Drug Testing and Analysis (accepted, uncorrected)

The synthesis and characterization of the 'research chemical' *N*-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(cyclohexylmethyl)-3-(4-fluorophenyl)-1*H*-pyrazole-5-carboxamide (3,5-AB-CHMFUPPYCA) and differentiation from its 5,3-regioisomer.

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Abstract

This study presents the identification of *N*-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(cyclohexylmethyl)-3-(4-fluorophenyl)-1*H*-pyrazole-5-carboxamide that was termed 3,5-AB-CHMFUPPYCA. This compound was obtained from a UK-based Internet vendor, who erroneously advertised this 'research chemical' as AZ-037 and which would have been associated with (*S*)-*N*-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(5-fluoropentyl)-5-(4-fluorophenyl)-1*H*-pyrazole-3-carboxamide. The presence of the pyrazole core indicates a bioisosteric replacement of an indazole ring that is frequently associated with synthetic cannabinoids of the "PINACA", "FUBINACA" and "CHMINACA" series. The pyrazole ring system present in 3,5-AB-CHMFUPPYCA gives rise to the regioisomer *N*-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(cyclohexylmethyl)-5-(4-fluorophenyl)-1*H*-pyrazole-3-carboxamide (named 5,3-AB-CHMFUPPYCA) and both isomers were synthesized using two specific routes which supported the correct identification of the 'research chemical' as 3,5-AB-CHMFUPPYCA. Both isomers could be conveniently differentiated. Interestingly, a route specific chlorine-containing by-product also was observed during the synthesis of 3,5-AB-CHMFUPPYCA and identified as *N*-(1-amino-3-methyl-1-oxobutan-2-yl)-4-chloro-1-(cyclohexylmethyl)-3-(4-fluorophenyl)-1*H*-pyrazole-5-carboxamide. An extensive analytical characterization included chromatographic, spectroscopic, mass spectrometric platforms as well as crystal structure analysis. The syntheses and analytical characterizations of both AB-CHMFUPPYCA isomers are reported for the first time and it serves as a reminder that the possibility of mislabeling of 'research chemicals' cannot be excluded. The pharmacological activities of both AB-CHMFUPPYCA isomers remain to be explored.

Introduction

Over the past decade, the phenomenon linked to new psychoactive substances (NPS) has attracted great interest from various communities and stakeholders that are concerned with public health, law enforcement and a range of fundamental sciences. The diversity of compounds that show, or are suspected to show, psychoactive properties in humans and that are made available to the general public by various routes include synthetic replacements for controlled substances, designer drugs in the traditional sense, regulated and unregulated medicinal products and derivatives of various biological activities developed by the pharmaceutical industry.[1] The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) is currently monitoring over 450 NPS via the European Union (EU) Early warning system (EWS) on NPS and it has become clear that the number of notified synthetic cannabinoids has risen to over 130.[2,3] The number of substances, the diversity in their chemical structure and the rate of their emergence make synthetic cannabinoids one of the largest family of NPS monitored at European level and it is equally obvious that this is a more widespread phenomenon (e.g.[4,5]). A number of recently occurring synthetic cannabinoids included the emergence of substituted indazole core structures that carry a valinamide component. Examples may be found in the so-called “PINACA”, “FUBINACA” and “CHMINACA” series (Figure 1A) and a range of studies have been published[6-20] in addition to recent developments in the United States of America where some of these newly emerging substances were subjected to temporary placement into Schedule 1.[21,22]

A more recent example in which the indazole core is replaced by a pyrazole ring, thus creating a bioisosteric system that mimics the indazole ring system, can be found in (*S*)-*N*-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(5-fluoropentyl)-5-(4-fluorophenyl)-1*H*-pyrazole-3-carboxamide that is currently advertised by a number of Internet retailers as the synthetic cannabinoid AZ-037. Following previously suggested conventions in naming structurally related substances, the name 5,3-5F-AB-FUPPYCA is suggested (Figure 1B). Although the identification of the structurally related 3,5-5F-ADB-FUPPYCA has been recently reported[23] (Figure 1B), detailed data on AZ-037 have not yet been described. The present study describes the characterization of a sample advertised as AZ-037 that was donated in January 2015 by an online retailer based in the United Kingdom. This sample was subjected to analytical characterization by gas chromatography (GC) and high performance liquid

chromatography (HPLC) coupled to various forms of mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR). These investigations revealed that the material was inconsistent with the structural features associated with AZ-037. Instead, the substance was characterized as *N*-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(cyclohexylmethyl)-3-(4-fluorophenyl)-1*H*-pyrazole-5-carboxamide, which was termed 3,5-AB-CHMFUPPYCA. Incidentally, the detection of this compound has just been reported to the EMCDDA by members of the EU EWS on NPS, and was termed AB-CHMFUPPYCA.[24] Further confirmation was obtained from organic synthesis of this substance along with its alternative regioisomer *N*-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(cyclohexylmethyl)-5-(4-fluorophenyl)-1*H*-pyrazole-3-carboxamide (5,3-AB-CHMFUPPYCA) that arises from presence of the pyrazole core structure. Both isomers were synthesized using two specific routes and subjected to extensive analytical characterization using chromatographic, spectroscopic, mass spectrometric platforms as well as crystal structure analysis. The 'research chemical' obtained from the Internet vendor was consistent with a compound isolated from plant material in Japan where it was termed AB-CHFUPYCA although further confirmation by synthesis of the two possible isomers was not obtained.[25]

Experimental

Reagents and standards

All reagents and dry solvents used in the syntheses were obtained from Sigma Aldrich Ltd. (Arklow, Ireland). LC-MS grade solvents were obtained from Fisher Scientific (Dublin, Ireland). A sample, advertised as AZ-037, was donated by a UK based 'research chemical' vendor in January 2015.

