Supporting Information

Multicomponent synthesis and binding mode of imidazo[1,2-*a*]pyridine-capped selective HDAC6 inhibitors

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Content

1. Supplemental Tables and Figures

Table S1. *In vitro* activity against asexual blood stages of *P. falciparum* parasites, cytotoxicity and selectivity indices of **4a-m**.

entry	R^1	R^2	*P. falciparum*[a] IC_{50} [µM]		HEK293	SI[b]	
			3D7	Dd2	IC_{50} [µM]	3D7	Dd2
4a	4-Me_2N-Ph	H	0.970(±0.277)	1.726(±0.386)	2.43(±1.19)	3	1
4b	Ph	H	1.246(±0.437)	3.740(±0.348)	9.12(±0.84)	7	2
4c	4-F-Ph	H	1.123(±0.439)	3.616(±0.479)	6.91(±1.01)	6	2
4d	3,5-Me-Ph	H	1.350(±0.644)	2.448(±0.446)	11.7(±1.27)	9	5
4e	4-Me_2N-Naphtyl	H	0.524(±0.131)	1.461(±0.040)	1.73(±0.31)	3	1
4f	4-Me-Ph	H	1.152(±0.153)	3.098(±0.650)	8.03(±0.87)	7	3
4g	3,5-*t*-Butyl-Ph	H	22.9 (±0.921)	>25	>100	>4	>4
4h	4-(HO-Et)$_2$N-Ph	H	1.678(±0.744)	3.777(±0.017)	>100	>60	>27
4i	H	H	2.297(±1.099)	5.366(±0.214)	11.7(±1.24)	5	2
4j	H	7-Me	3.441(±1.561)	3.362(±2.061)	10.2(±1.68)	3	3
4k	H	6-Me	1.885(±0.540)	6.830(±0.158)	5.48(±1.53)	3	0.8
4l	*n*-Propyl	H	1.347(±0.301)	5.035(±0.173)	9.02(±1.55)	7	2
4m	*c*-Hex	H	0.517(±0.094)	2.797(±1.070)	7.32(±1.04)	14	3
		Vorinostat	0.209(±0.103)	0.297(±0.180)	1.49(±0.20)	7	5
		Chloroquine	0.006(±0.006)	0.256(±0.042)	n.d.	n.d.	n.d.

[a]Two independent assays were carried out, each carried out in duplicate wells. [b]SI= IC_{50}(HEK293)/ IC_{50}(*P. falciparum*) – larger values indicate greater parasite selectivity.

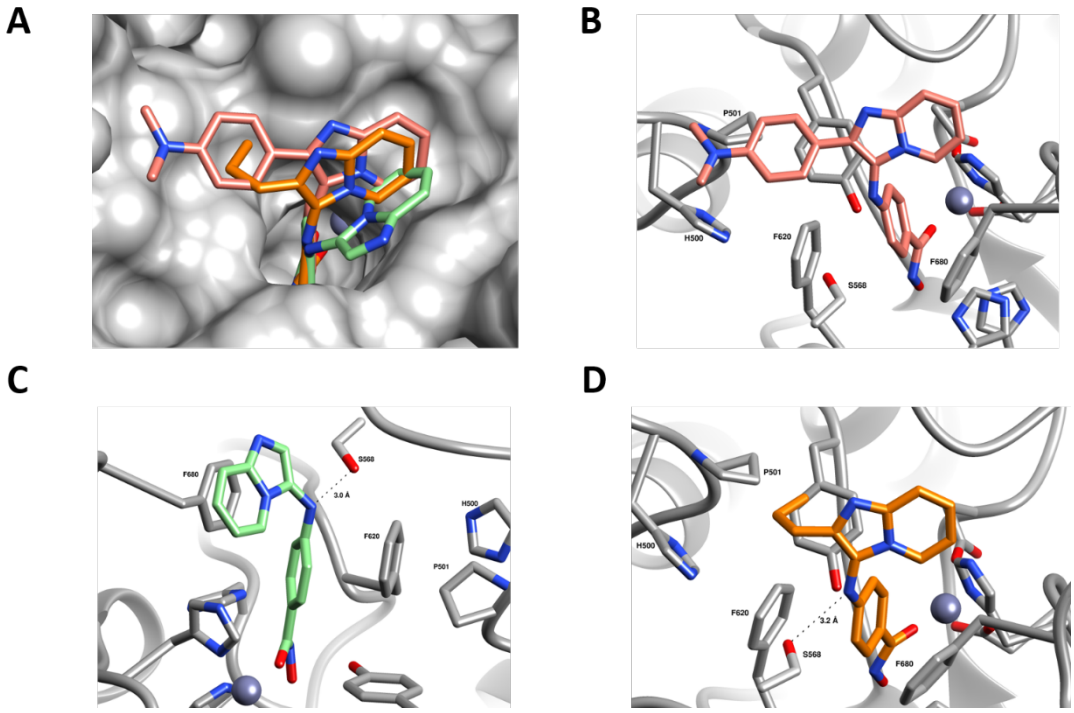


Fig S1. Docking poses of compounds **4a** (salmon), **4h** (green) and **4l** (orange) in human HDAC6 (PDB ID: 5EDU, nitrogen = blue, oxygen = red). Zinc is shown as a sphere. (A) Surface representation and superimposition of **4a**, **4h** and **4l**. (B, C, D) View of the active site of the respective ligands. Hydrogen bond interaction with S568 (corresponding to S531 in *Danio rerio* HDAC6) are indicated by dotted black lines.

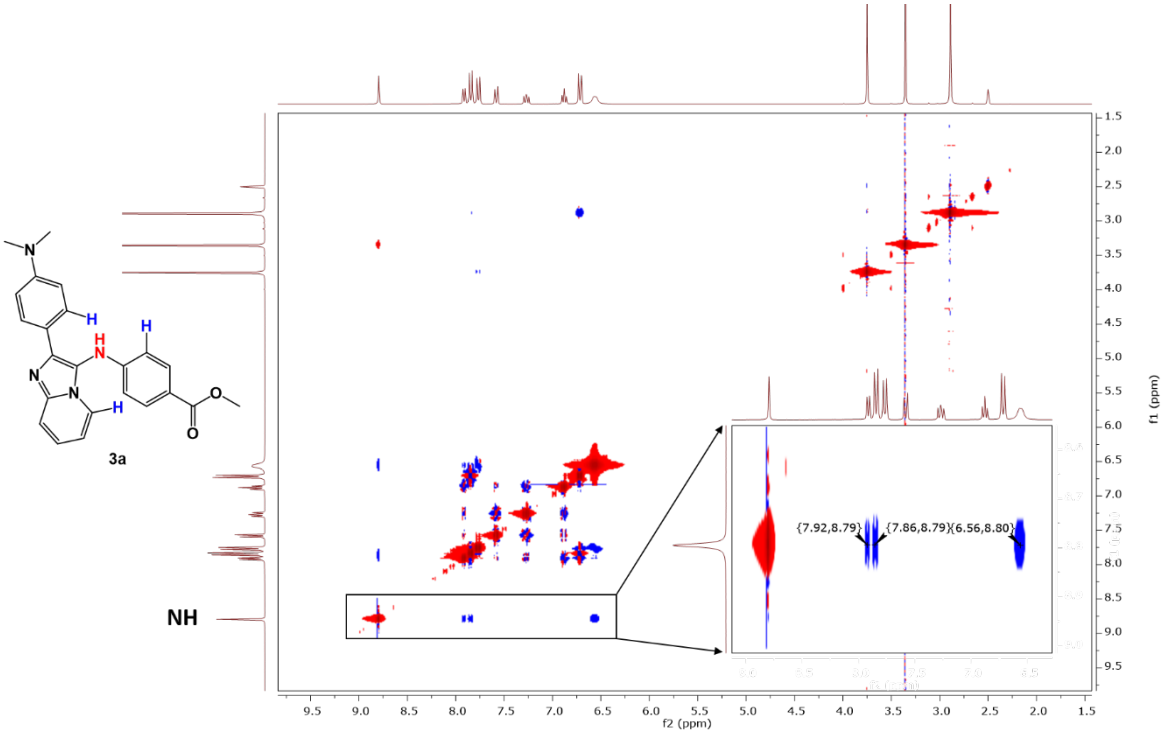


Fig S2. ^{1}H, ^{1}H-NOESY-NMR spectrum of compound **3a** in DMSO-d_6 as a representative example. Cross coupling signals of the secondary amine proton with the aromatic protons are highlighted.

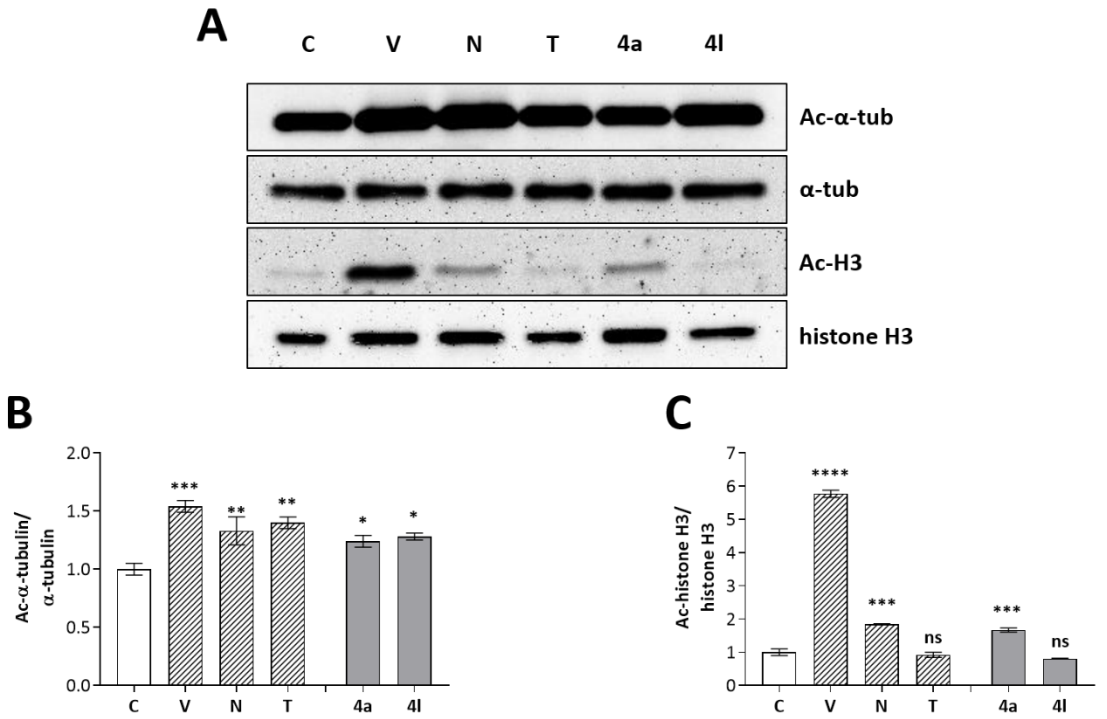


Fig. S3. Compound-induced α-tubulin and histone H3 acetylation in Cal27. (A) Representative immunoblot analysis of acetylated α-tubulin (Ac-α-tub), α-tubulin (α-tub), acetylated histone H3 (Ac-H3), and histone H3 (H3). Cal27 cells were incubated for 24 h with vehicle (DMSO 0.1%, C), vorinostat 1 µM (V), nexturastat A 1 µM (N), tubastatin A 1 µM (T), **4a** and **4l** 1 µM, respectively. (B-C) Densitometric analysis of tubulin acetylation in Cal27 (B) and histone H3 acetylation (C) was performed by ImageJ software (NIH). All values have been normalized to α-tubulin (B) or histone H3 (C). Statistical analysis was performed using one-way ANOVA test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and ns = not significant).

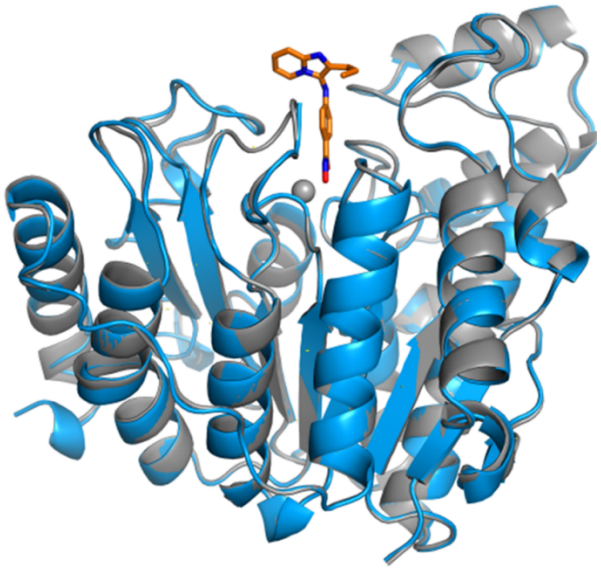


Fig. S4. Superposition of the HDAC6-**4l** complex (blue) with unliganded HDAC6 (dark grey, PDB 5EEM). No major structural changes occur upon inhibitor binding. The Zn^{2+} ion is shown as a grey sphere and **4l** is shown as a stick-figure with C = orange, N = dark blue, O = red.

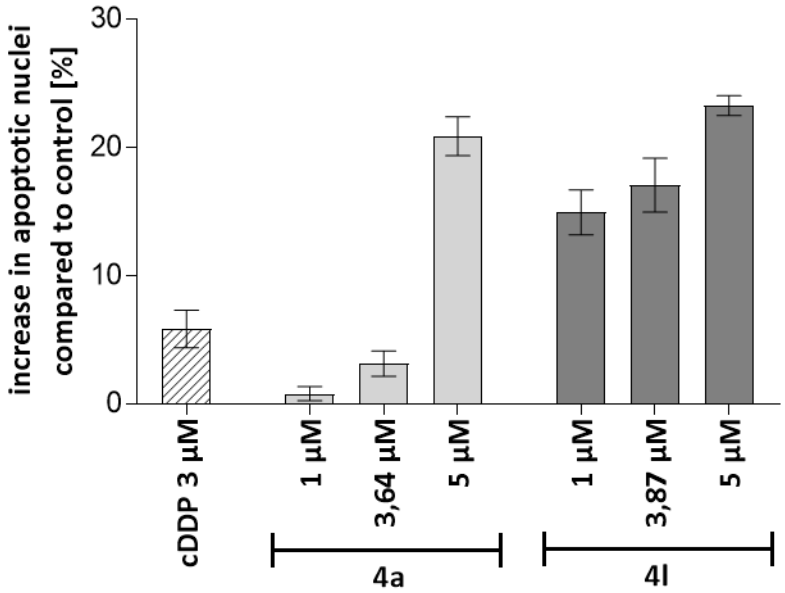


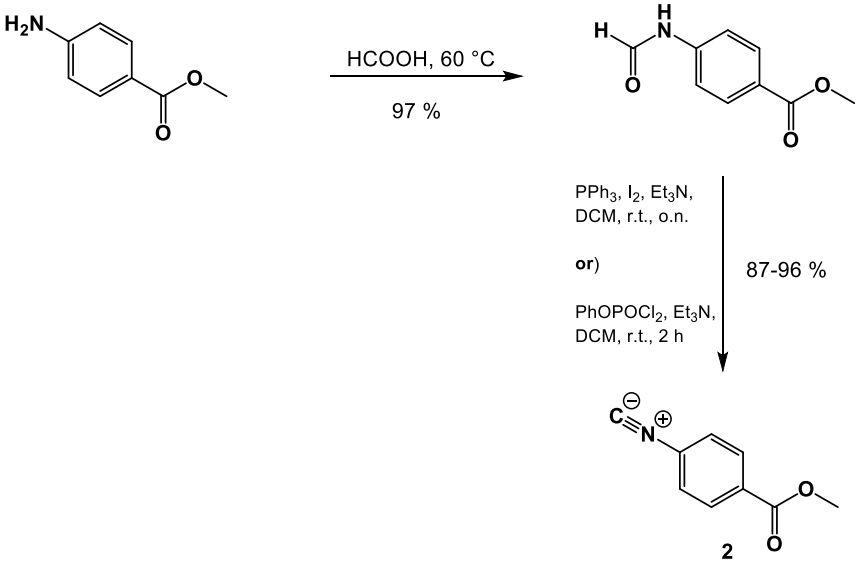
Fig. S5. 4a and **4l** induced apoptosis in Cal27 in a concentration dependent manner. Cal27 cells were incubated with 1 µM, 5 µM or the corresponding IC_{50} of **4a** or **4l** for 48h or with 3 µM of cisplatin (cDDP) for 24 h. The amount of apoptotic nuclei in the control was subtracted from treated samples. The effect was more pronounced for **4l** in comparison to **4a**. Data shown is a representative experiment performed in triplicate.

2. Chemistry

2.1 General Information

All chemicals and solvents were obtained from commercial suppliers (Sigma-Aldrich, Acros Organics, Carbolution Chemicals) and used as purchased without further purification. The progress of all reactions was monitored by thin layer chromatography (TLC) using Merck precoated silica gel plates (with fluorescence indicator UV254). Components were visualized by irradiation with ultraviolet light (254 nm) or staining in potassium permanganate solution following heating. Flash column chromatography was performed using prepacked silica cartridge with the solvent mixtures specified in the corresponding experiment. Melting points (mp) were taken in open capillaries on a Mettler FP 5 melting-point apparatus and are uncorrected. Proton (^{1}H) and carbon (^{13}C) NMR spectra were recorded on a Bruker Avance 300, 500 or 600 using DMSO-d_6 or $CDCl_3$ as solvents. Chemical shifts are given in parts per million (ppm), relative to residual solvent peak for ^{1}H and ^{13}C. ^{1}H-NMR signals marked with an asterisk (*) correspond to peaks assigned to the minor rotamer conformation. High resolution mass spectra (HRMS) analysis was performed on a UHR-TOF maXis 4G (Bruker Daltonics, Bremen) by electrospray ionization (ESI). UHPLC-MS was performed on a UHR-TOF maXis 4G (Bruker Daltonics, Bremen) in combination with Ultimate 3000 RS (Dionex, Sunnyvale). Microwave-assisted syntheses were carried out with a CEM Focused Microwave System, Model Discover SP. Analytical HPLC analysis were carried out on a Knauer HPLC system equipped with a Knauer UV detector K-2600 (254nm) using a Vertex Plus Column (length 150 x 4 mm with precolumn, packing material of the column was Eurospher II 100-5 C18). UV absorption was detected at 254 nm with a isocratic gradient of 10% B to 100% B in 30 min using HPLC-grade water +0.1% TFA (solvent A) and HPLC-grade acetonitrile +0.1% TFA (solvent B) for elution at a flow rate of 1 mL/min. The purities of all final compounds were 95% or higher.

2.2 Synthesis of Isocyanide 2



Scheme S1. Synthesis of isocyanide **2.**

Synthesis of Methyl 4-formamidobenzoate:

Methyl 4-aminobenzoate (0.5 g, 3.31 mmol, 1 eq.) was added to formic acid (0.5 mL, 13.2 mmol, 4 eq.) and the solution was stirred until the complete consumption of the starting material (controlled by TLC, *n*-hexane/EtOAc = 2:1). Afterwards, the solution was treated with destilled water and extracted with EtOAc (2 x 30 mL). The organic Phase was washed wit sat. aq. $NaHCO_3$ (2 x 15 mL) and dried over Na_2SO_4. After filtration and evaporation of the solvent under reduced pressure methyl 4-formamidobenzoate was obtained as a white solid (576 mg, 179.175 g/mol, 3.21 mmol, Yield = 97 %) The spectral data obtained for the compound were in accordance with the literature:[1] ^{1}H-NMR ($CDCl_3$, 300 MHz): δ [ppm] = 3.90 (s, 3 H), 3.91* (2 x s, 3 H), 7.12-7.15 (m, Ar-H, 1 H)*, 7.54 (*br*s, NH, 1 H), 7.62-7.65* (m, Ar-H, 1 H), 8.00-8.03 (m, Ar-H, 2 H), 8.03-8.05* (m, Ar-H, 2 H), 8.26-8.30* (*br*d, *J* = 10.8 Hz, NH, 1 H), 8.44 (d, *J* = 1.6 Hz, HC=O, 1 H), 8.85* (d, *J* = 11.2 Hz, HC=O, 1 H); ^{13}C-NMR ($CDCl_3$, 75 MHz): δ [ppm] = 52.26, 52.35, 117.33, 119.21, 126.36, 126.82, 131.07, 131.72, 140.97, 141.06, 159.06, 161.86, 166.40, 166.61.

*Synthesis of Isocyanide **2**:*

Methyl 4-formamidobenzoate (2.5 g, 14 mmol, 1 eq.) was dissolved in a triethylamine/dichloromethane mixture (1:1, 10 mL), followed by the addition of $PhOPOCl_2$ (CAUTION!, 3.13 mL, 21 mmol, 1.5 eq.). After stirring the reaction for 2 hours, *brine* (100 mL) was added and the mixture was extracted with EtOAc (2 x 300 mL). The combined organic extracts were washed with 1 M HCl (125 mL), sat. aq. $NaHCO_3$ (125 mL) and *brine* (125 mL). After drying over $MgSO_4$, the solvent was removed under reduced pressure. Column chromatography (*n*-hexane/EtOAc, *gradient*) yielded the desired isocyanide as a dark green solid (2.17 g, 13.5 mmol, Yield = 96 %).[2]

or

Methyl 4-formamidobenzoate (362 mg, 2.02 mmol, 1 eq.), iodine (769 mg, 3.03 mmol, 1.5 eq.) and triphenylphosphine (795 mg, 3.03 mmol, 1.5 eq.) were dissolved in dichloromethane (6 mL), followed by the dropwise addition of Et_3N (0.84 mL, 6.06 mmol, 3 eq.). The solution was stirred over night at room temperature. Because of incomplete consumption, further equivalents of iodine (0.5 eq.), triphenylphosphine (0.5 eq.) and Et_3N (1 eq.) were added and the solution was stirred over night at room temperature. Then, the mixture was diluted with dichloromethane and washed with ice-cold sat. aq. $Na_2S_2O_3$ (25 mL). The aqueous phase was extracted with dichlormethane (2 x 25 mL). The combined organic extracts were washed with dest. water (40 mL) and *brine* (40 mL). After drying over Na_2SO_4 and filtration, the solvent was removed under reduced pressure. Column chromatography (*n*-hexane/EtOAc, *gradient*) gave the product as a dark green solid (282 mg, 1.75 mmol, Yield = 87 %).[3] The spectral data obtained for the compound were in accordance with the data reported:[4] ^{1}H-NMR ($CDCl_3$, 300 MHz): δ [ppm] = 3.94 (s, 3 H), 7.43-7.46 (m, 2 H), 8.07-8.10 (m, 2 H).

2.3 Synthesis of 3a-3m

Optimization of the Groebke-Blackburn-Bienaymé three-component reaction (GBB-3CR):

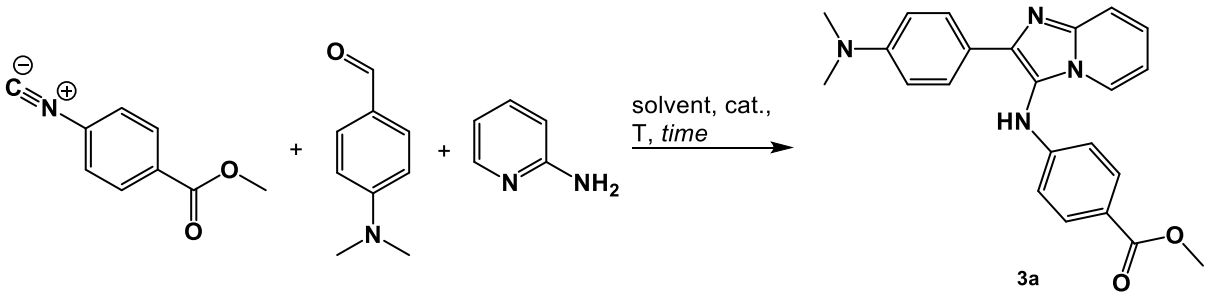


Table S2: Optimization of the Groebke-Blackburn-Bienaymé Reaction.[a, b]

entry	solvent	temp. (°C)	time (h)	catalyst	yield (%)
1	MeOH	rt	24	AcOH	15
2	MeOH	rt	24	$HClO_4$	0[c]
3	Ethanol	rt	24	AcOH	12
4	Acetonitril	rt	24	AcOH	0[d]
5	MeOH	rt	48	AcOH	35
6	MeOH	rt	48	$HClO_4$	24[e]
7	MeOH	rt	72	AcOH	54
8	MeOH	85 °C (MW)[f]	1	AcOH	35
9	MeOH	85 °C (MW)[f]	3	AcOH	52

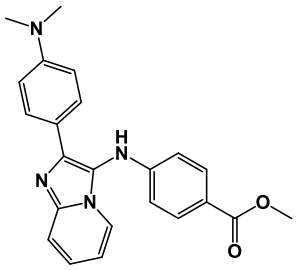
[a] Reaction conditions (0.5 mmol scale): MeOH (1.5 mL), 2-aminopyridine (1 eq.), aldehyde (1.2 eq.), catalyst (2 eq.) and isocyanide (1 eq.).
[b] Isolation by simple filtration and no recovery from mother liquor; hence, the total yield may be higher.
[c] Some unresolved side products were formed.
[d] No precipitate, treating the residue with MeOH gave the product in just 90 % purity.
[e] 10 mol% of $HClO_4$ were used.
[f] MW = Microwave (150 W).

A series of experiments were carried out to determine the optimal and most convenient conditions for the Groebke-Blackburn-Bienaymé three-component reaction using **3a** as our model compound (Table S1). The reactions were carried out in a 0.5 mmol scale with a typical equivalent pattern[5a,5b] of the involved starting materials. A convenient isolation by simple filtration was the aim of the synthesis, hence, no efforts for further recovery of the product from the mother liquor has been made. For all reactions a pre-formation of the imine intermediate was allowed to form, using 4-(dimethylamino)benzaldehyde and 2-aminopyridine in presence of the particular catalyst. This was followed by the addition of methyl 4-isocyanobenzoate. For this optimization, different solvents, catalysts, temperatures and reaction times were investigated. Among the used solvents, MeOH gave the best results. The best catalyst for this reaction was AcOH. Furthermore, the reaction time seemed to be very slow at room temperature, which was shown by increasing yields for longer reaction times. This led us to use microwave irradiation and a higher temperature. The microwave-assisted synthesis at 85 °C shortens the reaction time from 72 h to 3 h (entry 9) with similar yield.

*General Procedure for the synthesis of ester **3a-m**:*

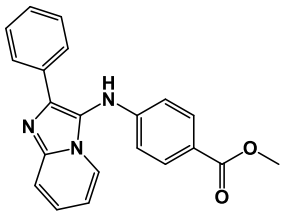
A solution of 2-aminopyridine (1 eq.), aldehyde (1.2 eq.) and AcOH (2 eq.) in methanol (≈ 0.5 mol/L) was stirred for 25 min. at room temperature in a 10 mL glass pressure microwave tube. This was followed by the addition of methyl 4-isocyanobenzoate and the mixture was subjected to microwave irradiation (Discover mode; power: 150 W; hold time: 3 h; temperature: 85 °C; PowerMax-cooling mode) under medium speed magnetic stirring. The reaction mixture was allowed to cool to rt and kept in the refrigerator overnight.[1] After filtration, the product could be obtained as a solid. If no crystallization occurred, the solvent was evaporated and the crude product was purified by column chromatography (*n*-hexane/EtOAc, *gradient*) to give the desired product.

*Methyl 4-((2-(4-(dimethylamino)phenyl)imidazo[1,2-a]pyridin-3-yl)amino)benzoate **3a**:*



The compound was obtained as described in the general procedure using 2-aminopyridine (47 mg), 4-(dimethylamino)benzaldehyde (90 mg), AcOH (57 µL) and methyl 4-isocyanobenzoate (81 mg) in 52 % yield as a yellow solid (100 mg): mp. = 248-249; ^{1}H-NMR (DMSO-d_6, 300 MHz): δ [ppm] = 2.89 (s, 6 H), 3.75 (s, 3 H), 6.55-6.57 (m, 2 H) 6.70-6.73 (m, 2 H), 6.86-6.90 (m, 1 H), 7.24-7.30 (m, 1 H), 7.57- 7.60 (m, 1 H), 7.75-7.78 (m, 2 H), 7.83-7.86 (m, 2 H), 7.90-7.92 (m, 1 H), 8.79 (s, 1 H) ;^{13}C-NMR (DMSO-d_6, 75 MHz): δ [ppm] = 39.93, 51.49, 112.04, 112.47, 115.63, 116.68, 119.24, 121.14, 122.62, 124.73, 127.32, 131.44, 138.58, 141.86, 149.87, 150.47, 166.08; LC-MS (ESI): m/z (%) [M =$C_{23}H_{22}N_4O_2$] = 387.2 $[M+H]^+$, t_R = 3.2 min.

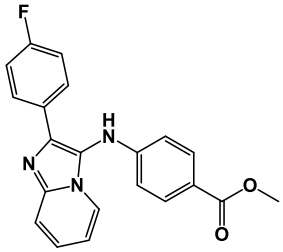
*Methyl 4-((2-phenylimidazo[1,2-a]pyridin-3-yl)amino)benzoate **3b**:*



The compound was obtained as described in the general procedure using 2-aminopyridine (151 mg), benzaldehyde (0.19 mL), AcOH (0.18 mL) and methyl 4-isocyanobenzoate (250 mg) in 47 % yield as a yellow solid (258 mg): mp. = 260-261; ^{1}H-NMR (DMSO-d_6, 600 MHz): δ [ppm] = 3.75 (s, 3 H), 6.42-6.75 (m, 2 H), 6.92-6.95 (m, 1 H), 7.27-7.30 (m, 1 H), 7.32-7.35 (m, 1 H), 7.38-7.40 (m, 2 H), 7.64-7.66 (m, 1 H), 7.76-7.78 (m, 2 H), 7.96-7.97 (m, 1 H), 8.00-8.01 (m, 2H), 8.89 (s, 1 H); ^{13}C-NMR (DMSO-d_6, 151 MHz): δ [ppm] = 51.51, 112.56, 117.24, 117.51, 119.48, 123.02, 125.40, 126.42, 127.69, 128.53, 131.45, 133.40, 137.61, 142.02, 150.13, 166.03; LC-MS (ESI): m/z (%) [M = $C_{21}H_{17}N_3O_2$] = 344.1 $[M+H]^+$, t_R = 2.8 min.

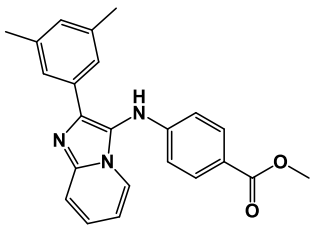
[1] Most of the products precipitated during the reaction. Some products just crystallized after storage in the refrigerator or slowly at room temperature.

Methyl 4-((2-(4-fluorophenyl)imidazo[1,2-a]pyridin-3-yl)amino)benzoate ***3c****:*



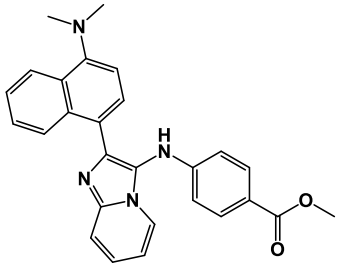
The compound was obtained as described in the general procedure using 2-aminopyridine (151 mg), 4-fluorobenzaldehyde (0.20 mL), AcOH (0.18 mL) and methyl 4-isocyanobenzoate (250 mg) in 33 % yield as a white solid (191 mg): mp. = 220-221; ^{1}H-NMR (DMSO-d_6, 600 MHz): δ [ppm] = 3.75 (s, 3 H), 6.44-6.73 (m, 2 H), 6.93-6.95 (m, 1 H), 7.23-7.26 (m, 2 H), 7.32-7.35 (m, 1 H), 7.64-7.65 (m, 1 H), 7.77-7.78 (m , 2 H), 7.96-7.97 (m, 1 H), 8.02-8.04 (m, 2 H), 8.89 (s, 1 H); ^{13}C-NMR (DMSO-d_6, 151 MHz): δ [ppm] = 51.51, 112.60, 115.41, 115.56, 117.20, 117.28, 119.59, 123.07, 125.51, 128.34, 128.39, 129.92, 129.94, 131.47, 136.80, 142.02, 150.00, 160.90, 162.53, 166.02; LC-MS (ESI): m/z (%) [M = $C_{21}H_{16}FN_3O_2$] = 362.1 $[M+H]^+$, t_R = 3.0 min.

