博士論文

自己免疫疾患治療薬を指向した

新規RORγt逆作動薬の合成研究

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略語表

Ac : acetyl

AF2 : activation function-2

AUC : area under the blood concentration time curve

BA : bioavailability

b.i.d. bis in die (twice a day)

Bn : benzyl

Boc : *tert*-butoxycarbonyl

Bu : butyl

CPME : cyclopentyl methyl ether

cPr : cyclopropyl

CL : clearance

DBD : DNA binding domain

DIEA : *N,N*-diisopropylethylamine

DMF : *N,N*-dimethylformamide

DMARDs : disease-modifying antirheumatic drugs

DMSO : dimethyl sulfoxide

EAE : Experimental autoimmune encephalomyelitis

Et : ethyl

Et_3N : triethylamine

EtOAc : Ethyl acetate

H : Helix

HATU : *O*-(7-azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate

HOBt : 1-hydroxybenzotriazole

HPLC : high performance liquid chromatography

i.v. : intravenous

IPE : diisopropyl ether

JP2 : 日本薬局方溶出試験第2液

LBD : ligand binding domain

Me : methyl

NSAIDs : non-steroidal anti-inflammatory drugs

NT : not tested

PBS : phosphate Buffered Saline

PD : pharmacodynamic

p.o. : per os

PMA : phorbol 12-myristate 13-acetate

ROR : Retinoic-acid-related orphan receptor

SAR : structure–activity relationship

SUMO : small ubiquitin-related modifier

TFA : trifluoroacetic acid

THF : tetrahydrofuran

TPSA : topological polar surface area

T3P : propylphosphonic anhydride

WSC : 1-ethyl-3-(3’-dimethylaminopropyl)carbodiimide

序章

第１節　自己免疫疾患と治療薬の現状

自己免疫疾患は、免疫系が自分自身の細胞を攻撃し、ダメージを与える炎症性の疾患である。免疫反応は本来バクテリア、ウイルス、毒素など外部の異物を認識し、排除するためのシステムであり、通常は自己の細胞に対する免疫応答は免疫寛容により回避されている。しかし、何らかの原因により免疫寛容が破綻し、自身の細胞を攻撃してしまうことで自己免疫疾患を発症する。現在のところ自己免疫疾患の発症に至る原因は解明されておらず、遺伝的要因、ホルモンバランスの乱れ、バクテリアやウイルス等への感染、ストレス、化学物質、食事等の環境要因など、複合的な要素が関わっているとされる[1-2]。自己免疫疾患は心臓、脳、神経、筋肉、皮膚、目、関節、肺、腎臓、リンパ節、消化管や血管など全身で発症し、症状は発症の部位により異なる。例えば関節で自己免疫疾患が発症した場合は関節性リウマチと呼ばれ、関節痛、関節の硬化、機能の喪失などが主な症状である。他にも、全身性エリマトーデス、1型糖尿病、多発性硬化症、乾癬、潰瘍性大腸炎など80種類以上の病気が知られている。米国では人口の約7%にあたる2350万人が自己免疫疾患に罹患していると推定されている[3]。日本でも、リウマチ患者が人口の0.6-1%にあたる100万人程度、乾癬の患者も50万人程度存在すると推計され、他の自己免疫疾患の患者も多い[4-5]。従来の薬物療法の中心は、アスピリンやイブプロフェンのような非ステロイド性抗炎症薬（NSAIDs）

による疼痛の緩和、あるいはステロイドやメトトレキサート、タクロリムス等の免疫抑制薬による免疫反応・炎症の抑制による症状の低減であった（Figure 1）。しかしながら、NSAIDsによる治療は症状の緩和に留まり、またステロイドや免疫抑制薬の長期間に渡る投与は高血糖、感染症等の副作用が懸念となる。1990年代後半から、TNFα阻害薬や、IL-6阻害薬などの生物学的製剤が関節リウマチの治療に用いられ、炎症性サイトカインの抑制が自己免疫疾患の治療に有効であることが示されてきた[6]。これらの抗体医薬は効果が高い一方で、高額な薬剤費や、静脈注射や点滴による投与が必要であるために患者の頻繁な通院が必要になる等のデメリットがある。そのため、経口投与可能な新たな低分子治療薬の開発が望まれている。

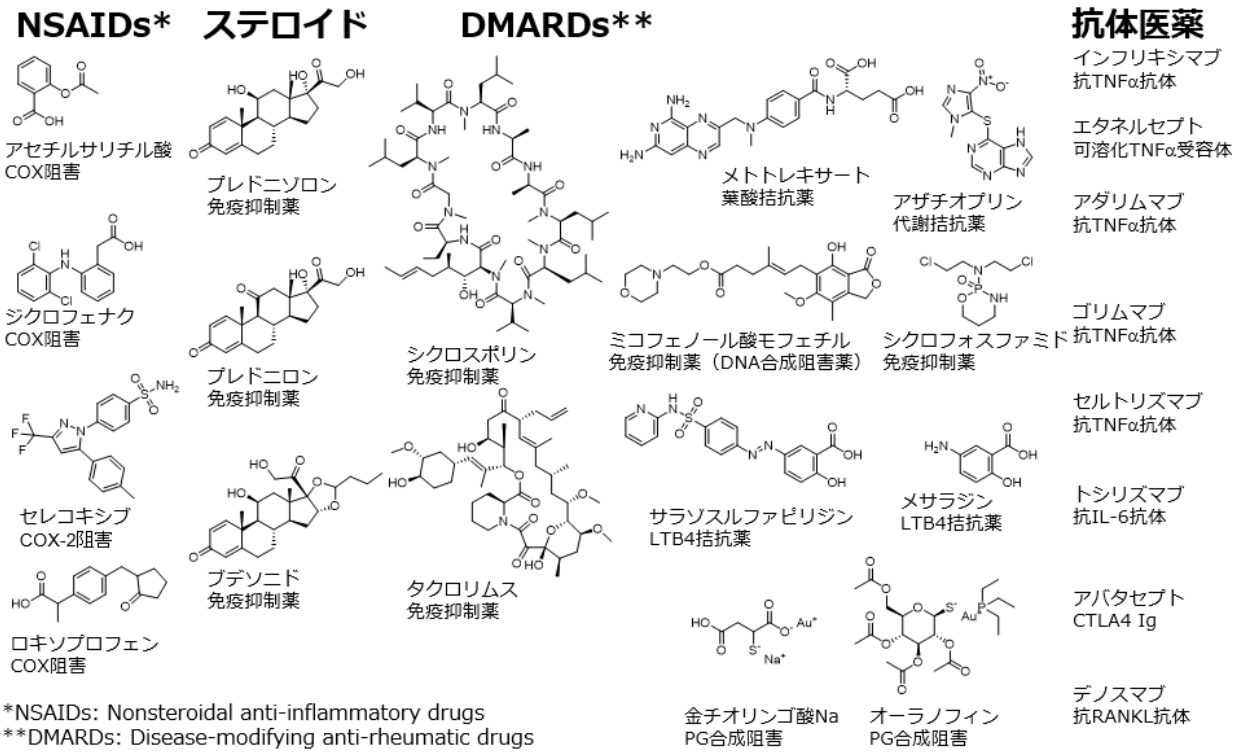


Figure 1. 自己免疫疾患治療で使用されている薬物の概要

第２節　Th17細胞とRORγt

Th17細胞はTh1、Th2に続いて発見されたCD4陽性のヘルパーT細胞のサブセットである[7-8]。Th17細胞、およびTh17細胞から産生される炎症性サイトカインのIL-17AやIL-17Fが潰瘍性大腸炎、リウマチ、多発性硬化症、乾癬などの自己免疫疾患の病態に関与している[9-14]。臨床的にも、Th17細胞から産生されるIL-17に対する抗IL-17A抗体セクキヌマブやイキセキズマブ、Th17レセプターに対するブロダルマブがTNFα阻害薬と同等の良好な臨床成績を収めており[15-17]、乾癬においてはTNF阻害薬に次いで選択される抗体医薬としてガイドライン[18]に記載されている。このTh17細胞の分化および活性化において重要な役割を果たしているのが、レチノイン酸関連オーファン受容体γt（Retinoic-acid-related orphan receptor γt、RORγt）である。RORは1993年にクローニングされた核内受容体スーパーファミリーに属する転写因子であり[19-21]、RORγおよびRORγtはそのサブファミリーである[22]。RORγは多くの細胞で発現しており、概日リズムや免疫システムの調節等に関与している。一方、RORγtは主に胸腺に発現しており、主に免疫システムの発達に関わっている。RORγtはCD4陽性のヘルパーT細胞をTh17細胞へと分化させること、Th17細胞を活性化し、IL-17A、IL-17F等のTh17由来サイトカイン産生のマスターレギュレーターとして機能すること、さらにCCR6受容体およびIL-23受容体等の発現にも関与するなど、Th17細胞の分化と活性化の双方に関与することが示されている。これらのことから、RORγtの阻害はIL-17A等の炎症

性サイトカインの産生を抑制し、新規の自己免疫疾患治療法となる可能性がある[23]（Figure 2）。RORγtノックアウトマウスはTh17細胞の分化の不全を引き起こし、大腸炎や実験的自己免疫性脳脊髄炎（Experimental autoimmune encephalomyelitis, EAE）等の種々の自己免疫疾患モデルに抵抗性であることが示されている[24-27]。

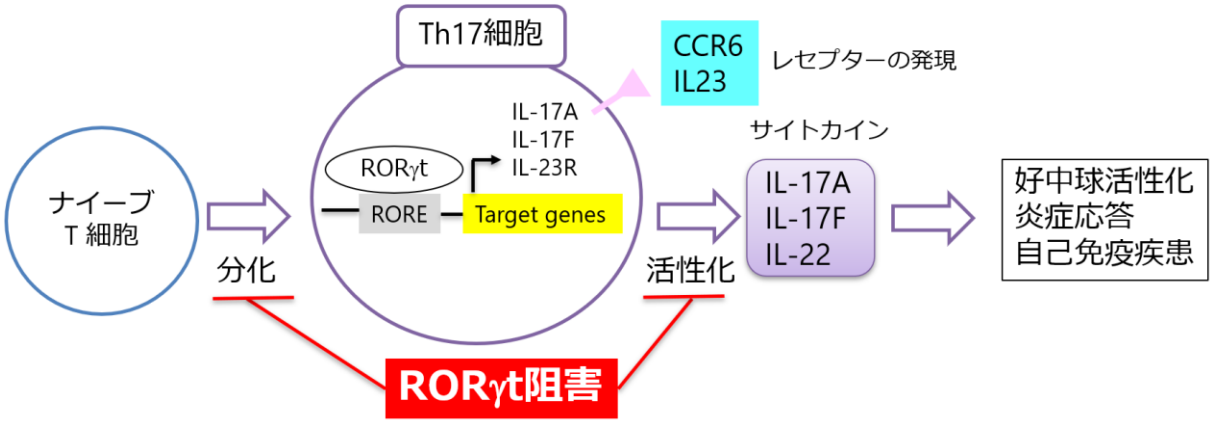


Figure 2. Th17細胞の分化・機能とRORγt阻害薬の作用点

ヒトのRORγは分子量58kDa、518残基のアミノ酸からなるタンパクであり、RORγtはN末端側が21アミノ酸残基短いアイソフォームである[21, 26, 28, 29]（Figure 3）。N末端側からA/B領域、DNA結合部位（DBD）、ヒンジ領域、C末端側のリガンド結合部位（LBD）、Activation function-2領域（AF2）となっており、12のαヘリックス（H1-H12）および、短い2つのヘリックス（H2'およびH11'）から構成される[30]。RORγtはAF2領域を通じてコアクティベータをリクルートし、恒常的に活性化されている。

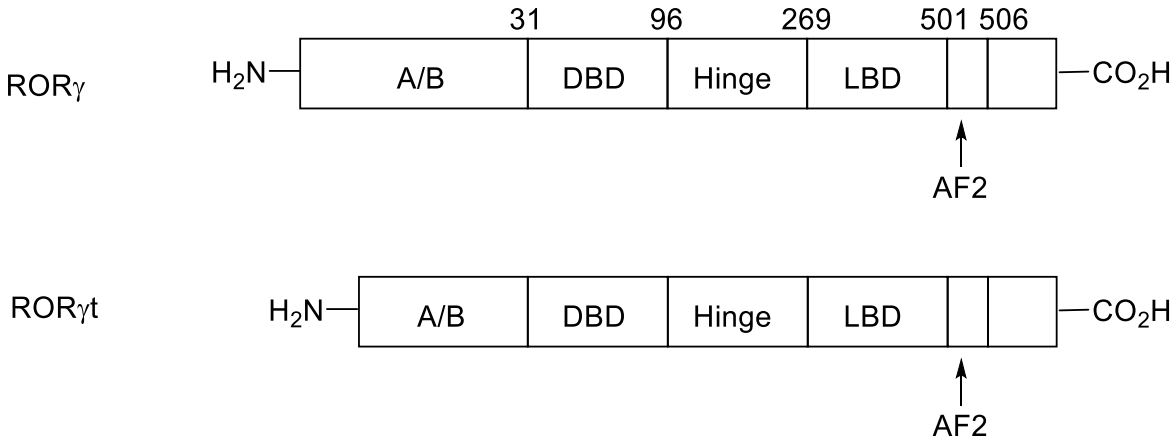


Figure 3. RORγおよびRORγtの構造

一般に受容体は不活性型で存在しており、内因性リガンド等の作動薬により活性化され、機能を発現する（Figure 4）。拮抗薬は、作動薬と受容体の結合に競合し、活性化を抑止する。これに対し、作動薬非存在下においても恒常的に活性化されている受容体も存在し、この作用を抑制するには受容体を不活性型へと変化させる逆作動薬が必要となる[27, 32-33]。RORγtは恒常的に活性化されているため、機能を阻害するには逆作動薬が必要である。

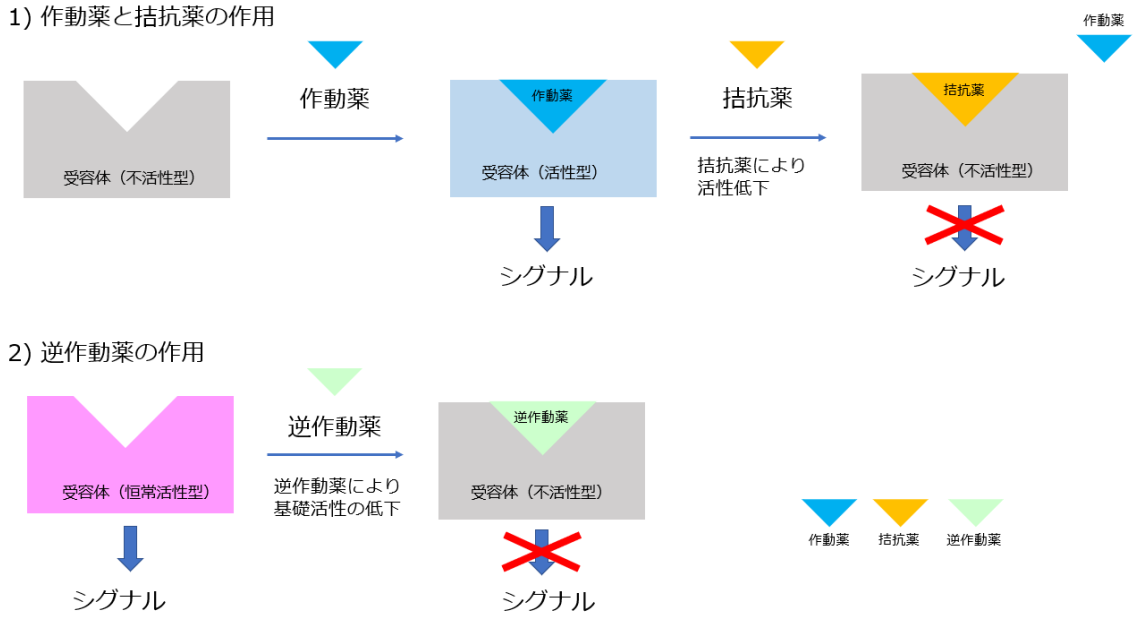


Figure 4. 受容体に対する作動薬、拮抗薬および逆作動薬の作用の模式図

RORγtの内因性リガンドはコレステロールの代謝物やビタミンD誘導体であるとされている[31, 34-35]（Figure 5）。7β, 27-ジヒドロキシコレステロール（7β, 27-OHC）および7α, 27-ジヒドロキシコレステロールがRORγtの作動薬、7αヒドロキシコレステロールが逆作動薬として働く。RORγtの調節は、内因性リガンドに加えて、リン酸化、ユビキチン化、SUMO化等によっても制御されている[34, 36-37]。

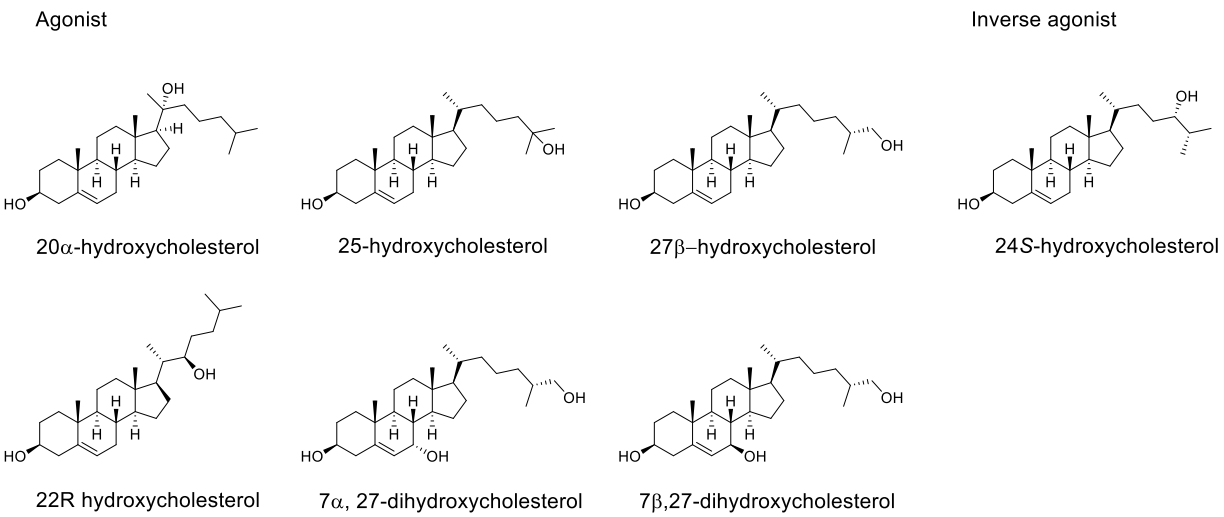


Figure 5. RORγtの内因性リガンドとされるコレステロール代謝物

これまでに多くのRORγタンパク―低分子複合体の結晶構造が公共データベースに登録されている[28]。研究開始当時には25-ヒドロキシコレステロールおよびジゴキシンとの結晶構造が報告されていた[35, 38]（Figure 6）。25-ヒドロキシコレステロールはRORγt作動薬であり、ジゴキシンはRORγt逆作動薬であるが、どちらも同じLBDに結合する。2つの結晶を比較した結果、アゴニスト活性発現には、ヘリックス11上のHis479とヘリックス12上のTyr502との水素結合、およびPhe506によるHis479およびTyr502の相互作用安定化（アゴニストロック）によるヘリックス12のコンフォメーション固定が重要であるとされる[39, 40]（Figure 6B）。逆作動薬のジゴキシンとRORγとの結晶では、His479とTyr502との水素結合形成が阻害され、ヘリックス12に存在するAF2領域とコアクティベータとの相互作用ができないように構造が変化することが逆作動薬の活性発現のメカニズムとされており[38, 40]、その模式図をFigure 7に示す。低分子のRORγt

逆作動薬とHis479との相互作用は多くの結晶構造で確認されており、逆作動活性発現の機構も議論されている[39, 40]。

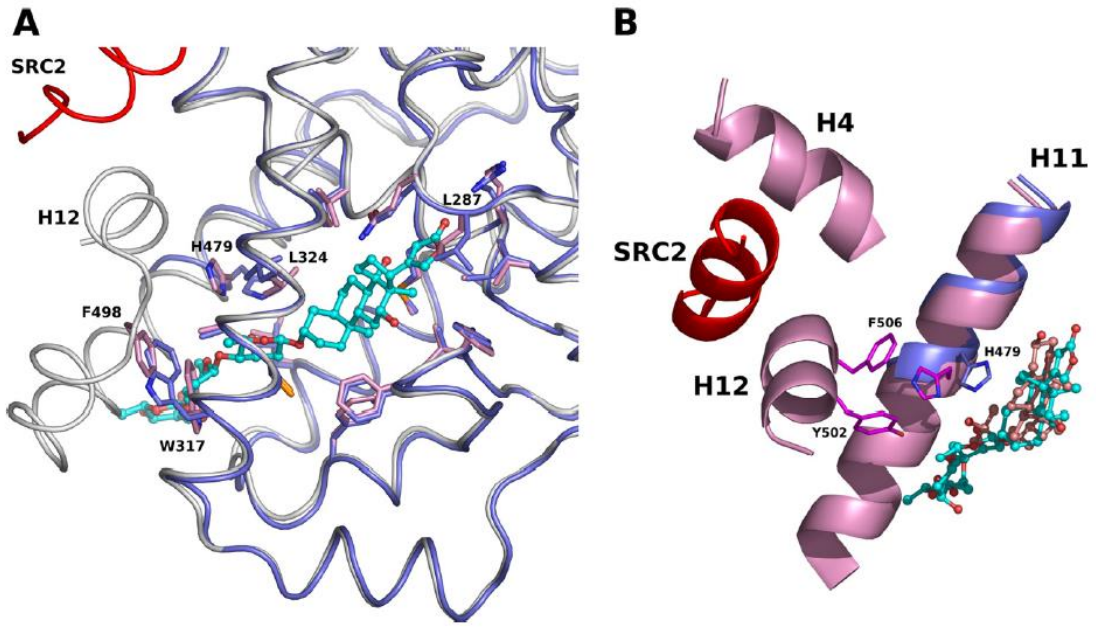


Figure 6. RORγ-ジゴキシン（逆作動薬）およびRORγ-ヒドロキシコレステロール（作動薬）共結晶構造のLBD周辺での重ね合わせ; A：ジゴキシン（Cyan）、ジゴキシンのLBD（dark blue）およびヒドロキシコレステロールのLBD（white）; B：ジゴキシン（Cyan）がHis479（dark blue）と相互作用することにより、ヒドロキシコレステロールで観測されているHis479、Tyr502およびPhe506の相互作用（magenta）形成を阻害している[38]（Fujita-Satoら、*J. Biol. Chem.* 2011, Fig. 4より引用）.

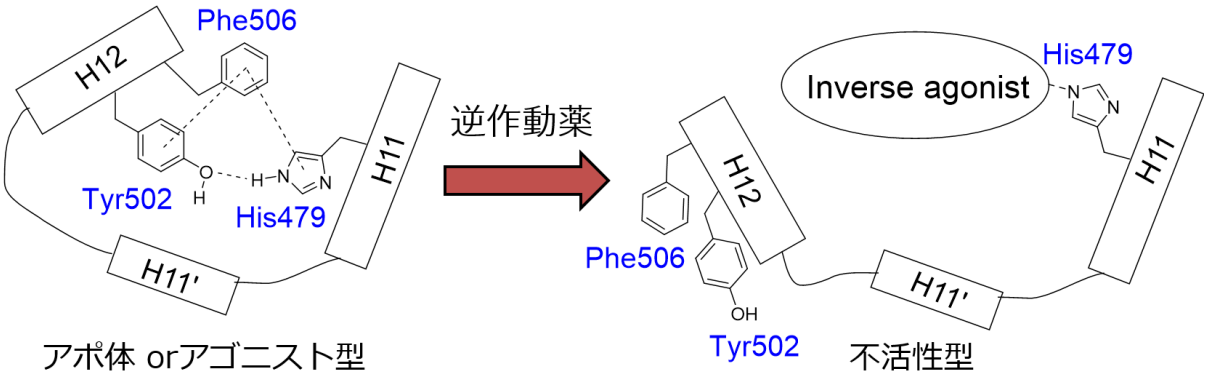


Figure 7. RORγt逆作動薬の機能発現のメカニズムを示した模式図

第３節　本研究の目的

研究開始当時、低分子のRORγt逆作動薬としてジゴキシンやSR1001が報告され、マウスおよびヒトのTh17細胞分化を抑制し、実験的自己免疫性脳脊髄炎（EAE）マウスモデルで薬効を示すことが明らかにされていた[29, 38, 41-42]（Figure 8）。しかしながら、ジゴキシンには悪心・嘔吐、下痢等の消化器症状や徐脈、不整脈、房室ブロック等の症状が発現するジギタリス中毒が副作用として知られており、併用禁忌の薬剤も多いため、長期にわたる投与が想定される自己免疫疾患の治療薬としては懸念がある。また、ジゴキシンは阻害活性が1.98 μM、SR1001は結合活性が111 nMと活性が比較的弱く、治療薬として開発するには課題があると考えられた。著者は新規のRORγt逆作動薬が炎症性自己免疫疾患に対する治療薬の新たな選択肢の一つになるものと考え、経口投与可能な新規RORγt逆作動薬の合成研究に着手した。なお、現在に至るまで多くのRORγt逆作動薬が報告されており[13, 29-30, 37, 41, 43-58]、Figure 8にその一部を示す。

以下、RORγt逆作動活性を有する化合物創製至る経緯と、その生物活性について述べる。

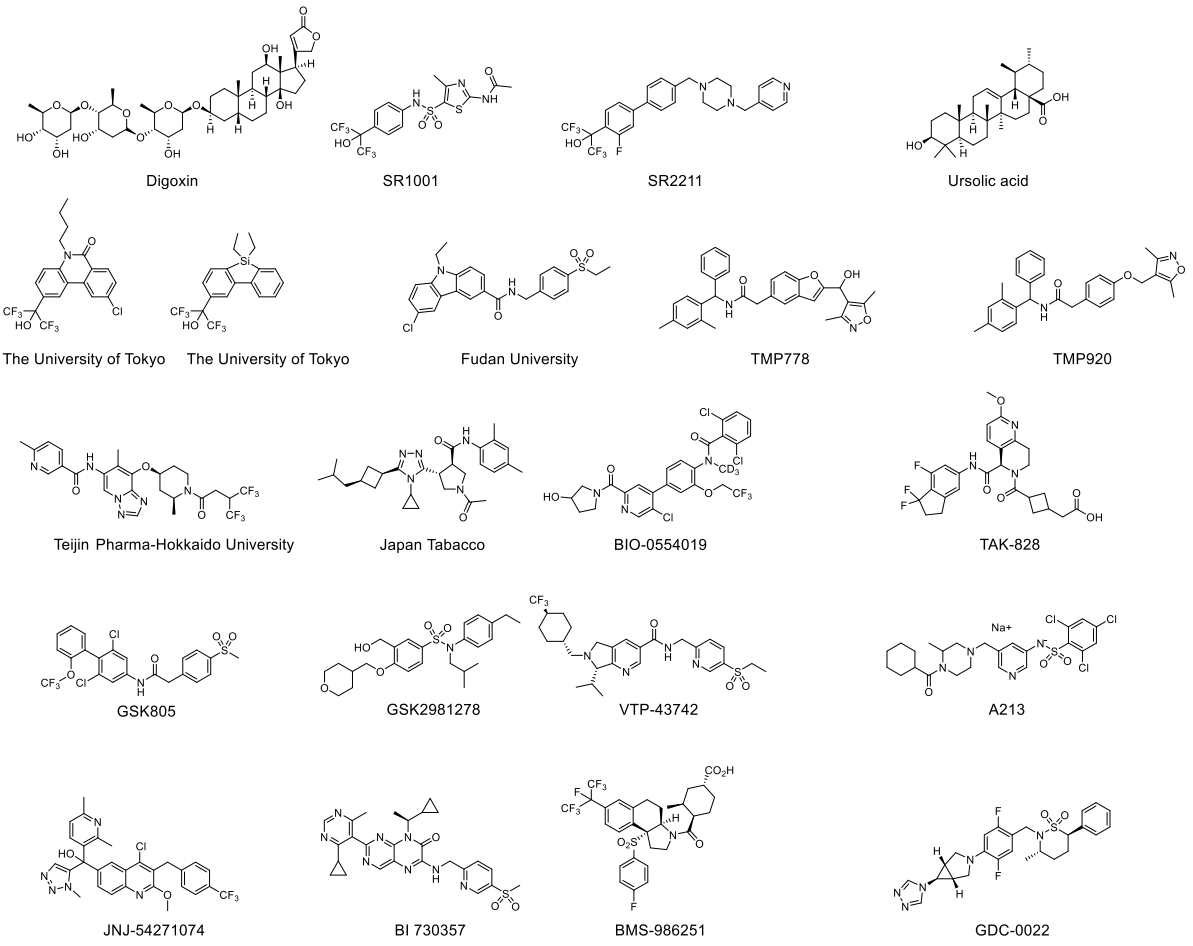


Figure 8. RORγt逆作動薬

第 1 章　直鎖型RORγt逆作動薬のデザイン、合成とその生物活性

第 1 節　研究方針および化合物のデザイン

新規RORγt逆作動薬の開発にあたり、化合物ライブラリを用いたハイスループットスクリーニングを実施し、カルバゾール環を有する化合物**1**がRORγt逆作動活性を有することを見出した（Figure 9）。本化合物は*in vitro*での結合活性が7.5 nMと強く、レポーター活性も92 nMと良好であった。また、既存のRORγt逆作動薬であるジゴキシンやSR1001とは異なる特徴的な骨格を有し、分子量が438と薬の出発点として適したサイズであったため、ヒット化合物**1**を出発点として研究を開始することとした。本化合物はラットの経口吸収性が低く、十分な血漿中濃度を確保できないことが課題として挙げられた。原因として、高い脂溶性（clogP:5.8）に起因する低い溶解度が要因の一つとして考えられた。そこで、物性改善によって経口吸収性を高める合成戦略をとることとした。物性改善にあたり、リピンスキー則[59]、特にclogP<5を指標に変換することを考慮した。

合成展開する新規RORγt逆作動薬は、化合物**1**を出発として以下のようにデザインした。まず、化合物を左端カルバゾール環 (a)、リンカー部 (b)、シアノフェニル基 (c) と3つの部分に分割（Scaffold A）、各部分の構造活性相関（SAR）を取得し、そこから

得られる情報を用いながら構造を変換して良好な物性を有する化合物へと最適化する戦略を立案した。最初に変換が比較的容易であるリンカー部および右端フェニル基について最適な構造を決定した後、高い脂溶性に寄与していると考えられる三環性のカルバゾール環の変換を行うこととした。化合物**1**に対して、ジゴキシンとRORγtタンパクとの結晶構造[20]（PDB:3B0W）を元にドッキングを行った。結果、Figure 10に示すように、シアノフェニル基がジゴキシンのラクトン環上カルボニルに対応するポケット奥のArg367やCys285との相互作用を形成、カルバゾール環がTrp317やLeu324等が形成する脂溶性ポケットを占有し、さらに逆作動活性発現に重要とされるHis479とCH-π相互作用をしていることが推定された。そこで、最適なリンカー長、シアノ基の最適な位置および隣接基導入による活性の向上、カルバゾール環の変換では周囲の脂溶性アミノ酸残基との相互作用を保つ変換による物性の改善を行うこととした。

