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Nitrogen-Containing Constituents of Black Cohosh: Chemistry, Structure Elucidation, and Biological Activities

Dejan Nikoli , David C. Lankin, Tamara Cisowska, Shao-Nong Chen, Guido F. Pauli, and Richard B. van Breemen

UIC/NIH Center for Botanical Dietary Supplements Research, Department of Medicinal Chemistry and Pharmacognosy, University of Illinois College of Pharmacy, 833 S Wood Street, Chicago, IL 60612-7231, USA

Dejan Nikoli : dnikol1@uic.edu

Abstract

The roots/rhizomes of black cohosh (*Actaea racemosa* L. syn. *Cimicifuga racemosa* [L]. Nutt., Ranunculaceae) have been used traditionally by Native Americans to treat colds, rheumatism, and a variety of conditions related to women's health. In recent years black cohosh preparations have become popular dietary supplements among women seeking alternative treatments for menopausal complaints. The popularity of the plant has led to extensive phytochemical and biological investigations, including several clinical trials. Most of the phytochemical and biological research has focused on two abundant classes of compounds: the triterpene glycosides and phenolic acids. A third group of phytoconstituents that has received far less attention consists of the alkaloids and related compounds that contain nitrogen. This chapter summarizes the current state of knowledge of the chemistry and biological activities associated with this group of constituents and provides some perspective on their significance for future research on this interesting plant.

Keywords

Black cohosh; *Cimicifuga racemosa*; Triterpene glycosides; Alkaloids; MassBank; Guanidino alkaloids; Cimipronidine; Arginine; γ -Guanidinobutyric acid; Acylated arginines; Isoquinoline alkaloids; Salsolinol; Benzyloisoquinoline alkaloids; Reticuline; Aporphine alkaloids; Magnoflorine; Protoberberine alkaloids; Protopine; Indole alkaloids; β -Carbolines; Monoamine oxidase; N_{ω} -Methylserotonin; 5-HT₇ receptor; Pictet-Spengler reaction; Methylcimitrypazepine; Cinnamides; *N*-Feruloyl arginine; *N*-Feruloyl tyramine glucoside; Choline; Betains; Trigonelline

2.1 Introduction

Black cohosh (*Actaea racemosa* L. syn. *Cimicifuga racemosa* [L]. Nutt., Ranunculaceae) is a flowering plant native to North America. The roots/rhizomes have been used traditionally by Native Americans to treat colds, rheumatism, and a variety of conditions related to women's health [1]. Because of the risks associated with hormone replacement therapy, black cohosh preparations have become popular dietary supplements among women seeking alternative

treatments for menopausal complaints [2, 3]. The most recent sales figures place black cohosh as seventh best-selling herb with annual sales of approximately \$17 million [4]. The popularity of the plant has led to extensive phytochemical and biological investigations, including numerous clinical trials, some of which date back to the 1950s. Despite such extensive research, the clinical efficacy of black cohosh products remains controversial [2]. Early studies suggested that black cohosh extracts were effective in reducing the frequency and intensity of hot flashes among premenopausal and postmenopausal women [5–8], while several recent trials including a Phase II double-blind placebo-controlled trial conducted at the authors' UIC/NIH Center for Botanical Dietary Supplements Research demonstrated no vasomotor symptoms benefits [9–12]. Clinical efficacy is not the only controversy surrounding this plant. The question of long-term safety of black cohosh came to light after initial reports of liver failure allegedly associated with the use of black cohosh had appeared in the literature [13]. The concern was serious enough to warrant two workshops organized by the NIH Office of Dietary Supplements to discuss issues related to safety of black cohosh. A recent review sponsored by the US Pharmacopoeia summarized 30 cases of liver damage associated with black cohosh use and recommended that black cohosh products carry a cautionary statement that they may adversely affect the liver [14]. In contrast, several recent reviews of randomized controlled clinical trials concluded that there is no evidence of hepatotoxicity [15–18], and recent in vitro and animal studies seem to support this conclusion [19, 20].

When it comes to phytochemical investigations, the past several decades of research have focused almost exclusively on two abundant classes of compounds: triterpene glycosides and phenolic acids. Triterpene glycosides represent the major constituents of all hydroalcoholic black cohosh extracts and have been extensively studied from both the phytochemical and biological side. More than 40 triterpenes have been isolated and structurally characterized to date [21, 22]. They represent a particular analytical challenge due to their close structural similarities and overall complexity of distinguishing these structures, which include numerous stereocenters. The most abundant triterpenes, particularly actein (**1**) and 23-*epi*-26-deoxyactein (**2**), are often used as markers for the standardization of black cohosh preparations [23–25]. Extensive reviews on the chemistry, rational naming system, and biological activities of the triterpenes have been published recently [22, 26, 27].

The major phenolic constituents of black cohosh are the hydroxycinnamic acids, caffeic acid (**3**), ferulic acid (**4**), and isoferulic acid (**5**), as well as their condensation products with glycoloyl phenylpropanoids, commonly known as cimicifugic acids (e.g., fukinolic acid (**6**)). Numerous members of this class have also been isolated and fully characterized, and a rational naming system has been proposed [22, 28, 29].

A third group of phytoconstituents that has received far less attention consists of the alkaloids and related compounds that contain nitrogen. These constituents represent a largely unexplored part of the black cohosh metabolome, and most of the work in this area has been confined to our laboratories in the UIC/NIH Center for Botanical Dietary Supplements Research. This review summarizes the current state of knowledge of the chemistry and biological activities associated with this group of diverse constituents and provides some perspective on their significance for future research on this interesting and widely used medicinal plant.

2.2 Historical Overview

It is somewhat surprising that the alkaloid metabolome of black cohosh has been overlooked for so long, considering that from a chemotaxonomic perspective the Ranunculaceae family is a well-known producer of alkaloids. The authoritative review on alkaloids written by Cordell et al. [30] lists Ranunculaceae as the third-most producing family, with more than 1,500 alkaloids isolated. Sporadic and inconclusive reports of the presence of alkaloids in black cohosh can be traced back to the beginning of the last century [27, 31]. However, due to changing taxonomic classification and varying degrees of documentation for some of those reports, it was not always clear whether the tested species was indeed *A. racemosa*. One of the first notable clues that black cohosh may contain alkaloids can be found in a report by Crum et al. who tested different parts of the plant using five alkaloid detection reagents and concluded that seeds contained alkaloids but not roots/rhizomes [32]. No identification of a specific compound was mentioned in that early report. Scattered reports of the presence of alkaloids in other *Actaea* species have been published over the years, and some examples of reported alkaloids and their sources are shown in Fig. 2.1 [33–35].

The first report on the isolation and full structural characterization of an alkaloid in black cohosh was published in 2005 by Fabricant et al., who reported on the identification of a new cyclic guanidine-type alkaloid named cimipronidine (**7**) to reflect its source (*Cimicifuga racemosa*), the (homo) proline base structure, and the occurrence of a guanidine group [36].

7 Cimipronidine

This was an interesting discovery not only because of the novelty of the structure (new chemical entity with seven carbon atoms), but also because guanidine alkaloids are relatively rare in higher plants. Furthermore, cimipronidine was the first reported guanidine alkaloid from the Ranunculaceae family. It turned out that cimipronidine was not an odd occurrence, as it was subsequently revealed that guanidine alkaloids and other compounds containing a guanidino group represent a significant part of the nitrogenous metabolome of black cohosh.

Subsequently, two other guanidine alkaloids cyclocimipronidine (**8**) and dopargine (**9**) were isolated and structurally characterized [37].

Importantly, the identification of highly basic guanidino compounds led to modifications in the separation procedure which allowed the effective separation of the complexes of the alkaloids with abundant phenolic acids and enabled effective dereplication procedure to be carried out [28]. This process led to the identification of *N*_ω-methylserotonin (**10**) as the active serotonergic principle [38]. Most recently, extensive mass spectrometric investigations led to conclusive or tentative identification of 73 nitrogenous primary or secondary metabolites [39]. This study unequivocally confirmed that alkaloids are well represented in the black cohosh metabolome.

2.3 Structure Elucidation by Mass Spectrometry

Of all the currently known nitrogenous metabolites of black cohosh, only **7**, **8**, and **9** were isolated and fully characterized using full sets of mass spectrometry and 1D and 2D NMR spectroscopy data; the rest was identified or tentatively identified by a mass spectrometry-based dereplication approach that will be described here in more detail.

Detection of alkaloids by mass spectrometry is relatively straightforward. The presence of basic nitrogen allows for sensitive detection using positive ion electrospray ionization. In addition, the presence of a basic center fixes the initial charge site, which allows for easier interpretation of the tandem mass spectra. Another benefit of the basic nitrogen is that molecular species observed upon electrospray ionization are typically protonated molecules. However, one should be mindful that some alkaloids contain quaternary nitrogen. Thus, if the analyst routinely subtracts a proton to arrive at the molecular formula, quaternary alkaloids can be easily missed. Indeed, we have encountered many such cases in black cohosh, which were easily solved once a possibility of quaternary nitrogen was considered.

The dereplication process starts with the determination of the elemental composition by accurate mass measurement. The determination of the correct formula is the critical step in the process, since an incorrect formula will ultimately lead to a wrong assignment. The validity of the elemental composition can be established using additional criteria such as the isotope pattern, the elemental composition of fragment ions, as well as general plausibility based on general knowledge of natural product chemistry and biosynthetic relationships. The latter point should not be underestimated as the analyst ultimately has to reconcile the mass spectrometry data with what is realistically possible (or probable) in nature. There is ample discussion in the literature regarding determination of elemental composition using accurate mass measurement, and the interested reader is referred to those publications for more details [40, 41].

The molecular formula and the product ion spectra can be searched in the SciFinder and Reaxys databases as well as in the MassBank (www.massbank.jp) database of tandem mass spectra. If a match is obtained in the MassBank database, the final confirmation of compound identity can be achieved by comparing the retention time and fragmentation pattern with those of authentic standards. This cautious approach considers the well-known fact that different types of instruments often generate different product ion spectra of the same compound, which is one of the main obstacles in the development of the standardized databases similar to those obtained by electron ionization [42]. During our work on black cohosh, the MassBank database was of limited utility and was useful mostly for identification of primary metabolites. For compounds for which there were no spectra in the MassBank database, the hits obtained in the SciFinder or Reaxys databases provided clues on possible structures. As alkaloids are comparatively rare natural products, database searches usually return only a handful of possible structures that need to be considered. We observed that SciFinder provided a somewhat broader coverage since it revealed compounds that have been identified or putatively identified in plants, but not necessarily derived from formal isolation. Based on the interpretation of product ion spectra and taking into account all available information, a proposed structure can be tested by comparison with an authentic standard. This iterative process is repeated until a conclusive assignment can be made. One challenging aspect of elucidating compounds from black cohosh was that database searches in many instances returned no hits, suggesting an entirely new structure. This necessitated a tedious process of de novo interpretation of tandem mass spectra to come up with a plausible structure that could be compared with authentic standards, most of which had to be synthesized.