Syntheses

Methyl 4-(4-fluorophenyl)-2,4-dioxobutanoate

Potassium *tert*-butoxide (11.20 g, 100 mmol) was added to a solution of dimethyl oxalate (11.80 g, 100 mmol) in tetrahydrofuran (75 mL). The mixture was stirred at room temperature for 30 min. A solution of 4-fluoroacetophenone (6.4 g, 50 mmol) in tetrahydrofuran (25 mL) was added and stirring was continued for 1 h. The reaction mixture was then added to 0.5 M aqueous hydrochloric acid (600 mL) and the

precipitated solid was collected by filtration to afford a light yellow solid (9.54 g, 43 mmol, 86 %): ESI-HRMS *m/z* found 225.0568 (*m/z* theor. for M+H, $C_{11}H_{10}O_4F$, 225.0558). ^{1}H NMR (d_6-$CDCl_3$) δ 7.77-7.69 (m, 2H, 2 x Ar-CH), 7.23-7.14 (m, 2H, 2 x Ar-CH), 7.05 (s, 1H, OH), 3.96 (d, *J* = 1.2 Hz, 3H), ^{13}C NMR (d_6-$CDCl_3$) δ 189.71 (C=O), 168.67-167.05 (Ar-CF), 165.35 (C(OH)), 162.53 (C=O), 131.27 (Ar-C), 130.50 (Ar-CH), 116.34 (Ar-CH), 53.20 (CH_3), ^{19}F (d_6-$CDCl_3$) δ –103.91 ppm.

Methyl 5-(4-fluorophenyl)-1H-pyrazole-3-carboxylate

A mixture of methyl 4-(4-fluorophenyl)-2,4-dioxobutanoate (4.48 g, 20 mmol) and hydrazine hydrate (1.10 g, 22 mmol) in acetic acid (20 mL) was heated (oil bath at 100 ^{o}C) for 2 h. The mixture was allowed to cool to room temperature and the product was collected by filtration to afford colorless crystals that were used without further purification (3.08 g, 14.0 mmol, 70 %): ESI-HRMS *m/z* found 221.0725 (*m/z* theor. for M+H, $C_{11}H_{10}O_2N_2F$, 221.0721).

Cyclohexylmethyl)hydrazine trifluoroacetate salt

A mixture of *tert*-butyl carbazate (5.89 g, 44.6 mmol) and cyclohexanecarboxaldehyde (5.00 g, 44.6 mmol) in methanol (140 mL) was stirred at room temperature for 1 h. The solution was then evaporated to dryness. Aqueous 50 % v/v aqueous acetic acid (125 mL) was added and, with constant mixing, sodium cyanoborohydride (2.80 g, 44.6 mmol) was added. The mixture was stirred for 2 h at room temperature, diluted with water, neutralized with sodium hydroxide and extracted with dichloromethane. Drying (anhydrous magnesium sulfate) and removal of the solvent afforded a colorless oil, which was dissolved in a mixture of dichloromethane (30 mL) and trifluoroacetic acid (30 mL). The mixture was stirred for 1 h at room temperature and the volatiles were removed under vacuum to afford a light brown, viscous oil (15.27 g which was used without further purification).

N-*(1-Amino-3-methyl-1-oxobutan-2-yl)-1-(cyclohexylmethyl)-5-(4-fluorophenyl)-1*H-*pyrazole-3-carboxamide and* N-*(1-amino-3-methyl-1-oxobutan-2-yl)-1-(cyclohexylmethyl)-3-(4-fluorophenyl)-1*H-*pyrazole-5-carboxamide (5,3-AB-CHMFUPPYCA) (Figure 2A)*

A mixture of crude cyclohexylmethyl)hydrazine trifluoroacetate (2.00 g) and methyl 4-(4-fluorophenyl)-2,4-dioxobutanoate (1.12 g, 10 mmol) in methanol (20 mL) was refluxed for 3 h. The mixture was allowed to cool to room temperature, the volatiles removed under vacuum and the residue partitioned between aqueous sodium carbonate and dichloromethane. Drying (anhydrous magnesium sulfate) followed by solvent removal gave a yellow oil. Tetrahydrofuran (20 mL), water (10 mL) and lithium hydroxide (2.00 g) were added. The mixture was heated (oil bath at 80 ^{o}C) for 2 h and then allowed to stir overnight at room temperature. The mixture was diluted with water, washed with diethyl ether, made acidic (concentrated aqueous hydrochloric acid) and extracted with dichloromethane. Drying (anhydrous magnesium sulfate) and removal of the solvent afforded light a light yellow solid (936 mg). Thionyl chloride (5 mL) was added to the solid (836 mg) and the mixture was refluxed for 1 hr. The volatiles were then removed under vacuum. *N,N*-Dimethylformamide (5 mL), *L*-valinamide hydrochloride (0.50 g, 3.3 mmol) and *N,N*-diisopropylethylamine (3 mL) were added, and the mixture was stirred at room temperature for 4 h. The mixture was diluted with water and extracted with dichloromethane. Drying (anhydrous magnesium sulfate) and removal of the solvent afforded a light a brown oil (689 mg). This was purified by preparative thin layer chromatography (silica gel; ethyl acetate/hexane, 9/1; 2 runs) to afford a beige solid (74 mg, 0.19 mmol, 2.1 % from methyl 4-(4-fluorophenyl)-2,4-dioxobutanoate): ESI-HRMS *m/z* found 401.2346 (*m/z* theor. for M+H, $C_{22}H_{30}O_2N_4F$, 401.2347); Melting point: 58-62 ^{o}C. ^{1}H NMR (d_6-DMSO) δ 7.63 (s; 1 H; one H from NH_2), 7.82-7.85 (m; 3 H; Ar H and one H from NH_2), 7.36 (t; *J* = 8.8 Hz; 2 H; Ar H), 6.73 (s; 1 H; pyrazole H), 4.36 (dd; *J* = 9.0, 6.3 Hz; NH-C<u>H</u>-CO), 4.02 (d; *J* = 7.2 Hz; 2 H; CyC<u>H</u>$_2$N), 2.03-2.10 (m; 1 H; C<u>H</u>$(CH_3)_2$), 1.71-1.79 (m; 1 H; cyclohexyl H), 1.49-1.59 (m; 3 H; cyclohexyl H), 1.34-1.42 (m; 2 H; cyclohexyl H), 1.02-1.14 (m; 3 H; cyclohexyl H), 0.92 (d; *J* = 6.8 Hz; 3 H; CH_3), 0.88 (d; *J* = 6.8 Hz; 3 H; CH_3) and 0.70-0.8 (m; 2 H; cyclohexyl H) ppm; ^{13}C NMR (d_6-DMSO) δ 172.54 (C=O), 162.26 (d; $^{1}J_{CF}$ = 245 Hz; Ar C), 160.63 (C=O), 144.95 (pyrazole C), 144.21 (pyrazole C), 131.23 (d; $^{3}J_{CF}$ = 8 Hz; Ar CH), 126.19 (Ar C), 115.86 (d; $^{2}J_{CF}$ = 22 Hz; Ar CH), 106.31 (pyrazole CH), 56.78 (CO-<u>C</u>H-NH), 55.34 (Cy<u>C</u>H$_2$N), 38.09 (cyclohexyl CH), 30.53 (<u>C</u>H$(CH_3)_2$), 29.72/29.67 (2 x cyclohexyl CH_2), 25.67 (cyclohexyl CH_2), 24.98/24.95 (2 x cyclohexyl CH_2), 19.29 (CH_3) and 17.92 (CH_3) ppm; ^{19}F NMR (d_6-DMSO) δ –112.98 ppm.

