A TANGLED WEB: EFFECTS OF CATHINONE-DERIVED NEW PSYCHOACTIVE SUBSTANCES (NPS') ON THE ORB-WEAVING BEHAVIOUR OF *ARANEUS DIADEMATUS* AND THE HUNTING ABILITIES OF *PARDOSA AGRESTIS*

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Acknowledgements

Contents

Abstract

New Psychoactive Substance (NPS) use has been increasing in popularity ever since mephedrone was reintroduced onto the drugs market in 2007. Legislation in the form of the Psychoactive Substances Act (PSA; 2016) in the UK aimed to outlaw NPS' and whilst they are now illegal under this Act, the nature of the market means that they are still constantly evolving. This project discusses the synthesis and characterisation of the mephedrone analog and synthetic cathinone (±)-4'-(trifluoromethyl)methylcathinone (4-TFMMC) as well as three of its novel metabolites as racemic mixtures. NPS development takes place rapidly so robust reference standards were created pre-emptively to aid analytical bodies with the identification of these compounds before their anticipated debut onto the black market. Both mephedrone and 4-TFMMC were administered to *Araneus diadematus* to identify whether either of the compounds would have a significant effect on webbuilding, as the spider system is one that is well established. Though web-building is only governed by a few simple rules, it is a complex and intricate behavioural mechanism that can be easily disrupted. Mephedrone was also administered to *Pardosa agrestis* to assess whether the compound would affect hunting behaviour. Both spiders catch prey in drastically different ways and so the author wanted to evaluate the plasticity of *P. agrestis* and whether this system could be considered comparable to *A. diadematus.* It was found that 4-TFMMC did not significantly affect the webs that *A. diadematus* spun. Mephedrone seemed to affect the faculties of both spider systems in a similar way; *A. diadematus* spun webs with smaller free zone and capture spiral areas and *P. agrestis* exhibited diminished movement of the capture legs whilst capturing prey items. This study was not designed to establish the potential effects of the compounds on the spiders' central nervous systems (CNS), though this is a route for future work to facilitate a more comprehensive understanding of how a spider system may be affected by synthetic cathinones.

Chapter 1

Introduction

Mephedrone and New Psychoactive Substance development

(±)-4'-(Methyl)methcathinone, also known as mephedrone, is a synthetic cathinone that became a popular drug of abuse from 2007-onwards, per its reintroduction to the drugs market (Deluca *et al.*, 2012). Mephedrone was arguably the first 'legal high' and was sold under non-descript pseudonyms marked with 'plant food' or 'not for human consumption' (Vardakou, Pistos & Spiliopoulou, 2011). Despite the PSA (UK Government, 2016) outlawing all present and future NPS' the market is still evolving (UNODC, 2013), as such it is the duty of analytical bodies to try and anticipate these up-and-coming drugs of abuse. Mephedrone was fully characterised by Santali *et al.* (2011); Khreit *et al.* (2013) built on this as part of their research, synthesising (±)-4'(trifluoromethyl)methcathinone (4-TFMMC) based on mephedrone's metabolic pathways. They found that the structural alterations many NPS' like mephedrone undergo may only be slight but can drastically alter the metabolism of the compounds. Data is almost always lacking for these novel compounds and so it is crucial that we have a comprehensive understanding and complete characterisations before they emerge onto the market (Hadlock *et al.,* 2011). In this way identification of a substance can be expedited and *thus* any potential damage to a drug user and other exposed individuals minimised.

Spiders as behavioural drug-testing systems

Using spiders as a drug testing system, whilst unconventional, is by no means a novel idea. One group of compounds with a wealth of historical testing in a spider system is amphetamines (Peters & Witt, 1949; Witt & Reed, 1965; Witt, 1971; Reed, Witt & Scarboro, 1982), they are structurally similar and have broadly similar effects as cathinones *in vivo* (Ellefsen, Concheiro & Huestis, 2016). Peters and Witt (1949) were the first to publish work concerning the administration of Pervitin (methamphetamine) to orb-weavers to try and alter their circadian rhythms, though

they quickly realised that their rhythms remained unchanged and that the spiders producing highly disjointed webs as a side-effect instead. Witt proceeded after this initial study to administer other substances, including other amphetamines, to spiders to see how their webs were affected, comparing and contrasting the effects between classes and individual compounds (Witt & Reed, 1965; Witt, 1971; Reed, Witt & Scarboro, 1982). A paper published by NASA (Noever, Cronise & Relwani, 1995) described administering different classes of substances to spiders, including amphetamines, and using computer software to evaluate the quality of webs spun. They concluded that the more toxic a chemical was, the more disjointed the web would be; though this was an arbitrary and very basic assessment of the effects of each compound only going off the number of cells within a web that a spider had completed. In more modern publications, specific measurements were taken from photographs of webs to give a more accurate overall assessment of a web's characteristics (Hesselberg & Vollrath, 2004; Zschokke & Herberstein, 2005; Wyman, Rodenhouse & Bank, 2011; Zschokke, 2011) using formulae developed to precisely calculate these desired web metrics (Herberstein & Tso, 2000). Spider systems seem to be more frequently used to evaluate the impacts of pesticides on wildlife as they are key predators vital for pest control (Shaw, Waddicor & Langan, 2005; Hanna & Hanna, 2013; Benamú *et al.,* 2013; Pasquet *et al.*, 2016) but the versatility of the research means that a lot of the methodology can be applied to testing other substances. Though a spider system cannot be compared to humans or rodents, administering drugs to spiders reveals a wealth of information on the function of their complex behavioural patterns (Witt, 1971).

Thesis Structure

The two main data chapters (Chapters 2-3) of this thesis are sequential and individual elements, written in the style of manuscripts for scientific journals and adhering to subject specific formatting conventions where possible. This layout has been chosen with the knowledge that it may seem unconventional but due to the cross-

disciplinary nature of the study, the author feels it is the most suitable way to present the research whilst maintaining continuity. Chapter 2 is an extended discussion of the synthesis and characterisation of an analog of mephedrone and three of its novel metabolites. Therein several presumptive tests used by analytical bodies are also evaluated within the scope of the work. Chapter 3 focuses on the administration of mephedrone and 4-TFMMC to *A. diadematus*. The effects of the drugs on the spiders' webs are discussed. Mephedrone was also administered to *P. agrestis* to compare the effects the drug had on their behaviour to *A. diadematus* to determine whether a hunting spider system (*P. agrestis*) is plastic enough for use in behavioural drug testing. Chapter 4 summarises the findings and implications of the thesis, suggesting avenues for further study.

Chapter 2

The Complete Synthesis and Characterisation of Three Metabolites of the Synthetic Cathinone (±)-4'-(Trifluoromethyl)methylcathinone (4-TFMMC)

Abstract – New Psychoactive Substances (NPS') have been popular drugs of abuse ever since mephedrone (**1b**) surfaced onto the drugs market in 2007. Its widespread use highlighted the loopholes within the Misuse of Drugs Act (MDA, 1971) in the UK, prompting new legislation in the form of the Psychoactive Substances Act (PSA, 2016). (±)-4'-(trifluoromethyl)methcathinone (4-TFMMC, **2**) is an analog of mephedrone (**1b**) synthesised ahead of its expected black market debut. 4-TFMMC (**2**) has been designed specifically to restrict one of mephedrone's key metabolic pathways (oxidation of the aromatic methyl moiety) and, theoretically, increase its half-life *in vivo*. Three novel metabolites were synthesised according to the metabolism of mephedrone (**1b**) and thus the assumed metabolism of 4-TFMMC (**2**), following the two unrestricted metabolic pathways remaining (*N*-demethylation, reduction). This study provides robust reference standards and comprehensive characterisation for 4-TFMMC (**2**) and the three novel synthetic metabolites. The possibility for the use of presumptive testing to aid in the detection of these compounds by analytical bodies is also discussed.

Introduction

Substance abuse is a pervasive societal problem, one that weighs heavily on our health systems and even more so on the users themselves (Dargan, Albert & Wood, 2010; James *et al.*, 2010). Whilst substance abuse isn't anything new, what is are the novel substances crafted to target a drug-using market of, often vulnerable, individuals (Every-Palmer, 2010; Giese *et al.*, 2015; Wastnedge, 2014). NPS' are based on pre-existing drugs of abuse manufactured originally to evade legislation, often existing as 'enhanced' and considerably more dangerous versions of their parent

compounds (Ellefsen, Concheiro & Huestis, 2016). Supply and manufacture were officially outlawed in May 2016 as part of a blanket-ban in the UK per the introduction of the PSA which defines a psychoactive substance as something which "by stimulating or depressing the person's central nervous system affects the person's mental functioning or emotional state" (UK Government, 2016). The Act was introduced as a way to combat the loopholes and oversights of the MDA (UK Government, 1971) which outlawed compounds based on specific structures and classes. The MDA separated these compounds into three classes: A, B and C, as well as into two separate Schedules; these relate to sentencing severity and societal impact (Class) and their potential for therapeutic use (Schedule; The Misuse of Drugs Regulations 2001). A critique of the Act is that substances such as alcohol and tobacco were not included in the Act despite their known detrimental effects, a move that was described as "from a scientific perspective, arbitrary…." (Nutt *et al.*, 2007). In several cases, a drug's class was decided from a purely political perspective; cannabis is one such substance that has been reclassified twice (The Misuse of Drugs Act 1971 (Modification) (No. 2) Order 2003; The Misuse of Drugs Act 1971 (Amendment) Order 2008) as a result of shifting of opinion in British Parliament and of the serving Home Secretary, who retains the authority to upgrade, downgrade, or add substances based on recommendations from an advisory council. An important note is that NPS development takes place rapidly, which is something the MDA wasn't developed to combat. It was because of this short-coming that more legislation, in the form of the PSA, was put in place so that drug legislature could become more proactive when targeting novel substances.

Table 1. Summary of nomenclature and structures of the compounds discussed within this chapter, excluding intermediates.

Name	Structure	Cmpd No.
Cathinone		1
Methcathinone		1a
Mephedrone: (±)-4'-(methyl)methcathinone		1b
(±)-4'-(trifluoromethyl)methcathinone		2
(±)-4'-(trifluoromethyl)ephedrine		3
(±)-4'-(trifluoromethyl)cathinone		4
(±)-4'-(trifluoromethyl)norephedrine		5
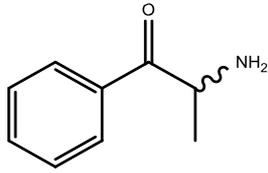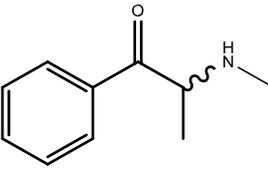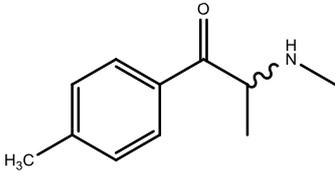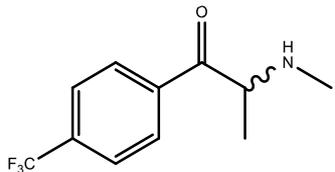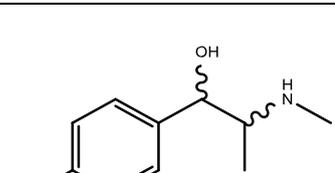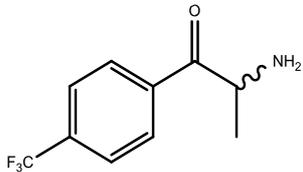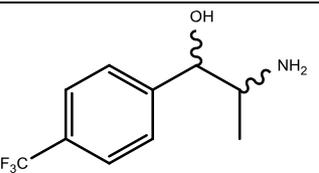
Synthetic cathinones have been popular drugs of abuse ever since mephedrone (**1b**; Table 1) surfaced onto the drugs market in 2007 (Deluca *et al.*, 2012), coining the term New Psychoactive Substance. (±)-4'-(Trifluoromethyl)methcathinone (4TFMMC, **2**) is structurally similar to mephedrone (**1b**) and methcathinone (**1a**), both of which are derived from the parent drug cathinone (**1**, Fig. 1) originally isolated from *Catha edulis* (Khat; Kalix & Braenden, 1985).

Cathinone (**1**): R=H, R'=H
MethCathinone (**1a**): R=H, R'=CH_3
Mephedrone (**1b**): R =CH_3, R'=CH_3
4-TFMMC (**2**): R=CF_3, R'=CH_3
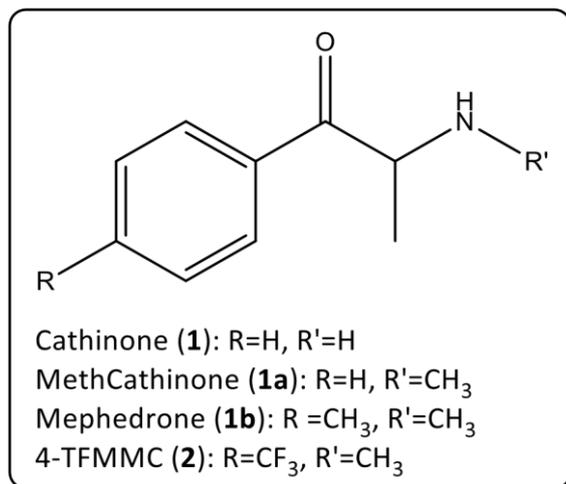
Fig 1. The core structure of three cathinone derived analogs.

As with long-standing drugs of abuse (e.g. cocaine, MDMA) NPS' are frequently confiscated by police and other legal bodies, though unlike established drugs of abuse there is a stark lack of metabolic and analytical data. Cathinones were controlled under the MDA and have been placed under further restrictions with the implementation of the PSA. Despite further restrictions, NPS use is becoming more popular (EMCDDA, 2015) yet for the most part, we cannot be certain of the effects of these compounds on the human body (Capriola, 2013; UNODC, 2013). That is why, arguably, it is more important now than ever to anticipate and fully characterise compounds that we believe are up and coming NPS' before they enter the market and are used by people without any knowledge of what they're taking and how it may affect them.

Mephedrone (**1b**) has three main routes of metabolism and these are as follows: Ndemethylation; reduction of the keto functionality; and CH oxidation of the

parramethyl functionality (Khreit et al., 2013). Khreit et al. demonstrated that the metabolism of 4-TFMMC (**2**; Scheme 1) shares some similarities with its parent compound, though due to the structural modification of the *para*-methyl moiety (CH$_3$ → -CF$_3$), its overall pharmacokinetics cannot be considered analogous to mephedrone (**1b**). This is due to the termination of the CH oxidation pathway which almost entirely eliminates any hydroxymethyl-groups used for glucuronidation, drastically slowing the metabolism of the compound. As a consequence of diminished metabolic pathways due to CH oxidation removal, half-life *in vivo* was shown to increase. The metabolites synthesised in this study are based on the two main routes of metabolism for mephedrone (**1b**) and the proposed metabolism of 4-TFMMC (**2**; *N*-demethylation and reduction). As the C-F bond would not be metabolised *via* conventional means, any aromatic hydroxymethyl-groups or other suspected metabolites produced from CH oxidation pathway have not been pursued in this study.
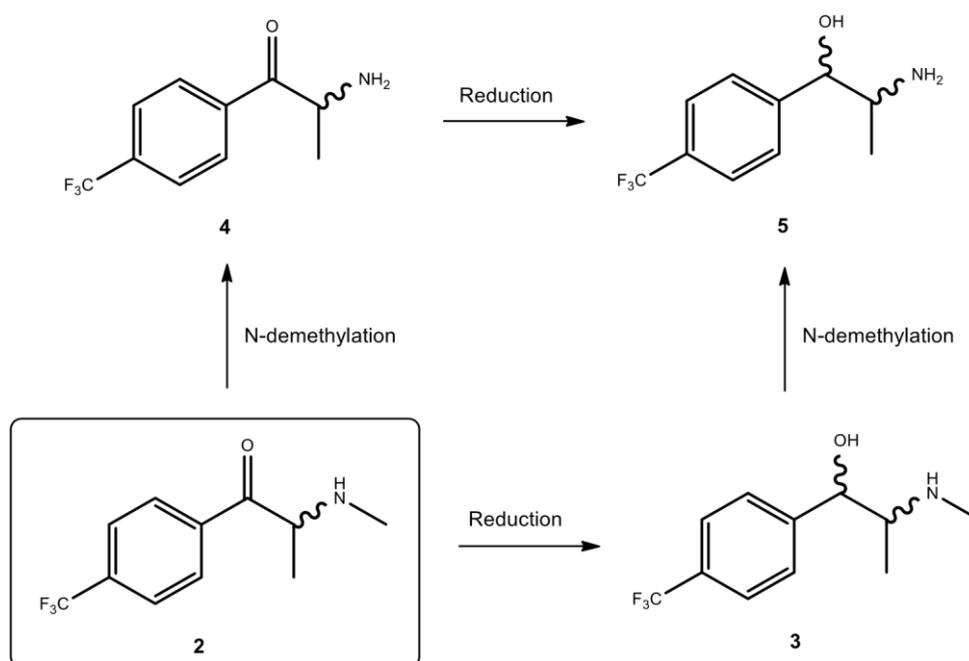
Scheme 1. The two proposed routes of metabolism of (±)-4'-(trifluoromethyl)methcathinone (**2**) pursued. Reduction and *N*-demethylation yield three metabolites: (±)-4'-(trifluoromethyl)cathinone (**4**); (±)-4'-(trifluoromethyl)ephedrine (**3**); and (±)-4'-(trifluoromethyl)norephedrine (**5**).

The aim of this study is to report a comprehensive analysis (Mpt.; TLC; IR; ^{1}H, ^{13}C, ^{19}F NMR; LC-MS; Raman) of (±)-4'-(trifluoromethyl)methcathinone and three of its proposed metabolites (Table 1) pre-emptively synthesised in anticipation of the introduction of the parent compound (4-TFMMC, **2**) onto the illegal drugs market.

Methods

All reagents were of commercial quality (from Sigma–Aldrich, Gillingham, UK), used without any further purification. Where necessary, solvents (from Fisher Scientific, Loughborough, UK) were dried and. Melting points were determined using closed capillary melting tubes on Stuart SMP10 MP apparatus (Sigma-Aldrich, Gillingham, UK). Thin-layer chromatography (TLC) was carried out on aluminium backed SiO_2 plates (Merck, Darmstadt, Germany), the spots were visualised using ultra-violet light (254 nm). TLC experiments were carried out at 5 mg mL^{-1} and presumptive tests were carried out at 20 mg mL^{-1} in accordance with UNODC guidelines (4.6; UNODC, 2015) for the identification of synthetic cathinones. Reagents for these experiments were prepared per the same guidelines and refrigerated to prevent degradation. Infrared spectra were obtained in the range 4000–400 cm^{-1} on a ThermoScientific Nicolet iS10ATRFTIR instrument (ThermoScientific, Rochester, USA). High-field ^{1}H, ^{13}C and ^{19}F NMR spectra were collected on JEOL ECS-400 Multinuclear FT-NMR (JEOL, Tokyo, Japan) at 400 MHz, 101 MHz and 376 MHz respectively, chemical shifts are reported as δ values. LC-MS data was collected on an Agilent 1260 infinity interfaced to a QTOF 6540 UHV accurate tandem mass spectrometer; all compounds were subject to ESI at 70 eV and values are reported as *m/z* ratios. Raman spectra were collected on a DXR Raman Microscope at 532 nm (ThermoScientific, Rochester, USA) and processed with OMNIC software (2008).

See supplementary information for all data not discussed in the main text (Figure S.I-1.0 onwards).

2.1 Synthesis of (±)- 4'-(trifluoromethyl)-2-bromopropiophenone (8)

The title compound was synthesised per the method reported by Kalendra (Kalendra *et al.*, 2003). To a solution of dichloromethane (DCM; 5 mL) and propiophenone (**7**, 20 mmol) was added one drop of hydrobromic acid (48% aq. solution) and one drop of bromine. The mixture was stirred and heated (30 minutes). When the bromine colour discharged, bromine (1 eq., including the original drop) was added dropwise whilst stirring. The reaction mixture was then concentrated *in vacuo.* Due to the bromopropiophenone's lachrymatory properties it was not purified before being used in the amination step so yield and spectral data were not obtained.

2.2 Synthesis of (±)-4'-(trifluoromethyl)methcathinone hydrochloride (2)

The title compound was synthesised per the method reported by Santali (Santali *et al.*, 2011). A suspension of (±)- 4'-(trifluoromethyl)-2-bromopropiophenone (1 eq.) and methylamine (2M, 1 eq.) in DCM (40 mL) was prepared. The mixture was stirred overnight at room temperature and then acidified (pH $\approx$ 1) with hydrochloric acid (6M, 50 mL). The aqueous layer was washed with DCM (3 × 50 mL) and then basified (pH $\approx$ 10) with potassium hydroxide (5M, *ca.* 100 mL) before being re-extracted with DCM (3 × 50 mL). The combined organic fractions were dried (MgSO$_4$) and concentrated *in vacuo* to attain a yellow oil then suspended in diethyl ether and HCl in dioxane (1 eq.) before being stirred gently for 5 minutes. The resulting solid was recrystallized in reagent grade acetone to give an off-white powder (1.48 g, 28% calculated from step 2.1). Mpt. (acetone) 225˚C; R$_f$ [SiO$_2$, EtOAc:*n*-hexane (1:3)] = 0.42; IR (ATR-FTIR) ν_{max} (cm^{-1}): 2900 (C-H), 2700 (N-H), 1689 (C=O), 1328 (C-F), 856 (*p*Ar) ; ^{1}H NMR (400 MHz, D$_2$O) δ 8.91 (d, 2H, *J* = 8.4 Hz, Ar**H**), 7.96 (d, 2H, *J* = 8.4 Hz, Ar**H**), 5.18 (q, 1H, *J* = 7.3 Hz, C**H**R$_2$(CH$_3$)), 2.86 (s, 3H, RNH(C**H$_3$**)), 1.64 (d, 3H, *J* = 7.3 Hz, CHR$_2$(C**H$_3$**)); ^{13}C NMR (101 MHz, D$_2$O) δ 199.72 (s, **C**=O), 138.18 (s, Ar**C**), 137.94 (s, Ar**C**), 132.29 (s, Ar**C**), 129.15 (s, Ar**C**), 129. 12 (s, Ar**C**), 127.70 (s, Ar**C**), 124.99 (s, **C**-F$_3$), 62.79 (s, **C**HR$_2$(CH$_3$)), 33.79 (s, RNH(**C**H$_3$)), 17.77 (s, CHR$_2$(**C**H$_3$)). ^{19}F NMR (376 MHz, D$_2$O) δ -64.22 (C**F$_3$**, s); LC-MS (ESI+, 70 eV): calculated for [M+H] C$_{11}$H$_{13}$NOF$_3$:

232.0950, found 232.0949; RAMAN v_{max} (cm^{-1}): 3089.07, 2964.92, 2939.53, 1691.14, 1619.46, 795.35, 633.37.