Methyl 4-((2-(3,5-dimethylphenyl)imidazo[1,2-a]pyridin-3-yl)amino)benzoate ***3d****:*



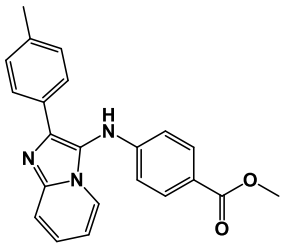
The compound was obtained as described in the general procedure using 2-aminopyridine (151 mg), 3,5-dimethylbenzaldehyde (0.26 mL), AcOH (0.18 mL) and methyl 4-isocyanobenzoate (250 mg) in 55 % yield as a yellow solid (328 mg): mp. = 234-235; ^{1}H-NMR (DMSO-d_6, 600 MHz): δ [ppm] = 2.23 (s, 6 H), 3.75 (s, 3 H), 6.55-6-57 (m, 2 H), 6.91-6.93 (m, 2 H), 7.30-7.33 (m, 1 H), 7.62-7.63 (m, 3 H), 7.76-7.78 (m, 2 H), 7.95-7.96 (m, 1 H), 8.86 (s, 1 H); ^{13}C-NMR (DMSO-d_6, 151 MHz): δ [ppm] = 21.10, 51.49, 112.46, 112.56, 117.14, 117.56, 119.39, 122.96, 124.39, 125.26, 129.06, 131.42, 133.22, 137.25, 137.71, 141.89, 150.26, 166.06; LC-MS (ESI): m/z (%) [M = $C_{23}H_{21}N_3O_2$] = 372.2 $[M+H]^+$, t_R = 3.4 min.

Methyl 4-((2-(4-(dimethylamino)naphthalen-1-yl)imidazo[1,2-a]pyridin-3-yl)amino)benzoate ***3e****:*



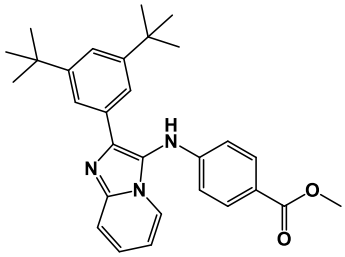
The compound was obtained as described in the general procedure using 2-aminopyridine (151 mg), 4-dimethylaminonaphthaldehyde (379 mg), AcOH (0.18 mL) and methyl 4-isocyanobenzoate (250 mg) in 45 % yield as a brown solid (317 mg): mp. = 214-216; ^{1}H-NMR (DMSO-d_6, 600 MHz): δ [ppm] = 2.82 (s, 6 H), 3.74 (s, 3 H), 6.51-6.52 (m, 2 H), 6.97-6.99 (m, 1 H), 7.07-7.08 (m, 1 H), 7.35-7.38 (m, 1 H), 7.42-7.45 (m, 1 H), 7.47-7.52 (m, 2 H), 7.70-7.72 (m, 3 H), 7.90-7.95 (m, 1 H), 8.18-8.19 (m, 1 H), 8.43-8.45 (m, 1 H), 8.75 (s, 1 H); ^{13}C-NMR (DMSO-d_6, 151 MHz): δ [ppm] = 44.73, 51.45, 112.44, 113.31, 117.30, 118.25, 118.93, 119.06, 123.16, 123.92, 124.92, 124.98, 125.05, 125.74, 126.99, 127.65, 128.16, 130.25, 131.29, 132.67, 139.39, 141.79, 150.49, 150.70, 166.02; LC-MS (ESI): m/z (%) [M = $C_{27}H_{24}N_4O_2$] = 437.2 $[M+H]^+$, t_R = 3.2 min.

Methyl 4-((2-(p-tolyl)imidazo[1,2-a]pyridin-3-yl)amino)benzoate ***3f****:*



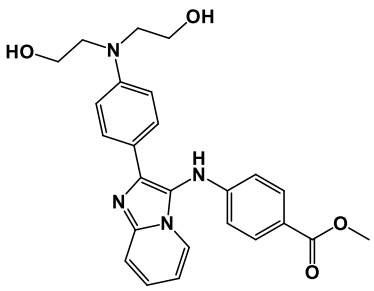
The compound was obtained as described in the general procedure using 2-aminopyridine (151 mg), 4-methylbenzaldehyde (379 mg), AcOH (0.22 mL) and methyl 4-isocyanobenzoate (250 mg) in 42 % yield as a white solid (239 mg): mp. = 227-228; ^{1}H-NMR (DMSO-d_6, 600 MHz): δ [ppm] = 2.28 (s, 3 H), 3.75 (s, 3 H), 6.41-6.72 (m, 2 H), 6.91-6.93 (m, 1 H), 7.19-7.20 (m, 2 H), 7.30-7.33 (m, 1 H), 7.62-7.64 (m, 1 H), 7.76-7.77 (m, 2 H), 7.89-7.91 (m, 2 H), 7.95-7.96 (m, 1 H), 8.86 (m, 1 H); ^{13}C-NMR (DMSO-d_6, 151 MHz): δ [ppm] = 20.81, 51.48, 112.41, 112.49, 117.11, 119.41, 122.93, 125.22, 126.35, 129.09, 130.61, 131.42, 137.01, 137.77, 141.94, 150.17, 166.03; LC-MS (ESI): m/z (%) [M = $C_{22}H_{19}N_3O_2$] = 358.2 $[M+H]^+$, t_R = 3.2 min.

Methyl 4-((2-(3,5-di-tert-butylphenyl)imidazo[1,2-a]pyridin-3-yl)amino)benzoate ***3g****:*



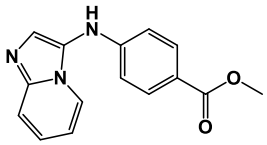
The compound was obtained as described in the general procedure using 2-aminopyridine (151 mg), 3,5-di-*tert*-butylbenzaldehyde (415 mg), AcOH (0.18 mL) and methyl 4-isocyanobenzoate (250 mg) in 42 % yield as a yellowish solid (317 mg): mp. = 295-296; ^{1}H-NMR (DMSO-d_6 / TFA-d (6/1, v/v), 600 MHz): δ [ppm] = 1.17 (s, 18 H), 3.71 (s, 3 H), 6.79-6.80 (m, 2 H), 7.43-7.46 (m, 1H), 7.47-7.47 (m, 1 H), 7.65-7.66 (m, 2 H), 7.78-7.80 (m, 2 H), 7.93-7.99 (m, 2 H), 8.51-8.52 (m, 1 H); ^{13}C-NMR (DMSO-d_6, 151 MHz): δ [ppm] = 31.51, 35.54, 52.11, 113.31, 114.20, 118.35, 120.30, 122.15, 122.53, 125.06, 126.00, 126.01, 131.19, 132.26, 134.80, 138.76, 150.00, 152.69, 167.01.; LC-MS (ESI): m/z (%) [M = $C_{29}H_{33}N_3O_2$] = 456.3 $[M+H]^+$, t_R = 4.7 min.

Methyl-4-((2-(4-(dimethylamino)naphthalen-1-yl)imidazo[1,2-a]pyridin-3-yl)amino)benzoate ***3h****:*



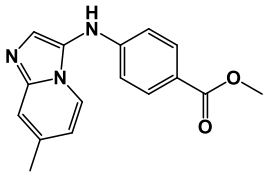
The compound was obtained as described in the general procedure using 2-aminopyridine (151 mg), 4-[N,N-Bis(2-hydroxyethyl)amino]benzaldehyde (398 mg), AcOH (0.18 mL) and methyl 4-isocyanobenzoate (250 mg) in 46 % yield as a brown solid (325 mg): mp. = 221-222; ^{1}H-NMR (DMSO-d_6, 600 MHz): δ [ppm] = 3.41 (t, J = 6.3 Hz, 4 H), 3.50-3.53 (m, 4 H), 3.75 (s, 3 H), 4.72 (t, J = 5.1 Hz, 2 H), 6.41-6.65 (m, 2 H), 6.68-6.69 (m, 2 H), 6.86-6.88 (m, 1 H), 7.25-7.27 (m, 1 H), 7.56-7.58 (m, 1 H), 7.76-7.80 (m, 4 H), 7.90-7.91 (m, 1 H), 8.77 (s, 1 H); ^{13}C-NMR (DMSO-d_6, 151 MHz): δ [ppm] = 51.48, 53.17, 58.15, 111.08, 111.97, 112.45, 115.42, 116.61, 119.22, 120.20, 122.56, 124.65, 127.47, 131.43, 138.64, 141.82, 147.53, 150.51, 166.07; LC-MS (ESI): m/z (%) [M = $C_{25}H_{26}N_4O_4$] = 447.2 $[M+H]^+$, t_R = 2.4 min.

Methyl 4-(imidazo[1,2-a]pyridin-3-ylamino)benzoate ***3i****:*



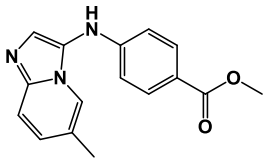
The compound was obtained as described in the general procedure using 2-aminopyridine (113 mg), glyoxylic acid monohydrate (184 mg), AcOH (0.14 mL) and methyl 4-isocyanobenzoate (200 mg) in 56 % yield as a brown solid (180 mg): mp. = 170-173; ^{1}H-NMR (DMSO-d_6, 600 MHz): δ [ppm] = 3.76 (s, 3 H), 6.60-6.62 (m, 2 H), 6.92-6.94 (m, 1 H), 7.26-7.29 (m, 1 H), 7.55 (m, 1 H), 7.59-7.61 (m, 1 H), 7.76-7.78 (m, 2 H), 7.96-7.97 (m, 1 H), 8.68 (s, 1 H); ^{13}C-NMR (DMSO-d_6, 151 MHz): δ [ppm] = 51.48, 112.28, 112.53, 117.56, 119.20, 122.13, 122.98, 124.30, 128.12, 131.26, 142.53, 150.56, 166.05; LC-MS (ESI): m/z (%) [M = $C_{15}H_{13}N_3O_2$] = 268.1[M+H]$^+$, t_R = 1.7 min.

Methyl 4-((7-methylimidazo[1,2-a]pyridin-3-yl)amino)benzoate ***3j****:*



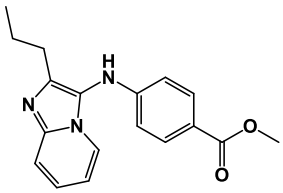
The compound was obtained as described in the general procedure using 4-Methyl-2-aminopyridine (151 mg), glyoxylic acid monohydrate (249 mg), AcOH (0.18 mL) and methyl 4-isocyanobenzoate (250 mg) in 45 % yield as a brown solid (202 mg): mp. = 173-174; ^{1}H-NMR (DMSO-d_6, 600 MHz): δ [ppm] = 2.36 (s, 3 H), 3.76 (s, 3 H), 6.58-6.59 (m, 2 H), 6.76-6.77 (m, 1 H), 7.37 (m, 1 H), 7.44 (m, 1 H), 7.76-7.77 (m, 2 H), 7.83-7.84 (m, 1 H), 8.63 (s, 1 H); ^{13}C-NMR (DMSO-d_6, 151 MHz): δ [ppm] = 20.69, 51.47, 112.46, 114.75, 115.81, 119.10, 121.58, 122.25, 127.83, 131.24, 134.75, 142.99, 147.14, 147.28, 150.74, 159.79, 166.06; LC-MS (ESI): m/z (%) [M = $C_{16}H_{15}N_3O_2$] = 282.1 [M+H]$^+$, t_R = 2.0 min.

Methyl 4-((6-methylimidazo[1,2-a]pyridin-3-yl)amino)benzoate ***3k****:*



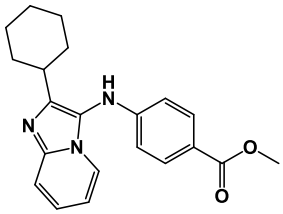
The compound was obtained as described in the general procedure using 5-methyl-2-aminopyridine (162 mg), glyoxylic acid monohydrate (239 mg), AcOH (0.17 mL) and methyl 4-isocyanobenzoate (240 mg) in 54 % yield as a brown solid (227 mg): mp. = 182-183; ^{1}H-NMR (DMSO-d_6, 600 MHz): δ [ppm] = 2.25 (s, 3 H), 3.76 (s, 3 H), 6.59-6.61 (m, 2 H), 7.12-7.14 (m, 1 H), 7.49-7.52 (m, 2 H), 7.76-7.78 (m, 3 H), 8.63 (s, 1 H); ^{13}C-NMR (DMSO-d_6, 151 MHz): δ [ppm] = 17.61, 51.47, 112.47, 117.01, 119.10, 120.19, 121.60, 121.79, 127.30, 128.05, 131.24, 141.65, 150.75, 166.06; LC-MS (ESI): m/z (%) [M = $C_{16}H_{15}N_3O_2$] = 282.1 [M+H]$^+$, t_R = 2.0 min.

Methyl 4-((2-propylimidazo[1,2-a]pyridin-3-yl)amino)benzoate ***3l****:*



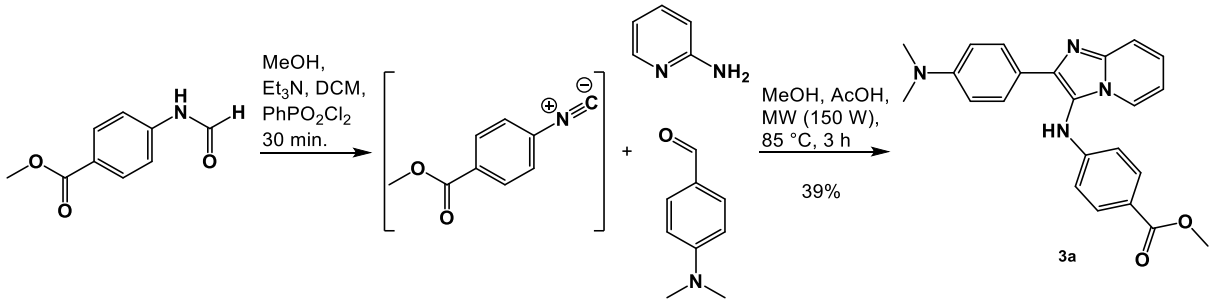
The compound was obtained as described in the general procedure using 2-aminopyridine (151 mg), butanal (0.17 mL), AcOH (0.18 mL) and methyl 4-isocyanobenzoate (250 mg) in 44 % yield as a yellow solid (217 mg): mp. = 182-184; ^{1}H-NMR (DMSO-d_6, 600 MHz): δ [ppm] = 0.86 (t, J = 7.4 Hz, 3 H), 1.65 (sext, J = 7.4, 2 H), 2.55 (t, J = 7.5 Hz, 2 H), 3.75 (s, 3 H), 6.49-6.51 (m, 2 H), 6.84-6.87 (m, 1 H), 7.21-7.24 (m, 1 H), 7.51-7.52 (m, 1 H), 7.75-7.76 (m, 2 H), 7.85-7.86 (m, 1 H), 8.51 (s, 1 H); ^{13}C-NMR (DMSO-d_6, 151 MHz): δ [ppm] = 13.94, 21.77, 28.76, 51.45, 111.78, 112.29, 116.72, 117.76, 119.00, 122.65, 124.00, 131.26, 141.31, 141.61, 150.88, 166.07; LC-MS (ESI): m/z (%) [M = $C_{18}H_{19}N_3O_2$] = 310.2 $[M+H]^+$, t_R = 2.5 min.

Methyl 4-((2-cyclohexylimidazo[1,2-a]pyridin-3-yl)amino)benzoate ***3m****:*



The compound was obtained as described in the general procedure using 2-aminopyridine (151 mg), cyclohexanecarboxaldehyde (0.23 mL), AcOH (0.18 mL) and methyl 4-isocyanobenzoate (250 mg) in 73 % yield as a yellow solid (410 mg): mp. = 221-223; ^{1}H-NMR (DMSO-d_6, 600 MHz): δ [ppm] = 1.16-1.29 (m, 3 H), 1.59-1.65 (m, 3 H), 1.72-1.77 (m, 4 H), 2.63-2.67 (m, 1 H), 3.75 (s, 3 H), 6.48-6.49 (m, 2 H), 6.83-6.85 (m, 1 H), 7.20-7.23 (m, 1 H), 7.51-7.53 (m, 1 H), 7.74-7.76 (m, 2 H), 7.81-7.82 (m, 1 H), 8.49 (s, 1 H); ^{13}C-NMR (DMSO-d_6, 151 MHz): δ [ppm] = 25.68, 26.01, 32.05, 36.05, 51.44, 111.79, 112.24, 116.38, 116.84, 118.97, 122.58, 123.95, 131.29, 141.55, 145.96, 151.03, 166.07; LC-MS (ESI): m/z (%) [M = $C_{21}H_{23}N_3O_2$] = 350.2 $[M+H]^+$, t_R = 3.2 min.

*<u>Synthesis of **3a** via the GBB three-component reaction with in situ isocyanide formation:</u>*



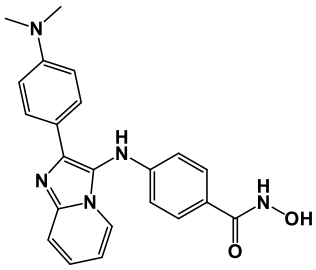
Synthesis of **3a** was adapted to the isocyanide-less GBB protocol by *Dömling* and co-workers.[6] To a stirred solution of methyl 4-formamidobenzoate (160 mg, 1 mmol, 1 eq.) in dichloromethane (2 mL) was added triethylamine (0.40 mL, 2.90 mmol, 2.9 eq.). Subsequently, $PhPO_2Cl_2$ (0.15 mL, 1 mol, 1 eq.) was slowly added and the mixture was stirred for 1 h. Afterwards, 2-aminopyridine (47 mg, 0.5 mmol, 0.5 eq.), 4-(dimethylamino)benzaldehyde (90 mg, 0.6 mmol, 0.6 eq.), AcOH (0.11 mL, 2 mmol, 2 eq.) and methanol was added and the mixture was subjected to microwave irridation (150 W) at 85 °C for 3 h. The reaction mixture was kept in the refrigerator overnight. After filtration, the product **3a** was obtained as a yellow solid (75 mg) in 39 % yield. For analytical data of **3a** see *General Procedure for the synthesis of ester* **3a-m**.

2.4 Synthesis of hydroxamic acids 4a-m

*General Procedure for the synthesis of hydroxamic acids **4a-m**:*

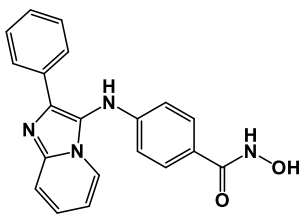
The respective ester was dissolved in dichloromethane/methanol (1:2) and cooled to 0 °C. Subsequently, hydroxylamine (50 wt % in water, 30 eq.) and sodium hydroxide (10 eq.) were added and the mixture was stirred for 15 min at 0 °C. The reaction was allowed to warm to room temperature and stirred until complete consumption of the ester (monitored by TLC, DCM/MeOH = 9:1). The solvent was then removed under reduced pressure and the residue was dissolved/suspended in water (10-15 mL). The pH was adjusted using 1N $HCl_{aq.}$ and the resulting precipitate was filtered and washed with diethylether to give the desired hydroxamic acid. In some cases, the respective carboxylic acid has formed as a side product, which was removed by using the anion exchange sorbent Isolute PE-AX® (with methanol as the eluent) or column chromatography (dichloromethane/methanol = *gradient*).[7]

*4-((2-(4-(Dimethylamino)phenyl)imidazo[1,2-a]pyridin-3-yl)amino)-N-hydroxybenzamide **4a**:*



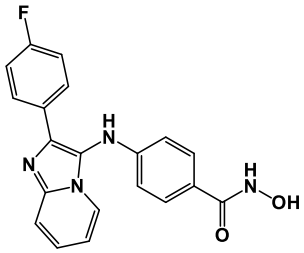
The compound was obtained as described using the general procedure in 53 % yield as a white solid (79 mg): mp. = 184-185 °C; ^{1}H-NMR (DMSO-d_6, 300 MHz): δ [ppm] = 2.89 (s, 6 H), 6.47-6.50 (m, 2 H), 6.69-6.74 (m, 2 H), 6.84-6.89 (m, 1 H), 7.23-7.29 (m, 1 H), 7.55-7.58 (m, 3 H), 7.83-7.88 (m, 2 H), 7.89-7.92 (m, 1 H), 8.51 (s, 1 H), 9.11 (*br*s, 1 H), 10.67 (*br*s, 1 H).^{13}C-NMR (DMSO-d_6, 151 MHz): δ [ppm] = 40.06, 111.90, 112.04, 112.17, 113.91, 115.57, 116.16, 116.64, 121.28, 122.62, 122.69, 124.61, 127.30, 128.71, 138.52, 141.76, 148.54, 149.84, 164.33; HRMS calculated for $C_{22}H_{21}N_5O_2$: 388.1768 $[M+H]^+$, found 388.1770; HPLC purity: 98.4 %.

*N-Hydroxy-4-((2-phenylimidazo[1,2-a]pyridin-3-yl)amino)benzamide **4b**:*



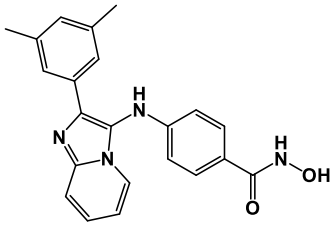
The compound was obtained as described using the general procedure in 52 % yield as a white solid (78 mg): mp. = 173-175; ^{1}H-NMR (DMSO-d_6, 600 MHz): δ [ppm] = 6.51-6.52 (m, 2 H), 6.92-6.94 (m, 1 H), 7.27-7.30 (m, 1 H), 7.31-7.34 (m, 1 H), 7.38-7.40 (m, 2 H), 7.57-7.59 (m, 2 H), 7.64-7.65 (m, 1 H), 7.95-7.96 (m, 1 H), 8.01-8.03 (m, 2 H), 8.64 (s, 1 H), 8.79 (s, 1 H), 10.88 (s, 1 H); ^{13}C-NMR (DMSO-d_6, 151 MHz): δ [ppm] = 112.25, 112.44, 117.21, 118.00, 122.83, 123.02, 125.29, 126.41, 127.62, 128.50, 128.81, 133.51, 137.56, 141.94, 148.26; HRMS calculated for $C_{20}H_{16}N_4O_2$: 345.1346 $[M+H]^+$, found 345.1348; HPLC purity: 99.0 %.

*4-((2-(4-Fluorophenyl)imidazo[1,2-a]pyridin-3-yl)amino)-N-hydroxybenzamide **4c**:*



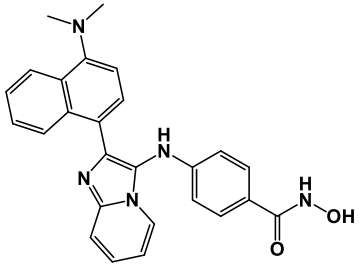
The compound was obtained as described using the general procedure in 82 % yield as a white solid (124 mg): mp. = 164-166; ^{1}H-NMR (DMSO-d_6, 600 MHz): δ [ppm] = 6.51-6.52 (m, 2 H), 6.92-6.94 (m, 1 H), 7.22-7.26 (m, 2 H), 7.31-7.34 (m, 1 H), 7.58-7.59 (m, 2 H), 7.63-7.64 (m, 1 H), 7.95-7.96 (m, 1 H), 8.03-8.07 (m, 2 H), 8.66 (s, 1 H), 8.88 (*br*s, 1 H), 10.91 (*br*s, 1 H); ^{13}C-NMR (DMSO-d_6, 151 MHz): δ [ppm] = 112.29, 112.51, 115.40, 115.54, 117.19, 117.80, 122.98, 123.10, 125.43, 128.34, 128.39, 128.83, 130.05, 130.07, 136.76, 141.95, 148.13, 160.89, 162.51, 164.40; HRMS calculated for $C_{20}H_{15}FN_4O_2$: 363.1252 $[M+H]^+$, found 363.1256; HPLC purity: 97.0 %.

*Methyl 4-((2-(3,5-dimethylphenyl)imidazo[1,2-a]pyridin-3-yl)amino)benzoate **4d**:*



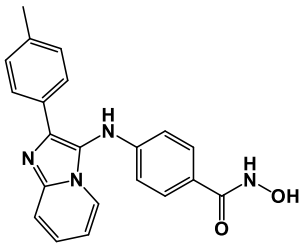
The compound was obtained as described using the general procedure in 44 % yield as a white solid (71 mg): mp. = 168-170; ^{1}H-NMR (DMSO-d_6, 500 MHz): δ [ppm] = 2.24 (s, 6 H), 6.48-6.50 (m, 2 H), 6.90-6.95 (m, 2 H), 7.29-7.32 (m, 1 H), 7.56-7.58 (m, 2 H), 7.60-7.62 (m, 1 H), 7.66 (s, 2 H), 7.93-7.94 (m, 1 H), 8.58 (s, 1 H), 10.06 (*br*s, 2 H); ^{13}C-NMR (DMSO-d_6, 126 MHz): δ [ppm] = 20.99, 112.14, 112.18, 117.00, 118.13, 122.85, 123.11, 124.32, 124.94, 128.48, 128.85, 133.28, 137.06, 137.60, 141.69, 148.04, 164.00; HRMS calculated for $C_{22}H_{20}N_4O_2$: 373.1659 $[M+H]^+$, found 373.1661; HPLC purity: > 99 %.

*4-((2-(4-(Dimethylamino)naphthalen-1-yl)imidazo[1,2-a]pyridin-3-yl)amino)-N-hydroxybenzamide **4e**:*



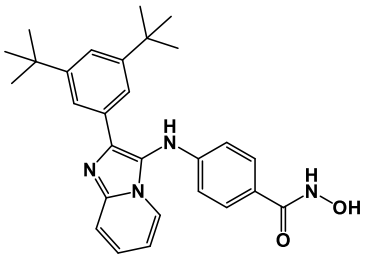
The compound was obtained as described using the general procedure in 43 % yield as a brown solid (65 mg): mp. = 176-177; ^{1}H-NMR (DMSO-d_6, 600 MHz): δ [ppm] = 2.83 (s, 6 H), 6.38-6.46 (m, 2 H), 6.96-6.98 (m, 1 H), 7.08-7.09 (m, 1 H), 7.34-7.37 (m, 1 H), 7.43-7.45 (m, 1 H), 7.49-7.54 (m, 4 H), 7.70-7.71 (m, 1 H), 7.93-7.94 (m, 1 H), 8.18-8.19 (m, 1 H), 8.46-8.47 (m, 1 H), 8.51 (s, 1 H), 9.22 (*br*s, 1 H), 10.74 (*br*s, 1 H); ^{13}C-NMR (DMSO-d_6, 151 MHz): δ [ppm] = 44.76, 112.10, 112.33, 113.34, 117.28, 119.41, 122.52, 123.19, 123.92, 124.83, 124.97, 125.17, 125.74, 127.05, 127.64, 128.19, 128.62, 132.72, 139.39, 141.69, 148.61, 150.67, 164.22; HRMS calculated for $C_{26}H_{23}N_5O_2$: 438.1925 $[M+H]^+$, found 438.1927; HPLC purity: >99 %.

N-Hydroxy-4-((2-(p-tolyl)imidazo[1,2-a]pyridin-3-yl)amino)benzamide ***4f****:*



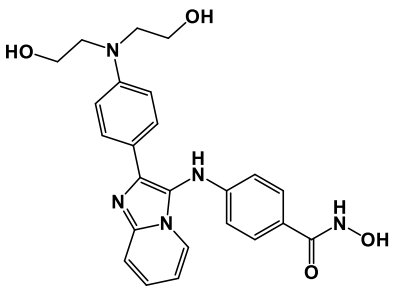
The compound was obtained as described using the general procedure in 59 % yield as a white solid (88 mg): mp. = 178-179; ^{1}H-NMR (DMSO-d_6, 500 MHz): δ [ppm] = 2.29 (s, 3 H), 6.49-6.50 (m, 2 H), 6.89-6.92 (m, 1 H), 7.18-7.20 (m, 2 H), 7.28-7.32 (m, 1 H), 7.56-7.58 (m, 2 H), 7.60-7.62 (m, 1 H), 7.91-7.95 (m, 3 H), 8.57 (s, 1 H), 9.93 (*br*s, 2 H); ^{13}C-NMR (DMSO-d_6, 126 MHz): δ [ppm] = 20.70, 112.14, 116.97, 117.64, 122.82, 123.07, 124.95, 126.29, 128.54, 128.94, 130.67, 136.79, 137.69, 141.67, 141.76, 148.02, 164.13; HRMS calculated for $C_{21}H_{18}N_4O_2$: 359.1503 $[M+H]^+$, found 359.1504; HPLC purity: >99%.

4-((2-(3,5-di-tert-Butylphenyl)imidazo[1,2-a]pyridin-3-yl)amino)-N-hydroxybenzamide ***4g****:*



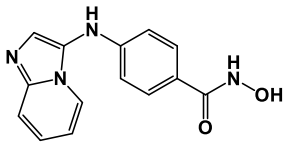
The compound was obtained as described using the general procedure in 47 % yield as a green solid (90 mg): mp. = 138-139; ^{1}H-NMR (DMSO-d_6, 600 MHz): δ [ppm] = 1.21 (s, 18 H), 6.52-6.53 (m, 2 H), 6.92-6.94 (m, 1 H), 7.30-7.33 (m, 2 H), 7.59-7.61 (m, 2 H), 7.66-7.68 (m, 1 H), 7.83-7.84 (m, 2 H), 8.03-8.04 (m, 1 H), 8.63 (s, 1 H), 8.77 (*br*s, 1 H), 10.90 (*br*s, 1 H); ^{13}C-NMR (DMSO-d_6, 151 MHz): δ [ppm] = 31.17, 34.49, 112.20, 112.35, 117.12, 117.89, 120.91, 121.16, 122.49, 122.87, 125.08, 128.69, 132.53, 138.34, 141.81, 148.67, 150.15, 164.29; HRMS calculated for $C_{28}H_{32}N_4O_2$: 457.2598 $[M+H]^+$, found 457.2600; HPLC purity: 96.1 %.

4-((2-(4-(bis(2-Hydroxyethyl)amino)phenyl)imidazo[1,2-a]pyridin-3-yl)amino)-N-hydroxybenzamide ***4h****:*



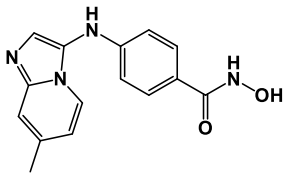
The compound was obtained as described using the general procedure in 83 % yield as a brown solid (142 mg): mp. = 176-178; ^{1}H-NMR (DMSO-d_6, 600 MHz): δ [ppm] = 3.41 (t, J = 6.3 Hz, 4 H), 3.50-3.53 (m, 4 H), 4.72 (t, J = 5.5 Hz, 2 H), 6.49 (m, 2 H), 6.67-6.69 (m, 2 H), 6.85-6.88 (m, 1 H), 7.24-7.27 (m, 1 H), 7.55-7.58 (m, 3 H), 7.80-7.81 (m, 2 H), 7.89-7.90 (m, 1 H), 8.50 (s, 1 H), 8.78 (s, 1 H), 10.86 (s, 1 H); ^{13}C-NMR (DMSO-d_6, 75 MHz): δ [ppm] = 53.18, 58.18, 111.10, 111.92, 112.19, 115.96, 116.58, 120.31, 122.59, 124.62, 127.50, 128.80, 138.60, 141.75, 147.53, 148.67, 164.60; HRMS calculated for $C_{24}H_{25}N_5O_4$: 448.1979 $[M+H]^+$, found 448.1976; HPLC purity: 96.0 %.

