Synthesis and biological evaluation of 8-hydroxy-2,7-naphthyridin-2-ium salts as novel inhibitors of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE)

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Supplementary Methods

Synthesis

**General remarks:** Starting materials (chemicals) were purchased from commercial suppliers and used without any further purification. Solvents were used in p.a. quality and dried according to common procedures, if necessary. Thin-layer chromatography (TLC) for reaction monitoring was performed with alumina plates coated with Merck silica gel 60 F254 (layer thickness: 0.2 mm) or Merck silica gel 60 RP-18 F254 (layer thickness: 0.2 mm) and analyzed under UV-light (254 nm). Yields were not optimized. For UV-Vis measurements we used a SI Analytics UviLine 9400 Spectrometer, for fluorescence measurements a PerkinElmer LS45 Luminescence Spectrometer. NMR spectra were recorded using a Bruker Avance DRX Spectrometer (\(^1\)H: 400 MHz, \(^{13}\)C: 100 MHz) instrument. The spectra are referenced against the NMR solvent and are reported as follows: \(^1\)H: chemical shift \(\delta\) (ppm), number of equivalent nuclei (by integration), multiplicity (s = singlet, d = doublet, dd = doublet of doublets, t = triplet, m = multiplet, br = broad), coupling constant (J in Hz), assignment. \(^{13}\)C: chemical shift \(\delta\) (ppm). The assignment resulted from HMBC and HSQC experiments. IR spectra were measured with Spectrum One FT-IR from PerkinElmer. Mass spectra were measured with a LCQ-Advantage or Exactive device (Thermo Fisher Scientific, Waltham, MA). Purity was determined for all tested compounds by HPLC and UV detection (\(\lambda = 210\) nm) and was >95%. HPLC methods, including semi-preparative HPLC purification and analyses of purity, were performed using the following protocols: M1: semi-preparative HPLC system comprises Alliance 2695 Separations Module, Alliance 2487 Dual \(\lambda \)Absorbance Detector, and Synergi 10 \(\mu\)m Hydro-RP 80 Å, 250 x 15 mm as the semi-preparative column. Compounds were dissolved in methanol (10 mg/mL) for injection. Elution was performed at room temperature under gradient conditions. Eluent A was water containing 0.05% (v/v) TFA; eluent B was MeCN, also containing 0.05% (v/v) TFA. Linear gradient conditions were as follows: 0–0.1 min, A=90%, B=10%; 0.1–12 min, linear increase to B=100%; 29–41 min, B=100%; 41–43 min, linear decrease to B=10%; 43–55 min A=90%, B=10%. A flow rate of 2 mL·min\(^{-1}\) was maintained during the entire elution. M2: analytical HPLC system comprises Alliance 2695 Separations Module, Alliance 2487 Dual \(\lambda \)Absorbance Detector, and Synergi 4 \(\mu\)m Max-RP 80 Å, 150 x 4.6 mm as the analytical column. Compounds were dissolved in methanol (1 mg/mL) for injection. Elution was performed at room temperature under gradient conditions. Eluent A was water containing 0.05% (v/v) TFA; eluent B was MeCN, also containing 0.05% (v/v) TFA. Linear gradient conditions were as follows: 0–4 min, A=90%, B=10%; 4–29 min, linear increase to B=100%; 29–31 min, B=100%; 31–31.5 min, linear decrease to B=10%; 31.5–40 min A=90%, B=10%. A flow rate of 1 mL·min\(^{-1}\) was maintained during the entire elution. M3: same conditions as Method M2, except for the analytical column, a LiChrospher 60 RP-select B (5 \(\mu\)m) LiChroCART 250 x 4 mm.
**Compound synthesis and characterization:**

The synthesis of compounds 5a-b has already been reported. Because of changes in the experimental procedure and to show unpublished characterization data, we have outlined the synthesis and the characterization data for those compounds as well.

8-hydroxy-2-methyl-6-phenyl-2,7-naphthyridin-2-ium chloride (3a)

3a was synthesized from 1-methyl-3-carbamoylpyridin-1-ium chloride (0.35 g, 2 mmol) and acetophenone (0.48 g, 4 mmol) according to the general procedure published by Ukawa-Ishikawa et al.1 Both starting materials were dissolved in a mixture of MeOH/H₂O (5 mL, 1:1 v/v) and treated with a KOH solution (1.5 mL, 5M). The reaction mixture was stirred for 2 h at 0 °C. Then, formic acid (8 mL) was added, and the mixture was heated for 10 min under reflux. After cooling down to room temperature, the volume of the reaction mixture was reduced by approximately 50% under reduced pressure. The reaction mixture was washed with cyclohexane. Subsequently, the pH of the aqueous layer was adjusted with KOH (5M) to a value of 11. The precipitate was separated by filtration, washed with ice-cold water, and dried under reduced pressure. The final purification step was a recrystallization from ethanol acidified with HCl. Yield: (181 mg, 33%); Appearance: yellow powder; IR absorptions (ν_max/cm⁻¹): 3347w (υ -OH), 3047w (υ -C=CH), 1683s (υ –C=N- imidic acid), 1645s (υ -C=C-), 1608s (υ –C=N+), 1193m (υ –C-O), 862s, 779s, 687s; 1H NMR (DMSO-D₆, 400 MHz, δ [ppm]): 12.65 (1H, br s, -OH), 9.58 (1H, s, H-1), 8.82 (1H, d, 3J = 6.7 Hz, H-3), 7.83-7.23 (1H, s, H-5), 4.37 (3H, s, -CH₃); 13C NMR (DMSO-D₆, 100 MHz, δ [ppm]): 161.22 (C-8), 152.38 (C-6), 147.95 (C-4a), 147.56 (C-7), 144.23 (C-3), 132.48 (phenyl C-1), 132.07 (phenyl C-4), 129.58 (phenyl C-3,5), 128.20 (phenyl C-2,6), 123.91 (C-4), 121.15 (C-8a), 101.68 (C-5), 47.71 (-CH₃); MS (ESI+): m/z = 237.3 [M]+; Purity: 99.3% (9.37 min, M2); 99.2% (11.47 min, M3).

