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Full Paper

Thiol-Reactive Analogues of Galanthamine, Codeine, and Morphine as Potential Probes to Interrogate Allosteric Binding within Nicotinic Acetylcholine Receptors

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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 synthesized 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, to elucidate the allosteric binding sites of galanthamine and codeine at nAChRs.

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Introduction

Galanthamine (**1**, Fig. 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] galanthamine is currently approved in many countries worldwide 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 the activity of nicotinic acetylcholine receptors (nAChRs).[1a,3] It increases acetylcholine (ACh) levels by competitively inhibiting acetylcholinesterase (AChE), the enzyme responsible for ACh hydrolysis (half-maximal inhibitory concentration (IC_{50}) ≈ 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] The binding site of galanthamine (**1**) on the AChE enzyme is well established;[6] in contrast, its binding site within nAChRs has not been located with certainty, although several 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]

In the absence of high-resolution structural information, the identification and validation of allosteric binding sites in proteins, such as 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 minimize non-selective labelling and can be complicated by the broad range of reactivity associated with different amino acid side chains.[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

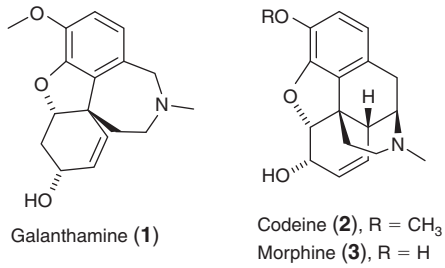


Fig. 1. Structures of galanthamine (**1**), codeine (**2**), and morphine (**3**).

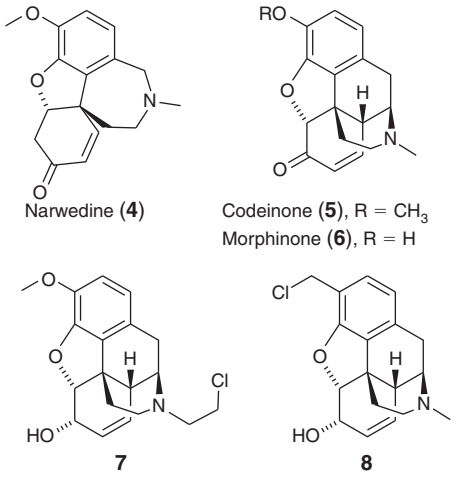
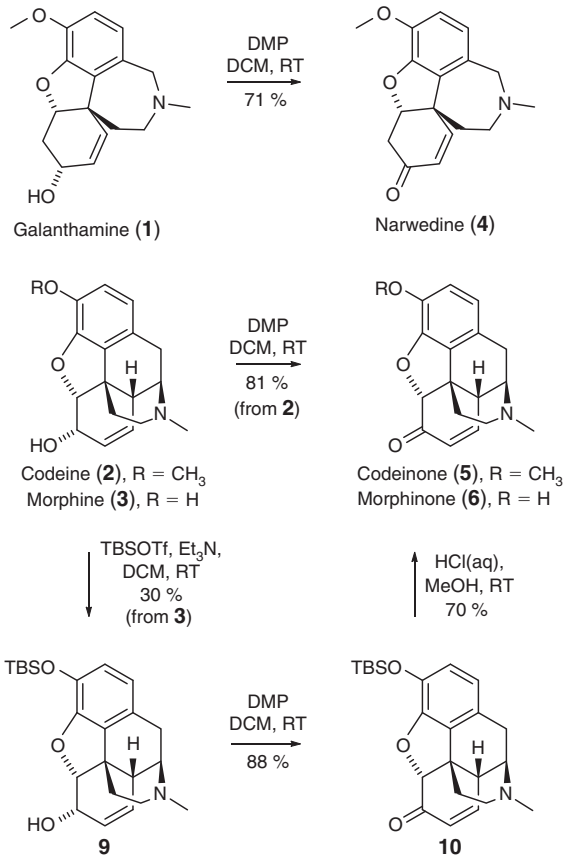


Fig. 2. Structures of the thiol-reactive analogues.