N-Hydroxy-4-(imidazo[1,2-a]pyridin-3-ylamino)benzamide ***4i****:*



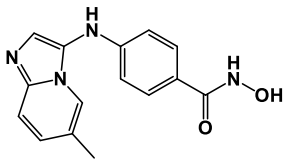
The compound was obtained as described using the general procedure in 50 % yield as a white solid (56 mg): mp. = 194-196; ^{1}H-NMR (DMSO-d_6, 600 MHz): δ [ppm] = 6.54-6.55 (m, 2H), 6.91-6.93 (m, 1 H), 7.25-7.28 (m, 1 H), 7.52 (s, 1 H), 7.58-7.60 (m, 3 H), 7.94-7.95 (m, 1 H), 8.43 (s, 1 H), 8.83 (*br*s, 1 H), 10.90 (*br*s, 1 H); ^{13}C-NMR (DMSO-d_6, 75 MHz): δ [ppm] = 112.17, 112.34, 117.55, 122.59, 122.72, 122.99, 124.17, 127.92, 128.59, 142.43, 148.72, 164.38; HRMS calculated for $C_{14}H_{12}N_4O_2$: 269.1033 $[M+H]^+$, found; 269.1036 HPLC purity: 96.5 %.

N-Hydroxy-4-((7-methylimidazo[1,2-a]pyridin-3-yl)amino)benzamide ***4j****:*



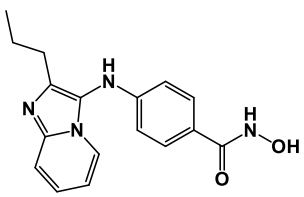
The compound was obtained as described using the general procedure in 63 % yield as a brown solid (89 mg): mp. = 198-199; ^{1}H-NMR (DMSO-d_6, 600 MHz): δ [ppm] = 2.36 (s, 3 H), 6.51-6.53 (m, 2 H), 6.75-6.77 (m, 1 H), 7.36 (s, 1 H), 7.41 (s, 1 H), 7.57-7.59 (m, 2 H), 7.82-7.83 (m, 1 H), 8.36 (s, 1 H), 8.80 (*br*s, 1 H), 10.90 (*br*s, 1 H); ^{13}C-NMR (DMSO-d_6, 151 MHz): δ [ppm] = 20.71, 112.26, 114.66, 115.80, 122.15, 122.27, 122.45, 127.66, 128.59, 134.64, 142.91, 148.93, 164.43; HRMS calculated for $C_{15}H_{14}N_4O_2$: 283.1190 $[M+H]^+$, found; 283.1187 HPLC purity: 96.0 %.

N-Hydroxy-4-((6-methylimidazo[1,2-a]pyridin-3-yl)amino)benzamide ***4k****:*



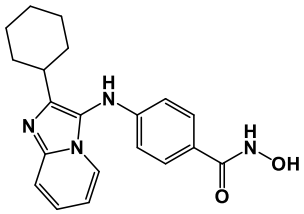
The compound was obtained as described using the general procedure in 72 % yield as a beige solid (109 mg): mp. = 161-162; ^{1}H-NMR (DMSO-d_6, 500 MHz): δ [ppm] = 2.26 (s, 3 H), 6.53-6.55 (m, 2 H), 7.11-7.13 (m, 1 H), 7.45 (s, 1 H), 7.49-7.51 (m, 1 H), 7.58-7.60 (m, 2 H), 7.76 (s, 1 H), 8.34 (s, 1 H), 8.78 (*br*s, 1 H), 10.89 (*br*s, 1 H); ^{13}C-NMR (DMSO-d_6, 126 MHz): δ [ppm] = 17.54, 112.21, 116.90, 120.07, 121.32, 122.32, 122.44, 127.03, 127.74, 128.47, 141.47, 148.84, 164.40; HRMS calculated for $C_{15}H_{14}N_4O_2$: 283.1190 $[M+H]^+$, found; 283.1186 HPLC purity: 96.6 %.

N-Hydroxy-4-((2-propylimidazo[1,2-a]pyridin-3-yl)amino)benzamide ***4l****:*



The compound was obtained as described using the general procedure in 83 % yield as a beige solid (125 mg): mp. = 157-158; ^{1}H-NMR (DMSO-d_6, 600 MHz): δ [ppm] = 0.86 (t, J = 7.4 Hz , 3 H), 1.66 (tq, J = 7.4 Hz, 2 H), 2.55 (t, J = 7.5 Hz, 2 H), 6.43-6.44 (m, 2 H), 6.83-6.86 (m, 1 H), 7.20-7.23 (m, 1 H), 7.50-7.51 (m, 1 H), 7.56-7.58 (m, 2 H), 7.83-7.84 (m, 1 H), 8.26 (s, 1 H), 8.83 (*br*s, 1 H), 10.89 (*br*s, 1 H); ^{13}C-NMR (DMSO-d_6, 126 MHz): δ [ppm] = 13.98, 21.84, 28.77, 111.68, 112.03, 116.69, 118.24, 122.37, 122.67, 123.90, 128.60, 141.26, 141.52, 149.04, 164.47; HRMS calculated for $C_{17}H_{18}N_4O_2$: 311.1503 $[M+H]^+$, found 311.1506; HPLC purity: 98.7 %.

4-((2-Cyclohexylimidazo[1,2-a]pyridin-3-yl)amino)-N-hydroxybenzamide ***4m****:*



The compound was obtained as described using the general procedure in 90 % yield as a white solid (172 mg): mp. = 178-179; ^{1}H-NMR (DMSO-d_6, 600 MHz): δ [ppm] = 1.16-1.30 (m, 3 H), 1.59-1.66 (m, 3 H), 1.72-1.77 (m, 4 H), 2.64-2.69 (m, 1 H), 6.41-6.42 (m, 2 H), 6.82-6.84 (m, 1 H), 7.19-7.22 (m, 1 H), 7.51-7.52 (m, 1 H), 7.55-7.57 (m, 2 H), 7.79-7.80 (m, 1 H), 8.24 (s, 1 H), 8.85 (*br*s, 1 H), 10.88 (*br*s, 1 H); ^{13}C-NMR (DMSO-d_6, 126 MHz): δ [ppm] = 25.70, 26.04, 32.09, 32.20, 36.03, 111.69, 111.96, 116.82, 116.86, 122.39, 122.61, 123.85, 128.62, 141.46, 145.95, 149.16, 164.48; HRMS calculated for $C_{20}H_{22}N_4O_2$: 351.1816 $[M+H]^+$, found 351.1815; HPLC purity: 98.0 %.

3. X-Ray crystallography

Materials and Methods

The MBP-TEV-z6CD2-Pet28a(+) vector prepared by Hai and Christianson[8] was used to express HDAC6 catalytic domain 2 (CD2) from *Danio rerio* (zebrafish) and purified as described for crystallization and X-ray structure determination.[8,9] Briefly, zebrafish HDAC6 CD2 (henceforth, simply "HDAC6") was expressed in *Escherichia coli* BL21 (DE3) cells in 2xYT media with 50 mg/L kanamycin. Cells were grown at 37° C until OD_{600} reached 1.0, after which the temperature was decreased to 18° C for an additional 18 h. Cells were supplemented with 200 μM $ZnSO_4$ and induced with 75 μM IPTG. Cells were sonicated in buffer A [20 mM Tris (pH 8.0), 100 mM NaCl, 1 mM tris(2-carboxyethyl)phosphine (TCEP), 5% glycerol] and centrifuged at 15,000 *g* for 1 h (4° C). Supernatant was purified on an amylose column (New England BioLabs), and protein was eluted using buffer A plus 10 mM maltose. Protein fractions were dialyzed overnight in buffer A plus 10 mg/mL TEV protease to cleave the MBP tag. The protein digest was applied to a Ni-NTA column (Qiagen) in Buffer B [50 mM K_2HPO_4 (pH 8.0), 1 mM TCEP, 300 mM NaCl, 5% glycerol] and protein was eluted using a gradient of 0–300 mM imidazole. HDAC6-containing fractions were applied to a HiLoad superdex 200 column in buffer C [50 mM HEPES (pH 7.5), 100 mM KCl, 1 mM TCEP, 5% glycerol] and concentrated to approximately 10 mg/mL. Protein was flash-cooled in liquid nitrogen and stored at -80 ˚C prior to use.

The HDAC6–**4l** complex was crystallized using the sitting drop vapor diffusion method at 4° C by equilibrating a drop containing 0.35 μL protein solution [10 mg/mL HDAC6, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.5), 2.0 mM **4l**, 100 mM KCl, 1 mM TCEP, 5% glycerol] and 0.35 μL precipitant solution [0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES) monohydrate (pH 6.0), 14% (w/v) polyethylene glycol 4,000] against a reservoir of 100 μL precipitant solution. Thin needle-like crystals appeared within 2 days. Crystals were harvested and soaked in crystallization buffer augmented with 15% ethylene glycol as a cryoprotectant prior to flash cooling in liquid nitrogen.

X-ray diffraction data were collected at the Stanford Synchrotron Radiation Lightsource, beamline 12-2. Diffraction data were indexed and integrated using iMOSFLM[10] and scaled using the CCP4 program suite.[11] The crystal structure of the HDAC6–**4l** complex was solved by molecular replacement using unliganded HDAC6 (PDB 5EEM) as a search probe in routines implemented in the crystallographic software Phaser.[12] The program Phenix[13] was used for crystallographic refinement, and the graphics software Coot[14] was used for visualizing the electron density map and protein model between refinement runs. Inhibitor and solvent molecules were added in the later stages of refinement. Occasionally, spurious electron density peaks were observed in electron density maps; such peaks could not be interpreted satisfactorily and were left uninterpreted. The programs MolProbity[15] and PROCHECK[16] were used to evaluate the quality of the final model. Final refinement statistics are recorded in Table 1. Polder omit electron density maps shown in Figure 1 were calculated as described.[17] Final refined coordinates and

structure factor amplitudes for the final model have been deposited in the Protein Data Bank (www.rcsb.org) with accession code 6CGP.

Table S3: Data collection and refinement statistics for the HDAC6–4l complex.

Data Collection	
Space group	$P22_12_1$
Unit cell dimensions	
a, b, c (Å)	45.3, 65.0, 140.5
α, β, γ (°)	90.0, 90.0, 90.0
Resolution (Å)	70.25 – 2.60 (2.59 – 2.50)[a]
R_{merge}[b]	0.220 (1.51)[a]
R_{pim}[c]	0.109 (0.782)[a]
$CC_{1/2}$[d]	0.988 (0.476)[a]
Redundancy	4.8 (4.5)[a]
I / σ	5.7 (1.1)[a]
Completeness (%)	99.3 (98.5)[a]
Refinement	
Resolution (Å)	38.08 – 2.50 (2.59 – 2.50)[a]
No. reflections	14843 (1429)[a]
R_{work}/R_{free}[e]	0.213/0.266 (0.334/0.362)[a]
No. atoms[f]	
Protein	2739
Ligands	30
Solvent	14
Average B factors (Å^2)	
Protein	42
Ligand	50
Solvent	35
R.m.s. deviations	
Bond lengths (Å)	0.002
Bond angles (°)	0.7
Ramachandran plot (%)[g]	
Favored	95.2
Allowed	3.7
Outliers	1.1

[a]Values in parentheses refer to the highest-resolution shell indicated. [b]$R_{merge} = \sum_{hkl}\sum_i |I_{i,hkl} - \langle I\rangle_{hkl}| / \sum_{hkl}\sum_i I_{i,hkl}$, where $\langle I\rangle_{hkl}$ is the average intensity calculated for reflection *hkl* from replicate measurements. [c]$R_{p.i.m.} = (\sum_{hkl}(1/(N-1))^{1/2}\sum_i |I_{i,hkl} - \langle I\rangle_{hkl}|) / \sum_{hkl}\sum_i I_{i,hkl}$, where $\langle I\rangle_{hkl}$ is the average intensity calculated for reflection *hkl* from replicate measurements and N is the number of reflections [d]Pearson correlation coefficient between random half-datasets. [e]$R_{work} = \sum ||F_o| - |F_c|| / \sum |F_o|$ for reflections contained in the working set. $|F_o|$ and $|F_c|$ are the observed and calculated structure factor amplitudes, respectively. R_{free} is calculated using the same expression for reflections contained in the test set held aside during refinement. [f]Per asymmetric unit. [g]Calculated with PROCHECK.

4. Biological evaluation

4.1 Reagents

Cisplatin was purchased from Sigma-Aldrich (Germany), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Serva (Germany). Tubastatin A and nexturastat were obtained from Tocris (United Kingdom). Vorinostat was synthesized according to known procedures.[18] All other reagents were supplied by PAN Biotech (Germany) unless otherwise stated.

4.2 Cell lines and cell culture

The human tongue squamous cell carcinoma cell line Cal27 and the human embryonic kidney cell line HEK293 were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany). All cell lines were grown at 37°C in a humidified atmosphere containing 5% CO_2 in DMEM containing 10% fetal calf serum, 120 IU/mL penicillin, and 120 µg/mL streptomycin. The cells were grown to 80% confluency before using them for the appropriate assays.

4.3 MTT cell viability assay

The rate of cell-survival under the action of test compounds was evaluated by an improved MTT assay as previously described.[19] In brief, HEK293 or Cal27 were seeded at a density of 3,000 and 2,500 cells/well in 96well plates (Corning, Germany). After 24 h, cells were exposed to increased concentrations of test compounds. Incubation was ended after 72 h and cell survival was determined by addition of MTT solution (5 mg/mL in phosphate buffered saline). The formazan precipitate was dissolved in DMSO (VWR, Germany). Absorbance was measured at 544 nm and 690 nm in a FLUOstar microplate-reader (BMG LabTech, Germany). All compounds were evaluated in three independent experiments each performed in triplicates.

4.4 Whole-cell HDAC inhibition assay

The cellular HDAC assay was based on an assay published by Ciossek *et al.*[20] and Bonfils *et al.*[21] with minor modificationsas described in reference.[19] Briefly, Cal27 cells were seeded in 96-well tissue culture plates (Corning, Germany) at a density of 1.5 x 10^4 cells/well in a total volume of 90 µL medium. After 24 h, cells were incubated for 18 h with increasing concentrations of test compounds. The reaction was started by adding 10 µL of 3 mM Boc-Lys(ε-Ac)-AMC (Bachem, Germany) to reach a final concentration of 0.3 mM. Cells were incubated with Boc-Lys(ε-Ac)-AMC for 3 h under cell culture conditions. After this incubation, 100 µl/well of stop solution (25 mM Tris-HCl (pH 8), 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1% NP40, 2.0 mg/mL trypsin, 10 µM vorinostat) was added and the reaction was developed for 3 h under cell culture conditions. Fluorescence intensity was measured at excitation of 320 nm and emission of 520 nm in a NOVOstar microplate-reader (BMG LabTech, Offenburg, Germany). All compounds were evaluated in triplicates in three independent experiments.

4.5 Immunoblotting

Cells were treated with 1 μM of 4a and 4l or vehicle (0.1% DMSO) for 24 h. The pan-HDACi vorinostat and the HDAC-6-selective inhibitors tubastatin A and nexturastat were used as controls. Cell pellets were dissolved with RIPA lysis buffer and clarified by centrifugation. Equal amounts of total protein (20 μg) were resolved by SDS-PAGE and transferred to polyvinylidene fluoride membranes. Blots were incubated with primary antibodies against acetylated α-tubulin, α-tubulin, acetylated histone H3, and histone H3 (biotechne, Germany). Immunoreactive proteins were visualized using luminol reagent (Santa Cruz, Heidelberg, Germany) with an Intas Imager (Intas, Germany).

4.6 Measurement of apoptotic cells

Apoptotic cells were determined by propidium iodide staining as previously described.[22]

4.7 In vitro testing on HDAC1 and -6.

The in vitro inhibitory activity of compounds **4a-m** against two human HDAC isoforms (1 and 6) were performed as followed. OptiPlate-96 black microplates (Perkin Elmer) were used with an assay volume of 50 μL. 5 μL test compound or control, diluted in assay buffer (50 mM Tris-HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 0.1 mg/mL BSA), were incubated with 35 μL of the fluorogenic substrate ZMAL (Z-(Ac)Lys-AMC)[23] (21.43 μM in assay buffer) and 10 μL of human recombinant HDAC1 (1 ng/μL in assay buffer; BPS Bioscience, Catalog# 50051) or HDAC6 (3.5 ng/μL in assay buffer; BPS Bioscience, Catalog# 50006) at 37 °C. After an incubation time of 90 min, 50 μL of 0.4 mg/mL trypsin in trypsin buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl) were added, followed by further incubation at 37 °C for 30 min. Fluorescence was measured with an excitation wavelength of 355 nm and an emission wavelength of 460 nm using a Fluoroskan Ascent microplate reader (Thermo Scientific). All compounds were evaluated in duplicate in at least two independent experiments.

4.8 In vitro testing on HDAC2, -3 and -8.

The in vitro inhibitory activity of **4l** against HDAC2, -3 and -8 was determined at Reaction Biology Corp. (Malvern, PA) with a fluorescent based assay according to the company's standard operating procedure using RHKK(Ac)AMC (HDAC2 and -3) or RHK(Ac)K(Ac)AMC (HDAC8) as substrates. The IC_{50} values were determined in duplicate using 10 different concentrations with 3-fold serial dilution starting at 10 μM. TSA (HDAC2 IC_{50}: 26.7 nM, $HDAC_3$ IC_{50}: 12.7 nM and HDAC8 IC_{50}: 609 nM) was used as reference compound.

4.9 In vitro drug sensitivity assay of P. falciparum blood stages

The P. falciparum laboratory strains 3D7 (chloroquine sensitive) and Dd2 (multi-resistant) were kept in continuous culture in complete culture medium (RPMI 1640, 25 mM HEPES, 2 mM L-glutamine, 50 μg/ml gentamicin and 0.5% w/v AlbuMAX).

All test compounds were dissolved in DMSO at stock concentrations between 12.5 mM to 100 mM and further dilutions were prepared in complete culture medium. SAHA and chloroquine diphosphate (Sigma) were used as controls. Antiplasmodial activity of the compounds was assessed against the two laboratory strains using the histidine-rich protein 2 (HRP2) assay as described previously.[24,25] In brief: 96 well plates were pre-coated with the compounds in a threefold serial dilution before ring stage parasites were added in complete culture medium at a hematocrit of 1.5% and a parasitemia of 0.05%. After three days of incubation plates were frozen until analyzed by HRP2-ELISA. All compounds were evaluated in duplicate in at least two independent experiments.

The 50% inhibitory concentrations (IC 50) were determined by analysing the nonlinear regression of log concentration–response curves using the drc-package v0.9.0 of R v3.2.2 (Vienna Austria 2015).

5. Molecular Docking

5.1 Ligand Preparation

Drawing and energy minimization of the ligands were performed in Maestro Elements (Schrödinger Release 2016-1: Maestro, Schrödinger, LLC, New York, NY, 2018) using the OPLS_2005 force field.[26] Then, the AutodockTools-1.5.6 program[27] was used to add hydrogens, compute Gasteiger charges, merge nonpolar hydrogens, and choose torsions. Except amide bonds, all acyclic bonds were made rotatable and the pdbqt file was generated.

5.2 Protein Preparation

The crystal structure of human HDAC6 was downloaded from the RCSB Protein Data Bank (PDB ID: 5EDU) and the chain A, Trichostatin A, potassium, maltose and water were removed. AutodockTools-1.5.6 program[27] was used to add all hydrogen atoms, modify histidine protonation (H610 and H611, adding only HD1)[28], compute gasteiger charges, and merge all non-polar hydrogens. After the generation of the pdbqt output file, the charge of the zinc atom was manually changed to +2.

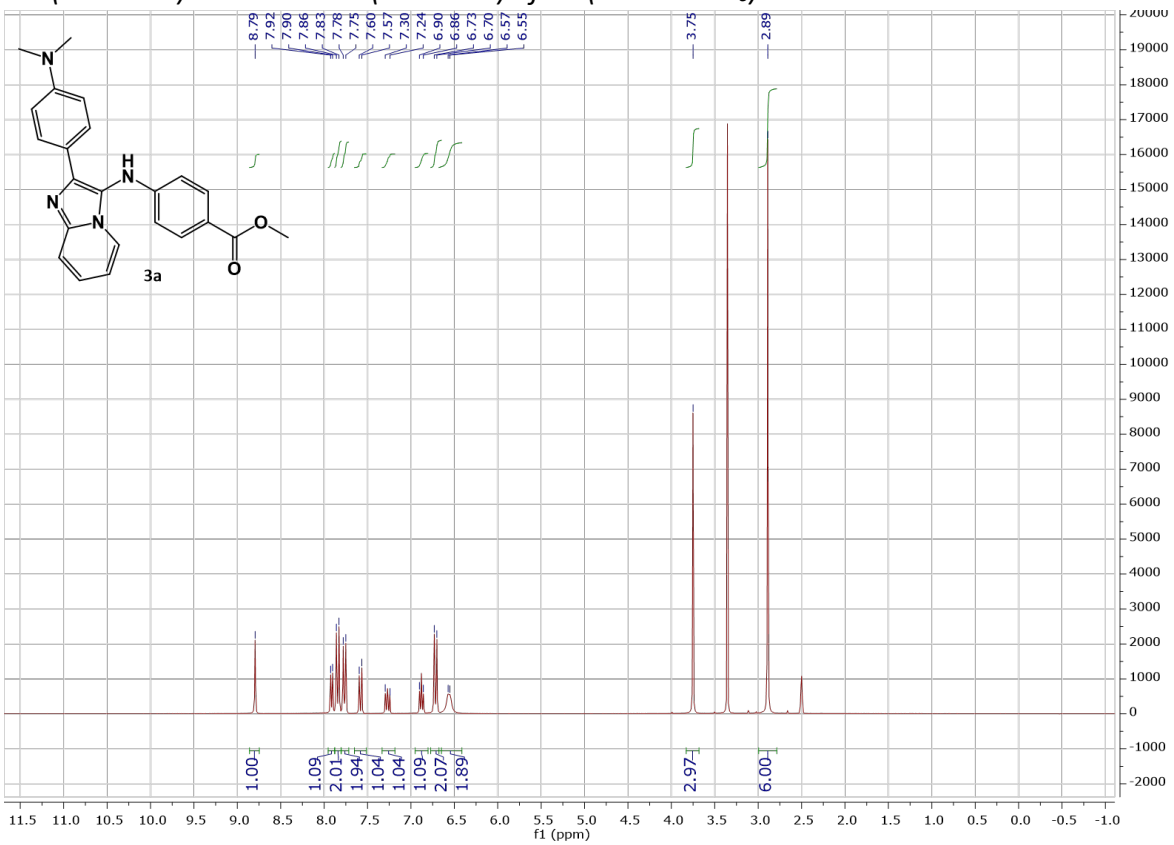
5.3 Docking

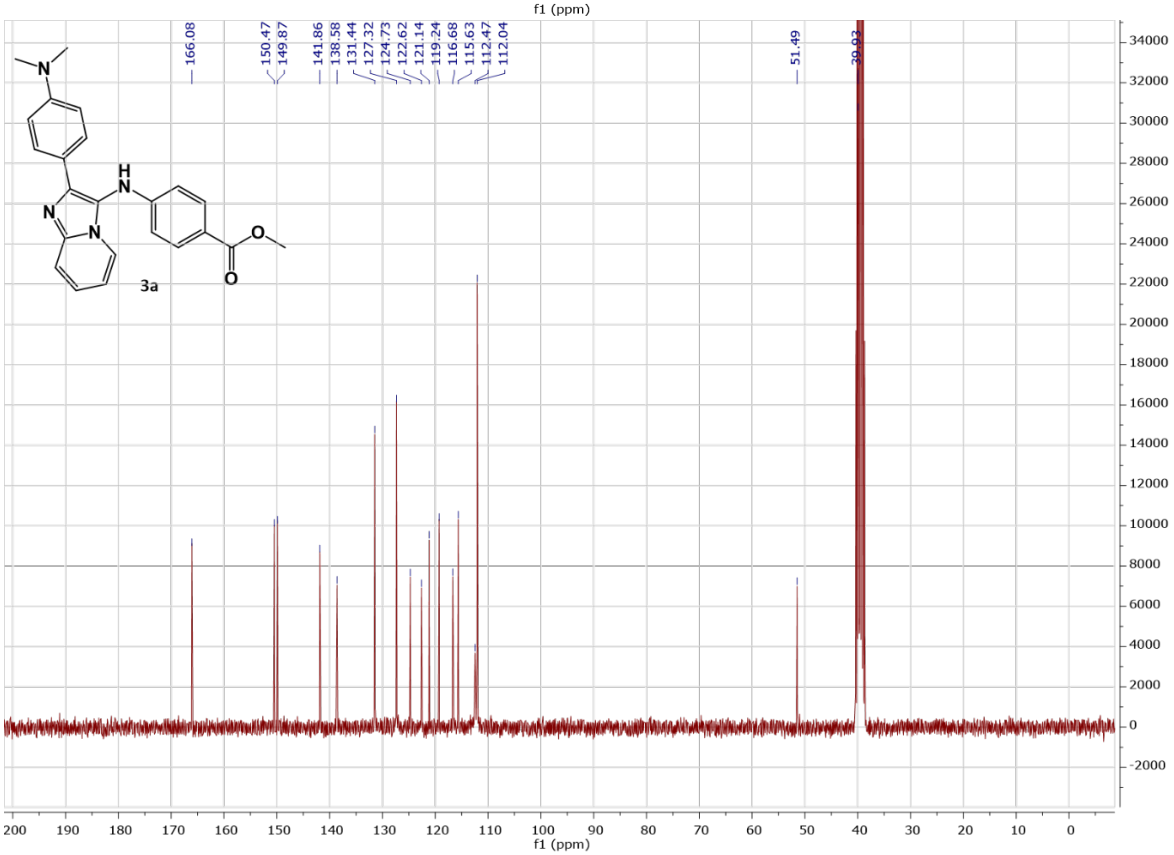
Autogrid 4.2.6[27] was utilized to generate the grid files using a grid box with the following settings: spacing of 0.375 Å, grid box size of 48 X 48 X 45 and coordinates for the center of the grid box (-1.102, 8.703, 6.641). Autodock 4.2.6[27] was used to perform the docking calculations. The generated pdbqt file of the enzyme was set as a rigid macromolecule, while Ser568 was set as a flexible residue to enhance binding mode predictions. The genetic algorithm search parameters were set to 100 GA runs for each ligand with a population size of 150, a maximum number of 2.5 X 10^6 energy evaluations, a maximum number of 2.7 X 10^4 generations, a mutation rate of 0.2, and a crossover rate of 0.8. Docking solutions with more than 20 % of all configurations and binding to the zinc ion were considered sufficiently converged. The lowest-energy conformer of the cluster with the best predicted binding energy was used as a representative predicted binding pose (see Figure S1). Molecular graphics were created with the UCSF Chimera package.[29]

6. Spectral Data

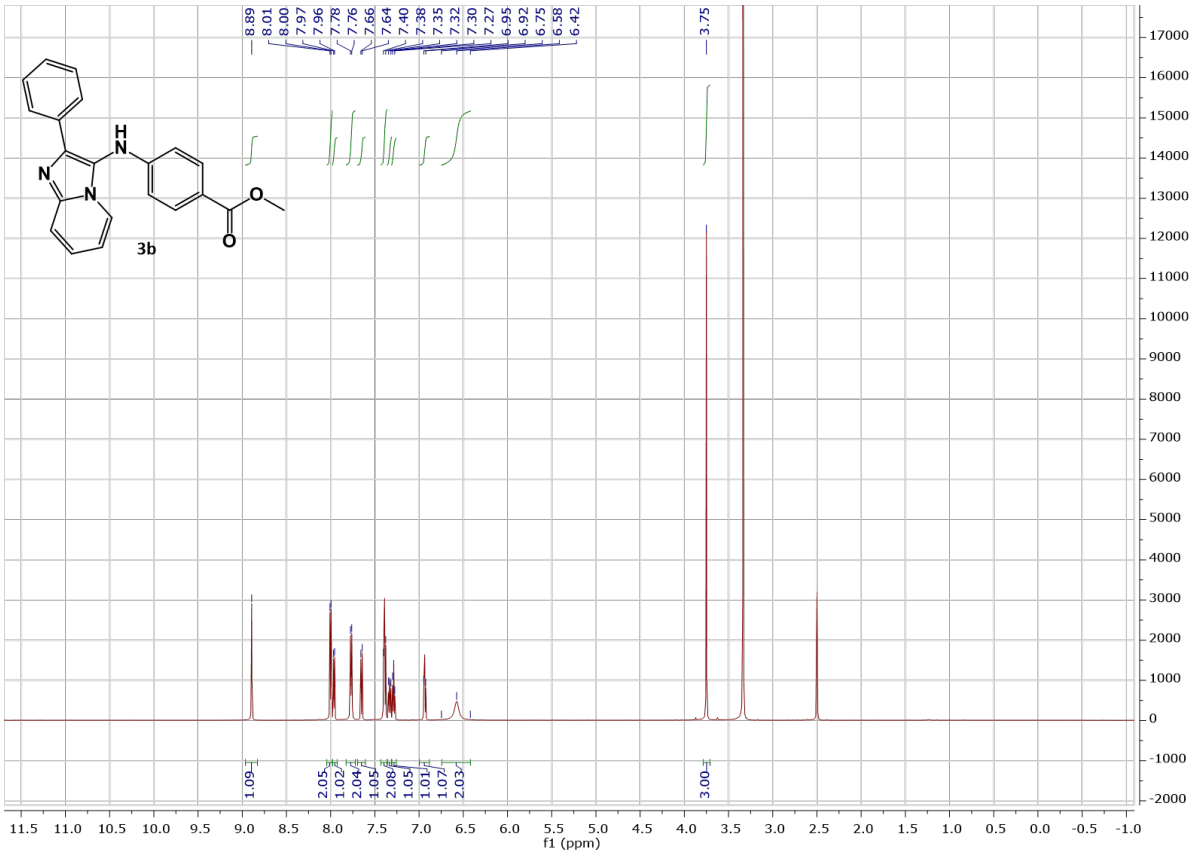
6.1 Copies of ^{1}H-NMR and ^{13}C-NMR spectra of compounds 3a-m and 4a-m

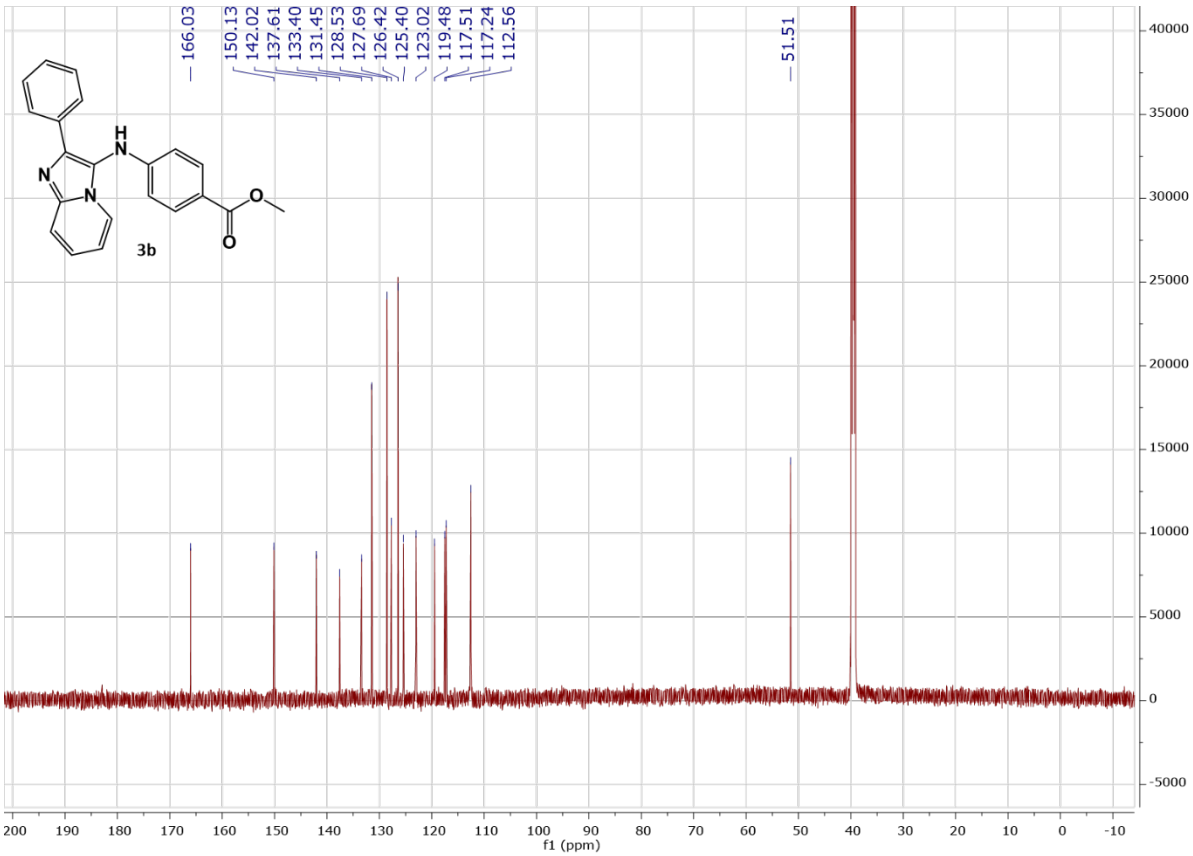
*^{1}H- (300 MHz) and ^{13}C-NMR (151 MHz) of **3a** (in DMSO-d_6):*



