Supplementary Information

Synthesis of 2-methoxybenzamide derivatives and evaluation of their hedgehog signaling pathway inhibition

Chiyu Sun,* Dajun Zhang, Tian Luan, Youbing Wang, Wenhu Zhang, Lin Lin, Meihua Jiang, Ziqian Hao, Ying Wang

School of Pharmacy, Shenyang Medical College, Shenyang 110034, China

Experimental Section

1.1.General

All chemicals were of analytical grade and purchased from commercial sources. All the reactions were monitored using thin layer chromatography (TLC) on silica gel plates. Melting points were determined on a Melting Points SGWX-4B apparatus (INESA, Shanghai, China). ^{1}H NMR spectra and ^{13}C NMR spectra have been acquired with a Bruker Avance 400 spectrometer (Bruker Bioscience, MA, USA). Mass spectra (MS) were recorded on Agilent 1100 LC-MS spectrometer (Palo Alto, CA, USA.). Elemental analysis was determined on a Carlo-Erba 1106 Elemental analysis instrument (Carlo Erba, Milan, Italy).

1.2. Synthesis

Procedure for preparation of **5a**-**g**

To a solution of substituted benzoic acid or nicotinic acid (30 mmoL) in anhydrous toluene was added thionyl chloride (4.7 g, 40 mmoL) and catalytic amount of pyridine. The mixture was stirred under reflux for 6 hours at 80 °C. The solvent was evaporated in vacuum to give white solids, which redissolved in 30 mL of DMF for next step without further purification. The crude acyl chloride were added into a solution of methyl 4-amino-2-methoxybenzoate (5.4 g, 30 mmoL) and TEA (40 mmoL) in DMF. The reaction mixture was stirred at room temperature overnight. The mixture was filtered and the filtrate was concentrated in vacuum. The residue was rinsed with water and dried to afford **5a**-**g** as white solids.

Procedure for preparation of **6a**-**g**

A mixture of intermediates **5a**-**g** (30 mmoL), sodium hydroxide (4.3 g, 0.1 moL), aqueous ethanol (60 mL) was stirred at 40 °C for 5 hours. The solvent was evaporated in vacuum and the residue was poured into water. The mixture was neutralized to pH = 6 with saturated citric acid solution. The mixture was filtered to give white solids **6a**-**g**.

4-(2-chlorobenzamido)-2-methoxybenzoic acid (**6a**): Yield 88%. ESI-MS m/z: 304.0 $[M-H]^+$. ^1H NMR (400 MHz, DMSO) δ 11.92 (s, 1H), 9.76 (s, 1H), 8.31 (s, 1H), 7.59 – 7.62 (m, 2H), 7.51 – 7.56 (m, 4H), 3.95 (s, 3H).

4-(2,4-dichlorobenzamido)-2-methoxybenzoic acid (**6b**) : Yield 85%. ESI-MS m/z: 338.0 $[M-H]^+$.

4-(3-fluorobenzamido)-2-methoxybenzoic acid (**6c**) : Yield 92%. ESI-MS m/z: 288.0 $[M-H]^+$.

4-(4-fluorobenzamido)-2-methoxybenzoic acid (**6d**) : Yield 90%. ESI-MS m/z: 288.0 $[M-H]^+$.

4-(2-chloronicotinamido)-2-methoxybenzoic acid (**6e**) : Yield 87%. ESI-MS m/z: 305.0 $[M-H]^+$.

4-(6-chloronicotinamido)-2-methoxybenzoic acid (**6f**): Yield 83%. ESI-MS m/z: 305.0 $[M-H]^+$.

2-methoxy-4-(nicotinamido)benzoic acid (**6g**): Yield 91%. ESI-MS m/z: 271.0 $[M-H]^+$.

Preparation of 2-chloro-5-nitrobenzimidamide hydrochloride (**7**)

To a solution of 2-chloro-5-nitrobenzonitrile (14.5g, 80 mmol) in ethanol (150 mL) was added sodium methoxide in methanol (0.8 g, 16mmol), and the mixture was stirred at -20 °C for 2h. Ammonium chloride (5 g, 90 mmol) was added, and the mixture was further stirred at 40 °C for another 3h. The reaction mixture was concentrated in vacuum, the residue was dissolved in water and acidified with 1 M HCl. The precipitated solid was collected by filtration, washed with ethyl acetate to give the title compound (7.3 g, 46%) as a white solid.

Preparation of 2-(2-chloro-5-nitrophenyl)-5-phenyl-1H-imidazole (**8**)

To a mixture of **7** (7 g, 30 mmol), sodium bicarbonate (4.9 g, 50 mmol) and THF (80 mL) was added bromoacetophenone (5.94 g, 30 mmol) at 40 °C in batches, and the mixture was stirred overnight. The mixture was poured into water (300 mL) and extracted with ethyl acetate. The organic phase was washed with brine, dried over sodium sulfate and concentrated in vacuum. After recrystallization with dichloromethane, the title compound (5.6 g, 65 %) was obtained as a pale yellow solid. ESI-MS (m/z) 621.13$[2M+Na]^+$. ^1H NMR(400 MHz, DMSO) δ 12.43 (s, 1H), 8.72 (d, J=2.7 Hz, 1H), 8.28 (dd, J=8.7 Hz, 1H), 7.92 (d, J=8.7Hz, 1H) , 7.91 (d, J = 7.5 Hz, 2H), 7.57 (d, J = 8.8 Hz, 1H), 7.46-7.52 (m, 3H).

Preparation of 4-chloro-3-(5-phenyl-1H-imidazol-2-yl)aniline (**9**)

To a solution of **8** (6 g, 20 mmol) in ethanol (100 ml) was added stannous chloride (5.8 g, 30 mmol) and 1M HCl (10 ml). The mixture refluxed at 80 °C for 3h.

The solvent was evaporated in vacuum, neutralized with 3M sodium hydroxide solution and then extracted with ethyl acetate (200 mL) twice. The combined organic phase was washed with saturated sodium chloride solution and then dried over sodium sulfate. The mixture was filtered and the filtrate was concentrated in vacuum to give the title compound (4.62 g, 86%) as pale yellow solid. ESI-MS (m/z) 270.08$[M+H]^+$. ^{1}H NMR(400 MHz, DMSO) δ 12.45 (s, 1H), 7.88 (d, J = 7.5 Hz, 2H), 7.42-7.50 (m, 4H), 7.56 (d, J = 8.8 Hz, 1H), 7.11 (d, J=3.0 Hz, 1H), 6.70 (dd, J=8.7 Hz, 1H), 5.45 (s, 2H).

General procedure for preparation of target compounds of **10**-**23**

A solution of 3-(1H-benzo[d]imidazol-2-yl)-4-chloroaniline or **9** (5 mmoL), **6a**-**g** (5 mmoL), HATU (1.9g, 5 mmoL) and N,N-diisopropylethylamine (5 mmoL) in dichloromethane (20 mL) stirred at room temperature for 24h. After filtration, the mixture was evaporated in vacuum. The residue was chromatographed on a silica gel column, eluted with petroleum ether /ethyl acetate (8:1, V/V) to give compounds **10**-**23**.

N-(3-(1H-benzo[d]imidazol-2-yl)-4-chlorophenyl)-4-(2-chlorobenzamido)-2-methoxybenzamide (**10**): white solid, 75% yield, m.p. 280-283 °C. ESI-MS (m/z): 531.0 $[M+H]^+$. ^{1}H NMR(400 MHz, DMSO) δ 12.74 (s, 1H), 10.80 (s, 1H), 10.26 (s, 1H), 8.37 (s, 1H), 7.94 (d, J = 8.6 Hz, 1H), 7.77 – 7.71 (m, 3H), 7.63 (d, J = 8.6 Hz, 2H), 7.59 (s, 1H), 7.55 (t, J = 7.5 Hz, 1H), 7.50 (d, J = 7.4 Hz, 1H), 7.41 (d, J = 8.2 Hz, 1H), 7.26 (t, J = 7.8 Hz, 2H), 3.94 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 169.7, 166.5, 157.9, 153.5, 141.5, 138.3(2C), 136.1, 133.5, 133.2, 132.3, 131.6, 130.5, 130.1, 128.9, 128.7, 128.1, 127.9, 126.4 (2C), 125.8, 123.2, 122.7, 115.5 (2C), 114.8, 108.6, 55.7. Anal. calcd. for $C_{28}H_{20}Cl_2N_4O_3$ (%): C, 63.29; H, 3.79; N, 10.54. Found (%): C, 63.32; H, 3.83; N, 10.51.