N-*(1-Amino-3-methyl-1-oxobutan-2-yl)-1-(cyclohexylmethyl)-5-(4-fluorophenyl)-1*H-*pyrazole-3-carboxamide and* N-*(1-amino-3-methyl-1-oxobutan-2-yl)-1-*

*(cyclohexylmethyl)-3-(4-fluorophenyl)-1*H*-pyrazole-5-carboxamide (3,5-AB-CHMFUPPYCA) (Figure 2B)*

A mixture of methyl 5-(4-fluorophenyl)-1*H*-pyrazole-3-carboxylate (250 mg, 1.14 mmol), (bromomethyl)cyclohexane (404 mg, 2.28 mmol), cesium carbonate (371 mg, 2.28 mmol) in dimethylformamide (3 mL) was stirred overnight at room temperature. The mixture was then diluted with saturated aqueous ammonium chloride solution and extracted with dichloromethane. Drying (anhydrous magnesium sulfate) and removal of the solvent afforded a colorless oil (428 mg). Lithium hydroxide (480 mg) and tetrahydrofuran (5 mL) were added, the mixture refluxed for 6 h and then allowed to stir overnight at room temperature. The mixture was diluted with water, washed with diethyl ether, made acidic (conc. aqueous hydrochloric acid) and extracted with dichloromethane. Drying (anhydrous magnesium sulfate) and removal of the solvent afforded light a colorless solid (230 mg, 0.76 mmol). Thionyl chloride (3 mL) was added to the solid and the mixture was refluxed for 1 h. The volatiles were then removed under vacuum. *N,N*-Dimethylformamide (2.5 mL), *L*-valinamide hydrochloride (304 mg, 2 mmol) and *N,N*-diisopropylethylamine (1 mL) were added, and the mixture was stirred at room temperature for 4 h. This was then diluted with water and extracted with dichloromethane. Drying (anhydrous magnesium sulfate) and removal of the solvent afforded a beige solid (257 mg). This was purified by preparative thin layer chromatography (silica gel; ethyl acetate/hexane, 9/1) to afford an almost colorless solid (115 mg, 0.29 mmol, 25 % from methyl 5-(4-fluorophenyl)-1*H*-pyrazole-3-carboxylate). This was recrystallized (ethanol) to give a colorless solid (49 mg): ESI-HRMS *m/z* found 401.2344 (*m/z* theor. for M+H, $C_{22}H_{30}O_2N_4F$, 401.2347); Melting point: 172-178 ^{o}C. ^{1}H NMR (d_6-DMSO) δ 8.20 (d; *J* = 8.8 Hz; 1 H; NH), 7.82-7.85 (m; 2 H; Ar H), 7.48 (s; 1 H; one H from NH_2), 7.40 (s; 1 H; pyrazole H), 7.27 (tr; *J* = 8.9 Hz; 2 H; Ar H), 7.13 (s; 1 H; one H from NH_2), 4.37 (dd; *J* = 7.2, 2.4 Hz; 2 H; Cy$C\underline{H}_2$N), 4.26 (dd; *J* = 8.4, 8.1 Hz; NH-C<u>H</u>-CO), 2.07-2.14 (m; 1 H; C<u>H</u>$(CH_3)_2$), 1.77-1.85 (m; 1 H; cyclohexyl H), 1.47-1.67 (m; 5 H; cyclohexyl H), 1.10-1.17 (m; 3 H; cyclohexyl H) and 0.91-0.99 (m; 8 H; 2 cyclohexyl H and 2 CH_3) ppm; ^{13}C NMR (d_6-DMSO) δ 172.87 (C=O), 161.10 (d; $^{1}J_{CF}$ = 243 Hz; Ar C), 159.71 (C=O), 147.62 (pyrazole C), 137.40 (pyrazole C), 129.64 (Ar C), 127.27 (d; $^{3}J_{CF}$ = 8 Hz; Ar CH), 115.96 (d; $^{2}J_{CF}$ = 21 Hz; Ar CH), 104.84 (pyrazole CH), 58.49 (CO-<u>C</u>H-NH), 56.47 (Cy<u>C</u>H_2N), 39.94 (cyclohexyl CH), 30.35 (<u>C</u>H$(CH_3)_2$), 30.23 (cyclohexyl CH_2), 26.19 (cyclohexyl CH_2), 25.50 (cyclohexyl CH_2), 19.68 (CH_3) and 18.73 (CH_3) ppm; ^{19}F NMR (d_6-DMSO) δ –115.00 ppm. A sample of the product was recrystallized from ethyl acetate/cyclohexane for x-ray crystallography.

Instrumentation

Liquid chromatography - mass spectrometry

LC-MS analyses, equipped with an electrospray ionization source, were performed on an Agilent 1100 LC system. The column (Allure PFP Propyl, 5 µm, 50 × 2.1 mm) was from Restek (Bellefonte, PA, USA) and the aqueous mobile phase A consisted of 0.1% formic acid in water whereas mobile phase B was prepared from 0.1% formic acid in acetonitrile, respectively. The Agilent LC-MSD settings were as follows: positive electrospray mode, capillary voltage 3000 V, drying gas (N_2) 12 L/min at 350 °C, nebulizer gas (N_2) pressure 60 psi, *m/z* 50-500, fragmentor voltage either 50 V, 130 V or 150 V. Samples for LC-MS analysis (2 µL injection volume) were dissolved in acetonitrile/water (1:1, containing 0.1% formic acid) at a concentration of 5 µg/mL. The following gradient elution program was used: 0-5 min 12% A and then increased to 35% over 30 min using a linear gradient. The flow rate was 1 mL/min and the column temperature was 30 °C.