Scheme 1. Synthesis of narwedine (**4**), codeine (**5**), and morphinone (**6**). DCM = dichloromethane; TBSOTf = *tert*-butyldimethylsilyl triflate; Et_3N = triethylamine; MeOH = methanol; RT = room temperature.

$(\alpha4)_3(\beta2)_2$ 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. Furthermore, 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**, Fig. 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.

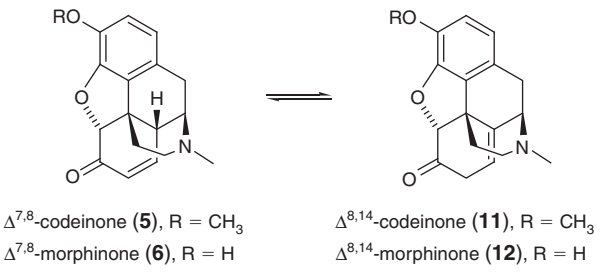
Results and Discussion

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 workup DMP oxidations, narwedine (**4**) can racemize. 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 racemization results from the inherent basicity of narwedine (**4**) itself. Given the facile racemization 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 crystallization involving dynamic kinetic resolution, as performed in the industrial synthesis of galanthamine.[18]

When codeine (**2**) was oxidized with freshly prepared DMP, codeinone (**5**)[19] was obtained as the product in 81 % yield. It was observed that when aged samples of DMP were used for the oxidation, a small portion of codeinone (**5**) was further oxidized 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 hydrolyzed by adventitious moisture.

Attempts to directly oxidize 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 *tert*-butyldimethylsilyl (TBS) ether at the phenolic position to afford compound **9**[21] in 30 % yield.



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

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

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 %.

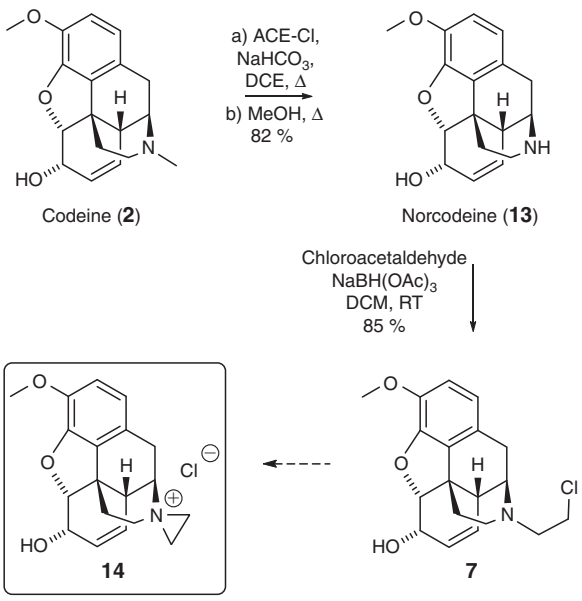
A minor impurity detected in samples of codeinone and morphinone resulted from deconjugation of the enone (Scheme 2). In aqueous solutions, an equilibrium is established between codeinone (**5**) or morphinone (**6**) and their deconjugated isomers **11** and **12**, which are unreactive towards thiol nucleophiles. The equilibrium between codeinone (**5**) and its deconjugated isomer **11** is well known,[22] whereas 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.

