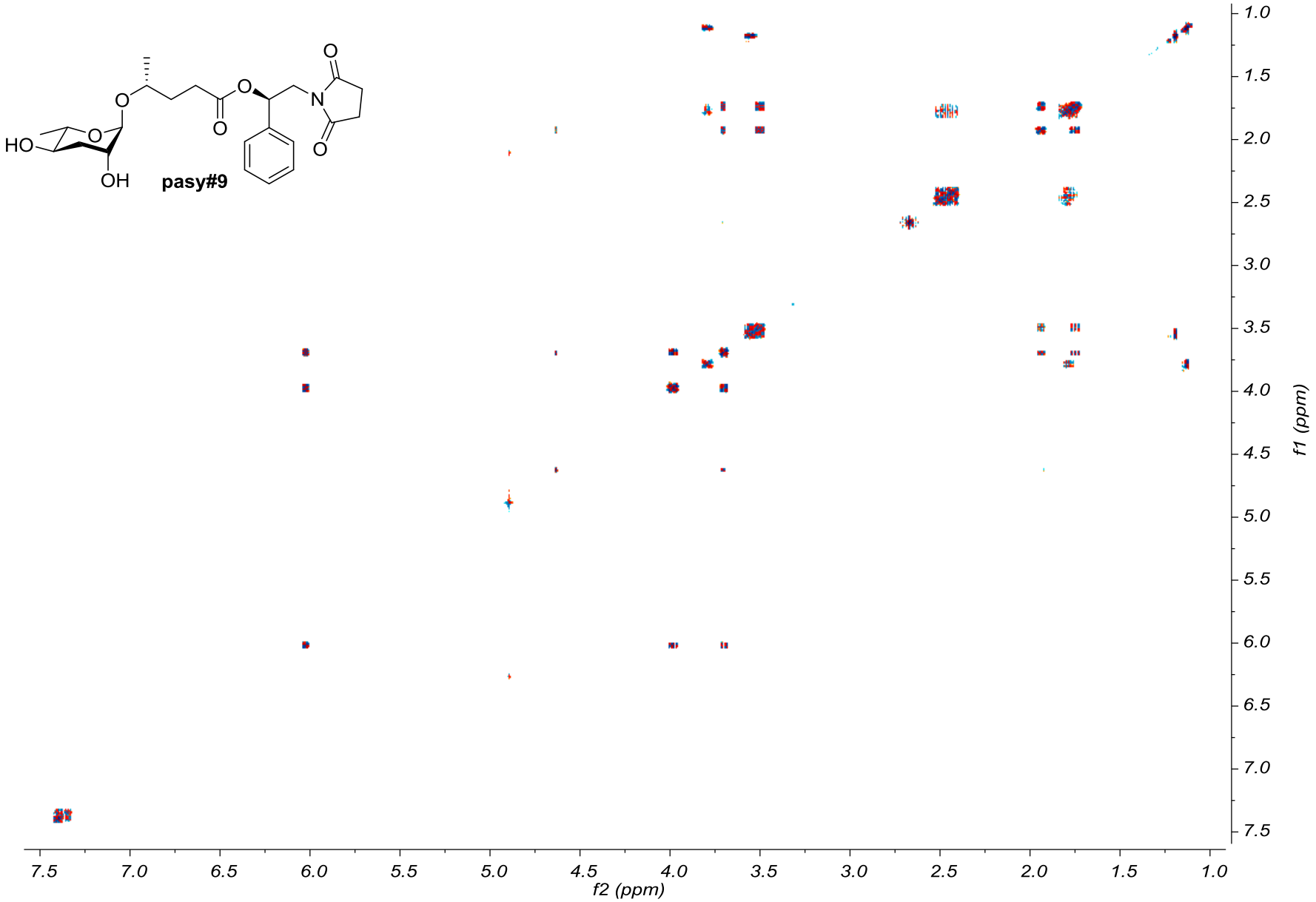
**HMBC Spectrum (600 MHz for $^{1}H$, 151 MHz for $^{13}C$, methanol-$d_4$) of pasa#9**



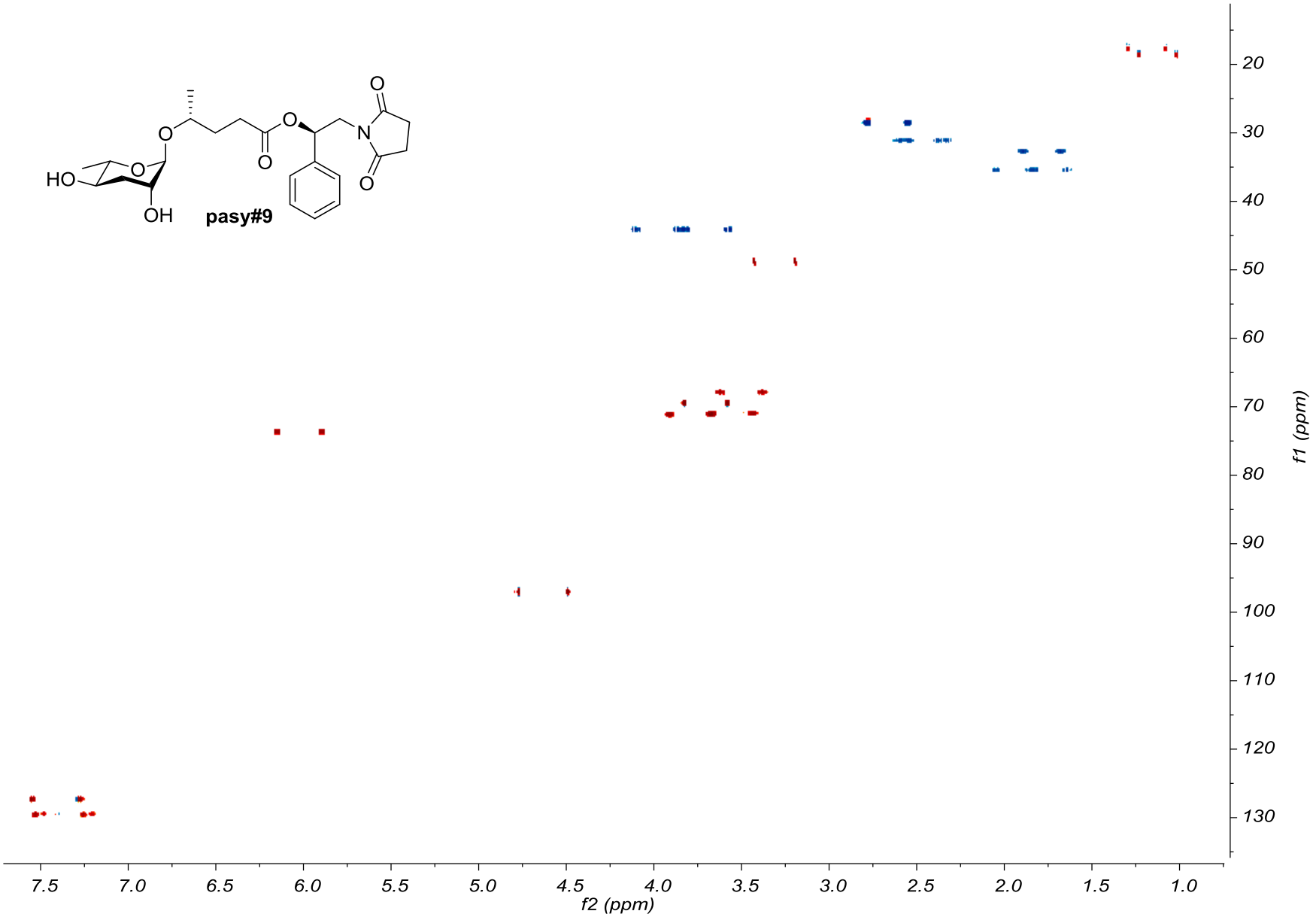
**$^1$H NMR Spectrum (600 MHz, methanol-$d_4$) of pasy#9**



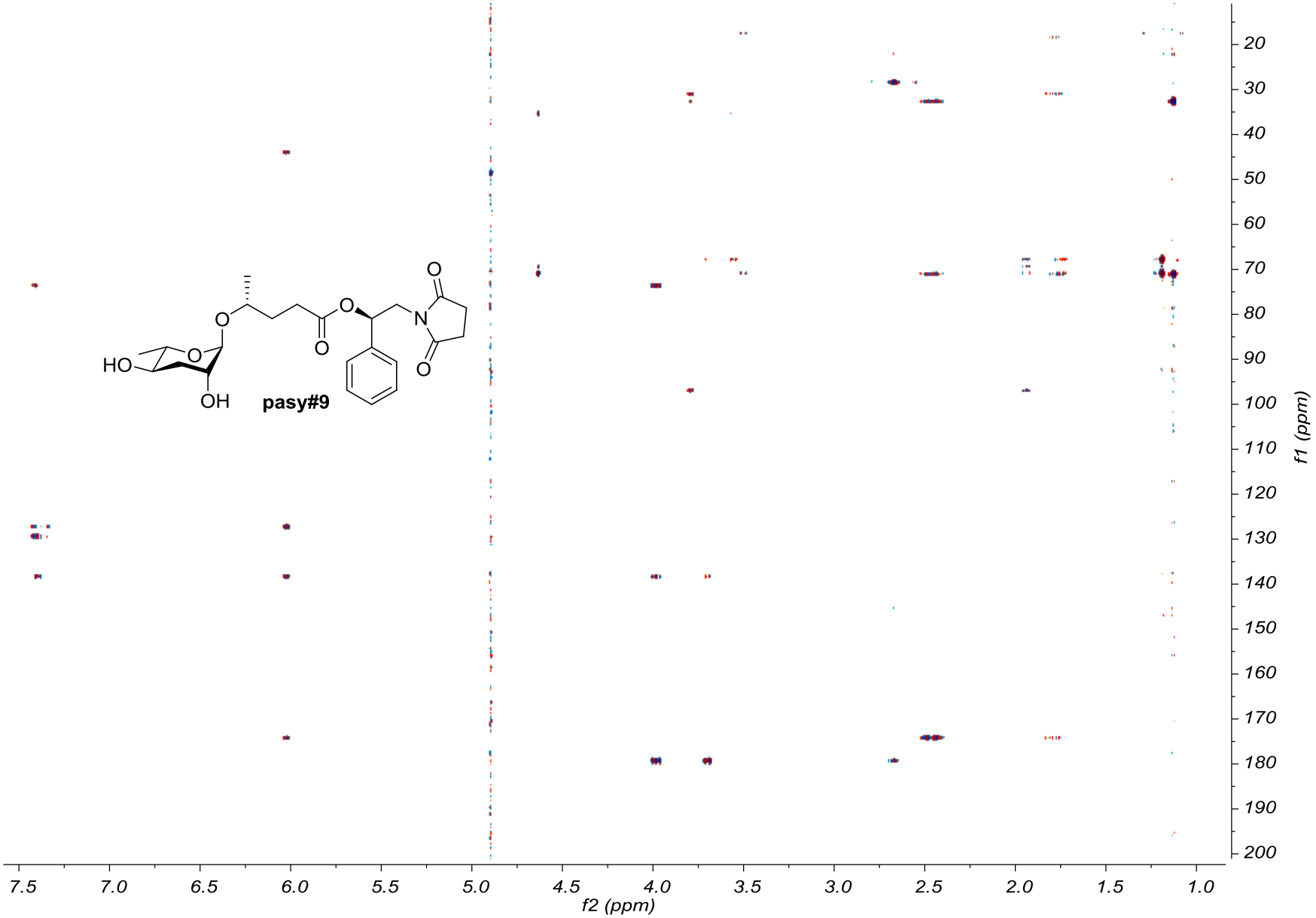
**dqfCOSY Spectrum (600 MHz, methanol-$d_4$) of pasy#9**



