Thiol-reactive analogues of galanthamine, codeine and morphine as potential probes to interrogate allosteric binding within nAChRs

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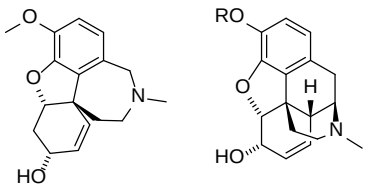
Abstract:

Alkaloids including galanthamine (**1**) and codeine (**2**) are reported to be positive allosteric modulators of nicotinic acetylcholine receptors (nAChRs) but the binding sites responsible for this activity are not known with certainty. Analogues of galanthamine (**1**), codeine (**2**) and morphine (**3**) with reactivity towards cysteine thiols were synthesised including conjugated enone derivatives of the three alkaloids **4**-**6** and two chloro-alkane derivatives of codeine **7** and **8**. The stability of the enones was deemed sufficient for use in buffered aqueous solutions and their reactivity towards thiols was assessed by determining the kinetics of reaction with a cysteine derivative. All three enone derivatives were of sufficient reactivity and stability to be used in covalent trapping, an extension of the substituted cysteine accessibility method (SCAM), to elucidate the allosteric binding sites of galanthamine and codeine at nAChRs.

Introduction:

Galanthamine (**1**, Figure 1) is an alkaloid present in many plant species from the Amaryllidaceae family including *Galanthus*, *Narcissus* and *Leucojum*. Initially used as a curare reversal agent in anaesthetic practice and to assist in recovery from paralysis [1], it is currently approved in many countries world-wide to provide symptomatic relief in Alzheimer's disease [2]. Galanthamine (**1**) has a dual mode of action on the cholinergic system with the overall effect of increasing nicotinic acetylcholine receptor (nAChR) activity [1a, 3]. It increases acetylcholine (ACh) levels by competitively inhibiting acetylcholinesterase (AChE), the enzyme responsible for ACh hydrolysis ($IC_{50} \approx 3$ μM) [4]. At low concentrations galanthamine (0.02–2 μM) has been reported to be a positive allosteric modulator (PAM) of nAChRs, increasing the response of the receptor to endogenous ACh. However, at higher concentrations (>10 μM) it acts as a nAChR inhibitor [5]. While the binding site of galanthamine (**1**) on the AChE enzyme is well established [6], its binding site within nAChRs has not been located with certainty, although a number of potential sites have been proposed [7]. The structurally similar alkaloid codeine (**2**), used as an analgesic and found in the opium poppy (*Papaver somniferum*), has also been reported to be a PAM of nAChRs without inhibiting AChE [8]. Based on detailed studies of structure and hydrogen bonding properties, it has been proposed that codeine (**2**) binds at the same location as galanthamine (**1**) on nAChRs [9]. In contrast to codeine, morphine (**3**) is not a PAM of nAChRs [8].



galanthamine (**1**) codeine (**2**), R = CH_3
morphine (**3**), R = H

Figure 1: Structures of galanthamine (**1**), codeine (**2**) and morphine (**3**).

In the absence of high-resolution structural information, the identification and validation of allosteric binding sites in proteins like nAChRs presents considerable challenges. Approaches include probing ligand receptor interactions through site-directed mutagenesis or ligand competition experiments within the substituted cysteine accessibility method (SCAM) [10]. However, these approaches provide indirect evidence of binding site location and may be compromised by conformational changes influencing ligand interaction at some distance from the putative site under investigation [11]. More direct evidence of allosteric ligand

binding can be achieved by photoaffinity labelling of receptors using photoactive ligands [12]. However, this method typically requires high protein concentrations to minimise non-selective labelling and can be complicated by the broad range of reactivity associated with different amino acid sidechains [13].

Covalent trapping is an affinity labelling method with the potential to provide concrete evidence of allosteric binding sites [14]. The technique extends the SCAM and employs cysteine mutagenesis in combination with thiol-reactive ligands. The formation of a covalent bond between the ligand and binding site results in an irreversible change in receptor function that can generally be detected by sensitive analytical techniques such as two electrode voltage clamp electrophysiology [10]. Covalent trapping has been successfully applied in the neuronal nAChR field to covalently attach methyllycaconitine (MLA) in the α7-α7 interface of the α7 nAChR [15], to identify the binding site of small analogues of MLA at α7 and α4β2 nAChRs [15-16], and to demonstrate that MLA binds at the α4-α4 interface of $(\alpha4)_3(\beta2)_2$ nAChRs at a site distinct from the canonical α4-β2 interface agonist binding site [17].

The development of thiol-reactive probes for covalent trapping is subject to a range of constraints. Ideally, the thiol-reactive analogue will closely resemble the parent ligand so that it binds at the same allosteric site and even exerts the same biological activity. Further, the thiol-reactive ligand must, after equilibrium binding, undergo reaction with a suitably positioned cysteine residue to irreversibly forge the covalent bond. It follows that the probe reactivity must be adequate to promote covalent trapping, but not so great as to impose solution instability or non-selective reactions with the receptor protein. In order to investigate the allosteric binding sites for galanthamine (**1**) and codeine (**2**) we targeted the conjugated enone analogues narwedine (**4**, Figure 2), codeinone (**5**) and morphinone (**6**), together with the mustard **7** and benzyl chloride **8** derivatives of codeine (**2**). These derivatives provide a topologically varied range of minor structural changes to the parent ligands. The details of their synthesis and the evaluation of their reactivity by examining the solution kinetics of their reaction with *N*-acetyl-L-cysteine methyl ester are presented herein.

