博士論文

新規尿酸排泄促進剤の創薬研究

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Development of novel uricosuric agents with highly potent uric acid reabsorption inhibitory activity

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本論文は、学術雑誌に収録された次の報文を基礎とするものである。

1. Uda J.; Kobashi S.; Miyata S.; Ashizawa N.; Matsumoto K.; Iwanaga T. Discovery of Dotinurad (FYU-981), a New Phenol Derivative with Highly Potent Uric Acid Lowering Activity. *ACS Med. Chem. Lett.,* **2020**, *11*, 2017–2023.

2. Uda J.; Kobashi S.; Ashizawa N.; Matsumoto K.; Iwanaga, T. Novel Monocyclic Amide-Linked Phenol Derivatives without Mitochondrial Toxicity Have Potent Uric Acid-Lowering Activity. *Bioorganic Med. Chem. Lett.*, **2021**, *40*, 127900.

3. Uda J.; Ashizawa N.; Iwanaga T. An Evaluation Method for Uric Acid Uptake Inhibition Using Primary Human Proximal Tubule Epithelial Cells Treated with Insulin. *Nucleosides Nucleotides Nucleic Acids*, **2022**, *41*, 724–735.

目次

略 語 表

DMF	*N*,*N*-dimethylformamide
THF	tetrahydrofuran
MOM	methoxymethyl
EDC·HCl	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
HOBt	1-hydroxybenzotriazole
m-CPBA	*m*-chloroperbenzoic acid
TFA	trifluoroacetic acid
CYP	cytochrome P450
XOI	xanthine oxidoreductase inhibitor
BBR	benzbromarone
URAT1	urate transporter 1
RPTECs	renal proximal tubule epithelial cells
ABCG2	ATP-binding cassette sub-family G member 2
OAT	organic anion transporter
MRP4	multidrug resistance protein 4
GLUT9	glucose transporter 9
MIA	mitochondrial inhibitory activity
RCR	mitochondrial respiratory control ratio
UUI	uric acid uptake inhibition
PZA	2-pyrazinecarboxylic acid
NMDG	*N*-methyl-D-glucamine
DPM	disintegration per minute
SURI	selective urate reabsorption inhibitor
NMR	nuclear magnetic resonance
TMS	tetramethylsilane
MS	mass spectrum
HRMS	high resolution mass spectrum
ESI	electrospray ionization

序論

　本論文は、痛風および高尿酸血症の治療に用いる尿酸排泄促進剤の開発を目指した創薬研究に関するものである。

　高尿酸血症は過食等による尿酸の過剰産生を原因とした「尿酸産生過剰型」、腎機能の低下等による尿酸の排泄低下を原因とした「尿酸排泄低下型」[1]、それらの混合した「混合型」に分類される。国内の調査によると、高尿酸血症患者のうち約60％は「尿酸排泄低下型」であり[2]、約25％の「混合型」を含めると、約85％が尿酸排泄機能の低下を伴っている。しかし、新たに腎外（主に腸管）からの排泄低下がもう一つの原因であることが明らかとなった。そのため、現在では、従来の「尿酸産生過剰型」を、真の「尿酸産生過剰型」および「腎外排泄低下型」の二つのタイプからなる「腎負荷型」と定義し、新たな病型分類では高尿酸血症を「尿酸排泄低下型」、「腎負荷型」および「混合型」に分類している[3]。

　尿酸は、核酸を構成するプリンの代謝物の一つであるキサンチンから、キサンチン酸化還元酵素による酸化的代謝により生成する（**Figure 1**）。

　ヒトを含む大型類人猿（チンパンジー、ゴリラ、オランウータン）や小型類人猿（テナガザル）は他の哺乳類とは異なり、尿酸の代謝酵素であるウリカーゼを有しておらず、尿酸がプリンの最終代謝産物である。特にヒトの血中尿酸濃度は他の哺乳動物と比較して著しく高い（ヒト　4–7 mg/dL、イヌ　0.2–0.3 mg/dL、ラット　0.3–0.6 mg/dL）[4]。尿酸には強い抗酸化作用があり、酸化ストレスを減らす効果がある。これが、ヒトの寿命が他の動物種に対し比較的長いことの要因であるとする説がある。しかし、血漿中で尿酸の濃度が高くなると、血中もしくは組織中で尿酸ナトリウム 1 水和物の結晶が析出して痛風関節炎の原因となる。また、高尿酸血症は、腎機能や循環器系の障害に関与する独立した危険因子でもある[5-7]。日本の高尿酸血症・痛風治療のガイドラインでは高尿酸血症を「血清尿酸値 > 7.0 mg/dL」と定義しており[8]、治療にあたり高尿酸血症の原因に即したメカニズムの薬物（尿酸降下薬）を使用することを推奨している。基本的には尿酸産生過剰型にはキサンチン酸化還元酵素阻害剤（XOI）を、尿酸排泄低下型には尿酸排泄促進剤を使用する。
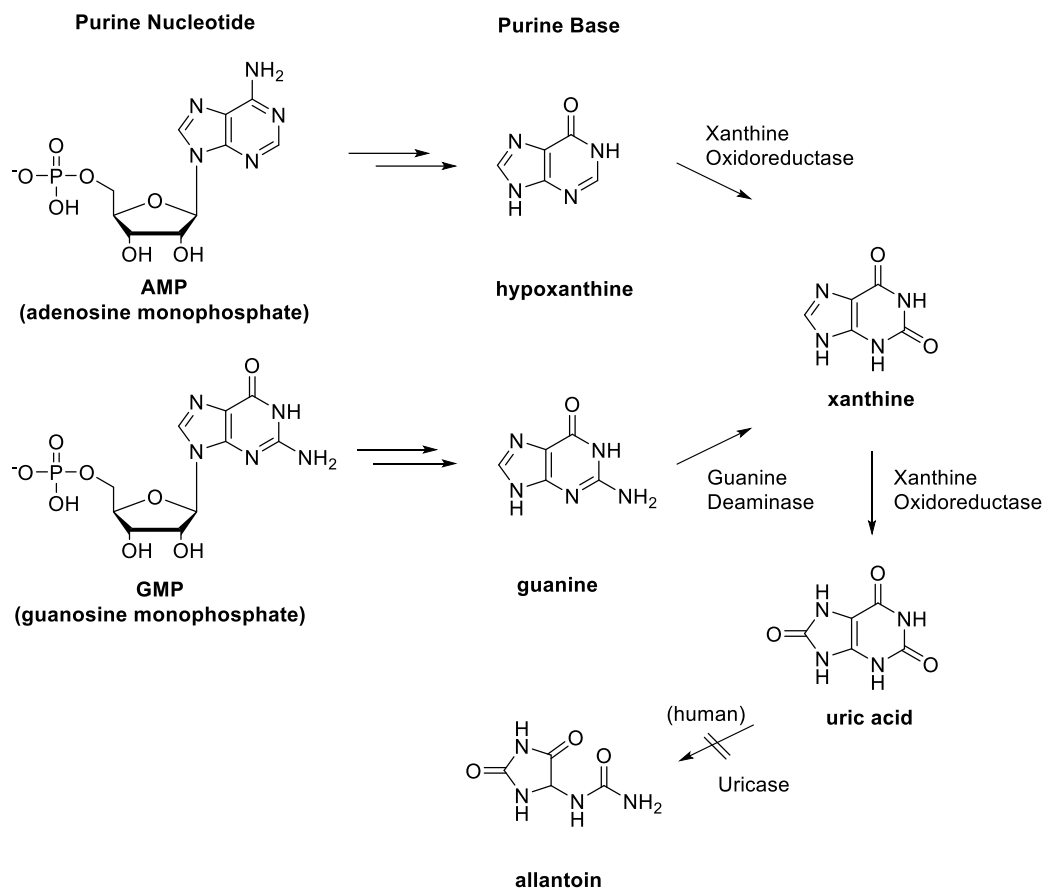
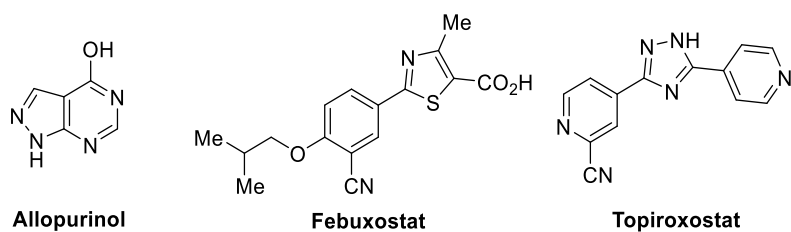**Figure 1.** Metabolism of purine nucleotide to uric acid.

Figure 2. Xanthine oxidoreductase inhibitors.

XOI は、キサンチンから尿酸への変換を担うキサンチン酸化還元酵素（XOR）を阻害する。古典的な XOI であるアロプリノールは腎排泄型の薬物であるため、特に、腎機能が低下した患者に使用した場合には、その代謝物であるオキシプリノールの排泄が困難となり、ときに重篤な皮膚症状（中毒性表皮壊死症）をひき起こす[9]。これに対して、新たな XOI であるフェブキソスタットおよびトピロキソスタットは、肝からの代謝排泄経路も有するため、腎機能が低下した患者にも使用される標準的な薬物となった。これら薬物の化学構造を **Figure 2** に示す。

　一方、尿酸排泄促進剤は、腎の尿酸輸送に関与するトランスポーターの阻害により尿酸の尿中排泄を促進する[10]。そのため、XOI と比較して早く作用し強力な薬効を示す。尿酸は腎糸球体によりろ過されたのち、その約 90% が尿細管で再吸収される。特に腎の近位尿細管上皮細胞（renal proximal tubule epithelial cells；RPTECs）には尿酸を輸送するトランスポーターが高発現しており、尿酸再吸収に urate transporter 1（URAT1）、organic anion transporter 4（OAT4）、OAT-10、glucose transporter 9（GLUT9）が、分泌に multidrug resistance protein 4（MRP4）、ATP-binding cassette sub-family G member 2（ABCG2）、OAT1、OAT3、NPT-1、NPT-4 が関与している[11-13]（**Figure 3**）。特に尿酸再吸収における URAT1 の関与は大きく、URAT1 遺伝子の欠損により腎性低尿酸血症（血清尿酸値 ≦ 2.0 mg/dL）を生じる。
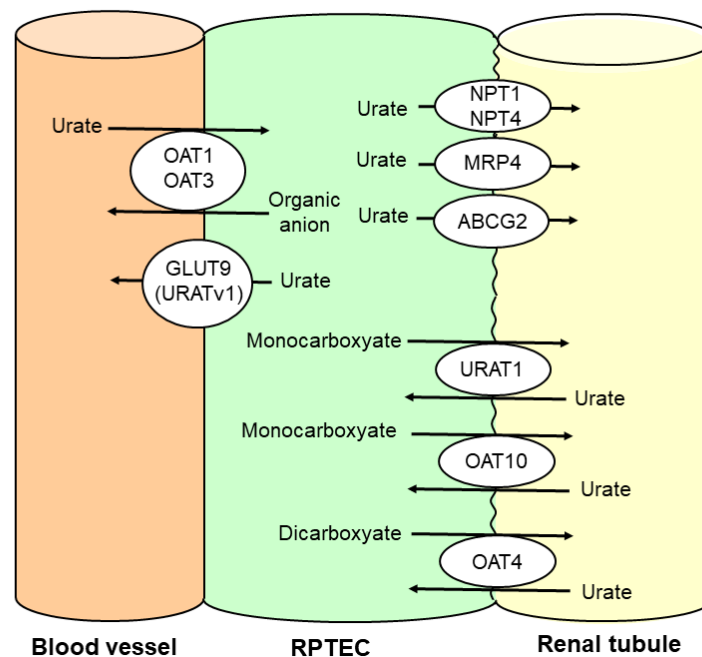

Figure 3. Transporters involved in the uptake and excretion of uric acid in PRTECs.

尿酸排泄促進剤にはプロベネシド、ベンズブロマロン（BBR）およびレシヌラドがある（**Figure 4**）。プロベネシドは非選択的なアニオントランスポーター阻害剤であり、尿酸の排泄を促進するだけでなく、他の薬物の排泄にも関与し薬物相互作用を引き起こす[14, 15]。BBR は尿酸排泄を強く促進するため、日本、ヨーロッパ、ブラジルなどの一部の国で使用されてきた。しかし、致死性の劇症肝炎が報告されたのち、日本では規制当局の警告によりその使用が制限され、ヨーロッパでは販売中止となった[16]。また、BBR は、遺伝子多型が存在する酵素であるチトクロム P450 2C9（CYP2C9）の阻害剤かつ基質にもなる（本酵素により代謝を受ける）。CYP2C9 を欠損している患者はこの酵素の基質となる薬物を代謝できないため、BBR の薬効や安全性[17] に影響を受けることが問題となる。レシヌラドは米国および EU で承認されたが、トランスポーターの選択性や薬理活性は低いうえ、BBR と同様に CYP2C9 の基質となる[18-20]。このような背景から臨床上のデメリットが少なく、標準的に使用される新たな薬物の開発が望まれていた。
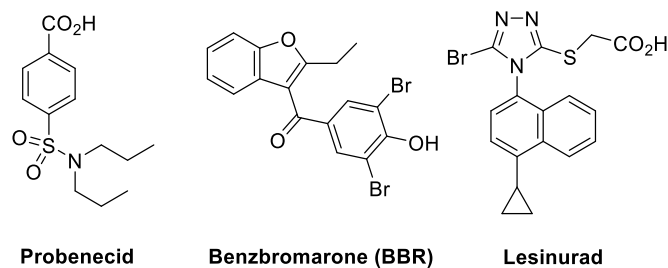

Figure 4. Uricosuric agents.

　BBR は前述のデメリットを有するが、強い薬理活性を示す点では魅力的である。そこで著者は、BBR の毒性が構造と関係する可能性に着目し、この化合物を出発点とする構造展開によって、強い薬理活性と高い安全性を両立する、新たな薬物を創製することを目指した。

　以下、本論にて、その結果の詳細について述べる。

第一章
尿酸排泄促進剤 Dotinurad の創出

第一節　ベンズブロマロンの構造的特徴とミトコンドリア毒性

　ベンズブロマロン（BBR）は URAT1 を強力に阻害し、これが尿酸排泄促進作用の主なメカニズムである [21]。BBR は電子求引性置換基であるブロモ基をフェノール環上に二つ有し、尿酸に近い酸性度（BBR の pK_a 値：5.17 vs. 尿酸の pK_a 値：5.75）を示す。また、平面状となり得る化学構造をもつ点でも、尿酸と特徴を共有する（**Figure 5**）。これらの特徴は、BBR がトランスポーターを阻害するうえで重要な役割を果たしている。

pK_a 5.17
Benzbromarone

pK_a 5.75
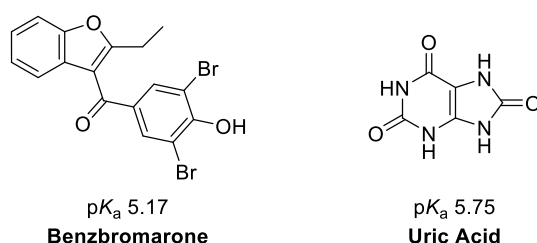**Uric Acid**

Figure 5. pKa values and the structures of BBR and uric acid.

　前述のように、BBR は稀に致死的な劇症肝炎を引き起こすが、その発症メカニズムは必ずしも明らかになっていない。代謝過程や代謝物そのものに原因があるとする説もあるが、もう一つの有力な説はミトコンドリア毒性に起因するというものである [22–27]。BBR は部分的にビスアリールケトン構造を有するが、同じ部分構造を有する薬物には、ミトコンドリア毒性および肝毒性を示すものがいくつか存在する。アミオダロン（抗不整脈薬）、トルカポン（パーキンソン病治療薬）およびフェノフィブラート（高脂血症治療薬）などがそうであり [27–29]（**Figure 6**）、これら薬物の毒性はそのビスアリールケトン構造に起因すると推測される。
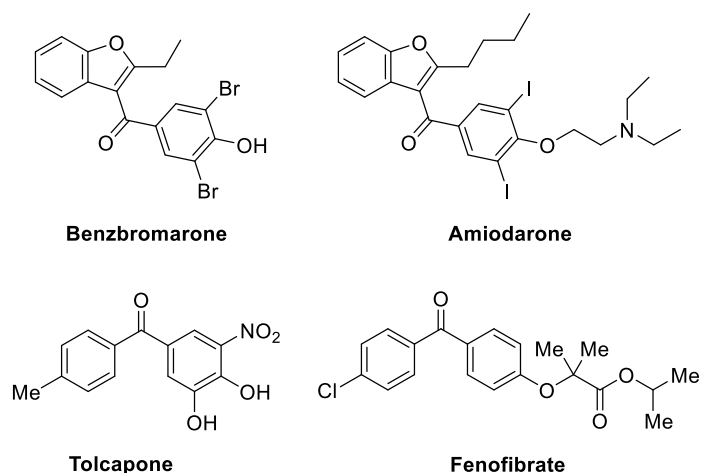
Figure 6. Medicines having bis-aryl ketone structure.

BBR およびアミオダロンとその類縁化合物は、ミトコンドリア毒性の指標となるミトコンドリア呼吸鎖阻害活性（MIA）についての構造活性相関が報告されている [25–27]。**Figure 7** にまとめたように、アミオダロンの部分構造に相当する安息香酸ユニットおよびベンゾフランユニットは MIA をほとんど示さない一方で、アミオダロン、ベンザロンおよび BBR の MIA は強い。したがって、ビスアリールケトン構造から脱却すれば、ミトコンドリア毒性を回避できることが期待される。また、ベンザロンと BBR を比較すると、フェノール環上のブロモ基により MIA が増すことがわかる。さらに、このブロモ基は反応性代謝物を生成する原因となり肝毒性につながるという説もあり、リスク因子である。
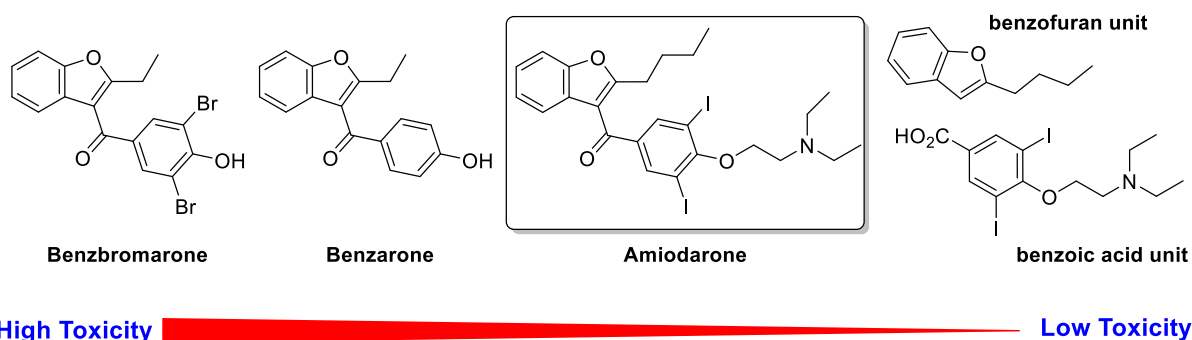
Figure 7. Structure-activity relationship between the structure of BBR related derivatives and the inhibition activity of mitochondrial respiratory chain.

ビスアリールケトン構造をもつ化合物から生成するラジカル種は安定化される。そしてそのことが毒性発現に関与することが知られている。例えば、ベンゾフェノンの光感作作用による皮膚毒性には、ラジカルの関与するメカニズムが提唱されている（**Figure 8**）[30]。
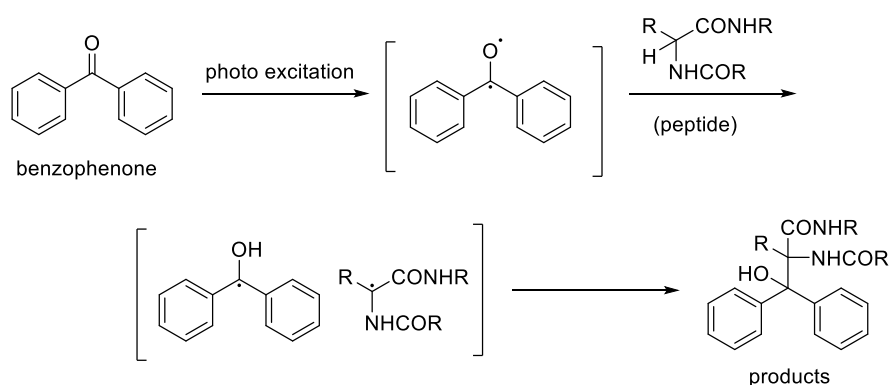
Figure 8. Mechanism of the photoinduced toxicity of benzophenones involving radical intermediates.

　このようなことから著者は、比較的安定なラジカル種を生じ得る化合物は、細胞中で酸化還元反応が行われているミトコンドリア呼吸鎖に影響をあたえると推測した。そこで、BBR のビスアリールケトン構造に変更を加えることにより、毒性を回避し、かつ薬理活性を維持する化合物を創製することを目指すこととした。

第二節　インドリンの 4-ヒドロキシベンズアミド誘導体の合成と尿酸取り込み阻害活性

　BBR を起点とした構造展開によって薬理活性の発現と毒性回避を両立させるため、まず、以下の戦略で臨むこととした（**Figure 9**）。

（1）ケトン構造をアミド構造へと代える：BBR のビスアリールケトン構造 [Ar-(CO)-Ar']をアミド型構造[Ar-(CO)-N]へと変え、ラジカルの安定化を抑制し、ミトコンドリア毒性を軽減する。

（2）アミド構造 [Ar-(CO)-N] のアシル部位 [Ar-(CO)-] としては、BBR の 4-ヒドロキシフェニル基とカルボニル基とを含む構造（4-hydroxylbenzoyl）を採用し、かつ、ブロモ基を他の電子求引基に代えることによりリスク回避を図る。

（3）アミド構造 [Ar-(CO)-N] のアミン部位としては、BBR のベンゾフラン部位と同じ二環性構造をもつインドールやインドリン等のアミンを採用する。
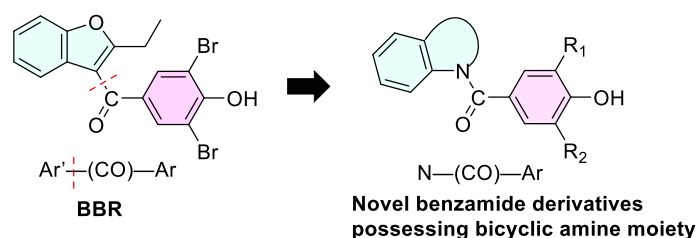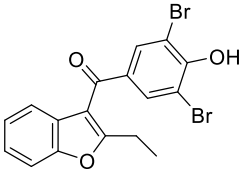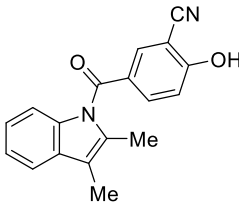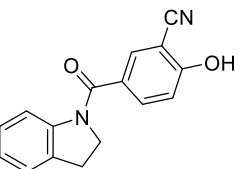
Figure 9. Design of novel benzamide derivatives based on BBR structure.

　上記の戦略にもとづき誘導体の合成をすすめ、ヒト腎において尿酸の輸送（排出と取り込み）に関与している近位尿細管上皮細胞（RPTECs）への尿酸取り込み阻害活性（UUI）を評価した。この評価系は、本研究のために新たに構築したものであり、その確立については第三章に詳細を記載する。また、ミトコンドリア毒性の指標として、酸素消費速度を指標としたミトコンドリア呼吸鎖阻害活性（MIA）を評価した。

　はじめに、ブロモ基に代わる電子求引性基としてシアノ基をもつインドール誘導体 1 およびインドリン誘導体 **2a** を合成し（合成法は後述）、UUI、MIA を評価した（**Table 1**）。

Table 1. UUI and MIA of BBR, **1**, and **2a**

Benzbromarone (BBR) **Indole analog 1** **Indoline analog 2a**

Compound	UUI [a] IC$_{50}$ (μM)	MIA [b] IC$_{50}$ or inhibition (%)
BBR	6.8	3.1 μM
1	10.8	3.6 μM
2a	2.5	(43%)

[a] UUI: Inhibitory activity of urate uptake into RPTECs.

[b] MIA: Mitochondrial respiratory control ratio (RCR), IC$_{50}$ (μM), or inhibition (%) at 100 μM.

　その結果、二つの化合物は *in vitro* 薬理試験において BBR と同等の UUI を示した。一方、MIA については、インドール誘導体 **1** は BBR と同等であったが、インドリン誘導体 **2a** では減弱し、100 μM でも 50%以上の活性を示さなかった。そのため、**Table 1** には 100 μM における阻害率（43%）を表記した。以降も MIA については、50%阻害濃度（IC$_{50}$）が 100 μM 以上になる場合には、適宜、100 μM における阻害率（%）を記すこととする。上記の結果は、芳香族であるインドールを含むアミド構造がビスアリールケトン様の性質を示すことによると推測できる。

　そこで、インドリン誘導体 **2a** の 4-hydroxybenzoic acid 部位に置換基を追加した各種誘導体 **2b–2i** を合成し、評価することとした。ここで、前述の化合物 **1** および **2a–2i** の合成法を述べる。

Scheme 1. Synthesis of indole analog **1**
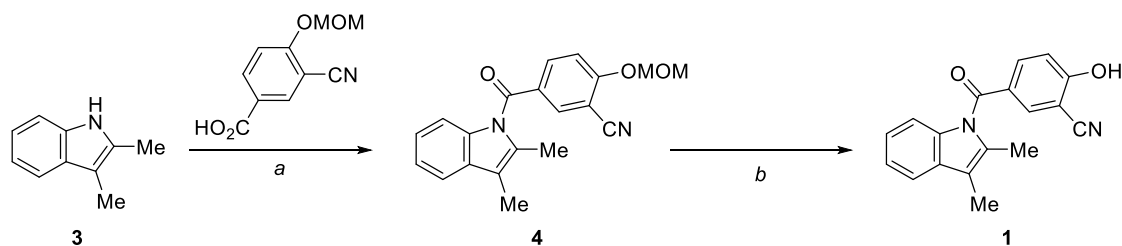
Reagents and conditions: (*a*) EDC·HCl, HOBt in DMF, CHCl₃; (*b*) 4 M HCl in 1,4-dioxane, THF, 35% yield over two steps.

　化合物 **1** の合成法を **Scheme 1** に示した。まず、ジメチルホルムアミド（DMF）とクロロホルムの混合溶媒中、1–エチル–3–（3–ジメチルアミノプロピル）カルボジイミド塩酸塩（EDC·HCl）と 1-ヒドロキシベンゾトリアゾール（HOBt）を用いて、2,3–ジメチルインドールを 4–メトキシメトキシ–3–シアノ安息香酸 ³¹⁾ でアシル化した。次に、THF 溶媒中で HCl の 1,4–ジオキサン溶液を作用させてメトキシメチル（MOM）基を除去し、化合物 **1** を得た（**Scheme 1**）。

　Scheme 2 には、化合物 **2a**–**2i** の合成法を示した。

Scheme 2. Synthesis of indoline analogs **2**

R₁ = H, R₂ = MOM (**a**)
R₁ = OMe , R₂ = MOM (**b**)
R₁ = I, R₂ = Me (**c**)
R₁ = CN, R₂ = Me (**d**)
R₁ = SMe, R₂ = Me (**e**)
R₁ = *tert*-Bu, R₂ = Me (**f**)
R₁ = Cyclopropyl, R₂ = Me (**g**)
R₁ = Cl, R₂ = Me (**h**)
R₁ = CF₃, R₂ = Me (**i**)

R₁ = H (**a**)
R₁ = OMe (**b**)
R₁ = I (**c**)
R₁ = CN (**d**)
R₁ = SMe (**e**)
R₁ = *tert*-Bu (**f**)
R₁ = Cyclopropyl (**g**)
R₁ = Cl (**h**)
R₁ = CF₃ (**i**)
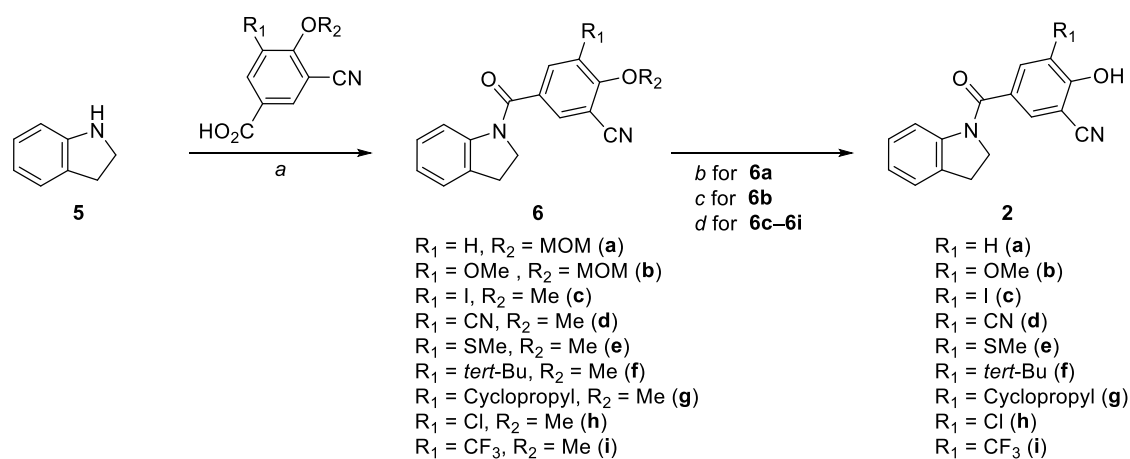
Reagents and conditions: (*a*) EDC·HCl, HOBt in DMF, CHCl₃; (*b*) 4 M HCl in 1,4-dioxane, THF; (*c*) TFA, CH₂Cl₂; (*d*) LiCl, DMF, 35–91% yield over two steps.

まず、インドリン **5** と各々の安息香酸誘導体 [31)]を上記と同様の方法で縮合させた。続いてフェノール部位保護基 R_2 がメトキシメチル基である場合には、THF溶媒中で HCl の 1,4-ジオキサン溶液を作用させる（**6a**）、もしくは、塩化メチレン溶液中でトリフルオロ酢酸を作用させる（**6b**）ことによりフェノールを遊離させた。また、フェノール部位の保護基 R_2 がメチル基である場合には DMF 溶媒中で塩化リチウムを作用させることにより、フェノールを遊離させた。

　Table 2 に、これらの誘導体の UUI および MIA のデータに加えて LogP（計算値）を記した。

Table 2. UUI and MIA of compounds **1** and **2**

Indole analog **1**　　　　Indoline analog **2**
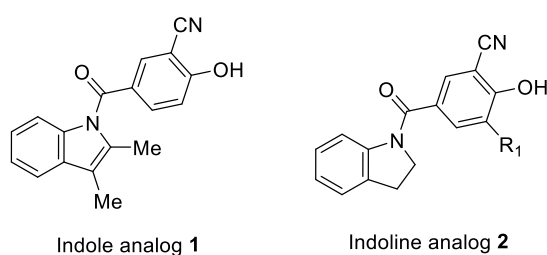
Compound	R_1	UUI [a]		MIA [b]	LogP [c]
		IC$_{50}$ (μM)	%	IC$_{50}$ or inhibition (%)	
1	-	10.8	52.1	3.6 μM	3.89
2a	H	2.5	67.5	(43%)	2.70
2b	OMe	> 100	21.7	> 100 μM (-10%)	2.57
2c	I	5.3	65.1	53 μM	4.05
2d	CN	83.9	26.3	(47%)	2.73
2e	SMe	> 100	24.2	40 μM	3.14
2f	*tert*-Butyl	31.6	32.4	6.6 μM	4.40
2g	Cyclopropyl	8.8	67.8	24 μM	3.43
2h	Cl	14.7	38.2	31 μM	3.26
2i	CF$_3$	> 100	30.0	(40%)	3.62
BBR	-	6.8	51.0	3.1 μM	4.95

[a] UUI: Inhibitory activity of urate uptake into RPTECs IC$_{50}$ and % inhibition at 10 μM.

[b] MIA: Mitochondrial respiratory control ratio (RCR), IC$_{50}$ (μM), or inhibition (%) at 100 μM.

[c] LogP was calculated using ChemBioDraw UltraVer.14.

これら薬理活性およびミトコンドリア毒性について、構造活性相関を考察する。まず、化合物 **2a**（R_1 = H）、**2c**（R_1 = I）、**2g**（R_1 = Cyclopropyl）および **2h**（R_1 = Cl）は比較的強い UUI 活性（IC_{50} < 10 μM）を示したが、電子供与性置換基（R_1 = OMe）を有する **2b** および電子求引性の強い置換基を有する **2d**（R_1 = CN）、**2i**（R_1 = CF$_3$）の活性は低下した。また、かさ高い置換基（*tert*-Butyl）を有する誘導体 **2f** も活性が著しく低下した。これらの結果は、単にフェノール部位の酸性度だけでなく、その近傍の立体的環境等も活性に大きな影響を与えることを示している。また、R_1 が極端にかさ高い *tert*-Butyl 基である **2f**、および R_1 が水素原子である **2a** などの例外もあるものの、概して脂溶性（LogP）が高い方が UUI 活性も高い傾向が見られる。

　一方、各化合物の脂溶性とミトコンドリア毒性との間には相関があることが判明した。**Figure 10** は、LogP 値（計算値）と MIA 値の間の関係を表したものである。

　なお、化合物 **2a**, **2d** および **2i** は 100 μM での MIA がいずれも約 50%であったことから IC_{50} を 100 μM とした。また、化合物 **2b** は 100 μM でほとんど活性を示さなかったが、ここでは暫定的に IC_{50} を 200 μM とした。
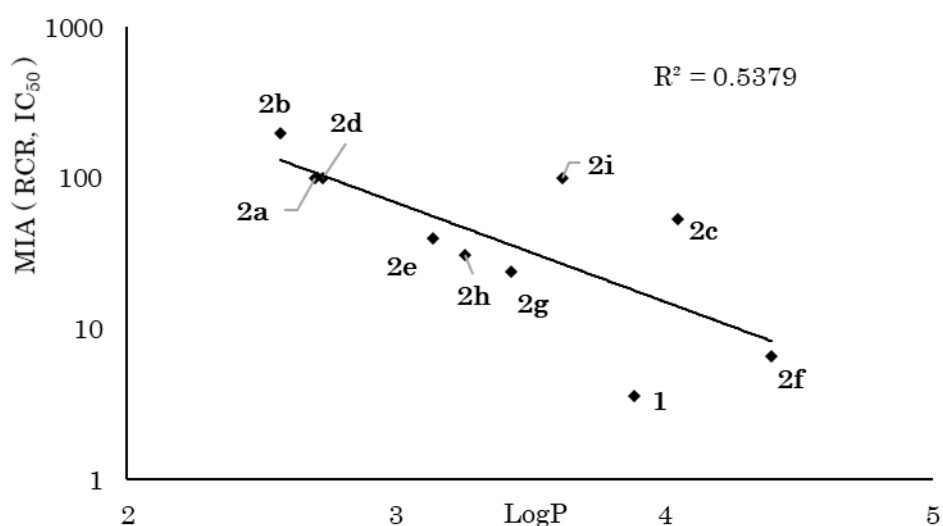
Figure 10. Correlation between MIA (RCR, IC_{50}) and LogP in Table 2. Since compound **2a**, **2d**, and **2i** showed ca. 50% MIA at 100 μM, these IC_{50} values are assigned as 100 μM. Though compound **2b** showed no MIA at 100 μM, the IC_{50} value is tentatively assigned as 200 μM.