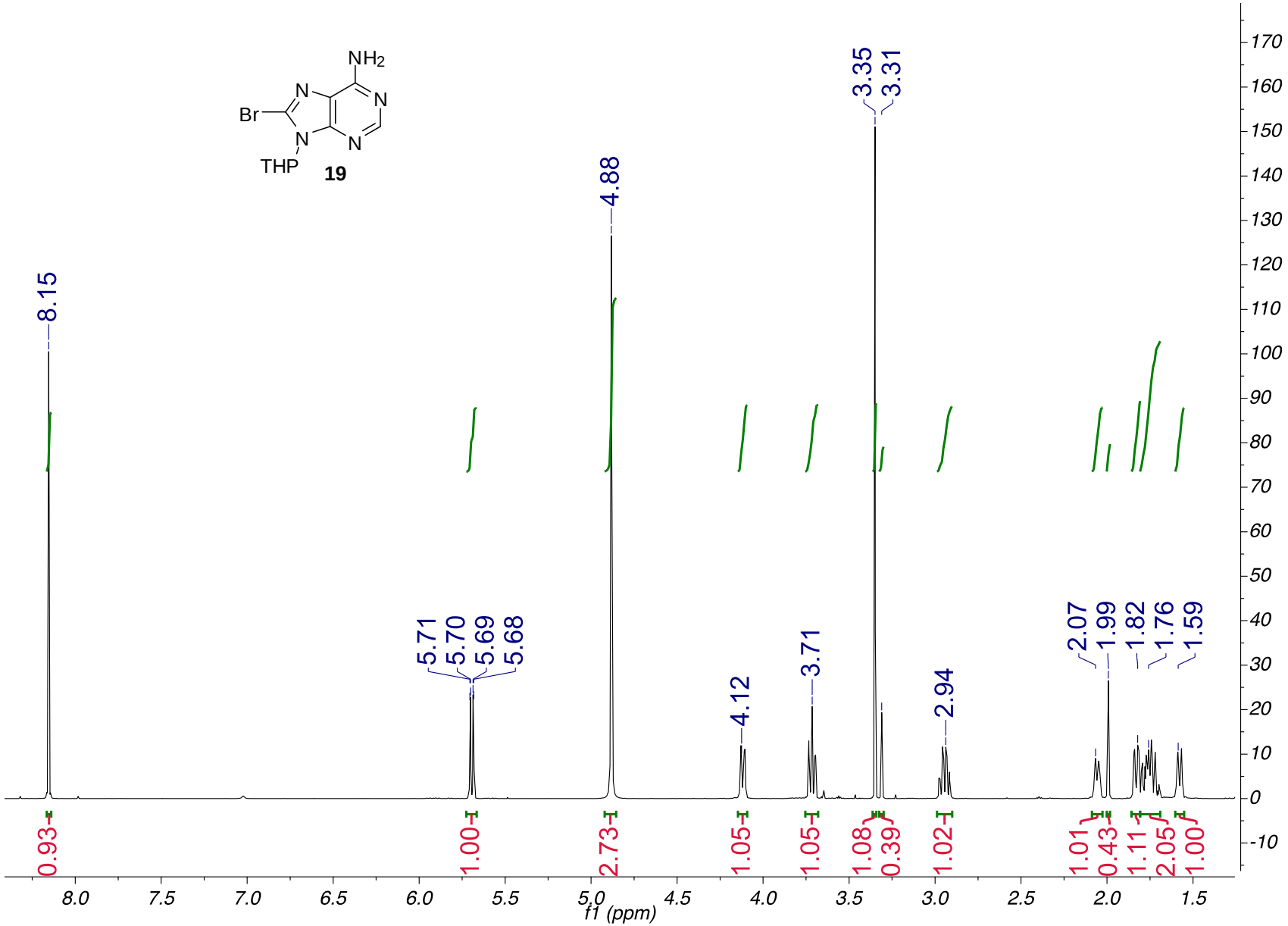
**HSQC Spectrum (600 MHz for $^{1}H$, 151 MHz for $^{13}C$, methanol-$d_4$) of pasy#9**



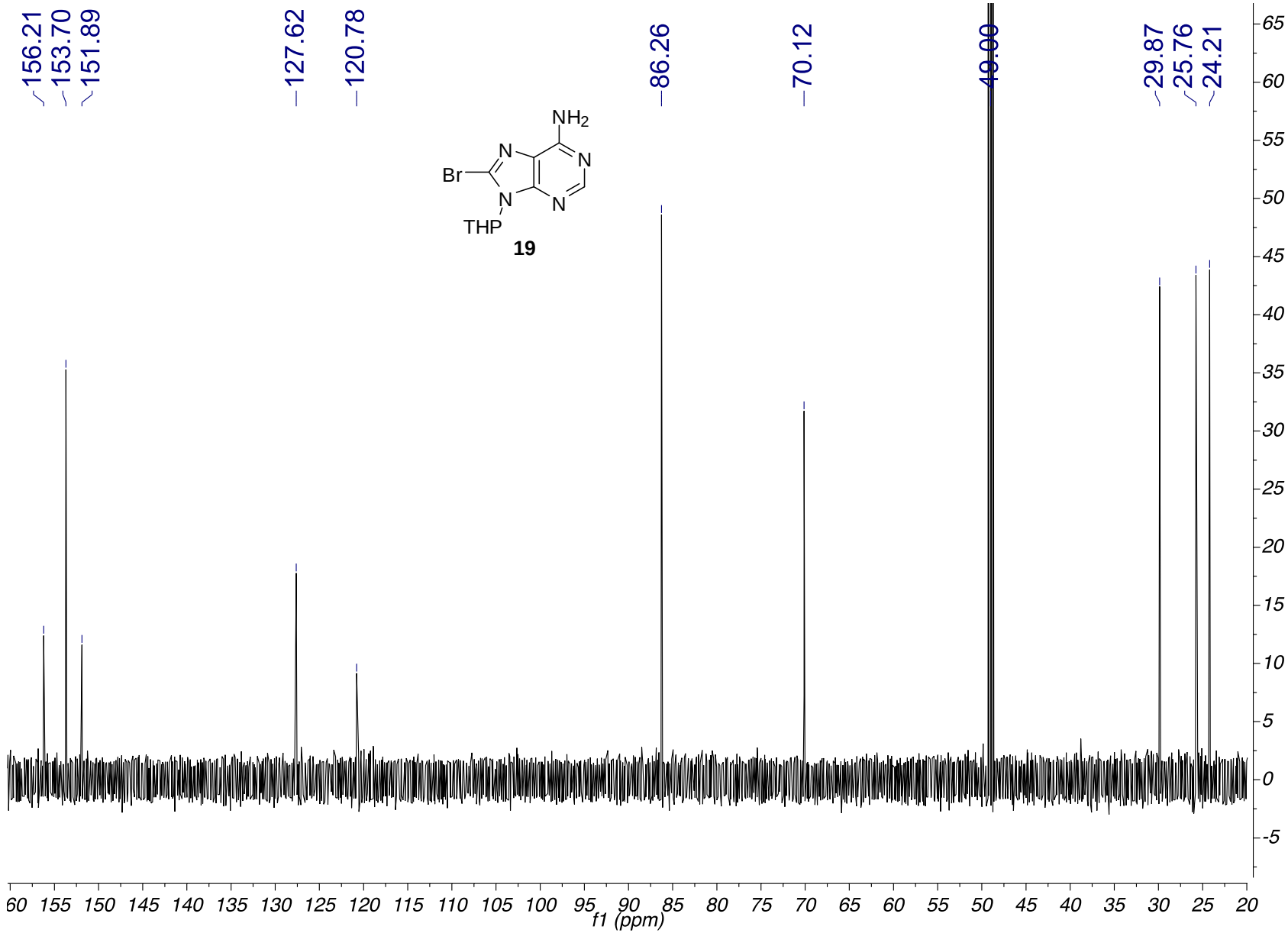
**HMBC Spectrum (600 MHz for $^{1}H$, 151 MHz for $^{13}C$, methanol-$d_4$) of pasy#9**



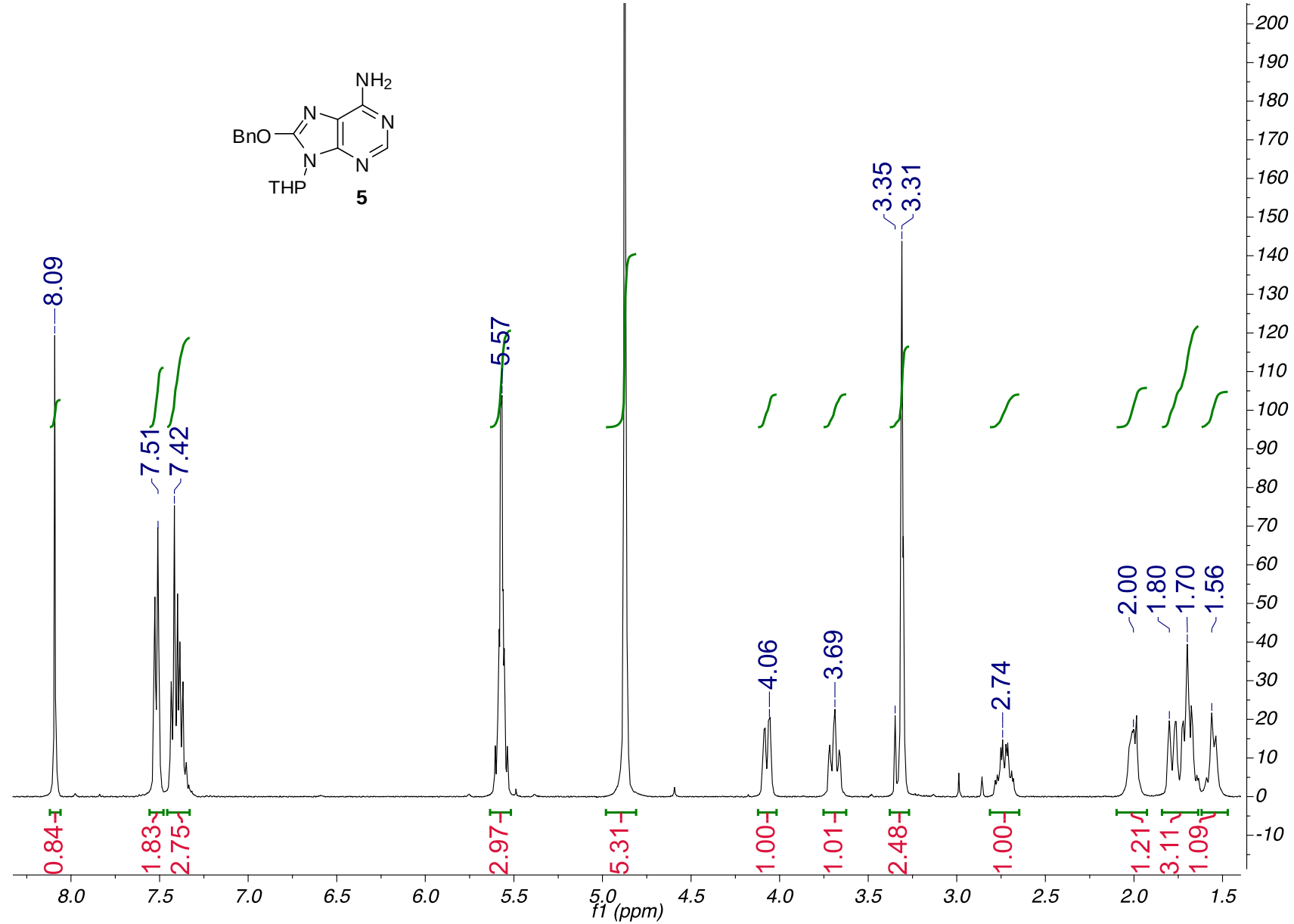
**$^1$H NMR Spectrum (600 MHz, methanol-$d_4$) of 19**



