Supporting information

Disrupting the PCSK9/LDLR Protein-Protein Interaction by an Imidazole-based Minimalist Peptidomimetic

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General information

All commercial materials (Aldrich, Fluka) were used without further purification. All solvents were of reagent grade or HPLC grade. All reactions were carried out under a nitrogen atmosphere unless otherwise noted. All reactions were monitored by thin layer chromatography (TLC) on precoated silica gel 60 F254; spots were visualized with UV light or by treatment with a 1% aqueous $KMnO_4$ solution. Products were purified by flash chromatography on silica gel 60 (230–400 mesh). ^1H NMR spectra and ^{13}C NMR spectra were recorded on 300 and 400 MHz spectrometers. Chemical shifts are reported in parts per million relative to the residual solvent. ^{13}C NMR spectra have been recorded using the APT pulse sequence. Multiplicities in ^1H NMR are reported as follows: s = singlet, d = doublet, t = triplet, m = multiplet, br s = broad singlet. High-resolution MS spectra were recorded with a Waters Micromass Q-ToF micro TM mass spectrometer, equipped with an ESI source.

General Experimental Procedures

General procedure A: van Leusen three-component reaction (vL-3CR)

Aldehyde (benzaldehyde or **2**, **4**, **6**) (1 eq) was dissolved in DMF (Conc. as indicated below). Methylamine aqueous solution 40 wt. % (2 eq) was added and the resulting mixture was kept under stirring for 2h at room temperature. Potassium carbonate (1.5 eq) and tosylmethyl isocyanide (1.2 eq) were sequentially added and the reaction was stirred for additional 24h at 50 °C. The resulting mixture was then partitioned between ethyl acetate/water, and the organic phase was washed with brine (x5), dried over Na_2SO_4 and concentrated under reduced pressure, to give a residue that was purified by flash chromatography (FC) as indicated below.

General procedure B: imidazoles C2-formylation

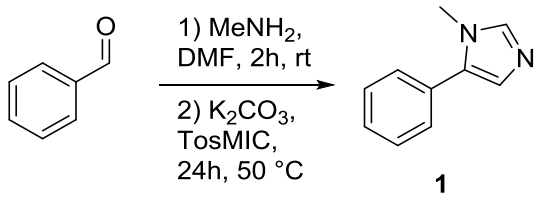
To a solution of compound **1** (or **3**) (1 eq) in dry THF under nitrogen atmosphere (Conc. as indicated below) cooled to -78 °C, was added dropwise a solution of *n*-butyl lithium in hexane(1.5 eq), and the resulting mixture was kept under stirring for 2h at the same temperature. Freshly distilled DMF (2eq) was added and the reaction was stirred for additional 24h at room temperature. The resulting mixture was then quenched with ice-water and extracted with ethyl acetate (x2). The organic phase was washed with brine (x4), dried over Na_2SO_4 and concentrated under reduced pressure, to give a residue that was purified by flash chromatography (FC) as indicated below.

General procedure C: imidazoles C2-formylation (with TMEDA)

To a solution of compound **5** (1 eq) and TMEDA (2 eq) in dry THF under nitrogen atmosphere (0.05 M) cooled to -78 °C, was added dropwise a solution of *n*-butyl lithium in hexane (1.5 eq), and the resulting mixture was kept under stirring for 2h at the same temperature. Freshly distilled DMF (2eq) was added and the reaction was stirred for additional 24h at room temperature. The resulting mixture was then quenched with ice-water and extracted with ethyl acetate (x2). The organic phase was washed with brine (x4), dried over Na_2SO_4 and concentrated under reduced pressure, to give a residue that was purified by flash chromatography (FC) as indicated below.

Preparation of imidazoles (compounds 1, 3, 5, 7)

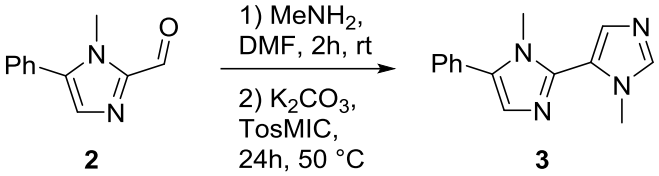
Compound 1



1

Prepared according to **general procedure A** from benzaldehyde (9.8 mmol, 1.040 g); Conc: 1 M; FC: ethyl acetate; yellowish solid (1.285 g); yield: 83%; spectroscopic data in agreement with literature.[1]

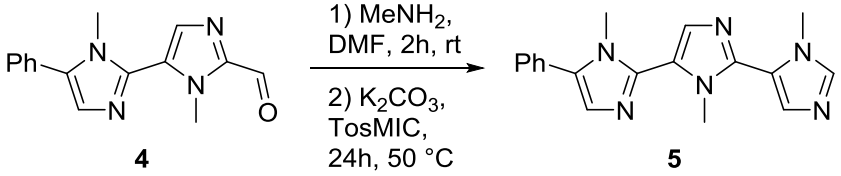
Compound 3



2 **3**

Prepared according to **general procedure A** from **2** (3.8 mmol, 700 mg); Conc: 1 M; FC: CH_2Cl_2:methanol 98:2 to 90:10; yellowish solid (633 mg); yield: 70%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.60 (s, 1H), 7.51 – 7.48 (m, 1H), 7.48 – 7.45 (m, 4H), 7.32 (br, d, J = 1.0 Hz, 1H), 7.23 (s, 1H), 3.93 (s, 3H), 3.71 (s, 3H); ^{13}C NMR (100 MHz,) δ 139.8, 139.6, 134.9, 130.0, 129.9, 128.8 (2C), 128.7 (2C), 128.1, 127.7, 122.9, 33.4, 33.2; HRMS (ESI) calcd for $C_{14}H_{15}N_4^+$ $[MH]^+$ 239.1291, found 239.1279.

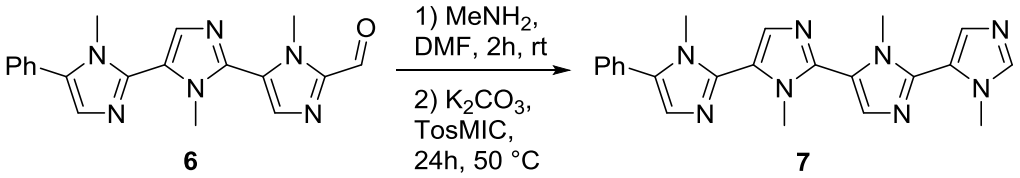
Compound 5



4 **5**

Prepared according to **general procedure A** from **4** (1.1 mmol, 292 mg); Conc: 0.5 M; FC: ethyl acetate:methanol 96:4 to 90:10 + 1% Et_3N; yellowish solid (217 mg); yield: 62%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.61 (s, 1H), 7.54 – 7.43 (m, 5H), 7.42 (s, 1H), 7.36 (d, J = 0.8 Hz, 1H), 7.26 (s, 1H), 3.94 (s, 3H), 3.91 (s, 3H), 3.74 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 140.4, 140.0, 139.5, 135.1, 130.7, 130.0, 129.8, 128.8 (2C), 128.7 (2C), 128.2, 128.0, 123.9, 122.4, 33.5, 33.4, 33.2; HRMS (ESI) calcd for $C_{18}H_{19}N_6^+$ $[MH]^+$ 319.1666, found 319.1659.

Compound 7

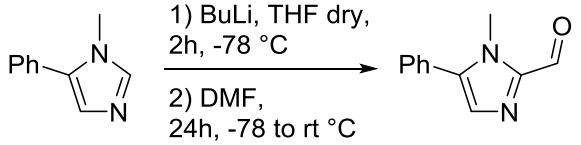


6 **7**

Prepared according to **general procedure A** from **6** (0.12 mmol, 42 mg); Conc: 0.25 M; FC: ethyl acetate:methanol 85:15 + 1 % Et_3N; yellowish solid (20 mg); yield: 43%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.64 (s, 1H), 7.54 – 7.49 (m, 1H), 7.48 (d, J = 2.2 Hz, 4H), 7.46 (s, 1H), 7.45 (s, 1H), 7.39 – 7.34 (br, m, 1H), 7.27 (s, 1H), 3.98 (s, 3H), 3.93 (s, 3H), 3.92 (s, 3H), 3.76 (s, 3H); 13C NMR (101 MHz,) δ 140.6, 140.3, 140.0, 139.4, 135.1, 130.5, 130.1, 129.7, 128.9 (2C), 128.7 (2C), 128.3 (2C), 127.9, 124.1, 123.4, 122.3, 33.6, 33.5, 33.4, 33.3; HRMS (ESI) calcd for $C_{22}H_{23}N_8^+$ $[MH]^+$ 399.2040, found 399.2046.

