**Full Title**: **DARK Classics in Chemical Neuroscience: Bucinnazine**

**Running Head:** new synthetic opioids, bucinnazine, AP-237

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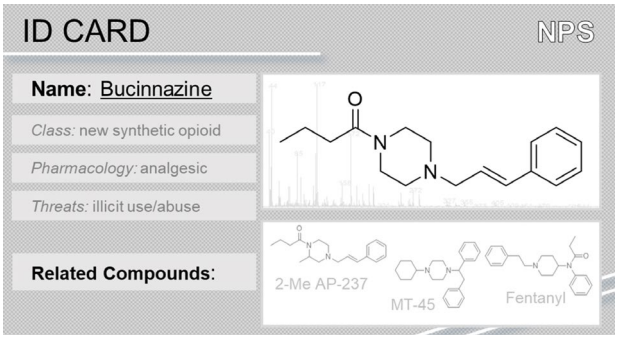
## TABLE OF CONTENTS (TOC) GRAPHIC




## ABSTRACT

Bucinnazine (1-butyryl-4-cinnamylpiperazine) is a synthetic opioid recently discovered in heroin seized samples in the U.S and in Europe. It was first synthesized in the late 60s and has been used for the treatment of cancer-associated chronic pain in China for many years. Bucinnazine is one of the most potent compounds among the series of piperazines, which also include other relevant compounds, such as MT-45, AD-1211, and 2-methyl-AP-237, a methylated derivative of bucinnazine. Bucinnazine is considered a $\mu$-selective opioid, binding primarily to the $\mu$-opioid receptor. However, bucinnazine also may share several characteristics with other piperazines, which act primarily on dopamine, serotonin, and norepinephrine neurotransmission. At the present, bucinnazine is not scheduled in the U.S., as it is not a therapeutic choice for the treatment of pain. Nevertheless, with the advent of the crypto currency and the easy access of substances on the Darknet, bucinnazine is a real threat to the public health. This review discusses the main aspects of bucinnazine's chemistry, pharmacology, and toxicology, and brings attention to the risk of the presence of this opioid in seized samples. Further studies on bucinnazine are still required to better evaluate its toxicity mechanisms, potential for drug-drug interactions, and abuse liability. Such information will be of utmost importance to guide future policies concerning the legal status of bucinnazine in the U.S.

*Keywords*: new synthetic opioids, bucinnazine, AP-237, toxicology

## INTRODUCTION

Opioid analgesics have been widely used as effective therapeutic options for pain management, and their global consumption has increased steadily in recent years. In the past 15 years, the rate of opioid pain reliever use increased drastically in the United States, and the country has experienced an increase rate in deaths from opioid overdose, opioid use disorder (OUD), and other harms as a consequence of the high use and misuse of opioids. In 2015, 140,077 emergency department visits were attributed to opioid toxidrome[1]. In 2016, a total of 63,632 drug overdose deaths occurred, corresponding to a 21.4% increase compared to 2015[2,3]. About two thirds (66.4%) of drug overdose deaths in 2016 involved prescription opioids, illicit opioids, or a combination of both, resulting in an increase of 27.7% compared to data from 2015[2,4]. In 2018, approximately 70% of the 67,367 overdose deaths were due to the use of opioids, and from 2017 to 2018, the number of synthetic opioid-involved deaths had increased by 10%, even as other opioid-involved deaths decreased slightly[3]. The Center for Disease Control (CDC) estimates that 75,500 overdose deaths occurred between March 2019 and March 2020, an increase of approximately 10% when compared to the same period one year before[5]. Synthetic opioids are currently the main cause for the drug overdose fatalities in the U.S[5].

## NEW SYNTHETIC OPIOIDS

Synthetic opioids are a subset of a larger group of substances classified as novel psychoactive substances (NPS). They are defined by the United Nations Office on Drugs and Crime (UNODC) as "substances of abuse, either in pure form or a preparation, that are not controlled by the 1961 Single Convention on Narcotic Drugs or the 1971 Convention on Psychotropic Substances, but which may pose a public health threat"[6]. Novel psychoactive substances in general, and synthetic opioids in particular, are a threat due to the lack of knowledge about their clinical effects or toxicities. The risks rely on the fact that most of these substances have not undergone the appropriate and necessary evaluation required by different authorities[7]. Several synthetic opioids have been developed and marketed by the pharmaceutical industry, for medicinal and veterinary use, while some were developed but never marketed as pharmaceutical opioids. Even though some synthetic opioids were not released for clinical treatment, they were published in

patents or in the scientific literature as part of the drug discovery and development efforts. Nefarious enterprise groups took advantage of this published knowledge to make these synthetic opioids and introduce them into the illicit market. Most synthetic opioids were originally discovered as part of the efforts undertaken to find an analgesic drug that was as potent as morphine, but less addictive[8,9].