As mass spectrometry does not have the ability of de novo structure elucidation of unknown compounds, the proposed structural assignment always comes with a degree of uncertainty [43]. In other words, the assignment can be made with various degrees of confidence. How to best grade the level of confidence in the assignment is a subject of active discussion in the metabolomics community [44]. The most commonly used nomenclature is that proposed by Sumner et al. [45], which assigns three levels of confidence. Identification at level 1 is established by comparing the retention time and fragmentation pattern of an unknown with those of an authentic standard. This level of evidence provides the highest degree of confidence in the assignment and is a widely accepted criterion for positive identification of compounds not only in the research domain but also in forensic and regulatory areas. Some compounds were identified at level 2 by comparing their product ion tandem mass spectra either with published spectra or with tandem mass spectra of close structural analogs. Comparison of tandem mass spectra of close chemical analogs is a viable approach commonly used in drug metabolism or chemical degradation studies. The basic idea behind this approach is the long-established “shift technique” pioneered by Klaus Biemann [46, 47]. This approach relies on the correct interpretation of tandem mass spectrum of a template molecule and tries to assign where the structural difference(s) is(are) with the unknown. Perhaps fittingly, the original approach was developed to determine the structure of alkaloids.

Finally, for level 3 characterization, only a chemical class of the unknown compound can be ascertained based on the similarity of its tandem mass spectrum relative to known

compounds belonging to the same class. Because direct comparison with the authentic standard is not accomplished, identifications at level 2 and 3 should always be considered tentative. Even if the measured spectrum of the unknown matches a published spectrum, without comparison of retention times under identical conditions, there is always a possibility that the two compounds may just be close structural analogs. Indeed, there are many classes of compounds where fragmentation pattern is dominated by ions from one portion of molecule producing nearly identical spectra for compounds with different structures. It is also important to point out that the strength of analytical evidence is not related to the novelty of the compound. Indeed, many of the compounds discussed in this review identified at level 1 are new natural products, for which synthetic standards were prepared after interpretation of the mass spectrometric data. If a natural product is identical to the synthetic standard regarding retention time and mass spectral characteristics, it may still not be identical due to differences in stereochemistry. Table 2.1 lists the mass spectrometric data for all of the compounds discussed in this review. The table is adapted from Nikolic et al. [39] and includes data for new compounds reported here for the first time. For easier referencing, the compounds are listed in the order in which they are discussed in the text. In the following sections, the black cohosh alkaloids with guanidine (2.4), isoquinoline (2.5), indole (2.6) structures will be discussed, together with the accompanying cinnamides (2.7), cholines and betains (2.8), as well as other primary and secondary products (2.9).

2.4 Guanidino Alkaloids

The presence of a substantial number of guanidino compounds is one of distinguishing features of the black cohosh metabolome. Cimipronidine (**7**), a cyclic guanidine alkaloid, was the first isolated alkaloid from black cohosh [36]. Guanidine alkaloids are quite rare in nature, and it is interesting to find a plant with such extensive diversity of this class of compounds.

Guanidino compounds are readily detected by mass spectrometry. The highly basic guanidine moiety allows for very sensitive detection using electrospray ionization. A molecular formula with three or more nitrogens is a strong indicator for the presence of a guanidino group, as there are few other possibilities that can account for so many nitrogens in the molecule, particularly if the molecular mass is modest (<400 Da). During collision-induced dissociation, acyclic guanidines display a characteristic loss of neutral guanidine (–59 Da; CH₅N₃). Accurate mass measurements are particularly useful to distinguish this loss from other isobaric losses of 59 Da, such as elimination of acetamide (CH₃CONH₂) originating from an acetylated amino group or trimethylamine originating from a quaternary nitrogen. In addition to the loss of neutral guanidine, protonated guanidine (CH₆N₃) of *m/z* 60 may be observed, albeit only at low collision energies. Formation of protonated guanidine is thought to proceed via an ion-neutral complex. When collision energy is low, the ion-neutral complex survives long enough for proton transfer to occur; otherwise the complex simply dissociates without proton transfer [48]. It should also be noted that, for many small guanidine molecules, no useful tandem mass spectra could be obtained with collision energies above 25 eV using a quadrupole/time of flight instrument.

The prototype acyclic guanidino compound and a likely biosynthetic precursor of the guanidine alkaloids is the amino acid arginine (**11**). Fragmentation pathways of arginine have been extensively studied [48–50] and have been used as a template to discover numerous analogs (**12–21**).

As an example of the identification strategy, Fig. 2.2 shows tandem mass spectra of γ -guanidinobutyric acid (**13**) and its oxidation analogs γ -guanidinobutanol (**12**) and γ -guanidinobutyraldehyde (**14**).

In the tandem mass spectrum of **13** the ion of m/z 87 is likely to be protonated butyrolactone, and its formation can be rationalized by an S_N2 attack of the carbonyl oxygen on the carbon atom bearing the guanidino group (Fig. 2.3). Direct attack of the carbonyl oxygen is supported by observation of protonated guanidine at m/z 60, which is formed by proton transfer in the ion-neutral complex between guanidine and protonated butyrolactone. An additional minor pathway for formation of the ion of m/z 87 is by elimination of ammonia from protonated γ -aminobutyric acid (GABA) (m/z 104), as determined in separate ion-trap experiments. Similarly, the product ion of m/z 86 has an elemental composition of C₄H₈NO corresponding to protonated butyrolactam. Ion-trap experiments indicated that the main pathways for formation of this ion are a loss of methylenediamine (NH=C=NH) from the ion of m/z 128 [MH-H₂O]⁺ and a loss of water from protonated GABA. Similarly, in the tandem mass spectrum of **12** the hydroxyl group participates in the S_N2 attack to form protonated tetrahydrofuran at m/z 73. The spectrum of **14** can be explained as being derived from both the cyclic and acyclic forms of this aldehyde (Fig. 2.4c). The presence of the cyclic form can explain the ready loss of water, as well as the base peak of m/z 70 which has the structure of protonated dihydropyrrole.

Using similar spectral arguments, other analogs such as δ -guanidinovaleric acid and its methyl ester (**17–18**) and acylated arginines (**19–21**) could be identified. The identification of *N*-benzoyl arginine (**21**) is reported here for the first time. Its tandem mass spectrum (Fig. 2.4) has all the characteristic features described above for acyclic guanidines.

In addition, the ions of m/z 105 and 77 suggested the presence of a benzoyl derivative. Combined with the molecular formula of C₁₃H₁₈N₄O₃, the most plausible structure consistent with the data is that of α-*N*-benzoyl arginine which was confirmed by comparison with authentic synthetic standard. Interestingly, database searches revealed that this compound has not been reported in plants even though the ethyl ester of α-*N*-benzoyl arginine is a widely studied compound. Its most common use is as a trypsin substrate to measure enzymatic activity of trypsin preparations [51]. As ethyl ester it has also been used as a model for arginine-containing peptides, and at least one study found that it induced

relaxation of blood vessels independent of its properties as a substrate for nitric oxide synthase [52].

In contrast to acyclic guanidines, the predominant fragmentation pathway of cyclic guanidines proceeds via loss of methylenediamine (-42 Da; $\text{NH}=\text{C}=\text{NH}$). Using spectra of **7** and **8**, various esters of cimipronidine could be identified in the black cohosh extracts depending on the solvent used to extract the plant or to redissolve the sample prior to injection. In addition, *N*-methylcyclocimipronidine (**22**) could be tentatively identified.

22 *N*-methylcyclocimipronidine

The biosynthetic origin of cimipronidine has not yet been established, but is most certainly linked to arginine metabolism. A possible biosynthetic scheme for production of guanidino alkaloids is shown in Fig. 2.5. The proposed central intermediate *N*-guanidinopyrroline is a cyclic hemiacetal form of **14**, which could be formed by action of an amine oxidase on agmatine, a decarboxylation product of arginine.

A candidate gene encoding an unspecified amine oxidase has been identified by Spiering et al. [53]. *N*-Guanidinopyrroline may through yet to be identified steps be converted into cimipronidine. Cimipronidine easily cyclizes into cyclocimipronidine under acidic conditions, which implies that cyclocimipronidine may be an isolation artifact. The acid–base interconversion between **7** and **8** in aqueous medium was demonstrated by Gödecke et al. [37].

Since the guanidine alkaloids were identified in black cohosh only recently, it cannot yet be assessed if any of these compounds are unique to *A. racemosa* or whether they are more widespread in the genus *Actaea*, but in any event they are certainly strong candidates for further investigation as possible novel standardization markers.

2.5 Isoquinoline Alkaloids

The isoquinoline alkaloids represent a large group of alkaloids that are biosynthetically derived from Pictet-Spengler condensation of dopamine with various aldehydes. Among all the alkaloids identified from various Ranunculaceae, isoquinolines represent the second-most common class [30]. Thus, it is not surprising that a significant diversity of this class was identified in black cohosh.

2.5.1 Dihydro- and Tetrahydroisoquinoline Alkaloids

The simplest structures within the isoquinoline alkaloids are the tetrahydroisoquinoline alkaloids. Dopargine (**9**), mentioned above, was the first alkaloid from this class isolated from black cohosh [37]. This alkaloid is likely derived from condensation of dopamine with γ -butyraldehyde (see Fig. 2.5). Salsolinol (**23**), a condensation product of dopamine and acetaldehyde, can be considered a prototype molecule of this class. It was initially identified

based on spectral database searches and subsequently confirmed by comparison with an authentic standard [39]. Salsolinol is widely distributed in the plant kingdom and can be found in many dietary sources including alcoholic beverages, bananas, cheese, beef, milk, and cocoa [54–56]. Importantly, salsolinol can be synthesized endogenously in dopaminergic neurons of mammals including humans [57]. Pharmacologists have extensively studied salsolinol for its neuropharmacological effects, such as modulation of catecholaminergic transmission, as well as for a possible role in the etiology of alcoholism [58]. Dopaminergic activity of salsolinol is often invoked as a mechanism for addictive effects of chocolate. However, recent studies showed that salsolinol does not cross the blood brain barrier [59]. Although it is unlikely that exogenously administered salsolinol can exhibit activities on the central nervous system (CNS), it still may show peripheral activities mediated by dopamine D2 receptors [60]. Salsolinol may be at least partially responsible for the observed in vitro dopaminergic activity of black cohosh extracts [61].

In addition to salsolinol, norsalsolinol (**24**) and 1,2-dehydrosalsolinol (**25**) have been identified at level 1. Both of these compounds have been identified as dopamine metabolites in the brain [62] and in urine [63]; however, they have not been reported in other higher plants. At this point it is not clear whether **24** is an isolation artifact or a genuine natural product. Traces of formaldehyde might have been present in the extraction solvent, which may have led to a nonenzymatic condensation with dopamine [57].

