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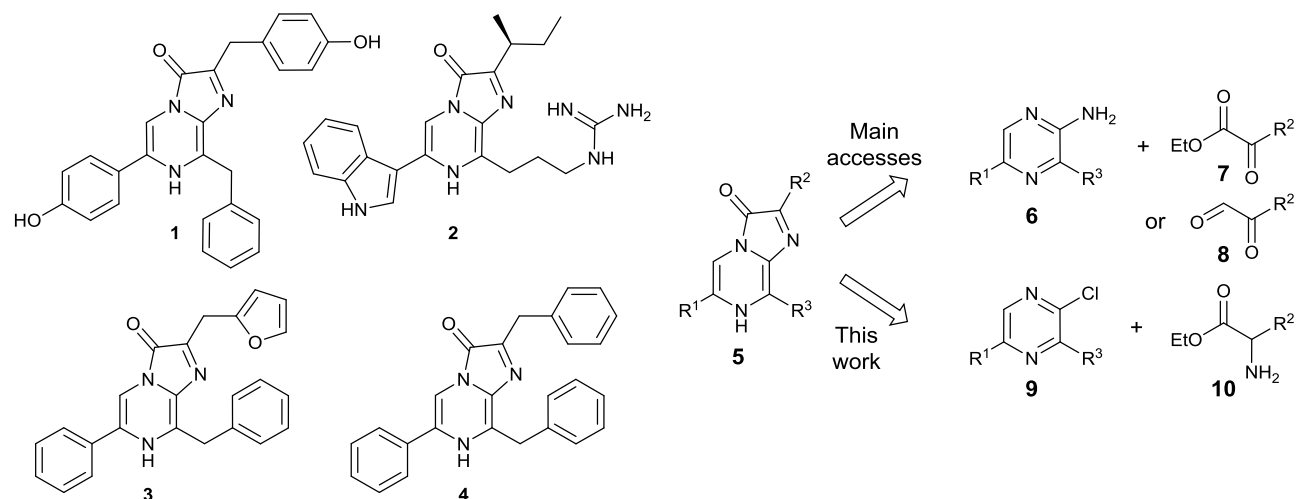
Gram-Scale Synthesis of Luciferins Derived from Coelenterazine and Original Insights in Their Bioluminescence Properties

Eloi P. Coutant Sophie Goyard Vincent Hervin Glwadys Gagnot Racha Baatallah Yves Jacob Thierry Rose* and Yves L. Janin*

An original gram-scale synthesis of O-acetylated forms of coelenterazine, furimazine or hydroxy-bearing analogues of these luciferins is described. The comparisons over two hours of their bioluminescence, using the nanoKAZ/NanoLuc luciferase, is providing remarkable insights useful for the selection of a substrate adapted for a given application.

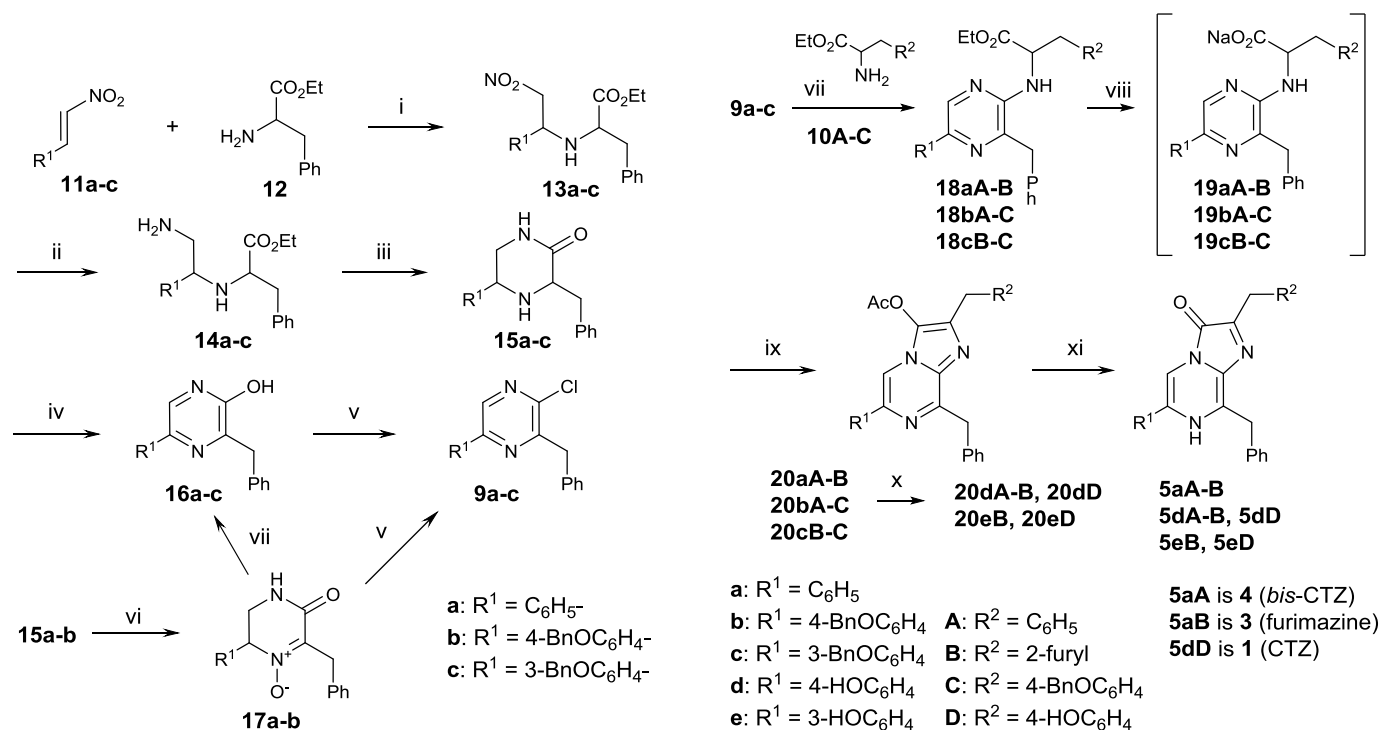
Introduction

Bioluminescence is based on the combination of at least oxygen, a small chemical cofactor and an enzyme. As depicted in scheme 1, extensive research,¹ led to the discovery that many sea-dwelling bioluminescent species^{2, 3} are using coelenterazine (CTZ, **1**) or varguline (**2**) and harbor a large variety of luciferases or photoproteins (which are calcium-dependent luciferases) to produce light with these. Across the years, because of the many uses of bioluminescence-based tools in life sciences,⁴⁻¹⁰ attempts were made to improve the signal intensity, duration and/or the emission wavelength. This started with the isolation of a wide range of coelenterazine-using luciferases and their combination with analogues of coelenterazine (**1**). It then moved to the analysis of the effect of luciferase mutations on the bioluminescence properties and the best results obtained so far were achieved by the association of mutated luciferases and luciferins analogues.¹¹⁻²⁵ It is the combinations of an extensively mutated form of the catalytic subunit of *Oplophorus gracilirostris* luciferase (nanoKAZ/NanoLuc)^{20, 21} and furimazine (**3**),²⁰ or bisdeoxycoelenterazine (bis-CTZ, **4**),²¹ which appears to be the current state of the art in regard with signal intensity and duration.



Scheme 1. Structures of coelenterazine (1), varguline (2), furimazine (3), and bisdeoxycoelenterazine (4) and retrosynthesis of imidazo[1,2-a]pyrazine-3(7H)-ones.

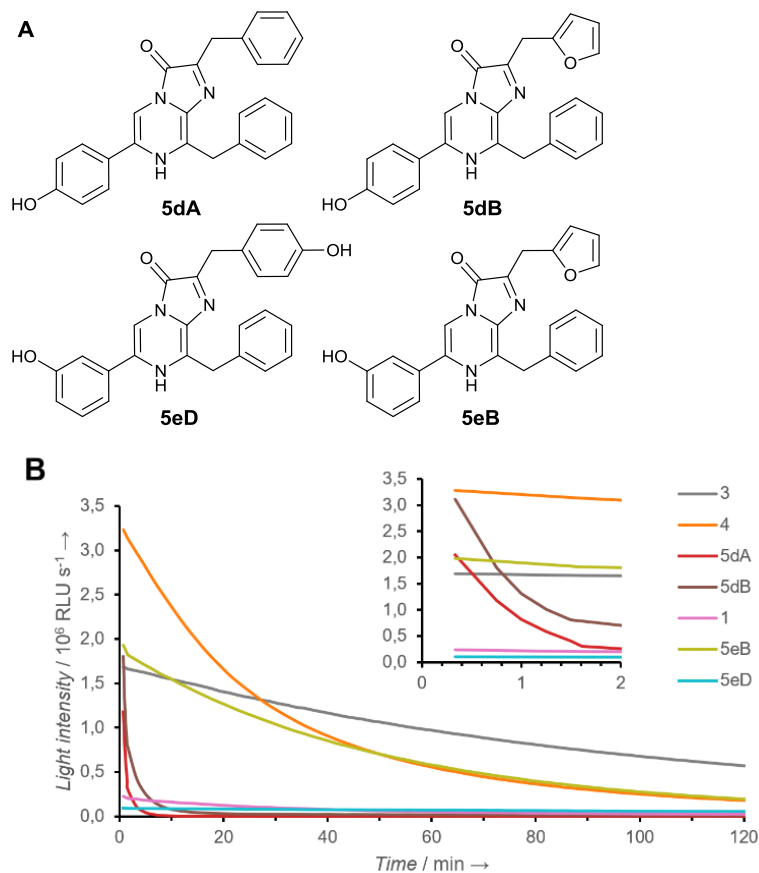
Concerning the chemistry of these luciferins, they are oxygen-sensitive and readily decompose in solution especially in the presence of a base, or upon light exposure.²⁶ As depicted above, many²⁷ if not all²⁸⁻³⁰ the reported preparations of imidazo[1,2-a]pyrazin-3(7H)-ones **5** are requiring an aminopyrazine (**6**) prior to the construction of the imidazole ring via a condensation with α-ketoesters (**7**) or α-ketoaldehydes (**8**). Recent improvements have extended this access to original luciferins^{24, 26} but to avoid some of its inherent limitations, we focused on an alternative initially explored on a model compound.³¹ This path not only avoids the use of the non-trivial intermediates **7-8**, but it also offers, via a key N-arylation of halogenopyrazines (**9**), the recourse to a wide range of the far more available α-amino esters (**10**).³²⁻³⁴ As depicted in scheme 2, an original preparation of chloropyrazines **9a-c** was achieved starting with a 1,4-addition reaction between β-nitrostyrenes **11a-c** and phenylalanine ethyl ester (**12**).



Scheme 2. i: neat. ii: Zn, H₃O⁺, Cl⁻, dioxane. iii: 130 °C, neat. iv: S₈, 1,3-Cl₂C₆H₄, reflux. v: PhPOCl₂, 100 °C. vi: AcOOH, AcOEt. vii: chlorobenzene, reflux or NaOH, EtOH, reflux. . viii: Cs₂CO₃, Pd(OAc)₂, BINAP, MeCN, 60 °C. ix: Ac₂O, 20 °C. x: H₂, Pd/C, AcOEt, AcOH, EtOH. xi: EtOH, DMSO, H₃O⁺, Cl⁻, 50 °C.

Until recently,³⁵ there were only very few precedents for this reaction,³⁶⁻⁴¹ probably because of a lack of such 1,4-addition in solution. Indeed, in our cases, it is only when removing the solvent that the 1,4 adducts **13a-c** were formed. The use of zinc and hydrochloric acid in dioxane for their reduction provided an access to the diamines **14a-c** including a compatibility with the benzyloxy groups of **13b-c**. Their cyclization to give the (separable) piperazinone diastereoisomers **15a-c** was then achieved with heat. The previously unreported use of sulfur as an oxidant was initially essential for their aromatization into the corresponding hydroxypyrazines **16a-c**. Later on, we found an alternative via an original dehydration of the N-oxide **17a-b** (obtained by the peroxyacetic acid treatment of **15a-b**) using either heat or more preferably sodium hydroxide. Finally, from the hydroxypyrazines **16a-c**, hot phenylphosphonic dichloride⁴² was essential to prepare the chloropyrazines **9a-c**, and this reagent could also be used to directly transform, for instance, N-oxide **17a** into the chloropyrazine **9a**. This was followed by the key Buchwald-Hartwig palladium-catalyzed N-arylation of the readily available^{33, 34} α -amino esters **10A-C** by chloropyrazines **9a-c**. Starting from related precedents,⁴³⁻⁴⁸ it quickly turned out that a mild temperature was required. The best conditions we found, 60 °C in acetonitrile with cesium carbonate for 12 hours using BINAP and palladium(II) acetate, led to the N-arylestes **18** in 69-90% yields. For the next step, we observed that the inherent instability of the target luciferins **5aB** limited its purification to a precipitation. To avoid this, we prepared the far more stable O-acetylated derivatives **20** in one pot from the N-arylestes **18** via the acid salts **19**, generated in situ, and an ensuing treatment with an excess of acetic anhydride. These pro-luciferins turned out

to be stable enough to withstand a chromatography but, even better, a simple recrystallization provided compounds **20aA-B** in up to grams amount. For the synthesis of the phenol-bearing luciferins, a catalytic hydrogenation of O-benzyl-bearing compounds **20bA-bC** and **20cB-cC** provided the corresponding O-acetylated luciferins **20dA-dB**, **20dD**, **20eB** and **20eD**. Then, as seen by LC/MS (supporting information, figure S1), a treatment of these O-acetylated luciferins **20** with a mixture of hydrochloric acid, ethanol and DMSO at 50 °C provided concentrated solutions of the pure luciferins **5** which could be used immediately, upon a dilution in the relevant buffer, or stored at low temperature. As depicted in figure 2, and described in the supporting information section, the bioluminescence properties of these luciferins were then studied using a purified recombinant nanoKAZ/NanoLuc luciferase. In comparison with the very low intensity of coelenterazine (**1**), or for that matter “isocoelenterazine” (**5eD**), furimazine (**3**) and bisdeoxycoelenterazine (**4**) were, as previously reported,^{20, 21} providing vastly improved bioluminescence signals lasting at least two hours. Interestingly, the two monohydroxy-bearing analogues h-coelenterazine (**5dA**) and **5dB** led to at least twice more intense signals but which lasted only minutes (figure 2B). Such initial intensity has actually been reported before for h-coelenterazine (**5dA**).²¹ On the other hand, the isomeric mono hydroxy-bearing compound **5eB** displayed a far more stable bioluminescence profile pretty much identical with the one observed for furimazine (**3**). With all these luciferins, the light intensity decreased with time: rather quickly for the “flash” and much more slowly for the “glow” substrates. For the “flash” substrates, luciferins **5dA** and **5dB**, the decrease fitted with a first order equation.



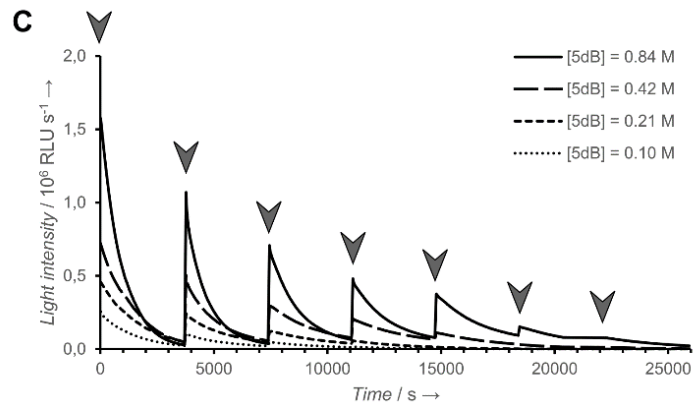


Figure 2. Bioluminescence signals of the luciferins **5** using recombinant nanoKAZ/NanoLuc. (A) Luciferin structures; compounds **1**, **3**, and **4** are depicted in figure 1. (B) Light intensity in RLU s⁻¹ plotted vs. time over two hours, the insert zooms on the first two minutes of the reaction. (C) Bioluminescence profiles of compound **5dB** at different concentrations along with the repeated addition (arrows) of luciferase.

Adding further substrate after losing 90% of the initial light emission intensity did not, at best, produce any changes and could even cause a decrease of the remaining signal intensity. On the other hand, as depicted in figure 2C, adding instead the same amount of enzyme used at the start led to a recovery of the signal. For the other substrates, this enzyme death was less pronounced but took place anyway (see supporting information).

Accordingly, the enzyme is irreversibly inactivated by a reaction product in all the cases with a constant (k_{inact}) dependent on the substrate used. Of note is that when all the substrate has been consumed, the area below these curves is providing a molecules consumed per RLU produced ratio (see table 1). Concerning the kinetics of these reactions, they are fitting with a Michaelis-Menten model, if we assume that the number of detected photons per consumed substrate molecule is constant whatever the substrate concentration (see the supporting information for a discussion). The K_M and V_{max} values were computed considering: 1) the luciferins (**S**) as the limiting substrates, O_2 as saturating substrate 2) an inhibition of the enzyme **E** by excess of substrate through the binding of a second substrate (**ESS**) on the Michaelis' complex (**ES**) with the dissociation constant K_I and 3) a stochastic inactivation of the enzyme (**E***) with the kinetic constant k_{inact} . As seen in table 1 and figure 3, coelenterazine (**1**) has a very high k_{cat} but a poor photon emission efficiency. Interestingly, furimazine (**3**) and its isomer **5eB** are the most efficient photon emitter per substrate molecule catalysis with the same k_{cat} but furimazine (**3**) is somehow providing a longer life time of active enzyme (low k_{inact}). All substrates are sensitive to substrate concentration beyond the K_M , but two luciferins, coelenterazine (**1**) and **5eD**, are more affected than others as seen with their low dissociation constants (K_I). Of note is that the strong light intensity produced by the catalyzed oxidation of **5dB** in a very short time (flash) does not mean the production of a high level of light intensity cumulated in two hours (ΣI) and the very long half-life ($t_{1/2}$) with low light intensity (glow) provided by compound **5eD** neither. Among these substrates, furimazine (**3**) is, so far, providing the best compromise. Also quite unexpected is the reason behind the fact that the natural substrate coelenterazine (**1**) leads to a signal two orders of magnitudes less intense than furimazine (**3**). Indeed, it is not because of a lesser catalytic activity

but it is mostly due to a pretty much counter intuitive lesser number of photons detected by luciferin consumed.

Table 1. Kinetic parameters for each substrate.							
	3	4	5dA	5dB	1	5eB	5eD
I_{max}							
(10^6 RLU s^{-1})	1.69	3.28	2.05	3.11	0.23	1.98	0.10
$t_{1/2}$ (min)	74.29	19.34	3.22	0.33	20.96	32.27	148
ΣI (10^6 RLU)	84.98	80.85	5.61	11.57	6.29	62.97	6.04
K_M (10^{-6} M)	3.40	2.33	3.71	3.45	8.8	4.23	7.02
k'_{cat}							
(10^{18} RLU s^{-1})	1.80	3.50	2.32	3.60	0.44	2.33	0.17
Molecules/RLU	1775	4581	12067	4463	101169	1804	70020
K_I (10^{-6} M)	104.94	115.21	140.55	101.64	22.48	84.02	35.42
K_M (10^{-6} M)	2.22	3.02	5.90	3.88	6.80	3.30	2.94
k_{cat} ($mol.s^{-1} .mole^{-1}$)	106	534	932	535	1500	140	362
k_{inact} (10^{-4} s $^{-1}$)	1.5	5.2	425	375	3.8	4.5	3.1
An alternative relative depiction of some of these variables is provided figure 3.							

Figure 3 is probably providing a better visual representation of these differences. In comparison with the central equidistant triangle representing three characteristics of furimazine (**3**): maximum intensity (I_{max}), the sum of the signal (ΣI), and half-life of the signal over two hours ($t_{1/2}$), the values for the other substrates can vary widely. Accordingly, the next stage of our research will be to find out how specific structural features of the luciferins have an influence on these characteristics and more importantly, can such changes lead to even better luciferins.

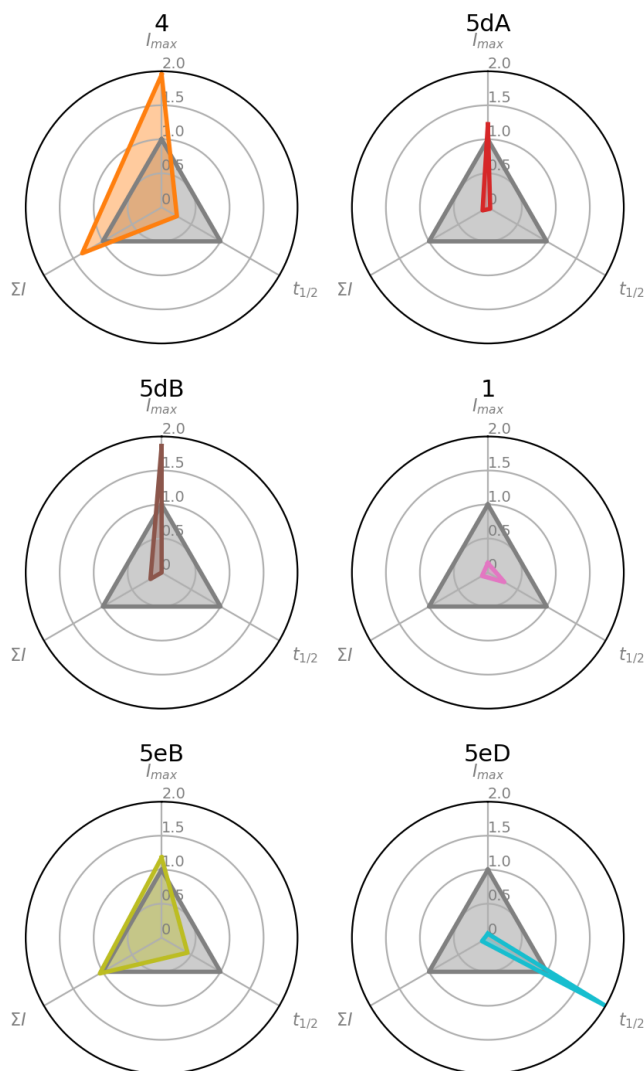


Figure 3. Depiction of the maxima of intensities (I_{max}), integrated signals (ΣI), and half-time durations ($t_{1/2}$) for all the luciferins, relative to furimazine (**3**) represented in light grey.

Conclusion

In conclusion, the results of this work should herald more researches focusing/based on bioluminescent reporting systems using imidazo[1,2-*a*]pyrazine-3(7*H*)-one luciferins. Indeed, we describe here a simple mean to prepare these rather expensive luciferins in such amount that it should provide scientists with many more opportunities to design and use such reporting systems. The kinetic analyses of light emission presented here are providing rather unexpected insights for distinct applications. The glowing property of some substrates is appropriate for high throughput in vitro bioassays and long imaging dynamics in vivo or in cellulo, whereas the flashier profile of other is appropriate for high sensitivity acquisition systems requiring more light in a short time. Moreover, it appears that if the actual catalytic efficiency of nanoKaz/NanoLuc is high, as it is in the 10^2 - 10^3 mol_s/s·mol_e range, the detection of emitted photons is remarkably modest: only one

RLU for more than 1800 decarboxylated molecules at best. Also of note is the fast stochastic inactivation seen for some substrate in contrast with other which may reflect the existence of two distinct inactivation mechanisms which we are also trying to investigate. In any case, in view of all this, we believe that further luciferases mutagenesis and/or design of original luciferin analogues could lead to even more improved bioluminescence profiles.

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Supplementary information

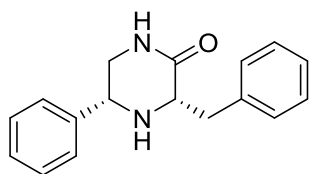
Chemistry

^1H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance 400 spectrometer at 400 MHz and 100 MHz, respectively. Shifts (δ) are given in ppm with respect to the TMS signal and cross-coupling constants (J) are given in Hertz. Column chromatography were performed either on Merck silica gel 60 (0.035 - 0.070 mm) or neutral alumina containing 1.5% of added water using a solvent pump and an automated collecting system driven by a UV detector set to 254 nm unless required otherwise. Sample deposition was carried out by absorption of the mixture to be purified on a small amount of the solid phase followed by its deposition of the top of the column. The low resolution mass spectra were obtained on an Agilent 1100 series LC/MSD system using an atmospheric electrospray ionization system or an Agilent 1200 series LC/MSD system using an Agilent Jet-Stream atmospheric electrospray ionization system and the high resolution mass spectra (HRMS) were obtained using a Waters Micromass Q-ToF with an electrospray ion source. When specified, anhydrous solvents used were purchased. Unless stated otherwise, a purity of at least 95% was obtained for all the compounds by means of chromatography, recrystallization or distillation and this level of purity was established by TLC, LC/MS and NMR spectroscopy.

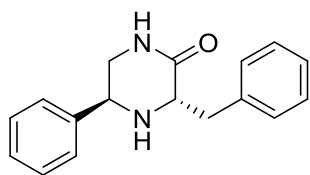
Synthesis and separation of (3S,5R) and (3S,5S)-3-benzyl-5-phenylpiperazin-2-ones (15a).

First step: preparation of nitroethylamine **13a**. Commercially available 2-nitrovinylbenzene (**11a**) (3.78 g, 0.025 mol) was added to a freshly extracted free base of L-phenylalanine ethyl ester (4.9 g, 0.025 mol). The suspension was made homogeneous by the addition of a small amount of dichloromethane and the solvent was then removed under vacuum to give a thick oil. Upon standing at least 10 minutes, an ^1H NMR sample pointed out the occurrence of the 1,4 adducts. A complete conversion could never be observed even if the oil was left overnight at room temperature. However, a more than 95% conversion was routinely achieved if this oil was left for one hour in the present case. Second step: preparation of diamine **14a**. The oil mentioned above was dispersed in a cold (10 °C) mixture of dioxane (100 mL) and 37% hydrochloric acid (38.7 mL, 0.33 mol). Zinc dust (7.45 g, 0.11 mol, < 10 μm) was added portion-wise in the course of 10 minutes while stirring rapidly. The suspension was then allowed to warm to room temperature

and stirred for 2 hours. This was diluted in water, made basic with an excess of 22% ammonia and extracted with ethyl acetate. The organic layer was washed with a small amount of 22% ammonia, water, brine, dried over sodium carbonate and concentrated to dryness to give the crude aminoester **14a** as an oil. Third step: preparation of compounds **15a**. This oil was heated at 140 °C under argon for 3 hours. The resulting ethanol was removed under vacuum, the solid was dispersed in boiling cyclohexane and filtered to remove unreacted L-phenylalanine ester. At this stage, the resulting solid can be used directly in the next step or further purified by a chromatography over silica gel (dichloromethane / ethanol 96/4 to 95/5) to separate the two diastereoisomers of **15a** as described below. *Note:* their structure attribution was easily performed by checking for the existence for the first diastereoisomer (or not, for the second diastereoisomer) of a nOe effect between their H-3 and H-5.



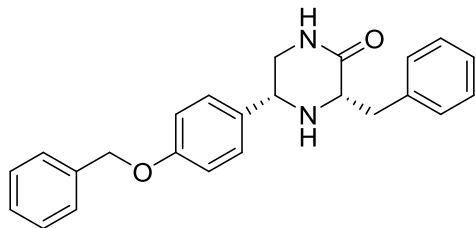
(3S,5R)-3-benzyl-5-phenylpiperazin-2-one (**15a, first dia**): Obtained as a white solid (2.05 g, 35%). ¹H NMR (CDCl₃): 7.42 – 7.29 (m, 9H), 7.23 (m, 1H), 6.27 (s, 1H), 4.06 (dd, 1H, *J* = 9.7, 4.7 Hz), 3.84 (dt, 1H, *J* = 10.1, 3.4 Hz), 3.60 (dd, 1H, *J* = 13.6, 3.1 Hz), 3.32 (m, 2H), 2.91 (dd, 1H, *J* = 13.6, 10.1 Hz), 1.78 (s, 1H). ¹³C NMR (CDCl₃): 171.0, 140.2, 138.4, 129.4, 128.7, 128.7, 128.2, 126.8, 126.6, 60.9, 57.8, 50.0, 38.4. HRMS (*m/z*): [M+H]⁺ calcd for C₁₇H₁₉N₂O, 267.1497, found: 267.1459.



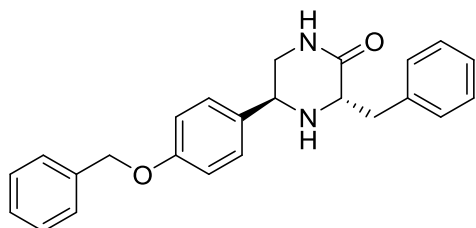
(3S,5S)-3-benzyl-5-phenylpiperazin-2-one (**15a, second dia**): Obtained as a white solid (1.29 g, 22%). ¹H NMR (CDCl₃): 7.46 – 7.18 (m, 10H), 6.44 (s, 1H), 4.27 (dd, 1H, *J* = 9.6, 4.0 Hz), 3.89 (dd, 1H, *J* = 10.7, 3.6 Hz), 3.50 (m, 1H), 3.41 (dt, 1H, *J* = 11.5, 4.0 Hz), 3.32 (dd, 1H, *J* = 13.8,

3.6 Hz), 3.18 (dd, 1H, $J = 13.8, 10.7$ Hz), 1.79 (s, 1H). ^{13}C NMR (CDCl_3): 171.8, 139.8, 138.0, 129.3, 128.9, 128.8, 128.2, 126.9, 126.8, 59.4, 51.7, 49.3, 37.8. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{17}\text{H}_{19}\text{N}_2\text{O}$, 267.1497, found: 267.1435.

Synthesis and separation of (3S,5R) and (3S,5S)-3-benzyl-5-(4-(benzyloxy)phenyl)piperazin-2-one (15b). First step: preparation of nitroethylamine **13b**. The 1-(benzyloxy)-4-(2-nitrovinyl)benzene¹ (**11b**) (3.54 g, 0.013 mol) was added to a freshly extracted free base of L-phenylalanine ethyl ester (2.68 g, 0.013 mol). The suspension was made homogeneous by the addition of a small amount of dichloromethane (30 mL) and the solvent was then removed under vacuum to give a thick oil. This was left standing overnight at room temperature to ensure a most complete conversion. Second step: preparation of diamine **14b**. The oil mentioned above was dispersed in a cold (10 °C) mixture of dioxane (50 mL) and 37% hydrochloric acid (20 mL, 0.16 mol). Zinc dust (3.65 g, 0.055 mol) was added portion-wise in the course of 10 minutes while stirring rapidly. The suspension was then allowed to warm to room temperature and stirred for 2 hours. This was diluted in water, made basic with an excess of 22% ammonia and extracted with ethyl acetate. The organic layer was washed with a small amount of 22% ammonia, water and brine, dried over sodium carbonate and concentrated to dryness to give the crude aminoester **14b** as an oil. Third step: preparation of compounds **15b**. This oil was heated at 140 °C under argon for 3 hours. The resulting ethanol was removed under vacuum and the diastereoisomers of compound **15b** could be separated as described below. On a larger scale (i.e.: from 12.19 g of compound **11b**), the crude aminoester **14b** was heated at 190 °C in an oil bath under high vacuum for how long as it took (20 minutes) to remove the unreacted phenylalanine ethyl ester which plagued any purification as well as the aromatization step when using sulfur. Accordingly, this oil could be used in the next step (with sulfur) without further purification. Alternatively, also on a large scale (22.25 g of compound **11b**), the crude compound **15b** obtained was then oxidized directly into the N-oxide **17b** as described below.

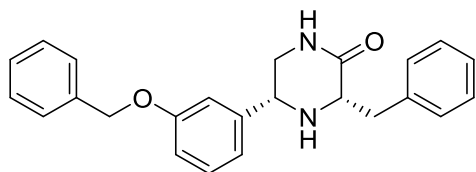


(3S,5R)-3-benzyl-5-(4-(benzyloxy)phenyl)piperazin-2-one (**15b, first dia**): This isomer was obtained using the first procedure described above as a white powder (0.92 g, 17%) after two chromatography over silica gel (dichloromethane - ethanol 95/5 to 92/8) and (cyclohexane - ethyl acetate 1/2) and a recrystallization in cyclohexane. ^1H NMR (CDCl_3): 7.45 – 7.21 (m, 12H), 6.94 (m, 2H), 6.47 (s (br), 1H), 5.06 (s, 2H), 4.00 (dd, 1H, $J = 4.3, 10.1$ Hz), 3.83 (dd, 1H, $J = 3.0, 10.1$ Hz), 3.59 (dd, 1H, $J = 3.0, 13.6$ Hz), 3.32 (m, 2H), 2.90 (dd, 1H, $J = 10.1, 13.6$ Hz), 1.73 (s (br), 1H). ^{13}C NMR (CDCl_3): 171.1, 158.7, 138.4, 136.9, 132.5, 129.4, 128.7, 128.6, 128.0, 127.9, 127.4, 126.6, 115.0, 70.1, 60.9, 57.2, 50.0, 38.5. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{25}\text{H}_{25}\text{N}_2\text{O}_3$: 373.1916, found: 373.1932.



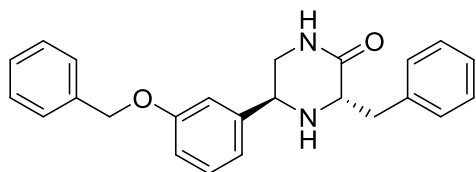
(3S,5S)-3-benzyl-5-(4-(benzyloxy)phenyl)piperazin-2-one (**15b, second dia**): This isomer was obtained using the first procedure described above as a white powder (0.59 g, 11%) after two chromatography over silica gel (dichloromethane - ethanol 95/5 to 92/8) and (ethyl acetate - ethanol 1/0 to 99/1). ^1H NMR (CDCl_3): 7.45 – 7.23 (m, 12H), 6.96 (m, 2H), 6.67 (s (br), 1H), 5.07 (s, 2H), 4.21 (dd, 1H, $J = 4.0, 9.6$ Hz), 3.85 (dd, 1H, $J = 3.5, 10.7$ Hz), 3.40 (m, 2H), 3.31 (dd, 1H, $J = 3.5, 13.8$ Hz), 3.18 (dd, 1H, $J = 10.7, 13.7$ Hz), 1.76 (s (br), 1H). ^{13}C NMR (CDCl_3): 171.9, 158.7, 138.0, 136.9, 132.2, 129.3, 128.9, 128.6, 128.1, 128.0, 127.4, 126.8, 115.0, 70.1, 59.4, 51.1, 49.3, 37.6. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{25}\text{H}_{25}\text{N}_2\text{O}_3$: 373.1916, found: 373.1907.

Synthesis and separation of (3S,5R) and (3S,5S)-3-benzyl-5-(3-(benzyloxy)phenyl)piperazin-2-one (15c). First step: preparation of nitroethylamine **13c**. The 1-(benzyloxy)-3-(2-nitrovinyl)benzene (**11c**)² (10.77 g, 0.042 mol), L-phenylalanine ethyl ester hydrochloride (9.69 g, 0.042 mol) and triethylamine (4.48 g, 0.044 mol) were stirred in dichloromethane (50 mL) for 10 minutes and the solvent was removed under vacuum to give a thick oil. This was left standing overnight at room temperature to ensure a most complete conversion. Second step: preparation of diamine **14c**. The oil mentioned above was dispersed in a cold (10 °C) mixture of dioxane (100 mL) and 37% hydrochloric acid (41 mL, 0.506 mol). Zinc dust (10.9 g, 0.168 mol) was added portion-wise in the course of 10 minutes while stirring rapidly. The suspension was then allowed to warm to room temperature and stirred for 2 hours. This was diluted in water, made basic with an excess of 22% ammonia and extracted with ethyl acetate. The organic layer was washed with a small amount of 22% ammonia, water and brine, dried over sodium carbonate and concentrated to dryness to give the crude aminoester **14c** as an oil. Third step: preparation of compounds **15c**. This oil was heated at 140 °C under argon for 3 hours. The resulting ethanol was removed under vacuum and the residue extracted with boiling cyclohexane, the extract left to crystallize to yield (in two crops) compound **15c** as a mixture of the two diastereoisomers (4.92 g, 31%). The insoluble material and the filtrates were concentrated to dryness and additional amount of the two (separated) diastereoisomers (1.64 g and 1.77 g, 21% in total) were obtained from this residue as described below.



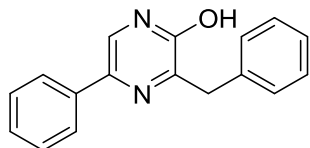
(3S,5R)-3-benzyl-5-(3-(benzyloxy)phenyl)piperazin-2-one (**15c, first dia**): This isomer was obtained from the recrystallization residue described above as a white powder (1.64 g), still containing 3% of an unidentified material, after two chromatography over silica gel (dichloromethane - ethanol 97/3 to 96/4), (cyclohexane – ethyl acetate 2/3) and a recrystallization in cyclohexane. ¹H NMR (DMSO-*d*₆): 7.75 (d (br), 1H, *J* = 4.0 Hz), 7.46-7.15 (m, 11H), 7.05 (m, 1H), 6.95 (m, 1H), 6.90 (m, 1H), 5.08 (s, 2H), 3.97 (m, 1H), 3.67 (m, 1H), 3.27 (dd, 1H, *J* = 13.8, 3.63 Hz), 3.20 (dt, 1H, *J* = 11.3, 4Hz), 3.03 (t, 1H, *J* = 10.9 Hz), 2.82 (dd, 1H, *J* = 13.9, 8.6 Hz),

2.31 (m, 1H). ^{13}C NMR (DMSO- d_6): 170.2, 158.8, 143.4, 139.7, 137.5, 129.9, 129.8, 128.9, 128.5, 128.3, 128.1, 126.4, 119.6, 114.1, 113.8, 69.6, 60.3, 56.7, 49.2, 38.1. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{24}\text{H}_{25}\text{N}_2\text{O}_2$: 373.1916, found: 373.1919.



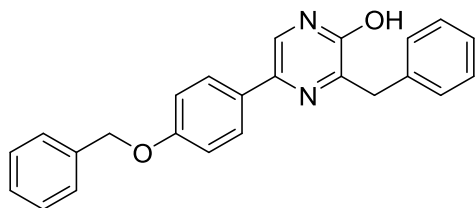
(3S,5S)-3-benzyl-5-(3-(benzyloxy)phenyl)piperazin-2-one (**15c**, **second dia**): This isomer was obtained from the recrystallization residue described above as a white powder (1.77 g) after a chromatography over silica gel (dichloromethane - ethanol 97/3 to 96/4) and a dispersion in boiling cyclohexane. ^1H NMR (DMSO- d_6): 7.81 (d (br), 1H, $J = 3.0$ Hz), 7.45-7.31 (m, 5H), 7.28 – 7.16 (m, 6H), 7.04 (m, 1H), 6.91 (m, 2H), 5.05 (s, 2H), 4.06 (m, 1H), 3.52 (m, 1H), 3.29 (m, 1H), 3.21 (m, 1H), 3.03 (m, 2H), 2.45 (s (br), 1H). ^{13}C NMR (DMSO- d_6): 171.2, 158.9, 143.3, 139.9, 137.6, 129.8, 129.7, 128.9, 128.6, 128.3, 128.1, 126.5, 119.6, 114.0, 113.8, 69.6, 58.7, 51.2, 48.3, 37.8. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{24}\text{H}_{25}\text{N}_2\text{O}_2$: 373.1916, found: 373.1926.

General procedure for the synthesis of compounds 16a-c using sulfur. The considered piperazin-2-one (0.011 mol) and sulfur (0.72 g, 0.0225 mol) were heated to reflux in 1,3-dichlorobenzene (40 mL) for 10 hours. This was concentrated to dryness and the residue purified as described below. On a larger scale, the residue was routinely taken up directly in the next step to obtain the chloropyrazines **9** as described.

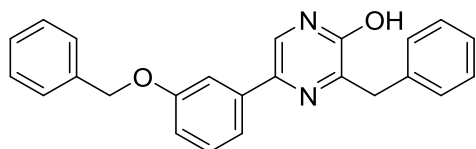


3-Benzyl-5-phenylpyrazin-2-ol (**16a**): This compound was obtained as a white powder (2.28 g, 73%) after a chromatography over silica gel (dichloromethane – ethanol 98/2 to 97/3). ^1H NMR

(DMSO-*d*₆) 12.41 (s, 1H), 7.85 (m, 3H), 7.40 (m, 4H), 7.30 (m, 3H), 7.20 (m, 1H), 4.07 (s, 2H). ¹³C NMR (DMSO-*d*₆): 157.4, 155.4, 138.4, 136.4, 131.4, 129.6, 129.1, 128.7, 127.7, 126.7, 124.9, 122.9, 39.3. HRMS (*m/z*): [M+H]⁺ calcd for C₁₇H₁₅N₂O: 263.1184, found, 263.1118.

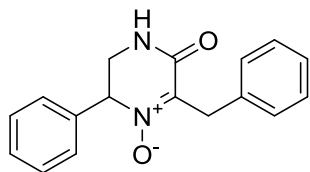


3-Benzyl-5-(4-(benzyloxy)phenyl)pyrazin-2-ol (**16b**): This compound was obtained as a powder (0.8 g, 62%) after a chromatography over silica gel (dichloromethane - ethanol 98/2). ¹H NMR (DMSO-*d*₆) 12.31 (s, 1H), 7.78 (m, 2H), 7.40 (m, 4H), 7.37 (m, 9H), 7.19 (m, 1H), 7.03 (m, 2H), 5.13 (s, 2H), 4.06 (s, 2H). ¹³C NMR (DMSO-*d*₆): 157.8, 156.5, 154.8, 137.9, 137.0, 131.2, 129.0, 128.8, 128.4, 128.2, 127.7, 127.6, 126.1, 125.8, 121.5, 114.9, 69.2, 38.8. HRMS (*m/z*): [M+H]⁺ calcd for C₂₄H₂₂N₂O₂: 369.1603, found, 369.1603.

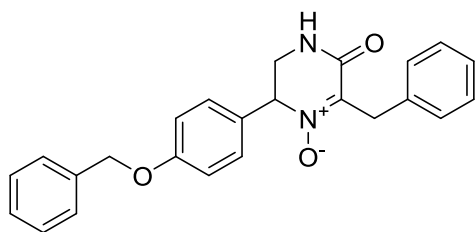


3-Benzyl-5-(3-(benzyloxy)phenyl)pyrazin-2-ol (**16c**): This compound was obtained as beige solid (1.41 g, 54%) as described above but using boiling decaline (30 mL) instead of 1,3-dichlorobenzene after a chromatography over silica gel (cyclohexane – ethyl acetate 2/1 to 1/1). ¹H NMR (DMSO-*d*₆) 12.42 (s (br), 1H), 7.92 (s, 1H), 7.50-7.27 (m, 12H), 7.20 (m, 1H), 6.94 (m, 1H), 5.13 (s, 2H), 4.07 (s, 2H). ¹³C NMR (DMSO-*d*₆): 159.2, 157.3, 155.5, 138.3, 137.9, 137.6, 131.0, 130.2, 129.7, 128.9, 128.7, 128.3, 128.1, 126.6, 123.1, 117.5, 114.2, 111.3, 69.7, 39.3. HRMS (*m/z*): [M+H]⁺ calcd for C₂₄H₂₂N₂O₂: 369.1603, found, 369.1609.

General procedure for the synthesis of the N-oxide 17a-b:



6-Benzyl-5-oxo-2-phenyl-2,3,4,5-tetrahydropyrazine 1-oxide (**17a**): To 3-benzyl-5-phenylpiperazin-2-one (0.44 g, 1.65 mmol) dissolved in acetic acid (5 mL) was added a 36% solution of peracetic acid in acetic acid (0.77 g, 3.63 mmol). This was stirred overnight, diluted in ethyl acetate, washed with water, brine, dried over molecular sieve and concentrated to dryness. The residue was purified by a chromatography over silica gel (cyclohexane – ethyl acetate 1/1 to 1/2) to yield the N-oxide (0.26 g, 56%) as a white powder. NMR, COSY correlations firmly established the structures of this compound. ^1H NMR (CDCl_3): 7.43 (m, 2H), 7.35-7.20 (m, 6H), 7.15 (m, 2H), 6.84 (s (br), 1H), 5.12 (t, 1H, $J = 4.4$ Hz), 4.13 (s, 2H), 4.00 (dd, 1H, $J = 4.4, 13.3$ Hz), 3.63 (dt, 1H, $J = 4.4, 13.3$ Hz). ^{13}C NMR (CDCl_3): 161.2, 141.7, 136.0, 134.2, 129.6, 129.1, 129.0, 128.4, 126.7, 126.6, 71.8, 43.1, 30.2. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{17}\text{H}_{17}\text{N}_2\text{O}_2$: 281.1190, found, 281.1234.



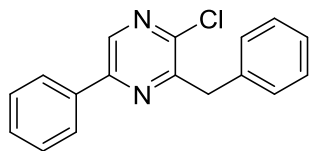
6-Benzyl-2-(4-(benzyloxy)phenyl)-5-oxo-2,3,4,5-tetrahydropyrazine 1-oxide (**17b**): The crude piperazin-2-one **16b** obtained from compound **11b** (0.0871 mol) as described above was dissolved in ethyl acetate (600 mL), 35% peracetic acid in acetic acid (33.8 mL, 0.174 mol) was added and this was stirred for 24 hours. The precipitate was filtered, washed with ethyl acetate and dried to yield compound **17b** (5.40 g, 16% from nitrostyrene **11b**). The filtrate was washed with water, brine, dried over magnesium sulfate, concentrated to dryness and the residue purified by chromatography over silica gel (cyclohexane – ethyl acetate 1/1 to 1/2) to yield more of compound **17b** (7.31 g, 21% from nitrostyrene **11b**). ^1H NMR ($\text{DMSO}-d_6$) 8.31 (d (br), 1H, $J =$

3.8 Hz), 7.44-7.17 (m, 10H), 7.14 (m, 2H), 6.97 (m, 2H), 5.17 (m, 1H), 5.10 (s, 2H), 3.94 (m, 3H), 3.53 (dt, 1H, $J = 4.5, 14.0$ Hz). ^{13}C NMR (DMSO- d_6): 160.7, 158.8, 140.7, 137.4, 137.2, 129.3, 128.9, 128.6, 128.5, 128.3, 128.1, 128.0, 126.7, 71.0, 69.7, 42.6, 30.2 (one signal missing). HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{24}\text{H}_{23}\text{N}_2\text{O}_3$: 387.1709, found, 387.1714.

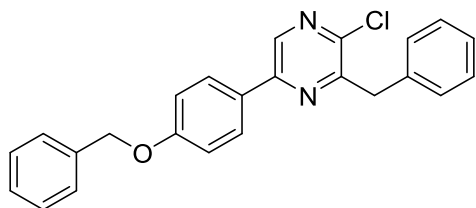
Procedure for the synthesis of compounds 16a via a thermal dehydration of the N-oxide 17a. In a microwave-adapted vial, a crude batch of 6-benzyl-5-oxo-2-phenyl-2,3,4,5-tetrahydropyrazine 1-oxide (0.41 g, 1.46 mmol) obtained as described above was dissolved in chlorobenzene (5 mL). This was sealed and heated in a microwave oven at 190 °C for two hours. The solvent was removed in vacuum and the residue purified by a chromatography over silica gel (cyclohexane – ethyl acetate 3/2) to give 3-benzyl-5-phenylpyrazin-2-ol **16a** (0.20 g, 52% from 3-benzyl-5-phenylpiperazin-2-one **15a**) with analytical data identical with the one described above.

Procedure for the synthesis of compounds 16a-b via a sodium hydroxide - based rearrangement of the N-oxides 17a-b, representative procedure. The N-oxide **17b** (7.31 g, 0.0189 mol) and sodium hydroxide (2.26 g, 0.0567 mol) were heated to reflux in ethanol (90 mL) for one hour. This was diluted in water, made acid with hydrochloric acid, the precipitate was filtered, washed with water and dried under vacuum at 60 °C to yield compound **16b** (6.66 g, 95%) as described above. Note: from a sample of compound **17a** (0.11 g, 0.39 mmol), the same protocol gave compound **16a** (0.09 g, 87%) as described above.

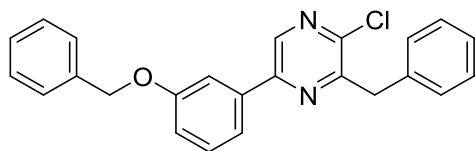
General procedure for the synthesis of compounds 9a-c: under a calcium-protected atmosphere, the considered 2-hydroxypyrazine (0.02 mol) was dispersed in phenylphosphonic dichloride (10 mL) and the suspension was heated at 100 °C for the indicated time. The resulting solution was diluted in ethyl acetate and poured onto an excess of crushed ice and stirred for 15 min. This was made basic with 22% ammonia and extracted with ethyl acetate. The organic layer was washed with water, brine, dried over magnesium sulfate and concentrated to dryness. The resulting residues were purified as described below.



3-benzyl-2-chloro-5-phenylpyrazine (**9a**): Obtained as a yellowish solid (1.60 g, 82%) after a chromatography over silica gel (cyclohexane-dichloromethane 3:2). Alternatively, this compound was obtained in 36% yield under the same reaction conditions but using the N-oxide **17a**. Note: From the crude mixture of compound **15a** obtained by a simple dispersion in boiling cyclohexane as described above and undertaking the next two synthetic steps (sulfur-based aromatization and chlorodehydroxylation) without any chromatography, batches of the pure compound **9a** (20.04 g, 62% from **11a**) were routinely obtained after a single chromatography over silica gel (dichloromethane-ethanol 97/3 to 96.5/3.5). ^1H NMR (CDCl_3): 8.68 (s, 1H), 8.04 (m, 2H), 7.55 (m, 3H), 7.42 (m, 2H), 7.35 (m, 2H), 7.28 (m, 1H), 4.41 (s, 2H). ^{13}C NMR (CDCl_3): 153.6, 150.4, 146.9, 138.6, 137.1, 135.4, 130.0, 129.2, 129.1, 128.5, 126.9, 126.8, 41.2. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{17}\text{H}_{14}\text{ClN}_2$, 281.0846, found, 281.0730.

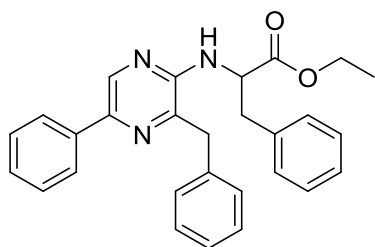


3-Benzyl-5-(4-(benzyloxy)phenyl)-2-chloropyrazine (**9b**): Obtained as a yellow solid (5.66 g, 62%) after heating for 18 hours and a chromatography over silica gel (cyclohexane-dichloromethane 1/1). ^1H NMR (CDCl_3): 8.61 (s, 1H), 8.0 (m, 2H), 7.47-7.20 (m, 10H), 7.11 (m, 2H), 5.17 (s, 2H), 4.38 (s, 2H). ^{13}C NMR (CDCl_3): 161.5, 153.3, 150.1, 145.9, 138.0, 137.2, 136.6, 129.2, 128.7, 128.5, 128.3, 128.1, 127.9, 127.4, 126.7, 115.4, 70.1, 41.2 (one signal missing). HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{24}\text{H}_{20}\text{ClN}_2\text{O}$, 387.1264, found, 387.1266.

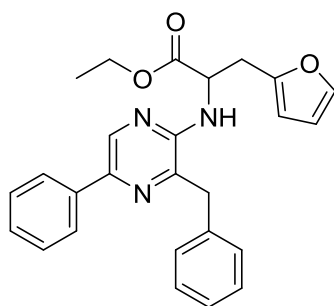


3-Benzyl-5-(3-(benzyloxy)phenyl)-2-chloropyrazine (**9c**): Obtained as a slowly solidifying oil (0.77 g, 61%) after heating for 18 hours and a chromatography over silica gel (cyclohexane-ethyl acetate 97/3). ^1H NMR (CDCl_3): 8.65 (s, 1H), 7.69 (m, 1H), 7.60 (m, 1H), 7.50 (m, 2H), 7.45-7.32 (m, 8H), 7.27 (m, 1H), 7.11 (m, 1H), 5.17 (s, 2H), 4.40 (s, 2H). ^{13}C NMR (CDCl_3): 159.4, 153.6, 150.0, 147.0, 138.6, 137.1, 136.8, 136.7, 130.1, 129.3, 128.7, 128.5, 128.1, 127.6, 126.8, 119.4, 116.7, 113.4, 70.2, 41.2. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{24}\text{H}_{20}\text{ClN}_2\text{O}$, 387.1264, found, 387.1262.

