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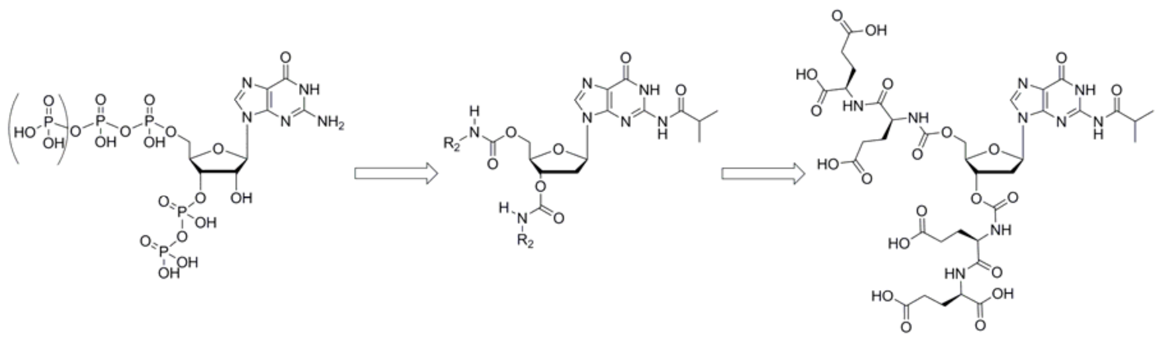

(p)ppGpp

General structure
of RelA inhibitors

Compound 2d

Design, Synthesis and Structure-Activity Relationship of novel Relacin analogues as inhibitors of Rel proteins

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1. Abstract

Rel proteins in bacteria synthesize the signal molecules (p)ppGpp that trigger the Stringent Response, responsible for bacterial survival. Inhibiting the activity of such enzymes prevents the Stringent Response, resulting in the inactivation of long-term bacterial survival strategies, leading to bacterial cell death. Herein, we describe a series of deoxyguanosine-based analogues of the Relacin molecule that inhibit *in vitro* the synthetic activity of Rel proteins from Gram positive and Gram negative bacteria, providing a deeper insight on the SAR for a better understanding of their potential interactions and inhibitory activity. Among the inhibitors evaluated, compound **2d** was found to be more effective and potent than our previously reported Relacin.

Keywords: Antibacterial, Stringent response, (p)ppGpp, RelA, Purine

2. Introduction

The Stringent Response is a process crucial for bacterial viability as it activates a series of long term bacterial survival pathways including the switch into stationary phase of growth, sporulation, and biofilm formation[1-4]. This response is triggered by the accumulation of the signal molecules 5'-triphosphate-3'-diphosphate and 5'-3'-bis-diphosphate, collectively called (p)ppGpp[5] (Figure 1A). In Gram negative bacteria (p)ppGpp is mostly synthesized by RelA and hydrolyzed by SpoT, while in Gram positive bacteria a bifunctional enzyme, Rel/Spo, both synthesizes and hydrolyses (p)ppGpp[6, 7].

RelA is a ribosome-associated (p)ppGpp synthetase activated in response to amino-acid starvation[8]. During amino-acid deprivation the binding of uncharged tRNAs to the ribosomal ‘A’ site stalls protein synthesis, stimulating a reaction in which RelA transfers a pyrophosphoryl group comprising the β- and γ-phosphates of an ATP donor to the 3′-hydroxy group of GTP or GDP to form pppGpp and ppGpp, respectively[8]. Despite the low abundance of RelA, up to mM levels of (p)ppGpp are rapidly produced. This is possible since (p)ppGpp synthesis evokes dissociation of RelA from the ribosome, allowing the enzyme to “hop” to another stalled ribosome and repeat the reaction[9].

Recently, the crystal structure of the catalytic N-terminal fragment of the bifunctional RelA homologue from the Gram positive bacterium *Streptococcus equisimilis* (Rel*seq*385) was determined[10]. In contrast to Gram negative bacteria, where RelA and SpoT are distinct proteins, this fragment displays both the hydrolase and synthetase active sites. The crystallographic analysis revealed two conformations for Rel*seq*385 that typify the opposing hydrolase-OFF/synthetase-ON and hydrolase-ON/synthetase-OFF states. The crystal structure also suggested a mechanism for the attack of the 3’-OH group of GDP (or GTP) onto the β-phosphorus atom of ATP. Most likely, Glu323 of Rel*seq*385 activates the hydroxyl group by proton abstraction. In addition, Glu323 also is suggested to coordinate the essential Mg^{2+} ion which is not present in the crystals and is expected to be bound to the pyrophosphate donor, ATP. Support for the critical role of Glu323 in the synthetic mechanism derives from a mutational study showing that a mutant Rel*seq*385 bearing a Glu323Gln substitution exhibited severely defective synthetase activity[10].

In the past few years, several efforts have been made toward the development of antibacterial agents based on nucleosides and their analogues. Such compounds have shown antimicrobial activity by inhibiting cell wall biosynthesis[11], DNA ligases[12], riboflavin synthesis[13], polyamine biosynthesis and quorum sensing[14], nucleoside phosphorylases[15], and siderophore biosynthesis[16] among others.

In the search for novel strategies to combat multi-drug resistant bacteria, we have explored the Stringent Response as a potential target for the development of a new generation of antibiotics[17, 18]. We have recently reported on Relacin[18] (Figure 1B), a novel ppGpp analog that prevents the activation of the Stringent Response by inhibiting (p)ppGpp production. Relacin perturbs the switch into stationary phase in Gram positive bacteria and leads to cell death. Furthermore, Relacin inhibits sporulation and biofilm formation; additional bacterial long term survival strategies used by such bacteria. In this report we present the synthesis of a series of symmetrically and asymmetrically substituted analogues of Relacin, designed at gaining a deeper understanding on the importance of the moieties at positions 5' and 3' on the deoxyribose ring and their potential interactions within the active site of Rel Proteins. The inhibitory potential of the prepared compounds was biochemically evaluated on the (p)ppGpp synthetase activity of RelA and Rel/Spo purified from *Escherichia coli* (*E. coli*) and *Deinococcus radiodurans* (*D. radiodurans*), respectively.

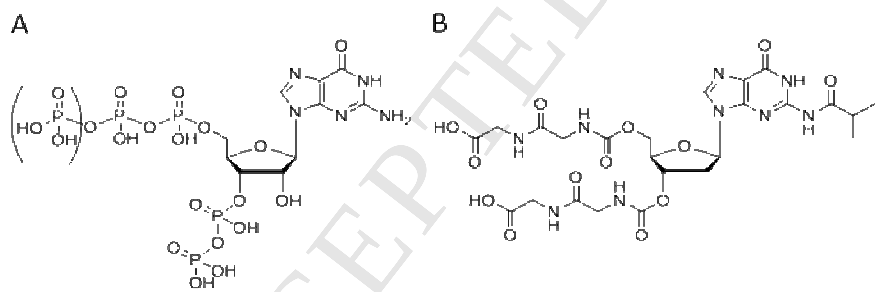


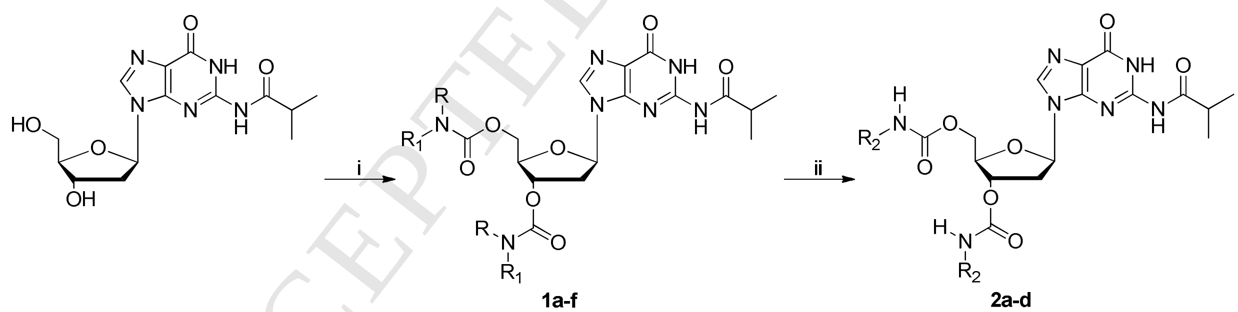
Figure 1

3. Results and Discussion

Design of ppGp(p) analogs.

Relacin, our model compound, has a di-peptide (Gly-Gly) at both 3' and 5' positions of the deoxyguanosine. This active analog has the following features: (1) symmetry in the choice of the di-peptide added to both 3’ and 5’ positions and, (2) two negative charges (at physiological pH) from the two acidic C-termini.

Our first goal was to compare the activity of Relacin to other symmetrically substituted compounds. As shown in Scheme 1, such analogues were prepared by mixing 2-isobutyryl-2’-deoxyguanosine with an excess of carbonyldiimidazole (CDI) in acetonitrile overnight. After the addition of CDI, the suspension completely dissolved and eventually the di-imidazolide derivative precipitated. After stirring overnight, the precipitate was filtered and re-suspended in dichloromethane (DCM). The different amino substituents (Table 1) were dissolved in DCM containing an excess of diisopropylethylamine (DIEA) and added to the former suspension. The mixture was stirred at room temperature until the reaction ended (according to TLC analysis). The final products were obtained after hydrogenolysis of the benzyl ester group to afford compounds **2 a-d**.



Scheme 1

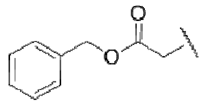
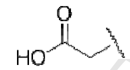
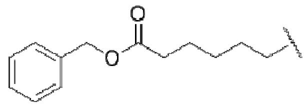
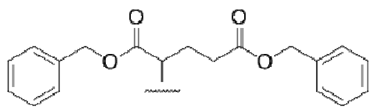
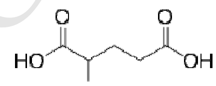
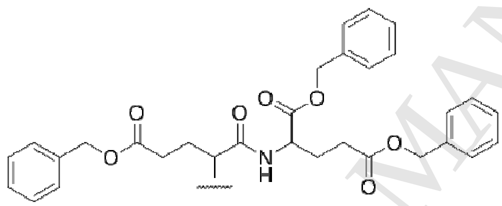
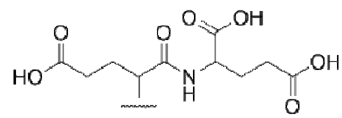
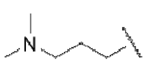
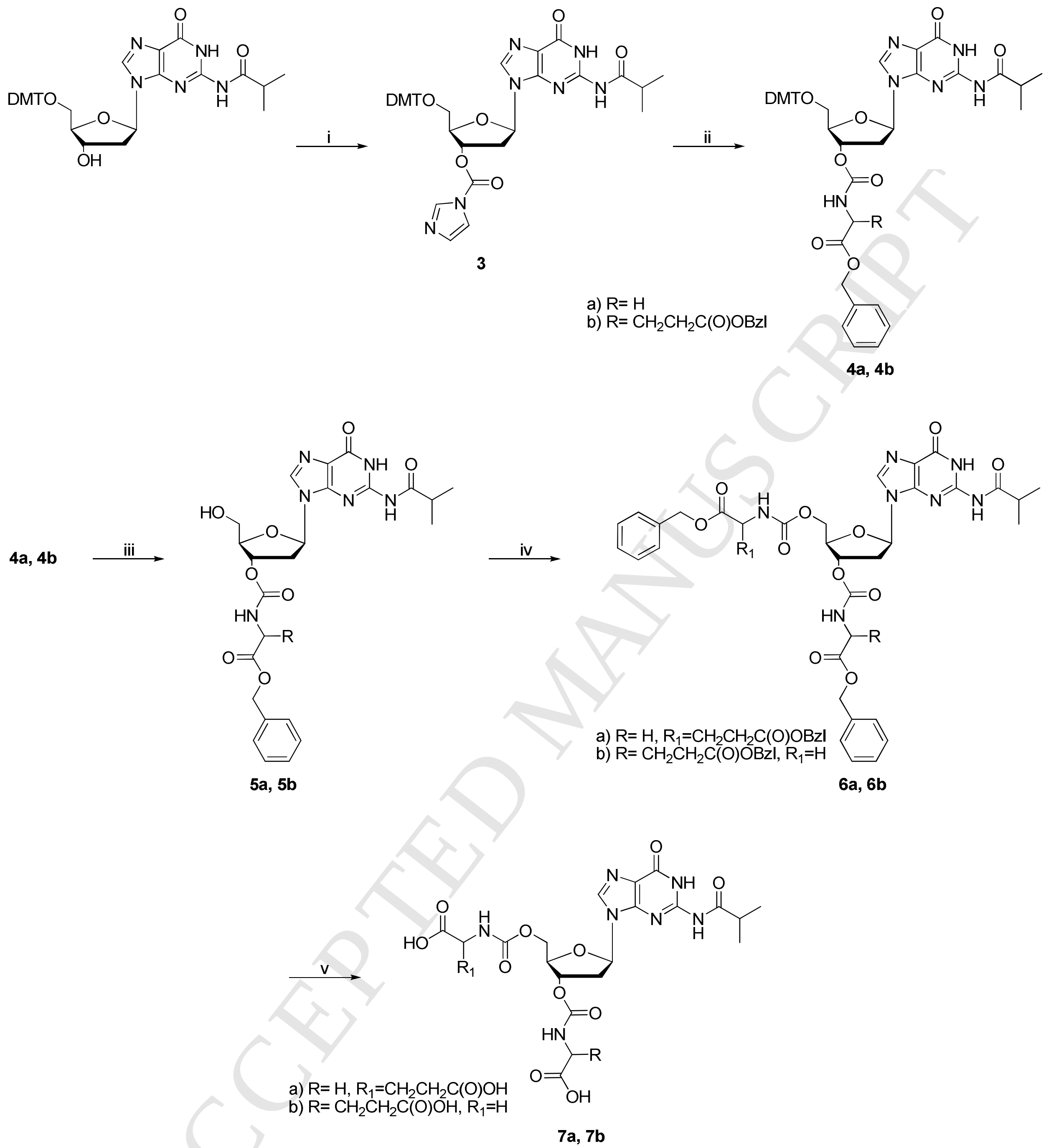
Comp.	R	R1	Comp.	R2
1a	H		2a	
1b	H		2b	
1c	H		2c	
1d	H		2d	
1e	H			
1f				

Table 1. Symmetric substituents on the 3' and 5' positions of the deoxyguanosine.