Synthesis of Chlorinated Analogues

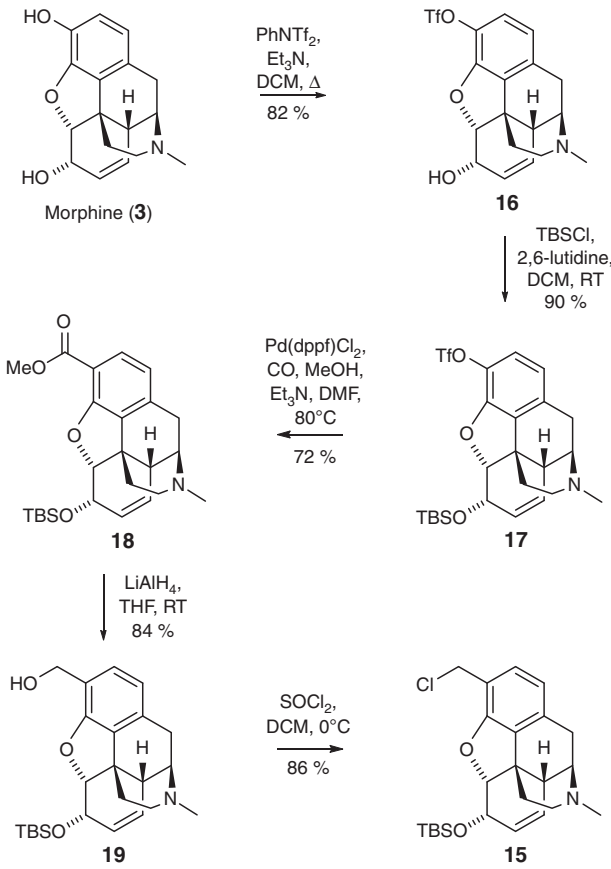
The codeine mustard **7** was prepared in two steps from codeine (**2**) as shown in Scheme 3. Codeine (**2**) was treated with α-chloroethyl chloroformate (ACE-Cl) to generate an intermediate carbamate that was hydrolyzed to norcodeine (**13**)[23] in methanol in 82 % yield over two steps. In the absence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

Synthesis of the codeine mustard **7** via reductive amination with chloroacetaldehyde was complicated by the ready formation of the reactive aziridinium ion **14** through intramolecular nucleophilic substitution. Reductive amination of norcodeine (**13**) with sodium cyanoborohydride as the reducing agent failed to generate the desired mustard **7**. Instead, a product with a mass spectrum consistent with that of the ethyl-bridged dimer was generated as the sole 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 (half-life ($t_{1/2}$) ≈ 2 h). Additionally, dissolving the compound in aqueous buffer resulted in rapid hydrolysis, generating the amino alcohol ($t_{1/2}$ ≈ 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

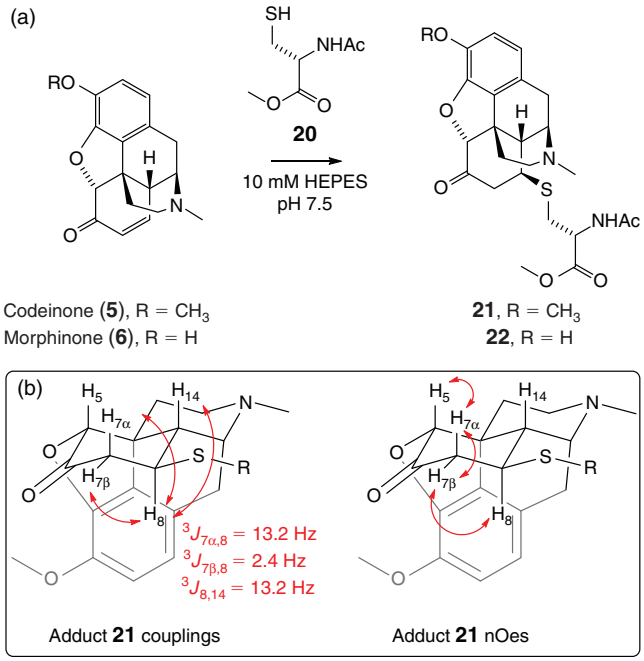


Scheme 3. Synthesis of codeine mustard **7**. DCE = 1,2-dichloroethane.



Scheme 4. Synthesis of the protected benzyl chloride **15** from morphine (**3**). $PhNTf_2$ = *N*-phenyltriflimide; $Pd(dppf)Cl_2$ = [1,1′-bis(diphenylphosphino)ferrocene]dichloropalladium.

prepared in five 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-catalyzed carbonylative coupling gave methyl ester **18** (72 %), which was reduced to



