Discovery of Dotinurad (FYU-981), a New Phenol Derivative with Highly Potent Uric Acid Lowering Activity

Junichiro Uda,[*,†] Seiichi Kobashi,[†] Sachiho Miyata,[†] Naoki Ashizawa,[‡] Koji Matsumoto,[‡] and Takashi Iwanaga[‡]

[†] Research Laboratories 1, Medical R&D Division, Fuji Yakuhin Co., Ltd., Saitama 331-0078, Japan;

[‡] Research Laboratories 2, Medical R&D Division, Fuji Yakuhin Co., Ltd., Saitama 331-0068, Japan

KEYWORDS: uricosurics, mitochondrial toxicity, Benzbromarone, primary human renal proximal tubule epithelial cells, Dotinurad

Contents

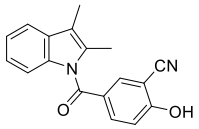
1. **General Information**
2. **Synthetic procedure and characterization for new compounds and key compounds**
3. **NMR Spectrum of Dotinurad (FYU-981)**
4. **Methods for in vitro, and pharmacokinetic studies**
5. **References**

1. General Information

^{1}H and ^{13}C were recorded on JEOL JNM-EX270 (^{1}H: 270 MHz) or JNM-ECZ400S (^{1}H: 400 MHz, ^{13}C: 101 MHz) spectrometers. Chemical shift (δ) are given in parts per million (ppm) relative to tetramethylsilane (TMS) as an internal standard. Coupling constant (J) are reported in Hertz (Hz). J: coupling constant, m: multiplet, sevent: seventet, quint: quintet, q: quartet, dt: double triplet, dd: double doublet, ddd: double double doublet, t: triplet, d: doublet, s: singlet, brs: broad singlet. $CDC1_3$: deuterium chloroform, DMSO-d_6: deuterium dimethyl sulfoxide, CD_3OD: deuterium methanol. Electrospray ionization (ESI) mass spectra (MS) were determined on Agilent 1100 Series LC/MSD G1946B (Agilent Technologies), high resolution mass spectra (HRMS) were obtained on LC/MS system (Thermo Fisher Scientific) composed with Q Exactive Focus MS system and Ultimate3000 HPLC system.

2. Synthetic procedure and characterization for new compounds and key compounds.

5-(2,3-Dimethyl-1*H*-indole-1-carbonyl)-2-hydroxybenzonitrile (1)



(i) 4-(methoxymethoxy)isophthalonitrile

To a solution of 4-nitrobenzonitrile (40.1 g, 0.271 mol) in dimethyl sulfoxide (400 mL), potassium cyanide (26.45 g, 0.406 mol) was added, and the mixture was stirred at 90°C for 1.5 h. The reaction solution was added potassium dicarbonate (74.9 g, 0.542 mol) and chloromethyl methyl ether (20.4 mL, 0.271 mol) at room temperature, and the mixture was stirred at for 0.5 h. The reaction solution was added H_2O, and insoluble matter was removed by celite filtration. The filtrate was diluted by ethyl acetate, washed with H_2O and brine, the organic layer was dried over anhydrous sodium sulfate. The solvent was evaporated, and the obtained residue was purified by silica gel column chromatography to obtain a pale yellow solid (17.8 g, 34.8% yield).

^{1}H-NMR (270 MHz, $CDCl_3$) δ: 7.88 (1H, d, J = 2.2 Hz), 7.80 (1H, dd, J = 8.6, 2.2 Hz), 7.36 (1H, d, J = 8.6 Hz), 5.37 (2H, s), 3.54 (3H, s).

(ii) Methyl 3-cyano-4-(methoxymethoxy)benzoate

To a solution of 4-(methoxymethoxy)isophthalonitrile (2.97 g, 15.8 mmol) in methanol (150 mL), sodium methoxide (853 mg, 15.8 mmol) was added, and the mixture was stirred at room temperature for 15 h. The solvent was evaporated, and diluted by ethyl acetate. The organic layer was washed with H_2O and brine, dried over anhydrous sodium sulfate. The residue was added 1,4-dioxane (30 mL), H_2O (15 mL), glacial acetic acid (4 mL), and the mixture was stirred at room temperature for 5 h. Then the solvent was evaporated, saturated sodium hydrogen carbonate solution was added, extracted with ethyl acetate, and the organic layer was dried over anhydrous sodium sulfate. The solvent was evaporated, and the obtained residue was purified by silica gel column chromatography to obtain a colorless solid (1.19 g, 34.1% yield).

^{1}H-NMR (270 MHz, $CDCl_3$) δ: 8.27 (1H, d, J = 2.2 Hz), 7.80 (1H, dd, J = 8.9, 2.2 Hz), 7.28 (1H, d, J = 8.9 Hz), 5.36 (2H, s), 3.93 (3H, s), 3.54 (3H, s).

(iii) 3-Cyano-4-(methoxymethoxy)benzoic acid

To a solution of methyl 3-cyano-4-(methoxymethoxy)benzoate (449 mg, 2.03 mmol) in tetrahydrofuran (5 mL) and H_2O (2.5 mL), lithium hydroxide monohydrate (128 mg, 3.05 mmol) was added, and the mixture was stirred at room temperature for 2 h. The reaction solution was added 1M HCl (6 mL) and H_2O (30 mL), extracted with ethyl acetate. The organic layer was washed with H_2O and brine, dried over anhydrous sodium sulfate. The solvent was evaporated to obtain a colorless solid (372 mg, 88.5% yield).

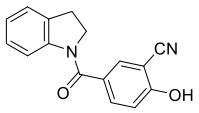
^{1}H-NMR (270 MHz, DMSO-d_6) δ: 8.22 (1H, d, J = 2.2 Hz), 8.19 (1H, dd, J = 8.9, 2.2 Hz), 7.42 (1H, d, J = 8.6 Hz), 5.46 (2H, s), 3.45 (3H, s).

(iv) 5-(2,3-Dimethyl-1*H*-indole-1-carbonyl)-2-(methoxymethoxy)benzonitrile

To a solution of 3-cyano-4-(methoxymethoxy)benzoic acid (300 mg, 1.45 mmol) in chloroform (9 mL) and *N*,*N*-dimethylformamide (1 mL), 1-hydroxybenzotriazole monohydrate (222 mg, 1.45 mmol) and 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (278 mg, 1.45 mmol) were added, and the mixture was stirred at room temperature for 10 min. The reaction solution was added H_2O (10 mL), extracted with ethyl acetate, and the organic layer was dried over anhydrous sodium sulfate. The solvent was evaporated to obtain HOBt ester. To a solution of 2,3-dimethylindole (252 mg, 1.73 mmol) in *N*, *N*-dimethylformamide (5 mL), the solution of HOBt ester in *N*, *N*-dimethylformamide (5 mL) was added, and the mixture was stirred at room temperature for 1 h. The reaction mixture was diluted by ethyl acetate. The organic layer was washed with H_2O and brine, dried over anhydrous sodium sulfate. The mixture was concentrated and the obtained residue was purified by silica gel column chromatography to obtain a colorless solid (135 mg, 44.9% yield).
^{1}H-NMR (270 MHz, $CDCl_3$) δ: 7.97 (1H, d, J = 2.2 Hz), 7.90 (1H dd, J = 8.9, 2.2 Hz), 7.46 (1H, d, J = 7.8 Hz), 7.33 (1H, d, J = 8.9 Hz), 7.20 (1H, dt, J = 1.1, 7.0 Hz), 7.07 (1H, dt, J = 1.1, 8.1 Hz), 6.99 (1H, d, J = 8.4 Hz), 5.39 (2H, s), 3.57 (3H, s), 2.34 (3H, s), 2.24 (3H, s).