Many synthetic opioids are substances structurally related to fentanyl (Figure 1). Nevertheless, new substances that share neither the fentanyl nor the classical morphinan chemical structures are available in the illicit market[10]. The virtually infinite possibilities for structural modifications generating new and more potent molecules make the identification of synthetic opioids very challenging, in both drug seizures and in victims or patients exhibiting an opioid toxidrome.

One specific synthetic opioid that recently re-emerged into the illicit drug market and is causing some concern is AP-237, also known as bucinnazine (1-butyryl-4-cinnamylpiperazine) (Figure 1). In 2019, bucinnazine, along with its main structural analog, 2-methyl AP-237 (Figure 1), were detected in Europe through the UNODC Early Warning System (EWA), and in the United States soon after[11,12].

## CHEMISTRY OF BUCINNAZINE

AP-237 or bucinnazine (1-butyryl-4-cinnamylpiperazine) is a synthetic opioid categorized as a new psychoactive substance due to its potential for abuse[8,11]. It was first synthesized in Japan in the late 1960s and it is one of the most potent compounds among the piperazine-amides series[9]. Bucinnazine is primarily used in China for the treatment of cancer pain as an alternative to morphine, but it is not approved for clinical use within the U.S. Currently, bucinnazine and its analog 2-methyl bucinnazine (2-methyl AP-237) are not scheduled in the U.S. presenting a public health concern.

Chemically, bucinnazine is a white crystalline powder with a molecular weight of 272.4 g/mol, and a melting point of 184-185°C[13]. Bucinnazine is often present in the salt form (hydrochloride), which is easily soluble in water. Bucinnazine has high lipophilicity, which allows rapid diffusion through membranes such as the blood-brain barrier (BBB) binding directly to opioid receptors in the brain.

Several compounds containing a piperazine ring are described as physiologically active including antihistaminic, tranquilizing, ganglion stimulating, and antitumor

agents[14]. Irikura *et al.* described the importance of the substitution pattern in the piperazine ring for the analgesic potential of several compounds synthesized by the authors. Compounds containing 1,4-bis butyryl substitutions were described as the most potent, and replacing the butyryl group by a smaller or longer chain led to a considerable diminution of the activity. Compounds substituted with a cinnamyl group at N4 of the piperazine ring in 1-acyl-4-substituted piperazines showed potent analgesic activity proving the importance of these substitutions. Among the compounds tested, bucinnazine showed to be the most potent[14] (see Pharmacology and Toxicology of Bucinnazine Section for further details).

## SYNTHESIS OF BUCINNAZINE

The first synthesis of bucinnazine has been reported in 1968, by Ito and co-workers, in a work developed in the Kyorin Pharmaceutical Company. They reported a library of 1-acyl-4-substituted piperazine derivatives (31 examples), evaluated their analgesic activity and established a structure-activity relationship. Among the derivatives prepared, bucinnazine stood out as the most promising analgesic drug candidate. A total of three synthetic pathways were reported by this group to attain this relevant molecule. One consisted of the condensation between n-butyryl chloride with 1-cinnamylpiperazine (achieved by reacting cinnamyl bromide or chloride with 1-formylpiperazine, in the presence of $NaHCO_3$, leading to the formation of 1-cinnamyl-4-formylpiperazine, which further underwent deformylation in heated aqueous NaOH 30%) in the presence of $NaHCO_3$ (Scheme 1A). A second synthetic route was established by reacting 1-butyrylpiperazine (prepared through the reaction between butyryl chloride and 1-formylpiperazine in the presence of $NaHCO_3$, generating 1-butyryl-4-formylpiperazine, which was further deprotected in the presence of NaH) and cinnamyl bromide in the presence of $NaHCO_3$, in refluxing benzene (Scheme 1B). A third methodology reported by this group consisted of the reaction between cinnamaldehyde and 1-butyrylpiperazine in heated formic acid (Scheme 1C)[14].

