

Supplementary Information

Discovery of *Plasmodium vivax* N-Myristoyltransferase Inhibitors: Screening, Synthesis, and Structural Characterization of their Binding Mode

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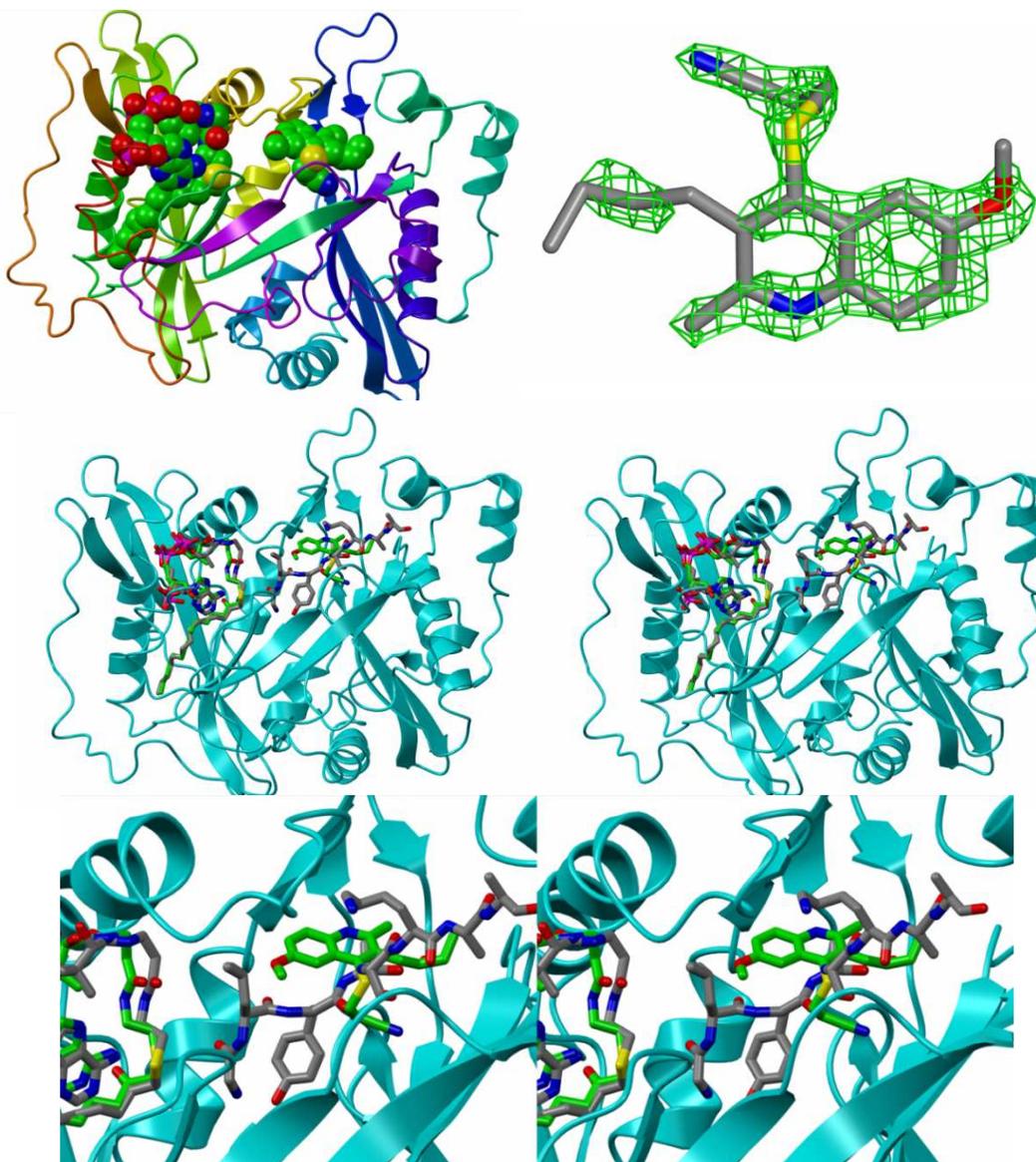
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Table of Contents

1. Supplementary Figure 1.....	2
2. High Throughput Screening.....	3
2.1. Chemical library.....	3
2.2. Reagents.....	3
2.3. Material.....	3
2.4. Library screening.....	3
3. X-Ray crystallography.....	4
3.1. Protein purification and crystallization.....	4
3.2. Supplementary Figure 2. Sequence of <i>Plasmodium vivax</i> N-myristoyltransferase ^a	5
3.3. Data collection, processing and structure solution.....	5
3.4. Supplementary Table 1. X-ray data collection and refinement statistics.....	6
4. Chemistry.....	7
4.1. General.....	7
4.2. Synthesis of compounds 1 , 7a-11a , 20a-24a , 7b-11b , 20b-24b , 7-11 and 20-24	7
5. References.....	18

1. Supplementary Figure 1.

(*Top panel*) Ribbon representation of chain A of the crystal structure of PvNMT color-ramped from the N-terminus (red) to the C-terminus (magenta) displayed with bound NHM (left) and **1** (right). The ligands are shown as spheres colored by atom; carbon (green), oxygen (red), nitrogen (blue), sulfur (yellow) and phosphorus (magenta). An initial, unbiased 'omit' difference density map (Fo-Fc contoured at a level of 3σ) associated with **1** is shown. (*Middle panel*) Stereo ribbon representation of chain A of PvNMT with bound NHM and **1** shown in cylinder representation, with the myristoyl-coenzyme A and the peptide (GLYASKLA) ligands from the crystal structure of a ScNMT ternary complex superposed. The ligand atoms are colored as above but distinguished by green carbons for the PvNMT ligands and grey carbons for the ScNMT ligands. The two structures were overlaid using the SSM superpose routine in CCP4mg applied to the A chains in the respective crystal structures. (*Lower panel*) Stereo view of the peptide/co-factor binding region showing overlapping binding sites for Compound **1** and peptide.



2. High Throughput Screening

2.1. Chemical library

The MRCT 60K diversity set was compiled from commercially available compound collections from multiple vendors. Both physico-chemical and structural filters were applied to select “lead-like” compounds and cluster analysis performed to maximize the diversity of the collection.

2.2. Reagents

All chemicals were purchased from Sigma-Aldrich Ltd (Gillingham, UK), Acros Organics (Geel, Belgium) and Alfa Aesar (Heysham, UK). Ultrapure water was obtained from MilliQ® Millipore water purification system. Myristoyl-coenzyme A was synthesized according to the literature and stored in DMSO at -20 °C.¹ 7-diethylamino-3-(4-maleimido-phenyl)-4-methylcoumarin (CPM) was supplied by Sigma-Aldrich and stored in DMSO (10 mM) at -20 °C.