その結果、化合物 **1**（インドール誘導体）は相関直線から乖離し、LogP に対し強い MIA を示した。対照的に、化合物 **2c**（R_1 = I）および **2i**（R_1 = CF$_3$）は、LogP に対し弱い MIA を示した。しかし、LogP 値と MIA との間には相関が見られ、決定係数は R^2 = 0.54 であり多くの化合物が相関直線の近くに位置した。これは、標的タンパク質への親和性や膜透過性が重要な鍵を握っていることを示唆している。

　次に UUI［10 μM における阻害率（%）］と MIA（IC$_{50}$）の関係を示す（**Figure 10-1**）。概して、MIA が小さくなると UUI も弱くなる傾向が見られる。しかし、化合物 **2a**，**2c**，**2g** はこの傾向から外れ、同等の MIA を示す他の化合物と比べて UUI が高く、薬物として有用であることがわかった。

　この傾向はより高い濃度（30 または 100 μM）で UUI を評価した場合でも同様であった（**Figure 10-2**, **3**）。
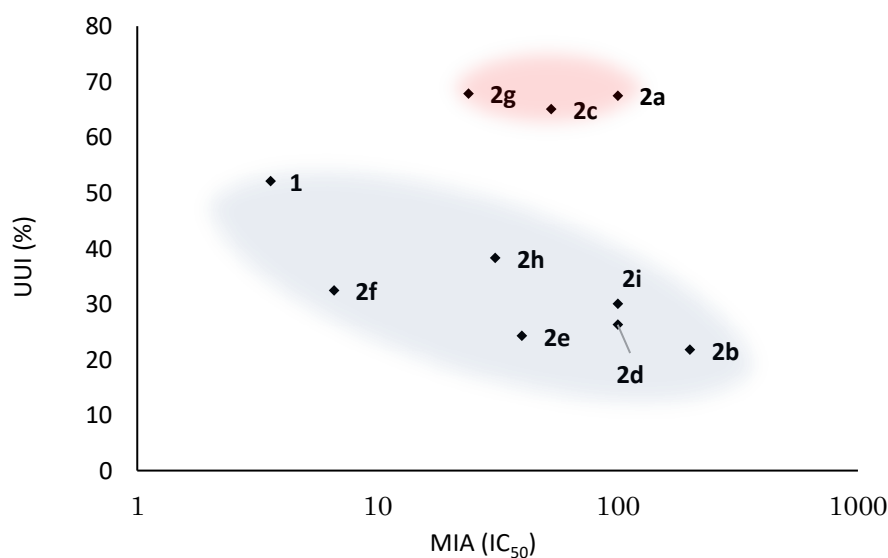
Figure 10-1. Relationship between UUI (% inhibition at 10 μM) and MIA (RCR, IC$_{50}$). Since compound **2a**, **2d**, and **2i** showed ca. 50% MIA at 100 μM, the IC$_{50}$ values are assigned as 100 μM. Though compound **2b** showed no MIA at 100 μM, the IC$_{50}$ value is assigned tentatively as 200 μM.
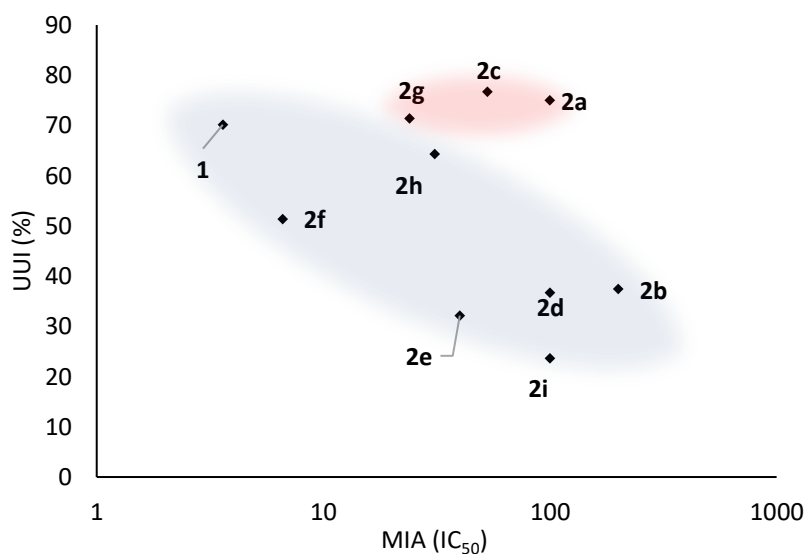
Figure 10-2. Relationship between UUI (% inhibition at 30 μM) and MIA (RCR, IC$_{50}$). Since compound **2a**, **2d**, and **2i** showed ca. 50% MIA at 100 μM, the IC$_{50}$ values are assigned as 100 μM. Though compound **2b** showed no MIA at 100 μM, the IC$_{50}$ value is assigned tentatively as 200 μM.
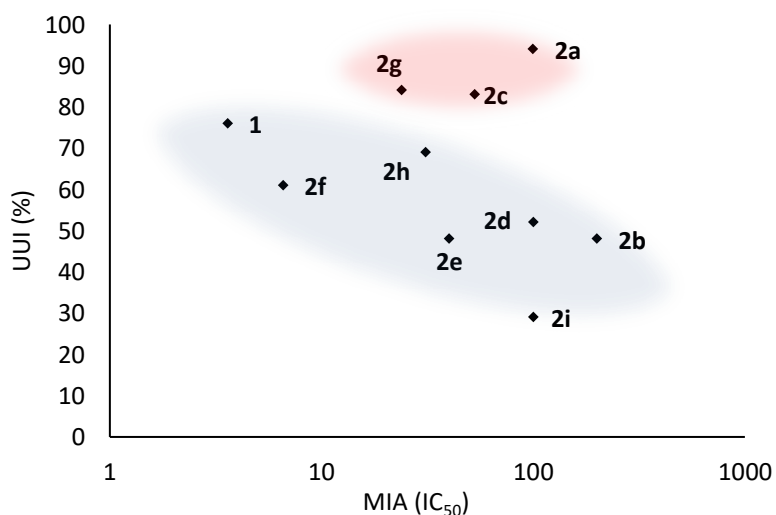
Figure 10-3. Relationship between UUI (% inhibition at 100 μM) and MIA (RCR, IC$_{50}$). Since compound **2a**, **2d**, and **2i** showed ca. 50% MIA at 100 μM, the IC$_{50}$ values are assigned as 100 μM. Though compound **2b** showed no MIA at 100 μM, the IC$_{50}$ value is assigned tentatively as 200 μM.

そこで、化合物 **2a**, **2c** および **2g** の薬物としての可能性をさらに評価するため、ラットでの薬物動態（PK）を調べることとした。**Table 3** には、各化合物の最高血中濃度（$C_{\max}$）と未変化体の尿中排泄率（Exc.）をまとめた。

Table 3. Pharmacokinetic profiles of **2a**, **2c**, **2g**, and BBR

2
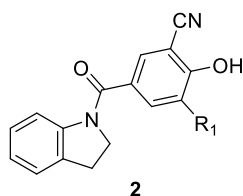
Compound	R₁	$C_{\max}$ [a] (μg/mL)	Exc. [b] (%)
2a	H	0.89	5.9
2c	I	5.95	0.02
2g	Cyclopropyl	1.36	0.35
BBR	–	4.42	0

[a] Maximum concentration ($C_{\max}$).

[b] Excretion rate in urine (%, 0–4 h) after oral administration of 3 mg/kg to rats.

ところで Jian らは、臨床試験において BBR の作用が 48 時間持続したという結果を報告している [32]。一方、及川らの報告によると [35-37]、BBR 投与後の未変化体は、48 時間後には血漿から消失し、尿中にはほとんど排泄されないが、主代謝物である 6 位水酸化体（**Figure 11**, 6-OH-BBR）[33, 34] は、血漿中で 72 時間残存し尿中に 1.2%が排泄される。
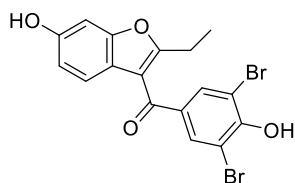
Figure 11. 6-OH-benzbromarone (6-OH-BBR)

6-OH-BBR は BBR に比較し弱いが、薬効を示すには十分な URAT1 阻害活性を示す（6-OH-BBR IC$_{50}$: 0.20 μM、BBR IC$_{50}$: 0.0345 μM）。さらに、URAT1 は尿細管上皮細胞の管腔側（尿側）に発現しており、上述のように 6-OH-BBR の動態が薬効に関連していることから、6-OH-BBR が活性代謝物であり、管腔側で薬理作用を発現することが示唆されている[35-37]。

しかし、BBR を 6-OH-BBR に代謝するのは遺伝子多型を有する CYP2C9 であるので、前述のようにその薬効や安全性に個人差を生じる。したがって、本研究の目指す化合物は、BBR とは異なり未変化体として尿中に排泄され薬効を発現することが望ましい。

ここで著者は、上述の Jian らの報告および及川らの報告を考慮し、最高血中濃度（C_{max}）が BBR と同程度であり、かつ、未変化体の 0–4 時間の尿中排泄率（Exc.）> 1.0%であることを、十分な薬効を期待し得る当面の基準として設定することとした。

この基準であらためて **Table 3** を見ると、化合物 **2a** は C_{max} が不十分である。一方、化合物 **2c** は C_{max} の基準を満たすが、尿中排泄率が低い。また、化合物 **2g** はいずれの値も十分ではない。著者は、これら化合物の薬物動態が不十分であるのは、一般的に酸化を受けやすいインドリン環のベンジル位メチレンの酸化的代謝が原因の一つであると推定し（**Figure 12**）、この部位の構造を変えることとした。その結果を第三節で述べる。
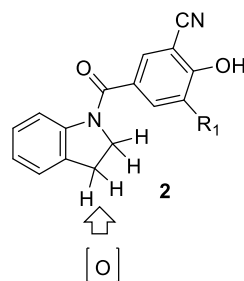
Figure 12. Potential oxidizable part of indoline.

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第三節　1,1-ジオキソ-1,2-ジヒドロ-3*H*-1,3-ベンゾチアゾールの 4-ヒドロキシベンズアミド誘導体の合成と尿酸取り込み阻害活性

　前節で述べたとおり、インドリンの 4-ヒドロキシ安息香酸アミド誘導体 **2a**, **2c**, **2g** が BBR に匹敵する薬理活性（UUI）を示し、かつミトコンドリア毒性も低いことを見出した。しかし、残念ながら、これら化合物の PK 特性は十分ではなく、その原因としてインドリンのメチレン部位の酸化的代謝が疑われた。

　一般的にベンジル位 CH_2 は酸化的な代謝を受けやすい。そこでメチレン基 CH_2 を、スルホニル基 SO_2 にまずは置き替え、直接的な酸化代謝を受けないようにすることを思い描いた。

　その効果を試すため、まず、化合物 **2g** のインドリン部位を 1,1-ジオキソ-1,2-ジヒドロ-3*H*-1,3-ベンゾチアゾールに変換した誘導体 **7** を合成することとした。この化合物の LogP 値（計算値）は 2.85 であり、化合物 **2g** の 3.43 より小さく、相対的に脂溶性が減少するため Exc.が向上することも期待した。

　化合物 **7** は **Scheme 3** に示す方法で合成した。

Scheme 3. Synthesis of 1,1-dioxo-1,2-dihydro-3*H*-1,3-benzothiazole derivative **7**
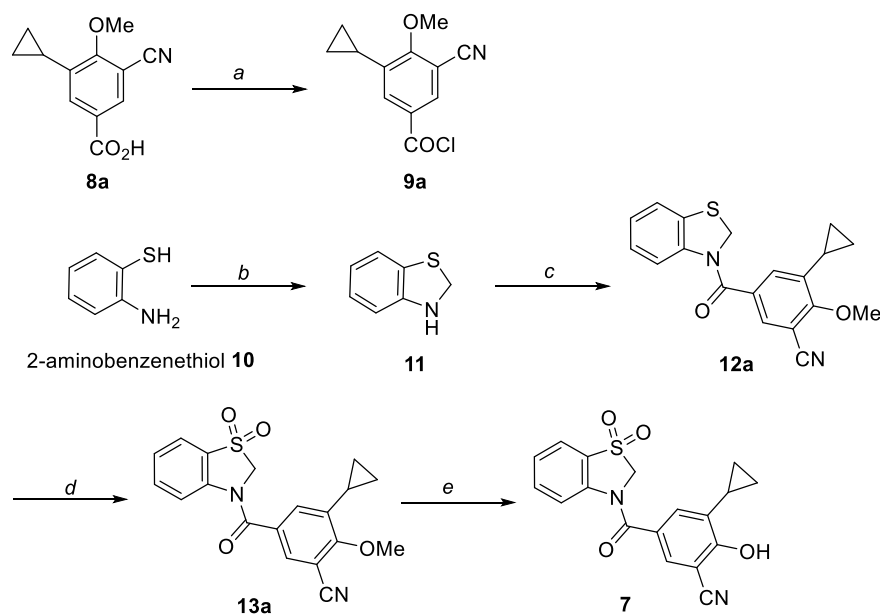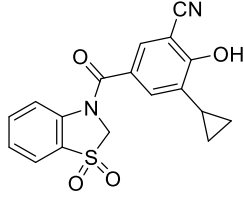
Reagents and conditions: (*a*) SOCl$_2$; (*b*) HCHO, H$_2$O, diisopropyl ether; (*c*) **9a**, triethylamine, CHCl$_3$; (*d*) *m*-CPBA, 43% yield from **8a**; (*e*) LiCl, DMF, 81% yield.
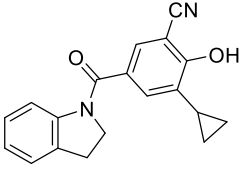
はじめに 2-アミノベンゼンチオール **10** とホルムアルデヒドの反応により、ジヒドロベンゾチアゾール **11** を得た。この中間体は安定性が低いため、精製操作を行わずに次工程に用いた。一方、安息香酸誘導体 **8a** [31)]を、$SOCl_2$ との反応により対応する酸塩化物 **9a** に誘導した。この酸塩化物と上記の **11** とを $CHCl_3$ 溶媒中、トリエチルアミンの存在下に縮合させてアミド **12a** を得た。続いて、このアミドに $CHCl_3$ 溶媒中、*m*-CPBA を作用させることにより 1,1-ジオキソ-1,2-ジヒドロ-3*H*-1,3-ベンゾチアゾール誘導体 **13a** へと酸化した後、DMF 溶媒中、LiCl を作用させて 100 ℃ に加熱することによりメチルエーテル部位のメチル基を除去し、化合物 **7** を **8a** からの通算収率 35%で得た。

　化合物 **7** の UUI、MIA、PK（C_{max} および Exc.）、および CYP2C9 阻害を化合物 **2g** および BBR と比較した結果をまとめた（**Table 4**）。

Table 4. Properties of compound **7**

7　LogP: 2.85　　　　　　**2g**　LogP: 3.43

Compound	UUI [a] IC$_{50}$ (μM)	MIA [b] IC$_{50}$ (μM)	C_{max} [c] (μg/mL)	Exc. [d] (%)	CYP2C9 IC$_{50}$ (μM)
7	19.2	59	3.81	0.6	57
2g	8.8	24	1.36	0.35	-
BBR	6.8	3.1	4.42	0	0.041

[a] UUI: Inhibitory activity of urate uptake into RPTECs.

[b] MIA: Mitochondrial respiratory control ratio (RCR), IC$_{50}$ (μM).

[c] Maximum concentration (C_{max}).

[d] Excretion rate in urine (%, 0–4 h) after oral administration of 3 mg/kg to rats.

　Table 4 が示すとおり、化合物 **7** の UUI 活性は化合物 **2g** および BBR よりもやや劣るものの十分に高いレベルを保っている。また、MIA はより弱く、良好である。そして、わずかではあるが期待通り、PK（C_{max} および Exc.）が改善された。

すなわち、C_{max} は 3.81 µg/mL と向上し、Exc. も 0.6% となった。さらに、化合物 **7** は CYP2C9 阻害活性が弱く（IC_{50} = 57 µM）、有望な特性を有している。そこで、Exc. のさらなる改善を目指し化合物 **7** をリード化合物として、フェノール環上の置換基の異なる各種誘導体 **14–26**（**Figure 13**）を合成することとした。
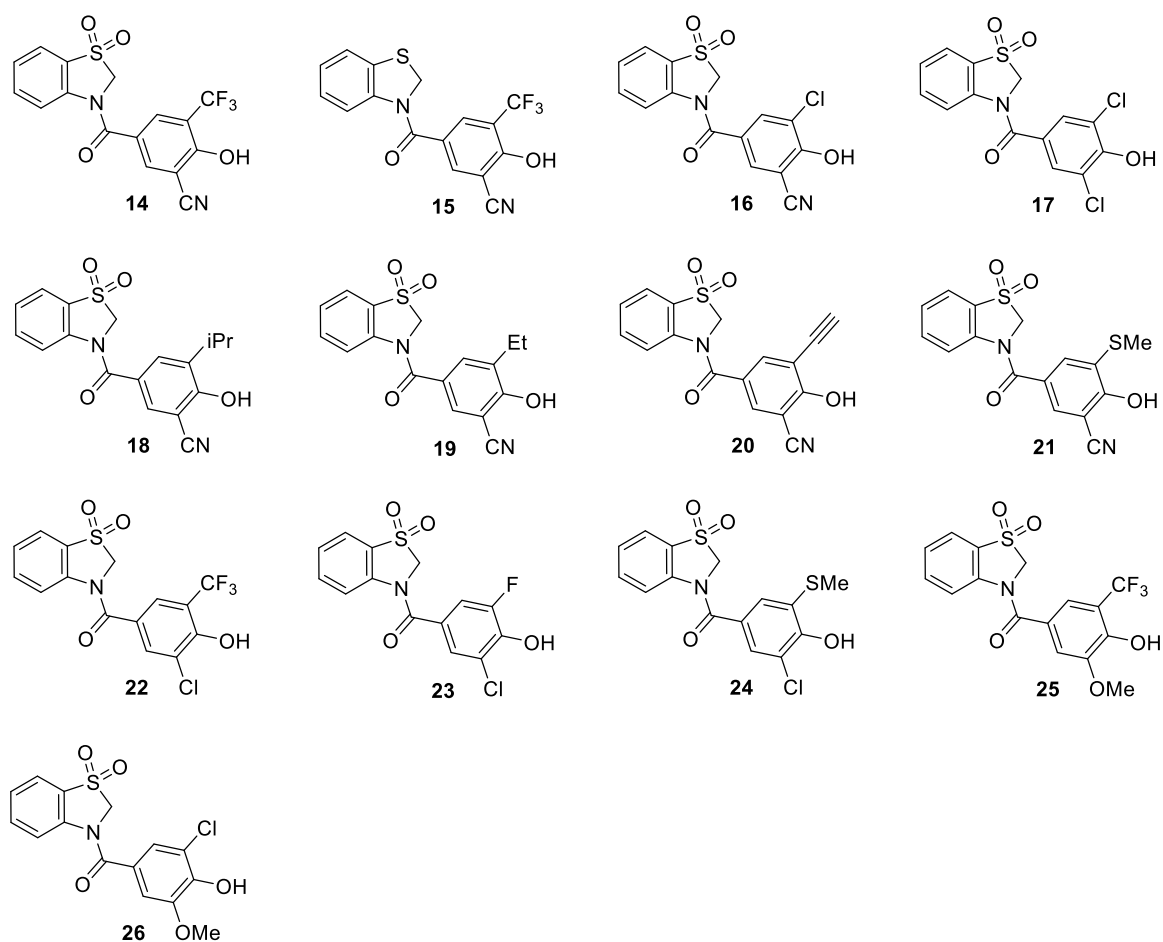
Figure 13. 1,2-Dihydro-3*H*-1,3-benzothiazole derivatives.

これら化合物の合成方法は **Scheme 4**（化合物 **14–24**）および **Scheme 5**（化合物 **25, 26**）に示す。まず、化合物 **14, 16–20, 21'**（R_1 = CN, R_2 = I）, **22, 23, 24'**（R_1 = Cl, R_2 = I）を前述の化合物 **7** と同様の経路で、出発物質となる安息香酸誘導体を代えることによって合成した。続いて、**21'** および **24'** に対して DMF 溶媒中で 2,2'-bipyridine（8 mol%）、$NiBr_2$（8 mol%）の存在下、Zn（1.6 eq.）および MeSSMe（0.5 eq.）を作用させることにより、各々メチルスルファニル誘導体 **21**（R_1 = CN, R_2 = SMe）、

24（R$_1$ = Cl, R$_2$ = SMe）へと変換した。また、1,2-ジヒドロ-3*H*-1,3-ベンゾチアゾール誘導体 **15** を中間体 **12b** から硫黄原子の酸化を経ることなく、メチルフェニルエーテルのメチル基を除去することで合成した（**Scheme 4**）。

Scheme 4. Synthesis of 1,2-dihydro-3*H*-1,3-benzothiazole derivatives
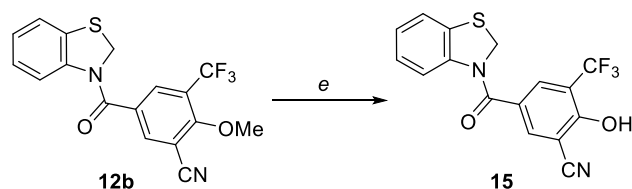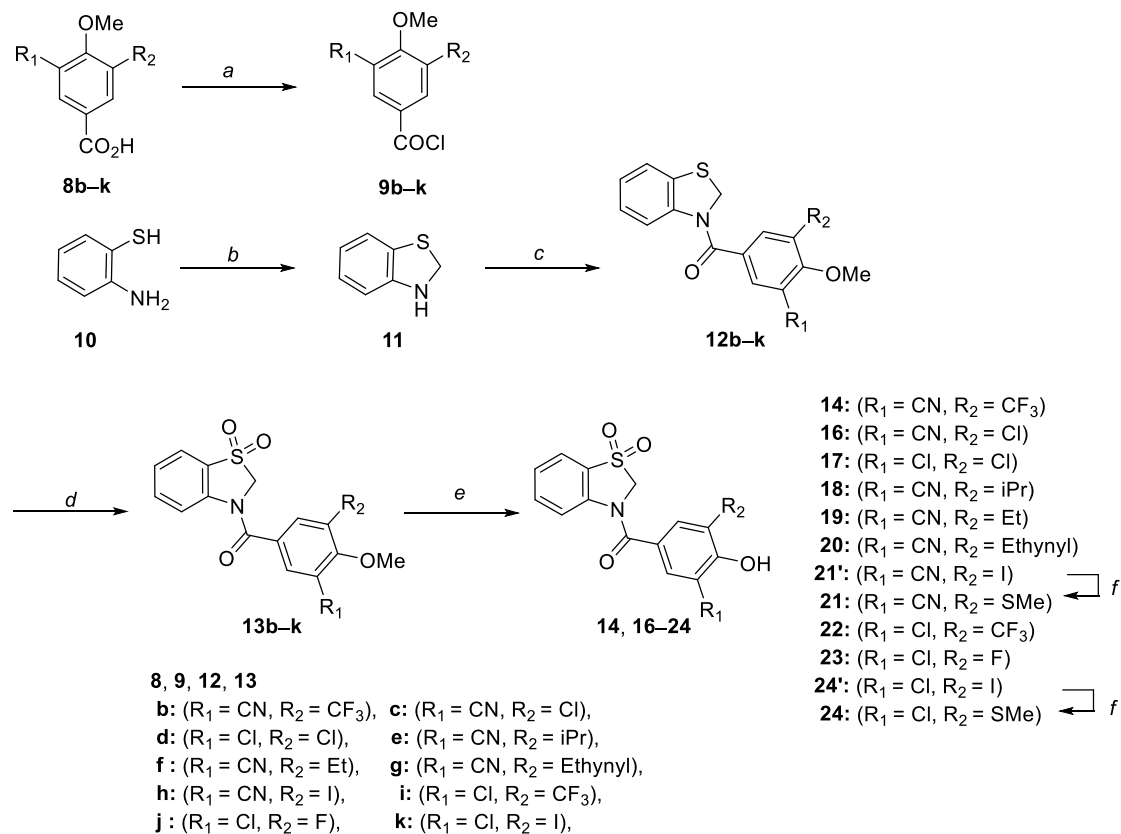
14: (R$_1$ = CN, R$_2$ = CF$_3$)
16: (R$_1$ = CN, R$_2$ = Cl)
17: (R$_1$ = Cl, R$_2$ = Cl)
18: (R$_1$ = CN, R$_2$ = iPr)
19: (R$_1$ = CN, R$_2$ = Et)
20: (R$_1$ = CN, R$_2$ = Ethynyl)
21': (R$_1$ = CN, R$_2$ = I)
21: (R$_1$ = CN, R$_2$ = SMe) ← *f*
22: (R$_1$ = Cl, R$_2$ = CF$_3$)
23: (R$_1$ = Cl, R$_2$ = F)
24': (R$_1$ = Cl, R$_2$ = I)
24: (R$_1$ = Cl, R$_2$ = SMe) ← *f*

8, 9, 12, 13
b: (R$_1$ = CN, R$_2$ = CF$_3$), **c:** (R$_1$ = CN, R$_2$ = Cl),
d: (R$_1$ = Cl, R$_2$ = Cl),   **e:** (R$_1$ = CN, R$_2$ = iPr),
f : (R$_1$ = CN, R$_2$ = Et),   **g:** (R$_1$ = CN, R$_2$ = Ethynyl),
h: (R$_1$ = CN, R$_2$ = I),   **i:** (R$_1$ = Cl, R$_2$ = CF$_3$),
j : (R$_1$ = Cl, R$_2$ = F),   **k:** (R$_1$ = Cl, R$_2$ = I),

Reagents and conditions: (*a*) SOCl$_2$; (*b*) HCHO, H$_2$O, diisopropyl ether; (*c*) **9**, triethylamine, CHCl$_3$; (*d*) *m*-CPBA, 15–79% yield from **8**; (*e*) LiCl, DMF, 53–99% yield; (*f*) 2,2'-bipyridine, Zn, NiBr$_2$ and MeSSMe, DMF, 100 °C, 23–29% yield.

フェノール上のオルト置換基としてメトキシ基を有する化合物 **25** および **26** の合成については、出発物質として 4 位ベンジルオキシ安息香酸誘導体 **8l** および **8m** を用い、前述と同様の経路（**Scheme 5**）で合成した。すなわち、まず、安息香酸 **8l** および **8m** より調製した酸塩化物 **9l** および **9m** をジヒドロベンゾチアゾール **11** と縮合させた後、*m*-CPBA を用いてスルフィド部位をスルホンへと酸化した。最後に、Pd/C を用いた接触水素化反応によりベンジル保護を除去して **25** および **26** を得た。

Scheme 5. Synthesis of compound **25** and **26**
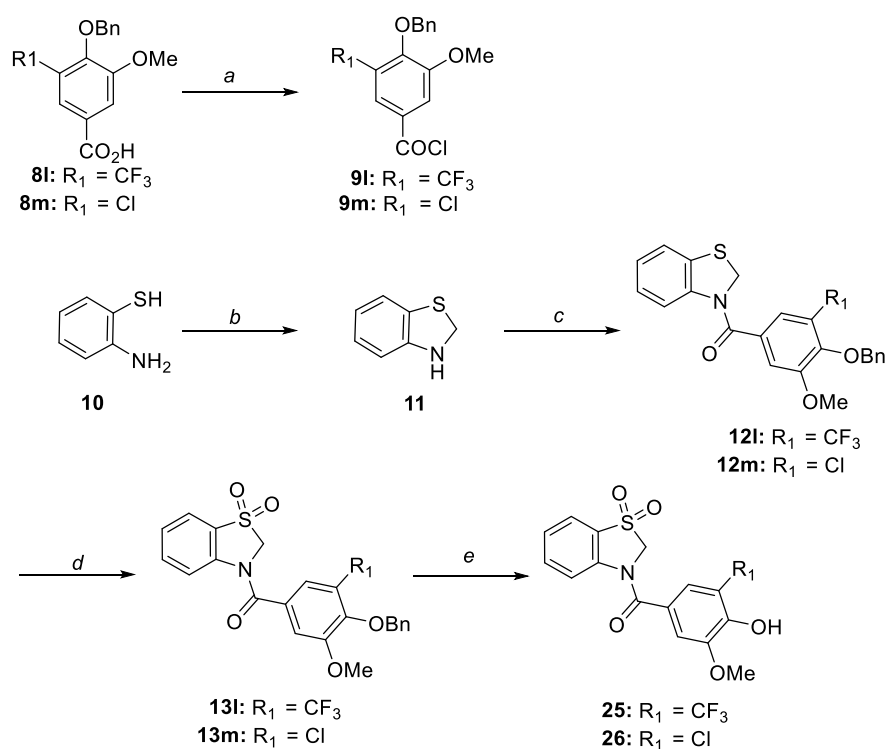
Reagents and conditions: (*a*) SOCl$_2$; (*b*) HCHO, H$_2$O, diisopropyl ether; (*c*) **9**, triethylamine, CHCl$_3$; (*d*) *m*-CPBA, 64–65% yield from **8**; (*e*) H$_2$, 5% Pd/C, 45–76%.

これら化合物の UUI、MIA、PK（C_{max} および Exc.）の結果をまとめた（**Table 5**）。

Table 5. UUI, MIA, and pharmacokinetic profiles of 1,2-dihydro-3H-1,3-benzothiazole derivatives

14, 16–26   15
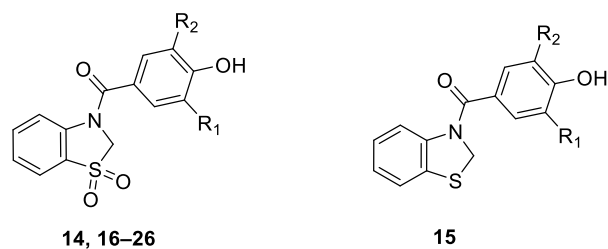
Compound	R_1	R_2	UUI [a] IC_{50} (μM)	MIA [b] IC_{50} or inhibition (%)	C_{max} [c] (μg/mL)	Exc. [d] (%)
14	CN	CF$_3$	> 100	(12%)	-	-
15	CN	CF$_3$	13.1	55 μM	3.15	0.1
16	CN	Cl	> 100	(20%)	1.35	23
17	Cl	Cl	6.8	27 μM	9.14	1.1
18	CN	Isopropyl	83.3	12 μM	4.46	0.1
19	CN	Et	> 100	37 μM	4.24	1.7
20	CN	Ethynyl	3.2	(13%)	1.58	12.4
21	CN	SMe	7.1	(21%)	1.08	9.4
22	CF$_3$	Cl	2.6	18 μM	6.41	0.1
23	F	Cl	33.9	63 μM	7.70	4.3
24	Cl	SMe	5.7	59 μM	4.64	0.2
25	CF$_3$	OMe	17.8	(47%)	3.99	0
26	Cl	OMe	4.3	(49%)	1.06	0.1
BBR	-	-	6.8	3.1 μM	4.42	0

[a] UUI: Inhibitory activity of urate uptake into RPTECs.

[b] MIA: Mitochondrial respiratory control ratio (RCR), IC_{50} (μM) or inhibition (%) at 100 μM.

[c] Maximum concentration (C_{max}).

[d] Excretion rate in urine (%, 0–4 h) after oral administration of 3 mg/kg to rats.

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以上のように、化合物 **17, 20, 21, 22, 24, 26** は UUI の IC_{50} 値が < 10 µM という強力な活性を示した。その中でも化合物 **17, 20** および **21** は Exc. > 1.0%であり、特に **17** については C_{max} が 9.14 µg/mL と良好であった。

そこで、作用機序について情報を得るため、いくつかの化合物の URAT1 阻害活性を調べた。その結果、強い UUI 活性を示す化合物ほど強い URAT1 阻害活性を示す傾向がみられた（**Table 6**）。したがって、これら化合物の UUI 活性は主としてRPTECs 上の URAT1 阻害によるものと推定される。

Table 6. URAT1 inhibition and UUI of 1,2-dihydro-3*H*-1,3-benzothiazole derivatives and BBR

Compound	URAT1 inhibition [a] (%)	UUI [b] (%)
7	30	42
15	71	49
16	29	25
17	78	81
19	36	32
21	44	57
BBR	37	51

[a] URAT1: inhibitory activity, % inhibition at 0.1 µM.

[b] UUI: inhibitory activity of urate uptake into RPTECs, % inhibition at 10 µM.

ジクロロ置換誘導体 **17** は、BBR と比較し同等以上の強い UUI および URAT1 阻害活性を示す一方、MIA は低く、目標とした C_{max} および Exc.（> 1.0%、0–4 時間）を達成した。また、**17** は CYP2C9 を阻害したが（$IC_{50} = 5.7$ µM）、BBR（$IC_{50} = 0.041$ µM）に比較して弱かった。上記の結果から、臨床における尿酸排泄活性は少なくとも BBR と同程度以上であり、CYP2C9 の阻害は薬効と十分に乖離していると推測される。このことから、本化合物を開発候補化合物として選択した。

化合物 **17** の性質をより詳細に調査するため、著者が所属する（株）富士薬品、生物研究部の共同研究者および別チームにおいて、各種 *in vitro* および *in vivo* 試験を行い BBR と比較した。

なお、化合物 **17** の pK_a は 4.69 であった [38]。BBR （pK_a 5.17）と比較しさらに酸性度が高まっている。

　Table 7 は URAT1 を過剰発現した細胞を用い、化合物の尿酸取り込み阻害（URAT1 阻害活性）を評価した結果である。化合物 **17** の IC$_{50}$ は 37.2 nM であり，BBR（IC$_{50}$ = 190 nM）より約 5 倍強いことがわかる。また、ラット PK 試験を 0.3 mg/kg 経口投与（p.o.）および静脈注射（i.v.）で行ったところ（**Table 8**）、良好な $C_{\max}$（1085.8 ng/mL ± 100.4）および AUC（5744.7 ± 188.8 ng·h/mL）を示し、生物学的利用率（BA）は 86.8%と高いものであった。

Table 7. URAT1 inhibition activity of **17** and BBR

17
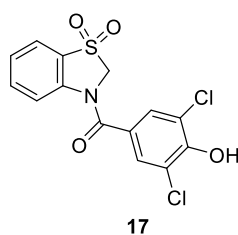
In vitro evaluation	URAT1 inhibition IC$_{50}$ (nM)
17	37.2
BBR	190

Table 8. Rat PK profile of **17**

dose	$C_{\max}$ or C_0 [a] (ng/mL)	AUC$_{0-inf}$ [b] (ng·h/mL)	BA [c]
0.3 mg/kg p.o.	1085.8 ± 100.4	5744.7 ± 188.8	86.8%
0.3 mg/kg i.v.	2401.3 ± 65.6	6619.5 ± 2041.8	

[a] Maximum concentration ($C_{\max}$ or C_0).

