Chapter 5

Bioactive Alkaloids of Hallucinogenic Mushrooms

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Chapter Outline

INTRODUCTION

Alkaloids are defined as a group of naturally occurring chemical compounds that mostly contain basic nitrogen atoms [1]. Alkaloid molecules are extremely important for biomedical science. They have a unique property – an ability to work as either hydrogen-acceptor or hydrogen-donor for hydrogen bonding, depending on the type of amine functionality present in alkaloids. This property is critically important for the interaction (binding) between targets (enzymes, proteins, and receptors) and drugs (ligands) possessing alkaloid scaffold [2]. Certain drugs with alkaloid structural features were synthesized by naturally occurring alkaloids, to which belong indole, indolinone, isoindole, isoxazole, imidazole, indazole, thiazole, pyrazole, oxazolidinone, oxadiazole, benzazepine, and many others [2].

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From a pharmaceutical and industrial point of view, alkaloids are probably the most important fungal metabolites. Our work concentrates on two groups of naturally occurring alkaloids in higher fungi (indoles and isoxazoles). In nature, indoles are probably the most often occurring heterocyclic compounds, having medicinal importance [3]. Two simple indole alkaloids: psilocin (3-[2 (dimethylamino) ethyl]-4-indolol) and psilocybin ([3-(2-dimethylaminoethyl)-1*H*-indol-4-yl] dihydrogen phosphate) are present in many mushroom species. These mushrooms are called hallucinogenic, psychedelic, entheogenic, magic, medicinal, neurotropic, psychoactive, sacred, or saint mushrooms [4]. Also other analogs of psilocybin, known as baeocystin, norbaeocystin, bufotenin, and aeruginascin, were found in hallucinogenic mushrooms. Hallucinogenic compounds were chemically identified in mushrooms belonging to various genera, e.g., *Agrocybe*, *Conocybe*, *Galerina*, *Gymnopilus*, *Hypholoma*, *Inocybe*, *Panaeolus*, *Psilocybe*, *Pholiotina*, *Pluteus*, and *Weraroa* [5]. Allen et al. listed 206 fungal species containing tryptamine alkaloids; however, some of them are false positives, which were noted down at the end of that update [5]. In the case of around 90 species mentioned by Allen, the presence of psilocin or psilocybin was proven by chemical analyzes.

They are also psychoactive species of genus *Amanita*, which contain the alkaloids (muscimol, ibotenic acid, muscazone, and muscarine) reacting with neurotransmitter receptors in the central nervous system. These alkaloids, except muscarine, belong to the chemical group of isoxazoles. Isoxazoles often exhibit extensive and pharmacologically important biological activities. Muscarine is the principal alkaloid toxin in the fungi of genera *Inocybe* and *Clitocybe* [6,7]. Although it does not belong to isoxazoles, in our opinion this alkaloid is important and is therefore described in our chapter.

Our work analyzes the origin, chemistry, biological activity, and the biomedical importance of bioactive alkaloids found in hallucinogenic mushrooms. In the present review, we aimed to cover the most important scientific papers within this field of science.

CHEMICAL CLASSES OF ALKALOIDS FOUND IN MUSHROOMS

There are four hallucinogenic substances in *Amanita* sp., which can cause hallucinogenic effect: muscimol, ibotenic acid, muscazone, and muscarine (Fig. 5.1a–d). All of these substances except muscarine are isoxazoles [8]. They are quickly absorbed through the digestive system [9]. Ibotenic acid (α-amino-3-hydroxy-5-isoxazoloacetic acid) is a substance soluble in water [7]. Pure ibotenic acid is colorless and unstable in solution [8]. The melting point of ibotenic acid is 150–152°C. The largest amount of this substance in *Amanita* mushrooms is located in the red cap and yellow tissue under the cap (*Amanita muscaria*) [7]. Muscimol (5-(aminomethyl)-3-hydroxyisoxazole) is more hydrophobic than ibotenic acid, but it is also soluble in cold water [7,8]. Muscimol is colorless and the melting point is 175°C. It is a product of decarboxylation of ibotenic acid [7,10]. Both substances ibotenic acid and muscimol have structures similar to human neurotransmitters: glutamic acid and GABA

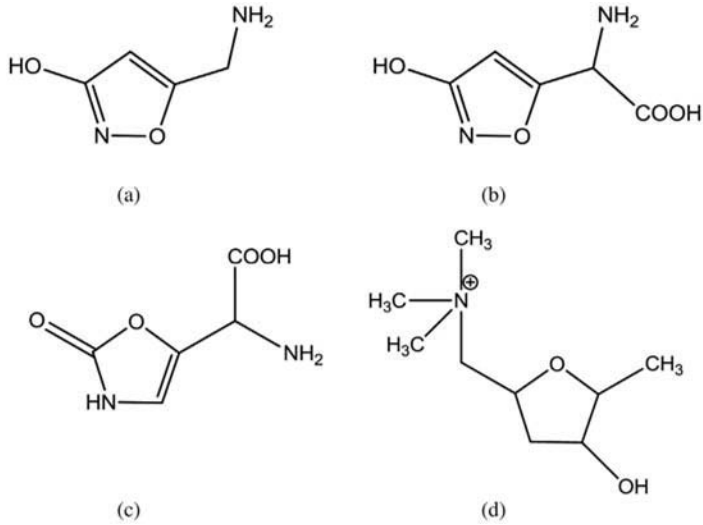

FIGURE 5.1 Chemical structures of (a) ibotenic acid, (b) muscimol, (c) muscazone, and (d) muscarine.

[11]. Muscazone (α-amino-2,3-dihydro-2-oxo-5-oxazoloacetic acid) is a lactam isomer of muscimol [7,11]. The melting point of muscazone is 190°C. It is also a colorless substance. The hallucinogenic properties of muscazone are weaker than ibotenic acid or muscimol [7]. Muscarine (2,5-anhydro-1,4,6-trideoxy-6-(trimethylammonio)-D-ribo-hexitol) is a substance soluble in water. The melting point of that compound is 180°C [7]. This alkaloid exhibits less hallucinogenic activity than ibotenic acid and muscimol [12]. Muscarine has three chiral centers and eight configurational isomers [13].

The main alkaloids tryptamine/indolamine derivatives in mushrooms, exhibiting hallucinogenic properties, are psilocybin and psilocin (Fig. 5.2a, b) [14–18]. In addition to the mentioned alkaloids, mushrooms also contain baeocystin, which is a derivative of the psilocybin and norbaeocystin (Fig. 5.2c, d) [18,19]. It is also known that there are such psychoactive compounds as aeruginascin and bufotenine (Fig. 5.2 e, f) [18,20,21]. Psilocybin (O-phosphoryl-4-hydroxy-*N*,*N*-dimethyltryptamine) and its main dephosphorylated metabolite psilocin (*N*,*N*-dimethyltryptamine) belong to tryptamine hallucinogens and are structurally similar to serotonin [15,19]. It is worth emphasizing that psilocybin is one of the naturally occurring alkaloids, which contain a phosphorus atom in its structure [16]. Psilocybin and psilocin in its purest of forms are white crystalline powder [15]. Psilocybin is soluble in water, while psilocin is a more lipid soluble. Both substances are soluble in methanol and ethanol [15,18]. The melting point of psilocybin is 185–195°C, and of psilocin, it is 173–176°C [8,18]. Both of this indole psychedelic compounds are unstable in light; their stability is good in low temperatures and in the dark [14,15]. Other compounds belonging to alkaloids with hallucinogenic properties are baeocystin (4-phosphoryloxy-*N*-methyltryptamine) and norbaeocystin. These compounds are less explored than psilocybin

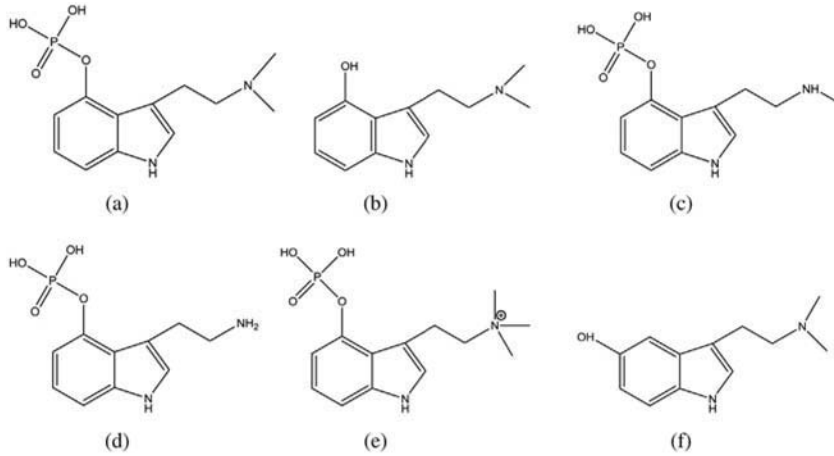

FIGURE 5.2 Chemical structures of (a) psilocybin, (b) psilocin, (c) baeocystin, (d) norbeaocystin, (e) aeruginascin, and (f) bufotenine.

and psilocin [22]. Baeocystin is a mono-methyl analog of psilocybin, showing a UV spectrum identical to psilocybin, indicating it to be a 4-substituted indole derivative. The melting point of this substance is 254–258°C [23]. Norbaeocystin is a demethylated equivalent of psilocybin [18]. The melting point of norbaeocystin is 188–192°C [24]. Both baeocystin and norbaeocystin are tryptophan derivatives formed through decarboxylation, indole ring hydroxylation at position 4, and N-methylation through the activity of *S*-adenosylmethionine and O-phosphorylation [8]. Bufotenine (*N*,*N*-dimethyl-5-hydroxytryptamine) is a positional isomer of psilocin [21]. Bufotenine has low lipid solubility [25]. The melting point of bufotenine is 146.5°C. The last hallucinogenic compound mentioned in this chapter – aeruginascin (Fig. 5.2e) – is a trimethyl analog of psilocybin [22]. It is a quaternary ammonium compound *N*,*N*,*N*-trimethyl-4-phosphoryloxytryptamine. Aeruginascin is stable at the room temperature and is more polar than psilocybin [20].