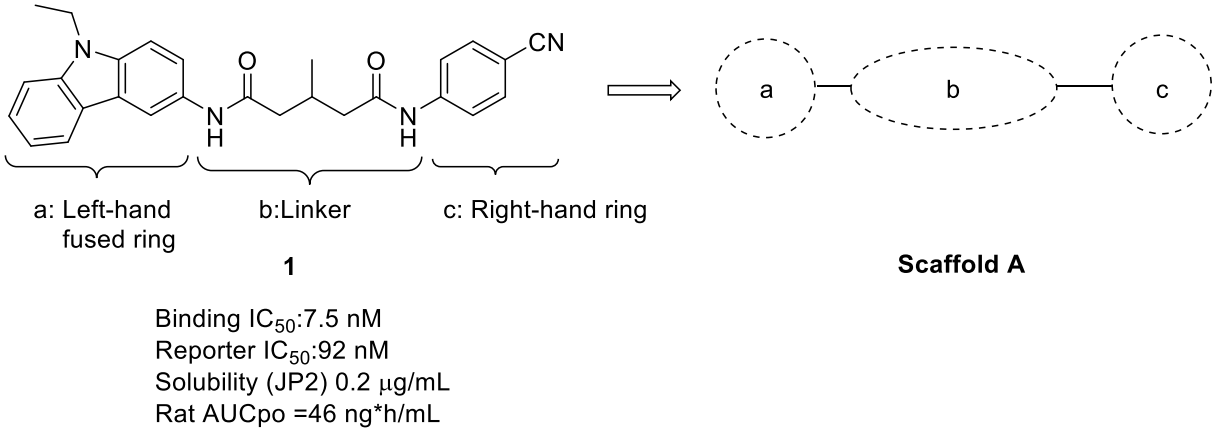


Figure 9. Hit化合物**1**およびそのプロファイル

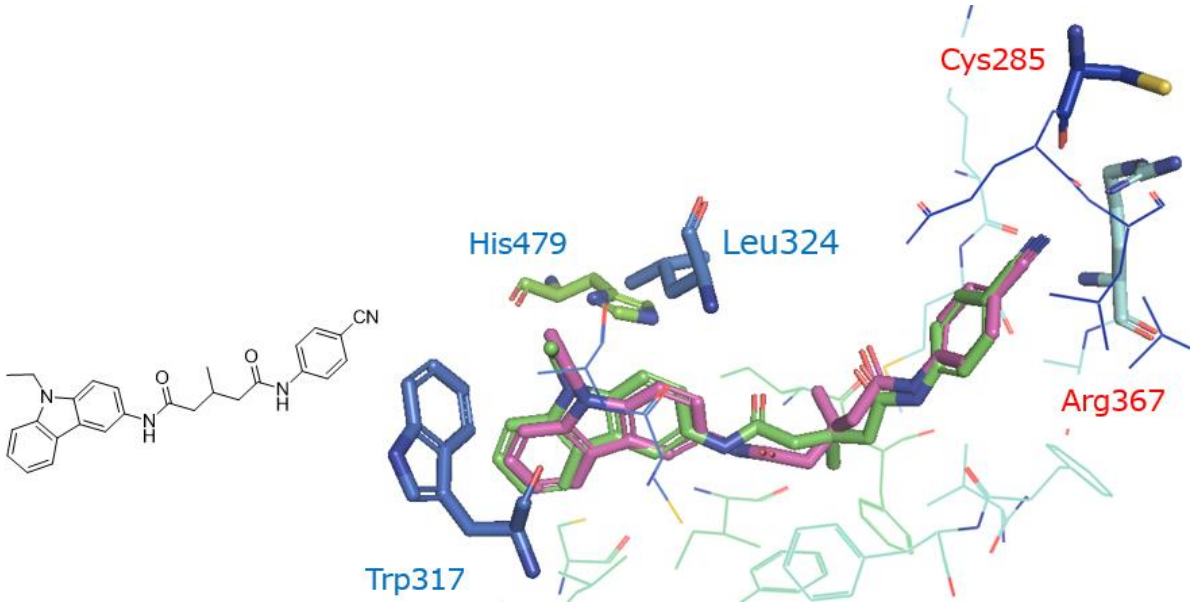


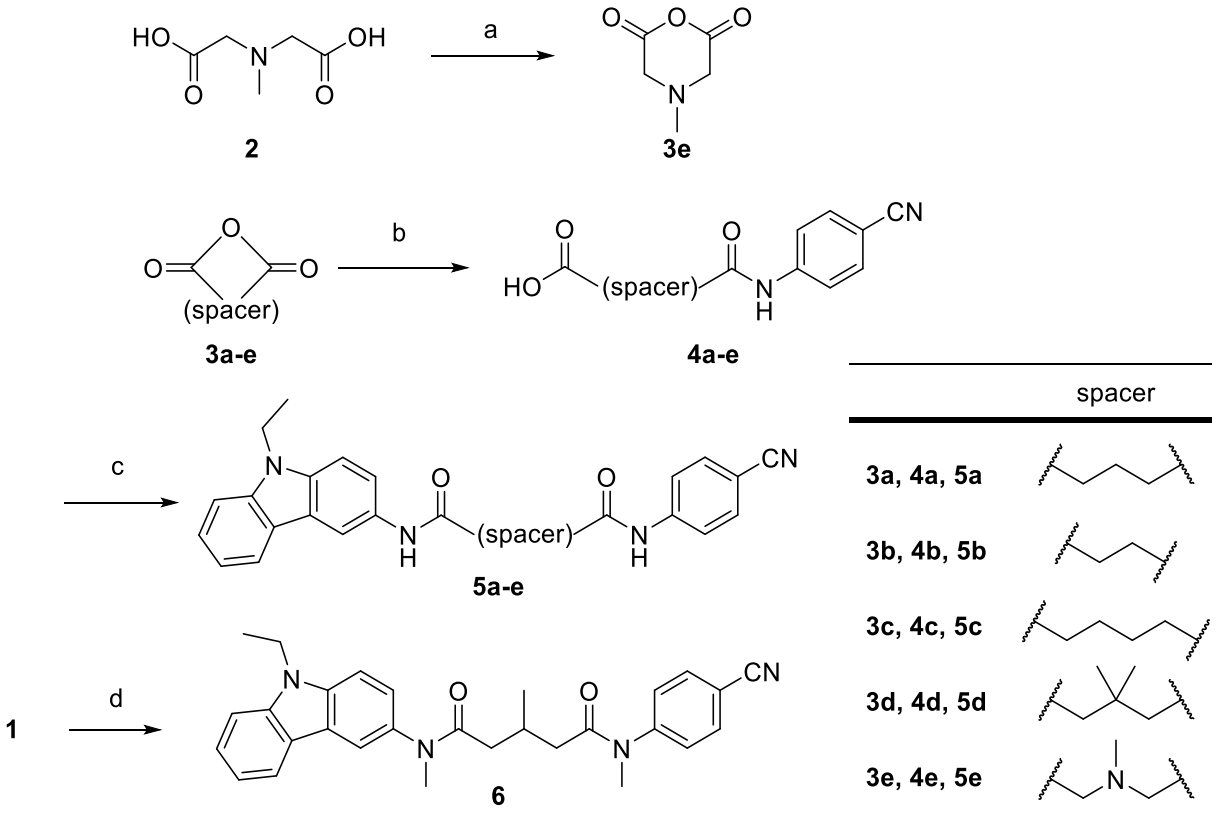
Figure 10.　ジゴキシンとRORγtとの共結晶（3B0W）を用いた化合物**1**の予測結合様式（マゼンタ: S体、グリーン: R体）

第２節　直鎖型化合物の合成

カルバゾール誘導体の合成

Scheme 1にリンカー部を変換したカルバゾール誘導体**5a–e**および**6**の合成について示す。ジカルボン酸**2**を無水酢酸により処理し、酸無水物**3e**を合成した。市販の酸無水物**3a–d**、あるいは先ほど調製した**3e**と4-シアノアニリンとを反応させカルボン酸**4a–e**を合成した。これらに対し、9-エチル-9H-カルバゾール-3-アミンを縮合することにより対応するカルバゾール誘導体を合成した。ビスメチルアミド体**6**は化合物**1**の2箇所のアミド基の窒素原子をメチル化することにより合成した。

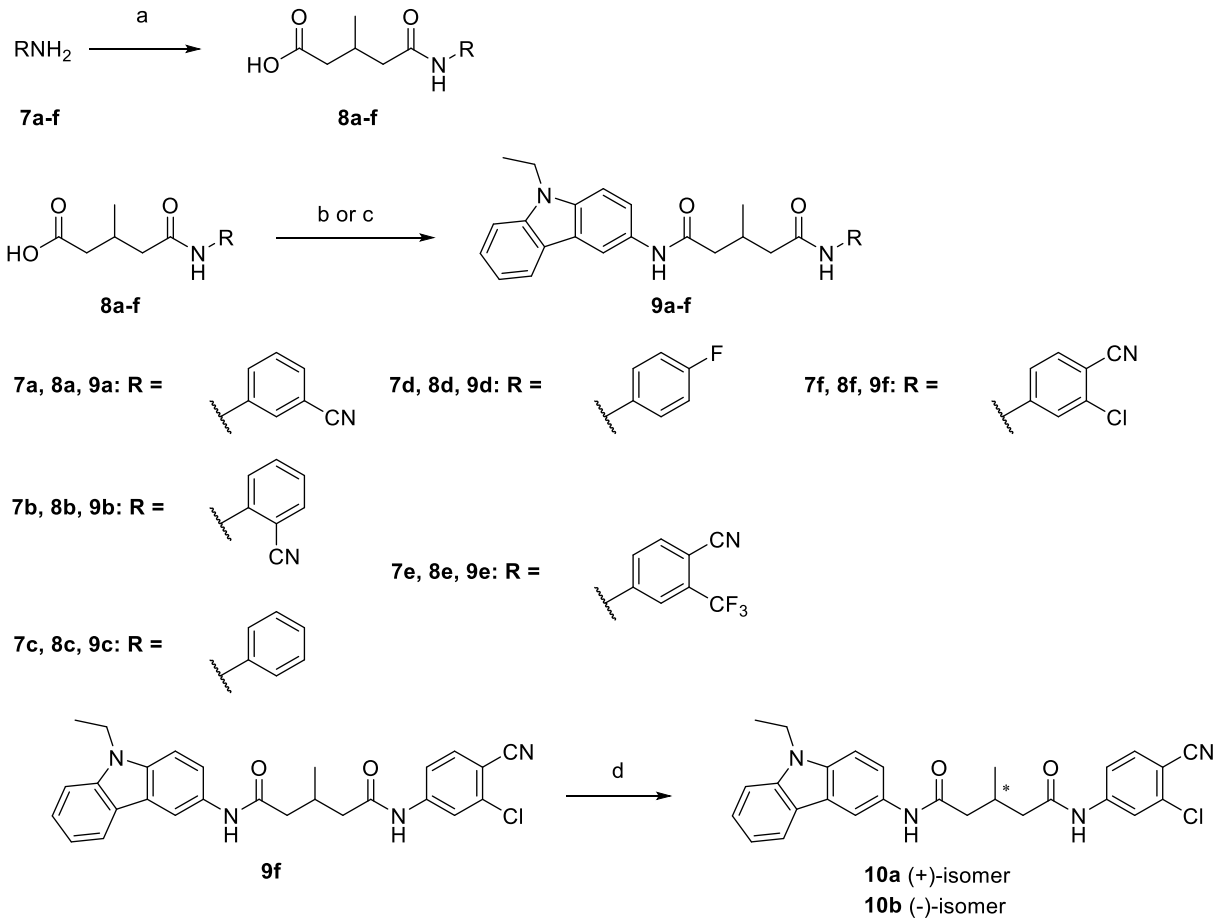
Scheme 1. Synthesis of compounds **5a–e** and **6**.



Reagents and conditions: (a) Ac_2O, reflux, quant.; (b) 4-aminobenzonitrile, THF, 90 °C, 52%-quant.; (c) 9-ethyl-9H-carbazol-3-amine, HATU, DIEA, DMF, rt, 14-71%; (d) MeI, NaH, DMF, 0 °C to rt, 10%.

右端を変換したカルバゾール誘導体は、Scheme 2に示す経路で合成した。アニリン**7a–g**と3-メチルグルタル酸無水物とを加熱することによりカルボン酸**8a–f**とし、これと9-エチル-9H-カルバゾール-3-アミンとのアミド化により**9a–f**を合成した。**9f**をキラルカラムクロマトグラフィーで精製することにより、光学活性体**10a**と**10b**を得た。

Scheme 2. Synthesis of carbazole derivatives **9a**–**f**, and chiral resolution of **9f**.



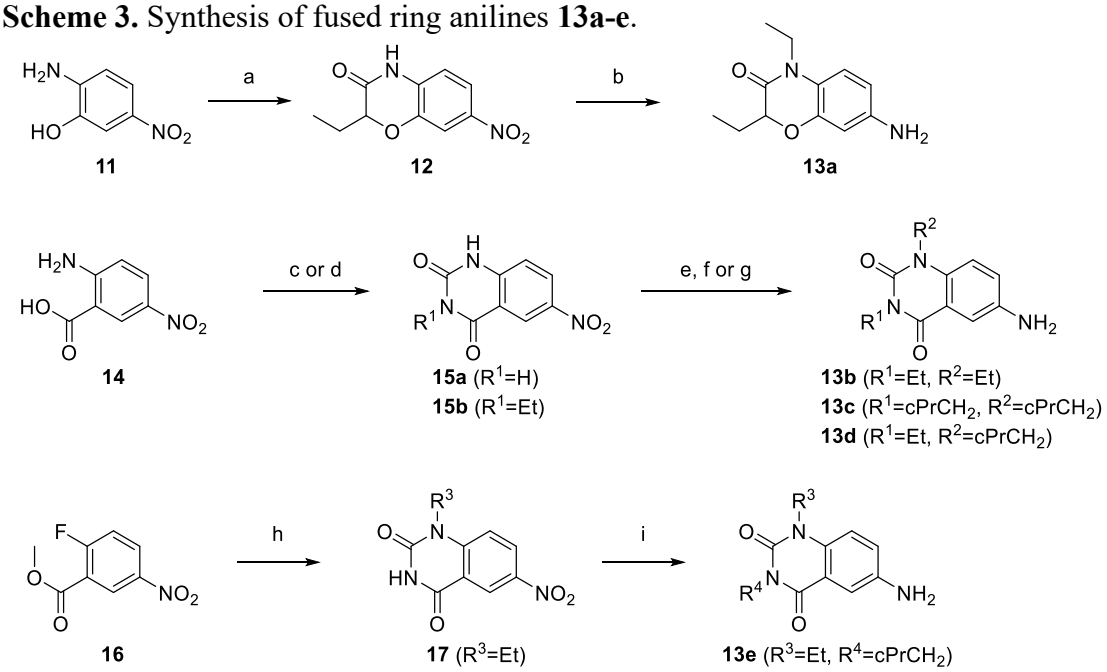
Reagents and conditions: (a) 4-methyldihydro-2H-pyran-2,6(3H)-dione, THF, reflux, 76%-quant.; (b) 9-ethyl-9H-carbazol-3-amine, WSC, Et_3N, HOBt, DMF, rt, 27-65% for **9a-d**; (c) 9-ethyl-9H-carbazol-3-amine, HATU, DIEA, DMF, rt, 39-45% for **9e** and **9f**; (d) chiral column separation by HPLC.

二環性化合物の合成

アニリン**13a-e**の合成をScheme 3に示す。2-アミノ5-ニトロフェノール（**11**）を2-ブロモ酪酸メチルと処理することで環化し、ベンズオキサジン**12**を得た。**12**のアミド基上窒素原子をエチル化した後、ニトロ基を還元することによりアニリン**13a**を合成し

た。キナゾリジンジオン誘導体**15a**は2−アミノ−5−ニトロ安息香酸**14**を尿素と反応させて閉環して合成した。化合物**15a**に対し、ジエチル化、続くニトロ基の還元を行うことにより**13b**を合成した。化合物**13c**も同様にして合成した。シクロプロピルメチル基を導入した**13d**は、安息香酸**14**をアミド化した後、尿素で環化することにより**15b**を得て、これをエチル化、還元することにより合成した。フルオロ安息香酸メチル誘導体**16**に対し、炭酸カリウム存在下エチルアミンをS_NAr反応することによりエチルアミノ基を導入した後、尿素と反応させ閉環体**17**とした。続いてシクロプロピルメチル化およびニトロ基の還元を行うことにより、**13e**を合成した。

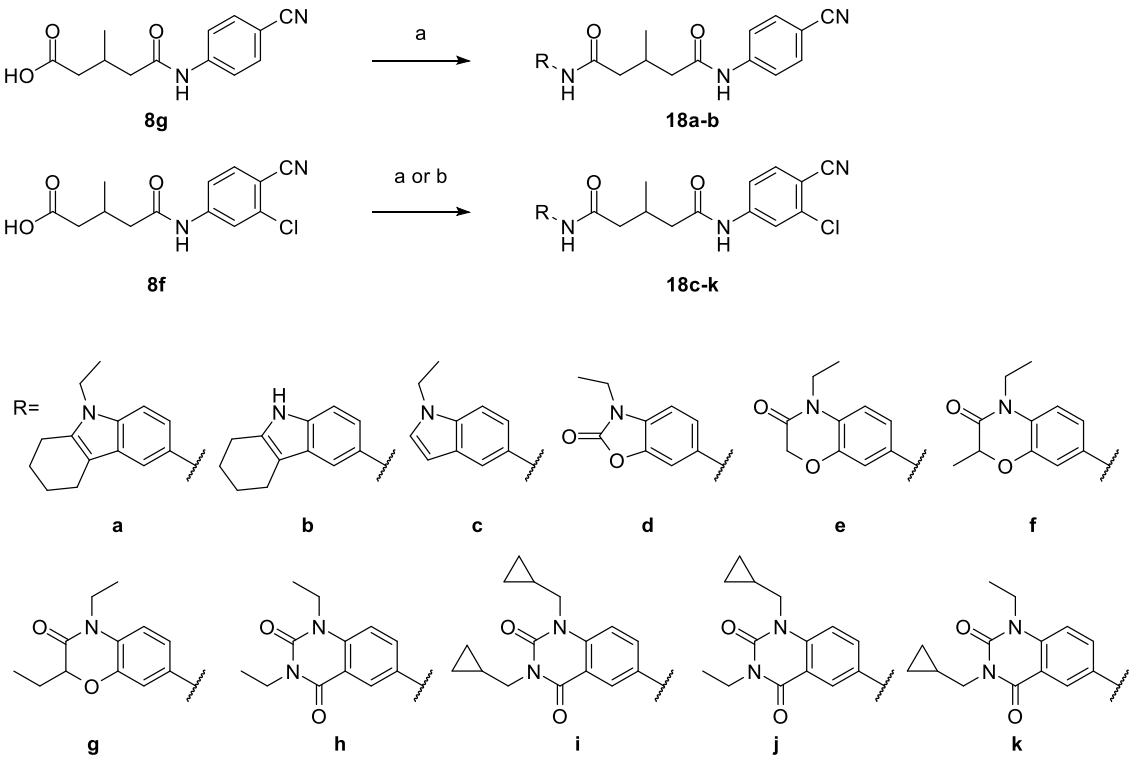
Scheme 3. Synthesis of fused ring anilines **13a-e**.



Reagents and conditions: (a) methyl 2-bromobutylate, KF, DMF, 60 °C, 83%; (b) (1) EtI, K_2CO_3, DMF, rt, 88%. (2) Pd-C, H_2, MeOH, rt, 76%; (c) Urea, 160 °C, 53% for **15a**; (d) (1) $EtNH_2$, HATU, DIEA, DMF, rt, 93%. (2) 20% phosgene in toluene, THF, Et_3N, 0 °C to reflux, 74% for **15b**; (e) (1) EtI, K_2CO_3, DMF, 70 °C, 70%. (2) Pd-C, H_2, MeOH-EtOAc, rt, 99% for **15a** to **13b**; (f) (1) (bromomethyl)cyclopropane, K_2CO_3, DMF, rt, 79%. (2) Pd-C, H_2, EtOH, rt, 77% for **15a** to **13c**; (g) (1) (bromomethyl)cyclopropane, K_2CO_3, DMF, 80 °C, 49%. (2) Pd-C, H_2, EtOAc, rt, 97% for **15b** to **13d**; (h) (1) $EtNH_2$, K_2CO_3, THF, 60 °C, 89%. (2) Urea, 160 °C, 57%; (i) (1) (bromomethyl)cyclopropane, NaH, DMF, rt, 81%. (2) Pd-C, H_2, EtOAc, rt, 78%.

左端側を変換した化合物**18a–k**の合成をScheme 4に示す。種々のアニリンをカルボン酸**8g**または**8f**と縮合することにより、**18a–k**を合成した。

Scheme 4. Synthesis of fused ring derivatives **18a**–**k**.



Reagent and conditions: (a) RNH_2, HATU, DIEA, DMF, rt, 9-69% for **18a-f**; (b) RNH_2, T3P, DIEA, EtOAc, reflux, 45-77% for **18g-k**.

第３節　カルバゾール誘導体の構造活性相関

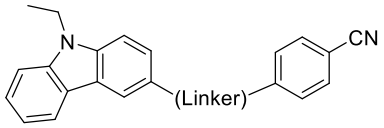
合成した化合物は次に示す*in vitro*および*in vivo*生物試験によりその生物活性を評価した。化合物のRORγtに対する親和性は蛍光標識したリガンドのRORγt結合阻害試験（TR-FRET）により測定した。化合物による*in vitro* RORγt逆作動活性は、ヒトJurkat細胞を用いたレポータージーンアッセイによりIL-17のmRNAの発現量を測定することにより評価した。化合物のラットにおける薬物動態は、選択した化合物を1 mg/kg

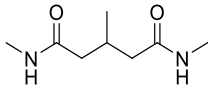
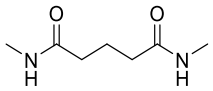
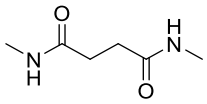
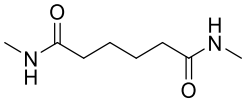
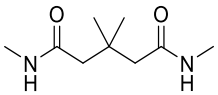
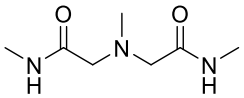
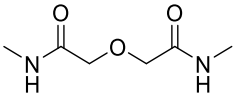
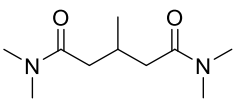
でのラットカセットドーズを用いて検討した。*in vitro* PD試験はPMAおよびIonomycin刺激によるラット全血中のIL-17A濃度を測定することにより実施した。*in vivo* PD試験は実験的自己免疫性脊髄炎（EAE）ラットにおける5日目の膝窩リンパ節から得られたIL-17AのmRNA量測定を行い、化合物の効果を検証した。

まず、*in vitro*におけるRORγtに対する親和性およびレポーター遺伝子試験において強力な逆作動活性を示す化合物を指向して検討を行った。はじめに、リンカー部位の構造活性相関（SAR）を検証した結果をTable 1に示す。無置換のアルキル鎖を用いて検討した結果、リンカーの長さについては、7原子長の化合物**5a**に対し、6原子長の化合物**5b**あるいは8原子長の化合物**5c**では結合活性が減弱し、7原子がリンカー長として好ましいことが示唆された。そこでリンカー長を7原子として更なる最適化検討を行った。リンカー中央部の変換では、中央部にメチル基を有する化合物**1**に対し、メチル基を持たない化合物**5a**、あるいはジメチル基を有する化合物**5d**では、結合試験では活性を保持するものの、レポーター試験で活性が減弱した。また、リンカーに窒素原子を導入した化合物**5e**や酸素原子を導入した化合物**5f**では、結合活性は維持するものの、レポーター活性が減弱した。アミド結合部位の変換については、リンカー部分のアミドをビスメチル化した化合物**6**では、RORγtへの結合活性が大きく減弱した。これらの構造活性相関から、リンカーはヒット化合物**1**が有していた3-メチルペンタン-1,5-ジアミド構造が最適であることを確認した。ここで、結合試験とレポーター活性の構

造活性相関で乖離がみられたが、これは化合物がRORγtタンパクに結合しても、生物学的機能を発現するための必須な条件を満たしていないためと考察される。

Table 1. リンカー部の構造活性相関

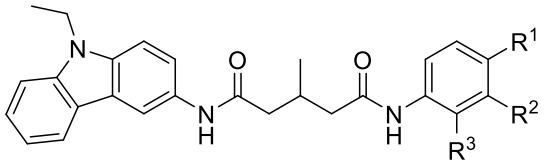


化合物	Linker	Binding IC_{50} (nM)	Reporter IC_{50} (nM)
1[a]		7.5	92
5a		4.8	200
5b		410	>30000
5c		140	>30000
5d		290	7800
5e		57	1600
5f		81	4200
6[a]		6700	15000

[a]Racemic.

次に、化合物**1**のリンカーを用いた右端フェニル基の検討結果をTable 2に示す。まずシアノフェニル部位の重要性を検証した。シアノ基の位置を変換した化合物**9a**、**9b**では結合活性が減弱した。シアノ基を有さない化合物**9c**や、シアノ基の代わりにフッ素原子を導入した化合物**9d**ではレポーター活性が減弱した。これらのことから、4位シアノ基が生物活性に大きく影響することが判明した。続いて、シアノ基に隣接する置換基の影響を調査した。核内受容体ファミリーであるアンドロゲン受容体阻害薬の研究において、Arg752と相互作用しているニトロ基あるいはシアノ基のオルト位に、トリフルオロメチル基やクロロ基等脂溶性の高い置換基を導入することで、活性が増強されることが報告されている[60-61]。これはタンパクとの相互作用を獲得するアンカーとして働く置換基のオルト位に脂溶性基を配置することで、付近に存在する疎水性アミノ酸残基との相互作用を獲得したためと推察された。この知見を利用し、RORγtのArg367と相互作用を取っていると想定されたシアノ基のオルト位にトリフルオロメチル基およびクロロ基の導入を行った。4-シアノ-3-トリフルオロフェニル基を有する化合物**9e**では活性は減弱したが、クロロ基を導入した化合物**9f**では結合活性およびレポーター活性の増強が見られた。これらの結果より、3-クロロ-4-シアノフェニル基が右端の最適構造として選出された。化合物**9f**は中央部メチル基に不斉中心を有しているため、光学分割を行った。興味深いことに、それぞれの光学活性体**10a**および**10b**は同等の活性を有していた。そこで、今後の検討をラセミ体で行うこととした。

Table 2. 右端フェニル基上置換基の構造活性相関[a].



化合物	R^1	R^2	R^3	Binding IC_{50} (nM)	Reporter IC_{50} (nM)
1	CN	H	H	7.5	92
9a	H	CN	H	190	6300
9b	H	H	CN	>10000	>30000
9c	H	H	H	6300	>30000
9d	F	H	H	840	>30000
9e	CN	CF_3	H	15	190
9f	CN	Cl	H	2.1	11
10a[b]	CN	Cl	H	1.3	13
10b[c]	CN	Cl	H	1.6	12

[a]All the compounds are racemic except for **10a** and **10b**. [b](+)-isomer. [c](-)-isomer

第４節　カルバゾール環変換の検討

化合物**9f**は、強力なRORγt親和性およびレポーター活性を示したが、本化合物はラットにおける経口投与時の血中曝露が、*in vivo* PD試験で検証するには十分ではないものと考えられた（ラットカセットドーズ：1mg/kg経口投与時、AUC＝8 ng•h/ml）。ま

た、化合物**9f**は非常に低い溶解度を示し（0.15 μg/mL）、この低溶解度が曝露の低い要因の1つとして考えられる。この低溶解度はカルバゾール環に由来する高い脂溶性によるものと考えた（clogP: 6.44）。脂溶性の高い化合物は、ADME-Tox（吸収、分布、代謝、排泄、毒性）プロファイルに好ましくないことが多数の研究から明らかになっている[62]。そこで、カルバゾール環を変換し、分子の脂溶性を低減させながら活性発現に必須な相互作用を保つ分子設計を行い、活性および物性を両立した化合物を目指すこととした（Figure 11）。まず、末端ベンゼン環を飽和環へ変換した化合物**18a**、除去した化合物**18c**では結合活性が保持された（Table 3）。一方、**18a**の窒素上エチル基を除去した化合物**18b**では活性が大きく減弱した。これらの結果より、脂溶性相互作用部位として窒素上置換基Xの必要性が示唆された。末端Y部については、芳香環でなくても（化合物**18a**, **18c**）活性を示したことから、末端芳香環をアルキル基へ変換できる可能性も考えられた。

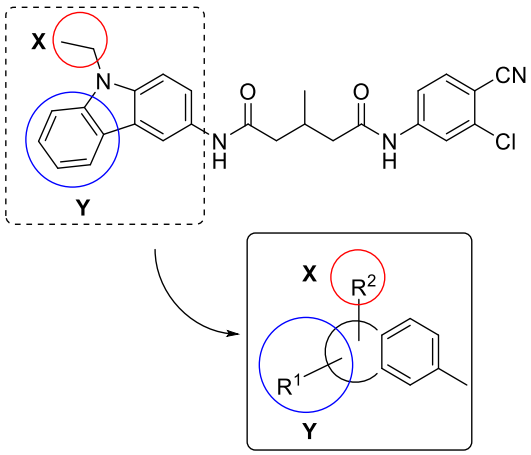
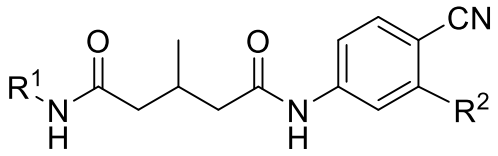
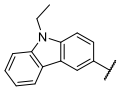
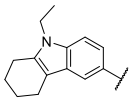
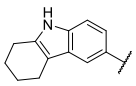
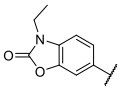
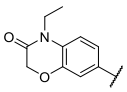
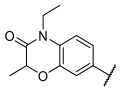
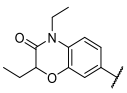
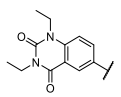


Figure 11. カルバゾール環からの構造変換

そこで、末端ベンゼン環をアルキル基へと変換した二環性化合物の合成を考えた。カルバゾール環を二環性の環へと変換とすることによって脂溶性低減が期待された。X部およびY部の部分構造を有する化合物をデザイン・合成したところ、ベンズオキサゾロン化合物**18d**や、ベンズオキサジノン化合物**18e**で結合活性が認められた。これらはカルバゾール環を有する**9f**と比較してclogPが低下し、溶解度の改善傾向が認められた。ベンズオキサジノン環のカルボニルα位に置換基を付与するとレポーター活性が向上することも明らかになり（化合物**18f**、**18g**）、Y部の脂溶性の重要性が示唆された。さらに、2,4-ジオキソ-1,2,3,4-テトラヒドロキナゾリン環を導入した**18h**は強力な結合活性並びにレポーター活性を有することを見いだした。**18h**は溶解度が大きく改善していた（JP2 : 42 μg/mL）。

Table 3. 三環性構造の変換[a]

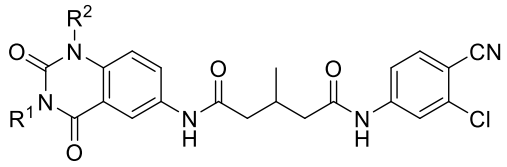


化合物	R^1	R^2	Binding IC_{50} (nM)	Reporter IC_{50} (nM)	clogP	Solubility (μg/mL)[b]
9f		Cl	2.1	11	6.44	0.15
18a		H	5.9	57	5.96	0.06
18b		H	5500	7700	4.75	0.19
18c		Cl	170	4000	5.06	0.1
18d		Cl	260	5200	3.34	0.9
18e		Cl	110	1400	3.45	12
18f		Cl	13	200	3.96	23
18g		Cl	7.5	100	4.50	5.6
18h		Cl	3.6	47	4.52	42

[a]Racemic. [b]Kinetic solubility in the 2nd fluid of disintegration test of the Japanese Pharmacopoeia (pH 6.8).

18hについての更なる最適化を考え、2,4-ジオキソ-1,2,3,4-テトラヒドロキナゾリン環上の置換基変換を検討した（Table 4）。まず、R^2をEt基に固定してR^1部分を変換した。R^1部分にシクロプロピルメチル基を導入した**18k**では活性が向上した。活性向上が認められた**18k**について、R^2を変換した。その結果、シクロプロピルメチル基を導入した**18i**が強い活性を示した。加えて、**18i**はPKプロファイルの改善が認められた（AUC＝106 ng•h/ml、BA:7%）。これらの結果から、化合物**18i**を用いて*in vitro*および*in vivo* PD試験を行うこととした。

Table 4. キナゾリジンジオン環上の置換基最適化および薬物動態 [a]



化合物	R^1	R^2	Binding IC_{50} (nM)	Reporter IC_{50} (nM)	Rat PK[b] AUC, *p.o.* (ng•h/mL)	CL (mL/h/kg)	BA (%)
18h	Et	Et	3.6	47	39	1609	6.2
18k		Et	2.5	5.0	10	2639	2.7
18j	Et		3.9	32	33	1702	5.5
18i			4.9	7.2	106.2	672	7.1

[a]Racemic. [b]The values are shown as means of three determinations (0.1 mg/kg, *i.v.*, and 1.0 mg/kg, *p.o.*).

第５節　化合物**18i**の*in vitro*および*in vivo* PD試験による評価

in vitro PD試験（Whole blood assay）

9週目のLewisラット腹部大静脈より採血し、血液を培地（RPMI1640 + 10% FBS + 1% PS）にて2倍希釈した。そこに**18i**を添加し、1時間後に培地にて希釈したPMA10 ng/ ml+ Ionomycin 1 μg/ mlで刺激した。24時間後に培養上清を回収し、ELISA にてIL-17A濃度を測定した。その結果、**18i**はIL-17Aの産生を用量依存的に抑制し、そのIC_{35}は300 nMであった。

in vivo PD試験

化合物**18i**投与によるIL-17A mRNAの抑制を調べ、薬効を評価した。すなわち、7週齢のメスのLewis ratsに対し、ミエリン塩基性タンパク（Myelin basic protein）および結核死菌（Mycobacterium tuberculosis）を含むアジュバント（Freund’s adjuvant）を用いて実験的自己免疫性脊髄炎（Experimental autoimmune encephalomyelitis: EAE）に感作させ、このラットのリンパ節を用いた*in vivo* PDアッセイにおいて**18i**（30、100、300 mg/kg, *p.o.*, b.i.d.）を評価した（Figure 12）。その結果、感作5日後のリンパ節でのIL-17A mRNA発現量は用量依存的に有意に減少し、100 mg/kgで薬効を示した。一方、IFN-γ

の発現には影響しなかった。以上より、**18i**が*in vivo*でRORγtを阻害することによってTh17細胞の活性化・分化を抑制する効果があることが示された。

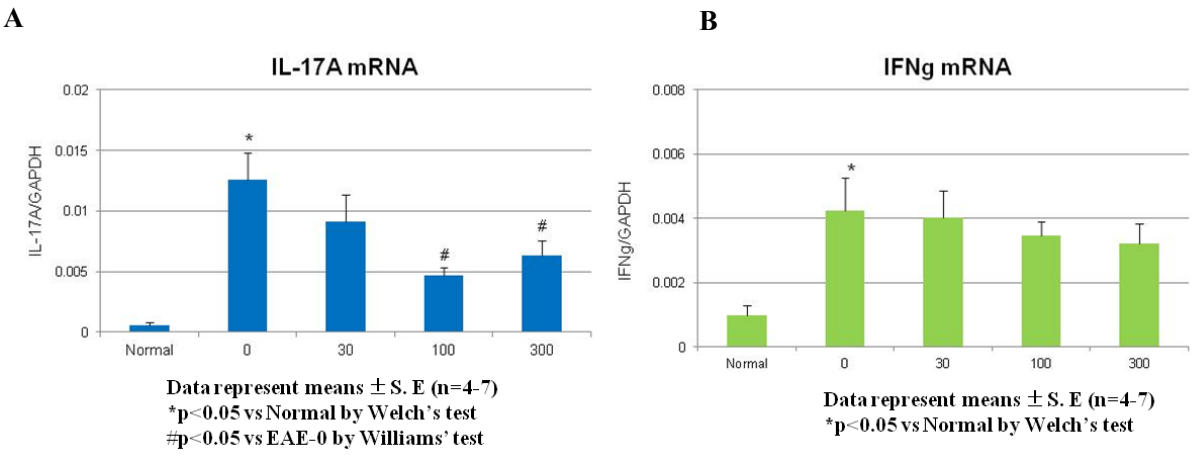


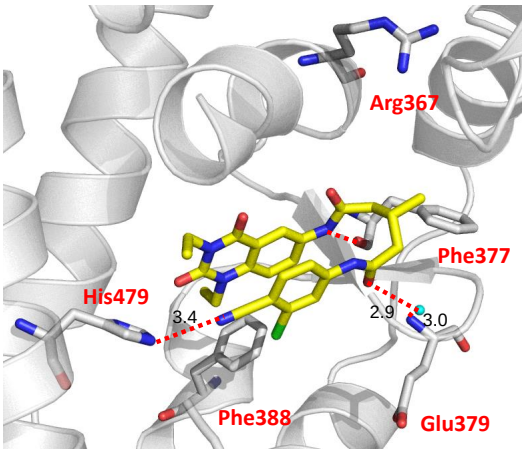
Figure 12. 18i のラット EAE モデルでの評価; (A) Dose dependent suppression of IL-17A mRNA; (B) Effect of **18i** on IFNγ level.