The three synthetic pathways described lead to the formation of the free base in good to very good yields, without requiring tedious and time-consuming work-up procedures (chromatography-free protocols). Furthermore, bucinnazine could easily be converted to its hydrochloride salt version at the end of the synthetic routes[14].

The great potential of this compound as an analgesic agent made other researchers focus on the development of novel derivatives to assess their biological activity. Protiva *et al*. reported the synthesis of several 1-aminoacyl-4-cinnamylpiperazines and bucinnazine to compare this parent compound to the new derivatives. The synthetic methodology they used was the one already described in Scheme 1. Nevertheless, the new derivatives proved to be less active than the parent compound, bucinnazine[13].

In recent years, the synthesis of aliphatic carboxamides has captured the attention of several researchers in the field of synthetic organic chemistry and the example of bucinnazine is still reported as a model for the application of these reactions. Two examples of NN2 nickel pincer complexes-mediated aminocarbonylation reactions have been recently described by Skrydstrup and co-workers, using slightly different synthetic protocols, suitable for carbon-isotope labeling. In the first example, a two-chamber setup is used, with one chamber being charged with the nickel(II) complex and n-propylzinc bromide in THF, which are allowed to stir at room temperature for 30 minutes to form in situ an alkyl-nickel complex. To this chamber is then added 1-cinnamylpiperazine and DBU (1,8-diazabicyclo(5.4.0)undec-7-ene). To the second chamber, pre-filled with argon and while performing the experiment in an argon filled glovebox, SilaCOgen, a solid CO source with a labeled carbon isotope, was added, in the presence of KF and using DMF as a solvent. In this second chamber, the release of labeled carbon monoxide occurs, leading to the formation of a stable acyl-nickel complex in the first chamber, with the acyl group being then transferred to the secondary amine group of the piperazine cycle, leading to the formation of 13C-labeled bucinnazine in very good yield (87%) (Scheme 2A)[15]. In the second example, using a similar nickel(II) pincer complex, the same solid carbon monoxide source (this time experiments were conducted using SilaCOgen with and without 13C-labeling), also in a two-chamber setup, the target compound was achieved in very good yields (69% for the unlabeled version, 79% for the labeled compound). In this approach, 1,6-bis(2-pyridyl)-2,5-dithiahexane (Py2S2) is introduced to the system and is quickly acylated leading to the formation of the 2-pyridil thioester, which further reacts with the 1-cinnamylpiperazine to afford the final product (Scheme 2B). These approaches showcase the potential of nickel(II)-promoted aminocarbonylation reactions in the preparation of active pharmaceutical ingredients and, at the same time, constitute alternative and effective synthetic routes for the preparation of bucinnazine[16].

## PHARMACOLOGY OF BUCINNAZINE

Bucinnazine is a synthetic opioid analgesic drug classified as new synthetic opioid (NSO). NSOs are classified in four sub-chemical classes namely phenylpiperidines, cyclohexylbenzamides, acetamides, and piperazines. Bucinnazine is one of the most potent compounds among the series of piperazines which also include MT-45 (Figure 2), AD-1211 (Figure 2), and 2-methyl-AP-237 (Figure 1), a methylated derivative of bucinnazine.

Bucinnazine presents higher therapeutic index, being a good alternative for the treatment of pain[17]. Its analgesic activity has been investigated in several studies since the 1970s[9,14,18], including its potential to cause dependence. Currently, bucinnazine is the analgesic of choice for the treatment of cancer-associated chronic pain in China.

Synthetic opioids can bind to the $\mu$-opioid receptors, the $\kappa$-opioid receptors, and the $\delta$-opioid receptors in the brain. Bucinnazine is considered a $\mu$-selective opioid, meaning that it primarily binds to the $\mu$-opioid receptor[8]. However, bucinnazine also may share several characteristics with other piperazines, which act primarily on dopamine, serotonin, and norepinephrine neurotransmission[19].

Bucinnazine is able to activate both protein G and β-arrestin pathways when binding to the μ-opioid receptor (MOR). Vandeputte *et al.* demonstrated the capacity of bucinnazine in activating both pathways using the nanoluciferase assay in HEK293 cells expressing either the MOR-βarr2-GRK2 or MOR-mini-Gi system. Comparing to the response triggered by hydromorphone (considered 100% response), bucinnazine activated both pathways with 50% of response[12].