[b] Area under the blood concentration-time curve (AUC); inf: infinity.

[c] Bioavailability.

Table 9 および **Figure 14** はフサオマキザル（*Cebus apella*）を用いて化合物 **17** の PK および薬効を調べた結果である [39]。なお、フサオマキザルは、尿中尿酸排泄率がヒトと同様に低く、かつ尿酸（UA）とその酸化代謝物であるアラントイン（Alla）の血漿中濃度比率（Alla/UA）が 0.05–0.15 であり、ヒト（0.1 以下）や類人猿（0.14 以下）と同程度であることから [40]、尿酸排泄促進作用をヒトに外挿するのに有用である。

　　尿中への尿酸排泄量の指標である、0–4 h における尿酸排泄分画（FE_{UA}: Fractional excretion of uric acid ＝尿酸クリアランス/クレアチニンクリアランス）は、BBR では 30 mg/kg 経口投与（p.o.）において 11.5%であるのに対し、化合物 **17** では 5 mg/kg p.o.において 16.5%であった。さらに、化合物 **17** は BBR よりも強く血漿尿酸濃度を減少させた。投与 0–8 時間における血中尿酸値低下量は化合物 **17**（5 mg/kg p.o.）が 0.97 mg/dL、BBR（30 mg/kg p.o.）が 0.46 mg/dL であった。この強力な効果は、従来の薬剤には報告されていないものである。

Table 9. PK profile and pharmacodynamics of **17**

Cebus apella PK	AUC_{0-24h} [a] (μg·h/mL)	FE_{UA} [b] (%)	Decrease in ΔP_{UA} [c] from control (mg/dL)
17 (5 mg/kg p.o.)	108.55 ± 52.94	16.5 ± 4.2	0.97
BBR (30 mg/kg p.o.)	95.24 ± 28.12	11.5 ± 7.9	0.46

[a] Area under the blood concentration-time curve (AUC).

[b] FE_{UA}: Fractional excretion of urate at 0–4 h; FE_{UA} value of control: $8.9 \pm 4.0\%$.

[c] ΔP_{UA}: Changes in plasma urate level at 0–8 h; value of control: 1.65 ± 0.78 mg/dL.
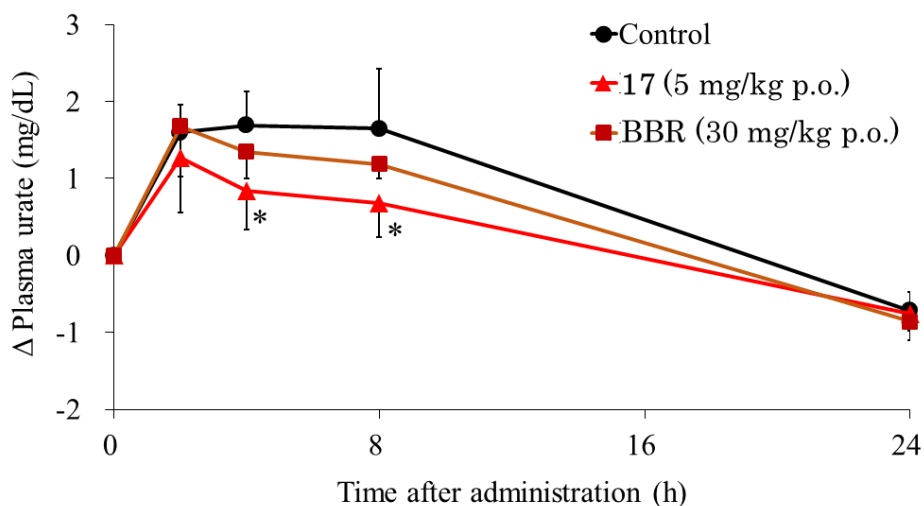
Figure 14. Changes in plasma urate level compared with the pre-dosing value in *Cebus apella* after oral administration of **17** (5 mg/kg) or BBR (30 mg/kg). Values are the means ± S.D. of five animals. The average pre-dosing value of control: 2.98 mg/dL. * P < 0.05, significantly different from control at each time point by Dunnett's test.

　さらに、化合物 **17** の選択性を確認するため、複数のトランスポーターへの阻害活性が検討された。その結果、**17** は、OAT1/3 や腸管での尿酸排泄を担っているトランスポーターである ATP-binding cassette sub-family G member 2（ABCG2）に対する阻害活性は弱く、選択的な尿酸再吸収阻害剤（selective urate reabsorption inhibitor, SURI）であることが示された [39]。ABCG2 に対する阻害は BBR よりも弱いことから、腸管での尿酸の排泄を阻害しない点でも BBR に比較し有利である。

　ヒトにおける化合物 **17** の代謝について、主要な代謝物は、フェノールのグルクロン酸抱合体と硫酸抱合体であった。マスバランス試験において、各々の代謝物の 0–72 h の尿中排泄率はそれぞれ 44.3%および 20.0%であり [41]、その他の代謝物は各 5.8%以下であった。また、前述のように、BBR には反応性代謝物が肝毒性の原因であるとする仮説がある。BBR の反応性代謝物として、カテコール体（CAT）、ヒドロキノン体（DBH）あるいはその酸化型（DBBQ）が報告されているが（**Figure 15**）[42]、これまでのところ化合物 **17** にはそのような代謝物は認められていない。化合物 **17** が安全な代謝経路をもっていることが示唆される。
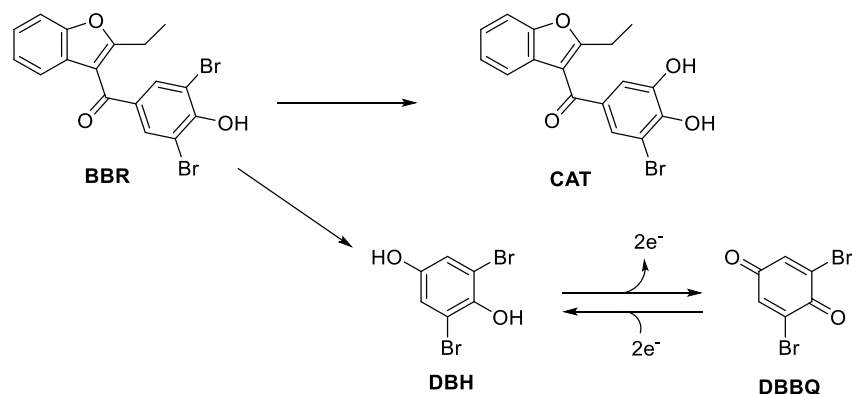
Figure 15. Proposed reactive metabolites of BBR.

　さらに化合物**17**は、のちに行われた第Ⅲ相臨床試験において優れた結果を示し、日本において Dotinurad として承認された。Dotinurad 2 mg の薬理活性は BBR 50 mg と同等であり [43)]、BBR の約 20～30 倍程度強いといえる。また、ヒトの臨床実績において、Dotinurad の最大臨床用量は 4 mg であるが、BBR で引き起こされるような薬剤性の劇症肝炎は 1 日用量が 10 mg 未満の医薬品では確認されたことはない。Dotinurad は BBR に比較し *in vitro* ミトコンドリア呼吸鎖阻害活性（MIA）が約 10 倍弱かったが、投与量を考慮すると、ヒトで実質数百倍のミトコンドリア阻害活性差があるといえる。

　以上のように、劇症肝炎のリスクと考えられている、ミトコンドリア毒性、反応性代謝物の存在および投与量の観点から、Dotinurad が劇症肝炎を引き起こすリスクは低いと推察される。

第四節　小括

　　BBR は稀に重篤な肝毒性を引き起こす点が問題であるが、薬効の強い有用な薬物である。そこで、BBR の化学構造に着目し、強い薬効を示すこと、および毒性の発現を回避することを両立する薬物の創製を試みることとした。BBR の化学的特徴を解析し、薬効を発現するには尿酸と同様の平面性の高い構造と適度な酸性度が必要であると仮定した。また、肝毒性を発現する原因はミトコンドリア毒性であり、ラジカルを安定化し得る部分構造であるビスアリールケトン構造に起因すると仮定した。そこで、BBR のビスアリールケトン型構造 [Ar-(CO)-Ar] を、Ar 基とケトンとの共役を部分的に回避したアミド型 [N-(CO)-Ar] 構造に変換することとした。すなわち、二環性のアミン（インドリン等）とフェノールをカルボニル基で介した、二環性アミンを部分構造とするベンズアミド誘導体を種々合成した。*In vitro* の薬効試験として、ヒト腎において尿酸の排泄や再吸収を担っている近位尿細管上皮細胞（RPTECs）を用いた尿酸取り込み阻害活性（UUI）、および尿酸の再吸収において主な役割を担っている尿酸トランスポーター（URAT1）の阻害活性を評価した。同時に、肝障害の指標として、ミトコンドリア呼吸鎖阻害活性（MIA）を評価した。その結果、強い *in vitro* の薬効の発現と低いミトコンドリア毒性を両立するインドリン誘導体（**2g** 等）を得ることに成功した。この過程で、アミド型へ構造を代えること、および脂溶性を下げることがミトコンドリア毒性を弱くするために重要であることも明らかとなった。しかし、これら誘導体の薬物動態（PK）の特性（C_{max} および尿中排泄率）は十分ではなかった。そこで、*in vivo* での特性を向上させるため、二環性アミン部位を代謝安定性のある構造へ変換することとした。結果として、*in vitro* 活性および PK 特性のバランスに優れた 1,1-ジオキソ-1,2-ジヒドロ-3*H*-1,3-ベンゾチアゾール誘導体 **17** 等を見出した（**Figure 16**）。選択した代表化合物 **17** は、ヒトの薬理作用を予測できるフサオマキザル（*Cebus apella*）をもちいた *in vivo* 試験において、BBR よりも強い尿酸低下作用が確認された。本化合物を医薬品の候補化合物として選択し、臨床開発をすすめた結果、日本国内で医薬品（Dotinurad）として承認され、現在海外での開発も進められている。
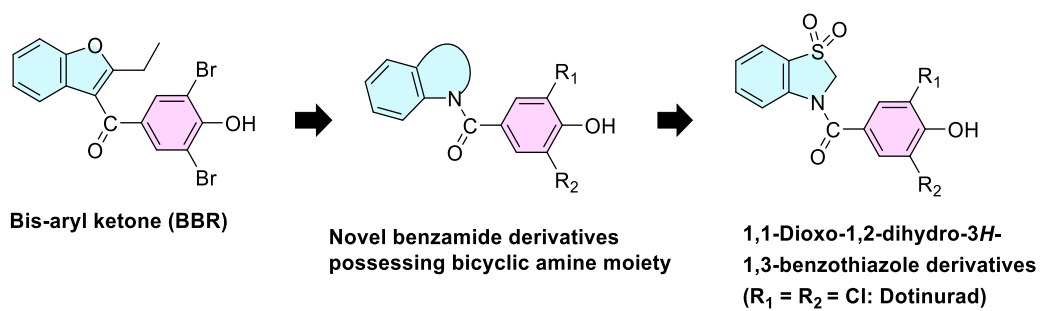
Figure 16. Strategy and results of the novel benzamide derivatives possessing bicyclic amine moiety.

In the figure, the structures shown are labeled as follows: Bis-aryl ketone (BBR); Novel benzamide derivatives possessing bicyclic amine moiety; 1,1-Dioxo-1,2-dihydro-3*H*-1,3-benzothiazole derivatives ($R_1 = R_2 = Cl$: Dotinurad).

第二章
ミトコンドリア毒性を低減したベンズアミド誘体の創製

第一節　単環性アミンを部分構造にもつベンズアミド誘導体のミトコンドリア毒性

　　第一章で述べたとおり、肝毒性が問題となっていた BBR に代わる医薬品を開発するという目標は Dotinurad の開発をもって達成された [44]。またその開発過程で、ビスアリールケトンという化学構造がミトコンドリア毒性の原因である可能性が示されたが、同時にその毒性が分子の脂溶性（LogP）と相関することも見出した。そこで著者は、この知見をもとにミトコンドリア毒性をさらに抑えその懸念を完全に払しょくするような化合物を創出するべく研究を続けた。

　　基本戦略としては、分子の脂溶性を低下させること、および分子の平面性を確保することを念頭に置き、Dotinurad の二環性アミン部位を単環性の脂環式アミンに置きかえることを計画した（**Figure 17**）。
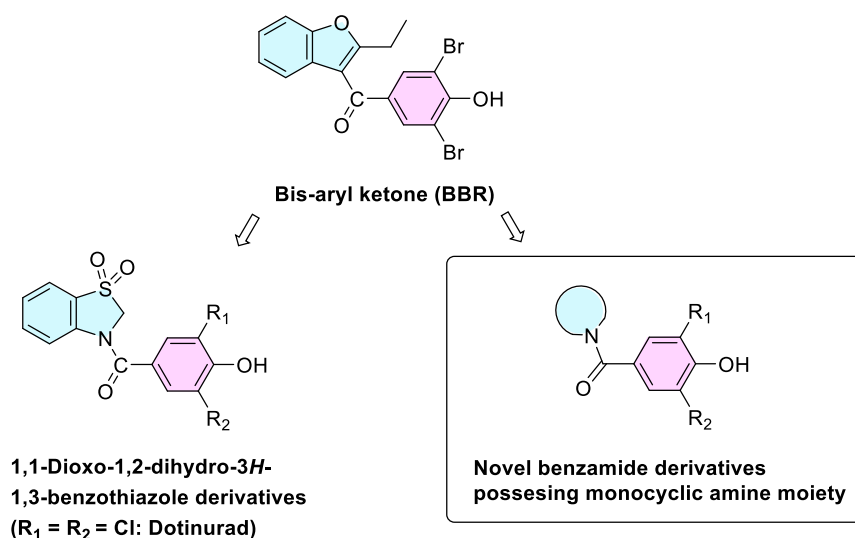
Figure 17. Strategy for designing novel uricosuric compounds.

実際にアミド分子を設計、合成するに先立ち、まず、MIA が分子構造に起因するという仮説をあらためて検証するため、フェニルビニルケトン誘導体 **30a**, **b** を合成し、MIA を評価することとした（**Scheme 6**）。

Scheme 6. Synthetic pathway for cyclopentene derivatives **30a**, **b**

27a: X = Cl
27b: X = Br

29

30a: X = Cl, two steps 54%
30b: X = Br, two steps 75%
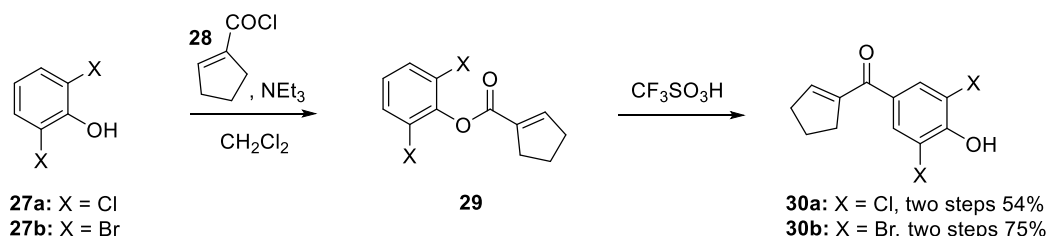
合成には、フェノールの 4 位アシル体を得るための典型的な方法である Fries 転位を利用した。すなわち、まず 2,6 位をハロゲン（クロロ基およびブロモ基）で置換したフェノールを、各々、CH$_2$Cl$_2$ 溶媒中、トリエチルアミンの存在下でシクロペンテンカルボン酸クロリドを用いて O–アシル化した。続いて、その粗生成物をトリフルオロメタンスルホン酸に溶解して 0 °C で撹拌すると、目的とする Fries 転位が進行し、ケトン **30a**, **b** を得ることができた。

Table 10. Structure-MIA relationships associated with the cyclopentene derivatives.

30a: X = Cl
30b: X = Br

Compound	LogP ^{a}	MIA ^{b} IC$_{50}$ (μM)
30a	3.39	10
30b	3.93	6.6
BBR	4.95	3.1

^{a} LogP was calculated using ChemDraw v19.

^{b} MIA: mitochondrial respiratory control ratio (RCR), IC$_{50}$ (μM).

Table 10 に示すように、ケトン **30a** および **30b** は、LogP 値（計算値）が BBR（LogP：4.95）より低いにもかかわらず、強い MIA を示した。著者は、これら化合物のアリールビニルケトン構造がビスアリールケトン構造と同様に生じるラジカルを安定化し得るため MIA に影響したものと推察した。

　そこで、二重結合とカルボニル基との共役を避けるために、やはりアミドを含む構造を採用することとし、ピロール誘導体 **32**（ベンゾイル芳香族アミン）、3−ピロリン誘導体 **34a**, **b**（ベンゾイル脂肪族アミン）、およびピロリジン誘導体 **36a**（ベンゾイル脂肪族アミン）を合成し、MIA を評価することとした。

　ピロール誘導体 **32** は **Scheme 7** に示す方法で合成した。すなわち、まず、水素化ナトリウムを塩基として、ピロールを安息香酸塩化物により *N*−アシル化したのち、DMF 溶媒中で塩化リチウムを作用させることによりフェノールを遊離させた。

Scheme 7. Synthetic pathway for pyrrole derivative **32**
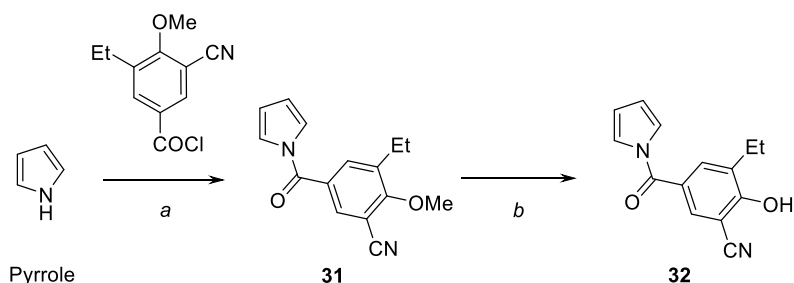
　Reagents and conditions: (*a*) NaH, THF; (*b*) LiCl, DMF, 110 °C, 13% yield over two steps.

　3−ピロリン誘導体 **34a**, **b** およびピロリジン誘導体 **36a** は **Scheme 8** に示す方法で合成した。化合物 **34a**, **36a** については、まず、3 および 5 位に置換基をもつ 4−メトキシ安息香酸とピロリジンまたは 3−ピロリンを、1−エチル−3−（3−ジメチルアミノプロピル）カルボジイミド塩酸塩（EDC·HCl）を用いて縮合したのち、得られた *N*−アシル体を DMF 溶媒中で塩化リチウムを作用させることによりフェノールを遊離させることで合成した。化合物 **34b** については 4−（メトキシメトキシ）安息香酸を用い、**34a** と同様にして EDC·HCl を用いて縮合したのち、*N*−アシル体を 4 M 塩酸　酢酸エチル溶液にて脱保護することで得た。

Scheme 8. Synthetic pathway for 3-pyrroline and pyrrolidine derivatives **34a**, **b** and **36**

33a (3-Pyrroline derivative):
R₁ = Et, R₂ = CN
35a (Pyrrolidine derivative):
R₁ = *tert*-Bu, R₂ = CN

34a (3-Pyrroline derivative):
R₁ = Et, R₂ = CN
36a (Pyrrolidine derivative):
R₁ = *tert*-Bu, R₂ = CN

Reagents and conditions: (*a*) EDC·HCl, CH₂Cl₂; (*b*) LiCl, DMF (**34a**: 73%, **36a**: 61% yield over two steps); (*c*) 4 M HCl solution in AcOEt, 68% yield over two steps.

　Table 11 に示す通り、ピロール誘導体 **32** は、BBR よりも LogP が低いにもかかわらず、MIA が強かった。一方、3‐ピロリンアミド誘導体 **34a**, **34b** および **36a** の MIA は極めて弱かった。第一章において、インドール誘導体（化合物 **1**）の MIA が強い一方で、インドリン誘導体（化合物 **2a**）の MIA は減弱することを述べたが、この傾向と一致する結果となった。

　そこで化合物 **34** および **36** の構造をさらに最適化することにより新しいタイプの尿酸排泄促進薬の創製を目指すこととした（第二節）。

Scheme 8. Synthetic pathway for 3-pyrroline and pyrrolidine derivatives **34a**, **b** and **36**

33a (3-Pyrroline derivative):
R₁ = Et, R₂ = CN
35a (Pyrrolidine derivative):
R₁ = *tert*-Bu, R₂ = CN

34a (3-Pyrroline derivative):
R₁ = Et, R₂ = CN
36a (Pyrrolidine derivative):
R₁ = *tert*-Bu, R₂ = CN

3-Pyrroline or Pyrrolidine

3-Pyrroline

37b

34b

Table 11. Structure-MIA relationships associated with compounds **32**, **34a**, **b**, and **36a**

Compound	LogP [a]	MIA [b] IC$_{50}$ or inhibition (%)
32	2.92	6.7 μM
34a	2.22	> 100 μM (17%)
34b	2.95	> 100 μM (22%)
36a	3.08	> 100 μM (22%)

[a] LogP was calculated using ChemDraw v19.

[b] MIA: mitochondrial respiratory control ratio (RCR), IC$_{50}$ (μM) or inhibition (%) at 100 μM.

第二節　単環性アミンを部分構造にもつベンズアミド誘導体の合成と尿酸取り込み阻害作用

　前節で述べた結果を踏まえ、ミトコンドリア毒性を示さない新たなタイプの尿酸排泄促進剤の創製を目指すべく、まずは、ピロール誘導体 **32**、3-ピロリン誘導体 **34a**、ピロリジン誘導体 **36a** の *in vitro* 薬理活性（URAT1 阻害および UUI）および MIA を BBR と比較した（**Table 12**）。

Table 12. URAT1 inhibition, UUI, and MIA of compound **32**, **34a**, and **36a**

Compound	URAT1 % inhibition at 3 μM	UUI [a] IC_{50} (μM)	MIA [b] IC_{50} or inhibition (%)
32	83%	> 1000	6.7 μM
34a	54%	> 1000	> 100 μM (17%)
36a	84%	> 1000	> 100 μM (22%)
BBR	91%	6.8	3.1 μM

[a] UUI: Inhibitory activity of urate uptake into RPTECs.

[b] MIA: mitochondrial respiratory control ratio (RCR), IC_{50} (μM) or inhibition (%) at 100 μM.

　その結果、これら誘導体の UUI は、二環性のアミンから誘導したベンズアミド誘導体と比較して著しく低いものとなった。これ自体は残念な結果であったが、前節でのべたように、化合物 **34a** および **36a** の MIA は弱く、100 μM という高濃度においてもほとんど阻害活性を示さない。さらに、薬効の本質である URAT1 阻害活性を示していることや、脂溶性が低下して薬物の尿中濃度が高まることによって、生体で高活性を示す可能性がある点に希望を見出し、さらに誘導体の合成をこころみることとした。

　具体的には、フェノール上の置換基を変えた化合物（**34c**–**34l**, **36b**）を合成し、*in vitro* 薬理活性（URAT1 阻害および UUI）および MIA を評価することとした。
　これら化合物の合成は、第一節 **Scheme 8** に示した化合物 **34a** および **36a** の合成と同様に行った。

ただし、置換基としてシクロプロピル基およびシアノ基をもつ化合物 **34l** については、**Scheme 9** に示す方法で合成した。すなわち、まず、トリエチルアミンの存在下、3-ピロリンに安息香酸クロリドを作用させアミド誘導体 **33l** を得た。続いて、DMF 溶媒中で、塩化リチウムを作用させることでフェノールを遊離させた。
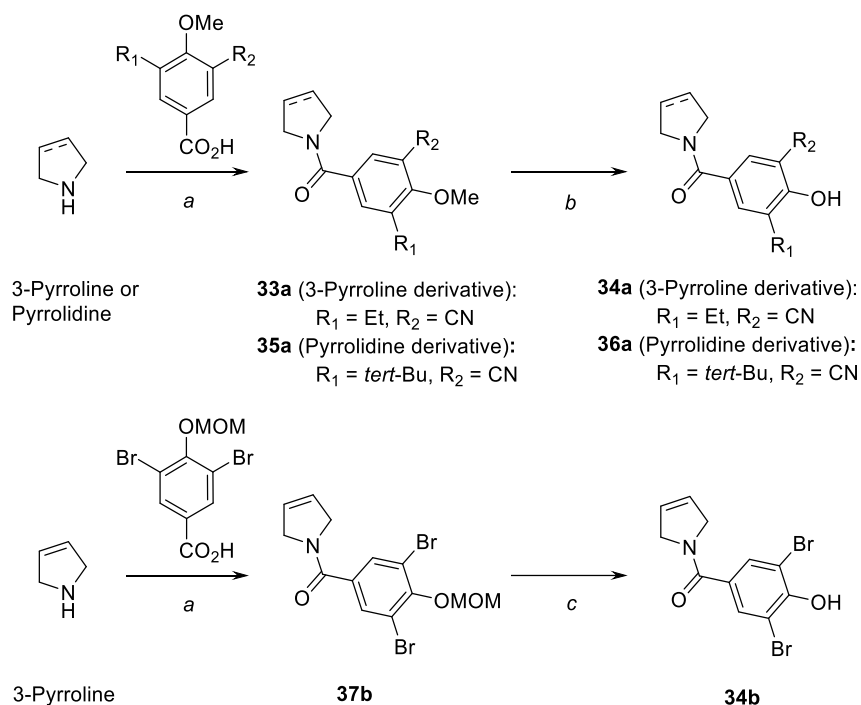
Scheme 9. Synthetic pathway for 3-pyrroline derivatives **34l**
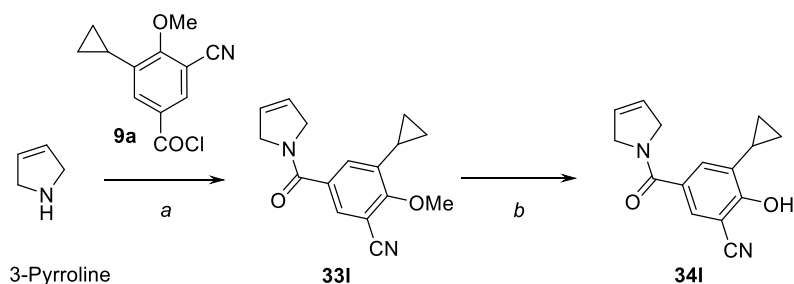
Reagents and conditions: (*a*) Et$_3$N, THF; (*b*) LiCl, DMF, 130 °C, 55% yield over two steps.

　Table 13 に示すとおり、ほとんどすべての誘導体は URAT1 を阻害し、かつ、例外（化合物 **34e**, **34k**）を除いて 100 µM で 50%程度以上の MIA を示さなかった。化合物 **34k** は強力に URAT1 を阻害し、かつ中程度の UUI を示したが、MIA（IC$_{50}$: 64 µM）を示した。**34e** や **34k** は、LogP が他の化合物に比較して高い（LogP > 3.0）ことから、これら単環性アミンを部分構造に持つベンズアミド誘導体においても、二環性化合物と同様に脂溶性が MIA に影響していることが推測される。また、LogP が低い（LogP < 2.0）化合物 **34d**, **34g**, **34h** は URAT1 阻害や UUI が弱く、薬理活性にも脂溶性が影響している可能性がある。一方、化合物 **34f** の URAT1 阻害および UUI は **34k** よりも弱いが、MIA を示さずリード化合物として有望であると考えられた。

Table 13. URAT1 inhibition, UUI, and MIA of compound **34c–34l** and **36b**
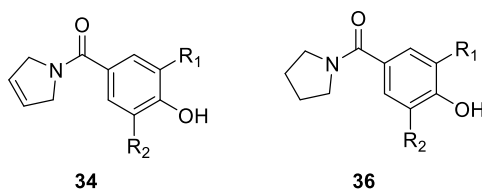
Compound	R_1, R_2	URAT1 % Inhibition at 3 μM	UUI [a] IC$_{50}$ μM	MIA [b] inhibition (%) or IC$_{50}$ (μM)	LogP [c]
34c	I, CN	79%	> 1000	8%	2.68
34d	CN, SMe	58%	> 1000	6%	1.77
34e	Pr, CF$_3$	49%	903	47%	3.54
34f	CF$_3$, CN	83%	818	-1%	2.25
34g	Cl, CN	66%	> 1000	5%	1.89
34h	Me, CN	25%	> 1000	-10%	1.81
34i	Br, CN	69%	747	0%	2.16
34j	Pr, CN	70%	> 1000	7%	2.65
34k	*tert*-Bu, CN	92%	311	60% (64 μM)	3.03
34l	Cyclopropyl, CN	69%	> 1000	-7%	2.06
36b	CF$_3$, CN	66%	> 1000	18%	2.29

[a] UUI: inhibitory activity of urate uptake into RPTECs.

[b] MIA: mitochondrial respiratory control ratio (RCR), inhibition (%) at 100 μM or IC$_{50}$ (μM).

[c] LogP was calculated using ChemDraw v19.

　次に、有望と考えた化合物 **34f** の脂環式アミンを、アゼチジン、3-メチル-3-ピロリン、チアゾリジンおよびジオキソチアゾリジンに変換した誘導体 **40a**, **41**, **42a**, **43** を合成することとした。また、脂環式アミンの変換により脂溶性が変化し、MIA や薬理活性に影響することが予想されたため、これら化合物のフェノール上の置換基を変換した誘導体についても同時に合成し評価することとした。

アゼチジン誘導体 **40a, b** および 3-メチル-3-ピロリン誘導体 **41** は、**Scheme 7** と同様に酸クロリドを用い、塩基としてトリエチルアミンを用いて合成した。一方、チアゾリジンジオン誘導体 **42a, b, c, d, e** は、**Scheme 8** と同様に EDC·HCl を用いた縮合反応を経て合成した。

また、ジオキソチアゾリジン誘導体 **43** は、化合物 **42a** を合成する中間体 **38** をメタクロロ過安息香酸（*m*-CPBA）にて酸化し、ジオキソ体 **39** を得たのち、フェノールの保護基（-OMe）を脱保護し合成した（**Scheme 10**）。

Scheme 10. Synthetic pathway for 1,1-dioxothiazolidine derivative **43**
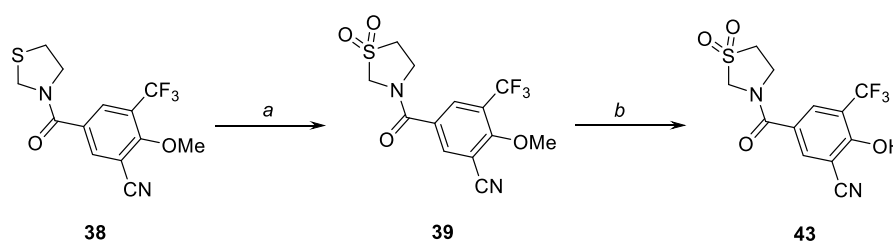
Reagents and conditions: (*a*) *m*-CPBA, CH₂Cl₂, 99% yield; (*b*) LiCl, DMF, 84% yield.

これら化合物の UUI、URAT1 阻害活性、MIA を評価し化合物 **34f** と比較した（**Table 14**）。

その結果、ほとんどすべての化合物が URAT1 阻害活性を示した。一方、UUI については、アゼチジン誘導体の活性は弱かったが、3-メチル-3-ピロリン、チアゾリジンおよびジオキソチアゾリジン誘導体は **34f** に比較して強い活性を示した。チアゾリジン誘導体の *tert*-Bu 置換体 **42d** は、URAT1 阻害活性が最も強かったが、比較的強い MIA を示した。この活性の傾向は比較的高い脂溶性（LogP > 3）によるものと推察している。

これら化合物の中で特に 4 つの化合物 **34f, 41, 42a** および **42c** は、薬理活性のパラメータ（URAT1 阻害および UUI）と毒性指標のパラメーター（MIA）の間に十分な乖離を示し、有望であることから、薬物動態（PK）の特性（$C_{\max}$ および Exc.）を確認することとした。

Table 14. URAT1 inhibition, UUI, and MIA of compound **40**, **41**, **42**, **43**

R$_1$: CF$_3$, *tert*-Bu, Br, Et, CN

A: **40** **41** **42** **43**
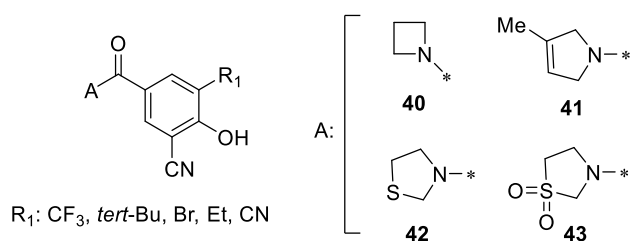
Compound	R$_1$	A	URAT1 % at 3 μM	UUI[a] IC$_{50}$ μM	MIA[b] inhibition (%) or IC$_{50}$ (μM)	LogP[c]
34f		3-Pyrroline	83	818	-1%	2.25
40a		Azetidine	75	>1000	17%	1.84
41	CF$_3$	3-Methyl-3-pyrroline	88	137	9%	2.42
42a		Thiazolidine	84	202	29%	2.58
43		1,1-Dioxo-thiazolidine	55	272	-3%	1.15
40b	*tert*-Butyl	Azetidine	71	>1000	41%	2.62
42b	Br		72	401	23%	2.48
42c	Et	Thiazolidine	67	124	25%	2.56
42d	*tert*-Butyl		92	257	77% (27 μM)	3.36
42e	CN		62	614	23%	1.69

[a] UUI: Inhibitory activity of urate uptake into RPTECs.
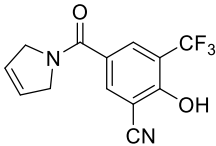
[b] MIA: mitochondrial respiratory control ratio (RCR), inhibition (%) at 100 μM or IC$_{50}$ (μM).

[c] LogP was calculated using ChemDraw v19.
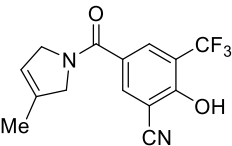
　第一章で述べたように、URAT1 は尿細管の上皮細胞の管腔側に位置するため、薬理活性発現には尿中の活性化合物の存在が必要であり、尿中排泄率（Exc.%）が重要である。また、CYP2C9 阻害は弱い方が好ましい。

　34f, **41**, **42a** および **42c** の PK 特性および CYP2C9 の阻害評価結果を **Table 15** に示す。
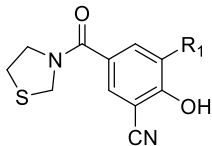
Table 15. PK profiles of compound **34f**, **41**, **42a**, and **42c** in rats

Compound	C_{max} [a] (μg/mL)	Exc. [b] %	CYP2C9 IC_{50} (μM)
34f	2.61	23	52
41	2.22	10	94
42a	3.70	9.0	> 100
42c	-	9.5	-
Dotinurad	9.14	1.1	5.7
BBR	4.42	0	0.041

[a] Maximum concentration (C_{max}).