第６節　化合物**18h**のX線構造解析による考察

X線構造解析により、化合物**18h**とRORγtタンパクとの相互作用を解析した。化合物**18h**はコレステロール結合サイトにあり、興味深いことにジゴキシンとは異なるU字構造の結合様式を取っていた（Figure 13A）。ジゴキシンの結晶がArg364やArg367およびHis479を活用していたことから（Figure 13B）、 化合物**18h**の結合モードが直線状と予想していたが、**18h**はRORγt逆作動活性発現の鍵となる相互作用と考えられるHis479のみと相互作用を取っており、ジゴキシンのラクトン環上のカルボニル基が活用しているArg367との相互作用は見られなかった。本化合物がU字構造を取る原動力として、

末端フェニル基同士のπ-π相互作用およびリンカー部分での畳み込みによるPhe377およびGlu379との水を介した相互作用が考えられる。この結合モードにより、これまで取得したSARが説明可能になった。すなわち、His479と相互作用しているシアノ基の除去、あるいはフッ素原子への変換で逆作動活性が減弱する。シアノ基のオルト位へのクロロ基の導入による活性増強効果（化合物**9f**）は、クロロ基とCys320のチオール基との距離が2.3Åであり、さらにPhe378, Phe388等の脂溶性側鎖と近接することで相互作用を獲得したことによるものと考えられる。リンカー中央にメチル基を導入すると活性が向上するが、光学異性体（化合物**10a**および**10b**）間で活性に差異が見られないことから、このメチル基は脂溶性相互作用獲得の効果よりも、Thorpe-Ingold効果[63]によりU字構造を取りやすくすることで活性向上に寄与していると考えられる。リンカー中央部にジメチル基を導入した化合物**5d**で活性の低下がみられた。ジメチル体**5d**の方がモノメチル体**1**よりもU字構造を取りやすいと考えられるものの、末端の２つの芳香環同士が適切な二面角で配置できていない可能性が想定される。リンカー部アミドの窒素原子上へのメチル化（化合物**6**）では、Phe377との水を介した相互作用形成が阻害されるため、活性が減弱したと考えられる。さらに、キナゾリジンジオン上の置換基がPhe388等の脂溶性ポケットに囲まれていることから、シクロプロピルメチル基の導入による活性向上が説明される。U字構造のファーマコフォアの解明により、キナゾリジンジオン誘導体**18h**の新たな展開が可能になった。

A



B

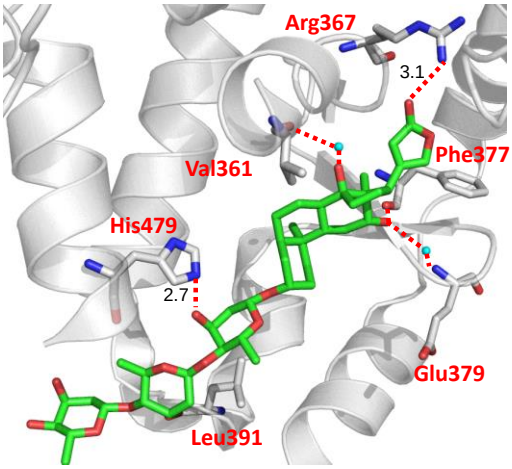


Figure 13. A: 逆作動薬**18h**とC末端を切り取ったRORγtタンパクとの結晶構造 （PDB code : 6B31）; B: ジゴキシンとRORγtタンパクとの結晶構造[37] （PDB code : 3B0W）.

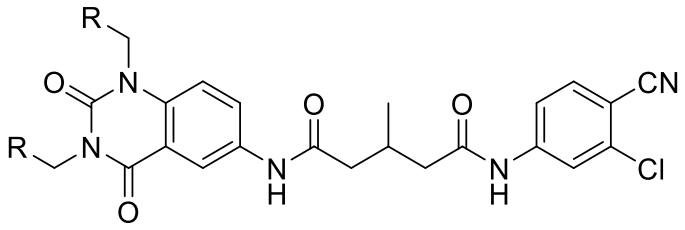
第７節　小括

新規RORγt逆作動薬の開発を目的として、ライブラリ化合物から見出したカルバゾール誘導体**1**を元にリード創出研究を行った。化合物**1**を左端カルバゾール環、リンカー部、シアノフェニル基と3つの部分に分割、各部分の構造活性相関を取得し、得られた情報を総合して最適な化合物へと導く戦略を立案した。まず、リンカー部では3-メチルペンタン-1,5-ジアミド構造が最適であること、右端のシアノフェニル基部位では、3-クロロ-4-シアノベンゾニトリル構造を有する化合物**9f**が強いレポーター活性を示すことを発見した。化合物**9f**は溶解度に課題があったため、カルバゾール環の脂溶性を低減させながら活性発現に必須な相互作用を保つ分子設計を行い、溶解度が改善された二環性の2,4-ジオキソ-1,2,3,4-テトラヒドロキナゾリン環へと変換した**18h**へと導いた。化合物**18h**のキナゾリジンジオン環上置換基を最適化し、血中曝露改善と共に強い活性を示す**18i**の発見に至った。化合物**18i**はラットEAEモデルにおいて100 mg/kgでリンパ節IL-17A mRNAの上昇を抑制した。また、RORγtタンパクと**18h**のX線共結晶構造解析の結果、ジゴキシン等既存の逆作動薬とは異なるU字型活性コンフォメーションを取るという知見を得た。

第２章　環化型RORγt逆作動薬のデザイン、合成とその生物活性

第１節　研究方針、並びに化合物のデザイン

化合物 **18i** はラット EAE モデルで 100 mg/kg の経口投与で作用を示したものの、投与量低減のため、活性増強および薬物動態改善が望まれる（Figure 14）。さらに、高次の評価をマウス自己免疫疾患モデルで行うにあたり、マウスカセットドーズ（1 mg/kg、*p.o.*）で薬物動態を評価したところ、経口吸収性に課題があることが判明した。そこで、**18h** や **18i** のリンカー部に対してヘテロ原子の導入を行い、化合物の脂溶性低下等による薬物動態改善を試みたものの、活性が保持されなかった。この結果から、直鎖型化合物のリンカーではヘテロ原子導入による脂溶性低減が困難であり、リンカー部分の変換が必須であると考えた。



18h (R=Me)
Binding IC_{50}:3.6 nM
Reporter IC_{50}:47 nM
Rat cassette (1 mg/kg): AUC: 39 ng*h/ml
Mouse cassette (1 mg/kg): AUC: 9 ng*h/ml

18i (R=cPr)
Binding IC_{50}:4.9 nM
Reporter IC_{50}:7.2 nM
Rat cassette (1 mg/kg): AUC: 106 ng*h/ml
Mouse cassette (1 mg/kg): AUC: 27 ng*h/ml

Figure 14. リード化合物 **18h**、**18i** の構造とそのプロファイル

一般的に薬物動態を改善するアプローチとして、Table 5 のような戦略が活用されている[64-66]。脂溶性の低減については既に実施しており、さらにリンカー部分の環化による回転可能結合を削減し、分子のフレキシビリティを下げる分子設計を行うこととした。化合物のコンフォメーションを固定化することで薬物動態が改善、さらに活性が向上するという報告もあり[67-68]、活性コンフォメーションである U 字型構造を固定化するデザインを行うこととした。リンカー上 2 か所のアミド基窒素原子上のメチル化体（化合物 **6**）で活性が低下したことから、環化の位置としてはリンカー部の炭素原子上 a を想定した（Figure 15）。さらに、代謝部位を見出し代謝に弱い部位をブロックすることによる薬物動態の改善も合わせて行うこととした。

Table 5. 薬物動態を改善する戦略

薬物動態を改善する戦略
•脂溶性の低減　(clogP<5)
•極性表面積を下げる (TPSA <140 Å^2)
•環化等により分子のフレキシビリティを下げる　(Rotatable bond≦10)
•代謝部位および周辺への置換基導入による代謝回避
•代謝に弱い官能基を除去、変換
•電子豊富な環の電子密度を下げる

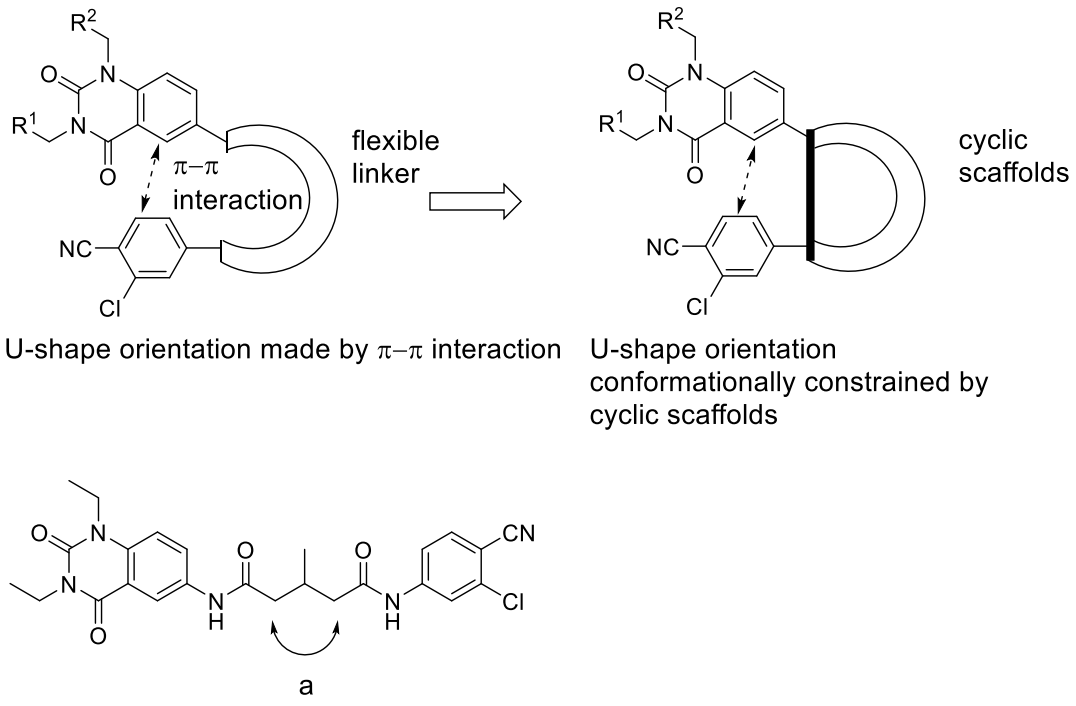


Figure 15. U字構造を取る化合物の模式図および環化の戦略

分子モデリングや文献情報、自社の知見より、シスのシクロペンタン、ジアゼパン、および1,3置換ピペリジン環A-DをU字構造模倣リンカーとしてデザインした[69-71]（Figure 16）。これらの化合物について、**18h**の結晶構造を鋳型としてドッキングモデルを作成し、U字型構造を取り得るか検証した。結果、デザインAのシス-1,2-置換シクロペンタン誘導体はU字構造であり、一方でデザインBのトランスのシクロペンタン体は伸長したコンフォメーションを取ることが推察された（Figure 16Aおよび16B）。U字構造の重要性を確認するため、U字構造が取れないと考えられたトランス体**B**も合わせて合成することとした。ジアゼパン環および1,3置換ピペリジン環についてはU字

構造を取ることが報告されており[70-71]、ドッキングモデル上でもU字構造を取ることが期待された（Figure16Cおよび16D）。

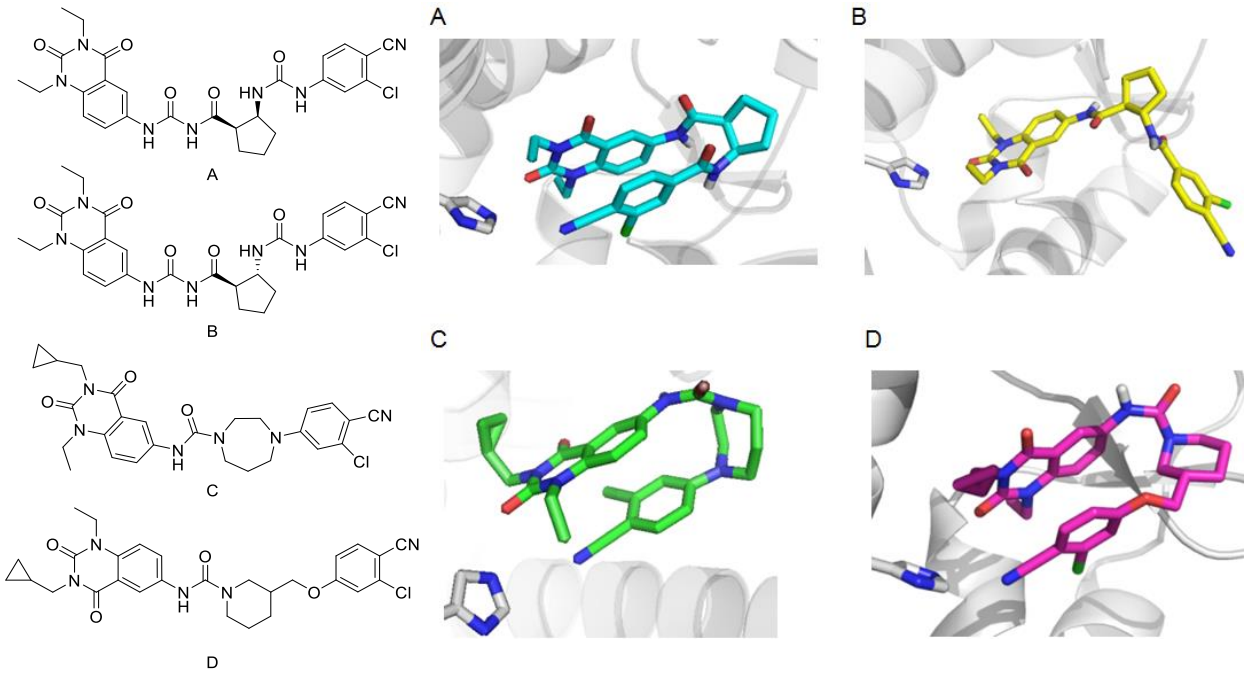


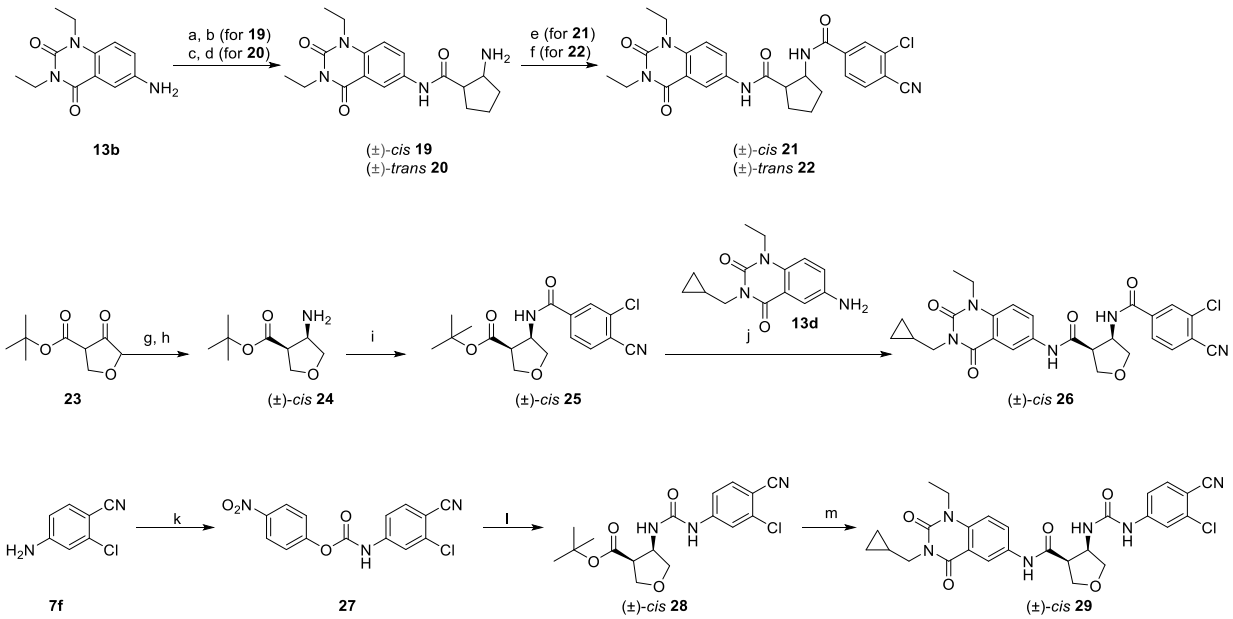
Figure 16. 化合物A-DのRORγtリガンド結合部位とのドッキングモデル

第２節　環状化合物の合成

シクロペンタン誘導体 **21**、**22**、テトラヒドロフラン誘導体 **26**、および **29** の合成を示す。アシル化と Boc 基の除去により化合物 **19** あるいは **20** を合成し、アミンに 3-クロロ 4-安息香酸を縮合させることにより化合物 **21** および **22** を得た。ケトン **23** を還元的にアミンにし、3-クロロ 4-安息香酸を縮合させることにより化合物 **25** とし[72]、カルボン酸の保護基の除去と縮合により化合物 **26** を得た[73]。4-アミノ 3-クロロベン

ゾニトリル（**7f**）から導いた中間体 **27** を **24** と反応させることにより **28** を得た。TFA で保護基を除去しカルボン酸とした後、**13d** と縮合することにより化合物 **29** を得た。

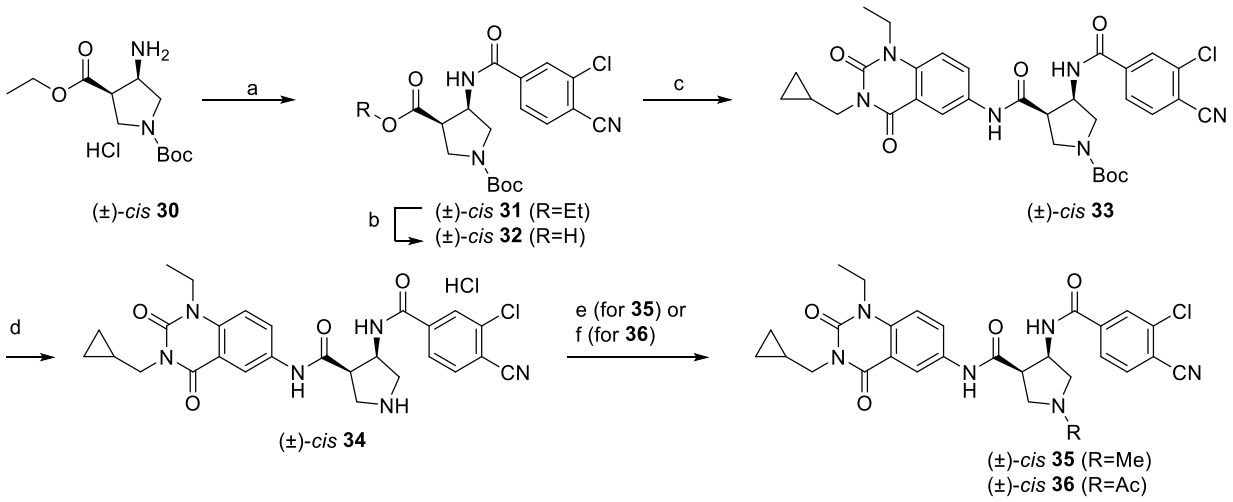
Scheme 5. Synthesis of **21, 22, 26** and **29**.



Reagents and conditions: (a) (±)-*cis* 2-((*tert*-butoxycarbonyl)amino)cyclopentanecarboxylic acid, T3P, DIEA, EtOAc, 60 °C, 70%; (b) TFA, rt, 97%; (c) (±)-*trans* 2-((t*ert*-butoxycarbonyl)amino)cyclopentanecarboxylic acid, T3P, DIEA, EtOAc, 60 °C, 75%; (d) TFA, rt, quant.; (e) 3-chloro-4-cyanobenzoic acid, T3P, DIEA, EtOAc, 60 °C, 48% (from **19** to **21**); (f) 3-chloro-4-cyanobenzoic acid, T3P, DIEA, EtOAc, 70 °C, 11% (from **20** to **22**); (g) $BnNH_2$, AcOH, $NaBH(OAc)_3$, $NaBH_4$, THF-DMF, 65%; (h) $Pd(OH)_2$-C, H_2, MeOH, 42%; (i) 3-chloro-4-cyanobenzoic acid, T3P, DIEA, EtOAc, 60 °C, 69%; (j) (1) TFA, 0 °C to rt; (2) 6-amino-3-(cyclopropylmethyl)-1-ethylquinazoline-2,4(1*H*,3*H*)-dione (**10**), T3P, DIEA, EtOAc, 60 °C, 19%; (k) 4-nitrophenyl chloroformate, pyridine, rt, quant.; (l) **24**, DIEA, DMA, rt, 61%; (m) (1) TFA, rt; (2) **13d** (Scheme 3), T3P, DIEA, EtOAc-THF, 22%.

ピロリジン誘導体 **35** および **36** の合成を Scheme 6 に示す方法で行った。**30** を 3-クロロ 4-シアノ安息香酸と縮合して **31** へと変換、引き続く加水分解によりカルボン酸 **32** とした。**32** のアミド化によりジアミド誘導体 **33** を合成した。**33** の Boc 基を除去した後、*N*-メチル体 **35** やアシル体 **36** へと導いた。

Scheme 6. Synthesis of pyrrolidine compounds **35** and **36**.

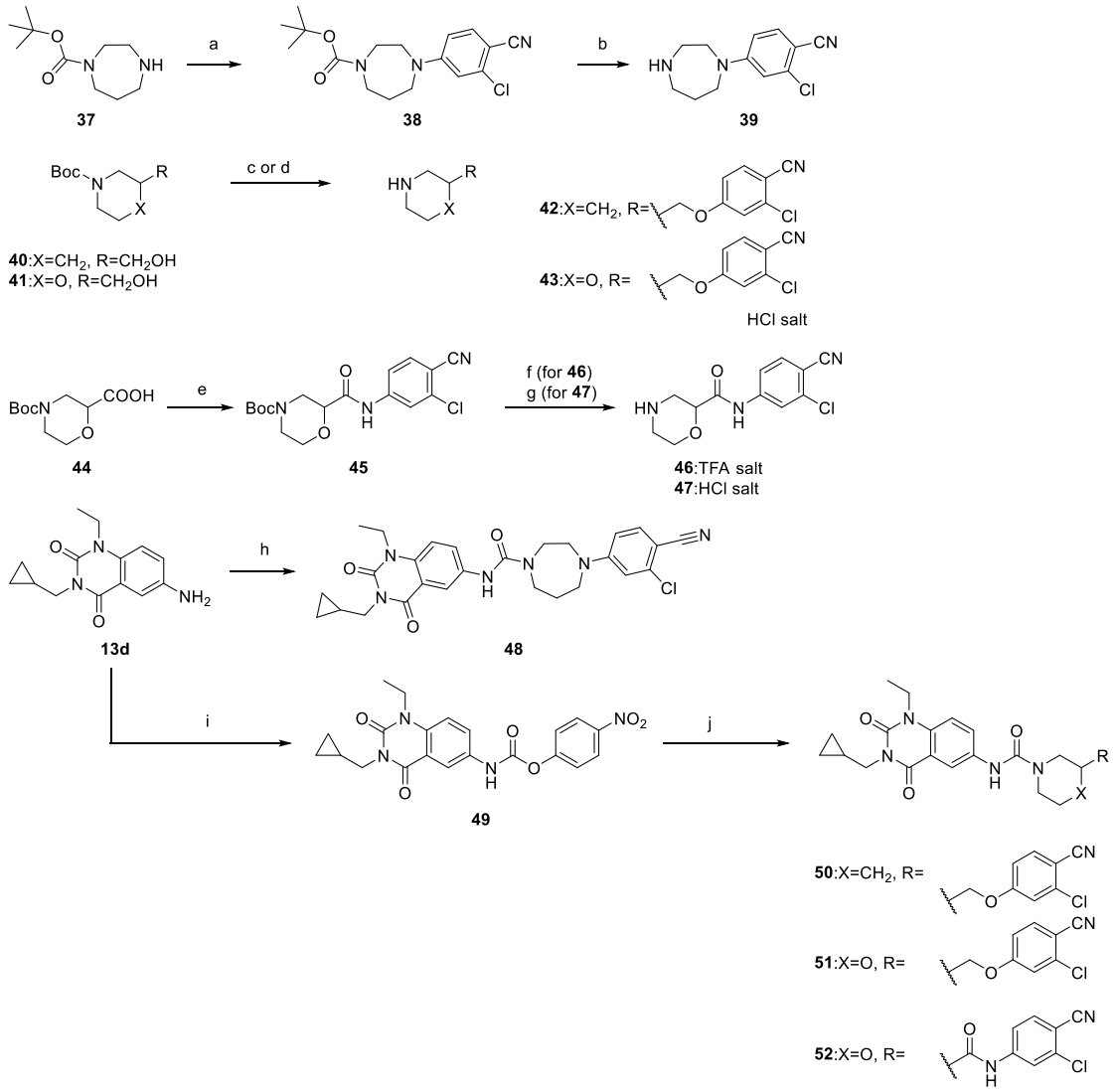


Reagents and conditions: (a) T3P, DIEA, 3-chloro-4-cyanobenzoic acid, EtOAc, rt, 74%; (b) LiOH, THF, H_2O, EtOH, rt, 99%; (c) **13d**, T3P, DIEA, EtOAc, 60 °C, 65%; (d) 4N HCl in CPME, MeOH, rt, 69%; (e) aq. HCHO, $NaBH(OAc)_3$, MeOH, 35%; (f) Ac_2O, pyridine, THF, 68%.

Scheme 7 にはジアゼパン、ピペラジン、モルホリン等環化誘導体 **48** および **50**－**52** の合成法を示す。市販の 1,4-ジアゼパン-1-カルボン酸 *tert*-ブチル **37** を 2-クロロ-4-フ

ルオロベンゾニトリルと反応させることにより **38** を得た。Boc 基の除去によりアミン **39** を合成した。アミン **42** および **43** も同様に合成を行った。*N*-Boc-モルホリン-2-カルボン酸 **44** と 4-アミノ-2-クロロベンゾニトリルを縮合させアミド **45** とした。Boc 基の除去をトリフルオロ酢酸、あるいは塩酸で行うことにより、アミン **46** および **47** を合成した。アニリン **13d** に炭酸ビス（トリクロロメチル）を作用させ、引き続きアミンと反応させることにより **48** を合成した。また、**13d** をクロロギ酸 4-ニトロフェニルと反応させることにより、カルバメート **49** へと変換し、これを各種アミンと反応させることによりウレア **50**、**51**、**52** を合成した。

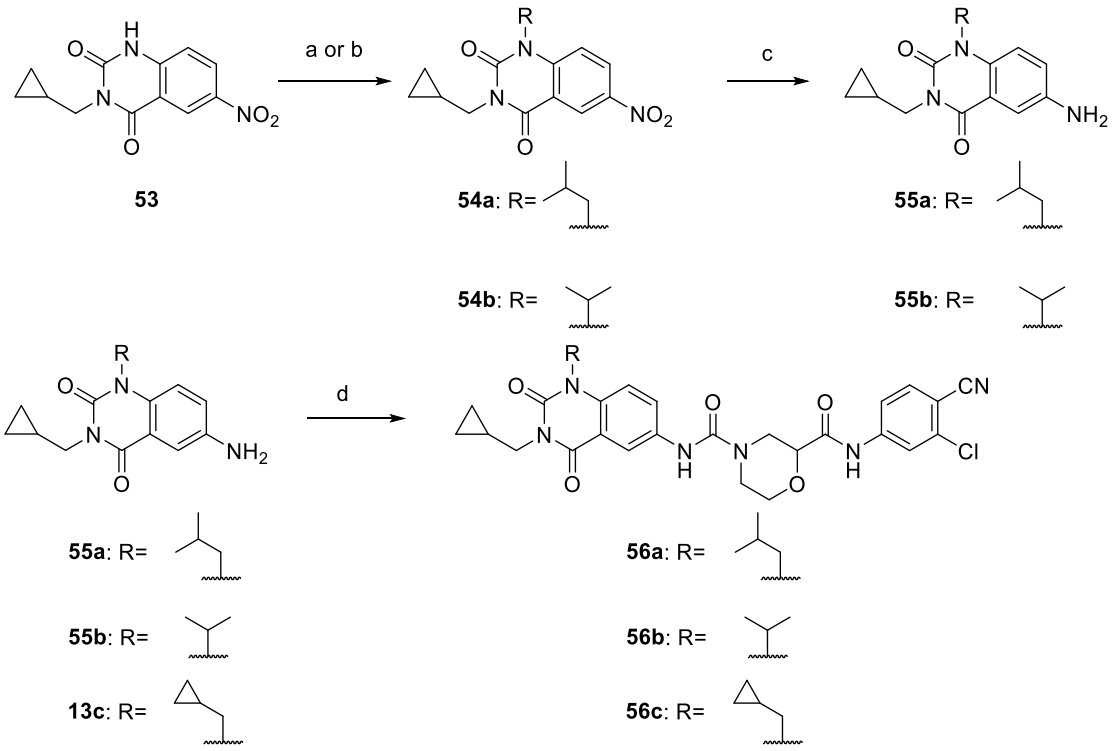
Scheme 7. Synthesis of quinazoline derivatives **48** and **50-52**.



Reagents and conditions: (a) 2-chloro-4-fluorobenzonitrile, K_2CO_3, DMF, 60 °C, quant.; (b) 4N HCl in EtOAc, EtOAc, 77%; (c) (1) 2-chloro-4-fluorobenzonitrile, K_2CO_3, DMSO, 50 °C. (2) TFA, rt, 34% (2 steps); (d) (1) 2-chloro-4-fluorobenzonitrile, K_2CO_3, DMF, 60 °C, 52%. (2) 4N HCl in CPME, MeOH, rt, 21% (2 steps); (e) 4-amino-2-chlorobenzonitrile, T3P, DIEA, EtOAc, 66%; (f) TFA, 68%; (g) 4N HCl in EtOAc, EtOAc, 89%; (h) (1) bis(trichloromethyl)carbonate, DIEA, THF, 0 °C; (2) **39**, DIEA, THF, rt, 11% (2 steps); (i) 4-nitrophenyl carbonochloridate, pyridine, THF, rt, 98%; (j) **42** (for **50**), **43** (for **51**), or **46** (for **52**), DIEA, DMF, 52-80%.

キナゾリジンジオン 1 位の置換基の変換を Scheme 8 に記載した方法で行った。**53** を対応するアルキルハライドと反応させキナゾリジン誘導体 **54a** および **54b** を合成した。ニトロ基を還元することでアニリン **55a** および **55b** を合成した。**55a**、**55b** および **13c** と 炭酸ビス（トリクロロメチル）を反応させた活性中間体と **47** を反応させることにより **56a–c** を得た。

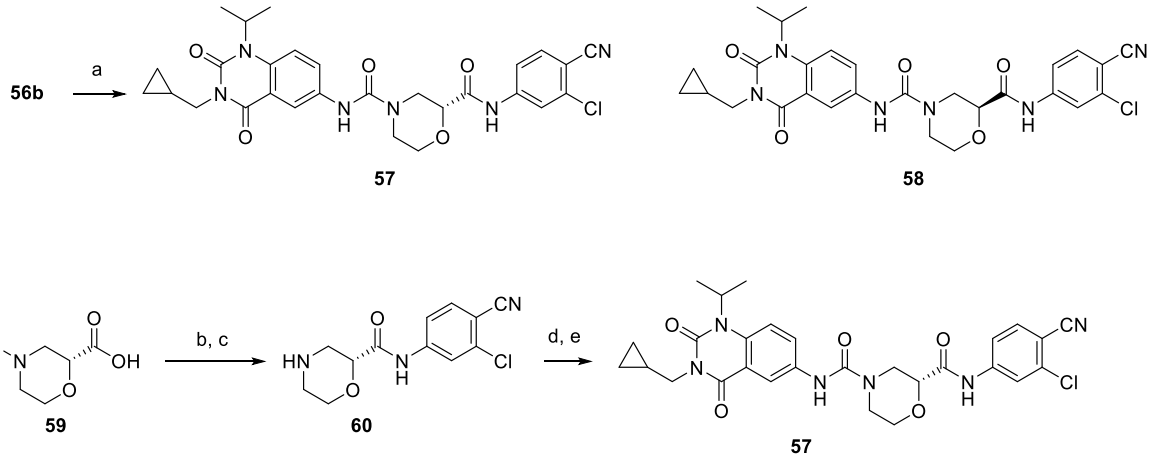
Scheme 8. Conversion of the substituent on quinazoline ring.



