Gram-scale synthesis of luciferins derived from coelenterazine and original insights into their bioluminescence properties

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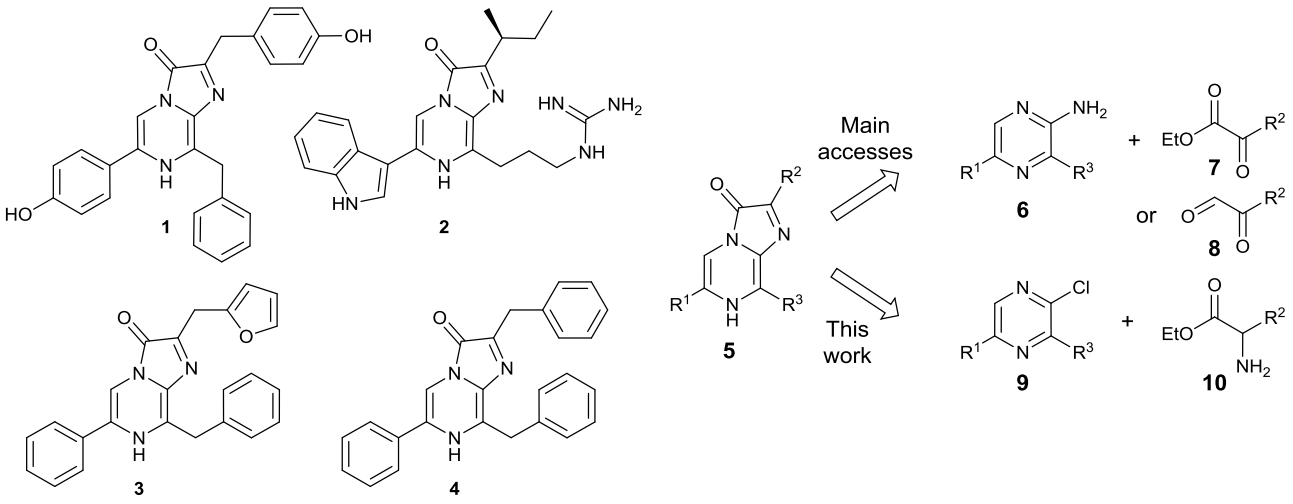
An original gram-scale synthesis of O-acetylated forms of coelenterazine, furimazine or hydroxy-bearing analogues of these luciferins is described. The comparisons over two hours of their bioluminescence, using the nanoKAZ/NanoLuc luciferase, is providing remarkable insights useful for the selection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

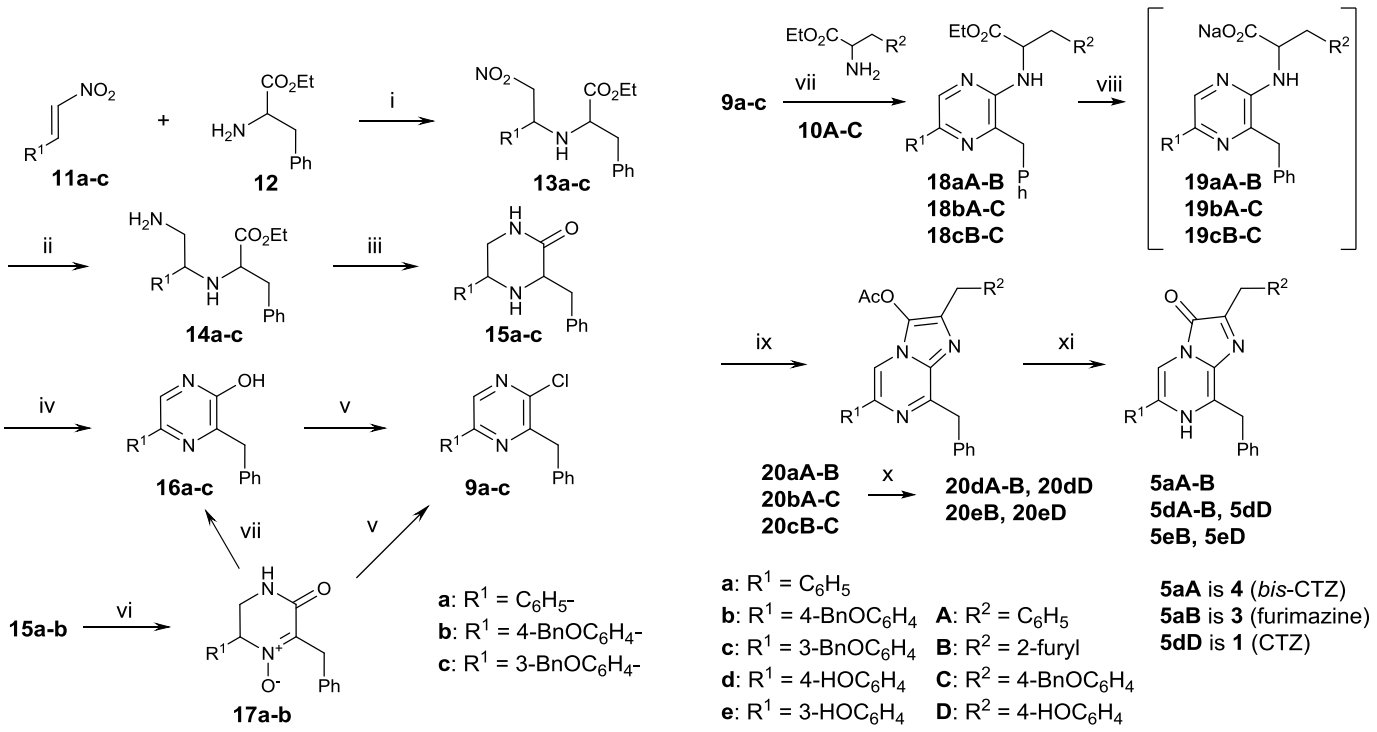
Introduction

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.

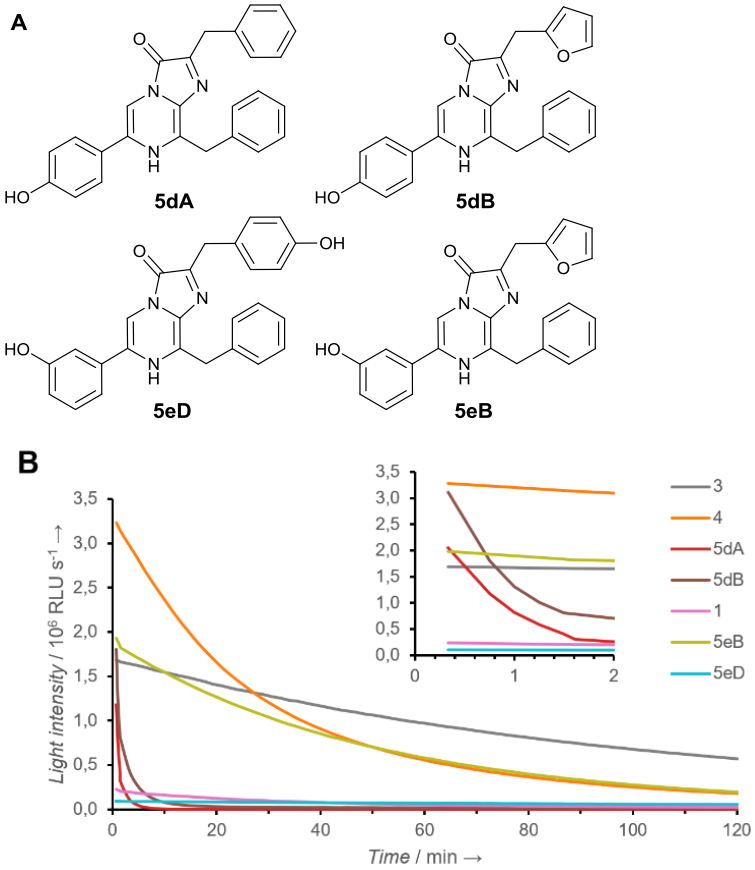
Concerning the chemistry of these luciferins, they are oxygen-sensitive and readily decompose in solution especially in the presence of a base, or upon light exposure.[26] As depicted above, many[27] if not all[28-30] the reported preparations of imidazo[1,2-a]pyrazin-3(7H)-ones **5** are requiring an 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, H_3O^+, Cl^-, dioxane. iii: 130 °C, neat. iv: S_8, 1,3-$Cl_2C_6H_4$, reflux. v: $PhPOCl_2$, 100 °C. vi: AcOOH, AcOEt. vii: chlorobenzene, reflux or NaOH, EtOH, reflux. . vii: Cs_2CO_3, $Pd(OAc)_2$, BINAP, MeCN, 60 °C. viii: NaOH, THF, 20 °C. ix: Ac_2O, 20 °C. x: H2, Pd/C, AcOEt, AcOH, EtOH. xi: EtOH, DMSO, H_3O^+, 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 dichloride[42] 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-arylesters **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-arylesters **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.



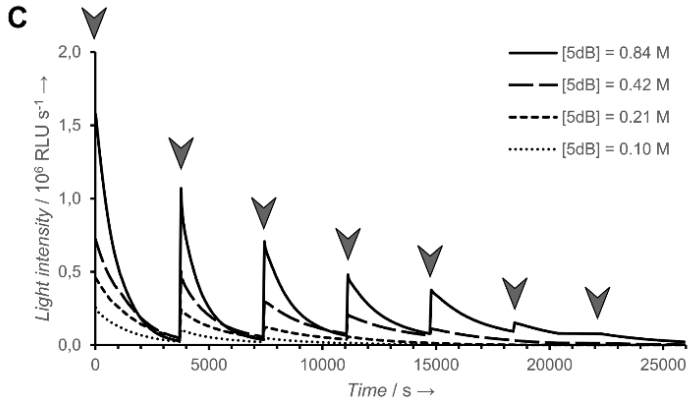


Figure 2. Bioluminescence signals of the luciferins 5 using recombinant nanoKAZ/NanoLuc. (A) Luciferin structures; compounds 1, 3, and 4 are depicted in figure 1. (B) Light intensity in RLU s-1 plotted vs. time over two hours, the insert zooms on the first two minutes of the reaction. (C) Bioluminescence 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 causes 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 others 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**
Imax							
(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} ($mol.s^{-1}$.$mol\varepsilon^{-1}$)	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 (Σ_I), 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.

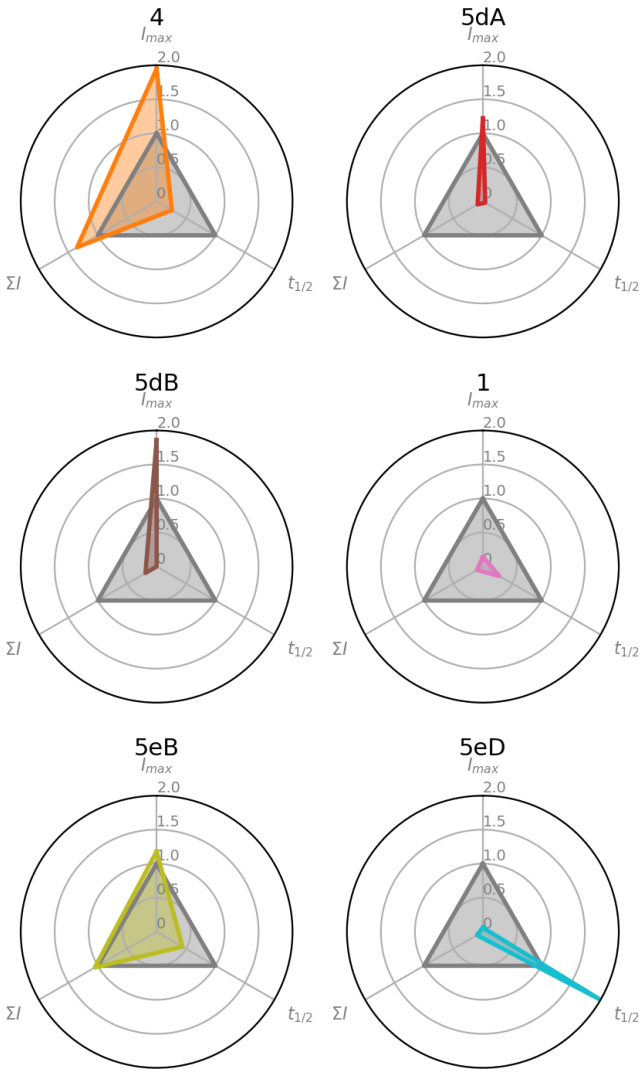


Figure 3. Depiction of the maxima of intensities (I_{max}), integrated signals (Σ_I), and half-time durations ($t_{1/2}$) for all the luciferins, relative to furimazine (**3**) represented in light grey.

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(*7H*)-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 $mol_S/s \cdot mol_\varepsilon$ 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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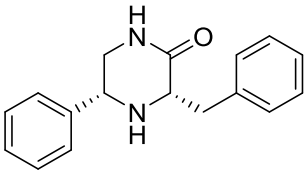
Supplementary information

Chemistry

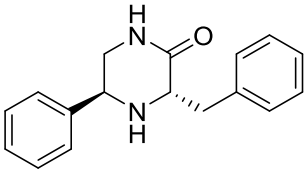
^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance 400 spectrometer at 400 MHz and 100 MHz, respectively. Shifts (δ) are given in ppm with respect to the TMS signal and cross-coupling constants (*J*) are given in Hertz. Column chromatography were performed either on Merck silica gel 60 (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 ^{1}H 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 diasteroisomers 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.



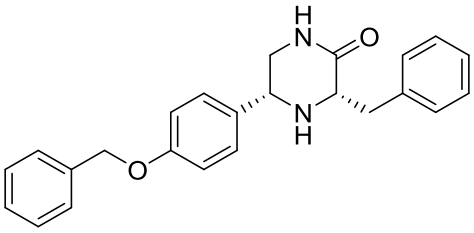
(3S,5R)-3-benzyl-5-phenylpiperazin-2-one (**15a, first dia**): Obtained as a white solid (2.05 g, 35%). ^{1}H NMR ($CDCl_3$): 7.42 – 7.29 (m, 9H), 7.23 (m, 1H), 6.27 (s, 1H), 4.06 (dd, 1H, *J* = 9.7, 4.7 Hz), 3.84 (dt, 1H, *J* = 10.1, 3.4 Hz), 3.60 (dd, 1H, *J* = 13.6, 3.1 Hz), 3.32 (m, 2H), 2.91 (dd, 1H, *J* = 13.6, 10.1 Hz), 1.78 (s, 1H). ^{13}C NMR ($CDCl_3$): 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 $C_{17}H_{19}N_2O$, 267.1497, found: 267.1459.



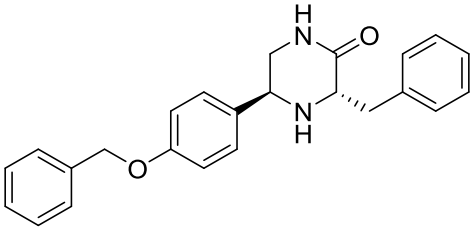
(3S,5S)-3-benzyl-5-phenylpiperazin-2-one (**15a, second dia**): Obtained as a white solid (1.29 g, 22%). ^{1}H NMR ($CDCl_3$): 7.46 – 7.18 (m, 10H), 6.44 (s, 1H), 4.27 (dd, 1H, *J* = 9.6, 4.0 Hz), 3.89 (dd, 1H, *J* = 10.7, 3.6 Hz), 3.50 (m, 1H), 3.41 (dt, 1H, *J* = 11.5, 4.0 Hz), 3.32 (dd, 1H, *J* = 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*): $[M+H]^+$ calcd for $C_{17}H_{19}N_2O$, 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)benzene[1] (**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 diasteroisomers 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.

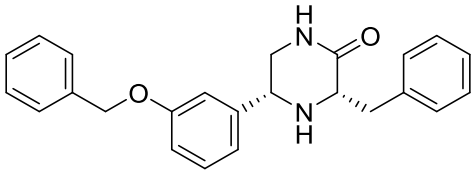


(3S,5R)-3-benzyl-5-(4-(benzyloxy)phenyl)piperazin-2-one (**15b, first dia**): This isomer was obtained using the first procedure described above as a white powder (0.92 g, 17%) after two chromatography over silica gel (dichloromethane - ethanol 95/5 to 92/8) and (cyclohexane - ethyl acetate 1/2) and a recrystallization in cyclohexane. ^{1}H 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*): $[M+H]^+$ calcd for $C_{25}H_{25}N_2O_3$: 373.1916, found: 373.1932.



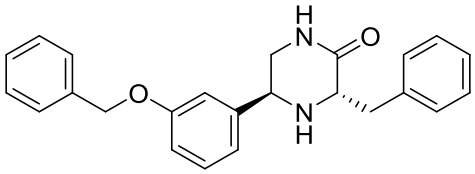
(3S,5S)-3-benzyl-5-(4-(benzyloxy)phenyl)piperazin-2-one (**15b, second dia**): This isomer was obtained using the first procedure described above as a white powder (0.59 g, 11%) after two chromatography over silica gel (dichloromethane - ethanol 95/5 to 92/8) and (ethyl acetate - ethanol 1/0 to 99/1). ^{1}H 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*): $[M+H]^+$ calcd for $C_{25}H_{25}N_2O_3$: 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 diasteroisomers (4.92 g, 31%). The insoluble material and the filtrates were concentrated to dryness and additional amount of the two (separated) diasteroisomers (1.64 g and 1.77 g, 21% in total) were obtained from this residue as described below.



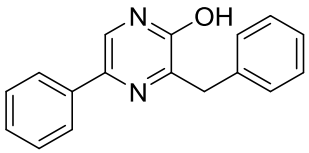
(3S,5R)-3-benzyl-5-(3-(benzyloxy)phenyl)piperazin-2-one (**15c, first dia**): This isomer was obtained from the recrystallization residue described above as a white powder (1.64 g), still containing 3% of an unidentified material, after two chromatography over silica gel (dichloromethane - ethanol 97/3 to 96/4), (cyclohexane – ethyl acetate 2/3) and a recrystallization in cyclohexane. ^{1}H 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*): $[M+H]^+$ calcd for $C_{24}H_{25}N_2O_2$: 373.1916, found: 373.1919.



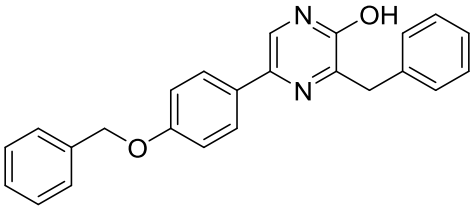
(3S,5S)-3-benzyl-5-(3-(benzyloxy)phenyl)piperazin-2-one (**15c, second dia**): This isomer was obtained from the recrystallization residue described above as a white powder (1.77 g) after a chromatography over silica gel (dichloromethane - ethanol 97/3 to 96/4) and a dispersion in boiling cyclohexane. ^{1}H 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*): $[M+H]^+$ calcd for $C_{24}H_{25}N_2O_2$: 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.

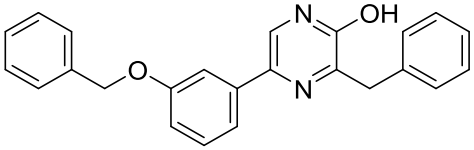


3-Benzyl-5-phenylpyrazin-2-ol (**16a**): This compound was obtained as a white powder (2.28 g, 73%) after a chromatography over silica gel (dichloromethane – ethanol 98/2 to 97/3). ^{1}H NMR

(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). ^{13}C 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 $C_{17}H_{15}N_2O$: 263.1184, found, 263.1118.

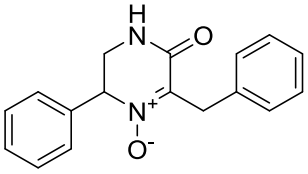


3-Benzyl-5-(4-(benzyloxy)phenyl)pyrazin-2-ol (**16b**): This compound was obtained as a powder (0.8 g, 62%) after a chromatography over silica gel (dichloromethane - ethanol 98/2). ^{1}H NMR (DMSO-d_6) 12.31 (s, 1H), 7.78 (m, 2H), 7.40 (m, 4H), 7.37 (m, 9H), 7.19 (m, 1H), 7.03 (m, 2H), 5.13 (s, 2H), 4.06 (s, 2H). ^{13}C 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 $C_{24}H_{22}N_2O_2$: 369.1603, found, 369.1603.

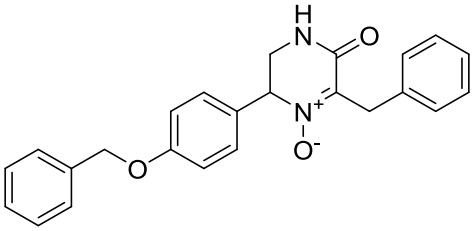


3-Benzyl-5-(3-(benzyloxy)phenyl)pyrazin-2-ol (**16c**): This compound was obtained as beige solid (1.41 g, 54%) as described above but using boiling decaline (30 mL) instead of 1,3-dichlorobenzene after a chromatography over silica gel (cyclohexane – ethyl acetate 2/1 to 1/1). ^{1}H NMR (DMSO-d_6) 12.42 (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). ^{13}C 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 $C_{24}H_{22}N_2O_2$: 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. Nota, COSY correlations firmly established the structures of this compound. ^{1}H 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*): $[M+H]^+$ calcd for $C_{17}H_{17}N_2O_2$: 281.1190, found, 281.1234.



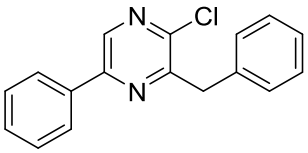
6-Benzyl-2-(4-(benzyloxy)phenyl)-5-oxo-2,3,4,5-tetrahydropyrazine 1-oxide (**17b**): The crude piperazin-2-one **16b** obtained from compound **11b** (0.0871 mol) as described above was dissolved in ethyl acetate (600 mL), 35% peracetic acid in acetic acid (33.8 mL, 0.174 mol) was added and this was 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**). ^{1}H NMR (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*): $[M+H]^+$ calcd for $C_{24}H_{23}N_2O_3$: 387.1709, found, 387.1714.

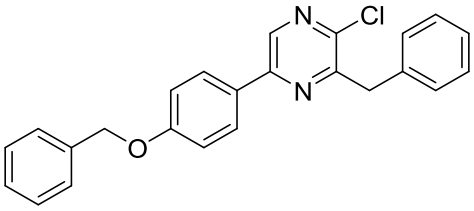
Procedure for the synthesis of compounds 16a via a thermal dehydration of the N-oxide 17a. In a microwave-adapted vial, a crude batch of 6-benzyl-5-oxo-2-phenyl-2,3,4,5-tetrahydropyrazine 1-oxide (0.41 g, 1.46 mmol) obtained as described above was dissolved in chlorobenzene (5 mL). This was sealed and 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.

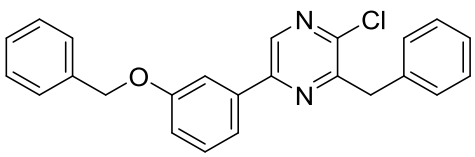
General procedure for the synthesis of compounds 9a-c: under a calcium-protected atmosphere, the considered 2-hydroxypyrazine (0.02 mol) was dispersed in phenylphosphonic dichloride (10 mL) and the suspension was heated at 100 °C for the indicated time. The resulting solution was diluted in ethyl acetate 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). Alernatively, 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 dispertion in boiling cyclohexane as described above and undertaking the next two synthetic steps (sulfur-based aromatization and chlorodehydroxylation) whithout 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). ^{1}H 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*): $[M+H]^+$ calcd for $C_{17}H_{14}ClN_2$, 281.0846, found, 281.0730.

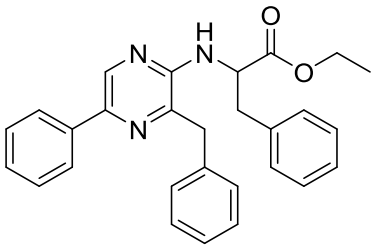


3-Benzyl-5-(4-(benzyloxy)phenyl)-2-chloropyrazine (**9b**): Obtained as a yellow solid (5.66 g, 62%) after heating for 18 hours and a chromatography over silica gel (cyclohexane-dichloromethane 1/1). ^{1}H NMR ($CDCl_3$): 8.61 (s, 1H), 8.0 (m, 2H), 7.47-7.20 (m, 10H), 7.11 (m, 2H), 5.17 (s, 2H), 4.38 (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*): $[M+H]^+$ calcd for $C_{24}H_{20}ClN_2O$, 387.1264, found, 387.1266.

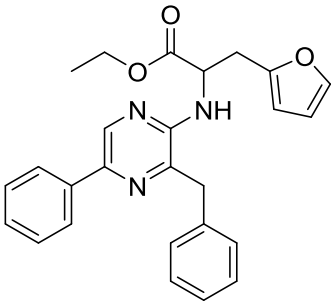


3-Benzyl-5-(3-(benzyloxy)phenyl)-2-chloropyrazine (**9c**): Obtained as a slowly solidifying oil (0.77 g, 61%) after heating for 18 hours and a chromatography over silica gel (cyclohexane-ethyl acetate 97/3). ^{1}H NMR ($CDCl_3$): 8.65 (s, 1H), 7.69 (m, 1H), 7.60 (m, 1H), 7.50 (m, 2H), 7.45-7.32 (m, 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*): $[M+H]^+$ calcd for $C_{24}H_{20}ClN_2O$, 387.1264, found, 387.1262.

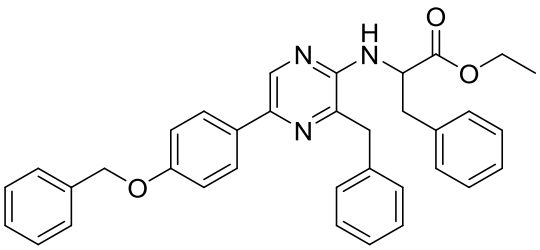
General procedure for the N-arylation of chloropyrazines 9a-c by α-aminoesters. In a 20 mL sealable vial, the considered 2-halogenopyrazine (1 mmol), the considered α-aminoesters[3, 4] (1.1 mmol, either as a free base or as a hydrochloride salt), cesium carbonate (1.04 g, 3.2 mmol), palladium acetate (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.



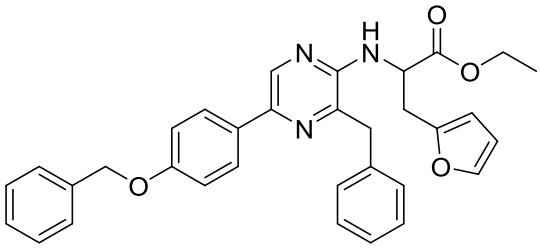
Ethyl (3-benzyl-5-phenylpyrazin-2-yl)phenylalaninate (**18aA**): Obtained as an oil (1.07 g, 69%) after a chromatography over silica gel (dichloromethane). ^{1}H NMR ($CDCl_3$): 8.43 (s, 1H), 7.96 (m, 2H), 7.49 (m, 1H), 7.37 (m, 1H), 7.33 – 7.20 (m, 8H), 7.90 (m, 2H), 4.97 (m, 2H), 4.14 (s, 2H), 4.13 (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*): $[M+H]^+$ calcd for $C_{28}H_{28}N_3O_2$, 438.2182, found, 438.2185.



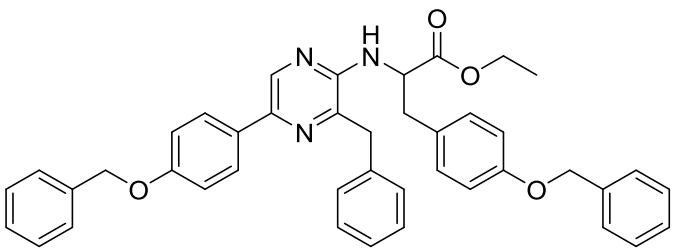
Ethyl 2-((3-benzyl-5-phenylpyrazin-2-yl)amino)-3-(furan-2-yl)propanoate (**18aB**): obtained as an oil (6.31 g, 89%) after a chromatography over silica gel (cyclohexane-ethyl acetate 95:5). ^{1}H NMR ($CDCl_3$): 8.42 (s, 1H), 7.95 (m, 2H), 7.46 (m, 2H), 7.39 – 7.28 (m, 5H), 7.22 (m, 2H), 6.22 (dd, 1H, *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*): $[M+H]^+$ calcd for $C_{26}H_{26}N_3O_3$, 428.1974, found: 428.1965.



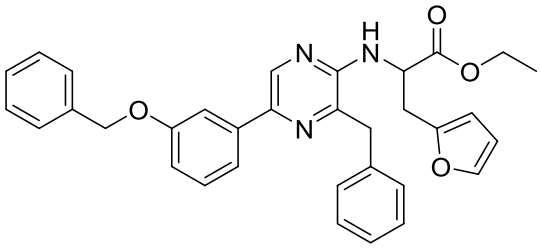
Ethyl (3-benzyl-5-(4-(benzyloxy)phenyl)pyrazin-2-yl)phenylalaninate (**18bA**): Obtained, as a white solid (4.47 g, 87%) after a chromatography over silica gel (cyclohexane – ethyl acetate 95/5 to 94/6). ^{1}H NMR ($CDCl_3$): 8.36 (s, 1H), 7.89 (m, 2H), 7.49 (m, 2H), 7.42 (m, 2H), 7.35 (m, 1H), 7.32-7.20 (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). ^{13}C NMR ($CDCl_3$): 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 $C_{35}H_{34}N_3O_3$, 544.2600, found, 544.2609.



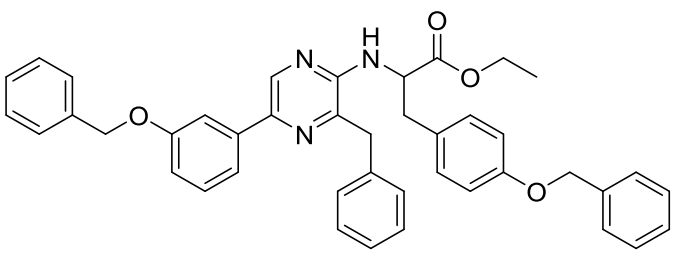
Ethyl 2-((3-benzyl-5-(4-(benzyloxy)phenyl)pyrazin-2-yl)amino)-3-(furan-2-yl)propanoate (**18bB**): Obtained as an oil (0.83 g, 79%) after a chromatography over silica gel (cyclohexane – ethyl acetate 94/6 to 93/7). ^{1}H NMR ($CDCl_3$): 8.35 (s, 1H), 7.89 (m, 2H), 7.48 (m, 2H), 7.42 (m, 2H), 7.36 (m, 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). ^{13}C NMR ($CDCl_3$): 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 $C_{33}H_{32}N_3O_4$, 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). ^{1}H 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*): $[M+H]^+$ calcd for $C_{42}H_{40}N_3O_4$, 650.3019, found, 650.3049.

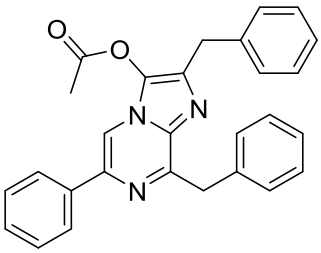


Ethyl 2-(3-benzyl-5-(3-(benzyloxy)phenyl)pyrazin-2-ylamino)-3-(furan-2-yl)propanoate (**18cB**): Obtained as an oil (0.63 g, 90%, still containing traces of EtOAc) after a chromatography over silica gel (cyclohexane – ethyl acetate 97/3 to 96/4). ^{1}H NMR ($CDCl_3$): 8.39 (s, 1H), 7.63 (m, 1H), 7.50 (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*): $[M+H]^+$ calcd for $C_{33}H_{32}N_3O_4$, 534.2393, found, 534.2402.



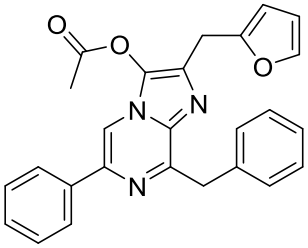
Ethyl 2-(3-benzyl-5-(3-(benzyloxy)phenyl)pyrazin-2-ylamino)-3-(4-(benzyloxy)phenyl)propanoate (**18cC**): Obtained as an oil (0.50 g, 78%) after a chromatography over silica gel (cyclohexane – ethyl acetate 97/3 to 96/4). ^{1}H NMR ($CDCl_3$): 8.40 (s, 2H), 7.65 (m, 1H), 7.65-7.20 (m, 17H), 6.99 (m, 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). ^{13}C NMR ($CDCl_3$): 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 $C_{42}H_{40}N_3O_4$, 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.

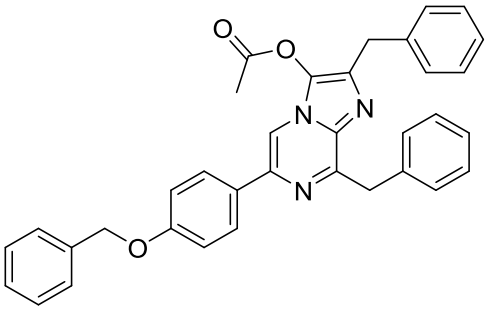


2,8-Dibenzyl-6-phenylimidazo[1,2-a]pyrazin-3-yl acetate (**20aA**): Obtained as a yellowish solid (0.17 g, 50%) after a recrystallization in cyclohexane. ^{1}H NMR ($CDCl_3$): 7.92 (m, 2H), 7.80 (s, 1H), 7.64 (m, 2H), 7.47 (m, 2H), 7.40 (m, 1H), 7.33 (m, 5H), 7.25 (m, 2H), 4.65 (s, 2H), 4.23 (s, 2H), 2.18 (s, 3H). ^{13}C NMR ($CDCl_3$): 167.1, 152.9, 139.1, 137.9, 137.8, 136.8, 135.1, 133.5,

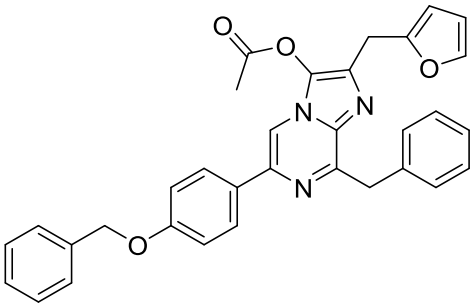
129.8, 129.1, 128.9, 128.8, 128.6, 128.5, 128.3, 126.5 (two signals), 126.4, 108.8, 39.4, 34.2, 19.9. HRMS (*m/z*): $[M+H]^+$ calcd for $C_{28}H_{24}N_3O_2$, 434.1869, found, 434.1825.



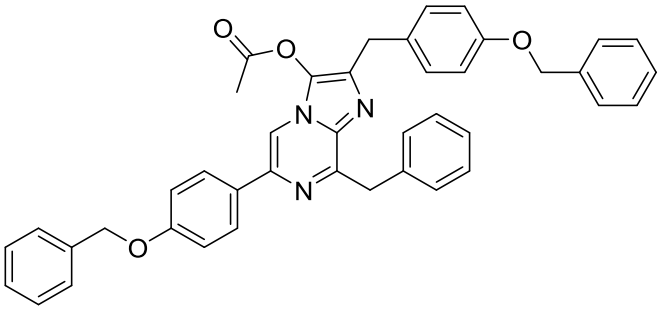
8-benzyl-2-(furan-2-ylmethyl)-6-phenylimidazo[1,2-a]pyrazin-3-yl acetate (**20aB**): Obtained as an off-white solid (4.02 g, 72%) after a recrystallization in cyclohexane. ^{1}H NMR ($CDCl_3$): 7.92 (m, 2H), 7.83 (s, 1H), 7.63 (m, 2H), 7.47 (m, 2H), 7.42-7.30 (m, 4H), 7.23 (m, 1H), 6.35 (m, 1H), 6.16 (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. ^{1}H 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.

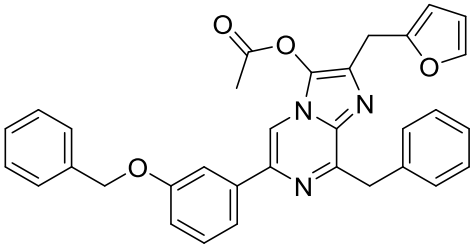


8-Benzyl-6-(4-(benzyloxy)phenyl)-2-(furan-2-ylmethyl)imidazo[1,2-a]pyrazin-3-yl acetate (**20bB**): Obtained as an off-white solid (1.13 g) pure enough (traces of acetic acid) to be used directly in the next step. ^{1}H NMR ($CDCl_3$): 7.85 (m, 2H), 7.75 (s, 1H), 7.61 (m, 2H), 7.47 (m, 2H), 7.44-7.21 (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*): $[M+H]^+$ calcd for $C_{33}H_{28}N_3O_4$, 530.2080, found, 530.2070.

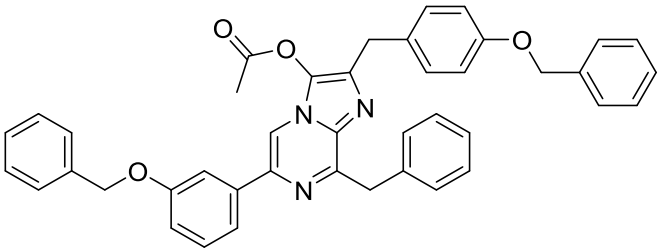


8-Benzyl-2-(4-(benzyloxy)benzyl)-6-(4-(benzyloxy)phenyl)imidazo[1,2-a]pyrazin-3-yl acetate (**20bC**): Obtained as an off-white solid (0.51 g) which was used directly in the next step. ^{1}H NMR ($CDCl_3$): 7.84 (m, 2H), 7.71 (s, 1H), 7.61 (m, 2H), 7.48 – 7.29 (m, 12H), 7.25 – 7.20 (m, 3H), 7.06 (m, 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.

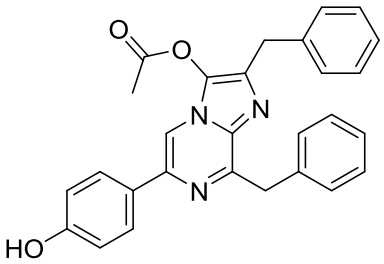


8-Benzyl-6-(3-(benzyloxy)phenyl)-2-(furan-2-ylmethyl)imidazo[1,2-a]pyrazin-3-yl acetate (**20cB**): Obtained as an oil (0.55 g) still containing a small amount of cyclohexane which was used in the next step without further purification. ^{1}H NMR ($CDCl_3$): 7.83 (s, 1H), 7.61 (m, 3H), 7.49 (m, 3H), 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.

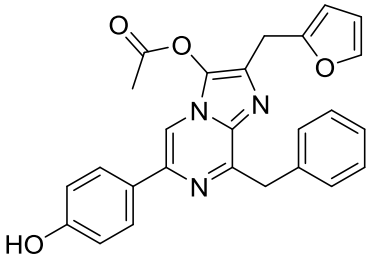


8-Benzyl-2-(4-(benzyloxy)benzyl)-6-(3-(benzyloxy)phenyl)imidazo[1,2-a]pyrazin-3-yl acetate (**20cC**): Obtained as a white solid (0.5 g, 77%) after a recrystallization in cyclohexane. ^{1}H NMR ($CDCl_3$): 7.79 (s, 1H), 7.61 (m, 3H), 7.51-7.30 (m, 14H), 7.23 (3H), 7.01 (m, 1H), 6.94 (m, 2H), 5.14 (s, 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.

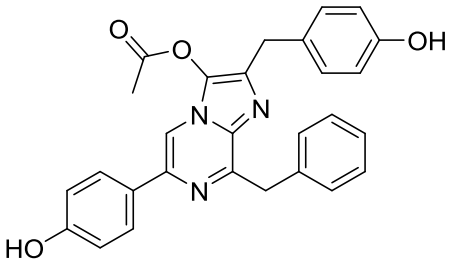
General protocol for the debenzylation of compounds 20bA-C, **20cB-C** into compounds **20dA-B, 20dD**, **20eB** and **20eD**. The considered O-acetylated luciferin (1.05 mmol) and 10% palladium over charcoal (0.11 g, 0.105 mmol) were dispersed in ethyl acetate (20 mL), ethanol (20 mL) and acetic acid (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.



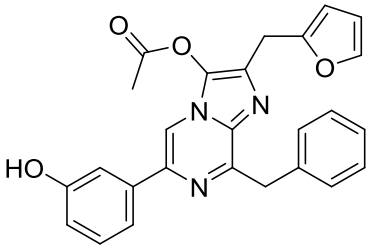
2,8-Dibenzyl-6-(4-hydroxyphenyl)imidazo[1,2-a]pyrazin-3-yl acetate (**20dA**): This compound was obtained as a white cotton (0.1 g, 27% from **18aA**) after a chromatography over silica gel (cyclohexane – ethyl acetate 4/1 to 3/1). ^{1}H NMR (DMSO-d_6): 9.64 (s, 1H), 8.54 (s, 1H), 7.89 (m, 2H), 7.48 (m, 2H), 7.26 (m, 8H), 6.87 (m, 2H), 4.46 (s, 2H), 4.08 (s, 2H), 2.37 (s, 3H). ^{13}C 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 $C_{28}H_{24}N_3O_3$: 450.1818, found, 450.1825.



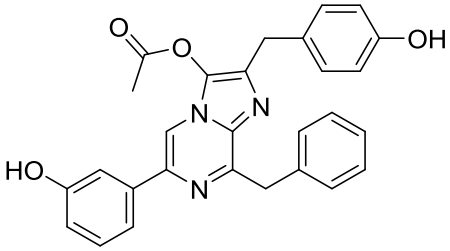
8-Benzyl-2-(furan-2-ylmethyl)-6-(4-hydroxyphenyl)imidazo[1,2-a]pyrazin-3-yl acetate (**20dB**): This compound was obtained as a white cotton (0.37 g, 39% from **18aB**) after a chromatography over silica gel (cyclohexane – ethyl acetate 3/1). ^{1}H NMR (DMSO-d_6): 9.65 (s, 1H), 8.55 (s, 1H), 7.90 (m, 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). ^{13}C 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 $C_{26}H_{22}N_3O_4$, 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). ^{1}H 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). ^{13}C 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 $C_{28}H_{24}N_3O_4$: 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). ^{1}H 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). ^{13}C 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 $C_{26}H_{22}N_3O_4$: 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). ^{1}H 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). ^{13}C 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 $C_{28}H_{24}N_3O_4$: 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 µl) 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.

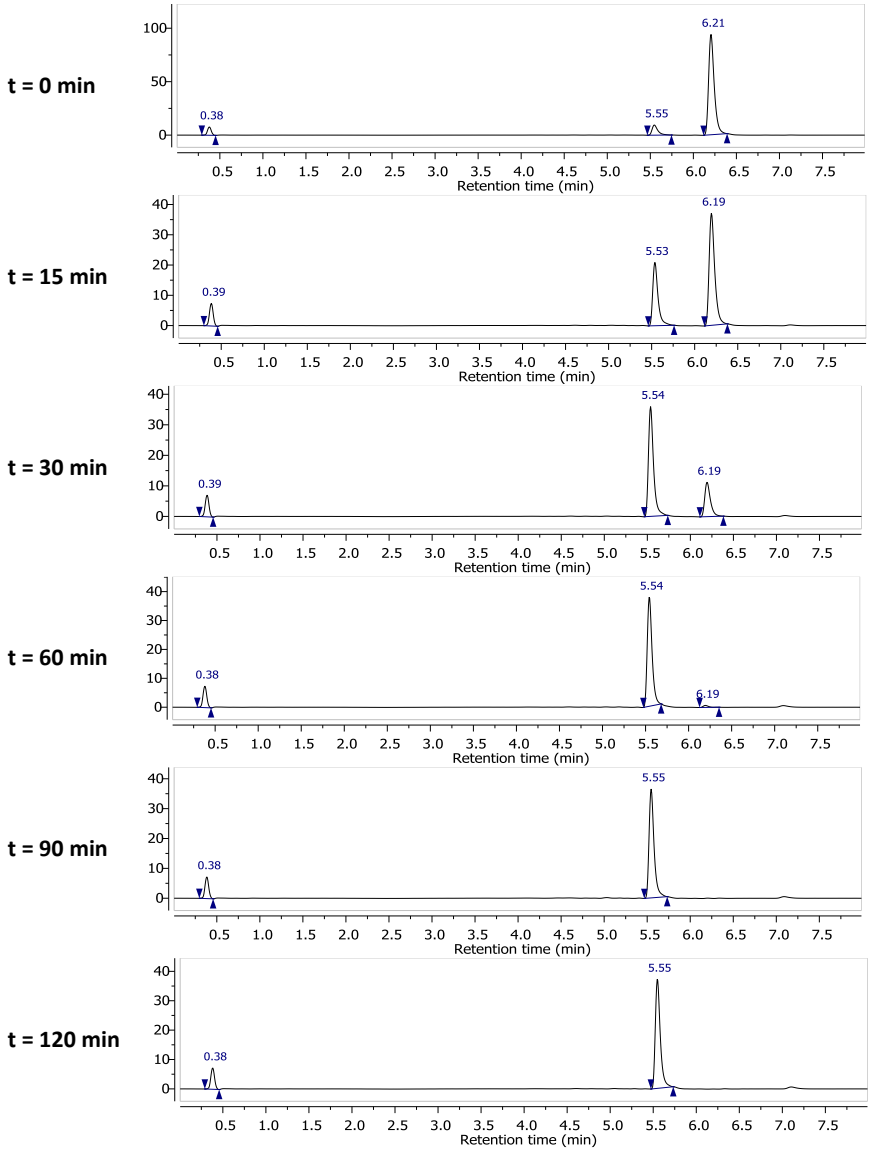


Figure S1. HPLC profiles, obtained on an Agilent apparatus, with a 3.5 µm XDB-C18 column, elution: 5:95 to 95:5 H_2O/MeOH in 3.5 min, with constant 5 mM ammonium formate. Peak at t_R = 5.5 is compound **5a-B** (*m/z* $[M+H]^+$ = 382), peak at t_R = 6.2 min is the O-acetylated compound **20a-B** (*m/z* $[M+H]^+$ = 424).

Biochemistry

NanoKAZ expression, purification and quality validation

The nanoKAZ luciferase[5] was expressed from a codon-optimized sequence of the gene coding for the previously reported[6] NanoLuc, the mutated catalytic domain of the luciferase from *Oplophorus gracilostris* featuring 16 amino acid substitutions. The gene *nanoKAZ* was synthetized by Eurofins (Germany) with carboxy-end His_6-tag (in caps) and flanking region corresponding to the pET23 sequence (Novagen).

Atggtcttcacactcgaagatttcgttggggactggcgacagacagccggctacaacctggaccaagtccttgaacagggaggtgtgtcca
gtttgtttcagaatctcggggtgtccgtaactccgatccaaaggattgtcctgagcggtgaaaatgggctgaagatcgacatccatgtcatcat
cccgtatgaaggtctgagcggcgaccaaatgggccagatcgaaaaaatttttaaggtggtgtaccctgtggatgatcatcactttaaggtgat
cctgcactatggcacactggtaatcgacggggttacgccgaacatgatcgactatttcggacggccgtatgaaggcatcgccgtgttcgacg
gcaaaaagatcactgtaacagggaccctgtggaacggcaacaaaattatcgacgagcgcctgatcaaccccgacggctccctgctgttccg
agtaaccatcaacggagtgaccggctggcggctgtgcgaacgcattctggcgCTCGAGCACCACCACCACCACCAC
TGA

The pET23 plasmid was amplified with the forward and reverse oligonucleotides (Fwd:5'CTCGAGCACCACCACCACCAC3';
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 □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 □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 □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 Hippel[7] from the residue sequence of nanoKAZ (ε_{280nm} = 24750 $M^{-1}.cm^{-1}$). 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 □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).

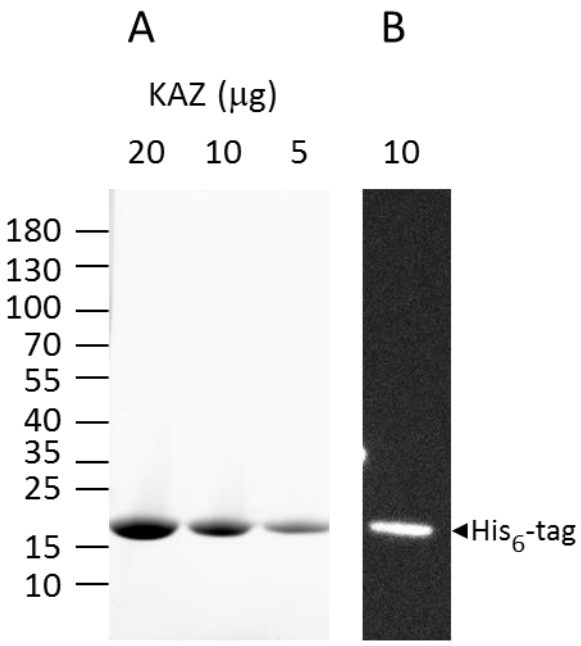


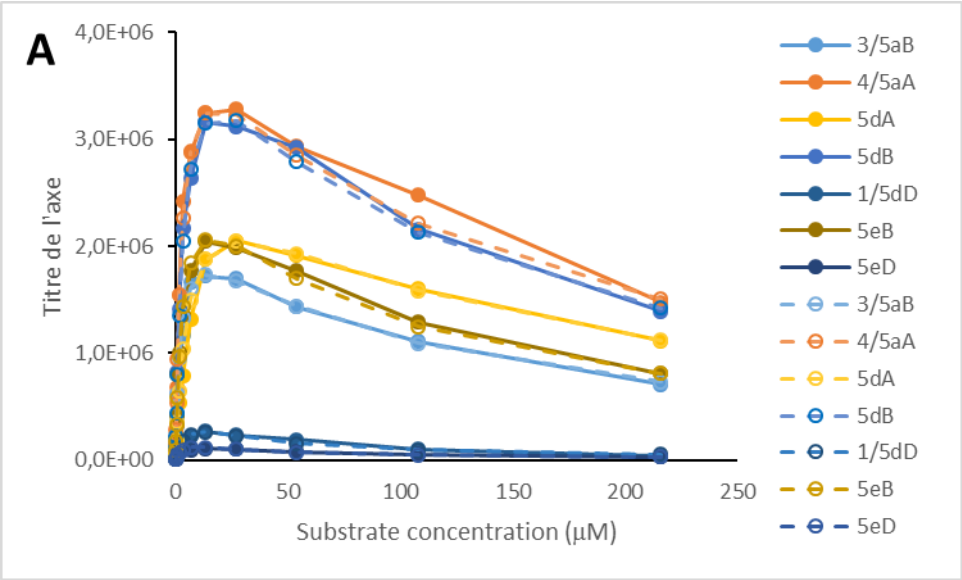
Figure S2. SDS-PAGE and western blot of purified nanoKAZ luciferase. The molecular weight scale (kDa) is indicated on the left. (A) The lanes from left to right show stained nanoKAZ bands (19 kDa) corresponding to 20, 10 and 5 □g solution samples respectively. (B) The lane shows the immunoprint of nanoKAZ band (19 kDa, 10 □g) revealed through its C-end His_6-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 10^6 times in PBS/tween buffer and 50 □L was added per well. The plate was orbitally shaked 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.