[b] Excretion rate in urine (%, 0–4 h) after oral administration of 3 mg/kg to rats.

　その結果、これらの化合物は生体での活性に重要な尿中排泄が Dotinurad（Exc. 1.1%）と比較して高かった。化合物 **34f** は CYP2C9 を弱く阻害したが、尿中への排泄率はこれら化合物の中でも高かった。これら化合物は生体において十分な活性を示す可能性がある。例えば、**34f** の RPTEC アッセイによる UUI 活性（818 μM, **Table 13**）は Dotinurad（IC_{50} = 6.8 μM）の約 120 分の 1 であるが、そのラット尿中排泄率は Dotinurad（Exc. = 1.1%）の約 20 倍である。Dotinurad がヒト（一日最大用量 4 mg）において高活性を示すことを考慮すれば、**34f** も数十 mg 程度の用量で十分な尿酸排泄促進作用を示す可能性がある。

　そこで、フサオマキザル（*Cebus apella*）における **34f** の薬効を検討することとした（**Figure 18**）。なお、活性があることを確かめるため、高い用量（300 mg/kg）で試験を行った。
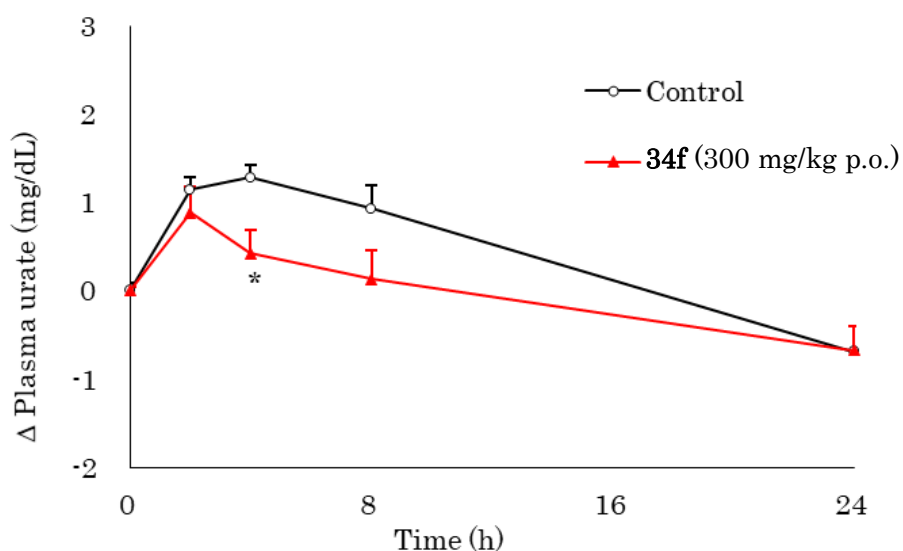
Figure 18. Changes in plasma urate level compared with the pre-dosing value in *Cebus apella* after oral administration of 300 mg/kg **34f**. Values are the means + S.D. of three animals. The average pre-dosing value of control: 2.82 mg/dL. *P < 0.05, significantly different from control at each time point by Dunnett's test.

　その結果、化合物 **34f** の投与により、血漿中尿酸濃度は有意に低下したが、Dotinurad に比較しその作用は弱かった。しかし、本化合物はミトコンドリア毒性をほとんど示さず（-1% at 100 μM）、CYP2C9 を阻害する活性も弱いことから、もうひとつの新たな尿酸排泄促進薬となり得る有望な化合物である。

　Dotinurad が開発されたことから、化合物 **34f** を含め、**Table 13** で示した化合物 **41**, **42a**, **42c** の十分な検証を行っていないが、これら化合物が尿酸排泄促進剤となり得る潜在的な性質を有していることが推察される。

　以上のように、二環性から単環性に変換し分子の脂溶性をコントロールすることで、化合物のミトコンドリア毒性を低下させることに成功した。化合物 **34f** は二環性化合物に比べて *in vitro* 薬理活性が低下したが、薬物の未変化体尿中排泄率が高まった結果として *in vivo* での活性を示したと推察される。

　また、第二章の研究結果は、化学構造に起因する毒性知見にもとづき、毒性を弱める創薬戦略が有効であることをあらためて示した。生物学の進歩とともに、副作用発現のメカニズムが分子レベルや細胞レベルで明らかになってきている。薬理作用は十分ではあるが副作用を持つ薬物に対し、原因となる化学構造を推定し、構造変換により活性を維持したうえで副作用を軽減する戦略は、他の薬剤に適用することが可能であり、新薬の創製につながる有用な手法と考えられる。

第三節　小括

　第一章で述べた Dotinurad の開発においては、脂溶性（LogP）とミトコンドリア呼吸鎖阻害（MIA）が相関することをみいだしていた。そこで、Dotinurad のアミン構造の構造を二環性から単環性の脂環式アミンに変更して脂溶性を低下させることで、ミトコンドリア毒性を示さずに薬理活性を発現する化合物の創製を目指した。

　アミン部位として芳香族性のピロールを持つ 4-ヒドロキシベンズアミド誘導体は強い MIA を示したが、脂環式の 3-ピロリン、ピロリジン、アゼチジン、チアゾリジンをアミン部位とする誘導体の MIA は期待通り低減した。これら誘導体は、脂溶性の低下に伴い、*in vitro* 薬効（URAT1 阻害および UUI）も低下したが、一方で未変化体の尿中への排泄率が高まり、*in vivo* では薬理活性の低下を補うことが期待された。特に 3-ピロリン誘導体やチアゾリジン誘導体は活性のバランスが良く、代表的な化合物 **34f** は、フサオマキザル（*Cebus apella*）による試験を実施したところ、300 mg/kg（p.o.）で血漿中尿酸値を有意に低下させた。化合物 **34f** をはじめとして、これら単環性アミンを部分構造とする誘導体は、ミトコンドリア毒性のない新しい尿酸排泄促進剤となり得る[45]（**Figure 19**）。

　第二章の研究結果は、第一章の研究結果も含め、化学構造に起因する毒性知見にもとづき、毒性を弱める創薬戦略が有効であることを示した。同様の方法を他の薬剤に適用することで新薬の創製につながる可能性があり、既存薬から出発する点で効率的であり、成功確率の高い有用な手法と考えられる。
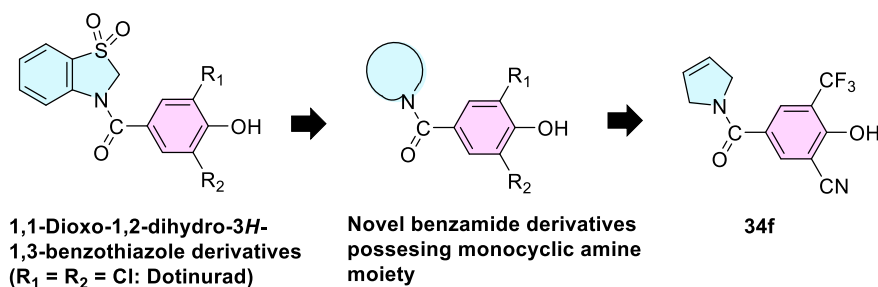
Figure 19. Strategy and results of the novel benzamide derivatives possessing monocyclic amine moiety.

第三章
ヒト近位尿細管上皮細胞（RPTECs）を用いた尿酸取り込み阻害評価系の構築

第一章で述べたとおり、尿酸は腎糸球体で一度ろ過され、約 90％が尿細管から再吸収される。腎における尿酸の再吸収はヒトの血中尿酸濃度を高く維持することに寄与しており [46)]、近位尿細管の管腔側に発現する URAT1 が重要な役割を果たしている。これは、URAT1 遺伝子の欠損により腎性低尿酸血症を発症することや、URAT1 阻害剤である BBR [47)] および Dotinurad [39)] による治療により、血中尿酸値が低下することからも確認できる。

尿酸取り込み阻害作用は、一般的に URAT1 を過剰発現した細胞、例えばアフリカツメガエル卵母細胞または HEK293 細胞 [48, 49)] への尿酸取り込み阻害活性（URAT1 阻害として）によって評価される [50)]。BBR や Dotinurad はこのような URAT1 過剰発現系において尿酸の取り込みを阻害する。しかし、過剰発現系での評価は一つのメカニズムによる作用を観察しているのみであり、必ずしも実際の生体における薬理作用を反映しているとは限らない。そこで、著者らはヒト腎臓での尿酸の取り込みを担っている近位尿細管上皮細胞（RPTECs）を用いて、より生体に近い新たな評価系を構築することを目的として研究を行うこととした。

これまでにも、ラットとヒトの RPTECs についての薬理学的研究がなされているが [51-56)]、尿酸の取り込みや、薬物による取り込み阻害は十分に研究されていなかった。また、RPTECs を用いて十分な尿酸取り込みを実現するには、腎での生理的な条件を模した系を *in vitro* で再現することが必要である。すなわち、この系の最適化は、生体で RPTECs が尿酸を取り込むための条件を確認することでもあり、それにより得られた情報はヒトの血中尿酸濃度を維持するための機能や、尿酸濃度に影響する因子、さらには排泄低下型高尿酸血症の病因を理解することにも役立つ。

以下、系の確立について詳細を述べる。

第一節　RPTECs を用いた尿酸取り込み阻害評価系の検討

　まず、ヒト初代培養 RPTECs への尿酸取り込みのための試験条件を設定するため、尿酸を輸送するトランスポーターが機能するための適切な交換基質および媒体（buffer）の検討を行った（Method A）。

　URAT1 を介して尿酸を RPTECs 内に輸送するにはモノカルボン酸が交換基質となる。ここでは、URAT1 が尿酸を取り込む際に交換基質となることが知られている 2-ピラジンカルボン酸（PZA）[48)]を使用し、系の構築を試みた。なお、PZA は抗結核薬ピラジナミドの代謝物である。ピラジナミドは臨床において、腎臓による尿酸クリアランスを低下させて高尿酸血症を引き起こすことが知られている [57)]。

Method A
＜方法＞
　上述のように、交換基質として PZA を用い、取り込み試験に使用する緩衝液として、細胞を用いた実験等に一般的に使用される *N*–メチル–D–グルカミン（NMDG）buffer [140 mM NMDG, 2 mM potassium gluconate, 1 mM magnesium gluconate, 1.8 mM calcium gluconate, 5 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), and 5 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.4] および transport buffer [96 mM sodium gluconate, 2 mM potassium gluconate, 1 mM magnesium gluconate, 1.8 mM calcium gluconate, 5 mM HEPES, and Tris, pH 7.4] を検討した。本条件下で RPTECs への ^{14}C 標識した尿酸の取り込みを測定し、BBR による阻害を評価した。

＜結果＞
　NMDG buffer を使用した場合、PZA 1 mM 存在下、もしくは PZA 5 mM 存在下において BBR 感受性の [^{14}C]-尿酸の取り込みは観察されなかった（**Figure 20**）。
　一方、transport buffer を使用した場合、PZA 1 mM 存在下（1 時間のインキュベーション）、もしくは PZA 5 mM 存在下（1 時間および 2 時間のインキュベーション）において BBR 感受性の[^{14}C]-尿酸取り込みが観察された（**Figure 21**）。

Method A
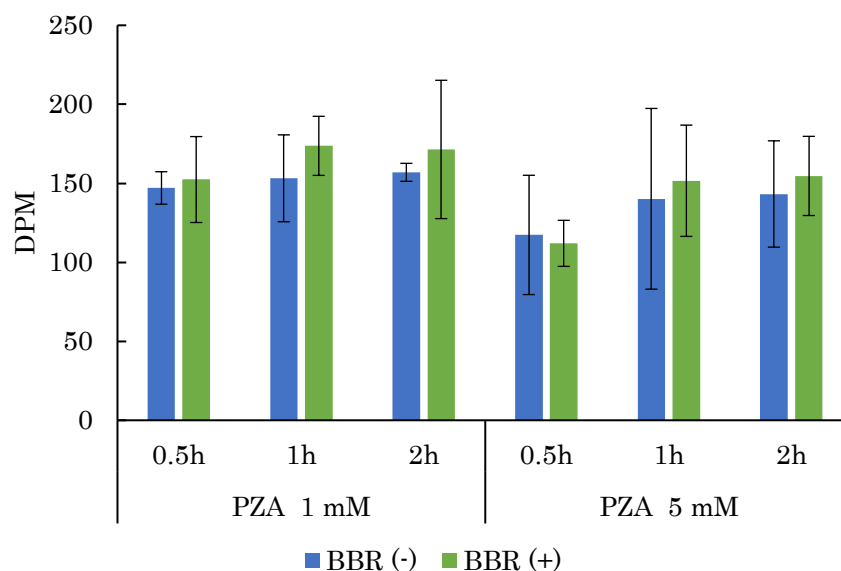
Figure 20. [^{14}C]-Uric acid uptake into primary human renal proximal tubule epithelial cells (RPTECs) in *N*-methyl-D-glucamine buffer and effect of BBR (100 µM) conducted by method A using three wells. DPM, disintegration per minute; PZA, 2-pyrazinecarboxylic acid. Values are the means ± S.D.
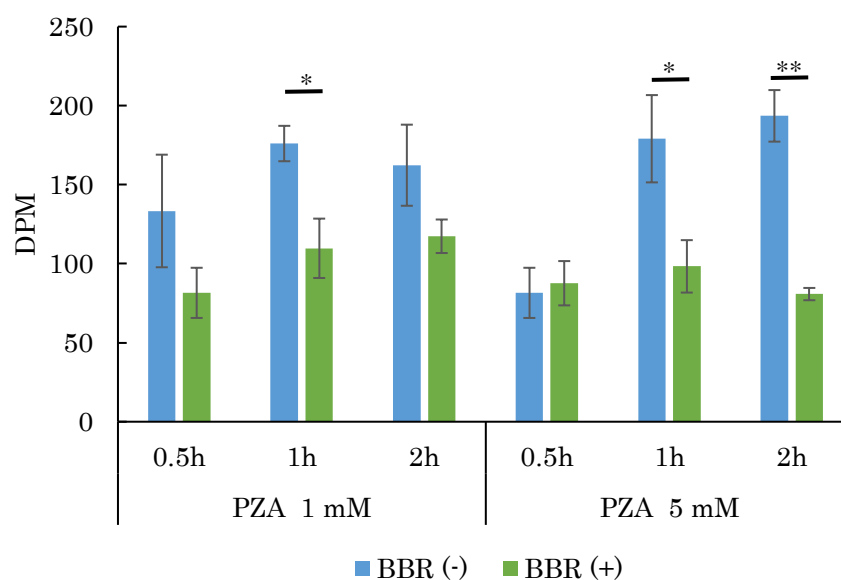
Figure 21. [^{14}C]-Uric acid uptake into primary human renal proximal tubule epithelial cells (RPTECs) in transport buffer and effect of BBR (100 µM) conducted by method A using three wells. DPM, disintegration per minute; PZA, 2-pyrazinecarboxylic acid. *$p < 0.05$, **$p < 0.01$; significant difference between the groups. Values are the means ± S.D.

＜考察＞

　Transport buffer は NMDG buffer とは異なり、グルコン酸ナトリウムが高濃度に（96 mM）含まれている。URAT1 を介した尿酸輸送においては、尿酸の交換基質となる PZA がナトリウム依存性モノカルボン酸トランスポーター（SMCT）[58] により細胞に取り込まれている必要がある。さらに SMCT による細胞内への PZA 共輸送ではナトリウムイオンが必要である。したがって、NMDG buffer を使用した場合（**Figure 20**）、[^{14}C]-尿酸が細胞に取り込まれなかった原因として、ナトリウムイオンが含まれないことが推測される。また、グルコン酸塩が尿酸の輸送に重要であるとの報告（Enomoto et al.）[49] からも、transport buffer が有用であることが支持される。

　以上のように、使用する buffer の組成の違いが理論通りに尿酸の取り込み量に影響することから、本評価系が URAT1 を介した尿酸取り込みを反映していると推測できる。しかし、transport buffer を使用した場合においても尿酸の取り込みが不十分であった（PZA 5 mM, 2 時間における＋BBR と－BBR の差：112.7 DPM, **Figure 21**）。そこで、尿酸の取り込みを増やすため、ヒト RPTECs をインスリンで前処理することを試みることとした（第二節、Method B）。インスリンは高尿酸血症に関与することが示唆されており [59, 60]、ラット RPTECs において URAT1 の発現を増加させることが報告されている [51]。

第二節　インスリン添加による系の構築

　第一節において、RPTECs を用いる評価系の交換基質として PZA が、緩衝液として transport buffer が適切であることを見出した。しかし、化合物を適切に評価するためには、その尿酸の取り込み量は不十分であった。

　臨床研究において、インスリンの投与により尿酸の尿中排泄が減少することが、Muscelli らにより報告されている [59, 60]。さらに、I 型糖尿病モデルである STZ ラットへのインスリンの投与や、ラット腎尿近位尿細管細胞（NRK-E52）へのインスリンの添加により、腎細胞の URAT1 発現が増加することが Toyoki らにより報告されている [51]。そこで、これらの知見に基づき、ヒト RPTECs において URAT1 の発現と尿酸の取り込みを増加させることを目的として、本評価系にインスリンを添加することを試みた（Method B）。

Method B
＜方法＞
　緩衝液と交換基質については、Method A にて確立した方法を用いた。RPTECs にインスリンを 24 時間作用させたのち、化合物を加え 2 時間後に $[^{14}C]$–尿酸の取り込みを測定した（Method B）。阻害活性測定が可能な十分な尿酸取り込み量とするため、Method B では RPTECs の数を Method A の 4 倍とした。

＜結果＞
インスリンの添加により RPTECs への尿酸の取り込みは増加した。また、この尿酸の取り込みは BBR により有意に阻害された（**Figure 22**）。

＜考察＞
　この結果における細胞への尿酸の取り込み量は、化合物の尿酸再吸収阻害活性を評価するには十分であると推察された。なお、このインスリンの作用は、前述したラット腎における Toyoki らの結果 [51] に矛盾しない。したがって、インスリンによる尿酸取り込み活性の促進は、ラットでの結果と同じようにヒトの細胞表面での URAT1 の発現を増したことによる可能性がある。この結果から、ヒトにおいてインスリンが RPTECs を介した尿酸の取り込みに役割を担っており、直接的に高尿酸血症に関与している可能性が示唆された。
　なお、URAT1 の機能亢進は食餌誘発性肥満マウスにインスリン抵抗性を誘発することが報告されている [61]。このようなことから、メタボリックシンドロームにおいて、URAT1 の機能亢進とインスリン抵抗性が悪循環していると推測され、URAT1 阻害剤はこのサイクルを断ち切る可能性がある（**Figure 23**）。今後、RPTECs でのトランスポーターの発現と機能的変化をさらに詳細に研究することにより、

尿酸代謝におけるインスリンの生理的な機能がより明らかになることが期待される。
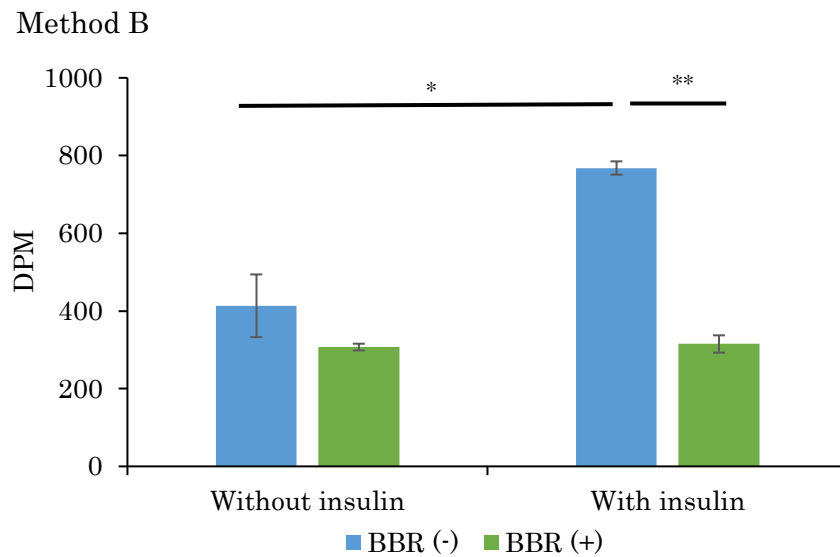
Method B

Figure 22. BBR (100 μM)-sensitive uric acid uptake into primary human RPTECs pretreated with or without insulin in transport buffer conducted by method B using three wells. *$p < 0.05$, **$p < 0.01$; significant difference between the groups. DPM, disintegration per minute. Values are the means ± S.D.
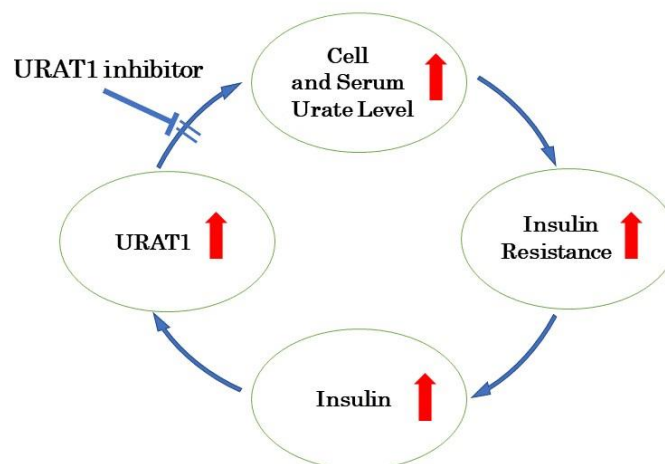
Figure 23. Vicious circle between insulin resistance and URAT1 activation, and the role of URAT1 inhibitor.

第三節　化合物の尿酸取り込み阻害活性評価と考察

　前節において、RPTECs への尿酸の取り込みを *in vitro* で再現し、化合物の尿酸取り込み阻害活性を評価することができる系を構築したことを述べた。

　そこで、次に本評価系の特性を確認すべく、Method B にて代表的な化合物として、BBR、Dotinurad および化合物 **34f**（**Figure 24**）の阻害活性（UUI）を 5 濃度で評価した（**Figure 25**）。
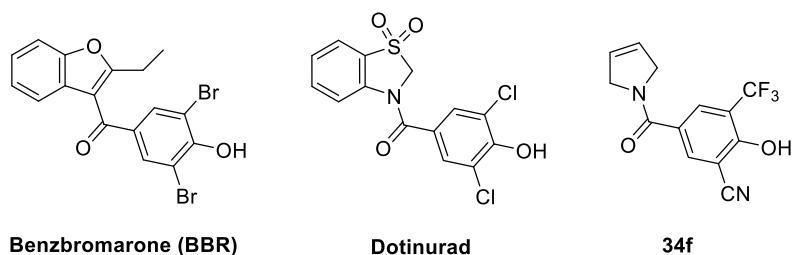
Figure 24. Chemical structures of benzbromarone (BBR), Dotinurad, and **34f**.
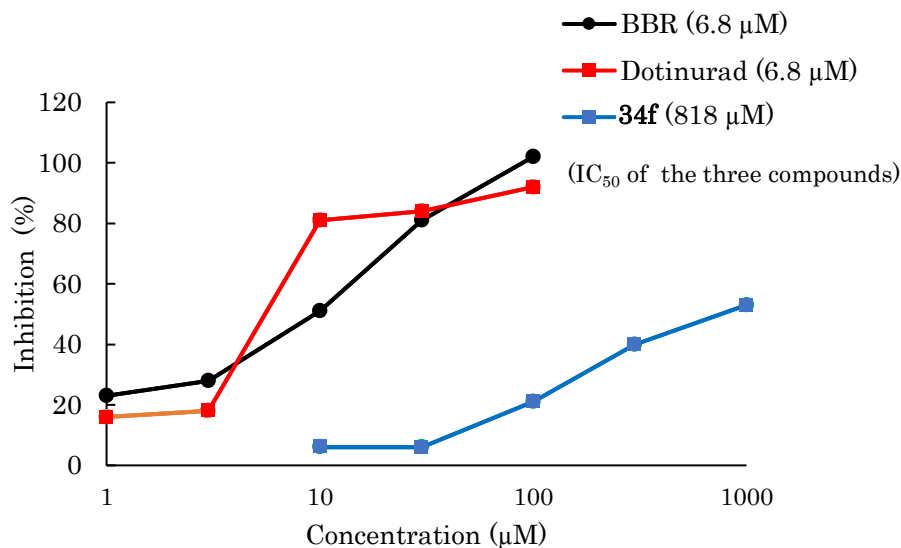
Figure 25. Inhibition of uric acid uptake into primary human renal proximal tubule epithelial cells (RPTECs) conducted by method B using three wells.

その結果、いずれの化合物も濃度依存的に尿酸取り込みを阻害し、この系が評価系として確立されていることを確認した。BBR は IC_{50}：6.8 μM、選択的尿酸再吸収阻害剤（SURI）である Dotinurad は IC_{50}：6.8 μM、化合物 **34f** は IC_{50}：818 μM であった。なお、第一節で実施した同条件による試験と今回の評価試験で尿酸取り込み量に差があったが、これは細胞ロットの違いによるものと推測される。

化合物 **34f**（IC_{50}：818 μM）の阻害活性は、他の 2 つの化合物よりも弱く Dotinurad の約 120 分の 1 であったが、URAT1 阻害活性は、Dotinurad の阻害活性の約 27 分の 1 であった（第一および第二章参照，IC_{50}：1.0 μM vs. 0.0373 μM）[43, 44]。一方、フサオマキザルを使用した化合物 **34f** の血中尿酸濃度の低下活性（300 mg/kg 経口投与）は、5 mg/kg の Dotinurad のそれとほぼ同等であり、活性比は約 60 倍であった。前章で論じたように、尿細管上皮細胞の管腔側に URAT1 が発現している。そのため、URAT1 阻害活性を有する化合物は尿中に排泄にされることで薬効を示す。化合物 **34f** の高い尿中排泄率が *in vivo* 活性に関与すると推察され、尿中排泄率の低い Dotinurad との *in vivo* 活性の薬効差は、*in vitro* 活性の差から想定されるより小さくなるはずである。したがって、27 倍差であった URAT1 阻害よりも 120 倍差であった UUI の方が実際の薬効（60 倍差）における薬理活性をより表していると推察される。

さらに、本系における URAT1 以外のトランスポーターに対する化合物の効果を明らかにするために、本評価系の阻害活性（UUI）と URAT1 阻害活性の IC_{50} 比を計算した。UUI/URAT1 阻害活性の IC_{50} 比について、BBR は（6.8 μM/0.19 μM）= 35.8、Dotinurad は（6.8 μM/0.0372 μM）= 183、化合物 **34f** は（818 μM/1.0 μM）= 818 であり、化合物により比が異なる。したがって、本評価系は URAT1 以外のトランスポーターが関与している。すなわち、化合物によるトランスポーター阻害の選択性が本評価系の活性に影響すると推測される。

これらのことから、本評価系は URAT1 阻害の評価系に比較して、より生体に近い評価系であると推察される。

ただし、本研究では以下のような点について未検討であり、尿酸取り込みの詳細なメカニズムを明らかにするためには、さらなる検討が必要である。

初代培養ヒト RPTECs を使用したため、尿酸取り込みの程度や化合物の阻害は細胞ロットによって異なる。また、インスリンによる尿酸の取り込み増加を確認しているが、URAT1 について mRNA やタンパクの発現量、および細胞内局在などを検討していない。上記の OAT、ABCG2、GLUT9 などの他のトランスポーターについても同様である。

第四節　小括

　本論第三章では、ヒト RPTECs を用いた尿酸取り込み阻害試験法の確立について述べた。上記第一章及び第二章における化合物評価に用いるために新たに構築したものである。

　一般的に、尿酸排泄促進剤の *in vitro* 評価は、ヒト尿酸トランスポーター1（URAT1）を過剰発現した細胞への尿酸取り込み阻害を指標とする。しかし、生体では複数のトランスポーターが尿酸の輸送に関与している。そこで、ヒトの腎において尿酸の再吸収を担っている近位尿細管上皮細胞（RPTECs）を用いた、新たな尿酸の取り込み阻害を評価する系の構築を試みた。当初、試験系の緩衝液として transport buffer を選択することで、細胞への尿酸の取り込みを確認した。しかし、阻害活性を評価するためには取り込み量が不十分であったことから、ラット腎尿細管上皮細胞にて URAT1 の発現をたかめるインスリンを添加した。結果として、細胞への尿酸の取り込みが増加し、URAT1 阻害剤である BBR によって濃度依存的に阻害された。これにより、この試験系は尿酸排泄促進剤を評価する方法として機能することが確認された。また、化合物により URAT1 阻害活性に対する UUI の比（UUI／URAT1 阻害）が異なることから、本評価系では化合物のトランスポーター阻害の選択性が活性に影響することが推測される。このようなことから、本評価系は、一般的な URAT1 阻害活性評価系に比較して、より生理的条件に近い尿酸取り込みを反映していると推察される。

　また、インスリンの添加による RPTECs への作用は、インスリンが血中の尿酸濃度の恒常性に関与することを示唆している。URAT1 および他のトランスポーターの発現と機能変化をさらに研究することにより、尿酸代謝におけるインスリンの生理的な意義を解明することにつながる。

結　語

　本論文は、高尿酸血症の治療に用いる新規な尿酸排泄促進剤の開発を目指した創薬研究に関するものである。古典的な尿酸排泄促進剤である BBR の毒性は、BBRの構造的な特徴に起因するミトコンドリア毒性が原因であると推測した。そこで、BBR の構造を変換することで、安全性が高く、かつ薬理活性が強い新たな薬物の創製が可能と考え本創薬研究をおこなった。

　第一章では、新規な尿酸排泄促進剤 Dotinurad の創出について述べた。まず、BBR の構造的特徴を解析し、薬効発現には尿酸と共通する特徴（平面性および適度な酸性度）が必要であることを仮定した。また、肝毒性の発現には、ミトコンドリア毒性が原因であり、ラジカルを安定化し得るビスアリールケトン構造に起因すると仮定した。そこで、BBR を基本構造として、酸性基であるフェノール性水酸基を保ったまま、そのビスアリールケトン型構造 [Ar-(CO)-Ar] を、アリール基とケトンとの共役を部分的に回避したアミド型構造 [N-(CO)-Ar] へと変換することをこころみた。その結果、構造をアミド型へ代えること、および脂溶性を下げることがミトコンドリア毒性を弱くするために重要であることを見出した。結果として、*in vitro* 系において薬効を発現しながら、ミトコンドリア毒性が弱い誘導体を複数得ることに成功した。さらに研究を進めた結果、PK 特性も優れている 1,1-ジオキソ-1,2-ジヒドロ-3*H*-1,3-ベンゾチアゾール誘導体を見出した。代表化合物（Dotinurad）は、薬効をヒトに外挿できるフサオマキザル（*Cebus apella*）をもちいた *in vivo* 試験において、BBR より強い血中尿酸値低下作用が確認された [43]。Dotinurad は日本国内で医薬品として承認され、現在海外での開発も進められている。

　第一章で述べたとおり、肝毒性が問題となっていた BBR に代わる医薬品を開発するという目標は Dotinurad の開発をもって達成された。また、その過程で分子の脂溶性（LogP）とミトコンドリア呼吸鎖阻害（MIA）が相関することが見出されていた。そこで、著者はミトコンドリア毒性をさらに抑えその懸念を完全に払しょくするような化合物を創出するべく研究を続けた。第二章では、その結果について述べた。アミン部位の構造を二環性から単環性の脂環式アミン（3-ピロリン、ピロリジン、アゼチジン、チアゾリジン）に代えると期待したとおり弱い MIA を示した。また、脂溶性を低下させたことで、*in vitro* 薬効（URAT1 阻害および UUI）も低下したが、未変化体の尿中への排泄率が高まり、*in vivo* では薬理活性の低下をおぎなうことが期待された。特に 3-ピロリンやチアゾリジンをアミン部位とする 4-ヒドロキシベンズアミド誘導体は活性のバランスが良く、例えば化合物 **34f** は、フサオマキザル（*Cebus apella*）による薬理試験において、300 mg/kg（p.o.）

で血漿中尿酸値を有意に低下させた。化合物 **34f** はミトコンドリア毒性のない、尿酸排泄促進作用を有する新しい化合物である [45]。

第一章、第二章の研究結果は、分子構造についての深い考察と適切な構造改変によって既存薬物の毒性低減が果たされた事例であり、企業における創薬戦略の一つとして示唆に富むものである。また、近年実施が容易になっている、タンパクや遺伝子の発現といった生物学的な特性評価や、人工知能（AI）およびビッグデータの活用により、化学構造と活性の相関について新たな知見を得ることが可能となっている。本手法にこれら新たな方法論を組み合わせ、薬理活性および毒性の両面で応用することで、新たな創薬の展開が可能となることが期待される。

本論第三章では、ヒト腎細胞を用いた尿酸取り込み阻害試験法の確立について述べた。上記第一章及び第二章における化合物評価に用いるために新たに構築したものである。また、本研究で得られた、ヒト RPTECs に対するインスリンの影響についても考察した。

一般的に、尿酸排泄促進剤の *in vitro* 評価は、ヒト尿酸トランスポーター1（URAT1）を過剰発現した細胞への尿酸取り込み阻害を指標とする。しかし、生体では複数のトランスポーターが尿酸の輸送に関与していることから、実際の活性を正しく評価するためには、複数のトランスポーターの関与を含めて評価する必要がある。そこで、ヒトの腎において尿酸の再吸収を担っている近位尿細管上皮細胞（RPTECs）を用いて、尿酸の取り込み阻害を評価する系の構築をこころみた。当初、試験系の緩衝液として transport buffer を選択することで、細胞への尿酸の取り込みを確認した。しかし、阻害活性を評価するためには取り込み量が不十分であったことから、ラット腎尿細管上皮細胞にて URAT1 の発現をたかめるインスリンを添加した。その結果、細胞への尿酸の取り込みは有意に増加し、URAT1 阻害剤である BBR によって濃度依存的に阻害された。これにより、この試験系は尿酸排泄促進剤を評価する方法として機能することが確認された。化合物によって URAT1 阻害活性に対する UUI 活性の比（UUI／URAT1 阻害）が異なることから、UUI の評価では URAT1 以外のトランスポーターの阻害が活性値に影響する。すなわち、化合物によるトランスポーター阻害の選択性が反映されると推察される。これらの結果から、新たに構築した本評価系は、一般的な URAT1 阻害活性評価系に比較して、より生体に近い尿酸取り込みを反映している。

以上、新たな尿酸排泄促進剤の開発を目指した研究を進めた結果、新規な尿酸排泄促進剤 Dotinurad を得ることに成功した。また、ミトコンドリア毒性をさらに抑えその懸念を完全に払しょくするような化合物を創出するべく研究を続けた結果、アミン部位の構造を 3-ピロリンに改変した化合物（**34f**）を見出だした。

今回おこなった、毒性を低減するための創薬手法は、新たな医薬品開発にも応用可能なものである。

　また、より生体に近い尿酸取り込みを反映した、ヒト RPTECs を用いた尿酸取り込み阻害を評価する系を構築することに成功した。確認された RPTECs への尿酸取り込みに対するインスリンの効果は、インスリンが血中の尿酸濃度の恒常性に関与することを示唆している。本評価系において URAT1 および他のトランスポーターの発現と機能変化をさらに研究することにより、尿酸代謝におけるインスリンの生理的な意義を解明することにつながる。

　Dotinurad は日本で 2020 年 5 月に医薬品（ユリス錠）として上市され、現在、中国および欧米等、海外での開発も進められている。Dotinurad は臨床での使用実績が拡大しつつあり、今後、世界標準の尿酸排泄促進薬として使用が広がり知見が蓄積されることを期待している。

合 成 に 関 す る 実 験

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 shifts (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. CDCl$_3$: deuterium chloroform, DMSO-d_6: deuterium dimethyl sulfoxide, CD$_3$OD: 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.