ANALYTICAL METHODS USED FOR ALKALOIDS ISOLATION AND DETECTION

It is difficult to analyze mushrooms extract using available chromatographic techniques because of the complex composition of mushrooms. In most cases, before carrying out the proper analysis, it is necessary to use appropriate sample preparation methods for isolation, purification, and concentration of the mushrooms hallucinogens analytes and biological samples.

There are many methods used to isolate substances from samples. These include liquid–liquid extraction (LLE), Soxhlet extraction, solid-phase extraction (SPE), or supercritical fluid extraction (SFE). The most popular extraction methods for isolation hallucinogenic substances from mushrooms are ultrasonic-assisted extraction and SPE (Table 5.1) [10,26–37]. Ultrasounds are

TABLE 5.1 The Methods of Extraction and Identification of Ibotenic Acid, Muscimol, Psilocin, and Psilocybin in Mushroom Samples

No.	Analyte	Matrix	Extraction method	Recovery (*R*%)	Detection technique	LOD LOQ	Amounts of alkaloids	References
1.	Muscimol	*A. muscaria* *A. pantherina*	UAE	96–101.1	HPLC–MS	1.4 mg/L 4.6 mg/L	Intraassay 41.3–1988 mg/kg Interassay 39.4–1987 mg/kg	[25]
2.	Ibotenic acid	*A. muscaria* *A. pantherina*	UAE	99.4–100.9	HPLC–MS	7.8 mg/L 25.9 mg/L	Intraassay 52.6–1978 mg/kg Interassay 60.7–1901 mg/kg	[25]
3.	Muscimol	*A. muscaria* *A. pantherina*	UAE	–	GC–MS	– –	*A. muscaria* 284–1052 mg/kg (cap) *A. pantherina* 1554–1880 mg/kg (cap)	[10]
4.	Ibotenic acid	*A. muscaria* *A. pantherina*	UAE	–	GC–MS	– –	*A. muscaria* <10–2845 mg/kg (cap) *A. pantherina* 188–269 mg/kg (cap)	[10]
5.	Muscimol	*A. muscaria*	UAE	–	HPLC–UV	30 μg/L	0.38 g/kg (cap), 0.08 g/kg (stem)	[27]
6.	Ibotenic acid	*A. muscaria*	UAE	–	HPLC–UV	18 μg/L	0.99 g/kg (cap), 0.23 g/kg (stem)	[27]
7.	Muscimol	*A. muscaria* *A. pantherina*	UAE, SPE	Intraday: 101 Interday: 101	HPLC–MS	2.4 mg/kg 7.9 mg/kg	*A. muscaria* 133 mg/kg	[26]

(Continued)

TABLE 5.1 The Methods of Extraction and Identification of Ibotenic Acid, Muscimol, Psilocin, and Psilocybin in Mushroom Samples *(cont.)*

No.	Analyte	Matrix	Extraction method	Recovery (*R*%)	Detection technique	LOD LOQ	Amounts of alkaloids	References
8.	Ibotenic acid	*A. muscaria* *A. pantherina*	UAE, SPE	Intraday: 99.8 Interday: 94.8	HPLC–MS	4.9 mg/kg 16 mg/kg	*A. muscaria* 146 mg/kg	[26]
9.	Muscimol	*Amanita* mushrooms	SPE	>80	HPLC–MS	<10 mg/kg	107 mg/kg	[30]
10.	Ibotenic acid	*Amanita* mushrooms	SPE	>80	HPLC–MS	<10 mg/kg	210 mg/kg	[30]
11.	Psilocybin	*P. semilanceata*	UAE	98	CE–UV	0.045 g/kg 0.225 g/kg	–	[28]
12.	Psilocin	*P. cubensis* *P. tampanensis* *P. cyanescens*	UAE	98.8	HPLC–DAD	60 mg/kg 220 mg/kg	0.12–2.52 g/kg	[29]
13.	Psilocybin	*P. cubensis* *P. tampanensis* *P. cyanescens*	UAE	97.3	HPLC–DAD	70 mg/kg 280 mg/kg	0.38–10.48 g/kg	[29]
14.	Psilocin	*P. cubensis* *P. tampanensis* *P. cyanescens*	UAE	~96.8	HPLC–ESI–MS	6.4 mg/kg –	6.42–12.67 mg/kg	[31]
15.	Psilocybin	*P. cubensis* *P. tampanensis* *P. cyanescens*	UAE	~96.8	HPLC–ESI–MS	6.4 mg/kg –	–	[31]
16.	Psilocybin	*Psilocybe* genus	UAE	–	HPLC–FL	– –	4.8–20 mg/kg	[33]

17.	Psilocybin		UAE	93–98	GC–MS	–	–	[32]
						–		
18.	Psilocin	*P. cubensis* *Copelandia* genus	UAE	–	HPLC–UV	–	–	[34]
						–		
19.	Psilocybin	*P. cubensis* *Copelandia* genus	UAE	–	HPLC–UV	–	–	[34]
						–		
20.	Psilocin	*P. semilanceata* *P. cubensis* *P. tampanesis* *P. cyanescens*	UAE	–	HPLC	–	–	[36]
						–		
21.	Psilocybin	*P. semilanceata* *P. cubensis* *P. tampanesis* *P. cyanescens*	UAE	–	HPLC	–	–	[36]
						–		
22.	Psilocin	*P. subcubensis*	UAE	–	GC–MS	Cap: 0.2 g/kg, stem: 0.2 g/kg	0.2 g/kg (cap), 0.3 g/kg (stem)	[35]
						–		
23.	Psilocybin	*P. subcubensis*	UAE		GC–MS	Cap: 8.6 g/kg, stem: 8.0 g/kg	8.6 g/kg (cap), 8.0 g/kg (stem)	[35]
						–		
24.	Psilocin	Mushrooms material	LLE	–	GC–MS	–	–	[37]
						–		
25.	Psilocybin	Mushrooms material	LLE	–	GC–MS	–	–	[37]
						–		

the source of additional energy, which allows separating the analytes from the sample matrix by destroying mushroom cells. The ultrasonication technique is faster, inexpensive, and more effective than other traditional methods used for natural substances isolation. Other extraction methods, which are also used, are SPE [27,31] and LLE [38]. In later sections, we present brief descriptions of some works on the isolation and determination of hallucinogenic substances. The amounts of alkaloids in different mushrooms are shown in Table 5.1 (for some studies these data are not available).

Amanita Mushrooms

Tsujikawa et al. isolated muscimol and ibotenic acid from *A. muscaria* and *Amanita pantherina*. The dried mushrooms were grounded to a fine powder. Next, 2 mL of 70% methanol was added to 50 mg of powdered mushrooms. After shaking (1 min) and ultrasonication (5 min), the sample was centrifuged (3000 rpm, 3 min), and the supernatant was transferred to another test tube. For residues, all procedure was repeated in the same way, and 100 μL of combined extract was evaporated to dryness under the stream of nitrogen. After evaporation, the sample was subjected to dansylation reaction. The dried residue was dissolved in 100 μL of 25 mM borax solution pH 9.5 and 50 μL of 20 mM DNS-Cl in acetonitrile. This mixture reacted at room temperature for 90 min. The reaction was stopped by the addition of 10 μL of ethanoloamine solution into the aforementioned borax solution. The next step included a conversion of DNS–IBO to DNS–IBO–Et because DNS–IBO could not be separated from the matrices. 1 mL of borax solution and 3 mL of ethyl acetate were added to dansylated solution. After shaking (5 min) and centrifugation (3 min), the ethyl acetate layer was transferred to another flask. The aqueous layer was extracted two more times with ethyl acetate (3 mL × 2), and the procedure was conducted in the same way. The combined ethyl acetate layer (9 mL) was evaporated to dryness under a stream of nitrogen, and the residue became a derivative with 100 μL of 1.25 M hydrogen chloride in ethanol. The mixture reacted at a temperature of 55°C for 60 min. The reaction was stopped by the evaporation of the reagent under a stream of nitrogen. The residue was dissolved in 100 μL mixture of ethanol/water ($1{:}1_{v/v}$). The sample was filtered before the HPLC analysis. The obtained recoveries for ibotenic acid and muscimol were 99.4–100.9% and 96.0–101.1%, respectively. The limits of detection were 7.8 mg/L for ibotenic acid and 1.4 mg/L for muscimol. The limits of quantification were 25.9 mg/L for ibotenic acid and 4.6 mg/L for muscimol [26].

Tsujikawa et al. also determined hallucinogenic substances in *A. pantherina* and *A. muscaria*. The procedure of extraction was conducted in the same way. After extraction, 200 μL of the sample was evaporated to dryness under a stream of nitrogen, and the residue was derivatized by a reaction with a mixture of 50 μL of BSTFA with 10% of TMCS and 50 μL of ethyl acetate containing 20 μg/mL *n*-pentadecane (IS).

The process of creating a derivative was performed at 80°C for 30 min. Next, ibotenic acid and muscimol were determined by GC–MS, using a selective ion-monitoring (SIM) mode [10].

Gennaro et al. extracted the powdered *A. muscaria* mushroom with ultrapure water in ultrasonic bath lasting 15 min. After sonification, the supernatant was transferred to another flask, and the residue was washed twice with ultrapure water in order to improve the recovery. The combined supernatant was diluted in ultrapure water and filtered before the HPLC analysis. The limits of detection were 18 μg/L for ibotenic acid and 30 μg/L for muscimol [28].