Bucinnazine is a weak opioid receptor agonist[20]. This makes bucinnazine a moderate to weak analgesic, as its analgesic effect is approximately one third of the one observed with morphine[21]. In fact, this is an indication that bucinnazine may be less addictive than morphine and other opioids, which is an advantage when it comes to clinical pain treatment. As a fast-acting analgesic, bucinnazine is most effective in shorter periods of time. The defined daily dose of bucinnazine was determined to be 30 mg as a bucinnazine hydrochloride tablet, or 1g as a bucinnazine hydrochloride injection for the pain treatment with desired effects of analgesia and sedation[21]. However, it has been noted that users of bucinnazine have experienced adverse effects as well, such as addiction and dependence, respiratory depression, and allergic reactions[22]. Most of these adverse effects come from

improper use of bucinnazine. Additionally, bucinnazine may share some adverse effects with other piperazines, such as agitation, headache, and insomnia[19].

Pharmacologically, bucinnazine is classified as a synthetic opioid. However, differently from fentanyl and its analogs, bucinnazine belongs to the chemical family of piperazines. Piperazines are compounds that contain a six-membered ring containing nitrogen atoms in opposing positions (N1 and N4 in bucinnazine). The variety of possible substitutions in both nitrogen atoms makes the piperazine ring an excellent skeleton for the development of new compounds. Indeed, different substitutions lead to different biological activities in the piperazine substituted molecules. Piperazines were previously described to be applied as antihistaminic, anthelmintic, antidepressants, among others applications[23,24].

Bucinnazine is a disubstituted piperazine in both nitrogen atoms of the heterocycle. Generally, piperazines containing 1,4-bis butyryl substitutions were described as the ones possessing more potent biological activity, and replacing the butyryl group by a smaller or a longer acyl group led to a considerable diminution of the activity. Compounds substituted with a cinnamyl group at N4 of the piperazine ring in 1-acyl-4-substituted piperazines showed potent analgesic activity proving the importance of these substitutions.

In general, the pharmacology of bucinnazine indicates both a depression and a stimulation of the central nervous system. There is possible ganglionic blocking and anti-serotonin activity, and bucinnazine has some tranquilizing effects in addition to its analgesic activity. Studies performed by Carrano *et al.* on rats, dogs, and cats, showed that bucinnazine caused behavior effects similar to those caused by morphine[9,25]. The maximum sublethal dose (MSLD) in rats was determined to be greater than 1.0 g/kg, a higher dose when compared to morphine's 130 mg/Kg[26].

In studies carried out by Irikura *et al.*[14], the authors described the analgesic effects of bucinnazine in mice, rats, and guinea pigs. The pressure, the hot plate, the D'Armour-Smith's, and the benzoquinone writhing methods were applied to identify the analgesic potency of bucinnazine. In all animals tested, the administration of bucinnazine resulted in a marked elevation of pain threshold. A gender difference in the analgesic potency of bucinnazine was observed only in albino rats, being 9 times more potent in female than in male rats, possibly due to the hepatic metabolism of bucinnazine. This gender-dependent metabolism in rats is the result of differences in expression of hepatic enzymes.

The sex-specific cytochrome P450s CYP2C11, CYP2C13, and CYP3A2 are expressed in males whereas CYP2C12 is expressed in females[27]. Furthermore, in this study, animals were treated using different routes of administration, and in the experiments carried out using *per os* administration, bucinnazine showed no less potency, and prompt onset of the effect, evidencing its good gastrointestinal and good overall bioavailability[28]. Bucinnazine has also demonstrated circadian variation in its effects. In a study by Yu *et al.*, it was found that bucinnazine is more potent in the morning than in the evening, compared to morphine and fentanyl[20].

## METHODS OF ANALYSIS

Most of the synthetic opioids that have been re-introduced into the illicit markets cannot be analyzed by standard immunoassays, mostly due to their unique structures and low concentrations[2]. In addition, piperazines specifically cannot be detected by routine urine immunoassays[19]. Several ELISA screening kits are available for the detection of synthetic opioids such as fentanyl that give adequate cross reactivity with its analogues. However, bucinnazine does not share structural similarity with fentanyl preventing these kits from working as efficiently as needed for the detection of synthetic opioids with different structural moieties[8]. Although immunoassays for detecting non-fentanyl analogs were recently developed, the difficult process for their development and the fast increase in the number and variety of new synthetic opioids remain a challenge[29]. Therefore, the development of new methods of analysis is a mandatory task for further studies. The most common methods applied for the detection of opioids in forensic samples are gas chromatography coupled to mass spectrometry (GC-MS), liquid chromatography coupled to mass spectrometry (LC-MS), and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)[30–32], although thin-layer chromatography (TLC), Raman Spectroscopy, and chromatography with detectors other than MS detectors are also applied[33,34].