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

(a) 4-(Methoxymethoxy)isophthalonitrile

To a solution of 4-nitrobenzonitrile (40.1 g, 0.271 mol) in dimethyl sulfoxide (400 mL) was added potassium cyanide (26.45 g, 0.406 mol), and the mixture was stirred at 90 °C for 1.5 h. KHCO$_3$ and chloromethyl methyl ether (20.4 mL, 0.271 mol) were added at room temperature, and the stirring was continued for 0.5 h. Water was added, and insoluble materials were removed by filtration through a pad of Celite. The filtrate was diluted withAcOEt, and the organic layer was washed with H$_2$O and brine, dried over anhydrous Na$_2$SO$_4$. The solvent was evaporated, and the obtained residue was purified by silica gel column chromatography to afford a pale-yellow solid (17.8 g, 35% yield).

^{1}H-NMR (270 MHz, CDCl$_3$) δ: 3.54 (3H, s), 5.37 (2H, s), 7.36 (1H, d, J = 8.6 Hz), 7.80 (1H, dd, J = 8.6, 2.2 Hz), 7.88 (1H, d, J = 2.2 Hz).

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

To a solution of 4-(methoxymethoxy)isophthalonitrile (2.97 g, 15.8 mmol) in methanol (150 mL) was added sodium methoxide (853 mg, 15.8 mmol), and the mixture was stirred at room temperature for 15 h. The solvent was evaporated, and the residue was dissolved with AcOEt. The solution was washed with H$_2$O and brine, dried over anhydrous Na$_2$SO$_4$. The residue was dissolved with 1,4-dioxane (30 mL), H$_2$O (15 mL) and glacial acetic acid (4 mL), and the mixture was stirred at room temperature for 5 h. The solvent was evaporated, and saturated NaHCO$_3$ solution was added. The products were extracted with AcOEt, and the combined extracts were dried over anhydrous Na$_2$SO$_4$. The solvent was evaporated, and the obtained residue was purified by silica gel column chromatography to afford a colorless solid (1.19 g, 34% yield).

^{1}H-NMR (270 MHz, CDCl$_3$) δ: 3.54 (3H, s), 3.93 (3H, s), 5.36 (2H, s), 7.28 (1H, d, J = 8.9 Hz), 7.80 (1H, dd, J = 8.9, 2.2 Hz), 8.27 (1H, d, J = 2.2 Hz).

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

To a solution of methyl 3-cyano-4-(methoxymethoxy)benzoate (449 mg, 2.03 mmol) in THF (5 mL) and H_2O (2.5 mL) was added lithium hydroxide monohydrate (128 mg, 3.05 mmol). After the mixture was stirred at room temperature for 2 h, 1 M HCl (6 mL) and H_2O (30 mL) were added. The products were extracted with AcOEt, and the organic layer was washed with H_2O and brine, dried over anhydrous Na_2SO_4. The solvent was evaporated to afford a colorless solid (372 mg, 89% yield).

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

(d) 5-(2,3-Dimethyl-1H-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 DMF (1 mL) were added 1-hydroxybenzotriazole monohydrate (222 mg, 1.45 mmol) and EDC·HCl (278 mg, 1.45 mmol). After the mixture was stirred at room temperature for 10 min, H_2O (10 mL) was added. The products were extracted with AcOEt, and the organic layer was dried over anhydrous Na_2SO_4. The solvent was evaporated to afford HOBt ester.

To a solution of 2,3-dimethylindole (252 mg, 1.73 mmol) in DMF (5 mL) was added the solution of HOBt ester in DMF (5 mL). After the mixture was stirred at room temperature for 1 h, the reaction mixture was diluted with AcOEt. The solution was washed with H_2O and brine, dried over anhydrous Na_2SO_4. The mixture was concentrated, and the obtained residue was purified by silica gel column chromatography to afford a colorless solid (135 mg, 45% yield).

^{1}H-NMR (270 MHz, CDCl$_3$) δ: 2.24 (3H, s), 2.34 (3H, s), 3.57 (3H, s), 5.39 (2H, s), 6.99 (1H, d, J = 8.4 Hz), 7.07 (1H, dt, J = 1.1, 8.1 Hz), 7.20 (1H, dt, J = 1.1, 7.0 Hz), 7.33 (1H, d, J = 8.9 Hz), 7.46 (1H, d, J = 7.8 Hz), 7.90 (1H dd, J = 2.2, 8.9 Hz), 7.97 (1H, d, J = 2.2 Hz).

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

To a solution of 5-(2,3-dimethyl-1H-indole-1-carbonyl)-2-(methoxymethoxy)benzonitrile (130 mg, 0.389 mmol) in THF (6 mL) was added 4 M HCl 1,4-dioxane solution (2 mL), and the mixture was stirred at room temperature for 20 h. The solvent was evaporated, and the obtained residue was diluted with AcOEt. The solution was washed with H_2O and brine, dried over anhydrous Na_2SO_4. The solvent was evaporated to afford a colorless solid (87 mg, 77% yield).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 2.20 (3H, s), 2.26 (3H, s), 7.02 (1H, d, J = 8.1 Hz), 7.09 (1H, dt, J = 7.8, 1.4 Hz), 7.15 (1H, d, J = 8.6 Hz), 7.17 (1H, dt, J = 7.3, 1.1 Hz), 7.51 (1H, d, J = 7.6 Hz) , 7.79 (1H, dt, J = 8.6, 2.2 Hz), 7.98 (1H, d, J = 2.2 Hz); the phenol-OH was not observed.

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 8.2, 12.4, 99.5, 113.2, 113.9, 115.8, 116.6, 118.0, 122.0, 122.7, 125.6, 130.1, 132.5, 135.6, 135.7, 135.9, 164.2, 166.8.

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)

Compound **2a** was prepared from indoline instead of 2,3-dimethylindole in a similar way that used for compound **1** (66% yield).

^{1}H-NMR (400 MHz, DMSO-d_6) δ: 3.11 (2H, t, J = 8.2 Hz), 4.08 (2H, t, J = 8.2 Hz), 7.06 (1H, d, J = 7.2Hz), 7.11 (1H, d, J = 8.7Hz), 7.20 (1H, t, J = 7.2 Hz), 7.31 (1H, d, J = 7.2 Hz), 7.74 (1H, brs), 7.77 (1H, dd, J = 8.7, 1.8 Hz), 7.92 (1H, d, J = 1.8 Hz); the phenol-OH was not observed.

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 27.9, 50.5, 98.8, 116.3, 116.56, 116.62, 123.9, 125.1, 126.9, 128.0, 132.7, 132.9, 134.2, 142.7, 162.0, 166.3.

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)

(a) 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) was added bromine (0.87 mL, 16.9 mmol), and the mixture was stirred at room temperature for 15 h. The reaction mixture was quenched by 10% sodium thiosulfate aqueous solution. The products were extracted with AcOEt, and the organic layer was washed with brine and dried over anhydrous Na_2SO_4. The solvent was evaporated to afford a pale-yellow solid (4.30 g, quant.).

(b) 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 DMF (30 mL) was added copper (I) cyanide (781 mg, 8.72 mmol). After the mixture was stirred at 150 °C for 18 h, KHCO$_3$ (1.06 g, 7.67 mmol) and chloromethyl methyl ether (0.30 mL, 3.99 mmol) were added at room temperature. After the stirring was continued at room temperature for 1 h, the mixture was diluted with AcOEt, and insoluble materials were removed by filtration through a pad of Celite. The filtrate was washed with H_2O and brine, then dried over anhydrous Na_2SO_4. The solvent was evaporated, and the obtained residue was purified by silica gel column chromatography to afford 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 THF (10 mL) and H_2O (5 mL) was added lithium hydroxide monohydrate (610 mg, 14.5 mmol). After the mixture was stirred at room temperature for 4 h, 1M HCl (25 mL) was added. The products were extracted with AcOEt, and the organic layer was dried over anhydrous Na_2SO_4. The solvent was evaporated to afford a colorless crystal (678 mg, 39% yield).

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

(c) 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 CH_2Cl_2 (9 mL) and DMF (1 mL) were added indoline (151 mg, 1.27 mmol) and EDC·HCl (292 mg, 1.52 mmol). After the mixture was stirred at room temperature for 1 h, the solvent was evaporated, and the residue was

diluted with AcOEt. The solution was washed with H_2O and brine, then dried over anhydrous Na_2SO_4. The solvent was evaporated, and the residue was dissolved with CH_2Cl_2 (5 mL) and trifluoroacetic acid (5 mL). After the mixture was stirred at room temperature for 2 h, the solvent was evaporated and the residue was crystalized with *n*-hexane/AcOEt to afford a colorless solid (222 mg, 60% yield).

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

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

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)

(a) Methyl 3-cyano-4-methoxy-5-vinylbenzoate

To a solution of methyl 3-cyano-5-ethynyl-4-methoxybenzoate (782 mg, 3.63 mmol) in THF (10 mL) was added 5% palladium on barium sulfate (78 mg), and the mixture was stirred under a hydrogen atmosphere at room temperature for 4 h. The insoluble materials were removed by filtration through a pad of Celite, and the filtrate was concentrated under reduced pressure. The obtained residue was purified by silica gel column chromatography (n-hexane/AcOEt = 5:1) to afford the title compound as a colorless solid (705 mg, 89% yield).

^{1}H-NMR (270 MHz, CDCl$_3$) δ: 3.94 (3H, s), 4.10 (3H, s), 5.49 (1H, d, J = 11.2 Hz), 5.90 (1H, d, J = 17.7 Hz), 6.95 (1H, d, J = 11.2, 17.7 Hz), 8.17(1H, d, J = 2.1 Hz), 8.35 (1H, d, J = 2.1 Hz).

(b) Methyl 3-cyano-5-formyl-4-methoxybenzoate

To a solution of methyl 3-cyano-4-methoxy-5-vinylbenzoate (350 mg, 1.61 mmol) in acetone (8 mL), acetonitrile (4 mL) and water (4 mL) were added 4-methylmorpholine *N*-oxide (943 mg, 8.05 mmol) and 10% microencapsulated osmium tetroxide (201 mg, 0.08 mmol). After the mixture was stirred for 18 h at room temperature, the insoluble materials were removed by filtration through a pad of Celite.

To the filtrate was added sodium periodate (1.72 g, 8.04 mmol), and the mixture was stirred at room temperature for 30 min. Water was added, and the mixture was extracted with AcOEt. The combined extracts were washed with brine and dried over anhydrous Na_2SO_4. The solvent was evaporated, and the obtained residue was purified by silica gel column chromatography (n-hexane/AcOEt = 3:1) to afford the title compound as a pale brown oil (308 mg, 87% yield).

^{1}H-NMR (270 MHz, CDCl$_3$) δ: 3.96 (3H, s), 4.39 (3H, s), 8.49 (1H, d, J = 2.2 Hz), 8.67 (1H, d, J = 2.2 Hz), 10.40 (1H, s).

(c) Methyl 3,5-dicyano-4-methoxybenzoate

To a solution of methyl 3-cyano-5-formyl-4-methoxybenzoate (308 mg, 1.41 mmol) in THF (10 mL) was added hydroxylamine hydrochloride (117 mg, 1.68 mmol). After stirring at room temperature for 20 h,

the mixture was concentrated under reduced pressure, then water was added. The products were extracted with AcOEt, and the organic layer was washed with brine and dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure, the residual solid was washed with n-hexane/AcOEt (1:1, 5 mL).

To a solution of the obtained material in THF (5 mL) were added triethylamine (1.0 mL, 7.21 mmol) and 2-chloro-1-methylpyridinium iodide (370 mg, 1.45 mmol). After the mixture was stirred at room temperature for 1 h, the solvent was evaporated under reduced pressure. 1 M HCl was added, and the products were extracted with AcOEt. The combined extracts were washed with 1 M aqueous NaOH, 10% aqueous sodium thiosulfate and brine, and dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure to afford the title compound as a pale-yellow solid (234 mg, 77% yield).

^{1}H-NMR (270 MHz, CDCl$_3$) δ: 3.97 (3H, s), 4.46 (3H, s), 8.43 (2H, s).

(d) 3,5-Dicyano-4-methoxybenzoic acid

To a solution of methyl 3,5-dicyano-4-methoxybenzoate (331 mg, 1.53 mmol) in THF (10 mL) and water (5 mL) was added lithium hydroxide monohydrate (128 mg, 3.05 mmol). After the mixture was stirred at room temperature for 90 min, the organic solvent was evaporated under reduced pressure. Water was added to the residue, and the aqueous solution was acidified with 1 M HCl. The products were extracted with AcOEt, the organic layer was washed with brine and dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure to afford the title compound as a pale-yellow solid (312 mg, 100% yield).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 4.36 (3H, s), 8.49 (2H, s). MS (m/z): 201 (M − H)$^-$.

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

To a solution of 3,5-dicyano-4-methoxybenzoic acid (250 mg, 1.16 mmol) in CH_2Cl_2 (9 mL) and DMF (1 mL) were added indoline (138 mg, 1.16 mmol) and EDC·HCl (267 mg, 1.39 mmol), and the mixture was stirred at room temperature for 1 h. Then the solvent was evaporated, and the residue was dissolved with AcOEt. The organic solution was washed with H_2O and brine, and dried over anhydrous Na_2SO_4. The solvent was evaporated, and the residue was dissolved with DMF (10 mL). Lithium chloride (445 mg, 10.5 mmol) was added, and the mixture was stirred at room temperature for 1 h. 1 M HCl (40 mL) was added, and the products were extracted with AcOEt, and the organic layer was dried over anhydrous Na_2SO_4. The solvent was evaporated to afford a light brown solid (267 mg, 80% yield).

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

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

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)

(a) Methyl 3-bromo-4-hydroxybenzoate

     To a solution of methyl 4-hydroxybenzoate (25.0 g, 164 mmol) in chloroform (225 mL) and methanol (25 mL) was added a solution of bromine (8.46 mL, 164 mmol) in chloroform (30 mL).   After the mixture was stirred at room temperature for 2 h, the solution was diluted with chloroform. The solution was washed with water, 10% aqueous sodium thiosulfate and brine, then dried over anhydrous Na_2SO_4. The solvent was evaporated to afford the title compound as a colorless solid (37.8 g, 100% yield).

  ^{1}H-NMR (270 MHz, CDCl$_3$) δ: 3.90 (3H, s), 7.05 (1H, d, J = 8.6 Hz), 7.92 (1H, dd, J = 2.0, 8.6 Hz), 8.19 (1H, d, J = 2.0 Hz). The ^{1}H-NMR spectroscopic data of the obtained material was fully consistent with that reported in the literature. [62)

  MS (m/z): 230 (M − H)$^-$.

(b) Methyl 3-cyano-4-hydroxybenzoate

     To a solution of methyl 3-bromo-4-hydroxybenzoate (37.8 g, 164 mmol) in DMF (250 mL) was added copper cyanide (22.0 g, 246 mmol), the mixture was stirred at 150 °C for 16 h. Potassium carbonate (68.0 g, 492 mmol) and chloromethyl methyl ether (14.8 mL 195 mmol) were added under ice cooling, and the stirring was continued for 2 h. The insoluble materials were removed by filtration through a pad of Celite. Water was added to the filtrate, the products were extracted with AcOEt. The combined extracts were washed with water and brine, and dried over anhydrous Na_2SO_4. The solvent was evaporated, and the obtained residue was dissolved in chloroform (30 mL). Trifluoroacetic acid (30 mL) was added, and the mixture was stirred at room temperature for 2 h. The reaction mixture was concentrated under reduced pressure, and the obtained solid was washed with n-hexane/AcOEt (2:1, 45 mL) to afford the title compound as a pale-yellow solid (6.53 g, 22% yield).

  ^{1}H-NMR (270 MHz, DMSO-d_6) δ: 3.78 (3H, s), 7.07 (1H, d, J = 8.8 Hz), 8.02 (1H, dd, J = 2.1, 8.8 Hz), 8.10 (1H, d, J = 2.1 Hz), 12.06 (1H, brs). The ^{1}H-NMR spectroscopic data of the obtained material was fully consistent with that reported in the literature. [63)

  MS (m/z): 176 (M − H)$^-$.

(c) Methyl 3-cyano-4-hydroxy-5-iodobenzoate

     To a solution of methyl 3-cyano-4-hydroxybenzoate (6.47 g 36.5 mmol) in chloroform (80 mL) and methanol (10 mL) were added *N*-iodosuccinimide (8.63 g 38.4 mmol) and trifluoromethanesulfonic acid (2.5 mL). After the mixture was stirred at room temperature for 1 h, the solvent was evaporated, and the obtained solid was washed with water (100 mL) to afford the title compound as a pale-yellow solid (11.1 g, quant.).

  ^{1}H-NMR (270 MHz, DMSO-d_6) δ: 3.60 (3H, s), 8.15 (1H, d, J = 2.1 Hz), 8.45 (1H, d, J = 2.2 Hz).

  MS (m/z): 302 (M − H)$^-$.

(d) Methyl 3-cyano-5-iodo-4-methoxybenzoate

     To a solution of methyl 3-cyano-4-hydroxy-5-iodobenzoate (11.1 g, 36.5 mmol) in DMF (230 mL) were added potassium carbonate (49.2 g, 0.356 mol) and dimethyl sulfate (17.0 mL 0.179 mol). After the mixture was stirred at room temperature for 18 h, the insoluble materials were removed by filtration. Water

(920 mL) was added to the filtrate, and a precipitated solid was collected by filtration to afford the title compound as a pale-yellow solid (8.99 g, 78% yield).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 4.20 (3H, s), 4.40 (3H, s), 8.63 (1H, d, J = 1.9 Hz), 8.88 (1H, d, J = 1.9 Hz).

(e) 3-Cyano-5-iodo-4-methoxybenzoic acid

To a solution of methyl 3-cyano-5-iodo-4-methoxybenzoate (8.00 g, 25.2 mmol) in THF (100 mL) and water (50 mL) was added lithium hydroxide monohydrate (4.23 g, 101 mmol). After the mixture was stirred at room temperature for 4 h, the solvent was evaporated under reduced pressure. Water was added, and the aqueous solution was washed with n-hexane and acidified with 2 M HCl. The products were extracted with AcOEt, and the organic layer was washed with brine and dried over anhydrous Na_2SO_4. The solvent was evaporated to afford the title compound as a colorless solid (7.26 g, 95% yield).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 4.38 (3H, s), 8.58 (1H, d, J = 2.0 Hz), 8.86 (1H, d, J = 2.0 Hz), 13.89 (1H, brs).

MS (m /z): 302 (M − H)$^-$.

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

Compound **2c** was prepared from 3-cyano-5-iodo-4-methoxybenzoic acid in a similar way that used for compound **2d** (66% yield).

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

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

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)

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

To a solution of methyl 3-cyano-4-hydroxy-5-iodobenzoate (p. 60) (1.00 g, 3.30 mmol) in DMF (10 mL) were added 2,2'-bipyridine (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). After the mixture was stirred at 90 °C for 16 h under an argon atmosphere, 1 M HCl was added, and insoluble materials were removed by filtration through a pad of Celite. The filtrate was diluted with AcOEt, and the solution was washed with H_2O and brine, and dried over anhydrous Na_2SO_4. The solvent was evaporated, and the residue was dissolved with DMF (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. The insoluble materials were removed by filtration through a pad of Celite. The filtrate was diluted with AcOEt, the solution was washed with H_2O and brine, and dried over anhydrous Na_2SO_4. The solvent was evaporated, and the obtained residue was purified by silica gel column chromatography to afford a colorless solid (276 mg, 35% yield).

^{1}H-NMR (270 MHz, CDCl$_3$) δ: 2.50 (3H, s) 3.94 (3H, s), 4.18 (3H, s,), 7.95 (1H, d, J = 1.9 Hz), 8.03 (1H, d, J = 1.9 Hz).

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

To a solution of methyl 3-cyano-4-methoxy-5-methylsulfanylbenzoate (270 mg, 1.14 mmol) in THF (4 mL) and H$_2$O (2 mL) was added lithium hydroxide monohydrate (192 mg, 4.56 mmol), and the mixture was stirred at room temperature for 1.5 h. 1 M HCl (10 mL) was added, and the products were extracted with AcOEt. The combined extracts were washed with H$_2$O and brine, and dried over anhydrous Na$_2$SO$_4$. The solvent was evaporated to afford a colorless solid (241 mg, 95% yield).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 2.52 (3H, s), 4.05 (3H, s), 7.92 (1H, d, J = 2.2 Hz), 8.01 (1H, d, J = 2.2 Hz), 13.49 (1H, brs).

MS m/z: 222 (M − H)$^-$.

(c) 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 CH$_2$Cl$_2$ (9 mL) and DMF (1 mL) were added indoline (126 mg, 1.06 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (242 mg, 1.26 mmol). After the mixture was stirred at room temperature for 1.5 h, 1 M HCl (40 mL) was added, and the products was extracted with AcOEt. The organic layer was washed with H$_2$O and brine, dried over anhydrous Na$_2$SO$_4$. The solvent was evaporated to afford the title compound.

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

To a solution of 5-(indoline-1-carbonyl)-2-methoxy-3-methylsulfanylbenzonitrile (c) in *N,N*-dimethylformamide (10 mL) was added lithium chloride (445 mg, 10.5 mmol). After the mixture was stirred at 100 °C for 1.5 h, 1 M HCl (40 mL) was added. The products were extracted with AcOEt, and the organic layer was washed with H$_2$O and brine, dried over anhydrous Na$_2$SO$_4$. The solvent was evaporated to obtain a colorless crystal a colorless solid (267 mg, 82% yield from indoline).

^{1}H-NMR (400 MHz, DMSO-d_6) δ: 2.43 (3H, s), 3.08 (2H, t, J = 8.2 Hz), 4.04 (2H, t, J = 8.2 Hz), 7.03 (1H, t, J = 7.5 Hz), 7.16 (1H, t, J = 7.5 Hz), 7.27 (1H, d, J = 7.5 Hz), 7.62 (1H, d, J = 1.8 Hz), 7.67 (1H, d, J = 1.8 Hz), 7.73(1H, brs); the phenol-OH was not observed.

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 14.6, 27.6, 50.2, 99.9, 116.2, 116.3, 123.6, 124.8, 126.6, 128.4, 129.1, 129.2, 130.2, 132.6, 142.5, 157.8, 165.8.

MS m/z: 309 (M − H)$^-$.

HRMS (ESI) m/z: calcd for C$_{17}$H$_{13}$N$_2$O$_2$S (M − H)$^-$ 309.0703, found 309.0710.

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

(a) Methyl 3-*tert*-butyl-4-hydroxybenzoate

To a solution of methyl 4-hydroxybenzoate (3.0 g, 19.7 mmol) in methanesulfonic acid (15 mL) was added 2-bromo-2-methylpropane (11.1 ml, 98.8mmol). After the mixture was stirred at 70 °C overnight, methanol (20 ml) was added, and the stirring was continued at 50 °C for 3 h. 30% Potassium hydroxide

aqueous solution was added, and the products were extracted with AcOEt. The combined extracts were washed with 10% aqueous potassium carbonate and brine, dried over anhydrous Na_2SO_4. The solvent was evaporated, and the residue was purified by silica gel column chromatography to afford the title compound as a pale-yellow solid (1.83 g, 45% yield).

^{1}H-NMR (270 MHz, CDCl$_3$) δ: 1.42 (9H, s), 3.88 (3H, s), 5.37 (1H, s), 6.69 (1H, d, J = 8.4 Hz), 7.79 (1H, dd, J = 8.4, 1.9 Hz), 7.99 (1H, d, J = 1.9 Hz).

(b) Methyl 3-*tert*-butyl-4-hydroxy-5-iodobenzoate

To a solution of methyl 3-*tert*-butyl-4-hydroxybenzoate (1.83 g, 8.78 mmol) in CH_2Cl_2 (24 mL) and methanol (3 mL) was added *N*-iodosuccinimide (2.08 g, 9.24 mmol) and trifluoromethanesulfonic acid (3 ml). After the mixture was stirred at room temperature for 15 min, water was added. The products were extracted with CH_2Cl_2, and the organic layer was washed with 10% aqueous sodium thiosulfate and brine, then dried over anhydrous Na_2SO_4. The solvent was evaporated to afford the title compound as a brown solid (2.77 g, 94% yield).

^{1}H-NMR (270 MHz, CDCl$_3$) δ: 1.41 (9H, s), 3.88 (3H, s), 5.92 (1H, s), 7.95 (1H, d, J = 1.9 Hz), 8.24 (1H, d, J = 1.9 Hz).

(c) Methyl 3-*tert*-butyl-5-iodo-4-methoxybenzoate

To a solution of methyl 3-*tert*-butyl-4-hydroxy-5-iodobenzoate (2.77 g, 8.29 mmol) in DMF (50 ml) were added potassium carbonate (12.0 g, 86.8 mmol) and dimethylsulfate (4.1 ml, 43.2 mmol). After the reaction mixture was stirred at room temperature overnight, water was added. The products were extracted with AcOEt, and the organic layer was washed with water and brine, then dried over anhydrous Na_2SO_4. The solvent was evaporated to afford the title compound (2.77 g, 96% yield).

^{1}H-NMR (270 MHz, CDCl$_3$) δ: 1.40 (9H, s), 3.90 (3H, s), 3.93 (3H, s), 8.01 (1H, d, J = 2.2 Hz), 8.36 (1H, d, J = 2.2 Hz).

(d) Methyl 3-*tert*-butyl-5-cyano-4-methoxybenzoate

To a solution of methyl 3-*tert*-butyl-5-iodo-4-methoxybenzoate (2.77 g, 7.96 mmol) in DMF (30 ml) was added copper cyanide (965 mg, 10.8 mmol). After the mixture was stirred at 150 °C for 2.5 h, 10% aqueous potassium carbonate was added. The products were extracted with AcOEt, and the organic layer was washed with water and brine, then dried over anhydrous Na_2SO_4. The solvent was evaporated, and the residue was purified by silica gel column chromatography to afford the title compound (1.48 g, 75% yield).

^{1}H-NMR (270 MHz, CDCl$_3$) δ: 1.39 (9H, s), 3.92 (3H, s), 4.22 (3H, s), 8.15 (1H, d, J = 2.2 Hz), 8.19 (1H, d, J = 2.2 Hz).

(e) 3-*tert*-Butyl-5-cyano-4-methoxybenzoic acid

To a solution of methyl 3-*tert*-butyl-5-cyano-4-methoxybenzoate (1.48 g, 5.98 mmol) in methanol (20 ml), THF (5 ml) and water (5 ml) was added lithium hydroxide monohydrate (753 mg, 17.9 mmol). After the mixture was stirred at room temperature for 2 h, 10% aqueous HCl was added. The products were extracted with AcOEt, and the organic layer was washed with brine, then dried over anhydrous Na_2SO_4. The solvent was evaporated to afford the title compound (1.18 g, 85% yield).

¹H-NMR (270 MHz, CDCl₃) δ: 1.41 (9H, s), 4.26 (3H, s), 8.23 (1H, d, J = 2.2 Hz), 8.25 (1H, d, J = 2.2 Hz).

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

Compound **2f** was prepared from 3-*tert*-butyl-5-cyano-4-methoxybenzoic acid in a similar way that used for compound **2d** (67% yield).

¹H-NMR (400 MHz, DMSO-*d₆*) δ: 1.36 (9H, s), 3.08 (2H, t, J = 8.2 Hz), 4.05 (2H, t, J = 8.2 Hz), 7.03 (1H, t, J = 7.3 Hz), 7.17 (1H, d, J = 7.3 Hz), 7.27 (1H, d, J = 7.3 Hz), 7.65 (1H, d, J = 2.3 Hz), 7.76 (1H, brs), 7.77 (1H, d, J = 2.3 Hz); the phenol-OH was not observed.

¹³C-NMR (101 MHz, DMSO-*d₆*) δ: 27.8, 29.1, 34.9, 50.5, 101.9, 116.5, 117.1, 123.8, 125.0, 126.8, 127.2, 130.3, 130.9, 132.8, 139.7, 142.7, 159.9, 166.5.

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)

(a) Methyl 3-cyano-5-cyclopropyl-4-methoxybenzoate

To a solution of methyl 3-cyano-4-hydroxy-5-iodobenzoate (p. 60) (1.00 g, 3.30 mmol) in 1,4-dioxane (15 mL) were added potassium carbonate (1.31 g, 9.48 mmol), cyclopropylboronic acid (325 mg, 3.78 mmol) and [1,3-bis-(2,6-diisopropylphenyl)imidazole-2-ylidene](3-chloropyridyl)palladium dichloride (108 mg, 0.158 mmol). After the mixture was stirred at 95 °C for 22 h, the insoluble materials were removed by filtration through a pad of Celite. The filtrate was concentrated, and the obtained residue was purified by silica gel column chromatography using a n-hexane/AcOEt (4:1) to afford the title compound as a pale-yellow solid (348 mg, 46% yield).

¹H-NMR (270 MHz, CDCl₃) δ: 0.74–0.80 (2H, m), 1.04–1.12 (2H, m), 2.14–2.25 (1H, m), 3.91 (3H, s), 4.15 (3H, s), 7.70 (1H, d, J = 2.1 Hz), 8.07 (1H, d, J = 2.1 Hz).

(b) 3-Cyano-5-cyclopropyl-4-methoxybenzoic acid

To a solution of methyl 3-cyano-5-cyclopropyl-4-methoxybenzoate (491 mg, 2.12 mmol) in THF (7.5 mL) and water (2.5 mL) was added lithium hydroxide monohydrate (359 mg, 8.56 mmol), and the mixture was stirred for 20 h at room temperature. After the organic solvent was evaporated, the residue was diluted with water, and the aqueous solution was washed with n-hexane and acidified with 1 M HCl. The products were extracted with AcOEt, and the organic layer was washed with brine, then dried over anhydrous Na_2SO_4. The solvent was evaporated to afford the title compound as a pale brown solid (394 mg, 86% yield).

¹H-NMR (270 MHz, DMSO-*d₆*) δ: 0.74–0.79 (2H, m), 1.03–1.10 (2H, m), 2.13–2.23 (1H, m), 4.06 (3H, s), 7.65 (1H, d, J = 2.0 Hz), 8.04 (1H, d, J = 2.0 Hz).

MS (m/z): 216 (M − H)⁻.

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

Compound **2g** was prepared from 3-cyano-5-cyclopropyl-4-methoxybenzoic acid in a similar way that used for compound **2d** (76% yield).

^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.65–0.71 (2H, m), 0.93–0.99 (2H, m), 2.07–2.14 (1H, m), 3.07 (2H, t, J = 8.2 Hz), 4.01 (2H, t, J = 8.0 Hz), 7.03 (1H, t, J = 7.3 Hz), 7.16 (1H, t, J = 7.3 Hz), 7.27 (1H, d, J = 7.3 Hz), 7.32 (1H, d, J = 1.8 Hz), 7.70 (1H, d, J = 1.8 Hz), 7.71 (1H, brs); the phenol-OH was not observed.

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 7.9, 9.7, 27.8, 50.3, 99.8, 116.5, 116.9, 123.8, 125.0, 126.8, 128.7, 129.4, 129.6, 132.0, 132.8, 142.7, 160.1, 166.3.

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)

(a) Methyl 3-chloro-5-cyano-4-hydroxybenzoate

To a solution of methyl 3-cyano-4-hydroxybenzoate (2.00 g, 11.3 mmol) in chloroform (15 mL) and methanol (5 mL) were added N-chlorosuccinimide (3.62 g, 27.1 mmol) and 4 M HCl AcOEt solution. After the mixture was stirred at room temperature for 1 h, the solvent was evaporated, and 10% aqueous methanol (40 mL) was added. The precipitated solid was collected by filtration and washed with water (10 mL) and isopropyl alcohol (4 mL) to afford the title compound as a colorless solid (1.27 g, 53% yield).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 4.16 (3H, s), 8.45 (1H, d, J = 2.0 Hz), 8.46 (1H, d, J = 2.0 Hz).

MS (m/z): 210 (M − H)$^-$, 212 (M + 2 − H)$^-$.

(b) Methyl 3-chloro-5-cyano-4-methoxybenzoate

To a solution of methyl 3-chloro-5-cyano-4-hydroxybenzoate (1.27 g, 6.00 mmol) in DMF (20 mL) were added potassium carbonate (5.00 g, 36.2 mmol) and dimethyl sulfate (1.70 mL, 17.9 mmol). After the mixture was stirred at room temperature for 18 h, the insoluble materials were removed by filtration, and water was added to the filtrate. The products were extracted with AcOEt, and the organic layer was washed with water and brine, then dried over anhydrous Na_2SO_4. The solvent was evaporated to afford the title compound as a colorless solid (1.03 g, 76% yield).

^{1}H-NMR (270 MHz, CDCl$_3$) δ: 3.94 (3H, s), 4.19 (3H, s), 8.17 (1H, d, J = 2.1 Hz), 8.25 (1H, d, J = 2.1 Hz).

(c) 3-Chloro-5-cyano-4-methoxybenzoic acid

To a solution of methyl 3-chloro-5-cyano-4-methoxybenzoate (1.02 g, 4.52 mmol) in THF (15 mL) and water (6 mL) was added lithium hydroxide monohydrate (759 mg, 18.1 mmol). After the mixture was stirred at room temperature for 90 min, the organic solvent was evaporated. Water was added, and the aqueous solution was washed with n-hexane and acidified with 1 M HCl. The products were extracted with AcOEt, and the organic layer was washed with brine and dried over anhydrous Na_2SO_4. The solvent was evaporated to afford the title compound as a colorless solid (946 mg, 99% yield).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 4.43 (3H, s), 8.55 (2H, s), 14.00 (1H, brs).

MS (m/z): 210 (M − H)$^-$, 212 (M + 2 − H)$^-$.

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

Compound 2h was prepared from 3-chloro-5-cyano-4-methoxybenzoic acid in a similar way that used for compound 2d (89% yield).

^{1}H-NMR (400 MHz, DMSO-d_6) δ: 3.08 (2H, t, J = 8.2 Hz), 4.06 (2H, t, J = 8.2 Hz), 7.05 (1H, t, J = 7.3 Hz), 7.18 (1H, t, J = 7.3 Hz), 7.29 (1H, d, J = 7.3 Hz), 7.78 (1H, brs), 7.92 (1H, d, J = 2.3 Hz), 7.94 (1H, d, J = 2.3 Hz); the phenol-OH was not observed.