Yoshioka et al. extracted ibotenic acid and muscimol, as well as seven other toxins, from mushrooms as follows: 200 mg of mushrooms was homogenized in 2.5 mL of 0.5% formic acid in methanol and ultrasonicated for 0.5 and 1 min, respectively. After centrifugation (1000 rpm, 3 min), the supernatant was transferred into another flask. The residue was dissolved in 2.5 mL of water, and the procedure was repeated. The combined extract was adjusted to 5 mL by 50% aqueous methanol solution. The next step was the SPE. One milliliter of extract was loaded onto a preconditioned Oasis HLB cartridge. The first 0.5 mL of the eluate was discarded, and the remaining part of the solution was collected and diluted 20-fold with 50% aqueous methanol solution. This sample was used for determination of ibotenic acid and muscimol by the LC–TOF–MS system. The intraday recoveries were 99.8% for ibotenic acid and 101% for muscimol, and the interday recoveries were 94.8% for ibotenic acid and 101% for muscimol. The limits of detection were 4.9 and 2.4 mg/kg, and the limits of quantification were 16 and 7.9 mg/kg for ibotenic acid and muscimol [27]. Yoshioka et al. identified ibotenic acid and muscimol in *A. pantherina* in concentration amounts of 146 and 133 μg/g, respectively. The weak point of these results was that they were based on a single sampling of the mushrooms. Nevertheless, it is a simple and rapid analytical method, which makes it possible to simultaneously identify nine mushroom toxins.

Gonmori et al. also identified ibotenic acid and muscimol in *Amanita* mushrooms using the LC–MS/MS technique [31]. They used acivicin as an internal standard. One hundred milligrams of mushroom was homogenized with 10 μg of acivicin in 10 mL 50% methanol aqueous solution for 5 min. After centrifugation (3000 rpm, 10 min), 1 mL of supernatant was mixed with 100 μL of 0.5% ammonia aqueous solution. This sample was loaded onto Oasis MAX 3 cc (60 mg), which was preconditioned with 1 mL of methanol and 1 mL of 5% ammonia aqueous solution. The cartridge was washed with a 2 mL mixture of acetonitryle/water ($9{:}1_{v/v}$). Ibotenic acid, muscimol, and internal standard were eluted with 6 mL of 2% formic acid in methanol. After evaporation to dryness under the stream of nitrogen, the residue was dissolved in a 1 mL mixture of 0.5% formic acid aqueous solution/methanol ($1{:}4_{v/v}$). As a next step, 5 μL of sample was injected to the LC–MS system. Selected reaction monitoring (SRM) mode of acquiring LC–MS/MS data was used in this case. The obtained recoveries for ibotenic acid and muscimol were above 80%. The limit of detection

was lower than 10 mg/kg for both compounds [31]. The use of acivicin as an internal standard was the novel point of this method, as well as the use of anion exchange SPE and no requirement for derivatizing before LC–MS/MS analysis. The authors stressed the fact that this method is much simpler than the method presented by Tsujikawa et al. [26]. However, the obtained muscimol (MUS) and ibotenic acid (IBO) recoveries are better in the previous work.

Tsujikawa et al. found that isoxazole compounds tend to be more concentrated in the flesh than in the red cuticle of *A. muscaria* and *A. pantherina*. They analyzed *Amanita* mushrooms that were circulating in the drug market [26]. The total contents of IBO/MUS in the caps were <10–2845/46–1052 ppm in *A. muscaria* and 188–269/1554–1880 ppm in *A. pantherina*. These results were in general agreement with the data reported in the past [39,40]; however, the muscimol concentrations were higher than in most of the previous reports. Authors proposed explanation that *A. muscaria* sold in the drug market were dried in the sun or with a heater because drying *A. muscaria* in the sun or with a heater causes an increase in MUS in the mushroom by decarboxylation of IBO [26]. The MUS/IBO contents also depended on the growing environment and genotype as is described in the review by Michelot and Melendez-Howell [7].

Psilocybe Mushrooms

Psilocybin and psilocin, the main hallucinogens in *Psilocybe* mushrooms, are often extracted using ultrasonic-assisted extraction. Pedersen-Bjergaard et al. extracted psilocybin from *Psilocybe semilanceata* before the CE analysis. The procedure of extraction was as follows: 100 mg of powdered mushrooms were extracted in an ultrasonic bath lasting 15 min with 3 mL of methanol and with the addition of 0.5 mg/mL of barbital, which was used as an internal standard. After extraction, the sample was centrifuged (3200 rpm, 10 min), the supernatant was transferred to another flask, and the extraction process was repeated with 2 mL of methanol containing barbital. The combined extract was diluted with run buffer ($1{:}1_{v/v}$) before CE analysis. The obtained recovery was 98% for psilocybin. The detection limit was 0.045 g/kg mushrooms for psilocybin and the quantification limit was 0.225 g/kg mushrooms for psilocybin [29].

Laussmann et al. extracted psilocybin and psilocin from *Psilocybe cubensis*, *Psilocybe tampanensis*, and *Panaelous cyanescens*. The extraction process was carried out for fresh, air-dried, and freeze-dried mushrooms. In the case of dried mushrooms, the samples were homogenized. After the homogenization process, 100 mg of sample was extracted with 100 mL of methanol containing 10 mM hydrogen chloride and 10 μg/mL of tryptamine as an internal standard in ultrasonic bath at 20–25°C lasting 60 min. In the case of fresh mushrooms, 5 g of mushroom material was extracted in the same solvent, using a laboratory mixer at 20–25°C for 15 min. After extraction, in both cases the extracts were filtered before the HPLC analysis. The obtained recoveries were 97.3 and 98.8% for

psilocybin and psilocin, respectively. The limits of detection were 70 mg/kg for psilocybin and 60 mg/kg for psilocin. The quantification limits were 280 mg/kg for psilocybin and 220 mg/kg for psilocin [30]. Authors also conducted tests in order to find optimal sample treatment conditions. They revealed that highest alkaloid concentrations are obtained when samples are freeze-dried prior to extraction. They showed that drying at an elevated temperature (60°C) leads to decomposition of 90% of psilocin. It is an important piece of information when comparing results of hallucinogenic substances analyzes content from different studies. One should take into account not only the growing environment of the mushrooms, but also the method of extraction and sample treatment.

Saito et al. determined psilocybin and psilocin in the same kind of mushrooms used by Lausmann et al. [30]. Twenty miligrams of dried, powdered mushrooms were added to 1 mL of ethyl acetate containing 5 μg of bufotenin, which was used as an internal standard. The sample was placed in an ultrasonic bath for 30 min at a temperature lower than 50°C. After sonification, the sample was centrifuged (3000 rpm, 5 min), and the supernatant was transferred to another flask. The residue was extracted two more times, using the same method. The combined extract was evaporated and dissolved in 4% pyridine in 0.2 mL of acetonitrile, and it was filtered. Then the 50 μL of extract reacted with 50 μL of 10 mM (*R*)(+)-4-(*N*, *N*-dimethyloaminosulfonyl)-7-(2-chloroformylpyrrolidin-1-yl)-2,1,3-benzoxadiazole in acetonitrile 60°C for 10 min before HPLC–FL or HPLC–ESI–MS using SIM mode. The recovery was approximately 96.8% for both compounds. The limit of detection was 0.64 μg/kg for psilocybin and psilocin [32].

Furthermore, 20 mg of dried, powdered mushroom was added to 1 mL of methanol containing 4.28 μg/mL 3-indoxyl-phosphate disodium salt as an internal standard. After 30 min ultrasonification at temperatures lower than 50°C, the sample was centrifuged (3000 rpm, 5 min), the supernatant was transferred to another flask and the residue was extracted two more times with 1 mL of methanol, using the same procedure. The combined extract was evaporated, and the residue was dissolved in 200 μL of 100 mM 1-methylimididazole buffer (pH 7) and filtered. Next, 10 μL of the extract reacted with 40 μL of 10 mM 5-dimethylaminonaphthalene-1-[*N*-(2-aminoethyl)] sulfonamide (DNS-ED) in *N*,*N*-dimethylformamide and 10 μL of 100 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride in pH 7 buffer 60°C for 4 h. After the reaction, the sample was diluted 20 times with 50 mM mixture of ammonium acetate/acetonitrile ($73{:}27_{v/v}$) and 5 μL was injected to the HPLC–FL [34]. Although the selectivity increases with the native FL detection after HPLC separation, the sensitivity seems not to be good enough for a real sample analysis. The detection sensitivity was about two orders of magnitude higher after derivatizing with DNS–ED as was shown by Saito et al.

Kikura-Hanajiri et al. determined 19 hallucinogenic substances, using the GC–MS and LC–MS. GC–MS was used for qualitative analysis and LC–ESI–MS – for qualitative and quantitative analysis. Five of these substances were

legally controlled tryptamines and phenethylamines originally found in fungi or plants. In case of plant/mushroom materials, 100 mg of dried mushroom sample was extracted with 2 mL of methanol containing 50 μL of the internal standard solution (0.2 mg/mL) in an ultrasonic bath lasting 10 min. The extract was centrifuged (3000 rpm, 5 min) and filtered before the GC-MS analysis. Developing a LC separation of the 19 compounds in one run was challenging. These investigations revealed that samples, mostly sold as "mixtures of mushrooms/plants" via the Internet, possessed the synthesized compounds, 5-MeO-DIPT (5-methoxy-diisopropyltryptamine) and AMT (α-methyltryptamine) and contained no psilocin or psilocybin.

Tsujikawa et al. identified psilocybin and psilocin in *P. cubensis* and *Copelandia* genus. The extraction procedure was as follows: 10 mg of dried, powdered mushrooms were double-extracted with 1 mL of methanol in an ultrasonic bath lasting 30 min. Then the extract was centrifuged (3000 rpm, 2 min), and the supernatant was transferred to another flask. The combined supernatant was evaporated under a stream of nitrogen. The residue was dissolved in 100 μL of mobile phase with 4-hydroxyindole (25 μg/mL), which was used as an internal standard [35]. Then a 10 mL aliquot was used for the HPLC analysis. Tsujikawa noted that psilocin and psilocybin have a tendency in *P.cubensis* to be contained in the cap more than the stem. In *Copelandia*, this tendency was not so noticeable. Moreover, it was shown that *P. cubensis* is psilocybin-rich, whereas *Copelandia* is psilocin-rich. However, only two samples of *Copelandia* were analyzed within this study, and these samples could be different species because their appearance and scale of spores were apparently different [35].