Peptide Hs pp60^{src}₍₂₋₁₆₎ (GSNKSKPKDASQRRR-NH₂) was obtained by automated solid-phase peptide synthesis on a ResPep SL apparatus (Intavis, Germany). Synthesis was carried out on Tentagel S Ram resin (0.42 mmol scale). Semi-preparative purification of the crude peptide was performed by RP-HPLC on a Waters 2767 system equipped with a photodiode array, an ESI mass spectrometer and a XBridge prep C₁₈ (5 µm, 19 mm × 100 mm) OBD column equipped with an XBridge Prep C18 guard column (5 µm, 19 mm × 10 mm). The following elution method was used: Gradient of H₂O and MeOH (solvents were supplemented with 0.1% formic acid): 0-10 min 2-50% MeOH, 10-12min 50% MeOH, 12-13min 50 to 2% MeOH, 13-15 min 2% MeOH; Flow rate: 20 mL/min. The peptide Hs pp60^{src}₍₂₋₁₆₎ was isolated as a white powder (158 mg, 18%). Purity > 96%; MS ESI *m/z* calcd for C₆₈H₁₂₄N₃₀O₂₂ [M + 2H]²⁺ 857.5, found 857.6.

2.3. Material

Assays were carried out using black 384-well polystyrene microplates with non-binding surface (Corning, #3575). Dilutions were performed in 384-well polypropylene microplates (Matrix Technology, #4340) and deepwell 384-well polypropylene microplates (Matrix Technology, #4325). Reagents and tested compounds were dispensed with Biomek FX and Biomek NX robots (Beckman Coulter) and a Flexdrop IV Ex device (Perkin Elmer). Fluorescence readings were performed with a Tecan SAFIRE II (λ_{exc} : 390 nm; λ_{em} : 470 nm) or a BMG Labtech FluoStar Optima F (λ_{exc} : 390 nm; λ_{em} : 460 nm) plate reader.

2.4. Library screening

The assay for *Plasmodium vivax* NMT activity was directly adapted from the one described by Goncalves *et al.* Similar buffers and solutions were employed. Primary screening was conducted as follows: 15 µL of a solution containing myristoyl-coenzyme A (final concentration: 4 µM) and PvNMT (final concentration: 400 ng/mL) in buffer 1% DMSO were added to the assay well and combined with 2 µL of a 110 µM solution of inhibitor (final concentration: 10 µM). The mixture was

incubated at 20 °C for 10-20 min. Then, 5 µL of a solution containing the peptide Hs pp60^{src}₍₂₋₁₆₎ and CPM (final concentrations: 4 µM and 8 µM respectively) were added and the plate was incubated for 60 min at room temperature. Finally, the reaction was stopped by adding 15 µL of “quenching solution” and the fluorescence intensity was measured. Columns 1-2 were used for positive controls and columns 23-24 for negative controls (without PvNMT). All compounds were tested in duplicate. The Z' factor was calculated for each 384-well plate.⁴ Plates with a Z' value < 0.5 were repeated. The percentage of remaining activity was calculated as follows: Percentage of activity = [Signal - Mean(Negative control)] / [Mean(Positive control) - Mean(Negative control)] × 100. Data were elaborated using Microsoft Office Excel 2007. Positive compounds on each assay plate were defined as compounds giving a percentage of remaining activity inferior to [mean(Positive control) – 4 × SD(Positive control)]. These compounds were selected and re-tested for confirmation. Confirmed compounds were defined as compounds giving more than 50% inhibition of the PvNMT activity at 10 µM. Fluorescence quenchers were removed from the hit compounds.

To assess the potency of the hits, 12-point dose-response curves (final concentrations of 195 nM - 100 µM) were measured in duplicate. IC₅₀ values were determined using GraFit 7.0 (Erithacus Software Ltd, UK) by non-linear regression fitting.

Apparent K_i^{app} values were calculated using Cheng-Prusoff equation⁵. The apparent K_m (K_m^{app}) of the peptide substrate Hs pp60^{src}₍₂₋₁₆₎ for PvNMT is $5.71 \pm 0.6 \mu\text{M}$. The K_m^{app} of the peptide Hs pp60^{src}₍₂₋₉₎, substrate of human NMTs, is $2.66 \pm 0.20 \mu\text{M}$ for HsNMT1 and $3.25 \pm 0.22 \mu\text{M}$ for HsNMT2.

3. X-Ray crystallography

3.1. Protein purification and crystallization

A region of the PvNMT gene encoding residues 27 – 410 was subcloned from a synthetic gene codon-optimised for bacterial expression (GeneArt) into a pET-expression vector that appends coding sequence for an N-terminal His₆-tag which can be cleaved with HRV 3C protease. Expression and purification was carried out essentially as described for *L. donovani* NMT⁶ except that the ion exchange step was substituted by cation exchange chromatography (GE Healthcare 1 mL HiTrap S HP) with elution by a linear gradient of 50 mM – 1 M NaCl in phosphate buffer (pH 6.5). PvNMT (6 mg/mL in 10 mM Tris-HCl pH 7.0, 125 mM NaCl, 1 mM DTT) was incubated at 4°C with 1/20th volume of *S*-(2-oxo)pentadecyl-coenzyme A (NHM)⁶ and ligand (both 10 mM in 50% dimethylsulphoxide). Crystals were grown by the vapour diffusion method. Hanging drops consisting of 1 µL protein mixture and 1 µL of reservoir solution containing 0.2 M ammonium sulphate and 25% (w/v) PEG 3350 in 0.1 M Bis-Tris buffer, pH 6.0 were equilibrated against 0.8 mL reservoir solution at 20°C. Crystals belong to the orthorhombic space group $P2_12_12_1$ with cell dimensions $a = 57.49 \text{ \AA}$, $b = 121.56 \text{ \AA}$, $c = 179.01 \text{ \AA}$. With three molecules per asymmetric unit, the Matthews's coefficient is $2.32 \text{ \AA}^3/\text{Da}$ corresponding to a solvent content of 47%. Crystals were flash-vitrified directly in liquid nitrogen before storage.