Reagents and conditions:(a) 1-bromo-2-methylpropane, K_2CO_3, DMF, 70 °C, 51% (for **54a**); (b) 2-iodopropane, Cs_2CO_3, DMF, 50 °C, 39%; (for **54b**); (c) Pd/C, H_2, MeOH or MeOH-THF, rt, 80-87%; (d) (1) 4-nitrophenyl carbonochloridate, pyridine, THF, rt; (2) **47** (Scheme 7), DIEA, DMF, rt, 37-78%.

Scheme 9 に光学活性体の合成およびその立体配置の決定について示す。**56b** をキラルカラムで分割することにより **57** および **58** を得た。**57** の絶対立体配置は、市販の光学活性体（*R*）-4-（*tert*-ブトキシカルボニル）モルホリン-2-カルボン酸（**59**）より合成した標品と、キラルカラム HPLC で保持時間を比較することにより R 体、逆の立体配置を有する **58** は S 体と決定した。

Scheme 9. The preparation of the optically pure compound **57** and **58**.



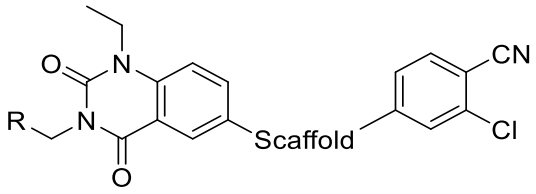
Reagents and conditions:(a) chiral column separation by HPLC; (b) 4-amino-2-chlorobenzonitrile, HATU, DIEA, DMF, rt, 24%; (c) TFA, rt, quant.; (d) 4-nitrophenyl carbonochloridate, **55b**, pyridine, THF, rt; (e) **60**, DIEA, DMF, 62% (2 steps).

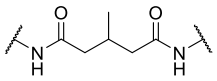
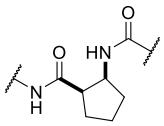
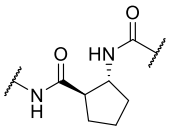
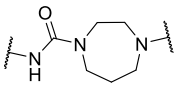
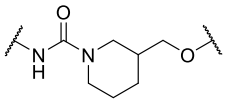
第３節　環状化合物の構造活性相関

活性を評価した結果、U字構造が予想されるシスのシクロペンタン体**21**は直鎖型の化合物**18h**と同等の活性を有していた（Table 6）。一方、直鎖型を取ると予想されるトランスのシクロペンタン体**22**は活性が弱かった。この結果より、U字構造が活性発現に好ましいことが確認できた。環化によるコンフォメーション固定による活性向上も期待したが、環化体**21**は化合物**18h**と同等の活性であった。**18h**について、Macromodel（Schrödinger, Inc）のmixed torsional/low-modeサンプリング法を用い、OPLS3e力場における真空中での最安定構造探索を計算したところ、最安定構造はU字構造であり、伸張した構造とのエネルギー差は17 kJ/molであった。脂肪族炭素鎖のゴーシュコンフォメーションは安定であることが報告されており[74-75]、鎖状構造でもU字型コンフォメーションが安定であったために、環化が活性向上につながらなかったと考えられる。また、ジアゼパン構造を有する化合物**48**は、結合活性は保持したものの、レポーター活性が低下した。検討した中で1,2-置換シスのシクロペンタン環誘導体**21**および1,3置換ピペリジン誘導体**50**が強いレポーター活性を示した。しかしながらこれらの化合物のマウス薬物動態試験（マウス1 mg/kgカセットドーズ、経口投与）でのAUCはそれぞれ化合物**21**で10 ng•h/mL、化合物**50**では32 ng•h/mLと不十分であった。これらの化合物の代謝部位はリンカー中央部のシクロペンタン環およびピペリジン環であると

文献情報から予測されたため[76-77]、化合物**21**および化合物**50**のさらなる変換による薬物動態改善の検討を行った。

Table 6. 環状リンカーの探索



化合物	Scaffold	R	Binding IC_{50} (nM)	Reporter IC_{50} (nM)
18h[a]		Me	3.6	47
21 (*cis*)[a]		Me	3.9	88
22 (*trans*)[a]		Me	>10000	>30000
48		cPr	36	630
50[a]		cPr	14	87

[a]Racemic.

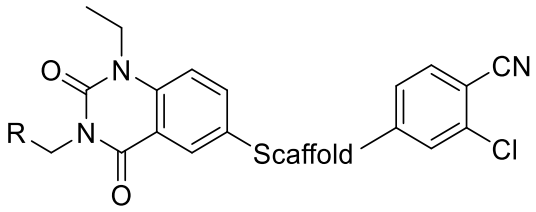
薬物動態を改善するため、想定された代謝部位であるリンカー部中央環上に、酸素原子や窒素原子等のヘテロ原子を導入し、代謝部位をブロックすることとした。キナ

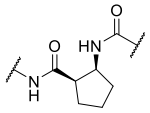
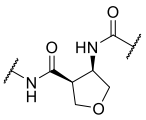
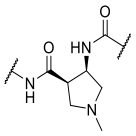
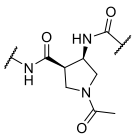
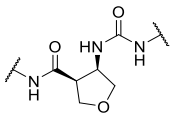
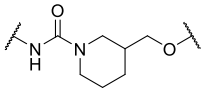
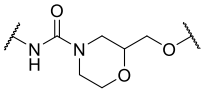
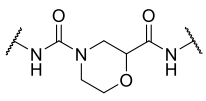
ゾリジンジオン環 3 位窒素原子上の置換基としては、レポーター活性を高めるシクロプロピルメチル基を利用した。初めに、シクロペンタン誘導体 **21** の変換を行った。酸素原子を導入した化合物 **26** では脂溶性も低減し（clogP: 3.99、log D=2.69）、活性も維持された。窒素原子を導入したピロリジン誘導体（化合物 **35** および **36**）ではレポーター活性が減弱した。直鎖型リンカーの場合、最適なリンカー長が 7 原子であることを初期の検討（Table 1）で明らかにしている。リンカー長を 7 原子長に合わせるため、アミド結合をウレア結合に変換した化合物 **29** では活性が増強され、実測での脂溶性も低減した（log D=2.67）。しかしながら、化合物 **29** は溶解度が非常に悪く、経口吸収性が改善されなかった（溶解度: 0.1 μg/mL 未満）。

一方、ピペリジン誘導体（化合物 **50**）の変換も同様に行った。酸素原子を導入して脂溶性が低下（clogP=4.31、logD=3.41）したモルホリン誘導体 **51** ではレポーター活性が減弱したものの、マウス薬物動態の改善が見られ、*in vivo* クリアランスも低下した。化合物 **51** のエーテル結合を、直鎖型リンカーで活性が強かったアミド結合へと変換した化合物 **52** ではレポーター活性が増強され、薬物動態もさらに向上した。

以上の検討より、環状リンカーにすることで、直鎖型リンカーでは許容されなかったヘテロ原子の導入が可能になることが判明した。ヘテロ原子を導入することによる代謝部位のブロックおよび脂溶性の低減効果により、活性を保持しながら薬物動態の改善を行うことが可能になるという知見を得た。

Table 7. 環状構造のリンカー[a]



化合物	R	Scaffold	Binding IC_{50} (nM)	Reporter IC_{50} (nM)	cLogP	LogD[b]	Mouse AUC *p.o.* [c] (ng•h/mL)	Mouse CL[d] (mL/h/kg)
21[e]	Me		3.9	88	4.24	2.76	10.2	3447
26[e]	cPr		5.6	90	3.99	2.69	<1	5093
35[e]	cPr		15	280	4.40	2.76	<3	11028
36[e]	cPr		24	6800	3.73	2.48	NT	NT
29[e]	cPr		8.4	26	4.38	2.67	<1	9079
50	cPr		14	87	4.82	3.72	31.9	2063
51	cPr		63	780	4.31	3.41	638.6	617
52	cPr		19	180	3.76	3.35	755.7	515

[a]Racemic. [b]Measured at pH 7.4. [c]The values are shown as means of three determinations (1.0 mg/kg, *p.o.*). [d]The values are shown as means of three determinations (0.1 mg/kg, *i.v.*). [e]*cis*.

リード化合物 **18h** から、環状リンカーを導入し、薬物動態改善を行った展開の概要を Figure 17 に示す。以上の検討より、各種環状リンカーから、1,3-モルホリン誘導体を選出し、さらなる最適化を行うこととした。

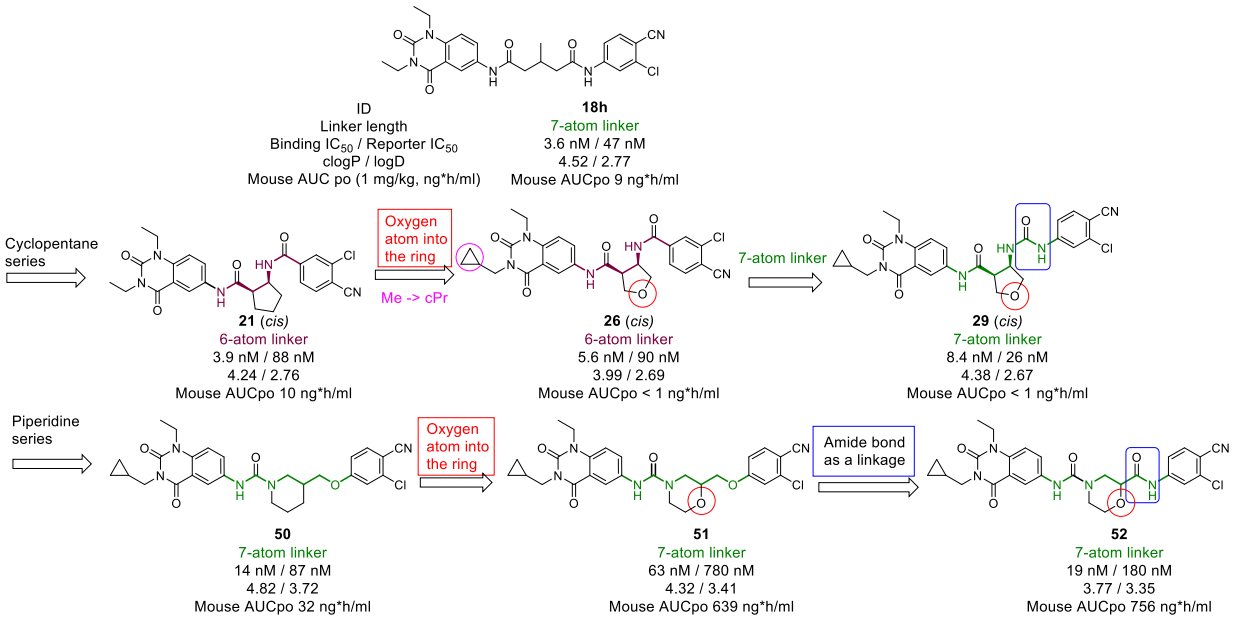


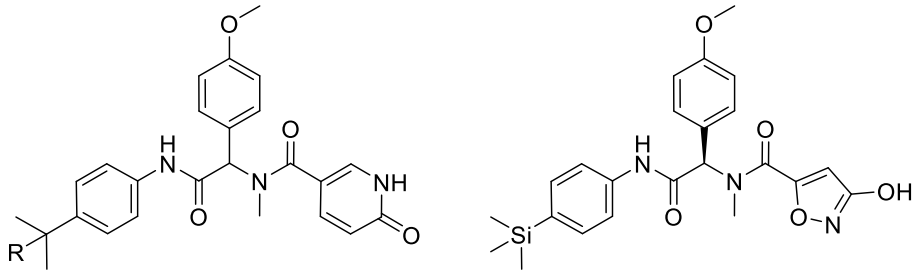
Figure 17. 骨格変換の概要

第 4 節　SBDDによるPhe388との相互作用獲得

化合物**52**はレポーター活性が170 nMであったため、薬効量低減のためにさらなる活性の向上が必要であった。ここで、Phe388との相互作用を獲得することで活性が向上するという知見を別の骨格の化合物における研究から得ていたため[73]、この知見を利用した化合物デザインができるかどうかを検討した。すなわち、フェニルグリシン誘

導体**61**にメチル基を付加した化合物**62**では、Phe388とCH–π相互作用を獲得し、結合活性が10倍、レポーター活性が59倍増強している（Figure18A）。化合物**62**と同様な結合様式を有すると考えられる化合物**63**のトリメチルシリル基と、化合物**18h**のキナゾリジンジオンジオン環上1位窒素原子上エチル基が同じポケットを占めていることから（Figure18B）、化合物**52**について同様な活性向上作用が得られることを期待し、最適化検討を行った。

A



61 (R=H): binding:270 nM, reporter:16000 nM　　**63**: binding:3.7 nM, reporter:44 nM
62 (R=Me): binding:26 nM, reporter:270 nM

B

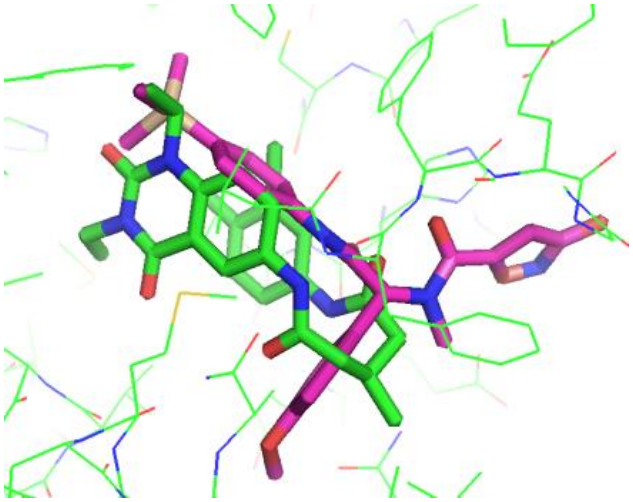
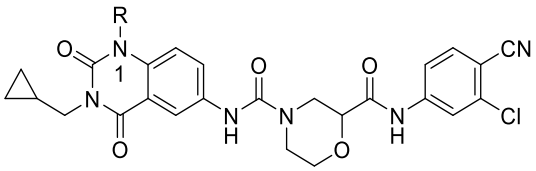


Figure 18. A: フェニルグリシン誘導体の構造活性相関情報; B: **18h**（green, PDB code:6B31）および**63**（magenta, PDB code:6B30）の重ね合わせ.

まず、シクロプロピルメチル基の導入を行った（**Table8**、**56c**）ところ、レポーター活性の向上および薬物動態の改善が見られた。シクロプロパン環を開環したイソブチル基（**56a**）では活性は向上しなかったが、α位で分岐したイソプロピル基（**56b**）を導入したところ、大幅なレポーター活性の向上が見られた。化合物**56b**を光学分割したR体（化合物**57**）では薬物動態が大きく改善した（AUC: 1289 ng•h/mL）。一方で、エナンチオマーのS体（化合物**58**）では薬物動態が悪かった。原因として、マウス代謝安定性が異なることが示唆された（マウス*in vitro*クリアランス: **57** μL/min/mg、**58**: 193 μL/min/mg）。以上の結果より、化合物**57**を*in vivo*試験の候補化合物に選出した。

Table 8. キナゾリジンジオン環１位窒素原子上置換基の効果



化合物	Stereo	R	Binding IC_{50} (nM)	Reporter IC_{50} (nM)	MouseAUC[a] (ng•h/mL)	Mouse CL (mL/h/kg)[b]
52	Racemate		19	180	755.7	515
56c	Racemate		28	110	1080.1	292
56a	Racemate		44	250	NT	NT
56b	Racemate		16	8.9	NT	NT
57	*R*		7.5	3.6	1289.3	131
58	*S*		370	900	3.5	12446

[a]The values are shown as means of three determinations (1.0 mg/kg, *p. o.*). [b]The values are shown as means of three determinations (0.1 mg/kg, *i.v.*).

第５節　マウス自己免疫疾患モデルにおける有効性の確認

マウスIL-23誘発自己免疫疾患モデルを用いた*in vivo* PD試験を行った（Figure 19）。マウス耳介にIL-23を投与すると炎症が惹起され、RORγt関連遺伝子の発現が増加する。そこで、耳介中のIL-17AのmRNAを定量し、化合物投与によるIL-17Aの産生抑制作用を評価した。結果、化合物**57**は30 mg/kgの経口投与でIL-17AのmRNA発現量を有意に

抑制した。以上より、化合物**57**が*in vivo*でRORγtを阻害することにより、Th17細胞の活性化・分化を抑制する効果があることが示された。

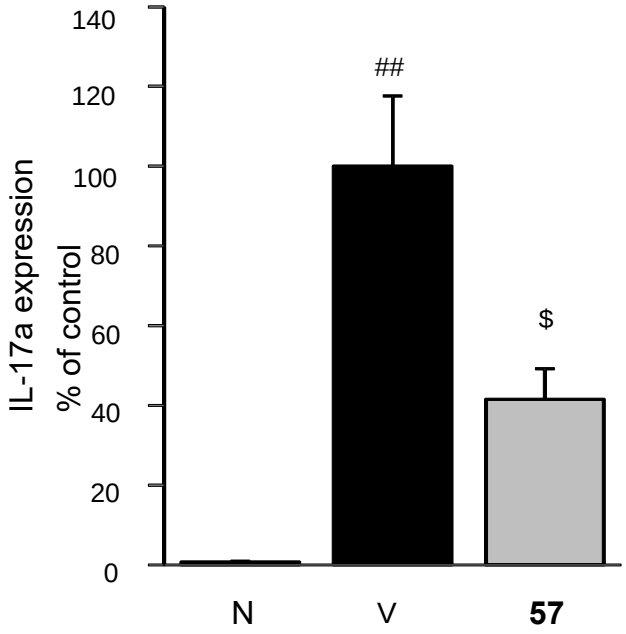


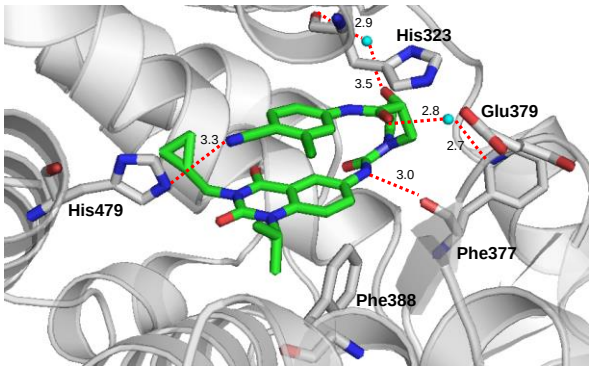
Figure 19. IL-23 投与マウス耳介における IL-17a 遺伝子発現の化合物 **57** による阻害（30 mg/kg, b.i.d.）．データは PBS 処置マウス:N=5, IL-23 処置マウス: N= 8 の平均値±標準誤差．##p<0.01 (Aspin-welch test) compared with PBS-treated group (N). $p<0.05 (Aspin-welch test) compared with vehicle-treated group (V).

第６節　化合物**57**のX線構造解析による考察

RORγtタンパクとのX線構造解析により、モルホリンアミド誘導体**57**がリード化合物の直鎖型化合物**18h**と同様にコレステロール結合部位に結合し、U字型を取ることが明らかになった（Figure 20A）。キナゾリジンジオン環とクロロベンゾニトリル環において、末端フェニル基同士がπ-π相互作用を形成しているためと考えられる。また、

His479とシアノ基が相互作用することで逆作動活性を示すと推察された。キナゾリジンジオン環に隣接しているアミド基はPhe377のカルボニル基と相互作用し、モルホリン環上の酸素原子はGln286やHis323と水を介した水素結合を取っている。キナゾリン環上一位窒素原子上の*i*Pr基は、別ケモタイプの化合物**63**のトリメチルシリル基と同じポケットを占めている（Figure 20B）。導入したメチル基が仮説通りPhe388との脂溶性相互作用を獲得し（Figure 20c）、活性向上につながったと考えられる。

A



B C

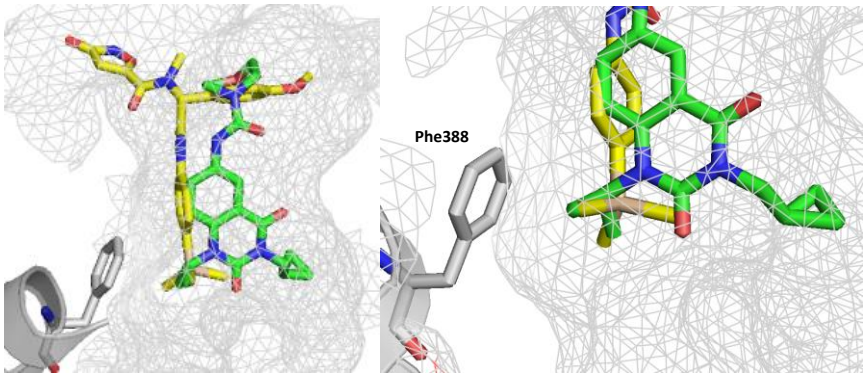


Figure 20. A: 化合物**57**（green）がRORγtタンパクに結合している結晶構造; B: 化合物**57**（green）および**63**（yellow）の重ね合わせ; C: Phe388周辺拡大図.

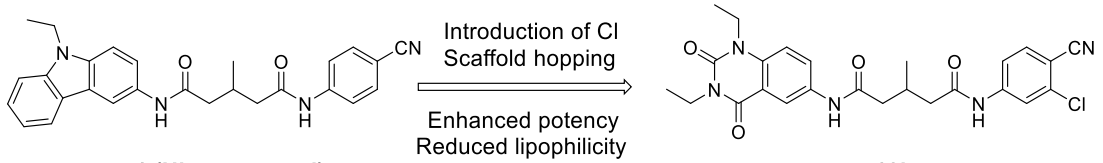
第 7 節　小括

リード化合物 **18h** は薬効試験に用いるマウスでの薬物動態が課題であった。回転可能結合数が少ないほど経口吸収性が良い化合物の割合が高いという報告[62]を参考に、活性コンフォメーションの U 字型構造をとることが期待される環状リンカーを有する化合物をデザイン・合成したところ、シスのシクロペンタン誘導体 **21** およびピペリジン誘導体 **50** が強い活性を示した。それぞれについて代謝部位ブロックおよび脂溶性低減の戦略を用いて薬物動態改善の検討を行った結果、モルホリンアミド誘導体 **52** で薬物動態が大きく改善されることが判明した。さらなるレポーター活性の向上のため、Phe388 との相互作用獲得を狙ったキナゾリジンジオン環上からの置換基の導入を行ったところ、イソプロピル基を導入した光学活性体 **57** はレポーター活性が向上し、薬物動態も良好であった。化合物 **57** をマウス自己免疫疾患モデルにより評価したところ、30 mg/kg の経口投与で IL-17A RNA の発現を 59%抑制した。X 線結晶構造解析により、モルホリンアミド体 **57** がリード化合物 **18h** と同様に U 字型活性コンフォメーションを取ること、キナゾリジンジオン環では、α 位に分岐したイソプロピル基により、Phe388 との脂溶性相互作用を獲得していることも明らかになった。

第３章　総括

筆者は自己免疫疾患に有効な薬剤を指向した化合物として、新規RORγt逆作動薬の合成研究を展開し、以下の研究成果を得た。

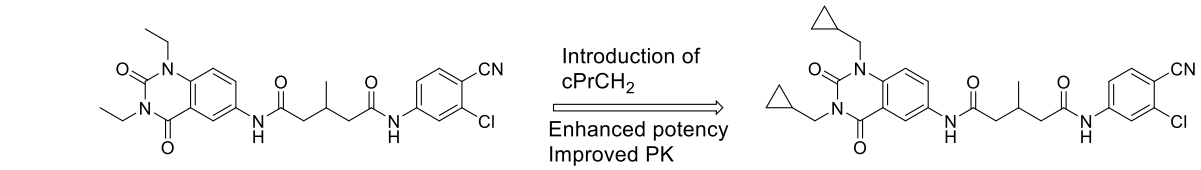
1. ハイスループットスクリーニングで見出したカルバゾール誘導体**1**の活性増強・薬物動態の改善を目的としたリード創出研究を行った。化合物**1**を左端カルバゾール環、リンカー部、シアノフェニル基と3つの部分に分割、各部分の構造活性相関を取得し、得られた情報を統合して最適な化合物へと導く戦略を立案し、実践した。結果、リンカー部では3-メチルペンタン-1,5-ジアミド構造、右端部では3-クロロ-4-シアノフェニル基が最適であることを明らかにした。カルバゾール環を有する化合物は溶解度に課題があったため、カルバゾール環の脂溶性を低減させながら活性発現に必須な相互作用を保つ分子設計を行い、溶解度が改善した二環性の2,4-ジオキソ-1,2,3,4-テトラヒドロキナゾリン環へと変換した**18h**を創出した。



1 (Hit compound)
Binding / Reporter IC_{50} 7.5 nM / 92 nM
clogP 5.8
Solubility (JP2) 0.2 μg/mL

18h
3.6 nM / 47 nM
clogP 4.5
Solubility (JP2) 42 μg/mL

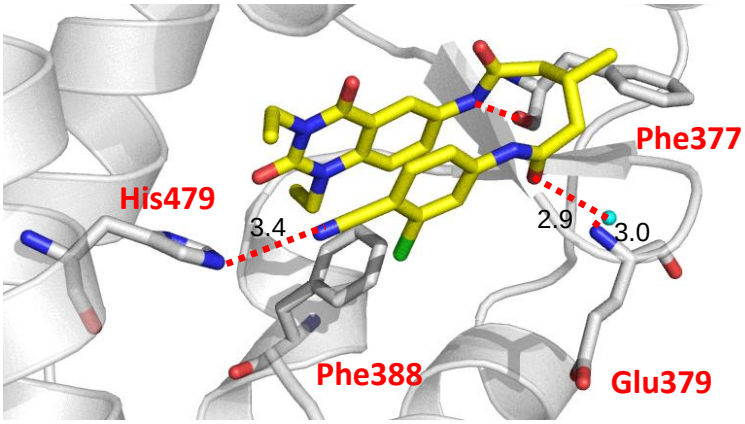
2. 化合物**18h**のキナゾリジンジオン環上置換基を最適化し、血中曝露改善と共に強いレポーター活性を示す化合物**18i**を創製した。化合物**18i**はラットEAEモデルにおいて100 mg/kgでリンパ節IL-17A mRNAの発現を抑制した。



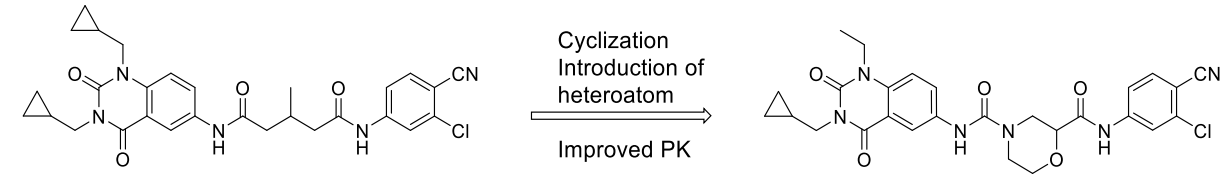
18h
Binding / Reporter IC_{50} 3.6 nM / 47 nM
clogP 4.5
Rat AUCpo 39 ng*h/ml

18i
4.9 nM / 7.2 nM
clogP 5.4
Rat AUCpo 106 ng*h/ml

3. RORγtタンパクと**18h**のX線共結晶構造解析の結果、ジゴキシン等既存の逆作動薬とは異なるU字型活性コンフォメーションを取るという知見を得た。



4. 直鎖型化合物のU字型活性コンフォメーションに着目し、U字型環状リンカーを設計することで、複数のターンミメティクス分子を発見した。さらに、代謝部位のブロックおよび脂溶性低減の手法により、薬物動態を改善したモルホリンアミド誘導体を取得するに至った。

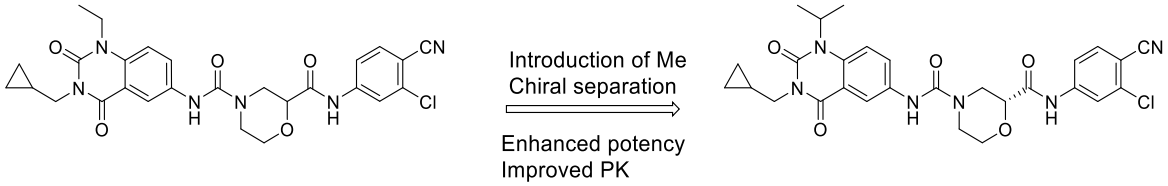


Binding / Reporter IC_{50}

18i
4.9 nM / 7.2 nM
clogP 4.5
Mouse AUCpo 27 ng*h/ml

52
19 nM / 180 nM
clogP 3.8
Mouse AUCpo 756 ng*h/ml

5. キナゾリジンジオン環上において、結晶構造および他の骨格のSAR情報を利用したSBDDによる置換基の設計を行った。脂溶性ポケットへのメチル基導入によりPhe388との脂溶性相互作用を獲得することで活性が大幅に向上し、経口投与でマウス自己免疫疾患モデルにおいて薬効を示す化合物**57**を創製することに成功した。



Binding / Reporter IC_{50}

52
19 nM / 180 nM
clogP 3.8
Mouse AUCpo 756 ng*h/ml

57
7.5 nM / 3.6 nM
clogP 4.1
Mouse AUCpo 1289 ng*h/mL
in vivo PD model effective@30 mg/kg

本研究では、低溶解度の原因と考えられた三環性構造を有するヒット化合物に対し、SAR及びX線構造解析から逆作動活性発現や活性向上に重要な相互作用を特定し、環の数を削減しながら必須な相互作用を獲得する置換基導入を行った。さらに極性基を

適切に配置することにより、活性及び物性に優れた二環性構造に効果的に変換する分子設計法を確立した。

また、安定的にU字構造を保持する化合物として、リンカー部分に環構造を導入することが有効であることを見出した。環構造導入により化合物変換の幅が広がり、直鎖型リンカーでは困難だったヘテロ原子の導入が許容された。ヘテロ原子導入による代謝部位のブロックおよび脂溶性低減効果により、*in vivo*クリアランスが低下し、薬物動態改善を達成した。

上記の検討の結果、経口投与で自己免疫疾患モデルにおいて薬効を示す新規RORγt逆作動薬逆作動薬を創製し、本メカニズムでの新規自己免疫疾患治療薬創出の可能性を示した。

実験の部

1. Chemistry

Reagents and solvents were obtained from commercial sources and used without further purification. Reaction progress was determined by thin layer chromatography (TLC) analysis on Merck Kieselgel 60 F254 plates or Fuji Silysia NH plates. Silica gel column chromatography was performed on Purif-Pack (SI or NH, SHOKO SCIENTIFIC). Proton nuclear magnetic resonance (1H NMR) spectra were recorded on Bruker Ultra Shield-300 (300 MHz) instruments. Chemical shifts are given in parts per million (ppm) with tetramethylsilane as an internal standard. Abbreviations are used as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublets of doublet, br = broad. Coupling constants (J values) are given in hertz (Hz). LC−MS analysis was performed on a Shimadzu LC-20AD separations module, Agilent 1200 series, or Applied Biosystems API 2000, operating in ESI (+ or −) ionization mode. Analytes were eluted using a linear gradient of 0.05% TFA containing H_2O/CH_3CN or 0.01% TFA containing H_2O/CH_3CN or 5 mM ammonium acetate containing H_2O/CH_3CN or 10 mM NH_4HCO_3 containing H_2O/CH_3CN or 0.01% heptafluorobutyric acid/1.0% isopropyl alcohol containing H_2O/CH_3CN mobile phase. Preparative HPLC was performed on a Waters 2525 separations module (L-column2 ODS (20 × 150 mm ID), CERI, Japan) or Waters auto purification instrument (X Terra RP18 OBD Prep Column (19 × 250 mm ID); MS spectra were recorded using a Waters ZQ2000 with electrospray ionization or on a GILSON system, equipped with a L-column2 ODS (20 × 150 mm ID, CERI, Japan) or on a Waters Deltaprep 300 system or on a Shimadzu 10A VP system. Samples were eluted using a linear gradient of 0.1% TFA in H_2O/CH_3CN or 10 mM NH_4HCO_3 in H_2O/CH_3CN, or H_2O/CH_3CN. Purity data were collected by a HPLC with Corona CAD (Charged Aerosol Detector), Nano quantity analyte detector (NQAD), or photo diode array detector. The column was a Capcell Pak C18AQ (50 mm × 3.0 mm ID, Shiseido, Japan) or L-column 2 ODS (30 mm × 2.0 mm ID, CERI, Japan) with a temperature of 50 °C and a flow rate of 0.5 mL/min. Mobile phases A and B under a neutral condition were a mixture of 50 mM ammonium acetate, H_2O, and CH_3CN (1:8:1, v/v/v) and a mixture of 50 mM ammonium acetate and CH_3CN (1:9, v/v), respectively. The ratio of mobile phase B was increased linearly from 5% to 95% over 3 min, 95% over the next 1 min. Mobile phases A and B under an acidic condition were a mixture of 0.2% formic acid in 10 mM ammonium formate and 0.2% formic acid in CH_3CN, respectively. The ratio of mobile phase B was increased linearly from 14% to 86% over 3 min, 86% over the next 1 min. All final compounds were purified to >95% purity unless otherwise noted as determined by analytical HPLC. Elemental analyses (Anal.) was carried out at Takeda Analytical Laboratories, Ltd. Yields were not optimized.

5-[(4-cyanophenyl)amino]-5-oxopentanoic acid (4a).