2.5.2 Benzyloisoquinoline Alkaloids

A large group of isoquinoline alkaloids is derived from condensation of dopamine and 4-hydroxyphenylacetaldehyde to form a benzyl tetrahydroisoquinoline skeleton that can be further coupled into a plethora of alkaloids including aporphines, protoberberines, and protopines [64]. Norcoclaurine (**26**), the prototype molecule of this group of alkaloids, was tentatively identified based on comparison with the published product ion tandem mass spectra [65, 66]. Another critical molecule in the biosynthetic pathways of isoquinoline alkaloids is reticuline (**27**), which was identified at level 1 by comparison with authentic standard. The study by Spiering et al. identified a candidate gene for *N*-methylcoclaurine 3-hydroxylase, an enzyme involved in the biosynthesis of reticuline, providing a mechanistic basis for the origin of this group of alkaloids in black cohosh [53].

	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	
26	OH	OH	OH	H	H	H	Norcoclaurine
27	OH	OMe	OMe	OH	H	Me	Reticuline
28	OH	OMe	OH	H	Me	Me	Magnocurarine
29	OMe	OH	OH	H	Me	Me	Oblongine

Reticuline is an interesting molecule, not only because it represents a branching point in the synthesis of complex isoquinoline alkaloids in plants, but also because recent studies provide evidence that it can serve as a biological precursor for the biosynthesis of endogenous morphine via action of the cytochrome P4502D6 [67–70]. In addition, reticuline has biological activities of its own, as a dopamine antagonist [71] and a mild activator of the mu opioid receptor [72].

Compounds **28** and **29** produced nearly identical product ion spectra (Table 2.1) but had different retention times, suggesting two isomeric structures. Loss of dimethylamine (m/z 271) suggested that these compounds contain quaternary nitrogen (see below). Database searches with this structural constraint revealed that these compounds are likely analogs of the alkaloid magnocurarine. Comparison with authentic magnocurarine led to assignment of **28** as magnocurarine, whereas **29** is likely one of the known positional isomers of magnocurarine such as lotusine or oblongine.

2.5.3 Aporphine Alkaloids

Aporphine alkaloids represent one of the largest groups of the isoquinoline alkaloids, with more than 600 known compounds. They are biosynthetically derived from oxidative *o*, *p* coupling of benzyloisoquinolines. In order to identify aporphines in black cohosh extracts, we relied on the previous studies that examined fragmentation behavior of this class of compounds [73, 74]. Due to their rigid structure, tandem mass spectra of aporphines are characterized by losses of small molecules such as water, CO, or CO₂, with cation radical fragment ions frequently present. The degree of substitution on the nitrogen can be easily distinguished based on the loss of nitrogen in the form of ammonia (secondary nitrogen), methylamine (tertiary nitrogen), or dimethylamine (quaternary nitrogen). For example, in the product ion spectrum of magnoflorine (**30**) (Fig. 2.6) loss of dimethylamine (–45 Da) was the second most abundant peak, typical for a quaternary aporphine. Interestingly, most of the aporphines identified in black cohosh (**30–35**) were quaternary alkaloids.

Aporphine alkaloids have been extensively studied for their wide range of biological activities and have been subjected to extensive medicinal chemistry modifications to produce

more active analogs for a specific indication. The activities relevant for medicinal uses of black cohosh include vasorelaxing, antinociceptive, antispasmodic, or neuroleptic effects [75–77]. Mechanistically, these effects can be explained by strong affinity of aporphinoids for α -adrenergic, dopamine, and serotonin receptors. Both agonistic and antagonistic activities have been described, depending on the particular aporphinoid. An excellent compilation of activities of natural and synthetic aporphinoids against dopamine receptors has been provided by Zhang et al. [78]. Laurolitsine (**32**), for example, displayed IC_{50} values of 2.6 and 6.8 μM against dopamine D1 and D2 receptors, respectively. Among serotonergic activities, most interest has focused on 5-HT_{1A} and 5-HT₇ receptors, and natural aporphinoids have been used as model pharmacophores to develop novel synthetic analogs [79, 80]. For example, *N*-methyllaurotetanine, a methyl analog of laurotetanine identified in black cohosh, is a potent ligand against 5-HT_{1A} [81]. Interestingly, a recent study by de la Peña et al. claims that magnoflorine possesses sedative and anxiolytic effects mediated by the GABAergic system [82]. Aporphinoids also act as α -adrenergic antagonists [83], and those with quaternary nitrogen have affinity for neuronal nicotinic receptors. For example, xanthoplanine (**35**) bound to the $\alpha 4\beta 2$ subtype of nicotinic receptors with a K_i of 0.91 μM [84]. Overall, as a class, aporphinoids are likely to contribute significantly to the overall pharmacological profile of black cohosh and are strong candidates for inclusion in the standardization process.

	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇	
30	OH	OMe	H	OMe	OH	Me	Me	Magnoflorine
31	OH	OMe	OH	OMe	H	Me	Me	Laurifoline
32	OMe	OH	OH	OMe	H	H	H	Laurolitsine
33	OMe	OMe	H	OMe	OH	Me	Me	Menisperine
34	OMe	OMe	OH	OMe	H	H	H	Laurotetanine
35	OMe	OMe	OH	OMe	H	Me	Me	Xanthoplanine

2.5.5 Protoberberine Alkaloids

Protoberberines are a widely distributed class of alkaloids, with some of the most prolific producers in the Ranunculaceae. Based on limited mass spectrometric evidence, three alkaloids from this class were tentatively identified at level 3 in black cohosh. The main difficulty in assigning the structures is due to the limited fragmentation that does not allow differentiation between 9,10- and 10,11-substitution pattern.

36a $R_1=OH, R_2=H$ Phellodendrine

36b $R_1=H, R_2=OH$ Cyclanoline

37 $R_1=H, R_2=OMe$ N-methyl tetrahydrocolumbamine

2.5.6 Protopine-Type Alkaloids

Two members of the protopine subclass of the isoquinoline alkaloids, protopine (**38**) and allocryptopine (**39**), were identified in black cohosh based on MassBank database searches and confirmed by comparison with authentic standards. These two alkaloids were among the few for which public databases were useful for identification.

38 Protopine

39 Allocryptopine

Protopine (**38**) and allocryptopine (**39**) are common in the genera *Corydalis* and *Fumaria* of the Papaveraceae. In the Ranunculaceae they have been reported only in the genus *Thalictrum*. Biological activities of protopine and allocryptopine have been extensively studied (for a review see [85]). Of relevance to black cohosh medicinal use, protopine has been reported to have benzodiazepine-like, analgesic, antidepressant, and anticholinergic activities in vitro and in animal studies [86–88]. The presence of this alkaloid might explain anecdotal reports of “vivid dreams” and opioid-like activities observed by patients taking black cohosh [89], as well as the observed in vitro opioid activity [90]. Li et al. found that protopine and allocryptopine were potent competitive inhibitors of the cytochrome P4502D6, with K_i values of 78 nM and 122 nM, respectively. This inhibition was studied in the context of potential drug-herb interactions with tamoxifen [91]. One of the side effects of tamoxifen therapy is occurrence of hot flashes, and patients often use black cohosh to alleviate such symptoms. However, since tamoxifen is a prodrug that is metabolically activated by the cytochrome P4502D6, there is concern that inhibition of this enzyme may render tamoxifen therapy less effective. Based on the measured content of protopine and allocryptopine (0.0063 % and 0.0088 %, respectively), it was concluded that these two compounds alone accounted for approximately half of the total activity of the active fraction [91].

2.6 Indole Alkaloids

Indole alkaloids are a large group of alkaloids biosynthetically derived from the amino acid tryptophan. Several compounds belonging to the subclasses of indole alkylamines and tetrahydro- β -carbolines have been detected in black cohosh.

2.6.1 Tryptophan-Derived Pictet-Spengler Adducts

Compounds **40** and **41** are Pictet-Spengler adducts formed by condensation of tryptophan with formaldehyde and acetaldehyde, respectively. Compound **40** was initially identified based on its mass spectrum characterized by an abundant fragment ion of m/z 144, with the elemental composition of $C_{10}H_{13}N_2$ corresponding to protonated tryptamine. In-source fragmentation followed by MS-MS product ion analysis of m/z 144 showed a fragmentation pattern identical to authentic tryptamine, suggesting that this compound is a tryptamine derivative. The neutral loss of iminoacetic acid ($C_2H_3NO_2$) combined with database searching suggested that **41** might be a tetrahydro- β -carboline carboxylic acid. Subsequent synthesis of two possible positional isomers (1- and 3-substituted) identified **40** as 1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid [39]. By similar reasoning, **41** was identified as 1-methyl-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid and confirmed by comparison with authentic standard. The presence of **41** in black cohosh is reported here for the first time.

The main fragmentation pathway of these adducts involves retro Diels-Alder cleavage to eliminate the neutral iminoacetic acid mentioned above. At low collision energies, loss of ammonia is also observed, which can be rationalized by the mechanism proposed in Fig. 2.7.

Both **40** and **41** are widespread alkaloids found in many dietary products such as fruits, wine, beer, chocolate, soy sauce, cheese, smoked meat products, and raisins [92–95]. **40** has also been detected in the human brain, which has generated interest in its contribution to the pathogenesis of alcoholism and psychiatric disorders [96]. There are scarce data on the potential biological activities of these compounds. β -Carboline is a common structural motif for monoamine oxidase (MAO) inhibition; however, **40** and **41** do not inhibit MAO [97]. There have been some speculations that **41** is a causative substance of eosinophilia-myalgia syndrome associated with ingestion of L-tryptophan [98, 99]. However, definitive proof for this has yet to be provided. Since **40** and **41** are common dietary ingredients, it would be difficult to assign any particular significance of these compounds to the overall clinical effects of black cohosh. Women are likely to ingest significantly more of these alkaloids through diet than from consuming black cohosh extracts

2.6.2 N_ω -Methylserotonin and Corresponding Pictet-Spengler Adducts

Identification of N_ω -methylserotonin (**10**) represents an important chapter in the black cohosh alkaloid story. The serotonergic activity of black cohosh extracts was first described

by Burdette et al. [100]. In that study, black cohosh extracts prepared by extraction with different solvents (methanol, 75 % ethanol, and 40 % isopropanol) were screened for binding to multiple serotonin receptor subtypes. The highest activity was observed for 5-HT_{1A}, 5-HT_{1D}, and 5-HT₇ receptors and, given the involvement of 5-HT_{1A} and 5-HT₇ receptors in thermoregulation, these two receptor subtypes were pursued further. In the radioligand displacement assay, the methanolic extract was found to inhibit binding of receptor probes 8-OHDPAT (5-HT_{1A}) and LSD (5-HT₇) with IC₅₀ values of 2.5 and 2.2 µg/mL, respectively. The 75 % ethanolic extract was somewhat more selective, with IC₅₀ values of 13.0 and 3.1 µg/mL for the 5-HT_{1A} and 5-HT₇ receptors, respectively. These initial results could not be fully reproduced in a subsequent study which found that methanolic and 75 % ethanolic extracts inhibited binding of LSD to 5-HT₇ receptor with IC₅₀ values of 87 and 55 µg/mL, respectively [38]. The bioassay-guided fractionation and identification of active principle(s) proved to be quite a challenge, and only development of an elaborate fractionation scheme involving use of pH-zone refined centrifugal partition chromatography in combination with mass spectrometric dereplication enabled identification of the active principle as *N*_ω-methylserotonin (Fig. 2.8).