N-(3-(1H-benzo[d]imidazol-2-yl)-4-chlorophenyl)-4-(2,4-dichlorobenzamido)-2-methoxybenzamide (**11**): white solid, 71% yield, m.p. 230-232°C. ESI-MS (m/z): 565.0 $[M+H]^+$. ^{1}H NMR(400 MHz, DMSO) δ 10.90 (s, 1H), 10.45 (s, 1H), 8.50 (d, *J* = 2.5 Hz, 1H), 8.06 (d, *J* = 8.9 Hz, 1H), 7.89 (dd, *J* = 6.1, 3.1 Hz, 2H), 7.81 – 7.75 (m, 3H), 7.72 (s, 1H), 7.68 (d, *J* = 8.2 Hz, 1H), 7.61 – 7.57 (m, 3H), 7.41 (d, *J* = 9.5 Hz, 1H), 3.96 (s, 3H). Anal. calcd. for $C_{28}H_{19}Cl_3N_4O_3$ (%): C, 59.44; H, 3.38; N, 9.90. Found (%): C, 59.46; H, 3.41; N, 9.96.

N-(3-(1H-benzo[d]imidazol-2-yl)-4-chlorophenyl)-4-(3-fluorobenzamido)-2-methoxybenzamide (**12**): white solid, 83% yield, m.p. 195-197 °C. ESI-MS (m/z): 515.1$[M+H]^+$. ^{1}H NMR(400 MHz, DMSO) δ 12.74 (s, 1H), 10.56 (s, 1H), 10.28 (s, 1H), 8.37 (d, J = 2.0 Hz, 1H), 7.94 (dd, J = 8.9, 2.2 Hz, 1H), 7.84 (dd, J = 15.6, 8.8 Hz, 3H),

7.78 – 7.74 (m, 3H), 7.57 – 7.51 (m, 2H), 7.48 (d, J = 6.9 Hz, 1H), 7.29 – 7.23 (m, 2H), 3.96 (s, 3H). Anal. calcd. for $C_{28}H_{20}ClFN_4O_3$ (%): C, 65.31; H, 3.92; N, 10.88. Found (%): C, 65.37; H, 3.89; N, 10.91.

N-(3-(1H-benzo[d]imidazol-2-yl)-4-chlorophenyl)-4-(4-fluorobenzamido)-2-methoxybenzamide (**13**): white solid, 85% yield, m.p. 234-235 °C. ESI-MS (m/z): 515.1 $[M+H]^+$. ^1H NMR(400 MHz, DMSO) δ 12.77 (s, 1H), 10.54 (s, 1H), 10.28 (s, 1H), 8.36 (d, *J* = 2.3 Hz, 1H), 8.08 (dd, *J* = 8.6, 5.5 Hz, 2H), 7.94 (dd, *J* = 8.8, 2.3 Hz, 1H), 7.78 – 7.75 (m, 2H), 7.73 (d, *J* = 7.7 Hz, 1H), 7.62 (d, *J* = 8.8 Hz, 1H), 7.59 (s, 1H), 7.54 (dd, *J* = 8.5, 1.2 Hz, 1H), 7.40 (t, *J* = 8.8 Hz, 2H), 7.26 (t, *J* = 7.8 Hz, 2H), 3.95 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 169.3, 166.0, 162.7, 157.3, 154.3, 141.1, 137.4(2C), 135.8, 133.0, 130.7, 129.8, 129.3, 129.1, 127.8 (2C), 127.5, 126.5 (2C), 123.6, 122.9, 116.5 (2C),115.9 (2C), 115.3, 109.1, 55.2. Anal. calcd. for $C_{28}H_{20}ClFN_4O_3$ (%): C, 65.31; H, 3.92; N, 10.88. Found (%): C, 65.35; H, 3.97; N, 10.93.

N-(4-((3-(1H-benzo[d]imidazol-2-yl)-4-chlorophenyl)carbamoyl)-3-methoxyphenyl)-2-chloronicotinamide (**14**): white solid, 80% yield, m.p. 192-193 °C. ESI-MS *m/z*: 532.0 $[M+H]^+$. ^1H NMR (400 MHz, DMSO) δ 12.76 (s, 1H), 10.49 (s, 1H), 10.27 (s, 1H), 8.36 (d, *J* = 5.0 Hz, 2H), 8.07 (dd, *J* = 7.4, 1.8 Hz, 1H), 7.95 (dd, *J* = 8.8, 2.5 Hz, 1H), 7.76 (d, *J* = 8.5 Hz, 2H), 7.64 (t, *J* = 8.4 Hz, 3H), 7.40 (d, *J* = 8.4 Hz, 1H), 7.26 (dd, *J* = 6.0, 3.1 Hz, 2H), 7.18 (dd, *J* = 7.3, 5.0 Hz, 1H), 3.95 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 169.3, 166.7, 157.2, 154.5, 146.5, 145.2, 141.3, 137.8(2C), 137.5, 135.8, 134.3, 133.1, 130.2, 129.1, 128.8, 127.6, 126.3 (2C), 123.9, 123.6, 122.6, 115.7 (2C), 115.2, 108.9, 55.8. Anal. calcd. for $C_{27}H_{19}Cl_2N_5O_3$ (%): C, 60.91; H, 3.60; N, 13.16. Found (%): C, 60.88; H, 3.67; N, 13.22.

N-(4-((3-(1H-benzo[d]imidazol-2-yl)-4-chlorophenyl)carbamoyl)-3-methoxyphenyl)-6-chloronicotinamide (**15**): white solid, 73% yield, m.p. 182-183 °C. ESI-MS (m/z): 532.09$[M+H]^+$. ^1H NMR(400 MHz, DMSO) δ 12.76 (s, 1H), 10.49 (s, 1H), 10.27 (s, 1H), 8.36 (d, *J* = 5.0 Hz, 2H), 8.07 (dd, *J* = 7.4, 1.8 Hz, 1H), 7.95 (dd, *J* = 8.8, 2.5 Hz, 1H), 7.76 (d, *J* = 8.5 Hz, 2H), 7.64 (t, *J* = 8.4 Hz, 3H), 7.40 (d, *J* = 8.4 Hz, 1H), 7.26 (dd, *J* = 6.0, 3.1 Hz, 2H), 7.18 (dd, *J* = 7.3, 5.0 Hz, 1H), 3.95 (s, 3H). Anal. calcd. for $C_{27}H_{19}Cl_2N_5O_3$ (%): C, 60.91; H, 3.60; N, 13.16. Found (%): C, 60.93; H, 3.64; N, 13.20.

