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Aminoalkoxy-substituted coumarins: Synthesis and evaluation for reactivation of inhibited human acetylcholinesterase

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Abstract

Reactivation of inhibited acetylcholinesterase remains an important therapeutic strategy for the treatment of poisoning by organophosphorus compounds, such as nerve agents or pesticides. Although drugs like obidoxime or pralidoxime have been used with considerable success, there is a need for new substances capable of reactivating acetylcholinesterase with a broader scope and increased efficacy. Possible screening candidates must fulfill two fundamental requirements: They must (i) show an affinity to acetylcholinesterase well balanced between sufficient binding and competitive inhibition and (ii) facilitate the nucleophilic cleavage of the phosphorylated catalytic serine residue. We attached a variety of nonaromatic primary and secondary amines to a coumarin core through selected alkoxy side linkers attached at coumarin positions 6 or 7 to obtain a small set of possible reactivators. Evaluation of their inhibition and reactivation potential in vitro showed some activity with respect to acetylcholinesterase inhibited by cyclosarin.

KEYWORDS
acetylcholinesterase, coumarins, enzyme reactivation, Mitsunobu reaction, organophosphorus compounds

1 | INTRODUCTION

Known for decades and stockpiled during cold war times, chemical weapons pose a frightening, underestimated threat to public health. Despite the Chemical Weapons Convention and ongoing efforts of the Organization for the Prohibition of Chemical Weapons to permanently and verifiably eliminate chemical weapons, recent events of the intentional release of these deadly substances are well documented.[1–3] In developing countries also self-poisoning by organophosphorus (OP) pesticides is a major medical problem causing more than 100,000 deaths per year.[4] In the face of this alarming situation, there is a strong need to deliver adequate therapeutic measures to patients within a short time. Moreover, current therapeutic options need to be extended and improved to avoid future casualties.

Toxicity of both nerve agents and OP pesticides mainly results from inhibition of acetylcholinesterase (AChE) causing accumulation of

acetylcholine and corresponding muscarinic and nicotinic symptoms like salivation, lacrimation, fasciculation, and respiratory distress.[5] If untreated, OP poisoning usually leads to death within minutes. Oximes capable of reactivating inhibited AChE are one rationale applied to counteract intoxications by nerve agents or OP pesticides. They act as nucleophiles cleaving the phosphylated (phosphorylated or phosphonylated) active serine and thus restoring enzyme function.[6] Obidoxime and pralidoxime are two well-established representatives that show reasonable reactivation potency against a variety of OP compounds, but lack broad-spectrum activity, especially for cyclosarin-inhibited AChE. Innumerable structural modifications have been introduced to modify their common pyridine aldoxime core to increase the reactivating scope.[6,7]

A major drawback of all currently fielded oximes is their permanent charge impairing tissue distribution and preventing sufficient blood–brain barrier crossing. To circumvent this, uncharged nonoxime reactivators derived from activated phenols or other α-nucleophiles like hydroxamates have been employed, but with limited success.[8,9] However, a completely different approach has recently been reported that aims to facilitate spontaneous reactivation instead of delivering tailored nucleophiles.[9] Spontaneous reactivation refers to hydrolytic liberation of the active serine, which has been observed for V-type nerve agents and OP pesticides, but not for G-type nerve agents.[10] It normally occurs only at a low rate but may be significantly increased by catalysis, which appears an interesting therapeutic approach. Proton transfer in an acid–base promoted manner is expected to activate an appropriately located water molecule and facilitates nucleophilic attack of the phosphylated active serine. In this respect, imidazole and pyridine-derived reactivators have been identified from high throughput screening of commercially available libraries.[11,12] This novel strategy of nonoxime reactivators offers not only the opportunity to deliver neutral compounds across the blood–brain barrier but also unlocks chemical space, which until now has been unexplored.

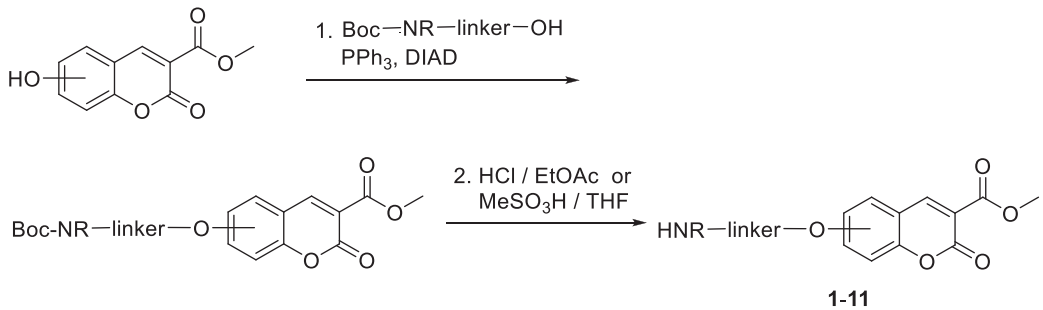
As with traditional oximes, any other reactivating agent will face the immanent dilemma of proper affinity to AChE. While sufficient affinity is required for fast enough formation of the enzyme-reactivator complex, too high affinity may cause too slow dissociation after reactivation. To avoid competitive inhibition, as a result, affinity needs to be carefully balanced and inhibition concentrations (IC_{50}) should not exceed the mid-micromolar range. Fortunately, AChE offers two binding sites that allow for modulation of affinity: the active site, where acetylcholine is cleaved, and a peripheral anionic site (PAS) involved in ligand guiding, which may still be addressed when the active site serine is occupied by phosphylation. Numerous ligands of AChE have been reported, mainly inhibitors, and many of them follow the strategy of binding simultaneously to the active and peripheral site to gain additional affinity through an increase in local or "effective" concentration.[13] Nonoxime reactivators, as mentioned above, also follow this dual binding site concept combining a substructure targeting the peripheral site and a heteroaromatic base catalyzing dephosphylation through acid–base promoted hydrolysis.[11,12]

In this study, we designed a series of aminoalkoxy-substituted coumarins combining this PAS-directed motif with a diverse, linker-connected set of amino moieties as the acting principle of dephosphylation. Cholinesterase inhibition and reactivation were subsequently investigated in vitro with tabun, sarin, cyclosarin, VX, and paraoxon-ethyl as a representative set of OP nerve agents and pesticides.

2 | RESULTS AND DISCUSSION

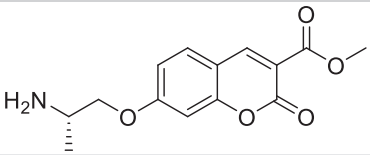
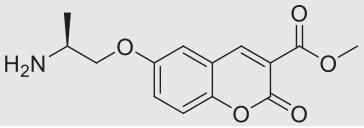
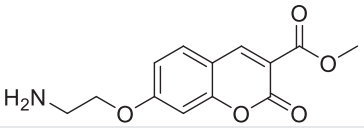
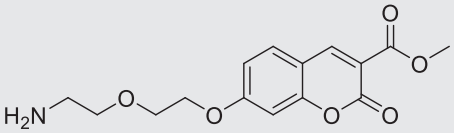
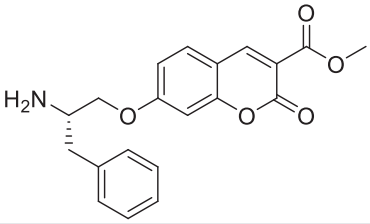
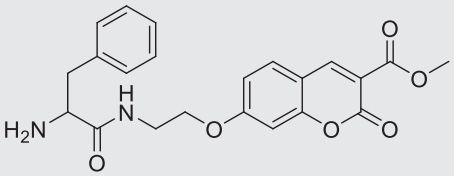
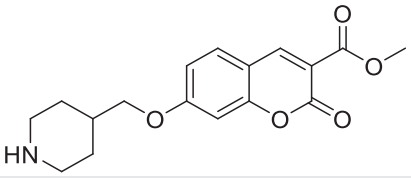
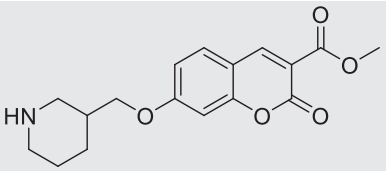
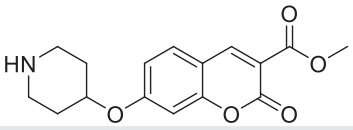
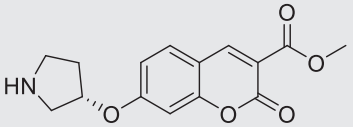
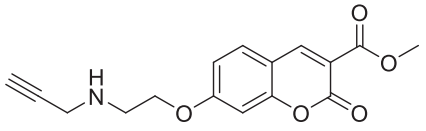
The dual binding site design reported in this study started from the coumarin motif, which constitutes a privileged and versatile scaffold for bioactive compounds,[14–17] in particular for the assembly of inhibitors of AChE, where coumarins are known to bind to the peripheral site mainly by π–π interactions.[18–27] To discover further bases capable of efficient dephosphylation, we replaced the imidazole or pyridine functional groups of reported nonoxime reactivators with nonaromatic primary or secondary amines, which we attached through alkoxy-linkers of various lengths at position 6 and 7 of the coumarin ring system. The resulting primary amines **1**–**6** (Table 1) contained a linear, amino acid-like substructure, whereas the secondary amines in compounds **7**–**11** were mostly cyclic, that is, derived from piperidine or pyrrolidine. In general, the basic nitrogen was placed at the terminal position to allow for interaction in dephosphylation and the alkoxy-linkers, as in **7**–**10**, were partly derived from methylene and methine groups of the piperidine or pyrrolidine ring. This diverse set of substructures was chosen to allow for additional interactions throughout the mid-gorge architecture of AChE.[28]