*N*_ω-Methylserotonin is a rare indole alkylamine that occurs both in plants and in animals. In plants, it has been reported in *Ranunculus sceleratus* (Ranunculaceae) [101] and, interestingly, in several *Citrus* species [102]. In its glucosidated form, it has been identified in *Zanthoxylum piperitum* seeds [103]. The biosynthetic pathway has not been yet established, but may involve either methylation of serotonin by an amine *N*-methyltransferase or hydroxylation of *N*-methyltryptamine by tryptamine 5-hydroxylase. Interestingly, a gene discovery study by Spiering et al. [53] identified two tryptophan decarboxylase-related genes. However, enzyme expression was found limited to immature flowers and young leaves, and no expression was detected in roots/rhizomes. The authors speculate that *N*_ω-methylserotonin translocates to the roots from aerial parts. In support of this hypothesis, we did detect *N*_ω-methylserotonin in aerial parts (submitted manuscript). *N*_ω-Methylserotonin is one of the few alkaloids in black cohosh for which some data on concentrations in plant material exist. The reported content in the study of Powell et al. is 0.0031 % which would translate into a daily dose of 3.7 µg under typical 120 mg extract/day dosing regimen.

Biological activities of *N*_ω-methylserotonin were evaluated in a battery of bioassays designed to evaluate its properties as a serotonergic compound. In the radioligand displacement assay, *N*_ω-methylserotonin showed greatest selectivity for 5-HT_{1A} and 5-HT₇ receptors [38]. The affinity for 5-HT₇ was particularly strong, with an IC₅₀ of 23 pM, which makes *N*_ω-methylserotonin one of the most potent known ligands to any receptor, not just to 5-HT₇. For comparison, the natural ligand serotonin showed an IC₅₀ value of 0.86 nM for the same receptor. In the functional assay using production of cAMP as a marker of receptor stimulation, *N*_ω-methylserotonin acted as agonist (EC₅₀ = 22 nM). Unfortunately, insufficient data were presented to determine whether it is a full or partial agonist. However, given the structural similarity to serotonin, it is reasonable to assume that *N*_ω-methylserotonin is a full agonist.

Two separate studies have determined that N_ω -methylserotonin binds to the serotonin transporter and thus may be regarded as selective serotonin reuptake inhibitor (SSRI). Chang et al. reported an IC_{50} of 1 μ M [104], while Powell et al. reported an IC_{50} of 0.49 μ M [38]. Direct comparison between these values is difficult, since the two studies used very different concentrations of radiolabeled serotonin (100 vs. 20 nM). However, in both studies the observed inhibition was likely an apparent one, since it is possible that N_ω -methylserotonin acted as a substrate for the serotonin transporter and not necessarily as an inhibitor like the classical therapeutic SSRIs. This is reminiscent of the situation in enzymatic assays where alternative enzyme substrates are sometimes erroneously interpreted as inhibitors.

To gain more insights into its potential in vivo activity, we recently studied the metabolism of N_ω -methylserotonin using human liver microsomes [105]. The experiments showed that this compound is predominantly metabolized by MAO-A. The kinetic parameters (K_m and V_{max}/K_m) were very similar to those of serotonin, suggesting that orally taken N_ω -methylserotonin is unlikely to reach the CNS in any appreciable amount. Thus, N_ω -methylserotonin shares the same fate as other biogenic amines, most of which are not orally active due to degradation by MAO. Overall, these data, combined with the very low content in black cohosh, make it unlikely that N_ω -methylserotonin contributes to the pharmacological activity of black cohosh extracts.

Once the presence of N_ω -methylserotonin was established, it was possible to identify a number of compounds that can be regarded as Pictet-Spengler adducts of N_ω -methylserotonin with various aldehydes.

Previously, we reported the identification of the formaldehyde-derived adducts 6-hydroxy-2-methyl-1,2,3,4-tetrahydro- β -carboline (**42**) and 3,4,5,6-tetrahydro-7-hydroxy-5-methyl-1H-azepino[5,4,3-cd]indole, trivially named cimitrypazepine (**43**). In addition, a dihydro- β -carboline compound $N(2)$ -methyl-6-hydroxy-3,4-dihydro- β -carboline (**45**) was also identified. Here we report the identification of the acetaldehyde-derived azepine adduct 3,4,5,6-tetrahydro-7-hydroxy-5,6-dimethyl-1H-azepino[5,4,3-cd]indole (**44**), trivially named methylcimitrypazepine, as a new natural product. The presence of **44** was confirmed by comparison of retention times and fragmentation pattern with the authentic standard (Fig. 2.9). In contrast to the β -carbolines, azepine structures cannot produce retro Diels-Alder fragments and fragment instead by opening of the azepine ring followed by elimination of methylene imine to produce the base peak (m/z 188 in **44** and 174 in **43**).

Further confirmation of the structure of **44** was derived from NMR analysis. A synthetic sample [106] was examined by 1H NMR (qHNMR, gCOSY, 900 MHz, DMSO- d_6 /0.5–0.5 mg/600 μ L), giving a relatively clean proton NMR spectrum (Fig. 2.10) with evidence for the presence of all the expected structural elements. An isolated 4-spin system A_3X , (CH_3 –

CH) is present, with the methyl resonance appearing at δ 1.318 ppm and the CH at δ 4.526 ppm ($^3J_{H,H} = 6.95$ Hz). An ethyl linkage ($-\text{CH}_2-\text{CH}_2-$), contained in the 7-membered ring, exhibits an NMR pattern consistent with protons occupying pseudoaxial and pseudoequatorial orientation, suggesting that the 7-membered ring assumes a puckered (non-planar) conformation. The presence of the aromatic AB pattern (δ 6.619 and δ 6.977 ppm; $^3J_{H,H} = 8.37$ Hz) confirms that cyclization to **44** has proceeded as desired.

Table 2.2 summarizes the ^1H NMR data acquired in DMSO- d_6 together with those previously reported by Somei et al. [106] for comparison. Two points of particular note relate to the $^1J_{H,C}$ coupling constants, which are measured from the ^{13}C -satellites in the ^1H spectrum. From the magnitude of the one-bond C,H couplings to the aliphatic carbons, it is clear which carbons are attached to the nitrogen in the 7-membered ring, especially the methylene carbons, and these are consistent with the indicated structure. Furthermore, a detailed examination of the signal from the indole proton (δ 10.579 br. s), which is attached to both ^{14}N (99.64 % isotopic abundance, spin = 1) and ^{15}N (0.36 % isotopic abundance, spin = $1/2$), reveals the presence of a ^{15}N satellite at natural abundance appearing at the base of the broad $^1\text{H}-^{14}\text{N}$ resonance (see inset in Fig. 2.10). From the ^{15}N , ^1H satellites, values for $^1J_{N-^{15}\text{H}-1} = 97.26$ Hz and $^3J_{H-1,H2} = 2.78$ Hz may be extracted and are consistent with the indole structure. The ^{15}N satellite information is typically observed when the NMR spectrum of the N-H compound is acquired in DMSO- d_6 , where chemical exchange, in this case of the indole H-1 proton, is slow, and the associated resonance of the proton attached to ^{14}N is not so broad ($<^1J_{N, H-1}^{15}$) as to completely obscure the ^{15}N satellite.

While β -carboline are known natural products, the azepine structures found in black cohosh have not been reported before. As noted by Somei et al. [106], Pictet-Spengler condensation of aldehydes with serotonin or N_ω -methylserotonin can produce both the carboline and azepine products (see Fig. 2.11).

The azepine cyclization occurs due to activation by the 7-hydroxyl group. Which cyclization pathway will predominate depends both on the experimental conditions and on the type of aldehyde. Typically, acidic conditions favor β -carboline, while basic conditions favor azepine. Interestingly, we could not conclusively demonstrate the presence of a β -carboline adduct with acetaldehyde, although there were indications that this adduct might also be present. The main difficulty arose due to co-elution of another compound with the same molecular formula, resulting in a tandem mass spectrum that had the same ions as the β -carboline standard but in a slightly different ratio. In any case, the abundance of the β -carboline product was much lower compared to the azepine adduct.

Given that **42–44** can be formed by nonenzymatic condensation, it is unclear whether they represent isolation artifacts or genuine natural products. Both formaldehyde and acetaldehyde may be present as impurities in organic solvent used for extraction, thus this reaction could conceivably occur during sample processing. Alternatively, it is possible that formation of the azepine ring can also be catalyzed by enzymes, as Pictet-Spengler reactions are proven steps in biosynthetic pathways leading to other natural products. Tests for such a reaction leading to the formation of **42–44** may represent an interesting area of future research.

Compound **45** had an elemental composition containing two hydrogens less than **42** and **43** ($C_{12}H_{12}N_2O$), suggesting a dihydro- β -carboline structure. The ready loss of a methyl radical (m/z 186), along with the fragment ion of m/z 170 corresponding to the loss of methylamine ($MH-CH_3NH_2$)⁺, indicated that the *N*(2) nitrogen on the β -carboline ring was methylated. Biosynthetic considerations were used to deduce the position of the double bond on the β -carboline ring. Accordingly, the most likely position of the double bond is 1,2 which was confirmed by comparison of retention time and fragmentation pattern with authentic *N*(2)-methyl-6-hydroxy-3,4-dihydro- β -carboline. Biosynthetically, this compound is likely formed by dehydrogenation of **42** and represents a new natural product. It should be noted that dihydro- β -carbolines are often by-products of Pictet-Spengler condensation [107]. Thus, it is possible that **45** is an isolation artifact. Since **42–45** are rare natural products, their biological activities are largely unexplored. In our laboratory, **42** and **43** did not bind to the 5-HT₇ receptor, nor did they inhibit CYP2D6 or CYP3A4.

2.7 Cinnamides

Alkamides are a large group of natural products occurring in at least 33 plant families. They possess a broad range of biological activities including analgesic, cannabimimetic, and immunomodulatory properties. Interestingly, two recent extensive reviews on alkamides did not list Ranunculaceae as an alkamide-producing family [108, 109]. Cinnamides can be considered a subclass of alkamides in which the acid portion is derived from hydroxycinnamic acids such as caffeic, ferulic, or isoferulic acid, and the amine part is derived from amino acids or biogenic amines. The identification of black cohosh cinnamides, many of which are new natural products, was accomplished by a simple strategy that will be described here using the unique black cohosh cinnamides caffeoyl (**46**) and feruloyl/isoferuloyl arginine (**47**, **48**) as exemplary compounds.