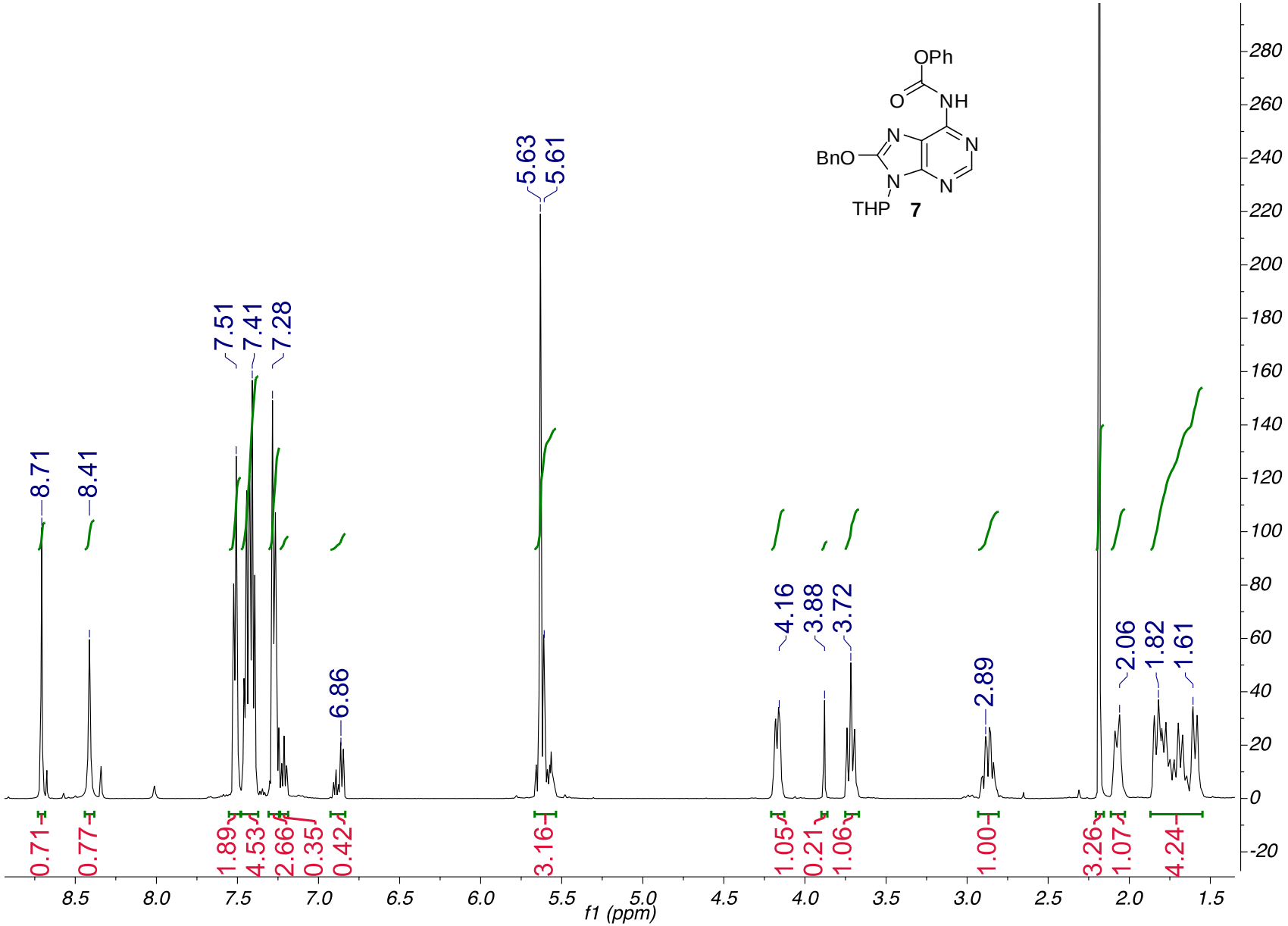
**$^{13}$C NMR Spectrum (125 MHz, methanol-$d_4$) of 19**



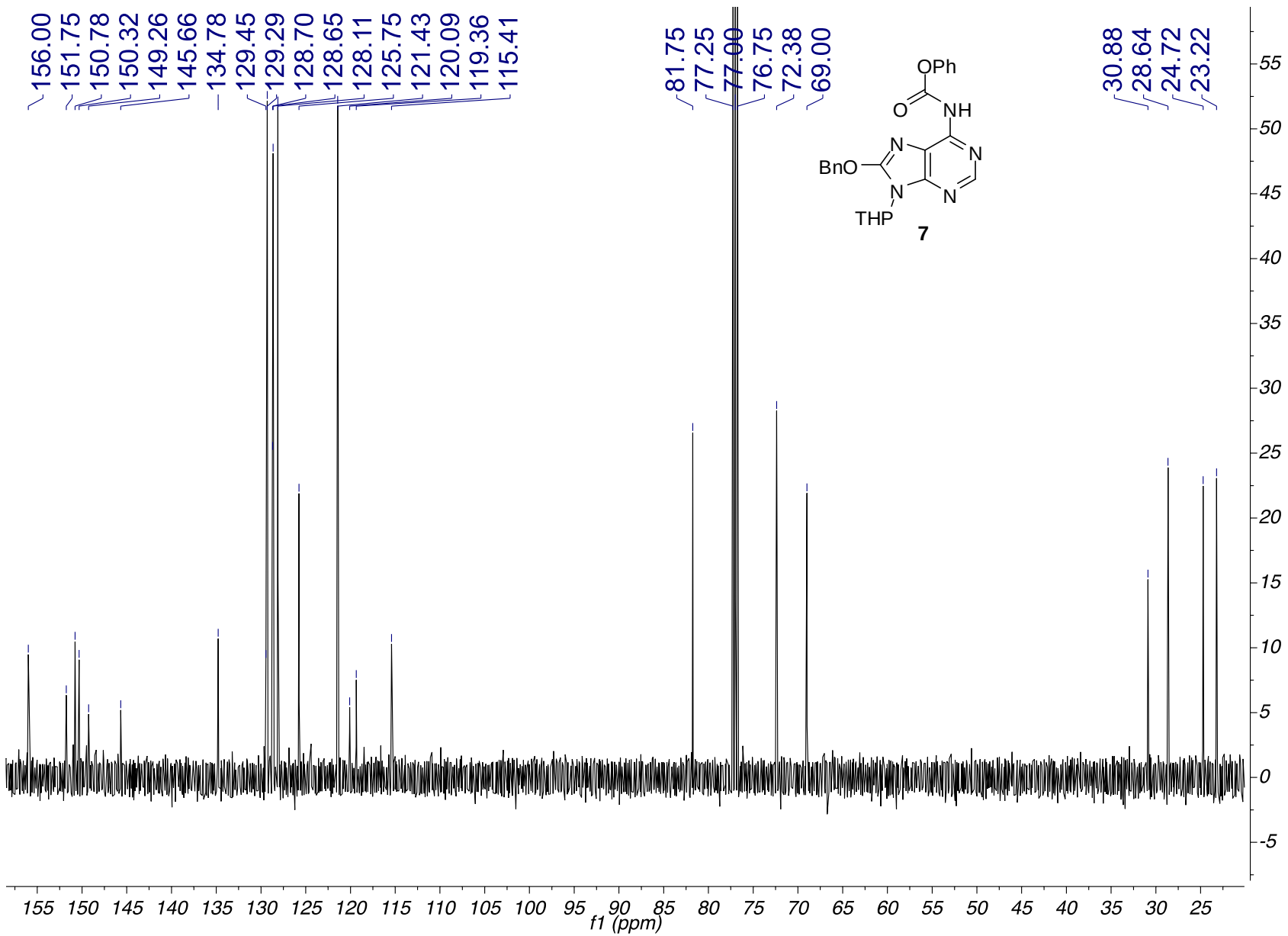
**$^1$H NMR Spectrum (400 MHz, methanol-$d_4$) of 5**



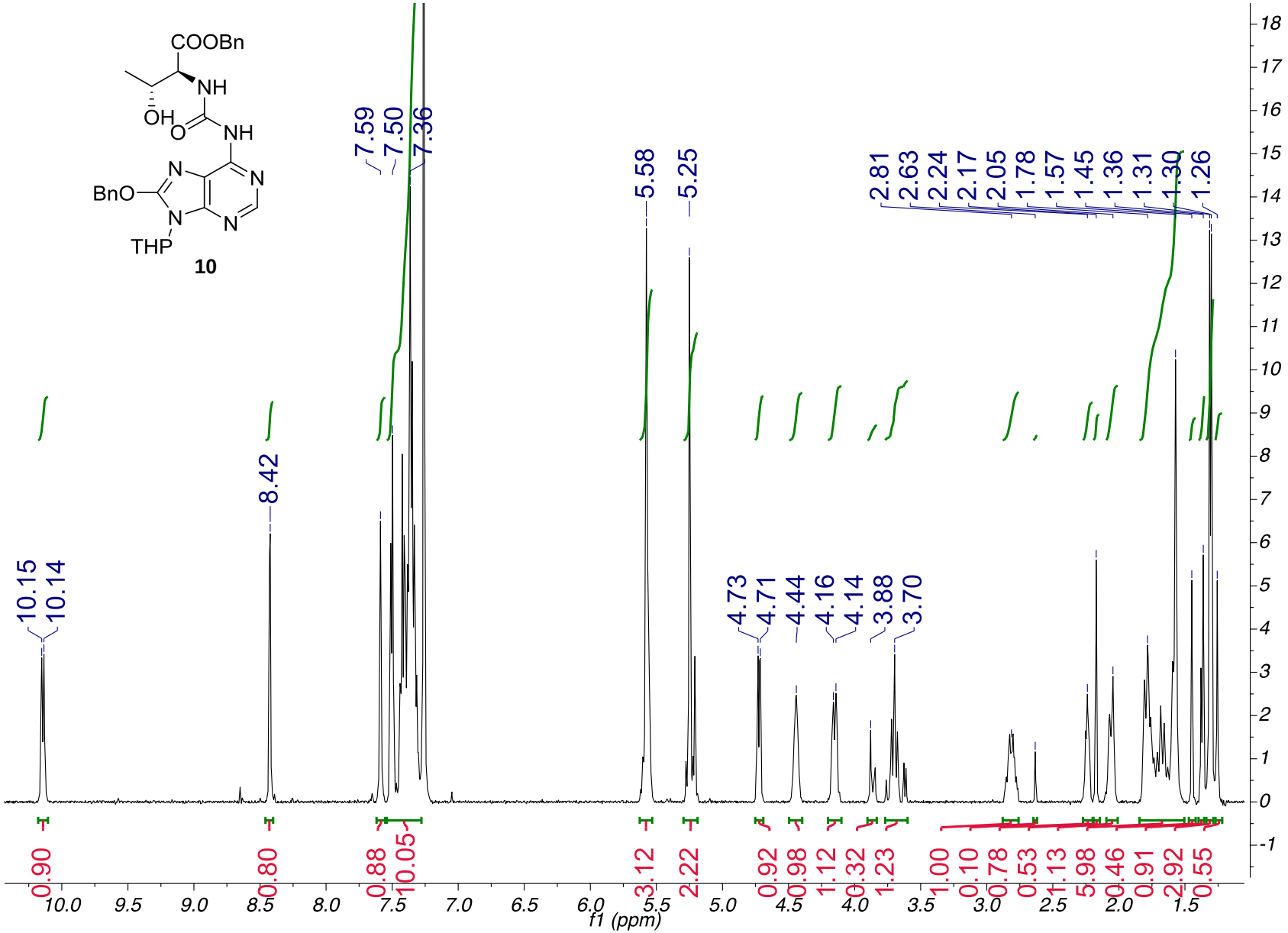
**$^1$H NMR Spectrum (500 MHz, chloroform-*d*) of 7**



