Identification of the novel synthetic opioid *N*-pyrrolidino isotonitazene at an Australian drug checking service.

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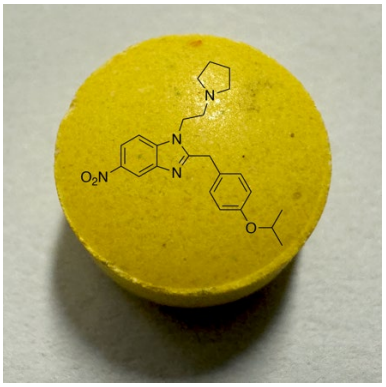


Abstract

2-Benzylbenzimidazole opioids and related derivatives, also known as ‘nitazenes’, present a growing threat to public health. Emerging in Europe in 2019, the nitazene group of drugs is a recent addition to the novel synthetic opioid class and has been associated internationally with adverse effects in drug users, overdose clusters and significant mortality. The high potency of many nitazene derivatives, which can in many cases exceed that of fentanyl, poses a significant challenge to the public health and early warning systems used to detect and respond to the emergence of new high-risk substances. This report describes close collaboration between an Australian drug checking service and a nearby university laboratory to identify and characterise the novel synthetic opioid *N*-pyrrolidino isotonitazene in an expected oxycodone sample presented by a member of the public. Though no prior publications are available describing the presence of this nitazene in the drug market, previously reported *in vitro* evaluation of this compound reveals it to be among the most potent nitazene opioid agonists known. The study highlights the rapid response possible

though engaging drug users with drug checking services as a market monitor and early warning system to alert health services and the broader community to the presence of unexpected, high-risk substances. Integration of well-resourced and supported drug checking services provides a powerful approach to tackle the public health threats associated with new synthetic opioids and other drugs of concern.

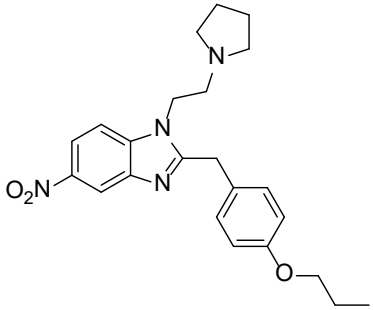
Keywords

N-pyrrolidino isotonitazene, isotonitazepyne, novel synthetic opioid, drug checking, harm reduction

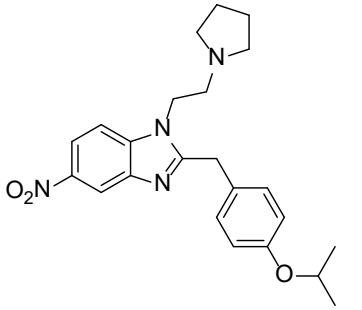
1. Introduction

2-Benzylbenzimidazole opioids and related derivatives, also known as 'nitazenes', present a growing threat to public health. Emerging in the recreational drug market in Europe in 2019,[1] the nitazene group of drugs is a recent addition to the novel synthetic opioid class characterised by variable structures and differing potencies that in many cases exceed that of fentanyl.[2–6] The variation of nitazene structure and potency poses a significant challenge to early warning systems that seek to protect public health by detecting and responding to the emergence of new high-risk substances. Internationally, the adulteration or substitution of the opioid drug supply with nitazenes has been associated with adverse effects for drug users and significant mortality.[6–11] Reports from late 2023 of significant overdose clusters in Dublin and Cork in Ireland associated with the nitazene *N*-pyrrolidino protonitazene (**NPP**, protonitazepyne, **Figure 1**) highlight the challenges that attend the introduction of novel synthetic opioids into local drug markets.[12]

In Sep 2024, a client of an Australian drug checking service presented round, mottled yellow pills with expectation that they contained the morphinan opioid oxycodone, accompanied by reports of significant non-fatal drug-related harm. Chemical analysis at the service failed to detect oxycodone, instead providing evidence indicating the presence of a nitazene drug related to but distinct from **NPP**, leading the client to discard the samples. Further analysis at an affiliated university laboratory the next day identified *N*-pyrrolidino isotonitazene (**NPI**, isotonitazepyne, **Figure 1**) as a major component leading to notification of the detection to clinical first responders, and the public. Given the limited information available in the primary literature associated with **NPI**, this report presents a full characterisation of this compound. This study highlights the prompt response possible though the integration of well-resourced drug checking services within early warning systems designed to monitor drug markets for the emergence of high-risk substances in the community.