Gas chromatography – mass spectrometry

An Agilent 6980 GC coupled to an Agilent 5973 MSD (HP-5ms column, 30 m x 0.25 mm x 0.25 µm) using helium as the carrier gas at a constant flow of 1 mL/min was employed in splitless mode. Injection port and transfer line temperatures were set at 250 ^{o}C and 280 ^{o}C, respectively. Oven temperature: 150 ^{o}C held for 2 min, ramped at 20 ^{o}C/min to 300 ^{o}C and held for 10.5 min. The total run time was 20 min. The samples for analysis were dissolved in acetonitrile and the injection volume was 1 µL.

X-Ray Crystallography

Intensity data were collected at 100(2) K using a MiTeGen micromount on a Bruker APEX Duo CCD diffractometer equipped with an Oxford Cobra cryosystem. Data were collected using ω and ϕ scans, corrected for Lorentz and polarization effects, and integrated using the Bruker APEX program suite. Structures were solved by direct methods and refined with least squares procedures. All non-hydrogen atoms were refined anisotropically and hydrogen atoms were placed geometrically in the calculated positions using a riding model except for H21, H28a, and H28b which were located and refined.

Data collected using Cu Kα radiation (1.54178 Å) for a colorless plate crystal 0.26 × 0.11 × 0.08mm^3, $C_{24}H_{32}FN_5O_2$, M = 441.54, Monoclinic, $P2_1$, a = 12.1979(5), b=7.0566(3), c = 14.6230(6)Å, β = 111.6180(10)°, V = 1170.15(8)Å^3, Z=2, ρ = 1.253mg/m^3, μ =0.709mm^{-1}, Reflections collected 18953 (θ_{max} = 68.49°), independent reflections 4247, $R_{(int)}$ = 0.0321, S = 1.025, R1 = 0.0290, wR2 = 0.0753.* CCDC deposition number 1405051. (* $R1=\Sigma||Fo|-|Fc|| / \Sigma |Fo|$ and $wR2 = \Sigma w(|Fo|^2-|Fc|^2)^2 / \Sigma w|Fo|^2)^{1/2}$)

Nuclear magnetic resonance spectroscopy

Samples were prepared in d_6-DMSO and ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectra were recorded on a Bruker AV600 NMR spectrometer using a 5 mm TCI cryoprobe. ^{1}H NMR spectra were referenced to an external TMS reference at δ = 0 ppm.

High-resolution electrospray mass spectrometry

HR-ESI mass spectra were recorded by direct injection into a LTQ Orbitrap Discovery (Thermo Fisher, UK). Samples were dissolved in acetonitrile/water (1:1, containing 0.1% formic acid) and infused at a rate of 5 μL/min. Full accurate high-resolution (30000) mass scans were performed in positive electrospray mode. Measured accurate masses were within ± 5ppm of the theoretical masses. The following conditions were used: drying gas (N_2) 10000 mL/min, capillary temperature 310 °C, spray voltage 4 V, capillary voltage 22 V and tube lens 77 V.

Results and discussion

The structural diversity associated with substances considered as so-called synthetic cannabinoids can create challenges to forensic, clinical, law enforcement and regulatory communities.[2-5,26-28] Some of the recently occurring indazole derivatives (Figure 1A) are based on the patent literature which point towards appreciable CB_1 receptor affinity and [^{35}S]GTPγS activity[29,30] but others remain to be fully explored to assess the extent to which these substances show psychopharmacological similarities to compounds present in cannabis. A compound that appeared to deviate from currently reported core structures, i.e. carrying a

pyrazole moiety instead of a more established indole, benzimidazole, pyrrole or indazole core, has been advertised on a number of 'research chemicals' websites as AZ-037, which represents (*S*)-*N*-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(5-fluoropentyl)-5-(4-fluorophenyl)-1*H*-pyrazole-3-carboxamide (Figure 1B). Any information about its psychoactive or pharmacological properties seems to be currently unavailable although it appears to be advertised as a synthetic cannabinoid on various websites. The EMCDDA has recently received a notification on its detection in an EU Member State via the EU EWS on NPS.[31]

The present investigation was initiated following the donation of a vendor sample to the authors' laboratories labeled as AZ-037. During initial characterization it was revealed that the analytical data were inconsistent with the expected structure. Inspection of the UK-based website also indicated that the suggested IUPAC name for AZ-037 was also not in agreement with the shown structure. The shown structure associated with AZ-037 was 1-(5-fluoropentyl)-1*H*-indole-3-carboxylic acid 8-quinolinyl ester, i.e. commonly referred to as the synthetic cannabinoid 5F-PB-22. Interestingly, the analytical characterization of a substance very closely related to AZ-037 has been published recently. In this case, the reported compound was *N*-(1-amino-3,3-dimethyl-1-oxobutan-2-yl)-1-(5-fluoropentyl)-3-(4-fluorophenyl)-pyrazole-5-carboxamide and the suggested name for this substance is 3,5-5F-ADB-FUPPYCA (Figure 1B). The presence of the pyrazole ring can give rise to a number of isomers, which complicates matters significantly and this meant that the chromatographic separation and characterization of isomers was deemed necessary. The preparation of both isomers associated with positions 5 and 3 confirmed unambiguously that the sample received from the Internet supplier was consistent with 3,5-AB-CHMFUPPYCA (Figure 2).

Synthesis

Both regioisomers 5,3-AB-CHMFUPPYCA and 3,5-AB-CHMFUPPYCA were synthesized using two specific routes that are outlined in Figure 2. The synthesis of 5,3-AB-CHMFUPPYCA involved the reaction of methyl 4-(4-fluorophenyl)-2,4-dioxobutanoate (a) with (cyclohexylmethyl)-hydrazine (b), which gave rise to the pyrazole intermediate (c). This intermediate was then reacted with lithium hydroxide, which provided the carboxylic acid species (d). This entity was reacted with thionyl chloride and subsequently *N,N*-dimethylformamide, *L*-valinamide hydrochloride and *N,N*-diisopropylethylamine to yield the 5,3-AB-CHMFUPPYCA

product (Figure 2A). The preparation of 3,5-AB-CHMFUPPYCA involved reacting 5-(4-fluorophenyl)-1*H*-pyrazole-3-carboxylate (e) with (bromomethyl)-cyclohexane (f) and cesium carbonate yielding the cyclohexylmethyl intermediate (g). This intermediate was reacted with lithium hydroxide, which induced the formation of the carboxylic acid species (h). This entity was reacted with thionyl chloride and subsequently *N,N*-dimethylformamide, *L*-valinamide hydrochloride and *N,N*-diisopropylethylamine, which yielded the 3,5-AB-CHMFUPPYCA product (Figure 2B).