As RelA may accommodate different size substituents at the 3' and 5' positions of deoxyguanosine, we decided to explore the activity of a family of Relacin analogues that are asymmetrical. Scheme 2 depicts the synthesis of the asymmetrically substituted compounds. The first step was the formation of an imidazolide intermediate at position 3' of the suitable DMTprotected 2'-deoxyguanosine (**3**). This intermediate was prepared adapting the procedure reported by Korshun et al. [19]. Next, the benzyl esters of either glycine or glutamic acid were added to a solution of the freshly prepared imidazolide in DCM containing DIEA. The mixture was stirred overnight. After acidic workup and column chromatography, the dimethoxytrityl (DMT) group was cleaved from compounds **4 a-b** to yield compounds **5 a-b** and the free hydroxyl moiety at position 5' was reacted with a slight excess of CDI in DCM. When all the starting material was consumed, (typically after 15 hours) an excess of the benzyl esters of either glycine or glutamic acid was added together with DIEA and the mixture was stirred overnight until the completion of the reaction to afford compounds **6 a-b**. After a further chromatographic purification step, the benzyl protecting groups were cleaved as described for the symmetric analogues resulting in the final products **7 a-b**.



Scheme 2

The potential inhibitors were tested *in vitro* on purified Rel proteins from both Gram positive and Gram negative bacteria using reported methods[18] . The results, summarized in Table 2, show that addition of basic moieties (**1 e-f**) lowers the inhibitory action of the compounds. Moreover, compound **1f**, with four dimethylamino groups is less active than compound **1e**, bearing only two such moieties. Replacement of this dimethylamino group by a carboxylic acid (compound **2a**) markedly improved the inhibitory activity while the addition of a second carboxylic acid (compound **2c**) did not result in a significant change in the inhibitory efficacy in comparison to **2a**. In addition, combining a glycine derivative moiety with a derivative of glutamic acid as in the asymmetric compounds (**7 a-b**) showed a similar result regardless of the position of the mono or di-acidic substitutents.

Out of the 8 new analogs prepared, compound **2d** had the highest inhibitory activity (80.4% at 1 mM, see table 2) whereas 4 out of the newly prepared compounds (**2a**, **2c**, **7a**, **7b**) had all about a 50% inhibitory effect (see table 2). Therefore, the inhibitory activity of the compounds **2a** and **2d** were further tested and compared. While compound **2a** features two glycine moieties linked by a carbamate bridge to carbons C3' and C5', compound **2d** presents two glutamyl-glutamic acid moieties at the same positions. The main differences between these two compounds are in their bulkiness and in the number of negatively charged sites (at physiological pH) for potential interactions within the catalytic site of the enzymes. Compound **2a** decreased (p)ppGpp levels in *B.subtillis* cells *in vivo* after the onset of the stringent response, by the addition of serine hydroxamate (SHX) (Figure 2A, gray bars). In comparison, compound **2d** was significantly more active in reducing (p)ppGpp levels (Figure 2A, white bars).

We next evaluated the in-vitro activity at varying concentrations of compound **2d** as an inhibitor of Rel enzymes from Gram positive (*D. radiodurans)* and Gram negative (*E. coli*) bacteria. Figure 2B presents the data resulting in a dose dependent inhibitory pattern for both enzymes. Notably compound **2d** was significantly more effective inhibiting RelA than Rel/Spo (Figure 2B), suggesting different interactions within their active sites.

Further, compound **2b** with a hexanoic acid substituent similar in length to Relacin, is a much less effective inhibitor. This indicates the importance of the amide bond, most likely involved in the formation of hydrogen bonds at the active site. Thus, compound **2d** bearing two glutamyl-glutamic acid moieties, i.e. six carboxylic acids in total, was found to be a more effective inhibitor and more potent than our previously reported Relacin. Compound **2d** is more acidic and voluminous than Relacin, providing more potential interactions including hydrogen bonds and electrostatic salt bridges. The isobutyryl group at position N2 in the nucleobase was predicted to form important hydrophobic interactions between Relacin and the active site of Rel/Spo from *Streptococcus equisimilis* [18]. However, removing it from our inhibitors didn't clearly point at an increase or a decrease in the inhibitory activity (data not shown).

Compound	1e	1f	2a	2b	2c	**2d**	7a	7b	Relacin[a]
% Inhibition	31.2	12.0	51.1	25.6	50.6	**80.4**	52.1	50.4	66.3
(±SD)	9.4	1.7	4.5	5.7	4.3	**4.1**	2.8	0.1	2.1

Table 2. Comparison of inhibitory potential (1 mM of inihbitor) on RelA isolated from *E. Coli.*

[a] data published by Wexselblatt et al. [18]

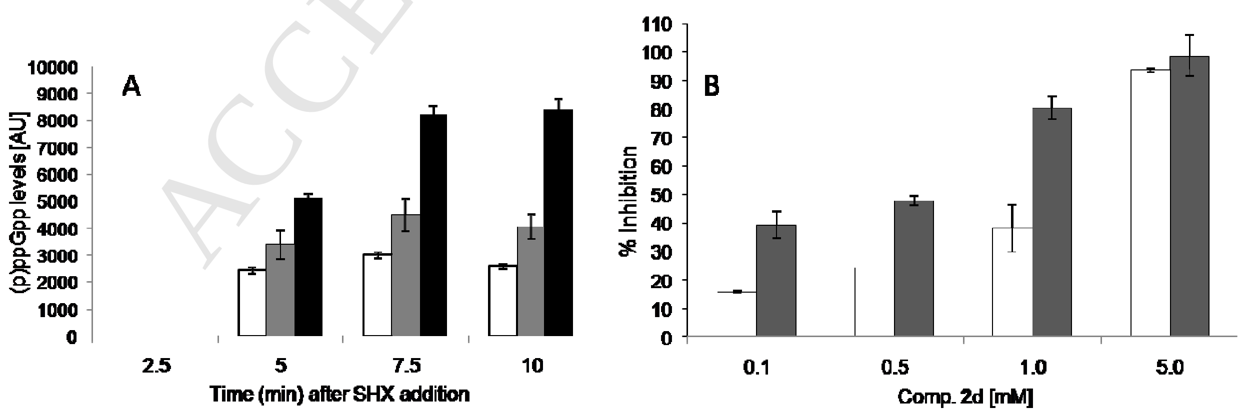


Figure 2

Taken together, the data presented in this work will be useful for the future design of new generations of potential antibacterial agents aimed at inhibiting the Stringent Response. The effect of compound **2d** on bacterial survival will be the subject of future work.

4. Experimental

4.1 Materials and Instruments

All chemicals and reagents were purchased from Sigma-Aldrich Ltd. and used without further purification. Ultra-dry solvents stored with molecular sieves under an argon atmosphere were purchased from Acros Ltd. HPLC solvents were acquired from Bio Lab Ltd.

NMR:

NMR data were collected with a Varian VXR-300 MHz spectrometer equipped with a 5-mm switchable probe. Data were processed using the VNMR and MestreNova software. NMR abbreviations are as follows: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br (broad).

High Resolution Mass Spectroscopy (HRMS):

HRMS was carried out with an Orbi-trap MS (Thermo Finnigen) using nanospray attachment. MRFA (met-arg-phe-ala) peptide was used as internal standard. Data were processed using bioworks 3.3 package.

Electron Spray Ionization Mass Spectroscopy (ESI-MS):

Electrospray ionization mass spectrometry was carried out using a ThermoQuest Finnigan LCQ-Duo in the positive ion mode. Data were processed using ThermoQuest Finnigan's Xcalibur™ Biomass Calculation and Deconvolution software.

HPLC:

Analytical HPLC was performed using a Shimadzu LC-2010 *HT* HPLC system equipped with a UV detector using a Merck RP-C18 25X4.6-mm column. Preparative and semi-preparative chromatography fractions were collected on a SpectraSystem SCM 1000 HPLC system equipped with a Gilson 117 UV detector, using Phenomenex RP-C18 25X10-mm and 25X22-mm columns.

4.2 Chemical synthesis

General procedure for the preparation of compounds 1 a-f

2-Isobutyryl-2'-deoxyguanosine (200 mg, 0.6 mmol) was suspended in dry acetonitrile (10 mL) and four equivalents of carbonyldiimidazole were added. After stirring overnight at room temperature, the precipitate was filtered and re-suspended in dry dichloromethane (10 mL). Four equivalents of the carboxy-protected R,R'-NH_2 and eight equivalents of diisopropylethylamine were added. The suspension was stirred at room temperature from two to 48 hours.

At the end of the reaction water was added and the organic phase was washed three times with 5% aqueous citric acid (20 mL). After drying and evaporating, the crude material was purified by column chromatography (compounds **1 a-d,** DCM to MeOH/DCM 1:10) or by RP-HPLC (compounds **1 e-f**). The desired compounds were obtained as white powders.

Benzyl ((((2R,5R)-3-(((2-(benzyloxy)-2-oxoethyl)carbamoyl)oxy)-5-(2-isobutyramido-6-oxo-1,6-dihydro-9H-purin-9-yl)tetrahydrofuran-2-yl)methoxy)carbonyl)glycinate (1a)

White powder, 563 mg, 65% (0.78 mmol)

ESI-MS: Calc: 719.70 Found $[M+H^+]$: 720.83

^{1}H NMR (DMSO-d6): 8.24 (s, 1H, H-8), 7.87 (m, 2H, N*H*CH_2), 7.33 (m, 10H, Ar), 6.2 (t, *J*=15 Hz, 1H, H-1'), 5.13 (d, *J*=5.7 Hz, 1H, H-3'), 5.12 (s, 4H, C*H*$_2$-Phenyl), 4.17 (s, 3H, H4', H5'), 3.84 (t, *J*= 12.9 Hz, 4H, C*H*$_2$), 2.9 (m, 1H, H-2'), 2.7 (m,1H, C*H* (iBu)), 2.4 (m, 1H, H-2'), 1.1 (d, *J*=6.9 Hz, 6H, C*H*$_3$ (iBu))

Benzyl 6-(((((2R,3S,5R)-3-(((6-(benzyloxy)-6-oxohexyl)carbamoyl)oxy)-5-(2-isobutyramido-6-oxo-1,6-dihydro-9H-purin-9-yl)tetrahydrofuran-2-yl)methoxy)carbonyl)amino)hexanoate (1b)

White powder, 380 mg, 77% (0.46 mmol)

ESI-MS: Calc: 831.38 Found [$M+H^+$]: 832.0

^{1}H NMR (DMSO-d6): 8.19 (s, 1H, H-8), 7.33 (m, 12H, 10H-Ar and 2H-N*H*CH_2), 6.2 (t, *J*=15 Hz, 1H, H-1'), 5.13 (d, *J*=5.7 Hz, 1H, H-3'), 5.05 (s, 4H, CH_2-Phenyl), 4.17 (m, 3H, H4', H5'), 2.94 (m, 5H, H-2' and CH_2), 2.7 (m,1H, C*H* (iBu)), 2.3 (m, 5H, H-2' and CH_2), 1.5-1.2 (m, 16H, CH_2), 1.1 (d, *J*=6.9 Hz, 6H, CH_3 (iBu))

Dibenzyl ((((2R,5R)-3-(((1,5-bis(benzyloxy)-1,5-dioxopentan-2-yl)carbamoyl)oxy)-5-(2-isobutyramido-6-oxo-1,6-dihydro-9H-purin-9-yl)tetrahydrofuran-2-yl)methoxy)carbonyl) glutamate (1c)

White powder, 220 mg, 35% (0.21 mmol)

ESI-MS: Calc: 1043.39 Found [$M+H^+$]: 1044.16

^{1}H NMR (DMSO-d6): 8.24 (s, 1H, H-8), 7.91 (m, 2H, N*H*CH_2), 7.33 (m, 20H, Ar), 6.15 (t, *J*=15 Hz, 1H, H-1'), 5.13-5.04 (m, 9H, H-3' and CH_2-Phenyl), 4.15 (m, 5H, H4', H5' and C*H*), 2.9 (m, 1H, H-2'), 2.7 (m,1H, C*H* (iBu)), 2.4 (m, 5H, H-2' and CH_2), 2.1-1.8 (m, 4H, CH_2), 1.1 (d, *J*=6.9 Hz, 6H, CH_3 (iBu))

Tribenzyl ((((2R,5R)-3-(((glutamylglutamate)carbamoyl)oxy)-5-(2-isobutyramido-6-oxo-1,6-dihydro-9H-purin-9-yl)tetrahydrofuran-2-yl)methoxy)carbonyl)glutamylglutamate (1d)

For the preparation of compound **1d**, dipeptide H-Glu(Bzl)-Glu(Bzl)-OBzl was synthesized as previously reported by Nakata et al. [20]

White powder, 320 mg, 36% (0.22 mmol)

ESI-MS: Calc: 1482.54 Found $[M+H^+]$: 1483.43

^{1}H NMR (DMSO-d6): 8.46 (m, 2H, N*H* (peptide bond)), 8.26 (m, 2H, H-8 and N*H* carbamate), 7.64 (d, *J*=7.8 Hz, 1H, N*H* carbamate), 7.33 (m, 30H, Ar), 6.18 (t, *J*=15 Hz, 1H, H-1'), 5.17 (d, *J*=5.8 Hz, H-3'), 5.12 (bs, 12H, CH_2-Phenyl), 4.42 (m, 2H, C*H*), 4.17 (m, 3H, H4'and H5'), 3.89 (m, 2H, C*H*), 2.9 (m, 1H, H-2'), 2.7 (m,1H, C*H* (iBu)), 2.48 (m, 8H, CH_2), 2.4 (m, 1H, H-2'), 2.0 (m, 8H, CH_2), 1.1 (d, *J*=6.9 Hz, 6H, CH_3 (iBu))

(2R,5R)-2-((((2-(dimethylamino)ethyl)carbamoyl)oxy)methyl)-5-(2-isobutyramido-6-oxo-1,6-dihydro-9H-purin-9-yl)tetrahydrofuran-3-yl (2-(dimethylamino)ethyl)carbamate (1e)

White powder, 73 mg, 21% (0.13 mmol)

HRMS: Calc $[M+H^+]$: 566.3045 Found: 566.3045

^{1}H NMR (DMSO-d6): 7.89 (s, 1H, H-8), 7.35 (m, 2H, N*H*CH_2), 6.2 (t, *J*=15 Hz, 1H, H-1'), 5.11 (d, *J*=5.8 Hz, H-3'), 4.1 (m, 3H, H4'and H5'), 3.06 (q, *J*=18.5 Hz, 5H, H-2' and CH_2CH_2NH), 2.85 (m,1H, C*H* (iBu)), 2.25 (t, J=12.9 Hz, 4H, CH_2CH_2NH), 2.5 (m, 1H, H-2'), 2.11 (s, 12H, NCH_3), 1.1 (d, *J*=6.9 Hz, 6H, CH_3 (iBu)) ^{13}C NMR (75 MHz, DMSO-d6) δ 156.40, 155.78, 135.41, 82.86, 82.71, 75.30, 64.26, 58.95, 58.80, 45.67, 38.95, 36.81, 20.70.