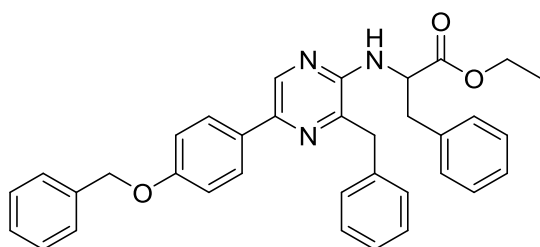
General procedure for the N-arylation of chloropyrazines 9a-c by α -aminoesters. In a 20 mL sealable vial, the considered 2-halogenopyrazine (1 mmol), the considered α -aminoesters^{3, 4} (1.1 mmol, either as a free base or as a hydrochloride salt), cesium carbonate (1.04 g, 3.2 mmol), palladium acetate (0.011 g, 0.05 mmol,) and 1,1'-binaphthalene-2,2'-diyl)bis(diphenylphosphine) (BINAP) (0.047 g, 0.075 mmol) were weighted. The air was replaced by argon and dry acetonitrile (4 mL) was injected. This was heated under an inert atmosphere (a balloon inflated with argon) at 60 °C for 12 hours using an oil bath along with fast stirring to break up any clumps of cesium hydrogen carbonate forming. The resulting dark red or black suspension was dispersed in dichloromethane, this was filtered, rinsed with dichloromethane and the filtrate was concentrated to dryness prior further purifications as described below. Note: on larger scale, a flask fitted with a rubber septum and a balloon inflated with argon worked as fine.



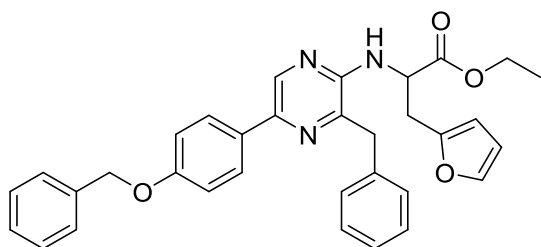
Ethyl (3-benzyl-5-phenylpyrazin-2-yl)phenylalaninate (**18aA**): Obtained as an oil (1.07 g, 69%) after a chromatography over silica gel (dichloromethane). ^1H NMR (CDCl_3): 8.43 (s, 1H), 7.96 (m, 2H), 7.49 (m, 1H), 7.37 (m, 1H), 7.33 – 7.20 (m, 8H), 7.90 (m, 2H), 4.97 (m, 2H), 4.14 (s, 2H), 4.13 (q, 2H, $J = 7.1$ Hz), 3.18 (dd, 1H, $J = 13.8, 5.4$ Hz), 3.09 (dd, 1H, $J = 13.8, 5.9$ Hz), 1.20 (t, 3H, $J = 7.1$ Hz). ^{13}C NMR (CDCl_3): 172.4, 150.4, 141.2, 141.1, 137.5, 136.9, 136.5, 136.3, 129.3, 128.8, 128.7, 128.6, 128.4, 127.8, 126.9, 126.9, 125.7, 61.2, 54.9, 40.9, 37.8, 14.1. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{28}\text{H}_{28}\text{N}_3\text{O}_2$, 438.2182, found, 438.2185.



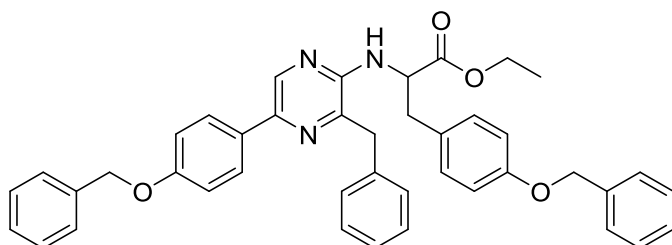
Ethyl 2-((3-benzyl-5-phenylpyrazin-2-yl)amino)-3-(furan-2-yl)propanoate (**18aB**): obtained as an oil (6.31 g, 89%) after a chromatography over silica gel (cyclohexane-ethyl acetate 95:5). ^1H NMR (CDCl_3): 8.42 (s, 1H), 7.95 (m, 2H), 7.46 (m, 2H), 7.39 – 7.28 (m, 5H), 7.22 (m, 2H), 6.22 (dd, 1H, $J = 3.2, 1.9$ Hz), 5.85 (dd, 1H, $J = 3.2, 0.7$ Hz), 5.13 (d, 1H, $J = 7.5$ Hz), 4.95 (dt, 1H, $J = 7.5, 5.3$ Hz), 4.16 (m, 4H), 3.21 (m, 2H), 1.22 (t, 3H, $J = 7.1$ Hz). ^{13}C NMR (CDCl_3): 172.0, 150.6, 150.3, 141.9, 141.2, 141.1, 137.5, 136.8, 136.6, 128.8, 128.7, 128.7, 127.8, 126.8, 125.7, 110.3, 107.7, 61.3, 53.2, 40.9, 30.4, 14.1. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{26}\text{H}_{26}\text{N}_3\text{O}_3$, 428.1974, found: 428.1965.



Ethyl (3-benzyl-5-(4-(benzyloxy)phenyl)pyrazin-2-yl)phenylalaninate (**18bA**): Obtained, as a white solid (4.47 g, 87%) after a chromatography over silica gel (cyclohexane – ethyl acetate 95/5 to 94/6). ¹H NMR (CDCl₃): 8.36 (s, 1H), 7.89 (m, 2H), 7.49 (m, 2H), 7.42 (m, 2H), 7.35 (m, 1H), 7.32-7.20 (m, 8H), 7.08 (m, 2H), 7.00 (m, 2H), 5.15 (s, 2H), 4.97 (m, 1H), 4.86 (d(br), 1H, *J* = 8.1 Hz), 4.14 (m, 4H), 3.17 (dd, 1H, *J* = 5.5, 13.8 Hz), 3.08 (dd, 1H, *J* = 6.0, 13.8 Hz), 1.19 (t, 3H, *J* = 7.2 Hz). ¹³C NMR (CDCl₃): 172.4, 158.8, 149.9, 141.1, 141.0, 137.0, 136.6, 136.3, 136.1, 130.5, 129.3, 128.8, 128.6, 128.5, 128.4, 128.0, 127.4, 126.9, 126.8, 126.7, 115.2, 70.1, 61.2, 54.9, 40.8, 37.9, 14.1. HRMS (*m/z*): [M+H]⁺ calcd for C₃₅H₃₄N₃O₃, 544.2600, found, 544.2609.



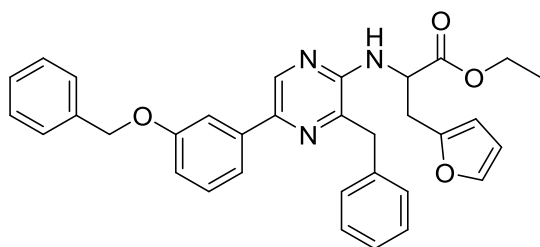
Ethyl 2-((3-benzyl-5-(4-(benzyloxy)phenyl)pyrazin-2-yl)amino)-3-(furan-2-yl)propanoate (**18bB**): Obtained as an oil (0.83 g, 79%) after a chromatography over silica gel (cyclohexane – ethyl acetate 94/6 to 93/7). ¹H NMR (CDCl₃): 8.35 (s, 1H), 7.89 (m, 2H), 7.48 (m, 2H), 7.42 (m, 2H), 7.36 (m, 1H), 7.32-7.20 (m, 6H), 7.08 (m, 2H), 6.22 (dd, 1H, *J* = 2.0, 3.0 Hz), 5.85 (dd, 1H, *J* = 0.7, 3.0 Hz), 5.15 (s, 2H), 5.07 (d(br), 1H, *J* = 7.6 Hz), 4.94 (m, 1H), 4.16 (m, 4H), 3.20 (d, 2H, *J* = 5.3 Hz), 1.21 (t, 3H, *J* = 7.2 Hz). ¹³C NMR (CDCl₃): 172.0, 158.8, 150.6, 149.9, 141.9, 141.1, 141.0, 137.0, 136.6, 136.1, 130.5, 128.8, 128.7, 128.6, 127.9, 127.4, 126.9, 126.8, 115.2, 110.3, 107.7, 70.1, 61.2, 53.2, 40.8, 30.5, 14.1. HRMS (*m/z*): [M+H]⁺ calcd for C₃₃H₃₂N₃O₄, 534.2393, found, 534.2410.



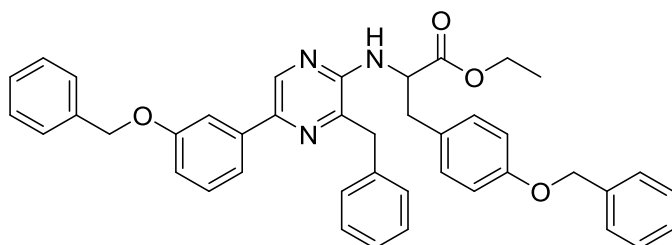
Ethyl

2-((3-benzyl-5-(4-(benzyloxy)phenyl)pyrazin-2-yl)amino)-3-(4-

(benzyloxy)phenyl)propanoate (**18bC**): Obtained as an oil (1.17 g, 70%) after a chromatography over silica gel (cyclohexane – ethyl acetate 91/9 to 9/1). ^1H NMR (CDCl_3): 8.34 (s, 1H), 7.88 (m, 2H), 7.45 (m, 4H), 7.40 (m, 4H), 7.34 (m, 2H), 7.25 (m, 3H), 7.20 (m, 2H), 7.06 (m, 2H), 6.91 – 6.77 (m, 4H), 5.13 (s, 2H), 5.05 (s, 2H), 4.90 (m, 1H), 4.83 (m, 1H), 4.11 (m, 4H), 3.10 (dd, 1H, $J = 13.9, 5.4$ Hz), 3.01 (dd, 1H, $J = 13.9, 5.9$ Hz), 1.19 (t, 3H, $J = 7.1$ Hz). ^{13}C NMR (CDCl_3): 172.7, 158.9, 157.9, 150.1, 141.2, 141.1, 137.2, 136.8, 136.3, 130.7, 130.4, 128.9, 128.8, 128.7 (3 signals), 128.1, 127.6, 127.0, 115.4, 115.0, 70.2, 61.2, 55.1, 41.0, 37.1, 14.3. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{42}\text{H}_{40}\text{N}_3\text{O}_4$, 650.3019, found, 650.3049.

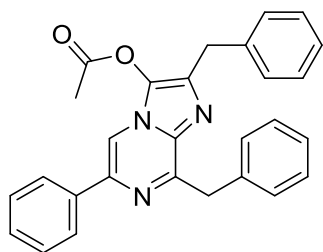


Ethyl 2-(3-benzyl-5-(3-(benzyloxy)phenyl)pyrazin-2-ylamino)-3-(furan-2-yl)propanoate (**18cB**): Obtained as an oil (0.63 g, 90%, still containing traces of EtOAc) after a chromatography over silica gel (cyclohexane – ethyl acetate 97/3 to 96/4). ^1H NMR (CDCl_3): 8.39 (s, 1H), 7.63 (m, 1H), 7.50 (m, 3H), 7.43-7.24 (m, 11H), 6.99 (m, 1H), 6.23 (dd, 1H, $J = 3.1, 2.0$ Hz), 5.85 (d, 1H, $J = 3.2$ Hz), 5.16 (s, 2H), 5.15 (d, 1H, $J = 6.1$ Hz), 4.96 (m, 1H), 4.17 (s, 2H), 4.16 (q, 2H, $J = 7.1$ Hz), 3.21 (m, 2H), 1.22 (t, 3H, $J = 7.1$ Hz). ^{13}C NMR (CDCl_3): 171.9, 159.4, 150.6, 150.3, 141.9, 141.2, 140.7, 138.9, 137.1, 136.7, 136.5, 129.8, 128.8, 128.7, 128.6, 127.9, 127.6, 126.9, 118.2, 114.5, 112.2, 110.3, 107.7, 70.1, 61.3, 53.2, 40.8, 30.4, 14.1 (one signal missing). HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{33}\text{H}_{32}\text{N}_3\text{O}_4$, 534.2393, found, 534.2402.



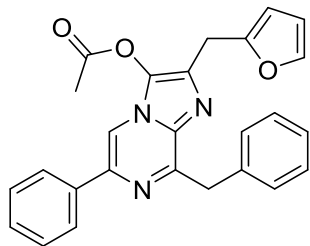
Ethyl 2-(3-benzyl-5-(3-(benzyloxy)phenyl)pyrazin-2-ylamino)-3-(4-(benzyloxy)phenyl)propanoate (**18cC**): Obtained as an oil (0.50 g, 78%) after a chromatography over silica gel (cyclohexane – ethyl acetate 97/3 to 96/4). ¹H NMR (CDCl₃): 8.40 (s, 2H), 7.65 (m, 1H), 7.65-7.20 (m, 17H), 6.99 (m, 1H), 6.88 (m, 2H), 6.83 (m, 2H), 5.17 (s, 2H), 5.07 (s, 2H), 4.94 (m, 2H), 4.14 (m, 4H), 3.13 (m, 1H), 3.013 (m, 1H), 1.21 (t, 3H, *J* = 7.1 Hz). ¹³C NMR (CDCl₃): 172.4, 159.4, 157.8, 150.2, 141.3, 140.6, 138.8, 137.2, 137.1, 136.4, 136.3, 130.3, 129.8, 128.8, 128.7, 128.6, 128.4, 128.0, 127.9, 127.6, 127.4, 126.9, 118.2, 114.9, 114.5, 112.2, 70.1, 70.0, 61.2, 55.0, 40.8, 36.9, 14.1 (one signal missing). HRMS (*m/z*): [M+H]⁺ calcd for C₄₂H₄₀N₃O₄, 650. 3019, found, 650. 3016.

Procedure for the synthesis of O-acetylated compounds 20aA-B, 20b-C, 20cB-C. In a sealable vessel, the considered N-pyrazyl aminoester **18** (1.0 mmol) and sodium hydroxide (0.16 g, 4 mmol) were weighted. The air was replaced with argon and anhydrous THF (5 mL) was injected. This was stirred at 20 °C under an inert atmosphere (a balloon inflated with argon) overnight and acetic anhydride (1.4 mL, 15.0 mmol) was then injected. After stirring an additional two hours under an inert atmosphere at room temperature, this was diluted in ethyl acetate, washed with water, brine and concentrated to dryness. The traces of acetic acid and acetic anhydride were removed by co-evaporation with toluene and then cyclohexane and the residue further purified as described below.

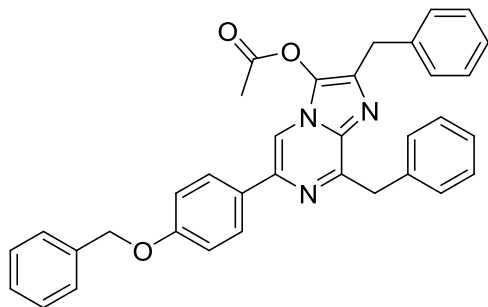


2,8-Dibenzyl-6-phenylimidazo[1,2-a]pyrazin-3-yl acetate (**20aA**): Obtained as a yellowish solid (0.17 g, 50%) after a recrystallization in cyclohexane. ¹H NMR (CDCl₃): 7.92 (m, 2H), 7.80 (s, 1H), 7.64 (m, 2H), 7.47 (m, 2H), 7.40 (m, 1H), 7.33 (m, 5H), 7.25 (m, 2H), 4.65 (s, 2H), 4.23 (s, 2H), 2.18 (s, 3H). ¹³C NMR (CDCl₃): 167.1, 152.9, 139.1, 137.9, 137.8, 136.8, 135.1, 133.5,

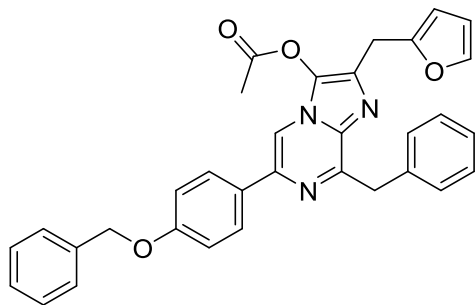
129.8, 129.1, 128.9, 128.8, 128.6, 128.5, 128.3, 126.5 (two signals), 126.4, 108.8, 39.4, 34.2, 19.9. HRMS (m/z): $[M+H]^+$ calcd for $C_{28}H_{24}N_3O_2$, 434.1869, found, 434.1825.



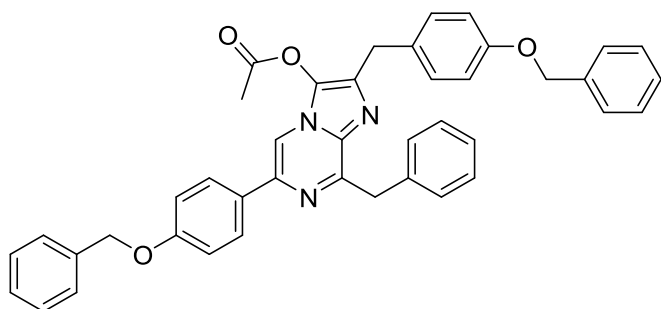
8-benzyl-2-(furan-2-ylmethyl)-6-phenylimidazo[1,2-a]pyrazin-3-yl acetate (**20aB**): Obtained as an off-white solid (4.02 g, 72%) after a recrystallization in cyclohexane. 1H NMR ($CDCl_3$): 7.92 (m, 2H), 7.83 (s, 1H), 7.63 (m, 2H), 7.47 (m, 2H), 7.42-7.30 (m, 4H), 7.23 (m, 1H), 6.35 (m, 1H), 6.16 (m, 1H), 4.64 (s, 2H), 4.24 (s, 2H), 2.36 (s, 3H). ^{13}C NMR ($CDCl_3$): 167.0, 153.0, 151.5, 141.5, 139.2, 137.8, 136.7, 133.5, 132.5, 129.7, 128.9, 128.8, 128.6, 128.3, 126.5, 126.4, 110.5, 108.9, 106.8, 39.3, 27.2, 20.1. HRMS (m/z): $[M+H]^+$ calcd for $C_{26}H_{22}N_3O_3$, 424.1661, found, 424.1607.



2,8-Dibenzyl-6-(4-(benzyloxy)phenyl)imidazo[1,2-a]pyrazin-3-yl acetate (**20bA**): Obtained as an off-white solid (0.44 g) pure enough (traces of THF and acetic acid) to be used directly in the next step. 1H NMR ($CDCl_3$): 7.83 (m, 2H), 7.72 (s, 1H), 7.63 (m, 2H), 7.47 (m, 2H), 7.48-7.21 (m, 13H), 5.15 (s, 2H), 4.64 (s, 2H), 4.22 (s, 2H), 2.18 (s, 3H). ^{13}C NMR ($CDCl_3$): 167.1, 159.3, 152.7, 139.0, 138.1, 137.9, 136.9, 135.0, 133.4, 129.7, 129.6, 129.1, 128.8, 128.6, 128.5, 128.3, 128.0, 127.7, 127.4, 126.5, 126.4, 115.2, 107.9, 70.1, 39.4, 34.2, 19.8. HRMS (m/z): $[M+H]^+$ calcd for $C_{35}H_{30}N_3O_3$, 540.2287, found, 540.2275.

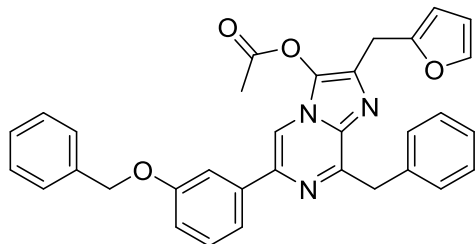


8-Benzyl-6-(4-(benzyloxy)phenyl)-2-(furan-2-ylmethyl)imidazo[1,2-a]pyrazin-3-yl acetate (**20bB**): Obtained as an off-white solid (1.13 g) pure enough (traces of acetic acid) to be used directly in the next step. ^1H NMR (CDCl_3): 7.85 (m, 2H), 7.75 (s, 1H), 7.61 (m, 2H), 7.47 (m, 2H), 7.44-7.21 (m, 7H), 7.07 (m, 2H), 6.37 (m, 1H), 6.16 (m, 1H), 5.15 (s, 2H), 4.62 (s, 2H), 4.24 (s, 2H), 2.35 (s, 3H). ^{13}C NMR (CDCl_3): 167.1, 159.3, 152.8, 151.5, 141.6, 139.1, 137.8, 136.8, 133.4, 132.3, 129.7, 129.6, 128.8, 128.6, 128.3, 128.0, 127.7, 127.4, 126.4, 115.2, 110.5, 107.9, 106.8, 70.1, 39.3, 27.2, 20.1. HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{33}\text{H}_{28}\text{N}_3\text{O}_4$, 530.2080, found, 530.2070.

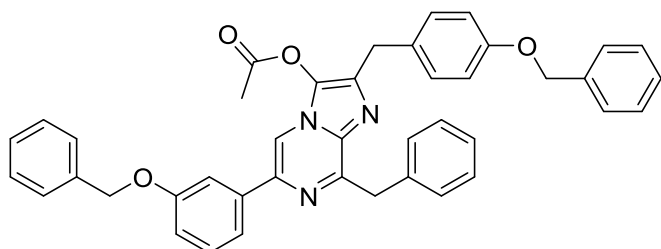


8-Benzyl-2-(4-(benzyloxy)benzyl)-6-(4-(benzyloxy)phenyl)imidazo[1,2-a]pyrazin-3-yl acetate (**20bC**): Obtained as an off-white solid (0.51 g) which was used directly in the next step. ^1H NMR (CDCl_3): 7.84 (m, 2H), 7.71 (s, 1H), 7.61 (m, 2H), 7.48 – 7.29 (m, 12H), 7.25 – 7.20 (m, 3H), 7.06 (m, 2H), 6.94 (m, 2H), 5.14 (s, 2H), 5.09 (s, 2H), 4.62 (s, 2H), 2.17 (s, 3H). ^{13}C NMR (CDCl_3): 167.1, 159.3, 157.9, 152.7, 138.9, 137.9, 137.1, 136.9, 135.4, 133.5, 130.4, 130.0, 129.7, 128.7, 128.6, 128.5, 128.2, 128.0, 127.9, 127.7, 127.5, 127.4, 126.4, 115.2, 114.9, 107.8,

70.1, 70.0, 39.4, 33.4, 26.9, 19.9. HRMS (m/z): $[M+H]^+$ calcd for $C_{42}H_{36}N_3O_4$, 646.2706, found, 646.2728.

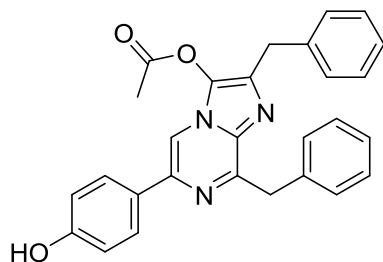


8-Benzyl-6-(3-(benzyloxy)phenyl)-2-(furan-2-ylmethyl)imidazo[1,2-a]pyrazin-3-yl acetate (**20cB**): Obtained as an oil (0.55 g) still containing a small amount of cyclohexane which was used in the next step without further purification. 1H NMR ($CDCl_3$): 7.83 (s, 1H), 7.61 (m, 3H), 7.49 (m, 3H), 7.45-7.21 (m, 8H), 7.02 (m, 1H), 6.36 (dd, 1H, $J = 3.1, 1.9$ Hz), 6.17 (dd, 1H, $J = 3.2, 0.8$ Hz), 5.14 (s, 2H), 4.65 (s, 2H), 4.25 (s, 2H), 2.36 (s, 3H). ^{13}C NMR ($CDCl_3$): 167.0, 159.3, 152.9, 151.4, 141.7, 138.9, 138.1, 137.7, 136.9, 133.4, 132.4, 129.8 (two signals), 128.9, 128.6, 128.3, 128.0, 127.6, 126.5, 118.8, 115.1, 113.3, 110.5, 109.0, 106.9, 70.2, 39.3, 27.1, 20.1. HRMS (m/z): $[M+H]^+$ calcd for $C_{33}H_{28}N_3O_4$, 530.2080, found, 530.2084.

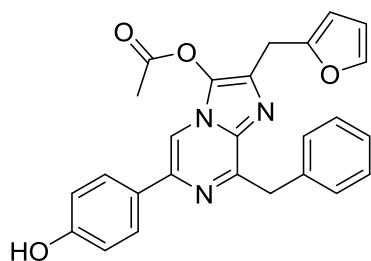


8-Benzyl-2-(4-(benzyloxy)benzyl)-6-(3-(benzyloxy)phenyl)imidazo[1,2-a]pyrazin-3-yl acetate (**20cC**): Obtained as a white solid (0.5 g, 77%) after a recrystallization in cyclohexane. 1H NMR ($CDCl_3$): 7.79 (s, 1H), 7.61 (m, 3H), 7.51-7.30 (m, 14H), 7.23 (3H), 7.01 (m, 1H), 6.94 (m, 2H), 5.14 (s, 2H), 5.09 (s, 2H), 4.64 (s, 2H), 4.16 (s, 2H), 2.18 (s, 3H). ^{13}C NMR ($CDCl_3$): 167.1, 159.3, 157.5, 152.8, 138.7, 138.3, 137.8, 137.2, 137.0, 135.5, 133.6, 130.3, 130.1, 129.8, 129.7, 128.8, 128.6, 128.5, 128.3, 128.0, 127.9, 127.6, 127.4, 126.4, 118.8, 115.0, 114.9, 113.2, 108.9, 70.2, 70.1, 39.4, 33.3, 19.9. HRMS (m/z): MIA.

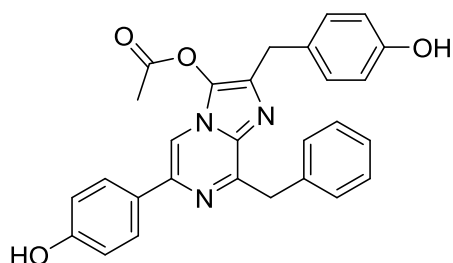
General protocol for the debenylation of compounds 20bA-C, 20cB-C into compounds **20dA-B, 20dD, 20eB** and **20eD**. The considered O-acetylated luciferin (1.05 mmol) and 10% palladium over charcoal (0.11 g, 0.105 mmol) were dispersed in ethyl acetate (20 mL), ethanol (20 mL) and acetic acid (6 mL). This was charged in hydrogen and stirred for 10-20 hours. Note: depending on the quality of the catalyst used, over-hydrogenation was sometime an issue, especially with the furan-bearing substrates. The resulting suspension was filtered, concentrated to dryness and the residue purified by chromatography as described below. Note: pre-adsorption of this residue over a small portion of silica gel before the chromatography was made using ethyl acetate without heating and no delay was taken before undertaking the purification by chromatography.



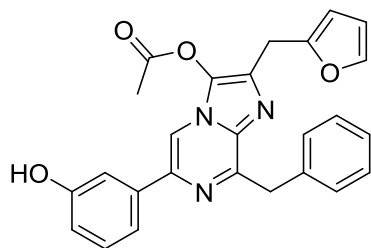
2,8-Dibenzyl-6-(4-hydroxyphenyl)imidazo[1,2-a]pyrazin-3-yl acetate (**20dA**): This compound was obtained as a white cotton (0.1 g, 27% from **18aA**) after a chromatography over silica gel (cyclohexane – ethyl acetate 4/1 to 3/1). ¹H NMR (DMSO-*d*₆): 9.64 (s, 1H), 8.54 (s, 1H), 7.89 (m, 2H), 7.48 (m, 2H), 7.26 (m, 8H), 6.87 (m, 2H), 4.46 (s, 2H), 4.08 (s, 2H), 2.37 (s, 3H). ¹³C NMR (DMSO-*d*₆): 168.5, 158.5, 151.9, 139.0, 138.4, 138.2, 135.3, 133.3, 129.7, 129.3, 129.1, 128.8, 128.7, 127.9, 127.5, 126.8, 126.7, 115.9, 109.4, 39.2, 33.0, 20.7. HRMS (*m/z*): [M+H]⁺ calcd for C₂₈H₂₄N₃O₃: 450.1818, found, 450.1825.



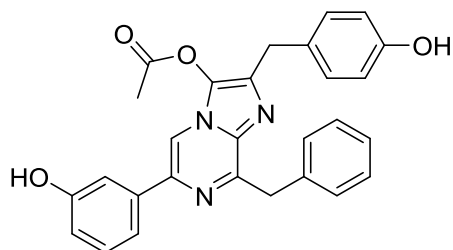
8-Benzyl-2-(furan-2-ylmethyl)-6-(4-hydroxyphenyl)imidazo[1,2-a]pyrazin-3-yl acetate (**20dB**): This compound was obtained as a white cotton (0.37 g, 39% from **18aB**) after a chromatography over silica gel (cyclohexane – ethyl acetate 3/1). ¹H NMR (DMSO-*d*₆): 9.65 (s, 1H), 8.55 (s, 1H), 7.90 (m, 2H), 7.55 (m, 1H), 7.47 (m, 2H), 7.27 (m, 2H), 7.22 (m, 1H), 6.88 (m, 2H), 6.39 (dd, 1H, *J* = 1.8, 3.1 Hz), 6.19 (dd, 1H, *J* = 0.8, 3.1 Hz), 4.46 (s, 2H), 4.13 (s, 2H), 2.40 (s, 3H). ¹³C NMR (DMSO-*d*₆): 168.4, 158.5, 152.0, 151.9, 142.4, 138.4, 138.3, 133.1, 132.6, 129.6, 129.2, 128.8, 127.9, 127.5, 126.8, 115.9, 111.0, 109.4, 107.2, 39.1, 26.8, 20.8. HRMS (*m/z*): [M+H]⁺ calcd for C₂₆H₂₂N₃O₄, 440.1610, found, 440.1603.



8-Benzyl-2-(4-hydroxybenzyl)-6-(4-hydroxyphenyl)imidazo[1,2-a]pyrazin-3-yl acetate (**20dD**): This compound was obtained as a beige solid (0.10 g, 27% from **20bC**) after a chromatography over silica gel (cyclohexane – ethyl acetate 2/1). ¹H NMR (DMSO-*d*₆) 9.65 (s, 1H), 9.20 (s, 1H), 8.53 (s, 1H), 7.89 (m, 2H), 7.48 (m, 2H), 7.24 (m, 3H), 7.07 (m, 2H), 6.87 (m, 2H), 6.69 (m, 2H), 4.46 (s, 2H), 3.96 (s, 2H), 2.37 (s, 3H). ¹³C NMR (DMSO-*d*₆): 168.5, 158.4, 156.2, 151.8, 138.5, 138.2, 136.0, 133.2, 130.2, 129.7, 129.1, 128.9, 128.7, 127.9, 127.6, 126.8, 115.9, 115.5, 109.3, 39.2, 32.3, 20.7. HRMS (*m/z*): [M+H]⁺ calcd for C₂₈H₂₄N₃O₄: 466.1767, found, 466.1758.



8-Benzyl-2-(furan-2-ylmethyl)-6-(3-hydroxyphenyl)imidazo[1,2-a]pyrazin-3-yl acetate (**20eB**): This compound was obtained as a red powder (0.12 g, 26% from **20cB**) after a chromatography over silica gel (cyclohexane – ethyl acetate 2/1). ¹H NMR (DMSO-*d*₆) 9.53 (s, 1H), 8.67 (s, 1H), 7.55 (m, 1H), 7.52 (m, 1H), 7.48 (m, 3H), 7.32-7.19 (m, 4H), 6.80 (ddd, 1H, *J* = 7.6, 2.0, 1.0 Hz), 6.39 (dd, 1H, *J* = 3.2, 2.0 Hz), 6.19 (dd, 1H, *J* = 3.2, 1.0 Hz), 4.48 (s, 2H), 4.15 (s, 2H), 2.40 (s, 3H). ¹³C NMR (DMSO-*d*₆): 168.4, 158.2, 152.1, 151.9, 142.5, 138.3, 138.0, 133.4, 132.8, 130.0, 129.6, 129.7, 129.4, 128.8, 126.9, 117.3, 116.0, 113.7, 111.1, 111.0, 107.2, 39.1, 20.8, 14.5. HRMS (*m/z*): [M+H]⁺ calcd for C₂₆H₂₂N₃O₄: 440. 1610, found, 440.1619.



8-Benzyl-2-(3-hydroxybenzyl)-6-(4-hydroxyphenyl)imidazo[1,2-a]pyrazin-3-yl acetate (**20eD**): This compound was obtained as a yellow powder (0.11 g, 32%) after a chromatography over silica gel (cyclohexane – ethyl acetate 2/1). ¹H NMR (DMSO-*d*₆) 9.56 (s, 1H), 9.24 (s, 1H), 8.64 (s, 1H), 7.50 (m, 4H), 7.25 (m, 4H), 7.08 (m, 2H), 6.83 (m, 1H), 6.70 (m, 2H), 4.48 (s, 2H), 3.97 (s, 2H), 2.37 (s, 3H). ¹³C NMR (DMSO-*d*₆): 168.6, 158.2, 156.2, 152.0, 138.4, 138.1, 137.9, 136.2, 133.4, 130.2, 129.7, 129.1, 129.0, 128.8, 126.9, 117.4, 116.0, 115.6, 115.5, 113.7, 111.0, 39.2, 32.3, 20.7. HRMS (*m/z*): [M+H]⁺ calcd for C₂₈H₂₄N₃O₄: 466. 1767, found, 466.1767.

General protocol for the generation of solutions of luciferins from the O-acetylated luciferin.

The considered O-acetylated luciferin (1 mg) was dissolved in DMSO (0.2 mL) and then diluted by adding a solution of acidic ethanol (0.3 ml) made from the addition of 37 % hydrochloric acid (100 μ l) on 100 % ethanol (12 mL). This 0.5 mL solution was incubated at 50°C for 2 to 3h to give a stock solution which was then stored at -80°C. As depicted in figure S1, the LC/MS monitoring of the hydrolysis of the O-acetylated luciferin **20a-B** into the corresponding luciferin **5a-B** was complete and clean in less than two hours.

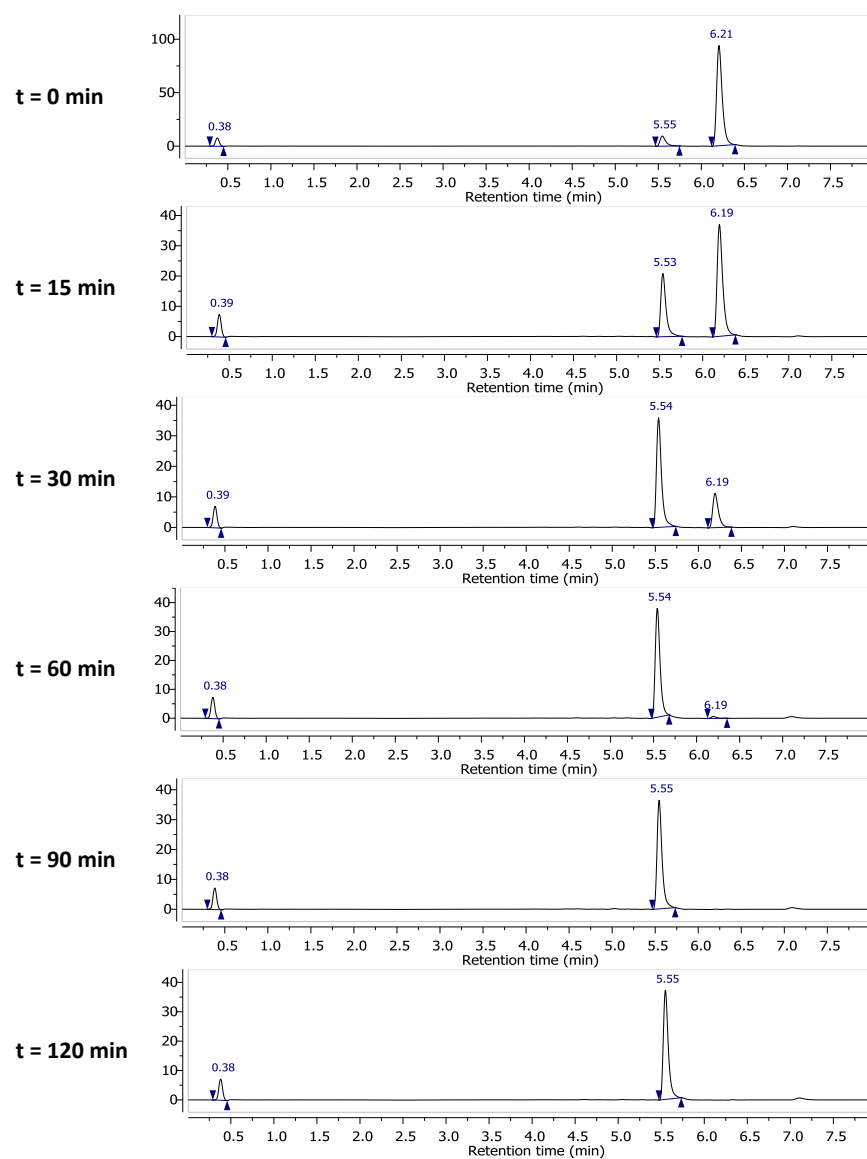


Figure S1. HPLC profiles, obtained on an Agilent apparatus, with a 3.5 μm XDB-C18 column, elution: 5:95 to 95:5 $\text{H}_2\text{O}/\text{MeOH}$ in 3.5 min, with constant 5 mM ammonium formate. Peak at $t_R = 5.5$ is compound **5a-B** (m/z $[\text{M}+\text{H}]^+ = 382$), peak at $t_R = 6.2$ min is the O-acetylated compound **20a-B** (m/z $[\text{M}+\text{H}]^+ = 424$).

Biochemistry

NanoKAZ expression, purification and quality validation

The nanoKAZ luciferase⁵ was expressed from a codon-optimized sequence of the gene coding for the previously reported⁶ NanoLuc, the mutated catalytic domain of the luciferase from *Oplophorus gracilostriis* featuring 16 amino acid substitutions. The gene *nanoKAZ* was synthesized by Eurofins (Germany) with carboxy-end His₆-tag (in caps) and flanking region corresponding to the pET23 sequence (Novagen).

```
Atggtcttcacactcgaagatttcgttggggactggcgacagacagccggctacaacctggaccaagtccttgaacagggaggtgtgtcca  
gtttgttcagaatctcgggggtgtccgtaactccgatccaaaggattgtcctgagcgggtgaaaatgggctgaagatcgacatccatgtcatcat  
cccgtatgaaggtctgagcggcgaccaaatgggccagatcgaaaaattttaaggtgggtgtaccctgtggatgatcatcactttaaggtgat  
cctgcactatggcactggtaatcgacggggttacgccgaacatgatcgactatttcggacggccgtatgaaggcatcgccgtgttcgacg  
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agtaaccatcaacggagtgaccggctggcggctgtgcgaacgcattctggcgCTCGAGCACCACCACCACCACCAC  
TGA
```

The pET23 plasmid was amplified with the forward and reverse oligonucleotides (Fwd:5'CTCGAGCACCACCACCACCACCAC3';

Rvr:5'GGTATATCTCCTTCTTAAAGTTAAAC3', Eurofins) using a Q5 DNA polymerase, dNTP mix (New England BioLabs). PCR product was purified by electrophoresis on agarose gel (1%, Macherey-Nagel). Purified pET23 vector and the synthetic gene were assembly using NEBuilder HiFi assembly master mix (New England BioLabs). The assembled product (5 μ L) was used to transform NEB 5-alpha competent *E. coli* and grown overnight on LB/Agar/ampicillin in Petri dish. Isolated colonies were grown in liquid medium, plasmid was isolated and nucleotide sequence was performed to confirm the presence of the insert (Eurofins).

The pET23-*kaz* was used to transform *E. coli* BL21 (DE3) to achieve high expression in *E. coli*. Cell were grown at 18 °C and IPTG was added to induce nanoKAZ production. After harvesting

the cells by centrifugation (1.5 L), pellet was resuspended in 50 mM Tris-HCl pH 8.0, 50 mM NaCl with protease inhibitors (Sigma) and lysozyme (0.1 mg/mL). Cells were disrupted by freezing-thawing cycle lysis method. DNase I (Sigma-Aldrich) was then added to remove DNA from the sample.

The crude extract was centrifuged 30 min at 1250 g. The supernatant was collected and NaCl (500 mM), imidazole (20 mM) and Triton X100 (0.1%) were added. Then this cleared lysate was loaded on a His-Trap HP column (5 mL, GE-Healthcare) at a flow rate of 4 mL/min using an AKTA pure chromatography system (GE-Healthcare). The column was washed with 20 volumes of column with a running buffer (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 20 mM imidazole) at 5 mL/min. The nanoKAZ was eluted with a gradient of imidazole from 20 mM to 200 mM in 50 mM Tris-HCl pH 8.0, 50 mM NaCl at 5 mL/min and fractions of 1 mL were collected in 96-deepwell plate (GE-Heath). Catalytic activity of fractions was profiled using a luminometer Hydex by diluting 10^7 folds the fractions in PBS with 27 μ M of furimazine (8-benzyl-2-(furan-2-ylmethyl)-6-phenylimidazo[1,2-*a*]pyrazin-3(7*H*)-one). The fractions of high activity were pooled, and loaded on a HiTrap Q column (1 mL, GE-Healthcare) equilibrated in 50 mM Tris-HCl pH 8.0, NaCl 50 mM. The protein was eluted in 50 mM MES pH 6.5, 50 mM NaCl at 1 mL/min at 18°C using the AKTA pure chromatography system. The fractions of 500 μ L were collected in 96-deepwell plates and their activities were assayed as described above. The fractions of high activity were pooled. An UV-spectrum (240-300nm) was acquired for evaluating the concentration of nanoKAZ from the solution absorption at 280 nm. The extinction coefficient was estimated by the method of Gill and von Hippel⁷ from the residue sequence of nanoKAZ ($\epsilon_{280\text{nm}} = 24750 \text{ M}^{-1}\cdot\text{cm}^{-1}$). The specific activity is about $92 \cdot 10^9$ acquired photons / s / mg of nanoKAZ.

As depicted in figure S2, the quality of the purified protein was assessed by loading a 2 μ L aliquot on a stain-free SDS gel (4–15% Mini-PROTEAN® TGX Stain-Free™ Protein Gels, Bio-Rad). The gel was activated by UV trans-illumination for 5 min (Bio-Gel Doc XR Imaging System) and fluorescence of tryptophan in protein bands were imaged. The gel was then transferred on nitrocellulose (ECL membrane, GE-Healthcare) and revealed using a mouse HRP-coupled IgG anti-His-tag (Invitrogen).

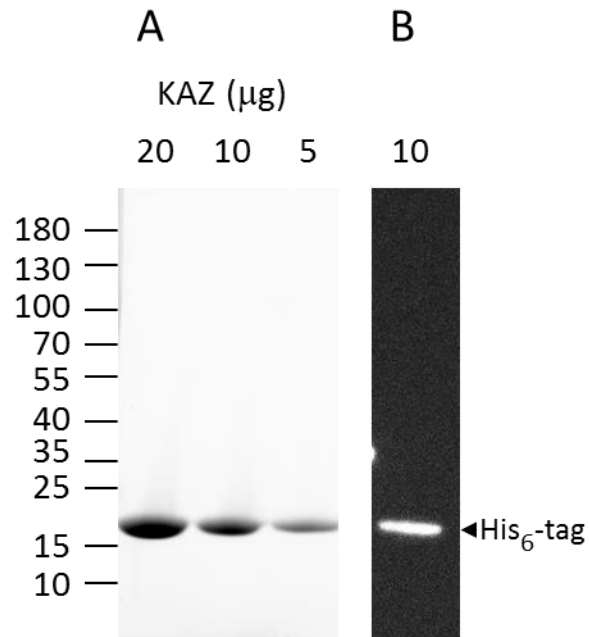


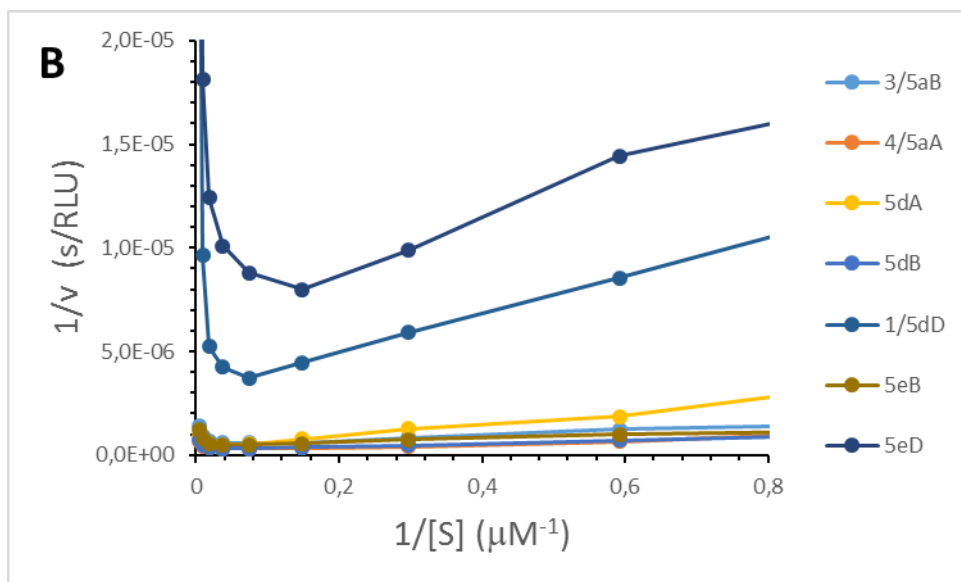
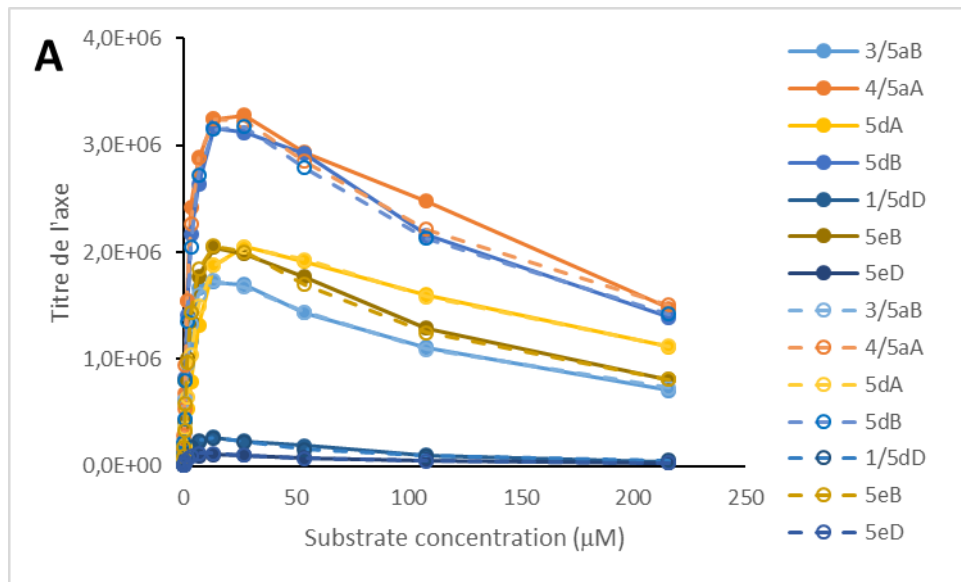
Figure S2. SDS-PAGE and western blot of purified nanoKAZ luciferase. The molecular weight scale (kDa) is indicated on the left. (A) The lanes from left to right show stained nanoKAZ bands (19 kDa) corresponding to 20, 10 and 5 μ g solution samples respectively. (B) The lane shows the immunoprint of nanoKAZ band (19 kDa, 10 μ g) revealed through its C-end His₆-tag by a HRP-coupled anti-His tag in the presence of bioluminescent HRP substrate (Supersignal West Femto, ThermoFisher Scientific).

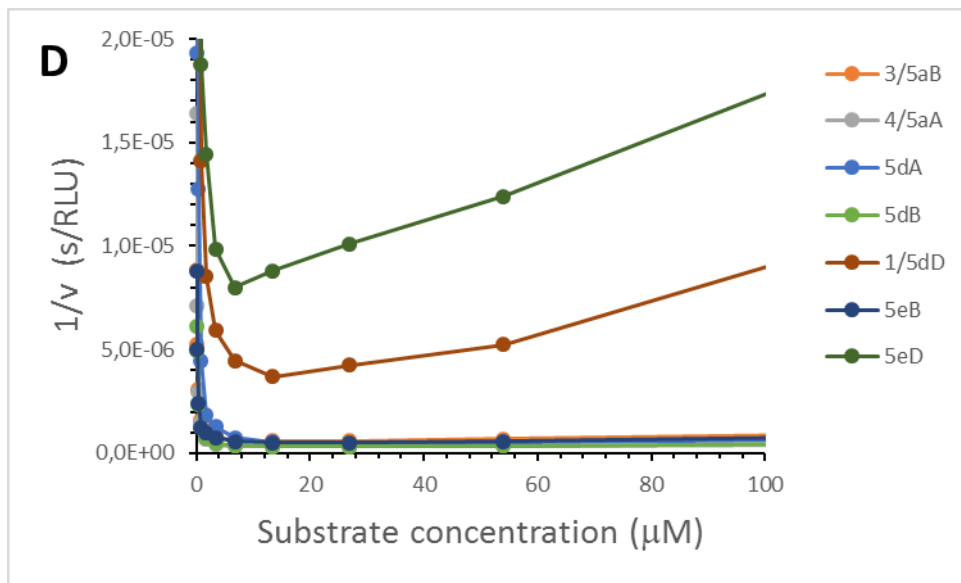
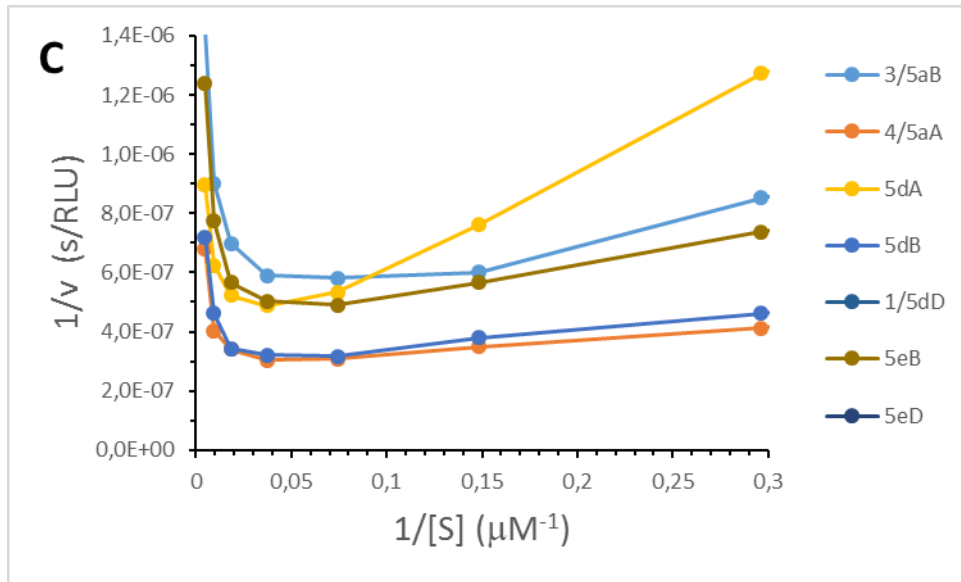
Bioluminescence assays

In 96-well white flat bottom plate (Costar), 50 μL /well were loaded from a 1/50th dilution of the stock (5.4 mM) imidazo[1,2-*a*]pyrazin-3(7*H*)-ones solution in DPBS (Gibco) + 0.1% (v/v) Tween 20 (Sigma-Aldrich). Note: the pH of DPBS (Gibco) is between 7.0 and 7.3; results may vary with a different pH. Spontaneous luminescence emission intensity displayed by the different luciferin analogues was measured on 3 points using a Berthold CentroXS luminometer (integration time of 1 second, 22°C). The nanoKAZ solution (at 50 ng/L) was diluted 10⁶ times in PBS/tween buffer and 50 μL was added per well. The plate was orbitally shaken for 5 seconds, then the luminescence signal intensity was measured per well over 2 hours, integrating 1 s/well every 3 minutes at 22°C. Given the design of the experiment, the first measurement of luminescence occurred 20 seconds after addition of the luciferase. In order to get the maximum intensities for extremely fast-decaying substrates, we acquired individually kinetics for each of substrates for 4 minutes, starting 1 second after the injection of the enzyme in the well of the plate inside the plate reader.