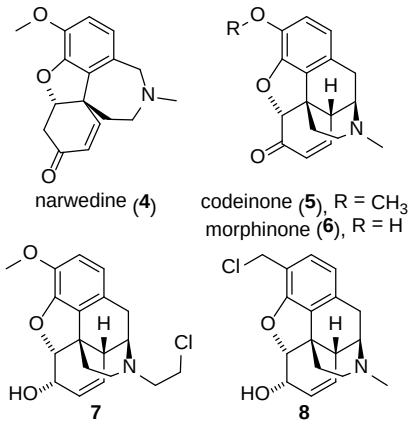


Figure 2: Structures of the thiol-reactive analogues.

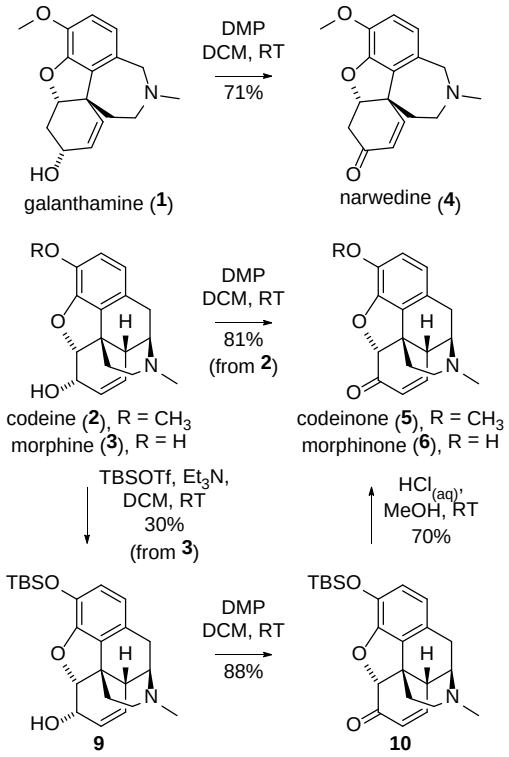
Results and Discussion:

Synthesis of conjugated enone analogues

Racemic narwedine (**4**) was obtained from the oxidation of galanthamine (**1**) with Dess-Martin periodinane (DMP) in 71 % yield (Scheme 1). The enantiomeric purity was estimated by comparison of the optical rotation with the optical rotation of resolved samples reported in the literature [18]. An enantiomerically enriched sample of narwedine (**4**), with an estimated 79:21 enantiomeric ratio could be obtained using manganese dioxide as the oxidant. Under basic conditions and in protic solvents, including those commonly used to work-up DMP oxidations, narwedine (**4**) can racemise. Following a base promoted retro-Michael reaction the resulting phenoxide ion can add to either of the two alkenes of the resulting dienone intermediate to regenerate either enantiomer of narwedine (**4**). Under milder conditions such as those employed in the oxidation with manganese dioxide partial racemisation results from the inherent basicity of narwedine (**4**) itself. Given the facile racemisation of narwedine in protic solvents, racemic narwedine was deemed suitable to undertake the solution kinetics for this study. If required, enantiomerically pure narwedine can be obtained by crystallisation involving dynamic kinetic resolution as performed in the industrial synthesis of galanthamine [18].

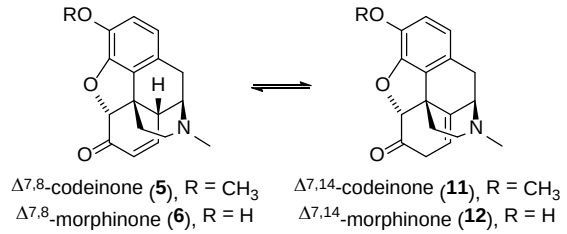
When codeine (**2**) was oxidised with freshly prepared DMP, codeinone (**5**) [19] was obtained as the sole product in 81 % yield. It was observed that when aged samples of DMP were used for the oxidation a small portion of the codeinone (**5**) was further oxidised to afford 14-hydroxycodeinone, identified by NMR comparison with the literature [20]. This over-oxidation is believed to result from traces of 2-iodoxybenzoic acid formed when DMP is hydrolysed by adventitious moisture.

Attempts to directly oxidise morphine (**3**) to morphinone (**6**) led to decomposition and the desired product could not be isolated from reaction mixtures. Instead, a route involving protection of the phenol was employed. Morphine (**3**) was selectively protected as the TBS ether at the phenolic position to afford compound **9** [21] in 30 % yield. Oxidation of the allylic alcohol with DMP afforded the protected enone **10** (88%) [21], which was then deprotected with aqueous hydrochloric acid to give morphinone (**6**) [21b] in a yield of 70%.



Scheme 1: Synthesis of narwedine (**4**), codeine (**5**) and morphinone (**6**).

A minor impurity detected in samples of codeinone and morphinone resulted from deconjugation of the enone (Scheme 2). In aqueous solutions, an equilibrium is established between codeinone (**5**) or morphinone (**6**) and their deconjugated isomers **11** and **12** which are unreactive towards thiol nucleophiles. While the equilibrium between codeinone (**5**) and its deconjugated isomer **11** is well known [22], the corresponding equilibrium for morphinone has not been reported in the literature. Based on the ^{1}H NMR analysis of the product mixtures, the deconjugated enone isomers **11** and **12** formed an estimated 5-10% of the final products. The presence of this non-reactive impurity could be readily accounted for in the subsequent kinetic analysis.



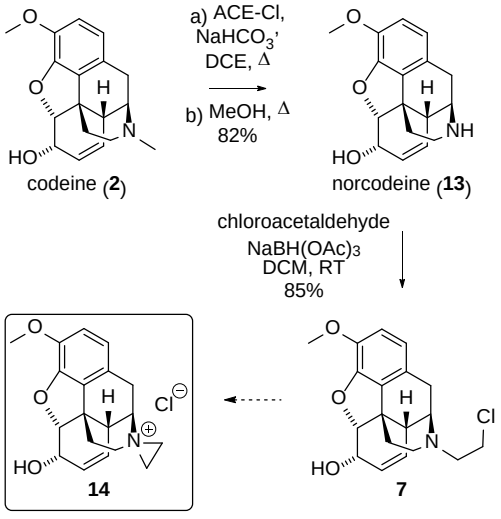
$\Delta^{7,8}$-codeinone (**5**), R = CH_3 $\Delta^{7,14}$-codeinone (**11**), R = CH_3
$\Delta^{7,8}$-morphinone (**6**), R = H $\Delta^{7,14}$-morphinone (**12**), R = H

125

126 **Scheme 2:** Deconjugation of codeinone (**5**) and morphinone (**6**) in aqueous solution.