N-(4-((3-(1H-benzo[d]imidazol-2-yl)-4-chlorophenyl)carbamoyl)-3-methoxyphenyl)nicotinamide (**16**): white solid, 86% yield, m.p. 242-244 °C. ESI-MS *m/z*: 498.1 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6) (ppm): δ 12.73 (s, 1H), 10.69 (s, 1H), 10.28 (s, 1H), 9.15 (s, 1H), 8.80 (d, *J* = 4.9 Hz, 1H), 8.37 (d, *J* = 2.5 Hz, 1H), 8.34 (d, *J* = 8.2 Hz, 1H), 7.94 (dd, *J* = 8.8, 2.4 Hz, 1H), 7.77 (d, *J* = 8.1 Hz, 2H), 7.73 (d, *J* = 7.0 Hz,

1H), 7.65-7.62 (m, 1H), 7.60 (d, *J* = 5.1 Hz, 2H), 7.53 (dd, *J* = 8.5, 1.5 Hz, 1H), 7.30 - 7.22 (m, 2H), 3.96 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 169.6, 166.4, 157.7, 153.8, 146.5, 145.6, 141.6, 138.2(2C), 135.9, 134.2, 133.5, 131.8, 130.2, 129.3, 129.1, 128.2, 126.1 (2C), 124.9, 123.7, 122.5, 115.6 (2C), 115.3, 108.4, 55.3. Anal. calcd. for $C_{27}H_{20}ClN_5O_3$ (%): C, 65.13; H, 4.05; N, 14.06. Found (%): C, 65.16; H, 3.97; N, 14.10.

N-(4-chloro-3-(5-phenyl-1H-imidazol-2-yl)phenyl)-4-(2-chlorobenzamido)-2-methoxybenzamide (**17**): white solid, 79% yield, m.p. 157-158 °C. ESI-MS m/z: 557.0 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO) δ 12.45 (s, 1H), 10.78 (s, 1H), 10.22 (s, 1H), 8.28 (d, J = 2.5 Hz, 1H), 7.92 - 7.83 (m, 3H), 7.80 (d, J = 2.1 Hz, 1H), 7.78 - 7.70 (m, 2H), 7.62 (ddd, J = 12.3, 7.6, 1.3 Hz, 2H), 7.56 (d, J = 8.6 Hz, 2H), 7.50 (dd, J = 7.4, 1.3 Hz, 1H), 7.40 (dt, J = 13.8, 7.1 Hz, 3H), 7.23 (t, J = 7.4 Hz, 1H), 3.94 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 169.4, 166.5, 153.5, 149.0, 141.5, 140.7, 135.2, 133.2, 133.1, 132.6, 132.5, 131.8, 130.7 (2C), 130.4, 130.2, 129.6, 129.4, 128.8, 128.6(2C), 128.1, 127.8, 125.7, 123.5, 123.2, 122.1, 115.0, 108.6, 55.7. Anal. calcd. for $C_{30}H_{22}Cl_2N_4O_3$ (%): C, 64.64; H, 3.98; N, 10.05. Found (%): C, 65.16; H, 3.97; N, 10.09.

N-(4-chloro-3-(5-phenyl-1H-imidazol-2-yl)phenyl)-4-(2,4-dichlorobenzamido)-2-methoxybenzamide (**18**): white solid, 76% yield, m.p. > 280 °C. ESI-MS m/z: 591.0 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO) δ 12.50 (s, 1H), 10.83 (s, 1H), 10.23 (s, 1H), 8.25 (s, 1H), 7.91 - 7.84 (m, 3H), 7.80 (d, J = 1.9 Hz, 1H), 7.76 (d, J = 8.4 Hz, 2H), 7.71 - 7.66 (m, 2H), 7.62 - 7.53 (m, 2H), 7.40 (t, J = 7.7 Hz, 3H), 7.24 (t, J = 7.3 Hz, 1H), 3.94 (s, 3H). Anal. calcd. for $C_{30}H_{21}Cl_3N_4O_3$ (%): C, 60.88; H, 3.58; N, 9.47. Found (%): C, 60.95; H, 3.64; N, 9.51.

N-(4-chloro-3-(5-phenyl-1H-imidazol-2-yl)phenyl)-4-(3-fluorobenzamido)-2-methoxybenzamide (**19**): white solid, 84% yield, m.p. 245 °C. ESI-MS m/z: 541.1 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO) δ 12.48 (s, 1H), 10.60 (s, 1H), 10.25 (s, 1H), 8.29 (s, 1H), 7.86 (dd, J = 9.4, 4.4 Hz, 4H), 7.79 (dd, J = 18.8, 6.7 Hz, 4H), 7.63 (dd, J = 13.9, 7.9 Hz, 1H), 7.56 (d, J = 8.6 Hz, 2H), 7.49 (td, J = 8.6, 2.0 Hz, 1H), 7.39 (t, J = 7.4 Hz, 2H), 7.23 (t, J = 7.0 Hz, 1H), 3.96 (s, 3H). Anal. calcd. for $C_{30}H_{22}ClFN_4O_3$ (%): C, 66.61; H, 4.10; N, 10.36. Found (%): C, 66.70; H, 4.05; N, 10.40.

N-(4-chloro-3-(5-phenyl-1H-imidazol-2-yl)phenyl)-4-(4-fluorobenzamido)-2-methoxybenzamide (**20**): white solid, 82% yield, m.p. 164-165 °C. ESI-MS (m/z): 541.1$[M+H]^+$. ^{1}H NMR(400 MHz, DMSO) δ 12.45 (s, 1H), 10.51 (s, 1H), 10.23 (s, 1H), 8.27 (s, 1H), 8.11 – 8.06 (m, 2H), 7.91 – 7.78 (m, 4H), 7.78 – 7.74 (m, 2H), 7.55 (t, J = 8.3 Hz, 2H), 7.44 – 7.37 (m, 4H), 7.23 (t, J = 7.2 Hz, 1H), 3.96 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 169.9, 166.7, 163.1, 154.1, 149.6, 142.1, 140.5, 135.4, 133.2, 132.3,

131.0 (2C), 130.8, 129.8, 129.3, 129.0, 128.7(2C), 128.5, 128.2, 127.3(2C), 123.1, 122.9, 122.6, 116.8(2C), 115.3, 108.2, 55.1. Anal. calcd. for $C_{30}H_{22}ClFN_4O_3$ (%): C, 66.61; H, 4.10; N, 10.36. Found (%): C, 66.59; H, 4.12; N, 10.32.

N-(4-chloro-3-(5-phenyl-1H-imidazol-2-yl)phenyl)-4-(2-chloronicotinamido)-2-methoxybenzamide (**21**): white solid, 81% yield, m.p. 237-239 °C. ESI-MS m/z: 558.1 $[M+H]^+$. ^1H NMR (400 MHz, DMSO) δ 12.45 (s, 1H), 10.48 (s, 1H), 10.23 (s, 1H), 8.37 (dd, J = 4.9, 1.9 Hz, 1H), 8.27 (d, J = 2.5 Hz, 1H), 8.07 (dd, J = 7.4, 1.9 Hz, 1H), 7.87 (dd, J = 8.7, 4.9 Hz, 3H), 7.80 (d, J = 2.1 Hz, 1H), 7.75 (d, J = 8.4 Hz, 2H), 7.56 (d, J = 8.7 Hz, 1H), 7.39 (t, J = 7.8 Hz, 3H), 7.23 (t, J = 7.3 Hz, 1H), 7.18 (dd, J = 7.4, 5.0 Hz, 1H), 3.95 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 169.5, 166.4, 153.8, 149.2, 146.1, 144.9, 141.6, 140.6, 137.2, 135.3, 134.6, 132.5, 133.2, 130.9 (2C), 130.5, 129.6, 129.1, 128.9, 128.7(2C), 127.9, 123.6, 123.3, 123.8, 122.4, 115.2, 108.3, 55.7. Anal. calcd. for $C_{29}H_{21}Cl_2N_5O_3$ (%): C, 62.38; H, 3.79; N, 12.54. Found (%): C, 62.43; H, 3.82; N, 12.56.