Knoevenagel condensation of 2,4-dihydroxy- or 2,5-dihydroxybenzaldehyde with dimethyl malonate catalyzed by piperidine[17] afforded the appropriate coumarins, which were subjected to subsequent Mitsunobu reactions (Scheme 1). Briefly, either methyl



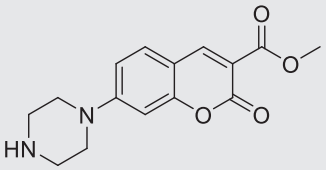
SCHEME 1 Synthesis of aminoalkoxy-substituted coumarins **1**–**11** from Boc-protected precursors. For detailed structures, see Table 1.

TABLE 1 Inhibition of human acetyl- (hAChE) and butyrylcholinesterase (hBChE) by **1**–**12** and obidoxime for reference

Compd	Free base	Acid/salt	Inhibition at 100 µM (%)[a]	
			hAChE	hBChE
1		HCl	63.6	13.7
2		HCl	51.5	9.9
3		HCl	50.8	11.4
4		HCl	64.4	14.6
5		HCl	81.8	40.0
6		HCl	65.7	11.4
7		$MeSO_3H$	59.0	68.6
8		$MeSO_3H$	57.0	67.1
9		$MeSO_3H$	21.5	11.6
10		$MeSO_3H$	59.9	10.6
11		HCl	64.1	25.7

(Continues)

TABLE 1 (Continued)

Compd	Free base	Acid/salt	Inhibition at 100 μM (%)[a] hAChE	hBChE
12		HCl	85.9	86.0
Obidoxime		HCl	7.4	10.3

[a]Cholinesterase inhibition was determined by a modified Ellman assay, which is based on a thiol-disulfide exchange reaction of 5,5′-dithiobis-2-nitrobenzoic acid and thiocholine released from the substrates acetylthiocholine or butyrylthiocholine. Compounds **1**–**12** themselves did not cleave acetylthiocholine under assay conditions.

6- or 7-hydroxy-2-oxo-2*H*-chromene-3-carboxylate, triphenylphosphine, and the respective Boc-protected primary amino-alcohol were dissolved in tetrahydrofuran (THF) and diisopropyl azodicarboxylate (DIAD) was added dropwise. Purification of the intermediates was achieved by column chromatography. Precursors for **1**–**5** and **7**–**11** were obtained that way. Boc-deprotection using hydrochloric or methanesulfonic acid directly precipitated **1**,[29] **2**–**10**, and **11**[30] as their respective hydrochlorides or methanesulfonates. Amide **6** (Table 1) was obtained by a uronium salt-mediated coupling of amine **3** (Table 1) with Boc-Phe-OH, followed by deprotection. Compound **12** (Table 1) was prepared as reported elsewhere.[31]

As pointed out above, it is very important to achieve a balanced cholinesterase affinity when designing reactivators to avoid counteracting competitive inhibition. This holds true not only for the target AChE but also for butyrylcholinesterase present in serum, which in case of high affinity might catch reasonable amounts of the applied reactivator from free circulation. Therefore, inhibition of human acetyl- and butyrylcholinesterase by compounds **1**–**12** was evaluated in vitro at 100 μM concentration, which has been shown to provide a good estimate of a possible mixed reactivation–inhibition behavior.[32]

First, the observed inhibition rates (Table 1) revealed that all compounds have no or no significant selectivity towards butyrylcholinesterase, which renders a negative impact on free circulation by butyrylcholinesterase binding unlikely. Second, there was no significant difference in AChE inhibition with respect to the location (position 6 or 7 of the coumarin moiety) and type of attachment. This suggests that affinity is mainly driven by the common coumarin moiety and not by additional interaction of the primary or secondary amine attached. These side chains should therefore be free to facilitate dephosphylation by proton abstraction from a suitably colocalized water molecule. This was subsequently evaluated for a representative set of nerve agent inhibited human AChE using an established reactivation assay.[33]

Tabun (GA), sarin (GB), cyclosarin (GF), VX as well as paraoxon-ethyl (PXE) were selected as representatives of nerve agents and OP pesticides to obtain inhibited human AChE with properties allowing for reasonable reactivation. Soman (GD) was excluded because aging (spontaneous hydrolytic loss of a second alkyl radical from the phosphylated enzyme) occurs within minutes rendering the inhibited enzyme futile towards reactivation. As shown in Table 2, most of the compounds **1**–**12** showed no or only very low reactivating power, but some activity was observed with human AChE inhibited by sarin and cyclosarin, especially with compounds **2**, **3**, **4**, and **9**. These compounds carry less bulky and mostly primary amino-alkoxy side chains that appear to fit more easily into the narrow mid-gorge region of AChE while reaching out to the phosphylated serine. Although reactivation does not occur at a rate considerable for further development, the obtained results show that next to established (hetero)aromatic structures also aliphatic or alicyclic primary and secondary amines may contribute to the design of nonoxime reactivators.

In addition to these findings, also stereochemical aspects become apparent when comparing the results obtained for the enantiomeric

TABLE 2 Reactivation of human acetylcholinesterase inhibited by tabun (GA), sarin (GB), cyclosarin (GF), VX, and paraoxon-ethyl (PXE) by compounds **1**–**12** and obidoxime after 60 min

Compd	Reactivation at 100 μM (%) GA	GB	GF	VX	PXE
1	n.r.	9.1	0.7	1.1	0.3
2	0.6	1.6	10.3	1.0	0.3
3	0.7	1.9	10.2	1.4	0.4
4	1.0	2.0	18.7	3.6	n.r.
5	0.9	0.6	7.3	1.0	n.r.
6	1.0	0.2	7.2	0.9	0.7
7	n.r.	7.5	7.0	n.r.	2.3
8	n.r.	8.6	4.7	n.r.	n.r.
9	1.3	3.1	11.9	0.8	1.1
10	1.8	1.8	6.1	1.5	1.5
11	n.r.	8.3	1.3	0.5	0.1
12	0.7	1.6	7.0	0.9	0.3
Obidoxime	35.8	44.9	66.5	81.3	87.4

Abbreviation: n.r., no reactivation.

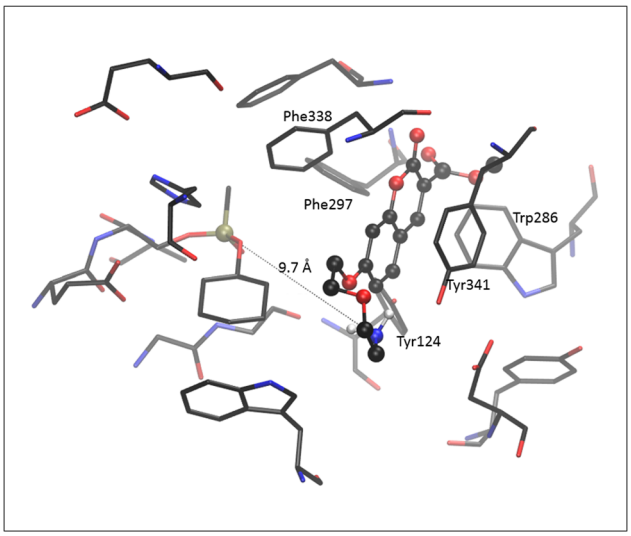


FIGURE 1 The most favorably oriented conformation of compound **4** docked into the active site of cyclosarin-inhibited AChE. The accommodation of **4** is stabilized by π–π stacking between its coumarin fragment and Tyr341 in the PAS at a distance of 3.7 Å from the phenyl core of coumarin. Two further PAS residues, Tyr124, and Trp286, are situated at 6.7 and 6.3 Å from the coumarin, respectively, in positions that enable T-shaped tilted π–π interaction. The NH_2 group is 9.7 Å away from the phosphorus atom of the modified Ser203. AChE, acetylcholinesterase; PAS, peripheral anionic site.

compounds **1** and **2** carrying an alaninol side chain in either (*R*)- or (*S*)-configuration. While **1** exhibited higher reactivation potential for sarin-inhibited AChE, compound **2** showed the opposite behavior at the cyclosarin-inhibited enzyme. The only difference between sarin and cyclosarin is that in cyclosarin the *O*-isopropyl radical is replaced by a larger *O*-cyclohexyl. This suggests that the water molecule involved in hydrolytic reactivation takes a different position or spatial orientation, which gives rise to this phenomenon. No selectivity was observed for AChE inhibited by VX or paraoxon-ethyl having an *O*-ethyl side chain in the corresponding position that is probably small and flexible enough to receive either enantiomer.