The most recent report of seized samples containing bucinnazine was published in 2019. GC-MS and LC-QTOF were applied for the identification of the unknown compound further identified as bucinnazine[11]. The mass spectra results showed *m/z* 272, *m/z* 172, and *m/z* 117 as the three most abundant peaks. The major fragment ion on the spectra occurs at *m/z* 117 and corresponds to the cinnamyl cation, and the m/z 172 is the

result of the formation of the N-methylene-3-phenyl-N-vinylprop-en-1-aminium with the fragmentation of the piperazine ring. A proposed mass spectral fragmentation pattern of bucinnazine based on the common mass spectral fragmentation pattern of several piperazines[35] is showed in Figure 3.

Zhang *et al.* described the use of surface enhanced Raman spectroscopy (SERS) to detect bucinnazine in water and urine samples. The authors showed a successful application of the method, with a linear relationship between bucinnazine concentrations and the Raman peak intensity in both water and urine samples. The method showed lower detection limits and good linearity, being suitable for quantitative analysis[36]. While this method was successful, it involves instrumentation that are not broadly available in forensic laboratories.

The use of HPLC-DAD was also described as a suitable method for the analysis of bucinnazine. Zhang *et al.* reported the detection of bucinnazine and 60 other compounds in a systematic toxicological analysis (STA) evaluating the selectivity of the method in forensically relevant biological samples. The method described was effective for the identification of bucinnazine in biological samples. However, HPLC-DAD has poor sensitivity when compared to MS, and for this reason other methods may be more beneficial[37].

In forensic cases, LC-MS/MS is currently the technique of choice for the detection of bucinnazine. Methods based in reversed-phase chromatography using gradient mobile phase are the best option for bucinnazine analysis in different samples. With the reintroduction of bucinnazine and other synthetic opioids to the illicit market, determining methods of analysis that are efficient and effective is now more important than ever.

## METABOLISM

Bucinnazine was first synthesized in the late 60s, and has been prescribed for pain treatment in China for many years. Despite its long clinical history, few data are available concerning its metabolism. The study of drug metabolites has been applied for the identification of biomarkers of drug abuse in blood and urine of patients and it shows to be a useful tool for the identification of new synthetic opioids when more powerful techniques are applied[2,38,39]. Metabolites play an important role in forensic cases as they

can be not only biomarkers of previous use but can also be more active or toxic then the parent drug.

Initial studies on bucinnazine metabolism were reported in rats and rabbits where metabolites were identified using gas chromatography and isotope tracing techniques[40,41]. Seven main metabolites were found; 1-butyryl-4-(4-hydroxycinnamyl)piperazine, 1-cinnamylpiperazine, 1-(4-hydroxycinnamyl)piperazine, benzoic acid, hippuric acid, 4-hydroxybenzoic acid, and 4-hydroxyhippuric acid[40,41]. Both authors described the main bucinnazine metabolic pathway as through *p*-hydroxylation.

Bucinnazine metabolism can also be compared to the metabolism of other cinnamylpiperazines and piperazines. Most piperazines undergo extensive phase I metabolism including CYP3A4-dependent N-dealkylation and CYP2D6-dependent oxidation to hydroxylates[42].

MT-45 is a piperazine and a synthetic opioid that shares some structural similarities with bucinnazine. MT-45 phase I metabolites are obtained via *N*-dealkyation and hydroxylation, and a lower proportion of phase II metabolites are produced via glucuronidation[43]. Cinnarizine and its fluorine derivative, flunarizine are both compounds which share structural similarity with bucinnazine, and have been the subject of metabolic studies in humans. Cinnarizine undergoes *N*-dealkyation and hydroxylation of the cinnamyl phenyl ring and/or in the diphenylmethyl group in phase I metabolism[44]. Scheme 3 shows a proposed bucinnazine metabolic pathway.