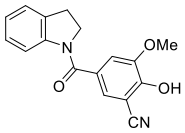
(v) 5-(2,3-dimethyl-1*H*-indole-1-carbonyl)-2-hydroxybenzonitrile (**1**)
To a solution of 5-(2,3-dimethyl-1*H*-indole-1-carbonyl)-2-(methoxymethoxy)benzonitrile (130 mg, 0.389 mmol) in tetrahydrofuran (6 mL), 4M HCl 1,4-dioxane solution (2 mL) was added, and the mixture was stirred at room temperature for 20 h. The solvent was evaporated, and the obtained residue was diluted by ethyl acetate. The organic layer was washed with H_2O, brine and dried over anhydrous sodium sulfate. The solvent was evaporated to obtain a colorless solid (87 mg, 77.0% yield).
^{1}H-NMR (270 MHz, DMSO-d_6) δ: 7.98 (1H, d, J = 2.2 Hz) , 7.79 (1H, dt, J = 8.6, 2.2 Hz), 7.51 (1H, d, J = 7.6 Hz) , 7.17 (1H, dt, J = 7.3, 1.1 Hz), 7.15 (1H, d, J = 8.6 Hz) 7.09 (1H, dt, J = 7.8, 1.4 Hz), 7.02 (1H, d, J = 8.1 Hz), 2.26 (3H, s), 2.20 (3H, s). (The phenol –OH was not observed)
^{13}C-NMR (101 MHz, DMSO-d_6) δ: 166.8, 164.2, 135.9, 135.7, 135.6, 132.5, 130.1, 125.6, 122.7, 122.0, 118.0, 116.6, 115.8, 113.9, 113.2, 99.5, 12.4, 8.2.
MS m/z: 289 $(M-H)^-$. HRMS (ESI) m/z: calcd for $C_{18}H_{13}N_2O_2$ $(M-H)^-$ 289.0982, Found 289.0987.

2-Hydroxy-5-(indoline-1-carbonyl)benzonitrile (2a)



2a was synthesized from indoline instead of 2,3-dimethylindole in the same manner as in the synthesis of **1** (66.3% yield) .
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 7.92 (1H, d, J = 1.8 Hz,), 7.77 (1H, dd, J = 8.7, 1.8Hz), 7.74(1H, brs), 7.31 (1H, d, J = 7.2 Hz), 7.20 (1H, t, J = 7.2 Hz), 7.11(1H, d, J = 8.7Hz), 7.06 (1H, d, J = 7.2Hz), 4.08 (2H, t, J = 8.2 Hz), 3.11 (2H, t, J = 8.2 Hz). (The phenol –OH was not observed)
^{13}C-NMR (101 MHz, DMSO-d_6) δ: 166.3, 162.0, 142.7, 134.2, 132.9, 132.7, 128.0, 126.9, 125.1, 123.9, 116.62, 116.56, 116.3, 98.8, 50.5, 27.9.
HRMS (ESI) m/z: calcd for $C_{16}H_{11}N_2O_2$ $(M-H)^-$ 263.0826, found 263.0830.

2-Hydroxy-5-(indoline-1-carbonyl)-3-methoxybenzonitrile (2b)



(i) Ethyl 3-bromo-4-hydroxy-5-methoxybenzoate
To a solution of ethyl 4-hydroxy-3-methoxybenzoate (3.00 g, 15.3 mmol) in chloroform (30 mL), bromine (0.87 mL, 16.9 mmol) was added and the mixture was stirred at room temperature for 15 h. The reaction solution was quenched by 10% sodium thiosulfate aqueous solution, and extracted with ethyl acetate. The organic layer was washed with brine, dried over anhydrous sodium sulfate. The solvent was evaporated to obtain a pale yellow crystal (4.30 g, quant.).

(ii) 3-Cyano-5-methoxy-4-(methoxymethoxy)benzoic acid

To a solution of ethyl 3-bromo-4-hydroxy-5-methoxybenzoate (2.00 g, 7.27 mmol) in *N*,*N*-dimethylformamide (30 mL), copper (I) cyanide (781 mg, 8.72 mmol) was added and the mixrure was stirred at 150°C for 18 h. To the reaction solution, potassium dicarbonate (1.06 g, 7.67 mmol) and chloromethyl methyl ether (0.30 mL, 3.99 mmol) were added at room temperature, and the mixture was stirred at room temperature for 1 h. The reaction mixture was diluted with ethyl acetate, and insoluble matter was removed by celite filtration. The filtrate was washed with H_2O and brine. The organic layer was dried over anhydrous sodium sulfate. The solvent was evaporated, and the obtained residue was purified by silica gel column chromatography to obtain a pale yellow oil (860 mg) as ethyl 3-cyano-5-methoxy-4-(methoxymethoxy)benzoate. To a solution of ethyl 3-cyano-5-methoxy-4-(methoxymethoxy)benzoate (860 mg, 3.24 mmol) in tetrahydrofuran (10 mL) and H_2O (5 mL), lithium hydroxide monohydrate (610 mg, 14.5 mmol) was added, and the mixture was stirred at room temperature for 4 h. The reaction solution was added 1M HCl (25 mL), extracted with ethyl acetate, and the organic layer was dried over anhydrous sodium sulfate. The solvent was evaporated to obtain a colorless crystal (678 mg, 39.3% yield).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 13.43 (1H, brs) , 7.83 (1H, d, J = 1.9 Hz), 7.80 (1H, d, J = 1.9 Hz), 5.33 (2H, s), 3.93 (3H, s), 3.55 (3H, s). MS m/z: 236 (calcd for $C_{11}H_{10}NO_5$ 236 $(M-H)^-$)

(iii) 2-Hydroxy-5-(indoline-1-carbonyl)-3-methoxybenzonitrile (**2b**)

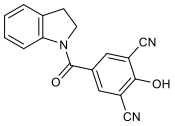
To a solution of 3-cyano-5-methoxy-4-(methoxymethoxy)benzoic acid (300 mg, 1.26 mmol) in dichloromethane (9 mL) and *N*,*N*-dimethylformamide (1 mL), indoline (151 mg, 1.27 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (292 mg, 1.52 mmol) were added, and the mixture was stirred at room temperature for 1 h. The solvent was evaporated, the residue was diluted by ethyl acetate, washed with H_2O and brine, and the organic layer was dried over anhydrous sodium sulfate. The solvent was evaporated, dichloromethane (5 mL) and trifluoroacetic acid (5 mL) were added, and the mixture was stirred at room temperature for 2 h. The solvent was evaporated and crystalized by *n*-hexane and ethyl acetate to obtain a colorless crystal (222 mg, 59.9% yield).

^{1}H -NMR (400 MHz, DMSO-d_6) δ 7.81 (1H, brs), 7.46 (1H, d, J = 1.8 Hz,), 7.44 (1H, d, J = 1.8 Hz,), 7.28 (1H, d, J = 7.3 Hz), 7.18 (1H, t, J = 7.3 Hz,), 7.04 (1H, t, J = 7.3 Hz), 4.07 (2H, t, J = 8.2 Hz), 3.89 (3H, s), 3.08 (2H, t, J = 8.2 Hz). (The phenol –OH was not observed)

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 166.2, 151.9, 147.9, 142.7, 132.8, 128.4, 126.8, 125.0, 123.8, 123.0, 116.5, 116.3, 115.2, 98.7, 56.4, 50.4, 27.8.

MS m/z: 293 $(M-H)^-$. HRMS (ESI) m/z: calcd for $C_{17}H_{13}N_2O_3$ $(M-H)^-$ 293.0931, found 293.0937.

2-Hydroxy-5-(indoline-1-carbonyl)isophthalonitrile (2d)



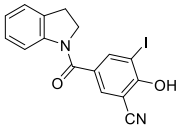
To a solution of 3,5-dicyano-4-methoxybenzoic acid (250 mg, 1.16 mmol) in dichloromethane (9 mL) and *N*,*N*-dimethylformamide (1 mL), indoline (138 mg, 1.16 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (267 mg, 1.39 mmol) were added, and the mixture was stirred at room temperature for 1 h. Then solvent was evaporated, the residue was diluted by ethyl acetate, washed with H_2O and brine, and the organic layer was dried over anhydrous sodium sulfate. The solvent was evaporated, *N*,*N*-dimethylformamide (10 mL) and lithium chloride (445 mg, 10.5 mmol) was added, and the mixture was stirred at room temperature for 1 h. The reaction solution was added 1M HCl (40 mL), extracted with ethyl acetate, and the organic layer was dried over anhydrous sodium sulfate. The solvent was evaporated to obtain a light brown solid (267 mg, 79.6% yield).