In general, tandem mass spectra of cinnamides are dominated by the ions originating from the acid part of the molecules. Tandem mass spectra of many cinnamic acids have been published, thus the coupling acid can be identified by comparing the ion series in the unknown amide with referenced spectra of free acids in positive ion mode. Fragment ions corresponding to the protonated amine may also be observed, but their abundance is usually low and strongly depends on the type of amine and the applied collision energy. As illustrated in Fig. 2.12, the fragmentation pattern of α -N-feruloyl/isoferuloyl arginine (**47**, **48**) is dominated by fragment ions of m/z 177, 149, 145, 117, and 89 originating from the ferulic/isoferulic acid portion of the amide. Similarly, α -N-caffeoyl arginine (**46**) produces a

similar ion series at m/z 163, 145, 135, 117, and 89. Differentiation between ferulic and isoferulic acid amides can be accomplished based on two characteristic spectral features. The first is that amides of isoferulic acid tend to produce less of the secondary fragment ions (m/z 149, 145 and 117) originating from elimination of small neutrals (H_2O , MeOH , and CO) from the primary acylium ion (m/z 177) (compare Fig. 2.12a, b). Another distinguishing feature is the presence of a small, but diagnostic fragment ion of m/z 163 with the elemental composition of $\text{C}_9\text{H}_7\text{O}_3$. This ion was observed only during fragmentation of protonated isoferulic acid, but not ferulic acid ([110] and our own observations). Our investigations on both quadrupole/time-of-flight and ion trap instruments revealed that this fragment ion originates from an ion-molecule reaction in the collision cell and is formed by addition of water to the ion of m/z 145.

Once the diagnostic ions from cinnamic acid are observed in the product ion spectrum of an unknown compound, the amine portion of the amide can be deduced based on database searching of the elemental composition of the remainder of the molecule. For example, the molecular formula of feruloyl arginine is $\text{C}_{16}\text{H}_{22}\text{N}_4\text{O}_5$. After subtracting ferulic acid ($\text{C}_{10}\text{H}_{10}\text{O}_4$) and adding a water molecule to account for the fact that water is lost during formation of the amide, the formula of the amine is predicted as $\text{C}_6\text{H}_{14}\text{N}_4\text{O}_2$. A database search of this formula yielded only one reasonable hit, arginine. The presence of arginine can then be ascertained by observation of m/z 175 (protonated arginine) along with other ions originating from the fragmentation of arginine, such as m/z 158, 70, and 60. The presence of the latter two ions, along with the low-abundance ions of m/z 278 (**46**) or m/z 292 (**47**, **48**) which originate from the loss of neutral guanidine, indicates a free guanidino group and confirms that acylation occurred at the α -amino group. These structural assignments were confirmed by synthesis of authentic standards (see Table 2.3 for the summary of 900 MHz ^1H NMR data). The NMR spectra obtained consist of two spectral elements: (1) the ferulic acid/isoferulic acid components and (2) the arginine component. The spectral elements of the former component consists of signals (an AMX pattern in the case of the ferulic acid moiety and an ABX pattern in the case of the isoferulic acid moiety) associated with the tri-substituted aromatic ring and an AM pattern (first order analysis) associated with the double bond. In all cases, the geometry of the double bond was shown to be *trans* as characterized by the large coupling constant ($^3J_{\text{H,H}} = \sim 15.7$ Hz, average) between the olefinic protons. The signals of the arginine component were complex, exhibiting non-first order behavior upon resolution enhancement, and consisted of a signal from the CH proton α to the carboxyl group of the amino acid, which appears as a doublet of triplets (dt) with a pattern arising from spin coupling to the diastereotopic protons H-2' and an amide NH proton. This represents the key bonding link between the two structural components in the molecule. The proton pairs H-3' and H-4' are also diastereotopic, as a result of the chiral center at C-1'. Because the molecules exist with the free acid in the arginine residue, it may be presumed that both the carboxyl and the guanidino group are largely ionic at physiological pH, and thus zwitterions will predominate in solution.

The above-described mass spectrometric identification strategy also led to identification of feruloyl (**49**) and isoferuloyl (**50**) putrescine, and to the tentative identification of the two new cinnamides α -*N*-isoferuloyl histidine (**51**) and α -*N*-isoferuloyl glutamic acid (**52**).

Although mass spectrometry cannot distinguish between the *cis* and *trans* configurations of the double bonds in these compounds, based on co-elution with the synthetic standards the configuration was determined to be *trans* in all cases. It is interesting to note that in all the cinnamoyl amino acids identified here isoferulic acid analogs were more abundant than the corresponding ferulic or caffeic acid analogs.

Another part of the cinnamide metabolome consists of glycosidated analogs. Interestingly, glycosidation was observed only for cinnamides where the coupling amine originates from the tyrosine metabolites tyramine and dopamine. Identification of glycosidated analogs can in principle be accomplished using the same basic strategy described above. In general, identification of glycosidated cinnamides involves assignment of three structural components: the amide moiety, the type of sugar, and the position of glycosidation. All glycosides detected in black cohosh were hexosides which can be ascertained based on tandem mass spectra characterized by a common loss of 162 units ($C_6H_{10}O_5$); however, the type of hexose or its configuration (α or β) cannot be determined based on this information. After subtracting the hexose from the elemental composition of the entire molecule, the cinnamide core can be identified based on database searches. Using this strategy, hexosides of *N*-feruloyltyramine (**53**), *N*-feruloyldopamine (**54**), and *N*-feruloyl methoxytyramine (**55**) were tentatively identified. The identity of the hexose at this point is unknown as it cannot be determined based on the mass spectrometric data alone. Interestingly, a recent publication by Yim et al. described identification of *cis* and *trans* *N*-feruloyl tyramine-4'-*O*- β -D-allopyranoside and *trans* *N*-feruloyl methoxytyramine-4'-*O*- β -D-allopyranoside from *Actaea* (syn. *Cimicifuga*) *heracleifolia* [111]. Similarly, *N*-feruloyl methoxytyramine-4'-*O*-galactoside has been identified in *Actaea* (syn. *Cimicifuga*) *dahurica* [112]. The possibility that black cohosh produces allosides is intriguing, as these hexosides are quite rare and detailed mass spectrometric investigations of this type of compounds are lacking to date.

When it comes to establishing the position of glycosidation, we initially proposed that the presence of the fragment ion corresponding to the hexosidated acylium ion of ferulic/isoferulic acid at m/z 339 and the secondary fragment formed by a loss of water (m/z 321) is an indication that the sugar is attached to the acid portion [39]. To obtain experimental confirmation for this hypothesis, we synthesized standards of *N*-feruloyl dopamine-4'-*O*- β -D-glucopyranoside and *N*-feruloyl tyramine-4'-*O*- β -D-glucopyranoside. Interestingly, only the dopamine analog fragmented to produce the glucosidated feruloyl ion in low abundance (Fig. 2.13b). In both cases, loss of glucose (m/z 314 and 330) was the dominant fragmentation pathway. From these limited studies we can conclude that the *presence* of m/z 339 and 321 may be used as an indication of glycosidation on the acid part, but the *absence* of such ions does not exclude glycosidation on the ferulic acid, particularly if a weak precursor ion signal precludes detection of these weak fragments. Another important observation was that fragment ions corresponding to the protonated amine (m/z 138 and m/z 154 for tyramine and dopamine, respectively) were not observed (Fig. 2.12a, b). This

observation can be explained by a lack of readily available acidic hydrogen that needs to be transferred to the amine during fragmentation.

In contrast, the tandem mass spectrum of a metabolite whose molecular formula nominally corresponds to a glycosidated feruloyl/isoferuloyl dopamine analog (**56**) (Fig. 2.13c) clearly shows the dopamine-related ions at m/z 154 and 137. The presence of protonated dopamine suggests that the amino group of dopamine is free, thus this compound is not a cinnamide. The spectrum also shows an ion of m/z 339 representing feruloyl/isoferuloyl hexose [113, 114]. These spectral features are consistent with a dopamine hexoside where the hexose unit is acylated with ferulic/isoferulic acid. Based on the presence of a weak, but diagnostic fragment ion of m/z 163 we can ascertain that the hexose is acylated with isoferulic acid [39]. However, neither the site of attachment of hexose to dopamine nor the position of acylation of the hexose can be determined based on the mass spectrometric data, although position 6 on the hexose part appears to be the most common acylation site [114, 115]. In general, cinnamate conjugates with amines and amino acids are widely distributed throughout the plant kingdom, with coffee being the major dietary source [116, 117]. Biosynthetically, they are formed by transfer of an acyl group from hydroxycinnamoyl-SCoA onto the corresponding amine, catalyzed by hydroxycinnamoyl-CoA acyltransferases. A gene for benzoyl/hydroxycinnamoyl transferase was identified by Spiering et al. and found expressed in all tested tissues including roots/rhizomes [53]. A glycosyl transferase gene was also detected that could be involved in the synthesis of glycosidated analogs. Although cinnamides in general are ubiquitous compounds, the metabolome found in black cohosh is unique. The abundance of arginine amides in particular is quite distinctive. Along with observation of numerous guanidine alkaloids, this implies that arginine processing represents a significant component of the plant's metabolic activity. Since many of the cinnamoyl amino acids are new natural products, it is still early to say whether they are unique to the genus of *Actaea*, but they are certainly good candidates for inclusion as new standardization markers. In contrast to their alkyl counterparts, which display a number of interesting biological activities, hydroxycinnamoyl amides have been mostly studied for their antioxidant activity [118]. However, given the uniqueness and novelty of the compounds identified in black cohosh, there is a lot to be learned about their biological activities.

2.8 Cholines and Betains

Compounds derived from amino alcohol choline (**57**) and containing a quaternary ammonium cation are generally designated as cholines. Choline itself is an essential nutrient present in egg yolks, meats, milk, and other animal products. It is also sold as dietary supplement in free form or as phosphatidylcholine. Choline was identified in black cohosh by mass spectra database searching and by comparison with an authentic standard. A number of choline esters with aromatic acids such as benzoic, ferulic, and isoferulic acid (**58–60**) were also identified by comparison with authentic standards. During CID, cholines display a characteristic loss of trimethylamine from the precursor ion, which can be used for selective detection of this class of compounds. For example, in the spectrum of benzoyl choline (**58**), the precursor ion of m/z 208 loses trimethylamine to form an ion at m/z 149, which can further fragment to lose CO₂ and produce an acylium ion of m/z 105, typical for

benzoyl derivatives (see Table 2.1). A more detailed discussion of the fragmentation pathways of choline esters can be found in Böttcher et al. [119].