Katz et al.[11] obtained a crystal structure of murine AChE inhibited with diisopropylfluorophosphate in a ternary complex with a nonoxime reactivator. Its imidazole group was appropriately positioned to activate a water molecule for a nucleophilic attack at the phosphorus atom. To get a closer insight into the binding mode of our selected reactivator **4**, we performed molecular docking of compound **4** bound to the active site of cyclosarin-inhibited human AChE.[34] As expected, given the relatively ample conformational space of compound **4** due to the expanded amino-alkoxy chain, several distinctive conformational clusters were obtained (see Supporting Information: Figure S1). They predominately share the placement of the coumarin core in the PAS, where it is surrounded by the aromatic residues of Tyr124, Tyr341, and Trp286 enabling stabilization through π–π stacking interactions (Figure 1). Such interactions have also been proposed for the binding modes in complexes of nonoxime[11] and oxime-based reactivators with inhibited AChE.[32,35–37] The amino-alkoxy chains of **4** belonging to different conformations, although not mutually aligned, were always directed towards the phosphylated active serine with a distance of the amino nitrogen from the phosphorus of 9.7–11.5 Å. The orientation of the flexible amino-alkoxy chain of **4** within the active site was much more volatile than that of the coumarin skeleton, which may facilitate the collision of the amino group with a suitably oriented water molecule to achieve enzyme reactivation.

3 | CONCLUSIONS

Reactivation of inhibited AChE is pivotal in poisoning by OP compounds like nerve agents or pesticides. However, the current toolbox still relies on oximes of limited broad-spectrum activity and lacks central nervous system penetration. Some nonoxime reactivators have been disclosed with limited success but still need further development. To extend the scope of amines beyond currently reported imidazole and pyridine derivatives, we prepared a small set of coumarins, connected to primary and secondary aliphatic and alicyclic amines. While their cholinesterase inhibition was well balanced, their reactivation potential was too low for further development. Nevertheless, our results show that not only certain phenols and *N*-heteroaromatic structures may serve in nonoxime reactivators, which we will further address in future developments.

4 | EXPERIMENTAL

4.1 | Chemistry

4.1.1 | General remarks

Thin-layer chromatography was carried out on Merck aluminum sheets, silica gel 60 F254. Detection was performed with ultraviolet (UV) light at 254 nm. Preparative column chromatography was performed on Merck silica gel (0.063–0.200 mm, 60 Å). Melting points were determined on a Büchi 510 oil bath apparatus. Nuclear magnetic resonance (NMR) spectra (see Supporting Information) were recorded in dimethyl sulfoxide (DMSO)-d_6 on a Bruker Avance DRX 500 spectrometer (^{1}H NMR: 500 MHz, ^{13}C NMR: 125 MHz) or, where indicated, on a Bruker Avance III 600 NMR spectrometer (^{1}H NMR: 600 MHz, ^{13}C NMR: 150 MHz). Chemical shifts δ are given in ppm, referring to the signal center using the solvent peaks for reference: DMSO-d_6 2.49/39.7 ppm. Elemental analyses were performed with a Vario EL apparatus. Liquid chromatography–mass spectrometry (LC–MS) analyses were carried out on an API2000 (Applied Biosystems) mass spectrometer coupled to an Agilent 1100 LC system using a Phenomenex Luna C18 column (Phenomenex; 50 × 2.0 mm, particle size 3 µm). The purity of the compounds was determined using the diode array detector (DAD) of the LC–MS instrument between 200 and 400 nm.

The InChI codes of the investigated compounds, together with some biological activity data, are provided as Supporting Information.

4.1.2 | Chemicals and reagents

Solvents, reagents, and bulk materials were obtained from either Acros, Alfa Aesar, Activate Scientific, Merck, or Sigma-Aldrich in analytical grade and were used without further purification. Tabun (ethyl *N*,*N*-dimethyl phosphoramidocyanidate, GA), sarin (*O*-isopropyl methylphosphonofluoridate, GB), cyclosarin (*O*-cyclohexyl methylphosphonofluoridate, GF) and VX (2-(diisopropylamino)ethyl-*O*-ethyl methylphosphonothioate) were made available by the German Ministry of Defense. Paraoxon-ethyl (diethyl 4-nitrophenyl phosphate, PXE) was from Dr. Ehrenstorfer GmbH and obidoxime hydrochloride from Merck. Packed red blood cells and fresh frozen plasma as sources of human acetyl- and butyrylcholinesterase were supplied by the Bavarian Red Cross Blood Donation Service. (*S*)-Methyl 7-(2-aminopropoxy)-2-oxo-2*H*-chromene-3-carboxylate hydrochloride (**1**), methyl 2-oxo-7-(2-(prop-2-ynylamino)ethoxy)-2*H*-chromene-3-carboxylate hydrochloride (**11**), and methyl 2-oxo-7-(piperazin-1-yl)-2*H*-chromene-3-carboxylate hydrochloride (**12**) were prepared as described by Mertens et al.[29–31]

4.1.3 | Mitsunobu reaction of hydroxy-2*H*-chromenes

Methyl 7-hydroxy-2-oxo-2*H*-chromene-3-carboxylate (440 mg, 2.00 mmol) or methyl 6-hydroxy-2-oxo-2*H*-chromene-3-carboxylate (440 mg, 2.00 mmol), triphenylphosphine (1.04 g, 4.00 mmol) and the respective alcohol (3.00 mmol) were dissolved in THF (30 ml) and cooled to 0°C. DIAD (0.59 ml, 607 mg, 3.00 mmol) was added dropwise and the solution was stirred at room temperature overnight. After evaporating the solvent in vacuo, the residue was redissolved in EtOAc (100 ml) and the organic phase was washed with 1 N NaOH (3 × 100 ml) and brine (100 ml). The organic phase was dried using Na_2SO_4, filtered and evaporated to dryness. The crude residue was either purified by column chromatography on silica gel using mixtures of petroleum ether and ethyl acetate or by recrystallization as indicated.

4.1.4 | HCl-promoted Boc deprotection

The Boc-protected chromene derivative (3.50 mmol) was dissolved in anhydrous EtOAc (20 ml) and cooled to 0°C. To the solution was added 1 N HCl in EtOAc (21 ml) and stirred at room temperature overnight. The precipitate was filtered off and washed with Et_2O (20 ml) to give the final product.

4.1.5 | Boc deprotection with methanesulfonic acid

The Boc-protected chromene derivative (3.50 mmol) was dissolved in dry THF (22 ml). Under ice-cooling, dry methanesulfonic acid (2.02 g, 21 mmol) was added and the resulting reaction mixture was stirred for 5 days at room temperature. The precipitated product was filtered off, washed with petroleum ether, and dried to obtain the final product. In the ^{1}H NMR spectra, integrals for the methanesulfonate protons were not detected. In the ^{13}C NMR spectra, the methanesulfonate carbon signals were obscured by the DMSO signal.