^{1}H-NMR (400 MHz, DMSO-d_6) δ: 8.19 (2H, s), 7.80 (1H, brs), 7.29 (1H, d, J = 6.9 Hz), 7.20 (1H, t, J = 7.5 Hz), 7.06 (1H, dd, J = 6.9, 7.5 Hz,), 4.07 (2H, t, J = 8.5 Hz), 3.09 (2H, t, J = 8.2 Hz). (The phenol –OH was not observed)

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 164.6, 163.9, 142.5, 137.9, 132.8, 127.5, 126.9, 125.1, 124.0, 116.6, 115.7, 102.4, 50.3, 27.8.

MS m/z: 288 $(M-H)^-$. HRMS (ESI) m/z: calcd for $C_{17}H_{10}N_3O_2$ $(M-H)^-$ 288.0778, found 288.0783.

2-Hydroxy-5-(indoline-1-carbonyl)-3-iodobenzonitrile (2c)



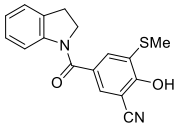
2c was synthesized from 3-cyano-4-methoxy-5-iodobenzoic acid in the same manner as in the synthesis of **2d** (66.3 % yield) .

^{1}H-NMR (400 MHz, DMSO-d_6) δ: 8.22 (1H, d, J = 2.3 Hz), 7.95 (1H, d, J = 2.3 Hz), 7.74(1H, brs), 7.28 (1H, d, J = 6.9 Hz), 7.17 (1H, d, J = 6.9 Hz), 7.05 (1H, t, J = 6.9 Hz), 4.05 (2H, t, J = 8.0 Hz), 3.08 (2H, t, J = 8.0 Hz). (The phenol –OH was not observed)

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 164.7, 160.2, 142.9, 142.5, 132.8, 132.6, 126.8, 125.0, 124.9, 124.0, 116.5, 116.0, 100.2, 88.8, 50.3, 27.8.

HRMS (ESI) m/z: calcd for $C_{16}H_{10}IN_2O_2$ $(M-H)^-$ 388.9792 found 388.9801.

2-Hydroxy-5-(indoline-1-carbonyl)-3-methylsulfanylbenzonitrile (2e)



(i) Methyl 3-cyano-4-methoxy-5-methylsulfanyl benzoate

To a solution of methyl 3-cyano-4-hydroxy-5-iodobenzoate (1.00 g, 3.30 mmol) in *N*,*N*-dimethylformamide (10 mL), 2,2'-bipyridyl (52.0 mg, 0.333 mmol), zinc powder (432 mg, 6.61 mmol), nickel (II) bromide (72.0 mg, 0.329 mmol) and dimethyl disulfide (156 mg, 1.66 mmol) were added, and the mixture was stirred at 90°C for 16 h under Ar. The reaction solution was added 1M HCl, and insoluble matter was removed by celite filtration. The filtrate was extracted with ethyl acetate, and the organic layer was dried over anhydrous sodium sulfate. Then solvent was evaporated, *N*,*N*-dimethylformamide (20 mL), potassium carbonate (4.56 g, 33.0 mmol) and dimethyl sulfate (1.60 mL, 16.9 mmol) were added, and the mixture was stirred at room temperature for 1 h. Then insoluble matter was removed by celite filtration, the filtrate was added H_2O, extracted with ethyl acetate. The organic layer was washed with H_2O and brine, dried over anhydrous sodium sulfate, and the solvent was evaporated and the obtained residue was purified by silica gel column chromatography to obtain a colorless crystal (276 mg, 35.2% yield).

^{1}H-NMR (270 MHz, $CDCl_3$) δ: 8.03 (1H, d, J = 1.9 Hz), 7.95 (1H, d, J = 1.9 Hz), 4.18 (3H, s,), 3.94 (3H, s), 2.50 (3H, s).

(ii) 3-Cyano-4-methoxy-5-methylsulfanylbenzoic acid

To a solution of methyl 3-cyano-4-methoxy-5-(methylthio)benzoate (270 mg, 1.14 mmol) in tetrahydrofuran (4 mL) and H_2O (2 mL), lithium hydroxide monohydrate (192 mg, 4.56 mmol) was added, and the mixture was stirred at room temperature for 1.5 h. The reaction solution was added 1M HCl (10 mL), and extracted with ethyl acetate. The organic layer was washed with H_2O and brine, dried over anhydrous sodium sulfate. The solvent was evaporated to obtain a colorless crystal (241 mg, 94.7% yield).

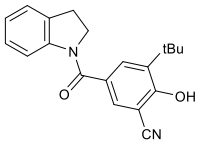
^{1}H-NMR (270 MHz, DMSO-d_6) δ:13.49 (1H, brs), 8.01 (1H, d, J = 2.2 Hz), 7.92 (1H, d, J = 2.2 Hz), 4.05 (3H, s), 2.52 (3H, s). MS m/z: 222 (calcd for $C_{10}H_8NO_3S$ $(M-H)^-$ 222).

(iii) 5-(Indoline-1-carbonyl)-2-methoxy-3-methylsulfanylbenzonitrile

To a solution of 3-cyano-4-methoxy-5-methylsulfanylbenzoic acid (235 mg, 1.05 mmol) in dichloromethane (9 mL) and *N*,*N*-dimethylformamide (1 mL), indoline (126 mg, 1.06 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (242 mg, 1.26 mmol) were added, and the mixture was stirred at room temperature for 1.5 h. The reaction solution was added 1M HCl (10 mL), extracted with ethyl acetate. The organic layer was washed with H_2O and brine, dried over anhydrous sodium sulfate. The solvent was evaporated to obtain the title compound.

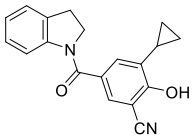
(iv) 2-Hydroxy-5-(indoline-1-carbonyl)-3-methylsulfanylbenzonitrile (**2e**)
To a solution of 5-(indoline-1-carbonyl)-2-methoxy-3--methylsulfanylbenzonitrile (iii) in *N*,*N*-dimethylformamide (10 mL), lithium chloride (445 mg, 10.5 mmol) was added and the mixture was stirred at room temperature for 1.5 h. The reaction solution was added 1M HCl (40 mL), extracted with ethyl acetate. The organic layer was washed with H_2O and brine, dried over anhydrous sodium sulfate. The solvent was evaporated to obtain a colorless crystal (267 mg, 81.9% yield from indoline).
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 7.73(1H, brs), 7.67 (1H, d, J = 1.8 Hz), 7.62 (1H, d, J = 1.8 Hz,) , 7.27 (1H, d, J = 7.5 Hz), 7.16 (1H, t, J = 7.5 Hz), 7.03 (1H, t, J = 7.5 Hz,), 4.04 (2H, t, J = 8.2 Hz), 3.08 (2H, t, J = 8.2 Hz), 2.43 (3H, s). (The phenol –OH was not observed)
^{13}C-NMR (101 MHz, DMSO-d_6) δ: 165.8, 157.8, 142.5, 132.6, 130.2, 129.2, 129.1, 128.4, 126.6, 124.8, 123.6, 116.3, 116.2, 99.9, 50.2, 27.6, 14.6.
MS m/z: 309 $(M-H)^-$. HRMS (ESI) m/z: calcd for $C_{17}H_{13}N_2O_2S$ $(M-H)^-$ 309.0703, found 309.0710.

3-*tert*-Butyl-2-hydroxy-5-(indoline-1-carbonyl)benzonitrile (2f)



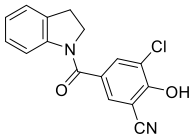
2f was synthesized from 5-*tert*-butyl-3-cyano-4-methoxybenzoic acid in the same manner as in the synthesis of **2d**. (67.4% yeild)
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 7.77 (1H, d, J = 2.3 Hz), 7.76(1H, brs) 7.65 (1H, d, J = 2.3 Hz), 7.27 (1H, d, J = 7.3 Hz), 7.17 (1H, d, J = 7.3 Hz), 7.03 (1H, t, J = 7.3 Hz), 4.05 (2H, t, J = 8.2 Hz), 3.08 (2H, t, J = 8.2 Hz), 1.36 (9H, s). (The phenol –OH was not observed)
^{13}C-NMR (101 MHz, DMSO-d_6) δ: 166.5, 159.9, 142.7, 139.7, 132.8, 130.9, 130.3, 127.2, 126.8, 125.0, 123.8, 117.1, 116.5, 101.9, 50.5, 34.9, 29.1, 27.8 .
HRMS (ESI) m/z: calcd for $C_{20}H_{19}N_2O_2$ $(M-H)^-$ 319.1452, found 319.1457.