2.3 Synthesis of (±)-4'-(trifluoromethyl)ephedrine hydrochloride (3)

The title compound was synthesised per the method reported by Pozo *et al*. (2015). (±)-4'-(Trifluoromethyl)methcathinone hydrochloride (300 mg, 1.4 mmol) was dissolved in anhydrous methanol (15 mL) and then chilled in an ice bath. NaBH$_4$ (1.32 g, 25 eq.) was added slowly and once effervescence had ceased the mixture was stirred for 24 hours under inert atmosphere. The reaction mixture was then concentrated *in vacuo* and then dissolved in deionised water (approx. 50 mL). This solution was then extracted with DCM (3 × 30 mL). The organic fractions were combined and dried (MgSO$_4$) then filtered. The drying agent was also washed with DCM (2 × 15 mL) and the collective filtrates combined. These were concentrated *in vacuo* to attain a viscous yellow oil which was subsequently dissolved in ethyl acetate (approx. 2 mL) and HCl in dioxane (4M, 3 eq.) added slowly. This solution was left stirring for 30 minutes before the solvents were removed *in vacuo* and the resulting solid triturated with acetone (3 × 20 mL) to produce an off-white powder (261 mg, 87%). Mpt. (acetone) 192-194°C; R_f [SiO$_2$, EtOAc:*n*-hexane (1:3)] = 0.10; IR (ATR-FTIR) v_{max} (cm^{-1}): 3354 (O-H), 2979 (C-H), 2803 (N-H), 1325 (C-F), 1112 (C-O), 852 (*p*-Ar); ^{1}H NMR (400 MHz, D$_2$O) δ 7.78-7.59 (m, 4H, Ar**H**), 5.27 (d, 1H, *J* = 3.1 Hz, C**H**R$_2$(CH$_3$)), 3.61 (qd, 1H, *J* = 6.8, 3.3 Hz, RC**H**(OH)), 2.81 (s, 3H, RNH(C**H$_3$**)), 1.10 (d, 3H, *J* = 6.8 Hz, CHR$_2$(C**H$_3$**)); ^{13}C NMR (101 MHz, D$_2$O) δ 145.89 (s, Ar**C**), 132.83 (s, Ar**C**), 132.51 (s, Ar**C**), 129.45 (s, Ar**C**), 128.63 (s, Ar**C**), 128.59 (s, Ar**C**), 125.75 (s, **C**-F$_3$), 73.62 (s, **C**-OH), 62.58 (s, **C**HR$_2$(CH$_3$)), 33.69 (s, RNH(**C**H$_3$)), 12.33 (s, CHR$_2$(**C**H$_3$)). ^{19}F NMR (376 MHz, D$_2$O) δ 63.32 (C**F$_3$**, s); LC-MS (ESI+, 70 eV): calculated for [M+H] C$_{11}$H$_{15}$NOF$_3$: 234.1110, found 234.1106; RAMAN v_{max} (cm^{-1}): 3076.34, 1621.08 , 1324.11, 792.89, 766.80, 633.84.

2.4 Synthesis of (±)-4'-(trifluoromethyl)cathinone hydrochloride (4)

The title compound was synthesised per the method reported by Santali (Santali *et al*., 2011). The di-tert-butyl-iminodicarboxylate potassium starting material (7.21 g,

1.2 eq.) was added to a solution of (±)- 4'-(trifluoromethyl)-2-bromopropiophenone (7.68 g, 1 eq.) in dimethylformamide (17.5 mL) and stirred at room temperature overnight. The reaction mixture was then diluted with water (approx. 100 mL) and extracted with methyl tert-butyl ether (MTBE; 4 × 50 mL). The crude solid (±)-N, N(BOC)2-4'-(trifluoromethyl)cathinone was dissolved in DCM before adding HCl in dioxane (5 eq.) and stirring overnight. The resulting solution was filtered to get an off-white solid which was then decolourised with acetone to yield a white solid (1.13 g, 22% calculated from step 2.1). (acetone) 238°C; R_f [SiO$_2$, EtOAc:n-hexane (1:3)] = 0.47; IR (ATR-FTIR) v_{max} (cm^{-1}): 2883 (C-H), 2700 (N-H), 1691 (C=O), 1329 (C-F), 855 (pAr); ^{1}H NMR (400 MHz, D$_2$O) δ 8.18-7.95 (m, 4H, Ar**H**), 5.26 (q, 1H, J = 7.3 Hz, C**H**R$_2$(CH$_3$)), 1.62 (d, 3H, J = 7.3 Hz, CHR$_2$(C**H$_3$**)). ^{13}C NMR (101 MHz, D$_2$O) δ 200.22 (s, **C**=O), 138.26 (s, Ar**C**), 137.88 (s, Ar**C**), 133.01 (s, Ar**C**), 132.34 (s, Ar**C**), 130.70 (s, Ar**C**), 129.18 (s, Ar**C**), 125.11 (s, **C**-F$_3$), 55.23 (s, **C**HR$_2$(CH$_3$)), 19.27 (s, CHR$_2$(**C**H$_3$)); ^{19}F NMR (376 MHz, D$_2$O) δ -64.17 (C**F$_3$**, s); LC-MS (ESI+, 70 eV): calculated for [M+H] C$_{10}$H$_{11}$NOF$_3$: 218.0793, found 218.0793; RAMAN v_{max} (cm^{-1}): 3079.88, 2962.66, 1692.33, 1618.93, 788.10, 633.11.

2.5 Synthesis of (±)-4'-(trifluoromethyl)norephedrine hydrochloride (5)

The title compound was synthesised per the method reported by Pozo *et al.* (2015) as in methods section 2.3 (219 mg, 73%). Mpt. (acetone) 210°C; R_f [SiO$_2$, EtOAc:$n$hexane (1:3)] = 0.15; IR (ATR-FTIR) v_{max} (cm^{-1}): 3268 (O-H), 2852 (C-H), 1320 (C-F), 1065 (C-O), 849 (p-Ar); ^{1}H NMR (400 MHz, D$_2$O) δ 7.78 (d, 2H, J = 8.1 Hz, Ar**H**), 7.59 (d, 2H, J = 8.0 Hz, Ar**H**), 5.10 (d, 1H, J = 3.7 Hz, C**H**R$_2$(CH$_3$)), 3.85 – 3.63 (m, 1H, C**H**(OH)), 1.14 (d, 3H, J = 6.8 Hz, CHR$_2$(C**H$_3$**)). ^{13}C NMR (101 MHz, D$_2$O) δ 145.79 (s, Ar**C**), 132.97 (s, Ar**C**), 132.65 (s, Ar**C**), 129.71 (s, Ar**C**), 128.68 (s, Ar**C**), 128.64 (s, Ar**C**), 125.74 (s, **C**F$_3$), 75.16 (s, **C**-OH), 54.89 (s, **C**HR$_2$(CH$_3$)), 14.83 (s, CHR$_2$(**C**H$_3$)). ^{19}F NMR (376 MHz, D$_2$O) δ -63.32 (C**F$_3$**, s); LC-MS (ESI+, 70 eV): calculated for [M+H] C$_{10}$H$_{13}$NOF$_3$:

220.0950, found 220.0949; RAMAN v_{max} (cm^{-1}): 3075.02, 2946.66, 1622.44, 803.70, 766.48, 632.91.

Results & Discussion
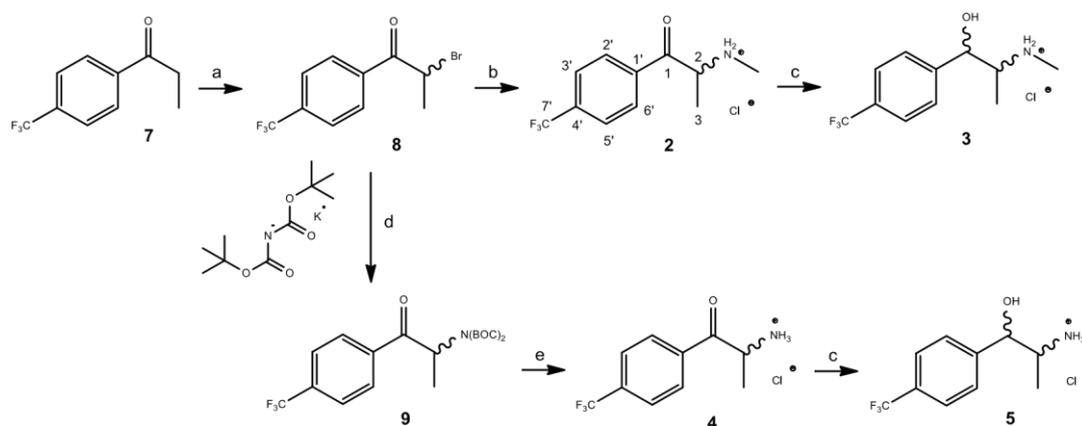
Scheme 2. Synthesis of target compounds/key metabolites of 4-TFMMC (**2**). Reagents and conditions: (a) Br$_2$/HBr (48% in water)/DCM/rt/1 h; (b) MeNH$_2$/DCM/rt/24 h; (c) MeOH/NaBH$_4$/rt/24 h; (d) DMF/(BOC)$_2$N$^-$K$^+$/rt/24 h; (e) DCM/HCl (4M in dioxane)/rt/24 h.

Synthesis of the parent compound (**2;** Scheme 2) from the commercially available starting material (**7**) was carried out *via* an α-bromination (a) and subsequent amination (b), before being isolated as its corresponding hydrochloride salt. The three metabolites synthesised (**3**, **4**, **5**) were also converted to salts to prevent degradation in their freebase forms (Nigg & Seigler, 2013). To produce products **4** and **5**, compound **8** was aminated *via* a di-tert-butyl-iminodicarboxylate protecting group (d) to yield a primary amine after deprotection (**7**; e). It was shown that Santali (Santali *et al.*, 2011) method used could be optimised by directly deprotecting **7** without the need for purification *via* column chromatography to produce **4**. Compounds **2** and **4** were reduced (c) with 25 eq. of NaBH$_4$ to accommodate for any residual water that may have been present. Acetone was used for purification (*via* recrystallization) for all products instead of diethyl ether and proved effective as another modification to the original method. Stereochemistry was not controlled in any reaction as it is not expected that enantiopure products will enter the market.

The first step in the synthesis (a) is an α-bromination of the commercially available propiophenone (**7**). The hydrobromic acid added to the reaction flask catalyses the formation of the enolate intermediate that subsequently attacks the δ^+ centre of the bromine. Progression of the bromination can easily be observed as the bromine in the reaction flask decolourises. This reaction produces a brominated propiophenone (**8**) and installs a good leaving group which can be exploited in further reactions. If 4TFMMC (**2**) or R.4-TFMMC (**3**) are the desired products, the next step of the reaction is an amination proceeding *via* an S_N2 reaction using methylamine (b) to produce the parent compound 4-TFMMC (**2**). If 4-TFMC (**4**) or R.4-TFMC (**5**) are wanted, the bromopropiophenone (**8**) must first be aminated using a di-tert-butyl-iminodicarboxylate salt (d) to form a protected intermediate (**9**). Deprotection of the intermediate (**8**; Scheme 3; e) yields synthetic metabolite 4-TFMC (**4**). The reduction steps (c) to produce synthetic metabolites R.4-TFMMC (**3**) and R.4-TFMC (**5**) are the same in both instances, using sodium borohydride to reduce the carbonyl functionality, yielding an ephedrine (**3**) and a norephedrine (**5**) respectively.
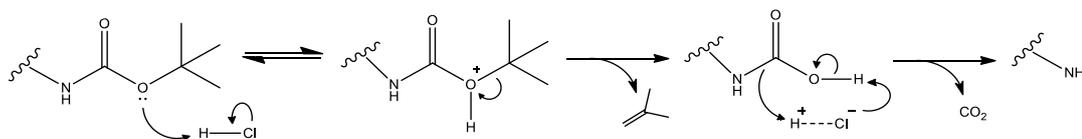
Scheme 3. Mechanism for the acid catalysed deprotection of a BOC protecting group (e). The tert-butyl cation can either be quenched, polymerised, or deprotonated to form isobutylene (gas) as in this example. The CO_2 produced should also be allowed to leave the reaction flask.

Due to the novel nature of the synthesis carried out in this study, no published work exists pertaining to the Nuclear Magnetic Resonance data of 4-TFMMC (**2**) and its metabolites in D_2O. As such, when assigning structural positions for the parent compound (**2**), data was compared with spectra obtained in d_6-DMSO (Khreit et al., 2013) and cross-referenced against structural data of mephedrone (**1b**) collected in D_2O (Alotaibi, Husbands & Blagbrough, 2015). The spectra of the synthetic metabolites (**3**, **4**, **5**) were assigned in a similar way, corroborating the absence and appearance of peaks between structures (Santali *et al.,* 2011; Khreit *et al.,* 2013; Pozo *et al.,* 2015). A comparison of the primary and secondary amine peaks cannot be

made due to the deuterium exchange of the samples with the NMR solvent. D_2O was chosen for analysis despite this because of the large library of sample spectra already obtained allowing for ease of comparison between pre-existing analogs.

The ^1H NMR spectrum of the 4-TFMMC hydrochloride salt (**2**; obtained in D_2O; Fig. 2) showed five proton environments, accounting for the loss of the secondary amine peak due to deuteration. The asymmetrically *para*-disubstituted aromatic ring (8.91 ppm, 2H, *J* = 8.4 Hz, AA'BB'; 7.96 ppm, 2H, *J* = 8.4 Hz, AA'BB'); a deshielded single hydrogen quartet (5.18 ppm, 1H, *J* = 7.3 Hz, C**H**(CH$_3$)) pertaining to the chiral carbon as a result of the neighbouring methyl moiety. A deshielded methyl singlet appears at 2.86 ppm (NHC**H$_3$**), and finally the methyl doublet attached to the chiral centre (1.64 ppm, 3H, *J* = 7.3 Hz, CH(C**H$_3$**)). The ^{13}C NMR spectrum (obtained in D_2O) supports the supposition that the compound is sufficiently pure, with 11 peaks overall and allows for the identification of groups not visible in the ^1H NMR such as the key peaks at 199.72 ppm (**C**=O) and 124.99 ppm (Ar**C**(CF$_3$)) respectively. Peaks at 62.79 ppm (**C**H(CH$_3$) and 33.79 ppm (NH(**C**H$_3$)) confirm the methyl groups observed in the ^1H NMR spectrum. The CF$_3$ group present at the *para* position gives rise to a singlet at 64.22 ppm in the ^{19}F NMR spectrum (obtained in D_2O; Fig. 3) confirming the signal seen in the ^{13}C NMR spectrum. Synthesis of 4-TFMMC (**2**) was confirmed *via* LC-MS with the molecular ion being found as 232.0949 *m/z*.

All Raman spectra were assigned based on the analysis of cathinone regioisomers carried out by Christie *et al.* (Christie *et al.*, 2013). The Raman spectrum of 4-TFMMC (**2**, Fig. 4) clearly shows several characteristic peaks associated with cathinones. The first peak corresponds to the C=O stretch at 1691.14 cm^{-1}; the second to the C=C stretch at 1619.46 cm^{-1}, the sharp intensity of the latter would generally be considered unusual but occurs as a result of the unconjugated 1,4-disubstitution on the aromatic ring. The peak observed at 795.35 cm^{-1} arises due to v_4 C-H out-of-plane bending and puckering around the aromatic ring. A relatively sharp signal is also present at 633.37 cm^{-1} that we tenuously assign as *p*-C-Ar stretching; this deviates considerably from literature value for the CH$_3$-Ar stretch seen in mephedrone (**1b**;

517.9 cm^{-1}). We believe the band occurs at higher energy as a result of the added steric bulk and electronic properties of the highly electronegative trifluoromethyl moiety.
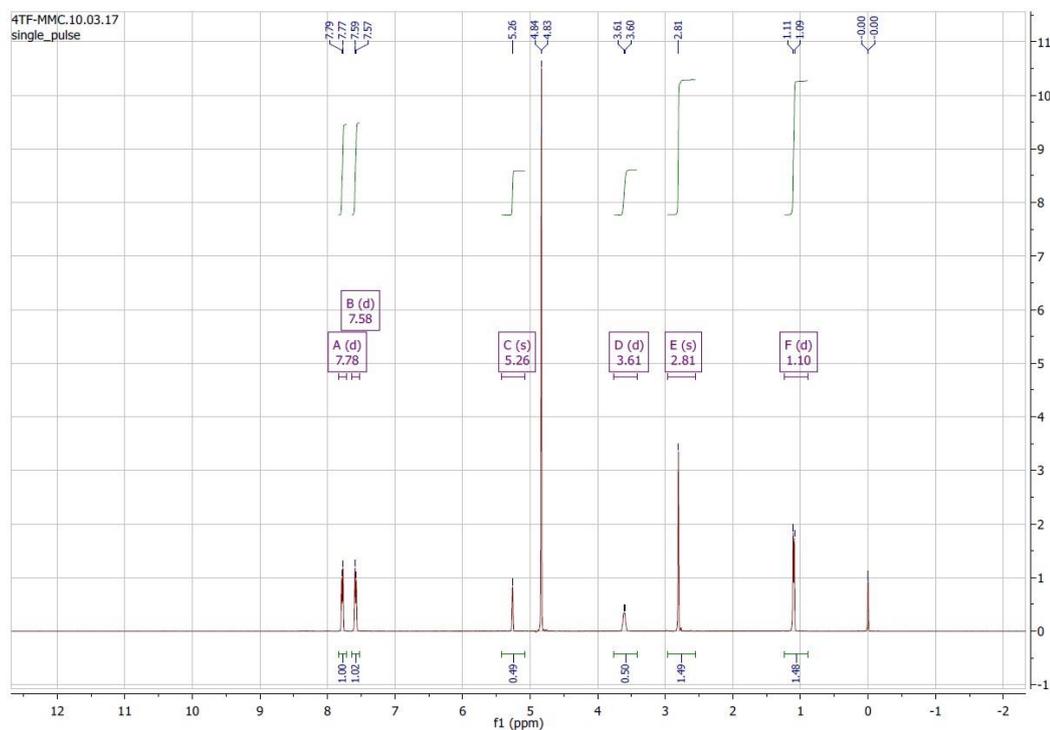

Fig. 2. ^{1}H NMR spectrum (400 MHz, D$_2$O) of (±)-4'-(trifluoromethyl)methcathinone (**2**).
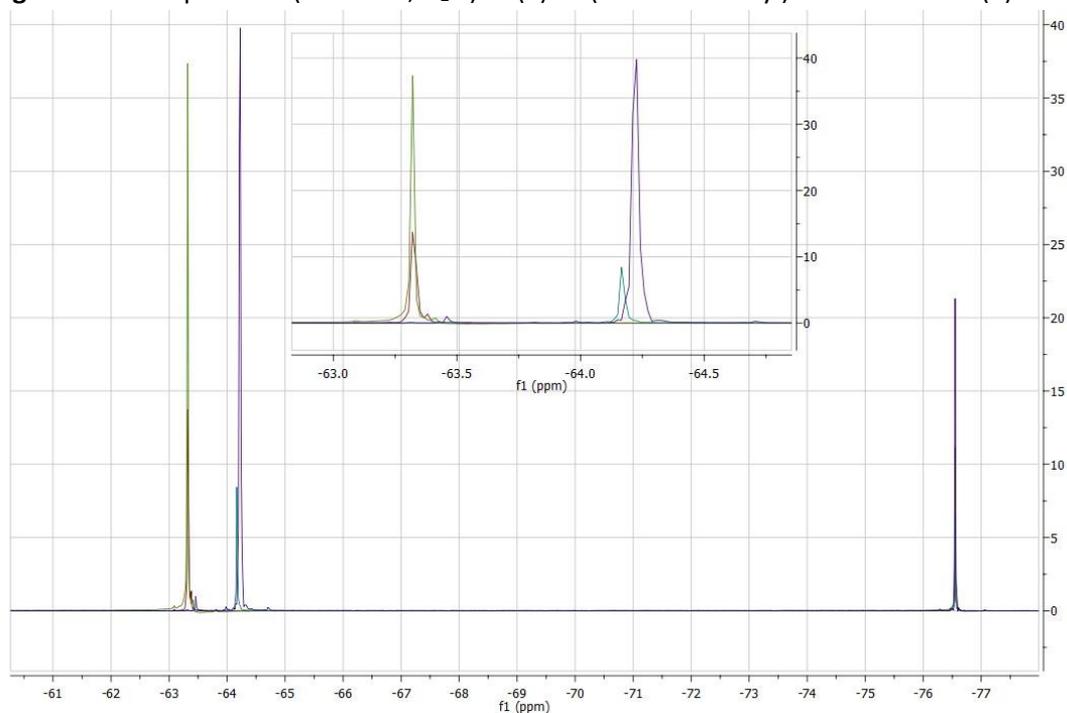

Fig. 3. Compiled and overlaid ^{19}F NMR spectra (376 MHz, D$_2$O) of all four compounds (L-R): R.4-TFMMC (**3**; purple), R.4-TFMC (**5**; red), 4-TFMC (**4**; blue), 4-TFMMC (**2**; purple).
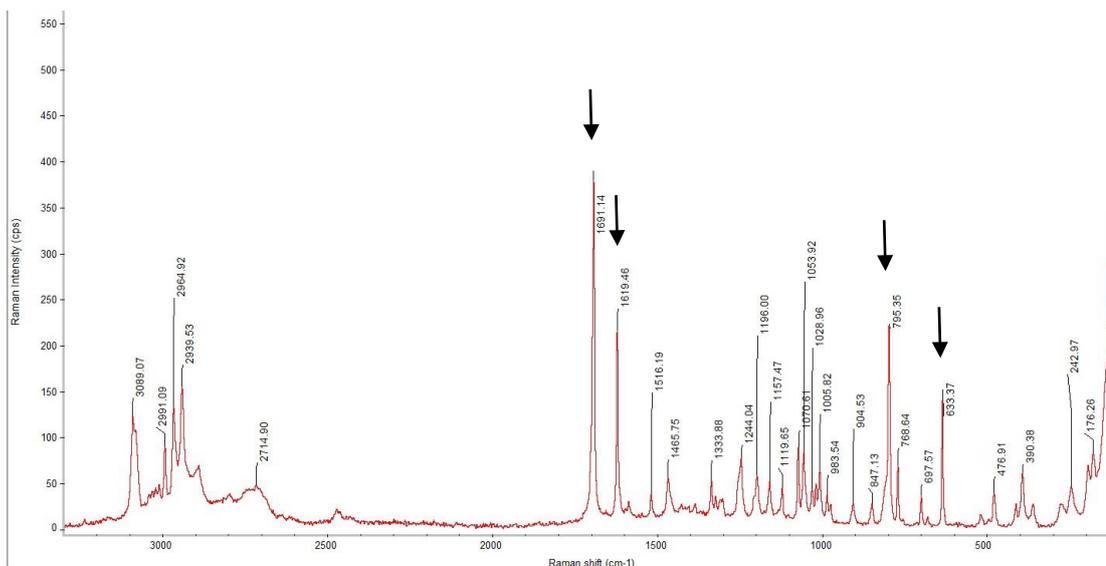
Fig. 4. Raman spectrum of (±)-4'-(trifluoromethyl)methcathinone (**2**). Black arrows indicate peaks 1691.14 cm^{-1} (C=O), 1619.46 cm^{-1} (C=C), 795.35 cm^{-1} (ArC-H), and 633.37 cm^{-1} (Ar-CF$_3$) respectively.