A mixture of dihydro-2H-pyran-2,6(3H)-dione **3a** (483 mg, 4.23 mmol) and 4-aminobenzonitrile (500 mg, 4.23 mmol) in THF (15 mL) was refluxed overnight. The resulting precipitations were filtered and rinsed with EtOAc to give **4a** (782 mg, 3.37 mmol, 80 %) as pale yellow powder. MS (ESI) m/z: 233.2 $(M+H)^+$. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.81 (2H, m), 2.17–2.35 (2H, m), 2.41 (2H, t, J = 7.4 Hz), 7.56–7.96 (4H, m), 10.33 (1H, s), 12.08 (1H, br s).

4-[(4-cyanophenyl)amino]-4-oxobutanoic acid (4b).

Compound **4b** was prepared from **3b** in 52% yield by a procedure similar to that described for **4a** as an off-white powder. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.53–2.69 (4H, m), 7.54–7.92 (4H, m), 10.40 (1H, s), 12.15 (1H, br s).

6-[(4-cyanophenyl)amino]-6-oxohexanoic acid (4c).

Compound **4c** was prepared from **3c** in 90% yield by a procedure similar to that described for **4a** as a colorless powder. MS (ESI) m/z: 247.1 $(M+H)^+$. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.42–1.70 (4H, m), 2.13–2.30 (2H, m), 2.36 (2H, t, J = 7.0 Hz), 7.58–7.95 (4H, m), 10.32 (1H, d, J = 3.0 Hz), 12.00 (1H, br s).

5-[(4-cyanophenyl)amino]-3,3-dimethyl-5-oxopentanoic acid (4d).

Compound **4d** was prepared from **3d** in quantitative yield by a procedure similar to that described for **4a** as a pale yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.00–1.14 (6H, m), 2.25–2.47 (4H, m), 6.10 (1H, br s), 6.51–6.69 (1H, m), 7.29–7.46 (1H, m), 7.76 (2H, d, J = 3.0 Hz), 11.99 (1H, br s).

({2-[(4-cyanophenyl)amino]-2-oxoethyl}(methyl)amino)acetic acid (4e).

Step 1: **4-Methylmorpholine-2,6-dione (3e)**.

A mixture of **2e** (500 mg, 3.40 mmol) and acetic anhydride (5 mL) was stirred at 165 °C for 30 min. The reaction mixture was cooled to rt and evaporated in vacuo to give **3e** as brown oil. This compound was used for next reaction without further purification.

Step 2: **({2-[(4-Cyanophenyl)amino]-2-oxoethyl}(methyl)amino)acetic acid (4e)**.

A mixture of **3e** (439 mg, 3.40 mmol), *p*-aminobenzonitrile (401 mg, 3.40 mmol) in THF (10 mL) was refluxed for 22 h. The reaction mixture was evaporated in vacuo, and the precipitates were washed with MeOH and Et_2O to afford **4e** (467 mg, 56%, from **2e**) as a light brown solid.

MS (ESI) m/z: 248.1 $(M+H)^+$. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.42 (3H, s), 3.41 (4H, s), 7.76–7.85 (4H, m), 10.30 (1H, s).

***N*-(4-Cyanophenyl)-*N′*-(9-ethyl-9*H*-carbazol-3-yl)pentanediamide (5a)**.

To a solution of **4a** (200 mg, 0.86 mmol), 9-ethyl-9*H*-carbazol-3-amine (217 mg, 1.03 mmol) and DIEA (0.451 mL, 2.58 mmol) in DMF (6 mL) was added HATU (491 mg, 1.29 mmol), and the mixture was stirred at rt overnight. The mixture was quenched with water at rt and extracted with EtOAc. The organic layer was separated, washed with water and brine, dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by column chromatography (silica gel, eluted with 5%−75% EtOAc in hexane), followed by recrystallization from EtOAc to give **5a** (229 mg, 0.540 mmol, 63%) as colorless powder. MS (ESI) m/z: 425.2 $(M+H)^+$. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.30 (3H, t, J = 7.0 Hz), 1.85–2.11 (2H, m), 2.31–2.44 (1H, m), 2.49–2.63 (3H, m), 4.41 (2H, q, J = 6.8 Hz), 7.17 (1H, t, J = 7.4 Hz), 7.36–7.65 (4H, m), 7.69–7.90 (4H, m), 8.04 (1H, d, J = 7.6 Hz), 8.43 (1H, s), 9.92 (1H, s), 10.37 (1H, s).

***N*-(4-Cyanophenyl)-*N′*-(9-ethyl-9*H*-carbazol-3-yl)succinamide (5b)**.

Compound **5b** was prepared from **4b** in 71% yield by a procedure similar to that described for **5a** as a colorless powder. MS (ESI) m/z: 411.2 $(M+H)^+$. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.30 (3H, t, J = 7.0 Hz), 2.60-2.82 (4H, m), 4.41 (2H, q, J = 6.8 Hz), 7.16 (1H, t, J = 7.4 Hz), 7.43 (1H, t, J = 7.4 Hz), 7.50–7.64 (3H, m), 7.69–7.89 (4H, m), 8.04 (1H, d, J = 8.0 Hz), 8.42 (1H, s), 10.02 (1H, s), 10.48 (1H, s).

***N*-(4-Cyanophenyl)-*N′*-(9-ethyl-9*H*-carbazol-3-yl)hexanediamide (5c)**.

Compound **5c** was prepared from **4c** in 14% yield by a procedure similar to that described for **5a** as a colorless powder. MS (ESI) m/z: 439.1 $(M+H)^+$. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.30 (3H, t, J = 7.0 Hz), 1.68 (4H, br s), 2.27–2.46 (4H, m), 4.41 (2H, q, J = 7.1 Hz), 7.07–7.25 (1H, m), 7.36–7.63 (4H, m), 7.68–7.85 (4H, m), 8.04 (1H, d, J = 7.6 Hz), 8.40 (1H, s), 9.89 (1H, s), 10.35 (1H, s).

***N*-(4-Cyanophenyl)-*N′*-(9-ethyl-9*H*-carbazol-3-yl)-3,3-dimethylpentanediamide (5d)**.

Compound **5d** was prepared from **4d** in 55% yield by a procedure similar to that described for **5a** as a pale yellow solid. MS (ESI) m/z: 453.4 $(M+H)^+$. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.06–1.33 (6H, m), 1.42 (3H, t, J = 7.2 Hz), 2.45 (2H, s), 2.56 (2H, s), 4.36 (2H, q, J = 7.2 Hz), 7.12-7.32 (1H, m), 7.32–7.66 (6H, m), 7.76 (2H, d, J = 8.7 Hz), 7.94 (1H, s), 8.08 (1H, d, J = 7.9 Hz), 8.25 (1H, d, J = 1.5 Hz), 10.56 (1H, s).

2-({2-[(4-Cyanophenyl)amino]-2-oxoethyl}(methyl)amino)-*N*-(9-ethyl-9*H*-carbazol-3-yl)acetamide (5e).

Compound **5e** was prepared from **4e** in 57% yield by a procedure similar to that described for **5a** as a colorless powder. MS (ESI) m/z: 440.2 $(M+H)^+$. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.31 (3H, t, J = 7.2 Hz), 2.49 (3H, s), 3.43 (2H, s), 3.48 (2H, s), 4.42 (2H, q, J = 7.2 Hz), 7.19 (1H, t, J = 7.5 Hz), 7.42–7.48 (1H, m), 7.56–7.67 (3H, m), 7.81 (2H, d, J = 8.7 Hz), 7.91 (2H, d, J = 8.7 Hz), 8.07 (1H, d, J = 7.8 Hz), 8.44 (1H, d, J = 2.1 Hz), 10.07 (1H, s), 10.55 (1H, s).

***N*-(4-Cyanophenyl)-*N′*-(9-ethyl-9*H*-carbazol-3-yl)-*N*,*N′*,3-trimethylpentanediamide (6)**.

To an ice-cooled solution of **1** (200 mg, 0.46 mmol) and DMF (3 mL) were added NaH (219 mg, 4.56 mmol) and iodomethane (0.142 mL, 2.28 mmol). After being stirred at rt overnight, the mixture was quenched with water at rt and extracted with EtOAc. The organic layer was separated, washed with water and brine, dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by column chromatography (silica gel, eluted with 50−100% EtOAc in hexane) to give **6** (21.00 mg, 0.045 mmol, 10.0 %) as a colorless amorphous solid. MS (ESI) m/z: 467.3 $(M+H)^+$. ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.72 (3H, d, J = 6.2 Hz), 1.34 (3H, t, J = 7.2 Hz), 1.83 (1H, dd, J = 15.0, 7.7 Hz), 1.89 (1H, brs), 2.02 (1H, dd, J = 15.2, 5.7 Hz), 2.12 (1H, d, J = 10.6 Hz), 2.32 (1H, m), 3.13 (3H, s), 3.21 (3H, s), 4.47 (2H, q, J = 7.0 Hz), 7.22 (1H, t, J = 7.3 Hz), 7.29 (1H, dd, J = 8.4, 1.5 Hz), 7.39 (2H, d, J = 8.4 Hz), 7.49 (1H, t, J = 7.5 Hz), 7.64 (2H, d, J = 8.4 Hz), 7.73 (2H, d, J = 8.1 Hz), 8.06 (1H, d, J = 1.8 Hz), 8.16 (1H, d, J = 7.7 Hz). ^{13}C NMR (151 MHz, DMSO-d_6): δ 13.7, 19.8, 27.5, 36.6, 37.0, 37.4, 39.8, 40.0, 109.2, 109.3, 109.8, 118.3, 118.9, 119.2, 120.6, 121.8, 122.6, 124.9, 126.2, 127.7, 133.2, 135.3, 138.2, 140.0, 147.9, 170.7, 171.0.

5-[(3-Cyanophenyl)amino]-3-methyl-5-oxopentanoic acid (8a).

A mixture of 4-methyldihydro-2*H*-pyran-2,6(3*H*)-dione (1.28 g, 10.00 mmol) and **7a** (1.181 g, 10 mmol) in THF (20 mL) was stirred at 90 °C for 20 h. After being cooled to rt, the reaction mixture was concentrated in vacuo. The residue was crystallized from EtOAc−hexane to give compound to give compound **4a** (2.210 g, 8.97 mmol, 90 %) as white powder. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.96 (3 H, d, J = 6.0 Hz), 2.05–2.45 (5 H, m), 7.43–7.62 (2 H, m), 7.78 (1 H, dt, J = 6.7, 2.3 Hz), 8.10 (1 H, s), 10.25 (1 H, s), 12.10 (1 H, br s). *Anal.* Calcd for $C_{13}H_{14}N_2O_3$: C, 63.40; H, 5.73; N, 11.38. Found: C, 63.35; H, 5.77; N, 11.28.

5-[(2-Cyanophenyl)amino]-3-methyl-5-oxopentanoic acid (8b).

Compound **8b** was prepared from **7b** in 91% yield by a procedure similar to that described for **8a** as a white powder. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.99 (3 H, d, J = 5.7 Hz), 2.08–2.22 (1 H, m), 2.22–2.46 (4 H, m), 7.30–7.41 (1 H, m), 7.52 (1 H, d, J = 7.9 Hz), 7.63–7.73 (1 H, m), 7.80 (1 H, d, J = 7.9 Hz), 10.16 (1 H, s), 12.10 (1 H, br s). *Anal.* Calcd for $C_{13}H_{14}N_2O_3$: C, 63.40; H, 5.73; N, 11.38. Found: C, 63.38; H, 5.77; N, 11.33.

5-Anilino-3-methyl-5-oxopentanoic acid (8c).

Compound **8c** was prepared from **7c** in 89% yield by a procedure similar to that described for **8a** as a white powder. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.95 (3 H, d, J = 6.0 Hz), 2.02–2.43 (5 H, m), 6.96–7.08 (1 H, m), 7.28 (2 H, t, J = 7.9 Hz), 7.58 (2 H, d, J = 7.6 Hz), 9.88 (1 H, s), 12.10 (1 H, br s). *Anal*. Calcd for $C_{12}H_{15}NO_3$: C, 65.14; H, 6.83; N, 6.33. Found: C, 65.04; H, 6.91; N, 6.28.

5-[(4-Fluorophenyl)amino]-3-methyl-5-oxopentanoic acid (8d).

Compound **8d** was prepared from **7d** in 76% yield by a procedure similar to that described for **8a** as a white powder. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.95 (3 H, d, J = 6.1 Hz), 2.00–2.43 (5 H, m), 7.06–7.20 (2 H, m), 7.53–7.65 (2 H, m), 9.94 (1 H, s), 12.08 (1 H, br s). Anal. Calcd for $C_{12}H_{14}FNO_3$: C, 60.24; H, 5.90; N, 5.85. Found: C, 60.32; H, 5.93; N, 5.83.

5-{[4-Cyano-3-(trifluoromethyl)phenyl]amino}-3-methyl-5-oxopentanoic acid (8e).

Compound **8e** was prepared from **7e** in quantitative yield by a procedure similar to that described for **8a** as pale brown viscous oil. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.80–1.08 (3H, m), 2.08–2.47 (5H, m), 7.88–8.16 (2H, m), 8.28 (1H, d, J = 1.9 Hz), 10.68 (1H, s), 12.09 (1H, s).

5-[(3-Chloro-4-cyanophenyl)amino]-3-methyl-5-oxopentanoic acid (8f).

Compound **8f** was prepared from **7f** in quantitative yield by a procedure similar to that described for **8a** as pale brown oil. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.82–1.07 (3H, m), 2.05–2.42 (5H, m), 7.60 (1H, dd, J = 8.7, 1.9 Hz), 7.88 (1H, d, J = 8.7 Hz), 8.06 (1H, d, J = 1.5 Hz), 10.50 (1H, s), 12.08 (1H, s).

5-[(4-Cyanophenyl)amino]-3-methyl-5-oxopentanoic acid (8g).

Compound **8g** was prepared from **7g** in 87% yield by a procedure similar to that described for **8a** as a pale yellow powder. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.80–1.08 (3H, m), 2.05–2.45 (5H, m), 7.57–7.97 (4H, m), 10.34 (1H, s), 12.11 (1H, br s).

***N*-(3-Cyanophenyl)-*N′*-(9-ethyl-9*H*-carbazol-3-yl)-3-methylpentanediamide (9a)**.

A mixture of **8a** (246 mg, 1 mmol), 9-ethyl-9*H*-carbazol-3-amine (210 mg, 1.00 mmol), 1H-benzo[d][1,2,3]triazol-1-ol hydrate (306 mg, 2.00 mmol) and WSC (383 mg, 2.00 mmol) in DMF (5 mL) was stirred at rt for 20 h. The reaction was poured into aqueous $NaHCO_3$ and EtOAc. The separated organic layer was washed with water and brine, dried over $MgSO_4$ and concentrated. The residue was purified by column chromatography (silica gel, eluted with 66−80% (EtOAc in hexane) to give compound **9a** (283 mg, 0.645 mmol, 65%) as a white powder. MS (ESI) m/z: 439.1 $(M+H)^+$. mp 164–166 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.03 (3 H, d, J = 6.0 Hz), 1.23–1.34 (3 H, m), 2.23–2.50 (5 H, m), 2.72–2.83 (1 H, m), 4.41 (1 H, d, J = 7.2 Hz), 7.13–7.23 (1 H, m), 7.52 (7 H, d, J = 12.1 Hz), 8.04 (1 H, d, J = 7.5 Hz), 8.13 (1 H, s), 8.41 (1 H, s), 9.93 (1 H, s), 10.25–10.34 (1 H, m). *Anal.* Calcd for $C_{27}H_{26}N_4O_2 \cdot H_2O$: C, 72.46; H, 6.08; N, 12.52. Found: C, 72.58; H, 6.02; N, 12.35.

***N*-(2-Cyanophenyl)-*N′*-(9-ethyl-9*H*-carbazol-3-yl)-3-methylpentanediamide (9b)**. Compound **9b** was prepared from 8b in 40% yield by a procedure similar to that described for **9a** as a white powder. MS (ESI) m/z: 439.2 $(M+H)^+$. mp 212–214 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.08 (3 H, d, J = 6.4 Hz), 1.30 (3 H, d, J = 7.2 Hz), 2.23–2.47 (5 H, m), 4.41 (2 H, d, J = 7.2 Hz), 7.13–7.22 (1 H, m), 7.32–7.40 (1 H, m), 7.40–7.49 (1 H, m), 7.51–7.62 (4 H, m), 7.64–7.73 (1 H, m), 7.77–7.87 (1 H, m), 8.05 (1 H, d, J = 7.5 Hz), 8.43 (1 H, s), 9.85–10.35 (2 H, m). *Anal.* Calcd for $C_{27}H_{26}N_4O_2 \cdot 0.1H_2O$: C, 73.65; H, 6.00; N, 12.72. Found: C, 73.51; H, 6.04; N, 12.58.

***N*-(9-Ethyl-9*H*-carbazol-3-yl)-3-methyl-*N′*-phenylpentanediamide (9c)**.

Compound **9c** was prepared from **8c** in 39% yield by a procedure similar to that described for **9a** as a white powder. MS (ESI) m/z: 414.1 $(M+H)^+$. mp 236–238 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.03 (3 H, d, J = 6.4 Hz), 1.30 (3 H, t, J = 7.2 Hz), 2.19–2.47 (5 H, m), 4.41 (2 H, q, J = 7.0 Hz), 6.98–7.07 (1 H, m), 7.17 (1 H, t, J = 7.3 Hz), 7.29 (2 H, t, J = 7.9 Hz), 7.44 (1 H, t, J = 7.2 Hz), 7.50–7.67 (5 H, m), 8.05 (1 H, d, J = 7.9 Hz), 8.42 (1 H, s), 9.93 (2 H, s). Anal. Calcd for $C_{26}H_{27}N_3O_2 \cdot 0.1H_2O$: C, 75.19; H, 6.60; N, 10.12. Found: C, 75.17; H, 6.65; N, 10.16.

***N*-(9-Ethyl-9*H*-carbazol-3-yl)-*N′*-(4-fluorophenyl)-3-methylpentanediamide (9d)**.

Compound **9d** was prepared from **8d** in 27% yield by a procedure similar to that described for **9a** as a white powder. MS (ESI) m/z: 432.1 $(M+H)^+$. m .p. 250-252 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.02 (3 H, d, J = 6.4 Hz), 1.30 (3 H, t, J = 7.2 Hz), 2.21–2.47 (5 H, m), 4.41 (2 H, d, J = 7.2 Hz), 7.06–7.23 (3 H, m), 7.44 (1 H, t, J = 7.7 Hz), 7.49–7.71 (5 H, m),

8.05 (1 H, d, J = 7.5 Hz), 8.41 (1 H, s), 9.93 (1 H, s), 10.00 (1 H, s). *Anal*. Calcd for $C_{26}H_{26}FN_3O_2 \cdot H_2O$: C, 70.89; H, 6.18; N, 9.54. Found: C, 71.06; H, 6.08; N, 9.46.

***N*-[4-Cyano-3-(trifluoromethyl)phenyl]-*N'*-(9-ethyl-9*H*-carbazol-3-yl)-3-methylpentanediamide (9e)**.

To a solution of **8e** (300 mg, 0.95 mmol), 9-ethyl-9H-carbazol-3-amine (241 mg, 1.15 mmol), and DIEA (0.500 mL, 2.86 mmol) in DMF (6 mL) was added HATU (544 mg, 1.43 mmol), and the mixture was stirred at rt overnight. The mixture was quenched with water at rt and extracted with EtOAc. The organic layer was separated, washed with water and brine, dried over MgSO4 and concentrated in vacuo. The residue was purified by column chromatography (silica gel, eluted with 5%−75% EtOAc in hexane, then NH-silica gel, eluted with 5%−100% EtOAc in hexane) to give **9e** (189 mg, 0.372 mmol, 39.0 %) as colorless amorphous solid. MS (ESI) m/z: 507.1 $(M+H)^+$. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.05 (3H, d, J = 6.0 Hz), 1.30 (3H, t, J = 7.0 Hz), 2.24–2.48 (3H, m), 2.56 (2H, d, J = 10.6 Hz), 4.41 (2H, q, J = 6.8 Hz), 7.17 (1H, t, J = 7.4 Hz), 7.33–7.67 (4H, m), 7.89–8.16 (3H, m), 8.30 (1H, s), 8.41 (1H, s), 9.93 (1H, s), 10.72 (1H, s).

***N*-(3-Chloro-4-cyanophenyl)-*N*'-(9-ethyl-9*H*-carbazol-3-yl)-3-methylpentanediamide (9f)**.

9f was synthesized in a manner similar to **9e**. 45% yield, colorless powder. MS (ESI) m/z: 473.1 $(M+H)^+$. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.03 (3H, d, J = 6.0 Hz), 1.20–1.40 (4H, m), 2.19–2.48 (3H, m), 2.54 (1H, br s), 4.41 (2H, q, J = 7.1 Hz), 7.07–7.27 (1H, m), 7.36–7.69 (5H, m), 7.87 (1H, d, J = 8.7 Hz), 7.97–8.14 (2H, m), 8.39 (1H, s), 9.91 (1H, s), 10.54 (1H, s).

Chiral resolution of 9f.

500 mg of **9f** was separated by chiral resolution using AD column. Column and conditions: Column: CHIRALPAK AD (Lot No. AF001) 50 mm ID X 500 mm. Mobile phase: n-Hex/ EtOH = 500/500 (v/v), temperature: 40 °C. Flow rate: 80ml/min. Detection: UV 220 nm. Injection concentration: 1.5 mg/mL (in n-hexane/EtOH = 500/500). Injection volume: 100 mL load.

(+)-*N*-(3-Chloro-4-cyanophenyl)-*N*'-(9-ethyl-9*H*-carbazol-3-yl)-3-methylpentanediamide (10a).

39% yield, colorless powder. MS (ESI) m/z: 473.2 $(M+H)^+$. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.03 (3H, d, J = 6.0 Hz), 1.30 (3H, t, J = 7.0 Hz), 2.22–2.48 (3H, m), 2.52–2.64

(2H, m), 4.41 (2H, q, *J* = 6.9 Hz), 7.17 (1H, t, *J* = 7.2 Hz), 7.36–7.66 (5H, m), 7.87 (1H, d, *J* = 8.7 Hz), 7.97–8.14 (2H, m), 8.39 (1H, s), 9.92 (1H, s), 10.55 (1H, s). $[\alpha]^{25}_{D}$ +4.3 (c 2.54, DMSO).

(-)-*N*-(3-Chloro-4-cyanophenyl)-*N*'-(9-ethyl-9*H*-carbazol-3-yl)-3-methylpentanediamide (10b).

40% yield, colorless powder. MS (ESI) m/z: 473.1 $(M+H)^+$. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.03 (3H, d, *J* = 6.0 Hz), 1.30 (3H, t, *J* = 7.0 Hz), 2.18-2.48 (3H, m), 2.52–2.64 (2H, m), 4.41 (2H, q, *J* = 7.2 Hz), 7.08–7.24 (1H, m), 7.37–7.69 (5H, m), 7.87 (1H, d, *J* = 8.7 Hz), 7.96-8.16 (2H, m), 8.39 (1H, s), 9.92 (1H, s), 10.55 (1H, s). $[\alpha]^{25}_{D}$ −4.0 (c 2.50, DMSO).

2-Ethyl-7-nitro-2*H*-1,4-benzoxazin-3(4*H*)-one (12).

To a stirred suspension of potassium fluoride (2.36 g, 40.55 mmol) in anhydrous DMF (15.0 mL) was added methyl 2-bromobutylate (2.06 mL, 17.84 mmol) and reaction mixture was stirred at rt for 15 min. 2-amino-5-nitrophenol (11, 2.5 g, 16.22 mmol) was added to the mixture and heated at 60 °C for 6 h followed by stirring at rt for additional 14 h. The reaction mixture was combined with another same quantity batch [potassium fluoride (2.36 g, 40.55 mmol), DMF (15.0 mL), methyl 2-bromobutylate (2.06 mL, 17.84 mmol) and 2-amino-5-nitrophenol (2.5 g, 16.22 mmol)] and crushed ice (175 g) was added to the combined mixture. White solid formed from the reaction mixture was filtered, washed with water (25 mL × 3) followed by hexane (25 mL × 3) and dried under reduced pressure to afford **12** (6.0 g, 83%) as white solid. MS (ESI) m/z: 221.0 $(M-H)^-$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.00 (t, 3H, *J* = 7.40 Hz), 1.75–1.89 (m, 2H), 4.70 (dd, 1H, *J* = 4.52 Hz, 7.64 Hz), 7.05 (d, 1H, *J* = 8.72 Hz), 7.79 (d, 1H, *J* = 2.44 Hz), 7.91 (dd, 1H, *J* = 2.44 Hz, 8.76 Hz), 11.28 (br s, 1H).

7-Amino-2,4-diethyl-2*H*-1,4-benzoxazin-3(4*H*)-one (13a).

Step A: **2,4-diethyl-7-nitro-2*H*-1,4-benzoxazin-3(4*H*)-one**.

To a stirred solution of **12** (3.0 g, 13.62 mmol) in anhydrous DMF (45 mL) was added K_2CO_3 (4.14 g, 29.97 mmol) at 0 °C under nitrogen atmosphere. After stirring at rt for 15 minutes, ethyl iodide (1.63 mL, 20.44 mmol) was added. The resulting reaction mixture was stirred at rt for 14 h. Reaction mixture was quenched with water (30 mL) and extracted with ethyl acetate (50 mL × 3). Combined organic layers was washed with water (30 mL) and brine (30 mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to afford 2,4-diethyl-7-nitro-2*H*-1,4-benzoxazin-3(4*H*)-one (3.0 g, 88%) as white solid. MS (ESI) m/z:

251.2 $(M+H)^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.99 (t, 3H, J = 7.36 Hz), 1.15 (t, 3H, J = 7.04 Hz), 1.73–1.88 (m, 2H), 3.99 (q, 2H, J = 7.08 Hz), 4.74 (dd, 1H, J = 4.64 Hz, 7.92 Hz), 7.43 (d, 1H, J = 9.00 Hz), 7.82 (d, 1H, J = 2.60 Hz), 7.96 (dd, 1H, J = 2.52 Hz, 8.92 Hz).

Step B: **7-Amino-2,4-diethyl-2*H*-1,4-benzoxazin-3(4*H*)-one**.

To a stirred deoxygenated solution of 2,4-diethyl-7-nitro-2*H*-1,4-benzoxazin-3(4*H*)-one (1.50 g, 6.00 mmol) in dry methanol (30 mL) was added 10% Pd-C (0.225 g) under nitrogen atmosphere and stirred at rt for 14 h under hydrogen atmosphere (balloon pressure). The reaction mixture was filtered through celite bed and washed with methanol (10 mL × 2). Combined organic layers was collected and concentrated under reduced pressure. The residue was triturated with pentane to afford **13a** (1.0 g, 76%) as brown solid. MS (ESI) m/z: 221.0 $(M+H)^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.95 (t, 3H, J = 7.36 Hz), 1.07–1.10 (m, 3H), 1.62–1.76 (m, 2H), 3.81 (q, 2H, J = 7.04 Hz), 4.35 (dd, 1H, J = 4.64 Hz, 8.20 Hz), 4.98 (s, 2H), 6.22–6.26 (m, 2H), 6.83 (d, 1H, J = 8.40 Hz).

6-Nitroquinazoline-2,4(1*H*,3*H*)-dione (15a).

A mixture of **14** (10.0 g, 54.91 mmol) and urea (98.93 g, 1647.2 mmol) was heated to 160 °C for 48 h. After complete consumption of starting material, the reaction mixture was cooled to 100 °C and water (10 mL) was added to the reaction mixture. The reaction mixture was filtered and washed with water; solid residue was dissolved in 0.5N NaOH solution. Then the reaction mixture was heated at 100 °C for 40 minutes. The reaction mixture was cooled to 0 °C and 1N HCl (aq.) was added to adjust pH = 5. Light yellow precipitate was filtered, washed with water and dried under vacuum to give compound **15a** (6.0 g, 53%) as a light yellow solid. MS (ESI) m/z: 206.1 $(M-H)^-$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.31 (d, 1H, J = 9.04 Hz), 8.44 (dd, 1H, J = 2.60 Hz, 8.96 Hz), 8.57 (d, 1H, J = 2.64 Hz), 11.66 (br s, 2H).

3-Ethyl-6-nitroquinazoline-2,4(1*H*,3*H*)-dione (15b).

Step A: **2-Amino-*N*-ethyl-5-nitrobenzamide**.

To a stirred solution of **14** (5.0 g, 27.45 mmol) in dry DMF (100 mL) at 25 °C were added $EtNH_2$ (2 M sol in THF, 41.18 mL, 82.35 mmol), DIPA (47.81 mL, 274.51 mmol) and HATU (13.57 g, 35.69 mmol). Reaction mixture was stirred at rt for 24 h. After complete consumption of 14, DMF was distilled off under reduced pressure to get the crude. Crude mass was quenched with ice-water and organic part was extracted with ethyl acetate (5 × 100 mL), washed with water (3 × 100 mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give crude which was purified by normal silica gel column chromatography using 10-20% ethyl acetate in hexane as an eluent to afford

2-amino-*N*-ethyl-5-nitrobenzamide (5.0 g, 93.32%) as yellow solid. MS (ESI) m/z: 208.0 $(M-H)^-$. ^1H NMR (400 MHz, DMSO-d_6): δ 1.12 (t, 3H, J = 7.20 Hz), 3.22-3.32 (m, 2H), 6.78 (d, 1H, J = 9.24 Hz), 7.77 (br s, 2H), 8.01 (dd, 1H, J = 9.16 Hz, 2.36 Hz), 8.48 (d, 1H, J = 2.36 Hz), 8.69 (br s, 1H).

Step B: **3-Ethyl-6-nitroquinazoline-2,4(1*H*,3*H*)-dione**.

To a solution of 2-amino-*N*-ethyl-5-nitrobenzamide (6 g, 28.7 mmol) in THF (60 mL) was added triethyl amine (8.7 mL) at rt. Phosgene (20% in toluene) (18.6 mL, 37.3 mmol) was added at rt and refluxed for 16 h. Reaction was monitored by TLC. Starting material was present and again 20% phosgene (7.1 mL, 14.35 mmol) was added. The reaction mixture was heated to reflux for 2 h. Starting material was consumed. The reaction mixture was concentrated, extracted with ethyl acetate (2 x 100 mL), dried (Na_2SO_4) and purified by 15% ethyl acetate in hexane as an eluent to afford **15b** (5g, 74.13%) as yellow solid. MS (ESI) m/z: 234.0 $(M-H)^-$. ^1H NMR (400 MHz, DMSO-d_6): δ 1.17 (t, 3H, J = 7.08 Hz), 3.90–3.95 (m, 2H), 7.32 (d, 1H, J = 9.00 Hz), 8.45 (dd, 1H, J = 9.04 Hz, 2.68 Hz), 8.62 (d, 1H, J = 2.60 Hz), 12.03 (br s, 1H).

6-Amino-1,3-diethylquinazoline-2,4(1*H*,3*H*)-dione (13b).

Step A: **1,3-Diethyl-6-nitroquinazoline-2,4(1*H*,3*H*)-dione**.

To a solution of **15a** (750 mg, 3.62 mmol) and iodoethane (1.448 mL, 18.10 mmol) in DMF (15 mL) was added K_2CO_3 (2.502 g, 18.10 mmol), and the mixture was stirred at 70 °C for 16 h. After being cooled to rt, the mixture was quenched with water at ambient temperature and extracted with EtOAc. The organic layer was separated, washed with water and brine, dried over $MgSO_4$ and concentrated in vacuo. The residual powder was triturated with DMF−EtOAc−hexane, and the insoluble materials were filtered to give 1,3-diethyl-6-nitroquinazoline-2,4(1*H*,3*H*)-dione (664 mg, 2.52 mmol, 70%) as a pale yellow powder. ^1H NMR (300 MHz, DMSO-d_6): δ 1.24 (3H, t, J = 7.2 Hz), 1.18 (3H, t, J = 7.2 Hz), 3.99 (2H, q, J = 6.9 Hz), 4.20 (2H, q, J = 6.9 Hz), 7.72 (1H, d, J = 9.4 Hz), 8.51 (1H, dd, J = 9.3, 2.8 Hz), 8.72 (1H, d, J = 3.0 Hz).

Step B: **6-Amino-1,3-diethylquinazoline-2,4(1*H*,3*H*)-dione**.

A mixture of 1,3-diethyl-6-nitroquinazoline-2,4(1*H*,3*H*)-dione (655 mg, 2.49 mmol) and Pd-C (50 mg, 0.47 mmol) in MeOH (15 mL) and EtOAc (15 mL) was stirred at rt under H_2 (balloon, 3L) for 16 h. Pd catalyst was removed by celite filtration. The filtrate was concentrated in vacuo to give compound **13b** (572 mg, 2.452 mmol, 99%) as a gray powder.

^{1}H NMR (300 MHz, DMSO-d_6): δ 1.01–1.32 (6H, m), 3.84–4.16 (4H, m), 5.28 (2H, s), 7.04 (1H, dd, J = 8.7, 2.6 Hz), 7.15–7.32 (2H, m).

6-Amino-1,3-bis(cyclopropylmethyl)quinazoline-2,4(1*H*,3*H*)-dione (13c).

Step A: **1,3-bis(cyclopropylmethyl)-6-nitroquinazoline-2,4(1*H*,3*H*)-dione**.