2-ethyl-8-hydroxy-6-phenyl-2,7-naphthyridin-2-ium chloride (3b)

3b was synthesized from 5a (0.57 g, 2 mmol) and acetophenone (0.48 g, 4 mmol) according to the general procedure published by Ukawa-Ishikawa et al.1 (see 3a). Yield: (52 mg, 9%); Appearance: yellow powder; IR absorptions (ν_max/cm⁻¹): 3367w (υ -OH), 3051w (υ -C=CH), 1679s (υ –C=N- imidic acid), 1641s (υ –C=C-), 1613m (υ –C=N+), 1173m (υ –C-O), 778s; 1H NMR (DMSO-D₆, 400 MHz, δ [ppm]): 12.67 (1H, br s, -OH), 9.66 (1H, s, H-1), 8.96-8.91 (1H, m, H-3), 8.21 (1H, d, 3J = 6.6 Hz, H-4), 8.00-7.80 (2H, m, phenyl H-2,6), 7.68-7.52 (3H, m, phenyl H-3,4,5), 7.24 (1H, s, H-5), 4.68 (2H, q, 3J = 7.1 Hz, -CH₂-CH₃), 1.56 (3H, t, 3J = 7.1 Hz, -CH₂-CH₃); 13C NMR (DMSO-D₆, 100 MHz, δ [ppm]): 161.25 (C-8), 152.53 (C-6), 148.24 (C-4a), 146.62 (C-1), 142.98 (C-3), 132.50 (phenyl C-1), 132.10 (phenyl C-4), 129.60 (phenyl C-3,5), 128.20 (phenyl C-2,6), 124.42 (C-4), 121.52 (C-8a), 101.71 (C-5), 56.15 (-CH₂-CH₃), 16.68 (-CH₂-CH₃); MS (ESI+): m/z = 251.2 [M]+; Purity: 98.9% (10.18 min, M2); 98.5% (12.31 min, M3).
2-benzyl-8-hydroxy-6-phenyl-2,7-naphthyridin-2-i um chloride (3c)

3c was synthesized from 5b (0.50 g, 2 mmol) and acetophenone (0.48 g, 4 mmol) according to the general procedure published by Ukawa-Ishikawa et al. \(^1\) (see 3a). Yield: (52 mg, 8%); Appearance: yellow powder; IR absorptions (\(\nu_{\text{max}}/\text{cm}^{-1}\)): 3066w (\(\nu \text{-C} = \text{C-H}\)), 2977w (\(\nu \text{-C-H}\)), 1682s (\(\nu \text{-C=N-N-imidic acid}\)), 1637s (\(\nu \text{-C=N+}\)), 1594s (\(\nu \text{-C=C-}\)), 1197 (\(\nu \text{-C=O}\)), 877m, 771s, 731s, 707m, 691s; \(^1\)H NMR (DMSO-D\(_6\), 400 MHz, \(\delta [\text{ppm}]\)): 12.75 (1H, br s, -O-H), 9.87 (1H, s, H-1), 8.95-8.90 (1H, m, H-3), 8.18 (1H, d, \(3J = 6.8 \text{ Hz}\), H-4), 7.92-7.86 (2H, m, phenyl H-2,6), 7.67-7.53 (5H, m, phenyl H-3,4,5 & benzyl H-2,6), 7.49-7.40 (3H, m, benzyl H-3,4,5), 7.25 (1H, s, 5-H), 5.90 (2H, s, -CH\(_2\)); 13C NMR (DMSO-D\(_6\), 100 MHz, \(\delta [\text{ppm}]\)): 161.30 (C-8), 153.03 (C-6), 148.50 (C-4a), 146.88 (C-1), 143.10 (C-3), 135.01 (benzyl C-1), 132.48 (phenyl C-1), 132.14 (phenyl C-4), 129.65 (benzyl C-4), 129.62 (benzyl C-3,5), 129.56 (phenyl C-3,5), 129.16 (benzyl C-2,6), 128.22 (phenyl C-2,6), 124.63 (C-4), 124.63 (C-4), 121.73 (C-8a), 101.75 (C-5), 62.81 (-CH\(_2\)); MS (ESI+): \(m/z = 313.1 \ [M]^+\); Purity: 99.9% (13.26 min, M2); 98.7% (16.08 min, M3).

