博 士 論 文

クマリン骨格を有する選択的 MTHFD2 阻害薬の合成研究

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目次

1

本博士論文中では便宜上、以下の略語を用いた。

Ac	acetyl
ADME	absorption, distribution, metabolism, and excretion
Admin.	administration
AICAR	5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside
AMP	adenosine monophosphate
APCI	atmospheric-pressure chemical ionization
aq.	aqueous
Ar	aryl
AUC	area under the curve
BID	*bis in die* (twice a day)
Boc	*tert*-butoxycarbonyl
Bu	butyl
ca.	*circa* (about)
C_{max}	maximum concentration
C_p	plasma concentration
$C_{p,free}$	unbound plasma concentration
d.r.	diastereomeric ratio
DCM	dichloromethane
DIPEA	*N,N*-diisopropylethylamine
DMF	*N,N*-dimethylformamide
DMSO	dimethylsulfoxide
DMT-MM	4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride
dppf	1,1'-bis(diphenylphosphino)ferrocene
ESI	electrospray ionization
Et	ethyl
f_u	unbound fraction
gem-	geminal
GI_{50}	50% inhibitory concentration of cell growth
GMP	guanosine monophosphate
h	hour(s)
HBA	hydrogen bond acceptor
HBD	hydrogen bond donor
HMBC	heteronuclear multiple-bond correlation spectroscopy
HOAt	3*H*-1,2,3-triazolo[4,5-*b*]pyridin-3-ol
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography

HRMS	High resolution mass spectrometry
HTS	high-throughput screening
IC$_{50}$	50% inhibitory concentration
IMP	inosine monophosphate
i-Pr	isopropyl
IR	infrared
LC/MS	liquid chromatography/mass spectrometry
LE	ligand efficiency
LipE	lipophilic efficiency
LogD	logarithm of ocatanol−water distribution coefficient
LogP	logarithm of ocatanol−water partition coefficient
Me	methyl
min	minute(s)
mRNA	messenger ribonucleic acid
Ms	methanesulfonyl
MS	mass spectrometry
MTHFD1	Methylenetetrahydrofolate dehydrogenase/cyclohydrolase/synthase 1
MTHFD2	Methylenetetrahydrofolate dehydrogenase/cyclohydrolase 2
Mw	molecular weight
n, *n-*	normal
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NBS	*N*-bromosuccinimide
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
p.o.	*per os* (oral administration)
PAMPA	parallel artificial membrane permeability assay
PEG	polyethylene glycol
Ph	phenyl
Pin	pinacolato
PK	pharmacokinetics
pK_a	logarithm of acidity constant
PMB	*p*-methoxybenzyl
PSA	polar surface area
quant.	quantitative yield
rt	room temperature
RuPhos	2-dicyclohexylphosphino-2',6'-diisopropoxybiphenyl
SAR	structure−activity relationship
sat.	saturated
t, *t-*, *tert-*	tertiary

t$_{1/2}$	elimination half-life
Tf	trifluoromethanesulfonyl
TFA	trifluoroacetic acid
TGI	tumor growth inhibition
THF	tetrahydrofolate or tetrahydrofuran
TLC	thin-layer chromatography
tPSA	topological polar surface area
UV	ultraviolet
v	volume
WSCI·HCl	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride

序論

　癌は実に 38 年もの間、我が国の死因第一位の疾患であり、死因別の死亡率では昭和 22 年以降増加の一途を辿っている。令和元年には日本人の全死亡者のおよそ 3.7 人に 1 人、37 万人余りが癌により命を落としている[1]。医学の進歩により、癌の「三大治療」、すなわち外科手術、放射線治療、薬物治療が広く用いられるようになった。かつて「不治の病」であった癌も、就業継続率が低いといった社会的課題は残るものの、現在では多くの患者が社会生活を送りながら治療を続けられるまでになった[2]。しかしながら既存の治療が奏功しない癌により未だ多くの命が失われており、癌に対する新規治療法、特に新規抗癌剤に対するアンメットニーズは非常に高いと言える。

　癌に対する薬物治療は長らく化学療法剤が中心であったが、その高い薬効の反面、非選択的な細胞毒性による副作用の強さが課題であった。近年は疾病メカニズム研究の発展により、癌細胞の増殖や転移に関わる因子に選択的に作用する「分子標的薬」の開発が盛んであり、化学療法剤に比べて正常細胞に対する副作用の少ない薬剤であることが期待されている。代表的な分子標的薬であるイマチニブ（グリベック）は、2001 年に米国で上市されて以降、慢性骨髄性白血病などへの治療選択肢を大きく広げてきた[3]。また「第四の治療」と評される免疫療法も、免疫チェックポイント阻害薬のニボルマブ（オプジーボ）に代表されるように、近年では新たな画期的治療として受け入れられてきている[4,5]。一方でこれらの優れた薬剤も万能ではなく、奏功する患者群とそうでない患者群が存在するため、恩恵を受けられる患者群は一部に留まる。既存治療に不応な患者層には別のメカニズムを有する薬剤が奏功する可能性があり、そのため現在も新たな標的分子の探索が世界中で行われている。

　MTHFD2（Methylenetetrahydrofolate dehydrogenase/cyclohydrolase 2）は、2010 年代に入ってから癌の悪性度に関わる因子として特定された酵素である。MTHFD2 の高発現は癌患者の予後不良と相関することが報告されており、その阻害剤は新規抗癌剤としての可能性を秘めているものと期待されている。しかしながら MTHFD2 に選択的な阻害剤がこれまで知られていなかったことから、阻害剤が癌に対する作用を実際に示すのかどうか、十分に検討されていなかった。

　本論文ではこれまでに報告の無かった MTHFD2 選択性を与えるクマリン骨格の発見と、構造最適化による選択的 MTHFD2 阻害薬の獲得、およびその抗腫瘍活性について述べる。第 1 章では、HTS ヒットを基にした新規クマリン骨格の獲得について述べる。第 2 章では、クマリン上の置換基最適化による誘導体の高活性化について述べる。第 3 章では、さらなる構造最適化研究による DS18561882 の獲得とその薬理評価について述べる。構造生物学的手法の活用による有望なクマリン母骨格の早期獲得と、酸性基周辺の精密な変換による膜透過性および細胞系での活性の飛躍的向上が、創薬化学における課題解決の好例となった。

DS18561882 の獲得により、選択的 MTHFD2 阻害薬の経口投与によって抗腫瘍活性が認められることが初めて明らかとなった。MTHFD2 は癌の標的として長らく有望視されていたが、MTHFD1 阻害作用を乖離した低分子阻害薬はこれまでに報告がなく、本剤を投与することによって癌の増殖抑制が見られたことは、今後の薬剤開発に重要な知見となる。本研究による知見が創薬研究の加速に繋がることを願ってやまない。

本論

第 1 章　クマリン骨格を有するリード化合物の獲得
1-1　背景

　MTHFD2（Methylenetetrahydrofolate dehydrogenase/cyclohydrolase 2）は one carbon (1C) metabolism という葉酸代謝経路に関わる酵素であり、核酸生合成に重要な役割を果たしているとされる[6]。主にヒトのミトコンドリアに局在し、葉酸代謝における以下の 2 反応に関与する。すなわち NAD^+ 共存下での 5,10–メチレンテトラヒドロ葉酸（CH_2–THF）に対する脱水素化と、続く 5,10–メテニルテトラヒドロ葉酸（CH^+=THF）に対する加水分解を触媒し、10–ホルミルテトラヒドロ葉酸（CHO–THF）の生成を促す[7]。この CHO–THF から産生するギ酸（formate）が細胞質へ移行した後に、プリン塩基の 2 位構築時に 1C ユニットとして利用されることで核酸の生合成が行われている（Figure 1-1）。
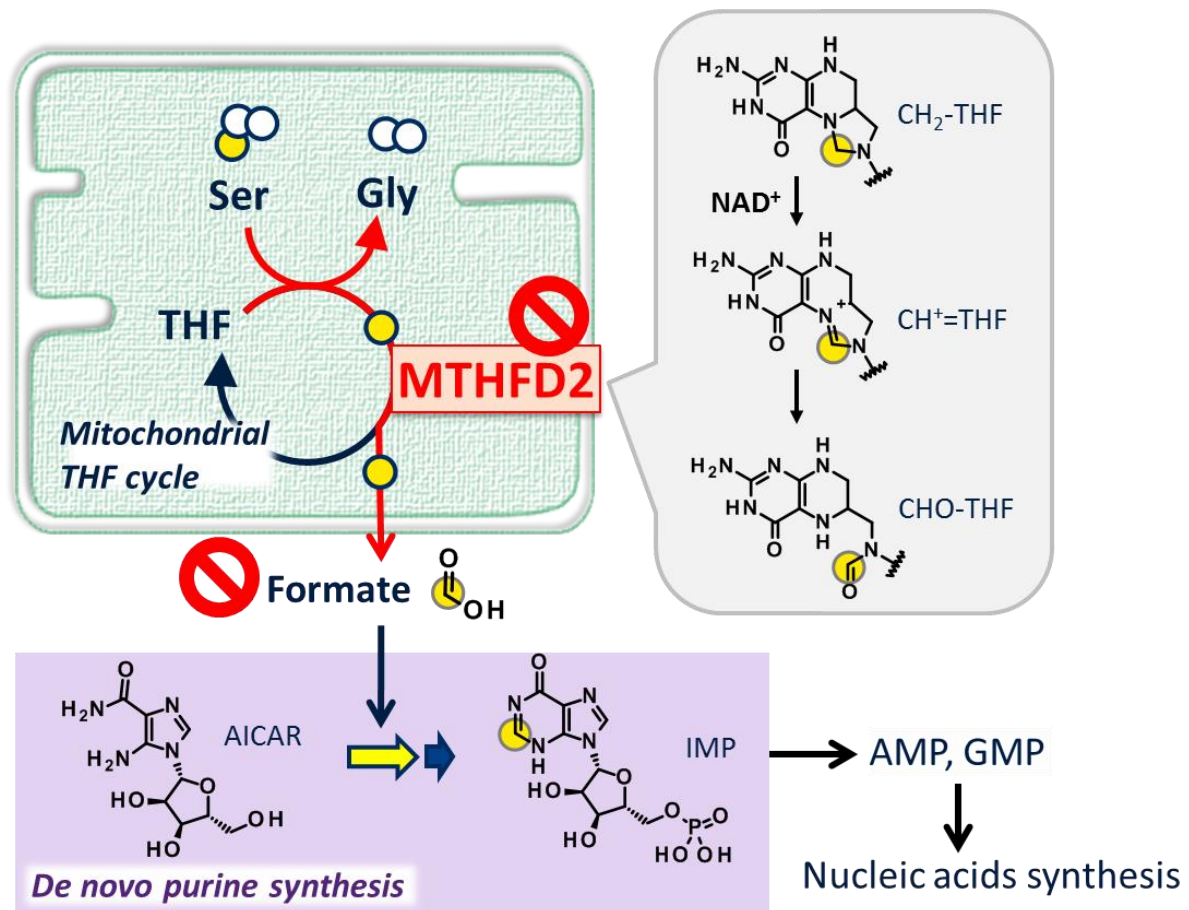

Figure 1-1. Role of MTHFD2 in mitochondrial 1C metabolism

MTHFD2 は近年、癌の悪性度との関連性が数多く報告されており、抗癌剤の新たな標的分子として注目されている [8]。2014 年に Nilsson らは MTHFD2 の mRNA およびタンパクの発現量が多くの癌種において亢進しており、特に MTHFD2 の高発現と乳癌における予後不良が相関することを報告している [9]。MTHFD2 高発現と予後不良との相関はその後、大腸癌 [10]、腎細胞癌 [11]、肝細胞癌 [12] でも報告され、膀胱癌では発症リスクを上昇させる可能性が示唆されている [13]。また MTHFD2 は発生初期を除く正常細胞ではほとんど発現しておらず、癌細胞に選択的に存在する標的であることが示唆されている。これらのことから、MTHFD2 が癌の新たな治療標的になり得ると期待されている [6,14]。

　MTHFD2 と同様の生理活性を有するアイソザイムとして MTHFD1 が広く知られている。両者は構造的に同一のフォールディングを有する一方で、いくつかの相違点が知られている [7]。すなわち、(1) MTHFD2 は主にミトコンドリアに、MTHFD1 は主に細胞質に存在する、(2) MTHFD2 は補酵素として NAD^+ を用いる一方、MTHFD1 は $NADP^+$ を用いる、(3) MTHFD2 の有する dehydrogenase 活性と cyclohydrolase 活性に加えて MTHFD1 は synthase 活性も有する、(4) MTHFD2 は正常細胞には発現が少ないが、MTHFD1 は正常細胞を含めて広く発現している [15]、といった点である。特に 4 つ目の相違点により、MTHFD1 を阻害することは正常細胞にも潜在的に悪影響を及ぼす可能性が考えられる。このことから、MTHFD1 を阻害せず MTHFD2 を選択的に阻害する薬剤が得られれば、正常細胞への影響の少ない新規抗癌剤として有用であると考え、選択的 MTHFD2 阻害薬の合成研究に着手した。

1-2　既知 MTHFD2 阻害剤の総括

　これまでに複数のグループから MTHFD2 阻害剤の報告があるものの、アイソザイムに対して選択性を有する化合物は知られていない。20 年以上前に Lilly 社から MTHFD1 阻害剤として報告された葉酸誘導体 LY345899[16]（Figure 1-2）は、MTHFD2 の阻害活性も有することが近年報告されている（MTHFD1 IC_{50} = 96 nM, MTHFD2 IC_{50} = 663 nM）[7]。大腸癌を移植したマウス xenograft モデルにおいて、この化合物の腹腔内投与による腫瘍増殖抑制作用が確認されている [14]。同様に carolacton という天然物も高活性な MTHFD1/MTHFD2 デュアル阻害剤であることが報告されている（Figure 1-2）[17]。このように MTHFD2 に対して高い阻害活性を有する化合物は知られているものの、アイソザイム間の高い構造類似性のためか、MTHFD1 阻害能を乖離した化合物は報告されていなかった。なお論文報告はないものの、複数のグループより MTHFD2 を阻害する葉酸誘導体の特許出願 [18,19] があるほか、Raze Therapeutics 社はカフェインあるいは類似の複素環を有する MTHFD2 阻害剤を特許出願 [20,21] している。
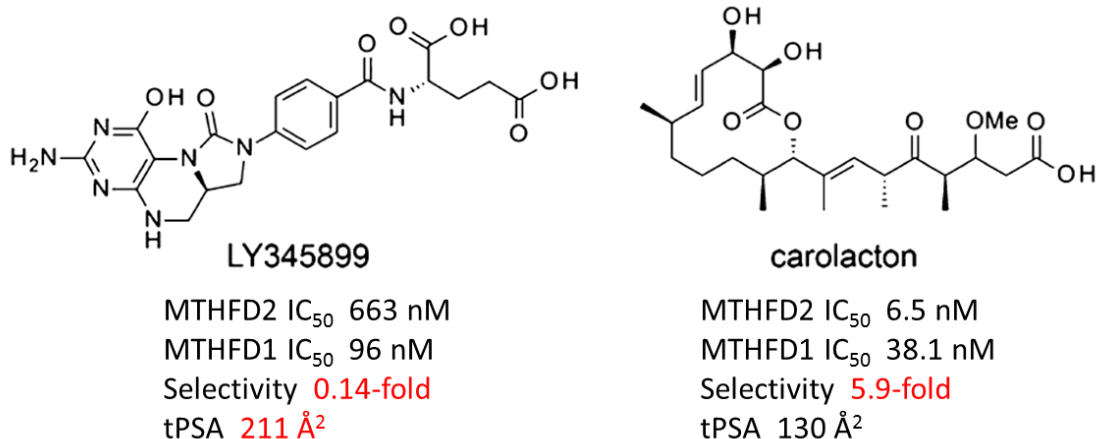
Figure 1-2. Structures and properties of the reported MTHFD1/MTHFD2 dual inhibitors

　以上示したように、carolacton と Raze 社の特許情報を除くと、既知 MTHFD2 阻害剤のほとんどが葉酸誘導体である。LY345899 に代表される葉酸誘導体には、アイソザイム選択性に乏しいほか、極性表面積（PSA）が高いため受動的な細胞膜透過に不利であるという特徴がある[22]。一般に服薬コンプライアンスに長けるとされる経口薬では、消化管上皮からの薬物吸収が循環血への薬物移行に必要であるため[23]、経口薬を目指すにあたり細胞膜透過性が低いという特徴は致命的な課題になる可能性が危惧される。そこで私は、アイソザイム選択性の獲得、および経口薬を見据えた十分な膜透過性の確保を目的に、既知の葉酸誘導体とは異なる化合物獲得を目指すことにした。すなわち、化合物ライブラリーを用いる high-throughput screening（HTS）により、PSA のより小さい新規骨格を有する阻害剤を探索することとした。

1-3　HTS ヒットの獲得と複合体構造情報の考察

　新たな骨格を有する阻害剤を獲得するために、化合物ライブラリーを用いた thermal shift assay による high-throughput screening（HTS）を実施した。これにより、化合物 **1** に代表される tetrahydropyrido[4,3-*d*]pyrimidin-4-one 誘導体がスクリーニングヒットとして得られた。化合物 **1** は MTHFD2 に対して中程度の阻害活性（MTHFD2 IC$_{50}$ = 8.3 µM）を有しつつ、興味深いことに MTHFD1 に対しては全く阻害活性を示さない（MTHFD1 IC$_{50}$ > 100 µM）という所望の選択性を有していた（Figure 1-3）。

HTS hit **(1)**
MTHFD2 IC$_{50}$ = 8.3 µM
MTHFD1 IC$_{50}$ > 100 µM
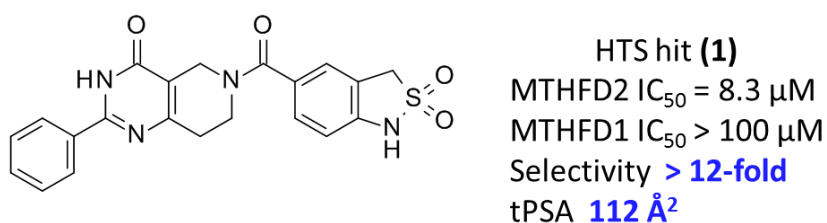Selectivity **> 12-fold**
tPSA **112 Å^2**

Figure 1-3. Structure and properties of HTS hit **1**

化合物 **1** は MTHFD2 の葉酸結合ポケットを占有するが、結合様式は葉酸誘導体と一部異なることが分かった。Soaking 法により得られた化合物 **1**/MTHFD2 複合体の X 線結晶構造解析の結果を Figure 1-4 に、化合物 **1** と LY345899 の MTHFD2 への結合様式の比較を Figure 1-5 にそれぞれ示す。LY345899 のプテリジン部位は MTHFD2 の Val131、Leu133、Asp155 などから成るポケットを占有し、これらの残基と高度な水素結合ネットワークを形成している。一方で化合物 **1** はこのプテリジンの存在する空間は占有せずに、末端フェニル基を除いた分子全体が、LY345899 のグルタミル安息香酸部位と同じポケットを占めている。化合物 **1** と MTHFD2 との間の主な相互作用は次の通りである：①4 つの水素結合（Gln132/Lys88…ピリミジノン C=O、Asn87…安息香酸アミド C=O、および Gly310…スルタム S=O）、②π−π 相互作用（Tyr84…ピリミジノン環）。これらはいずれも LY345899 にも共通して見られるものである。なお水素結合のうち Asn87 および Gly310 の関与する 2 つは結合長が 3.3 Å とやや長く観測されている。
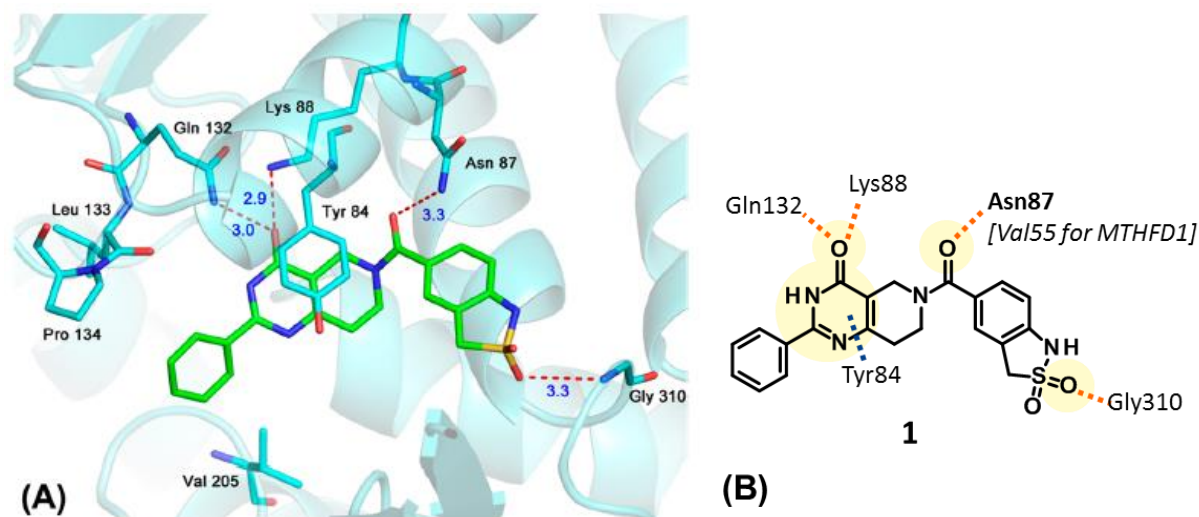

Figure 1-4. (A) X-ray structure of the compound **1**/MTHFD2 complex refined at 2.5 Å resolution (PDB ID: 6JID). Dashed red lines represent hydrogen bonding. Distances of the interactions are in ångströms. R-value = 0.2032. (B) Summary of the observed interaction of **1**/MTHFD2 (orange: hydrogen bonding, blue: π–π interaction).
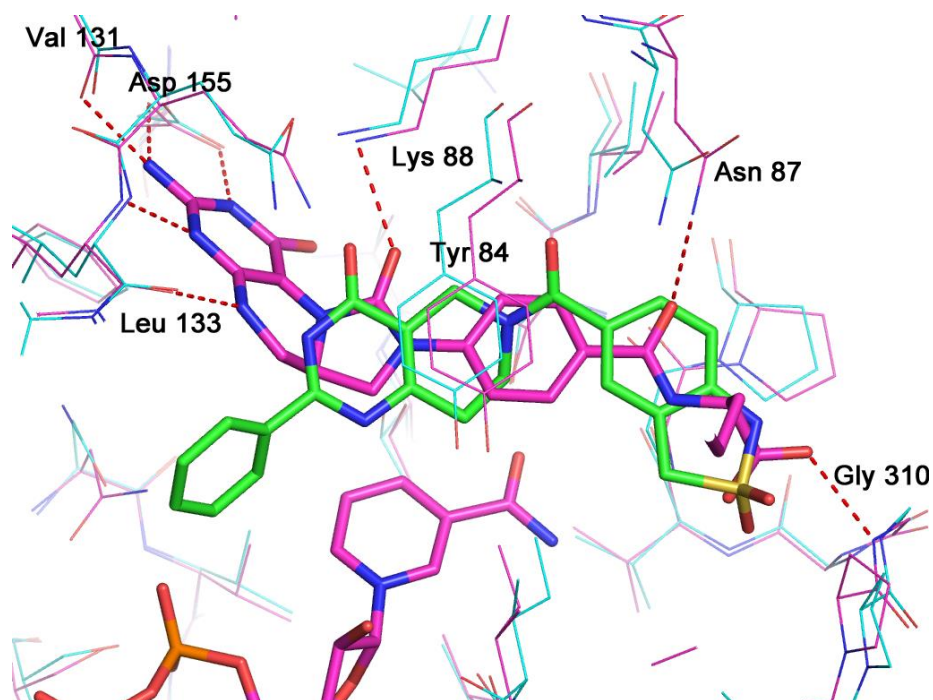
Figure 1-5. Superposition of the LY345899/NAD$^+$/MTHFD2 complex (PDB ID: 5TC4, magenta) and the compound **1**/MTHFD2 complex (green). Dashed red lines represent hydrogen bonding for the complex of LY345899.
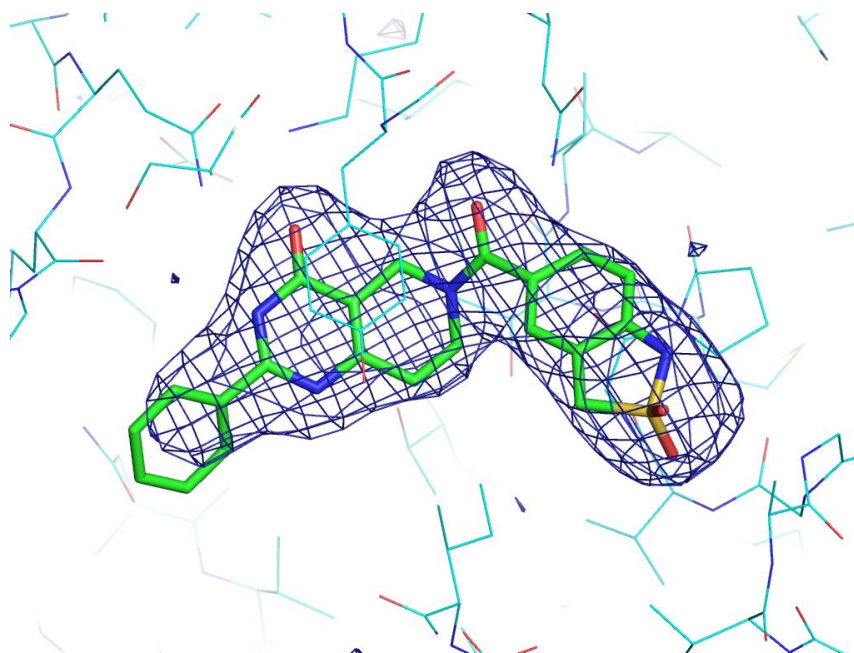

Figure 1-6. Electron density of the ligand in the **1**/MTHFD2 complex. Blue mesh represents Fo-Fc map contoured at 3.0σ which is calculated without model-bias of bound compound.

化合物 **1** のピリミジノン部位は、4 位のカルボニル基が 2 つの強固な水素結合に関わるとともに Tyr84 と π-π 相互作用をしていることから、結合能に重要であることが強く示唆された。一方で化合物 **1** の安息香酸アミド部位のカルボニル基はアイソザイム選択性に寄与しているものと推察された。Figure 1-4 (B)に示すように、化合物 **1** との主要な相互作用に関わる残基のうち、Asn87 のみがアイソザイム間で保存されておらず、MTHFD1 では Val55 に対応する[7]。このため MTHFD1 では化合物 **1** のカルボニル基とアミノ酸側鎖との水素結合が形成できず結合が弱まるため、MTHFD2 への選択性が生じていると考察した。後の検討により、実際にこのカルボニル基を除去した化合物 **S23** では MTHFD2 の阻害活性が消失することが分かっている（MTHFD2 IC$_{50}$ > 30 μM）。なお相互作用していない化合物 **1** 周辺残基には、Asn87 の他にも複数の非保存残基が存在する（Asn78 (Arg46 for MTHFD1), Ala80 (Asp48), Ser83 (Leu51), Glu141 (Thr111), Phe157 (Leu127), Asn204 (Ile176)など）。これらの残基とリガンドとの直接的な相互作用は見られないものの、これらの変化が選択性に影響を与えている可能性も十分に考えられる。

　化合物 **1** の末端フェニル基は Leu133, Pro134, Val205, Ile276 などの疎水性アミノ酸に囲まれた空間を占めている。化合物 **1** のフェニル基の電子密度は明確でなく、単結合の回転が見られることから、この空間の充填度には余裕があることが示唆された（Figure 1-6）。

1-4　末端スルタム部位の構造活性相関

　前節で得られた情報を念頭に、阻害剤の構造活性相関を取得することにした。

　はじめに化合物 **1** の末端スルタム部位を種々の置換基へと変換した（Table 1-1）。まずバイオアイソスターとしてテトラゾール誘導体 **2** と安息香酸誘導体 **3** を評価した[24]。テトラゾール誘導体では阻害活性は向上しなかったが、安息香酸誘導体では MTHFD2 阻害活性が 3 倍向上した。続いてより酸性度の低い置換基であるスルホンアミド誘導体（**4, 5**）、1,2,3-ベンゾトリアゾール（**6**）、1,2,4-オキサジアゾール-5-オン（**7**）をそれぞれ合成した。しかしながら、いずれの化合物も MTHFD2 の阻害活性を示さなかった。

　次に化合物 **3** の安息香酸周辺を変換した。オルト位への置換基導入は許容であり（**8, 9**）、特に電子求引基であるクロロ基を導入した化合物 **8** では阻害活性がさらに 3 倍程度向上した。1-ナフタレンカルボン酸への変換も許容であり、2 倍程度の活性向上が見られた（**10**）。一方でピリジン環の導入（**11, 12**）やシクロヘキサン環（**13**）への変換では阻害活性が低下した。また化合物 **1** のスルタムの *N*-メチル体（**14**）や、化合物 **3** のエステル体（**15**）およびカルバモイル体（**16**）を合成したが、いずれも阻害活性を示さなかった。

　これらの結果より、末端部位の酸性度が阻害活性に重要であること、および同部位には平面的な広がりを持つ置換基を許容しやすいことが明らかとなった。

Table 1-1. Structure–activity relationship of the sultam transformation.[a]
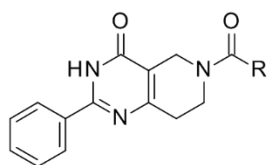
Compound	R	MTHFD2 IC$_{50}$ (μM)	Compound	R	MTHFD2 IC$_{50}$ (μM)
1		8.3	9		3.1
2		10	10		1.4
3		2.7	11		15
4		>30	12		10
5		>30	13		>30
6		>30	14		>30
7		>30	15		>30
8		0.94	16		>30

[a]IC$_{50}$ values for enzymatic assays. The method is described in the experimental section.

1-5　ピリミジノン母核の変換：合成

　続いて、前節にて得られた安息香酸誘導体 **3** を基に、ピリミジノン母核の変換を実施した。基本的な構造活性相関の取得に加えて、スキャフォールドホッピングによる母核の変換を行うことにした（Figure 1-7）[25]。ピリミジノンなどの環状アミド構造は、特に弱酸性を示す剥き出しの N-H の存在により、しばしば化合物の物性面に悪い影響を与えることが知られている [26]。そこでこのようなフリーの N-H を持たない母核を新たにデザインすることによって、潜在的な物性面の懸念を解消できると考えた。

　母核の変換に際しては、ピリミジノン 4 位のカルボニル基および芳香族性は維持することにした。これは、化合物 **1**/MTHFD2 複合体の X 線解析結果から、ピリミジノンのカルボニル基はタンパクとの 2 つの水素結合に、芳香族性は Tyr84 とのπ-π相互作用にそれぞれ関与しているため、同様の相互作用様式が阻害活性に必要だと考えたためである。他方、ピリミジノン 3 位のフリーの N-H についてはタンパクや水との主要な相互作用は見られないことから、変換可能であると推察した。さらに Figure 1-6 で見られたように末端フェニル基の電子密度は明瞭でなく、ポケットに密に充填されていないと推測されることから、様々な変換が許容されうると考えた。これらの情報を参考に、種々の母核のデザイン、合成を実施した。
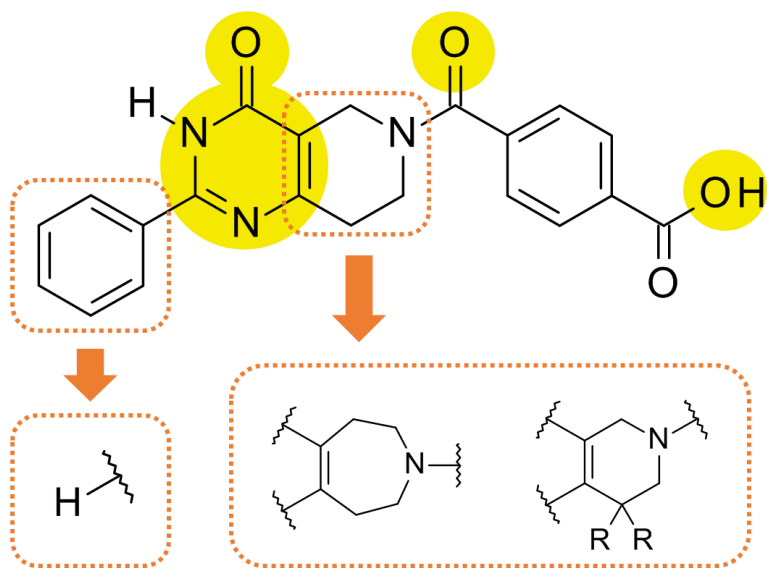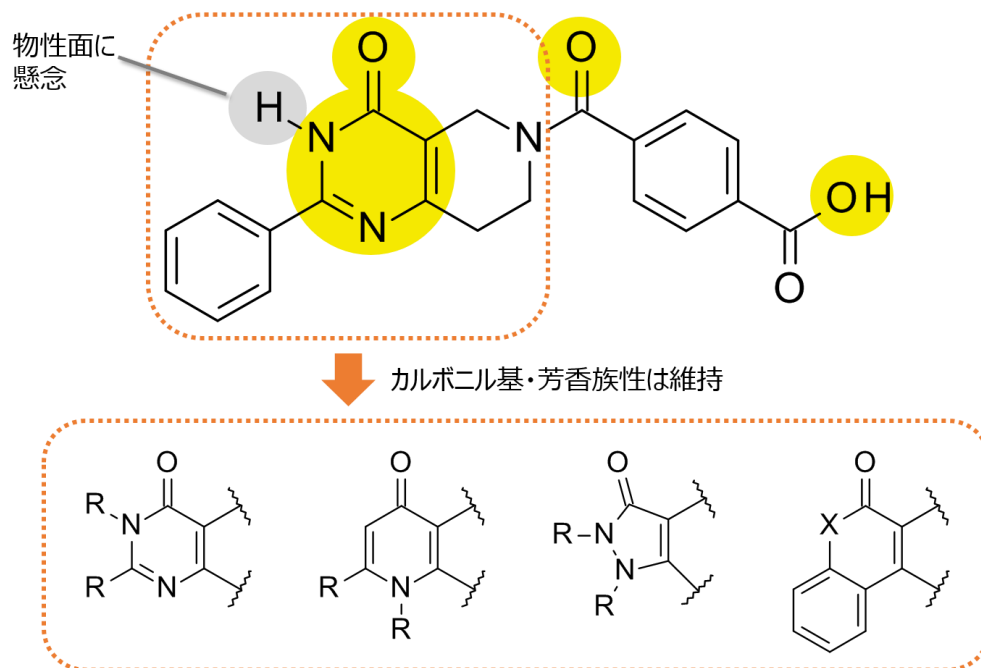
**Figure 1-7.** Summary of compound design in scaffold hopping (R: alkyl/aryl substituent(s); X: O or NH)

各種誘導体の合成法を Scheme 1-1 に示す。ピリミジノン誘導体については、対応するβ-ケトエステル **17** とアミジン **18** との縮合により骨格を構築後[27]、Boc 基の除去、テレフタル酸ハーフエステルとの縮合、続くメチルエステルの加水分解によって、化合物 **25–27, 29** に示す誘導体を得た。市販化合物より 1 工程で得られる中間体 **22** を用いることで、より短工程にて同様の誘導体（**24, 28**）を得ることもできる。同じ中間体 **22** をヒドラジン誘導体 **30** と縮合させることにより、ピラゾリノン骨格も構築可能であり、誘導体 **32–34** を得た[28]。4-ピリドン誘導体 **38** は以下の通り合成した。まず *N*-ベンジル-4-ピペリジノン **35** をエナミンへと変換した後[29]、ベンゾイル酢酸エチルと反応させることで二環性 4-ピラノン誘導体 **36** を得た[30]。メチルアミンによる置換反応により 4-ピリドン骨格を構築した後[31]、ベンジル基の除去、アミド化、および脱保護によって **38** が得られた。

Scheme 1-1. Synthesis of compounds with various core scaffolds.[a]
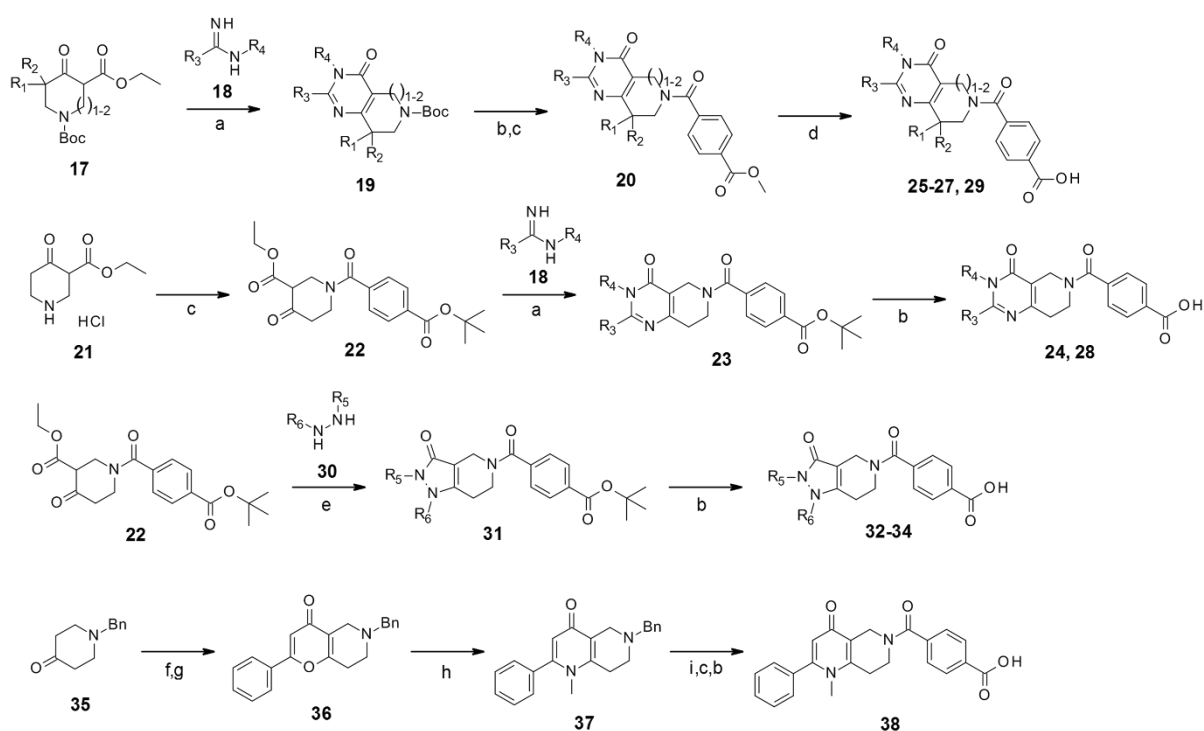
[a]Reagents and conditions: (a) K$_2$CO$_3$, EtOH; (b) HCl, 1,4-dioxane or TFA, CH$_2$Cl$_2$; (c) carboxylic acid, WSCI·HCl, HOBt, DMF or CH$_2$Cl$_2$; (d) 1 M NaOH, THF, rt; (e) EtOH, Et$_3$N; (f) morpholine, toluene, reflux; (g) ethyl benzoylacetate, xylenes, reflux; (h) MeNH$_2$, MeOH, 80ºC–100ºC; (i) Pd(OH)$_2$, H$_2$, 1M HCl, MeOH, rt.

Scheme 1-2. Synthesis of tricyclic coumarin derivatives.[a]
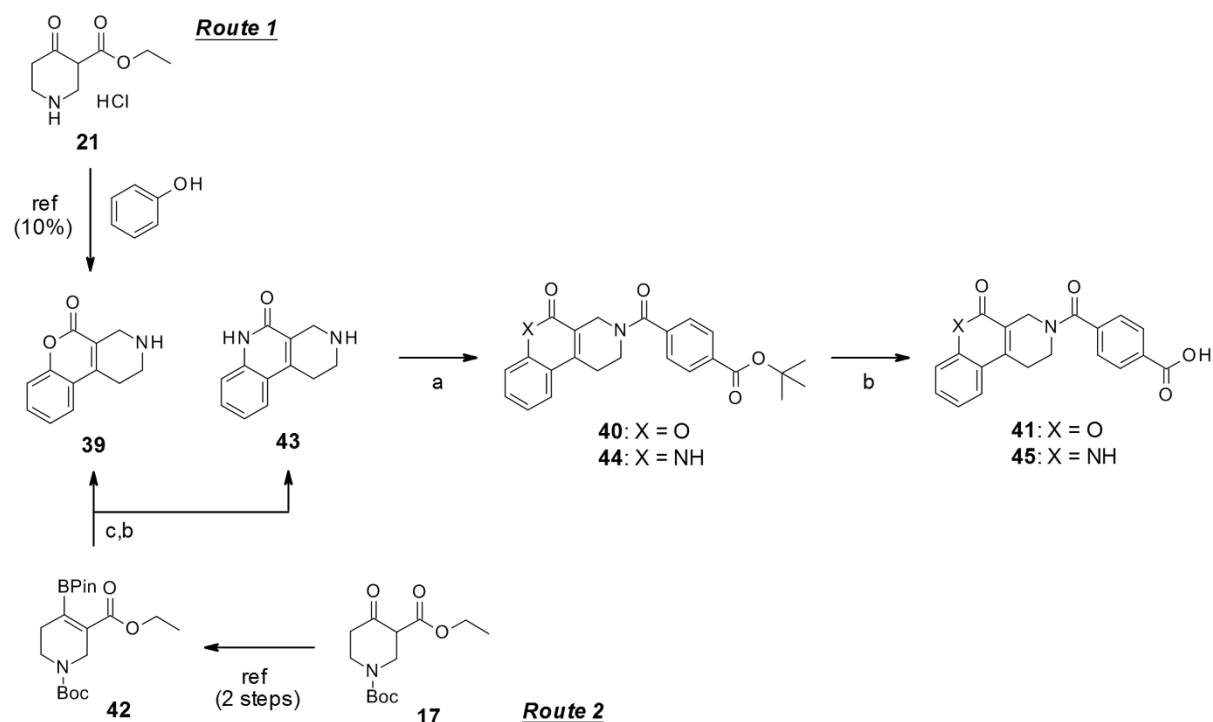
[a]Reagents and conditions: (a) 4-(*tert*-butoxycarbonyl)benzoic acid, WSCI·HCl, HOBt, CH$_2$Cl$_2$ or DMT-MM, MeOH; (b) HCl, 1,4-dioxane or TFA, CH$_2$Cl$_2$; (c) Pd(dppf)Cl$_2$, *o*-bromophenol or *o*-bromoaniline, NaHCO$_3$, THF–H$_2$O.

　クマリン誘導体の合成法をScheme 1-2に示す。三環性母核**39**はroute 1に示すようにβ-ケトエステル（**21**）を用いた既知のPechmann縮合反応により得ることができた[32]。しかしながら原料であるフェノールの反応性が低いために低収率（~10%）に留まった。

　そこでroute 2に示す別法を開発した。まずBoc保護したβ-ケトエステル（**17**）から既知の2工程によりボロン酸ピナコールエステル誘導体（**42**）を合成した[33,34]。**42**および*o*-ブロモフェノールを鈴木カップリング条件に付すことで、カップリング反応と6員環ラクトン形成が続けて進行し、Boc基を除去することで所望の三環性クマリン母核**39**が単一生成物として得られた。クマリン母核**39**から定法に従い化合物**41**を合成した。同様に環化反応の基質として*o*-ブロモフェノールに代えて*o*-ブロモアニリンを用いることで、ラクタムを有する誘導体（**45**）も合成することができた[35]。

18

1-6　ピリミジノン母核の変換：構造活性相関

　母格周辺を変換した化合物の酵素阻害活性をTable 1-2に示す。化合物**3**のフェニル基を除去した誘導体（**24**）ではMTHFD2の阻害活性が消失したことから、疎水性領域に存在するフェニル基が結合に一定程度寄与していることが示唆された。母核のピペリジンをホモピペリジンへと環拡大した誘導体（**25**）や、ピペリジン上にメチル基を導入した誘導体（**26**）、および*gem*-ジメチル基を導入した誘導体（**27**）はいずれもMTHFD2阻害活性を示さなかった。

　次に潜在的な物性改善を視野に、ピリミジノン3位のN-Hをアルキル化した化合物を評価した。先に述べた通り、このN-Hは特段の相互作用をしていないことが化合物**1**のX線構造解析によって示唆されている。そこで3位にメチル基を導入した誘導体（**28**）、および3位からメチレン鎖を2位へと伸ばした三環性誘導体（**29**）を評価した。その結果、化合物**28**では化合物**3**と比べて9倍ほどMTHFD2阻害活性が減弱したものの、化合物**29**では3倍ほどの減弱に留まり、弱いながらもMTHFD2阻害活性を維持した。さらに、同様にフリーのN-Hを持たない4-ピリドン誘導体（**38**）は、化合物**3**と同等の阻害活性を示した。これらの結果より、母核部分の水素結合ドナーは活性発現には必須でないと判断した。

　より構造の異なる母核であっても阻害活性が残ることが分かった。ピリミジノンに代えて5員環を有する3-ピラゾリノン誘導体**32**–**34**を評価した。その結果、置換基の嵩高さが増加するごとにMTHFD2阻害活性の向上が見られ、最も嵩高い化合物**34**では化合物**3**と同等以上の阻害活性を与えることが分かった。ピリミジノン2位におけるフェニル置換基の効果（**24** vs **3**）と同じく、化合物末端の脂溶性置換基の重要性が示唆された。最後に、化合物**3**のフェニル基を組み替えて三環性クマリン構造とした誘導体（**41**）を評価した。化合物**41**は評価した中で最も強いMTHFD2阻害活性を示した。同様にラクタム誘導体（**45**）も阻害活性を認めたが、阻害活性値は元のピリミジノン（**3**）より弱い結果となった。

　Table 1-2にてMTHFD2阻害活性を示した化合物に対しては、アイソザイムであるMTHFD1に対する阻害活性も評価した。その結果、評価したいずれの化合物もMTHFD1に対する阻害活性は認められず（$IC_{50} > 30$ μM）、母核変換後もMTHFD2に対する高い選択性を維持していることが分かった。このことから、合成した誘導体がいずれも化合物**1**で見られたタンパクとの結合様式を維持しているものと推察される。得られた誘導体の中から、阻害活性や分子量、合成展開の容易性などを総合的に考慮し、クマリン誘導体**41**をリード化合物として選抜した。

Table 1-2. Scaffold hopping of the core.[a]
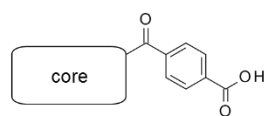
Compound	core	MTHFD2 IC$_{50}$ (μM)	MTHFD1 IC$_{50}$ (μM)
3		2.7	>30
24		>30	-
25		>30	-
26		>30	>30
27		>30	-
28		24	>30
29		8.2	>30
32		11	>30
33		6.6	>30
34		1.9	>30
38		2.3	>30
41		1.6	>30
45		4.6	>30

[a]IC$_{50}$ values for enzymatic assays. The method is described in the experimental section.