127 *Synthesis of chlorinated analogues*

128 The codeine mustard **7** was prepared in two steps from codeine (**2**) as shown in Scheme 3.
129 Codeine (**2**) was treated with α-chloroethyl chloroformate (ACE-Cl) to generate an
130 intermediate carbamate that was hydrolysed to norcodeine (**13**) [23] in methanol in 82 % yield
131 over two steps. In the absence of base, the initial reaction with ACE-Cl was very slow with
132 residual codeine observed after three days. This may result from generation of acid within the
133 reaction mixture rendering the tertiary amine less nucleophilic. Addition of solid sodium
134 bicarbonate to the reaction mixture resulted in a significant increase in rate and complete
135 conversion was achieved in one day. Removal of the base prior to methanolysis was required
136 to avoid the formation of a by-product, believed to be the methyl carbamate.



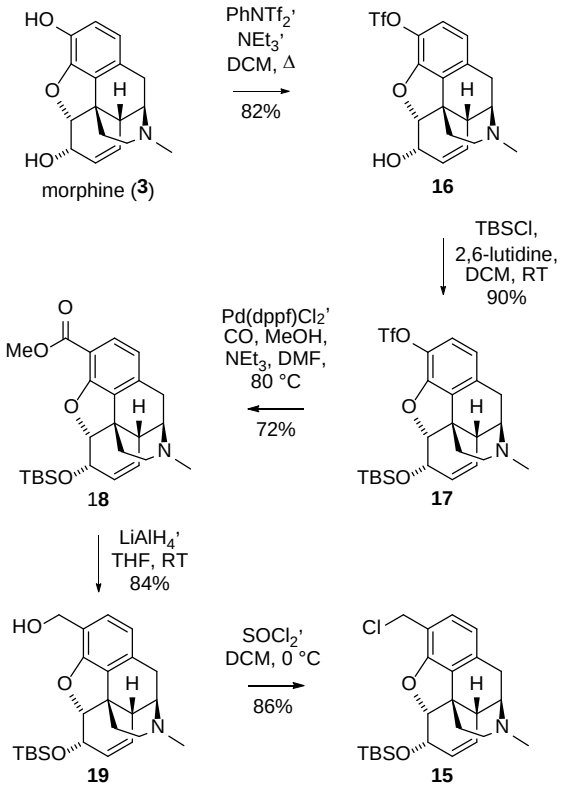
137

138 **Scheme 3:** Synthesis of codeine mustard **7**.

139 Synthesis of the codeine mustard **7** *via* reductive amination with chloroacetaldehyde was
140 complicated by the ready formation of the reactive aziridinium ion **14** through intramolecular
141 nucleophilic substitution. Reductive amination of norcodeine (**13**) with sodium
142 cyanoborohydride as the reducing agent failed to generate the desired mustard **7**. Instead, a
143 product with mass spectrum consistent with the ethyl bridged dimer was generated as the sole
144 product. Reductive amination with sodium triacetoxyborohydride afforded the codeine

mustard **7** as the sole product in 85 % yield. Attempts to obtain the ^{1}H-NMR spectrum of the codeine mustard **7** in deuterated methanol led to the rapid formation of the d_3-methyl ether product ($t_{½} \approx 2$ h). Additionally, dissolving the compound in aqueous buffer resulted in rapid hydrolysis generating the amino alcohol ($t_{½} \approx 30$ min). Based on this reactivity it was determined that the codeine mustard **7** would be too unstable to be useful as a reactive probe in covalent trapping experiments.

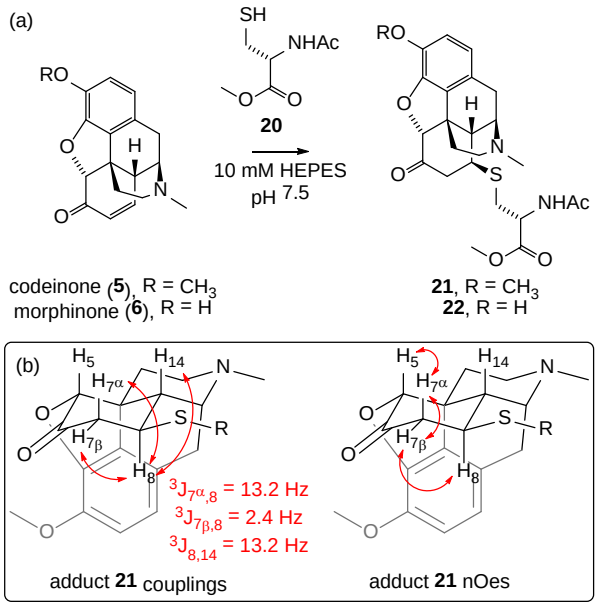
While the targeted benzyl chloride **8** derivative of codeine could not be prepared in pure form, a protected analogue **15** was prepared in 5 steps from morphine (Scheme 4). Selective triflation of the phenol afforded the morphine triflate **16** [24] in 82 % yield, which was then protected as the TBS ether **17** (90 %) [25]. Subsequent palladium catalysed carbonylative coupling gave methyl ester **18** (72%), which was reduced to give the benzyl alcohol **19** in 84 % yield. The protected benzyl chloride derivative **15** was afforded by treatment with thionyl chloride (86%). However, attempts to deprotect the silyl ether to afford the desired benzyl chloride derivative **8** failed due to the reactivity of the benzyl chloride moiety. Deprotection with aqueous hydrochloric acid, as was applied in the synthesis of morphinone, led to complete hydrolysis of the benzyl chloride. Deprotection using tetrabutylammonium fluoride (TBAF) led to an inseparable and complex mixture of products. Finally, it was observed that dissolving the protected benzyl chloride **15** in deuterochloroform led to slow dimerisation and dissolving in aqueous buffer led to the rapid formation of the benzyl alcohol ($t_{½} < 1$ min). Based on these results it was determined that even if the benzyl chloride derivative **8** could be obtained through desilylation, it would be too unstable to be useful as a thiol-reactive probe and its synthesis was not pursued further.



