SUPPORTING INFORMATION

(*S*)-*N*-methyldihydroquinazolinones are the Active Enantiomers of Retro-2 Derived Compounds against Toxins

Neetu Gupta, Valérie Pons, Romain Noël, David-Alexandre Buisson, Aurélien Michau, Ludger Johannes, Daniel Gillet, Julien Barbier, and Jean-Christophe Cintrat

Table of contents:

General procedures
All chemicals and solvents used in the syntheses were reagent grade and were used without additional purification. THF and CH_2Cl_2 were distilled respectively from sodium/benzophenone ketyl and calcium hydride before use. Glassware was flame-dried under vacuum and cooled under nitrogen to room temperature. All reactions were performed under dry nitrogen gas and monitored by TLC. Thin-layer chromatography was performed with precoated TLC silica gel 60 F254, and organic compounds were visualized by UV light (254 nm), iodine vapor, phosphomolybdic acid [10% (w/v) in ethanol] staining with heating.
The large-scale purification was performed on a CombiFlash with a UV-vis detector with RediSep columns. The samples were adsorbed on Celite or silica and loaded into solid load cartridges. An ethyl acetate/cyclohexane or methanol/methylene chloride gradient was employed. Fractions were collected based on detection at 254 nm.
Chemicals for *in vitro* Experiments. The following products were purchased from the indicated commercial sources: [^{14}C]-leucine (Perkin-Elmer), Shiga-like toxin 1 (Stx, List Biological Laboratories, Inc.), DMSO (Sigma), fetal bovine serum (Sigma), glutamine, pyruvate, non-essential amino acids and antibiotics solutions (Gibco). Ricin was from Bruno Beaumelle.

HPLC-MS analysis and purification were performed using a Waters system (2525 binary gradient module, in-line degasser, 2767 sample manager, 2996 Photodiode Array Detector) with a binary gradient solvent delivery system. The eluent was a gradient of (99.9% water / 0.1% HCOOH) and (99.9% MeCN / 0.1% HCOOH) or (99.9% water / 0.1% HCOOH) and (99.9% MeOH / 0.1% HCOOH). Each compound was applied to a 100-4.6mm (5mm) Zorbax SBC18 column equilibrated with H_2O/MeCN or H_2O/MeOH 95:5.
This system was coupled with was a Waters Micromass ZQ system with a ZQ2000 quadrupole analyzer. The ionization was performed by electrospray and the other parameters were as follows: source temperature 120 °C, cone voltage 20 V, and continuous sample injection at 0.3 mL⁄ min flow rate. Mass spectra were recorded in both positive and negative ion mode in the m/z 100-2,000 range and treated with the Mass Lynx 4.0 software.

One-dimensional and two-dimensional NMR experiments were performed on a Bruker Avance 400 Ultrashield spectrometer. ^{1}H-NMR and ^{13}C spectra were recorded at room temperature at 400 MHz and 100 MHz respectively; samples were dissolved in $CDCl_3$ at a concentration of approximately 5 mM. The $CHCl_3$ singlet signal was set up at 7.27 ppm. Chemical shifts are given in ppm and the coupling constants in Hz. Spectral data are consistent with assigned structures.
Purity of compounds has been assessed by RP-HPLC. All compounds showed >95% purity.

Synthesis
Purity of compounds has been assessed by RP-HPLC. All compounds showed >95% purity.

6-Fluoro-1-methyl-2-(5-(2-methylthiazol-4-yl)thiophen-2-yl)-3-phenyl-2,3-dihydroquinazolin-4(1H)-one (**5**)

To a solution of 4-fluoro anthranilic acid (1.55 g, 10 mmol) in anhydrous THF (50 mL) was added triphosgene (3 g, 10 mmol). The resulting suspension was heated at 70 °C for 5 hr and then allowed to cool to room temperature. THF was evaporated and the resulting mixture was triturated with anhydrous ether, filtered and washed with ether to afford 1.7 g of 4-fluoro isatoic

anhydride as off-white flakes. This compound was carefully added to a suspension of NaH (60 % in mineral oil, prewashed with hexanes and dried under vacuum) in anhydrous DMF (10 mL). The resulting mixture was stirred at room temperature for 1 hr, MeI (20 mmol) was added dropwise and the reaction was allowed to proceed overnight at room temperature. The reaction flask was cooled down to 0 °C, crushed ice was carefully added and the mixture was filtered on a fritted glass. The precipitate was washed with cold water and dried overnight over P_2O_5 under vacuum to afford *N*-methyl 4-fluoro isatoic anhydride as a yellowish solid that was immediately engaged in the next step. *N*-methyl 4-fluoro isatoic anhydride was placed with aniline (10 mmol, 930 mg, 900 µL) in a sealed tube and the resulting mixture was heated at 130 °C overnight. Upon cooling to room temperature, ethyl acetate was added to the mixture, sonicated to dissolve the entire residue and silica was added before evaporation to dryness. Flash chromatography (elution with cyclohexane/ethyl acetate, 95/5 to 80/20) afforded 5-fluoro-2-(methylamino)-*N*-phenylbenzamide (1.7 g, 69 % yield over three steps) as a white powder. 5-Fluoro-2-(methylamino)-*N*-phenylbenzamide (1 g, 4.1 mmol) and commercially available 5-(2-methylthiazol-4-yl)thiophene-2-carbaldehyde (1.2 g, 5.74 mmol) were dissolved in THF (100 mL) and a catalytic amount of PTSA (68 mg, 0.4 mmol) was added. The redish solution was refluxed overnight. After evaporation to dryness the crude mixture was purified by flash chromatography on silica gel using cyclohexane/ethyl acetate (80/20 to 0/100) to yield the expected compound 6-fluoro-1-methyl-2-(5-(2-methylthiazol-4-yl)thiophen-2-yl)-3-phenyl-2,3-dihydroquinazolin-4(1H)-one (**5**) as a slightly yellow powder (1 g, 56 %).