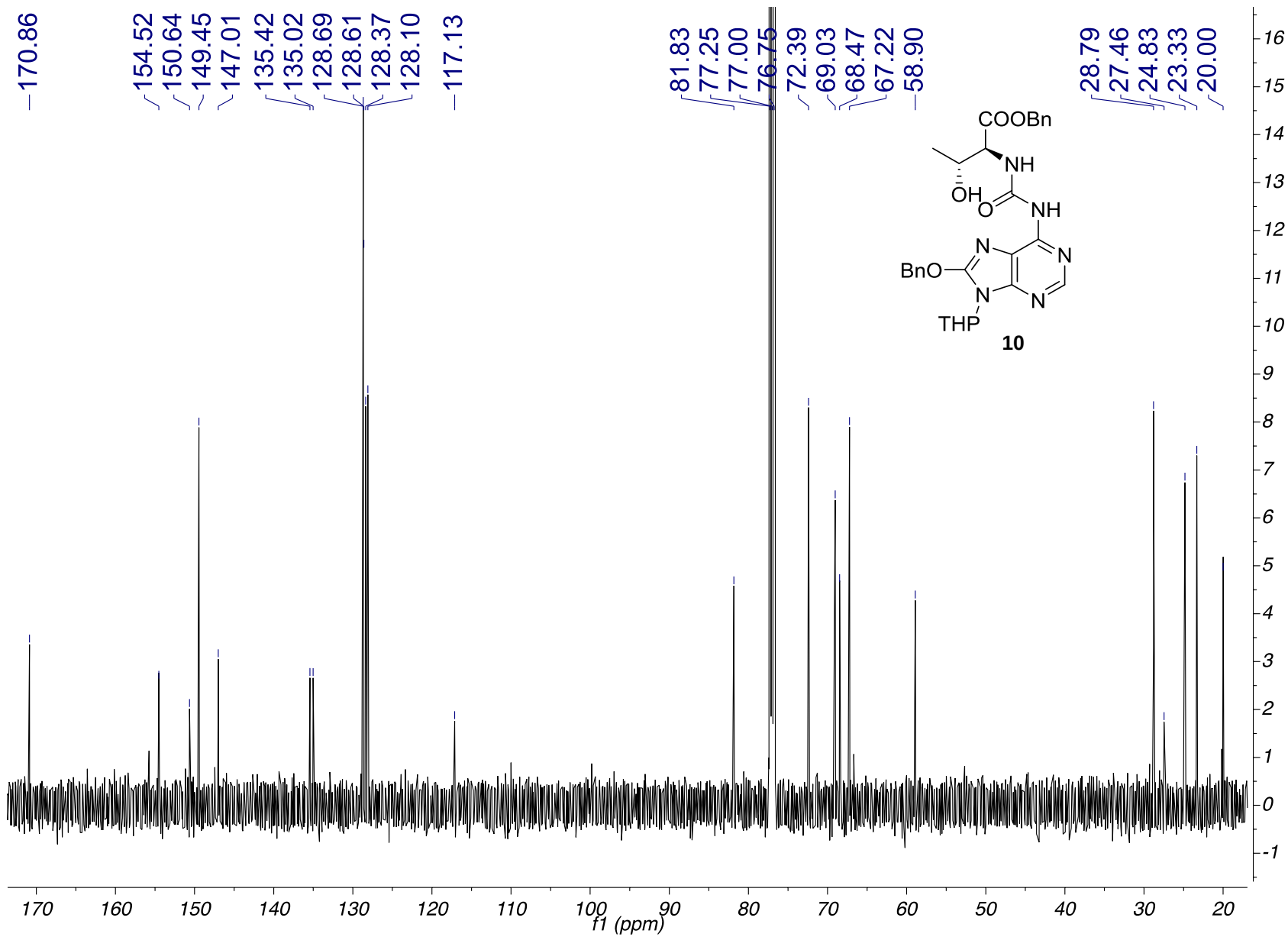
**$^{13}$C NMR Spectrum (125 MHz, chloroform-*d*) of 7**



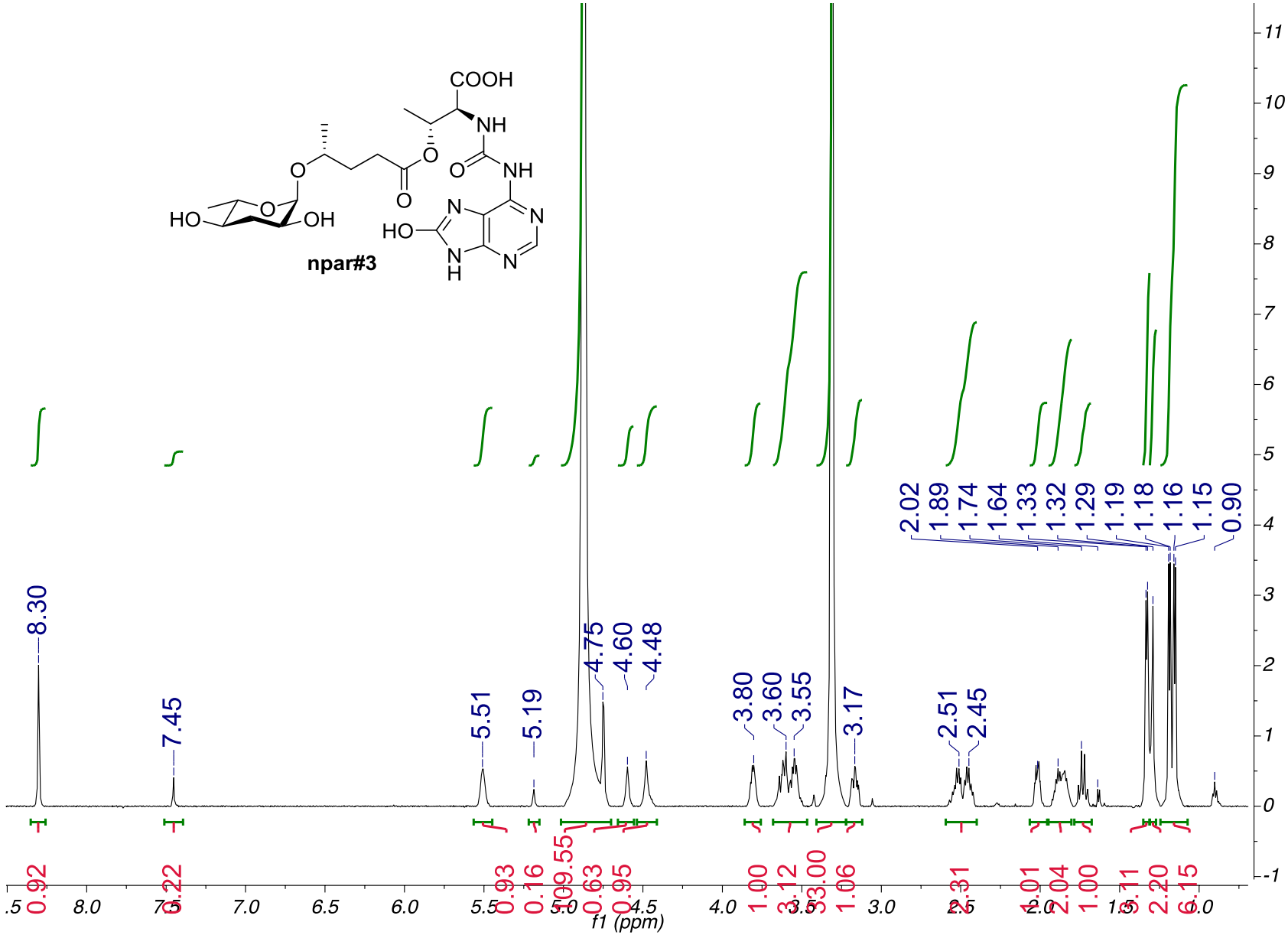
**$^1$H NMR Spectrum (500 MHz, chloroform-*d*) of 10**



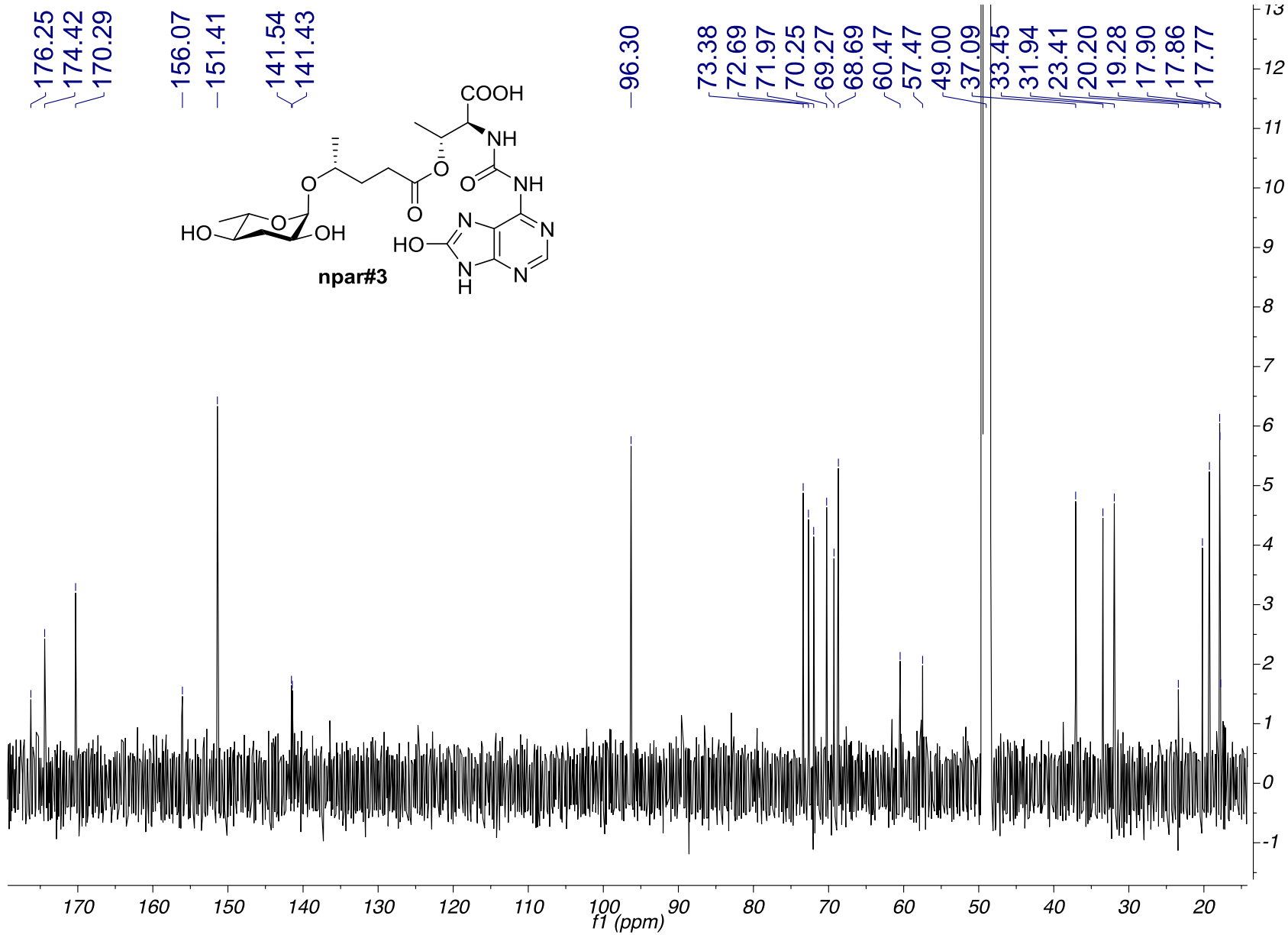
**$^{13}$C NMR Spectrum (125 MHz, chloroform-*d*) of 10**