2-ethyl-8-hydroxy-6-(naphthalen-2-yl)-2,7-naphthyridin-2-im chloride (3d)

3d was synthesized from 5a (0.57 g, 2 mmol) and 1-(naphthalen-2-yl)ethan-1-one (0.68 g, 4 mmol) according to the general procedure published by Ukawa-Ishikawa et al. \(^1\) Both starting materials were dissolved in a mixture of MeOH/H\(_2\)O (5 mL, 1:1 v/v) and treated with a KOH solution (1.5 mL, 5M). The reaction mixture was stirred for 2 h at 0 °C. Then, formic acid (8 mL) was added, and the mixture was heated for 10 min under reflux. After cooling down to room temperature, the precipitate was separated by filtration, washed with an ice-cold mixture of H\(_2\)O/MeOH (9:1 v/v), and dried under reduced pressure. The dry powder was dissolved in a minimal amount of a MeOH/H\(_2\)O mixture (2:1 v/v) and the solution was acidified by the addition of HCl conc. to a pH-value of 2. This mixture was washed with cyclohexane (3x) and dried under reduced pressure. The final purification step was performed by using the semi-preparative HPLC method M1. Yield: (66 mg, 10%); Appearance: yellow powder; IR absorptions (\(\nu_{\text{max}}/\text{cm}^{-1}\)): 3060w (\(\nu \text{-C} = \text{C-H}\)), 1675s (\(\nu \text{-C=N-Imidsäre}\)), 1636s (\(\nu \text{-C=C-}\)), 1607s (\(\nu \text{-C=N+}\)), 1127s (\(\nu \text{-C=O}\)), 797m, 718m; \(^1\)H NMR (CD\(_3\)OD, 400 MHz, \(\delta [\text{ppm}]\)): 9.62 (1H, s, H-1), 8.82-8.73 (1H, m, H-3), 8.45 (1H, s, naphthyl 1-H), 8.21-7.87 (5H, m, 4-H & naphthyl H-3,4,5,8), 7.72-7.61 (2H, m, naphthyl H-6,7), 7.27 (1H, s, 5-H), 4.64 (2H, q, \(3J = 7.3 \text{ Hz}\), -CH\(_2\)-CH\(_3\)), 1.72 (3H, t, \(3J = 7.3 \text{ Hz}\), -CH\(_2\)-CH\(_3\)); \(^{13}\)C NMR (DMSO-D\(_6\), 100 MHz, \(\delta [\text{ppm}]\)): 161.54 (C-8), 152.67 (C-6), 148.15 (C-4a), 146.85 (C-1), 142.91 (C-3), 134.47 (naphthyl C-4a), 132.84 (naphthyl C-8a), 129.41 (naphthyl C-8), 129.28 (naphthyl C-4), 128.74 (naphthyl C-1,6), 128.21 (naphthyl C-5), 127.76 (naphthyl C-7), 124.66 (naphthyl C-3), 124.28 (C-4), 121.50 (C-8a), 101.94 (C-5), 56.05 (-CH\(_2\)-CH\(_3\)), 16.76 (-CH\(_2\)-CH\(_3\)); MS (ESI+): \(m/z = 313.1 \ [M]^+\); Purity: 97.8% (13.39 min, M2); 97.0% (15.98 min, M3).

2-benzyl-8-hydroxy-6-(naphthalen-2-yl)-2,7-naphthyridin-2-im chloride (3e)

3e was synthesized from 5b (0.50 g, 2 mmol) and 1-(naphthalen-2-yl)ethan-1-one (0.68 g, 4 mmol) according to the general procedure published by Ukawa-Ishikawa et al. \(^1\) Both starting materials were dissolved in a mixture of MeOH/H\(_2\)O (5 mL, 1:1 v/v) and treated with a KOH solution (1.5 mL, 5M). The reaction mixture was stirred for 2 h at 0 °C. Then, formic
acid (8 mL) was added, and the mixture was heated for 10 min under reflux. After cooling down to room temperature, the volume of the reaction mixture was reduced by approximately 50% under reduced pressure. The reaction mixture was washed with cyclohexane. Subsequently, the pH of the aqueous layer was adjusted with KOH (5M) to a value of 11. Solvents were evaporated under reduced pressure. The residue was dissolved in EtOH (10 mL) and cooled for 12 h at 4 °C. The inorganic precipitate (KCl, K(HCOO)) was separated by filtration. The ethanolic filtrate was acidified with HCl conc. to a pH-value of 2 and stored at -20 °C for 24 h. The precipitate was separated by filtration and dried under reduced pressure. The final purification step was performed by using the semi-preparative HPLC method M1. Yield: (139 mg, 17%); Appearance: yellow powder; IR absorptions (υmax/cm⁻¹): 3067w (υ – C=C-H), 1664s (υ – C=N- imidic acid), 1613s (υ – C=O-H), 1129s (υ – C-O-H), 795m, 719m, 703s; ¹H NMR (DMSO-D₆, 400 MHz, δ [ppm]): 12.83 (1H, br s, -O-H), 9.84 (1H, s, H-1), 8.90 (1H, d, 3J = 6.8 Hz, H-3), 8.56-8.52 (1H, m, naphthyl H-1′), 8.18 (1H, d, 3J = 6.8 Hz, 4-H), 8.11 (1H, d, 3J = 8.7 Hz, naphthyl H-4), 8.08-7.99 (2H, m, naphthyl H-5,8), 7.95 (1H, d, 3J = 8.7 Hz, naphthyl H-3), 7.67-7.62 (2H, m, naphthyl H-6,7), 7.60-7.55 (2H, m, benzyl H-2,6), 7.51-7.44 (3H, m, benzyl H-3,4,5), 7.37 (1H, s, 5-H), 5.89 (2H, s, -CH₂(CH₃); ¹³C NMR (DMSO-D₆, 100 MHz, δ [ppm]): 161.31 (C-8), 152.30 (C-6), 148.50 (C-4a), 146.82 (C-7), 143.10 (C-3), 134.95 (benzyl C-1), 134.51 (naphthyl C-4a), 132.80 (naphthyl C-8a), 129.76 (benzyl C-4), 129.71 (benzyl C-3,5), 129.65 (naphthyl C-2), 129.41 (naphthyl C-8), 129 (benzyl C-2,6 & naphthyl C-4), 128.83 (naphthyl C-1), 127.75 (naphthyl C-7), 124.70 (C-4), 124.61 (naphthyl C-3), 121.76 (C-8a), 102.13 (C-5), 62.96 (-CH₂); MS (ESI⁺): m/z = 363.2 [M⁺]; Purity: 96.0% (15.68 min, M2); 98.0% (18.83 min, M3).