^{1}H-NMR (400MHz, $CDCl_3$) δ (ppm) = 2.72 (s, 3H), 3.0 (s, 3H), 5.98 (s, 1H), 6.66 (dd, 1H, $^4J_{HF}$ = 4, $^3J_{HH}$ = 8), 6.83 (d, 1H, $^3J_{HH}$ = 3.6), 7.14 (s, 1H), 7.17 (d, 1H, $^3J_{HH}$ = 3.6), 7.17 (ddd, 1H, $^4J_{HH}$ = 3, $^3J_{HH}$ = 8, $^3J_{HF}$ = 8.9), 7.3-7.42 (m, 5H), 7.81 (dd, 1H, $^4J_{HH}$ = 3, $^3J_{HF}$ = 8.7).
^{13}C-NMR (100MHz, $CDCl_3$) δ (ppm) = 19.17, 37.03, 78.18, 112.06, 115.45 (d, $^3J_{CF}$ = 7), 115.54 (d, $^2J_{CF}$ = 24), 119.5 (d, $^3J_{CF}$ = 7), 121.41 (d, $^2J_{CF}$ = 23), 123.08, 126.68, 127.49, 127.86, 129.45, 138.98, 139.25, 140.53, 142.98, 148.92, 157.02 (d, $^1J_{CF}$ = 239), 161.17, 166.57.
MS (ESI) $[M+H]^+$ = 436.1
HRMS m/z $[(M+H)^+]$ calcd for $C_{23}H_{19}FN_3OS_2$ 436.0948 found 436.0937
LC/MS (X-bridge 100 × 4.6 mm): t_R = 8.88 min
IR (neat, cm^{-1}) 1656 (s), 1621 (w), 1591 (w), 1501 (s), 1447 (s), 1399, 1356, 1313, 1264 (m), 1164 (m), 814 (m), 724 (m)

Chiral Chromatography Separation
Analysis
An HPLC system (Shimadzu: controller CBM-20A, pumps LC-10ADvp) equipped with UV detector (SPD-10Avp) was employed. A 250 mm x 4.6 mm stainless steel column packed with Daicel IB was used for all purifications/analysis. The mobile phase was a binary mixture of hexane/ethanol (50/50). Flow rate was 1 mL/min. Injection volume was 20 µL. All chromatograms were recorded at 220 nm. The retention times for the first (**5a**) and second (**5b**) eluted enantiomers were 13.05 and 17.14 min, respectively.
Purification
Daicel IB 250x30 mm column, eluent hexane/isopropanol 60/40, flow 42.5 mL/min, DAD 200-400 nm, t_{R1a}= 8.2 min, t_{R1b} = 11.7 min
Compound **5a**
$[\alpha]^D_{20}$= + 42.63 (c = 5.97, CH_2Cl_2)
Compound **5b**
$[\alpha]^D_{20}$= - 43.0 (c = 2.69, CH_2Cl_2)

Crystal Structure Determination of Compound 5. The crystal data of both enantiomers of compound **5** were collected at 150(2) K using a Nonius Kappa CCD diffractometer with graphite-monochromated Mo Kα radiation. The data sets were corrected for Lorentz and polarization effects. Both structures were solved by direct methods and refined by full-matrix least-squares with all non-hydrogen atoms anisotropic and hydrogens included on calculated positions riding on their carrier atoms. The absolute configuration was determined from the value of the refined Flack parameter.{Flack, 1983 #24} All calculations were performed using SHELXL-97. {Sheldrick, 2008 #25}