Scheme 4: Synthesis of the protected benzyl chloride **15** from morphine (**3**)

Reaction Kinetics

In this work reactivity of the reactive probe candidates was evaluated by monitoring the solution kinetics of the reaction with *N*-acetyl-L-cysteine methyl ester (**20**, Scheme 5a). The comparison of solution phase data with that obtained from covalent trapping experiments may be used to establish future guidelines on desired levels of the reactivity and stability for reactive probes. In this manner, compounds that react are likely to react unselectively or too slowly with thiols can be excluded before deploying resources on the covalent trapping experiment. Such investigations could also aid in the design of new reactive probes or provide information on the stereochemical course of reactions that could aid in the selection of cysteine mutants for the covalent trapping experiments.



Scheme 5: (a) Reaction of codeinone (**5**) or morphinone (**6**) with *N*-acetyl-L-cysteine methyl ester (**20**) to form adducts **21** or **22**. (b) Selected ^{1}H NMR coupling constants and nOes for adduct **21** (boxed section).

The pseudo first order kinetics of the reaction between codeinone (**5**) or morphinone (**6**) and a 20-fold excess of *N*-acetyl-L-cysteine methyl ester (**20**) were studied under conditions as close as possible to those employed in covalent trapping experiments. Reactions were conducted in triplicate with enone concentration determined by LCMS. Due to the enone tautomerisation discussed earlier (Scheme 2) the stock solutions of codeinone (**5**) or morphinone (**6**) contained a small amount of the deconjugated isomers **11** and **12** which eluted together with their respective conjugated enones. Since the deconjugated isomers **11** and **12** do not react with thiols and the rate of tautomerisation was observed to be slow relative to the rate of addition, the exponential decay relationship for total codeinone (**5** + **11**) or morphinone (**6** + **12**) concentration has a non-zero asymptote corresponding to the concentration of the deconjugated isomers. The relationship between total codeinone or morphinone concentration and time is therefore given by Equation 1 where $[A]_0$ is the initial concentration of the conjugated enone, [B] is the concentration of the deconjugated enone and k_{obs} is the pseudo-first order rate constant:

$$total\ enone\ concentration = [A]_0 e^{-k_{obs}t} + [B]$$

Equation 1

The kinetics of the reaction between narwedine (**4**) and a 20-fold excess of *N*-acetyl-L-cysteine methyl ester (**20**) were monitored under slightly different conditions. Since the reaction was much slower under buffered aqueous conditions and concentrations (25 μM)

typically used during LC-MS analysis of codeinone or morphinone, the reaction was monitored using ^{1}H-NMR which allows for much higher concentrations (mM). Reactions were conducted in triplicate in deuterated methanol. Unlike codeinone (**5**) and morphinone (**6**), narwedine (**4**) does not isomerise to an unreactive product and the relationship between the concentration of narwedine (**4**) and time is a simple exponential decay.

A plot of the total codeinone concentration over time is shown in Figure 3. The corresponding plots for total morphinone (**6**) and narwedine (**4**) can be found in the supporting information. The starting concentration of codeinone (**5**) was 25 μM and the concentration of *N*-acetyl-L-cysteine methyl ester (**20**) was 500 μM. The observed pseudo-first order rate constant (k_{obs}) is (2.0 ± 0.1) × 10^{-3} s^{-1} (Table 1). The corresponding second order rate constant (k) is 4.0 ± 0.2 M^{-1} s^{-1}. Morphinone provided similar results. By comparison, the second order rate constant for the reaction of SCAM reagent 2-aminoethyl methanethiosulfonate (MTSEA) with 2-mercaptoethanol is reported as (7.6 ± 0.4) × 10^{4} M^{-1} s^{-1} under similar conditions (58 mM sodium phosphate buffer, pH 7.0, 20 °C).[10] With a starting concentration of 10 mM narwedine (**4**) and 200 mM *N*-acetyl-L-cysteine methyl ester the observed pseudo-first order rate constant (k_{obs} = [2.26 ± 0.04] × 10^{-4} s^{-1}) corresponded to a significantly smaller second-order rate constant (k) of (1.13 ± 0.02) × 10^{-3} M^{-1} s^{-1}. The second order rate constants for codeinone (**5**) and morphinone (**6**) are three orders of magnitude larger. Based on solution stability and reactivity of the enones, they appear suitable for use as thiol-reactive probes. The proposed deployment of enones as probes in covalent trapping also receives support from the observation of covalent attachment between the structurally distinct enone natural products gracilioether B and plakilactone C and the cysteine containing binding site of the peroxisome proliferator-activated receptor γ (PPARγ) under physiologically relevant conditions.[26]