3-Cyclopropyl-2-hydroxy-5-(indoline-1-carbonyl)benzonitrile (2g)



2g was synthesized from 3-cyano-5-cyclopropyl-4-methoxybenzoic acid in the same mannar as in the synthesis of **2d** (75.6 % yield).
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 7.71 (1H, brs), 7.70 (1H, d, J = 1.8 Hz), 7.32 (1H, d, J = 1.8 Hz), 7.27 (1H, d, J = 7.3 Hz), 7.16 (1H, t, J = 7.3 Hz), 7.03 (1H, t, J = 7.3 Hz), 4.01 (2H, t, J = 8.0 Hz), 3.07 (2H, t, J = 8.2 Hz), 2.14-2.07 (1H, m), 0.99-0.93 (2H, m), 0.71-0.65 (2H, m). (The phenol –OH was not observed)
^{13}C-NMR (101 MHz, DMSO-d_6) δ: 166.3, 160.1, 142.7, 132.8, 132.0, 129.6, 129.4, 128.7, 126.8, 125.0, 123.8, 116.9, 116.5, 99.8, 50.3, 27.8, 9.7, 7.9.
HRMS (ESI) m/z: calcd for $C_{19}H_{15}N_2O_2$ $(M-H)^-$ 303.1139, found 303.1145.

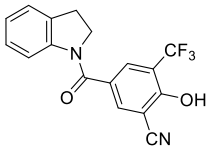
3-Chloro-2-hydroxy-5-(indoline-1-carbonyl)benzonitrile (2h)



2h was synthesized from 3-chloro-5-cyano-4-methoxybenzoic acid in the same manner as in the synthesis of **2d** (89.0 % yield).

^{1}H-NMR (400 MHz, DMSO-d_6) δ: 7.94 (1H, d, J = 2.3 Hz), 7.92 (1H, d, J = 2.3 Hz), 7.78 (1H, brs), 7.29 (1H, d, J = 7.3 Hz), 7.18 (1H, t, J = 7.3 Hz), 7.05 (1H, t, J = 7.3 Hz), 4.06 (2H, t, J = 8.2 Hz), 3.08 (2H, t, J = 8.2 Hz). (The phenol –OH was not observed)
^{13}C-NMR (101 MHz, DMSO-d_6) δ: 164.9, 157.4, 142.5, 133.8, 132.8, 131.4, 129.0, 126.9, 125.1, 124.0, 122.1, 116.6, 115.9, 101.8, 50.4, 27.8.
HRMS (ESI) m/z: calcd for $C_{16}H_{10}ClN_2O_2$ $(M-H)^-$ 297.0436, found 297.0442.

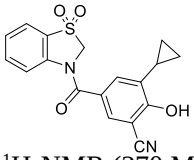
2-Hydroxy-5-(indoline-1-carbonyl)-3-trifluoromethylbenzonitrile (2i)



2i was synthesized from 5-cyano-4-methoxy-3-trifluoromethylbenzoic acid in the same manner as in the synthesis of **2d** (91.2% yield).
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 8.23 (1H, d, J = 1.8 Hz), 8.06 (1H, d, J = 1.8 Hz), 7.81 (1H, brs), 7.28 (1H, d, J = 7.5 Hz), 7.18 (1H, t, J = 7.5 Hz), 7.05 (1H, t, J = 7.5 Hz), 4.06 (2H, t, J = 8.2 Hz), 3.08 (2H, t, J = 8.2 Hz). (The phenol –OH was not observed)
^{13}C-NMR (101 MHz, DMSO-d_6) δ: 164.9, 159.1, 142.5, 136.9, 132.9, 131.3(q, J = 4.8 Hz), 128.3, 126.9, 125.1, 124.1, 122.9(q, J = 274 Hz), 119.1(q, J = 30.0 Hz), 116.7, 115.6, 102.8, 50.4, 27.8
HRMS (ESI) m/z: calcd for $C_{17}H_{10}F_3N_2O_2$ $(M-H)^-$ 331.0699, found 331.0705.

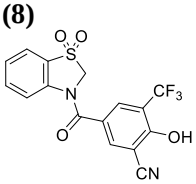
Although the synthetic method for compounds **7-20** was described previously,[1] the additional information is described below. Additionally, the synthetic procedure and method of **11** (Dotinurad) are shown at the end of this chapter.

3-(3-Cyano-5-cyclopropyl-4-hydroxy-benzoyl)-1,1-dioxo-1,2-dihydro-3*H*-1,3-benzothiazole (7)



^{1}H-NMR (270 MHz, DMSO-d_6) δ: 7.97(1H, d, J = 8.4 Hz), 7.87 (1H, d, J = 7.8 Hz), 7.79 (1H, d, J = 2.1 Hz), 7.73 (1H, dd, J = 8.4, 7.8 Hz), 7.42 (1H, dd, J = 7.8, 7.8 Hz), 7.38 (1H, d, J = 2.1Hz), 5.26 (2H, s), 2.05-2.15 (1H, m), 0.93-1.00(2H, m), 0.67-0.73 (2H, m). (The phenol –OH was not observed)
^{13}C-NMR (101 MHz, DMSO-d_6) δ; 166.3, 161.5, 139.4, 134.2, 132.3, 130.6, 130.0, 128.8, 125.5, 125.3, 121.0, 120.3, 116.4, 100.1, 67.0, 9.6, 7.6.
MS (m/z): 353 $(M-H)^-$.
HRMS (ESI): m/z calcd for $C_{18}H_{13}N_2O_4S$ $(M-H)^-$ 353.0601, found 353.0609.

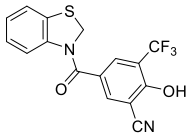
3-(3-Cyano-4-hydroxy-5-trifluoromethylbenzoyl)-1,1-dioxo-1,2-dihydro-3*H*-1,3-benzothiazole (8)



^{1}H-NMR (270 MHz, DMSO-d_6) δ: 8.27 (1H, d. J = 2.1 Hz), 8.10 (1H, d, J = 2.1 Hz), 8.09 (1H, d, J = 7.9 Hz), 7.91 (1H, dd, J = 7.8, 1.3 Hz), 7.77 (1H, ddd, J = 79, 7.8, 1.3 Hz), 7.44 (1H, dd, J = 7.8, 7.8 Hz), 5.37 (2H, s). (The phenol –OH was not observed)
^{13}C-NMR (101 MHz, DMSO-d_6) δ: 165.4, 161.0, 139.4, 138.0, 134.6, 132.3, 128.8, 126.1, 124.7, 123.1(q, J = 278Hz), 121.4, 120.6, 119.3(q, J = 31.0Hz), 115.7, 103.0, 66.9.

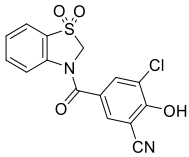
MS (m/z): 381 $(M-H)^-$. HRMS (ESI) m/z: calcd for $C_{16}H_8F_3N_2O_4S$ $(M-H)^-$ 381.0162, found 381.0167.

3-(3-Cyano-4-hydroxy-5-trifluoromethylbenzoyl)-1,2-dihydro-3*H*-1,3-benzothiazole (9)



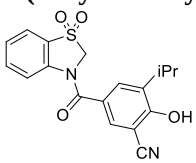
^{1}H-NMR (270 MHz, DMSO-d_6) δ: 8.22 (1H, d, J = 2.1 Hz), 8.05 (1H, d, J = 2.1 Hz), 7.55 (1H, br), 7.32-7.38 (1H, m), 7.04-7.14 (2H, m), 5.38 (2H, s). (The phenol –OH was not observed)
^{13}C-NMR (101 MHz, DMSO-d_6) δ: 165.6, 160.6, 138.7, 137.5, 131.8(q, J=4.8Hz), 131.1, 125.8, 125.5, 124.9, 122.94(q, J = 275Hz), 122.85, 119.2(q, J = 30.0Hz), 119.1, 115.8, 102.9, 53.7.
MS (m/z): 349 $(M-H)^-$. HRMS (ESI) m/z: calcd for $C_{16}H_8F_3N_2O_2S$ $(M-H)^-$ 349.0264, found 349.0270.