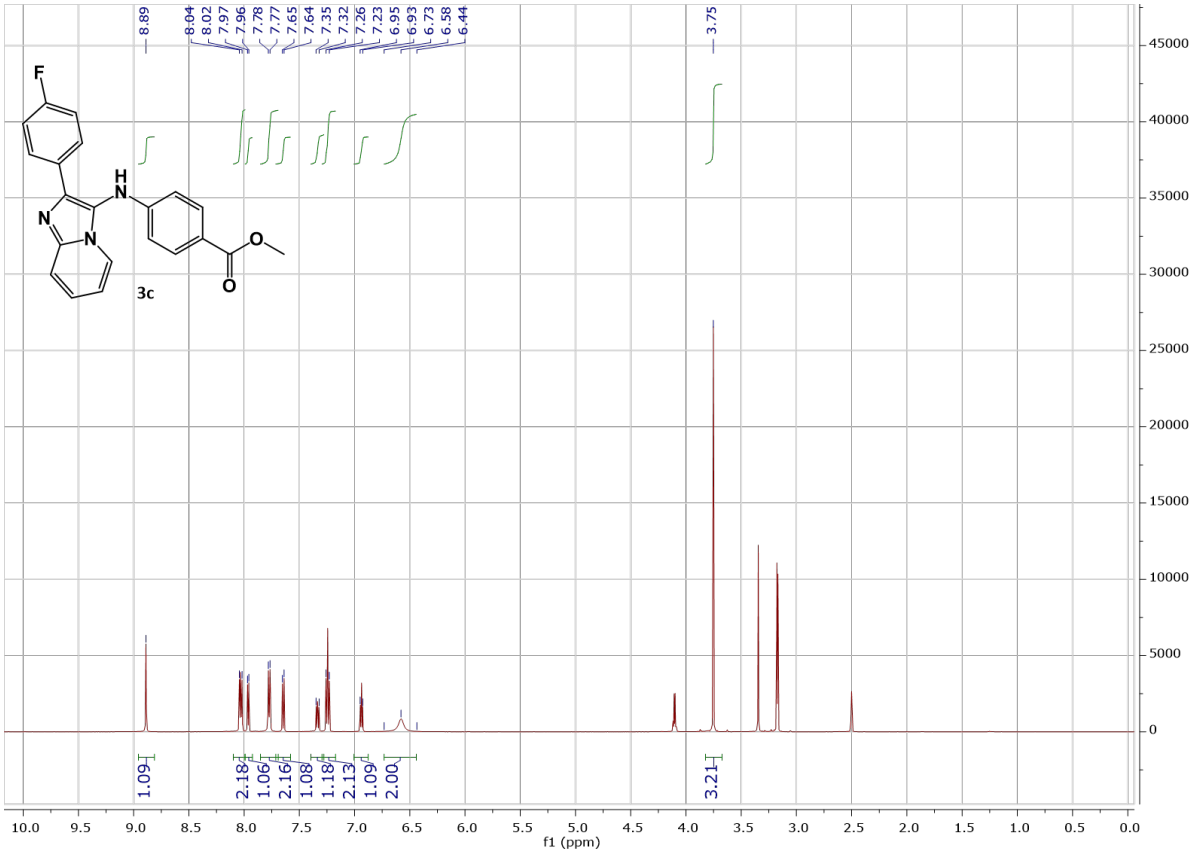


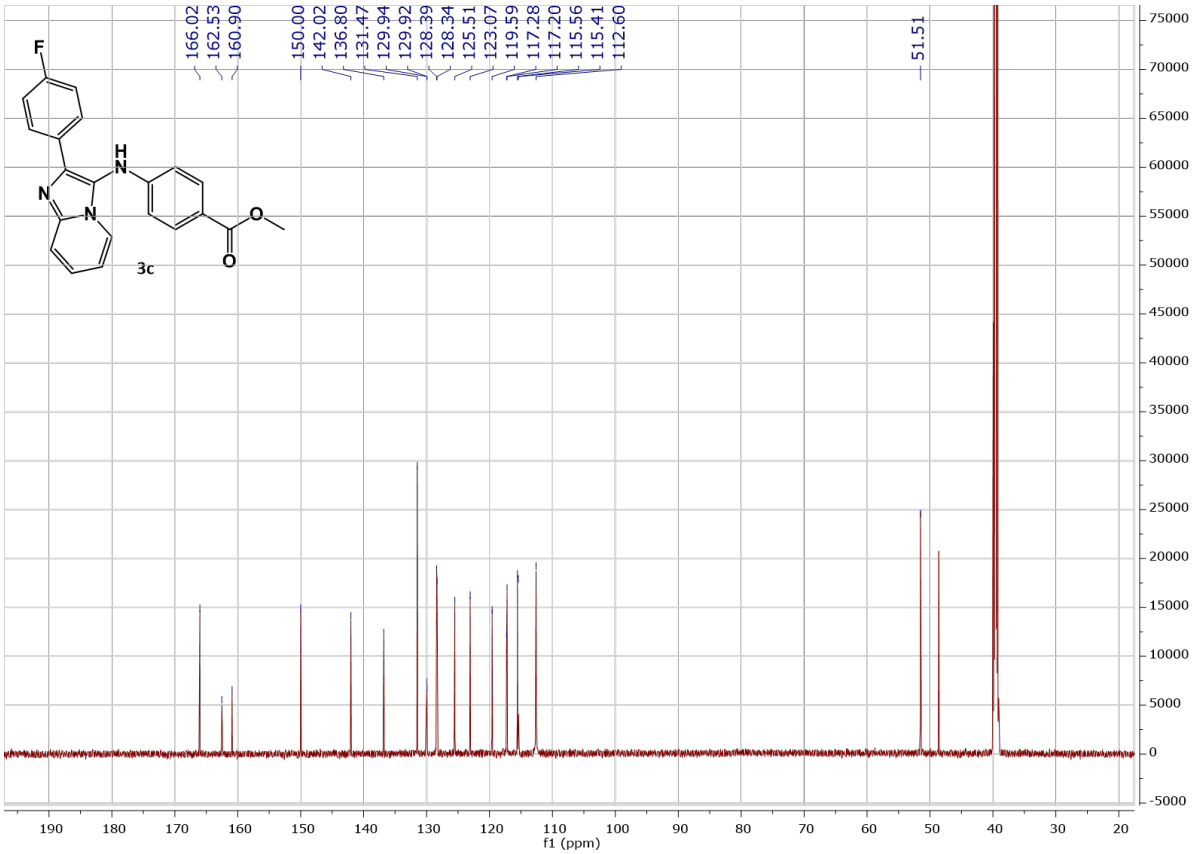
*^{1}H- (600 MHz) and ^{13}C-NMR (151 MHz) of **3b** (in DMSO-d_6):*



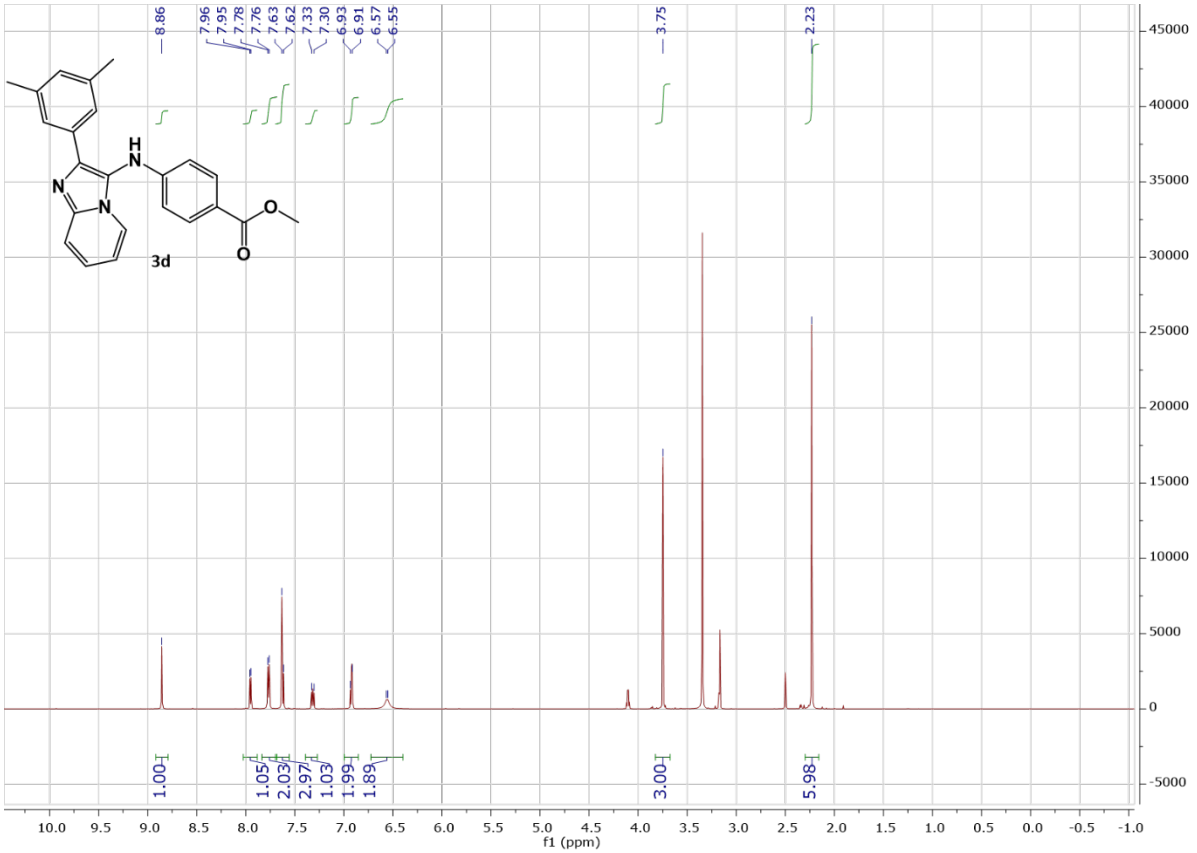


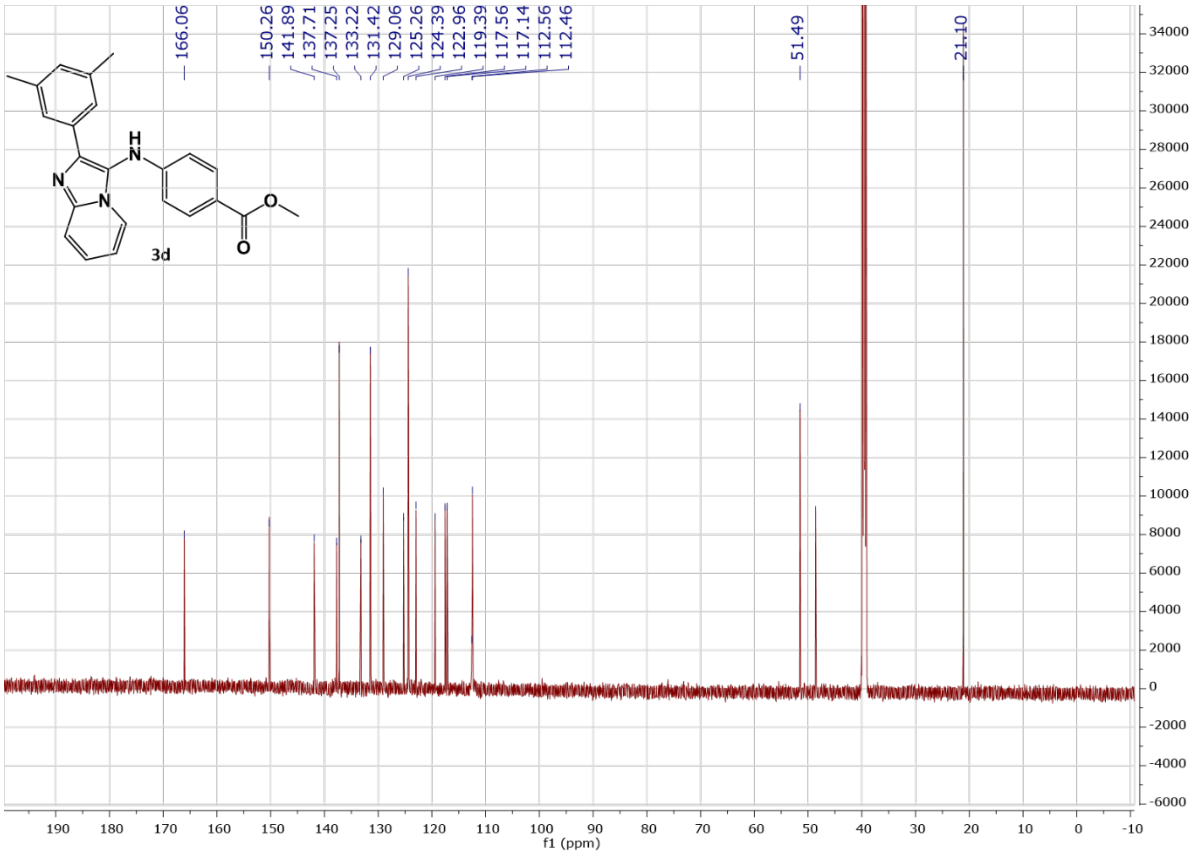
*^{1}H- (600 MHz) and ^{13}C-NMR (151 MHz) of **3c** (in DMSO-d_6):*



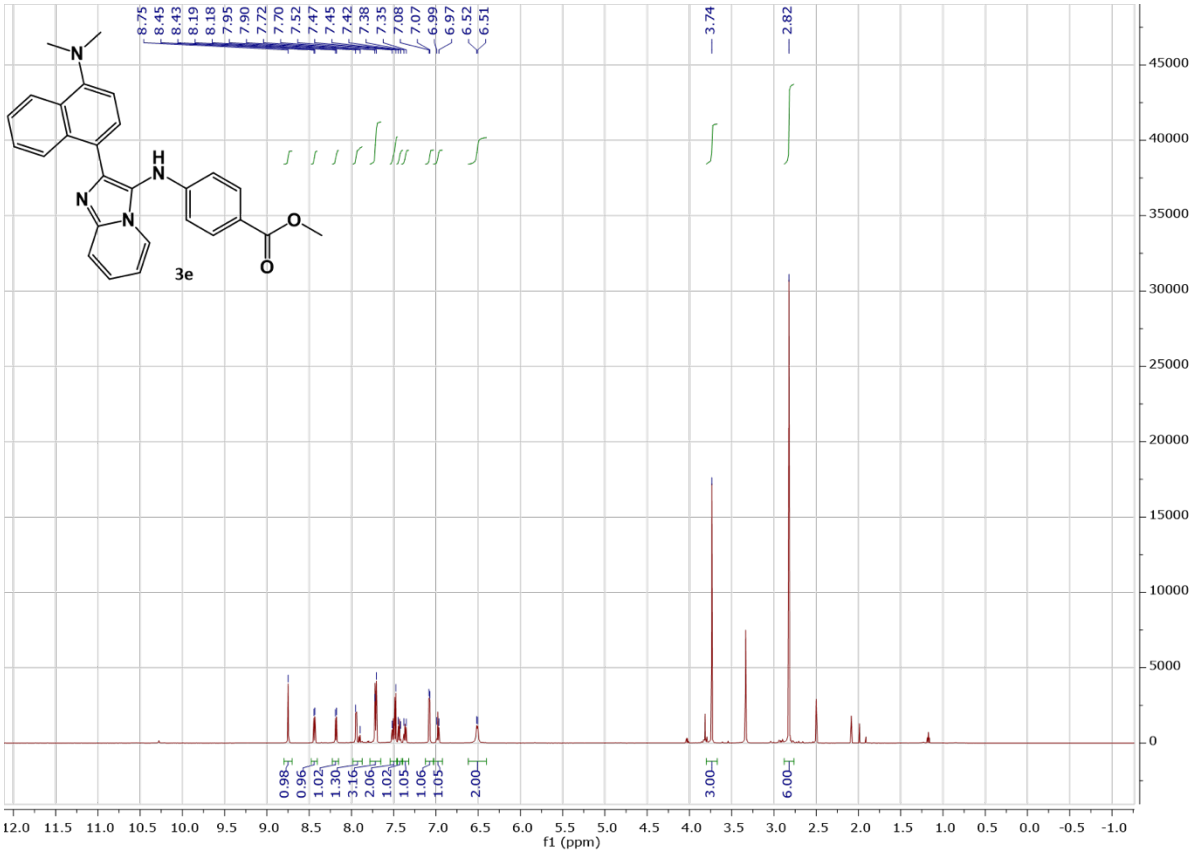


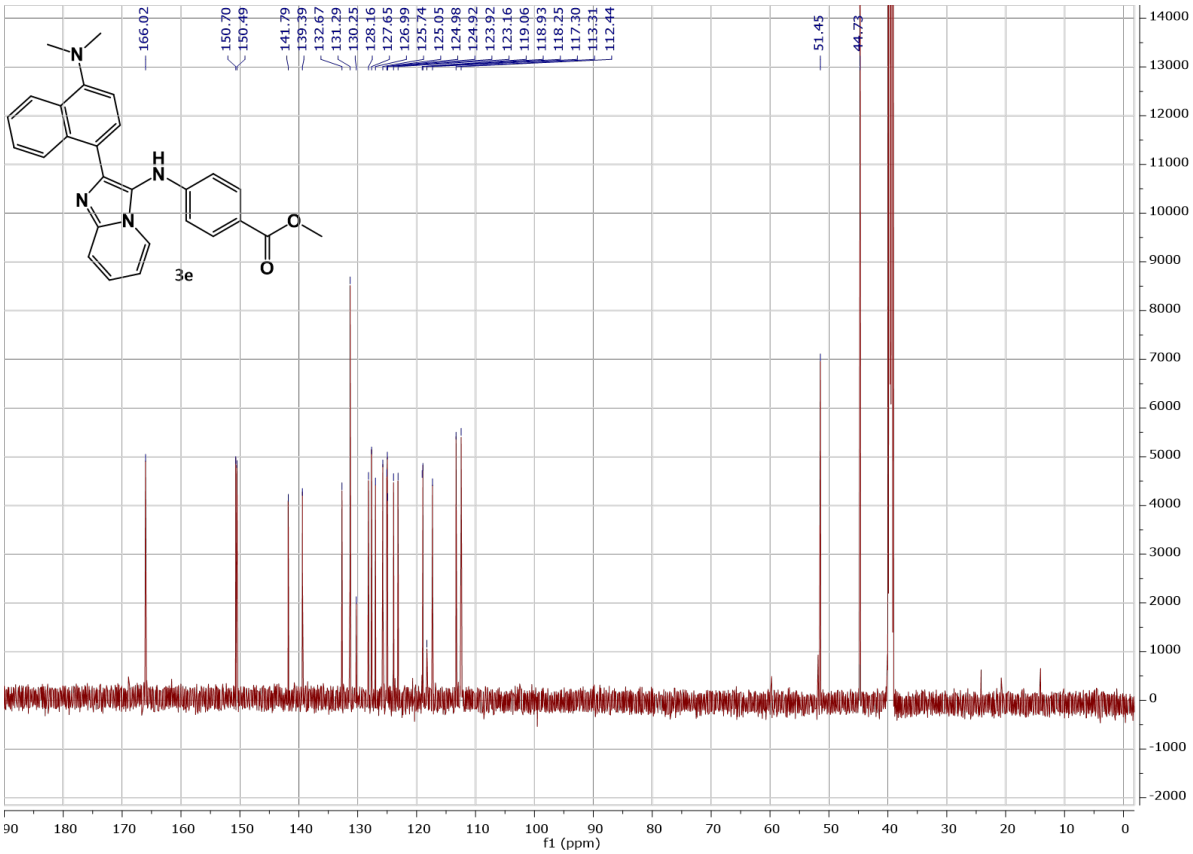
*^{1}H- (600 MHz) and ^{13}C-NMR (151 MHz) of **3d** (in DMSO-d_6):*



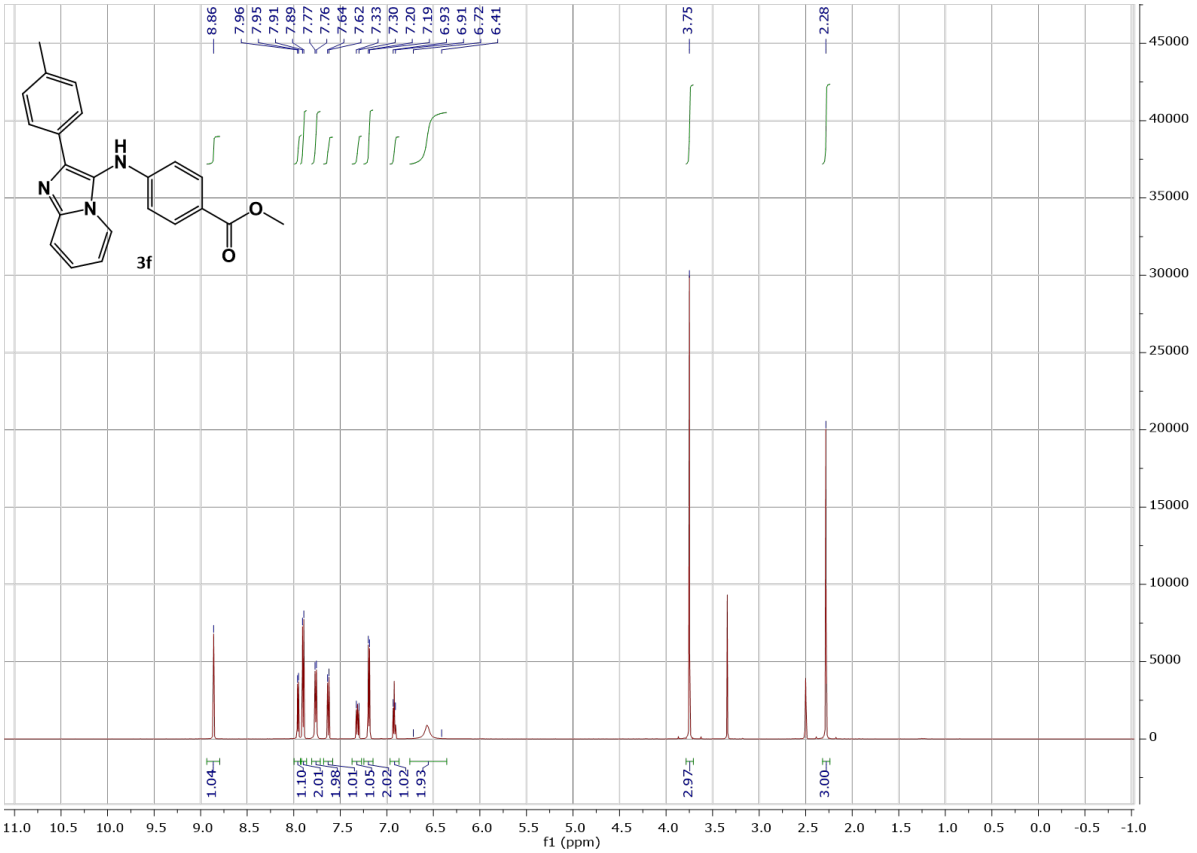


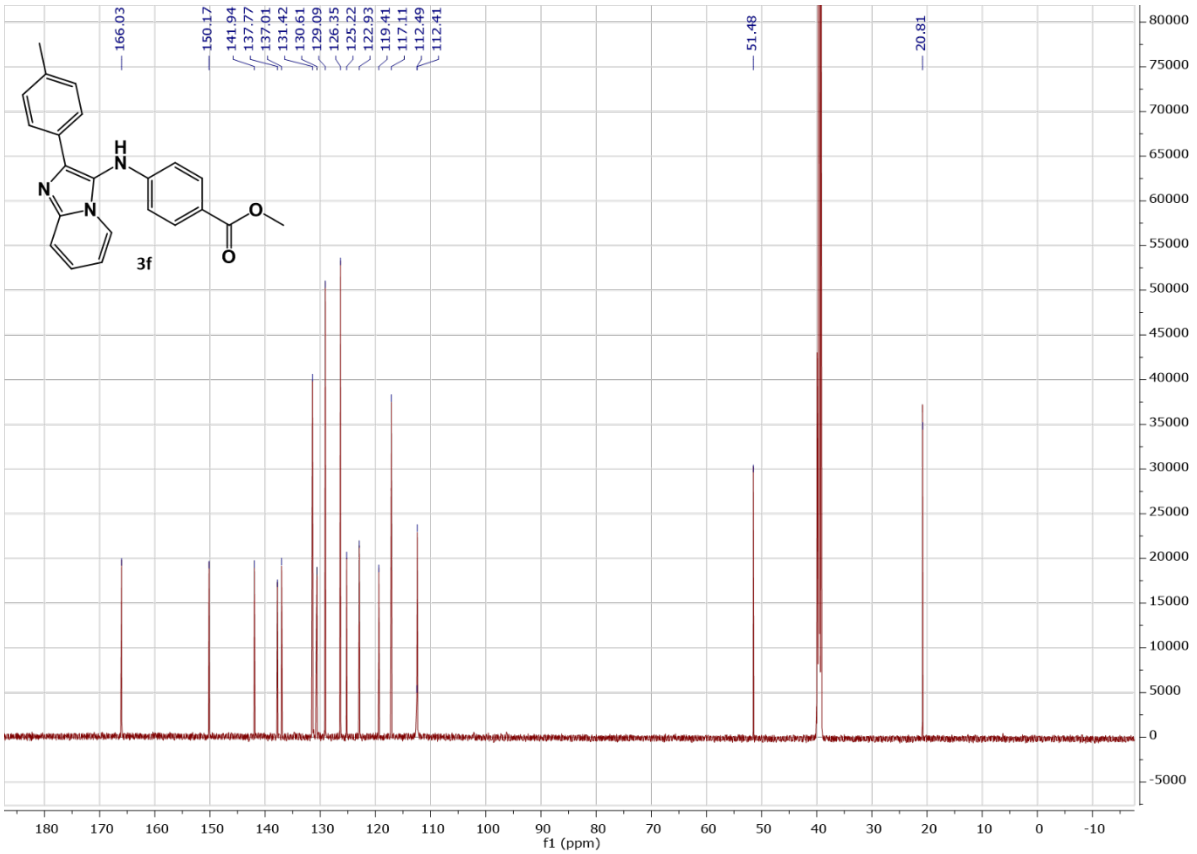
*^{1}H- (600 MHz) and ^{13}C-NMR (151 MHz) of **3e** (in DMSO-d_6):*



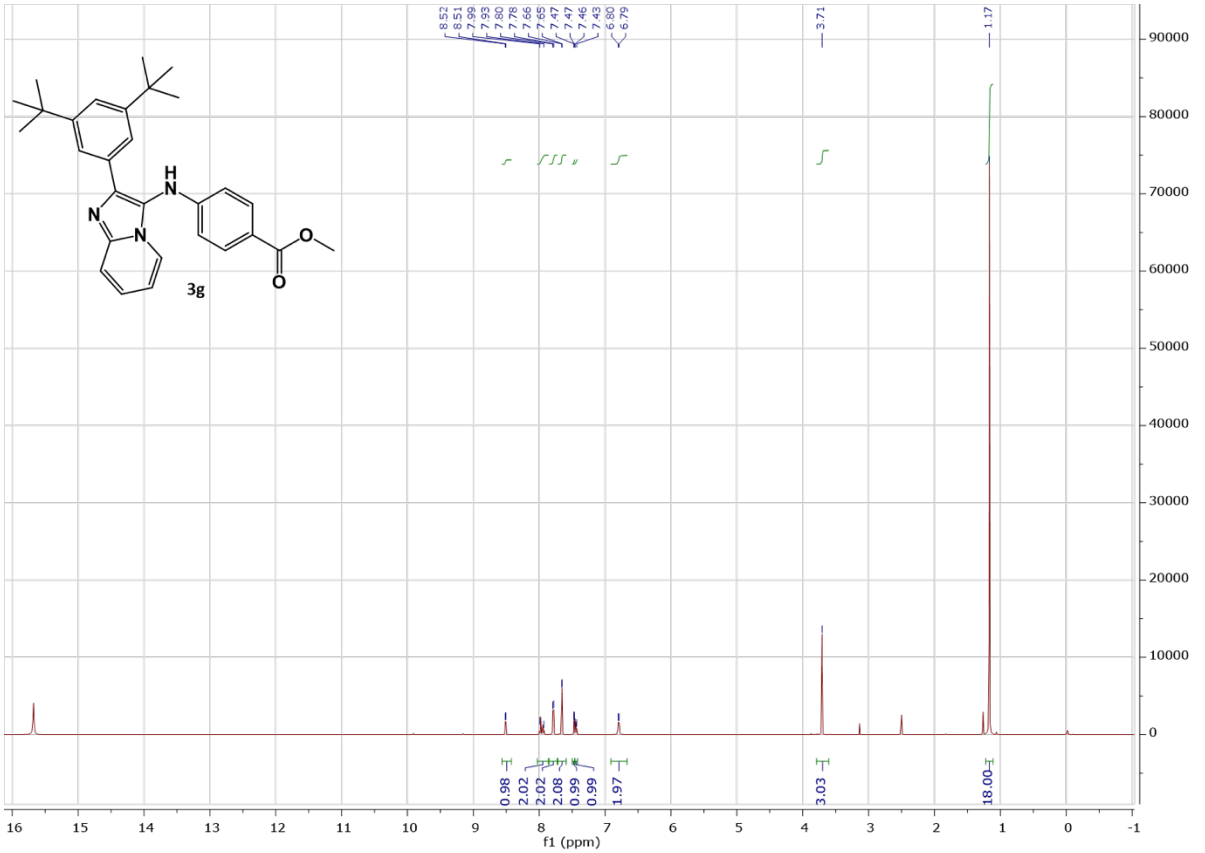


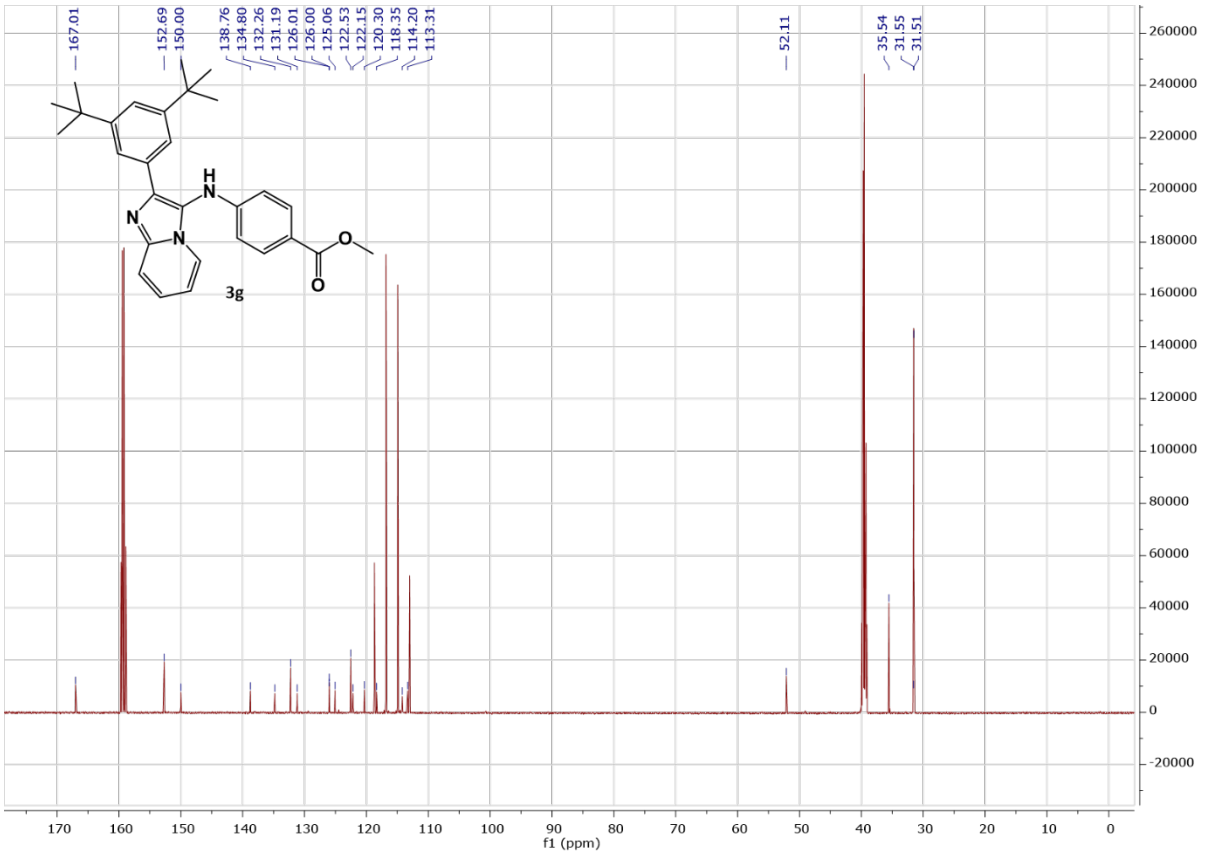
*^{1}H- (600 MHz) and ^{13}C-NMR (151 MHz) of **3f** (in DMSO-d_6):*