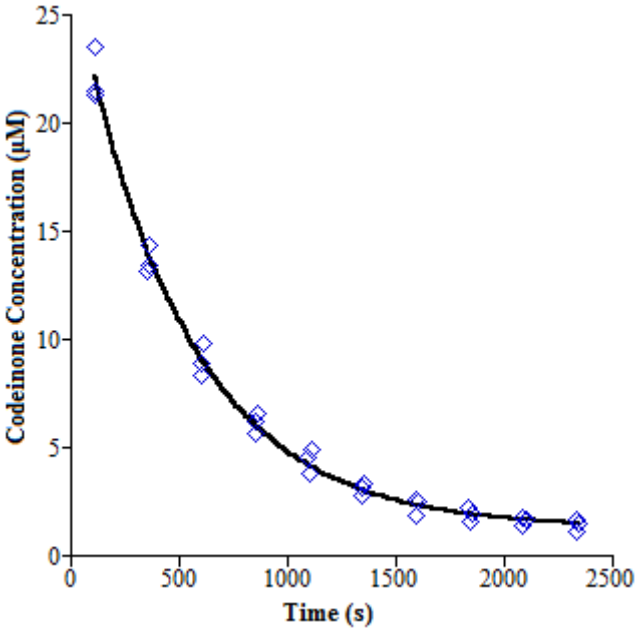
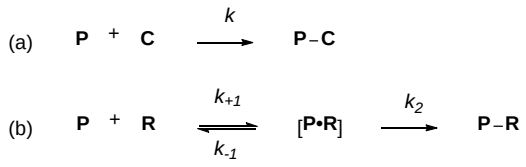


Figure 3: Plot of concentration of total codeinone (**5** + **11**) during the reaction with *N*-acetyl-L-cysteine methyl ester (**20**) with a starting concentration of 25 µM (10 mM HEPES buffer, pH 7.5, 20 °C)

Although the kinetic investigations provide useful information regarding probe stability and relative reactivity, care should be exercised in extrapolating the magnitudes of the second order rate constants *k* measured in solution with the performance in covalent trapping experiments with nAChR mutants. The rate constants measured in this work involve a second order reaction of a reactive probe (**P**) and a cysteine derivative (**C**, Scheme 6a). The covalent trapping experiment of a thiol-reactive probe (**P**) with a cysteine mutant receptor (**R**) is characterised by equilibrium binding followed by irreversible covalent bond formation, trapping the ligand within the binding site (Scheme 6b). This kinetic scheme provides a basis for understanding covalent trapping data and in part is determined by the absolute reactivity of the probe for the cysteine mutant. However, the rate constant k_2 defines the first order reaction of the reactive probe-receptor complex ([**P•R**]) involving covalent bond formation and cannot be directly compared with the second order rate constant *k* measured in solution. The formation of a probe-receptor complex will influence the rate of reaction due to proximity effects. If the cysteine residue in the receptor binding site is positioned favourably for reaction with the probe, the rate of covalent bond formation may be significantly greater than expected based on measures of absolute reactivity. Conversely, if the cysteine residue in the binding site is in an unfavourable position for reaction with the probe, the rate of covalent trapping may be significantly lower.



Scheme 6: (a) Second order addition of reactive probe (**P**) with a cysteine derivative (**C**); (b) Kinetic scheme for the covalent trapping of a thiol-reactive probe (**P**) by a cysteine mutant receptor (**R**).

The stereochemistry of the adduct **21** was determined by NMR analysis of a pure sample obtained in 94 % yield from the reaction of codeinone (**5**) with *N*-acetyl-L-cysteine methyl ester (**20**) in methanol. The 8*S*-stereochemistry observed (Scheme 5) was that predicted based on steric considerations. These alkaloid derivatives adopt a T-shaped conformation with the piperidine and cyclohexenone rings forming a plane perpendicular to the furan and phenyl rings. As a result the lower *si* face of the cyclohexenone ring is blocked by the steric bulk of the furan and phenyl rings, favouring addition to the top *re* face and leading to an equatorial disposition of the cysteine substituent in the cyclohexanone ring. The stereochemistry of adduct **21** was supported by consideration of coupling constants and nOe interactions of protons in the cyclohexanone ring. The H8 proton appeared as a triplet of doublets with two large coupling constants (13.2 Hz) and one small coupling constant (2.4 Hz). The large couplings are consistent with axial-axial couplings between the H8 proton and the adjacent H14 and H7α protons. The smaller coupling is consistent with an axial-equatorial coupling between the H8 proton and the adjacent H7β proton. The observed nOe interactions were also consistent with the proposed structure.

Table 1: Experimentally determined parameters for the integrated rate equations.

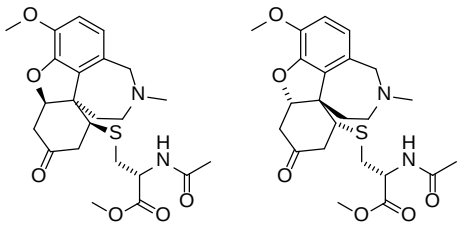
Enone	$[A]_0$ (M)	[B] (M)	k_{obs} (s^{-1})	k ($M^{-1}s^{-1}$)
codeinone[a]	$(26 \pm 1) \times 10^{-6}$	$(1.2 \pm 0.2) \times 10^{-6}$	$(2.0 \pm 0.1) \times 10^{-3}$	4.0 ± 0.2
morphinone[a]	$(21 \pm 1) \times 10^{-6}$	$(1.3 \pm 0.2) \times 10^{-6}$	$(1.8 \pm 0.1) \times 10^{-3}$	3.6 ± 0.2
narwedine[b]	$(8.8 \pm 0.2) \times 10^{-3}$	-[c]	$(2.26 \pm 0.04) \times 10^{-4}$	$(1.13 \pm 0.02) \times 10^{-3}$

[a] HEPES buffer (10 mM), pH 7.5, 20 °C; [b] deuterated methanol, 25 °C; [c] Not applicable.

The 8*S*-adduct **21** had been reported in the literature previously, reacting codeinone (**5**) with *N*-acetyl-L-cysteine methyl ester (**20**) in acetonitrile under mildly basic conditions [27].