Preparation of C2-formyl imidazoles (compounds 2, 4, 6)

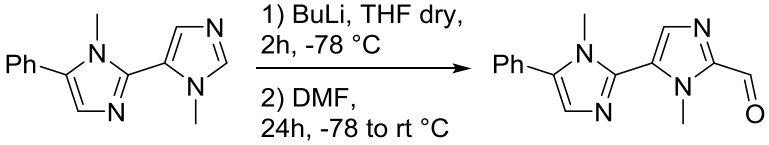
Compound 2



1 **2**

Prepared according to **general procedure B** from **1** (6.3 mmol, 997 mg); Conc: 0.5 M; FC: ethyl acetate:*n*-hexane 4:6; yellowish solid (727 mg); yield: 62%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.87 (s, 1H), 7.56 – 7.46 (m, 3H), 7.44 (dd, J = 7.7, 1.7 Hz, 2H), 7.36 (s, 1H), 4.00 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 182.9, 145.2, 140.5, 131.7, 130.0, 129.7 (2C), 129.7 (2C), 128.5, 33.8; HRMS (ESI) calcd for $C_{11}H_{11}N_2O^+$ $[MH]^+$ 187.0866, found 187.0874.

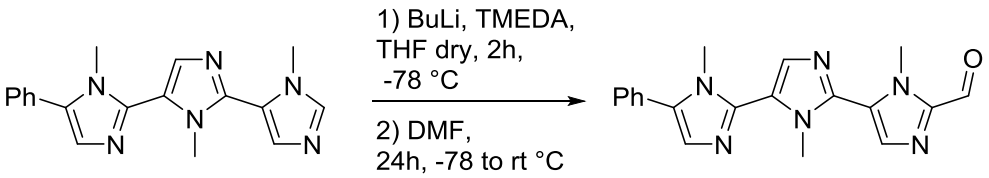
Compound 4



3 **4**

Prepared according to **general procedure B** from **3** (2.5 mmol, 595 mg); Conc: 0.25 M; FC: ethyl acetate:*n*-hexane 6:4; yellowish solid (345 mg); yield: 52%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.88 (s, 1H), 7.51 (s, 1H), 7.50 – 7.40 (m, 5H), 7.28 (s, 1H), 4.23 (s, 3H), 3.70 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 182.2, 144.6, 137.6, 135.9, 132.0, 129.3 (2C), 128.9 (2C), 128.8 (2C), 128.5, 33.7, 33.3 (1 quaternary carbon is missed); HRMS (ESI) calcd for $C_{15}H_{15}N_4O^+$ $[MH]^+$ 267.1240, found 267.1245.

Compound 6

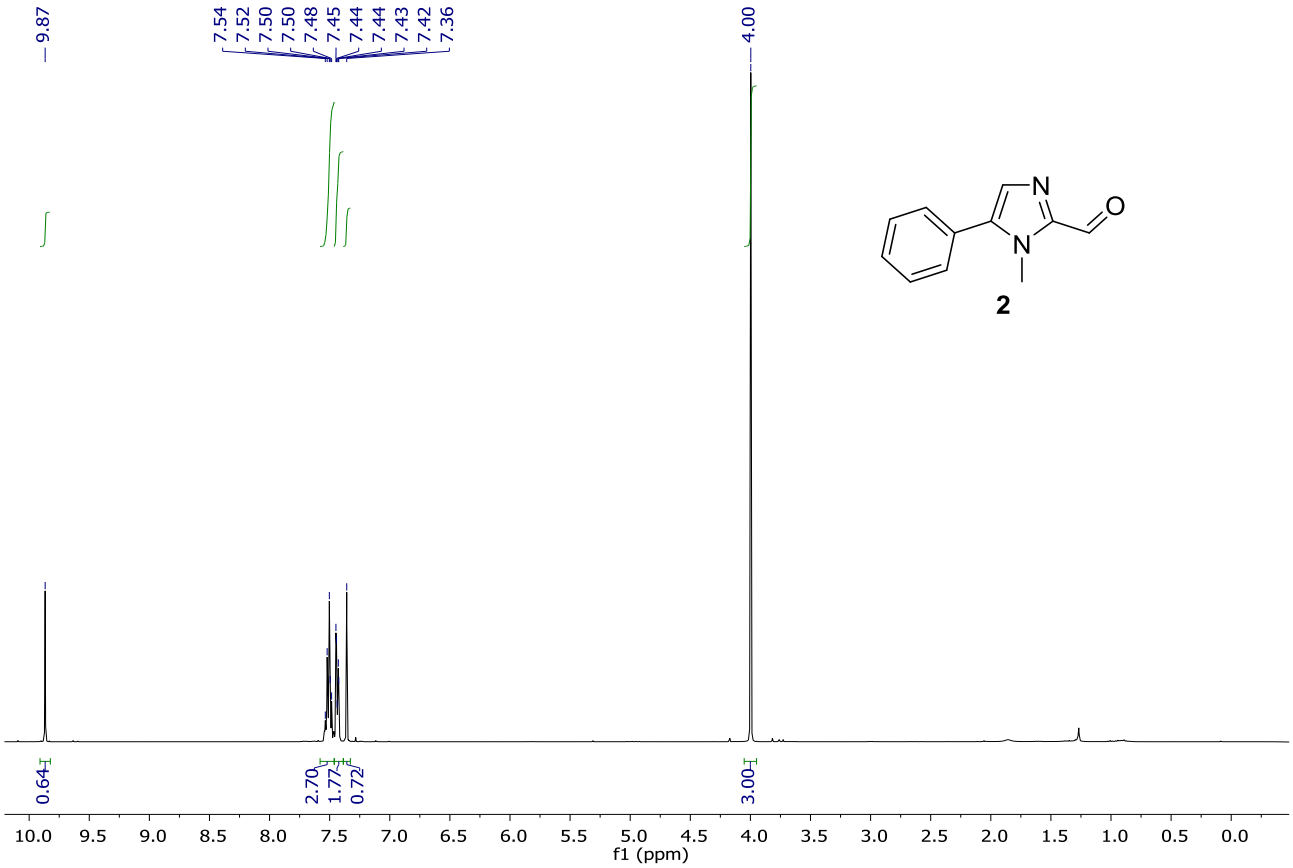


5 **6**

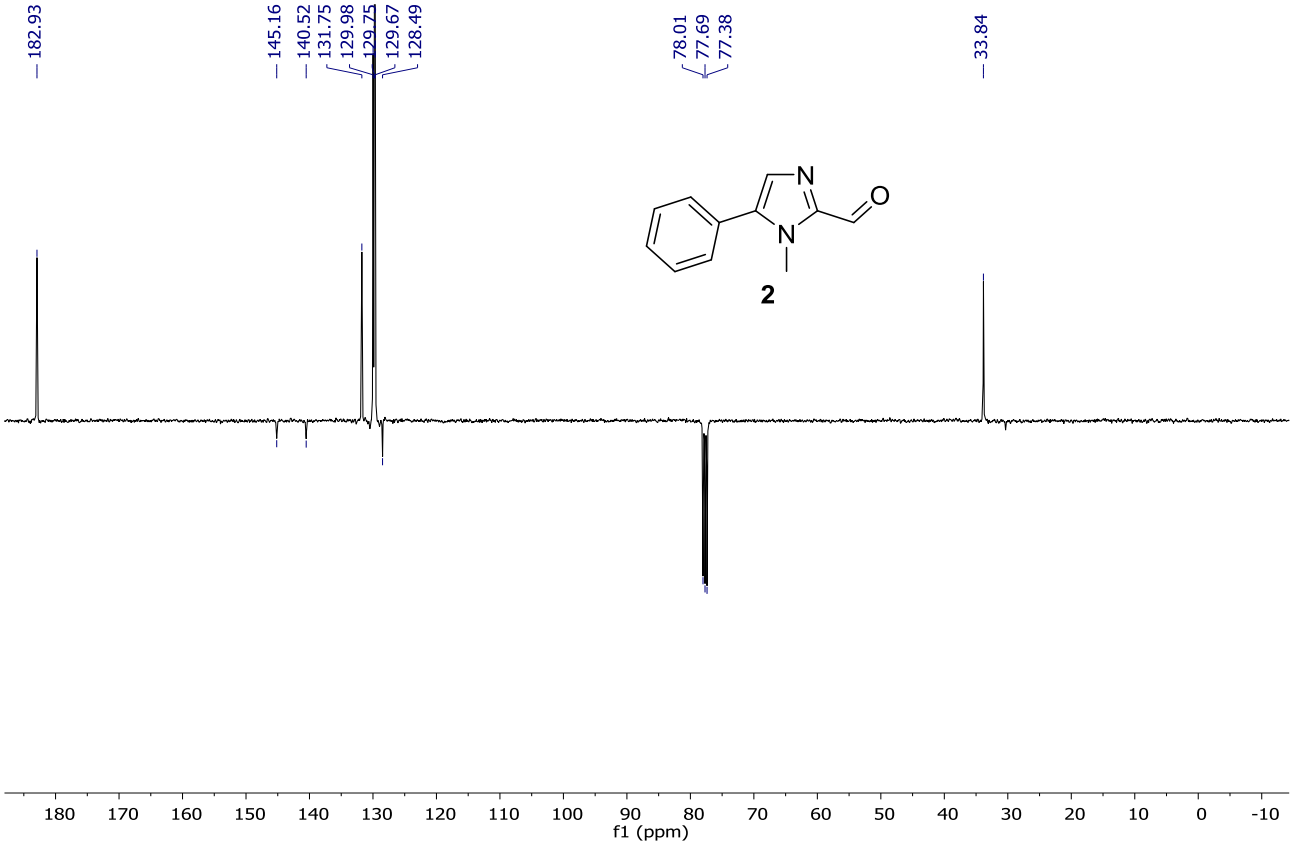
Prepared according to **general procedure C** from **5** (0.37 mmol, 118 mg); FC: ethyl acetate:methanol 97.5:2.5 to 95:5; yellowish solid (52 mg); yield: 41%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.89 (s, 1H), 7.53 (s, 1H), 7.51 – 7.32 (m, 6H), 7.24 (s, 1H), 4.19 (s, 3H), 3.94 (s, 3H), 3.72 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 182.91, 145.47, 139.18, 135.77, 133.18, 131.12, 130.30, 129.52 (2C), 129.42 (2C), 128.99, 128.88, 34.34, 34.15, 33.88 (3 quaternary carbons are missed); HRMS (ESI) calcd for $C_{19}H_{19}N_6O^+$ $[MH]^+$ 347.1615, found 347.1608.