The structural differences in the regioisomers included the positioning of the substituents around the pyrazole core, which were dictated by the positioning of the double bonds within the pyrazole ring structure. In the 5,3-AB-CHMFUPPYCA isomer (Figure 2A) the 4-fluorophenyl component was attached to position 5 whereas the secondary carboxamide structure was attached to position 3 of the pyrazole ring. In case of the 3,5-AB-CHMFUPPYCA isomer, the opposite arrangement was present. A route specific chlorine containing by-product, *N*-(1-amino-3-methyl-1-oxobutan-2-yl)-4-chloro-1-(cyclohexylmethyl)-3-(4-fluorophenyl)-1*H*-pyrazole-5-carboxamide, was encountered during the synthesis of the 3,5-AB-CHMFUPPYCA isomer (supplemental information) and this was not observed during the preparation of the regioisomeric form.

Gas chromatography mass spectrometry

Separation of both isomers was successfully achieved using gas chromatography (GC). The retention times for 5,3-AB-CHMFUPPYCA and 3,5-AB-CHMFUPPYCA were 14.74 and 15.20 min, respectively, and a comparison with the vendor sample was in agreement with the identity of the latter (Figure 3A-C). A comparison of both EI mass spectra also provided sufficient evidence that both isomers could be differentiated based on fragmentation patters (Figure 3E-F). In the EI mass spectrum for 5,3-AB-CHMFUPPYCA, the base peak was observed at *m/z* 285 and indicated the loss of 115 Da from the parent molecule, which represented the formation of an oxonium species (Figure 3G). Another dominant peak was observed at *m/z* 356 that may be described by the loss of formamide from the molecular ion. Another minor fragment encountered in the EI mass spectrum was *m/z* 189, which might have been consistent with the loss of cyclohexylmethyl species from the oxonium ion at *m/z* 285. In the EI mass spectrum of 3,5-AB-CHMFUPPYCA, the base peak was observed at

m/z 257, which was thought to represent the 1-(cyclohexylmethyl)-3-(4-fluorophenyl)-1*H*-pyrazole fragment and its formation might have been facilitated by the position of the double bonds within the pyrazole ring of the 3,5-AB-CHMFUPPYCA isomer (Figure 3G). The positioning of the double bonds in the pyrazole ring might have facilitated the formation of a temporary double bond between the nitrogen at position 1 of the pyrazole ring and the carbon at position 5, which might have resulted in the formation of a thermodynamically stable species (Figure 4). The formation of this species would not have been possible with the 5,3-AB-CHMFUPPYCA isomer due to the positioning of the double bonds within the pyrazole ring. Also present in the EI mass spectrum of the 3,5-AB-CHMFUPPYCA isomer was the detection of the molecular ion at *m/z* 400. The fragment at *m/z* 356 was presumably formed by the loss of formamide (Figure 3G). The chlorinated by-product formed during the synthesis of 3,5-AB-CHMFUPPYCA was detected under GC-MS analysis conditions and a retention time of 16.07 min was obtained for this compound (supplemental data).

Liquid chromatography mass spectrometry

Analysis of both synthesized isomers and the vendor sample by high performance liquid chromatography (HPLC) confirmed satisfactory separation. A retention time of 13.41 min was obtained for the 5,3-AB-CHMFUPPYCA isomer, whereas a retention time of 13.85 min was obtained for the 3,5-AB-CHMFUPPYCA isomer (Figure 5). The electrospray ionization (ESI) single quadrupole mass spectra obtained from in-source collision-induced dissociation (CID) of the synthesized regioisomers (150 V fragmentor voltage) shared similar product ions but key features that allowed for differentiation between the two substances were also apparent (Figure 6). For example, the in-source CID spectrum of 5,3-AB-CHMFUPPYCA displayed the sodiated adduct $[M + Na]^+$ at *m/z* 423 as the base peak (Figure 6A), which was not the case with 3,5-AB-CHMFUPPYCA where the relative abundance was around 40% (Figure 6B). A major difference, however, was observed in the mass spectrum of 3,5-AB-CHMFUPPYCA that formed a base peak at *m/z* 260 and which was absent in the 5,3-AB-CHMFUPPYCA (Figure 6A). Figure 6C shows a proposed mechanism of its formation that may be rationalized by the loss of 2-cyclohexylacetamide from the protonated molecule. Product ions common in both spectra included *m/z* 356 by way of cleaving formamide from the protonated molecule and the subsequent formation of the *m/z* 285 oxonium ion. The *m/z* 189 ion may have then been formed by the loss of the cyclohexylmethyl species from the oxonium fragment, resulting in a species with

chemical formula $C_{10}H_6FN_2O^+$. The chlorinated by-product was observed during the HPLC-ESI-MS analysis of 3,5-AB-CHMFUPPYCA. A retention time of 14.49 min was obtained for this compound (supplemental data). Analysis by high-resolution mass spectrometry was employed, which yielded formation of identical product ions for each isomer (supplemental data).

Nuclear magnetic resonance spectrometry

In addition to one-dimensional proton (^{1}H), carbon (^{13}C) and fluorine (^{19}F) NMR analyses, the implementation of two-dimensional experiments proved helpful for the characterization of both isomers and the vendor sample. For example, in the case of 5,3-AB-CHMFUPPYCA, heteronuclear multiple-bond correlation (HMBC) experiments revealed a 3-bond ^{1}H/^{13}C correlation between the respective carbon atom at position 5 of the pyrazole ring and the protons at the *ortho* position of the fluorinated phenyl ring. A correlation was also observed between the aforementioned carbon and the protons of the methylene group attached to the cyclohexyl ring (Figure 7A). As far as 3,5-AB-CHMFUPPYCA was concerned, the 3-bond correlation between the carbon atom at position 5 of the pyrazole ring and the protons on the methylene group attached to the cyclohexyl ring (Figure 7B) was also present. As expected, the 3-bond correlation with the protons on the fluorinated phenyl ring was not detected. The vendor sample was also subjected to HMBC analysis and confirmed the 3,5-AB-CHMFUPPYCA assignment (supplemental data). Further information was obtained from nuclear Overhauser effect spectroscopy (NOESY), which provided additional insights into distinguishing features between the two isomers. A qualitative nuclear Overhauser experiment (NOE) showed connectivity between the methylene group attached to the cyclohexyl ring and the protons attached to the *ortho* position of the fluorinated phenyl ring present in the 5,3-AB-CHMFUPPYCA isomer. This nOe was not observed with 3,5-AB-CHMFUPPYCA (supplemental data).