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

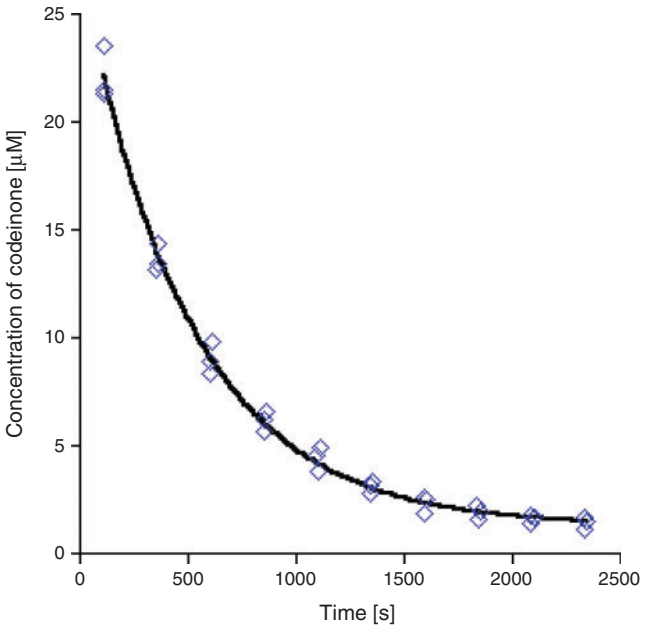


Fig. 3. Plot of the concentration of total codeinone (**5** + **11**) during reaction with *N*-acetyl-L-cysteine methyl ester (**20**) at a starting concentration of 25 μM (10 mM HEPES buffer, pH 7.5, 20°C).

give the benzyl alcohol **19** in 84 % yield. The protected benzyl chloride derivative **15** was obtained 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 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 dimerization, and dissolving in aqueous buffer led to the rapid formation of the benzyl alcohol ($t_{1/2} < 1$ 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.

Reaction Kinetics

In this work, the 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 the desired levels of reactivity and stability for reactive probes. In this manner, compounds that 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.

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, and the enone concentration was determined by liquid chromatography–mass spectrometry (LCMS). Due to the enone tautomerization 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. As the deconjugated isomers **11** and **12** do not react with thiols and the rate of tautomerization 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 Eqn 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:

$$\text{Total enone concentration} = [A]_0 e^{-k_{obs}t} + [B] \qquad (1)$$

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. Because the reaction was much slower under the buffered aqueous conditions and concentrations (25 μM) typically used during LCMS analysis of codeinone or morphinone, the reaction was monitored using ^{1}H NMR spectroscopy, 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 isomerize 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 Fig. 3. The corresponding plots for total morphinone (**6**) and narwedine (**4**) can be found in the Supplementary Material. The starting concentration of codeinone (**5**) was

Table 1. Experimentally determined parameters for the integrated rate equations

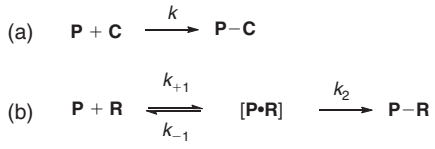
Enone	$[A]_0$ [M]	[B] [M]	k_{obs} [s^{-1}]	k [$M^{-1} s^{-1}$]
Codeinone[A]	$(26 \pm 1) \times 10^{-6}$	$(1.2 \pm 0.2) \times 10^{-6}$	$(2.0 \pm 0.1) \times 10^{-3}$	4.0 ± 0.2
Morphinone[A]	$(21 \pm 1) \times 10^{-6}$	$(1.3 \pm 0.2) \times 10^{-6}$	$(1.8 \pm 0.1) \times 10^{-3}$	3.6 ± 0.2
Narwedine[B]	$(8.8 \pm 0.2) \times 10^{-3}$	–[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.

25 μM, and the concentration of *N*-acetyl-L-cysteine methyl ester (**20**) was 500 μM. The experimentally determined pseudo-first order rate constant k_{obs} is $(2.0 \pm 0.1) \times 10^{-3}\ s^{-1}$ (Table 1). The corresponding second-order rate constant (k) is $4.0 \pm 0.2\ M^{-1}\ s^{-1}$. Morphinone provided similar results. By comparison, the second-order rate constant for the reaction of SCAM reagent 2-aminoethyl methanethiosulfonate with 2-mercaptoethanol is reported as $(7.6 \pm 0.4) \times 10^4\ M^{-1}\ s^{-1}$ 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 ($k_{obs} = (2.26 \pm 0.04) \times 10^{-4}\ s^{-1}$) corresponded to a significantly smaller k of $(1.13 \pm 0.02) \times 10^{-3}\ M^{-1}\ s^{-1}$. The second-order rate constants for codeinone (**5**) and morphinone (**6**) are three orders of magnitude larger. Based on the 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 under physiologically relevant conditions.[26]

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 constant 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 characterized 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 higher than expected based on measures of solution 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.