1-7　クマリン骨格を有する化合物 41 の詳細

　化合物**41**/MTHFD2複合体のX線結晶構造をFigure 1-8に示す。化合物**41**の結合モードは化合物**1**とほぼ同様であり、同一の残基との水素結合およびπ-π相互作用を維持していた。スルホンアミドの酸素原子の片方のみが水素結合を形成している化合物**1**と対照的に、化合物**41**のカルボン酸は2つの酸素原子がともにGly310主鎖のN-Hから3 Å程度に位置しており、いずれも結合に寄与していると推察される。
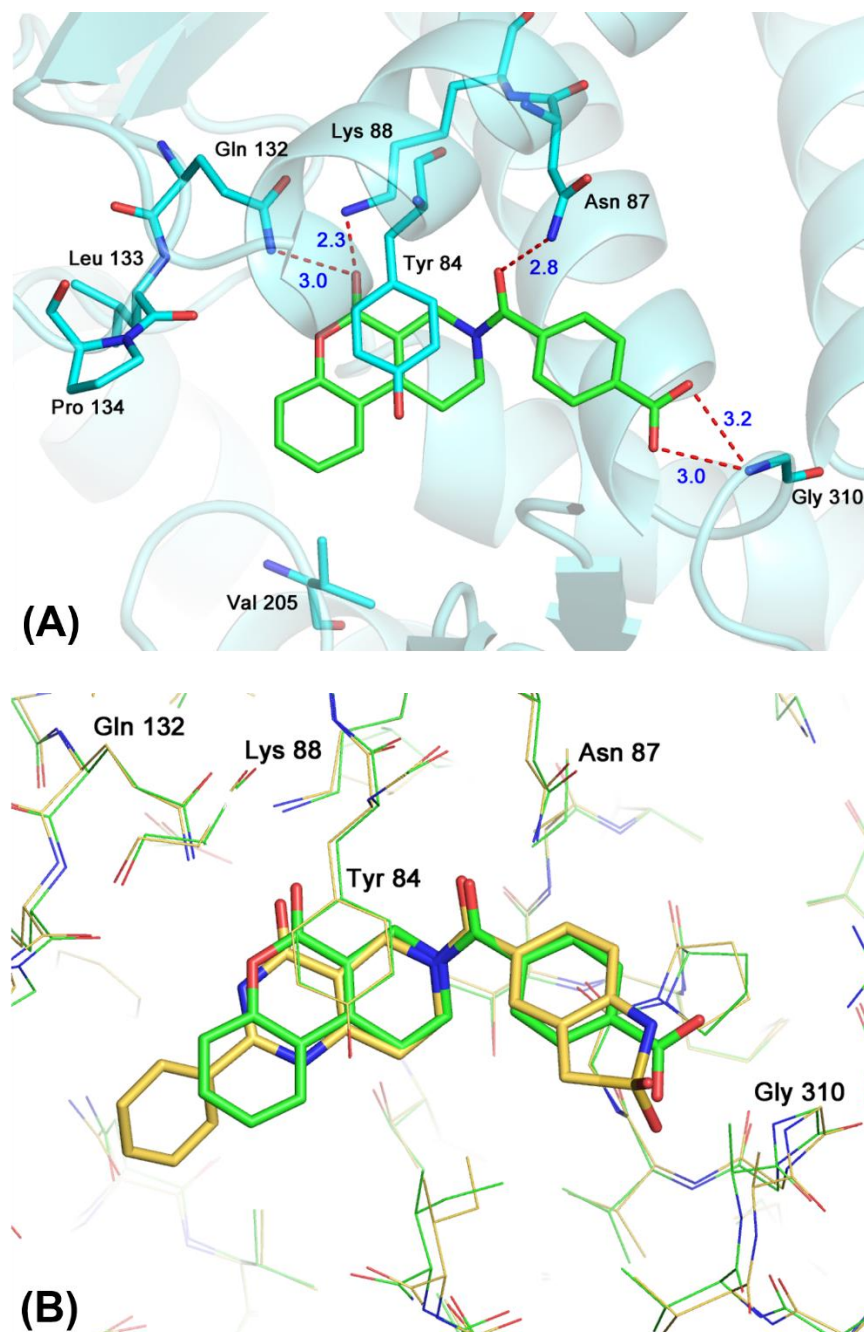

Figure 1-8. (A) X-ray analysis of the compound **41**/MTHFD2 complex refined at 2.25 Å resolution (PDB ID: 6JIB). Dashed red lines represent hydrogen bonding. Distances of the interactions are in ångströms. *R*-value = 0.2034. (B) Superposition of **41**/MTHFD2 (green) and **1**/MTHFD2 (yellow).

ヒット化合物**1**と同様、化合物**41**もMTHFD1を阻害せずMTHFD2のみ阻害する選択性を有していた。化合物**41**/MTHFD2複合体と報告されているLY345899/MTHFD1複合体の比較をFigure 1-9に示す。化合物**1**での考察と同じく、MTHFD2/MTHFD1選択性は主に化合物**41**とMTHFD2のAsn87（MTHFD1ではVal55に対応）との水素結合の存在に起因するものと考えられる。しかしながら、明確な相互作用は確認されないものの、阻害剤近傍の他の非保存残基も実際には選択性に寄与しているものと考えられる。
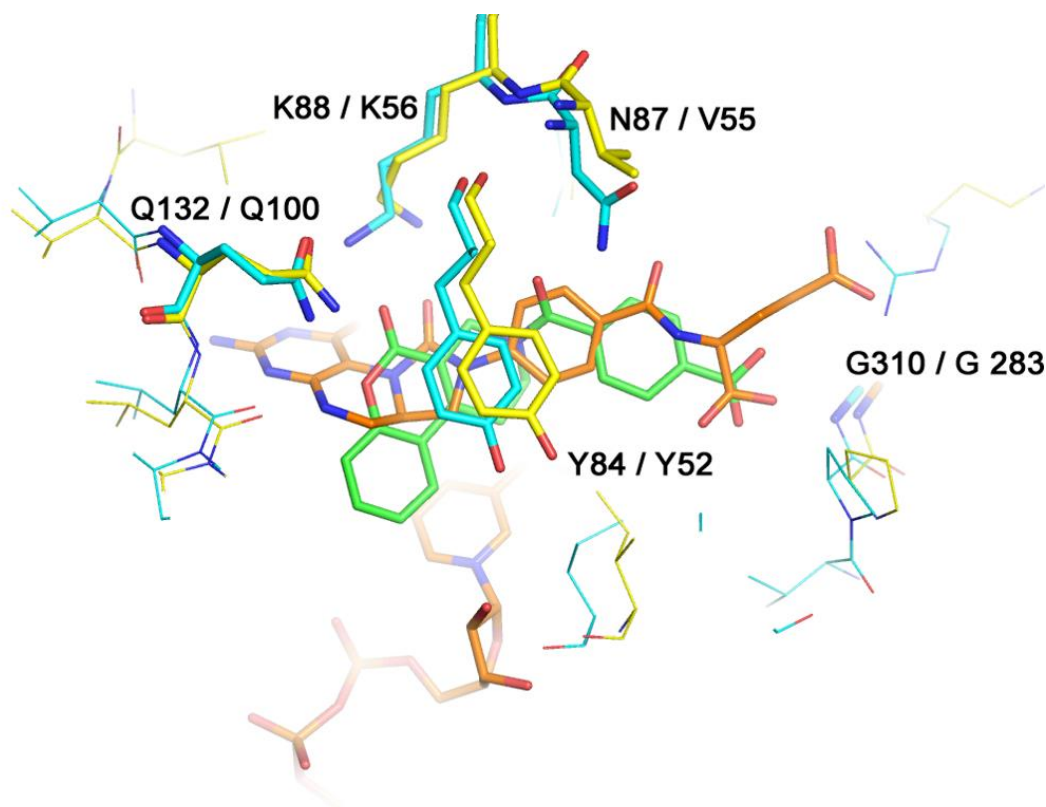

Figure 1-9. Superposition of **41**/MTHFD2 (PDB ID: 6JIB, green) and LY345899/NADP⁺/MTHFD1 (PDB ID: 6ECQ, orange). Amino acid residues within 5 Å from the ligand are displayed as line. The four residues described in the manuscript are highlighted with a caption of residue name for MTHFD2 (blue) / MTHFD1 (yellow).

　化合物**41**とHTSヒット**1**の基本プロファイルの比較をTable 1-3に示した。化合物**41**は化合物**1**と比べて阻害活性が向上し（MTHFD2 IC_{50} = 1.6 vs 8.3 μM）、分子量が低下している（349 vs 422）。これによりリガンド効率（LE）は0.24から0.31へ上昇している。一般にLE ≥ 0.3が良好なバインダーの指標とされることから[36,37]、化合物**41**はより有望なバインダーであると判断できる。同時に化合物**41**への変換によって極性表面積（tPSA）や水素結合ドナー数（#HBD）、水素結合アクセプター数（#HBA）も低下している。このことは化合物**41**が細胞膜透過により有利であり、経口吸収性がより期待できることを示唆している[22,38]。以上より、化合物**41**はヒット化合物と比べて、初期リード化合物として優れたプロファイルを有しており、最適化研究の出発点として適するものと考えられた。

Table 1-3. Profiles of compound **1** and **41**
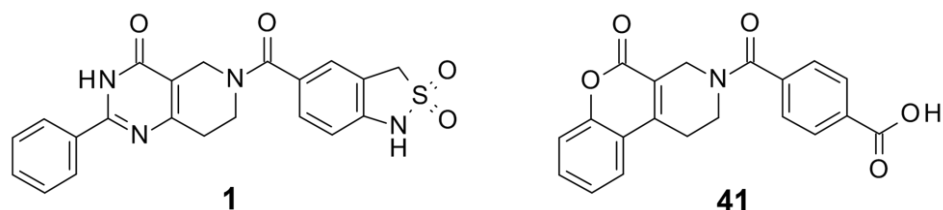
Compound	MTHFD2 IC$_{50}$	Mw	LE	tPSA	#HBD	#HBA
1	8.3 μM	422	0.24	112	2	7
41	1.6 μM	349	0.31	88	1	6

1-8　小括

　MTHFD1を阻害せずMTHFD2を選択的に阻害する新規骨格の獲得を目指して研究を実施した。HTSにより葉酸ポケットを占有する新規ピリミジノン誘導体1がヒットとして得られ、MTHFD2に対する中程度の阻害活性、およびMTHFD1に対する高い選択性が確認された。複合体のX線結晶構造解析の結果、化合物1は葉酸誘導体のプテリジンを除いた部分と同様の結合様式を取ることが判明した。

　基本SARを取得したところ、阻害活性の維持には末端の酸性置換基が重要であることが示唆され、安息香酸誘導体がより高活性を与えることを見出した。さらに阻害活性の向上と潜在的な物性改善を目的に、母核の変換を実施し、ピリミジノンに代えてクマリン骨格を有する化合物41を獲得した。化合物41は化合物1と同様の結合モードでMTHFD2タンパクに結合することが確認され、MTHFD1に対する選択性も維持することが分かった。さらにヒット化合物と比べて、低分子量ながらMTHFD2阻害活性が向上し、膜透過性に関わるとされるin silicoパラメータの改善が見られた。これらのことから、化合物41は選択的MTHFD2阻害剤として有用な初期リード化合物であると考えられる。

第 2 章　Structure-based drug design による高活性な MTHFD2 阻害薬の獲得

2-1　背景

　第1章にてMTHFD2選択的な阻害剤である新規クマリン誘導体**41**を獲得した。しかしながら化合物**41**は、既報のMTHFD2阻害剤に比べて阻害活性が弱く、阻害剤の薬理作用を検証する目的ではまだ不十分であると考えられる。そこで、さらなる阻害活性の向上を目指し、化合物**41**を起点とした誘導体展開を実施することにした。

　クマリン骨格周辺のSARを取得するにあたり、まず化合物**41**/MTHFD2複合体の結晶構造情報を整理することにする（Figure 2-1）。第1章にて指摘したように、化合物**41**はMTHFD2の葉酸ポケットを占有するが、その結合モードはやや異なる。すなわち、葉酸誘導体では末端プテリジン部位がVal131、Leu133、Asp155からなるポケットを占有して高度な水素結合ネットワークを形成しているが（Figure 1-5）、化合物**41**はこの親水性の領域を占有することなく、葉酸誘導体のグルタミル安息香酸部位と重なるようにポケットを占有している（Figure 1-8）。化合物**41**の主要な相互作用は第1章で示した4つの水素結合およびTyr84とのπ-π相互作用であり、特にクマリン部位において芳香族性と水素結合アクセプターとなるカルボニル酸素の存在が活性発現に重要であることを指摘した。このような背景から、阻害活性の向上を目指すにあたっては、化合物**41**の三環性クマリン構造を維持して展開することとした。

　一方で、クマリンの炭素上は修飾可能であると考えた。クマリンのベンゼン環周辺には比較的広い空間が存在している（Figure 2-1）。この空間はLeu133、Pro134、Val205、Ile276などの疎水性アミノ酸残基に囲まれているため、クマリン部位に疎水性置換基を導入して空間を埋めることができれば、阻害活性が向上するものと期待した。さらに空間の端はタンパク外側へと抜けているため、クマリン8位あるいは9位の方向から置換基を伸長することで、近傍の疎水性残基だけでなく、遠隔位の親水性残基との相互作用も狙うことができると考えた。
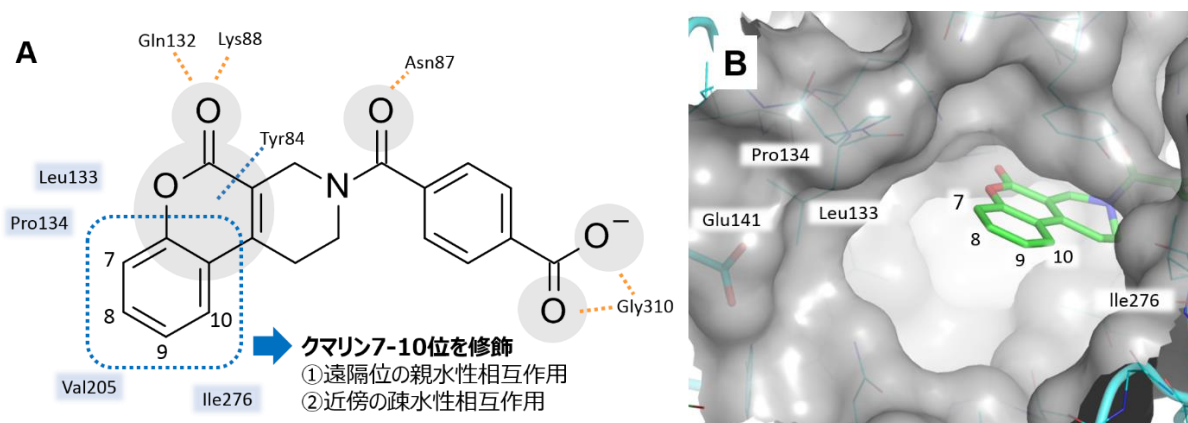

Figure 2-1. Detailed structure analysis around the benzene ring of the coumarin core in **41**/MTHFD2 complex (PDB ID: 6JIB). (A) Summary of the interactions and location of key amino acid residues. (B) Protein surface composed of the amino acid residues within 12 Å around the ligand. Location of key amino acid residues is also displayed.

以上を元に、クマリン上への置換基検討として以下の2つの戦略を実践することにした。すなわち、①Glu141との遠隔位静電相互作用を狙いクマリン8位からアミン側鎖を伸長する、②クマリン周辺の疎水性環境を埋めるため7, 9, 10位へメチル基を導入する、という戦略である（Figure 2-2）。
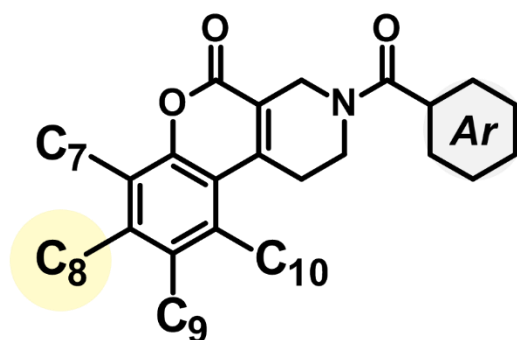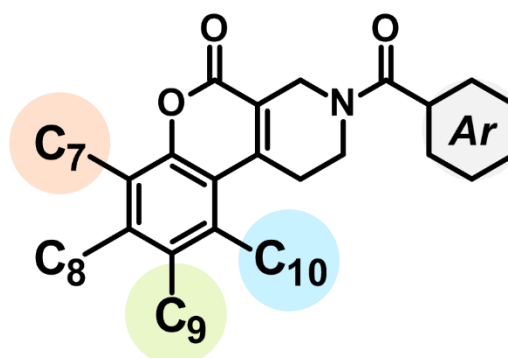

Figure 2-2. Strategies for modification of 7–10 position of the coumarin core

2-2　クマリン 8 位への置換基導入検討

1つ目の戦略として、クマリン骨格8位にアミノ基を有する側鎖の導入を検討した。化合物41/MTHFD2のX線構造情報により、この部分に塩基性のアミノ基を付与することで、遠隔位ではあるがGlu141残基との水を介した静電相互作用が期待できると考えた。

検討結果をTable 2-1に示す。まずクマリン骨格8位に*N,N*-ジメチル-2-アミノエトキシ基を導入した化合物**46**を合成し、評価した。この結果、化合物**46**は化合物**41**に比べてMTHFD2に対する阻害活性値が4.7倍向上することが見出された。この際、MTHFD1に対する阻害活性も化合物**41**に比べて強くなってしまったものの（MTHFD1 IC_{50} = 18.7 vs >30 µM）、それでも55倍という高いアイソザイム選択性を維持していた。側鎖を*N,N*-ジメチル-3-アミノプロポキシ基へと伸長した化合物**47**や、さらにピペリジンへと環化した化合物**48**においても、化合物**46**と同様の結果を示した。ベンジルアミン型の側鎖を有する化合物**49**では、MTHFD2への阻害活性は化合物**46**と同等であったものの、アイソザイム選択性がおよそ16倍へと低下した。より塩基性の低いアニリン誘導体**50**では、置換基導入による阻害活性向上効果は見られず、活性向上には一定程度に強い塩基性が必要であることが示唆された。最後に4-メチルピペラジニル基を導入した化合物**51**を評価した。その結果、化合物**51**は化合物**46–49**と同等のMTHFD2阻害活性を示し、さらにMTHFD1に対する阻害活性が低下したことで、アイソザイム選択性が>93倍へと向上することが見出された。

Table 2-1. SAR of the 8-position of the tricyclic coumarin.[a,b]
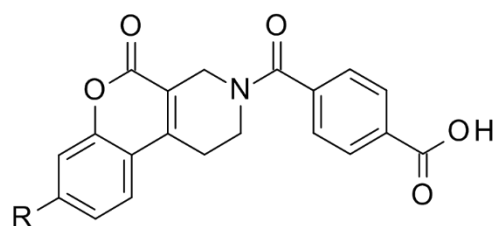
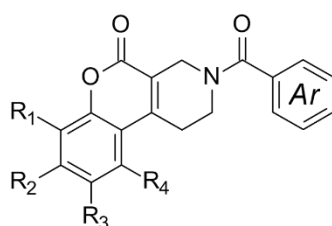| Compound	R	MTHFD2 IC$_{50}$ (µM)	MTHFD1 IC$_{50}$ (µM)	Selectivity
41	H	1.6	>30	>18
46		0.34	18.7	55
47		0.36	27	75
48		0.39	23	59
49		0.34	5.4	16
50		3.8	>30	>7
51		0.32	>30	>93

[a]IC$_{50}$ values for enzymatic assays. The method is described in the experimental section.
[b]Selectivity represents the ratio of MTHFD1 IC$_{50}$/MTHFD2 IC$_{50}$.

2-3 クマリン 7–10 位に対するメチルスキャンの検討

　次に2つ目の戦略として、クマリンの他の位置、すなわち7位、9位、および10位における置換基効果を検討した。先に述べたとおり、化合物**41**/MTHFD2のX線結晶構造解析により、クマリンのベンゼン環は疎水性のポケットを十分に埋めていないことが推察される。そこでクマリン7, 9, 10位に対するメチルスキャンを実施した[39]。

Table 2-2. Methyl scanning on coumarin and combination with the sultam.[a,b]

Compound	R_1	R_2	R_3	R_4	Ar	MTHFD2 IC_{50} (μM)	MTHFD1 IC_{50} (μM)	Selectivity
41	H	H	H	H		1.6	>30	>18
46	H		H	H		0.34	18.7	55
52	H	H	H	Me		1.3	>30	>23
53	H		Me	H		>30	>30	ND
54	Me		H	H		0.049	5.0	102
55	Me		H	H		0.053	8.4	158
56	Me		H	H		0.018	5.2	289

[a]IC_{50} values for enzymatic assays. The method is described in the experimental section. [b]Selectivity represents the ratio of MTHFD1 IC_{50}/MTHFD2 IC_{50}.

検討結果をTable 2-2に示す。まず化合物**41**の10位（R$_4$）にメチル基を導入した化合物**52**を合成、評価したところ、阻害活性は元の化合物**41**と同等であり、10位へのメチル基導入に大きな効果は見られなかった。続いて化合物**46**の9位（R$_3$）にメチル基を導入した化合物**53**、および7位（R$_1$）にメチル基を導入した化合物**54**をそれぞれ合成し、阻害活性を化合物**46**と比較した。9位にメチル基を導入した化合物**53**では予想に反し、MTHFD2阻害活性が消失した。一方で7位にメチル基を導入した化合物**54**を評価したところ、MTHFD2阻害活性は化合物**46**に比べて7倍向上することが判明した。これらの結果より、クマリン7位へのメチル基導入のみが阻害活性を大きく向上させることを見出した。7位メチル基がクマリン周辺の疎水性空間を適切に埋めたことで、阻害活性の向上に繋がったものと考察している。

　更なる活性向上を狙い、置換基の組み合わせ最適化を検討した。第1章にてHTSヒットとして得られたスルタム型置換基を7-メチルクマリン構造に導入し、クマリン8位に*N,N*-ジメチル-2-アミノエトキシ基を有する化合物**55**、および4-メチルピペラジニル基を有する化合物**56**をそれぞれ合成した。化合物**55**は化合物**54**と同等のMTHFD2阻害活性、およびより高いアイソザイム選択性を与えた。さらに化合物**56**は、これまでに検討した化合物の中で最も高いMTHFD2阻害活性（IC$_{50}$ = 0.018 μM）およびアイソザイム選択性（289倍）を与えた。

　ここで末端酸性基と母核の組み合わせについて整理する。第1章にてピリミジノン母核に対して検討した際には、末端置換基としては安息香酸が最適であり、スルタムに比べて3倍ほど強いMTHFD2 IC$_{50}$値を示した（**3** (2.7 μM) vs **1** (8.3 μM), Table 1-1）。一方で今回、7-メチルクマリン構造に対する検討では、安息香酸型（**54**, 0.049 μM）とスルタム型（**55**, 0.053 μM）で阻害活性がほとんど変化していない。明確な理由は不明なものの、母核の選択によってSARが若干変化している可能性が考えられる。

　本検討で得られた化合物**56**は、リード化合物**41**と比べてMTHFD2阻害活性が89倍向上し、高い選択性を示す、理想的な阻害活性プロファイルを有する化合物であると考えられる。

2-4　7–10位置換クマリン誘導体の合成

　クマリン8位に置換基を有する誘導体の合成法をScheme 2-1に示す。まずβ-ケトエステル**21**とレゾルシノール**57**の混合物に64%硫酸を作用させ、Pechmann縮合体**58**を硫酸塩として得た[40]。なお第1章にてフェノールを用いて同様の反応を行った際には目的物は低収率にとどまったが、反応性の高いレゾルシノールを用いた場合には高収率で目的物が得られた。続いてテレフタル酸モノ*tert*-ブチルとの縮合により、共通中間体であるアミド体**59**を得た。化合物**59**のフェノール性水酸基に対して、S_N2反応あるいは光延反応によりアルキル基を導入して**60a–c**とし、酸性条件にて*tert*-ブチル基を除去することでクマリン8位に炭素－酸素結合を有する**46–48**を得た。炭素－炭素結合あるいは炭素－窒素結合を有する誘導体については、化合物**59**を対応するトリフラート**61**へと変換した後、アリールボロン酸ないしボロン酸エステルを用いる鈴木カップリングあるいはBuchwaldアミノ化反応を行い、上述と同様に脱保護することにより**49–51**の誘導体を得ることができた。
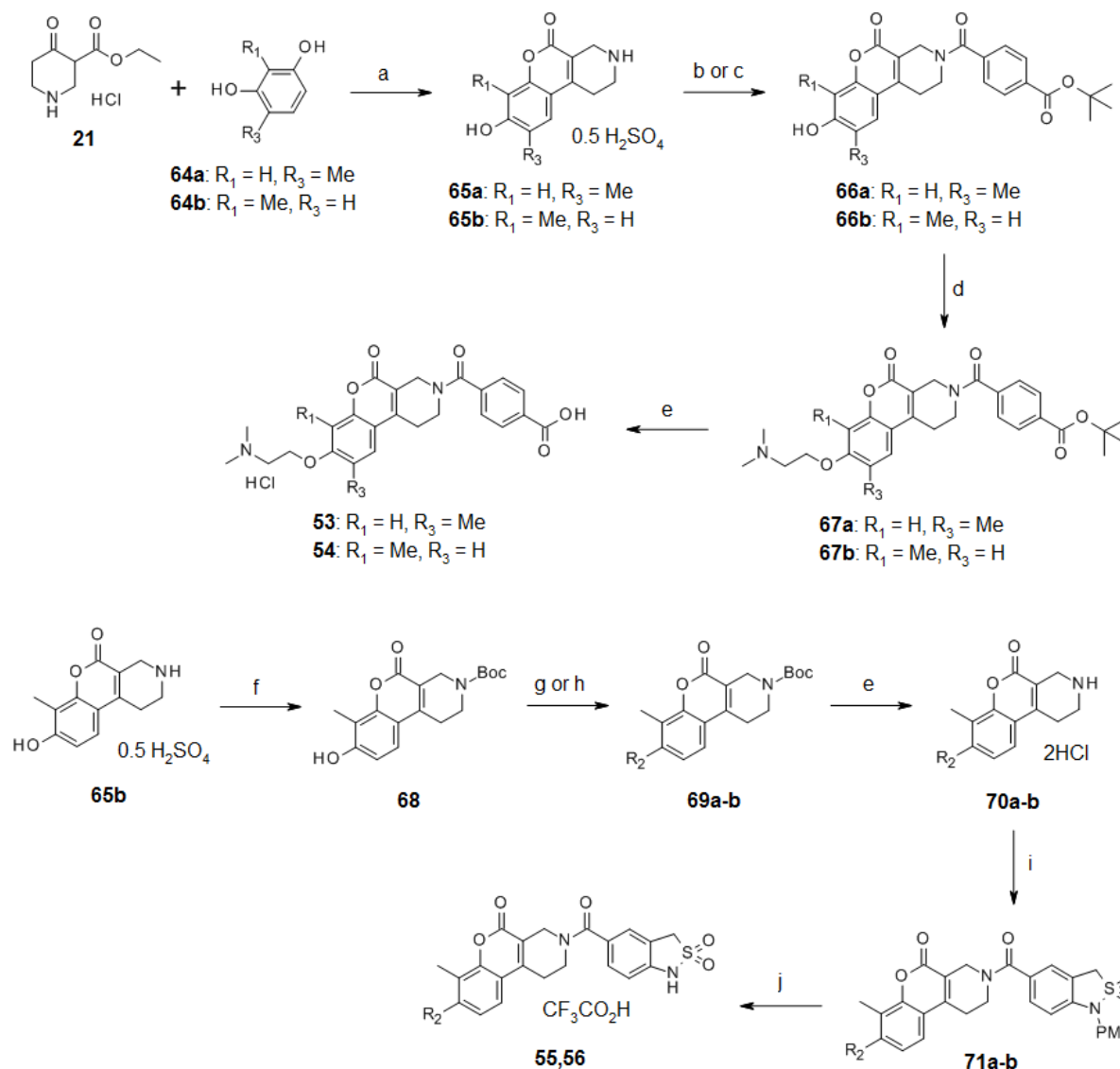
Scheme 2-1. Syntheses of the initial C8-substituted derivatives.[*a*]
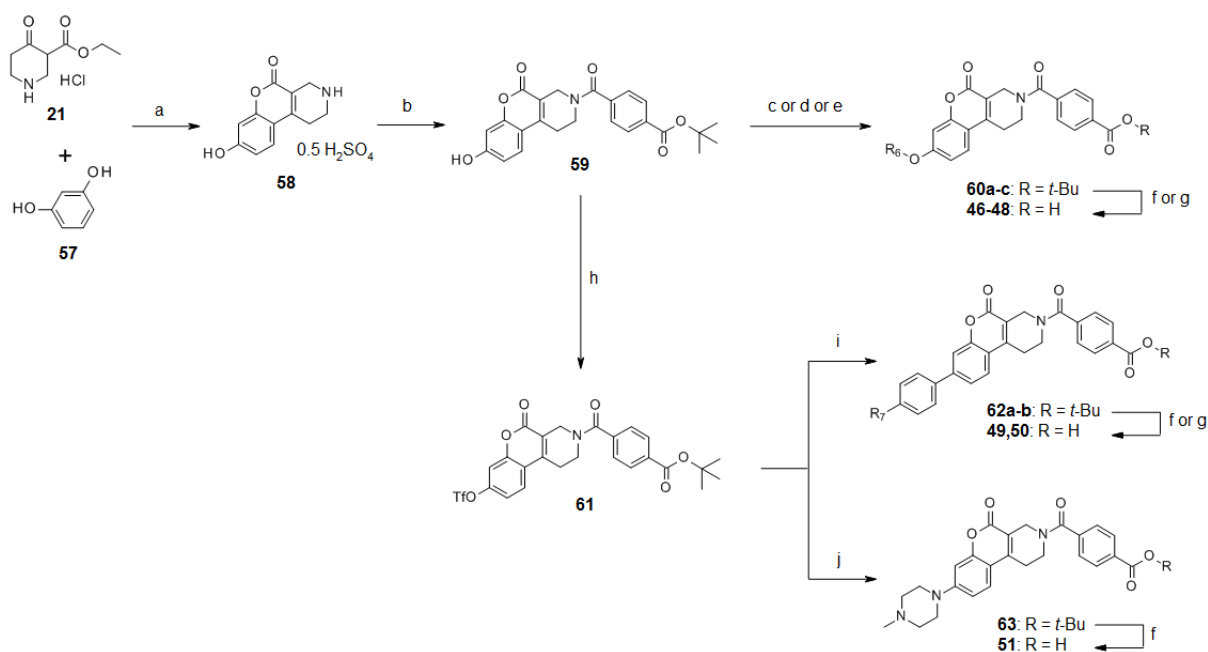
[*a*]Reagents and conditions: (a) 64% H_2SO_4, rt; (b) 4-(*tert*-butoxycarbonyl)benzoic acid, DMT-MM, 4-methylmorpholine, MeOH, rt; (c) 2-dimethylaminoethyl chloride hydrochloride, K_2CO_3, DMF, 120 °C; (d) 3-dimethylamino-1-propanol, diisopropyl azodicarboxylate, PPh_3, THF, rt; (e) 4-hydroxy-1-methylpiperidine, 1,1'-(azodicarbonyl)dipiperidine, *n*Bu_3P, THF, 50–60 °C; (f) 4N HCl in 1,4-dioxane, rt; (g) TFA, DCM, rt; (h) Tf_2O, pyridine, DCM, 0 °C; (i) boronic acid or boronic ester, Pd(dppf)Cl_2–DCM adduct, $NaHCO_3$, 1,4-dioxane, water, 90–100 °C; (j) 1-methylpiperazine, RuPhos Pd–G1 *t*BuOMe adduct, RuPhos, Cs_2CO_3, toluene, 110 °C.

9位あるいは7位にメチル基を有する誘導体（**53, 54**）は、化合物**46**と同様の合成法により調製した（Scheme 2-2）。すなわち、レゾルシノールに代えて4-メチルレゾルシノール（**64a**）、あるいは2-メチルレゾルシノール（**64b**）を用いることで、8-ヒドロキシ-9-メチルクマリン誘導体（**65a**）、および8-ヒドロキシ-7-メチルクマリン誘導体（**65b**）をそれぞれ得た。アミド化、光延反応、続く*tert*-ブチル基の除去により、対応する化合物**53, 54**を合成した。スルタムを有する誘導体の合成では、合成経路に若干の変更を加えた。すなわち、まず8-ヒドロキシ-7メチルクマリン誘導体**65b**の二級アミンに対して、二炭酸ジ*tert*-ブチルを作用させてBoc基を導入した（**68**）。Scheme 2-1と同様の条件を用いてフェノール性水酸基をアルキル化した後[41]、Boc基を除去して**70a–b**とした。*p*-メトキシベンジル（PMB）基で保護されたスルタムを有する安息香酸誘導体と縮合した後、トリフルオロ酢酸にてPMB基を除去することで所望のスルタム誘導体**55, 56**を得た。

Scheme 2-2. Syntheses of C7-, C9-, and C10-substituted derivatives.[a]

[a]Reagents and conditions: (a) 64% H_2SO_4, rt; (b) 4-(*tert*-butoxycarbonyl)benzoic acid, DMT-MM, 4-methylmorpholine or Et_3N, MeOH, rt; (c) 4-(*tert*-butoxycarbonyl)benzoic acid, WSCI·HCl, HOBt, Et_3N, DMF, rt; (d) 2-(dimethylamino)ethanol, diisopropyl azodicarboxylate, PPh_3, THF, rt–60 °C; (e) 4N HCl in 1,4-dioxane, rt; (f) Boc_2O, 1N aq. NaOH, aq. $NaHCO_3$, THF, rt; (g) 2-dimethylaminoethyl chloride hydrochloride, K_2CO_3, acetone, reflux; (h) i. Tf_2O, pyridine, DCM, 0 °C; ii. 1-methylpiperazine, RuPhos Pd–G1 *t*BuOMe adduct, RuPhos, Cs_2CO_3, toluene, 110 °C; (i) 1-[(4-methoxyphenyl)methyl]-2,2-dioxo-3*H*-2,1-benzothiazole-5-carboxylic acid, WSCI·HCl, HOBt, Et_3N, DCM, rt; (j) TFA, rt.

Scheme 2-2と同様の方法にて8-ヒドロキシ-10-メチルクマリン誘導体の合成を試みた。しかしながら、対応する5-メチルレゾルシノール（**64c**）を用いたPechmann縮合では、望みの異性体（**72a**）は生成されず、望まない10-ヒドロキシ-8-メチルクマリン誘導体（**72b**）が生成された（Scheme 2-3）。そこで10-メチル基の効果については、8位が無置換の誘導体を合成して検証することにした。10-メチルクマリン誘導体**52**の合成には、第1章にて開発した別法を用いた。まずピナコールボレート誘導体**42**と2-ブロモ-3-メチルフェノールを鈴木カップリング条件に付すことで、三環性骨格が構築された化合物**73**を単一生成物として得た。すでに述べた合成法と同様に、Boc基の除去、アミド化、続く*tert*-ブチル基の除去により所望の誘導体**52**を合成した。

Scheme 2-3. Synthesis of compound **52**.*a*
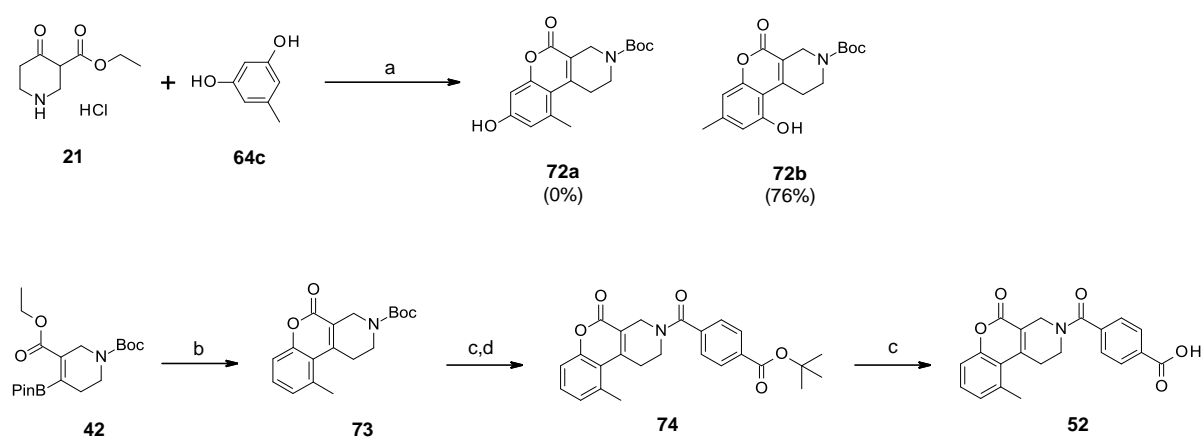
*a*Reagents and conditions: (a) i. 70% perchloric acid, rt; ii. 5N aq. NaOH, Boc$_2$O, THF, rt; (b) 2-bromo-3-methylphenol, Pd(dppf)Cl$_2$–DCM adduct, NaHCO$_3$, THF, water, 100 °C.; (c) 4N HCl in 1,4-dioxane, rt; (d) 4-(*tert*-butoxycarbonyl)benzoic acid, DMT-MM, Et$_3$N, MeOH, rt.

2-5　高活性化合物 56 の細胞増殖阻害評価

　良好なMTHFD2酵素阻害活性および高いアイソザイム選択性を与える化合物**56**が得られたことから、細胞系を用いた化合物の評価を行うこととした。乳癌由来細胞株であるMDA-MB-231株を用いた細胞増殖阻害試験に付したところ、選択的MTHFD2阻害剤**56**は細胞増殖阻害活性を示すことが明らかとなった（$GI_{50} = 19\,\mu M$）。しかしながらその増殖阻害活性は酵素阻害活性（$IC_{50} = 0.018\,\mu M$）に比べて3桁ほど弱く、細胞系での高活性発現には化合物の物性面の改善など、さらに検討を要することが示唆された（Figure 2-3）。
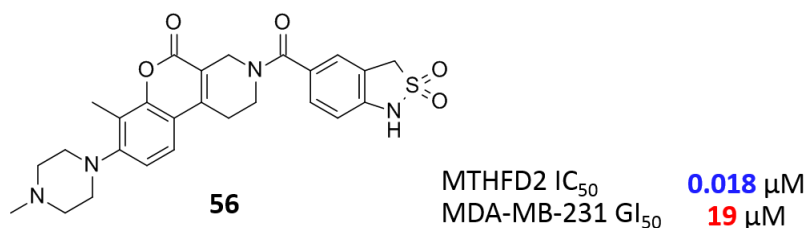

MTHFD2 IC_{50}	**0.018** μM
MDA-MB-231 GI_{50}	**19** μM

Figure 2-3. Significant gap was observed between cell-free MTHFD2 IC_{50} and cell-based GI_{50} values for the potent inhibitor **56**.

2-6　小括

　リード化合物**41**の高活性化を目指し、クマリン上の置換基を検討した。結晶構造を参考に、8位に塩基性置換基を、7位にメチル基をそれぞれ導入することで、MTHFD2の酵素阻害活性が向上することを発見した。さらに末端酸性部位をスルタムとすることで、高活性かつ高いアイソザイム選択性を示す化合物**56**を得た。しかしながら得られた化合物**56**の細胞増殖阻害活性は酵素阻害活性に比して1,000倍以上弱く、細胞系での作用確認には酵素阻害活性以外のパラメータの改善が必要であると考えられた。

第3章　In vivo で抗腫瘍効果を示す MTHFD2 阻害薬の獲得

3-1　背景

　第2章にて獲得した化合物**56**は高い酵素阻害活性にも関わらず、細胞系にて高い活性を示さなかった。細胞系での活性減弱の原因は化合物**56**の物性、特に膜透過性にあると推察し、物性改善を図ることとした（Figure 3-1）。

　膜透過性の指標であるPAMPAを測定したところ、化合物**56**は非常に低い値を示すことがわかった（PAMPA Pe (pH 7.4): < 0.1）。膜透過性の低さは、化合物**56**のスルタム部位の高い酸性度（pK_a 5.7）に起因すると考察した。このような酸性基を有する化合物は、生理的条件化にて容易に脱プロトン化してアニオンとなるため、膜透過性低下の原因になり得ることが一般的に知られている[42]。化合物**56**の膜透過性を改善させる方策として、スルタムの酸性度を低下させることで、生理的条件化における分子形の比率を上げることが有効であると考えた。そこでMTHFD2阻害活性に影響を与えない、スルタムの代替基を探索することにした。
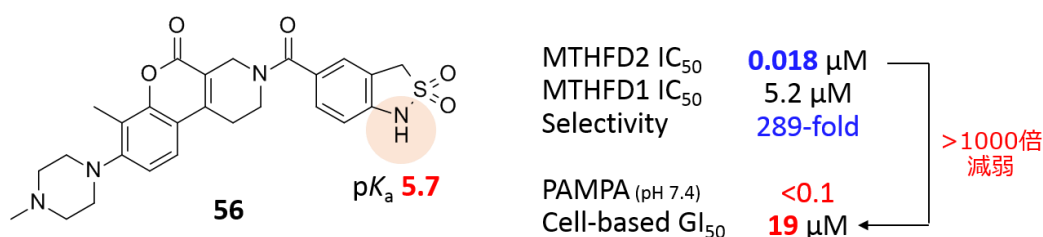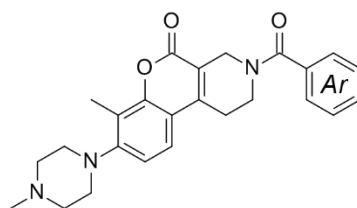

MTHFD2 IC$_{50}$　**0.018** μM
MTHFD1 IC$_{50}$　5.2 μM
Selectivity　289-fold

PAMPA (pH 7.4)　<0.1
Cell-based GI$_{50}$　**19** μM

>1000倍減弱

Figure 3-1. Summary of the profiles of compound **56**.

3-2　スルタムを代替する置換基の探索

　第1章での検討により、スルタム部分を非酸性基へと変換すると、多くの場合MTHFD2の酵素阻害活性が大きく減弱することが分かっている。そこでTable 1-1での検討結果を注意深く再考したところ、スルホンアミドを導入した化合物**5**（MTHFD2 IC$_{50}$ > 30 μM）について、最大用量の30 μMにて弱いながらも阻害活性が見られることに注目した。そこで一定程度の活性が維持できるのではないかと期待して、高活性な7-メチルクマリン骨格に対してスルホンアミドを導入した誘導体を合成することにした。

Table 3-1. SAR of the sultam and sulfonamide derivatives.[a,b]

Compound	Ar	MTHFD2 IC$_{50}$ (µM)	MTHFD1 IC$_{50}$ (µM)	selectivity	pK_a (acid)	PAMPA Pe pH 7.4 (×10^{-6} cm/s)	MLogP/LipE	MDA-MB-231 GI$_{50}$ (µM)
56	(benzisothiazole sultam)	0.018	5.2	289	5.7	<0.1	1.6/6.1	19
75	(phenyl methanesulfonamide)	0.2	>30	>150	8.2	5.4	1.6/5.1	6.3
76	(Me-substituted)	0.53	>30	>56	8.3	23.1	1.8/4.4	7.7
77	(OMe-substituted)	0.4	>30	>75	8.4	44.2	1.6/4.8	10
78	(F-substituted)	0.038	8	211	6.9	16.9	1.7/5.7	2.0
79	(Cl-substituted)	0.048	6.4	133	6.8	31.1	1.8/5.5	0.94
80	(Br-substituted)	0.033	4.2	127	6.8	>50	1.9/5.5	1.6
81	(OCF$_3$-substituted)	0.021	2.9	138	6.8	43.7	1.9/5.8	0.38
82	(Cl-substituted)	0.089	>30	>337	N.D.	6.6	1.8/5.2	5.5
83	(Br-substituted sultam)	0.017	3.1	182	N.D.	N.D.	1.9/5.8	>30

[a]IC$_{50}$ values for enzymatic assays. The method is described in the experimental section.
[b]Selectivity represents the ratio of MTHFD1 IC$_{50}$/MTHFD2 IC$_{50}$. N.D.: no data.