3-(3-Chloro-5-cyano-4-hydroxybenzoyl)-1,1-dioxo-1,2-dihydro-3*H*-1,3-benzothiazole (10)



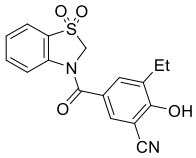
^{1}H-NMR (270 MHz, DMSO-d_6) δ: 8.06 (1H, d. J = 8.6 Hz), 8.00 (1H, d. J = 2.2 Hz), 7.99 (1H, d, J = 2.2 Hz), 7.88 (1H, dd, J = 8.4, 1.4 Hz), 7.75 (1H, ddd, J = 8.6, 7.3, 1.4 Hz), 7.44 (1H, dd, J = 8.4, 7.3 Hz), 5.32 (2H, s). (The phenol –OH was not observed)
MS (m/z): 347 $(M-H)^-$. HRMS (ESI) m/z: calcd for $C_{15}H_8ClN_2O_4S$ 346.9898 $(M-H)^-$, found 346.9899.

3-(3-Cyano-4-hydroxy-5-isopropylbenzoyl)-1,1-dioxo-1,2-dihydro-3*H*-1,3-benzothiazole (12)



^{1}H-NMR (270 MHz, DMSO-d_6) δ: 8.00 (1H, d, J = 8.4 Hz), 7.90 (1H, dd, J = 7.8, 0.8 Hz), 7.87 (1H, d, J = 2.3 Hz), 7.76 (1H, d, J = 2.3Hz), 7.75 (1H, dd, J = 8.4, 7.8), 7.43 (1H, ddd, J = 7.8, 7.8, 0.8Hz), 5.34 (2H, s), 3.35 (1H, sevent, J = 6.8 Hz), 1.18 (6H, d, J = 6.8 Hz). (The phenol –OH was not observed)
^{13}C-NMR (101 MHz, DMSO-d_6) δ: 166.5, 159.8, 139.5, 137.4, 134.4, 131.2, 128.9, 125.8, 125.7, 121.3, 120.6, 116.7, 100.7, 67.1, 26.1, 22.2.
MS (ESI) m/z: 355 $(M-H)^-$. HRMS (ESI): m/z calcd for $C_{18}H_{15}N_2O_4S$ $(M-H)^-$ 355.0758, found 355.0765.

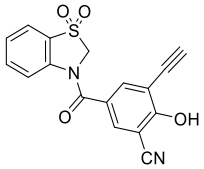
3-(3-Cyano-5-ethyl-4-hydroxy-benzoyl)-1,1-dioxo-1,2-dihydro-3*H*-1,3-benzothiazole (13)



^{1}H-NMR (270 MHz, DMSO-d_6) δ:11.01 (1H, brs), 8.00 (1H, d, J = 8.4 Hz), 7.90 (1H, d, J = 7.6 Hz), 7.88 (1H, d, J = 2.2 Hz), 7.75 (1H, dd, J = 8.4, 7.6 Hz), 7.74(1H, d, J = 2.2 Hz), 7.43 (1H, dd, J = 7.6, 7.6 Hz), 5.34 (2H, s), 2.68 (2H, q, J = 7.6 Hz), 1.15 (3H, t, J = 7.6 Hz).
^{13}C-NMR (101 MHz, DMSO-d_6) δ: 166.5, 160.8, 139.6, 134.5, 134.0, 132.9, 131.5, 129.0, 125.9, 125.3, 121.3, 120.7, 116.8, 100.4, 67.2, 22.5, 13.7.

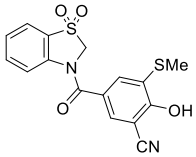
MS (m/z): 341 $(M-H)^-$. HRMS (ESI): m/z calcd for $C_{17}H_{13}N_2O_4S$ $(M-H)^-$ 341.0601, found 341.0608.

3-(3-Cyano-5-ethynyl-4-hydroxybenzoyl)-1,1-dioxo-1,2-dihydro-3*H*-1,3-benzothiazole (14)



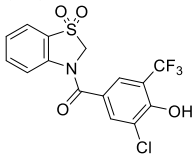
^{1}H-NMR (270 MHz , DMSO-d_6) δ: 8.05 (1H, d, J = 7.6 Hz), 8.04 (1H, d, J = 2.4Hz), 7.93 (1H, d. J = 2.4 Hz), 7.91 (1H, d, J = 8.4 Hz), 7.76 (1H, dd, J = 8.4, 7.6 Hz), 7.44 (1H, dd, J = 7.6, 7.6 Hz), 5.35 (2H, s), 4.58 (1H, s). (The phenol –OH was not observed)
MS (m/z): 337 $(M-H)^-$. HRMS (ESI): m/z calcd for $C_{17}H_9N_2O_4S$ $(M-H)^-$ 337.0288, found 337.02902.

3-(3-Cyano-4-hydroxy-5-methylsulfanylbenzoyl)-1,1-dioxo-1,2-dihydro-3*H*-1,3-benzothiazole (15)



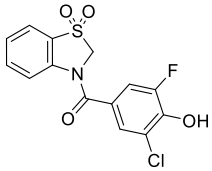
^{1}H-NMR (270 MHz, DMSO-d_6) δ: 8.08 (1H, d, J = 8.4 Hz), 7.91 (1H, d, J = 7.3 Hz), 7.82(1H, d, J = 2.2 Hz), 7.77 (1H, dd, J = 8.4, 7.6 Hz), 7.68 (1H, d, J = 2.2 Hz), 7.45 (1H, dd, J = 7.6, 7.3 Hz), 5.34 (2H, s), 2.46 (3H, s). (The phenol –OH was not observed)
MS (m/z): 359 $(M-H)^-$. HRMS (ESI): m/z calcd for $C_{16}H_{11}N_2O_4S_2$ $(M-H)^-$ 359.0165, found 359.0166.

3-(3-Chloro-4-hydroxy-5-trifluoromethylbenzoyl)-1,1-dioxo-1,2-dihydro-3*H*-1,3-benzothiazole (16)



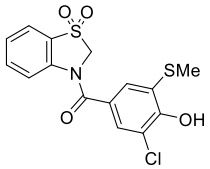
^{1}H-NMR (270 MHz, DMSO-d_6) δ: 8.07 (1H, d, J = 8.2 Hz), 8.06 (1H, d, J = 2.1 Hz), 7.91 (1H, dd, J = 7.8, 0.8 Hz), 7.86 (1H, d, J = 2.1 Hz), 7.77 (1H, ddd, J = 8.2, 7.8, 1.3 Hz), 7.44 (1H, ddd, J = 7.8, 7.8, 0.8 Hz), 5.36 (2H, s). (The phenol –OH was not observed)
MS (m/z): 390 $(M-H)^-$. HRMS (ESI): m/z calcd for $C_{15}H_8ClF_3NO_4S$ $(M-H)^-$ 389.9820, found 389.9829.

3-(3-Chloro-5-fluoro-4-hydroxybenzoyl)-1,1-dioxo-1,2-dihydro-3*H*-1,3-benzothiazole (17)



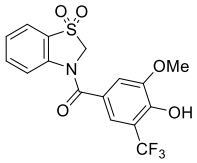
^{1}H-NMR (270 MHz, DMSO-d_6) δ: 11.35 (1H, brs), 8.02 (1H, d, J = 8.4Hz), 7.90 (1H, d, J = 7.4 Hz), 7.76 (1H, ddd, J = 8.4, 7.4, 1.2 Hz), 7.61 (1H, s), 7.59 (1H, dd, J = 11.1, 1.8 Hz).7.43 (1H, dd, J = 7.4, 7.4 Hz), 5.35 (2H, s).
^{13}C-NMR (101 MHz, DMSO-d_6) δ: 165.8, 151.3 (d, J = 245Hz), 145.4(d, J = 15.6Hz), 139.4, 134.5, 129.0, 125.9, 125.8(d, J = 17.5Hz), 124.6, 122.3(d, J = 3.8Hz), 121.3, 120.6, 115.1(d, J = 21.3Hz), 67.1.
MS (m/z): 340 $(M-H)^-$, 342 $(M+2-H)^-$. HRMS (ESI): m/z calcd for $C_{14}H_8ClFNO_4S$ $(M-H)^-$ 339.9852, found 339.9852.