The stereochemistry of the adduct **21** was determined by NMR analysis of a pure sample obtained in 94 % yield from the



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**).

reaction of codeinone (**5**) with *N*-acetyl-L-cysteine methyl ester (**20**) in methanol. The observed 8*S*-stereochemistry (Scheme 5) was expected 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 nuclear Overhauser effect (nOe) interactions of protons in the cyclohexanone ring. The H_8 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 H_8 proton and the adjacent H_{14} and $H_{7\alpha}$ protons. The smaller coupling is consistent with an axial–equatorial coupling between the H_8 proton and the adjacent $H_{7\beta}$ proton. The observed nOe interactions were also consistent with the proposed structure.

The 8*S*-adduct **21** had been reported in the literature previously by 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 ^1H NMR and optical rotation to that prepared in methanol. A comparison of the ^1H and ^{13}C NMR data for the two reports is given in the Supplementary Material 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

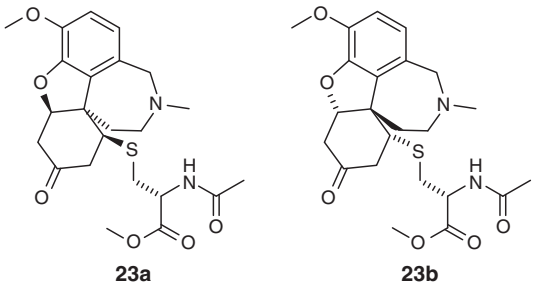


Fig. 4. Proposed structures of the two diastereomers formed when racemic narwedine (**4**) reacts with *N*-acetyl-L-cysteine methyl ester (**20**).

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** (Fig. 4), 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 Supplementary Material.

Conclusion

Thiol-reactive analogues of galanthamine (**1**), codeine (**2**), and morphine (**3**) were synthesized 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 constant k values of 4.0 ± 0.2 and $3.6 \pm 0.2\ M^{-1}\ s^{-1}$, respectively. Narwedine (**4**) reacted with a second-order rate constant k of $(1.13 \pm 0.02) \times 10^{-3}\ M^{-1}\ s^{-1}$. 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.