Note concerning the assumption that the number of detected photons per consumed substrate molecule is constant whatever the substrate concentration.

As depicted in figure S3, the maximum measured light intensity is plotted versus initial substrate concentration we noticed that, for all the substrates studied, there is a signal intensity decrease at high concentration. We suggest the following three, not mutually exclusive, hypothesis to explain this observation. First, the substrate and reaction products absorb the light emission. Second, as for many enzymes, an excess of substrate inhibits the oxidation catalysis via an uncompetitive mechanism. Third, the photon emission occurring in the active site may be quenched by the excess of substrate present outside the active site.





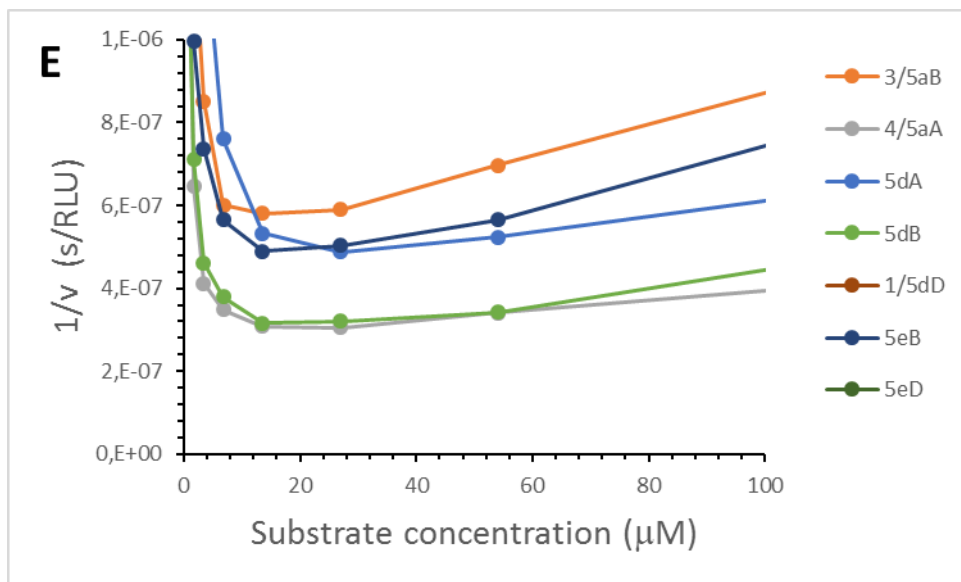


Figure S3. Maximal light intensity vs substrate concentration. (A) The maximal light intensity (RLU/s) is plotted in plain lines versus substrate concentrations (10^{-6}M) with the same enzyme concentration ($1.24 \cdot 10^{-12}\text{M}$). The kinetics fit with a Michaelis-Menten model (dashed lines). All substrates inhibit the detected light intensity, by extrapolation the initial reaction velocity v , at high concentration, suggesting an uncompetitive inhibition by excess of substrate. (B, C) The Lineweaver-Burk plots of $1/v$ (1/Light intensity in s/RLU) versus $1/S$ (M^{-1}) are shown with two scales. The curvature along the $1/v$ axis is characteristic of uncompetitive inhibition by substrate excess. The extrapolation of the linear part is intercepted by the x-axis at the $-1/K'_M$ value, the inverse of the apparent Michaelis constant, and by the y-axis at the $1/V'_{\max}$. (D,E) In the same plots shown with two scales of $1/v$ versus S , the extrapolation of the linear part is intercepted by the x-axis at the K_I value, the dissociation constant of the second substrate molecule from the complex ES.

Light absorption of all the substrates

Concerning the first hypothesis to explain this signal intensity decrease at high concentration, we measured the substrates absorption characteristics between 400 and 600 nm (table S1).

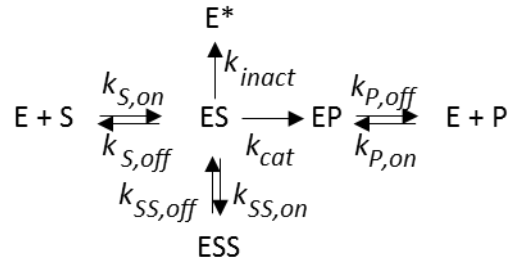
Table S1, light absorption of all the substrates between 400 and 600 nm.

Luciferins	3/5aB	4/5aA	5dA	5dB	1/5dD	5eB	5eD
λ_{\max} (nm)	413	426	426	418	426	412	418
ϵ_{\max} (M ⁻¹ cm ⁻¹)	6294	7881	6973	7754	8950	6415	7692
$\epsilon_{440\text{ nm}}$ (M ⁻¹ cm ⁻¹)	5010	7453	6594	6811	8326	5013	6496
$\epsilon_{450\text{ nm}}$ (M ⁻¹ cm ⁻¹)	3946	6715	5779	5633	7137	4003	5244
$\epsilon_{460\text{ nm}}$ (M ⁻¹ cm ⁻¹)	2964	5832	4781	4409	5734	3050	3981
$\epsilon_{440-460\text{ nm}}$ (M ⁻¹ cm ⁻¹)	3974	6666	5718	5618	7066	4022	5240

The light emissions observed with these substrates peak is between 440-460 nm. The absorptions of these compounds in solution peak between 413 and 426 nm with averaged molar extinction coefficients (ϵ) in between 440 and 460 nm of 3974 and 7066 M⁻¹cm⁻¹. At micromolar concentration, the absorption of light emitted by the substrate catalyzed oxidation is thus very weak in view of Beer-Lambert's law (emitted light intensity = measured light intensity / e^(ϵ L c) where L is the half height of the solution in the plate well (mm-range) and c the substrate concentration in the 10⁻⁶-10⁻⁴ M range). Accordingly, substrate absorption should contribute but is too weak to account for the loss of light observed beyond 27 μ M of concentration for any tested substrate.

Kinetics analysis

The apparent reaction rate (v') is given by the measured light intensity (RLU/s) reflecting counted photons/s, depending on the real catalytic rate (k_{cat}) and the yield of the measurement (\square , the number of molecules catalyzed per RLU). The kinetics have been fitted using the Michaelis-Mentel model drawn below considering 1) the luciferins (S) as the limiting substrates and O₂ as saturating substrate in the experimental assay conditions (100 \square L) in a 6 mm-diameter well of multi-well plates, 2) the inhibition of the enzyme E by excess of substrate through the binding of a second substrate (ESS) on the Michaelis' complex (ES) with the dissociation constant K_I and the on ($k_{SS,on}$) and off ($k_{SS,off}$) binding constants, and 3) the stochastic inactivation of the enzyme (E*) along the reaction turn over decreasing exponentially the active enzyme population with the kinetic constant k_{inact} . K'_M is the apparent constant of Michaelis and V'_{max} the apparent maximal reaction velocity.



With

$$\frac{d[ES]}{dt} = [E][S]k_{S,on} - [ES]k_{S,off} - [ES]k_{cat} - [ES][S]k_{SS,on} + [ESS]k_{SS,off} - [ES]k_{inact}$$

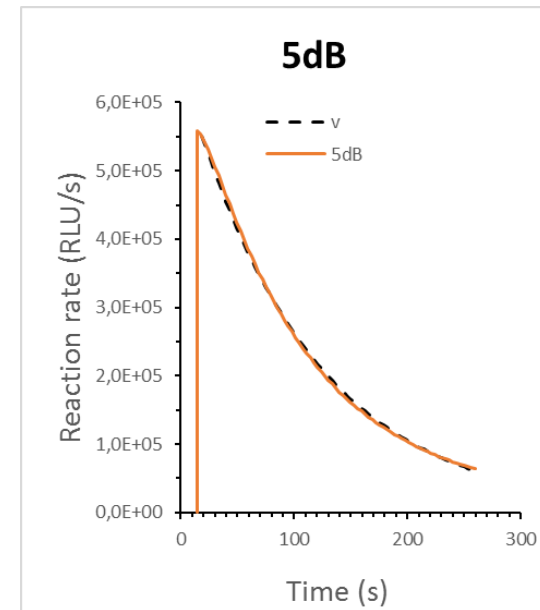
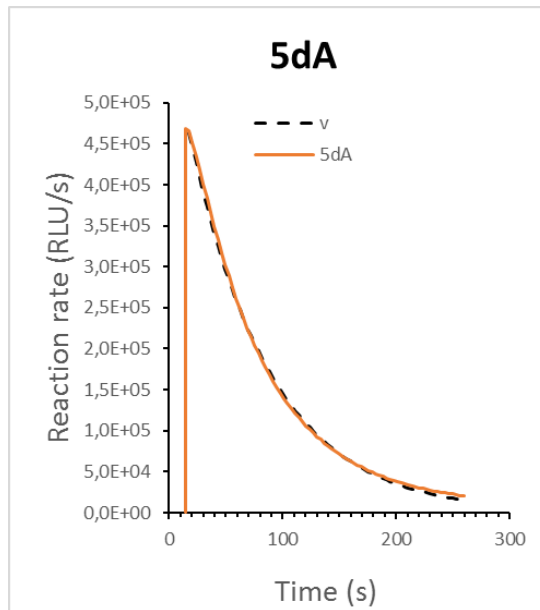
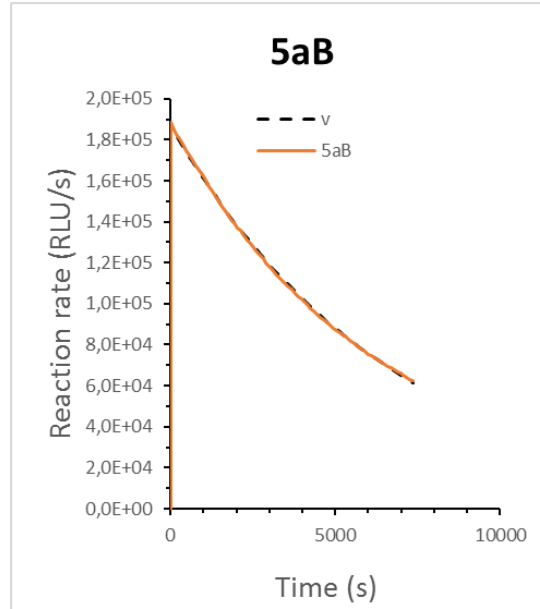
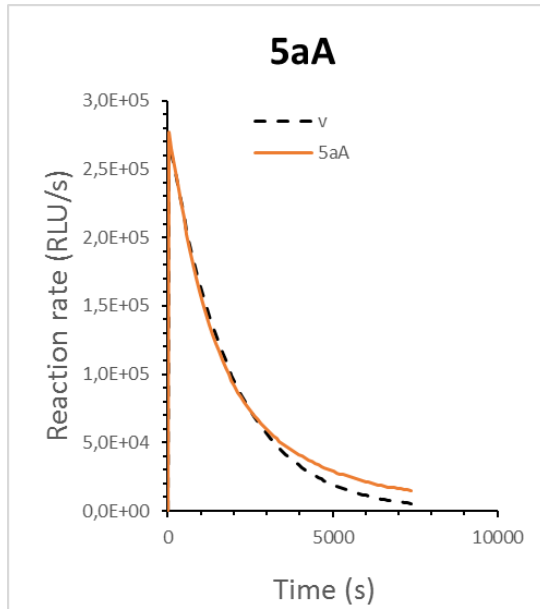
$$K_I = \frac{k_{SS,off}}{k_{SS,on}} \text{ (mol L}^{-1}\text{)}; K_M = \frac{k_{S,off} + k_{cat}}{k_{S,on}} = \frac{K'_M}{1 + \frac{[S]^2}{K_I}} \text{ (mol L}^{-1}\text{)}$$

$$V_{max} = [E_0][S]k_{cat} = V'_{max} \frac{[S] + K_M + \frac{[S]^2}{K_I}}{[S]} \text{ (mol s}^{-1}\text{)}$$

$$v' = \frac{[E][S]k_{cat}}{[S] + K_M + \frac{[S]^2}{K_I}} \text{ (RLU s}^{-1}\text{)}$$

$$[E] = [E_0]e^{-k_{inact}t}, \text{ with } k_{inact} \text{ in } s^{-1} \text{ and the time } t \text{ in } s$$

$$v = v'\rho \text{ (mol } s^{-1}\text{) and } \rho = \frac{[S]N_A vol}{\Sigma I} \text{ (molecules. RLU}^{-1}\text{), } N_A = 6.02 \cdot 10^{23} \text{ and } vol \text{ the volume (L)}$$



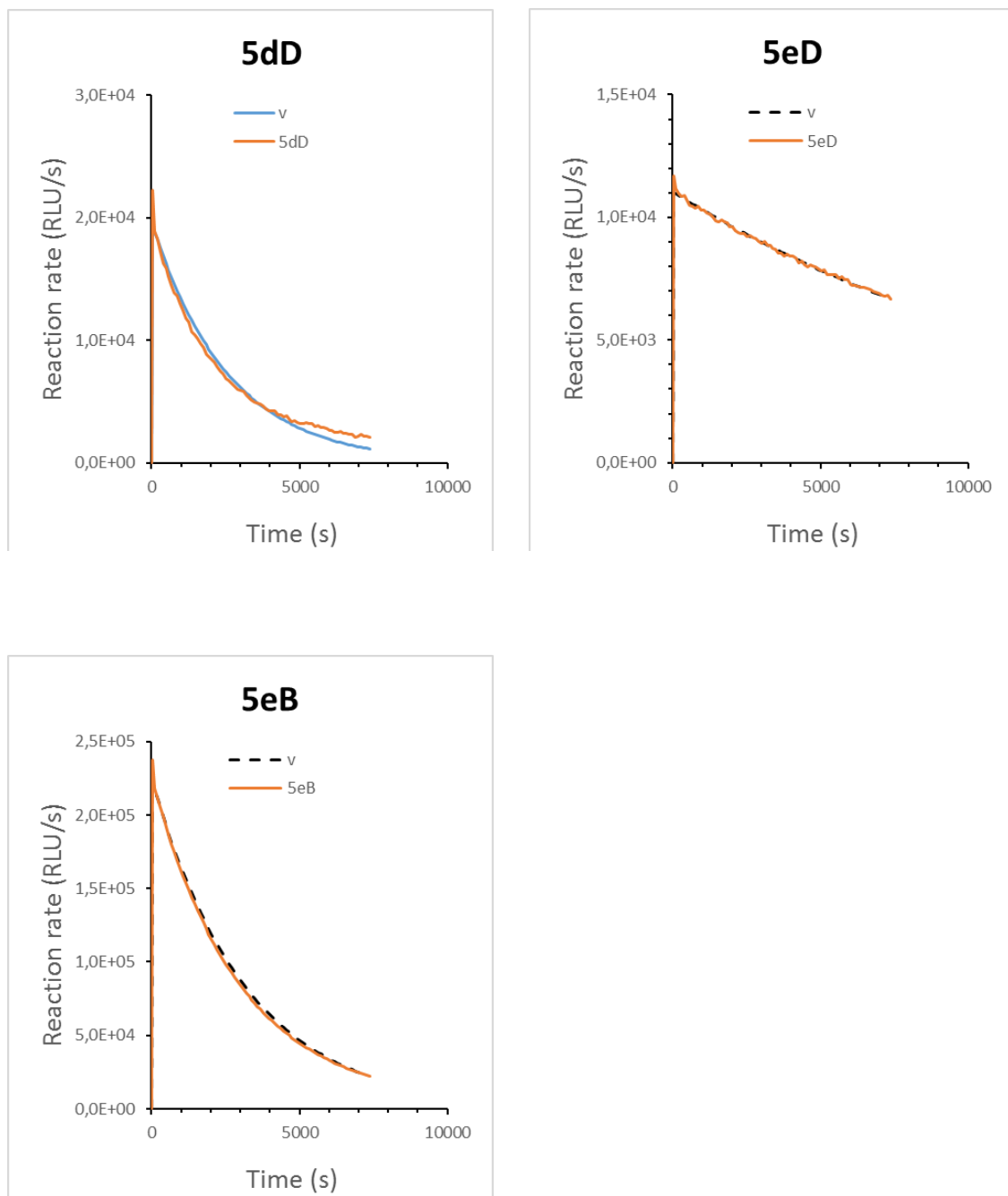
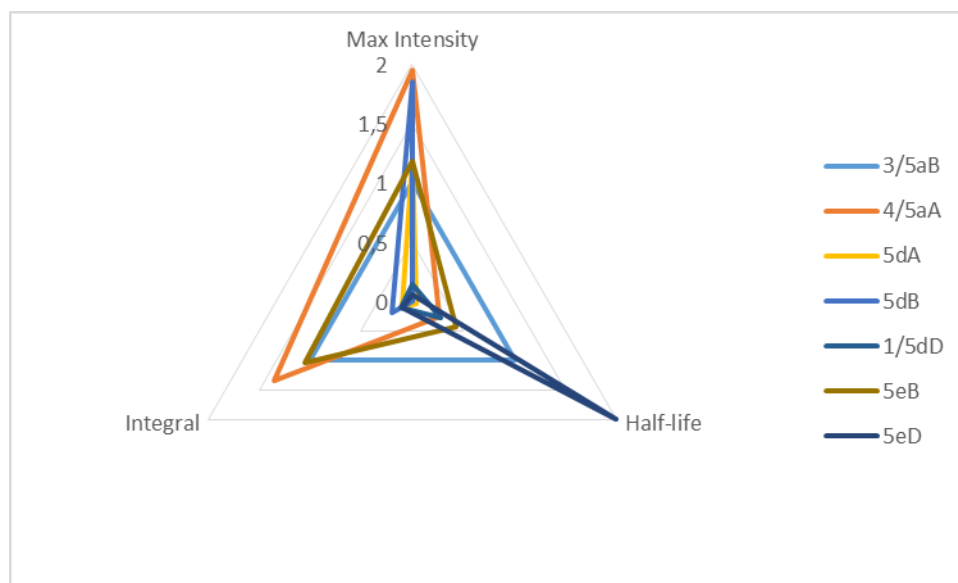


Figure S4: Enzyme inactivation kinetics. The kinetics are plotted versus time with the same enzyme concentration and the same substrate concentration. These plots compare the fitting of the experimental data (red plain line) with the theoretical reaction velocity computed according to the described Michaelis-Menten model taking into consideration the irreversible inactivation of the enzyme (dashed black lines).

From these computations, the following kinetic parameters were obtained:

	3/5aB	4/5aA	5dA	5dB	1/5dD	5eB	5eD
I_{\max} (10^6 RLU/s)	1.69	3.28	2.05	3.11	0.23	1.98	0.10
$t_{1/2}$ (min)	74.29	19.34	3.22	0.33	20.96	32.27	148
ΣI (10^6 RLU/s)	84.98	80.85	5.61	11.57	6.29	62.97	6.04
K'_M (10^{-6} M)	3.40	2.33	3.71	3.45	8.8	4.23	7.02
k'_{cat} (10^{18} RLU/s)	1.80	3.50	2.32	3.60	0.44	2.33	0.17
molecules/RLU	1775	4581	12067	4463	101169	1804	70020
K_I (10^{-6} M)	104.94	115.21	140.55	101.64	22.48	84.02	35.42
K_M (10^{-6} M)	2.22	3.02	5.90	3.88	6.80	3.30	2.94
k_{cat} (mol/s·mol E)	106	534	932	535	1500	140	362
k_{inact} (10^{-4} s $^{-1}$)	1.5	5.2	425	375	3.8	4.5	3.1



Plotting of the luciferase inactivation in the course of substrate catalyzed oxidation

As depicted in figure S5, the light emission intensity decreases with time: steep for “flash” (**5dB**) and slow (**3/5aB**) for “glow” substrates. For verifying if the reaction rate indicated by the photon emission (RLU/s) decreases with time, the same enzyme amount ($62 \cdot 10^{-18}$ mol) was added when indicated by the arrows for different concentrations of substrates indicated in the legend between brackets (10^{-6} M). This demonstrates that enzyme is irreversibly inactivated during the kinetics as shown for luciferin **5dB** an **3/aB**.

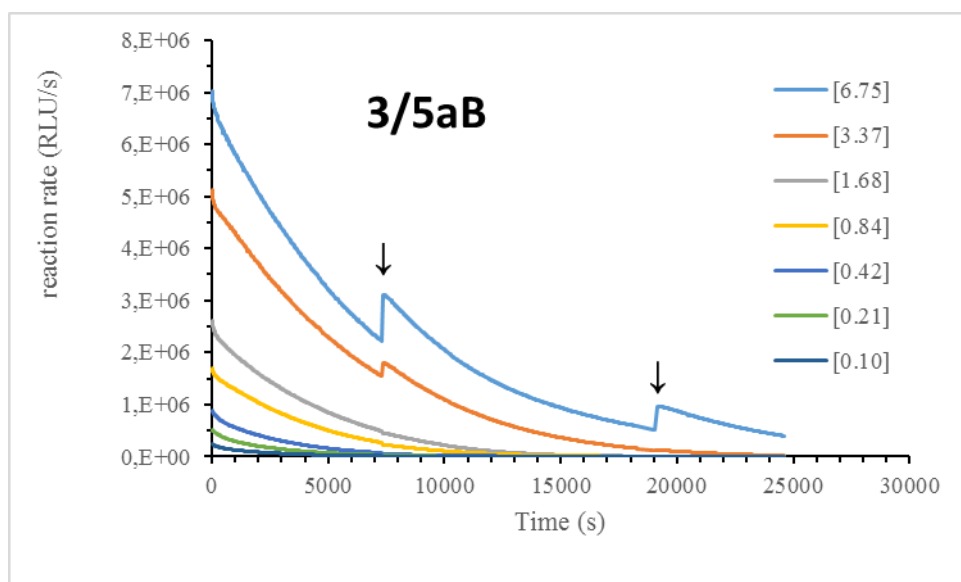
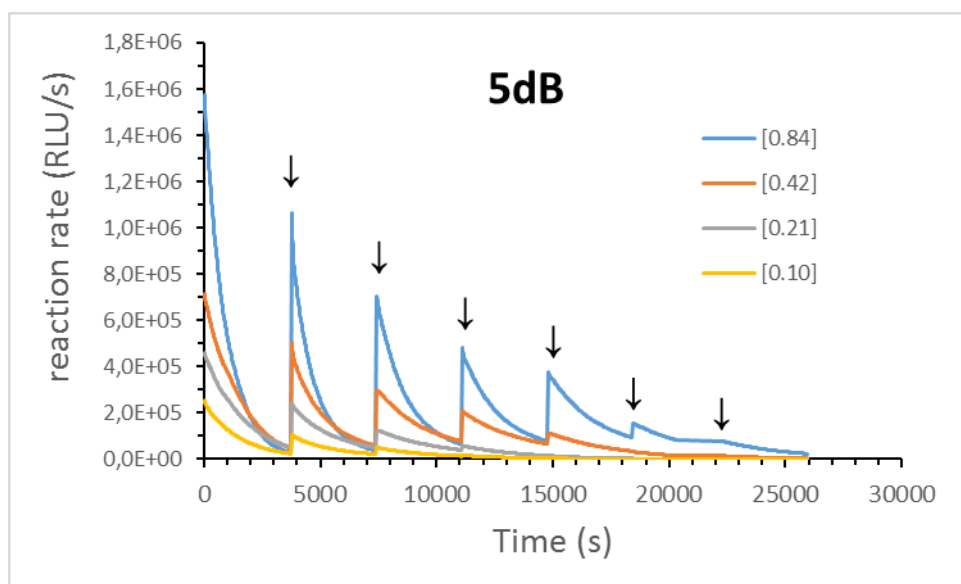
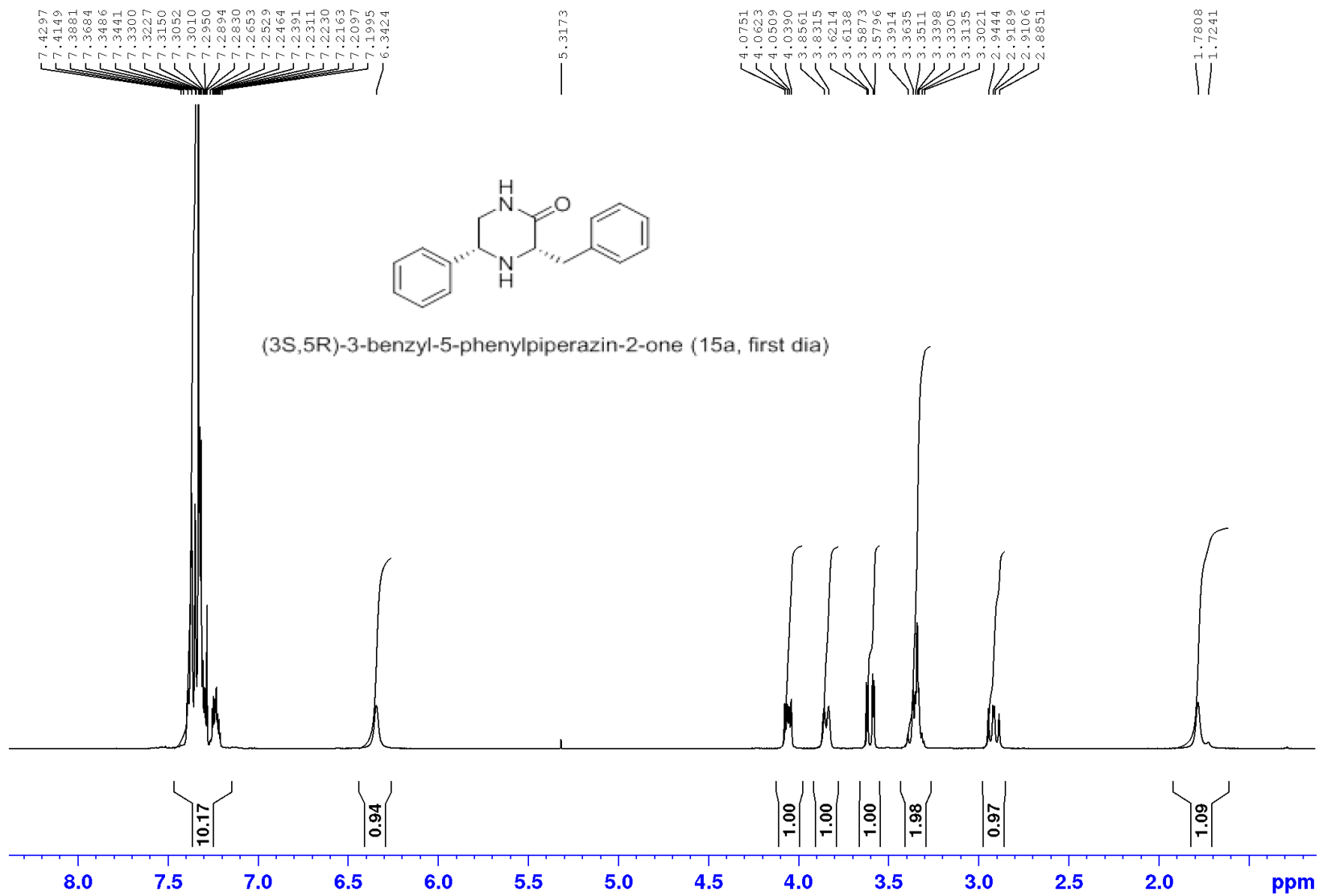


Figure S5. Luciferase inactivation in the course of the catalyzed oxidation of 5dB and 3/5aB.

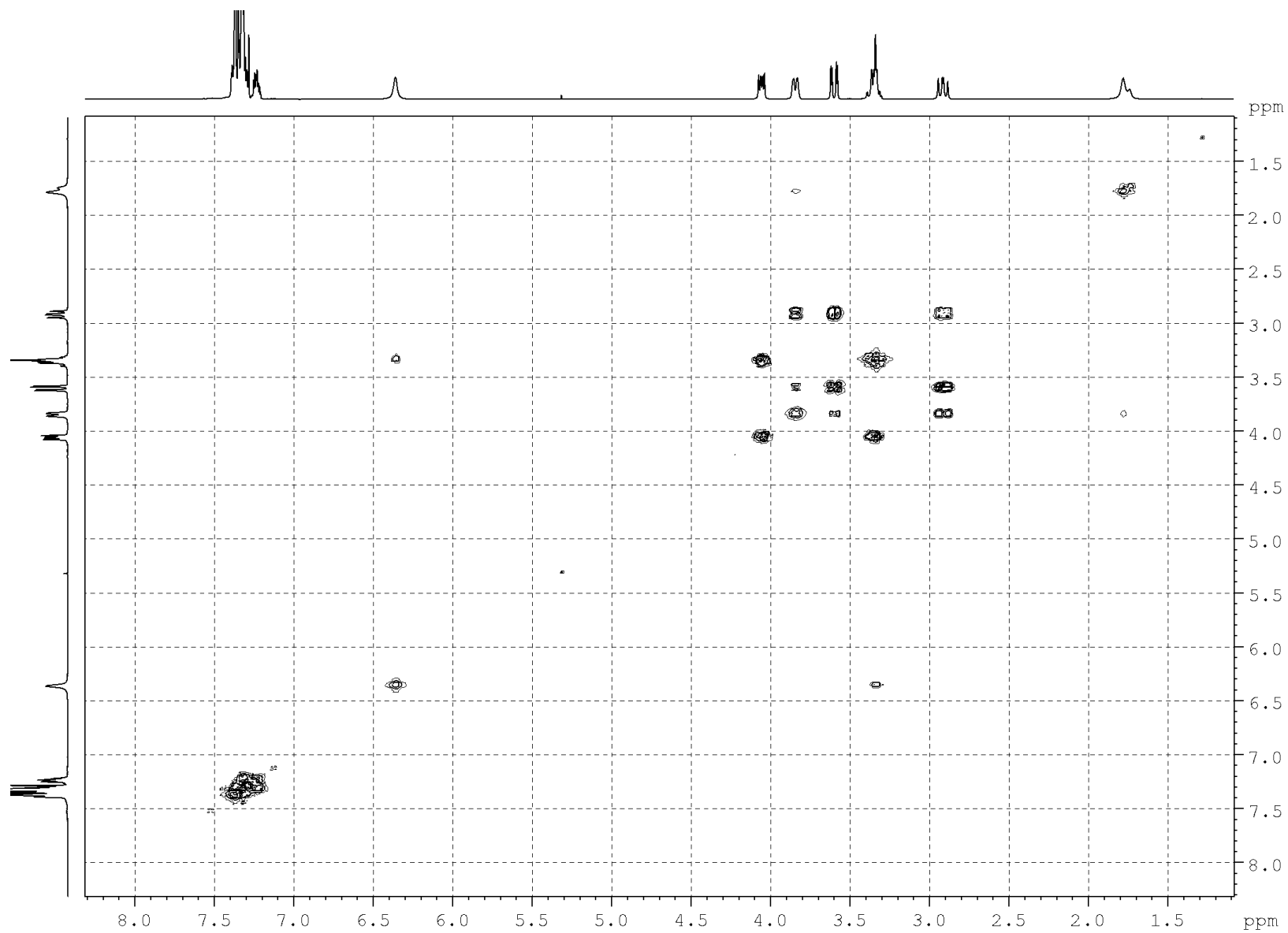
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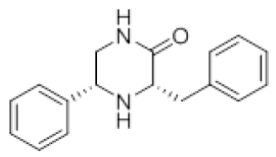
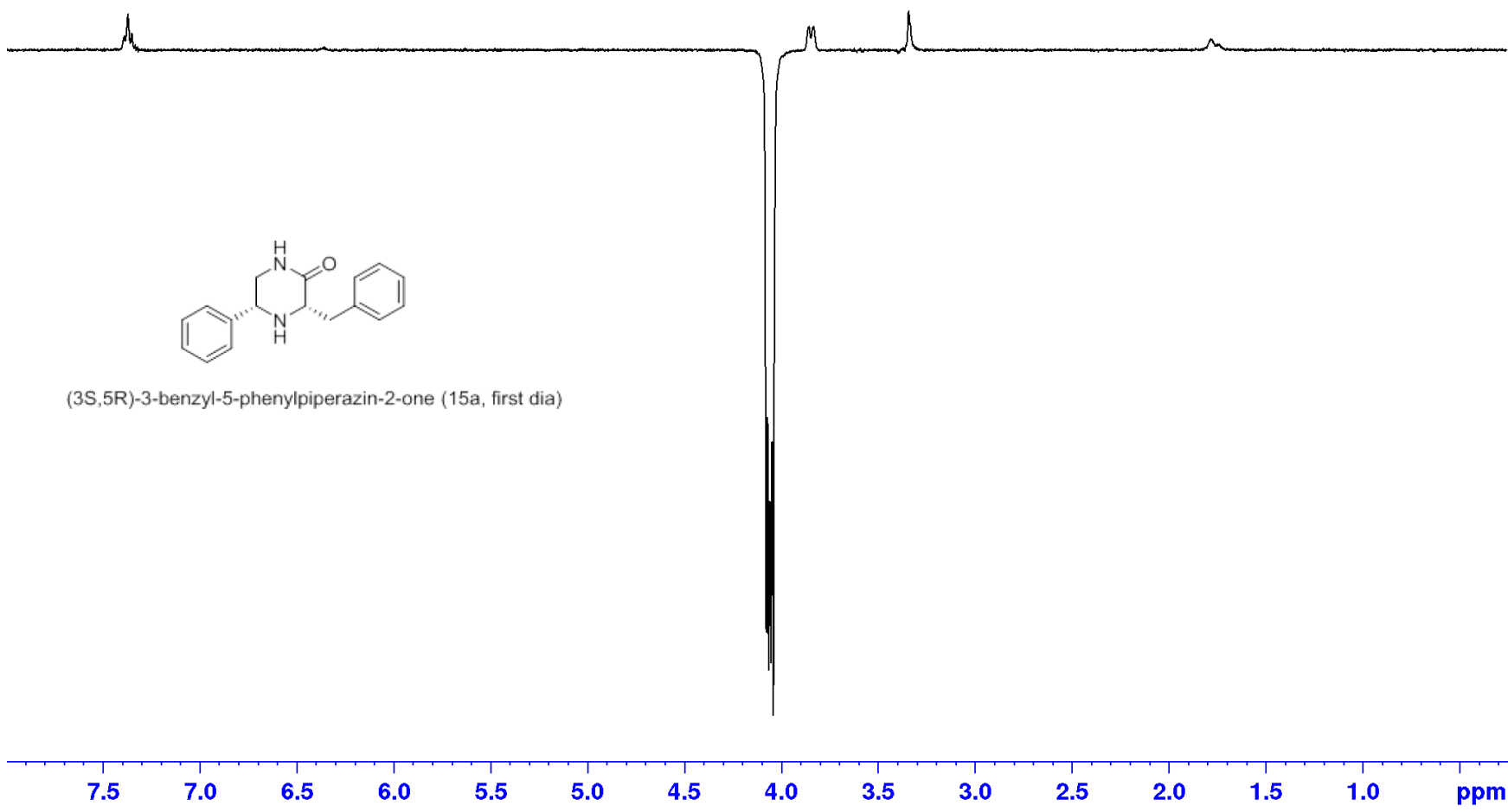
¹H and ¹³C spectra



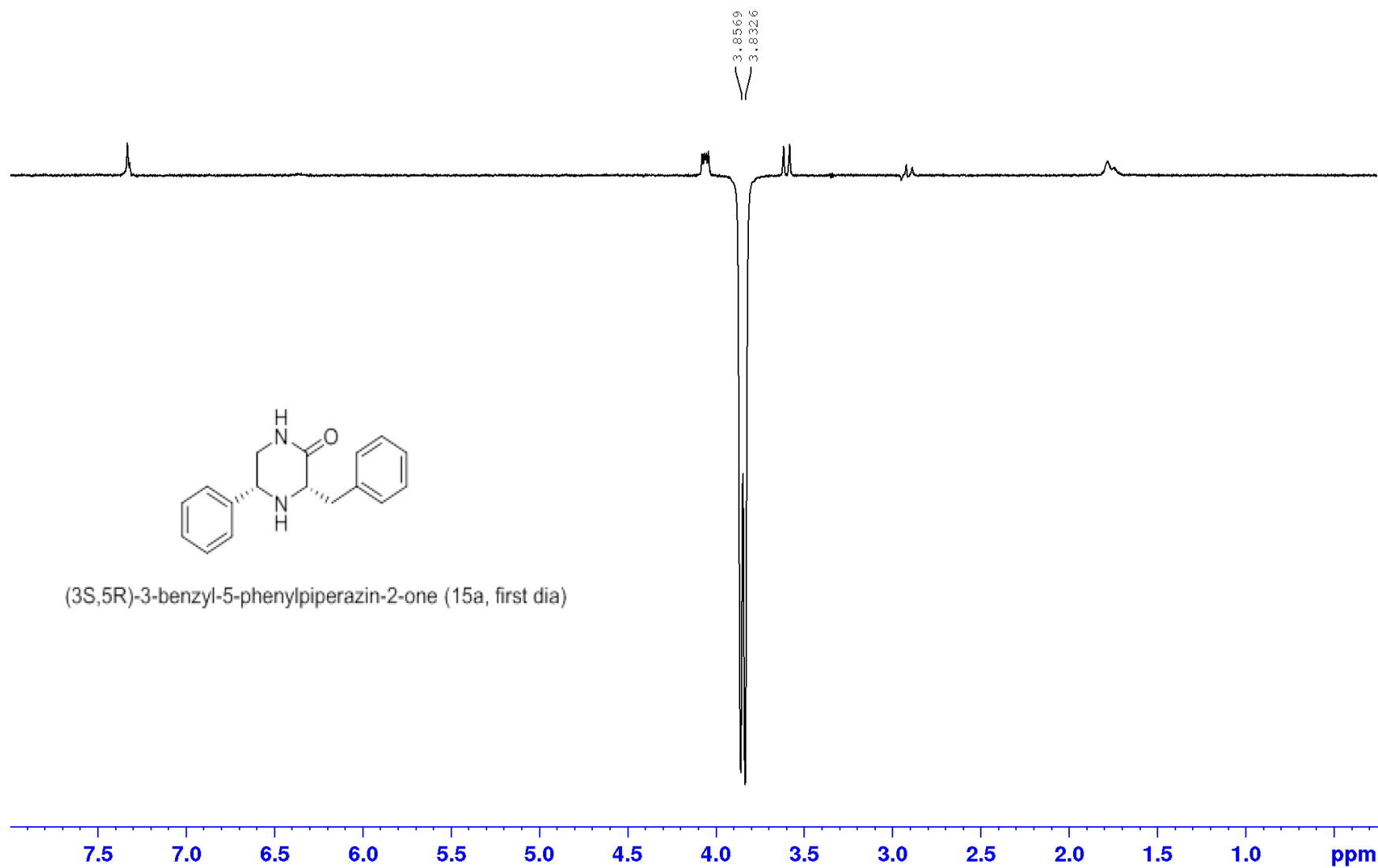
(3S,5R)-3-benzyl-5-phenylpiperazin-2-one (15a, first dia)



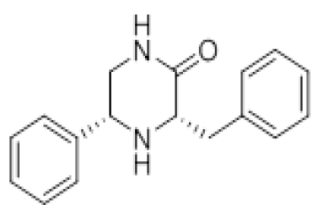
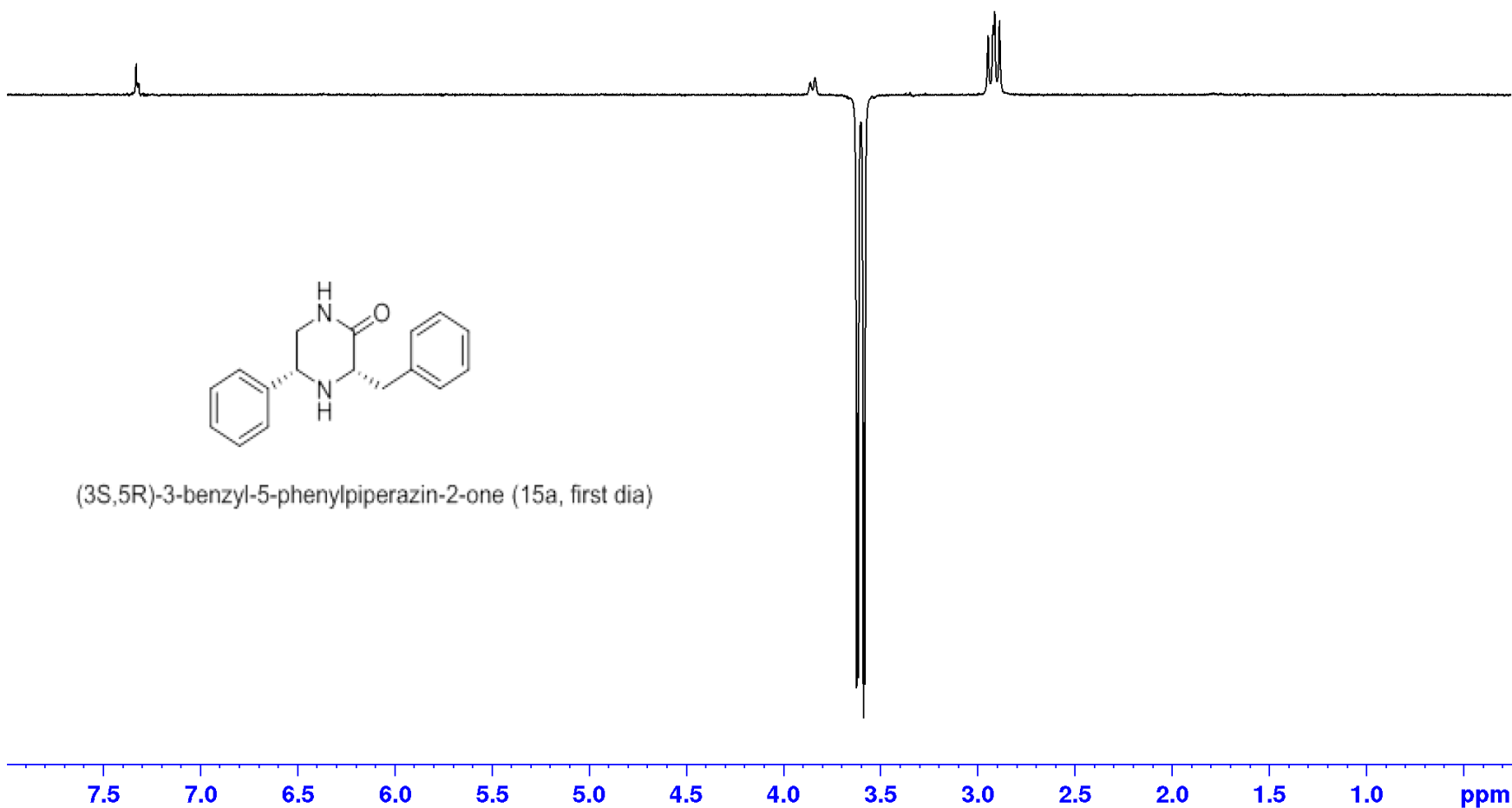
4.0764
4.0638
4.0525
4.0405



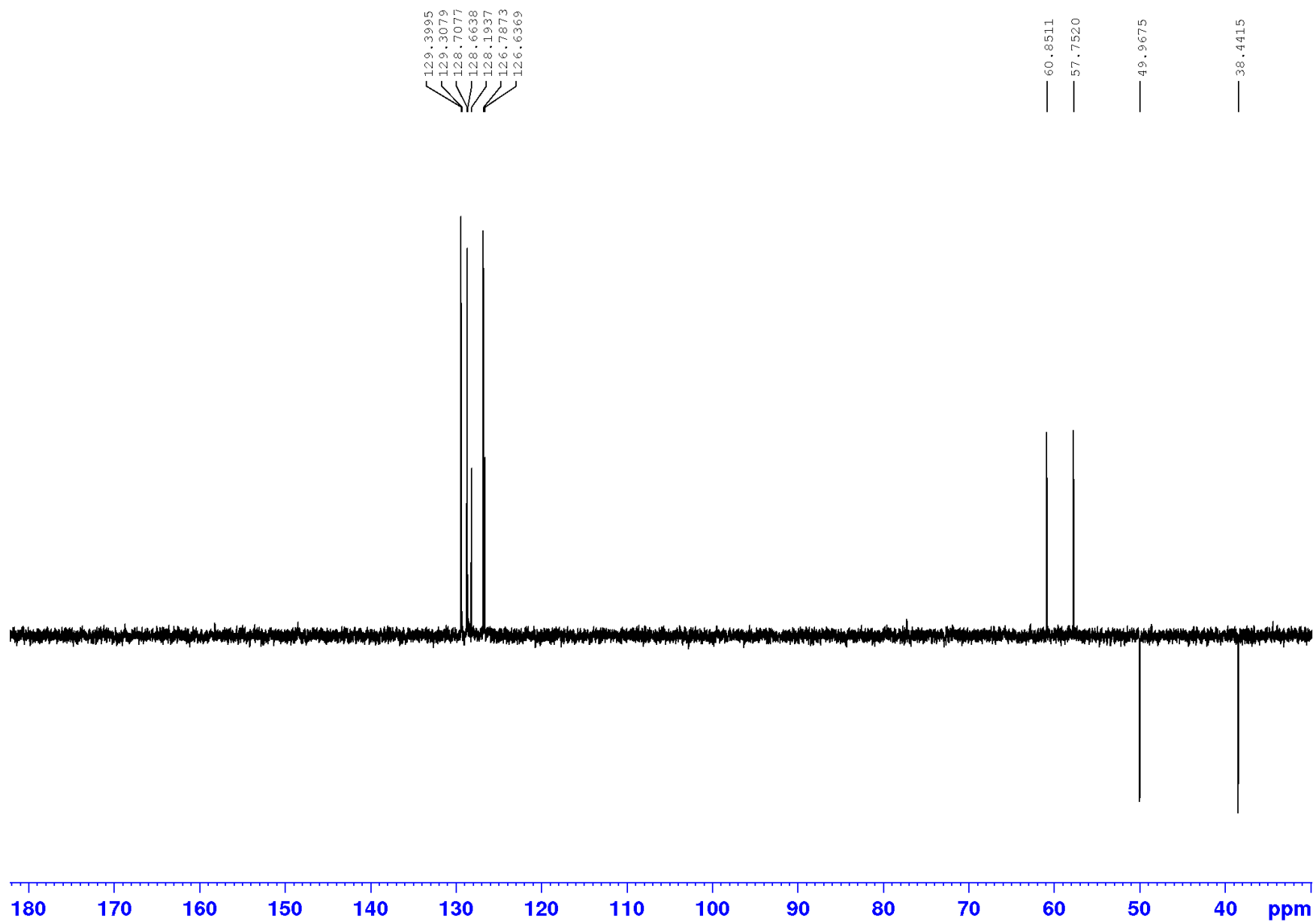
(3S,5R)-3-benzyl-5-phenylpiperazin-2-one (15a, first dia)