Aromatic choline esters have been predominantly reported from seeds of plants from the Brassicaceae. Choline esters and other compounds with quaternary nitrogen are prototype agents acting on the cholinergic system. Benzoyl choline, for example, has been of interest to pharmacologists since the early 1950s, primarily as a model substrate for butyrylcholinesterase [120] and as a pharmacological tool to investigate various actions of acetyl choline. In the cholinergic system, benzoyl choline shows weak muscarinic and strong nicotinic activity [121]. Similar cholinergic activities have not been investigated for ferulic and isoferulic acid choline esters. However, the biological activity of choline esters after oral ingestion is low due to rapid hydrolysis into acid and choline catalyzed by butyrylcholinesterase.

Another interesting compound (**61**) from this class was detected in the highly polar water fraction of black cohosh. The elemental composition and product ion tandem mass spectrometric analysis of **61** indicated that it also contains a choline moiety, but the remainder of the molecule corresponded to that of a hexose sugar, which suggests a tentative assignment of this compound as *O*-hexosyl choline. This identification is also consistent with late elution of **61** under HILIC conditions, indicative of a highly polar molecule. There have been no reports of this type of compound occurring in plants.

In addition to choline derivatives, several betaines (**62–66**) have been identified in black cohosh. Chemically, betains can be considered permanent zwitterions. In plant cells, betains serve important roles as osmoprotectors balancing the osmotic pressure difference between cells and their surroundings.

Among the identified betains, L-carnitine (**64**) and trigonelline (**66**) have been most extensively studied. L-carnitine is widely distributed throughout the plant and animal kingdoms. In living cells, it plays an important role in energy production, since it helps transport fatty acids from the cytoplasm into the mitochondria, where their degradation takes place. Although many plants contain L-carnitine, meat products are the main source of L-carnitine in the human diet. L-Carnitine is also sold as a dietary supplement for its purported beneficial roles in the contexts of cardiovascular disease, diabetes, or weight loss.

Trigonelline is a widespread plant alkaloid formed by methylation of nicotinic acid. Among other plants, it has been identified in coffee [122], and it has been extensively investigated for a number of purported beneficial properties such as antioxidant, neuroprotective, or hypoglycemic (reviewed in [123]). Interestingly, a recent study by Allred et al. identified trigonelline as a novel phytoestrogen capable of stimulating growth of MCF-7 cells in very low doses [124]. This is a potentially significant finding, given that this is such a common compound. However, these results have not been confirmed by others, and black cohosh is not estrogenic as determined in numerous in vitro and in vivo studies [11, 125–128].

2.9 Miscellaneous Primary and Secondary Metabolites

Mass spectrometric dereplication has also been used to identify a number of primary metabolites in black cohosh extracts. Those include the nonessential amino acids pipercolic acid (**67**) and pyroglutamic acid (**68**), vitamins (**69–71**) and methylated nucleosides (**72, 73**). Two unusual compounds **74** and **75** were tentatively identified at level 3 based on interpretation of their tandem mass spectra. The origin of these constituents is unclear, and it is possible that they are impurities from extraction solvents.

2.10 Concluding Remarks and Future Directions

As the number of identified nitrogenous compounds in black cohosh approaches one hundred, the overall picture of this important part of the *A. racemosa* metabolome is gradually taking shape. Nitrogenous metabolites show great chemical diversity, including the presence of some unique and rare compounds. In particular, this plant appears to extensively process arginine, as manifested in the presence of numerous acylated derivatives of arginine, as well as in the abundance of guanidine-type alkaloid diversity. Guanidine alkaloids are rare in the plant kingdom, and we think that new compounds and even chemical templates are likely to be discovered from this part of the metabolome in the future. Another interesting observation is the presence of a significant number of quaternary amines. These, along with highly basic and essentially permanently positively charged guanidines, offer a potential explanation for the biological relevance of phenolic acids which might act as counter ions for positively charged alkaloids. Formation of strong ion pairs between abundant phenolic acids and alkaloids should be taken into account, not only during the isolation of both the acids and the alkaloids, but also when interpreting biological observations related to both classes of phytoconstituents.

The presence of glycosidated cinnamides is also an interesting discovery. This type of compounds is, again, not very common in the plant kingdom. While the identity of the specific hexose remains to be shown, based on chemotaxonomic considerations it seems

plausible that allose is the coupling sugar. This hypothesis is very intriguing, as such compounds have not been explored in detail either chemically or biologically.

When it comes to biological relevance, the discovery of alkaloids represents an important breakthrough towards a better understanding of the pharmacological properties of black cohosh. Alkaloids are flagship natural products with proven biological activities, particularly as CNS agents, and as such they are likely the active principles responsible for the observed CNS activities of black cohosh. It is important to note that, as alkaloids are minor but potent constituents, small differences in their quantity may lead to large differences in the observed activities of crude extracts. In this regard, the alkaloids may indeed be a missing link that can explain at least some of the variability in various in vitro studies or clinical trials of black cohosh. As all clinical trials to date were carried out without consideration and/or knowledge of the existence of these alkaloids, there is no way of knowing how much (if any) of these compounds were present in the tested extracts.

In an indirect way, the discovery of alkaloids in black cohosh is also relevant for a better understanding of the biological activities of other classes of *Actaea/Cimicifuga* compounds, particularly phenolic acids. As noted above, these acids tend to form relatively strong ion pairs with alkaloids, making the isolation of pure acids free from alkaloids a demanding task. If the N_{ω} -methylserotonin case is any guide, even traces of a very potent compound may produce positive results in a bioassay, which may then be erroneously attributed to the major compound. This is particularly true if compounds are tested at high concentration, as is frequently the case. For example, at 100 μ M of the test compound, even 99 % purity would result in a 1 μ M concentration of impurity. At this level, many alkaloids described here exhibit significant biological activity. Therefore, the present findings of a prominent alkaloid metabolome in black cohosh make it important to check any isolated compound, especially phenolic acids, for possible alkaloid impurities. A prominent example of a minor alkaloid impurity being responsible for the observed prominent biological effect has recently been reported by Fitch et al. [129].

Future research in this area is promising and is likely to produce exciting results. Even though a large number of nitrogenous compounds have already been identified, it is likely that the diversity reported here is still only the tip of the iceberg. Many more new compounds or even chemical templates will likely be discovered. As many of the identified compounds are either new to black cohosh or even new natural products, there is plenty to learn about them. Potential new insights may include biosynthetic pathways, chemotaxonomic relationships and, most certainly, a more comprehensive understanding of their biological properties. Finally, given the known broad range of biological activities of alkaloids in general and the already large variety of alkaloids in black cohosh in particular, it is reasonable to propose that the inclusion of some of these compounds will improve future standardization protocols for black cohosh preparations.

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Fig. 2.1.
Reported alkaloids from *Actaea* species

Fig. 2.2. Tandem mass spectra of (a) γ -guanidinobutyric acid (**13**), (b) γ -guanidinobutyraldehyde (**14**), and (c) γ -guanidinobutanol (**12**). Reproduced from Nikolic et al. [39] with permission

Fig. 2.3.
Proposed fragmentation pathways for (a) γ -guanidinobutyric acid (**13**); (b) γ -guanidinobutanol (**12**); and (c) γ -guanidinobutyraldehyde (**14**)

Fig. 2.4.
Tandem mass spectrum of α -N-benzoyl arginine (**21**)

Fig. 2.5.

Possible biosynthetic pathways for formation of guanidino compounds in black cohosh. As a central intermediate, **14** could be formed via action of arginine decarboxylase (*ADC*) and diaminopropane amine oxidase (*DAO*). A candidate gene for an unspecified amine oxidase has been identified by Spiering et al. [53]. **14** may be further oxidized into **13** by a γ -guanidinobutyraldehyde dehydrogenase. One such dehydrogenase has been identified in leaves of *Vicia faba* [130], and Spiering et al. identified a candidate gene for an aldehyde dehydrogenase in black cohosh roots/rhizomes. In addition, the same study identified an alcohol dehydrogenase which could convert **14** into **12**. **14** can also serve as the coupling aldehyde for biosynthesis of **9** via Pictet-Spengler condensation. Arginine could also serve as a substrate for acyl transferases that produce a number of acylated arginine derivatives (**19–21**). Adapted from Gödecke et al. [37]

Fig. 2.6.

Tandem mass spectrum of magnoflorine (**30**). Note the presence of numerous radical cations in the spectrum (m/z 284, 282, 222). Such radicals are stabilized by delocalization of the free electron onto the extended aromatic system

Fig. 2.7.
Proposed origins of abundant fragment ions of **41**

Fig. 2.8. Identification of N_{ω} -methylserotonin (**10**) in black cohosh. (a) LC-MS chromatogram of N_{ω} -methylserotonin standard; (b) LC-MS chromatogram of 5-HT₇-active fraction; (c) and (d) tandem mass spectra of N_{ω} -methylserotonin standard and the compound eluting at the same retention time in the active fraction, respectively. Reproduced from [38] with permission

Fig. 2.9. Identification of methylcimitrypazepine **44**, a new azepine from black cohosh. The stereochemistry at the chiral center is unknown

Fig. 2.10. 900 MHz ¹H NMR spectrum of synthetic methylcimitrypazepine (**44**), a new azepine identified in black cohosh. The Asterisk indicates the presence of a trace of formic acid used in the purification procedure

Fig. 2.11.

Proposed mechanism for formation of β -carboline and azepine adducts of *N* ω -methylserotonin (**10**) and aldehydes (adapted from [39])

Fig. 2.12.

Product ion tandem mass spectra of amides of hydroxycinnamic acids; (a) α -N-feruloyl arginine (47); (b) α -N-isoferuloyl arginine (48); and (c) α -N-caffeoyl arginine (46). Ion series corresponding to the acid portion are labeled with “asterisks,” while those corresponding to the amine portion are labeled with a “diamond.” Note the low abundance but diagnostic fragment ion of m/z 163, which is formed by amides of isoferulic but not ferulic acid. Adapted from reference [39], with permission

Fig. 2.13.