3-ethyl-5-hydroxy-11H-indeno[1,2-c][2,7]naphthyridin-3-im chloride (3f)

3f was synthesized from 5a (0.57 g, 2 mmol) and 1-indanone (0.53 g, 4 mmol) according to the general procedure published by Ukawa-Ishikawa et al.¹ (see 3e). Yield: (11 mg, 2%); Appearance: yellow powder; IR absorptions (υmax/cm⁻¹): 1683s (υ – C=N- imidic acid), 1640w (υ – C=C-), 1614w (υ – C=N’-), 1140m (υ – C-O-H), 772w, 728m, 708s; ¹H NMR (CD3OD, 400 MHz, δ [ppm]): 9.55 (1H, d, 3J = 1.27 Hz, H-4), 8.74 (1H, dd, 3J = 6.9 Hz, 4J = 1.27 Hz, H-2), 8.13 (1H, d, 3J = 6.9 Hz, H-1), 8.08 (1H, d, 3J = 7.7 Hz, H-7), 7.79 (1H, d, 3J = 7.5 Hz, H-10), 7.68-7.62 (1H, m, H-9), 7.60-7.54 (1H, m, H-8), 4.67 (2H, q, 3J = 7.4 Hz, -CH₂(CH₃), 4.09 (2H, s, H-11), 1.70 (3H, t, 3J = 7.4 Hz, -CH₂-CH₃); ¹³C NMR (DMSO-D₆, 100 MHz, δ [ppm]): 161.52 (C-5), 152.19 (C-6a), 147.00 (C-4), 146.30 (C-10a), 144.64 (C-11b), 142.90 (C-2), 134.97 (6b-C), 131.36 (C-9), 128.10 (C-8), 126.13 (C-10), 122.58 (C-7), 121.26 (C-1), 120.94 (C-4a), 114.19 (C-11a), 55.91 (-CH₂-CH₃), 33.03 (C-11), 16.67 (-CH₂-CH₃); MS (ESI⁺): m/z = 263.3 [M⁺]; Purity: 96.3% (11.39 min, M2); 95.8% (13.51 min, M3).

2-benzyl-12-hydroxy-5,6,6a,10a-tetrahydronaphtho[1,2-c][2,7]naphthyridin-2-im chloride (3g)

3g was synthesized from 5b (0.50 g, 2 mmol) and 1-tetralone (0.58 g, 4 mmol) according to the general procedure published by Ukawa-Ishikawa et al.¹ (see 3a). Yield: (22 mg, 3%); Appearance: yellow powder; IR absorptions (υmax/cm⁻¹): 3056w (υ – C=C-H), 1676s (υ – C=N- imidic acid), 1636s (υ – C=C-), 1609m (υ – C=N’-), 1134 (υ – C-O-), 800m, 775s, 706s;
$^1$H NMR (CD$_3$OD, 400 MHz, $\delta$ [ppm]): 9.66 (1H, d, $^4J = 1.5$ Hz, H-1), 8.77 (1H, dd, $^3J = 7.1$ Hz, $^4J = 1.5$ Hz, H-3), 8.24 (1H, d, $^3J = 7.1$ Hz, H-4), 7.96-7.92 (1H, m, H-10), 7.59-7.45 (8H, m, H-7,8,9 & benzyl H-2,3,4,5,6), 5.86 (2H, s, N+-CH$_2$-), 3.10-2.96 (4H, m, H-5,6); 13C NMR (CD$_3$OD, 100 MHz, $\delta$ [ppm]): 159.59 (C-12), 146.67 (C-4a), 145.64 (C-10b), 145.85 (C-7), 141.09 (C-3), 139.20 (C-6a), 132.64 (benzyl C-1), 131.16 (C-8), 128.69 (benzyl C-4), 128.47 (benzyl C-3,5), 127.81 (benzyl C-2,6), 127.56 (C-7 or C-9), 126.42 (C-7 or C-9), 126.16 (C-10a), 123.70 (C-10), 120.95 (C-12a), 120.19 (C-4), 108.61 (C-4b), 62.81 (N+-CH$_2$-), 26.24 (C-6), 19.61 (C-5); MS (ESI+): $m/z = 339.2$ [M]$^+$; Purity: 98.8% (14.38 min, M2), 98.2% (17.42 min, M3).