N-pyrrolidino protonitazene (**NPP**)



N-pyrrolidino isotonitazene (**NPI**)

Figure 1. Structures of *N*-pyrrolidino protonitazene (**NPP**) and *N*-pyrrolidino isotonitazene (**NPI**)

2. Experimental

2.1 General

Chemical analysis and compound characterisation was conducted at the CanTEST Health and Drug Checking Service (CanTEST) located in central Canberra, Australia, and the nearby Australian National University (ANU).

CanTEST offers drug checking to members of the public in possession of drugs intended for personal use. The service employs a model of client-facing on-site testing to provide free and confidential chemical analysis of drugs combined with tailored harm reduction and health interventions. Modes of analysis available on site at CanTEST (CanTEST analysis) include Fourier Transform Infra-Red (FTIR, Section 2.2), Ultra-Performance Liquid Chromatography-Photo-Diode Array (UPLC-PDA, Section 2.3) analysis and immunoassay test strips (Section 2.4). Additional information about the development and operation of the CanTEST service has been described elsewhere.[13,14]

The CanTEST service is affiliated with the nearby ANU providing additional testing capability (ANU analysis). Modes of analysis include High Resolution Ultra-Performance Liquid Chromatography-Electrospray Ionisation-Tandem Mass Spectrometry (UPLC-ESI-MS/MS Section 2.5), High Resolution Gas Chromatography-Electron Ionisation-Mass Spectrometry (GC-EI-MS, Section 2.6) and Nuclear Magnetic Resonance (NMR, Section 2.7). Both fentanyl and nitazene test strips were obtained from BTNX (Ontario, Canada). All solvents used

throughout sample preparation and analysis were liquid chromatography grade (Fisher scientific). A sample containing **NPP** was acquired from a client submission to CanTEST, matching the previously reported ^{1}H NMR (**Figure S3.1**) and GC-EI-MS characterisation data.[12]

2.2 FTIR Analysis

The FTIR analysis was performed as previously described in Algar *et al* 2024.[13] One whole pill was homogenised by crushing and mixing in a mortar with pestle. Following cleaning and a background scan, approximately 1-2 mg of the sample powder was loaded onto the instrument for analysis.

2.3 UPLC-PDA Analysis

The UPLC-PDA analysis was conducted as previously described.[13] Chromatographic separation was achieved using an alternative gradient consisting of the following mobile phases: 0.1% formic acid (Scharlau) in water and methanol at a flow rate of 0.5 mL/min where the percentage of organic solvent was linearly changed: 0 min, 3%; 0.01 min, 35%; 3.50 min, 55%; 3.80 min, 3%; 6.0 min, 3%.

2.4 Immunoassay Test Strip Analysis

Sample homogenate was prepared in 0.1% formic acid in water/methanol (97:3) in a 2 mL glass sample vial to a final concentration of 1.00 mg/mL. BTNX Rapid Response fentanyl and nitazene test strips were applied to the solution for 15 seconds and then placed on a non-absorbent surface to develop. After 60 seconds the results were recorded as either positive, negative, or invalid (**Table S1.1**).

2.5 UPLC-ESI-MS/MS Analysis

A sample of homogenised pill (10.0 mg) was weighed into a 2 mL glass vial, suspended in methanol (1.5 mL) and vortexed for 30 seconds. The suspension was allowed to settle, and the supernatant was transferred into a syringe and filtered through a PTFE 13 mm 0.2 µm syringe filter (1x dilution). The resulting solution was diluted 100x in 0.1% formic acid in water/methanol (97:3), and dicyclohexylamine (0.0050 mg/mL final concentration) added as internal standard. A sample containing **NPP** (approximately 0.001-0.002 mg/mL) and dicyclohexylamine (0.0050 mg/mL) was prepared and run alongside for comparison.