A
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3,0E+06
2,0E+06
1,0E+06
0,0E+00
Titre de l'axe
0
50
100
150
200
250
Substrate concentration (µM)
3/5aB
4/5aA
5dA
5dB
1/5dD
5eB
5eD
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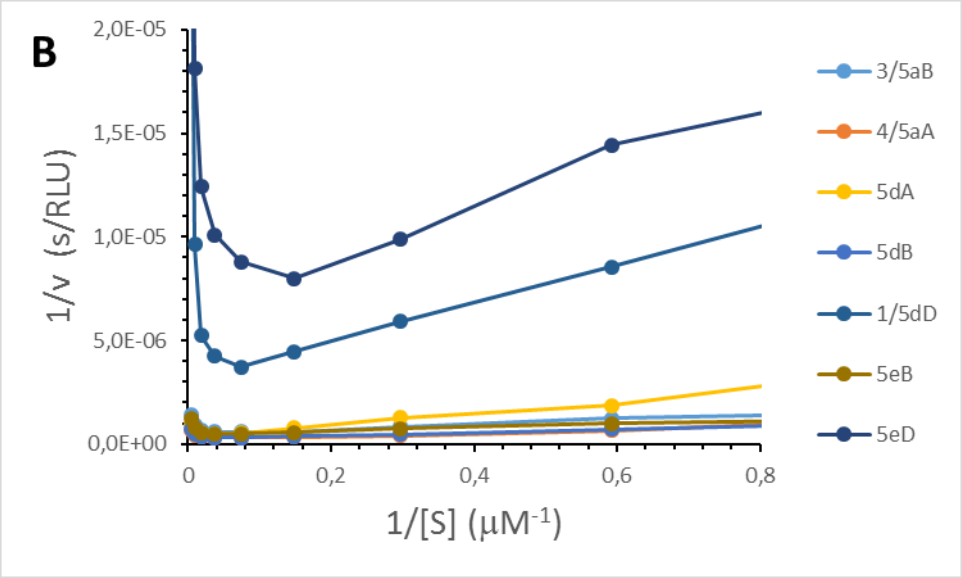
4/5aA
5dA
5dB
1/5dD
5eB
5eD


B
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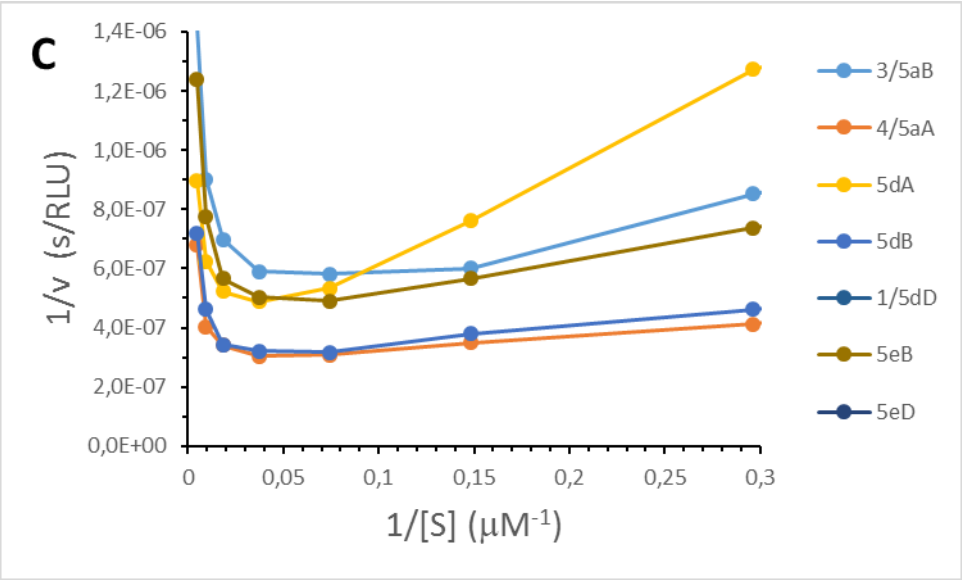
1,5E-05
1,0E-05
5,0E-06
0,0E+00
1/v (s/RLU)
0
0,2
0,4
0,6
0,8
1/[S] (µM-1)
3/5aB
4/5aA
5dA
5dB
1/5dD
5eB
5eD


C
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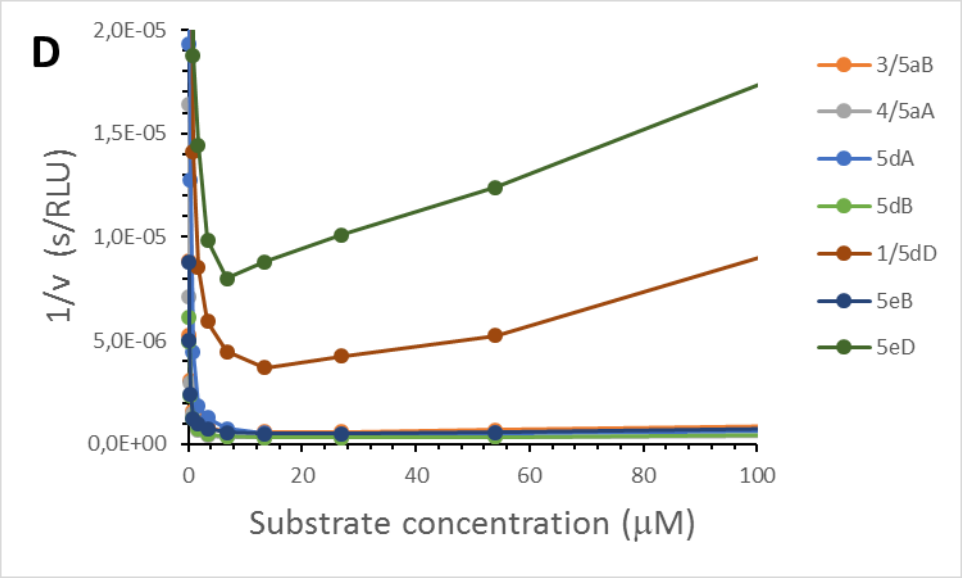
1,0E-06
8,0E-07
6,0E-07
4,0E-07
2,0E-07
0,0E+00
1/v (s/RLU)
0
0,05
0,1
0,15
0,2
0,25
0,3
1/[S] (µM-1)
3/5aB
4/5aA
5dA
5dB
1/5dD
5eB
5eD


D
2,0E-05
1,5E-05
1,0E-05
5,0E-06
0,0E+00
1/v (s/RLU)
0
20
40
60
80
100
Substrate concentration (µM)
3/5aB
4/5aA
5dA
5dB
1/5dD
5eB
5eD

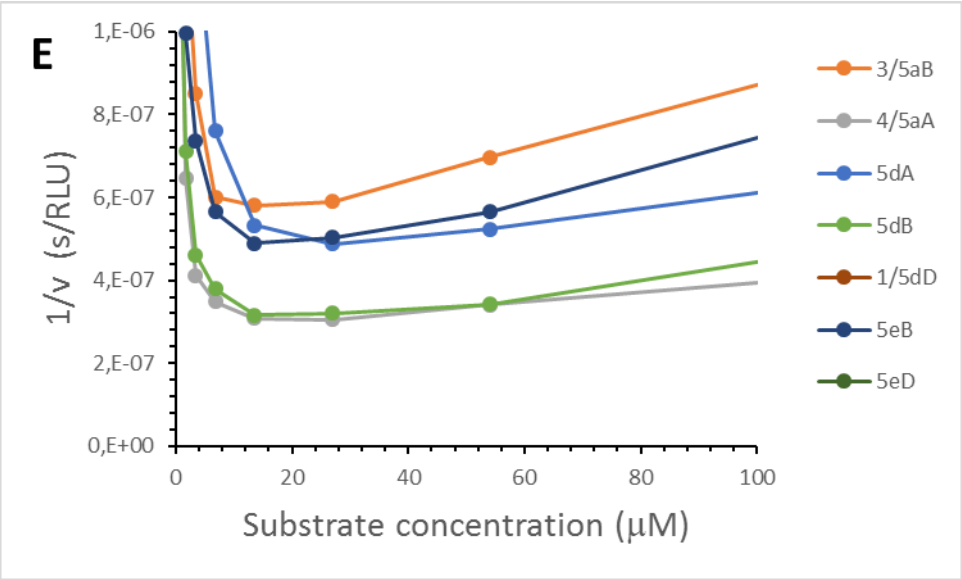


Figure S3. Maximal light intensity vs substrate concentration. (**A**) The maximal light intensity (RLU/s) is plotted in plain lines versus substrate concentrations (10^{-6}M) with the same enzyme concentration ($1.24 \cdot 10^{-12}$M). The kinetics fit with a Michaelis-Menten model (dashed lines). All 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).

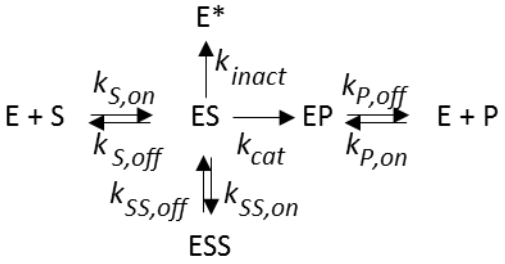
Table S1, light absorption of all the substrates between 400 and 600 nm.

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
$\varepsilon_{440\ nm}$ ($M^{-1}cm^{-1}$)	5010	7453	6594	6811	8326	5013	6496
$\varepsilon_{450\ nm}$ ($M^{-1}cm^{-1}$)	3946	6715	5779	5633	7137	4003	5244
$\varepsilon_{460\ nm}$ ($M^{-1}cm^{-1}$)	2964	5832	4781	4409	5734	3050	3981
$\varepsilon_{440\text{-}460\ nm}$ ($M^{-1}cm^{-1}$)	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 $M^{-1}cm^{-1}$. 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 / $e^{(\varepsilon L c)}$ 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 μM 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 (□, 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 O_2 as saturating substrate in the experimental assay conditions (100 □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

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

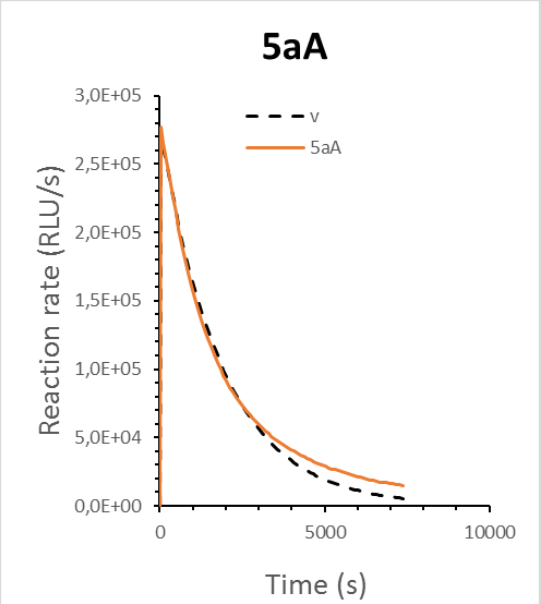
$$K_I = \frac{k_{SS,off}}{k_{SS,on}} \text{ (mol L}^{-1}\text{)} \; ; \; K_M = \frac{k_{S,off} + k_{cat}}{k_{S,on}} = \frac{K'_M}{1 + \frac{[S]^2}{K_I}} \text{ (mol L}^{-1}\text{)}$$

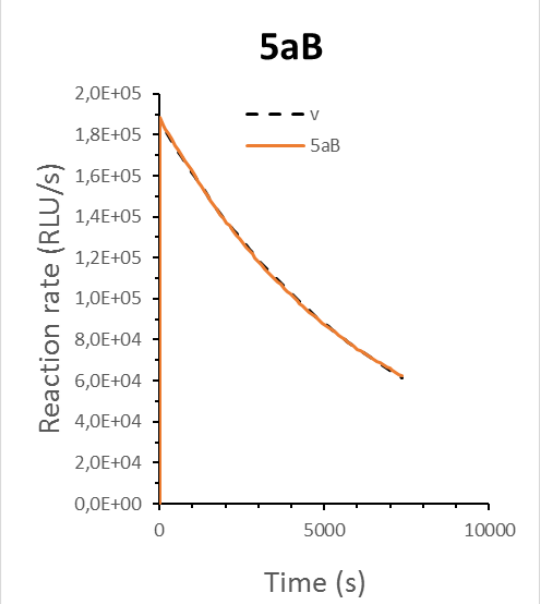
$$V_{max} = [E_0][S]k_{cat} = V'_{max}\frac{[S] + K_M + \frac{[S]^2}{K_I}}{[S]} \text{ (mol s}^{-1}\text{)}$$

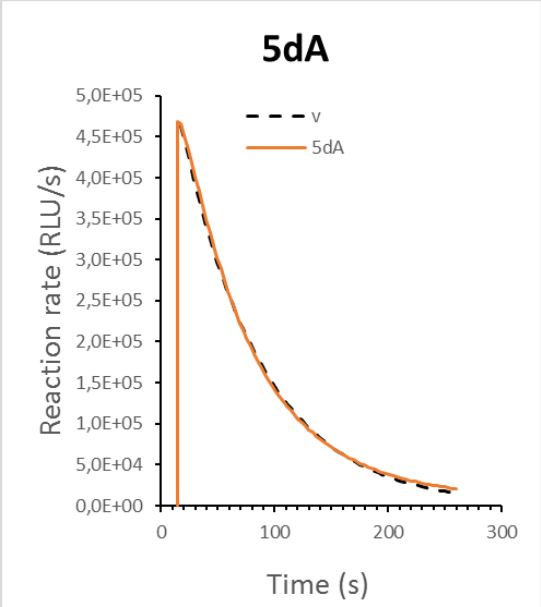
$$v' = \frac{[E][S]k_{cat}}{[S] + K_M + \frac{[S]^2}{K_I}} \text{ (RLU s}^{-1}\text{)}$$

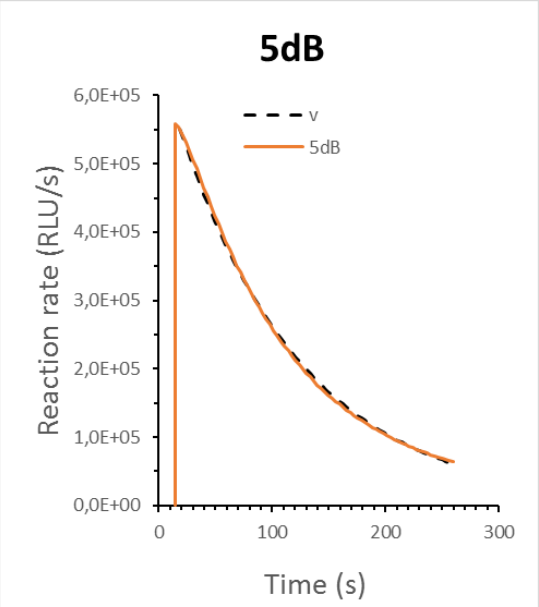
$[E] = [E_0]e^{-t k_{inact}}$, with k_{inact} in s^{-1} and the time t in s

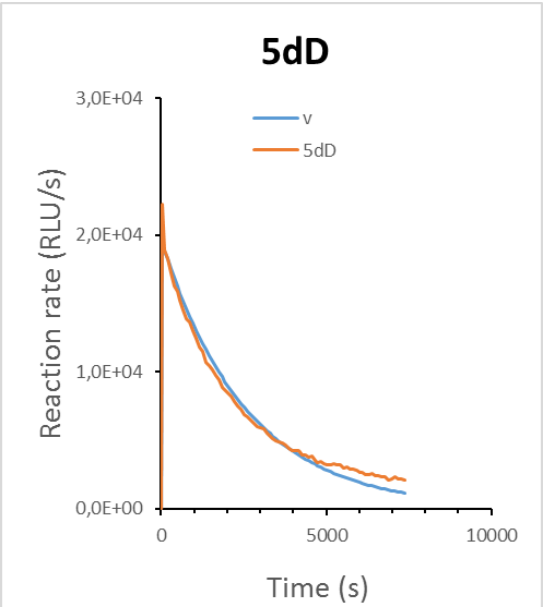
$v = v'\rho$ (mol s^{-1}) and $\rho = \frac{[S]N_A vol}{\Sigma I}$ (molecules. RLU^{-1}), $N_A = 6.02\ 10^{23}$ and vol the volume (L)

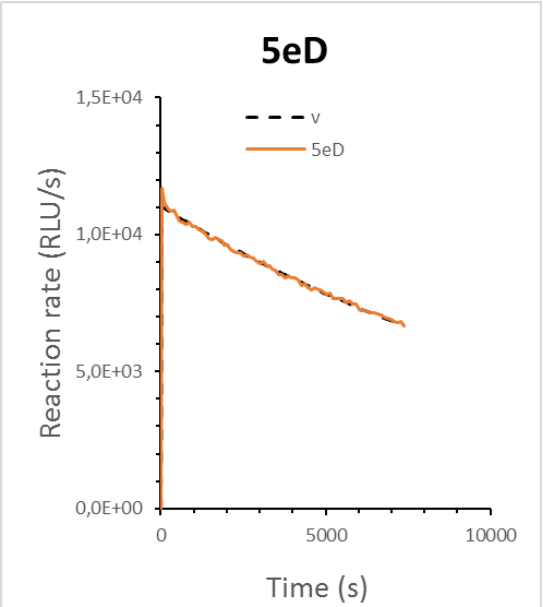












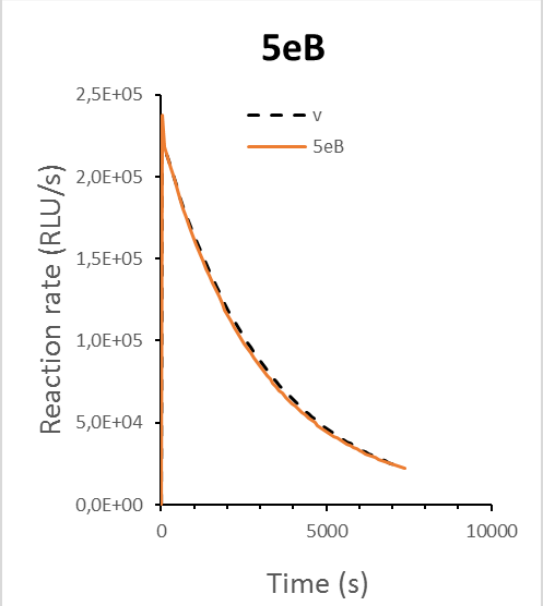
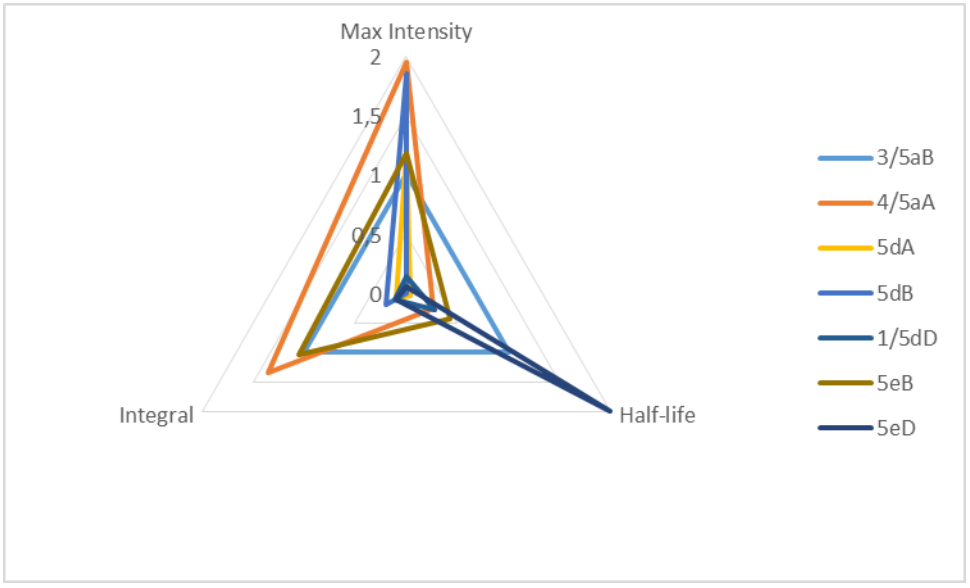


Figure S4: Enzyme inactivation kinetics. The kinetics are plotted versus time with the same enzyme concentration and the same substrate concentration. These plots compare the fitting of the experimental data (red plain line) with the theoretical reaction velocity computed according to the described Michaelis-Menten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

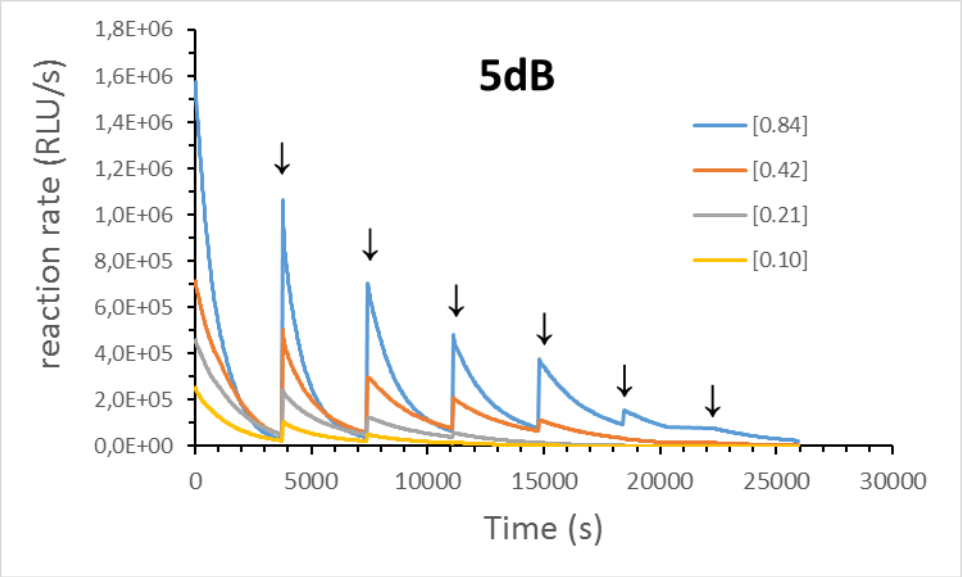
From these computations, the following kinetic parameters were obtained:

	3/5aB	4/5aA	5dA	5dB	1/5dD	5eB	5eD
$\mathbf{I_{max}}$ (10^6 RLU/s)	1.69	3.28	2.05	3.11	0.23	1.98	0.10
t½ (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
$\boldsymbol{K'_M}$ (10^{-6} M)	3.40	2.33	3.71	3.45	8.8	4.23	7.02
$\boldsymbol{k'_{cat}}$ (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
$\boldsymbol{K_I}$ (10^{-6} M)	104.94	115.21	140.55	101.64	22.48	84.02	35.42
$\boldsymbol{K_M}$ (10^{-6} M)	2.22	3.02	5.90	3.88	6.80	3.30	2.94
$\boldsymbol{k_{cat}}$ (mol/s·mol E)	106	534	932	535	1500	140	362
$\boldsymbol{k_{inact}}$ (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**.



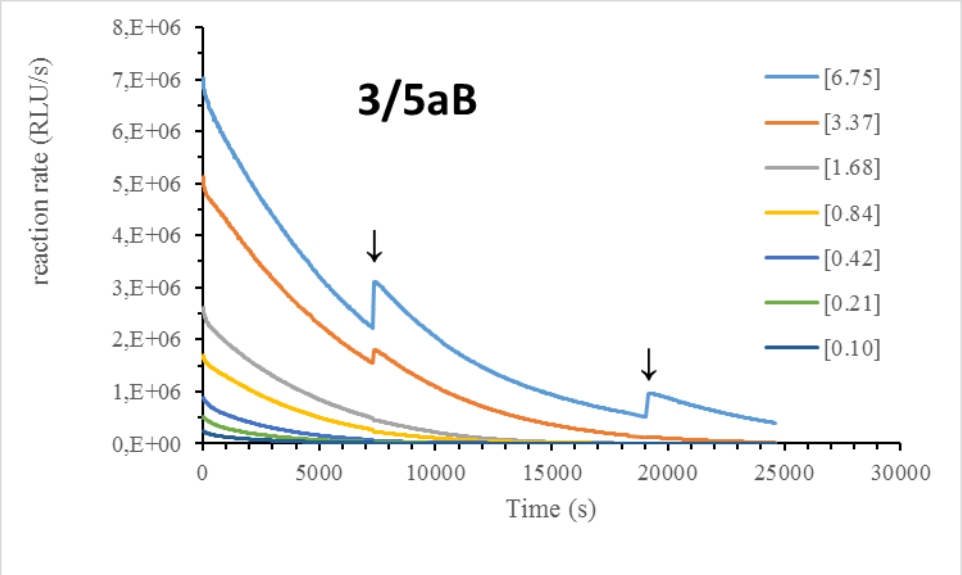


Figure S5. Luciferase inactivation in the course of the catalyzed oxidation of 5dB and 3/5aB.

References

1. K. Tanaka, E. Yamamoto and N. Watanabe, US 20090082403, 2009.
2. A. Conte-Mayweg, H. Kuehne, T. Luebbers, C. Maugeais, W. Mueller and P. Pflieger, US 2006030613, 2006.
3. E. P. Coutant, V. Hervin, G. Gagnot, C. Ford, R. Baatallah and Y. L. Janin, *Beilstein J. Org. Chem.*, 2018, 2853-2859.
4. G. Gagnot, V. Hervin, E. P. Coutant, S. Desmons, R. Baatallah, V. Monnot and Y. L. Janin, *Beilstein J. Org. Chem.*, 2018, **14**, 2846-2852.
5. S. Inouye, J. Sato, Y. Sahara-Miura, S. Yoshida, H. Kurakata and T. Hosoya, *Biochem. Biophys. Res. Commun.*, 2013, **437**, 23-28.
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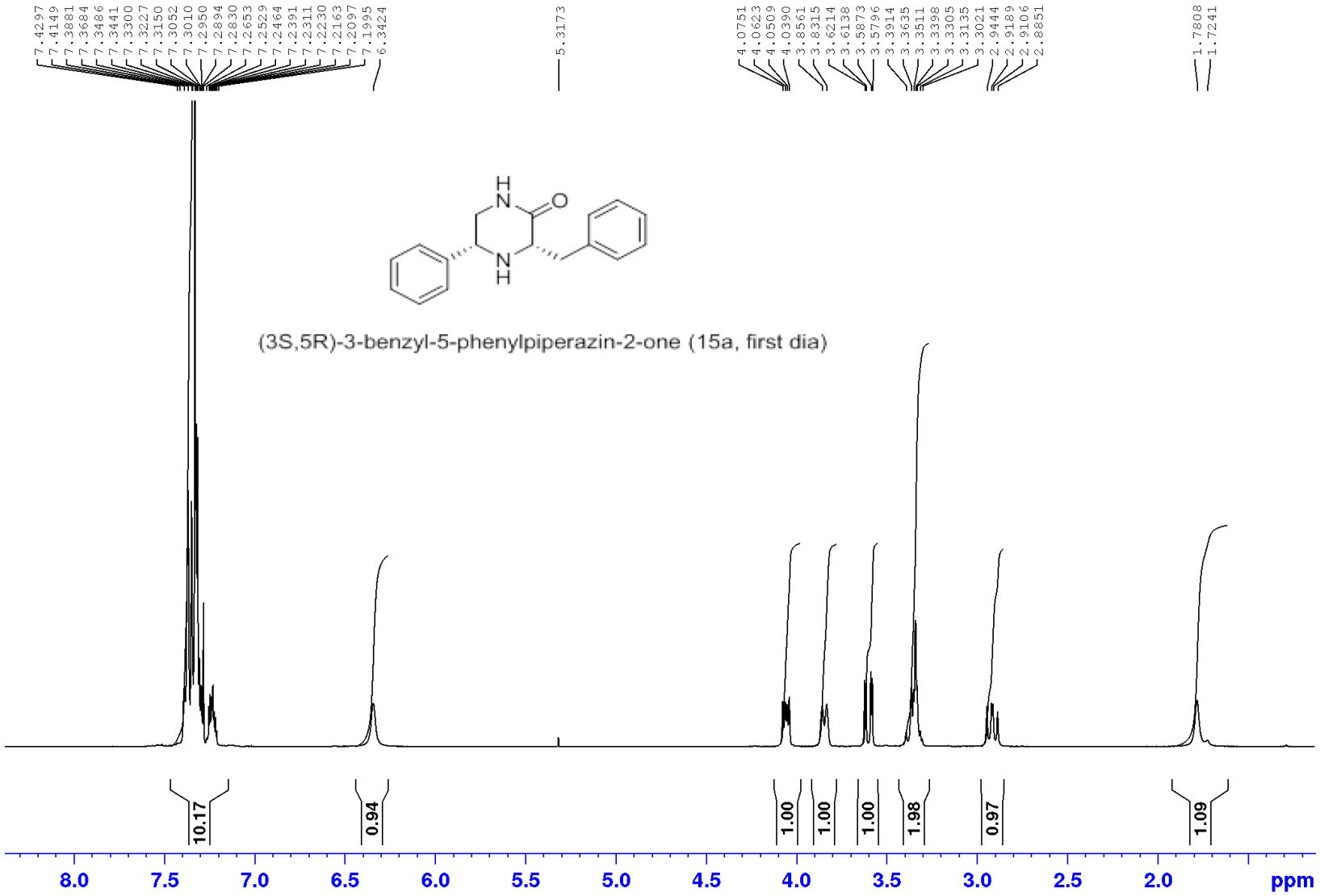
7. S. C. Gill and P. H. von Hippel, *Anal. Biochem.*, 1989, **182**, 319-326.

^{1}H and ^{13}C spectra



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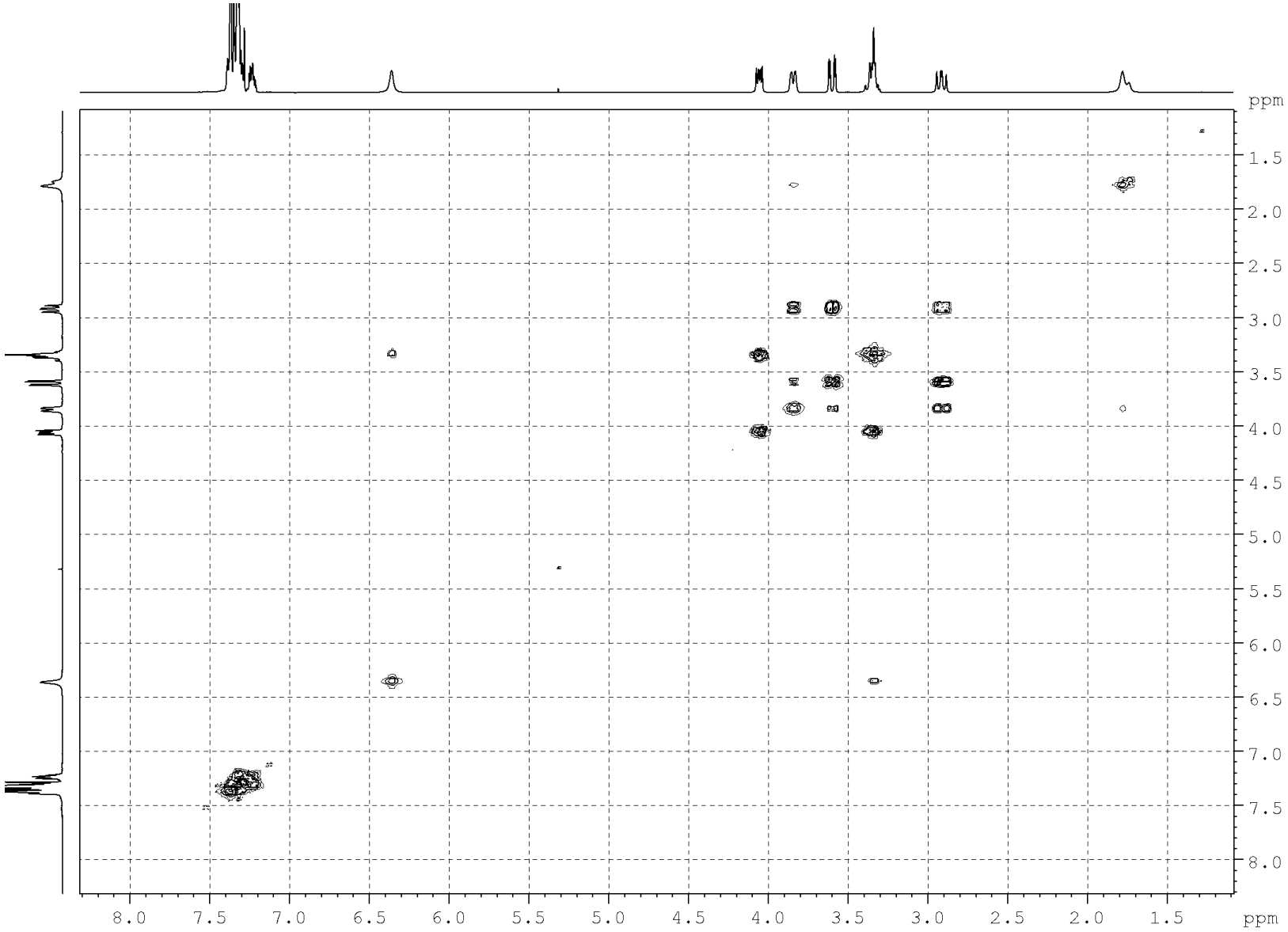

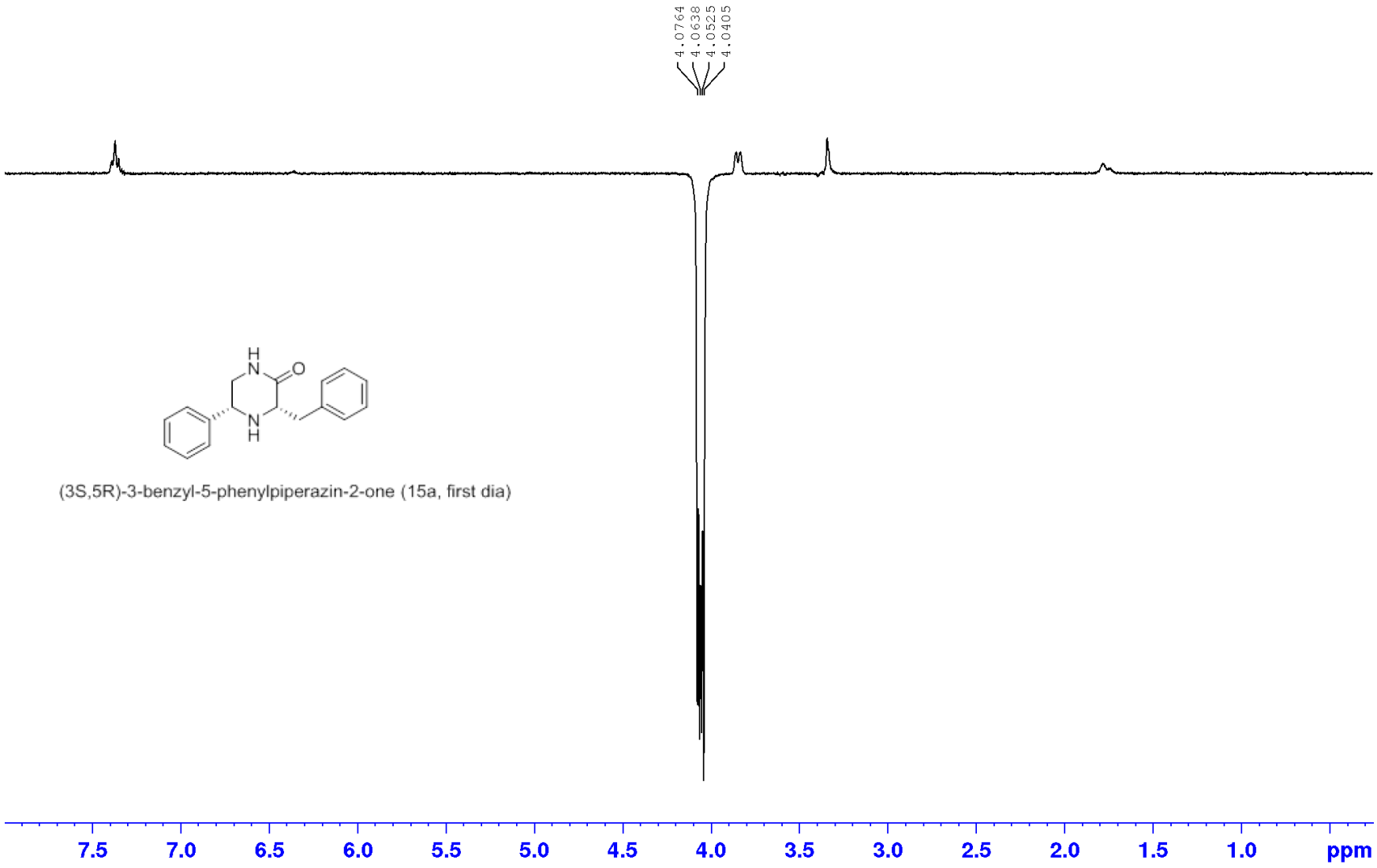
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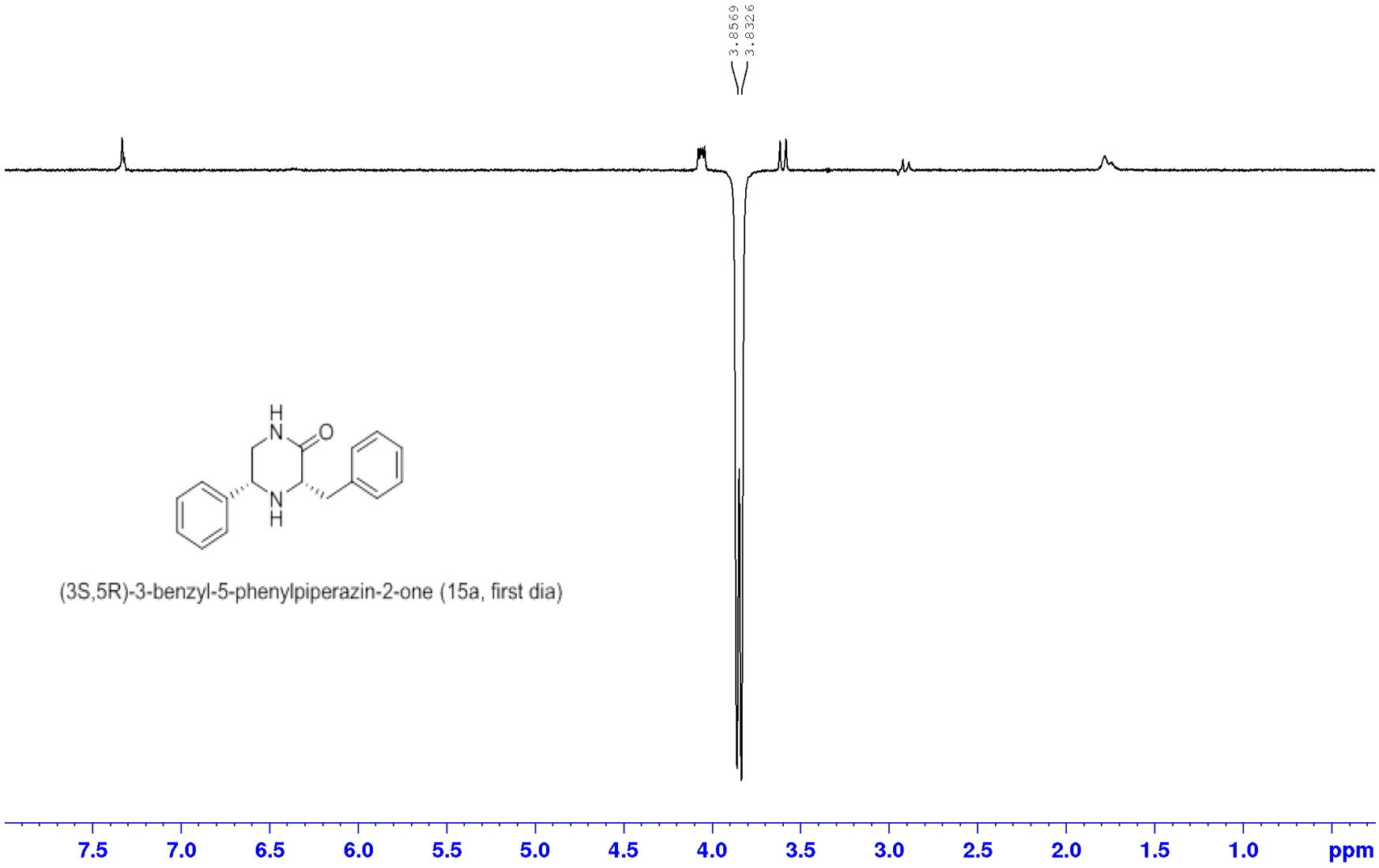
4.0638
4.0525
4.0405
H
N
O
N
H
(3S,5R)-3-benzyl-5-phenylpiperazin-2-one (15a, first dia)
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
ppm


3.8569
3.8326
H
N
O
N
H
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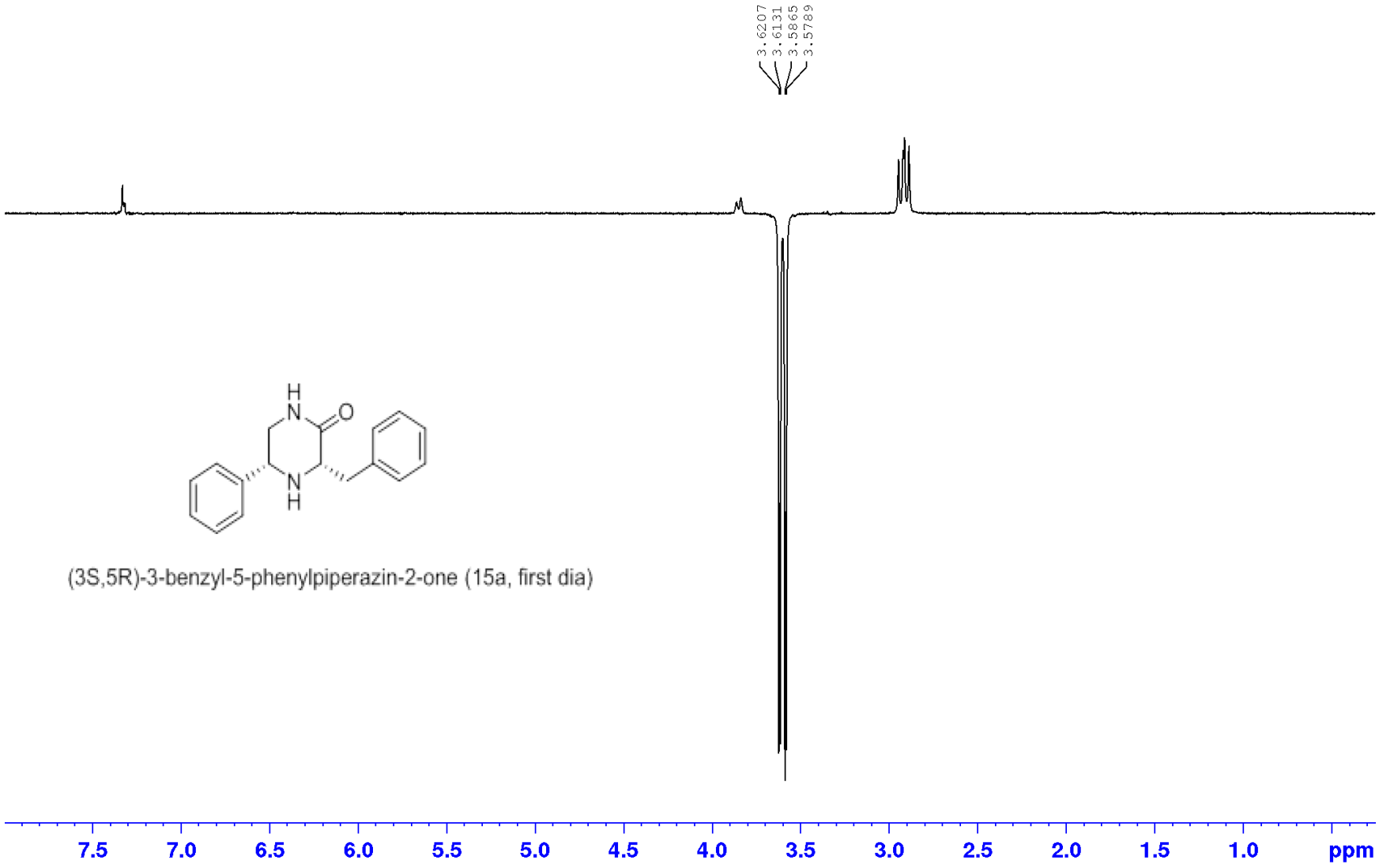
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
ppm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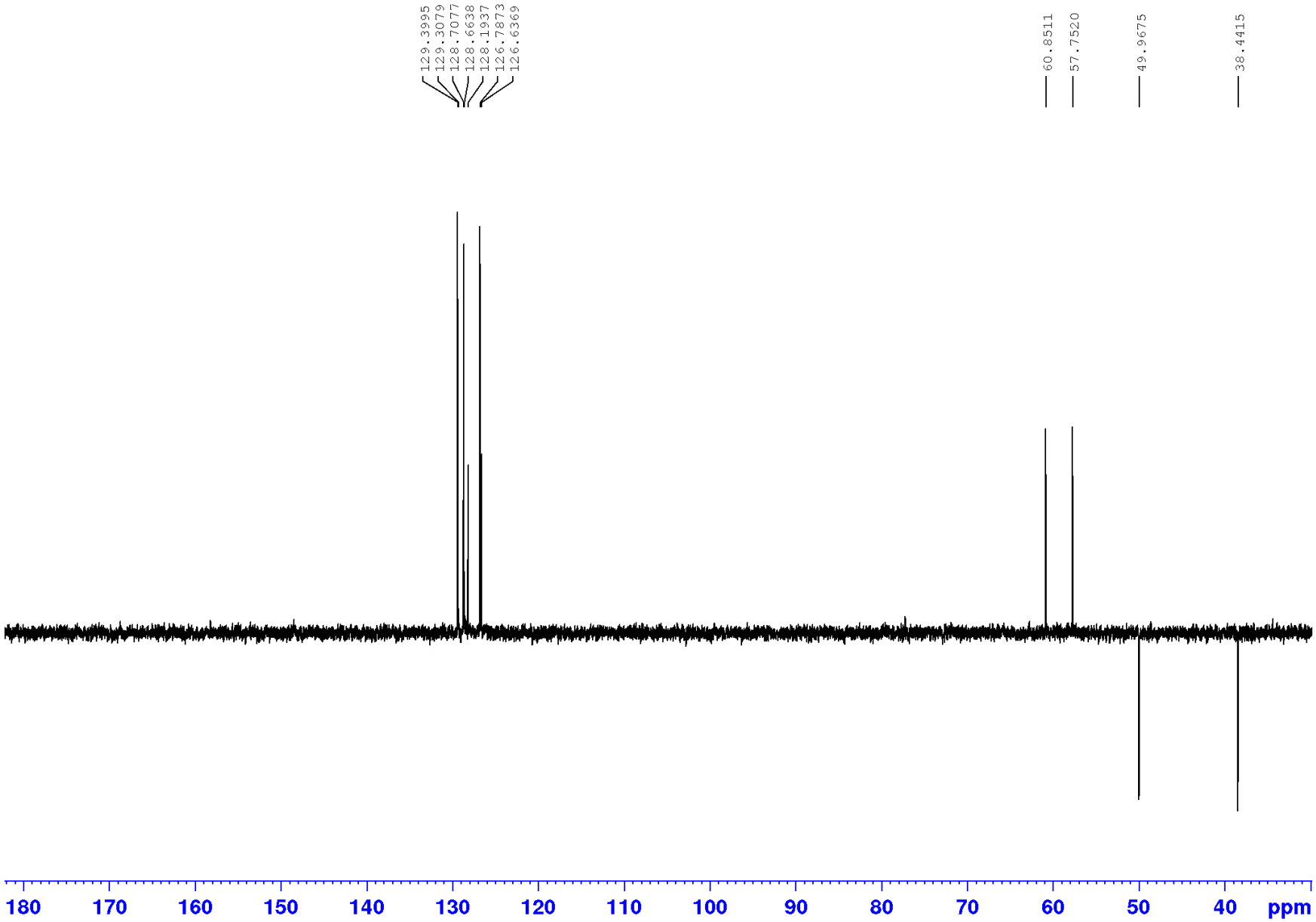
3.6131
3.5865
3.5789
H
N
O
N
H
(3S,5R)-3-benzyl-5-phenylpiperazin-2-one (15a, first dia)
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
ppm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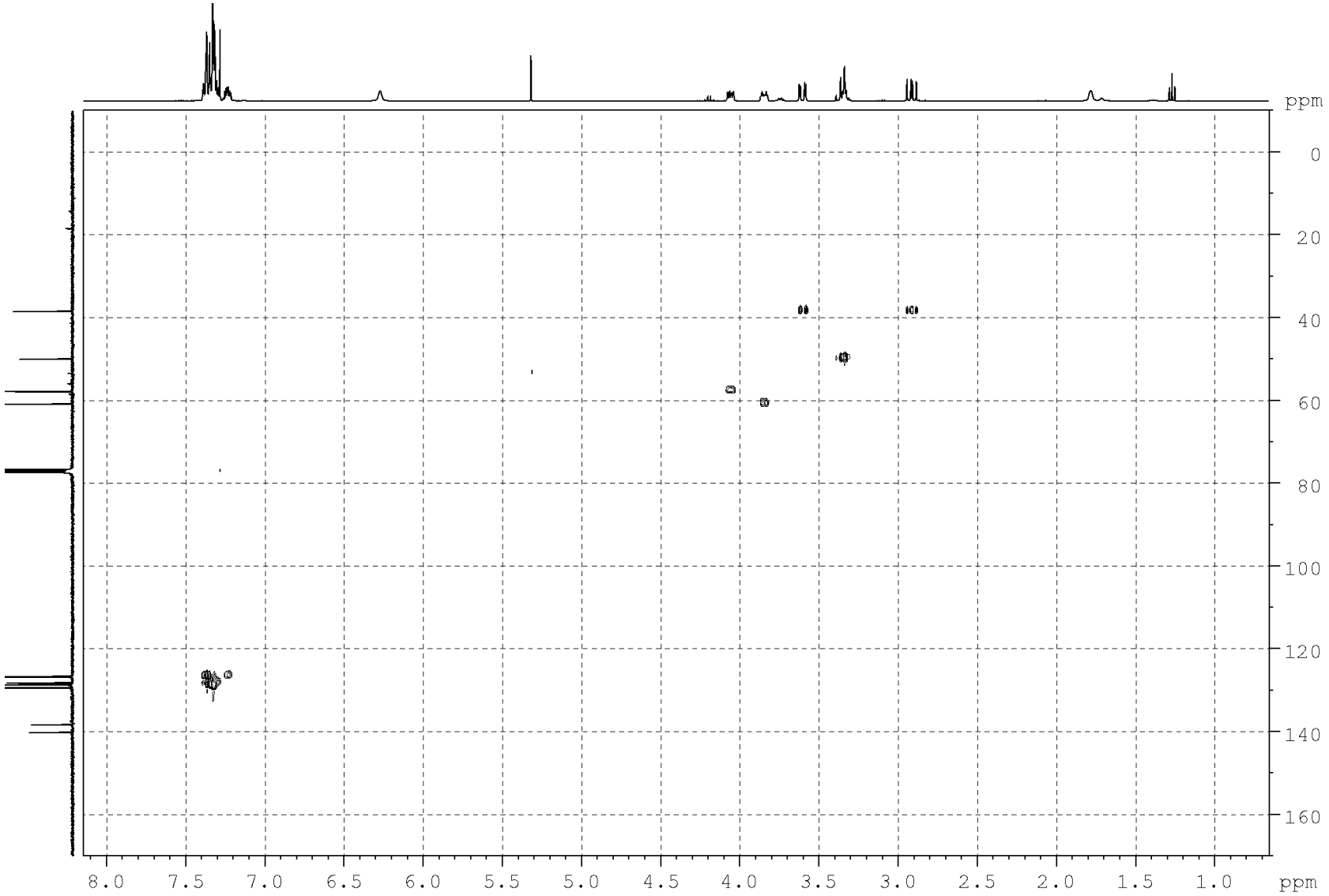
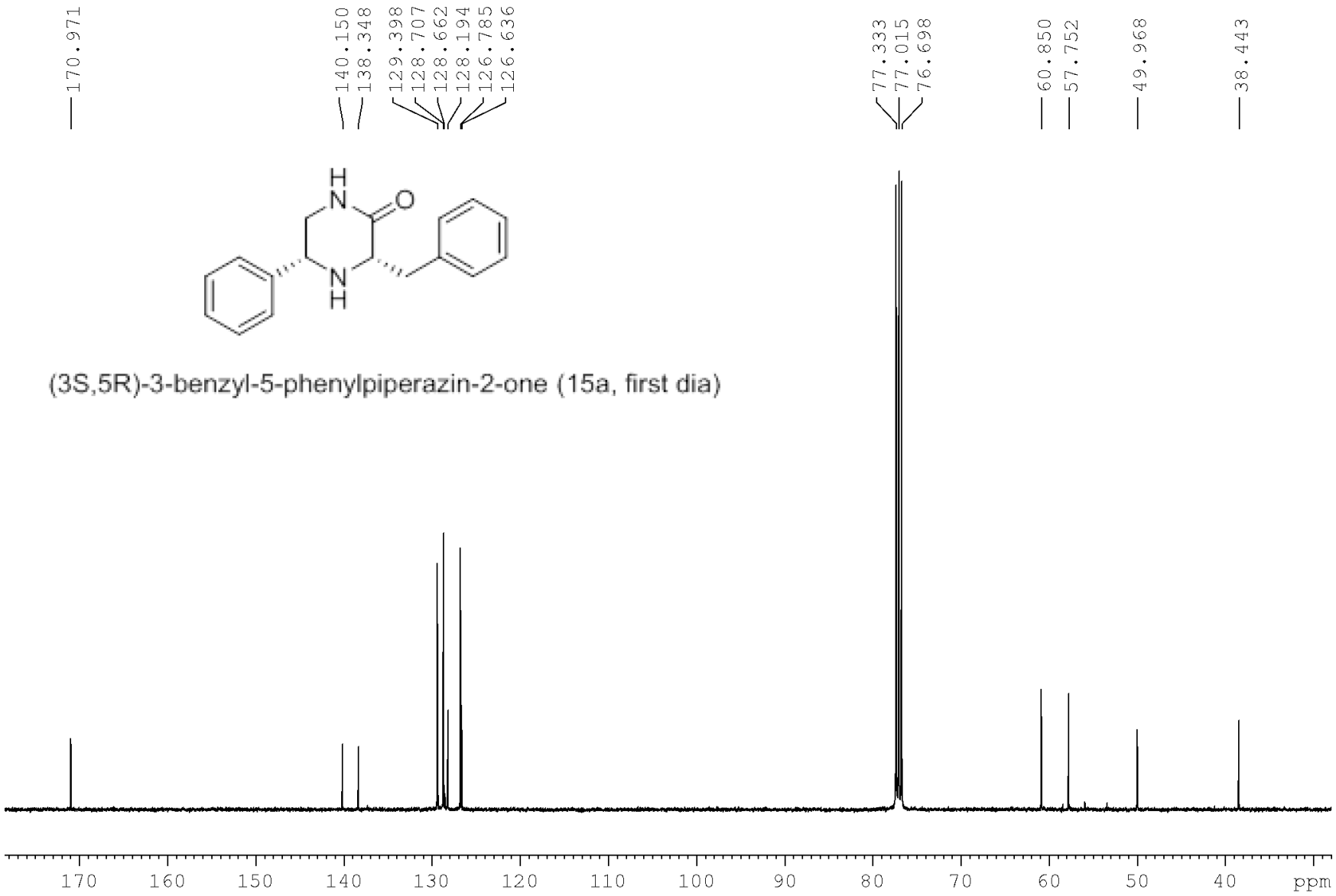
129.3079
128.7077
128.6638
128.1937
126.7873
126.6369
60.8511
57.7520
49.9675
38.4415
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
ppm