スルホンアミド誘導体の検討結果をTable 3-1に示す。化合物56のスルタムに代えてスルホンアミドを導入した化合物75は、予想通りMTHFD2阻害活性が11倍減弱した。一方で、膜透過の指標であるPAMPAの値は非常に大きく改善することが分かった（Pe (pH 7.4): 5.4）。この結果、酵素阻害活性が11倍低いにも関わらず、化合物75のMDA-MB-231細胞に対する増殖阻害活性は、化合物56に比べてむしろ向上するという重要な知見を得た。化合物75のスルホンアミド部位の酸性度はpK_a 8.2と、化合物56のスルタム（pK_a 5.7）に比べて大きく低下しており、この酸性度の低下が化合物75における膜透過性の向上、しいては細胞増殖阻害活性の向上に繋がったと考察している。

　第1章で得られた知見より、酸性基のオルト位への置換基導入により活性が向上する可能性があると考え、スルホンアミドのオルト位の置換基を検討した。まずオルト位に電子供与基を有するスルホンアミド（化合物76, 77）を評価した。両化合物のMTHFD2阻害活性は無置換体75よりさらに2~3倍減弱したものの、酸性度のさらなる低下（pK_a 8.3–8.4）および膜透過性の大幅な向上が見られた。しかしながら化合物76および77の細胞増殖阻害活性は化合物75に比べて向上しなかった。このことから膜透過性の向上のみではこれ以上の高活性化は期待できないと考えた。続いてオルト位に電子求引基を有するスルホンアミド（化合物78–81）を評価したところ、酸性度の上昇が見られたものの（pK_a 6.8–6.9）、どれも中程度から高い膜透過性を維持していた。活性評価の結果、いずれの化合物も無置換のスルホンアミド（75）と比べてMTHFD2阻害活性が大きく向上し、元のスルタム56と同程度まで阻害活性が回復することが分かった。特に嵩高いトリフルオロメトキシ基を有する化合物81が最も高い活性を与える結果となった。高い酵素阻害活性と膜透過性を両立できたことで、予想通り、化合物78–81の細胞増殖阻害活性は大きく向上した。

　化合物79のスルホンアミド基をメタ位に移動した化合物82では、化合物79と比べてMTHFD2阻害活性が2倍減弱、GI$_{50}$は6倍減弱する結果となり、無置換体75と同等程度の活性に留まった。また元のスルタム56のオルト位にブロモ基を導入した化合物83も評価した。化合物83は非常に強いMTHFD2阻害活性を示したものの、対応するスルホンアミド体80で見られた効果とは対照的に、細胞増殖阻害活性は無置換のスルタムよりもさらに減弱する結果となった（GI$_{50}$ > 30 μM）。

3-3　スルホンアミドの効果に関する考察

　ここで化合物**78–81**の膜透過性が高い理由について考察する。化合物**78–81**のスルホンアミドのpK_aは6.8–6.9と、無置換体**75**（pK_a 8.2）に比べて酸性度が上昇している。しかしながら膜透過性は無置換体**75**と比べて大きく向上しており、電子供与基を有する化合物**76, 77**と同程度であった。さらにブロモ基、トリフルオロメトキシ基といった嵩高い置換基を有する化合物が、立体的に小さいフルオロ基を有する化合物よりも膜透過性が高かった。これらのことから、膜透過性はスルホンアミドの酸性度のみならず、オルト位の立体的嵩高さにも影響されることが示唆された。加えて、スルホンアミド基をメタ位に移した化合物**82**がオルト位異性体（**79**）と比較し、脂溶性が同等（MLogP 1.8）にも関わらず膜透過性が大きく低下することから、膜透過性の向上は脂溶性増加だけでは説明されない。すなわち上述した膜透過性の向上には、置換位置がオルト位であることが必須である。この理由としては、酸性度を持つスルホンアミドの水素結合ドナーをオルト位置換基が立体的にマスクすることで、化合物の極性表面積を下げる作用があるのではないかと考察している。

　スルタム**56**のオルト位にブロモ基を導入した化合物**83**では、スルホンアミド誘導体で見られた現象と対照的に、細胞増殖阻害活性が元のスルタム**56**に比べても低下する結果となった。ブロモ基はスルタムのN-Hを立体的にマスクする部位に位置しているものの、N-Hの酸性度があまりに高いため、生理的条件では大半の分子が脱プロトン化されており、オルト位置換基が膜透過性および細胞増殖阻害活性に与える影響は限定的であったものと推察した。

　化合物**79**/NAD$^+$/MTHFD2三者複合体のX線結晶構造解析により、その相互作用形式が明らかとなった（Figure 3-2）。化合物**79**の母核の結合様式は、化合物**41**/MTHFD2二者複合体におけるリード化合物**41**の結合様式（Figure 1-7）を保持していた。化合物**41**では末端のカルボン酸がGly310と水素結合を形成していたが、化合物**79**ではスルホンアミドの片方の酸素原子とGly310との間で同様の相互作用が観察された。

　一方でクマリン上に導入した置換基については、三者複合体の解析により興味深い知見が得られた。化合物**79**のクマリン8位に導入した4-メチルピペラジニル基について、外側のアミン部位は補酵素であるNAD$^+$のリン酸リンカー部位と塩橋を形成していることが明らかとなった。すなわち、Table 2-1で観察されたクマリン8位への塩基性置換基導入による活性向上効果は、当初期待していたGlu141との遠隔位静電相互作用によるものではなく、NAD$^+$との塩橋形成によるものであることが強く示唆された。

　NAD$^+$の存在を考えることで、第2章におけるクマリン上のメチルスキャンの結果も説明されうる。クマリン7位へのメチル基導入（**54**, Table 2-2）が活性を向上させるのに対し、9位への導入（**53**, Table 2-2）ではMTHFD2阻害活性が消失した。7位メチル基は脂溶性の空間を埋めることで活性向上に寄与した一方で、9位置換基は隣接するNAD$^+$分子との立体反発によって活性が消失した可能性が示唆された。
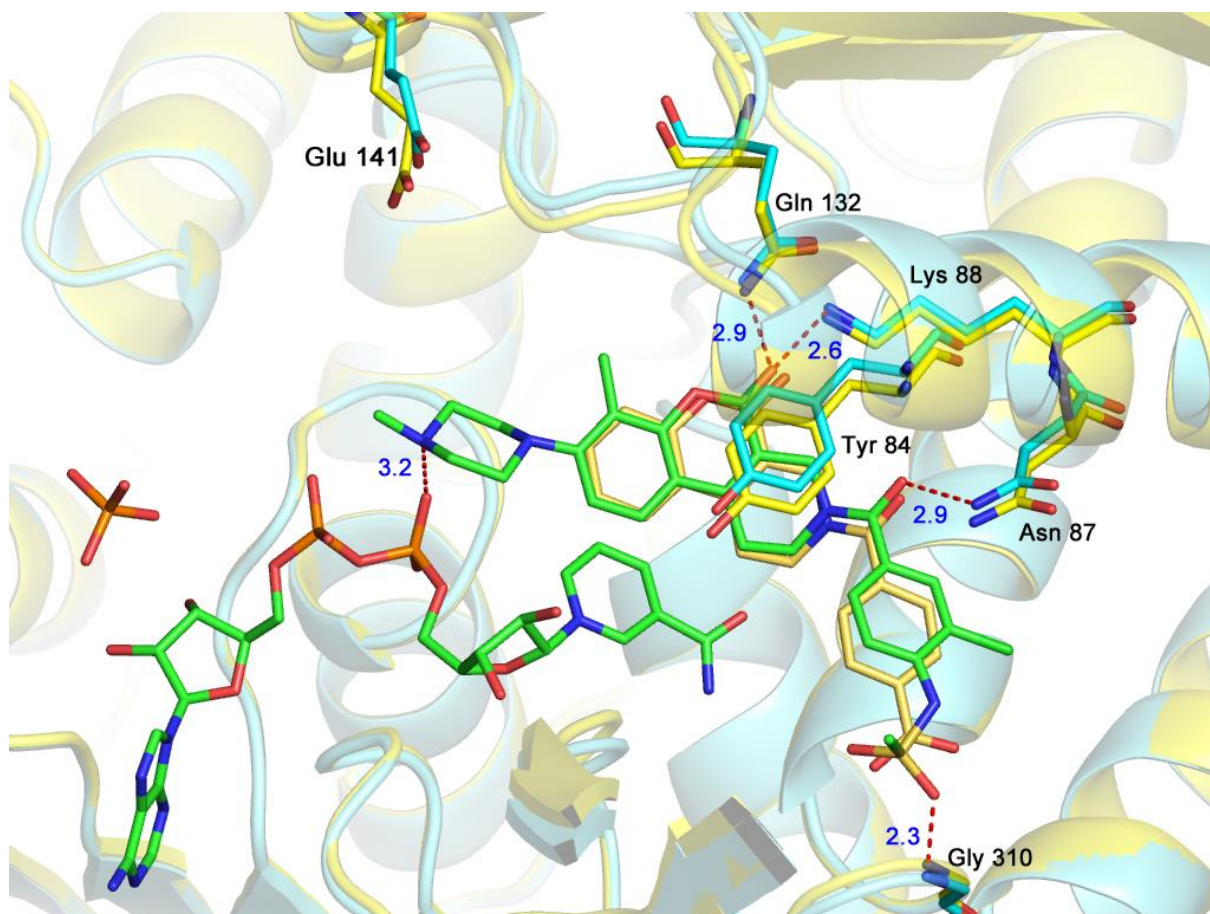
Figure 3-2. Superposition of the **79**/NAD$^+$/MTHFD2 complex (PDB ID: 6KG2, ligands: green, protein: blue) and the **41**/MTHFD2 complex (PDB ID: 6JIB, yellow). Dashed red lines represent hydrogen bonding observed for the complex of **79**. Distances of the interactions are in ångströms.

3-4 4-メチルピペラジニル基の最適化

さらなる構造最適化を目指し、クマリン8位の4-メチルピペラジニル基の修飾を行った（Table 3-2）。

ポケットに最適な部分構造を探索するため、まず化合物**79**のピペラジン部位に*cis*-3,5-ジメチル基を導入した化合物**84**を合成し、評価した。化合物**84**は化合物**79**よりも若干高活性であったが、2つのメチル基によって脂溶性が大きく上昇し（LogD 1.7 vs 1.3）、最適化研究で用いられる指標である脂溶性効率（LipE）は低下した（5.2 vs 5.5）[43]。そこで化合物**79**のピペラジン部位の3位にメチル基を1つだけ導入した誘導体（**85, 86**）を合成した。その結果、(*R*)-体の**85**は化合物**79**に比べて活性が減弱したが、(*S*)-体の**86**では阻害活性が5倍向上し、MDA-MB-231細胞に対するGI$_{50}$およびLipEも向上することが分かった。この部分構造に、Table 3-1で見出された高活性なトリフルオロメトキシ基を導入することで、酵素阻害活性および細胞増殖阻害活性が最も強い化合物**87**（DS18561882）を獲得した。DS18561882はMTHFD1に対して90倍の選択性を維持していた。

Table 3-2. SAR of the piperazine moiety.[a,b]
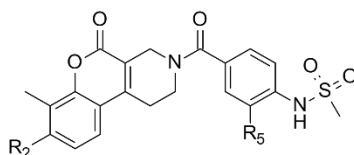
Compound	R$_2$	R$_5$	MTHFD2 IC$_{50}$ (μM)	MTHFD1 IC$_{50}$ (μM)	selectivity	MLogP/LipE	LogD	MDA-MB-231 GI$_{50}$ (μM)
79		Cl	0.048	6.4	133	1.8/5.5	1.3	0.94
84		Cl	0.037	4.9	132	2.2/5.2	1.7	0.78
85		Cl	0.13	12	92	2.0/4.8	ND	1.7
86		Cl	0.0093	1.1	118	2.0/6.0	1.6	0.20
87		OCF$_3$	0.0063	0.57	90	2.1/6.1	1.9	0.14

[a]IC$_{50}$ values for enzymatic assays. The method is described in the experimental section.
[b]Selectivity represents the ratio of MTHFD1 IC$_{50}$/MTHFD2 IC$_{50}$. ND: no data.

3-5　誘導体の酵素阻害および細胞増殖阻害活性の相関とその考察

　合成した化合物の酵素阻害活性と細胞増殖阻害活性には良好な相関傾向が見られた。

　Figure 3-3は、横軸にMTHFD2 IC_{50}を、縦軸にMDA-MB-231細胞に対するGI_{50}を取り、各々の化合物をPAMPA Pe値によって色分けしてプロットしたものである。橙色の楕円で囲んだ膜透過性のある化合物群はいずれもスルホンアミド誘導体であるが、これらについてはIC_{50}値が低い高活性な化合物ほどGI_{50}値が低くなるという、活性値の相関傾向が見られた。このことはMDA-MB-231細胞に対する増殖阻害活性がMTHFD2阻害に起因することを示唆している。一方で膜透過性の非常に低いスルタム（**56**）および類縁体（**83**）は上記の相関から外れてプロットされている。これは細胞膜透過性が低いために、細胞系において阻害活性を十分に発揮できていないと解釈することができる。酸性度の低いスルホンアミドを導入して膜透過性を向上させるというアプローチが、細胞系での活性向上に寄与したことがFigure 3-3からも示された。
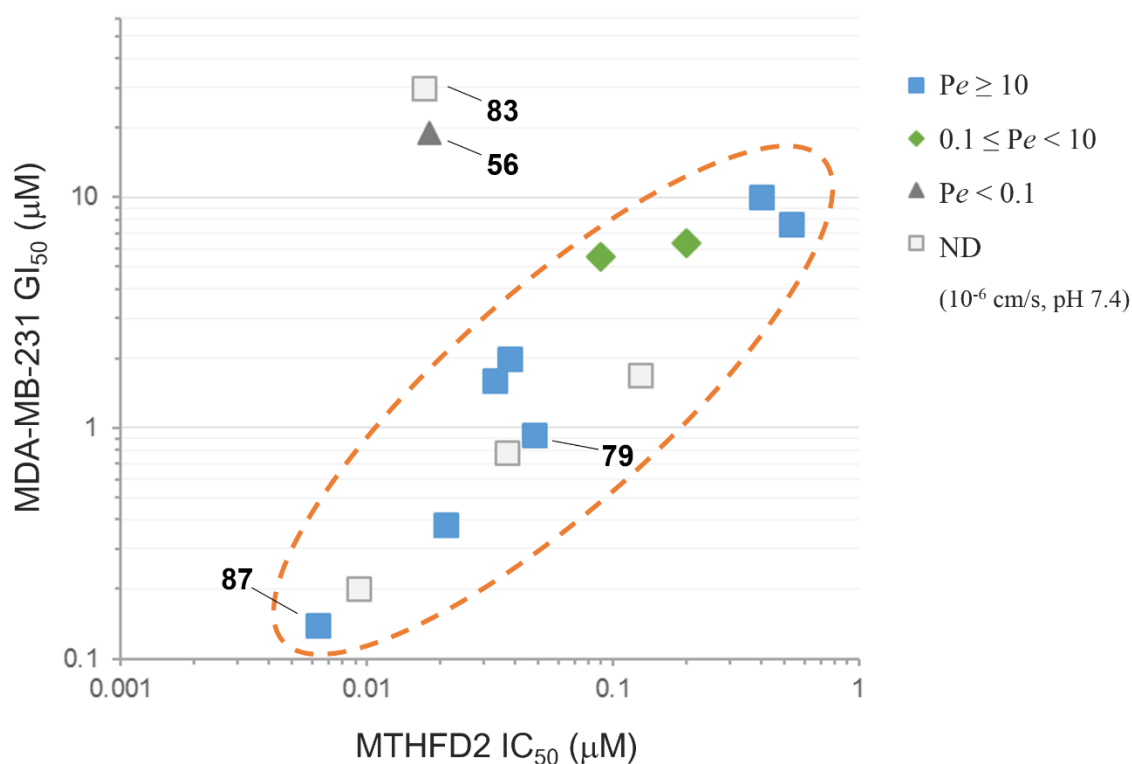
Figure 3-3. Correlation between cell-free MTHFD2 IC_{50} (μM) and cell-based GI_{50} (μM) against the MDA-MB-231 cell line. Compounds **56**, **75–87** are plotted and each color in the plot indicates the range of PAMPA Pe (pH 7.4). ND: no data.

3-6 スルホンアミド誘導体等の合成

　Scheme 3-1にはスルタム誘導体**83**の合成法を示す。原料のスルタム**88**に対して*N*-ブロモスクシンイミドを作用させることでブロモ基を導入した（**89**）。メチルエステルを加水分解してカルボン酸**90**を得た後、**70b**と縮合させることで**83**を得た。

Scheme 3-1. Syntheses of sultam 83.*a*
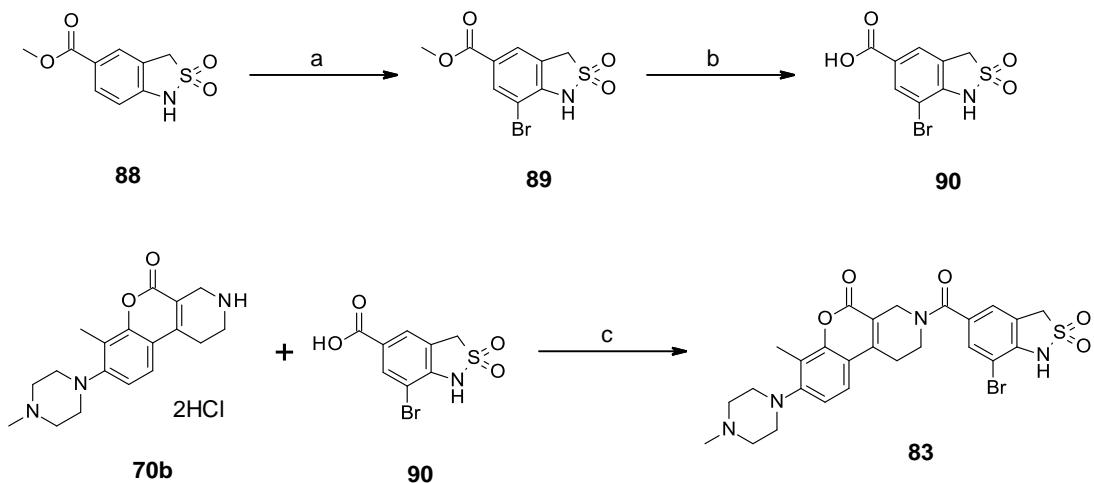
*a*Reagents and conditions: (a) NBS, DMF, 0 °C to rt; (b) 1N NaOH aq., MeOH–THF, 0 °C to rt; (c) WSCI·HCl, HOAt, DIPEA, DMF, rt.

　Scheme 3-2にはスルホンアミド誘導体の合成法を示す。化合物**75–82**はそれぞれ共通中間体である**70b**から誘導した。まず4-あるいは3-アミノ安息香酸誘導体（**91a–h**）との縮合反応によりアミド結合を導入し、対応するアニリン誘導体**92a–h**を得た。次にアニリンのメシル化を実施した。一部のアニリンに対しては、小過剰のメタンスルホニルクロリドおよびピリジンを作用させることで所望の誘導体を合成した。しかしながら本手法では、アニリン窒素上にメシル基が2つ導入された副生成物の生成が競合した。そこでモノメシル体を単一生成物として得るために、過剰量のメタンスルホニルクロリドを作用させてジメシル体を合成した後、ワンポットで加水分解する段階的手法を取った。これにより所望のモノメシル体を選択的に得ることが可能となった。

　94a, **94c**, **94d**はそれぞれ対応するピペラジン誘導体とトリフラート（**93**）とのBuchwaldアミノ化反応により調製した。**94b**は**94a**に対して還元的アミノ化によりメチル基を導入することで合成した。**94b–d**の各誘導体は、上述の**75–82**合成時と同様の手法にて最終生成物**84–87**へと導くことができた。

Scheme 3-2. Syntheses of sulfonamide derivatives.[a]
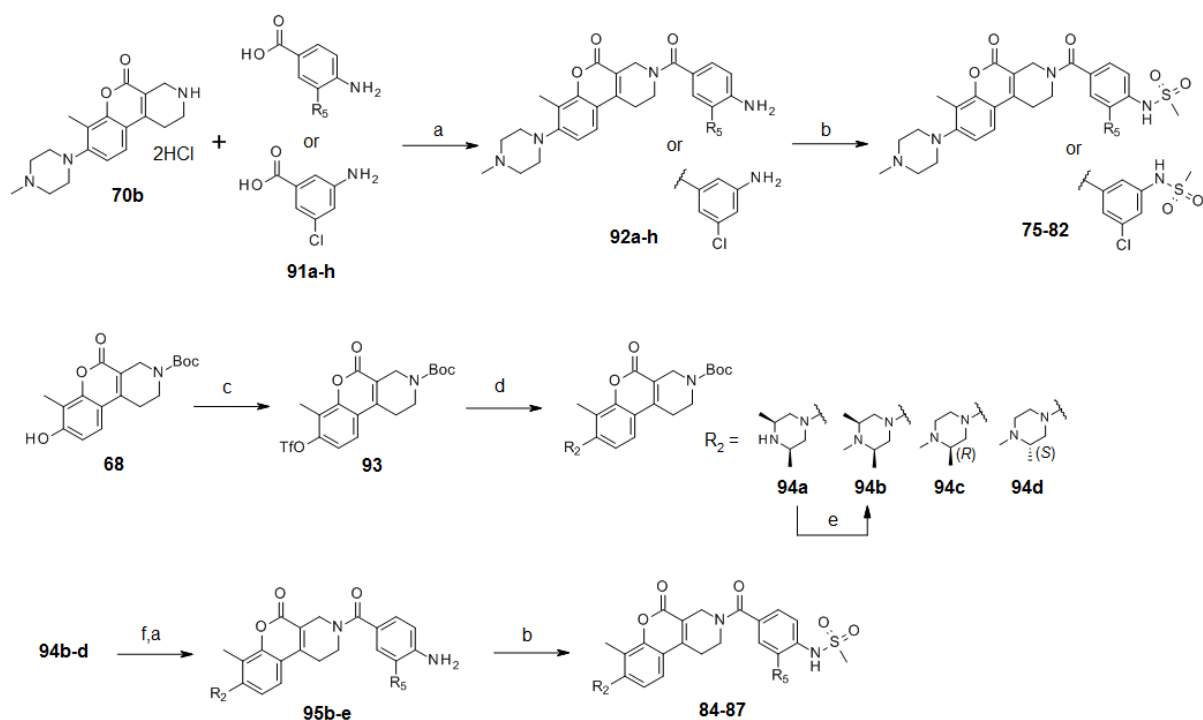
[a]Reagents and conditions: (a) benzoic acids, WSCI·HCl, HOAt, DIPEA, DMF, rt; (b) MsCl, pyridine, DCM, rt or MsCl, Et$_3$N, DCM, rt, then 1N aq. NaOH, THF, rt; (c) Tf$_2$O, pyridine, DCM, 0 °C; (d) piperazines, RuPhos Pd–G1 *t*BuOMe adduct, RuPhos, Cs$_2$CO$_3$, toluene, 110 °C; (e) sodium triacetoxyborohydride, 37% formalin, MeOH–DCM, 0 °C–rt; (f) 4N HCl in 1,4-dioxane, rt.

3-7　代表化合物のin vitroおよびin vivo薬物動態プロファイル

　有望化合物**79**および**87**について、各種in vitro ADMEパラメータをTable 3-3に、マウス経口投与時におけるPKプロファイルをFigure 3-4およびTable 3-4に、さらにフリー体換算したPKプロファイルをFigure 3-5にそれぞれ示す。両化合物ともに経口薬として十分な膜透過性、水溶液溶解性、および代謝安定性を示した（Table 3-3）。マウスへの経口投与では両化合物とも高い血漿中曝露が観測され、特に化合物**87**では顕著に高曝露であった（Figure 3-4）。一方で脂溶性の低い化合物**79**は化合物**87**と比べて血漿中フリー体分率（f_u）が5倍高いため、マウス血漿中におけるフリー体薬物濃度（$C_{p,free}$）は両化合物でほぼ同等であった。両化合物とも10 mg/kgから300 mg/kgにかけて投与量依存的な曝露の増加が見られた。

　MTHFD2の阻害により強い抗腫瘍活性を得るためには、腫瘍中の核酸生合成を常に阻害し続けることが重要であると考えた。そこで化合物の血漿中濃度推移がMTHFD2阻害に十分であるかどうか検討するため、各時点における$C_{p,free}$を化合物のGI$_{50}$と比較した。その結果、化合物**79**の100 mg/kgおよび300 mg/kg投与群では0~7 hにおいて$C_{p,free}$がGI$_{50}$を上回ることが分かった。同様に、化合物**87**の30 mg/kgおよび100 mg/kg投与群では0~8 hにおいて、さらに300 mg/kg投与群では0~24 hの全ての測定時点において、それぞれ$C_{p,free}$がGI$_{50}$を上回った。すなわち両化合物とも投与から7~8時間後まではGI$_{50}$を越える血漿中曝露があるものの、投与24時間後には化合物はいずれも血漿中からほとんど消失しており、化合物**87**の最大用量以外では投与24時間後には十分にMTHFD2を阻害できていない可能性が示唆された。このことから、in vivoにおいてもMTHFD2を継続して阻害するために、1日2回経口投与による抗腫瘍試験を実施することにした。

Table 3-3. In vitro ADME parameters of compound **79** and **87**.

Compound	PAMPA Pe pH 5.0/7.4 ($\times 10^{-6}$ cm/s)	Solubility (pH 1.2/pH 6.8) (µg/mL)[a]	Metabolic stability (% remaining)[b]	Unbound fraction in mouse plasma (f_u) (%)
79	7.2/31.1	240/120	91	15.3
87	17.2/>50	1100/630	66	3.1

[a]Solubility: µg/mL in pH 1.2 and pH 6.8 buffer. [b]Remaining rate of tested compounds (1.0 µM) after incubation for 0.5 h in mouse liver microsomes (0.5 mg/mL).
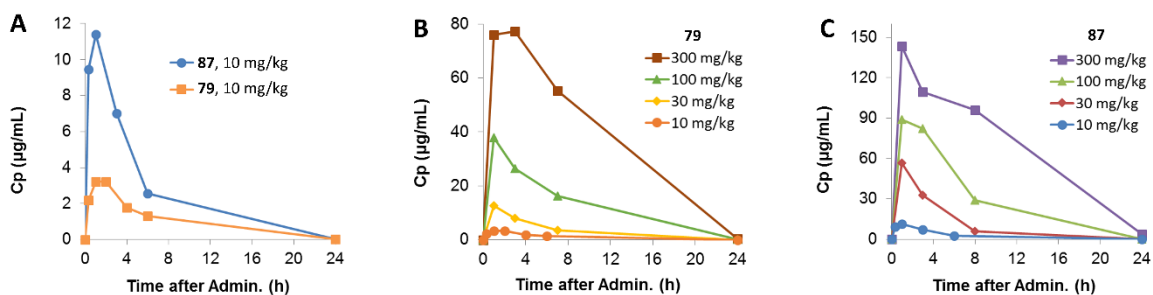
Figure 3-4. Time profiles of plasma concentration (C_p) in mice after oral administration. (A) Comparison of time profiles of C_p of **79** and **87** at 10 mg/kg p.o. dosing. (B) Dose dependence of **79** at 10, 30, 100, and 300 mg/kg p.o. dosing. (C) Dose dependence of **87** at 10, 30, 100, and 300 mg/kg p.o. dosing.
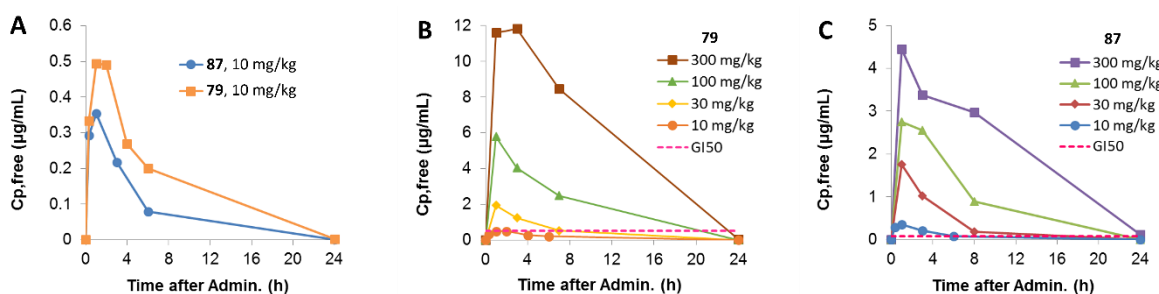

Figure 3-5. Time profiles of the free plasma concentration ($C_{p, \, free}$) in mice after oral administration, calculated from C_p and f_u. (A) Comparison of time profiles of $C_{p,free}$ of compounds **79** and **87** at 10 mg/kg p.o. dosing. (B) Dose dependence of compound **79** at 10, 30, 100, and 300 mg/kg p.o. dosing. GI_{50} value (0.94 µM) is shown in a dashed pink line. (C) Dose dependence of compound **87** at 10, 30, 100, and 300 mg/kg p.o. dosing. GI_{50} value (0.14 µM) is shown in a dashed pink line.

Table 3-4. Pharmacokinetic parameters of **79** and **87** in mice after oral administration ($N = 2$)[a].

Dose	**79**			**87**		
	AUC	C_{max}	$t_{1/2}$	AUC	C_{max}	$t_{1/2}$
(mg/kg)	(µg·h/mL)	(µg/mL)	(h)	(µg·h/mL)	(µg/mL)	(h)
10	25.3	3.66	2.73	64.4	11.4	2.21
30	79.2	12.7	3.35	264	56.5	2.16
100	306	37.9	3.51	726	90.1	2.32
300	929	82.6	2.32	1630	143	4.00

[a]Animal: male BALBc mice.

3-8　代表化合物のin vivo抗腫瘍試験

　MDA-MB-231細胞株をマウスの皮下に移植したxenograftモデルを用いて、化合物**79**および**87**の抗腫瘍活性を経口投与にて評価した（Figure 3-6）。前節での検討に基づき1日2回投与とし、投与後11日目までの腫瘍径と体重変化を対照群と比較した。

　化合物**79**の300 mg/kg, BID投与群では、中程度の腫瘍増殖抑制作用が観察された（TGI: 43%）。より高活性なMTHFD2阻害薬である化合物**87**（DS18561882）投与群では、30, 100, 300 mg/kg, BIDで用量依存的な腫瘍増殖抑制が確認された。特に最高用量である300 mg/kg, BID投与群では腫瘍増殖のほぼ完全な抑制が見られた（TGI: 67%）。またいずれの投与群においても有意な体重減少は見られず、化合物の高い安全性が示唆された。以上より、選択的MTHFD2阻害薬の経口投与によって抗腫瘍効果が見られることが明らかとなった。
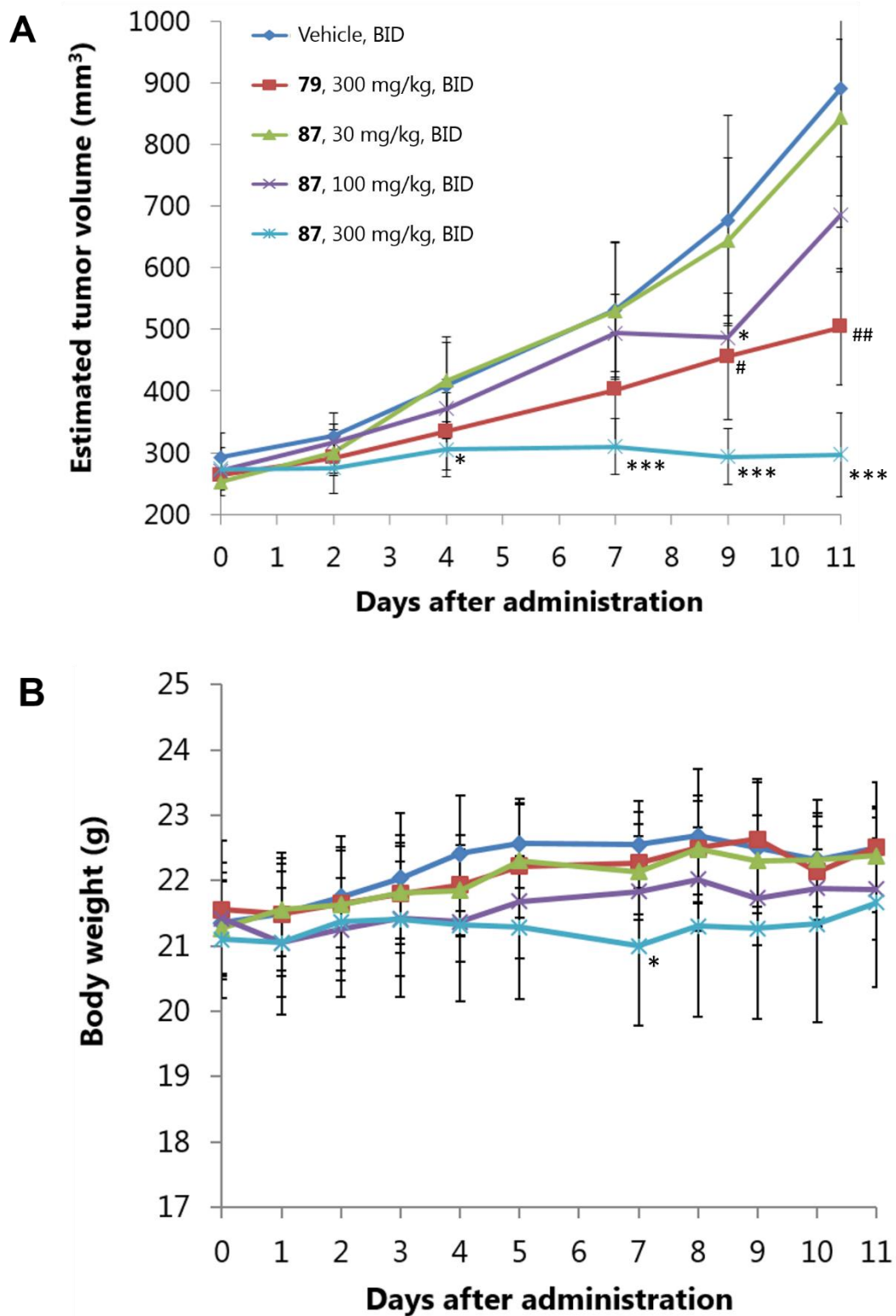
Figure 3-6. Anti-tumor effect of **79** and **87** (DS18561882) in mouse xenograft model ($N = 6$, mean $\pm$ SD). (A) Tumor volume of each group. (B) Body weight change during the study. #$p < 0.05$ and ##$p < 0.01$ vs. control (*t*-test). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ vs. control (Dunnett's test).

3-9 小括

　化合物**56**の膜透過性が低い原因がスルタムの高い酸性度にあると考え、スルタムを代替する置換基の検討を行った。その結果、酸性度の低いスルホンアミド基でスルタムを代替することで、膜透過性の大幅な向上に繋がることが分かった。置換基効果を詳細に検討し、スルホンアミドのオルト位置換基が活性と膜透過性の双方に重要であることを見出し、化合物**79**など細胞増殖阻害活性の向上した化合物群を獲得した。さらにピペラジニル基の最適化により、非常に強い酵素阻害活性と細胞増殖阻害活性を有し、MTHFD1に対して90倍の選択性を示す化合物**87**（DS18561882）を獲得することができた。

　化合物**79**/NAD$^+$/MTHFD2三者複合体のX線結晶構造により、SARの理解には補酵素NAD$^+$の存在が重要であることが判明した。クマリン8位の塩基性基はNAD$^+$のリン酸部位と塩橋を形成することで高活性化に寄与したこと、およびクマリン9位へのメチル基導入が許容されない原因はNAD$^+$との立体反発であったことが、それぞれ強く示唆された。

　代表化合物**79**および**87**はいずれも経口剤として良好なin vitro ADMEパラメータを有し、マウスにおいて高い経口吸収性を示した。さらにMDA-MB-231株を皮下移植したマウスを用いるxenograftモデルにおいて、両化合物は経口投与において腫瘍増殖抑制作用を示し、選択的MTHFD2阻害薬の経口投与による抗腫瘍効果を初めて確認することができた。

Figure 3-7. Summary

56
MTHFD2 IC$_{50}$ = 0.018 μM
MTHFD1 IC$_{50}$ = 5.2 μM
MDA-MB-231 GI$_{50}$ = 19 μM

87
(DS18561882)
MTHFD2 IC$_{50}$ = 0.0063 μM
MTHFD1 IC$_{50}$ = 0.57 μM
MDA-MB-231 GI$_{50}$ = 0.14 μM
In vivo anti-tumor activity
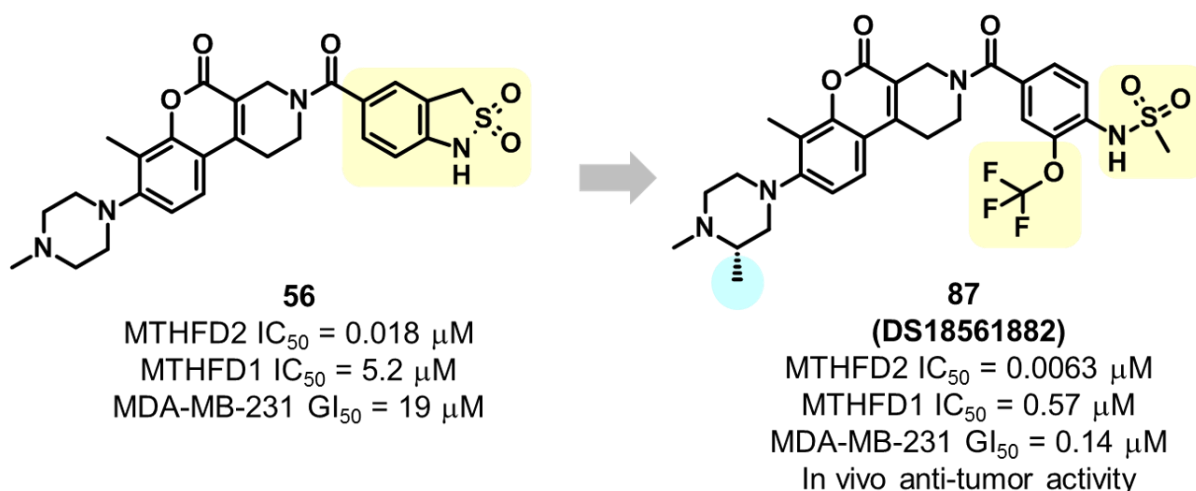
総論

　本論文では、新規抗癌剤としての可能性を秘める選択的MTHFD2阻害薬を初めて獲得し、複合体構造情報や物性値を指標にした構造最適化を経て、マウスへの経口投与により抗腫瘍効果を認めるDS18561882を創出した。

　第1章では、MTHFD2への選択性を有するHTSヒットを獲得後、末端置換基や母核を変換することで、三環性クマリン骨格を有する化合物**41**を獲得した。化合物**41**はHTSヒットと同じく良好な選択性を有しつつ、各種in vitro/in silicoパラメータが改善されたことから、選択的MTHFD2阻害剤として有用な初期リード化合物であると判断した。

　第2章では、複合体構造情報を活用したクマリン上の置換基検討を行い、8位に塩基性置換基を、7位にメチル基をそれぞれ導入することで、MTHFD2の酵素阻害活性が向上することを発見した。高活性置換基の組み合わせにより、初期リードから阻害活性が89倍向上し、高いアイソザイム選択性を示す化合物**56**を獲得することができた。

　第3章では、末端置換基の酸性度およびオルト位の立体的嵩高さが、膜透過性と細胞系での増殖阻害活性の改善に繋がることを発見し、細胞系で高活性を示すスルホンアミド誘導体を見出した。この過程において、クマリン8位の塩基性基による活性向上効果が補酵素NAD^+との塩橋によって説明できることを、三者複合体のX線構造解析により明らかとした。
　構造最適化を経て獲得した高活性・高選択性を示す代表化合物**87**（DS18561882）は、マウスにおいて経口吸収性を示し、高用量ながらマウスxenograftモデルにおいて経口投与で抗腫瘍効果を示すことがわかった。

　MTHFD2と癌との関連性は多く報告されているものの、選択的MTHFD2阻害薬によって抗腫瘍効果を示した例はこれが初めてであり、DS18561882による癌の増殖抑制が見られたことは、今後の研究開発にとって重要な一歩である。本研究において得られた新たな知見が、今後のMTHFD2阻害薬の研究開発に繋がることを強く願っている。

1. Chemistry

General

Unless otherwise noted, commercial reagents and solvents were obtained from suppliers and used as purchased. Normal-phase column chromatography was performed on silica gel (SiO_2) or amino-silica gel using prepackaged cartridges. Preparative reverse-phase high performance liquid chromatography (HPLC) was performed with a GILSON prepHPLC system. Conditions [column: Develosil Combi-RP-5 28 mm × 100 mm, gradient elution: 0.1% HCO_2H–H_2O / 0.1% HCO_2H–MeCN, flow rate: 25 mL/min, UV detection: 254 nm]. Analytical thin-layer chromatography (TLC) was performed on Merck pre-coated TLC glass sheets with silica gel 60 F_{254}. ^{1}H and ^{13}C NMR spectra were recorded on JEOL JNM-EX400 or Bruker AVANCE III 500 spectrometers, and chemical shifts are given in ppm from tetramethylsilane as an internal standard. Infrared spectra were recorded on KBr discs with a Jasco FT/IR-6100 type A and are reported in wavenumbers (cm^{-1}). Optical rotations were recorded on a Rudolph Autopol V plus or a Jasco P-1030 polarimeter. ESI/APCI mass spectra were recorded on Agilent Infinity 1260 series LC/MS. Purities of ≥ 90% were confirmed by the LC/MS for all test compounds. Conditions [column: Develosil Combi-RP-5 2.0 mm × 50 mm, gradient elution: 0.1% HCO_2H–H_2O / 0.1% HCO_2H–MeCN = 98/2 – 0/100 (v/v), flow rate: 1.2 mL/min, UV detection: 254 nm, column temperature: 40 °C, ionization: APCI/ESI]. High resolution mass spectra (HRMS) were obtained on LC/MS system composed of Waters Xevo Q-Tof MS system and Acuity UPLC system. Elemental analyses are indicated only by the symbols of the elements; analytical results were within 0.4% of the theoretical values. Ligand efficiency (LE) was calculated using the reported IC_{50} values[7,17] by the following equation: LE = 1.4 × pIC_{50}/heavy atom count (HAC). MLogP and tPSA were calculated by ADMET Predictor™ version 8.5 (Simulations Plus, Inc., Lancaster, CA). LipE was calculated by the following equation: LipE = pIC_{50} − MLogP.
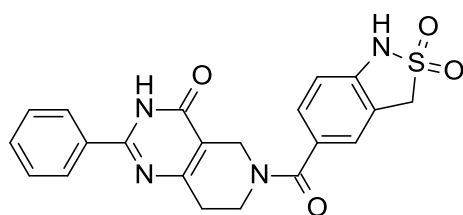

6-[(2,2-dioxido-1,3-dihydro-2,1-benzothiazol-5-yl)carbonyl]-2-phenyl-5,6,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-4(3*H*)-one (1)

A DMF solution of 1,3-dihydro-2,1-benzothiazole-5-carboxylic acid 2,2-dioxide (21 mg, 0.10 mmol), 2-phenyl-5,6,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-4(3*H*)-one (27 mg, 0.12 mmol), WSCI-HCl (19 mg, 0.10 mmol), HOBt (14 mg, 0.10 mmol), and *N,N*-diisopropylethylamine (17 μL, 0.10 mmol) was stirred at rt for 18 h. Concentration of the crude mixture and purification by preparative reverse-phase HPLC afforded **1** (3.91 mg, 9% yield) as a powder. ^{1}H-NMR (DMSO-D$_6$) δ: 12.81 (1H, br s), 10.91 (1H, br s), 8.08 (2H, d, J = 7.3 Hz), 7.62-7.38 (5H, m), 6.87 (1H, d, J = 7.9 Hz), 4.59 (2H, s), 4.47-4.34 (2H, br m), 3.87-3.68 (2H, br m), 2.83-2.73 (2H, br m). MS (ESI/APCI) m/z: 423.2 (calcd for $C_{21}H_{19}N_4O_4S$ (M+H)$^+$: 423.1).
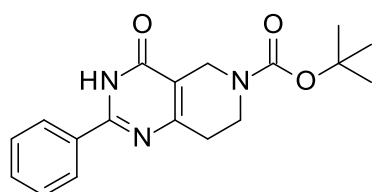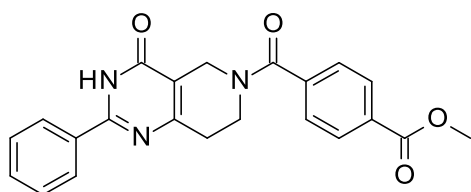

***tert*-butyl 4-oxo-2-phenyl-3,5,7,8-tetrahydropyrido[4,3-*d*]pyrimidine-6(4*H*)-carboxylate (S1)**

To a suspension of *N*-Boc-3-carboethoxy-4-piperidone (2.0 g, 7.37 mmol) and benzamidine hydrochloride (1.27 g, 8.11 mmol) in ethanol (20 mL) was added potassium carbonate (2.24 g, 16.2 mmol). The mixture was stirred at rt for 10 h. Insoluble solid was removed by filtration, and the filtrate was concentrated *in vacuo*. Purification by column chromatography (SiO_2, MeOH/CH_2Cl_2 = 0/100 − 5/95 (v/v)) afforded **S1** (2.51 g, quant.) as a colorless solid. ^{1}H-NMR (CDCl$_3$) δ: 8.05 (2H, d, J = 6.1 Hz), 7.59-7.50 (3H, m), 4.45 (2H, s), 3.77-3.71 (2H, m), 2.83 (2H, s), 1.51 (9H, s). MS (ESI/APCI) m/z: 328.2 (calcd for $C_{18}H_{22}N_3O_3$ (M+H)$^+$: 328.2).
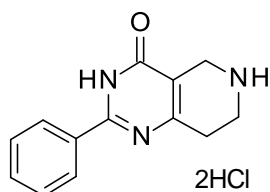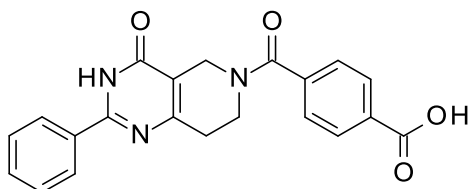
2-phenyl-5,6,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-4(3H)-one 2HCl (S2)

To a suspension of **S1** (2.50 g, 7.6 mmol) in THF (20 mL) and MeOH (10 mL) was added 4N HCl 1,4-dioxane solution (20 mL). After stirring at 40 ℃ for 3 h, the solvent was removed *in vacuo*. The resulting residue was washed with Et$_2$O, filtrated, and dried to give **S2** (2.167 g, 95% yield) as a white solid. ^{1}H-NMR (DMSO-D$_6$, 80 ℃) δ: 9.47 (2H, br s), 8.10 (2H, d, J = 6.7 Hz), 7.50-7.60 (3H, m), 3.96 (2H, s), 3.37-3.43 (2H, m), 2.90-2.94 (2H, m). MS (ESI/APCI) m/z: 228.2 (calcd for C$_{13}$H$_{14}$N$_3$O (M+H)$^+$: 228.1).

methyl 4-[(4-oxo-2-phenyl-3,5,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-6(4*H*)-yl)carbonyl]benzoate (15)

To a suspension of **S2** (200 mg, 0.67 mmol) and 4-methoxycarbonylbenzoic acid (133 mg, 0.73 mmol) in DMF were added WSCI-HCl (154 mg, 0.80 mmol), HOBt (91 mg, 0.67 mmol) and triethylamine (0.22 mL, 1.60 mmol) at 0 ℃. After stirring for 6 h, the solution was concentrated *in vacuo* and diluted with CH$_2$Cl$_2$. The organic layer was washed with water, dried over anhydrous Na$_2$SO$_4$, and filtrated. After evaporating the solvent, the residue was purified by column chromatography (SiO$_2$, MeOH/CH$_2$Cl$_2$ = 0/100 – 5/95 (v/v)) to give **15** (219 mg, 84% yield) as a colorless solid. ^{1}H-NMR (CDCl$_3$) δ: 8.14 (2H, d, J = 7.9 Hz), 8.04 (2H, br s), 7.60-7.52 (3H, m), 7.49-7.44 (2H, m), 4.85-4.35 (2H, m), 4.13-3.62 (2H, m), 3.96 (3H, s), 3.00-2.79 (2H, m). MS (ESI/APCI) m/z: 390.2 (calcd for C$_{22}$H$_{20}$N$_3$O$_4$ (M+H)$^+$: 390.1).