Analysis was conducted using a Dionex RSLC Nano liquid chromatograph (LC) coupled to a Thermo-Fisher Orbitrap Fusion ETD mass spectrometer via a Heated Electrospray Ionisation (H-ESI) ion source, operated in positive mode with a collision energy of 40 eV. Acquisition was run without quadrupole mass selection but otherwise using conditions as previously described.[13] Fragment ion ratios were taken by normalising the peak intensity of each precursor and fragment ion abundance to the most abundant ion (*m/z* 98) in the product ion spectrum.

2.6 GC-EI-MS Analysis

To the initial sample prepared for UPLC-ESI-MS/MS (1 x dilution), was added dicyclohexylamine (0.05 mg/mL final concentration) as internal standard for GC-EI-MS analysis. A sample containing **NPP** (approximately 0.10 - 0.20 mg/mL) and dicyclohexylamine (0.05 mg/mL) was prepared and run alongside for comparison.

Analysis was conducted using an Agilent 8890 GC coupled to an Agilent 7250 quadrupole time-of-flight (QToF) high resolution mass spectrometer equipped with a Gerstel MPS preparative autosampler. The specific conditions used were as previously described, with minor adjustments.[13] The GC inlet was operated in splitless mode, and the final temperature of 325 ºC was held for 10 min, for a total analysis time of 22 min. Fragment ion ratios were taken by normalising the area under the curve of extracted ion chromatograms for each precursor and fragment ion to that of the most abundant ion (*m/z* 84) in the spectra (**Figure S2.1-S2.2**, **Table S2.1**).

2.7 NMR Analysis

A sample of the homogenised pill (100 mg) was weighed into a 20 mL glass scintillation vial, suspended in methanol (1.8 mL) and vortexed for 30 seconds. The suspension was allowed to settle, and the supernatant (~1.5 mL) was transferred by micropipette to an Eppendorf tube. Following centrifugation for 30 seconds, the supernatant was again aspirated by micropipette, transferred to a 2 mL glass vial and dried in a heating block at 60 ºC under a constant flow of nitrogen gas, resulting in a crude mixed yellow and white solid (4.1 mg).

One and two dimensional ^{1}H and ^{13}C NMR spectra were acquired in DMSO-d_6 at 298 K on a Bruker Avance III HD 800 spectrometer (800.13 MHz ^{1}H, 201.22 MHz ^{13}C) equipped with 5 mm TCI cryoprobe. Chemical shifts (δ) are reported in parts per million with ^{1}H shifts referenced to the residual solvent peaks (DMSO-d_5: ^{1}H δ 2.50) and ^{13}C shifts referenced to the solvent peak (DMSO-d_6: ^{13}C δ 39.52). Coupling constants (*J*) are reported in Hertz (Hz). Standard abbreviations indicating multiplicity were used as follows: m = multiplet, hept = heptet, q = quartet, t = triplet, d = doublet, s = singlet. Deuterated solvents were supplied by Cambridge Isotope Laboratories (MA, USA), Inc. Analysis of these spectra was completed using the MestReNOVA (version 14.2.1) and Bruker Topspin (version 3.6.3) software packages.

Three individual pills were prepared as above for QNMR analysis; the total recoverable mass of each crushed pill was weighed and then used in the extraction step. The volume of methanol for extraction was adjusted to reflect the larger sample mass (6.0 mL). All recoverable liquid was transferred followed by one washing with methanol (1.0 mL). The extracted samples were dissolved in DMSO-d_6 to a total volume of 1000 μL and transferred to 5 mm NMR tubes; an external standard NMR sample was also prepared by dissolving 18.2 mg of hydroquinone (National Measurement Institute, 99.7 ± 0.3%) in DMSO-d_6 to a total volume of 1000 μL. ^{1}H spectra were acquired at 298 K using QNMR conditions, of the three samples and the external standard; a blank DMSO-d_6 sample was also run to ensure that there were no underlying signals from the solvent contamination (**Figure S2.17**). The molar concentrations of **NPI** were determined by integrating the **NPI** proton at δ 8.485 – 8.450 (1H)

and comparing this to the integral of the hydroquinone aromatic peak at δ 6.575 – 6.525 in the external standard**,** using the ERETIC function in the Topspin 3.6.3 package. The total mass of **NPI** per original pill was then determined from the concentrations and volumes of the NMR samples, and the recoverable and original pill masses (**Table S2.3**).