4.1.6 | Synthesis of (*S*)-methyl 6-(2-aminopropoxy)-2-oxo-2*H*-chromene-3-carboxylate hydrochloride (**2**)

According to Section 4.1.3, methyl 6-hydroxy-2-oxo-2*H*-chromene-3-carboxylate was reacted with (*S*)-*tert*-butyl 1-hydroxypropan-2-ylcarbamate (526 mg) and the product purified by column chromatography (petroleum ether/ethyl acetate 50:50 [% vol/vol]) to give a yellow powder; yield 41%; mp 128–131°C. ^{1}H NMR δ 1.13 (d, ^3J = 6.3 Hz, 3H, CH-C*H*$_3$), 1.37 (s, 9H, C(CH$_3$)$_3$), 3.78–3.87 (m, 5H, O-CH$_3$, C*H*H-O, NH-C*H*), 3.92 (dd, ^3J = 8.3 Hz, ^3J = 3.6 Hz, 1H, CH*H*-O), 6.88 (d, ^3J = 7.3 Hz, 1H, NH), 7.32 (dd, ^3J = 9.1 Hz, ^4J = 2.9 Hz, 1H, 7-H), 7.37 (d, ^3J = 9.1 Hz, 1H, 8-H), 7.47 (d, ^4J = 2.9 Hz, 1H, 5-H), 8.70 (s, 1H, 4-H). ^{13}C NMR δ 17.42 (CH-CH$_3$), 28.35 (C(*C*H$_3$)$_3$), 45.34 (NH-CH), 52.54 (O-CH$_3$), 71.39 (CH$_2$-O), 77.83 (*C*(CH$_3$)$_3$), 112.92 (C-5), 117.39, 117.76, 118.30, 122.87, 148.84, 149.18 (C-3, C-4, C-4a, C-7, C-8, C-8a), 155.06, 155.17, 156.19 (O-CO-NH, C-2, C-6), 163.30 (*C*O-O-CH$_3$). Anal. calcd for $C_{19}H_{23}NO_7$: C, 60.47; H, 6.14; N, 3.71. Found: C, 59.96; H, 6.33; N, 3.96. LC–MS (ESI) (90% H_2O to 100% MeOH in 10 min, then 100% MeOH for 10 min, DAD 220–400 nm), 99% purity, *m/z* = 378.1 ([M+H]$^+$), 395.2 ([M+NH$_4$]$^+$).

(*S*)-Methyl 6-(2-(*tert*-butoxycarbonylamino)propoxy)-2-oxo-2*H*-chromene-3-carboxylate (1.32 g) was used according to Section 4.1.4 to obtain compound **2** as a yellow solid; yield 74%; mp 238–239°C. ^{1}H NMR δ 1.31 (d, ^3J = 6.5 Hz, 3H, CH-C*H*$_3$), 3.57–3.65 (m, 1H, C*H*-CH$_3$), 3.83 (s, 3H, O-CH$_3$), 4.07 (dd, ^2J = 10.2 Hz, ^3J = 6.7 Hz, 1H, C*H*H-O), 4.18 (dd, ^2J = 10.2 Hz, ^3J = 3.7 Hz, 1H, CH*H*-O), 7.39 (dd, ^3J = 9.1 Hz, ^4J = 2.8 Hz, 1H, 7-H), 7.42 (d, ^3J = 9.1 Hz, 1H, 8-H), 7.54 (d, ^4J = 2.7 Hz, 1H, 5-H), 8.32 (s, 3H, NH$_3^+$), 8.73 (s, 1H, 4-H). ^{13}C NMR δ 15.14 (CH-*C*H$_3$), 46.10 ($^+$H$_3$N-CH), 52.59 (O-CH$_3$), 69.58 (CH$_2$-O), 113.10 (C-5), 117.56, 117.96, 118.32, 123.09, 148.71, 149.54 (C-3, C-4, C-4a, C-7, C-8, C-8a), 154.45, 156.16 (C-2, C-6), 163.30 (*C*O-O-CH$_3$). LC–MS (ESI) (90% H_2O to 100% MeOH in 10 min, then 100% MeOH for 10 min, DAD 220–400 nm), 100% purity, *m/z* = 278.2 ([M+H]$^+$), 295.3 ([M+NH$_4$]$^+$).

4.1.7 | Synthesis of methyl 7-(2-aminoethoxy)-2-oxo-2*H*-chromene-3-carboxylate hydrochloride (**3**)

According to Section 4.1.3, methyl 7-hydroxy-2-oxo-2*H*-chromene-3-carboxylate was reacted with *tert*-butyl 2-hydroxyethylcarbamate (484 mg) and the product purified by column chromatography (petroleum ether/ethyl acetate 67:33 [% vol/vol] to ethyl acetate [100%]) to give a white powder; yield 74%; mp 160–162°C. ^{1}H NMR δ 1.37 (s, 9H, C(CH$_3$)$_3$), 3.32 (q, ^3J = 5.7 Hz, 2H, NH-C*H*$_2$), 3.80 (s, 3H, O-CH$_3$), 4.11 (t, ^3J = 5.7 Hz, 2H, CH$_2$-O), 6.98 (dd, ^3J = 8.6 Hz, ^4J = 2.4 Hz, 1H, 6-H), 7.00–7.03 (m, 2H, NH, 8-H), 7.82 (d, ^3J = 8.6 Hz, 1H, 5-H), 8.73 (s, 1H, 4-

H). ^{13}C NMR δ 28.33 ($C(CH_3)_3$), 52.30 (O-CH_3), 67.60 (CH_2-O), 77.99 ($C(CH_3)_3$), 100.90 (C-8), 111.61 (C-4a), 113.19, 113.74 (C-3, C-6), 131.83, 149.53, 155.79, 156.33, 157.09 (O-CO-NH, C-2, C-4, C-5, C-8a), 163.47, 164.14 (C-7, *C*O-O-CH_3). One carbon signal (NH-CH_2) was obscured by the solvent peak. Anal. calcd for $C_{18}H_{21}NO_7$: C, 59.50; H, 5.83; N, 3.85. Found: C, 59.46; H, 6.37; N, 3.51. LC-MS (ESI) (90% H_2O to 100% MeOH in 10 min, then 100% MeOH for 10 min, DAD 220–600 nm), 98% purity, m/z = 364.3 ($[M+H]^+$), 381.3 ($[M+NH_4]^+$).

Methyl 7-[2-(*tert*-butoxycarbonylamino)ethoxy]-2-oxo-2*H*-chromene-3-carboxylate (1.27 g) was used according to Section 4.1.4 to obtain compound **3** as a white solid; yield 88%; mp 231–233°C. ^{1}H NMR (600 MHz) δ 3.20–3.27 (m, 2H, ^+H_3N-CH_2), 3.80 (s, 3H, CH_3), 4.35 (t, ^3J = 5.0 Hz, 2H, CH_2-O), 7.04 (dd, ^3J = 8.6 Hz, ^4J = 2.4 Hz, 1H, 6-H), 7.06 (d, ^4J = 2.3 Hz, 1H, 8-H), 7.87 (d, ^3J = 8.6 Hz, 1H, 5-H), 8.36 (s, 3H, NH_3^+), 8.75 (s, 1H, 4-H). ^{13}C NMR (150 MHz) δ 38.15 (^+H_3N-CH_2), 52.40 (CH_3), 65.45 (CH_2-O), 101.20 (C-8), 112.02 (C-4a), 113.62, 113.78 (C-3, C-6), 131.94, 149.53, 156.32, 156.97 (C-2, C-4, C-5, C-8a), 163.38, 163.46 (C-7, *C*O-O-CH_3). LC–MS (ESI) (90% H_2O to 100% MeOH in 10 min, then 100% MeOH for 10 min, DAD 220–400 nm), 100% purity, m/z = 264.2 ($[M+H]^+$), 281.3 ($[M+NH_4]^+$).

4.1.8 | Synthesis of methyl 7-[2-(2-aminoethoxy)-ethoxy]-2-oxo-2*H*-chromene-3-carboxylate hydrochloride (**4**)

According to Section 4.1.3, methyl 7-hydroxy-2-oxo-2*H*-chromene-3-carboxylate was reacted with *tert*-butyl 2-(2-hydroxyethoxy) ethylcarbamate (616 mg) and the product recrystallized from MeOH to give a white powder; yield 65%; mp 108–109°C (MeOH). ^{1}H NMR δ 1.35 (s, 9H, $C(CH_3)_3$), 3.08 (q, ^3J = 6.0 Hz, 2H, NH-CH_2), 3.45 (t, ^3J = 6.1 Hz, 2H) and 3.72–3.77 (m, 2H, CH_2-O-CH_2), 3.80 (s, 3H, O-CH_3), 4.21–4.26 (m, 2H, CH_2-O-CH_2-CH_2-O), 6.73 (t, ^3J = 5.5 Hz, 1H, NH), 6.99–7.03 (m, 2H, 6-H, 8-H), 7.82 (d, ^3J = 8.5 Hz, 1H, 5-H), 8.73 (s, 1H, 4-H). ^{13}C NMR δ 28.35 ($C(CH_3)_3$), 52.31 (O-CH_3), 68.38, 68.40, 69.43 (CH_2-O-CH_2-CH_2-O), 77.75 ($C(CH_3)_3$), 100.93 (C-8), 111.61 (C-4a), 113.19, 113.76 (C-3, C-6), 131.82, 149.55, 155.72, 156.34, 157.09 (O-CO-NH, C-2, C-4, C-5, C-8a), 163.48, 164.19 (C-7, *C*O-O-CH_3). One carbon signal (NH-CH_2) was obscured by the solvent peak. Anal. calcd for $C_{20}H_{25}NO_8$: C, 58.96; H, 6.18; N, 3.44. Found: C, 58.88; H, 6.38; N, 3.38. LC–MS (ESI) (90% H_2O to 100% MeOH in 10 min, then 100% MeOH for 10 min, DAD 220–400 nm), 100% purity, m/z = 408.3 ($[M+H]^+$), 425.4 ($[M+NH_4]^+$).