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, and 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 temperatures at 0°C. Removal of solvent under vacuum 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 PerkinElmer 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 per 100 mL and solvent. Infrared (IR) absorption spectra were obtained using a PerkinElmer Spectrum One Fourier transform infrared spectrometer. All compounds were analyzed as a thin film on NaCl plates. Key absorbance bands are reported in wavenumber (cm^{-1}). NMR spectra were obtained on a Bruker 400 (400 MHz) or a Bruker 800 (800 MHz) NMR spectrometer. Samples were analyzed 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 analysis. Chemical shifts (δ) are reported in ppm relative to TMS (δ = 0), and the splitting of the ^{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 (ax) or equatorial (eq) 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 correlation spectroscopy (COSY), nuclear Overhauser effect correlation spectroscopy (NOESY), heteronuclear multiple-bond correlation (HMBC), and heteronuclear single-quantum correlation (HSQC) NMR. Low-resolution mass spectrometry (LRMS) and high-resolution mass spectrometry (HRMS) were performed using positive electron ionization (EI) on a Micromass VG Autospec mass spectrometer or using positive electrospray ionization (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 equiv.) and *N*-acetyl-L-cysteine methyl ester (2 equiv.) were dissolved in methanol ($100\ \mu L\ mg^{-1}$ of enone), and the resulting solution was stirred at room temperature overnight. The following morning, the solvent was removed under vacuum 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), and after purification by flash chromatography (9 : 1 chloroform/methanol), the title compound **21** (30 mg, 94 %) was obtained as a white solid, mp 86–88°C. $[\alpha]_D^{20}$ +22 (*c* 1.0 in $CHCl_3$) (lit$[\alpha]_D^{20}$ −127 (*c* 0.5 in $CHCl_3$)[27]). ν_{max} (NaCl)/cm^{-1} 3287, 1731, 1667, 1277, 1259. δ_H ($CDCl_3$, 800 MHz) 6.70–6.71 (1H, m, H_2), 6.67–6.69 (1H, m, H_1), 6.32 (1H, d, br, *J* 6.8, $H_{3'}$), 4.81 (1H, m, $H_{2'}$), 4.69 (1H, s, H_5), 3.89 (3H, s, H_{3a}), 3.63 (1H, s, br, H_9), 3.46 (3H, s, $H_{1a'}$), 3.04 (1H, dd, *J* 14.0, 4.4, $H_{2a'}$), 3.01 (1H, d, *J* 18.8, $H_{10\alpha}$), 2.98 (1H, dd, *J* 14.0, 4.0, $H_{2a'}$), 2.70 (1H, dd, *J* 13.2, 2.4, $H_{7\beta}$), 2.57 (1H, d, br, *J* 11.6, H_{16eq}), 2.53 (1H, t, *J* 13.2, $H_{7\alpha}$), 2.44–2.49 (4H, m, H_{14}, H_{17a}), 2.34 (1H, dd, *J* 18.4, 5.2, $H_{10\beta}$), 2.30 (1H, td, *J* 12.8, 2.4, H_8), 2.20 (1H, td, *J* 12.0, 2.8, H_{16ax}), 2.06 (1H, td, *J* 12.0, 4.0, H_{15ax}), 1.97 (3H, s, $H_{3b'}$), 1.82 (1H, d, br, *J* 12.0, H_{15eq}). δ_C ($CDCl_3$, 100 MHz) 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 (15 %, $[M]^{+\bullet}$), 299 (35), 298 (100, $[M - C_6H_{10}NO_3S]^{+}$), 297 (30, $[M - C_6H_{11}NO_3S]^{+\bullet}$). HRMS *m/z* (EI) 474.1827; calcd for $C_{24}H_{30}N_2O_6S$ 474.1825.

The literature procedure[27] was applied to codeinone (**5**, 20 mg, 67 μmol) using *N*-acetyl-L-cysteine methyl ester

(2 equiv.) and sodium bicarbonate (6 equiv.) to give a yellow oil, $[\alpha]_D^{20}$ +31 (*c* 1.0 in $CHCl_3$), which was 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 in $CHCl_3$).

Narwedine-N-Acetyl-L-cysteine Methyl Ester Adducts

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

*Adduct **23a** (or **23b**)*

$[\alpha]_D^{20}$ +3 (*c* 0.4 in $CHCl_3$). ν_{max} (NaCl)/cm^{-1} 3289, 1744, 1721, 1675, 1286, 1204. δ_H ($CDCl_3$, 800 MHz) 6.68 (1H, d, *J* 8.0, H_2), 6.64 (1H, d, *J* 8.0, H_1), 6.29 (1H, d, br, *J* 7.6, $H_{3'}$), 4.86 (1H, ddd, *J* 7.6, 5.6, 4.0, $H_{2'}$), 4.68 (1H, t, *J* 2.8, H_{4a}), 4.11 (1H, d, *J* 14.8, $H_{12\beta}$), 3.83 (3H, s, H_{3a}), 3.78 (3H, s, $H_{1a'}$), 3.63 (1H, d, *J* 14.8, $H_{12\alpha}$), 3.58 (1H, s, br, H_8), 3.41 (1H, t, br, *J* 13.6, $H_{10\beta}$), 3.14 (1H, dd, *J* 13.6, 4.0, $H_{2a'}$), 3.10 (1H, d, br, *J* 14.4, $H_{10\alpha}$), 2.98–3.01 (1H, m, $H_{5\beta}$), 2.94–2.96 (1H, m, $H_{5\alpha}$), 2.88 (1H, dd, *J* 13.6, 5.6, $H_{2a'}$), 2.58 (1H, dd, *J* 16.8, 3.6, $H_{7\beta}$), 2.50 (1H, dd, *J* 16.8, 2.8, $H_{7\alpha}$), 2.32 (3H, s, H_{11a}), 2.09 (1H, t, br, *J* 14.0, $H_{9\alpha}$), 2.07 (3H, s, $H_{3b'}$), 1.96 (1H, dd, *J* 14.0, 3.2, $H_{9\beta}$). δ_C ($CDCl_3$, 100 MHz) 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 (<1 %, $[M]^{+\bullet}$), 286 (35), 285 (100, $[M - C_6H_{11}NO_3S]^{+\bullet}$), 242 (40), 216 (25), 199 (25), 174 (45), 118 (20), 88 (45), 76 (50). *m/z* (HRMS ESI) 463.1904; $[M + H]^+$ requires 463.1903.