4-[(4-oxo-2-phenyl-3,5,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-6(4*H*)-yl)carbonyl]benzoic acid (3)

To a suspension of **15** (121 mg, 0.31 mmol) in MeOH (5 mL) was added 1M NaOH aq. (5 mL), and the mixture was stirred overnight at rt. 1M aq. HCl (5 mL) was added to it, and the resulting precipitate was filtrated and dried at 60 ℃ under reduced pressure to afford **3** (101 mg, 87% yield) as a colorless solid. ^{1}H-NMR (DMSO-D$_6$) δ: 13.19 (1H, s), 12.86 (1H, s), 8.14-7.99 (4H, m), 7.63-7.46 (5H, m), 4.55-4.19 (2H, m), 3.98-3.52 (2H, m), 2.77 (2H, s). MS (ESI/APCI) m/z: 376.2 (calcd for C$_{21}$H$_{18}$N$_3$O$_4$ (M+H)$^+$: 376.1). HRMS (ESI): m/z calcd for C$_{21}$H$_{18}$N$_3$O$_4$ (M+H)$^+$ 376.1292. Found 376.1288. IR (KBr) 3435, 3081, 2899, 2498, 1898, 1682, 1644, 1605, 1557, 1509, 1444, 1324, 1239 cm^{-1}. Anal. Calcd for C$_{21}$H$_{17}$N$_3$O$_4$: C, 67.19; H, 4.56; N, 11.19. Found: C, 66.81; H, 4.67; N, 11.16.
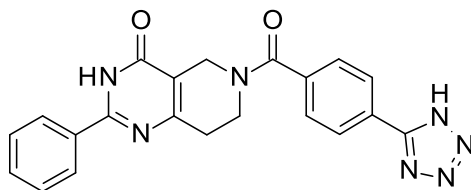
2-phenyl-6-[4-(1*H*-tetrazol-5-yl)benzoyl]-5,6,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-4(3*H*)-one (2)

To a DMF (2 mL) solution of 4-(1*H*-tetrazol-5-yl)benzoic acid (77 mg, 0.40 mmol) were added WSCI-HCl (77 mg, 0.40 mmol), HOBt (46 mg, 0.34 mmol), **S2** (100 mg, 0.33 mmol) and triethylamine (0.092 mL, 0.67 mmol). The mixture was stirred at rt overnight. After concentration *in vacuo*, MeOH was added to the residue. The resulting solid was collected by filteration, washed with water, MeOH, and EtOAc to give **2** (90 mg, 68% yield) as a pale yellow solid. ^{1}H-NMR (DMSO-D$_6$) δ: 12.83 (1H, s), 8.17-8.03 (4H, m), 7.72 (2H, d, J = 7.9 Hz), 7.62-7.49 (3H, m), 4.58-4.24 (2H, m), 4.01-3.56 (2H, m), 2.80 (2H, s). MS (ESI/APCI) m/z: 400.2 (calcd for C$_{21}$H$_{18}$N$_7$O$_2$ (M+H)$^+$: 400.1).
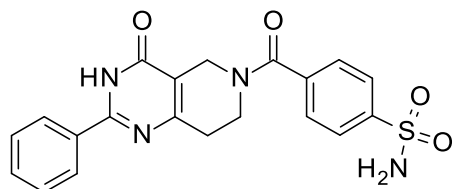
4-[(4-oxo-2-phenyl-3,5,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-6(4*H*)-yl)carbonyl]benzenesulfonamide (4)

To a DMF (2 mL) solution of 4-sulfamoylbenzoic acid (74 mg, 0.37 mmol) were added WSCI-HCl (77 mg, 0.40 mmol), HOBt (46 mg, 0.34 mmol), **S2** (100 mg, 0.33 mmol) and triethylamine (0.092 mL, 0.67 mmol). The mixture was stirred at rt for 3 days. After concentration *in vacuo*, MeOH/CH$_2$Cl$_2$ (1:9) was added to the residue. The resulting solid was collected by filtration, washed with water and CH$_2$Cl$_2$, and dried at 60 °C under reduced pressure to give **4** (95 mg, 69% yield) as a colorless solid. ^{1}H-NMR (DMSO-D$_6$) δ: 12.85 (1H, s), 8.08 (2H, s), 7.92 (2H, d, J = 7.6 Hz), 7.69 (2H, d, J = 7.6 Hz), 7.61-7.48 (5H, m), 4.57-4.21 (2H, m), 3.98-3.51 (2H, m), 2.79 (2H, s). MS (ESI/APCI) m/z: 411.3 (calcd for C$_{20}$H$_{19}$N$_4$O$_4$S (M+H)$^+$: 411.1).
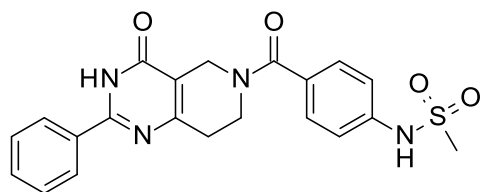
N-{4-[(4-oxo-2-phenyl-3,5,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-6(4*H*)-yl)carbonyl]phenyl}me-thanesulfonamide (5)

To a DMF (2 mL) solution of *p*-(methanesulfonamido)benzoic acid (86 mg, 0.40 mmol) were added WSCI-HCl (77 mg, 0.40 mmol), HOBt (46 mg, 0.34 mmol), **S2** (100 mg, 0.33 mmol) and triethylamine (0.092 mL, 0.67 mmol). The mixture was stirred at rt for 2 days. After concentration *in vacuo*, the residue was purified by column chromatography (SiO$_2$, MeOH/CH$_2$Cl$_2$ = 0/100 − 10/90 (v/v)). The obtained solid was washed with EtOAc and dried at 60 °C under reduced pressure to give **5** (120 mg, 85% yield) as a colorless solid. ^{1}H-NMR (DMSO-D$_6$) δ: 12.84 (1H, s), 10.11 (1H, s), 8.07 (2H, d, J = 6.7 Hz), 7.61-7.47 (5H, m), 7.28 (2H, d, J = 8.5 Hz), 4.52-4.29 (2H, m), 3.98-3.58 (2H, m), 3.09 (3H, s), 2.78 (2H, s). MS (ESI/APCI) m/z: 425.0 (calcd for C$_{21}$H$_{21}$N$_4$O$_4$S (M+H)$^+$: 425.1).
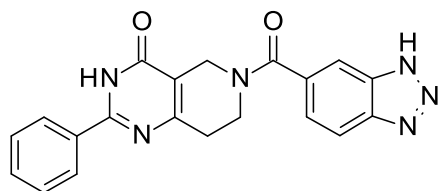
6-(1*H*-benzotriazol-6-ylcarbonyl)-2-phenyl-5,6,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-4(3*H*)-one (6)

To a DMF (2 mL) solution of benzotriazole-5-carboxylic acid (60 mg, 0.37 mmol) were added WSCI-HCl (77 mg, 0.40 mmol), HOBt (46 mg, 0.34 mmol), **S2** (100 mg, 0.33 mmol) and triethylamine (0.092 mL, 0.67 mmol). The mixture was stirred at rt overnight. After concentration *in vacuo*, the residue was diluted with

MeOH/CH$_2$Cl$_2$, washed with water, and dried over anhydrous Na$_2$SO$_4$. Filtration, removal of solvents by evaporation, and purification by column chromatography (SiO$_2$, MeOH/CH$_2$Cl$_2$ = 0/100 – 10/90 (v/v)) afforded a colorless solid. The obtained solid was washed with CH$_2$Cl$_2$ and dried at 60 °C under reduced pressure to give **6** (50 mg, 40% yield) as a colorless solid. ^{1}H-NMR (DMSO-D$_6$) δ: 12.82 (1H, s), 8.28-7.84 (4H, m), 7.67-7.42 (4H, m), 4.59-4.25 (2H, m), 4.01-3.54 (2H, m), 2.80 (2H, s). MS (ESI/APCI) m/z: 373.1 (calcd for C$_{20}$H$_{17}$N$_6$O$_2$ (M+H)$^+$: 373.1).
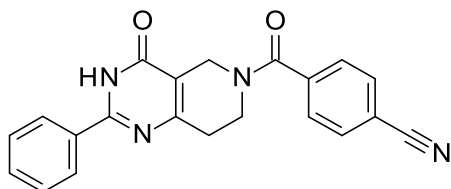
4-[(4-oxo-2-phenyl-3,5,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-6(4*H*)-yl)carbonyl]benzonitrile (S3)

To a DMF (5 mL) solution of 4-cyanobenzoic acid (165 mg, 1.12 mmol), S2 (313 mg, 1.04 mmol) and *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluorophosphate (451 mg, 1.19 mmol) was added *N*,*N*-diisopropylethylamine (0.9 mL, 5 mmol). After stirring at rt for 3 days, the mixture was diluted with MeOH/CHCl$_3$ (1:9). The solution was washed with water (3 times), dried over anhydrous MgSO$_4$, filtered, and concentrated *in vacuo*. Trituration with EtOAc/*n*-hexane (1:1) gave **S3** (318.5 mg, 86% yield) as a white powder. ^{1}H-NMR (DMSO-D$_6$) δ: 12.90-12.81 (1H, m), 8.13-8.03 (2H, m), 7.97 (2H, d, J = 7.9 Hz), 7.69 (2H, d, J = 7.9 Hz), 7.61-7.49 (3H, m), 4.56-4.16 (2H, m), 3.98-3.50 (2H, m), 2.77 (2H, br s). MS (ESI/APCI) m/z: 357.1 (calcd for C$_{21}$H$_{17}$N$_4$O$_2$ (M+H)$^+$: 357.1).
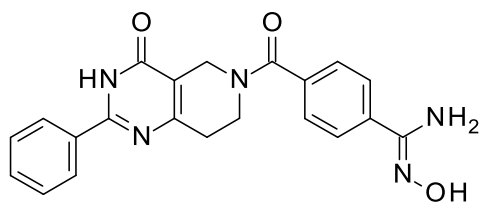
N'-hydroxy-4-[(4-oxo-2-phenyl-3,5,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-6(4*H*)-yl)carbonyl]benzene-carboximidamide (S4)

50% aq. hydroxylamine (0.8 mL) was added to the suspension of **S3** (80 mg, 0.22 mmol) in dimethylsulfoxide (1.5 mL) at rt. The mixture was stirred at 90 °C for 13 h. After cooling down to rt, water (5 mL) was added to the reaction mixture, and the resulting precipitate was collected by filtration. The solid was washed with water and EtOAc/*n*-hexane (1:1), and dried at 60 °C to give **S4** (68.2 mg, 78% yield) as a white solid. ^{1}H-NMR (DMSO-D$_6$) δ: 12.74 (1H, s), 9.80 (1H, br s), 8.08 (2H, br s), 7.77 (2H, d, J = 7.9 Hz), 7.60-7.48 (5H, m), 5.92 (2H, s), 4.57-4.23 (2H, m), 4.00-3.55 (2H, m), 2.78 (2H, br s). MS (ESI/APCI) m/z: 390.1 (calcd for C$_{21}$H$_{20}$N$_5$O$_3$ (M+H)$^+$: 390.1).
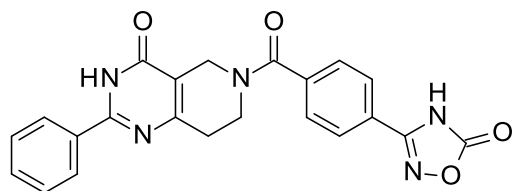
6-[4-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl)benzoyl]-2-phenyl-5,6,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-4(3*H*)-one (7)

To a DMF (1.5 mL) suspension of **S4** (63.2 mg, 0.162 mmol) was added 1,1'-carbonyldiimidazole (35 mg, 0.22 mmol) and the mixture was stirred at 65 °C for 9 days. During the reaction, the same amount of reagent (1,1'-carbonyldiimidazole) was added to the mixture for another three times until most of the starting material was consumed. Preparative reverse-phase HPLC, evaporation of volatile solvents in the obtained fractions, and following lyophilization afforded **7** (7.2 mg, 11% yield) as a pale brown powder. ^{1}H-NMR (DMSO-D$_6$) δ: 12.83

(1H, br s), 8.12-8.01 (2H, m), 7.91-7.81 (2H, m), 7.69-7.43 (5H, m), 4.58-4.22 (2H, m), 3.98-3.54 (2H, m), 2.77 (2H, s). MS (ESI/APCI) m/z: 416.2 (calcd for $C_{22}H_{18}N_5O_4$ (M+H)$^+$: 416.1).
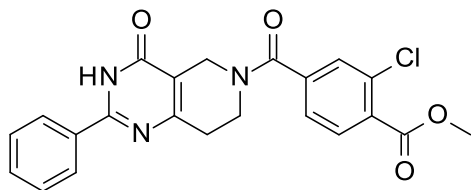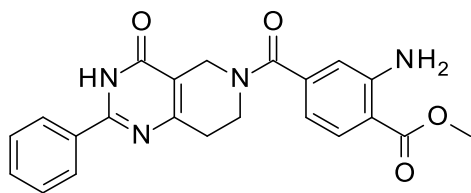

methyl 2-chloro-4-[(4-oxo-2-phenyl-3,5,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-6(4*H*)-yl)carbonyl]benzoate (S5)

To a DMF (3 mL) suspension of 3-chloro-4-(methoxycarbonyl)benzoic acid (72 mg, 0.33 mmol) were added WSCI-HCl (76 mg, 0.40 mmol), HOBt (45 mg, 0.33 mmol), **S2** (100 mg, 0.33 mmol) and triethylamine (0.11 mL, 0.80 mmol) at 0 °C. The mixture was stirred at rt for 2 days. Concentration *in vacuo* and purification by column chromatography (SiO$_2$, MeOH/CH$_2$Cl$_2$ = 0/100 – 5/95 (v/v)) afforded **S5** (119 mg, 84% yield) as a colorless solid. ^{1}H-NMR (DMSO-D$_6$) δ: 8.08 (2H, d, J = 7.3 Hz), 7.87 (1H, d, J = 7.9 Hz), 7.65 (1H, s), 7.56-7.47 (4H, m), 4.36 (2H, br s), 3.89 (3H, s), 3.73 (2H, br s), 2.80-2.74 (2H, m). MS (ESI/APCI) m/z: 424.3 (calcd for $C_{22}H_{19}ClN_3O_4$ (M+H)$^+$: 424.1).
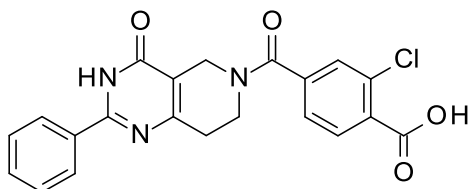
2-chloro-4-[(4-oxo-2-phenyl-3,5,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-6(4*H*)-yl)carbonyl]benzoic acid (8)

To a MeOH (10 mL) solution of **S5** (117 mg, 0.28 mmol) was added 1M NaOH aq. (5 mL). The mixture was stirred at rt for 2 h. 1M aq. HCl (5 mL) was added to it, and the resulting precipitate was filtrated and dried at 60 °C under reduced pressure to afford **8** (94 mg, 83% yield) as a colorless solid. ^{1}H-NMR (DMSO-D$_6$) δ: 8.08 (2H, d, J = 7.9 Hz), 7.84 (1H, d, J = 7.9 Hz), 7.62-7.47 (5H, m), 4.37 (2H, s), 3.74 (2H, br s), 2.77 (2H, t, J = 5.5 Hz). MS (ESI/APCI) m/z: 410.1 (calcd for $C_{21}H_{17}ClN_3O_4$ (M+H)$^+$: 410.1).


methyl 2-amino-4-[(4-oxo-2-phenyl-3,5,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-6(4*H*)-yl)carbonyl]benzoate (S6)

To a DMF (3 mL) suspension of 2-aminoterephthalic acid 1-methyl ester (66 mg, 0.33 mmol) were added WSCI-HCl (76 mg, 0.40 mmol), HOBt (45 mg, 0.33 mmol), **S2** (100 mg, 0.33 mmol) and triethylamine (0.11 mL, 0.80 mmol) at 0 °C. The mixture was stirred at rt for 2 days. Concentration *in vacuo* and purification by column chromatography (SiO$_2$, MeOH/CH$_2$Cl$_2$ = 0/100 – 5/95 (v/v)) afforded **S6** (125 mg, 93% yield) as a colorless solid. ^{1}H-NMR (DMSO-D$_6$) δ: 8.08 (2H, d, J = 7.3 Hz), 7.77 (1H, d, J = 8.5 Hz), 7.57-7.47 (3H, m), 6.83 (1H, s), 6.70-6.54 (3H, m), 4.36 (2H, s), 3.82 (3H, s), 3.74 (2H, br s), 2.79-2.73 (2H, m). MS (ESI/APCI) m/z: 405.2 (calcd for $C_{22}H_{21}N_4O_4$ (M+H)$^+$: 405.1).
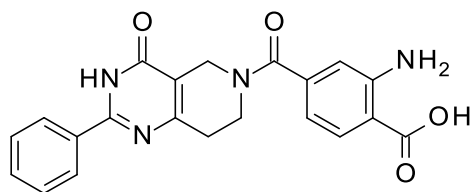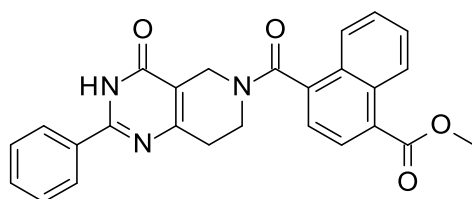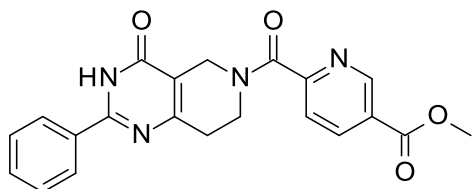
2-amino-4-[(4-oxo-2-phenyl-3,5,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-6(4*H*)-yl)carbonyl]benzoic acid (9)

To a MeOH (6 mL) solution of **S6** (48 mg, 0.12 mmol) was added 1M NaOH aq. (6 mL). The mixture was stirred overnight at rt. 1M aq. HCl (6 mL) was added to it, and the resulting precipitate was filtrated, washed with water and MeOH, and dried at 60 °C under reduced pressure to afford **9** (36 mg, 78% yield) as a colorless solid. ^{1}H-NMR (DMSO-D$_6$) δ: 8.08 (2H, d, J = 7.3 Hz), 7.76 (1H, d, J = 7.9 Hz), 7.59-7.45 (3H, m), 6.80 (1H, d, J = 1.8 Hz), 6.54 (1H, dd, J = 7.9, 1.8 Hz), 4.36 (2H, s), 3.74 (2H, br s), 2.75 (2H, t, J = 5.5 Hz). MS (ESI/APCI) m/z: 391.2 (calcd for C$_{21}$H$_{19}$N$_4$O$_4$ (M+H)$^+$: 391.1).
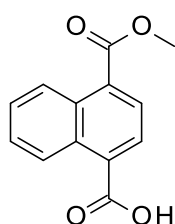
4-(methoxycarbonyl)naphthalene-1-carboxylic acid (S7)

To a mixture of dimethyl naphthalene-1,4-dicarboxylate (100 mg, 0.41 mmol), MeOH (3 mL) and THF (3 mL) was added 1M NaOH aq. (0.6 mL). The mixture was stirred overnight at rt. 1M aq. HCl (3 mL) was added to it, and the resulting precipitate was collected by filtration and dried at 60 °C under reduced pressure to afford crude **S7** (78 mg, 83% yield) as a colorless solid, which was used for the next step without further purification. MS (ESI/APCI) m/z: 229.1 (calcd for C$_{13}$H$_9$O$_4$ (M–H)$^-$: 229.1).

methyl 4-[(4-oxo-2-phenyl-3,5,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-6(4*H*)-yl)carbonyl]naphthalene-1-carboxylate (S8)

To a DMF (3 mL) solution of crude **S7** (67 mg, 0.23 mmol) were added WSCI-HCl (55 mg, 0.28 mmol), HOBt (32 mg, 0.23 mmol), **S2** (70 mg, 0.23 mmol) and triethylamine (0.08 mL, 0.56 mmol). The mixture was stirred at rt overnight. Concentration *in vacuo* and purification by column chromatography (SiO$_2$, MeOH/CH$_2$Cl$_2$ = 0/100 − 5/95 (v/v)) afforded **S8** (87 mg, 85% yield) as a colorless solid. ^{1}H-NMR (DMSO-D$_6$) δ: 8.76 (1H, d, J = 8.5 Hz), 8.18-8.01 (3H, m), 7.94-7.44 (7H, m), 4.75-3.95 (2H, m), 3.98 (3H, s), 3.45-2.60 (4H, m). MS (ESI/APCI) m/z: 440.3 (calcd for C$_{26}$H$_{22}$N$_3$O$_4$ (M+H)$^+$: 440.2).
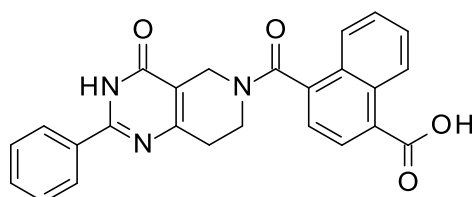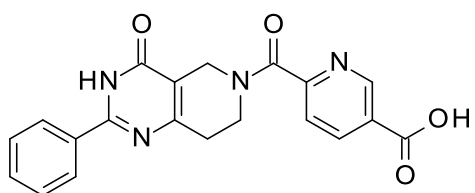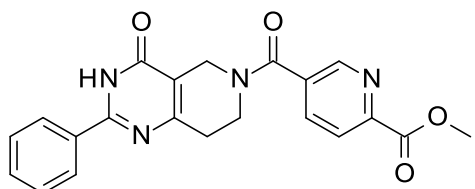

4-[(4-oxo-2-phenyl-3,5,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-6(4*H*)-yl)carbonyl]naphthalene-1-car-boxylic acid (10)

To a MeOH (10 mL) solution of **S8** (85 mg, 0.19 mmol) was added 1M NaOH aq. (3 mL). The mixture was stirred overnight at rt. 1M aq. HCl (3 mL) was added to it, and the resulting precipitate was collected by filtration, washed with water and MeOH, and dried at 60 °C under reduced pressure to afford **10** (68 mg, 83% yield) as a colorless solid. ^{1}H-NMR (DMSO-D$_6$) δ: 12.78 (1H, s), 8.87 (1H, d, J = 8.5 Hz), 8.17-8.03 (3H, m), 7.90-7.78 (1H, m), 7.71-7.45 (6H, m), 4.77-3.86 (2H, m), 3.45-2.46 (4H, m). MS (ESI/APCI) m/z: 426.2 (calcd for C$_{25}$H$_{20}$N$_3$O$_4$ (M+H)$^+$: 426.1).

methyl 6-[(4-oxo-2-phenyl-3,5,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-6(4*H*)-yl)carbonyl]pyridine-3-carboxylate (S9)

To a DMF (5 mL) solution of 5-methoxycarbonylpyridine-2-carboxylic acid (50 mg, 0.28 mmol) were added WSCI-HCl (107 mg, 0.55 mmol), HOBt (38 mg, 0.28 mmol), **S2** (100 mg, 0.33 mmol) and triethylamine (0.09 mL, 0.61 mmol). The mixture was stirred at rt overnight. Then it was concentrated *in vacuo*, dissolved with EtOAc, and washed with 10% aq. citric acid, sat. aq. NaHCO$_3$, and brine, respectively. Filtration, concentration *in vacuo* and tritulation with EtOAc/*n*-hexane afforded **S9** (107 mg, 99% yield) as a colorless solid. ^{1}H-NMR (CDCl$_3$) δ: 9.24 (1H, d, J = 8.5 Hz), 8.45-8.42 (1H, m), 8.12-8.03 (2H, m), 7.86-7.78 (1H, m), 7.60-7.49 (3H, m), 4.83-4.58 (2H, m), 4.14-3.82 (2H, m), 4.01-4.00 (3H, m), 3.02-2.96 (2H, m). MS (ESI) m/z: 391.1 (calcd for C$_{21}$H$_{19}$N$_4$O$_4$ (M+H)$^+$: 391.1).

6-[(4-oxo-2-phenyl-3,5,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-6(4*H*)-yl)carbonyl]pyridine-3-carbox-ylic acid (11)

To a MeOH (4 mL) solution of **S9** (105 mg, 0.27 mmol) was added 1M NaOH aq. (2.5 mL). The mixture was stirred overnight at rt. 10% aq. citric acid was added to it and MeOH was removed under reduced pressure. The resulting precipitate was collected by filtration, washed with water, and dried under reduced pressure to afford **11** (82 mg, 81% yield) as a colorless solid. ^{1}H-NMR (DMSO-D$_6$) δ: 13.59 (1H, br s), 12.93-12.78 (1H, br m), 9.10 (1H, br s), 8.43-8.39 (1H, m), 8.08 (2H, t, J = 8.8 Hz), 7.78 (1H, t, J = 7.6 Hz), 7.61-7.49 (3H, m), 4.58-4.26 (2H, m), 4.01-3.60 (2H, m), 2.84-2.73 (2H, m). MS (ESI) m/z: 377.2 (calcd for C$_{20}$H$_{17}$N$_4$O$_4$ (M+H)$^+$: 377.1).

methyl 5-[(4-oxo-2-phenyl-3,5,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-6(4*H*)-yl)carbonyl]pyridine-2-carboxylate (S10)

To a DMF (3 mL) solution of 6-methoxycarbonylpyridine-3-carboxylic acid (47 mg, 0.26 mmol) were added WSCI-HCl (55 mg, 0.28 mmol), HOBt (32 mg, 0.23 mmol), **S2** (70 mg, 0.23 mmol) and triethylamine (0.08 mL, 0.56 mmol). The mixture was stirred at rt for 2 days. Concentration *in vacuo* and purification by column chromatography (SiO$_2$, MeOH/CH$_2$Cl$_2$ = 0/100 − 5/95 (v/v)) afforded **S10** (21 mg, 23% yield) as a colorless solid. ^{1}H-NMR (DMSO-D$_6$) δ: 8.80 (1H, s), 8.13-8.05 (4H, m), 7.57-7.48 (3H, m), 4.49-4.32 (2H, m), 3.92 (3H,

s), 3.85-3.73 (2H, m), 2.79 (2H, t, J = 6.1 Hz). MS (ESI/APCI) m/z: 391.2 (calcd for $C_{21}H_{19}N_4O_4$ (M+H)$^+$: 391.1).
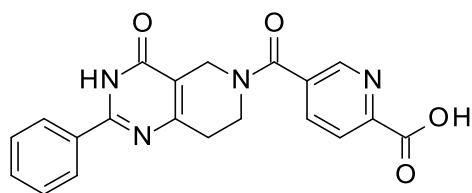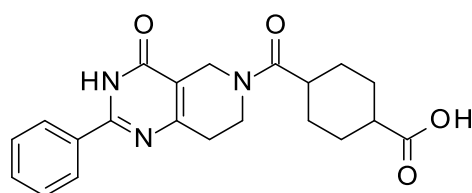

5-[(4-oxo-2-phenyl-3,5,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-6(4*H*)-yl)carbonyl]pyridine-2-carboxylic acid (12)

To a mixture of **S10** (21 mg, 0.054 mmol) and MeOH (5 mL) was added 1M NaOH aq. (2 mL). The mixture was stirred overnight at rt. 1M aq. HCl (2 mL) was added to it, and the resulting precipitate was collected by filtration, washed with water and MeOH, and dried at 60 °C under reduced pressure to afford **12** (16 mg, 79% yield) as a colorless solid. ^{1}H-NMR (DMSO-D$_6$) δ: 8.78 (1H, d, J = 1.2 Hz), 8.13-8.04 (4H, m), 7.59-7.47 (3H, m), 4.41 (2H, br s), 3.77 (2H, br s), 2.80 (2H, t, J = 5.5 Hz). MS (ESI/APCI) m/z: 377.2 (calcd for $C_{20}H_{17}N_4O_4$ (M+H)$^+$: 377.1).
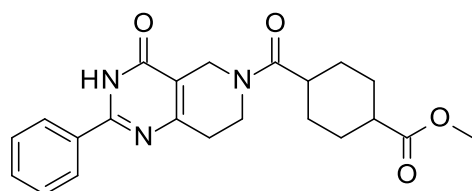
methyl 4-[(4-oxo-2-phenyl-3,5,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-6(4*H*)-yl)carbonyl]cyclohexanecarboxylate (S11)

To a solution of **S2** (199 mg, 0.67 mmol) and 4-(methoxycarbonyl)cyclohexane-1-carboxylic acid (136 mg, 0.73 mmol) in CH$_2$Cl$_2$ (4 mL) were added WSCI-HCl (153 mg, 0.80 mmol), HOBt-H$_2$O (103 mg, 0.67 mmol) and triethylamine (0.28 mL, 2.0 mmol). After stirring for 3 days at rt, the solution was diluted with MeOH-CHCl$_3$. The organic layer was washed with 10% aq. citric acid, sat. aq. NaHCO$_3$, and brine, dried over anhydrous Na$_2$SO$_4$, and filtrated. After evaporating the solvent, the residue was purified by column chromatography (SiO$_2$, MeOH/CH$_2$Cl$_2$ = 1/99 – 8/92 (v/v)) to give *cis/trans* mixture of **S11** (270 mg, quant.) as a white solid (*d.r.* = *ca.* 3:2). MS (ESI/APCI) m/z: 396.3 (calcd for $C_{22}H_{26}N_3O_4$ (M+H)$^+$: 396.2).


4-[(4-oxo-2-phenyl-3,5,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-6(4*H*)-yl)carbonyl]cyclohexanecarboxylic acid (13)

To a MeOH (6 mL) suspension of *cis/trans* mixture of **S11** (270 mg, 0.68 mmol) was added 1M NaOH aq. (6 mL). The mixture was stirred at rt for 4 h. 1M aq. HCl (6 mL) was added to it, and the resulting precipitate was collected by filtration. The solid was washed with water and dried at 60 °C under reduced pressure to afford *cis/trans* mixture of **13** (184 mg, 71% yield) as a white solid (*d.r.* = *ca.* 3:2). ^{1}H-NMR (DMSO-D$_6$) δ: 12.09 (2H, br s), 8.08 (2H, d, J = 7.3 Hz), 7.57-7.46 (3H, m), 4.35 (2H, s), 3.78-3.72 (2H, m), 2.78-2.64 (3H, m), 2.23-1.35 (9H, m). MS (ESI/APCI) m/z: 382.3 (calcd for $C_{21}H_{24}N_3O_4$ (M+H)$^+$: 382.2).
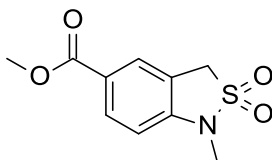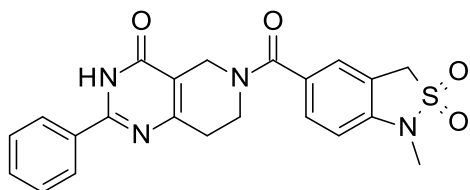
methyl 1-methyl-1,3-dihydro-2,1-benzothiazole-5-carboxylate 2,2-dioxide (S12)

To a DMF (1.5 mL) solution of methyl 1,3-dihydro-2,1-benzothiazole-5-carboxylate 2,2-dioxide (100 mg, 0.44 mmol) and potassium carbonate (60.8 mg, 0.44 mmol) was added iodomethane (0.110 mL, 1.76 mmol) and the mixture was stirred at rt for 3.5 h. Sat. aq. NH_4Cl was added to the reaction mixture and it was extracted with EtOAc/n-hexane (3:1). The organic layer was washed with water (twice) and brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure to afford **S12** (100 mg, 94% yield) as a brown solid. ^{1}H-NMR (CDCl$_3$) δ: 8.08-8.05 (1H, m), 7.96-7.94 (1H, m), 6.75 (1H, d, J = 8.5 Hz), 4.39 (2H, s), 3.91 (3H, s), 3.20 (3H, s).
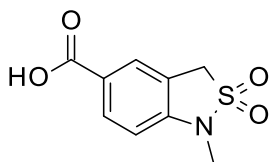
1-methyl-1,3-dihydro-2,1-benzothiazole-5-carboxylic acid 2,2-dioxide (S13)

To a mixture of **S12** (98 mg, 0.41 mmol), MeOH (5 mL) and THF (5 mL) was added 1M NaOH aq. (5 mL). After stirring for 1 h at rt, 1M aq. HCl (6 mL) was added to the mixture, and it was extracted with EtOAc. The organic layer was washed with water and brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure to afford **S13** (61 mg, 66% yield) as a brown solid. ^{1}H-NMR (DMSO-D$_6$) δ: 7.97-7.93 (1H, m), 7.89-7.88 (1H, m), 7.03 (1H, d, J = 8.5 Hz), 4.77 (2H, s), 3.11 (3H, s).

6-[(1-methyl-2,2-dioxido-1,3-dihydro-2,1-benzothiazol-5-yl)carbonyl]-2-phenyl-5,6,7,8-tetrahydro-pyrido[4,3-*d*]pyrimidin-4(3*H*)-one (14)

To a suspension of **S2** (27 mg, 0.090 mmol) and **S13** (21 mg, 0.092 mmol) in CH_2Cl_2 (2 mL) were added WSCI-HCl (22.1 mg, 0.108 mmol), HOBt-H_2O (13.7 mg, 0.090 mmol) and triethylamine (0.0374 mL, 0.270 mmol). After stirring overnight at rt, the solution was diluted with CHCl$_3$, washed with 10% aq. citric acid, sat. aq. $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4, and filtrated. After evaporating the solvent, the residue was purified by column chromatography (SiO$_2$, MeOH/CH_2Cl_2 = 1/99 – 8/92 (v/v)) to give **14** (24.4 mg, 62% yield) as a white solid. ^{1}H-NMR (DMSO-D$_6$) δ: 12.84 (1H, s), 8.10-8.04 (2H, m), 7.60-7.48 (5H, m), 7.03 (1H, d, J = 8.5 Hz), 4.74 (2H, s), 4.46-4.33 (2H, m), 3.94-3.60 (2H, m), 3.10 (3H, s), 2.81-2.75 (2H, m). MS (ESI/APCI) m/z: 437.2 (calcd for $C_{22}H_{21}N_4O_4S$ (M+H)$^+$: 437.1).
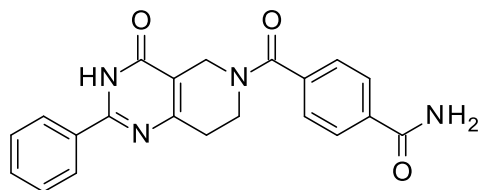
4-[(4-oxo-2-phenyl-3,5,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-6(4*H*)-yl)carbonyl]benzamide (16)

To a DMF (3 mL) suspension of terephthalamic acid (61 mg, 0.37 mmol) were added WSCI-HCl (76 mg, 0.40 mmol), HOBt (45 mg, 0.33 mmol), **S2** (100 mg, 0.33 mmol) and triethylamine (0.11 mL, 0.80 mmol). The

mixture was stirred at rt for 2 days. After concentration *in vacuo*, the residue was purified by column chromatography (SiO$_2$, MeOH/CH$_2$Cl$_2$ = 5/95 – 15/85 (v/v)). The obtained solid was washed with EtOAc to afford **16** (52 mg, 42% yield) as a colorless solid. ^{1}H-NMR (DMSO-D$_6$) δ: 12.90-12.80 (1H, br m), 8.13-7.93 (5H, m), 7.61-7.48 (6H, m), 4.53-4.22 (2H, m), 3.96-3.56 (2H, m), 2.78 (2H, s). MS (ESI/APCI) m/z: 375.2 (calcd for C$_{21}$H$_{19}$N$_4$O$_3$ (M+H)$^+$: 375.1).
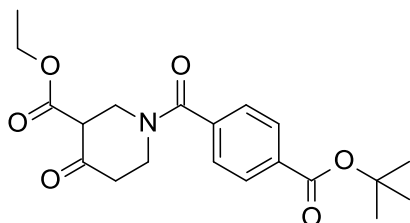

ethyl 1-[4-(*tert*-butoxycarbonyl)benzoyl]-4-oxopiperidine-3-carboxylate (22)

To a DMF (30 mL) solution of ethyl 4-oxo-3-piperidinecarboxylate hydrochloride (3.0 g, 14.4 mmol) and 4-(*tert*-butoxycarbonyl)benzoic acid (3.2 g, 14.4 mmol) were added WSCI-HCl (5.4 g, 28.9 mmol), HOBt-H$_2$O (2.22 g, 14.4 mmol) and triethylamine (3.0 mL, 21.7 mmol). The mixture was stirred at rt for 2 days. After concentration *in vacuo*, water (100 mL) was added to the mixture, and it was extracted with EtOAc. The organic layer was dried over anhydrous Na$_2$SO$_4$, filtered, concentrated under reduced pressure, and purified by column chromatography (SiO$_2$, EtOAc/*n*-hexane = 21/79 – 42/58 (v/v)) to give **22** (3.5 g, 65% yield) as a colorless oil. ^{1}H-NMR (CDCl$_3$) δ: 8.07-8.02 (2H, m), 7.53-7.43 (2H, m), 4.43-3.44 (6H, m), 2.58-2.34 (2H, m), 1.61 (9H, s), 1.39-1.15 (4H, m). MS (ESI/APCI) m/z: 376.3 (calcd for C$_{20}$H$_{26}$NO$_6$ (M+H)$^+$: 376.2).
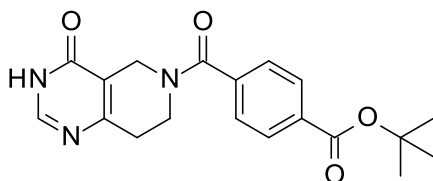

tert*-butyl 4-[(4-oxo-3,5,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-6(4*H*)-yl)carbonyl]benzoate (23a)

To a solution of **22** (58 mg, 0.15 mmol) in ethanol (1.5 mL) were added formamidine hydrochloride (14 mg, 0.17 mmol) and potassium carbonate (47 mg, 0.34 mmol). The mixture was stirred at rt for 3 days. After concentration *in vacuo*, 10% aq. citric acid was added to the mixture. The resulting precipitate was filtered, washed with water, dissolved with ethanol, and concentrated under reduced pressure to give **23a** (19 mg, 35% yield) as a colorless solid. MS (ESI/APCI) m/z: 356.2 (calcd for C$_{19}$H$_{22}$N$_3$O$_4$ (M+H)$^+$: 356.2).
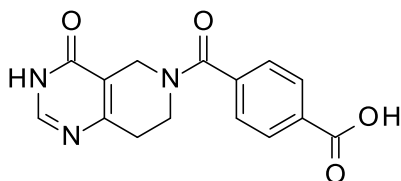

4-[(4-oxo-3,5,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-6(4*H*)-yl)carbonyl]benzoic acid (24)

To a CH$_2$Cl$_2$ (5 mL) solution of **23a** (19 mg, 0.053 mmol) was added trifluoroacetic acid (5 mL) at rt. The mixture was stirred at rt overnight. Concentration *in vacuo* and tritulation with EtOAc/*n*-hexane afforded **24** (13 mg, 81% yield) as a colorless solid. ^{1}H-NMR (DMSO-D$_6$) δ: 12.54 (1H, br s), 8.11-8.05 (1H, m), 8.03-8.00 (2H, m), 7.61-7.54 (2H, m), 4.45-4.13 (2H, m), 3.91-3.48 (2H, m), 2.71-2.64 (2H, m). MS (ESI/APCI) m/z: 300.2 (calcd for C$_{15}$H$_{14}$N$_3$O$_4$ (M+H)$^+$: 300.1).
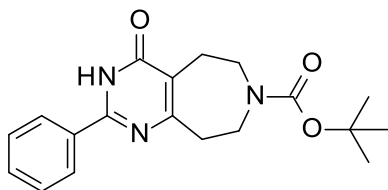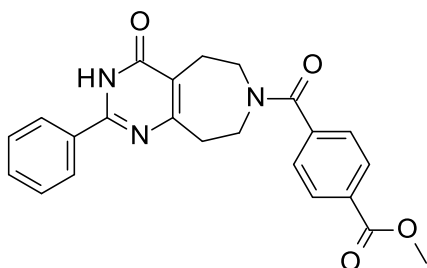
tert-butyl 4-oxo-2-phenyl-3,4,5,6,8,9-hexahydro-7*H*-pyrimido[4,5-*d*]azepine-7-carboxylate (19a)

To a suspension of ethyl 1-Boc-5-oxoazepane-4-carboxylate (500 mg, 1.75 mmol) and benzamidine hydrochloride (302 mg, 1.93 mmol) in ethanol (10 mL) was added potassium carbonate (533 mg, 3.86 mmol) at rt. The mixture was stirred at rt overnight. Insoluble materials were removed by filteration, and the filtrate was concentrated *in vacuo*. Purification by column chromatography (SiO$_2$, MeOH/CH$_2$Cl$_2$ = 0/100 − 5/95 (v/v)) afforded **19a** (433 mg, 72% yield) as a colorless solid. ^{1}H-NMR (CDCl$_3$) δ: 8.06 (2H, d, J = 6.7 Hz), 7.60-7.50 (3H, m), 3.72-3.55 (4H, m), 3.07 (2H, br s), 2.97 (2H, br s), 1.50 (9H, s). MS (ESI/APCI) m/z: 342.3 (calcd for C$_{19}$H$_{24}$N$_3$O$_3$ (M+H)$^+$: 342.2).
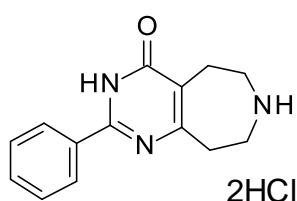
2-phenyl-3,5,6,7,8,9-hexahydro-4*H*-pyrimido[4,5-*d*]azepin-4-one 2HCl (S14)

To a MeOH (10 mL) suspension of **19a** (432 mg, 1.27 mmol) was added 4M HCl in 1,4-dioxane (5 mL) at rt. The mixture was stirred at rt for 2 h. Concentration *in vacuo* and trituration with EtOAc afforded **S14** (398 mg, quant.) as a colorless solid. MS (ESI/APCI) m/z: 242.2 (calcd for C$_{14}$H$_{16}$N$_3$O (M+H)$^+$: 242.1).


methyl 4-[(4-oxo-2-phenyl-3,4,5,6,8,9-hexahydro-7*H*-pyrimido[4,5-*d*]azepin-7-yl)carbonyl]benzoate (20a)

To a DMF (6 mL) solution of **S14** (100 mg, 0.32 mmol) and 4-methoxycarbonylbenzoic acid (64 mg, 0.35 mmol) were added WSCI-HCl (74 mg, 0.38 mmol), HOBt (44 mg, 0.32 mmol) and triethylamine (0.11 mL, 0.77 mmol) at 0 °C. The mixture was stirred at rt for 2 days. After evaporating solvents, the residue was dissolved with CH$_2$Cl$_2$, and the organic layer was washed with water and dried over anhydrous Na$_2$SO$_4$. Filtration, concentration *in vacuo*, and purification by column chromatography (SiO$_2$, MeOH/CH$_2$Cl$_2$ = 0/100 − 3/97 (v/v)) afforded **20a** (110 mg, 86% yield) as a colorless solid. ^{1}H-NMR (CDCl$_3$) δ: 8.15-8.04 (4H, m), 7.61-7.48 (5H, m), 4.02-3.90 (5H, m), 3.63-3.50 (2H, m), 3.26-3.09 (2H, m), 3.02-2.87 (2H, m). MS (ESI/APCI) m/z: 404.3 (calcd for C$_{23}$H$_{22}$N$_3$O$_4$ (M+H)$^+$: 404.2).
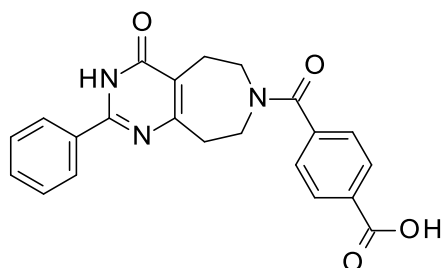
4-[(4-oxo-2-phenyl-3,4,5,6,8,9-hexahydro-7H-pyrimido[4,5-d]azepin-7-yl)carbonyl]benzoic acid (25)