3. Results

3.1 CanTEST Analysis

FTIR analysis of the homogenised sample produced a relatively featureless spectrum (**Figure S1.1**) consistent with complex mixture of pill fillers/binders with no confident drug identification made using the OPUS Drug ID software.

UPLC-PDA analysis indicated a single component producing a UV spectrum with maximum absorptions at 239 and 309 nm, matching the spectrum of a previously identified **NPP** sample (**Figure S1.2**) when both spectra are manually overlayed. However, the retention times of the client sample (3.06 min) and **NPP** (3.56 min) differed (15% difference). The near identical UV spectra suggested the analyte contained a nitrobenzimidazole opioid of similar structure to **NPP**.

BTNX fentanyl and nitazene test strips were recorded as negative and positive respectively for the client sample (**Table S1.1**). This indicated the likely absence of fentanyl or a fentanyl derivative but the likely presence of a nitazene derivative. The analytical findings obtained at the service were relayed to the client within minutes and led to the discard of the samples, which were transported to the nearby ANU laboratory for further analysis.

3.2 ANU Analysis

The GC-EI-MS analysis of the client sample returned a peak (RT 19.4 min), differing from that of **NPP** (RT 20.6 min, 6% difference). The mass spectrum (**Figure 2**, **S2.1-S2.4**, **Table 1**, **S2.1**) revealed a molecular ion at *m/z* 408.2106 ($C_{23}H_{28}O_3N_4^{\bullet+}$, +0.9 ppm) and fragments at *m/z* 84.0808 ($C_5H_{10}N^+$, +0.0 ppm) consistent with an *N*-methylene pyrrolidinium fragment, and *m/z* 107.0492 ($C_7H_7O^+$, +0.9 ppm).[1] The **NPP** sample showed similar a similar mass spectrum.[12] The observation of different retention times but similar mass spectra suggested the presence of a constitutional isomer of **NPP**.