(2R,5R)-2-(((bis(3-(dimethylamino)propyl)carbamoyl)oxy)methyl)-5-(2-isobutyramido-6-oxo-1,6-dihydro-9H-purin-9-yl)tetrahydrofuran-3-yl bis(3-(dimethylamino)propyl) carbamate (1f)

Colorless oil, 53 mg, 17% (0.07 mmol)

HRMS: Calc: 764.5141 Found: 764.5147

^{1}H NMR (DMSO-d6): 8.03 (s, 1H, H-8), 6.19 (t, *J*=15 Hz, 1H, H-1'), 5.19 (d, *J*=5.8 Hz, H-3'), 4.16 (m, 3H, H4'and H5'), 3.1 (m, 18H, H-2' and $Me_2NCH_2CH_2CH_2N$), 2.85 (m,1H, C*H* (iBu)), 2.25 (m, 8H, $Me_2NCH_2CH_2CH_2N$), 2.5 (m, 1H, H-2'), 2.11 (s, 24H, NCH_3), 1.1 (d, *J*=6.9 Hz, 6H, CH_3 (iBu)) 13C NMR (75 MHz, DMSO-d6) δ 180.85, 155.88, 155.33, 154.84, 149.29, 137.53, 120.85, 83.69, 82.76, 75.39, 64.66, 56.85, 56.76, 45.92, 45.65, 45.54, 45.46, 36.56, 35.50, 26.84, 26.20, 19.73, 19.47.

General procedure for the preparation of compounds 2 a-d:

Compounds **(1 a-d)** were dissolved in methanol. 10% Pd on activated carbon was added cautiously and the mixture was shaken for three hours under hydrogen atmosphere at 30 psi. The reaction mixture was then filtered and evaporated to yield the free carboxylic acids as white crispy compounds.

((((2R,5R)-3-(((carboxymethyl)carbamoyl)oxy)-5-(2-isobutyramido-6-oxo-1,6-dihydro-9H-purin-9-yl)tetrahydrofuran-2-yl)methoxy)carbonyl)glycine (2a)

White powder, 190 mg, 90% (0.35 mmol)

HRMS: Calc [$M+H^+$]: 540.1685 Found: 540.1685

^{1}H NMR (DMSO-d6): 8.24 (s, 1H, H-8), 7.64 (m, 2H, $NHCH_2$), 6.2 (t, *J*=15 Hz, 1H, H-1'), 5.16 (d, *J*=5.7 Hz, 1H, H-3'), 4.17 (s, 3H, H4', H5'), 3.66 (t, *J*= 12.3 Hz, 4H, CH_2), 2.9 (m, 1H, H-2'), 2.7 (m,1H, C*H* (iBu)), 2.4 (m, 1H, H-2'), 1.1 (d, *J*=6.9 Hz, 6H, CH_3 (iBu)) 13C NMR (75 MHz, DMSO-d6) δ 180.55, 171.88, 156.64, 156.10, 155.24, 149.13, 148.73, 137.67, 120.65, 83.22, 82.94, 75.66, 64.58, 42.62, 36.78, 35.23, 19.28.

6-(((((2R,5R)-3-(((5-carboxypentyl)carbamoyl)oxy)-5-(2-isobutyramido-6-oxo-1,6-dihydro-9H-purin-9-yl)tetrahydrofuran-2-yl)methoxy)carbonyl)amino)hexanoic acid (2b)

White powder, 250 mg, 88% (0.38 mmol)

HRMS: Calc [$M+H^+$]: 652.2937 Found: 652.2940

^{1}H NMR (DMSO-d6): 8.21 (s, 1H, H-8), 7.33 (m, 2H, N*H*CH_2), 6.1 (t, *J*=15 Hz, 1H, H-1’), 5.14 (d, *J*=5.7 Hz, 1H, H-3’), 4.16 (m, 3H, H4', H5'), 2.94 (m, 5H, H-2’ and CH_2), 2.7 (m,1H, C*H* (iBu)), 2.42 (m, 1H, H-2’), 2.3 (m, 4H, CH_2), 1.5-1.2 (m, 16H, CH_2), 1.1 (d, *J*=6.9 Hz, 6H, CH_3 (iBu)) 13C NMR (75 MHz, DMSO-d6) δ 180.51, 174.86, 156.22, 155.70, 155.24, 149.08, 148.67, 137.67, 135.55, 122.10, 120.71, 83.34, 83.09, 75.12, 64.13, 36.89, 35.24, 34.08, 29.54, 26.26, 24.65, 19.28.

((((2R,5R)-3-(((1,3-dicarboxypropyl)carbamoyl)oxy)-5-(2-isobutyramido-6-oxo-1,6-dihydro-9H-purin-9-yl)tetrahydrofuran-2-yl)methoxy)carbonyl)glutamic acid (2c)

White powder, 95 mg, 90% (0.14 mmol)

HRMS: Calc [$M+H^+$]: 684.2107 Found: 684.2108

^{1}H NMR (DMSO-d6): 8.25 (s, 1H, H-8), 7.65 (m, 2H, N*H*CH), 6.22 (t, *J*=15 Hz, 1H, H-1’), 5.13 (d, *J*=5.3 Hz, 1H, H-3’), 4.15 (m, 3H, H4', H5'), 3.97 (m, 2H, C*H*), 2.9 (m, 1H, H-2’), 2.7 (m,1H, C*H* (iBu)), 2.2 (m, 5H, H-2’ and CH_2), 2.0-1.7 (m, 4H, CH_2), 1.1 (d, *J*=6.9 Hz, 6H, CH_3 (iBu)) 13C NMR (75 MHz, DMSO-d6) δ 180.55, 174.15, 173.83, 156.28, 155.78, 155.25, 149.12, 148.74, 137.81, 125.34, 120.63, 83.16, 82.94, 75.57, 67.46, 64.65, 53.50, 36.94, 35.23, 30.88, 30.60, 29.44, 26.70, 26.59, 25.58, 19.30.

((((2R,5R)-3-(((glutamylglutamic acid)carbamoyl)oxy)-5-(2-isobutyramido-6-oxo-1,6-dihydro-9H-purin-9-yl)tetrahydrofuran-2-yl)methoxy)carbonyl)glutamylglutamic acid (2d)

White powder, 102 mg, 83% (0.1 mmol)

HRMS: Calc [M+H$^+$]: 942.2959 Found: 942.2921

^{1}H NMR (DMSO-d6): 8.46 (m, 2H, N*H* (peptide bond)), 8.26 (m, 2H, H-8 and N*H* carbamate), 7.64 (d, *J*=7.8 Hz, 1H, N*H* carbamate), 6.18 (t, *J*=15 Hz, 1H, H-1’), 5.17 (d, *J*=5.8 Hz, H-3’), 4.42 (m, 2H, C*H*), 4.17 (m, 3H, H4'and H5'), 3.89 (m, 2H, C*H*), 2.9 (m, 1H, H-2’), 2.7 (m,1H, C*H* (iBu)), 2.48 (m, 8H, CH_2), 2.4 (m, 1H, H-2’), 2.0 (m, 8H, CH_2), 1.1 (d, *J*=6.9 Hz, 6H, CH_3 (iBu)) 13C NMR (75 MHz, DMSO-d6) δ 180.62, 177.85, 174.36, 174.29, 174.23, 173.51, 172.95, 171.77, 168.27, 155.26, 149.11, 148.72, 137.82, 120.61, 83.18, 61.92, 55.80, 54.23, 53.67, 51.84, 51.74, 35.19, 30.75, 30.68, 29.79, 29.58, 28.61, 27.83, 26.92, 25.51, 19.30.

(2R,3S,5R)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-5-(2-isobutyramido-6-oxo-1,6-dihydro-9H-purin-9-yl)tetrahydrofuran-3-yl 1H-imidazole-1-carboxylate (3):

2-Isobutyryl-5’-DMT-2’deoxyguanosine (10.6 g, 16.5mmol) was dissolved in 140 mL dry DCM and carboyldiimidazole (13.4 g, 82.5 mmol) was added. The mixture was stirred at room temperature for four hours and washed with water. After drying and evaporating the organic phase, the desired compound was obtained as a yellowish foam. No further purification was required. Yield: 11.2 g, 92.7% (15.3 mmol)

ESI-MS: Calc: 733.29 Found [M+H$^+$]: 734.04

^{1}H NMR (DMSO-d6): 8.34 (s, 1H, Imidazole), 8.18 (s, 1H, H-8), 7.69 (s, 1H, Imidazole), 7.31 (d, *J*=7.4 Hz, 2H, DMT), 7.18 (d, *J*=8.4 Hz, 8H, DMT), 7.09 (s, 1H, Imidazole), 6.77 (t, *J*=17.7 Hz, 3H, DMT), 6.37 (t, *J*=14.3 Hz, 1H, H-1'), 5.5 (d, *J*=5.9 Hz, 1H, H-3'), 4.45 (m, 1H, H-4'), 3.7 (s, 6H, OCH_3), 3.41 (t, *J*= 18 Hz, 1H, H-5'), 3.19 (m, 2H, H-5' and H-2'), 2.84-2.7 (m, 2H, H-2' and CH (iBu)), 1.1 (d, *J*=6.74 Hz, 6H, CH_3 (iBu))

General procedure for the preparation of compounds 4 a-b:

Compound (**3**) was dissolved in dry DCM. 2 equivalents of either H-Gly-OBzl or H-Glu(OBzl)-OBzl and 4 equivalents of diisopropylethylamine were added. After stirring overnight, the mixture was washed with citric acid, dried, evaporated and purified by column chromatography (DCM to MeOH/DCM 1:10) yielding white powders.

Benzyl ((((2R,3S,5R)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-5-(2-isobutyramido-6-oxo-1,6-dihydro-9H-purin-9-yl)tetrahydrofuran-3-yl) oxy) carbonyl) glycinate (4a)

White powder, 350 mg, 68% (0.4 mmol)

ESI-MS: Calc: 830.88 Found [M+H$^+$]: 831.71

^{1}H NMR (DMSO-d6): 8.13(s, 1H, H-8), 7.81 (t, *J*=12.36 Hz, 1H, *NH*CH), 7.33 (m, 7H, Ar and DMT), 7.18 (d, *J*=8.4 Hz, 8H, DMT), 6.77 (t, *J*=17.7 Hz, 3H, DMT), 6.25 (t, *J*=13.6 Hz, 1H, H-1'), 5.16-5.12 (s+bs, 3H, CH_2-Phenyl and H3'), 4.1 (m, 1H, H-4'), 3.83 (d, *J*=6.12 Hz, 2H, CH_2), 3.7 (s, 6H, OCH_3), 3.15-2.95 (m, 2H, H-5'), 2.74 (m, 1H, CH (iBu)), 1.1 (d, *J*=6.74 Hz, 6H, CH_3 (iBu))

dibenzyl((((2R,3S,5R)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-5-(2-isobutyramido-6-oxo-1,6-dihydro-9H-purin-9-yl)tetrahydrofuran-3-yl)oxy)carbonyl)-L-glutamate (4b)

White powder, 3.6 g, 53% (3.6 mmol)

ESI-MS: Calc: 992.4 Found [M+H$^+$]: 993.20

^{1}H NMR (DMSO-d6): 8.11 (s, 1H, H-8), 7.90 (d, *J*=7.31 Hz, 1H, *NH*CH), 7.36 (d, *J*=7.4 Hz, 2H, DMT), 7.33 (m, 10H, Ar), 7.16 (d, *J*=8.4 Hz, 8H, DMT), 6.77 (t, *J*=17.7 Hz, 3H, DMT), 6.22 (t, *J*=14.3 Hz, 1H, H-1'), 5.14 (s, 2H, CH_2-Phenyl) 5.1 (d, *J*=4.4 Hz, 1H, H-3'), 5.06 (s, 2H, CH_2-Phenyl), 4.24-4.1 (m, 2H, H-4' and NH*CH*CH_2), 3.7 (s, 6H, OCH_3), 3.15-2.95 (m, 2H, H-5'), 2.72 (m, 1H, CH (iBu)), 2.45-1.8 (m, 6H, H-2' and CHCH_2CH_2), 1.1 (d, *J*=6.74 Hz, 6H, CH_3 (iBu))

General procedure for the preparation of compounds 5 a-b:

Compounds (**4 a-b**) were dissolved in 5% dichloroacetic acid in DCM and stirred for 15 minutes. After evaporation, the crude material was purified by column chromatography (DCM to MeOH/DCM 1:10) yielding white foams.