3.2. Supplementary Figure 2. Sequence of *Plasmodium vivax* N-myristoyltransferase^a

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MGSSHHHHHSSGLEVLFGQPHMDYKFWYTQPVPKINDEFNESVNEPFIS 50
DNKVEDVRKDEYKLPPGYSWYVCDVKDEKDRSEIYTLTLDNYVEDDDNIF 100
RFNYSAEFLLWALTSPNYLKTWHIGVKYDASNKLIGFISAIPDICIHKR 150
TIKMAEVNFLCVHKTLRSKRLAPVLIKEITRRINLENIWQAIYTAGVYLP 200
KPVSDARYYHRSINVKKLIEIGFSSLNSRLTMSRAIKLYRVEDTLNIKNM 250
RLMKKKDVEGVHKL LGSYLEQFNLYAVFTKEEIAHWFLPIENVIYTYVNE 300
ENGIKIDMISFYSLPSQILGNDKYSTLNAAYSFYNVTTTATFKQLMQDAI 350
LLAKRNNFDVFNALVEMQNKSVFEDLKFEGEGDGLKYLYLNWKCASFAPA 400
HVGIVLL
```

^a Sequence of the protein used in *in vitro* assays. X-ray structure was obtained from cleaved protein (cleavage after the first 19 amino acids, between Q and G). The numbering in the manuscript and the PDB file is based on the full length protein.

3.3. Data collection, processing and structure solution

X-ray diffraction data were collected on synchrotron beamline ID14-4 ($\lambda = 0.9393 \text{ \AA}$) at the ESRF (Grenoble) using an ADSC detector and processed using XDS⁷ implemented within *xia2*.⁸ Data collection and refinement statistics are summarized in Supplementary Table 1. All data were used in molecular replacement calculations in the program MOLREP⁹ using the coordinates of a single chain of PvNMT in complex with NHM as the search model (structure to be published). Three solutions were found, and the corresponding structure comprising 3 protein chains was refined using maximum likelihood methods implemented in REFMAC.¹⁰ 5% of the data were excluded for R_{free} calculations. The model was refined by iterative cycles of REFMAC using anisotropic temperature factors, interspersed with manual modeling and adjustments carried out in COOT.¹¹ Refinement was concluded with an R -factor of 18.6% (R_{free} 23.6%) for data in the range 72 - 1.55 \AA . Complete chains (corresponding to residues 27 - 410, numbering as in full-length protein) can be traced for two molecules. N-terminal residues (remaining from the tag) in all 3 chains and loop 226 - 243 in chain C have not been modeled and these are assumed to be disordered. The final model consists of 11498 atoms (Supplementary Table 1). The protein has good geometry with 96.5% of the residues in the preferred region of the Ramachandran plot, with an additional 3.22% in allowed regions and 0.28% (corresponding to residue Phe336 in all 3 chains) as outliers. The coordinates and structure factor files have been deposited in the Protein Data Bank under the accession code **4a95**.

3.4. Supplementary Table 1. X-ray data collection and refinement statistics

PDB code 4a95	PvNMT- Compound 1
Data collection	
Space group	P2 ₁ 2 ₁ 2 ₁
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	57.49, 121.56, 179.01
<i>α</i> , <i>β</i> , <i>γ</i> (°)	90, 90, 90
Resolution (Å)	72 – 1.55 (1.59 – 1.55) ^a
<i>R</i> _{sym} (%) ^b	8.6 (35.3)
<i>I</i> / <i>σI</i>	15.6 (2.3)
Completeness (%)	85.2 (46.6) ^c
Redundancy	8.1 (2.6)
Refinement	
No. unique reflections	154218
<i>R</i> _{work} / <i>R</i> _{free} ^d	18.6 / 23.6
No. atoms	11498
Protein	9681
Ligand	66
Co-factor	192
Ion	11
Water	1542
B-factors (Å ²)	
Protein	15.3
Ligand	19.6
Co-factor	12.4
Water	27.6
R.m.s deviations ^e	
Bond lengths (Å)	0.024
Bond angles (°)	2.117
Chiral volumes (Å ³)	0.015

^a Highest resolution shell is shown in parenthesis.

^b $R_{\text{sym}} = \sum_h \sum_l |I_h - \langle I_h \rangle| / \sum_h \sum_l \langle I_h \rangle$, where I_l is the l^{th} observation of reflection h and $\langle I_h \rangle$ is the weighted average intensity for all observations l of reflection h .

^c The processing statistics suggest that the diffraction data is essentially 100% complete to 1.9 Å spacing. The relatively low measure of completeness to the reported resolution is due to detector shape effects.

^d $R_{\text{cryst}} = \sum \|F_o\| - |F_c| / \sum |F_o|$ where F_o and F_c are the observed and calculated structure factor amplitudes, respectively. R_{free} is the R_{cryst} calculated with 5% of the reflections chosen at random and omitted from refinement.

^e Root-mean-square deviation of bond lengths, bond angles or chiral atom volumes from ideal geometry.

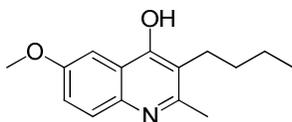
4. Chemistry

4.1. General

All chemicals were purchased from Sigma-Aldrich Ltd (Gillingham, UK), Acros Organics (Geel, Belgium) and Alfa Aesar (Heysham, UK) and used without further purification. Moisture sensitive reactions were performed under nitrogen atmosphere using dried glassware and standard syringe/septa techniques. Silica gel column chromatography was performed on an Isolera (Biotage, Uk) automated apparatus with SNAP silica cartridges (Biotage, Uk). NMR spectra were recorded on 400MHz Bruker instruments at room temperature and were referenced to TMS or residual solvent signals. Data are presented as follows: chemical shift, multiplicity (br s = broad singlet, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet) and integration.