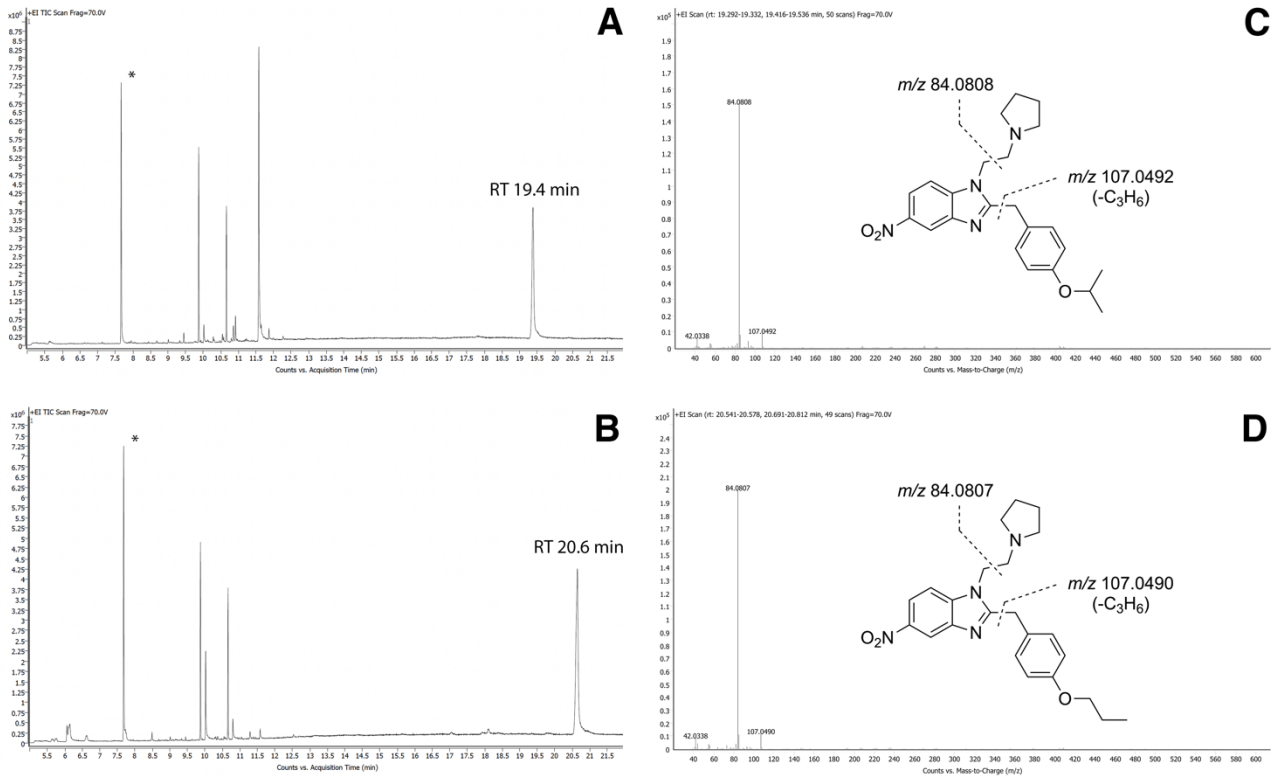


Figure 2. GC-EI-MS (EI+, 70 eV) total ion chromatogram for **A**) client sample (**NPI**, RT 19.4 min), and **B**) **NPP** (RT 20.6 min). Dicyclohexylamine (RT 7.7 min) internal standard is indicated by an asterisk (*). Mass spectrum for **C**) client sample (**NPI**), and **D**) **NPP**.

During UPLC-ESI-MS/MS, a peak was observed at a retention time of 7.37 min with *m/z* 402.2247, ($C_{23}H_{29}O_3N_4^+$, +3.2 ppm) for the client sample, corresponding to the protonated molecule of **NPP** or an isomer (**Table 1**, **S2.2**). Following collision induced dissociation, the client sample returned fragment ions consistent with an *N*-ethylene pyrrolidine ion at *m/z* 98.0963 ($C_6H_{12}N^+$, -1.0 ppm)[15] and an ion corresponding to that observed in GC-EI-MS at *m/z* 107.0493 ($C_7H_7O^+$, +1.9 ppm).[1] While the precursor and fragment ions were consistent between both the client sample and **NPP** (**Table 1**), the latter returned a different retention time of 7.50 min (3.8% difference) also consistent with an isomeric species. Spectra and chromatograms can be found in supporting information (**Figures S2.5-S2.6**).

Table 1. High resolution GC-EI-MS and LC-ESI-MS/MS analysis of the client sample (**NPI**) and **NPP**.

Method	Sample	Retention time (min)	Observed ion (m/z)	Chemical formula	Error (ppm)	Relative abundance (%)

GC-EI-MS	Client sample (**NPI**)	19.4	84.0808 107.0492 408.2160	$C_5H_{10}N^+$ $C_7H_7O^+$ $C_{23}H_{28}O_3N_4^{\bullet+}$	+0.0 +0.9 +0.9	100[a] 4.8[a] 0.5[a]
	NPP	20.6	84.0807 107.0490 408.2155	$C_5H_{10}N^+$ $C_7H_7O^+$ $C_{23}H_{28}O_3N_4^{\bullet+}$	-1.2 -0.9 -0.2	100[a] 4.8[a] 0.4[a]
LC-ESI-MS/MS	Client sample (**NPI**)	7.37	98.0963 107.0493 409.2247	$C_6H_{12}N^+$ $C_7H_7O^+$ $C_{23}H_{29}O_3N_4^+$	-1.0 +1.9 +3.2	100[b] 5.2[b] 0.2[b]
	NPP	7.50	98.0964 107.0494 409.2243	$C_6H_{12}N^+$ $C_7H_7O^+$ $C_{23}H_{29}O_3N_4^+$	+0.0 +2.8 +2.2	100[b] 4.3[b] 0.2[b]

[a]Derived from scan MS extracted ion chromatogram peak areas. [b]Derived from product ion MS/MS spectrum peak heights.