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

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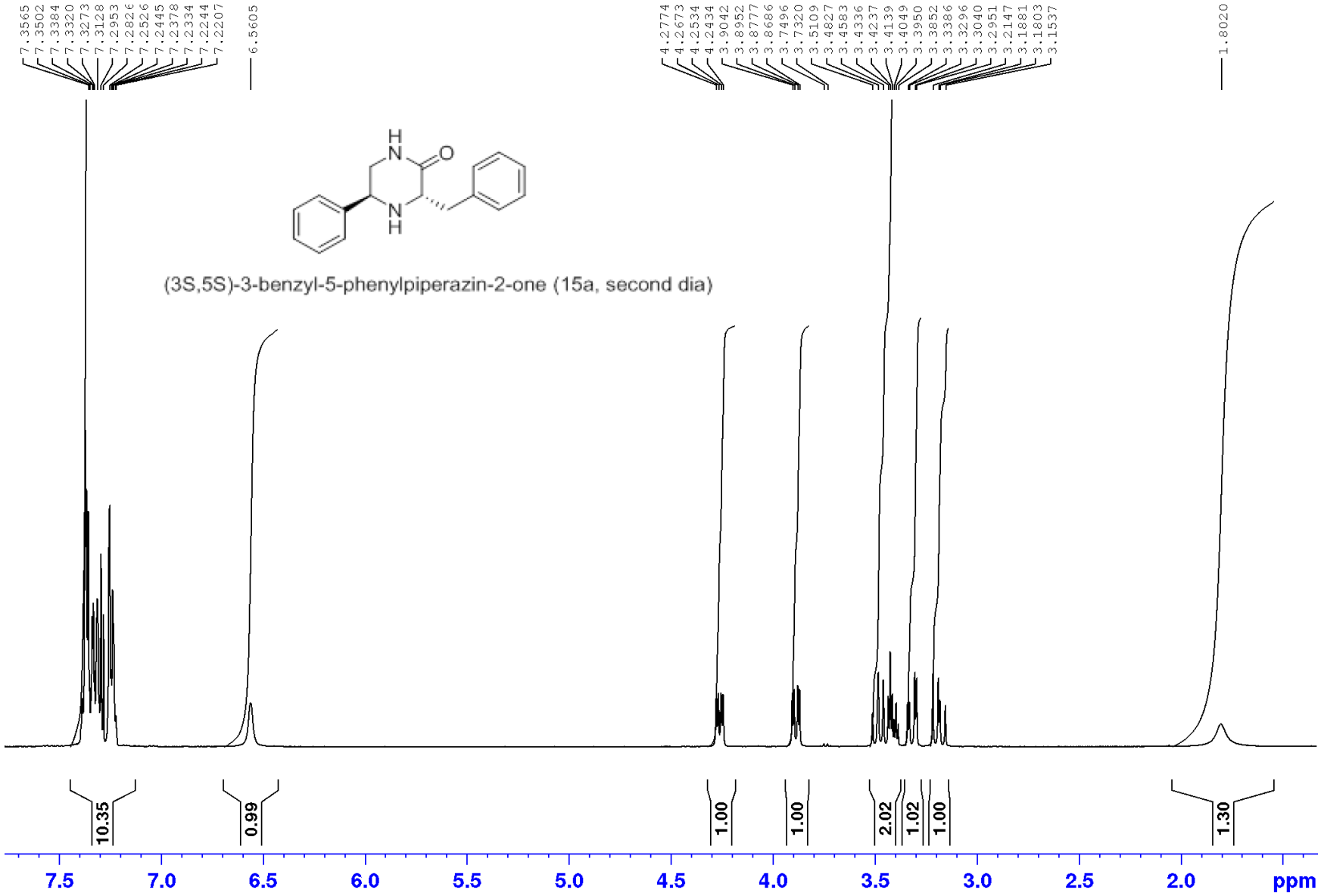
138.348
129.398
128.707
128.662
128.194
126.785
126.636
77.333
77.015
76.698
60.850
57.752
49.968
38.443
H
N
O
N
H
(3S,5R)-3-benzyl-5-phenylpiperazin-2-one (15a, first dia)
170
160
150
140
130
120
110
100
90
80
70
60
50
40
ppm



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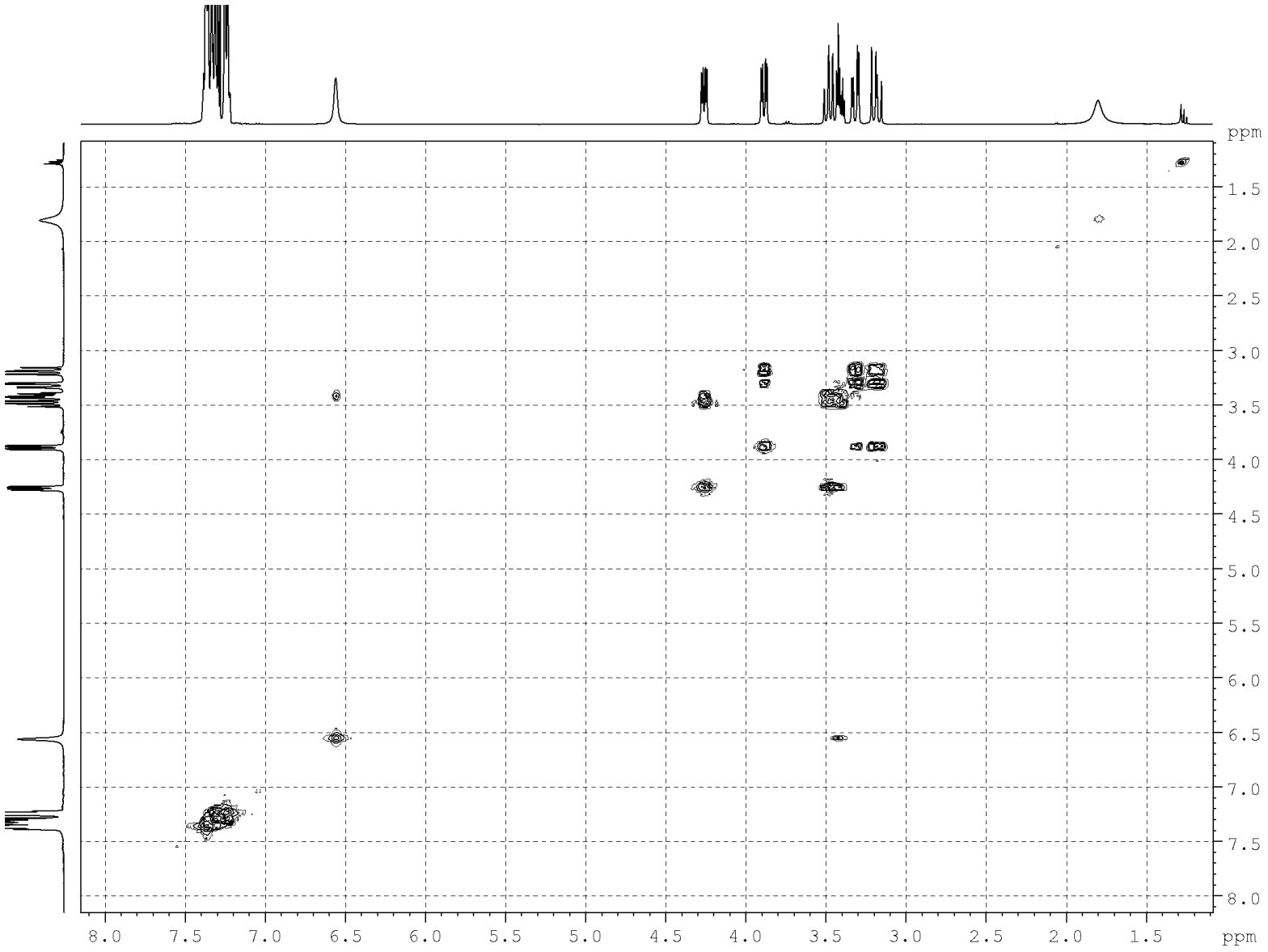
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7.5
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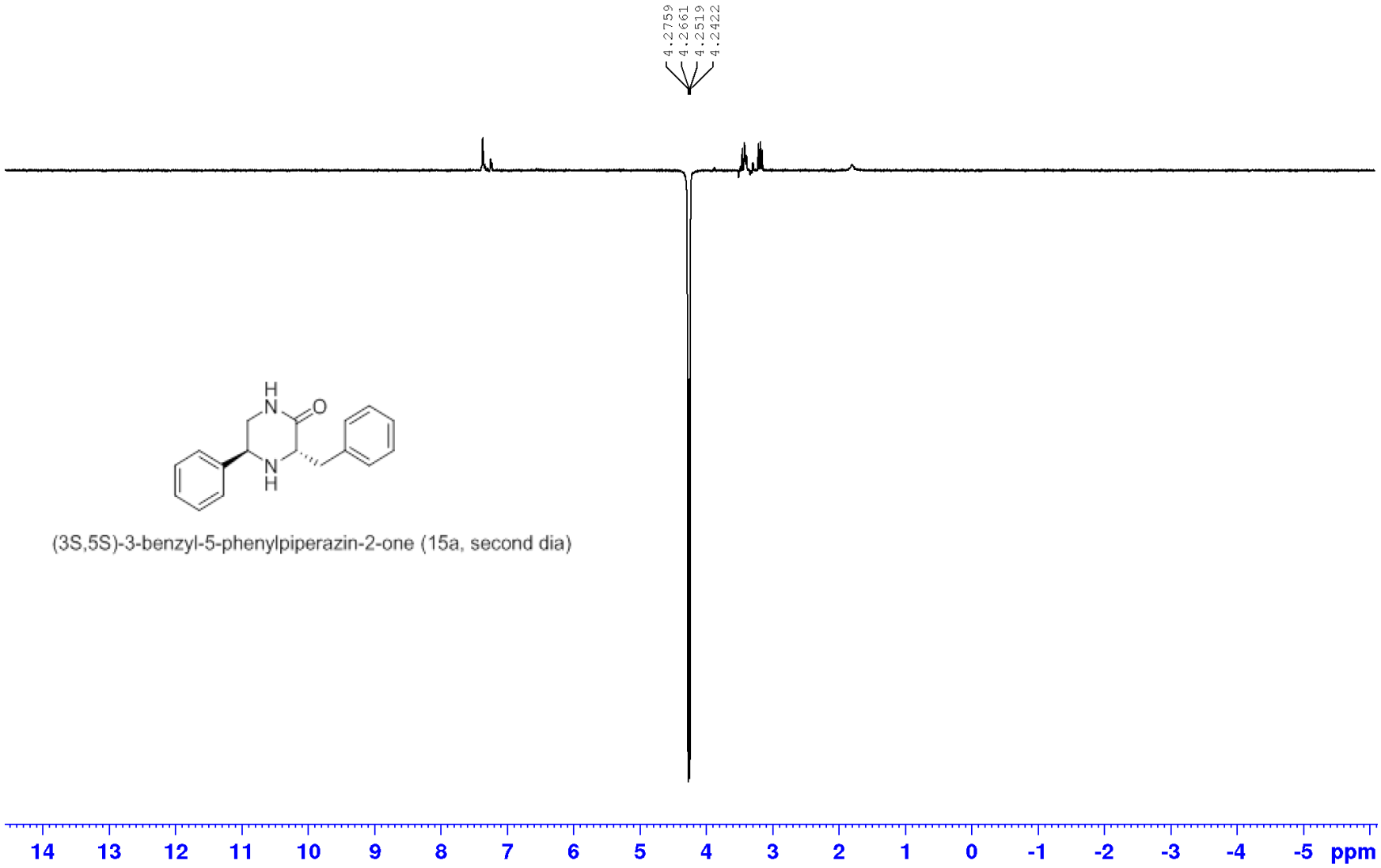
2.0
1.5
ppm



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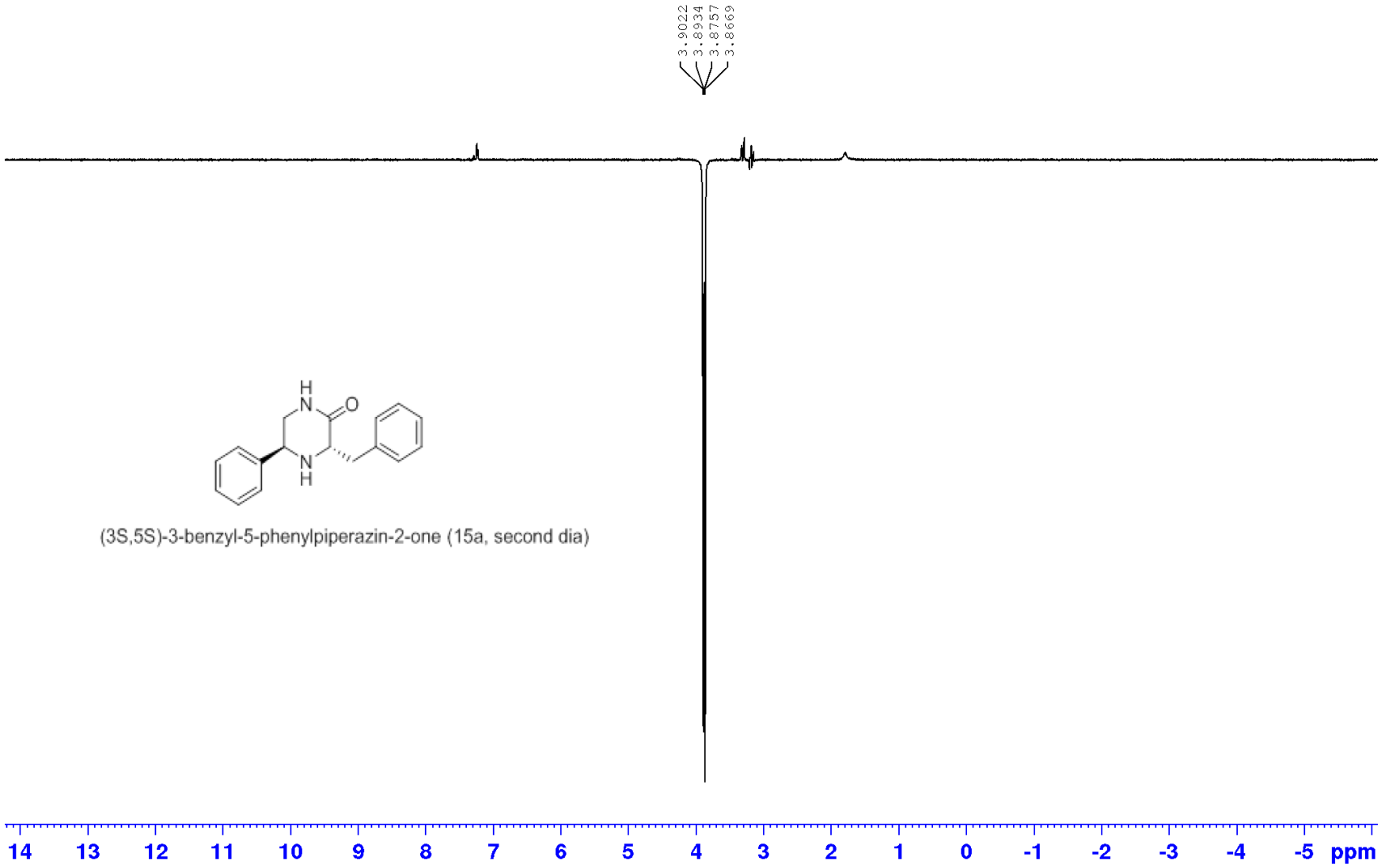
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H
N
O
N
H
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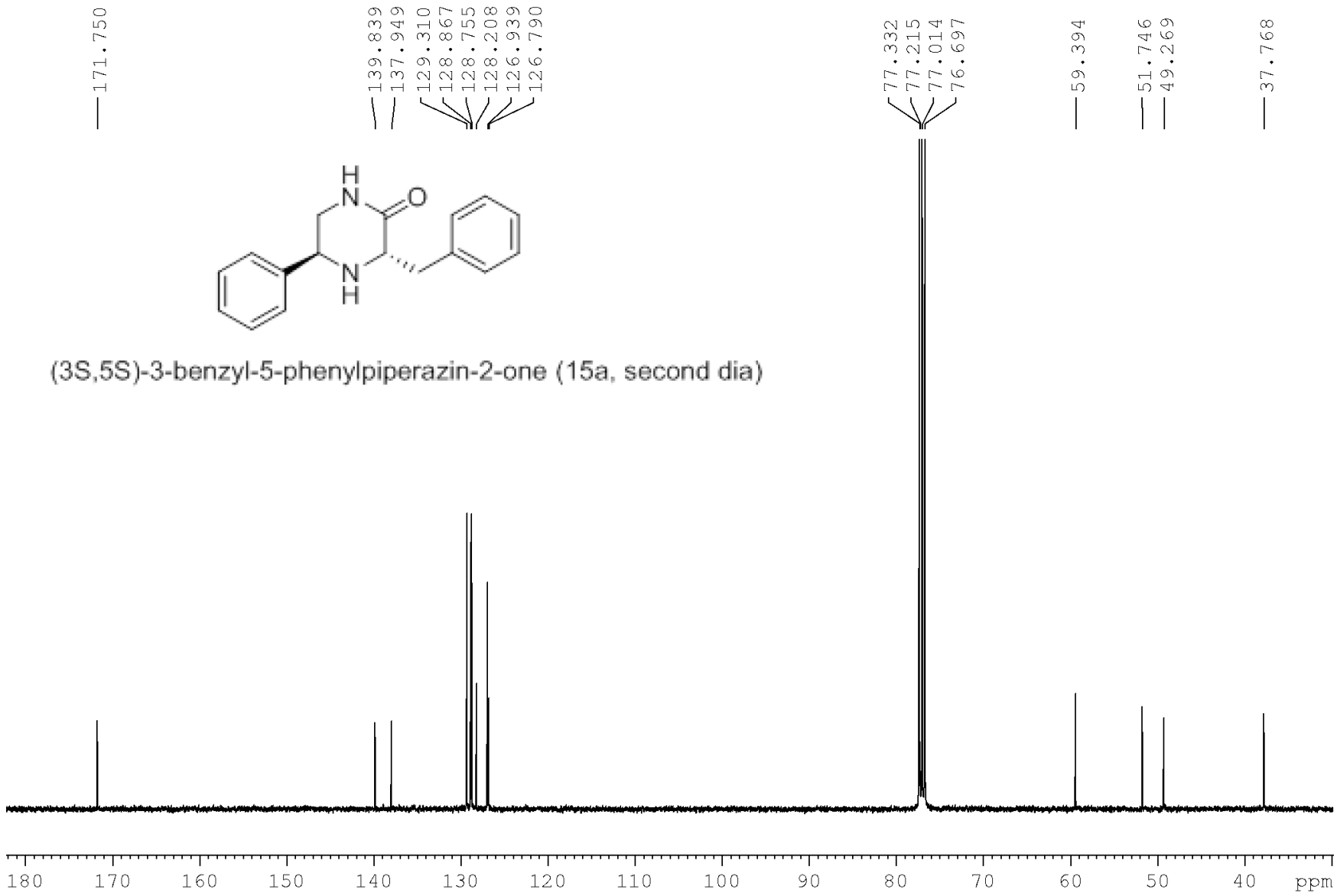
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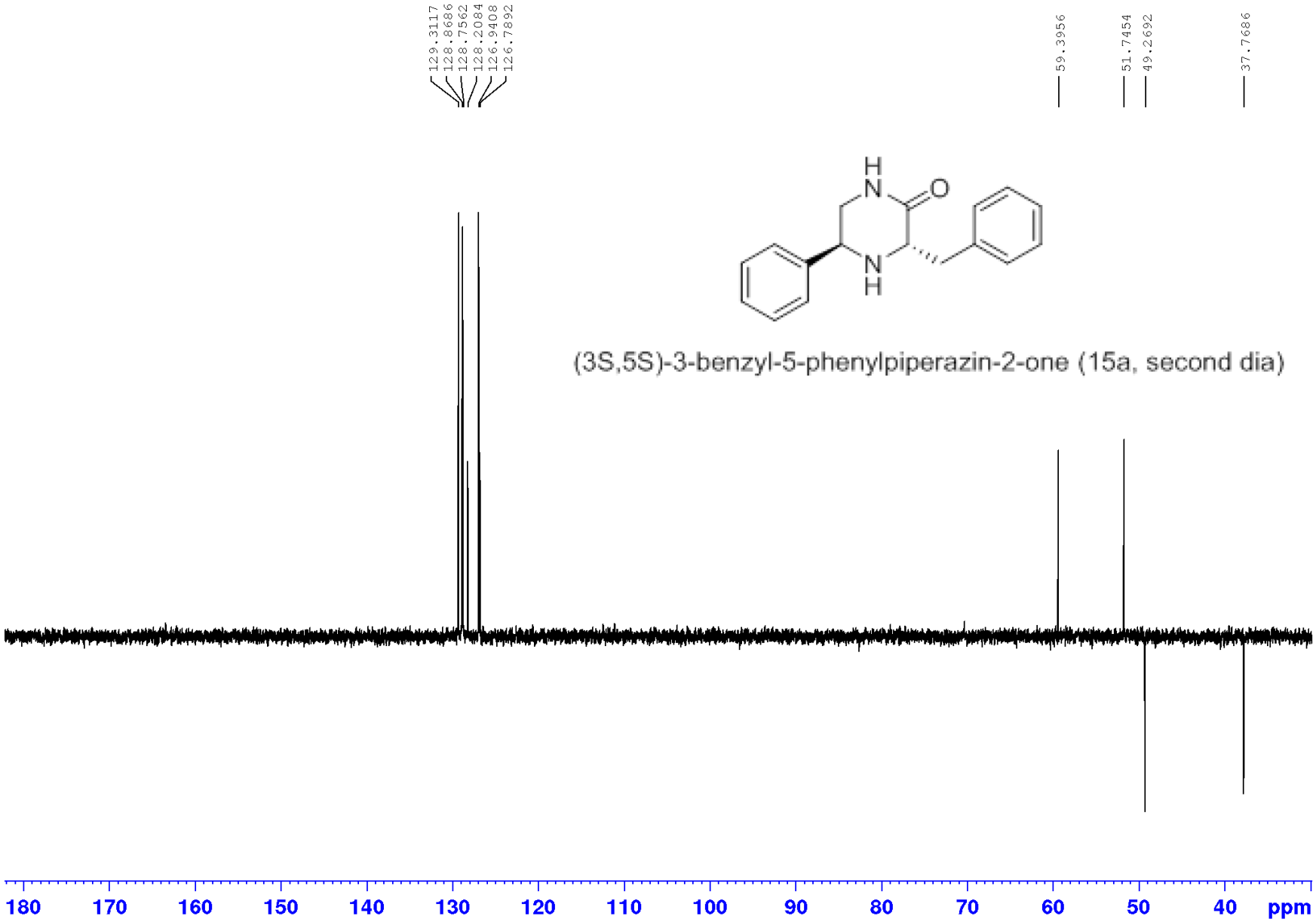
126.790
77.332
77.215
77.014
76.697
59.394
51.746
49.269
37.768
H
N
O
N
H
(3S,5S)-3-benzyl-5-phenylpiperazin-2-one (15a, second dia)
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
ppm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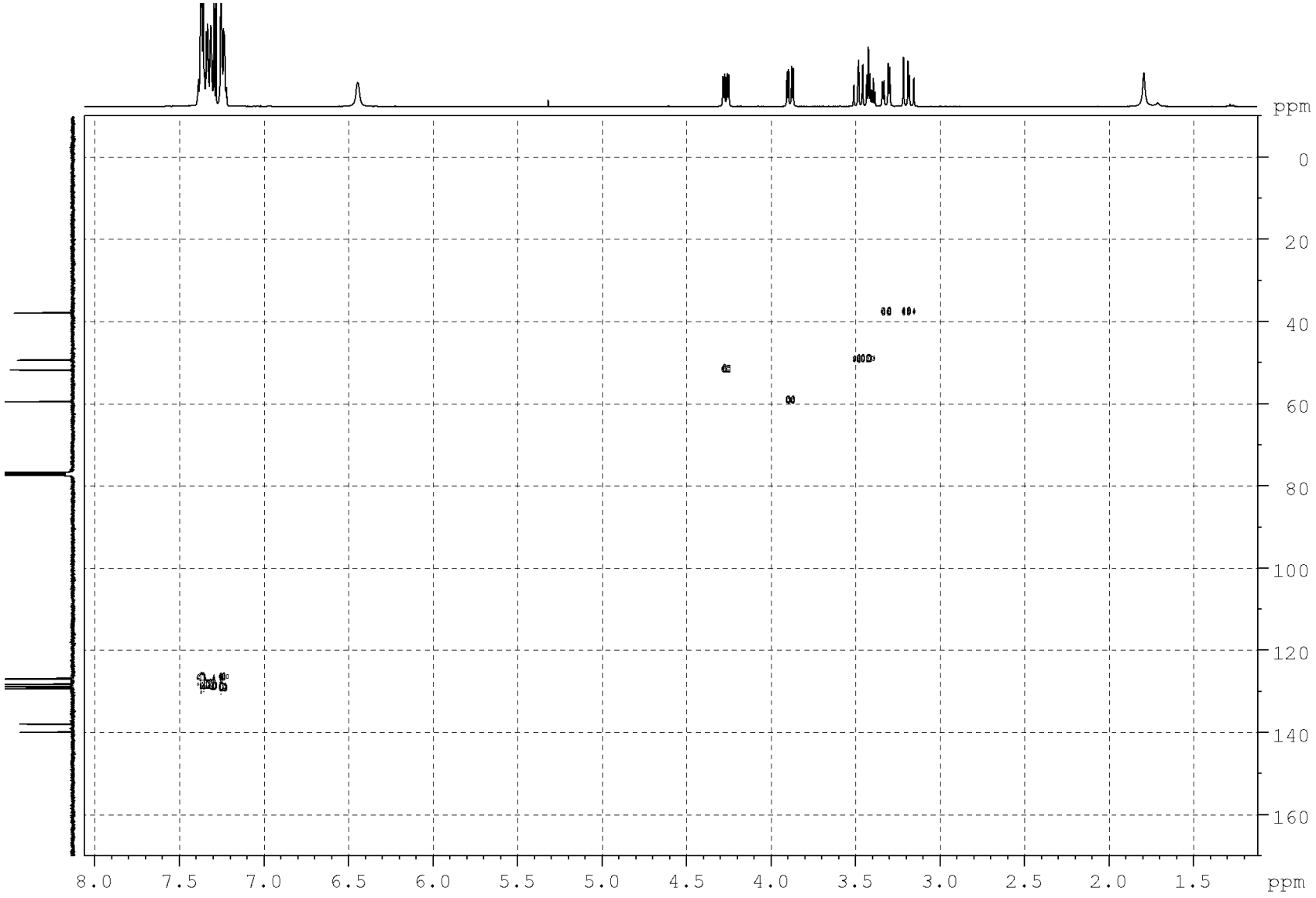
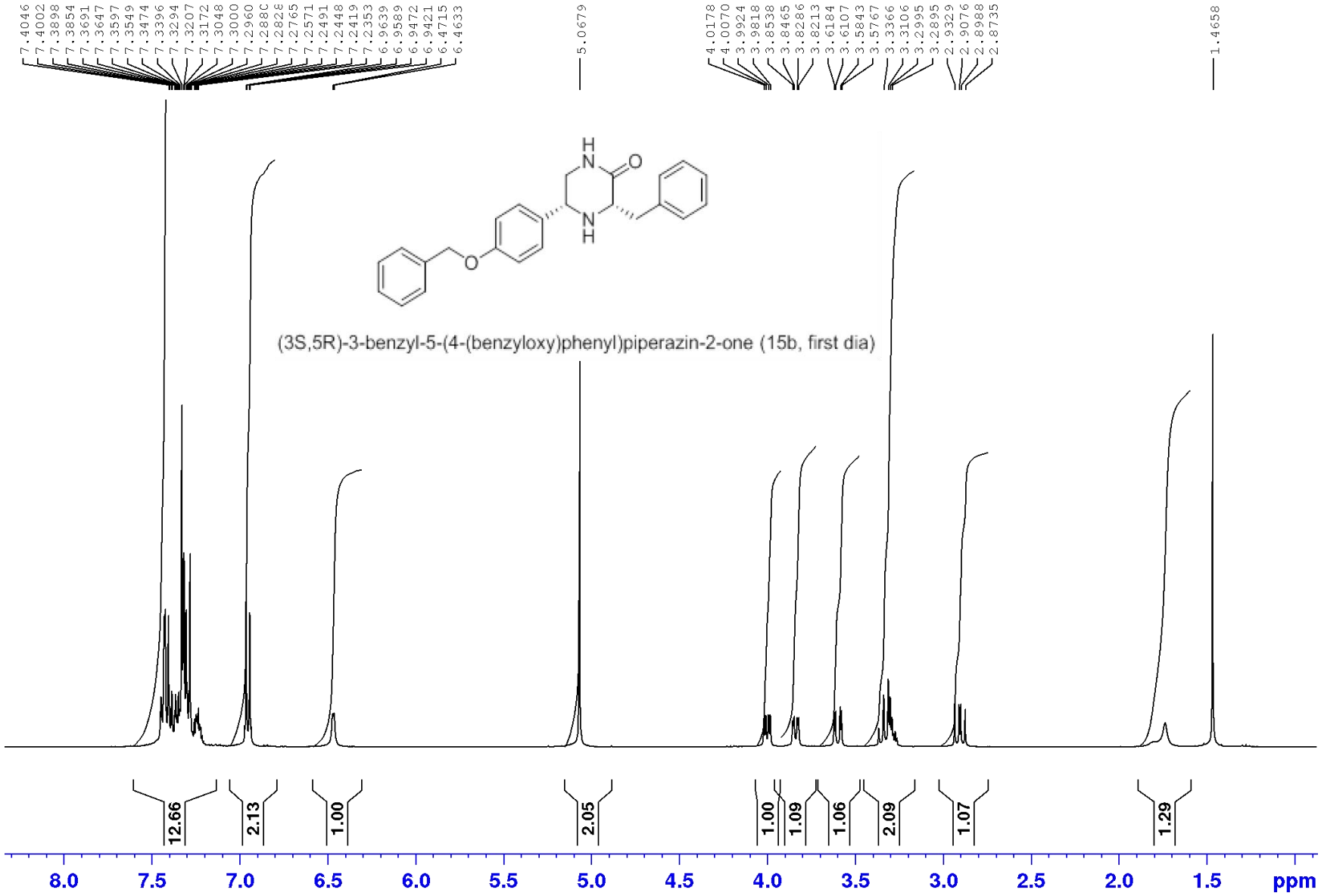
H
N
O
N
H
(3S,5S)-3-benzyl-5-phenylpiperazin-2-one (15a, second dia)
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
ppm

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

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7.2448
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7.2353
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2.9076
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(3S,5R)-3-benzyl-5-(4-(benzyloxy)phenyl)piperazin-2-one (15b, first dia)
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2.13
1.00
2.05
1.00
1.09
1.06
2.09
1.07
1.29
8.0
7.5
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4.5
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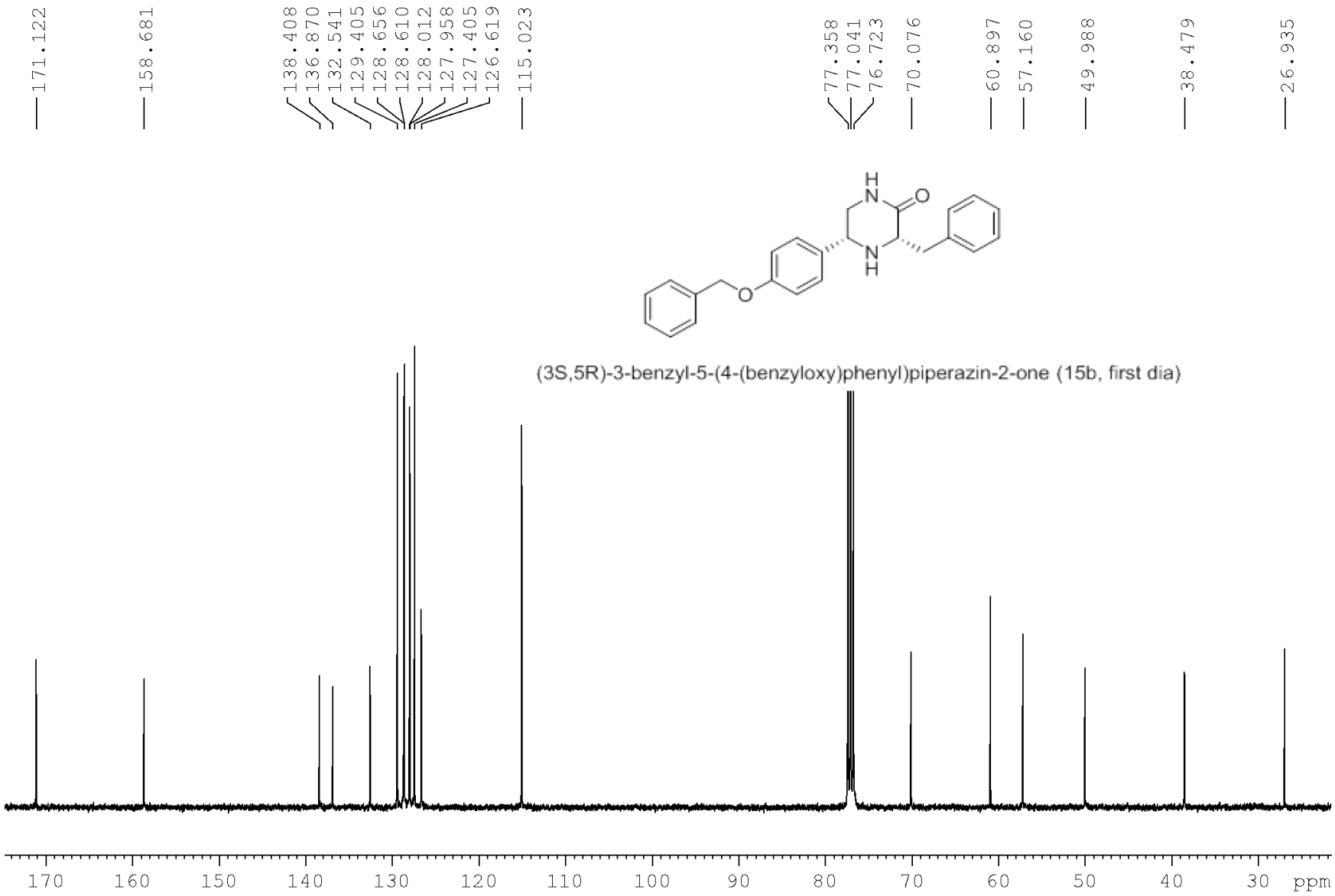
2.0
1.5
ppm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77.358
77.041
76.723
70.076
60.897
57.160
49.988
38.479
26.935
(3S,5R)-3-benzyl-5-(4-(benzyloxy)phenyl)piperazin-2-one (15b, first dia)
170
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140
130
120
110
100
90
80
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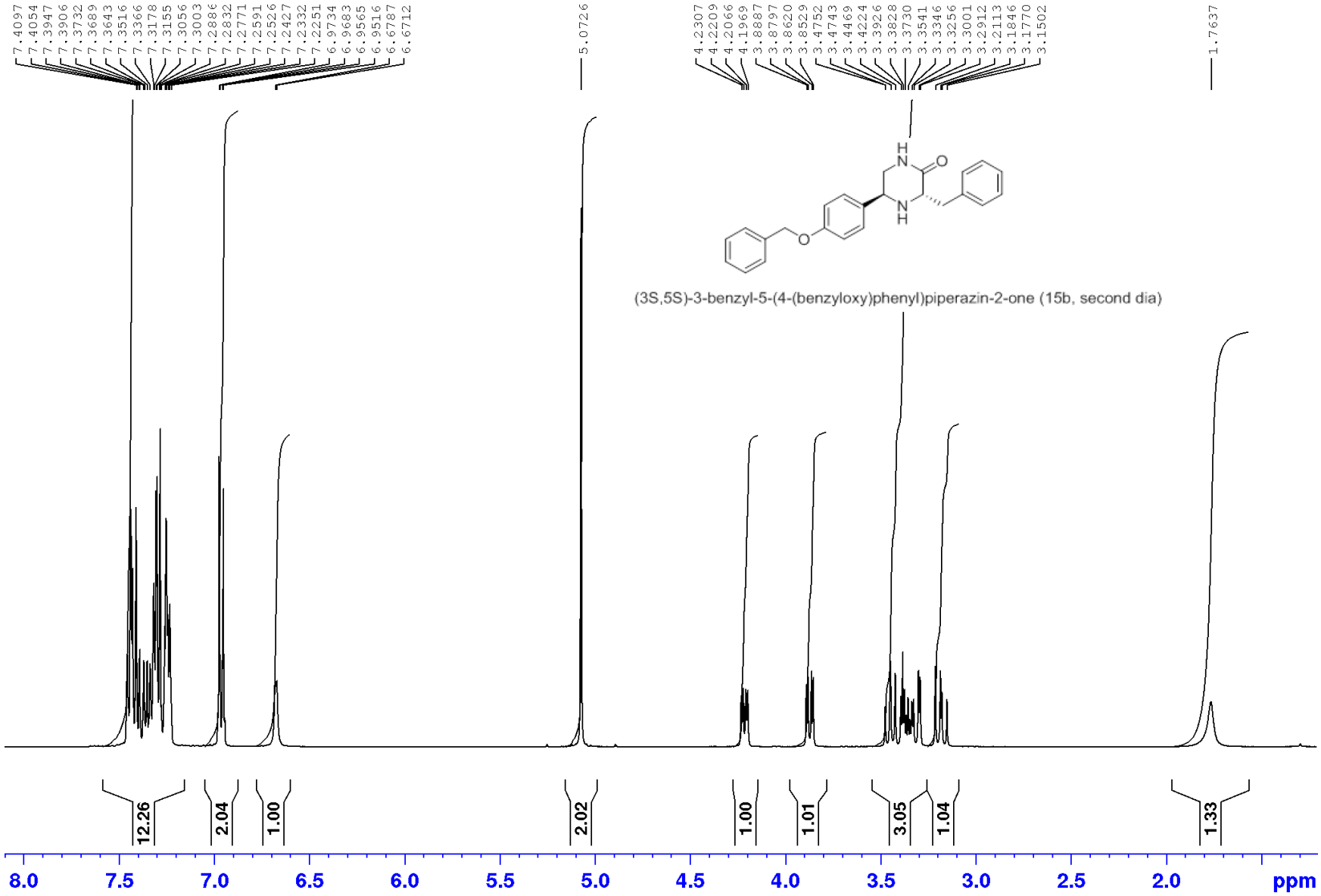
60
50
40
30
ppm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7.2332
7.2251
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6.9683
6.9565
6.9516
6.6787
6.6712
5.0726
4.2307
4.2209
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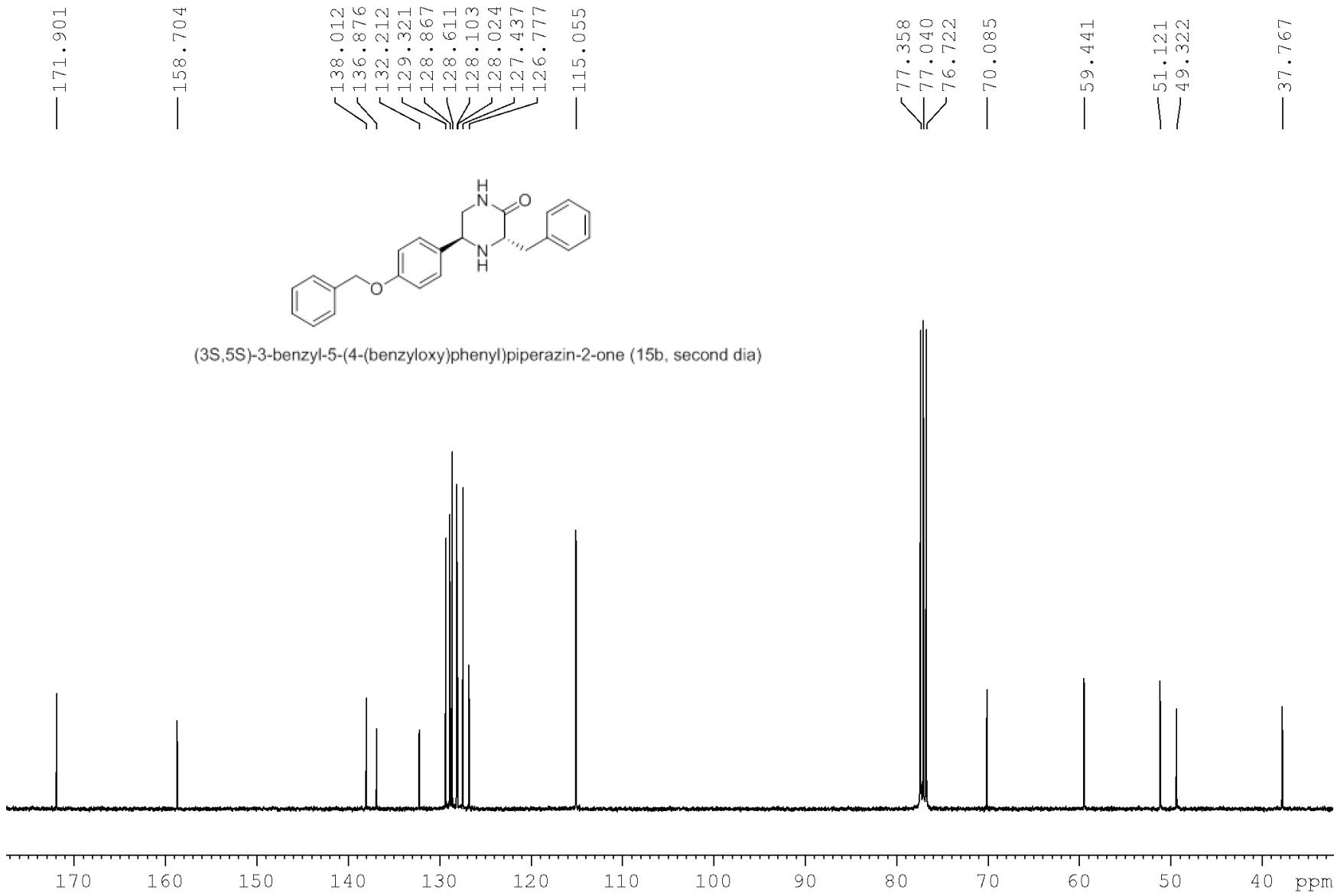
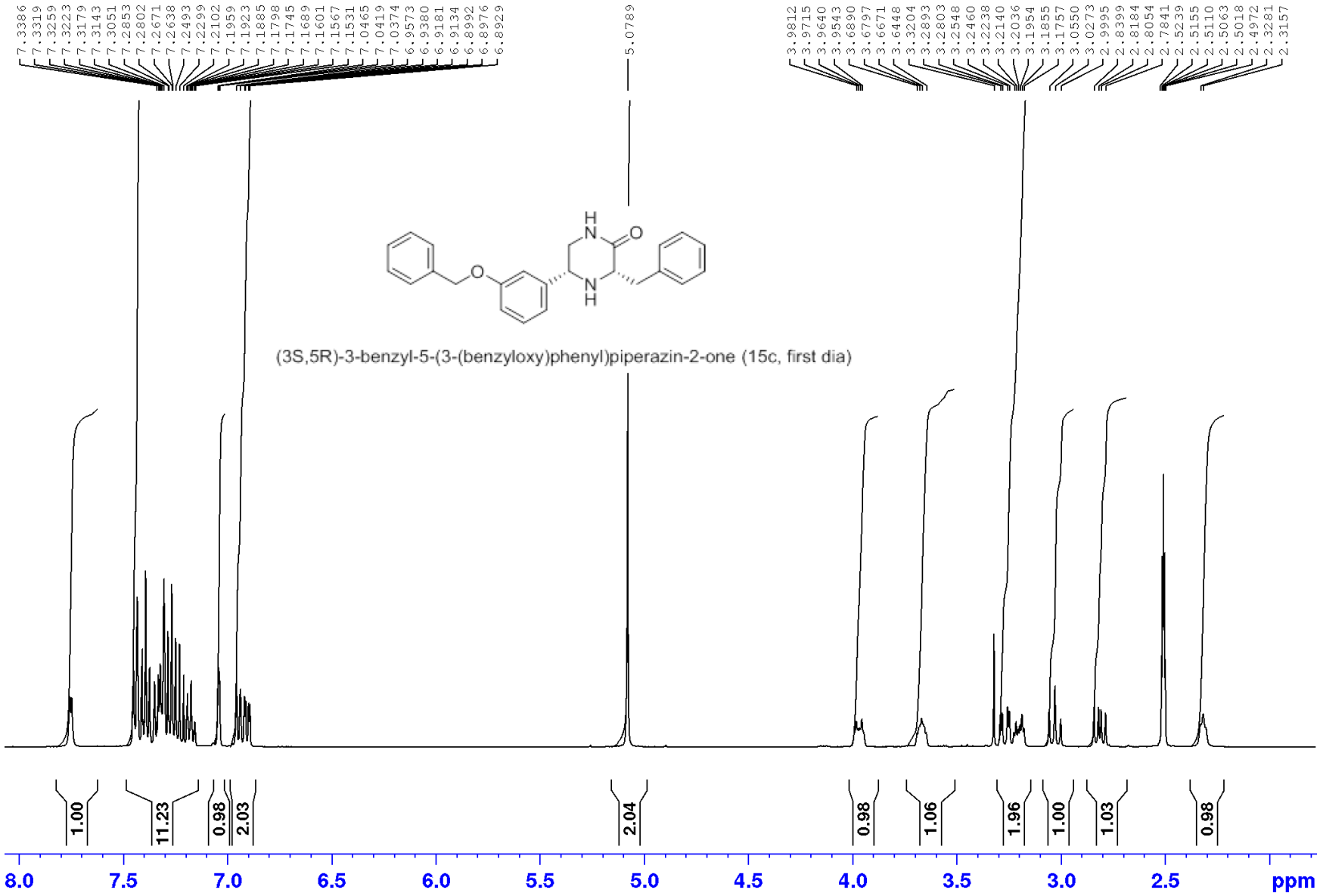
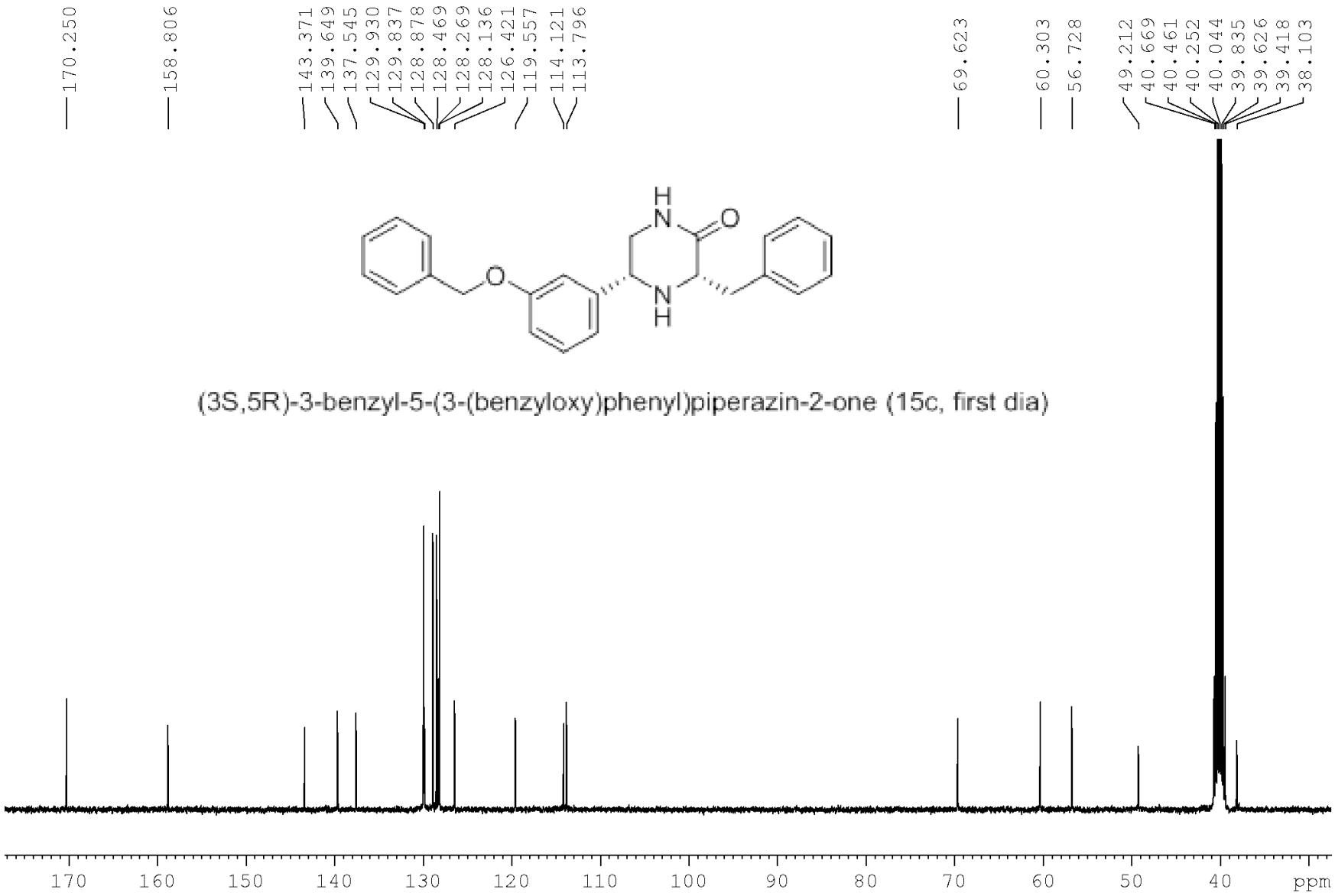
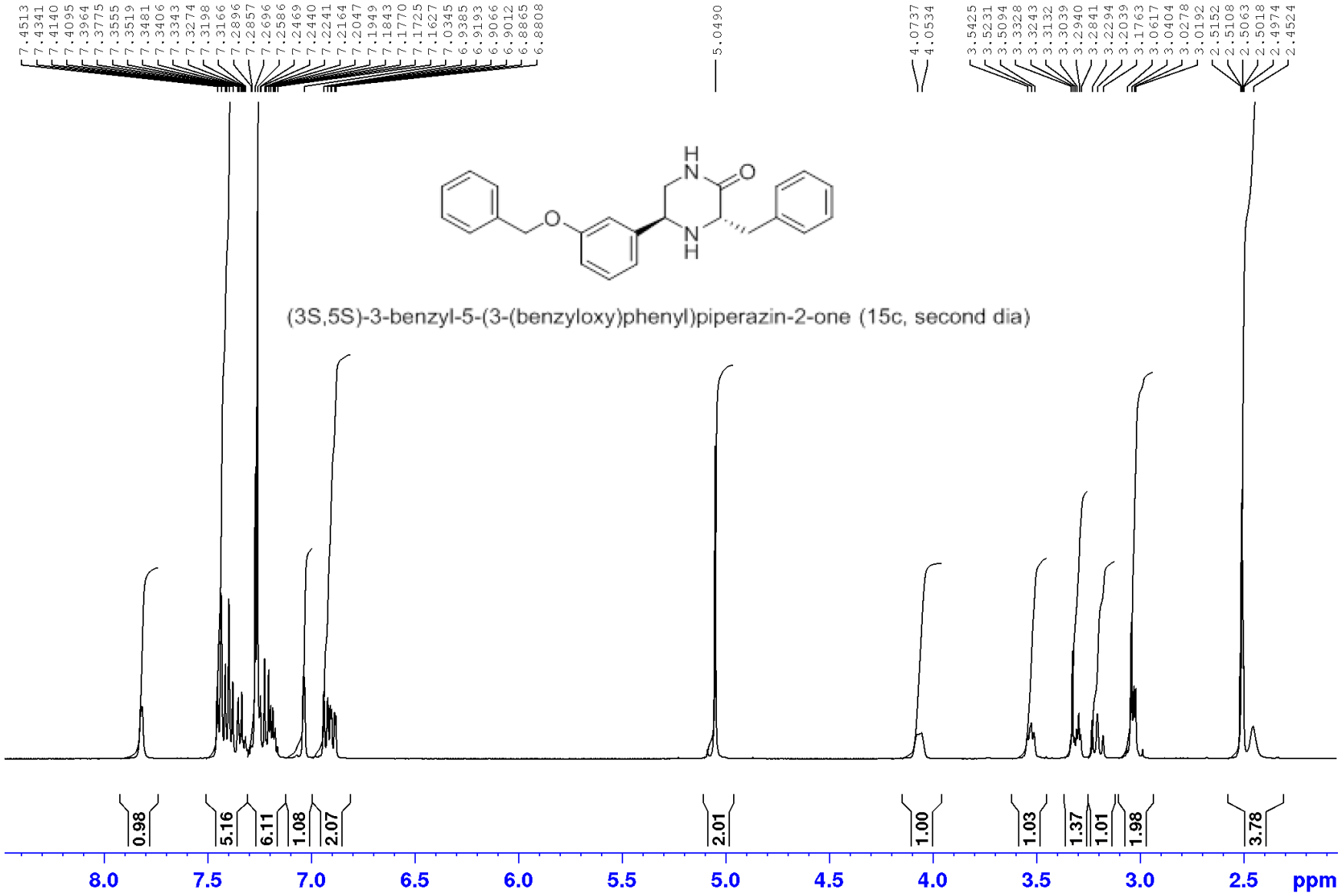
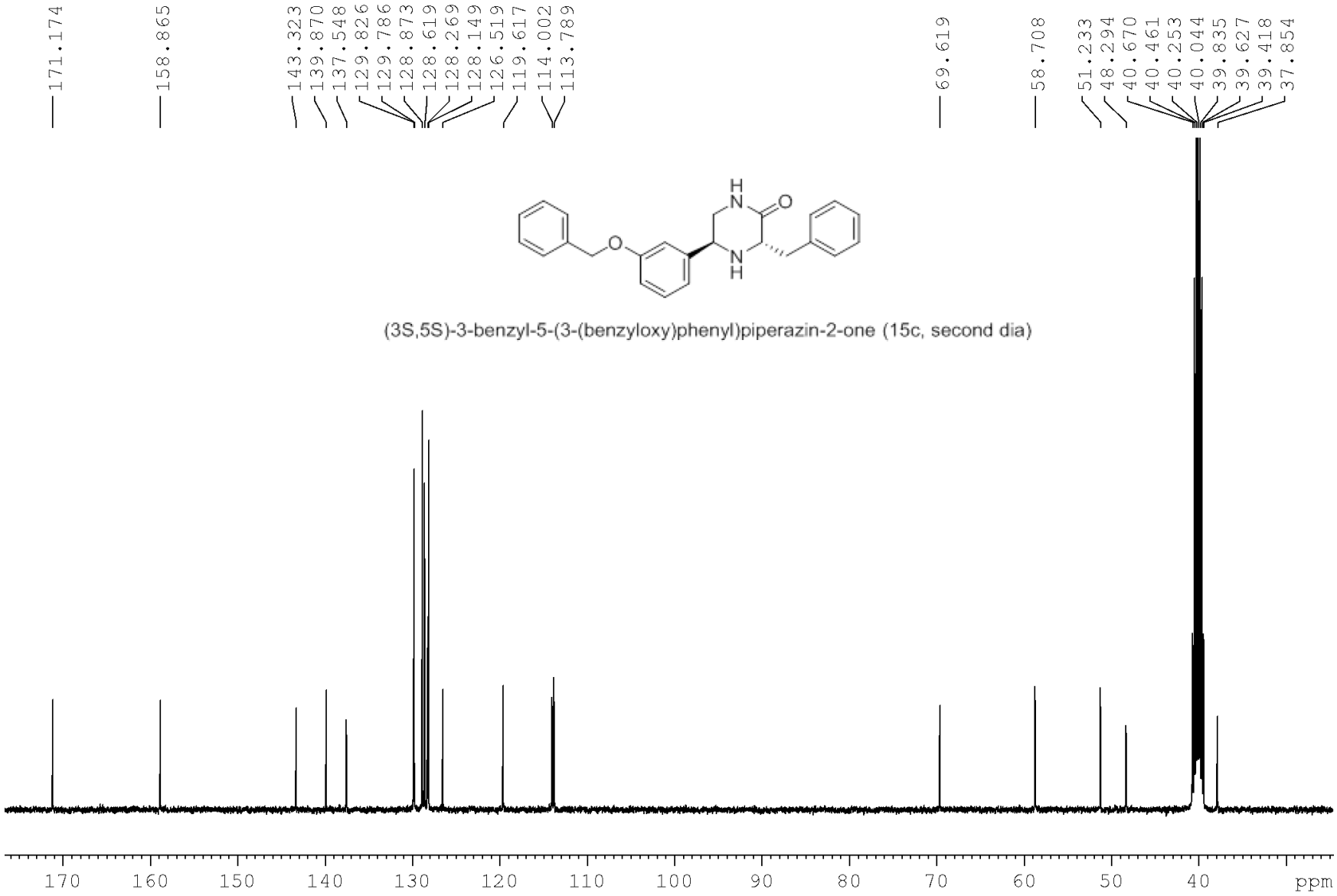
3.3828
3.3730
3.3541
3.3346
3.3256
3.3001
3.2912
3.2113
3.1846
3.1770
3.1502
1.7637
(3S,5S)-3-benzyl-5-(4-(benzyloxy)phenyl)piperazin-2-one (15b, second dia)
12.26
2.04
1.00
2.02
1.00
1.01
3.05
1.04
1.33
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
ppm