Mushoff et al. determined psilocybin and psilocin in *P. semilanceata*, *P. cubensis*, *P. tampanensis* and *P. cyanescens*. One hundred milligrams of dried, powdered mushrooms were extracted with 9 mL of methanol in an ultrasonic bath lasting 120 min. After centrifugation, the supernatant was used to the HPLC analysis [37]. The alkaloids content was determined with <0.003–1.15% of psilocybin and 0.01–0.90% psilocin. The authors also provided detailed morphological characteristics of investigated hallucinogenic mushrooms, which is important when analyzing mushrooms.

Keller et al. determined hallucinogenic substances in *Psilocybe subcubensis* and *Agrocybe praecox*, which does not contain hallucinogens such as psilocybin and psilocin. The mushrooms were lyophilized and cut into small pieces. Fifty milligrams of mushroom was extracted with 1 mL of chloroform in an ultrasonic bath lasting 60 min. After centrifugation (14,000 rpm, 10 min) and filtration, 0.5 mL of clear, spiked supernatant was transferred to vial and was evaporated under a stream of nitrogen. The hallucinogenic mushrooms were extracted through the same procedure, but only 0.05 mL of supernatant was evaporated. The residues were dissolved in 30 μL of *N*-methyl-*N*-(trimethylsilyl)-2,2,2-trifluoroacetamide. The mixtures were heated for 30 min at 70°C. After heating, the mixtures were cooled and 1 μL of samples were injected into the GC–MS system. They used SIM mode of data acquiring. The limits of detection in the cap of *P. subcubensis* were 8.6 and 0.2 g/kg for psilocybin and psilocin,

respectively. The limits of detection in the stem of *P. subcubensis* were 8.0 g/kg for psilocybin and 0.3 g/kg for psilocin [36].

Sarwar et al. determined psilocybin and psilocin in mushrooms. About 200–500 mg of dried, powdered mushrooms was extracted in 10% solution of acetic acid and 5 mL of deionized water. After centrifugation (3 min), the supernatant was transferred into a beaker and neutralized by adding a small volume of sodium bicarbonate. This solution was extracted with an equal volume of chloroform. The diphasic solution was centrifuged and the chloroform layer was transferred to another flask and concentrated under air before GC–MS analysis [38].

As we can see in Table 5.1, the high-performance liquid chromatography is the most widely used analytical technique in the scope of analysis of hallucinogenic substances. Although several other techniques have been applied to identify these substances in mushrooms, the detection limits are much better for the HPLC technique than GC or CE methods, especially when using electrochemical or MS detection or flow injection analysis [14].

Biological Samples

The leading techniques allowing separation of the main hallucinogenic alkaloids, i.e., psilocybin, psilocin, bufotenin, ibotenic acid and muscimol, from biological fluids are LLE [9,41–43] and SPE [16,44–50] (Table 5.2). These methods allow the satisfactory recovery of trace amounts of the analyte in physiological samples. The most frequently used analytical methods for the identification of selected psychoactive compounds from the samples of urine, plasma, and serum are chromatographic methods, such as high-performance liquid chromatography (HPLC) with a diode array detector (DAD) [51] or electrochemical detection [14,42,43] gas chromatography (GC) with a MS detector [16,45,50,52] and electro-migration method-capillary electrophoresis (CE) with a UV detector [48] (Table 5.2).

In order to detect and identify the analytes in a physiological fluid sample with suitable sensitivity, the treatment of samples prior to analysis is necessary for toxicological studies. The extraction of selected hallucinogenic alkaloids from complicated fluid samples should include the purification of a sample matrix. This step can be taken, using hydrolysis or precipitation of proteins in the plasma or urine samples. For the urine or blood plasma, which contains a very large amount of conjugated metabolites, the use of hydrolysis allows satisfactory results to be obtained and allows a lower-level of the analyte detection.

The main hallucinogenic isoxazoles (muscimol and ibotenic acid) in biological samples.

As mentioned above, the main *Amanita* hallucinogenic alkaloids were extracted from a biological matrix, such as serum and urine. There are only several reports that describe the extraction procedure and the process of ibotenic acid and muscimol identification in such samples. The main extraction procedures used for isolation of these compounds are LLE and SPE [9,49].

TABLE 5.2 The Methods of Extraction and Identification of Ibotenic Acid, Muscimol, Psilocin, Psilocybin, and Bufotenine in Biological Samples

No.	Analyte	Matrix	Extraction method	Recovery (*R*%)	Detection	LOD LOQ	References
1.	Psilocin	Serum	LLE	–	LC–MS/MS	0.5 μg/L –	[41]
2.	Psilocin	Urine	LLE	88	HPLC–ECD	– 10 μg/L	[43]
3.	Psilocin	Plasma	SPE LLE	100 88	HPLC–ECD	– 10 μg/L	[42]
4.	Psilocin	Urine Serum	SPE	98	GC–MS	5 μg/L –	[16]
5.	Psilocin	Serum Blood Urine	SPE	80	LC–MS	2 μg/L –	[44]
6.	Psilocin	Urine	SPE	–	GC–MS	– 10 μg/L	[45]
7.	Psilocin	Urine	SPE	88	LC–MS/MS	5 μg/L 10 μg/L	[46]
8.	Psilocybin	Urine	SPE	92	LC–MS/MS	5 μg/L 10 μg/L	[46]

9.	Psilocin	Urine	SPE	87.6	LC–MS/MS	0.5 μg/L 10 μg/L	[47]
10.	Psilocin	Urine	SI–SPE	84	CE–UV	7 μg/L 13 μg/L	[48]
11.	Psilocin	*G. spectabilis* Rat plasma	SPE	97.7–104.8	UPLC–PDA	0.05 μg/L 0.12 μg/L	[53]
12.	Psilocin	Urine	–	–	LC–MS/MS	4 μg/L –	[54]
13.	Psilocin	Urine	SPE	86.6	LC–MS/MS	0.2 μg/L 0.2 μg/L	[21]
14.	Psilocin	Plasma	SPE	89.1	LC–MS/MS	0.05 μg/L 0.15 μg/L	[21]
15.	Psilocin	Serum	SPE	89.5	LC–MS/MS	0.05 μg/L 0.17 μg/L	[21]
16.	Bufotenine	Urine	SPE	88.8	LC–MS/MS	0.10 μg/L 0.14 μg/L	[21]
17.	Bufotenine	Plasma	SPE	91.3	LC–MS/MS	0.07 μg/L 0.27 μg/L	[21]
18.	Bufotenine	Serum	SPE	91.6	LC–MS/MS	0.05 μg/L 0.11 μg/L	[21]

(Continued)

TABLE 5.2 The Methods of Extraction and Identification of Ibotenic Acid, Muscimol, Psilocin, Psilocybin, and Bufotenine in Biological Samples *(cont.)*

No.	Analyte	Matrix	Extraction method	Recovery (*R*%)	Detection	LOD LOQ	References
19.	Psilocin	Plasma	SPE	≥86	LC–MS/MS	0.1 μg/L 0.34 μg/L	[55]
20.	Psilocin	Serum Blood	SPE	88	GC–MS	3 ng/g 5 ng/g	[52]
21.	Ibotenic acid	Urine	LLE–SPE	74	GC–MS	0.1 mg/L –	[9]
22.	Muscimol	Urine	LLE–SPE	80	GC–MS	0.1 mg/L –	[9]
23.	Ibotenic acid	Serum	SPE	87.9–103	LC–MS/MS	1 μg/L 2.5 μg/L	[49]
24.	Muscimol	Serum	SPE	89.8–96.4	LC–MS/MS	1 μg/L 2.5 μg/L	[49]

In the first case [9], the authors described the extraction method for ibotenic acid and muscimol from the urine sample. The extraction procedure included LLE and SPE. The first step of the proposed procedure was the SPE. For concentration and purification of the analytes exchange, the Dowex 50W X8 sorbent was used. The urine sample was shaken with an ion exchange sorbent in a mixture of 0.1 M hydrochloric acid: methanol. Then, it was centrifuged and again shaken with brine and ethyl chloroformate, i.e., the second step of the LLE, and the organic layer was finally collected. The sample was then analyzed using a gas chromatograph with a mass spectrometer (GC–MS). The recovery was 80% for ibotenic acid and 74% for muscimol. The detection limit was 1000 μg/L for both muscimol and ibotenic acid [9].

The SPE was also used in the case of human serum samples. The ion exchange Oasis MAX 3cc extraction cartridges were used for extraction of the ibotenic acid and muscimol from serum [49]. A 100 μL aliquot of human serum containing ibotenic acid and muscimol was mixed with 100 ng of acivicin (internal standard, IS) dissolved in methanol, distilled water, and in 0.5% ammonia aqueous solution; the mixture was vortexed. Then the mixture was loaded on an extraction cartridge (preconditioned with methanol and 0.5% ammonia aqueous solution). The cartridge was washed with distilled water and then with methanol. The target compounds and IS were eluted with 0.05% TFA in methanol. The extract was evaporated to dryness. Then, the residue was reconstituted in methanol and subjected to the LC–MS/MS analysis. The recovery was 87.9–103% (depending on concentration) for ibotenic acid and 89.8–96.4% for muscimol. These good recoveries were obtained by Haegawa et al. after testing various compositions for the washing and eluting solutions. The detection limits were 1.0 and 2.5 μg/L for ibotenic acid and muscimol, respectively [49]. They are much better than detection limits showed in studies of Stribrny et al. [9].

Very recently, the CE–ESI–MS/MS method used to identify, separate, and determine the mushroom's ibotenic acid, muscimol, and muscarine from urine, has been developed [56]. The obtained LOD values were at the nanomolar concentration level showing the usefulness of this method for identification and quantification of the studied toxins in the human urine.