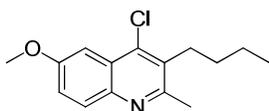
The purity of title compounds was verified by RP-HPLC on a Waters 2767 system equipped with a photodiode array and an ESI mass spectrometer. Separation was achieved using a XBridge C₁₈ (5 μm, 4.6 mm × 100 mm) column, equipped with an XBridge C₁₈ guard column (5 μm, 4.6 mm × 20 mm). The following elution method was used: Gradient of H₂O and MeOH (solvents were supplemented with 0.1% formic acid): 0-10 min 5-98% MeOH, 10-12 min 98% MeOH, 12-13 min 98 to 5% MeOH, 13-17 min 5% MeOH. Flow rate: 1.2 mL/min. Purity of tested compounds was ≥ 95%, unless specified.

4.2. Synthesis of compounds 1, 7a-11a, 20a-24a, 7b-11b, 20b-24b, 7-11 and 20-24



3-butyl-6-methoxy-2-methylquinolin-4-ol 1a

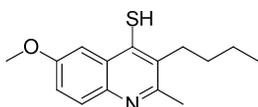
4-methoxy-aniline (3.08 g, 25 mmol), ethyl 2-acetylhexanoate (4.66 g, 25 mmol) and *para*-toluene sulfonic acid (238 mg, 1.25 mmole) were heated under reflux in toluene (25 mL) overnight in a flask equipped with a Dean-Stark apparatus. Toluene was evaporated under reduced pressure and the residue was heated under reflux with 10 mL Ph₂O for 1 h. After cooling of the reaction mixture, Et₂O (150 mL) was added. The suspension was stirred for 30 min, filtered and the precipitate washed with Et₂O to give **1a** as a yellow powder (2.39 g, 39%). ¹H NMR (MeOD, δ, ppm) 7.63 (d, 1H), 7.46 (d, 1H), 7.26 (dd, 1H), 3.89 (s, 3H), 2.66 (t, 2H), 1.48 (m, 4H), 0.97 (t, 3H). ¹³C NMR (MeOD, δ, ppm): 120.94, 118.93, 118.08, 117.38, 101.87, 53.06, 29.46, 23.28, 21.09, 15.07, 11.49. HRMS *m/z* calcd for C₁₅H₁₉NO₂ [M + H]⁺ 246.1494, found 246.1493.



3-butyl-4-chloro-6-methoxy-2-methylquinoline 1b

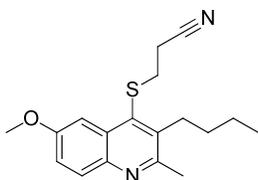
1a (2.085 g, 8.5 mmol), thionyl chloride (15 mL) and DMF (1 mL) were heated at 80 °C for 1 h. The solvent was removed under reduced pressure and the residue was partitioned between CH₂Cl₂ and a

saturated aqueous solution of NaHCO₃. The organic phase was washed with water and dried over CaSO₄. The solvent was removed under reduced pressure and the residue was purified by automated flash chromatography on silica (gradient from pure *n*-hexane to 80/20 *n*-hexane/EtOAc). **1b** was isolated as a beige powder (1.65 g, 74%). ¹H NMR (MeOD, δ, ppm) 7.75 (d, 1H), 7.31 (d, 1H), 7.27 (dd, 1H), 3.90 (s, 3H), 2.85 (t, 2H), 2.64 (s, 3H), 1.48 (m, 4H), 0.98 (t, 3H). ¹³C NMR (MeOD, δ, ppm) 158.43, 155.49, 141.62, 139.83, 132.77, 126.35, 121.81, 101.62, 54.70, 30.34, 29.79, 22.63, 21.76, 12.77. HRMS *m/z* calcd for C₁₅H₁₈ClNO [M + H]⁺ 264.1155, found 264.1145.



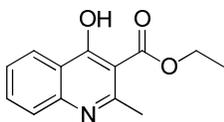
9-mercapto-7-methoxy-1,2,3,4-tetrahydroacridin-10-ium chloride **1c**

NaSH (4.00 g, 71 mmol) was added to a solution of **1b** (1.50 g, 5.69 mmol) in DMF (40 mL). The suspension was refluxed for 4 h. After cooling to room temperature, 60 mL of ethyl acetate were added and the mixture was filtered through a silica plug. Solvents were evaporated under reduced pressure and the residue was dissolved in 500 mL of Et₂O. Acidification to pH~2 with HCl 2 N in Et₂O led to the formation of a fine precipitate which was filtered and washed with Et₂O. The product **1c** was isolated as a yellow powder (1.40 g, 83%) and used without further purification. ¹H NMR (MeOD, δ, ppm) 7.90 (d, 1H), 7.80 (d, 1H), 7.59 (dd, 1H), 4.00 (s, 3H), 3.04 (t, 2H), 2.80 (s, 3H), 1.52 (m, 4H), 1.00 (t, 3H). HRMS *m/z* calcd for C₁₅H₁₉NOS [M + H]⁺ 262.1266, found 262.1251.



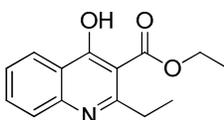
3-butyl-4-((2-cyanoethyl)thio)-6-methoxy-2-methylquinolin-1-ium chloride **1**

To **1c** (60 mg, 0.20 mmol) and K₂CO₃ (56.0 mg, 0.40 mmol) were added 2 mL of dry DMF. The suspension was stirred for 10 min at room temperature, followed by the dropwise addition of 3-bromopropionitrile (25.2 μL, 0.40 mmol). The mixture was stirred for 1 h at 110 °C and cooled to room temperature. The solution was partitioned between water and EtOAc. The organic layer was dried over CaSO₄ and the product purified by flash chromatography (gradient of *n*-hexane/EtOAc from 90/10 to 70/30). After evaporation of the solvent, the residue was diluted Et₂O and precipitated as a chlorhydrate salt by adding HCl 2 N in Et₂O. The product was recovered by filtration as a yellow powder (34 mg, 48%). ¹H NMR (CDCl₃, δ, ppm) 8.94 (d, 1H), 7.78 (s, 1H), 7.24 (d, 1H), 4.03 (s, 3H), 3.14-3.22 (m, 7H), 2.58 (m, 2H), 1.52 (m, 4H), 1.00 (t, 3H). ¹³C NMR (CDCl₃, δ, ppm) 160.86, 142.27, 125.97, 124.34, 104.38, 56.29, 32.61, 31.15, 22.95, 19.22, 13.78. HRMS *m/z* calcd for C₁₈H₂₂N₂OS [M + H]⁺ 315.1531, found 315.1530.



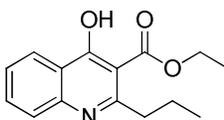
ethyl 4-hydroxy-2-methylquinoline-3-carboxylate 9a