NMR spectra

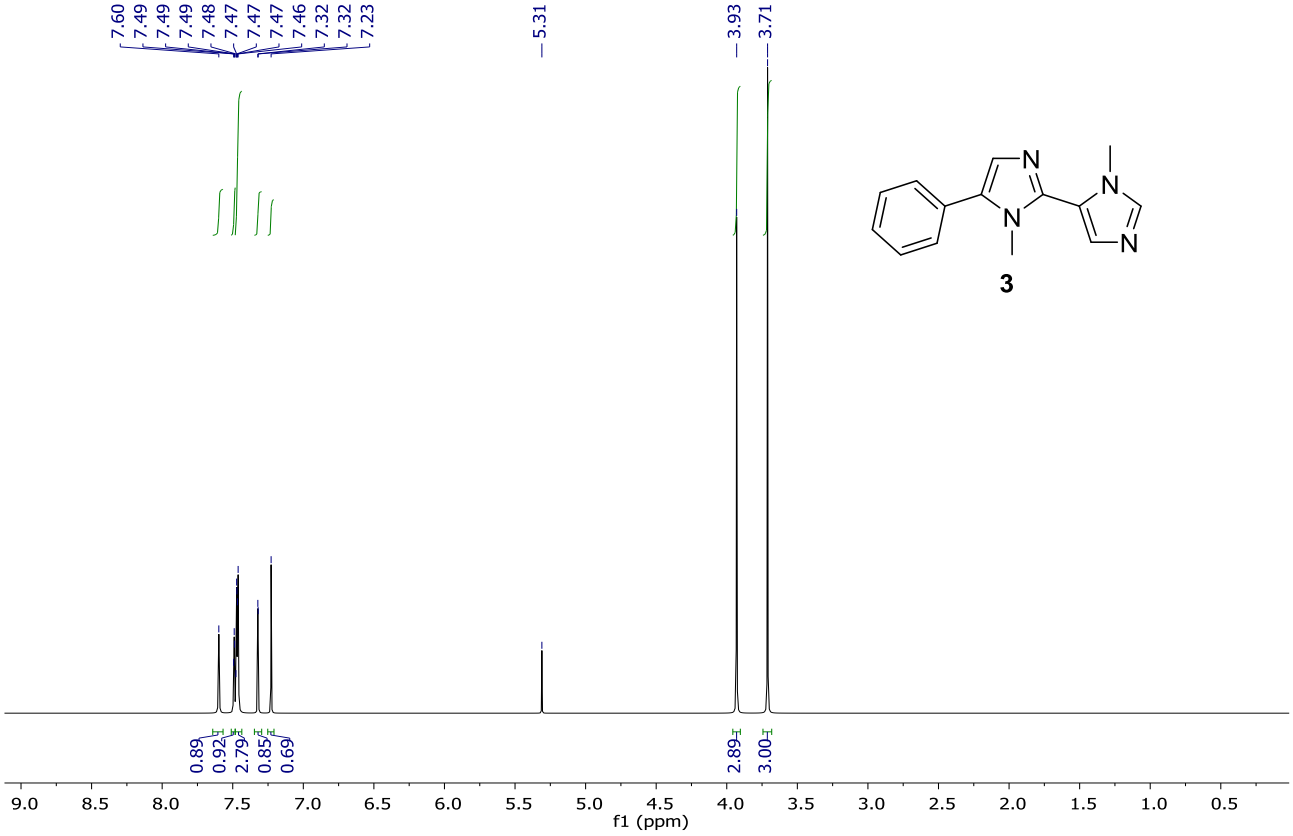
^{1}H NMR spectra of **2** (400 MHz, $CDCl_3$)



^{13}C NMR spectra of **2** (100 MHz, $CDCl_3$)

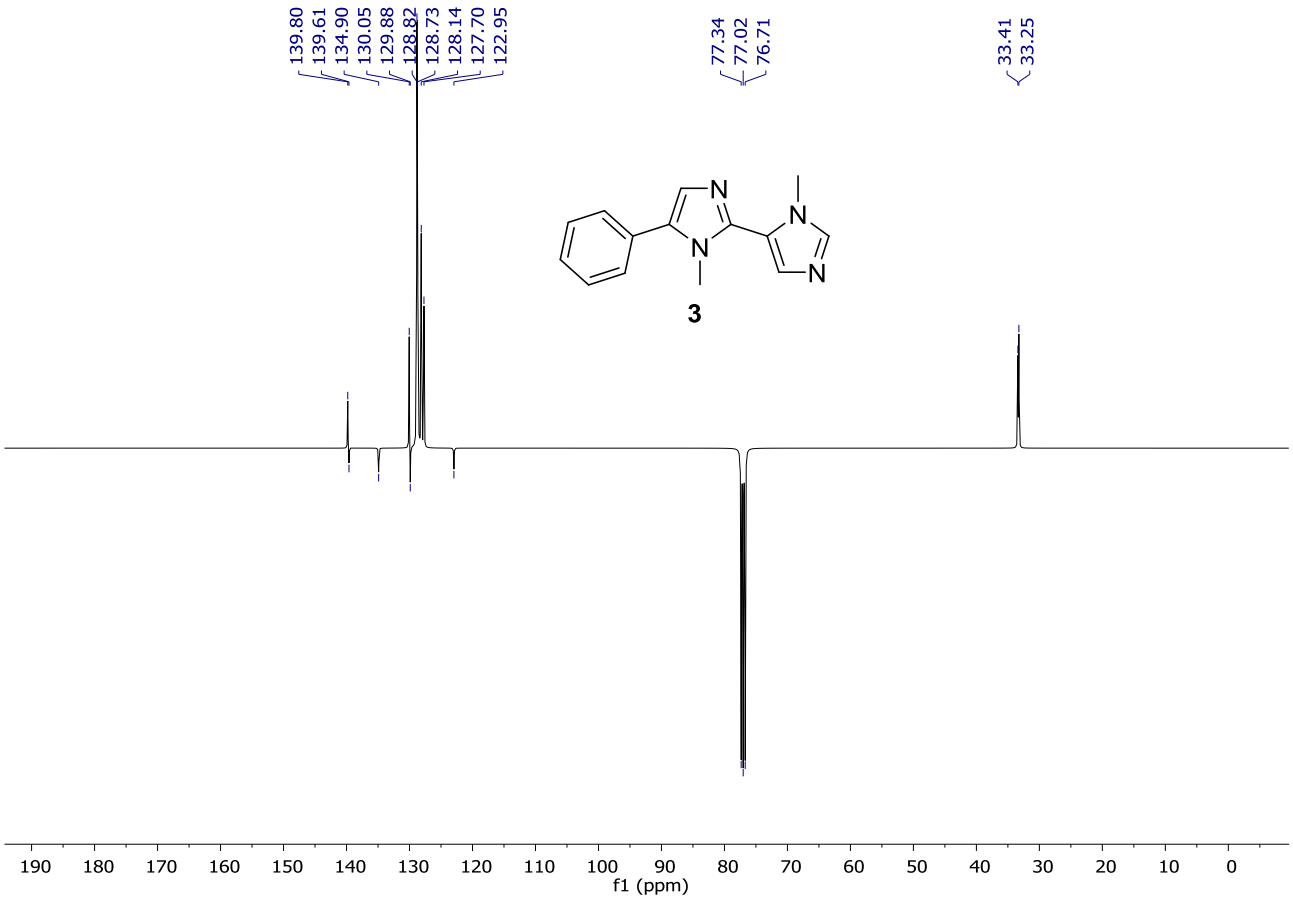


^{1}H NMR spectra of **3** (400 MHz, $CDCl_3$)