Product ion tandem mass spectra of (a) synthetic *N*-feruloyl tyramine-4'-*O*- β -D-glucopyranoside; (b) synthetic *N*-feruloyl tyramine-4'-*O*- β -D-glucopyranoside; and (c) a putative dopamine-*O*-(isoferuloyl)-hexoside from black cohosh (**56**). Note the absence of protonated amine in the spectra of *N*-feruloyl tyramine glucoside (m/z 138) and *N*-feruloyl dopamine glucoside (m/z 154). In the spectrum of the unknown compound **56**, fragment ions originating from dopamine (m/z 154 and 137) are clearly visible suggesting that it is not a cinnamide, but most likely a dopamine hexoside where the hexose unit is acylated with ferulic/isoferulic acid. The site of hexose attachment to dopamine was arbitrarily drawn to position 3 for clarity. Spectra were taken at 15 eV collision energy on a Waters SYNAPT quadrupole/time-of-flight mass spectrometer

Table 2.1
Mass spectrometry data for compounds identified in 75 % ethanolic extract of roots/rhizomes of black cohosh

No.	<i>m/z</i> [M+H] ⁺	Formula	Error (ppm)	Major fragments ^d	Identification	Level
7	172.1086	C ₇ H ₁₃ N ₃ O ₂	0.0	172.1086(100); 154.0972(64); 137.0704(18); 130.0863(70); 119.0611(15); 112.0762(25); 95.0568(21); 94.0538(16); 70.0657(60)	Cimipronidine	1 ^b
8	154.0978	C ₇ H ₁₁ N ₃ O	-1.3	154.0978(100); 112.0768(20); 95.0547(6); 94.0656(16); 70.0676(16); 67.0512(10)	Cyclocimipronidine	1 ^b
9	265.1673	C ₁₃ H ₂₀ N ₄ O ₂	3.0	206.1178(100); 137.0613(18); 70.0560(10); 60.0448(5)	Dopargine	1 ^b
10	191.1192	C ₁₁ H ₁₄ N ₂ O	4.2	160.0745(100); 148.0725(18); 132.0824(22); 115.0588(20)	N _ω -Methylserotonin	1
11	175.1191	C ₆ H ₁₄ N ₄ O ₂	-1.5	175.1191(12); 158.0938(12); 130.0978(75); 116.0693(35); 112.0840(18); 71.0493(25); 70.0653(100); 60.0565(10)	Arginine	1
T12	132.1127	C ₅ H ₁₃ N ₃ O	-7.6	132.1127(100); 90.0960(15); 73.0617(12); 60.0570(7); 55.0575(8)	γ-Guanidino butanol	2 ^c
13	146.0925	C ₅ H ₁₁ N ₃ O ₂	-3.4	146.0969(100); 128.0856(20); 111.0585(12); 104.0720(12); 87.0440(40); 86.0601(35); 69.0307(7); 60.0570(6)[15 eV]	γ-Guanidino butyric acid	1
T14	130.0971	C ₅ H ₁₁ N ₃ O	-6.9	130.0971(60); 112.0872(30); 71.0502(12); 70.0660(100); 60.0570(7) [15 eV]	γ-Guanidino butyraldehyde	2
15	160.1081	C ₆ H ₁₃ N ₃ O ₂	-3.1	160.1098(70); 128.0857(5); 118.0834(5); 101.0598(100); 86.0602(5); 59.0504(9)	γ-Guanidino butyric acid methyl ester	1
16	174.1244	C ₇ H ₁₅ N ₃ O ₂	0.6	174.1244(100); 146.0953(9); 132.1046(9); 128.0857(11); 15.0753(73); 87.0432(85); 86.0611(12)[15 eV]	γ-Guanidino butyric acid ethyl ester	1
T17	160.1077	C ₆ H ₁₃ N ₃ O ₂	-5.4	160.1077(10); 101.0026(100); 100.0532(88)	δ-Guanidinovaleic acid	2
T18	174.1243	C ₇ H ₁₅ N ₃ O ₂	0.0	174.1244(100); 132.1034(8); 115.0762(78); 100.0771(8); 73.0659(18); 55.0568(15)	δ-Guanidinovaleic acid methyl ester	2
19	203.1144	C ₇ H ₁₅ N ₄ O ₃	0.0	203.1144(100); 186.0820(175.1172(50); 158.0940(30); 144.0661(45); 143.0809(10); 130.1028(10); 116.0690(20); 112.0845(15); 98.0640(18); 71.0493(10); 70.0653(30); 60.0660(6) [15 eV]	α-N-Formyl arginine	1
20	217.1297	C ₈ H ₁₆ N ₄ O ₃	-1.8	217.1297(9); 175.1200(22); 158.0935(100); 130.0978(17); 116.0693(60); 115.0881(35); 112.0840(50); 74.0231(18); 71.0505(32); 70.0653(90); 60.0565(5)	α-N-Acetyl arginine	1
21	279.1461	C ₁₃ H ₁₈ N ₄ O ₃	1.4	279.1465(43); 262.1194(34); 220.0967(74); 219.1130(72); 216.1135(10); 201.1028(19); 175.1249(9); 174.0921(11); 73.1082(19); 159.0781(8); 122.0607(16); 113.0716(13); 105.0342(100); 70.0659(30)	α-N-Benzoyl arginine	1 ^e

No.	m/z [M+H] ⁺	Formula	Error (ppm)	Major fragments ^d	Identification	Level
T22	168.1128	C ₈ H ₁₃ N ₃ O	-5.4	168.1128(100); 112.0768(13); 94.0658(6); 70.0678(12); 67.0512(4)	N-Methyl cyclocimipronidine	2
23	180.1017	C ₁₀ H ₁₃ NO ₂	-4.4	180.1017(3); 163.0752(12); 151.0727(10); 145.0651(46); 117.0698(100); 115.0540(95); 105.0962(8); 91.0541(20)	Salsolinol	1
24	166.0869	C ₉ H ₁₁ NO ₂	0.6	166.0869(18); 149.0608(18); 137.0618(100); 121.0641(17); 121.0649(90); 103.0530(28); 91.0560(37); 77.0390(12)	Nonsalsolinol	1
25	178.0882	C ₁₀ H ₁₁ NO ₂	7.9	178.0882(100); 176.0704(5); 163.0645(44); 162.0566(54); 160.0765(5); 137.0614(11); 135.0453(16); 117.0354(8); 115.0569(8); 89.0410(4)	1,2-Dehydrosalsolinol	1
26	314.1749	C ₁₉ H ₂₄ NO ₃ ⁺	-2.2	314.1749(100); 271.1338(10); 269.1193(50); 239.1040(10); 237.0931(46); 211.1048(15); 209.0978(28); 192.1030(15); 175.0763(35); 151.0752(10); 145.0646(18); 143.0534(18); 137.0611(18); 107.0484(40)	Magnocurarine	1
T27	272.1287	C ₁₆ H ₁₇ NO ₃	0.0	255.0926(20); 237.0890(40); 161.0591(30); 143.0712(30); 115.0583(37); 107.0503(100); 77.0449(18)	Norocclaurine	2 ^d
28	330.1719	C ₁₆ H ₂₃ NO ₄	4.2	330.1720(6); 299.1310(6); 267.1067(6); 192.1037(100); 177.0803(11); 175.0783(20); 143.0502(20); 137.0599(24); 115.0526(10)	Reticuline	1
T29	314.1750 [M ⁺]	C ₁₉ H ₂₄ NO ₃ ⁺	-1.9	314.1767(100); 271.1332(10); 269.1196(50); 239.1010(10); 237.0925(25); 211.1153(5); 209.0938(20); 192.1030(18); 175.0801(15); 145.0646(15); 143.0513(14); 137.0603(10); 115.0569(20); 107.0506(40)	Isomer of magnocurarine (oblongine)	3
30	342.1705 [M ⁺]	C ₂₀ H ₂₄ NO ₄ ⁺	0.0	342.1725(28); 299.1301(11); 297.1129(84); 282.0892(34); 279.1039(8); 265.0876(100); 237.0920(30); 222.0700(10); 219.0804(14); 209.0981(8); 207.0806(8); 191.0882(10)	Magnoflorine	1
T31	342.1702 [M ⁺]	C ₂₀ H ₂₄ NO ₄ ⁺	-0.9	297.1237(28); 282.1075(10); 265.1015(100); 237.0991(40); 250.0738(10); 237.0991(42); 233.0677(22); 205.0789(20)	Laurifoline	2 ^d
32	314.1392	C ₁₈ H ₁₉ NO ₄	1.3	297.1128(18); 282.0860(25); 265.0874(78); 237.0914(100); 222.0710(15); 205.0646(50); 177.0729(15)	Laurolitsine	1
33	356.1861 [M ⁺]	C ₂₁ H ₂₆ NO ₄ ⁺	-0.3	356.1865(30); 313.1440(8); 311.1273(38); 296.1041(35); 281.0831(17); 279.1023(100); 280.1116(30); 265.0888(15); 251.1107(21); 264.0786(42); 248.0844(40); 236.0838(16)	Menisperine	1
T35	356.1874 [M ⁺]	C ₂₁ H ₂₆ NO ₄ ⁺	3.4	356.1865(6); 311.1273(40); 296.1041(60); 281.0831(16); 280.1116(100); 265.0868(13)	Xanthoplanine	2 ^d
T36aT36b	342.1705 [M ⁺]	C ₂₀ H ₂₄ NO ₄ ⁺	0.0	342.17105(5); 192.1018 (100); 177.0802(10)	Phellodendrine or cyclanoline	3 ^d

No.	m/z [M+H] ⁺	Formula	Error (ppm)	Major fragments ^d	Identification	Level
T37	356.1870 [M ⁺]	C ₂₁ H ₂₆ NO ₄ ⁺	2.2	356.1870(5); 192.1016(100); 177.0803(10)	N-Methyl tetrahydrocolumbamine or isomer	3
38	354.1359	C ₂₀ H ₁₉ NO ₅	5.1	354.1359(90); 336.1168(20); 323.0955(9); 275.0661(20); 247.0757(15); 206.0807(15); 189.0783(64); 188.0702(85); 149.0609(25); 119.0476(6); 91.0582(6)	Protopine	1
39	370.1653	C ₂₁ H ₂₃ NO ₅	-0.3	370.1653(100); 352.1522(42); 290.0963(42); 206.0807(34); 189.0783(38); 188.0702(100); 165.0932(10); 149.0609 (10)	Allocryptopine	1
40	217.0981	C ₁₂ H ₁₂ N ₂ O ₂	1.8	144.0806(100); 143.0723(12); 130.0654(6); 117.0685(8)	1,2,3,4,-Tetrahydro-β-carboline-3-carboxylic acid	1
41	233.1136	C ₁₃ H ₁₄ N ₂ O ₂	0.9	231.1136(7); 214.0850(31); 188.0695(31); 168.0788(40); 158.0945(100); 154.0654(12); 146.0593(30); 144.0792(10); 143.0718(16); 130.0647(15); 118.0653(7)[15 eV]	Harmine, 1,2,3,4-tetrahydro-3-carboxylic acid	1 ^e
42	203.1185	C ₁₂ H ₁₄ N ₂ O	0.5	160.0758(100); 159.0696(6); 132.0865(6); 117.0614(6)	N(2)-Methyl-6-hydroxy-1,2,3,4-tetrahydro-β-carboline	1
43	203.1185	C ₁₂ H ₁₄ N ₂ O	0.5	188.0953(5); 174.0938(100); 162.0894(30); 160.0758(20); 159.0696(50); 147.0688(46); 146.0600(22); 131.0760(12); 130.0651(10); 129.0706(6)	Cimicripazepine	1
44	217.1338	C ₁₃ H ₁₆ N ₂ O	-1.4	217.1338(86); 188.1071(100); 186.0913(70); 176.1099(20); 174.0920(16); 171.0657(14); 160.1110(8); 159.0771(11); 158.0914(5) [15 eV]	Methylcimicripazepine	1 ^e
45	201.1031 [M ⁺]	C ₁₂ H ₁₃ N ₂ O ⁺	1.5	201.1031(100); 186.0808(29); 185.0730(6); 172.0771(11); 171.0578(40); 170.0608(52); 160.0777(12); 142.0662(12); 115.0558(15)	N(2)-Methyl-6-hydroxy-3,4-dihydro-β-carboline	1
46	337.1514	C ₁₃ H ₂₀ N ₄ O ₅	0.6	337.1514(6); 278.1035(6); 175.1211(15); 163.0399(100); 158.0932(10); 145.0291(30); 135.0449(20); 117.0340(19); 89.0396(10); 70.0672(6)	α-N-Caffeoyl arginine	1
47	351.1664	C ₁₆ H ₂₂ N ₄ O ₅	-1.1	351.1678(6); 292.1192(5); 177.0554(100); 175.1200(16); 158.0932(8); 149.0603(8); 145.0282(83); 130.0983(5); 117.0342(32); 116.0710(5); 89.0396(11); 70.0671(7); 60.0572(5)	α-N-Feruloyl arginine	1
48	351.1664	C ₁₆ H ₂₂ N ₄ O ₅	-1.1	351.1678(6); 292.1192(5); 177.0554(100); 175.1200(11); 163.0394(8); 158.0932(8); 149.0603(17); 145.0282(24); 130.0983(5); 117.0342(18); 116.0710(5); 89.0396(11); 70.0671(5); 60.0572(4)	α-N-Isoferuloyl arginine	1
49	265.1543	C ₁₄ H ₂₀ N ₂ O ₃	-3.4	177.0558(90); 149.0595(18); 145.0300(100); 117.0347(90); 89.0390(55)	Feruloyl putrescine	1
50	265.1543	C ₁₄ H ₂₀ N ₂ O ₃	-3.4	177.0553(100); 163.0356(4); 149.0590(22); 145.0289(31); 134.0355 (7); 117.0343(30); 89.0385(25)	Isoferuloyl putrescine	1
T51	332.1257	C ₁₆ H ₁₈ N ₃ O ₅	3.3	177.0551(100); 163.0395(3); 149.0597(16);	α-N-Isoferuloyl histidine	2