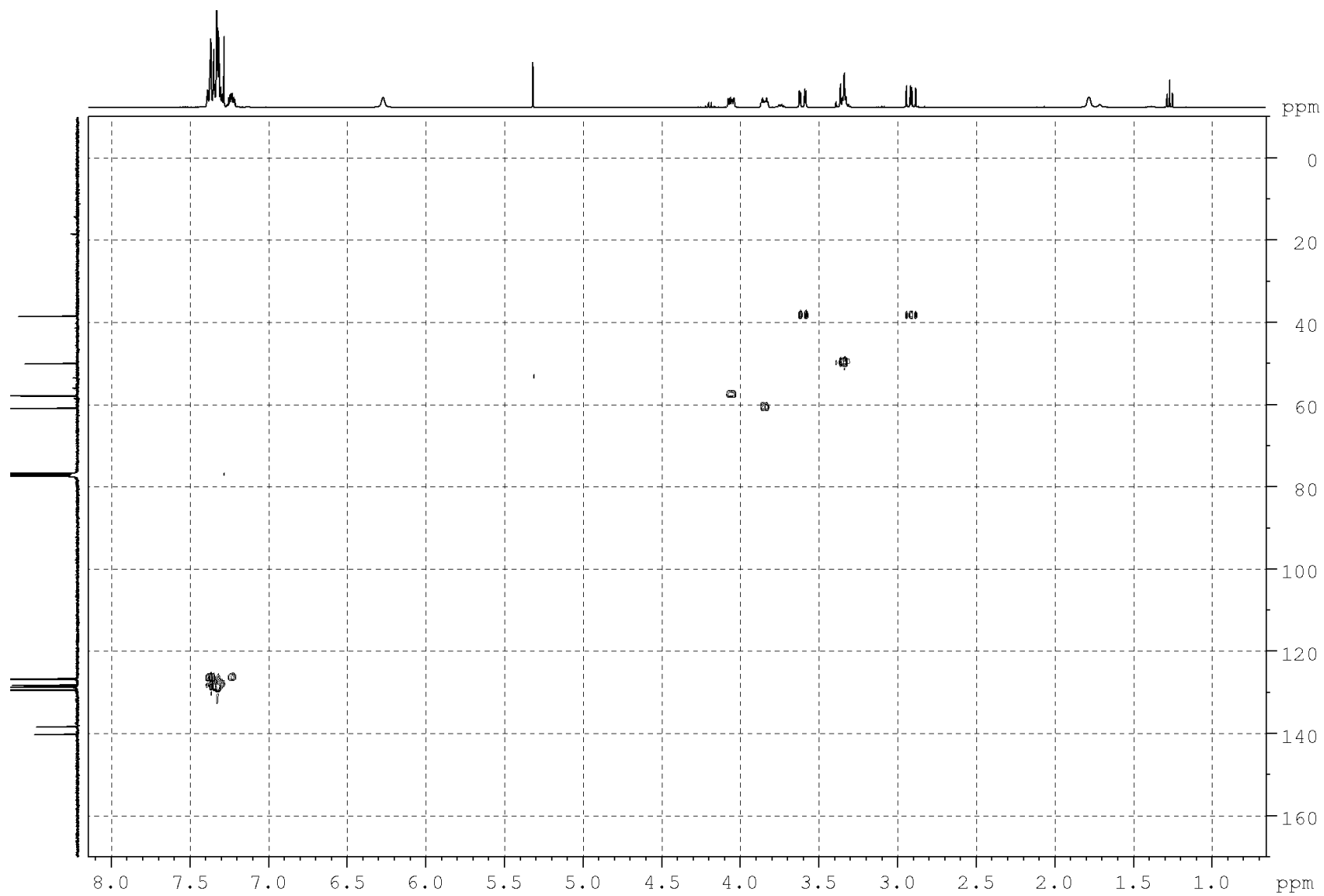
3.6207
3.6131
3.5865
3.5789

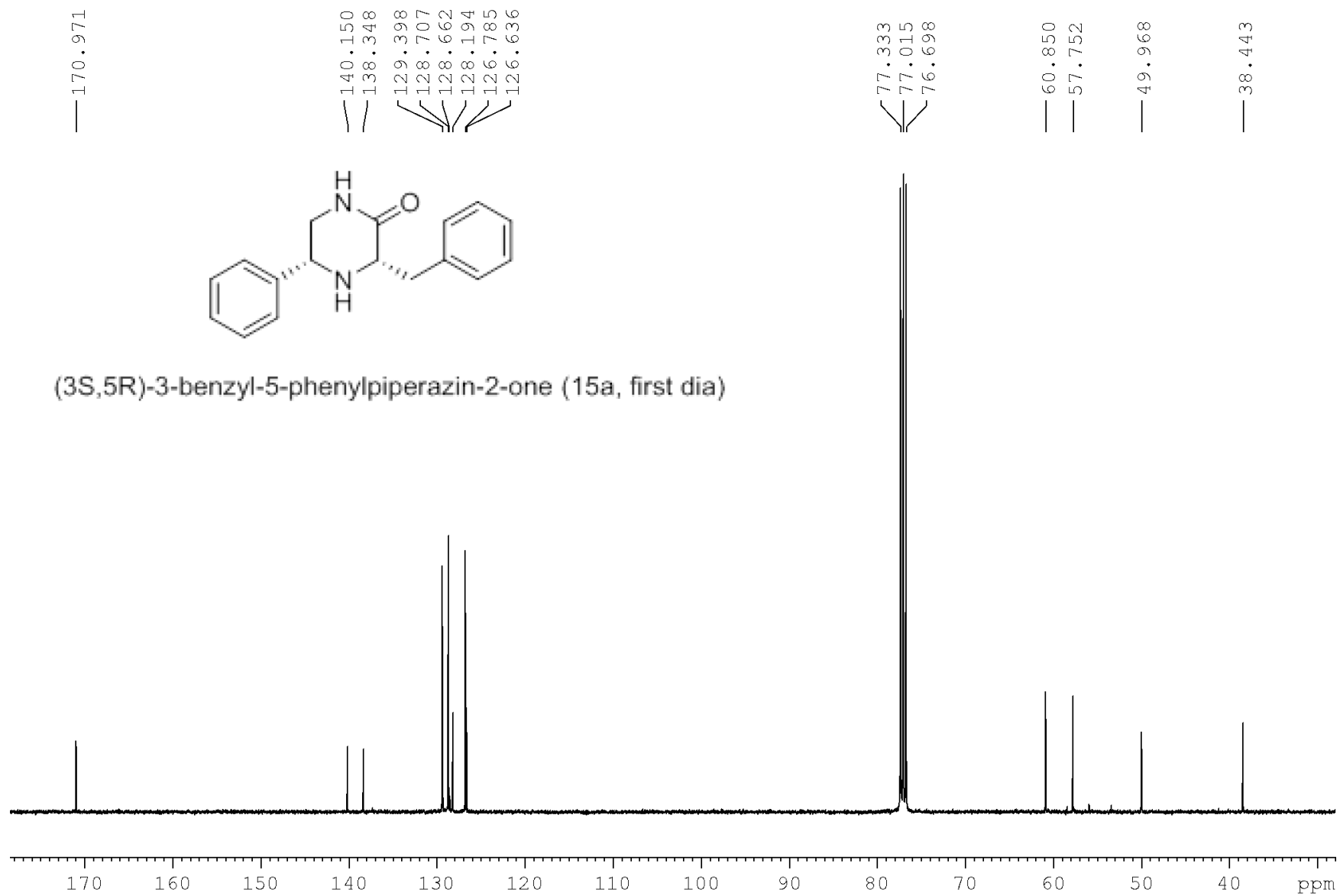


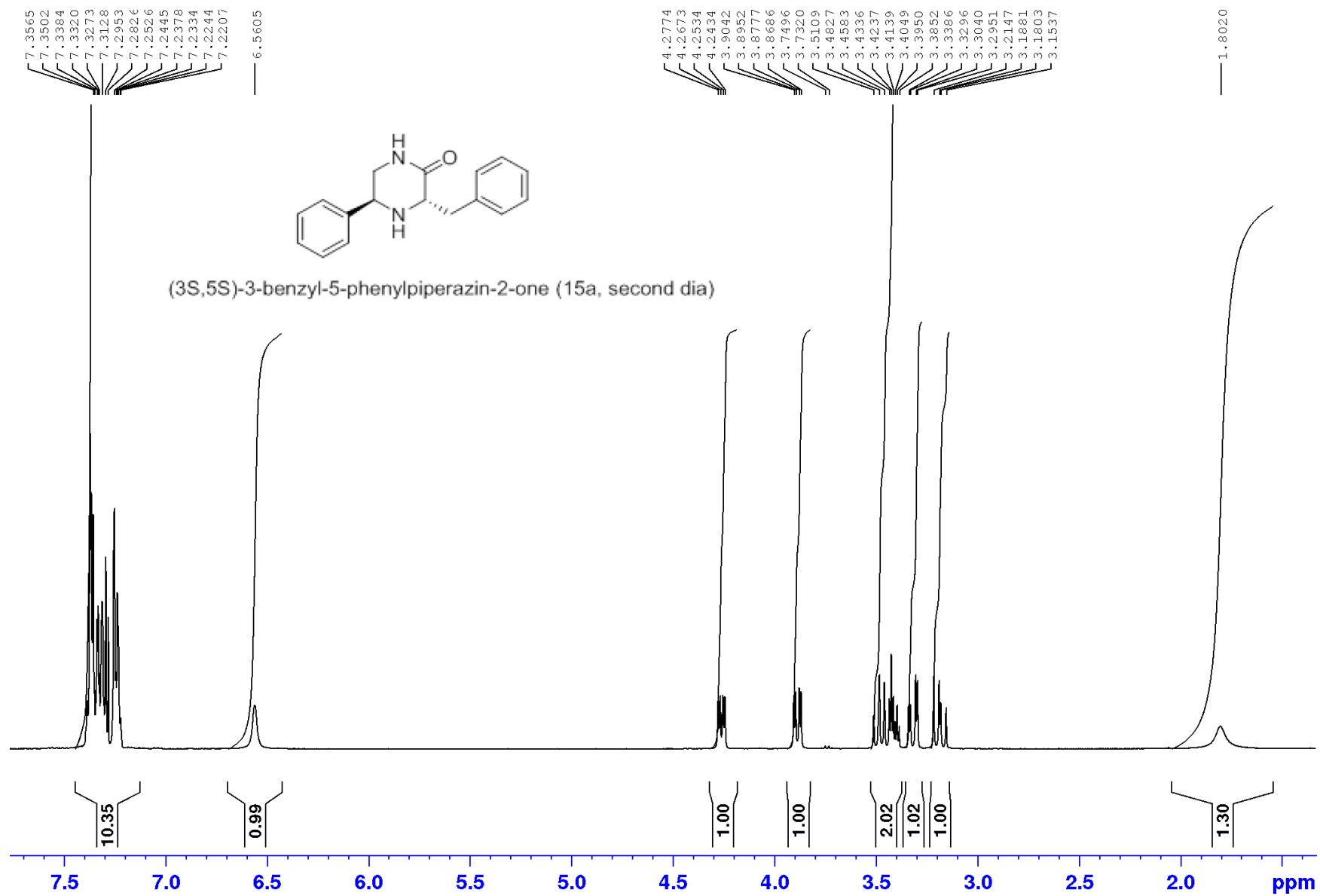
(3S,5R)-3-benzyl-5-phenylpiperazin-2-one (15a, first dia)



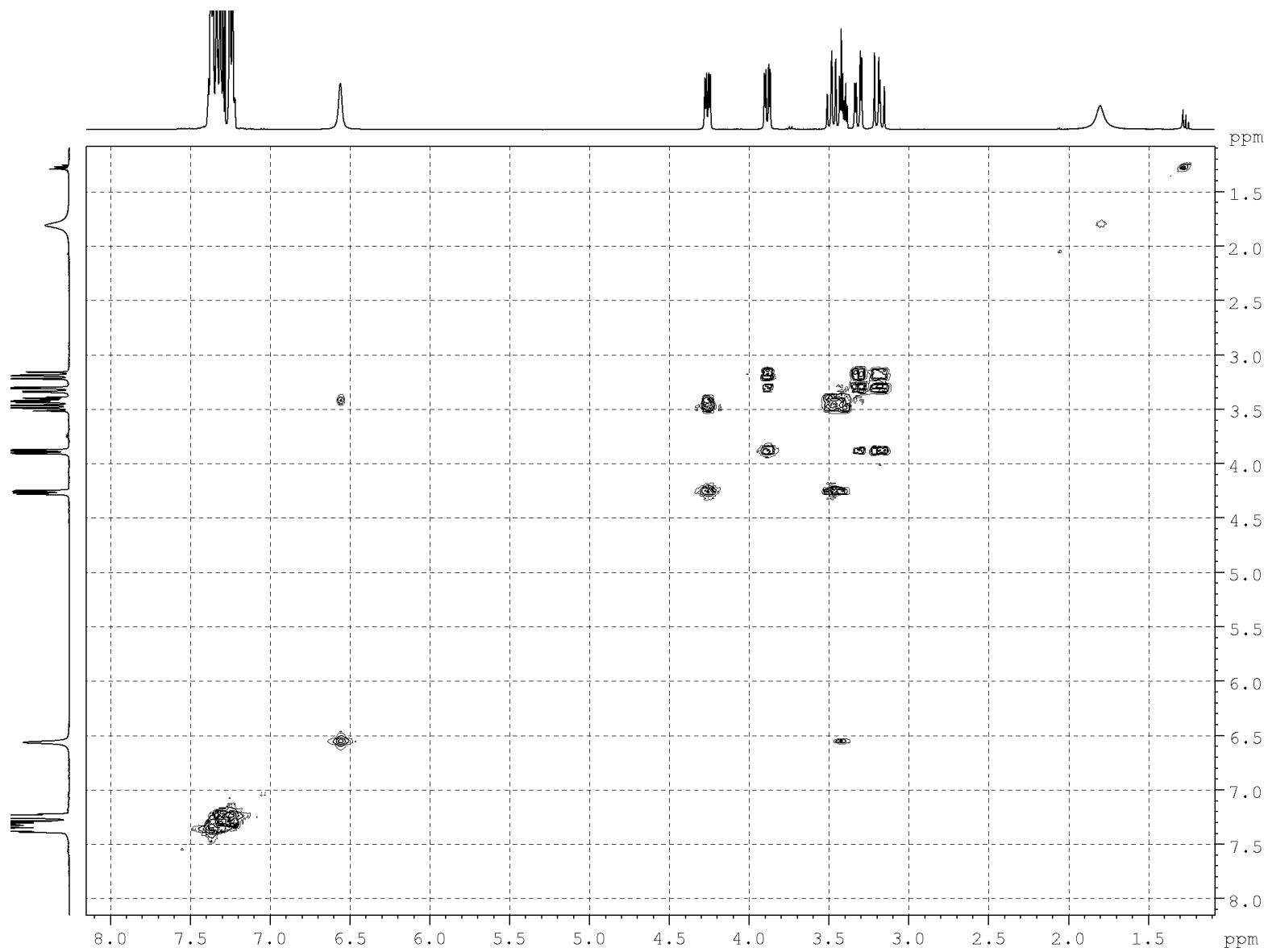
(3S,5R)-3-benzyl-5-phenylpiperazin-2-one (15a, first dia)



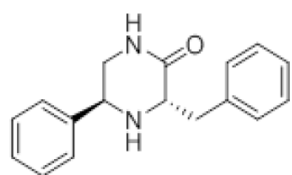
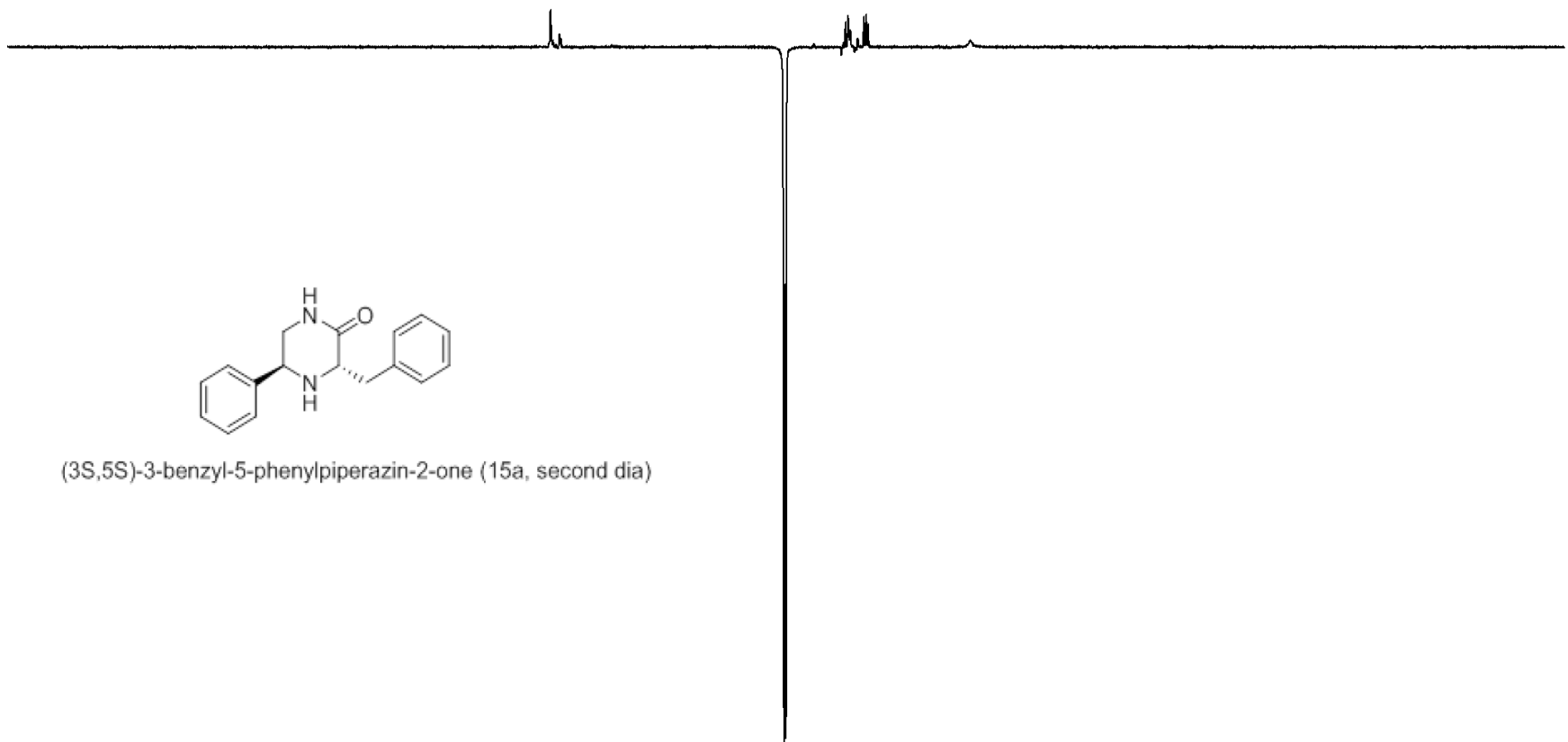




(3S,5S)-3-benzyl-5-phenylpiperazin-2-one (15a, second dia)



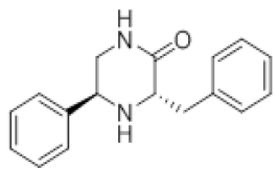
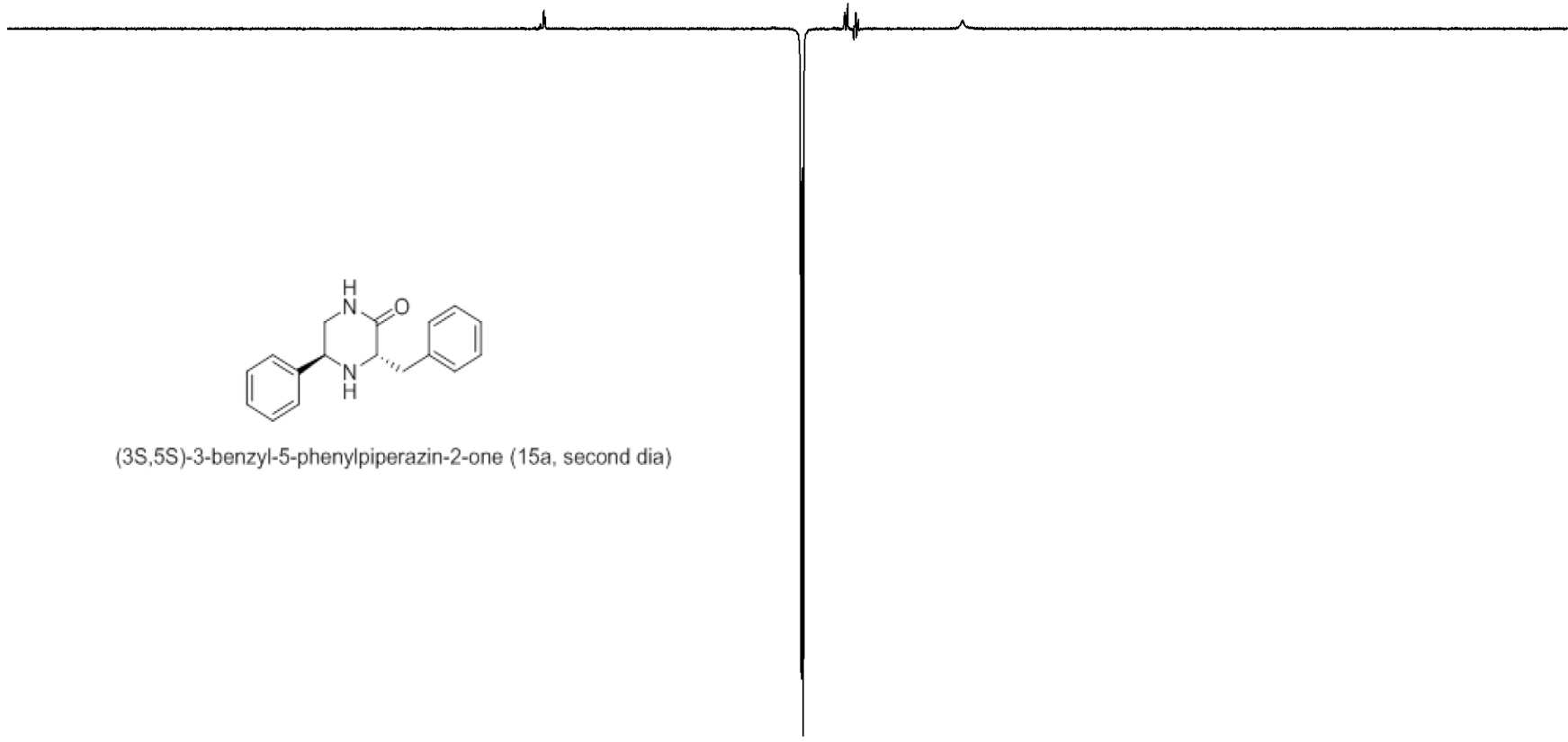
4.2759
4.2661
4.2519
4.2422



(3S,5S)-3-benzyl-5-phenylpiperazin-2-one (15a, second dia)

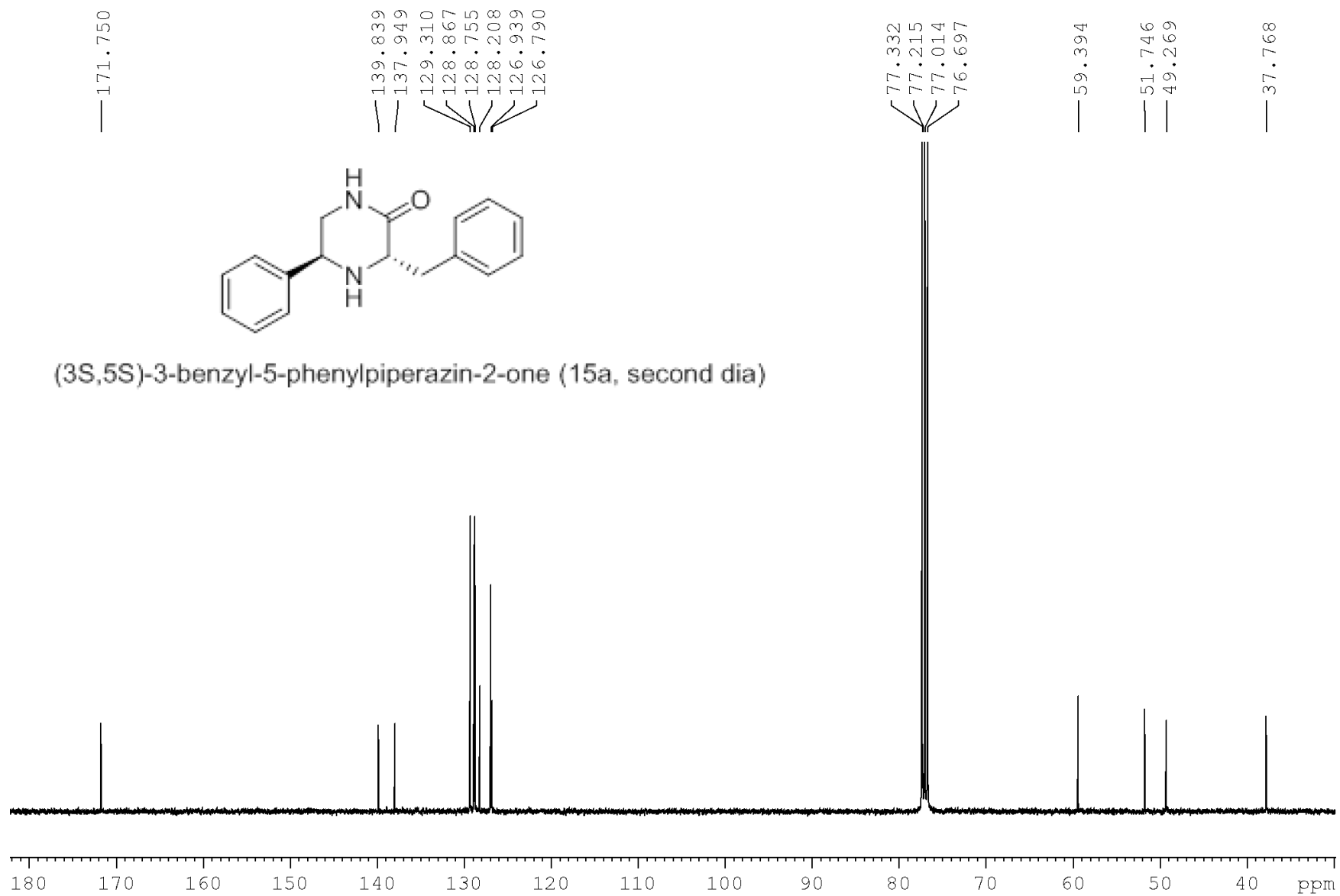
14 13 12 11 10 9 8 7 6 5 4 3 2 1 0 -1 -2 -3 -4 -5 ppm

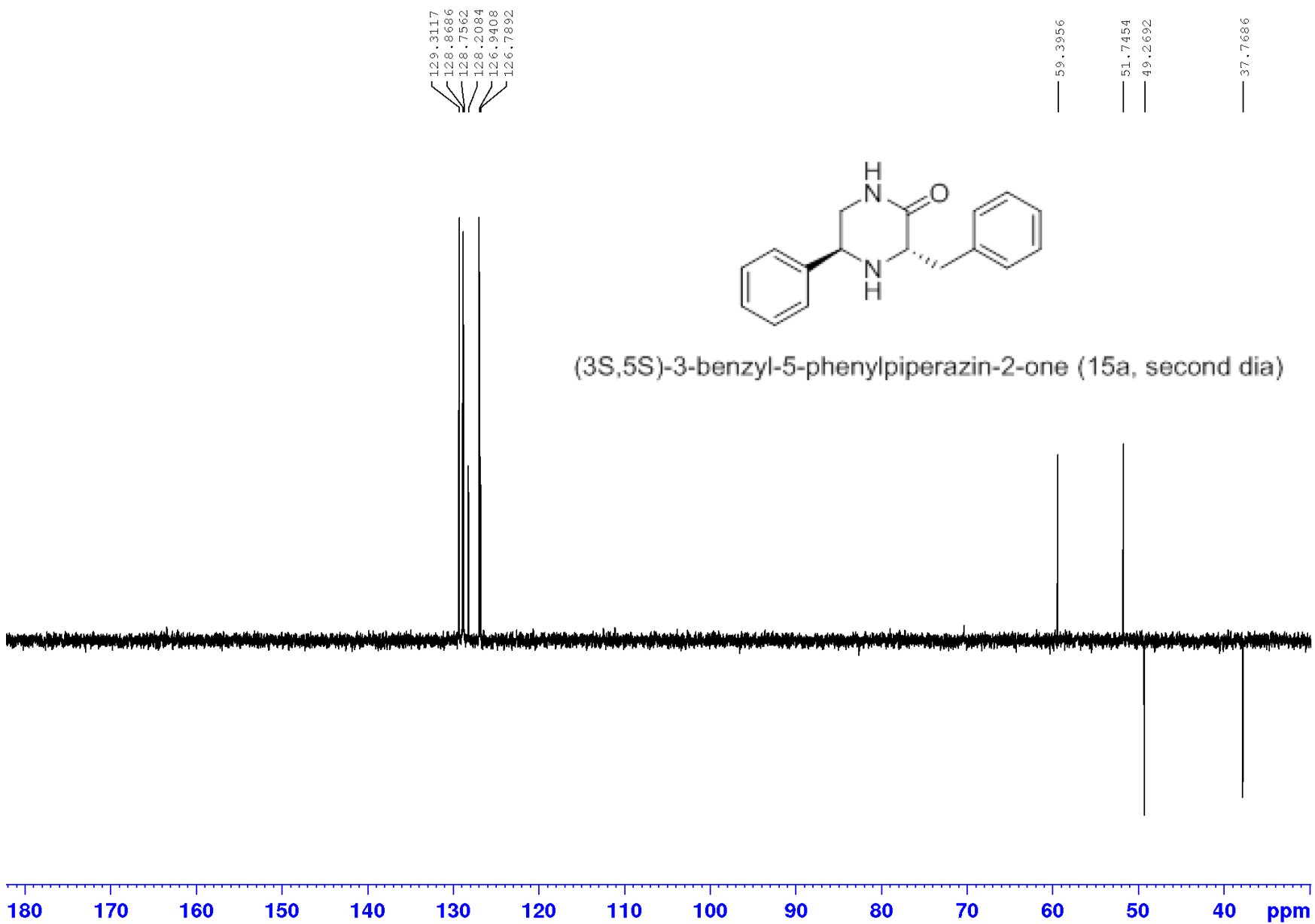
3.9022
3.8934
3.8757
3.8669



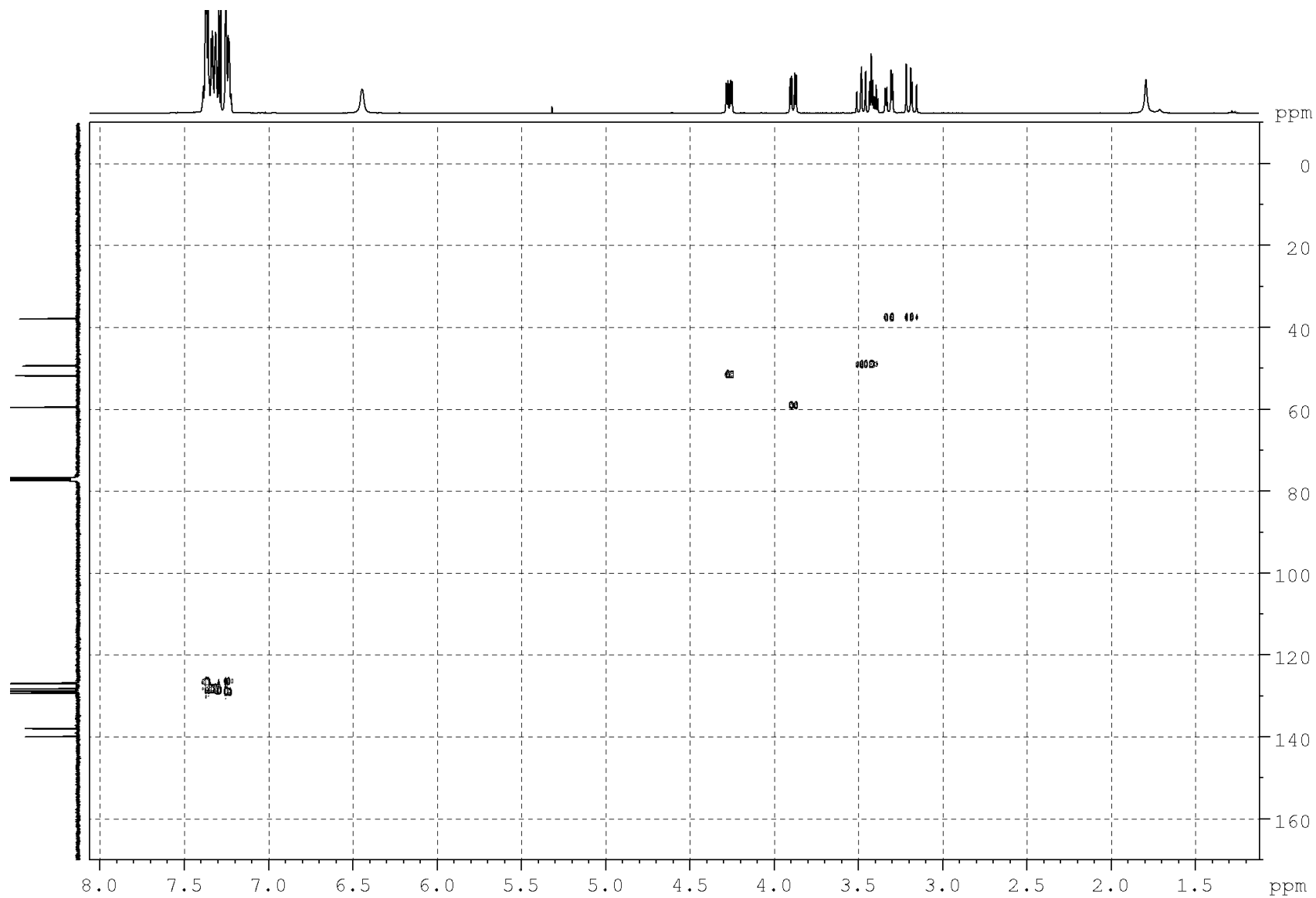
(3S,5S)-3-benzyl-5-phenylpiperazin-2-one (15a, second dia)

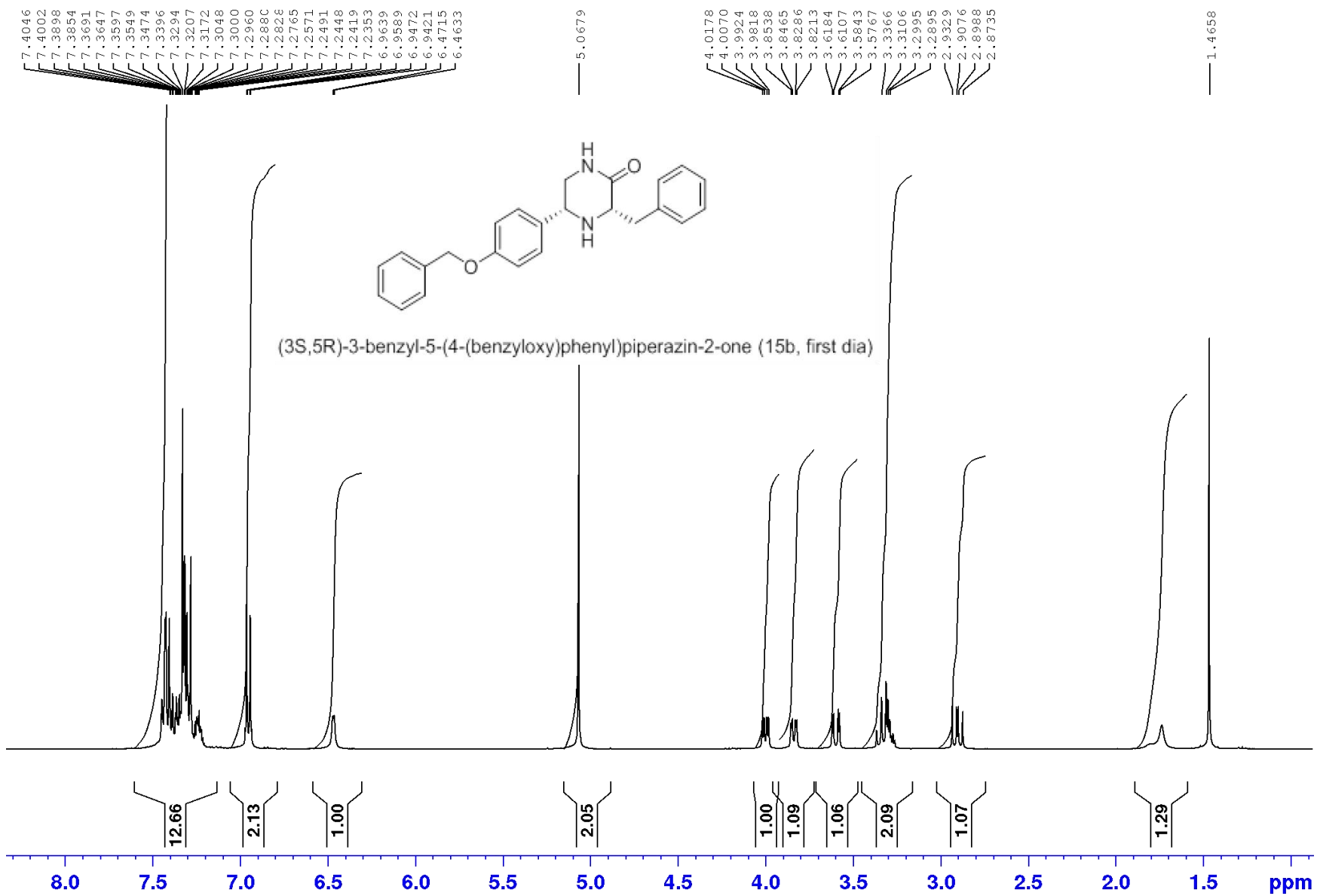
14 13 12 11 10 9 8 7 6 5 4 3 2 1 0 -1 -2 -3 -4 -5 ppm

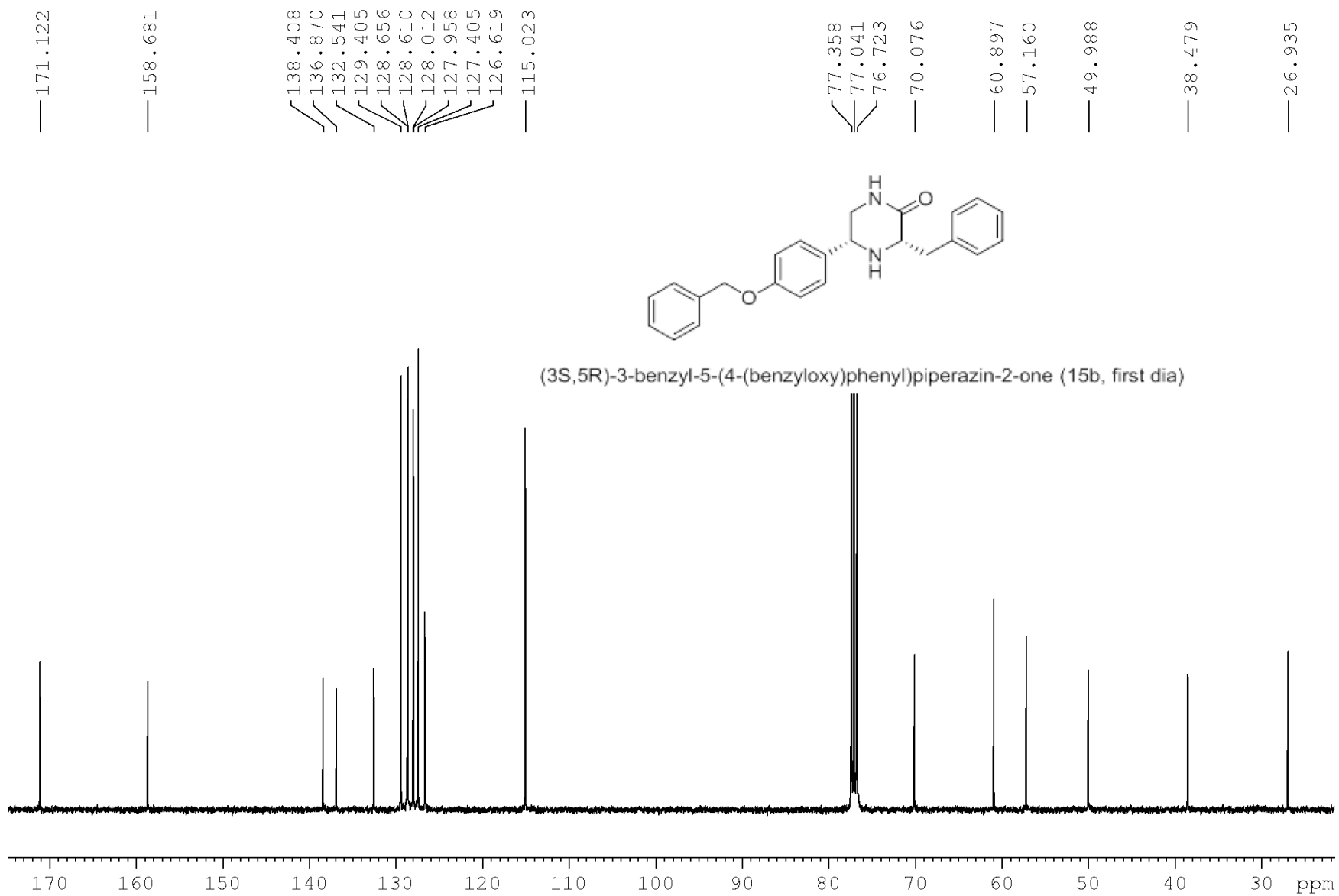


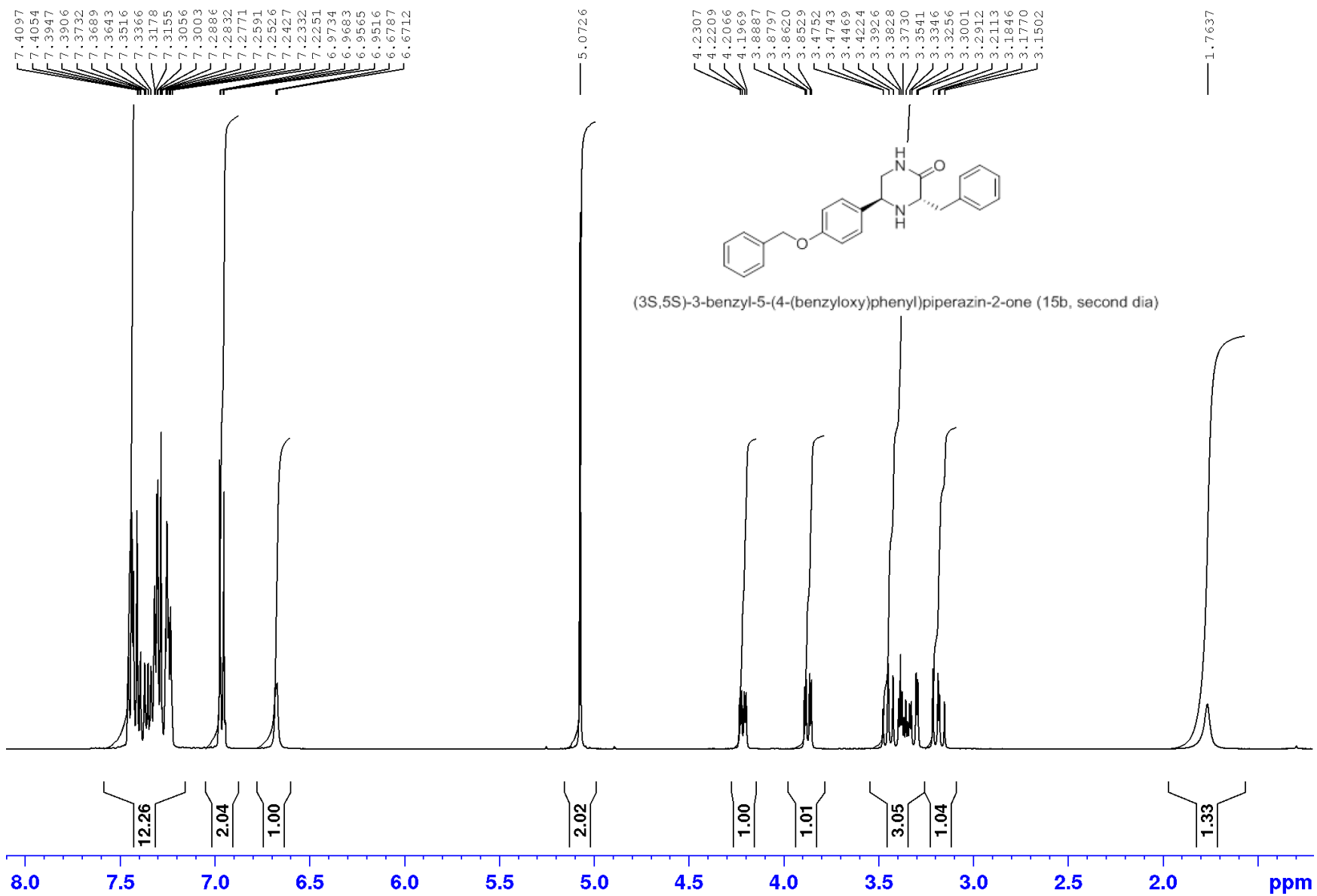


(3S,5S)-3-benzyl-5-phenylpiperazin-2-one (15a, second dia)









— 171.901

— 158.704

138.012
136.876
132.212
129.321
128.867
128.611
128.103
128.024
127.437
126.777
— 115.055

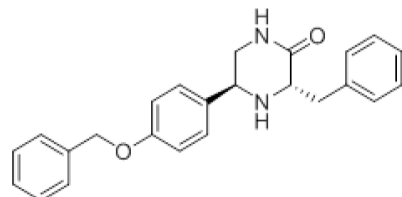
77.358
77.040
76.722

— 70.085

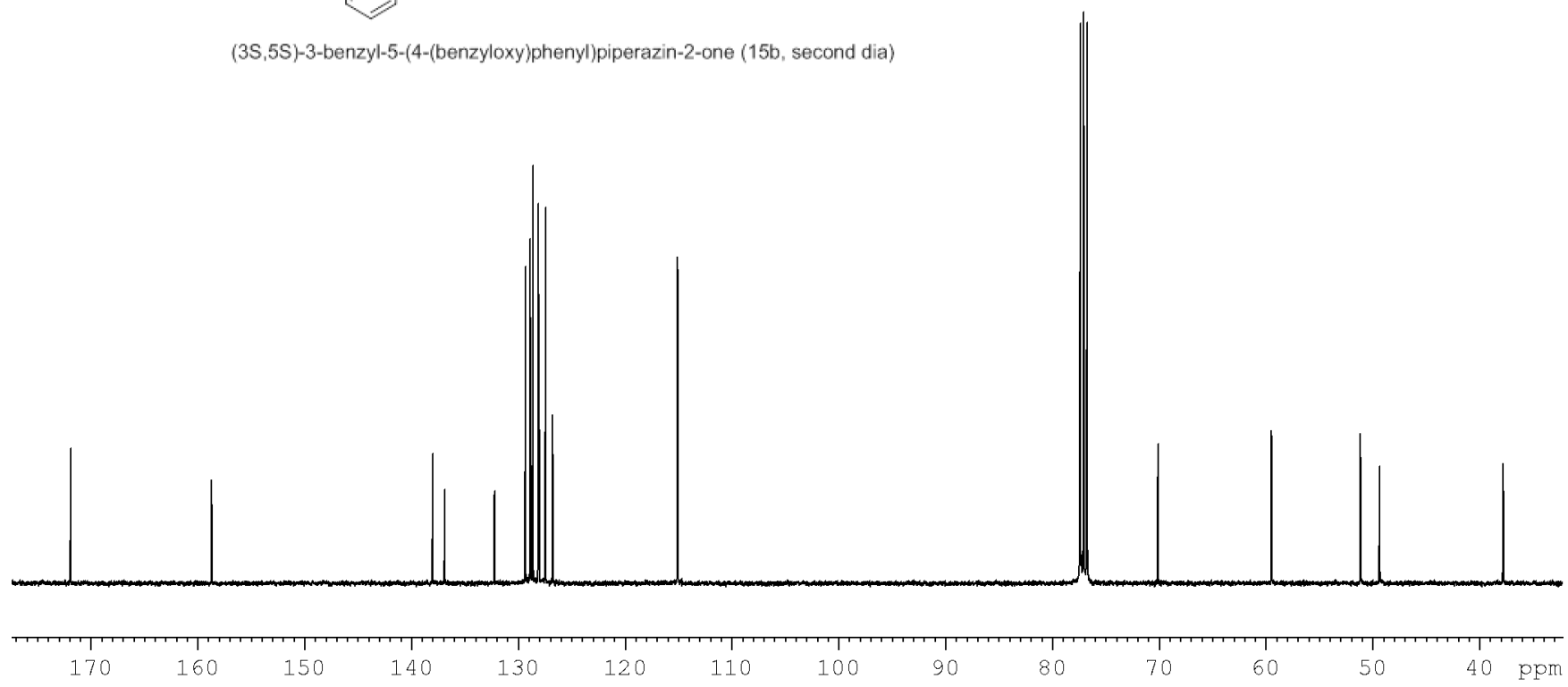
— 59.441

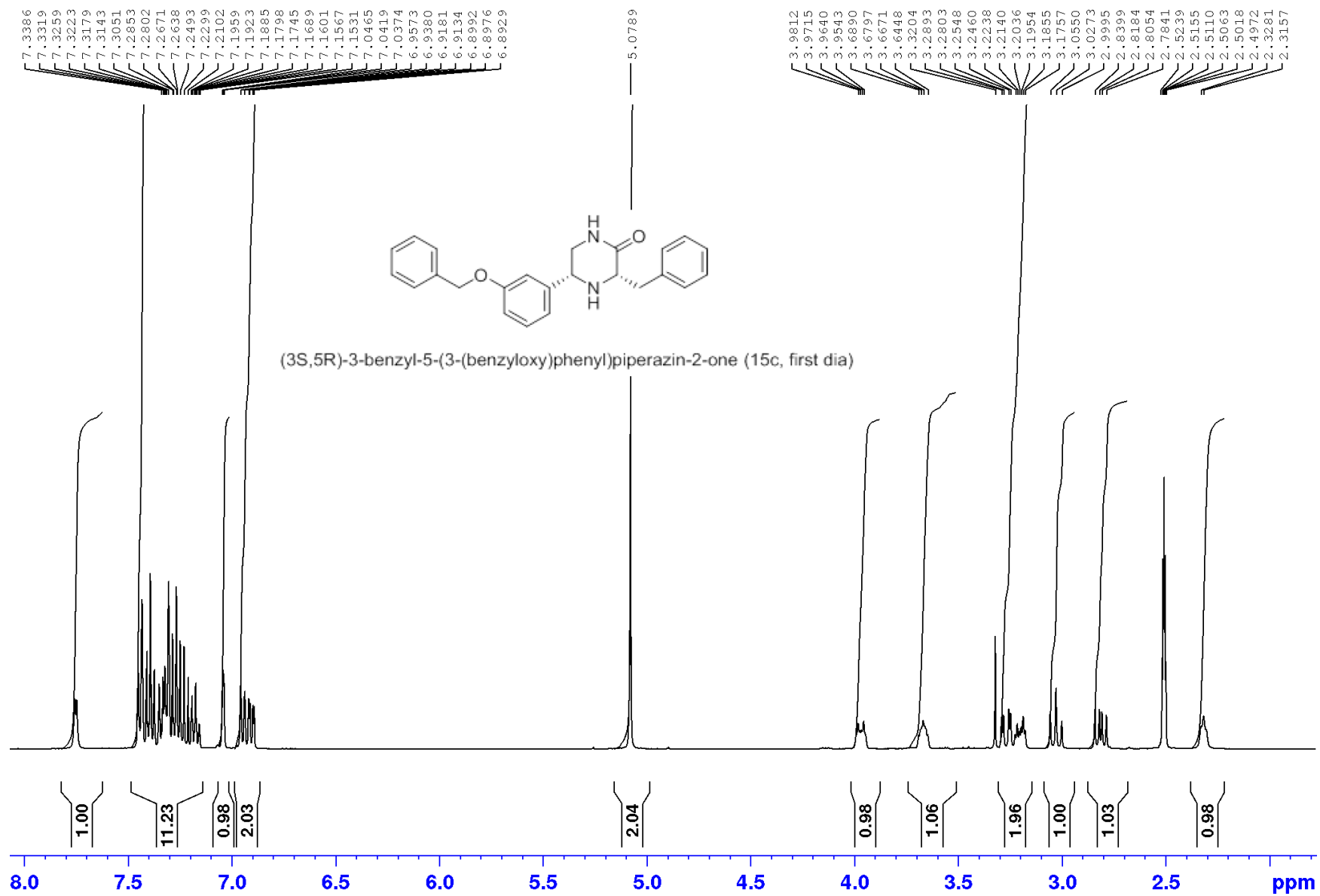
51.121
49.322

— 37.767



(3S,5S)-3-benzyl-5-(4-(benzyloxy)phenyl)piperazin-2-one (15b, second dia)





— 170.250

— 158.806

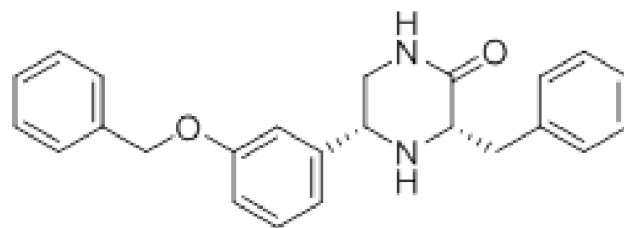
— 143.371
— 139.649
— 137.545
— 129.930
— 129.837
— 128.878
— 128.469
— 128.269
— 128.136
— 126.421
— 119.557
— 114.121
— 113.796

