Journal Name

COMMUNICATION

Small Molecule Recognition of Mephedrone using An Anthracene Molecular Clip

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

K. Kellett,[a] J. H. Broome[b], M. Zloh[a], S. B. Kirton[a], S. Fergus[a], U. Gerhard[a], J. L. Stair[a]* and K. J. Wallace*[b]

An anthracene molecular probe has been synthesised and shown to target mephedrone, a stimulant drug from the cathinone class of new psychoactive substances (NPS). A protocol has been developed to detect mephedrone via the probe using NMR spectroscopy in a simulated street sample containing two of the most common cutting agents, benzocaine and caffeine.

There is continuing interest in utilising host-guest recognition in different disciplines, for example, biological transportation within cells[1], the materials world,[2] and forensic science, which is now employing host-guest chemistry in portable devices for rapid and onsite identification of illicit substances.[3] At present, there is a lack of rapid screening approaches for new drugs of abuse, a necessity for both law enforcement and healthcare workers. Although a number of approaches have been investigated[4] there remains a need to improve selectivity over chemical analogues and common cutting agents.

New psychoactive substances (NPS), also referred to as designer drugs or 'legal highs', are newly available substances not controlled by the United Nations drug conventions but may cause serious negative health effects. Cathinones, such as mephedrone and flephedrone (Fig. 1A), are stimulants and one of the most abused class of NPS.[5] Due to their lipophilic nature, they can easily pass through the blood brain barrier; thereby, stimulating the central nervous system by releasing dopamine and inhibiting the re-uptake of epinephrine, norepinephrine and serotonin.[6] Mephedrone is of particular concern due to its negative health implications and continued popularity despite efforts to control the substance.[5]

A major challenge in the field of drug detection is the preferential recognition of a specific NPS over related analogues possessing similar organic frameworks. For example, flephedrone differs from mephedrone by only one 4-fluoro motif, while methamphetamine differs by a carbonyl and tolyl moiety (Fig. 1A). These small structural differences have potential to significantly impact binding to a host molecule. The work presented in this study demonstrates that the concerted effort of multiple interaction sites between an anthracene molecular 'clip' and the NPS mephedrone results in a molecular probe that preferentially targets mephedrone over related substances.

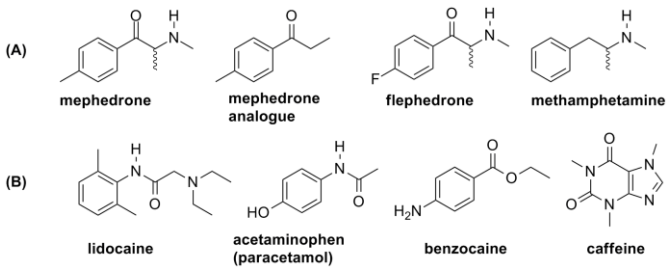


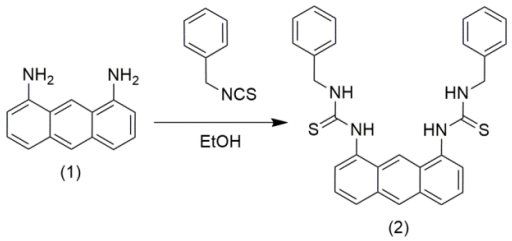
Fig. 1. Structures of mephedrone, and related chemical analogues (A) and cutting agents (B).

There is a plethora of molecular probes utilising the concept of host-guest chemistry[7] for the detection of cation,[8] anion,[9] neutral[10] and simultaneous cation-anion[11] species. At present, there are no studies investigating small molecule recognition for mephedrone or any NPS for that matter. It is known that amphetamines can interact with proteins *via* non-covalent interactions, in particular, π-π stacking and hydrogen bonding interactions.[12] Examination of the Brookhaven Protein DataBank[13] was carried out to identify high-quality protein-ligand complexes between receptors, and drugs of abuse/common adulterants/endogenous psychoactive substances (i.e. dopamine and serotonin) similar to mephedrone. These were used to develop a consensus pharmacophore of mephedrone-receptor binding to support host molecule selection (ESI Fig. S1 and S2). With this in mind, a molecular probe was designed that utilised these interactions to bind mephedrone. The rigid anthracene scaffold was chosen to enable π-π stacking with aromatic