The main advantages of CE are an extremely small injection volume, high separation efficiency, and short analysis time [56]. This method is environmentally friendly due to low solvent consumption in comparison with LC.

The main hallucinogenic indoles in biological samples.

Psilocybin and psilocin have free hydroxyl groups, so it is likely that they are excreted in the urine as conjugated forms. The literature shows that psilocin concentration in urine increases with the process of enzymatic hydrolysis with β-glucuronidase. This indicates that this substance can conjugate by metabolic pathway to glucuronide conjugate forms [41,43,54,57]. Thus, in this case hydrolysis forms an important part of proper treatment of the sample prior to extraction.

Suitable enzyme sources and the power of the enzyme used for enzymatic hydrolysis is an important factor in the process of obtaining a high concentration of the analyte free form. Among the conjugates formed in the body with psilocin, the best proved is a β-glucuronidase derived from *Escherichia coli* by enzymatic treatment units from 12500 to 25,000 units per 5 mL of urine samples [45]. In the first case, of extraction of psilocin from urine, hydrolysis was used before the LLE extraction. After purification, the resulting supernatant was used for the LLE. The process of hydrolysis was carried out with β-glucuronidase at a pH of 5, 40°C for 5 h. Then, after the hydrolysis, all samples were extracted with methanol and membrane filtered and then analyzed by the HPLC with an electrochemical detector. The recovery of free psilocin was 88–106.2% (depending on concentration) [43].

The LLE have been also used for the extraction of psilocin and its conjugate in serum samples. β-glucuronidase was used for the hydrolysis of glucuronide conjugates of psilocin and the psilocybin analysis of serum samples. Incubation temperature was 37° C for 2 h. The hydrolysis was carried out at a pH of 5. The extraction was made on both hydrolyzed and nonhydrolyzed samples. The serum sample was adjusted to pH 8 and then extracted with two portions of chloroform. The resulting mixture was centrifuged (10 min, 15,000 g). Then, the organic layer was separated, the solvent was evaporated and the residue was dissolved in a mobile phase and analyzed, using a liquid chromatography coupled with a tandem mass spectrometer (LC–MS/MS). The multiple reaction monitoring (MRM) mode of acquiring LC–MS data was used. With this method, the satisfactory 0.5 μg/L detection limit was obtained [41].

The second leading technique used for the hallucinogenic indoles isolation from the biological matrix is SPE. It is applied mainly to psilocybin, psilocin, and bufotenine. SPE was applied to the samples of urine and serum, using commercially available columns with different chemical properties of sorbent. The extraction used, among other sorbents, copolymer (Clean Screen CSDAU206, ZCDAU020), including ion exchange properties (Oasis MCX, XAD nonionix, OSP-2a CBA Certifty Varian Bond Elut LRC, Waters MAX), which is widely used both for polar compounds and nonpolar silica deposits octadecyl modified with C18 (Bond Elut C18) [16,42,44–48]. In the case of ion exchange sorbents, the main solution used for the elution had the pH > 7; however, the most commonly used one was the ammonium base solution having a concentration from 2% to 5% in methanol or ethyl acetate. Application of the principle as an eluent and ion exchange sorbent gives high-recovery values from 87% to 100% [21,45,46,52,55].

The example of a procedure based on SPE, using ion exchange columns Oasis MCX (30 mg, 1 mL), is psilocin extraction from urine samples. In the first extraction stage, the sorbent was conditioned with methanol, water, sodium acetate (pH 4). Then, the sample of urine (pH 4) was loaded onto the extraction cartridge. Next, the cartridge was washed with acetate buffer, methanol,

or a buffer, a mixture of methanol: water (80:20 v/v) or buffer, and a mixture of methanol: water (50:50 v/v). After the interferents were washed, the sorbent was dried and then the analytes were eluted with 5% ammonium solution in methanol. The resulting extract was evaporated to dryness, and the residue was taken up in a mobile phase and analyzed, using the LC–MS/MS. The obtained psilocin recovery was 87.6%, the detection limit was 0.5 μg/L, and the limit of quantification 10 μg/L [47].

Bogusz described the determination of psilocybin and psilocin in serum, blood, and urine [44]. Before precipitation of the samples, the mixture was centrifuged (5 min, 14,000 g). Then, the ammonium buffer (pH 9.3) was added to the obtained supernatant, and the mixture was stirred at vortex. The solution prepared in this way was centrifuged again (10 min, 5000 g). In this case, the silica sorbent with octadecyl group C18 was used for extraction of psilocybin and psilocin. The cartridge was conditioned with methanol, water, and ammonium buffer pH 9.3. The supernatant obtained during precipitation was then applied onto the C18 cartridge and washed with ammonium buffer (pH 9.3). Then the sorbent was dried and the substances were eluted with methanol: acetic acid (9:1 v/v). The extract was evaporated to dryness, and then dissolved in a mobile phase and analyzed by LC-MS with a SIM mode of data acquiring. The recovery of psilocin was 90% and 80% for psilocybin; the LOD for both substances was 2 μg/L [44].

Definitely higher recoveries, lower limits of detection and quantification are achieved, using online SPE. This system was used to isolate and concentrate psilocin from blood plasma. The procedure utilizes a cation exchange extraction cartridge (OSP-2a CBA). Methanol was used as an eluent. After the extraction, the sample was analyzed by the HPLC with electrochemical detection. The recovery achieved by this method was 100%. A satisfactory value limit of quantification at the level of 10 μg/L was obtained [42]. SPE can be also directly connected to the analytical system, such as the capillary zone electrophoresis (CE–UV), in order to improve the selectivity and the rate of extraction. This system can be based on sequential injection, SI, of the sample and flow control injection with a thousand concentrations of sample. Sequential injection solid phase extraction (SI–SPE) was used for extraction and concentration of psilocin from urine. The C18 extraction cartridge was used for purification, separation, and concentration of psilocin. The extract was analyzed using the CE–UV. The recovery was 84–1.2% and a satisfactory detection limit of 7 μg/L was achieved with this method [48,58].

BIOSYNTHESIS OF ISOXAZOLES

No precursors to ibotenic acid can be shown without doubts. Ibotenic acid, muscimol, and muscazone originate probably from the same precursor, β-hydroxyglutamic acid. Biosynthetic pathway including ring closures and decarboxilation determines the structures of these products [7].

BIOSYNTHESIS OF TRYPTAMINE ALKALOIDS IN MUSHROOM

Psilocybin was demonstrated by many studies to be derived from tryptophan [59–61]. The precise mechanism of psilocybin biosynthesis is debated by many authors. In 1959, Hofmann et al. showed that radioactive tryptophan is incorporated into psilocin and psilocybin in *Psilocibe semperviva* [62]. Agurell and Nilsson investigated the biosynthesis of psilocybin by feeding labeled precursor to *P. cubensis*. The study was concerned with the sequence of events, which leads from tryptophan to psilocybin. They showed that 4-hydroxytryptophan appears to be a poor precursor of psilocybin and indicated that *P. cubensis* can utilize two paths to psilocybin [60]. The first is shown in Fig. 5.3. This sequence involves chemical modifications as follows: decarboxylation, N-methylation, 4-hydroxylation, and phosphorylation of the 4-hydroxyindole moiety. Agurell and Nilsson suggested that an alternative route is a conversion of 4-hydroxytryptamine to psilocybin.

Since tryptamine operated as a better precursor for psilocybin synthesis than tryptophan in cultured *P. cubensis*, it seems that decarboxylation of tryptophan to tryptamine is the first step in the psilocybin biosynthesis [64]. Leung and Paul revealed incompletely methylated psilocybins (these are baeocystin and norbaeocystin) in *Psilocybe baeocystis* [65].

These results suggest that methylation might occur as the last step in biosynthesis of psilocybin. Chilton et al. proposed a mechanism by which tryptophan is transformed to bufotenine and another one showing biotransformation of tryptophan to psilocin (Fig. 5.4) [66]. They revealed that tryptamine-4, 5-epoxide can be intermediate between tryptamine and psilocin, but it was unknown if it is also a precursor of serotonin and bufotenine. Another investigation showed that

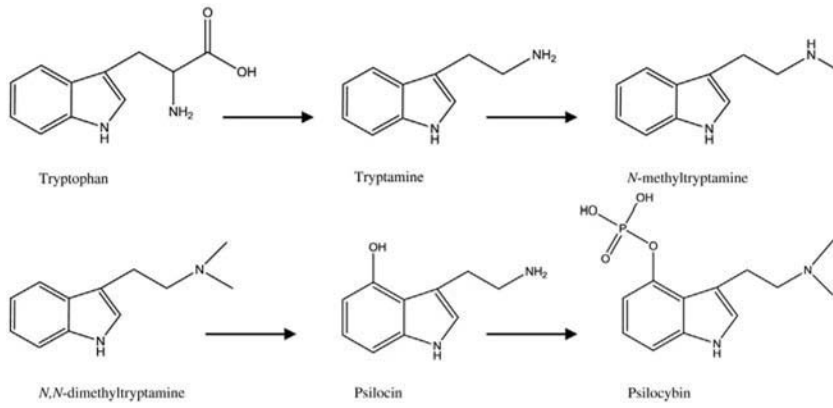

FIGURE 5.3 The model is based on the data obtained in studies provided by Agurell and Nilsson [63].