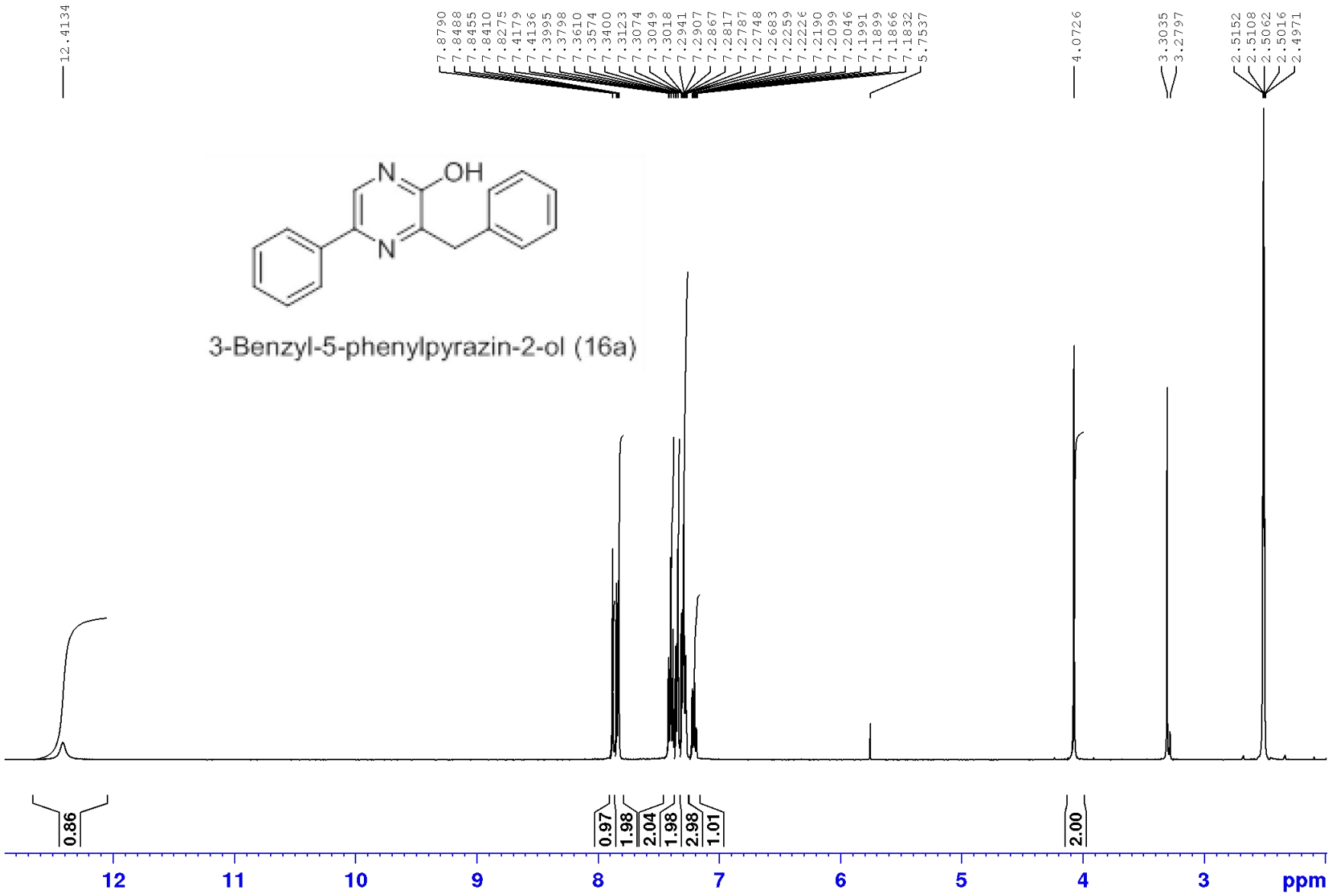

171.901
158.704
138.012
136.876
132.212
129.321
128.867
128.611
128.103
128.024
127.437
126.777
115.055
77.358
77.040
76.722
70.085
59.441
51.121
49.322
37.767
(3S,5S)-3-benzyl-5-(4-(benzyloxy)phenyl)piperazin-2-one (15b, second dia)
170
160
150
140
130
120
110
100
90
80
70
60
50
40
ppm

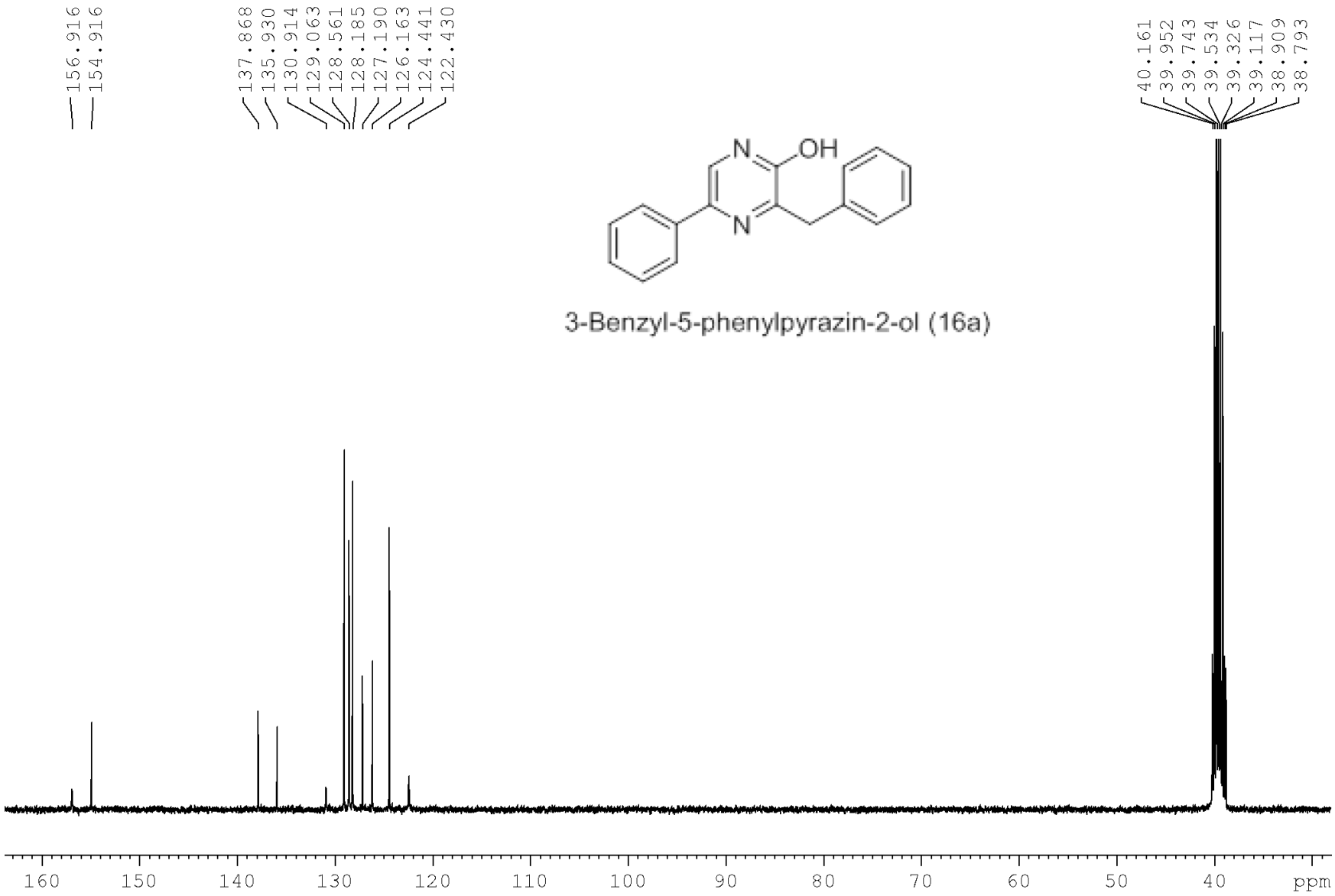

7.3386
7.3319
7.3259
7.3223
7.3179
7.3143
7.3051
7.2853
7.2802
7.2671
7.2638
7.2493
7.2299
7.2102
7.1959
7.1923
7.1885
7.1798
7.1745
7.1689
7.1601
7.1567
7.1531
7.0465
7.0419
7.0374
6.9573
6.9380
6.9181
6.9134
6.8992
6.8976
6.8929
5.0789
3.9812
3.9715
3.9640
3.9543
3.6890
3.6797
3.6671
3.6448
3.3204
3.2893
3.2803
3.2548
3.2460
3.2238
3.2140
3.2036
3.1954
3.1855
3.1757
3.0550
3.0273
2.9995
2.8399
2.8184
2.8054
2.7841
2.5239
2.5155
2.5110
2.5063
2.5018
2.4972
2.3281
2.3157
(3S,5R)-3-benzyl-5-(3-(benzyloxy)phenyl)piperazin-2-one (15c, first dia)
1.00
11.23
0.98
2.03
2.04
0.98
1.06
1.96
1.00
1.03
0.98
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
ppm


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
H
N
O
O
N
H
(3S,5R)-3-benzyl-5-(3-(benzyloxy)phenyl)piperazin-2-one (15c, first dia)
170
160
150
140
130
120
110
100
90
80
70
60
50
40
ppm

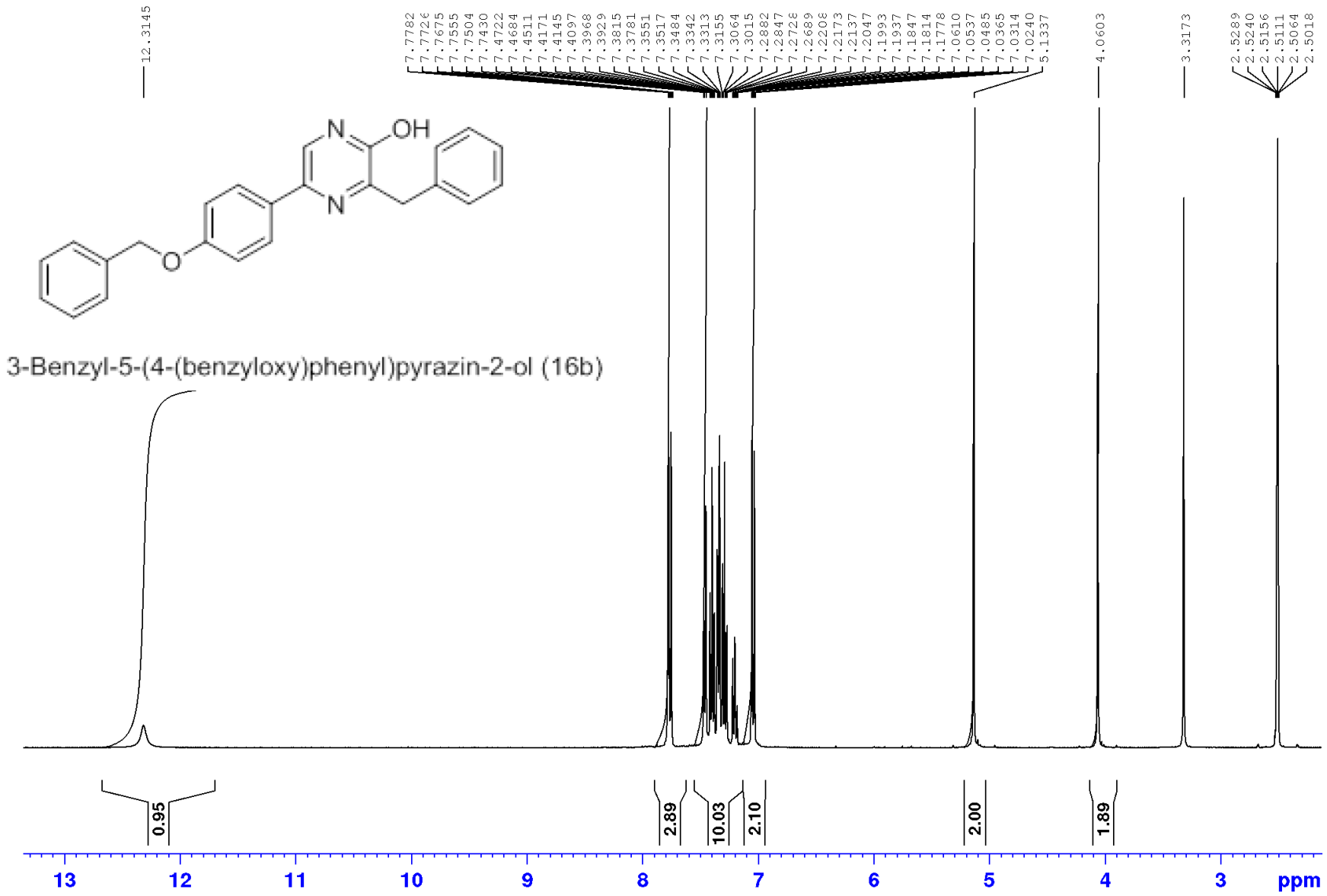

7.4513
7.4341
7.4140
7.4095
7.3964
7.3775
7.3555
7.3519
7.3481
7.3406
7.3343
7.3274
7.3198
7.3166
7.2896
7.2857
7.2696
7.2586
7.2469
7.2440
7.2241
7.2164
7.2047
7.1949
7.1843
7.1770
7.1725
7.1627
7.0345
6.9385
6.9193
6.9066
6.9012
6.8865
6.8808
5.0490
4.0737
4.0534
3.5425
3.5231
3.5094
3.3328
3.3243
3.3132
3.3039
3.2940
3.2841
3.2294
3.2039
3.1763
3.0617
3.0404
3.0278
3.0192
2.5152
2.5108
2.5063
2.5018
2.4974
2.4524
H
N
O
O
N
H
(3S,5S)-3-benzyl-5-(3-(benzyloxy)phenyl)piperazin-2-one (15c, second dia)
0.98
5.16
6.11
1.08
2.07
2.01
1.00
1.03
1.37
1.01
1.98
3.78
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
ppm

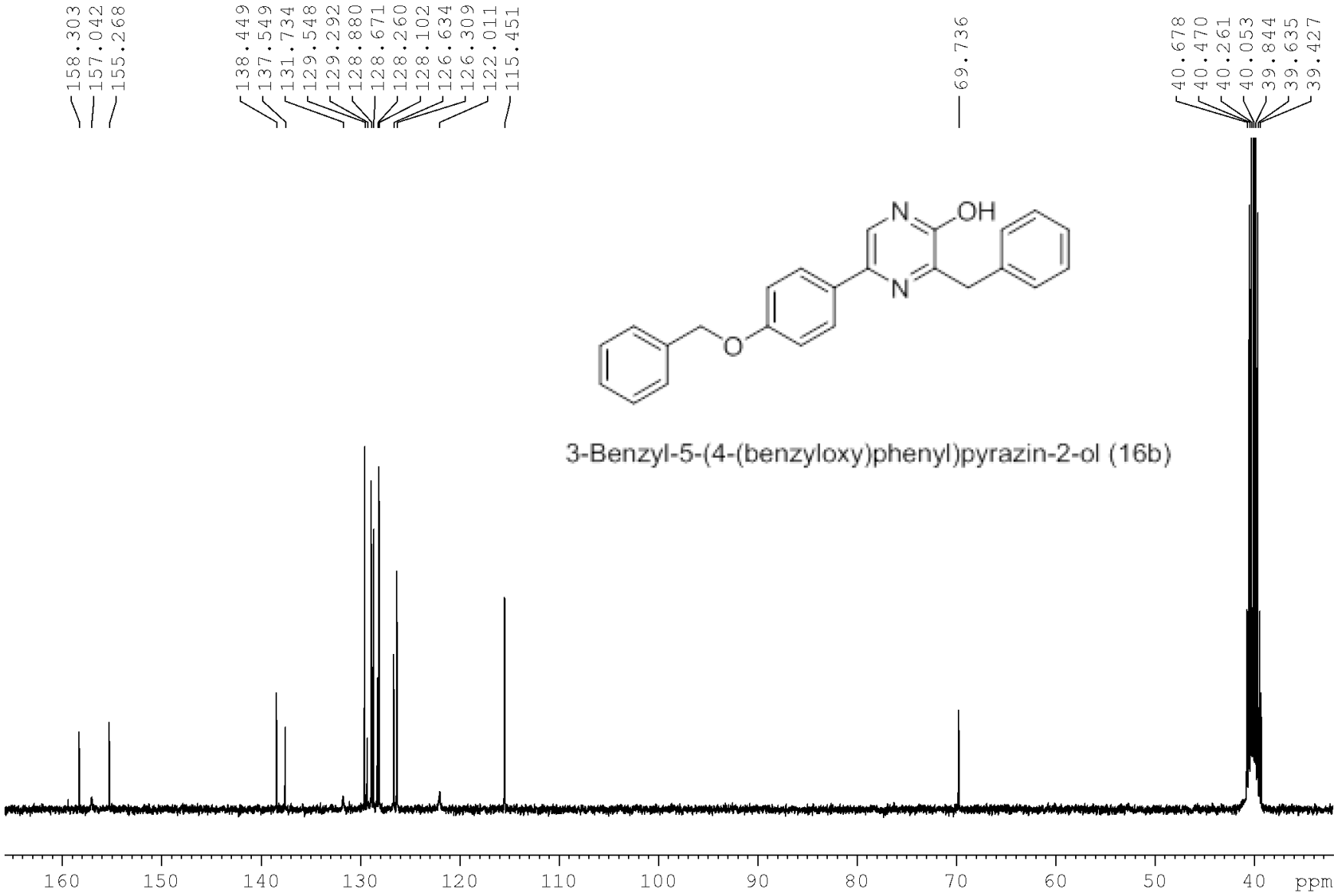

171.174
158.865
143.323
139.870
137.548
129.826
129.786
128.873
128.619
128.269
128.149
126.519
119.617
114.002
113.789
69.619
58.708
51.233
48.294
40.670
40.461
40.253
40.044
39.835
39.627
39.418
37.854
H
N
O
O
N
H
(3S,5S)-3-benzyl-5-(3-(benzyloxy)phenyl)piperazin-2-one (15c, second dia)
170
160
150
140
130
120
110
100
90
80
70
60
50
40
ppm

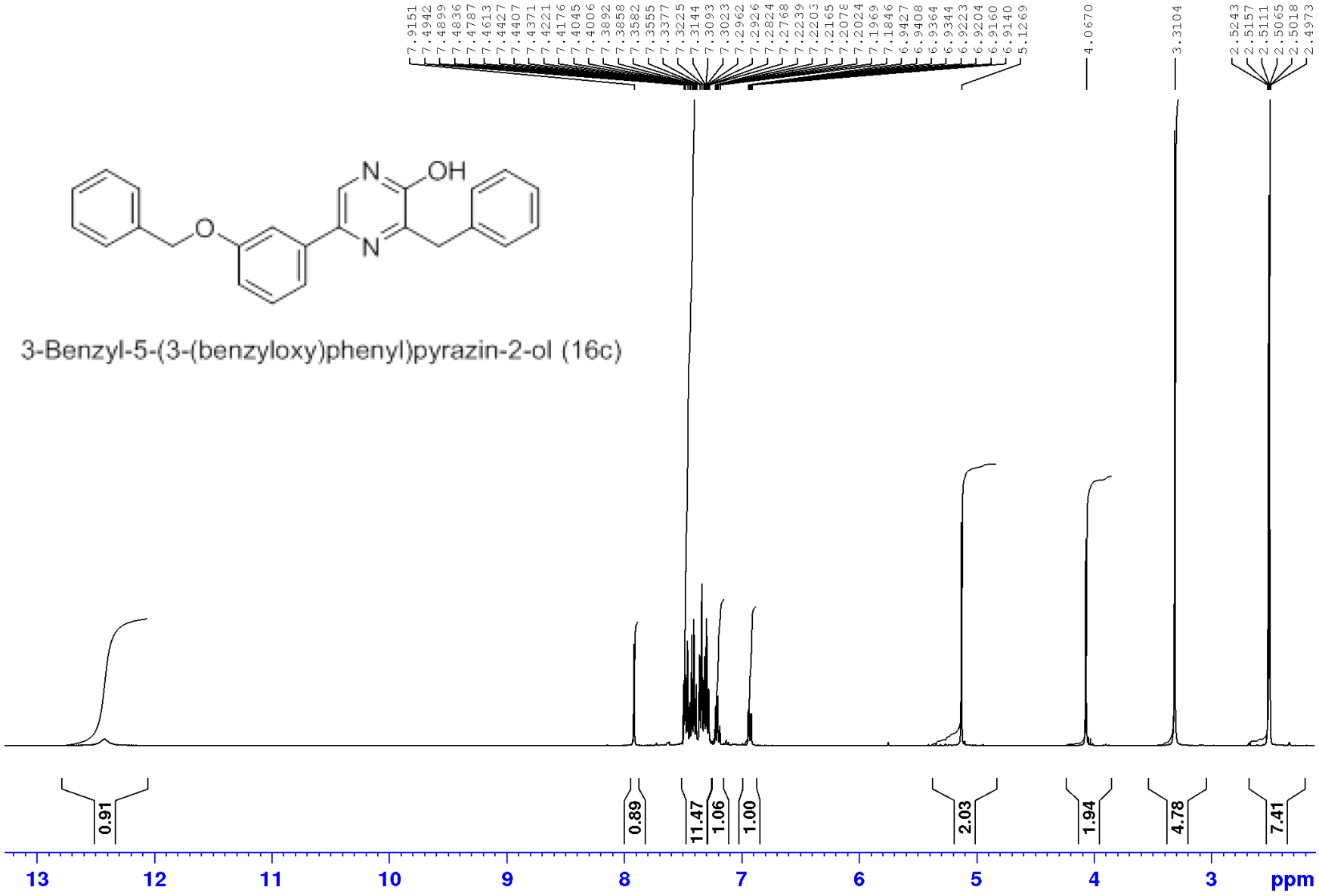

12.4134
7.8790
7.8488
7.8455
7.8410
7.8275
7.4179
7.4136
7.3995
7.3798
7.3610
7.3574
7.3400
7.3123
7.3074
7.3049
7.3018
7.2941
7.2907
7.2867
7.2817
7.2787
7.2748
7.2683
7.2259
7.2226
7.2190
7.2099
7.2046
7.1991
7.1899
7.1866
7.1832
5.7537
4.0726
3.3035
3.2797
2.5152
2.5108
2.5062
2.5016
2.4971
N
OH
N
3-Benzyl-5-phenylpyrazin-2-ol (16a)
0.86
0.97
1.98
2.04
1.98
2.98
1.01
2.00
12
11
10
9
8
7
6
5
4
3
ppm

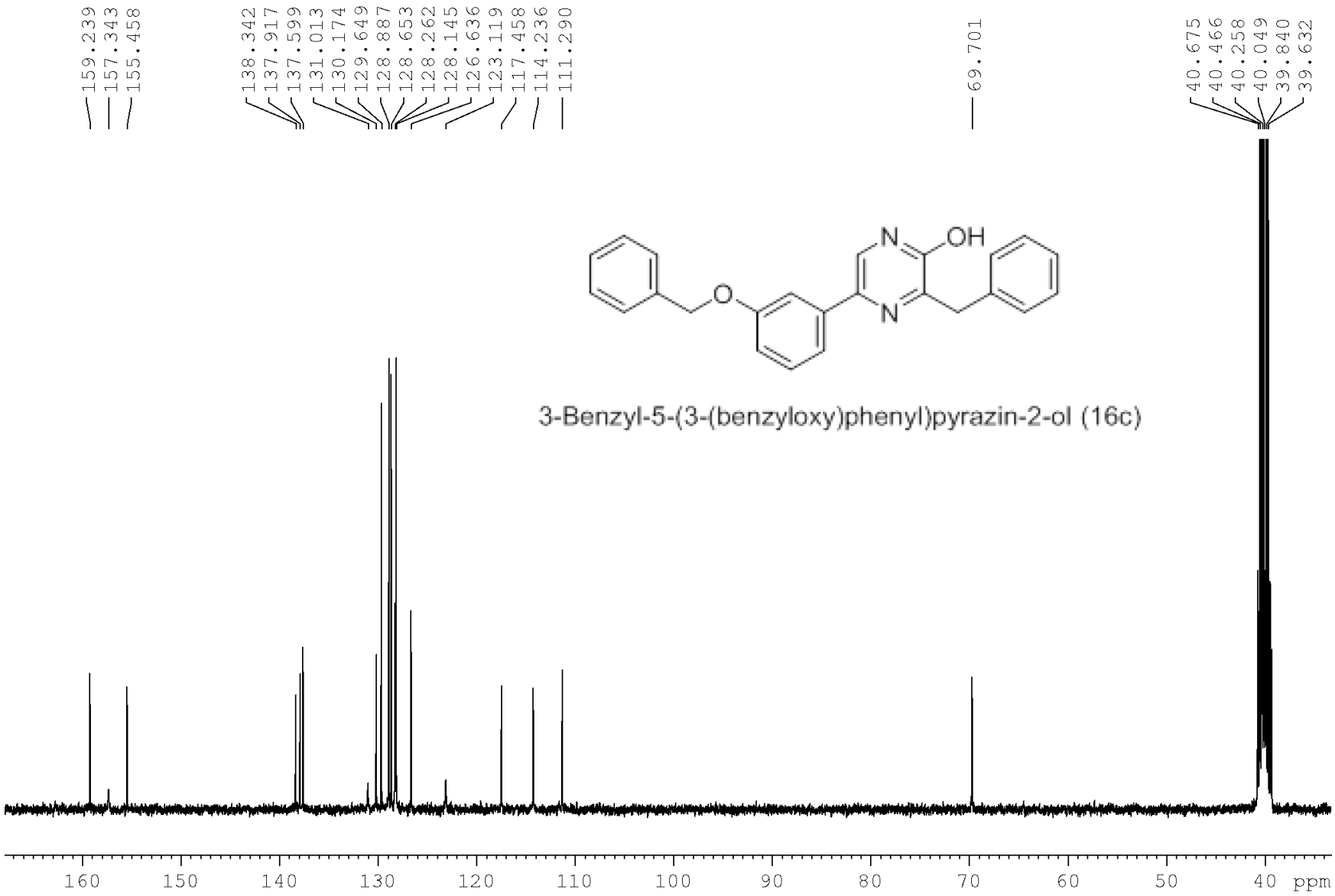


3-Benzyl-5-phenylpyrazin-2-ol (16a)

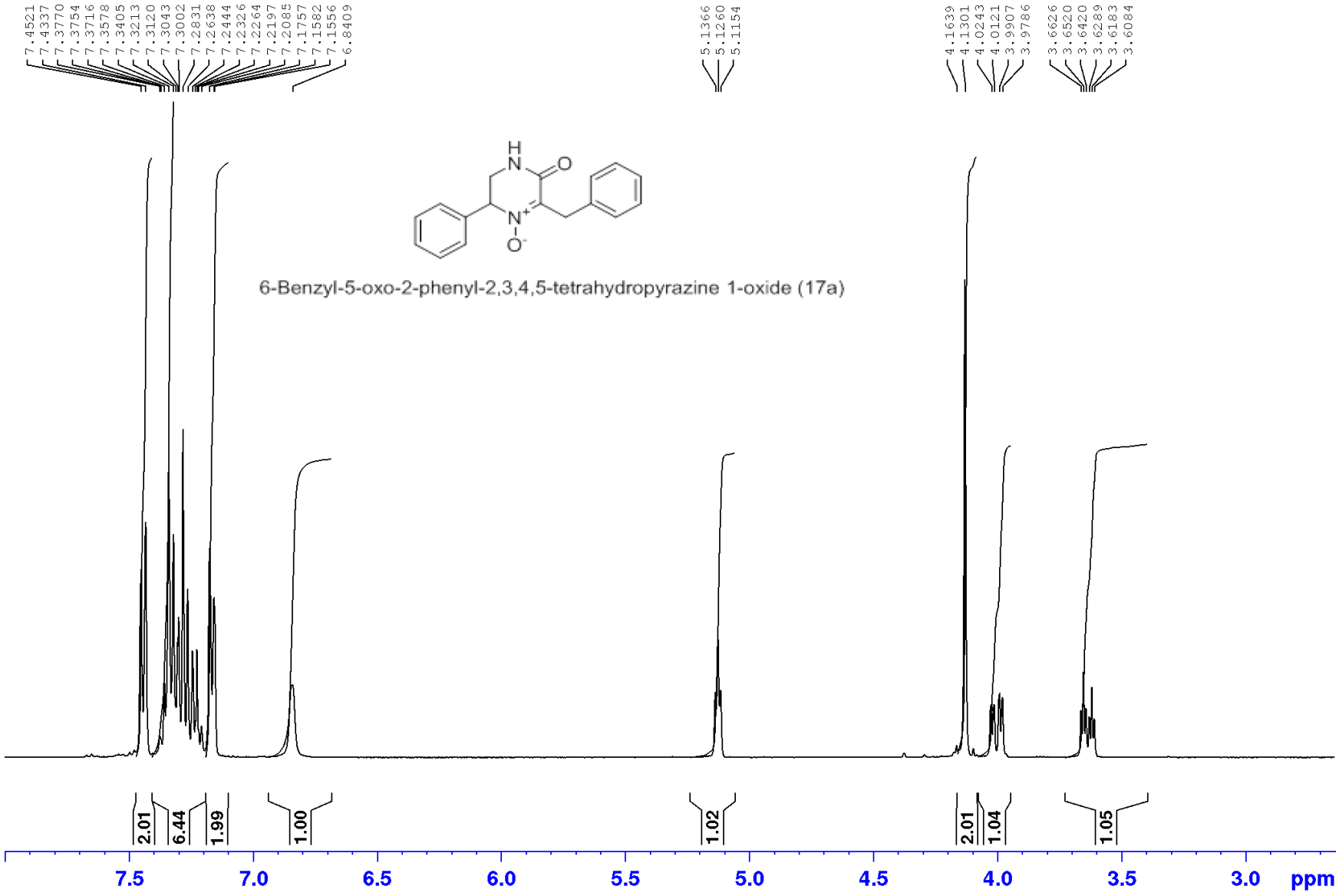

12.3145
7.7782
7.7726
7.7675
7.7555
7.7504
7.7430
7.4722
7.4684
7.4511
7.4171
7.4145
7.4097
7.3968
7.3929
7.3815
7.3781
7.3551
7.3517
7.3484
7.3342
7.3313
7.3155
7.3064
7.3015
7.2882
7.2847
7.2728
7.2689
7.2208
7.2173
7.2137
7.2047
7.1993
7.1937
7.1847
7.1814
7.1778
7.0610
7.0537
7.0485
7.0365
7.0314
7.0240
5.1337
4.0603
3.3173
2.5289
2.5240
2.5156
2.5111
2.5064
2.5018
N
OH
N
O
3-Benzyl-5-(4-(benzyloxy)phenyl)pyrazin-2-ol (16b)
0.95
2.89
10.03
2.10
2.00
1.89
13
12
11
10
9
8
7
6
5
4
3
ppm


158.303
157.042
155.268
138.449
137.549
131.734
129.548
129.292
128.880
128.671
128.260
128.102
126.634
126.309
122.011
115.451
69.736
40.678
40.470
40.261
40.053
39.844
39.635
39.427
N
OH
N
O
3-Benzyl-5-(4-(benzyloxy)phenyl)pyrazin-2-ol (16b)
160
150
140
130
120
110
100
90
80
70
60
50
40
ppm

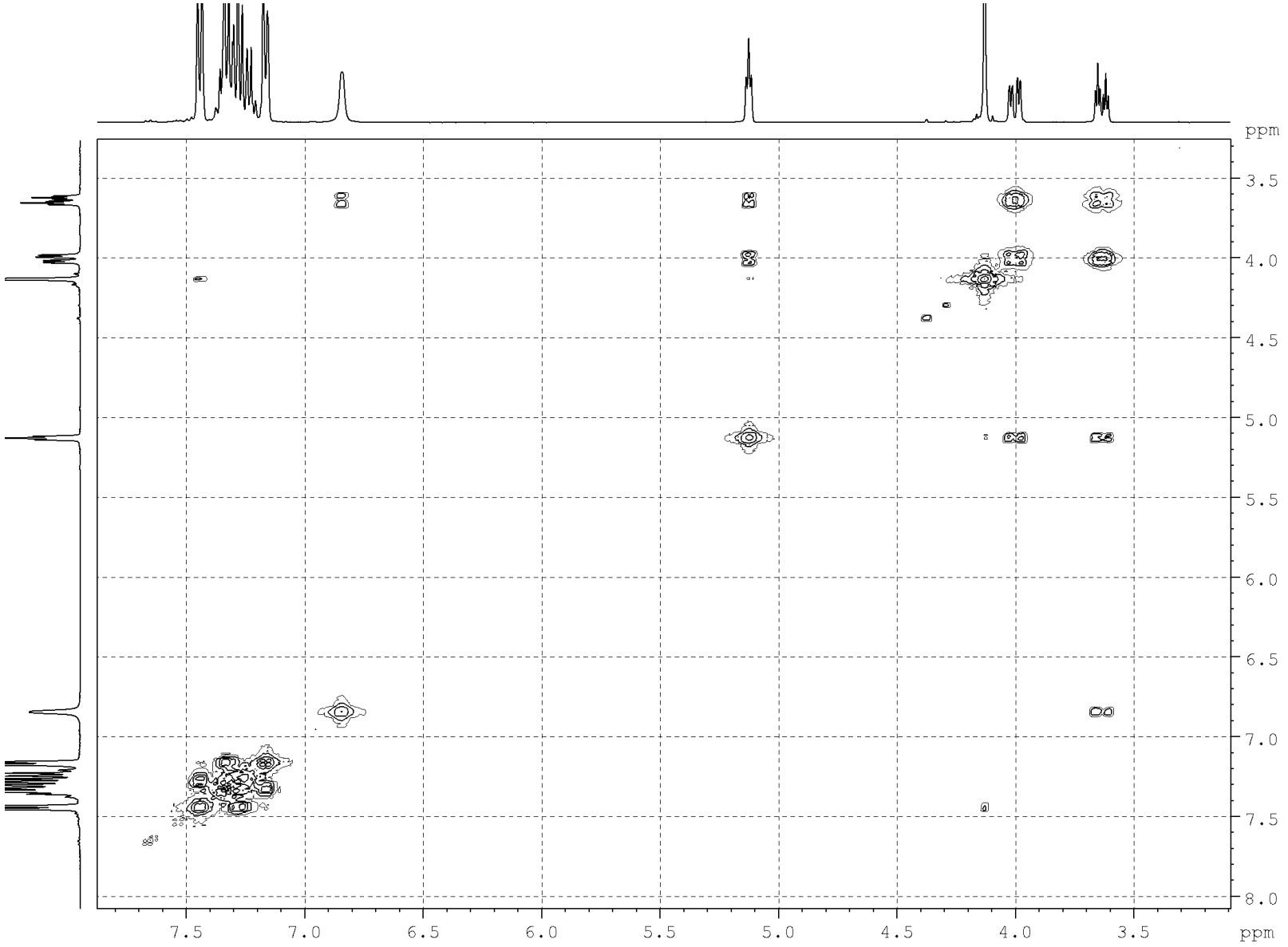

7.9151
7.4942
7.4899
7.4836
7.4787
7.4613
7.4427
7.4407
7.4371
7.4221
7.4176
7.4045
7.4006
7.3892
7.3858
7.3582
7.3555
7.3377
7.3225
7.3144
7.3093
7.3023
7.2962
7.2926
7.2824
7.2768
7.2239
7.2203
7.2165
7.2078
7.2024
7.1969
7.1846
6.9427
6.9408
6.9364
6.9344
6.9223
6.9204
6.9160
6.9140
5.1269
4.0670
3.3104
2.5243
2.5157
2.5111
2.5065
2.5018
2.4973
N
OH
O
N
3-Benzyl-5-(3-(benzyloxy)phenyl)pyrazin-2-ol (16c)
0.91
0.89
11.47
1.06
1.00
2.03
1.94
4.78
7.41
13
12
11
10
9
8
7
6
5
4
3
ppm

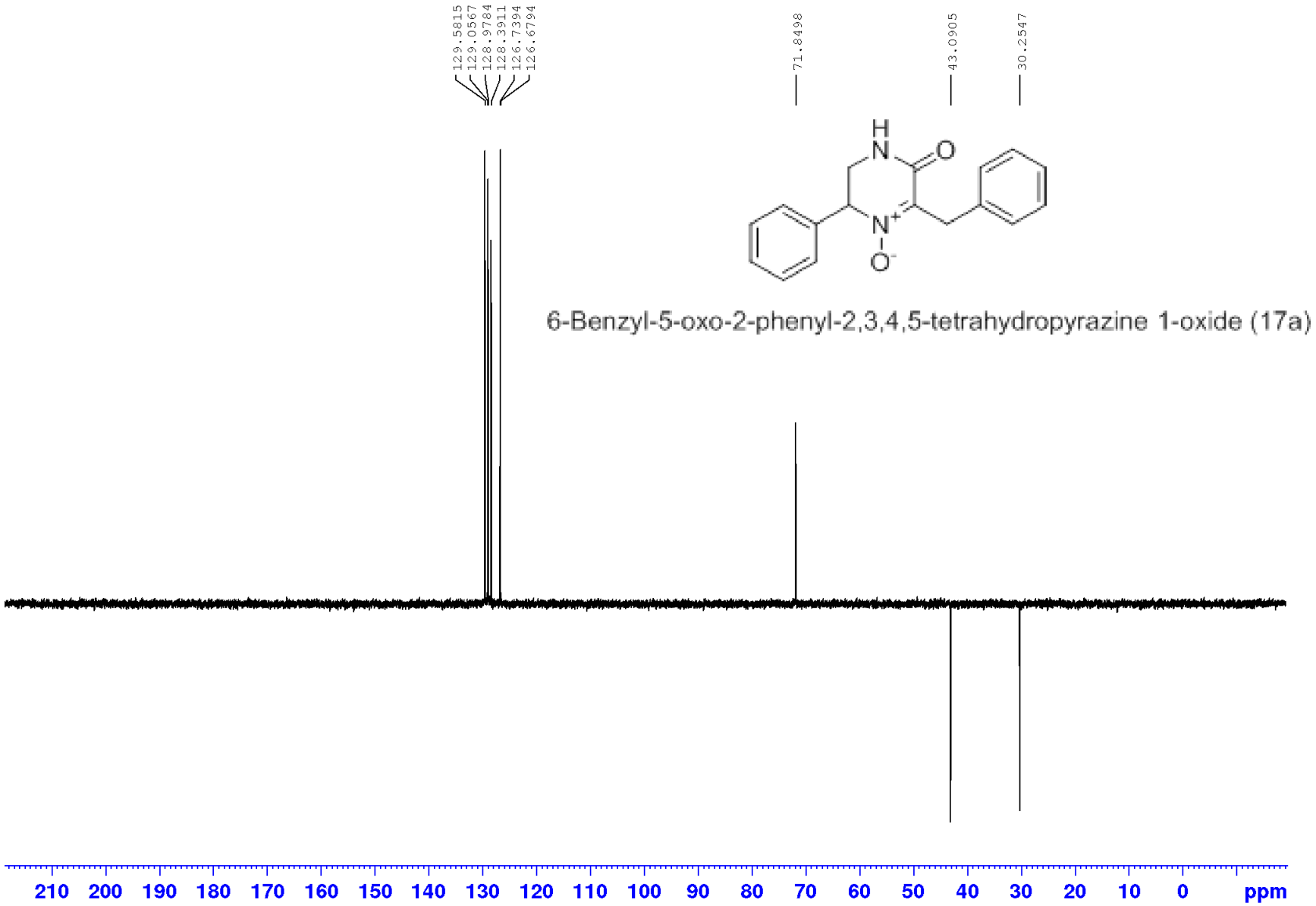


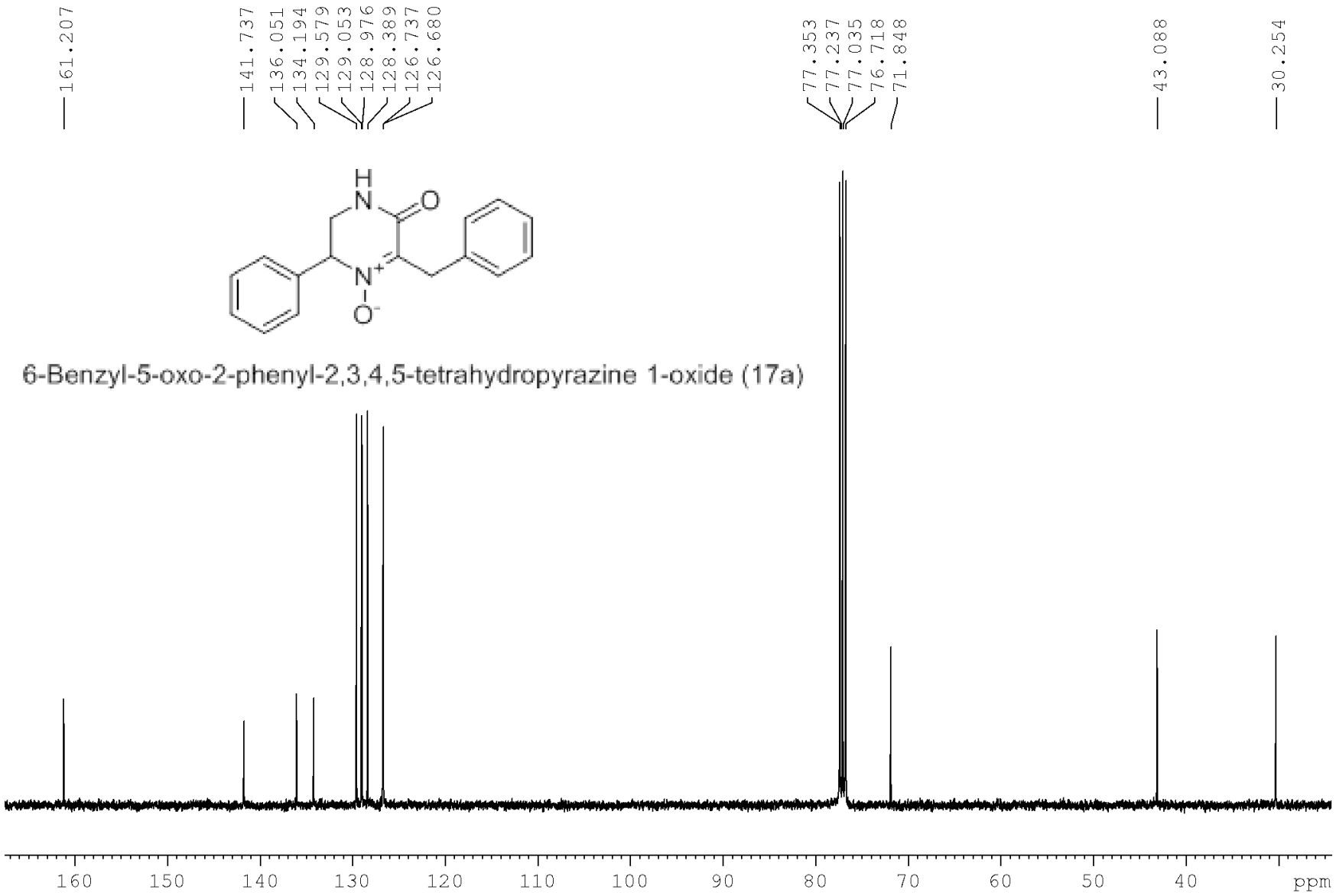
3-Benzyl-5-(3-(benzyloxy)phenyl)pyrazin-2-ol (16c)