3-(3-Chloro-4-hydroxy-5-methylsulfanylbenzoyl)-1,1-dioxo-1,2-dihydro-3*H*-1,3-benzothiazole (18)



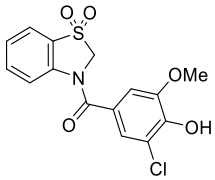
^{1}H-NMR (270 MHz, DMSO-d_6) δ: 10.54 (1H, s), 8.02(1H, d, J = 8.6 Hz), 7.90 (1H, d, J = 7.6 Hz), 7.76 (1H, dd, J = 8.6, 7.8 Hz), 7.54 (1H, s), 7.43 (1H, dd, J = 7.8, 7.6 Hz), 7.38(1H, s), 5.34 (2H, s), 2.44 (3H, s).
^{13}C-NMR (101 MHz, DMSO-d_6) δ: 166.6, 151.9, 139.6, 134.5, 129.7, 129.0, 126.8, 125.9, 124.1, 124.0, 121.3, 120.7, 120.1, 67.3, 14.1.
MS (m/z): 368 $(M-H)^-$. HRMS (ESI): m/z calcd for $C_{15}H_{11}ClNO_4S_2$ $(M-H)^-$ 367.9823, found 367.9831.

3-(4-Hydroxy-3-methoxy-5-trifluoromethylbenzoyl)-1,1-dioxo-1,2-dihydro-3*H*-1,3-benzothiazole (19)



^{1}H-NMR (DMSO-d_6, 270 MHz) δ: 10.68 (1H, s). 8.02(1H, d, J = 8.1 Hz), 7.90 (1H, d, J = 7.3 Hz), 7.76 (1H, dd, J = 7.3, 7.3 Hz), 7.54 (1H, s), 7.47 (1H, s), 7.43 (1H, dd, J = 8.1, 7.3 Hz), 5.35 (2H, s), 3.93 (3H, s).
^{13}C-NMR (101 MHz, DMSO-d_6) δ: 167.0, 149.3, 148.4, 139.7, 134.4, 129.0, 125.8, 123.6(q, J = 274Hz), 121.3, 120.7, 118.9, 115.7(d, J = 30.0Hz), 114.8, 70.4, 67.3, 56.6.
MS (m/z): 386 $(M-H)^-$. HRMS (ESI): m/z calcd for $C_{16}H_{11}F_3NO_5S$ $(M-H)^-$ 386.0315, found 386.0324.

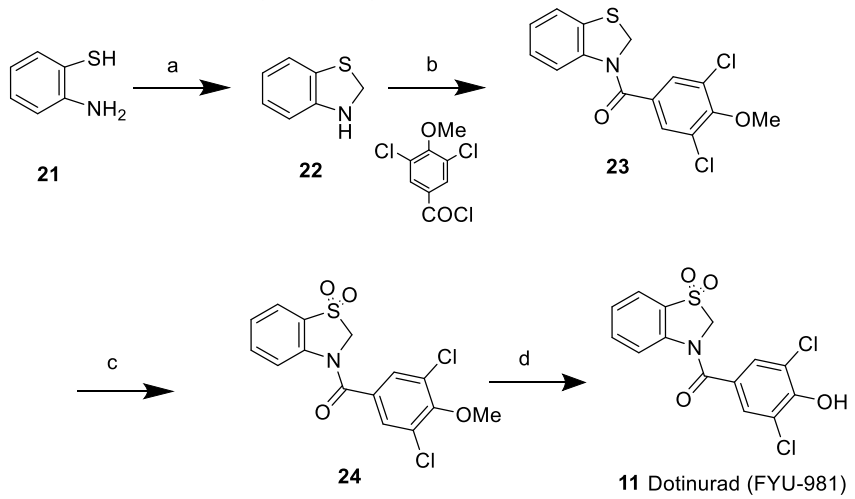
3-(3-Chloro-4-hydroxy-5-methoxybenzoyl)-1,1-dioxo-1,2-dihydro-3*H*-1,3-benzothiazole (20)



^{1}H-NMR (270 MHz, DMSO-d_6) δ: 7.99 (1H, d, J = 8.4 Hz), 7.90 (1H, d, J = 7.6 Hz), 7.75 (1H, dd, J = 8.4, 7.6 Hz), 7.43 (1H, dd, J = 7.6, 7.6 Hz), 7.35 (1H, s), 7.27 (1H, s), 5.34 (2H, s), 3.88 (3H, s). (The phenol –OH was not observed)
^{13}C-NMR (101 MHz, DMSO-d_6) δ: 166.9, 148.5, 146.7, 139.7, 134.4, 129.0, 125.8, 124.5, 122.2, 121.3, 120.7, 119.9, 110.5, 67.4, 56.5.
MS (m/z): 352 $(M-H)^-$. HRMS (ESI): m/z calcd for $C_{15}H_{11}ClNO_5S$ $(M-H)^-$ 352.0051, found 352.0053.

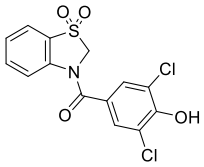
Synthesis of Dotinurad, FYU-981

Synthetic procedure for Dotinurad (FYU-981) [a]



[a] Reagents and conditions: (a) HCHO, Water, Diisopropyl ether; (b) Triethylamine, $CHCl_3$; (c) m-CPBA, 57% from **23**; (d) LiCl, DMF, 78%.

3-(3,5-Dichloro-4-hydroxybenzoyl)-1,1-dioxo-1,2-dihydro-3*H*-1,3-benzothiazole (11)



(i) **1,2-Dihydro-3*H*-1,3-benzothiazole**

37% Formalin (5.2 mL) was diluted with water (80 mL), and diisopropylether (80 mL) and 2-aminobenzenethiol (7.84 g, 62.6 mmol) were added, and then the mixture was stirred at room temperature for 30 minutes. The organic layer was separated, and the aqueous layer was extracted with diisopropylether. The organic layers were combined, washed with brine, dried over anhydrous sodium sulfate, and then concentrated under reduced pressure. The obtained residue was used for the step (iii).

(ii) **3,5-Dichloro-4-methoxybenzoyl chloride**

To 3,5-dichloro-4-methoxybenzoic acid (8.81 g, 39.9 mmol), toluene (170 mL), *N*,*N*-dimethylformamide (5 droplets) and thionyl chloride (6.0 mL) were added, and then the mixture was stirred at 60°C for 16 hours. After the solvent was distilled off under reduced pressure, toluene was added and concentrated. The obtained residue was used for the step (iii).

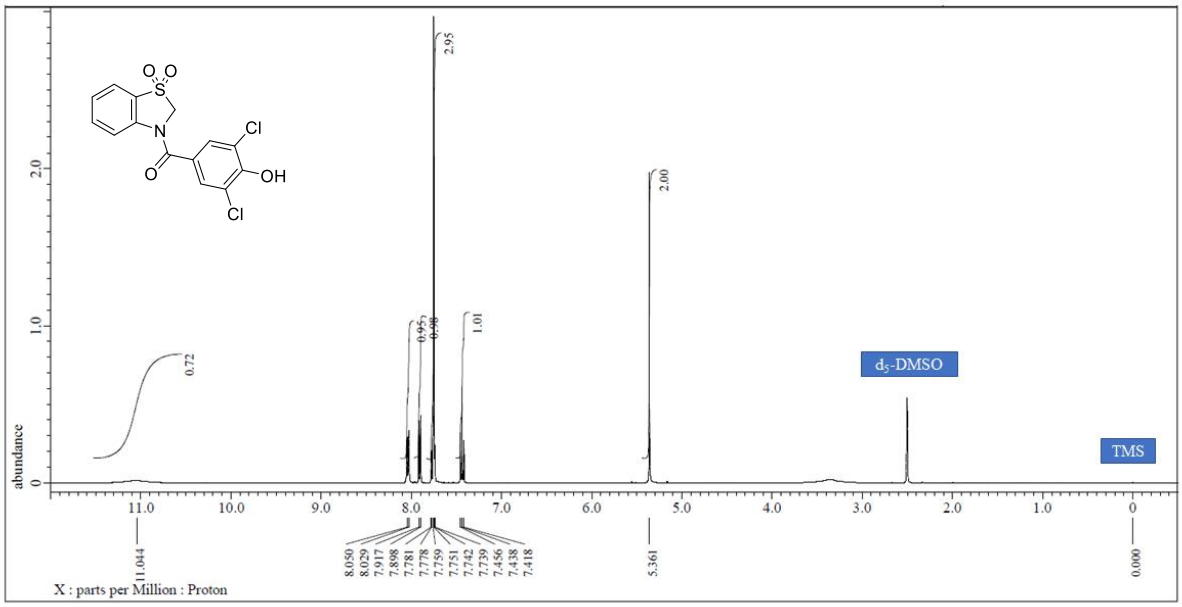
(iii) **3-(3,5-Dichloro-4-methoxybenzoyl)-1,2-dihydro-3*H*-1,3-benzothiazole**