2-ethyl-8-hydroxy-6-(pyridin-2-yl)-2,7-naphthyridin-2-ium chloride (3h)

3h was synthesized from 5a (0.57 g, 2 mmol) and 1-(2-pyridyl)ethanone (0.48 g, 4 mmol) according to the general procedure published by Ukawa-Ishikawa et al. Both starting materials were dissolved in a mixture of MeOH/H$_2$O (5 mL, 1:1 v/v) and treated with a KOH solution (1.5 mL, 5M). The reaction mixture was stirred for 2 h at 0 °C. Then, formic acid (8 mL) was added, and the mixture was heated for 10 min under reflux. After cooling down to room temperature, the mixture was evaporated to dryness. The residue was recrystallized from MeOH. The precipitate was separated by filtration and dried under reduced pressure. Then, the product was recrystallized from ethanol acidified with HCl by the addition of petroleum ether. The final purification step was performed by using the semi-preparative HPLC method M1. Yield: (34 mg, 6%); Appearance: yellow powder; IR absorptions ($\nu$ max/cm$^{-1}$): 3226w ($\nu$-O-H), 3078w ($\nu$-C=C-H), 2989w ($\nu$-C-H), 1684m ($\nu$-C=N- imidic acid), 1638m ($\nu$-C=C-), 1622m ($\nu$-C=N+), 1179m ($\nu$-C-O), 1120m, 871m, 788s, 702s; 1H NMR (DMSO-D$_6$, 400 MHz, $\delta$ [ppm]): 12.00 (1H, br s, -O-H), 9.66 (1H, s, H-1), 8.91-8.77 (2H, m, pyridinyl H-6), 8.31-8.18 (2H, m, H-4 & pyridinyl H-3), 8.11-8.03 (1H, m, pyridinyl H-4), 7.70-7.58 (2H, m, H-5 & pyridinyl H-5), 4.67 (2H, q, $^3J = 7.3$ Hz, -CH$_2$-CH$_3$), 1.57 (3H, t, $^3J = 7.3$ Hz, -CH$_2$-CH$_3$); 13C NMR (DMSO-D$_6$, 100 MHz, $\delta$ [ppm]): 160.28 (C-8), 150.28 (pyridinyl C-6), 148.50 (C-6), 148.44 (C-4a), 147.93 (pyridinyl C-2), 146.65 (C-1), 143.15 (C-3), 138.62 (pyridinyl C-4), 126.87 (pyridinyl C-5), 125.02 (C-4), 122.89 (pyridinyl C-3), 122.82 (C-8a), 101.55 (C-5), 56.45 (-CH$_2$-CH$_3$), 16.54 (-CH$_2$-CH$_3$); MS (ESI+): $m/z = 252.2$ [M]$^+$; Purity: 95.2% (9.02 min, M2), 96.7% (11.10 min, M3).

3-carbamoyl-1-ethylpyridin-1-ium iodide (5a)$^2$

5a was synthesized from nicotinamide and ethyl iodide according to a previously published procedure.$^2$ Yield: (29%); Appearance: colorless needles; IR absorptions ($\nu$ max/cm$^{-1}$): 3332w ($\nu$-NH$_2$), 3163w ($\nu$-C=C-H), 1672s ($\nu$-amide I), 1633m ($\nu$-amide II), 1606s ($\nu$-C=N$^-$), 1383m, 1194m, 759m, 675s; $^1$H NMR (DMSO-D$_6$, 400 MHz, $\delta$ [pppm]): 9.48 (1H, s, H-2), 9.24-9.19 (1H, m, H-6), 8.94-8.87 (1H, m, H-4), 8.54 (1H, br s, -NH$_2$), 8.30-8.24 (1H, m, H-5), 8.17 (1H, s br, -NH$_2$), 4.69 (2H, q, $^3J = 7.3$ Hz, -CH$_2$-CH$_3$), 1.57 (3H, t, $^3J = 7.3$ Hz, -CH$_2$-CH$_3$); 13C NMR (D$_2$O, 100 MHz, $\delta$ [ppm]): 165.92 (C=O), 146.26 (C-6), 144.09 (C-2), 143.81 (C-4), 133.88 (C-3), 128.40 (C-5), 58.00 (-CH$_2$-CH$_3$), 15.59 (-CH$_2$-CH$_3$); MS (ESI+): $m/z = 151.1$ [M]$^+$.
1-benzyl-3-carbamoylpyridin-1-ium chloride (5b)\(^3\)