[a] *Department of Pharmacy, Pharmacology and Postgraduate Medicine, School of Life and Medical Sciences, University of Hertfordshire, Al10 9AB UK. Email: J.Stair@herts.ac.uk, Fax: +44(0)1707284506 Tel:+44(0)1707283367*
[b] *Department of Chemistry and Biochemistry, 118 College Drive, Hattiesburg, MS 39406, USA. E-mail: karl.wallace@usm.edu; Fax: 601 266 6075; Tel: 601 266 4715*
† Footnotes relating to the title and/or authors should appear here.
Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

Please do not adjust margins

functionalities on the mephedrone as well as to provide an organic backbone and incorporate hydrogen-bonding motifs (thiourea moieties),[14] interactions demonstrated as being important by the pharmacophore analysis (see ESI). Anthracene analogues have a planar structure complemented by a conjugated π network, making them excellent fluorophores, due to their photoluminescence properties.[9] These unique photophysical and structural properties allow the anthracene moiety to double as a signalling unit, while being an integral part of the chemosensor's rigid scaffold. The guest is anticipated to bind within the cleft formed by the anthracene and the thiourea arms, which are functionalized in the 1 and 8 position of the anthracene unit. Thus, compound **2** was prepared by reacting 1,8-diaminoanthracene with two equivalents of benzylisothiocyanate in ethanol (see ESI). The solid was filtered, dried and washed with ethanol to afford the desired molecular probe **2** in 40% yield (scheme 1).



Scheme 1. Synthesis of molecular probe **2**.

In order to scrutinize the binding affinity of probe **2** towards mephedrone, ^{1}H-NMR spectroscopy was used extensively to determine which hydrogen atom environment on the molecular probe was perturbed upon the addition of the drug. As it is well known that thioureas are excellent binding motifs for anions, the free amine form of mephedrone was utilized to exclude any interaction between the counter-anion (Cl^-) and the host.[15] Once this was achieved aliquots of mephedrone in acetone were added to a 0.02 mol·dm^{-3} solution of probe **2** in acetone-d_6. When adding mephedrone, the two NH protons on the thiourea group observed at 9.87 and 8.29 ppm were significantly shifted down-field (ESI Table S2 and Fig. 2). These chemical shift changes can be rationalised by close contact of a single hydrogen-bonding interaction between the carbonyl group on the mephedrone molecule and the hydrogen atom on one of the NH groups of the thiourea. Additionally, the C(9)H and the C(10)H, 8.78 and 8.67 ppm respectively, of the molecular probe's scaffold also showed chemical shift changes. This suggests these hydrogen atoms are also influenced by the drug being bound in close proximity. Neutral molecule detection can be difficult with molecular 'clips' as there is often a high degree of flexibility where a multitude of non-covalent interactions are required to work in a concerted fashion to overcome any entropic considerations. However, an advantage of neutral guest recognition is that the guest has an organic framework, whereby chemical shift changes of the guest can also be used to aid understanding of the close contacts. In addition to chemical shift changes seen on the host, there were also noteworthy changes seen for mephedrone from the methine centre, two methyl groups, and tolyl methyl moiety (ESI Fig. S5). The methine and two methyl groups are in close proximity to the β-carbonyl and amine functionalities, which undergo hydrogen bonding with the host. This causes a decrease in electron density around these groups on mephedrone, which results in downfield ^{1}H-NMR shifts. Conversely, the tolyl methyl experiences an up-field shift. Interestingly, the sigmoidal behaviour seen in the binding isotherm (Fig. 2) suggests that cooperativity is occurring. Whereby, the three non-covalent interactions (2 × CH···π and NH···N) with compound **2**, facilitate the NH···OC binding event in a cooperative manner. This is reasonable as these interactions are missing in the model compound, whereby no chemical shifts are seen (Fig 2, entries E and F)