N-(4-chloro-3-(5-phenyl-1H-imidazol-2-yl)phenyl)-4-(6-chloronicotinamido)-2-methoxybenzamide (**22**): white solid, 77% yield, m.p. 254-255°C. ESI-MS (m/z): 558.1$[M+H]^+$. ^1H NMR(400 MHz, DMSO) δ 12.48 (s, 1H), 10.72 (s, 1H), 10.25 (s, 1H), 9.00 (d, J = 2.0 Hz, 1H), 8.40 (dd, J = 8.3, 2.3 Hz, 1H), 8.26 (s, 1H), 7.87 (d, J = 6.2 Hz, 3H), 7.78 (s, 1H), 7.74 (s, 2H), 7.56 (d, J = 8.7 Hz, 1H), 7.52 (d, J = 8.5 Hz, 1H), 7.40 (t, J = 7.5 Hz, 3H), 7.25 (d, J = 7.3 Hz, 1H), 3.96 (s, 3H). Anal. calcd. for $C_{29}H_{21}Cl_2N_5O_3$ (%): C, 62.38; H, 3.79; N, 12.54. Found (%): C, 62.36; H, 3.77; N, 12.58.

N-(4-chloro-3-(5-phenyl-1H-imidazol-2-yl)phenyl)-4-nicotinamido-2-methoxybenzamide (**23**): white solid, 83 % yield, m.p. > 280 °C. ESI-MS m/z: 524.2 $[M+H]^+$. ^1H NMR (400 MHz, DMSO) δ 12.45 (s, 1H), 10.69 (s, 1H), 10.25 (s, 1H), 9.15 (s, 1H), 8.80 (d, J = 4.7 Hz, 1H), 8.34 (d, J = 8.1 Hz, 1H), 8.27 (d, J = 2.3 Hz, 1H), 7.88 (d, J = 7.3 Hz, 3H), 7.80 (d, J = 2.0 Hz, 1H), 7.78 - 7.74 (m, 2H), 7.61 (dd, J = 7.9, 4.9 Hz, 1H), 7.54 (dd, J = 12.2, 8.7 Hz, 2H), 7.39 (t, J = 7.6 Hz, 2H), 7.23 (t, J = 7.3 Hz, 1H), 3.96 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 169.4, 166.5, 153.9, 149.3, 146.4, 145.7, 141.7, 140.7, 135.1, 134.2, 133.1, 132.6, 131.3, 130.7 (2C), 130.4, 129.5, 129.2, 128.8, 128.6(2C), 128.1, 124.5, 123.5, 123.2, 122.3, 115.1, 108.5, 55.6. Anal. calcd. for $C_{29}H_{22}ClN_5O_3$ (%): C, 66.48; H, 4.23; N, 13.37. Found (%): C, 66.51; H, 4.19; N, 13.35.

1.3 Gli Luciferase reporter assay

NIH3T3 cells stably containing Gli-dependent firefly luciferase with renilla luciferase were cultured with calf serum culture medium (10 % calf serum, DMEM, Pen/Strep/Glutamine) until the cells were extremely confluent. Then, cell culture media were replaced with low calf serum culture medium (0.5 % calf serum, DMEM,

Pen/Strep/Glutamine). NIH3T3-Gli-Luc cells were plated in 96-well plates with an density of 2×10^4 cells per well and grew at 37 °C overnight. Various test compounds and Shh-conditioned medium treated the cells in the plates for 48 h. The firefly and renilla luciferase activity in the cell lysates was examined with a dual-luciferase reporter assay system (Promega E1901) in a luminometer (Glomax 20/20, Promega, USA) according to the manufacturer's instructions. The firefly luciferase values were normalized to the renilla values.

1.4 Analysis of Smo ciliary localization

Smo-EGFP stable cell lines were generated from NIH3T3 cells using a pUB6-Smo-EGFP construct. Medium (DMEM; HyClone, Logan, UT) with 10% FBS. Cells grown on coverslips were fixed in 4% (w/v) paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 10 min at room temperature and washed twice with PBS. Fixed cells were incubated with blocking buffer (1% horse serum, 0.1% Triton X-100 in PBS) for 30 min at 5 °C. Cells were incubated with the following primary antibodies overnight at 4 °C: mouse antiacetylated tubulin (Sigma, St. Louis, MO) and guinea pig anti-Gli2. After washes with blocking buffer, cells were further incubated with Cy3-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) and DAPI (Sigma) for 2 h at room temperature. All fluorescent images were acquired on confocal laser scanning fluorescence microscope (Olympus Fluorescence FV1200, Japan).

1.5 Cell infection

The wild-type (WT) Smo produced drug-resistant Smo D477G through site-directed mutagenesis kit (Yeasen Biotech, Shanghai, China). The primer sequences were 5'-CTTCAGCTGCCACTTCTATGGCTTCTTCAACCAGGCTG, and 5'-CAGCCTGGTTGA AGA AGCCATAGAAGTGGCAGCTGAAG. Green fluorescent protein was tagged in the Smo via GFP ORF (Vigene Biosciences, Shandong, China). 293T cells were transfected with viral packaging vector and Smo-GFP (WT and D477G) by utilizing PolyLea reagent (Biorike, Hunan, China) according to the manufacturer's instructions. After 48h, the collection and purification of the supernatants were carried out with ultra centrifugal filter. Then, NIH3T3-Gli-Luc cells were infected with the purified viral Smo-GFP vector. And the cells overexpressing Smo-GFP were gathered via SH800S cell sorter (Sony Biotech, Japan). The Hh inhibition of vismodegib and compound 21 was further evaluated in the NIH3T3-Gli-Luc reporter assay with D477G cell line.

1.6 Cell proliferation assay

The antiproliferative activity of compounds was evaluated against DAOY cells by MTT assay in vitro. DAOY cells were seeded in 96-well microplate at a density of 5 ×

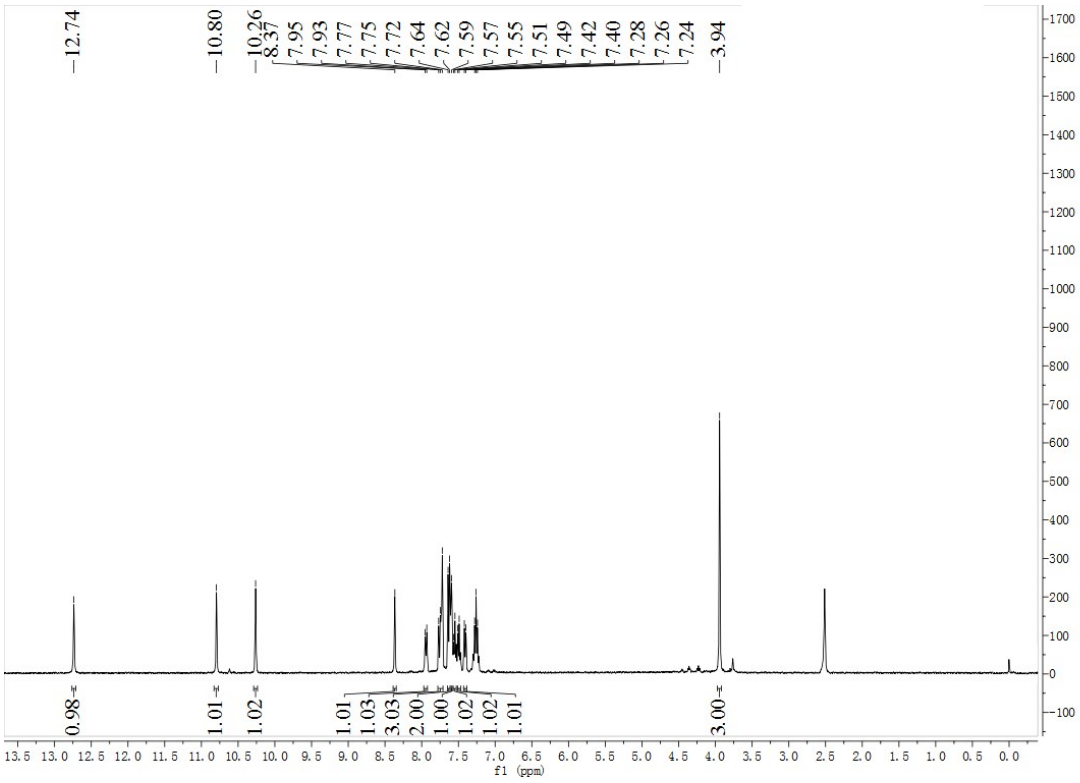
10^3 cells per well and cultured in DMEM supplemented with 10 % FBS until adherent for 24 h. Vismodegib or compound 21 dissolved in DMSO and diluted in medium to the specified concentration. The cells were treated with the indicated concentrations of test compounds and incubated in 5% CO_2 at 37 °C for 48 h. Fresh MTT solution (1 mg/mL) was added to each well, then the plate was incubated at 37 °C for another 4 h. The formazan crystals dissolved in 100 μL DMSO per well. The absorbance was measured at 570 nm by ELISA reader.