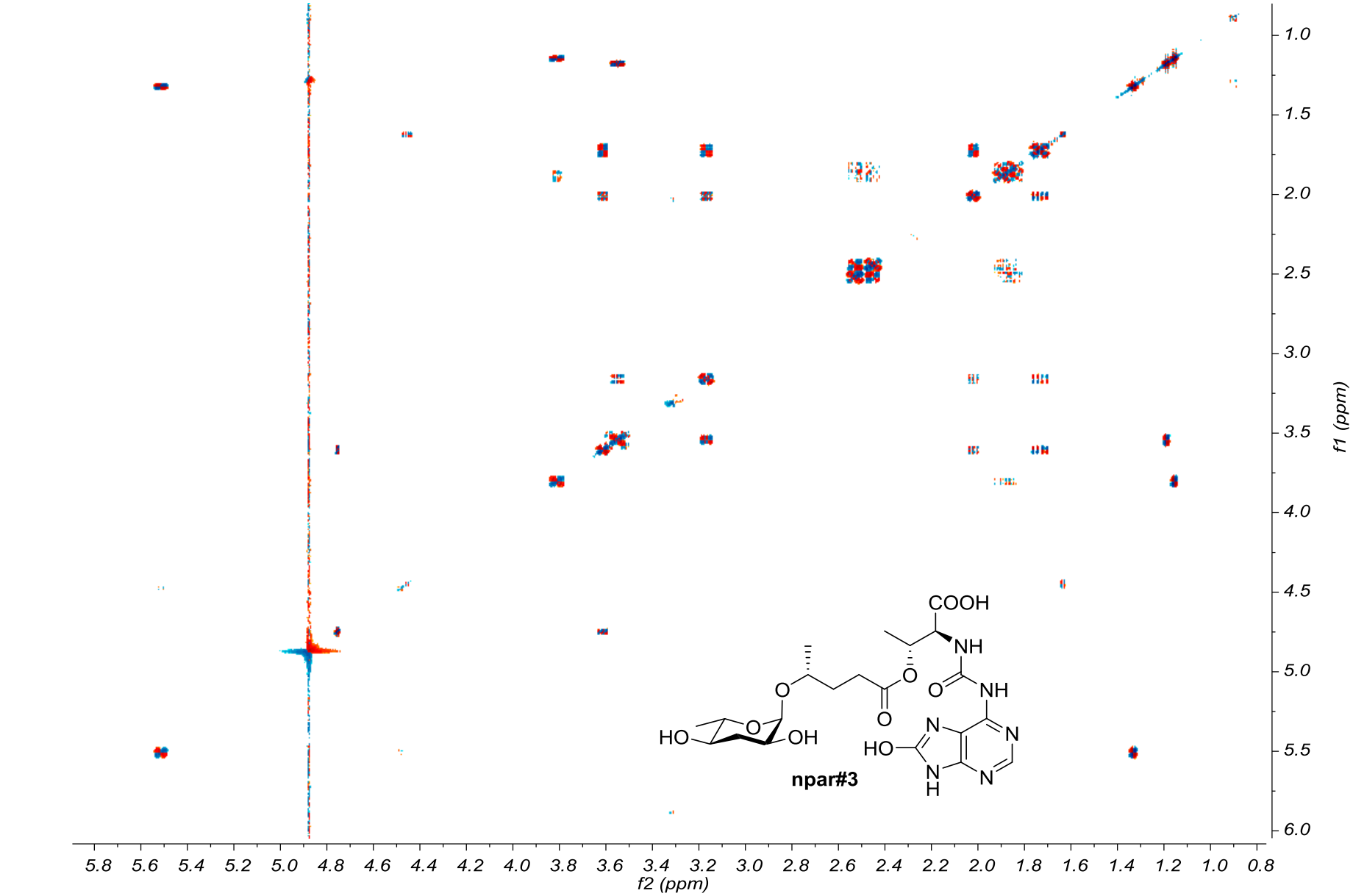
**$^1$H NMR Spectrum (600 MHz, methanol-$d_4$) of npar#3**



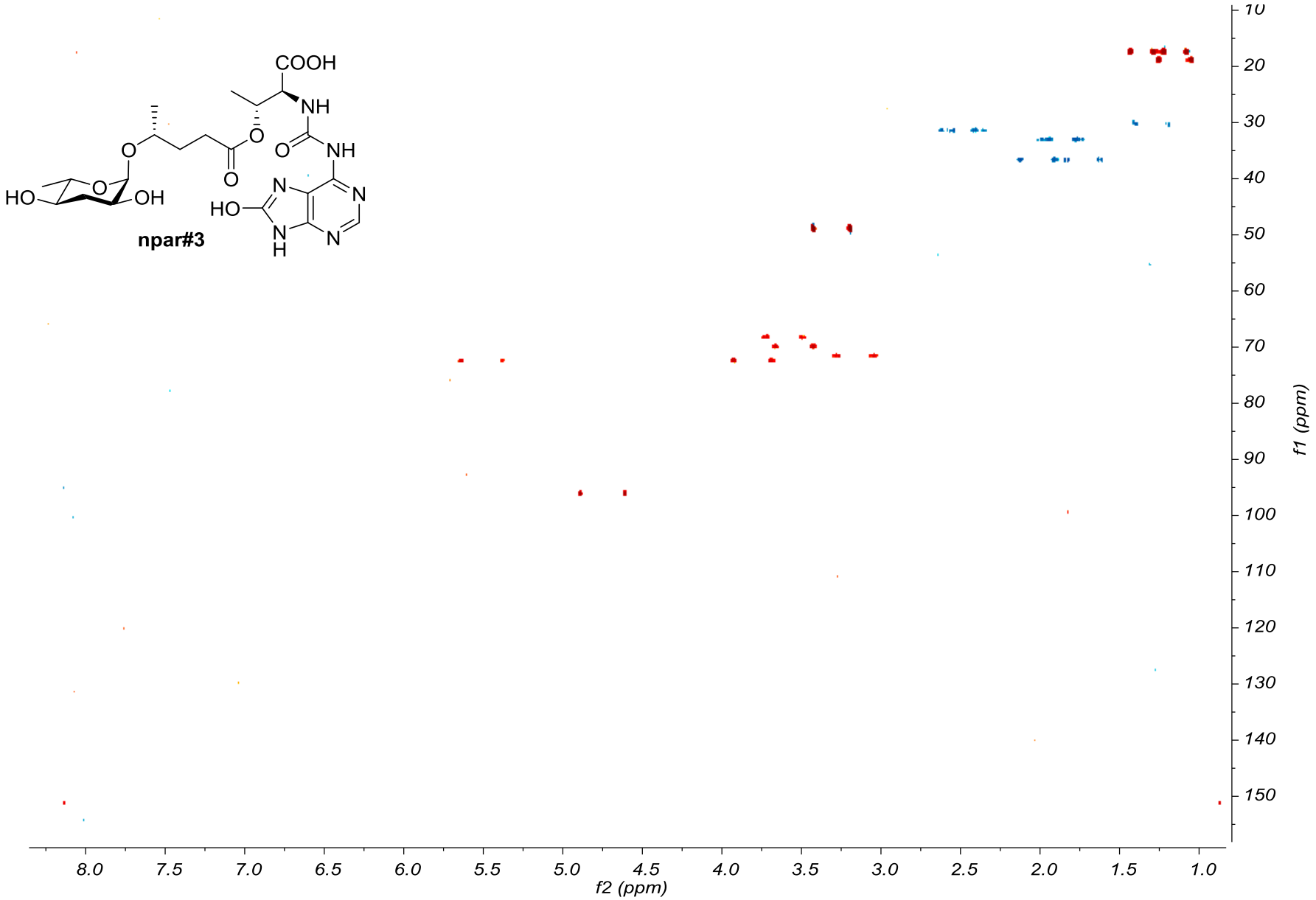
**$^{13}$C NMR Spectrum (125 MHz, methanol-$d_4$) of npar#3**