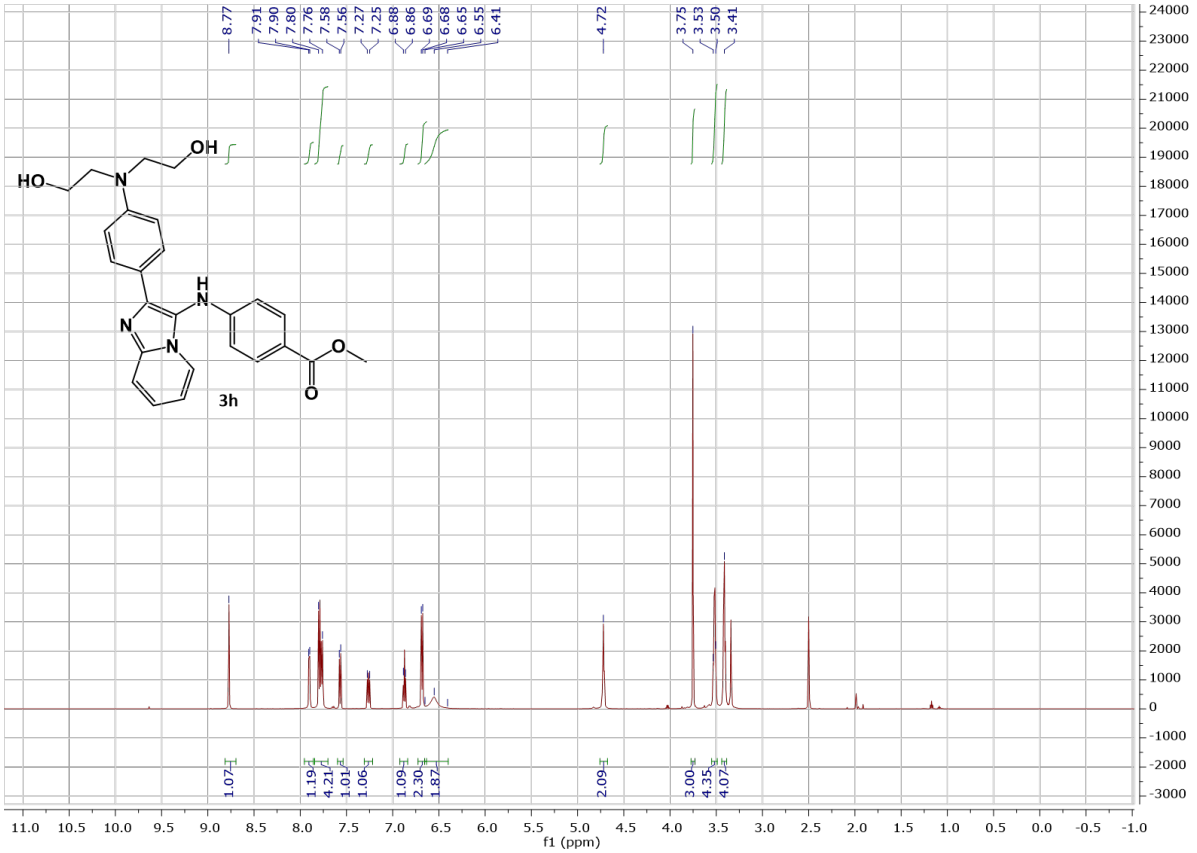


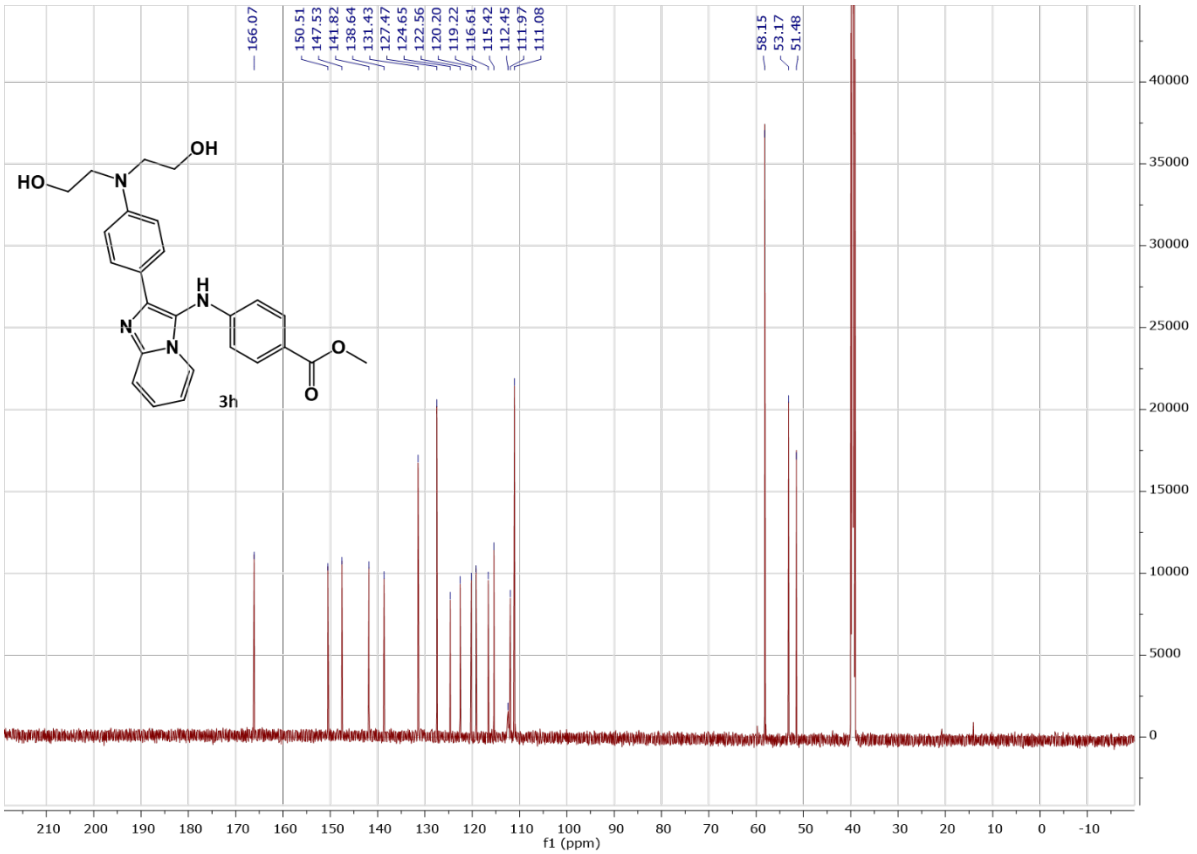
*^{1}H- (600 MHz) and ^{13}C-NMR (151 MHz) of **3g** (in DMSO-d_6/ TFA-d (6/1, v/v)):*



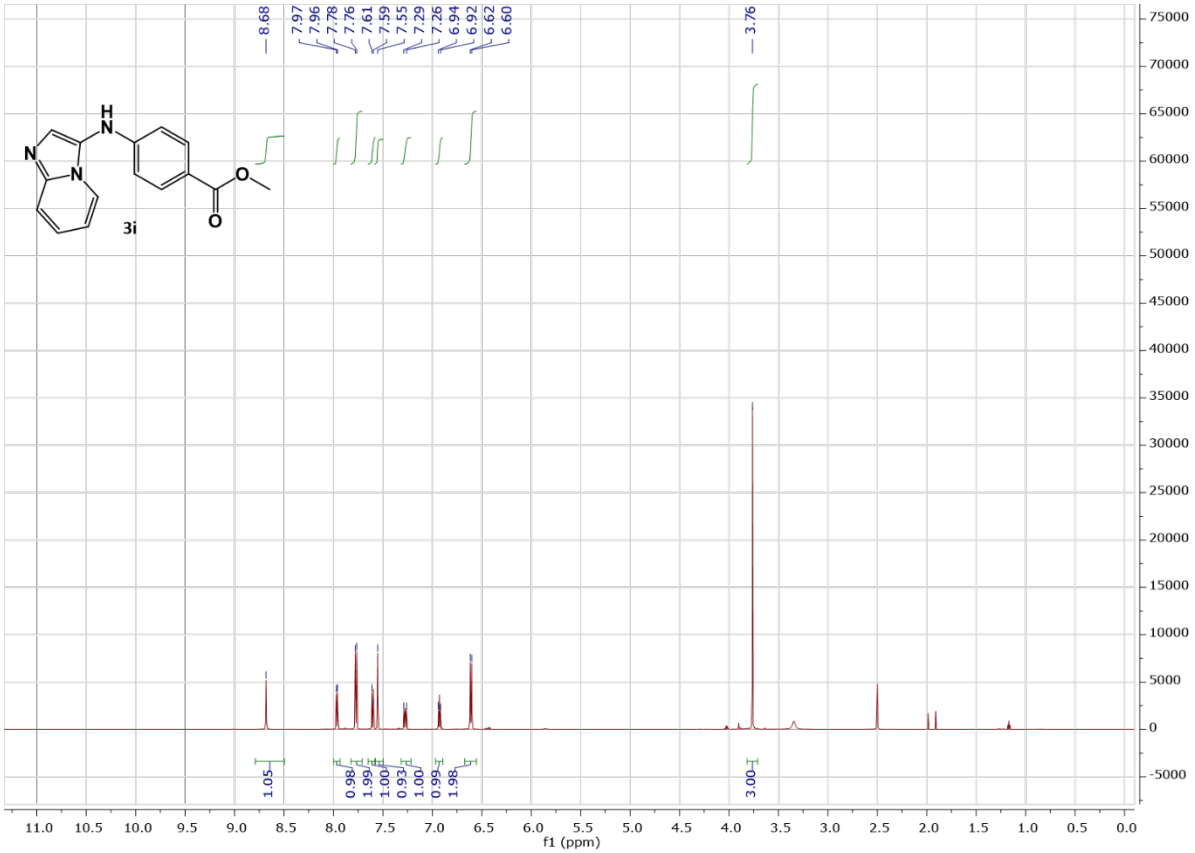


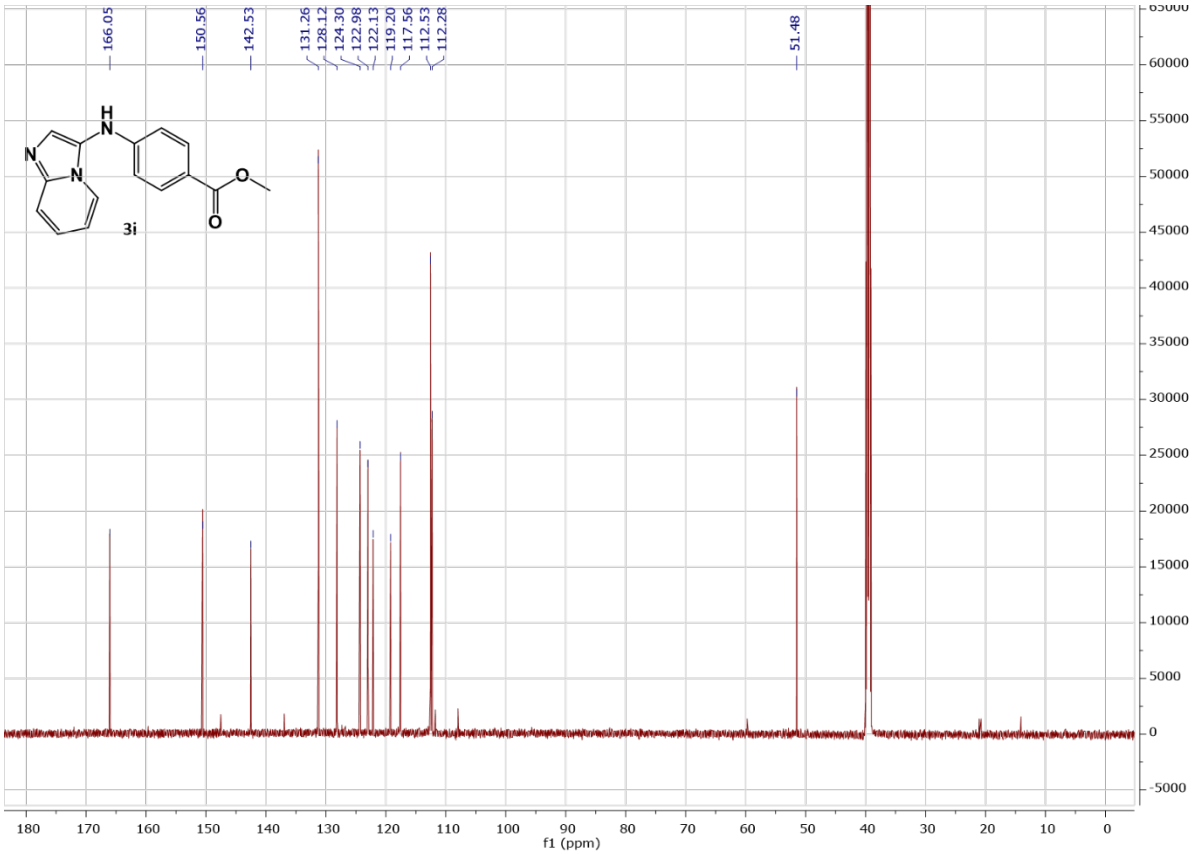
*^{1}H- (600 MHz) and ^{13}C-NMR (151 MHz) of **3h** (in DMSO-d_6):*



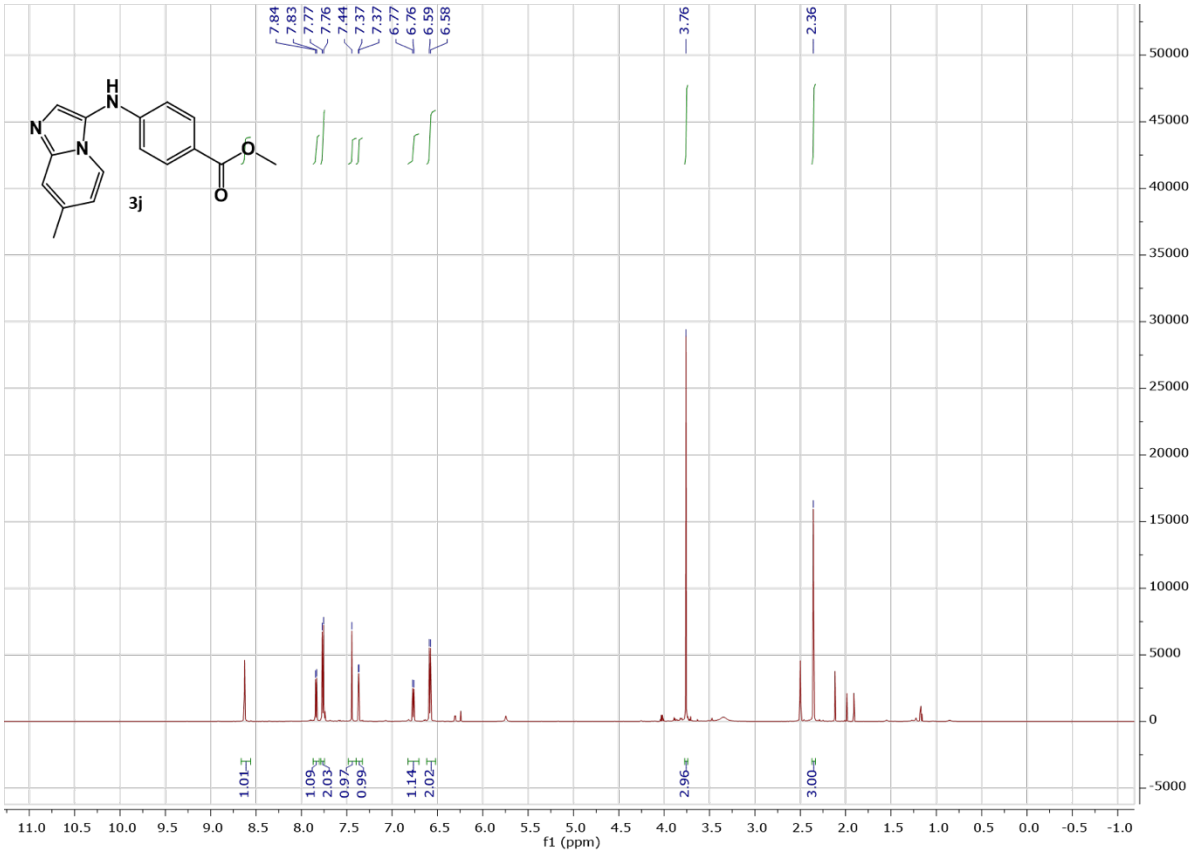


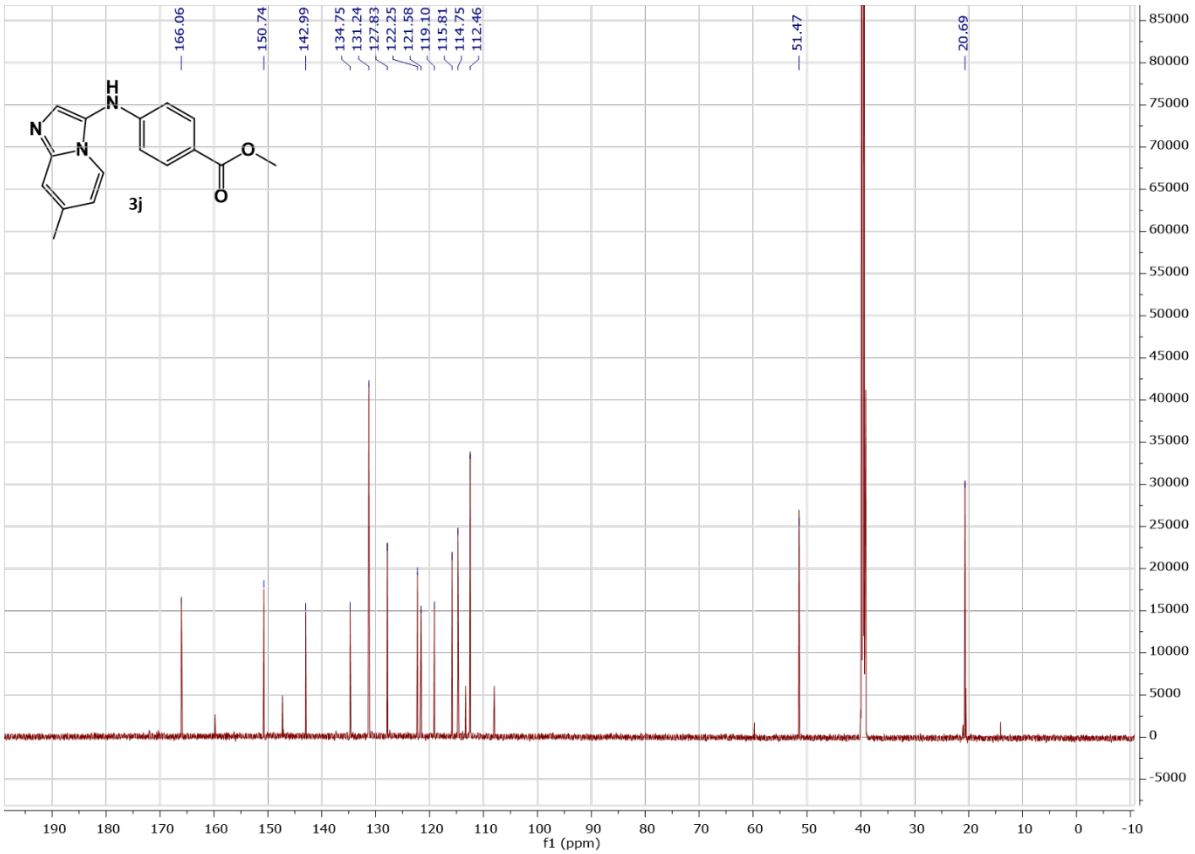
*^{1}H- (600 MHz) and ^{13}C-NMR (151 MHz) of **3i** (in DMSO-d_6):*



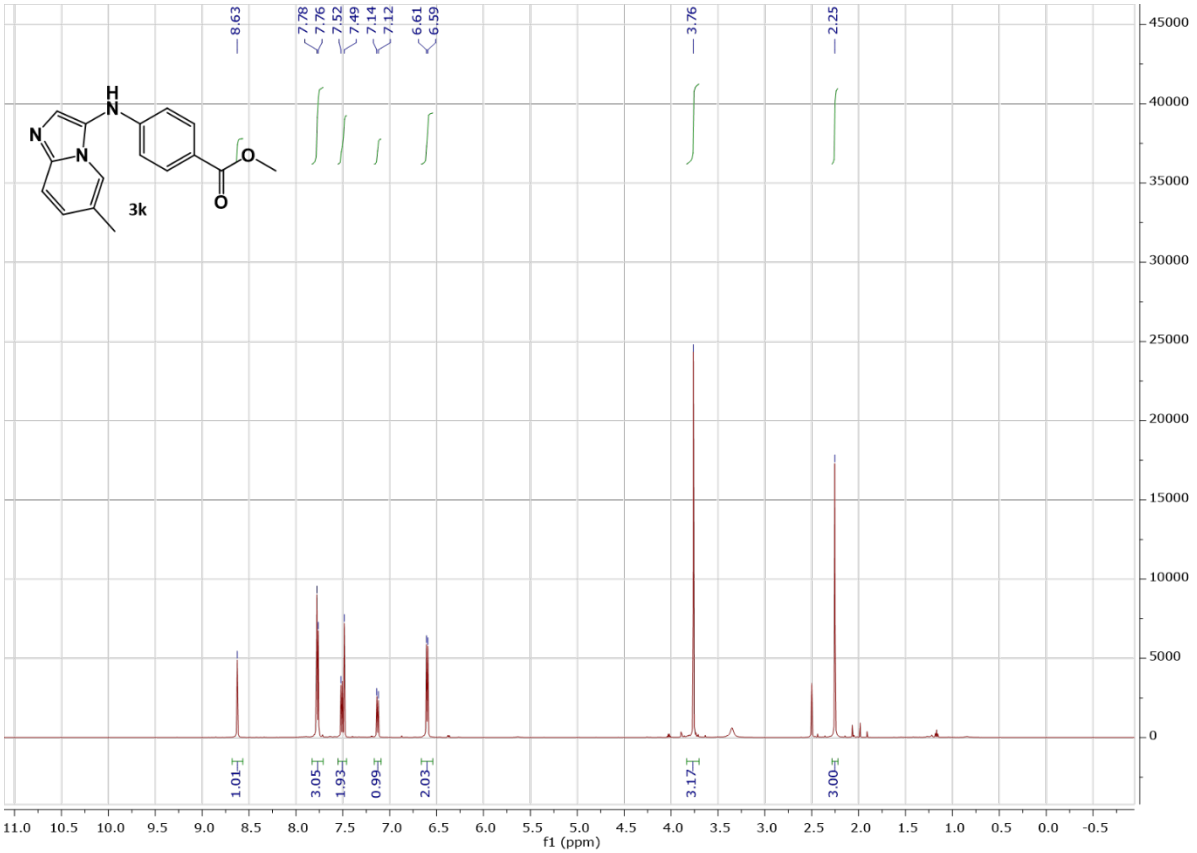


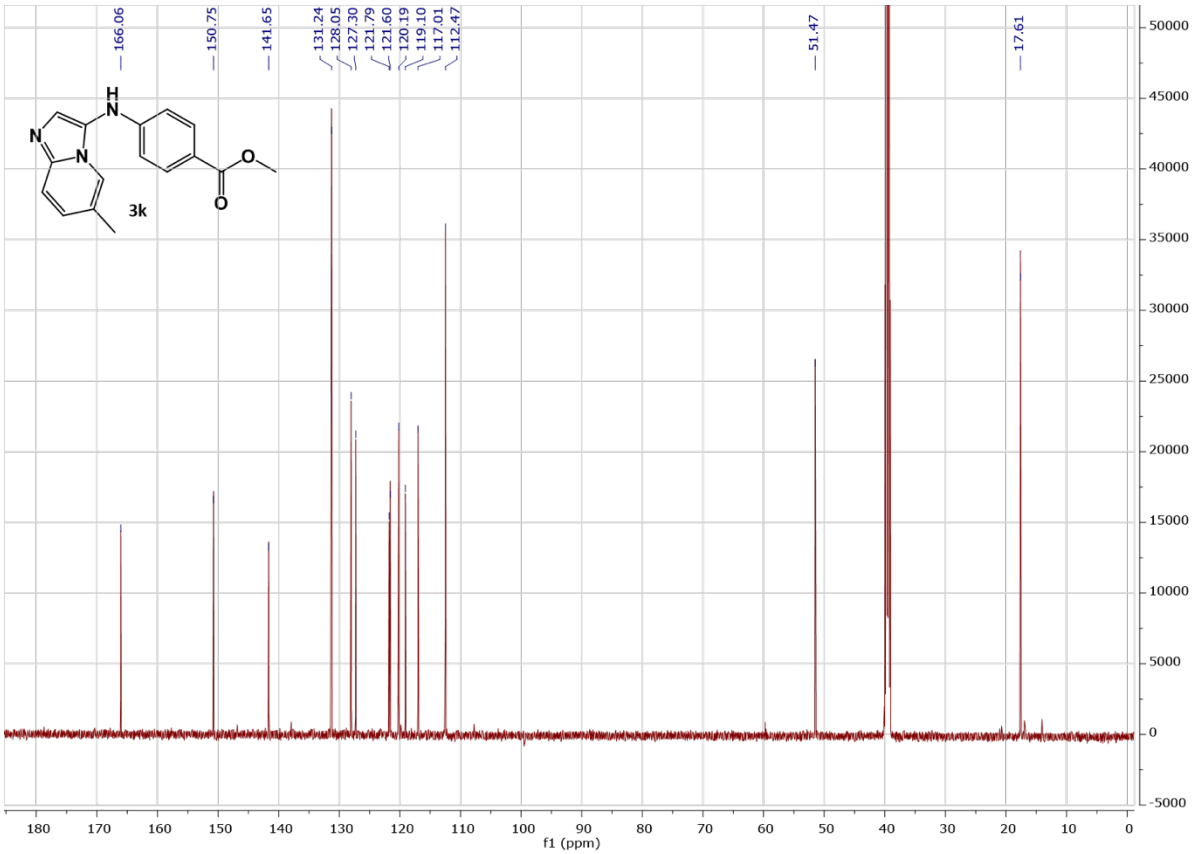
*^{1}H- (600 MHz) and ^{13}C-NMR (151 MHz) of **3j** (in DMSO-d_6):*