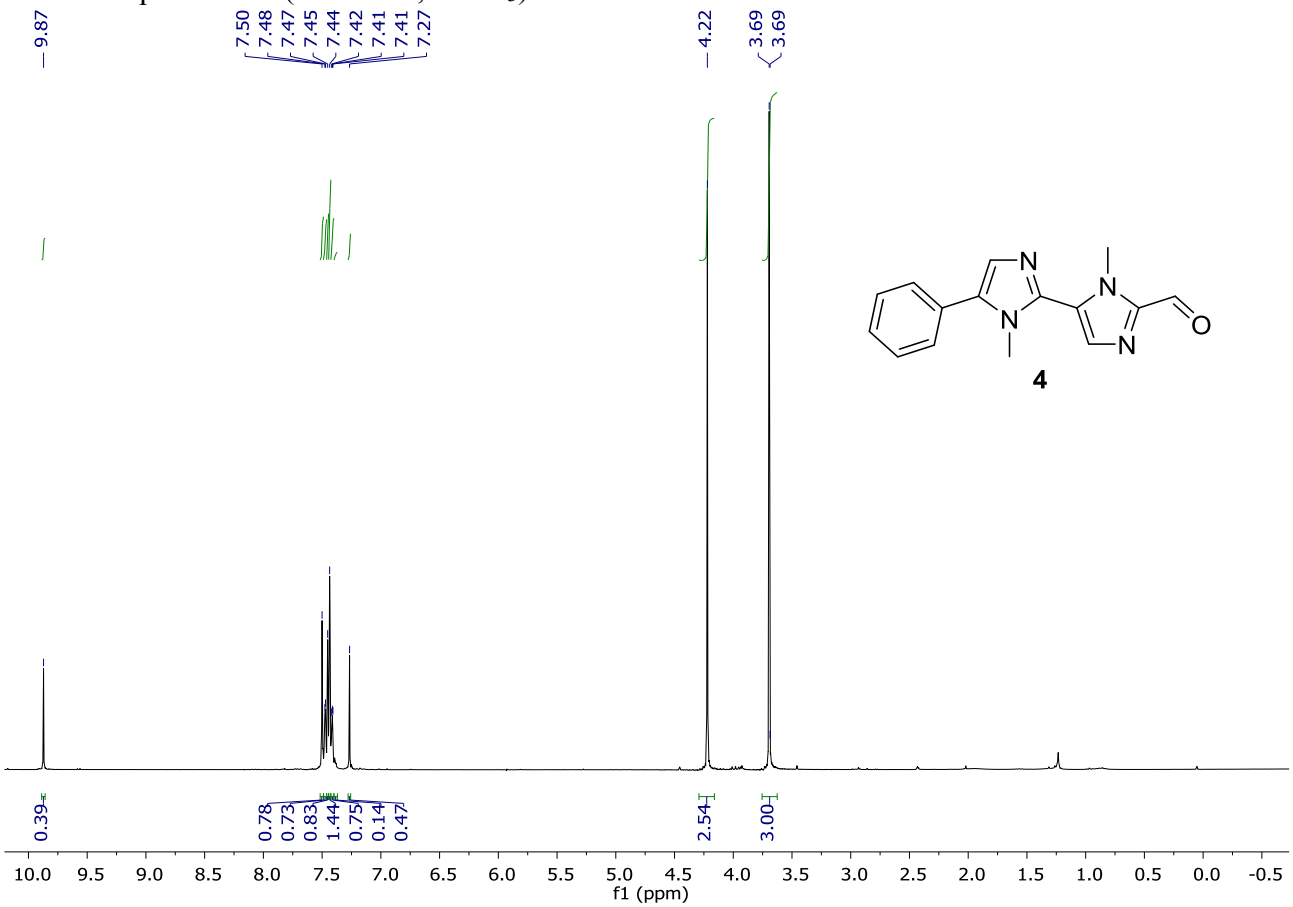
3

^{13}C NMR spectra of **3** (100 MHz, $CDCl_3$)



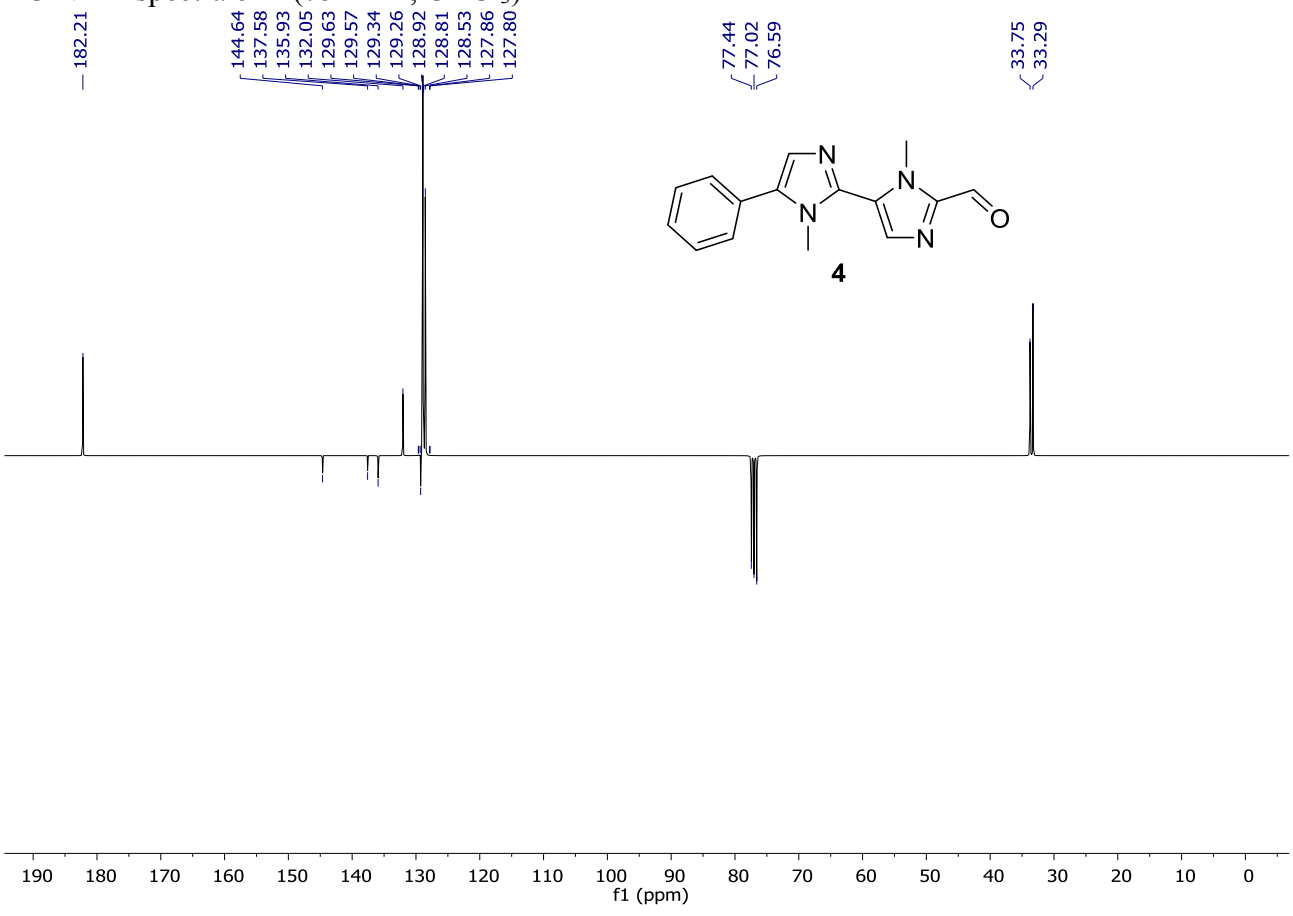
3

^{1}H NMR spectra of **4** (300 MHz, $CDCl_3$)