The ^{1}H NMR spectrum of the client sample closely resembled that for **NPP**,[12] producing broad peaks at 2.39 ppm (4H) and 1.36 ppm (4H) with observed ^{1}H→^{1}H COSY and ^{1}H→^{13}C HMBC cross peaks between the two resonances, matching those expected for a pyrrolidine substituent. The presence of a heptet at 4.56 ppm (1H, J = 6.0 Hz) and doublet at 1.23 ppm (6H, J = 6.0 Hz) with expected two-dimensional ^{1}H→^{1}H COSY coupling indicated substitution by an isopropoxy group consistent with an isotonitazene rather than a protonitazene or other nitazene derivative (**Figure 3**, **S2.7**). Due to overlap with impurities, likely resulting from pill binding agents or excipients in DMSO-d_6, integration of the observed doublet at 1.23 ppm was instead taken from a second spectrum taken using chloroform-*d* ($CDCl_3$) as solvent, where the signal was better resolved (**Figure S2.8**). Two-dimensional NMR spectra in DMSO-d_6 solvent were acquired, and a complete assignment for the client sample is presented in **Table 2**. Reference spectra can be found in supporting information (**Figure S2.7-2.12**). Together, the analysis revealed the client sample to contain *N*-pyrrolidino isotonitazene (**NPI**, isotonitazepyne), a constitutional isomer of **NPP**. The low resolution GC-MS spectrum and a reference material for **NPI** is available from Cayman Chemical but was not obtained as part of this study due to a projected 3- to 4-month delay for supply.