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 27.8, 50.4, 101.8, 115.9, 116.6, 122.1, 124.0, 125.1, 126.9, 129.0, 131.4, 132.8, 133.8, 142.5, 157.4, 164.9.

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)

(a) Methyl 3-cyano-4-methoxy-5-trifluoromethylbenzoate

To a solution of methyl 3-cyano-5-iodo-4-methoxybenzoate (p. 60) (5.65 g, 18.6 mmol) in DMF (110 mL) were added copper iodide (679 mg, 3.57 mmol) and methyl fluorosulfonyldifluoroacetate (6.85 g, 35.7 mmol). After the mixture was stirred at 130 °C for 90 min, insoluble materials were removed by filtration through a pad of Celite. The filtrate was concentrated, and the residue was purified by silica gel column chromatography (n-hexane/AcOEt = 6:1) to afford the title compound as a colorless solid (4.17 g, 87% yield).

^{1}H-NMR (270 MHz, CDCl$_3$) δ: 3.97 (3H, s), 4.30 (3H, s), 8.45 (2H, s).

(b) 3-Cyano-4-methoxy-5-trifluoromethylbenzoic acid

To a solution of methyl 3-cyano-4-methoxy-5-trifluoromethylbenzoate (4.17 g, 16.1 mmol) in THF (60 mL) and water (20 mL) was added lithium hydroxide monohydrate (2.70 g, 64.3 mmol).   After the mixture was stirred at room temperature for 3 h, the solvent was evaporated, and the residue was acidified with 2 M HCl. The products were extracted with AcOEt, the organic layer was washed with brine and dried over anhydrous Na$_2$SO$_4$. The solvent was evaporated to afford the title compound as a colorless solid (3.92 g, 99% yield).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 4.23 (3H, s), 8.33 (1H, d, J = 2.2 Hz), 8.54 (1H, d, J = 2.2 Hz).

MS (m/z): 244 (M − H)$^-$.

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

Compound **2i** was prepared from 3-cyano-4-methoxy-5-trifluoromethylbenzoic acid in a similar way that used for compound **2d** (91% 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: 27.8, 50.4, 102.8, 115.6, 116.7, 119.1 (q, J = 30.0 Hz), 122.9 (q, J = 274 Hz), 124.1, 125.1, 126.9, 128.3, 131.3 (q, J = 4.8 Hz), 132.9, 136.9, 142.5, 159.1, 164.9.

HRMS (ESI) m/z: calcd for $C_{17}H_{10}F_3N_2O_2$ (M − H)$^-$ 331.0699, found 331.0705.

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

(a) 3-Cyano-5-cyclopropyl-4-methoxybenzoyl chloride (**9a**)

To a solution of 3-cyano-5-cyclopropyl-4-methoxybenzoic acid (p. 64) (200 mg, 0.921 mmol) in toluene (2 mL) were added DMF (1 droplet) and thionyl chloride (0.10 mL, 1.39 mmol). After the mixture was stirred at 60 °C for 16 h, the solvent was evaporated. The obtained residue was dissolved with toluene (2mL), and the solvent was evaporated. The obtained residue was used for the step (c).

(b) 1,2-Dihydro-3*H*-1,3-benzothiazole (**11**)

37% Formalin (0.23 mL) was diluted with water (3.5 mL), and diisopropyl ether (3.5 mL) and 2-aminobenzenethiol (346 mg, 2.76 mmol) were added. After the mixture was stirred at room temperature for 30 min, the organic layer was separated. The aqueous phase was extracted with diisopropyl ether. The combined organic layers were washed with brine and dried over anhydrous Na_2SO_4. The solvent was evaporated, and the obtained residue was used for the next step.

(c) 3-(3-Cyano-5-cyclopropyl-4-methoxybenzoyl)-1,2-dihydro-3*H*-1,3-benzothiazole (**12a**)

To a solution of 1,2-dihydro-3*H*-1,3-benzothiazole in chloroform (3 mL) were added triethylamine (0.38 mL, 2.74 mmol) and 3-cyano-5-cyclopropyl-4-methoxybenzoyl chloride. After the mixture was stirred at room temperature for 2 h, the solvent was evaporated. Water was added, and the products were extracted with AcOEt. The combined extracts were washed with 1 M HCl, 1 M aqueous NaOH and brine, then dried over anhydrous Na_2SO_4. The solvent was evaporated, and the obtained residue was used for the next step.

(d) 3-(3-Cyano-5-cyclopropyl-4-methoxybenzoyl)-1,1-dioxo-1,2-dihydro-3*H*-1,3-benzothiazole (**13a**)

To a solution of 3-(3-cyano-5-cyclopropyl-4-methoxybenzoyl)-1,2-dihydro-3*H*-1,3-benzothiazole in chloroform (5 mL) was added 70% 3-chloroperbenzoic acid (422 mg, 1.71 mmol). After the mixture was stirred at room temperature for 16 h, 10% sodium thiosulfate was added. The solvent was evaporated, then 1 M aqueous NaOH was added. The products were extracted with AcOEt, and the organic layer was washed with 1 M aqueous NaOH and brine, and then dried over anhydrous Na_2SO_4. The solvent was evaporated, and the obtained residue was crystallized from n-hexane/AcOEt/MeOH to afford the title compound as a colorless solid (147 mg, 43% yield).

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

To a solution of 3-(3-cyano-5-cyclopropyl-4-methoxybenzoyl)-1,1-dioxo-1,2-dihydro-3*H*-1,3-benzothiazole (142 mg, 0.385 mmol) in DMF (2 mL) was added lithium chloride (163 mg, 3.85 mmol). After the mixture was stirred at 100 °C for 23 h, 1 M HCl was added. The products were extracted with AcOEt, the combined extracts were washed with 1 M HCl and brine, and dried over anhydrous Na_2SO_4. The solvent was evaporated, and the obtained residue was crystallized from n-hexane/AcOEt to afford the title compound as a colorless solid (115 mg, 81% yield).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 0.670.73 (2H, m), 0.93–1.00 (2H, m), 2.05–2.15 (1H, m), 5.26 (2H, s), 7.38 (1H, d, J = 2.1 Hz), 7.42 (1H, dd, J = 7.8, 7.8 Hz), 7.73 (1H, dd, J = 7.8, 8.4 Hz), 7.79 (1H, d, J = 2.1 Hz), 7.87 (1H, d, J = 7.8 Hz), 7.97 (1H, d, J = 8.4 Hz); the phenol-OH was not observed.

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 7.6, 9.6,. 67.0, 100.1, 116.4, 120.3, 121.0, 125.3, 125.5, 128.8, 130.0, 130.6, 132.3, 134.2, 139.4, 161.5,166.3.

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 (14)

Compound **14** was prepared from 3-cyano-4-methoxy-5-trifluoromethylbenzoic acid (p. 66) in a similar way that used for compound **7** (18% over all yield).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 5.37 (2H, s), 7.44 (1H, dd, J = 7.8, 7.8 Hz), 7.77 (1H, ddd, J = 1.3, 7.8, 7.9 Hz), 7.91 (1H, dd, J = 1.3, 7.8 Hz), 8.09 (1H, d, J = 7.9 Hz), 8.10 (1H, d, J = 2.1 Hz), 8.27 (1H, d. J = 2.1 Hz); the phenol-OH was not observed.

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 66.9, 103.0, 115.7, 119.3 (q, J = 31.0 Hz), 120.6, 121.4, 123.1 (q, J = 278 Hz), 124.7, 126.1, 128.8, 132.3, 134.6, 138.0, 139.4, 161.0, 165.4.

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 (15)

3-(3-Cyano-4-methoxy-5-trifluoromethylbenzoyl)-1,2-dihydro-3*H*-1,3-benzothiazole (**12b**) was prepared from 3-cyano-4-methoxy-5-trifluoromethylbenzoic acid (p. 66) in a similar way that used for compound **12a**. The title compound was prepared from compound **12b** in a similar way that used for compound **7** (56% yield).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 5.38 (2H, s), 7.04–7.14 (2H, m), 7.32–7.38 (1H, m), 7.55 (1H, br), 8.05 (1H, d, J = 2.1 Hz), 8.22 (1H, d, J = 2.1 Hz); the phenol-OH was not observed.

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 53.7, 102.9, 115.8, 119.1, 119.2 (q, J = 30.0 Hz), 122.85, 122.94 (q, J = 275 Hz), 124.9, 125.5, 125.8, 131.1, 131.8 (q, J = 4.8Hz), 137.5, 138.7, 160.6, 165.6.

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 (16)

Compound **16** was prepared from 3-chloro-5-cyano-4-methoxybenzoic acid (p. 65) in a similar way that used for compound **7** (30% over all yield).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 5.32 (2H, s), 7.44 (1H, dd, J = 8.4, 7.3 Hz), 7.75 (1H, ddd, J = 8.6, 7.3, 1.4 Hz), 7.88 (1H, dd, J = 8.4, 1.4 Hz), 7.99 (1H, d, J = 2.2 Hz), 8.00 (1H, d. J = 2.2 Hz), 8.06 (1H, d. J = 8.6 Hz); 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-(3,5-Dichloro-4-hydroxybenzoyl)-1,1-dioxo-1,2-dihydro-3*H*-1,3-benzothiazole (17, Dotinurad)

(a) 1,2-Dihydro-3*H*-1,3-benzothiazole (**11**)

37% Formalin (5.2 mL) was diluted with water (80 mL), and diisopropyl ether (80 mL) and 2-aminobenzenethiol (7.84 g, 62.6 mmol) were added. After the mixture was stirred at room temperature for 30 minutes, the organic layer was separated, and the aqueous phase was extracted with diisopropyl ether. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4. The solvent was evaporated, and the obtained residue was used for the next step (c).

(b) 3,5-Dichloro-4-methoxybenzoyl chloride (**9d**)

To a solution of 3,5-dichloro-4-methoxybenzoic acid (8.81 g, 39.9 mmol) in toluene (170 mL) were added DMF (5 droplets) and thionyl chloride (6.0 mL, 83.2 mmol). After the mixture was stirred at 60 °C for 16 h, the solvent was evaporated. The residue was dissolved with toluene, and the solution was concentrated. The obtained residue was used for the next step.

(c) 3-(3,5-Dichloro-4-methoxybenzoyl)-1,2-dihydro-3*H*-1,3-benzothiazole (**12d**)

To a solution of 1,2-dihydro-3*H*-1,3-benzothiazole (a) in chloroform (50 mL) were added triethylamine (17.4 mL, 126 mmol) and 3,5-dichloro-4-methoxybenzoyl chloride (b). After the mixture was stirred at room temperature for 1 h, the solvent was concentrated. The obtained residue was diluted with AcOEt, and the solution was washed with 1 M HCl, 1 M aqueous NaOH and brine, and then dried over anhydrous Na_2SO_4. The solvent was evaporated, and the obtained residue was used for the next step.

^{1}H-NMR (400 MHz, DMSO-d_6) δ: 3.90 (3H, s) 5.33 (2H, s), 7.06-7.14 (2H, m), 7.35 (1H, dd, J = 7.6, 2.4 Hz), 7.63 (1H, brs), 7.78 (2H, d. J = 8.4 Hz).

(d) 3-(3,5-Dichloro-4-methoxybenzoyl)-1,1-dioxo-1,2-dihydro-3*H*-1,3-benzothiazole (**13d**)

To a solution of 3-(3,5-dichloro-4-methoxybenzoyl)-1,2-dihydro-3*H*-1,3-benzothiazole in chloroform (230 mL) was added 70% 3-chloroperbenzoic acid (43.25 g, 175 mmol) at 0 °C.   After the mixture was stirred at room temperature for 20 h, the reaction was quenched with 10% sodium thiosulfate. The solvent was removed under reduced pressure, and the residue was dissolved with AcOEt. The solution was washed with 1 M aqueous NaOH and brine, then dried over anhydrous Na_2SO_4. The solvent was evaporated to afford the title compound as a colorless solid (13.25 g, 57% yield from the step (a)).

^{1}H-NMR (400 MHz, DMSO-d_6) δ: 3.91 (3H, s), 5.33 (2H, s), 7.47 (1H, dd, J = 7.2, 7.6 Hz), 7.79 (1H, dd, J = 7.2, 7.2 Hz), 7.86 (2H, s), 7.93 (1H, d, J = 7.2 Hz), 8.15 (1H, d, J = 7.6 Hz).

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

To a solution of 3-(3,5-dichloro-4-methoxybenzoyl)-1,1-dioxo-1,2-dihydro-3*H*-1,3-benzothiazole (1.00 g, 2.69 mmol) in DMF (5 mL) was added lithium chloride (570 mg, 13.4 mmol). After the mixture was stirred at 130 °C for 2 h, 1 M HCl was added. The products were extracted with AcOEt, and the organic layer was washed with 1 M HCl and brine, then dried over anhydrous Na_2SO_4. The solvent was evaporated, and the obtained residue was crystallized from ethanol to afford the title compound as a colorless solid (749 mg, 78% yield).

^{1}H-NMR (400 MHz, DMSO-d_6) δ: 5.36 (2H, s), 7.44 (1H, t, J = 8.0 Hz), 7.75 (2H, s), 7.76 (1H, td, J = 1.2, 8.0 Hz), 7.91 (1H, dd, J = 1.2, 8.0 Hz), 8.04 (1H, d, J = 8.0 Hz), 11.05 (1H, brs).

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 66.98, 120.57, 121.33, 122.22, 125.97, 126.59, 128.66, 128.97, 134.51, 139.34, 152.20, 165.58.

MS (ESI): 356 (M − H)$^-$, 358 (M + 2 − H)$^-$.

HRMS (ESI): m/z calcd for C$_{14}$H$_8$Cl$_2$NO$_-$S (M − H)$^-$ 355.9556, found 355.9564.

3-(3-Cyano-4-hydroxy-5-isopropylbenzoyl)-1,1-dioxo-1,2-dihydro-3H-1,3-benzothiazole (18)

Compound **18** was prepared from 3-cyano-4-methoxy-5-isopropybenzoic acid in a similar way that used for compound **7** (66% over all yield).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 1.18 (6H, d, J = 6.8 Hz), 3.35 (1H, sevent, J = 6.8 Hz), 5.34 (2H, s), 7.43 (1H, ddd, J = 0.8, 7.8, 7.8 Hz), 7.75 (1H, dd, J = 7.8, 8.4 Hz), 7.76 (1H, d, J = 2.3Hz), 7.87 (1H, d, J = 2.3 Hz), 7.90 (1H, dd, J = 7.8, 0.8 Hz), 8.00 (1H, d, J = 8.4 Hz); the phenol-OH was not observed.

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 22.2, 26.1, 67.1, 100.7, 116.7, 120.6, 121.3, 125.7, 125.8, 128.9, 131.2, 134.4, 137.4, 139.5, 159.8, 166.5.

MS (ESI) m/z: 355 (M − H)$^-$.

HRMS (ESI): m/z calcd for C$_{18}$H$_{15}$N$_2$O$_-$S (M − H)$^-$ 355.0758, found 355.0765.

3-(3-Cyano-5-ethyl-4-hydroxybenzoyl)-1,1-dioxo-1,2-dihydro-3H-1,3-benzothiazole (19)

Compound **19** was prepared from 3-cyano-5-ethyl-4-methoxybenzoic acid in a similar way that used for compound **7** (66% over all yield).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 1.15 (3H, t, J = 7.6 Hz), 2.68 (2H, q, J = 7.6 Hz), 5.34 (2H, s), 7.43 (1H, dd, J = 7.6, 7.6 Hz), 7.74(1H, d, J = 2.2 Hz), 7.75 (1H, dd, J = 7.6, 8.4Hz), 7.88 (1H, d, J = 2.2 Hz), 7.90 (1H, d, J = 7.6 Hz), 8.00 (1H, d, J = 8.4 Hz), 11.01 (1H, brs).

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 13.7, 22.5, 67.2, 100.4, 116.8, 120.7, 121.3, 125.3, 125.9, 129.0, 131.5, 132.9, 134.0, 134.5, 139.6, 160.8, 166.5.

MS (m/z): 341 (M − H)$^-$.

HRMS (ESI): m/z calcd for C$_{17}$H$_{13}$N$_2$O$_-$S (M − H)$^-$ 341.0601, found 341.0608.

3-(3-Cyano-5-ethynyl-4-hydroxybenzoyl)-1,1-dioxo-1,2-dihydro-3H-1,3-benzothiazole (20)

Compound **20** was prepared from 3-cyano-5-ethynyl-4-methoxybenzoic acid in a similar way that used for compound **7** (8% over all yield).

^{1}H-NMR (270 MHz , DMSO-d_6) δ: 4.58 (1H, s), 5.35 (2H, s), 7.44 (1H, dd, J = 7.6, 7.6 Hz), 7.76 (1H, dd, J = 7.6, 8.4 Hz), 7.91 (1H, d, J = 8.4 Hz), 7.93 (1H, d. J = 2.4 Hz), 8.04 (1H, d, J = 2.4 Hz), 8.05 (1H, d, J = 7.6 Hz); the phenol-OH was not observed.

MS (m/z): 337 (M − H)$^-$.

HRMS (ESI): m/z calcd for C$_{17}$H$_9$N$_2$O$_-$S (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 (21)

3-(3-Cyano-4-hydroxy-5-iodobenzoyl)-1,1-dioxo-1,2-dihydro-3*H*-1,3-benzothiazole (**21'**) was prepared from 3-cyano-5-iodo-4-methoxybenzoic acid (p. 61) in a similar way that used for compound **7** (27% over all yield).

To a solution of compound **21'** (334 mg, 0.927 mmol) in DMF (3.5 mL) were added 2,2'-bipyridine (11 mg, 0.070 mmol), zinc powder (95 mg, 1.45 mmol), nickel (II) bromide (16 mg, 0.073 mmol) and dimethyl disulfide (0.04 mL, 0.44 mmol). After the mixture was stirred at 80 °C for 1 h, the insoluble matters were removed by filtration through a pad of Celite. The filtrate was diluted with AcOEt, and the solution was washed with 1 M HCl and brine, then dried over anhydrous Na_2SO_4. The solvent was evaporated, and the obtained residue was dissolved in DMF (3 mL). Potassium carbonate (298 mg, 2.16 mmol) and dimethyl sulfate (0.13 mL 1.37 mmol) was added. After the mixture was stirred at room temperature for 1 h, water was added. The products were extracted with AcOEt, and the organic layer was washed with water and brine, then dried over anhydrous Na_2SO_4. The solvent was evaporated, and the obtained residue was purified by silica gel column chromatography (n-hexane/AcOEt).

To the obtained products in DMF (1 mL) was added lithium chloride (64 mg, 1.51 mmol). After stirring at 100 °C for 1.5 h, the mixture was diluted with AcOEt. The solution was washed with 1 M HCl and brine, then dried over anhydrous Na_2SO_4. The solvent was concentrated under reduced pressure, and the obtained residue was crystallized from n-hexane/chloroform to afford the title compound as a yellow solid (95 mg, 29% yield).

^{1}H-NMR (270 MHz, DMSO-*d₆*) δ: 2.46 (3H, s), 5.34 (2H, s), 7.45 (1H, dd, *J* = 7.6, 7.3 Hz), 7.68 (1H, d, *J* = 2.2 Hz), 7.77 (1H, dd, *J* = 7.6, 8.4 Hz), 7.82(1H, d, *J* = 2.2 Hz), 7.91 (1H, d, *J* = 7.3 Hz), 8.08 (1H, d, *J* = 8.4 Hz); 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 (22)

Compound **22** was prepared from 3-chloro-4-methoxy-5-trifluoromethybenzoic acid in a similar way that used for compound **7** (14% over all yield).

^{1}H-NMR (270 MHz, DMSO-*d₆*) δ: 5.36 (2H, s) 7.44 (1H, ddd, *J* = 0.8, 7.8, 7.8 Hz), 7.77 (1H, ddd, *J* = 1.3, 7.8, 8.2 Hz), 7.86 (1H, d, *J* = 2.1 Hz), 7.91 (1H, dd, *J* = 0.8, 7.8 Hz), 8.06 (1H, d, *J* = 2.1 Hz), 8.07 (1H, d, *J* = 8.2 Hz); 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 (23)

Compound **23** was prepared from 3-chloro-5-fluoro-4-methoxybenzoic acid in a similar way that used for compound **7** (61% over all yield).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 5.35 (2H, s), 7.43 (1H, dd, J = 7.4, 7.4 Hz), 7.59 (1H, dd, J = 1.8, 11.1 Hz), 7.61 (1H, s), 7.76 (1H, ddd, J = 1.2, 7.4, 8.4 Hz), 7.90 (1H, d, J = 7.4 Hz), 8.02 (1H, d, J = 8.4 Hz), 11.35 (1H, brs).

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 67.1, 115.1 (d, J = 21.3Hz), 120.6, 121.3, 122.3 (d, J = 3.8 Hz), 124.6, 125.8 (d, J = 17.5 Hz), 125.9, 129.0, 134.5, 139.4, 145.4 (d, J = 15.6 Hz), 151.3 (d, J = 245 Hz), 165.8.

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-3H-1,3-benzothiazole (24)

3-(3-Chloro-4-hydroxy-5-iodobenzoyl)-1,1-dioxo-1,2-dihydro-3H-1,3-benzothiazole (**24'**) was prepared from 3-chloro-5-iodo-4-methoxybenzoic acid in a similar way that used for compound **7** (19% over all yield). The title compound was prepared from compound **24'** in a similar way that used for compound **21** (23% yield).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 2.44 (3H, s), 5.34 (2H, s), 7.38 (1H, s), 7.43 (1H, dd, J = 7.6, 7.8 Hz), 7.54 (1H, s), 7.76 (1H, dd, J = 7.8, 8.6 Hz), 7.90 (1H, d, J = 7.6 Hz), 8.02 (1H, d, J = 8.6 Hz), 10.54 (1H, s).

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 14.1, 67.3, 120.1, 120.7, 121.3, 124.0, 124.1, 125.9, 126.8, 129.0, 129.7, 134.5, 139.6, 151.9, 166.6.

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-3H-1,3-benzothiazole (25)

3-(4-Benzyloxy-3-methoxy-5-trifluoromethylbenzoyl)-1,1-dioxo-1,2-dihydro-3H-1,3-benzothiazole (**13l**) was prepared from 4-benzyloxy-3-methoxy-5-trifluoromethylbenzoic acid in a similar way that used for compound **13a** (65% over all yield).

To a solution of compound **13l** (574 mg) in THF (6 mL) was added 5% palladium on carbon (310 mg), and the mixture was stirred at room temperature for 22 h under a hydrogen atmosphere. The mixture was filtered through a pad of Celite, and the filtrate was concentrated under reduced pressure. The obtained residue was crystallized from n-hexane/chloroform to afford the title compound as a colorless solid (353 mg, 76% yield).

^{1}H-NMR (DMSO-d_6, 270 MHz) δ: 3.93 (3H, s), 5.35 (2H, s), 7.43 (1H, dd, J = 7.3, 8.1 Hz), 7.47 (1H, s), 7.54 (1H, s), 7.76 (1H, dd, J = 7.3, 7.3 Hz), 7.90 (1H, d, J = 7.3 Hz), 8.02 (1H, d, J = 8.1 Hz), 10.68 (1H, s).

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 56.6, 67.3, 70.4, 114.8, 115.7 (d, J = 30.0 Hz), 118.9, 120.7, 121.3, 123.6 (q, J = 274 Hz), 125.8, 129.0, 134.4, 139.7, 148.4, 149.3, 167.0

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 (26)

      3-(4-Benzyloxy-3-chloro-5-methoxybenzoyl)-1,1-dioxo-1,2-dihydro-3*H*-1,3-benzothiazole (**13m**)   was prepared from 4-benzyloxy-3-chloro-5-methoxybenzoic acid in a similar way that used for compound **13a** (64% over all yield).   The title compound was prepared from compound **13m** in a similar way that used for compound **25** (45% yield).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 3.88 (3H, s), 5.34 (2H, s), 7.27 (1H, s), 7.35 (1H, s), 7.43 (1H, dd, J = 7.6, 7.6 Hz), 7.75 (1H, dd, J = 7.6, 8.4 Hz), 7.90 (1H, d, J = 7.6 Hz), 7.99 (1H, d, J = 8.4 Hz); the phenol-OH was not observed.

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 56.5, 67.4, 110.5, 119.9, 120.7, 121.3, 122.2, 124.5, 125.8, 129.0, 134.4, 139.7, 146.7, 148.5, 166.9.

MS (m/z): 352 (M − H)$^-$.

HRMS (ESI): m/z calcd for C$_{15}$H$_{11}$ClNO$_5$S (M − H)$^-$ 352.0051, found 352.0053.

Cyclopent-1-en-1-yl(3,5-dichloro-4-hydroxyphenyl)methanone (30a)

(a) 2,6-Dichlorophenyl cyclopent-1-ene-1-carboxylate

      To a solution of cyclopent-1-ene-1-carboxylic acid (1.00 g, 8.92 mmol) in CH$_2$Cl$_2$ (30 mL) were added thionyl chloride (1.3 mL, 17.8 mmol) and catalytic amount of DMF. After the mixture was refluxed for 30 min, the solution was concentrated under reduced pressure to afford a crude acid chloride.

      To a solution of 2,6-dichlorophenol (1.45 g, 8.92 mmol) and triethylamine (1.94 mL, 17.8 mmol) in CH$_2$Cl$_2$ (30 mL) was added the acid chloride in CH$_2$Cl$_2$ (10 mL) under ice cooling. After the mixture was stirred for 1 h, the solution was diluted with chloroform. The solution was washed with 10% aqueous potassium carbonate solution and bine, then dried over anhydrous Na$_2$SO$_4$. The solvent was evaporated, and the obtained residue was purified by silica gel column chromatography (n-hexane/AcOEt = 3:1) to afford the title compound as a colorless oil (2.16 g, 94% yield).

(b) Cyclopent-1-en-1-yl(3,5-dichloro-4-hydroxyphenyl)methanone (**30a**)

      2,6-Dichlorophenyl cyclopent-1-ene-1-carboxylate (1.00 g, 3.89 mmol) was added to trifluoromethanesulfonic acid (5 mL) at 0 °C. After stirring at room temperature overnight, the reaction mixture was poured into ice-water. The products were extracted with chloroform, and the organic layer was washed with brine and dried over anhydrous Na$_2$SO$_4$. The solvent was evaporated under reduced pressure. The obtained residue was purified by silica gel column chromatography (n-hexane/AcOEt = 3:1) to afford the title compound as a colorless solid (542mg, 54% yield).

^{1}H-NMR (400 MHz, CDCl$_3$) δ: 2.02 (2H, ddd, J = 7.3, 7.8, 15.1 Hz), 2.60–2.77 (4H, m), 6.22 (1H, s), 6.52–6.56 (1H, m), 7.73 (2H, s). ^{13}C-NMR (101 MHz, DMSO-d_6) δ: 22.2, 31.9, 34.1, 122.0, 129.2, 130.6, 142.7, 146.8, 152.7, 189.5.

MS (m/z): 255 (M − H)$^-$, 257 (M + 2 − H)$^-$.

Cyclopent-1-en-1-yl(3,5-dibromo-4-hydroxyphenyl)methanone (30b)

(a) 2,6-Dibromophenyl cyclopent-1-ene-1-carboxylate

To a solution of cyclopent-1-ene-1-carboxylic acid (1.00 g, 8.92 mmol) in CH_2Cl_2 (30 mL) were added thionyl chloride (1.3 mL, 17.8 mmol) and catalytic amount of DMF. After the mixture was refluxed for 30 min, the solution was concentrated under reduced pressure to afford a crude acid chloride.

To a mixture of 2,5-dibromophenol (2.25g, 8.92 mmol) and triethylamine (1.94 mL, 17.8 mmol) in CH_2Cl_2 (30 mL) were added the obtained acid chloride in CH_2Cl_2 (10 mL) under ice cooling. After stirring for 1 h, the mixture was diluted with chloroform. The solution was washed with 10% potassium carbonate and bine, and dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure, and the obtained solid was washed with n-hexane/diisopropyl ether (1:1) and dried under reduced pressure at 60 °C to afford the compound as a colorless solid (2.35 g, 76% yield). The obtained compound was used for the next step (b).

(b) Cyclopent-1-en-1-yl(3,5-dibromo-4-hydroxyphenyl)methanone (**30b**)

To trifluoromethanesulfonic acid (5 mL) was added 2,6-dibromophenyl cyclopent-1-ene-1-carboxylate (1.00 g, 2.89 mmol) at 0 °C. After stirring at room temperature overnight, the reaction mixture was poured into ice-water. The products were extracted with chloroform, and the organic layer was washed with brine, dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure, and the obtained residual solid was washed with n-hexane/diisopropyl ether (1:1) and dried under reduced pressure at 60 °C to afford the title compound as a colorless solid (745 mg, 75% yield).

^{1}H-NMR (400 MHz, CDCl$_3$) δ: 2.01 (2H, ddd, J = 7.6, 7.8, 15.1 Hz), 2.60–2.77 (4H, m), 6.22 (1H, s), 6.52–6.56 (1H, m), 7.90 (2H, s).

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 22.2, 31.9, 34.1, 111.5, 131.9, 132.9, 142.7, 146.8, 154.4, 189.3.

MS (m/z): 343 (M − H)$^-$ 345 (M + 2 − H)$^-$, 347(M + 4 − H)$^-$.

3-Ethyl-2-hydroxy-5-(pyrrole-1-carbonyl)benzonitrile (32)

To a solution of pyrrole (67.0 mg, 1.00 mmol) in THF (2 mL) was added sodium hydride 60% in oil (50 mg, 1.25 mmol) and 3-cyano-5-ethyl-4-methoxybenzoyl chloride (223 mg, 1.00 mmol) at 0 °C. After the mixture was stirred at room temperature overnight, ice-water and 1 M HCl were added. The products were extracted with AcOEt, the organic layer was washed with water and brine, and dried over magnesium sulfate. The solvent was evaporated, and the obtained residue was purified by silica gel column chromatography (n-hexane/AcOEt = 5:1) to afford a solid (55 mg, 22%).

To a solution of the obtained compound in DMF (1.5 mL) was added lithium chloride (55mg, 1.30 mmol). After the mixture was stirred at 110 °C for 5 h, ice-water and 1 M HCl was added. The products were extracted with n-hexane/AcOEt (1:1), and the organic layer was washed with 1 M HCl and brine, then dried over magnesium sulfate. The solvent was evaporated, and the residue was washed with diisopropyl ether to afford the title compound as a brown solid (30 mg, 59% yield).

^{1}H-NMR (270 MHz, CDCl$_3$) δ: 1.29 (3H, t, J = 7.5 Hz), 2.75 (2H, q, J = 7.5 Hz), 6.39 (2H, dd, J = 2.2, 2.4 Hz), 7.24 (2H, dd, J = 2.2, 2.4 Hz), 7.80 (1H, d, J = 1.9 Hz), 7.82 (1H, d, J = 1.9 Hz).

MS (m/z): 239 (M − H)$^-$.

HRMS (ESI) m/z: calcd for C$_{14}$H$_{11}$N$_2$O$_2$ (M − H)$^-$ 239.0826, found 239.0823.

5-(2,5-Dihydro-1*H*-pyrrole-1-carbonyl)-3-ethyl-2-hydroxybenzonitrile (34a)

(a) 5-(2,5-Dihydro-1*H*-pyrrole-1-carbonyl)-3-ethyl-2-methoxybenzonitrile

To a solution of 3-cyano-5-ethyl-4-methoxybenzoic acid (206 mg, 1.00 mmol) in CH$_2$Cl$_2$ (10 mL) were added EDC·HCl (230 mg 1.20 mmol) and 2,5-dihydro-1*H*-pyrrole (70 mg, 1.01 mmol). After the mixture was stirred at room temperature for 1 h, the solvent was evaporated, and the residue was dissolved with AcOEt. The solution was washed with 1 M HCl, 1 M aqueous NaOH and brine, and dried over anhydrous Na$_2$SO$_4$. The solvent was evaporated, and the obtained residue was used for the next step.

(b) 5-(2,5-Dihydro-1*H*-pyrrole-1-carbonyl)-3-ethyl-2-hydroxybenzonitrile (**34a**)

To a solution of 5-(2,5-dihydro-1*H*-pyrrole-1-carbonyl)-3-ethyl-2-methoxybenzonitrile in DMF (5 mL) was added lithium chloride (212 mg, 5.00 mmol). After the mixture was stirred at 150 °C for 2 h, the solvent was evaporated, and 1 M HCl was added. The products were extracted with AcOEt, the organic layer was washed with 1 M HCl and brine, dried over anhydrous Na$_2$SO$_4$. The solvent was evaporated to afford the title compound as a pale brown solid (178 mg, 73% yield over two steps).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 1.33 (3H, t, J = 7.4 Hz), 2.66 (2H, q, J = 7.4 Hz), 4.26 (4H, s), 5.84 (1H, d, J = 6.5 Hz), 5.94 (1H, d, J = 6.5 Hz), 7.63 (1H, d, J = 2.2 Hz), 7.72 (1H, d, J = 2.2 Hz), 10.65 (1H, brs).

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 13.94, 22.50, 53.52, 55.25, 99.95, 116.89, 125.35, 126.14, 128.53, 129.80, 132.29, 133.35, 158.61, 166.74.

MS (m/z): 241 (M − H)$^-$.

(3,5-Dibromo-4-hydroxyphenyl)(2,5-dihydro-1*H*-pyrrol-1-yl)methanone (34b)

(a) [3,5-Dibromo-4-(methoxymethoxy)phenyl](2,5-dihydro-1*H*-pyrrol-1-yl)methanone

To a solution 3,5-dibromo-4-(methoxymethoxy)benzoic acid (340 mg, 1.00 mmol) in CH$_2$Cl$_2$ (10 mL) were added EDC·HCl (230 mg, 1.20 mmol) and 2,5-dihydro-1*H*-pyrrole (70 mg, 1.01 mmol). After the mixture was stirred at room temperature for 1 h, the solvent was evaporated, and the residue was dissolved with AcOEt. The solution was washed with 1 M HCl, 1 M aqueous NaOH and brine, and dried over anhydrous Na$_2$SO$_4$. The solvent was evaporated, and the obtained residue was used for the next step.

(b) (3,5-Dibromo-4-hydroxyphenyl)(2,5-dihydro-1*H*-pyrrol-1-yl)methanone (**34b**)

To a solution of 4 M HCl in AcOEt (6 mL) was added (3,5-dibromo-4-(methoxymethoxy)phenyl)(2,5-dihydro-1*H*-pyrrol-1-yl)methanone (a), and the mixture was stirred at room temperature for 1 h. The solvent was evaporated, and the residue was purified by silica gel column chromatography (n-hexane/AcOEt = 1:1) to afford the title compound as a pale brown solid (235 mg, 68% yield over two steps).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 4.26 (4H, s), 5.84 (1H, d, J = 5.9 Hz), 5.93 (1H, d, J = 5.9 Hz), 7.76 (2H, s).