— 69.623

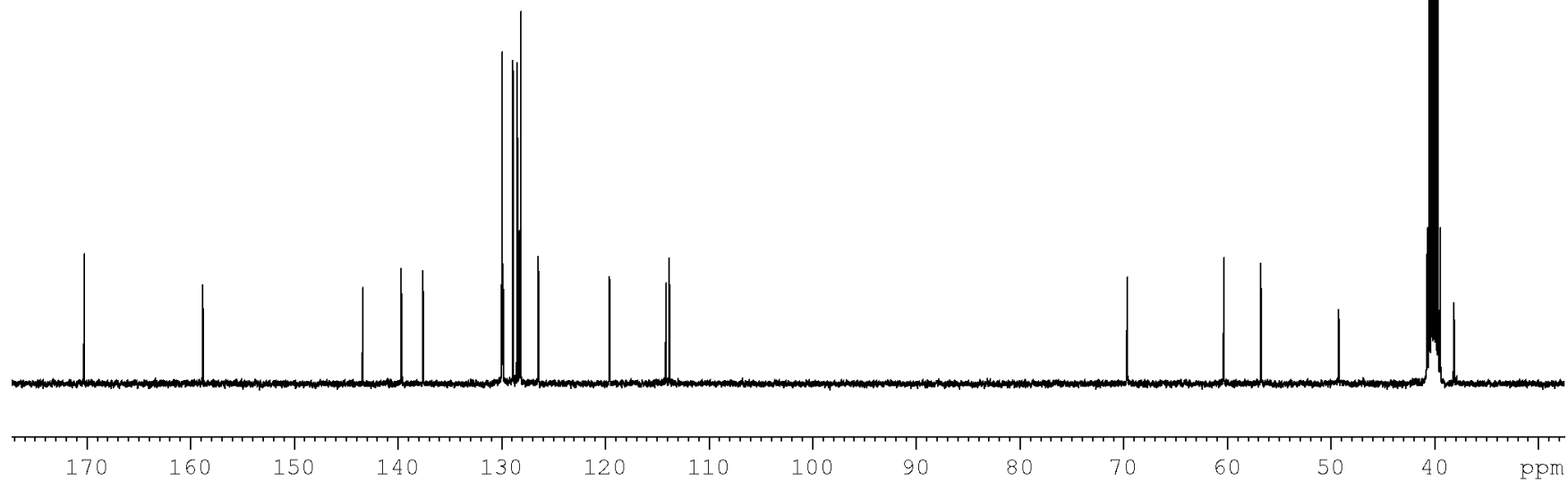
— 60.303

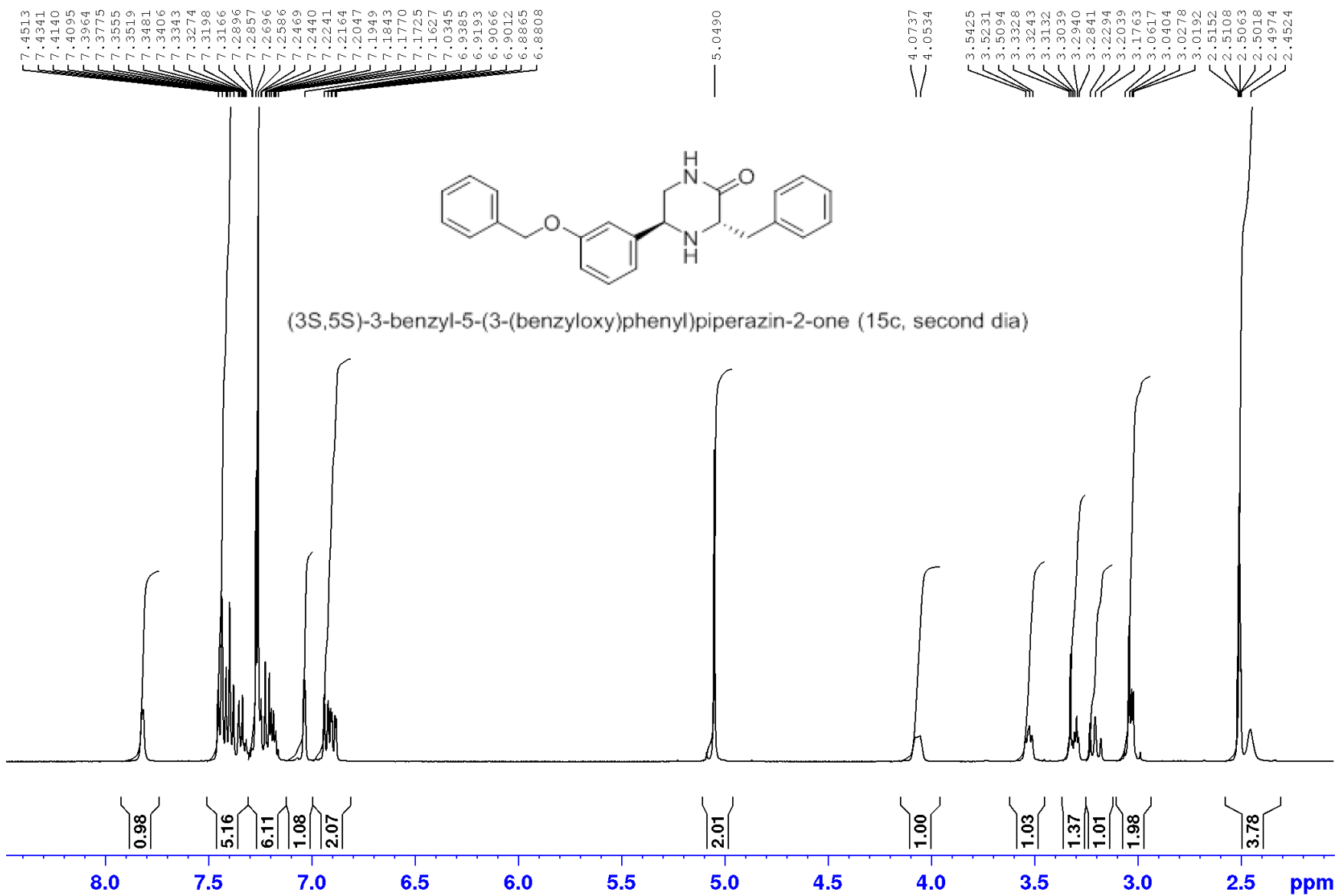
— 56.728

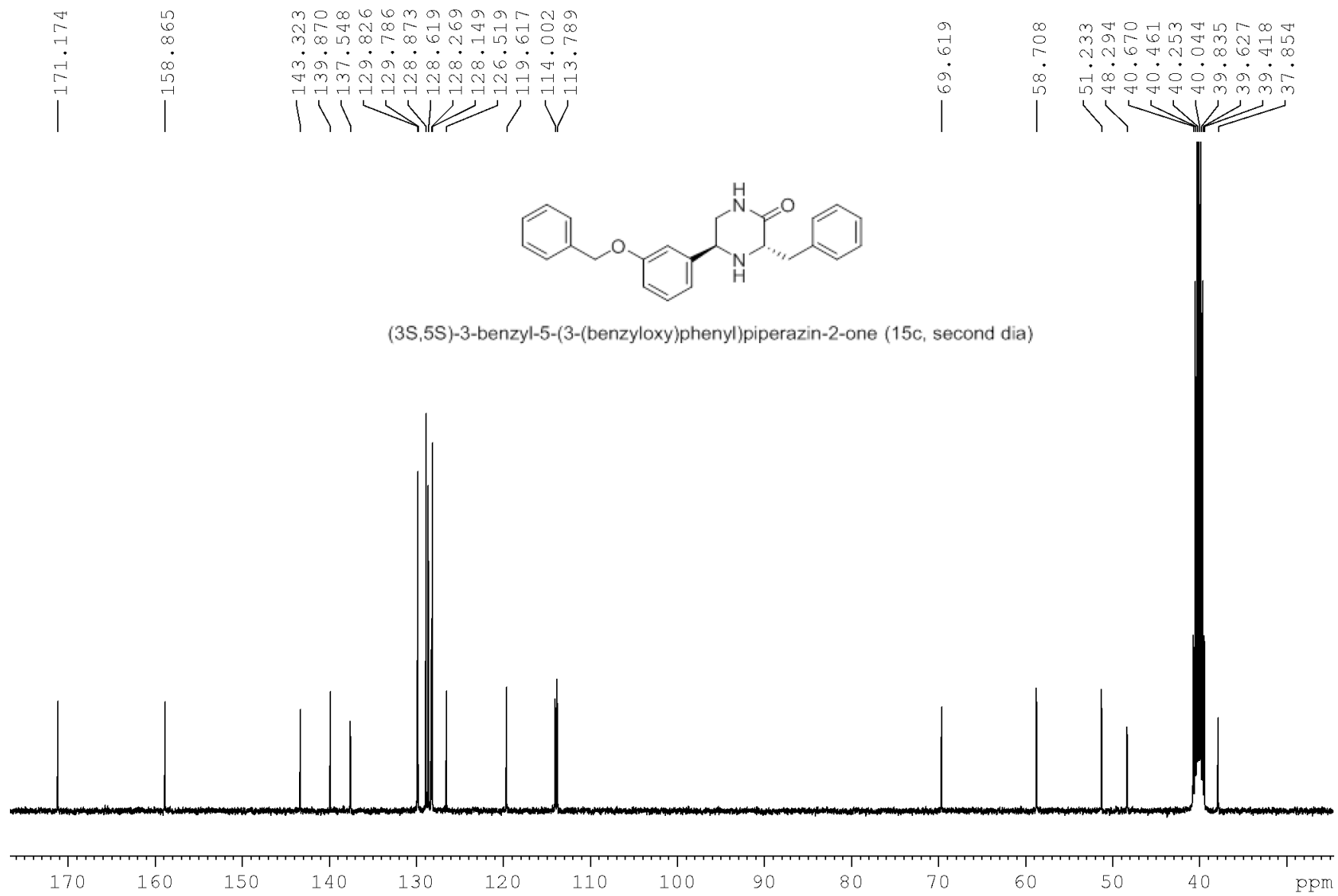
— 49.212
— 40.669
— 40.461
— 40.252
— 40.044
— 39.835
— 39.626
— 39.418
— 38.103

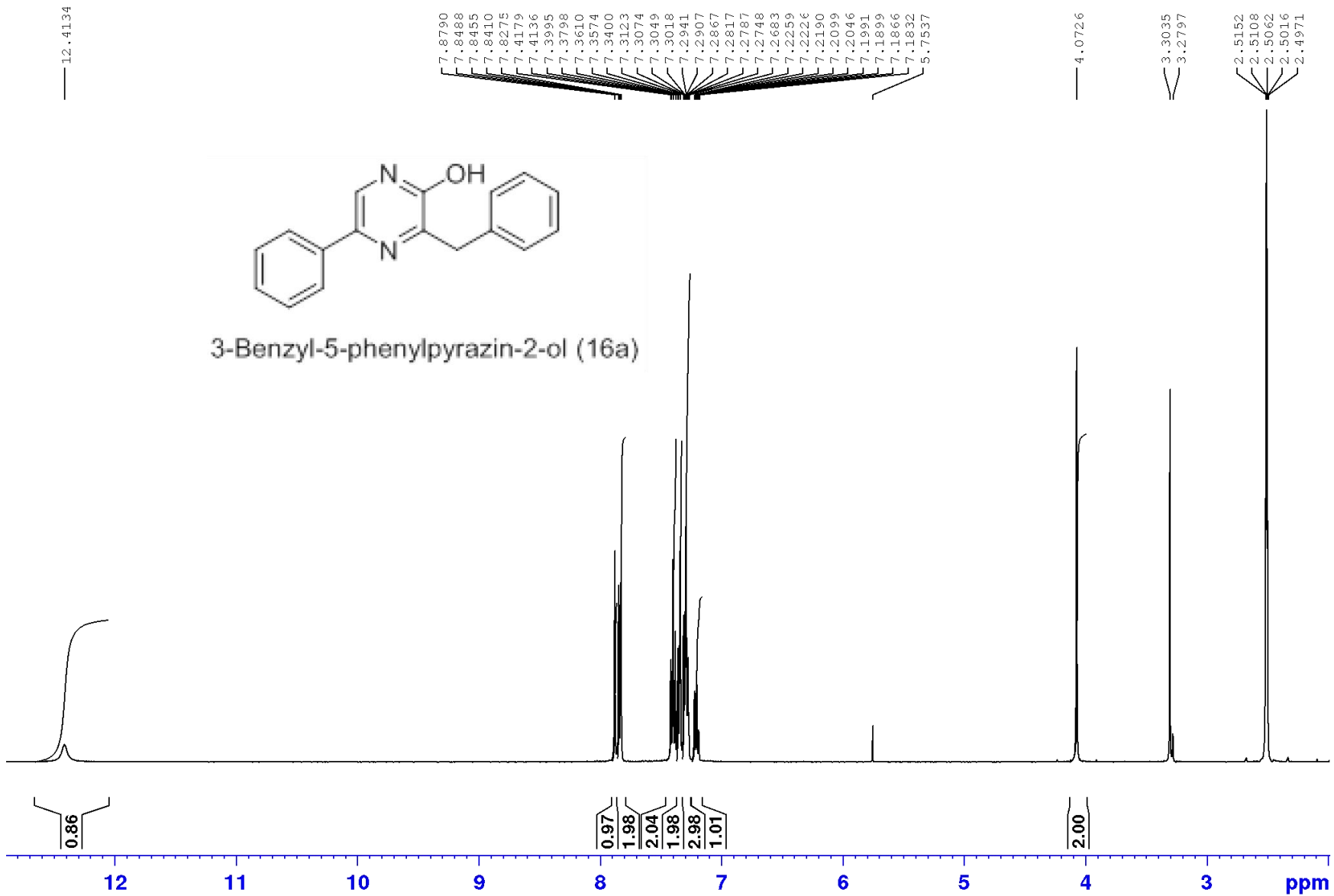


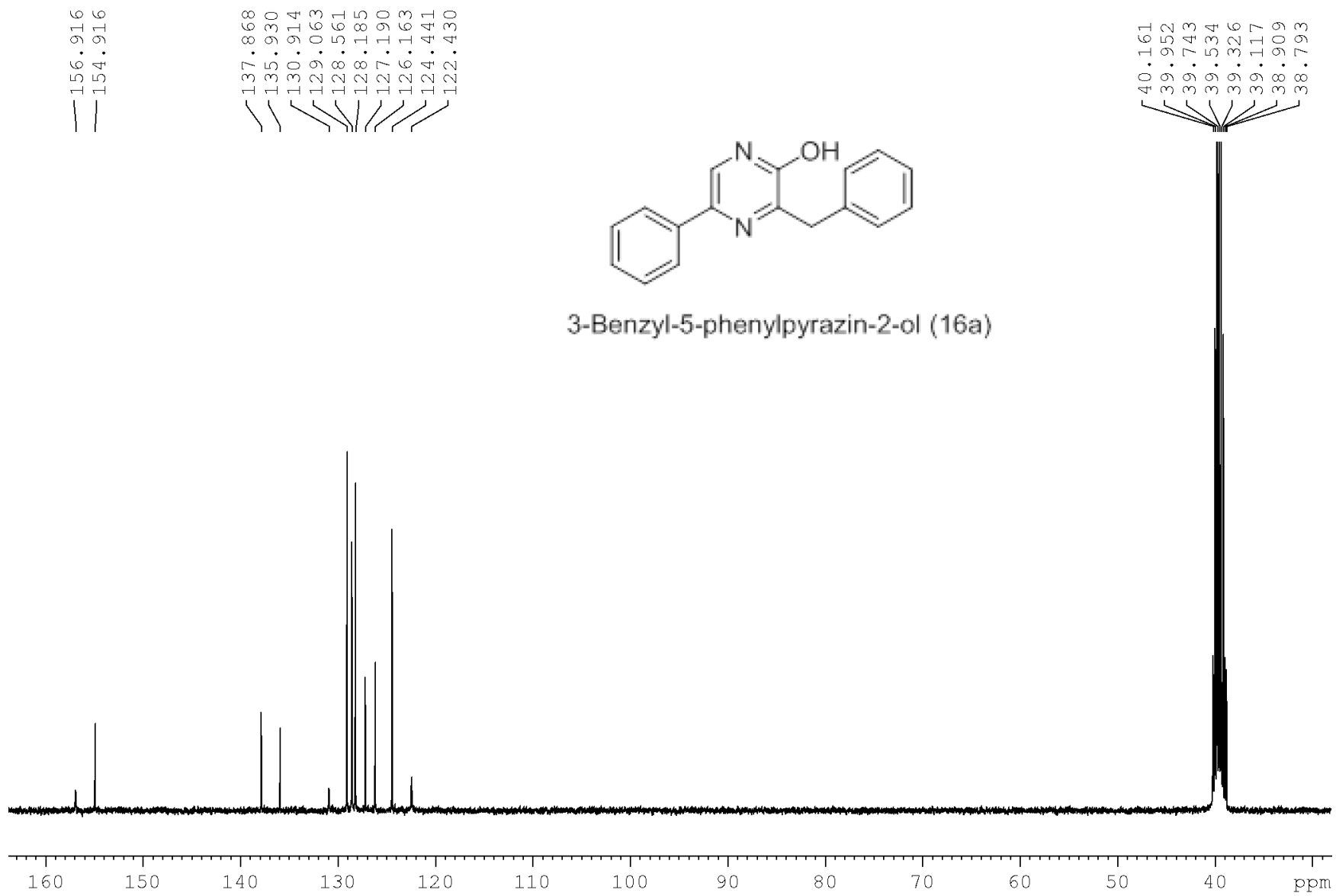
(3S,5R)-3-benzyl-5-(3-(benzyloxy)phenyl)piperazin-2-one (15c, first dia)

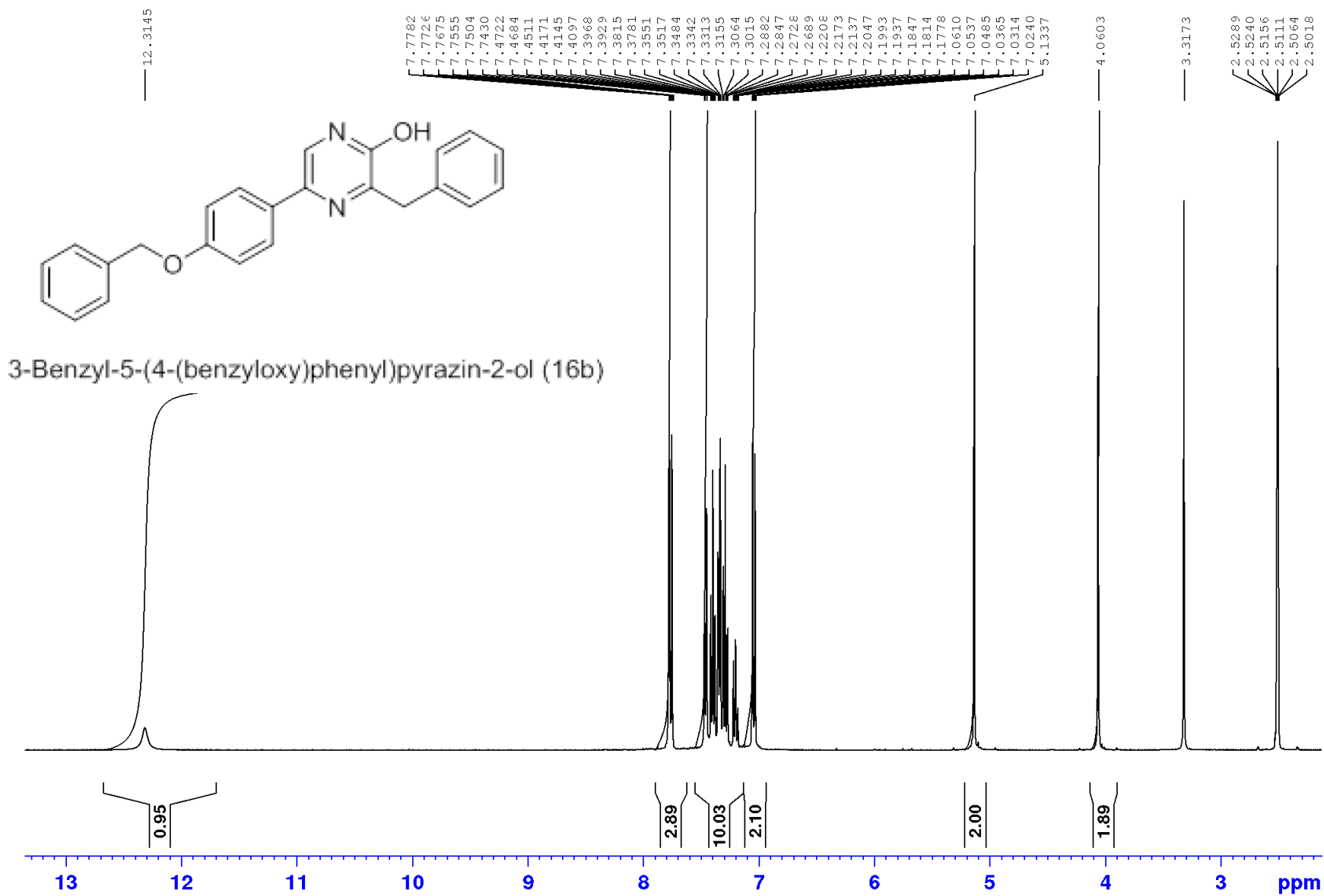


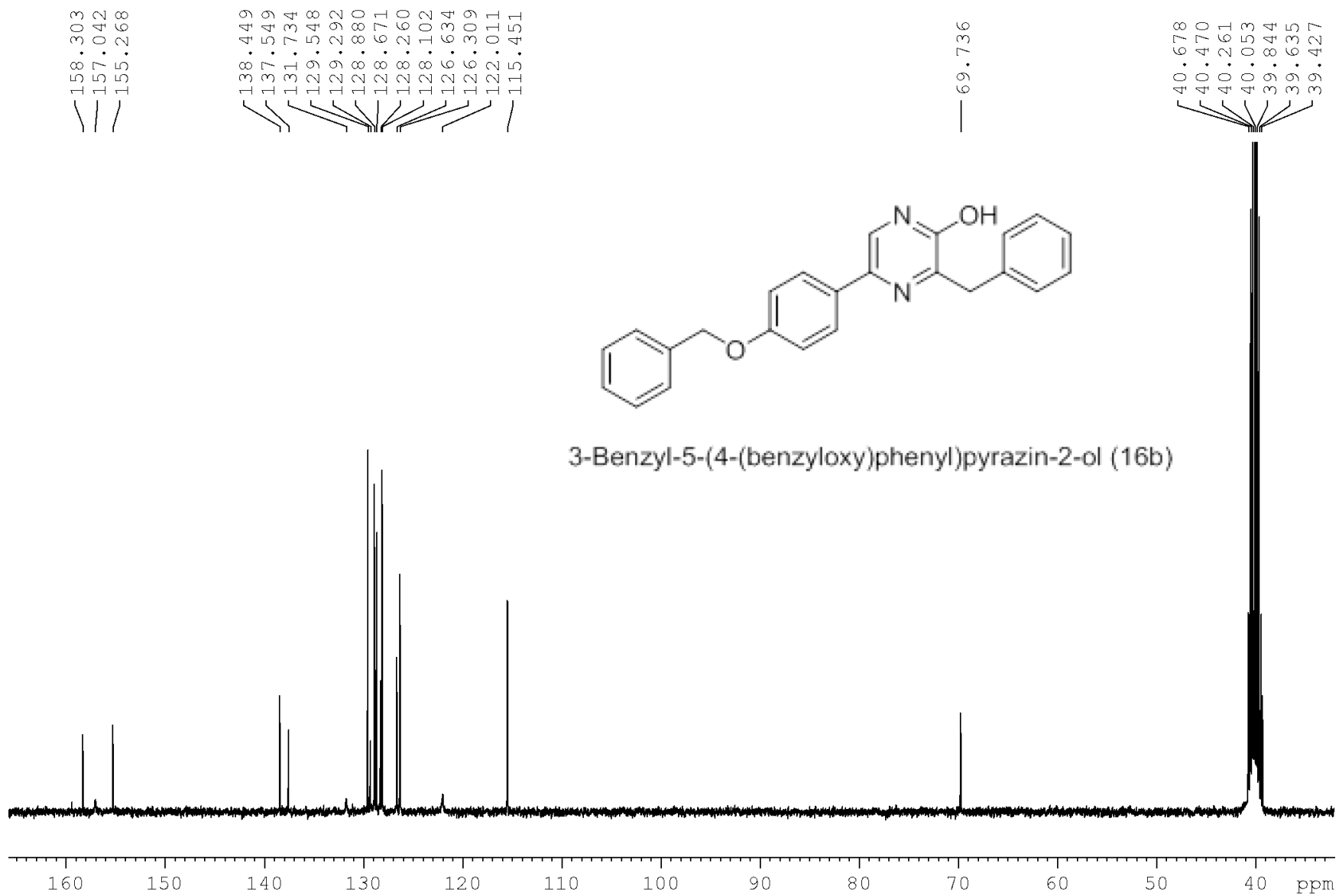


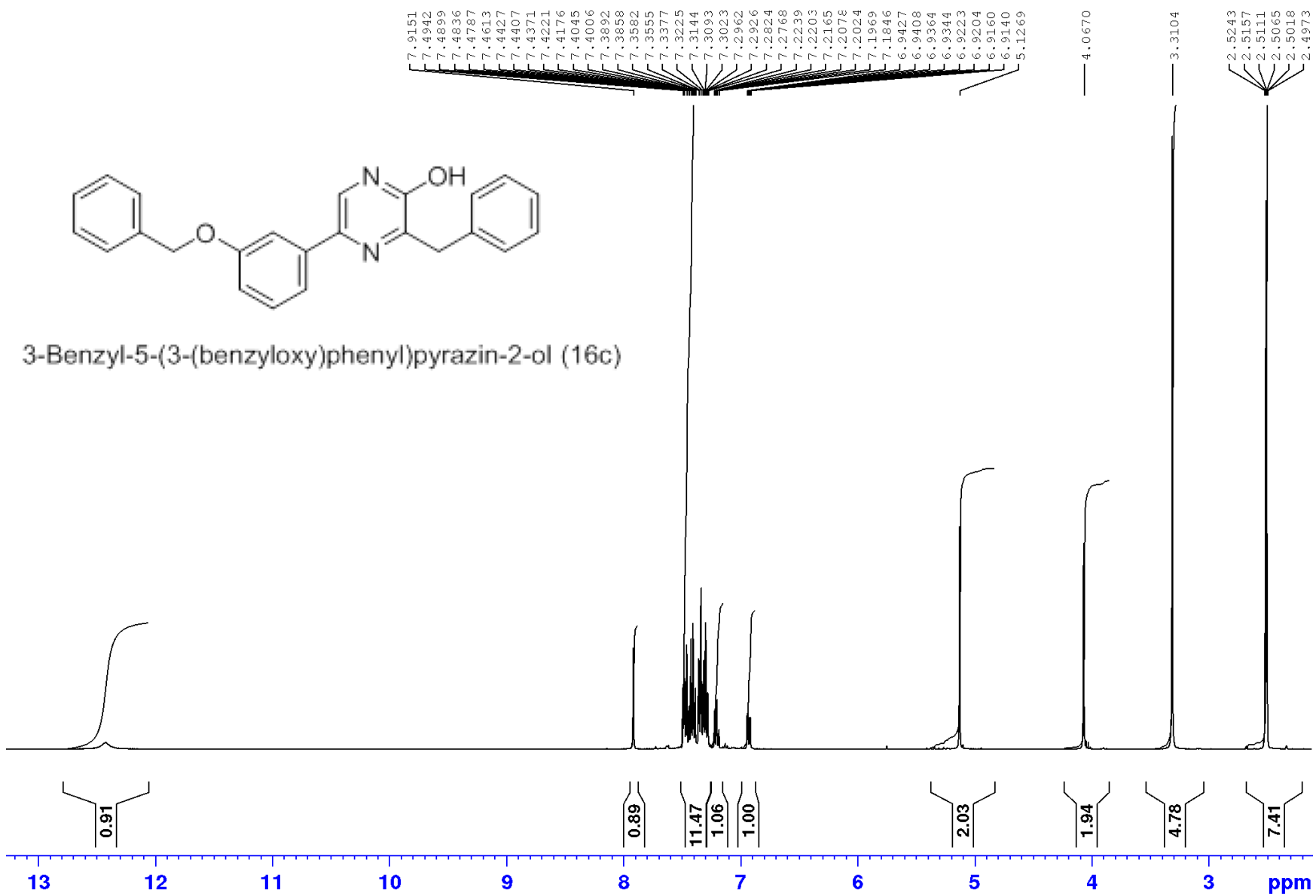


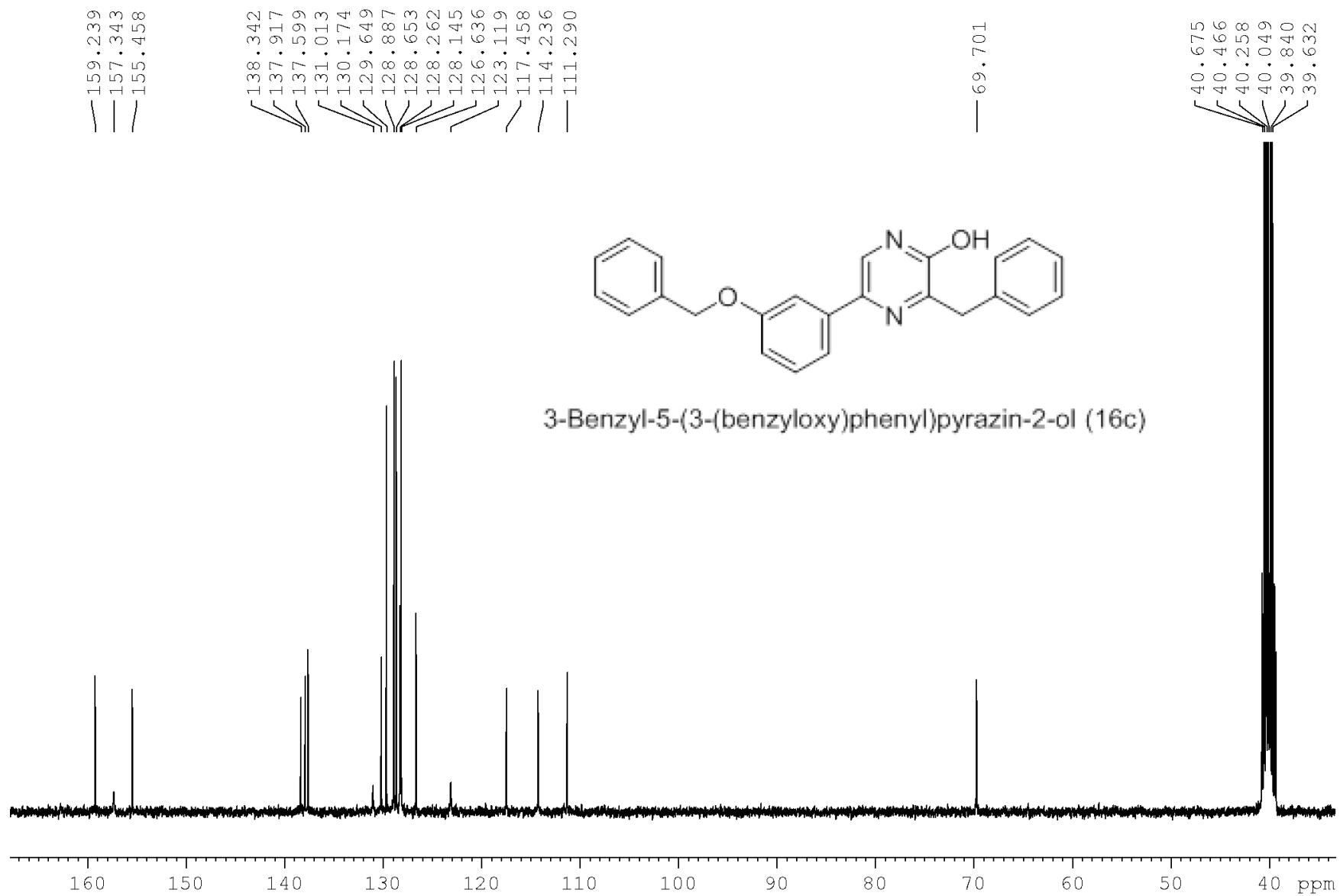


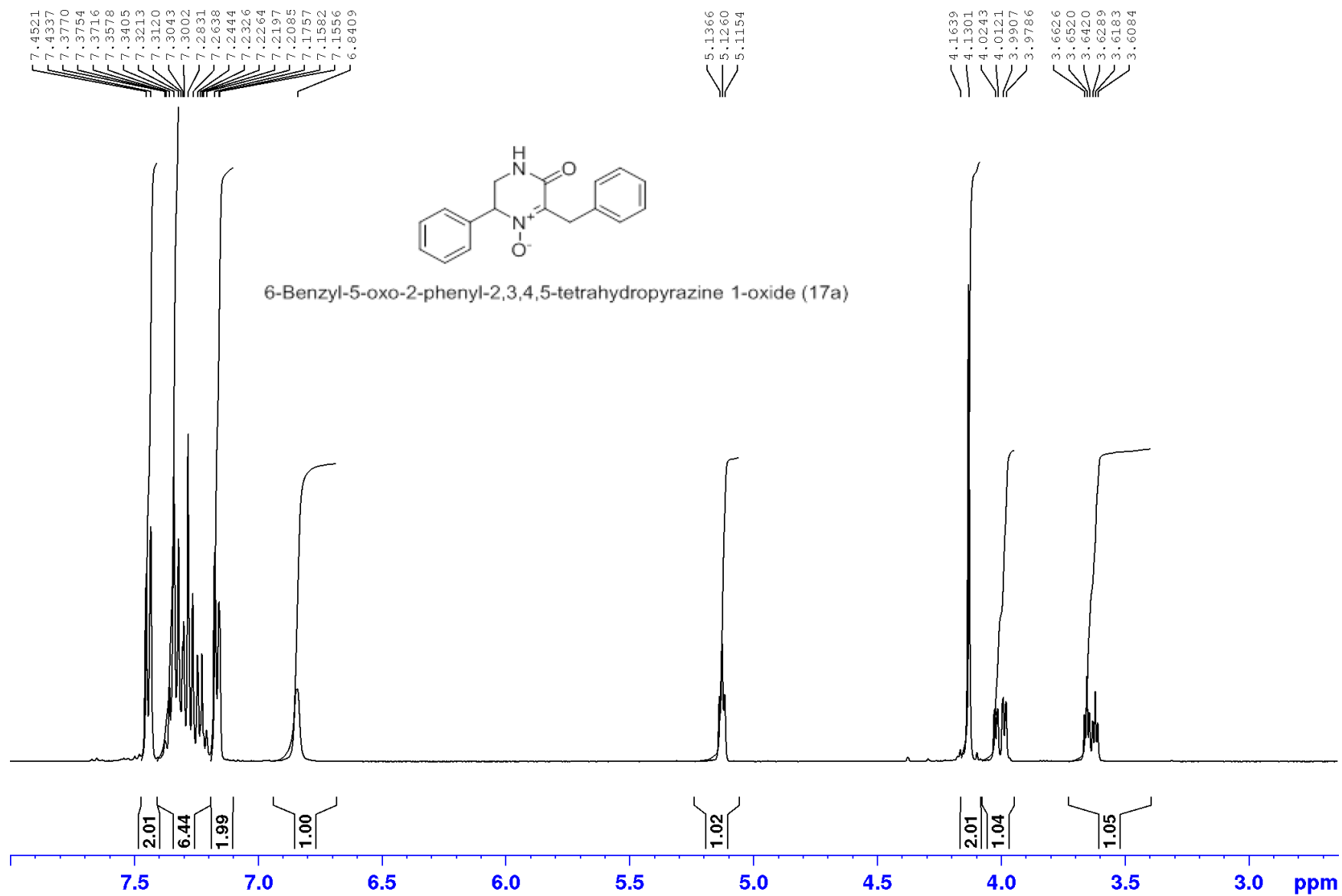




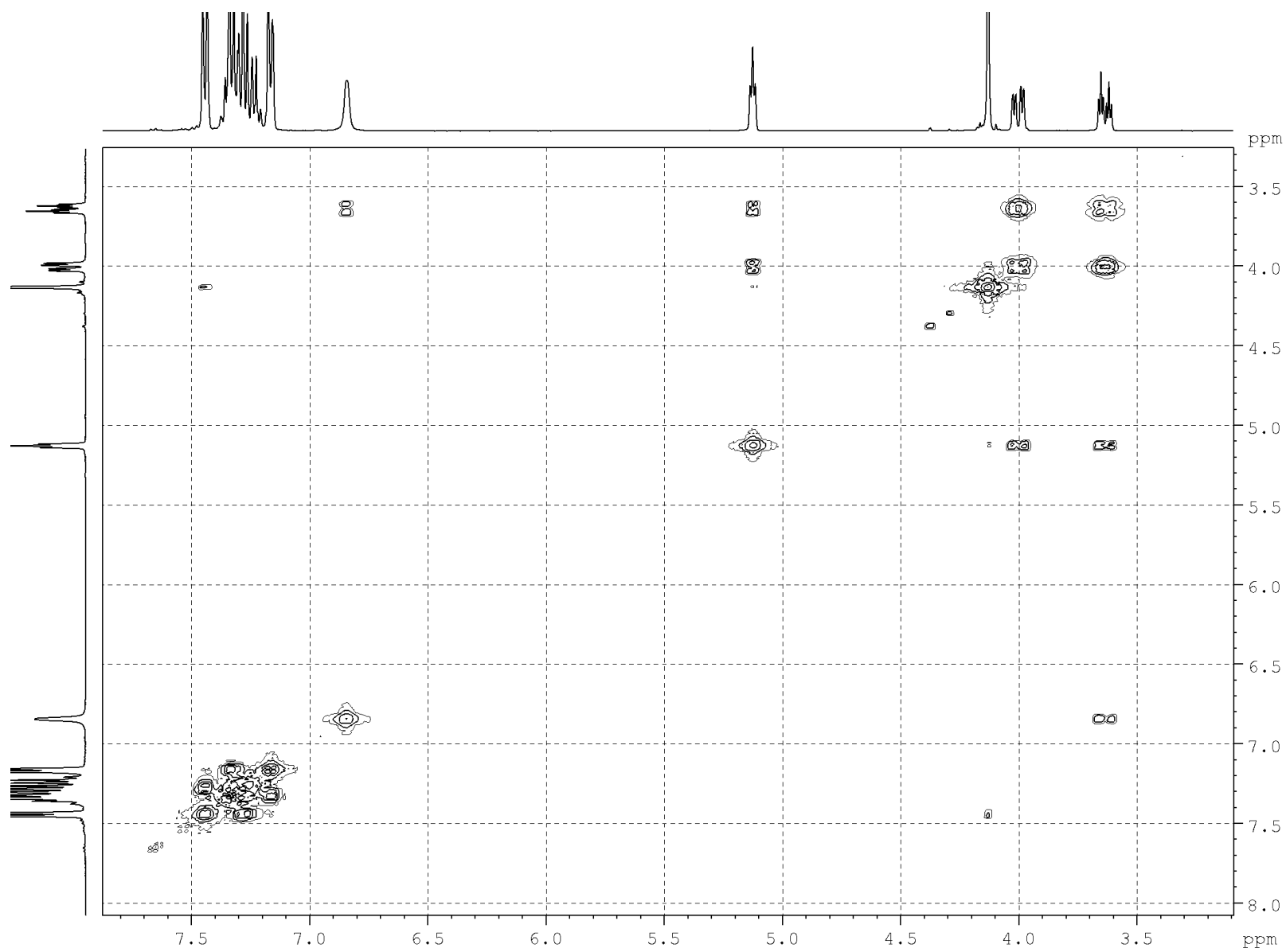


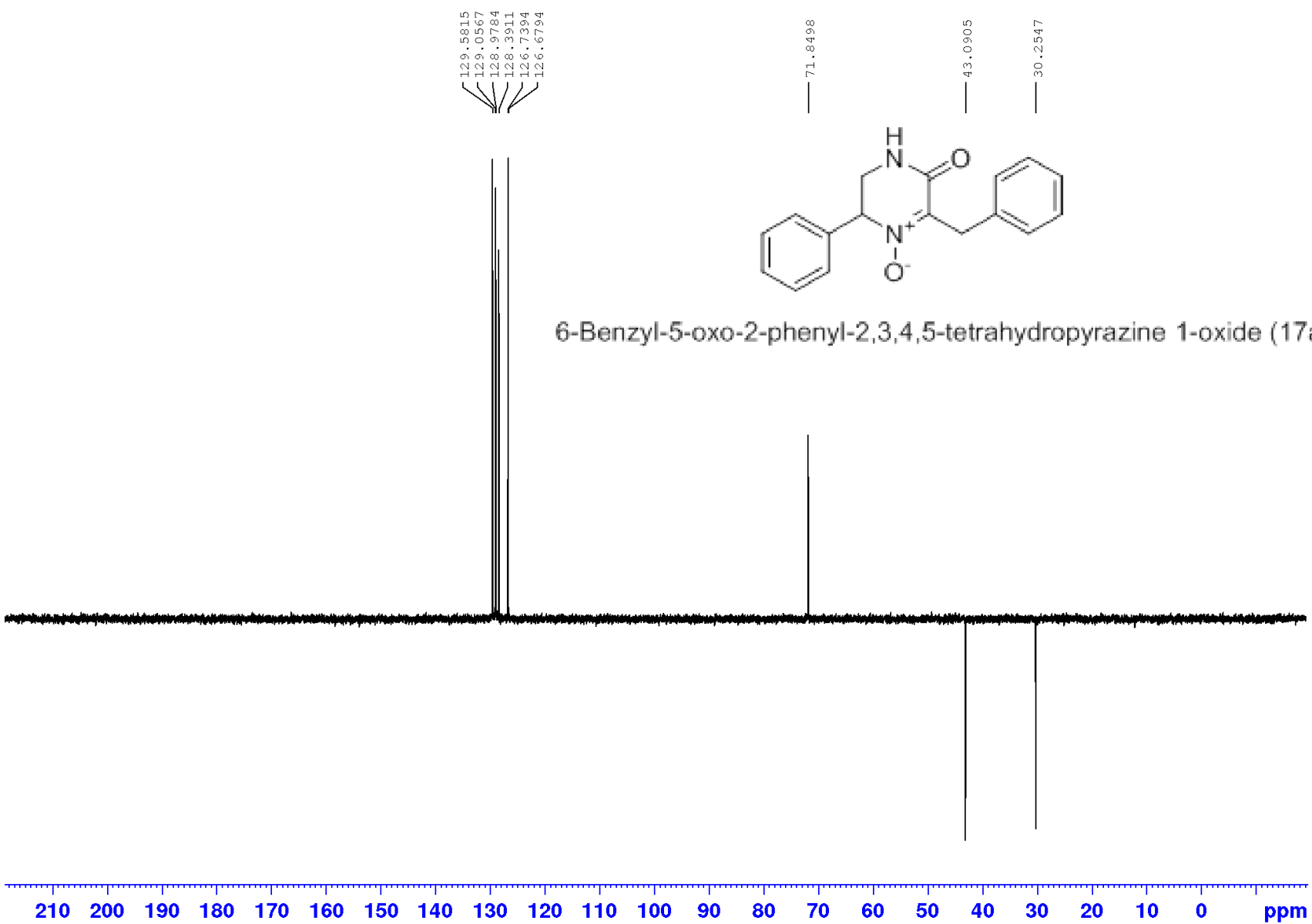


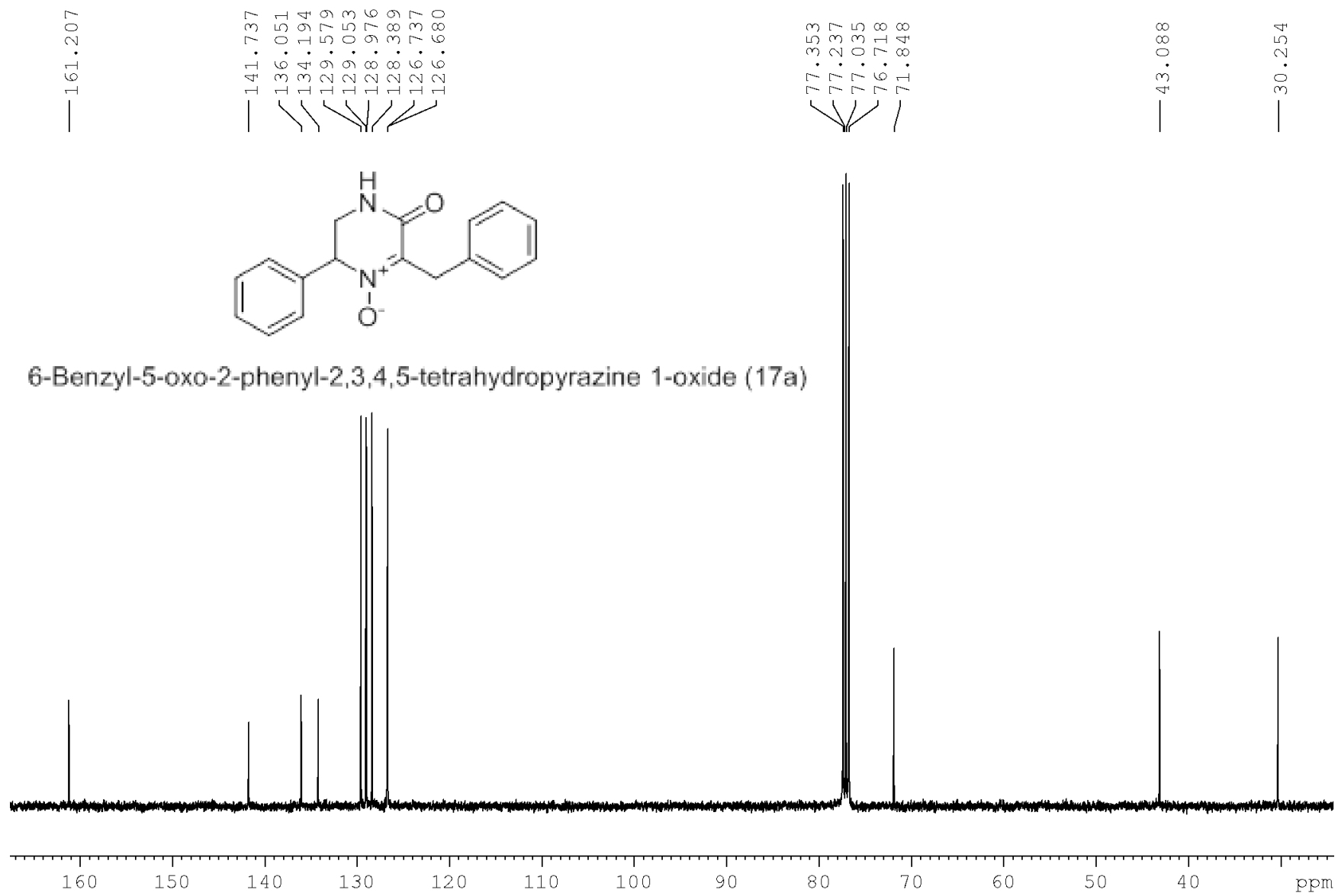


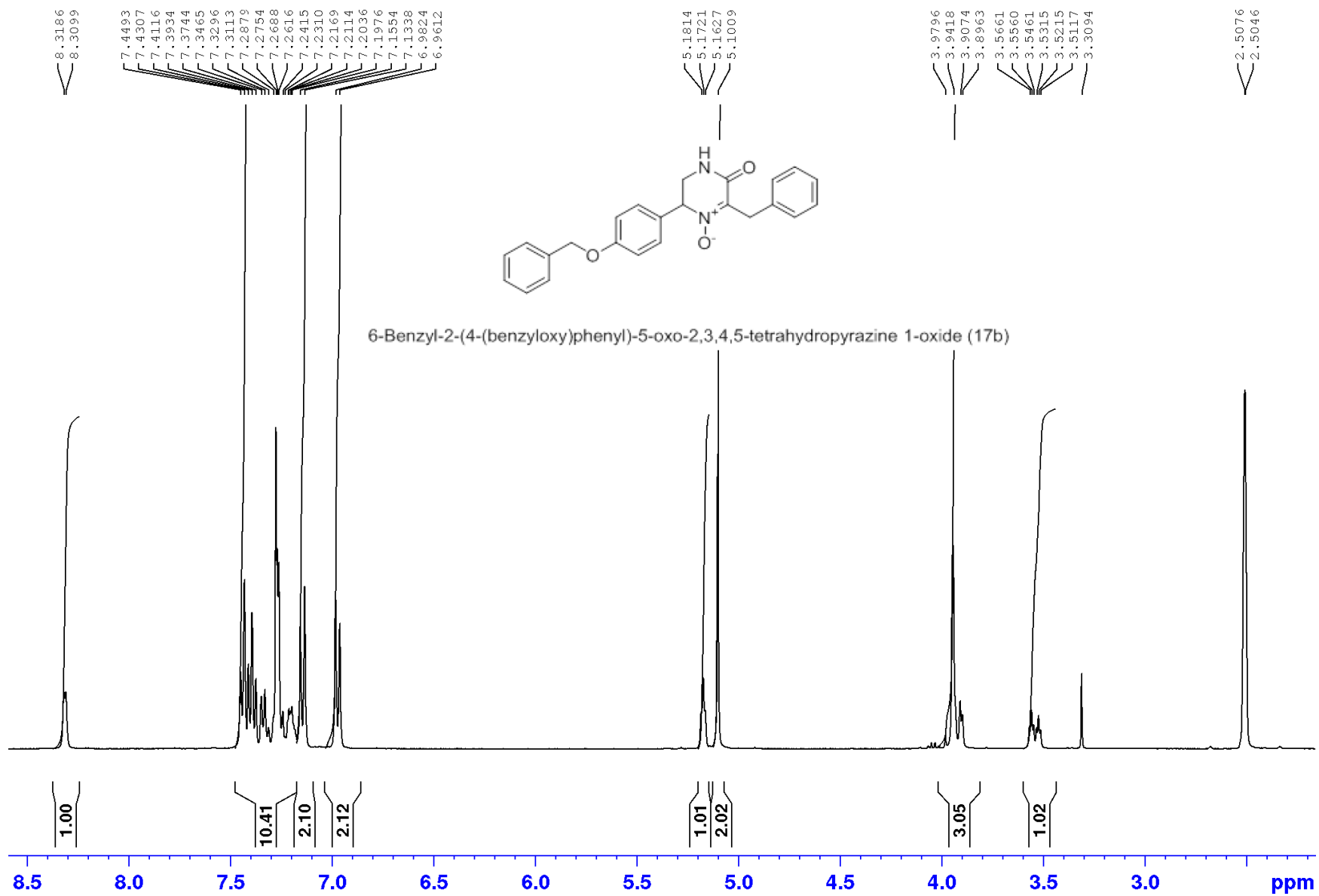


6-Benzyl-5-oxo-2-phenyl-2,3,4,5-tetrahydropyrazine 1-oxide (17a)

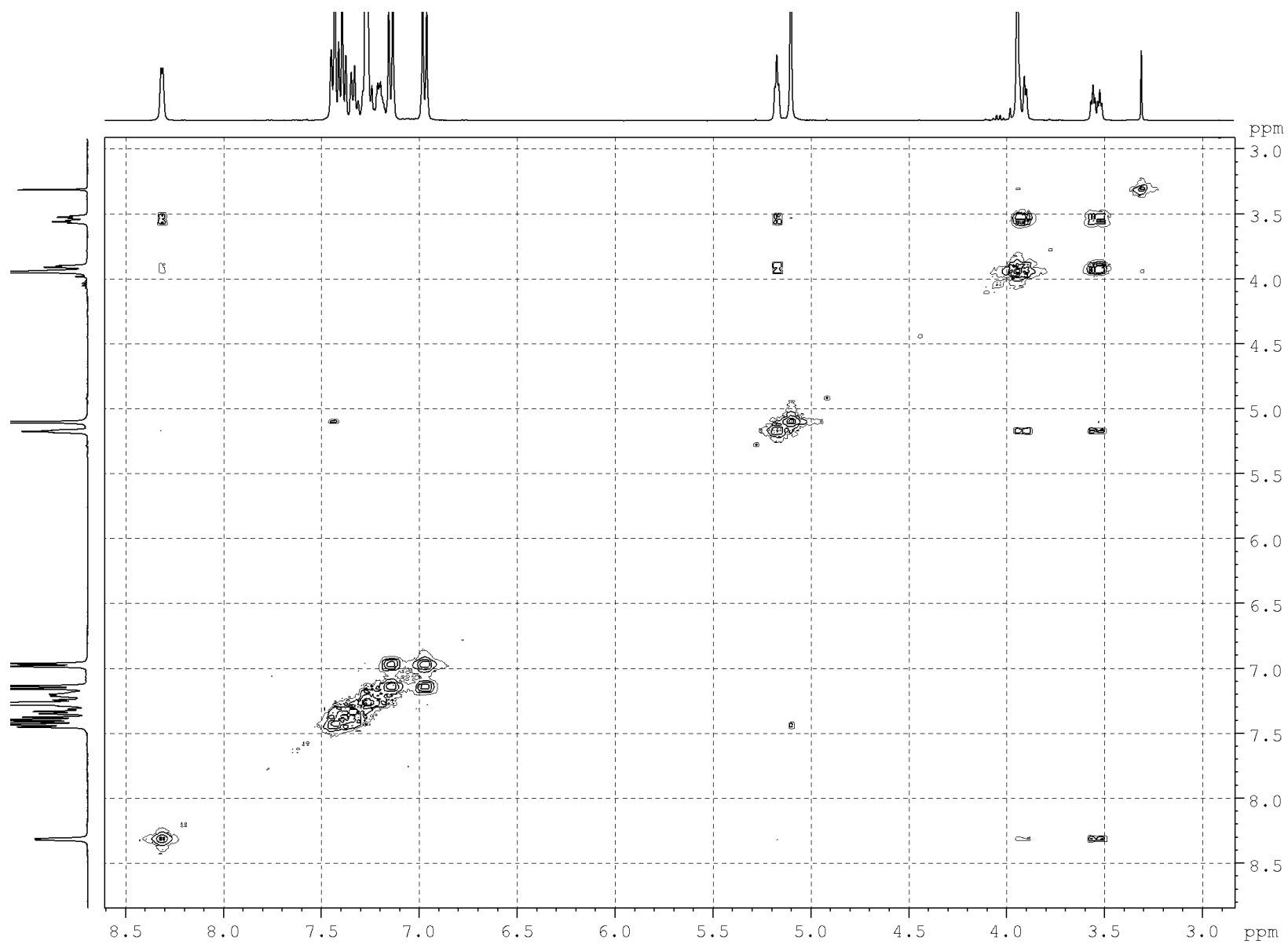








6-Benzyl-2-(4-(benzyloxy)phenyl)-5-oxo-2,3,4,5-tetrahydropyrazine 1-oxide (17b)