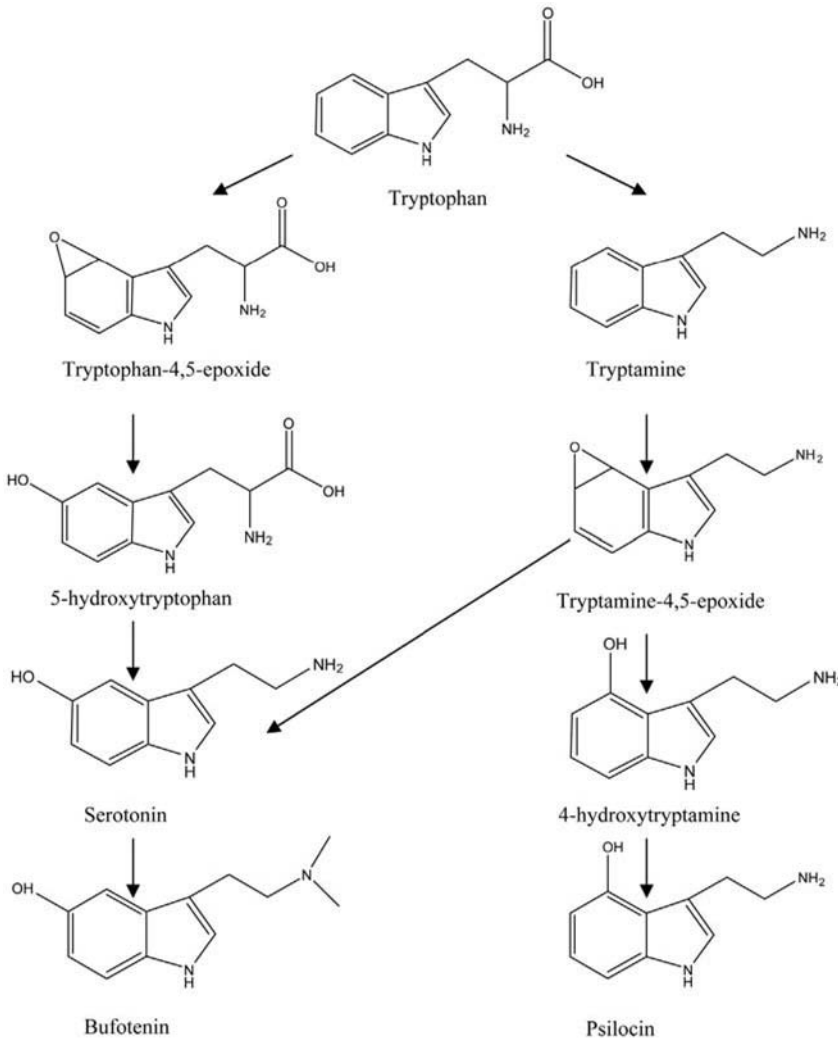

FIGURE 5.4 Possible paths of biosynthesis of psilocin and bufotenine.

the mushroom efficiently hydroxylated tryptamine to psilocin but *N*,*N*-diethyltryptamine was transformed to 4-hydroxy-*N*,*N*-diethyltryptamine [67,68].

Baeocystin and norbaeocystin are natural psilocybin analogs first isolated from *P. baeocystis* [23,65]. Baeocystin was also detected in some other species from genera *Psilocybe*, *Conocybe, and Panaeolus* [69]. The presence of baeocystin and norbaeocystin in *P. baeocystis* suggests that the alternative pathway is probably utilized by some fungi. The possible paths leading to psilocybin and its analogs are presented in Fig. 5.5.

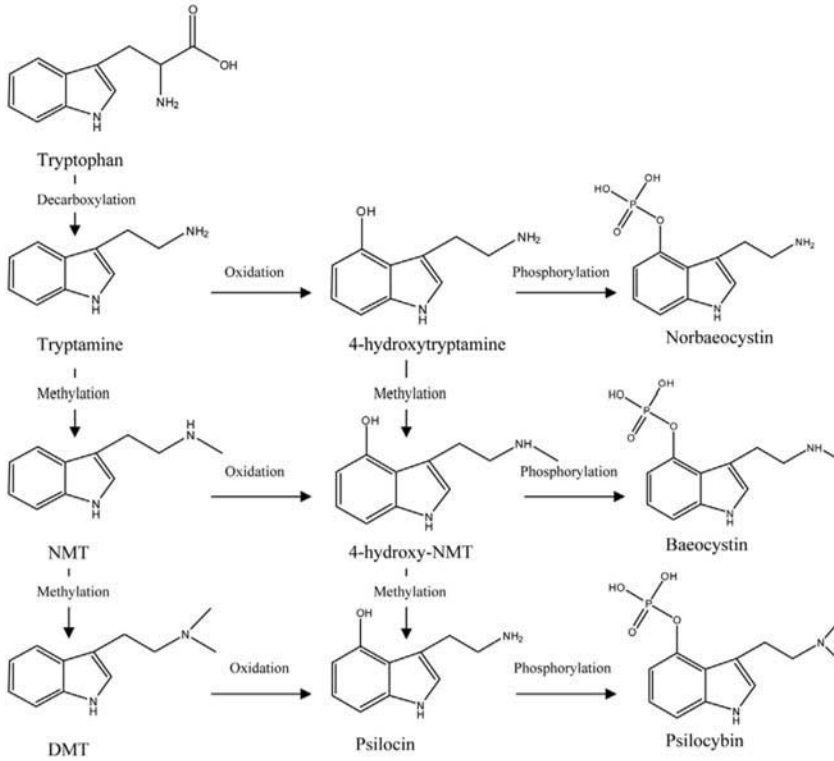

FIGURE 5.5 Alternative pathway for the biosynthesis of psilocybin and its analogs from tryptophan.

BIOLOGICAL ACTIVITY AND PSYCHOPHARMACOLOGICAL EFFECTS

Indoleamine Hallucinogenic Pharmacodynamics

Many of the indoleamines have significant biological activity. One of the most highly studied indoleamines is hormone serotonin (5-hydroxytryptamine or 5-HT). This neuromodulator is mainly synthesized by intestinal cells that regulate overall intestinal physiology. Serotonin has been involved in the regulation of many physiological and pathological events. This neurotransmitter plays an important role in the central nervous system as well as in the cardiovascular and gastrointestinal systems [3]. Seven different serotonin receptors and 14 different receptor subclasses were identified [70]. Numerous factors were used to investigate the 5-HT receptors. Reliable pharmacological investigation on the hallucinogenic indoleamines are possible since selective antagonists for relevant serotonergic receptors were discovered and cloned. Indoleamine hallucinogens cause extremely similar experiences in humans. The similarity of

their psychopharmacological effects and their ability to produce cross-tolerance indicate that indolealkylamines (to which psilocin and psilocybin belong) act through a common receptor mechanism.

It is widely accepted that the unitary mechanism responsible for the effects of serotonergic hallucinogens is activation of the 5-HT_{2A} receptor; however, it does not exclude the probability that the interaction of indoleamines with non-5-HT_2 receptors does have also psychopharmacological and behavioral consequences [71].

It was shown that stimulus properties of tryptamines could be blocked by the 5-HT_2 receptor antagonists such as pirenperone [72], which formed the basis for the theory proposed by Glennon et al. claiming that hallucinogenic drugs act specifically at the 5-HT_2 receptor subtypes [73]. Many other works strongly indicate that the stimulus effects of hallucinogens (mainly LSD–lysergic acid diethylamide) are mediated by the 5-HT_{2A} receptor [74,75]. Indeed, Vollenweider et al. reported that most of the subjective effects of psilocybin are blocked by pretreatment with the 5-HT_{2A} antagonist ketanserin [76]. Nevertheless, ketanserin had no influence on other drug associated symptoms like reduction of arousal and vigilance and impairment of multiple-object tracking, suggesting that psilocin activates several serotonin receptor subtypes [77]. Recently, it has been noticed that binding affinity of psilocin to the 5-HT receptors is quite different than LSD (see Table 5.3). McKenna et al. investigated the affinities of 21 indolealkylamine derivatives to the 5-HT_{1A}, 5-HT_{2A}, and 5-HT_{2B} receptors,

TABLE 5.3 Binding of Psilocin and (+)-LSD to 5-HT Receptors on the Basis of Ref. [71]

	K_i (nM)	
Binding site	**Psilocin**	**(+)-LSD**
5-HT_{1A}	567.4 (49)*	1.1
5-HT_{1B}	219.6	3.9
5-HT_{1D}	36.4	–
5-HT_{1e}	–	93.0
5-HT_{2A}	107.2 (25)*	3.5
5-HT_{2B}	4.6	30.0
5-HT_{2C}	97.3 (10)*	5.5
5-HT_3	>10,000	–
5-HT_5	83.7	9.0
5-HT_6	57.0	6.9
5-HT_7	3.5	6.6

K_i in brackets reported by Blair et al. [79].

using radioligand competition studies [78]. This study demonstrated that hallucinogenic 4-hydroxy-indolealkylamines, like psilocin, bind potently and selectively to the 5-HT_{2A} receptor. The 5-hydroxylated derivatives (e.g., bufotenine) displayed approximately equal potency at the 5-HT_{1A} and 5-HT_{2A} sites. Other serotonin analogs display moderate to high affinity for the 5-HT_1 and 5-HT_2 subtypes [79–81]. It is worth mentioning that when hallucinogens are tested in competition with the receptor antagonist, it may profoundly influence apparent affinity [82]. In spite of the fact that the abuse rate is still growing, especially among young people, we know relatively little about how the hallucinogenic tryptamines affect the brain. Most probably, the risk associated with hallucinogen administration is commonly known as a "bad trip" and is characterized by anxiety, fear/panic, dysphoria, and/or paranoia [83]. Visual hallucinations are caused probably by increasing cortical excitability and altering visual-evoked cortical responses after activation of the 5-HT_{2A} receptors [84]. Current knowledge about the 5-HT receptor ligands, also in preclinical research and clinical trials, was reviewed by Filip and Bader [85]. The recent investigations concerning the influence of psilocin and its derivatives on the brain will be described briefly in subsequent sections.