To a MeOH (10 mL) suspension of **20a** (108 mg, 0.27 mmol) was added 1M NaOH aq. (5 mL). The mixture was stirred at rt for 2 h. 1M aq. HCl (5 mL) was added to it, and the resulting precipitate was collected by filtration. The solid was washed with water and MeOH, and dried at 60 °C under reduced pressure to afford **25** (85 mg, 82% yield) as a colorless solid. ^{1}H-NMR (DMSO-D$_6$) δ: 13.16 (1H, br s), 12.79 (1H, br s), 8.16-7.98 (4H, m), 7.61-7.44 (5H, m), 3.91-3.73 (2H, m), 3.53-3.39 (2H, m), 3.14-2.76 (4H, m). MS (ESI/APCI) m/z: 390.1 (calcd for C$_{22}$H$_{20}$N$_3$O$_4$ (M+H)$^+$: 390.1).
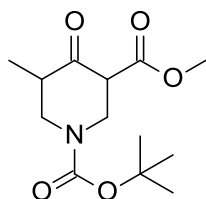
1-tert-butyl 3-methyl 5-methyl-4-oxopiperidine-1,3-dicarboxylate (17b)

To a solution of Boc$_2$O (378 mg, 1.73 mmol) in CH$_2$Cl$_2$ (5 mL) were added 3-methyl-5-methoxycarbonyl-4-piperidone hydrochloride (300 mg, 1.44 mmol) and triethylamine (0.208 mL, 2.02 mmol), and the mixture was stirred at rt until the starting material was consumed. The solution was washed with water, dried over anhydrous Na$_2$SO$_4$, concentrated *in vacuo*, and purified by column chromatography (SiO$_2$, EtOAc/n-hexane = 1/4 (v/v)) to afford **17b** (392 mg, quant.) as an oil. MS (ESI/APCI) m/z: 172.3, 216.2 (calcd for C$_8$H$_{14}$NO$_3$ (M–Boc+H)$^+$: 172.1, for C$_9$H$_{14}$NO$_5$ (M–tBu+H)$^+$: 216.1).
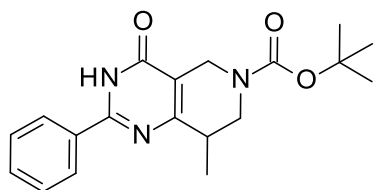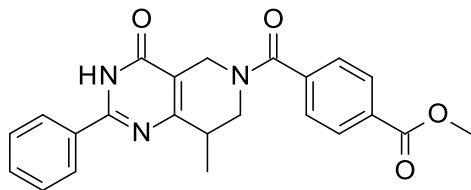

tert-butyl   8-methyl-4-oxo-2-phenyl-3,5,7,8-tetrahydropyrido[4,3-d]pyrimidine-6(4H)-carboxylate (19b)

To a suspension of **17b** (387 mg, 1.43 mmol) and benzamidine hydrochloride (246 mg, 1.57 mmol) in ethanol (6 mL) was added potassium carbonate (435 mg, 3.14 mmol) at rt. The mixture was stirred at rt overnight. Insoluble materials were removed by filteration, and the filtrate was concentrated *in vacuo*. Purification by column chromatography (SiO$_2$, EtOAc/CH$_2$Cl$_2$ = 1/9 – 1/1 (v/v)) afforded **19b** (303 mg, 62% yield) as a white solid. ^{1}H-NMR (DMSO-D$_6$) δ: 12.80 (1H, s), 8.12-8.06 (2H, m), 7.60-7.49 (3H, m), 4.44-4.06 (2H, m), 3.60-3.49 (2H, m), 2.82-2.73 (1H, m), 1.44 (9H, s), 1.22 (3H, d, J = 6.7 Hz). MS (ESI/APCI) m/z: 342.2 (calcd for C$_{19}$H$_{24}$N$_3$O$_3$ (M+H)$^+$: 342.2).
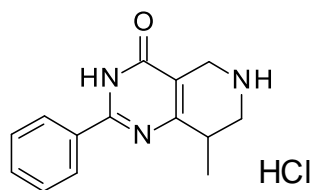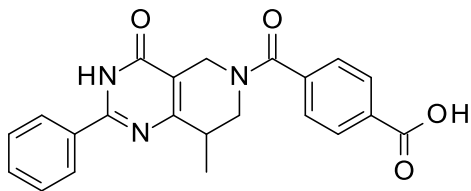
8-methyl-2-phenyl-5,6,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-4(3*H*)-one HCl (S15)

To a MeOH (1.5 mL) suspension of **19b** (157 mg, 0.46 mmol) was added 4M HCl in 1,4-dioxane (1.5 mL) at rt. The mixture was stirred at rt for 1.5 h. Concentration under reduced pressure gave crude **S15** (128 mg, quant.) as a white solid, which was used for the next step without further purification. MS (ESI/APCI) m/z: 242.1 (calcd for $C_{14}H_{16}N_3O$ (M+H)$^+$: 242.1).


methyl 4-[(8-methyl-4-oxo-2-phenyl-3,5,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-6(4*H*)-yl)carbonyl]benzoate (20b)

To a mixture of **S15** (128 mg, 0.46 mmol) and 4-methoxycarbonylbenzoic acid (91.4 mg, 0.51 mmol) in CH_2Cl_2 (4 mL) were added WSCI-HCl (107.5 mg, 0.55 mmol), HOBt-H_2O (70.6 mg, 0.46 mmol) and triethyl-amine (0.192 mL, 1.38 mmol). After stirring at rt overnight, the solution was diluted with CH_2Cl_2. The organic layer was washed with 10% aq. citric acid, sat. aq. $NaHCO_3$, and brine, dried over anhydrous Na_2SO_4, and filtrated. After evaporating solvents, the residue was purified by column chromatography (SiO_2, MeOH/CH_2Cl_2 = 0/100 − 5/95 (v/v)) to give **20b** (180 mg, 97% yield) as a white solid. ^{1}H-NMR (DMSO-D_6) δ: 12.92-12.74 (1H, m), 8.14-8.04 (4H, m), 7.65-7.49 (5H, m), 4.64-4.41 (1H, m), 4.25-4.19 (1H, m), 3.94-3.60 (5H, m), 2.96-2.81 (1H, m), 1.36-1.10 (3H, m). MS (ESI/APCI) m/z: 404.3 (calcd for $C_{23}H_{22}N_3O_4$ (M+H)$^+$: 404.2).
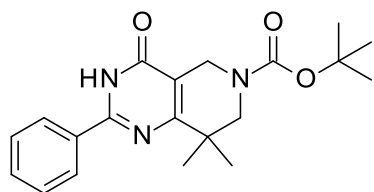

4-[(8-methyl-4-oxo-2-phenyl-3,5,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-6(4*H*)-yl)carbonyl]benzoic acid (26)

To a MeOH (5 mL) suspension of **20b** (144 mg, 0.36 mmol) was added 1M NaOH aq. (5 mL). The mixture was stirred at rt for 2 h. 1M aq. HCl (5 mL) was added to it, and the resulting precipitate was collected by filtration, washed with water, and dried at 60 °C under reduced pressure to afford **26** (109 mg, 78% yield) as a white solid. ^{1}H-NMR (DMSO-D_6, 80 °C) δ: 12.63 (2H, br s), 8.13-7.99 (4H, m), 7.58-7.47 (5H, m), 4.50-4.28 (2H, m), 3.82-3.51 (2H, m), 2.89 (1H, br s), 1.25 (3H, s). MS (ESI/APCI) m/z: 390.3 (calcd for $C_{22}H_{20}N_3O_4$ (M+H)$^+$: 390.1).
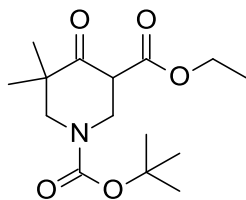

1-*tert*-butyl 3-ethyl 5,5-dimethyl-4-oxopiperidine-1,3-dicarboxylate (17c)

To a THF (5 mL) solution of 1-Boc-3,3-dimethyl-4-oxopiperidine (200 mg, 0.88 mmol) at −78 °C was added lithium bis(trimethylsilyl)amide (1.0 M in THF, 1.06 mL), and the mixture was stirred for 1 h at the same temperature. Ethyl cyanoformate (0.104 mL, 1.06 mmol) was added to the solution and the mixture was stirred for 1 h at −78 °C. Water was added to the mixture, and it was extracted with EtOAc (3 times). The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure to afford crude **17c** (263 mg, quant.), which was used for the next step without further purification. MS (ESI/APCI) m/z: 200.3, 244.2 (calcd for $C_{10}H_{18}NO_3$ (M−Boc+H)$^+$: 200.1, for $C_{11}H_{18}NO_5$ (M−*t*Bu+H)$^+$: 244.1).
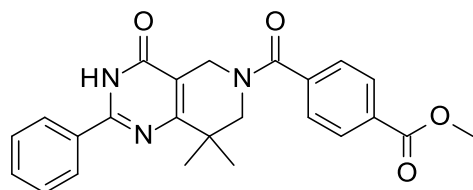
***tert*-butyl 8,8-dimethyl-4-oxo-2-phenyl-3,5,7,8-tetrahydropyrido[4,3-*d*]pyrimidine-6(4*H*)-carboxylate (19c)**

To a suspension of **17c** (263 mg, 0.88 mmol) and benzamidine hydrochloride (152 mg, 0.97 mmol) in ethanol (10 mL) was added potassium carbonate (267 mg, 1.93 mmol) at rt. The mixture was stirred at rt overnight. Insoluble materials were removed by filteration, and the filtrate was concentrated *in vacuo*. Purification by column chromatography (SiO$_2$, MeOH/CH$_2$Cl$_2$ = 0/100 – 5/95 (v/v)) afforded **19c** (233 mg, 75% yield) as a colorless solid. ^{1}H-NMR (DMSO-D$_6$) δ: 12.79 (1H, s), 8.11 (2H, d, J = 7.3 Hz), 7.58-7.51 (3H, m), 4.27 (2H, s), 3.42 (2H, s), 1.44 (9H, s), 1.22 (6H, s). MS (ESI/APCI) m/z: 356.3 (calcd for C$_{20}$H$_{26}$N$_3$O$_3$ (M+H)$^+$: 356.2).
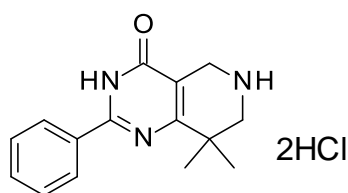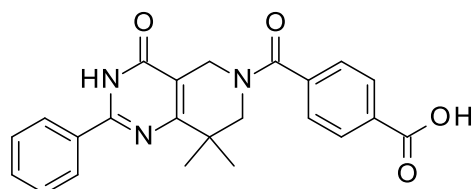

8,8-dimethyl-2-phenyl-5,6,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-4(3*H*)-one 2HCl (S16)

To a MeOH (10 mL) suspension of **19c** (232 mg, 0.65 mmol) was added 4M HCl in 1,4-dioxane (5 mL) at rt. The mixture was stirred at rt for 2 h. Concentration *in vacuo* and trituration with EtOAc gave **S16** (190 mg, 89% yield) as a colorless solid. MS (ESI/APCI) m/z: 256.3 (calcd for C$_{15}$H$_{18}$N$_3$O (M+H)$^+$: 256.1).

methyl 4-[(8,8-dimethyl-4-oxo-2-phenyl-3,5,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-6(4*H*)-yl)carbonyl]benzoate (20c)

To a DMF (6 mL) solution of **S16** (100 mg, 0.30 mmol) and 4-methoxycarbonylbenzoic acid (61 mg, 0.34 mmol) were added WSCI-HCl (71 mg, 0.37 mmol), HOBt (41 mg, 0.30 mmol) and triethylamine (0.11 mL, 0.73 mmol). The mixture was stirred at rt for 2 days. Concentration *in vacuo* and purification by column chromatography (SiO$_2$, MeOH/CH$_2$Cl$_2$ = 0/100 – 3/97 (v/v)) afforded **20c** (95 mg, 75% yield) as a colorless solid. ^{1}H-NMR (CDCl$_3$) δ: 8.13 (2H, d, J = 8.5 Hz), 8.11-8.05 (2H, m), 7.57-7.51 (3H, m), 7.48-7.43 (2H, m), 4.87-4.39 (2H, m), 3.96 (3H, s), 3.89-3.41 (2H, m), 1.48-1.18 (6H, m). MS (ESI/APCI) m/z: 418.2 (calcd for C$_{24}$H$_{24}$N$_3$O$_4$ (M+H)$^+$: 418.2).

4-[(8,8-dimethyl-4-oxo-2-phenyl-3,5,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-6(4*H*)-yl)carbonyl]benzoic acid (27)

To a MeOH (10 mL) suspension of **20c** (95 mg, 0.23 mmol) was added 1M NaOH aq. (5 mL). The mixture was stirred at rt for 2 h. 1M aq. HCl (5 mL) was added to it, and the resulting precipitate was collected by

filtration. The solid was washed with water and MeOH, and dried at 60 °C under reduced pressure to afford **27** (87 mg, 95% yield) as a white solid. ^{1}H-NMR (DMSO-D$_6$) δ: 12.63 (1H, br s), 8.11 (2H, d, J = 7.9 Hz), 8.02 (2H, d, J = 7.9 Hz), 7.59-7.48 (5H, m), 4.39 (2H, s), 3.61 (2H, s), 1.25 (6H, s). MS (ESI/APCI) m/z: 404.2 (calcd for C$_{23}$H$_{22}$N$_3$O$_4$ (M+H)$^+$: 404.2).
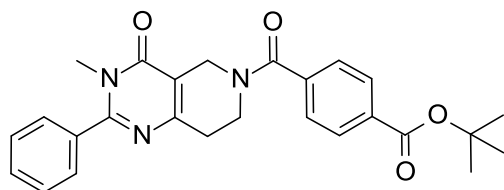

tert-butyl   4-[(3-methyl-4-oxo-2-phenyl-3,5,7,8-tetrahydropyrido[4,3-_d_]pyrimidin-6(4_H_)-yl)car-bonyl]benzoate (23b)

To a solution of **22** (201 mg, 0.53 mmol) and *N*-methylbenzenecarboximidamide hydrochloride (100 mg, 0.59 mmol) in ethanol (3.2 mL) was added potassium carbonate (163 mg, 1.17 mmol). The reaction mixture was stirred at 70 °C for 6 h. The mixture was diluted with ethanol, and insoluble materials were removed by filtration. The filtrate was concentrated *in vacuo* and purified by column chromatography (SiO$_2$, EtOAc/CH$_2$Cl$_2$ = 1/9 – 4/6 (v/v)) to afford **23b** (82.5mg, 35% yield) as a colorless solid. ^{1}H-NMR (DMSO-D$_6$) δ: 8.02-7.97 (2H, m), 7.63-7.58 (4H, m), 7.56-7.51 (3H, m), 4.54-4.22 (2H, m), 3.96-3.51 (2H, m), 3.36-3.23 (3H, m), 2.76-2.68 (2H, m), 1.57 (9H, s). MS (ESI/APCI) m/z: 446.2 (calcd for C$_{26}$H$_{28}$N$_3$O$_4$ (M+H)$^+$: 446.2).
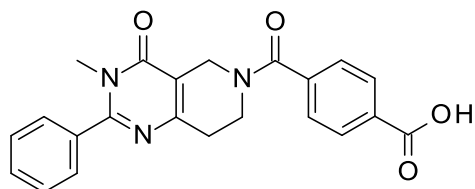

4-[(3-methyl-4-oxo-2-phenyl-3,5,7,8-tetrahydropyrido[4,3-_d_]pyrimidin-6(4_H_)-yl)carbonyl]benzoic acid (28)

To a CH$_2$Cl$_2$ (1.5 mL) solution of **23b** (38.5 mg, 0.086 mmol) was added trifluoroacetic acid (0.5 mL) at rt. The mixture was stirred at rt for 6 h. Concentration *in vacuo* and tritulation with Et$_2$O afforded **28** (19.4 mg, 58% yield) as a white solid. ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 8.03-8.00 (2H, m), 7.59-7.50 (7H, m), 4.43-4.37 (2H, m), 3.82-3.66 (2H, m), 3.30 (3H, s), 2.73-2.69 (2H, m). MS (ESI/APCI) m/z: 390.3 (calcd for C$_{22}$H$_{20}$N$_3$O$_4$ (M+H)$^+$: 390.1).
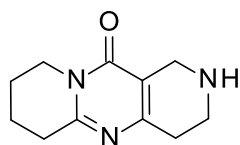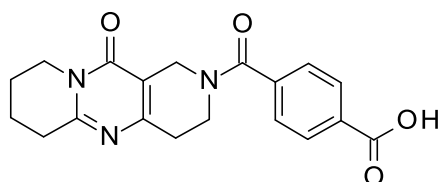

1,2,3,4,6,7,8,9-octahydro-11_H_-dipyrido[1,2-_a_:4',3'-_d_]pyrimidin-11-one (S17)

To a suspension of ethyl 4-oxo-3-piperidinecarboxylate hydrochloride (1.00 g, 4.82 mmol) and 2-iminopiperidine hydrochloride (650 mg, 4.82 mmol) in ethanol (9 mL) was added sodium ethoxide solution (6 mL, *ca.* 20% in ethanol, 15.5 mmol) at rt. Insoluble salt was removed by filtration, and the filtrate was stirred at 85–90 °C for 6 h. The resulting precipitate was collected by filtration, washed with CH$_2$Cl$_2$, dried under reduced pressure, and purified by column chromatography (SiO$_2$, MeOH/CH$_2$Cl$_2$ = 0/100 – 20/80 (v/v)) to afford **S17** (384 mg, 39% yield) as a yellow solid. ^{1}H-NMR (CDCl$_3$) δ: 3.95 (2H, t, J = 6.1 Hz), 3.81 (2H, s), 3.14-3.10 (2H, m), 2.91-2.87 (2H, m), 2.63-2.59 (2H, m), 2.00-1.85 (4H, m). MS (ESI/APCI) m/z: 206.2 (calcd for C$_{11}$H$_{16}$N$_3$O (M+H)$^+$: 206.1).
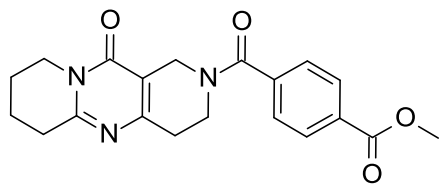
methyl 4-[(11-oxo-4,6,7,8,9,11-hexahydro-1*H*-dipyrido[1,2-*a*:4',3'-*d*]pyrimidin-2(3*H*)-yl)car-bonyl]benzoate (20d)

To a solution of **S17** (78.3 mg, 0.38 mmol) and 4-methoxycarbonylbenzoic acid (76 mg, 0.42 mmol) in CH$_2$Cl$_2$ (4 mL) were added WSCI-HCl (88.2 mg, 0.46 mmol), HOBt-H$_2$O (58.2 mg, 0.38 mmol) and triethyla-mine (0.159 mL, 1.14 mmol). After stirring overnight at rt, the solution was diluted with CH$_2$Cl$_2$. The organic layer was washed with 10% aq. citric acid, sat. aq. NaHCO$_3$, and brine, dried over anhydrous Na$_2$SO$_4$, and filtrated. After evaporating solvents, the residue was purified by column chromatography (SiO$_2$, MeOH/CH$_2$Cl$_2$ = 4/96 – 12/88 (v/v)) to give **20d** (126 mg, 90% yield) as a white solid. ^{1}H-NMR (DMSO-D$_6$) δ: 8.04 (2H, d, J = 7.9 Hz), 7.62-7.56 (2H, m), 4.45-4.12 (2H, m), 3.93-3.81 (5H, m), 3.77-3.46 (2H, m), 2.84-2.76 (2H, m), 2.69-2.60 (2H, m), 1.93-1.71 (4H, m). MS (ESI/APCI) m/z: 368.3 (calcd for C$_{20}$H$_{22}$N$_3$O$_4$ (M+H)$^+$: 368.2).


4-[(11-oxo-4,6,7,8,9,11-hexahydro-1*H*-dipyrido[1,2-*a*:4',3'-*d*]pyrimidin-2(3*H*)-yl)carbonyl]benzoic acid (29)

To a MeOH (5 mL) solution of **20d** (107 mg, 0.29 mmol) was added 1M NaOH aq. (5 mL). The mixture was stirred at rt for 2 h. After 1M aq. HCl (6 mL) was added to it until pH 2, the mixture was extracted with CHCl$_3$ (3 times) and MeOH-CHCl$_3$ (twice), respectively. The combined organic layers were dried over anhydrous Na$_2$SO$_4$, filtrated, concentrated *in vacuo*, and triturated with EtOAc to afford **29** (69 mg, 67% yield) as a white solid. ^{1}H-NMR (DMSO-D$_6$) δ: 13.19 (1H, s), 8.01 (2H, d, J = 8.5 Hz), 7.60-7.52 (2H, m), 4.45-4.13 (2H, m), 3.91-3.81 (2H, m), 3.76-3.47 (2H, m), 2.83-2.77 (2H, m), 2.68-2.59 (2H, m), 1.95-1.72 (4H, m). MS (ESI/APCI) m/z: 354.2 (calcd for C$_{19}$H$_{20}$N$_3$O$_4$ (M+H)$^+$: 354.1).
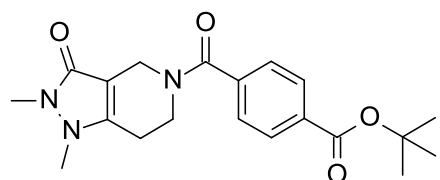

***tert*-butyl 4-[(1,2-dimethyl-3-oxo-1,2,3,4,6,7-hexahydro-5*H*-pyrazolo[4,3-*c*]pyridin-5-yl)carbonyl]ben-zoate (31a)**

A mixture of **22** (91 mg, 0.24 mmol), 1,2-dimethylhydrazine dihydrochloride (39 mg, 0.29 mmol) and tri-ethylamine (0.17 mL, 1.2 mmol) in ethanol (5 mL) was refluxed overnight. After evaporating solvents, water was added to the residue and it was extracted with EtOAc. The organic layer was dried over anhydrous Na$_2$SO$_4$, filtered, concentrated *in vacuo*, and purified by column chromatography (SiO$_2$, MeOH/CH$_2$Cl$_2$) to afford **31a** (55 mg, 61% yield) as a colorless solid. MS (ESI/APCI) m/z: 372.4 (calcd for C$_{20}$H$_{26}$N$_3$O$_4$ (M+H)$^+$: 372.2).
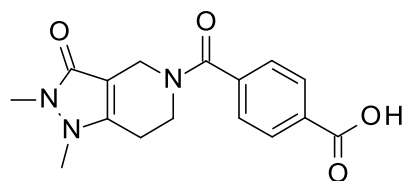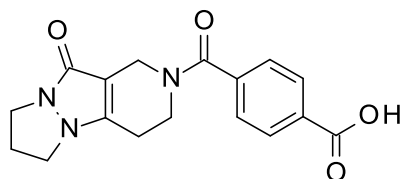
4-[(1,2-dimethyl-3-oxo-1,2,3,4,6,7-hexahydro-5H-pyrazolo[4,3-c]pyridin-5-yl)carbonyl]benzoic   acid (32)

To a CH$_2$Cl$_2$ (2 mL) solution of **31a** (55 mg, 0.15 mmol) was added trifluoroacetic acid (2 mL) at rt. The mixture was stirred at rt for 6 h. Concentration *in vacuo* and trituration with Et$_2$O afforded **32** (18 mg, 39% yield) as a pale brown solid. ^{1}H-NMR (DMSO-D$_6$) δ: 8.01 (2H, d, J = 7.9 Hz), 7.61-7.51 (2H, m), 4.34-3.45 (4H, m), 3.29-3.19 (6H, m), 2.71-2.58 (2H, m). MS (ESI/APCI) m/z: 316.3 (calcd for C$_{16}$H$_{18}$N$_3$O$_4$ (M+H)$^+$: 316.1).
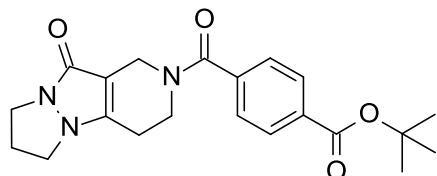

tert-butyl   4-[(10-oxo-4,7,8,10-tetrahydro-1H,6H-pyrazolo[1',2':1,2]pyrazolo[4,3-c]pyridin-2(3H)-yl)carbonyl]benzoate (31b)

A mixture of **22** (84 mg, 0.22 mmol), pyrazolidine dihydrochloride (39 mg, 0.27 mmol) and triethylamine (0.155 mL, 1.11 mmol) in ethanol (5 mL) was refluxed overnight. After evaporating solvents, water was added to the residue and it was extracted with EtOAc. The organic layer was dried over anhydrous Na$_2$SO$_4$, filtered, concentrated *in vacuo*, and purified by column chromatography (SiO$_2$, EtOAc/n-hexane) to afford **31b** (86 mg, quant.) as a pale yellow oil, which was used for the next step without further purification. MS (ESI/APCI) m/z: 384.3 (calcd for C$_{21}$H$_{26}$N$_3$O$_4$ (M+H)$^+$: 384.2).


4-[(10-oxo-4,7,8,10-tetrahydro-1H,6H-pyrazolo[1',2':1,2]pyrazolo[4,3-c]pyridin-2(3H)-yl)carbonyl]benzoic acid (33)

To a CH$_2$Cl$_2$ (2 mL) solution of **31b** (86 mg, 0.22 mmol) was added trifluoroacetic acid (2 mL) at rt. The mixture was stirred at rt for 6 h. Concentration *in vacuo* and trituration with Et$_2$O afforded **33** (22 mg, 24% yield) as a pale brown solid. ^{1}H-NMR (DMSO-D$_6$) δ: 8.06-7.95 (2H, m), 7.62-7.47 (2H, m), 4.35-3.45 (10H, m), 2.69-2.52 (2H, m). MS (ESI/APCI) m/z: 328.3 (calcd for C$_{17}$H$_{18}$N$_3$O$_4$ (M+H)$^+$: 328.1).
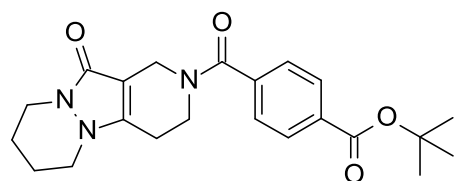

tert-butyl   4-[(11-oxo-4,6,7,8,9,11-hexahydro-1H-pyrido[3',4':4,5]pyrazolo[1,2-a]pyridazin-2(3H)-yl)carbonyl]benzoate (31c)

A mixture of **22** (72 mg, 0.19 mmol), hexahydropyridazine dihydrochloride (40 mg, 0.23 mmol) and triethylamine (0.140 mL, 1.01 mmol) in ethanol (5 mL) was refluxed overnight. After evaporating solvents, water was added to the residue and it was extracted with EtOAc. The organic layer was dried over anhydrous Na$_2$SO$_4$, filtered, concentrated *in vacuo*, and purified by column chromatography (SiO$_2$, EtOAc/n-hexane) to afford **31c** (57 mg, 75% yield) as a pale yellow oil. MS (ESI/APCI) m/z: 398.2 (calcd for C$_{22}$H$_{28}$N$_3$O$_4$ (M+H)$^+$: 398.2).
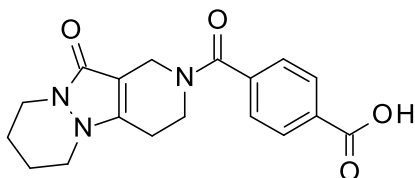
4-[(11-oxo-4,6,7,8,9,11-hexahydro-1*H*-pyrido[3',4':4,5]pyrazolo[1,2-*a*]pyridazin-2(3*H*)-yl)car-bonyl]benzoic acid (34)

To a CH$_2$Cl$_2$ (2 mL) solution of **31c** (57 mg, 0.14 mmol) was added trifluoroacetic acid (2 mL) at rt. The mixture was stirred at rt for 6 h. Concentration *in vacuo* and trituration with Et$_2$O afforded **34** (25 mg, 51% yield) as a pale brown solid. ^{1}H-NMR (DMSO-D$_6$) δ: 8.07-8.00 (2H, m), 7.59-7.52 (2H, m), 4.31-3.99 (2H, m), 3.92-3.38 (6H, m), 2.67-2.56 (2H, m), 1.87-1.69 (4H, m). MS (ESI/APCI) m/z: 342.1 (calcd for C$_{18}$H$_{20}$N$_3$O$_4$ (M+H)$^+$: 342.1).
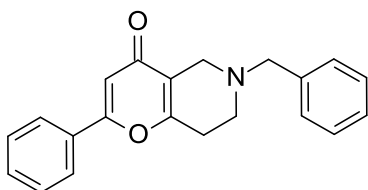

6-benzyl-2-phenyl-5,6,7,8-tetrahydro-4*H*-pyrano[3,2-*c*]pyridin-4-one (36)

A toluene (25 mL) solution of 1-benzyl-4-piperidone (1.89 mL, 10.6 mmol) and morpholine (0.921 mL, 10.6 mmol) was refluxed for 9 h under Dean-Stark apparatus. After the removal of the solvent, the residue and morpholine (0.46 mL, 5.28 mmol) were dissolved in toluene (35 mL) again and refluxed for 8 h under the same conditions. After concentrated under reduced pressure, crude 4-(1-benzyl-3,6-dihydro-2*H*-pyridin-4-yl)mor-pholine (2.71 g, quant.) was obtained as a yellow oil. This crude material (943.6 mg, 3.65 mmol) and ethyl benzoylacetate (1.27 mL, 7.31 mmol) were dissolved in xylenes (10 mL) and refluxed for 5 h under Dean-Stark apparatus. The mixture was poured into 1M aq. HCl, and the resulting precipitate was collected by filtration, washed with a small portion of water and CH$_2$Cl$_2$, dried at 60 °C to afford HCl salt of **37** (215 mg, 17% yield) as a yellow solid. The aqueous phase was washed with CH$_2$Cl$_2$, and NaOH pellets were added into it until the aqueous phase became basic indicated by pH test paper. It was extracted with CH$_2$Cl$_2$ (three times), and the combined organic layer was washed with brine, dried over anhydrous Na$_2$SO$_4$, filtered, and concentrated *in vacuo*. Purification by column chromatography (SiO$_2$, EtOAc/*n*-hexane = 1/3 − 1/1 (v/v)) afforded a free form of **36** (104 mg, 9% yield) as a yellow solid. ^{1}H-NMR (CDCl$_3$, for free form) δ: 7.78-7.72 (2H, m), 7.51-7.44 (3H, m), 7.39-7.27 (5H, m), 6.72 (1H, s), 3.74 (2H, s), 3.51 (2H, s), 2.79 (4H, s). MS (ESI/APCI) m/z: 318.2 (calcd for C$_{21}$H$_{20}$NO$_2$ (M+H)$^+$: 318.1).
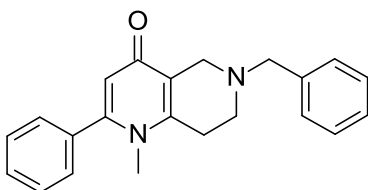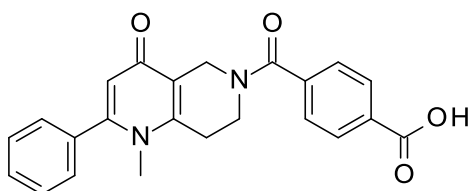

6-benzyl-1-methyl-2-phenyl-5,6,7,8-tetrahydro-1,6-naphthyridin-4(1*H*)-one (37)

A mixture of **36** (103 mg, 0.32 mmol) and methylamine solution (40% in water, 11 mL) was refluxed for 12 h. After evaporating the solvent, the residue was dissolved in 40% MeOH solution of methylamine (4 mL) and stirred at 100 °C for 1 h under microwave irradiation. The mixture was concentrated *in vacuo*, and the residue was purified by column chromatography (SiO$_2$, MeOH/CH$_2$Cl$_2$ = 1/99 − 10/90 (v/v)) to afford **37** (93.4 mg, 87% yield) as a pale yellow solid. ^{1}H-NMR (CDCl$_3$) δ: 7.50-7.25 (10H, m), 6.29 (1H, s), 3.75 (2H, s), 3.65 (2H, s), 3.32 (3H, s), 2.81-2.72 (4H, m). MS (ESI/APCI) m/z: 331.4 (calcd for C$_{22}$H$_{23}$N$_2$O (M+H)$^+$: 331.2).
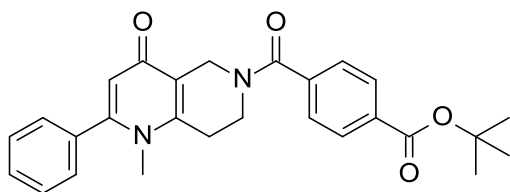
tert-butyl 4-[(1-methyl-4-oxo-2-phenyl-1,5,7,8-tetrahydro-1,6-naphthyridin-6(4_H_)-yl)carbonyl]benzoate (S18)

To a MeOH (8 mL) solution of **37** (93.4 mg, 0.28 mmol) and 1M aq. HCl (0.311 mL) was added 20% Pd(OH)$_2$/C (44.7 mg) under N$_2$ atmosphere. The mixture was vigorously stirred at rt under H$_2$ atmosphere (balloon) for 50 min. Under N$_2$, the mixture was diluted with MeOH, and filtered over a glass-fiber filter (twice). The filtrate was concentrated at 35 °C until the liquid volume became *ca.* 5 mL. To this solution were added 4-(*tert*-butoxycarbonyl)benzoic acid (114 mg, 0.51 mmol), WSCI-HCl (135 mg, 0.71 mmol), HOBt-H$_2$O (78.3 mg, 0.51 mmol) and triethylamine (0.157 mL, 1.13 mmol). The mixture was stirred at rt for 2 h. After evaporating solvents, the residue was diluted with CH$_2$Cl$_2$, washed with 10% aq. citric acid, sat. aq. NaHCO$_3$, and brine, dried over anhydrous Na$_2$SO$_4$, and filtrated. Concentration under reduced pressure followed by column chromatography (SiO$_2$, MeOH/CH$_2$Cl$_2$ = 0/100 – 10/90 (v/v)) afforded **S18** (113 mg, 90% yield) as a colorless solid. ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 8.00-7.95 (2H, m), 7.58-7.54 (2H, m), 7.53-7.49 (3H, m), 7.43-7.38 (2H, m), 5.94-5.91 (1H, m), 4.36-4.28 (2H, m), 3.90-3.64 (2H, m), 3.30 (3H, s), 2.93-2.86 (2H, m), 1.57 (9H, s). MS (ESI/APCI) m/z: 445.2 (calcd for C$_{27}$H$_{29}$N$_2$O$_4$ (M+H)$^+$: 445.2).


4-[(1-methyl-4-oxo-2-phenyl-1,5,7,8-tetrahydro-1,6-naphthyridin-6(4_H_)-yl)carbonyl]benzoic acid (38)

To a CH$_2$Cl$_2$ (3 mL) solution of **S18** (92.7 mg, 0.21 mmol) was added trifluoroacetic acid (1 mL) at rt. The mixture was stirred at rt for 2 h. Concentration *in vacuo* and trituration with Et$_2$O afforded **38** (81 mg, quant.) as a white solid. ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 8.05-8.01 (2H, m), 7.60-7.53 (5H, m), 7.47-7.42 (2H, m), 6.34 (1H, s), 4.51-4.44 (2H, m), 3.83-3.76 (2H, m), 3.46 (3H, s), 3.04-2.98 (2H, m). MS (ESI/APCI) m/z: 389.2 (calcd for C$_{23}$H$_{21}$N$_2$O$_4$ (M+H)$^+$: 389.1).
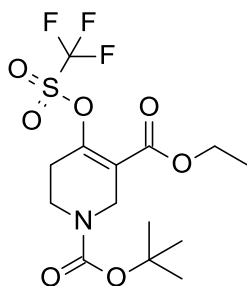

1-_tert_-butyl 3-ethyl 4-{[(trifluoromethyl)sulfonyl]oxy}-5,6-dihydropyridine-1,3(2_H_)-dicarboxylate (S19)

To a CH$_2$Cl$_2$ (200 mL) solution of *N*-Boc-4-oxo-3-piperidinecarboxylic acid ethyl ester (7.22 g, 26.6 mmol) and *N,N*-diisopropylethylamine (6.83 mL, 39.9 mmol) was added trifluoromethanesulfonic anhydride (5.37 mL, 31.9 mmol) in CH$_2$Cl$_2$ (25 mL) dropwise for 1 h at 0 °C. After stirring for 3 h at the same temperature, sat. aq. NaHCO$_3$ solution was added to the reaction mixture and it was extracted with CH$_2$Cl$_2$. The organic layer was washed with brine, dried over anhydrous Na$_2$SO$_4$, filtered and concentrated *in vacuo*. The residue was purified by column chromatography (SiO$_2$, EtOAc/*n*-hexane = 3/97 – 20/80 (v/v)) to give **S19** (7.80 g, 73% yield) as a yellow oil. The ^{1}H-NMR spectrum was matched with the reported one[33].
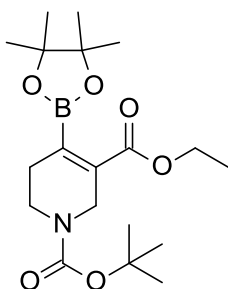
1-*tert*-butyl 3-ethyl 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-5,6-dihydropyridine-1,3(2*H*)-dicarboxylate (42)

A mixture of **S19** (4.59 g, 11.4 mmol), bis(pinacolato)diboron (3.47 g, 13.7 mmol), potassium acetate (3.37 g, 34.3 mmol) and Pd(dppf)Cl$_2$ CH$_2$Cl$_2$ adduct (930 mg, 1.14 mmol) in 1,4-dioxane (50 mL) was stirred at 110 °C for 3 h under N$_2$ atmosphere. After cooling down to rt, the mixture was filtered through Celite and concentrated *in vacuo*. Purification by column chromatography (SiO$_2$, EtOAc/*n*-hexane = 1/9 – 1/3 (v/v)) afforded **42** (3.43 g, 79% yield) as a pale yellow oil. The ^{1}H-NMR spectrum was matched with the reported one[34].
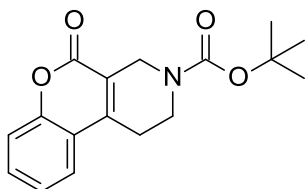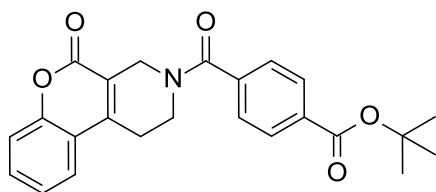

***tert*-butyl 5-oxo-1,5-dihydro-2*H*-chromeno[3,4-*c*]pyridine-3(4*H*)-carboxylate (S20)**

A mixture of **42** (500 mg, 1.31 mmol), 2-bromophenol (0.145 mL, 1.38 mmol), Pd(dppf)Cl$_2$ CH$_2$Cl$_2$ adduct (53.5 mg, 0.066 mmol), NaHCO$_3$ (275 mg, 3.28 mmol), THF (10 mL) and water (2.5 mL) was refluxed for 5.5 h. After cooling down to rt, the mixture was diluted with EtOAc, and filtered over Celite. The filtrate was concentrated *in vacuo*, and purified by column chromatography (SiO$_2$, EtOAc/*n*-hexane = 1/9 – 4/6 (v/v)) to afford **S20** (177 mg, 45% yield) as a pale yellow solid. ^{1}H-NMR (CDCl$_3$) δ: 7.54 (1H, d, J = 7.3 Hz), 7.52-7.47 (1H, m), 7.33 (1H, d, J = 8.3 Hz), 7.32-7.28 (1H, m), 4.40 (2H, s), 3.73 (2H, t, J = 5.9 Hz), 2.90-2.85 (2H, m), 1.47 (9H, s).
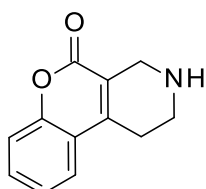

1,2,3,4-tetrahydro-5*H*-chromeno[3,4-*c*]pyridin-5-one (39)

To a solution of **S20** (174 mg, 0.58 mmol) in CH$_2$Cl$_2$ (3 mL) was added trifluoroacetic acid (1 mL) at 0 °C. The mixture was stirred at rt for 3.5 h. After evaporating volatile materials, sat. aq. NaHCO$_3$ was added to the residue, and it was extracted with CH$_2$Cl$_2$ (twice). The combined organic layer was dried over anhydrous Na$_2$SO$_4$, filtrated, and concentrated under reduced pressure to give **39** (123 mg, quant.) as a pale yellow solid. The analytical spectra were confirmed to be the same as the product **39** obtained from the 1st generation route. ^{1}H-NMR (DMSO-D$_6$) δ: 7.69 (1H, d, J = 7.8 Hz), 7.55 (1H, t, J = 7.1 Hz), 7.38-7.33 (2H, m), 3.54 (2H, s), 2.96 (2H, t, J = 5.6 Hz), 2.72 (2H, t, J = 5.6 Hz). MS (ESI/APCI) m/z: 202.2 (calcd for C$_{12}$H$_{12}$NO$_2$ (M+H)$^+$: 202.1).

tert-butyl 4-[(5-oxo-1,5-dihydro-2H-chromeno[3,4-c]pyridin-3(4H)-yl)carbonyl]benzoate (40)

To a suspension of **39** (120 mg, 0.596 mmol), 4-(tert-butoxycarbonyl)benzoic acid (146 mg, 0.656 mmol), HOBt-H_2O (92 mg, 0.596 mmol) and WSCI-HCl (138 mg, 0.716 mmol) in CH_2Cl_2 (5 mL) was added triethyl-amine (0.248 mL, 1.79 mmol) at rt. After stirring at rt for 4.5 h, the reaction mixture was diluted with CH_2Cl_2, and washed with 10% aq. citric acid, sat. aq. $NaHCO_3$, and brine, respectively. The organic layer was dried over anhydrous Na_2SO_4, and purified by column chromatography (SiO_2, EtOAc/CH_2Cl_2 = 4/96 – 20/80 (v/v)) to give **40** (227 mg, 94% yield) as a colorless solid. ^{1}H-NMR (DMSO-D_6) δ: 8.02-7.97 (2H, m), 7.82-7.71 (1H, m), 7.66-7.58 (3H, m), 7.49-7.39 (2H, m), 4.56-4.24 (2H, m), 3.99-3.56 (2H, m), 3.06-2.97 (2H, m), 1.57 (9H, s). MS (ESI/APCI) m/z: 350.3, 406.1 (calcd for $C_{20}H_{16}NO_5$ (M–tBu+H)$^+$: 350.1, for $C_{24}H_{24}NO_5$ (M+H)$^+$: 406.2).
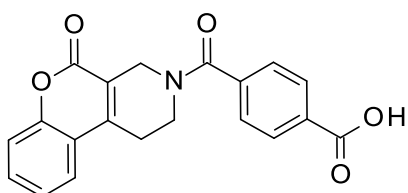

4-[(5-oxo-1,5-dihydro-2H-chromeno[3,4-c]pyridin-3(4H)-yl)carbonyl]benzoic acid (41)

To a CH_2Cl_2 (3 mL) solution of **40** (184 mg, 0.45 mmol) was added trifluoroacetic acid (1 mL) dropwise at rt. After stirring for 1.5 h at rt, the resulting mixture was concentrated in vacuo, and triturated with EtOAc. The solid was collected by filtration, and washed with EtOAc to afford **41** (125.7 mg, 79% yield) as a white solid. ^{1}H-NMR (DMSO-D_6) δ: 13.21 (1H, br s), 8.06-8.01 (2H, m), 7.83-7.72 (1H, m), 7.67-7.58 (3H, m), 7.49-7.39 (2H, m), 4.56-4.26 (2H, m), 3.99-3.57 (2H, m), 3.05-2.98 (2H, m). ^{13}C-NMR (DMSO-D_6, as a mixture of rota-mers) δ: 168.8, 168.4, 166.7, 158.9, 151.5, 146.6, 146.3, 139.6, 131.8, 131.4, 129.5, 127.3, 127.0, 124.6, 124.2, 119.3, 118.8, 116.4, 45.1, 42.6, 40.2, 37.5, 24.9, 23.9. MS (ESI/APCI) m/z: 350.3 (calcd for $C_{20}H_{16}NO_5$ (M+H)$^+$: 350.1). HRMS (ESI): m/z calcd for $C_{20}H_{14}NO_5$ (M–H)$^-$ 348.0878. Found 348.0895. IR (KBr) 3408, 2895, 2673, 2554, 1709, 1648, 1607, 1573, 1511, 1456, 1433, 1397, 1320, 1299, 1258, 1142, 1089, 1042, 749 cm^{-1}. Anal. Calcd for $C_{20}H_{15}NO_5 \cdot 0.2H_2O$: C, 68.06; H, 4.40; N, 3.97. Found: C, 67.94; H, 4.48; N, 3.97.
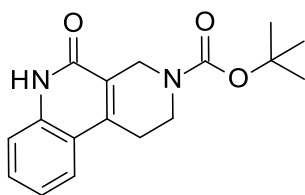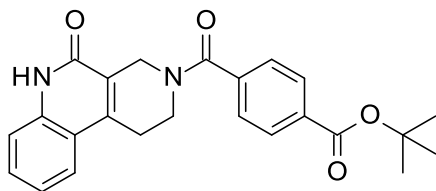

tert-butyl 5-oxo-1,4,5,6-tetrahydrobenzo[c][2,7]naphthyridine-3(2H)-carboxylate (S21)

A mixture of **42** (100 mg, 0.26 mmol), 2-bromoaniline (46.4 mg, 0.27 mmol), Pd(dppf)Cl_2 CH_2Cl_2 adduct (10.6 mg, 0.013 mmol), $NaHCO_3$ (56.7 mg, 0.67 mmol), THF (2 mL) and water (0.5 mL) was stirred at 100 ºC for 1.5 h under microwave irradiation. After cooling down to rt, the mixture was diluted with EtOAc, washed with water (twice) and brine, and dried over anhydrous Na_2SO_4. Filtration, concentration under reduced pres-sure, and trituration with ethanol afforded **S21** (45 mg, 57% yield) as a white solid. ^{1}H-NMR (DMSO-D_6) δ: 11.85 (1H, s), 7.71 (1H, d, J = 7.3 Hz), 7.49 (1H, t, J = 7.3 Hz), 7.33 (1H, d, J = 7.9 Hz), 7.22 (1H, t, J = 7.3 Hz), 4.26 (2H, s), 3.66-3.62 (2H, m), 2.94-2.88 (2H, m), 1.44 (9H, s). MS (ESI/APCI) m/z: 245.1, 301.2 (calcd for $C_{13}H_{13}N_2O_3$ (M–tBu+H)$^+$: 245.1, for $C_{17}H_{21}N_2O_3$ (M+H)$^+$: 301.1).
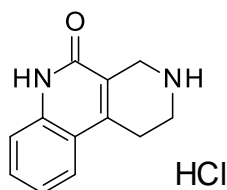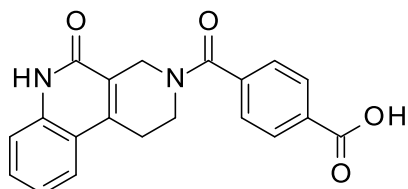
2,3,4,6-tetrahydrobenzo[*c*][2,7]naphthyridin-5(1*H*)-one HCl (43)

To a suspension of **S21** (42.7 mg, 0.14 mmol) in MeOH (2 mL) was added 4M HCl in 1,4-dioxane (1 mL) at rt. The mixture was stirred at rt for 1 h. Concentration under reduced pressure gave crude **43** (33.7 mg, quant.) as a white solid, which was used for the next step without further purification.