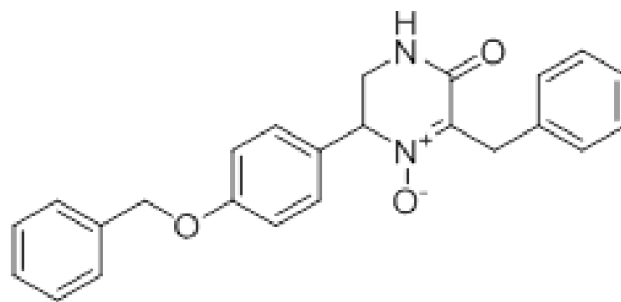


160.709
158.800

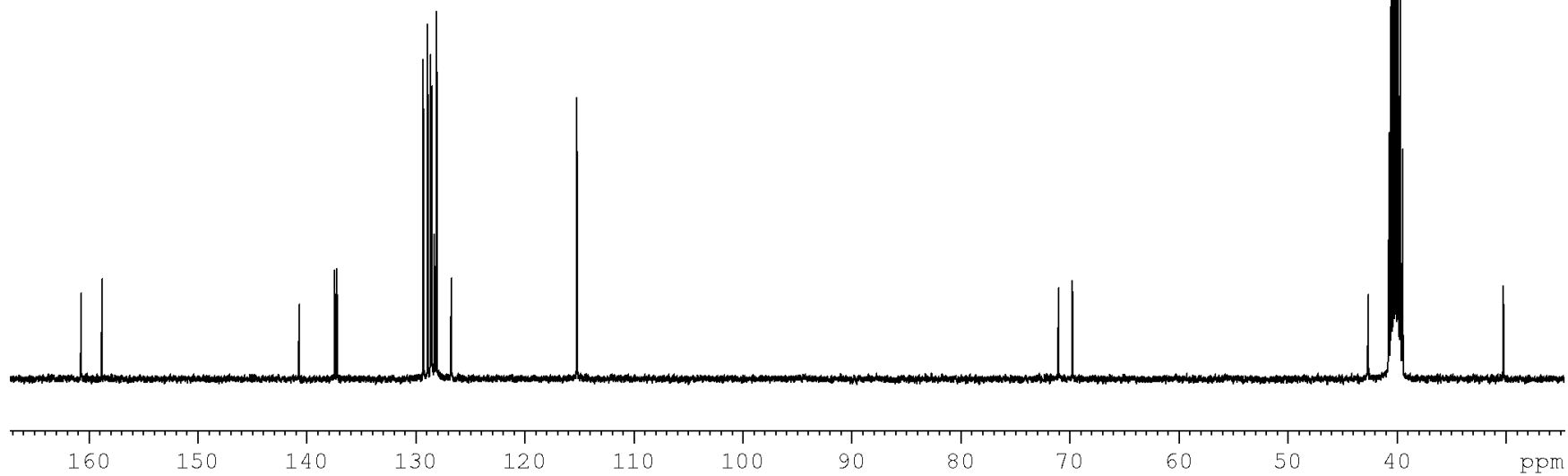
140.707
137.425
137.235
129.303
128.896
128.644
128.532
128.294
128.095
128.055
126.743
115.208

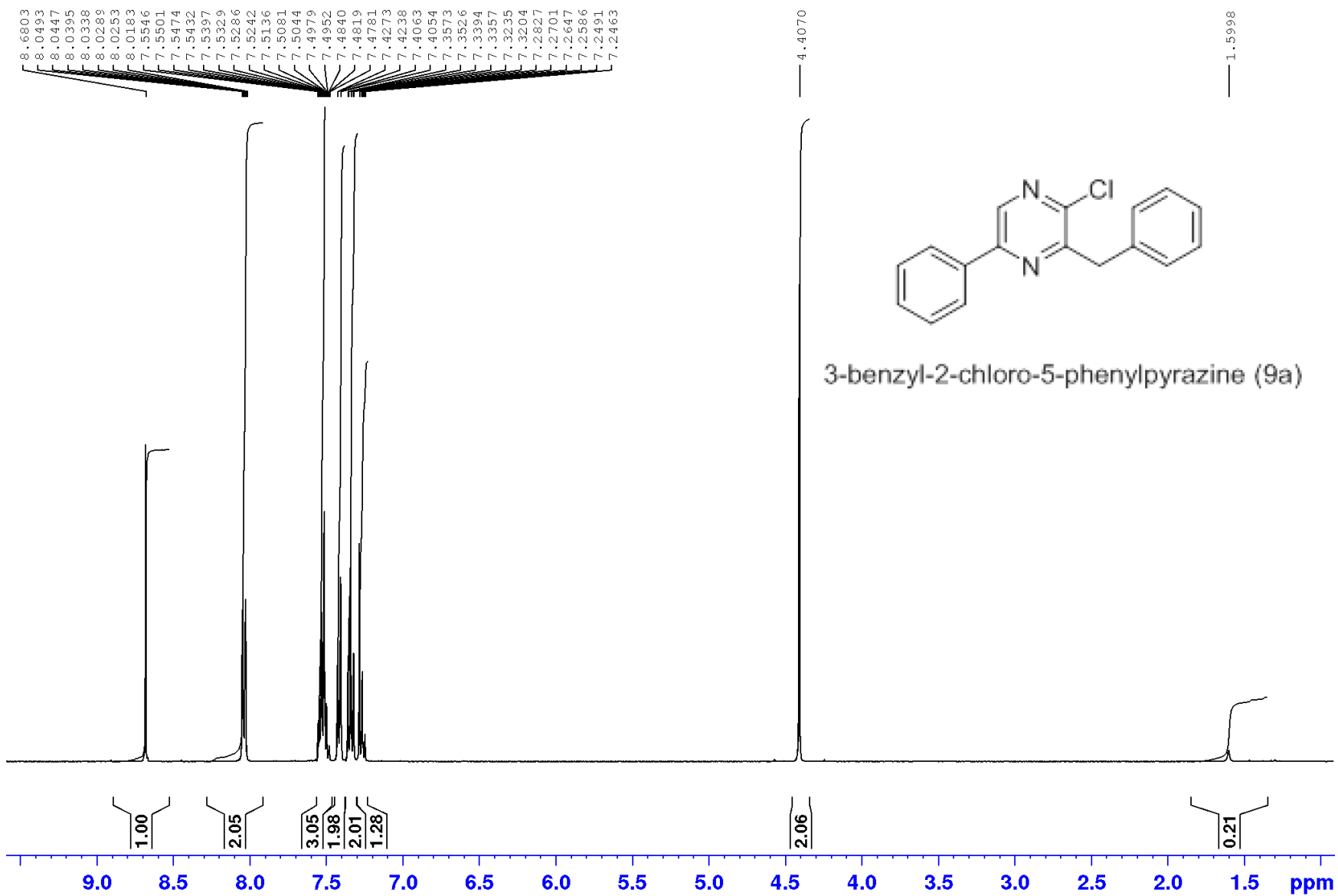
71.019
69.729

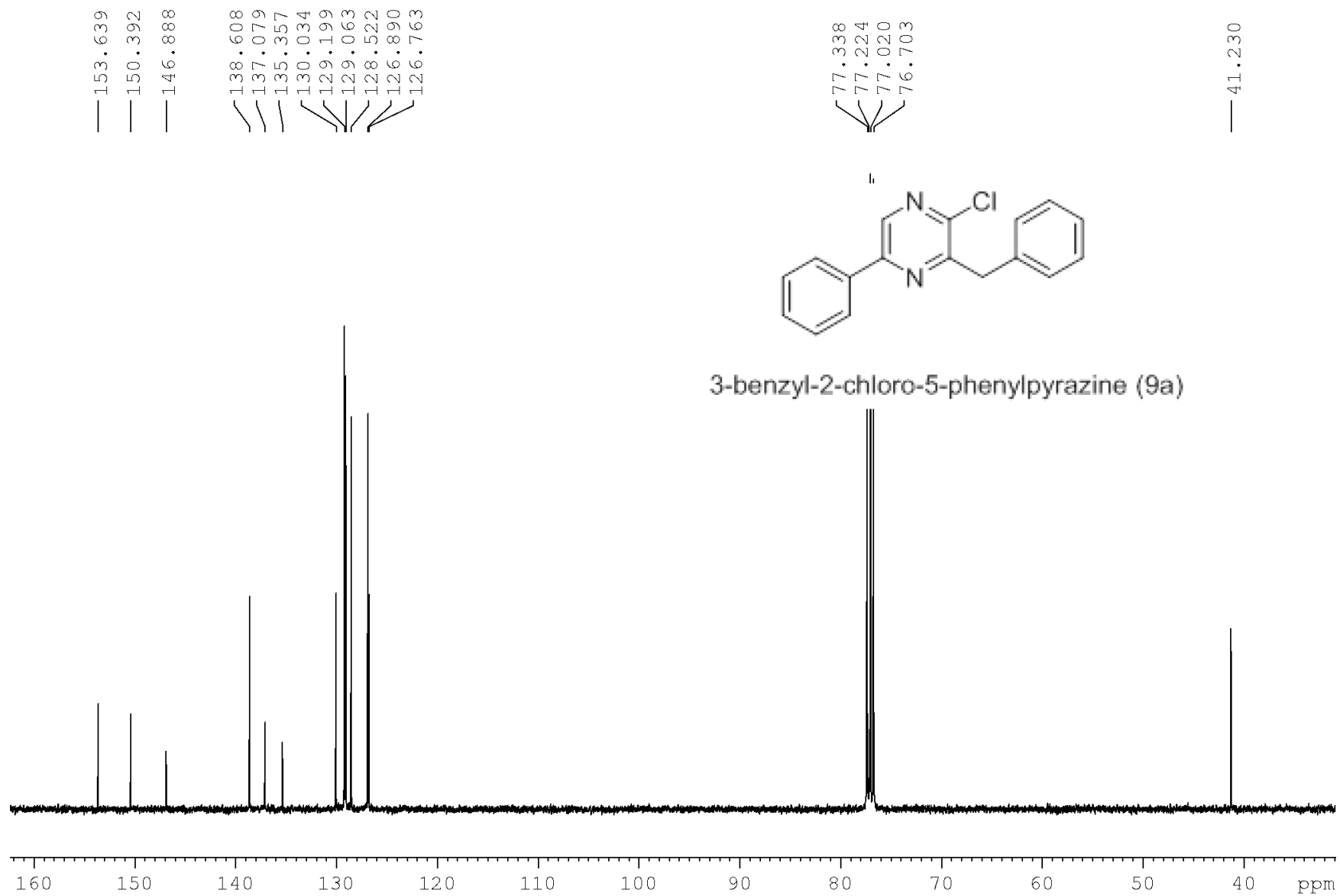
42.595
40.675
40.467
40.258
40.049
39.841
39.632
39.423
30.193

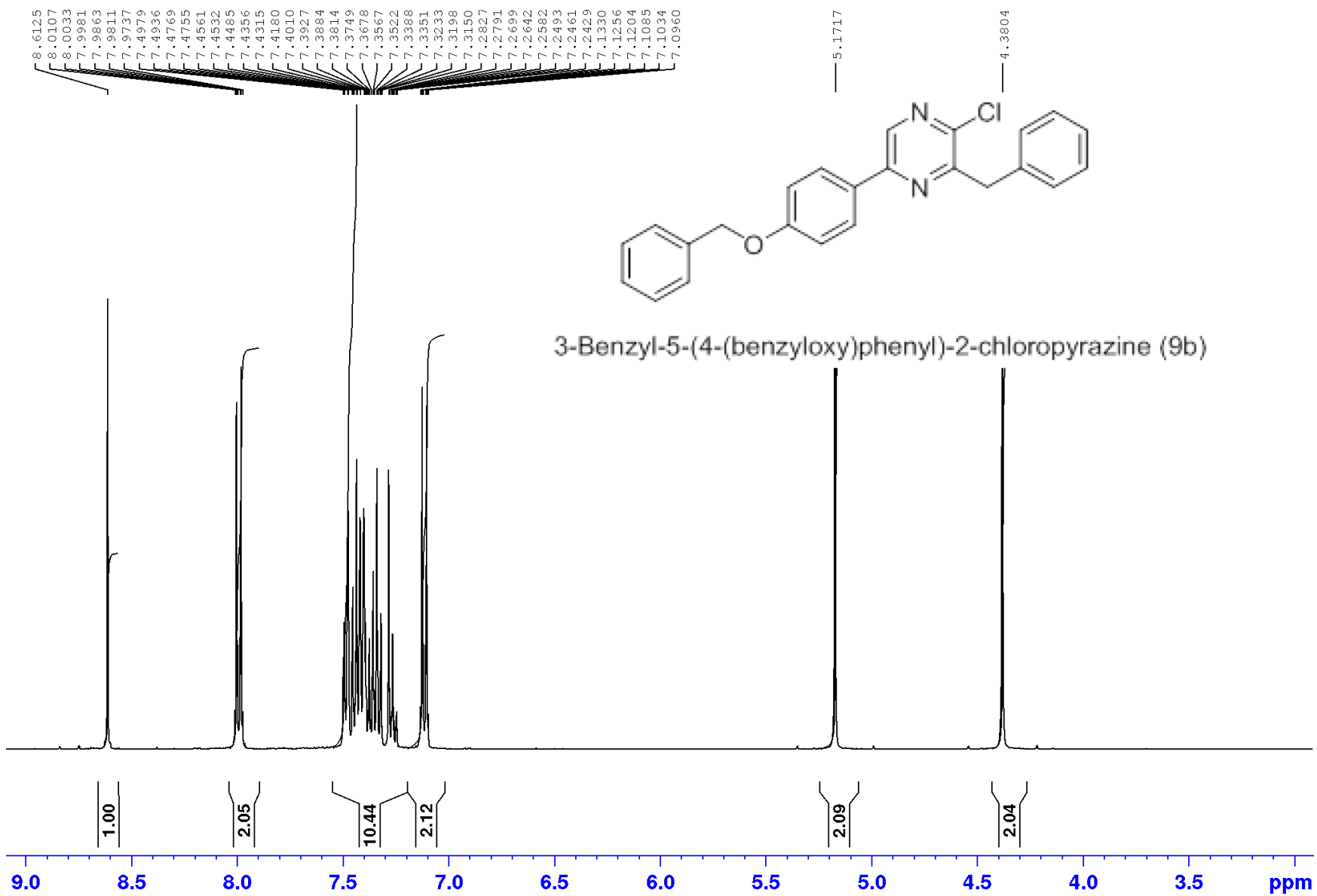


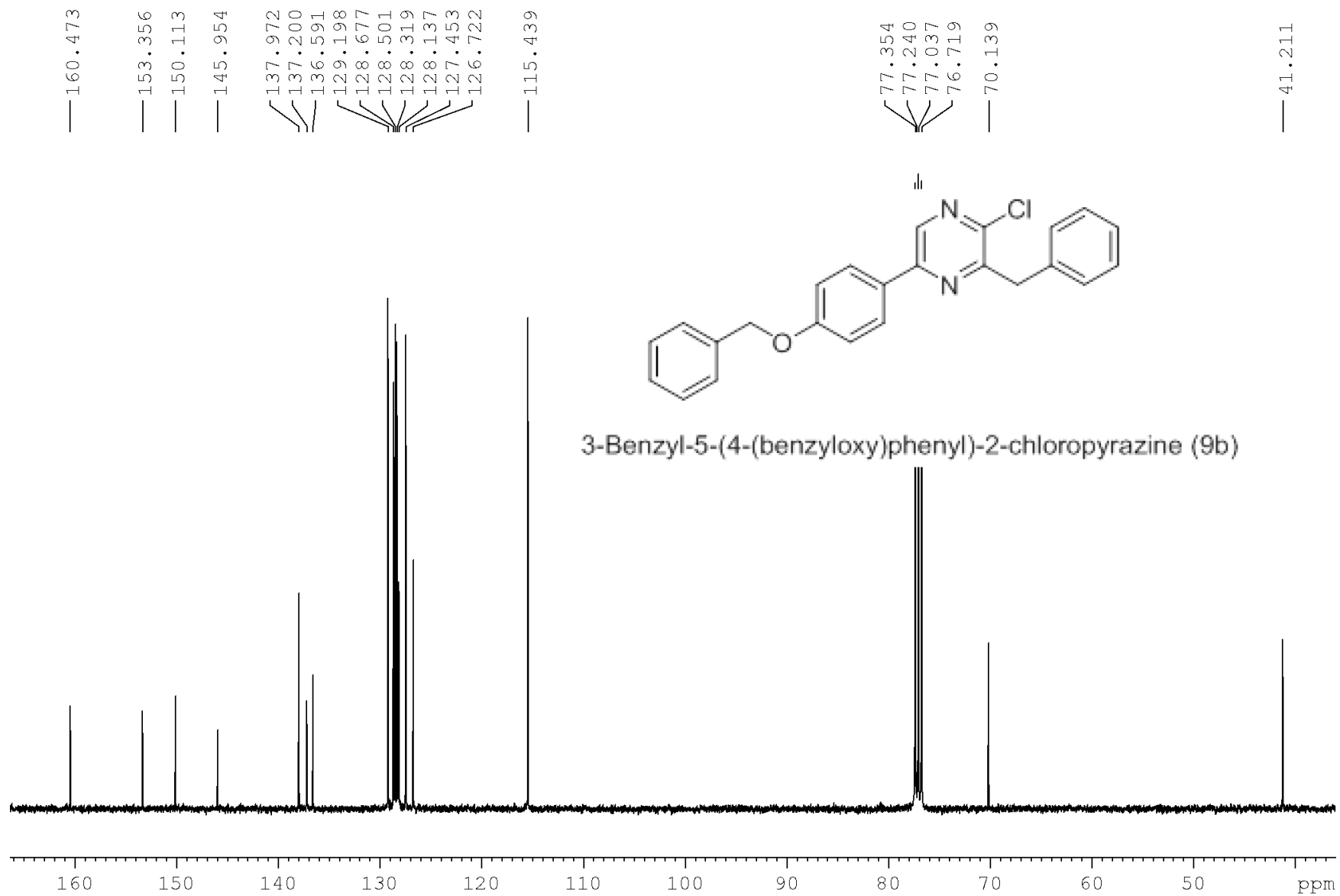
6-Benzyl-2-(4-(benzyloxy)phenyl)-5-oxo-2,3,4,5-tetrahydropyrazine 1-oxide (17b)

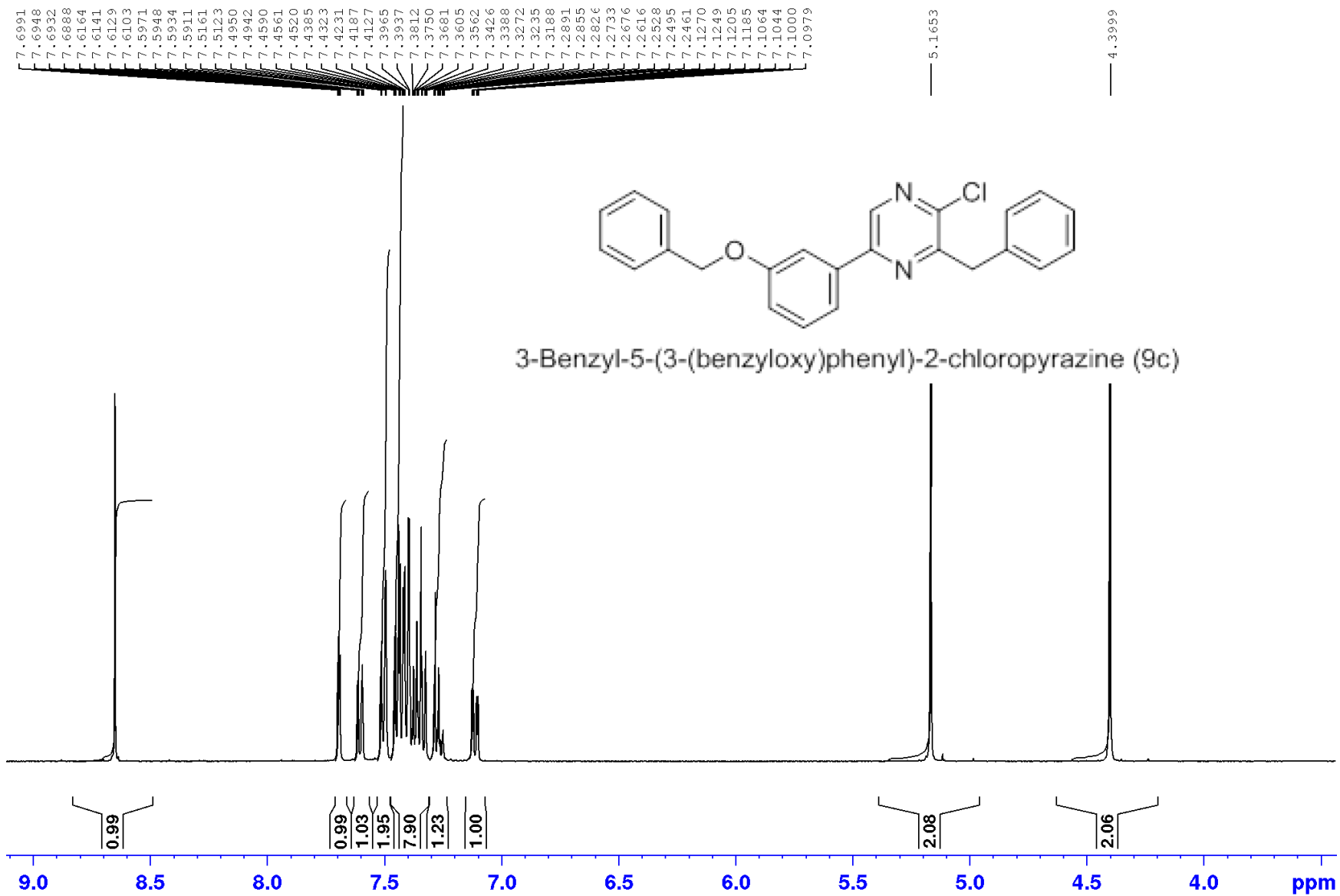








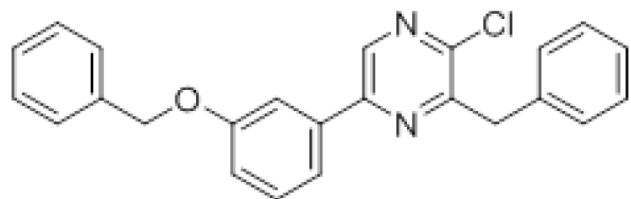




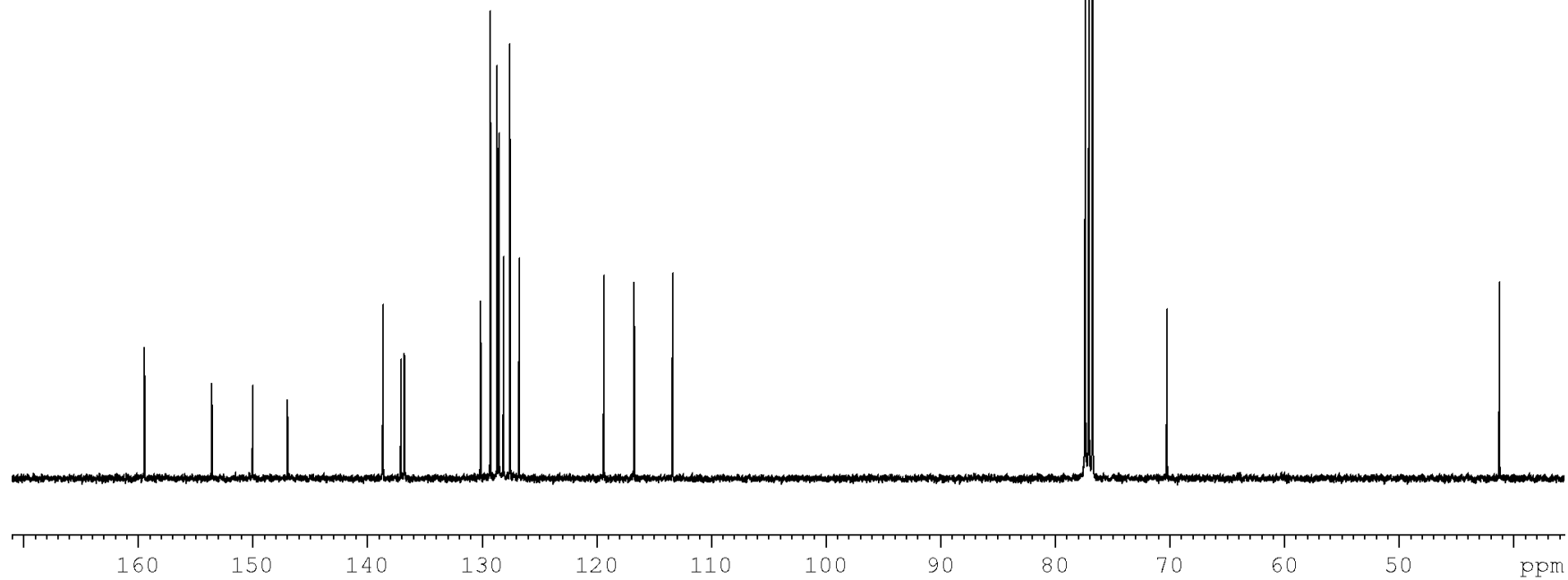
— 159.443
— 153.561
— 150.001
— 146.961
— 138.626
— 137.052
— 136.774
— 136.741
— 130.123
— 129.270
— 128.665
— 128.504
— 128.103
— 127.556
— 126.753
— 119.352
— 116.717
— 113.358

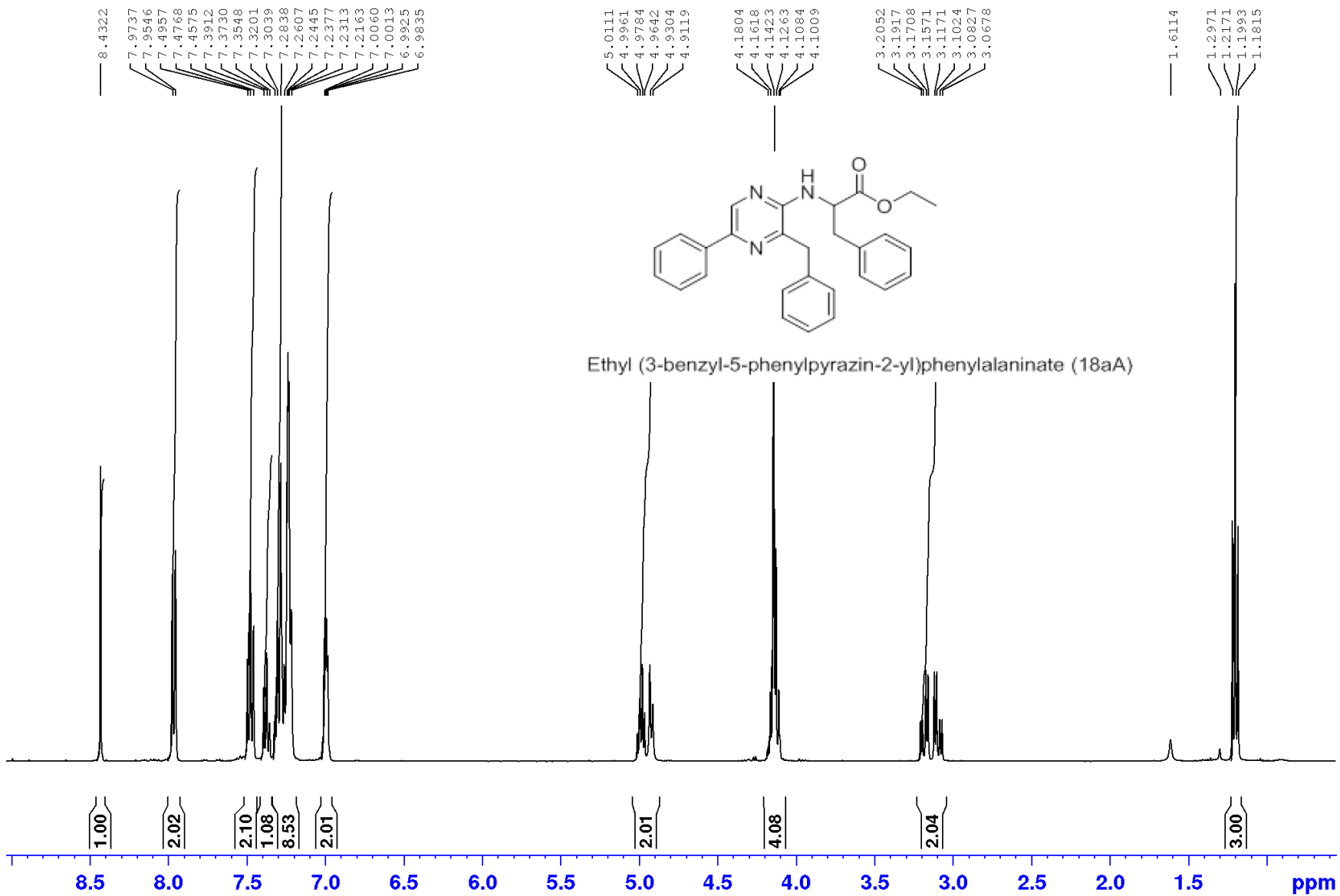
— 77.340
— 77.023
— 76.705
— 70.212

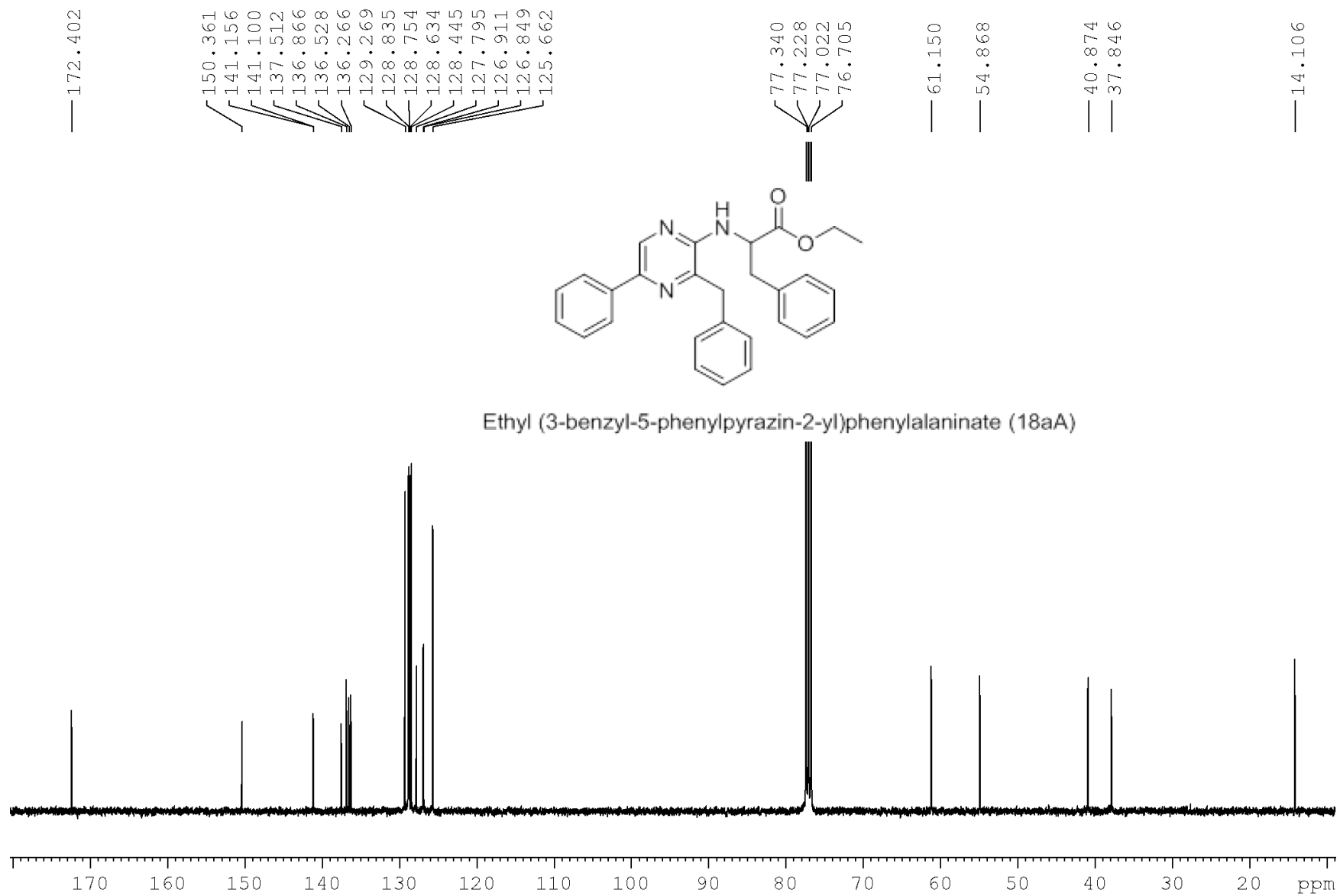
— 41.205

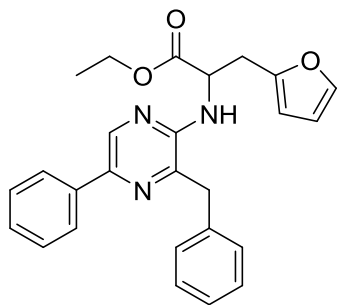


3-Benzyl-5-(3-(benzyloxy)phenyl)-2-chloropyrazine (9c)

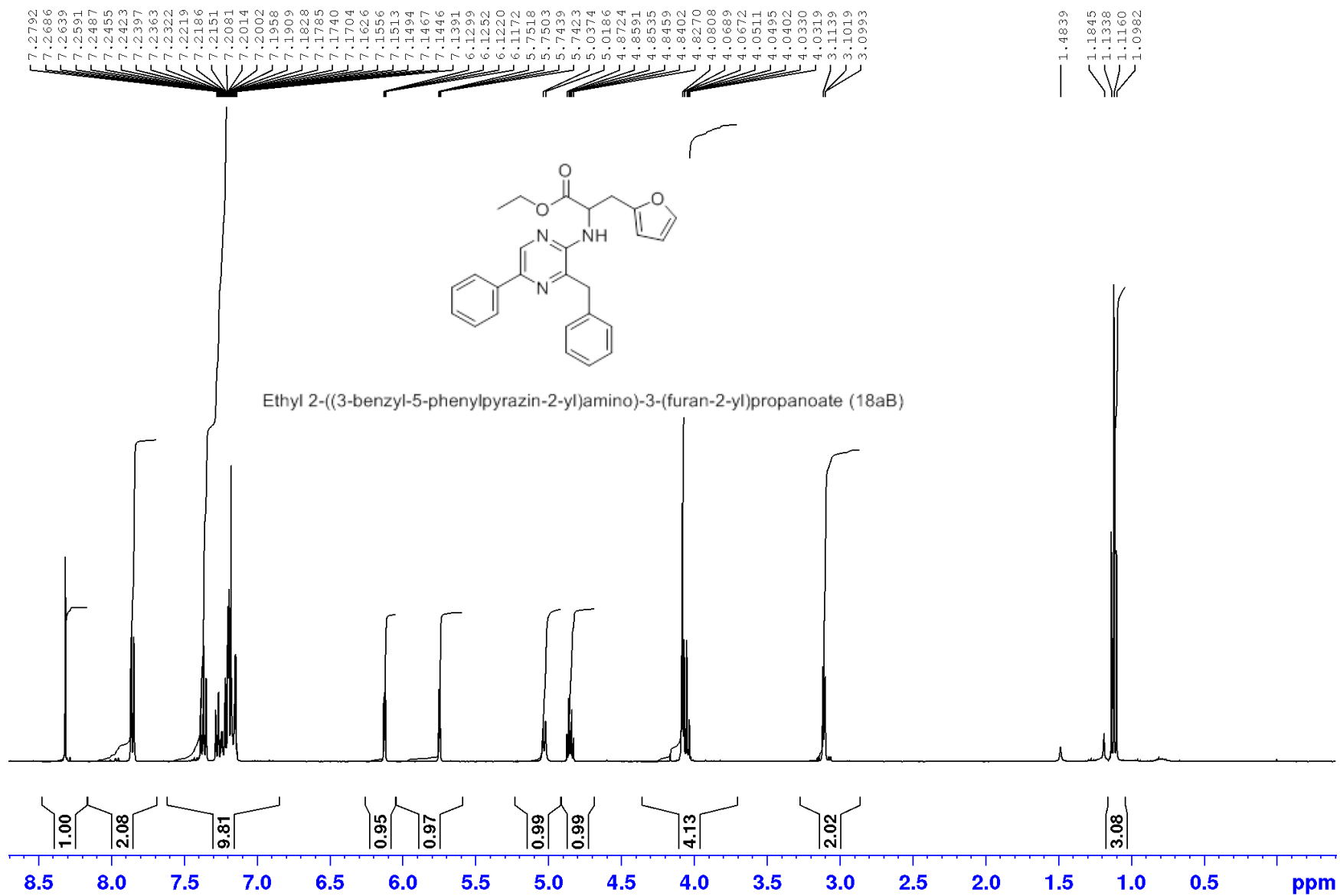


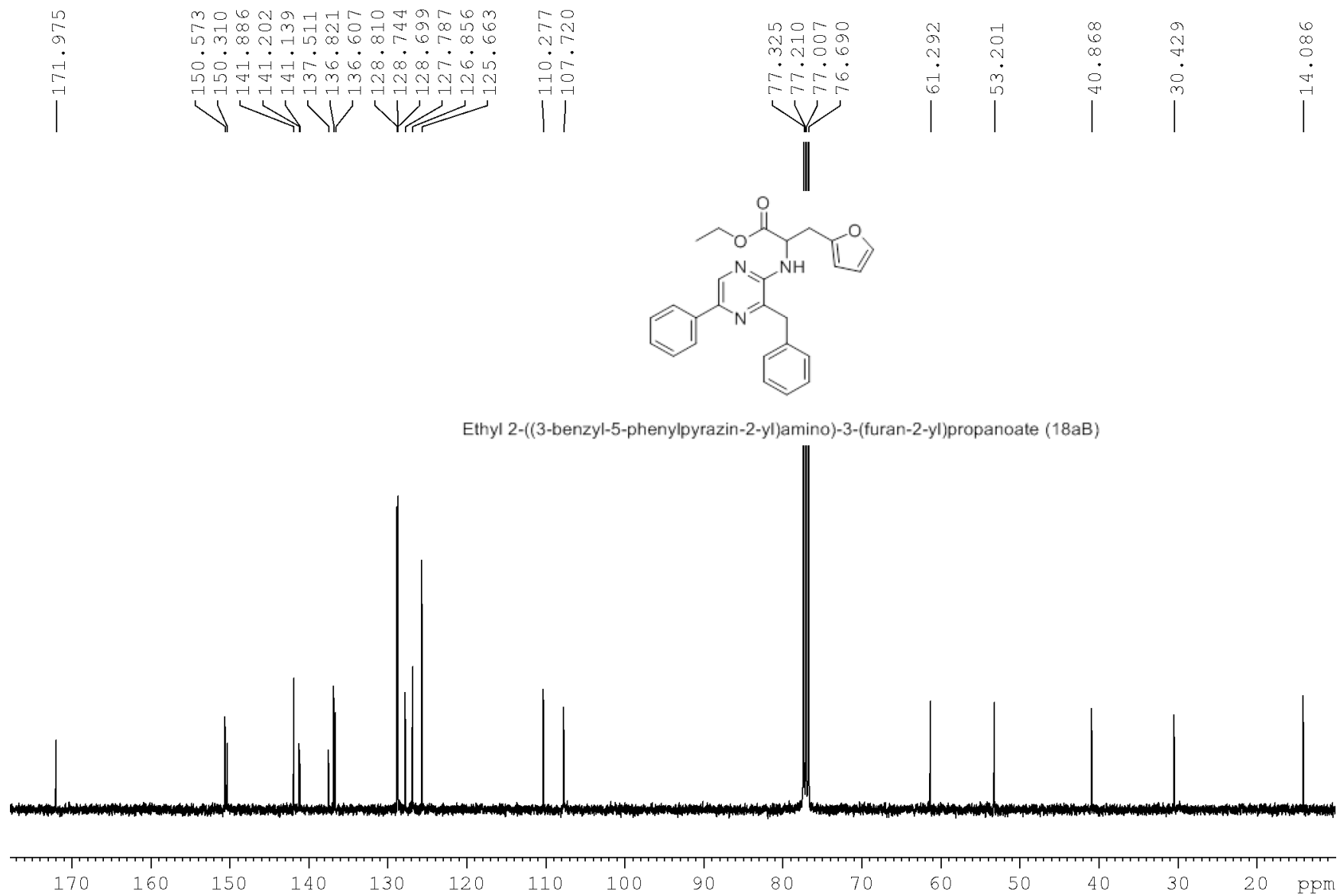


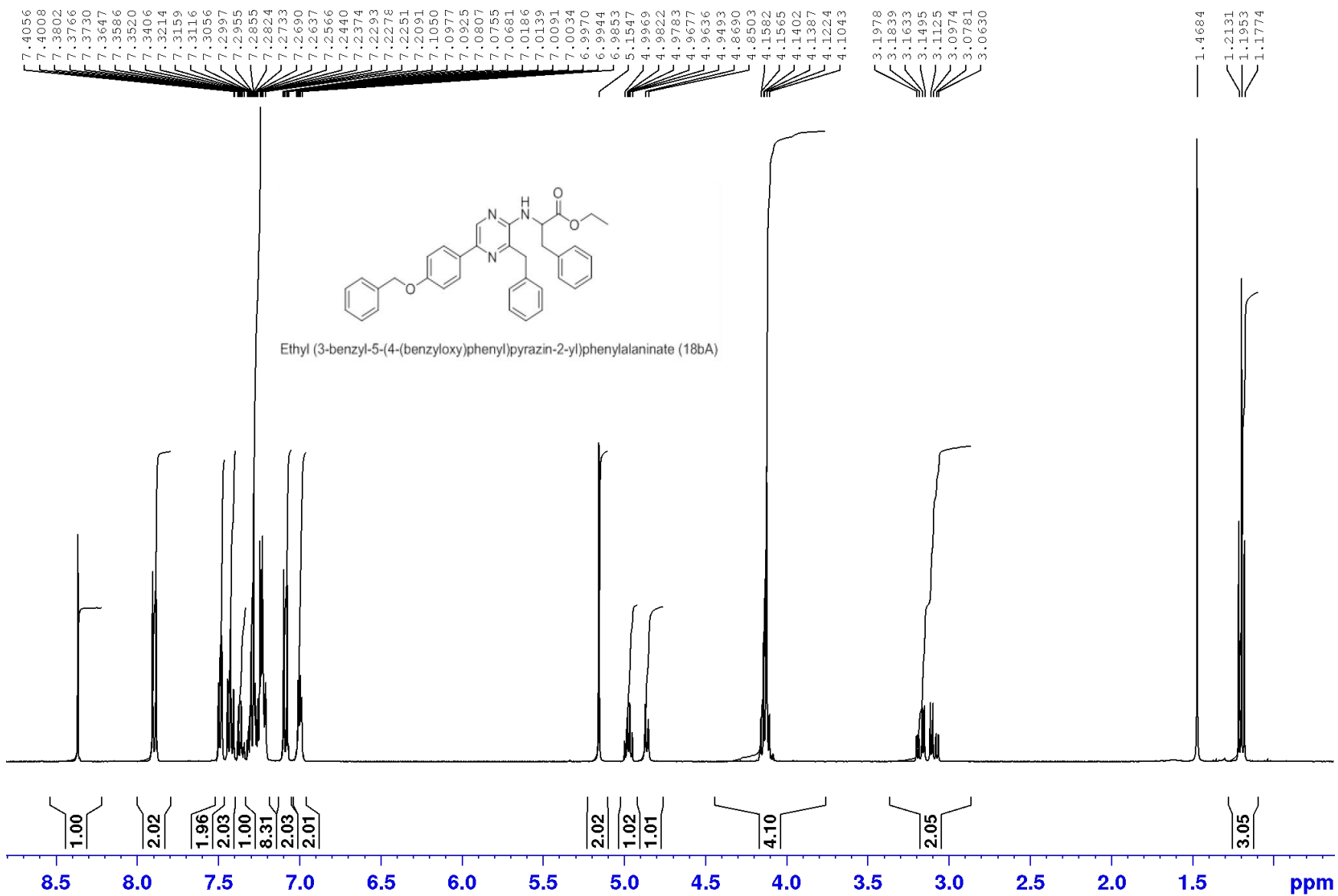


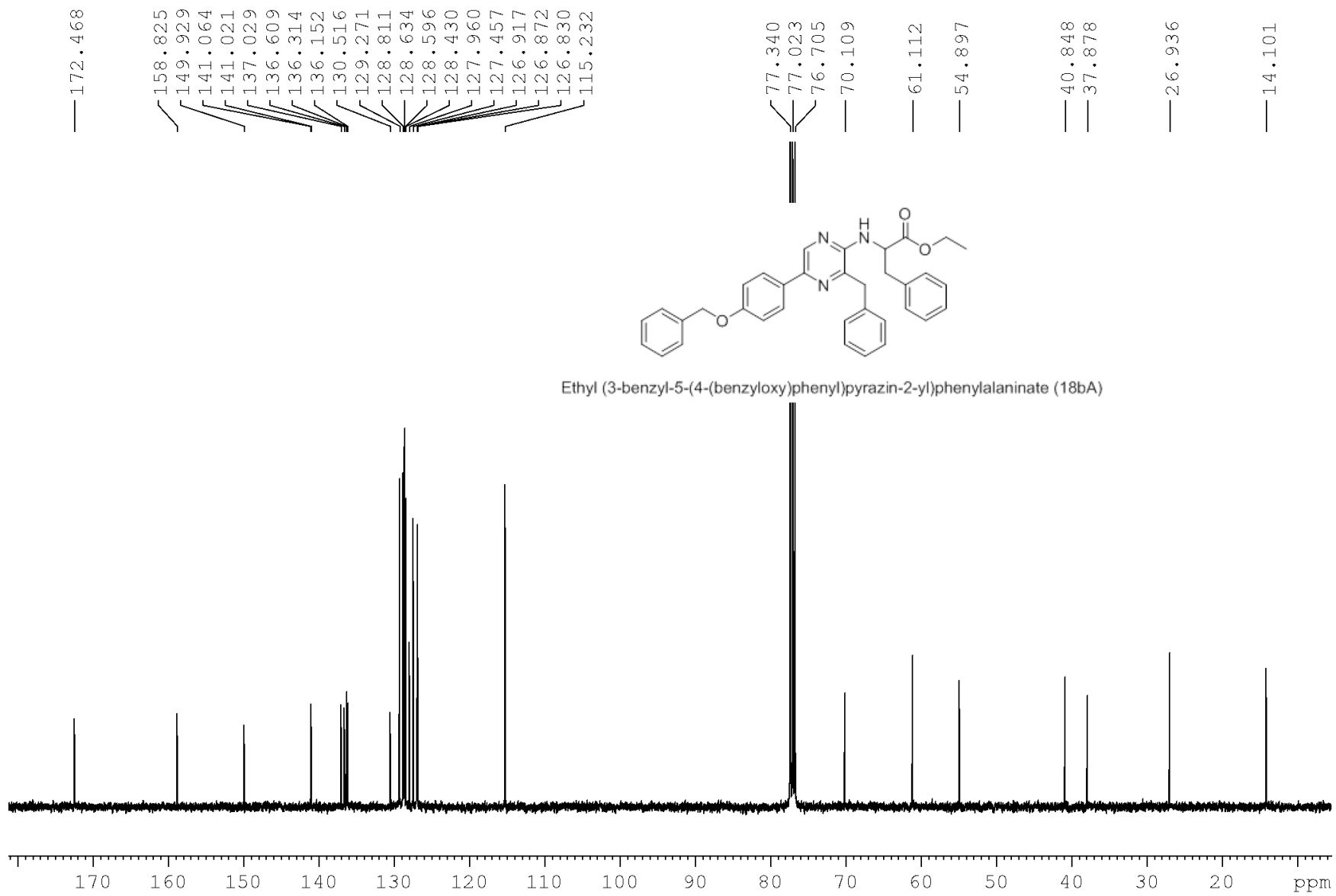


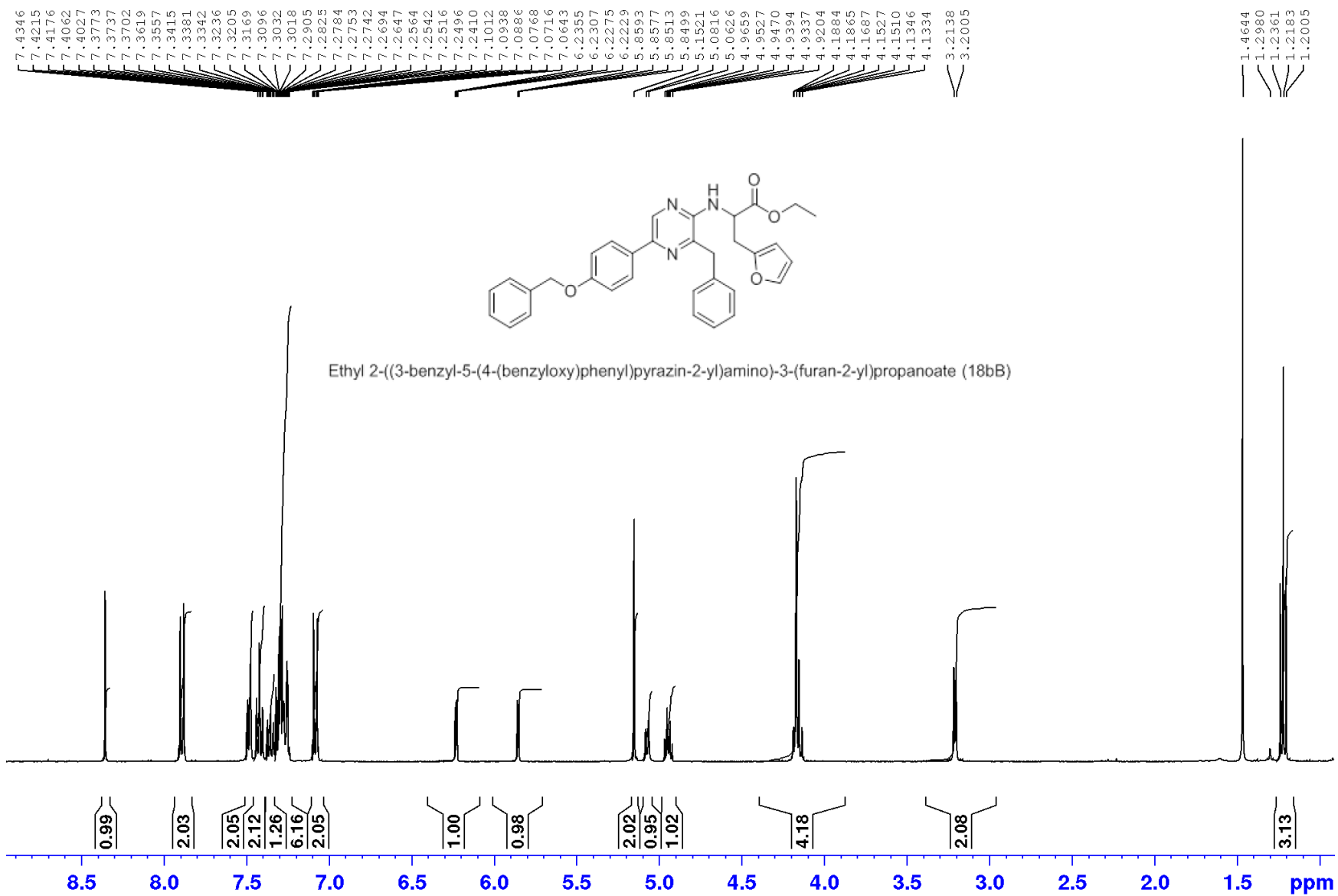
Ethyl 2-((3-benzyl-5-phenylpyrazin-2-yl)amino)-3-(furan-2-yl)propanoate (18aB)