However, the NMR data and optical rotation reported differed considerably from that obtained for compound **21** prepared in methanol solution as described in this work. Employing the previously reported experimental procedure [27] provided a sample with identical ^{1}H NMR and optical rotation to that prepared in methanol. A comparison of the ^{1}H and ^{13}C NMR data for the two reports is given in the supporting information together with copies of 1D and 2D NMR spectra.

The reaction between racemic narwedine (**4**) and enantiomerically pure *N*-acetyl-L-cysteine methyl ester (**20**) afforded a more complex stereochemical outcome. Given the racemic nature of the enone under investigation, diastereomers resulting from the two alkaloid enantiomers were expected. In addition, the enone double bond provides two faces accessible for nucleophilic addition leading to the formation of up to four diastereomers. Two separable diastereomers were obtained in 38% and 43% yield from the reaction of narwedine (**4**) with *N*-acetyl-L-cysteine methyl ester (**20**) in methanol. These showed similar ^{1}H and ^{13}C NMR spectra. Based on the steric considerations and NMR analysis we tentatively assigned these adducts as diastereomers **23a** and **23b** arising from addition *cis* to the conformationally constrained and planar aromatic ring of narwedine (**4**).[28] It was not possible to assign the relative configuration between the alkaloid core and the tethered amino acid. A discussion of the stereochemical assignment of the two diastereomers together with copies of 1D and 2D NMR spectra are provided in the supporting information.



23a **23b**

Figure 4: Structures of the two diastereomers formed when racemic narwedine (**4**) reacts with *N*-acetyl-L-cysteine methyl ester (**20**)

Conclusion:

Thiol-reactive analogues of galanthamine (**1**), codeine (**2**) and morphine (**3**) were synthesised as probes to study the binding site of these compounds at nAChRs. These included the conjugated enone derivatives of all three alkaloids **4**-**6**, a mustard derivative of codeine **7** and a protected benzyl chloride derivative of codeine **15**. The chlorinated derivatives of codeine **7** and **8** were deemed too reactive for use in covalent trapping studies, due to instability in

aqueous buffer. The kinetics of the reaction between the conjugated enones and *N*-acetyl cysteine methyl ester were studied as a model for their reactivity with cysteine residues in mutant nAChRs. Codeinone (**5**) and morphinone (**6**) reacted exclusively at the least hindered face of the cyclohexenone ring with second order rate constants (k) of 4.0 ± 0.2 M^{-1} s^{-1} and 3.6 ± 0.2 M^{-1} s^{-1} respectively. Narwedine (**4**) reacted at both faces of the cyclohexenone ring with a second order rate constant (k) of (1.13 ± 0.02) × 10^{-3} M^{-1} s^{-1}. Based on the solution stability and thiol reactivity the three enone derivatives appear suitable as thiol-reactive probes in covalent trapping experiments. Covalent trapping experiments will be pursued in the near future and the results of these studies will be reported in due course.

Acknowledgements:

We thank Tasmanian Alkaloids for the generous supply of codeine and morphine and Janssen Pharmaceutica for the generous supply of galanthamine hydrobromide. We thank Dr Sam Bailey and Dr Robert Rezaie from Tasmanian Alkaloids for helpful discussions, and Prof. Gottfried Otting for assistance with NMR analysis.

Experimental:

General Experimental:

All reactions were performed under an atmosphere of nitrogen unless otherwise stated. Codeine and morphine were supplied by Tasmanian Alkaloids, galanthamine hydrobromide was supplied by Janssen Pharmaceutica, dichloroethane was purchased from Ajax Finechem, all other solvents were purchased from Merck, all other chemicals were purchased from Sigma Aldrich. Reaction temperatures were controlled using oil baths for temperatures greater than room temperature or standard ice baths for 0 °C. Removal of solvent *in vacuo* refers to the concentration of samples by rotary evaporation under reduced pressure. Melting points were determined using an Optimelt automated melting point system. Optical rotations were determined using a Perkin-Elmer Model 343 Polarimeter set at the 589 nm sodium D line, in a 1.00 dm cell at 20 °C. The specific rotation is reported along with the concentration in g/100 mL and solvent. Infrared (IR) absorption spectra were obtained using a Perkin-Elmer Spectrum One FTIR spectrometer. All compounds were analysed as a thin-film on NaCl plates. Key absorbance bands are reported in wavenumbers (cm^{-1}). Nuclear Magnetic Resonance (NMR) spectra were obtained on a Bruker 400 (400 MHz) or a Bruker 800 (800 MHz) NMR spectrometer. Samples were analysed at room temperature and dissolved in

deuterated chloroform ($CDCl_3$). The machine was operated at 400 MHz or 800 MHz for ^{1}H-NMR or 100 MHz for ^{13}C-NMR. Chemical shifts (δ) are reported in ppm relative to TMS (δ = 0) and the splitting of ^{1}H-NMR peaks are reported with the following codes; s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublets, dt = doublet of triplets, dm = doublet of multiplets, td = triplet of doublets, ddd = doublet of doublet of doublets, br = broad. Where two protons are attached to the same carbon they are assigned as axial or equatorial where appropriate. Where axial or equatorial assignment is not appropriate the protons are assigned as α (top face) or β (bottom face). Assignment of chemical shifts (δ) is based on analysis of COSY, NOESY, HMBC and HSQC NMR. Low-resolution mass spectrometry (LRMS) and high-resolution mass spectrometry (HRMS) were performed using positive electron ionisation (EI) on a Micromass VG Autospec mass spectrometer, or using positive electrospray ionisation (ESI) on a Micromass ZMD ESI-Quad (LRMS) or a Waters LCT Premier XE mass spectrometer (HRMS).

General procedure for Michael addition reaction:

A solution of the enone (1 eq) and *N*-acetyl-L-cysteine methyl ester (2 eq) were dissolved in methanol (100 µL / mg of enone) and the resulting solution was stirred at room temperature overnight. The following morning the solvent was removed *in vacuo* to give the crude compound, which was purified by flash chromatography.