Benzyl((((2R,3S,5R)-2-(hydroxymethyl)-5-(2-isobutyramido-6-oxo-1,6-dihydro-9H-purin-9-yl)tetrahydrofuran-3-yl)oxy)carbonyl)glycinate (5a)

White foam, 250 mg, 95% (0.47 mmol)

ESI-MS: Calc: 528.51 Found [M+H$^+$]: 529.3

^{1}H NMR (DMSO-d6): 8.26 (s, 1H, H-8), 7.80 (t, *J*=12.36 Hz, 1H, *NH*CH), 7.35 (m, 5H, Ar), 6.18 (t, *J*=13.6 Hz, 1H, H-1'), 5.21-5.12 (s+bs, 4H, CH_2-Phenyl, OH5' and H3'), 4.1 (m, 1H, H-

4'), 3.83 (d, *J*=6.12 Hz, 2H, CH_2), 3.57 (t, *J*=8.5 Hz, 2H, H-5'), 2.74 (m, 2H, H2' and CH (iBu)), 2.38 (m, 1H, H-2'), 1.1 (d, *J*=6.74 Hz, 6H, CH_3 (iBu))

Dibenzyl ((((2R,3S,5R)-2-(hydroxymethyl)-5-(2-isobutyramido-6-oxo-1,6-dihydro-9H-purin-9-yl)tetrahydrofuran-3-yl)oxy)carbonyl)-L-glutamate (5b)

White foam, 1.5 g, 98% (2.2 mmol)

ESI-MS: Calc: 690.26 Found $[M+H^+]$: 691.20

^1H NMR (DMSO-d6): 8.25 (s, 1H, H-8), 7.88 (d, *J*=7.31 Hz, 1H, *NH*CH), 7.33 (m, 10H, Ar), 6.14 (t, *J*=14.3 Hz, 1H, H-1'), 5.17-5.07 (m, 6H, CH_2-Phenyl, OH5' and H3'), 4.14 (m, 1H, NH*CH*CH_2), 4.01(m, 1H, H-4'), 3.57 (m, 2H, H-5'), 2.74 (m, 2H, H2' and CH (iBu)), 2.32 (m, 1H, H-2'), 2.1-1.8 (m, 4H, CHCH_2CH_2), 1.1 (d, *J*=6.74 Hz, 6H, CH_3 (iBu))

General procedure for the preparation of compounds 6 a-b:

Compounds (**5 a-b**) were dissolved in dry DCM and 2 equivalents of carbonyldiimidazole were added. After stirring at room temperature for 16 hours, 4 equivalents of either H-Gly-OBzl or H-Glu(OBzl)-OBzl and 4 equivalents of diisopropylethylamine were added. After stirring overnight, the mixture was washed with citric acid, dried, evaporated and purified by column chromatography (DCM to MeOH/DCM 1:10) yielding white powders.

Dibenzyl ((((2R,3S,5R)-3-(((2-(benzyloxy)-2-oxoethyl)carbamoyl)oxy)-5-(2-isobutyramido-6-oxo-1,6-dihydro-9H-purin-9-yl)tetrahydrofuran-2-yl)methoxy)carbonyl)glutamate (6a)

756 mg, 37.3% (0.86 mmol)

ESI-MS: Calc: 881.32 Found $[M+H^+]$: 882.04

^{1}H NMR (DMSO-d6): 8.24 (s, 1H, H-8), 7.90 (d, *J*=7.31 Hz, 1H, *NH*CH), 7.84 (t, *J*=12.36 Hz, 1H, *NH*CH), 7.33 (m, 15H, Ar), 6.16 (t, *J*=13.6 Hz, 1H, H-1'), 5.16-5.09 (m, 7H, CH_2-Phenyl, and H3'), 4.1 (m, 4H, H-4', H5' and NH*CH*CH_2), 3.83 (d, *J*=6.12 Hz, 2H, CH_2), 2.9 (m, 1H, H-2'), 2.74 (m, 1H, CH (iBu)), 2.38 (m, 1H, H-2'), 2.1-1.8 (m, 4H, CHCH_2CH_2), 1.1 (d, *J*=6.74 Hz, 6H, CH_3 (iBu))

Dibenzyl ((((2R,5R)-2-((((2-(benzyloxy)-2-oxoethyl)carbamoyl)oxy)methyl)-5-(2-isobutyramido-6-oxo-1,6-dihydro-9H-purin-9-yl)tetrahydrofuran-3-yl)oxy) carbonyl) glutamate (6b)

2.6 g, 90% (2.95 mmol)

ESI-MS: Calc: 881.32 Found [$M+H^+$]: 881.94

^{1}H NMR (DMSO-d6):): 8.25 (s, 1H, H-8), 7.9-7.8 (m, 2H, *NH*CH), 7.33 (m, 15H, Ar), 6.18 (t, *J*=13.6 Hz, 1H, H-1'), 5.15-5.05 (m, 7H, CH_2-Phenyl, and H3'), 4.1 (m, 4H, H-4', H5' and NH*CH*CH_2), 3.85 (d, *J*=6.12 Hz, 2H, CH_2), 2.9 (m, 1H, H-2'), 2.75 (m, 1H, CH (iBu)), 2.31 (m, 1H, H-2'), 2.15-1.8 (m, 4H, CHCH_2CH_2), 1.1 (d, *J*=6.74 Hz, 6H, CH_3 (iBu))

General procedure for the preparation of compounds 7 a-b:

These compounds were prepared following the procedure used for the preparation of compounds (**2 a-d**), using compounds (**6 a-b**) as starting materials.

((((2R,5R)-3-(((carboxymethyl)carbamoyl)oxy)-5-(2-isobutyramido-6-oxo-1,6-dihydro-9H-purin-9-yl)tetrahydrofuran-2-yl)methoxy)carbonyl)glutamic acid (7a)

White powder, 250 mg, 79% (0.41 mmol)

HRMS: Calc [M+H$^+$]: 612.1896 Found: 612.1899

^{1}H NMR (DMSO-d6): 8.24 (s, 1H, H-8), 7.7 (m, 2H, *NH*CH), 6.23 (t, *J*=13.6 Hz, 1H, H-1'), 5.16 (d, *J*= 5.65 Hz, 1H, H3'), 4.2-4.01 (m, 4H, H-4', H5' and NH*CH*CH_2), 3.68 (d, *J*=6.12 Hz, 2H, CH_2), 2.93 (m, 1H, H-2'), 2.77 (m, 1H, CH (iBu)), 2.45 (m, 1H, H-2'), 2.31 (m, 2H, CHCH_2), 2.05-1.75 (2*m, 4H, CH$CH_2$$CH_2$), 1.1 (d, *J*=6.74 Hz, 6H, CH_3 (iBu)) 13C NMR (75 MHz, DMSO-d6) δ 180.54, 174.13, 173.82, 171.87, 171.67, 156.65, 155.80, 155.23, 149.13, 148.73, 137.70, 120.65, 83.20, 83.01, 75.64, 64.62, 53.48, 42.53, 36.87, 35.24, 30.59, 26.56, 19.29.

((((2R,5R)-2-((((carboxymethyl)carbamoyl)oxy)methyl)-5-(2-isobutyramido-6-oxo-1,6-dihydro-9H-purin-9-yl)tetrahydrofuran-3-yl)oxy)carbonyl)glutamic acid (7b)

White powder, 1.6 g, 95% (2.6 mmol)

HRMS: Calc [M+H$^+$]: 612.1896 Found: 612.1900

^{1}H NMR (DMSO-d6): 8.24 (s, 1H, H-8), 7.7 (m, 2H, *NH*CH), 6.23 (t, *J*=13.6 Hz, 1H, H-1'), 5.16 (d, *J*= 5.65 Hz, 1H, H3'), 4.2-4.01 (m, 4H, H-4', H5' and NH*CH*CH_2), 3.68 (d, *J*=6.12 Hz, 2H, CH_2), 2.93 (m, 1H, H-2'), 2.77 (m, 1H, CH (iBu)), 2.45 (m, 1H, H-2'), 2.31 (m, 2H, CHCH_2), 2.05-1.75 (2*m, 4H, CH$CH_2$$CH_2$), 1.1 (d, *J*=6.74 Hz, 6H, CH_3 (iBu)) 13C NMR (75 MHz, DMSO-d6) δ 180.61, 174.99, 173.68, 172.10, 171.86, 156.36, 155.67, 154.98, 149.14, 148.77, 137.91, 120.64, 83.22, 82.82, 75.34, 64.27, 54.56, 44.20, 36.99, 35.22, 32.56, 28.48, 19.31.

4.3 Inhibition tests

The potential inhibitors were tested *in vitro* using reported methods[18].

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Captions to Figures:

Figure 1: Structures of (A) (p)ppGpp and (B) Relacin.

Figure 2: Inhibition of Rel proteins by compounds **2a** and **2d**. **A)** (p)ppGpp synthesis in *B. subtillis* cells after the addition of serine hydroxamate. White: Cells grown with 2mM of **2d**. Gray: Cells grown with 2 mM of **2a**. Black: Cells grown without **2a** **B)** Inhibition of Rel proteins by **2d** *in vitro*. White: Rel/Spo from *D. Radiodurans*, Gray: RelA from *E. coli*.

Scheme 1: Synthetic route for the preparation of symmetric compounds. Reaction conditions: i) a) CDI, Acetonitrile, RT, Overnight; b) R,R_1-NH, DCM, DIEA, RT, 2 to 20 hours, 17-77%; ii) H_2-10% Pd/C, Methanol, RT, 3 hours, 30 psi, 80-90%.

Scheme 2: Synthetic route for the preparation of asymmetric compounds. Reaction conditions: i) CDI, DCM, RT, 4 hours, 92.7%; ii) H_2N-CHR-OBzl, DCM, DIEA, RT, overnight, 53-68%; iii) 5% DCA in DCM, RT, 15 minutes, 95-98%; iv) a) CDI, DCM, RT, Overnight; b) H_2N-CHR_1-OBzl, DCM, DIEA, RT, overnight,40-90%; v) H_2-10% Pd/C, methanol, 3 hours, RT, 30 psi, 79-95%.

6. Acknowledgements

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7. Supplementary data

Supplementary data (NMR spectra) related to this article can be found at…

Highlights:

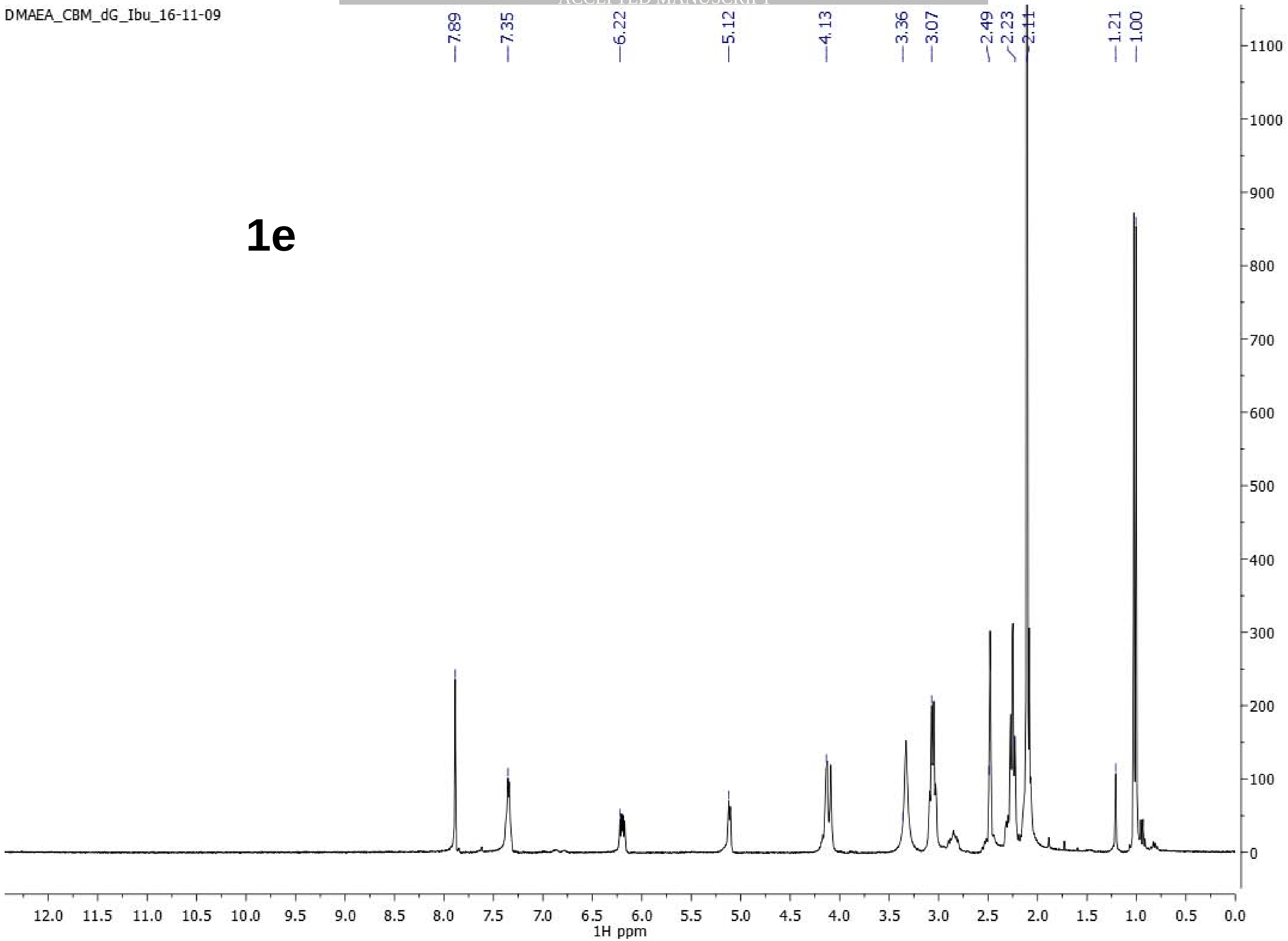
- We describe new antibacterial agents that inhibit the bacterial RelA proteins
- The most potent analog has antibacterial activity in both Gram negative & Gram positive bacteria
- SAR highlight the importance of the type of substituent added at positions 3’ and 5’ of Deoxyguanosine