1.7 Molecular modeling

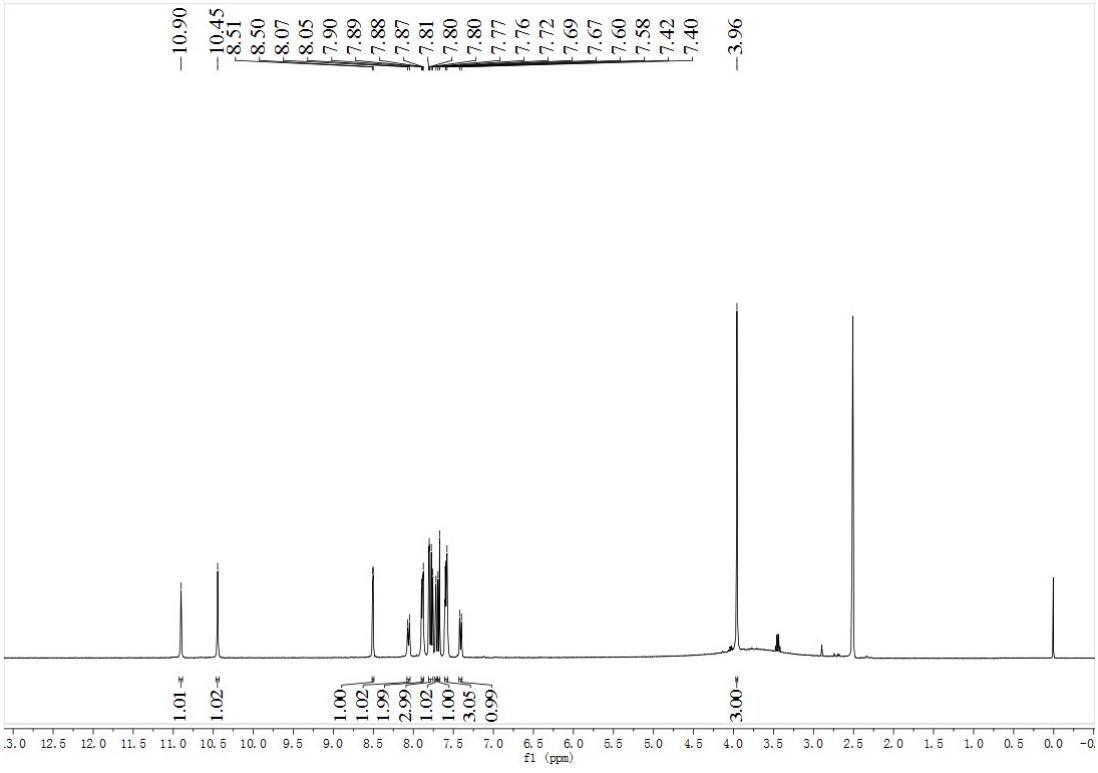
The crystal complex of vismodegib interacting with the Smo receptor was obtained from the PDB database (PDB code: 5L7I). The subunit B of Smo crystal saved as a molecular docking receptor model. The optimization of the protein mainly involved removal of cocrystal molecule and water molecules, amino acid protonation, adjustment of loop region and naming standardization. Then, the docking position of the ligand was defined at the center of vismodegib with radius of 10.0Å. The lowest energy comformations of the designed compounds were determined, and their the CHARMM force field parameters were minimized before the operation of AutoDock vina 1.1.2 in the binding site. The analysis of ligand-receptor simulation were implemented in Discovery Studio (version 4.5) .