Infrared data yielded several key peaks for 4-TFMMC (**2**): C-H methyl peaks at 2900 and 2700 cm^{-1}; a sharp ketone peak at 1689 cm^{-1}; C-F peak at 1328 cm^{-1}; and a *para*disubstituted aromatic peak at 856 cm^{-1}. Infrared and Raman analysis, whilst often only used to confirm a structure, become incredibly useful as diagnostic tools when compared against a library of reference standards. Both methods require only a small amount of sample when we compare them to Nuclear Magnetic Resonance spectroscopy, with the added advantage of having a lower instrument cost. NPS' generally undergo less sample modification than their illicit counterparts *ergo* suffer from less matrix interference with these techniques.

Demethylation of the parent compound (**2**) to a primary amine leads to an increase in polarity and would be expected to cause small but distinct chemical shift changes in the ^{1}H and ^{13}C NMR spectra. The synthetic metabolite 4-TFMC (**4**) differs in a couple of notable ways from its parent compound (**2**). Firstly, the ^{1}H NMR spectra (obtained in D$_2$O; Fig. 5) shows the disappearance of the methyl group attached to the amine functionality (Table 2), suggesting that the secondary amine of the parent compound is no longer present. This disappearance is reflected in the mass

reduction of the molecular ion (**2**: [M+H]: 232.0949 *m/z*, **4**: [M+H]: 218.0793 *m/z*) as well as the ^{13}C NMR, with the loss of the 62.79 ppm signal resulting in 10 instead of 11 carbon environments. Another noticeable difference between the parent compound and the demethylated metabolite (**4**) is the shifting of the quartet representing the chiral carbon to higher field, as well the doublet representing the methyl moiety on the chiral carbon moving to lower field, indicating increased polarity within the system due to the presence of the primary amine.
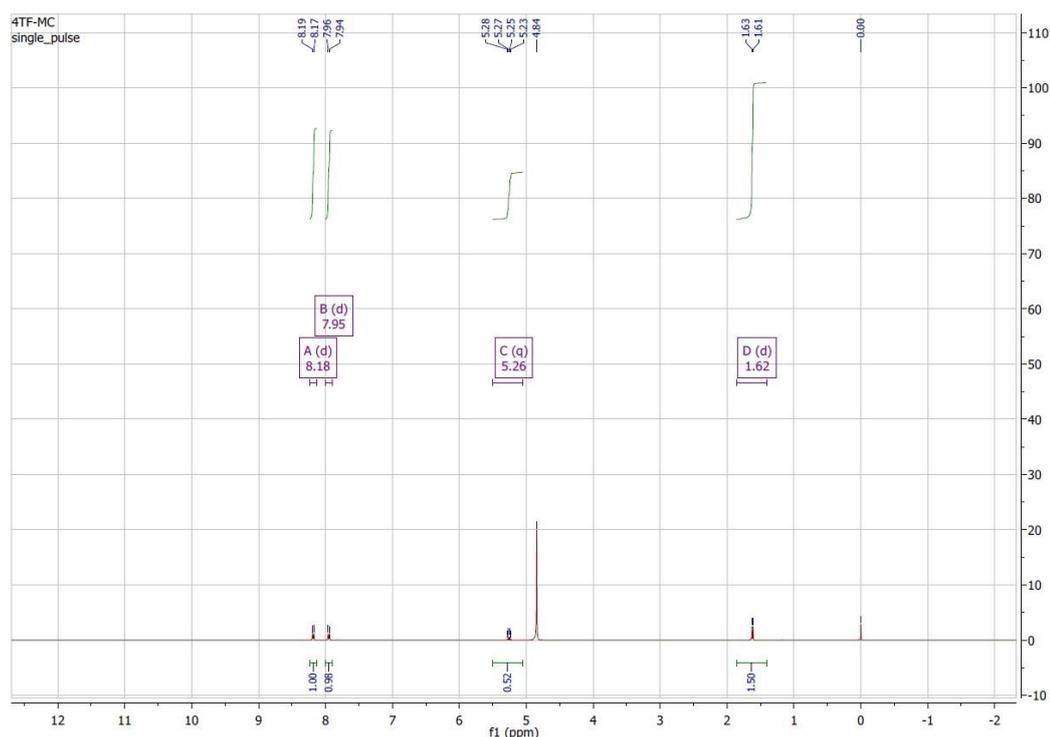
Fig. 5. ^{1}H NMR spectrum (400 MHz, D_2O) of (±)-4'-(trifluoromethyl)cathinone (**4**).

The Raman spectrum for **4** (Fig. 6) displays little variation when compared to that of the parent compound (**2**). The only peak that moves significantly is the one assigned to the v_4 C-H out-of-plane bending and puckering (788.10 cm^{-1}). We suggest that the shift may arise as a result of the afore mentioned increase in polarity of **4** as a result of demethylation. The Infrared spectrum for 4-TFMC is also very similar to **2**, with no loss or addition of peaks and all key peaks showing little-to-no variation.

21

Fig. 6. Raman spectrum of (±)-4'-(trifluoromethyl)cathinone (**4**). Black arrows indicate peaks 1692.33 cm^{-1} (C=O), 1618.93 cm^{-1} (C=C), 788.10 cm^{-1} (ArC-H), and 633.11 cm^{-1} (Ar-pC) respectively.

Table 2. ^{1}H, ^{13}C, ^{19}F NMR data (excluding the aromatic ring) comparing key peaks in ppm for all four compounds. Black boxes indicate no data is available for the specific peak.
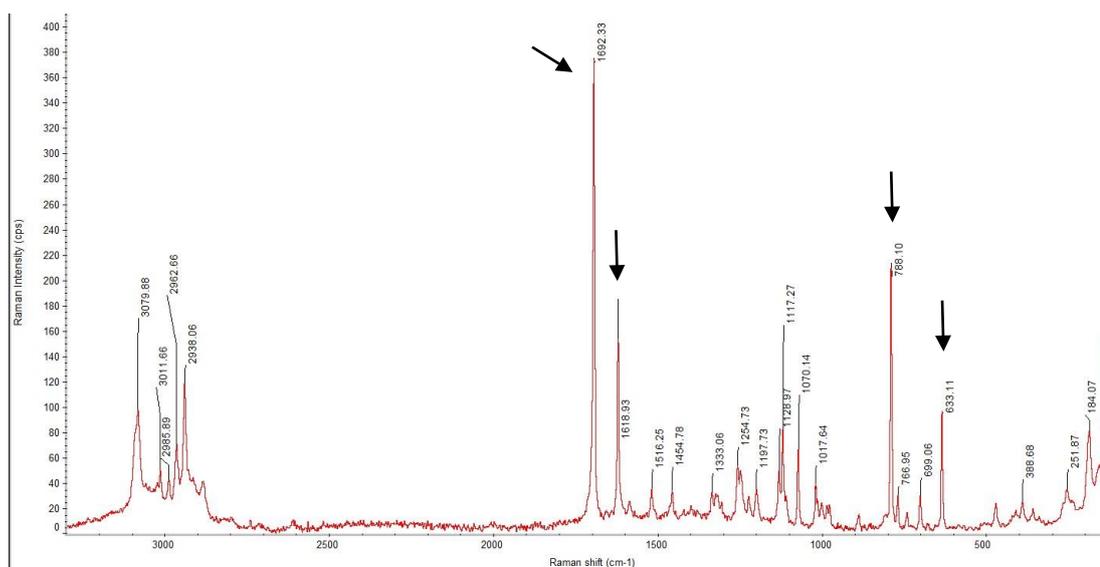
NMR	Peak	4-TFMMC (**2**)	**3**	4-TFMC (**4**)	**5**
^{1}H NMR	C**H**R$_2$(CH$_3$)	5.18	5.27	5.26	5.10
	C**H**(OH)	■	3.61	■	3.85-3.63
	RN**H**(C**H₃**)	2.86	2.81	■	■
	CHR$_2$(C**H₃**)	1.64	1.10	1.62	1.14
^{13}C NMR	**C**=O	199.72	■	200.22	■
	C-F$_3$	124.99	125.75	125.11	125.74
	C-OH	■	73.62	■	75.16
	CHR$_2$(CH$_3$)	62.79	62.58	55.23	54.89
	RNH(**C**H$_3$)	33.79	33.69	■	■
	CHR$_2$(**C**H$_3$)	17.77	12.33	19.27	14.83
^{19}F NMR	C-**F₃**	-64.22	-63.32	-64.17	-63.32

22

The ^{1}H-NMR for (±)-4'-(trifluoromethyl)ephedrine (**3**; obtained in D$_2$O; Fig. 7) displays six proton environments and bears resemblance to the parent compound (**2**): the *para*-disubstituted aromatic ring (7.78 ppm, 2H, *J* = 8.3 Hz, AA'BB'; 7.59 ppm, 2H, *J* = 8.1 Hz, AA'BB'); the proton at 5.27 ppm is now a doublet, suggesting a loss in coupling (5.27 ppm, 1H, *J* = 3.1) compared to its unreduced counterpart (**2**); a new signal appears at 3.61 ppm (3.61 ppm, *J* = 6.8, 3.3 Hz) corresponding to the CH of the alcohol group that presents as a multiplet due to the complex coupling that results from the free rotation around this bond. As with **2**, there is a deshielded methyl singlet present (2.81 ppm,3H, NHC**H₃**), and finally the methyl doublet attached to the chiral centre (1.10 ppm, 3H, *J* = 6.8 Hz, CH(C**H₃**)).
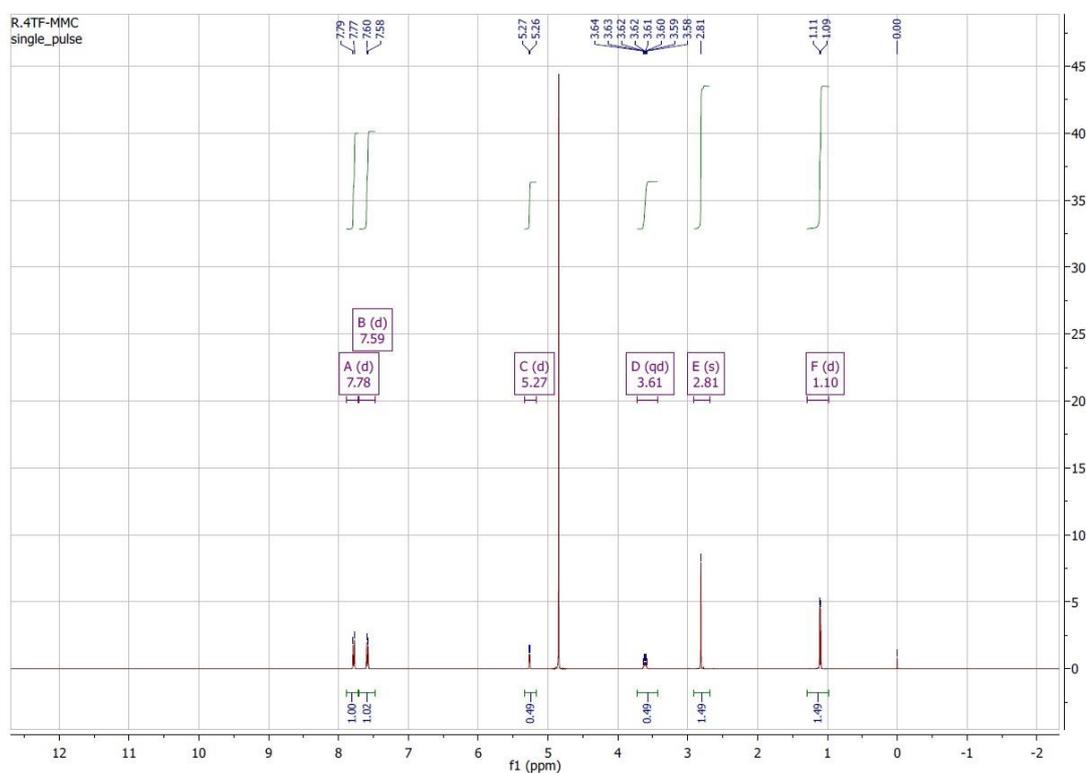
Fig. 7. ^{1}H NMR spectrum (400 MHz, D$_2$O) of (±)-4'-(trifluoromethyl)ephedrine (**3**).

The ^{1}H NMR for (±)-4'-(trifluoromethyl)norephedrine (**5**; obtained in D$_2$O; Fig. 8) displays five distinct proton environments. The *para*-disubstituted aromatic ring (7.78 ppm, 2H, *J* = 8.1 Hz, AA'BB'; 7.59 ppm, 2H, *J* = 8.0 Hz, AA'BB'); the proton at 5.10 ppm is a doublet (*J* = 3.7 Hz) echoing the loss in coupling we see in (±)-

4'(trifluoromethyl)ephedrine (**3**). Another similarity between synthetic metabolites **3** and **5** is the presence of the additional CH peak when compared to the cathinones **2** and **4** due to the reduction of the keto- moiety, because of this we witness the additional shift of this CH peak in the reduced metabolite **5** to lower field. No *N*methyl peak is observed for **5** (Table 2) in comparison to compound **3** due to the presence of a primary amine, this loss is confirmed by the reduction in the mass of the molecular ion (**3** [M+H]: 234.1106 *m/z*, **5** [M+H]: 220.0949). The methyl group attached to the chiral centre appears as a doublet at 1.14 ppm (*J* = 6.8 Hz).
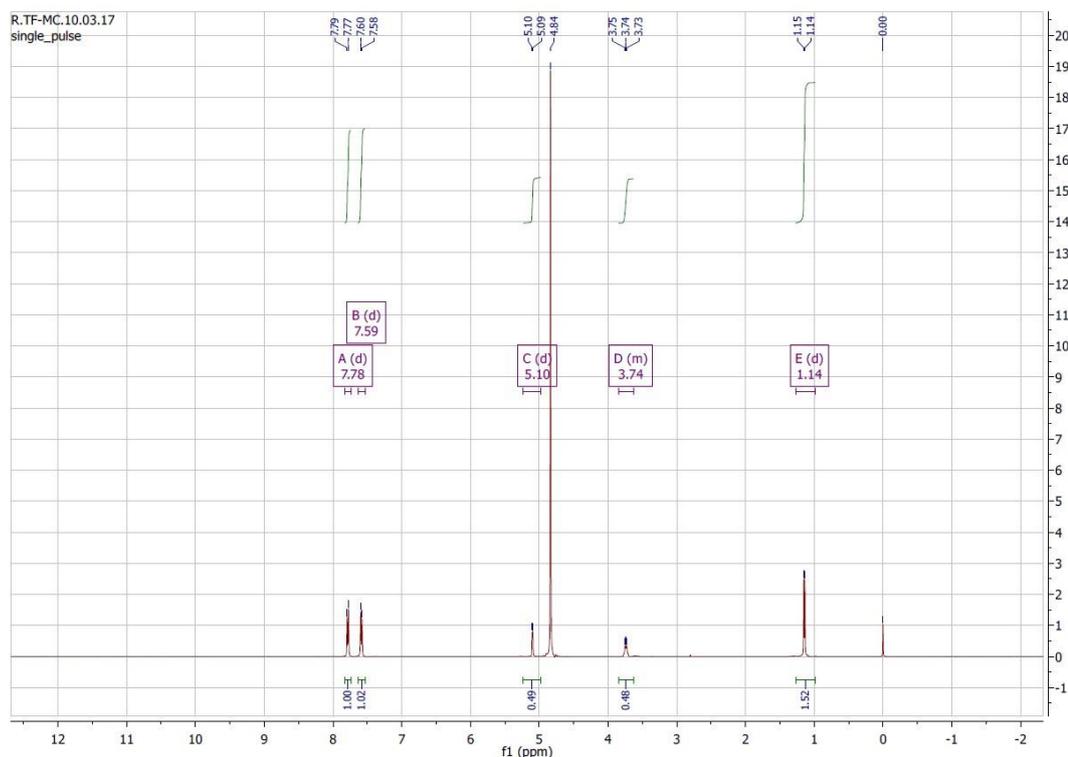
Fig. 8. ^{1}H NMR spectrum (400 MHz, D$_2$O) of (±)-4'-(trifluoromethyl)norephedrine (**5**).

Reduction of the keto-functionality reveals only a few changes with regards to the ^{13}C NMR spectra of (±)-4'-(trifluoromethyl)ephedrine (**3**; obtained in D$_2$O) and R.4-TFMC (**5**; obtained in D$_2$O): 11 environments are observed for **3**, as with **2**, and 10 environments are seen for **5**, as with **4**. The additional peak seen in the ^{1}H NMR of (±)-4'-(trifluoromethyl)ephedrine (**3**) is confirmed by the mass of the molecular ion, which also indicates that the parent drug (**2**) was successfully reduced (**2:** [M+H]:

232.0949 *m/z* → **3:** [M+H]: 234.1106 *m/z*). Notably there is the disappearance of the carbonyl peak (199.72 ppm) and the addition of an alcohol peak (73.62 ppm), typically seen in any reduction of a C=O bond. The same change is observed in the ^{13}C NMR spectrum of R.4-TFMC with the alcohol peak at 75.16 ppm. The carbon of the *para*-methyl functionality produces a peak at approximately 125 ppm for both **3** and **5**. One peak that differs considerably between the two reduced compounds is the chiral carbon at 62.58 ppm (**3**) and 54.89 ppm (**5**), the difference is because of the increased polarity of **5** due to demethylation and is a key peak when looking to differentiate the methylated and demethylated metabolites. The peak at 33.69 ppm for (±)-4'-(trifluoromethyl)ephedrine (**3**) indicates the *N*-methyl, confirming what is seen in the ^{1}H NMR spectrum; due to the lack of an *N*-methyl group in (±)-4'(trifluoromethyl)norephedrine (**5**) this peak does not appear (Table 2). The peak at 12.33 ppm represents the methyl moiety on the chiral carbon (CH(**C**H$_3$).

The ^{19}F NMR spectrum (obtained in D$_2$O; Fig. 3) shows a singlet peak at -63.32 ppm for both reduced metabolites (**3**, **5**), as such it is extremely unlikely that either reduced synthetic metabolite could be distinguished from the other using this technique.

Raman analysis confirms the loss of the keto- functionality by the disappearance of the corresponding C=O peak at 1691.14 cm^{-1} for both (±)-4'-(trifluoromethyl)ephedrine (**3**; Fig. 9) and (±)-4'-(trifluoromethyl)norephedrine (**5**; Fig. 10); the OH stretch does not seem to be Raman active for either reduced synthetic metabolite (**3, 5**). The C=C stretch is observed at approximately 1620 cm^{-1} across all compounds, its value changing only minutely. The ν_4 C-H out-of-plane bending and ring puckering remains at 792.62 cm^{-1} (**3**) and 803.70 cm^{-1} (**5**), with an additional peak at approximately 766 cm^{-1} for both reduced synthetic metabolites that we propose arises as a result of ring interactions with the trifluoromethyl moiety, as the system now has free rotation due to the ketone reduction.