Method 1: Ethylacetoacetate (6.50 g, 50 mmol) was dissolved in 100 mL of *N,N*-dimethylacetamide and sodium hydride (1.20 g, 50 mmol) was added portionwise under stirring over 10 min. To this solution was added a mixture of isatoic anhydride (9.79 g, 60 mmol) and *N,N*-dimethylacetamide (75 mL) followed by stirring at 120 °C for 20 min. The solution was concentrated under reduced pressure, followed by addition of water (500 mL). The suspension was submitted to ultrasonic waves and the resulting precipitate was collected by filtration, washed with water and dried to give compound **9a** as an off-white powder (7.80 g, 67%). ¹H NMR (MeOD, δ, ppm). 8.23 (dd, 1H), 7.70 (dt, 1H), 7.55 (d, 1H), 7.42 (dt, 1H), 4.37 (q, 2H), 2.53 (s, 3H), 1.38 (t, 3H).



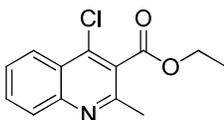
ethyl 2-ethyl-4-hydroxyquinoline-3-carboxylate 10a

Compound **10a** was synthesized by method 1 from propionylethylacetate (1.00 g, 6.94 mmol), NaH (166 mg, 6.94 mmol) and isatoic anhydride (1.36 g, 8.32 mmol). Off-white powder (0.83 g, 49%). ¹H NMR (MeOD, δ, ppm) 8.24 (dd, 1H), 7.72 (dt, 1H), 7.58 (dd, 1H), 7.42 (dt, 1H), 4.39 (q, 2H), 2.81 (q, 2H), 1.39 (t, 3H), 1.38 (t, 3H).



ethyl 4-hydroxy-2-propylquinoline-3-carboxylate 11a

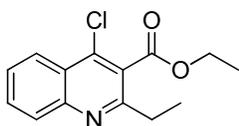
Compound **11a** was synthesized by method 1 from ethyl 3-oxohexanoate (3.92 g, 20.0 mmol), NaH (480 mg, 20.0 mmol) and isatoic anhydride (3.92 g, 24.0 mmol). Off-white powder (2.24 g, 36%). ¹H NMR (MeOD, δ, ppm) 8.24 (dd, 1H), 7.72 (dt, 1H), 7.58 (dd, 1H), 7.42 (dt, 1H), 4.38 (q, 2H), 2.78 (m, 2H), 1.80 (sextet, 2H), 1.38 (t, 3H), 1.04 (t, 3H).



ethylquinoline-3-carboxylate 9b

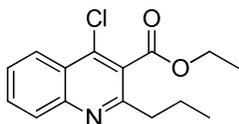
Method 2: To **9a** (3.00 g, 13 mmol) was added POCl₃ (2.42 mL, 26 mmol) and the mixture was heated at 110 °C for 20 min. The solution was poured into a mixture of ammonia (aq., 28-30%) and ice and stirred for 10 min. The mixture was extracted three times with ethyl acetate. Combined organic layers were washed with water and dried (MgSO₄), filtered, and concentrated under reduced pressure. The product was purified by flash chromatography (gradient hexane/EtOAc from 95/5 to

85/15) to give **9b** as a colorless oil (2.81 g, 86%). ¹H NMR (CDCl₃, δ, ppm) 8.23 (dd, 1H), 8.06 (d, 1H), 7.80 (dt, 1H), 7.65 (dt, 1H), 4.54 (q, 2H), 2.75 (s, 3H), 1.47 (t, 3H).



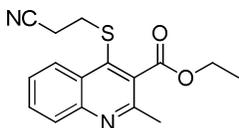
ethyl 4-chloro-2-ethylquinoline-3-carboxylate **10b**

Compound **10b** was synthesized by method 2 from **10a** (202 mg, 0.82 mmol) and POCl₃ (500 μL, 5.36 mmol). Colorless oil (183 mg, 84%). ¹H NMR (CDCl₃, δ, ppm) 8.24 (dd, 1H), 8.08 (d, 1H), 7.79 (dt, 1H), 7.64 (dt, 1H), 4.54 (q, 2H), 3.00 (q, 2H), 1.48 (t, 3H), 1.41 (t, 3H).



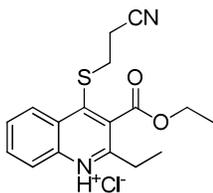
ethyl 4-chloro-2-propylquinoline-3-carboxylate **11b**

Compound **11b** was synthesized by method 2 from **11a** (1.60 g, 6.16 mmol) and POCl₃ (1.15 mL, 12.3 mmol). Colorless oil (1.60 g, 93%). ¹H NMR (CDCl₃, δ, ppm) 8.22 (dd, 1H), 8.06 (d, 1H), 7.77 (dt, 1H), 7.62 (dt, 1H), 4.52 (q, 2H), 2.93 (m, 2H), 1.86 (sextet, 2H), 1.46 (t, 3H), 1.03 (t, 3H).



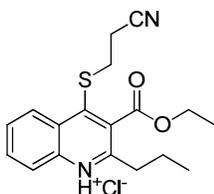
ethyl 4-((2-cyanoethyl)thio)-2-methylquinoline-3-carboxylate **9**

Method 3: 3-mercaptopropanenitrile¹² (810 mg, 9.30 mmol) and K₂CO₃ (1.29 g, 9.30 mmol) were stirred for 10 min at room temperature under argon. To this solution was added **9b** (1.16 g, 4.65 mmol) in 10 mL THF. The mixture was refluxed for 4 h, the solvent was removed under reduced pressure and the residue was extracted with EtOAc. The organic layer was dried (MgSO₄), concentrated and purified by flash chromatography (gradient hexane/EtOAc from 100/0 to 60/40) to give **9** as a colorless oil that solidifies on standing into an off-white solid (1.92 g, 92%). ¹H NMR (MeOD, δ, ppm) 8.55 (dd, 1H), 8.06 (d, 1H), 7.86 (dt, 1H), 7.75 (dt, 1H), 4.56 (q, 2H), 3.21 (t, 2H), 2.70 (s, 3H), 2.65 (t, 2H), 1.48 (t, 3H). ¹³C NMR (MeOD, δ, ppm) 167.38, 154.19, 147.36, 138.55, 135.23, 128.57, 127.77, 126.89, 125.55, 118.04, 62.21, 31.97, 21.72, 17.82, 13.08. HRMS *m/z* calcd for C₁₆H₁₆N₂O₂S [M + H]⁺ 301.1011, found 301.1013.



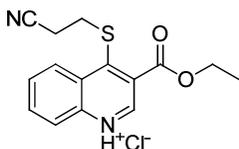
4-((2-cyanoethyl)thio)-3-(ethoxycarbonyl)-2-ethylquinolin-1-ium chloride **10**

Compound **10** was synthesized by method 3 from **10b** (100 mg, 0.38 mmol), 3-mercaptopropanenitrile (66 mg, 0.76 mmol) and K_2CO_3 (105 mg, 0.76 mmol). The product was isolated as a chlorohydrate salt. Yellow powder (97.4 mg, 73%). 1H NMR (MeOD, δ , ppm) 8.70 (d, 1H), 8.26 (d, 1H), 8.13 (dt, 1H), 7.98 (dt, 1H), 4.58 (q, 2H), 3.36 (t, 2H), 3.15 (q, 2H), 2.72 (t, 2H), 1.47 (m, 2 \times 3H). ^{13}C NMR (MeOD, δ , ppm) 164.75, 158.07, 149.76, 140.03, 134.67, 130.12, 126.83, 122.75, 117.79, 63.21, 32.65, 27.35, 18.18, 13.05, 12.96. HRMS m/z calcd for $C_{17}H_{18}N_2O_2S$ $[M + H]^+$ 315.1167, found 315.1159.



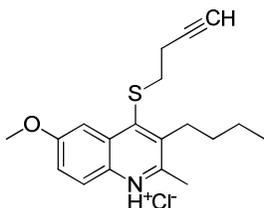
4-((2-cyanoethyl)thio)-3-(ethoxycarbonyl)-2-propylquinolin-1-ium chloride **11**