A mixture of **15a** (333 mg, 1.61 mmol), (bromomethyl)cyclopropane (0.624 mL, 6.43 mmol) and K_2CO_3 (489 mg, 3.54 mmol) in DMF (10 mL) was stirred at rt for 16 h. The mixture was quenched with brine at rt and extracted with EtOAc. The organic layer was separated, washed with brine, dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by column chromatography (silica gel, eluted with 5−30% EtOAc in hexane) to give 1,3-bis(cyclopropylmethyl)-6-nitroquinazoline-2,4(1*H*,3*H*)-dione (402 mg, 1.28 mmol, 79%) as a colorless solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.32–0.76 (8H, m), 1.11–1.44 (2H, m), 4.00 (2H, d, J = 7.2 Hz), 4.12–4.16 (2H, m), 7.45 (1H, d, J = 9.4 Hz), 8.50 (1H, dd, J =9.3, 2.8 Hz), 9.11 (1H, d, J = 2.6 Hz).

Step B: **6-Amino-1,3-bis(cyclopropylmethyl)quinazoline-2,4(1*H*,3*H*)-dione**.

A mixture of 1,3-bis(cyclopropylmethyl)-6-nitroquinazoline-2,4(1*H*,3*H*)-dione (400 mg, 1.27 mmol) and Pd-C (135 mg, 0.13 mmol) in EtOH (10 mL) was stirred at rt for 2 h under H_2 atmosphere. The mixture was filtered through celite pad and the filtrate was concentrated in vacuo to give compound **13c** (280 mg, 77%) as a colorless solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.30–0.68 (8H, m), 0.97–1.44 (2H, m), 3.40–3.86 (2H, m), 4.01 (4H, dd, J = 14.0, 6.8 Hz), 6.96–7.08 (1H, m), 7.08–7.22 (1H, m), 7.51 (1H, d, J = 3.0 Hz).

6-Amino-1-(cyclopropylmethyl)-3-ethylquinazoline-2,4(*1H*,*3H*)-dione (13d).

Step A: **1-(cyclopropylmethyl)-3-ethyl-6-nitroquinazoline-2,4(1*H*,3*H*)-dione**.

To a stirred solution of **15b** (250 mg, 1.06 mmol) in dry DMF (10 mL) under N_2-atmosphere were added K_2CO_3 (294 mg, 2.13 mmol), and after 5 min, (bromomethyl)cyclopropane (0.43 mL, 3.19 mmol) was added at rt. Reaction mixture was heated at 80 °C for 8 h. After complete consumption of **15b**, reaction was quenched with ice-water and organic part was extracted with EtOAc (4 × 100 mL), washed with water (2 × 50 mL), dried over anhydrous Na_2SO_4 and solvent was distilled off under reduced pressure. Crude mass was purified by washing with 2% EtOAc in hexane to give 1-(cyclopropylmethyl)-3-ethyl-6-nitroquinazoline-2,4(1*H*,3*H*)-dione (150 mg, 49%) as yellow sticky solid. MS (ESI) m/z: 290.2 $(M+H)^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.45–0.53 (m,

4H), 1.16–1.25 (m, 4H), 3.97–4.02 (m, 2H), 4.11 (d, 2H, *J* = 6.88 Hz), 7.81 (d, 1H, *J* = 9.28 Hz), 8.50 (dd, 1H, *J* = 9.28 Hz, 2.80 Hz), 8.73 (d, 1H, *J* = 2.72 Hz).

Step B: **6-Amino-1-(cyclopropylmethyl)-3-ethylquinazoline-2,4(1*H*,3*H*)-dione**.

To a stirred solution of 1-(cyclopropylmethyl)-3-ethyl-6-nitroquinazoline-

2,4(1*H*,3*H*)-dione (150 mg, 0.52 mmol) in ethyl acetate (15 mL) was added 10% palladium-carbon (80 mg). Reaction mixture was stirred at rt under hydrogen gas-pressure (30 Psi) for 3 h. After complete consumption of the starting material, reaction mixture was filtered off through celite pad and washed with ethyl acetate (100 mL). Filtrate was evaporated under reduced pressure to afford **13d** (130 mg, 97%) as a yellow solid. MS (ESI) m/z: 260.2 $(M+H)^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.42-0.47 (m, 4H), 0.75–0.85 (m, 1H), 1.12 (t, 3H, *J* = 7.04 Hz), 3.94–3.99 (m, 4H), 5.28 (s, 2H), 7.03 (dd, 1H, *J* = 8.88 Hz, 2.64 Hz), 7.24 (d, 1H, *J* = 2.64 Hz), 7.29 (d, 1H, *J* = 8.96 Hz).

1-Ethyl-6-nitroquinazoline-2,4(1*H*,3*H*)-dione (17).

Step A: **Methyl 2-(ethylamino)-5-nitrobenzoate**.

To a stirred solution of 2-fluoro-5-nitrobenzoic acid methyl ester (16, 5.0 g, 25.11 mmol) in dry THF (50 mL) at 25 °C in a sealed tube were added $EtNH_2$ (2 M solution in THF, 2.83 g, 62.77 mmol, 31.5 mL), and K_2CO_3 (3.47 g, 25.11 mmol). The reaction mixture was heated at 60 °C for 16 h. After complete consumption of 16, the reaction mixture was evaporated under reduced pressure to give crude which was purified by washing with 2% EtOAc in hexane to afford methyl 2-(ethylamino)-5-nitrobenzoate (5.0 g, 89%) as a light yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.20 (t, 3H, *J* = 7.04 Hz), 4.10–4.16 (q, 2H), 7.66 (d, 1H, *J* = 9.28 Hz), 8.48 (dd, 1H, *J* = 9.22 Hz, 2.76 Hz), 8.66 (d, 1H, *J* = 2.64 Hz), 11.00 (br s, 1H).

Step B: **1-Ethyl-6-nitroquinazoline-2,4(1*H*,3*H*)-dione**.

A mixture of methyl 2-(ethylamino)-5-nitrobenzoate (5.0 g, 22.32 mmol) and urea (40.58 g, 669.64 mmol) was heated at 160 °C for 48 h. After complete consumption of the starting methyl ester, the reaction mixture was cooled to rt and diluted with water. Precipitate was filtered off and crude was purified by washing with dichloromethane to afford compound **17** (3.0 g, 57%) as a light yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.20 (t, 3H, *J* = 7.04 Hz), 4.10–4.16 (q, 2H), 7.66 (d, 1H, *J* = 9.28 Hz), 8.48 (dd, 1H, *J* = 9.22 Hz, 2.76 Hz), 8.66 (d, 1H, *J* = 2.64 Hz), 11.00 (br s, 1H).

6-Amino-3-(cyclopropylmethyl)-1-ethylquinazoline-2,4(1*H*,3*H*)-dione (13e).

Step A: **3-(cyclopropylmethyl)-1-ethyl-6-nitroquinazoline-2,4(1*H*,3*H*)-dione**.

To a stirred solution of **17** (350 mg, 1.49 mmol) in dry DMF (10 mL) at 25 °C under N_2-atmosphere were added NaH (119.15 mg, 2.98 mmol) and after 5 min (bromomethyl)cyclopropane (0.239 mL, 2.98 mmol). The reaction mixture was stirred at rt for 5 h. After complete consumption of **17**, the reaction was quenched with ice-water and organic part was extracted with EtOAc (4 × 50 mL), washed with water (2 × 50 mL), dried over anhydrous Na_2SO_4 and solvent was distilled off under reduced pressure. The residue was purified by washing with 2% EtOAc in hexane to give 3-(cyclopropylmethyl)-1-ethyl-6-nitroquinazoline-2,4(1*H*,3*H*)-dione (350 mg, 81%) as a faint green sticky solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.37–0.46 (m, 4H), 1.17–1.20 (m, 1H), 1.24 (t, 3H, J = 7.04 Hz), 3.86 (d, 2H, J = 7.16 Hz), 4.19–4.24 (m, 2H), 7.74 (d, 1H, J = 9.32 Hz), 8.53 (dd, 1H, J = 9.24 Hz, 2.68 Hz), 8.75 (d, 1H, J = 2.64 Hz).

Step B: **6-amino-3-(cyclopropylmethyl)-1-ethylquinazoline-2,4(1*H*,3*H*)-dione**.

To a stirred solution of 3-(cyclopropylmethyl)-1-ethyl-6-nitroquinazoline-

2,4(1*H*,3*H*)-dione (420 mg, 1.45 mmol) in EtOAc (20 mL) was added 10% Pd-C (50 mg). The reaction mixture was stirred at rt under hydrogen gas-pressure (30 Psi) for 3 h. After complete consumption of the starting material, the reaction mixture was filtered off through celite pad and washed with EtOAc (100 mL). Filtrate was evaporated under reduced pressure to afford **13k** (292 mg, 78%) as a yellow solid. MS (ESI) m/z: 260.2 $(M+H)^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.32–0.40 (m, 4H), 1.17 (t, 3H, J = 7.00 Hz), 1.22–1.23 (m, 1H), 3.82 (d, 2H, J =7.04 Hz), 4.06 (q, 2H, J = 6.96 Hz), 5.27 (s, 2H), 7.02–7.05 (m, 1H), 7.21–7.25 (m, 2H).

***N*-(4-Cyanophenyl)-*N'*-(9-ethyl-2,3,4,9-tetrahydro-1*H*-carbazol-6-yl)-3-**

methylpentanediamide (18a).

To a solution of **8g** (172 mg, 0.70 mmol), 9-ethyl-2,3,4,9-tetrahydro-1*H*-carbazol-

6-amine (150 mg, 0.70 mmol), and DIEA (0.367 mL, 2.10 mmol) in DMF (8 mL) was added HATU (399 mg, 1.05 mmol), and the mixture was stirred at rt overnight. The mixture was quenched with water at rt and extracted with EtOAc. The organic layer was separated, washed with water and brine, dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by column chromatography (silica gel, eluted with 10−100% EtOAc in hexane) followed by crystallization from EtOAc to give compound **18a** (179 mg, 0.404 mmol, 58%) as a pale brown powder. MS (ESI) m/z: 443.2 $(M+H)^+$. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.99 (3H, d, J = 6.1 Hz), 1.19 (3H, t, J = 7.2 Hz), 1.65–1.94 (4H, m), 2.16–2.41 (3H, m),

2.41–2.48 (2H, m), 2.57 (2H, t, *J* = 5.3 Hz), 2.63–2.72 (2H, m), 4.04 (2H, q, *J* = 7.2 Hz), 7.16 (1H, dd, *J* = 8.7, 1.9 Hz), 7.26 (1H, d, *J* = 8.7 Hz), 7.62–7.87 (5H, m), 9.68 (1H, s), 10.37 (1H, s).

***N*-(4-Cyanophenyl)-3-methyl-*N′*-(2,3,4,9-tetrahydro-1*H*-carbazol-6-yl)pentanediamide (18b)**.

18b was synthesized in a manner similar to **18a**. 69% yield, pale brown powder. MS (ESI) m/z: 415.3 $(M+H)^+$. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.99 (3H, d, *J* = 6.4 Hz), 1.80 (4H, d, *J* = 4.5 Hz), 2.16–2.40 (3H, m), 2.41–2.47 (2H, m), 2.56 (2H, br s), 2.67 (2H, br s), 7.00–7.22 (2H, m), 7.64 (1H, s), 7.69–7.86 (4H, m), 9.64 (1H, s), 10.37 (1H, s), 10.51 (1H, s).

***N*-(3-Chloro-4-cyanophenyl)-*N′*-(1-ethyl-1*H*-indol-5-yl)-3-methylpentanediamide (18c)**.

18c was synthesized in a manner similar to **18a**. 61% yield, colorless solid. MS (ESI) m/z: 423.2 $(M+H)^+$. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.00 (3H, d, *J* = 6.0 Hz), 1.34 (3H, t, *J* = 7.2 Hz), 2.15-2.41 (3H, m), 2.43-2.49 (2H, m), 4.16 (2H, q, *J* = 7.2 Hz), 6.34 (1H, d, *J* = 2.6 Hz), 7.22 (1H, dd, *J* = 8.7, 1.9 Hz), 7.29-7.41 (2H, m), 7.61 (1H, dd, *J* = 8.5, 2.1 Hz), 7.82-7.91 (2H, m), 8.07 (1H, d, *J* = 1.9 Hz), 9.71 (1H, s), 10.53 (1H, s).

***N*-(3-chloro-4-cyanophenyl)-*N*'-(3-ethyl-2-oxo-2,3-dihydro-1,3-benzoxazol-6-yl)-3-methyl pentanediamide (18d)**. **18d** was synthesized in a manner similar to **18a**. 9% yield, colorless amorphous solid. MS (ESI) m/z: 439.2 $(M-H)^+$. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.99 (3H, d, *J* = 5.7 Hz), 1.25 (3H, t, *J* = 7.0 Hz), 2.13-2.48 (5H, m), 3.82 (2H, q, *J* = 6.8 Hz), 7.08-7.38 (2H, m), 7.59 (1H, d, J = 8.3 Hz), 7.72 (1H, s), 7.86 (1H, d, *J* = 8.7 Hz), 8.04 (1H, s), 10.02 (1H, br s), 10.55 (1H, br s).

***N*-(3-Chloro-4-cyanophenyl)-*N*'-(4-ethyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-7-yl)-3-methylpentanediamide (18e)**.

18e was synthesized in a manner similar to **18a**. 10% yield, colorless amorphous solid. MS (ESI) m/z: 453.2 $(M-H)^-$. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.98 (3H, d, *J* = 5.7 Hz), 1.13 (3H, t, *J* = 6.8 Hz), 2.15–2.47 (5H, m), 3.90 (2H, d, *J* = 6.8 Hz), 4.59 (2H, s), 7.11 (1H, d, *J* = 8.7 Hz), 7.21 (1H, d, *J* = 8.7 Hz), 7.36 (1H, s), 7.60 (1H, d, *J* = 8.3 Hz), 7.87 (1H, d, *J* = 8.3 Hz), 8.06 (1H, s), 9.92 (1H, s), 10.53 (1H, s).

***N*-(3-chloro-4-cyanophenyl)-*N*'-(4-ethyl-2-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-7-yl)-3-methylpentanediamide (18f)**.

18f was synthesized in a manner similar to **18a**. 10% yield, white solid. MS (ESI) m/z: 467.3 $(M-H)^-$. ^1H NMR (400 MHz, DMSO-d_6): δ 0.98 (d, 3H, J = 6.04 Hz), 1.11 (t, 3H, J = 6.96 Hz), 1.39 (d, 3H, J = 6.68 Hz), 2.21-2.44 (m, 5H), 3.88 (q, 2H, J = 8.04 Hz), 4.64 (q, 1H, J = 6.48 Hz), 7.10 (d, 1H, J = 8.84 Hz), 7.19 (d, 1H, J = 8.76 Hz), 7.37 (br s, 1H), 7.59 (d, 1H, J = 8.76 Hz), 7.86 (d, 1H, J = 8.56 Hz), 8.05 (br s, 1H), 9.89 (br s, 1H), 10.50 (br s, 1H).

***N*-(3-chloro-4-cyanophenyl)-*N*'-(2,4-diethyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-7-yl)-3-methylpentanediamide (18g).**

To a stirred solution of 6-amino-3-ethylbenzo[*b*][1,4]dioxin-2(3*H*)-one (0.35 g, 1.59 mmol) and **8f** (0.53 g, 1.91 mmol) in ethyl acetate (10 mL) was added DIEA (0.70 mL, 3.98 mmol) followed by T3P solution (50 wt% in ethyl acetate, 2.4 mL, 3.97 mmol) under an argon atmosphere. The reaction mixture was heated at reflux for 5 h. Reaction mixture was cooled to rt, diluted with ethyl acetate (100 mL), washed with cold aqueous $NaHCO_3$ (25 mL), water (25 mL) and brine (25 mL). Organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to obtain crude product. The crude product was purified by silica gel column chromatography using 60−70% ethyl acetate in hexane as an eluent to give **18g** (0.350 g, 46%) as white solid. MS (ESI) m/z: 483.4 $(M+H)^-$. ^1H NMR (400 MHz, DMSO-d_6): δ 0.97 (t, 6H, J = 7.16 Hz), 1.12 (t, 3H, J = 6.88 Hz), 1.67−1.78 (m, 2H), 2.23−2.44 (m, 5H), 3.87 (q, 2H, J = 6.96 Hz), 4.49 (dd, 1H, J = 4.52 Hz, J = 8.12 Hz), 7.09 (d, 1H, J = 8.68 Hz), 7.17 (d, 1H, J = 8.80 Hz), 7.39 (br s, 1H), 7.59 (d, 1H, J = 8.72 Hz), 7.87 (d, 1H, J = 8.60 Hz), 8.05 (br s, 1H), 9.91 (br s, 1H), 10.52 (br s, 1H).

***N*-(3-Chloro-4-cyanophenyl)-*N*′-(1,3-diethyl-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-6-yl)-3-methylpentanediamide (18h)**.

18h was synthesized in a manner similar to **18g**. 72% yield, colorless powder. MS (ESI) m/z: 496.4 $(M+H)^+$. ^1H NMR (300 MHz, DMSO-d_6): δ 1.00 (3H, d, J = 6.4 Hz), 1.09–1.35 (6H, m), 2.18–2.49 (5H, m), 3.84–4.24 (4H, m), 7.44 (1H, d, J = 9.4 Hz), 7.57 (1H, dd, J = 8.7, 1.9 Hz), 7.78–7.97 (2H, m), 8.02 (1H, d, J = 2.3 Hz), 8.34 (1H, d, J = 2.6 Hz), 10.14 (1H, s), 10.52 (1H, s). *Anal.* Calcd for $C_{25}H_{26}ClN_5O_4 \cdot$ 0.1EtOAc: C, 60.44; H, 5.35; N, 13.87. Found: C, 60.71; H, 5.63; N, 13.42.

***N*-[1,3-Bis(cyclopropylmethyl)-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-6-yl]-*N*′-(3-chloro-4-cyanophenyl)-3-methylpentanediamide (18i)**.

18h was synthesized in a manner similar to **18g**. 61% yield, colorless solid. MS (ESI) m/z: 546.3 $(M-H)^-$. mp 184–187 °C. ^1H NMR (300 MHz, DMSO-d_6): δ 0.27–0.55 (8H, m), 0.89–

1.08 (3H, m), 1.11–1.35 (2H, m), 2.19–2.48 (5H, m), 3.85 (2H, d, J = 7.2 Hz), 4.03 (2H, d, J = 6.8 Hz), 7.47–7.65 (2H, m), 7.85 (1H, d, J = 8.7 Hz), 7.92 (1H, dd, J = 9.1, 2.6 Hz), 8.03 (1H, d, J = 1.9 Hz), 8.36 (1H, d, J = 2.6 Hz), 10.15 (1H, s), 10.53 (1H, s). *Anal.* Calcd for $C_{29}H_{30}ClN_5O_4$: C, 63.56; H, 5.52; N, 12.78. Found: C, 63.41; H, 5.64; N, 12.49.

***N*-(3-Chloro-4-cyanophenyl)-*N*'-[1-(cyclopropylmethyl)-3-ethyl-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-6-yl]-3-methylpentanediamide (18j)**.

18j was synthesized in a manner similar to **18g**. 45% yield, off-white solid. MS (ESI) m/z: 522.2 $(M+H)^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.45−0.48 (m, 4H), 1.00 (d, 3H, J = 6.44 Hz), 1.15 (t, 3H, J = 6.96 Hz), 1.17−1.19 (m, 1H), 2.30−2.54 (m, 5H), 3.96−4.03 (m, 4H), 7.52 (d, 1H, J = 9.24 Hz), 7.57(dd, 1H, J = 8.66 Hz, 1.88 Hz), 7.84 (d, 1H, J = 8.60 Hz), 7.91 (d, 1H, J = 7.72 Hz), 8.02 (d, 1H, J = 1.84 Hz), 8.34 (d, 1H, J = 2.48 Hz), 10.15 (s, 1H), 10.53 (s, 1H). Purity was 89%.

***N*-(3-Chloro-4-cyanophenyl)-*N*′-[3-(cyclopropylmethyl)-1-ethyl-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-6-yl]-3-methylpentanediamide (18k)**.

18k was synthesized in a manner similar to **18k**. 77%, off-white solid. MS (ESI) m/z: 520.3 $(M-H)^-$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.35–0.36 (m, 2H), 0.39–0.43 (m, 2H), 0.99 (d, 3H, J = 6.16 Hz), 1.20 (t, 3H, J = 6.76 Hz), 1.22 (m, 1H), 2.26–2.50 (m, 5H), 3.83 (d, 2H, J = 6.96 Hz), 4.09–4.14 (m, 2H), 7.44 (d, 1H, J = 9.12 Hz), 7.56 (d, 1H, J = 9.32 Hz), 7.84 (d, 1H, J = 8.64 Hz), 7.90 (dd, 1H, J = 9.00 Hz, 1.80 Hz), 8.01 (s, 1H), 8.36 (s, 1H), 10.16 (s, 1H), 10.54 (s, 1H).

(±)-*cis*-2-Amino-*N*-(1,3-diethyl-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-6-yl)cyclopentanecarboxamide (19)

Step A: ***tert*-Butyl {(±)-*cis*-2-[(1,3-diethyl-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-6-yl)carbamoyl]cyclopentyl}carbamate**.

A mixture of 6-amino-1,3-diethylquinazoline-2,4(1*H*,3*H*)-dione (**13e**, 45 mg, 0.19 mmol), (±)-*cis*-2-[(*tert*-butoxycarbonyl)amino]cyclopentanecarboxylic acid (48.7 mg, 0.21 mmol) and T3P (0.125 mL, 0.21 mmol), DIEA (34 μL, 0.19 mmol) in EtOAc (10 mL) was stirred at 50 °C overnight. The mixture was quenched with brine at rt and extracted with EtOAc. The organic layer was separated, washed with brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, eluted with 10%–50%EtOAc in hexane) to give *tert*-butyl {(±)-*cis*-

2-[(1,3-diethyl-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-6-yl)carbamoyl]cyclopentyl}carbamat e (60.0 mg, 0.135 mmol, 70%) as colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.13–1.39 (14H, m), 1.51–2.03 (6H, m), 2.09–2.23 (1H, m), 2.87–3.24 (1H, m), 3.92–4.39 (5H, m), 4.78–5.18 (1H, m), 7.15 (1H, d, J = 9.1 Hz), 7.88–8.02 (1H, m), 8.03–8.11 (1H, m), 8.12–8.24 (1H, m); MS (ESI) m/z: 443.3 $[M-H]^-$.

Step B: **(±)-*cis*-2-Amino-*N*-(1,3-diethyl-2,4-dioxo-1,2,3,4-**

tetrahydroquinazolin-6-yl)cyclopentanecarboxamide.

A mixture of *tert*-butyl {(±)-*cis*-2-[(1,3-diethyl-2,4-dioxo-1,2,3,4-

tetrahydroquinazolin-6-yl)carbamoyl]cyclopentyl}carbamate (60 mg, 0.13 mmol) and TFA (3 mL) was stirred at rt for 3 h. The mixture was quenched with sat. $NaHCO_3$ aq. at rt and extracted with EtOAc. The organic layer was separated, washed with brine, dried over $MgSO_4$ and concentrated *in vacuo* to give **19** (45 mg, 0.13 mmol, 100%) as a colorless oil. The residue was used in next reaction without further purifications. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.15–1.40 (6H, m), 1.51–2.21 (8H, m), 2.60–2.82 (1H, m), 3.64 (1H, d, J = 5.3 Hz), 4.03–4.31 (4H, m), 7.16 (1H, d, J = 9.1 Hz), 7.96 (1H, d, J = 2.6 Hz), 8.37 (1H, dd, J = 9.1, 2.6 Hz), 10.80 (1H, brs); MS (ESI) m/z: 345.3 $[M+H]^+$.

(±)-*trans*-2-Amino-*N*-(1,3-diethyl-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-6-

yl)cyclopentane-1-carboxamide (20).

20 was synthesized from 13e in a manner similar to **19**.

Step A: ***tert*-butyl {(±)-*trans*-2-[(1,3-Diethyl-2,4-dioxo-1,2,3,4-**

tetrahydroquinazolin-6-yl)carbamoyl]cyclopentyl}carbamate.

75% yield, colorless solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.14–1.40 (6H, m), 1.44–1.54 (9H, m), 1.56–1.93 (3H, m), 1.94–2.14 (1H, m), 2.14–2.44 (1H, m), 2.88 (1H, brs), 3.87–4.37 (5H, m), 4.79 (1H, brs), 7.16 (1H, d, J = 9.1 Hz), 7.24 (1H, brs), 8.02–8.42 (2H, m), 10.36 (1H, brs); MS (ESI) m/z: 445.3 $[M+H]^+$.

Step B:**(±)-*trans*-2-Amino-*N*-(1,3-diethyl-2,4-dioxo-**

1,2,3,4-tetrahydroquinazolin-6-yl)cyclopentane-1-carboxamide.

Quant., colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.18–1.40 (6H, m), 1.56–1.90 (8H, m), 2.56–2.92 (1H, m), 2.95–3.19 (1H, m), 3.26–3.44 (1H, m), 4.03–4.26 (4H, m), 7.17 (1H, d, J

= 9.1 Hz), 7.97 (1H, d, *J* = 2.6 Hz), 8.40 (1H, dd, *J* = 9.1, 2.6 Hz); MS (ESI) m/z: 345.3 $[M+H]^+$.

3-Chloro-4-cyano-*N*-{(±)-*cis*-2-[(1,3-diethyl-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-6-yl)carbamoyl]cyclopentyl}benzamide (21).

A mixture of **19** (63 mg, 0.18 mmol), 3-chloro-4-cyanobenzoic acid (36.5 mg, 0.20 mmol), T3P (0.118 mL, 0.20 mmol) and DIEA (0.035 mL, 0.20 mmol) in EtOAc (3 mL) was stirred at 60 °C for 5 h. The mixture was quenched with brine at rt and extracted with EtOAc. The organic layer was separated, washed with brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, eluted with 10%–100% EtOAc in hexane) to give **21** (45.0 mg, 0.089 mmol, 48%) as a colorless solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.13–1.39 (6H, m), 1.50–1.69 (1H, m), 1.80–2.31 (5H, m), 3.17 (1H, q, *J* = 7.9 Hz), 3.99–4.31 (4H, m), 4.67 (1H, t, *J* = 7.4 Hz), 7.14 (1H, d, *J* = 9.8 Hz), 7.45 (1H, d, *J* = 7.6 Hz), 7.59–7.76 (2H, m), 7.83 (1H, d, *J* = 1.1 Hz), 7.98-8.10 (2H, m), 8.39 (1H, s); ^{13}C NMR (101 MHz, $CDCl_3$) δ 12.6, 13.1, 23.2, 29.2, 32.9, 37.2, 38.9, 48.6, 53.8, 114.1, 115.2, 115.8, 116.1, 119.6, 125.4, 127.5, 128.8, 132.7, 134.2, 136.4, 137.4, 139.4, 149.9, 161.1, 164.7, 172.8; MS (ESI) m/z: 508.4 $[M+H]^+$.

3-Chloro-4-cyano-*N*-{(±)-*trans*-2-[(1,3-diethyl-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-6-yl)carbamoyl]cyclopentyl}benzamide (22).

A mixture of **20** (180 mg, 0.52 mmol), 3-chloro-4-cyanobenzoic acid (104 mg, 0.57 mmol), T3P (0.338 mL, 0.57 mmol) and DIEA (0.100 mL, 0.57 mmol) in EtOAc (5 mL) was stirred at 70 °C overnight. The mixture was quenched with 1N HCl aq. at rt and extracted with EtOAc. The organic layer was separated, washed with 1N NaOH aq. and brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, eluted with 10%–100%EtOAc in hexane) to give **22** (30.0 mg, 0.059 mmol, 11%) as a colorless solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.17 (6H, m), 1.76 (4H, m), 2.02 (2H, m), 2.69–2.98 (1H, m), 3.85–4.26 (4H, m), 4.51 (1H, t, *J* = 7.0 Hz), 7.46 (1H, d, *J* = 9.4 Hz), 7.94 (2H, d, *J* = 8.3 Hz), 8.05–8.20 (2H, m), 8.31 (1H, d, *J* = 1.9 Hz), 8.82 (1H, d, *J* = 7.6 Hz), 10.14 (1H, s). ^{13}C NMR (101 MHz, DMSO-d_6) δ 12.5, 12.9, 23.8, 29.5, 32.2, 36.1, 38.1, 51.7, 54.7, 113.9, 114.8, 115.1, 115.6, 117.6, 126.7, 126.8, 128.4, 134.4, 134.7, 134.8, 135.4, 140.1, 149.4, 160.6, 163.4, 172.8; MS (ESI) m/z: 508.4 $[M+H]^+$.

***tert*-Butyl (±)-*cis*-4-aminotetrahydrofuran-3-carboxylate (24).**

Step A: ***tert*-Butyl 4-(benzylamino)-2,5-dihydrofuran-3-carboxylate**.

A solution of **23** (7.43 g, 39.91 mmol), phenylmethanamine (6.54 mL, 59.87 mmol) and AcOH (3.43 mL, 59.87 mmol) in THF (dry, 89 mL) and DMF (dry, 44.3 mL) was stirred at rt for 2 h, and then sodium triacetoxyborohydride (12.69 g, 59.87 mmol) was added thereto portionwise (over a period of 5 min). The mixture was stirred at rt overnight. Sodium borohydride (1.510 g, 39.91 mmol) was added. The mixture was stirred at rt overnight. The mixture was neutralized with sat. $NaHCO_3$ aq. at 0 °C and extracted with EtOAc. The organic layer was separated, washed with water and brine, dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, eluted with EtOAc in hexane) to give *tert*-butyl 4-(benzylamino)-2,5-dihydrofuran-3-carboxylate (7.09 g, 25.7 mmol, 65%) as colorless crystals. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.47 (9 H, s), 4.26 (2 H, d, J=6.42 Hz), 4.65–4.76 (4 H , m), 7.06–7.41 (m, 5 H).

Step B: ***tert*-Butyl (±)-*cis*-4-aminotetrahydrofuran-3-carboxylate**.

A solution of *tert*-butyl 4-(benzylamino)-2,5-dihydrofuran-3-carboxylate (7.09 g, 25.75 mmol) in MeOH (86 mL) was hydrogenated in the presence of 20% $Pd(OH)_2$-C (50% wet, 6.0 g, 42.72 mmol) at rt under high pressure (ca. 5 atm) for 3 days. After removal of the catalyst by filtration, the filtrate was evaporated to give pale yellow oil. The residue was purified by column chromatography (silica gel, eluted with MeOH in EtOAc) to give **24** (2.00 g, 10.68 mmol, 42 %) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.49 (9 H, s), 3.09 (1 H , m), 3.60 (1 H, dd, J = 8.69, 3.78 Hz), 3.78 (1 H, m) 3.92– 4.18 (3 H, m).

***tert*-Butyl (±)-*cis*-4-(3-chloro-4-cyanobenzamido)tetrahydrofuran-3-carboxylate (25)**. A mixture of 3-chloro-4-cyanobenzoic acid (102 mg, 0.56 mmol), **24** (105 mg, 0.56 mmol), T3P (0.330 mL, 0.56 mmol) and DIEA (98 µL, 0.56 mmol) in EtOAc (5 mL) was stirred at 60 °C for 5 h. The mixture was neutralized with sat. $NaHCO_3$ aq. at rt and extracted with EtOAc. The organic layer was separated, washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, eluted with 10%–50% EtOAc in hexane) to give **25** (135 mg, 0.385 mmol, 69%) as white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.44 (9H, s), 3.31 (1H, q, J = 7.6 Hz), 3.82 (1H, dd, J = 9.1, 3.8 Hz), 4.01 (1H, dd, J = 9.3, 5.5 Hz), 4.06–4.19 (2H, m), 4.87–5.02 (1H, m), 7.39 (1H, d, J = 7.9 Hz), 7.65–7.81 (2H, m), 7.93 (1H, d, J = 0.8 Hz).

(±)-*cis*-4-(3-Chloro-4-cyanobenzamido)-*N*-[3-(cyclopropylmethyl)-1-ethyl-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-6-yl]tetrahydrofuran-3-carboxamide (26).

Step A: **(±)-*cis*-4-(3-chloro-4-cyanobenzamido)tetrahydrofuran-3-carboxylic acid**. TFA (1766 μL, 22.92 mmol) was added to **25** (134 mg, 0.38 mmol) at 0 °C. The mixture was stirred at rt for 2 h. The mixture was evaporated and azeotroped with toluene to give crude (±)-*cis*-4-(3-chloro-4-cyanobenzamido)tetrahydrofuran-3-carboxylic acid as colorless oil (148 mg, quant.). MS (ESI) m/z: 293.1 $[M-H]^-$.

Step B: **(±)-*cis*-4-(3-Chloro-4-cyanobenzamido)-*N*-[3-(cyclopropylmethyl)-1-ethyl-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-6-yl]tetrahydrofuran-3-carboxamide**.

The mixture of crude (±)-*cis*4-(3-chloro-4-cyanobenzamido)tetrahydrofuran-3-carboxylic acid (117 mg, 0.398 mmol), 6-amino-3-(cyclopropylmethyl)-1-ethylquinazoline-2,4(1*H*,3*H*)-dione (**13d**, 114 mg, 0.44 mmol), T3P (0.258 mL, 0.44 mmol) and DIEA (0.076 mL, 0.44 mmol) in EtOAc (4 mL) and dry THF (2.00 mL) was stirred at 60 °C for 4 h. The mixture was neutralized with sat. $NaHCO_3$ aq. at rt and extracted with EtOAc. The organic layer was separated, washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, eluted with 50%–100% EtOAc in hexane) to give white solid. The solid was suspended in hexane/IPE and filtered to give **26** (45.0 mg, 0.084 mmol, 19%) as white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.27–0.49 (4H, m), 1.18 (4H, t, J = 7.0 Hz), 3.46 (1H, q, J = 7.8 Hz), 3.71–3.89 (3H, m), 3.97–4.21 (5H, m), 4.87–5.05 (1H, m), 7.38 (1H, d, J = 9.1 Hz), 7.65–7.84 (3H, m), 7.95 (1H, d, J = 7.9 Hz), 8.25 (1H, d, J = 2.3 Hz), 8.91 (1H, d, J = 8.3 Hz), 10.25 (1H, s); ^{13}C NMR (101 MHz, DMSO-d_6) δ 3.5, 9.8, 12.4, 38.2, 45.2, 49.1, 52.5, 68.7, 70.4, 113.8, 114.7, 114.9, 115.4, 117.6, 126.6, 126.7, 128.4, 133.9, 134.5, 135.0, 135.1, 139.8, 149.6, 160.9, 164.3, 168.5; MS (ESI) m/z: 536.4 $[M+H]^+$.