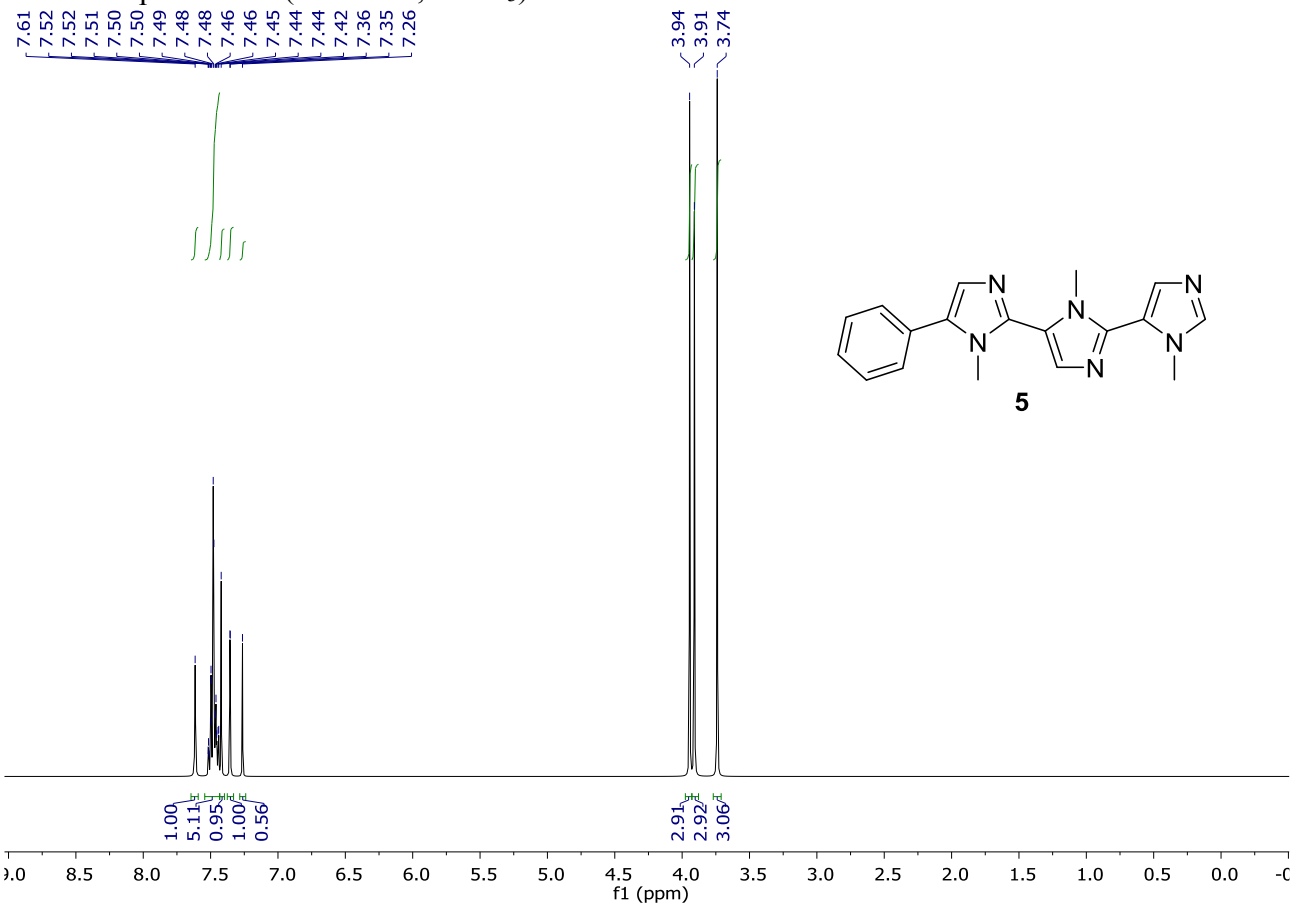
4

^{13}C NMR spectra of **4** (75 MHz, $CDCl_3$)



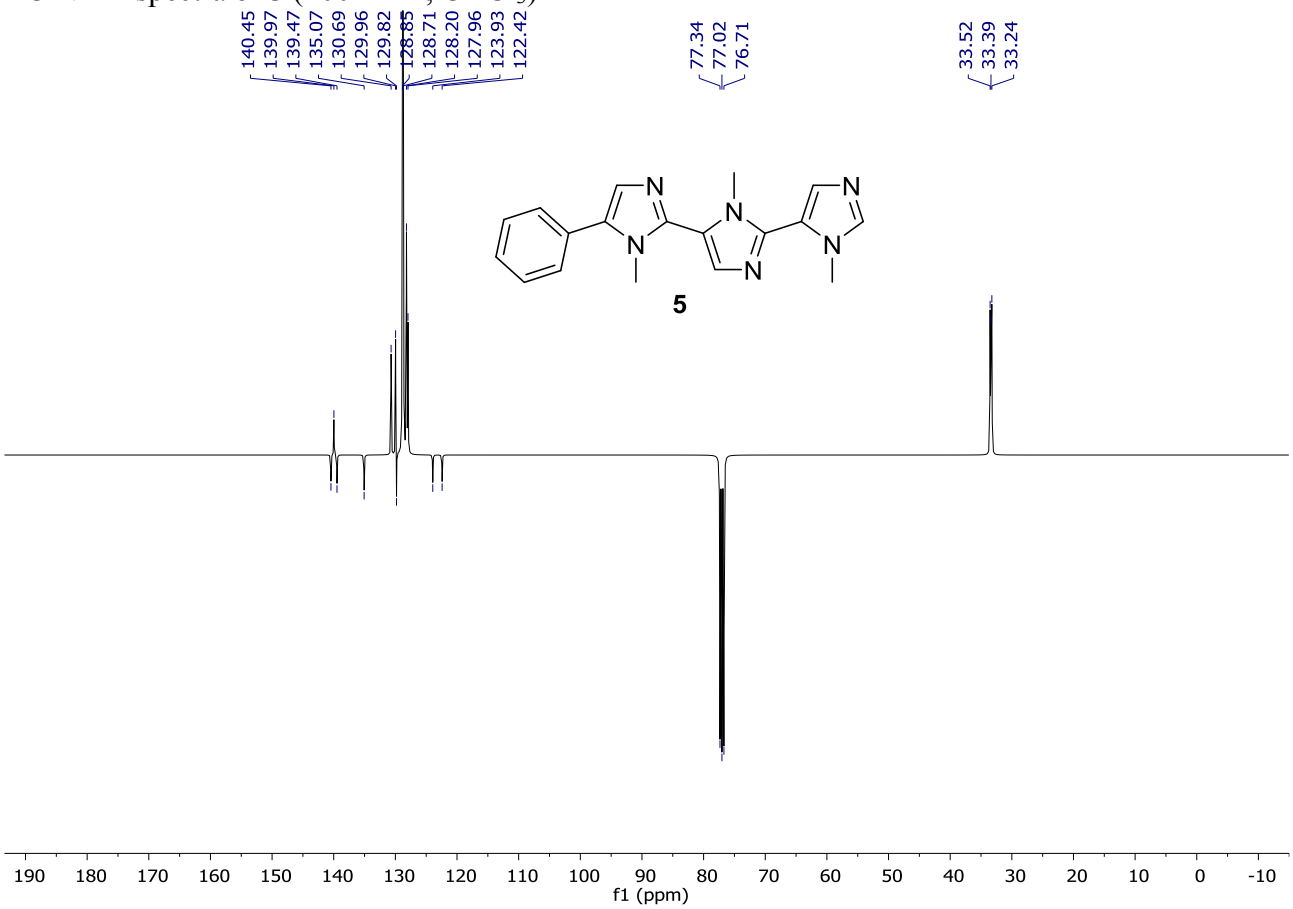
4

^{1}H NMR spectra of **5** (400 MHz, $CDCl_3$)