Supporting Information

Design, Synthesis and Structure-Activity Relationship of novel Relacin analogues as inhibitors of Rel proteins

Ezequiel Wexselblatt, Ilana Kaspy, Gad Glaser, Joshua Katzhendler and Eylon Yavin

^{1}H and ^{13}C NMR for representative compounds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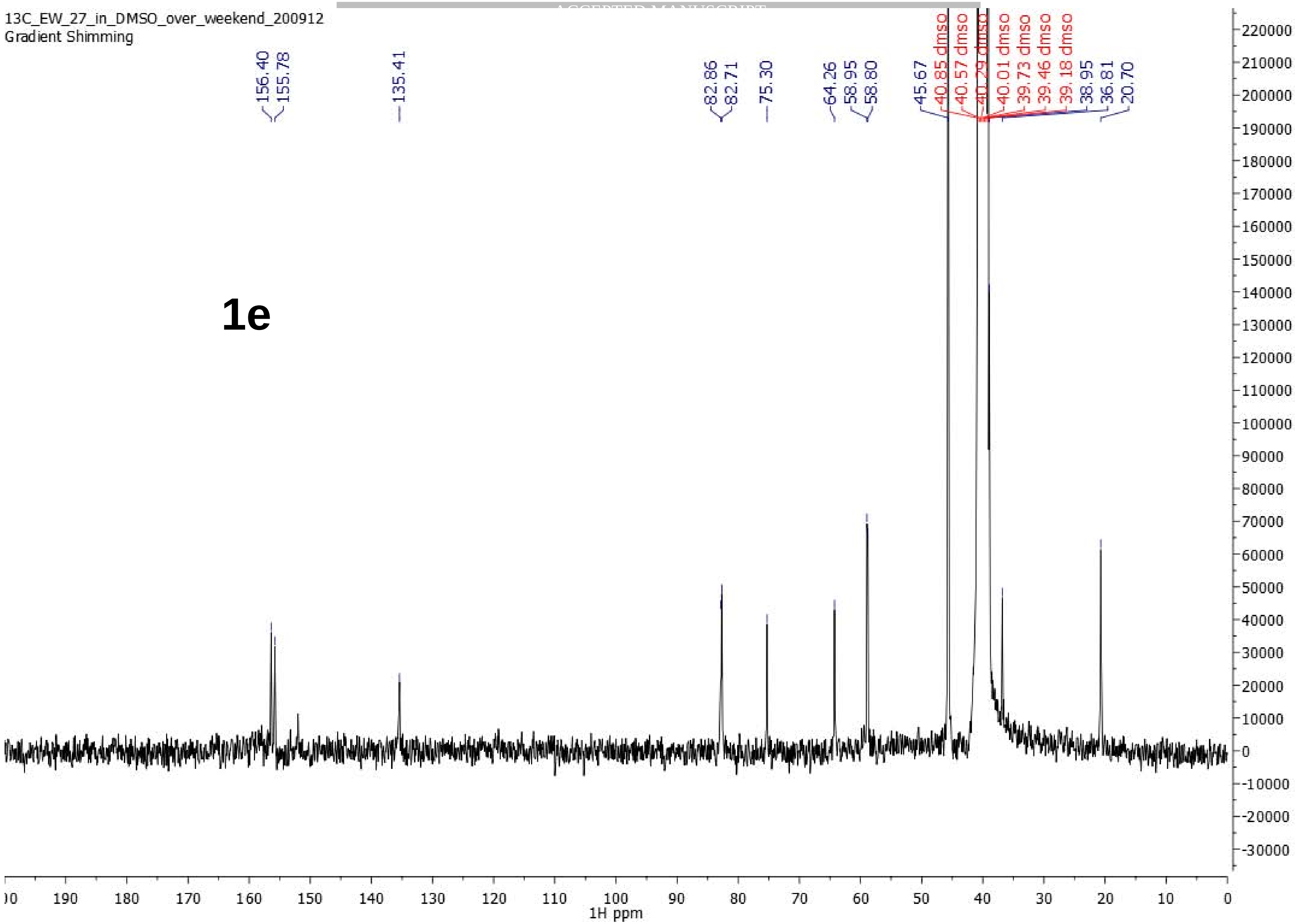
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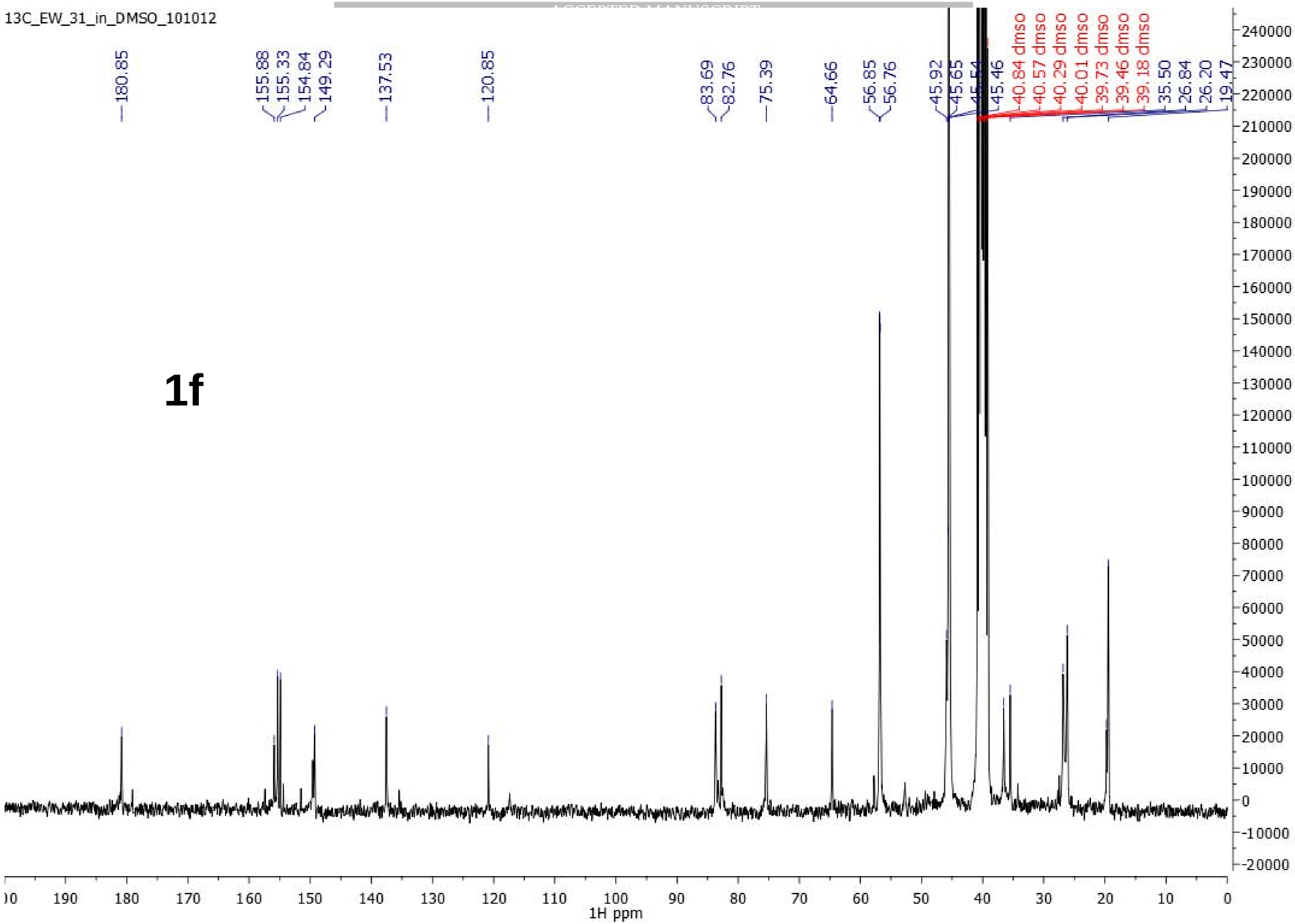
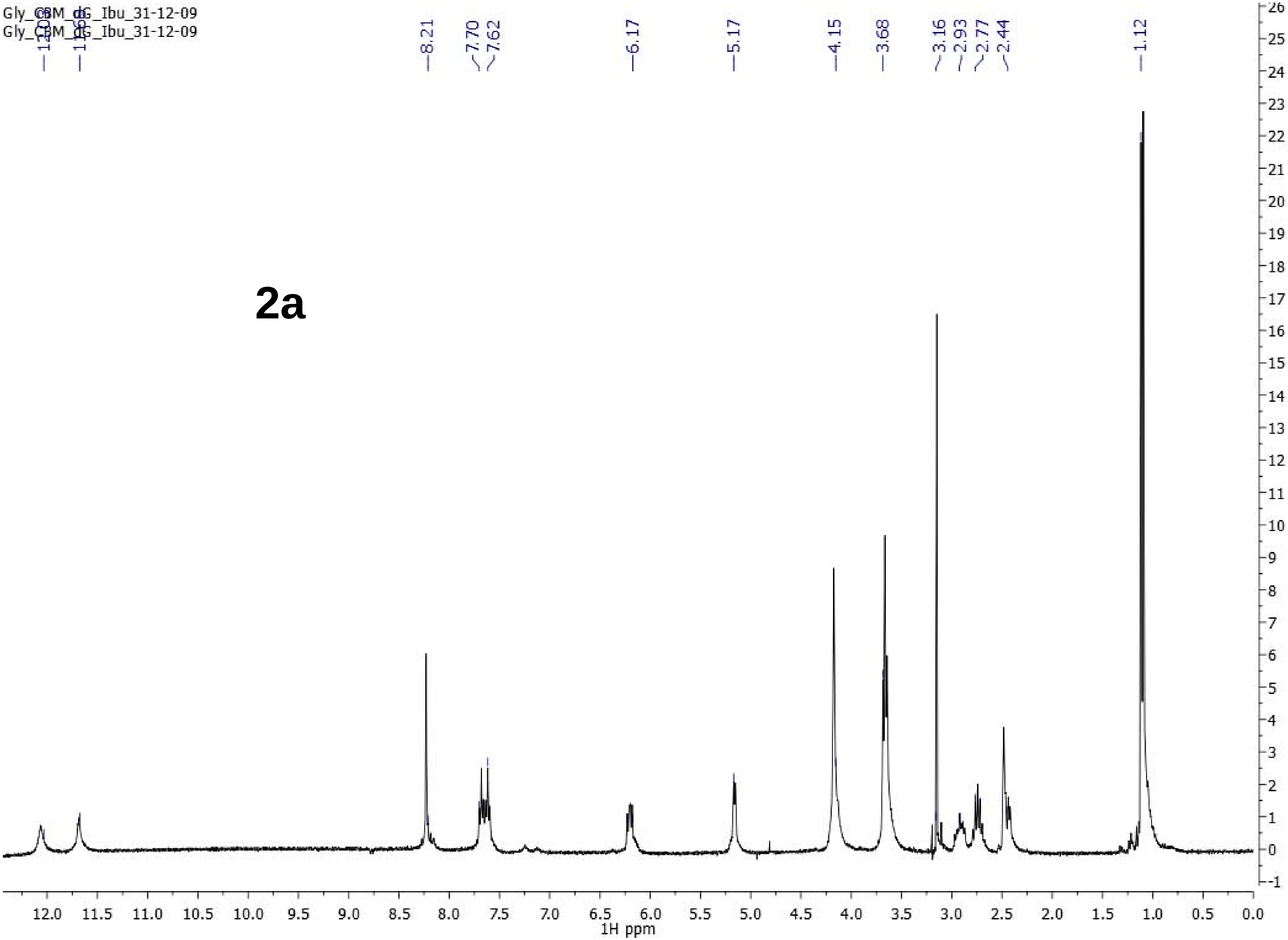
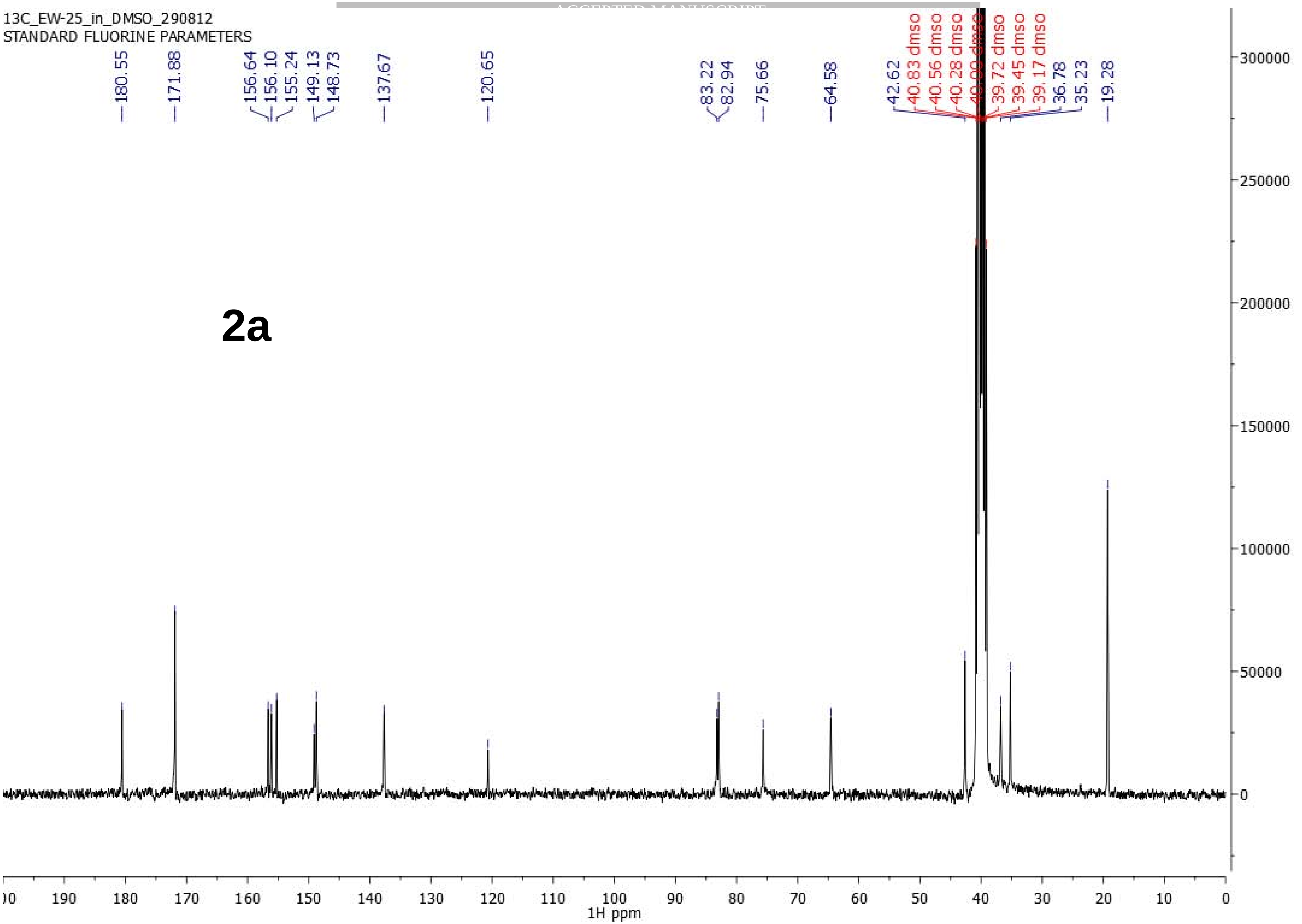
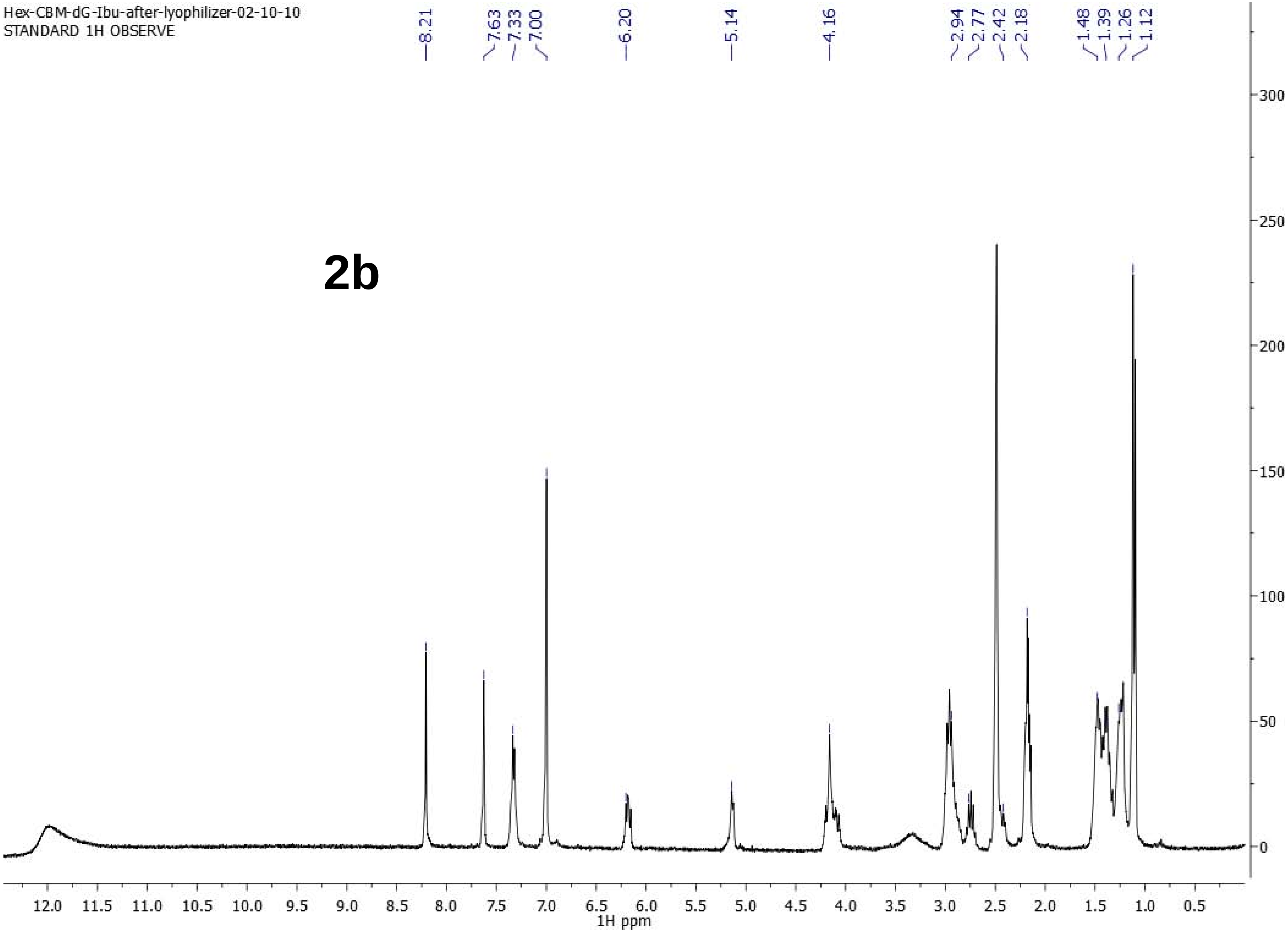
20.70
1H ppm