5b was synthesized from nicotinamide and benzyl chloride according to a previously published procedure.\(^3\) Yield: (71%); Appearance: colorless needles; IR absorptions (\(\nu_{\text{max}}/cm^{-1}\)): 3265w (\(\nu_{-NH_2}\)), 3128w (\(\nu_{-C=C-H}\)), 1698s (\(\nu_{-amide\ I}\)), 1651m (\(\nu_{-amide\ II}\)), 1390m, 1186w, 734s, 697s, 669s; \(^1\)H NMR (DMSO-D\(_6\), 400 MHz, \(\delta\) [ppm]): 9.95 (1H, s, H-2), 9.42 (1H, d, \(3J = 6.1\) Hz, H-6), 9.10 (1H, d, \(3J = 8.0\) Hz, H-4), 9.00 (1H, br s, -NH\(_2\)), 8.29 (1H, dd, \(3J = 8.0\) Hz, \(3J = 6.1\) Hz, H-5), 8.21 (1H, br s, -NH\(_2\)), 7.68-7.62 (2H, m, benzyl H-2,6), 7.48-7.40 (3H, m, benzyl H-3,4,5), 5.98 (2H, s, -CH\(_2\)); \(^{13}\)C NMR (DMSO-D\(_6\), 100 MHz, \(\delta\) [ppm]): 163.07 (C=O), 146.84 (C-6); 145.42 (C-2), 144.54 (C-4), 134.57 (C-3), 134.45 (benzyl C-1), 129.91 (benzyl C-4), 129.66 (benzyl C-3,5); 129.58 (benzyl C-2,6), 128.70 (C-5), 63.85 (-CH\(_2\)); MS (ESI+): \(m/z = 213.0\) [M]**

Biological tests

**Cholinesterase activity assay:** Inhibition of BChE and AChE by the 8-hydroxy-2,7-naphthyridin-2-ium salts was determined using a kinetic assay in 96-well microplate format based on Ellman’s reaction.\(^4\) Two pseudo substrates, butyrylthiocholine chloride (BTCCl) and acetylthiocholine iodide (ATCI), were utilized to measure the activities of BChE and AChE, respectively. The assay conditions have been previously detailed.\(^5\) The Ellman’s method (1961) uses 5,5’-dithiobis(2-nitro-benzoic acid) (DTNB, Ellman’s reagent) which forms a colored anion (5-thio-2-nitro-benzoic acid) after reacting with the product of cholinesterase reaction. Spontaneous and enzymatic hydrolysis was measured spectrophotometrically at \(\lambda = 412\) nm using Varioskan Flash multimode plate reader (Thermo Fisher Scientific, Finland). Compounds were tested at 10 \(\mu\)M in the primary screening. Compounds that displayed more than 75% inhibition against any of the target enzymes at 10 \(\mu\)M were further characterized with dose-response experiments. These trials were performed against a wide concentration range (8 replicates per concentration, with 2 biological replicates). \(IC_{50}\) values were calculated with Prism program (version 5 for Windows) using a non-linear regression (sigmoidal fitting, four parameters with variable slope). Physostigmine (in methanol, final concentration of 10 \(\mu\)M) was used as a positive control to assess the assay accuracy during the screening trials, while DMSO:buffer Tris–HCl 50 mM pH 8 was used as solvent control. Final concentration of DMSO in the assay did not exceed 0.05%. As blank controls for our 8-hydroxy-2,7-naphthyridin-2-ium salts we detected their absorption before the enzymatic reaction was started. The enzymatic reaction was started with the addition of the enzyme. Enzymes used and their final concentrations were: equine BChE (EC 3.1.1.8, from equine serum, Sigma–Aldrich, USA) 0.350 U/ml; electric eel AChE (EC 3.1.1.7, from Electric eel, Sigma–Aldrich, USA) 0.224 U/ml. Reactions took place at room temperature (25 °C).

**pK\(_a\) determination via UV-Vis spectroscopy**

To determine the pK\(_a\) value of the 8-hydroxy-2,7-naphthyridin-2-ium salts, by the example of compound 3a, we recorded absorption spectra (\(\lambda = 300 - 500\) nm) at different pH values (pH = 2.3, 7.3, 8.0, 8.5, 9.2, 12.3) at a compound concentration of 52 \(\mu\)M (Fig S1). We used the spectrometer Lambda 10 from PerkinElmer for absorption detection. The physical basics for
our pKₐ determination via UV-Vis spectroscopy is the Beer–Lambert law (Eq. 1) and the Henderson–Hasselbalch equation (Eq.2).

\[ A = \varepsilon \cdot d \cdot c \]

Equation 1: Beer–Lambert law

\[ pH = pK_a - \log \frac{c_a}{c_b} \]

Equation 2: Henderson–Hasselbalch equation

The calculation of the pKₐ value from the spectral data was based on following assumptions:

- At pH = 2.3 only the protonated state exists
- At pH = 12.3 only the deprotonated state exists
- Absorption at pH-values of 2.3 and 12.3 were used to determine the absorption coefficients of the protonated and deprotonated state, respectively
- For the deprotonated state we used the absorption values at a wavelength of 410 nm and not at the absorption maximum (λ = 405 nm)