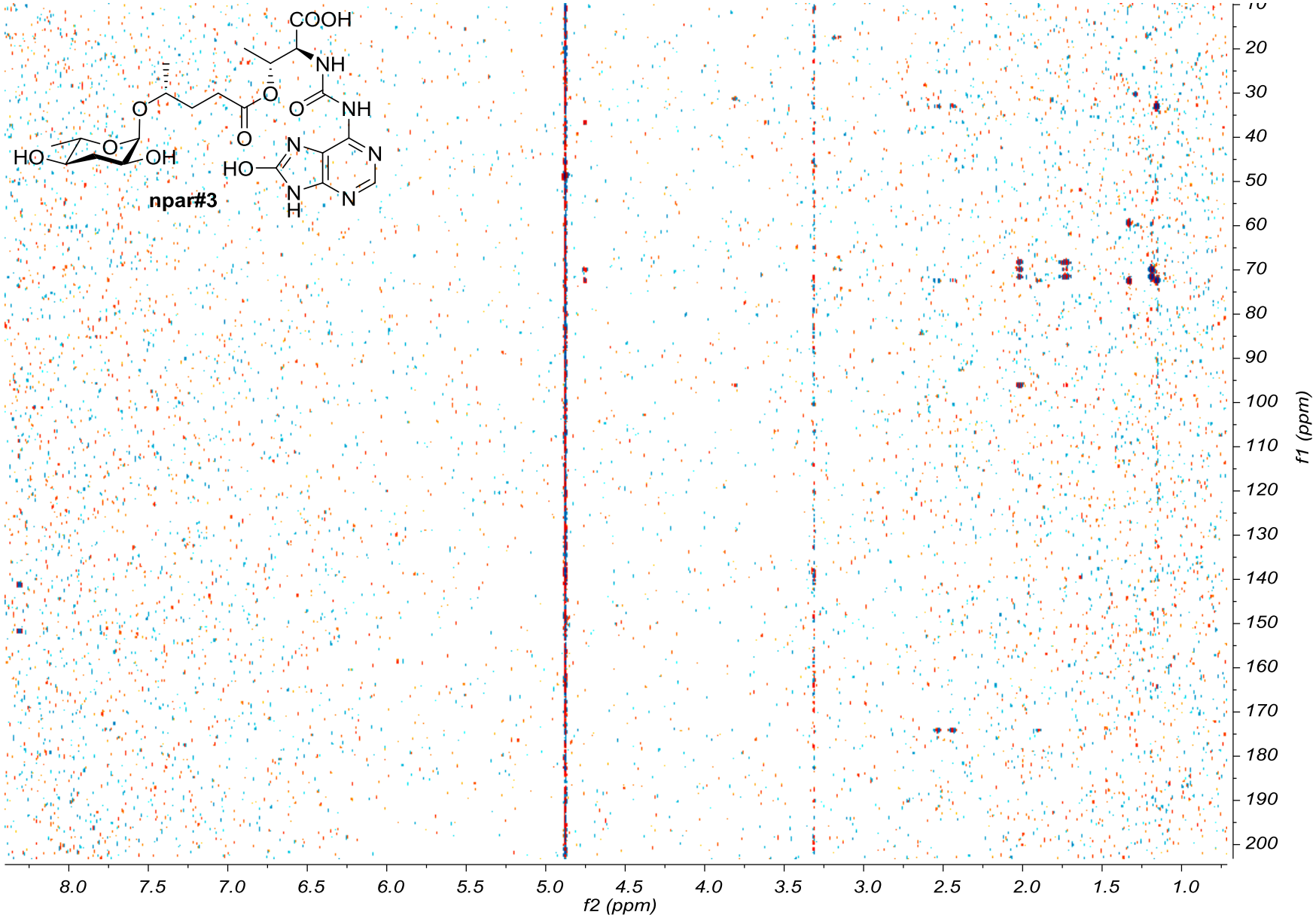
**dqfCOSY Spectrum (600 MHz, methanol-$d_4$) of npar#3**



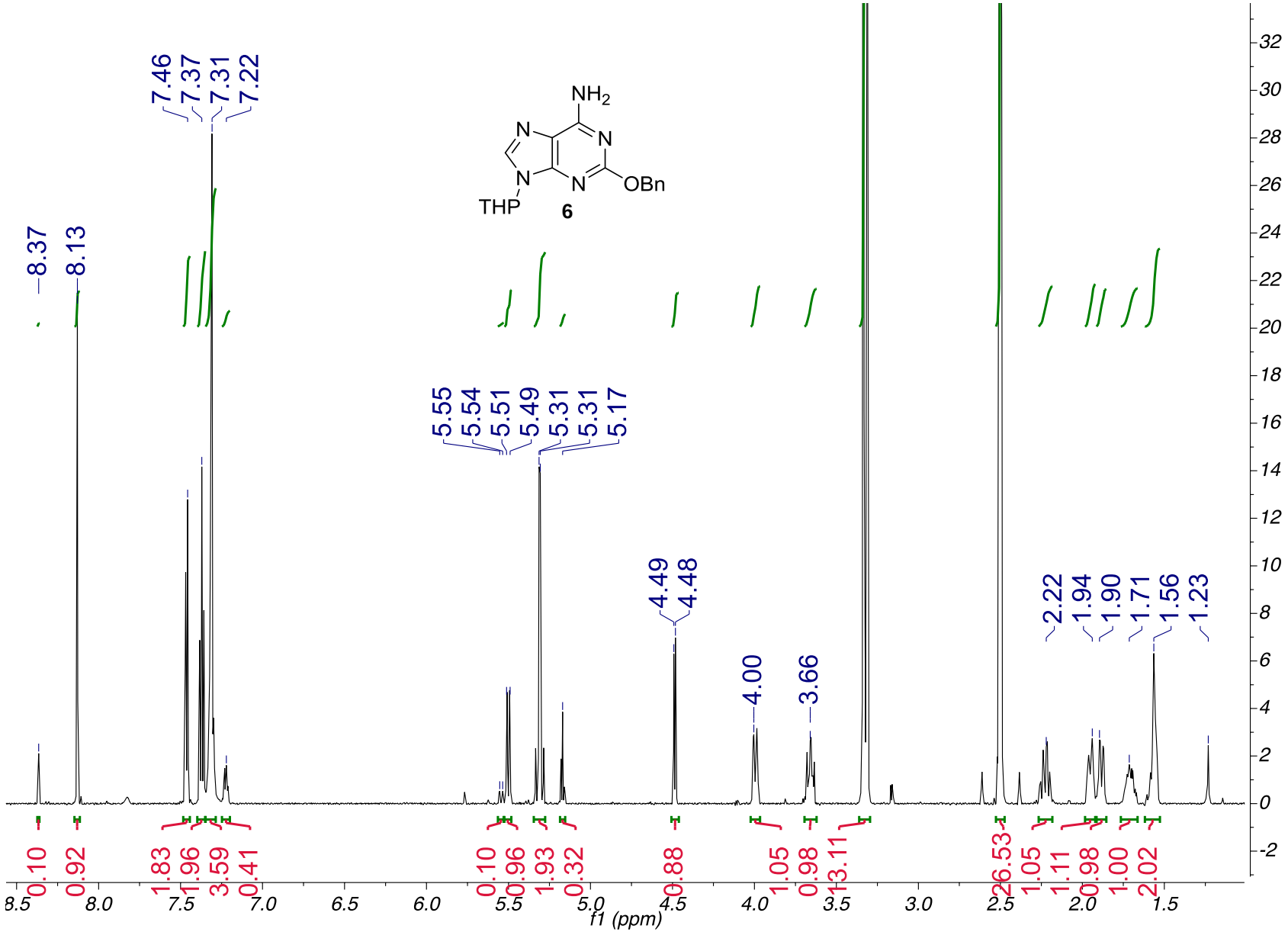
**HSQCAD Spectrum (600 MHz for $^{1}H$, 151 MHz for $^{13}C$, methanol-$d_4$) of npar#3**



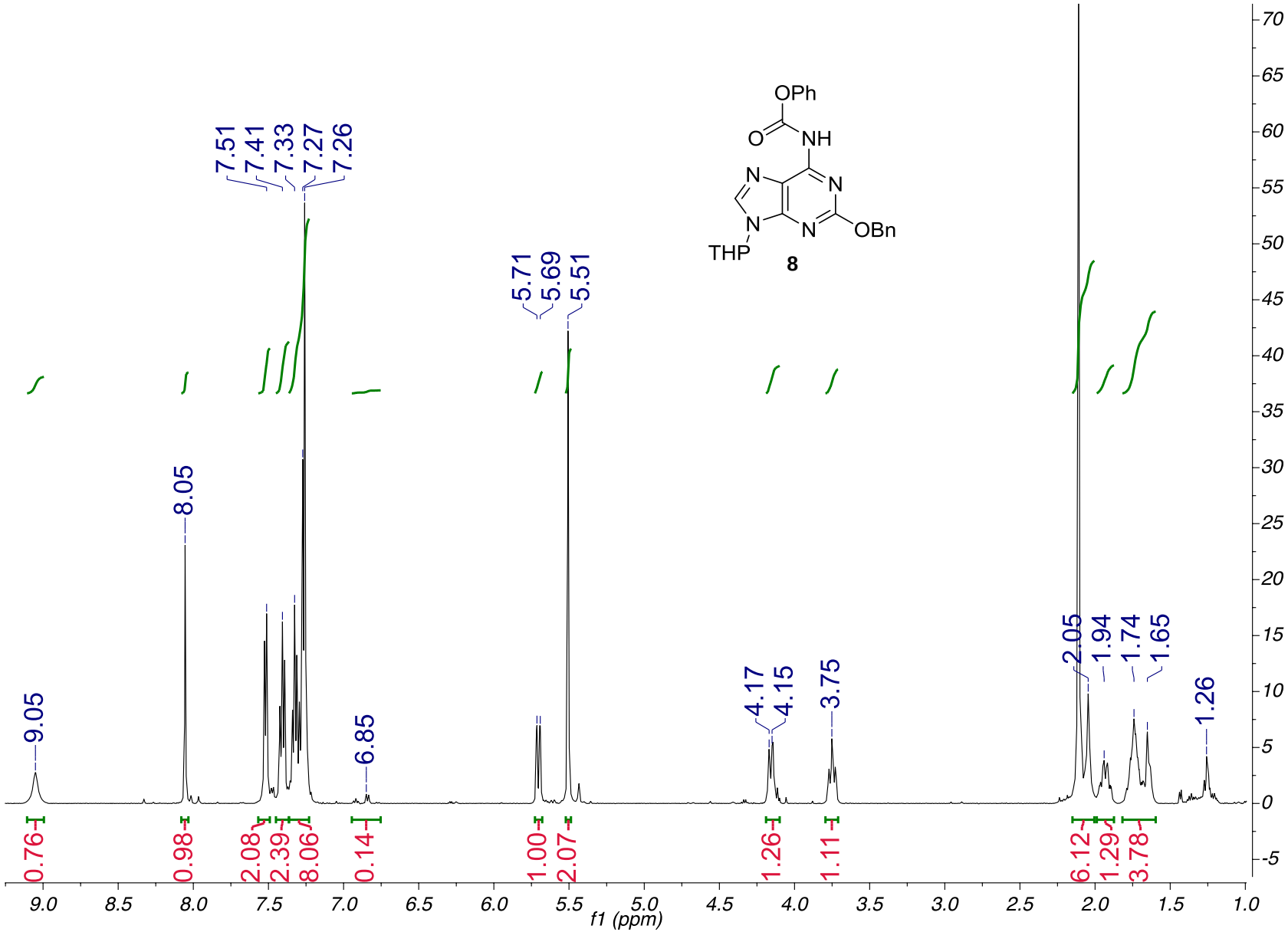
**HMBC Spectrum (600 MHz for $^{1}H$, 151 MHz for $^{13}C$, methanol-$d_4$) of npar#3**