Metabolism of Indoleamines Produced by Mushrooms and their Psychosomatic Effects

Psilocybin is a substituted indolealkylamine, which belongs to the group of hallucinogenic tryptamines. Psilocybin and psilocin are the main psychoactive compounds of hallucinogenic mushrooms. The toxicity of psilocybin is relatively low (LD_{50} = 280 mg/kg in rats and LD_{50} = 285 mg/kg in mice) [86]. This means that a 60 kg (1 kg = 2.2046 pounds) person must eat 1.7 kg of dried *P. cubensis* mushrooms to reach the LD_{50} value [87]. When administered intravenously in rabbits, psilocybin's LD_{50} is around 12.5 mg/kg (TOXNET, National Library of Medicine, USA). However, nondrug variables can significantly alter toxic reactions, e.g., diet, physical exertion, expectation, and stress.

Acute adverse effects as well as chronic toxicity of magic mushroom use has been recently briefly reviewed by van Amsterdam et al. [87]. It should be noticed that psilocybin is a prodrug for psilocin. This means that whenever a reference is made to the *in vivo* effects of psilocybin (e.g., in the following section), it means that the actual biologically active species is psilocin [88,89]. Indeed, the *in vivo* experiments on rats showed that psilocybin was rapidly hydrolyzed to psilocin. Then psilocin was well taken up by intestinal segments and transferred to the blood side [90]. The same study revealed that tissue uptake of intact psilocybin was negligible or absent.

The investigations by the HPLC technique indicated about 50% bioavailability of psilocin after oral administration of psilocybin [89]. Psilocin is detectable in the human plasma within 20–40 min after oral administration, and the plasma concentration ranges maximum after approximately 80–100 min

[42,89]. After 6 h of drug ingestion, the effects are completely worn out, as was shown by Hasler et al. [91]. The work by Lindenblatt et al. revealed a large interindividual variation as regards psilocin plasma concentration of healthy volunteers after oral administration of 0.2 mg psilocybin per kilogram of the body mass [42]. Recent results of pharmacokinetic investigations on rat plasma, after the oral administration of a *Gymnopilus spectabilis* extract, showed that psilocin was rapidly absorbed into blood and reached maximum concentration at 90 ± 2.1 min [51]. According to another study, the maximum amount of psilocin concentration in rat plasma, after an intraperitoneal injection of 5 mg/kg, was obtained after 15 min [92]. Psychopathological effects in humans occur with plasma levels of 4–6 μg/L [91].

According to another study, the threshold dose of 45 μg psylocybin per kg of the body weight was assessed clearly as a psychoactive by most of the volunteers. They reported slight amounts of drowsiness, increased sensitivity, and intensification of preexisting mood states [93]. Hallucinogenic potency of psilocin in men is much lower than LSD (10–20 mg for the first and 60–200 μg for the latter drug) [94]. After the ingestion of higher doses (up to 315 μg/kg of the body weight), the changes in the mood states, sensory perception (including colorful visual illusions, complex scenic hallucinations, and synesthesias) as well as alterations in perceptions of time and space are produced [93]. The authors explain that psilocybin is not hazardous with respect to somatic health. However, some cases are known about where the cause of death was from psilocin toxicity.

In 2012, "magic mushroom" ingestion was determined by court-appointed experts as a cause of death [95]. Plasma toxicology revealed the psilocin level of 30 μg/L in a 24-year-old heart transplant recipient as a consequence of consuming psychedelic mushroom. The fatal ventricular arrhythmia was caused by excessive sympathetic stimulation of the transplanted heart. Indoleamine hallucinogens in overdose can also produce a psychosis-like syndrome in humans that resembles first episodes of schizophrenia [76]. The case report presented by Holger et al. showed a sudden impairment of the left ventricular function, after *P. semilanceata* consumption, followed by a rapid recovery. The potential catecholamine- and serotonin-like characteristics of psilocin was considered as causative [96]. On the other hand, Beck et al. suggested that adverse reactions to *Psilocybe* mushroom intake are not caused by psilocybin but phenylethylamine (PEA), which was detected for instance in *P. semilanceata* [97]. This substance, structurally related to amphetamines, naturally occurs in the nervous system of mammals and acts probably as a neurotransmitter or neuromodulator. PEA might be responsible for the cardiovascular effects (tachycardia) and other adverse reactions (nausea and anxiety) of magic mushrooms [97]. However, PEA is metabolized too rapidly in the digestive system to reach the brain, and it does not have potential for abuse.

Recently, very interesting findings have been revealed by Carhart-Harris et al. The authors used functional magnetic resonance imaging (fMRI) to measure the

effect of psilocybin on the resting-state brain activity. Their results suggest a biological mechanism, in which the connectivity of the brain's connector hubs is decreased via the 5-HT_{2A} receptor stimulation by psilocybin [98]. Some preclinical studies show that stimulation of the 5-HT_{2A} receptors increases the GABAergic transmission and the pyramidal cells (excitatory glutamatergic cells) inhibition, which may explain the deactivations observed by Carhart-Harris et al. [99,100]. Their results are in agreement with the hypothesis that the 5-HT_{2A} receptor-mediated regulation of glutamate release is the mechanism through which hallucinogens activate the cerebral cortex [99].

The investigations of the fate of psilocin in the rat showed that after 24 h 65% of the dose of 10 mg/kg psilocin is excreted in the urine [101]. A controlled study in men showed that within 24 h, 3.4 ± 0.9% of the applied dose of psilocybin when excreted with urine was free psilocin. The limited amount (10 μg/L) was usually reached 24 h after the drug was administered. In this study, eight volunteers received psilocybin in psychoactive oral doses of 212 ± 25 μg/kg of the body weight [43]. Terminal elimination half-lives of psilocin calculated from plasma concentration–time data were estimated at (2.72 ± 1.06 h; n = 6) [89,91] and from cumulative urinary excretion rates at (3.29 ± 0.57 h; n = 8) [43]. Three other metabolites of psilocybin were identified: 4-hydroxyindole-3-yl-acetaldehyde (4H1A); 4-hydroxyindole-3-yl-acetic-acid (4-HIAA); and 4-hydroxytryptophol (4-HTP) [89]. Nevertheless, later pharmacokinetic and forensic studies revealed that psilocin is mostly eliminated by conjugative metabolism as psilocin glucuronide [16,45,57]. This is done by glucuronosyltransferases: microsomal enzymes originating from liver or intestinal tissues. In the study provided by Sticht et al., free psilocin was determined in urine at the concentration of 0.23 mg/L, while the total amount was 1.76 mg/L [16]. These reports suggest that glucuronidation seems to be an important detoxification step (Fig. 5.6).

Psilocin has two potential glucuronidation sites, but it was shown, with considerable confidence, that human UDP-glucuronosyltransferases (UGTs) only catalyze the glucuronidation of psilocin at its hydroxy group [102].

Bufotenine [3-(2-dimethylaminoethyl)-1*H*-indol-5-ol; 5-hydroxy-*N*,*N*-dimethyltryptamine; 5-HO-DMT] shows activity similar to LSD and other known hallucinogens. It has the ability to bind and activate hallucinogenic serotonin (5-HT) receptors, 5-HT_{2A} and 5-HT_{2C} [103]. McBride reported lack of the hallucinogenic response in the case of bufotenine in human experiments due to poor ability to cross the blood–brain barrier (BBB) [103]. Indeed, the experiments on rats showed that 1 h after injection of bufotenine (1, 30, or 100 mg/kg), its level was high in lung, heart and blood, and lower in brain and liver [104]. Bufotenine disappeared almost completely within 8 h. These experiments also indicated that after being injected into rats, bufotenine is rapidly eliminated, partly by monoamine oxidase A (MAO-A). On the other hand, it was found that 5-HO-DMT is positive for head-twitch response (rapid side-to-side head movement that occurs in mice and rats after the serotonin 5-HT_{2A} receptor is

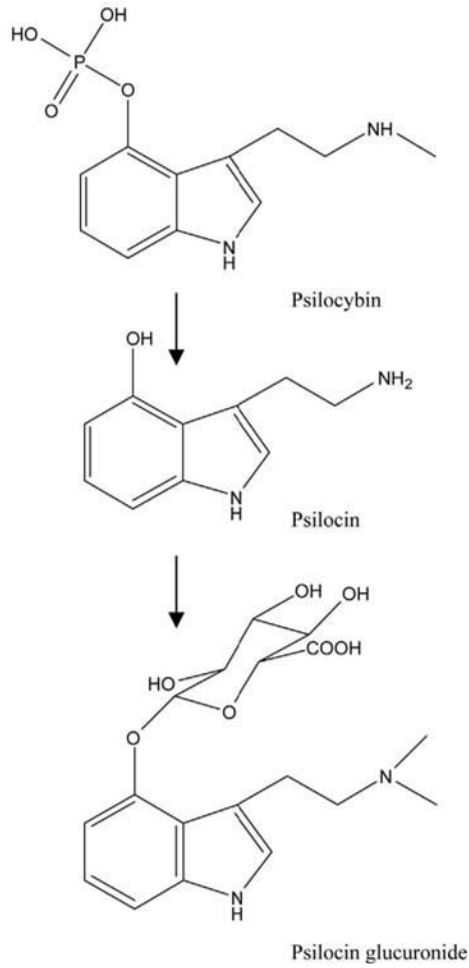

FIGURE 5.6 The psilocybin elimination from the body by glucuronidation process.

activated), which correlates well with hallucinogenic activity of a variety of drugs in humans [105].

The head-twitch response is probably the best assay to successively discriminate hallucinogenic drugs from closely related nonpsychoactive compounds [106]. The work of Fabing and Hawkins showed that 8–16 mg of intravenous injection of bufotenine in human volunteers resulted in primary visual disorders, permutation of space and time perception, and paresthesias [107]. At the highest dose, a generalized tingling of the body was observed.

Turner et al. examined the effects of some indolealkylamines on men. When 20 mg of bufotenine was injected into a patient during a 77-min interval, no psychological changes were shown.