**Molecular docking**

Computational Methods: The crystal structures of donepezil in complex with Torpedo californica (Tc) AChE (PDB-ID: 1EVE solved at 2.5 Å),⁶ electrophorus electricus (Ee) AChE (PDB-ID: 1EEA),⁷ and human BChE in complex with benzyl pyridinium-4-methyltrichloroacetimidate (PDB-ID: 4B0O solved at 2.35 Å)⁸ were taken from the Protein Data Bank.⁹ The crystal structure of EeAChE has been solved only in the apo form and at low resolution (4.2 Å). Since the sequence identity of the binding pocket of TcAChE and EeAChE is 100% as well as the structural overlay of both pockets showed a small deviation (0.30 Å) we took the protein from the TcAChE-donepezil structure for docking. In case of BuChE there is no crystal structure of the horse serum enzyme available, therefore we used the human enzyme which has been crystallized with a benzylpyridinium salt (PDB ID 4B0O) that shows similarity to the novel inhibitors developed in this work. Human and horse serum enzyme show an overall sequence identity of 90% and of 95% in the inhibitor binding pocket. Only one substitution is observed within the inhibitor binding pocket, Pro285 is mutated to Leu285 in horse serum BuChE. Both are hydrophobic residues with comparable side chain location. We generated a homology model of horse serum BuChE using the human enzyme using program MODELLER. The RMSD value of the superimposed binding pocket atoms was 0.1 Å indicating the nearly identical binding pockets. Redocking of the inhibitor cocrystallized in PDB ID 4B0O gave the same results as observed for the human
BuChE. For the docking study, the heteroatoms and water molecules (excluded the water molecule bound at the binding site) were removed and hydrogen atoms were added to the protein structures by using the program MOE 2012.10. All protein and ligand structures were built and protonated using the protonate 3D protocol and energy minimized using the MMFF94 force field in MOE 2012.10. For the 8-hydroxy-2,7-naphthyridin-2-ium salts the pyridone (9) and hydroxypyridine (3) tautomers were generated. Docking of the ligands was carried out using Glide SP Schrodinger 2014u2 with default settings. A sphere of 16 Å around the co-crystallized inhibitor was defined as the binding site for the ligand docking and 25 confirmations were generated for each ligand. Four conserved water molecules located in the BChE and five conserved water molecules in TcAChE binding pocket were considered for the inhibitor docking. The docking results were analyzed using MOE 2012.10. The protein-inhibitor complexes were finally minimized in MOE using the AMBER12_EHT force field and the GB/SA solvation model using default values. The protocol was able to reproduce the crystal structure of donepezil with 0.44 Å (PDB ID 1EVE) and the structure of benzyl pyridinium-4 methyltrichloroacetimidate with 0.88 Å (PDB ID 4B0O) by using the top-scored inhibitor pose.
Fig. S1. Absorption spectra of 3a at different pH-values.
Fig. S2. Downfield shifted signals in the $^1$H NMR spectra (400 MHz, DMSO-$D_6$) of compound 3b in its protonated (above) and deprotonated state (below). The proton signals of the 8-hydroxy-2,7-naphthyridin-2-ium scaffold and 2,7-naphthyridin-2-ium-8-olate scaffold, respectively, are labeled by their multiplicity and their assignment.
Fig S3. Docking pose of 3f (magenta) in comparison to 3e (cyan) derived for AChE. Both inhibitors are interacting in a similar way with the residues of the binding pocket.
Fig S4. Docking pose of 3f (magenta) in comparison to 3e (cyan) derived for BChE.
### Supplementary Table

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Table S1: Excitation and emission maxima ($\lambda_{\text{abs}}^\text{max}$ and $\lambda_{\text{em}}^\text{max}$), relative fluorescence intensity (FI) and fluorescence quantum yield ($\Phi_F$) of compound 3a-h. Measurements were performed using aqueous Tris buffer (25 mM, pH = 8) at room temperature. Relative fluorescence intensity was determined at a compound concentration of 15 nM.
Supplementary NMR spectra

$^1$H NMR (400 MHz, DMSO-D$_6$) for 8-hydroxy-2-methyl-6-phenyl-2,7-naphthyridin-2-ium chloride (3a):

$^{13}$C NMR (100 MHz, DMSO-D$_6$) for 8-hydroxy-2-methyl-6-phenyl-2,7-naphthyridin-2-ium chloride (3a):
$^1$H NMR (400 MHz, DMSO-$d_6$) for 2-ethyl-8-hydroxy-6-phenyl-2,7-naphthyridin-2-ium chloride (3b):

$^{13}$C NMR (100 MHz, DMSO-$d_6$) for 2-ethyl-8-hydroxy-6-phenyl-2,7-naphthyridin-2-ium chloride (3b):
$^1$H NMR (400 MHz, DMSO-$d_6$) for 2-benzyl-8-hydroxy-6-phenyl-2,7-naphthyridin-2-ium chloride (3c):

$^{13}$C NMR (100 MHz, DMSO-$d_6$) for 2-benzyl-8-hydroxy-6-phenyl-2,7-naphthyridin-2-ium chloride (3c):
$^1$H NMR (400 MHz, CD$_3$OD) for 2-ethyl-8-hydroxy-6-(naphthalen-2-yl)-2,7-naphthyridin-2-ium chloride (3d):