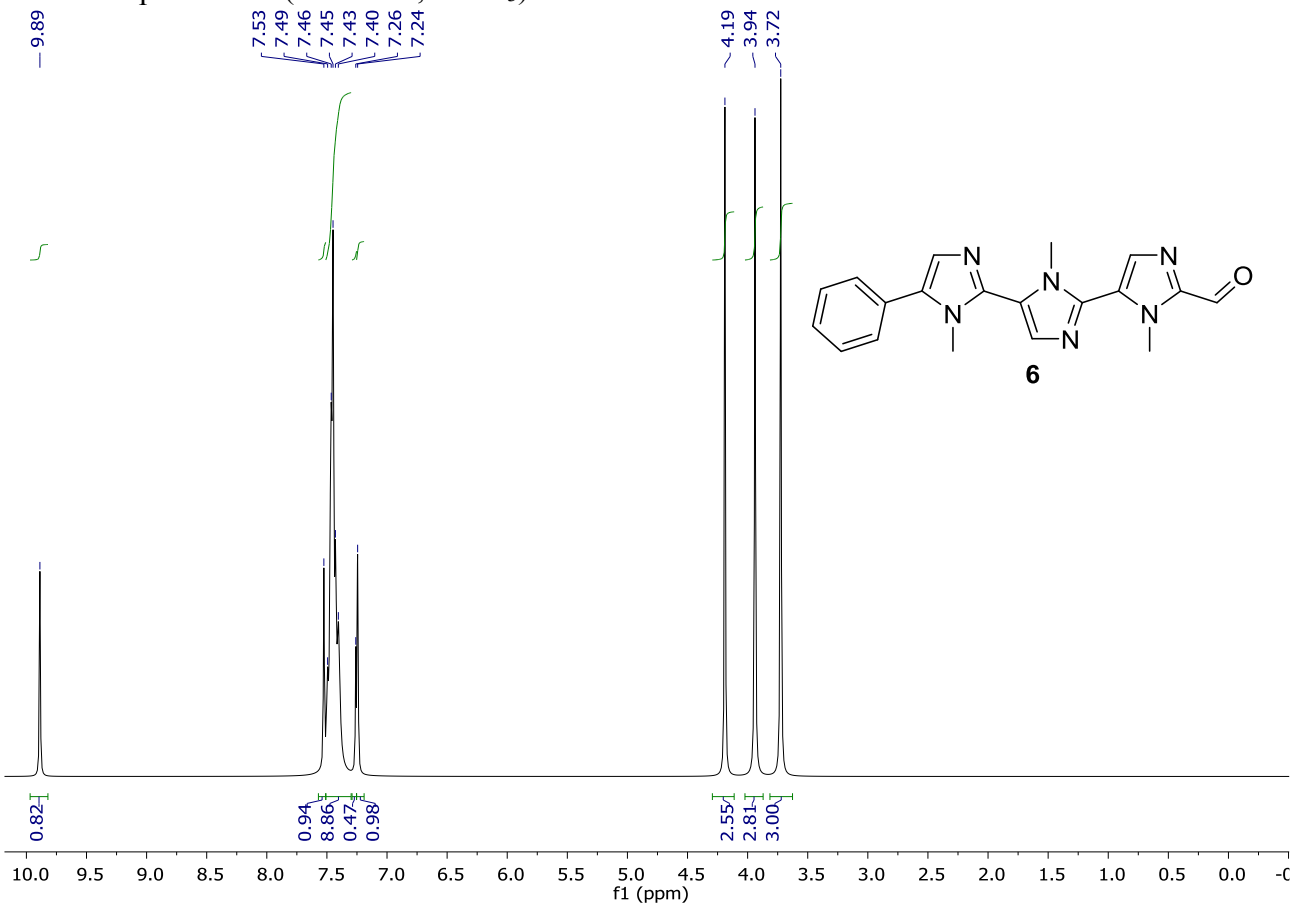
5

^{13}C NMR spectra of **5** (100 MHz, $CDCl_3$)



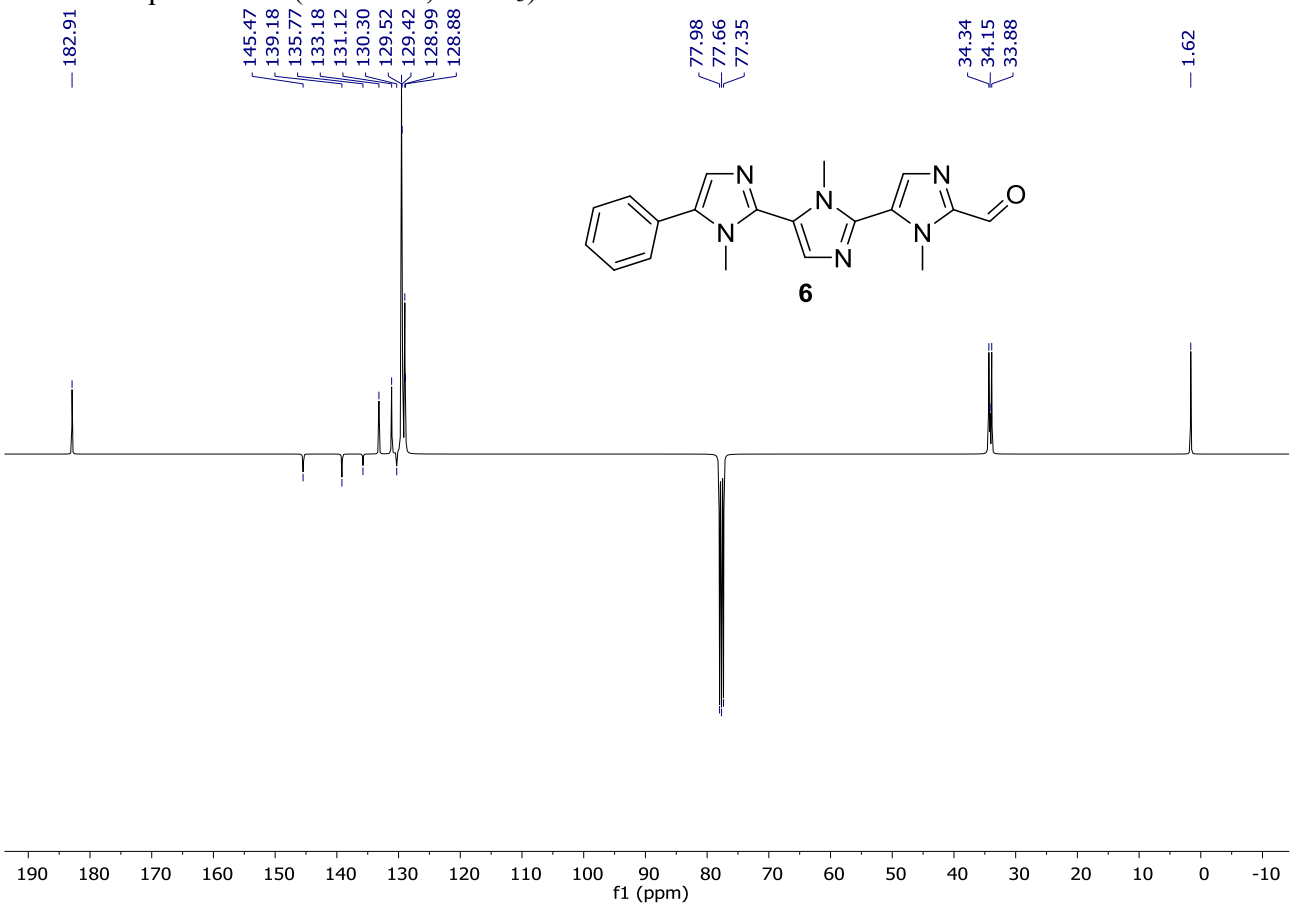
5

^{1}H NMR spectra of **6** (400 MHz, $CDCl_3$)