Codeinone – N-acetyl-L-cysteine methyl ester adduct:

The general procedure was applied to codeinone (**5**, 20 mg, 67 µmol), purifying by flash chromatography (9:1 chloroform : methanol), to yield the title compound **21** (30 mg, 94%) as a white solid, mp: 86-88 °C. $[\alpha]_D^{20}$ +22 (*c* 1.0, $CHCl_3$), (lit [27] $[\alpha]_D^{20}$ -127 (*c* 0.5, $CHCl_3$)). ν_{max} (NaCl)/cm^{-1} 3287, 1731, 1667, 1277, 1259. δ_H (800 MHz, $CDCl_3$) 6.70-6.71 (m, 1H, H_2), 6.67-6.69 (m, 1H, H_1), 6.32 (d, br, J = 6.8 Hz, 1H, $H_{3'}$), 4.81 (m, 1H, $H_{2'}$), 4.69 (s, 1H, H_5), 3.89 (s, 3H, H_{3a}), 3.63 (s, br, 1H, H_9), 3.46 (s, 3H, $H_{1a'}$), 3.04 (dd, J = 14.0 Hz, 4.4 Hz, 1H, $H_{2a'}$), 3.01 (d, J = 18.8 Hz, 1H, $H_{10\alpha}$), 2.98 (dd, J = 14.0 Hz, 4.0 Hz, 1H, $H_{2a'}$), 2.70 (dd, J = 13.2 Hz, 2.4 Hz, 1H, $H_{7\beta}$), 2.57 (d, br, J = 11.6 Hz, 1H, H_{16eq}), 2.53 (t, J = 13.2 Hz, 1H, $H_{7\alpha}$), 2.44-2.49 (4H, m, H_{14}, H_{17a}), 2.34 (dd, J = 18.4 Hz, 5.2 Hz, 1H, $H_{10\beta}$), 2.30 (td, J = 12.8 Hz, 2.4 Hz, 1H, H_8), 2.20 (td, J = 12.0 Hz, 2.8 Hz, 1H, H_{16ax}), 2.06 (td, J = 12.0 Hz, 4.0 Hz, 1H, H_{15ax}), 1.97 (s, 3H, $H_{3b'}$), 1.82 (d, br, J = 12.0 Hz, 1H, H_{15eq}). δ_C 204.8, 170.9, 169.9, 145.2, 143.1, 126.8, 126.5, 120.4, 115.0, 91.5, 57.0, 56.9, 52.6, 52.3, 47.5, 47.4, 47.3, 47.2,

43.0, 41.6, 35.7, 31.5, 23.2, 19.3. *m/z* (EI) = 474 ($M^{+\bullet}$, 15), 299 (35), 298 ($[M–C_6H_{10}NO_3S]^+$, 100), 297 ($[M–C_6H_{11}NO_3S]^{+\bullet}$, 30). *m/z* (EI) = 474.1827 ($M^{+\bullet}$, $C_{24}H_{30}N_2O_6S$ gives 474.1825)

The literature procedure [27] was applied to codeinone (**5**, 20 mg, 67 µmol) with 2 eq of *N*-acetyl-L-cysteine methyl ester and 6 eq of sodium bicarbonate to give a yellow oil, $[\alpha]_D^{20}$ +31 (*c* 1.0, $CHCl_3$), purified by flash chromatography (9:1 chloroform : methanol), to yield the title compound **21** (14 mg, 44%) as a white solid, mp: 90-92 °C. $[\alpha]_D^{20}$ +23 (*c* 1.0, $CHCl_3$).

Narwedine – N-acetyl-L-cysteine methyl ester adducts:

The general procedure was applied to narwedine (**4**, 50 mg, 175 µmol), purifying by flash chromatography (9:1 chloroform : methanol), to yield adducts **23a** (or **23b**) (31 mg, 38 %) and adducts **23b** (or **23a**) (35 mg, 43 %) as colourless oils.

Adduct **23a** (or **23b**). $[\alpha]_D^{20}$ +3 (*c* 0.4, $CHCl_3$). ν_{max} (NaCl)/cm^{-1} 3289, 1744, 1721, 1675, 1286, 1204. δ_H (800 MHz, $CDCl_3$) 6.68 (d, J = 8.0 Hz, 1H, H_2), 6.64 (d, J = 8.0 Hz, 1H, H_1), 6.29 (d, br, J = 7.6 Hz, 1H, $H_{3'}$), 4.86 (ddd, J = 7.6 Hz, 5.6 Hz, 4.0 Hz, 1H, $H_{2'}$), 4.68 (t, J = 2.8 Hz, 1H, H_{4a}), 4.11 (d, J = 14.8 Hz, 1H, $H_{12\beta}$), 3.83 (s, 3H, H_{3a}), 3.78 (s, 3H, $H_{1a'}$), 3.63 (d, J = 14.8 Hz, 1H, $H_{12\alpha}$), 3.58 (s, br, 1H, H_8), 3.41 (t, br, J = 13.6 Hz, 1H, $H_{10\beta}$), 3.14 (dd, J = 13.6 Hz, 4.0 Hz, 1H, $H_{2a'}$), 3.10 (d, br, J = 14.4 Hz, 1H, $H_{10\alpha}$), 2.98-3.01 (m, 1H, $H_{5\beta}$), 2.94-2.96 (m, 1H, $H_{5\alpha}$), 2.88 (dd, J = 13.6 Hz, 5.6 Hz, 1H, $H_{2a'}$), 2.58 (dd, J = 16.8 Hz, 3.6 Hz, 1H, $H_{7\beta}$), 2.50 (dd, J = 16.8 Hz, 2.8 Hz, 1H, $H_{7\alpha}$), 2.32 (s, 3H, H_{11a}), 2.09 (t, br, J = 14.0 Hz, 1H, $H_{9\alpha}$), 2.07 (s, 3H, $H_{3b'}$), 1.96 (dd, J = 14.0 Hz, 3.2 Hz, 1H, $H_{9\beta}$). δ_C 206.3, 171.1, 170.0, 146.8, 144.0, 131.7, 129.5, 123.0, 111.7, 87.9, 60.0, 56.1, 55.3, 53.0, 51.7, 51.4, 44.3, 41.8, 41.1, 40.2, 33.9, 32.8, 23.2. *m/z* (EI) = 462 ($M^{+\bullet}$, < 1), 286 (35), 285 ($[M–C_6H_{11}NO_3S]^{+\bullet}$, 100), 242 (40), 216 (25), 199 (25), 174 (45), 118 (20), 88 (45), 76 (50). *m/z* (ESI) = 463.1904 ($[M+H]^+$, $C_{23}H_{31}N_2O_6S$ gives 463.1903).