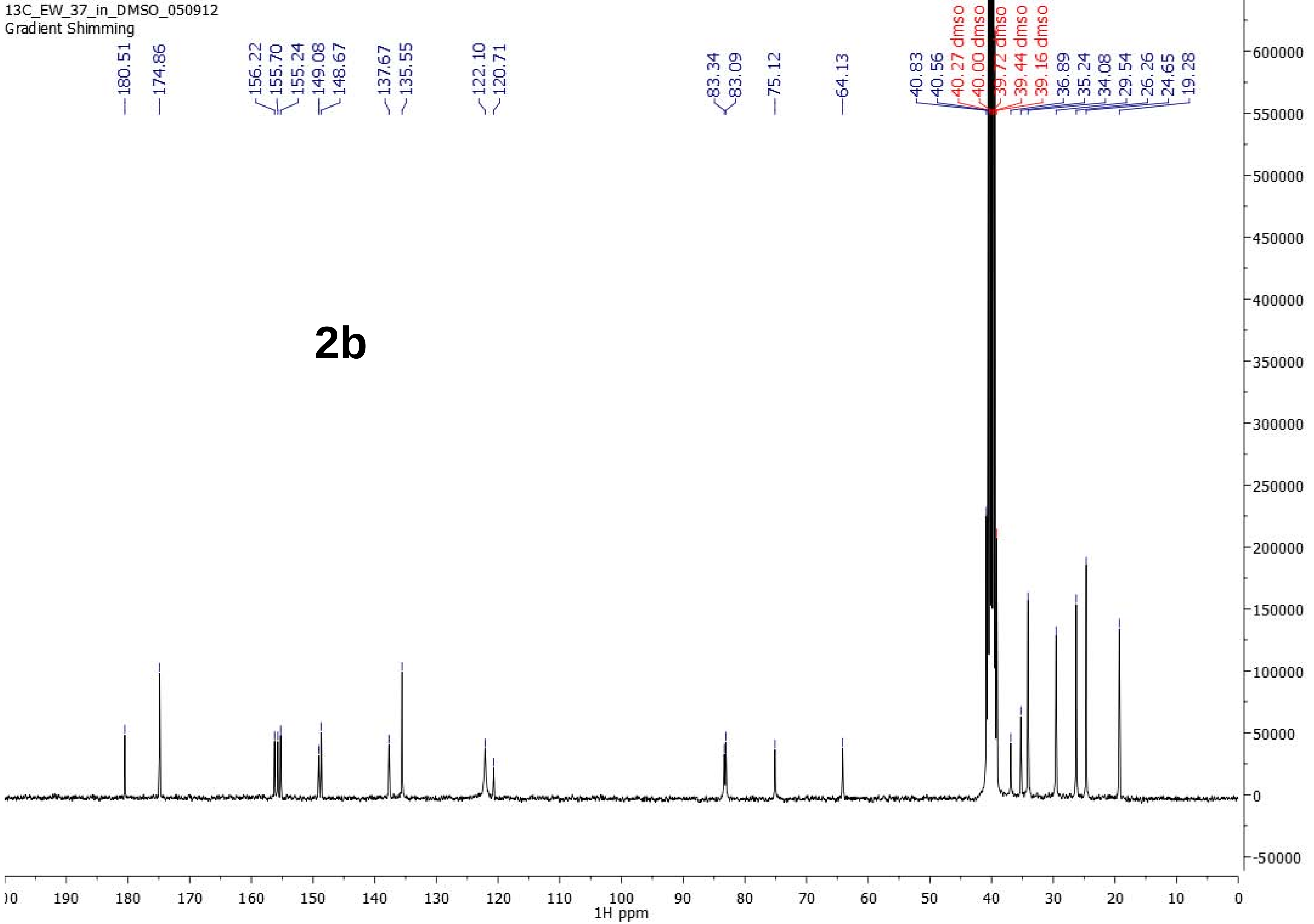

13C_EW_31_in_DMSO_101012
180.85
155.88
155.33
154.84
149.29
137.53
120.85
83.69
82.76
75.39
64.66
56.85
56.76
45.92
45.65
45.54
45.46
40.84 dmso
40.57 dmso
40.29 dmso
40.01 dmso
39.73 dmso
39.46 dmso
39.18 dmso
35.50
26.84
26.20
19.47
1f
1H ppm

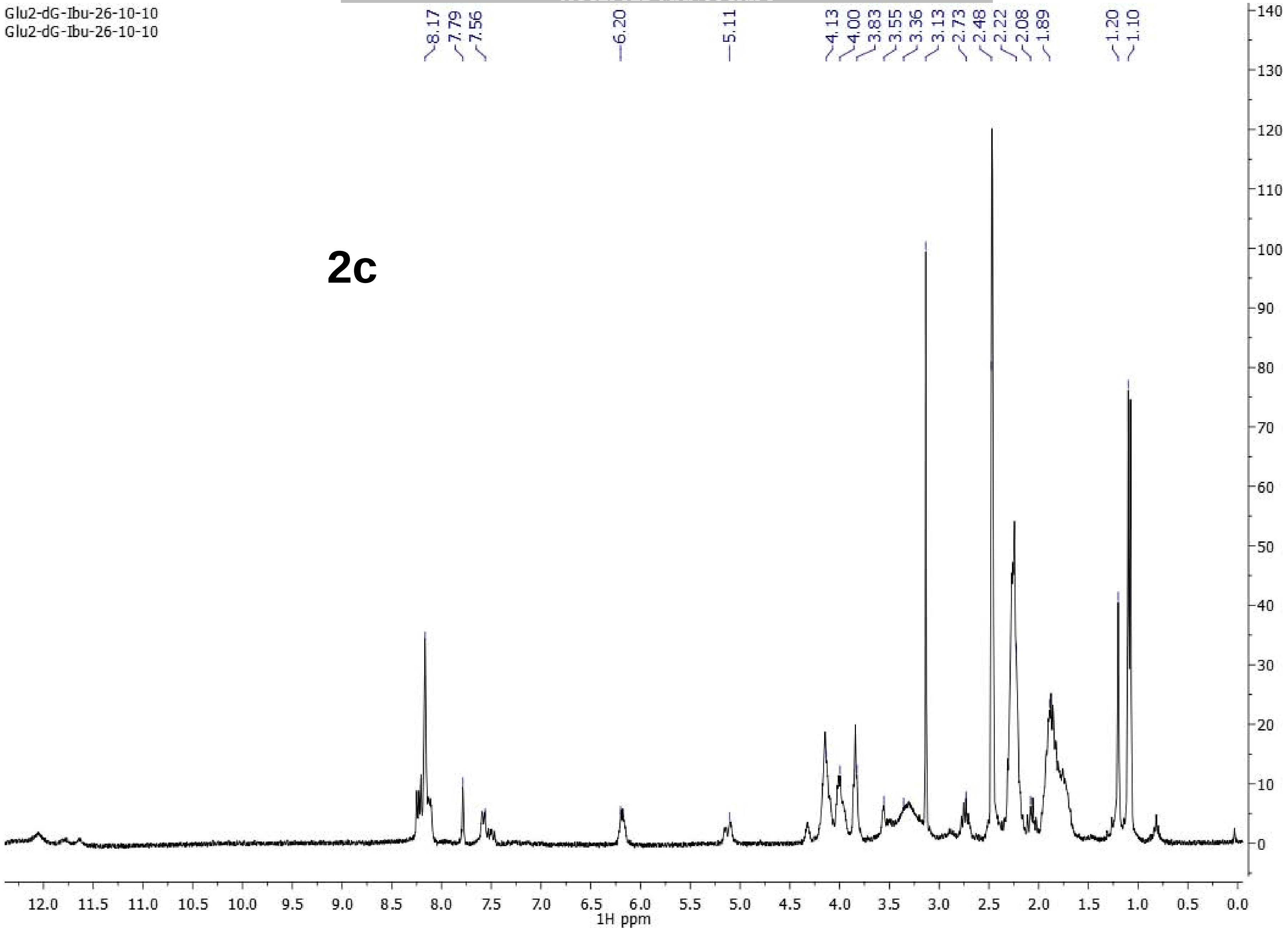

Gly_CBM_dG_Ibu_31-12-09
Gly_CBM_dG_Ibu_31-12-09
12.03
11.68
8.21
7.70
7.62
6.17
5.17
4.15
3.68
3.16
2.93
2.77
2.44
1.12
2a
1H ppm