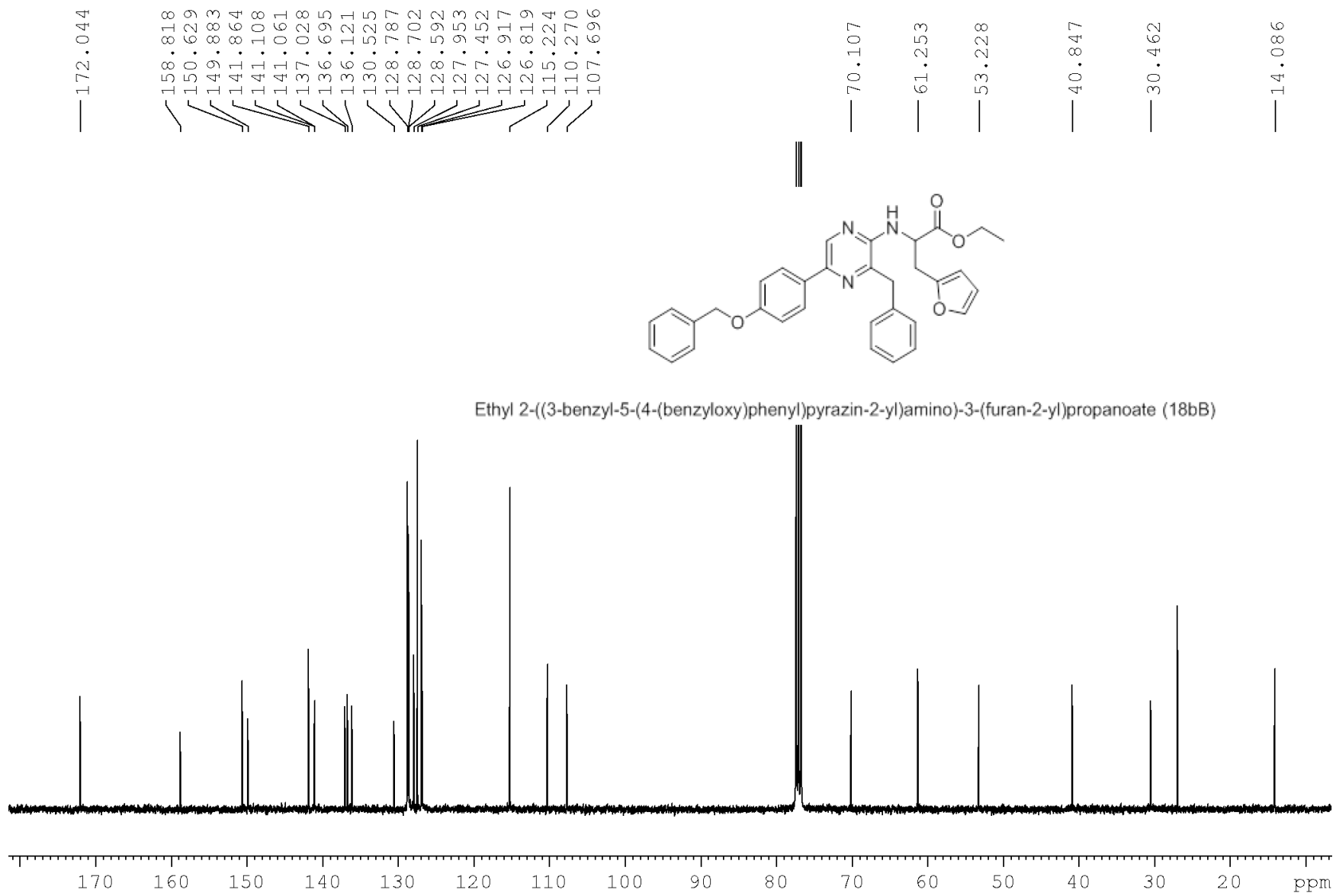


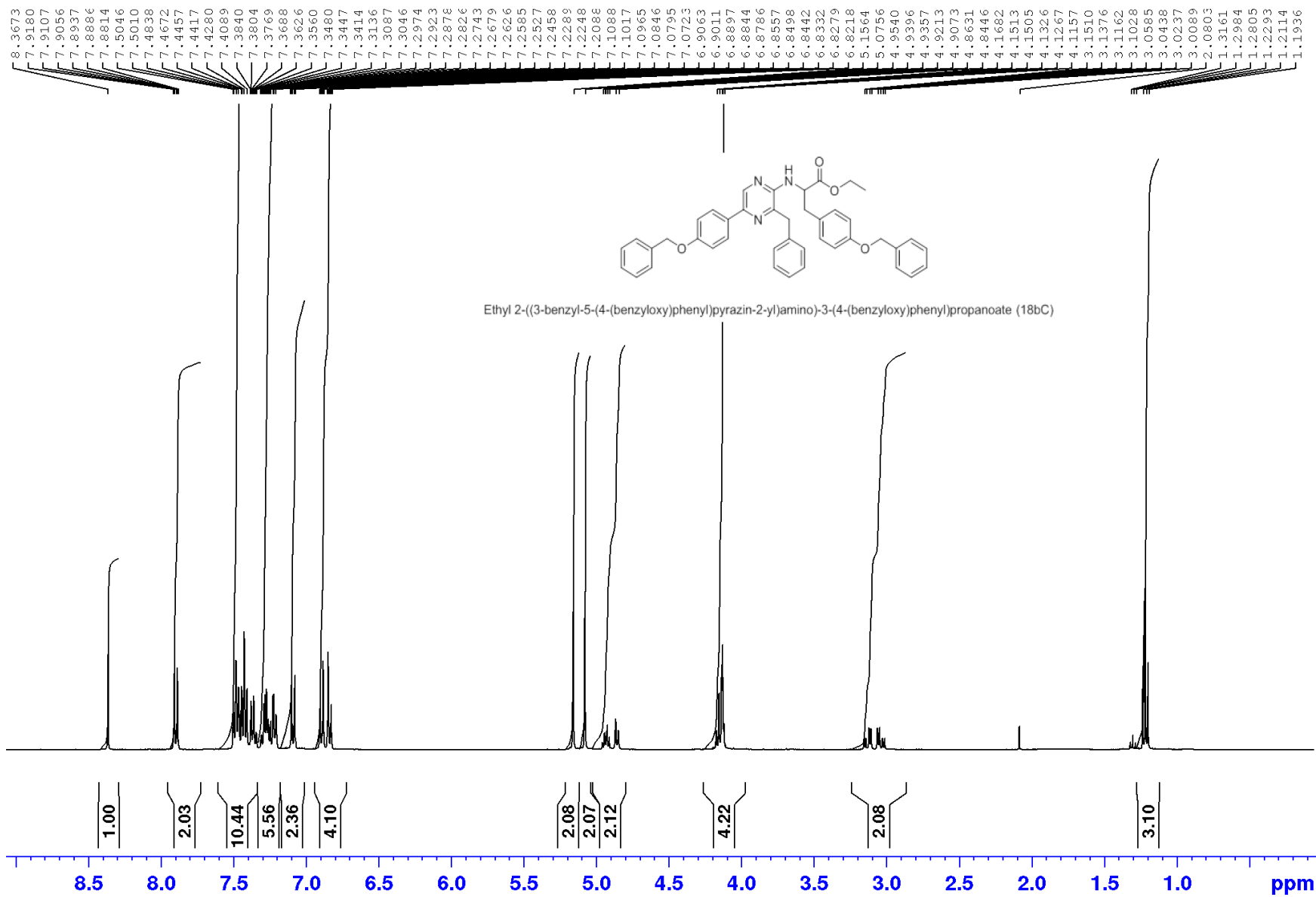


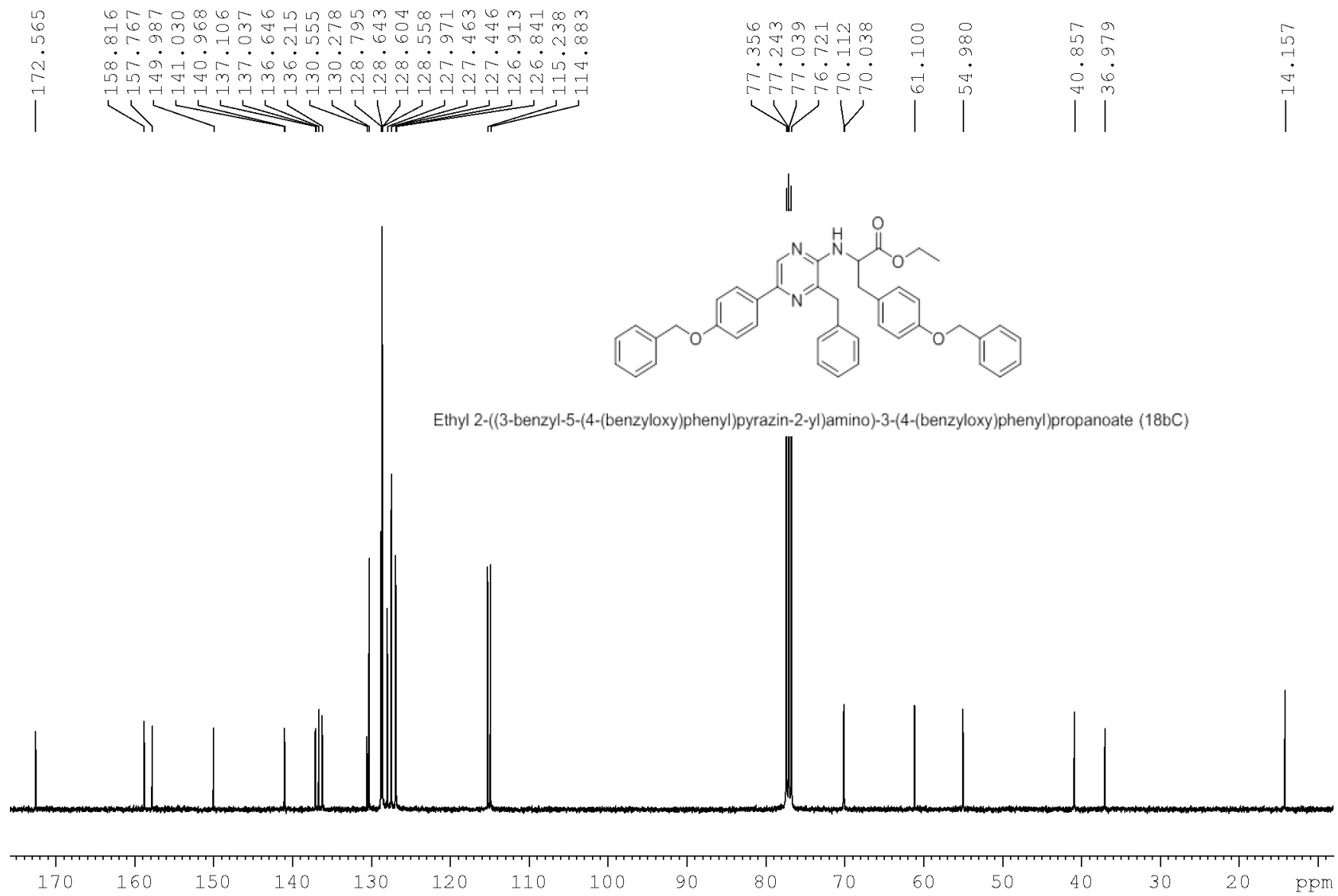


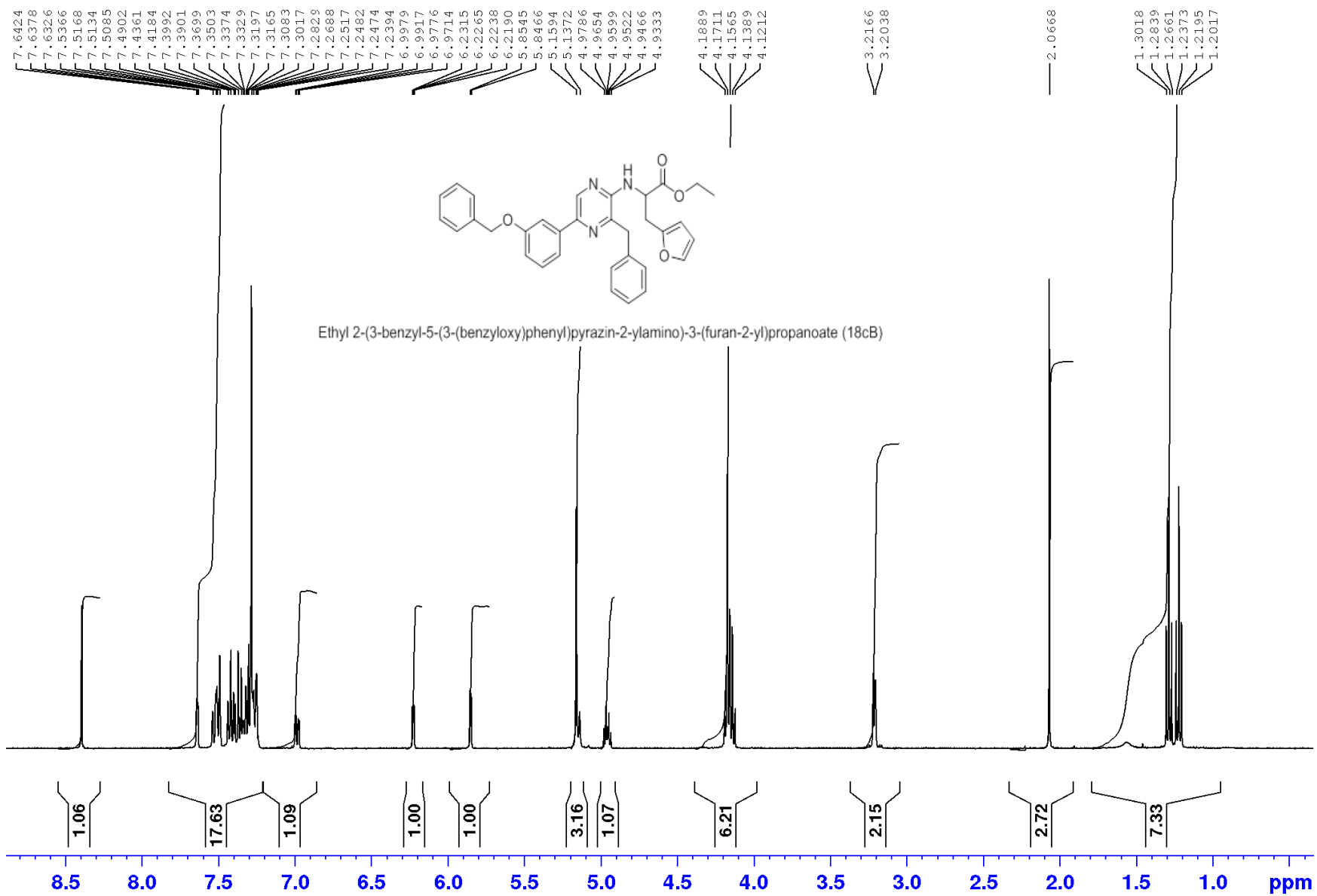


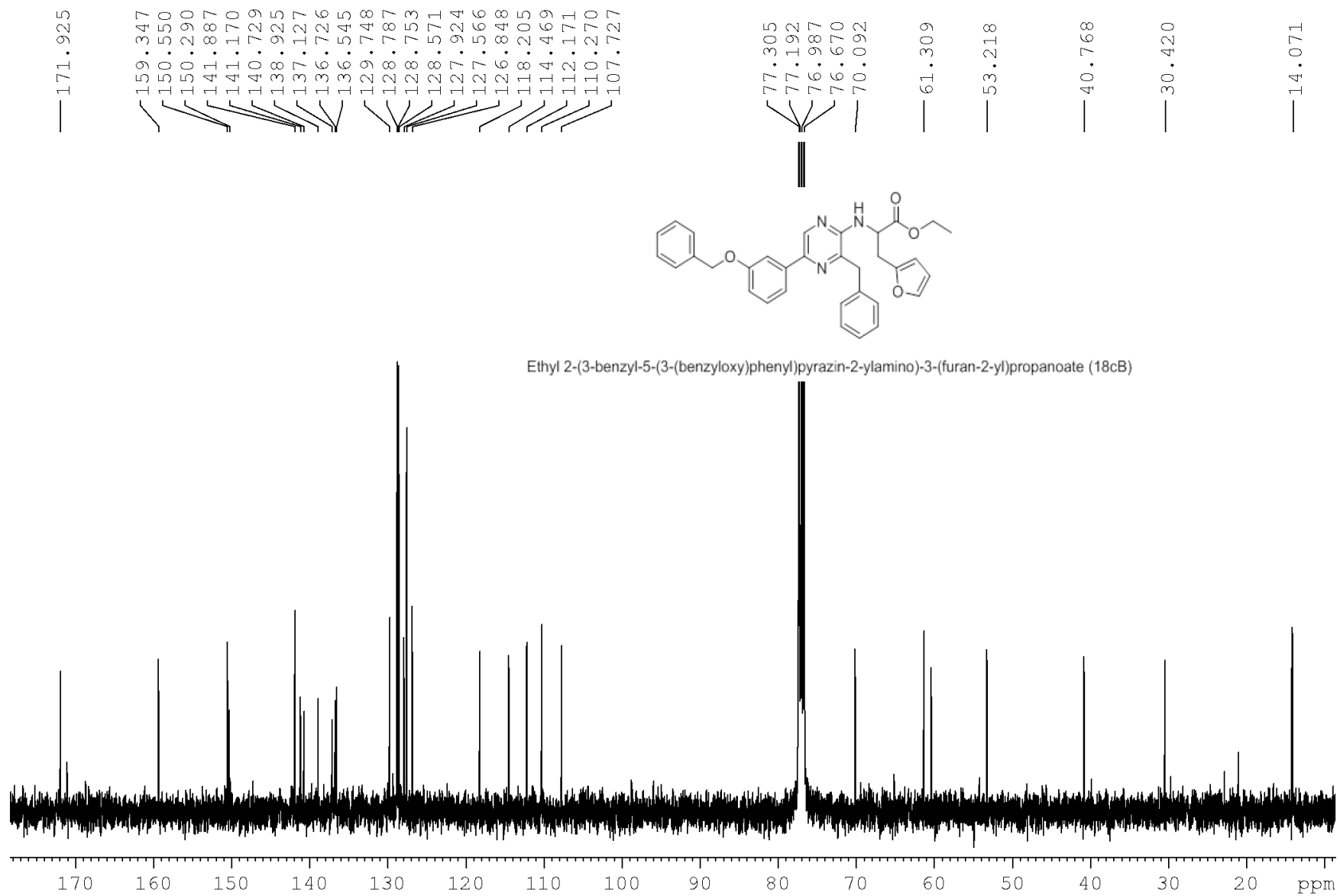


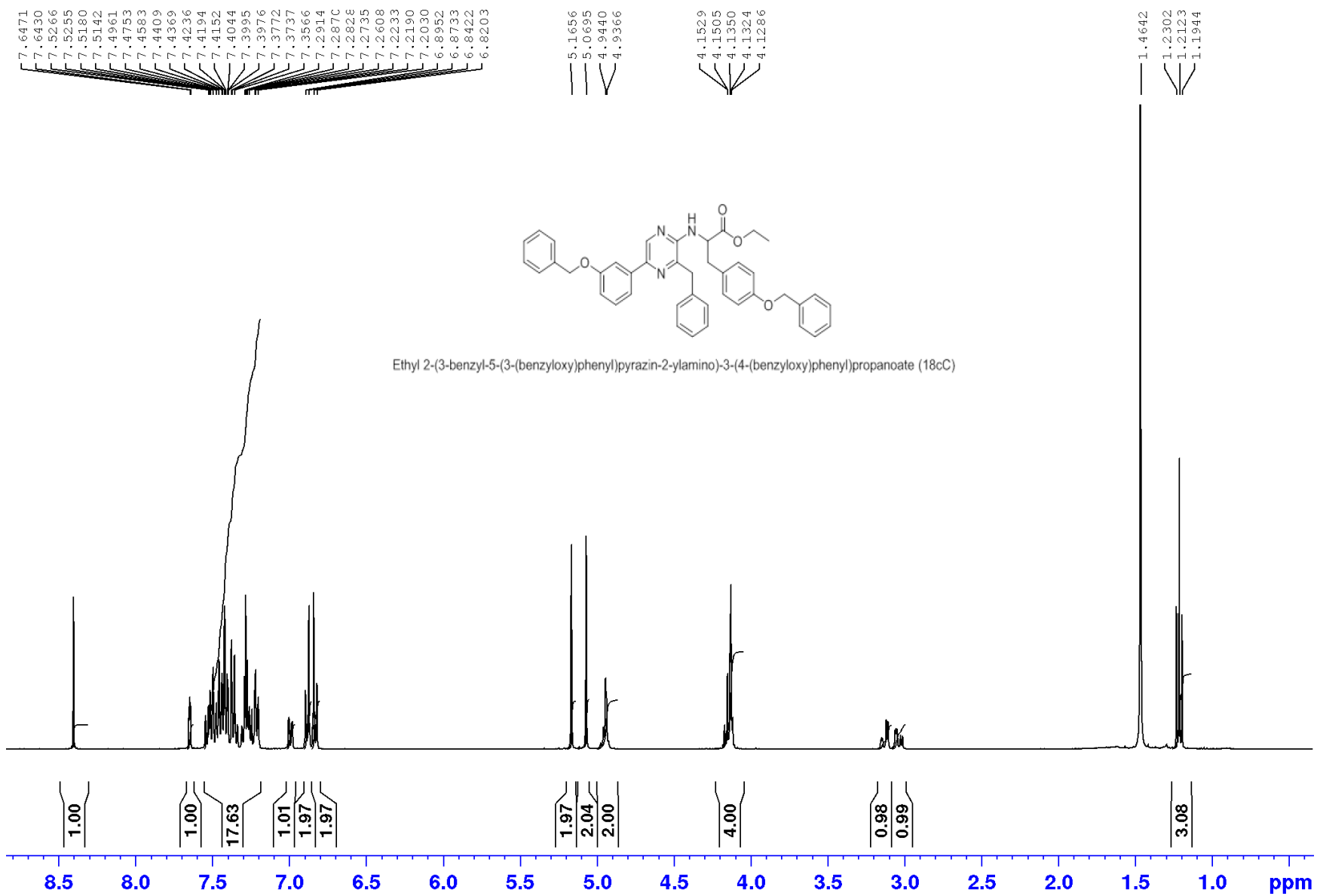


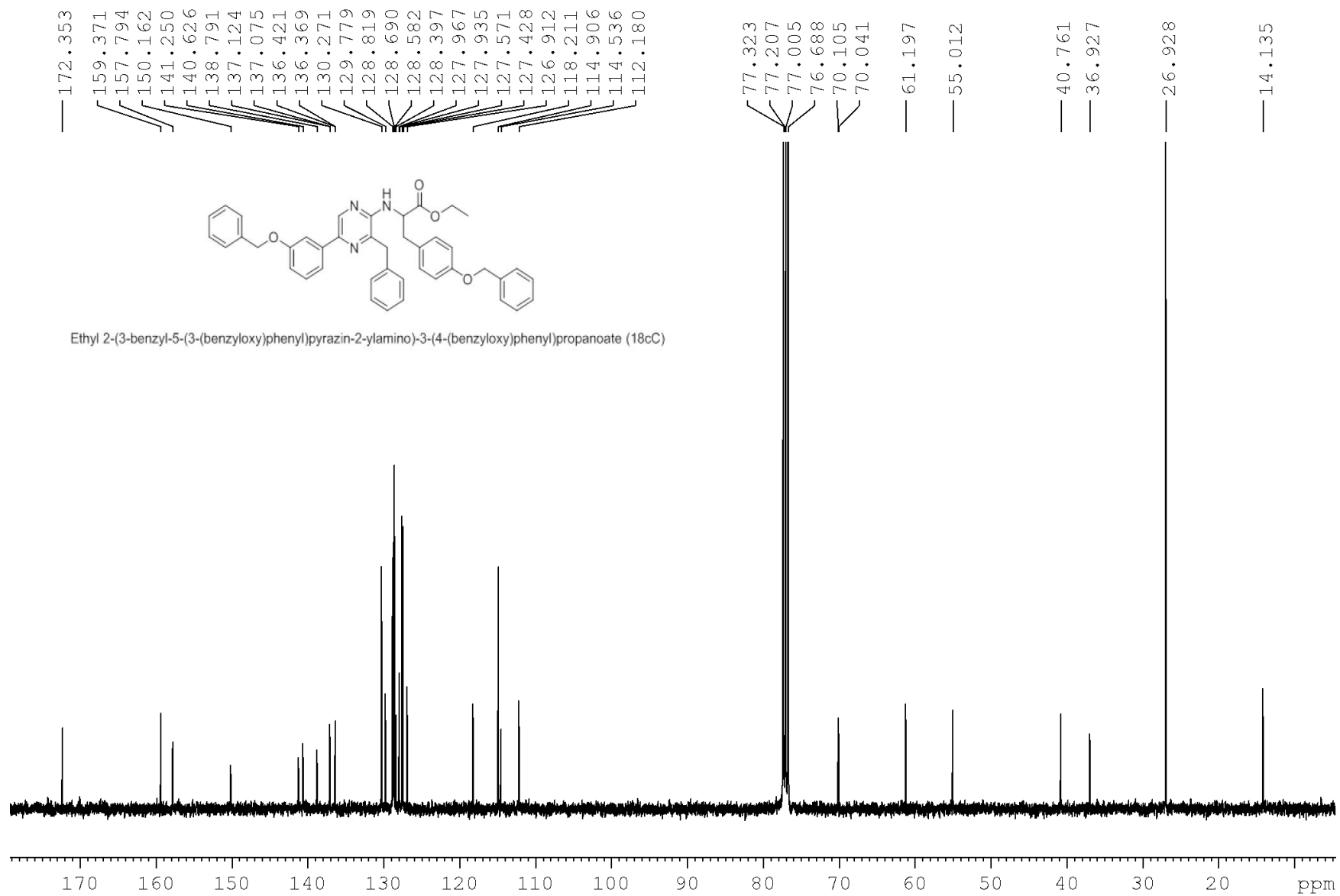


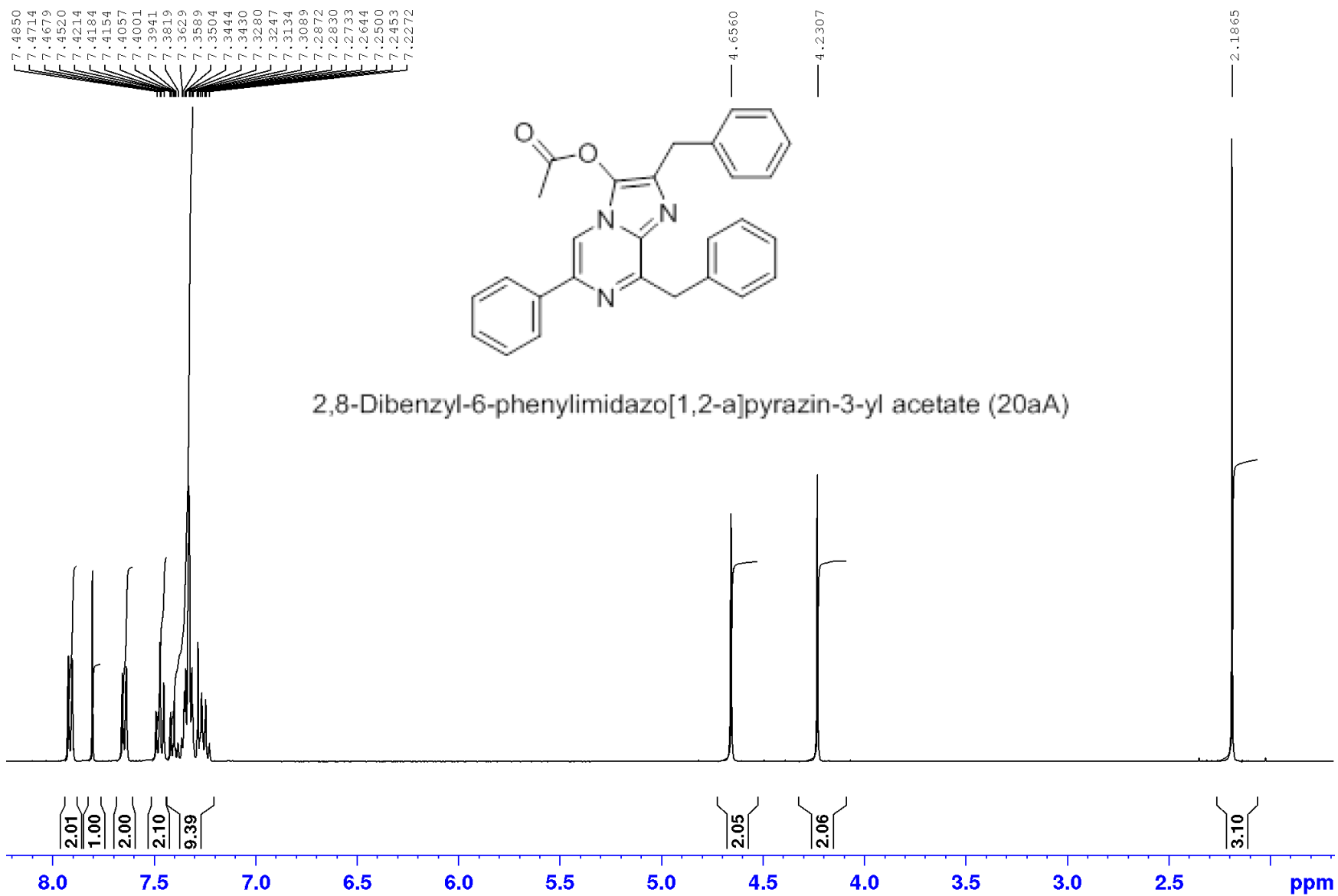


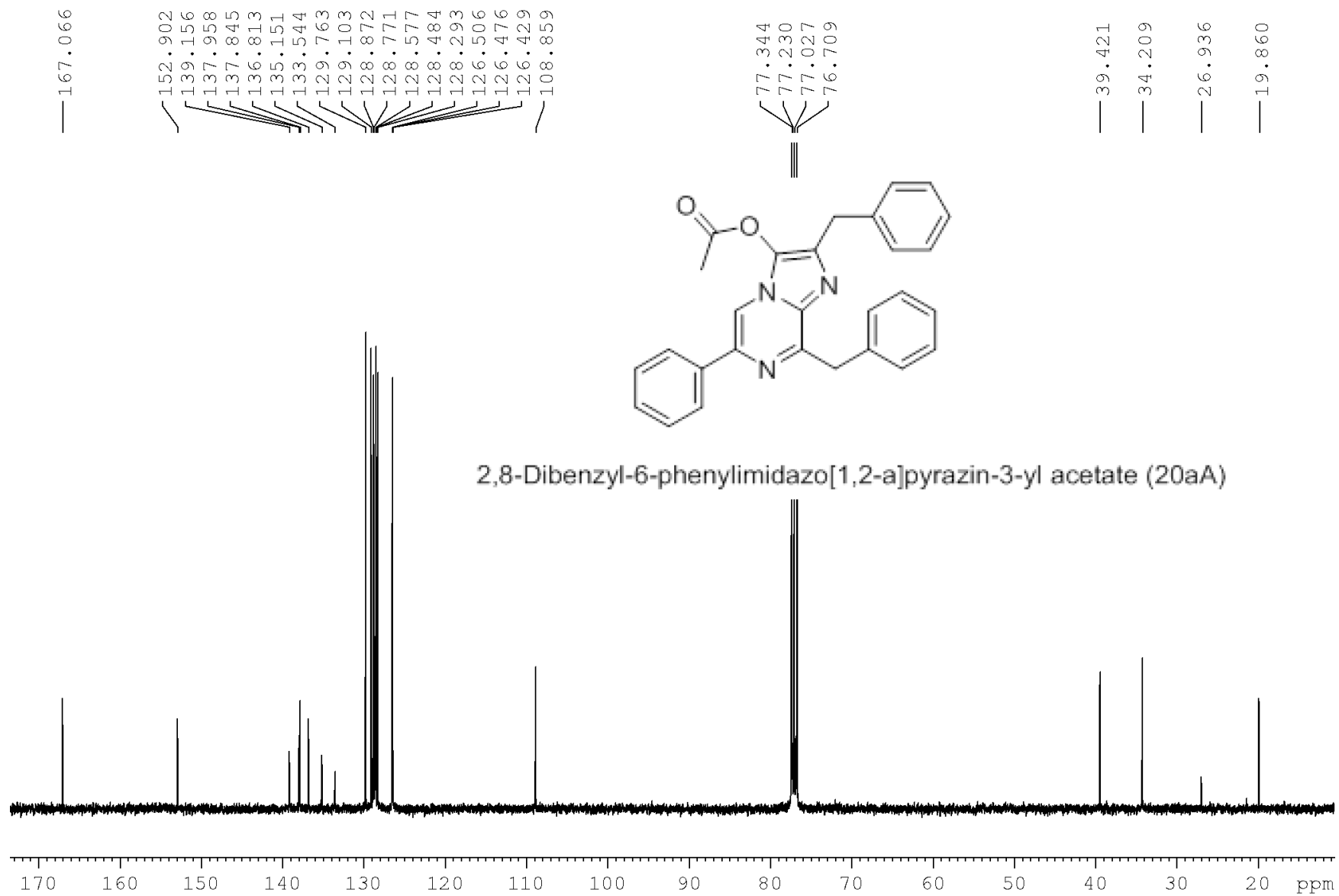


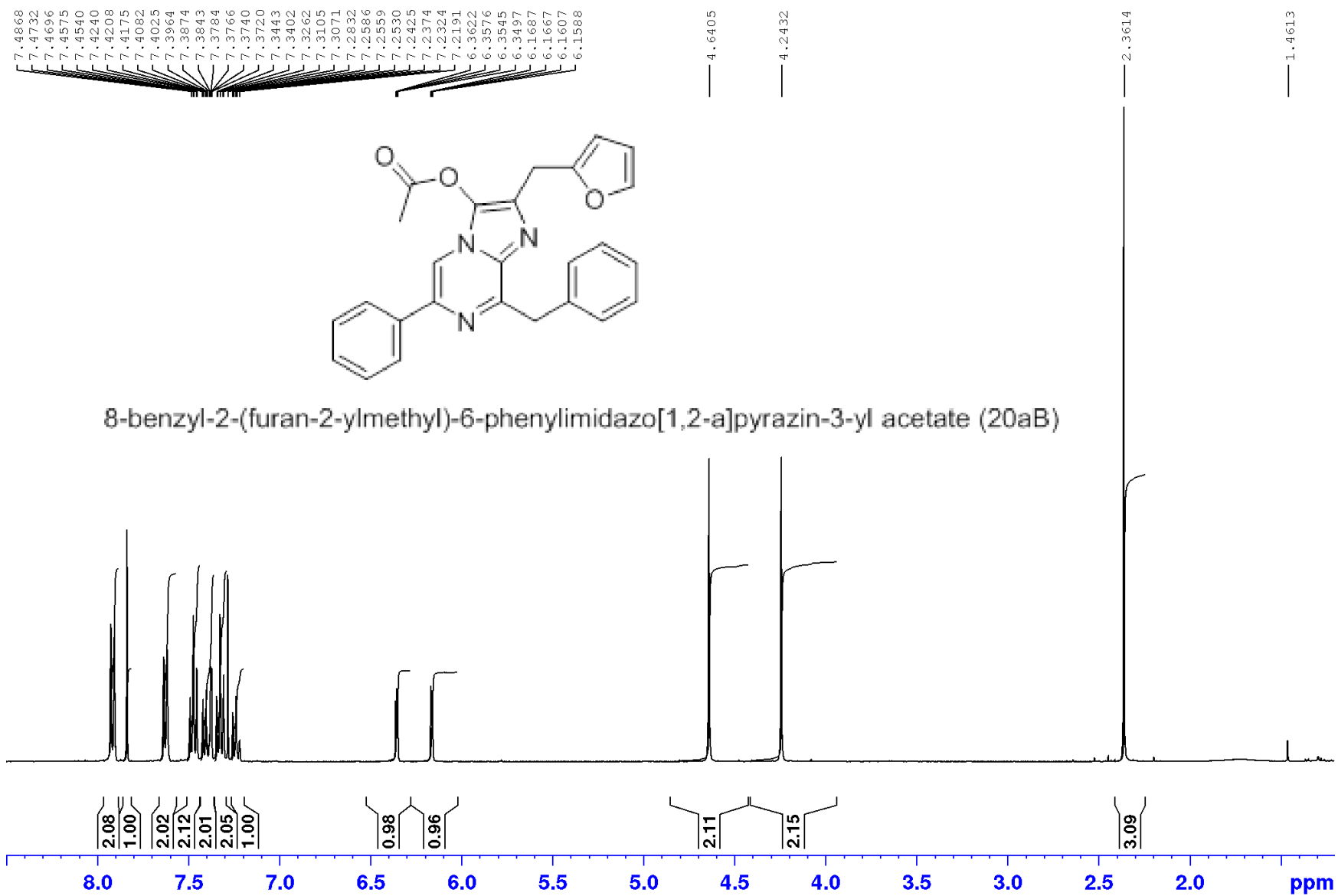


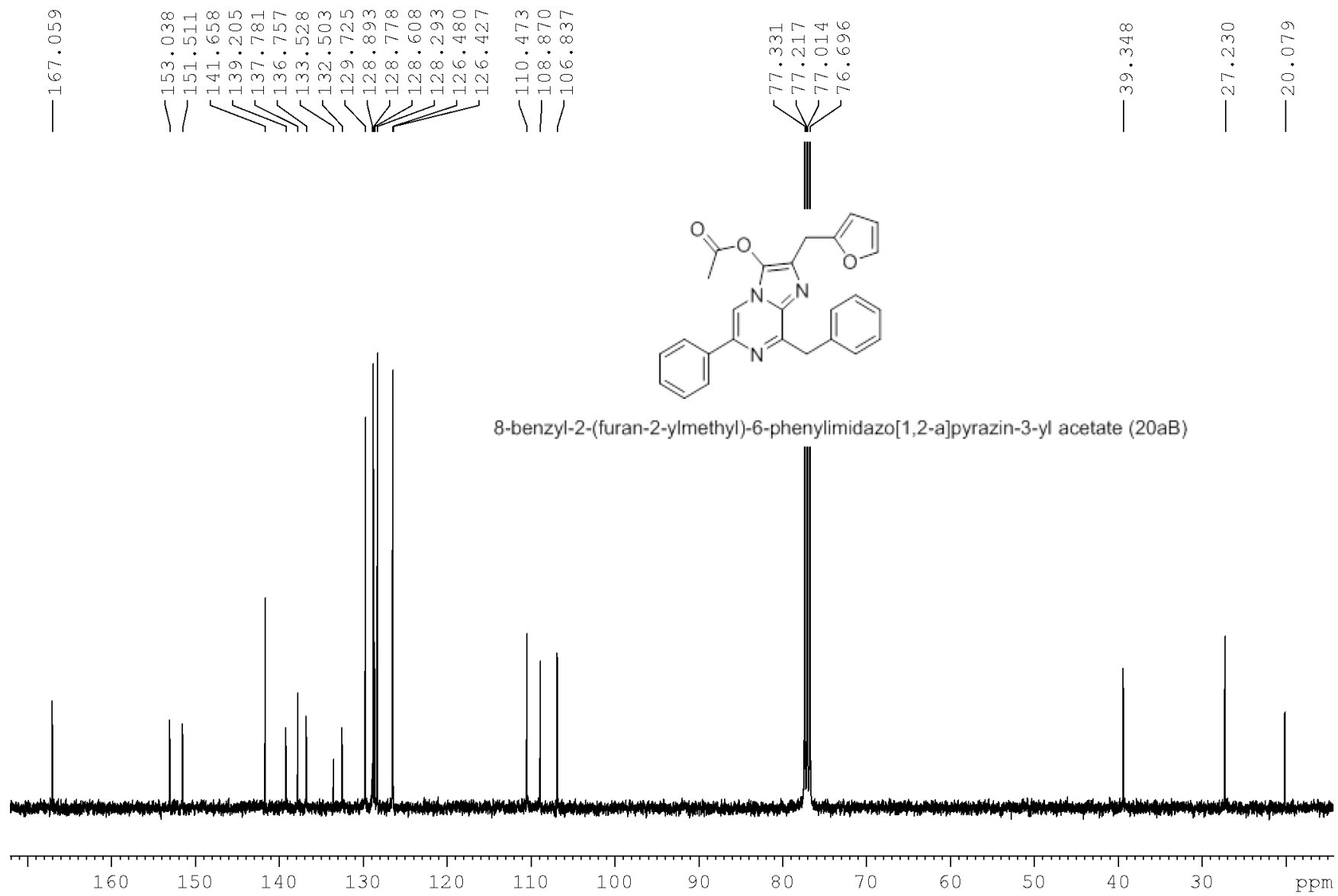


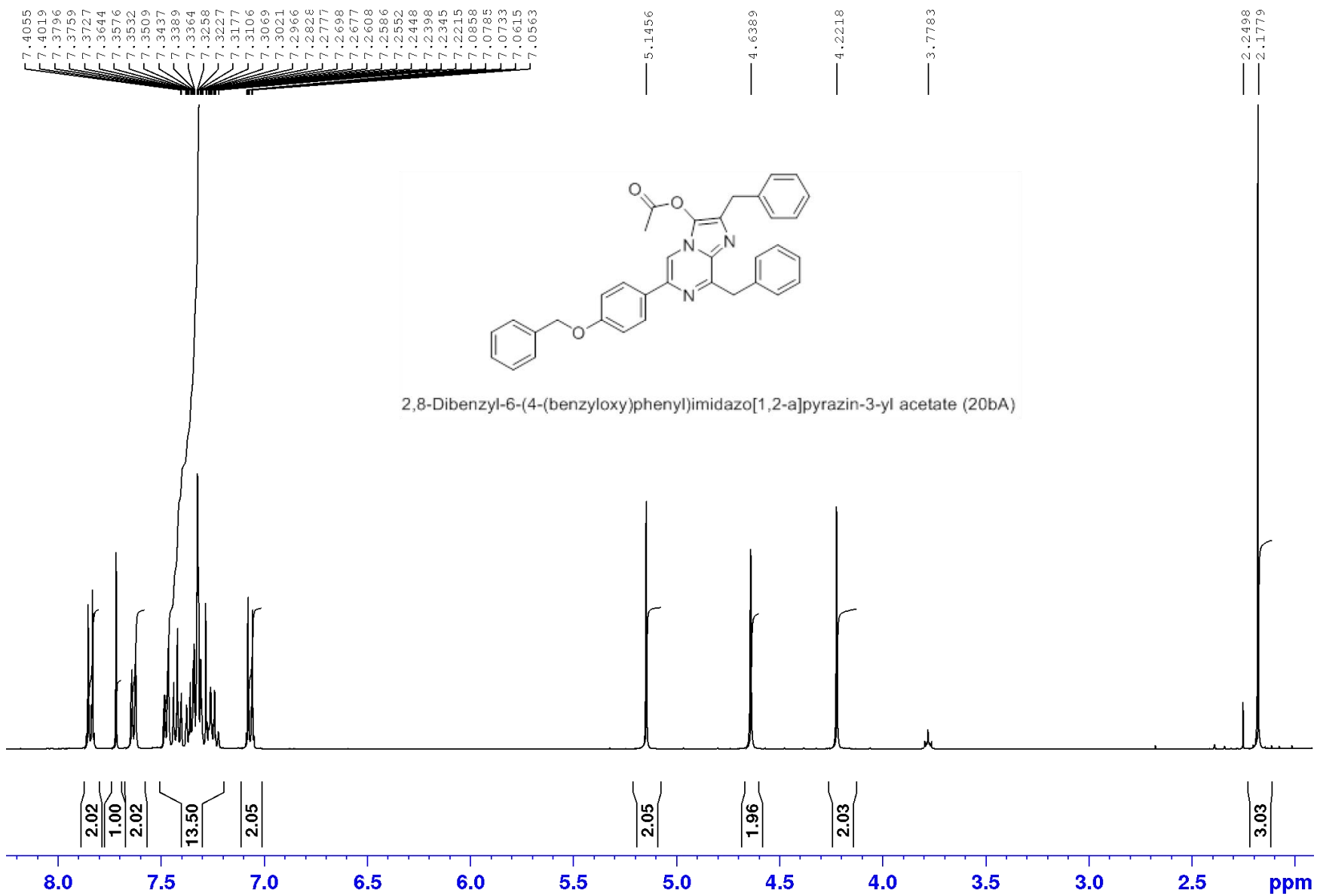


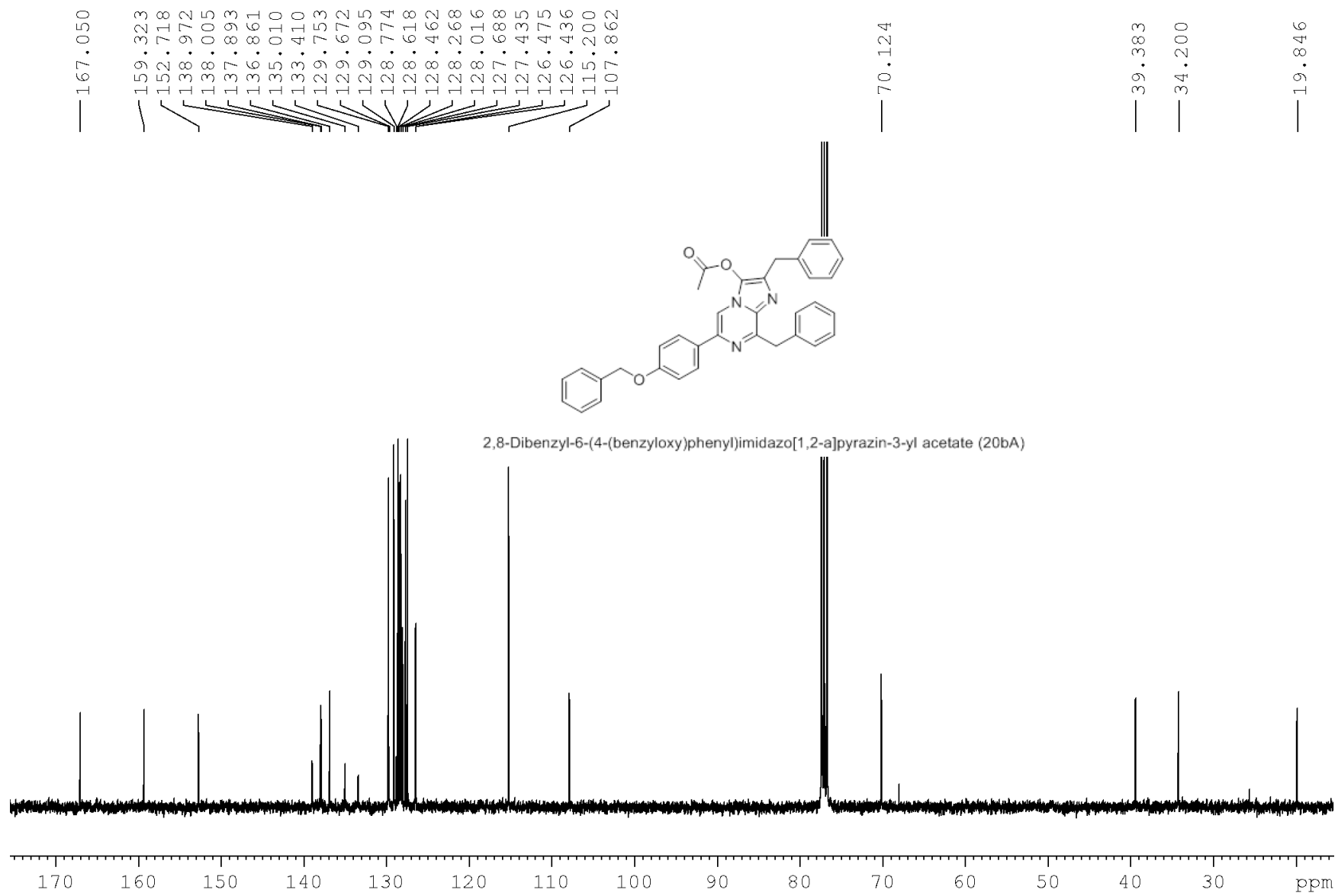


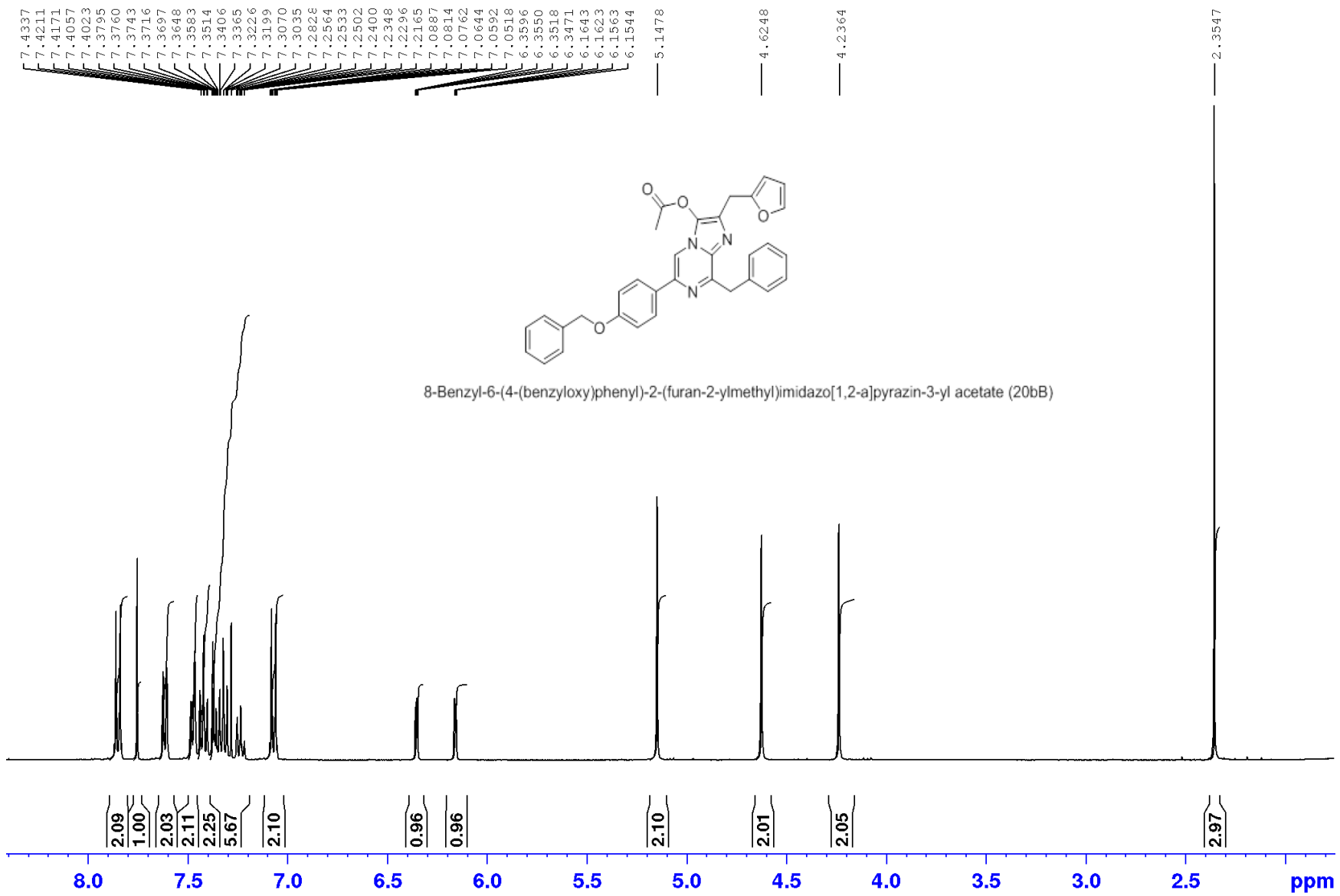


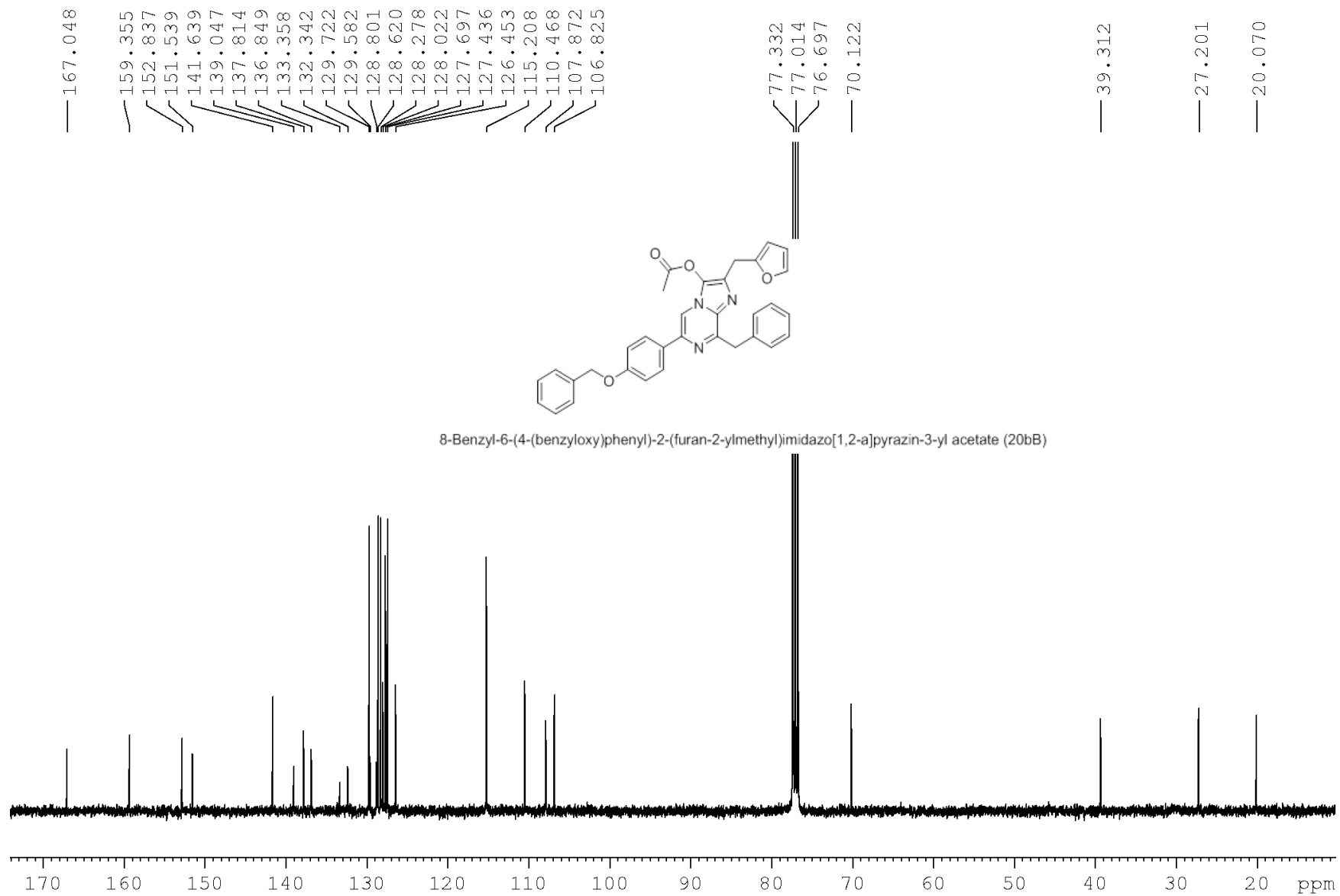


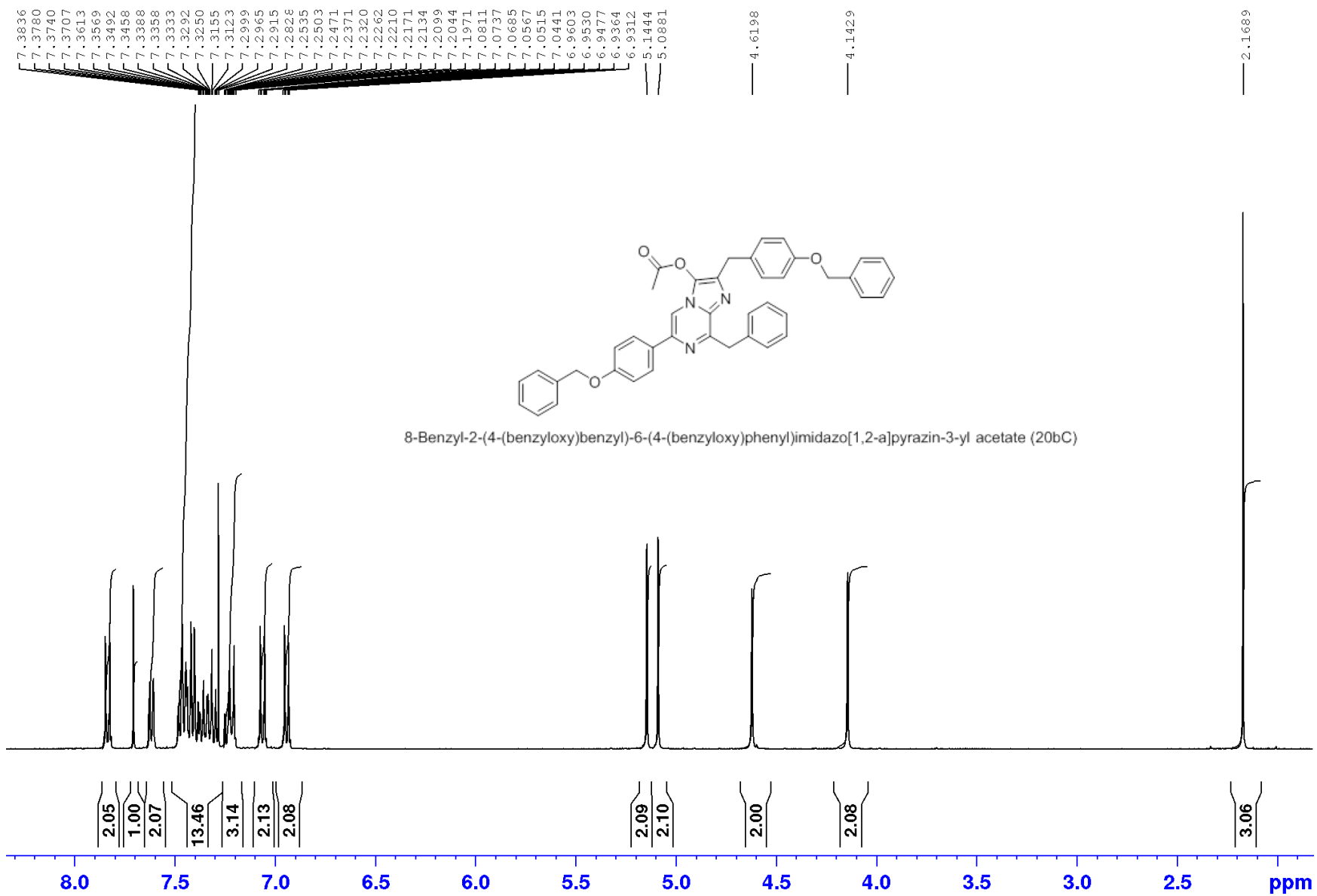


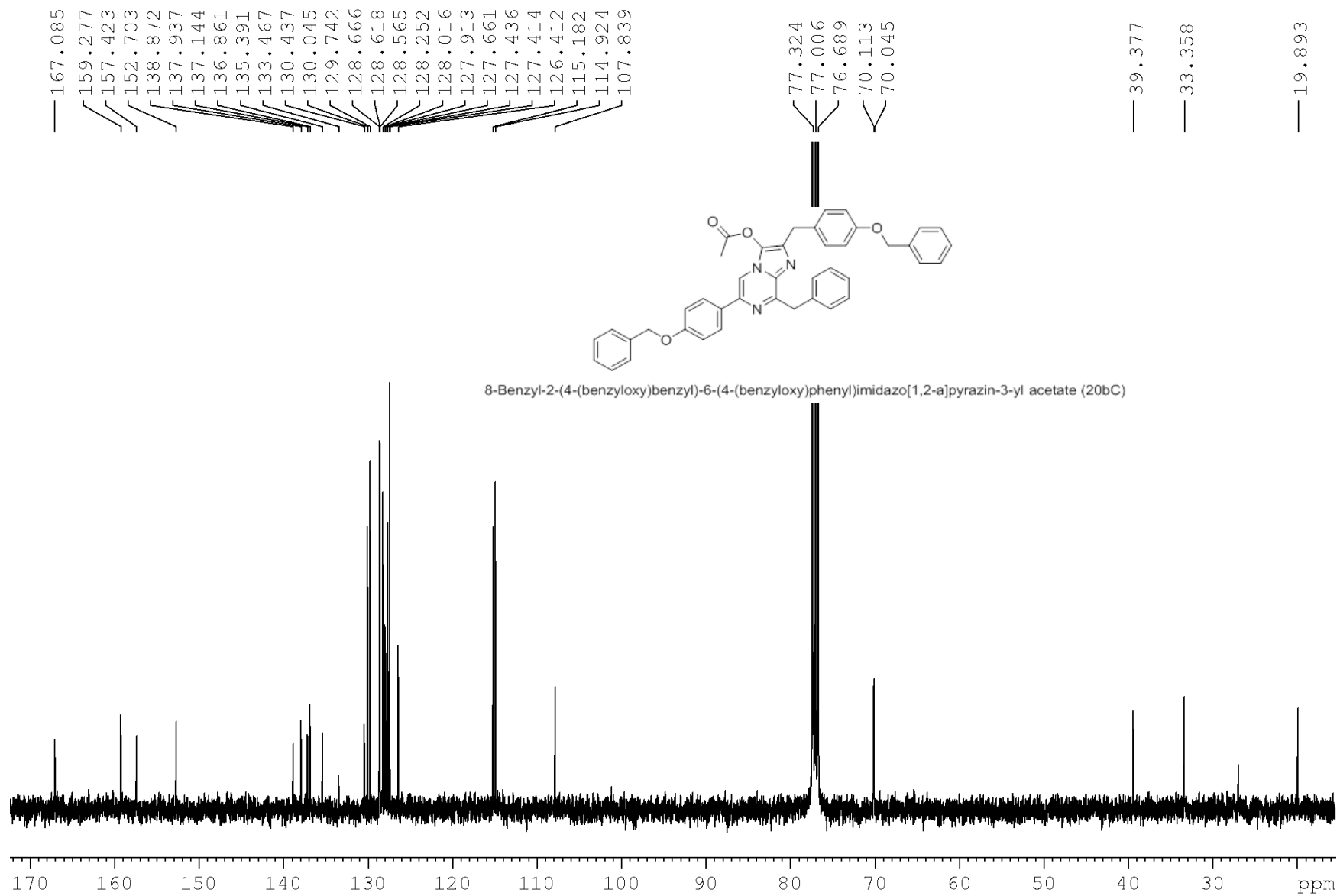


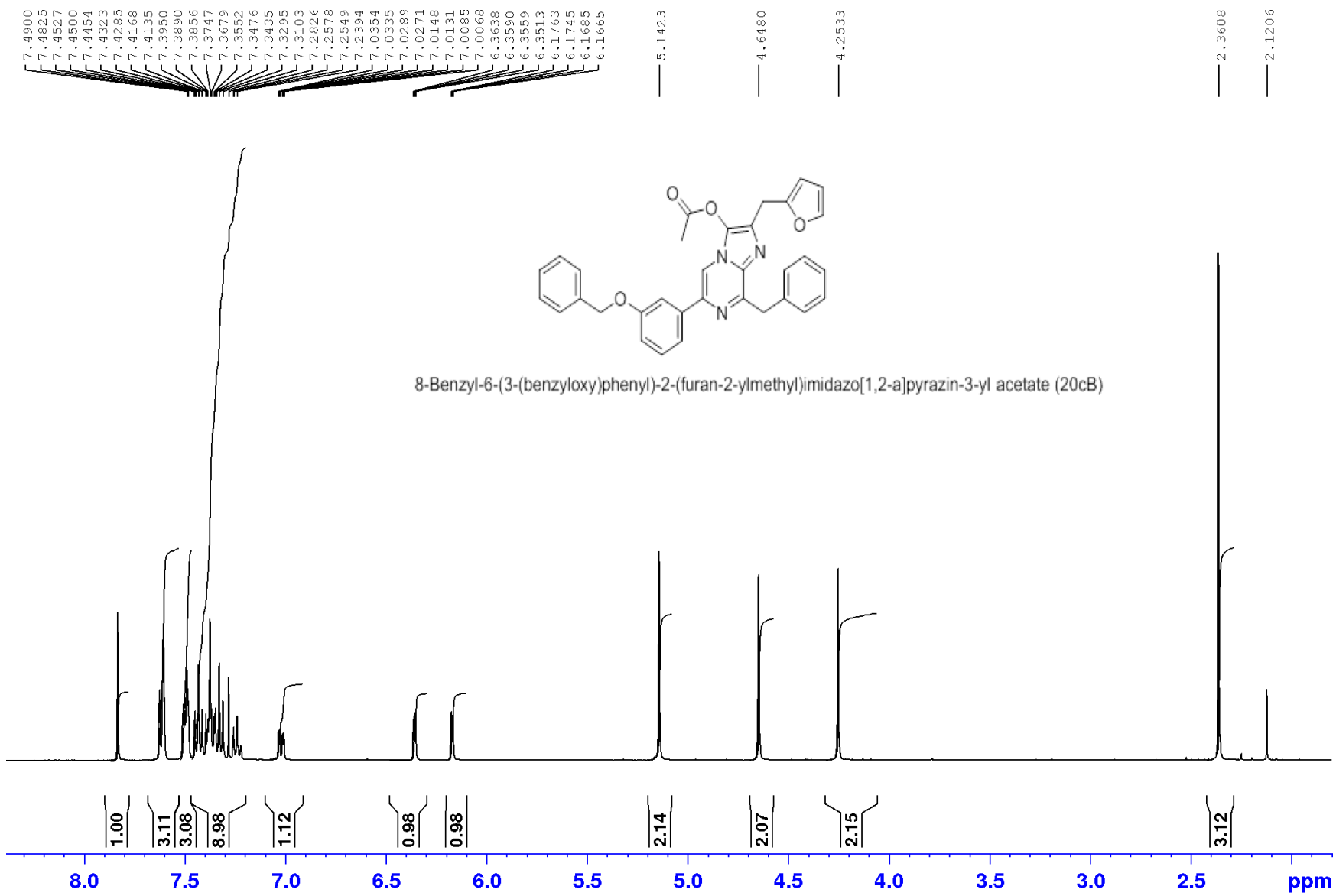


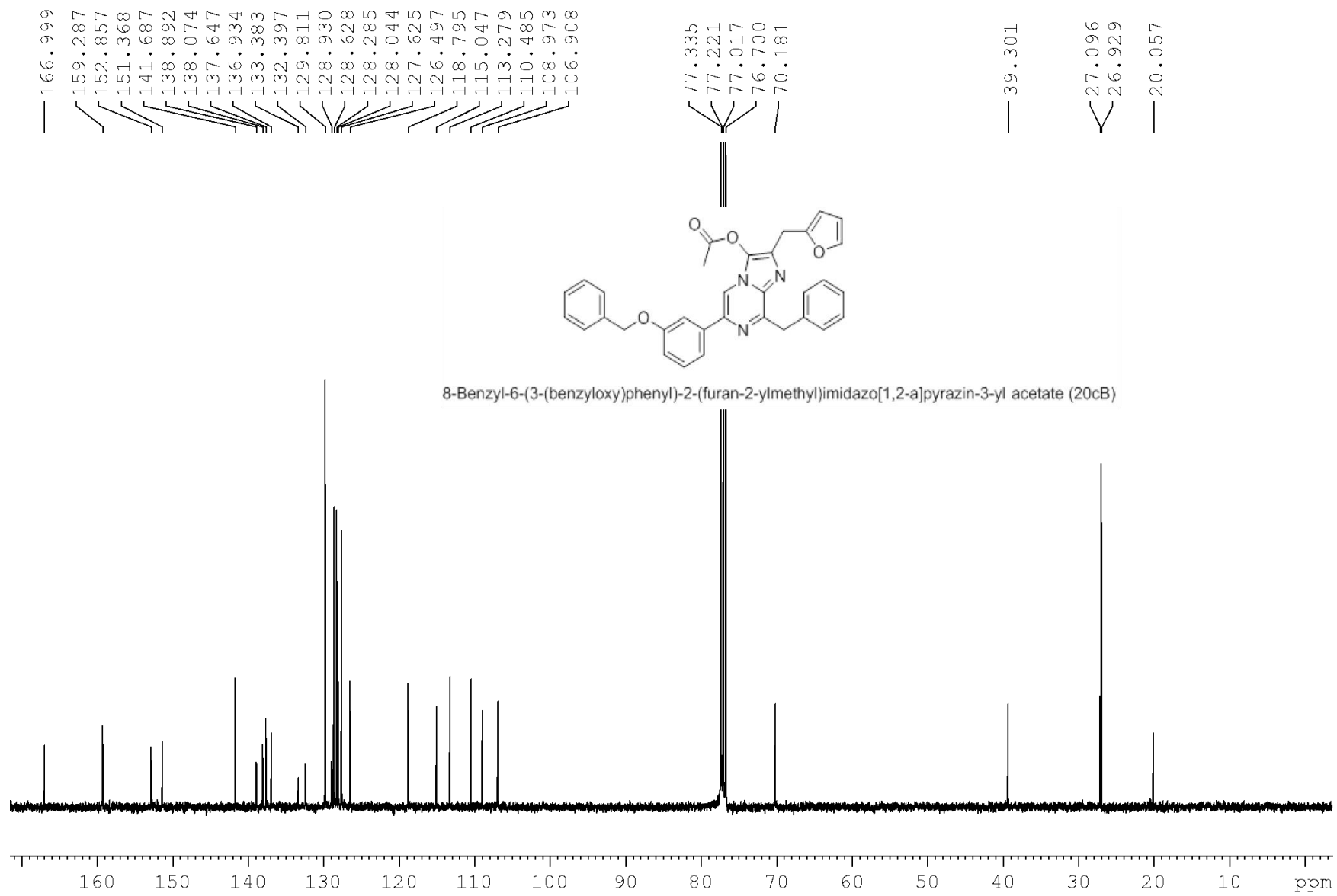