The Infrared spectra of **3** and **5,** similarly to the NMR data, show the loss of the ketone peak (1689 cm⁻¹) with the appearance of alcohol peaks at 3354 cm⁻¹ (**3**) and 3268 cm¹ (**5**) as well as C-O peaks at 1112 cm⁻¹ (**3**) and 1065 cm⁻¹ (**5**) per the reduction of the cathinones. Other signals observed are methyl peaks (**3**: 2979 cm⁻¹, **5**: 2803 cm⁻¹); carbofluoro peaks (**3**: 1325 cm⁻¹, **5**: 1320 cm⁻¹); and *para*-disubstituted aromatic peaks (**3**: 852 cm⁻¹, **5**: 849 cm⁻¹), all with very similar values to the parent compound (**2**).
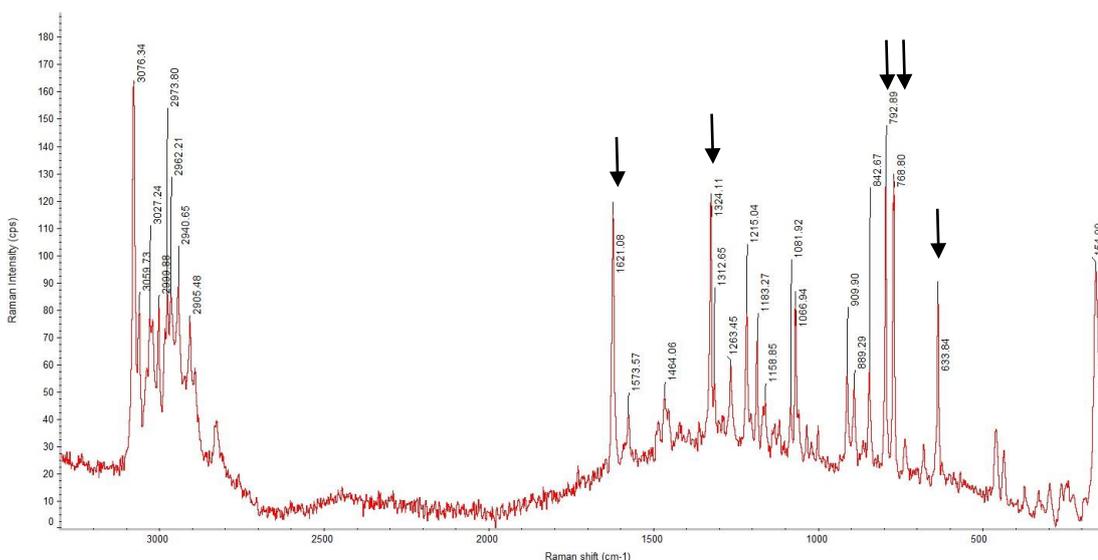
Fig. 9. Raman spectrum of (±)-4'-(trifluoromethyl)ephedrine (**3**). Black arrows indicate peaks 1621.08 cm⁻¹ (C=C), 1324.11 cm⁻¹ (ArC-H), 792.89 cm⁻¹ (ArC-H), 766.80 cm⁻¹ (Ar-CF₃), and 633.84 cm⁻¹ (Ar-*p*C) respectively**.**
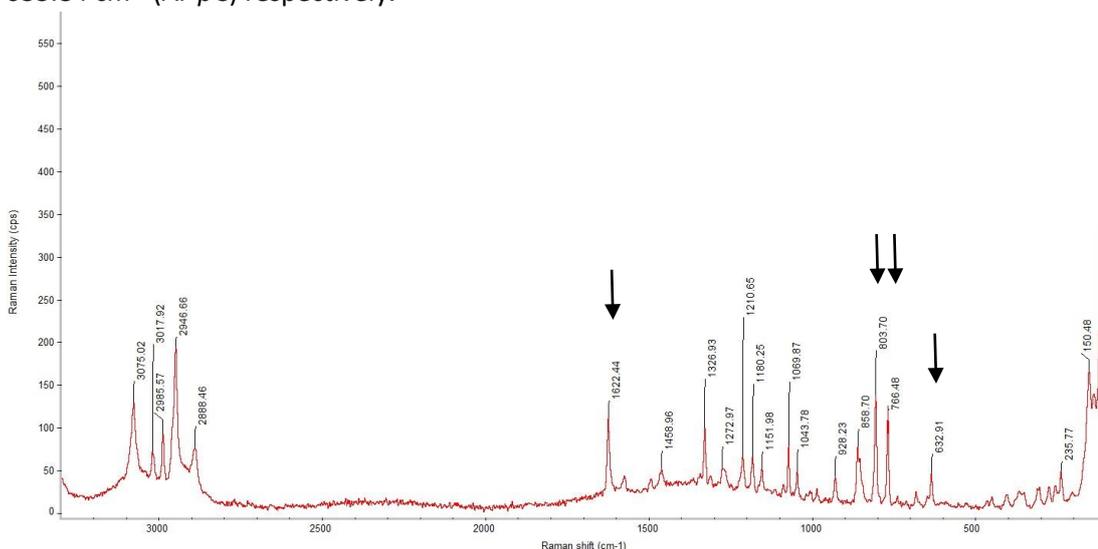

Fig. 10. Raman spectrum of (±)-4'-(trifluoromethyl)norephedrine (**5**). Black arrows indicate peaks 1622.44 cm⁻¹ (C=C), 803.70 cm⁻¹ (ArC-H), 766.48 cm⁻¹ (Ar-CF₃), and 632.91 cm⁻¹ (Ar-*p*C) respectively.

Using ^{1}H NMR and ^{19}F NMR, characteristic peaks can be used to distinguish between the cathinones and the ephedrines such as the CH of the alcohol groups that would only be present in the reduced metabolites. With these two techniques, it is not possible to discriminate between these four compounds directly. However, all four compounds display unique signals for the methyl moiety (C3) connected to the chiral carbon (C2) in their respective ^{13}C NMR spectra, *ergo* we suggest here that these peaks could be used to identify these compounds when in a mixture (Fig. 11). Unfortunately in practice, employing NMR to separate and identify NPS' isn't realistic when dealing with clinical samples as it requires significantly higher concentrations due to the low detection limits of the technique (mmol *vs.* nmol). In these cases LCMS, utilising a HILIC based column, would be better suited as it provides the most ideal limits of detection.
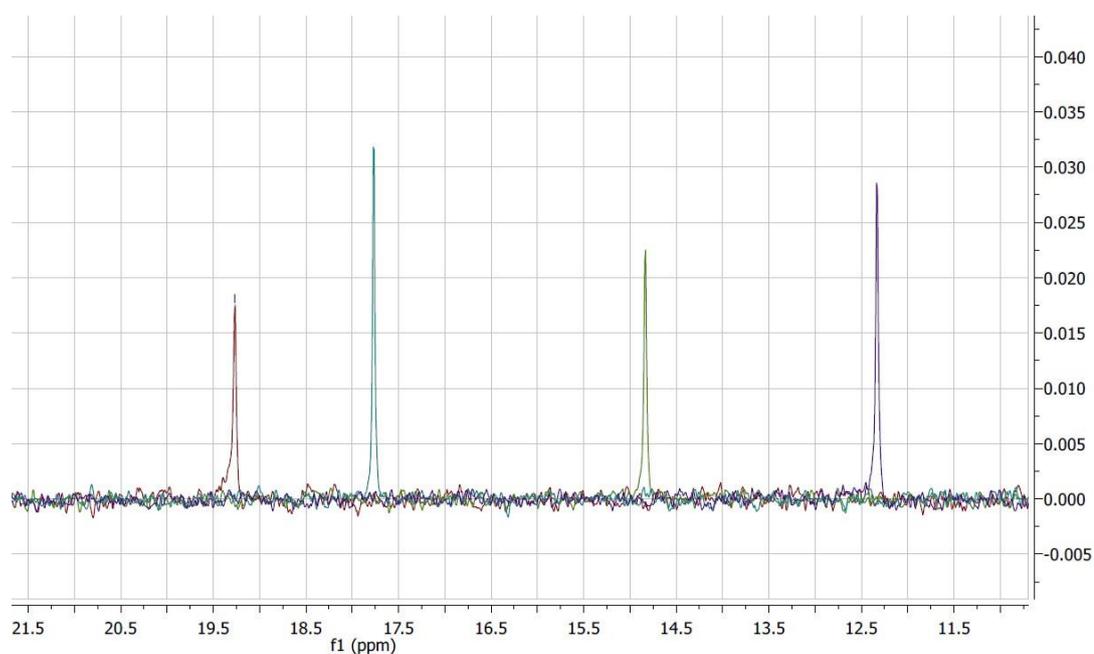

Fig. 11. A magnified section of the overlaid ^{13}C NMR spectra of 4-TFMMC (**2**, blue), R.4TFMMC (**3**, purple), 4-TFMC (**4**, red), and R.4-TFMC (**5**, green) showing the C3 peak for identification of the four compounds.

Presumptive Testing

Presumptive tests offer a cheap way for individuals without specialist equipment or expertise to rapidly test for specific compounds. The tests are incredibly useful as diagnostic tools, often employed by police to aid in the identification of unknown substances, giving an indication of whether a drug may be present (UNODC, 2005).

The presumptive tests used in this study have been selected for the testing of the fluorinated analogs because they have been shown to detect cathinones and derivatives in previous work (UNODC, 1994; UNODC, 2005; UNODC, 2015). It should be noted that a positive reaction will vary depending on the compound being tested; UNODC Guidelines should be consulted for officially observed colour changes.

Simon's Test: This test is generally used to detect secondary amines (UNODC, 2005). A positive reaction produces an array of colour changes depending on the substance being tested. The secondary amine of the analyte reacts under alkaline conditions with the acetaldehyde present before complexing with the sodium nitroprusside. Despite being designed to detect secondary amines, primary amines can also react, as shown in the case of 4-TFMC (**4**).

Robadope Test: The Robadope test is a variation of the Simon's test that uses acetone as a reaction partner instead of acetaldehyde (UNODC, 2005). The alteration is designed to detect primary amine functionality specifically. However, it should be noted that not all primary amines will react with the Robadope test, as in the case of cathinone (**1**), that produce a positive reaction with the Simon's test.

Zimmerman's Test: A test for alkaloids that can be used to detect cathinones. There will often be an initial colour change followed by a second colour change that becomes apparent after five minutes. Both colour changes are the differentiating criteria when identifying the compounds being tested (UNODC, 2015). The test operates *via* the formation of a Meisenheimer complex (UNODC, 1989) that is then

oxidised by the base present to produce a strongly coloured enolate (Wubbels, Winitz & Whitaker, 1990).

Liebermann's Test: A test used for the detection of cathinones, including methcathinone (**1a**) and mephedrone (**1b**; Toole *et al.*, 2012). A positive result for cathinones will be bright yellow, though the mechanism for this colour change remains unclear.

Chen-Kao Test: Used to distinguish ephedrines and methcathinones from amphetamines (UNODC, 2005). A result is considered to be positive with a colour change from the pale blue colour of the copper sulphate to violet or brown/orange. This is due to the formation of a complex between the ephedrine and the copper ions present in solution. The ephedrine or methcathinone act as bidentate ligands in the alkaline solution that form an intensely coloured four co-ordinate complex, variations in binding energy and structure lead to the difference in observed colour.

A limitation of presumptive tests is the requirement of a relatively large sample amount when compared to other analytical methods, so for trace analysis their use isn't possible. They may also generate false positives or false negatives depending on how the tests were prepared as well as the time from preparation to usage. It is fair to say that the tests are generally more helpful in identification when trying to determine the class of a compound rather than pinpoint a specific structure due to the broad spectrum of (often) subjective colour changes. Nevertheless, presumptive tests should not be discounted from an analyst's toolset if only for the minimal equipment and training requirements where more sophisticated instruments may not be available.

Colour changes should be recorded directly after samples have been added to test wells in addition to any further changes five minutes later. The fluorinated analogs generally reacted as expected, although there were some instances where unexpected positives or negatives were observed. One notable negative reaction is that of 4-TFMC (**4**) with the Liebermann test, though one possible explanation could

be that the amount of the metabolite was too low to be detected using standardised concentrations.

It is tenuously suggested that from these results (Table 3) the Chen-Kao test would be the most reliable for distinguishing between these compounds as both the cathinones and ephedrines give starkly different positive reactions. Both the Robadope and Simon's tests yielded more unexpected results than anticipated positives or negatives. As such they cannot be recommended for testing these compounds based on the results obtained, as the colour changes are very slight and could be seen as misleading.

Table 3. Summary of overall colour changes from 0 to 5 minutes after samples were added to wells. (-) indicates a negative result i.e. no colour change. (+) indicates a positive result, unexpected positive results are not marked specifically, reported only as a 'colour change (+)'.

Presumptive tests:	Robadope	Simon's	Zimmerman's	Liebermann's	Chen-Kao
4-TFMMC (**2**)	Olive/ brown (+)	(-)	Peach (+)	Yellow→ Grey (+)	Green→ orange/ brown (+)
3	(-)	(-)	(-)	Yellow→ Grey (+)	Violet→ Grey (+)
4-TFMC (**4**)	Light pink/ peach (+)	Light orange/ peach (+)	Light pink/peach (+)	(-)	Violet→ Orange (+)
5	Light pink/ peach (+)	(-)	(-)	Yellow→ Grey (+)	Violet (+)

Conclusions

The successful synthesis of (±)-4'-(trifluoromethyl)methcathinone and three of its novel metabolites: (±)-4'-(trifluoromethyl)ephedrine (**3**), (±)-4'-(trifluoromethyl)cathinone (**4**), (±)-4'-(trifluoromethyl)norephedrine (**5**) is reported. We report the first ^{1}H NMR, ^{13}C NMR, ^{19}F NMR, Raman, LC-MS, and FTIR data for compounds **3**, **4**, and **5**. It has also been demonstrated that all four compounds can be tenuously identified using presumptive testing. Future work should include the development of a chromatographic method using hydrophobic interaction chromatography (HILIC) and perhaps more comprehensive characterisation using presumptive tests with samples attained from matrices such as urine or blood. It is hoped that this work will be used to evaluate the potential metabolic pathways in humans as well as expedite identification in the case of police drug seizures and hospital admittance.

Chapter 3

Administration of Synthetic Cathinones Significantly Affects Some Prey-Capturing Behaviours of *Araneus diadematus* and *Pardosa agrestis.*

Abstract

The NPS market is constantly evolving and with these developments comes the introduction of novel compounds for which no metabolic data is available. Invertebrates have been shown to be good systems for chemical testing; arachnids are particularly useful when assessing the effects of compounds that affect the CNS. Administering substances to orb-weaving arachnid models such as *Araneus diadematus* can affect the webs they spin the following day due to the complexity and sensitivity of their behavioural patterns. We used *A. diadematus* to study the effects of the synthetic cathinone mephedrone (**1b**) and one of its analogs (±)-4'(trifluoromethyl)methylcathinone (4-TFMMC, **2**). We also administered mephedrone (**1b**) to *Pardosa agrestis* to judge whether hunting spiders could be used for behavioural drug testing similarly to *A. diadematus.* 4-TFMMC (**2**) was shown to have no significant effects on a web's appearance and mephedrone (**1b**) significantly affected some aspects of the spiders' webs. Mephedrone (**1b**) webs showed a general trend towards smaller capture spiral and free zone areas and though the latter seemed to recover with time, capture spiral areas did not return to pre-drug levels. Several spiders dosed with mephedrone (**1b**) spun multiple hubs per web and some webs produced were considered completely illegible because of their highly disjointed nature. Mephedrone (**1b**) significantly reduced the maximum amplitude of the capture legs in *P. agrestis* during prey capture, though back legs were not significantly affected. The velocity of the capture legs was also not significantly affected by mephedrone (**1b**). *A. diadematus* appears to be a suitable system for the testing of synthetic cathinones and *P. agrestis* shows promise for future arachnid based drug trials.

Introduction

Invertebrate models are an appealing prospect for chemically induced behavioural analysis as they offer low-risk testing and high sample volume whilst still being able to collect data in a controlled and concise manner (Cattaneo *et al.,* 2009). Arachnids in particular, exhibit precise and complex behaviours and movements when actively hunting and web building that can be measured and quantified. This identifies them as ideal candidates for the exploration of the biological effects of compounds such as mephedrone (**1b**) and other NPS', where human metabolic data may not be available (Hadlock *et al.*, 2011; Capriola, 2013).

Cathinone is a monoamine alkaloid derived from the African shrub *Catha edulis* (Khat; Kalix & Braenden, 1985) and has a similar structure to amphetamine and methcathinone. The NPS mephedrone (**1b**) is an analog of methcathinone and as such is part of the cathinone class of compounds. NPS' are compounds based on preexisting drugs of abuse that have been structurally modified, the aim being to mimic pharmacological effects whilst also avoiding legislation (Schifano *et al.*, 2011). In the UK, the PSA (UK Government 2016) has outlawed all NPS' not already legislated against in the MDA (UK Government, 1971). This blanket ban restricts any substance not otherwise classified under the MDA that has a psychological effect on the user, aiming to eradicate the widespread usage of these ex- 'legal highs' by combatting distribution and sales of substances under nondescript and ambiguous pseudonyms online and in head shops (Vardakou, Pistos & Spiliopoulou, 2011). In many cases a newly developed NPS will exist as an 'enhanced' analog of a pre-existing drug of abuse (Winstock *et al.*, 2010; Ellefsen, Concheiro & Huestis, 2016); this can often be hazardous to an unsuspecting user who may think they are taking something less potent or completely different (Camilleri, *et al.*, 2010).

One of many problems faced when trying to control the NPS market is its constant evolution (UNODC, 2013). (±)-4'-(trifluoromethyl)methylcathinone (4-TFMMC, **2**) is an NPS that is an analog of mephedrone (**1b**) that, to our knowledge, has not yet emerged onto the NPS market. It has been synthesised specifically to act as a more

potent version of its parent compound mephedrone (**1b**) and this has been achieved by the restriction of one of mephedrone's three primary metabolic pathways, preventing CH oxidation from taking place at the *para*-methyl moiety (Khreit *et al*., 2013). It is expected that because of this modification half-life *in vivo* will be greatly increased because of the almost complete prevention of glucuronidation due to a lack of hydroxymethyl-moieties. It was expected that this alteration would prolong the effects of the compound, possibly resulting in greater toxicity within the system.

Araneus diadematus (Cross Orb-weaver) and *Pardosa agrestis* (wolf spiders) were selected for this study due to their abundance in the wild. Both spiders have the ability to perform precise and complex behaviours: *A. diadematus* whilst webbuilding and *P. agrestis* whilst hunting prey, these behaviours are relatively easy to record by either filming or phtotgraphy (Peters & Witt, 1949; Witt & Reed, 1965; Witt, 1971; Reed, Witt & Scarboro, 1982; Noever, Cronise & Relwani, 1995; Hesselberg & Vollrath, 2004; Wyman, Rodenhouse & Bank, 2011; Benamú *et al*., 2013). Although both species are common and abundant predators, they utilise very different mechanisms to capture prey. *P. agrestis* are ground active, visually locating and 'pouncing' on prey (Hardman & Turnbull, 1980; Shaw, Waddicor & Langan, 2005) whilst *A. diadematus* are orb-weaving spiders that spin highly sophisticated and intricate webs (Witt, Reed & Peakall, 1968) based on only a few rudimentary rules (Krink & Vollrath, 1997).

There is a lack of literature concerning chemical testing of ground active species, as such, we will attempt to assess the plasticity of *P. agrestis* as a model system and whether its sensitivity is comparable to that of *A. diadematus* with the administration of mephedrone (**1b**). We also aim to establish whether 4-TFMMC (**2**) and/or mephedrone (**1b**) are biologically active in an orb-weaver system using a developed protocol. For *P. agrestis* we will record if there are any changes in how each leg moves in response to stimuli (prey items) with a focus on velocity of the capture legs and mean leg rotation (amplitude) with the administration of mephedrone (**1b**). For *A. diadematus* we will measure specific web parameters such as capture spiral height,

number of radii and number of spiral threads (Zschokke, 2011) to see whether mephedrone (**1b**) or 4-TFMMC (**2**) administration affects one or more of the analysed web features.

Methods

Experimental design

<u>Collection and Housing</u>

Orb-weavers

A. diadematus were collected by hand exclusively from gorse bushes (*Ulex sp.)* from Ribble Estuary (53.7112° N, 2.9642° W). Only females were used after being allowed to acclimatise to lab conditions (25 ±1 ˚C, 44 ± 5% relative humidity, 16:8 D:N) for 14 days before being dosed. Orb-weavers were individually housed in frames with removable side panels (30x5.5x30 cm, LxWxH) constructed from clear acrylic plastic. Both side panels (30x30 cm) were coated in a thin layer of petroleum jelly to ensure that the webs would not adhere to them during web construction. During the data recording period webs were photographed in the afternoon, sprayed and then left for 10 minutes to allow spiders to ingest droplets from the silk. Spiders were fed once a week, with 6-8 winged prey items (*Drosophila melanogaster*).

Wolf Spiders

Pardosa agrestis were selected due to their abundance in the wild and were collected from the same location as the orb-weavers using pitfall traps and by hand. Both male and female spiders were collected initially with the intent of having a 1:1 male to female ratio but since male mortality was so high, only female wolf spiders were used in the experiment. The wolf spiders were individually housed in pots (5x5x7 cm, LxWxH) and only mature females without egg sacs were used after being allowed to acclimatise to lab conditions (25 ±1 ˚C, 44 ± 5% relative humidity, 12:12 D:N) for at least 14 days. The wolf spiders were starved for seven days before being filmed in a 5.5 cm petri dish. During each filming period spiders were offered two wingless prey items (*D. melanogaster*). Each spider was filmed twice, once prior to dosing and then

again seven days later. The spiders were starved again before being dosed and filmed on the second occasion. Filming took place at the same time on each day of filming under the same temperature, humidity, and lighting conditions to ensure that this did not affect hunting behaviour. Due to the drug being passively ingested the amounts were too small to be measured accurately.

<u>Dosing and Drug Solutions:</u>

Orb-weavers

Before dosing, the spiders were carefully removed from their webs to be weighed; they were then placed back onto their webs and allowed to settle for 30 minutes before being dosed. 50 mmol drug solutions were made by dissolving the compounds in sugar solution composed of 1.5 g d-Fructose per 5 ml distilled water to ensure ready uptake of the drug (Hesselberg & Vollrath, 2004). The solutions were administered *via* a blunt syringe (Witt, 1971) with each spider receiving a single droplet (approx. 0.05 cm^3). After another 30 minutes, the spiders were weighed again to approximate uptake of the drug solution by percentage body mass. *A. diadematus* generally spin new webs every morning but are reluctant to do so unless the web has been compromised in some way (i.e. in the case of prey capture or voluntary ingestion after spraying with water). To ensure that the spiders built new webs each day that were representative of their drugged state, they were partially destroyed using a pyrography tool (Zschokke & Herberstein, 2005). Approximately 70% of the web was destroyed, taking care to leave the majority of the structural silk intact as destruction of too much has been shown to affect some aspects of the web (Zschokke & Vollrath, 2000). The silk debris was then left in the frame for the spider to ingest, as a normal part of web building includes destruction of the web and ingestion of the silk before a new web is spun (Witt & Reed, 1965).