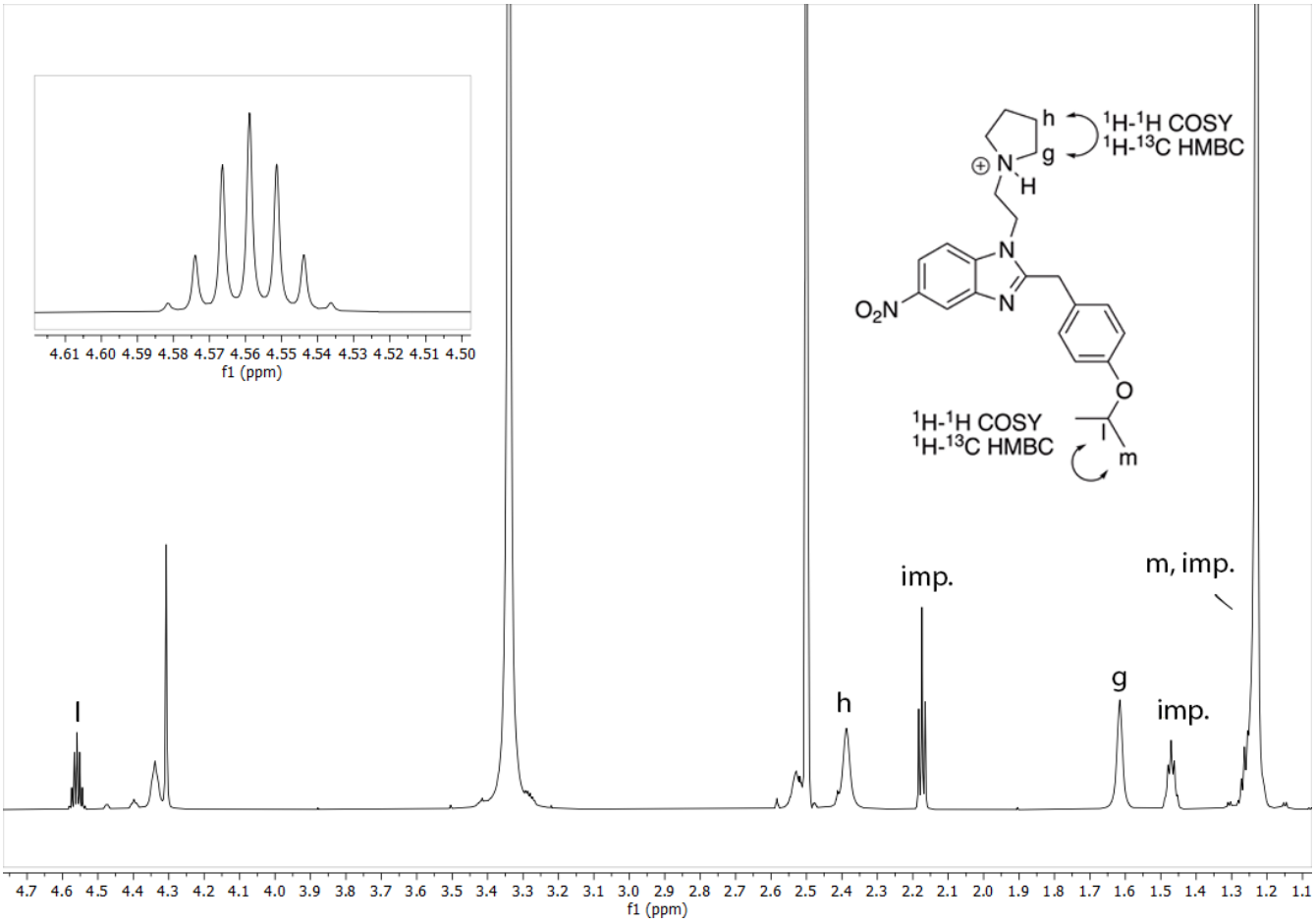


Figure 3. ^{1}H NMR of the client sample (**NPI**) in DMSO-d_6; 3.24 ppm = H_2O; 2.50 ppm DMSO-d_5. Peaks at 2.17 and 2.47 ppm correspond to impurities; The isopropoxy substituent doublet at 1.23 ppm is obscured by an alkane-like impurity.

Table 2. NMR data of client sample (**NPI**) in DMSO-d_6. Assignment for proton-coupled ^{13}C signals were determined using HSQC and HMBC ^{1}H $\rightarrow$^{13}C cross-peak analysis.

Structure	Label	Chemical shifts (ppm)		Multiple bond correlations	
		^{1}H	^{13}C	COSY ^{1}H$\rightarrow$^{1}H cross-peak	HMBC ^{1}H $\rightarrow$^{13}C cross-peak
	a	8.46	114.7	b (weak)	c, n, p, o
	b	8.13	117.6	c	a, n, o
	c	7.73	110.9	b	n, p
	d	4.34	43.1	e	e, o
	e	2.53	54.3	d	g
	f	11.95			
	g	2.39	53.8	h	h, g’
	h	1.62	23.2	g	g, h’
	i	4.31	32.3		j, q, r
	j	7.18	129.9	j	i, k, s, j’

	k	6.90 - 6.84	115.8	k	s, r, j, k’
	l	4.56	69.1	m	m, s
	m	1.23	21.8	l	l, m’
	n		142.6		
	o		139.9		
	p		141.4		
	q		158.4		
	r		127.8		
	s		156.3		

To establish an approximate quantity of **NPI** per pill, an extraction followed by quantitative-NMR (QNMR) was undertaken on three whole individual pills. The quantity of **NPI** in each pill ranged from 3.5 to 3.3 mg per pill (3.4 ± 0.1 mg, mean ± SD, **Table S2.3**, **Figures S2.13-S2.17**).

4. Discussion

First described in the illicit drug market 2019,[1] nitazenes were originally developed as experimental analgesic agents in the 1950s, but were never brought to market.[6,16] In September 2024, the nitazene opioid **NPI** was detected at an Australian drug checking service in an expected oxycodone sample. Of particular concern in this case was the misrepresentation of the product as a pharmaceutical agent that is commonly diverted in Australia. Although this nitazene analogue is available as a reference material from Cayman Chemical and has been investigated as a predicted NSO by several laboratories,[5,6] this is the first reported detection of this analogue within the illicit drug market.

Analysis by FTIR proved incapable of identifying any drug component within the presented pill, likely due to the low abundance of **NPI** (<2% w/w by QNMR) in the homogenised sample, a known limitation of this analytical technique.[17] Nitazene immunoassay test strips provided an initial indication of the presence of a nitazene, with reported limits of detection between 1000-9000 ng/mL for a range of common nitazene derivatives.[18] However, the inability of test strips to differentiate between nitazenes remains a limitation given the wide variation in reported *in vitro* potency reported for members of this class.[2–6]

Chromatographic analysis indicated the presence of a ‘nitazene like’ component (UPLC-PDA, CanTEST Analysis, Section 3.1) and gave structural information in the form of the molecular formula and the production of several characteristic fragment ions (GC-EI-MS and UPLC-ESI-MS/MS, ANU analysis, Section 3.2). The spectroscopic and spectrometric features of the two constitutional isomers **NPP** and **NPI** were essentially identical across the three chromatographic analytical techniques, but the compounds differed in their retention times. Such scenario provides obvious challenges to methods of analysis like direct MS that operate without chromatographic resolution of isomeric species.[19–22]

Access to an **NPI** reference material would conventionally be required to match chromatographic retention times. However, in the current context, the lengthy 3-4 month delay to acquisition of these materials, and the need for a rapid response, made this

approach impractical. NMR facilitated the rapid structural elucidation of **NPI** and clearly differentiated this from the previously observed constitutional isomer **NPP**. Furthermore, QNMR analysis found there to be a significant dosage of **NPI** in a single pill (3.4 ± 0.1 mg, mean ± SD), raising concerns regarding the risk to public health of this presentation.