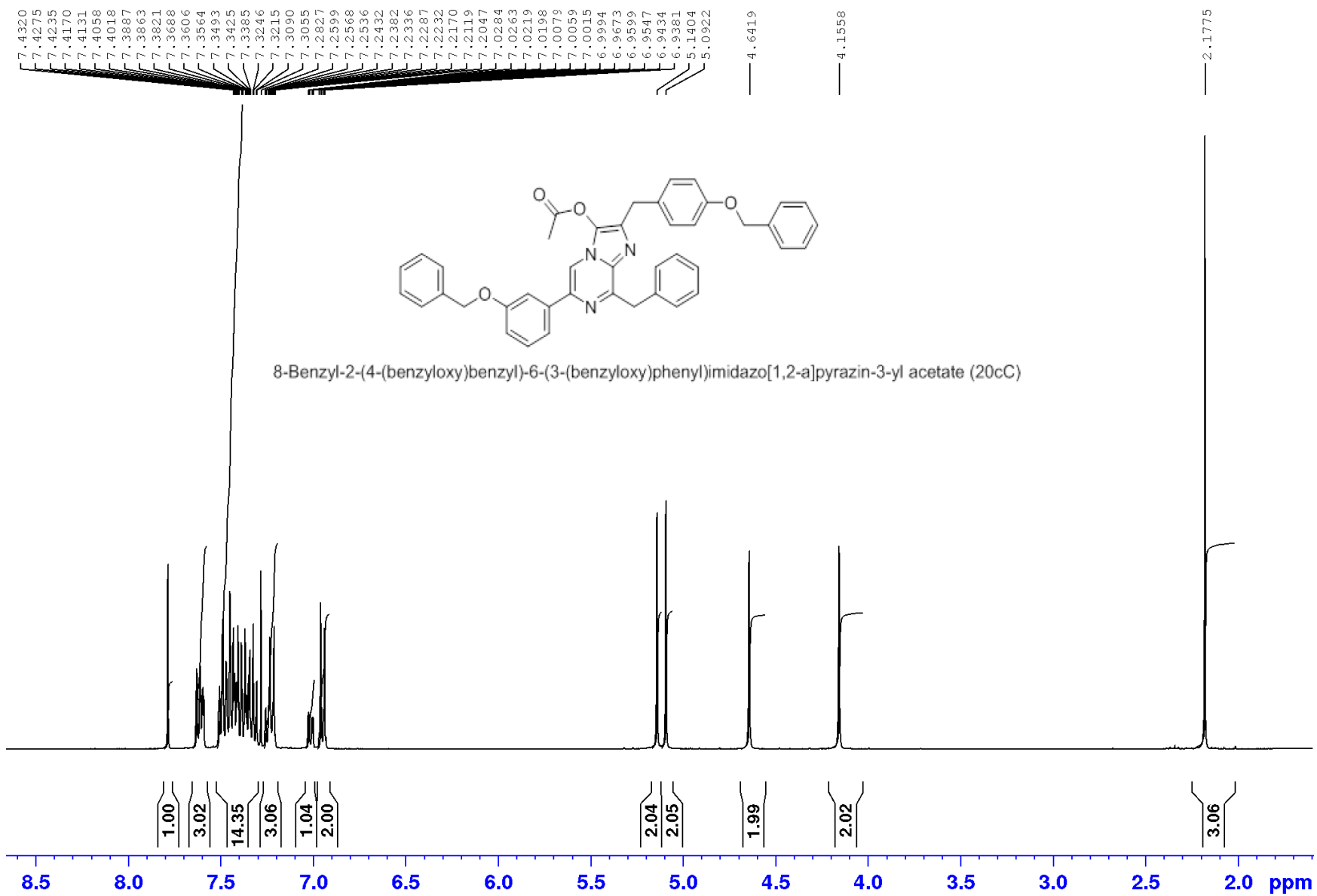


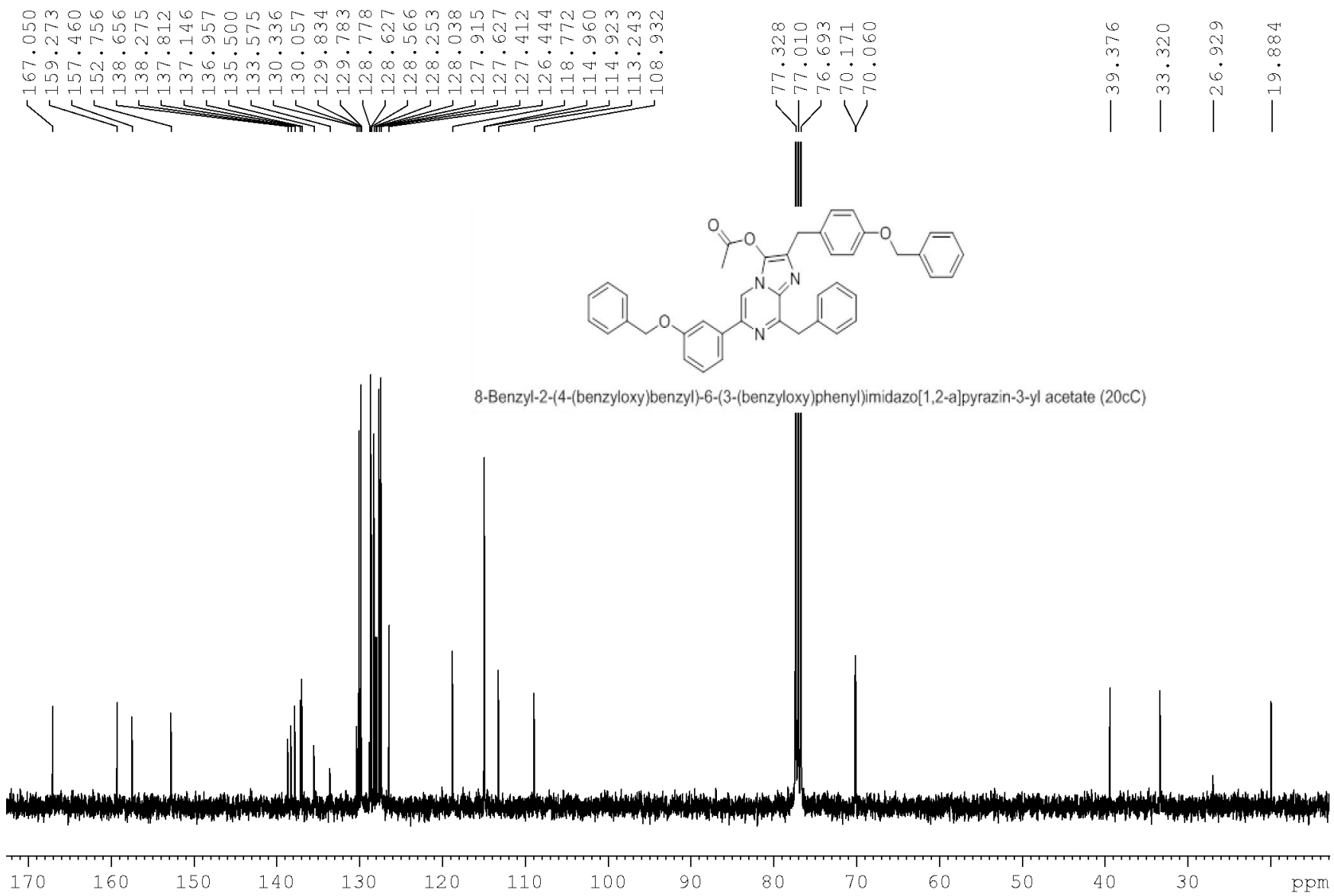


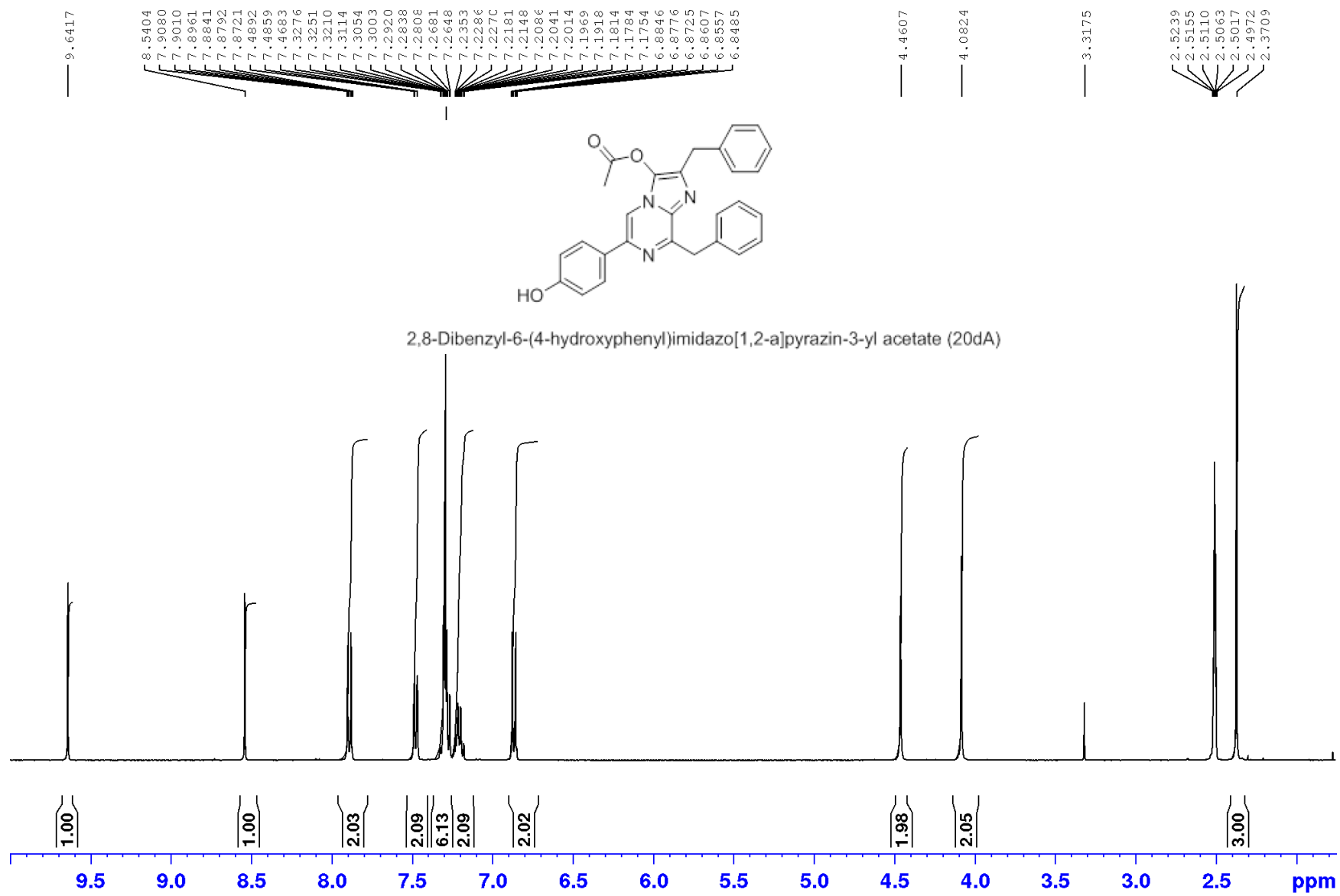


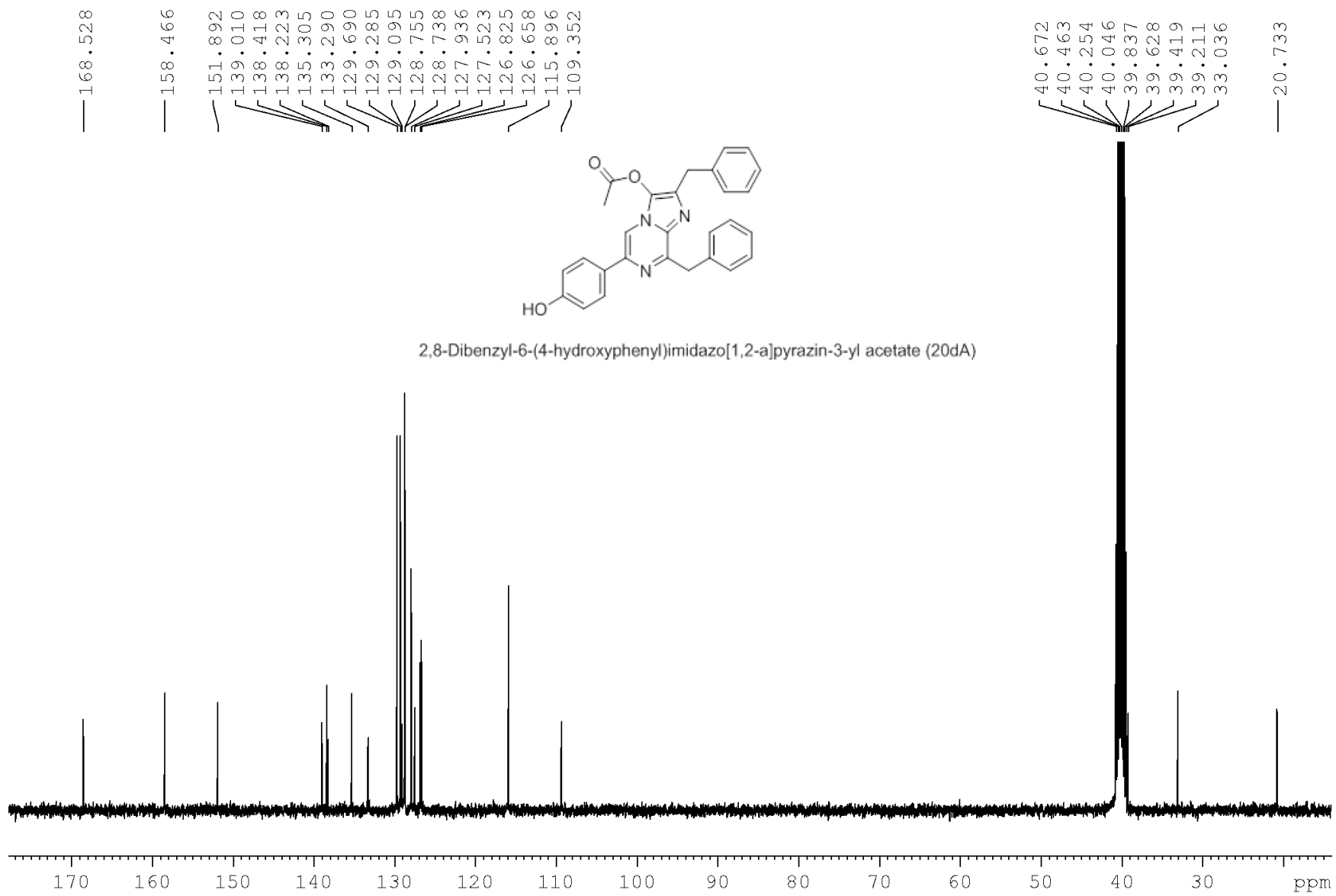


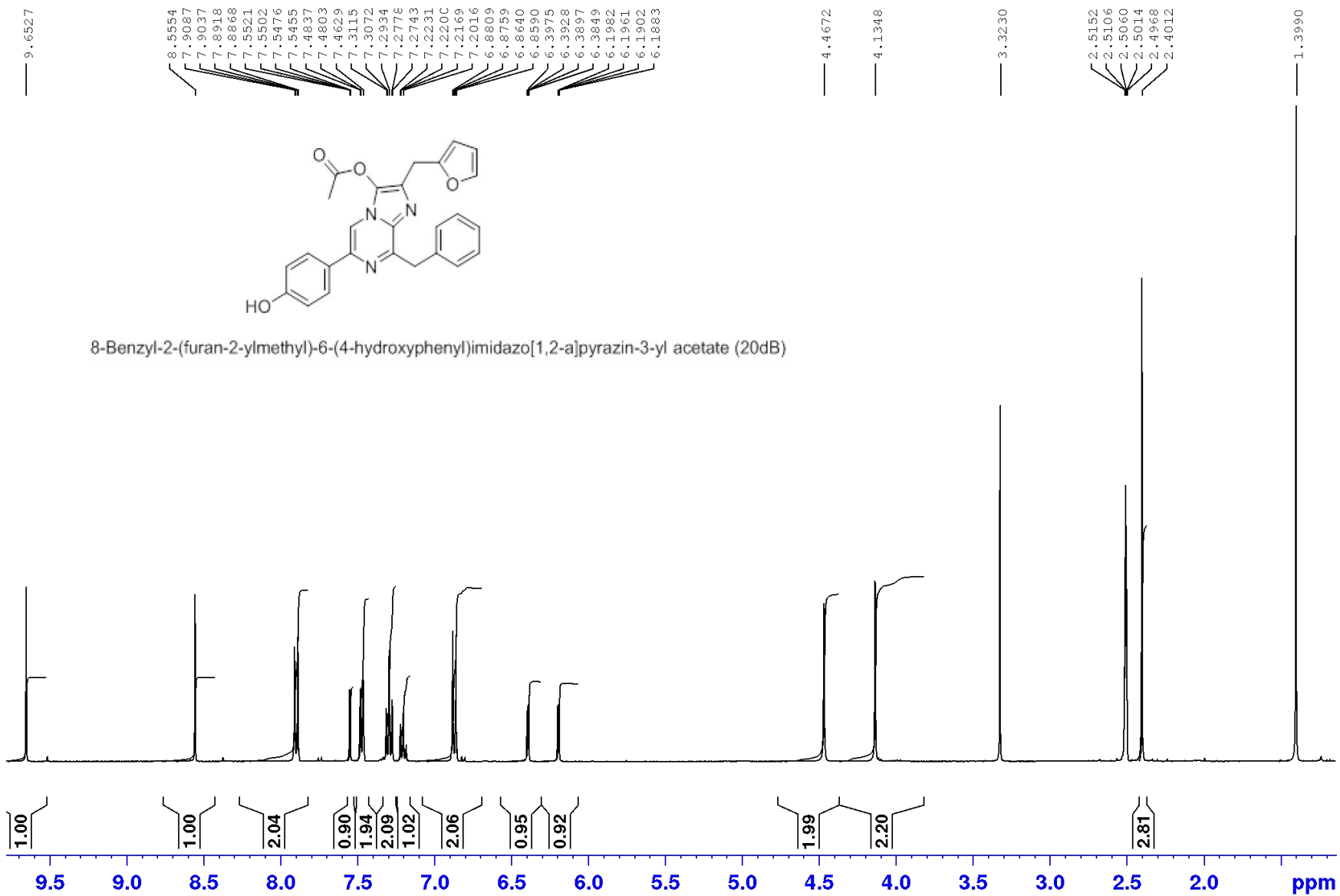


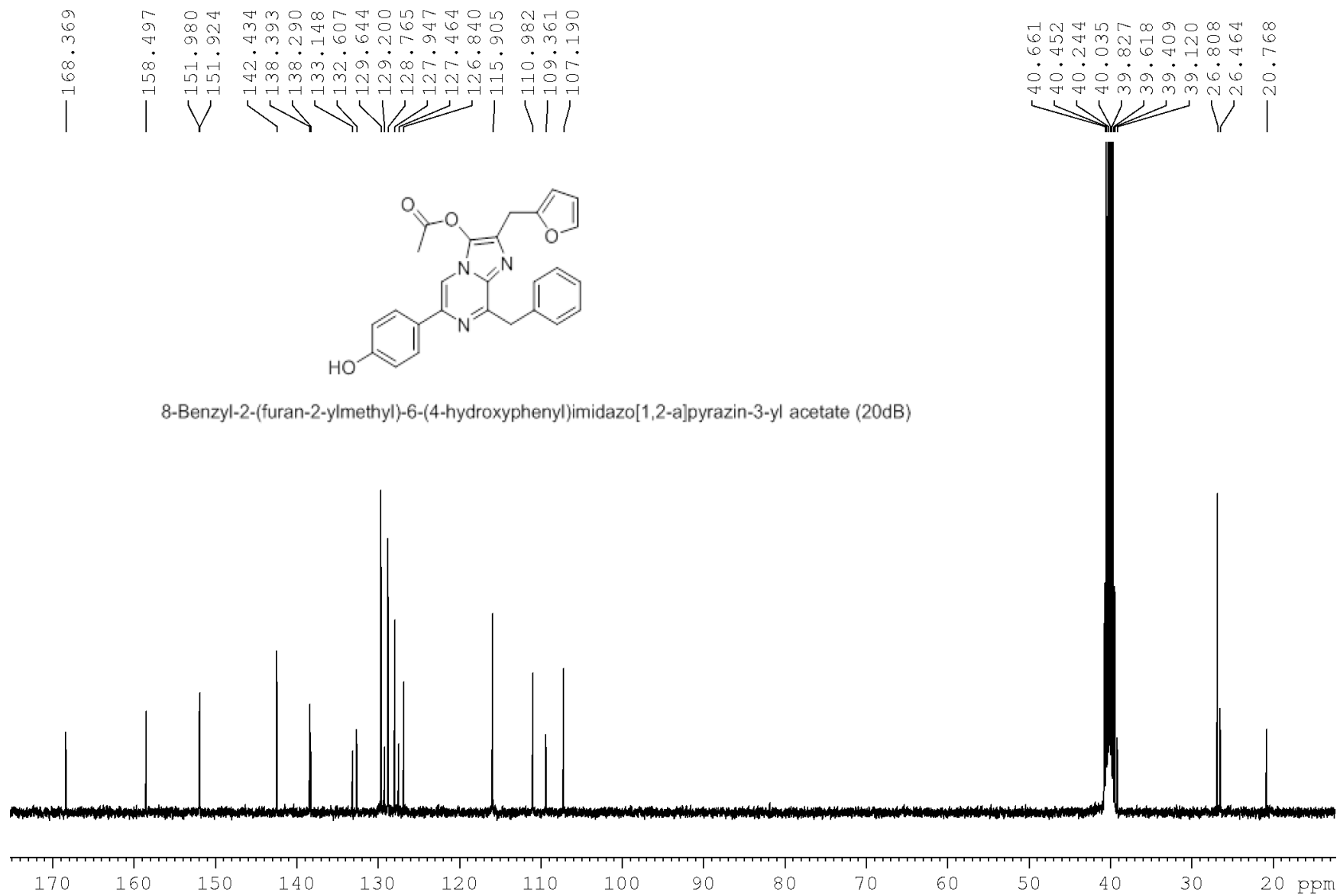


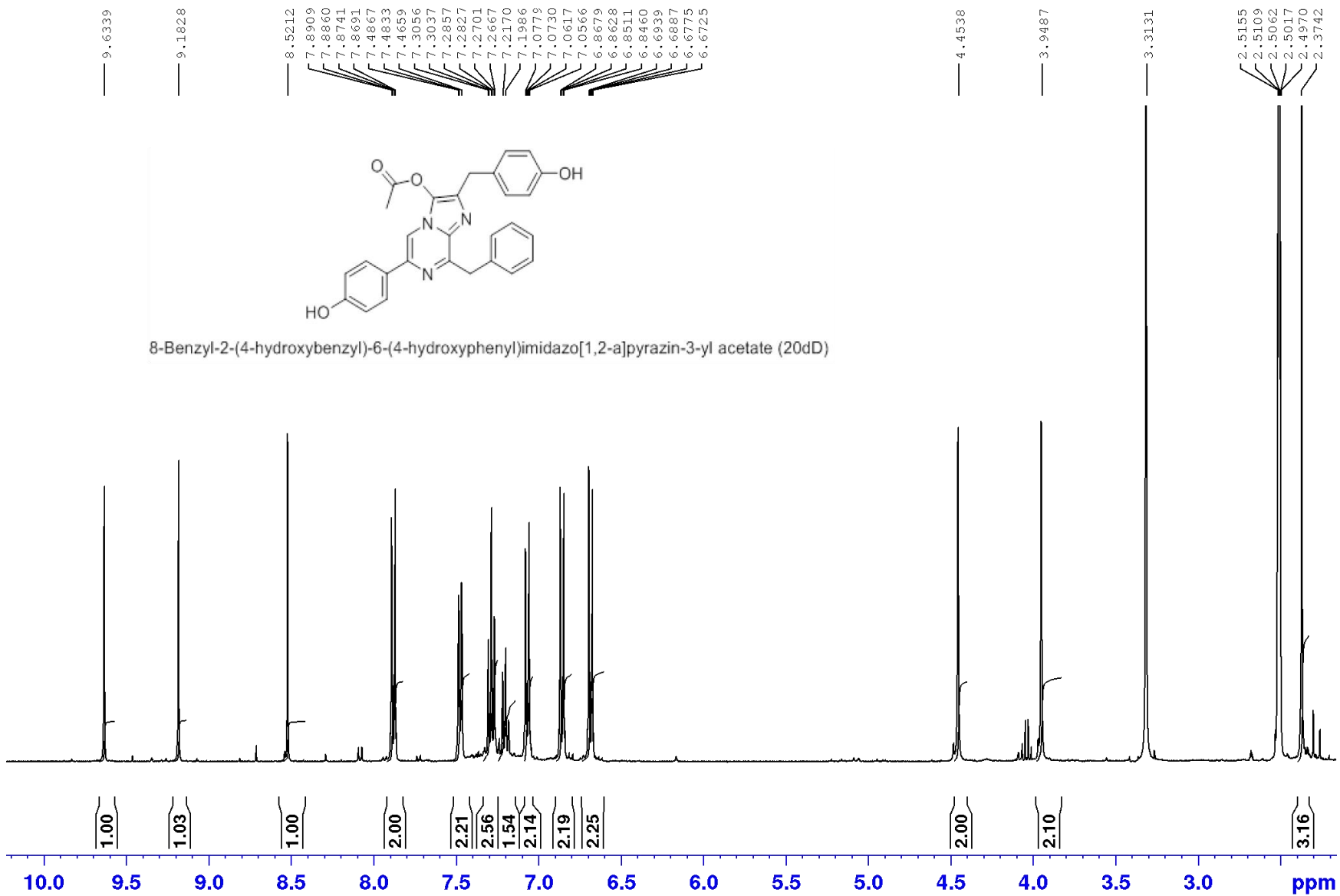


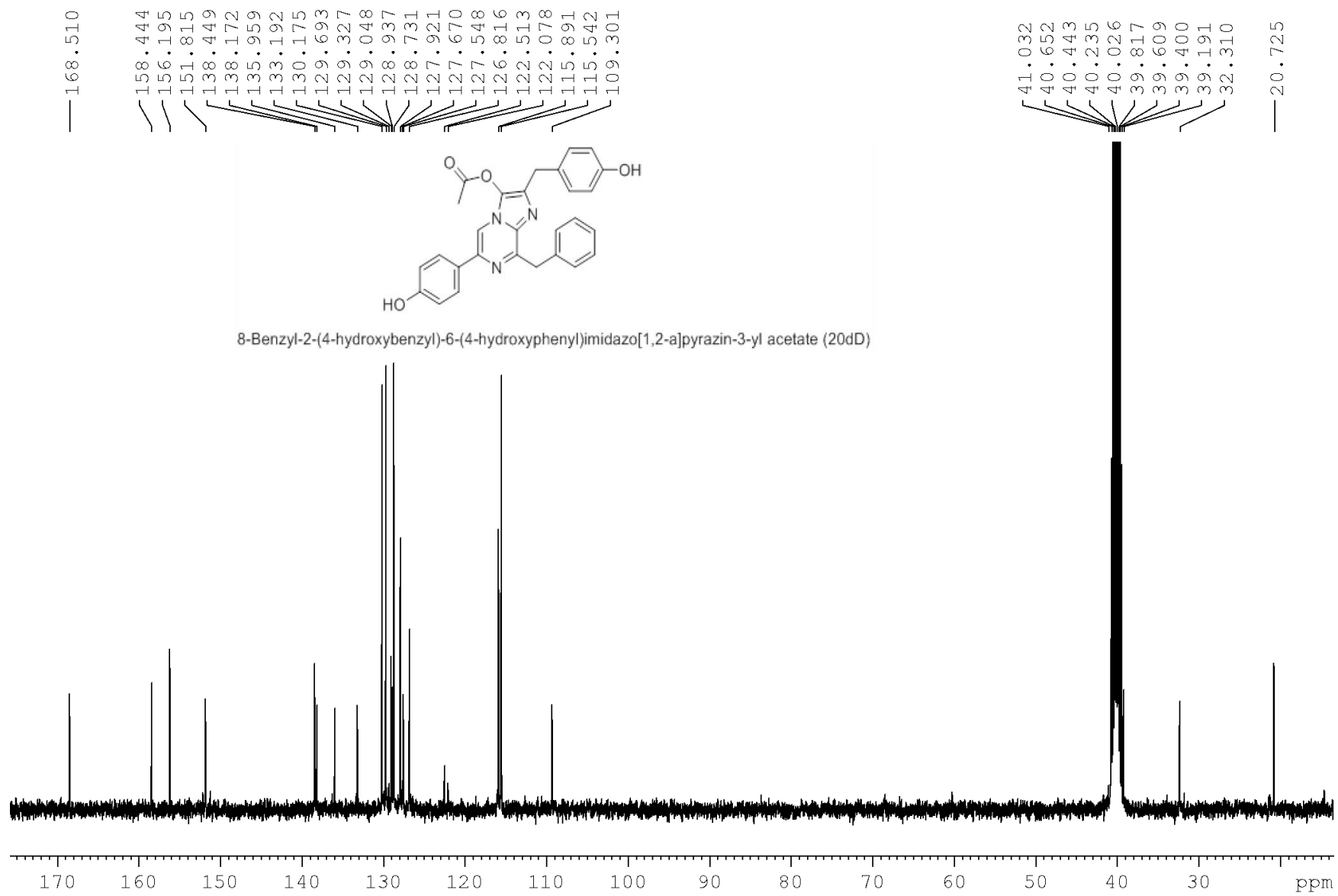


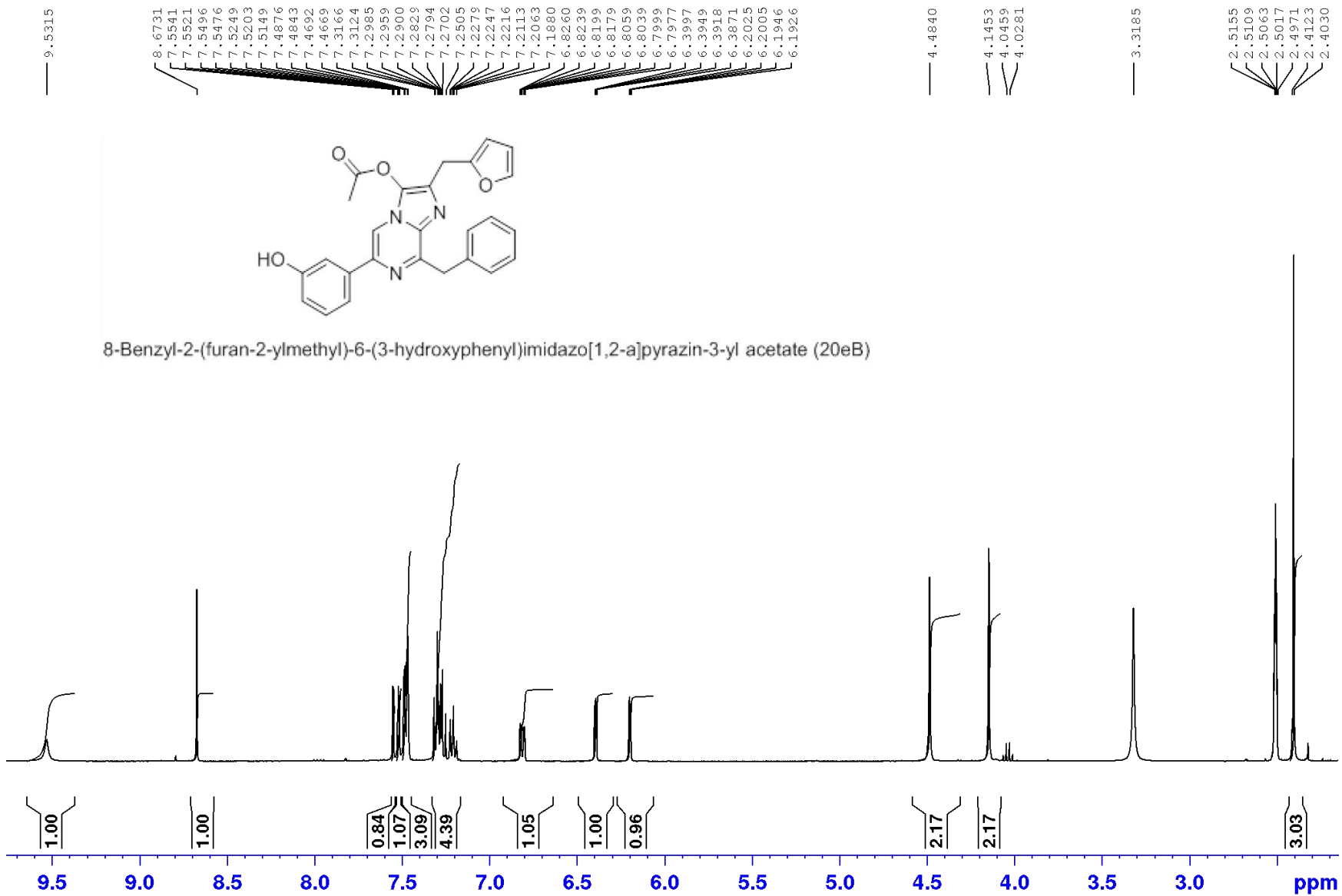






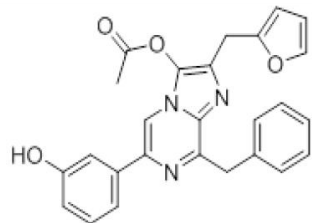






— 168.384
— 158.188
— 152.090
— 151.869
— 142.449
— 138.338
— 138.007
— 133.375
— 132.797
— 130.041
— 129.604
— 129.382
— 128.796
— 126.873
— 117.326
— 115.988
— 113.707
— 111.114
— 110.984
— 107.216

— 40.670
— 40.461
— 40.253
— 40.044
— 39.835
— 39.627
— 39.419
— 39.123
— 26.806
— 26.462
— 20.793
— 14.536



8-Benzyl-2-(furan-2-ylmethyl)-6-(3-hydroxyphenyl)imidazo[1,2-a]pyrazin-3-yl acetate (20eB)