Spectrum of the target compounds

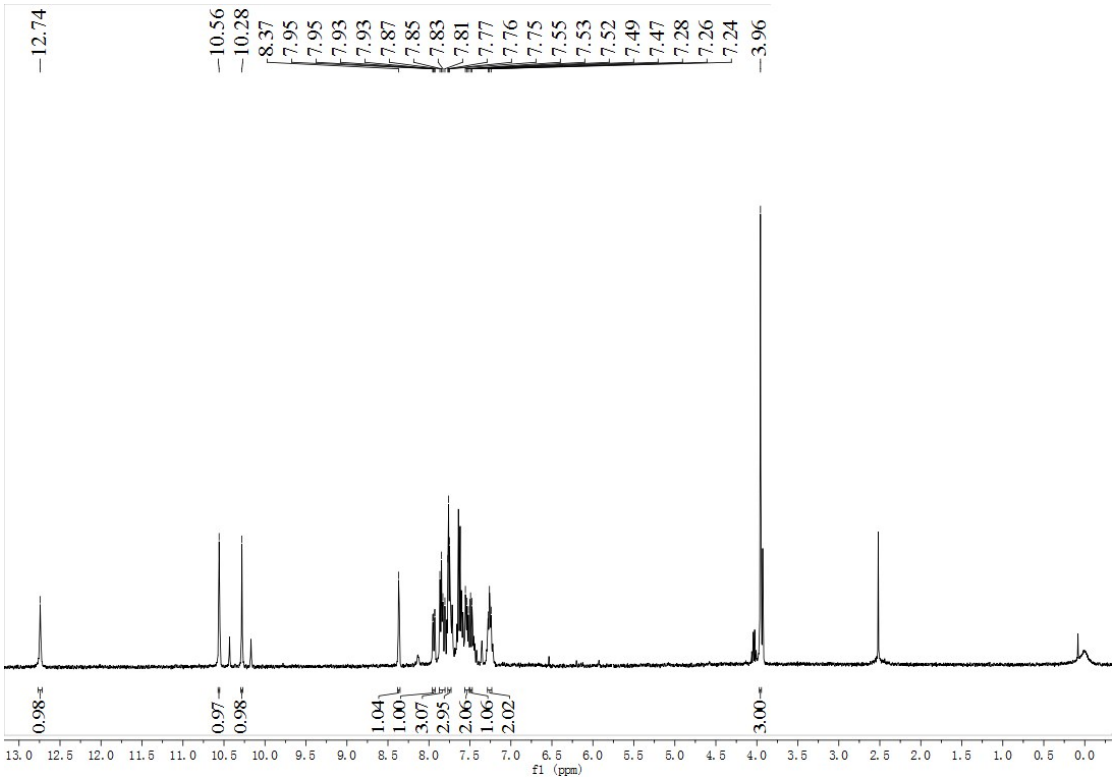
^{1}H NMR spectrum of compound 10



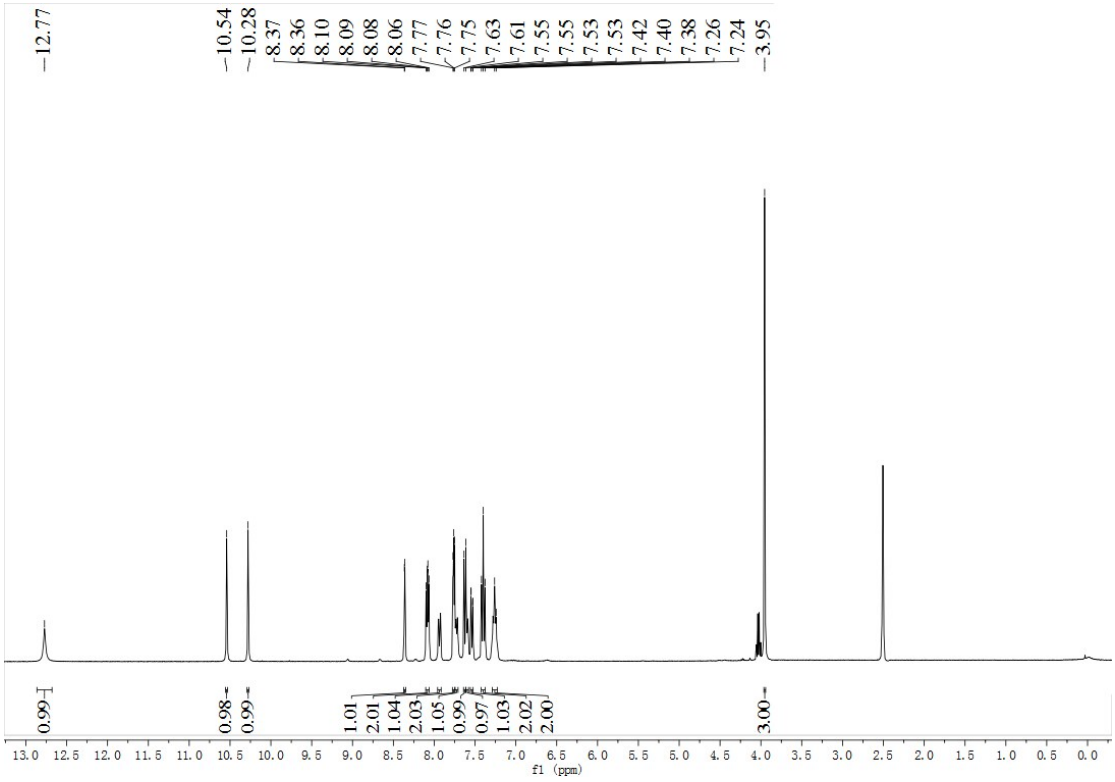
^{1}H NMR spectrum of compound 11



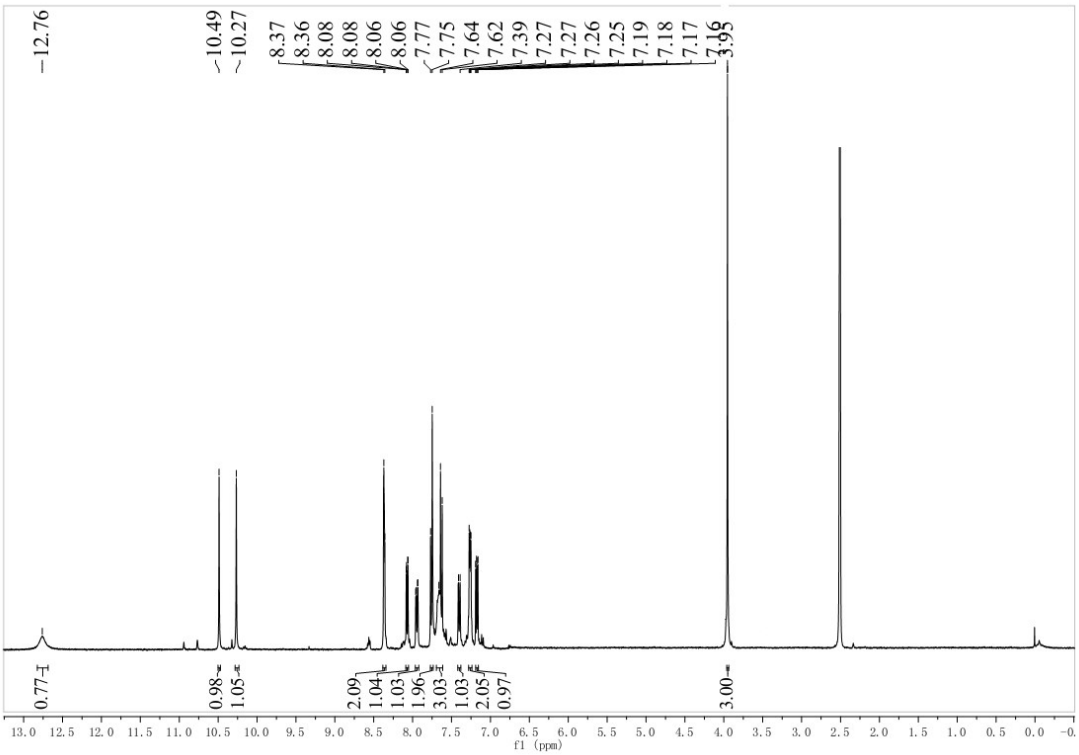
^{1}H NMR spectrum of compound 12



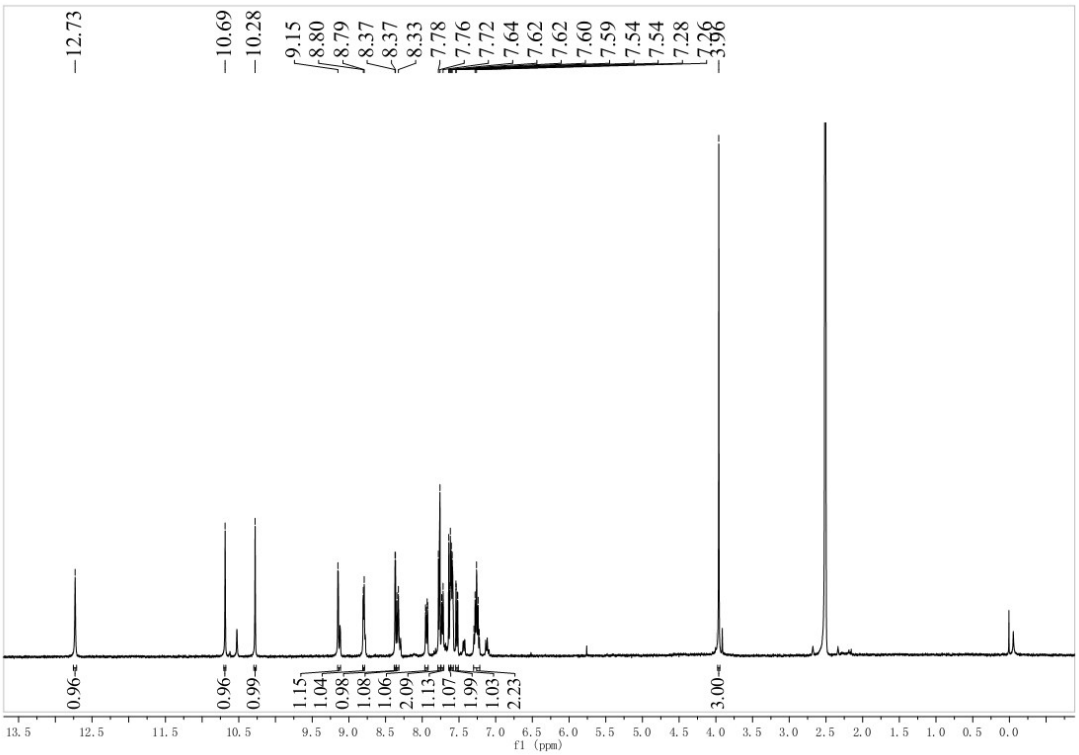