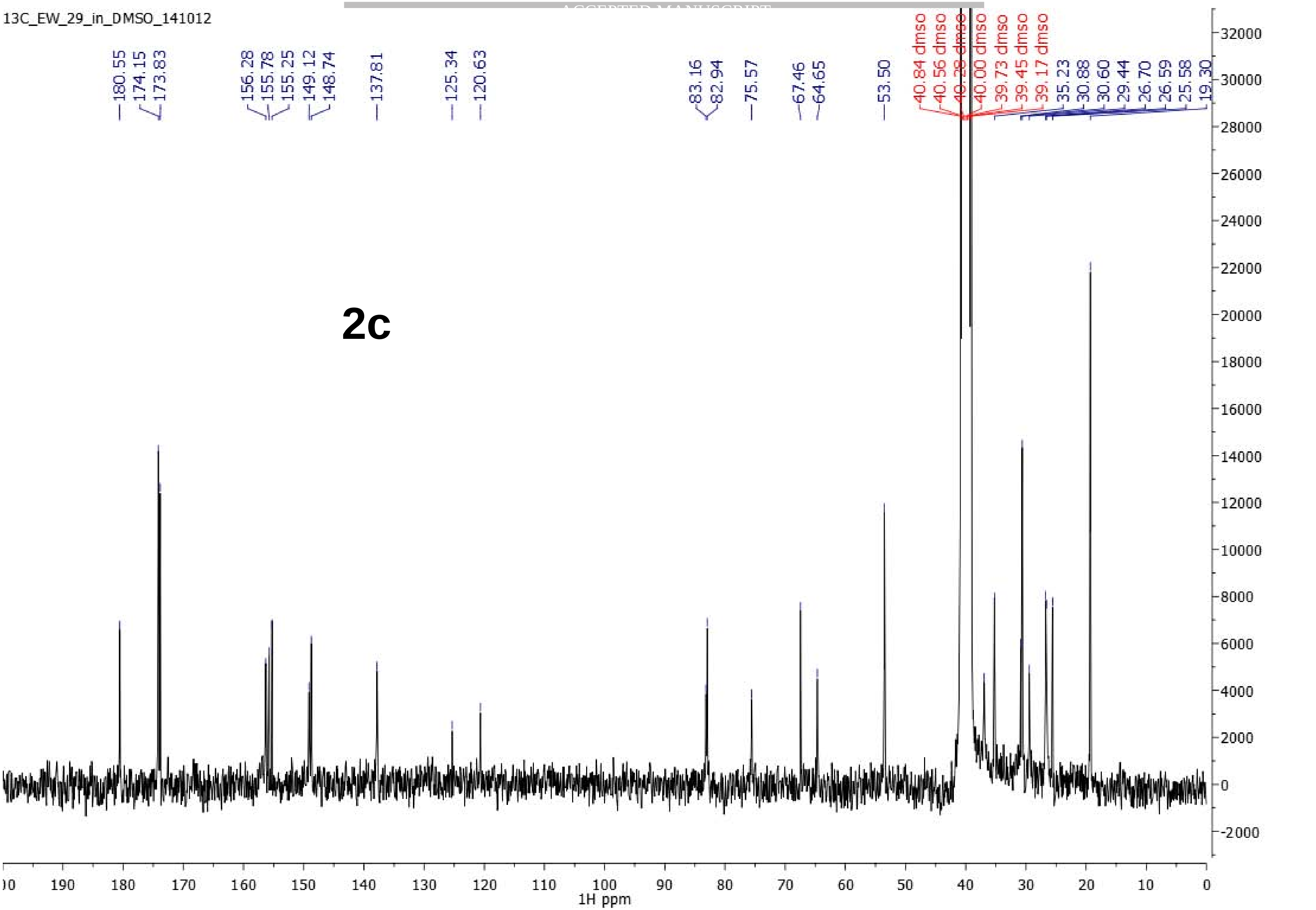

13C_EW-25_in_DMSO_290812
STANDARD FLUORINE PARAMETERS
180.55
171.88
156.64
156.10
155.24
149.13
148.73
137.67
120.65
83.22
82.94
75.66
64.58
42.62
40.83 dmso
40.56 dmso
40.28 dmso
40.00 dmso
39.72 dmso
39.45 dmso
39.17 dmso
36.78
35.23
19.28
2a
300000
250000
200000
150000
100000
50000
0
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
1H ppm

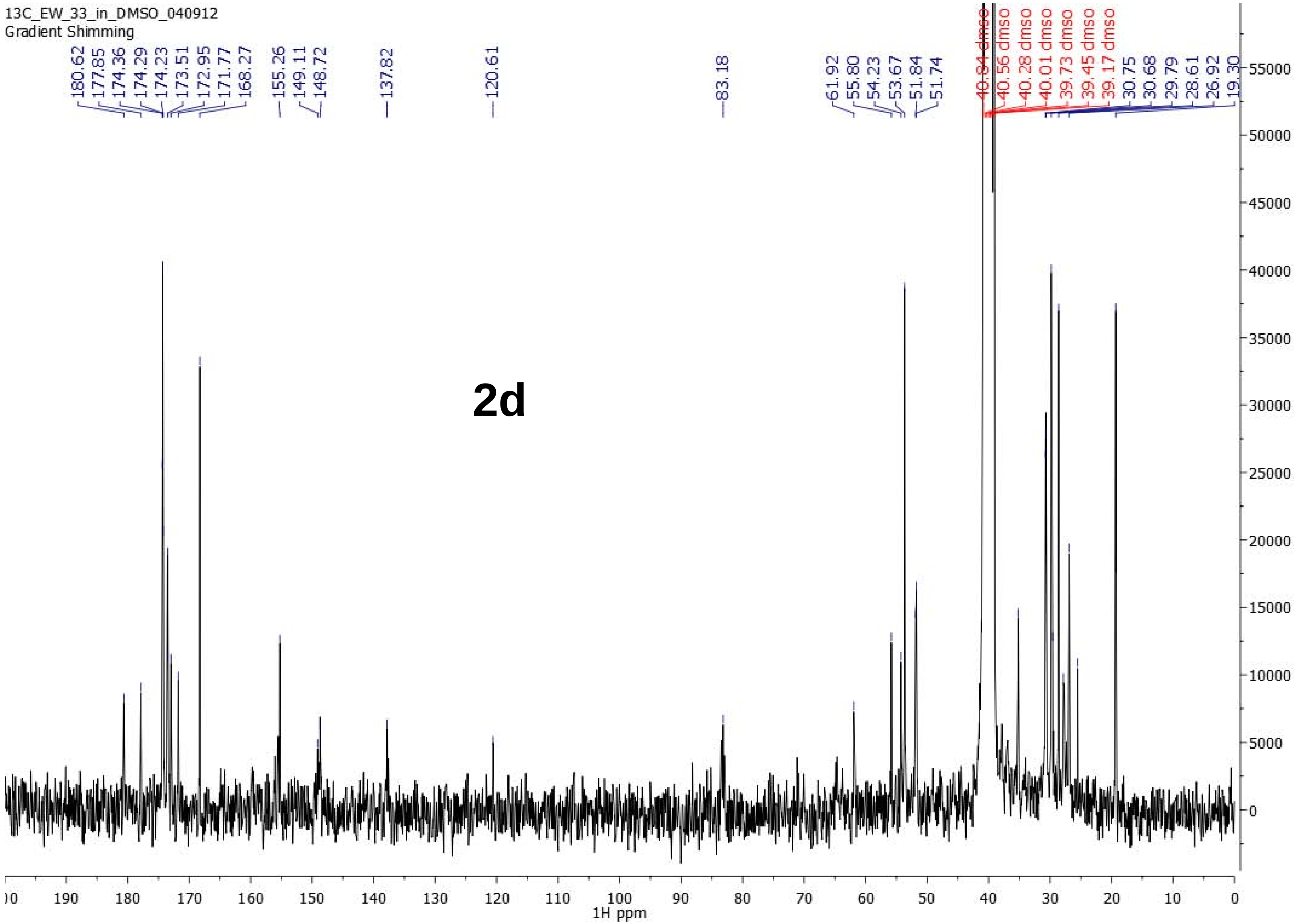


Hex-CBM-dG-Ibu-after-lyophilizer-02-10-10
STANDARD 1H OBSERVE
8.21
7.63
7.33
7.00
6.20
5.14
4.16
2.94
2.77
2.42
2.18
1.48
1.39
1.26
1.12
2b
300
250
200
150
100
50
0
12.0 11.5 11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5
1H ppm

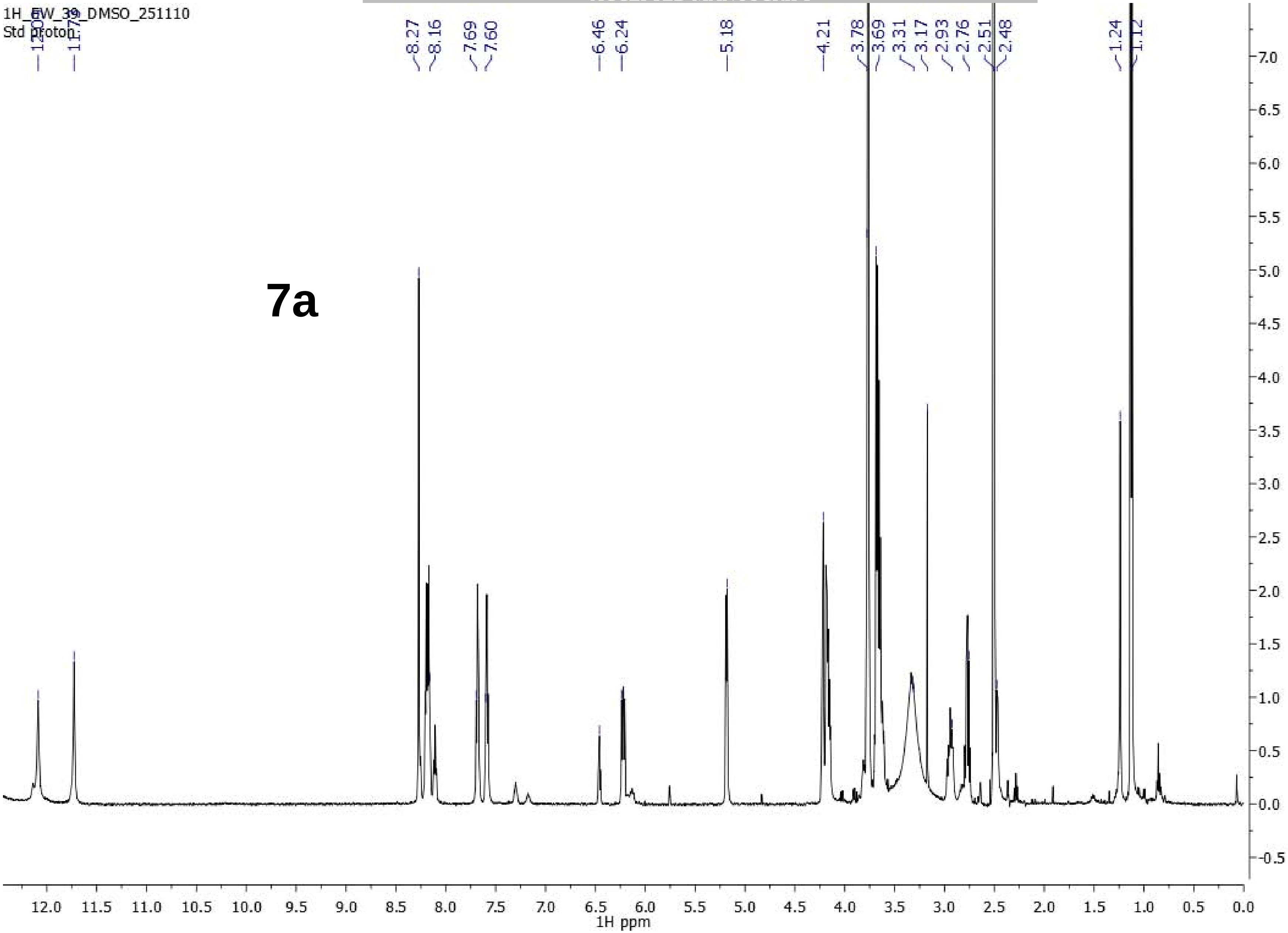

13C_EW_37_in_DMSO_050912
Gradient Shimming
180.51
174.86
156.22
155.70
155.24
149.08
148.67
137.67
135.55
122.10
120.71
83.34
83.09
75.12
64.13
40.83
40.56
40.27 dmso
40.00 dmso
39.72 dmso
39.44 dmso
39.16 dmso
36.89
35.24
34.08
29.54
26.26
24.65
19.28
2b
1H ppm


Glu2-dG-Ibu-26-10-10
Glu2-dG-Ibu-26-10-10
8.17
7.79
7.56
6.20
5.11
4.13
4.00
3.83
3.55
3.36
3.13
2.73
2.48
2.22
2.08
1.89
1.20
1.10
2c
1H ppm