When 10 mg or more was injected during a 50-s interval, the effects were extreme (hyperventilation persisting for about 2 min, salivation, and the reddish blue color of the face) [108]. It was shown that the elevated levels of endogenous bufotenine (as a product of the serotonin-degradative pathway) may play a role in autistic spectrum disorders (ASD) and schizophrenia, and can be correlated with hyperactivity scores in autism [109,110]. Little information exists about pharmacodynamic properties of baeocystin and norbaeocystin. The human pharmacology and toxicology of aeruginascin has not been tested yet, either. Aeruginascin is assumed to undergo a rapid metabolism into its dephosphorylation product by analogy to the known *Psilocybe* alkaloids [20].

Pharmacodynamics of Isoxazole Hallucinogens and Muscarine

The main psychedelic components of some *Amanita* mushroom species are muscimol (5-aminomethyl-3-OH-isoxazole) and ibotenic acid [(*S*)-2-amino-2-(3-hydroxyisoxazol-5-yl) acetic acid). Muscimol is a product of *in vivo* ibotenic acid decarboxylation [111]. Both muscimol and ibotenic acid induce a distinct anorexogenic action on mice (2–3 mg/kg oral) with sedation, hypnosis, and catalepsy [112,113]. Muscimol is some 6- to 10-fold more toxic than ibotenic acid in animals; its active dose given orally is 7.5–10 mg, and the LD_{50} (intraperitoneal) is 2.5 mg/kg for mice and 3.5 mg/kg for rats [114]. Chemical structure of these isoxasoles closely resembles the product of glutamic acid enzymatic decarboxylation, i.e., g-aminobutyric acid (GABA) [8]. This most important inhibitory neurotransmitter in the brain plays a significant role in regulating neuronal excitability throughout the nervous system. There are known to be three major classes of GABA receptors ($GABA_A$, $GABA_B$, and $GABA_C$). Gamma-aminobutyric acid regulates the brain excitability mainly via $GABA_A$ receptors, which comprises of five subunits classified into three major groups (alpha, beta, and gamma) [115]. The subunits of GABA receptors determine their pharmacological activity. Muscimol binds to both high- and low-affinity sites of $GABA_A$ receptors (K_ds of 10 nM and 0.27 μM), as was shown in a radioligand binding study using a bovine brain [116]. It is also a potent partial agonist at $GABA_C$ receptors [117].

Muscimol alters neuronal activity in multiple-brain regions due to wide distribution of $GABA_A$ receptors in the brain. However, brain regional distribution of muscimol high-affinity-binding sites partially differs from those of other binding sites of the $GABA_A$ receptors. High-affinity muscimol binding in the brain sections was determined by quantitative autoradiography and sedative/ataxic effects induced *in vivo* by muscimol, using a constant speed rotarod [118]. The results suggest that the behavioral effects of muscimol are preferentially mediated through high-affinity agonist binding sites of the forebrain $GABA_A$ receptors. The study of muscimol distribution in rats showed that this drug easily enters the brain [119]. Thirty minutes after intravenous administration of [^{3}H]muscimol (1 mg/kg) to rats, this compound was

indicated in brain at the concentration of 200 nmol/kg. The brain regions with the highest muscimol concentrations were the substantia nigra, the colliculi, and the hypothalamus [119]. The increase in the levels of radioactive muscimol metabolites in plasma was also noticed. Thus, as far as the metabolism of muscimol is concerned, the transamination appears to be one of its major pathways [119–122].

Muscazone, the lactam isomer of muscimol, exhibits minor pharmacological activities in comparison with the previous substances. The LD_{50} data for this compound are not available [123].

As mentioned earlier, many members of the genus *Amanita* are psychedelic. The recreational use of them is very risky due to low differences between the psychedelic and lethal dose of some mushroom species. They can cause annoying symptoms occurring after the mushroom ingestion like vomiting, hallucinations, restlessness, increased psychomotor drive, and central nervous system depression. Sometimes, other antycholinergic symptoms, like tachycardia and increased blood pressure, mydriasis, dry and red skin, can occur, too [124].

Another alkaloid – muscarine, firstly isolated from the mushroom *A. muscaria* in 1868, is highly toxic. It causes profound activation of the peripheral parasympathetic nervous system by simulating the action of the neurotransmitter acetylcholine at muscarinic acetylcholine receptors [125]. However, muscarine is only a trace compound in the fly agaric *A. muscaria*. As mentioned earlier, it is the principal toxin in the fungi of genera *Inocybe* and *Clitocybe* (up to 0.43%) [126,127]. Typical muscarinic syndrome within 15–30 min of ingestion include combinations of nausea, vomiting, diarrhea, abdominal pain, hypersalivation, diaphoresis, tachycardia, bradycardia, hypotension, lacrimation, blurred vision, miosis, tremor, restlessness, and syncope [126]. Death can be avoided completely by prompt diagnosis and treatment with atropine [128]. Other highly toxic alkaloids are amanitins. The fatal dose for humans is about 0.1 mg/kg [123]. An excellent review describing the unusual features associated with *A. muscaria* and *A. pantherina* species, their active components and toxins, was provided by Michelot and Melendez-Howell [7].

BIOMEDICAL IMPORTANCE OF MUSHROOMS' ISOXAZOLES AND INDOLES

Ligands for the $5\text{-}HT_{2A}$ receptor may be extremely useful tools for future cognitive neuroscience research [129]. The indole nucleus is an important element of many natural and synthetic molecules with significant biological activity. This double ring system contains seven positions that are open to chemical modification. However, the majority of medicinal chemists are focused mainly on modification of the 4- and 5-positions. It has been shown that modification of either the 6- or 7-positions significantly reduces the psychoactive effects of the resulting substance [130]. Many antimigraine drugs (e.g., almotriptan, zolmitriptan) are the indolealkylamine derivatives [131].

Pure synthetic psilocybin (Indocybin® Sandoz) has already been used and marketed for experimental and psychotherapeutic purposes in the 1960s [132]. Psilocybin as the 5-HT agonist is useful in studying the neurobiological basis of cognition and consciousness [93]. It is a valuable tool in the analysis of serotonin–dopamine interactions in acute psychotic states [133]. There are some works suggesting that psilocybin and other hallucinogens can reduce obsessive-compulsive disorder (OCD) symptoms in humans [134,135]. Recently, it has been shown that 1-methylpsilocin has the potential to evoke psilocybin-like effects on OCD, but is less likely than psilocybin to provoke unwanted hallucinogenic effects if administered at equivalent doses [136]. Psilocybin was also used to study its usefulness in treatment for anxiety in advanced-stage cancer patients [137] and for cluster headache [138]. In the latter study, 22 of 26 psilocybin users reported that psilocybin aborted attacks. However, participants in this research were not blind to their treatment, which raised the possibility of the placebo response. The investigators should take into account the fact that hallucinogen administration involves unique psychological risks like a "bad trip" or less common, but very harmful for the drug users, prolonged psychoses. Psilocin and psilocybin are currently regarded as dangerous drugs. The risks of hallucinogen administration, safeguards for minimizing these risks and new perspectives were reviewed by Johnson et al. [139] and Tyls et al. [140].

Naturally occurring isoxazoles were efficiently transformed into various classes of medicinally important molecules. They were found to inhibit voltage-gated sodium channels to control pain, enable the construction of tetracycline antibiotic derivatives, and as a treatment for depression [141]. Muscimol was shown to be a viable candidate for the transmeningeal pharmacotherapy of intractable focal epilepsy [142,143].

Also, the lipophilic bioisosteres of muscimol and GABA were synthesized and signed as a therapeutic agent for the treatment of epilepsy [144]. Muscimol is widely used as a ligand to probe GABA receptors. The development of muscimol and related compounds as a GABA agonist has been very recently reviewed by Johnston (2014) [145]. In 2013, the antiinflammatory activity of muscimol in endotoxemia was revealed [146]. This study in mice showed that muscimol (0.1 mg/kg) significantly decreased lipopolysaccharide-induced placental inflammation.

Ibotenic acid, the second main *Amanita* alkaloid, is a powerful neurotoxin used in many investigations as a "brain–lesion" causing agent [147,148]. Intrahippocampal injection of ibotenic acid causes severe neuronal loss, resulting in learning and memory deficit. Animal models that are relevant for Alzheimer's disease-like neurodegeneration are generated by injection of this excitotoxin [149–151].

CONCLUSIONS

Research on hallucinogenic alkaloids have gained noticeable importance and have been given a prominent position in the field of medicine, both with respect to their biological activity and role played in the introduction of new drugs.

Among the hallucinogenic alkaloids an important role play tryptophan indole-based alkaloids (psilocybin, psilocin), which have been identified in a large number of mushrooms, especially of the genus *Psilocybe*, but are still waiting for more control studies to ascertain their therapeutic role in some other conditions, apart from psychiatry.

Much less research has concerned hallucinogenic substances contained in mushroom *Amanita* species (muscimol, ibotenic acid). These isoxazole alkaloids have a long history for their use as sacraments in religious ceremonies along with medical and recreational purposes and thus need more attention to explore their therapeutic role. Although hallucinogenic mushrooms have been used by people for thousands of years, their intriguing features are still being discovered and their relevance to medical science is growing. The isoxazole derivatives are associated with important biological activities in different therapeutic areas. Some of them are used as antirheumatic drugs, inhibitors for ulcers, or anticonvulsant drugs. There are also trials for using these compounds as anticancer agents.

However, there is also a "dark side" to hallucinogenic mushroom use; they are most frequently used by young people, predominantly users of other drugs. Although it has been difficult to demonstrate the toxic effects of hallucinogenic mushroom use, it is well-established that such use can induce uncontrolled action in the user.

Collecting hallucinogenic mushrooms require substantial mycological knowledge as there are many look-a-likes (little brown mushrooms). Some of these look-a-likes mushrooms are toxic. In rare cases, when the intake of such mushrooms has been substantial, flashbacks of adverse experiences have been reported. For these reasons, and perhaps due to the fact that the use of hallucinogenic mushrooms is not uncommon for young people, restrictions have been ordained in many countries over the usage of these mushrooms.

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