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 53.6, 55.3, 111.4, 125.3, 126.1, 130.4, 131.3, 152.3, 165.5.

MS (m/z): 344 (M − H)$^-$, 346 (M + 2 − H)$^-$, 348 (M + 4 − H)$^-$.

3-*tert*-Butyl-2-hydroxy-5-(pyrrolidine-1-carbonyl)benzonitrile (36a)

(a) 3-*tert*-Butyl-2-methoxy-5-(pyrrolidine-1-carbonyl)benzonitrile

To a solution of 3-*tert*-butyl-5-cyano-4-methoxybenzoic acid (p. 63) (500 mg, 2.14 mmol) in DMF (15 mL) were added pyrrolidine (0.213 ml, 2.58 mmol) and EDC·HCl (822 mg, 4.29 mmol). After the mixture was stirred at room temperature overnight, 10% aqueous potassium carbonate solution was added. The products were extracted with AcOEt, the organic layer was washed with water and brine, and dried over anhydrous Na$_2$SO$_4$. The solvent was evaporated, and the residue was purified by silica gel column chromatography to afford the title compound as a colorless oil (340 mg, 56% yield).

(b) 3-*tert*-Butyl-2-hydroxy-5-(pyrrolidine-1-carbonyl)benzonitrile (**36a**)

To a solution of 3-*tert*-butyl-2-methoxy-5-(pyrrolidine-1-carbonyl)benzonitrile (610 mg, 2.13 mmol) in DMF (20 mL) was added lithium chloride (903 mg, 21.3 mmol). After the mixture was stirred at 120 °C overnight, 10% potassium carbonate aqueous solution was added. The aqueous solution was washed with AcOEt and acidified with 1 M HCl. The products were extracted with AcOEt, and the organic layer was washed with brine and dried over anhydrous Na$_2$SO$_4$. The solvent was evaporated, and the obtained residue was washed with diethyl ether to afford the title compound as a colorless solid (354 mg, 61% yield).

^{1}H-NMR (270 MHz, CDCl$_3$) δ: 1.42 (9H, s), 1.87–2.02 (4H, m), 3.45 (2H, t, J = 6.5 Hz), 3.64 (2H, t, J = 6.5 Hz), 6.78 (1H, s), 7.47 (1H, d, J = 2.2 Hz), 7.70 (1H, d, J = 2.2 Hz).

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 23.9, 26.1, 29.1, 34.9, 46.3, 49.1, 101.8, 117.2, 128.8, 130.2, 131.0, 139.4, 159.5, 166.6.

MS (m/z): 271 (M − H)$^-$.

HRMS (ESI) m/z: calcd for C$_{16}$H$_{19}$N$_2$O$_2$ 271.1452 (M − H)$^-$, found 271.1453.

2-Hydroxy-5-(pyrrolidine-1-carbonyl)-3-trifluoromethylbenzonitrile (36b)

(a) 2-Methoxy-5-(pyrrolidine-1-carbonyl)-3-trifluoromethylbenzonitrile

To a solution of 3-cyano-4-methoxy-5-trifluoromethylbenzoic acid (p. 66) (300 mg, 1.22 mmol) in CH$_2$Cl$_2$ (10 mL) were added EDC·HCl (280 mg, 1.46 mmol) and pyrrolidine (97 mg, 1.36 mmol). After the mixture was stirred at room temperature for 2 h, the solvent was evaporated, and 1 M HCl was added. The products were extracted with AcOEt, and the organic layer was washed with 1 M aqueous NaOH and brine, and dried over anhydrous Na$_2$SO$_4$. The solvent was evaporated, and the obtained residue was purified by silica gel column chromatography (n-hexane/AcOEt = 1:1) to afford the title compound as a colorless oil.

(b) 2-Hydroxy-5-(pyrrolidine-1-carbonyl)-3-trifluoromethylbenzonitrile (**36b**)

To a solution of 2-methoxy-5-(pyrrolidine-1-carbonyl)-3-trifluoromethylbenzonitrile in DMF (5 mL) was added lithium chloride (119 mg, 2.81 mmol). After the mixture was stirred at room temperature for 35 min, the solvent was evaporated. 1 M HCl was added, and the products were extracted with AcOEt. The organic layer was washed with 1 M HCl and brine, and dried over anhydrous Na_2SO_4. The solvent was evaporated to afford the title compound as a colorless solid (206 mg, 59% yield over two steps).

^{1}H-NMR (270 MHz, CDCl$_3$) δ: 1.88–2.07 (4H, m), 3.45 (2H, t, J = 6.3 Hz), 3.68 (2H, t, J = 6.7 Hz), 7.77 (1H, d, J = 2.1 Hz), 7.92 (1H, d, J = 2.1 Hz).

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 23.9, 26.0, 46.3, 48.8, 102.6, 115.6, 118.9 (J = 30.8 Hz), 122.9 (J = 272.6 Hz), 128.60, 131.9 (J = 4.8 Hz), 136.7, 158.7, 165.0.

MS (m/z): 283 (M − H)$^-$.

HRMS (ESI) m/z: calcd for $C_{13}H_{10}F_3N_2O_2$(M − H)$^-$ 283.0699, found 283.0700.

5-(2,5-Dihydro-1*H*-pyrrole-1-carbonyl)-2-hydroxy-3-iodobenzonitrile (34c)

(a) 5-(2,5-Dihydro-1*H*-pyrrole-1-carbonyl)-3-iodo-2-methoxybenzonitrile

To a solution of 3-cyano-5-iodo-4-methoxybenzoic acid (p. 61) (500 mg, 1.64mmol) in CH_2Cl_2 (9 mL) and DMF (1 mL) were added 2,5-dihydro-1*H*-pyrrole (114 mg, 1.65 mmol) and EDC·HCl (380 mg, 1.98 mmol). After the mixture was stirred at room temperature for 1 h, the solvent was evaporated, and the residue was dissolved with AcOEt. The solution was washed with 1 M HCl, 1 M aqueous NaOH and brine, then dried over anhydrous Na_2SO_4. The solvent was evaporated, and the obtained residue was used for the next step.

(b) 5-(2,5-Dihydro-1*H*-pyrrole-1-carbonyl)-2-hydroxy-3-iodobenzonitrile (**34c**)

To a solution of 5-(2,5-dihydro-1*H*-pyrrole-1-carbonyl)-3-iodo-2-methoxybenzonitrile in DMF (10 mL) was added lithium chloride (700 mg, 16.5 mmol). After the mixture was stirred at 120 °C for 3h, the solvent was evaporated, and the residue was diluted with AcOEt. The solution was washed with 1 M HCl and brine, and dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure to afford the title compound as a pale brown solid (540 mg, 96% yield over two steps).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 4.26 (4H, s), 5.85 (1H, d, J = 6.8 Hz), 5.93 (1H, d, J = 6.8 Hz), 7.93 (1H, d, J = 2.2 Hz), 8.19 (1H, d, J = 2.2 Hz).

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 53.5, 55.2, 88.7, 100.1, 116.0, 125.2, 126.1, 130.0, 132.4, 143.0, 160.0, 165.1.

MS (m/z): 339 (M − H)$^-$.

HRMS (ESI) m/z: calcd for $C_{12}H_8IN_2O_2$ (M − H)$^-$ 338.9636, found 338.9637.

5-(2,5-Dihydro-1*H*-pyrrole-1-carbonyl)-2-hydroxy-3-methylsulfanylbenzonitrile (34d)

(a) 5-(2,5-Dihydro-1*H*-pyrrole-1-carbonyl)-2-methoxy-3-methylsulfanylbenzonitrile

To a solution of 3-cyano-4-methoxy-5-methylsulfanylbenzoic acid (230 mg, 1.03mmol) in CH_2Cl_2 (9 mL) and DMF (1 mL) were added EDC·HCl (237 mg) and 2,5-dihydro-1*H*-pyrrole (75 mg, 0.39 mmol). After the mixture was stirred at room temperature for 1 h, the solvent was evaporated, and the residue was dissolved with AcOEt. The solution was washed with 1 M HCl, 1 M aqueous NaOH and brine, and dried over anhydrous Na_2SO_4. The solvent was evaporated, and the obtained residue was used for the next step.

(b) 5-(2,5-Dihydro-1*H*-pyrrole-1-carbonyl)-2-hydroxy-3-methylsulfanylbenzonitrile (**34d**)

To a solution of 5-(2,5-dihydro-1*H*-pyrrole-1-carbonyl)-2-methoxy-3-methylsulfanylbenzonitrile (a) in DMF (10 mL) was added lithium chloride (437 mg, 10.3 mmol). After the mixture was stirred at 120 °C for 3 h, the solvent was evaporated. The residue was dissolved with AcOEt, and the solution was washed with 1M HCl, brine, and dried over anhydrous Na_2SO_4. The solvent was evaporated to afford the title compound as a pale brown solid (238 mg, 89% yield over two steps).

[^1]H-NMR (270 MHz, DMSO-d_6) δ: 2.45 (3H, s), 4.27 (4H, s), 5.85 (1H, d, J = 6.4 Hz), 5.94 (1H, d, J = 6.4 Hz), 7.58 (1H, d, J = 2.1 Hz), 7.68 (1H, d, J = 2.1 Hz), 11.24 (1H, brs).

[^13]C-NMR (101 MHz, DMSO-d_6) δ: 14.4, 53.5, 55.3, 99.8, 116.5, 125.3, 126.2, 128.1, 129.4, 129.8, 157.1, 166.4.

MS (m/z): 259 (M − H)⁻.

HRMS (ESI) m/z: calcd for $C_{13}H_{11}N_2O_2S$ (M − H)⁻ 259.0547, found 259.0547.

(2,5-Dihydro-1*H*-pyrrol-1-yl)(4-hydroxy-3-propyl-5-trifluoromethylphenyl)methanone (34e)

(a) Methyl 4-hydroxy-3-iodo-5-propylbenzoate

To a solution of methyl 4-hydroxy-3-propylbenzoate (1.92 g, 9.89 mmol) in chloroform (20 mL) was added *N*-iodosuccinimide (2.25 g, 10.0 mmol). After the mixture was stirred at room temperature for 20 h, the solvent was evaporated. The residue was diluted with AcOEt, and the solution was washed with water, 10% aqueous sodium thiosulfate and brine, then dried over anhydrous Na_2SO_4. The solvent was evaporated, and the obtained residue was purified by silica gel column chromatography (n-hexane/AcOEt = 4:1) to afford the title compound as a pale yellow solid (2.72 g, 86% yield).

[^1]H-NMR (270 MHz, $CDCl_3$) δ: 0.96 (3H, t, J = 7.3 Hz), 1.58–1.72 (2H, m), 2.67 (2H, t, J = 7.6 Hz), 3.88 (3H, s), 5.67 (1H, s), 7.78 (1H, d, J = 1.9 Hz), 8.21 (1H, d, J = 1.9 Hz).

(b) Methyl 3-iodo-4-methoxy-5-propylbenzoate

To a solution of methyl 4-hydroxy-3-iodo-5-propylbenzoate (2.72 g, 8.50 mmol) in DMF (20 mL) were added potassium carbonate (3.52 g, 25.5 mmol) and dimethyl sulfate (1.21 mL, 12.8 mmol). After the reaction mixture was stirred at room temperature for 15 h, insoluble materials were removed by filtration through a pad of Celite. The filtrate was acidified with 1 M HCl. The products were extracted with AcOEt, and the organic layer was washed with water and brine, then dried over anhydrous Na_2SO_4. The solvent was evaporated to afford the title compound as a pale-yellow solid (2.74 g, 96% yield).

[^1]H-NMR (270 MHz, $CDCl_3$) δ: 0.97 (3H, t, J = 7.3 Hz), 1.59–1.73 (2H, m), 2.67 (2H, t, J = 7.8 Hz), 3.82 (3H, s), 3.89 (3H, s), 7.85 (1H, d, J = 2.1 Hz), 8.30 (1H, d, J = 2.1 Hz).

(c) Methyl 4-methoxy-3-propyl-5-trifluoromethylbenzoate

  To a solution of methyl 3-iodo-4-methoxy-5-propylbenzoate (1.43 g, 4.28 mmol) in DMF (30 mL) were added copper iodide (153 mg, 0.80 mmol) and methyl fluorosulfonyldifluoroacetate (1.50 g, 7.8 mmol). After the mixture was stirred at 150 °C for 5 h, the insoluble materials were removed by filtration through a pad of Celite. The filtrate was diluted with AcOEt, and the solution was washed with brine and dried over anhydrous Na_2SO_4. The solvent was evaporated, and the residue was purified by silica gel column chromatography (n-hexane/AcOEt = 6:1) to afford the title compound as a colorless oil (1.14 g, 96% yield).

 ^{1}H-NMR (270 MHz, CDCl$_3$) δ: 1.00 (3H, t, J = 7.3 Hz), 1.55–1.77 (2H, m), 2.70 (2H, t, J = 8.1 Hz), 3.88 (3H, s), 3.93 (3H, s), 8.08 (1H, d, J = 2.1 Hz), 8.12 (1H, d, J = 2.1 Hz).

(d) 4-Methoxy-3-propyl-5-trifluoromethylbenzoic acid

  To a solution of methyl 4-methoxy-3-propyl-5-trifluoromethylbenzoate (1.13 g, 4.09 mmol) in THF (14 mL) and water (7 mL) was added lithium hydroxide monohydrate (1.03 g, 24.5mmol). After the mixture was stirred at room temperature for 19 h, the solvent was evaporated, and the residue was acidified with 2 M HCl. The products were extracted with AcOEt, and the organic layer was washed with brine, and dried over anhydrous Na_2SO_4. The solvent was evaporated to afford the title compound as a colorless solid (1.07 g, 100% yield).

(e) (2,5-Dihydro-1H-pyrrol-1-yl)(4-methoxy-3-propyl-5-trifluoromethylphenyl)methanone

  To a solution of 4-methoxy-3-propyl-5-trifluoromethylbenzoic acid (274 mg, 1.04 mmol) in CH_2Cl_2 (10 mL) were added 2,5-dihydro-1H-pyrrole (72 mg, 1.04 mmol) and EDC·HCl (239 mg, 1.25 mmol). After the mixture was stirred at room temperature for 1 h, the solvent was evaporated, and the residue was diluted with AcOEt. The solution was washed with 1 M HCl, 1 M aqueous NaOH and brine, then dried over anhydrous Na_2SO_4. The solvent was evaporated, and the obtained residue was used for the next step.

(f) (2,5-Dihydro-1H-pyrrol-1-yl)(4-hydroxy-3-propyl-5-trifluoromethylphenyl)methanone (**34e**)

  To a solution of (2,5-dihydro-1H-pyrrol-1-yl)(4-methoxy-3-propyl-5-trifluoromethylphenyl)methanone synthesized in step (e) in DMF (10 mL) was added lithium chloride (440 mg, 10.4 mmol). After the mixture was stirred at 150 °C for 21 h, the solvent was evaporated. 1 M HCl was added, and the products were extracted with AcOEt. The combined extracts were washed with 1 M HCl and brine, then dried over anhydrous Na_2SO_4. The solvent was evaporated, and the residue was purified by silica gel column chromatography (n-hexane/AcOEt = 2:1) to afford the title compound as a pale brown solid (64 mg, 21% yield over two steps).

 ^{1}H-NMR (270 MHz, DMSO-d_6) δ: 0.91 (3H, t, J = 7.5 Hz), 1.55 (2H, tq, J = 7.5, 7.5 Hz), 2.67 (2H, t, J = 7.5 Hz), 4.27 (4H, s), 5.85 (1H, d, J = 6.1 Hz), 5.94 (1H, d, J = 6.1 Hz), 7.57 (1H, d, J = 2.0 Hz), 7.62 (1H, d, J = 2.0 Hz), 9.83 (1H, brs).

 MS (m/z): 298 (M − H)$^-$.

 HRMS (ESI) m/z: calcd for $C_{15}H_{15}F_3NO_2$ (M − H)$^-$ 298.1060, found 298.1060.

5-(2,5-Dihydro-1*H*-pyrrole-1-carbonyl)-2-hydroxy-3-trifluoromethylbenzonitrile (34f)

(a) 5-(2,5-Dihydro-1*H*-pyrrole-1-carbonyl)-2-methoxy-3-trifluoromethylbenzonitrile

To a solution of 3-cyano-4-methoxy-5-trifluoromethylbenzoic acid (245 mg, 1.00 mmol) (p. 66) in CH$_2$Cl$_2$ (10 mL) were added 2,5-dihydro-1*H*-pyrrole (70 mg, 1.01 mmol) and EDC·HCl (230 mg, 1.20 mmol). After the mixture was stirred at room temperature for 1 h, the solvent was evaporated, and the residue was acidified with 1 M HCl. The products were extracted with AcOEt, and the organic layer was washed with 1 M aqueous NaOH and brine, then dried over anhydrous Na$_2$SO$_4$. The solvent was evaporated, and the obtained residue was used for the next step.

(b) 5-(2,5-Dihydro-1*H*-pyrrole-1-carbonyl)-2-hydroxy-3-trifluoromethylbenzonitrile (**34f**)

To a solution of 5-(2,5-dihydro-1*H*-pyrrole-1-carbonyl)-2-methoxy-3-trifluoromethylbenzonitrile synthesized in step (a) in DMF (5 mL) was added lithium chloride (212 mg, 5.00 mmol). After the mixture was stirred at room temperature for 1 h, the solvent was evaporated. The residue was acidified with 1 M HCl, and the products were extracted with AcOEt. The organic layer was washed with 1 M HCl and brine, then dried over anhydrous Na$_2$SO$_4$. The solvent was evaporated, and the residue was washed with n-hexane/AcOEt (8 mL) to afford the title compound as a colorless solid (196 mg, 70% yield over two steps).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 4.29 (4H, s), 5.85 (1H, d, J = 6.5 Hz), 5.94 (1H, d, J = 6.5 Hz), 8.02 (1H, d, J = 2.1 Hz), 8.22 (1H, d, J = 2.1 Hz).

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 53.7, 55.2, 102.7, 115.6, 119.0 (q, J = 29.9 Hz), 122.9 (q, J = 273 Hz), 125.3, 126.1, 128.3, 131.3 (q, J = 4.8 Hz), 136.7, 158.8, 165.4.

MS (m/z): 281 (M − H)$^-$.

HRMS (ESI) m/z: calcd for C$_{13}$H$_8$F$_3$N$_2$O$_2$ (M − H)$^-$ 281.0543 (M − H)$^-$, found 281.0543.

3-Chloro-5-(2,5-dihydro-1*H*-pyrrole-1-carbonyl)-2-hydroxybenzonitrile (34g)

(a) 3-Chloro-5-(2,5-dihydro-1*H*-pyrrole-1-carbonyl)-2-methoxybenzonitrile

To a solution of 3-chloro-5-cyano-4-methoxybenzoic acid (p. 65) (269 mg, 1.27 mmol) and 2,5-dihydro-1*H*-pyrrole (88 mg, 1.27 mmol) in CH$_2$Cl$_2$ (10 mL) was added EDC·HCl (293 mg, 1.51 mmol). After the mixture was stirred at room temperature for 1 h, the solvent was evaporated, and 1 M HCl was added. The products were extracted with AcOEt, and the organic layer was washed with 1 M aqueous NaOH and brine, then dried over anhydrous Na$_2$SO$_4$. The solvent was evaporated, and the obtained residue was used for the next step.

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 4.43 (3H, s), 8.55 (2H, s), 14.00 (1H, brs).

MS (m/z): 210 (M − H)$^-$, 212 (M + 2 − H)$^-$.

(b) 3-Chloro-5-(2,5-dihydro-1*H*-pyrrole-1-carbonyl)-2-hydroxybenzonitrile (**34g**)

To a solution of 3-chloro-5-(2,5-dihydro-1*H*-pyrrole-1-carbonyl)-2-methoxybenzonitrile synthesized in the step (a) in DMF (10 mL) was added lithium chloride (269 mg, 6.35 mmol). After the mixture was stirred at 120 °C for 1 h, the solvent was evaporated. 1 M HCl was added to the residue, and the products were extracted with AcOEt. The organic layer was washed with 1 M HCl and brine, then dried over anhydrous

Na$_2$SO$_4$. The solvent was evaporated to afford the title compound as a pale brown solid (254 mg, 80% yield over two steps).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 4.27 (4H, s), 5.85 (1H, d, J = 6.2 Hz), 5.94 (1H, d, J = 6.2 Hz), 7.91 (1H, d, J = 2.1 Hz), 7.92 (1H, d, J = 2.1 Hz).

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 53.7, 55.2, 101.8, 115.8, 121.9, 125.3, 126.2, 129.1, 131.3, 133.9, 156.9, 165.4.

MS (m/z): 247 (M − H)$^-$, 249 (M + 2 − H)$^-$.

HRMS (ESI) m/z: calcd for C$_{12}$H$_8$ClN$_2$O$_2$ (M − H)$^-$ 247.0280, found 247.0278.

5-(2,5-Dihydro-1H-pyrrole-1-carbonyl)-2-hydroxy-3-methylbenzonitrile (34h)

(a) Methyl 3-cyano-4-methoxy-5-methylbenzoate

To a solution of methyl 3-cyano-4-hydroxy-5-iodobenzoate (p. 60) (1.50 g, 4.95 mmol) in 1,4-dioxane (15 mL) were added potassium carbonate (1.96 g, 14.2 mmol), methylboronic acid (340 mg, 5.68 mmol) and [1,3-bis(2,6-diisopropylphenyl)imidazole-2-ylidene](3-chloropyridyl)palladium dichloride (32 mg, 0.05 mmol). After the mixture was stirred at 60 °C for 24 h under an argon atmosphere, 1 M HCl was added. The products were extracted with AcOEt, and the organic layer was washed with 1 M aqueous NaOH and brine, then dried over anhydrous Na$_2$SO$_4$. The solvent was evaporated, and the obtained residue was purified by silica gel column chromatography (n-hexane/AcOEt = 6:1) to afford the title compound as a pale brown oil (412 mg, 41% yield).

^{1}H-NMR (270 MHz, CDCl$_3$) δ: 2.00 (3H, s), 3.60 (3H, s), 3.81 (3H, s), 7.73 (1H, d, J = 1.9 Hz), 7.80 (1H, d, J = 1.9 Hz).

(b) 3-Cyano-4-methoxy-5-methylbenzoic acid

To a solution of methyl 3-cyano-4-methoxy-5-methylbenzoate (412 mg, 2.01 mmol) in THF (6 mL) and water (3 mL) was added lithium hydroxide monohydrate (337 mg, 8.03 mmol). After the mixture was stirred at room temperature for 4 h, the solvent was evaporated. Water was added, and the aqueous solution was washed with diethyl ether, then acidified with 1 M HCl. The products were extracted with AcOEt, and the organic layer was washed with brine, and dried over anhydrous Na$_2$SO$_4$. The solvent was evaporated to afford the title compound as a light brown solid (378 mg, 98% yield).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 2.32 (3H, s), 4.01 (3H, s), 8.08 (2H, s), 13.34 (1H, br).

MS (m/z): 190 (M − H)$^-$.

(c) 5-(2,5-Dihydro-1H-pyrrole-1-carbonyl)-2-methoxy-3-methylbenzonitrile

To a solution of 3-cyano-4-methoxy-5-methylbenzoic acid (255 mg, 1.34 mmol) in CH$_2$Cl$_2$ (10 mL) was added 2,5-dihydro-1H-pyrrole (93 mg, 1.35 mmol) and EDC·HCl (306 mg, 1.60 mmol). After the mixture was stirred at room temperature for 1 h, the solvent was evaporated, then 1 M HCl was added. The products were extracted with AcOEt, and the organic layer was washed with 1 M aqueous NaOH and brine, and dried over anhydrous Na$_2$SO$_4$. The solvent was evaporated, and the obtained residue was purified by

silica gel column chromatography (n-hexane/AcOEt = 1:3) to afford the title compound as a colorless oil (278 mg, 86% yield).

(d) 5-(2,5-Dihydro-1*H*-pyrrole-1-carbonyl)-2-hydroxy-3-methylbenzonitrile (**34h**)

To a solution of 5-(2,5-dihydro-1*H*-pyrrole-1-carbonyl)-2-methoxy-3-methylbenzonitrile (278 mg, 1.16mmol) in DMF (5 mL) was added lithium chloride (282 mg, 6.65 mmol). After the mixture was stirred at 120 °C for 2 h, the solvent was evaporated, and acidified with 1 M HCl. The products were extracted with AcOEt, and the organic layer was washed with 1 M HCl and brine, then dried over anhydrous Na_2SO_4. The solvent was evaporated, and the obtained residue was washed with n-hexane/AcOEt (12 mL, 3:1) to afford the title compound as a colorless solid (205 mg, 67 % yield).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 2.25 (3H, s), 4.26 (4H, s), 5.84 (1H, d, J = 6.3 Hz), 5.94 (1H, d, J = 6.3 Hz), 7.64 (1H, d, J = 2.1 Hz), 7.71 (1H, d, J = 2.1 Hz), 10.61 (1H, br).

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 16.4, 53.6, 55.3, 99.7, 116.9, 125.4, 126.2, 126.5, 128.3, 129.9, 134.9, 159.3, 166.8.

MS (m/z): 227 (M − H)⁻.

HRMS (ESI) m/z: calcd for $C_{13}H_{11}N_2O_2$ (M − H)⁻ 227.0826, found 227.0822.

3-Bromo-5-(2,5-dihydro-1*H*-pyrrole-1-carbonyl)-2-hydroxybenzonitrile (34i)

(a) Methyl 3-bromo-5-formyl-4-hydroxybenzoate

To a solution of methyl 3-bromo-4-hydroxybenzoate (10.0 g, 43.3 mmol) in acetic acid (100 mL) was added hexamethylenetetramine (6.11 g, 43.6 mmol). After the mixture was refluxed for 16 h, the solvent was evaporated, and 1 M HCl was added. The products were extracted with AcOEt, and the organic layer was washed with brine, and dried over anhydrous Na_2SO_4. The solvent was evaporated, and the residue was purified by silica gel column chromatography (chloroform/n-hexane = 2:1) to afford the title compound as a colorless solid (6.12 g, 55% yield).

^{1}H-NMR (270 MHz, CDCl$_3$) δ: 3.95 (3H, s), 8.29 (1H, d, J = 2.0 Hz), 8.46 (1H, d, J = 2.0 Hz), 9.92 (1H, s), 12.02 (1H, s).

MS (m/z): 257 (M − 1 − H)⁻, 259 (M + 1 − H)⁻.

(b) Methyl 3-bromo-5-formyl-4-methoxybenzoate

To a solution of methyl 3-bromo-5-formyl-4-hydroxybenzoate (3.10 g, 12.0 mmol) in DMF (60 mL), were added potassium carbonate (16.5 g, 119 mmol) and dimethyl sulfate (5.7 mL, 60.1 mmol). After the mixture was stirred at room temperature for 2 h, the insoluble materials were removed by filtration through a pad of Celite. Water was added to the filtrate. The products were extracted with AcOEt, and the organic layer was washed with water and brine, dried over anhydrous Na_2SO_4. The solvent was evaporated, and the residue was purified by silica gel column chromatography (n-hexane/AcOEt = 6:1) to afford the title compound as a colorless solid (2.50 g, 76% yield).

^{1}H-NMR (270 MHz, CDCl$_3$) δ: 3.94 (3H, s), 4.05 (3H, s), 8.46 (1H, d, J = 2.1 Hz), 8.48 (1H, d, J = 2.1 Hz), 10.35 (1H, s).

(c) Methyl 3-bromo-5-hydroxyiminomethyl-4-methoxybenzoate

To a solution of methyl 3-bromo-5-formyl-4-methoxybenzoate (1.00 g, 3.66 mmol) (b) in ethanol (20 mL) and THF (5 mL) were added sodium acetate (900 mg, 11.0 mmol) and hydroxylamine hydrochloride (382 mg, 5.50 mmol). After the mixture was stirred at room temperature for 45 min, and the solvent was evaporated. Water was added, and the products were extracted with chloroform. The combined extracts were washed with brine and dried over anhydrous Na_2SO_4. The solvent was concentrated, and the obtained residue was used for the next step.

(d) Methyl 3-bromo-5-cyano-4-methoxybenzoate

To a solution of methyl 3-bromo-5-hydroxyiminomethyl-4-methoxybenzoate (c) in chloroform (20 mL) were added triethylamine (1.5 mL, 10.8 mmol) and 2-chloro-1-methylpyridinium iodide (1.12 g, 4.38 mmol). After the mixture was stirred at room temperature for 1 h, the solvent was evaporated, and 1 M HCl was added. The products were extracted with AcOEt, and the organic layer was washed with 10% aqueous sodium thiosulfate and brine, then dried over anhydrous Na_2SO_4. The solvent was evaporated to afford the title compound as a colorless solid (970 mg, 98% yield over two steps).

^{1}H-NMR (270 MHz, $CDCl_3$) δ: 3.95 (3H, s), 4.19 (3H, s), 8.22 (1H, d, $J = 2.1$ Hz), 8.43 (1H, d, $J = 2.1$ Hz).

(e) 3-Bromo-5-cyano-4-methoxybenzoic acid

To a solution of methyl 3-bromo-5-cyano-4-methoxybenzoate (970 mg, 3.59 mmol) in THF (20 mL) and water (10 mL) was added lithium hydroxide monohydrate (603 mg, 14.4 mmol). After the mixture was stirred at room temperature for 90 min, the organic solvent was evaporated. The obtained residue was acidified with 1M HCl. The products were extracted with AcOEt, and the organic layer was washed with brine, dried over anhydrous Na_2SO_4. The solvent was evaporated to afford the title compound as a colorless solid (891 mg, 97% yield).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 4.41 (3H, s), 8.59 (1H, d, $J = 2.0$ Hz), 8.69 (1H, d, $J = 2.0$ Hz).

MS (m/z): 254 (M − 1 − H)$^-$, 256 (M + 1 − H)$^-$.

(f) 3-Bromo-5-(2,5-dihydro-1H-pyrrole-1-carbonyl)-2-methoxybenzonitrile

To a solution of 3-bromo-5-cyano-4-methoxybenzoic acid (256 mg, 1.00 mmol) in CH_2Cl_2 (10 mL) were added EDC·HCl (230 mg, 1.20 mmol) and 2,5-dihydro-1H-pyrrole (69 mg, 1.00 mmol). After the mixture was stirred at room temperature for 1 h, the solvent was evaporated, and the residue was acidified with 1 M HCl. The products were extracted with AcOEt, and the organic layer was washed with 1 M aqueous NaOH and brine, then dried over anhydrous Na_2SO_4. The solvent was evaporated, and the obtained residue was used for the next step.

(g) 3-Bromo-5-(2,5-dihydro-1H-pyrrole-1-carbonyl)-2-hydroxybenzonitrile (**34i**)

To a solution of 3-bromo-5-(2,5-dihydro-1H-pyrrole-1-carbonyl)-2-methoxybenzonitrile (f) in DMF (10 mL) was added lithium chloride (212 mg, 5.00 mmol). After the mixture was stirred at 120 °C for 1 h, the solvent was evaporated, and 1 M HCl was added. The products were extracted with AcOEt, and the organic layer was washed with 1 M HCl and brine, then dried over anhydrous Na_2SO_4. The solvent was evaporated to afford the title compound as a pale brown solid (239 mg, 82 % yield over two steps).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 4.27 (4H, s), 5.85 (1H, d, J = 6.3 Hz), 5.93 (1H, d, J = 6.3 Hz), 7.94 (1H, d, J = 2.1 Hz), 8.04 (1H, d, J = 2.1 Hz).

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 53.6, 55.2, 101.6, 111.9, 115.9, 125.3, 126.1, 129.4, 131.8, 136.9, 157.8, 165.2.

MS (m/z): 291 (M − 1 − H)$^-$, 293 (M + 1 − H)$^-$.

HRMS (ESI) m/z: calcd for $C_{12}H_8BrN_2O_2$ (M − H)$^-$ 290.9775, found 290.9777.

5-(2,5-Dihydro-1H-pyrrole-1-carbonyl)-2-hydroxy-3-propylbenzonitrile (34j)

(a) Methyl 3-cyano-4-methoxy-5-propylbenzoate

To a solution of methyl 3-iodo-4-methoxy-5-propylbenzoate (1.36 g, 4.07 mmol) in DMF (30 mL) was added copper cyanide (400 mg, 4.47 mmol). After the mixture was stirred at 150 °C for 75 min, the insoluble materials were removed by filtration through a pad of Celite. Water was added to the filtrate, and the products were extracted with AcOEt. The organic layer was washed with water and brine, then dried over anhydrous Na$_2$SO$_4$. The solvent was evaporated, and the obtained residue was purified by silica gel column chromatography (n-hexane/AcOEt = 6:1) to afford the title compound as a pale-yellow solid (895 mg, 84% yield).

^{1}H-NMR (270 MHz, CDCl$_3$) δ: 0.96 (3H, t, J = 7.3 Hz), 1.56–1.70 (2H, m), 2.64 (2H, t, J = 7.8 Hz), 3.92 (3H, s), 4.14 (3H, s), 8.04 (1H, d, J = 2.1 Hz), 8.12 (1H, d, J = 2.1 Hz).

(b) 3-Cyano-4-methoxy-5-propylbenzoic acid

To a solution of methyl 3-cyano-4-methoxy-5-propylbenzoate (885 mg, 3.79 mmol) in THF (14 mL) and water (7 mL) was added lithium hydroxide monohydrate (318 mg, 7.58 mmol), and the mixture was stirred at room temperature for 3 h. The organic solvent was evaporated, and 2 M HCl was added. The products were extracted with AcOEt, and the organic layer was washed with brine and dried over anhydrous Na$_2$SO$_4$. The solvent was evaporated to afford the title compound as a colorless solid (713 mg, 86 % yield).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 0.91 (3H, t, J = 7.3 Hz), 1.51–1.65 (2H, m), 2.65 (2H, t, J = 7.6 Hz), 4.03 (3H, s), 8.06 (1H, d, J = 2.2 Hz), 8.09 (1H, d, J = 2.2 Hz).

MS (m/z): 218 (M − H)$^-$.

(c) 5-(2,5-Dihydro-1H-pyrrole-1-carbonyl)-2-methoxy-3-propylbenzonitrile

To a solution of 3-cyano-4-methoxy-5-propylbenzoic acid (285 mg, 1.30 mmol) in CH$_2$Cl$_2$ (10 mL) were added EDC·HCl (299 mg, 1.56 mmol) and 2,5-dihydro-1H-pyrrole (90 mg, 1.30 mmol). After the mixture was stirred at room temperature for 1 h, the solvent was evaporated, and 1 M HCl was added. The products were extracted with AcOEt, and the organic layer was washed with 1 M aqueous NaOH and brine, then dried over anhydrous Na$_2$SO$_4$. The solvent was evaporated, and the obtained residue was used for the next step.