^{1}H NMR spectrum of compound 13



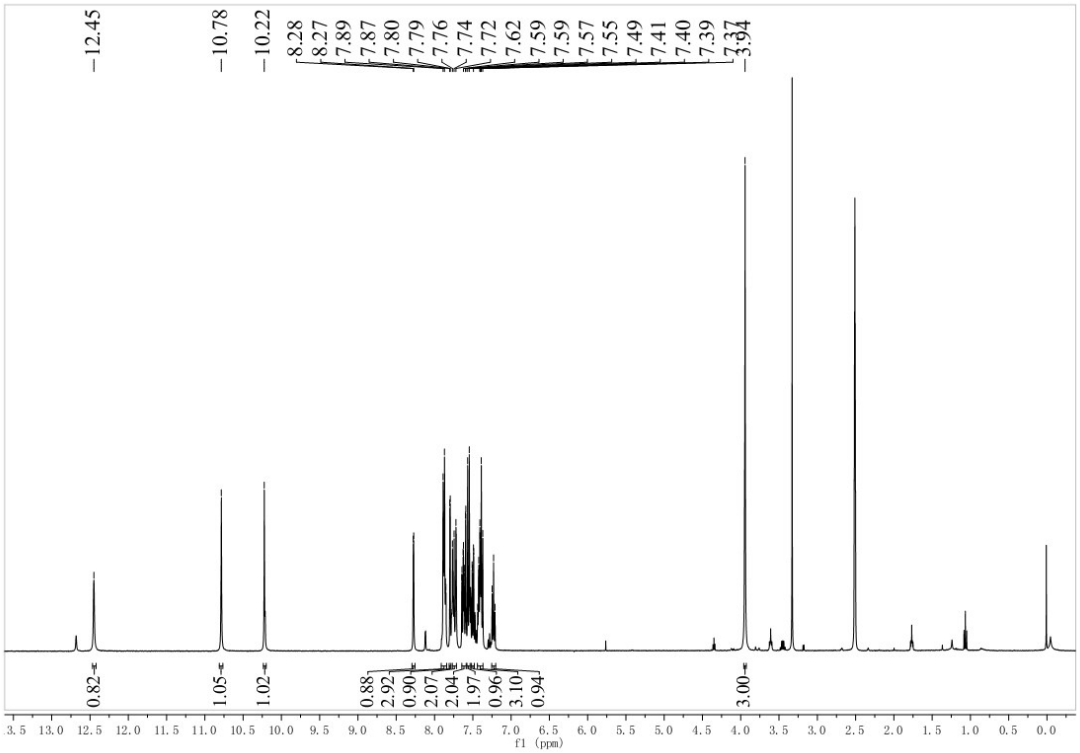
^{1}H NMR spectrum of compound 14



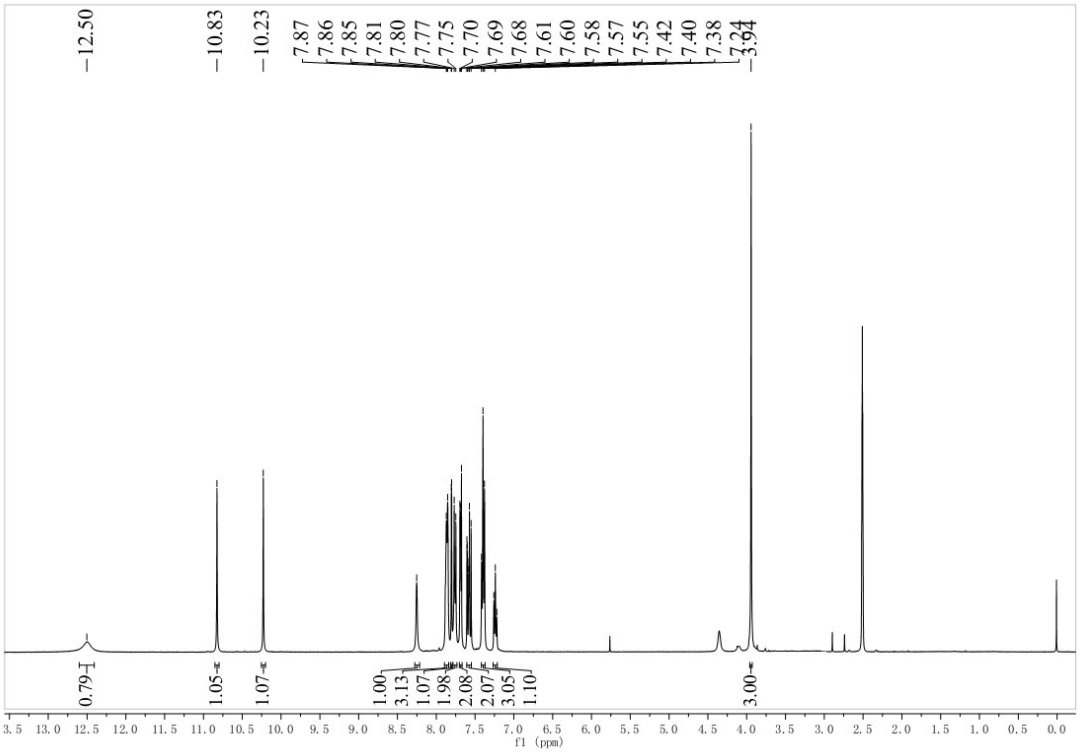
^{1}H NMR spectrum of compound 16



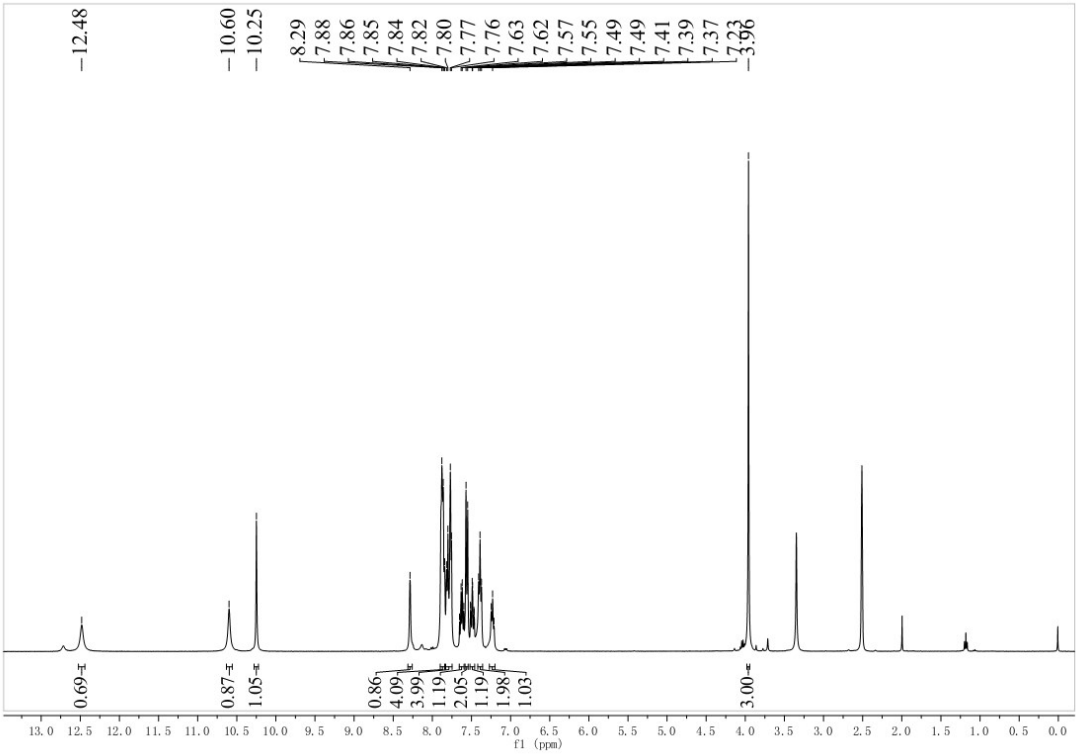
^{1}H NMR spectrum of compound 17



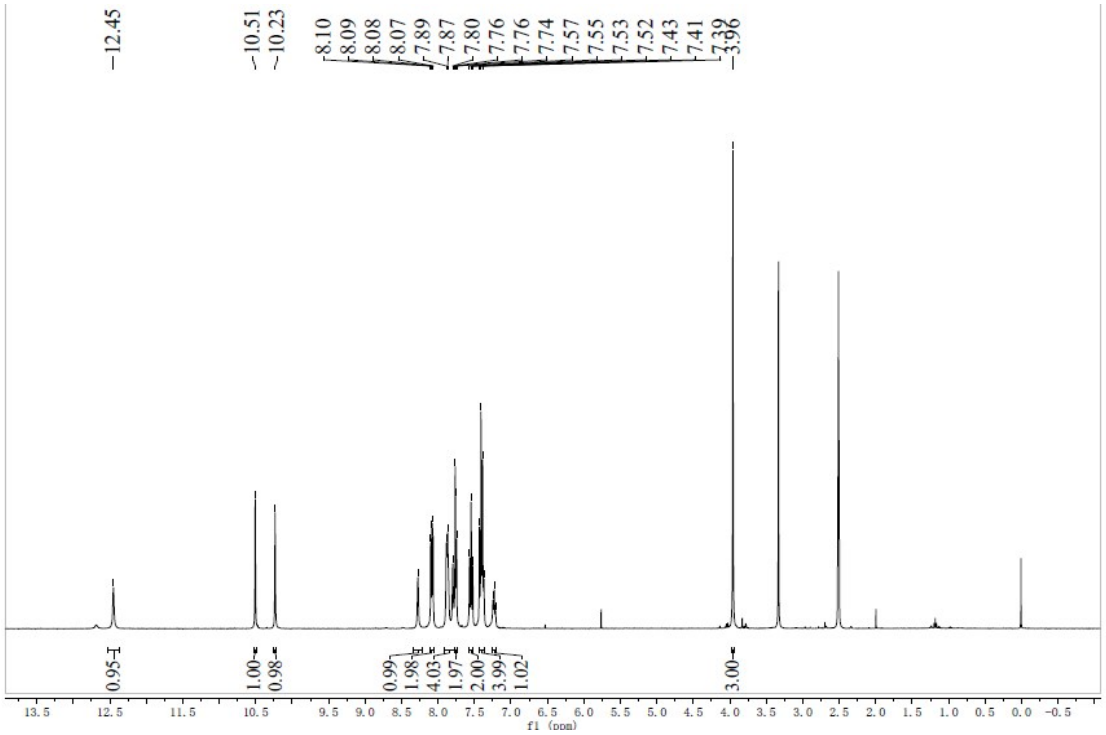