Methyl 7-{2-[2-(*tert*-butoxycarbonylamino)ethoxy]ethoxy}-2-oxo-2*H*-chromene-3-carboxylate (1.43 g) was used according to Section 4.1.4 to obtain compound **4** as a white solid; yield 97%; mp 189–190°C. ^{1}H NMR δ 2.97 (t, ^3J = 5.0 Hz, 2H, ^+H_3N-CH_2), 3.69 (t, ^3J = 4.9 Hz, 2H, CH_2-O-CH_2), 3.80 (s, 3H, CH_3), 3.81–3.84 (m, 2H, CH_2-O-CH_2), 4.26–4.31 (m, 2H, CH_2-O-CH_2-CH_2-O), 7.02 (dd, ^3J = 8.6 Hz, ^4J = 2.4 Hz, 1H, 6-H), 7.05 (d, ^4J = 2.4 Hz, 1H, 8-H), 7.84 (d, ^3J = 8.6 Hz, 1H, 5-H), 8.06 (s, 3H, NH_3^+), 8.74 (s, 1H, 4-H). ^{13}C NMR δ 38.55 (^+H_3N-CH_2), 52.32 (CH_3), 66.89, 68.26, 68.64 (CH_2-O-CH_2-CH_2-O), 100.95 (C-8), 111.66 (C-4a), 113.26, 113.73 (C-3, C-6), 131.86, 149.54, 156.33, 157.07 (C-2, C-4, C-5, C-8a), 163.46, 164.07 (C-7, *C*O-O-CH_3). LC–MS (ESI) (90% H_2O to 100% MeOH in 10 min, then 100% MeOH for 10 min, DAD 220–600 nm), 100% purity, m/z = 308.3 ($[M+H]^+$).

4.1.9 | Synthesis of (*S*)-methyl 7-(2-amino-3-phenylpropoxy)-2-oxo-2*H*-chromene-3-carboxylate hydrochloride (**5**)

According to Section 4.1.3, methyl 7-hydroxy-2-oxo-2*H*-chromene-3-carboxylate was reacted with (*S*)-*tert*-butyl 1-hydroxy-3-phenylpropan-2-ylcarbamate (754 mg) and the product recrystallized from petroleum ether/ethyl acetate to give a white powder; mp 122–123°C. ^{1}H NMR δ 1.31 (s, 9H, $C(CH_3)_3$), 2.76 (dd, ^2J = 13.7 Hz, ^3J = 8.5 Hz, 1H, C*H*H-C_6H_5), 2.88 (dd, ^2J = 13.7 Hz, ^3J = 5.6 Hz, 1H, CH*H*-C_6H_5), 3.80 (s, 3H, O-CH_3), 3.99 (tt, ^3J = 8.6 Hz, ^3J = 5.6 Hz, 1H, NH-C*H*), 4.06 (d, ^3J = 5.6 Hz, 2H, CH_2-O), 6.97–7.02 (m, 3H, NH, 6-H, 8-H), 7.16–7.21 (m, 1H, H_{para} C_6H_5), 7.21–7.30 (m, 4H, H_{ortho} C_6H_5, H_{meta} C_6H_5), 7.82 (d, ^3J = 8.6 Hz, 1H, 5-H), 8.73 (s, 1H, 4-H). ^{13}C NMR δ 28.32 ($C(CH_3)_3$), 36.85 (CH_2-C_6H_5), 51.20 (NH-CH), 52.32 (O-CH_3), 70.22 (CH_2-O), 77.93 ($C(CH_3)_3$), 100.97 (C-8), 111.68 (C-4a), 113.24, 113.79 (C-3, C-6), 126.24 (C_{para} C_6H_5), 128.29 (C_{ortho} C_6H_5), 129.25 (C_{meta} C_6H_5), 131.84, 138.53, 149.53, 155.31, 156.34, 157.09 (C_{ipso} C_6H_5, O-CO-NH, C-2, C-4, C-5, C-8a), 163.48, 164.16 (C-7, *C*O-O-CH_3). Anal. calcd for $C_{25}H_{27}NO_7$: C, 66.21; H, 6.00; N, 3.09. Found: C, 65.85; H, 6.00; N, 3.01. LC–MS (ESI) (90% H_2O to 100% MeOH in 10 min, then 100% MeOH for 10 min, DAD 220–400 nm), 96% purity, m/z = 454.30 ($[M+H]^+$).

(*S*)-Methyl 7-[2-(*tert*-butoxycarbonylamino)-3-phenylpropoxy]-2-oxo-2*H*-chromene-3-carboxylate (1.59 g) was used according to Section 4.1.4 to obtain compound **5** as a white solid; yield 54%; mp 224–226°C. ^{1}H NMR) δ 2.99 (dd, ^2J = 13.7 Hz, ^3J = 9.3 Hz, 1H, CH-C*H*H), 3.14 (dd, ^2J = 13.7 Hz, ^3J = 5.4 Hz, 1H, CH-CH*H*), 3.75–3.79 (m, 1H, ^+H_3N-C*H*), 3.80 (s, 3H, CH_3), 4.09 (dd, ^2J = 10.7 Hz, ^3J = 5.6 Hz, 1H, C*H*H-O), 4.24 (dd, ^2J = 10.7 Hz, ^3J = 3.1 Hz, 1H, CH*H*-O), 7.02–7.06 (m, 2H, 6-H, 8-H), 7.24–7.28 (m, 1H, H_{para} C_6H_5), 7.28–7.35 (m, 4H, H_{ortho} C_6H_5, H_{meta} C_6H_5), 7.83–7.88 (m, 1H, 5-H), 8.56 (s, 3H, NH_3^+), 8.74 (s, 1H, 4-H). ^{13}C NMR δ 35.03 (CH_2-C_6H_5), 51.17 (^+H_3N-CH), 52.37 (CH_3), 67.28 (CH_2-O), 101.26 (C-8), 112.11 (C-4a), 113.72 (C-3, C-6), 127.14 (C_{para} C_6H_5), 128.82 (C_{ortho} C_6H_5), 129.46 (C_{meta} C_6H_5), 131.90, 136.02, 149.44, 156.26, 156.89 (C_{ipso} C_6H_5, C-2, C-4, C-5, C-8a), 163.28, 163.43 (C-7, *C*O-O-CH_3). LC–MS (ESI) (90% H_2O to 100% MeOH in 10 min, then 100% MeOH for 10 min, DAD 220–400 nm), 99% purity, m/z = 354.3 ($[M+H]^+$).

4.1.10 | Synthesis of (*S*)-methyl 7-[2-(2-amino-3-phenylpropanamido)ethoxy]-2-oxo-2*H*-chromene-3-carboxylate hydrochloride (**6**)

A mixture of Boc-Phe-OH (265 mg, 1.00 mmol), *N*,*N*-diisopropylethylamine (DIPEA) (258 mg, 2.00 mmol), 1-[bis

(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate (HATU) (380 mg, 1.00 mmol) and methyl 7-(2-aminoethoxy)-2-oxo-2*H*-chromene-3-carboxylate hydrochloride (**3**, 264 mg, 0.88 mmol) in CH_2Cl_2 (10 ml) was stirred at room temperature for 2 h. It was diluted with CH_2Cl_2 (40 ml), washed with 10% citric acid (2 × 50 ml), saturated $NaHCO_3$ solution (2 × 50 ml), and brine (1 × 50 ml). The organic layer was evaporated and the residue was triturated with ethyl acetate to give a semi-solid material; yield 48%. ^{1}H NMR δ 1.27 (s, 9H, C(CH_3)$_3$), 2.74 (dd, ^2J = 13.8 Hz, ^3J = 9.8 Hz, 1H, CH-C*H*H), 2.91 (dd, ^2J = 13.5 Hz, ^3J = 4.9 Hz, 1H, CH-CH*H*), 3.38–3.47 (m, 1H, NH-C*H*H), 3.50 (dq, ^2J = 16.8 Hz, ^3J = 5.7 Hz, 1H, NH-CH*H*), 3.80 (s, 3H, O-CH_3), 4.05–4.13 (m, 2H, CH_2-O), 4.13–4.19 (m, 1H, NH-C*H*), 6.84 (d, ^3J = 8.6 Hz, 1H, O-CO-NH), 6.99 (dd, ^3J = 8.7 Hz, ^4J = 2.4 Hz, 1H, 6-H), 7.02 (d, ^4J = 2.3 Hz, 1H, 8-H), 7.11–7.18 (m, 1H, H_{para} C_6H_5), 7.18–7.26 (m, 4H, H_{ortho} C_6H_5, H_{meta} C_6H_5), 7.84 (d, ^3J = 8.7 Hz, 1H, 5-H), 8.19 (t, ^3J = 5.7 Hz, 1H, N*H*-CH_2), 8.73 (s, 1H, 4-H). ^{13}C NMR δ 28.25 (C(CH_3)$_3$), 37.79, 38.03 (CH-CH_2, NH-CH_2), 52.32 (O-CH_3), 55.81 (NH-CH), 67.40 (CH_2-O), 78.11 (*C*(CH_3)$_3$), 100.97 (C-8), 111.68 (C-4a), 113.26, 113.73 (C-3, C-6), 126.25 (C_{para} C_6H_5), 128.07 (C_{ortho} C_6H_5), 129.29 (C_{meta} C_6H_5), 131.86, 138.17, 149.54, 155.26, 156.34, 157.08 (C_{ipso} C_6H_5, O-CO-NH, C-2, C-4, C-5, C-8a), 163.47, 164.08 (C-7, *C*O-O-CH_3), 172.10 (CH-*C*O-NH). LC–MS (ESI) (90% H_2O to 100% MeOH in 10 min, then 100% MeOH for 10 min, DAD 220–400 nm), 99% purity, *m/z* = 511.3 ([M+H]$^+$), 528.3 ([M+NH_4]$^+$).