## CURRENT ISSUES AND CONCERNS ON BUCINNAZINE ABUSE

Synthetic opioids are broadly found in street samples most commonly sold as adulterations in mixtures with heroin and cocaine or as counterfeit pharmaceuticals resembling prescription pills such as hydrocodone or oxycodone[3,45,46]. Synthetic opioids can present different affinities for the main opioid receptors as well as different potencies. For example, carfentanil, a fentanyl analogue compound, is 10,000 times more potent than morphine, and despite its availability exclusively for veterinary applications, several deaths were connected to the presence of this substance[47,48].

The original purpose for the creation of synthetic opioids was to find a safer and more effective version of morphine. However, this has backfired and has instead created opportunities for illicit drug manufacturers and underground chemists to synthesize

modified products that avoid legal restrictions and increase their profits[8]. Bucinnazine is not a drug of choice in the U.S for the treatment of pain, thus it is not scheduled. Currently, with the advent of the crypto currency and the easy access of substances on the Darknet[6], bucinnazine is a real threat to the public health. The evidence of the role that cryptomarkets play in the drug market increased in the past years, showing an increase of the distribution of illicit substances in Western countries, even despite any law enforcement effort[49]. The identification of seized samples containing bucinnazine is part of a larger public health issue and the development of reliable analytical methods for this purpose has become a mandatory task for the monitoring of bucinnazine in the market.

Furthermore, the information about bucinnazine effects is still limited and many questions remain unanswered regarding its safety, toxicity, and abuse liability. Further work for the study of its toxic mechanisms, drug-drug interaction, and abuse liability would provide relevant data about toxic effects aiding to guide the policies concerning the legal status of this substance in the U.S.

## SUPPORTING INFORMATION AVAILABLE

No support information is available.

## ABBREVIATIONS:

BBB – blood-brain barrier
CDC – Center for Disease Control
CYP – cytochrome P450
DEA – drug enforcement agency
ELISA – enzyme-linked immunosorbent assay
EWA - Early Warning System
GC – gas chromatography
GPCR – G protein coupled receptors
HEK 293 – human embryonic kidney cells
HPLC-DAD – hig performance liquid chromatography coupled to diode array detector
LC – liquid chromatography
LC-QTOF - liquid chromatography with quadrupole and time of flight mass detector

MOR – μ-opioid receptor

MS – mass spectrometry

MSLD - maximum sublethal dose

MS/MS – tandem mass spectrometry

NPS – new psychoactive substances

NSO – new synthetic opioids

OUD – opioid use disorder

SERS - surface enhanced Raman spectroscopy

STA – systematic toxicological analysis

TLC – thin layer chromatography

UNODC – United Nations Office for Drugs and Crime

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Karissa Resnik, Pedro Brandão, and Emanuele Amorim Alves developed the theory and wrote the manuscript. Emanuele Amorim Alves was responsible for the final concept of the manuscript.

## FUNDING SOURCES/ACKNOWLEDGEMENTS

Authors declare no funding source or acknowledgements.

## CONFLICT OF INTEREST STATEMENT

Authors declare no conflict of interest, particularly no financial and personal relationships with other people or organizations that could inappropriately influence (bias) this work.

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## FIGURE LEGENDS

**Figure 1 –** Chemical structures of fentanyl, bucinnazine and 2-methyl-AP-237. The piperidine ring is showed in blue and the piperazine in red.

**Figure 2** – Chemical structures of new synthetic opioids MT-45 and AD-1211.

**Figure 3 –** Mass spectra and main fragments of bucinnazine.

## SCHEME LEGENDS

**Scheme 1 –** First synthetic pathways described for the preparation of bucinnazine.

**Scheme 2 –** Recent developments on bucinnazine synthesis using nickel(II) NN2-pincer complexes via aminocarbonylation reactions.

**Scheme 3 –** Proposed phase I metabolic pathway of bucinnazine considering the metabolism of piperazines as described by Baba *et al.* and Morishita *et al.*[40,41]. (1) 1-butyryl-4-(4-hydroxycinnamyl)piperazine; (2) 1-cinnamylpiperazine; (3) benzoic acid; (4) hippuric acid; (5) 1-(4-hydroxycinnamyl)piperazine; (6) 4-hydroxyhippuric acid; (7) 4-hydroxybenzoic acid.