The choice of analogous guests was important to this study in order to establish selectivity between related chemical analogues (Fig. 1A) and typical adulterants (Fig. 1B) found in 'street' samples.[16] Each compound from Fig. 1 was added to a 0.02 mol·dm^{-3} acetone-d_6 solution of probe **2** (see ESI). The mephedrone analogue (1-(*p*-tolyl)propan-1-one) and methamphetamine were evaluated in order to systematically isolate interaction from the carbonyl and amine functional groups on the guest. The addition of these two substances showed only subtle shift changes (ESI Fig. S6 and Fig. S7 respectively), while flephedrone showed interaction but still to a lesser degree than mephedrone (Fig. 2 and ESI Fig. S8). This suggests that the host interacts preferentially with cathinone-like structures (i.e. requiring both a carbonyl and amine on the guest). The adulterants tested (lidocaine, acetaminophen, benzocaine and caffeine) resulted in no proton shift changes on the host further supporting the need for a β-ketoamine arrangement to induce interactions (ESI Fig. S11-S14). A plot of the N(1)H signal versus concentration of mephedrone and analogues for titration experiments against probe **2** shows that mephedrone gave the largest chemical shift change, ≈ 2 ppm, when compared to the other compounds (Fig. 2 bottom). Upon closer inspection of the NMR spectra, the addition of mephedrone, flephedrone, or methamphetamine gave rise to new ^{1}H-NMR signals. We believe a pericyclic cycloaddition reaction is occurring in the C(9)H and C(10)H position on the anthracene ring, commonly seen in other anthracene systems.[17] As a consequence of the 4π+4π cycloaddition it is difficult to obtain reasonable *K* values by least-square regression.

To investigate whether two thiourea pendant side arms are necessary in order to establish the probe-drug interaction, model compound **3** (1-benzyl-3-phenylthiourea) was also synthesised and isolated (see ESI). The same set of ^{1}H-NMR titration experiments were carried out with the two cathinones. There were minimal chemical shift changes observed from compound **3** in the presence of mephedrone or flephedrone (ESI Fig. S15 and S17). Therefore, it is reasonable to assume that both thiourea functional groups are required for binding the drug in a concerted fashion and highlighting the importance of the chelate effect with probe **2** (see DFT discussion). As there is no anthracene moiety, a pericyclic

cycloaddition was not observed supporting the claim that a cycloaddition reaction is occurring with probe **2**.

It is known that solvent molecules often compete with the analyte. Therefore, mass spectrometry studies were carried out to support the formation of the host-guest complex, as solvent molecules are not normally a factor in gas phase MS. Samples were prepared in HPLC grade acetone, in which 10 equivalents of the free-amine drug were added. The mass spectra of probe **2** was analysed in the presence of mephedrone and flephedrone, giving ESI-MS (+'ve) m/z = 685 [(**2**)·mephedrone+H]$^+$, and m/z = 689 [(**2**)·flephedrone+H]$^+$ (ESI Fig. S19 and S20). To confirm that the probe **2**-drug mass signal was not an artefact, deuterated water was added to the sample to show an increase in the mass due to the exchange of the labile protons with deuterium. It is reasonable to assume that the aromatic π systems certainly aids binding in the gas phase, as there is no competing solvent molecules. This is also supported by MS-MS experiments, whereby the **2**-mephedrone host-guest complex fragments into the free-drug and probe **2** upon dissociation to form two distinct signals at m/z 179 and 507 respectively.

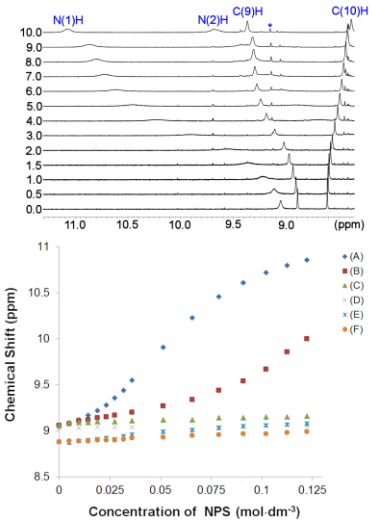


Fig. 2. (Top) Partial ^{1}H-NMR spectra showing titration of probe **2** with the addition of mephedrone; (*) is suspected cycloaddition product. (Bottom) Chemical shift changes observed for the NH(1) signal, upon the addition of (A) mephedrone, (B) flephedrone, (C) methamphetamine, (D) 1-(*p*-tolyl)propan-1-one (mephedrone analogue), (E) and (F) compound **3** upon the addition of mephedrone and flephedrone, respectively.