1,2-Dihydro-3*H*-1,3-benzothiazole obtained in step (i) was dissolved in chloroform (50 mL), and triethylamine(17.4 mL) and 3,5-dichloro-4-methoxybenzoyl chloride obtained in step (ii) were added to the solution, and then the mixture was stirred at room temperature for 1 hour. The solvent was distilled off under reduced pressure and water was added, and then the mixture was extracted with ethyl acetate. The organic layer was washed with 1N hydrochloric acid, 1N sodium hydroxide and saturated brine, and then dried over anhydrous sodium sulfate. The solvent was distilled off under reduced pressure and the obtained residue was used for the next step without further purification.
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 7.78(2H, d. J = 8.4 Hz), 7.63 (1H, brs), 7.35 (1H, dd, J = 7.6, 2.4 Hz), 7.06-7.14 (2H, m), 5.33 (2H, s), 3.90 (3H, s).

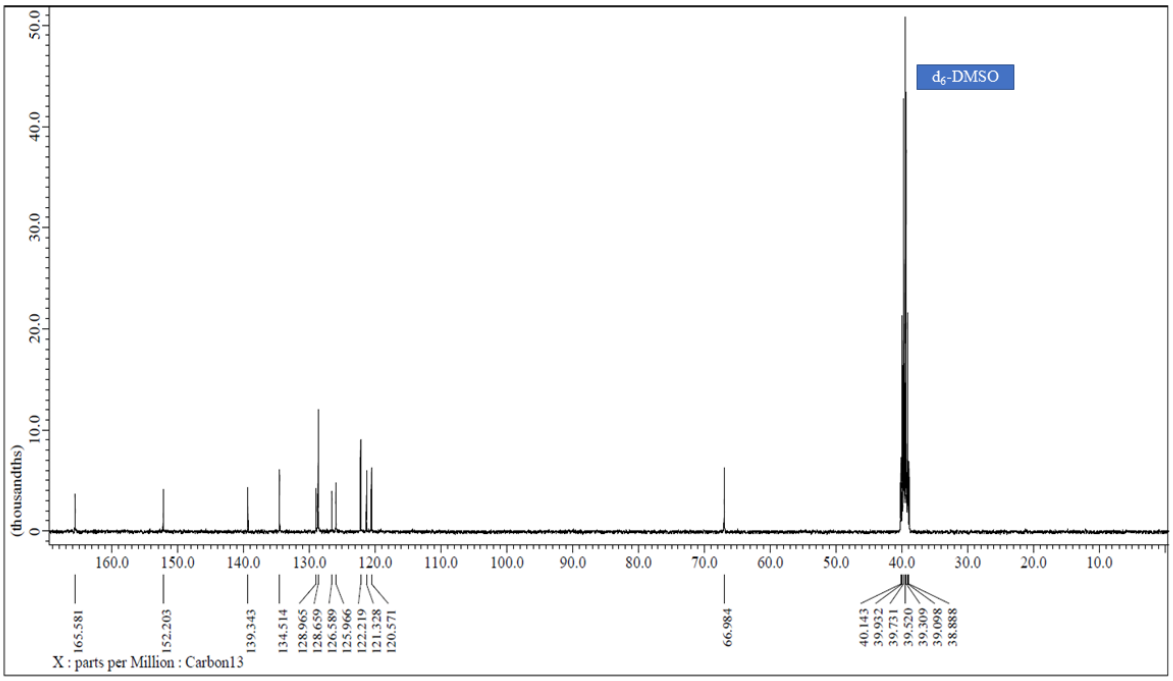
(iv) **3-(3,5-Dichloro-4-methoxybenzoyl)- 1,2-dihydro-3*H*-1,3-benzothiazole**
3-(3,5-Dichloro-4-methoxybenzoyl)-1,2-dihydro-3*H*-1,3-benzothiazole obtained in step (iii) was dissolved in chloroform (230 mL), 70% *m*-chloroperbenzoic acid (43.25 g, 175 mmol) was added to the solution at 0°C, the mixture was stirred at room temperature for 20 hours and quenched with 10% sodium thiosulfate. The solvent was distilled off under reduced pressure and 1N sodium hydroxide was added, and then the mixture was extracted with ethyl acetate. The organic layer was washed with 1N sodium hydroxide and brine, dried over anhydrous sodium sulfate. The mixture was concentrated under reduced pressure to obtain the title compound (13.25 g, 56.9% from step (i)) as a colorless crystal.
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 8.15(1H, d, J = 7.6 Hz), 7. 93 (1H, d, J = 7.2 Hz), 7.86 (2H, s), 7.79 (1H, dd, J = 7.2, 7.2Hz), 7.47 (1H, dd, J = 7.2, 7.6 Hz), 5.33 (2H, s), 3.91 (3H, s).
(v) **3-(3,5-Dichloro-4-hydroxybenzoyl)-1,1-dioxo-1,2-dihydro-3*H*-1,3-benzothiazole (11)**
3-(3,5-Dichloro-4-methoxybenzoyl)-1,1-dioxo-1,2-dihydro-3*H*-1,3-benzothiazole (1.00 g, 2.69 mmol) was dissolved in *N*,*N*-dimethylformamide (5 mL), and lithium chloride (570 mg, 13.4 mmol) was added, and the mixture was stirred at 130°C for 2 hours. To the reaction solution, 1N hydrochloric acid was added, and then the reaction mixture was extracted with ethyl acetate. The organic layer was washed with 1N hydrochloric acid and brine, and then dried over anhydrous sodium sulfate. The mixture was concentrated under reduced pressure and the obtained residue was crystallized from ethanol to obtain the title compound (749 mg, 77.8% yield) as a colorless crystal.
^{1}H-NMR (400 MHz, DMSO-d_6) δ: 11.05 (1H, brs). 8.04 (1H, d, J = 8.0 Hz), 7. 91 (1H, dd, J = 8.0, 1.2Hz), 7.76 (1H, td, J = 8.0, 1.2Hz), 7.75(2H, s), 7.44 (1H, t, J = 8.0 Hz), 5.36 (2H, s).
^{13}C-NMR (101 MHz, DMSO-d_6) δ: 165.58, 152.20, 139.34, 134.51, 128.97, 128.66, 126.59, 125.97, 122.22, 121.33, 120.57, 66.98.
MS (ESI): 356 $(M-H)^-$, 358 $(M+2-H)^-$. HRMS (ESI): m/z calcd for $C_{14}H_8Cl_2NO_4S$ $(M-H)^-$ 355.9556, found 355.9564.