Compound **11** was synthesized by method 3 from **11b** (100 mg, 0.36 mmol), 3-mercaptopropanenitrile (62.7 mg, 0.72 mmol) and K_2CO_3 (99.5 g, 0.72 mmol). The product was isolated as a chlorohydrate salt. Yellow powder (83.5 mg, 64%). Purity: 94.6%. 1H NMR (MeOD, δ , ppm) 8.78 (d, 1H), 8.31 (d, 1H), 8.23 (dt, 1H), 8.08 (dt, 1H), 4.62 (q, 2H), 3.42 (t, 2H), 3.15 (m, 2H), 2.76 (t, 2H), 1.92 (m, 2H), 1.48 (t, 3H), 1.10 (t, 3H). ^{13}C NMR (MeOD, δ , ppm) 164.18, 156.65, 152.34, 138.30, 134.83, 130.66, 128.70, 127.17, 121.36, 117.70, 63.43, 35.04, 32.78, 23.25, 18.25, 13.03, 12.88. HRMS m/z calcd for $C_{18}H_{20}N_2O_2S$ $[M + H]^+$ 329.1234, found 329.1236.



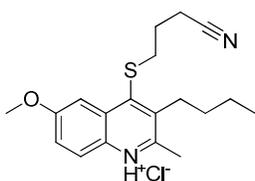
4-((2-cyanoethyl)thio)-3-(ethoxycarbonyl)quinolin-1-ium chloride **8**

Compound **8** was synthesized by method 3 from ethyl 4-chloroquinoline-3-carboxylate (100 mg, 0.42 mmol, Alfa Aesar), 3-mercaptopropanenitrile (73.2 mg, 0.84 mmol) and K_2CO_3 (116 mg, 0.84 mmol). The product was isolated as a chlorohydrate salt. Yellow powder (46.0 mg, 34%). Purity: 94.0%. 1H NMR (MeOD, δ , ppm) 9.42 (s, 1H), 8.90 (d, 1H), 8.28 (m, 2H), 8.09 (dt, 1H), 4.56 (q, 2H), 3.52 (t, 2H), 2.84 (t, 2H), 1.48 (t, 3H). ^{13}C NMR (MeOD, δ , ppm) 163.14, 158.49, 150.00, 144.16, 136.03, 130.77, 129.69, 128.39, 127.44, 121.91, 117.64, 63.15, 33.16, 18.03, 13.05. HRMS m/z calcd for $C_{15}H_{14}N_2O_2S$ $[M + H]^+$ 287.0854, found 287.0849.



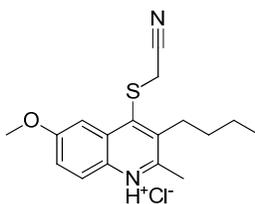
4-(but-3-yn-1-ylthio)-3-butyl-6-methoxy-2-methylquinolin-1-ium chloride **12**

Method 4: To **1c** (40 mg, 0.14 mmol) and potassium carbonate (39.1 mg, 0.28 mmol) were added 2 mL of dry DMF. The suspension was stirred for 10 min at room temperature, followed by the dropwise addition of 4-bromo-1-butyne (25.2 μ L, 0.27 mmol). The reaction mixture was stirred for 1 h at 110 $^{\circ}$ C and cooled to room temperature. The solution was diluted with water and extracted with EtOAc. The organic layer was dried over MgSO₄ and the product purified by flash chromatography (gradient of hexane/EtOAc from 90/10 to 70/30). After evaporation of the solvent, the residue was diluted in diethyl ether and precipitated as a chlorohydrate salt by adding HCl 2 N in Et₂O. The product was recovered by filtration as a yellow powder (39 mg, 83%). ¹H NMR (CDCl₃, δ , ppm) 8.96 (d, 1H), 7.80 (d, 1H), 7.54 (dd, 1H), 4.02 (s, 3H), 3.16 (m, 7H), 2.45 (t, 2H), 2.04 (s, 1H), 1.53 (m, 4H), 1.01 (t, 3H). ¹³C NMR (CDCl₃, δ , ppm) 125.63, 124.21, 104.83, 70.91, 35.87, 56.10, 35.87, 32.44, 31.20, 22.97, 20.17, 13.78. HRMS *m/z* calcd for C₁₉H₂₃NOS [M + H]⁺ 314.1579, found 314.1578.



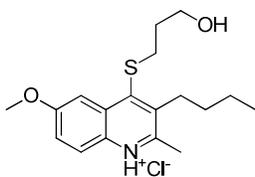
3-butyl-4-((3-cyanopropyl)thio)-6-methoxy-2-methylquinolin-1-ium chloride **13**

Compound **13** was synthesized by method 4 from **1c** (40 mg, 0.14 mmol), K₂CO₃ (39.1 mg, 0.28 mmol) and 4-bromobutyronitrile (27.0 μ L, 0.27 mmol). Yellow powder (31 mg, 63%). ¹H NMR (CDCl₃, δ , ppm) 8.96 (d, 1H), 7.71 (d, 1H), 7.54 (dd, 1H), 4.01 (s, 3H), 3.13 (m, 7H), 2.50 (t, 2H), 1.93 (m, 2H), 1.51 (m, 4H), 0.99 (t, 3H). ¹³C NMR (CDCl₃, δ , ppm) 160.62, 131.29, 125.69, 124.40, 104.71, 56.18, 32.49, 31.42, 26.16, 22.97, 16.76, 13.80. HRMS *m/z* calcd for C₁₉H₂₄N₂OS [M + H]⁺ 329.1688, found 329.1677.



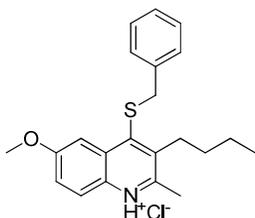
3-butyl-4-((cyanomethyl)thio)-6-methoxy-2-methylquinolin-1-ium chloride **14**

Compound **14** was synthesized by method 4 from **1c** (70 mg, 0.24 mmol), K₂CO₃ (68.2 mg, 0.49 mmol) and iodoacetonitrile (22.1 μ L, 0.31 mmol). Yellow powder (26 mg, 33%). ¹H NMR (CDCl₃, δ , ppm) 8.93 (d, 1H), 7.75 (d, 1H), 7.54 (dd, 1H), 4.06 (s, 3H), 3.74 (s, 2H), 3.18-3.26 (m, 5H), 1.56 (m, 4H + H₂O), 1.02 (t, 3H). ¹³C NMR (CDCl₃, δ , ppm) 160.92, 152.53, 145.34, 143.70, 132.75, 131.04, 125.86, 123.82, 115.42, 104.42, 56.37, 32.67, 31.45, 22.95, 21.64, 19.21, 13.76. HRMS *m/z* calcd for C₁₇H₂₀N₂OS [M + H]⁺ 301.1375, found 301.1370.



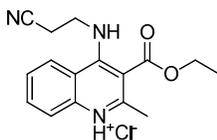
3-butyl-4-((3-hydroxypropyl)thio)-6-methoxy-2-methylquinolin-1-ium chloride 15

Compound **15** was synthesized by method 4 from **1c** (80 mg, 0.27 mmol), K_2CO_3 (103 mg, 0.57 mmol) and 3-bromo-1-propanol (24.0 μ L, 0.27 mmol). The product was purified using a gradient of hexane/EtOAc from 60/40 to 40/60. Yellow powder (76 mg, 80%). 1H NMR ($CDCl_3$, δ , ppm) 8.84 (d, 1H), 7.78 (d, 1H), 7.45 (dd, 1H), 4.02 (s, 3H), 3.75 (t, 2H), 3.10-3.19 (m, 7H), 1.86 (q, 2H), 1.52 (m, 4H), 1.00 (t, 3H). ^{13}C NMR ($CDCl_3$, δ , ppm) 160.25, 151.33, 141.36, 132.39, 131.10, 125.49, 123.82, 104.99, 60.55, 56.09, 34.24, 32.83, 32.31, 31.11, 22.96, 18.94, 13.80. HRMS m/z calcd for $C_{18}H_{25}NO_2S$ $[M + H]^+$ 320.1684, found 320.1681.



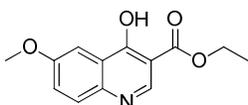
4-(benzylthio)-3-butyl-6-methoxy-2-methylquinolin-1-ium chloride 16