Adduct **23b** (or **23a**). $[\alpha]_D^{20}$ +50 (*c* 0.4, $CHCl_3$). ν_{max} (NaCl)/cm^{-1} 3271, 1720, 1659, 1286, 1204. δ_H (800 MHz, $CDCl_3$) 6.69 (d, J = 8.0 Hz, 1H, H_2), 6.64 (d, J = 8.0 Hz, 1H, H_1), 6.19 (d, J = 7.6 Hz, 1H, $H_{3'}$), 4.78 (td, J = 7.6 Hz, 4.0 Hz, 1H, $H_{2'}$), 4.69 (t, J = 2.8 Hz, 1H, H_{4a}), 4.19 (d, J = 14.8 Hz, 1H, $H_{12\beta}$), 3.84 (s, 3H, H_{3a}), 3.77 (s, 3H, $H_{1a'}$), 3.67 (s, br, 1H, H_8), 3.63 (d, J = 14.8 Hz, 1H, $H_{12\alpha}$), 3.39 (t, br, J = 13.2 Hz, 1H, $H_{10\beta}$), 3.15 (dd, J = 13.6 Hz, 4.0 Hz, 1H, $H_{2a'}$), 3.09 (d, br, J = 14.4 Hz, 1H, $H_{10\alpha}$), 3.03 (dd, J = 18.4 Hz, 2.8 Hz, 1H, $H_{5\alpha}$), 2.95 (dd, J = 18.4 Hz, 2.8 Hz, 1H, $H_{5\beta}$), 2.78 (dd, J = 13.6 Hz, 7.6 Hz, 1H, $H_{2a'}$), 2.61 (dd, J = 17.2 Hz, 3.6 Hz, 1H, $H_{7\beta}$), 2.52 (dd, J = 17.2 Hz, 2.8 Hz, 1H, $H_{7\alpha}$), 2.34 (s, 3H, H_{11a}), 2.08-2.12

(m, 4H, $H_{9\alpha}$, $H_{3b'}$), 1.98 (dd, J = 14.0 Hz, 3.2 Hz, 1H, $H_{9\beta}$). δ_C 206.0, 171.2, 170.2, 146.8, 144.0, 131.7, 129.3, 123.1, 111.7, 88.0, 59.9, 56.1, 55.3, 53.2, 51.3, 51.2, 43.3, 42.1, 40.8, 40.1, 33.3, 33.0, 23.4. *m/z* (EI) = 462 ($M^{+\bullet}$, < 1), 286 (35), 285 ($[M–C_6H_{11}NO_3S]^{+\bullet}$, 100), 242 (40), 216 (25), 199 (25), 174 (45), 118 (20), 88 (45), 76 (50). *m/z* (ESI) = 463.1900 ($[M+H]^+$, $C_{23}H_{31}N_2O_6S$ gives 463.1903).

Investigation of reaction kinetics by LC-MS:

The enones and their adducts with *N*-acetyl-L-cysteine methyl ester were separated using an Agilent 1260 UHPLC system with an Agilent C18 column (50 mm with a 5 mm guard column, 2.1 mm diameter, 1.8 μm particle size). The mobile phase consisted of 86% aqueous ammonium acetate (10 mM) adjusted to pH 5.5 and 14% acetonitrile with a flow rate of 0.5 mL min^{-1}. Analytes were ionized by atmospheric pressure electrospray ionisation (AP-ESI) with an Agilent 6120 quadrupole mass spectrometer and ions were monitored in positive mode for the protonated species ($[M+H]^+$). The capillary voltage was 1500 V and the fragmentor voltage was 150 V.

Reactions were carried out in 10 mM HEPES buffer adjusted to pH 7.5 at 20 °C. A solution of the enone (1 eq) was mixed with a solution of *N*-acetyl-L-cysteine methyl ester (20 eq) and the composition of the mixture was analysed by LC-MS at regular intervals. The concentration of the enone at each interval was determined with reference to a calibration curve and the pseudo first order rate constant for the reaction was estimated by least squares curve fitting from the plot of enone concentration against time using KaleidaGraph.

Investigation of reaction kinetics by ^{1}H-NMR:

Reactions were carried out in deuterated methanol at 25 °C. A solution of the enone (1 eq) was mixed with a solution of *N*-acetyl-L-cysteine methyl ester (20 eq) and the composition of the mixture was determined by ^{1}H-NMR analysis at regular intervals. The concentration of the enone at each interval was determined by comparing the relative integration of the H-7 olefinic proton in the starting material with the $H_{12\beta}$ benzylic proton in both the starting material and product. The pseudo first order rate constant for the reaction was estimated by least squares curve fitting from the plot of enone concentration against time using KaleidaGraph.

Supporting Information:

Experimental procedures for compounds **4-7**, **9**, **10**, **13**, **15-19**, together with ^{1}H-NMR and ^{13}C-NMR spectra for all compounds and low resolution mass spectra for new compounds.

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