Wolf Spiders

The dosing arena was composed of an inverted 5.5 cm petri dish with a 5.5 cm filter paper placed in the lid, soaked in 1.2 ml of 50 mmol drug solution made with distilled water (Hanna and Hanna, 2013). A dosage of 100 mmol was initially trialled but proved to be fatal so the dosage was lowered to 50 mmol to ensure only sub-lethal effects on the spiders were observed. The spiders were left for 25 minutes to allow them to passively ingest the drug *via* their natural grooming behaviour before being filmed.

Data Collection:

Orb-weavers

Photography:

Webs were photographed against a black backdrop with a Canon EOS 700D digital camera using a EF-S 18-135 mm f/3.5-5.6 IS STM lens. Speed-lights were positioned at either side of the frames to illuminate the web; the following settings gave consistently clear images: ISO: 200-400; aperture: 4.5; shutter-speed: 1/320. After the web was photographed it was misted and left for 10 minutes and then partially destroyed with the pyrography tool. The images were processed using ImageJ (Schindelin *et al.*, 2015) where measurements could be taken digitally (Fig. 1) with a set scale to calculate the desired web metrics (Table 1).

Measurements taken on ImageJ were split into 'web-spiral' metrics, defined as measurements that required a spiral or orb web, and 'non-web' metrics defined as measurements that could be recorded without a conventional web structure being produced. The web-spiral metrics investigated in this study were as follows: average cell height; eccentricity; capture spiral area; and free zone area. The non-web metrics were: number of hubs a spider created; the total number of radii in a structure; and the percentage of perfect radii spun.
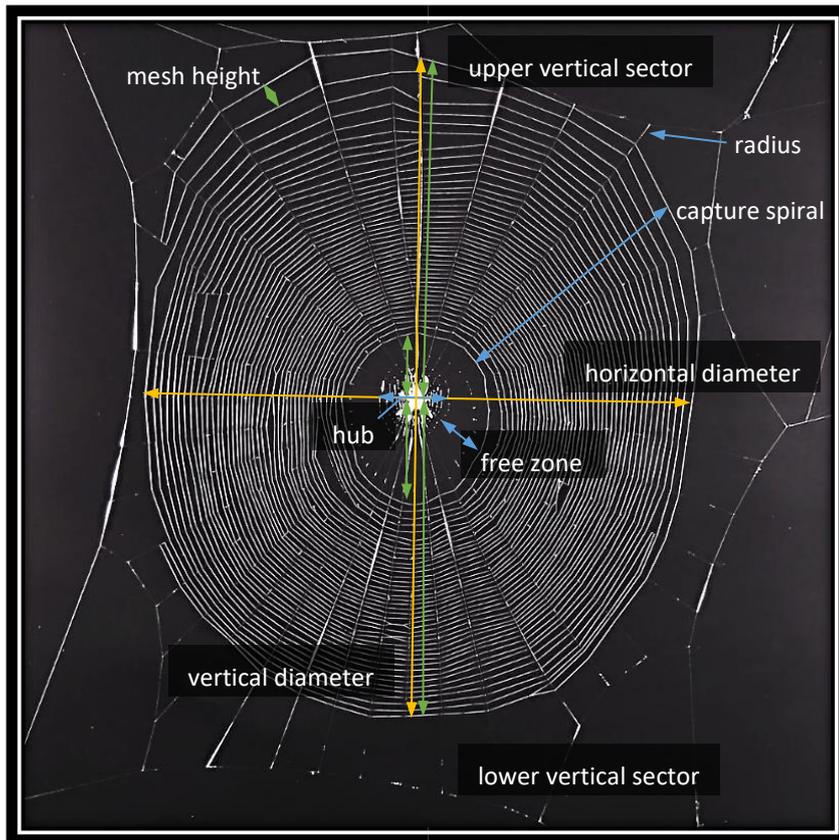
Fig. 1. Diagram of typical *A. diadematus* web features (blue) including metrics used to calculate eccentricity (yellow) and average mesh height (green).

Table 1. Definitions of web and non-web metrics analysed.

Average cell height (cm)	Approximate height of a cell in the capture spiral, a trapezoid shape created by two web radii and two strands of sticky capture silk.	$$\frac{1}{2}\left(\frac{r_u - Hr_u}{S_u - 1} - \frac{r_l - Hr_l}{S_l - 1}\right)$$ Where: r_u – upper vertical capture spiral length r_l – lower vertical capture spiral length Hr_u – upper vertical length of hub and free zone Hr_l – Lower vertical length of hub and free zone S_u – total number of capture spiral turns in the upper vertical sector S_l – total number of capture spiral turns in the lower vertical sector[1]
Mean capture spiral area (cm²)	Average area of the capture spiral of a web excluding the free zone and the hub.	
Eccentricity	A measure of web shape, can be used to gauge the normality of a web. A more positive value would indicate a more normal web and *vice versa*.	$$\sqrt{1 - \left(\frac{width}{height}\right)^2}$$ Where: $width$ - horizontal diameter of capture spiral $height$ – vertical diameter of capture spiral * If the width > height then the bracketed term was inverted and eccentricity assigned as a negative value[1]
Free zone area (cm²)	The area free of silk between the capture spiral and the hub, excluding the hub area.	
Number of hubs	The total amount of hub-like structures a spider constructed.	
Total Radii	The sum of all radii within a web.	
Perfect radii (%)	Undisrupted radii per web as a percentage of total radii. Distorted ('not-perfect') radii were defined as any radii which split into a 'Y' shape or curved more than 5˚ within the capture spiral.	

[1] **(**Herberstein & Tso, 2000)

Wolf Spiders

High-speed video analysis:

Wolf spiders were individually placed in a transparent 5.5 cm petri dish with two wingless prey items. The dish was placed on a glass table and lit from below using a bright infrared light panel (LEDW-BL-400/200-SLLUB-Q-1R-24V, PHLOX). Videoing took place in an artificially lit room to reduce the effects of diminished lighting on hunting behaviour (Baatrup and Bayley, 1993). A filming session generally lasted 1015 minutes, unless a spider caught the two prey items in quick succession. The spiders were videoed from above using a digital high-speed video camera (Phantom Miro ex2) recording at 200 frames per second with a shutter speed of 1 ms and a resolution of 640×480 pixels. Two clips were recorded for each spider by manual trigger: one clip per prey item capture. A total of 24 clips were recorded, and each one was trimmed to contain only the capture movements of the spider-generally 30-80 frames. The clips were then manually analysed using Manual Whisker Annotator (MWA; Hewitt, Yap & Grant, 2016), where each leg was treated as a whisker. Clips containing locomotory movements were also initially considered for analysis but unfortunately were not consistent enough across the sample to quantify. Using MWA, we could track the movement of each individual leg angle frame by frame. For each clip analysed the angle of the legs was determined using the centre line of the spider and the point marking the end of each leg (Fig. 2). The centre line was determined by the placement of a nose and orientation point marked in each tracking frame. Once the angle (θ) of each leg had been found angular velocity, defined as the speed at which a leg moves throughout the duration of a clip, could be calculated. This was done by smoothing the angle signal and then finding the nearest peak from the unsmoothed signal to identify the protraction/retraction cycles, though only protraction is discussed in this study. As both the leg angles and the protraction/retraction cycles had been established, maximum amplitude (˚), defined as the scope of movement of a leg throughout the duration of a clip, could then be

calculated. This was done by calculating the difference in leg angle between the beginning and the end of a movement cycle.
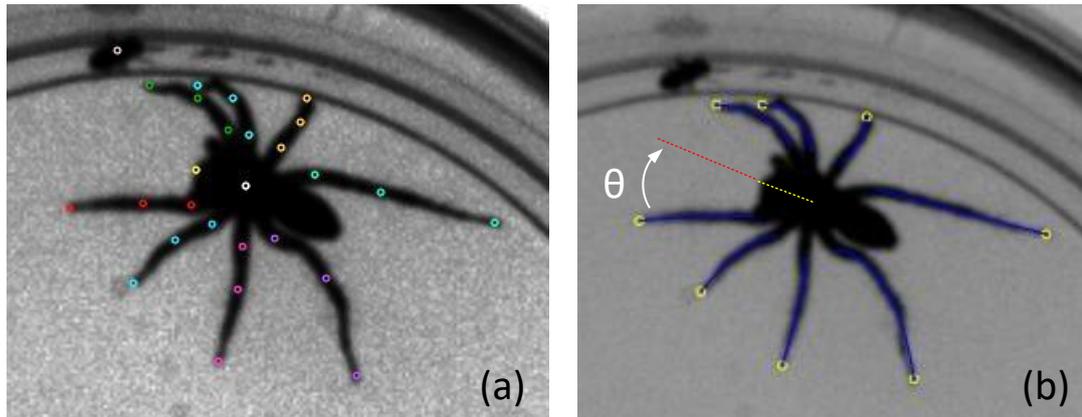

Fig. 2. Video data with overlaid leg tracking (a) showing the three points per leg as well as the nose point (yellow); the orientation point (white); and the prey item marker (pale brown). Analysis of individual leg angle (b) with legs highlighted (blue) as well as each leg tip (yellow). The centre line is marked (yellow) and has been artificially extended (red) to visually demonstrate how mean leg angle (θ) is calculated by the software (MWA).

Data Analysis:

Statistical comparisons between the effects of the drug treatments on web shape *versus* the control were made using a one-way ANOVA or a Kruskal-Wallis test followed by Tukey post hoc tests depending on the data distributions. Wilcoxon signed-rank tests were used to test prey capture variables of the wolf spiders. Comparisons of web building frequency *versus* shape were calculated using a chisquared test. All statistical tests were carried out using the software R v3.4.1 (R Development Core Team, 2012).

Results

Orb-weavers

Mortality rate was low over the course of the experiment, with only one casualty in the 4-TFMMC (**2**) group on day 2. One spider was also excluded from this group as it did not ingest the drug solution, this resulted in 7 spiders being used in the 4-TFMMC (**2**) treatment group.

The effects of mephedrone (**1b**) and 4-TFMMC (**2**) on web geometry are summarised in Table 2. A chi-squared test shows the total counts of web shapes across the whole test period. Mephedrone (**1b**) dosed spiders spun more illegible webs than expected, whilst the control and 4-TFMMC (**2**) treated spiders spun no illegible webs (Fig. 3). Mephedrone (**1b**) dosed spiders also spun less scaffold and corner webs than the other two groups, suggesting that ingestion of the drug has a significant effect on web geometry (X-squared=22.265, df = 3, p-value = 0.001).

Table 2. Observed Frequencies of web shape *vs.* treatment for the orb-weaving spiders across all days analysed.				
Treatment	1. Spiral	2. Illegible	3. Scaffold	4. Corner
Control	26	0	1	6
4-TFMMC	25	0	0	3
Mephedrone	23	6	4	0

Ingestion of the sugar solutions was not consistent between treatments (Fig. 4) with both drugged sugar solutions having a lower uptake than the control (11.63% ±se 0.01). As Hesselberg and Vollrath (2004) suggested, this may imply that the spiders could detect the drugs in the solution and that they may have had an unpleasant taste, 4-TFMMC (**2**; 5.87% ±se 0.01) even more so than mephedrone (**1b**; 9.28% ±se <0.001). It is possible that if a sweeter solution had been used then uptake may have been higher, though then problems with the solubility of the drug in the more saturated solution would have become an issue (Hesselberg & Vollrath, 2004).
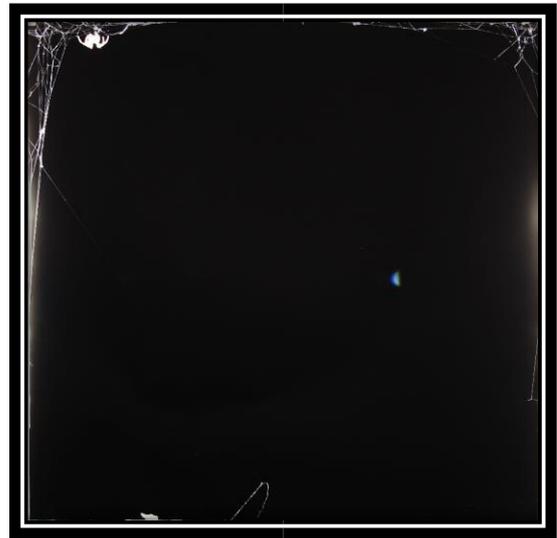
1. Spiral

2. Illegible

3. Scaffold

4. Corner

Fig. 3. Web categories based on their overall appearance: Spiral, where a capture spiral was visible; Illegible, where no meaningful measurements could be taken from the web due to its highly disjointed nature; Scaffold, where some structural silk was present, but no capture spiral; and Corner, where the spider had created a structure in the corner of a frame that could not be considered illegible or as structural silk.
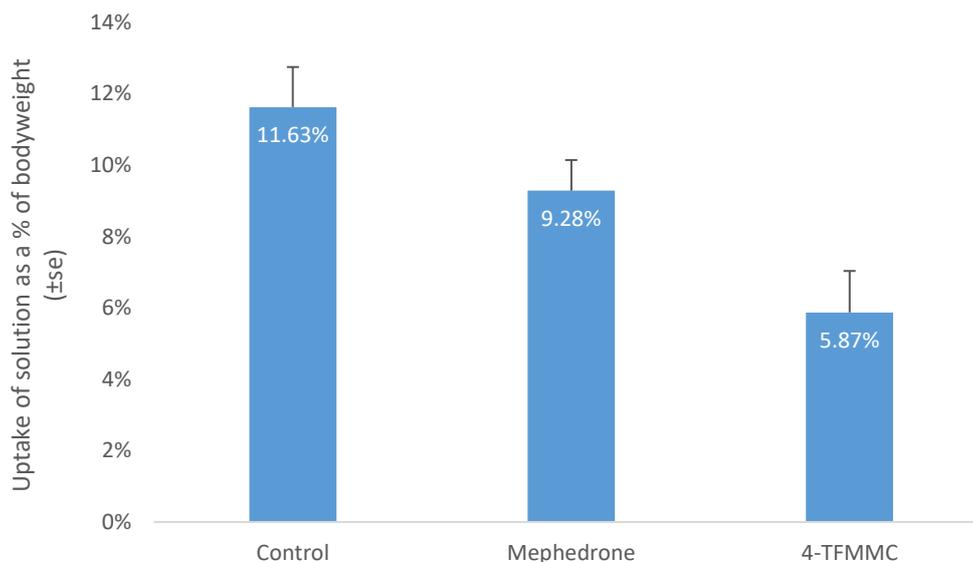
Fig. 4. Mean amount (±se) of sugar solution ingested by *A. diadematus* calculated as a percentage of bodyweight for all three treatments. The control treatment refers to a control sugar solution containing no drug.

Spiders dosed with mephedrone (**1b**) spun significantly less perfect radii than the controls and 4-TFMMC (**2**) dosed spiders on day 2 (Chi=6.71, p= 0.035, *df*= 2; Fig. 5). There was an upward pattern towards more perfect radii for the mephedrone (**1b**) dosed spiders over the course of the study; on day 16 the percentage of perfect radii of mephedrone (**1b**) treated spider was similar to controls. Although not significant, mephedrone (**1b**) spiders also spun more hubs on day 16 (Chi= 5.84, p=0.054) than the control and 4-TFMMC (**2**). There were no differences in the total number of radii spun between treatments across the recording period (Table S.I-2.0).

For the web-spiral metrics only capture spiral area and free zone area were found to show significant differences between treatments (Fig. 6; Table S.I-2.0). Free zone area was significantly smaller on day 2 for the mephedrone dosed spiders (**1b**; f=7.814, p=0.008, *df*=2) when compared to the control and 4-TFMMC (**2**) dosed spiders. There was also a significant reduction in free zone area for the mephedrone webs on day 4 (**1b**; f=3.663, p=0.049, *df*=2) though the post hoc test reported no significant difference between groups. The observed increase in free zone area across the recording period indicates that this aspect of the webs, for the mephedrone dosed

spiders, slowly became more regular. Throughout the study capture spiral area remained consistently small for the mephedrone (**1b**) treated spiders, with no apparent recovery to control levels. Although consistently smaller, only the webs spun by the mephedrone (**1b**) spiders on day 8 showed a significant reduction in capture spiral area compared to the control and 4-TFMMC (**2**, f=4.437, p=0.027, *df*= 2).
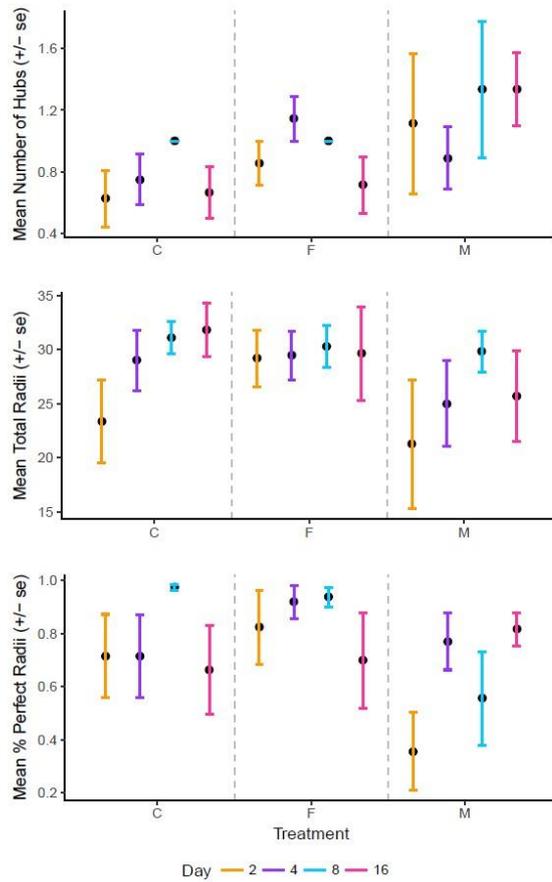

Fig. 5. Mean (±se) of non-web metrics across the four recording periods. Plots depict (topbottom) mean number of hubs; mean total radii; mean percentage perfect radii across all treatments: control (C), 4-TFMMC (**2**; F), and mephedrone (**1b**; M).
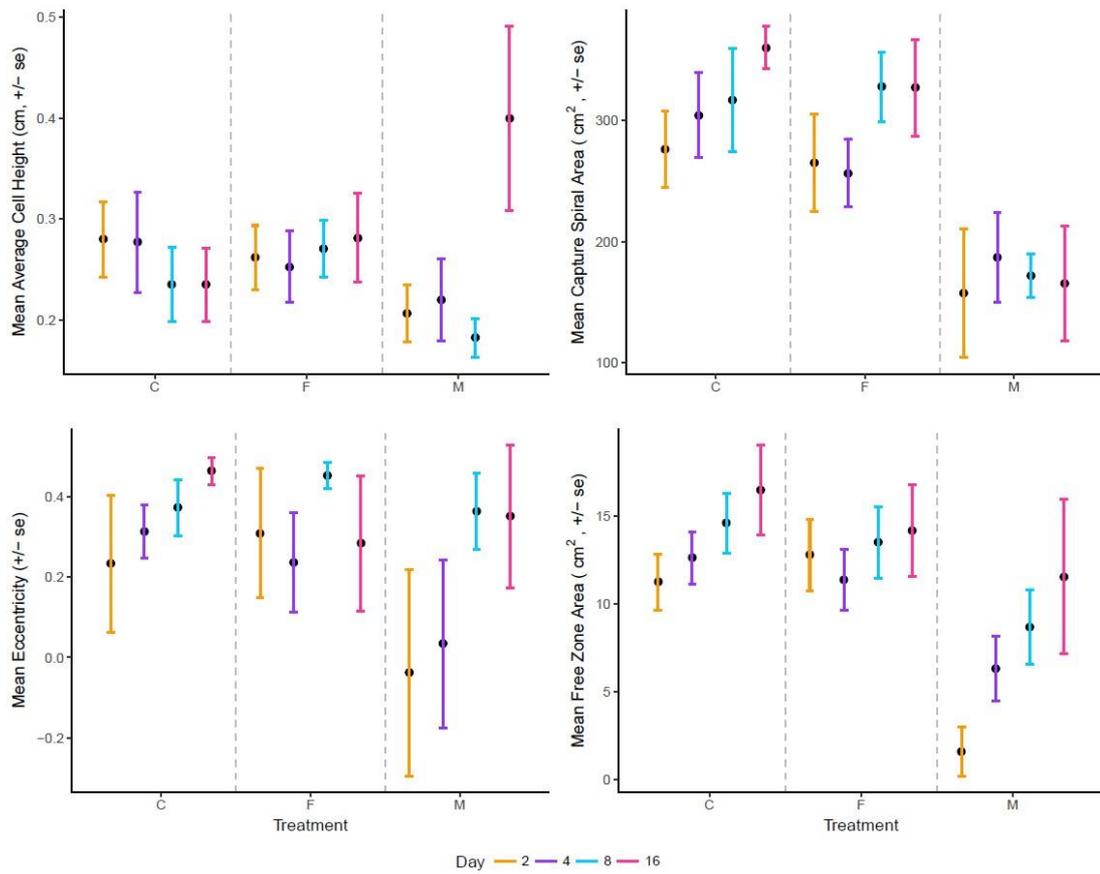
Fig. 6. Mean (±se) of web-spiral metrics depicting (L-R): mean average cell height (cm); mean capture spiral area (cm^2); mean eccentricity; mean free zone area (cm^2) across all treatments: control (C), 4-TFMMC (**2**; F), and mephedrone (**1b**; M).