No.	m/z [M+H] ⁺	Formula	Error (ppm)	Major fragments ^a	Identification	Level
T52	338.1234	C ₁₆ H ₁₈ NO ₇	-1.8	145.0283(21); 117.0346(20); 110.0744(5); 89.0395(10)	α -N-Isoferuloyl glutamic acid	2
T53	476.1929	C ₂₄ H ₂₉ NO ₉	1.7	314.1398(100); 177.0558(90); 149.0607(6); 145.0282(45); 121.0617(3); 117.0363(10); 89.0380(6)	N-Feruloyl tyramine hexoside	2
T54	492.1878	C ₂₄ H ₂₉ NO ₁₀	1.6	330.1343(24); 177.0533(100); 149.0657(4); 145.0320(52); 117.0320(12)	N-Feruloyl dopamine hexoside	2
T55	506.2026	C ₂₅ H ₃₁ NO ₁₀	2.4	344.1525(12); 177.0558(100); 149.0609(5); 145.0282(62); 117.0336(15); 89.0414(6)	N-Feruloyl-methoxytyraminehexoside	2
T56	492.1878	C ₂₄ H ₂₉ NO ₁₀	1.6	321.0980(15); 177.0558(100); 163.0390(3); 149.0657(10); 145.0328(17); 137.0606(22); 119.0501(5); 117.0375(10); 91.0558(7); 89.0421(6)	Dopamine- <i>O</i> -(isoferuloyl)-hexoside	3 f
57	104.1068 [M ⁺]	C ₅ H ₁₄ NO	-6.7	104.1068(100); 60.0818(14)	Choline	1
58	280.1341 [M ⁺]	C ₁₂ H ₁₈ NO ₂ ⁺	1.4	208.1341(6); 149.0584(90); 105.0357(100); 77.0374(18)	Benzoyl choline	1
59	280.1554 [M ⁺]	C ₁₃ H ₂₂ NO ₄ ⁺	1.8	221.0815(100); 206.0581 (20); 177.0550(60); 149.0604(8); 145.0325(30); 117.0372(20); 89.0386(8)	Feruloyl choline	1
60	280.1554 [M ⁺]	C ₁₃ H ₂₂ NO ₄ ⁺	1.8	221.0813(100); 206.0578(20); 177.0544(60); 163.0358(5); 162.0318(5); 149.0605(10); 145.0320(14); 134.0369(6); 117.0341(10); 89.0388(10)	Isoferuloyl choline	1
T61	266.1617 [M ⁺]	C ₁₁ H ₂₃ NO ₆ ⁺	4.9	266.1617(95); 104.1073(100); 60.0838(13)	Choline hexoside	3
62	118.0872 [M ⁺]	C ₅ H ₁₂ NO ₂ ⁺	3.4	118.0872(100); 59.0711(10)	Glycine betaine	1
63	144.1018 [M ⁺]	C ₇ H ₁₄ NO ₂ ⁺	-4.9	144.1018(100); 84.0823(10); 58.0672(10)	Proline betaine	1
64	162.1122 [M ⁺]	C ₇ H ₁₆ NO ₃ ⁺	-4.9	162.1122(100); 103.0406(20); 102.0930(8); 85.0300(7); 60.0829(8) [15 eV]	L-Carnitine	1
T65	198.1237 [M ⁺]	C ₉ H ₁₆ N ₃ O ₂ ⁺	-3.0	198.1237(14); 154.1338(100); 95.0619(60); 68.0517(6); 60.0829(8) [15 eV]	Histidine betaine	2
66	138.0547 [M ⁺]	C ₇ H ₈ NO ₂ ⁺	-5.8	138.0547(100); 136.0394(5); 110.0587(6); 94.0647(30)92.0494(24); 65.0377(6)	Trigonelline	1
67	130.0860	C ₆ H ₁₁ NO ₂	-6.1	84.0823(100)	Pipecolic acid	1
68	130.0503	C ₅ H ₇ NO ₃	-0.8	84.0449(100)	Pyroglutamic acid	1
70	332.1340	C ₁₄ H ₂₁ NO ₈	-1.5	332.1340(6); 314.1237(29); 152.0704(100); 136.0765(16); 134.0602(28); 124.0764(26); 108.0816(55); 106.0651(10)	5'- <i>O</i> -(β -D-glucopyranosyl) pyridoxine	3
71	220.1178	C ₉ H ₁₇ NO ₅	-3.2	220.1178(8); 142.0858(10); 124.0712(37); 103.0721(12); 98.0217(51); 90.0529(100); 85.0547(35); 72.0417(69); 70.0295(18)	Pantothenic acid	1

No.	m/z [M+H] ⁺	Formula	Error (ppm)	Major fragments ^d	Identification	Level
T72	282.1202	C ₁₁ H ₁₅ N ₅ O ₄	2.1	150.0783(100)	N-Methyladenosine	2
T73	282.1183	C ₁₁ H ₁₅ N ₅ O ₄	-6.7	136.0622(100); 119.0360(10)	2'-O-Methyladenosine	2
T74	178.0875	C ₁₀ H ₁₁ NO ₂	3.9	178.0879(20); 119.0476(40); 91.0547(100)	N-Phenylacetyl acetamide	3
T75	206.1550	C ₁₃ H ₁₉ NO	2.4	107.0504(100); 100.1135(5); 79.0544(18); 77.0405(16)	N-Cyclohexyl-4-hydroxy benzylamine	3

^aInstrument: waters SYNAPT quadrupole/time-of-flight. Spectra taken at 25 eV except where noted

^bIsolated compound

^cMost compounds annotated at levels 2 or 3 were tentatively (T) identified by comparison of their fragmentation patterns with those of structural analogs

^dIdentification based on published tandem mass spectra

^eCompound reported here for the first time

^f15 eV spectrum of **56** is shown in Fig. 2.13c. Adapted from Nikolic et al. [39]

Table 2.2

¹H NMR data for methylcimitrypazepine (**44**)

Proton	DMSO-d ₆ (900 MHz)					MeOH-d ₄ [106] (500 MHz)	
	δ Chemical shift (ppm)	² J(Hz)	³ J(Hz)	⁴ J(Hz)	¹ J _(H,C) (Hz)	δ Chemical shift (ppm)	³ J(Hz)
H-1	10.579 s, br., 1H		2.78			Not observed	
H-2	6.975 s, br., 1H		2.78	1.31	178.45	6.95 s	
H-3	2.868 dt, 1H	-16.1	3.03		126.2	3.19–3.27 m, 1H	
	3.107 dddd, 1H	-16.1	4.77, 5.75	1.31	123.2	2.97–3.04 m, 2H	
H-4	2.971 ddd, 1H	-13.73	4.72, 2.33		n.m. a		
	3.491 td, 1H	-13.73	3.47		135.71	3.60–3.68 m, 1H	
C-6 Me	1.318 d, 3H		6.95		126.6	1.44 d	6.8
H-6	4.526 q, 1H		6.95		137.1	4.73 q	6.8
H-8	6.619 d, 1H		8.37		157.01	6.65 d	8.6
H-9	6.977 d, 1H		8.37		158.95	7.03 d	8.6
N-Me	2.55 s, br, 3H				137.81	2.59 s	
7-OH	8.547 s, br, 1H					Not observed	

^aThe ¹³C satellites for the H-4 signal appearing at 82.971 ppm were heavily overlapped

Table 2.3¹H NMR data (900 MHz, DMSO-d₆) for arginine derivatives **47** and **48**

Proton	Feruloyl (47)		Isoferuloyl (48)	
	δ Chemical shift (ppm)	³ J(Hz)	δ Chemical shift (ppm)	³ J(Hz)
H-2	7.085 s, br, 1H		6.947 s, br, 1H	
H-5	6.741 d, 1H	7.998	6.9068 d, br, 1H	8.42
H-6	6.901 d, 1H	7.998	6.891 d, br, 1H	8.42
H-α	7.213 d, 1H	15.72	6.582 d, 1H	15.66
H-β	6.626 d, 1H	15.72	7.176 d, 1H	15.66
NH	7.907 d, 1H	7.51	7.907 d, br, 1H	6.75
MeO	3.787 s, 3H		3.779 s, 3H	
NH ₂	9.223 s, br, 2H		8.343 s, br	
NH ₂	9.390 s, br, 2H		9.140 s, br	
H-1'	4.101 dt	7.51, 5.64	4.069 dt, 1H	6.75, 4.07
H-2'	1.730 m, 1H		1.698 m, br, 1H	
	1.640 m, 1H		1.618 m, br, 1H	
H-3'	1.512 m, 1H		1.504 m, br, 1H	
	1.458 m, 1H		1.459 m, br, 1H	
H-4'	3.084 m, 1H		3.066 m, br, 2H	
	3.047 m, 1H			