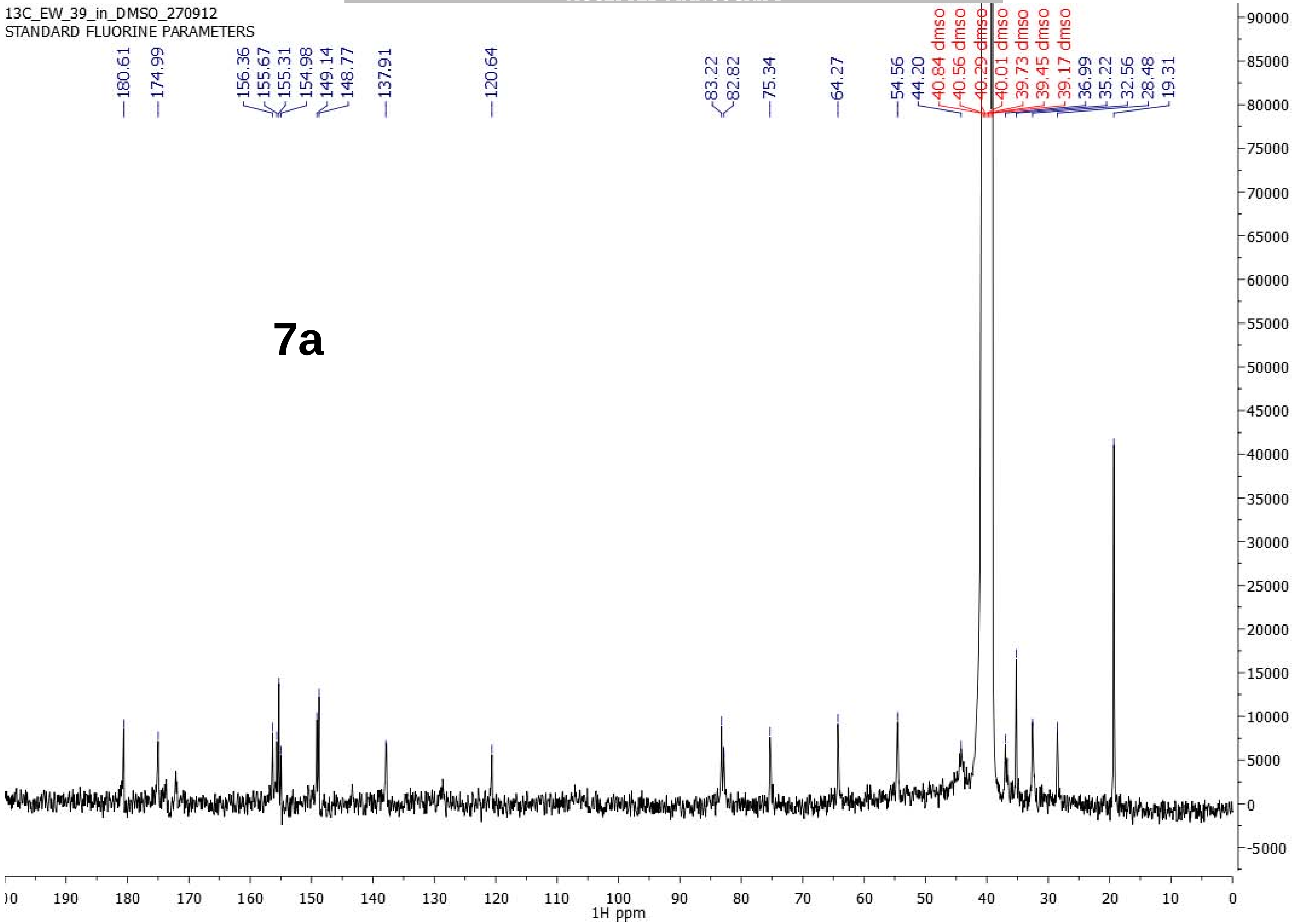

13C_EW_29_in_DMSO_141012
180.55
174.15
173.83
156.28
155.78
155.25
149.12
148.74
137.81
125.34
120.63
83.16
82.94
75.57
67.46
64.65
53.50
40.84 dmso
40.56 dmso
40.28 dmso
40.00 dmso
39.73 dmso
39.45 dmso
39.17 dmso
35.23
30.88
30.60
29.44
26.70
26.59
25.58
19.30
2c
1H ppm


13C_EW_33_in_DMSO_040912
Gradient Shimming
180.62
177.85
174.36
174.29
174.23
173.51
172.95
171.77
168.27
155.26
149.11
148.72
137.82
120.61
83.18
61.92
55.80
54.23
53.67
51.84
51.74
40.84 dmso
40.56 dmso
40.28 dmso
40.01 dmso
39.73 dmso
39.45 dmso
39.17 dmso
30.75
30.68
29.79
28.61
26.92
19.30
2d
1H ppm

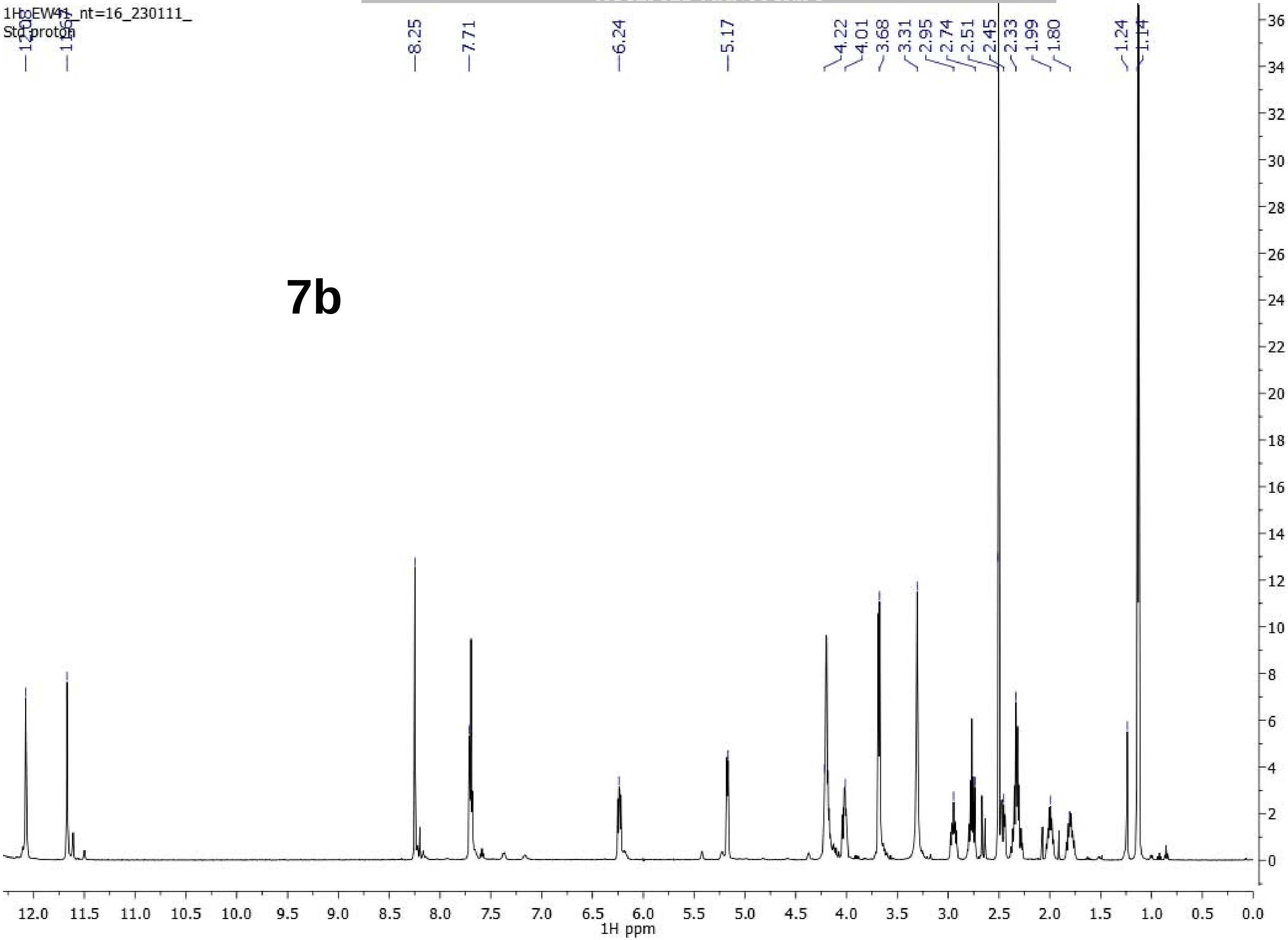
7a



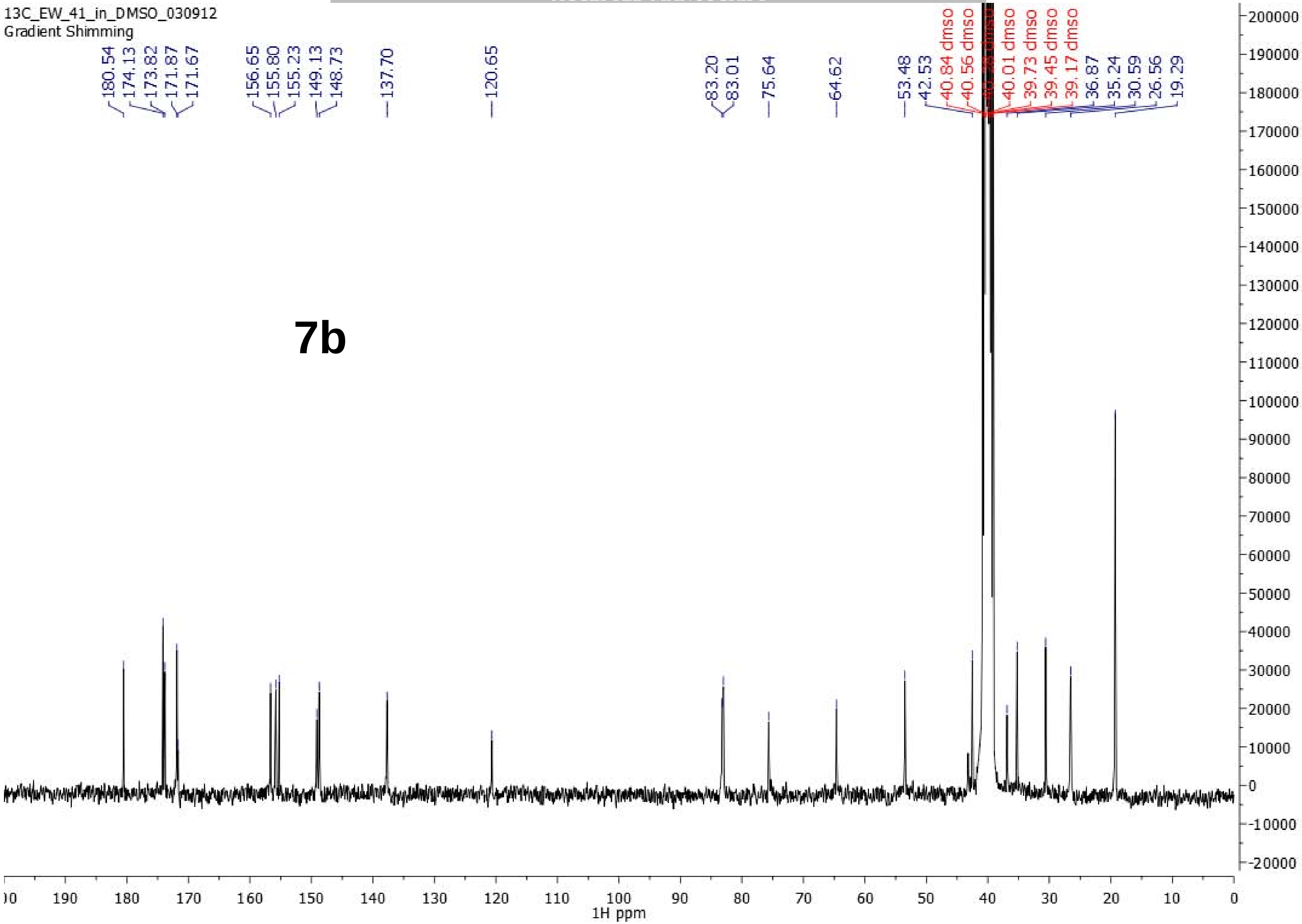
1H_EW_39_DMSO_251110
Std proton
12.09
11.73
8.27
8.16
7.69
7.60
6.46
6.24
5.18
4.21
3.78
3.69
3.31
3.17
2.93
2.76
2.51
2.48
1.24
1.12
1H ppm


13C_EW_39_in_DMSO_270912
STANDARD FLUORINE PARAMETERS
180.61
174.99
156.36
155.67
155.31
154.98
149.14
148.77
137.91
120.64
83.22
82.82
75.34
64.27
54.56
44.20
40.84 dmso
40.56 dmso
40.29 dmso
40.01 dmso
39.73 dmso
39.45 dmso
39.17 dmso
36.99
35.22
32.56
28.48
19.31
1H ppm

7a


1H_EW41_nt=16_230111_
Std proton
12.08
11.67
8.25
7.71
6.24
5.17
4.22
4.01
3.68
3.31
2.95
2.74
2.51
2.45
2.33
1.99
1.80
1.24
1.14
1H ppm

7b


13C_EW_41_in_DMSO_030912
Gradient Shimming
180.54
174.13
173.82
171.87
171.67
156.65
155.80
155.23
149.13
148.73
137.70
120.65
83.20
83.01
75.64
64.62
53.48
42.53
40.84 dmso
40.56 dmso
40.01 dmso
39.73 dmso
39.45 dmso
39.17 dmso
36.87
35.24
30.59
26.56
19.29
7b
1H ppm