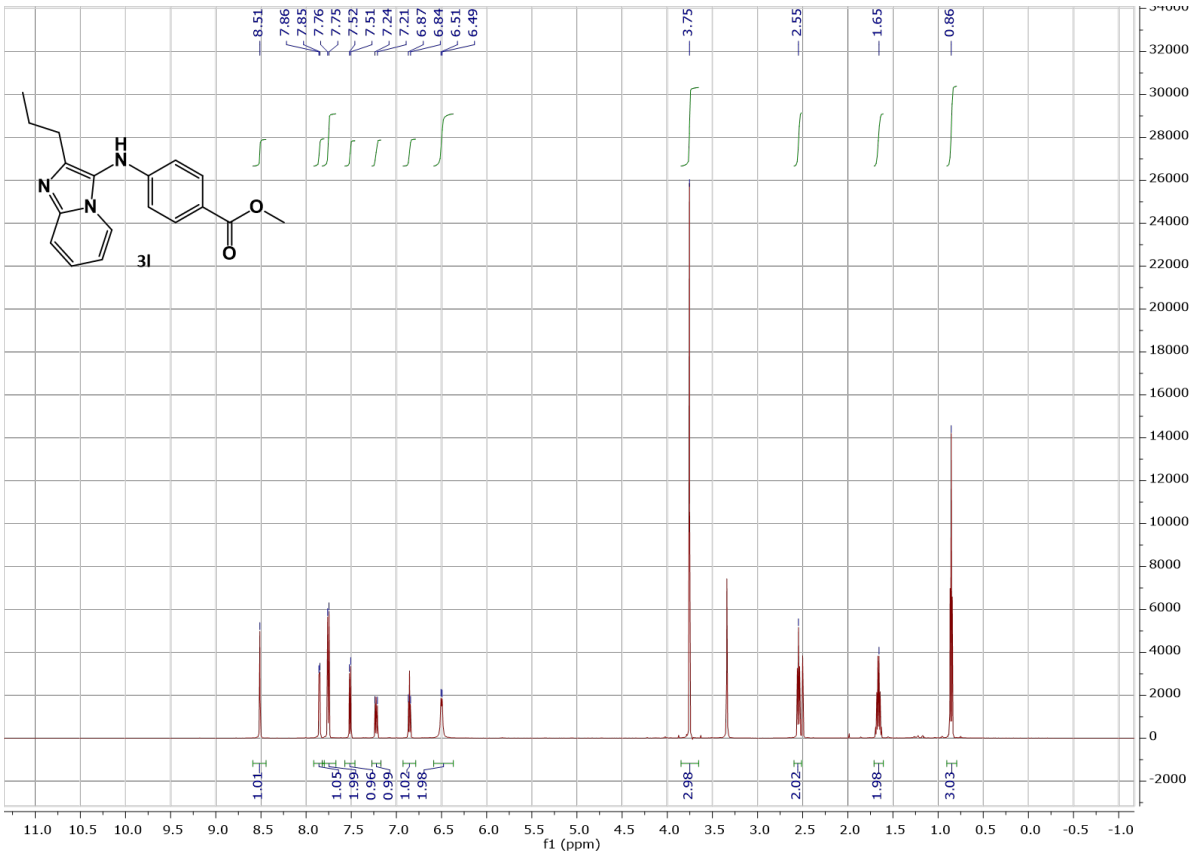


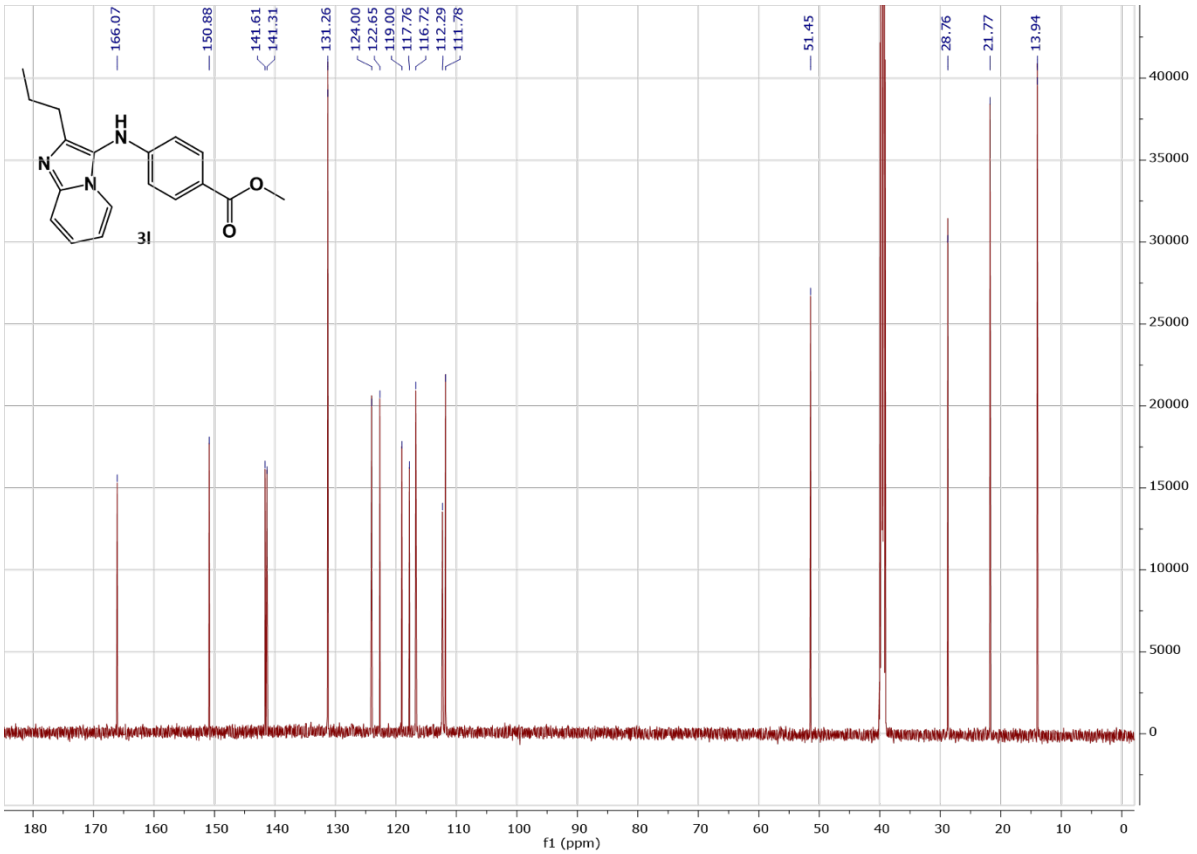
*^{1}H- (600 MHz) and ^{13}C-NMR (151 MHz) of **3k** (in DMSO-d_6):*



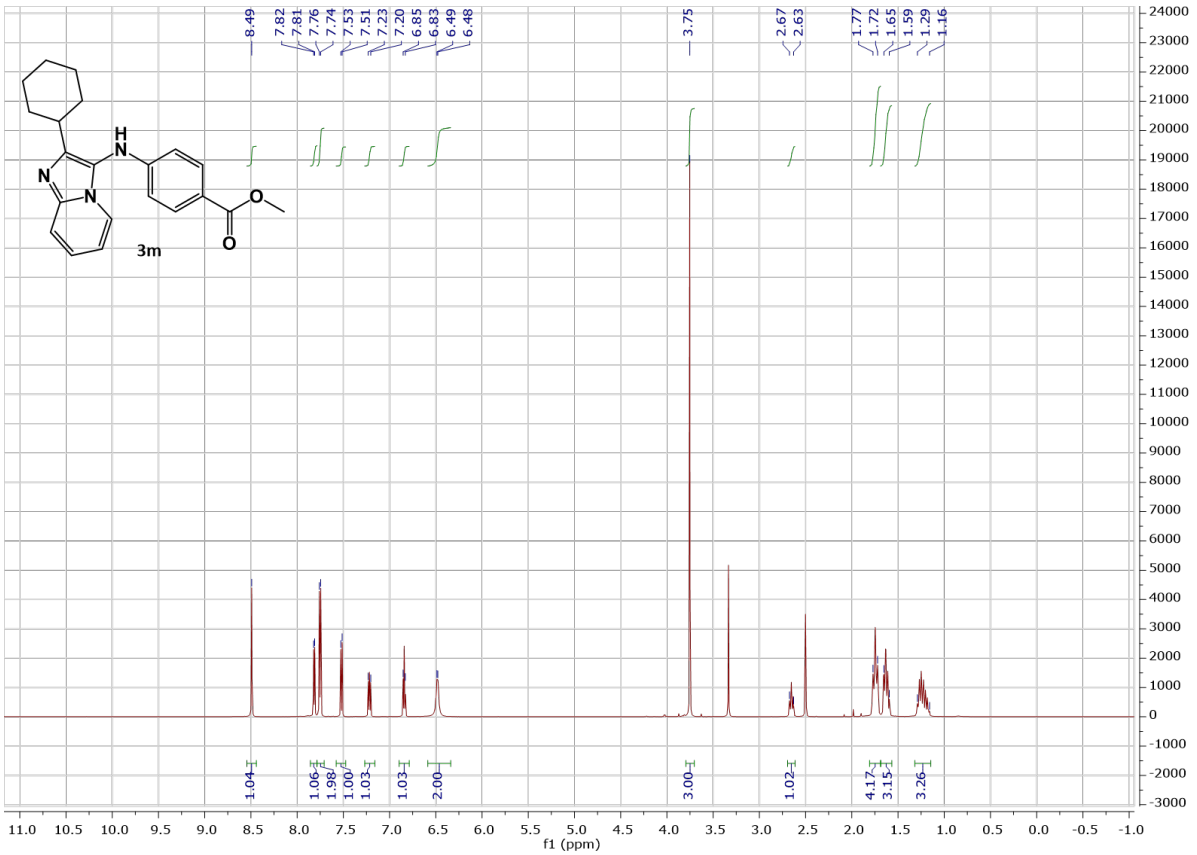


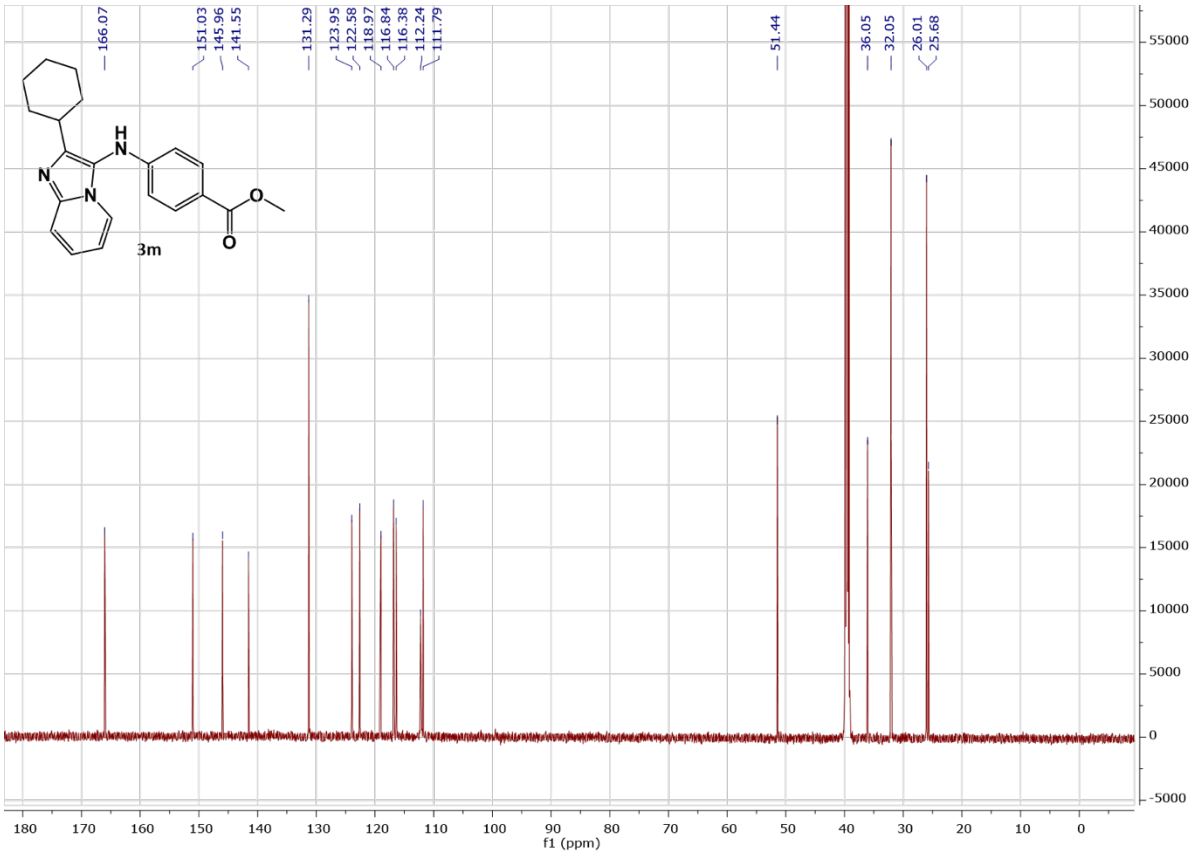
*^{1}H- (600 MHz) and ^{13}C-NMR (151 MHz) of **3l** (in DMSO-d_6):*



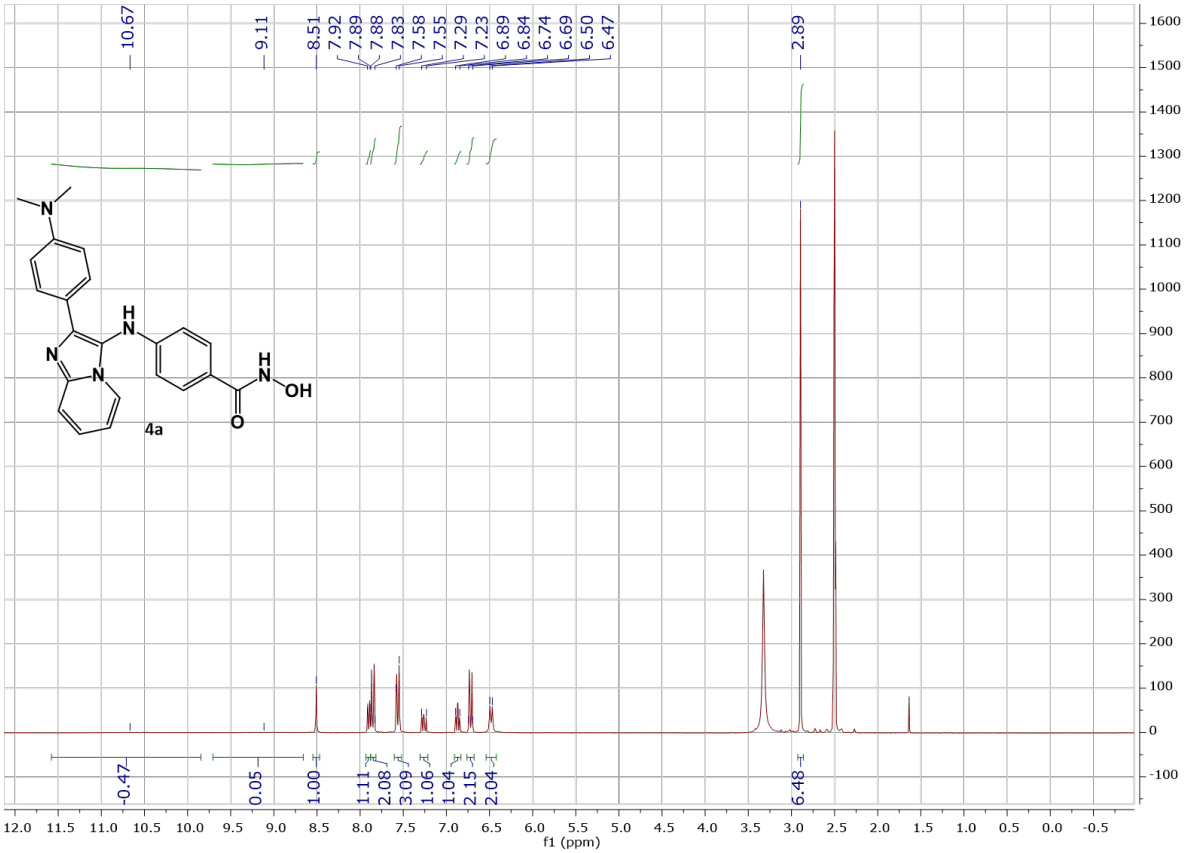


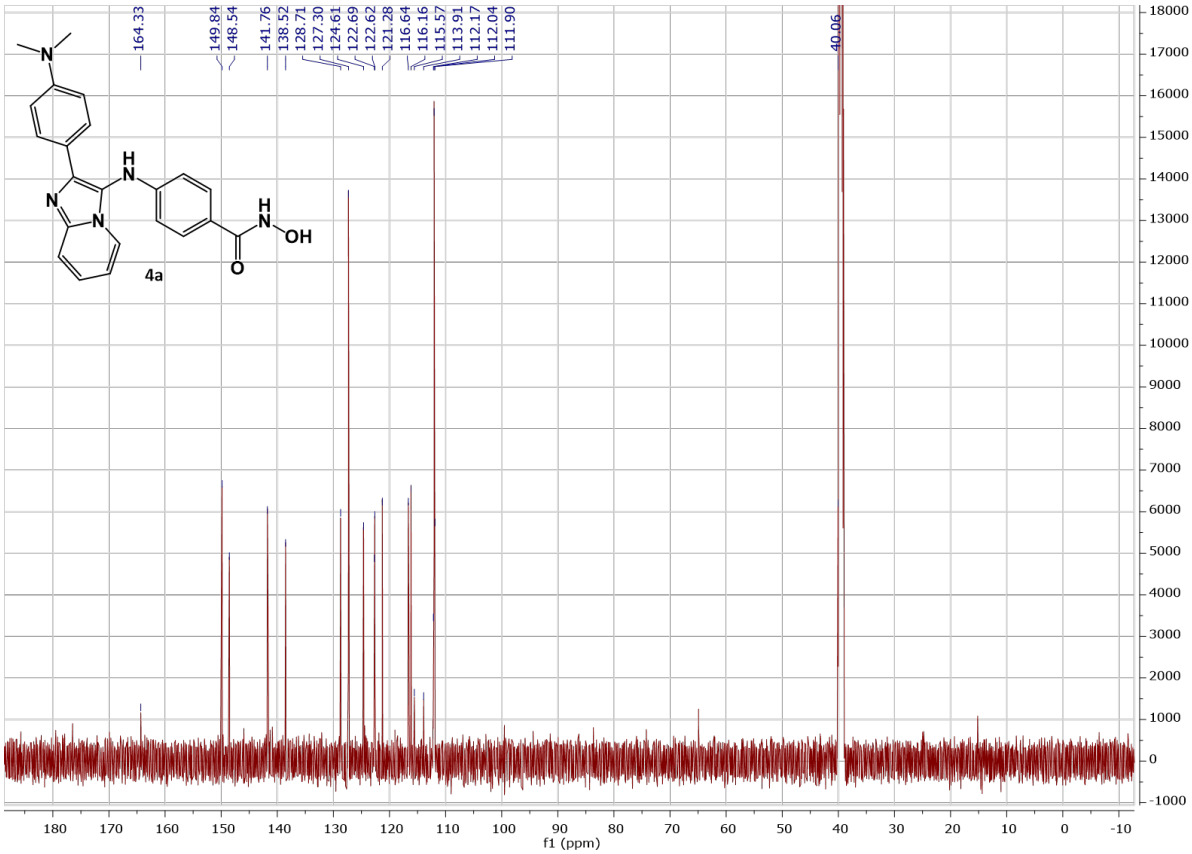
*^{1}H- (600 MHz) and ^{13}C-NMR (151 MHz) of **3m** (in DMSO-d_6):*



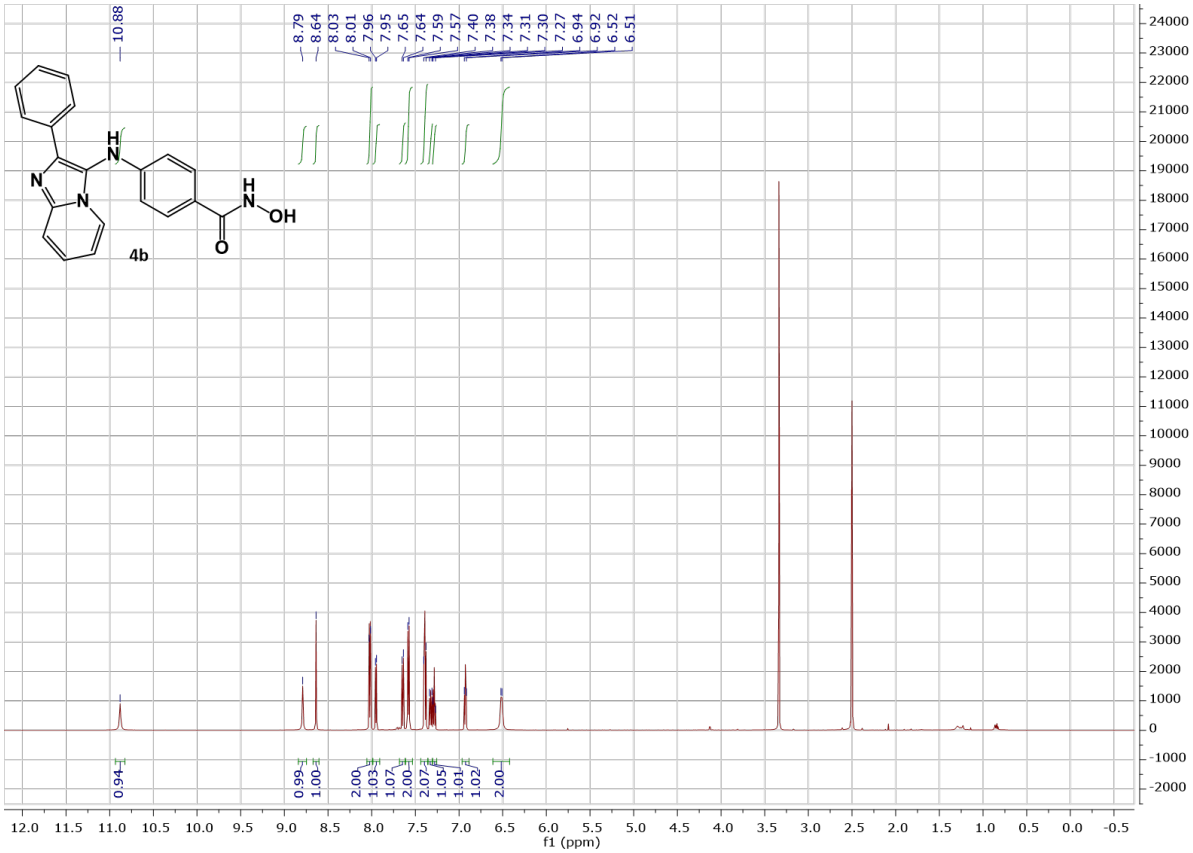


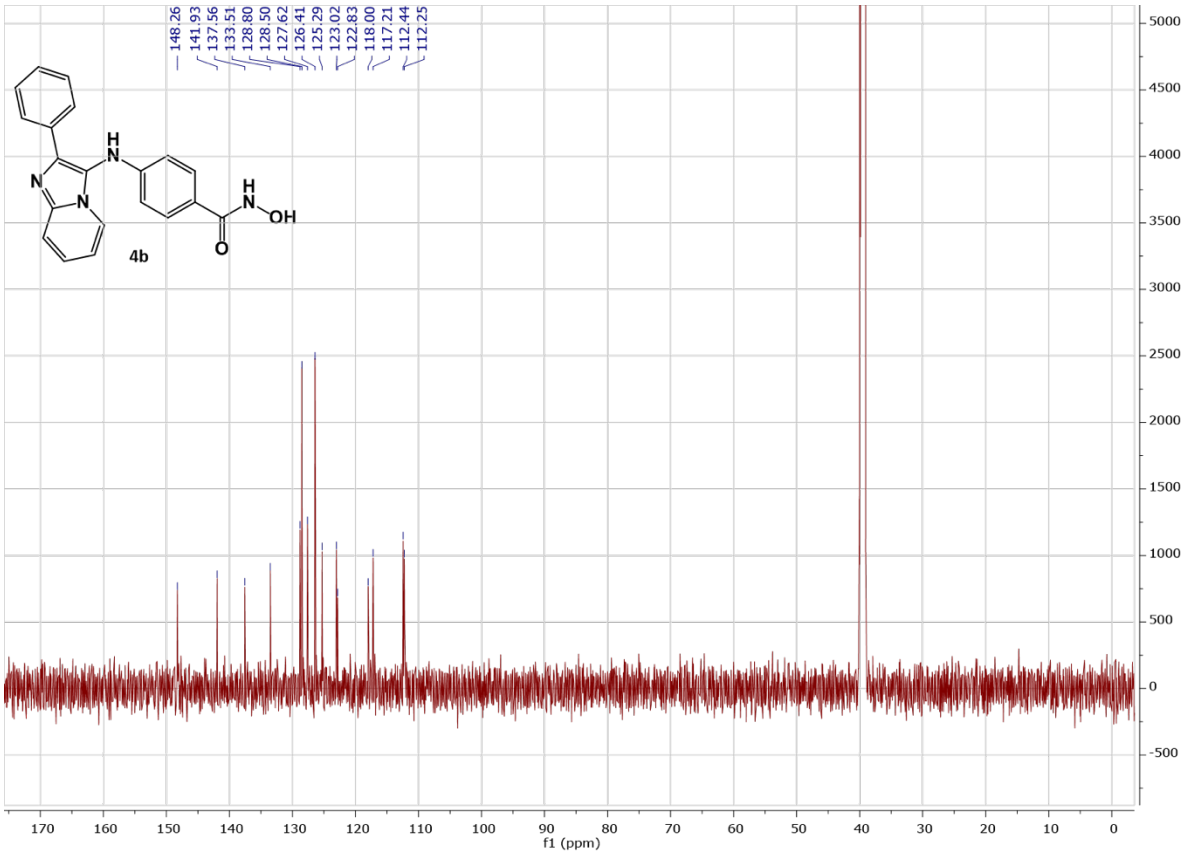
*^{1}H- (300 MHz) and ^{13}C-NMR (151 MHz) of **4a** (in DMSO-d_6):*



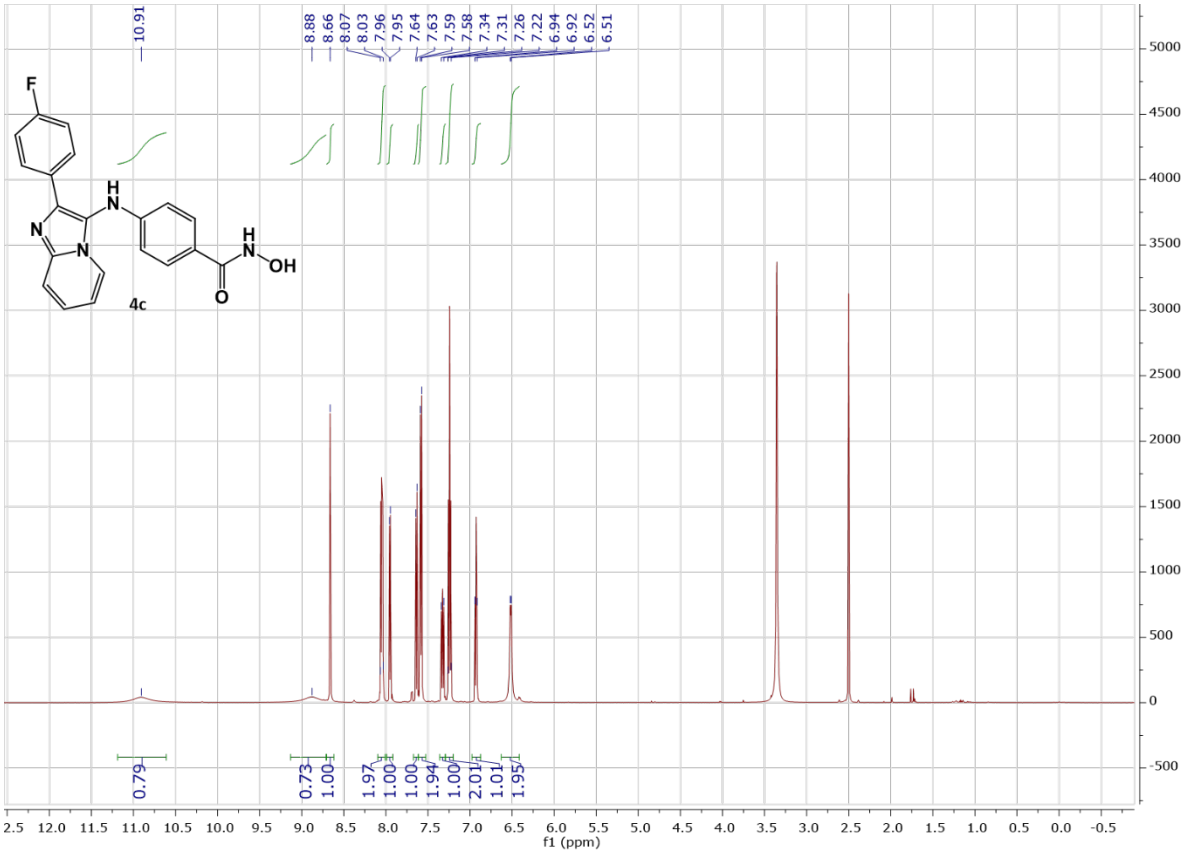


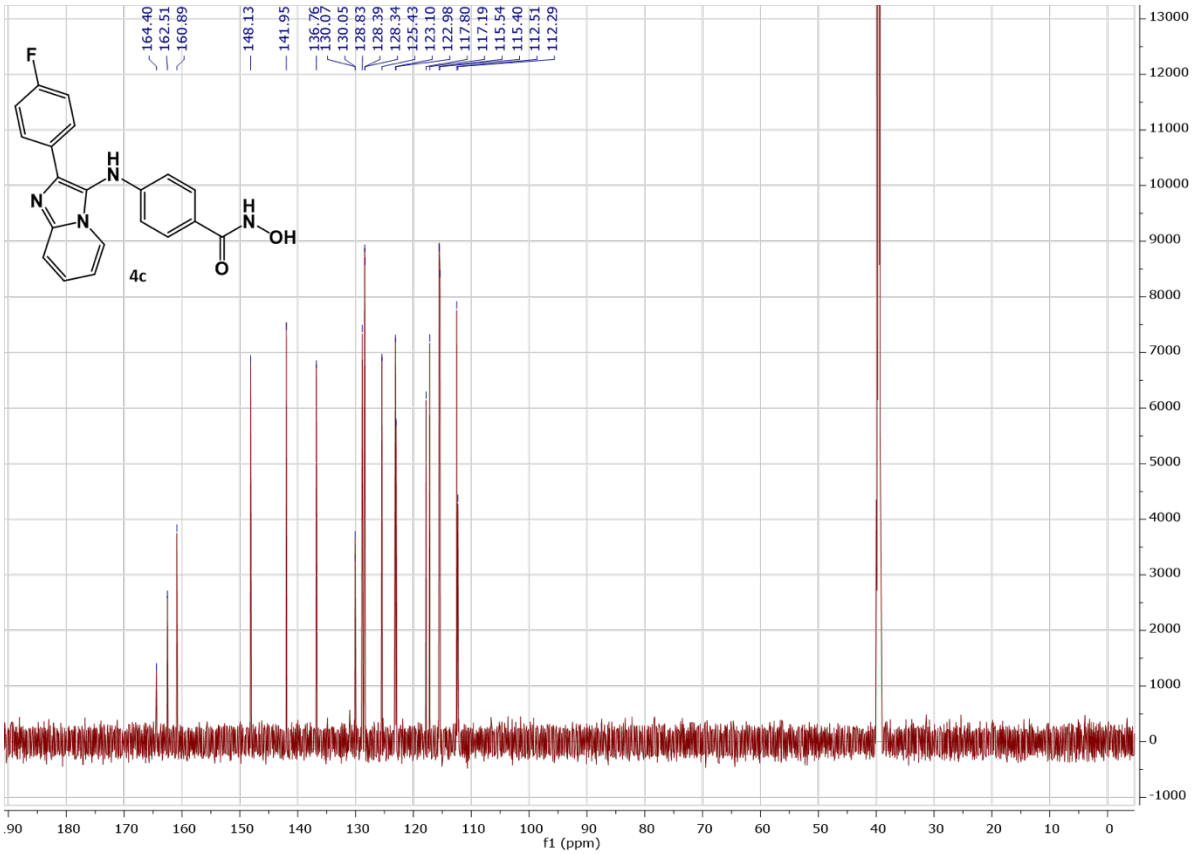
*^{1}H- (600 MHz) and ^{13}C-NMR (151 MHz) of **4b** (in DMSO-d_6):*