***tert*-butyl 4-[(5-oxo-1,4,5,6-tetrahydrobenzo[*c*][2,7]naphthyridin-3(2*H*)-yl)carbonyl]benzoate (44)**

To a solution of **43** (33.7 mg, 0.14 mmol), 4-(*tert*-butoxycarbonyl)benzoic acid (146 mg, 0.656 mmol) and triethylamine (0.0197 mL, 0.14 mmol) in MeOH (3 mL) was added DMT-MM hydrate (46.2 mg, 0.16 mmol) at rt. After stirring at rt for 2 h, the reaction mixture was concentrated *in vacuo*, and the residue was diluted with water. It was extracted with CHCl$_3$, and the organic layer was washed with brine, dried over anhydrous Na$_2$SO$_4$, and filtrated. Purification by column chromatography (SiO$_2$, MeOH/CH$_2$Cl$_2$ = 0/100 – 10/90 (v/v)) to give **44** (49.7 mg, 86% yield) as a white solid. ^{1}H-NMR (DMSO-D$_6$, 80 ℃) δ: 11.58 (1H, s), 7.99-7.96 (2H, m), 7.70-7.66 (1H, m), 7.57-7.54 (2H, m), 7.49-7.45 (1H, m), 7.35-7.32 (1H, m), 7.23-7.19 (1H, m), 4.45-4.39 (2H, m), 3.84-3.69 (2H, m), 3.01-2.98 (2H, m), 1.57 (9H, s). MS (ESI/APCI) m/z: 405.2 (calcd for C$_{24}$H$_{25}$N$_2$O$_4$ (M+H)$^+$: 405.2).


4-[(5-oxo-1,4,5,6-tetrahydrobenzo[*c*][2,7]naphthyridin-3(2*H*)-yl)carbonyl]benzoic acid (45)

A mixture of **44** (38.6 mg, 0.095 mmol), MeOH (1 mL), and 4M HCl in 1,4-dioxane (1 mL) was stirred at rt overnight. Concentration of the reaction mixture under reduced pressure gave methyl ester of **45**. The residue was dissolved in MeOH (1 mL) and 1M NaOH aq. (1 mL) was added to it. The mixture was stirred at rt for 1.5 h. 1M aq. HCl (1 mL) and MeOH (1 mL) was added to the mixture, and the resulting precipitate was collected by filtration, washed with water, and dried. Preparative reverse-phase HPLC and following trituration with ethanol at 50 ℃ afforded **45** (10.3 mg, 31% yield) as a white solid. ^{1}H-NMR (DMSO-D$_6$, 80 ℃) δ: 11.59 (1H, s), 8.03-8.00 (2H, m), 7.69 (1H, d, J = 7.9 Hz), 7.56 (2H, d, J = 7.9 Hz), 7.50-7.45 (1H, m), 7.34 (1H, d, J = 7.9 Hz), 7.23-7.19 (1H, m), 4.47-4.39 (2H, m), 3.84-3.73 (2H, m), 3.11-2.96 (2H, m). MS (ESI/APCI) m/z: 349.1 (calcd for C$_{20}$H$_{17}$N$_2$O$_4$ (M+H)$^+$: 349.1).
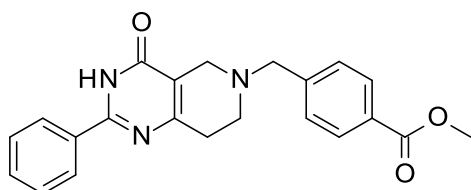

methyl 4-[(4-oxo-2-phenyl-3,5,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-6(4*H*)-yl)methyl]benzoate (S22)

Sodium triacetoxyborohydride (159 mg, 0.75 mmol) was added to the mixture of **S2** (150 mg, 0.50 mmol), methyl 4-formylbenzoate (91 mg, 0.55 mmol) and THF (10 mL). After stirring at rt overnight, sat. aq. NaHCO$_3$ was added to the mixture and it was extracted with CH$_2$Cl$_2$. The organic layer was washed with water, dried over anhydrous Na$_2$SO$_4$, filtered, and concentrated *in vacuo*. Purification by column chromatography (SiO$_2$, EtOAc/CH$_2$Cl$_2$ = 1/1 (v/v)) afforded **S22** (50 mg, 27% yield) as a colorless solid. ^{1}H-NMR (CDCl$_3$) δ: 11.83 (1H, s), 8.07-8.01 (4H, m), 7.55-7.39 (5H, m), 3.93 (3H, s), 3.80 (2H, s), 3.52 (2H, s), 2.91-2.78 (4H, m). MS (ESI/APCI) m/z: 376.1 (calcd for C$_{22}$H$_{22}$N$_3$O$_3$ (M+H)$^+$: 376.2).
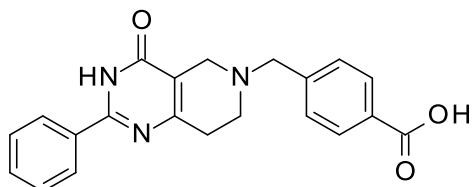

4-[(4-oxo-2-phenyl-3,5,7,8-tetrahydropyrido[4,3-*d*]pyrimidin-6(4*H*)-yl)methyl]benzoic acid (S23)

To a MeOH (10 mL) solution of **S22** (50 mg, 0.13 mmol) was added 1M NaOH aq. (3 mL). The mixture was stirred at rt for 3 days. 1M aq. HCl (3 mL) was added to it, and the resulting precipitate was filtrated and dried at 60 ºC under reduced pressure to afford **S23** (42 mg, 87% yield) as a colorless solid. ^{1}H-NMR (DMSO-D$_6$) δ: 12.87 (1H, br s), 12.67 (1H, br s), 8.07 (2H, d, J = 7.3 Hz), 7.94 (2H, d, J = 8.5 Hz), 7.60-7.47 (5H, m), 3.78 (2H, s), 3.27 (2H, s), 2.80-2.70 (4H, m). MS (ESI/APCI) m/z: 362.2 (calcd for C$_{21}$H$_{20}$N$_3$O$_3$ (M+H)$^+$: 362.1). The IC$_{50}$ value for MTHFD2 was found to be > 30 µM.
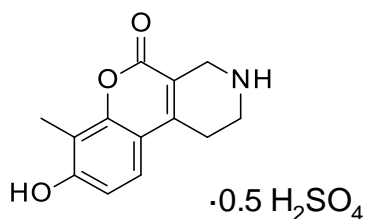
8-hydroxy-7-methyl-1,2,3,4-tetrahydro-5*H*-chromeno[3,4-*c*]pyridin-5-one·0.5H₂SO₄ (65b)

A mixture of ethyl 4-oxopiperidine-3-carboxylate hydrochloride (40.5 g, 195 mmol), 2-methylresorcinol (24.2 g, 195 mmol) and 64% sulfuric acid (200 mL) was stirred at rt for 6 h and allowed to stand still overnight. Ice water (300 mL) was added and the solution was stirred for 2 h. Insoluble material was collected by filtration, washed with a small volume of water and *n*-hexane, then dried at 50 °C under reduced pressure to give **65b** (45.1 g, 83% yield) as a solid. ^{1}H-NMR (DMSO-D$_6$, 50 °C) δ: 7.42 (1H, d, J = 8.5 Hz), 6.89 (1H, d, J = 8.5 Hz), 3.78 (2H, s), 3.23-3.18 (2H, m), 2.88 (2H, t, J = 5.8 Hz), 2.18 (3H, s). MS (ESI/APCI) m/z: 232.2 (M+H)$^+$. HRMS (ESI): m/z calcd for C$_{13}$H$_{14}$NO$_3$ (M+H)$^+$ 232.0968. Found 232.0975.
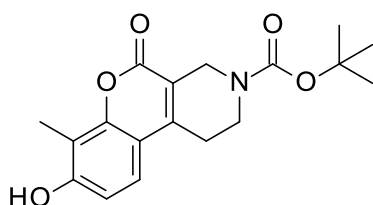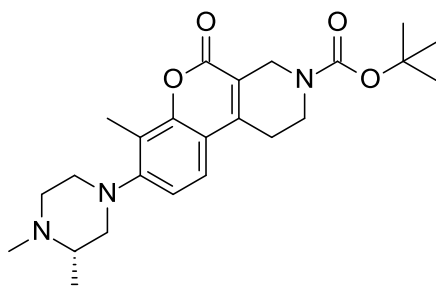

***tert*-butyl 8-hydroxy-7-methyl-5-oxo-1,5-dihydro-2*H*-chromeno[3,4-*c*]pyridine-3(4*H*)-carboxylate (68)**

To a suspension of **65b** (5.02 g, 15.2 mmol) in THF (100 mL) was added 1M aq. NaOH (15.2 mL), and the reaction mixture was stirred at rt for 30 min. After sat. aq. NaHCO₃ (30 mL) and Boc₂O (3.5 g, 16 mmol) were added, the resultant solution was stirred for 3 h and then allowed to stand still overnight. The mixture was diluted with EtOAc, and the organic layer was washed with water, dried over anhydrous Na₂SO₄, filtered and concentrated *in vacuo*. EtOAc was added to the residue and the resulting solid was collected by filtration, dried at 60 °C under reduced pressure to give **68** (4.32 g, 86% yield) as a solid. ^{1}H-NMR (DMSO-D$_6$) δ: 10.37 (1H, s), 7.41 (1H, d, J = 8.5 Hz), 6.87 (1H, d, J = 8.5 Hz), 4.19 (2H, s), 3.61 (2H, t, J = 5.8 Hz), 2.83 (2H, t, J = 5.8 Hz), 2.16 (3H, s), 1.44 (9H, s). MS (ESI/APCI) m/z: 276.2 (M–*t*Bu+H)$^+$. HRMS (ESI): m/z calcd for C$_{18}$H$_{20}$NO$_5$ (M–H)$^-$ 330.1347. Found 330.1342.
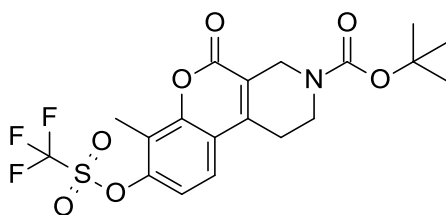

***tert*-butyl 7-methyl-5-oxo-8-{[(trifluoromethyl)sulfonyl]oxy}-1,5-dihydro-2*H*-chromeno[3,4-*c*]pyridine-3(4*H*)-carboxylate (93)**

To a suspension of **68** (6.43 g, 19.4 mmol) and pyridine (2.82 mL, 34.9 mmol) in DCM (100 mL) was added Tf₂O (4.24 mL. 25.2 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 30 min. Water was added to it, and the resultant solution was extracted with chloroform. The organic layer was washed with HCl and brine, dried over anhydrous Na₂SO₄ and then filtered. After the solvent was evaporated under reduced pressure, the residue was purified by column chromatography (SiO₂, MeOH/DCM = 0/100 – 2/98 (v/v)) to obtain **93** (8.09 g, 90% yield) as a pale-yellow solid. ^{1}H-NMR (CDCl₃) δ: 7.49 (1H, d, J = 8.5 Hz), 7.29-7.24 (1H, m), 4.43 (2H, s), 3.75 (2H, t, J = 5.8 Hz), 2.92-2.84 (2H, m), 2.49 (3H, s), 1.50 (9H, s). MS (ESI/APCI) m/z: 364.2 (M–Boc+H)$^+$, 408.1 (M–*t*Bu+H)$^+$. Anal. Calcd for C$_{19}$H$_{20}$F$_3$NO$_7$S·H$_2$O: C, 47.40; H, 4.61; N, 2.91; F, 11.84; S, 6.66. Found: C, 47.58; H, 4.36; N, 2.93; F, 12.15; S, 6.73.
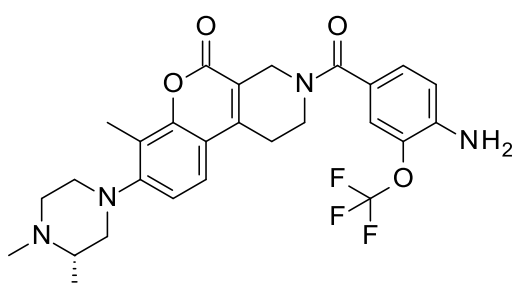
***tert*-butyl 8-[(3*S*)-3,4-dimethylpiperazin-1-yl]-7-methyl-5-oxo-1,5-dihydro-2*H*-chromeno[3,4-*c*]pyridine-3(4*H*)-carboxylate (94d)**

In a similar manner to that employed for the synthesis of **69b**, the title compound **94d** (507 mg, 76% yield) was obtained us-ing **93** (723 mg, 1.56 mmol) and (2*S*)-1,2-dimethylpiperazine dihydrochloride (583 mg, 3.12 mmol). ^{1}H-NMR (CDCl$_3$) δ: 7.36 (1H, d, J = 8.5 Hz), 6.98 (1H, d, J = 8.5 Hz), 4.40 (2H, s), 3.75-3.70 (2H, m), 3.14-2.82 (6H, m), 2.64-2.47 (2H, m), 2.43-2.32 (7H, m), 1.50 (9H, s), 1.13 (3H, d, J = 6.1 Hz). MS (ESI/APCI) m/z: 428.4 (M+H)$^+$. HRMS (ESI): m/z calcd for C$_{24}$H$_{34}$N$_3$O$_4$ (M+H)$^+$ 428.2544. Found 428.2537.
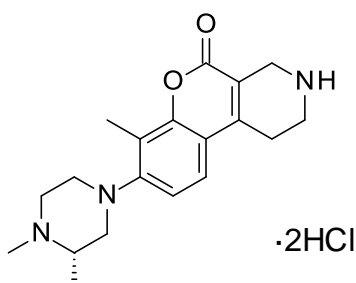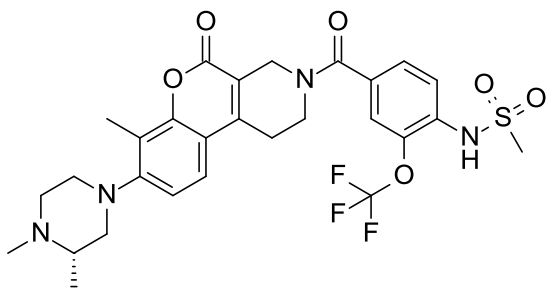

8-[(3*S*)-3,4-dimethylpiperazin-1-yl]-7-methyl-1,2,3,4-tetrahydro-5*H*-chromeno[3,4-*c*]pyridin-5-one·2HCl (S24)

In a similar manner to that employed for the synthesis of **70b**, the title compound **S24** (496 mg, quant.) was obtained from **94d** (505 mg, 1.18 mmol). ^{1}H-NMR (DMSO-D$_6$) δ: 10.77 (1H, br s), 9.53 (2H, br s), 7.63 (1H, d, J = 8.5 Hz), 7.16 (1H, d, J = 8.5 Hz), 4.01-3.96 (2H, m), 3.57-2.64 (14H, m), 2.32 (3H, s), 1.36 (3H, d, J = 6.1 Hz). MS (ESI/APCI) m/z: 328.2 (M+H)$^+$. HRMS (ESI): m/z calcd for C$_{19}$H$_{26}$N$_3$O$_2$ (M+H)$^+$ 328.2020. Found 328.2048.


3-[4-amino-3-(trifluoromethoxy)benzoyl]-8-[(3*S*)-3,4-dimethylpiperazin-1-yl]-7-methyl-1,2,3,4-tetrahydro-5*H*-chromeno[3,4-*c*]pyridin-5-one (95e)

In a similar manner to that employed for the synthesis of **92e**, the title compound **95e** (56 mg, 71% yield) was obtained using **S24** (65 mg, 0.15 mmol) and 4-amino-3-(trifluoromethoxy)benzoic acid (37 mg, 0.17 mmol). ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 7.51 (1H, d, J = 9.1 Hz), 7.26-7.21 (2H, m), 7.05 (1H, d, J = 8.5 Hz), 6.87 (1H, d, J = 8.5 Hz), 5.62 (2H, s), 4.40 (2H, s), 3.81-3.74 (2H, m), 3.04-2.79 (6H, m), 2.61-2.47 (3H, m), 2.29 (3H, s), 2.26 (3H, s), 1.05 (3H, d, J = 6.1 Hz). MS (ESI/APCI) m/z: 531.3 (M+H)$^+$. HRMS (ESI): m/z calcd for C$_{27}$H$_{30}$F$_3$N$_4$O$_4$ (M+H)$^+$ 531.2214. Found 531.2207.

N-[4-({8-[(3S)-3,4-dimethylpiperazin-1-yl]-7-methyl-5-oxo-1,5-dihydro-2H-chromeno[3,4-c]pyridin-3(4H)-yl}carbonyl)-2-(trifluoromethoxy)phenyl]methanesulfonamide (87, DS18561882)

In a similar manner to that employed for the synthesis of **79**, the title compound **87** (57 mg, 90% yield) was obtained using **95e** (55 mg, 0.10 mmol) and methanesulfonyl chloride (0.029 mL, 0.37 mmol). ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 7.63 (1H, d, J = 8.5 Hz), 7.53-7.44 (3H, m), 7.06 (1H, d, J = 8.5 Hz), 4.40 (2H, s), 3.80-3.72 (2H, m), 3.10 (3H, s), 3.04-2.81 (6H, m), 2.59-2.34 (3H, m), 2.31-2.26 (6H, m), 1.06 (3H, d, J = 6.1 Hz). ^{13}C-NMR (DMSO-D$_6$) δ: 167.6, 159.3, 153.7, 150.8, 146.8, 139.5, 133.3, 131.2, 126.8, 123.0, 121.9, 120.7, 120.1 (q, $^1J_{C-F}$ = 258 Hz), 117.9, 116.4, 115.0, 113.4, 57.7, 57.6, 54.9, 51.1, 41.9, 40.7, 24.5, 16.3, 10.8. MS (ESI/APCI) m/z: 609.3 (M+H)$^+$. HRMS (ESI): m/z calcd for C$_{28}$H$_{32}$F$_3$N$_4$O$_6$S (M+H)$^+$ 609.1989. Found 609.2015. IR (KBr) 3417, 2935, 2820, 1712, 1640, 1603, 1505, 1445, 1386, 1340, 1257, 1234, 1214, 1160, 1106 cm^{-1}. Anal. Calcd for C$_{28}$H$_{31}$F$_3$N$_4$O$_6$S·H$_2$O: C, 53.67; H, 5.31; N, 8.94; F, 9.10; S, 5.12. Found: C, 54.05; H, 5.40; N, 8.68; F, 8.94; S, 5.00. [α]$_D^{25}$ −8.92° (c = 1.01, CHCl$_3$).
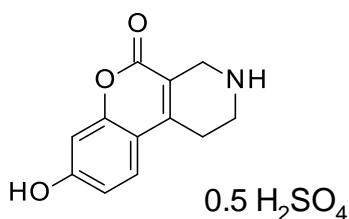

8-hydroxy-1,2,3,4-tetrahydro-5H-chromeno[3,4-c]pyridin-5-one·0.5H$_2$SO$_4$ (58)

In a similar manner to that employed for the synthesis of **65b**, the title compound **58** (5.76 g, 45% yield) was obtained using ethyl 4-oxopiperidine-3-carboxylate hydrochloride (10 g, 48.2 mmol) and resorcinol (5.31 g, 48.2 mmol). ^{1}H-NMR (DMSO-D$_6$) δ: 7.59 (1H, d, J = 8.5 Hz), 6.84 (1H, dd, J = 8.5, 2.4 Hz), 6.76 (1H, d, J = 2.4 Hz), 3.80 (2H, s), 3.24 (2H, t, J = 5.8 Hz), 2.95-2.88 (2H, m). MS (ESI/APCI) m/z: 218.2 (M+H)$^+$.
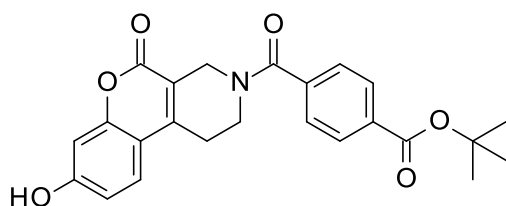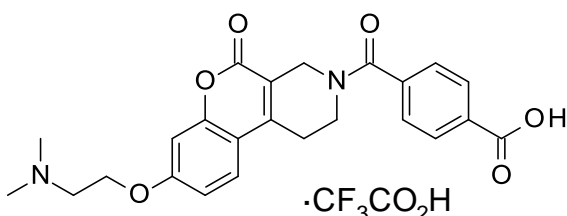

***tert*-butyl 4-[(8-hydroxy-5-oxo-1,5-dihydro-2H-chromeno[3,4-c]pyridin-3(4H)-yl)carbonyl]benzoate (59)**

To a suspension of **58** (1.0 g, 3.76 mmol) and 4-(*tert*-butoxycarbonyl)benzoic acid (835 mg, 3.76 mmol) in MeOH (30 mL) were added 4-methylmorpholine (1 mL, 9.01 mmol) and DMT-MM hydrate (1.16 g, 3.94 mmol) at rt. The mixture was stirred at rt for 4 h and allowed to stand still overnight. It was diluted with EtOAc and washed with sat. aq. NaHCO$_3$ and brine, dried over anhydrous Na$_2$SO$_4$, and filtered. Concentration under reduced pressure and purification by column chromatography (SiO$_2$, EtOAc/DCM = 0/100 − 50/50 (v/v)) afforded **59** (536 mg, 34% yield) as a colorless solid. ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 10.23 (1H, s), 7.97 (2H, d, J = 8.5 Hz), 7.57-7.52 (3H, m), 6.84-6.80 (1H, m), 6.74-6.71 (1H, m), 4.35 (2H, br s), 3.73 (2H, br s), 2.96-2.89 (2H, m), 1.57 (9H, s). MS (ESI/APCI) m/z: 422.2 (M+H)$^+$.
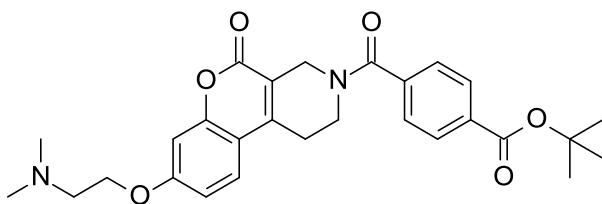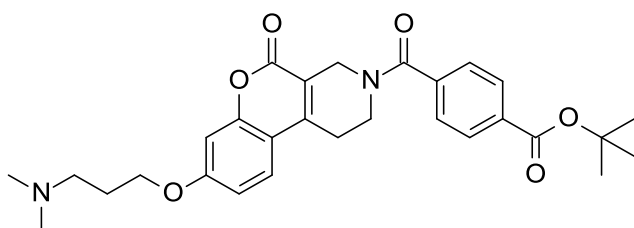
tert-butyl 4-({8-[2-(dimethylamino)ethoxy]-5-oxo-1,5-dihydro-2*H*-chromeno[3,4-*c*]pyridin-3(4*H*)-yl}carbonyl)benzoate (60a)

To a DMF (3 mL) solution of **59** (445 mg, 1.06 mmol) were added potassium carbonate (1.45 g, 10.6 mmol) and 2-dimethylaminoethyl chloride hydrochloride (760 mg, 5.28 mmol). The mixture was stirred at 120 °C for 7 h. After cooling down to rt, water was added to the mixture and it was extracted with EtOAc. The organic layer was washed with water, 1M aq. NaOH, water, and brine, and dried over anhydrous Na_2SO_4. Filtration, concentration *in vacuo*, and purification by column chromatography (SiO_2, MeOH/DCM = 3/97 – 15/85 (v/v)) afforded **60a** (165 mg, 32% yield) as a pale-yellow amorphous. ^{1}H-NMR (DMSO-D$_6$) δ: 8.01-7.97 (2H, m), 7.72-7.57 (3H, m), 7.09-6.98 (2H, m), 4.51-4.20 (2H, m), 4.19-4.13 (2H, m), 3.98-3.53 (2H, m), 3.01-2.92 (2H, m), 2.68-2.63 (2H, m), 2.22 (6H, s), 1.57 (9H, s). MS (ESI/APCI) m/z: 493.3 (M+H)$^+$.
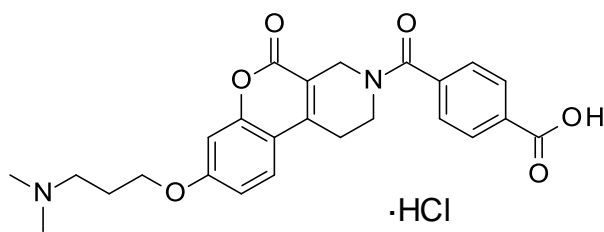

4-({8-[2-(dimethylamino)ethoxy]-5-oxo-1,5-dihydro-2*H*-chromeno[3,4-*c*]pyridin-3(4*H*)-yl}car-bonyl)benzoic acid·CF$_3$CO$_2$H (46)

To a DCM (0.2 mL) solution of **60a** (16.0 mg, 0.033 mmol) was added TFA (0.2 mL, 3 mmol) at rt. The mixture was stirred at rt for 1.5 h. Concentration under reduced pressure and trituration with EtOAc afforded **46** (3.8 mg, 21% yield) as a white solid. ^{1}H-NMR (DMSO-D$_6$) δ: 9.60 (1H, br s), 8.05-8.02 (2H, m), 7.78-7.66 (1H, m), 7.63-7.58 (2H, m), 7.16-7.09 (1H, m), 7.08-7.04 (1H, m), 4.52-4.23 (4H, m), 3.97-3.53 (4H, m), 3.02-2.96 (2H, m), 2.87 (6H, s). MS (ESI/APCI) m/z: 437.1 (M+H)$^+$. HRMS (ESI): m/z calcd for $C_{24}H_{25}N_2O_6$ (M+H)$^+$ 437.1707. Found 437.1725. IR (KBr) 3434, 3052, 2768, 2629, 2497, 1712, 1613, 1436, 1402, 1282, 1262, 1239, 1200, 1174, 1157, 1133, 1096 cm^{-1}.


tert-butyl 4-({8-[3-(dimethylamino)propoxy]-5-oxo-1,5-dihydro-2*H*-chromeno[3,4-*c*]pyridin-3(4*H*)-yl}carbonyl)benzoate (60b)

To a THF (2 mL) solution of **59** (88 mg, 0.21 mmol) and triphenylphosphine (109.3 mg, 0.42 mmol) were added 3-dimethylamino-1-propanol (48.9 µL, 0.42 mmol) and diisopropyl azodicarboxylate (82.2 µL, 0.42 mmol) at rt. The mixture was stirred at rt for 1.5 h and then concentrated under reduced pressure. Purification by column chromatography (SiO_2, MeOH/DCM = 6/94 – 15/85 (v/v)) afforded **60b** (92.5 mg, 87% yield) as a colorless solid. ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 7.98-7.95 (2H, m), 7.63-7.60 (1H, m), 7.58-7.55 (2H, m), 6.99-6.94 (2H, m), 4.39-4.34 (2H, m), 4.13 (2H, t, J = 6.4 Hz), 3.81-3.68 (2H, m), 2.97-2.93 (2H, m), 2.38 (2H, t, J = 7.0 Hz), 2.17 (6H, s), 1.90-1.84 (2H, m), 1.57 (9H, s). MS (ESI/APCI) m/z: 507.4 (M+H)$^+$.
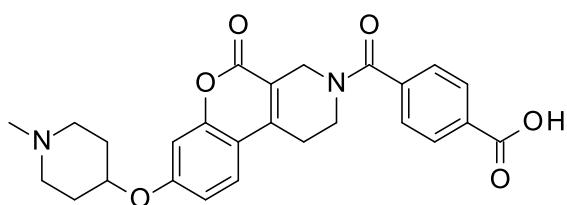
4-({8-[3-(dimethylamino)propoxy]-5-oxo-1,5-dihydro-2*H*-chromeno[3,4-*c*]pyridin-3(4*H*)-yl}car-bonyl)benzoic acid·HCl (47)

4M HCl in 1,4-dioxane (3 mL) was added to **60b** (62.0 mg, 0.12 mmol) and the mixture was stirred at rt overnight. After concentrated *in vacuo*, the resulting material was triturated with EtOAc. The obtained solid was collected by filtration and dried at 60 °C under reduced pressure to give **47** (29.7 mg, 50% yield) as a yellow solid. ¹H-NMR (DMSO-D₆, 80 °C) δ: 10.43 (1H, br s), 8.04-7.99 (2H, m), 7.67-7.63 (1H, m), 7.58-7.55 (2H, m), 7.02-6.97 (2H, m), 4.42-4.35 (2H, m), 4.20 (2H, t, J = 6.1 Hz), 3.80-3.71 (2H, m), 3.24-3.19 (2H, m), 2.99-2.95 (2H, m), 2.78 (6H, s), 2.22-2.15 (2H, m). MS (ESI/APCI) m/z: 451.2 (M+H)⁺. HRMS (ESI): m/z calcd for $C_{25}H_{27}N_2O_6$ (M+H)⁺ 451.1864. Found 451.1874.
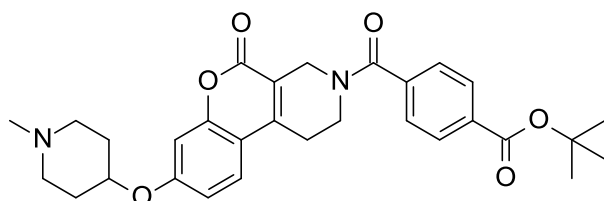

tert-butyl 4-({8-[(1-methylpiperidin-4-yl)oxy]-5-oxo-1,5-dihydro-2*H*-chromeno[3,4-*c*]pyridin-3(4*H*)-yl}carbonyl)benzoate (60c)

To a mixture of **59** (92.6 mg, 0.22 mmol) and 4-hydroxy-1-methylpiperidine (51.6 mg, 0.45 mmol) in THF (3 mL) were added tributylphosphine (0.109 mL, 0.44 mmol) and 1,1'-(azodicarbonyl)dipiperidine (111 mg, 0.44 mmol) at rt. The mixture was stirred at 50 °C for 3 h. Then, 4-hydroxy-1-methylpiperidine (47.6 mg, 0.42 mmol), 1,1'-(azodicarbonyl)dipiperidine (112 mg, 0.44 mmol) and tributylphosphine (0.109 mL, 0.44 mmol) were added and the mixture was stirred at 60 °C for 6 h. The mixture was concentrated *in vacuo* and purified by column chromatography (SiO₂, MeOH/DCM = 3/97 – 15/85 (v/v)) to afford **60c** (27.1 mg, 24% yield) as a pale-yellow solid. ¹H-NMR (DMSO-D₆, 80 °C) δ: 8.01-7.95 (2H, m), 7.64-7.54 (3H, m), 7.03-6.95 (2H, m), 4.55-4.32 (3H, m), 3.89-3.59 (2H, m), 2.98-2.91 (2H, m), 2.69-2.61 (2H, m), 2.31-2.19 (5H, m), 2.00-1.92 (2H, m), 1.76-1.66 (2H, m), 1.57 (9H, s). MS (ESI/APCI) m/z: 519.2 (M+H)⁺.


4-({8-[(1-methylpiperidin-4-yl)oxy]-5-oxo-1,5-dihydro-2*H*-chromeno[3,4-*c*]pyridin-3(4*H*)-yl}car-bonyl)benzoic acid (48)

To a MeOH (1 mL) solution of **60c** (25.3 mg, 0.049 mmol) was added 4M HCl in 1,4-dioxane (1 mL) and the mixture was stirred at rt overnight. After concentrated *in vacuo*, MeOH (0.5 mL) and 1M aq. NaOH (0.5 mL) were added to it, and the solution was stirred at rt for 2.5 h. 1M HCl (0.5 mL) was added to the solution, and the solvent was evaporated under reduced pressure. Preparative reverse-phase HPLC afforded **48** (6.8 mg, 25% yield) as a white solid. ¹H-NMR (DMSO-D₆, 80 °C) δ: 8.01 (2H, d, J = 7.9 Hz), 7.61 (1H, d, J = 9.2 Hz), 7.56 (2H, d, J = 7.9 Hz), 7.00-6.96 (2H, m), 4.55-4.49 (1H, m), 4.42-4.35 (2H, m), 3.79-3.71 (2H, m), 2.97-2.93 (2H, m), 2.66-2.60 (2H, m), 2.30-2.23 (2H, m), 2.21 (3H, s), 2.00-1.92 (2H, m), 1.74-1.64 (2H, m). MS (ESI/APCI) m/z: 463.2 (M+H)⁺. HRMS (ESI): m/z calcd for $C_{26}H_{27}N_2O_6$ (M+H)⁺ 463.1864. Found 463.1870.
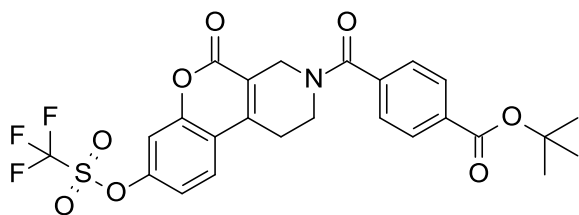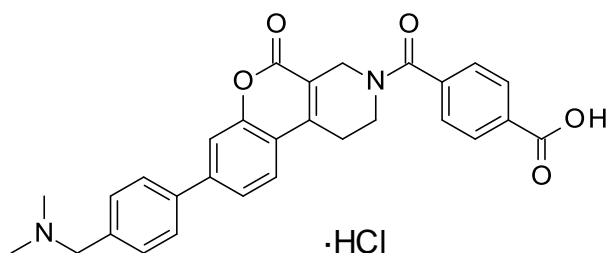
tert-butyl   4-{[5-oxo-8-{[(trifluoromethyl)sulfonyl]oxy}-1,5-dihydro-2H-chromeno[3,4-c]pyridin-3(4H)-yl]carbonyl}benzoate (61)

In a similar manner to that employed for the synthesis of **93**, the title compound **61** (619 mg, 77% yield) was obtained from **59** (614 mg, 1.46 mmol). ^{1}H-NMR (CDCl$_3$) δ: 8.07 (2H, d, J = 8.5 Hz), 7.72-7.65 (1H, m), 7.51 (2H, d, J = 8.5 Hz), 7.32-7.28 (2H, m), 4.86-4.35 (2H, m), 4.15-3.69 (2H, m), 3.08-2.85 (2H, m), 1.62 (9H, s). MS (ESI/APCI) m/z: 498.0 (M–*t*Bu+H)$^+$, 554.1 (M+H)$^+$.
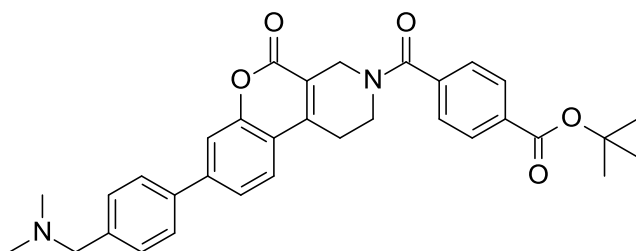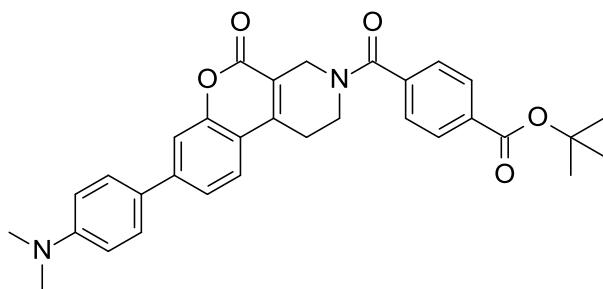

tert-butyl   4-{[8-{4-[(dimethylamino)methyl]phenyl}-5-oxo-1,5-dihydro-2H-chromeno[3,4-c]pyridin-3(4H)-yl]carbonyl}benzoate (62a)

A mixture of **61** (70 mg, 0.13 mmol), 4-[(N,N-dimethylamino)methyl]phenylboronic acid pinacol ester hydrochloride (42 mg, 0.14 mmol), Pd(dppf)Cl$_2$–DCM adduct (5.4 mg, 0.0066 mmol), NaHCO$_3$ (26.6 mg, 0.32 mmol), 1,4-dioxane (1 mL) and water (0.25 mL) was stirred at 90–100 °C for 1.5 h under N$_2$ atmosphere. After cooling down to rt, the mixture was diluted with EtOAc and insoluble materials were removed by filtration over Celite. The filtrate was concentrated *in vacuo* and purified by column chromatography (SiO$_2$, MeOH/DCM = 0/100 – 10/90 (v/v)) to afford **62a** (65.2 mg, 96% yield) as a brown solid. ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 7.99 (2H, d, J = 7.9 Hz), 7.81-7.69 (5H, m), 7.58 (2H, d, J = 7.9 Hz), 7.45 (2H, d, J = 7.9 Hz), 4.47-4.40 (2H, m), 3.82-3.75 (2H, m), 3.63-3.59 (2H, m), 3.08-2.99 (2H, m), 2.30 (6H, s), 1.58 (9H, s). MS (ESI/APCI) m/z: 539.3 (M+H)$^+$.
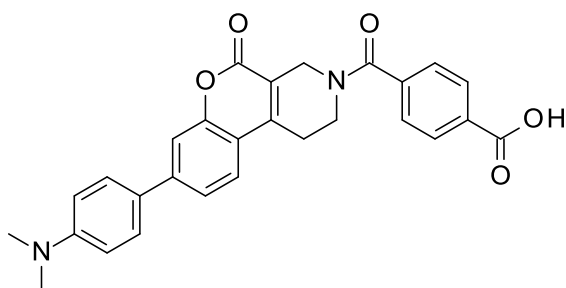

4-{[8-{4-[(dimethylamino)methyl]phenyl}-5-oxo-1,5-dihydro-2H-chromeno[3,4-c]pyridin-3(4H)-yl]carbonyl}benzoic acid·HCl (49)

In a similar manner to that employed for the synthesis of **47**, the title compound **49** (27.0 mg, 57% yield) was obtained from **62a** (49.4 mg, 0.092 mmol). ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 8.04-8.01 (2H, m), 7.91-7.88 (2H, m), 7.83-7.81 (1H, m), 7.77-7.74 (2H, m), 7.67-7.64 (2H, m), 7.60-7.57 (2H, m), 4.48-4.43 (2H, m), 4.30 (2H, s), 3.84-3.76 (2H, m), 3.11-3.02 (2H, m), 2.74 (6H, s). MS (ESI/APCI) m/z: 483.2 (M+H)$^+$. HRMS (ESI): m/z calcd for C$_{29}$H$_{27}$N$_2$O$_5$ (M+H)$^+$ 483.1915. Found 483.1909.
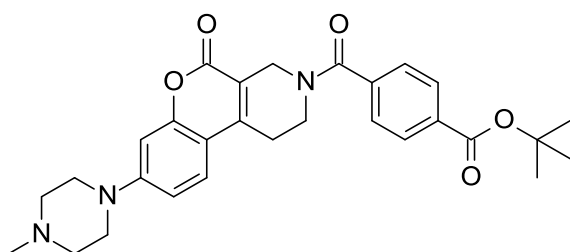
tert-butyl 4-({8-[4-(dimethylamino)phenyl]-5-oxo-1,5-dihydro-2H-chromeno[3,4-c]pyridin-3(4H)-yl}carbonyl)benzoate (62b)

In a similar manner to that employed for the synthesis of **62a**, the title compound **62b** (42.5 mg, 64% yield) was obtained from **61** (70 mg, 0.13 mmol) and 4-(*N*,*N*-dimethylamino)benzeneboronic acid (23 mg, 0.14 mmol). ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 7.99 (2H, d, J = 7.9 Hz), 7.72-7.69 (1H, m), 7.67-7.63 (3H, m), 7.60-7.56 (3H, m), 6.83-6.81 (2H, m), 4.46-4.38 (2H, m), 3.85-3.72 (2H, m), 3.02-2.99 (2H, m), 2.97 (6H, s), 1.58 (9H, s). MS (ESI/APCI) m/z: 525.4 (M+H)$^+$.