*Adduct **23b** (or **23a**)*

$[\alpha]_D^{20}$ +50 (*c* 0.4 in $CHCl_3$). ν_{max} (NaCl)/cm^{-1} 3271, 1720, 1659, 1286, 1204. δ_H ($CDCl_3$, 800 MHz) 6.69 (1H, d, *J* 8.0, H_2), 6.64 (1H, d, *J* 8.0, H_1), 6.19 (1H, d, *J* 7.6, $H_{3'}$), 4.78 (1H, td, *J* 7.6, 4.0, $H_{2'}$), 4.69 (1H, t, *J* 2.8, H_{4a}), 4.19 (1H, d, *J* 14.8, $H_{12\beta}$), 3.84 (3H, s, H_{3a}), 3.77 (3H, s, $H_{1a'}$), 3.67 (1H, s, br, H_8), 3.63 (1H, d, *J* 14.8, $H_{12\alpha}$), 3.39 (1H, t, br, *J* 13.2, $H_{10\beta}$), 3.15 (1H, dd, *J* 13.6, 4.0, $H_{2a'}$), 3.09 (1H, d, br, *J* 14.4, $H_{10\alpha}$), 3.03 (1H, dd, *J* 18.4, 2.8, $H_{5\alpha}$), 2.95 (1H, dd, *J* 18.4, 2.8, $H_{5\beta}$), 2.78 (1H, dd, *J* 13.6, 7.6, $H_{2a'}$), 2.61 (1H, dd, *J* 17.2, 3.6, $H_{7\beta}$), 2.52 (1H, dd, *J* 17.2, 2.8, $H_{7\alpha}$), 2.34 (3H, s, H_{11a}), 2.08–2.12 (4H, m, $H_{9\alpha}$, $H_{3b'}$), 1.98 (1H, dd, *J* 14.0, 3.2, $H_{9\beta}$). δ_C ($CDCl_3$, 100 MHz) 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 (<1 %, $[M]^{+\bullet}$), 286 (35), 285 (100, $[M - C_6H_{11}NO_3S]^{+\bullet}$), 242 (40), 216 (25), 199 (25), 174 (45), 118 (20), 88 (45), 76 (50). *m/z* (HRMS ESI) 463.1900; $[M + H]^+$ requires 463.1903.

Investigation of Reaction Kinetics by LCMS

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 ionization 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 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) buffer solution adjusted to pH 7.5 at 20°C. A solution of the enone (1 equiv.) was mixed with a solution of *N*-acetyl-L-cysteine methyl ester (20 equiv.), and the composition of the mixture was analyzed by LCMS 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 of the plot of enone concentration against time using *KaleidaGraph*.

Investigation of Reaction Kinetics by ^1H NMR

Reactions were carried out in deuterated methanol at 25°C. A solution of the enone (1 equiv.) was mixed with a solution of *N*-acetyl-L-cysteine methyl ester (20 equiv.), and the composition of the mixture was determined by ^1H NMR analysis at regular intervals. The concentration of the enone at each interval was determined by comparing the relative integration of the H7 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 of the plot of enone concentration against time using *KaleidaGraph*.

Supplementary Material

Experimental procedures for compounds **4**–**7**, **9**, **10**, **13**, **15**–**19**, ^1H NMR and ^{13}C NMR spectra for all compounds, and low-resolution mass spectra for new compounds are available on the Journal's website.

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