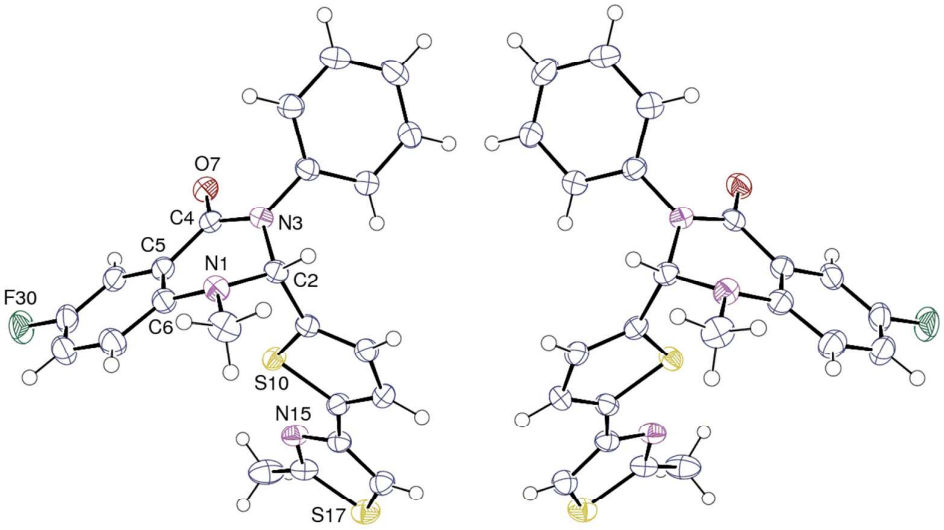
Compound **5a**

Crystal Data: $C_{23}H_{18}FN_3OS_2$, M = 435.52, orthorhombic, space group $P2_12_12_1$, a = 9.3645(5), b = 9.9300(3), c = 22.0458(11) Å, V = 2050.03(16) Å^3, Z = 4, Refinement of 274 parameters on 6250 independent reflections out of 43856 measured reflections (R_{int} = 0.031) led to $R1$ = 0.038, $wR2$ = 0.091, S = 1.019, $\Delta\rho_{min}$ = –0.22, $\Delta\rho_{max}$ = 0.23 e Å^{-3}, Flack parameter 0.00(5).

Compound **5b**

Crystal Data: $C_{23}H_{18}FN_3OS_2$, M = 435.52, orthorhombic, space group $P2_12_12_1$, a = 9.3608(3), b = 9.9287(2), c = 22.0495(7) Å, V = 2049.29(10) Å^3, Z = 4, Refinement of 274 parameters on 6240 independent reflections out of 90241 measured reflections (R_{int} = 0.021) led to $R1$ = 0.028, $wR2$ = 0.078, S = 1.066, $\Delta\rho_{min}$ = –0.20, $\Delta\rho_{max}$ = 0.19 e Å^{-3}, Flack parameter, 0.03(4).

ORTEP view of both enantiomers of compound **5** is shown below.



CCDC deposition number: 965990 and 965991.

Intoxication Assays.

HeLa and A549 cells were maintained at 37 °C under 5% CO_2 in DMEM (Dulbecco's modified Eagle's medium, Invitrogen), supplemented with 10% fetal bovine serum, 4.5 g/L glucose, 100 U/mL penicillin, 100 µg/mL streptomycin, 4 mM glutamine, 5 mM pyruvate. The cells were plated at a density of 50,000 cells per well in 96-well Cytostar-TTM scintillating microplates (Perkin-Elmer) with scintillator incorporated into the polystyrene plastic. After incubation with either 30 µM or various concentration of compounds (or 0.1 % DMSO) for 4 hr at 37 °C, cells were challenged with increasing doses of Stx (HeLa cells) or ricin (A549 cells) in the continued presence of compounds. After incubation for 20 hr, the medium was removed and replaced with DMEM without leucine (Eurobio) containing 10% fetal bovine serum, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1% Penicillin/Streptomycin supplemented with 0.5 µCi/mL

[^{14}C]-leucine. The cells were grown for an additional 6 hr at 37 °C in an atmosphere of 5% CO_2 and 95% air. Protein biosynthesis was then determined by measuring the incorporation of radiolabelled leucine into cells using a Wallac 1450 MicroBeta liquid scintillation counter (Perkin Elmer).
The mean percentage of protein biosynthesis was determined and normalized from duplicate wells. All values are expressed as means ± SEM. Data were fitted with Prism v5 software (Graphpad Inc., San Diego, CA) to obtain the 50% inhibitory toxin concentration (IC50) i.e. the concentration of toxin that is required to kill 50% of cells. IC_{50} values and protection factor R (R = IC_{50} drug/IC_{50} DMSO) were determined by the software's nonlinear regression "dose-response EC_{50} shift equation". The goodness of fit for toxin alone (carrier) or with drug was assessed by r^2 and confidence intervals.

Determination of EC_{50} Values. The EC_{50} represents the concentration giving 50% of its full inhibitory effect against Stx. EC_{50} ± SEM was used to compare compounds efficacy because it is more precise than R values and associated % protection. This is due to the fact that R values may fluctuate between cell experiments from different 96-wells plates corresponding to compounds tested on different days. In contrast EC_{50} value for a single compound is calculated from experimental data obtained on a single 96-well plate. Cell assays were performed with various concentrations of the inhibitor. For each concentration, a percentage of protection was determined from R values calculated with Prism software with Rmax corresponding to the higher value of R of the series:

$$\%\, protection = \frac{R-1}{Rmax-1} \times 100$$

Drug concentration was plotted against the corresponding percentage of protection of cells and the 50% efficacy concentration (EC_{50}) was calculated by non-linear regression using the Prism software package.