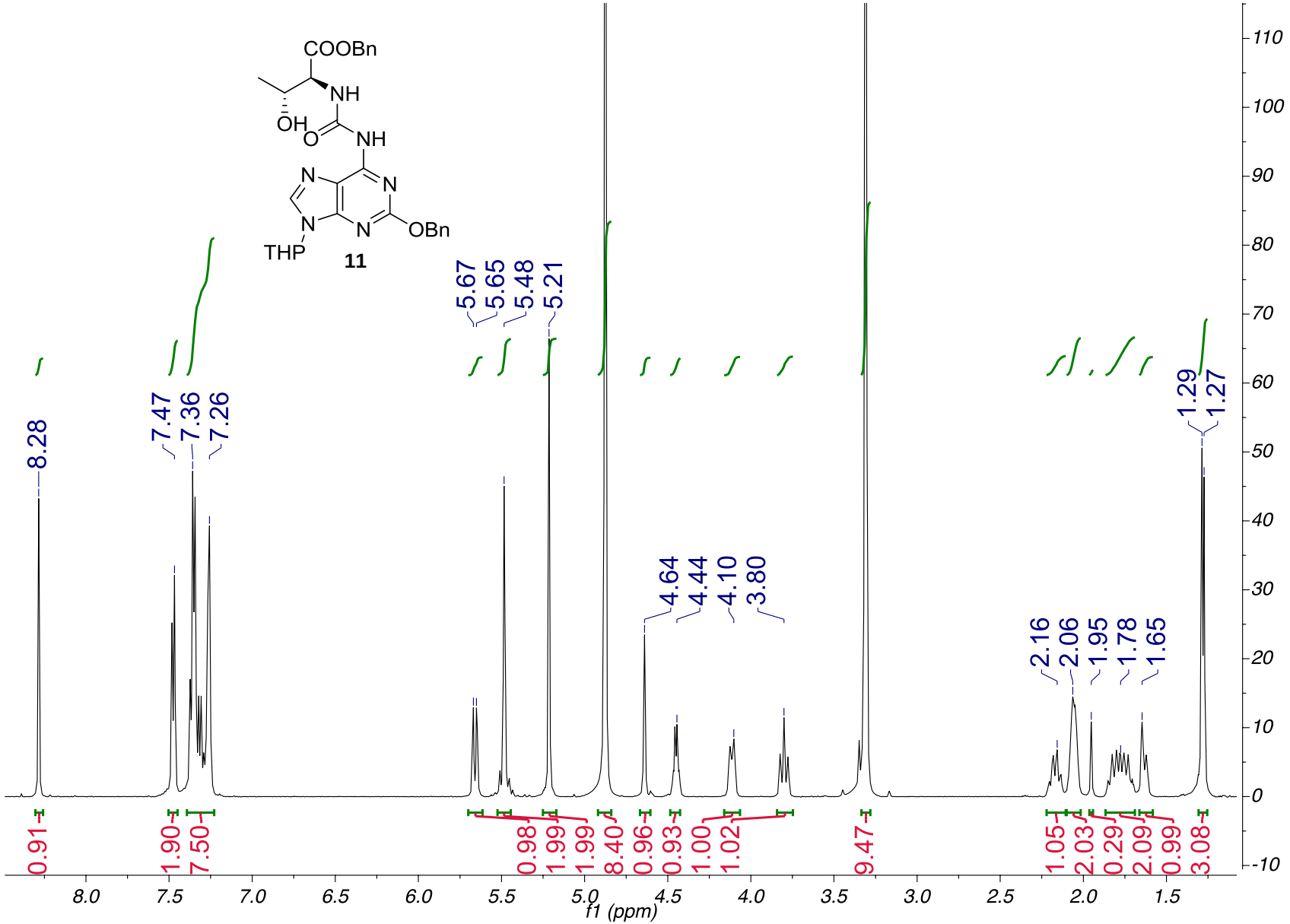
**$^1$H NMR Spectrum (600 MHz, DMSO-$d_6$) of 6**



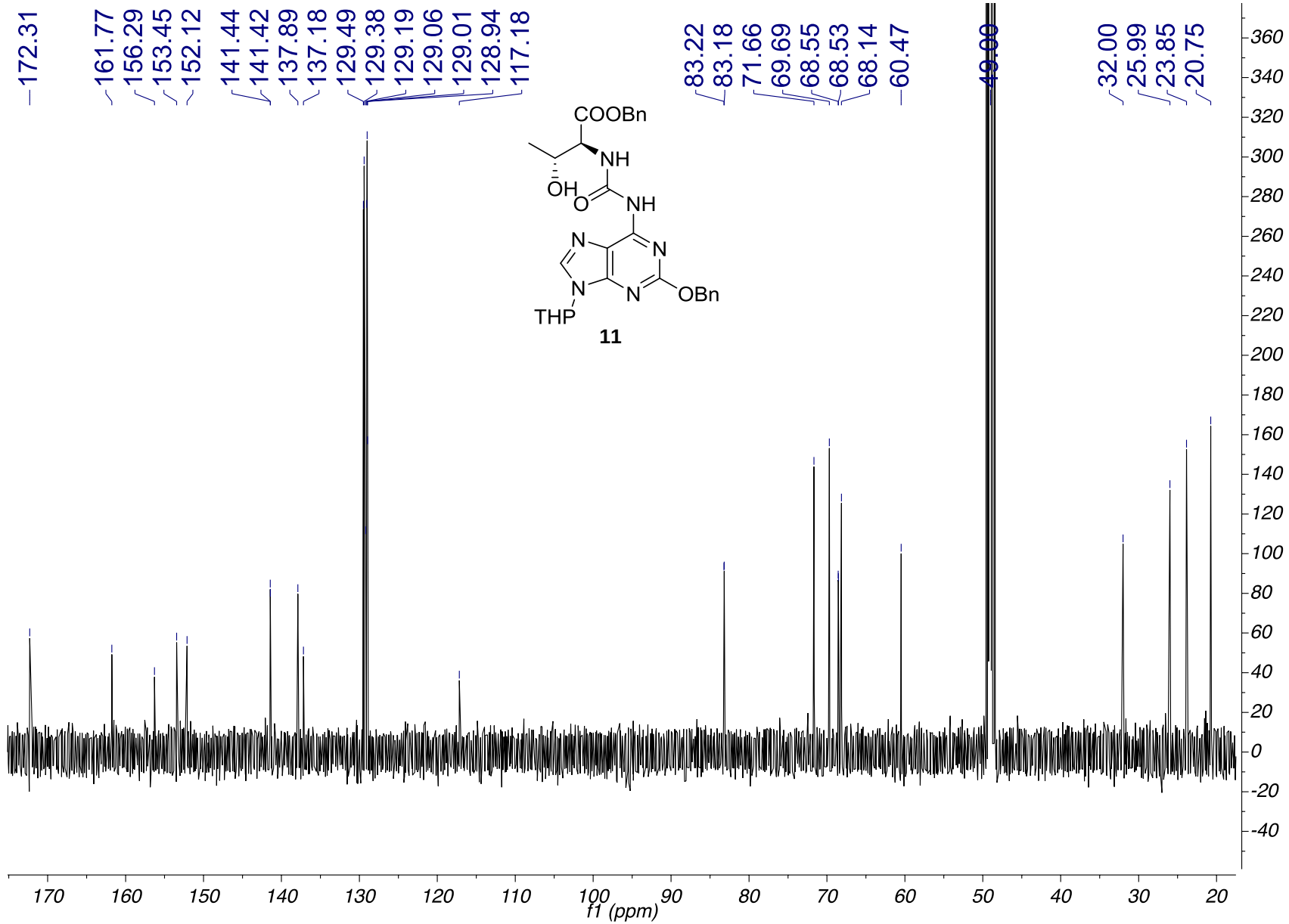
**$^{1}$H NMR Spectrum (500 MHz, chloroform-*d*) of 8**



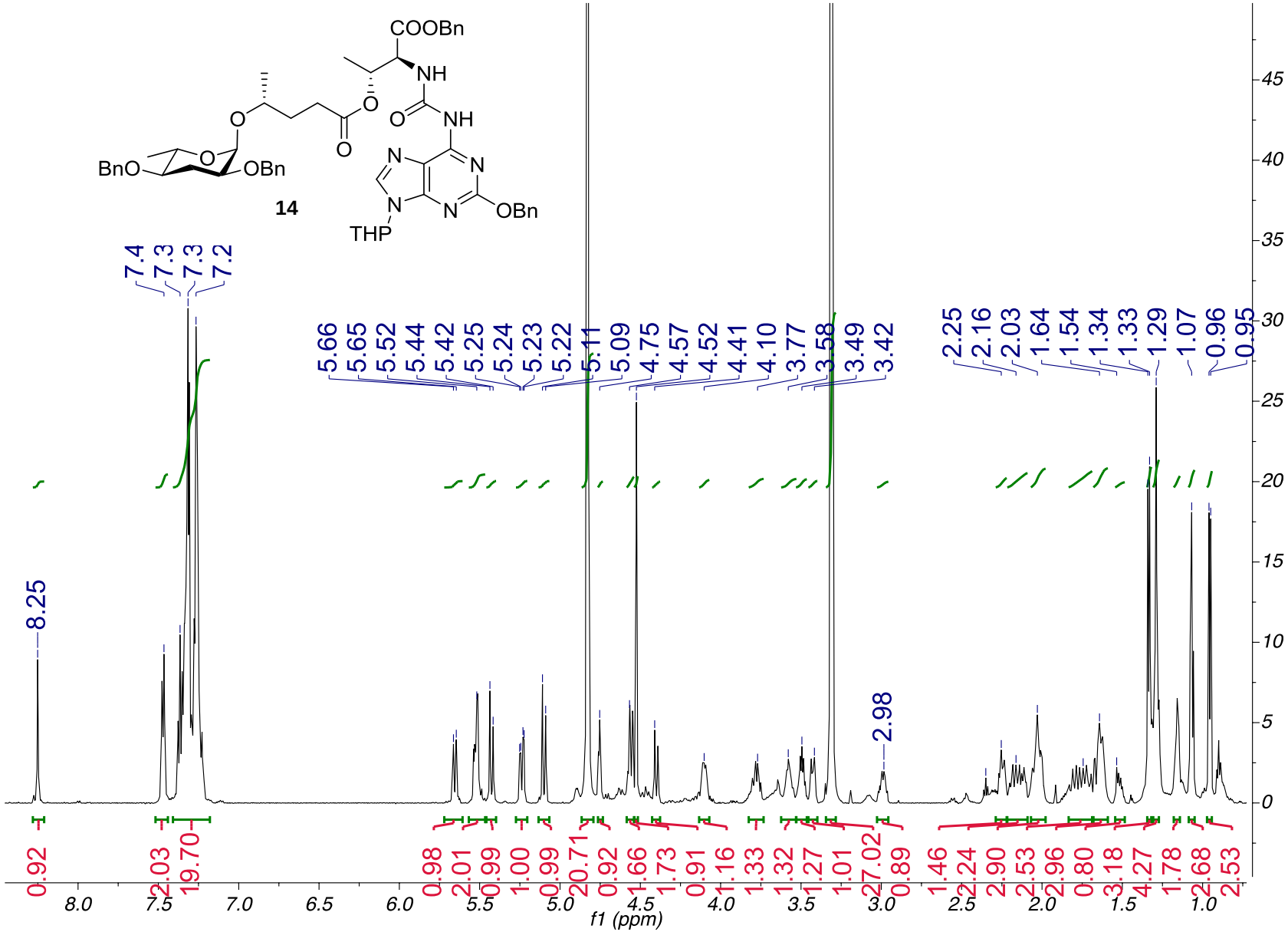
**$^1$H NMR Spectrum (500 MHz, methanol-$d_4$) of 11**