(*S*)-Methyl 7-{2-[2-(*tert*-butoxycarbonylamino)-3-phenylpropanamido]ethoxy}-2-oxo-2*H*-chromene-3-carboxylate (1.79 g) was used according to Section 4.1.4 to obtain compound **6** as a yellowish solid; yield 85%; mp 158–160°C. ^{1}H NMR (600 MHz) δ 3.06 (dd, ^2J = 13.7 Hz, ^3J = 7.4 Hz, 1H, CH-C*H*H), 3.11 (dd, ^2J = 13.7 Hz, ^3J = 6.3 Hz, 1H, CH-CH*H*), 3.36–3.40 (m, 1H, NH-C*H*H), 3.48–3.56 (m, 1H, NH-CH*H*), 3.79 (s, 3H, CH_3), 3.97–4.02 (m, 1H, $^+H_3$N-C*H*), 4.02–4.10 (m, 2H, CH_2-O), 6.96 (dd, ^3J = 8.7 Hz, ^4J = 2.4 Hz, 1H, 6-H), 6.98 (d, ^4J = 2.3 Hz, 1H, 8-H), 7.12–7.16 (m, 1H, H_{para} C_6H_5), 7.19 (t, ^3J = 7.4 Hz, 2H, H_{meta} C_6H_5), 7.23 (d, ^3J = 7.1 Hz, 2H, H_{ortho} C_6H_5), 7.85 (d, ^3J = 8.6 Hz, 1H, 5-H), 8.52 (s, 3H, NH_3^+), 8.75 (s, 1H, 4-H), 8.99 (t, ^3J = 5.6 Hz, 1H, CO-NH). ^{13}C NMR (150 MHz) δ 36.87, 38.06 (CH-CH_2, NH-CH_2), 52.39 (CH_3), 53.51 ($^+H_3$N-CH), 67.23 (CH_2-O), 100.95 (C-8), 111.74 (C-4a), 113.31, 113.79 (C-3, C-6), 127.09 (C_{para} C_6H_5), 128.46 (C_{ortho} C_6H_5), 129.68 (C_{meta} C_6H_5), 131.93, 135.18, 149.62, 156.42, 157.08 (C_{ipso} C_6H_5, C-2, C-4, C-5, C-8a), 163.52, 163.97 (C-7, *C*O-O-CH_3), 168.31 (CO-NH). LC–MS (ESI) (90% H_2O to 100% MeOH in 10 min, then 100% MeOH for 10 min, DAD 220–400 nm), 98% purity, *m/z* = 411.4 ([M+H]$^+$).

4.1.11 | Synthesis of 4-{[3-(methoxycarbonyl)-2-oxo-2*H*-chromen-7-yloxy]methyl}piperidinium methanesulfonate (**7**)

According to Section 4.1.3, methyl 7-hydroxy-2-oxo-2*H*-chromene-3-carboxylate was reacted with *tert*-butyl 4-(hydroxymethyl) piperidine-1-carboxylate (646 mg) and the product purified by column chromatography (petroleum ether/ethyl acetate 50:50 [% vol/vol]) to give a white powder; yield 56%; mp 150–152°C. ^{1}H NMR δ 1.11–1.21 (m, 2H, N(CH_2-C*H*H)$_2$), 1.39 (s, 9H, C(CH_3)$_3$), 1.69–1.78 (m, 2H, N(CH_2-CH*H*)$_2$), 1.90–2.00 (m, 1H, C*H*-CH_2), 2.66–2.82 (m, 2H, N(C*H*H-CH_2)$_2$), 3.80 (s, 3H, O-CH_3), 3.93–3.98 (m, 2H, N(CH*H*-CH_2)$_2$), 3.99 (d, ^3J = 6.4 Hz, 2H, CH_2-O), 6.98–7.00 (m, 1H, 6-H), 7.00–7.01 (m, 1H, 8-H), 7.82 (d, ^3J = 8.5 Hz, 1H, 5-H), 8.72 (s, 1H, 4-H). ^{13}C NMR δ 28.23 (C(CH_3)$_3$), 35.19 (CH-CH_2), 43.13 (N(CH_2-CH_2)$_2$), 52.29 (O-CH_3), 72.76 (CH_2-O), 78.64 (*C*(CH_3)$_3$), 100.87 (C-8), 111.53 (C-4a), 113.10, 113.73 (C-3, C-6), 131.82, 149.54, 153.99, 156.33, 157.12 (O-CO-NH, C-2, C-4, C-5, C-8a), 163.47, 164.34 (C-7, *C*O-O-CH_3). One carbon signal (N(CH_2-CH_2)$_2$) was not recognizable. Anal. calcd for $C_{22}H_{27}NO_7$: C, 63.30; H, 6.52; N, 3.36. Found: C, 63.11; H, 6.58; N, 3.56. LC–MS (ESI) (90% H_2O to 100% MeOH in 10 min, then 100% MeOH for 10 min, DAD 220–400 nm), 97% purity, *m/z* = 418.2 ([M+H]$^+$), 435.4 ([M+NH_4]$^+$).

tert-Butyl 4-{[3-(methoxycarbonyl)-2-oxo-2*H*-chromen-7-yloxy]methyl}piperidine-1-carboxylate (1.46 g) was used according to Section 4.1.4 to obtain compound **7** as a slightly rosy solid; yield 89%; mp 170–172°C. ^{1}H NMR δ 1.43–1.52 (m, 2H, N(CH_2-C*H*H)$_2$), 1.87–1.96 (m, 2H, N(CH_2-CH*H*)$_2$), 2.04–2.15 (m, 1H, C*H*-CH_2), 2.86–2.98 (m, 2H, N(C*H*H-CH_2)$_2$), 3.26–3.36 (m, 2H, N(CH*H*-CH_2)$_2$), 4.02 (d, ^3J =;6.3 Hz, 2H, CH_2-O), 6.99 (dd, ^3J = 8.7 Hz, ^4J = 2.4 Hz, 1H, 6-H), 7.03 (d, ^4J = 2.4 Hz, 1H, 8-H), 7.82 (d, ^3J = 8.7 Hz, 1H, 5-H), 8.20–8.62 (m, 2H, NH_2^+), 8.70 (s, 1H, 4-H). One proton signal (O-CH_3) was not recognizable. ^{13}C NMR δ 25.18 ($^+H_2$N(CH_2-CH_2)$_2$), 32.92 (CH-CH_2), 42.94 ($^+H_2$N(CH_2-CH_2)$_2$), 52.35 (O-CH_3), 72.17 (CH_2-O), 100.96 (C-8), 111.84 (C-4a), 113.71, 114.09 (C-3, C-6), 131.76, 149.11, 156.97, 157.33 (C-2, C-4, C-5, C-8a), 163.95, 164.24 (C-7, *C*O-O-CH_3). LC–MS (ESI) (90% H_2O to 100% MeOH in 10 min, then 100% MeOH for 10 min, DAD 220–400 nm), 99% purity, *m/z* = 317.9 ([M+H]$^+$).