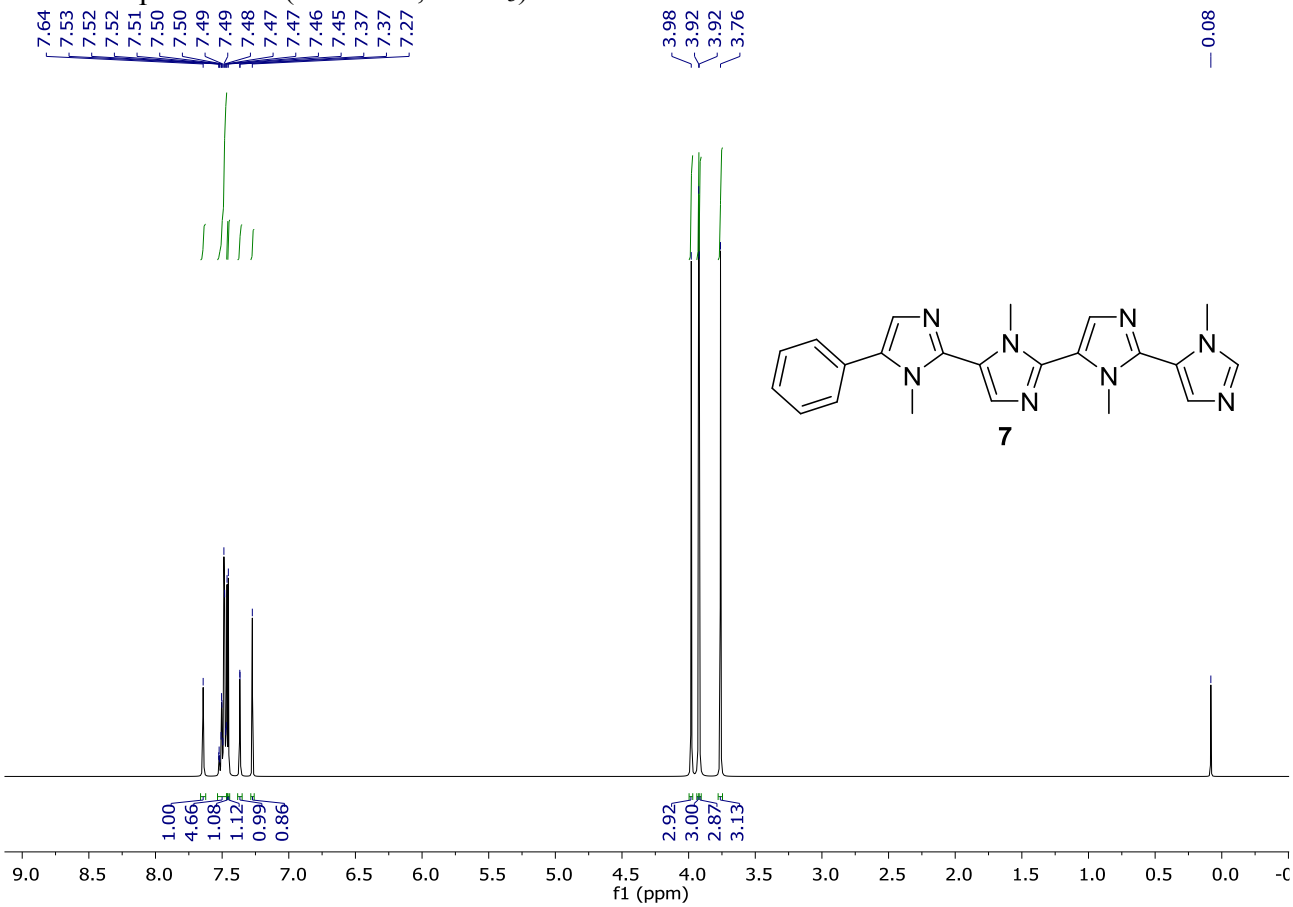
6

^{13}C NMR spectra of **7** (100 MHz, $CDCl_3$)

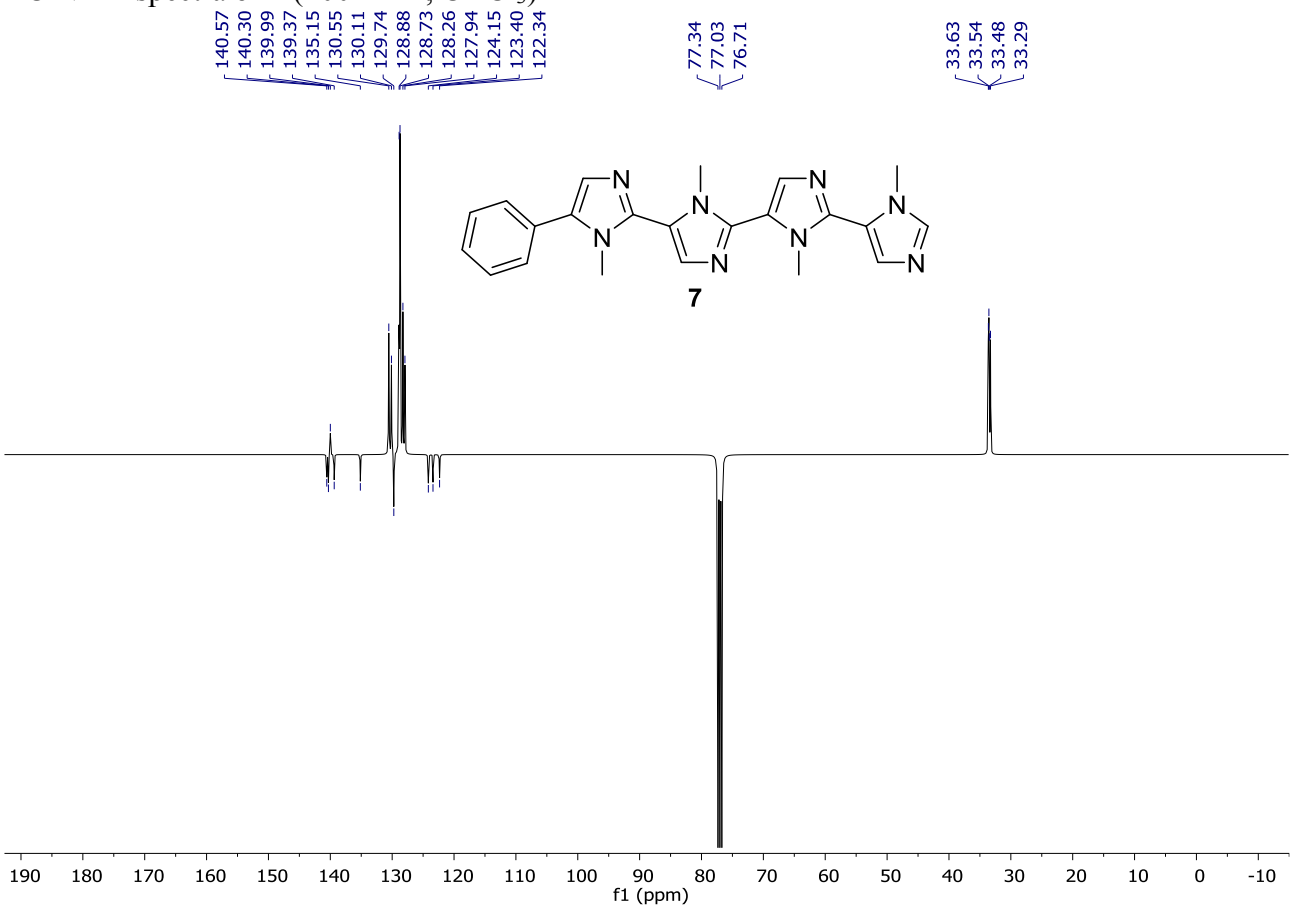


6

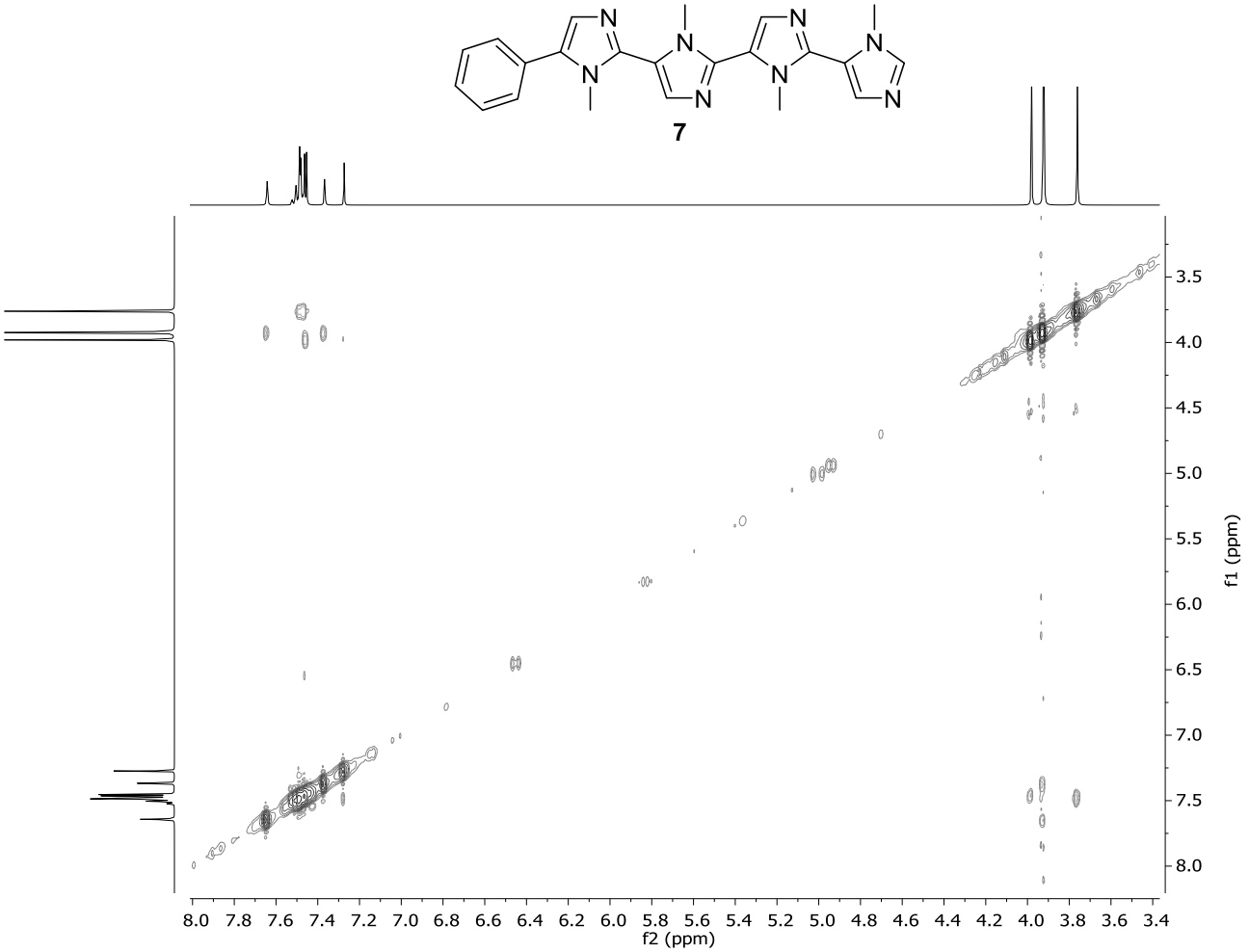
^{1}H NMR spectra of **7** (400 MHz, $CDCl_3$)