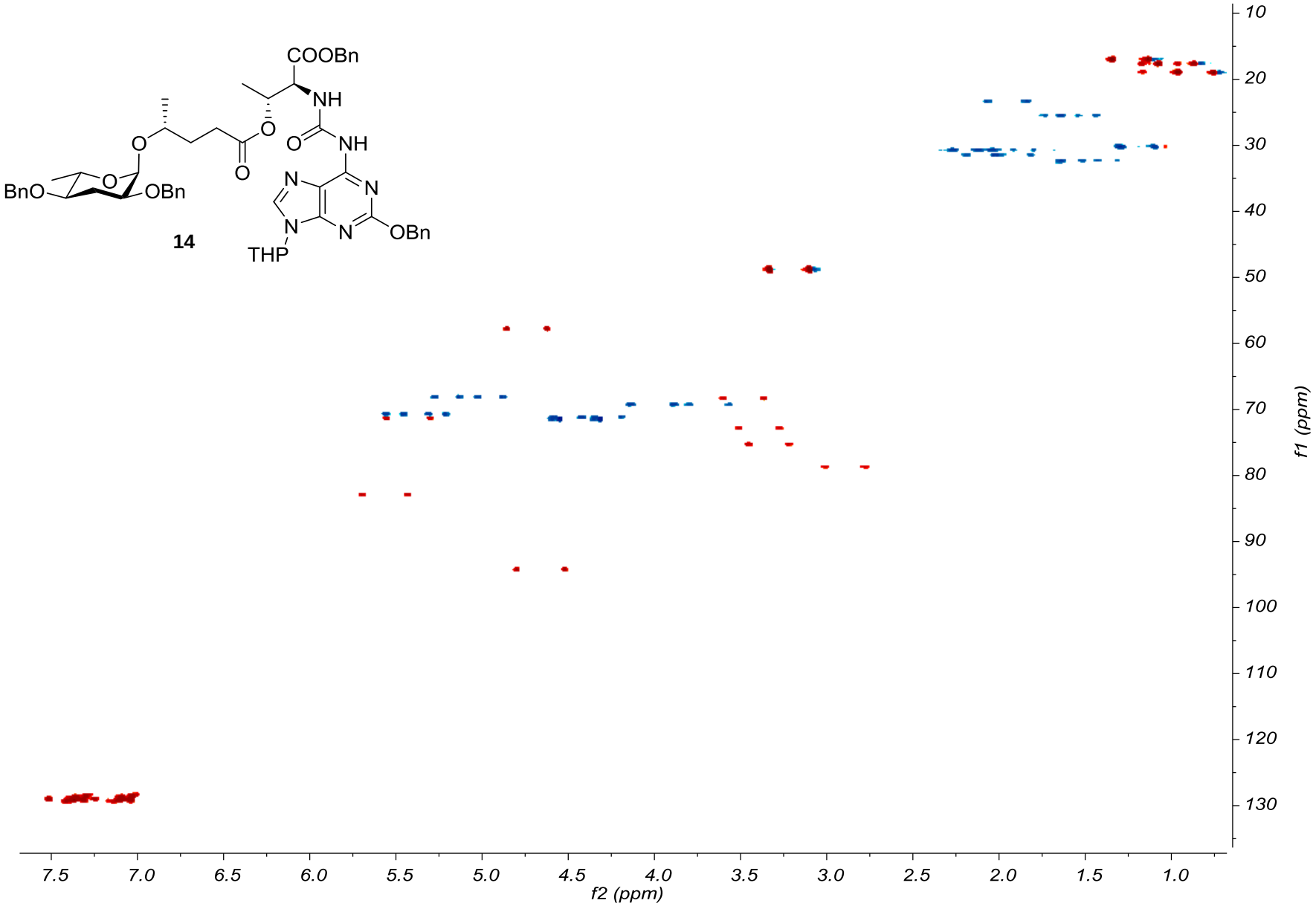
**$^{13}$C NMR Spectrum (125 MHz, methanol-$d_4$) of 11**



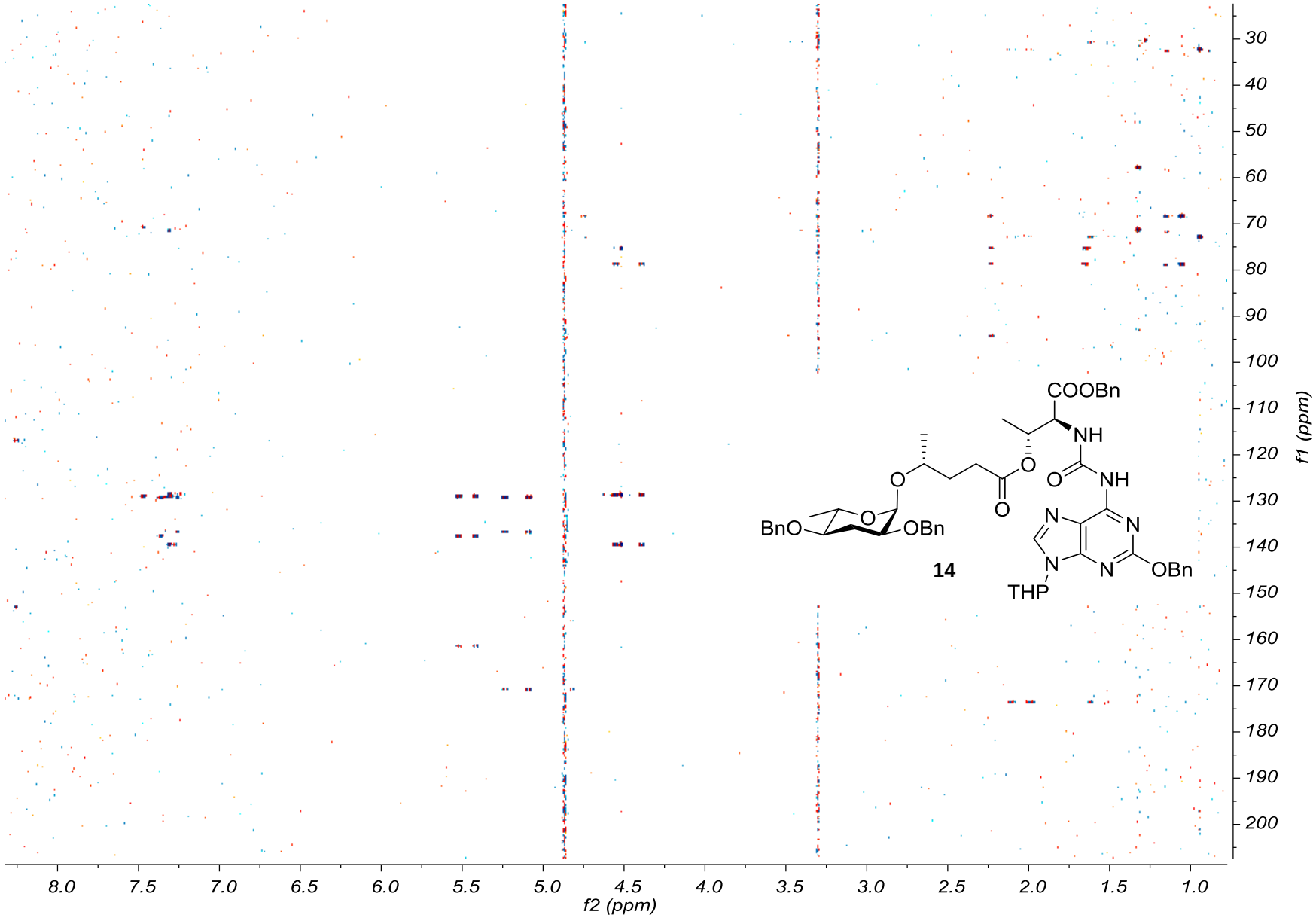
**$^1$H NMR Spectrum (600 MHz, methanol-$d_4$) of 14**



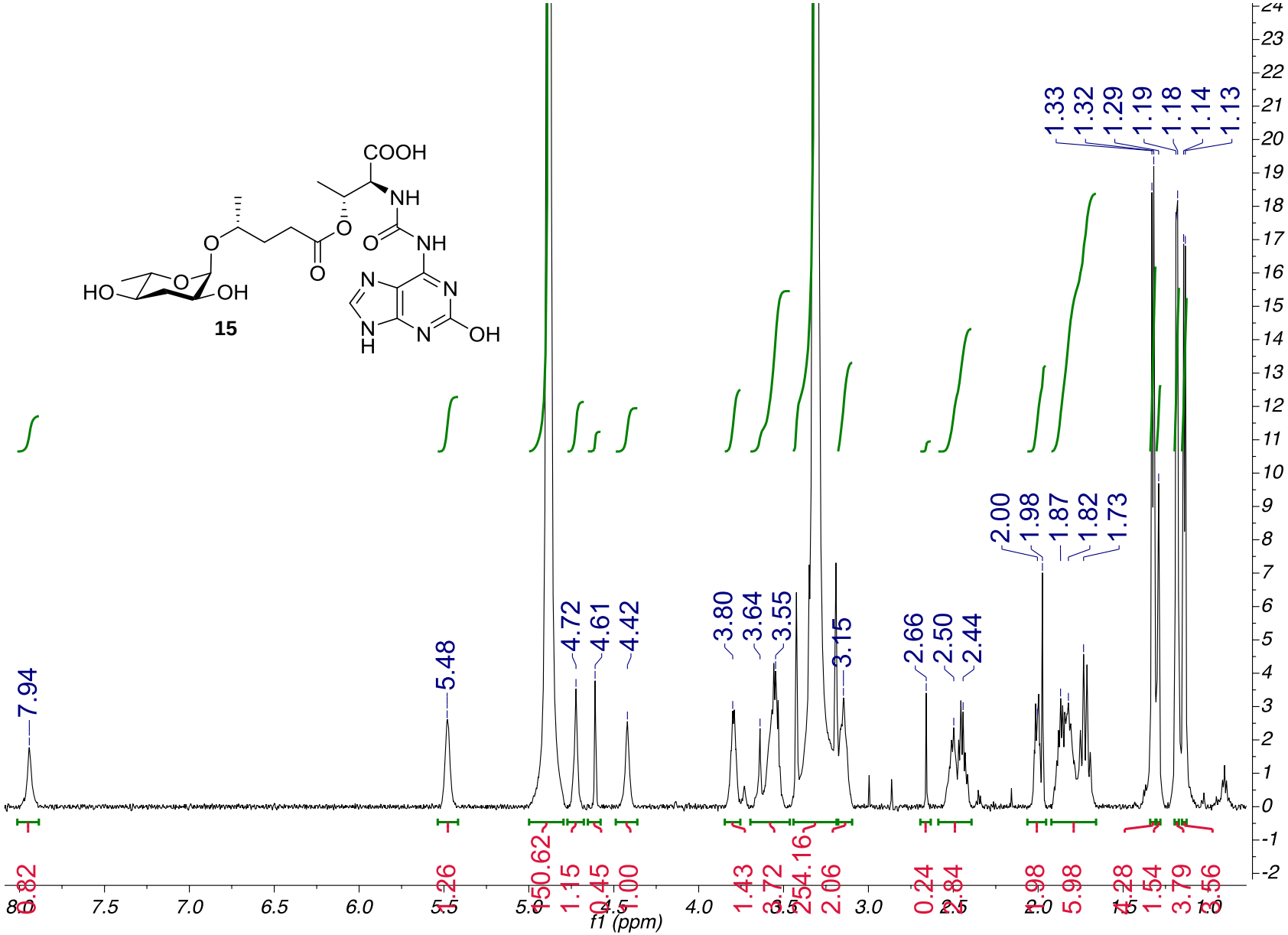
**HSQC Spectrum (600 MHz for $^{1}H$, 151 MHz for $^{13}C$, methanol-$d_4$) of 14**



**HMBC Spectrum (600 MHz for $^{1}H$, 151 MHz for $^{13}C$, methanol-$d_4$) of 14**



**$^1$H NMR Spectrum (600 MHz, methanol-$d_4$) of npar#3-isomer (2-oxo) (15)**



## 6. Supporting References

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3. Bose, N.; Ogawa, A.; von Reuss, S. H.; Yim, J. J.; Ragsdale, E. J.; Sommer, R. J.; Schroeder, F. C., Complex small-molecule architectures regulate phenotypic plasticity in a nematode. *Angew Chem Int Ed Engl* **2012,** *51*, 12438-43.

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