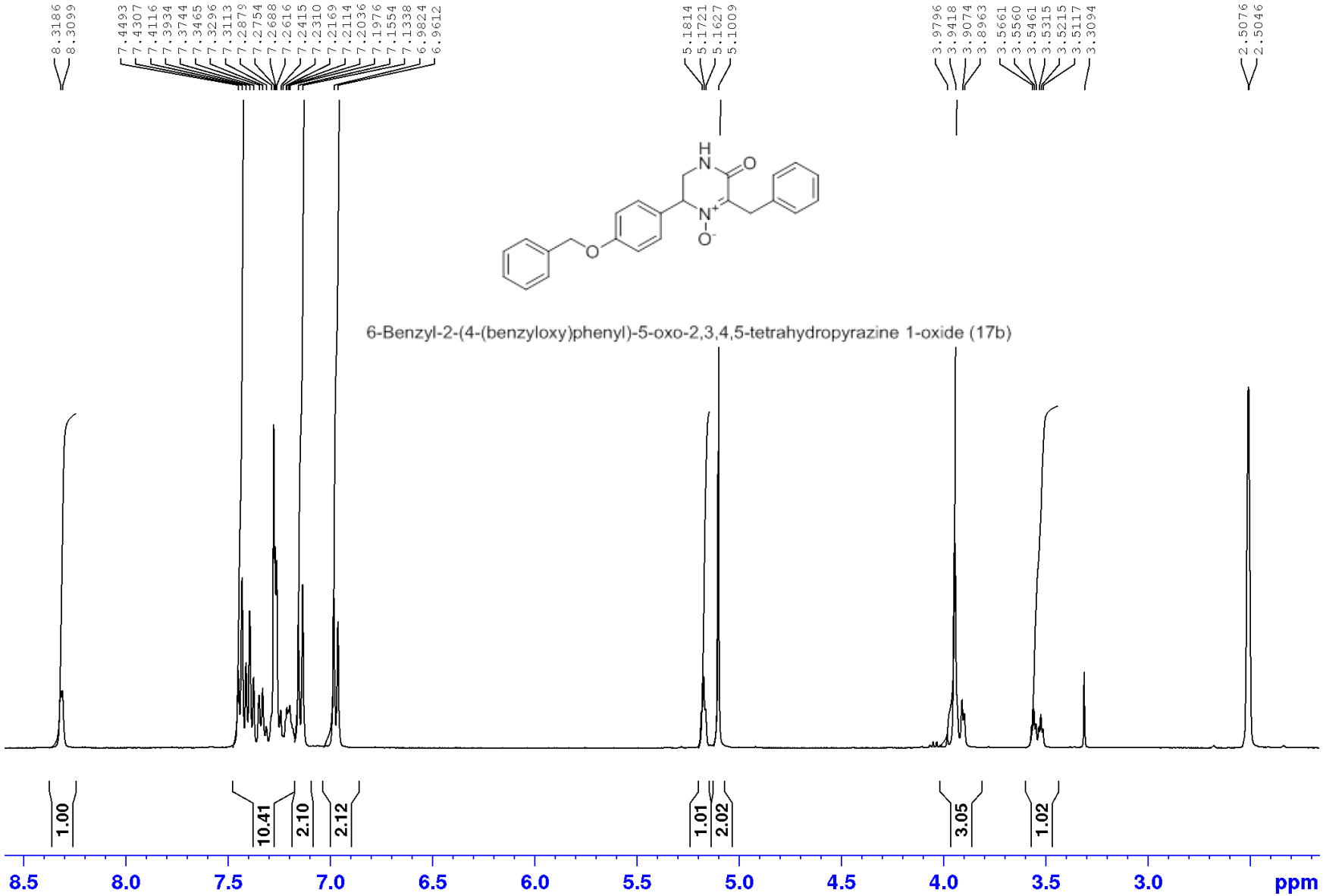

7.4521
7.4337
7.3770
7.3754
7.3716
7.3578
7.3405
7.3213
7.3120
7.3043
7.3002
7.2831
7.2638
7.2444
7.2326
7.2264
7.2197
7.2085
7.1757
7.1582
7.1556
6.8409
5.1366
5.1260
5.1154
4.1639
4.1301
4.0243
4.0121
3.9907
3.9786
3.6626
3.6520
3.6420
3.6289
3.6183
3.6084
H
N
O
N+
O-
6-Benzyl-5-oxo-2-phenyl-2,3,4,5-tetrahydropyrazine 1-oxide (17a)
2.01
6.44
1.99
1.00
1.02
2.01
1.04
1.05
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
ppm

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

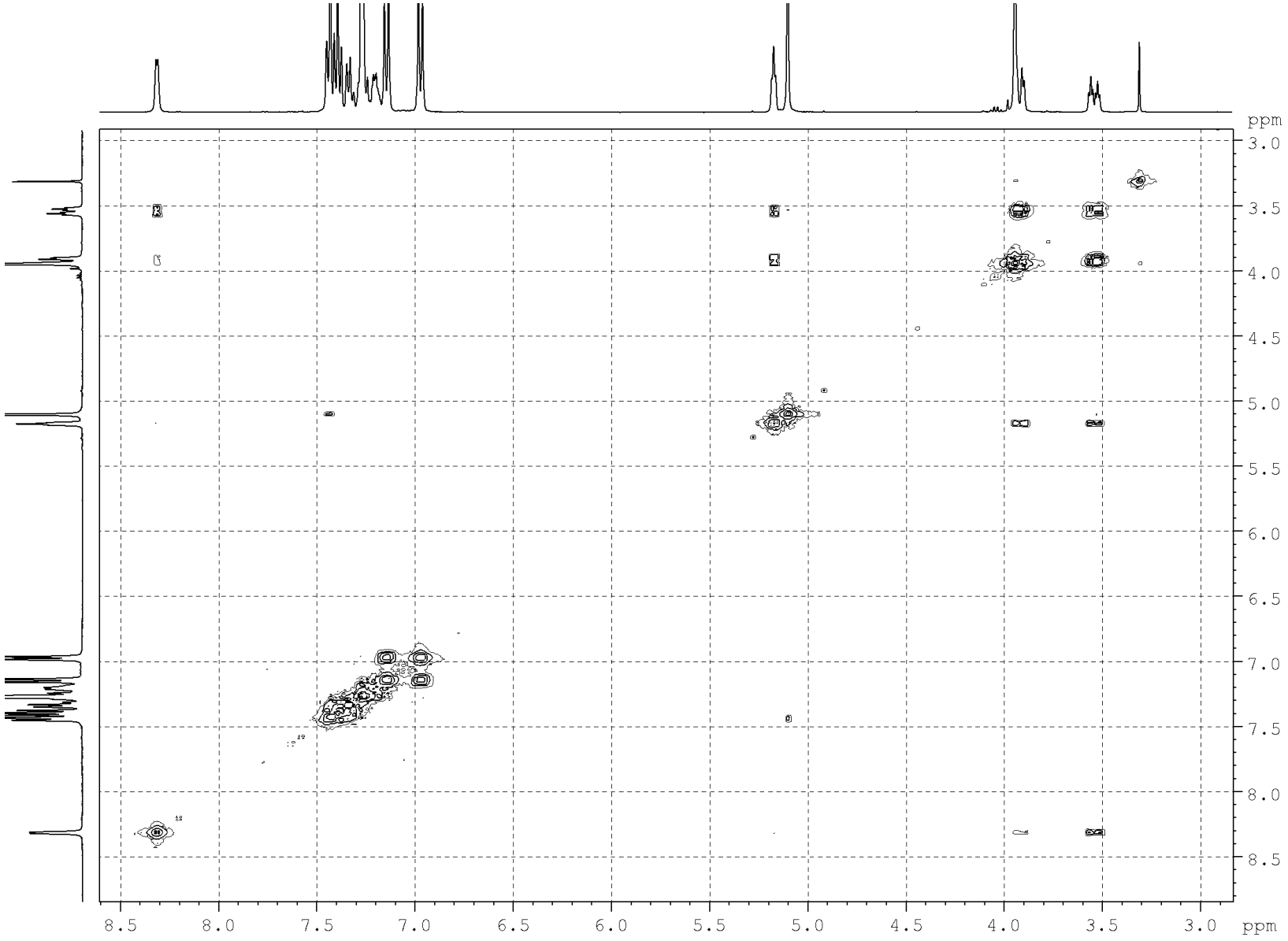


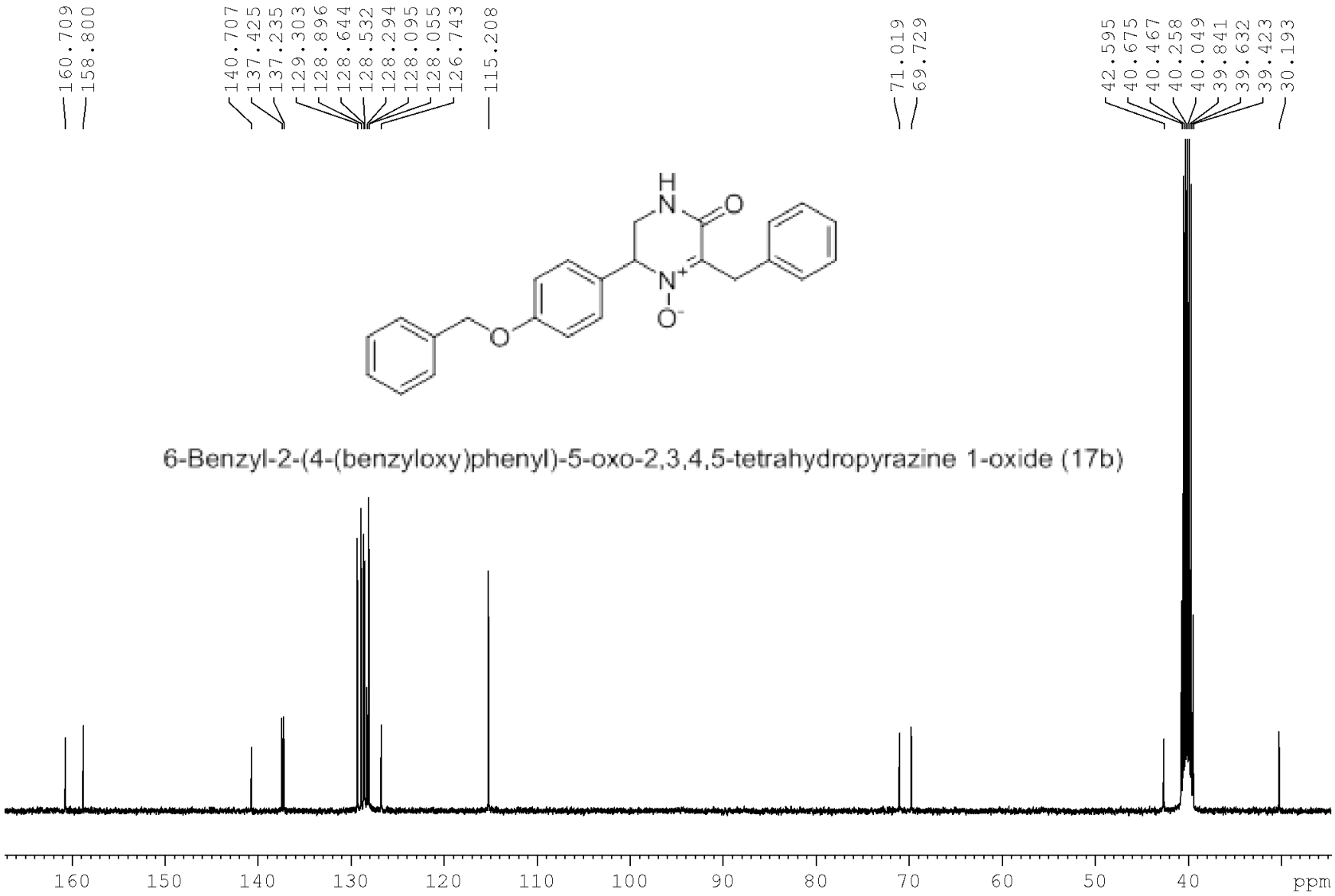

129.5815
129.0567
128.9784
128.3911
126.7394
126.6794
71.8498
43.0905
30.2547
H
N
O
N+
O-
6-Benzyl-5-oxo-2-phenyl-2,3,4,5-tetrahydropyrazine 1-oxide (17a)
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 ppm

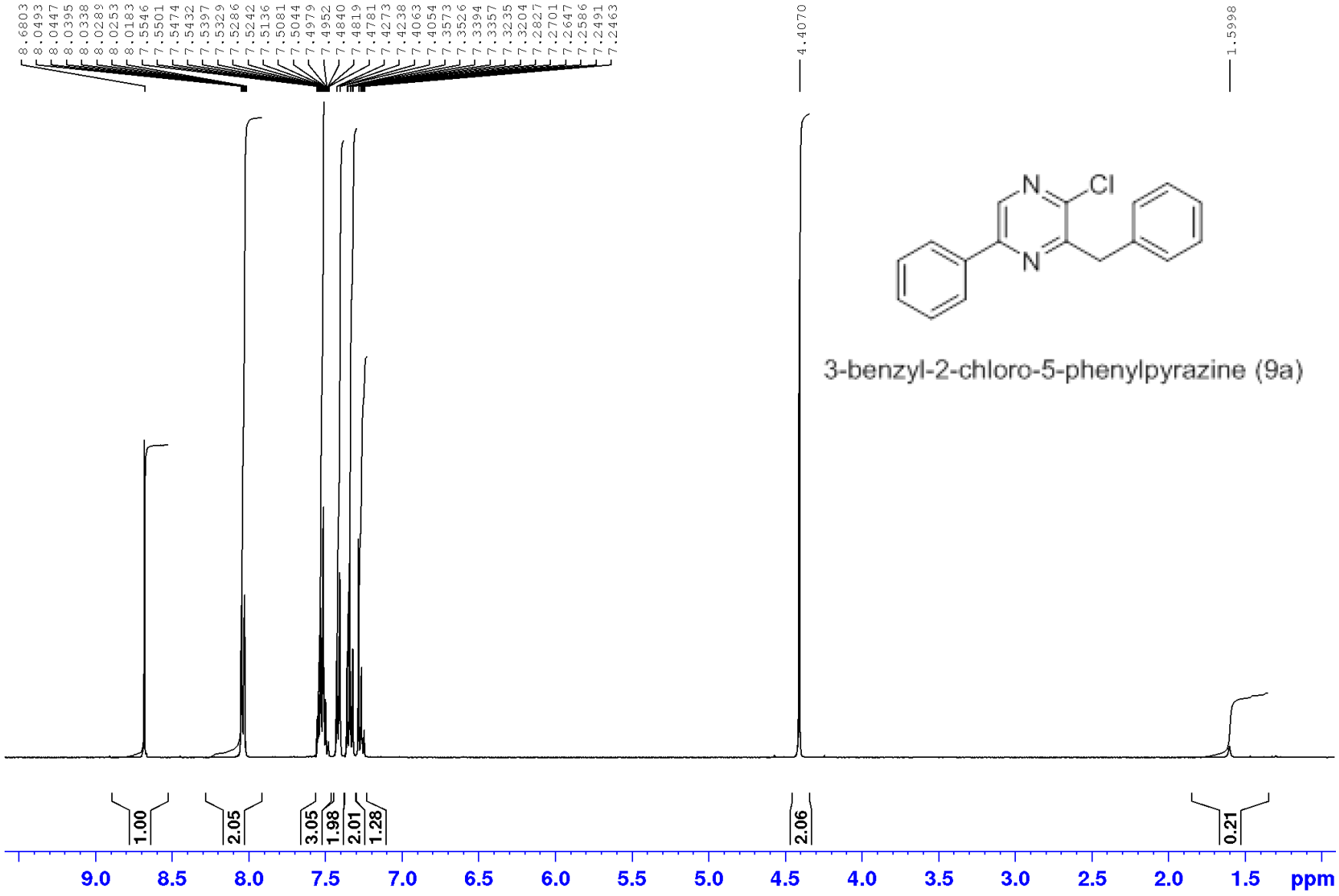

161.207
141.737
136.051
134.194
129.579
129.053
128.976
128.389
126.737
126.680
77.353
77.237
77.035
76.718
71.848
43.088
30.254
H
N
O
N+
O-
6-Benzyl-5-oxo-2-phenyl-2,3,4,5-tetrahydropyrazine 1-oxide (17a)
160
150
140
130
120
110
100
90
80
70
60
50
40
ppm

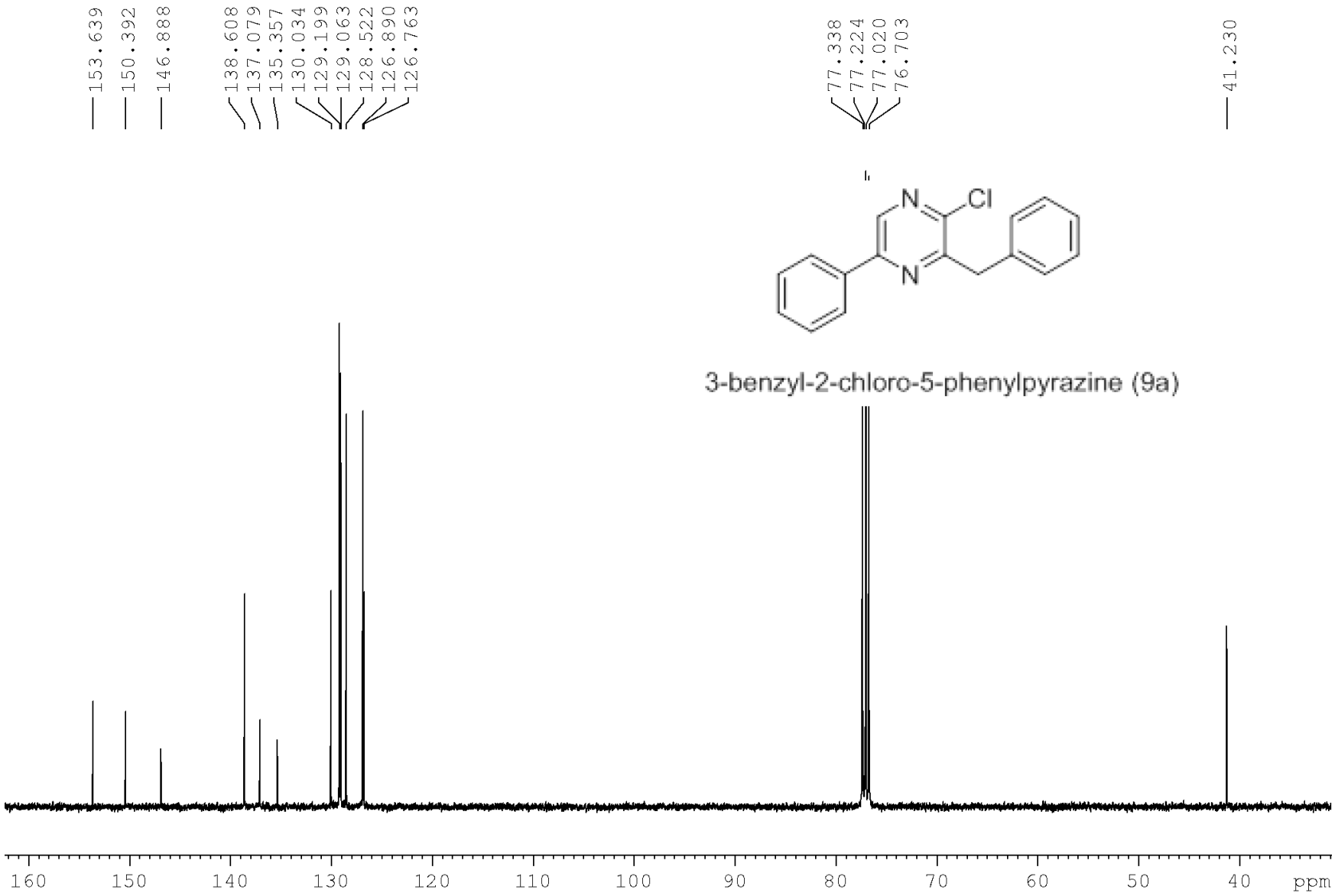

8.3186
8.3099
7.4493
7.4307
7.4116
7.3934
7.3744
7.3465
7.3296
7.3113
7.2879
7.2754
7.2688
7.2616
7.2415
7.2310
7.2169
7.2114
7.2036
7.1976
7.1554
7.1338
6.9824
6.9612
5.1814
5.1721
5.1627
5.1009
3.9796
3.9418
3.9074
3.8963
3.5661
3.5560
3.5461
3.5315
3.5215
3.5117
3.3094
2.5076
2.5046
6-Benzyl-2-(4-(benzyloxy)phenyl)-5-oxo-2,3,4,5-tetrahydropyrazine 1-oxide (17b)
1.00
10.41
2.10
2.12
1.01
2.02
3.05
1.02
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
ppm

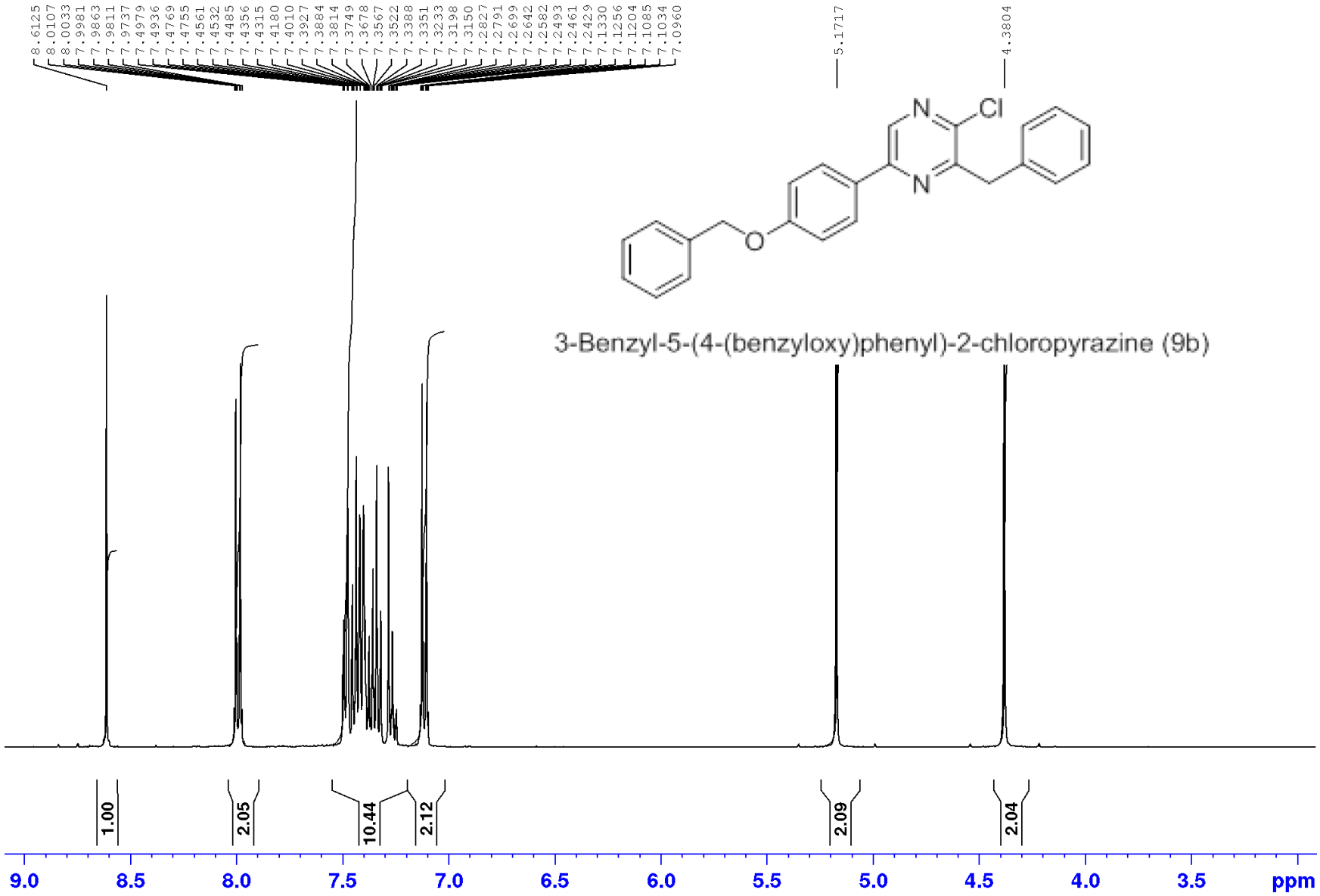
6-Benzyl-2-(4-(benzyloxy)phenyl)-5-oxo-2,3,4,5-tetrahydropyrazine 1-oxide (17b)



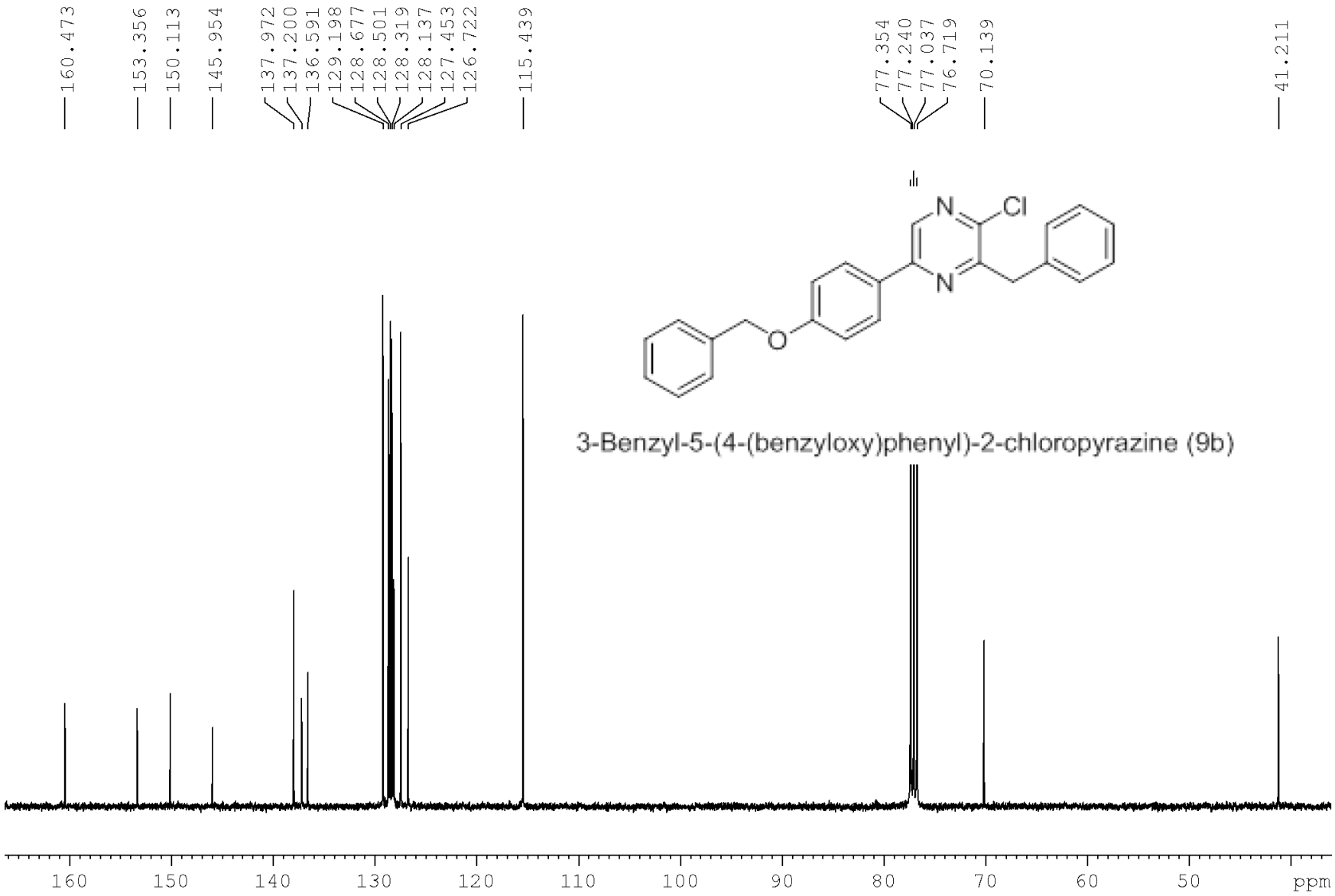

160.709
158.800
140.707
137.425
137.235
129.303
128.896
128.644
128.532
128.294
128.095
128.055
126.743
115.208
71.019
69.729
42.595
40.675
40.467
40.258
40.049
39.841
39.632
39.423
30.193
H
N
O
N+
O-
O
6-Benzyl-2-(4-(benzyloxy)phenyl)-5-oxo-2,3,4,5-tetrahydropyrazine 1-oxide (17b)
160
150
140
130
120
110
100
90
80
70
60
50
40
ppm


8.6803
8.0493
8.0447
8.0395
8.0338
8.0289
8.0253
8.0183
7.5546
7.5501
7.5474
7.5432
7.5397
7.5329
7.5286
7.5242
7.5136
7.5081
7.5044
7.4979
7.4952
7.4840
7.4819
7.4781
7.4273
7.4238
7.4063
7.4054
7.3573
7.3526
7.3394
7.3357
7.3235
7.3204
7.2827
7.2701
7.2647
7.2586
7.2491
7.2463
4.4070
1.5998
N
Cl
N
3-benzyl-2-chloro-5-phenylpyrazine (9a)
1.00
2.05
3.05
1.98
2.01
1.28
2.06
0.21
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
ppm


153.639
150.392
146.888
138.608
137.079
135.357
130.034
129.199
129.063
128.522
126.890
126.763
77.338
77.224
77.020
76.703
41.230
N
Cl
N
3-benzyl-2-chloro-5-phenylpyrazine (9a)
160
150
140
130
120
110
100
90
80
70
60
50
40
ppm



3-Benzyl-5-(4-(benzyloxy)phenyl)-2-chloropyrazine (9b)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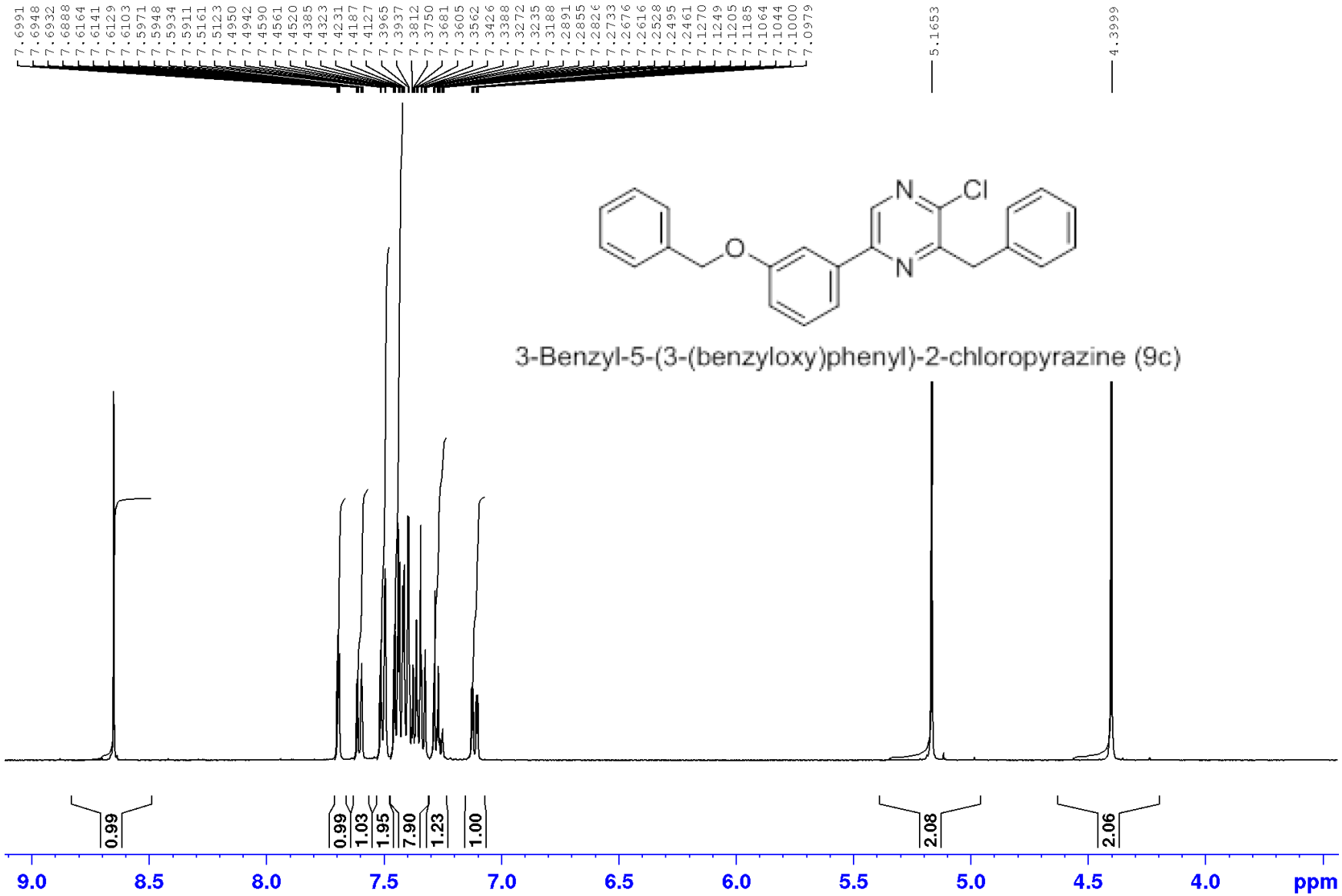
153.356
150.113
145.954
137.972
137.200
136.591
129.198
128.677
128.501
128.319
128.137
127.453
126.722
115.439
77.354
77.240
77.037
76.719
70.139
41.211
N
Cl
N
O
3-Benzyl-5-(4-(benzyloxy)phenyl)-2-chloropyrazine (9b)
160
150
140
130
120
110
100
90
80
70
60
50
ppm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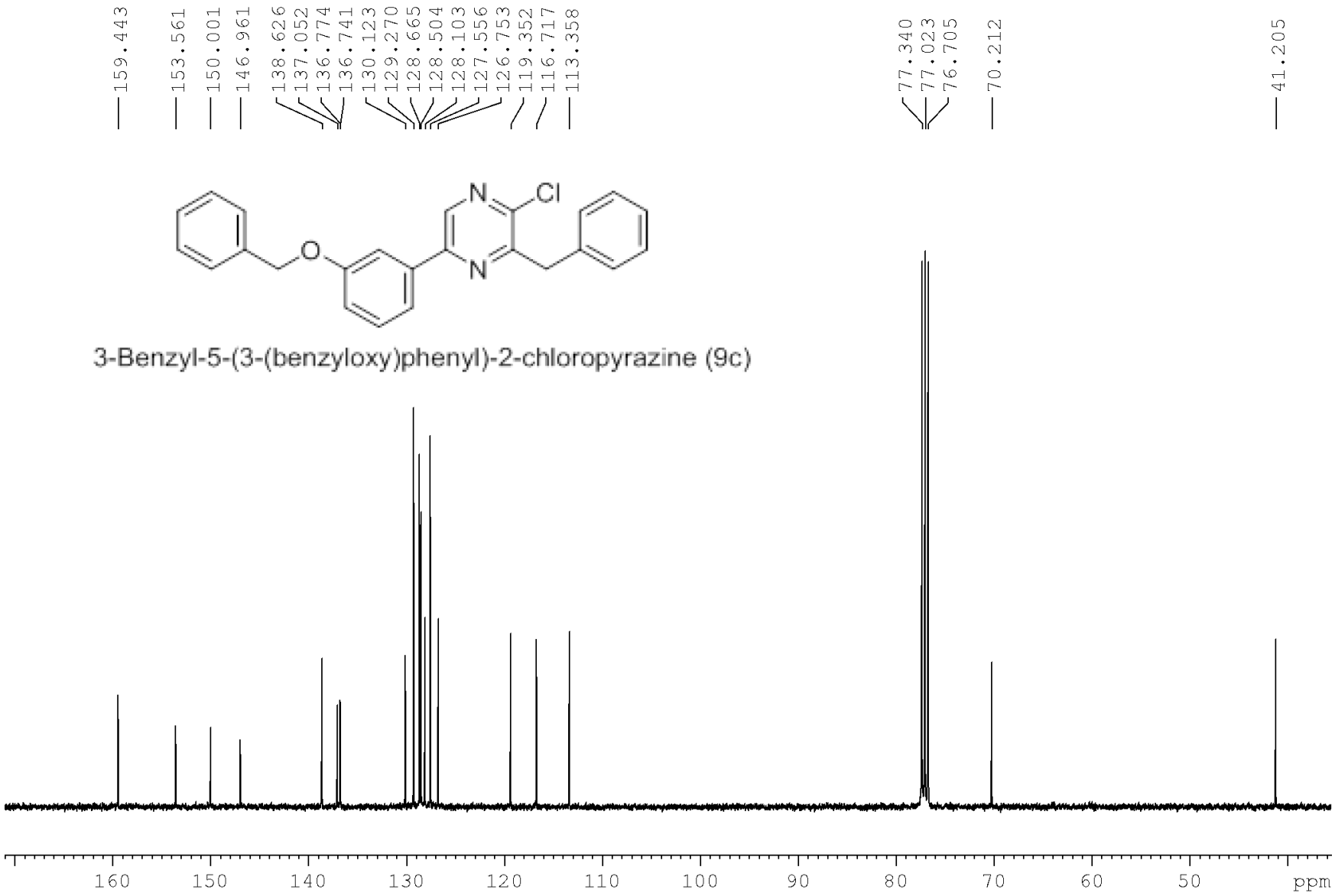
7.6932
7.6888
7.6164
7.6141
7.6129
7.6103
7.5971
7.5948
7.5934
7.5911
7.5161
7.5123
7.4950
7.4942
7.4590
7.4561
7.4520
7.4385
7.4323
7.4231
7.4187
7.4127
7.3965
7.3937
7.3812
7.3750
7.3681
7.3605
7.3562
7.3426
7.3388
7.3272
7.3235
7.3188
7.2891
7.2855
7.2826
7.2733
7.2676
7.2616
7.2528
7.2495
7.2461
7.1270
7.1249
7.1205
7.1185
7.1064
7.1044
7.1000
7.0979
5.1653
4.3999
N
Cl
O
N
3-Benzyl-5-(3-(benzyloxy)phenyl)-2-chloropyrazine (9c)
0.99
0.99
1.03
1.95
7.90
1.23
1.00
2.08
2.06
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
ppm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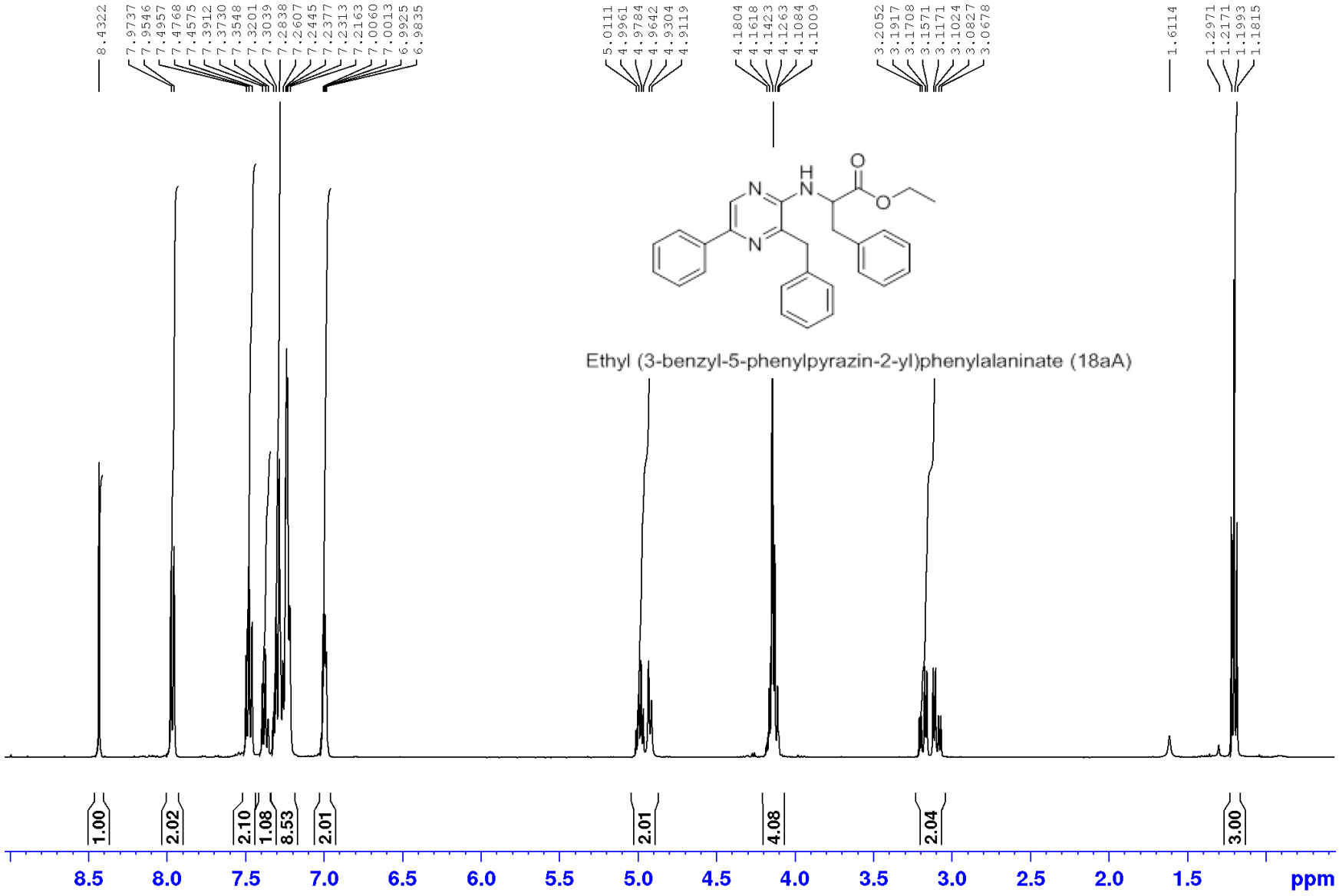
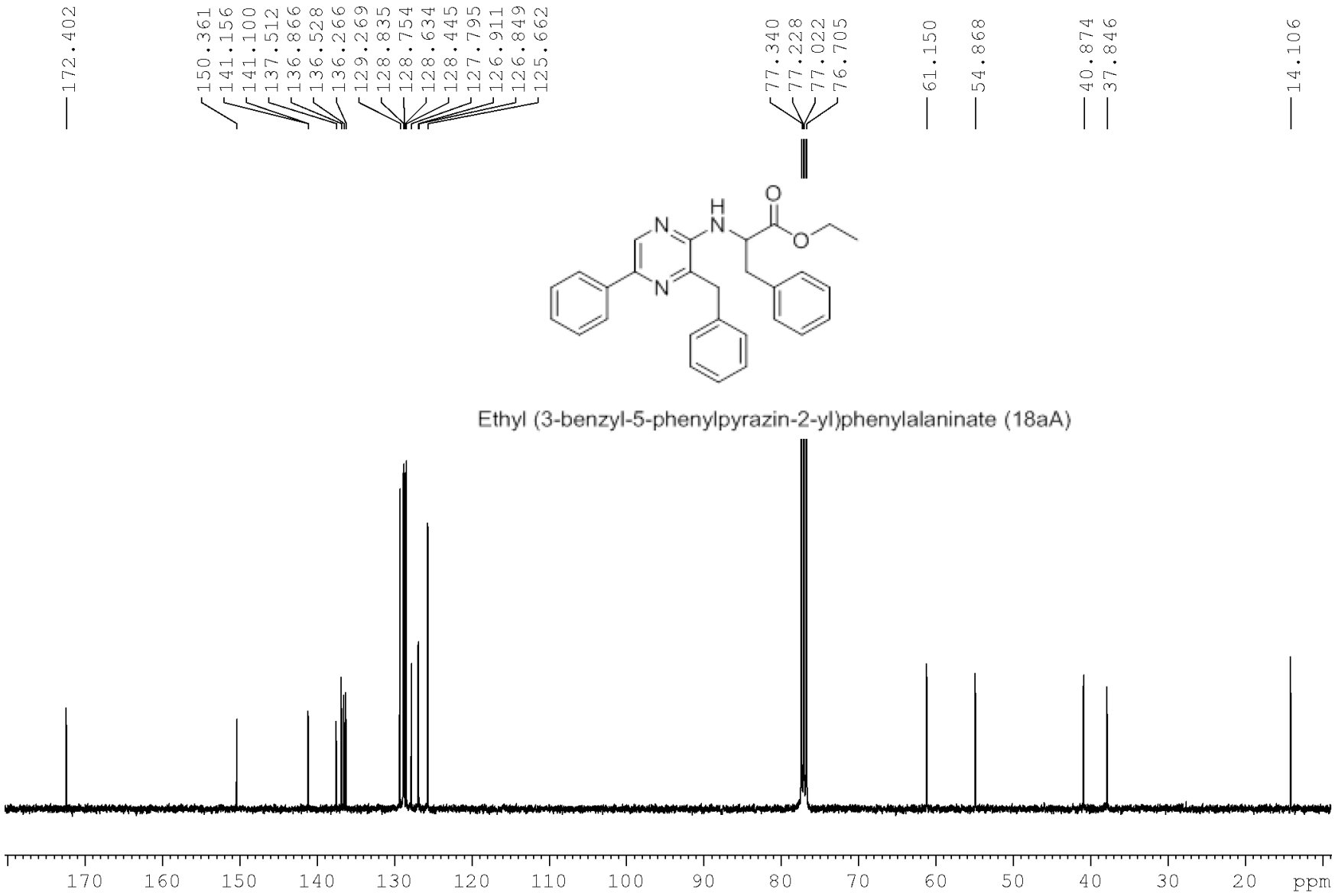
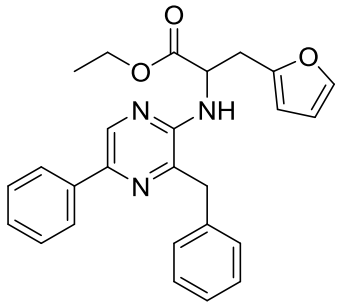
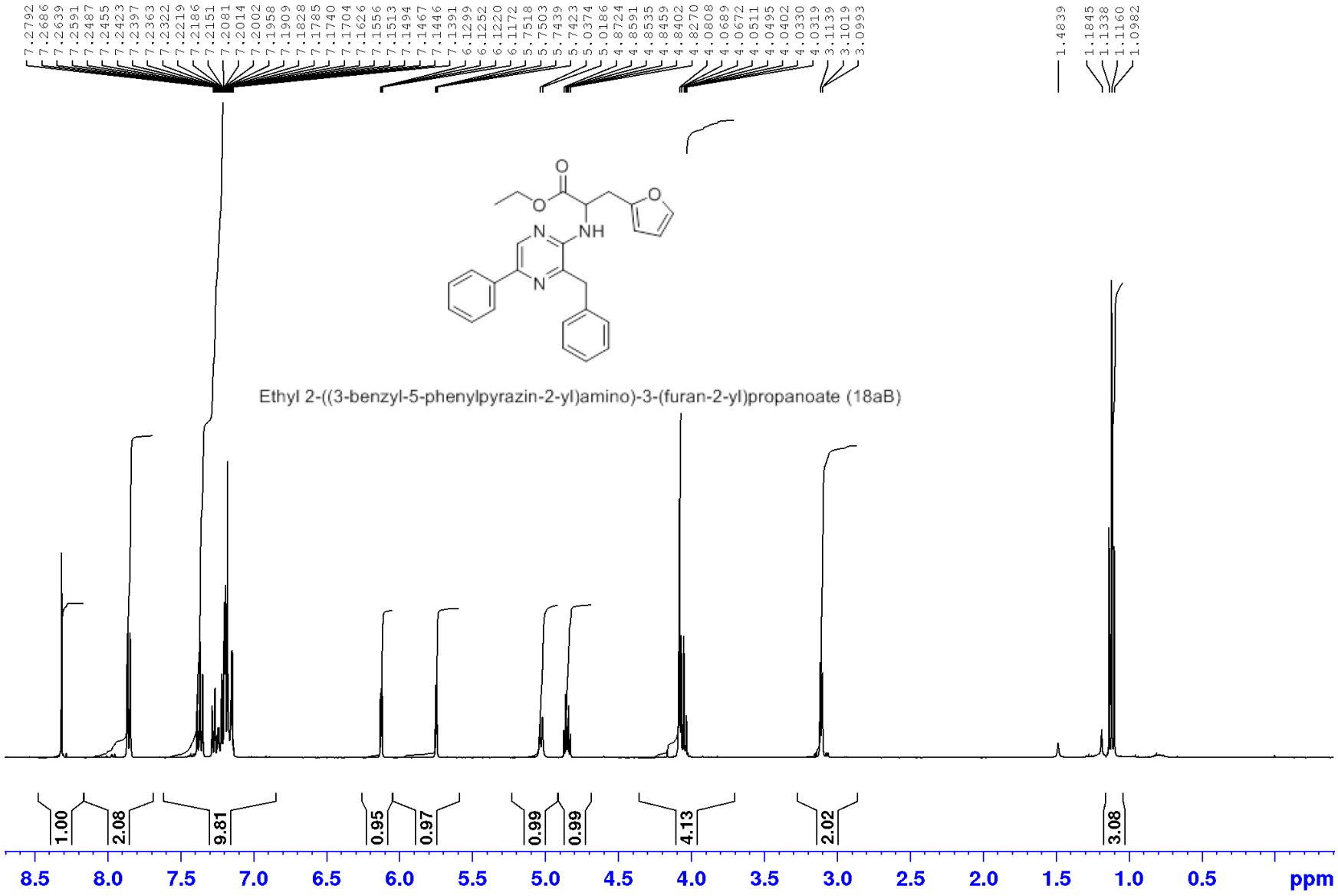
153.561
150.001
146.961
138.626
137.052
136.774
136.741
130.123
129.270
128.665
128.504
128.103
127.556
126.753
119.352
116.717
113.358
77.340
77.023
76.705
70.212
41.205
N
Cl
O
N
3-Benzyl-5-(3-(benzyloxy)phenyl)-2-chloropyrazine (9c)
160
150
140
130
120
110
100
90
80
70
60
50
ppm