Compound **16** was synthesized by method 4 from **1c** (40 mg, 0.14 mmol), K_2CO_3 (41.2 mg, 0.30 mmol) and benzyl bromide (21.0 μ L, 0.22 mmol). The product was purified using a gradient of hexane/EtOAc from 100/0 to 90/10. Yellow powder (48 mg, 91%). 1H NMR (DMSO, δ , ppm) 8.33 (d, 1H), 7.72 (d, 1H), 7.66 (dd, 1H), 7.20 (m, 3H), 7.04 (m, 2H), 4.23 (br s, 2H), 3.95 (s, 3H), 2.94 (m, 2H), 2.89 (s, 3H), 1.37 (m, 4H), 0.90 (t, 3H). ^{13}C NMR (DMSO, δ , ppm) 137.71, 129.26, 128.89, 127.94, 105.64, 56.30, 40.96, 31.63, 30.81, 22.86, 14.05. HRMS m/z calcd for $C_{22}H_{25}NOS$ $[M + H]^+$ 352.1730, found 352.1730.



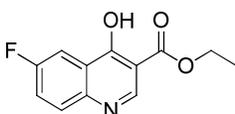
4-((2-cyanoethyl)amino)-3-(ethoxycarbonyl)-2-methylquinolin-1-ium chloride 17

9b (100 mg, 0.40 mmol), 3-aminopropanenitrile (148 μ L, 2.0 mmol) and K_2CO_3 (55.3 mg, 0.40 mmol) were heated under reflux in toluene (3 mL) for 16 h. The product was extracted from EtOAc/water, dried ($MgSO_4$) and concentrated under reduced pressure. The residue was purified by flash chromatography (gradient hexane/EtOAc from 60/40 to 30/70) and precipitated as an HCl salt using HCl 2 N in Et_2O . Yellow powder (9.8 mg, 8%). 1H NMR (MeOD, δ , ppm) 8.51 (d, 1H), 8.02 (dt, 1H), 7.92 (d, 1H), 7.79 (dt, 1H), 4.56 (q, 2H), 3.86 (t, 2H), 3.02 (t, 2H), 2.78 (s, 3H), 1.48 (t, 3H). ^{13}C NMR (MeOD, δ , ppm) 166.28, 154.24, 134.30, 127.50, 123.24, 119.48, 117.14, 62.90, 41.85, 18.58, 17.23, 12.87. HRMS m/z calcd for $C_{16}H_{17}N_3O_2$ $[M + H]^+$ 284.1399, found 284.1390.



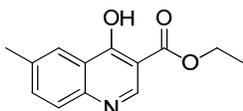
ethyl 4-hydroxy-6-methoxyquinoline-3-carboxylate 7a

Method 5: A mixture of 3-methoxyaniline (3.69 g, 30 mmol) and diethyl(ethoxymethylene) malonate (6.06 mL, 30 mmol) was heated in an oil bath at 140 °C for 1 h. The generated ethanol was collected. The reaction flask was moved away from the oil bath and 10 mL of diphenyl ether were added into the reaction mixture. The reaction was refluxed for 2 h, cooled to room temperature and the mixture was poured into 250 mL of hexane with vigorous stirring. The suspension was sonicated for 30 min and filtered. The precipitate was washed with hexane and dried to give **7a** as a brown powder (1.06 g, 14%). ¹H NMR (DMSO, δ , ppm) 12.29 (s, 1H), 8.47 (d, 1H), 7.56 (m, 2H), 7.31 (dd, 1H), 4.19 (q, 2H), 3.83 (s, 3H), 1.26 (t, 3H).



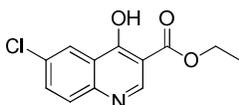
ethyl 6-fluoro-4-hydroxyquinoline-3-carboxylate 20a

Compound **20a** was synthesized by method 5 from 4-fluoroaniline (3.33 g, 30 mmol) and diethyl(ethoxymethylene) malonate (6.06 mL, 30 mmol). Grey powder (770 mg, 10.9%). ¹H NMR (DMSO, δ , ppm) 12.48 (s, 1H), 8.59 (d, 1H), 7.81 (dd, 1H), 7.72 (dd, 1H), 7.62 (dt, 1H), 4.22 (q, 2H), 1.29 (t, 3H).



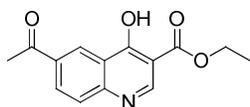
ethyl 4-hydroxy-6-methylquinoline-3-carboxylate 21a

Compound **21a** was synthesized by method 5 from *para*-toluidine (3.21 g, 30 mmol) and diethyl(ethoxymethylene) malonate (6.06 mL, 30 mmol). Beige powder (497 mg, 7.1%). ¹H NMR (DMSO, δ , ppm) 12.26 (s, 1H), 8.52 (d, 1H), 7.95 (s, 1H), 7.53 (m, 2H), 4.22 (q, 2H), 2.43 (s, 3H), 1.28 (t, 3H).



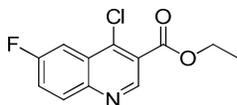
ethyl 6-chloro-4-hydroxyquinoline-3-carboxylate 22a

Compound **22a** was synthesized by method 5 from 4-chloroaniline (3.83 g, 30 mmol) and diethyl(ethoxymethylene) malonate (6.06 mL, 30 mmol). Grey powder (2.07 g, 27.4%). ¹H NMR (DMSO, δ , ppm) 12.47 (br s, 1H), 8.59 (s, 1H), 8.08 (d, 1H), 7.76 (dd, 1H), 7.68 (d, 1H), 4.23 (q, 2H), 1.28 (t, 3H).



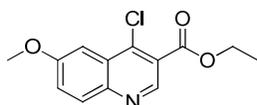
ethyl 6-acetyl-4-hydroxyquinoline-3-carboxylate 23a

Compound **23a** was synthesized by method 5 from 4-amino-acetophenone (4.01 g, 30 mmol) and diethyl(ethoxymethylene) malonate (6.06 mL, 30 mmol). Beige powder (7.27 g, 93%). ¹H NMR (DMSO, δ , ppm) 12.56 (s, 1H), 8.72 (d, 1H), 8.61 (s, 1H), 8.22 (dd, 1H), 7.71 (d, 1H), 4.24 (q, 2H), 2.66 (s, 3H), 1.30 (t, 3H).



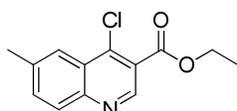
ethyl 4-chloro-6-fluoroquinoline-3-carboxylate 20b

Compound **20b** was synthesized by method 5 from **20a** (500 mg, 2.13 mmol) and POCl₃ (500 μ L, 5.36 mmol). The product was purified by flash chromatography (gradient hexane/EtOAc from 100/0 to 80/20) to give the compound **20b** as a white powder (477 mg, 88%). ¹H NMR (CDCl₃, δ , ppm) 9.17 (s, 1H), 8.16 (dd, 1H), 8.04 (dd, 1H), 7.64 (m, 1H), 4.52 (q, 2H), 1.48 (t, 3H).



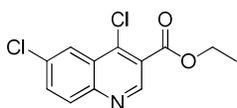
ethyl 4-chloro-6-methoxyquinoline-3-carboxylate 7b

Compound **7b** was synthesized by method 2 from **7a** (581 mg, 2.35 mmol) and POCl₃ (440 μ L, 4.70 mmol). The product was purified by flash chromatography (gradient hexane/EtOAc from 100/0 to 75/25) to give the compound **7b** as a white powder (497 mg, 80%). ¹H NMR (CDCl₃, δ , ppm) 9.06 (s, 1H), 8.05 (d, 1H), 7.61 (s, 1H), 7.49 (d, 1H), 4.51 (q, 2H), 4.01 (s, 3H), 1.47 (t, 3H).



ethyl 4-chloro-6-methylquinoline-3-carboxylate 21b

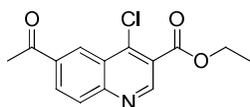
Compound **21b** was synthesized by method 2 from **21a** (500 mg, 2.16 mmol) and POCl₃ (500 μ L, 5.36 mmol). The product was purified by flash chromatography (gradient hexane/EtOAc from 100/0 to 80/20) to give the compound **21b** as an off-white powder (465 mg, 86%). ¹H NMR (CDCl₃, δ , ppm) 9.14 (s, 1H), 8.18 (s, 1H), 8.03 (d, 1H), 7.68 (dd, 1H), 4.51 (q, 2H), 2.62 (s, 3H), 1.47 (t, 3H).