X-Ray crystallography

The solid-state structure of the 3,5-AB-CHMFUPPYCA isomer was elucidated by single crystal X-ray diffraction and is shown below in Figure 8. The structure is a solvate with a co-crystallized molecule of acetonitrile present in the asymmetric unit. The molecule crystallizes in the chiral monoclinic space group $P2_1$ and the chiral

center C22 has been determined to be the *S*-enantiomer. The packing is dominated by the amide donor groups (N21, N28), which form hydrogen bonds with the terminal amido oxygen O27 (N28…O27 range 2.856(2) - 2.963(2) Å). These link the molecules into a strongly associated chain. A channel exists between the chains and the acetonitrile solvent molecules lies within it. A weak NH…N interaction (N28…N29, 3.039 Å) exists between 3,5-AB-CHMFUPPYCA and the solvent (supplemental information).

Conclusion

An in-depth analytical characterization of a 'research chemical' advertised as AZ-037 revealed mislabeling when it transpired that it was consistent with 3,5-AB-CHMFUPPYCA instead. The combination of analytical techniques and confirmation of compound identification by organic synthesis provided an effective approach to tackling an increasingly complex area of investigation where increasing demands are placed on investigators in the field of new psychoactive substances.

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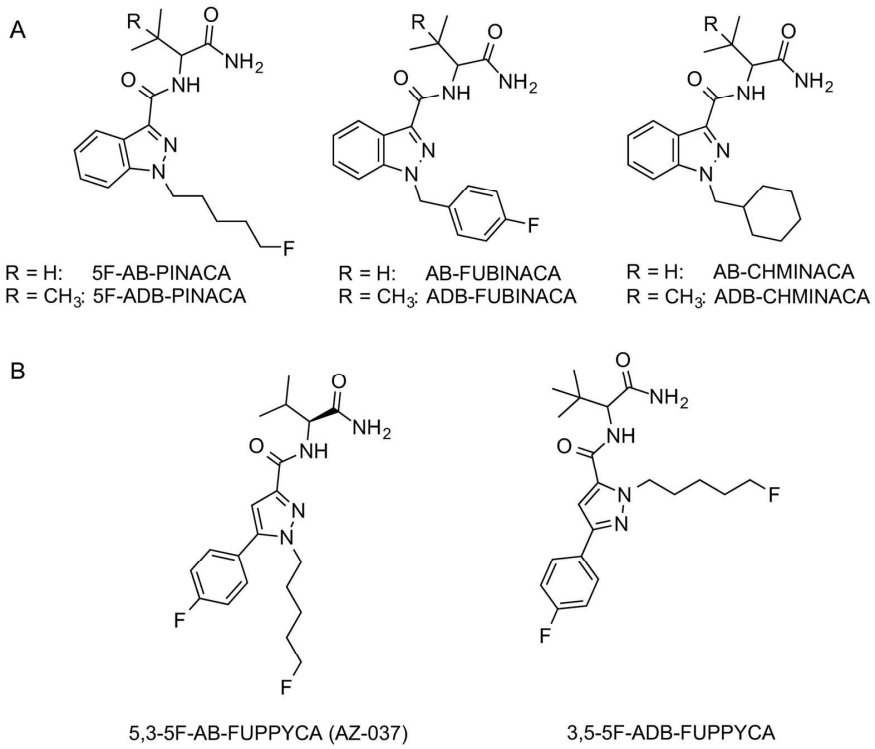
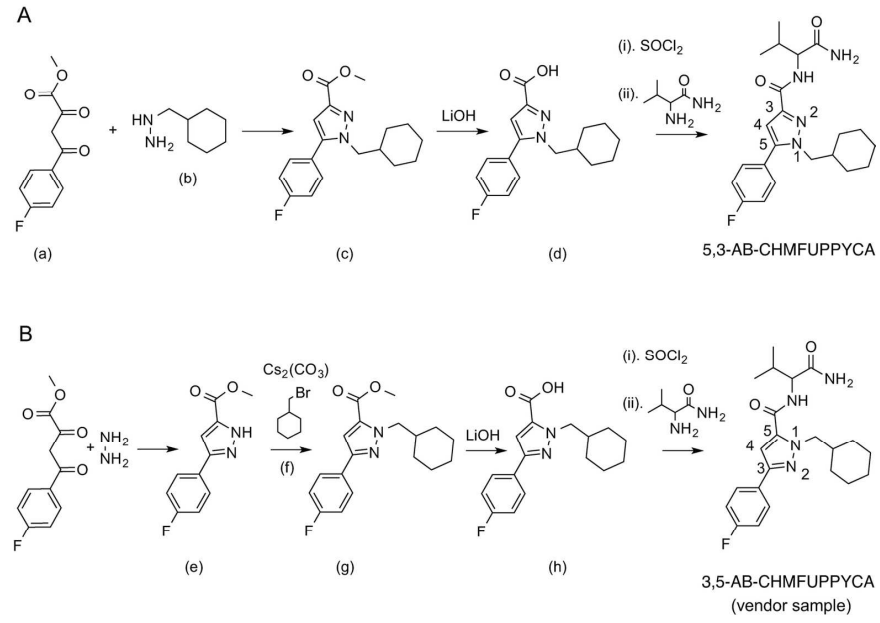
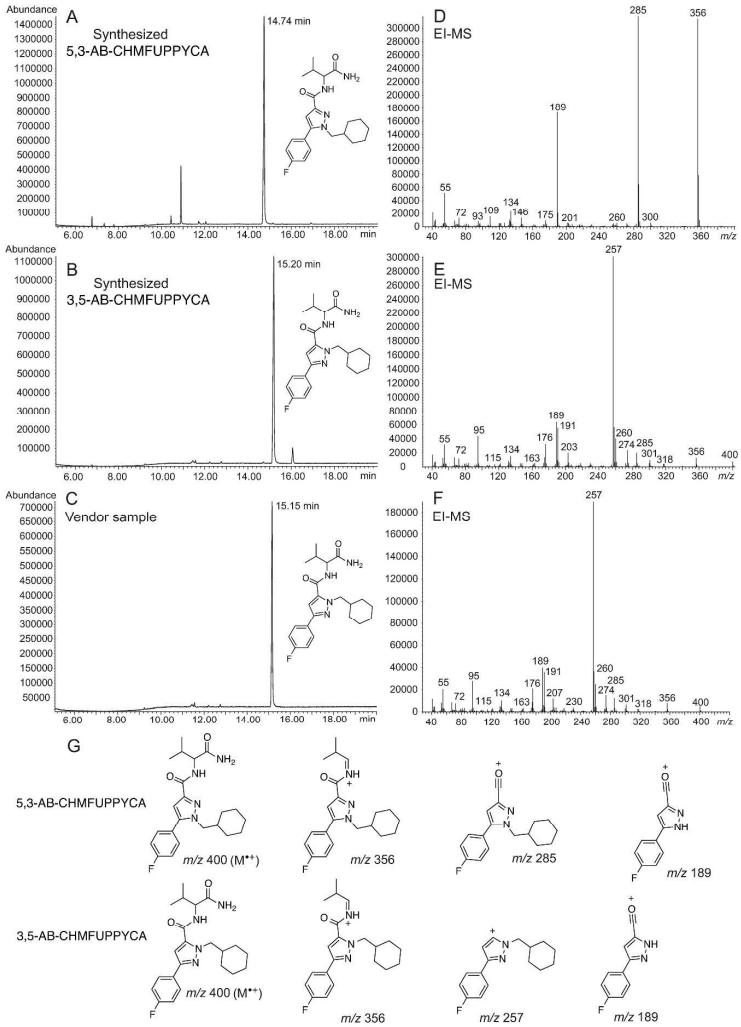