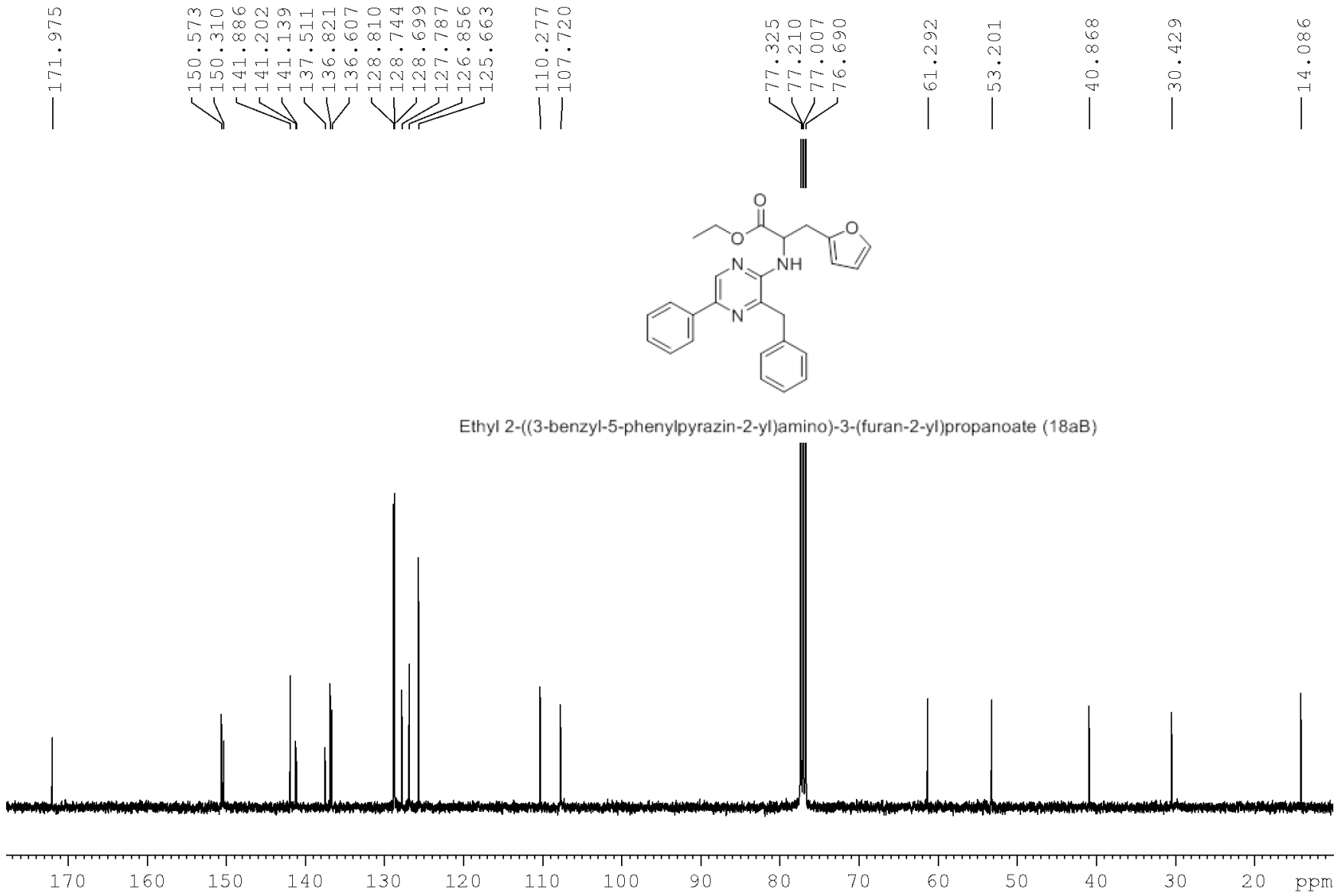

8.4322
7.9737
7.9546
7.4957
7.4768
7.4575
7.3912
7.3730
7.3548
7.3201
7.3039
7.2838
7.2607
7.2445
7.2377
7.2313
7.2163
7.0060
7.0013
6.9925
6.9835
5.0111
4.9961
4.9784
4.9642
4.9304
4.9119
4.1804
4.1618
4.1423
4.1263
4.1084
4.1009
3.2052
3.1917
3.1708
3.1571
3.1171
3.1024
3.0827
3.0678
1.6114
1.2971
1.2171
1.1993
1.1815
Ethyl (3-benzyl-5-phenylpyrazin-2-yl)phenylalaninate (18aA)
1.00
2.02
2.10
1.08
8.53
2.01
2.01
4.08
2.04
3.00
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
ppm

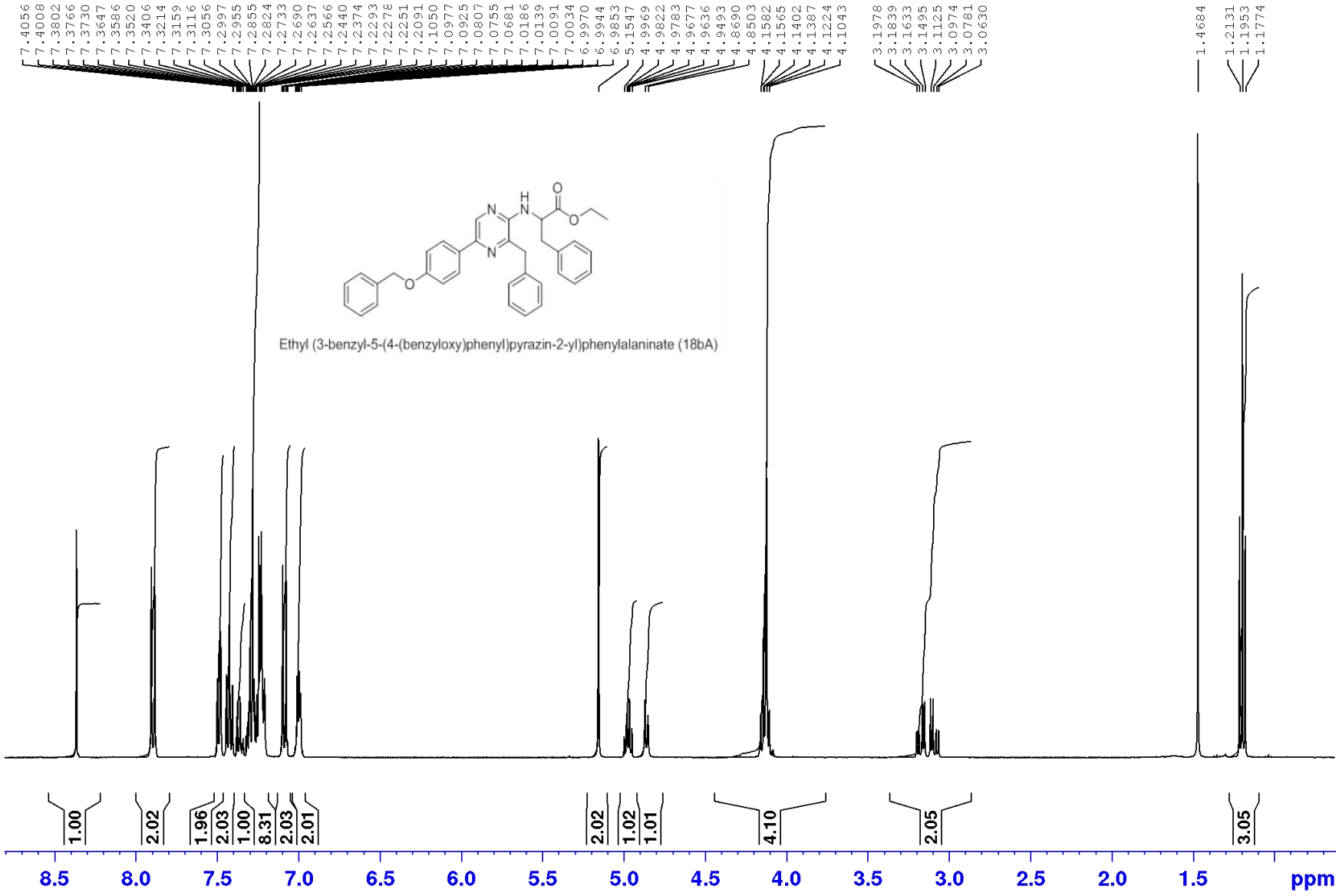

172.402
150.361
141.156
141.100
137.512
136.866
136.528
136.266
129.269
128.835
128.754
128.634
128.445
127.795
126.911
126.849
125.662
77.340
77.228
77.022
76.705
61.150
54.868
40.874
37.846
14.106
Ethyl (3-benzyl-5-phenylpyrazin-2-yl)phenylalaninate (18aA)
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
ppm

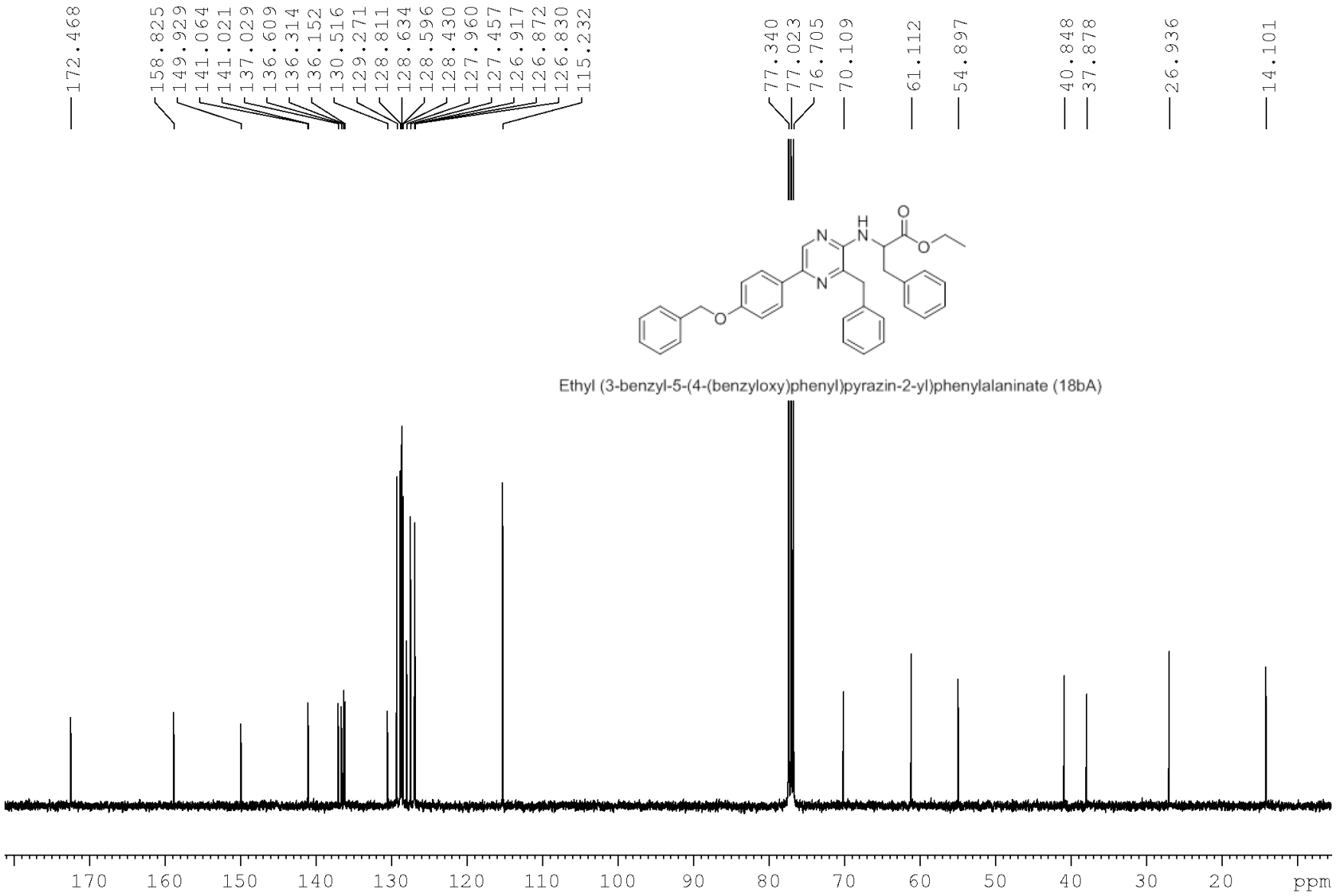


Ethyl 2-((3-benzyl-5-phenylpyrazin-2-yl)amino)-3-(furan-2-yl)propanoate (18aB)


7.2792
7.2686
7.2639
7.2591
7.2487
7.2455
7.2423
7.2397
7.2363
7.2322
7.2219
7.2186
7.2151
7.2081
7.2014
7.2002
7.1958
7.1909
7.1828
7.1785
7.1740
7.1704
7.1626
7.1556
7.1513
7.1494
7.1467
7.1446
7.1391
6.1299
6.1252
6.1220
6.1172
5.7518
5.7503
5.7439
5.7423
5.0374
5.0186
4.8724
4.8591
4.8535
4.8459
4.8402
4.8270
4.0808
4.0689
4.0672
4.0511
4.0495
4.0402
4.0330
4.0319
3.1139
3.1019
3.0993
1.4839
1.1845
1.1338
1.1160
1.0982
O
O
O
N
NH
N
Ethyl 2-((3-benzyl-5-phenylpyrazin-2-yl)amino)-3-(furan-2-yl)propanoate (18aB)
1.00
2.08
9.81
0.95
0.97
0.99
0.99
4.13
2.02
3.08
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
ppm


171.975
150.573
150.310
141.886
141.202
141.139
137.511
136.821
136.607
128.810
128.744
128.699
127.787
126.856
125.663
110.277
107.720
77.325
77.210
77.007
76.690
61.292
53.201
40.868
30.429
14.086
O
O
NH
N
N
O
Ethyl 2-((3-benzyl-5-phenylpyrazin-2-yl)amino)-3-(furan-2-yl)propanoate (18aB)
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
ppm


7.4056
7.4008
7.3802
7.3766
7.3730
7.3647
7.3586
7.3520
7.3406
7.3214
7.3159
7.3116
7.3056
7.2997
7.2955
7.2855
7.2824
7.2733
7.2690
7.2637
7.2566
7.2440
7.2374
7.2293
7.2278
7.2251
7.2091
7.1050
7.0977
7.0925
7.0807
7.0755
7.0681
7.0186
7.0139
7.0091
7.0034
6.9970
6.9944
6.9853
5.1547
4.9969
4.9822
4.9783
4.9677
4.9636
4.9493
4.8690
4.8503
4.1582
4.1565
4.1402
4.1387
4.1224
4.1043
3.1978
3.1839
3.1633
3.1495
3.1125
3.0974
3.0781
3.0630
1.4684
1.2131
1.1953
1.1774
Ethyl (3-benzyl-5-(4-(benzyloxy)phenyl)pyrazin-2-yl)phenylalaninate (18bA)
1.00
2.02
1.96
2.03
1.00
8.31
2.03
2.01
2.02
1.02
1.01
4.10
2.05
3.05
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
ppm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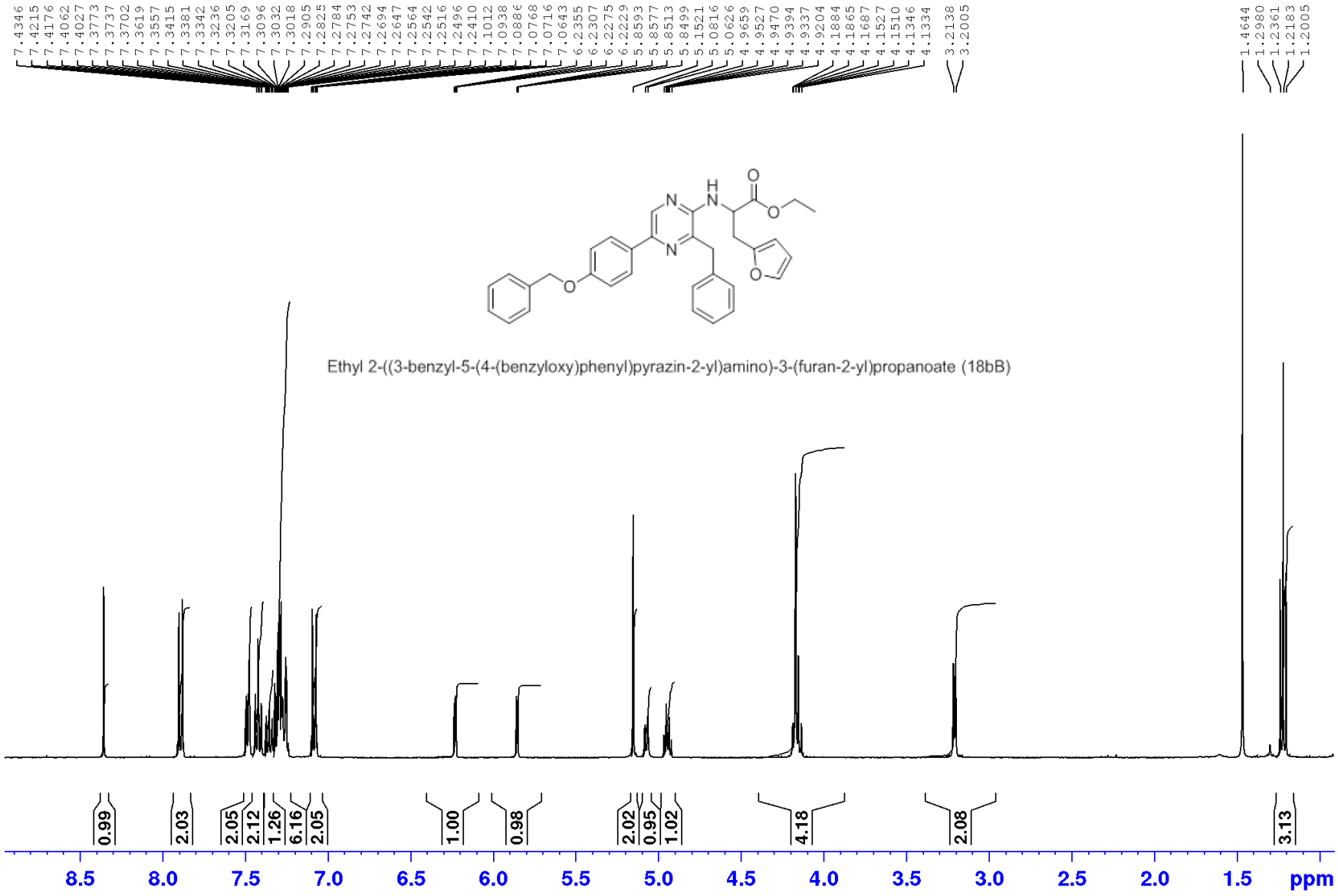
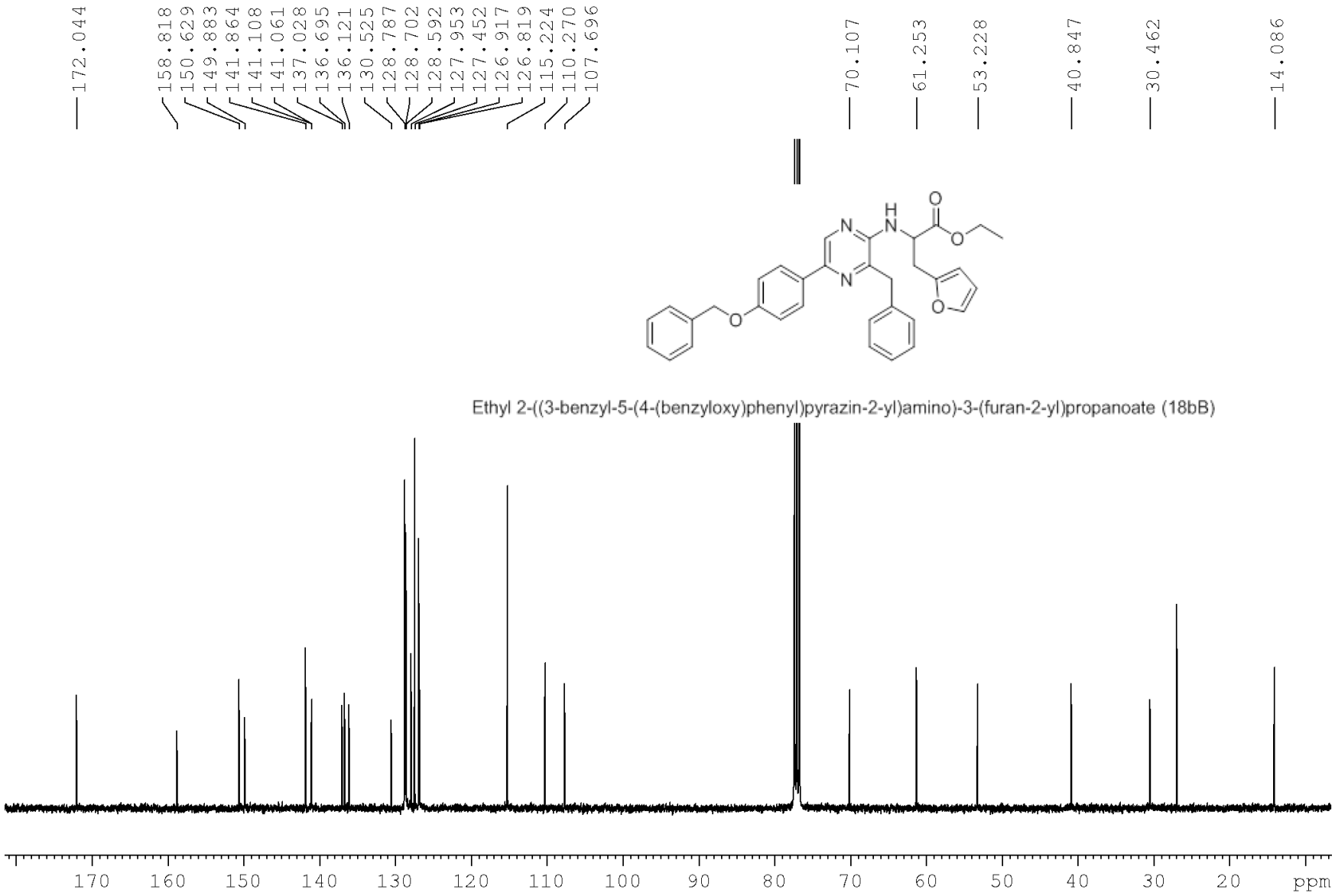
158.825
149.929
141.064
141.021
137.029
136.609
136.314
136.152
130.516
129.271
128.811
128.634
128.596
128.430
127.960
127.457
126.917
126.872
126.830
115.232
77.340
77.023
76.705
70.109
61.112
54.897
40.848
37.878
26.936
14.101
Ethyl (3-benzyl-5-(4-(benzyloxy)phenyl)pyrazin-2-yl)phenylalaninate (18bA)
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
ppm


7.4346
7.4215
7.4176
7.4062
7.4027
7.3773
7.3737
7.3702
7.3619
7.3557
7.3415
7.3381
7.3342
7.3236
7.3205
7.3169
7.3096
7.3032
7.3018
7.2905
7.2825
7.2784
7.2753
7.2742
7.2694
7.2647
7.2564
7.2542
7.2516
7.2496
7.2410
7.1012
7.0938
7.0886
7.0768
7.0716
7.0643
6.2355
6.2307
6.2275
6.2229
5.8593
5.8577
5.8513
5.8499
5.1521
5.0816
5.0626
4.9659
4.9527
4.9470
4.9394
4.9337
4.9204
4.1884
4.1865
4.1687
4.1527
4.1510
4.1346
4.1334
3.2138
3.2005
1.4644
1.2980
1.2361
1.2183
1.2005
Ethyl 2-((3-benzyl-5-(4-(benzyloxy)phenyl)pyrazin-2-yl)amino)-3-(furan-2-yl)propanoate (18bB)
0.99
2.03
2.05
2.12
1.26
6.16
2.05
1.00
0.98
2.02
0.95
1.02
4.18
2.08
3.13
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
ppm


172.044
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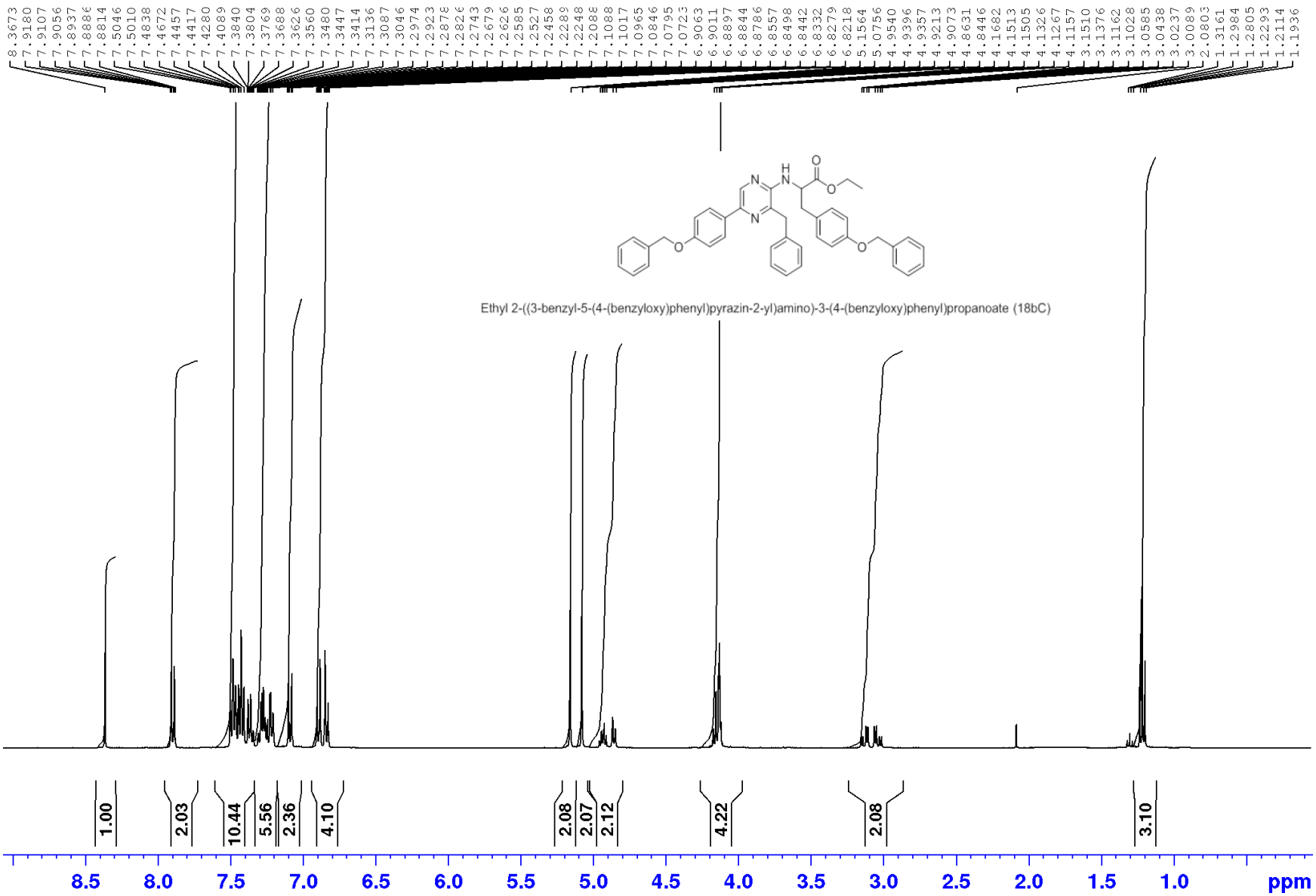
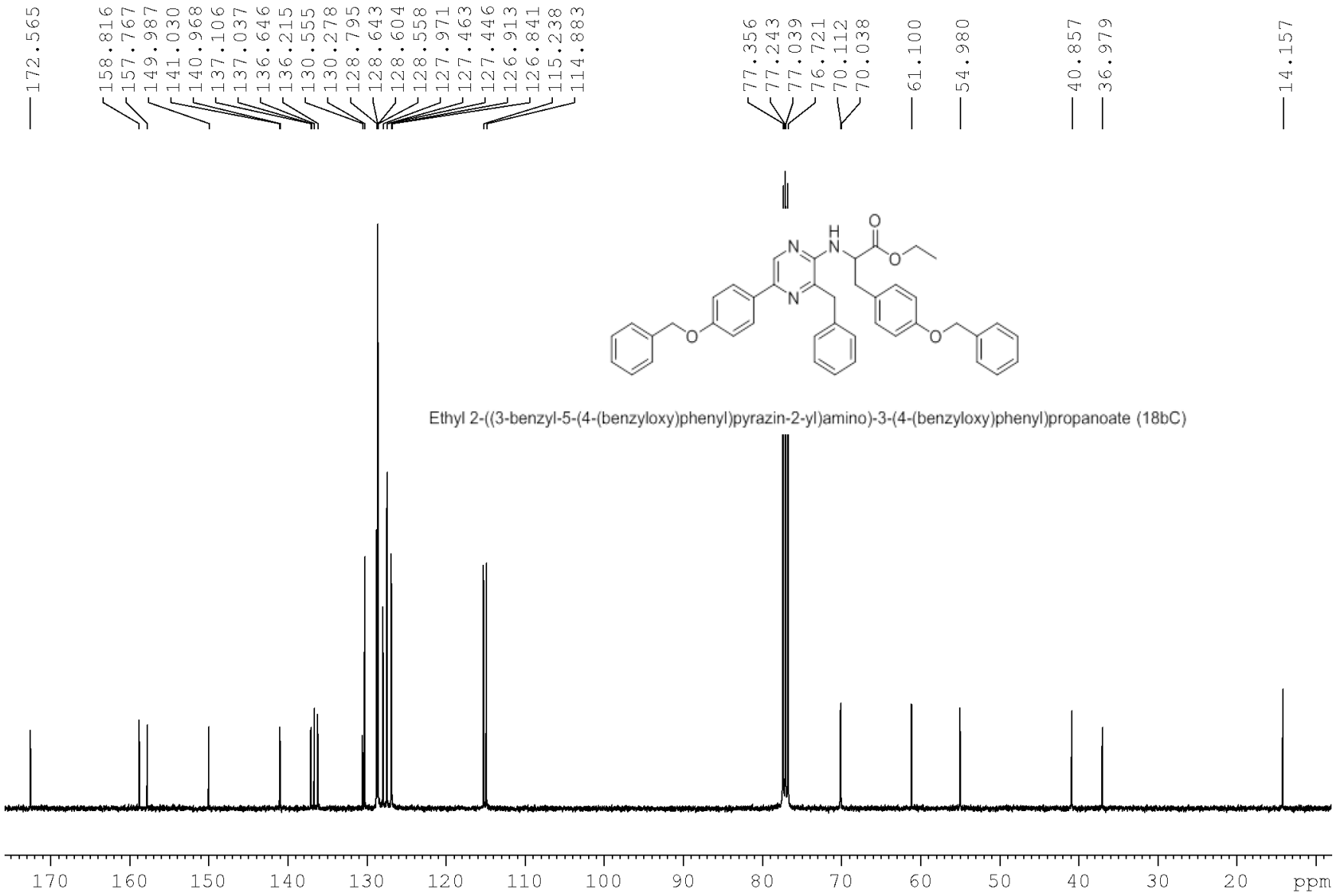
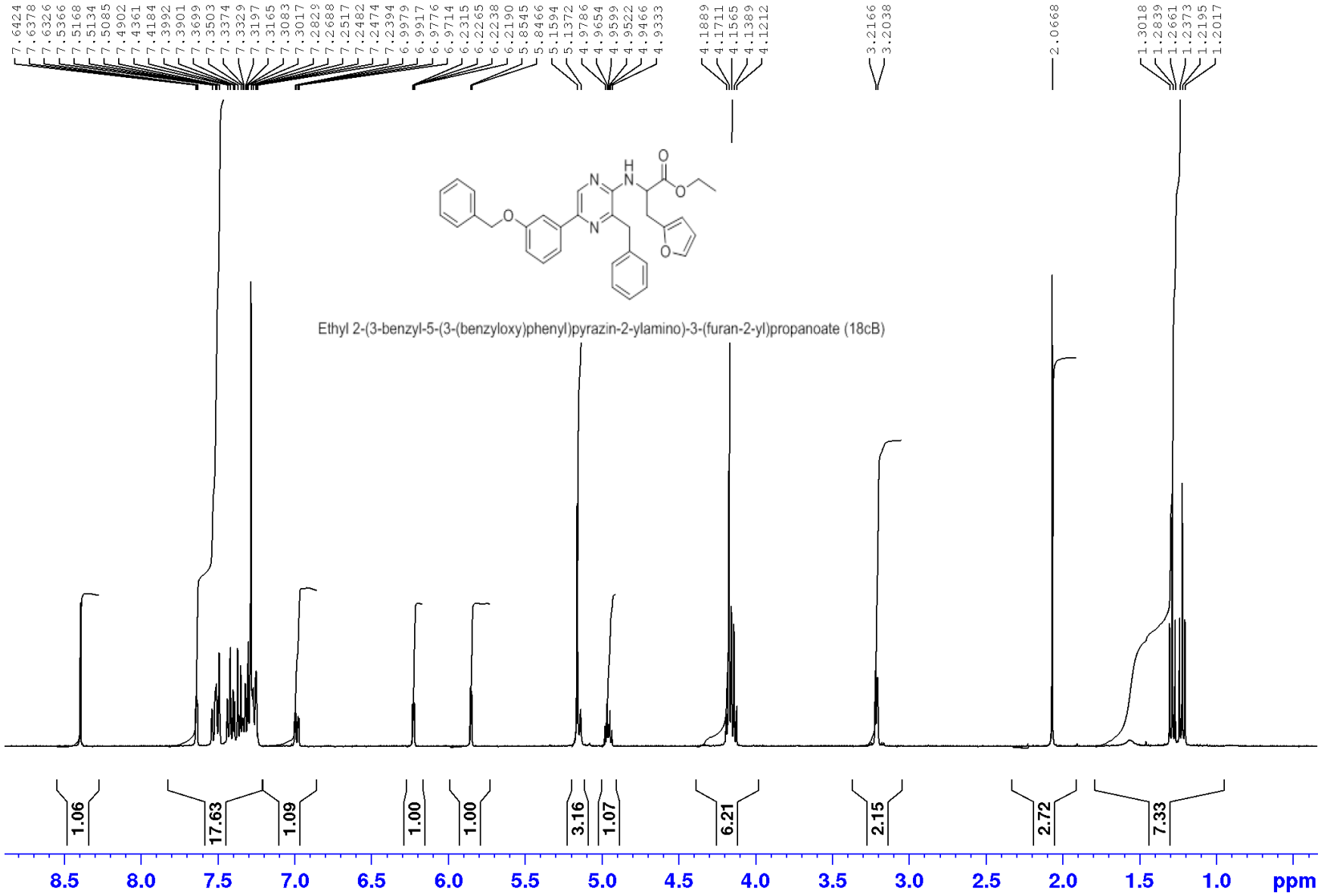
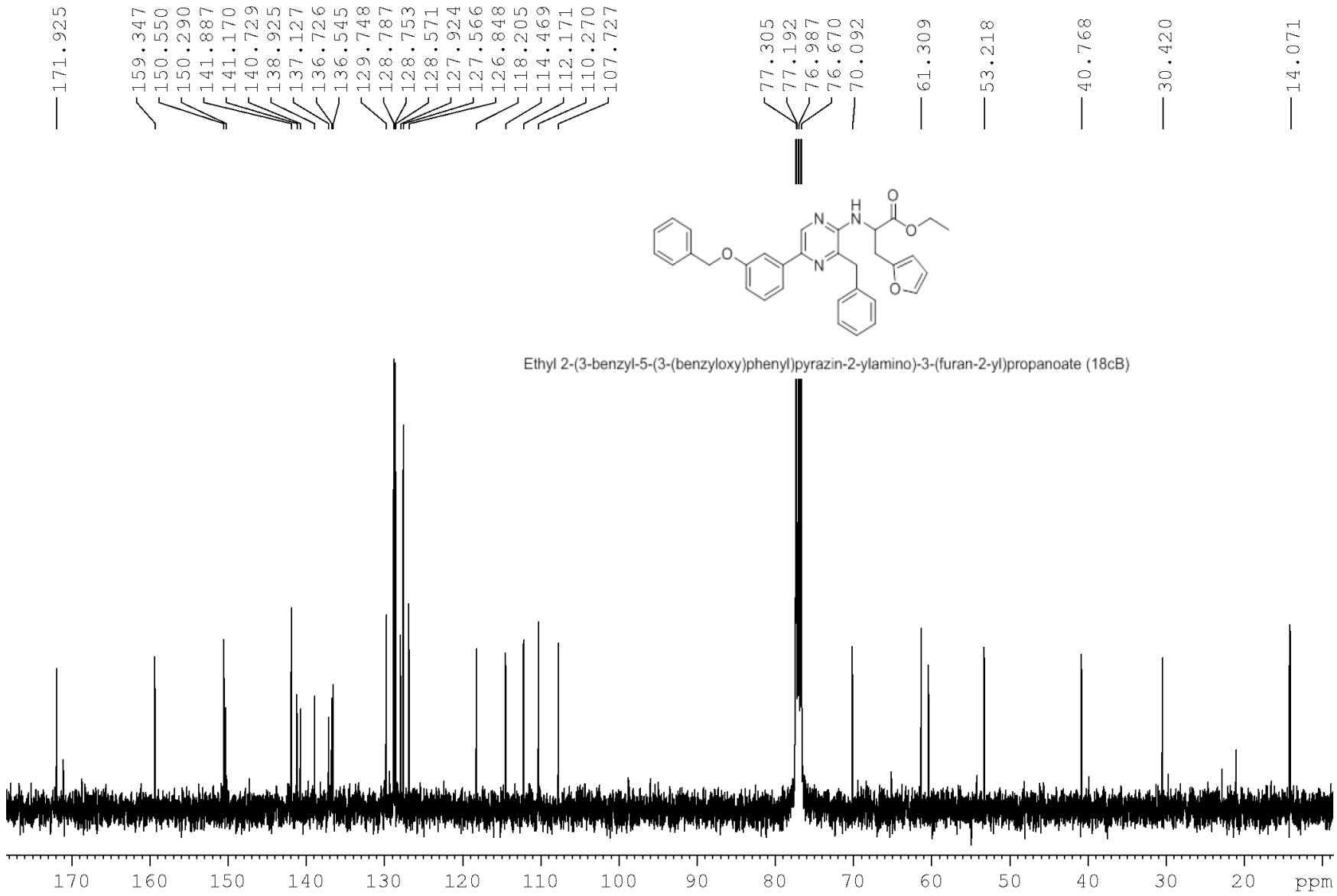
150.629
149.883
141.864
141.108
141.061
137.028
136.695
136.121
130.525
128.787
128.702
128.592
127.953
127.452
126.917
126.819
115.224
110.270
107.696
70.107
61.253
53.228
40.847
30.462
14.086
Ethyl 2-((3-benzyl-5-(4-(benzyloxy)phenyl)pyrazin-2-yl)amino)-3-(furan-2-yl)propanoate (18bB)
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
ppm


Ethyl 2-((3-benzyl-5-(4-(benzyloxy)phenyl)pyrazin-2-yl)amino)-3-(4-(benzyloxy)phenyl)propanoate (18bC)
1.00
2.03
10.44
5.56
2.36
4.10
2.08
2.07
2.12
4.22
2.08
3.10
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
ppm


172.565
158.816
157.767
149.987
141.030
140.968
137.106
137.037
136.646
136.215
130.555
130.278
128.795
128.643
128.604
128.558
127.971
127.463
127.446
126.913
126.841
115.238
114.883
77.356
77.243
77.039
76.721
70.112
70.038
61.100
54.980
40.857
36.979
14.157
Ethyl 2-((3-benzyl-5-(4-(benzyloxy)phenyl)pyrazin-2-yl)amino)-3-(4-(benzyloxy)phenyl)propanoate (18bC)
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
ppm


7.6424
7.6378
7.6326
7.5366
7.5168
7.5134
7.5085
7.4902
7.4361
7.4184
7.3992
7.3901
7.3699
7.3503
7.3374
7.3329
7.3197
7.3165
7.3083
7.3017
7.2829
7.2688
7.2517
7.2482
7.2474
7.2394
6.9979
6.9917
6.9776
6.9714
6.2315
6.2265
6.2238
6.2190
5.8545
5.8466
5.1594
5.1372
4.9786
4.9654
4.9599
4.9522
4.9466
4.9333
4.1889
4.1711
4.1565
4.1389
4.1212
3.2166
3.2038
2.0668
1.3018
1.2839
1.2661
1.2373
1.2195
1.2017
Ethyl 2-(3-benzyl-5-(3-(benzyloxy)phenyl)pyrazin-2-ylamino)-3-(furan-2-yl)propanoate (18cB)
1.06
17.63
1.09
1.00
1.00
3.16
1.07
6.21
2.15
2.72
7.33
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
ppm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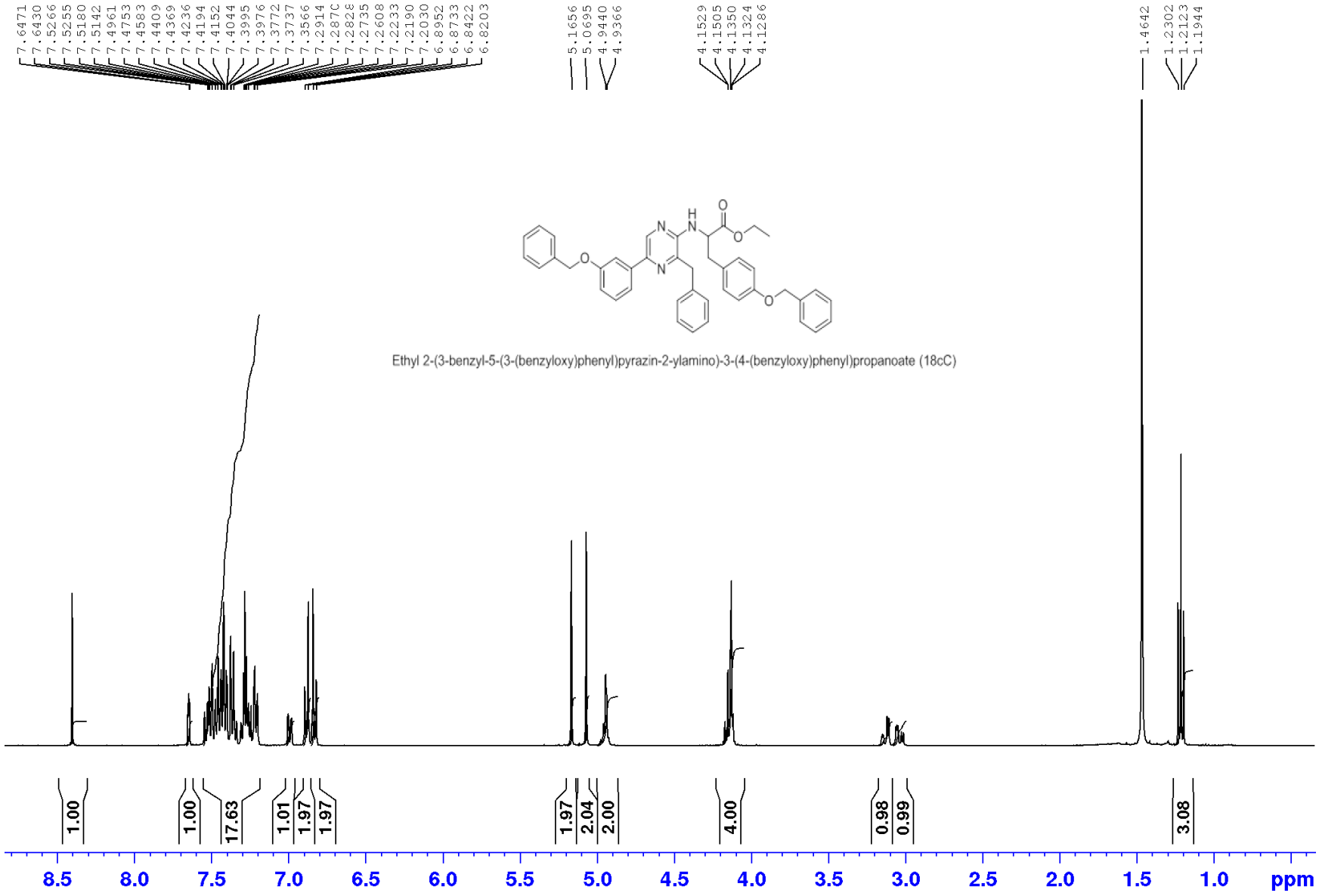
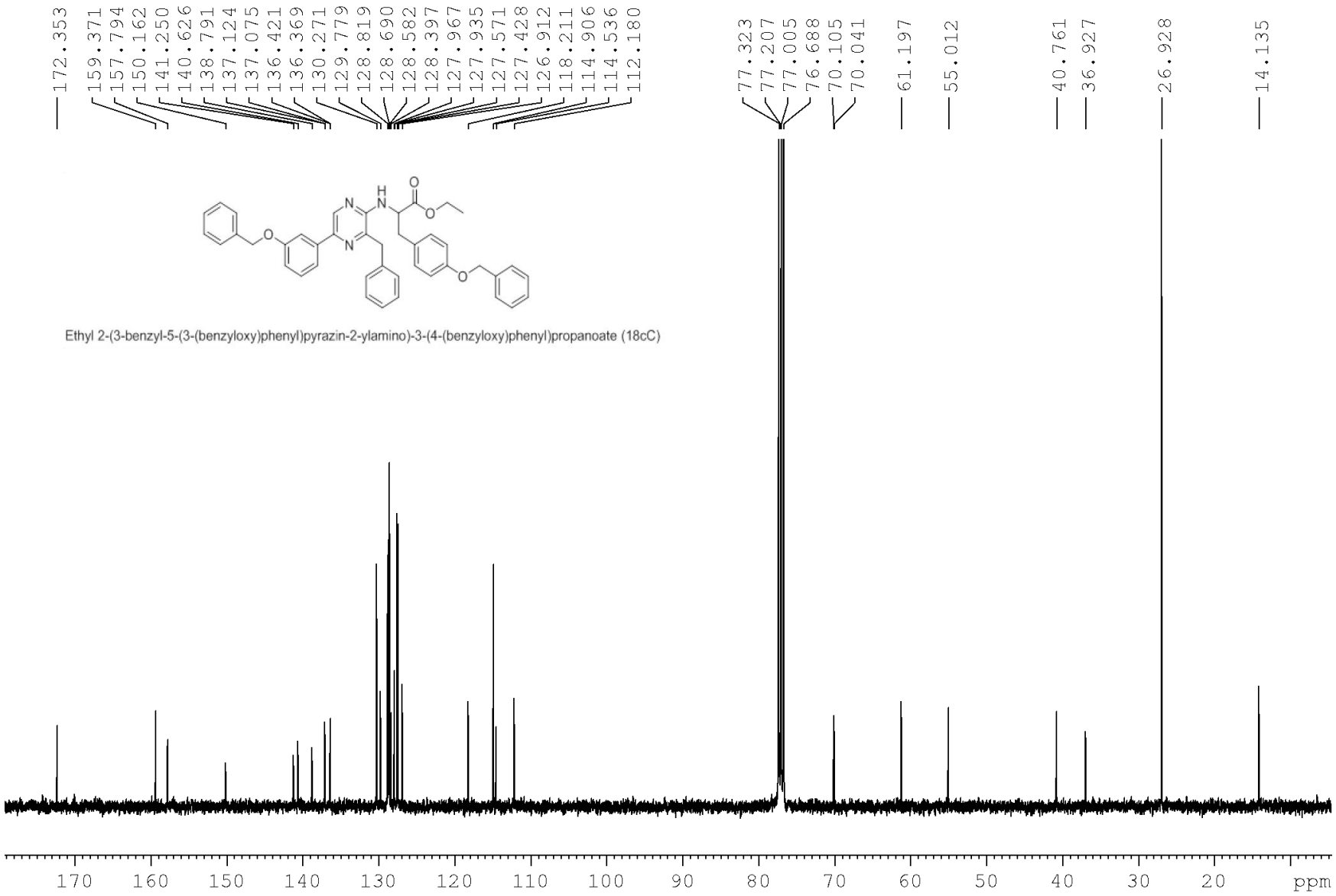
159.347
150.550
150.290
141.887
141.170
140.729
138.925
137.127
136.726
136.545
129.748
128.787
128.753
128.571
127.924
127.566
126.848
118.205
114.469
112.171
110.270
107.727
77.305
77.192
76.987
76.670
70.092
61.309
53.218
40.768
30.420
14.071
Ethyl 2-(3-benzyl-5-(3-(benzyloxy)phenyl)pyrazin-2-ylamino)-3-(furan-2-yl)propanoate (18cB)
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
ppm