4.1.12 | Synthesis of (*R,S*)-3-{[3-(methoxycarbonyl)-2-oxo-2*H*-chromen-7-yloxy]methyl}piperidinium methanesulfonate (**8**)

According to Section 4.1.3, methyl 7-hydroxy-2-oxo-2*H*-chromene-3-carboxylate was reacted with *tert*-butyl (*R,S*)-3-(hydroxymethyl)-piperidine-1-carboxylate (646 mg) and the product purified by column chromatography (petroleum ether/ethyl acetate 67:33 [% vol/vol]) to give a white powder; yield 75%; mp 188–190°C. ^{1}H NMR δ 1.24–1.46 (m, 11H, C(CH_3)$_3$, N-CH_2-C*H*H-CH*H*), 1.59–1.68 (m, 1H, N-CH_2-CH_2-CH*H*), 1.77–1.85 (m, 1H, N-CH_2-CH*H*), 1.86–1.96 (m, 1H, C*H*-CH_2), 2.78–3.02 (m, 2H, N(C*H*H)$_2$), 3.80 (s, 3H, O-CH_3), 3.98 (dd, ^2J = 10.2 Hz, ^3J = 7.9 Hz, 1H, C*H*H-O), 4.02 (dd, ^2J = 10.0 Hz, ^3J = 5.5 Hz, 1H, CH*H*-O), 7.01 (dd, ^3J = 8.7 Hz, ^4J = 2.4 Hz, 1H, 6-H), 7.03 (d, ^4J = 2.4 Hz, 1H, 8-H), 7.83 (d, ^3J = 8.7 Hz, 1H, 5-H), 8.73 (s, 1H, 4-H). Two proton signals (N(CH*H*)$_2$) were not recognizable. ^{13}C NMR δ 23.87 (N-CH_2-CH_2), 26.53 (N-CH_2-CH_2-CH_2), 28.15 (C(CH_3)$_3$), 35.26 (CH-CH_2), 46.32 (N-CH_2-CH), 52.31 (O-CH_3), 70.69 (CH_2-O), 78.65

($C(CH_3)_3$), 100.88 (C-8), 111.58 (C-4a), 113.18, 113.75 (C-3, C-6), 131.83, 149.54, 154.05, 156.34, 157.13 (O-CO-NH, C-2, C-4, C-5, C-8a), 163.47, 164.24 (C-7, *C*O-O-CH_3). One carbon signal (N-CH_2-CH_2) was not recognizable. Anal. calcd for $C_{22}H_{27}NO_7$: C, 63.30; H, 6.52; N, 3.36. Found: C, 63.03; H, 6.46; N, 3.31. LC–MS (ESI) (90% H_2O to 100% MeOH in 10 min, then 100% MeOH for 10 min, DAD 220–400 nm), 99% purity, m/z = 418.4 ($[M+H]^+$), 435.6 ($[M+NH_4]^+$).

tert-Butyl (*R,S*)-3-{[3-(methoxycarbonyl)-2-oxo-2*H*-chromen-7-yloxy]methyl}piperidine-1-carboxylate (1.46 g) was used according to Section 4.1.5 to obtain compound **8** as a slightly rosy solid; yield 100%; mp 163–164°C. ^{1}H NMR δ 1.30–1.42 (m, 1H, ^+H_2N-CH_2-CH_2-C*H*H), 1.59–1.72 (m, 1H, ^+H_2N-CH_2-CH_2-CH*H*), 1.80–1.89 (m, 2H, ^+H_2N-CH_2-CH_2), 2.17–2.29 (m, 1H, C*H*-CH_2), 2.73–2.87 (m, 2H, N(C*H*H)$_2$), 3.22–3.30 (m, 1H) and 3.33–3.40 (m, 1H, N(CH*H*)$_2$), 3.80 (s, 3H, O-CH_3), 4.03 (dd, ^2J = 10.1 Hz, ^3J = 7.0 Hz, 1H, C*H*H-O), 4.10 (dd, ^2J = 10.1 Hz, ^3J = 5.5 Hz, 1H, CH*H*-O), 7.01 (dd, ^3J = 8.6 Hz, ^4J = 2.4 Hz, 1H, 6-H), 7.04 (d, ^4J = 2.4 Hz, 1H, 8-H), 7.85 (d, ^3J = 8.6 Hz, 1H, 5-H), 8.33–8.71 (m, 2H, NH_2^+), 8.74 (s, 1H, 4-H). ^{13}C NMR δ 21.41 (^+H_2N-CH_2-CH_2), 24.65 (^+H_2N-CH_2-CH_2-CH_2), 33.14 (CH-CH_2), 43.65, 45.51 ($^+H_2N(CH_2)_2$), 52.35 (O-CH_3), 70.27 (CH_2-O), 101.01 (C-8), 111.78 (C-4a), 113.39, 113.71 (C-3, C-6), 131.91, 149.52, 156.32, 157.06 (C-2, C-4, C-5, C-8a), 163.47, 163.91 (C-7, *C*O-O-CH_3). LC–MS (ESI) (90% H_2O to 100% MeOH in 10 min, then 100% MeOH for 10 min, DAD 220–400 nm), 99% purity, m/z = 318.1 ($[M+H]^+$).

4.1.13 | Synthesis of 4-[3-(methoxycarbonyl)-2-oxo-2*H*-chromen-7-yloxy]piperidinium methanesulfonate (**9**)

According to Section 4.1.3, methyl 7-hydroxy-2-oxo-2*H*-chromene-3-carboxylate was reacted with *tert*-butyl 4-hydroxypiperidine-1-carboxylate (604 mg) and the product purified by column chromatography (petroleum ether/ethyl acetate 67:33 [% vol/vol] to ethyl acetate [100%]) to give a white powder; yield 62%; mp 169–171°C. ^{1}H NMR δ 1.40 (s, 9H, $C(CH_3)_3$), 1.54 (dtd, $2J$ = 12.8 Hz, ^3J = 8.8 Hz, ^3J = 3.9 Hz, 2H, N(CH_2-C*H*H)$_2$), 1.90–2.00 (m, 2H, N(CH_2-CH*H*)$_2$), 3.12–3.23 (m, 2H, N(C*H*H-CH_2)$_2$), 3.63–3.72 (m, 2H, N(CH*H*-CH_2)$_2$), 3.80 (s, 3H, O-CH_3), 4.77 (tt, ^3J = 7.8 Hz, ^3J = 3.7 Hz, 1H, CH-O), 7.02 (dd, ^3J = 8.7 Hz, ^4J = 2.4 Hz, 1H, 6-H), 7.10 (d, ^4J = 2.3 Hz, 1H, 8-H), 7.82 (d, ^3J = 8.7 Hz, 1H, 5-H), 8.72 (s, 1H, 4-H). ^{13}C NMR δ 28.17 (C(CH_3)$_3$), 30.26 (N(CH_2-CH_2)$_2$), 52.27 (O-CH_3), 73.20 (CH-O), 78.91 (*C*(CH_3)$_3$), 101.73 (C-8), 111.57 (C-4a), 113.14, 114.40 (C-3, C-6), 131.92, 149.48, 154.01, 156.31, 157.16 (O-*C*O-NH, C-2, C-4, C-5, C-8a), 162.78, 163.45 (C-7, CO-O-CH_3). One carbon signal (N(CH_2-CH_2)$_2$) was obscured by the solvent peak. Anal. calcd for $C_{21}H_{25}NO_7$: C, 62.52; H, 6.25; N, 3.47. Found: C, 61.48; H, 6.63; N, 4.27. LC–MS (ESI) (90% H_2O to 100% MeOH in 10 min, then 100% MeOH for 10 min, DAD 220–400 nm), 98% purity, m/z = 404.3 ($[M+H]^+$).

tert-Butyl 4-[3-(methoxycarbonyl)-2-oxo-2*H*-chromen-7-yloxy]-piperidine-1-carboxylate (1.41 g) was used according to Section 4.1.5 to obtain compound **9** as a slightly rosy solid; yield 100%; mp 96–97°C. ^{1}H NMR δ 1.80–1.91 (m, 2H, $^+H_2N(CH_2$-C*H*H)$_2$), 2.10–2.20 (m, 2H, $^+H_2N(CH_2$-CH*H*)$_2$), 3.05–3.17 (m, 2H, ^+H_2N(C*H*H-CH_2)$_2$), 3.22–3.33 (m, 2H, ^+H_2N(CH*H*-CH_2)$_2$), 3.80 (s, 3H, O-CH_3), 4.84 (tt, ^3J = 7.6 Hz, ^3J = 3.6 Hz, 1H, CH-O), 7.05 (dd, ^3J = 8.7 Hz, ^4J = 2.4 Hz, 1H, 6-H), 7.15 (d, ^4J = 2.3 Hz, 1H, 8-H), 7.85 (d, ^3J = 8.7 Hz, 1H, 5-H), 8.43–8.61 (m, 2H, NH_2^+), 8.73 (s, 1H, 4-H). ^{13}C NMR δ 27.23 ($^+H_2N(CH_2$-$CH_2)_2$), 40.92 ($^+H_2N(CH_2$-$CH_2)_2$), 52.36 (O-CH_3), 70.33 (CH-O), 101.82 (C-8), 111.86 (C-4a), 113.46, 114.41 (C-3, C-6), 132.05, 149.48, 156.33, 157.15 (C-2, C-4, C-5, C-8a), 162.36, 163.47 (C-7, *C*O-O-CH_3). LC–MS (ESI) (90% H_2O to 100% MeOH in 10 min, then 100% MeOH for 10 min, DAD 220–400 nm), 99% purity, m/z = 304.1 ($[M+H]^+$).