Molecular modelling calculations were carried out to rationalize the change of NMR chemical shifts observed in the solution phase. The minimum energy conformations were generated for probe **2** alone, mephedrone and flephedrone, and relevant host-guest complexes using conformational searching implemented in Hyperchem 8.10 and OPLS force field (ESI Fig. S21 to S25). Selected conformations were optimized using density functional theory (DFT) calculations (B3LYP 6-311++G(2d,2p) in Orca). Probe **2** had two low energy conformations in the gas phase, which were taken forward for analysis of the bound complexes using DFT calculations. The proposed geometry of the host-drug interaction for probe **2** is supported by the DFT calculations (Fig. 3). This optimised structure, which shows mephedrone bound within the cleft of probe **2** *via* an array of hydrogen bonding interactions[18] and a π-π interaction,[19] further supports the observed NMR chemical shift changes. Another interesting feature is the *trans-cis* rotamer of the thiourea group, typically seen in solution, observed in the solid state and supported by theoretical calculations, which suggests that *trans-cis* is the preferred rotamer, unlike the analogous urea group which is rarely seen in the *trans-cis* fashion.[20]

The interaction energies were calculated for probe **2** with both mephedrone and flephedrone based on the minimum energy conformation of the complexes (see ESI). Mephedrone positioned within the binding pocket of probe **2** suggested the formation of two hydrogen bonds with a favourable interaction energy of -2.88 kcal·mol^{-1} (Fig. 3). Flephedrone, a closely related mephedrone analogue, was found to bind to probe **2** outside of the binding pocket, leading to just one hydrogen bond forming and an interaction energy of -7.82 kcal·mol^{-1}. Interaction energies indicated that there is a clear preference for the lowest energy conformation for each complex. To ensure that the minimum conformation of both drugs was achieved, the cathinones were studied in their respective binding positions, i.e. mephedrone was positioned to bind to probe **2** outside of the binding pocket and *vice versa*. The interaction energy of mephedrone bound outside of the pocket was 14.67 kcal·mol^{-1}, while flephedrone bound inside of the pocket had an interaction energy of -2.06 kcal·mol^{-1}. This confirms that the lowest energy conformations of each cathinone are truly indicative of the way they bind to probe **2** and are in good agreement with the experimentally observed data.

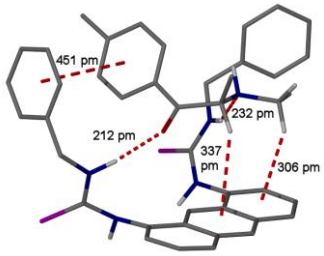


Fig. 3. DFT fully optimized structure of probe **2** complexed with mephedrone highlighting the array of intermolecular interactions (π-π, CH···π, NH···N and NH···OC).

In order to see how the chemosensor detected cathinones at low concentrations, the molecular probe's photophysical properties using fluorescence spectroscopy were investigated. A 5 × 10^{-6} mol·dm^{-3} acetone solution of probe **2** was prepared and excited at 410 nm. The fluorescence spectrum of probe **2** showed a featureless band at 485 nm. Addition of neat mephedrone or flephedrone (freebase) produced significant changes to the fluorescence emission of probe **2** (Fig. 4; see ESI Fig. S27 for mephedrone). Aliquots of the drug were added to the acetone solution of probe **2**. Addition of the first aliquot produced an increase in fluorescence intensity, which was a much larger increase for flephedrone than mephedrone. This is supported by the DFT calculations of the complexes; flephedrone forms a more stable π

stacking arrangement with the anthracene moiety, due to the electron withdrawing nature of the fluorine group. This generates an initial fluorescence emission increase resulting from the exciplex (Fig. 4-insert). DFT studies indicate mephedrone prefers to bind inside the pocket and interact with the benzyl π systems, rather than the anthracene moiety. Additional aliquots resulted in sequential quenching of the system.