4-({8-[4-(dimethylamino)phenyl]-5-oxo-1,5-dihydro-2H-chromeno[3,4-c]pyridin-3(4H)-yl}carbonyl)benzoic acid (50)

To a DCM (1.5 mL) solution of **62b** (36 mg, 0.069 mmol) was added TFA (0.5 mL) and the mixture was stirred at rt for 2 h. After concentrated *in vacuo*, the residue was purified by column chromatography (SiO$_2$, MeOH/DCM = 5/95 − 15/85 (v/v)) to afford **50** (9.6 mg, 30% yield) as a yellow solid. ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 8.02 (2H, d, J = 8.5 Hz), 7.73-7.56 (7H, m), 6.84-6.80 (2H, m), 4.46-4.38 (2H, m), 3.81-3.74 (2H, m), 3.03-3.00 (2H, m), 2.97 (6H, s). MS (ESI/APCI) m/z: 469.3 (M+H)$^+$. HRMS (ESI): m/z calcd for C$_{28}$H$_{25}$N$_2$O$_5$ (M+H)$^+$ 469.1758. Found 469.1753.

tert-butyl 4-{[8-(4-methylpiperazin-1-yl)-5-oxo-1,5-dihydro-2H-chromeno[3,4-c]pyridin-3(4H)-yl]carbonyl}benzoate (63)

In a similar manner to that employed for the synthesis of **69b**, the title compound **63** (54 mg, 85% yield) was obtained from **61** (70 mg, 0.13 mmol) and 1-methylpiperazine (0.0209 mL, 0.19 mmol). ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 7.97 (2H, d, J = 8.5 Hz), 7.56 (2H, d, J = 8.5 Hz), 7.50 (1H, d, J = 9.1 Hz), 6.96 (1H, dd, J = 9.1, 1.8 Hz), 6.79 (1H, d, J = 1.8 Hz), 4.37-4.31 (2H, m), 3.82-3.66 (2H, m), 3.33 (4H, t, J = 5.2 Hz), 2.93-2.90 (2H, m), 2.45 (4H, t, J = 5.2 Hz), 2.23 (3H, s), 1.57 (9H, s). MS (ESI/APCI) m/z: 504.3 (M+H)$^+$.
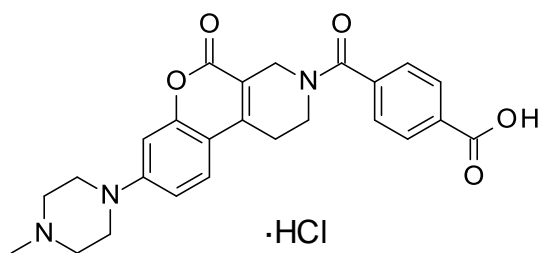
4-{[8-(4-methylpiperazin-1-yl)-5-oxo-1,5-dihydro-2*H*-chromeno[3,4-*c*]pyridin-3(4*H*)-yl]carbonyl}ben-zoic acid·HCl (51)

In a similar manner to that employed for the synthesis of **47**, the title compound **51** (21 mg, 58% yield) was obtained from **63** (38 mg, 0.076 mmol). ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 8.03-7.99 (2H, m), 7.59-7.54 (3H, m), 7.06-7.00 (1H, m), 6.95-6.91 (1H, m), 4.43-4.30 (2H, m), 3.83-3.65 (2H, m), 3.38-3.00 (8H, m), 2.96-2.91 (2H, m), 2.80 (3H, s). MS (ESI/APCI) m/z: 448.2 (M+H)$^+$. HRMS (ESI): m/z calcd for C$_{25}$H$_{26}$N$_3$O$_5$ (M+H)$^+$ 448.1867. Found 448.1872.
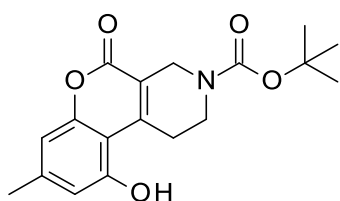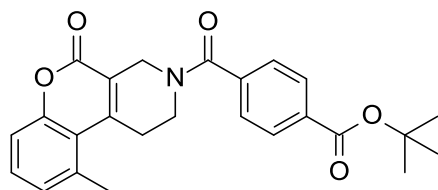

***tert*-butyl 10-hydroxy-8-methyl-5-oxo-1,5-dihydro-2*H*-[1]benzopyrano[3,4-*c*]pyridine-3(4*H*)-carbox-ylate (72b)**

To a mixture of 5-methylresorcinol (1.20 g, 9.63 mmol) and 70% perchloric acid (10 mL) was added ethyl 4-oxo-3-piperidinecarboxylate hydrochloride (2.00 g, 9.63 mmol) at rt. After stirring for 6 h at rt, 5-methylres-orcinol (1.20 g, 9.63 mmol) was added to the mixture. After stirring overnight at rt, ice water and Et$_2$O was added to the mixture, and insoluble material was obtained by filtration. Aqueous phase of mother liquid was separated, and mixed with the obtained solid. To this mixture was added 5N aq. NaOH (23 mL), THF (100 mL), and Boc$_2$O (4.20 g, 19.3 mmol) at 0 °C. After stirring for 1 h at rt, 5N aq. NaOH (3 mL) was added, and the mixture was stirred for another 15 min at rt. 5N HCl (2 mL) was added to the mixture, which was subsequently extracted with EtOAc. The organic layer was washed with 10% aq. citric acid, water, and brine, respectively, dried over Na$_2$SO$_4$, filtered, and concentrated in vacuo. The residue was suspended in EtOAc (100 mL) at 50 °C, and the resulting solid was collected by filtration to give **72b** (2.43 g, 76%) as a white solid. The structure was determined by ^{1}H-NMR, NOE, and HMBC. ^{1}H-NMR (DMSO-D$_6$) δ: 10.57 (1H, s), 6.65 (1H, br s), 6.59 (1H, br s), 4.18 (2H, s), 3.55-3.49 (2H, m), 3.19-3.13 (2H, m), 2.28 (3H, s), 1.43 (9H, s). MS (ESI/APCI) m/z: 276.2 (M–*t*Bu+H)$^+$, 232.2 (M–Boc+H)$^+$.
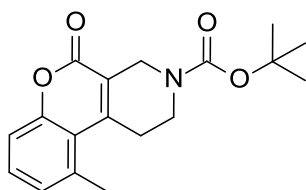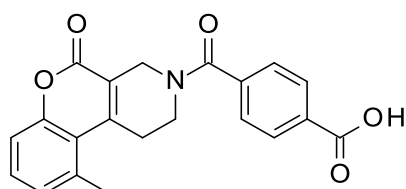

***tert*-butyl 10-methyl-5-oxo-1,5-dihydro-2*H*-chromeno[3,4-*c*]pyridine-3(4*H*)-carboxylate (73)**

A mixture of **42** (100 mg, 0.26 mmol), 2-bromo-3-methylphenol (49 mg, 0.26 mmol), Pd(dppf)Cl$_2$–DCM adduct (10.6 mg, 0.013 mmol), NaHCO$_3$ (55 mg, 0.66 mmol), THF (2 mL) and water (0.5 mL) was stirred at 100 °C for 1.5 h under microwave irradiation. After cooling down to rt, the mixture was diluted with EtOAc and insoluble materials were removed by filtration over Celite. The filtrate was concentrated *in vacuo* and purified by column chromatography (SiO$_2$, EtOAc/*n*-hexane = 1/9 – 1/3 (v/v)) to afford **73** (43.8 mg, 53% yield) as a white solid. ^{1}H-NMR (CDCl$_3$) δ: 7.37-7.33 (1H, m), 7.22 (1H, d, J = 7.9 Hz), 7.08 (1H, d, J = 7.3 Hz), 4.43 (2H, s), 3.65 (2H, t, J = 5.8 Hz), 3.15-3.11 (2H, m), 2.72 (3H, s), 1.51 (9H, s). MS (ESI/APCI) m/z: 260.2 (M–*t*Bu+H)$^+$, 216.2 (M–Boc+H)$^+$.

tert-butyl 4-[(10-methyl-5-oxo-1,5-dihydro-2_H_-chromeno[3,4-_c_]pyridin-3(4_H_)-yl)carbonyl]benzoate (74)

To a suspension of **73** (42.6 mg, 0.14 mmol) in MeOH (1 mL) was added 4M HCl in 1,4-dioxane (1 mL) and the mixture was stirred at rt. After 1 h, the reaction mixture was concentrated under reduced pressure. The obtained material was mixed with 4-(_tert_-butoxycarbonyl)benzoic acid (30.3 mg, 0.14 mmol), MeOH (2 mL), triethylamine (0.0187 mL, 0.14 mmol) and DMT-MM hydrate (44.5 g, 0.15 mmol) at rt. The mixture was stirred at rt for 2 h. After concentrated _in vacuo_, water was added to the residue and it was extracted with chloroform. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated. Purification by column chromatography (SiO_2, EtOAc/_n_-hexane = 1/3 – 1/1 (v/v)) afforded **74** (50.4 mg, 89% yield) as a white solid. ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 7.99 (2H, d, J = 7.9 Hz), 7.60 (2H, d, J = 7.9 Hz), 7.46-7.40 (1H, m), 7.22 (1H, d, J = 8.5 Hz), 7.17 (1H, d, J = 7.3 Hz), 4.46-4.38 (2H, m), 3.73-3.61 (2H, m), 3.25-3.22 (2H, m), 2.71 (3H, s), 1.57 (9H, s). MS (ESI/APCI) m/z: 420.3 (M+H)$^+$.
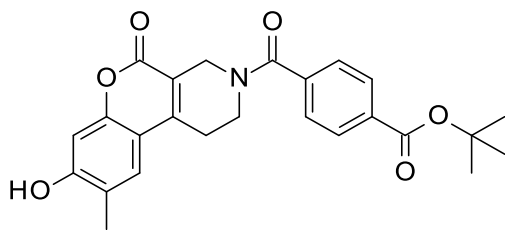

4-[(10-methyl-5-oxo-1,5-dihydro-2_H_-chromeno[3,4-_c_]pyridin-3(4_H_)-yl)carbonyl]benzoic acid (52)

4M HCl in 1,4-dioxane (1 mL) was added to **74** (44 mg, 0.11 mmol) and the mixture was stirred at rt overnight. Concentration under reduced pressure and following trituration with ethanol at 50 °C gave **52** (28 mg, 74% yield) as a white solid. ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 8.04-8.01 (2H, m), 7.61-7.58 (2H, m), 7.45-7.40 (1H, m), 7.23-7.21 (1H, m), 7.18-7.14 (1H, m), 4.46-4.39 (2H, m), 3.73-3.64 (2H, m), 3.27-3.22 (2H, m), 2.71 (3H, s). ^{13}C-NMR (DMSO-D$_6$, for a mixture of rotamers) δ: 168.6, 168.0, 166.7, 158.5, 152.6, 148.6, 139.5, 136.7, 131.9, 130.5, 129.5, 128.7, 127.2, 119.5, 118.6, 115.2, 45.7, 43.2, 40.9, 38.0, 30.0, 29.0, 23.9. MS (ESI/APCI) m/z: 364.2 (M+H)$^+$. HRMS (ESI): m/z calcd for $C_{21}H_{16}NO_5$ (M–H)$^-$ 362.1034. Found 362.1032. IR (KBr) 3407, 2934, 2673, 2556, 1709, 1647, 1513, 1437, 1323, 1252 cm^{-1}. Anal. Calcd for $C_{21}H_{17}NO_5\cdot0.4H_2O$: C, 68.06; H, 4.84; N, 3.78. Found: C, 68.06; H, 4.78; N, 4.00.
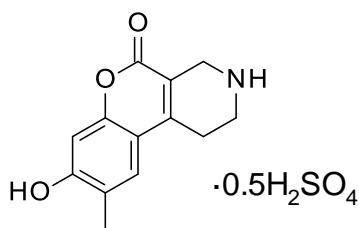
8-hydroxy-9-methyl-1,2,3,4-tetrahydro-5_H_-chromeno[3,4-_c_]pyridin-5-one·0.5H$_2$SO$_4$ (65a)

In a similar manner to that employed for the synthesis of **65b**, the title compound **65a** (1.15 g, 78% yield) was obtained using ethyl 4-oxopiperidine-3-carboxylate hydrochloride (1.08 g, 5.24 mmol) and 4-methylresorcinol (650 mg, 5.24 mmol). ^{1}H-NMR (DMSO-D$_6$) δ: 10.67 (1H, s), 9.03 (2H, br s), 7.52 (1H, s), 6.79 (1H, s), 3.97 (2H, s), 3.46-3.39 (2H, m), 3.09-3.04 (2H, m), 2.20 (3H, s). MS (ESI/APCI) m/z: 232.1 (M+H)$^+$.
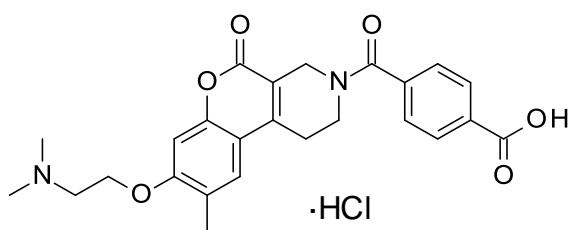
tert-butyl 4-[(8-hydroxy-9-methyl-5-oxo-1,5-dihydro-2_H_-chromeno[3,4-_c_]pyridin-3(4_H_)-yl)carbonyl]benzoate (66a)

In a similar manner to that employed for the synthesis of **59**, the title compound **66a** (406 mg, 52% yield) was obtained from **65a** (500 mg, 1.78 mmol) and 4-(_tert_-butoxycarbonyl)benzoic acid (396 mg, 1.78 mmol). ^{1}H-NMR (DMSO-D$_6$) δ: 10.53 (1H, s), 8.01-7.97 (2H, m), 7.63-7.42 (3H, m), 6.78-6.73 (1H, m), 4.50-4.16 (2H, m), 3.96-3.52 (2H, m), 2.99-2.91 (2H, m), 2.19 (3H, s), 1.57 (9H, s). MS (ESI/APCI) m/z: 380.1 (M–_t_Bu+H)$^+$.
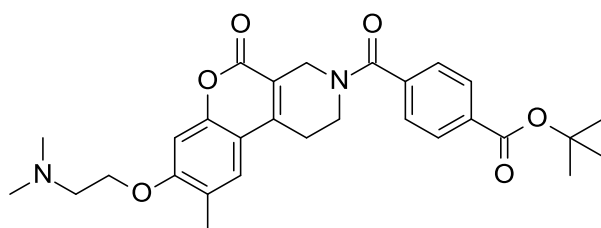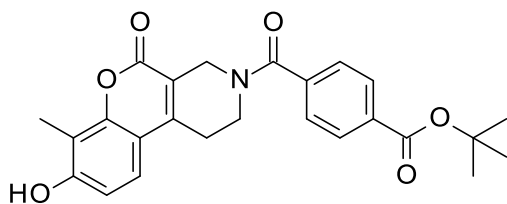

tert-butyl 4-({8-[2-(dimethylamino)ethoxy]-9-methyl-5-oxo-1,5-dihydro-2_H_-chromeno[3,4-_c_]pyridin-3(4_H_)-yl}carbonyl)benzoate (67a)

To a suspension of **66a** (56.6 mg, 0.13 mmol), 2-(dimethylamino)ethanol (0.026 mL, 0.26 mmol) and triphenylphosphine (68 mg, 0.26 mmol) in THF (1.5 mL) was added diisopropyl azodicarboxylate (0.0512 mL, 0.26 mmol) at rt under N$_2$ atmosphere. The mixture was stirred for 3 h at rt and for 1 h at 60 °C. Then, 2-(dimethylamino)ethanol (0.013 mL, 0.13 mmol), triphenylphosphine (36.1 mg, 0.13 mmol) and diisopropyl azodicarboxylate (0.0256 mL, 0.13 mmol) were added and the mixture was stirred for 4 h at 60 °C. Concentration under reduced pressure and purification by column chromatography (SiO$_2$, MeOH/DCM = 2/98 – 10/90 (v/v)) afforded **67a** (4.4 mg, 7% yield) as a white solid. ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 7.97 (2H, d, J = 8.5 Hz), 7.56 (2H, d, J = 8.5 Hz), 7.47 (1H, s), 7.00 (1H, s), 4.41-4.32 (2H, m), 4.18 (2H, t, J = 5.8 Hz), 3.80-3.68 (2H, m), 2.95 (2H, t, J = 5.5 Hz), 2.71 (2H, t, J = 5.5 Hz), 2.26 (6H, s), 2.22 (3H, s), 1.57 (9H, s). MS (ESI/APCI) m/z: 507.2 (M+H)$^+$.

4-({8-[2-(dimethylamino)ethoxy]-9-methyl-5-oxo-1,5-dihydro-2_H_-chromeno[3,4-_c_]pyridin-3(4_H_)-yl}carbonyl)benzoic acid·HCl (53)

In a similar manner to that employed for the synthesis of **47**, the title compound **53** (2.9 mg, 67% yield) was obtained from **67a** (4.4 mg, 0.0087 mmol). ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 8.02 (2H, d, J = 7.9 Hz), 7.57 (2H, d, J = 7.9 Hz), 7.53 (1H, s), 7.08 (1H, s), 4.47 (2H, t, J = 5.2 Hz), 4.42-4.36 (2H, m), 3.78-3.72 (2H, m), 3.56 (2H, t, J = 5.2 Hz), 2.98-2.95 (2H, m), 2.88 (6H, s), 2.27 (3H, s). MS (ESI/APCI) m/z: 451.3 (M+H)$^+$. HRMS (ESI): m/z calcd for C$_{25}$H$_{27}$N$_2$O$_6$ (M+H)$^+$ 451.1864. Found 451.1872.

tert-butyl 4-[(8-hydroxy-7-methyl-5-oxo-1,5-dihydro-2H-chromeno[3,4-c]pyridin-3(4H)-yl)carbonyl]benzoate (66b)

To a suspension of **65b** (1.5 g, 5.4 mmol) and 4-(*tert*-butoxycarbonyl)benzoic acid (1.2 g, 5.4 mmol) in DMF (20 mL) were added WSCI·HCl (1.23 g, 6.4 mmol), HOBt (720 mg, 5.4 mmol) and triethylamine (0.75 mL, 5.4 mmol) at 0 °C. The mixture was stirred at rt for 6 h. After concentrated *in vacuo*, the residue was diluted with DCM, and washed with water. The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated. Purification by column chromatography (SiO_2, MeOH/EtOAc/DCM = 0/0/100 – 0/24/76 – 5/0/95 (v/v)) followed by trituration with EtOAc–diisopropyl ether afforded **66b** (1.2 g, 53% yield) as a solid. ¹H-NMR (DMSO-D$_6$, 80 °C) δ: 10.08 (1H, s), 7.97 (2H, d, J = 7.9 Hz), 7.56 (2H, d, J = 7.9 Hz), 7.39 (1H, d, J = 8.5 Hz), 6.88 (1H, d, J = 8.5 Hz), 4.36 (2H, br s), 3.74 (2H, br s), 2.94-2.89 (2H, m), 2.18 (3H, s), 1.57 (9H, s). MS (ESI/APCI) m/z: 436.3 (M+H)$^+$.

tert-butyl 4-({8-[2-(dimethylamino)ethoxy]-7-methyl-5-oxo-1,5-dihydro-2H-chromeno[3,4-c]pyridin-3(4H)-yl}carbonyl)benzoate (67b)

In a similar manner to that employed for the synthesis of **67a**, the title compound **67b** (29.1 mg, 36% yield) was obtained from **66b** (70.2 mg, 0.16 mmol). ¹H-NMR (DMSO-D$_6$, 80 °C) δ: 7.99-7.95 (2H, m), 7.58-7.52 (3H, m), 7.10-7.06 (1H, m), 4.41-4.34 (2H, m), 4.20 (2H, t, J = 5.5 Hz), 3.81-3.68 (2H, m), 2.98-2.93 (2H, m), 2.70 (2H, t, J = 5.5 Hz), 2.26 (6H, s), 2.21 (3H, s), 1.57 (9H, s). MS (ESI/APCI) m/z: 507.4 (M+H)$^+$.
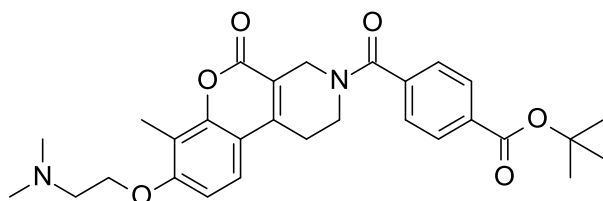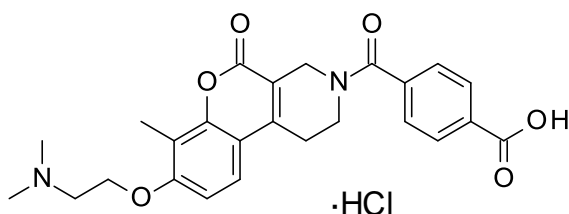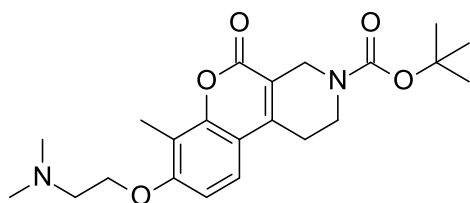

4-({8-[2-(dimethylamino)ethoxy]-7-methyl-5-oxo-1,5-dihydro-2H-chromeno[3,4-c]pyridin-3(4H)-yl}carbonyl)benzoic acid·HCl (54)

In a similar manner to that employed for the synthesis of **47**, the title compound **54** (17.7 mg, 77% yield) was obtained from **67b** (24.0 mg, 0.047 mmol). ¹H-NMR (DMSO-D$_6$, 80 °C) δ: 10.40 (1H, br s), 8.02 (2H, d, J = 7.9 Hz), 7.60-7.56 (3H, m), 7.12 (1H, d, J = 8.5 Hz), 4.50 (2H, t, J = 4.9 Hz), 4.43-4.38 (2H, m), 3.81-3.73 (2H, m), 3.57 (2H, t, J = 4.9 Hz), 2.99-2.96 (2H, m), 2.89 (6H, s), 2.27 (3H, s). MS (ESI/APCI) m/z: 451.2 (M+H)$^+$. HRMS (ESI): m/z calcd for $C_{25}H_{27}N_2O_6$ (M+H)$^+$ 451.1864. Found 451.1891.

***tert*-butyl 8-[2-(dimethylamino)ethoxy]-7-methyl-5-oxo-1,5-dihydro-2*H*-chromeno[3,4-*c*]pyridine-3(4*H*)-carboxylate (69a)**

To a suspension of **68** (622 mg, 1.88 mmol) and potassium carbonate (1.3 g, 9.4 mmol) in acetone (20 mL) was added 2-dimethylaminoethyl chloride hydrochloride (954 mg, 6.62 mmol). The mixture was refluxed for 8 h and allowed to stand still overnight. The resulting residue was diluted with acetone, filtered over Celite, and concentrated *in vacuo*. Purification by col-umn chromatography (SiO$_2$, MeOH/DCM = 0/100 – 8/92 (v/v)) afforded **69a** as a solid. ^{1}H-NMR (CDCl$_3$) δ: 7.36 (1H, d, J = 8.5 Hz), 6.85 (1H, d, J = 8.5 Hz), 4.39 (2H, s), 4.17 (2H, t, J = 5.5 Hz), 3.73 (2H, t, J = 5.8 Hz), 2.89-2.76 (4H, m), 2.38 (6H, s), 2.33 (3H, s), 1.50 (9H, s). MS (ESI/APCI) m/z: 403.3 (M+H)$^+$.
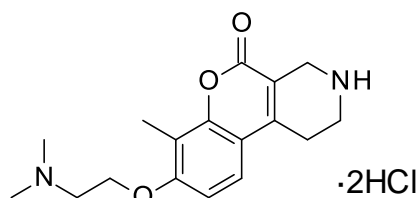
8-[2-(dimethylamino)ethoxy]-7-methyl-1,2,3,4-tetrahydro-5*H*-chromeno[3,4-*c*]pyridin-5-one·2HCl (70a)

In a similar manner to that employed for the synthesis of **70b**, the title compound **70a** (596 mg, 90% yield) was obtained from **69a** (714 mg, 1.77 mmol). ^{1}H-NMR (DMSO-D$_6$) δ: 10.65 (1H, br s), 9.66 (2H, br s), 7.68 (1H, d, J = 8.8 Hz), 7.17 (1H, d, J = 8.8 Hz), 4.52 (2H, t, J = 4.9 Hz), 3.97 (2H, s), 3.58 (2H, t, J = 4.9 Hz), 3.42 (2H, t, J = 5.8 Hz), 3.14 (2H, t, J = 5.8 Hz), 2.87 (6H, s), 2.27 (3H, s). MS (ESI/APCI) m/z: 303.3 (M+H)$^+$. Anal. Calcd for C$_{17}$H$_{22}$N$_2$O$_3$·2HCl·H$_2$O: C, 51.91; H, 6.66; N, 7.12; O, 16.27; Cl, 18.03. Found: C, 51.59; H, 6.56; N, 7.10; O, 16.56; Cl, 18.01.

8-[2-(dimethylamino)ethoxy]-3-{[1-(4-methoxybenzyl)-2,2-dioxido-1,3-dihydro-2,1-benzothiazol-5-yl]carbonyl}-7-methyl-1,2,3,4-tetrahydro-5*H*-chromeno[3,4-*c*]pyridin-5-one (71a)

To a suspension of **70a** (250 mg, 0.67 mmol) and 1-[(4-methoxyphenyl)methyl]-2,2-dioxo-3*H*-2,1-benzothi-azole-5-carboxylic acid (244 mg, 0.73 mmol) in DCM (7 mL) were added WSCI·HCl (153 mg, 0.80 mmol), HOBt hydrate (121 mg, 0.80 mmol) and triethylamine (0.203 mL, 1.47 mmol). The mixture was stirred at rt for 3 h and allowed to stand still overnight. The mixture was diluted with DCM, washed with sat. aq. NaHCO$_3$, water, and brine, respectively. The organic layer was dried over anhydrous Na$_2$SO$_4$, filtered, and concentrated. Purification by column chromatography (SiO$_2$, MeOH/DCM = 2/98 – 10/90 (v/v)) afforded **71a** (380 mg, 92% yield) as a white solid. ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 7.52 (1H, d, J = 8.5 Hz), 7.47 (1H, s), 7.41-7.36 (3H, m), 7.07 (1H, d, J = 9.1 Hz), 6.93 (2H, d, J = 8.5 Hz), 6.78 (1H, d, J = 7.9 Hz), 4.74 (4H, s), 4.38 (2H, s), 4.19 (2H, t, J = 5.5 Hz), 3.78-3.73 (5H, m), 2.95-2.92 (2H, m), 2.72 (2H, t, J = 5.5 Hz), 2.27 (6H, s), 2.21 (3H, s). MS (ESI/APCI) m/z: 618.2 (M+H)$^+$.

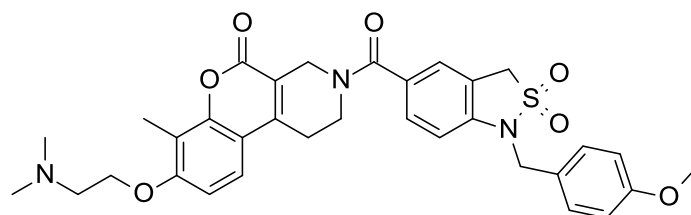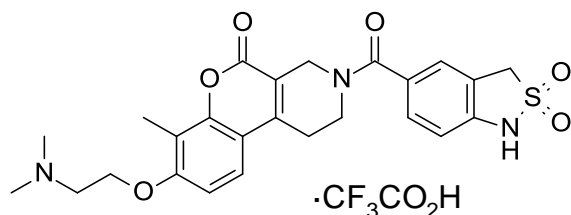**8-[2-(dimethylamino)ethoxy]-3-[(2,2-dioxido-1,3-dihydro-2,1-benzothiazol-5-yl)carbonyl]-7-methyl-1,2,3,4-tetrahydro-5*H*-chromeno[3,4-*c*]pyridin-5-one·CF$_3$CO$_2$H (55)**

A mixture of **71a** (334 mg, 0.54 mmol) and TFA (6 mL) was stirred at rt for 6 h and allowed to stand still overnight. Excess TFA was removed by azeotropic distillation with DCM (3 times). The residue was sonicated with MeOH at 50 °C and insoluble materials were removed by filtration. Evaporation of the solvent followed by trituration with EtOAc at 50 °C gave **55** (299 mg, 90% yield) as a pale-yellow solid. ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 7.58 (1H, d, J = 9.1 Hz), 7.42 (1H, s), 7.39 (1H, d, J = 7.9 Hz), 7.11 (1H, d, J = 9.1 Hz), 6.88 (1H, d, J = 7.9 Hz), 4.53 (2H, s), 4.45 (2H, t, J = 4.9 Hz), 4.41 (2H, s), 3.79 (2H, t, J = 5.8 Hz), 3.56 (2H, t, J = 4.9 Hz), 2.99-2.94 (2H, m), 2.90 (6H, s), 2.27 (3H, s). MS (ESI/APCI) m/z: 498.2 (M+H)$^+$. HRMS (ESI): m/z calcd for C$_{25}$H$_{28}$N$_3$O$_6$S (M+H)$^+$ 498.1693. Found 498.1716.
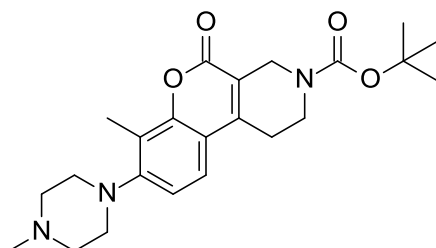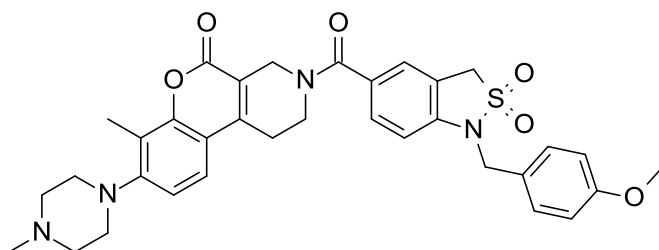

tert-butyl 7-methyl-8-(4-methylpiperazin-1-yl)-5-oxo-1,5-dihydro-2_H_-chromeno[3,4-_c_]pyridine-3(4_H_)-carboxylate (69b)

To a suspension of **93** (5.60 g, 12.1 mmol) in toluene (100 mL) were added cesium carbonate (5.91 g, 18.1 mmol), RuPhos Pd–G1 _t_BuOMe adduct (250 mg, 0.30 mmol), RuPhos (141 mg, 0.30 mmol) and 1-methylpiperazine (3.33 mL, 30.2 mmol). The mixture was stirred at 110 °C for 8 h under N$_2$ atmosphere. After dilution with chloroform–MeOH, insoluble materials were removed by filtration over Celite. Water and brine were added into the filtrate, and it was extracted with chloroform–MeOH. The organic layer was dried over anhydrous Na$_2$SO$_4$, filtered, concentrated, and purified by column chromatography (SiO$_2$, MeOH/DCM = 1/99 – 10/90 (v/v)) to afford **69b** (4.59 g, 92% yield) as a yellow solid. ^{1}H-NMR (CDCl$_3$) δ: 7.36 (1H, d, J = 8.5 Hz), 6.99 (1H, d, J = 8.5 Hz), 4.40 (2H, s), 3.72 (2H, t, J = 5.8 Hz), 3.02 (4H, t, J = 4.6 Hz), 2.88-2.82 (2H, m), 2.66-2.57 (2H, m), 2.38 (6H, s), 1.66-1.59 (2H, m), 1.49 (9H, s). MS (ESI/APCI) m/z: 414.3 (M+H)$^+$.
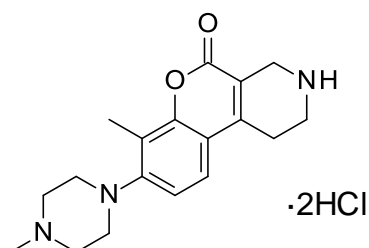
7-methyl-8-(4-methylpiperazin-1-yl)-1,2,3,4-tetrahydro-5_H_-chromeno[3,4-_c_]pyridin-5-one·2HCl (70b)

To a MeOH (50 mL) solution of **69b** (4.59 g, 11.1 mmol) was added 4M HCl in 1,4-dioxane (50 mL). The mixture was stirred at rt for 2 h. After concentrated _in vacuo_, EtOAc, _n_-hexane and a small volume of MeOH were added to the residue. The resulting solid was collected by filtration and dried under reduced pressure to afford **70b** (3.92 g, 91% yield) as a yellow solid. ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 9.79 (1H, br s), 7.60 (1H, d, J = 8.5 Hz), 7.14 (1H, d, J = 8.5 Hz), 3.97 (2H, s), 3.41 (2H, t, J = 6.1 Hz), 3.30-3.25 (4H, m), 3.17-3.10 (8H, m), 2.82 (3H, s), 2.33 (3H, s). MS (ESI/APCI) m/z: 314.2 (M+H)$^+$.

**3-{[1-(4-methoxybenzyl)-2,2-dioxido-1,3-dihydro-2,1-benzothiazol-5-yl]carbonyl}-7-methyl-8-(4-methylpiperazin-1-yl)-1,2,3,4-tetrahydro-5***H***-chromeno[3,4-*c*]pyridin-5-one (71b)**

To a suspension of **70b** (100 mg, 0.26 mmol), 1-[(4-methoxyphenyl)methyl]-2,2-dioxo-3*H*-2,1-benzothia-zole-5-carboxylic acid (87 mg, 0.26 mmol), HOAt (44 mg, 0.31 mmol) and WSCI·HCl (60 mg, 0.31 mmol) in DMF (3 mL) was added *N,N*-diisopropylethylamine (0.21 mL, 1.21 mmol). The mixture was stirred at rt for 7 h. After concentrated *in vacuo*, the residue was diluted with MeOH–chloroform, and washed with water. The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated. Purification by column chromatography (SiO_2, MeOH/DCM = 3/97 – 12/88 (v/v)) afforded **71b** (169 mg, quant.) as a pale yellow amorphous. ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 7.49 (1H, d, J = 8.5 Hz), 7.47 (1H, s), 7.41-7.36 (3H, m), 7.06 (1H, d, J = 8.5 Hz), 6.93 (2H, d, J = 8.5 Hz), 6.78 (1H, d, J = 8.5 Hz), 4.74 (4H, s), 4.38 (2H, s), 3.78-3.74 (5H, m), 2.97-2.91 (8H, m), 2.53-2.50 (2H, m), 2.28 (3H, s), 2.26 (3H, s). MS (ESI/APCI) m/z: 629.3 (M+H)$^+$.
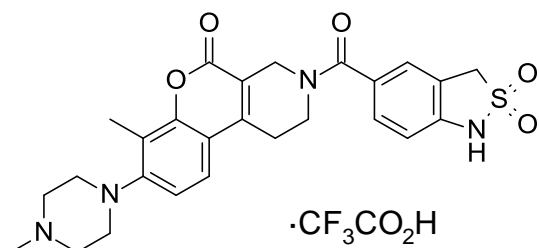

3-[(2,2-dioxido-1,3-dihydro-2,1-benzothiazol-5-yl)carbonyl]-7-methyl-8-(4-methylpiperazin-1-yl)-1,2,3,4-tetrahydro-5*H***-chromeno[3,4-*c*]pyridin-5-one·CF$_3$CO$_2$H (56)**

In a similar manner to that employed for the synthesis of **55**, the title compound **56** (90 mg, 54% yield) was obtained from **71b** (169 mg, 0.27 mmol). ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 7.55 (1H, d, J = 8.5 Hz), 7.44-7.37 (2H, m), 7.12 (1H, d, J = 8.5 Hz), 6.88 (1H, d, J = 7.9 Hz), 4.53 (2H, s), 4.42 (2H, s), 3.81-3.77 (2H, m), 3.38-3.30 (4H, m), 3.22-3.16 (4H, m), 2.98-2.94 (2H, m), 2.85 (3H, s), 2.32 (3H, s). ^{13}C-NMR (DMSO-D$_6$) δ: 169.1, 159.2, 158.1 (q, $^2J_{C-F}$ = 31.4 Hz), 152.3, 150.8, 146.7, 141.6, 128.4, 128.2, 125.1, 122.0, 119.9, 118.5, 117.3, 117.0 (q, $^1J_{C-F}$ = 299 Hz), 115.3, 114.7, 110.7, 52.8, 51.5, 48.3, 42.2, 24.9, 10.6. MS (ESI/APCI) m/z: 509.2 (M+H)$^+$. HRMS (ESI): m/z calcd for $C_{26}H_{29}N_4O_5S$ (M+H)$^+$ 509.1853. Found 509.1839.
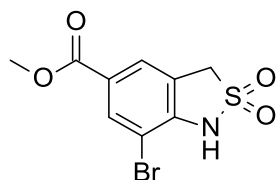
**methyl 7-bromo-2,2-dioxo-2,3-dihydro-1***H***-2λ^6,1-benzothiazole-5-carboxylate (89)**

To a solution of methyl 2,2-dioxo-2,3-dihydro-1H-2λ^6,1-benzothiazole-5-carboxylate (300 mg, 1.32 mmol) in DMF (4 mL) was added *N*-bromosuccinimide (258 mg, 1.45 mmol) at 0 °C. The mixture was stirred for 30 min at 0 °C and for 1 h at rt. After diluted with EtOAc, the organic layer was washed with water and brine, dried over Na_2SO_4, concentrated under reduced pressure, and purified by column chromatography (SiO_2, MeOH/DCM = 2/98 – 15/85 (v/v)) to afford **89** (358 mg, 89% yield) as a white solid. ^{1}H-NMR (CDCl$_3$) δ: 8.18 (1H, s), 7.90 (1H, s), 7.10 (1H, br s), 4.56 (2H, s), 3.92 (3H, s). MS (ESI/APCI) m/z: 304.0, 306.1 (M–H)$^-$.
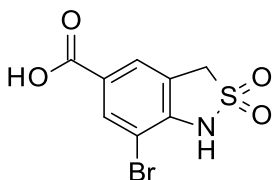
**7-bromo-2,2-dioxo-2,3-dihydro-1***H***-2λ^6,1-benzothiazole-5-carboxylic acid (90)**

To a solution of **89** (170 mg, 0.56 mmol) in THF (2 mL) and MeOH (2 mL) was added 1M NaOH aq. (0.221 mL) at 0 °C. After stirring for 2 h at rt, 1M NaOH aq. (0.221 mL) was added. After stirring for 2 h at 50 °C, 1M NaOH aq. (0.663 mL) was added. After stirring for 13 h at 75 °C, the mixture was cooled down to rt and a

half amount of the solvent was evaporated. The mixture was acidified into pH 4 by adding 1M HCl, and the resulting solid was collected by filtration. Drying under reduced pressure afforded **90** (145 mg, 89% yield) as a white solid. ^{1}H-NMR (DMSO-D$_6$) δ: 13.11 (1H, br s), 11.30 (1H, br s), 7.98-7.96 (1H, m), 7.82 (1H, s), 4.81 (2H, s). MS (ESI/APCI) m/z: 290.0, 291.9 (M–H)$^-$.
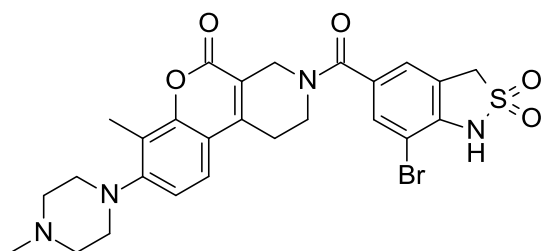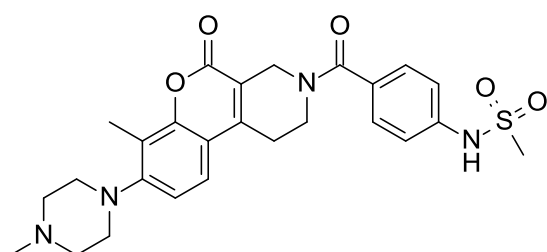
7-bromo-5-[7-methyl-8-(4-methylpiperazin-1-yl)-5-oxo-1,5-dihydro-2H-[1]benzopyrano[3,4-c]pyridine-3(4H)-carbonyl]-1,3-dihydro-2H-2λ^6,1-benzothiazole-2,2-dione (83)

To a suspension of **70b** (60 mg, 0.16 mmol), **90** (46 mg, 0.16 mmol), HOAt (23 mg, 0.17 mmol) and WSCI·HCl (33 mg, 0.17 mmol) in DMF (3 mL) was added N,N-diisopropylethylamine (0.135 mL. 0.78 mmol), and the mixture was stirred for 16 h at rt. After concentration in vacuo, the mixture was purified by column chromatography (SiO$_2$, MeOH/DCM = 3/97 – 20/80 (v/v)). The obtained crude material was diluted with DCM/MeOH and water, and then extracted with DCM/MeOH (4 times). The combined organic layer was dried over Na$_2$SO$_4$, filtrated, concentrated, and purified by column chromatography (SiO$_2$, MeOH/DCM = 13/87 – 20/80 (v/v)) to give **83** (20 mg, 22% yield) as a white solid. ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 7.53 (1H, d, J = 8.5 Hz), 7.47 (1H, s), 7.29 (1H, s), 7.09 (1H, d, J = 8.5 Hz), 4.42-4.39 (4H, m), 3.78 (2H, t, J = 5.8 Hz), 2.97-2.92 (2H, m), 2.86-2.81 (4H, m), 2.50-2.47 (7H, m), 2.30 (3H, s). MS (ESI/APCI) m/z: 587.2, 589.2 (M+H)$^+$.
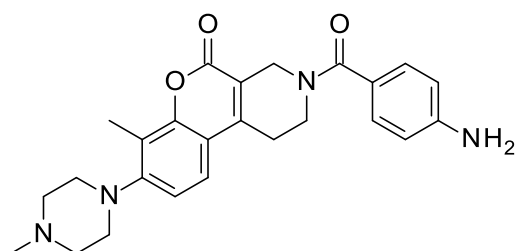
3-(4-aminobenzoyl)-7-methyl-8-(4-methylpiperazin-1-yl)-1,2,3,4-tetrahydro-5H-chromeno[3,4-c]pyridin-5-one (92a)

In a similar manner to that employed for the synthesis of **92e**, the title compound **92a** (45 mg, 50% yield) was obtained using **70b** (80 mg, 0.21 mmol) and 4-aminobenzoic acid (32 mg, 0.23 mmol). ^{1}H-NMR (DMSO-D$_6$) δ: 7.55 (1H, d, J = 8.5 Hz), 7.22 (2H, d, J = 8.5 Hz), 7.08 (1H, d, J = 8.5 Hz), 6.57 (2H, d, J = 8.5 Hz), 5.61 (2H, s), 4.39 (2H, s), 3.79-3.75 (2H, m), 2.98-2.92 (6H, m), 2.53-2.45 (4H, m), 2.27 (3H, s), 2.25 (3H, s). MS (ESI/APCI) m/z: 433.2 (M+H)$^+$.

N-(4-{[7-methyl-8-(4-methylpiperazin-1-yl)-5-oxo-1,5-dihydro-2H-chromeno[3,4-c]pyridin-3(4H)-yl]carbonyl}phenyl)methanesulfonamide (75)

To a solution of **92a** (45 mg, 0.10 mmol) in DCM (3 mL) were added pyridine (0.042 mL. 0.52 mmol) and methanesulfonyl chloride (0.041 mL. 0.52 mmol). The mixture was stirred at rt for 12 h and then allowed to stand still overnight. After evaporating the solvent, the residue was purified by column chromatography (SiO$_2$,

MeOH/DCM = 5/95 – 15/85 (v/v)), subsequently triturated with EtOAc–*n*-hexane, and dried at 60 °C under reduced pressure to afford **75** (38 mg, 72% yield). ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 9.80 (1H, br s), 7.52 (1H, d, J = 8.5 Hz), 7.47-7.42 (2H, m), 7.31-7.26 (2H, m), 7.08 (1H, d, J = 8.5 Hz), 4.40 (2H, s), 3.80-3.74 (2H, m), 3.05-2.92 (6H, m), 3.04 (3H, s), 2.67-2.57 (4H, m), 2.33 (3H, s), 2.29 (3H, s). MS (ESI/APCI) m/z: 511.3 (M+H)$^+$. HRMS (ESI): m/z calcd for C$_{26}$H$_{31}$N$_4$O$_5$S (M+H)$^+$ 511.2010. Found 511.2029. IR (KBr) 3433, 3183, 2936, 1696, 1604, 1428, 1386, 1338, 1287, 1237, 1152, 1105 cm^{-1}.

3-(4-amino-3-methylbenzoyl)-7-methyl-8-(4-methylpiperazin-1-yl)-1,2,3,4-tetrahydro-5*H*-chromeno[3,4-*c*]pyridin-5-one (92b)
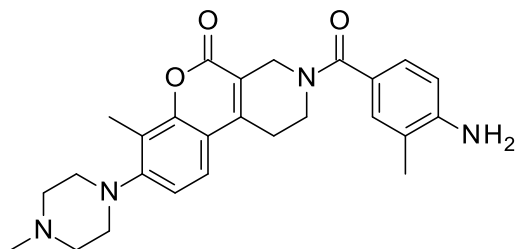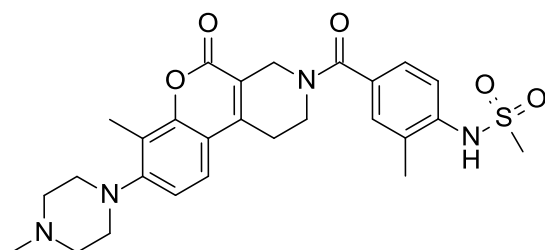
In a similar manner to that employed for the synthesis of **92e**, the title compound **92b** (41 mg, 44% yield) was obtained using **70b** (80 mg, 0.21 mmol) and 4-amino-3-methylbenzoic acid (35 mg, 0.23 mmol). ^{1}H-NMR (DMSO-D$_6$) δ: 7.55 (1H, d, J = 8.5 Hz), 7.13-7.05 (3H, m), 6.61 (1H, d, J = 8.5 Hz), 5.36 (2H, s), 4.39 (2H, s), 3.79-3.74 (2H, m), 2.97-2.92 (6H, m), 2.56-2.46 (4H, m), 2.27 (3H, s), 2.26 (3H, s), 2.07 (3H, s). MS (ESI/APCI) m/z: 447.3 (M+H)$^+$.

***N*-(2-methyl-4-{[7-methyl-8-(4-methylpiperazin-1-yl)-5-oxo-1,5-dihydro-2*H*-chromeno[3,4-*c*]pyridin-3(4*H*)-yl]carbonyl}phenyl)methanesulfonamide (76)**

In a similar manner to that employed for the synthesis of **75**, the title compound **76** (37 mg, 77% yield) was obtained using **92b** (41 mg, 0.092 mmol) and methanesulfonyl chloride (0.036 mL, 0.46 mmol). ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 8.99 (1H, br s), 7.52 (1H, d, J = 8.5 Hz), 7.39 (1H, d, J = 8.5 Hz), 7.35-7.33 (1H, m), 7.30-7.27 (1H, m), 7.07 (1H, d, J = 8.5 Hz), 4.40 (2H, s), 3.80-3.74 (2H, m), 3.03 (3H, s), 2.99-2.92 (6H, m), 2.60-2.55 (4H, m), 2.35 (3H, s), 2.31 (3H, s), 2.29 (3H, s). MS (ESI/APCI) m/z: 525.3 (M+H)$^+$. HRMS (ESI): m/z calcd for C$_{27}$H$_{33}$N$_4$O$_5$S (M+H)$^+$ 525.2166. Found 525.2161. IR (KBr) 3434, 3121, 2935, 2841, 2794, 1715, 1602, 1450, 1385, 1324, 1289, 1264, 1240, 1157, 1103 cm^{-1}.
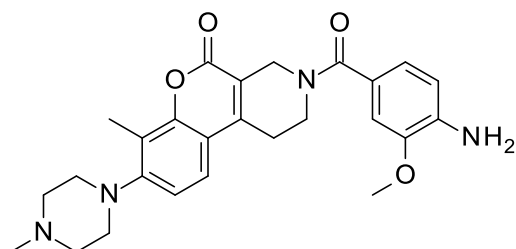

3-(4-amino-3-methoxybenzoyl)-7-methyl-8-(4-methylpiperazin-1-yl)-1,2,3,4-tetrahydro-5*H*-chromeno[3,4-*c*]pyridin-5-one (92c)

In a similar manner to that employed for the synthesis of **92e**, the title compound **92c** (108 mg, quant.) was obtained using **70b** (90 mg, 0.23 mmol) and 4-amino-3-methoxybenzoic acid (43 mg, 0.26 mmol). ^{1}H-NMR (DMSO-D$_6$, 60 °C) δ: 7.54-7.51 (1H, m), 7.07 (1H, d, J = 9.2 Hz), 6.93-6.87 (2H, m), 6.66 (1H, d, J = 7.9 Hz),

5.07 (2H, s), 4.41 (2H, s), 3.81-3.76 (5H, m), 2.98-2.92 (6H, m), 2.57-2.51 (4H, m), 2.28-2.25 (6H, m). MS (ESI/APCI) m/z: 463.3 (M+H)$^+$.