^{13}C NMR spectra of **7** (100 MHz, $CDCl_3$)



^{1}H-^{1}H NOESY NMR spectra of **7** (400, 400 MHz, $CDCl_3$)



Computational methods

The starting geometry of **7**, created by GaussView,[2] was energy minimized by conjugate gradient algorithm implemented in Gaussion09.[2] Thus, the optimized geometry was subjected to heating, equilibration, and molecular dynamics simulation by *sander* module of AMBER 12.[3] GAFF force field were used to assign the computational parameters of **7**, simulated as neutral compound in an implicit GB solvent model and a dielectric continuum of 80 (simulating water). With a time step 2 fs, the peptidomimetic was heated to 300 K, 700 K and then to 1000 K over 20 ps. After an equilibration phase of 2 ns, the model was then frozen to 700 K and finally to 300 K, over 20 ps. In the production run, 20 ns of the MD simulations were performed, recording frames every 10 ps.

The resulting trajectory was visually analyzed by VMD.[4] In this stage, the fluctuation of the torsion angles separating the imidazole rings were evaluated and the distribution percentage were calculated with a resolution of 30° (**Figure 3**, main text). The conformation presenting all dihedral angles of 180° was again minimized by Gaussian09 at DFT/B3LYP/6-31g(d) level of theory and, in this geometry, the C^{β}-C^{β} distances were measured.

Furthermore, the low energy conformer of **7** was superimposed on a generic poly-alanine β-strand (**Figure 4** in the main text).

Table S1. Average C^{β}-C^{β} distances, measured during MD simulations, for peptidomimetic **7** compared with common secondary structure. When the differences between the values measured for **7** and the ones known for the secondary structures are lower than 0.3 Å, boxes have been highlighted in red. Table was adapted from *ref*. 5.

Structure	Sequence	C^{β}-C^{β} distances (Å)	7
α-Helix	*i-i+1*	5,2	5.5
	i-i+2	7,1	7.3
	i-i+3	5,6	11.4
	i-i+4	6,5	
	i-i+5	9,8	
	i-i+6	10,8	
	i-i+7	10,7	
	i-i+8	12,9	
β-Sheet (parallel)	*i-i+1*	5,8	5.5
	i-i+2	7,1	7.3
	i-i+3	11,1	11.4
	i-i+4	13,2	
	i-i'	5,5	
	i-i'+1	7,2	
	i-i'+2	9	
	i-i'+3	11,5	
	i-i'+4	14,4	
β-Sheet (anti-parallel)	*i-i+1*	5,8	5.5
	i-i+2	6,5	7.3
	i-i+3	11.0	11.4
	i-i+4	12,8	
	i-i'	4,5	
	i-i'+1	7,6	
	i-i'+2	8,9	
	i-i'+3	12,7	
	i-i'+4	14,8	
γ-Turn (type-1)	*i-i+1*	5,7	5.5
	i-i+2	5,1	7.3
	i-i+3	5,4	11.4
	i+1-i+2	5,2	
	i+1-i+3	7,5	
	i+2-i+3	5,6	
γ-Turn (classic)	*i-i+1*	4,7	5.5
	i-i+2	7,2	7.3
	i+1-i+2	5,1	
γ-Turn (inverse)	*i-i+1*	5,7	5.5
	i-i+2	5,4	7.3
	i+1-i+2	6,2	

Biological studies

In vitro PCSK9-LDLR binding assay. The compound ability to interfere with the PCSK9-LDLR binding were tested using the in vitro PCSK9-LDLR binding assay (CycLex Co., Nagano, Japan) following the manufacture instructions as already described by us.[6] Briefly, **7**, dissolved in DMSO, is tested at the concentration range of 10^{-7} – 10^{-3} M. Plates are pre-coated with a recombinant LDLR-AB domain, which contains the binding site for PCSK9. Before starting the assay, tested compounds and/or the vehicle were diluted in reaction buffer and added in microcentrifuge tubes. Afterwards, the reaction mixtures were added in each well of the microplate and the reaction was started by adding His-tagged PCSK9 wild type solution (3 µl). The microplate was allowed to incubate for 2 h at room temperature (RT) shaking at 300 rpm on an orbital microplate shaker. Subsequently, wells were washed 4 times with wash buffer. After the last wash, the biotinylated anti-His-tag monoclonal antibody (100 µl) was added and incubated at RT for 1 h shaking at 300 rpm. After incubation, wells were washed for 4 times with wash buffer. After the last wash, 100 µl of HRP-conjugated streptavidin were added and the plate was incubated for 20 min at RT. After incubation, wells were washed 4 times with wash buffer. Finally, the substrate reagent (tetra-methylbenzidine) was added and the plate was incubated for 10 min at RT shaking at ca. 300 rpm. The reaction was stopped with 2.0 N sulfuric acid and the absorbance at 450 nm was measured using the Synergy H1 fluorescent plate reader (Biotek, Bad Friedrichshall, Germany).

Cell line culture. The HepG2 cell line was bought from ATCC (HB-8065, ATCC from LGC Standards, Milan, Italy). HepG2 cells were cultured in DMEM high glucose with stable L-glutamine supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin (complete growth medium) and incubated at 37 °C under 5% CO_2 atmosphere. HepG2 cells were used for no more than 20 passages after thawing, because the increase of the number of passages may change the cell characteristics and impair assay results.
MTT. 3 x 10^4 HepG2 cells/well were seeded in 96-well plates and treated with 1, 10, 50, and 100 µM of compound **7**, respectively, or vehicle (DMSO) in complete growth media for 24 h. Subsequently, the treatment solvent was aspirated and 100 µL/well of MTT filtered solution added for 2 h. After the incubation time, 0.5 mg/mL MTT solution was aspirated and 100 µL/well of MTT lysis buffer (8 mM HCl + 0.5% NP-40 in DMSO) added. After 5 min of slow shaking, the absorbance at 575 nm was read using the Synergy H1 fluorescent plate reader (Biotek, Bad Friedrichshall, Germany).

Fluorescent LDL uptake cell based assay. HepG2 cells (3 x 10^4/well) were seeded in 96-well plates and kept in complete growth medium for 2 d before treatment. The third day, they were treated with 1, 10, 50, and 100 µM of compound **7**, respectively, or vehicle (DMSO) for 24 h. At the end of the treatment, the culture medium was replaced with 50 µl/well LDL-DyLight™ 550 working solution (Cayman Chemical Company, Ann Arbor, MI, US). The cells were additionally incubated for 2 h at 37 °C and then the culture medium was aspirated and replaced with PBS (100 µl/well). The degree of LDL uptake was measured using the Synergy H1 fluorescent plate reader from Biotek (excitation and emission wavelengths 540 and 570 nm, respectively).

Statistical analysis of biological assays. Data are presented as mean ± standard error using GraphPad Prism 6 (GraphPad, La Jolla, CA, USA). Statistical analyses were carried out by one-way ANOVA followed by Dunnett's test. P-values < 0.05 were considered to be significant.

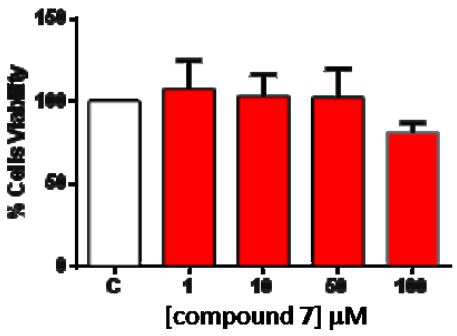


Figure S1. Bar graphs indicating the results of MTT cell viability assay of HepG2 cells after compound **7** treatment for 24 h. The data points represent the averages ± SEM of three independent experiments in triplicate.

References

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