7.6471
7.6430
7.5266
7.5255
7.5180
7.5142
7.4961
7.4753
7.4583
7.4409
7.4369
7.4236
7.4194
7.4152
7.4044
7.3995
7.3976
7.3772
7.3737
7.3566
7.2914
7.287C
7.2828
7.2735
7.2608
7.2233
7.2190
7.2030
6.8952
6.8733
6.8422
6.8203
5.1656
5.0695
4.9440
4.9366
4.1529
4.1505
4.1350
4.1324
4.1286
1.4642
1.2302
1.2123
1.1944
Ethyl 2-(3-benzyl-5-(3-(benzyloxy)phenyl)pyrazin-2-ylamino)-3-(4-(benzyloxy)phenyl)propanoate (18cC)
1.00
1.00
17.63
1.01
1.97
1.97
1.97
2.04
2.00
4.00
0.98
0.99
3.08
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
ppm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128.690
128.582
128.397
127.967
127.935
127.571
127.428
126.912
118.211
114.906
114.536
112.180
77.323
77.207
77.005
76.688
70.105
70.041
61.197
55.012
40.761
36.927
26.928
14.135
Ethyl 2-(3-benzyl-5-(3-(benzyloxy)phenyl)pyrazin-2-ylamino)-3-(4-(benzyloxy)phenyl)propanoate (18cC)
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160
150
140
130
120
110
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90
80
70
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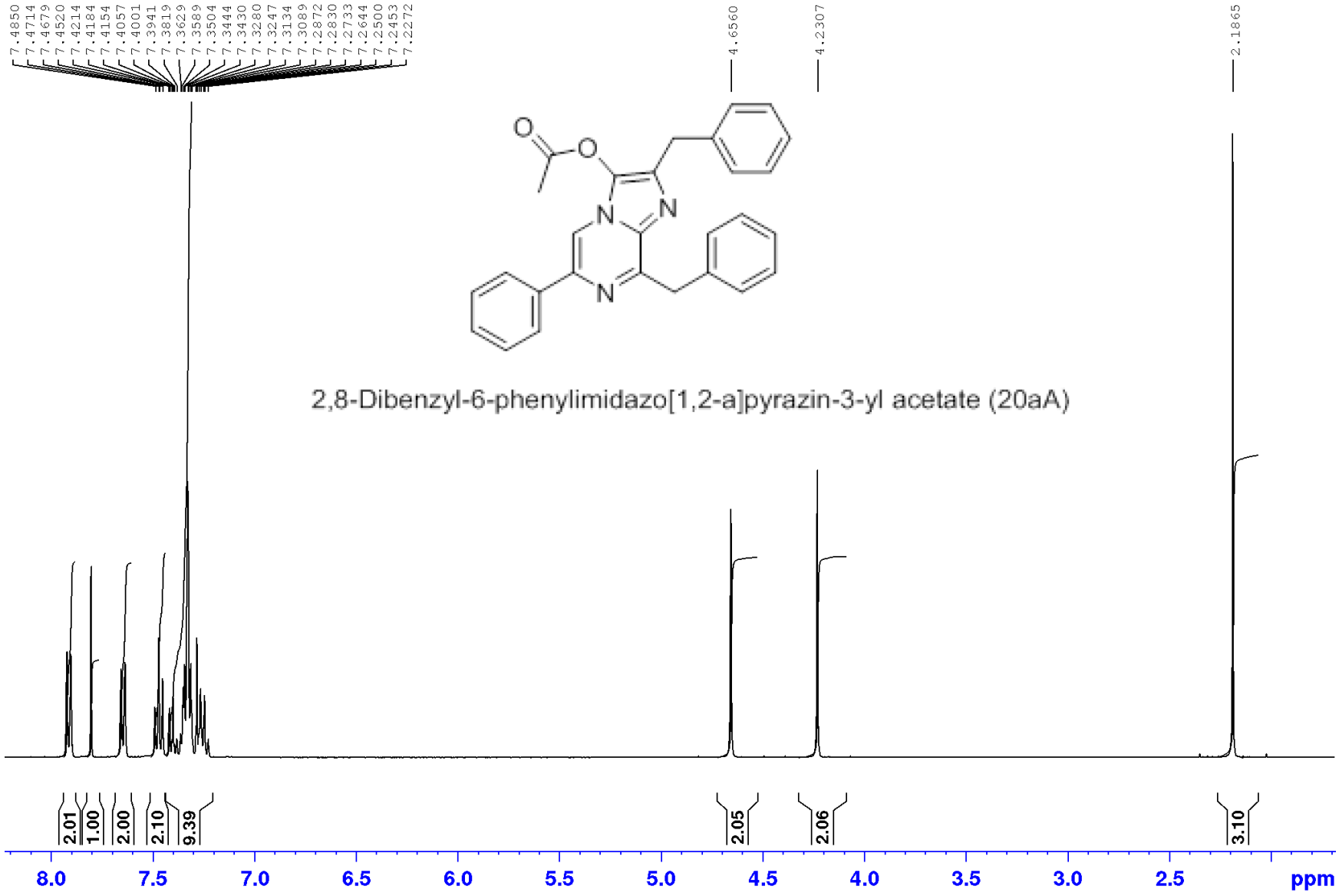
50
40
30
20
ppm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7.4154
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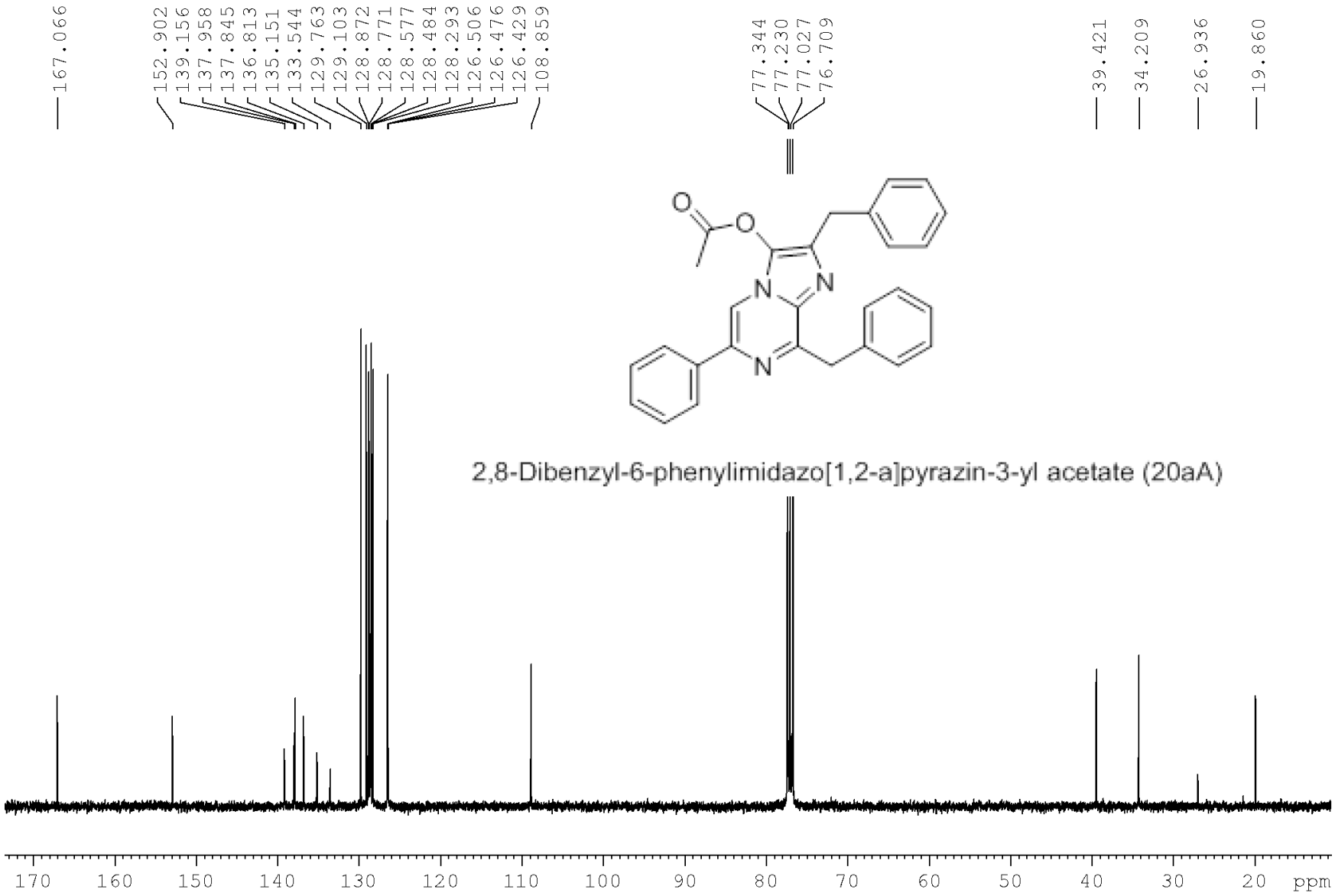
7.3819
7.3629
7.3589
7.3504
7.3444
7.3430
7.3280
7.3247
7.3134
7.3089
7.2872
7.2830
7.2733
7.2644
7.2500
7.2453
7.2272
4.6560
4.2307
2.1865
O
O
N
N
N
2,8-Dibenzyl-6-phenylimidazo[1,2-a]pyrazin-3-yl acetate (20aA)
2.01
1.00
2.00
2.10
9.39
2.05
2.06
3.10
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
ppm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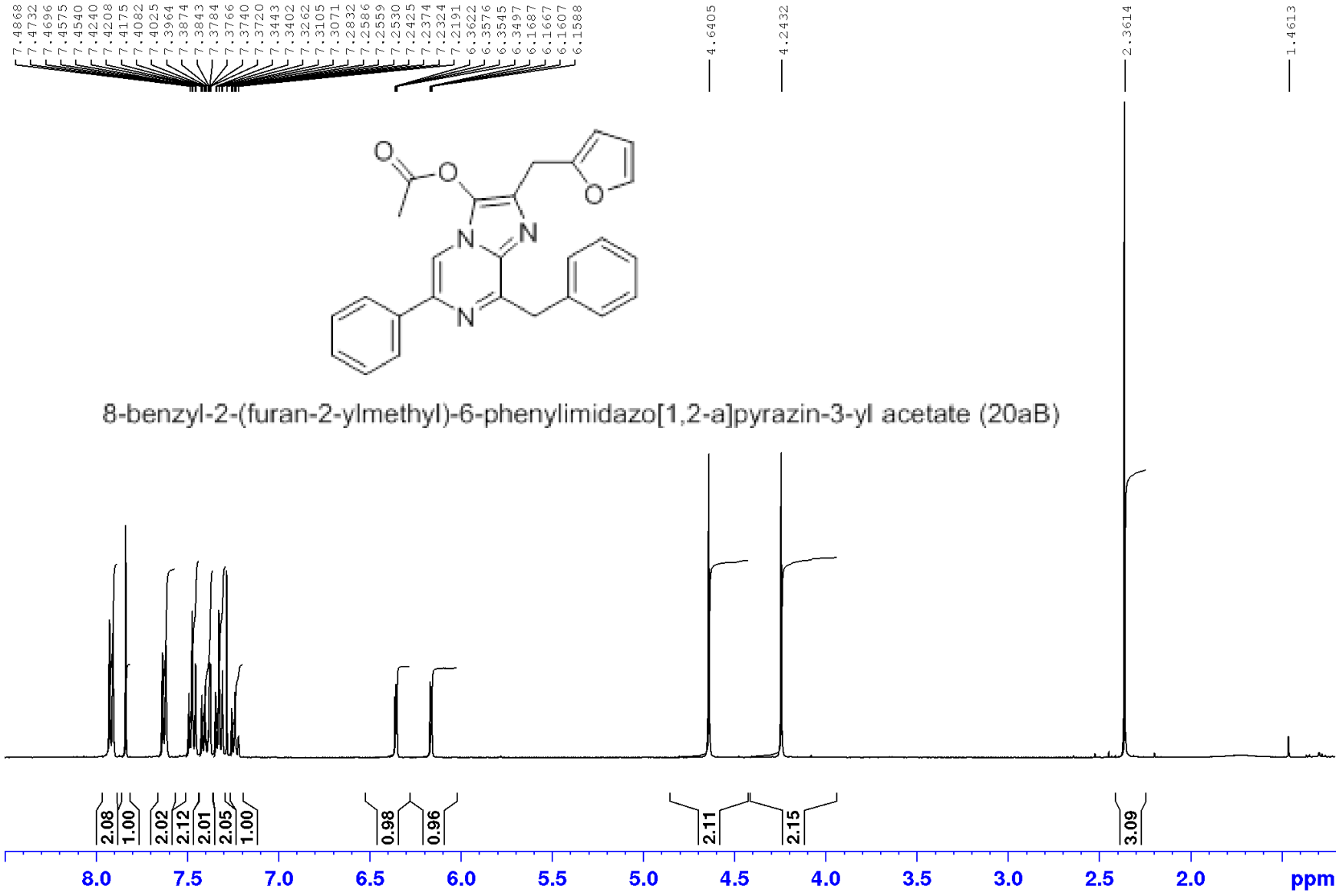
128.771
128.577
128.484
128.293
126.506
126.476
126.429
108.859
77.344
77.230
77.027
76.709
39.421
34.209
26.936
19.860
O
O
N
N
N
2,8-Dibenzyl-6-phenylimidazo[1,2-a]pyrazin-3-yl acetate (20aA)
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
ppm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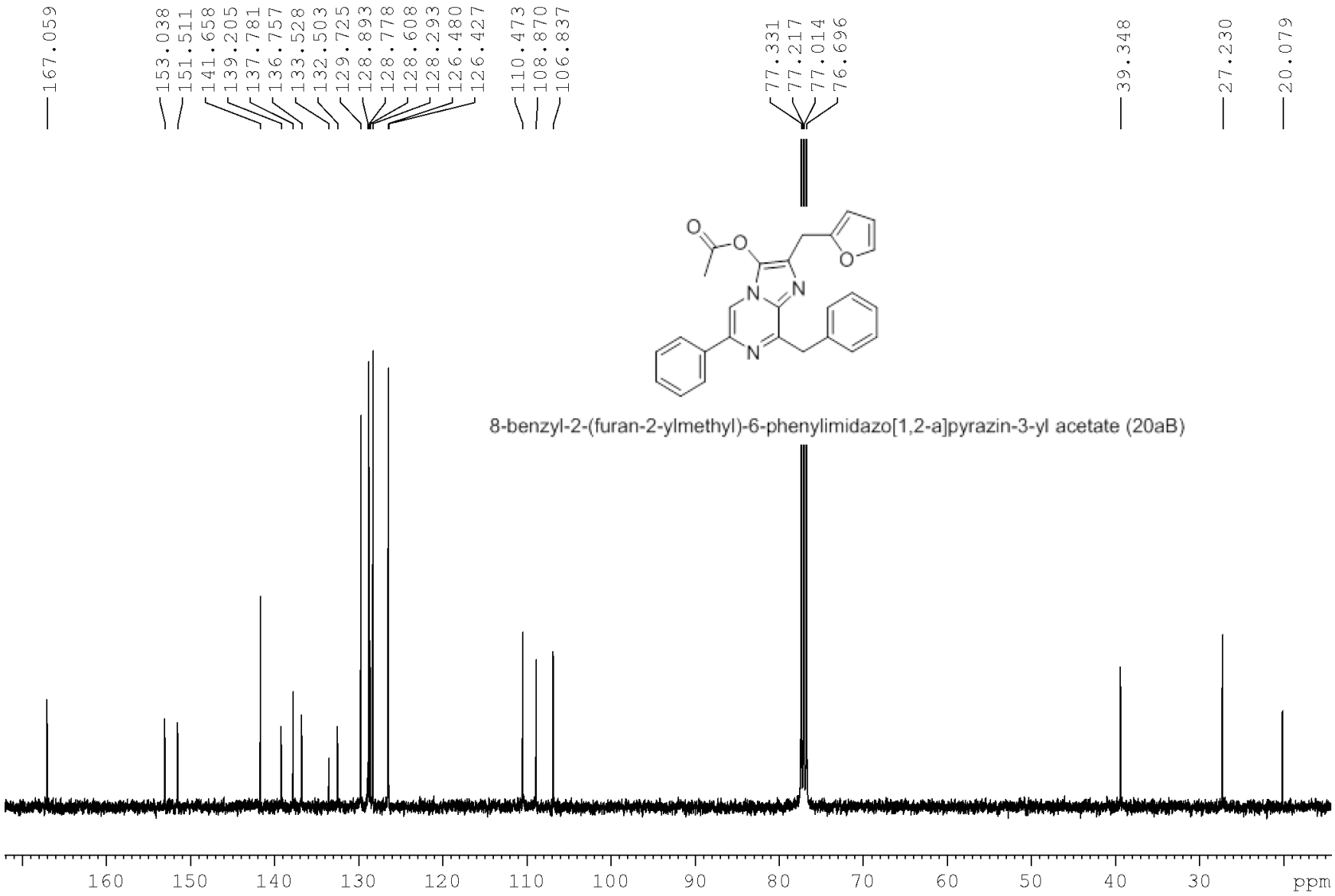
7.4540
7.4240
7.4208
7.4175
7.4082
7.4025
7.3964
7.3874
7.3843
7.3784
7.3766
7.3740
7.3720
7.3443
7.3402
7.3262
7.3105
7.3071
7.2832
7.2586
7.2559
7.2530
7.2425
7.2374
7.2324
7.2191
6.3622
6.3576
6.3545
6.3497
6.1687
6.1667
6.1607
6.1588
4.6405
4.2432
2.3614
1.4613
8-benzyl-2-(furan-2-ylmethyl)-6-phenylimidazo[1,2-a]pyrazin-3-yl acetate (20aB)
2.08
1.00
2.02
2.12
2.01
2.05
1.00
0.98
0.96
2.11
2.15
3.09
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
ppm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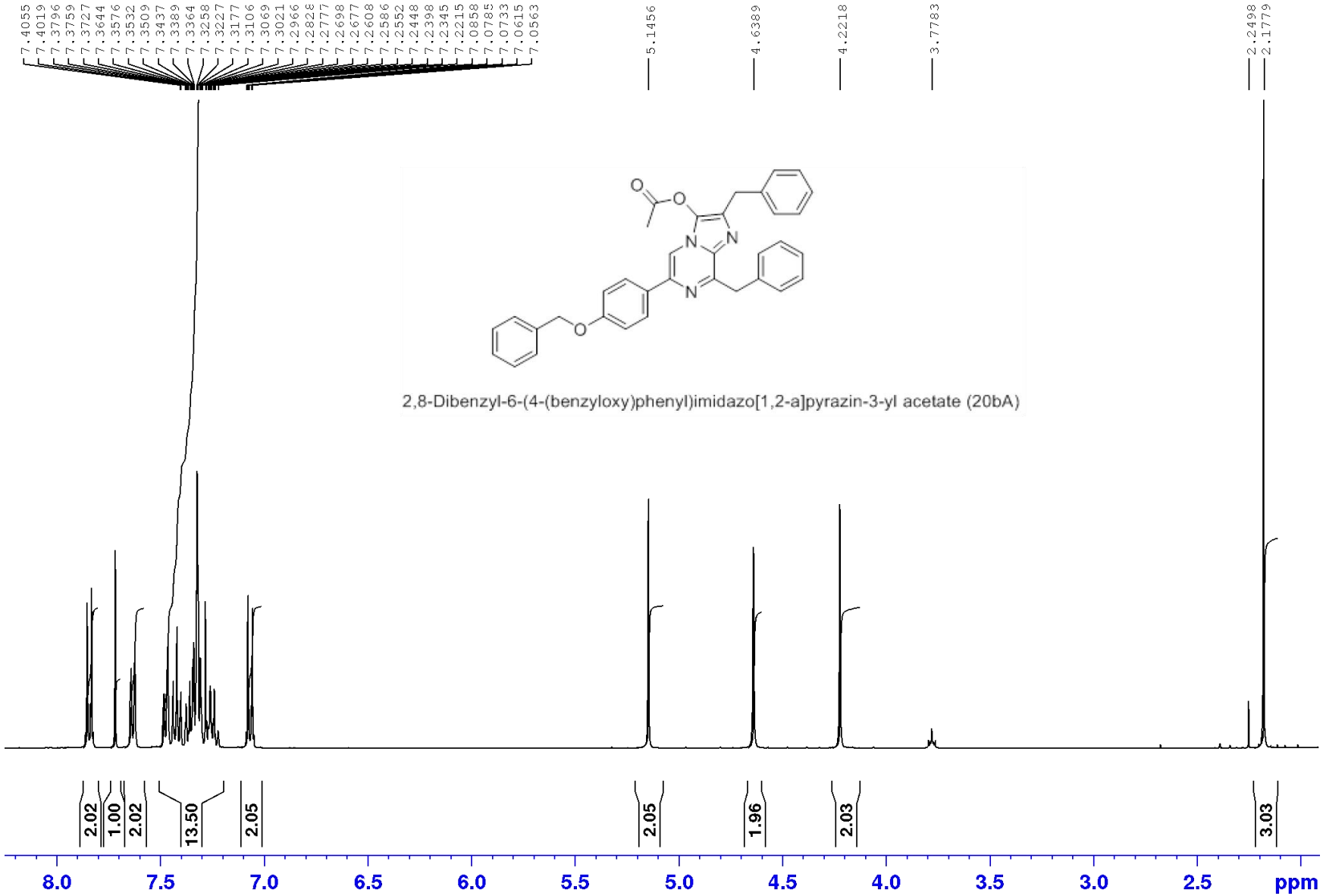
141.658
139.205
137.781
136.757
133.528
132.503
129.725
128.893
128.778
128.608
128.293
126.480
126.427
110.473
108.870
106.837
77.331
77.217
77.014
76.696
39.348
27.230
20.079
O
O
N
N
N
O
8-benzyl-2-(furan-2-ylmethyl)-6-phenylimidazo[1,2-a]pyrazin-3-yl acetate (20aB)
160
150
140
130
120
110
100
90
80
70
60
50
40
30
ppm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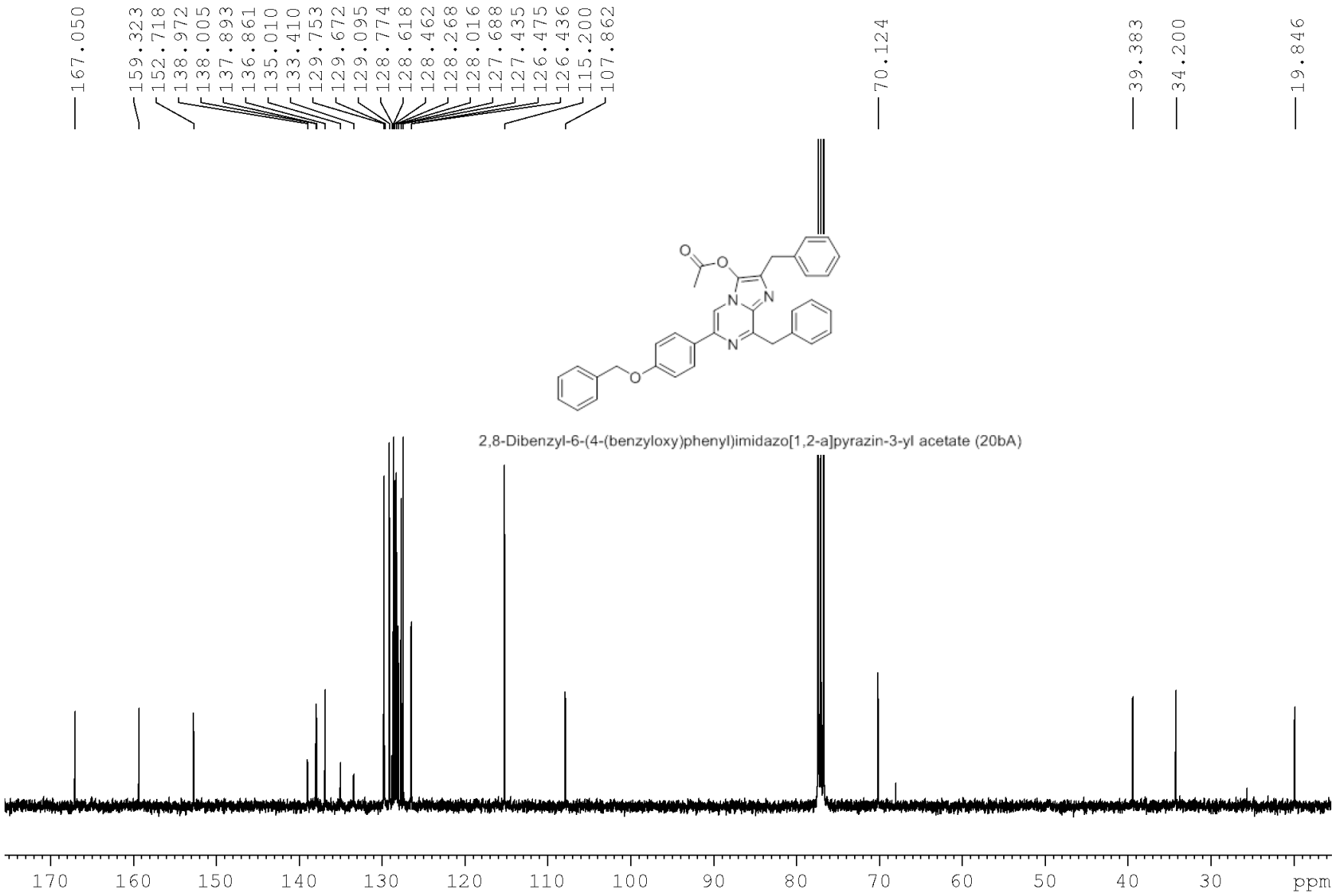
7.3796
7.3759
7.3727
7.3644
7.3576
7.3532
7.3509
7.3437
7.3389
7.3364
7.3258
7.3227
7.3177
7.3106
7.3069
7.3021
7.2966
7.2828
7.2777
7.2698
7.2677
7.2608
7.2586
7.2552
7.2448
7.2398
7.2345
7.2215
7.0858
7.0785
7.0733
7.0615
7.0563
5.1456
4.6389
4.2218
3.7783
2.2498
2.1779
2,8-Dibenzyl-6-(4-(benzyloxy)phenyl)imidazo[1,2-a]pyrazin-3-yl acetate (20bA)
2.02
1.00
2.02
13.50
2.05
2.05
1.96
2.03
3.03
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
ppm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137.893
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135.010
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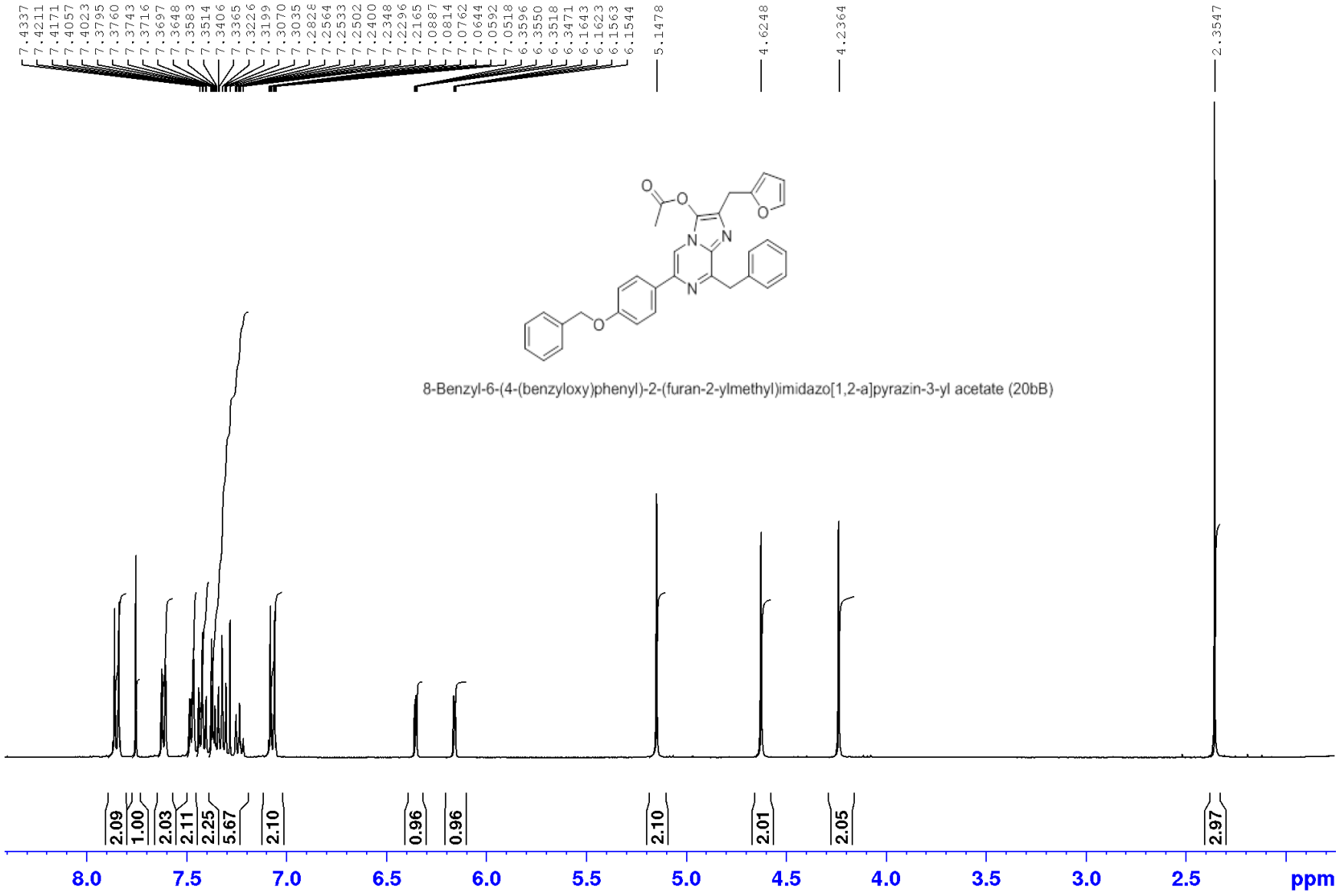
129.672
129.095
128.774
128.618
128.462
128.268
128.016
127.688
127.435
126.475
126.436
115.200
107.862
70.124
39.383
34.200
19.846
2,8-Dibenzyl-6-(4-(benzyloxy)phenyl)imidazo[1,2-a]pyrazin-3-yl acetate (20bA)
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
ppm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7.3760
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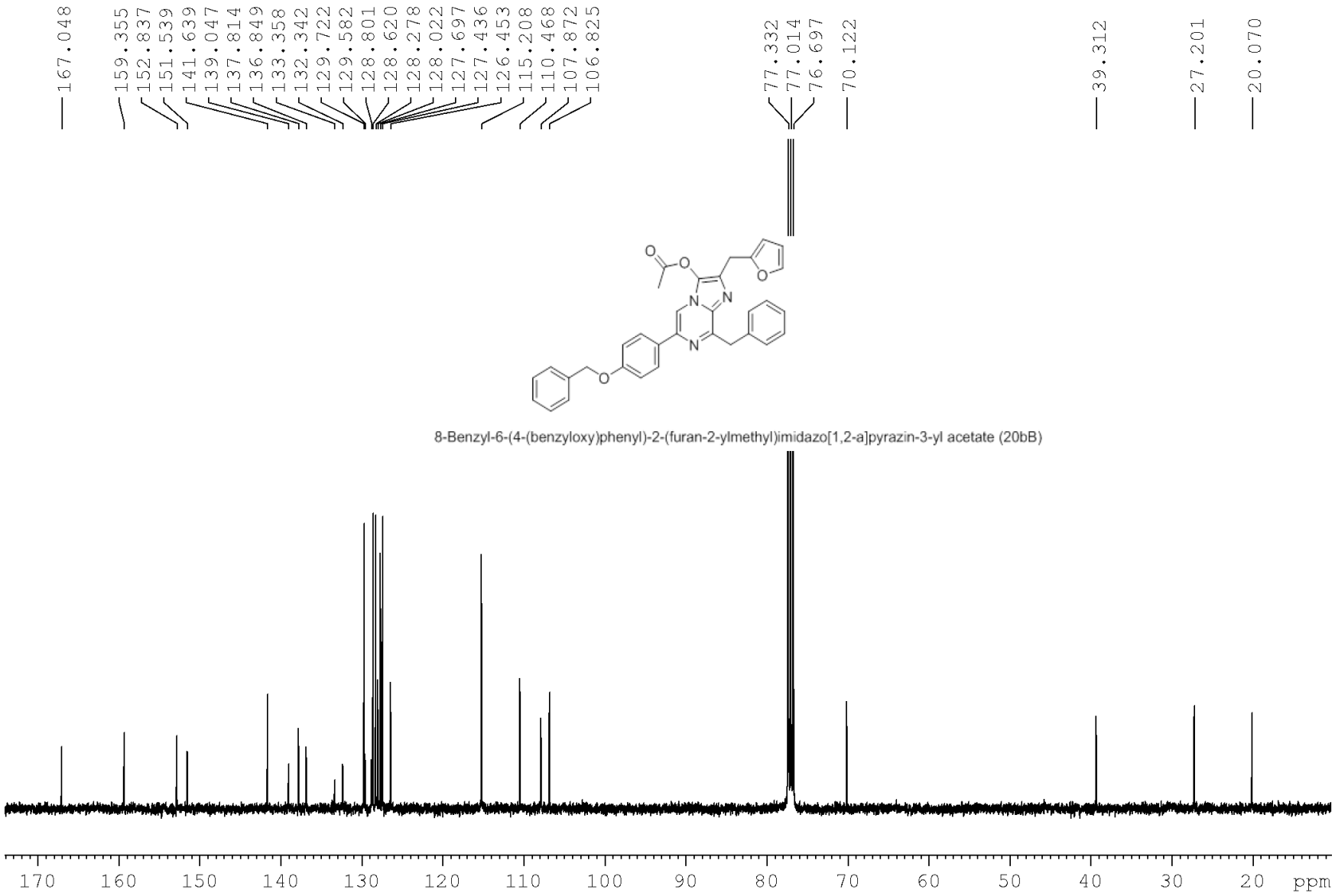
7.3716
7.3697
7.3648
7.3583
7.3514
7.3406
7.3365
7.3226
7.3199
7.3070
7.3035
7.2828
7.2564
7.2533
7.2502
7.2400
7.2348
7.2296
7.2165
7.0887
7.0814
7.0762
7.0644
7.0592
7.0518
6.3596
6.3550
6.3518
6.3471
6.1643
6.1623
6.1563
6.1544
5.1478
4.6248
4.2364
2.3547
8-Benzyl-6-(4-(benzyloxy)phenyl)-2-(furan-2-ylmethyl)imidazo[1,2-a]pyrazin-3-yl acetate (20bB)
2.09
1.00
2.03
2.11
2.25
5.67
2.10
0.96
0.96
2.10
2.01
2.05
2.97
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
ppm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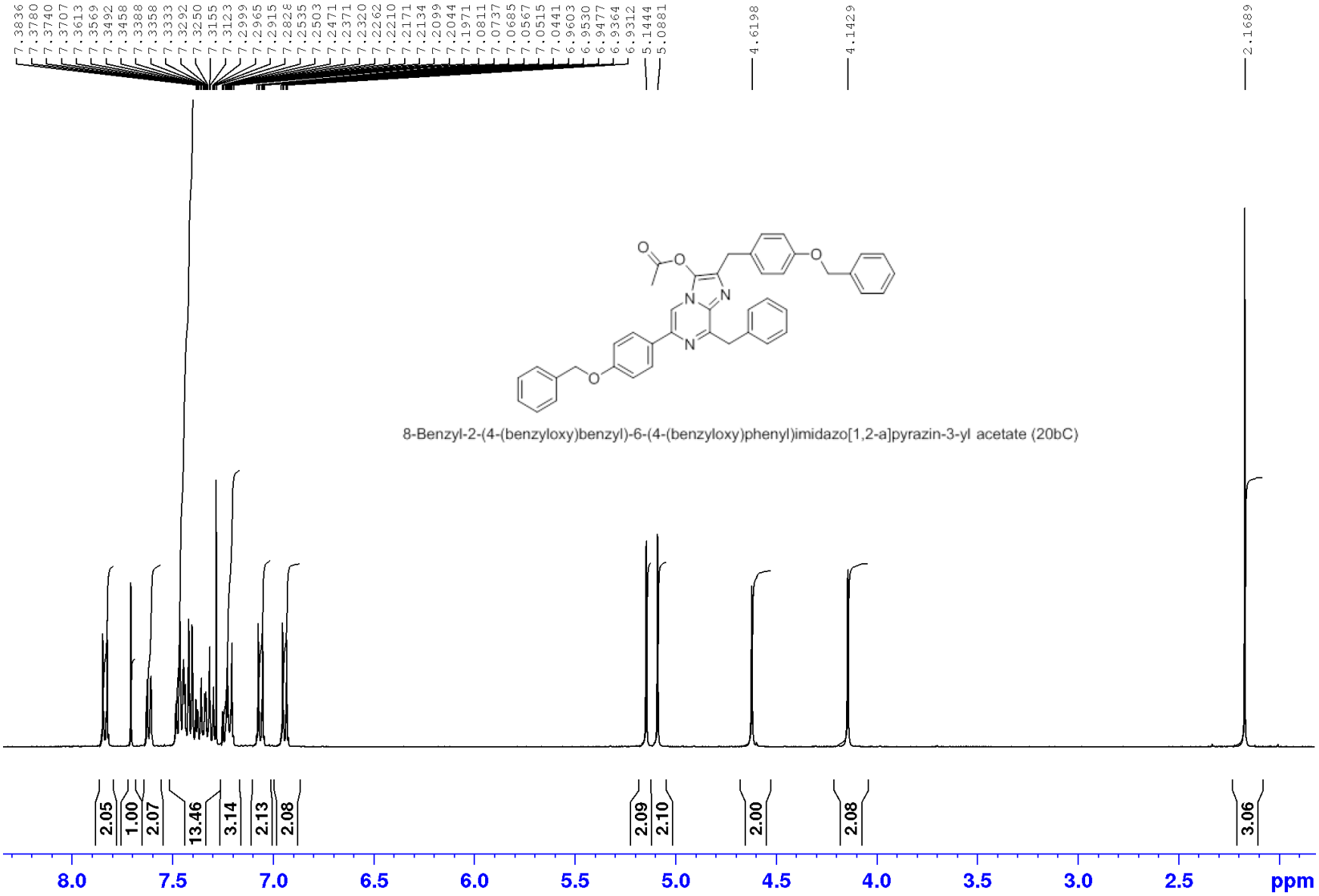
128.801
128.620
128.278
128.022
127.697
127.436
126.453
115.208
110.468
107.872
106.825
77.332
77.014
76.697
70.122
39.312
27.201
20.070
8-Benzyl-6-(4-(benzyloxy)phenyl)-2-(furan-2-ylmethyl)imidazo[1,2-a]pyrazin-3-yl acetate (20bB)
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
ppm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7.3707
7.3613
7.3569
7.3492
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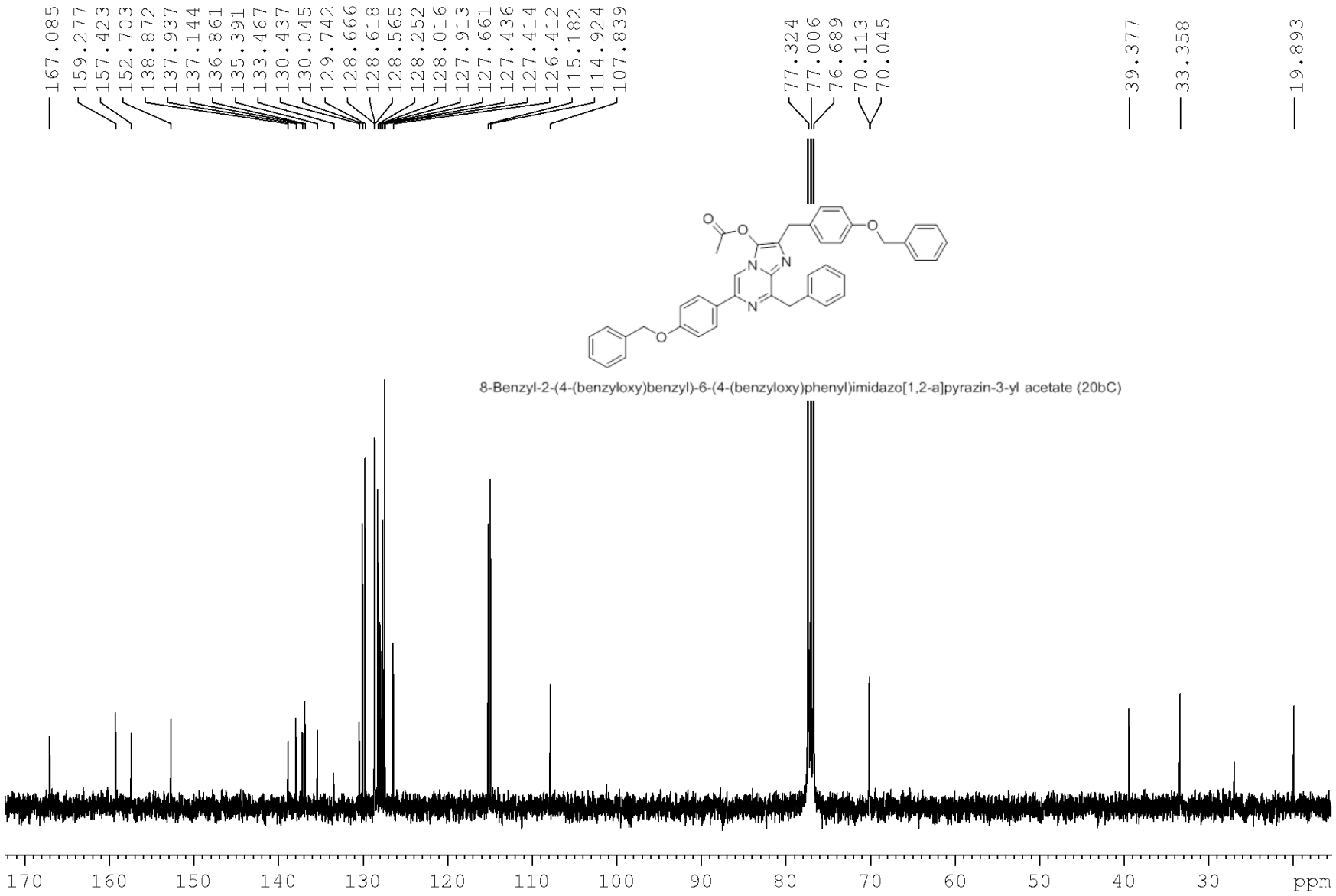
7.3388
7.3358
7.3333
7.3292
7.3250
7.3155
7.3123
7.2999
7.2965
7.2915
7.2828
7.2535
7.2503
7.2471
7.2371
7.2320
7.2262
7.2210
7.2171
7.2134
7.2099
7.2044
7.1971
7.0811
7.0737
7.0685
7.0567
7.0515
7.0441
6.9603
6.9530
6.9477
6.9364
6.9312
5.1444
5.0881
4.6198
4.1429
2.1689
8-Benzyl-2-(4-(benzyloxy)benzyl)-6-(4-(benzyloxy)phenyl)imidazo[1,2-a]pyrazin-3-yl acetate (20bC)
2.05
1.00
2.07
13.46
3.14
2.13
2.08
2.09
2.10
2.00
2.08
3.06
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
ppm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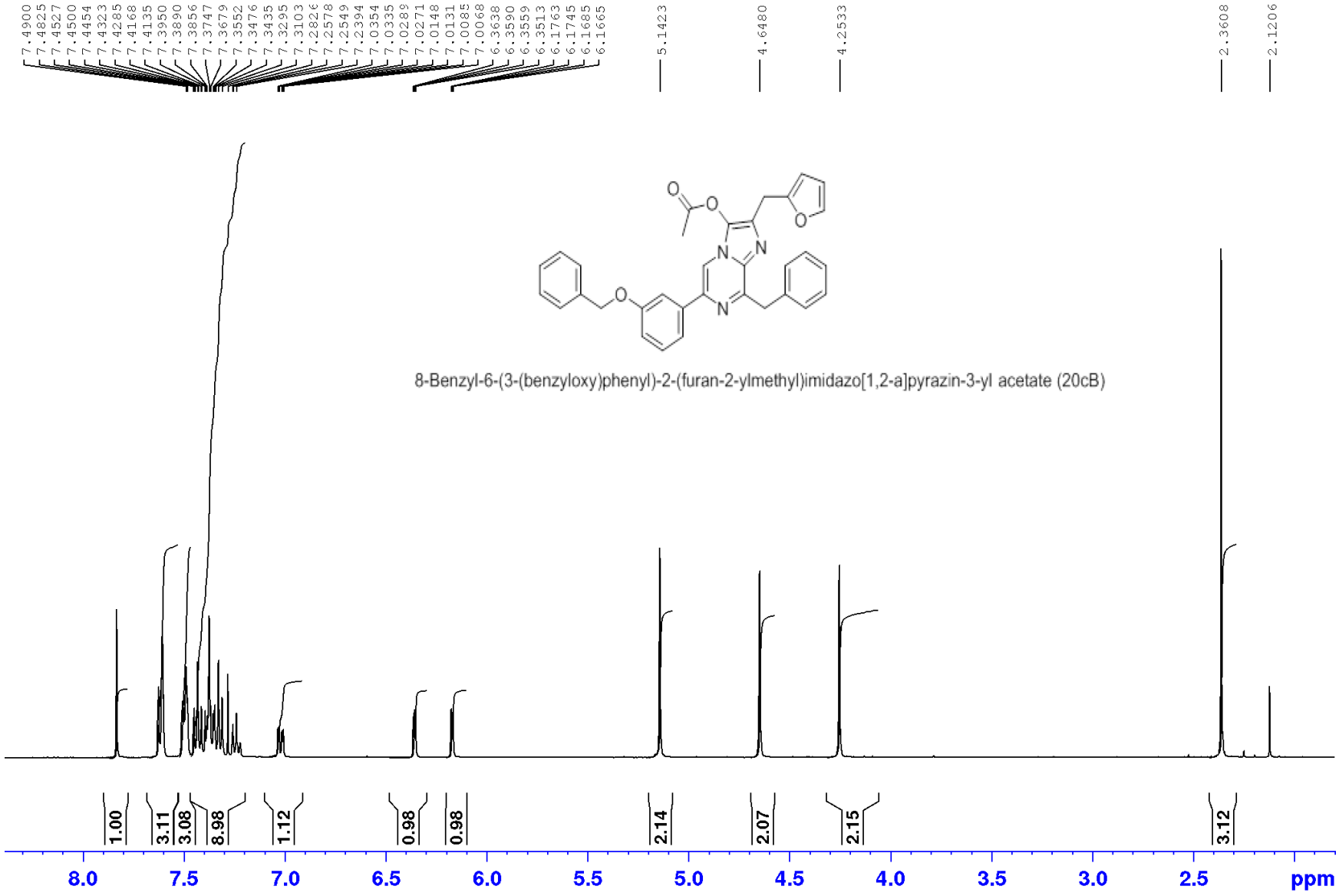
128.666
128.618
128.565
128.252
128.016
127.913
127.661
127.436
127.414
126.412
115.182
114.924
107.839
77.324
77.006
76.689
70.113
70.045
39.377
33.358
19.893
8-Benzyl-2-(4-(benzyloxy)benzyl)-6-(4-(benzyloxy)phenyl)imidazo[1,2-a]pyrazin-3-yl acetate (20bC)
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
ppm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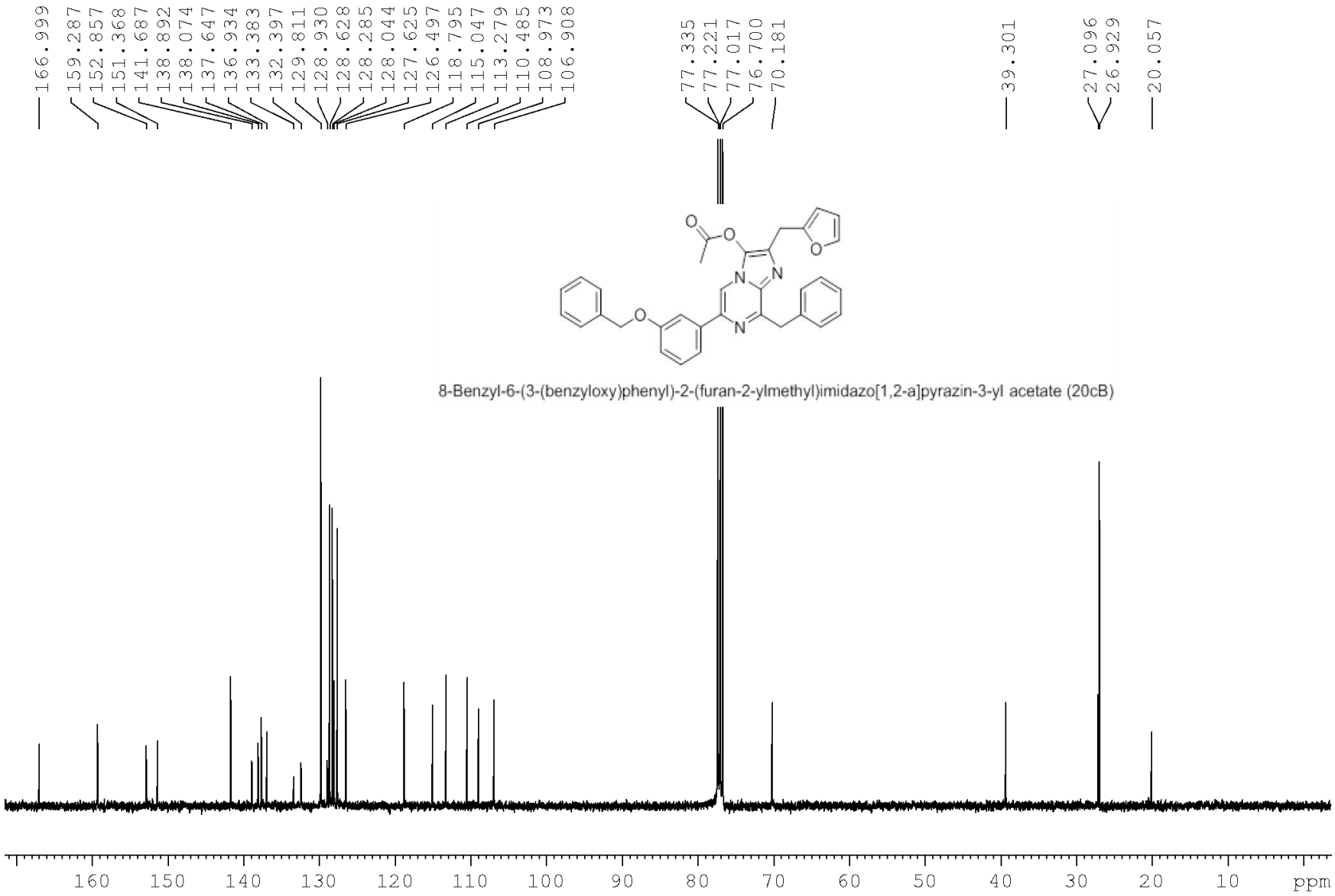
7.3950
7.3890
7.3856
7.3747
7.3679
7.3552
7.3476
7.3435
7.3295
7.3103
7.2826
7.2578
7.2549
7.2394
7.0354
7.0335
7.0289
7.0271
7.0148
7.0131
7.0085
7.0068
6.3638
6.3590
6.3559
6.3513
6.1763
6.1745
6.1685
6.1665
5.1423
4.6480
4.2533
2.3608
2.1206
8-Benzyl-6-(3-(benzyloxy)phenyl)-2-(furan-2-ylmethyl)imidazo[1,2-a]pyrazin-3-yl acetate (20cB)
1.00
3.11
3.08
8.98
1.12
0.98
0.98
2.14
2.07
2.15
3.12
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
ppm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137.647
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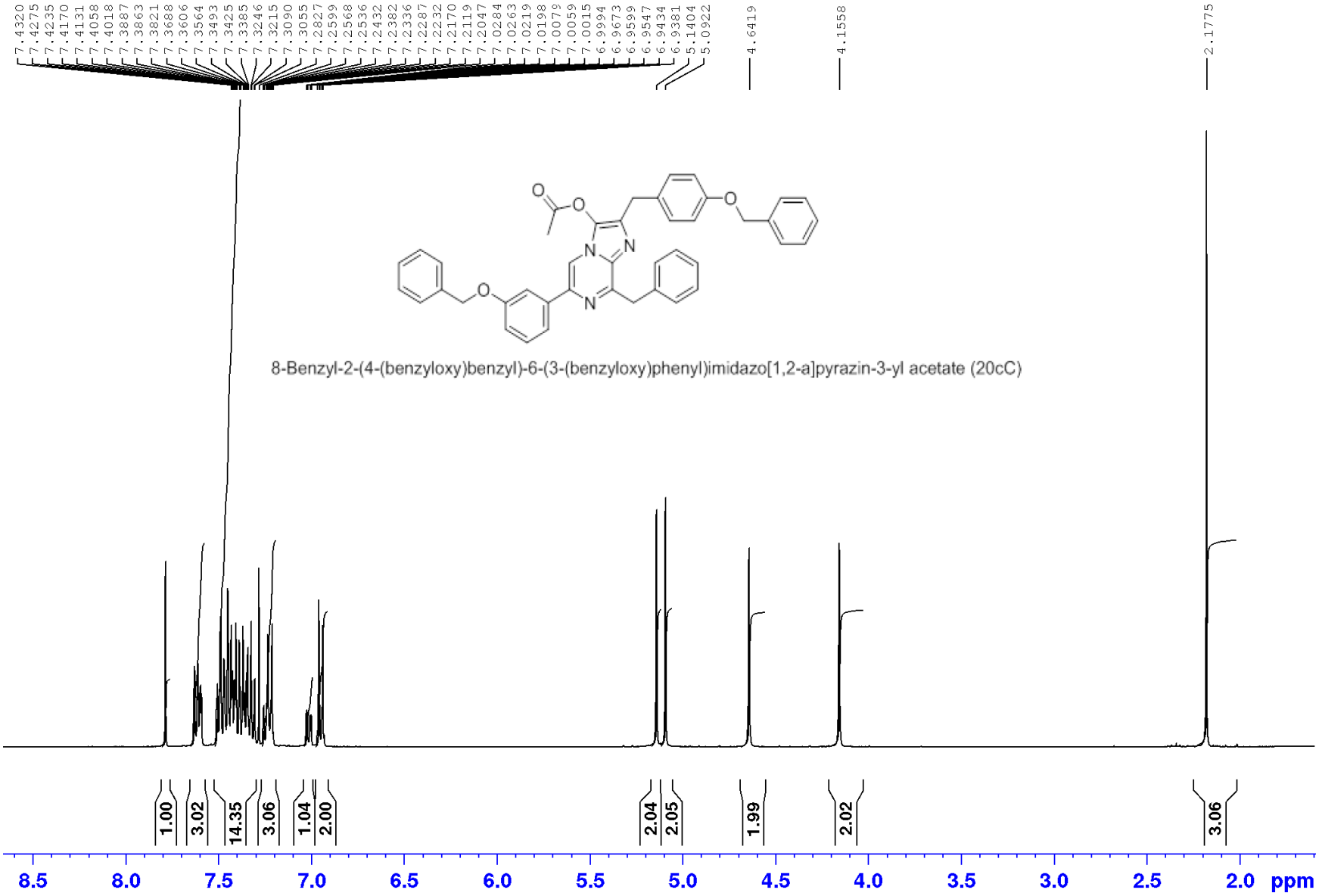
133.383
132.397
129.811
128.930
128.628
128.285
128.044
127.625
126.497
118.795
115.047
113.279
110.485
108.973
106.908
77.335
77.221
77.017
76.700
70.181
39.301
27.096
26.929
20.057
8-Benzyl-6-(3-(benzyloxy)phenyl)-2-(furan-2-ylmethyl)imidazo[1,2-a]pyrazin-3-yl acetate (20cB)
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
ppm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7.4018
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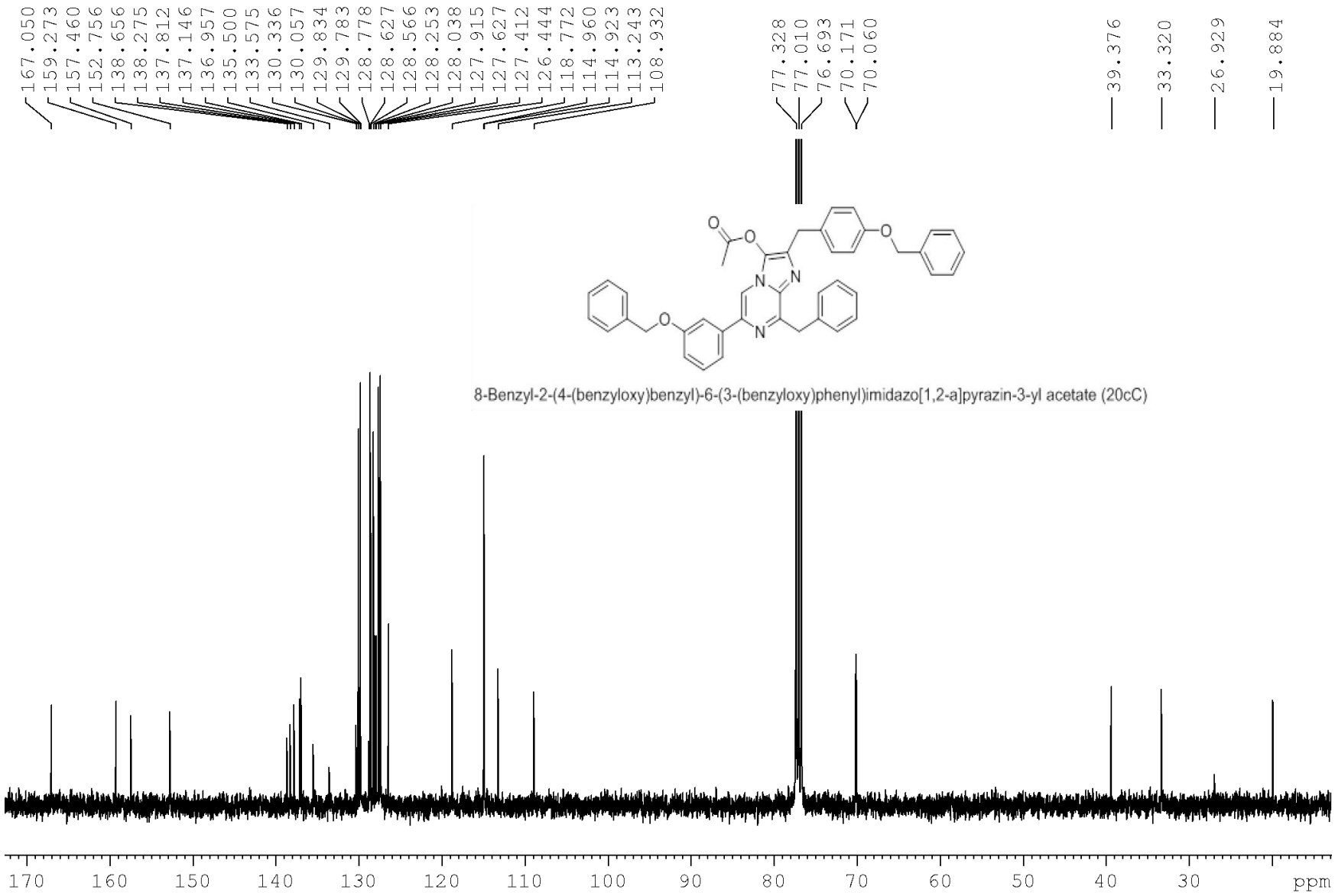
7.3863
7.3821
7.3688
7.3606
7.3564
7.3493
7.3425
7.3385
7.3246
7.3215
7.3090
7.3055
7.2827
7.2599
7.2568
7.2536
7.2432
7.2382
7.2336
7.2287
7.2232
7.2170
7.2119
7.2047
7.0284
7.0263
7.0219
7.0198
7.0079
7.0059
7.0015
6.9994
6.9673
6.9599
6.9547
6.9434
6.9381
5.1404
5.0922
4.6419
4.1558
2.1775
8-Benzyl-2-(4-(benzyloxy)benzyl)-6-(3-(benzyloxy)phenyl)imidazo[1,2-a]pyrazin-3-yl acetate (20cC)
1.00
3.02
14.35
3.06
1.04
2.00
2.04
2.05
1.99
2.02
3.06
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
ppm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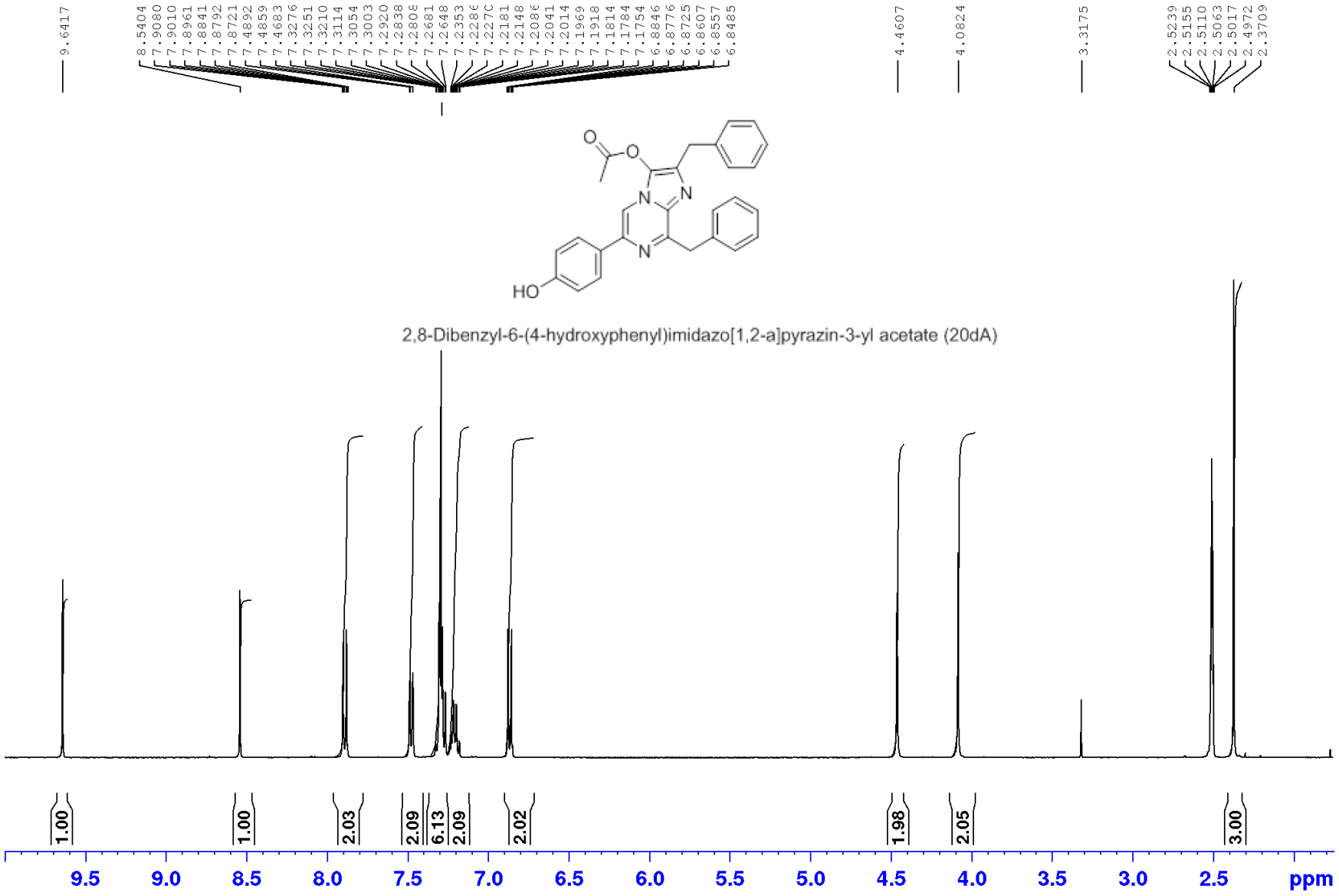
133.575
130.336
130.057
129.834
129.783
128.778
128.627
128.566
128.253
128.038
127.915
127.627
127.412
126.444
118.772
114.960
114.923
113.243
108.932
77.328
77.010
76.693
70.171
70.060
39.376
33.320
26.929
19.884
8-Benzyl-2-(4-(benzyloxy)benzyl)-6-(3-(benzyloxy)phenyl)imidazo[1,2-a]pyrazin-3-yl acetate (20cC)
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
ppm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7.8792
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7.4892
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7.3276
7.3251
7.3210
7.3114
7.3054
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7.2920
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6.8725
6.8607
6.8557
6.8485
4.4607
4.0824
3.3175
2.5239
2.5155
2.5110
2.5063
2.5017
2.4972
2.3709
O
O
N
N
N
HO
2,8-Dibenzyl-6-(4-hydroxyphenyl)imidazo[1,2-a]pyrazin-3-yl acetate (20dA)
1.00
1.00
2.03
2.09
6.13
2.09
2.02
1.98
2.05
3.00
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
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4.0
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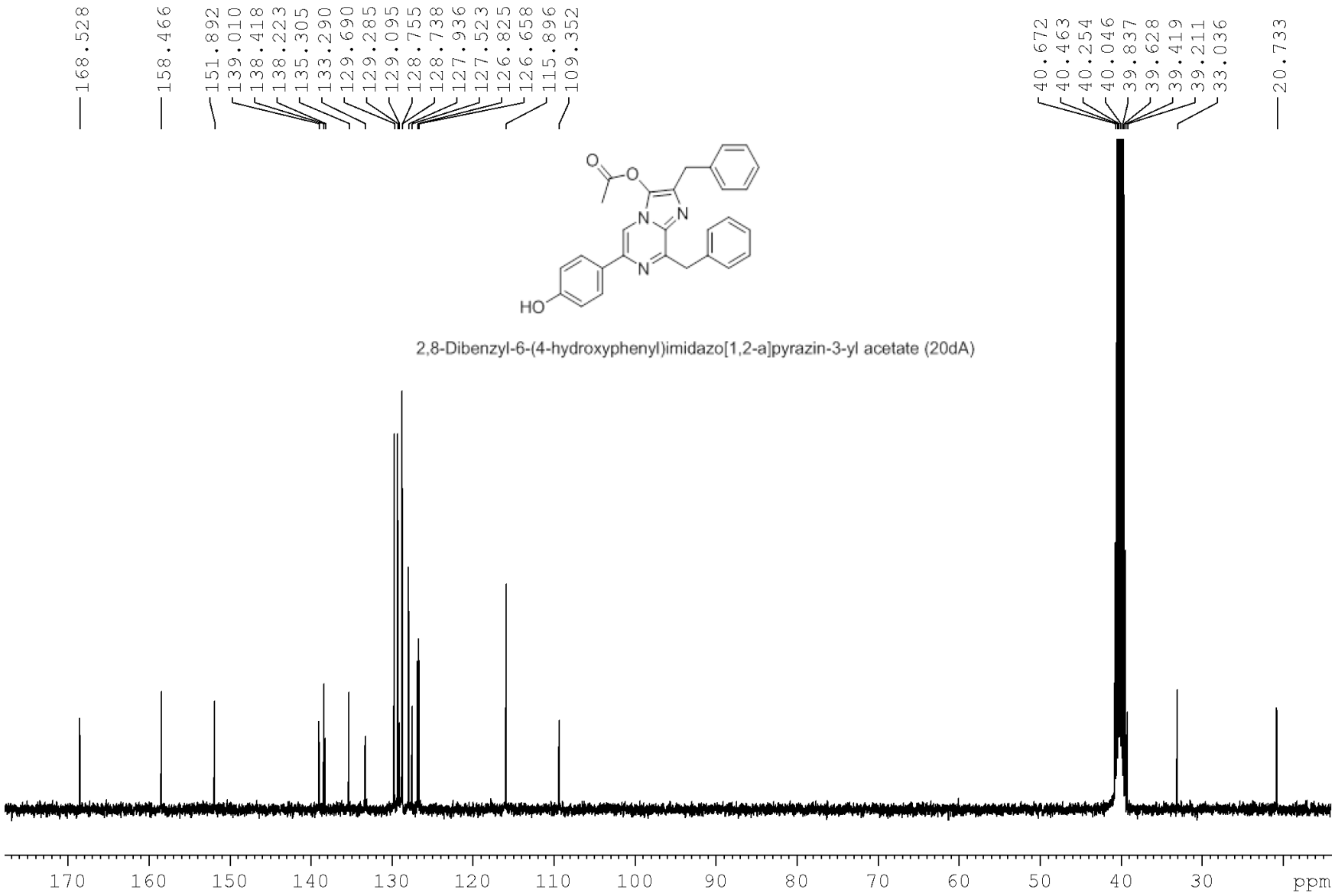
2.5
ppm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139.010
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133.290
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128.755
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127.523
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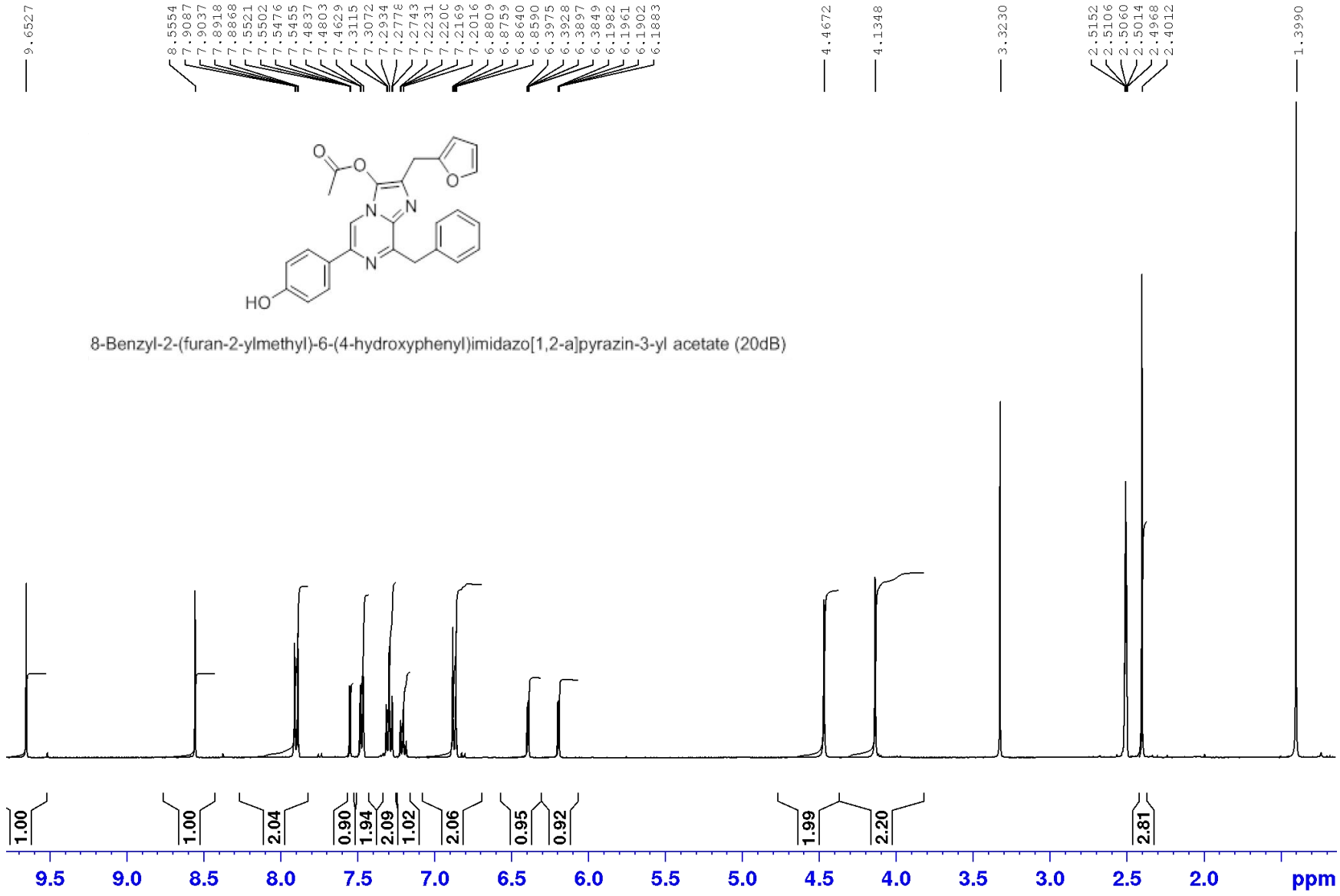
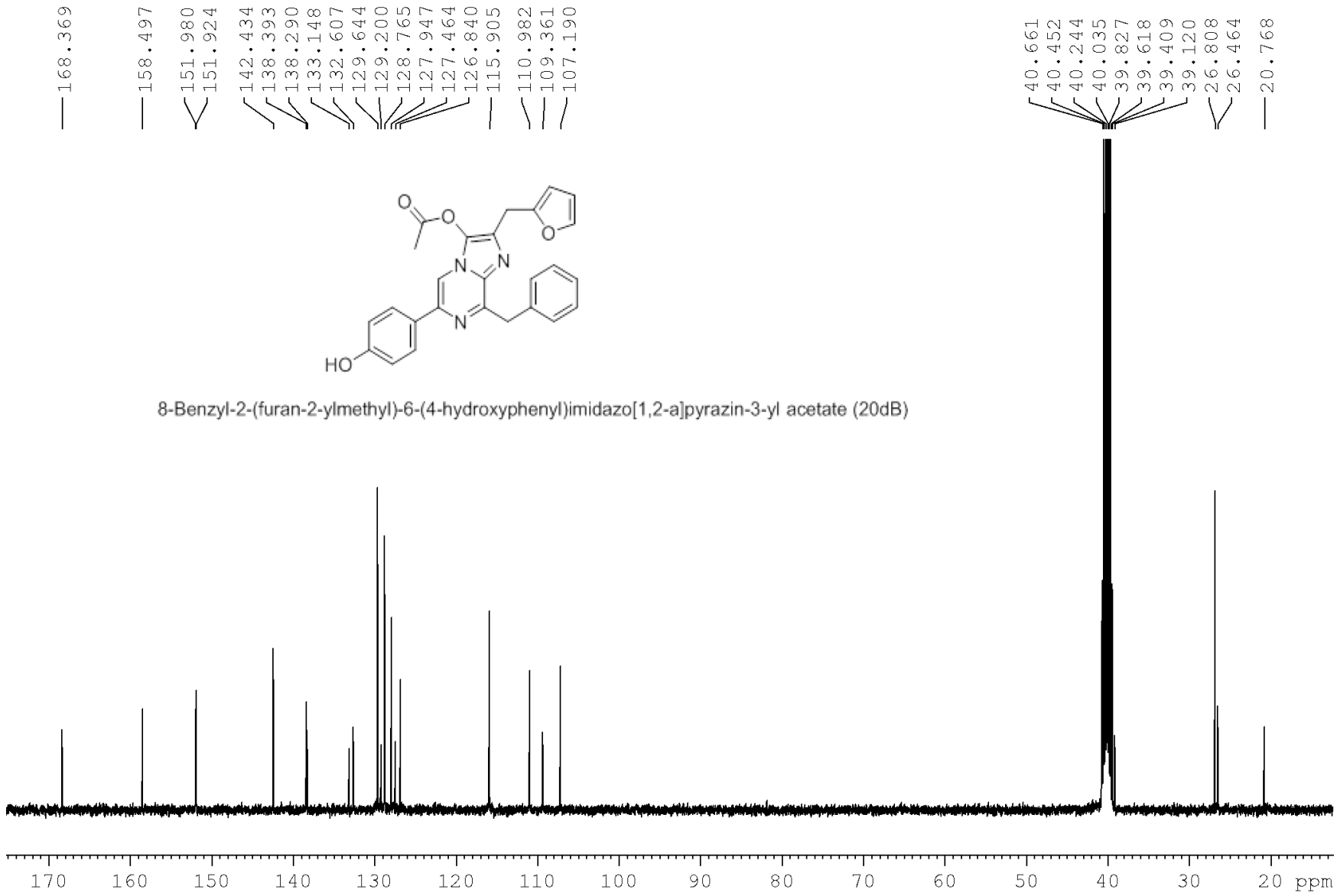
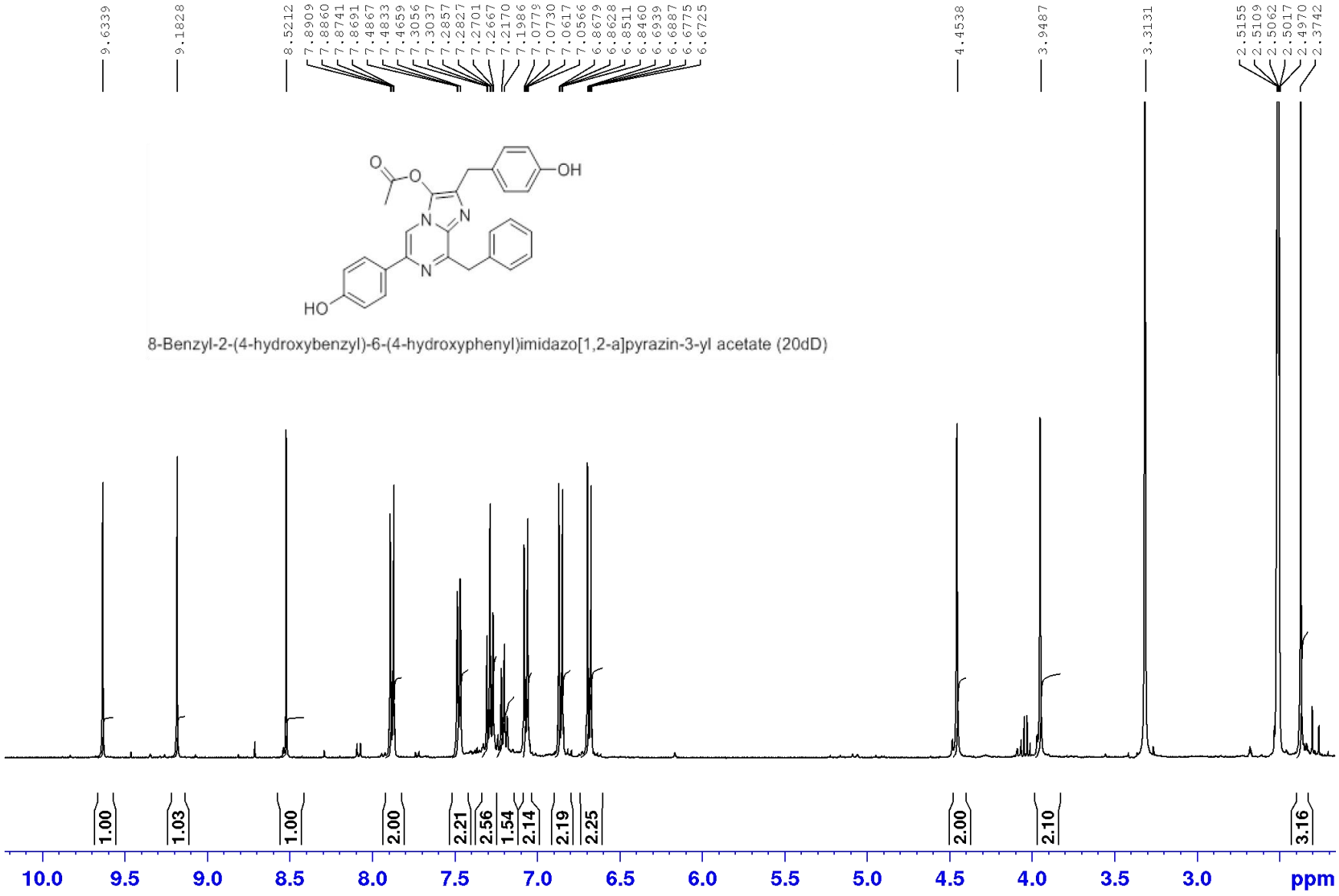
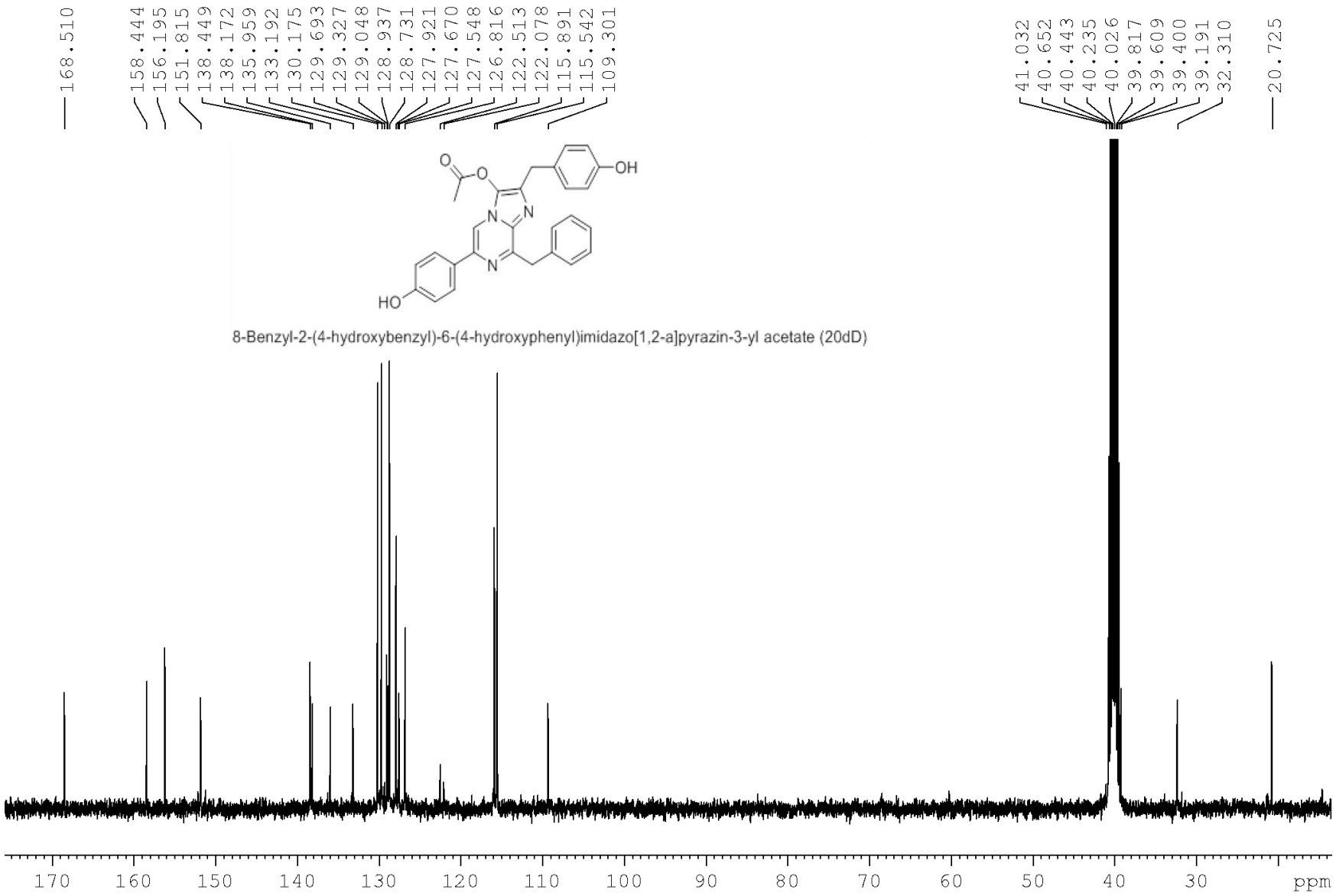
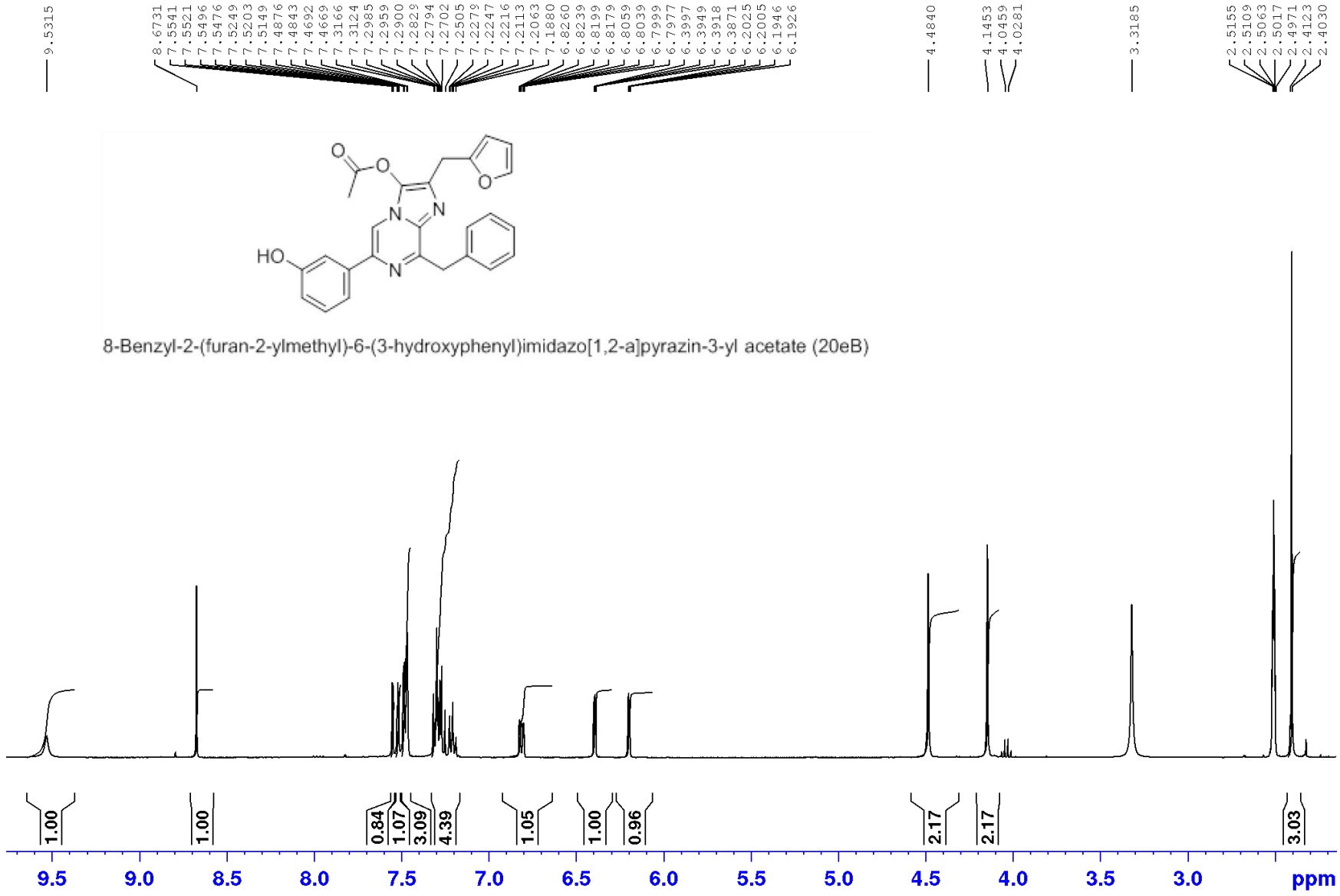
126.658
115.896
109.352
40.672
40.463
40.254
40.046
39.837
39.628
39.419
39.211
33.036
20.733
O
O
N
N
N
HO
2,8-Dibenzyl-6-(4-hydroxyphenyl)imidazo[1,2-a]pyrazin-3-yl acetate (20dA)
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
ppm