ethyl 4,6-dichloroquinoline-3-carboxylate 22b

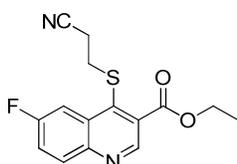
Compound **22b** was synthesized by method 2 from **22a** (500 mg, 1.99 mmol) and POCl₃ (500 μ L, 5.36 mmol). The product was purified by flash chromatography (gradient hexane/EtOAc from 100/0

to 80/20) to give the compound **22a** as a yellow powder (260 mg, 49%). ¹H NMR (CDCl₃, δ, ppm) 9.20 (s, 1H), 8.41 (d, 1H), 8.10 (d, 1H), 7.79 (dd, 1H), 4.52 (q, 2H), 1.49 (t, 3H).



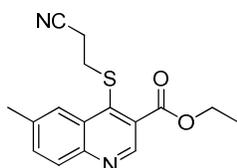
ethyl 6-acetyl-4-chloroquinoline-3-carboxylate 23b

Compound **23b** was synthesized by method 2 from **23a** (500 mg, 1.93 mmol) and POCl₃ (500 μL, 5.36 mmol). The product was purified by flash chromatography (gradient hexane/EtOAc from 100/0 to 80/20) to give the compound **23b** as a yellow powder (310 mg, 58%). ¹H NMR (CDCl₃, δ, ppm) 9.29 (s, 1H), 9.00 (d, 1H), 8.38 (dd, 1H), 8.21 (d, 1H), 4.52 (q, 2H), 2.79 (s, 3H), 1.48 (t, 3H).



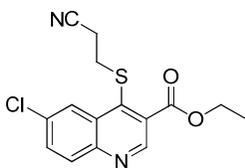
ethyl 4-((2-cyanoethyl)thio)-6-fluoroquinoline-3-carboxylate 20

Compound **20** was synthesized by method 4 from **20b** (76.1 mg, 0.30 mmol), 3-mercaptopropanenitrile (52.3 mg, 0.60 mmol) and K₂CO₃ (62.2 mg, 0.45 mmol). Purification by flash chromatography (gradient hexane/EtOAc from 90/10 to 55/45) to give **20** as an off-white powder (75.0 mg, 82%). Purity: 93.8%. ¹H NMR (CDCl₃, δ, ppm) 9.28 (s, 1H), 8.86 (dd, 1H), 8.45 (dd, 1H), 7.88 (m, 1H), 4.57 (q, 2H), 3.45 (t, 2H), 2.76 (t, 2H), 1.51 (t, 3H). ¹³C NMR (CDCl₃, δ, ppm) 164.12, 162.88, 161.56, 143.46, 136.81, 131.56, 128.48, 125.78, 116.89, 111.39, 63.73, 33.22, 18.71, 14.23. HRMS *m/z* calcd for C₁₅H₁₃N₂O₂SF [M + H]⁺ 305.0760, found 305.0748.



ethyl 4-((2-cyanoethyl)thio)-6-methylquinoline-3-carboxylate 21

Compound **21** was synthesized by method 4 from **21b** (74.9 mg, 0.3 mmol), 3-mercaptopropanenitrile (52.3 mg, 0.60 mmol) and K₂CO₃ (62.2 mg, 0.45 mmol). Purification by flash chromatography (gradient hexane/EtOAc from 90/10 to 55/45) to give **21** as a yellow powder (86.5 mg, 96%). ¹H NMR (CDCl₃, δ, ppm) 9.02 (s, 1H), 8.42 (s, 1H), 8.09 (d, 1H), 7.69 (dd, 1H), 4.52 (q, 2H), 3.24 (t, 2H), 2.64 (s, 3H), 2.53 (t, 2H), 1.47 (t, 3H). ¹³C NMR (CDCl₃, δ, ppm) 166.37, 147.58, 147.33, 139.17, 133.85, 128.93, 125.19, 117.75, 62.41, 32.26, 22.07, 18.74, 14.29. HRMS *m/z* calcd for C₁₆H₁₆N₂O₂S [M + H]⁺ 301.1011, found 301.1000.



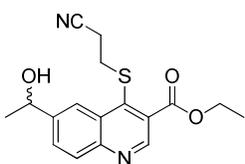
ethyl 6-chloro-4-((2-cyanoethyl)thio)quinoline-3-carboxylate 22

Compound **22** was synthesized by method 4 from **22b** (81.0 mg, 0.30 mmol), 3-mercaptopropanenitrile (52.3 mg, 0.60 mmol) and K_2CO_3 (62.2 mg, 0.45 mmol). Purification by flash chromatography (gradient hexane/EtOAc from 90/10 to 55/45) to give **22** as a white powder (93.0 mg, 96%). 1H NMR ($CDCl_3$, δ , ppm) 9.07 (s, 1H), 8.62 (d, 1H), 8.13 (d, 1H), 7.79 (dd, 1H), 4.53 (q, 2H), 3.24 (t, 2H), 2.57 (t, 2H), 1.48 (t, 3H). ^{13}C NMR ($CDCl_3$, δ , ppm) 165.95, 148.79, 147.25, 140.52, 135.09, 132.45, 132.04, 129.78, 125.28, 117.47, 62.66, 32.31, 18.79, 14.28. HRMS m/z calcd for $C_{15}H_{13}ClN_2O_2S$ $[M + H]^+$ 321.0465, found 321.0459.



ethyl 6-acetyl-4-((2-cyanoethyl)thio)quinoline-3-carboxylate 23

Compound **23** was synthesized by method 4 from **23b** (121.5 mg, 0.45 mmol), 3-mercaptopropanenitrile (78 μ L, 0.90 mmol) and K_2CO_3 (93.3 mg, 0.68 mmol). Purification by flash chromatography (gradient hexane/EtOAc from 70/30 to 50/50) to give **23** as a white powder (100.0 mg, 68%). 1H NMR ($CDCl_3$, δ , ppm) 9.34 (d, 1H), 9.21 (s, 1H), 8.42 (dd, 1H), 8.27 (d, 1H), 4.55 (q, 2H), 3.33 (t, 2H), 2.84 (s, 3H), 2.58 (t, 2H), 1.49 (t, 3H). ^{13}C NMR ($CDCl_3$, δ , ppm) 150.84, 130.86, 129.86, 128.43, 62.70, 32.84, 26.96, 18.84, 14.28. HRMS m/z calcd for $C_{17}H_{16}N_2O_3S$ $[M + H]^+$ 329.0960, found 329.0953.



ethyl 4-((2-cyanoethyl)thio)-6-(1-hydroxyethyl)quinoline-3-carboxylate 24

To a solution of **23** (33.0 mg, 0.15 mmol) in methanol (2 mL) was added sodium borohydride (5.7 mg, 0.15 mmol) in 2 portions. The mixture was stirred for 20 min at room temperature. Completion of the reaction was monitored by TLC. The mixture was filtered through celite. The solvent was evaporated and the residue was purified by flash chromatography (gradient $CH_2Cl_2/MeOH$ from 98/2 to 95/5) to give **24** as a yellow solid (21.2 mg, 43%). 1H NMR ($CDCl_3$, δ , ppm) 9.07 (s, 1H), 8.71 (d, 1H), 8.17 (d, 1H), 7.88 (dd, 1H), 5.20 (q, 1H), 4.53 (q, 2H), 3.27 (t, 2H), 2.53 (d, 2H), 1.61 (d, 3H), 1.48 (t, 3H). ^{13}C NMR ($CDCl_3$, δ , ppm) 166.25, 148.40, 146.67, 131.29, 130.50, 129.69, 129.01, 122.36, 117.96, 70.03, 62.46, 32.43, 25.48, 18.75, 14.29. HRMS m/z calcd for $C_{17}H_{18}N_2O_3S$ $[M + H]^+$ 331.1116, found 331.1105.

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