Even though there were only modest optical changes upon the binding between probe **2** and mephedrone, the ^{1}H-NMR spectra showed significant chemical shift changes with the drug and no changes with caffeine and benzocaine, two common cutting agents found in mephedrone street samples. Therefore, we used NMR spectroscopy to evaluate if probe **2** could be used to detect mephedrone in a simulated 'street' sample containing these two compounds. Therefore, a protocol was developed to produce the freebase of mephedrone in the presence of benzocaine and caffeine. Mephedrone hydrochloride, benzocaine and caffeine were combined in equal proportions and dissolved in water. The mixture was then filtered, as caffeine is sparingly soluble in water compared to benzocaine and mephedrone hydrochloride. The mephedrone freebase was then liberated with ammonium hydroxide (pH = 10) and extracted into diethyl ether. The NMR of this solution showed the presence of all compounds; however, reduced caffeine and benzocaine signals were seen relative to mephedrone, which was an advantageous consequence of the protocol. NMR titration of probe **2** against this extracted street sample mixture confirmed that mephedrone, indeed, still preferentially binds in the presence of caffeine and benzocaine (ESI Fig. S28 to S33).

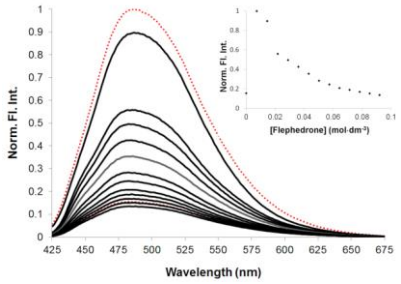


Fig. 4. Normalized fluorescent titration of probe **2** with neat flephedrone (red lines indicate probe 2 only and the first aliquot) Insert: Plot of flephedrone concentration and the quenching of fluorescence intensity at 485 nm (acetone, 5×10^{-6} mol·dm^{-3}, λex = 410 nm).

In summary, an anthracene molecular 'clip' displayed greater interaction with mephedrone *vs* methamphetamine and other related analogues via ^{1}H-NMR, suggesting a preference for a β-ketoamine arrangement. Interestingly, DFT calculations confirmed the NMR and fluorescence experimental results suggesting different binding geometries for mephedrone *vs* flephedrone. Addition of common cutting agents did not affect interaction between mephedrone and probe **2**, which is promising for use with 'street' samples. The development of an in-field chemosensor is a continuing endeavour; however, significant knowledge about the structural components necessary to selectively bind mephedrone has been gained.

Synthesis and characterization protocols are highlighted in the supporting information. Financial support for this work was provided by the NSF grant OCE-0963064, NSF GK-12 #0947944 for J. H. Broome's graduate studentship, the Winston Churchill Foundation and Santander Research Grant for K. Kellett's 12 week travel stipend to the Wallace lab, and the University of Hertfordshire for K. Kellett's graduate studentship. This paper was supported in part by grants of the European Commission (Drug Prevention and Information Programme 2014-16, contract no. JUST/2013/DPIP/AG/4823, EU-MADNESS project).