Wolf Spiders

A week after the spiders were treated and filmed only one of the mephedrone dosed wolf spiders had died. Although all three prey capture metrics show a reduction after mephedrone administration, only the maximum amplitude of the capture legs was significantly reduced compared to controls (W=1153, p=0.001; Fig. 7; Table S.I-3.0).
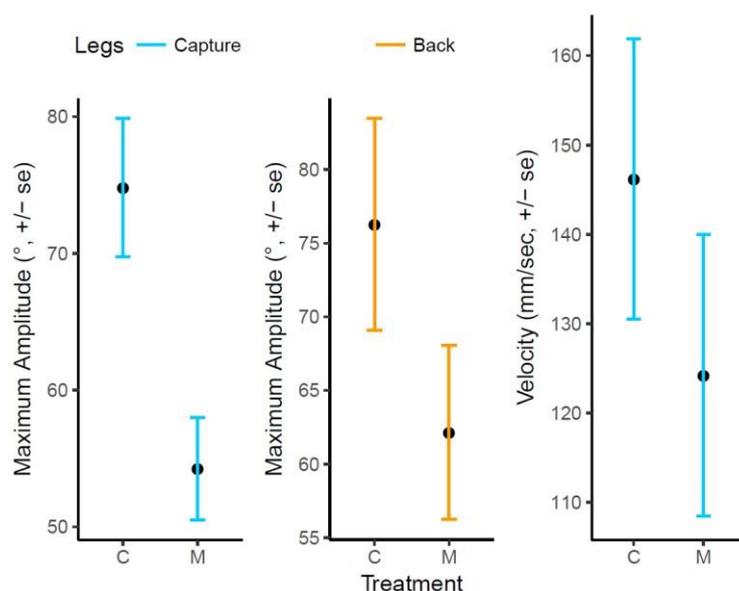
Fig. 7. Mean (±se) of maximum amplitude (°) for both capture (blue) and back (orange) legs, velocity (mm/sec) for the capture legs (blue) of the wolf spiders treated with either a control sugar solution (C), or a mephedrone dosed sugar solution (**1b**; M).

Discussion

This study investigated the effects of mephedrone (**1b**) and 4-TFMMC (**2**) on webbuilding in *A. diadematus*. Whilst the effects of 4-TFMMC (**2**) were not significantly different to controls, it was found that mephedrone (**1b**) significantly altered webs metrics. Recovery after 16 days was witnessed but not in all measured web metrics. We also aimed to test the plasticity of prey capture metrics in *P. agrestis* after administration of mephedrone (**1b**) as an alternative system to web-based chemical testing. It was found that the synthetic cathinone did have a significant effect on the capture metrics of *P. agrestis*, suggesting that it is a viable system for testing behavioural impairment.

Orb-weavers

Though webs were generally similar within treatment groups, individual variation occurred from spider to spider. Despite web-building being carried out in a very formulaic manner (Foelix, 2011), it is well known that the spinning habits of *A. diadematus* vary depending on numerous circumstances such as age, weight, where previous structural silk has been placed; temperature; and humidity (Witt, Reed & Peakall, 1968; Vollrath, Downes & Krackow, 1997). These biotic and abiotic factors can affect how a web is spun and thus its geometry. Some drugged individuals in the current study spun webs that resembled those of the control group, despite having ingested a similar amount of solution as a drugged spider that produced an illegible web. Despite this intragroup variation, the results of this study generally align with previous work with regards to the expected effects of mephedrone (**1b**) being similar to *d*-amphetamine (Witt & Reed, 1965; Reed, Witt & Scarboro, 1982; Hesselberg & Vollrath, 2004) in so much as that a trend towards smaller webs and reduced building frequency was observed.

Witt and Reed (1965) reviewed the effects of drug application on web-spinning behaviour and reported that methamphetamine increases building frequency and web size, a compound which arguably is more structurally similar to mephedrone (**1b**) than *d*-amphetamine. The reported effects of methamphetamine are actually more similar to what was observed when dosing spiders with 4-TFMMC (**2**); though not statistically significant, the individuals given 4-TFMMC (**2**) spun more uniform webs than the control on days 2 and 4. It should be noted however, that these similarities could simply be as a result of the decreased ingestion of the 4-TFMMC (**2**) solution having less of an effect on web-building (Reed & Witt, 1968). As well as the reduced dosage, the effects of the 4-TFMMC (**2**) in comparison to mephedrone (**1b**) could have arisen as a result of 4-TFMMC (**2**) being metabolised more rapidly than mephedrone (**1b**) and/or being metabolically unavailable in this particular spider system.

It was feared that the spiders would reject the drugged sugar solutions administered *via* syringe as they would when given distilled water in the same manner but it seems

that *A. diadematus* are more inclined to take a sweetened solution of water even when it contains a foreign substance. Studies have been carried out wherein spiders were starved of water prior to dosing to ensure ready uptake of the solution (Samu and Vollrath, 1992), though this option was not pursued as we did not want to risk desiccation of the subjects; as the spiders were only fed once a week and derive most of their liquid uptake from flies, it was feared that a significant loss of body mass might occur and affect the quality of the webs (Zschokke & Herberstein, 2005). The dose of mephedrone (**1b**) administered to the spiders, with an average uptake of 9.28%, was sufficient enough to have an effect on web spinning. The uptake of 4TFMMC (**2**) was considerably lower (5.87%) and compared to the control uptake (11.63%) suggests that the drug could be detected and potentially that it has a more noticeable taste than mephedrone (**1b**). The dosages of the drug solutions used in this paper were selected to be analogous to that of a human dose, as it was discovered that *A. diadematus* are surprisingly resistant to high doses of amphetamine type compounds (Witt, 1971).

The effects of a high dose of amphetamine (Witt & Reed, 1965; Hesselberg & Vollrath, 2004) are comparable to those seen when a spider was dosed with mephedrone (**1b**) in the current study, with smaller capture spirals and free zones being spun. During the recovery period, the area of the free zone steadily increased but capture spiral area remained at a diminished size for the remainder of the study, though this may be as a result of *A. diadematus* often building within the constraints of any previously laid structural silk (Zschokke & Vollrath, 2000). These observed patterns would suggest that mephedrone (**1b**) affects the sensory control of the spider, similarly to amphetamine (Peters, Witt, & Wolff, 1950). Visual observation approximately 20 minutes after administration of the drugged sugar solution appears to support this conclusion, with spiders observed hanging from their webs reaching for empty space whilst spinning and twitching sporadically. This effect was also noticed in the spiders' behaviour when they were reweighed, seeming more sluggish and less inclined to leave weighing pots. By day 8 there was a large change in web eccentricity of the

mephedrone (**1b**) webs, with spiders in this treatment group producing less eccentric webs (expressed as more positive values). This likely indicates a recovery from the mephedrone (**1b**) drugging at this time, expressed as the free zone area and the eccentricity returning to similar values compared to the controls. Conversely, average mesh size remains relatively consistent throughout the experiment regardless of treatment, possibly suggesting that it is not affected by reduced sensory control at these doses. The large value on day 16 for the mephedrone (**1b**) dosed spiders being the exception, resulting from several of the more severely affected spiders beginning to spin disjointed but interpretable spiral shaped webs, instead of illegible structures that prohibited the measurement of spiral metrics.

The non-web metrics recorded provide information on how consistent a web is despite not including any information about the capture spiral. For all non-web metrics both the control and 4-TFMMC (**2**) treatments produced very similar values. The number of hubs was generally 1 if a spider spun a web, and 0 if not. For the percentage of perfect radii, the control and 4-TFMMC (**2**) treatments consistently produced webs with values between 75-100%, with at least 20 radii per web. Combined with the results derived from the spiral web metrics, 4-TFMMC (**2**) dosed spiders spun webs that did not statistically deviate from the control group webs at a 50 mmol dosage. On the other hand, the webs spun by spiders dosed with mephedrone (**1b**) often contained multiple hubs per structure, with those structures recorded as illegible until recovery and production of more consistent spirals towards the end of the study. Multiple hubs was a unexpected and novel phenomenon that has not been reported in previous studies discussing the administration of psychoactive substances to *A. diadematus* (Peters & Witt, 1949; Witt & Reed, 1965; Witt, 1971; Reed, Witt & Scarboro, 1982; Noever, Cronise & Relwani, 1995; Hesselberg & Vollrath, 2004). The author speculates that multiple hubs may have arisen due to the sensory disorientation of the spiders, though it should be noted that its occurrence persisted throughout the study despite the percentage of perfect radii and the total number of radii showing a general trend towards recovery.

Wolf Spiders

As ground active spiders would not accept sugar droplets from a syringe, the drug solution was administered passively to *P. agrestis* following the methodology of Hanna and Hanna (2013). Coating prey items was considered as an alternative but prey could not be dosed without killing them and dead prey items were ignored by the wolf spiders, likely due to these visual hunters requiring prey movement for detection (Ford, 1978). Passive ingestion does appear to be an effective way to dose *P. agrestis*, though it is limited in its use due to the inability to calculate the exact dose ingested, as such it is more suitable for studies trying to assess contact dosage rather than the ingestion of compounds.

Administering mephedrone (**1b**) to *P. agrestis* significantly reduces capture leg movement during prey capture with a significant decrease in the maximum amplitude of capture legs after dosing, meaning a smaller arc of movement when compared to pre-drug controls. Although this pattern was similar in non-capture (back) legs the difference was not significant. Visual observations after dosing showed spiders exhibiting less exploratory behaviour, often only pouncing on prey items when they came into contact with the subject. There was also a slight decrease in the velocity of the capture legs but this result was not significant. If we assume that mephedrone (**1b**) has a similar effect on *P. agrestis* as *A. diadematus*, this could suggest that protraction velocity of the capture legs is not affected by sensory disruption or potentially that the speed at which the legs extend towards the prey is predetermined by the spider's individual physiology (Casas, Steinmann & Dangles, 2008). All spiders filmed caught both prey items offered, even with seemingly reduced mobility, indicating that appetite was not affected by the drug. This conclusion is in agreement with research carried out on wolf spiders exposed to cypermethrin that showed no effect on appetite despite suffering from locomotory sub-lethal effects (Shaw, Waddicor & Langan, 2005).

Conclusion

The administration of mephedrone (**1b**) had significant effects on the behaviours of both *A. diadematus* and *P. agrestis.* The latter shows promise as a novel system that could be used in behavioural drug studies. We recorded novel behaviour in the form of multiple hubs for dosed orb-weaving spiders and we also identified recovery of some, but not all, web metrics after day 16 of the study. The administration of 4TFMMC (**2**) did not have any significant effects on web building in *A. diadematus* at the given dosage (50 mmol). It is suggested that the effects of mephedrone (**1b**) on *P. agrestis* and *A. diadematus* are similar from the perspective of reduced movement and exploratory behaviour, though this link is only made tenuously with the knowledge that more in-depth analysis of the *P. agrestis* system is required. Using *P. agrestis* as a behavioural drug testing system does seem like a viable optionparticularly if using orb-weaving spiders is not feasible due to availability of lab resources or geographical restrictions, though the author suggests finding a more effective and quantifiable route of drug application. The present study was not designed to establish the effects the compounds would have on a spider's CNS, as such, an interesting extension of this research would be to examine the effects of novel synthetic cathinones on invertebrate neurobiology to further explore the mechanisms that drive the changes in behaviour.

Chapter 4

Concluding Remarks

Organic Synthesis

Previous work by Khreit *et al.* (2013) reported the synthesis and characterisation of 4-TFMMC (**2**); here we report the successful synthesis and characterisation of its novel metabolites: R.4-TFMMC (**3**), 4-TFMC (**4**), and R.4-TFMC (**5**). The author suggests that tenuous identification of the compounds using presumptive tests is possible and that all four compounds can be separately identified using ^{13}C NMR, though further work will need to be carried out to verify if separation and identification is possible from a mixture. Future work also includes developing a chromatographic method for the separation of the four compounds using a HILICbased column to aid in potential identification of samples by analytical bodies. The synthesis and characterisation of other metabolites of 4-TFMMC (**2**; Khreit *et al.,* 2013) and NPS' is also an interesting prospect when considering the fast-paced nature of the NPS market.

Animal Behaviour

The effects that mephedrone (**1b**) had on the webs of *A. diadematus* broadly agree with literature discussing *d*-amphetamine in the same system (Witt & Reed, 1965; Reed, Witt & Scarboro, 1982; Hesselberg & Vollrath, 2004). 4-TFMMC (**2**) had no significant effect on the webs but future work should include the trial of a sweeter solution to see if percentage ingestion and/or the effect of the drug on the system increases. The filming of the webs during construction (Hesselberg & Vollrath, 2004) and resulting time budgets would also be an ideal way to see if the drugs had any effects on the spiders that weren't reflected in the webs they produced. This technique could also be used to try and reveal the reasons behind occurrences such as multiple hubs and the how illegible webs were spun. Future work concerning the effects of mephedrone (**1b**) and 4-TFMMC (**2**) on the CNS' of the two spider models

would also aid in the understanding of exactly how the recorded behaviours were affected.

No previous work has been done using *P. agrestis* as a behavioural drug model, though this thesis establishes that they have the potential once a robust protocol has been put in place to not only evaluate metrics associated with hunting, but locomotory behaviours too. In addition, further investigation should be done into how to accurately quantify the amount of drug the spider ingests. Whether this would entail an adjustment of Hanna and Hanna's (2013) methodology, which was used to assess passive pesticide dosage, or dosage by some other means.

For studies carried out over a longer period of time, the author recommends using spiders bred within the lab to help eliminate environmental variation that may otherwise be present. It should be noted however, that it is a time-consuming process with a very high spiderling mortality rate (Zschokke & Herberstein, 2005). Once further development and refinement of these methods has taken place, the possibility for the testing of other compounds within the bounds of the spider systems discussed in this study seems like a reasonable next step. In addition to further drug testing, investigation into the effects of these substances on a spider's CNS would also aid the comprehensive understanding of how the spider system's behavioural mechanisms are affected.

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Appendix

Table SI-1.0 Compound Structure and accurate mass

Compound Name/Number	Structure	Exact mass found
4-TFMMC/2		232.0949
R.4-TFMMC/3		234.1108
4-TFMC/4		218.0793
R.4-TFMC/5		220.0949
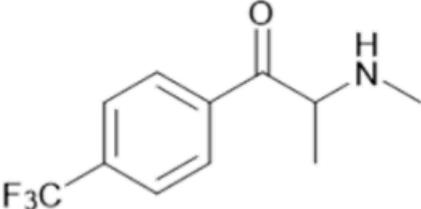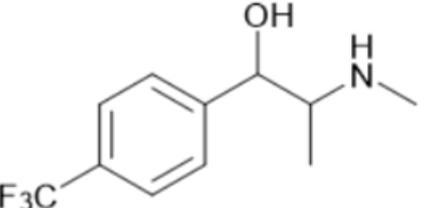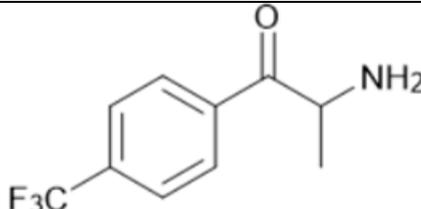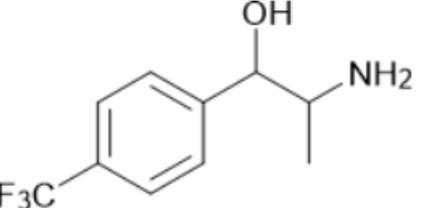
Table SI-2.0 Statistical results for *A. diadematus*

Day	Variable	Mean & SD for Treatment			Test Statistic		p value	Df
		Control	4-TFMMC	4-MMC				
D2	% Perfect Radii	0.72 ±0.44[a]	0.82 ±0.37[a]	0.36 ±0.44[b]	Chi	6.709	0.035	2
D2	Average Cell Height	0.28 ±0.08[a]	0.26 ±0.08[a]	0.21 ±0.05[a]	Chi	2.751	0.253	2
D2	# Hubs	0.63 ±0.52[a]	0.86 ±0.38[a]	1.11 ±1.36[a]	Chi	0.641	0.726	2
D2	# Radii	23.33 ±9.35[a]	29.17 ±6.49[a]	21.25 ±11.95[a]	f	1.069	0.372	2
D2	Free Zone Area	11.25 ±3.58[a]	12.78 ±4.98[a]	1.60 ±2.39[b]	f	7.814	0.008	2
D2	Eccentricity	0.23 ±0.38[a]	0.31 ±0.39[a]	−0.04 ±0.44[a]	Chi	1.989	0.370	2
D2	Capture Spiral Area	276.06 ±70.61[a]	265.30 ±98.51[a]	157.59 ±92.00[a]	f	1.945	0.189	2
D4	% Perfect Radii	0.71 ±0.44[a]	0.92 ±0.17[a]	0.77 ±0.32[a]	Chi	1.074	0.585	2
D4	Average Cell Height	0.28 ±0.12[a]	0.25 ±0.09[a]	0.22 ±0.10[a]	Chi	0.967	0.617	2
D4	# Hubs	0.75 ±0.46[a]	1.14 ±0.38[a]	0.89 ±0.60[a]	Chi	2.327	0.312	2
D4	# Radii	29.00 ±6.78[a]	29.43 ±6.02[a]	25.00 ±11.24[a]	Chi	0.770	0.681	2
D4	Free Zone Area	12.61 ±3.65[a]	11.37 ±4.56[a]	6.33 ±4.52[a]	f	3.663	0.049	2
D4	Eccentricity	0.31 ±0.16[a]	0.24 ±0.33[a]	0.03 ±0.51[a]	Chi	0.630	0.730	2
D4	Capture Spiral Area	304.22 ±85.93[a]	256.55 ±74.03[a]	186.92 ±90.64[a]	f	3.017	0.077	2
D8	% Perfect Radii	0.97 ±0.03[a]	0.94 ±0.10[a]	0.56 ±0.53[a]	Chi	0.548	0.760	2
D8	Average Cell Height	0.24 ±0.11[a]	0.27 ±0.07[a]	0.18 ±0.04[a]	Chi	4.480	0.107	2
D8	# Hubs	1.00 ±0.00[a]	1.00 ±0.00[a]	1.33 ±1.32[a]	Chi	0.000	1.000	2
D8	# Radii	31.11 ±4.46[a]	30.29 ±5.09[a]	29.80 ±4.15[a]	f	0.144	0.867	2
D8	Free Zone Area	14.59 ±5.13[a]	13.50 ±5.39[a]	8.68 ±4.69[a]	f	2.229	0.136	2
D8	Eccentricity	0.37 ±0.21[a]	0.45 ±0.09[a]	0.36 ±0.21[a]	Chi	0.451	0.798	2
D8	Capture Spiral Area	316.91 ±128.77[a]	327.64 ±75.78[a]	172.12 ±40.26[b]	f	4.437	0.027	2
D16	% Perfect Radii	0.66 ±0.50[a]	0.70 ±0.48[a]	0.82 ±0.18[a]	Chi	0.512	0.774	2
D16	Average Cell Height	0.24 ±0.09[a]	0.28 ±0.10[a]	0.40 ±0.27[a]	Chi	2.552	0.279	2
D16	# Hubs	0.67 ±0.50[b]	0.71 ±0.49[ab]	1.33 ±0.71[a]	Chi	5.840	0.054	2
D16	# Radii	31.11 ±6.05[a]	29.60 ±9.71[a]	25.67 ±12.56[a]	f	0.676	0.522	2
D16	Free Zone Area	16.47 ±6.23[a]	14.15 ±5.81[a]	11.54 ±12.40[a]	f	0.479	0.628	2
D16	Eccentricity	0.46 ±0.08[a]	0.28 ±0.37[a]	0.35 ±0.53[a]	Chi	1.061	0.588	2
D16	Capture Spiral Area	359.96 ±42.99[a]	326.75 ±88.79[ab]	165.67 ±141.47[b]	Chi	5.671	0.059	2

Table SI-3.0 Statistical results for *P. agrestis*

Test	Variable	Mean & SD for Treatment		Test Statistic		p value	Df
		Control	4-MMC				
1a	Max Amplitude	74.81 ±31.58[a]	54.25 ±24.26[b]	W	1153	0.001	1
1a	Velocity	146.18 ±98.05[a]	124.22 ±102.27[a]	W	954	0.204	1
1b	Max Amplitude	76.27 ±45.58[a]	62.14 ±39.19[a]	W	1046	0.139	1

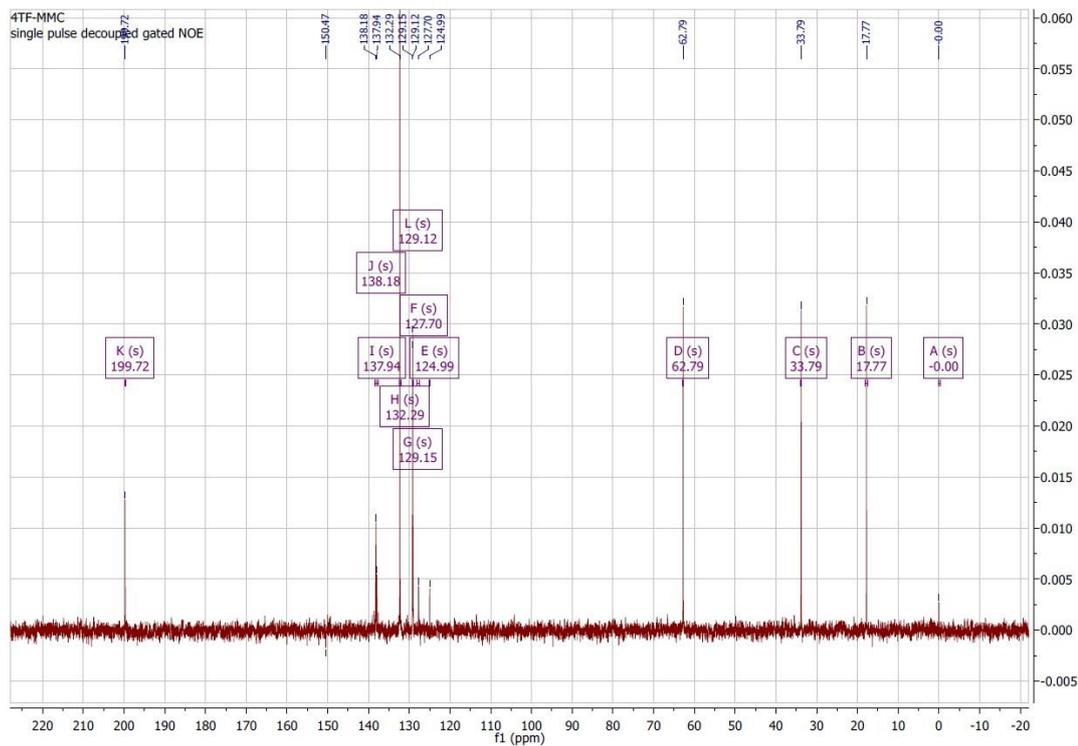Figure SI-1.0 ^{13}C NMR Spectrum of 4-TFMMC (**2**)