(d) 5-(2,5-Dihydro-1H-pyrrole-1-carbonyl)-2-hydroxy-3-propylbenzonitrile (**34j**)

To a solution of 5-(2,5-dihydro-1H-pyrrole-1-carbonyl)-2-methoxy-3-propylbenzonitrile (c) in DMF (10 mL) was added lithium chloride (276 mg, 6.51 mmol). After the mixture was stirred at 120 °C for 5 h,

the solvent was evaporated. 1 M HCl was added, and the mixture was extracted with AcOEt. The organic layer was washed with 1 M HCl and brine, dried over anhydrous Na_2SO_4. The solvent was evaporated, and the obtained residue was purified by silica gel column chromatography (chloroform/methanol = 19:1) to afford the title compound as a pale brown solid (224 mg, 67% yield over two steps).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 0.90 (3H, t, J = 7.3 Hz), 1.53 (2H, dt, J = 7.3, 7.3 Hz), 2.62 (2H, t, J = 7.3 Hz), 4.26 (4H, s), 5.85 (1H, d, J = 6.4 Hz), 5.94 (1H, d, J = 6.4 Hz), 7.62 (1H, d, J = 2.1 Hz), 7.71 (1H, d, J = 2.1 Hz), 10.58 (1H, br).

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 13.7, 22.4, 31.2, 53.5, 55.3, 100.0, 117.0, 125.4, 126.2, 128.1, 130.0, 130.7, 134.2, 159.0, 166.8.

MS (m/z): 255 (M − H)$^-$.

HRMS (ESI) m/z: calcd for $C_{15}H_{15}N_2O_2$ (M − H)$^-$ 255.1139, found 255.1138.

3-*tert*-Butyl-5-(2,5-dihydro-1*H*-pyrrole-1-carbonyl)-2-hydroxybenzonitrile (34k)

(a) 3-*tert*-Butyl-5-(2,5-dihydro-1*H*-pyrrole-1-carbonyl)-2-methoxybenzonitrile

To a solution of 3-*tert*-butyl-5-cyano-4-methoxybenzoic acid (p. 63) (300 mg, 1.29 mmol) in DMF (10 mL) were added EDC·HCl (493 mg, 2.57 mmol) and 2,5-dihydro-1*H*-pyrrole (0.117 ml, 1.54 mmol). After the mixture was stirred at room temperature overnight, water was added. The products were extracted with AcOEt, and the organic layer was washed with 5% aqueous citric acid and brine, and dried over anhydrous Na_2SO_4. The solvent was evaporated, and the residue was purified by silica gel column chromatography to afford the title compound as a colorless oil (280 mg, 77% yield).

(b) 3-*tert*-Butyl-5-(2,5-dihydro-1*H*-pyrrole-1-carbonyl)-2-hydroxybenzonitrile (**34k**)

To a solution of 3-*tert*-butyl-5-(2,5-dihydro-1*H*-pyrrole-1-carbonyl)-2-methoxybenzonitrile (280 mg, 0.992 mmol) in DMF (10 mL) was added lithium chloride (417 mg, 9.84 mmol).   After the mixture was stirred at 120 °C for 3 h, 10% aqueous potassium carbonate was added. The aqueous solution was washed with AcOEt and acidified with 1 M HCl. The products were extracted with AcOEt, and the organic layer was washed with water and brine, then dried over anhydrous Na_2SO_4. The solvent was evaporated, and the obtained solid was washed with diethyl ether and collected by filtration to afford the title compound as a colorless solid (163 mg, 61% yield over two steps).

^{1}H-NMR (270 MHz, CDCl$_3$) δ: 1.42 (9H, s), 4.25 (2H, brs), 4.45 (2H, brs), 5.73–5.81 (1H, m), 5.90–5.97 (1H, m), 6.41 (1H, s), 7.55 (1H, d, J = 2.2 Hz), 7.73 (1H, d, J = 2.2 Hz).

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 29.1, 34.9, 53.6, 55.3, 101.8, 117.2, 125.4, 126.2, 128.3, 130.0, 131.0, 139.5, 159.6, 166.9.

MS (m/z): 269 (M − H)$^-$.

HRMS (ESI) m/z: calcd for $C_{16}H_{17}N_2O_2$ (M − H)$^-$ 269.1296, found 269.1297.

3-Cyclopropyl-5-(2,5-dihydro-1H-pyrrole-1-carbonyl)-2-hydroxybenzonitrile (34l)

(a) 3-Cyano-5-cyclopropyl-4-methoxybenzoyl chloride

The mixture of 3-cyano-5-cyclopropyl-4-methoxybenzoic acid (p. 64) (190 mg, 0.875 mmol) in thionyl chloride (5 mL, 69.3 mmol) was stirred at 60 °C for 2 h. The reaction mixture was concentrated, and the residue was used for the next step.

(b) 3-Cyclopropyl-5-(2,5-dihydro-1*H*-pyrrole-1-carbonyl)-2-methoxybenzonitrile

To a suspension of 2,5-dihydro-1*H*-pyrrole hydrochloride (95 mg, 0.90 mmol) in CH_2Cl_2 (3 mL) were added triethylamine (0.60 mL, 4.33 mmol) and a solution of 3-cyano-5-cyclopropyl-4-methoxybenzoyl chloride in CH_2Cl_2 (3 mL) at 0 °C. After the mixture was stirred at room temperature for 3 h, the solvent was evaporated. 1 M HCl was added, and the products were extracted with AcOEt. The organic layer was washed with 1 M aqueous NaOH and brine, and dried over anhydrous Na_2SO_4. The solvent was evaporated, and the residue was used for the next step.

(c) 3-Cyclopropyl-5-(2,5-dihydro-1*H*-pyrrole-1-carbonyl)-2-hydroxybenzonitrile (**34l**)

To a solution of 3-cyclopropyl-5-(2,5-dihydro-1*H*-pyrrole-1-carbonyl)-2-methoxybenzonitrile (b) in DMF (10 mL) was added lithium chloride (340 mg, 8.02 mmol). After the mixture was stirred at 130 °C for 3 h, 1 M HCl was added. The products were extracted with AcOEt, and the organic layer was washed with water and brine, then dried over anhydrous Na_2SO_4. The solvent was evaporated, and the obtained crystal was washed with diethyl ether to afford the title compound as a light brown crystal (121 mg, 55% yield over three steps).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 0.65–0.71 (2H, m), 0.92–1.00 (2H, m), 2.03–2.13 (1H, m), 4.22 (2H, s), 4.24 (2H, s), 5.83 (1H, d, J = 6.8 Hz), 5.93 (1H, d, J = 6.8 Hz), 7.29 (1H, d, J = 2.0 Hz), 7.67 (1H, d, J = 2.0 Hz), 10.71 (1H, brs).

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 7.9, 9.7, 53.5, 55.2, 99.7, 116.9, 125.3, 126.2, 128.5, 129.2, 129.6, 131.8, 159.8, 166.7.

MS (m/z): 253 (M − H)$^-$.

HRMS (ESI) m/z: calcd for $C_{15}H_{13}N_2O_2$ (M − H)$^-$ 253.0983, found 253.0981.

5-(Azetidine-1-carbonyl)-2-hydroxy-3-trifluoromethylbenzonitrile (40a)

(a) 3-Cyano-4-methoxy-5-trifluoromethylbenzoyl chloride

To thionyl chloride (5 mL, 69.3 mmol) was added 3-cyano-4-methoxy-5-trifluoromethylbenzoic acid (p. 66) (300 mg, 1.14 mmol). After the mixture was stirred at 50 °C for 5 h, the solvent was evaporated. Toluene was added, and the solvent was evaporated, and the obtained residue was used for the next step.

(b) 5-(Azetidine-1-carbonyl)-2-methoxy-3-trifluoromethylbenzonitrile

To a suspension of azetidine hydrochloride (126 mg, 1.35 mmol) in CH_2Cl_2 (5 mL) were added triethylamine (0.85 mL, 6.13 mmol) and a solution of 3-cyano-4-methoxy-5-trifluoromethylbenzoyl chloride in CH_2Cl_2 (5 mL) at 0 °C. After the mixture was stirred at room temperature for 1 h, the solvent was evaporated. 1 M HCl was added, and the products were extracted with AcOEt. The organic layer was washed

with 1 M aqueous NaOH and brine, and dried over anhydrous Na_2SO_4. The solvent was evaporated to afford the title compound as a colorless oil.

(c) 5-(Azetidine-1-carbonyl)-2-hydroxy-3-trifluoromethylbenzonitrile (**40a**)

To a solution of 5-(azetidine-1-carbonyl)-2-methoxy-3-trifluoromethylbenzonitrile (356 mg, 1.25 mmol) in DMF (10 mL) was added lithium chloride (155 mg, 3.66 mmol). After the mixture was stirred at 100 °C for 30 min, the solvent was evaporated. 1 M HCl was added, and the products were extracted with AcOEt. The organic layer was washed with 1 M HCl and brine, and dried over anhydrous Na_2SO_4. The solvent was evaporated to afford the title compound as a colorless solid (297 mg, 90% yield over two steps).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 2.26 (2H, quint, J = 7.8 Hz), 4.04 (2H, brs), 4.37 (2H, brs), 8.01 (1H, d, J = 2.1 Hz), 8.12 (1H, d, J = 2.1 Hz).

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 15.56, 48.86, 53.00, 102.7, 115.8, 119.1 (q, J = 29.9 Hz), 122.9 (q, J = 271.7 Hz), 124.1, 131.5 (q, J = 4.8 Hz), 137.1, 160.2, 165.7.

MS (m/z): 269 (M − H)$^-$.

HRMS (ESI) m/z: calcd for $C_{12}H_8F_3N_2O_2$ (M − H)$^-$ 269.0543, found 269.0544.

5-(Azetidine-1-carbonyl)-3-*tert*-butyl-2-hydroxybenzonitrile (40b)

(a) 5-(Azetidine-1-carbonyl)-3-*tert*-butyl-2-methoxybenzonitrile

To a solution of 3-*tert*-butyl-5-cyano-4-methoxybenzoic acid (p. 63) (300 mg, 1.29 mmol) in CH_2Cl_2 (10 mL) were added thionyl chloride (0.188 ml, 2.61 mmol) and a catalytic amount of DMF. After the mixture was stirred at 60 °C for 1 h, the solvent was evaporated to afford the acid chloride.

To a solution of the obtained acid chloride in CH_2Cl_2 (5 ml) were added a solution of azetidine hydrochloride (132 mg, 1.41 mmol) and triethylamine (0.54 ml, 3.90 mmol) in CH_2Cl_2 (10 ml). After the mixture was stirred at room temperature for 2 h, water was added. The products were extracted with chloroform, and the organic layer was washed with brine, then dried over anhydrous Na_2SO_4. The solvent was evaporated, and the residue was purified by silica gel column chromatography to afford the title compound as a colorless solid (300 mg, 85% yield).

(b) 5-(Azetidine-1-carbonyl)-3-*tert*-butyl-2-hydroxybenzonitrile (**40b**)

To a solution of 5-(azetidine-1-carbonyl)-3-*tert*-butyl-2-methoxybenzonitrile (300 mg, 1.10 mmol) in DMF (10 mL) was added lithium chloride (467 mg, 11.0 mmol). After the mixture was stirred at 120 °C overnight, an aqueous 10% potassium carbonate solution was added. The aqueous solution was washed with AcOEt and acidified with 1 M HCl, and the products were extracted with AcOEt. The organic layer was washed with water and brine, and dried over anhydrous Na_2SO_4. The solvent was evaporated, and the residue was filtered and washed with diethyl ether to afford the title compound as a colorless solid (193 mg, 68% yield).

^{1}H-NMR (270 MHz, CDCl$_3$) δ :1.42 (9H, s), 2.37 (2H, quint, J = 7.6 Hz), 4.17–4.38 (4H, m), 6.26 (1H, s), 7.62 (1H, d, J = 2.2 Hz), 7.85 (1H, d, J = 2.2 Hz).

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 15.6, 29.1, 34.9, 48.7, 53.2, 101.9, 117.2, 124.8, 130.7, 131.3, 139.6, 160.36, 167.2.

MS (m/z): 257 (M − H)$^-$.

HRMS (ESI) m/z: calcd for C$_{15}$H$_{17}$N$_2$O$_2$ (M − H)$^-$ 257.1296, found 257.1295.

2-Hydroxy-5-(3-methyl-2,5-dihydro-1*H*-pyrrole-1-carbonyl)-3-trifluoromethylbenzonitrile (41)

(a) 2-Methoxy-5-(3-methyl-2,5-dihydro-1*H*-pyrrole-1-carbonyl)-3-trifluoromethylbenzonitrile

To a suspension of 3-methyl-2,5-dihydro-1*H*-pyrrole hydrochloride (170 mg, 1.42 mmol) in CH$_2$Cl$_2$ (5 mL) were added triethylamine (2 mL, 14.4 mmol) and a solution of 3-cyano-4-methoxy-5-trifluoromethylbenzoyl chloride (p.87, prepared from 3-cyano-4-methoxy-5-trifluoromethylbenzoic acid 300mg, 1.14 mmol) in CH$_2$Cl$_2$ (5 mL) at 0 °C. After the mixture was stirred at room temperature for 1 h, the solvent was evaporated. 1 M HCl was added, and the products were extracted with AcOEt. The organic layer was washed with 1 M aqueous NaOH and brine, and the dried over anhydrous Na$_2$SO$_4$. The solvent was evaporated under reduced pressure to afford the title compound as a pale-yellow solid.

(b) 2-Hydroxy-5-(3-methyl-2,5-dihydro-1*H*-pyrrole-1-carbonyl)-3-trifluoromethylbenzonitrile (**41**)

To a solution of 2-methoxy-5-(3-methyl-2,5-dihydro-1*H*-pyrrole-1-carbonyl)-3-trifluoromethyl-benzonitrile in DMF (10 mL) was added lithium chloride (246 mg, 5.80 mmol).   After the mixture was stirred at 130 °C for 30 min, the solvent was evaporated. 1 M HCl was added, and the products were extracted with AcOEt. The organic layer was washed with 1 M HCl and brine, and dried over anhydrous Na$_2$SO$_4$. The solvent was evaporated under reduced pressure, and the obtained solid was washed with n-hexane/AcOEt (1:1, 9 mL) to afford the title compound as a colorless solid (177 mg, 52% yield over three steps).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 1.70 and 1.77 (3H, brs), 4.18 and 4.23 (4H, brs), 5.46 and 5.54 (1H, brs), 8.00 (1H, d, J = 2.1 Hz), 8.20 (1H, d, J = 2.1 Hz).

MS (m/z): 295 (M − H)$^-$.

HRMS (ESI) m/z: calcd for C$_{14}$H$_{10}$F$_3$N$_2$O$_2$(M − H)$^-$ 295.0700, found 295.0699.

2-Hydroxy-5-(thiazolidine-3-carbonyl)-3-trifluoromethylbenzonitrile (42a)

(a) 2-Methoxy-5-(thiazolidine-3-carbonyl)-3-trifluoromethylbenzonitrile

To a solution of 3-cyano-4-methoxy-5-trifluoromethylbenzoic acid (p. 66) (300 mg, 1.22 mmol) and thiazolidine (120 mg, 1.35 mmol) in CH$_2$Cl$_2$ (10 mL) was added EDC·HCl (280 mg, 1.46 mmol). After the mixture was stirred at room temperature for 1 h, the solvent was evaporated. 1 M HCl was added, and the mixture was extracted with AcOEt. The combined extracts were washed with 1 M aqueous NaOH and brine, and dried over anhydrous Na$_2$SO$_4$. The solvent was evaporated to afford the title compound as a colorless oil (373 mg, 97% yield).

(b) 2-Hydroxy-5-(thiazolidine-3-carbonyl)-3-trifluoromethylbenzonitrile (**42a**)

To a solution of 2-methoxy-5-(thiazolidine-3-carbonyl)-3-trifluoromethylbenzonitrile (292 mg, 0.92 mmol) in DMF (10 mL) was added lithium chloride (212 mg, 5.00 mmol). After the mixture was stirred at

120 °C for 1h, the solvent was evaporated. 1 M HCl was added, and the products were extracted with AcOEt. The combined extracts were washed with 1 M HCl and brine, and dried over anhydrous Na_2SO_4. The solvent was evaporated, and the obtained solid was washed with n-hexane/AcOEt (1mL) to afford the title compound as a colorless solid (162 mg, 58% yield).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 3.03 (2H, t, J = 6.4 Hz), 3.78 (2H, t, J = 6.4 Hz), 4.61 (2H, s), 7.97 (1H, d, J = 2.1 Hz), 8.16 (1H, d, J = 2.1 Hz).

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 30.0 (brs), 48.8 (brs), 50.4 (brs), 102.7, 115.5, 119.0 (q, J = 30.8 Hz), 122.8 (q, J = 273.6 Hz), 127.8, 131.5 (q, J = 4.8 Hz), 137.1, 159.1, 165.3.

MS (m/z): 301 (M − H)$^-$.

HRMS (ESI) m/z: calcd for $C_{12}H_8F_3N_2O_2S$ (M − H)$^-$ 301.0264, found 301.0263.

3-Bromo-2-hydroxy-5-(thiazolidine-3-carbonyl)benzonitrile (42b)

(a) 3-Bromo-2-methoxy-5-(thiazolidine-3-carbonyl)benzonitrile

To a solution of 3-bromo-5-cyano-4-methoxybenzoic acid (p. 83) (200 mg, 0.781 mmol) and thiazolidine (70 mg, 0.785 mmol) in CH_2Cl_2 (10 mL) was added EDC·HCl (180 mg, 0.94 mmol). After the mixture was stirred at room temperature for 1 h, the solvent was evaporated under reduced pressure. 1 M HCl was added, and the products were extracted with AcOEt. The combined extracts were washed with 1 M aqueous NaOH and brine, and dried over anhydrous Na_2SO_4. The solvent was evaporated, and the obtained residue was used for the next step.

(b) 3-Bromo-2-hydroxy-5-(thiazolidine-3-carbonyl)benzonitrile (**42b**)

To a solution of 3-bromo-2-methoxy-5-(thiazolidine-3-carbonyl)benzonitrile (a) in DMF (10 mL) was added lithium chloride (166 mg, 3.92 mmol). After the mixture was stirred at 120 °C for 1 h, the solvent was evaporated. 1 M HCl was added, and the products were extracted with AcOEt. The combined extracts were washed with 1 M HCl and brine, and dried over anhydrous Na_2SO_4. The solvent was evaporated, and the obtained solid was washed with AcOEt (1 mL) to afford the title compound as a colorless solid (185 mg, 76% yield over two steps).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 3.02 (2H, t, J = 6.3 Hz), 3.77 (2H, t, J = 6.3 Hz), 4.59 (2H, s), 7.89 (1H, d, J = 2.1 Hz), 8.00 (1H, d, J = 2.1 Hz).

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 29.8, 49.6, 51.0, 101.7, 112.0, 115.8, 129.0, 132.2, 137.1, 158.1, 165.2.

MS (m/z): 311 (M − 1 − H)$^-$, 313 (M + 1 − H)$^-$.

HRMS (ESI) m/z: calcd for $C_{11}H_8BrN_2O_2S$ (M − H)$^-$ 310.9495, found 310.9496.

3-Ethyl-2-hydroxy-5-(thiazolidine-3-carbonyl)benzonitrile (42c)

(a) 3-Ethyl-2-methoxy-5-(thiazolidine-3-carbonyl)benzonitrile

To a solution of 3-cyano-5-ethyl-4-methoxybenzoic acid (300 mg, 1.46 mmol) and thiazolidine (130 mg, 1.46 mmol) in DMF (6 mL) were added EDC·HCl (420 mg, 2.20 mmol). After the mixture was stirred at room temperature for 5 h, water was added, and the products were extracted with AcOEt. The organic layer

was washed with water, aqueous NaHCO$_3$, 1 M HCl and brine, then dried over magnesium sulfate. The solvent was evaporated to afford the title compound as a solid (350 mg, 87% yield).

(b) 3-Ethyl-2-hydroxy-5-(thiazolidine-3-carbonyl)benzonitrile (**42c**)

To a solution of 3-ethyl-2-methoxy-5-(thiazolidine-3-carbonyl)benzonitrile (365 mg, 1.32 mmol) in DMF (5 mL) was added lithium chloride (470 mg, 11.1 mmol). After the mixture was stirred at 110 °C overnight, 1 M HCl was added, and the mixture was extracted with AcOEt. The combined extracts were washed with water and brine, then dried over magnesium sulfate. The solvent was evaporated, and the obtained residue was washed with diisopropyl ether to afford the title compound as a colorless solid (120 mg, 35% yield).

^{1}H-NMR (400 MHz, DMSO-d_6) δ: 1.13 (3H, t, J = 7.5 Hz), 2.70 (2H, t, J = 7.6 Hz), 3.01 (2H, d, J = 6.2 Hz), 3.76 (2H, t, J = 6.2 Hz), 4.59 (2H, brs), 7.59 (1H, d, J = 2.2 Hz), 7.69 (1H, d, J = 2.2 Hz).

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 13.9, 22.5, 29.4 (brs), 48.5 (brs), 50.6 (brs), 100.0, 116.8, 128.0, 130.3, 132.4, 133.6, 159.0, 166.8.

MS (m/z): 261(M − H)$^-$.

HRMS (ESI) m/z: calcd for C$_{13}$H$_{13}$N$_2$OS (M − H)$^-$ 261.0703, found 261.0702.

3-*tert*-Butyl-2-hydroxy-5-(thiazolidine-3-carbonyl)benzonitrile (42d)

(a) 3-*tert*-Butyl-2-methoxy-5-(thiazolidine-3-carbonyl)benzonitrile

To a solution of 3-*tert*-butyl-5-cyano-4-methoxybenzoic acid (p. 63) (300 mg, 1.29 mmol) and thiazolidine (0.12 ml, 1.52 mmol) in DMF (10 mL) was added EDC·HCl (493 mg, 2.57 mmol). After the mixture was stirred at room temperature overnight, water was added. The products were extracted with AcOEt, and the organic layer was washed with water and brine, then dried over anhydrous Na$_2$SO$_4$. The solvent was evaporated, and the residue was purified by silica gel column chromatography to afford the title compound as a colorless oil (350 mg, 89% yield). The obtained compound was used in the next step.

(b) 3-*tert*-Butyl-2-hydroxy-5-(thiazolidine-3-carbonyl)benzonitrile (**42d**)

To a solution of 3-*tert*-butyl-2-methoxy-5-(thiazolidine-3-carbonyl)benzonitrile (350 mg, 1.15 mmol) in DMF (10 mL) was added lithium chloride (487 mg, 11.5 mmol). After the mixture was stirred at 120 °C overnight, water was added. The aqueous solution was washed with AcOEt, then acidified with 1 M HCl. The products were extracted with AcOEt, and the organic layer was washed with water and brine, and dried over anhydrous Na$_2$SO$_4$. The solvent was evaporated, and the residue was collected by filtration and washed with diethyl ether to afford the title compound as a colorless solid (207 mg, 62% yield).

^{1}H-NMR (270 MHz, CDCl$_3$) δ: 1.42 (9H, s), 3.07 (2H, t, J = 5.9 Hz), 3.85–3.96 (2H, m), 4.56–4.68 (2H, m), 6.35 (1H, s), 7.55 (1H, d, J = 2.2 Hz), 7.71 (1H, d, J = 2.2 Hz).

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 29.0, 29.9 (brs), 34.9, 48.7 (brs), 50.5 (brs), 101.8, 117.0, 127.9, 130.6, 131.2, 139.6, 159.8, 166.8.

MS (m/z): 289 (M − H)$^-$.

HRMS (ESI) m/z: calcd for C$_{15}$H$_{17}$N$_2$O$_2$S (M − H)$^-$ 289.1016, found 289.1017.

2-Hydroxy-5-(thiazolidine-3-carbonyl)isophthalonitrile (42e)

(a) 2-Methoxy-5-(thiazolidine-3-carbonyl)isophthalonitrile

To a solution of 3,5-dicyano-4-methoxybenzoic acid (325 mg, 1.61 mmol) (p. 59) and thiazolidine (144 mg, 1.62 mmol) in CH_2Cl_2 (10 mL) was added EDC·HCl (370 mg, 1.93 mmol). After the mixture was stirred at room temperature for 1 h, the solvent was evaporated. 1 M HCl was added, and the products were extracted with AcOEt. The organic layer was washed with 1 M aqueous NaOH and brine, and dried over anhydrous Na_2SO_4. The solvent was evaporated, and the obtained residue was used for the next step.

(b) 2-Hydroxy-5-(thiazolidine-3-carbonyl)isophthalonitrile (**42e**)

To a solution of 2-methoxy-5-(thiazolidine-3-carbonyl)isophthalonitrile (a) in DMF (10 mL) was added lithium chloride (341 mg, 8.04 mmol). After the mixture was stirred at 120 °C for 1 h, the solvent was evaporated. 1 M HCl was added, and the products were extracted with AcOEt. The organic layer was washed with 1 M HCl and brine, and dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure, and the obtained solid was washed with n-hexane/AcOEt (1:2, 6 mL) and methanol (1 mL) to afford the title compound as a pale-yellow solid (198 mg, 47% yield over two steps).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 3.03 (2H, t, J = 6.3 Hz), 3.77 (2H, t, J = 6.3 Hz), 4.59 (2H, s), 8.13 (2H, s).

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 29.9, 48.8, 49.9, 102.4, 115.4, 127.6, 138.0, 163.3, 165.0.

MS (m /z): 258 (M − H)$^-$.

HRMS (ESI) m/z: calcd for $C_{12}H_8N_3O_2S$ (M − H)$^-$ 258.0343, found 258.0342.

5-(1,1-Dioxo-1λ^6-thiazolidine-3-carbonyl)-2-hydroxy-3-trifluoromethylbenzonitrile (43)

(a) 5-(1,1-Dioxo-1λ^6-thiazolidine-3-carbonyl)-2-methoxy-3-trifluoromethylbenzonitrile

To a solution of 2-methoxy-5-(thiazolidine-3-carbonyl)-3-trifluoromethylbenzonitrile (373 mg, 1.18 mmol) in CH_2Cl_2 (10 mL) was added 70% 3-chloroperbenzoic acid (611 mg, 2.48 mmol). After the mixture was stirred at room temperature for 30 min, 10% aqueous sodium thiosulfate was added. The organic solvent was evaporated, and 1 M aqueous NaOH was added. The products were extracted with AcOEt, and the organic layer was washed with brine, then dried over anhydrous Na_2SO_4. The solvent was evaporated to afford the title compound as a colorless solid (406 mg, 99% yield).

(b) 5-(1,1-Dioxo-1λ^6-thiazolidine-3-carbonyl)-2-hydroxy-3-trifluoromethylbenzonitrile (**43**)

To a solution of 5-(1,1-dioxo-1λ^6-thiazolidine-3-carbonyl)-2-methoxy-3-trifluoromethylbenzonitrile (400 mg, 1.15 mmol) in DMF (10 mL) was added lithium chloride (243 mg, 5.73 mmol). After the mixture was stirred at 130 °C for 30 min, the solvent was evaporated. 1 M HCl was added, and the products were extracted with AcOEt. The organic layer was washed with 1 M HCl and brine, and dried over anhydrous Na_2SO_4. The solvent was evaporated, and the residual solid was washed with n-hexane/AcOEt (1:1) to afford the title compound as a colorless solid (321 mg, 84% yield).

^{1}H-NMR (270 MHz, DMSO-d_6) δ: 3.46 (2H, t, J = 7.1 Hz), 4.05 (2H, t, J = 7.1 Hz), 4.73 (2H, s), 7.95 (1H, d, J = 2.1 Hz), 8.13 (1H, d, J = 2.1 Hz).

^{13}C-NMR (101 MHz, DMSO-d_6) δ: 48.2 (brs), 53.5 (brs), 102.8, 115.7, 119.2 (J = 31.0 Hz), 122.9 (J = 273.9 Hz), 124.8, 131.7 (J = 4.8 Hz), 137.4, 160.2, 166.4. (around 39: one peak is sealded in solvent peak).

MS (m/z): 333 (M − H)$^-$.

HRMS (ESI) m/z: calcd for $C_{12}H_8F_3N_2O_4S$ (M − H)$^-$ 333.0162, found 333.0162.

生物活性に関する実験

　以下、動物実験については、動物愛護の観点に配慮しながら、動物実験等の自主管理と科学的観点に基づく動物実験等が適正に実施されていることについて、外部機関にて認証された施設にて実施した。

　すべての実験操作は、各施設の実験動物委員会の承認を経て日本の法律で定められた動物実験のガイドラインに従って実施された。

　ラットを使用した PK 実験については、一般財団法人日本医薬情報センターの認証を受けた施設（株式会社富士薬品第二研究所、認証番号：20－123）にて実施した。

　フサオマキザルを用いた実験については、AAALAC International（The Association for Assessment and Accreditation of Laboratory Animal Care International、国際実験動物管理公認協会）の認証を受けた施設（株式会社新日本科学）にて実施した。

第一章および第二章の実験

1) RPTEC assay
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 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 h 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 0.1 M 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.

2) URAT1 assay
i) URAT1-mediated uric acid uptake assay

　　　General experimental method was similar to that by Anzai et al.[49] HEK293 cells overexpressing URAT1 were preincubated in serum- and chloride-free Hanks' Balanced Salt Solution (HBSS) buffer at 37 °C

for 10 min. To initiate uptake of [^{14}C]-uric acid, cells were incubated with uptake buffer containing 10 μM [^{14}C]-uric acid and test compounds at 37 °C. After 5 min, uptake was terminated by washing the cells three times with ice-cold HBSS buffer. Then, cells were solubilized with 0.1 M NaOH solution. Radioactivity was measured using a liquid scintillation counter (LSC6100, Aloka, Japan). Cellular protein level was also determined in a separate experiment and [^{14}C]-uric acid uptake was expressed as pmol/mg protein.

ii) URAT1 assay for Dotinurad

Methods for preparation of oocytes, *in vitro* synthesis of URAT1 (SLC22A12)-cRNA, and uptake experiments were described previously.[64] 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.

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.[65] Oxygen consumption was measured in a chamber equipped with a Clark-type oxygen electrode (Instec Laboratories, Inc., PA USA) at 30 °C.[66] 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).[67]

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) Pharmacodynamics in *Cebus monkeys*

Five *Cebus monkeys* that were fasted for 18 h before drug administration orally received 5 mg/kg dotinurad, 30 mg/kg benzbromarone, and 0.5% methylcellulose (MC) as a control. [38] The other experiment, three *Cebus monkeys* fasted for the same time orally received 300 mg/kg **34f** and 0.5% MC. Blood samples (about 1 mL) obtained from the saphenous vein at; before; and 2, 4, 8, and 24 h after drug administration using a heparinized needle were kept on ice. Plasma was obtained from the blood samples by centrifugation at 3000 rpm for 10 min at 4 °C. Urine samples were collected 0–4, 4–8, and 8–24 h after drug administration.

Urate and creatinine levels in the samples were measured by a U-3000 spectrophotometer using an Iatro LQ UAII (Mitsubishi Chemical Medience, Corp., Tokyo, Japan) and L-type Wako Creatinine F (Wako Pure Chemical Industries, Ltd.). Each treatment was administered in 13-day intervals to wash out drugs, and treatment and sample collection were performed as crossover experiments. Fractional excretion of urate (FE_{UA}) was calculated as the ratio of urate clearance to creatinine clearance. Urinary urate excretion was calculated as urinary urate concentration x urine volume.

第 三 章 の 実 験

Materials for cell assays

Primary human RPTECs, renal epithelial cell growth kits, renal epithelial cell basal medium, trypsin-EDTA for primary cells, trypsin-neutralizing solution for primary cells, and Dulbecco's phosphate-buffered saline were obtained from ATCC. Sodium gluconate, potassium gluconate, magnesium gluconate, calcium gluconate, NMDG, and insulin solution were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell assay, method A

Primary human RPTECs were cultured in T75 cell culture flasks until 80% confluence, trypsinized, counted, and plated. RPTECs were seeded at a density of 40,000 cells per well in a poly-D-lysine (50 μg/well)-coated tissue culture plate in 200 μL of complete medium and incubated at 37 °C in a 5% CO_2 incubator for 24 h. Cells were pre-incubated with 100 μM of the reference compound; BBR for 15 min at 37 °C in the respective buffers; transport buffer [96 mM sodium gluconate, 2 mM potassium gluconate, 1 mM magnesium gluconate, 1.8 mM calcium gluconate, 5 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), and 5 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.4], and NMDG buffer [140 mM NMDG, 2 mM potassium gluconate, 1 mM magnesium gluconate, 1.8 mM calcium gluconate, 5 mM HEPES, and 5 mM Tris, pH 7.4]. The cells were then treated with 20 μM [^{14}C]-uric acid and PZA at 1 mmol/L and 5 mmol/L in the respective buffers and incubated for 0.5, 1, and 2 h at 37 °C on a plate shaker set at 300 rpm. Cells were washed twice with 200 μL of ice-cold phosphate-buffered saline (pH 7.4), then lysed by the addition of 75 μL of 0.1 M NaOH and incubated for 10 min at 37 °C and 300 rpm. Microscint™-40 (PerkinElmer) was added to all wells, and the plate was incubated for 15 min at 37 °C and 300 rpm. The [^{14}C]-uric acid uptake by the cells was read in a MicroBeta^2 scintillation counter (PerkinElmer, Hopkinton, MA, USA) using counts per minute (CPM) mode and converted to disintegration per minute (DPM).

Cell assay, method B

Primary human RPTECs were cultured as described in method A. RPTECs were seeded at a density of 160,000 cells per well in 800 μL of complete medium with or without 0.1 μM insulin and incubated at 37 °C in a 5% CO_2 incubator for 24 h. The cells were then treated with 5 mM PZA adjusted to pH 7.4 by

adding 0.1 M NaOH, and incubated for 0.5 h at 37 °C with gentle shaking. Thereafter, the buffer containing PZA was discarded. In method B, only the transport buffer was used for uptake study. The cells were washed twice with 800 μL of ice-cold transport buffer without PZA. Pre-warmed transport buffer (600 μL, 37 °C) containing BBR, Dotinurad, and compound **34f** (final concentration: 1, 3, 10, 30, 100, 300, and 1000 μM) was added to each well and incubated for 15 min at 37 °C. Uptake was performed by adding $[^{14}C]$-uric acid at a final concentration of 20 μM and incubating at 37 °C for 2 h and 300 rpm. The cells were dissociated using trypsin, collected in tubes, washed twice with 300 μL of ice-cold phosphate-buffered saline, and lysed with 75 μL of 0.1 M NaOH by incubation for 10 min at 37 °C. The contents of the tubes were transferred to 96-well white opaque plates. Microscint^{TM}-40 was added, and the radioactivities were measured as per the method described for method A.

Statistics

Data are presented as means ± S.D. The statistical difference between the groups was determined by either Student's *t* test (normal distribution data) or Mann–Whitney U test (non-normal distribution data) and a *p* value of 0.05 or less was considered statistically significant. Percent inhibition of the test compounds was analyzed. IC_{50} values of compounds were determined by Prism statistical analysis software (GraphPad, La Jolla, CA, USA), with extrapolation of the IC_{50} value using Growth syntax from MS Excel.

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