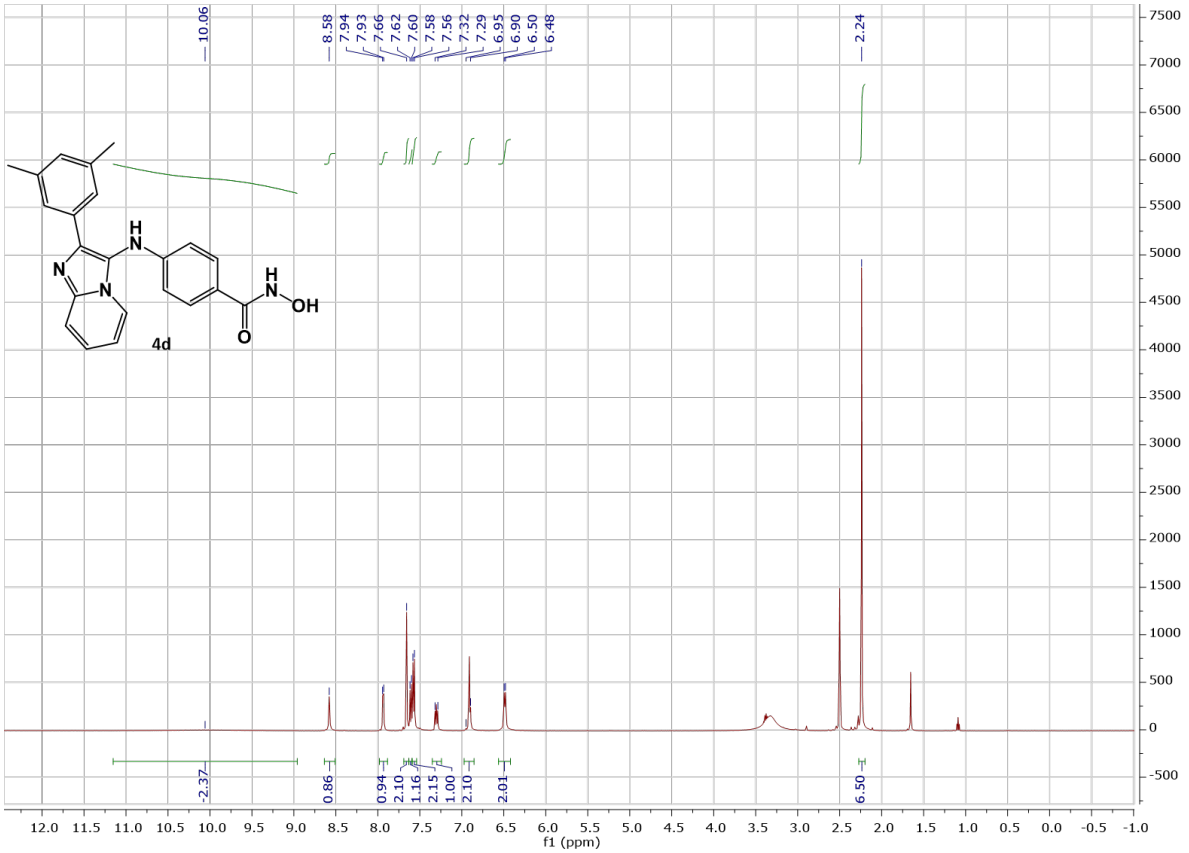


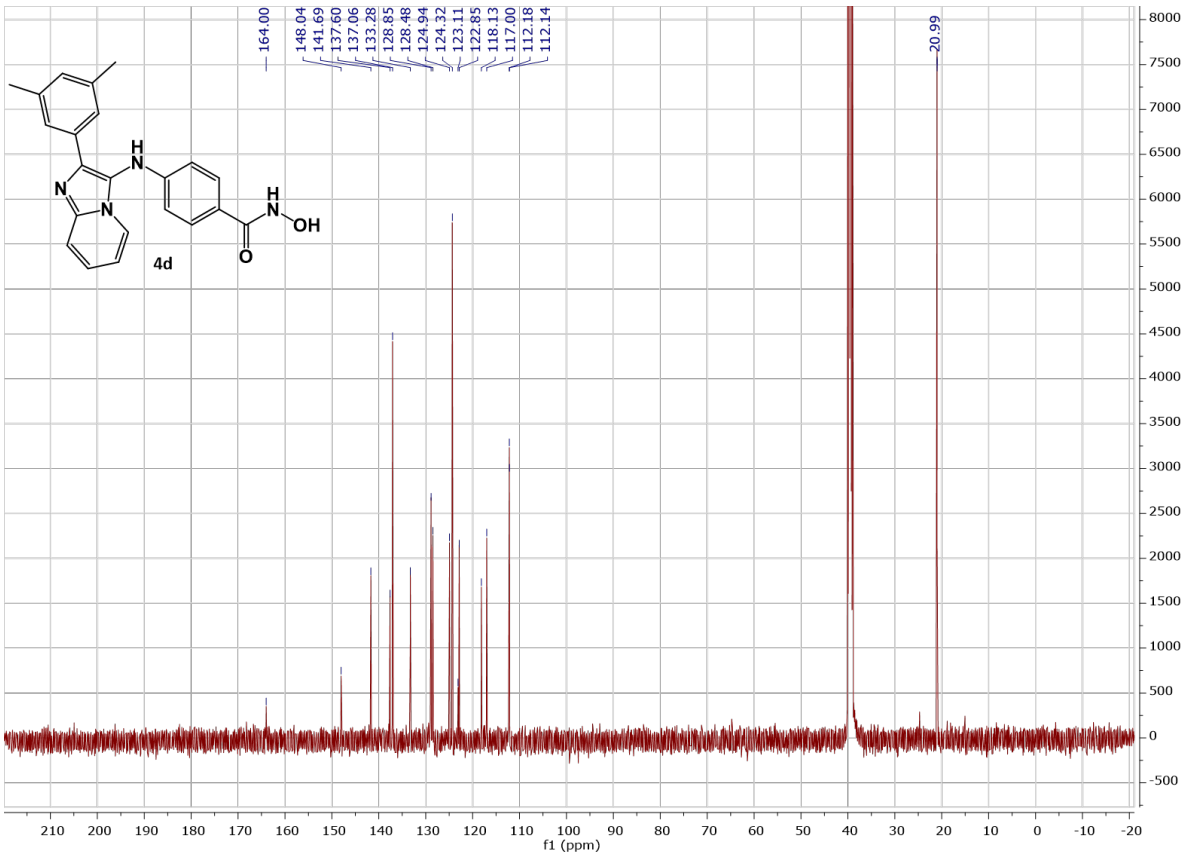
*^{1}H- (600 MHz) and ^{13}C-NMR (151 MHz) of **4c** (in DMSO-d_6):*



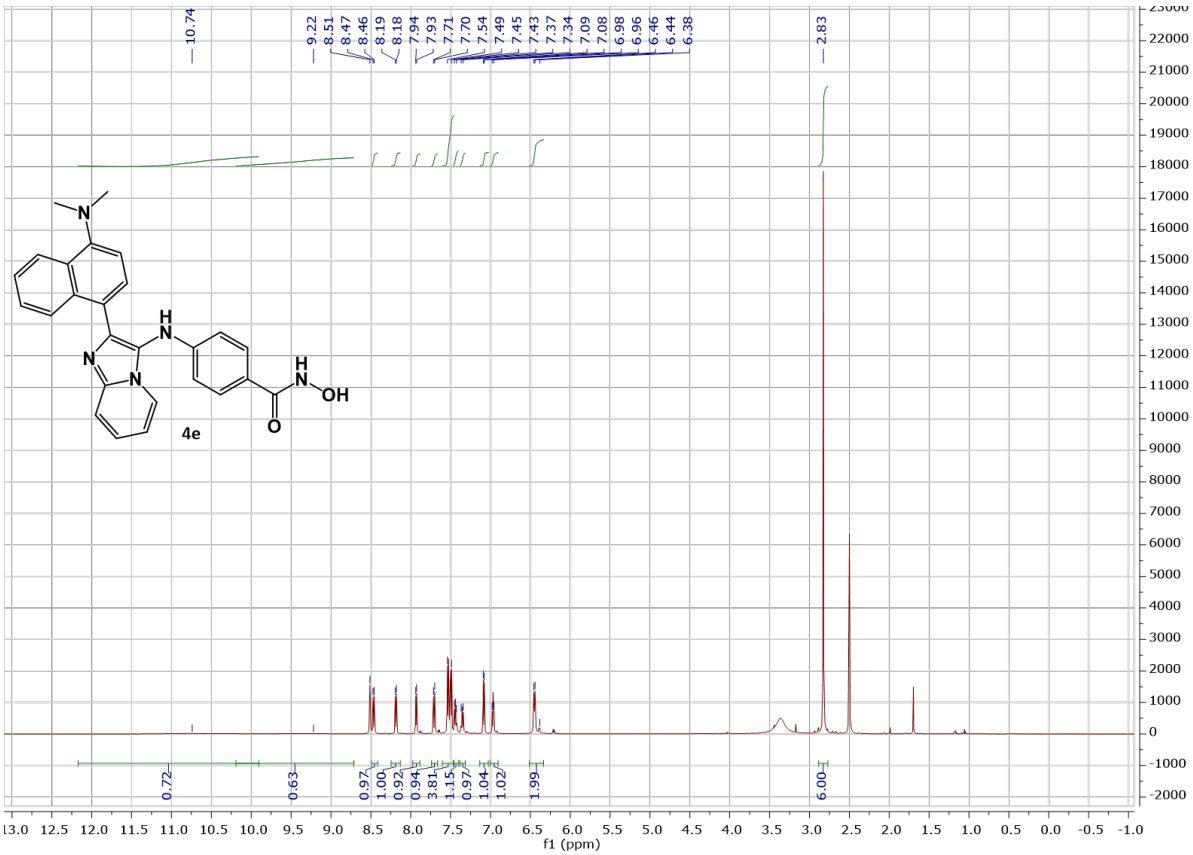


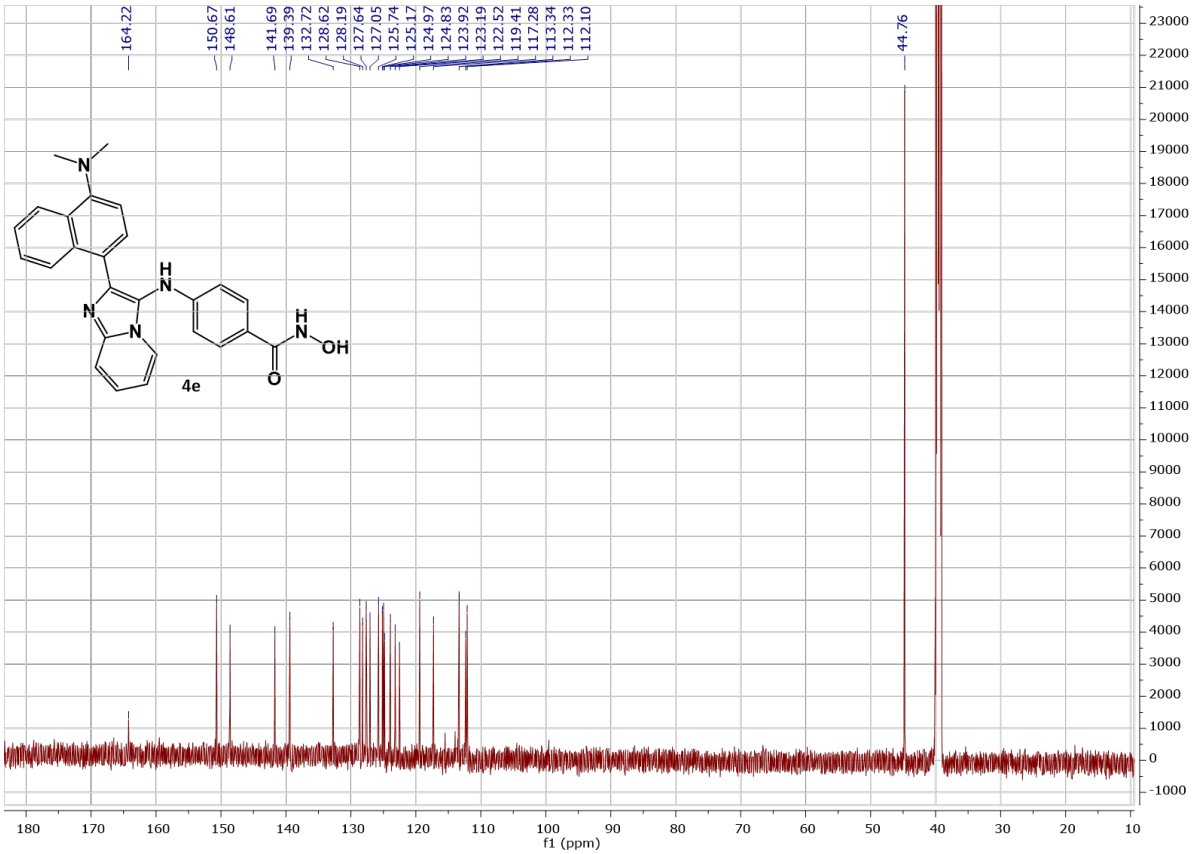
*^{1}H- (600 MHz) and ^{13}C-NMR (151 MHz) of **4d** (in DMSO-d_6):*



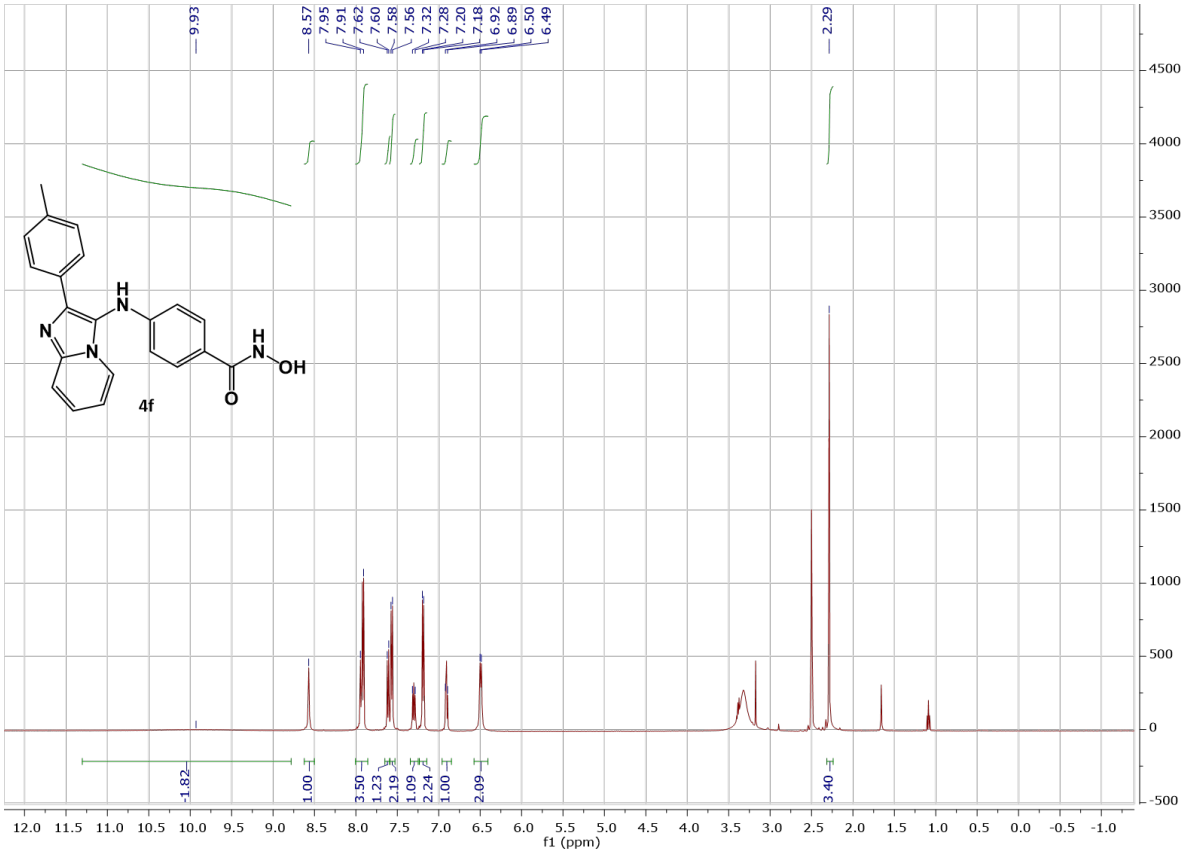


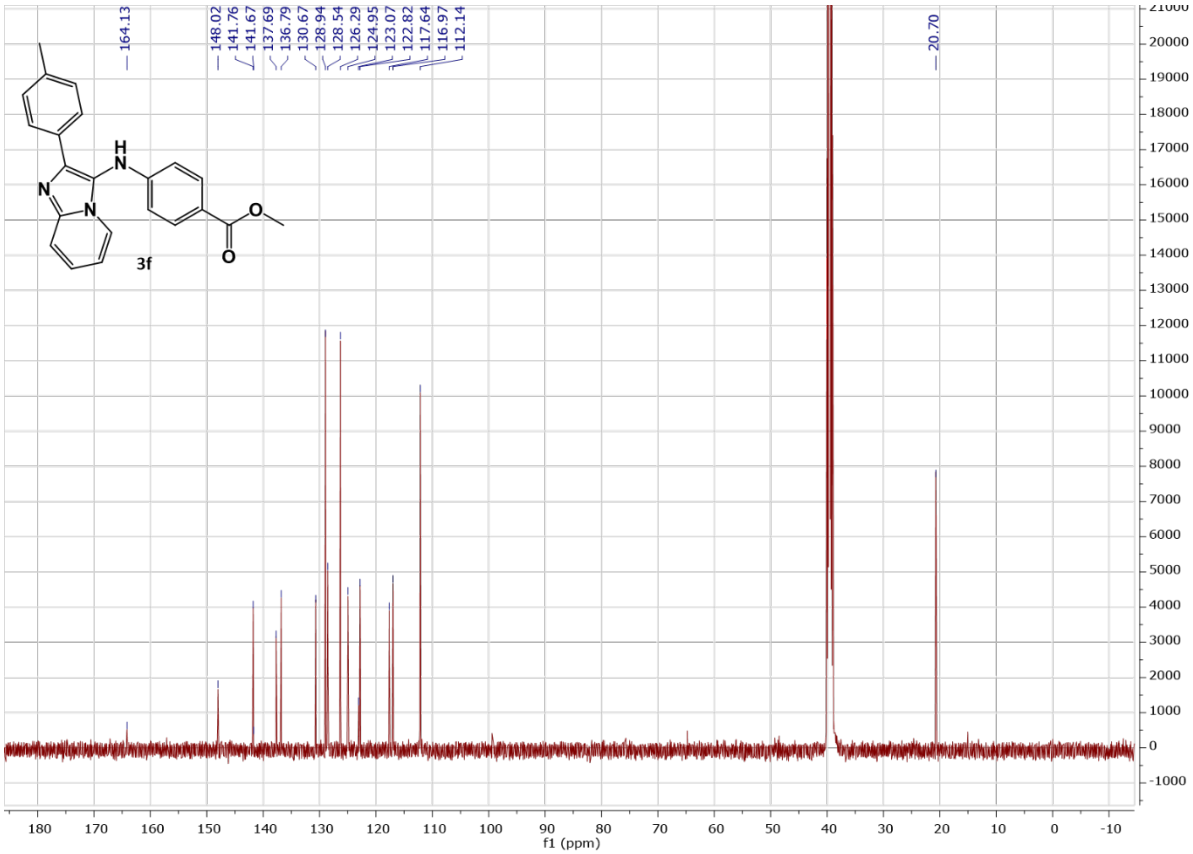
*^{1}H- (600 MHz) and ^{13}C-NMR (151 MHz) of **4e** (in DMSO-d_6):*



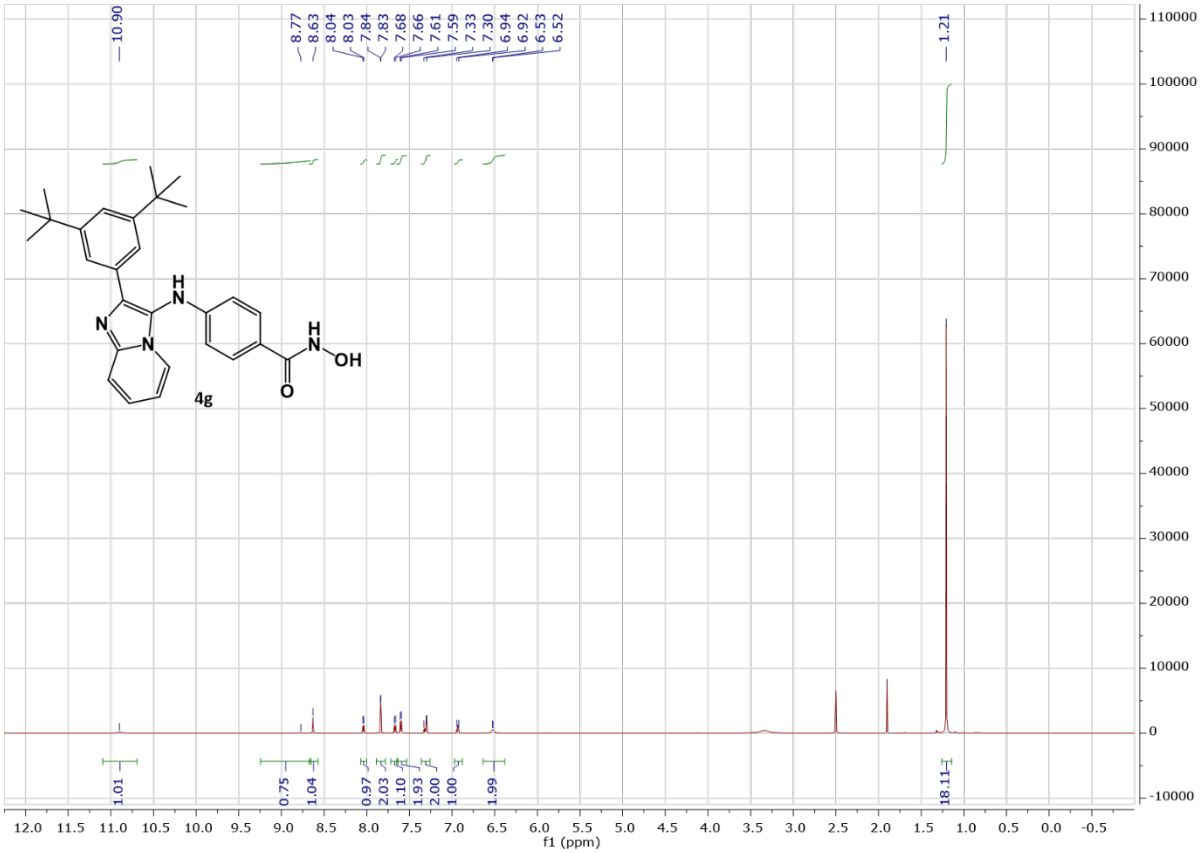


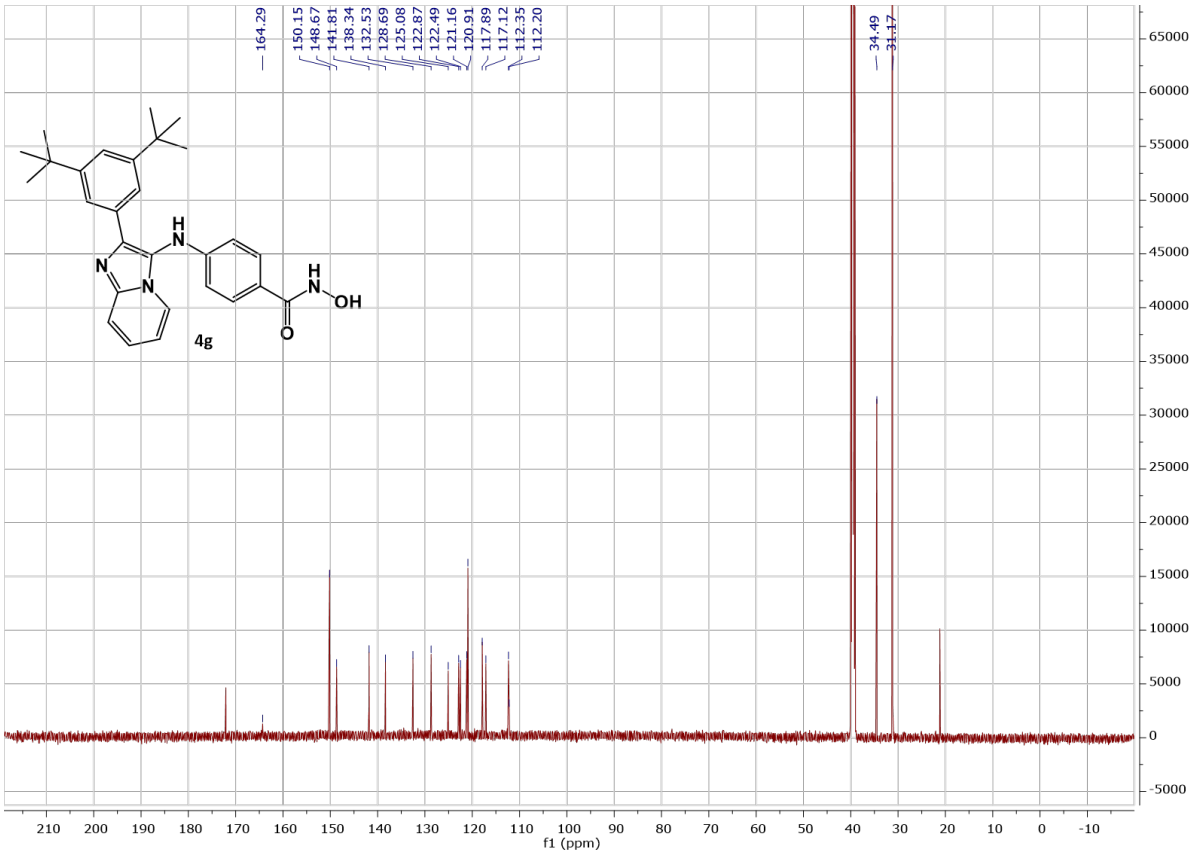
*^{1}H- (500 MHz) and ^{13}C-NMR (126 MHz) of **4f** (in DMSO-d_6):*



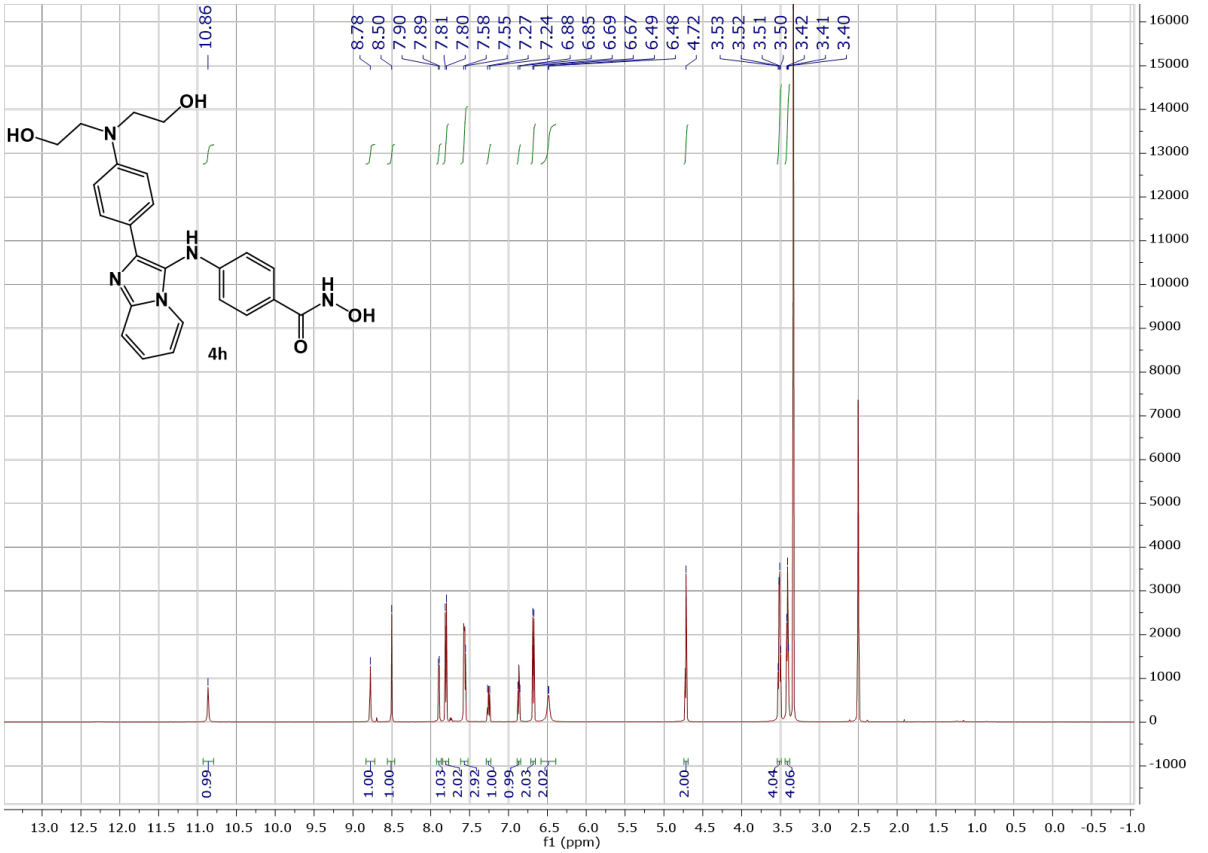


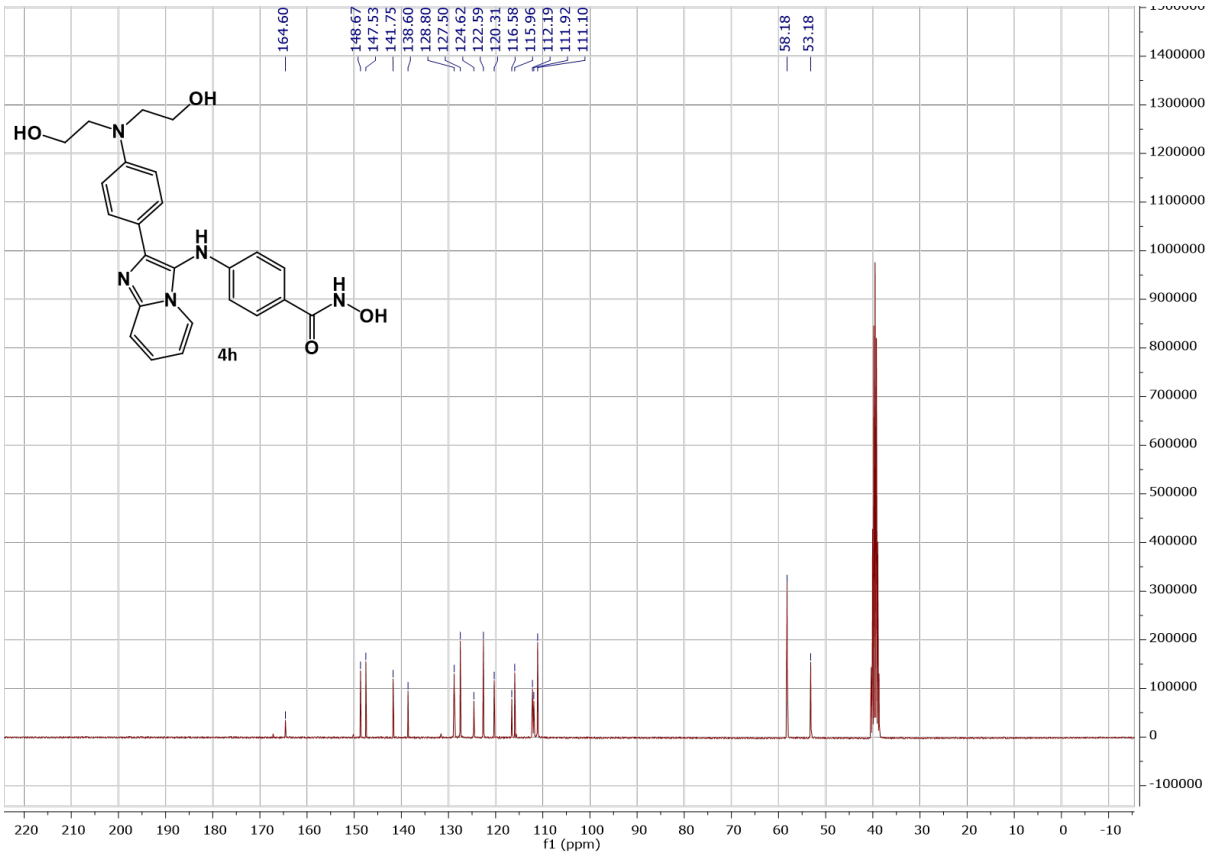
*^{1}H- (600 MHz) and ^{13}C-NMR (151 MHz) of **4g** (in DMSO-d_6):*