^{1}H NMR spectrum of compound 18



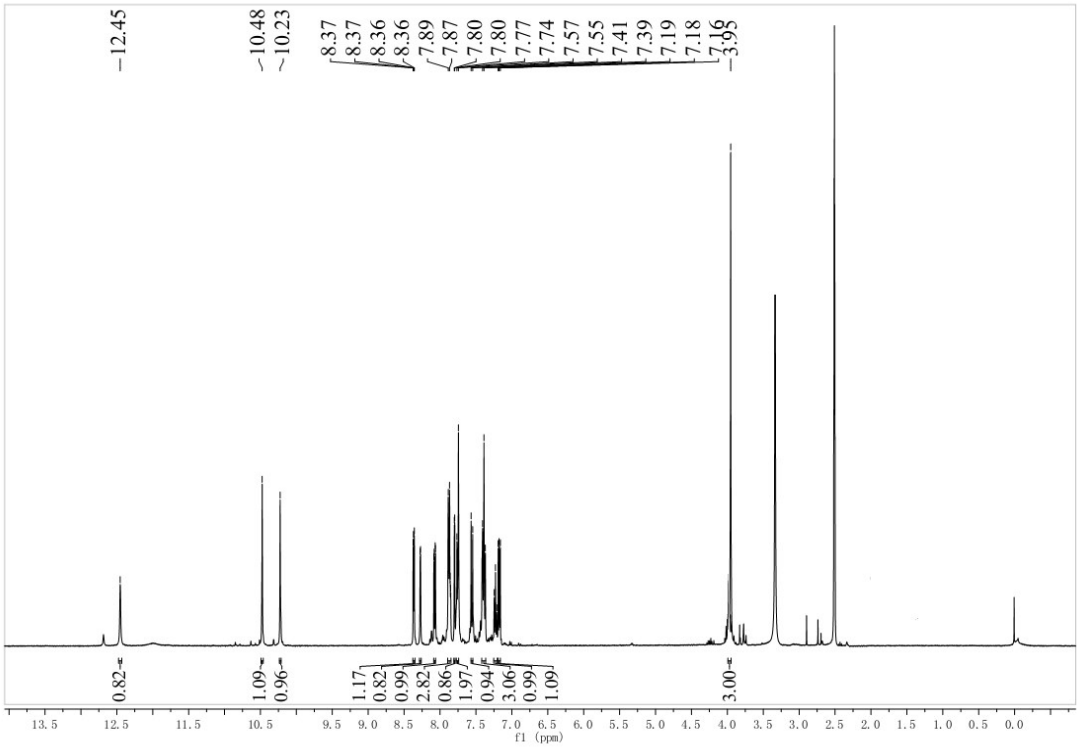
^{1}H NMR spectrum of compound 19



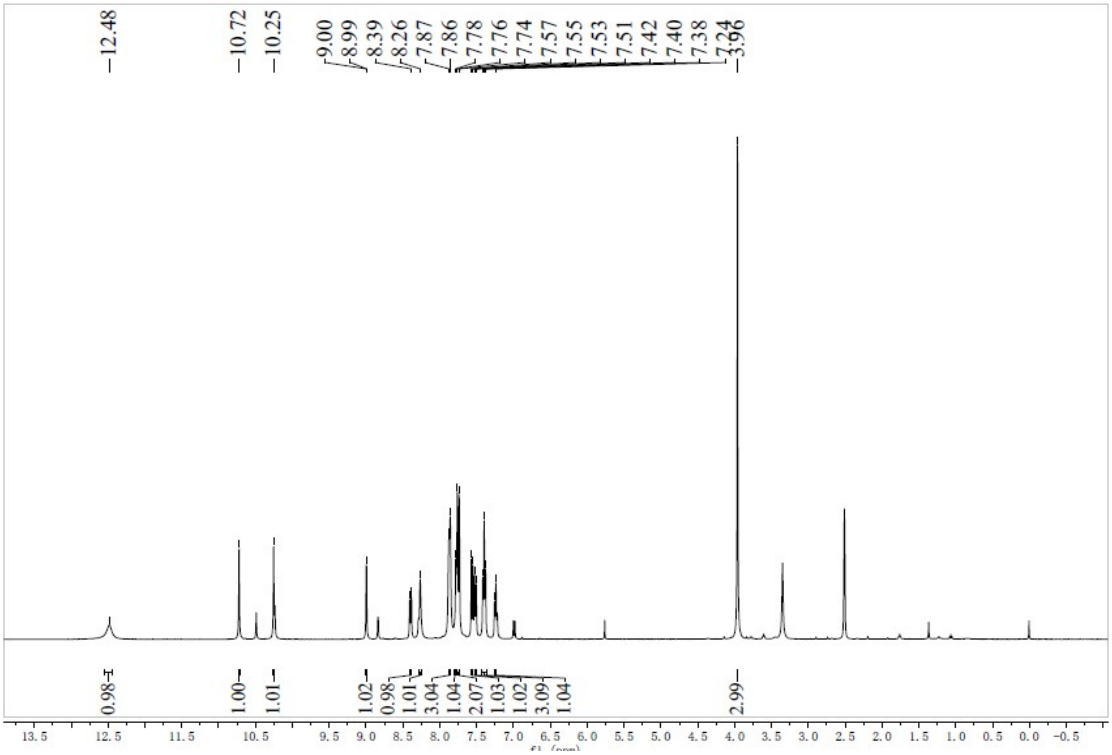
^{1}H NMR spectrum of compound 20



^{1}H NMR spectrum of compound 21



^{1}H NMR spectrum of compound 22



^{1}H NMR spectrum of compound 23