4.1.14 | Synthesis of (*S*)-3-[3-(methoxycarbonyl)-2-oxo-2*H*-chromen-7-yloxy]pyrrolidinium methanesulfonate (**10**)

According to Section 4.1.3, methyl 7-hydroxy-2-oxo-2*H*-chromene-3-carboxylate was reacted with (*S*)-*tert*-butyl 3-hydroxypyrrolidine-1-carboxylate (562 mg) and the product purified by column chromatography (petroleum ether/ethyl acetate 67:33 [% vol/vol]) to give a white powder; yield 67%; mp 144–146°C. ^{1}H NMR δ 1.31–1.46 (m, 9H, $C(CH_3)_3$), 2.01–2.12 (m, 1H, N-CH_2-C*H*H-CH), 2.13–2.27 (m, 1H, N-CH_2-CH*H*-CH), 3.29–3.47 (m, 3H, N-C*H*$_2$-CH_2-CH, N-C*H*H-CH), 3.54–3.67 (m, 1H, N-CH*H*-CH), 3.80 (s, 3H, O-CH_3), 5.12–5.26 (m, 1H, CH-O), 7.00 (dd, ^3J = 8.7 Hz, ^4J = 2.4 Hz, 1H, 6-H), 7.06 (d, ^4J = 2.4 Hz, 1H, 8-H), 7.84 (d, ^3J = 8.7 Hz, 1H, 5-H), 8.73 (s, 1H, 4-H). ^{13}C NMR δ 28.27 (*C*(CH_3)$_3$), 30.07, 30.84 (N-CH_2-CH_2), 43.77, 43.93 (N-*C*H$_2$-CH_2), 51.24, 51.43 (N-*C*H$_2$-CH), 52.32 (O-CH_3), 76.70, 77.57 (CH-O), 78.74 (*C*(CH_3)$_3$), 101.71 (C-8), 111.79 (C-4a), 113.41, 114.26 (C-3, C-6), 132.00, 149.48, 153.58, 156.29, 157.03 (O-CO-NH, C-2, C-4, C-5, C-8a), 162.57, 163.45 (C-7, CO-O-CH_3). Anal. calcd for $C_{20}H_{23}NO_7$: C, 61.69; H, 5.95; N, 3.60. Found: C, 61.83; H, 6.08; N, 3.63. LC–MS (ESI) (90% H_2O to 100% MeOH in 10 min, then 100% MeOH for 10 min, DAD 220–400 nm), 98% purity, m/z = 390.0 ($[M+H]^+$), 407.2 ($[M+NH_4]^+$).

(*S*)-*tert*-Butyl 3-[3-(methoxycarbonyl)-2-oxo-2*H*-chromen-7-yloxy] pyrrolidine-1-carboxylate (1.36 g) was used according to 4.1.5 to obtain compound **10** as a slightly rosy solid; yield 70%; mp 151–153°C. ^{1}H NMR δ 2.12–2.20 (m, 1H, ^+H_2N-CH_2-C*H*H-CH), 2.26–2.34 (m, 1H, ^+H_2N-CH_2-CH*H*-CH), 3.25–3.46 (m, 3H, ^+H_2N-C*H*$_2$-CH_2-CH, ^+H_2N-C*H*H-CH), 3.49–3.58 (m, 1H, ^+H_2N-CH*H*-CH), 3.80 (s, 3H, O-CH_3), 5.29–5.35 (m, 1H, CH-O), 7.01 (dd, ^3J = 8.7 Hz, ^4J = 2.4 Hz, 1H, 6-H), 7.09 (d, ^4J = 2.4 Hz, 1H, 8-H), 7.87 (d, ^3J = 8.7 Hz, 1H, 5-H), 8.75 (s, 1H, 4-H), 8.94–9.21 (m, 2H, NH_2^+). ^{13}C NMR δ 30.57 (^+H_2N-CH_2-CH_2), 43.90 (^+H_2N-*C*H$_2$-CH_2), 50.26 (^+H_2N-CH_2-CH), 52.38 (O-CH_3), 76.69 (CH-O), 101.95 (C-8), 112.11 (C-4a), 113.76, 114.35 (C-3, C-6), 132.06, 149.42, 156.24, 156.91 (C-2, C-4, C-5, C-8a), 161.90, 163.40 (C-7, *C*O-O-CH_3). LC–MS (ESI) (90% H_2O to 100% MeOH in 10 min, then 100% MeOH for 10 min, DAD 220–400 nm), 99% purity, m/z = 290.2 ($[M+H]^+$).

4.2 | Enzyme assays

4.2.1 | Enzyme inhibition assay

Stock solutions of **1**–**12** were prepared at 10 mM concentration in water (**1**–**10**, **12**) or 50% aqueous acetonitrile (**11**). Fresh frozen plasma was used as the source of butyrylcholinesterase (hBChE) as well as human erythrocyte ghosts from packed red blood cells as the source of acetylcholinesterase (hAChE), which were prepared according to the literature.[38] The activity of the AChE preparation was adjusted to the original whole blood activity by dilution with sodium phosphate buffer (100 mM, pH 7.4) as determined by a modified Ellman assay.[39] Aliquots of the plasma and erythrocyte preparation were stored at −80°C, thawed carefully on ice, and homogenized by sonication before analysis. Duplicate kinetic measurements were subsequently carried out in polystyrene cuvettes thermostated at 37°C.

In a typical experiment, 3000 µl sodium phosphate buffer (100 mM, pH 7.4) was placed into a cuvette followed by 10 µl of either the erythrocyte preparation (hAChE) or plasma (hBChE), 100 µl of 5,5′-dithiobis-(2-nitrobenzoic acid) (10 mM in sodium phosphate buffer [100 mM, pH 7.4]) and 32 µl of the stock solutions of **1**–**12** or obidoxime (for reference). After addition of 50 µl acetyl- (28.3 mM) or butyrylthiocholine (63.2 mM) in sodium phosphate buffer (100 mM, pH 7.4), UV/vis recordings (412 nm) were started at cyclic intervals.

To exclude any mock activity by nucleophilic cleavage of acetylthiocholine under assay conditions, compounds **1**–**12** (100 µM) were also subjected to kinetic analysis in the absence of human AChE. Less than 1% thiocholine release from acetylthiocholine was observed in comparison to corresponding experiments with **1**–**12** in the presence of human AChE set to 100%.

4.2.2 | Enzyme reactivation assay

Inhibited human AChE was obtained by incubating (30 min, 37°C) the erythrocyte preparation with a small volume (1%, vol/vol) of appropriate concentrations of GA, GB, GF, VX, or PXE to achieve AChE inhibition of >95%. Thereafter, the treated samples were dialyzed against sodium phosphate buffer (100 mM, pH 7.4) overnight at 4°C to remove any residual OP inhibitor. The absence of inhibitory activity was verified by incubating (30 min, 37°C) a mixture of dialyzed and native erythrocyte preparations followed by kinetic analysis as described above. Aliquots of the inhibited human AChE were stored at −80°C.

To screen for reactivation potential, 150 µl inhibited human AChE was mixed with an equal volume of phosphate buffer containing 0.2% gelatin to stabilize AChE activity during prolonged incubation at 37°C.[33] Subsequently, a 3 µl stock solution of **1**–**12** or obidoxime (for reference) was added (t = 0 min, 100 µM concentration in the reactivation assay) and 20 µl samples were taken at defined time intervals (t = 5, 15, 30, 45, 60 min) for measurement of AChE activity as reported above. The additional dilution resulted in a 0.6 µM concentration of the remaining reactivator in the cuvette, which had negligible influence on activity (see above). Reactivation was finally calculated from duplicate measurements by normalizing the gain in AChE activity with respect to control.

4.3 | Molecular docking

The crystal structure of the human AChE with inhibitor cyclosarin bound to the Ser203 was accessed from the Protein Data Bank (PDB structure 6WVP[34]). The docking of compound **4** into the active site of AChE was performed using the AutoDock program suite.[40] The Lamarckian Genetic Algorithm was utilized, with a maximum of 2,500,000 energy evaluations and 10 requested genetic algorithm dockings. Docking of molecule **4** resulted in 10 conformations; four of them are similar and belong to one cluster with the mean binding energy of −4.53 kcal mol^{-1}. The remaining six conformations are distributed in six distinctive conformational clusters of lower stability.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest. Design, performance, data interpretation and manuscript writing has not been influenced by the German Ministry of Defence.

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