4-Nitrophenyl (3-chloro-4-cyanophenyl)carbamate (27).

To a solution of 2-chloro-4-aminobenzonitrile (1.0 g, 6.55 mmol) in THF (15 mL) were added successively 4-nitrophenyl carbonochloridate (1.52 g, 7.54 mmol) and pyridine (0.607 mL, 7.54 mmol) at rt. After being stirred at rt for overnight, the reaction mixture was quenched with water at ambient temperature. The resulting precipitate was collected and rinsed with water to give crude **27** (3.08 g, 9.70 mmol, quant.) as off-white powder, which

was used in the next reaction without further purifications. ^{1}H NMR (300 MHz, DMSO-d_6) δ 6.85–7.05 (1H, m), 7.54–7.69 (2H, m), 7.77–7.90 (1H, m), 7.96 (1H, d, J = 8.7 Hz), 8.07–8.23 (1H, m), 8.25–8.43 (1H, m), 11.15 (1H, s).

***tert*-Butyl (±)-*cis*-4-[3-(3-chloro-4-cyanophenyl)ureido]tetrahydrofuran-3-carboxylate (28)**.

27 (161 mg, 0.51 mmol) and DIEA (0.089 mL, 0.51 mmol) were added to a solution of **24** (95 mg, 0.51 mmol) in DMA (5 mL) at rt. The mixture was stirred at rt for 3.5 h. The mixture was poured into water at rt and extracted with EtOAc. The organic layer was separated, washed with water and brine, dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, eluted with 10%–60% EtOAc in hexane) to give **28** (114 mg, 0.312 mmol, 61 %) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.44 (9H, s), 3.35 (1H, q, J = 7.4 Hz), 3.79–3.94 (2H, m), 3.99 (1H, t, J = 8.7 Hz), 4.08–4.22 (1H, m), 4.78-4.96 (1H, m), 5.95 (1H, d, J = 8.7 Hz), 7.33 (2H, dd, J = 8.7, 1.5 Hz), 7.53 (1H, d, J = 8.7 Hz), 7.66 (1H, d, J = 1.9 Hz); MS (ESI) m/z: 364.1 $[M-H]^-$.

(±)-*cis*-4-[3-(3-Chloro-4-cyanophenyl)ureido]-*N*-[3-(cyclopropylmethyl)-1-ethyl-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-6-yl]tetrahydrofuran-3-carboxamide (29).

Step A: **(±)-*cis*-4-[3-(3-Chloro-4-cyanophenyl)ureido]tetrahydrofuran-3-carboxylic acid.**

TFA (1.4 mL) was added to **28** (114 mg, 0.31 mmol) at 0 °C. The mixture was stirred at rt for 2 h. The mixture was evaporated and azeotroped with toluene to give crude (±)-*cis*-4-[3-(3-chloro-4-cyanophenyl)ureido]tetrahydrofuran-3-carboxylic acid (122 mg) as a yellow solid, which was used in the next step without further purification.

Step B: **(±)-*cis*-4-(3-(3-Chloro-4-cyanophenyl)ureido)-*N*-(3-(cyclopropylmethyl)-1-ethyl-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-6-yl)tetrahydrofuran-3-carboxamide**.

A mixture of crude (±)-*cis*-4-[3-(3-chloro-4-cyanophenyl)ureido]tetrahydrofuran-3-carboxylic acid (96 mg, 0.31 mmol), **13d** (88 mg, 0.34 mmol), T3P (0.201 mL, 0.34 mmol) and DIEA (0.060 mL, 0.34 mmol) in EtOAc (4 mL) and THF (dry, 2.00 mL) was stirred at 60 °C for 4 h. The mixture was neutralized with sat. $NaHCO_3$ aq. at rt and extracted with

EtOAc. The organic layer was separated, washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, eluted with 50%–100% EtOAc in hexane) to give white solid. The solid was suspended in hexane/IPE and filtered to give **29** (42.0 mg, 0.076 mmol, 22 %) as white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.27–0.51 (4H, m), 1.17 (4H, t, J = 6.8 Hz), 3.40–3.50 (1H, m), 3.58 (1H, dd, J = 8.7, 4.5 Hz), 3.82 (2H, d, J = 7.2 Hz), 3.90 (1H, dd, J = 8.7, 5.7 Hz), 3.95–4.03 (1H, m), 4.04–4.16 (3H, m), 4.72 (1H, brs), 6.65 (1H, d, J = 9.1 Hz), 7.09 (1H, d, J = 1.5 Hz), 7.35 (1H, d, J = 9.1 Hz), 7.61 (2H, d, J = 6.8 Hz), 7.82 (1H, dd, J = 8.7, 2.3 Hz), 8.36 (1H, d, J = 2.3 Hz), 9.34 (1H, s), 10.33 (1H, s); ^{13}C NMR (101 MHz, DMSO-d_6) δ 3.5, 9.8, 12.4, 38.1, 45.2, 48.7, 52.5, 68.6, 72.3, 102.8, 114.7, 114.9, 116.0, 116.4, 117.0, 117.5, 126.5, 133.9, 134.5, 134.9, 135.8, 145.4, 149.7, 153.9, 160.9, 168.8; MS (ESI) m/z: 551.4 $[M+H]^+$.

1-(*tert*-Butyl) 3-ethyl (±)-*cis*-4-(3-chloro-4-cyanobenzamido)pyrrolidine-1,3-dicarboxylate (31).

T3P (3.93 mL, 6.61 mmol) was added to a mixture of **30** (1.299 g, 4.41 mmol), 3-chloro-4-cyanobenzoic acid (0.800 mg, 4.41 mmol), DIEA(4.60 mL, 26.44 mmol) and EtOAc (35 mL) at rt. After being stirred at 60 °C for 15 h, the reaction mixture was poured into $NaHCO_3$ aq. (half-saturated, 100 mL) and extracted with EtOAc (3 times). The organic layer was washed with water and brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residual oil was purified by column chromatography (silica gel, eluted with 8%–45% EtOAc in hexane) to give **31** (1.38 g, 3.27 mmol, 74 %) as a colorless amorphous solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.28 (3H, t), 1.46 (9H, s), 3.29 (1H, brs), 3.46 (1H, brs), 3.66–3.88 (3H, m), 4.21 (2H, q, J = 6.9 Hz), 4.82-4.93 (1H, m), 7.12–7.51 (1H, m), 7.71 (1H, d), 7.77 (1H, d), 7.93 (1H, s); MS (ESI) m/z: 420.2 $[M-H]^-$.

(±)-*cis*-1-(*tert*-Butoxycarbonyl)-4-(3-chloro-4-cyanobenzamido)pyrrolidine-3-carboxylic acid (32).

2 N lithium hydroxide (9.81 mL, 19.63 mmol) was added to a solution of **31** (1.38 g, 3.27 mmol) in THF (11 mL)–EtOH (11 mL) at rt. After being stirred at rt for 1 h, the reaction mixture was poured into iced water (100 mL), acidified (pH 4) with 1 N HCl and extracted with EtOAc (3 times). The organic layer was washed with water and brine, dried over $MgSO_4$ and concentrated *in vacuo* to give **32** (1.28 g, 3.25 mmol, 99 %) as a colorless amorphous solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.45 (9H, s), 3.14-3.91 (6H, m), 4.69-5.00 (1H, m), 7.44 (1H, br s), 7.71-7.79 (2H, m), 7.89-7.96 (1H, m); MS (ESI) m/z: 392.2 $[M-H]^-$.

***tert*-Butyl (±)-*cis*-3-(3-chloro-4-cyanobenzamido)-4-{[3-(cyclopropylmethyl)-1-ethyl-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-6-yl]carbamoyl}pyrrolidine-1-carboxylate (33)**.

T3P (1.447 mL, 2.43 mmol) was added to a solution of **32** (639 mg, 1.62 mmol), **13d** (421 mg, 1.62 mmol) and DIEA (1.413 mL, 8.11 mmol) in EtOAc (10 mL) at rt. After being stirred at 60 °C for 15 h, the reaction mixture was poured into $NaHCO_3$ aq. (half-saturated, 70 mL) and extracted with EtOAc (3 times). The organic layer was washed with water and brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residual oil was purified by column chromatography on silica gel (eluted with 20%– 80% EtOAc in hexane) to give **33** (672 mg, 1.058 mmol, 65%) as a colorless solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.39–0.50 (4H, m), 1.33 (3H, t, J = 7.0 Hz), 1.46 (9H, s), 3.34–3.99 (8H, m), 4.17 (2H, q), 4.84–4.94 (1H, m), 7.17 (1H, d, J = 9.1 Hz), 7.61–7.96 (4H, m), 8.03 (1H, d, J = 9.1 Hz), 8.09 (1H, d, J = 2.3 Hz), 8.46–8.66 (1H, m); MS (ESI) m/z: 633.4 $[M-H]^-$.

(±)-*cis*-4-(3-Chloro-4-cyanobenzamido)-*N*-[3-(cyclopropylmethyl)-1-ethyl-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-6-yl]pyrrolidine-3-carboxamide hydrochloride (34).

4 N HCl in cyclopentyl methyl ether (7.0 mL, 28.00 mmol) was added to a mixture of **33** (665 mg, 1.05 mmol) and MeOH (2.8 mL) at rt. After being stirred at rt for 1 h, the reaction mixture was diluted with Et_2O (15 mL), and the crystals were collected by filtration and washed with Et_2O to give **34** (412 mg, 0.721 mmol, 69 %) as colorless powder. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.32–0.38 (2H, m), 0.39–0.48 (2H, m), 1.19 (3H, t, J = 6.8 Hz), 3.39–3.46 (2H, m), 3.51–3.72 (4H, m), 3.83 (2H, d, J = 7.2 Hz), 4.11 (2H, q), 5.01–5.12 (1H, m), 7.42 (1H, d, J = 9.1 Hz), 7.73 (1H, dd, J = 9.1, 2.3 Hz), 7.77–7.83 (2H, m), 8.01 (1H, d, J = 7.9 Hz), 8.29 (1H, d, J = 2.6 Hz), 9.08 (1H, d, J = 7.9 Hz), 9.41 (2H, brs), 10.47 (1H, s); MS (ESI) m/z: 535.4 $[M+H-HCl]^+$.

(±)-*cis*-4-(3-Chloro-4-cyanobenzamido)-*N*-[3-(cyclopropylmethyl)-1-ethyl-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-6-yl]-1-methylpyrrolidine-3-carboxamide (35).

Sodium triacetoxyborohydride (527 mg, 2.48 mmol) was added to a mixture of **34** (284 mg, 0.50 mmol), 36% formaldehyde (190 μL, 2.48 mmol) and MeOH (7 mL) at rt. After being stirred at rt for 1.5 h, the reaction mixture was poured into sat. $NaHCO_3$ aq. (60 mL) and extracted with EtOAc-THF (3:1, 3 times). The organic layer was washed with water and brine, dried over $MgSO_4$ and concentrated *in vacuo* to give crystals, which were collected by filtration and washed with Et_2O to give **35** (94.7 mg, 0.172 mmol, 35%) as colorless powder.

^{1}H NMR (300 MHz, DMSO-d_6) δ 0.32–0.39 (2H, m), 0.40–0.48 (2H, m), 1.13–1.25 (4H, m), 2.34 (3H, s), 2.83 (1H, t), 2.93–3.09 (2H, m), 3.43 (2H, q, J = 8.3 Hz), 3.83 (2H, d, J = 7.2 Hz), 4.08 (2H, q, J = 6.5 Hz), 4.90 (1H, quin, J = 8.2 Hz), 7.33 (1H, d, J = 9.1 Hz), 7.59 (1H, s), 7.65–7.72 (2H, m), 7.89 (1H, d, J = 7.9 Hz), 8.24 (1H, d, J = 2.6 Hz), 8.76 (1H, d, J = 8.3 Hz), 10.13 (1H, s); ^{13}C NMR (101 MHz, DMSO-d_6) δ 3.5, 9.8, 12.4, 41.6, 45.2, 48.8, 50.6, 51.4, 57.0, 59.2, 113.6, 114.6, 114.9, 115.3, 117.5, 126.4, 126.7, 128.3, 133.9, 134.3, 134.8, 134.9, 139.8, 149.7, 160.8, 164.1, 169.5; MS (ESI) m/z: 549.4 $[M+H]^+$.

(±)-*cis*-1-Acetyl-4-(3-chloro-4-cyanobenzamido)-*N*-[3-(cyclopropylmethyl)-1-ethyl-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-6-yl]pyrrolidine-3-carboxamide (36).

Ac_2O (56 μL, 0.59 mmol) was added to a mixture of **34** (170 mg, 0.30 mmol), pyridine (72.2 μL, 0.89 mmol) and THF (6 mL) at rt. After being stirred at rt for 3.5 h, the reaction mixture was poured into 0.1 N HCl (60 mL) and extracted with EtOAc-THF (3:1, 3 times). The organic layer was washed successively with sat. $NaHCO_3$ aq., water and brine, dried over $MgSO_4$ and concentrated *in vacuo* to give crystals, which were collected by filtration and washed with EtOAc/IPE to give **36** (117.4 mg, 0.203 mmol, 68 %) as colorless powder. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.32–0.38 (2H, m), 0.39–0.46 (2H, m), 1.14–1.25 (4H, m), 1.95–2.02 (3H, m), 3.37–3.94 (7H, m), 4.11 (2H, q, J = 6.8 Hz), 4.93–5.07 (1H, m), 7.43 (1H, d, J = 9.4 Hz), 7.75–7.82 (2H, m), 7.86 (1H, s), 8.02 (1H, d, J = 7.9 Hz), 8.26 (1H, dd, J = 6.6, 2.5 Hz), 8.97 (1H, t, J = 8.7 Hz), 10.28 (1H, d, J = 7.6 Hz); ^{13}C NMR (75 MHz, DMSO-d_6) δ 4.0, 10.3, 12.9, 23.3, 45.8, 47.3, 48.9, 50.8, 52.0, 67.8, 114.5, 115.3, 115.5, 116.0, 118.4, 127.4, 129.0, 134.4, 135.2, 135.6, 135.7, 140.4, 150.3, 161.4, 164.7, 168.5, 168.7, 169.0; MS (ESI) m/z: 577.4 $[M+H]^+$.

***tert*-Butyl 4-(3-chloro-4-cyanophenyl)-1,4-diazepane-1-carboxylate (38)**.

A mixture of *tert*-butyl 1,4-diazepane-1-carboxylate **37** (0.292 mL, 1.50 mmol), 2-chloro-4-fluorobenzonitrile (350 mg, 2.25 mmol), and K_2CO_3 (621 mg, 4.49 mmol) in DMF (6 mL) was stirred at 60 °C for 16 h. The reaction mixture was quenched with water at ambient temperature and extracted with EtOAc. The organic layer was separated, washed with water and brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, eluted with 10%–100% EtOAc in hexane) to give **38** (504 mg, 1.500 mmol, 100 %) as colorless viscous oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.28–1.52 (9H, m), 1.94 (2H, t, J = 5.9 Hz), 3.19–3.43 (2H, m), 3.49–3.70 (6H, m), 6.57 (1H, dd, J = 8.9, 2.5 Hz), 6.70 (1H, d, J = 2.3 Hz), 7.42 (1H, d, J = 8.7 Hz); MS (ESI) m/z: 236.2 $[M-Boc+H]^+$.

2-Chloro-4-(1,4-diazepan-1-yl)benzonitrile (39).

A mixture of **38** (490 mg, 1.46 mmol) in 4 M HCl in EtOAc (4 mL, 16.00 mmol) and EtOAc (8 mL) was stirred at rt overnight, and then concentrated *in vacuo*. The residue was suspended in EtOAc, and the insoluble materials were collected by filtration and rinsed with EtOAc. The hydrochloride salt thus obtained was suspended in EtOAc, and the mixture was washed with 1M aq. NaOH, water, and brine. The organic layer was dried over $MgSO_4$ and concentrated *in vacuo* to give **39** (266 mg, 1.128 mmol, 77 %) as colorless amorphous solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.72 (2H, quin, J = 5.9 Hz), 2.56-2.68 (2H, m), 2.77-2.90 (2H, m), 3.43-3.55 (2H, m), 3.59 (2H, t, J = 6.0 Hz), 6.76 (1H, dd, J = 9.1, 2.6 Hz), 6.90 (1H, d, J = 2.6 Hz), 7.56 (1H, d, J = 9.1 Hz) *NH was disappeared ; MS (ESI) m/z: 236.2 $[M+H]^+$.

2-Chloro-4-(piperidin-3-ylmethoxy)benzonitrile (42).

A mixture of 2-chloro-4-fluorobenzonitrile (636 mg, 4.09 mmol), K_2CO_3 (565 mg, 4.09 mmol) and **40** (800 mg, 3.72 mmol) in DMSO (5 mL) was stirred at 50 °C overnight. The mixture was quenched with brine at rt and extracted with EtOAc. The organic layer was separated, washed with brine, dried over $MgSO_4$ and concentrated *in vacuo*. To the residue was added TFA (3 mL), which was stirred at rt overnight. The mixture was concentrated *in vacuo*. The mixture was quenched with 1N NaOH aq. at rt and extracted with EtOAc. The organic layer was separated, washed with brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography (NH silica gel, eluted with 10%–100% EtOAc in hexane) to give **42** (320 mg, 1.276 mmol, 34%) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.13–1.39 (1H, m), 1.47–1.82 (2H, m), 1.81–1.96 (2H, m), 2.03 (1H,m), 2.39–2.78 (2H, m), 3.04 (1H, dt, J = 12.1, 3.8 Hz), 3.21 (1H, dd, J = 11.9, 3.6 Hz), 3.76-3.97 (2H, m), 6.84 (1H, dd, J = 8.9, 2.5 Hz), 7.00 (1H, d, J = 2.3 Hz), 7.56 (1H, d, J = 8.7 Hz); MS (ESI) m/z: 251.2 $[M+H]^+$.

2-Chloro-4-(morpholin-2-ylmethoxy)benzonitrile hydrochloride (43).

Step A: ***tert*-Butyl 2-[(3-chloro-4-cyanophenoxy)methyl]morpholine-4-carboxylate**.

To a mixture of *tert*-butyl 2-(hydroxymethyl)morpholine-4-carboxylate (**41**, 931 mg, 4.29 mmol) and K_2CO_3 (1184 mg, 8.57 mmol) in dry DMF (20 mL), 2-chloro-4-fluorobenzonitrile (667 mg, 4.29 mmol) was added at rt. After being stirred overnight, the reaction was allowed to warm to 60 °C and was stirred for overnight. The reaction mixture was quenched with water at rt and extracted with EtOAc. The organic layer was separated, washed with water and brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column

chromatography (silica gel, eluted with 5%–30% EtOAc in hexane) to give *tert*-butyl 2-[(3-chloro-4-cyanophenoxy)methyl]morpholine-4-carboxylate as a white solid.

Step B: **2-Chloro-4-(morpholin-2-ylmethoxy)benzonitrile hydrochloride**.

To a solution of *tert*-butyl 2-[(3-chloro-4-cyanophenoxy)methyl]morpholine-

4-carboxylate in MeOH (5 mL), 4 N HCl in cyclopentyl methyl ether (1.1 ml, 4.29 mmol) was added dropwise at rt. After being stirred over weekend, the reaction mixture was concentrated *in vacuo* to give **43** (259 mg, 0.896 mmol, 21 % in 2 steps) as a white amorphous solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.82–3.10 (2H, m), 3.20 (1H, d, J = 12.5 Hz), 3.30 (1H, s), 3.75–3.92 (1H, m), 3.92–4.34 (4H, m), 7.13 (1H, dd, J = 8.9, 2.5 Hz), 7.39 (1H, d, J = 2.3 Hz), 7.83–8.05 (1H, m), 9.70 (2H, brs); MS (ESI) m/z: 253.1 $[M\text{-}HCl+H]^+$.

***tert*-Butyl 2-[(3-chloro-4-cyanophenyl)carbamoyl]morpholine-4-carboxylate (45)**.

To a solution of 4-(*tert*-butoxycarbonyl)morpholine-2-carboxylic acid (**44**, 110 mg, 0.48 mmol), 4-amino-2-chlorobenzonitrile (72.6 mg, 0.48 mmol) and DIEA (0.125 mL, 0.71 mmol) in EtOAc (5 mL), T3P (0.308 mL, 0.52 mmol) was added at rt. After being stirred at 60 °C for 41 h, the reaction mixture was quenched with sat. NH_4Cl aq.at rt and extracted with EtOAc. The organic layer was separated, washed with water and brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, eluted with 10%–50%EtOAc in hexane) to give **45** (116 mg, 0.316 mmol, 66 %) as a white amorphous solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.49 (9H, s), 2.72–3.05 (2H, m), 3.66 (1H, td, J = 11.7, 2.6 Hz), 3.88–4.10 (3H, m), 4.42 (1H, d, J = 13.2 Hz), 7.48–7.59 (1H, m), 7.59–7.67 (1H, m), 7.93 (1H, d, J = 1.5 Hz), 8.51 (1H, s); MS (ESI) m/z: 364.2 $[M\text{-}H]^-$.

***N*-(3-Chloro-4-cyanophenyl)morpholine-2-carboxamide 2,2,2-trifluoroacetate (46)**.

To **45** (115.5 mg, 0.32 mmol), TFA (2 mL) was added at rt. After being stirred at rt for 4 h, the reaction mixture was concentrated *in vacuo* to give orange oil. The residual oil was dissolved in IPE (ca. 5 mL), filtered off and rinsed with IPE to give **46** (81 mg, 0.214 mmol, 68%) as a brown solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.98–3.32 (2H, m), 3.48 (2H, d, J = 2.3 Hz), 3.77–3.95 (1H, m), 4.12 (1H, d, J = 12.1 Hz), 4.48 (1H, dd, J = 10.0, 2.5 Hz), 7.74–7.85 (1H, m), 7.87–8.00 (1H, m), 8.13 (1H, d, J = 1.9 Hz), 9.11 (1H, brs), 10.63 (1H, s); MS (ESI) m/z: 266.2 $[M\text{-}TFA+H]^+$.

***N*-(3-Chloro-4-cyanophenyl)morpholine-2-carboxamide hydrochloride (47)**.

To a stirred solution of **45** (995 mg, 2.72 mmol) in EtOAc (10 mL) was added 4N HCl in EtOAc (10 mL, 40.00 mmol). After being stirred at rt overnight, the mixture was concentrated

in vacuo. The residue was triturated with EtOAc to give **47** (731.0 mg, 2.419 mmol, 89 %) as an off-white amorphous solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.92–3.26 (3H, m), 3.39–3.54 (1H, m), 3.83–4.00 (1H, m), 4.05–4.17 (1H, m), 4.58 (1H, dd, J = 10.6, 2.6 Hz), 7.75–7.88 (1H, m), 7.90–7.97 (1H, m), 8.14 (1H, d, J = 1.9 Hz), 9.61 (2H, brs), 10.78 (1H, s); MS (ESI) m/z: 266.2 $[M\text{-}HCl+H]^+$.

4-(3-Chloro-4-cyanophenyl)-*N*-[3-(cyclopropylmethyl)-1-ethyl-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-6-yl]-1,4-diazepane-1-carboxamide (48).

To an ice-cooled solution of **13d** (138 mg, 0.53 mmol) and DIEA (0.111 mL, 0.64 mmol) in THF (4 mL) was added bis(trichloromethyl) carbonate (189 mg, 0.64 mmol). After being stirred at 0 °C for 1 h, a solution of **39** (100 mg, 0.42 mmol) and DIEA (0.185 mL, 1.06 mmol) in THF (4.00 mL) was added, and the reaction mixture was stirred at rt for 3 days. The mixture was quenched with water at ambient temperature and extracted with EtOAc. The organic layer was separated, washed with water and brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, eluted with 10%–100% EtOAc in hexane) to give **48** (23.60 mg, 0.045 mmol, 11%) as a colorless amorphous solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.23–0.54 (4H, m), 1.07–1.37 (4H, m), 1.79–1.94 (2H, m), 3.37–3.52 (2H, m), 3.52–3.78 (6H, m), 3.78–3.92 (2H, m), 4.08–4.23 (2H, m), 6.84 (1H, d, J = 9.1 Hz), 6.99 (1H, s), 7.39 (1H, d, J = 9.4 Hz), 7.57 (1H, d, J = 9.1 Hz), 7.85 (1H, d, J = 9.1 Hz), 8.08–8.21 (1H, m), 8.57 (1H, s); ^{13}C NMR (101 MHz, DMSO-d_6) δ 3.5, 9.9, 12.5, 24.3, 38.1, 45.2, 45.2, 45.5, 47.9, 49.6, 96.3, 110.6, 111.6, 114.3, 114.9, 117.4, 118.0, 127.6, 134.0, 134.9, 135.7, 136.8, 149.8, 151.5, 154.5, 161.1; MS (ESI) m/z: 521.4 $[M+H]^+$.

4-Nitrophenyl (3-(cyclopropylmethyl)-1-ethyl-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-6-yl)carbamate (49).

To a solution of **13d** (650 mg, 2.51 mmol) in THF (10 mL) were added successively 4-nitrophenyl carbonochloridate (581 mg, 2.88 mmol) and pyridine (0.232 mL, 2.88 mmol) at rt overnight. The reaction mixture was quenched with water at ambient temperature. The resulting precipitate was collected and rinsed with water to give crude **49** (1042 mg, 2.455 mmol, 98 %) as off-white powder. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.23–0.55 (4H, m), 1.03–1.33 (4H, m), 3.74–4.01 (2H, m), 4.06–4.27 (2H, m), 7.48–7.69 (3H, m), 7.83–7.98 (1H, m), 8.21–8.43 (3H, m), 10.68 (1H, brs).

3-[(3-Chloro-4-cyanophenoxy)methyl]-*N*-[3-(cyclopropylmethyl)-1-ethyl-2,4-

dioxo-1,2,3,4-tetrahydroquinazolin-6-yl]piperidine-1-carboxamide (50).

A mixture of **42** (70 mg, 0.28 mmol), **49** (118 mg, 0.28 mmol) and DIEA (0.049 mL, 0.28 mmol) in DMF (5 mL) was stirred at 60 °C overnight. The mixture was quenched with brine at rt and extracted with EtOAc. The organic layer was separated, washed with brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography (NH silica gel, eluted with 10%–50%EtOAc in hexane) to give **50** (120 mg, 0.224 mmol, 80%) as a colorless amorphous solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.33–0.64 (4H, m), 1.13 (1H, d, J = 6.0 Hz), 1.20–1.52 (6H, m), 1.74–2.01 (2H, m), 2.18 (1H, m), 2.99 (1H, dd, J = 13.2, 9.4 Hz), 3.10–3.37 (1H, m), 3.82 (1H, d, J = 13.2 Hz), 3.91–4.03 (3H, m), 4.04–4.36 (3H, m), 6.57 (1H, s), 6.89 (1H, dd, J = 8.7, 2.6 Hz), 7.03 (1H, d, J = 2.3 Hz), 7.16 (1H, d, J = 9.1 Hz), 7.58 (1H, d, J = 8.7 Hz), 7.77 (1H, d, J = 2.3 Hz), 8.08 (1H, dd, J = 9.1, 2.3 Hz); ^{13}C NMR (101 MHz, $CDCl_3$) δ 3.8, 10.0, 12.6, 22.9, 24.1, 26.9, 35.5, 38.8, 45.2, 46.2, 47.1, 70.5, 105.4, 113.9, 114.2, 116.0, 116.2, 118.8, 128.0, 134.5, 135.1, 135.4, 138.4, 150.5, 154.8, 161.7, 162.4; MS (ESI) m/z: 536.5 $[M+H]^+$.

2-[(3-Chloro-4-cyanophenoxy)methyl]-N-(3-(cyclopropylmethyl)-1-ethyl-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-6-yl)morpholine-4-carboxamide (51).

A mixture of **43** (80 mg, 0.28 mmol), **49** (117 mg, 0.28 mmol) and DIEA (0.193 mL, 1.11 mmol) in dry DMF (10 mL) was stirred for 3 h at rt. The mixture was quenched with water at rt and extracted with EtOAc. The organic layer was separated, washed with water and brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, eluted with 20%–80% EtOAc in hexane) and concentrated. The resulting solid was recrystallized from hexane/EtOAc to give **51** (78 mg, 0.145 mmol, 52%) as a white amorphous solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.34–0.57 (4H, m), 1.28–1.39 (4H, m), 2.78–3.10 (2H, m), 3.19 (1H, t, J = 10.8 Hz), 3.70 (1H, t, J = 10.6 Hz), 3.92–4.20 (9H, m), 6.81–7.25 (4H, m), 7.58 (1H, d, J = 8.7 Hz), 7.89 (1H, brs), 8.04 (1H, d, J = 9.1 Hz); ^{13}C NMR (101 MHz, DMSO-d_6) δ 3.5, 9.9, 12.5, 38.2, 43.7, 44.9, 45.2, 65.6, 69.4, 73.1, 103.8, 114.6, 114.9, 115.0, 116.1, 116.3, 117.6, 127.3, 134.1, 135.7, 136.9, 149.8, 155.0, 161.1, 162.2, 162.5; MS (ESI) m/z: 538.4 $[M+H]^+$.

***N*^{2}-(3-Chloro-4-cyanophenyl)-*N*^{4}-[3-(cyclopropylmethyl)-1-ethyl-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-6-yl]morpholine-2,4-dicarboxamide (52)**.

To a solution of **46** (81.3 mg, 0.31 mmol) and DIEA (0.214 mL, 1.22 mmol) in dry DMF (3 mL) was added **49** (130 mg, 0.31 mmol) at rt. After being stirred at rt overweekend, the reaction mixture was quenched with sat. NH_4Cl aq. at rt and extracted with EtOAc. The

organic layer was separated, washed with water and brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, eluted with 30%–100% EtOAc in hexane) to give **52** (108 mg, 0.196 mmol, 64%) as an off-white amorphous solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.34–0.58 (4H, m), 1.22–1.34 (1H, m), 1.38 (4H, t, J = 7.2 Hz), 3.18-3.40 (2H, m), 3.73–3.90 (1H, m), 3.94–4.08 (3H, m), 4.12 (1H, dt, J = 11.4, 3.0 Hz), 4.18–4.33 (4H, m), 7.20 (1H, d, J = 9.1 Hz), 7.55–7.65 (1H, m), 7.65–7.71 (1H, m), 7.95 (2H, dd, J = 8.7, 2.3 Hz), 8.00–8.08 (1H, m), 8.59 (1H, s); ^{13}C NMR (75 MHz, DMSO-d_6) δ 4.0, 10.5, 12.8, 36.3, 38.7, 43.8, 45.7, 45.8, 66.3, 75.3, 115.1, 115.5, 116.6, 118.2, 119.1, 120.5, 127.8, 134.7, 135.6, 136.1, 136.2, 144.1, 150.3, 155.4, 161.6, 169.1; MS (ESI) m/z: 551.4 $[M+H]^+$.

3-(Cyclopropylmethyl)-1-isobutyl-6-nitroquinazoline-2,4(1*H*,3*H*)-dione (54a).

A mixture of **53** (500 mg, 1.91 mmol), K_2CO_3 (794 mg, 5.74 mmol), and 1-bromo-2-methylpropane (0.624 mL, 5.74 mmol) in DMF (10 mL) was stirred at 70 °C for 5 h. The mixture was quenched with water and extracted with EtOAc. The organic layer was separated, washed with water and brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, eluted with EtOAc in hexane) to give **54a** (308.1 mg, 0.971 mmol, 51 %) as a yellow powder. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.38–0.55 (4H, m), 1.03 (6H, d, J = 6.8 Hz), 1.20–1.39 (1H, m), 2.19 (1H, m), 4.01 (2H, d, J = 7.2 Hz), 4.07 (2H, d, J = 7.6 Hz), 7.29 (1H, d, J = 9.1 Hz), 8.44–8.50 (1H, m), 9.10 (1H, d, J = 3.0 Hz).

3-(Cyclopropylmethyl)-1-isopropyl-6-nitroquinazoline-2,4(1*H*,3*H*)-dione (54b). 2-Iodopropane (1.146 mL, 11.48 mmol) was added to a mixture of **53** (1.00 g, 3.83 mmol), Cs_2CO_3 (1.871 g, 5.74 mmol) and DMF (22 mL) at rt. After being stirred at 50 °C for 2 h, 2-iodopropane (1.146 mL, 11.48 mmol) and Cs_2CO_3 (1.871 g, 5.74 mmol) were added thereto, and the whole was stirred at 65 °C for further 18 h. The reaction mixture was poured into water (180 mL) and extracted with EtOAc (3 times). The organic layer was washed with water and brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residual oil was purified by column chromatography (silica gel, eluted with 2%–20% EtOAc in hexane) to give 3-(cyclopropylmethyl)-2-isopropoxy-6-nitroquinazolin-4(3*H*)-one (518 mg, 1.708 mmol, 45 %, less polar) as colorless powder and **54b** (447 mg, 1.474 mmol, 39 %, more polar) as colorless powder. Each crystal was collected by filtration and washed with cold hexane. 3-(cyclopropylmethyl)-2-isopropoxy-6-nitroquinazolin-4(3*H*)-one: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.42–0.57 (4H, m), 1.17–1.32 (1H, m), 1.47 (6H, d, J = 6.0 Hz), 3.99 (2H, d, J = 7.2 Hz), 5.59 (1H, m), 7.52 (1H, d, J = 8.7 Hz), 8.42 (1H, dd, J = 9.1, 2.6 Hz), 9.06 (1H, d, J = 2.6 Hz); MS (ESI) m/z: 304.2 $[M+H]^+$.