N-(2-methoxy-4-{[7-methyl-8-(4-methylpiperazin-1-yl)-5-oxo-1,5-dihydro-2H-chromeno[3,4-c]pyridin-3(4H)-yl]carbonyl}phenyl)methanesulfonamide (77)
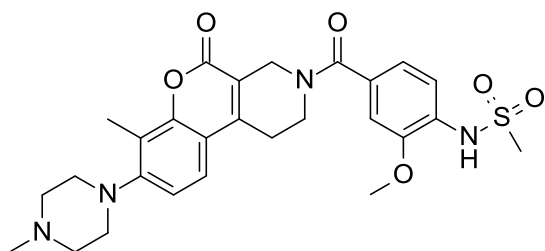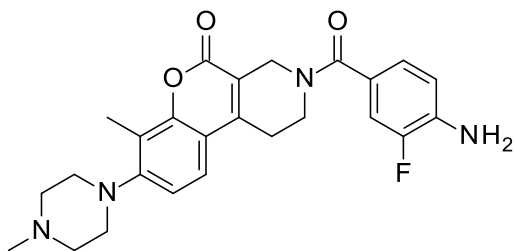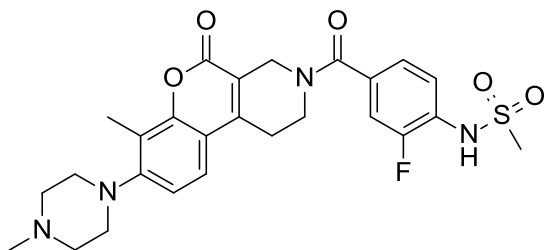
In a similar manner to that employed for the synthesis of **75**, the title compound **77** (95 mg, 80% yield) was obtained using **92c** (102 mg, 0.22 mmol) and methanesulfonyl chloride (0.107 mL, 1.37 mmol). ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 8.74 (1H, s), 7.52 (1H, d, J = 8.5 Hz), 7.37 (1H, d, J = 7.9 Hz), 7.14 (1H, d, J = 1.8 Hz), 7.07 (1H, d, J = 8.5 Hz), 7.04 (1H, dd, J = 7.9, 1.8 Hz), 4.41 (2H, s), 3.86 (3H, s), 3.81-3.73 (2H, m), 3.03 (3H, s), 2.98-2.93 (6H, m), 2.55-2.51 (4H, m), 2.29 (3H, s), 2.27 (3H, s). ^{13}C-NMR (DMSO-D$_6$, for a mixture of rotamers) δ: 169.1, 159.3, 154.0, 151.5, 150.8, 146.8, 133.0, 127.7, 123.6, 121.9, 119.1, 117.9, 116.5, 115.0, 113.8, 110.8, 56.0, 54.8, 51.0, 45.7, 45.2, 43.0, 40.4, 40.2, 37.9, 25.0, 23.9, 10.8. MS (ESI/APCI) m/z: 541.2 (M+H)$^+$. HRMS (ESI): m/z calcd for C$_{27}$H$_{33}$N$_4$O$_6$S (M+H)$^+$ 541.2115. Found 541.2116. IR (KBr) 3426, 3132, 2941, 2839, 2791, 1716, 1636, 1619, 1599, 1451, 1438, 1385, 1371, 1325, 1279, 1238, 1159, 1121, 1101 cm^{-1}.
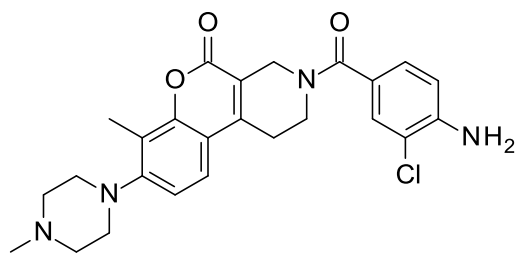

3-(4-amino-3-fluorobenzoyl)-7-methyl-8-(4-methylpiperazin-1-yl)-1,2,3,4-tetrahydro-5H-chromeno[3,4-c]pyridin-5-one (92d)

In a similar manner to that employed for the synthesis of **92e**, the title compound **92d** (81 mg, 99% yield) was obtained using **70b** (70 mg, 0.18 mmol) and 4-amino-3-fluorobenzoic acid (30 mg, 0.20 mmol). ^{1}H-NMR (DMSO-D$_6$) δ: 7.55 (1H, d, J = 8.5 Hz), 7.19-7.15 (1H, m), 7.11-7.06 (2H, m), 6.78 (1H, t, J = 8.5 Hz), 5.67 (2H, s), 4.39 (2H, s), 3.77 (2H, br s), 2.99-2.91 (6H, m), 2.55-2.47 (4H, m), 2.27 (3H, s), 2.25 (3H, s). MS (ESI/APCI) m/z: 451.2 (M+H)$^+$.

N-(2-fluoro-4-{[7-methyl-8-(4-methylpiperazin-1-yl)-5-oxo-1,5-dihydro-2H-chromeno[3,4-c]pyridin-3(4H)-yl]carbonyl}phenyl)methanesulfonamide (78)

In a similar manner to that employed for the synthesis of **75**, the title compound **78** (26 mg, 27% yield) was obtained using **92d** (81 mg, 0.18 mmol) and methanesulfonyl chloride (0.07 mL, 0.90 mmol). ^{1}H-NMR (DMSO-D$_6$) δ: 9.57 (1H, s), 7.53-7.47 (2H, m), 7.42-7.36 (1H, m), 7.32-7.28 (1H, m), 7.07 (1H, d, J = 8.5 Hz), 4.40 (2H, s), 3.76 (2H, br s), 3.09 (3H, s), 2.99-2.93 (6H, m), 2.56-2.51 (4H, m), 2.28 (3H, s), 2.28 (3H, s). MS (ESI/APCI) m/z: 529.3 (M+H)$^+$. HRMS (ESI): m/z calcd for C$_{26}$H$_{30}$FN$_4$O$_5$S (M+H)$^+$ 529.1916. Found 529.1921.

3-(4-amino-3-chlorobenzoyl)-7-methyl-8-(4-methylpiperazin-1-yl)-1,2,3,4-tetrahydro-5*H*-chromeno[3,4-*c*]pyridin-5-one (92e)

To a suspension of **70b** (600 mg, 1.55 mmol), 4-amino-3-chlorobenzoic acid (294 mg, 1.71 mmol), HOAt (233 mg, 1.71 mmol) and WSCI·HCl (372 mg, 1.94 mmol) in DMF (10 mL) was added *N,N*-diisopropylethyl-amine (1.35 mL, 7.77 mmol). The mixture was stirred at rt for 4 h and then allowed to stand still at rt for 3 days. Water was added to the solution, and the resulting solid was collected by filtration, washed with water and *n*-hexane, and dried at 60 °C under reduced pressure to give **92e** (650 mg, 90% yield) as a solid. ^{1}H-NMR (DMSO-D$_6$, 50 °C) δ: 7.53 (1H, d, J = 8.5 Hz), 7.35 (1H, d, J = 2.1 Hz), 7.22-7.18 (1H, m), 7.07 (1H, d, J = 9.2 Hz), 6.82 (1H, d, J = 8.5 Hz), 5.73 (2H, s), 4.40-4.38 (2H, m), 3.79-3.75 (2H, m), 2.97-2.93 (6H, m), 2.54-2.50 (4H, m), 2.28 (3H, s), 2.26 (3H, s). MS (ESI/APCI) m/z: 467.2 (M+H)$^+$.
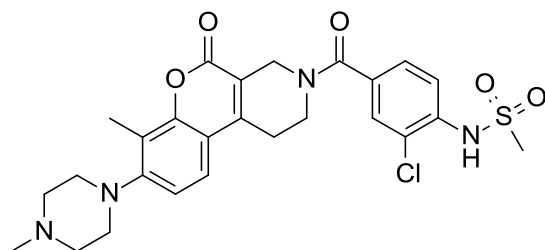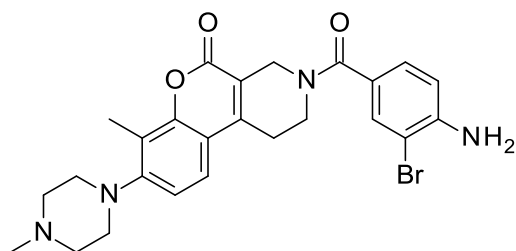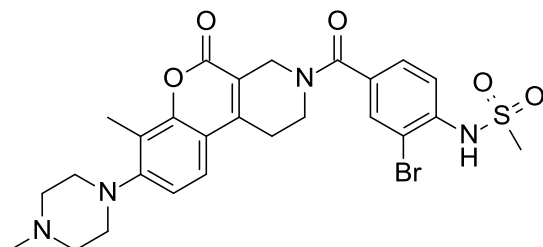

N-(2-chloro-4-{[7-methyl-8-(4-methylpiperazin-1-yl)-5-oxo-1,5-dihydro-2*H*-chromeno[3,4-*c*]pyridin-3(4*H*)-yl]carbonyl}phenyl)methanesulfonamide (79)

To a suspension of **92e** (650 mg, 1.39 mmol) in DCM (30 mL) were added triethylamine (0.98 mL, 6.96 mmol) and methanesulfonyl chloride (0.38 mL, 4.87 mmol) dropwise at 0 °C. After stirring at rt for 2 h, the solvent was evaporated under reduced pressure. The residue was dissolved in THF (15 mL), and 1M aq. NaOH (14 mL) was added to the solution at 0 °C. The mixture was stirred at rt overnight, and then neutralized to pH 7–8 by the addition of 1M HCl. After evaporating the volatile solvent, the solution was extracted three times with DCM. The combined organic layers were dried over anhydrous Na$_2$SO$_4$, filtered, concentrated, and puri-fied by column chromatography (SiO$_2$, MeOH/DCM = 4/96 – 12/88 (v/v)). The obtained solid was suspended in EtOAc and heated to reflux for 1 h. After cooling down to rt, insoluble materials were collected by filtration and dried at 60 °C under reduced pressure to afford **79** (566 mg, 75% yield) as a colorless solid. ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 9.33 (1H, s), 7.60 (1H, d, J = 1.8 Hz), 7.57-7.49 (2H, m), 7.43 (1H, dd, J = 8.5, 1.8 Hz), 7.07 (1H, d, J = 8.5 Hz), 4.40 (2H, s), 3.80-3.73 (2H, m), 3.10 (3H, s), 2.99-2.94 (6H, m), 2.58-2.53 (4H, m), 2.29 (6H, s). ^{13}C-NMR (DMSO-D$_6$) δ: 167.6, 159.3, 153.9, 150.8, 146.8, 136.7, 133.0, 128.7, 127.5, 126.6, 125.4, 121.9, 117.9, 116.4, 115.1, 113.8, 54.7, 50.9, 45.5, 41.0, 24.8, 10.8. MS (ESI/APCI) m/z: 545.2 (M+H)$^+$. HRMS (ESI): m/z calcd for C$_{26}$H$_{30}$ClN$_4$O$_5$S (M+H)$^+$ 545.1620. Found 545.1627. IR (KBr) 3437, 2841, 1703, 1637, 1624, 1601, 1503, 1434, 1387, 1333, 1261, 1151, 1105 cm^{-1}. Anal. Calcd for C$_{26}$H$_{29}$ClN$_4$O$_5$S: C, 57.29; H, 5.36; N, 10.28; Cl, 6.50; S, 5.88. Found: C, 57.00; H, 5.46; N, 10.17; Cl, 6.70; S, 5.87.
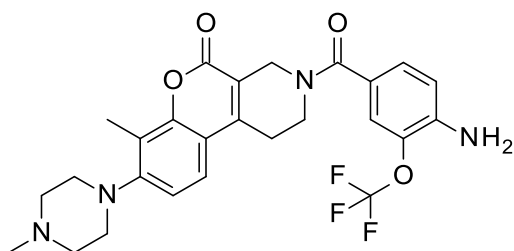
3-(4-amino-3-bromobenzoyl)-7-methyl-8-(4-methylpiperazin-1-yl)-1,2,3,4-tetrahydro-5*H*-chromeno[3,4-*c*]pyridin-5-one (92f)

In a similar manner to that employed for the synthesis of **92e**, the title compound **92f** (80.7 mg, 61% yield) was obtained using **70b** (100 mg, 0.26 mmol) and 4-amino-3-bromobenzoic acid (62 mg, 0.29 mmol). ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 7.54-7.48 (2H, m), 7.25-7.21 (1H, m), 7.09-7.05 (1H, m), 6.86-6.82 (1H, m), 5.56 (2H, s), 4.39 (2H, s), 3.78 (2H, t, J = 5.2 Hz), 2.97-2.93 (6H, m), 2.56-2.51 (4H, m), 2.30-2.26 (6H, m). MS (ESI/APCI) m/z: 511.2, 513.2 (M+H)$^+$.
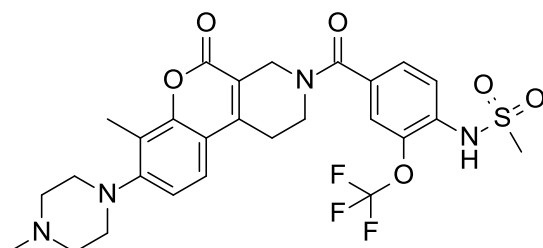

N-(2-bromo-4-{[7-methyl-8-(4-methylpiperazin-1-yl)-5-oxo-1,5-dihydro-2*H*-chromeno[3,4-*c*]pyridin-3(4*H*)-yl]carbonyl}phenyl)methanesulfonamide (80)

In a similar manner to that employed for the synthesis of **81**, the title compound **80** (29.6 mg, 42% yield) was obtained using **92f**(61 mg, 0.12 mmol) and methanesulfonyl chloride (0.093 mL, 1.19 mmol). ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 9.20 (1H, br s), 7.75 (1H, s), 7.55-7.45 (3H, m), 7.07 (1H, d, J = 8.5 Hz), 4.40 (2H, s), 3.79-3.74 (2H, m), 3.10 (3H, s), 2.99-2.93 (6H, m), 2.58-2.54 (4H, m), 2.30 (3H, s), 2.29 (3H, s). MS (ESI/APCI) m/z: 589.1, 591.3 (M+H)$^+$. HRMS (ESI): m/z calcd for C$_{26}$H$_{30}$BrN$_4$O$_5$S (M+H)$^+$ 589.1115. Found 589.1135.

3-[4-amino-3-(trifluoromethoxy)benzoyl]-7-methyl-8-(4-methylpiperazin-1-yl)-1,2,3,4-tetrahydro-5*H*-chromeno[3,4-*c*]pyridin-5-one (92g)

In a similar manner to that employed for the synthesis of **92e**, the title compound **92g** (126 mg, 94% yield) was obtained using **70b** (100 mg, 0.26 mmol) and 4-amino-3-(trifluoromethoxy)benzoic acid (63 mg, 0.28 mmol). ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 7.51 (1H, d, J = 8.5 Hz), 7.25-7.21 (2H, m), 7.07 (1H, d, J = 8.5 Hz), 6.87 (1H, d, J = 8.5 Hz), 5.63 (2H, s), 4.40 (2H, s), 3.78 (2H, t, J = 5.8 Hz), 2.99-2.92 (6H, m), 2.57-2.52 (4H, m), 2.28 (6H, s). MS (ESI/APCI) m/z: 517.3 (M+H)$^+$.

N-[4-{[7-methyl-8-(4-methylpiperazin-1-yl)-5-oxo-1,5-dihydro-2*H*-chromeno[3,4-*c*]pyridin-3(4*H*)-yl]carbonyl}-2-(trifluoromethoxy)phenyl]methanesulfonamide (81)

To a suspension of **92g** (100 mg, 0.19 mmol) in pyridine (2 mL) was added methanesulfonyl chloride (0.151 mL, 1.94 mmol). The mixture was stirred at 60 °C for 3 h, and then allowed to stand still at rt overnight. After concentrated *in vacuo*, THF (2 mL), MeOH (1 mL) and 1M aq. NaOH (3.8 mL) were added to the residue and

the mixture was stirred at rt for 1 h. 1M HCl was added to the solution, and volatile solvents were evaporated. Purification by column chromatography (amino-silica, MeOH/DCM = 2/98 – 14/86 (v/v)) followed by trituration with diisopropyl ether afforded **81** (56 mg, 49% yield) as a white solid. ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 7.65-7.61 (1H, m), 7.53-7.44 (3H, m), 7.09-7.06 (1H, m), 4.40 (2H, s), 3.80-3.74 (2H, m), 3.10 (3H, s), 2.99-2.93 (6H, m), 2.57-2.54 (4H, m), 2.29 (3H, s), 2.29 (3H, s). ^{13}C-NMR (DMSO-D$_6$) δ: 167.7, 159.3, 153.9, 150.8, 146.8, 139.5, 133.4, 131.2, 126.8, 123.0, 121.9, 120.7, 120.1 (q, $^1J_{C-F}$ = 258 Hz), 117.9, 116.4, 115.6, 115.1, 113.9, 54.7, 50.8, 45.4, 43.0, 40.7, 40.1, 24.5, 10.8. MS (ESI/APCI) m/z: 595.3 (M+H)$^+$. HRMS (ESI): m/z calcd for C$_{27}$H$_{30}$F$_3$N$_4$O$_6$S (M+H)$^+$ 595.1833. Found 595.1886. IR (KBr) 3434, 2923, 2849, 1708, 1637, 1603, 1504, 1449, 1387, 1338, 1288, 1253, 1239, 1214, 1159 cm^{-1}. Anal. Calcd for C$_{27}$H$_{29}$F$_3$N$_4$O$_6$S·H$_2$O: C, 52.93; H, 5.10; N, 9.15; F, 9.30; S, 5.23. Found: C, 53.06; H, 5.04; N, 9.06; F, 9.42; S, 5.25.

3-(3-amino-5-chlorobenzoyl)-7-methyl-8-(4-methylpiperazin-1-yl)-1,2,3,4-tetrahydro-5*H*-chromeno[3,4-*c*]pyridin-5-one (92h)
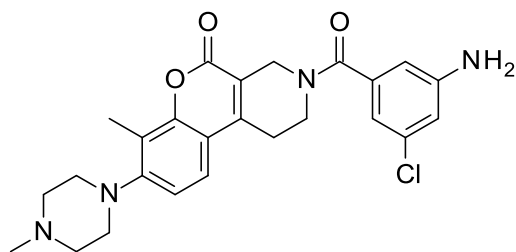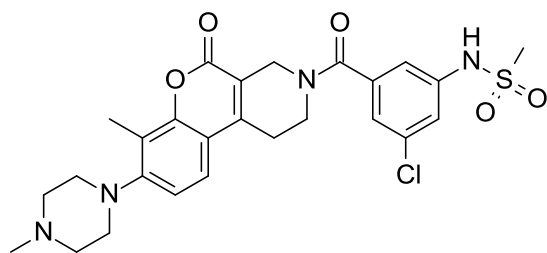
In a similar manner to that employed for the synthesis of **92e**, the title compound **92h** (122 mg, quant.) was obtained using **70b** (100 mg, 0.26 mmol) and 3-amino-5-chlorobenzoic acid (50 mg, 0.29 mmol). ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 7.51 (1H, d, J = 8.5 Hz), 7.07 (1H, d, J = 9.1 Hz), 6.69 (1H, s), 6.55 (1H, s), 6.54 (1H, s), 5.44 (2H, s), 4.36 (2H, s), 3.79-3.71 (2H, m), 2.99-2.90 (6H, m), 2.55-2.51 (4H, m), 2.28 (3H, s), 2.27 (3H, s). MS (ESI/APCI) m/z: 467.3 (M+H)$^+$.

***N*-(3-chloro-5-{[7-methyl-8-(4-methylpiperazin-1-yl)-5-oxo-1,5-dihydro-2*H*-chromeno[3,4-*c*]pyridin-3(4*H*)-yl]carbonyl}phenyl)methanesulfonamide (82)**

In a similar manner to that employed for the synthesis of **75**, the title compound **82** (42 mg, 63% yield) was obtained using **92h** (57 mg, 0.12 mmol) and methanesulfonyl chloride (0.0142 mL, 0.18 mmol). ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 9.96 (1H, br s), 7.51 (1H, d, J = 8.5 Hz), 7.34 (1H, s), 7.24-7.21 (2H, m), 7.07 (1H, d, J = 8.5 Hz), 4.38 (2H, s), 3.79-3.70 (2H, m), 3.07 (3H, s), 2.98-2.93 (6H, m), 2.54-2.51 (4H, m), 2.29 (3H, s), 2.27 (3H, s). MS (ESI/APCI) m/z: 545.3 (M+H)$^+$. HRMS (ESI): m/z calcd for C$_{26}$H$_{30}$ClN$_4$O$_5$S (M+H)$^+$ 545.1620. Found 545.1642.
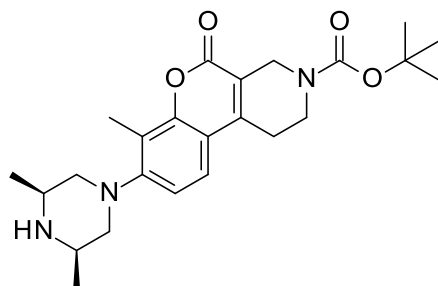
tert-butyl 8-[_meso_-3,5-dimethylpiperazin-1-yl]-7-methyl-5-oxo-1,5-dihydro-2_H_-chromeno[3,4-_c_]pyridine-3(4_H_)-carboxylate (94a)

In a similar manner to that employed for the synthesis of **69b**, the title compound **94a** (111 mg, 24% yield) was obtained using **93** (499 mg, 1.08 mmol) and _cis_-2,6-dimethylpiperazine (370 mg, 3.24 mmol). ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 7.49 (1H, d, J = 8.5 Hz), 7.02 (1H, d, J = 8.5 Hz), 4.22 (2H, s), 3.63 (2H, t, J = 5.8 Hz), 3.04-2.95 (4H, m), 2.87-2.83 (2H, m), 2.31-2.23 (5H, m), 1.44 (9H, s), 1.00 (6H, d, J = 6.1 Hz). MS (ESI/APCI) m/z: 428.3 (M+H)$^+$.
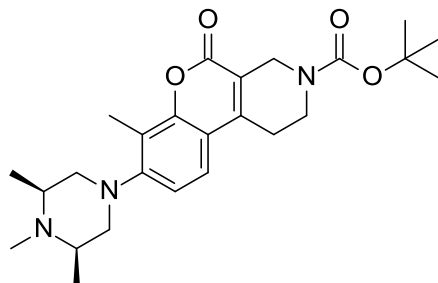
tert-butyl 7-methyl-5-oxo-8-[_meso_-3,4,5-trimethylpiperazin-1-yl]-1,5-dihydro-2_H_-chromeno[3,4-_c_]pyridine-3(4_H_)-carboxylate (94b)

To a solution of **94a** (99 mg, 0.23 mmol) in DCM (3 mL) and MeOH (0.3 mL) was added 37% formalin (0.0854 mL) at rt. After stirring at rt for 20 min, the reaction mixture was cooled down to 0 °C, and sodium triacetoxyborohydride (73 mg, 0.35 mmol) was added. The mixture was stirred at 0 °C for 5 min and at rt for 50 min. After diluted with DCM, the organic layer was washed with sat. aq. NaHCO$_3$ and separated through Phase Separator (Biotage AB). The obtained organic layer was concentrated _in vacuo_, and the residue was purified by column chromatography (SiO$_2$, MeOH/DCM = 2/98 – 10/90 (v/v)) to give **94b** (86 mg, 84% yield) as a yellow solid. ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 7.50 (1H, d, J = 8.5 Hz), 7.02 (1H, d, J = 8.5 Hz), 4.22 (2H, s), 3.63 (2H, t, J = 5.8 Hz), 3.04-2.98 (2H, m), 2.87-2.83 (2H, m), 2.57-2.51 (2H, m), 2.41-2.35 (2H, m), 2.29 (3H, s), 2.24 (3H, s), 1.44 (9H, s), 1.05 (6H, d, J = 6.1 Hz). MS (ESI/APCI) m/z: 442.4 (M+H)$^+$.
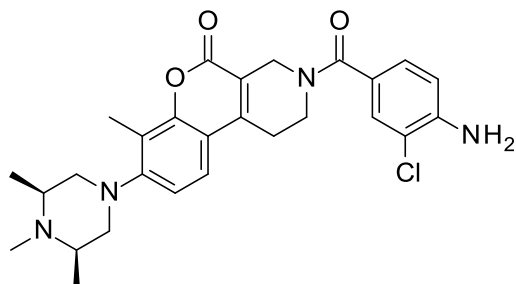

3-(4-amino-3-chlorobenzoyl)-7-methyl-8-[_meso_-3,4,5-trimethylpiperazin-1-yl]-1,2,3,4-tetrahydro-5_H_-chromeno[3,4-_c_]pyridin-5-one (95b)

To a MeOH (1 mL) solution of **94b** (84 mg, 0.19 mmol) was added 4M HCl in 1,4-dioxane (1 mL). The mixture was stirred at rt. After 1 h, the reaction mixture was concentrated under reduced pressure to give a crude intermediate. The title compound **95b** (79 mg, 84% yield) was obtained in a similar manner to that employed for the synthesis of **92e**, using the crude intermediate and 4-amino-3-chlorobenzoic acid (39.7 mg, 0.23 mmol). ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 7.50 (1H, d, J = 9.1 Hz), 7.34 (1H, d, J = 1.8 Hz), 7.21-7.16 (1H, m), 7.03 (1H, d, J = 8.5 Hz), 6.84 (1H, d, J = 8.5 Hz), 5.59 (2H, s), 4.39 (2H, s), 3.77 (2H, t, J = 5.5 Hz), 3.07-2.99 (2H, m), 2.96-2.91 (2H, m), 2.54 (2H, t, J = 10.9 Hz), 2.40-2.33 (2H, m), 2.28 (3H, s), 2.23 (3H, s), 1.05 (6H, d, J = 6.1 Hz). MS (ESI/APCI) m/z: 495.3 (M+H)$^+$.
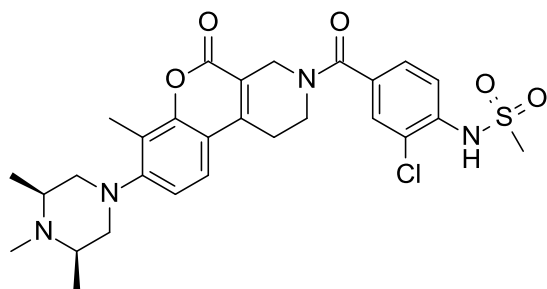
N-[2-chloro-4-({7-methyl-5-oxo-8-[*meso*-3,4,5-trimethylpiperazin-1-yl]-1,5-dihydro-2H-chromeno[3,4-c]pyridin-3(4H)-yl}carbonyl)phenyl]methanesulfonamide (84)

To a solution of **95b** (79 mg, 0.16 mmol) in pyridine (1.5 mL) was added methanesulfonyl chloride (0.0373 mL, 0.48 mmol). The mixture was stirred at 50 °C for 100 min. Methanesulfonyl chloride (0.0248 mL, 0.32 mmol) was added and the mixture was stirred for 30 min and then cooled to rt. Pyridine (1 mL) and methanesulfonyl chloride (0.0373 mL, 0.48 mmol) were added and the mixture was stirred at 55 °C for 1 h, cooled down to rt, concentrated under reduced pressure and stored in a refrigerator, overnight. The residue was diluted with chloroform and purified by column chromatography (SiO_2, MeOH/DCM = 2/98 – 15/85 (v/v)). The obtained material was dissolved in THF (5 mL) and MeOH (0.5 mL), and 1M aq. NaOH (0.318 mL) was added to it. The mixture was stirred at rt for 50 min. 1M HCl aq. (0.3 mL) was added, and the solution was concentrated under reduced pressure. The residue was purified by column chromatography (amino-silica, MeOH/DCM = 10/90 – 50/50 (v/v) and SiO_2, MeOH/DCM = 1/99 – 12/88 (v/v)). Trituration of the residue with diisopropyl ether gave **84** (37 mg, 42% yield) as a white solid. ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 9.30 (1H, br s), 7.59 (1H, s), 7.55 (1H, d, J = 7.9 Hz), 7.51 (1H, d, J = 7.9 Hz), 7.43 (1H, d, J = 7.9 Hz), 7.04 (1H, d, J = 8.5 Hz), 4.40 (2H, s), 3.77 (2H, br s), 3.09 (3H, s), 3.07-3.00 (2H, m), 2.99-2.93 (2H, m), 2.60-2.52 (2H, m), 2.46-2.40 (2H, m), 2.29 (3H, s), 2.26 (3H, s), 1.07 (6H, d, J = 5.5 Hz). ^{13}C-NMR (DMSO-D$_6$) δ: 167.7, 159.3, 153.4, 150.8, 146.8, 137.0, 132.7, 128.7, 127.4, 126.6, 125.3, 121.9, 117.8, 116.4, 114.9, 113.8, 58.2, 57.8, 40.9, 37.4, 24.5, 17.5, 10.8. MS (ESI/APCI) m/z: 573.3 (M+H)$^+$. HRMS (ESI): m/z calcd for $C_{28}H_{34}ClN_4O_5S$ (M+H)$^+$ 573.1933. Found 573.1957. IR (KBr) 3435, 2987, 2929, 2815, 1712, 1638, 1602, 1502, 1437, 1386, 1335, 1240, 1160, 1103, 1075, 1053 cm^{-1}. Anal. Calcd for $C_{28}H_{33}ClN_4O_5S \cdot H_2O \cdot 0.2CH_2Cl_2$: C, 55.87; H, 5.84; N, 9.18; Cl, 8.13; S, 5.25. Found: C, 55.57; H, 5.84; N, 9.03; Cl, 8.38; S, 5.22.
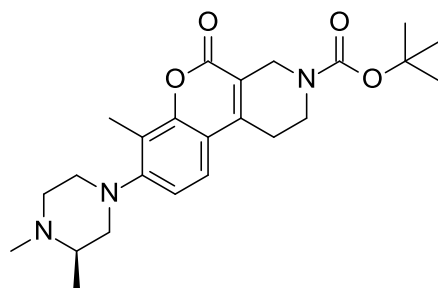
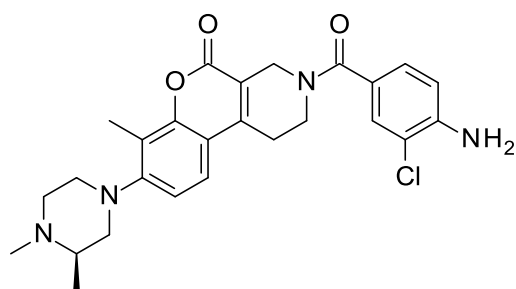
***tert*-butyl 8-[(3R)-3,4-dimethylpiperazin-1-yl]-7-methyl-5-oxo-1,5-dihydro-2H-chromeno[3,4-c]pyridine-3(4H)-carboxylate (94c)**

In a similar manner to that employed for the synthesis of its enantiomer **47d**, the title compound **47c** (29.5 mg, 21% yield) was obtained using **46** (151 mg, 0.32 mmol) and (2R)-1,2-dimethylpiperazine (159 mg, 1.39 mmol). MS (ESI/APCI) m/z: 428.1 (M+H)$^+$.
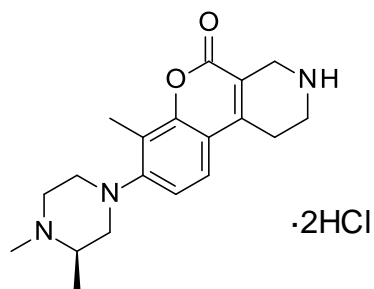
8-[(3*R*)-3,4-dimethylpiperazin-1-yl]-7-methyl-1,2,3,4-tetrahydro-5*H*-chromeno[3,4-*c*]pyridin-5-one·2HCl (S25)

In a similar manner to that employed for the synthesis of its enantiomer **S24**, the title compound **S25** (27.6 mg, quant.) was obtained from **94c** (29.5 mg, 0.069 mmol). MS (ESI/APCI) m/z: 328.3 (M+H)$^+$.

3-(4-amino-3-chlorobenzoyl)-8-[(3*R*)-3,4-dimethylpiperazin-1-yl]-7-methyl-1,2,3,4-tetrahydro-5*H*-chromeno[3,4-*c*]pyridin-5-one (95c)

In a similar manner to that employed for the synthesis of **92e**, the title compound **95c** (24.3 mg, 73% yield) was obtained using **S25** (27.6 mg, 0.069 mmol) and 4-amino-3-chlorobenzoic acid (13 mg, 0.076 mmol). ^{1}H-NMR spectrum was consistent with that of its enantiomer **95d**. MS (ESI/APCI) m/z: 481.3 (M+H)$^+$.
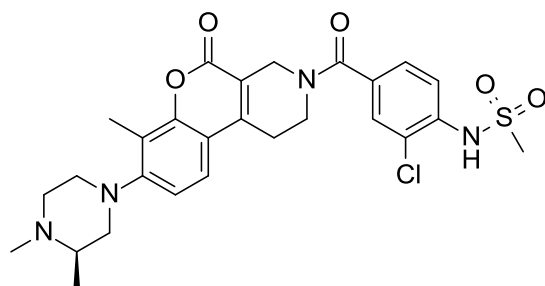
_N_-[2-chloro-4-({8-[(3*R*)-3,4-dimethylpiperazin-1-yl]-7-methyl-5-oxo-1,5-dihydro-2*H*-chromeno[3,4-*c*]pyridin-3(4*H*)-yl}carbonyl)phenyl]methanesulfonamide (85)

In a similar manner to that employed for the synthesis of its enantiomer **86**, the title compound **85** (13 mg, 48% yield) was obtained using **95c** (23 mg, 0.048 mmol) and methanesulfonyl chloride (0.0186 mL, 0.24 mmol). ^{1}H-NMR spectrum was consistent with that of its enantiomer **86**. MS (ESI/APCI) m/z: 559.1 (M+H)$^+$. [α]$_D^{20}$ +16.6° (c = 0.371, CHCl$_3$).

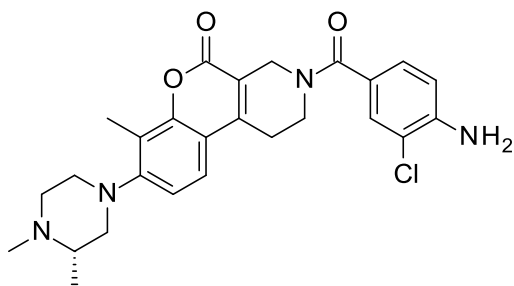**3-(4-amino-3-chlorobenzoyl)-8-[(3*S*)-3,4-dimethylpiperazin-1-yl]-7-methyl-1,2,3,4-tetrahydro-5*H*-chromeno[3,4-*c*]pyridin-5-one (95)**

To a solution of **94d** (21 mg, 0.049 mmol) in MeOH (1 mL) was added 4M HCl in 1,4-dioxane (1 mL). After stirring at rt for 1 h, the solvent was evaporated under reduced pressure. To the residue were added 4-amino-3-chlorobenzoic acid (10 mg, 0.058 mmol), WSCI·HCl (12 mg, 0.062 mmol), HOAt (9 mg, 0.066 mmol), DCM (1 mL), and *N,N*-diisopropylethylamine (0.033 mL, 0.19 mmol) at rt. The mixture was allowed to stand still at rt overnight, and then diluted with chloroform. The organic layer was washed with sat. aq. NaHCO$_3$ and separated through Phase Separator (Biotage AB). The obtained organic layer was concentrated *in vacuo*, and the residue was purified by column chromatography (SiO$_2$, MeOH/DCM = 2/98 – 12/88 (v/v)) to give **95d** (19.5 mg, 82% yield) as a white solid. ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 7.51 (1H, d, J = 8.5 Hz), 7.34 (1H, d, J = 1.8 Hz), 7.21-7.17 (1H, m), 7.05 (1H, d, J = 8.5 Hz), 6.84 (1H, d, J = 7.9 Hz), 5.60 (2H, s), 4.39 (2H, s), 3.77 (2H, t, J = 5.8 Hz), 3.03-2.80 (7H, m), 2.56-2.51 (1H, m), 2.40-2.33 (1H, m), 2.29 (3H, s), 2.25 (3H, s), 1.04 (3H, d, J = 6.1 Hz). MS (ESI/APCI) m/z: 481.3 (M+H)$^+$.
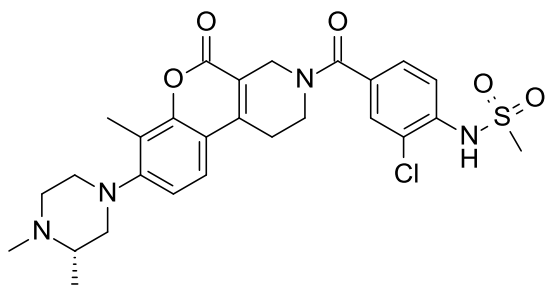

N-[2-chloro-4-({8-[(3*S*)-3,4-dimethylpiperazin-1-yl]-7-methyl-5-oxo-1,5-dihydro-2*H*-chromeno[3,4-*c*]pyridin-3(4*H*)-yl}carbonyl)phenyl]methanesulfonamide (86)

In a similar manner to that employed for the synthesis of **79**, the title compound **86** (8 mg, 41% yield) was obtained using **95d** (17 mg, 0.035 mmol) and methanesulfonyl chloride (0.014 mL, 0.18 mmol). ^{1}H-NMR (DMSO-D$_6$, 80 °C) δ: 7.59 (1H, d, J = 1.8 Hz), 7.57-7.50 (2H, m), 7.45-7.41 (1H, m), 7.06 (1H, d, J = 8.5 Hz), 4.40 (2H, s), 3.79-3.75 (2H, m), 3.09 (3H, s), 3.01-2.82 (7H, m), 2.59-2.51 (1H, m), 2.45-2.32 (1H, m), 2.29 (3H, s), 2.28 (3H, s), 1.05 (3H, d, J = 6.1 Hz). ^{13}C-NMR (DMSO-D$_6$) δ: 167.7, 159.3, 153.7, 150.8, 146.8, 136.7, 132.9, 128.7, 127.5, 126.6, 125.4, 121.9, 117.9, 116.4, 115.0, 113.8, 57.8, 57.5, 54.9, 51.1, 41.9, 41.0, 24.7, 16.3, 10.8. MS (ESI/APCI) m/z: 559.3 (M+H)$^+$. HRMS (ESI): m/z calcd for C$_{27}$H$_{32}$ClN$_4$O$_5$S (M+H)$^+$ 559.1777. Found 559.1806. IR (KBr) 3435, 2932, 2817, 1712, 1634, 1603, 1502, 1442, 1386, 1335, 1285, 1240, 1160, 1105 cm^{-1}. [α]$_D^{25}$ −11.8° (c 1.00, CHCl$_3$).

2. Biological evaluation

General

All experimental procedures for animals were performed in accordance with the in-house guideline of the Institutional Animal Care and Use Committee of Daiichi Sankyo Co., Ltd.

Enzymatic assay

For MTHFD2 NAD-dependent dehydrogenase assay, 0.125 μg/mL MTHFD2 recombinant protein, 100 μM NAD, 0.2 mg/mL tetrahydrofolate (THF), 2.5 mM formaldehyde, 5 mM $MgCl_2$, and 10% DMSO or compounds were mixed in 384-well plate (Greiner, 781801, UV transparent). The amount of mixture was 40 μL/well. After incubation for 30 min at room temperature, the reaction was stopped by adding HCl.

For MTHFD1 NADP-dependent dehydrogenase assay, 0.125 μg/mL MTHFD1 recombinant protein, 82.7 μM NADP, 0.3 mg/mL tetrahydrofolate (THF) , 2.5 mM formaldehyde, 5 mM $MgCl_2$, and 10% DMSO or compounds were mixed in 384-well plate (Greiner, 781801, UV transparent). The amount of mixture was 40 μL/well. After incubation for 30 min at room temperature, the reaction was stopped by adding HCl.

The product methenyl-THF was detected by the absorbance at 355 nm. IC_{50} values were calculated from quadruplicate experiments using GraphPad Prism. The revised structure of LY374571 was used for the standard for the assays[44].

Cell-based growth inhibition assay

MDA-MB-231 cells (MDA-MB-231-luc-D3H2LN, Caliper Life sciences, Inc) were seeded in a 96-well plate at 2,000 cells/90 μL/well. On the following day (Day 0), a test compound diluted with medium was added at 10 μL/well. For the medium for a growth test, MEM with 10% dialyzed FBS, 400 μM Serine and 250 μM Glycine were used. 3 days (Day 3) after the treatment with the compound, 100 μL of a cell-titer Glo (Promega) solution diluted 2.5 times with PBS was added and the amount of luminescence was measured by EnVision. In order to evaluate net effect on cell growth, the value on Day 0 was subtracted from the value on Day 3 after initiation of treatment with the compound. Based on the amount of growth by a sample containing no compound was regarded as 100, the 50% inhibitory concentration (GI_{50} value) was obtained.

Pharmacokinetics

The tested compounds were suspended in a 0.5 (w/v) % methyl cellulose 400 solution (Wako Pure Chemical Industries) and administered orally to male BALBc mice (Charles River Laboratories Japan, Inc.) at a dose of 10, 30, 100, or 300 mg/kg. The plasma samples were collected and the concentration at each time point were measured by LC-MS/MS. The pharmacokinetic parameters were calculated by a non-compartmental analysis.

In vivo anti-tumor test

Five-week old female BALB/cAJcl-nu/nu mice (CLEA Japan, Inc.) were inoculated subcutaneously with MDA-MB-231luc tumor cells (4×10^6 cells/mouse). From eleven days after implantation (day 0), the mice were orally administered vehicle (0.5% methylcellulose), compound **79** (300 mg/kg) or compound **87** (30, 100 or 300 mg/kg) twice daily (in morning and evening) until sacrificed at day 11. The long and short tumor diameters and body weight were assessed at 2–3- and 1–2-day intervals, respectively. Tumor dimensions were measured with a digital caliper and tumor volumes were calculated as long diameter $\times$ short diameter^2/2.

3. X-ray crystallography

Protein production of X-ray crystallography

A DNA fragment encoding human MTHFD2 (residues 36-338) was amplified by PCR and inserted into pET15b vector (Novagen) to produce an N-terminal 6xHis-tagged MTHFD2. The expression was performed in E. coli strain ArcticExpress (DE3) RIL (Agilent). After sonication and centrifugation of the cells, the supernatant was applied to a HisTrap FF crude column (GE Healthcare), and the protein was eluted with a gradient of from 20 mM to 500 mM imidazole. A subsequent gel filtration was carried out using a HiLoad 16/600 Superdex 200 pg column (GE Healthcare) with buffer consisting of 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM DTT. The MTHFD2 were collected and concentrated to 16 mg/mL.

Crystal preparation and structure determination for compound 1/MTHFD2 and compound 41/MTHFD2

Co-crystals of 4-[(2,4-diamino-6-hydroxy-pyrimidin-5-yl)carbamoylamino]benzoic acid[44] (**S26**, a weak binder, cell-free $IC_{50} > 30$ μM for the in-house MTHFD2 enzymatic assay) and MTHFD2 were prepared using the sitting-drop vapor diffusion method at 293K in 28% *i*-PrOH/ 0.1M bis-Tris, pH 6.5/ 3% PEG200/ 10 mM spermidine. Bound compound was removed from co-crystals by incubation in 10% *i*-PrOH/ 0.1M bis-Tris HCl, pH6.5/ 3% PEG200/ 25% glycerol/ 10 mM spermidine/ 10 mM K phosphate buffer, pH 8.5 for 1 day at 293K. Then, the test compounds were introduced to MTHFD2 crystals by soaking in the same solution containing 1 to 10 mM test compound and 2.5 mM $MgCl_2$ at 293K for 1 to 4 days. Obtained crystals were flash-frozen and stored in liquid nitrogen until use.

Diffraction data were collected at the beam-lines of Tsukuba Photon factory as shown in Table S1. After data processing, initial phase were determined by *PHASER*[45] using human MTHFD1 structure (PDB ID = 1DIA[16]) as a search model for molecular replacement. After that, phase refinement and model building were carried out using *REFMAC5*[46] and *COOT*[47]. Statistics of data processing and phase refinement are summarized in Supplementary Table S1. Figures describing crystal structures are drawn by *pymol*[48].

S26
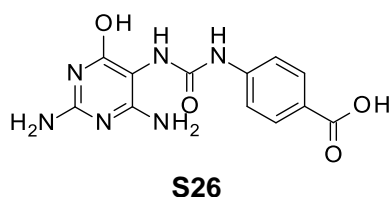
Crystal preparation and structure determination for compound 79/NAD^{+}/MTHFD2

Co-crystal composed of compound **79**, NAD^{+} and MTHFD2 was prepared as previously described with slight modification. Briefly, co-crystals of **S26**/MTHFD2 were prepared by vapor diffusion method. Bound compound was substituted with compound **79** and NAD^{+} by soaking in solution containing 1 mM compound **79** and 2.5 mM NAD^{+} for 40 h at 293 K. Diffraction data were collected at BL-17A of Tsukuba Photon factory as shown in Table S2. Phase refinement and model building were carried out using *REFMAC5* and *COOT*. Statistics of data processing and phase refinement are summarized in Table S2. Figures describing crystal structures are drawn by *pymol*.

Table S1. Statistics for data collection and phase refinement.

$^{a}R_{merge} = \Sigma h\Sigma j \,|{<}I(h){>} - I(h)j \,| / \Sigma h\Sigma j \,{<}I(h){>}$, where ${<}I(h){>}$ is the mean intensity of symmetry-related reflections. ^{b}R-value $= \Sigma \,| \,|Fobs| - |Fcalc| \,| / \Sigma \,|Fobs|$. R_{free} for 5.2% of reflections excluded from refinement. Values in parentheses are for the highest resolution shell.

	compound **1**/MTHFD2	compound **41**/MTHFD2
Data Collection		
X-ray source	PF BL-17A	PF AR NW-12A
Wavelength (Å)	0.98	1.00
Space group	$P6_5$	$P6_5$
Unit cell dimensions	$a=b$=116.5 Å, c=113.2 Å	$a=b$=116.4, c=113.2
	$\alpha=\beta$=90 °, γ=120 °	$\alpha=\beta$=90 °, γ=120 °
Resolution (Å)	49.36-2.50	46.05-2.25
	(2.57-2.50)	(2.31-2.25)
Total No. of observations	153418 (11519)	354192 (13351)
Unique reflections	30155 (2214)	41331 (3207)
Redundancy	5.1 (5.2)	8.6 (4.2)
Completeness (%)	99.9 (99.9)	99.4 (97.1)
I/σ(I)	20.6 (2.9)	13.8 (1.0)
R_{merge}^{a}	0.040 (0.470)	0.092 (0.937)
Refinement		
Resolution (Å)	25-2.5	25-2.25
No. of reflections	30124	41260
RMS Bonds (Å)	0.006	0.006
RMS Angles (°)	1.645	1.096
No. of atoms		
protein	4280	4395
water and solvent	66	181
ligand	60	104
Average B value (Å^2)		
protein	78.4	51.5
water and solvent	76.0	54.7
ligand	81.0	53.9
R-value^{b}	0.2032	0.2034
R_{free}^{b}	0.2542	0.2434

Table S2. Statistics for data collection and phase refinement.

[a]$R_{merge} = \Sigma h\Sigma j \ |<I(h)> - I(h)j \ | / \Sigma h\Sigma j <I(h)>$, where $<I(h)>$ is the mean intensity of symmetry-related reflections. [b]R-value $= \Sigma \ | |Fobs| - |Fcalc| \ | / \Sigma \ |Fobs|$. R_{free} for 5.2% of reflections excluded from refinement. Values in parentheses are for the highest resolution shell.

	compound **79**/NAD^{+}/MTHFD2
Data Collection	
X-ray source	PF BL-17A
Wavelength (Å)	0.98
Space group	$P6_5$
Unit cell dimensions (Å)	$a=b$=116.5, c=113.2
Resolution (Å)	2.25
Unique reflections	41534
Redundancy	3.4
Completeness (%)	99.9 (99.8)
I/σ(I)	8.1 (0.6)
Rmerge[a]	0.037 (0.547)
Refinement	
No. of reflections	41488
RMS Bonds (Å)	0.007
RMS Angles (°)	1.677
Average B value (Å^2)	59.1
R-value[b]	0.1953
R_{free}[b]	0.2497

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謝辞