Figure SI-1. 1 ^{13}C NMR Spectrum of R.4-TFMMC (**3**)
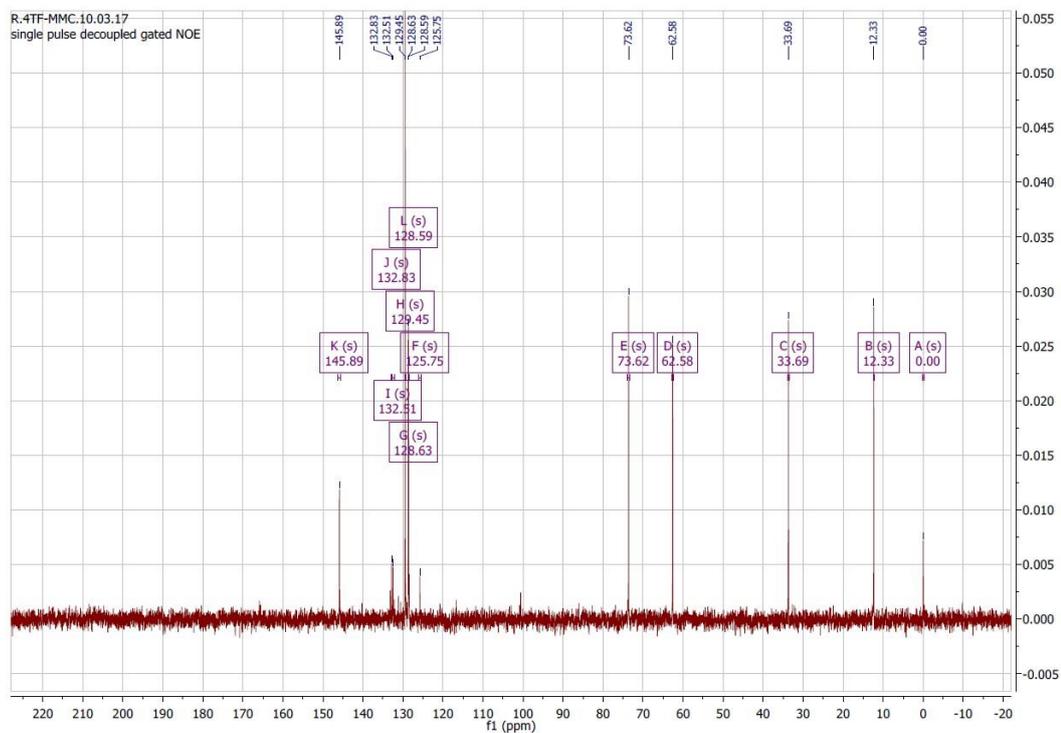
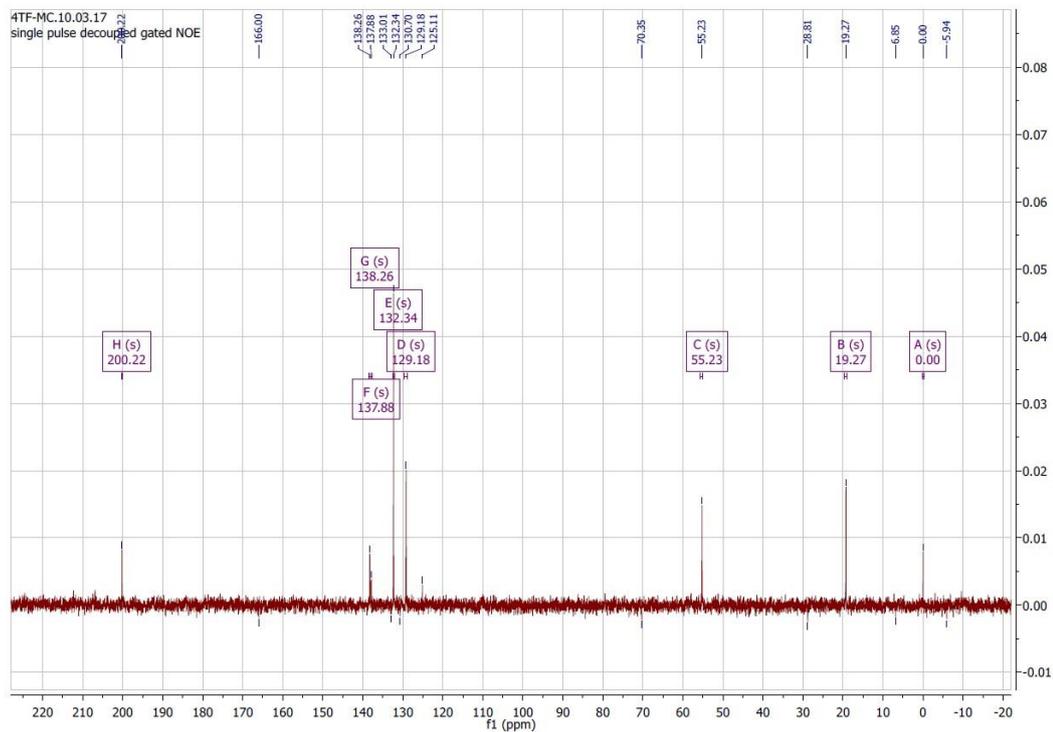Figure SI-1. 2 ^{13}C NMR Spectrum of 4-TFMC (**4**)

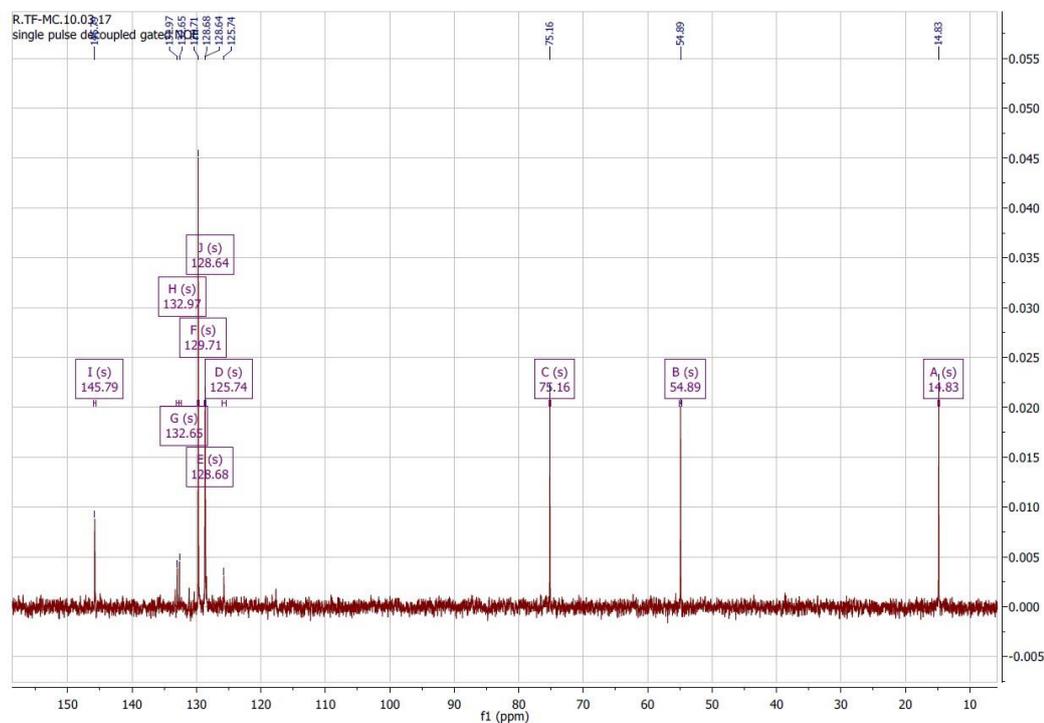Figure SI-1. 3 ^{13}C NMR Spectrum of R.4-TFMC (**5**)

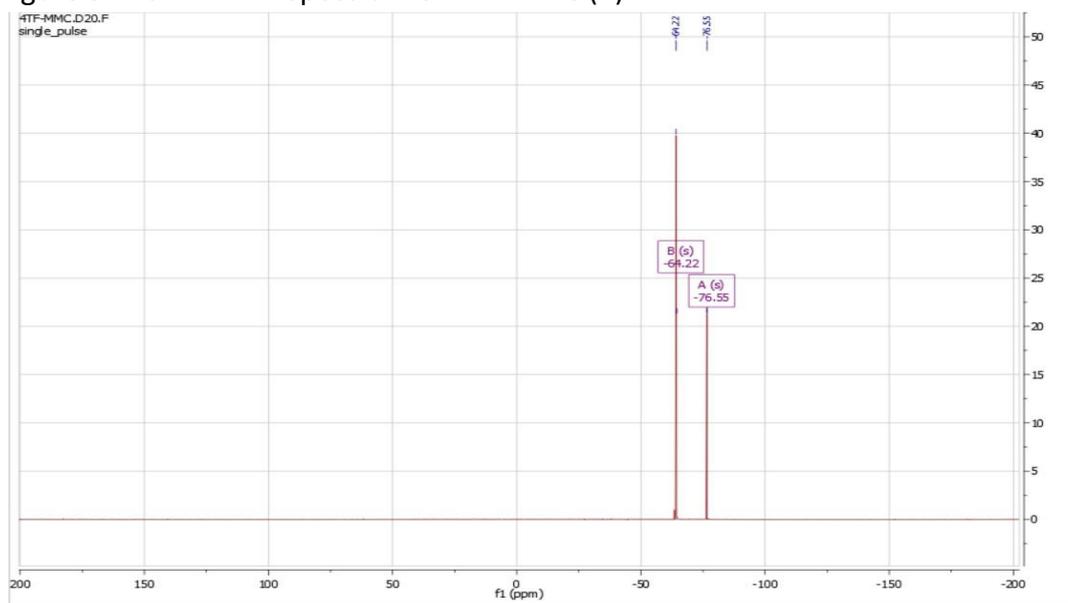Figure SI-2.0 ^{19}F NMR Spectrum of 4-TFMMC (**2**)
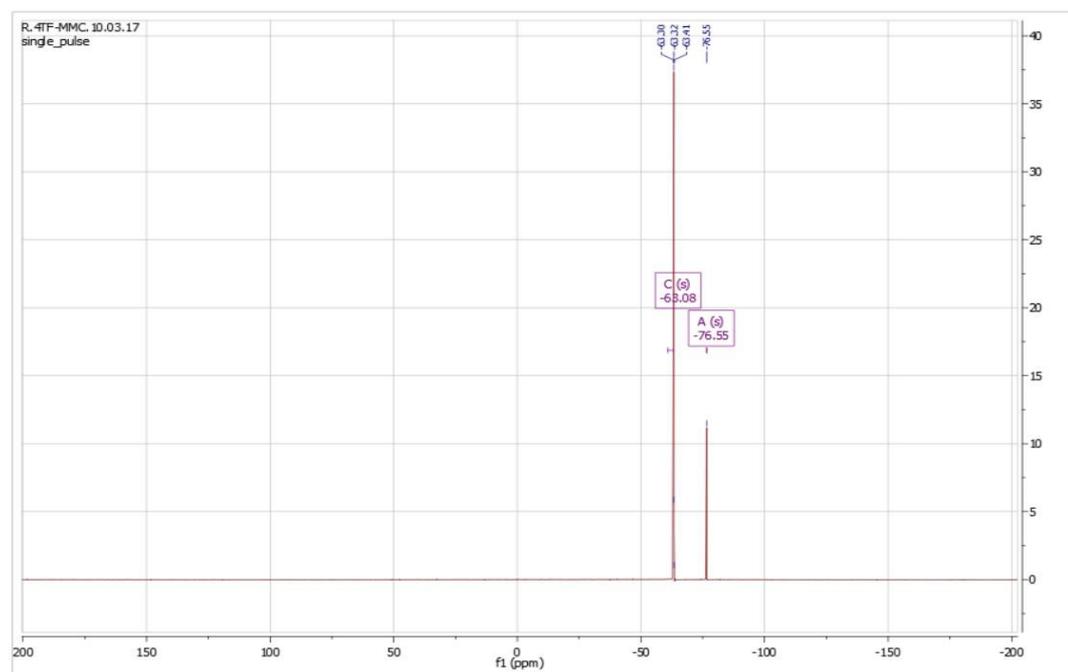

Figure SI-2.1 ^{19}F NMR Spectrum of R.4-TFMMC (**3**)
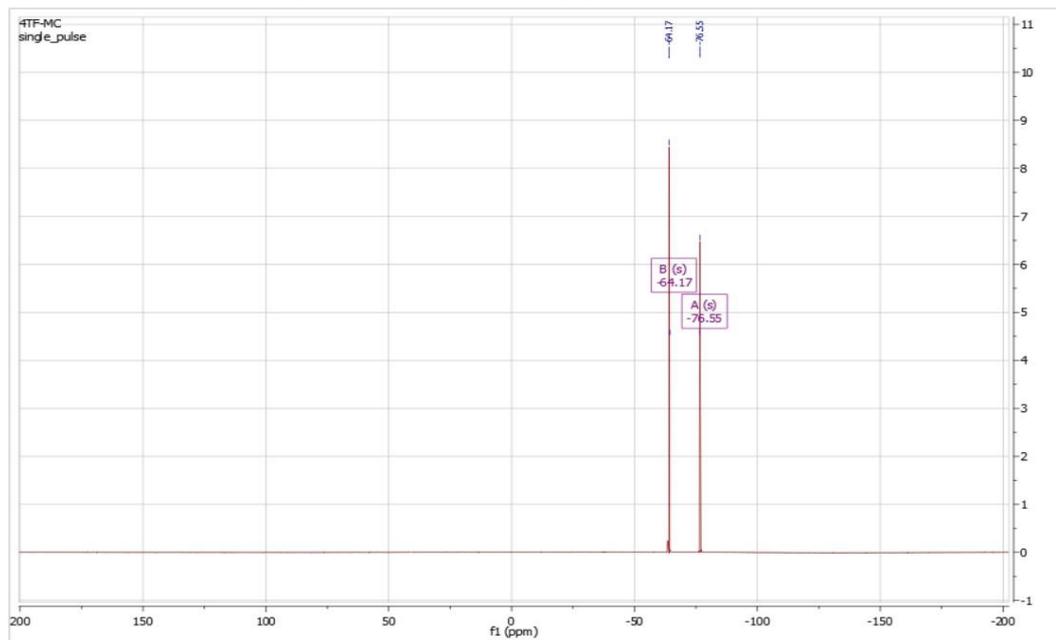
Figure SI-2.2 ^{19}F NMR Spectrum of 4-TFMC (**4**)
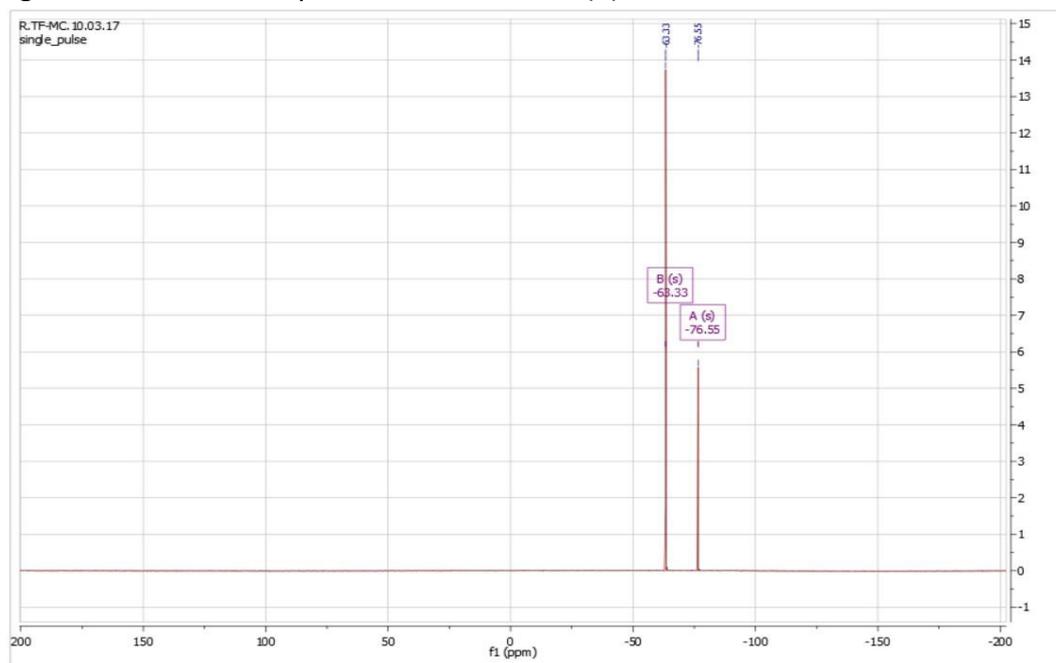

Figure SI-2.3 ^{19}F NMR Spectrum of R.4-TFMC (**5**)