54b: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.42–0.55 (4H, m), 1.24–1.36 (1H, m), 1.65 (6H, d, J = 6.8 Hz), 3.98 (2H, d, J = 7.2 Hz), 5.12 (1H, brs), 7.48 (1H, d, J = 9.1 Hz), 8.45 (1H, dd, J = 9.3, 2.8 Hz), 9.10 (1H, d, J = 2.6 Hz); MS (ESI) m/z: 304.3 $[M+H]^+$.

6-Amino-3-(cyclopropylmethyl)-1-isobutylquinazoline-2,4(1*H*,3*H*)-dione (55a).

To a solution of **54a** (300 mg, 0.95 mmol) in MeOH (6.0 mL) was added Pd-C (30 mg, 0.28 mmol) and the mixture was stirred at rt for 5 h under hydrogen atmosphere (1 atm). The mixture was filtered through celite pad. The filtrate was concentrated *in vacuo* to give **54a** (235.2 mg, 0.818 mmol, 87 %) as a gray solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.23-0.37 (2H, m), 0.36-0.45 (2H, m), 0.89 (6H, d, J = 6.8 Hz), 1.05-1.30 (1H, m), 2.07 (1H, dt, J = 13.7, 6.8 Hz), 3.83 (2H, d, J = 7.1 Hz), 3.90 (2H, d, J = 7.6 Hz), 5.12-5.43 (2H, m), 6.94-7.09 (1H, m), 7.13-7.37 (2H, m); MS (ESI) m/z: 288.3 $[M+H]^+$.

6-Amino-3-(cyclopropylmethyl)-1-isopropylquinazoline-2,4(1*H*,3*H*)-dione (55b).

A solution of **54b** (442 mg, 1.46 mmol) in MeOH (7 mL) - THF (3.5 mL) was hydrogenated in the presence of 10 % Pd-C (50 % wet, 150 mg, 1.27 mmol) at rt under ordinary pressure for 2 h. After removal of the catalyst by filtration, the filtrate was concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, eluted with 10%–50% EtOAc in hexane) to give crystals, which were collected by filtration and washed with IPE/hexane to give **55b** (318 mg, 1.163 mmol, 80 %) as pale yellow powder. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.42–0.48 (4H, m), 1.23–1.36 (1H, m), 1.59 (6H, d, J = 7.2 Hz), 3.73 (2H, brs), 3.96 (2H, d, J = 7.2 Hz), 5.01 (1H, brs), 6.99 (1H, dd, J = 8.9, 2.8 Hz), 7.20 (1H, d, J = 9.1 Hz), 7.51 (1H, d, J = 3.0 Hz); MS (ESI) m/z: 274.2 $[M+H]^+$.

***N*^{2}-(3-Chloro-4-cyanophenyl)-*N*^{4}-[3-(cyclopropylmethyl)-1-isobutyl-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-6-yl]morpholine-2,4-dicarboxamide (56a)**.

Step A: **4-Nitrophenyl [3-(cyclopropylmethyl)-1-isobutyl-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-6-yl]carbamate**.

To a stirred solution of **55a** (230 mg, 0.80 mmol) in THF (4.0 mL) were added successively 4-nitrophenyl carbonochloridate (186 mg, 0.92 mmol) and pyridine (0.074 mL, 0.92 mmol) at rt, and the mixture was stirred at rt for 2 h. The reaction mixture was quenched with water at ambient temperature. The mixture was quenched with water and extracted with EtOAc. The organic layer was separated and dried over $MgSO_4$ and concentrated *in vacuo*. The residue was triturated with Et_2O to give 4-nitrophenyl [3-(cyclopropylmethyl)-1-isobutyl-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-6-

yl]carbamate (288.3 mg, 0.637 mmol, 80 %) as an off-white solid. This product was subjected to the next reaction without further purification.

Step B: ***N*²-(3-Chloro-4-cyanophenyl)-*N*⁴-[3-(cyclopropylmethyl)-1-isobutyl-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-6-yl]morpholine-2,4-dicarboxamide**.

To a stirred solution of **47** (50 mg, 0.17 mmol) and 4-nitrophenyl (3-(cyclopropylmethyl)-1-isobutyl-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-6-yl)carbamate (82 mg, 0.18 mmol) in DMF (2 mL) was added DIEA (0.058 mL, 0.33 mmol), and the mixture was stirred at rt for 2 h. The mixture was quenched with water and extracted with EtOAc. The organic layer was separated and dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, eluted with EtOAc) and crystallized from EtOAc-hexane to give **56a** (74.4 mg, 0.128 mmol, 78 %) as colorless crystals. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.31–0.47 (4H, m), 0.92 (6H, d, J = 6.4 Hz), 1.13–1.32 (1H, m), 2.02–2.19 (1H, m), 3.09 (2H, dd, J = 12.8, 9.8 Hz), 3.59–3.73 (1H, m), 3.80–4.10 (6H, m), 4.17–4.37 (2H, m), 7.43 (1H, d, J = 9.1 Hz), 7.81–7.98 (3H, m), 8.19 (2H, dd, J = 12.5, 2.3 Hz), 8.96 (1H, s), 10.47 (1H, s); ^{13}C NMR (101 MHz, DMSO-d_6) δ 3.4, 9.7, 19.6, 26.5, 43.3, 45.2, 45.3, 49.3, 65.8, 74.8, 94.9, 106.1, 114.9, 115.2, 116.2, 117.7, 118.7, 120.1, 127.2, 134.7, 135.1, 135.6, 135.7, 143.6, 150.5, 154.9, 161.3, 168.6; MS (ESI) m/z: 579.5 $[M+H]^+$.

***N*²-(3-Chloro-4-cyanophenyl)-*N*⁴-[3-(cyclopropylmethyl)-1-isopropyl-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-6-yl]morpholine-2,4-dicarboxamide (56b)**. 4-Nitrophenyl carbonochloridate (61.4 mg, 0.30 mmol) was added to a solution of **55b** (72.4 mg, 0.26 mmol) and pyridine (25 μL, 0.31 mmol) in THF (1 mL) at rt, and the mixture was stirred at rt for 2 h. The reaction mixture was concentrated *in vacuo*. The residue was dissolved in DMF (3 mL), and **47** (80 mg, 0.26 mmol) and DIEA (115 μL, 0.66 mmol) were added thereto at rt for 1 h. The reaction mixture was poured into water (60 mL), acidified (pH 3) with 2 N HCl and extracted with EtOAc. The organic layer was washed with water and brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residual oil was purified by column chromatography on silica gel (30%–100% EtOAc in hexane) to give crystals, which were collected by filtration and washed with IPE/hexane to give **56b** (111.7 mg, 0.198 mmol, 75 %) as colorless powder. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.42–0.49 (4H, m), 1.26–1.35 (1H, m), 1.61 (6H, d, J = 7.2 Hz), 3.18–3.33 (2H, m), 3.74–3.84 (1H, m), 3.93–4.03 (3H, m), 4.05–4.12 (1H, m), 4.22 (2H, dd, J = 9.8, 3.0 Hz), 5.04 (1H, brs), 6.99 (1H, s), 7.33 (1H, d, J = 9.1 Hz), 7.57 (1H, dd), 7.64 (1H, d), 7.89–7.98 (3H, m), 8.55 (1H, s); ^{13}C NMR (101 MHz, DMSO-d_6) δ 3.5, 9.9,

19.4, 43.3, 45.0, 45.3, 48.0, 65.8, 74.8, 105.9, 115.1, 115.4, 116.1, 117.8, 118.6, 120.0, 127.0, 134.5, 135.1, 135.4, 135.7, 143.6, 149.6, 154.9, 161.2, 168.5; MS (ESI) m/z: 565.5 $[M+H]^+$.

N^4-[1,3-Bis(cyclopropylmethyl)-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-6-yl]-N^2-(3-chloro-4-cyanophenyl)morpholine-2,4-dicarboxamide (56c).

Step A: **4-Nitrophenyl [1,3-bis(cyclopropylmethyl)-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-6-yl]carbamate**.

To a solution of **13c** (200 mg, 0.70 mmol) in THF (4.0 mL) were added successively 4-nitrophenyl carbonochloridate (162 mg, 0.81 mmol) and pyridine (0.065 mL, 0.81 mmol) at rt, and the mixture was stirred at rt for 2 h. The mixture was quenched with water and extracted with EtOAc. The organic layer was separated and dried over $MgSO_4$ and concentrated *in vacuo*. The residue was triturated with Et_2O-hexane to give 4-nitrophenyl [1,3-bis(cyclopropylmethyl)-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-6-yl]carbamate (282.3 mg, 0.627 mmol, 89 %) as an off-white solid. This product was subjected to the next reaction without further purification.

Step B: **N^4-[1,3-Bis(cyclopropylmethyl)-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-6-yl]-N^2-(3-chloro-4-cyanophenyl)morpholine-2,4-dicarboxamide**.

To a mixture of **47** (50 mg, 0.17 mmol), 4-nitrophenyl [1,3-bis(cyclopropylmethyl)-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-6-yl]carbamate (74.5 mg, 0.17 mmol), and DMF (2 mL) was added DIEA (0.058 mL, 0.33 mmol), and the mixture was stirred at rt for 1 h. Water was added and the mixture was extracted with EtOAc. The organic layer was washed with water and brine, dried over $MgSO_4$, filtered and concentrated. The residue was purified by column chromatography (silica gel, eluted with 50%–100% EtOAc in hexane) and crystallized from EtOAc-hexane to give **56c** (34.9 mg, 0.060 mmol, 37%) as colorless crystals. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.31–0.53 (8H, m), 1.19–1.31 (2H, m), 3.02–3.17 (2H, m), 3.59–3.73 (1H, m), 3.86 (2H, d, J = 7.2 Hz), 3.92–4.11 (4H, m), 4.17–4.35 (2H, m), 7.53 (1H, d, J = 9.1 Hz), 7.82–7.98 (3H, m), 8.19 (2H, dd, J = 14.0, 2.3 Hz), 8.96 (1H, s), 10.47 (1H, s); ^{13}C NMR (101 MHz, DMSO-d_6) δ 3.4, 3.5, 9.6, 9.9, 43.3, 45.3, 46.8, 65.8, 74.8, 106.1, 107.2, 114.9, 115.1, 116.1, 117.6, 118.7, 120.0, 127.3, 134.6, 135.1, 135.6, 135.7, 143.6, 150.5, 154.9, 161.1, 168.5; MS (ESI) m/z: 577.5 $[M+H]^+$.

Optical resolution of 56b by chiral HPLC

100 mg of **56b** was was subjected to optical resolution using HPLC (column: CHIRALPAK IC, sample conc.: 10.0 mg/mL, injection volume: 10 mL (100 mg load), eluent: methanol/ethanol=50/50, pressure: 0.2 Mpa, flow rate: 80 mL/min, detecter: UV220 nm, 30 ^{o}C) to give **57** and **58** (47 mg each).

57: tR1=13.67 min. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.42-0.48 (4H, m), 1.25-1.35 (1H, m), 1.61 (6H, d, J = 6.8 Hz), 3.18-3.33 (2H, m), 3.74-3.84 (1H, m), 3.93-4.02 (3H, m), 4.09 (1H, dt, J = 11.7, 3.0 Hz), 4.19-4.26 (2H, m), 5.04 (1H, br s), 6.99 (1H, br s), 7.33 (1H, d, J = 9.1 Hz), 7.57 (1H, dd, J = 8.6, 1.9 Hz), 7.64 (1H, d, J = 8.6 Hz), 7.89 (1H, d, J = 1.9 Hz), 7.93 (1H, d, J = 2.5 Hz), 7.96 (1H, dd, J = 9.0, 2.5 Hz), 8.55 (1H, s); ^{13}C NMR (75 MHz, DMSO-d_6) δ 4.0, 10.4, 19.9, 43.8, 45.5, 45.8, 48.5, 66.3, 75.3, 106.6, 115.6, 115.9, 116.7, 118.3, 119.1, 120.5, 127.3, 135.0, 135.6, 135.9, 136.3, 144.1, 150.1, 155.4, 161.7, 169.1; MS (ESI) m/z: 565.5 $[M+H]^+$; $[\alpha]_D^{25}$ -66.5 (c 0.25, MeOH).

58: tR2=16.47 min. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.41-0.48 (4H, m), 1.25-1.34 (1H, m), 1.61 (6H, d, J = 6.8 Hz), 3.18-3.32 (2H, m), 3.74-3.83 (1H, m), 3.93-4.02 (3H, m), 4.08 (1H, dt, J = 11.4, 3.0 Hz), 4.18-4.27 (2H, m), 5.04 (1H, br s), 7.02 (1H, br s), 7.33 (1H, d, J = 9.1 Hz), 7.57 (1H, dd, J = 8.6, 1.9 Hz), 7.63 (1H, d, J = 8.6 Hz), 7.89 (1H, d, J = 1.9 Hz), 7.93 (1H, d, J = 2.5 Hz), 7.96 (1H, dd, J = 9.0, 2.7 Hz), 8.56 (1H, s); ^{13}C NMR (101 MHz, DMSO-d_6) δ 3.5, 9.9, 19.4, 43.3, 45.0, 45.3, 48.0, 65.8, 74.8, 106.1, 115.1, 115.4, 116.1, 117.8, 118.6, 120.0, 126.9, 134.6, 135.1, 135.4, 135.7, 143.5, 149.6, 154.9, 161.2, 168.5; MS (ESI) m/z: 565.5 $[M+H]^+$.

Asymmetric synthesis of compound 57

(*R*)-*N*-(3-Chloro-4-cyanophenyl)morpholine-2-carboxamide (60).

Step A: **(*R*)-*tert*-Butyl 2-[(3-chloro-4-cyanophenyl)carbamoyl]morpholine-4-carboxylate**.

HATU (3.86 g, 10.14 mmol) was added to a solution of 4-amino-2-chlorobenzonitrile (1.19 g, 7.80 mmol), **59** (1.984 g, 8.58 mmol) and DIEA (2.72 mL, 15.60 mmol) in DMF (40 mL) at rt. After being stirred at rt for 16 h, the reaction mixture was poured into $NaHCO_3$ aq. (half-saturated, 200 mL) and extracted with EtOAc (3 times). The organic layer was washed with water and brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residual oil was purified by column chromatography on silica gel (eluted with 10%–60% EtOAc in hexane) to give (*R*)-*tert*-butyl 2-[(3-chloro-4-cyanophenyl)carbamoyl]morpholine-4-carboxylate (0.690 g,

1.886 mmol, 24%) as a colorless amorphous solid. MS (ESI) m/z: 364.1 $(M-H)^-$. ^1H NMR (300 MHz, $CDCl_3$) δ 1.49 (9H, s), 2.78–3.00 (2H, m), 3.66 (1H, td, J = 11.7, 2.6 Hz), 3.95–4.09 (3H, m), 4.36–4.47 (1H, m), 7.54 (1H, dd), 7.62 (1H, d), 7.93 (1H, d, J = 1.9 Hz), 8.50 (1H, s).

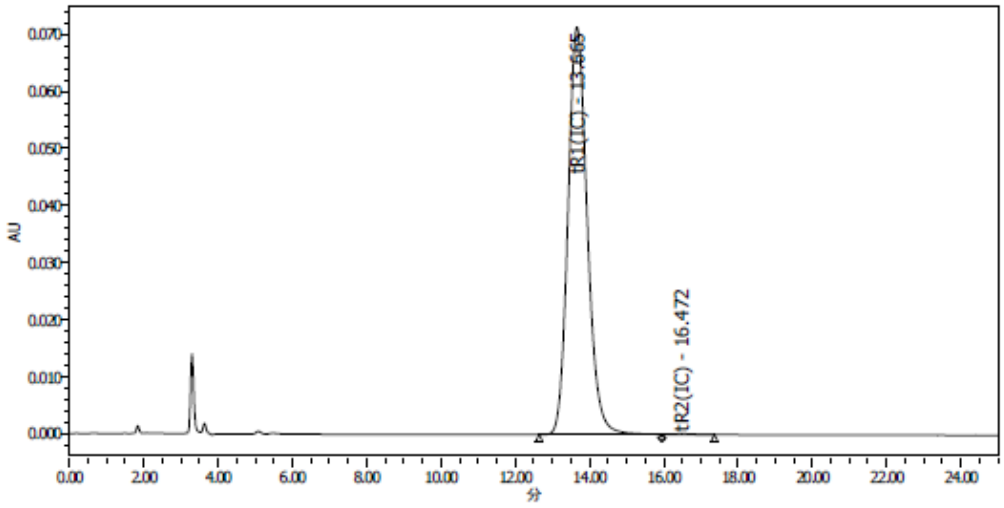
Step B: **(*R*)-*N*-(3-Chloro-4-cyanophenyl)morpholine-2-carboxamide**.

TFA (8.5 mL) was added to (*R*)-*tert*-butyl 2-[(3-chloro-4-cyanophenyl)carbamoyl]morpholine-4-carboxylate (685 mg, 1.87 mmol) at rt. After being stirred at rt for 20 min, the reaction mixture was poured into iced sat. $NaHCO_3$ aq. (80 mL), basified (pH 8) by the careful addition of K_2CO_3, saturated with NaCl and extracted with EtOAc-THF (3:1, 5 times). The organic layer was washed with brine, dried over $MgSO_4$ and concentrated *in vacuo* to give **60** (507 mg, 1.908 mmol, quant.) as a colorless amorphous solid. MS (ESI) m/z: 266.2 $(M+H)^+$. ^1H NMR (300 MHz, DMSO-d_6) δ 2.86–3.08 (3H, m), 3.31 (1H, dd, J = 12.8, 2.6 Hz), 3.73 (1H, td, J = 11.4, 2.8 Hz), 3.98–4.06 (1H, m), 4.30 (1H, dd, J = 10.0, 2.8 Hz), 7.32 (1H, brs), 7.83 (1H, dd), 7.93 (1H, d), 8.15 (1H, d, J = 1.9 Hz), 10.48 (1H, s).

(*R*)-N^2-(3-chloro-4-cyanophenyl)-N^4-[3-(cyclopropylmethyl)-1-isopropyl-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-6-yl]morpholine-2,4-dicarboxamide (57).

4-Nitrophenyl carbonochloridate (116 mg, 0.58 mmol) was added to a solution of **55b** (137 mg, 0.5 mmol) and pyridine (47 µL, 0.58 mmol) in THF (2 mL) at rt, and the mixture was stirred at rt for 1.5 h. The reaction mixture was concentrated *in vacuo*. The residue was dissolved in DMF (5.8 mL), and **60** (139 mg, 0.53 mmol) and DIEA (174 µL, 1.00 mmol) were added thereto at rt. After being stirred at rt for 1 h, the reaction mixture was poured into water (100 mL), acidified (pH 3) with 2 N HCl and extracted with EtOAc (3 times). The organic layer was washed with water and brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residual oil was purified by column chromatography on silica gel (eluted with 30%–100%EtOAc in hexane) to give **57** (174.2 mg, 0.308 mmol, 62 %) as a colorless amorphous solid. Chiral HPLC analysis showed that this compound has the same retention time as **57**, which was separated by chiral HPLC (tR1). MS (ESI) m/z: 565.5 $(M+H)^+$. ^1H NMR (300 MHz, $CDCl_3$) δ 0.42-0.49 (4H, m), 1.27–1.35 (1H, m), 1.61 (6H, d, J = 6.8 Hz), 3.17–3.33 (2H, m), 3.79 (1H, m), 3.93–4.03 (3H, m), 4.05–4.10 (1H, m), 4.19–4.28 (2H, m), 5.04 (1H, brs), 7.06 (1H, s), 7.33 (1H, d, J = 9.4 Hz), 7.57 (1H, dd), 7.64 (1H, d), 7.90 (1H, d, J = 1.9 Hz), 7.92 (1H, d), 7.96 (1H, dd), 8.57 (1H, s).

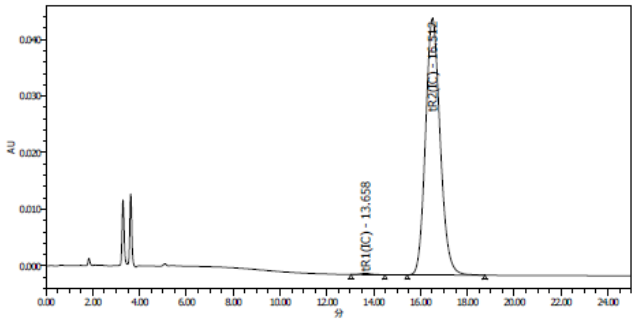
Chiral separation of compound 57 (tR1)



	成分名	保持時間 (分)	面積 (μV秒)	%面積	高さ (μV)
1	tR1(IC)	13.665	2571796	99.72	71480
2	tR2(IC)	16.472	7327	0.28	155

カラム：CHIRALPAK IC 4.6mmID×250mmL
移動相：Methanol/Ethanol=50/50 (v/v)
流　速：1.0 ml/min
温　度：30 ℃
検　出：UV220 nm
濃　度：0.5 mg/mL
注入量：0.010 mL

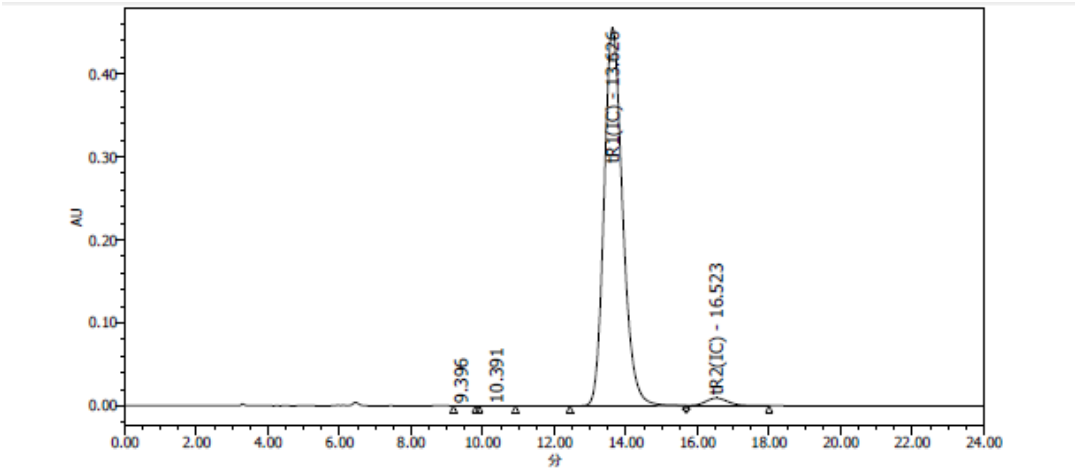
Chiral separation of compound 58 (tR2)



	成分名	保持時間 (分)	面積 (μV秒)	%面積	高さ (μV)
1	tR1(IC)	13.658	7466	0.38	225
2	tR2(IC)	16.512	1965551	99.62	45287

カラム：CHIRALPAK IC 4.6mmID×250mmL
移動相：Methanol/Ethanol=500/500 (v/v)
流　速：1.0 ml/min
温　度：30 ℃
検　出：UV220 nm
濃　度：0.5 mg/mL
注入量：0.010 mL

HPLC of synthesized compound 57

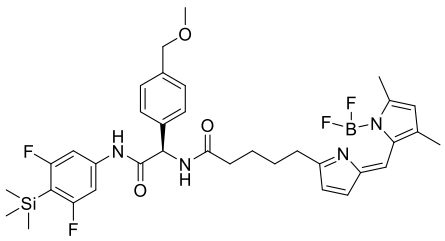


	成分名	保持時間 (分)	面積 (μV秒)	%面積	高さ (μV)
1		9.396	859	0.01	53
2		10.391	1348	0.01	55
3	tR1(IC)	13.626	16395208	97.58	455800
4	tR2(IC)	16.523	404410	2.41	9252

2. Biological evaluations

Binding assay

The binding activity of the test compound to RORγt was measured by a time resolved fluorescence resonance energy transfer method (TR-FRET) utilizing histidine-tagged RORγt, BODIPY-labeled compound **A**, and terbium-labeled anti-histidine tag antibody (Invitrogen). First, a test compound diluted with an assay buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 1 mM DTT, 0.1% BSA) was added to a 384 well plate by 3 μL each. Then, RORγt diluted with an assay buffer to 240 nM was added by 3 μL each, after which compound **A** diluted with the assay buffer to 160 nM was added by 3 μL each, and the mixture was stood at rt for 20 min. Thereafter, a terbium-labeled anti-histidine tag antibody diluted with the assay buffer to 8 nM was added by 3 μL each. The mixture was stood at rt for 20 min, and fluorescence intensity (excitation wavelength 320 nm, fluorescence wavelength 520 nm, delay time 100 microseconds) was measured by Envision (PerkinElmer).



A

Reporter gene assay

Preparation of a reaction medium and the transfection of the Jurkat cells with the reporter vector of RORγt response element and the RORγt expression vector were conducted according to published method[78]. The transfected cells were suspended in reaction medium (40 mL), and plated in a 96 well plate by 90 μL. The test compounds at various concentration in reaction medium (10 μL) were added, and the cells were incubated overnight. Bright-Glo (100 μL) was added to the mixture, and the mixture was stirred at room temperature for 10 min. The luminescence level was measured to calculate the transcriptional effect of the test compounds.

Whole blood assay

Blood was collected from abdominal vein of Lewis rats (9 weeks old, male) and was diluted into 2-fold by RPMI1640 containing 10% FBS and 1% PS (Life Technologies). **18i** was added to the diluted blood, and 1 hour later, the blood was stimulated with 10 ng/mL of PMA (Wako) and 1 mg/mL of ionomycin (Wako). Twenty four hours after the stimulation, culture supernatants were collected and concentrations of IL-17A in the culture supernatants were measured by ELISA (eBioscience).

***in vivo* PD assay (rat)**

Sensitization of rat

Female Lewis rats (7 weeks old) were immunized subcutaneously at footpad with 100 μL of emulsion containing 200 μg Myelin basic protein (Sigma) and 100 μg of Mycobacterium tuberculosis extract H37 Ra (Difco) in incomplete Freund's adjuvant (Difco).

Compound treatment and RT-qPCR

Ten, 30 or 100 mg/kg of **18i** was orally administrated twice a day to immunized rats from day 0 to day 5. On day 5, rats were euthanized and the popliteal lymph nodes were obtained. Total RNA was purified from the popliteal lymph nodes using Isogen (Wako) according to the manufacturer's method. mRNA levels were analyzed by reverse transcription-quantitative PCR (RT-qPCR) by using High-capacity RNA to cDNA kit and Taqman Gene Expression Master Mix (Life Technologies). Sequence of primers and probes using qPCR are follows.

GAPDH

Forward primer: 5'-GTGTTCCTACCCCCAATGTATCC-3'

Reverse primer: 5'-GATGTCATCATACTTGGCAGGTTT-3'

Probe: 5'-TTGTGGATCTGACATGCCGCCTG-3'

IL-17A

Forward primer: 5'-GCTCCAGAAGGCCCTCAGA-3'

Reverse primer: 5'-GTCCTCATTGCGGCTCAGA-3'

Probe: 5'-TACCTCAACCGTTCCACTTCACCCTGG-3'

IFN-γ

Forward primer: 5'-CACGCCGCGTCTTGGT-3'

Reverse primer: 5'-GAGTGTGCCTTGGCAGTAACAG-3'

Probe: 5'-TTGCAGCTCTGCCTCATGGCCC-3'

***in vivo* PD Assay (Mice)**

IL-23-induced cytokine production in mice

A mouse IL-23 solution (500 ng/10 μL, prepared by Takeda Pharmaceutical Company Limited) or PBS (10μL, negative control group) was administered intradermally in the ear of Balb/c mice (Charles River Japan, male, 7 weeks old). 24 h after administration, the ear was resected under isoflurane anesthesia. Compound **57** was suspended in 0.5% methylcellulose and administered orally 30 min before and 8 h after IL-23 administration.

RNA extraction from the ear tissue and quantitative PCR were performed as follows. Specifically, ear tissue was collected by 5-mm biopsy punch and was immersed in RNAlater (QIAGEN) for at least 18 hr. The RNAlater-treated ear tissue was homogenized in 350 μL of RLT buffer (RNeasy mini kit, QIAGEN) and treated (55°C, 10 min) with Proteinase K (QIAGEN). Total RNA was then extracted according to the RNeasy mini kit protocol. The RNA thus obtained was then reverse transcribed into cDNA using the High-Capacity RNA-to-cDNA kit (Applied Biosystems), and the amount of each gene expressions was measured by real-time PCR (Viia7TM, Applied Biosystems). The PCR buffer used was TaqMan Fast Advanced Master Mix (Applied Biosystems), and TaqMan Gene Expression Assays (Applied Biosystems, Mm00439618_m1 (IL-17A) and 4352341E (β-actin)) were used for each gene detection. The IL-17A gene expression level was normalized to the β-actin gene expression level, and the percent inhibition of IL-17A gene expression with the test compound was then calculated. The results (percent inhibition of IL-17A gene expression with oral administration of compound **57**) measured by the above-mentioned method are shown in Figure 19. Compound **57** inhibited 59% of IL-17A cytokine expression compared with vehicle-treated group.

3. Crystal structure of a eutomer of 18h and 57 with C-terminal truncated RORγt

A. Cloning, expression and purification of C-terminal truncated RORγt

For structure determination, the RORγt ligand binding domain (residues 261-494) was amplified from cDNA by PCR and cloned into the pSX70 vector. The human RORγt LBD was over-expressed in fusion with an N-terminal 6x poly-histidine tag and TEV cleavage site. Large scale production of recombinant protein was carried out in E. coli BL21 cells utilizing 5 L shake flasks.

RORγt purification was carried out from cell pellets re-suspended in lysis buffer consisting of 50 mM Tris-HCl (pH 7.6), 200 mM NaCl, 20mM imidazole, 0.25 mM TCEP, 3 Roche Complete tablets, and further lysed via polytron for 2-4 minutes. The lysate was centrifuged at 4200xg for 60 minutes and clarified supernatant was batch bound with 5 ml of Probond Ni resin (Invitrogen). The resin slurry was washed then eluted with buffer containing and additional 200 mM imidazole. Subsequent cleavage of the 6x poly-histidine tag was initiated by the addition of 1 mg of TEV followed by dialysed into buffer containing only 20 mM imidazole. TEV cleavage was confirmed by mass spectroscopy and the sample was incubated with an additional 5 ml of Probond Ni resin. The resin slurry was removed by centrifugation and the protein sample was further purified by size-exclusion chromatography utilizing a Superdex 200 column equilibrated in 25 mM Tris-HCl (pH 7.9), 200 mM NaCl, 5% Glycerol, 0.5 mM TCEP. Fractions containing the protein of interest were pooled and concentrated to 11 mg/ml utilizing YM10 centricon (Millipore) and flash-frozen in liquid nitrogen for storage at -80 ^{o}C.

B. *Crystallization, Data Collection and Structure Solution of RORγt*

Crystals of RORγt in complex with compounds RORγt were prepared by incubation of 11 mg/ml protein with 1mM compound from 50 mM DMSO stock solutions and left on ice for 1 h. Initial crystal trials were conducted utilizing Takeda San Diego's automated nanovolume crystallization technology platform. Large crystals suitable for data collection were obtained from reservoir solution containing 1-1.6M Sodium Formate, 3% MPD, and 100 mM HEPES (pH 7.5). Crystals selected for data collection were flash frozen in mother liquor with liquid nitrogen in an ALS compatible crystal mounting cassette. Diffraction data was collected from cryo-cooled crystals at the Advanced Light Source (ALS) beamline 5.0.3., and data reduction was performed using the HLK2000 software package[79]. The structure was determined by molecular replacement using the programs MOLREP[80] and PHASER[81] from the CCP4 program suite. Subsequent structure refinement and model re-building was conducted utilizing REFMAC[82] and XtalView[83] software packages. The coordinates and structure factors have been deposited in Protein Data Bank with accession code 6B31 and 6B33.

4. Solubility measurement

Kinetic solubility was measured based on a published method.[84-85] The test compounds were dissolved in DMSO to yield 10 mM DMSO solutions. The solutions (5 μL) were dispensed into a 96-well multi-filter plate, and JP2 solution (245 μL) was added. After

incubation at 25 °C for 24 h, precipitates were separated by filtration. The filtrates were analyzed by HPLC analysis.

5. Pharmacokinetics study in rats / mice

The PK parameters were evaluated by cassette dosing of the test compounds according to published method[86]. Five test compounds were simultaneously administered to a single rat or mouse. Plasma concentrations were determined after oral (1 mg/kg) and intravenous (0.1 mg/kg) administration. After administration, blood samples were collected in regular intervals for 8 h. The concentration of the plasma samples was determined by LC-MS/MS.

6. Evaluation of logD

LogD,(a partition coefficient between 1-octanol and aqueous buffer pH 7.4 of the test compounds) was measured by the retention time of reversed phase chromatographic procedure based on the published method[86].

7. Conformational Search and Docking Study

Conformational search of **18h** was conducted with the program Macromodel (Schrödinger, Inc.), mixed torsional/low-mode sampling method without solvent and OPLS3e as force field. The docking models of RORγt and compounds were built utilizing crystal structure of RORγt ligand-binding domain in complex with Digoxin (PDB code: 3B0W) or the RORγt crystal structure of compound **18h** (PDB code: 6B31). Docking was performed with the program Glide (Schrödinger, Inc.) in standard precision mode with further minimization.

8. Calculation of Chemical properties

The value of cLogP was calaculated by PerkinElmer ChemDraw Professional 18.

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