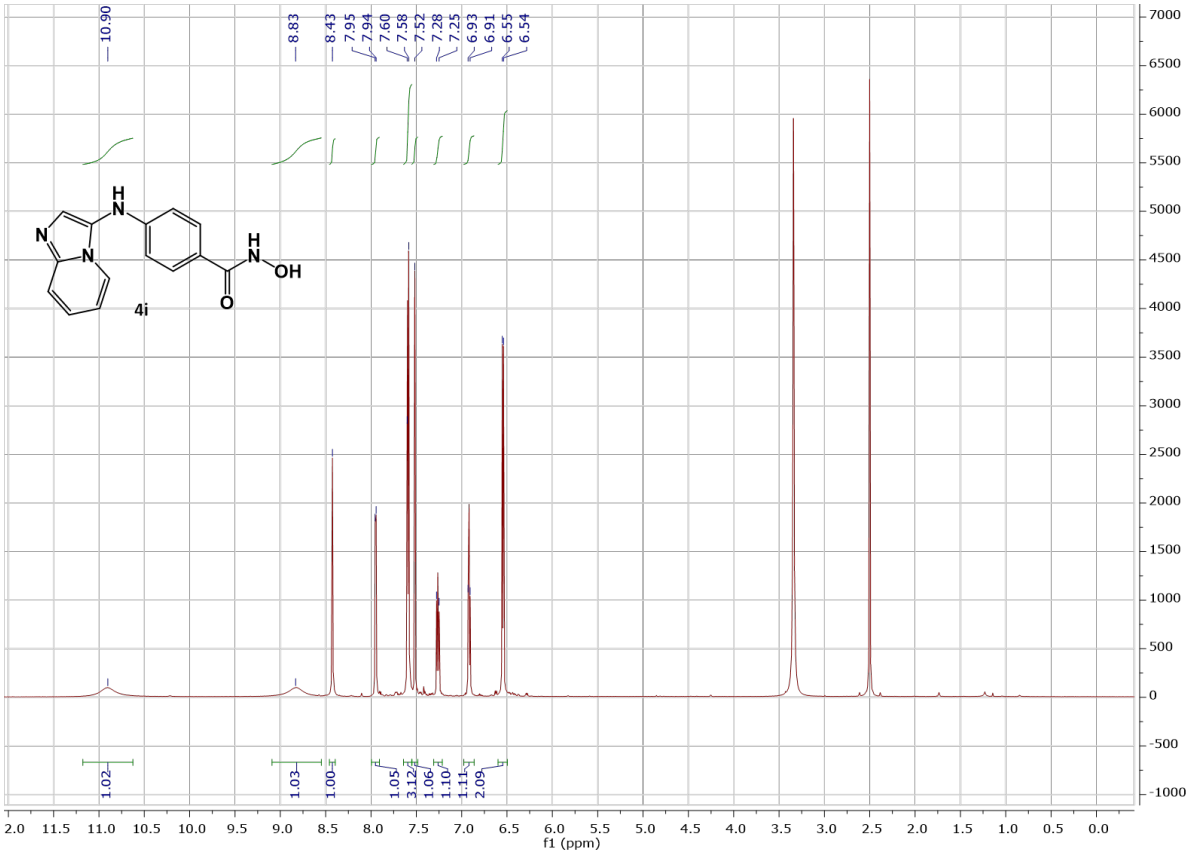


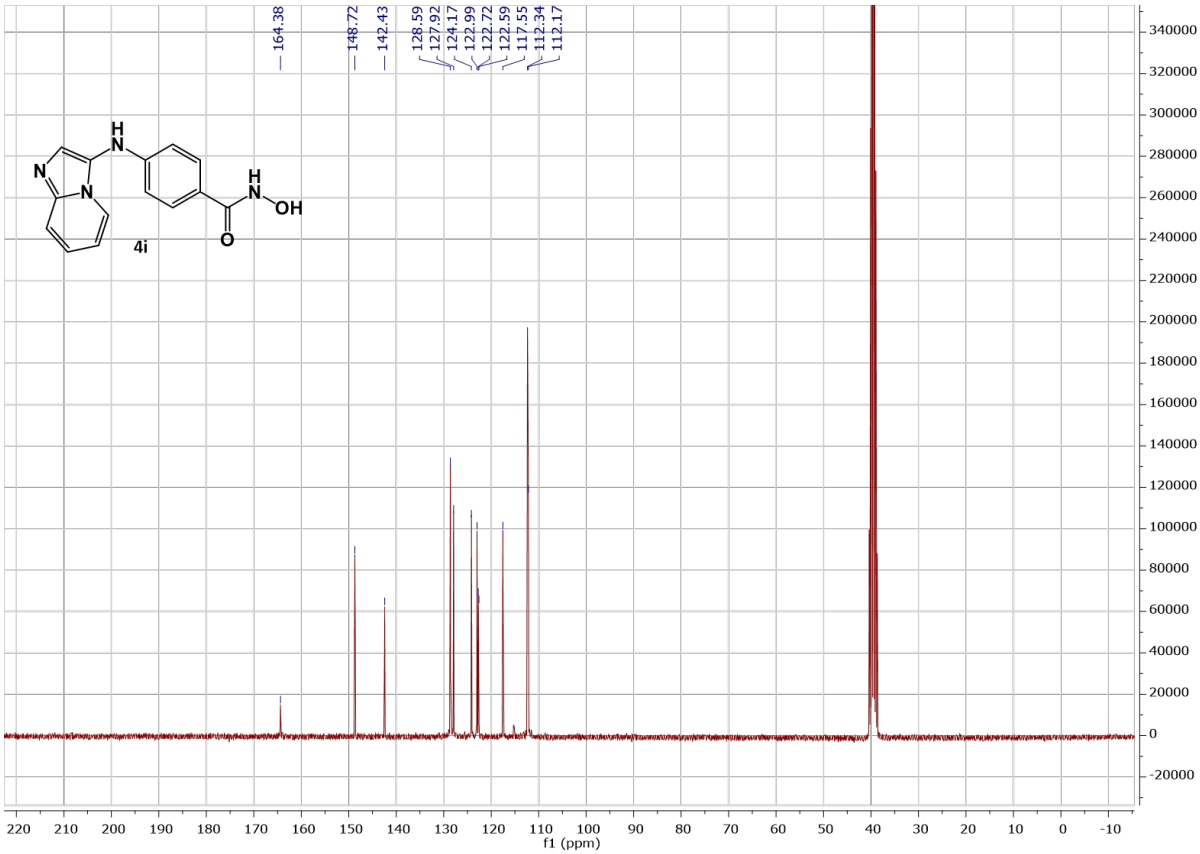
*^{1}H- (600 MHz) and ^{13}C-NMR (75 MHz) of **4h** (in DMSO-d_6):*



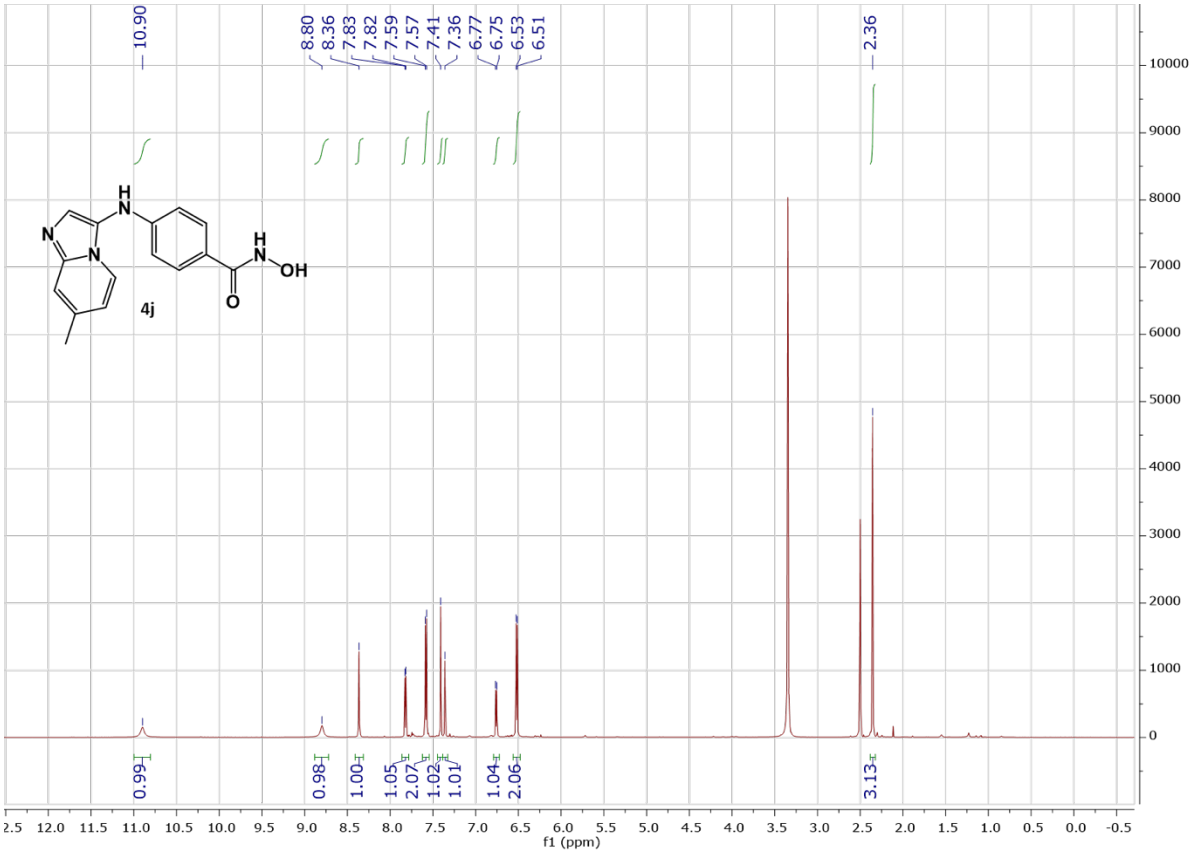


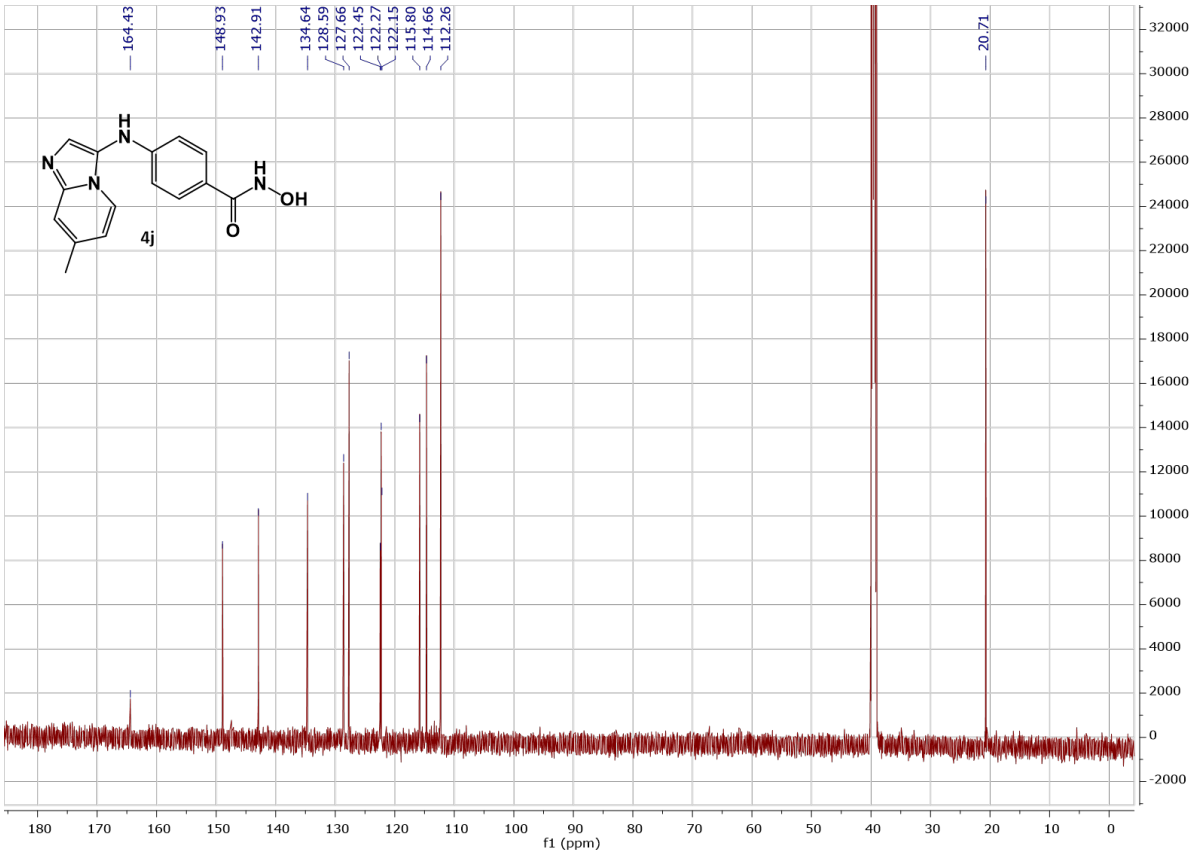
*^{1}H- (600 MHz) and ^{13}C-NMR (75 MHz) of **4i** (in DMSO-d_6):*



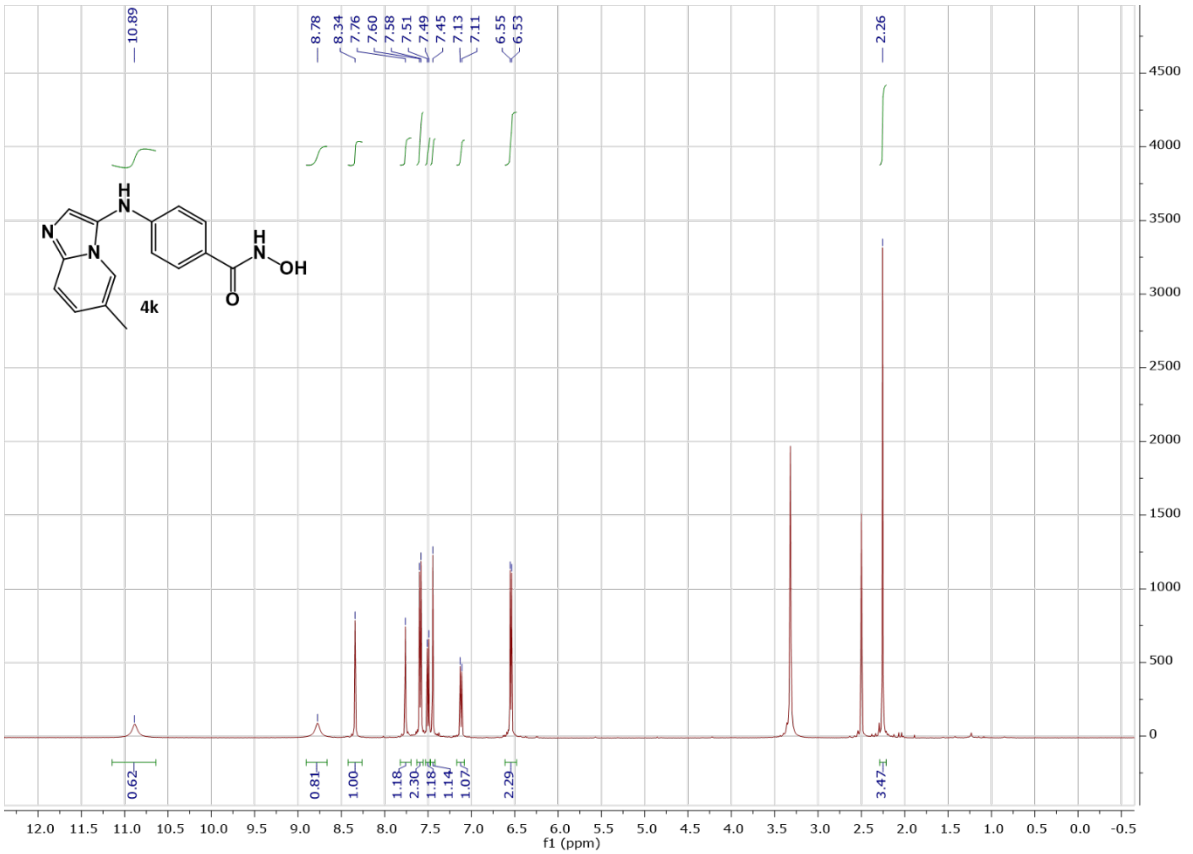


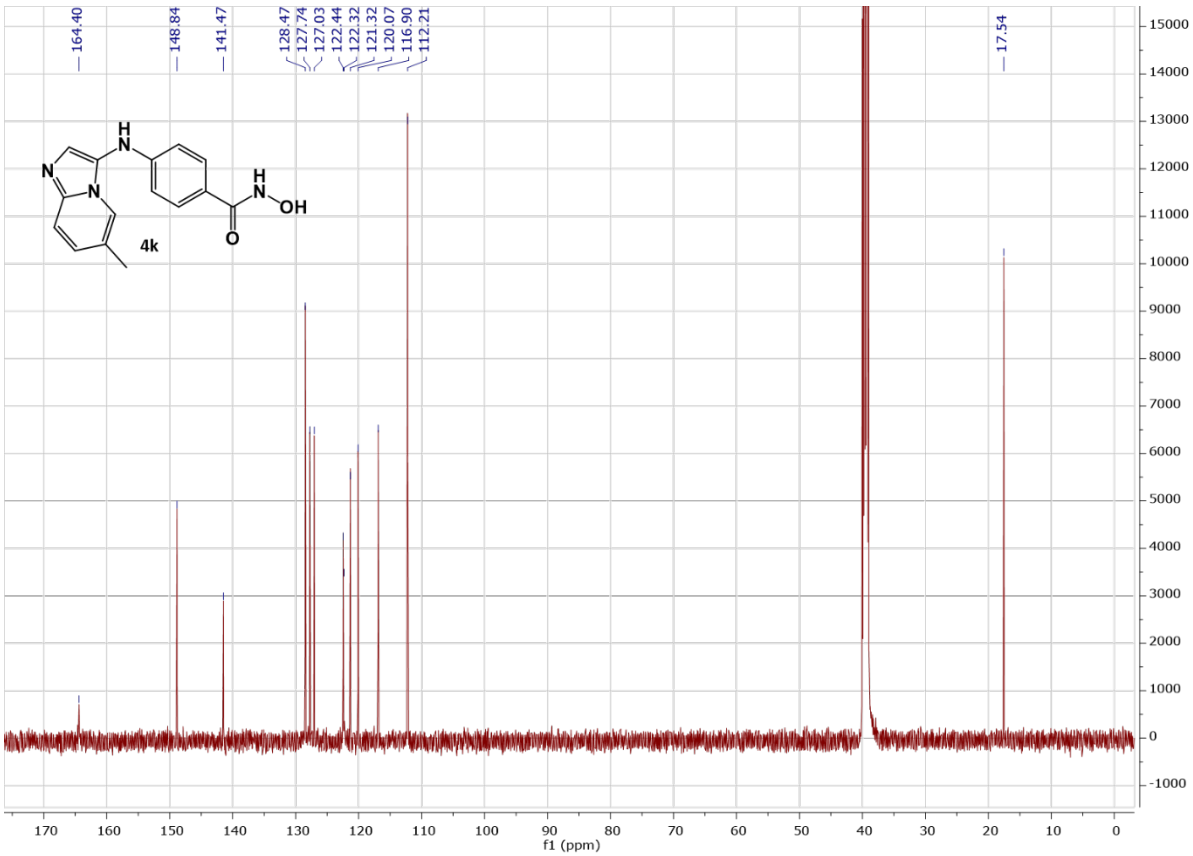
*^{1}H- (600 MHz) and ^{13}C-NMR (151 MHz) of **4j** (in DMSO-d_6):*



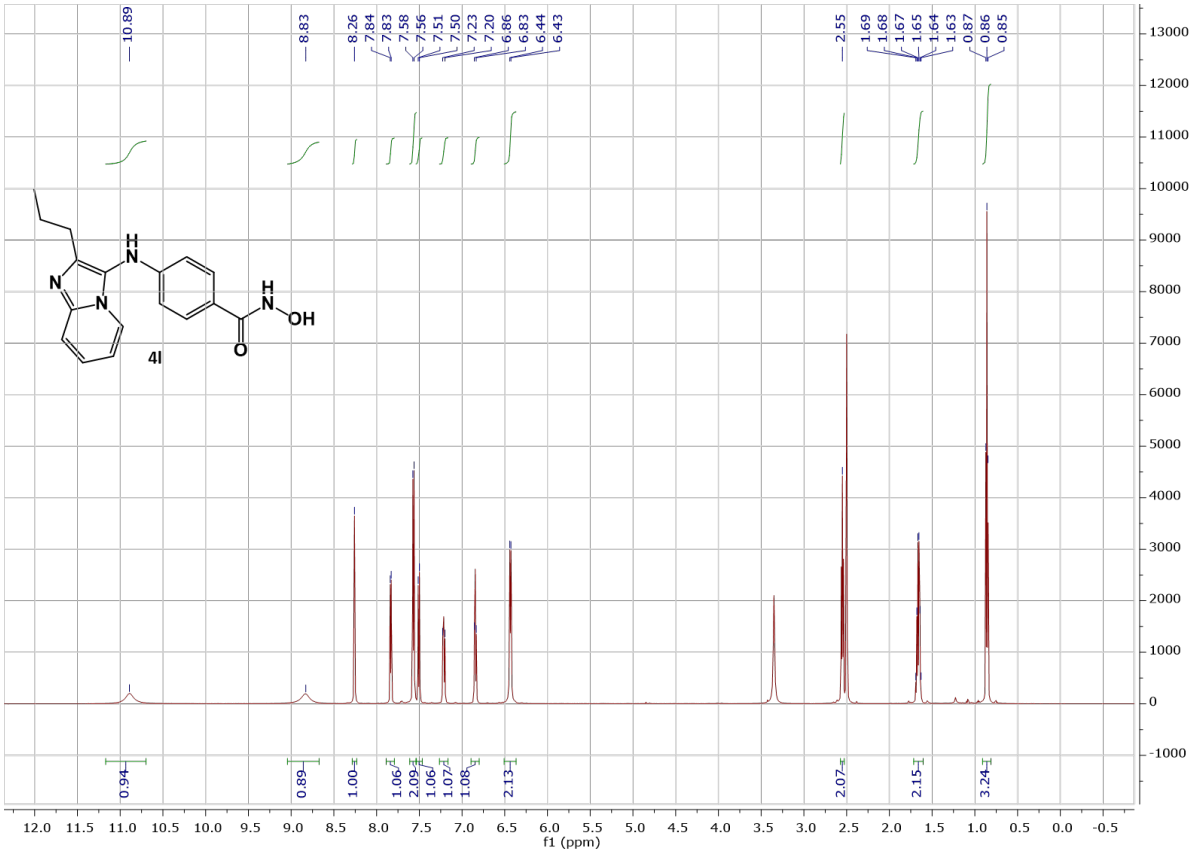


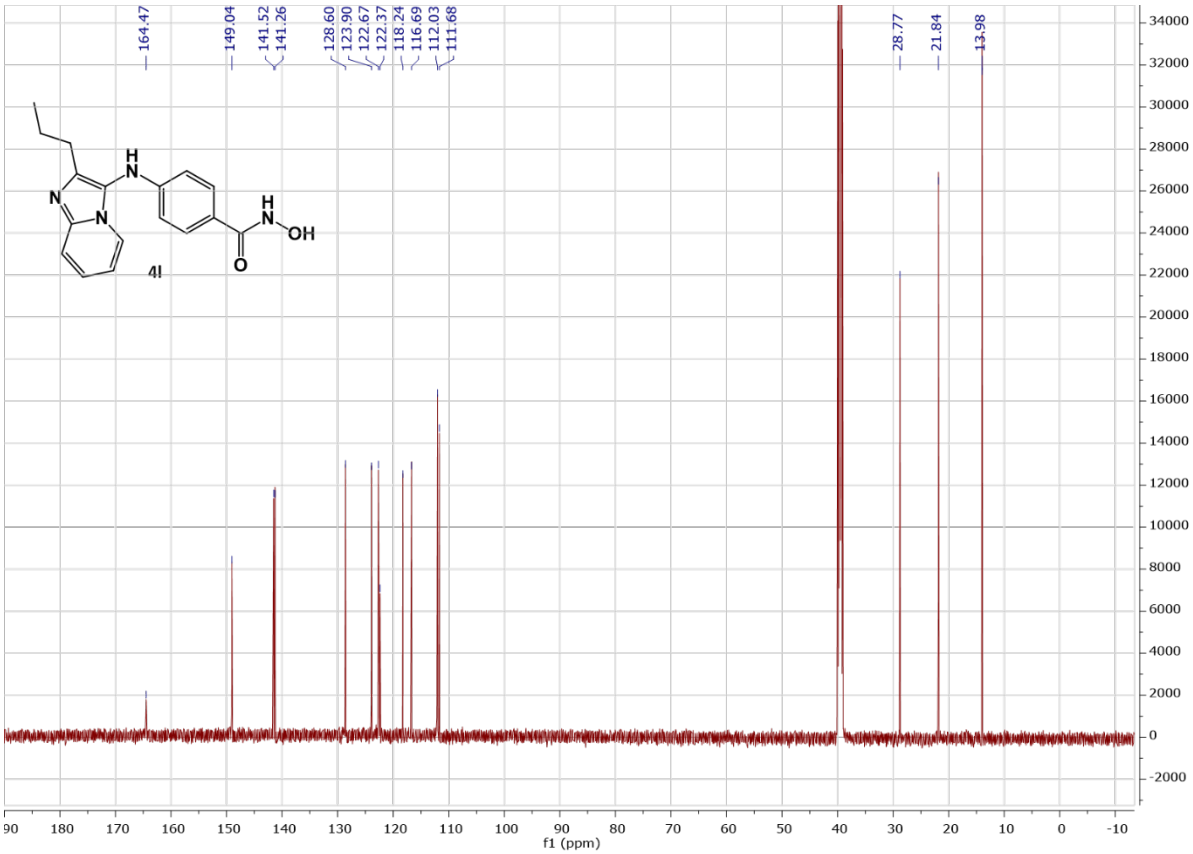
*^{1}H- (500 MHz) and ^{13}C-NMR (126 MHz) of **4k** (in DMSO-d_6):*



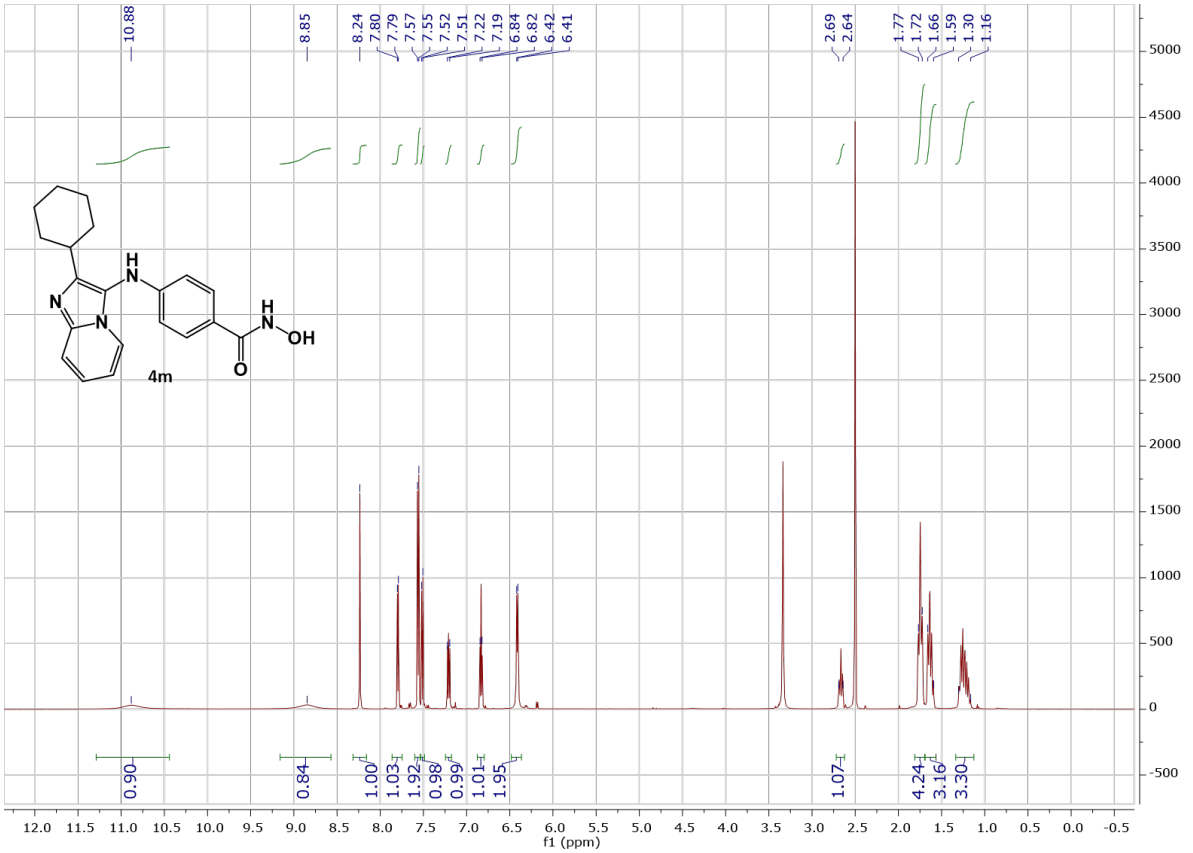


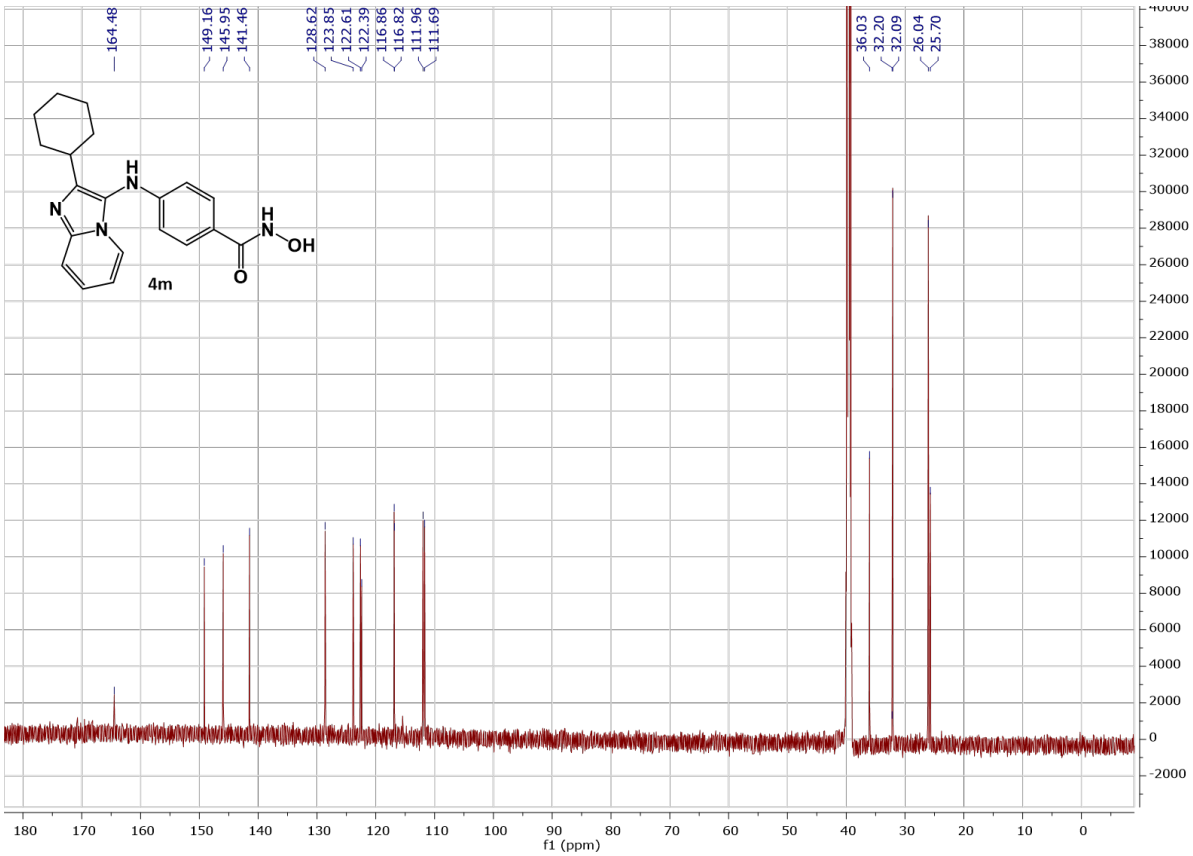
*^{1}H- (600 MHz) and ^{13}C-NMR (151 MHz) of **4l** (in DMSO-d_6):*





*^{1}H- (600 MHz) and ^{13}C-NMR (151 MHz) of **4m** (in DMSO-d_6):*





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