Figure SI-3.0 Gradient enhanced HMBC spectrum of 4-TFMMC (**2**)
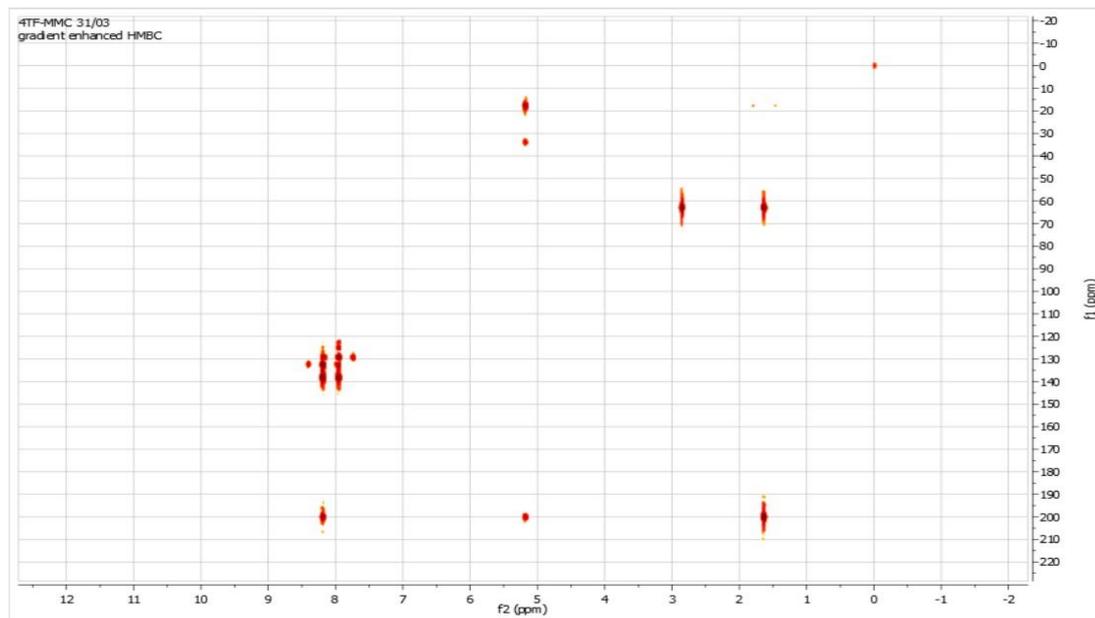
Figure SI-3.1 Gradient enhanced HMBC spectrum of R.4-TFMMC (**3**)
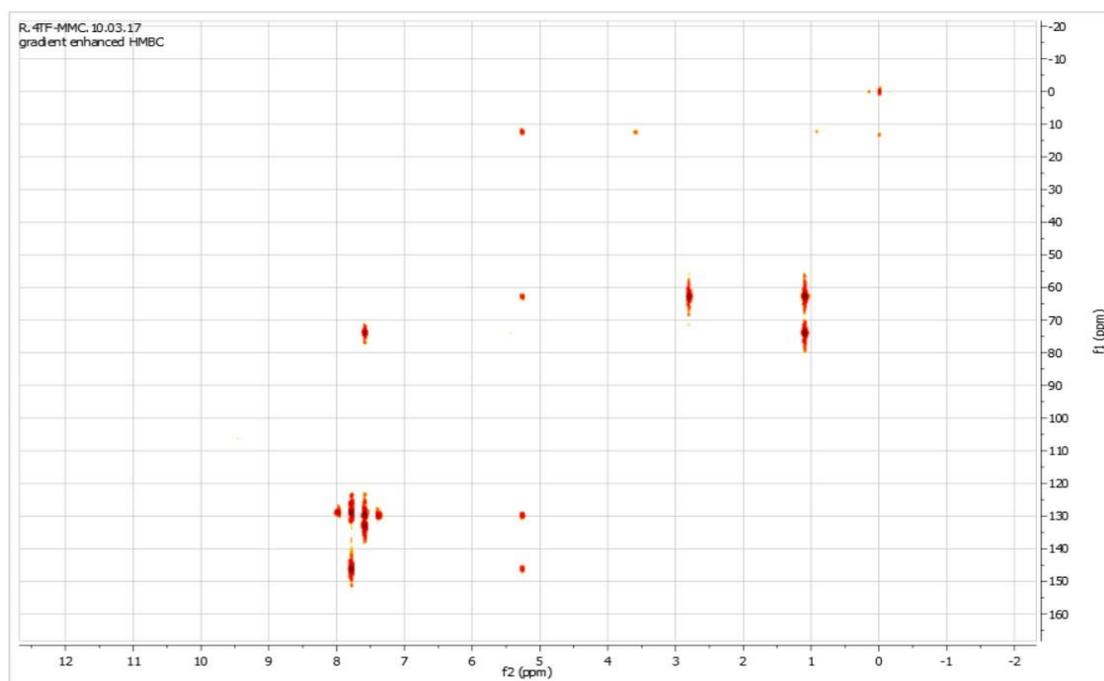
Figure SI-3.2 Gradient enhanced HMBC spectrum of 4-TFMC (**4**)
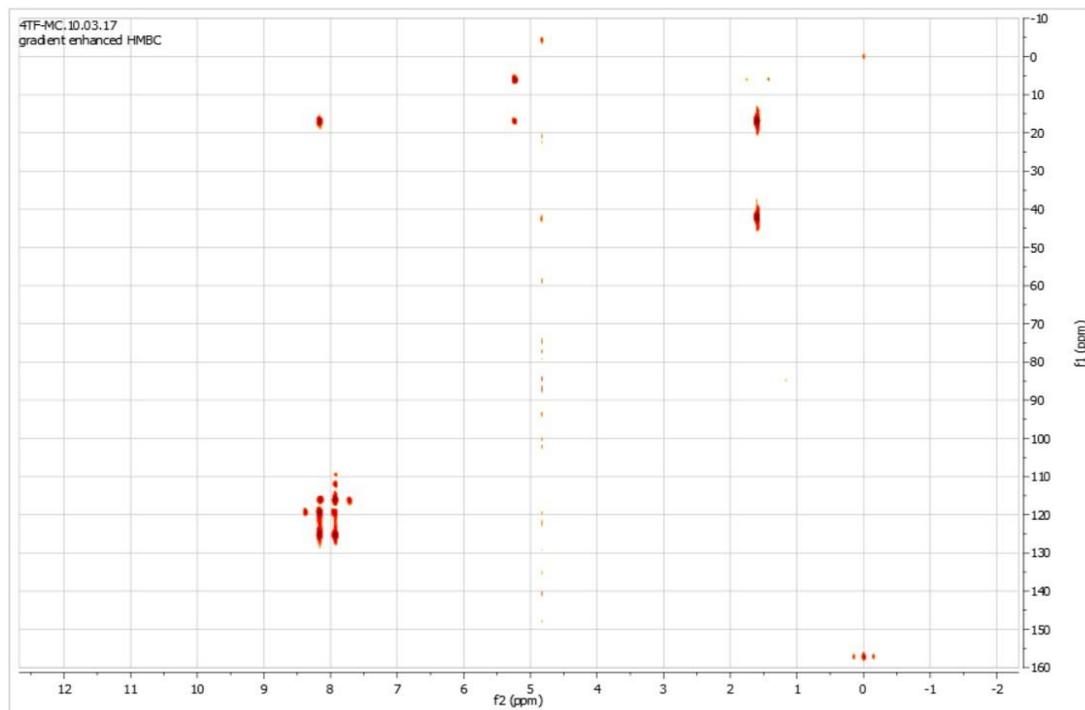
Figure SI-3.3 Gradient enhanced HMBC spectrum of R.4-TFMC (**5**)
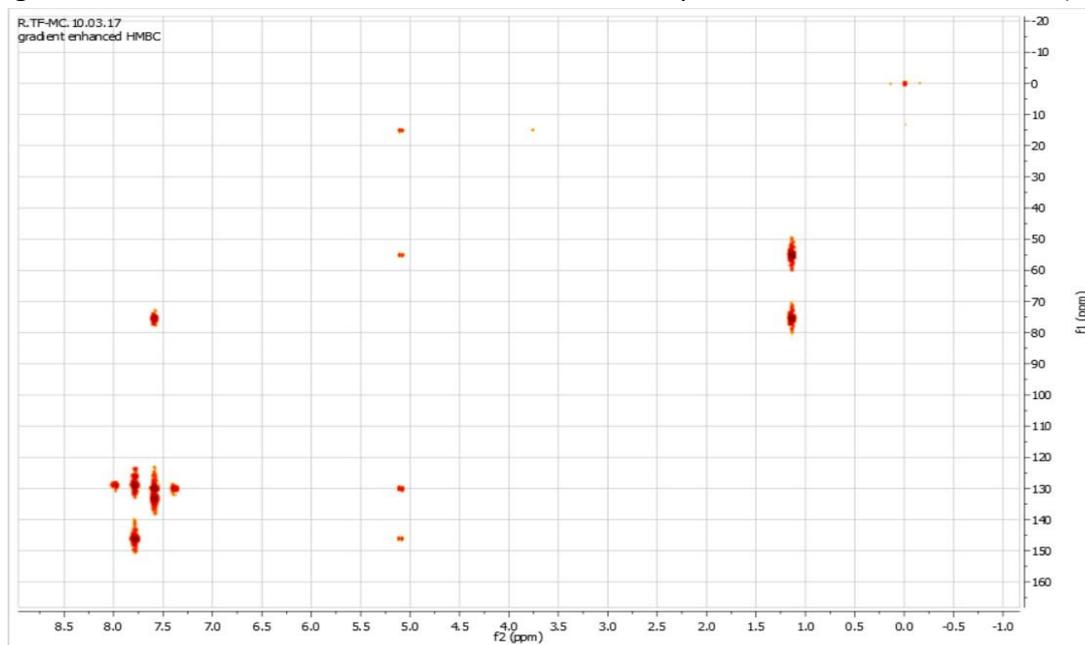
Figure SI-4.0 Gradient enhanced HMBC with X-decoupling spectrum of 4-TFMMC (**2**)
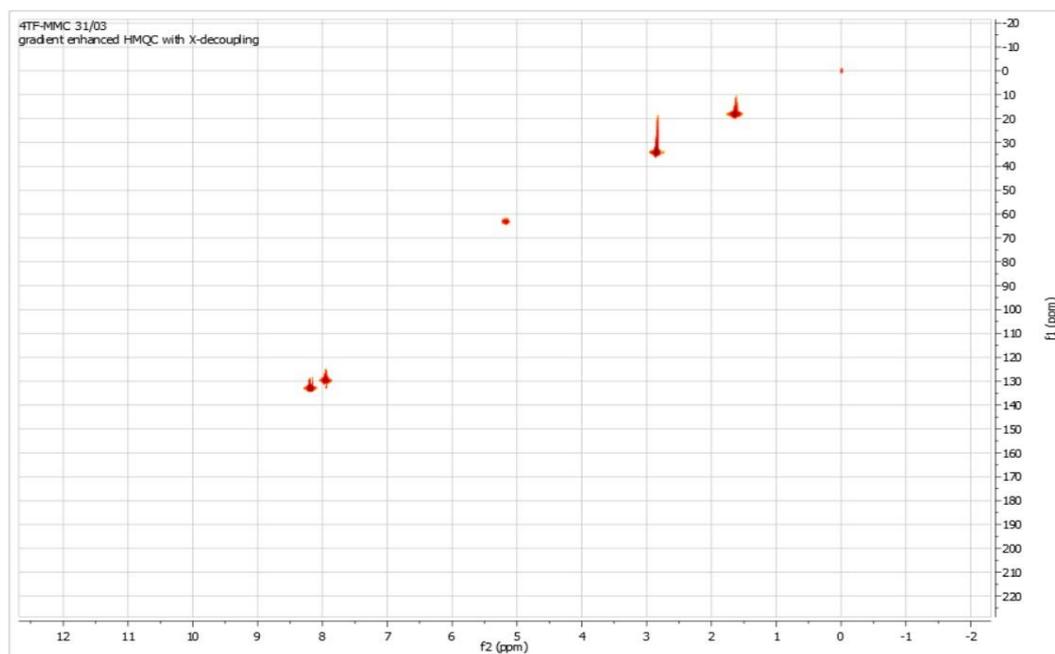
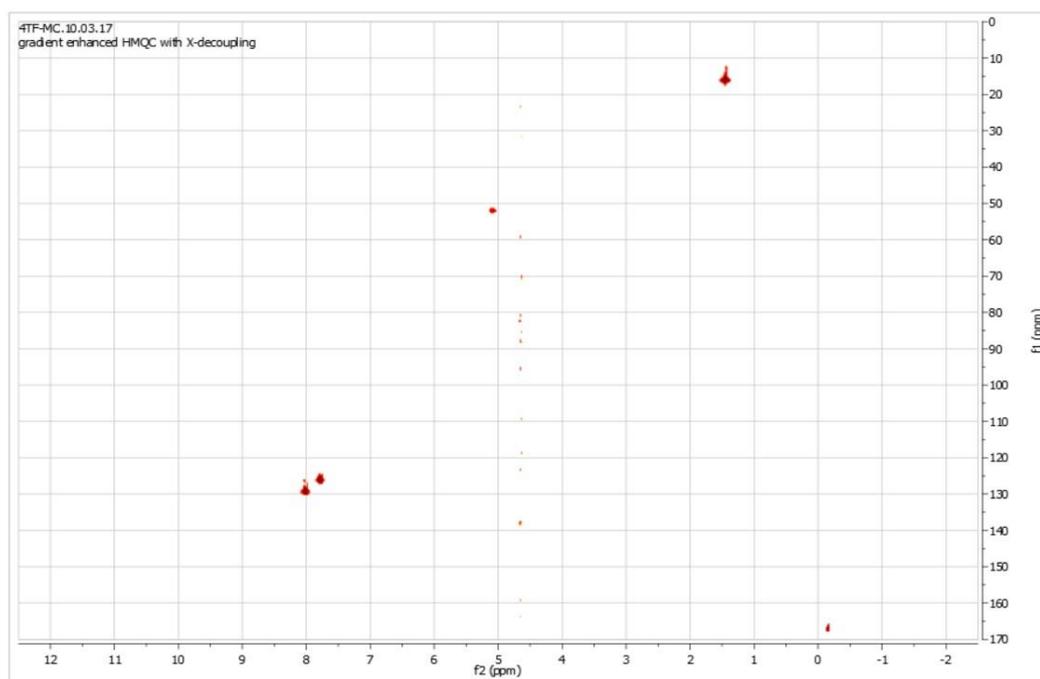Figure SI-4.1 Gradient enhanced HMBC with X-decoupling spectrum of R.4-TFMMC (**3**)
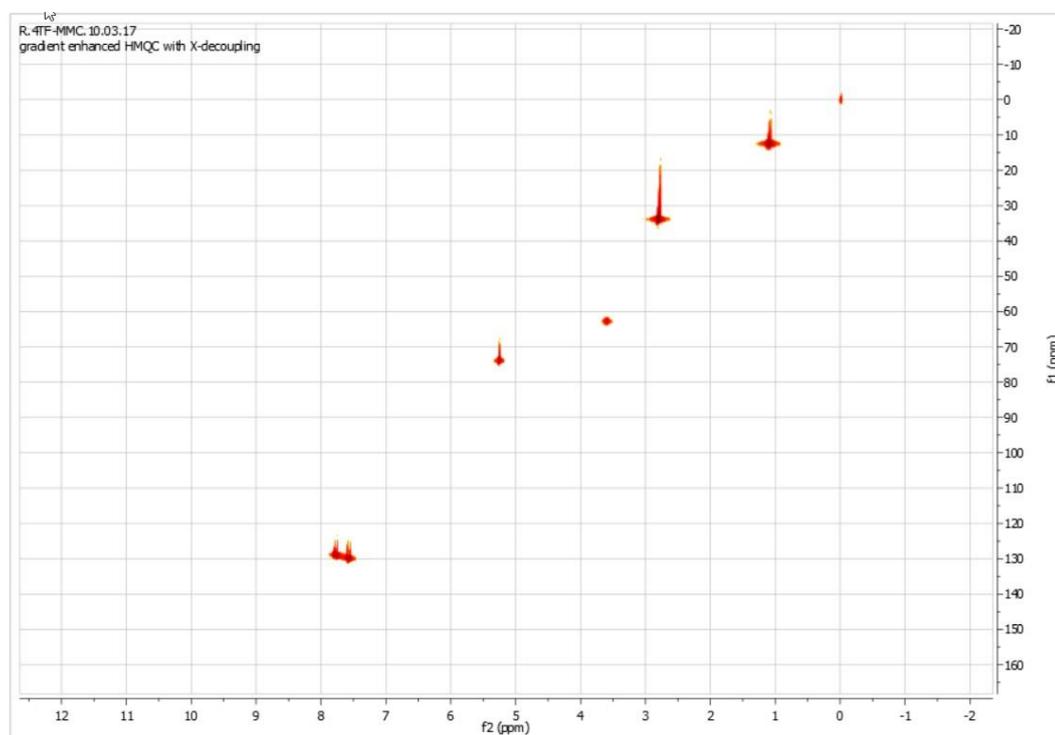
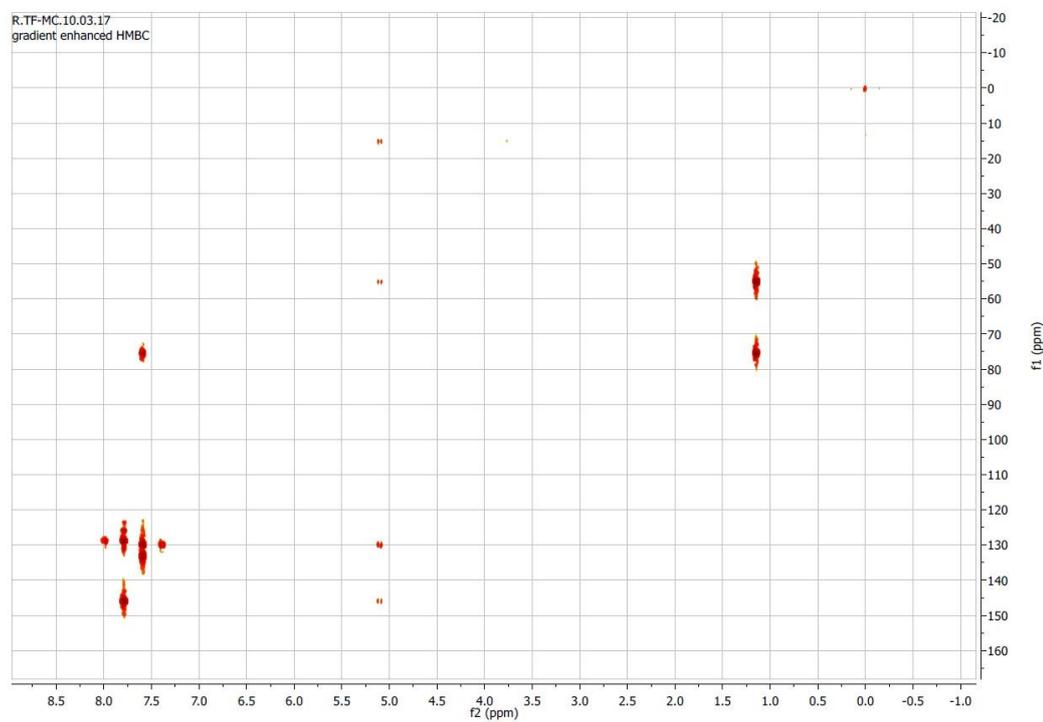Figure SI-4.2 Gradient enhanced HMBC with X-decoupling spectrum of 4-TFMC (**4**)

Figure SI-4.3 Gradient enhanced HMBC with X-decoupling spectrum of R.4TFMC (**5**)
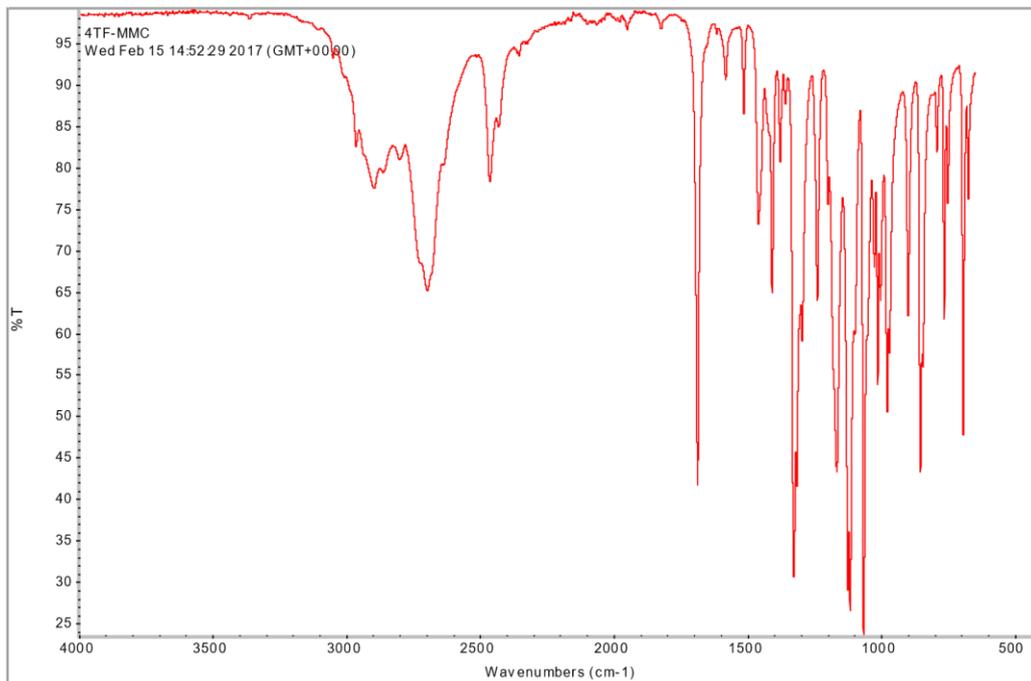
SI-5.0 FTIR Spectrum of 4-TFMMC (**2**)

4TF-MMC
Wed Feb 15 14:52:29 2017 (GMT+00:00)

Figure SI-5.1 FTIR Spectrum of R.4-TFMMC (**3**)

R.4TF-MMC
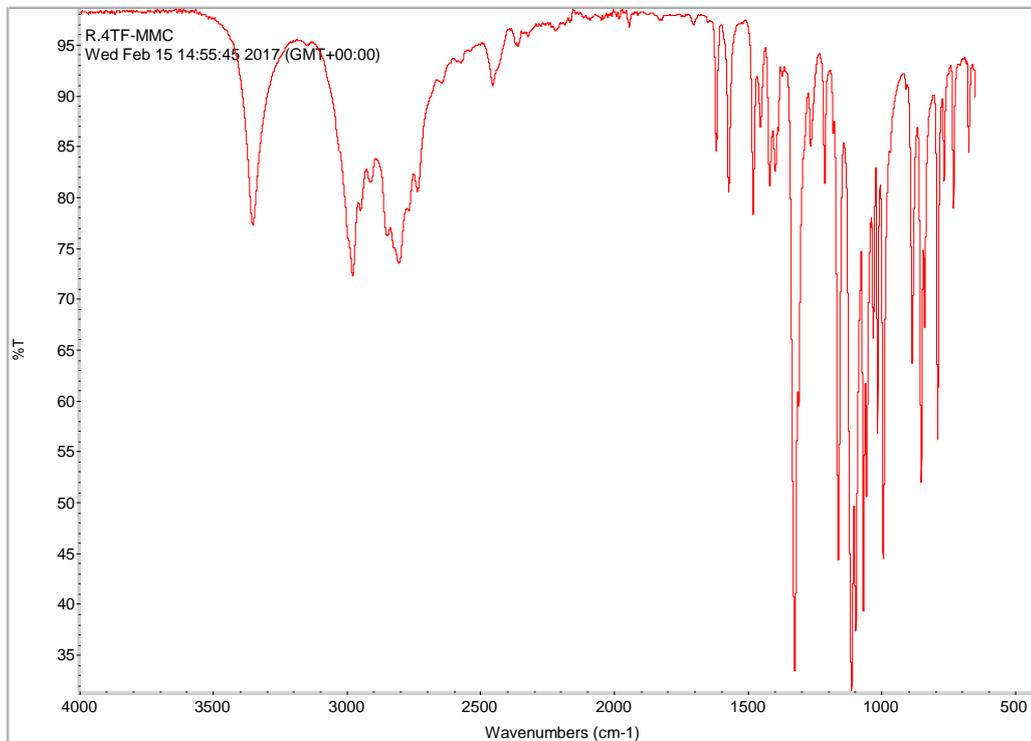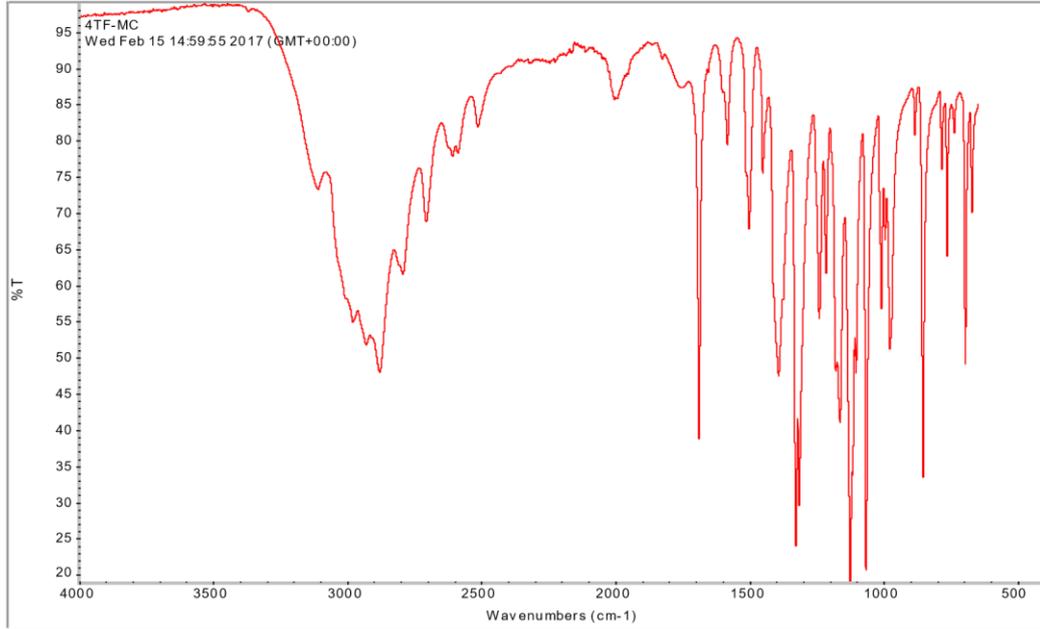Wed Feb 15 14:55:45 2017 (GMT+00:00)

Figure SI-5.2 FTIR Spectrum of 4-TFMC (**4**)

Figure SI-5.3 FTIR Spectrum of R.4-TFMC (**5**)