The potency of nitazenes is often reported relative to morphine or fentanyl. Both **NPP** and **NPI** are powerful activators of the μ-opioid receptor, as demonstrated by their *in vitro* EC_{50} values for stimulation of [^{35}S]GTPγS binding to activated G-proteins,[5] β-arrestin 2 recruitment (MOR-βarr2) or inhibition of cAMP accumulation.[6] One study reported **NPP** and **NPI** have MOR-βarr2 agonist potencies approximately 350 times and 1100 times that of morphine (**Table S4.1**), and 30 and 90 times that or fentanyl, respectively.[6] Though complicated by a range of factors, including drug bioavailability and metabolism, studies of nitazene drugs indicate that this high *in vitro* potency translates into *in vivo* activity in animal subjects.[4,6,23–25]

The adulteration or substitution of the opioid drug supply with nitazenes internationally has been associated with adverse effects for drug users and significant mortality.[6–11] Recent reports of overdose clusters in Dublin and Cork in Ireland associated with **NPP** highlight the public health threat that accompanies the introduction of novel synthetic opioids into the drug market.[12] In the Australian context, a range of nitazenes have been detected in the drug supply and subject to alerts from public health authorities.[11,26,27] These detections have not only been associated with opioid supply, but also concerningly, the adulteration or substitution of other drugs such as benzodiazepines, cocaine, ketamine, MDMA and methamphetamine.[11,26,27] The data for such alerts typically arises from the analysis of seized drugs or the toxicology samples obtained in association with emergency department presentations.[11,27,28] Wastewater analysis, which has limited coverage of NPS and targets only representative members of the nitazene family,[29] has reported on the presence of nitazenes in the United states, but not Australia to date.[30]

Drug checking provides an alternative pathway to monitor the illicit drug market and respond to the introduction of high-risk substances such as novel synthetic opioids. Working directly with members of the public, drug checking offers the potential to immediately respond to client reports of unexpected effects or drug related harm, and where appropriate, undertake public health messaging associated with high-risk detections. The detection of **NPI** in an expected oxycodone sample reported here, was achieved in a rapid time frame. Within 24 hours of sample presentation at the drug checking service, an alert was issued to local clinical first responders to communicate the detection of a nitazene and provide information on the clinical management of nitazene harms. In the following 48 hours, a community notice was released by the CanTEST service, distributed on social media and covered by the local media. Similarly rapid responses attended earlier detections of nitazene drugs at the service, including the detection of metonitazene in December 2022 and **NPP** in July 2024 (**Table S4.1**). Together, this series of detections highlights the unpredictability and rapid change within drug markets, with a local trend toward increasing potency and potential for harm for the nitazene drugs identified.

In addition to drug market monitoring and the issue of drug alerts, drug checking services form part of a broader response to the introduction high-risk substances like nitazenes. Drug checking services provide avenues for science communication and public education. The provision of analytical test results is accompanied by health and alcohol and other drug (AOD) interventions that can be tailored to individual circumstances. This could include advice on safer using practices or dissemination of information regarding fluctuations or concerning detections in the local illicit drug market. The service also provides access to and training in the use of the opioid reversal agent naloxone, one effective measure to address nitazene related overdose.[11,27,31]

5. Conclusion

Close collaboration between an Australian drug checking service and an affiliated university laboratory has identified and characterised the novel synthetic opioid **NPI** in an expected oxycodone sample, presented by a member of the public. Previously reported *in vitro* evaluations of this compound reveals it to be among the most potent nitazene opioid agonists known.[5,6] The study highlights the rapid response possible though engaging drug users with drug checking services as a market monitor and early warning system to alert health services and the broader community to the presence of unexpected, high-risk substances. Integration of well-resourced and supported drug checking services provides a powerful approach to tackle the public health threats associated with novel synthetic opioids and other drugs of concern.

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