3. NMR Spectrum of Dotinurad (FYU-981)

^{1}H-NMR (400 MHz, DMSO-d_6)



^{13}C-NMR (101 MHz, DMSO-d_6)



4. Methods for in vitro, and pharmacokinetic studies

1) RPTEC assay

i) Uric acid uptake inhibition assay using primary human renal proximal tubule epithelial cells

Primary human renal proximal tubule epithelial cells (RPTEC) were cultured in T75 cell culture flasks till 80% confluency. RPTEC were seeded in a 24 well plate at a density of 160,000 cells per well in 800 µL of complete medium with 0.1 µM insulin [2] and incubated at 37°C, 5% CO_2 incubator for 24 h. Following incubation, the cells were treated with 2-pyrazinecarboxylic acid (PZA) at a concentration of 5 mM with care taken that the pH of the solution was in the physiological range (pH 7.4). The assay plate was incubated for 0.5 hours at 37°C after gentle shaking. The buffer containing PZA was discarded and cells were washed twice with 800 µL of ice-cold transport buffers without PZA. For the inhibition assay, 600 µL of 37°C warmed transport buffer containing test compounds (final concentration: 1, 3, 10, 30, and 100 µM) were added and pre-incubate for 15 min at 37°C. Uptake assay initiated by adding 20 µM [^{14}C]-uric acid was performed for 2 h at 37°C on a plate shaker set at 300 rpm. Following uptake, the cells of all the wells were dissociated using cell dissociation buffer and collected in tubes. The cells were washed twice with 300 µL of ice-cold PBS and lysed with 75 µL of 0.1N NaOH, and incubated for 10 min at 37°C. The content of the tubes was transferred to 96 well white opaque plates. Microscint-40 was added to all the wells and the radioactivity was measured using scintillation counter.

ii) Dose-dependent inhibition of uric acid uptake into RPTEC by compound 11 (FYU-981)

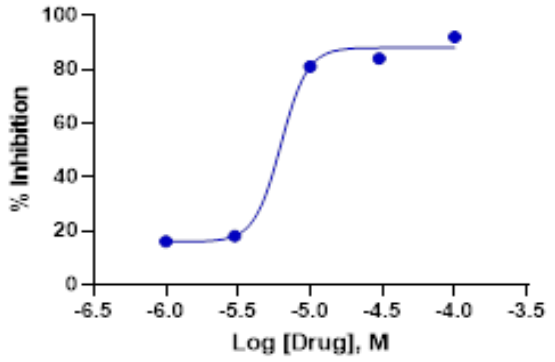


Figure 4-1 Dose-dependency of FYU-981 in RPTEC assay

2) URAT1 assay

i) URAT1-mediated uric acid uptake assay

Methods for preparation of oocytes, in vitro synthesis of URAT1 (SLC22A12)-cRNA, and uptake experiments were described previously.[3] In brief, the oocytes were injected with cRNA and cultured for 2 days and then preincubated in ND96 buffer at 25°C for 15 min. To initiate uptake of [^{14}C]-uric acid, the oocytes were incubated with uptake buffer containing 20 µM [^{14}C]-uric acid and test compounds at 25°C. After 60 min, uptake was terminated by washing the oocytes three times with ice-cold uptake buffer. The oocytes were solubilized with 5% sodium dodecyl sulfate solution. Radioactivity was measured using a liquid scintillation counter (Perkin Elmer, Boston, MA). Uptake was expressed as the cell/medium ratio (microliters per oocyte), obtained by dividing the uptake amount by the concentration of substrate in the uptake buffer.

ii) Dose-dependent inhibition of URAT1-mediated uric acid uptake by compound 11 (FYU-981)

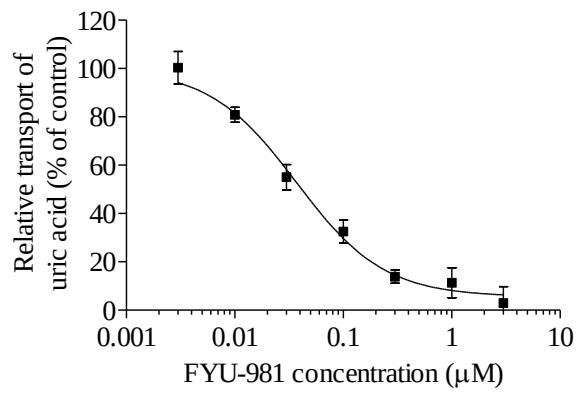


Figure 4-2 Dose-dependency of FYU-981 in URAT1 assay

iii) Correlation of inhibitory effect by compound 11 (FYU-981) between RPTEC and URAT1 assays

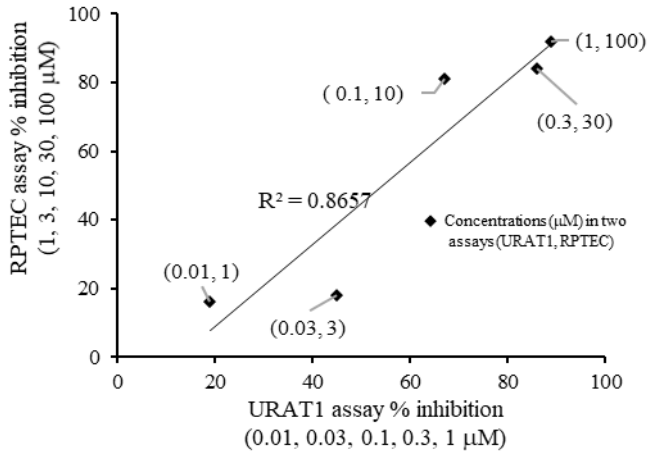


Figure 4-3 Correlation of inhibitory effect by FYU-981 between RPTEC and URAT1 assays

3) Oxygen consumption as an index of mitochondrial respiration

Rat mitochondria were isolated from the liver by differential centrifugation according to the method of Hoppel et al.[4] Oxygen consumption was measured in a chamber equipped with a Clark-type oxygen electrode (Instec Laboratories, Inc., PA USA) at 30°C.[5] The respiratory control ratio (RCR) was determined as a marker of the coupling of oxidative phosphorylation of the mitochondria. The RCR is the ratio of the rate of the oxygen consumption in the presence of a substrate and ADP (state 3) to the rate after complete conversion of ADP to ATP (state 4).[6]

4) Pharmacokinetics in rats

Each compound was orally administered to fasted rats at a dose of 3 mg/kg. Blood was collected from jugular vein at several time points after dosing and centrifuged to obtain plasma. In separate experiments, urine collection at 0–4 h was performed. Concentrations of the compound in plasma and urine were determined by HPLC. As an index of urinary excretion, the excretion rate in urine (%) per amount of compounds treated was determined.

5. References

(1) Kobashi, S.; Uda, J.; Miyata, S.; Inoue, T.; Ashizawa, N.; Matsumoto, K.; Taniguchi, T.; Iwanaga, T.; Nagata, O. Novel Phenol Derivative. WO/2011/040449

(2) Toyoki, D.; Shibata, S.; Kuribayashi-Okuma, E.; Xu, N.; Ishizawa, K.; Hosoyamada, M.; Uchida, S. Insulin Stimulates Uric Acid Reabsorption via Regulating Urate Transporter 1 and ATP-binding Cassette Subfamily G Member 2. *Am. J. Physiol. Renal Physiol*. **2017**, *313*, F826–F834.

(3) Iwanaga, T.; Kobayashi, D.; Hirayama, M.; Maeda, T.; Tamai, I. Involvement of Uric Acid Transporter in Increased Renal Clearance of the Xanthine Oxidase Inhibitor Oxypurinol Induced by a Uricosuric Agent, Benzbromarone. *Drug Metab. Dispos.* **2005**, *33*, 1791-1795.

(4) Hoppel, C.; DiMarco, J. P.; Tandler, B. Riboflavin and Rat Hepatic Cell Structure and Function. Mitochondrial Oxidative Metabolism in Deficiency States. *J. Biol. Chem.* **1979**, *254*, 4164-4170.

(5) Krahenbuhl, S.; Chang, M.; Brass, E. P.; Hoppel, C. L. Decreased Activities of Ubiquinol: Ferricytochrome c Oxidoreductase (Complex III) and Ferrocytochrome c: Oxygen Oxidoreductase (Complex IV) in Liver Mitochondria from Rats with Hydroxycobalamin[c-lactam]-induced Methylmalonic Aciduria. *J. Biol. Chem*. **1991**, *266*, 20998-21003.

(6) Estabrook, R. Mitochondrial Respiratory Control and Polarographic Measurement of ADP:O Ratios. *Methods Enzymol*. **1967**, *10*, 41-47.