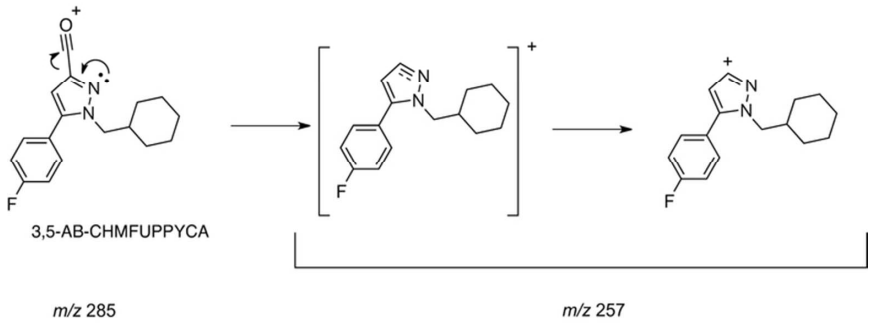
Chemical structures of synthetic cannabinoids from (A) the PINACA, FUBINACA, CHMINACA series and (B) the FUPPYCA series.



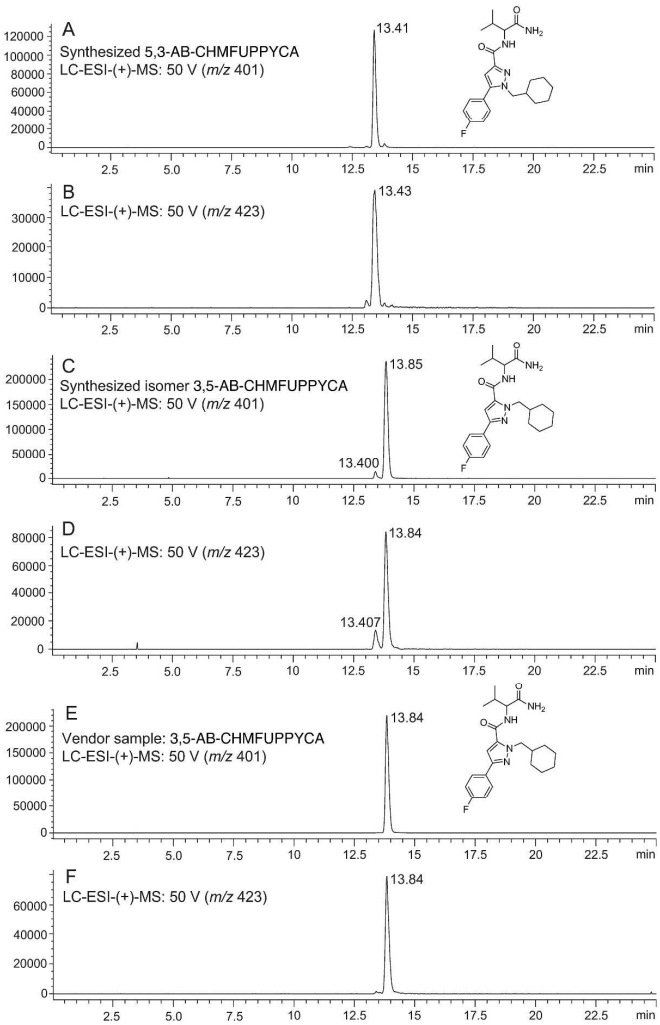
Synthesis protocol for the AB-CHMFUPPYCA isomers. (A) Specific synthetic route for 5,3-AB-CHMFUPPYCA. (B) Specific synthetic route for 3,5-AB-CHMFUPPYCA.
142x101mm (300 x 300 DPI)



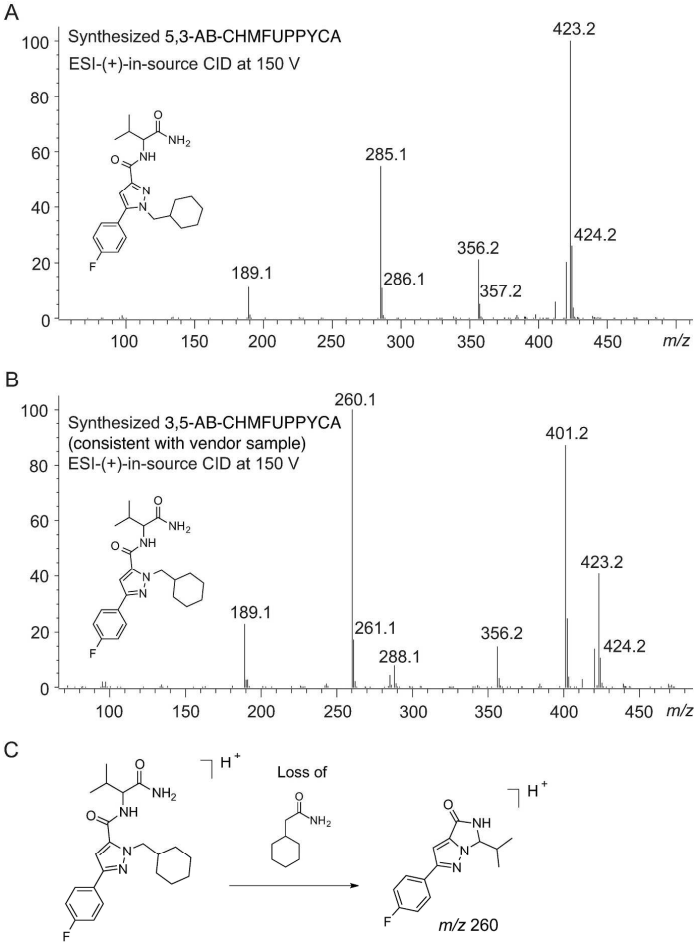
(A-F) GC-MS data obtained for both AB-CHMFUPPYCA isomers and vendor sample. (G) Proposed EI-MS fragmentation patterns for the AB-CHMFUPPYCA isomers.