$^{13}$C NMR (400 MHz, DMSO-D$_6$) for 2-ethyl-8-hydroxy-6-(naphthalen-2-yl)-2,7-naphthyridin-2-ium chloride (3d):
$^1$H NMR (400 MHz, DMSO-D$_6$) for 2-benzyl-8-hydroxy-6-(naphthalen-2-yl)-2,7-naphthyridin-2-ium chloride (3e):

$^{13}$C NMR (400 MHz, DMSO-D$_6$) for 2-benzyl-8-hydroxy-6-(naphthalen-2-yl)-2,7-naphthyridin-2-ium chloride (3e):
$^1$H NMR (400 MHz, CD$_3$OD) for 3-ethyl-5-hydroxy-11H-indeno[1,2-c][2,7]naphthyridin-3-i um chloride (3f):

$^{13}$C NMR (100 MHz, DMSO-D$_6$) for 3-ethyl-5-hydroxy-11H-indeno[1,2-c][2,7]naphthyridin-3-i um chloride (3f):
$^1$H NMR (400 MHz, CD$_3$OD) for 2-benzyl-12-hydroxy-5,6,6a,10a-tetrahydronaphtho[1,2-c][2,7]naphthyridin-2-ium chloride (3g):

$^{13}$C NMR (400 MHz, CD$_3$OD) for 2-benzyl-12-hydroxy-5,6,6a,10a-tetrahydronaphtho[1,2-c][2,7]naphthyridin-2-ium chloride (3g):
$^1$H NMR (400 MHz, DMSO-$d_6$) for 2-ethyl-8-hydroxy-6-(pyridin-2-yl)-2,7-naphthyridin-2-ium chloride (3h):

$^{13}$C NMR (100 MHz, DMSO-$d_6$) for 2-ethyl-8-hydroxy-6-(pyridin-2-yl)-2,7-naphthyridin-2-ium chloride (3h):
$^1$H NMR (400 MHz, DMSO-D$_6$) for 3-carbamoyl-1-ethylpyridin-1-ium iodide (5a):

$^{13}$C NMR (100 MHz, D$_2$O) for 3-carbamoyl-1-ethylpyridin-1-ium iodide (5a):
$^1$H NMR (400 MHz, DMSO-**D$_6$**) for 1-benzyl-3-carbamoylpyridin-1-ium chloride (**5b**):

$^{13}$C NMR (400 MHz, DMSO-**D$_6$**) for 1-benzyl-3-carbamoylpyridin-1-ium chloride (**5b**):
Supplementary MS spectra

8-hydroxy-2-methyl-6-phenyl-2,7-naphthyridin-2-ium chloride (3a):
2-ethyl-8-hydroxy-6-phenyl-2,7-naphthyridin-2-ium chloride (3b):
2-benzyl-8-hydroxy-6-phenyl-2,7-naphthyridin-2-ium chloride (3c):
2-ethyl-8-hydroxy-6-(naphthalen-2-yl)-2,7-naphthyridin-2-ium chloride (3d):
2-benzyl-8-hydroxy-6-(naphthalen-2-yl)-2,7-naphthyridin-2-ium chloride (3e):
3-ethyl-5-hydroxy-11H-indeno[1,2-c][2,7]naphthyridin-3-ium chloride (3f):
2-benzyl-12-hydroxy-5,6,6a,10a-tetrahydronaphtho[1,2-c][2,7]naphthyridin-2-ium chloride (3g)
2-ethyl-8-hydroxy-6-(pyridin-2-yl)-2,7-naphthyridin-2-ium chloride (3h):
3-carbamoyl-1-ethylpyridin-1-ium iodide (5a):
1-benzyl-3-carbamoylpyridin-1-ium chloride (5b):
Supplementary IR spectra

8-hydroxy-2-methyl-6-phenyl-2,7-naphthyridin-2-ium chloride (3a):

2-ethyl-8-hydroxy-6-phenyl-2,7-naphthyridin-2-ium chloride (3b):
2-benzyl-8-hydroxy-6-phenyl-2,7-naphthyridin-2-ium chloride (3c):

2-ethyl-8-hydroxy-6-(naphthalen-2-yl)-2,7-naphthyridin-2-ium chloride (3d):
2-benzyl-8-hydroxy-6-(naphthalen-2-yl)-2,7-naphthyridin-2-ium chloride (3e):

3-ethyl-5-hydroxy-11H-indeno[1,2-c][2,7]naphthyridin-3-ium chloride (3f):
2-benzyl-12-hydroxy-5,6,6a,10a-tetrahydronaphtho[1,2-c][2,7]naphthyridin-2-ium chloride (3g):

2-ethyl-8-hydroxy-6-(pyridin-2-yl)-2,7-naphthyridin-2-ium chloride (3h):
3-carbamoyl-1-ethylpyridin-1-ium iodide (5a):

1-benzyl-3-carbamoylpyridin-1-ium chloride (5b):
Supplementary References

10. Molecular Operating Environment (Moe), 2012.10; Chemical Computing Group Inc., 1010 Sherbooke St. West, Suite #910, Montreal, Qc, Canada, H3a 2r7, 2012.