9.6527
8.5554
7.9087
7.9037
7.8918
7.8868
7.5521
7.5502
7.5476
7.5455
7.4837
7.4803
7.4629
7.3115
7.3072
7.2934
7.2778
7.2743
7.2231
7.2200
7.2169
7.2016
6.8809
6.8759
6.8640
6.8590
6.3975
6.3928
6.3897
6.3849
6.1982
6.1961
6.1902
6.1883
4.4672
4.1348
3.3230
2.5152
2.5106
2.5060
2.5014
2.4968
2.4012
1.3990
O
O
O
N
N
N
HO
8-Benzyl-2-(furan-2-ylmethyl)-6-(4-hydroxyphenyl)imidazo[1,2-a]pyrazin-3-yl acetate (20dB)
1.00
1.00
2.04
0.90
1.94
2.09
1.02
2.06
0.95
0.92
1.99
2.20
2.81
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
ppm

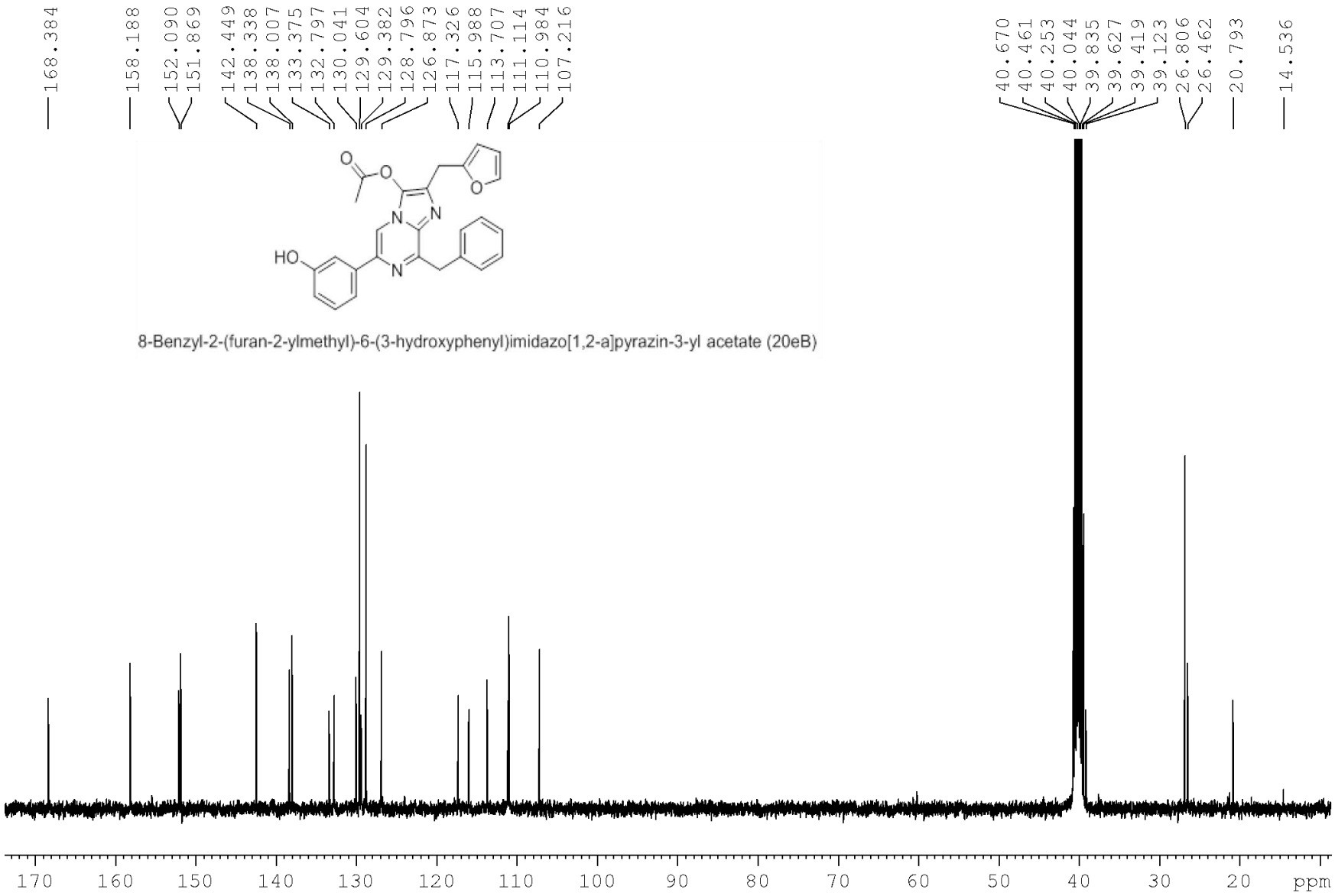

168.369
158.497
151.980
151.924
142.434
138.393
138.290
133.148
132.607
129.644
129.200
128.765
127.947
127.464
126.840
115.905
110.982
109.361
107.190
40.661
40.452
40.244
40.035
39.827
39.618
39.409
39.120
26.808
26.464
20.768
O
O
N
N
N
HO
8-Benzyl-2-(furan-2-ylmethyl)-6-(4-hydroxyphenyl)imidazo[1,2-a]pyrazin-3-yl acetate (20dB)
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20 ppm


9.6339
9.1828
8.5212
7.8909
7.8860
7.8741
7.8691
7.4867
7.4833
7.4659
7.3056
7.3037
7.2857
7.2827
7.2701
7.2667
7.2170
7.1986
7.0779
7.0730
7.0617
7.0566
6.8679
6.8628
6.8511
6.8460
6.6939
6.6887
6.6775
6.6725
4.4538
3.9487
3.3131
2.5155
2.5109
2.5062
2.5017
2.4970
2.3742
O
O
OH
N
N
N
HO
8-Benzyl-2-(4-hydroxybenzyl)-6-(4-hydroxyphenyl)imidazo[1,2-a]pyrazin-3-yl acetate (20dD)
1.00
1.03
1.00
2.00
2.21
2.56
1.54
2.14
2.19
2.25
2.00
2.10
3.16
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
ppm

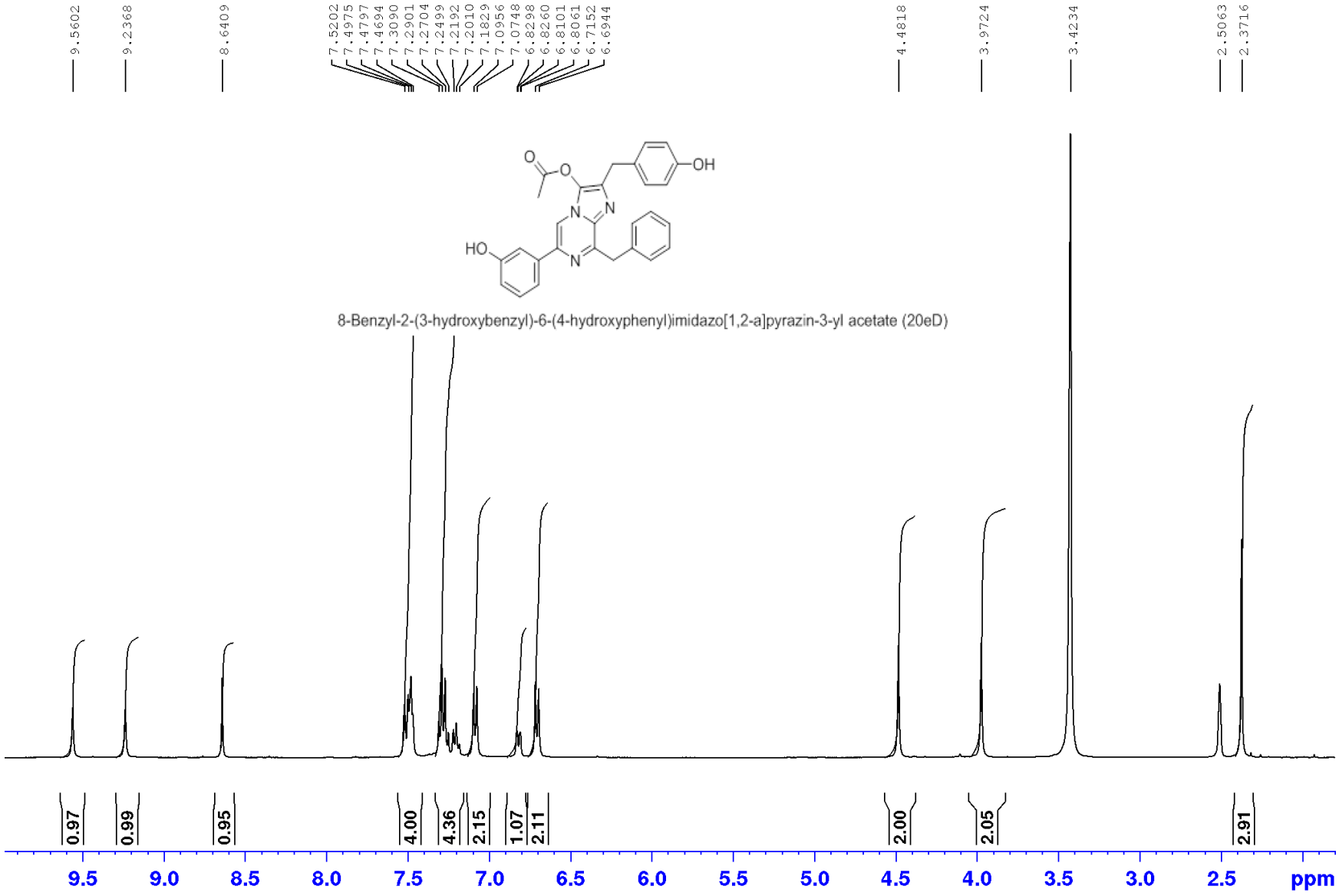

168.510
158.444
156.195
151.815
138.449
138.172
135.959
133.192
130.175
129.693
129.327
129.048
128.937
128.731
127.921
127.670
127.548
126.816
122.513
122.078
115.891
115.542
109.301
41.032
40.652
40.443
40.235
40.026
39.817
39.609
39.400
39.191
32.310
20.725
O
O
N
N
N
OH
HO
8-Benzyl-2-(4-hydroxybenzyl)-6-(4-hydroxyphenyl)imidazo[1,2-a]pyrazin-3-yl acetate (20dD)
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
ppm

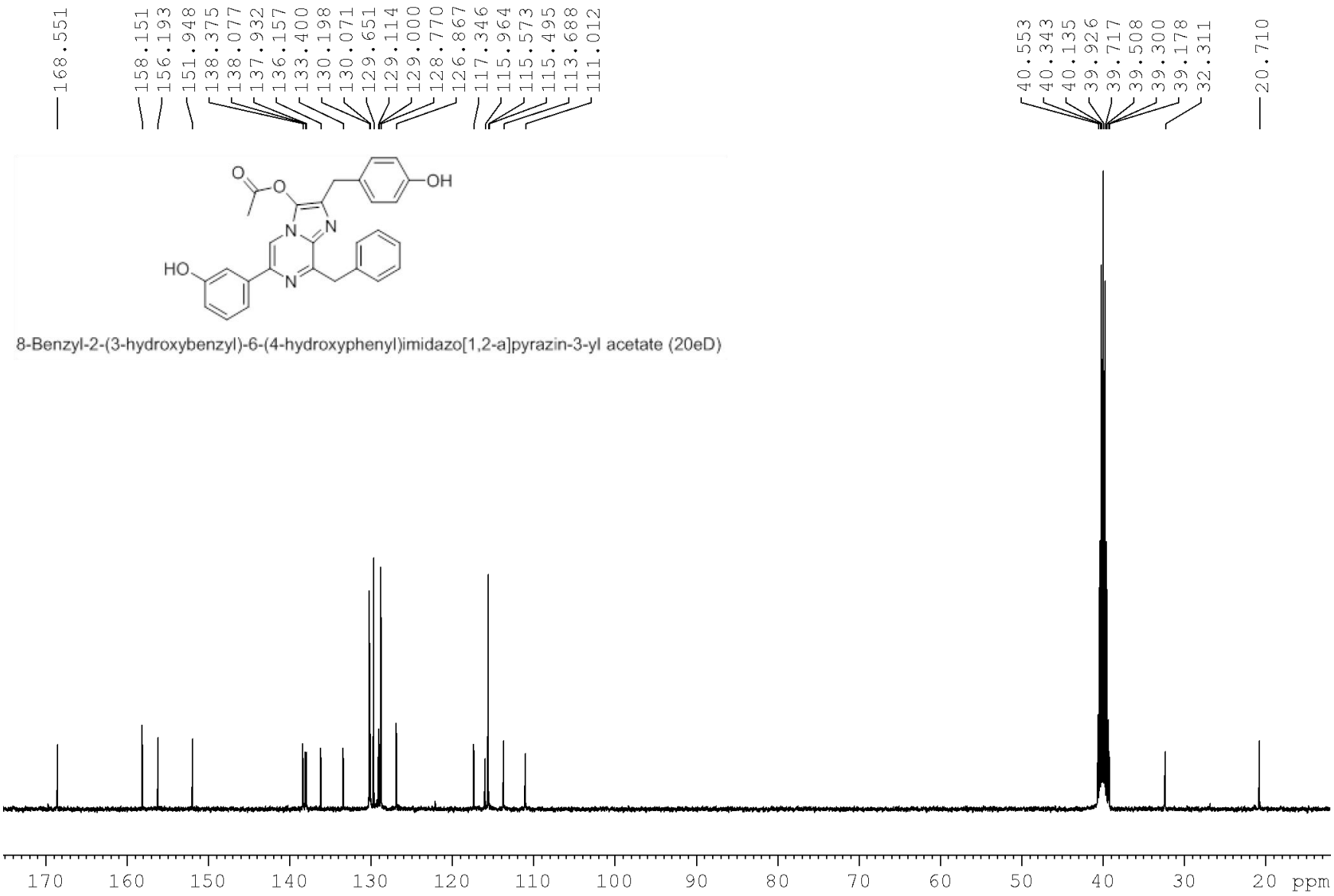


8-Benzyl-2-(furan-2-ylmethyl)-6-(3-hydroxyphenyl)imidazo[1,2-a]pyrazin-3-yl acetate (20eB)



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


9.5602
9.2368
8.6409
7.5202
7.4975
7.4797
7.4694
7.3090
7.2901
7.2704
7.2499
7.2192
7.2010
7.1829
7.0956
7.0748
6.8298
6.8260
6.8101
6.8061
6.7152
6.6944
4.4818
3.9724
3.4234
2.5063
2.3716
OH
HO
O
O
N
N
N
8-Benzyl-2-(3-hydroxybenzyl)-6-(4-hydroxyphenyl)imidazo[1,2-a]pyrazin-3-yl acetate (20eD)
0.97
0.99
0.95
4.00
4.36
2.15
1.07
2.11
2.00
2.05
2.91
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
ppm


168.551
158.151
156.193
151.948
138.375
138.077
137.932
136.157
133.400
130.198
130.071
129.651
129.114
129.000
128.770
126.867
117.346
115.964
115.573
115.495
113.688
111.012
40.553
40.343
40.135
39.926
39.717
39.508
39.300
39.178
32.311
20.710
OH
HO
O
O
N
N
N
8-Benzyl-2-(3-hydroxybenzyl)-6-(4-hydroxyphenyl)imidazo[1,2-a]pyrazin-3-yl acetate (20eD)
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
ppm