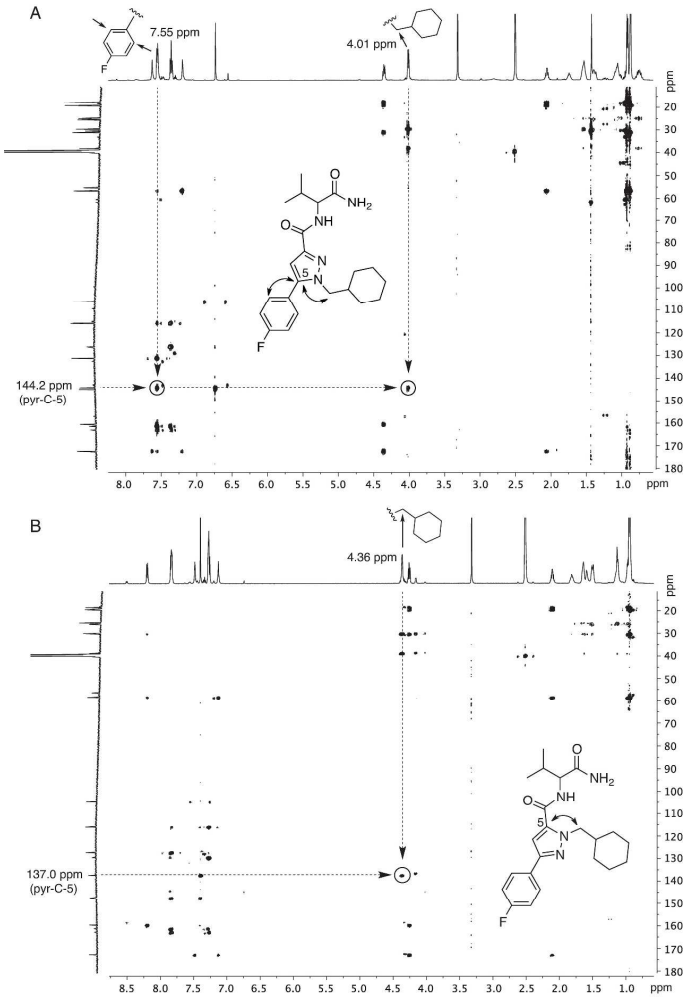
Proposed mechanism for the formation of 1-(cyclohexylmethyl)-3-(4-fluorophenyl)-1H-pyrazole species observed at m/z 257 in the EI-mass spectrum of 3,5-AB-CHMFUPPYCA.



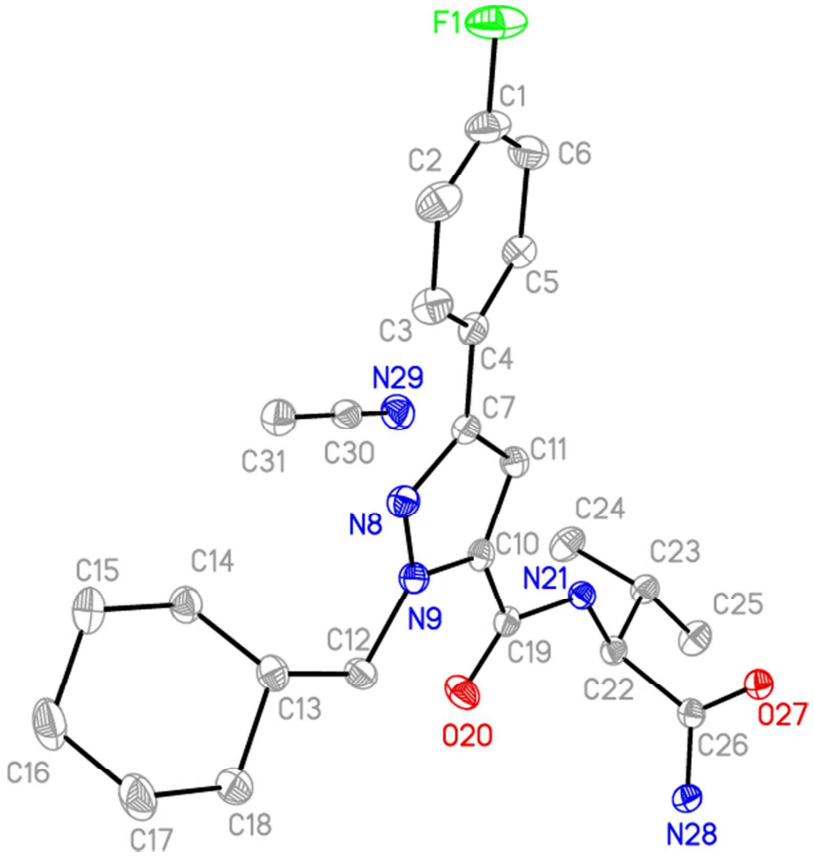
(A-F) Analysis of synthesized AB-CHMFUPPYCA isomers and vendor sample using high performance liquid chromatography selected ion monitoring (SIM) mass spectrometry at 50V.



(A-B) Electrospray ionization single quadrupole mass spectra following in-source collision-induced dissociation at 150 V. (C) Proposed mechanism for the formation of the species with m/z 260 that may be rationalized by the loss of 2-cyclohexylacetamide from the protonated molecule.



Heteronuclear multiple-bond correlation NMR spectra for (A) the 5,3-AB-CHMFUPPYCA isomer and (B) the 3,5-AB-CHMFUPPYCA isomer.



Molecular structure of 3,5-AB-CHMFUPPYCA (thermal displacement at 50% probability) with hydrogen atoms omitted for clarity.