Notes and references

(1) Gale, P. A. *Acc. Chem. Rev.* **2011**, *44*, 216.
(2) Zhou, X.; Han, S.; Zhang, Q.; Dou, Y.; Guo, J.; Che, L.; Li, X.; Zhang, J. *Polym. Chem.* **2015**, *6*, 3716.
(3) Izake, E. L. *Forensic Sci. Int.* **2010**, *202*, 1.
(4) (a) Assi, S.; Guirguis, A.; Halseu, S.; Fergus, S.; Stair, J. L. *Anal. Methods*, 736(b) Smith, J.; Metters, J.; Irving, C.; Sutcliffe, O. B.; Banks, C. E. *Analyst* **2014**, 389(c) Elie, L.; Baron, M.; Croxton, R.; Elie, M. *Forensic Sci. Int.* **2012**.
(5) European Monitoring Centre for Drugs and Drug Addiction and Europol, *EU Drug Markets Report In-depth Analysis,* Luxembourg: Publications Office of the European Union, 2016
(6) Kelly, J. P. *Drug Test Anal.* **2011**, *3*, 439.
(7) (a) Steed, J. W.; Turner, D. R.; Wallace, K. *Core concepts in supramolecular chemistry and nanochemistry*; John Wiley & Sons: Chitester, 2007(b) Bamfield, P.; Hutchings, M. G. *Chromic phenomena: technological applications of colour chemistry*; Royal Society of Chemistry: Cambridge, 2010.
(8) (a) Mallet, A.; Davis, A.; Davis, D.; Panella, J.; Wallace, K.; Bonizzoni, M. *Chem. Commun.* **2015**, *51*, 16948(b) Manandhar, E.; Cragg, P. J.; Wallace, K. J. *Supramol. Chem.* **2014**, *26*, 141(c) Orcutt, K. M.; Jones, W. S.; McDonald, A.; Schrock, D.; Wallace, K. J. *Sensors* **2010**, *10*, 1326(d) Manandhar, E.; Wallace, K. J. *Inorg. Chimi. Acta* **2012**, *381*, 15.
(9) (a) Davis, A. B.; Lambert, R. E.; Fronczek, F. R.; Cragg, P. J.; Wallace, K. J. *New J. Chem.* **2014**, *38*, 4678(b) Wallace, K. J.; Belcher, W. J.; Turner, D. R.; Syed, K. F.; Steed, J. W. *J. Am. Chem. Soc.* **2003**, *125*, 9699.
(10) Walton, I.; Davis, M.; Munro, L.; Catalano, V. J.; Cragg, P. J.; Huggins, M. T.; Wallace, K. J. *Org. Lett.* **2012**, *14*, 2686.
(11) Manandhar, E.; Broome, J. H.; Myrick, J.; Lagrone, W.; Cragg, P. J.; Wallace, K. J. *Chem. Commun.* **2011**, *47*, 8796.
(12) Gibbons, S.; Zloh, M. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 4135.
(13) Berman, H. M.; Westbrook, J.; Feng, Z.; Gilliland, G.; Bhat, T. N.; Weissig, H.; Shindyalov, I. N.; Bourne, P. E. *Nucl. Acids Res.* **2000**, *28*, 235.
(14) Hunter, C. A.; Sanders, J. K. *J. Am. Chem. Soc.* **1990**, *112*, 5525.
(15) Khansari, M. E.; Wallace, K. D.; Hossain, M. A. *Tetrahedron Lett.* **2014**, *55*, 438.
(16) (a) Brandt, S. D.; Sumnall, H. R.; Measham, F.; Coled, J. *Drug Test Anal.* **2010**, *2*, 377(b) Smith, J. P.; Metters, J. P.; Khreit, O. I. G.; Sutcliffe, O. B.; Banks, C. E. *Anal. Chem.* **2016**, *86*, 9985.
(17) (a) Wei, P.; Yan, X.; Huang, F. *Chem. Commun.* **2014**, *50*, 14105(b) Xu, J.-F.; Chen, Y.-Z.; Wu, L.-Z.; Tung, C.-H.; Yang, Q.-Z. *Org. Lett.* **2013**, *15*, 6148(c) Yang, N.-C. C.; Masnovi, J.; Chuag, W.-L.; Wang, T.; Shou, H.; Yang, D.-D. H. *Tetrahedron* **1981**, *37*, 3285.
(18) Umezawa, Y.; Tsuboyama, S.; Honda, K.; Uzawa, J.; Nishio, M. *Bull. Chem. Soc. Jpn* **1998**, *71*, 1207.
(19) Sinnokrot, M. O.; Valeev, E. F.; Sherrill, C. D. *J. Am. Chem. Soc.* **2002**, *124*, 10887.
(20) Custelcean, R. *Chem. Commun.* **2008**, 295.